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IMMUNE RECONSTITUTION IN PAEDIATRIC PATIENTS WITH ACQUIRED IMMUNODEFICIENCY

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ABSTRACT

Background: Immune system plays a crucial role in defending organism from pathogens. However, immunological functions may be severely impaired by a number of disorders including acquired immunodeficiencies, possibly due to infections, chronic diseases, immunosuppressive drugs or surgeon therapies. In order to favour the immune reconstitution of patients with acquired immunodeficiency, various therapeutic treatments are constantly under investigation for both adults and paediatric patients.

Aim of the study: To evaluate immune reconstitution in children with acquired immunodeficiency. Attention was focused on two groups of HIV-1 vertically infected children (subjected the first group to HAART from the third months of life, the second to a simplified treatment) and on a cohort a leukaemia children who underwent Umbilical Cord Blood Transplantation (UCBT).

Materials and Methods: Immune restoration was principally evaluated in terms of lymphoproliferative response and T-cell receptor (TCR) repertoire. Immunological studies were complemented by routine clinical and laboratory analyses.

Results: HIV-infected children showed a significant normalization of immune functions investigated, with a long-term maintaining of good clinical and immunological parameters. Also children who underwent UCBT showed a notable immune restoration.

Conclusions: In HIV-infected children, an early application of antiretroviral treatment from the third month of life favours immune reconstitution and the application of a simplified regimen seems to permit the maintenance of good immunological results obtained during the previous successful HAART. In the context of transplanted children our data underline applicability and advantages of UBC compared to Bone marrow (BM) transplantations.

Key words: Immune reconstitution, Acquired Immunodeficiency, HIV-1, HAART, Umbilical cord blood transplantation, T cell receptor, Spectratyping

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Doctoral thesis from the Department of Sanità pubblica e biologia cellulare,
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This thesis is based on the following articles and manuscript:

1) Palma P, Romiti ML, Cancrini C, **Pensieroso S,** Santucci M, Montesano C, Bernardi S, Amicosante M, Di Matteo G, Di Cesare S, Wahren B, Rossi P, Castelli-Gattinara G. Early Highly Active Antiretroviral treatment delayed over 3 months of life is associated with good clinical outcome, long-term viral control and persistent antiviral T-cell response in HIV-1 vertically infected infants. Submitted to Eur. J. of Pediatrics.

2) Palma P, Romiti ML, Cancrini C, **Pensieroso S**, Montesano C, Cantucci MB, Tozzi A, Bernardi S, Martino AM, Andreoni M, Amicosante M, Freda E, Rossi P & Castelli-Gattinara G. Successful simplification of protease inhibitor-based HAART with a triple nucleosides regimen in HIV-1 vertically infected children. Submitted to Antiviral therapy

3) **Pensieroso S**, Romiti ML, Palma P, Castelli-Gattinara G, Bernardi S, Freda E, Rossi P, Cancrini C. Switching from protease inhibitor-based HAART to a protease inhibitor-sparing regimen is associated with improved specific HIV-immune responses in HIV-infected children. AIDS. 20, 1893-1896 (2006).

4) Finocchi A, Romiti ML, Di Cesare S, Puliafito P, **Pensieroso S**, Rana I, Pinto R, Cancrini C, De Rossi G, Caniglia M, Rossi P. Rapid T-cell receptor CD4+ repertoire reconstitution and immune recovery in unrelated umbilical cord blood transplanted pediatric leukemia patients. J. Pediatr. Hematol. Oncol. 28, 403-411 (2006).

ABBREVIATIONS

3TC:	Lamivudine
Ab:	Antibody
ABC:	Abacavir
Ag:	Antigen
AIDS:	Acquired immunodeficiency syndrome
ALL:	Acute lymphoblastic leukaemia
AML:	Acute myeloblastic leukaemia
APC:	Antigen presenting cell
APC-Cy5	Allophycocyanin
AT-2:	Aldhitriol 2
ATG:	Antithymocyte globulins
AZT:	Zidovudine
BCR:	B cell receptor
BM:	Bone marrow
BU:	Busulfan
CCR5:	CC-chemokine receptor 5
CD40L:	CD40 ligand
CDR:	Complementary determining region
CLP:	Common lymphoid progenitor
CMV:	Cytomegalovirus
Cpm:	counts per minute
CSA:	Cyclosporine A
cTEC:	cortical thymic epithelial cell
CTL:	Cytotoxic T lymphocyte
CTX:	cyclophosphamide
CXCR4:	CXC-chemokine receptor 4
CY:	Cyclophosphamide
d4T	Stavudine
DC:	Dendritic cell
DC-SIGN:	DC-specific ICAM3-grabbing non integrin
ddI:	Didanosine
DN:	Double negative
DP:	Double positive
ER:	Endoplasmic reticulum
FasL:	Fas ligand
FDC:	Follicular dendritic cell
FISH:	Fluorescence in situ hybridization

FITC:	Fluorescein isothiocyanate
FK406:	Tacrolimus
GC:	Germinal centre
G-CSF:	Granulocyte Colony Stimulating Factor
GM-CSF:	Granulocyte/macrophage-colony stimulating factor
GVHD:	Graft versus host disease
HAART:	Highly Active Antiretroviral Therapy
HIV:	Human immunodeficiency virus
HSCT:	Haematopoietic stem cell transplantation
HSV-1:	Herpes virus-1
ICAM:	Intercellular adhesion molecule
IFN-γ:	Interferon γ
IL-4:	Interleukin 4
IL-5:	Interleukin 5
IL-6:	Interleukin 6
IL-12:	Interleukin 12
IVIG:	Intravenous Ig
LC:	Langherans cell
LPS:	Lypopolisaccaride
LPV:	Lopinavir
MDDC:	Monocyte-derived DC
MHC:	Major histocompatibility complex
MLR:	Leucocyte mixed reaction
mTEC:	medullar thymic epithelial cell
myDC:	Myeloid dendritic cell
NK:	Natural killer
NNRTI:	Non-nucleoside reverse transcriptase inhibitor
NRTI:	nucleoside reverse transcriptase inhibitor
PCR:	Polymerase chain reaction
pDCs:	Plasmacytoid dendritic cell
PDN:	Prednisolone
PE	R-Phycoerythrin
PHA:	Phytohaemaglutinin
PI:	Protease Inhibitor
PMA:	Phorbol myristate acetate
PWM:	Pokeweed mitogen
RAG:	Recombination activating gene
RT-PCR:	Reverse transcriptase PCR
RTV:	Ritonavir
SD:	Standard deviation

SI:	Stimulation index
SP:	Single positive
TBI:	Total body irradiation
TCR:	T cell receptor
Th:	T helper
TLR:	Toll like receptor
TNF:	Tumour necrosis factor
TREC:	TCR-excision circle
UCBT:	Umbilical Cord Blood Transplantation
VP16:	Etoposide

CONTENTS

INTRODUCTION

In healthy individuals the organism is protected from pathogens such as bacteria, viruses and parasites through the action of a specialized complex of cells constituting the immune system.

In particular conditions, the immune system could be altered leading to immunological disorders like lymphoproliferative disease, autoimmunity, hypersensitivity, and immunodeficiency.

The latter could be distinguished into two types: primary immunodeficiencies, due to genetic or developmental alterations and secondary immunodeficiencies, induced by viral or bacterial infections, by the use of immunosuppressive drugs, or by chronic disease or surgeon therapy.

Several treatments exist in order to favour the reconstitution of immune system in immune-compromised patients, and new therapeutic approaches are constantly under investigation.

Objective of this study is to evaluate the immune reconstitution in paediatric patients with secondary immunodeficiencies.

Particular attention was focused on immune restoration of:

- Cohorts of human immunodeficiency virus (HIV)-infected children:
  - Treated with early Protease Inhibitor Highly Active Antiviral Therapy (PI-HAART) started over three months of life.
  - Treated with a simplified regimen (PI-sparing-HAART), after a first line successful PI-HAART.
- A cohort of leukaemia children who undergone to unrelated umbilical cord blood transplantation (UCBT).

A number of techniques could be used to study the immune reconstitution: we performed several immunological and molecular analyses, with particular interest in the spectratyping technique, which measure the breadth of the antigenic diversity of the T-cell receptor (TCR) repertoire distribution.

The immune system

The immune system protects the body from infections by pathogenic organism. It is composed by cells, organs and tissues arranged in a dynamic interactive network that can be divided into two major arms: innate and adaptive immunity.

The first kind of protection is non-specific and immediately guaranteed by cells, like as granulocytes, monocytes and macrophages, activated by direct contact with various microbial products via toll-like receptors (TLR). Innate immune functions are also carried out by mast cells and natural killer (NK) beside to antibacterial enzymes and components of the complement system. Innate immunity does not confer long-term protection to the host because there is no creation of immunological memory.

The second kind of protection, the adaptive immunity, is pathogen-specific and requires the recognition of specific “non-self” antigens in the presence of “self”, during the process of antigen presentation. This antigen specificity is possible thanks to B cell receptor (BCR) and T cell receptor (TCR) respectively expressed on surface of B and T lymphocytes, and is necessary for the generation of specific responses, and for the development of an immunological memory able to mount a stronger attack each time that a particular pathogen is re-encountered. Besides T and B lymphocytes, also antigen presenting cells (APCs) play a pivotal role in adaptive immunity, being responsible of antigen presentation: overall these cells guarantee specificity, memory and diversity, which are the three main features of adaptive immunity.

B lymphocytes

B-cells mediate humoral responses via secretion of their BCR in the form of Antibodies (Abs), glycoproteins belonging to gammaglobulin class, able to bind pathogens, facilitating their destruction and neutralization in a specific way. B lymphocytes may also act as antigen presenting cells and as regulators of immunological responses, secreting cytokines and interacting with T cells.

B-cells are generated in the bone marrow (BM) from the Common Lymphoid Progenitor (CLP) (Ye 2007). In the bone marrow (BM) rearrangements of genes encoding for the BCR occur and transitional naïve B-cells still functionally immature are formed. (Chung J. 2003). At this point, naïve B-cells migrate in periphery and, following Antigen (Ag) encounter, they can immediately differentiate into short-life low-affinity immunoglobulin (Ig)-secreting cells (Martin 2001), that are responsible of the T-cell independent Ag responses, or can be transported by the bloodstream to secondary lymphoid organs, principally spleen and lymph nodes. Here they mature within germinal centres (GC), during the so called germinal centre reaction, to constitute long-life plasma-cells responsible of the T-cell dependent Ag responses (de Vinuesa 2000, Wolniak 2004). A prerequisite to the formation of a GC is the co-stimulatory interaction of antigen-specific follicular B and T-helper cells (Liu 1991) through their receptors CD40/CD40L: as a consequence of this interaction, GC precursor B-cells emerge and proliferate within the stromal environment created by a special network of dendritic cells (DCs) called follicular dendritic cells (FDCs) (Tew 1990, Kosco 1992), leading to the formation of GC. In a GC two zones could be distinguished: a dark zone and a light zone. In the dark zone B-cells undergo proliferation while their variable genes are subjected to diversification by the somatic hypermutation machinery

(Weigert 1970, Mac Lennan 1994). After somatic hypermutation, B-cells are directed to the light zone, where undergo a selection process based on the affinity of their BCR toward the antigens presented in the form of immune complexes by FDCs in the presence of helper T cells (Nayak 1999). The so formed high-affinity B-cells are positively selected, undergo isotype switching, and may become Abs secreting cells (plasma-cells) or memory B-cells that subsequently exit the GC through the surrounding mantle zone (Mac Lennan 1994). Memory B-cells could be identified by the expression of switched Ig and by the CD27 surface marker (Klein 1998, Agematsu 2000). Interestingly a new memory B-cell population $CD27^{+}IgM^{+}$, which can be generated in the absence of GC has been recently identified (Weller 2001, 2004). These IgM^{+} memory B-cells cannot be detected in splenectomised and asplenic individuals (Carsetti 2006), so that they could be related to B1a B-cells population found in mice that require the spleen for their survival and produce natural Abs (Wardemann 2002). Such B-cell population in human, called B-1 B-cells to distinguish from the classical B-2 B-cells, could be responsible for natural Abs production and for T-independent response (Kruetzmann 2003), constituting a bridge between innate and adaptive immunity.

B-cell development is schematized in Figure I.

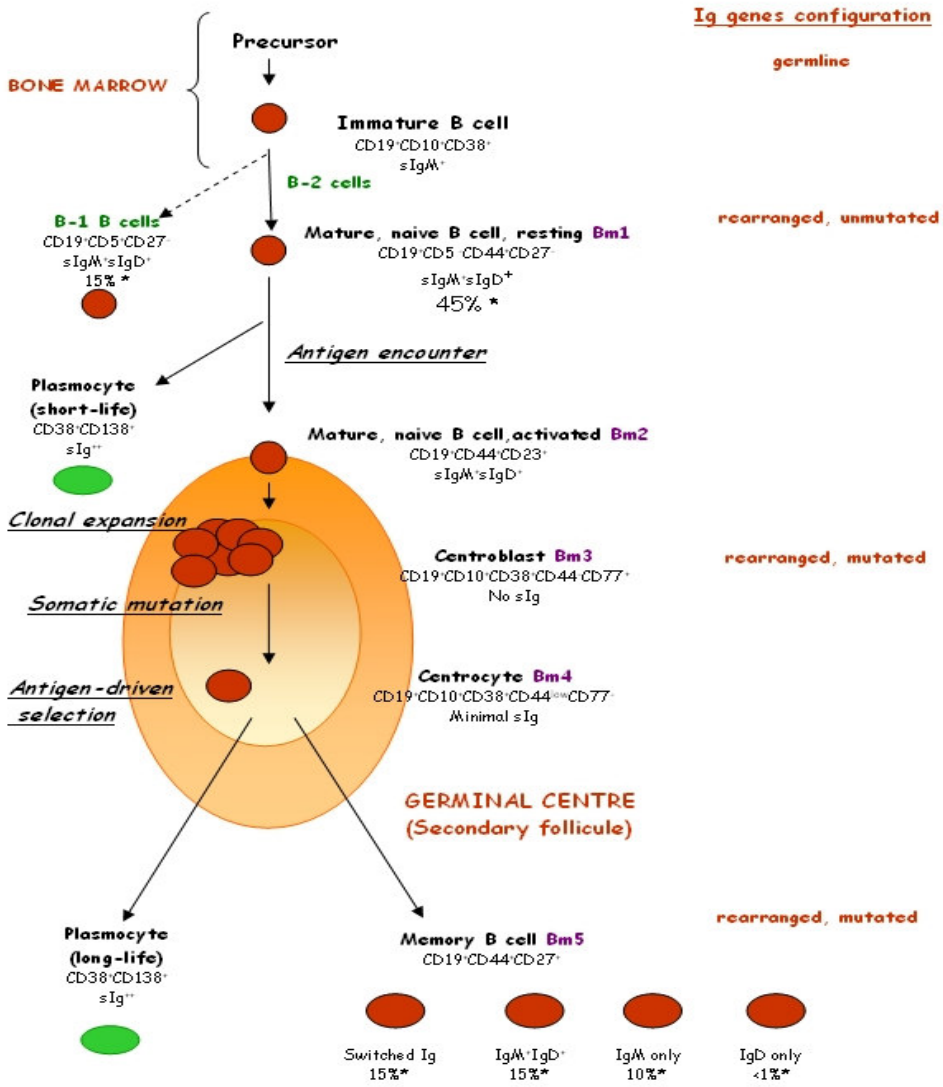


Figure I. B-cell development*. In BM rearrangement of genes encoding for BCR occur. Atypical B-1 B-cells are IgM+ and are immediately ready to produce natural Abs in a T-cell-independent manner, while classical B-2 B-cells mature in periphery and, following Ag encounter, become short-life plasma-cells, or migrate in GC, where, in a T-cell-dependent manner, undergo clonal expansion, somatic hypermutation, positive selection and isotype switching. Finally they leave secondary lymphoid organ becoming long-life plasma-cells or constituting the memory pool.*

T lymphocytes

T-cells mediate cellular responses through several action mechanisms depending on T-cell subtype involved. Indeed two subsets exist: cytotoxic and helper T-cells. A third subset is constituted by T-cells (expressing transcription factor Foxp3 and CD25 marker on their surface) with regulatory functions.

Cytotoxic T lymphocytes (CTLs), identified from surface expression marker $CD8^+$, kill infected cells via secretion of cytokines (like as TNF-α and IFN-α), granules (like as perforin and granzymes) or by the interaction of Fas ligand (FasL) with Fas expressed on target-cells (Berke 1994, Shresta 1998).

T helper (Th) lymphocytes, identified from surface expression marker $CD4^+$, could be distinguished in two further subset: $CD4^+$ Th1 lymphocytes act as cell stimulators via secretion of cytokines like as Interferon-γ (IFN-γ) and Iterleukin-12 (IL-12), while $CD4^+$ Th2 lymphocytes favour antibody production from B cells secreting Interleukin-4 (IL-4), Interleukin-5 (IL-5) and Interleukin-6 (IL-6).

T-cell activation occurs following the antigen presentation process: antigens are presented in form of peptides bound to the Major Histocompatibility Complex (MHC), constituted by molecules expressed on the surface of several autologous cells, mainly DCs.

Although the recent evidences of extrathymic T-cell development pathways in secondary lymphoid organs (Blais 2006), the thymus is the organ that supports the differentiation and selection of T cells (Bevan 1977).

After the CLP entrance into the thymus, thymocyte development follow with the expression of recombination activating gene RAG 1 and RAG 2, which permit the rearrangement of TCR β-chain, a process supported by signals derived from cortical thymic epithelial cells (cTEC) (Takahama 2006).

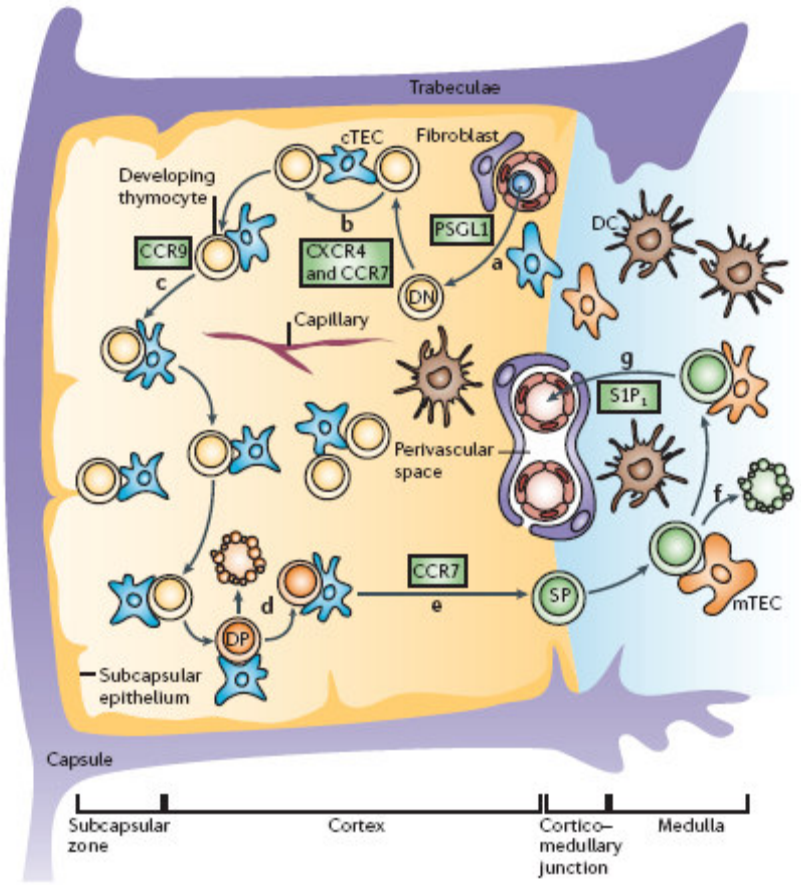


Figure II. T-cell development in the thymus. a) *T-lymphoid progenitor migrates into the thymus.* ***b-c)*** *DN thymocytes, after migration (regulated by chemokine signals) towards thymus cortex, become DP and* ***d)*** *undergo positive and negative selection.* ***e)*** *Positively selected thymocytes differentiate in $CD4^+$ or $CD8^+$ SP and* ***f)*** *in the medulla are further selected to delete tissue-specific-antigen-reactive T-cells and to generate regulatory T-cells.* ***g)*** *SP T-cells express shingosine-1-phosphate receptor 1 ($S1P_1$) and are directed back trough circulation that contain high concentration of sphingosine-1-phosphate. CXCR4: CXC-chemokine receptor 4; CCR7: CC-chemokine receptor 7; CCR9: CC-chemokine receptor 9; PSGL1: platelet-selectin glycoprotein ligand. (Adapted from Nature Rev. Takahama 2006).*

During this phase thymocyte are characterised by the surface expression of CD25, whereas are $CD44^-$ and CD4 and CD8 double negative (DN) (Pearse 1989, Shinkai 1992).

Only the cells that succeed in in-frame rearrangement of the gene encoding the TCR β-chain are selected for further differentiation, which consists in the rearrangement of genes encoding the TCR α-chain and thereby in the expression of functional TCRαβ antigen receptors (von Boehmer 1997). During this phase thymocytes become $CD4^+CD8^+$ double positive (DP) (Takahama 2006) and undergo positive selection (Kisielow 1988, Jameson 1995), which consists in the interaction between their TCR with peptide-MHC complexes expressed by stromal cells, such as cTECs and DCs in the cortex (Bousso 2002). Only tymocyte with a low-avidity interaction, receive survival signals and become single positive thymocytes (SP) for CD4 or CD8, while those without any interaction or with an high-avidity interaction die for apoptosis (negative selection) to avoid autoimmunity. Positively selected DP thymocytes then begin relocating from the cortex to the medulla (Witt 2005), where they spend approximately 12 days before being exported from the thymus (Egerton 1990). During this maturation period, central tolerance is obtained through deletion of self-reactive T-cells. Indeed medullar thymic epithelial cells (mTECs) express tissue-specific antigens promiscuously (Kiewski 2004) a process at least partially dependent on the transcriptional factor autoimmune regulator (AIRE) (Derbinski 2005). In addition medulla is thought to be the place for the production of regulatory T-cells (Fontenot 2005, Watanabe 2005).

Finally T-cells, that are mature, but still naïve (Reichert 1986) get to periphery and circulate continuously between peripheral blood and lymphoid tissue until they encounter any antigen.

Following the encounter of a peptide bound to a MHC-complex expressed on an APC, they receive activation and proliferation signals and become effector cells.
After their immunological action, a little portion of $CD4^+$ and $CD8^+$ will constitute a memory pool for that specific antigens, while all the others clonal lymphocytes will die trough apoptosis.
T-cell development in the thymus is schematized in Figure II.

As mentioned, the unique specificities of both B and T lymphocytes are the result of several recombination processes of gene segments encoding for their receptors (BCR and TCR respectively): such processes are very similar between the two cellular types, occur during lymphocytes maturation and lead to creation of a huge repertoire able to recognize an almost unlimited number of Ags. In the following section TCR recombination process will be described, considering that the same principle is valid for BCR.

T-cell receptor (TCR)

As mentioned, TCR is the molecule responsible for recognizing antigens in the context of MHC. In 95% of T-cells, TCR is a heterodimer consisting of α and β chain, while in the remaining 5% of T-cells TCRs consist of γ and δ chains.
Each chain of the TCR is a member of the immunoglobulin superfamily and is constituted by one N-terminal variable (V) domain, one constant (C) domain, a transmembrane/cell membrane-spanning region and a short cytoplasmic tail at the C-terminal end. The variable domains of the TCR α and β chain have each one three hypervariable or complementary determining regions (CDRs):

CDR3 is the most important because is the specific site for recognizing of processed antigen peptide bound to MHC.

Figure III schematizes TCR structure.

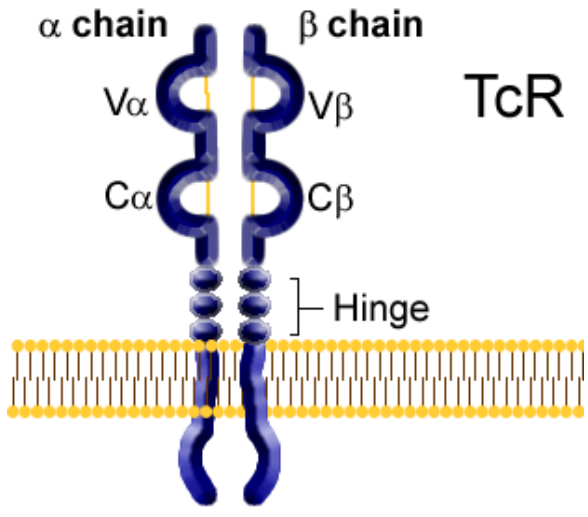


Figure III. TCR structure.

TCR α and β chains are created, as mentioned above, during T-cell development in the thymus, following the so called somatic recombination process between polymorphic genes (constituted by several gene segments) in the germinal line (Davis 1988). These polymorphic genes are located in the chromosome 7 and code for aminoterminal variable domain (V), for a diversity region (D) (only for β chain), and for the junction region (J). The TCR α chain is generated by V-J genes recombination while the β chain is generated by V-D-J genes recombination (Alt 1992). The intersection of these specific regions (V and J for the α, V D and J for the β chain) corresponds to the CDR3 region.

Recombination processes and final product are visualized in Figure IV for the β-chain rearrangement.

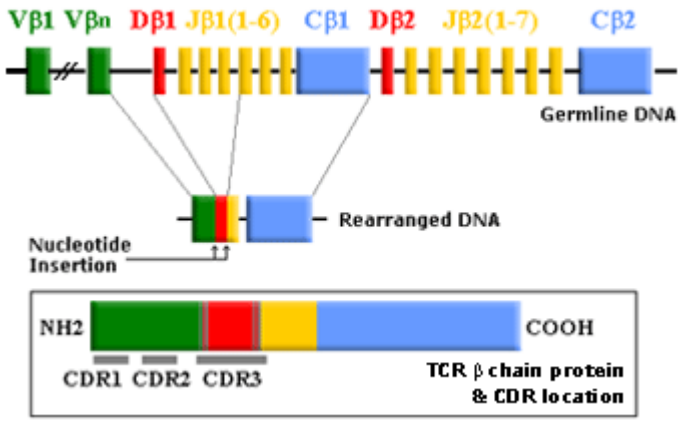


Figure IV. B-chain rearrangement*. A first recombination process occurs between genes encoding for diversity and junction region. In a second phase DJ segments are rearranged with a variable region V.*

TCR diversity is guaranteed either by somatic recombination, either by the combination of a particular α chain with a particular β chain (Lewis 1994): it's to note that while more of one α chain could be expressed on a T-cell, only one β chain could be expressed in each T-cell (allelic exclusion).

Furthermore during the recombination processes random palindromic P nucleotides are added (due to imprecise junction during recombination) and also T nucleotides are inserted by the action of terminal deossinucleotide transferase (TdT) enzyme. This imprecise junction produces CDR3 segments with diverse lengths in a range of 6-8 amino acids (Pannettier 1995) so that CDR3 lengths are a direct measure of TCR diversity.

Antigen presenting cells (APCs)

APCs are constituted by macrophages, which ingest microbes and particulate Ags, B cells, which take up soluble Ags by binding them with surface Igs, and DCs, which are the most potent and important APCs, having the unique function of antigen presentation (Banchereau 1998). In order to stimulate immunological responses, APCs present Ags trough peptide exposition on MHCs expressed on surface. Furthermore they provide co-stimulatory signals like B7-1 (CD80) and B7-2 (CD86) necessary to prime a naïve T-cell through interaction with CD28 (Chambers 1997).

APCs initiate their action trapping pathogens in peripheral lymphoid organs and presenting them to lymphocytes that continuously circulate from the blood stream to the lymphoid organs and back again, monitoring the Ags presented by APCs.

Migration of cells and cell-cell interactions require many adhesion molecules including selectins, integrins and intercellular adhesion molecules (ICAMs).

Dendritic cells (DCs)

DCs are cells dedicated to the Ags up-take and presentation. Consequently they are the best stimulators of naïve T lymphocytes to undergo clonal expansion (Banchereau 1998, Hart 1997). DCs can be classified into several subsets on the basis of their anatomical distribution, immunological function, and cell-surface marker expression. The main DCs subsets include myeloid DCs (myDCs) and plasmacytoid DCs (pDCs) in the blood, and Langherans cells (LCs) in tissue (Shortman 2002, Banchereau 2000). In general myDCs are characterized by their ability to secrete large amounts of interleukin 12 (IL-

12), whereas pDCs can prime adaptive immunity by producing large amounts of type I interferons (IFNs) (Cella 1999); LCs mainly express langerin (also known as CD207), a C-type lectin involved in cell migration (Valladeau 1999).

DCs develop in the BM from $CD34^+$ monocyte precursor. Until immature, they are specialized in the Ag up-take (Sallusto 1994) while, once activated, they mature and loss the Ag up-take ability becoming specialized in the Ag presentation on MHC. They migrate to the lymphoid tissues, interact with T-cells and B-cells, forming the so named "immunological synapse" and trigger the immune response (Dustin ML 2006).

Therefore the efficiency of an immunological response is dependent by a correct antigen presentation process and thereby by the correct expression of MHCs on DCs surface.

Major histocompatibility complex (MHC)

Two types of MHC molecules exist: class I MHC (MHC-I) and class II MHC (MHC-II).

Endogenous antigens (obtained from tumour and virus-infected cells) are presented to CTLs following the loading on MHC-I molecules after a proteolitic process performed by proteasome, followed by transport into the lumen of endoplasmic reticulum (ER) (Townsend 1985, Heemels 1995, Lehner 1996); exogenous antigens (obtained from bacteria or free virus particle) are presented to $CD4^+$ cells following the loading on MHC-II molecules inside acidified endosomes after a proteolitic process performed by phagosome and intracellular vesicles (Cresswell 1994, Germain 1994, Watts 1997, Geuze 1998). MHCs are both polygenic (several MHC-I and -II genes

exist) and polymorphic (there are multiple alleles of each gene) (Klein 1993) and both consist of two polypeptide chains. In humans, the most important class I α chain genes are called HLA-A, -B and –C. MHC-I molecule has 4 domains, of which three are formed by α chain and one by β_2-microglobulin (not encoded within the MHC). There are also three major pairs of MHC-II α- and β-chain genes, called HLA-DR, -DP and –DQ, and in addition an extra β-chain in the DR-cluster.

MHC-I and MHC-II general structures and their interaction with TCR molecules are reported in Figure V.

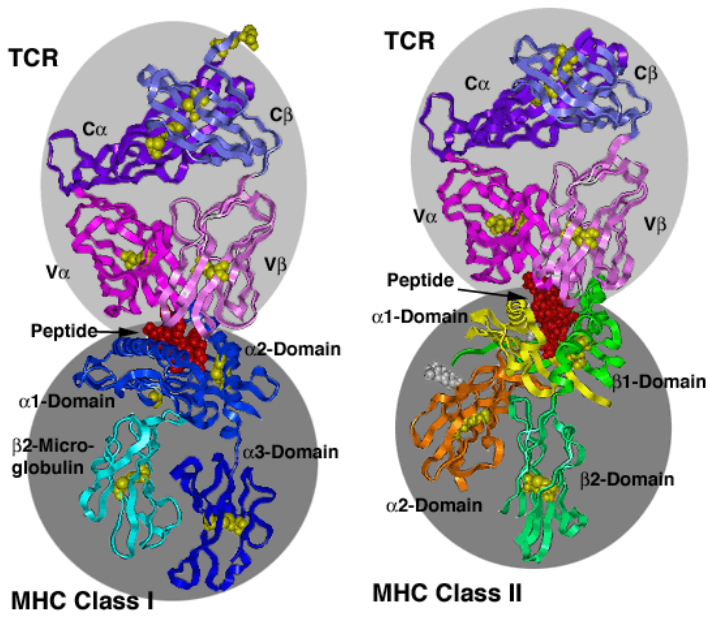


Figure V. MHC-I and –II structures and interaction with TCR*. MHCI is constituted by a α-chain associated with β_2-microglobulin. The α_1 and α_2 domains pair to form the peptide binding cleft. MHC-II is constituted by α and β chain each one composed by two domains: the α1-domani and the β1-domain form the site for peptide-binding.*

Children' immune system

Children immune system is not fully developed, making them more susceptible of infections. On the other hand the presence of the thymus (which disappears in adults) give them the possibility to obtain prompt immune reconstitution after eventual T-cell deletion, a characteristic that is to be considered when they undergo to various treatment or therapy. Before birth, children immune defences are mainly sustained by humoral arms transmitted from the mother via placenta and, in the first months of life in breast milk. This passive immunity is short-lived and is gradually substituted by longer-lasting immunity acquired following exposure to infection or through immunisation.

Immune system disorders and immunodeficiency

Despite the efficiency of immunological defence, failure of immune system may occur leading to a number of immunological disorders like lymphoproliferative disease, in the case of tumour, autoimmunity and hypersensitivity, when lymphocytes maturation is altered and responses are directed towards self-antigens and own tissues, and immunodeficiency, in the case of defective functioning of one or more immunological component.

As a consequence of immunodeficiency, responses to Ags are severely impaired or entirely absent so that patients become very vulnerable to opportunistic infections. It is possible to distinguish two types of immunodeficiencies: primary immunodeficiencies, which are congenital and have genetic origin, and secondary immunodeficiencies, which are acquired: an immune compromised status could be a consequence of chemotherapeutical drugs administration, and could be voluntarily induced in organ transplantation to avoid rejection. However, the most famous acquired immunodeficiency is the Acquired Immune Deficiencies Syndrome (AIDS) caused by HIV-infection.

Pathogenesis of HIV

HIV-1 is a complex retrovirus firstly identified in the early ’80 (Barrè-Sinoussi 1983, Gallo 1984, Levy 1984, Popovic 1984), which impairs immune system until to cause death of infected people in absence of therapy (Colebunders 1991). Its structure is schematized in Figure VI and its natural replication cycle is following described.

Natural replication cycle of HIV initiates with the entry of the virus in the host cell, and then by RNA release, retrotranscription and integration in the host genome (Brown 1990). After host T-cells activation, proviral transcription and RNA translation occur, leading to formation of new mature virions able to spread infection in the host (Felber 1989, Hadzopoulou-Cladaras 1989).

Infection results in a primary burst of viral replication (Albert, Gaines 1987) followed by immune adaptive response activation, which does not achieve complete virus eradication, but leads to a marked decrease of initial viraemia. After primary infection most patients enter into an asymptomatic phase which can last for months to more than 10 years (Lifson 1991). During this phase immune system is progressively deleted and individuals become subject for opportunistic infection, developing clinical manifestation of AIDS.

Either B, T and DCs compartments are impaired from HIV that is able to escape immune system through several mechanisms.

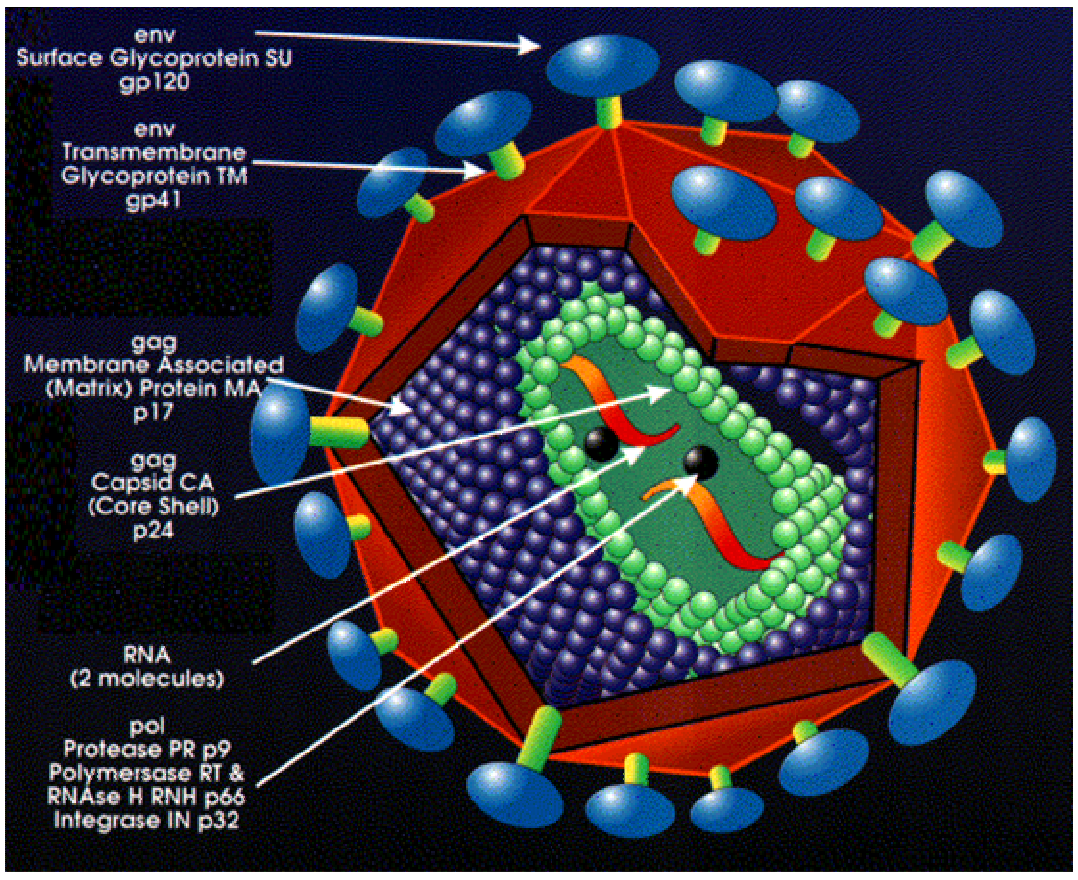


Figure VI. HIV-1 structure. *HIV is constituted by two copies of single-stranded RNA enclosed by a conical capsid comprising the viral protein p24. The single-strand RNA is tightly bound to enzymes that are indispensable for the development of the virion, such as reverse transcriptase and integrase. A matrix composed of an association of the viral protein p17 surrounds the capsid, ensuring the integrity of the virion particle. Also enclosed within the virion particle are the viral proteases. An external envelope is formed from host-cell membrane with host proteins and the viral glycoproteins gp120 associated with the transmembrane proteins gp41. p24 and p17 are encoded by gag gene; gp120 and gp41 are encoded by env gene.*

B-cell compartment

After primary infection an important defensive action is carried out by B-cells, which product antibodies predominantly raised against structural proteins released from destroyed infected cells and intact virions (Burton 1997, Poignard 1996). However, the genetic variability of HIV permits the virus to escape from the action of neutralizing antibodies. Indeed, as new variants arise, nAbs against those variants develop within three months, but by this time new viral variants resistant to neutralization by those antibodies has already arisen (Wey, Richman 2003). Other escape mechanisms from neutralizing antibodies include glycosylation, and steric and conformational block of receptor binding sites (Johnson 2002, Kwong 1998).

Furthermore, although during asymptomatic phase antibody titers remain elevated (Lang 1989), with disease progression B-cells become hyperactive (Vendrell 1991, Yarchoan 1986) causing hypergammaglobulinemia, increased expression of activation markers, and increased incidence of B-cell lymphomas (De Milito 2004). All these events lead B lymphocytes to undergo to apoptosis (De Milito 2001, Titanji 2003) causing a reduction in their number (Titanji 2006) and an impairment in the responsiveness to mitogens and antigens (Samuelsson 1997, De Milito 2004), specially in the memory subset (De Milito 2004). These alterations could be only partially recovered by current therapies (Titanji 2006).

T-cell compartment

HIV specifically targets T-lymphocytes expressing on their surface $CD4^+$ receptor (Klatzmann 1984) and during primary infection number of $CD4^+$ T-cells strongly, but transiently, decreases (Fauci 1996).

These events are followed by the activation of $CD4^+$ helper T-cells (Kalams 1999), which appear to be critical in containing viral replication through the release of the soluble mediator IL-2 (Kalams 1999), and the cytotoxic $CD8^+$ T-cells (Ariyoshi 1992, Koup 1994), which undergo a clonal expansion and release cytotoxic cytokines (IFN-γ) and granules (granzyme and perforin) (Berke 1994, Shresta 1998). These events lead to the control of viraemia and to the asymptomatic phase (Clark, Daar, Lifson 1991).

On the other hand, as in the case of B lymphocytes, the virus accumulates mutations during infection in order to escape from specific attack of CTLs that recognize specific viral epitopes (Walker 1991, Plata 1987, Riviere 1994). Furthermore, since HIV continuously triggers CTLs response, it causes the clonal exhaustion of specific TCRBV families and the progressive decrease in the $CD8^+$ number (Hoffenbach 1989).

During the course of disease, the persistent release of HIV-1 provokes the progressive disappearance of $CD4^+$ T-cells (Haase 1999), an increase in the frequency of activated cells undergoing apoptosis (Herbeuval JP 2005, Copeland 1996) and the lost of T-cell proliferative capability in response to recall antigens and mitogens (Schulick 1993). Furthermore a shift in T helper function from Th1 to Th2 has been observed (Clerici 1993, Graziosi 1994).

DCs compartment

Current data suggest that both myDCs and pDCs are susceptible of infection by HIV-1 (Smed-Sorensen 2005, Donaghy 2003, Cameron 1992, Patterson 1998, 2001, Macatonia 1990), although only 1-3% of the DCs population can be productively infected with HIV *in vitro* (Smed-Sorensen 2005) and HIV replication in DCs is 10- to 100-fold lower than in $CD4^+$ cells (Mcllroy 1995).
Virus entry may occur through lectins such as DC-specific intercellular adhesion molecule 3 (ICAM3)-grabbing non-integrin (DC-SIGN), langerin, mannose receptor (also known as CD206) and un unidentified trypsin-resistant C-type lectin (Geijtenbeek 2000, Turville 2001, 2002).
Two types of DC-mediated HIV transmission have been proposed (Wu 2006): *trans*-infection through formation of so called infectious synapse (Mc Donald 2003, Arrighi 2004, Turville 2004, Garcia 2005) or through HIV-associated exosomes (Wiley 2006), and *cis*-infection with *de novo* production and long-term transmission of the virus, after infection and replication in DCs (Nobile 2005, Burleigh 2006). DCs-mediated HIV transmission is schematized in Figure VII.
An important reservoir of HIV is also represented by FDCs of GC: they are not productively infected by HIV, but can trap and maintain large quantities of HIV early after infection (Heath 1995). In addition FDCs can promote the migration of resting $CD4^+$ cells to the germinal-centre microenvironment, favouring their infection (Heath 1995, Smith 2001).
The number of DCs decreases during the course of disease (Knight 1997, Pacanowsky 2001, Barron 2003), but it's still unclear if they are impaired in their functioning. Some studies indicated that DCs are able to stimulate a normal allogeneic T-cell response (Cameron 1992), or that they have impaired

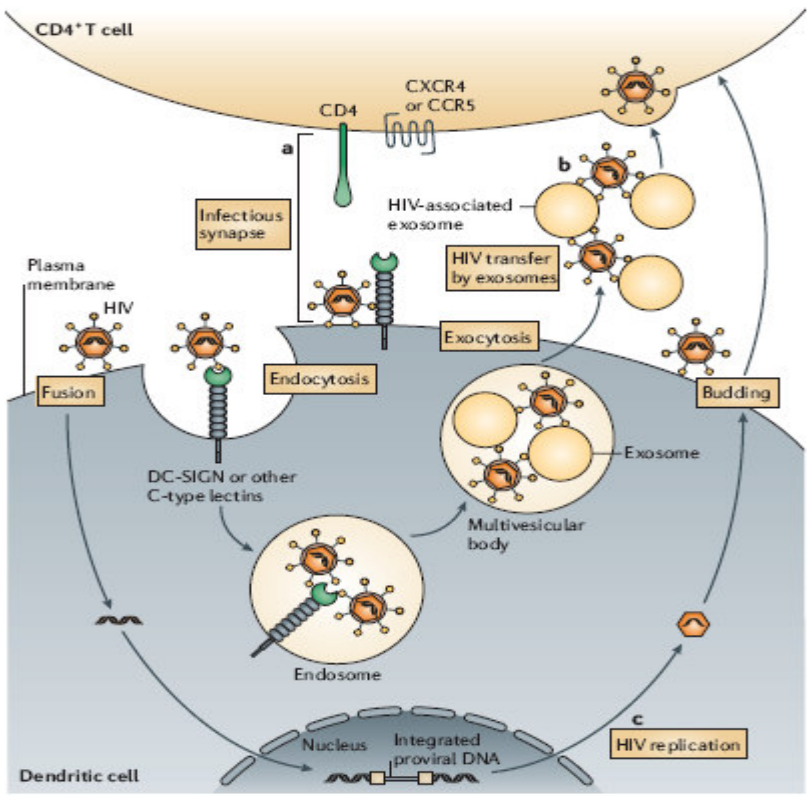


Figure VII. Mechanisms of dendritic-cell-mediated HIV transmission. a) *trans-infection with infectious synapse: DCs transfer captured HIV to target $CD4^+$ T-cells through a complex formed by cell-cell junctions involving DC-SIGN.* ***b)*** *Trans-infection with exocytotic pathway: endocytosed HIV can gain access to endosomal multivescicular bodies, enabling the release of HIV associated with exosomes: they are likely to be transmitted to $CD4^+$ T cells through membrane binding and fusion.* ***c)*** *Cis-infection: after initial exposure of HIV, HIV infection of DCs results in de novo production of viral particles. (Adapted from Nature Rev. Immunol. Wu 2006).*

function only at late-stage disease (Hsieh 2003), while others showed that both myDCs and pDCs are significantly less efficient at stimulating allogeneic T-cells (Donaghy 2003, Macatonia 1990), have a reduced maturation ability (Granelli-Piperno 2004) and a decreased expression of the co-stimulatory molecules CD80 and CD86 (Lore 2002).

Paediatric HIV-infection.

Paediatric HIV-1 infection is primarily due to mother-to-child virus transmission (vertical infection), which occurs during pregnancy or after delivery or during breast-feeding (Scarlatti 1996, Ehrnst 1991, Goedert 1991, Oxtoby 1988, Ziegler 1985). Influencing factors for transmission are immunological status of the mother (with inverted correlation between $CD4^+$ number and transmission risk) (Scarlatti 1991), the virus phenotype (Scarlatti 1993), and the presence of neutralizing antibodies in sera mother (Devash 1990, Goedert 1989, Rossi 1989). Caesarean section, the avoided breast-feeding and the use of antivirals during pregnancy reduce the risk for children to be infected (Eur. Coll. Study 1992, Mayaux 1997).

In the 25-30% of children heavy symptoms appear in the first year of life with immunodeficit and strong decrease of $CD4^+$ lymphocytes. In the remaining 70% disease course seems similar to the adults with symptoms variable and arising of similar opportunistic infections. 5-8% of these patients are asymptomatic for more than 7-8 years (Martin 1996).

It's possible that children with a rapid course of disease are infected during pregnancy, the others at the end of pregnancy or during delivery (Wilfert 1994).

Treatment

Positive results against HIV *in vitro* were firstly obtained by the use of an antiretroviral drug, the zidovudine (AZT) and subsequent clinical trials provided evidence that it could increase $CD4^+$ counts in AIDS patients. Then, other Nucleoside and Non-Nucleoside Reverse Transcriptase Inhibitors (NRTI

and NNRTI respectively) and Protease Inhibitors (PI) were developed.

In the following years became evident how the combined action of three or more antiretroviral drug led to strong positive effects in HIV-infected patients with also a reduction in the mutational load.

DRUG	CLASS	ACTION MECHANISM
Zidovudine or **Azidothymidine** (**ZDV** or **AZT**)	NRTI	AZT is a thymidine analogue
Stavudine (d4T)	NRTI	Stavudine is thymidine analogue.
Lamivudine (3TC)	NRTI	Lamivudine is a cytidine analogue.
Abacavir (ABC)	NRTI	Abacavir is a guanosine analogue
Didanosine (ddI)	NRTI	Didanosine (ddI) is an adenosine analogue.
Efavirenz (Efv)	NNRTI	Efavirenz binds to a Reverse Transcriptase pocket
Nevirapin (Nvr)	NNRTI	Nevirapin binds to a Reverse Transcriptase pocket
Ritonavir (RTV)	PI	Ritonavir inhibits a liver enzyme that normally metabolizes away protease inhibitors
Indinavir (IDV)	PI	Indinavir inhibits both HIV-1 and HIV-2 proteases
Nelfinavir (NFV)	PI	Nelfinavir inhibits both HIV-1 and HIV-2 proteases
Saquinavir (Sqv)	PI	Saquinavir inhibits both HIV-1 and HIV-2 proteases
Lopinavir (LPV)	PI	Lopinavir inhibits proteases binding plasma proteins

Tab I. Antiretrovirals. *NRTIs inhibit retrotranscription trough competitive binding to RNA. NNRTIs effect a direct inhibition towards Reverse transcriptase. PIs compete with viral protease for the same binding sites.*

Therefore Highly Active Antiretroviral Therapy (HAART), based on combination of two NRTI and one PI, was successful introduced.

Action mechanism of the main antiretroviral drugs are reported in Table I. Nowadays antiretroviral regimens are currently used: they permit to control viral replication and to increase $CD4^+$ counts leading to a general immune reconstitution of T-cell compartment (Chiappini 2006, Hainaut 2000, Romiti 2001)

Therefore, even if in B-cells and DCs positive effects are less evident and partial (Pastori 2002, Montefiori 2001, Titanji 2005, Gompels 1998, Pacanowsky 2001, Chehimi 2002, Barron 2003), introduction of HAART has strongly decreased morbidity and mortality in HIV-infection slowing down disease progression either in adult population (Berrey 2001, Palella 1998), either in paediatric patients (Romiti 2001, Resino 2002).

However, in paediatric HIV-infection a crucial point of discussion is represented by the initiation time of therapy, because there are no immunological markers that could suggest it (Abrams 2003).

Since the higher risk of disease progression and death in the first year of life, an early start of HAART was proposed (Dunn 2003, Faye 2004). On the other hand, some authors suggested that HAART applied from the first weeks of life, because of viral suppression, may reduce the availability of viral antigens for processing and presentation to the T-cell compartment, thus impairing the generation and the expansion of HIV-1 specific memory response during this period of relative immunological immaturity (Scott 2001, Spiegel 1999). Indeed humoral and cellular HIV-specific responses were not detected in several HIV-1 infected infants treated less than 3 months of age (Borkowsky 2004, Hainaut 2000, Luzuriaga 2000, Scott 2001, Spiegel 1999, Viganò 2006).

On the contrary a delayed initiation of HAART in the first year of life could favour the priming and the development of HIV-specific T-cell responses.

Other discussion points are related to the applicability of alternative regimens to avoid the numerous side effects of HAART.

Indeed, besides the positive effects of HAART, this therapy may lead to several serious adverse events, including diarrhoea, hepatitis, hypercholesterolemia, encephalomyelitis, neutropenia, pancreatitis (Carr, Wanke 1999). Furthermore poor compliance augment risk of viral failure and it's also possible that the virus develop antiviral resistance due to rapid genomic mutations. These limitations impact negatively on the patient' quality of life and are particularly worrisome in children, given their long-term expectative of life (McComsey 2004, Arpadi 2001, Jaquet 2000, Babl 1999, Amaya 2002).

As a consequence, in the last years, several HAART simplified regimens were developed (Martinez, Raffi 2000, Negredo 2002) either for adults, either for children.

In the last years, a number of studies are also focused on alternative treatments, including cellular therapies, in order to definitively substitute HAART regimens and to obviate to the most important restriction of HAART: indeed current antiretroviral regimens are not able to eradicate definitively HIV that could persist in latent state inside the host genome (Finzi 1999, Siliciano 2003) thereby making not possible any treatment interruption. In this context of particular relevance are the recent studies focused on DCs manipulation and pulsing to induce a strong $CD8^+$ HIV-specific cytotoxic response maintaining at the same time the control of viral replication and the number of $CD4^+$ lymphocytes (Andrieu 2007), in view of future efficient vaccine development.

Haematological disorders and transplantation: an other case of acquired immunodeficiency

A typical situation of immune system impairment is a consequence of haematological disorders like as Leukaemia, in which too many abnormal and not functional lymphocytes are produced. Such diseases are treated with chemotherapeutical drugs that kill all leucocytes, leading to a strong impairment of the host immune compartments, and to a high susceptibility to viruses and other pathogens.

Haematopoietic stem cell transplantation (HSCT) is used to favour immune reconstitution of these patients (Sullivan 2000) or for patients with metabolic diseases, autoimmune pathologies (Ikehara 1998) or HIV-associated lymphomas (De Paoli 2006).

Haematopoietic stem cells (HSCs) are pluripotent T-cells that originate since the embryo development and are continuously produced in BM in the entire course of life in physiological conditions (Peault 2003). Bone marrow transplantation (BMT) is the most common type of transplantation, but other sources of HSCs are the Peripheral Blood (PB) and the Cord Blood (CB).

Before transplant, BM could be *in vitro* treated to eliminate T-cells, to avoid the Graft Versus Host Disease (GVHD), or mature erythrocytes, in the case of erithrocytic incompatibility between donor and recipient. BM is infused in a peripheral vein of the host, and the HSCs spontaneously reach the host BM, through the homing process, involving adhesion molecules like as the ICAM (Lee 2004).

Transplantation could be autologous if HSCs used are that of the own patients taken before chemotherapy and re-administered after treatment. The reduction of GVHD and the elimination of the rejection risk are the most advantages of

this procedure, but there is an elevated risk to re-administer cancerous cells to the patients.

An ideal compatibility condition is represented by the singeneic transplantation: in this case the donor is recipient homozygous twin, but the risk is related to possibility that donor cells lead the same mutations favouring the disease development.

Allogeneic transplantation is the most applied: in this case transplanted cells become from an other person that could be related (if he comes from the same family), or not related (if he is a voluntary donor). Familiar donor could be genotypically identical if he has the same HLA alleles of the donor, or aploidentical if donor and recipient have identical only one out of two chromosomes containing genes coding for HLA. If the donor is not a familiar, transplantation could occur only if donor and recipient are fenotypically identical: therefore is necessary to find HLA-compatible donors through the HLA-typing.

Allogeneic transplantation major risks are correlated to rejection and, especially in children, to GVHD in which donor cells attack host immune system (Kondo 2001). To avoid GVHD before and after transplantation immunosuppressive drugs such as Cyclosporine A (CSA), alone or in association with other drugs, are included in conditioning regimens generally consisting in total body irradiation (TBI), in association with cyclophosphamide (CTX) or other chemotherapeutical agents used to create a place for the donors HSCs, to suppress recipient immune system, and to kill cancerous cells.

Since of the conditioning regimen and GVHD prophylaxis, after transplantation, a number of patients show complications related to the duration of immune-compromised status. Recovery of polymorphonuclear and

dendritic cells is relatively rapid, occurs within weeks and could be favoured by the administration of Granulocyte Colony Stimulating Factor (G-CSF) (Iori 2004). In contrast T lymphocytes reconstitute themselves less rapidly and often present a reduced functionality: functional numbers of T-cells might appear 3 to 4 months after transplantation and full recovery might take 6 to 12 months (Crooks et al 2006). Therefore is necessary to follow carefully patients especially within the two years after transplantation: during this time there is an elevated risk to develop viral and fungal infections, associated with defective cell-mediated immunity, or late bacterial infections, as a result of defective antibody production, respectively treated with antiviral and antifungal drugs, and antibiotics (Crooks 2006). Immunoglobulins are somministered in the case of ipogammaglobulinemy (AIEOP 2003).

Haematopoietic reconstitution is evaluated by emocromocytometric tests. The presence of genetically different cells, the so named chimerism, is evaluated by cytogenetic test like Fluorescence in situ hybridization (FISH) and molecular analyses like Polymerase Chain Reaction (PCR).

Engraftment failure occurs if after 90 days from transplant there is not autologous haematopoietic reconstitution (Iori 2004).

Umbilical cord blood transplantation (UCBT)

Recently UCBT, particularly from unrelated donors, has progressively become a widely employed treatment for patients with both malignant and non malignant haematological disorders (Wagner 1995, Locatelli 1999, Barker 2003).

Knudtzon et al. firstly observed the presence of stem cells within the UCB. (Knudtzon et al 1974). In the ‘80th UCB was proposed as alternative source of

HSCs after mieloablative therapy (Broxmeyer 1982) and, in 1988 the first UCBT was carried out (Gluckman 1989).

UCBT offers several advantages including simplicity to be obtained, rapid availability of biologic product, absence of risk for donors, reduced likelihood of transmitting infections, less stringent criteria for HLA matching for donor/recipient selection, and, overall, reduced incidence and severity of GVHD (Barker 2003, Madrigal 1997). Indeed T-cells are in immaturity state and seem functionally and phenotipically naïve (CD45 $RA^{+}RO^{-}$). Furthermore only a small fraction of them show the expression of activation marker HLA-DR and $CD25^{+}$ (D'Arena 1998), and it is demonstrated that they have a low T-cell mediated cytotoxic capacity, having a higher threshold for IL-2, IFN-γ and TNF-α cytokine stimulation, low reactivity to mitogens as Phytohaemaglutinin (PHA), and reduced ability to generate CTLs in Mixed Leucocyte Reaction (MLR) (Quian 1997, Harris 1992). Finally they show a widely polyclonal TCR (Garderet 1998, Moretta 1999).

Besides advantages, UCBT present also some disadvantages: indeed contains low HSCs number in comparison to BM, a characteristic that, accompanied by immaturity state of cells, could hamper the complete immune recovery and cause a delay in the reconstitution (Gluckman 2000, Thomson 2000, Barker 2001, Moretta 2001, Rocha 2001, Dalle 2004). On the other hand, the stem cells immaturity is a factor that could be determinant in the obtaining of a long-term immune-haematological reconstitution, given the stronger proliferative ability of these cells.

Regarding humoral immunity, it has been shown that B-cells progenitors in UCB are more than in BM (Locatelli 1996, Hoyt 1999), and B subpopulation reconstitute earlier as also Ig sera levels. Also NK show a rapid reconstitution and a reduced cytotoxicity (Moretta 1999, Thomson 2000).

Previous studies on immune reconstitution after UCBT showed prompt and efficient proliferative response to mitogens and recovery of NK functions (Locatelli 1996, Giraud 2000, Thompson 2000, Moretta 2001), while the only few data available on TCR repertoire reconstitution after unrelated UCBT showed a complete TCR repertoire normalization only after two or more years from transplantation (Klein 2001, Talvensaari 2002).

Immune reconstitution analysis

In the following paragraphs a general overview on the main techniques used to analyse patient' immune reconstitution will be presented, focusing attention on TCRBV spectratyping, that has been largely used for experiments of this thesis.

Cytofluorimetric analysis

In order to verify cell phenotypes and/or to establish cell counts of the various immunological compartments is very useful the cytofluorimetric analysis, through the use of monoclonal antibodies able to specifically bind differentiation, maturation and activation markers, expressed from the various cellular subpopulation.

TCRBV repertoire analysis - Spectratyping

In the context of T-cell immune reconstitution evaluation, one of the most important analysis tools is given by TCRBV repertoire analysis (Roux et al 1996).

Cytofluorimetric assays could be performed to quantify the expression of the various TCRBV families. Alternative molecular analyses are based on Reverse Transcriptase-PCR (RT-PCR) following by cloning and sequencing of TCRBV families, to obtain repertoire clonotype information (Rosemberg 1992, Choi 1989).

But the most useful technique is the spectratyping, which provides information on TCR diversity at the level of CDR3 length of Vβ chain, considering that length heterogeneity is representative of overall sequence heterogeneity.

TCR analyses are performed on β chain because it is the only one that is subjected to allelic exclusion.

The experimental procedure is briefly schematized in Figure VIII (for major details see materials and methods section).

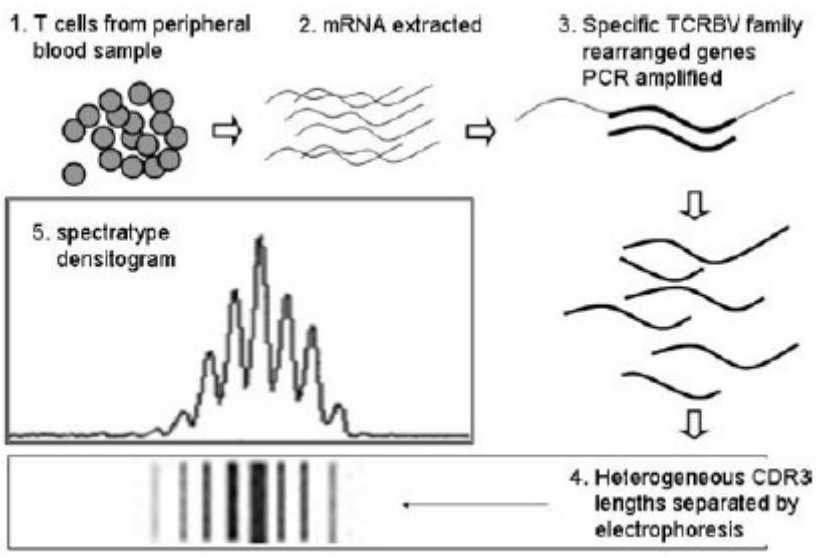


Figure VIII. Spectratyping experimental procedure. *mRNA is extracted from T-cells (eventually separated into $CD4^+$ and $CD8^+$ cellular subset) and subsequently reverse transcribed and amplified in reverse transcriptase PCR (RT-PCR) using 24 Vβ primers and one Cβ primer. PCR products are heterogeneous CDR3 lengths which are size-separated by electrophoresis. Finally, the quantity of material deposited in each band is quantified by densitometry and the spectratype trace is produced. (Adapted from Kepler, Bioinformatics 2005).*

Although there is consistency in the molecular method used to generate TCRBV spectratype data, a wide range of analysis methods exists to describe and quantify TCR diversity. One of the least sensitive, but more commonly used, methods is Visual Scoring, which characterizes TCRBV diversity as oligoclonal or polyclonal (Ferrand 2000, Bour 1999, Kook 2002).

To quantify the complexity of TCR repertoire, Bomberger et al (1998) developed a method in which all peaks greater than 10% of the total peak area are enumerated: distribution is considered normal if there are more than 6 peaks. Bomberger introduced also the Diversity Score, which value is given first calculating the ratio between the sum of the heights of the major peaks to the sum of all peak heights and then dividing this ratio by the number of major peaks present. An overall Complexity Score is given by the sum of all Vβ complexity numbers calculated (Bomberger 1998).

Others have developed various peak area calculation including peak counts for peaks greater than a determined percentage of the maximal peak area relative mean fluorescence divided by the peak area (Dumont-Girard 1998) and peak scoring (Lu 2004, Wu 2000).

One of the most used quantitative methods is that of Gorochov (1998): for this analysis peak areas in each Vβ are compared against the distribution of a normal standard determined by averaging values obtained from $CD4^+$ T-cell component of five normal tissues. The differences between the experimental peak areas and the normal values within each Vβ are averaged and reported as Gorochov values.

Alternative quantitative methods of analysis include the Diversity System that compares experimental values against the distribution profile of cord blood (Peggs 2003) and the system of Pilch who integrates data of genetic diversity with cytofluorimetric measures of Vβ surface expression (Pilch 2002).

Recently various softwares are being developed to facilitate a standardized analysis of TCRBV repertoire data: Immunoscope TM automatically calculates peak area and the Gorochov value (Collette et al 2001, 2003); TC Landscape TM integrates data measuring genetic diversity with data measuring Vβ surface expression (Pilch et al 2002); SPA (He 2005, Kepler 2005) and REPERTOIRE (Long et al. 2006) compare peak areas to normal peak areas which were based upon a theoretical normal Gaussian peak area. By adding the resulting peak amplitude disparities between the sample and the standard peak areas, an individual P value is generated for each Vβ family, and an overall P value is generated by averaging all analysed individual Vβ subfamilies giving equal weight to each analysed Vβ in a sample.

T-cell receptor excision circles (TRECs) analysis

Quantification of T cell receptor excision circle (TRECs) is an other molecular technique that gives information on the thymic output (Kong 1999, Douek 1998, 2000). TRECs are generated during genes rearrangement of V(D)J segments of TCR: they are little circles of excise DNA, which is not integrated into genome. TRECs are stables and do not undergo to cell division, so that are diluted in every cellular division (McFarland 2000). TRECs levels have been shown to correlate with recent thymic emigration and thus thymopoiesis (Douek 1998, Al-Harthi 2000), although also cellular proliferation and apoptosis influence TRECs levels, complicating data interpretation (Ye 2002, Berzins 2002, Hazenberg 2003).

T-cell proliferation

To evaluate T lymphocytes functionality and their ability to respond to common mitogens and common or specific Ags, lymphoproliferative tests are very useful (Romiti 2001), and to evaluate the efficiency of stimulators, MLRs test could be performed using purified DCs versus T-cells

Cause of the low abundance of DCs *in vivo* (Rissoan 1999), Monocyte-derived Dendritic Cells (MDDCs) are commonly used to model the immunological function of DCs (Steinman R.M. 2003, Romani 1994). $CD14^+$ monocytes from human peripheral blood differentiate into immature DCs after 4-6 days in culture in the presence of IL-4 and granulocyte/macrophage colony-stimulating factor (GM-CSF). These immature DCs have similar characteristics to myeloid DCs and can be converted into mature MDDCs by exposure to various stimuli, including lipopolysaccharide (LPS), Interferon-γ (IFN-γ), tumour-necrosis factor (TNF) and CD40 ligand (CD40L) (Banchereau 2000, 1998).

In the last years the use of MDDCs knew a great expansion, because DCs could be also used for pulsing experiments (Lu et al 2004), in view of cellular therapy, with the finality to prime more potent immunological responses in immune-compromised patients.

AIM OF THE STUDY

Aim of this study was to evaluate the immune reconstitution in different cohorts of paediatric patients with acquired immunodeficiencies.

In particular we studied a cohort of 9 HIV-infected children who started HAART after 3 months of age to analyse if a delayed initiation of therapy, could favour the immune recovery in these children.

Then we investigated in a larger cohort of 19 HIV-1 vertically infected children (9 of which from the precedent study) if the switching from a PI-based HAART to a PI-sparing regimen with 3 NRTI could maintain immune reconstitution obtained with previous HAART.

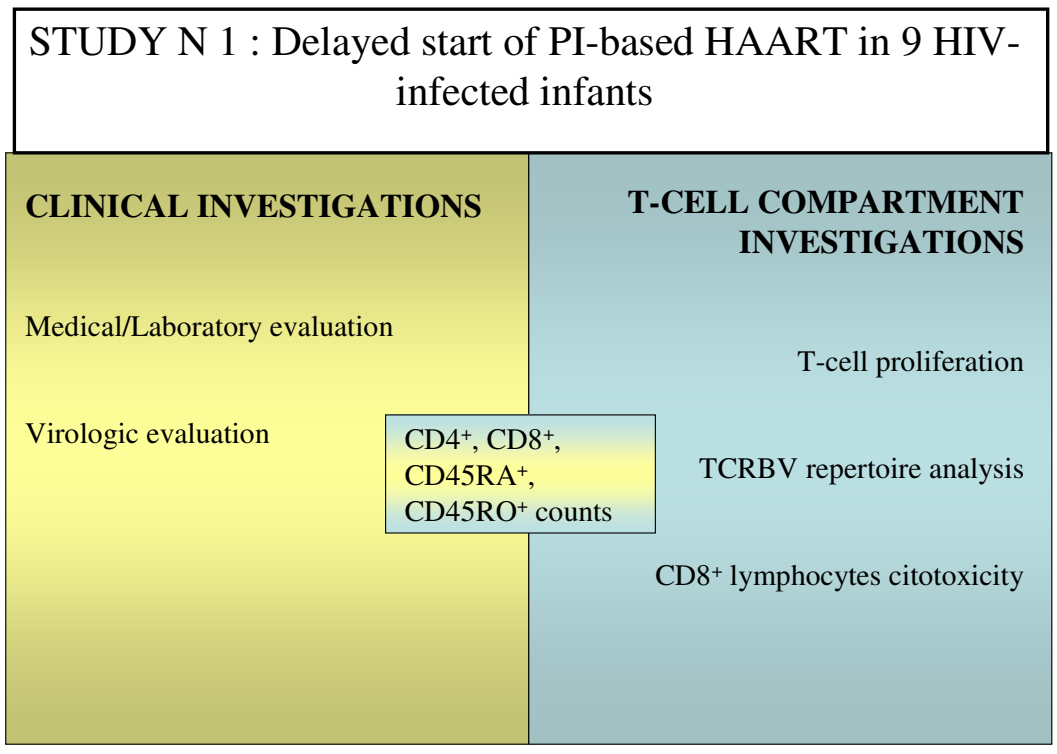
Finally immune reconstitution was evaluated in a cohort of 6 leukaemia children who underwent to cord blood transplantation.

DESIGN

Immune reconstitution in acquired immunodeficiency children was investigated following 3 main research branches based on the scheme below:

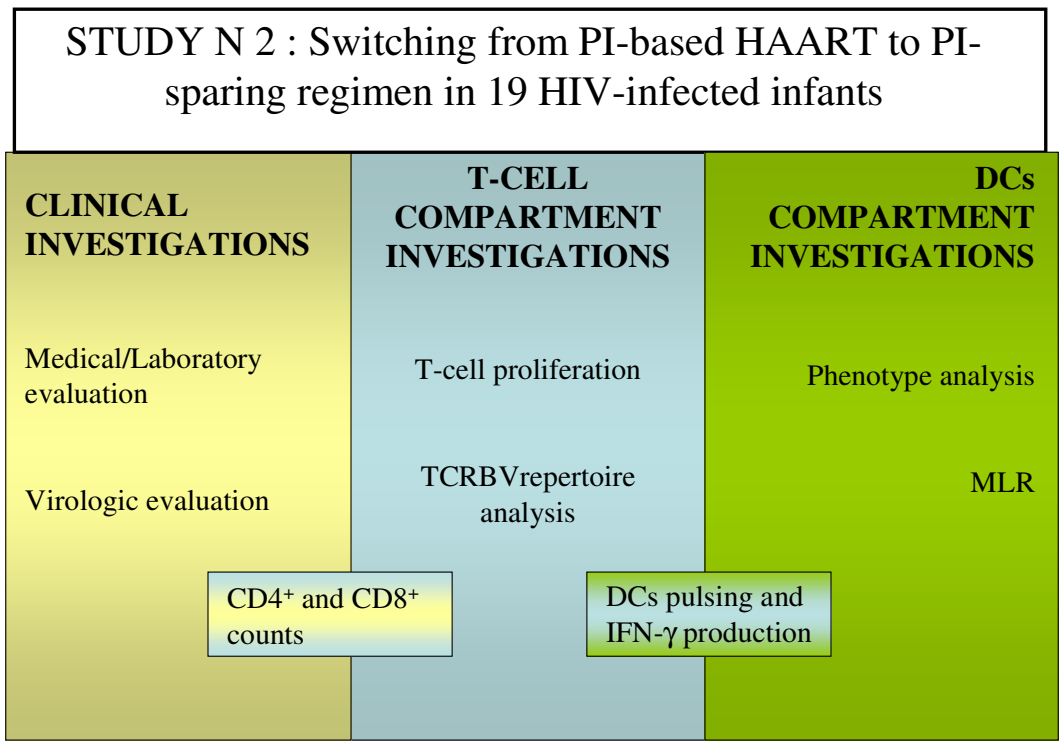
1) Study on delayed (after 3rd month of life) start of PI-based HAART in 9 HIV-infected infants (Follow up: 47, 9 ± 18, 5 months).

   - Clinical investigations: routine medical/laboratory analyses, virologic evaluation.
   - T-cell compartment investigations: $CD4^+$, $CD8^+$, $CD45RA^+$ and $CD45RO^+$ cell counts, T-cell proliferation, TCRBV repertoire, $CD8^+$ citotoxicity.



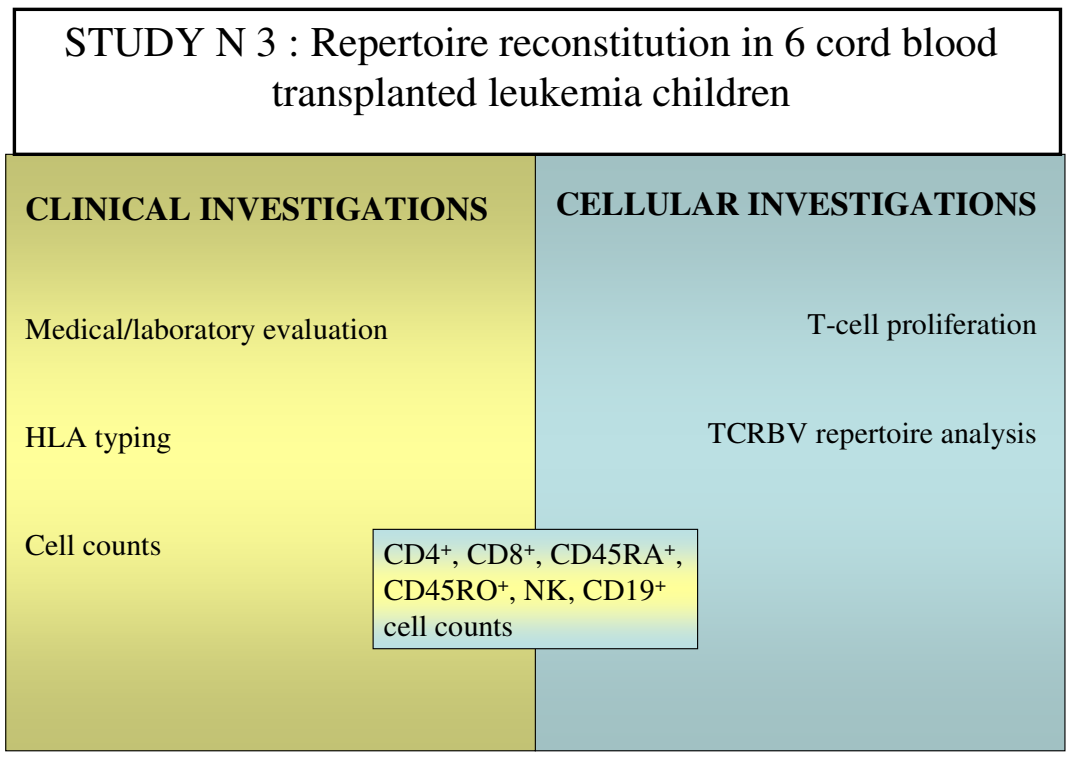
2) Study on switching from PI-based HAART to 3NRTI regimen in 19 HIV-infected children (Follow up: 96 weeks).

- Clinical investigations: routine medical/laboratory analyses, virologic evaluation
- T-cell compartment investigations: $CD4^+$ and $CD8^+$ counts, T-cell proliferation, TCRBV repertoire analysis.
- Dendritic cell compartment investigations: Phenotype analyses, MLR, DC-pulsing and $CD8^+$ cytotoxicity stimulation (analyses were performed on MDDCs).



3) Study on cord blood transplantation in leukaemia children.

- Clinical investigations: routine medical/laboratory analyses, virologic evaluation.
- Cellular investigations: $CD4^+$, $CD8^+$, $CD45RA^+$, $CD45RO^+$, NK, $CD19^+$ cell counts T-cell proliferation, TCRBV repertoire analysis.



MATERIALS & METHODS

Patients, therapies and clinical investigations

Children were enrolled followings specific criteria in each study. In all cases the institutional review ethical board approved the study and written informed consent was obtained from the patients' parents or legal guardian.
Periodical controls consisting in complete medical physical examinations and routine laboratory tests including $CD4^+$ and $CD8^+$ T-cell count were performed during follow up in all patients

Study 1

Nine HIV-vertically infected children (pA-pI), who started PI-based HAART between 3 and 8 months of age, were enrolled in the study. All infants were diagnosed as HIV-1 infected on the basis of at least two positive results for the HIV-1 DNA PCR and one viral isolation.
Infants underwent HAART with two or three NRTI plus NFV as a PI or, for the last two patients enrolled under recent guidelines, (Sharland et al 2004) LPV/RTV. Children were followed at Paediatric Hospital "Bambino Gesù", in Rome.
Individual patient characteristics and antiretroviral regimens at baseline are outlined in Table 1.1

Patient	Neonatal prophylaxis	Age at initiation of HAART (Months)	Antiretroviral treatment
Pa	AZT x 42 days	3.1	ABC+3TC+LPV/RTV
Pb	None	3.2	AZT+3TC+NFV
Pc	None	3.3	3TC+d4T+NFV
Pd	AZT x 42 days	3.3	AZT+3TC+LPV/RTV
Pe	None	4.2	3TC+d4T+NFV
Pf	None	5.9	AZT+3TC+NFV
Pg	None	6.6	ABC+AZT+3TC+NFV
Ph	None	7.0	3TC+d4T+NFV
Pi	None	7.6	3TC+d4T+NFV

Table 1.1. Individual patient treatments adopted. *AZT: azidovudine; ABC: Abacavir, 3TC: lamivudine; LPV: Lopinavir; RTV: Ritonavir; d4T: Stavudine.*

Study 2

Nineteen HIV vertically infected children (p1-p19), previous treated with a successful stable PI-based HAART for at least 12 months were enrolled in the study. These patients were from 2 to 18 years old, had normal value of $CD4^+$% for age before study entry, and did not have previous NNRTI therapy neither history of virological failure with others regimens. At study entry PI-based HAART was switched to a PI-sparing simplified protocol in which the PI was substituted with a third NRTI. Years of precedent PI-based HAART and drugs combination before and after simplification are summed for each patients in Table 2.1.

Of these children, four were the same of the precedent study: corresponding number are the sequent: pC=p15; pF=p17; pG=p18; pI=p16

Patients	Years on PI-based HAART	PI-based HAART	3 NRTI HAART
P1	4.5	3TC + AZT + NFV	3TC + AZT + ABC
P2	5.0	3TC + AZT + RTV	3TC + AZT + ABC
P3	4.7	3TC + AZT + NFV	3TC + AZT + ABC
P4	4.1	AZT + ABC + NFV	AZT + ABC + 3TC
P5	4.6	3TC + d4T + NFV	3TC + d4T + ABC
P6	4.6	AZT + ABC + NFV	AZT + ABC + 3TC
P7	4.6	3TC + d4T + NFV	3TC + d4T + ABC
P8	4.4	AZT + ABC + NFV	AZT + ABC + 3TC
P9	2.7	AZT + 3TC + IND	AZT + 3TC + ABC
P10	4.7	AZT + ABC + NFV	AZT + ABC + 3TC
P11	2.6	3TC + d4T + LPV/RTV	3TC + d4T + ddI
P12	3.5	AZT + ddI + NFV	AZT + ddI + 3TC
P13	4.4	3TC + AZT + NFV	3TC + AZT + ABC
P14	4.6	3TC + AZT + NFV	3TC + AZT + ABC
P15 (=PC)	4.9	3TC + ABC+ NFV	3TC + ABC+ AZT
P16 (=PI)	4.5	3TC + d4T + NFV	3TC + d4T + ABC
P17 (=PF)	2.1	3TC + AZT + NFV	3TC + AZT + ABC
P18 (=PG)	2.0	3TC + ABC + AZT + NFV	3TC + ABC + AZT
P19	4.2	AZT + ABC + NFV	AZT + ABC + 3TC

Table 2.1. Individual patient treatments adopted (previous PI-based HAART and PI-sparing regimens). *ddi: Didanosine.*

Study 3

Six children who underwent UCBT from unrelated donors with a 24 months observation time were enrolled in the study. Children were transplanted at the Haematology and Bone Marrow Transplantation Unit of Paediatric Hospital Bambino Gesù (Rome, Italy) since November 2000. Cord blood units for unrelated transplants were provided by the cord blood banks in Milan, Padua, and Dusseldorf.

Conditioning regimen consisted in chemotherapy in 2 patients and chemotherapy with TBI in 4 patients. All patients received horse antilymphocytes globulin (Lymphoglobuline, Sangstat, Lyon, France) during the conditioning regimen. GVHD prophylaxis included a combination of cyclosporine A (CSA) and steroids. Patient n° 1 intolerant of CSA received tacrolimus (FK506). Recombinant human G-CSF was administered after transplantation to all patients to accelerate myeloid recovery. No routine intravenous immunoglobulin (IVIG) was used unless serum Immunoglobulin G (IgG) level was less than 400 mg/dL and the patients had developed an infectious complication. Only 1 of the patients received IVIG for low level of IgG and concurrent infections. Patients' characteristics and treatments are summarized in Table 3.1

Pat	Age at transplant	Diagnosis	Conditioning	GVHD prophylaxis	Current status
P1	13	ALL	TBI/CY/VP16/ATG	CSA/FK506	Alive
P2	6	ALL	TBI/CY/VP16/ATG	CSA/PDN	Alive
P3	5	ALL	TBI/CY/VP16/ATG	CSA/PDN	Alive
P4	4	ALL	BU/CY/ATG	CSA/PDN	Alive
P5	1	AML	BU/CY/ATG	CSA/PDN	Alive
P6	14	ALL	BU/CY/ATG	CSA/PDN	Death

Tab 3.1. Individual patients conditioning regimen adopted. *ALL: acute lymphoblastic leukaemia; AML: acute myeloblastic leukaemia; TBI: Total body irradiation; CY: cyclophosphamide; VP16: etoposide; ATG: antithymocyte globulins; BU: busulfan; CSA: Cyclosporine A; FK505: tacrolimus; PDN: prednisolone.*

HLA typing and chimerism (Study 2)

HLA typing was performed by serology for HLA-A and B, and by high-resolution molecular typing for HLA-DRB1.

Chimerism was analysed by FISH for sex chromosomes, or restriction analysis fragment length polymorphisms analysis on peripheral blood leukocytes and mononuclear cells.

Plasma HIV-RNA determination (Study 1 and 2)

Plasma RNA was determined using a quantitative b-DNA assay (Quantiplex HIV-RNA 2.0 bDNA Assay, Chiron Diagnostic Corporation, Emerville, CA, USA) with a detection limit of 50 copies/ml of plasma viral load: values yielding more than 500.000 copies were confirmed by quantitative RT-PCR assay (Roche Amplicor, Roche Molecular Systems, Branchburg, NJ, USA). Viral values between 50 and 999 copies/mL, preceded and followed by measurements less than 50 copies/mL were defined as “blips” (Nettles 2005). Rate of blips was evaluated as the ratio between the number of detected blips and the number of determinations:

$$\text{Rate} = \frac{\text{n. of blips}}{\text{n. of determinations}}$$

Flow cytometry

Flow cytometric analyses were performed on PBMC and on MDDCs according to standard protocols with a FACScan flow cytometer (Becton

Dickinson, San Jose, CA, U.S.A.) using specific monoclonal antibodies Fluorescein isothiocyanate (FITC), R-Phycoerythrin (PE), Allophycocyanin-Cy5 (APC-Cy5) conjugated. CD3+, $CD4^+$, $CD8^+$ CD45RO and CD45RA, $CD16^+$, CD56+ $CD19^+$ CD1a, CD14, DC-SIGN, CD40, CD80, CD83, CD86, HLA-DR, and HLA I markers were studied in the various immune compartments (Pharmingen, San Diego CA, U.S.A.). Fluorescence was analysed using Cellquest software.

T-cell proliferation assay

Purified suspensions of PBMC were obtained by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. $2x10^5$ cells were triple cultured in microtiter plates for 3 and 6 days with mitogens and antigens respectively at 37° C with CO_2 0,5% in complete culture medium consisting of RPMI 1640 with L-glutamine (Sigma, St. Louis, MO) supplemented with 10% AB Human serum (Sigma), 50 IU/ml penicillin and 50 µg/ml of streptomycin (Sigma).

Mitogenic stimuli used were phytoaemmaglutinin (PHA) 5µg/ml, pokeweed mitogen (PWM) 0.35 µg/ml (Sigma) and OKT3 3µl (stock supernatant obtained from OKT3 cell line, ATCC-Sigma, MI, Italy). Common antigens used were antigen solutions of Candida Albicans, Cytomegalovirus (CMV) and Herpes virus 1 (HSV-1) 15 µl/well (AID GmbH-Strasberg-Germany). HIV-1 specific antigens used were p24 (0.1 µg/ml) (Intracel, London, UK), gp120 (0.15 µg/ml), gp41 (0.05 µg/ml) and p17 (0.08 µg/ml) (NIBSC, Blanche Lane, UK).

PBMC from the same samples were cultured for 3 and 6 days without any stimulus as negative controls in complete culture medium. To analyse T-cell proliferation, all the cultures were then pulsed with 0,5 μCi ^{3}H-thymidine for additional 6 hours and harvested onto glass fibre filters (Packard, MI, Italy) and analysed by B-counter scintillator (Canberra Packard Instrument Company, Meriden, CT, and U.S.A.). Data were expressed as count per minute (cpm) and elaborated as stimulation index (SI), defined as the ratio between the average counts of stimulated and unstimulated PBMC.

$$SI = \frac{\text{proliferation of PBMC with mitogen or antigen}}{\text{Spontaneous proliferation of PBMC}}$$

Lymphoproliferations towards mitogens were considered positive in relation to the sequent scheme:

PHA > 35.000 cpm

PWM > 18.000 cpm

OKT3 > 25.000 cpm

Lymphoproliferations towards common antigens (Tetanus, Candida) were considered positive if cpm > 5.000

Finally lymphoproliferations towards HIV1-specific antigens (p24, gp120, gp41 and p17) were considered positive if SI was > 3

TCR Spectratyping

Purified suspensions of PBMC were obtained from patients by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. PBMC were fractionated into $CD4^{+}$ and $CD8^{+}$ subsets by the use of anti-$CD4^{+}$ or anti-$CD8^{+}$

monoclonal antibody-coupled magnetic beads (Dynal AS, OSLO, Norway) following manufacturer's instructions. Total RNA was extracted by TRIzol® (Gibco-BRL/Life Technologies) in accordance with the manufacturer's instructions. 0.3 µl of RNA were used for transformation into cDNA and amplification by PCR for each one of 24 5'Vβ primers coupled with one 3'Cβ (y5 labelled in 5') (Maslanka 1995) both 10 µM, by a Superscript™ One-Step RT-PCR with PLATINUM Taq kit (Invitrogen, Mi, Italy) in 25 µl total volume, using 0.3µl RT/Platinum® Taq Mix according to mildly modified standard protocol. The temperature profile used in a GeneAmp 9600 DNA thermal cycler (Perkin Elmer Cetus) included the following steps: a 50° C cycle for 20'(cDNA synthesis) followed by "denaturation" at 94° C 45 s, "annealing" at 55 ° C 45 s, "extension" at 72 ° C 45 s, followed by a "final extension" at 72 ° C 10 min.

Sequences of primers used were the followings:

Vβ1: CAGTTCCCTGACTTGCACTC

Vβ2: GCTTCTACATCTGCAGGTGC

Vβ3: GAGAGAAGAAGGAGCGCTTC

Vβ4: GCAGCATATATCTCTGCAGC

Vβ5.1: CTCGGCCCTTTATCTTTGCG

Vβ5.3: CCCTAACTATAGCTCGAGC

Vβ6.1: GATCCAGCGCACACAGC

Vβ6.2: GATCCAGCGCACAGAGC

Vβ7: CCTGAATGCCCCAACAGC

Vβ8: GAACCCAGGGACTCAGCTG

Vβ9: GGAGCTTGGTGACTCTGCTG

Vβ11: CAGGCCCTCACATACCTCTCA

Vβ12: CAAAGACAGAGGATTTCCTCC

Vβ13: GTCGGCTGCTCCCTCCC

Vβ14: GTCTCTCGAAAAGAGAAGAGG

Vβ15: GTCTCTCGACAGGCACAGGC

Vβ16: GAACTGGAGGATTCTGGAGTT

Vβ17: CCAAAAGAACCCGACAGCTTTC

Vβ18: GTGCGAGGAGATTCGGCAGC

Vβ20: CCTCCTCAGTGACTCTGGC

Vβ21: GGCTCAAAGGAGTAGACTCC

Vβ22: GTTGAAAGGCCTGATGGATC

Vβ23: CAGTTCAGTGACTATCATTCTG

Vβ24: GGGGACGCAGCCATGTACC

1,5 µl of PCR products were diluted in a stop buffer (deionised formamide and TE pH 7.5) and denatured at 95° C for 4 min. 9 µl of each sample were loaded in wells of a 6% polyacrylamide gel (Pharmacia Ready Mix Gel) and run in electrophoresis in a DNA Automatic Fluorescence Sequencer (Pharmacia ALF DNA Sequencer, Sweden), and finally analysed by specific software (Pharmacia DNA Fragment Manager 2.0).

Data interpretation was done following the visual scoring method (Ferrand 2000, Bour 1999, Kook 2002). Based on this method three main pattern profiles of distribution were identified: Polyclonal/Gaussian profiles (p) with 5-9 peaks per TCRBV family and a Gaussian “bell shaped distribution”; Polyclonal altered profiles (pa) with 5 or more peaks with a non Gaussian-like distribution pattern; skewed/perturbed profiles (sk) with a number of peak comprised in a range from 1 to 4 peaks or with a multipeak pattern having one solitary peak >50% of the total peak area or one or more deleted peaks.

Polyclonal and Polyclonal altered profiles were considered normal. In studies 1 and 2 polyclonal altered profiles were considered as p, while in study 3 they were reported as “pa”. Skewed profiles (sk) were considered pathogenic.

Cloning and sequencing of a monoclonal spectratyping profile (Study 1)

In one patient, spectratyping analysis showed a persistent oligoclonal pattern of TCRBV9 family in the $CD8^+$ subset. Specific PCR product was cloned in pT7Blue-vector (kit Novagen, Darmstadt, Germany). About 15-20 clones for each family were amplified with Vβ 9 and 3’Cβ primers, using Go Taq Tm DNA polymerase (Promega) by 32 PCR cycles, with each cycle consisting of 95° C for 5 min, 55° C for 45 sec and 72° C for 45 sec, followed by a final extension period at 72° C for 10 min.

PCR products were purified (kit Promega Madison WI, USA) and sequenced (Beckman Ceek 8000, Fullerton, CA) in accordance with the producer’s protocol.

Sequences were analysed by specific software (ChromasPro, Technelysium PTY LTD, and Seq Ed, Applied Biosystems Inc., Foster city, CA, USA).

Analysis of HIV-1-specific $CD8^+$ T-lymphocytes. (Study 1 and 2)

HIV-1 clade independent and HLA-class I promiscuous peptides designed on HIV-1 GAG, TAT and NEF proteins (Sigma-Genosys, Cambridge, UK) were used as specific stimuli for the patients’ PBMC (Amicosante 2002). $1x10^6$

fresh PBMC in 1 ml of complete RPMI medium were incubated with 1 μg each of *anti*-CD28 and CD49d monoclonal antibodies and 1 μg pool of GAG, TAT and NEF peptides. The IFN-γ release induced by Phorbol myristate acetate (PMA) (50 ng/ml) plus ionomycin (10 μg/ml) was used as a positive control. The cultures were incubated at 37 °C in a 5% CO_2 incubator for 1 hour, followed by additional 5 hours incubation with 10 μg/ml of the secretion inhibitor Brefeldin-A (Sigma, St. Louis, MO). Cells were washed and then stained with monoclonal antibodies specific for anti-human CD3 (IgG1, clone RPA-T3); anti-human CD8 (IgG1 clone MOPC-21) (all antibodies from Becton Dickinson Immunocytometry Systems, San Jose, CA) for 15 min at 4°C. Samples were fixed in 1% paraformaldheide for 10 min at 4°C, incubated with anti-IFN)-γ mAb diluted in PBS 1X, BSA 1% and saponin 0.5%. Cells were acquired by FACScalibur (Becton Dickinson, Immunocytometry Systems). At least 200.000 live events were acquired, gated on small viable $CD3^+CD8^+$ lymphocytes. Data files were analysed using CellQuest software (Becton Dickinson). Results are expressed as frequency of IFN-γ producing $CD8^+$ T cells per 10^6 $CD8^+$ T cells. PBMCs were obtained from 5 HIV-negative healthy children and used as negative controls.

Generation of MDDCs (Study 2)

To obtain MDDCs, firstly monocytes were separated from patients' PBMC by adherence in 12 well-plates for 2 hours in complete culture medium (RPMI with L-glutamine, penicillin/streptomycin, 10% FCS) or by the use of magnetic anti-$CD14^+$ microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). Purified monocytes were then cultured for 6 days at concentration of $5x10^6$ cells/mL in complete culture medium supplemented with 100 ng/ml

of GM-CSF and 10 ng/ml of IL-4 (Sigma), to favour the differentiation of monocytes in MDDCs. To induce MDDCs maturation, immature MDDCs were incubated with LPS (1 μg/mL) on day 6^{th} for 24 additional hours.

MLR (Study 2)

To perform MLR tests, 10^4 patients' immature or mature MDDCs, generated as described above, were used as stimulators in each well. Allogeneic PBMCs, obtained from healthy donors by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, were used as responders. Two different healthy donors were used for each MLR test to avoid the possibility that a low proliferation was due to HLA compatibility. Allogeneic cells were added to each well with a ratio Stimulators/responders of 1:10. Negative controls included MDDCs alone and PBMCs alone; positive controls consisted in PMBCs stimulated with PHA (5μg/ml). Patients' MDDCs were co-cultured with healthy donors allogeneic PBMCs for 6 days. Then 0,5 μCi ^{3}H-Thymidine was added and the cells were cultured for additional 6 hours. Finally cells were harvested onto glass fibre filters (Packard, MI, Italy) and analysed by B-counter scintillator (Canberra Packard Instrument Company, Meriden, CT, and U.S.A.).

MDDCs pulsing and $CD8^+$ IFN-γ production (Study 2)

Four patients were analysed for the ability of their $CD8^+$ to produce IFNγ after stimulation with autologous MDDCs pulsed with HIV-1 inactivated by aldhitriol-2 (AT-2). IFNγ production was assessed by intracellular staining.

The entire procedure is schematized in Figure 2.1. and explained in the following text.

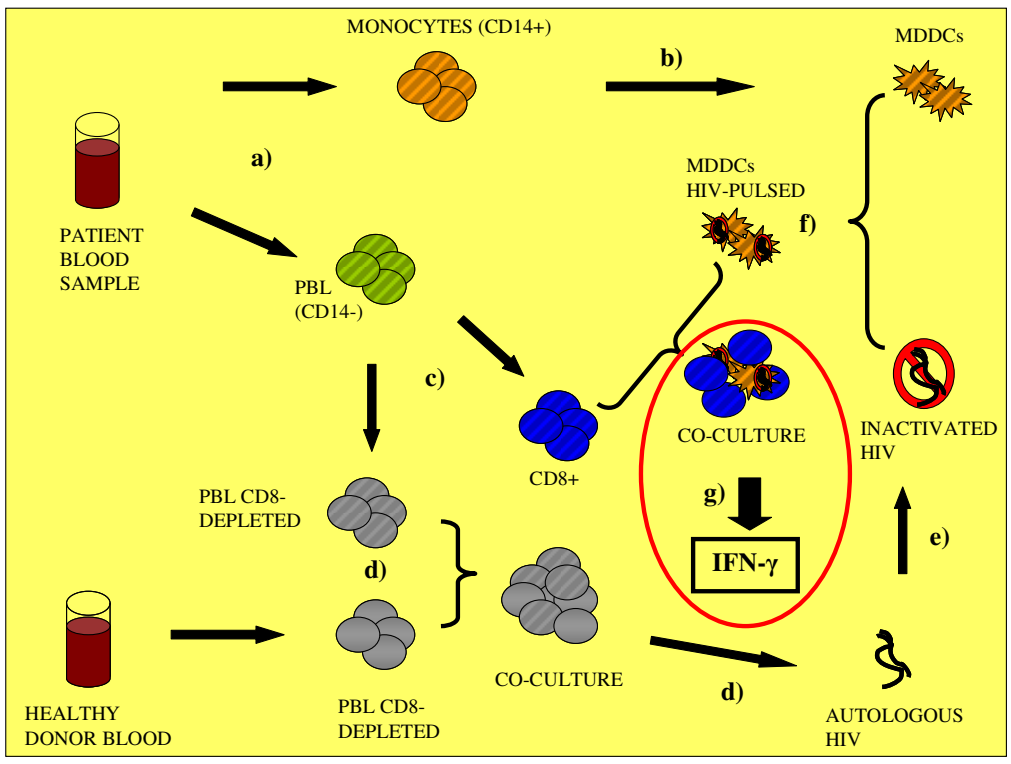


Figure 2.1. MDDCs pulsing and CD8$^+$ IFN-γ production. Experimental procedure. a), b), c) *Cell separations.* ***d)****Viral isolation* ***e), f)*** *Induction of virus specific T-cell response.* ***g)*** *Cytotoxic assay.*

PBMCs were fractionated in CD14$^+$ subset (monocytes corresponding to the positive fraction) and CD14$^-$ subset (corresponding to the negative fraction) using CD14$^+$ antibody-coupled microbeads (Miltenyi). Monocytes were cultured for 6 days at concentration of 5x10^6 cells/mL in complete culture medium supplemented with 100 ng/ml of GM-CSF and 10 ng/ml of IL-4 (Sigma), to obtain MDDCs. CD14$^-$ subset was furthermore fractionated in CD8$^+$ cells (positive fraction) and CD8$^-$ cells (negative fraction) using anti-

$CD8^+$ monoclonal antibody-coupled microbeads (Myltenyi). Virus was isolated by 28 days co-culture of PHA-stimulated CD8-depleted-PBMCs of healthy donors with patients CD8-depleted PBMCs. To facilitate HIV replication new PHA-stimulated-PBMC-CD8-depleted were added to the co-culture every 7 days. The presence of HIV-1 in the culture supernatants was confirmed by p24 ELISA assay for virus titer (Innogenetics). e) Viral isolates were inactivated with 250 µl aldrithiol-2 (AT-2) (Sigma) for 1 hour at 37° C in order to preserve the intact native conformation and fusogenic activity of HIV Env protein gp120 (Rossio et al 1998). f) Freshly immature MDDCs were pulsed with AT-2-inactivated autologous isolate (50ng of p24) for 2 hours at 37°C. After three washes with RPMI 1640 medium, virus-pulsed MDDCs were cultured in complete culture medium supplemented with 100 ng/ml GM-CSF, 5 ng/ml IL-4 (Sigma), 50 ng/ml TNF-α (R&D Systems) and 1000 U/ml IFN-α-2b (R&D Systems) for 3 days. PBL freshly prepared from non-adherent PBMC were stimulated on day 0 and restimulated on day 7 with virus-pulsed autologous MDDCs (at a stimulator/responder ration of 1:3). Exogenous IL-2 (R&D Systems) was added every 3-4 days. g) at day 14 of culture cells were harvested and intracellular stained for IFN-γ production analysis. PMA/Iono stimulated PBLs were used as positive control for intracellular staining.

Statistical analysis

Clinical data are presented as means $\pm$ standard deviation (SD) of the mean. The correlation between lymphoproliferative response and age (study 1) was analysed by the non-parametric Spearman rank test. Data were analysed by using GraphPad Prism Software version 4.00.

To calculate the patients' *z*-scores of lipids at each time interval (study 2) we used normal means and standard deviations for age (Christensen 1980) after controlling that all variables had a normal distribution. Average measures at multiple time points were compared to reference (i.e. initial values) during follow up. Student T-test for paired samples was used for assessing statistical significance. P values less than 0,01 were considered significant.

RESULTS AND DISCUSSION

Study n° 1 : Early highly active antiretroviral treatment delayed over 3 months of life is associated with good clinical outcome, long-term viral control and persistent antiviral T-cell response in HIV-1 vertically infected infants . (Paper 1)

Clinical parameters

Pat	Baseline			End of follow-up			
	Age (mon)	Plasma HIV RNA load (log copies/ml)	$CD4^+$ (%)	Age (mon)	Duration of viral load <50 copies/ml (mon)	$CD4^+$ (%)	$CD4^+$ Nadir
Pa	3.1	5.1	54	26	22	39	37
Pb	3.2	7.1	23	45	34	42	38
Pc	3.3	5.8	40	66	63	42	35
Pd	3.3	5.1	31	23	20	29	25
Pe	4.2	5.7	29	61	58	34	28
Pf	5.9	5.7	27	55	49	34	32
Pg	6.6	5.7	5	59	50	44	37
Ph	7.0	5.6	30	26	22	32	26
Pi	7.6	5.6	24	70	64	28	25
K	3-6		46 (35-56)	12-24		41 (32-51)	
K	6-12		46 (31-56)	24-72		38 (28-47)	

Table 1.2. Patients' characteristics. *K: Age-matched reference values according to Shearer 2003; cell values (expressed as percentages) are the medians, with ranger from 10^{th} and 90^{th} percentiles in parentheses. Mon: months*

In Table 1.2 a comparison between clinical patients' characteristics at baseline and at the end of follow-up is reported.

Clinical outcome

During follow-up of 47,9 ± 18,5 months, all patients maintained a good clinical status with normal linear growth. No serious adverse events occurred, neither signs of lipodystrophy were clinically documented in any of the infants enrolled in this study. Good compliance to the antiretroviral treatment was reported in all patients by periodic questionnaires and pill counts.

Virologic evaluation

The clearance of HIV-1 virions in plasma following the initiation of therapy was biphasic, as previously described (Luzuriaga 1999). Major part of the plasmatic virus was cleared during the firsts 4 weeks, with a slower second-phase decline, leading to complete viral suppression (<50 copies /ml) within a mean period of 5.4 ± 2.9 months of treatment. The HIV-1 viral load remained undetectable during the follow-up for a mean of 42.4± 18.2 months.

$CD4^+$ and $CD8^+$ counts

The percentage of $CD4^+$ T-cells increased from a mean value of 29±13.2% to 36±5.9% during the long-term follow-up (Table 1.2). A rapid and consistent increase in $CD4^+$ T-cell counts was observed within the first months even in the three infants (patients' pF, pG, pI) with a severe immunodeficiency at baseline. The subset distribution of $CD4^+$ and $CD8^+$ naïve and memory ($CD45RA^+$ and $CD45RO^+$ respectively) was maintained in the normal range values for age (Shearer 2003) (data not shown).

T-cell compartment analyses

T-cell proliferation

Proliferation to mitogens and recall antigens showed normal conservation during follow-up compared with age-matched healthy controls. In all children studied, an in vitro T-cell proliferation (SI>3) to at least one of the HIV-1 antigens tested (p24, p17, gp120 or gp41) was detected at multiple time points (data not shown). T-cell proliferation to HIV-1 specific antigens maintained during follow-up and did not result related with age (Figure 1.1).

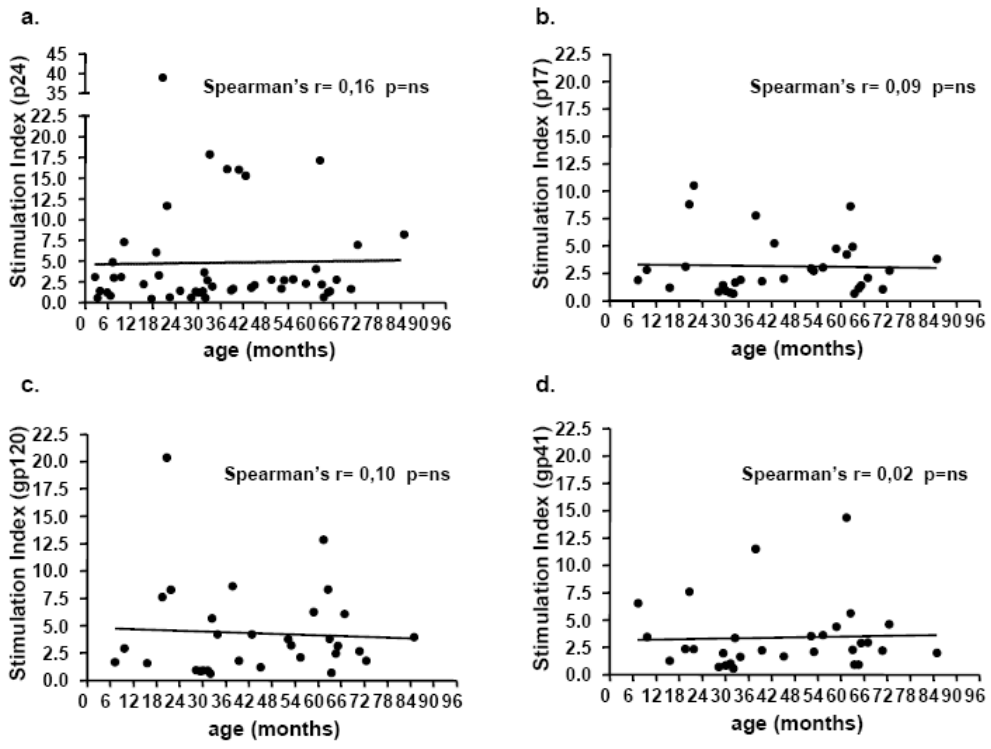


Figure 1.1. Absence of correlation between lymphoproliferative response and patients' age. *Correlations were analysed by the non-parametric Spearman rank test.*

TCRBV repertoire

In Table 1.3 the percentages of skewed TCRBV profiles on $CD4^+$ and $CD8^+$ subset at baseline and at the end of follow-up are reported.

Patient	**Baseline**		**End of follow-up**	
	$CD4^+$ TCRBV repertoire	$CD8^+$ TCRBV repertoire	$CD4^+$ TCRBV repertoire	$CD8^+$ TCRBV repertoire
Pa	4.0	19.2	0.0	27.2
Pb	0.0	25.0	0.0	4.2
Pc	0.0	64.0	0.0	0.0
Pd	54.2	56.0	16.7	12.5
Pe	73.7	84.6	0.0	8.7
Pf	0.0	93.7	0.0	11.54
Pg	0.0	6.2	0.0	0.0
Ph	8.3	44.0	0.0	26.1
Pi	0.0	45.8	15.4	43.5

Table 1.3: Drop of skewed TCRBV patterns percentages during follow-up. *TCRBV pattern distribution was analysed both in $CD4^+$ and in $CD8^+$ subset.*

At baseline, on $CD4^+$ subset, five patients showed normal polyclonal profiles of TCRBV repertoire, while the remaining subjects demonstrated a skewed pattern. At the end of follow-up almost all patients showed a repertoire normalization; only patient pI showed a little increase in skewed patterns. On $CD8^+$ subset, TCRBV repertoire distribution , as expected, was significantly altered at baseline in all infants analysed. At the end of follow-up, a significant trend towards normalization was observed (Tab 1.3 and Figure 1.2).

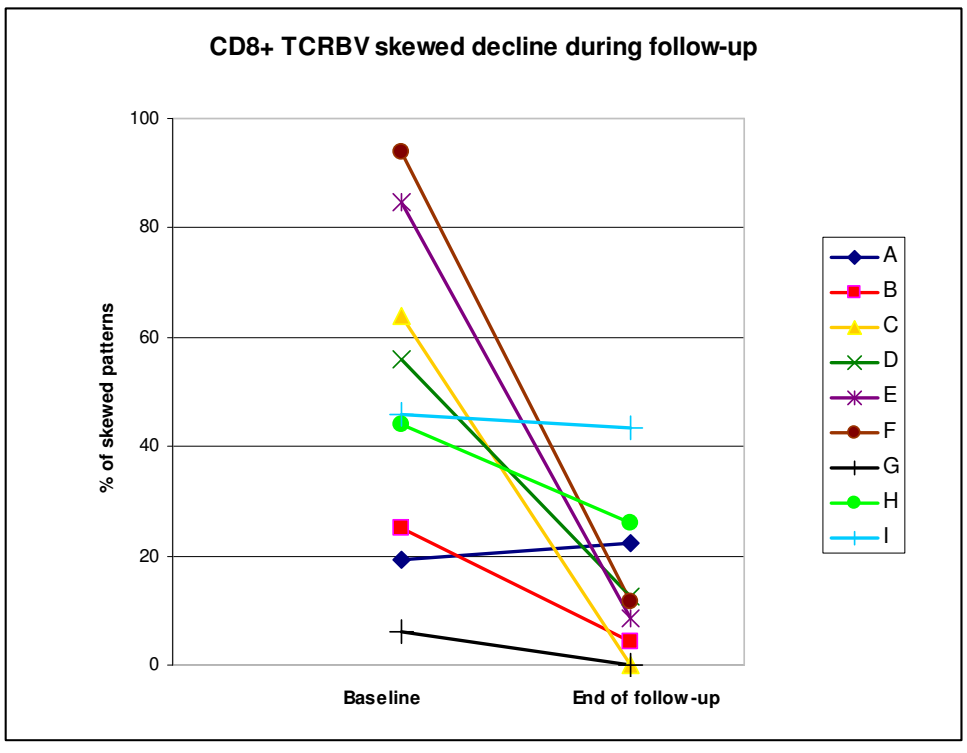
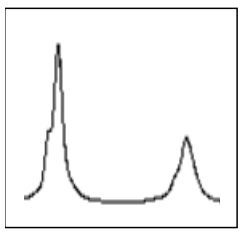


Figure 1.2. Drop of TCRBV skewed pattern percentages on CD8$^+$ subset.

Interestingly, in patient pI, which maintained skewed distributions in over 40% of TCRBV families, the analysis of TCRBV repertoire on CD8$^+$/CD45RA$^+$ and the CD8$^+$/CD45RO$^+$ subset, showed a higher prevalence of altered TCRBV in the CD45RO$^+$ subset (57,14%) compared to CD45RA$^+$ subset (12,50%).

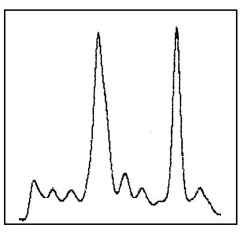
Furthermore, an oligoclonal persistence in the TCRBV9 family on CD8$^+$ subset of this patient was observed and to better understand the specific distribution sequence repertoire, CDR3 region was sequenced after amplification and cloning at the baseline and during follow-up. Results of sequencing are showed in Figure 1.3.

PROFILE



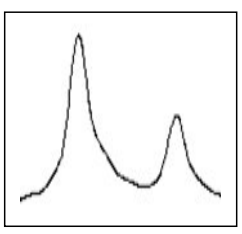
t=0

FREQ	Vβ	CDR3	Jβ	Cβ	%
6/12	CAS	SAPGSGGVYEQF	FGPGTRLTVL (2.1)	EDLKN (C2)	**60**
4/12	CAS	SQTLRGLAGGHETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**26**
1/12	CAS	SPGHFSYEQY	FGPGTRLTVT (2.7)	EDLKN (C2)	**7**
1/12	CAS	SQLRMGPTIPGGELF	FGEGSRLTVL (2.2)	EDLKN (C2)	**7**



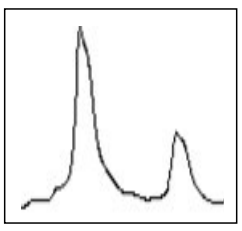
t=6

6/18	CAS	SAPGSGGVYEQF	FGPGTRLTVL (2.1)	EDLKN (C2)	**32**
2/18	CAS	NPGGGRQETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**10**
2/18	CAS	SQTLRGLAGGHETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**10**
1/18	CAS	SKGLARGETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**6**
1/18	CAS	SQGGNTGELL	FGEGSRLTVL (C1)	EDLNK (C1)	**6**
1/18	CAS	SPTSRNTEAF	FGQGTRLTVV (1.1)	EDLNK (C1)	**6**
1/18	CAS	SQGNAGPRDTQY	FGPGTRLTVL (2.3)	EDLKN (C2)	**6**
1/18	CAS	NSQGFVQGERETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**6**
1/18	CAS	SPRTGGTQYTQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**6**
1/18	CAS	SQESRGITDTQY	FGPGTRLTVL (2.3)	EDLKN (C2)	**6**
1/18	CAS	SPDRLETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**6**



t=12

10/22	CAS	NPGGGRQETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**45**
8/22	CAS	SAPGSGGVYEQF	FGPGTRLTVL (2.1)	EDLKN (C2)	**36**
3/22	CAS	SQTLRGLAGGHETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**14**
1/22	CAS	SQFSDRLGQETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**5**



t=24

11/17	CAS	NPGGGRQETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**64**
1/17	CAS	SAPGSGGVYEQF	FGPGTRLTVL (2.1)	EDLKN (C2)	**6**
1/17	CAS	SQTLRGLAGGHETQY	FGPGTRLLVL (2.5)	EDLKN (C2)	**6**
1/17	CAS	NQEVSPDQPQH	FGDGTRLSIL (1.5)	EDLNK (C1)	**6**
1/17	CAS	NATSGSTDTQY	FGPGTRLTVL (2.3)	EDLKN (C2)	**6**
1/17	CAS	NQLGWGNSYNEQF	FGPGTRLTVL (2.1)	EDLKN (C2)	**6**
1/17	CAS	NQERGRTDTQY	FGPGTRLTVL (2.3)	EDLKN (C2)	**6**

Figure 1.3. CD8$^+$ TCR-Vβ9 sequencing analysis during follow-up. *Left side: Pattern profile of TCR-Vβ9 distribution. Right side: Sequencing results: sequences of the region V-D-J-C are reported with frequencies and percentages.*

Two predominant CDR3 lengths of 12 and 15 amino acids were observed at baseline, and maintained during the entire follow-up. In agreement with the major TCR polyclonality, at 6 months of HAART the higher number of different sequences was observed. In addition at the same time point a new sequence of 12 aminoacids appeared, becoming the most frequent sequence at the last time point analysed (24 months).

CD8$^+$ Lymphocytes cytotoxicity

Intracellular staining to evaluate the frequency of IFN-γ positive CD8$^+$ T-cells after stimulation with HIV-1-specific peptide pools for GAG, TAT and NEF protein was performed in 7 out of the 9 subjects in the study at the end of follow-up period. In all patients, CD8$^+$ T lymphocytes had the capacity to respond to non-specific stimuli such as PMA/ionomycin (34674±31388 CD8$^+$/IFN- γ + T-cells/10^6 CD8$^+$ cells). Two of theme (pF and pI) presented a higher number of HIV-1-specific CD8$^+$/IFN-γ + T-cells in response to HIV-1 peptides (1200/10^6 CD8$^+$ cells and 3800/10^6 CD8$^+$ cells, respectively). The other study subjects (patients pA, pC, pD, pG and pH) did not show specific CD8$^+$ reactivity (all <100/106 CD8+ cells), similarly to HIV-1-negative control (data not shown).

Discussion

Despite evident advantages of HAART application in reducing morbidity and mortality of both adults and children in HIV-infection (Palella 1998, Romiti 2001, Resino 2002), for paediatric patients the exact timing of antiretroviral therapy initiation is a central argue (Faye 2004).

In our cohort of children, which started therapy after the third month of age, the virus clearance from the plasma was always observed and viral control was then maintained over time. This long-term viral control, that is not observed in previous studies in which therapy started in the first weeks of life (Buseyne 2002, Luzuriaga 2004), is probably due to a better compliance and to the development of a better immunological HIV-specific response, that is in accordance with literature data, which show a T-cell response mainly after 6 months of age (Chiappini 2006). It's possible that a period free of therapy before its application could favour the development of a more durable memory response (Luzuriaga 2000). Indeed if an early treatment decrease the risk of disease progression (Abrams 2003), on the other hand it could impair the availability of viral antigen for processing and presentation to the T-cell compartment and thereby the development of HIV-specific cellular immune responses (Dunn 2003, Faye 2002).

Our functional data are confirmed by TCRBV repertoire distribution analysis, which showed a strong normalization trend during the long-term follow-up, underlining the presence of an intact T-cell population with the potential to recognize a wide range of antigens (Romiti 2001).

However, a partially skewed repertoire on $CD8^+$ subset was observed in one patient of our cohort. The persistence of altered distributions in this patients could be explained by the persistence of specific $CD8^+$ memory T-cells

(Luzuriaga 2000). Indeed he was the only one with a significant HIV-specific CTL response and showed a prevalence of altered TCRBV repertoire distribution in the $CD45RO^+$ subset, data according to a previous study by Kou et al who explained these alterations with the presence of HIV-specific memory response (Kou 2003). This hypothesis is supported by the TCR Vβ9 analysis in the $CD8^+$ subset of patient pI: Vβ9 of this patient in fact, shows a clonal expansion, confirmed by sequencing during the follow-up. We could conclude that specific immune responses, in this patient, have been influenced by the longer exposure to virus replication. Indeed he was symptomatic at the enrolment and started HAART later than 6 months of age.

The non-recruitment of a specific CTL response in the other children could be explained by the low viral burden and their young age (Haridas 2003). Indeed HIV-specific $CD8^+$ T cell function is critically influenced by age and by the state of $CD4^+$ T-cell compartment (Sandberg 2003).

We could speculate that the application of HAART after the third month of life, could favour immune reconstitution in early-treated children because a transient viraemia would be sufficient to induce a strong HIV-specific immune response.

Study n° 2: Switching from protease inhibitor-based-HAART to a protease inhibitor-sparing regimen is associated with improved specific HIV-immune responses in HIV-infected children. (Paper 2 & 3)

Clinical parameters

Table 2.2 summarizes the clinical status of patients showing the virologic evaluations and the $CD4^+$ and $CD8^+$ counts, with a comparison between the baseline and the end of follow-up.

a)

Patient	**T=0 wks**				**T=96 wks**		
	Age (y)	Rate of blips	$CD4^+$ cell counts % (n°)	$CD8^+$ cell counts % (n°)	Rate of blips	$CD4^+$ cell counts % (n°)	$CD8^+$ cell counts % (n°)
P1	6.8	0.1	35 (1268)	33 (1182)	0	41 (1168)	29 (792)
P2	14.5	0	34 (594)	39 (730)	0	38 (676)	38 (676)
P3	10.6	0	25 (400)	36 (529)	0.1	29 (524)	38 (505)
P4	13.5	0.4	32 (698)	38 (765)	0.2	33 (565)*	37 (677)*
P5	7.4	0.1	27 (412)	26 (319)	0	29 (389)	26 (348)
P6	9.8	0.2	29	29	0.3	35	36

			(980)	(1300)		(1106)	(1137)
P7	9.3	0.3	33	26	0.1	34	27
			(833)	(770)		(1159)	(920)
P8	7.9	0.1	36	27	0.3	37	30
			(1172)	(935)		(1043)	(846)
P9	18.9	0.2	23	23	0	26	26
			(583)*	(1201)*		(654)	(1076)
P10	8.3	0	37	22	0.3	42	19
			(754)	(422)		(926)	(367)
P11	14.2	0	46	29	0	38	28
			(906)*	(552)*		(1181)	(870)
P12	6.7	0.1	40	29	0	32	36
			(708)	(557)		(499)	(561)
P13	12.9	0.1	28	32	0.2	30	34
			(423)	(452)		(513)	(581)
P14	11.5	0	46	27	0	44	26
			(1217)	(754)		(1107)	(595)
P15	5.0	0.2	37	21	0	41	20
			(1360)	(772)		(1115)	(544)
P16	5.1	0.1	27	29	0.1	28	27
			(812)	(933)		(935)	(955)
P17	5.2	0.2	34	31	0	32	26
			(2237)	(1956)		(1171)	(951)
P18	2.4	0.1	41	28	0	41	25
			(994)	(564)		(832)	(507)
P19	4.5	0.2	45	22	0.1	44	26
			(999)	(460)		(836)	(494)

b)

Age	**CD4+** **%** **Abs. N°**	**CD8+** **%** **Abs. N°**
2-6	38 (28-47)	23 (16-30)
	1380 (700-2200)	840 (490-1330)
6-12	37 (31-47)	25 (18-35)
	980 (650-1500)	680 (370-1100)
12-18	41 (31-52)	26 (18-35)
	840 (530-1300)	530 (330-920)

Tab 2.2 Clinical parameters at baseline and at the end of follow-up. a) *Individual patients' characteristics are reported. Cell counts are reported as percentages and absolute numbers*
** Values missing at the right time-point are relative to 48 wks time point.*
b) *Normal values: age-matched reference values according to Shearer 2003; values are expressed as percentages and absolute numbers with the medians, with ranger from 10th to 90th percentiles in parentheses.*

Clinical outcome

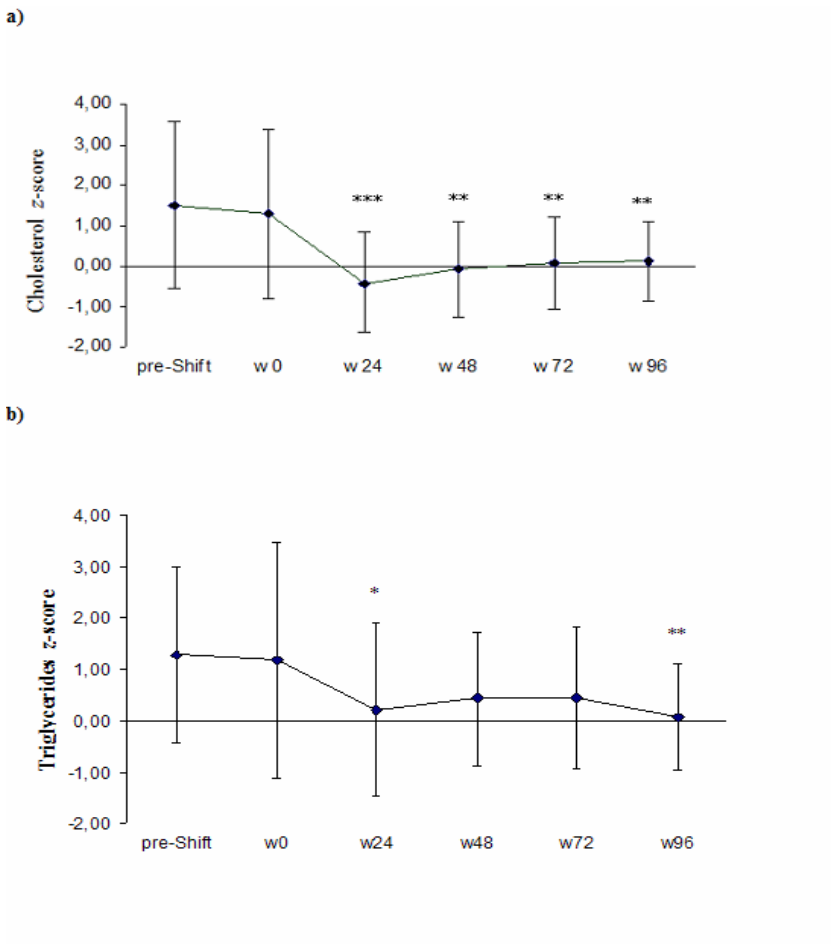


Figure 2.2 Variations from the baseline of Cholesterol and Triglycerides (means ± SD) ***a)*** *Cholesterol z-score and* ***b)*** *Triglycerides z-score.* ***P < *0.001,* ** P < *0.01,* *P <*0,05*

Generally the triple nucleoside regimen was well tolerated. None discontinued therapy due to toxicity. Symptoms of a hypersensitivity reaction were reported only in one patient switched to abacavir. The discontinuation of abacavir and shifting in ddI resulted in the resolution of symptoms in that patient. No clinical neither laboratory signs of hyperlactatemia were detected.
New lypodystrophy cases were not detected and, as reported in Figure 2.2, median total cholesterol and fasting triglycerides mean values significantly decreased.

Patients and their parents reported, through appropriate questionnaires, satisfaction with the new therapeutic combination, demonstrating a good adherence to the simplification protocol.

Virologic evaluation

Viral suppression was maintained over time in 18 out of 19 patients (child p4 had a viral failure at 48 weeks as a result of poor adherence). Intermittent episodes of detectable viraemia between 50 and 999 copies/ml preceded and followed by measurements under 50 copies/ml (blips) did not increase during the three NRTI regimen. Indeed the mean of blips rate was 0,12 during PI-based regimen and 0,09 during 3NRTI treatment.

$CD4^+$ and $CD8^+$ counts

Range of CD4+ and CD8+ counts were comparables to age-related healthy controls over time (Shearer 2003).

T-cell compartment analyses

T-cell proliferation

Pt	Lymphoproliferation t=0 ws SI (cpm)					Lymphoproliferation t = 96 ws SI (cpm)				
	k	**p 24**	**p 17**	**gp 120**	**gp 41**	**K**	**p 24**	**p 17**	**gp 120**	**gp 41**
P1	1150	1.67 (1924)	1.36 (1565)	1.90 (2194)	1.08 (1243)	688	**15.26** (10502)	**3.34** (2299)	**3.00** (2064)	**4.22** (2902)
P2	1074	**4.23** (4550)	2.17 (2329)	0.86 (919)	2.11 (2269)	567	**5.49** (3118)	**4.46** (2532)	**4.20** (2387)	**4.22** (2395)
P3	1108	1.03 (1143)	1.70 (1887)	1.34 (1484)	0.56 (621)	453	**3.74** (1696)	2.29 (1038)	**3.79** (1717)	2.34 (1062)
P4	1365	2.74 (3743)	0.99 (1349)	6.90 (8580)	1.79 (2438)	Nd	**nd**	**nd**	**nd**	nd
P5	1185	1.16 (1372)	0.80 (950)	0.92 (1096)	1.01 (1202)	400	2.73 (1093)	**7.78** (3112)	2.14 (857)	3.42 (1366)
P6	1180	**7.48** (8827)	2.61 (3080)	2.12 (2504)	1.81 (2131)	436	**4.01** (1752)	**8.29** (3617)	**3.80** (1660)	**12.22** (5329)
P7	489	**11.23** (5493)	2.02 (990)	**8.94** (4371)	2.29 (1120)	1157	0.90 (1043)	1.68 (1945)	1.13 (1310)	1.13 (1308)
P8	1478	**3.96** (5865)	1.30 (1923)	**3.75** (5554)	1.91 (2836)	451	**7.39** (3334)	**15.10** (6808)	**9.46** (4267)	**11.98** (5402)
P9	nd	nd	nd	nd	**nd**	708	**5.97** (4227)	**5.39** (3818)	**3.73** (2642)	**13.14** (9304)
P 10	729	1.39 (1010)	1.44 (1051)	**4.85** (3535)	1.73 (1261)	494	**7.64** (3776)	**4.61** (2276)	**8.32** (4111)	**8.32** (4112)
P 11	nd	nd	**nd**	nd	nd	1051	**9.31** (9785)	**23.88** (25101)	**7.39** (7777)	**22.26** (23397)
P 12	911	**8.12** (7405)	**9.32** (8488)	**6.67** (6072)	**5.20** (4736)	865	**5.88** (5089)	**3.06** (2648)	**3.18** (2755)	**9.01** (7794)
P 13	680	0.73 (496)	0.64 (437)	0.88 (596)	0.95 (648)	459	**7.96** (3653)	**4.14** (1902)	2.63 (1210)	2.53 (1161)

P 14	790	**3.54** (2793)	1.14 (902)	**4.71** (3722)	1.08 (851)	1126	**14.69** (16538)	**12.70** (14298)	**8.01** (9019)	**9.11** (10262)
P 15	1286	1.22 (1570)	1.10 (1419)	2.45 (3153)	0.91 (1166)	553	**3.30** (1825)	**4.74** (2623)	2.88 (1592)	0.99 (545)
P 16	603	**8.64** (5210)	**8.42** (5007)	**8.76** (5287)	**9.81** (5917)	782	**19.47** (15225)	**4.39** (3438)	1.74 (1357)	**6.77** (5295)
P 17	926	1.04 (965)	0.64 (597)	**5.52** (5110)	**5.55** (5140)	470	**9.94** (4670)	**9.31** (4377)	**8.14** (3828)	**6.27** (2946)
P 18	413	**4.03** (1665)	2.14 (883)	2.38 (984)	2.11 (871)	766	2.73 (2097)	2.66 (2042)	**3.21** (2464)	2.14 (1646)
P 19	597	1.14 (680)	1.50 (897)	1.26 (751)	1.34 (799)	542	**3.83** (2075)	**9.28** (5032)	**8.24** (4464	**3.53** (1914)

Table 2.3 T-cell proliferation. *SI and cpm responses are showed for each patient, yellow boxes represent SI $\geq$3.*
K= unstimulated background in medium alone

T-cell proliferation analyses were performed at multiple time point; values at baseline and at the end of follow-up are reported as cpm and SI in Table 2.3. Lymphoproliferative T-cell responses to mitogens (PHA, PWM, OKT3) and recall antigens (Candida Albicans, HSV-1) were maintained within laboratory values. Noteworthy, as showed in Tab 2.3, lymphoproliferative T-cell response to HIV-specific antigens (HIV core protein p24, p17, and envelope glycoprotein gp120, gp41) significantly increased ($p<0,01$) during follow up in almost all patients.

Of interest, a significant increase ($p<0,01$) in the total number of responder patients to at least two out of four HIV-specific antigens was observed during the follow-up as shown in Figure 2.3, relative to the baseline and to the end of follow-up

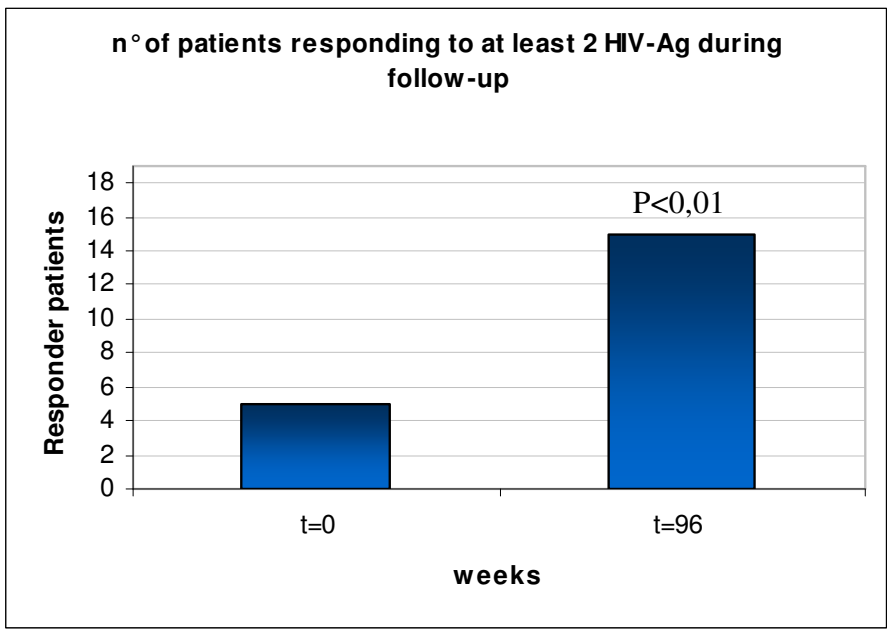


Figure 2.3. Number of responder patients to at least two out of four HIV-specific antigens during the follow-up.

TCRBV repertoire

During previous PI-based HAART, TCRBV repertoire distributions showed a normalization trend in $CD4^+$ subset and, to a less extent, in $CD8^+$ subset as reported in the study n°1 and showed for 2 children in Romiti et al 2001.

Switching from PI-based-HAART to PI-sparing regimen led to a strong increase in TCRBV repertoire polyclonality also in $CD8^+$ subset: indeed 79% (15/19) of children showed a statistically significant normalization trend ($p<0,01$) of TCRBV repertoire distribution in $CD8^+$ subset whereas in $CD4^+$ subset almost all TCRBV families remained polyclonal overtime. In Table 2.4 percentages of polyclonal families are reported at the initiation and at the end f the study. Patient p4, who failed protocol, was the only one with an increase of alterations both in $CD4^+$ and $CD8^+$ TCRBV subsets.

Patient	TCR $CD8^+$ polyclonality (%) t=0	TCR $CD8^+$ polyclonality (%) t=96
P1	91.7	95.8
P2	81.8	95.8
P3	75.0	75.0
P4	100	91,8 *
P5	66.7	95.7
P6	66.7	100
P7	61.9	100
P8	90.9	100
P9	50.0 *	90.9
P10	95.8	100
P11	72.2 *	87.5
P12	38.9	83.4
P13	73.9	86.4
P14	87.5	100
P15	100	100
P16	57.1	91.7
P17	82.6	96.8
P18	95.7	100
P19	38.9	100

Table 2.4. Normalization trend of TCRBV repertoire in $CD8^+$ subset.

In Figure 2.4 the decline in the mean percentages of skewed pattern profiles of all patients analysed during follow-up is reported.

An example of kinetic of $CD8^+$ TCRBV repertoire distribution of a patient (p17) since birth, during previous HAART and subsequent simplified regimen, is reported in Figure 2.5

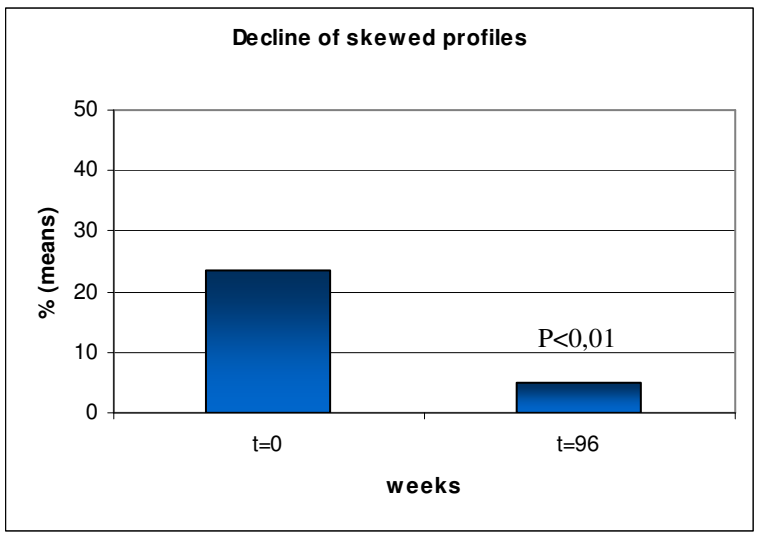


Figure 2.4. Drop of skewed profiles from baseline to the end of follow-up. *Percentages represent the means of skewed percentages of all patients at t=0 and t=96 weeks.*

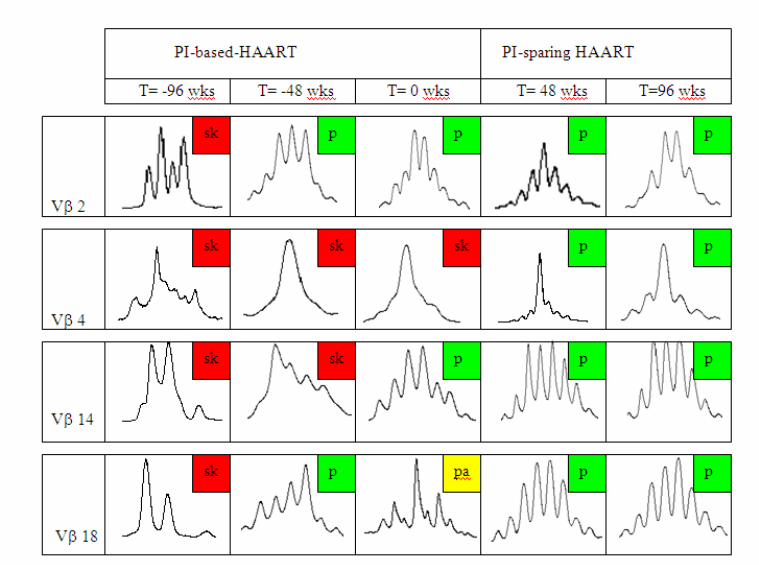
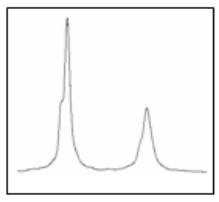


Figure 2.5. Kinetics of 4 TCRBV families in patient p17*. t=0 is the time of switching.*

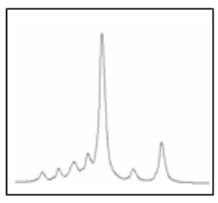
In the TCRBV9 of patient p16 (corresponding to patient pI of the previous study), despite the normalization trend, maintenance of some specific altered peaks was observed during the follow-up. Furthermore, spectratyping analysis performed 2 years after the end of follow-up on TCRBV9 from $CD8^+CD45^+RA$ naïve and $CD8^+CD45^+RO$ memory subsets, showed the same altered peaks observed previous during follow up, as reported in Figure. 2.6

$CD8^+$ Lymphocytes cytotoxicity

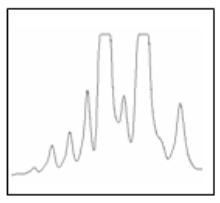
The frequency of IFN-γ positive $CD8^+$ T-cells after stimulation with HIV-1-specific peptide pools for GAG, TAT and NEF protein was evaluated by intracellular staining for all patients during follow-up. An increased percentage of HIV-specific IFN-γ producing $CD8^+$ T-cells was detected in 17/19 (89%) children. Mean values at baseline and at the end of follow-up are reported in Figure 2.7.



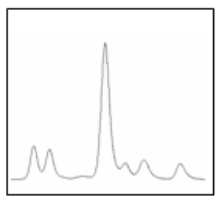
CD8⁺ Vβ9
PI-HAART



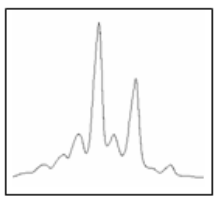
CD8⁺ Vβ9
t=0 ws



CD8⁺ CD45RA⁺ Vβ9
t=48 ws

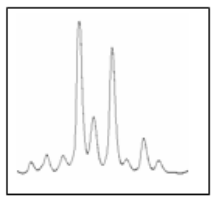


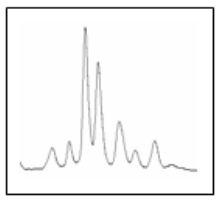
CD8⁺ CD45RO Vβ9
t=48 ws



CD8⁺ Vβ9
t=96 ws

PI-SPARING HAART





CD8⁺ CD45RA⁺ Vβ9 CD8⁺ CD45RO Vβ9

PI-SPARING HAART after follow-up

Figure 2.6. Maintenance of TCRBV9 alterations in patient p16. *Vβ9 TCR repertoire distributions are showed before, during and after the simplification therapy follow-up. At time t=48 weeks and after follow-up, analyses were performed both on CD45RA$^+$ naïve and CD45RO$^+$ memory subsets.*

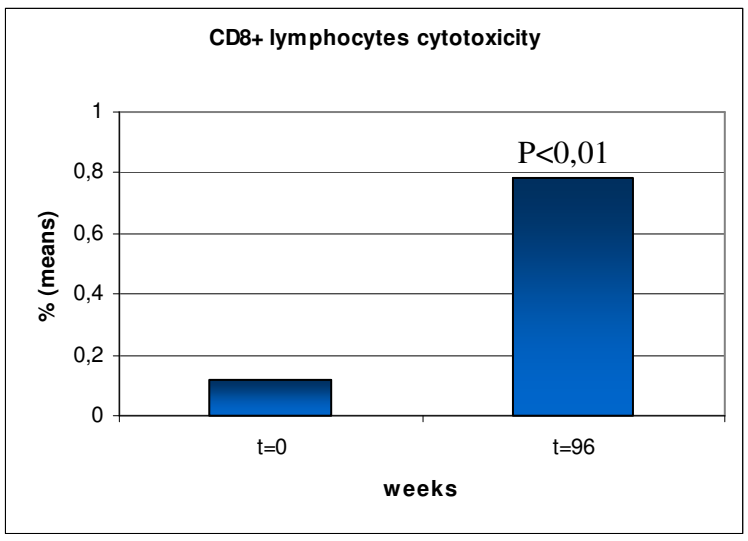


Figure 2.7. Mean percentages of IFN-γ producing CD8$^+$ cells. *Percentages are the means of IFN-γ producing CD8$^+$ cells percentages of all patients at t=0 and t=96 weeks.*

Dendritic cells compartment

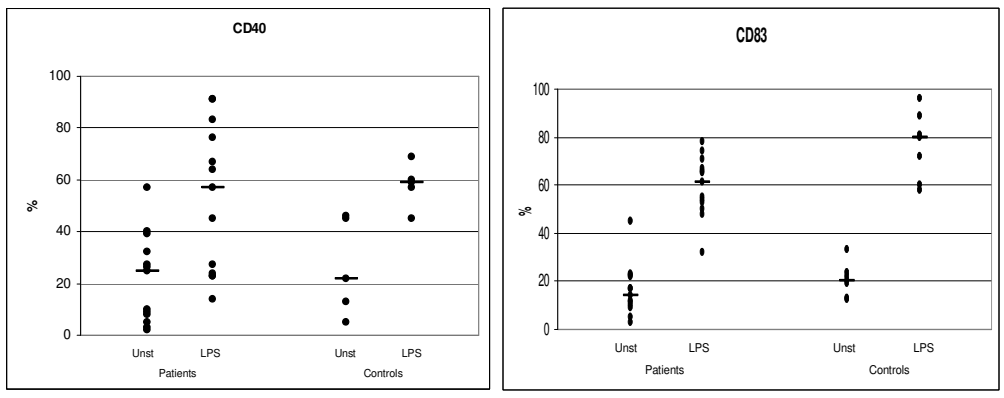
Phenotype analysis

Analyses performed on 13 out of 19 patients at the end of follow up, show MDDCs phenotypes, before and after stimulation with LPS, comparable to controls, although with individual different expression of any markers.

Percentages of MDDCs expressing specific differentiation and maturation markers are showed in Figure 2.8.

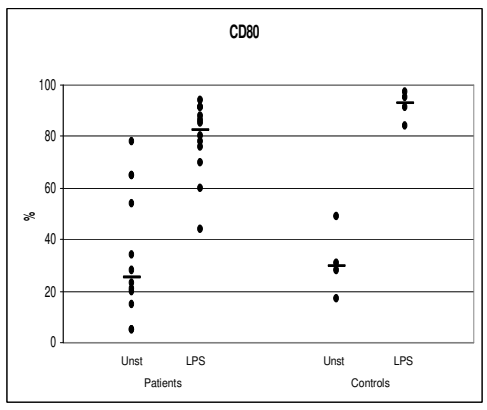
a)

b)



c)

d)



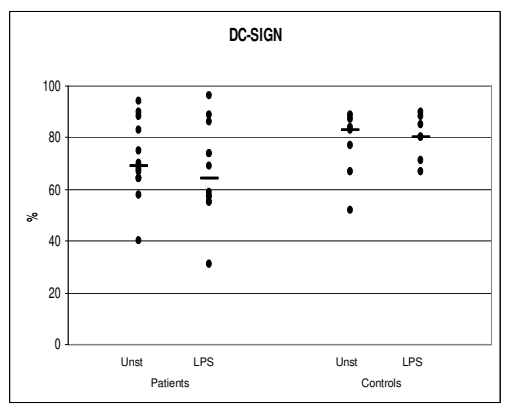


Figure 2.8. Markers surface expression. a) *CD40* ***b)*** *CD83* ***c)*** *CD80* ***d)*** *DC-SIGN Values refer to percentages of cells expressing the specific marker before and after treatment with maturative stimulus LPS. Patient's values are compared to Controls values. Medians are reported.*

A representative analysis of six surface markers is reported for patient p9 in Figure 2.9.

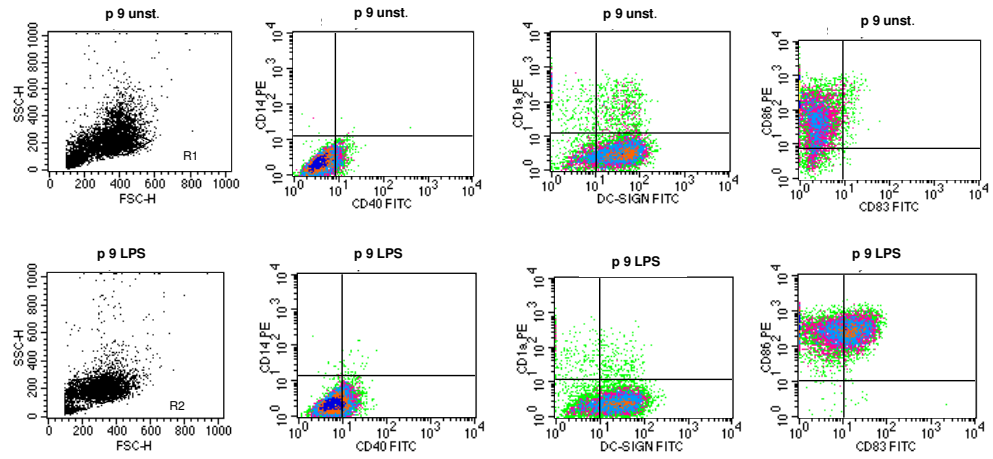


Figure 2.9. Phenotype analysis of MDDCs of patient p9. *Cells were analysed without any stimulus (UNST), or after stimulation with LPS. Values are reported as percentages of positive cells to specific surface expression marker.*

MLR

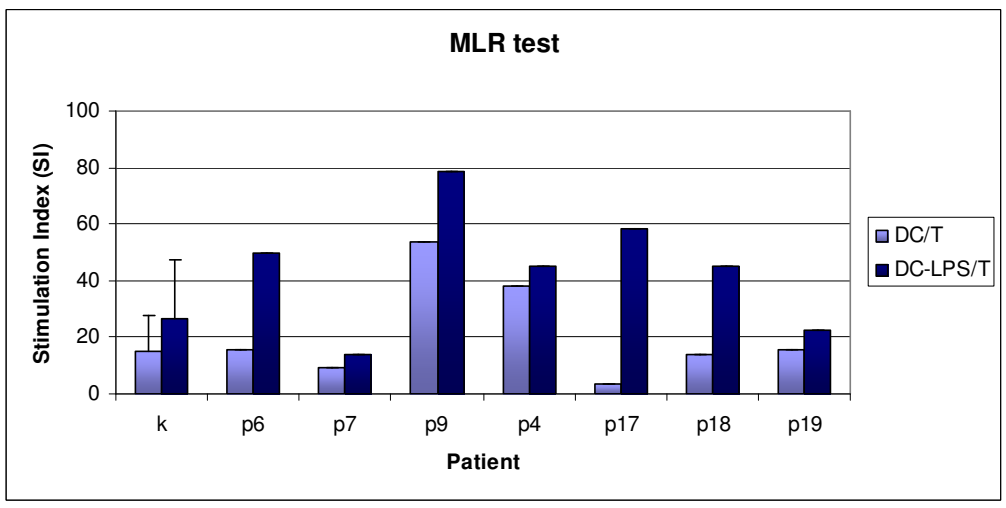


Figure 2.10. MLR test. T-cells proliferation was measured after co-culture with patients' unstimulated or LPS-stimulated MDDCs. K: controls. A total of 7 controls were used.

MLR tests were performed on 7 out of 19 patients at the end of follow-up. As showed in Figure 2.10, patients' MDDCs present normal allostimulatory functions, comparable with control MDDCs obtained from healthy donors.

DC pulsing and IFN-γ production.

To date autologous virus was isolated from PBMC of 6 patients. DC' pulsing with autologous virus was performed in 3 of them and specific IFN-γ production by $CD8^+$ T cells was analysed through intracellular staining. Results are showed in Tab 2.5.

Patient	**Gated Events**	**% IFN-γ producing unstimulated $CD8^+$**	**% IFN-γ producing PMA/Iono stimulated $CD8^+$**	**% IFN-γ producing HIV-DC-pulsed stimulated $CD8^+$**
P2	6.000	0,73	2,89	0,36
P18	100.000	0,12	5,72	1,84
P19	60.000	0,29	3,00	0,24

Table 2.5. IFN-γ production from CD8+ T-cells stimulated by HIV-pulsed-MDDCs. *Negative and positive controls are represented from IFN-γ producing unstimulated $CD8^+$ cells and IFN-γ producing PMA/Iono-stimulated $CD8^+$ cells respectively.*

One out of three patients showed the production of IFN-γ after stimulation with autologous HIV-pulsed dendritic cells whereas two patients did not produce IFN-γ.

Discussion

In the present study we followed 19 children, of which four were the same of the previous study. In this case the cohort was switched from a PI-based regimen to a PI-sparing regimen based on 3NRTI to avoid the numerous side effects of PI-based HAART, which are particularly heavy in paediatric patients with a long-term life expectative.

Previous studies in adults showed that switching the PI-based HAART to a simplified regimen is safe, but there are strong indications of an increased risk of virologic failure for patients with previous exposure to suboptimal NRTI therapy (Maggiolo et al 2005). In children more encouraging results were obtained: indeed after the switching, the maintenance of virologic suppression and a significant improvement of the lipid abnormalities besides the augmentation of HIV-specific immune response have been observed (McComsey 2003, Legrand 2005). It's possible that simplification efficacy is age-related and dependent from to the initiation time of HAART. Patients treated with a first-line HAART and then switched to 3NRTI protocols showed that simplification was as safe as continuation of the original regimen and better accepted (Rizzardini 2006).

Since our cohort consisted in children first-line treated with successful HAART, we hopefully presumed to observe the maintenance of good immunological parameters obtained with previous regimen. Interestingly, we observed even a significant amelioration in immune reconstitution of these children in terms of enhanced HIV-specific lymphoproliferative responses, and in terms of progressive normalization of TCRVB families in $CD8^+$ subset during the 96 weeks follow-up.

Interestingly, TCRBV normalization was observed, in a less extent, even in that child (p16=pI) who showed, in the previous study, the persistence of altered repertoire on the $CD8^+$ subset. Notably, in the context of TCRBV9, although a normalization trend was observed, an oligoclonal expansion, corresponding to the previous monoclonal expansion, was conserved after 96 weeks of PI-sparing regimen. This could reinforce the hypothesis for which antiretroviral therapy, followed by simplified regimen, could maintain the specificity of immunological response avoiding clonal exhaustion that would occur in absence of treatments, with the natural course of disease (Pantaleo 1997). Either TCRBV9 from $CD8^+CD45RA^+$ naïve subset either TCRBV9 from $CD8^+CD45RO^+$ memory subset showed the same altered spectrum (as shown in Figure 2.6); this could be explain by the fact that the same clone, if exposed to an Antigen, may express either $CD45RO^+$ or RA^+ (Wack 1998): indeed some $CD45RA^+$ cells are derived from $CD45RO^+$ cells reverted to RA^+ phenotype (Macallan 2004, Maini et al 2000). To clarify it, it could be necessary to perform cytofluorimetric analysis on truly naïve cells expressing $CD45RA^+$ $CD62L^+$ markers on their surface (Markert 2001, Hintzen 1993, Campbell 2001).

A broad TCR repertoire may supply a multipotential capacity to generate specific T-cells against a variety of antigens including HIV (Seder 2003) and a greater thymic function in children might explain the greater capacity to immune reconstitute their TCRBV repertoire compared with adults (Correa 2007). Since we observed, from these children, an increase in the cytotoxic response to HIV-antigens and the persistence of some specific alterations in the TCRBV repertoire (i.e. in the TCRBV9 of patient p16 as discussed), we could speculate that TCRBV normalization is not accompanied by a loss in the immunological memory.

These hypotheses are sustained by PBMC lymphoproliferations obtained after HIV-antigens exposure, a result confirmed and amplified by the recent work of Correa et al who demonstrate as in HIV-infected children, successfully treated with HAART, $CD4^+$ T-cells are able to produce IFN-γ and IL-2 after antigen-specific stimulation (Correa et al 2007).

Since enhancement in specific lymphoproliferative response and normalization of TCR distribution were not associated neither with a detectable increase of viral replication, as demonstrated by the maintenance of viral control, neither with the duration of previous HAART (which range was from 2 to 5 years), we hypothesized that the key element for the immunological improvement observed was the elimination of PI from the therapy. Indeed it has recently been shown a variety of immunomodulatory roles of PIs in the degradation of proteins (Piccinini 2005). These drugs may alter lymphoproliferative cytokines production (TNF-α, IL-2, IFN-γ), cause immune suppression and hamper antigen presentation (Andre 1998) impairing the immune reconstitution.

Since immunotherapeutic approaches are recently focused on DCs engineering and manipulating in order to improve the immune restoration of patients, we analysed, at the end of follow-up, phenotypes and functions of MDDCs obtained from our cohort.

We firstly verified that MDDCs obtained from HIV-infected patients show normal surface marker expression and allostimulatory capability comparable to healthy controls, as reported from literature data (Chougnet 1999, Sapp 1999)

Then we analysed whether was possible to induce an *in vitro* production of IFN-γ from $CD8^+$ T-cell stimulated by co-culture with autologous HIV(AT-2-inactivated)-pulsed-MDDCs.

Literature data are in contrast: remarkable studies of Lu et al showed an *in vitro* production of IFN-γ from $CD8^+$ stimulated with autologous-HIV-pulsed MDDCs, and *in vivo* data in macaque and humans confirmed a notably cellular and humoral responses (Lu et al 2001, 2003, 2004, 2007), while a pilot study by Kundu on HIV-infected humans showed no adverse reaction to vaccination with HIV-pulsed MDDCs, but also no significant improvements in HIV-specific immune response or decreases in the viral load (Kundu et al 1998).

Our preliminary results performed on three out of six patients, of which was possible to recover autologous HIV after co-culture of patients' $CD4^+$ with healthy donors $CD4^+$, showed the IFN-γ production from $CD8^+$ cells in one case. In the other two patients analysed, we had some problems related to the low number of events analysed (see Table 2.6).

Immunotherapy applied in spite of, or in concomitance with, antiretroviral regimens represent an exciting goal for future application in the field of immune reconstitution especially in HIV-infection for what complete and definitive viral eradication is not yet possible with current therapies.

Study n° 3 : Rapid T-cell receptor CD4+ repertoire reconstitution and immune recovery in unrelated umbilical cord blood transplanted paediatric leukaemia patients. (Paper 4)

Clinical parameters

In Table 3.2 clinical characteristics of patients are reported.

Pat	HLA compatibility	GVHD acute	GVHD chronic	Chimerism	CMV react.	Current status
P1	4/6	I	II	Full donor	Yes	Alive
P2	4/6	I	-	Full donor	Yes	Alive
P3	4/6	II	I	Full donor	Yes	Alive
P4	4/6	-	-	Full donor	No	Alive
P5	5/6	III	-	Full donor	No	Alive
P6 †	4/6	-	-	Mixed	No	Death

Table 3.2. Patients characteristic. *† : Dead patient.*

Clinical outcome

Successful engraftment was observed in all children enrolled. Five out of 6 children survived with a median follow-up duration of 36 months (range 31-48 months). One patient died from relapse of the original disease 18 months post transplantation. Acute GVHD was observed in 4 children. Limited chronic GVHD was detected in 2 children.

Three children, positive for cytomegalovirus (CMV) before transplantation, developed recurrent CMV reactivations after UCBT and were successfully treated with specific antiviral treatment.

HLA typing and chimerism

One patient (p5) was matched for 5 out of 6 antigens while the other 5 patients were matched for 4 out of 6 antigens. All patients except p6 were documented to be fully donor chimeric after transplantation.

Cell counts

All children analysed had sustained recovery of both neutrophil and platelet counts. The median time to neutrophil engraftment was 26 days (range 20-40 days). The median time to reach sustained platelet count of more than 20.000 was 65 days (range 33-184 days).

Changes in absolute values of total lymphocytes, T-cell subset, B-cells, and NK-cells are shown at 3, 12, and 24 months post-transplant in Table 3.3.

The absolute count of lymphocytes increased gradually during the first year post-transplantation and reached a normal count (Wagner 1996) within 12 months in 4 out of 6 patients The absolute numbers of $CD3^+$ T cells at this time point was also normal in 3 out of 6 patients. Similar to $CD3^+$ T cells, $CD4^+$ recovery was relatively rapid and 4 out of 6 patients restored $CD4^+$ T-cell counts within the normal range by 12 months post-UCBT.

$CD8^+$ T cell reconstitution was less rapid than $CD4^+$ T-cell recovery with 5 out of 6 patients having $CD8^+$ T-cell count below the normal range until 12 months post-UCBT. At 12 months post UCBT the absolute numbers of $CD4^+$ T-cells expressing the $CD45RA^+$ were within the normal range in the majority of patients, whereas the absolute numbers of $CD8^+$ $CD45RA^+$ T-cells were lower.

Pat	Age at CBT (y)	N° Lymphocytes 3-4 mo	N° Lymphocytes 12 mo	N° Lymphocytes 24 mo	N° CD3$^+$ 3-4 mo	N° CD3$^+$ 12 mo	N° CD3$^+$ 24 mo	N° CD4$^+$ 3-4 mo	N° CD4$^+$ 12 mo	N° CD4$^+$ 24 mo	N° CD8$^+$ 3-4 mo	N° CD8$^+$ 12 mo	N° CD8$^+$ 24 mo	N° NK 3-4 mo	N° NK 12 mo	N° CD19$^+$ 3-4 mo	N° CD19$^+$ 12 mo
P1	13	1020	2000	2310	184	1040	1317	163	680	485	10	240	462	704	540	163	360
P2	6	640	3120	2870	205	1872	1837	90	1248	1119	109	562	631	358	218	64	998
P3	5	590	1930	3500	159	1177	1995	65	733	1400	83	290	525	83	232	136	502
P4	4	910	2600	2660	Nd	1664	1383	Nd	1248	851	Nd	338	293	Nd	78	Nd	832
P5	1	920	1360	6040	368	272	1872	230	217	1872	129	54	785	460	285	10	557
P6 †	14	1010	1110	-	50	288	-	40	200	-	10	78	-	848	200	151	544
K	1-2	5500 (3600-8900)			3550 (2100-6200)			2160 (1300-3400)			1040 (620-2000)			360 (180-920)		1310 (720-2600)	
K	2-6	3600 (2300-5400)			2390 (1400-3700)			1380 (700-2200)			840 (490-1300)			300 (130-720)		750 (390-1400)	
K	6-12	2700 (1900-3700)			1820 (1200-2600)			980 (650-1500)			680 (370-1100)			230 (100-480)		480 (270-860)	
K	12-18	2200 (1400-3300)			1480 (1000-2200)			840 (530-1300)			530 (330-920)			190 (70-480)		300 (110-570)	

Table3.3. Table 3.3 Immunological recovery of patients treated with UCBT:
laboratory findings at different time points after transplantation.
Values are reported as absolute numbers (x 10^3/ml)
K : Age-matched reference values according to Shearer 2003; values are the medians, with ranger from 5 to 95 percentiles in parentheses.
† : Dead patient
ND : not determined

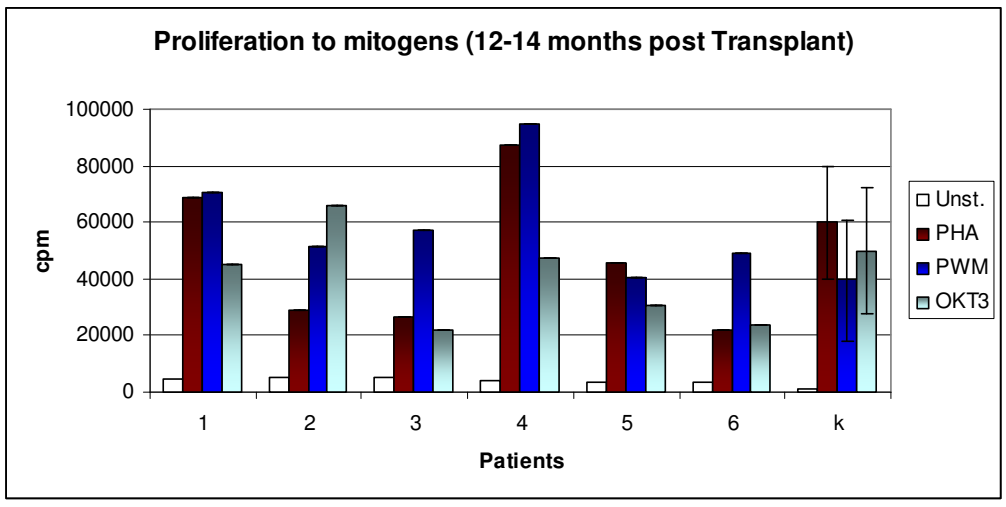
Similar analysis at 24 months post-UCBT showed a normalization of the naïve compartment in all patients either in $CD4^+$, either in $CD8^+$ subset, as reported in Table 3.4.

Patients	**Age at CBT (y)**	**N° $CD4^+$ $CD45RA^+$**			**N° $CD8^+$ $CD45RA^+$**		
		3-4 mo	12 mo	24 mo	3-4 mo	12 mo	24 mo
P1	13	34	238	243	83	144	328
P2	6	9	1023	705	30	478	505
P3	5	10	542	1190	29	209	404
P4	4	Nd	890	Nd	Nd	281	Nd
P5	1	23	80	1741	77	40	746
P6 †	14	Nd	73	-	nd	55	-
K	1-2	1650 (1000-2900)			940 (490-1700)		
K	2-6	980 (430-1500)			670 (380-1100)		
K	6-12	570 (320-1000)			540 (310-900)		
K	12-18	400 (230-770)			400 (240-710)		

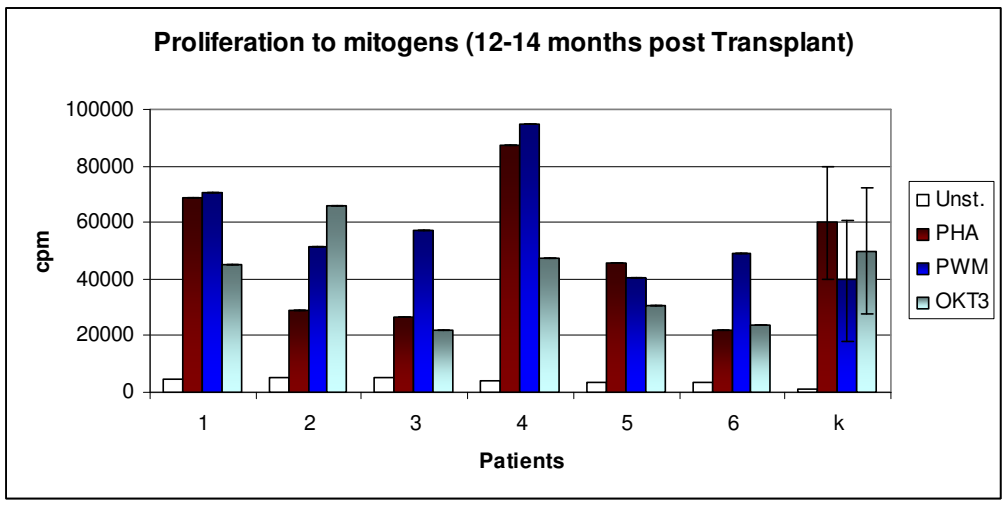
Table 3.4. Absolute numbers of $CD4^+CD45RA^+$ and $CD8^+CD45RA^+$ T-cell subsets. *Values are reported as absolute number (x10^3/ml)*
K : Age-matched reference values according to Shearer 2003; values are the medians, with ranger from 10^{th} to 90^{th} percentiles in parentheses.
† : Dead patient
ND : not determined

T-cell compartment analyses

T-cell proliferation



a)



b)

Figure 3.1 : Proliferative response to mitogens. *3^H-thymidine incorporation after mitogen stimuli of patients' PBMC in response to PHA, PWM and OKT3 was evaluated* ***a)*** *3-4 months after transplantation and* ***b)*** *10-12 months after transplantation. Values are expressed as cpm, control proliferations are reported in mean ± SD. For patient p4 data relative to t=3-4 months are missing.*

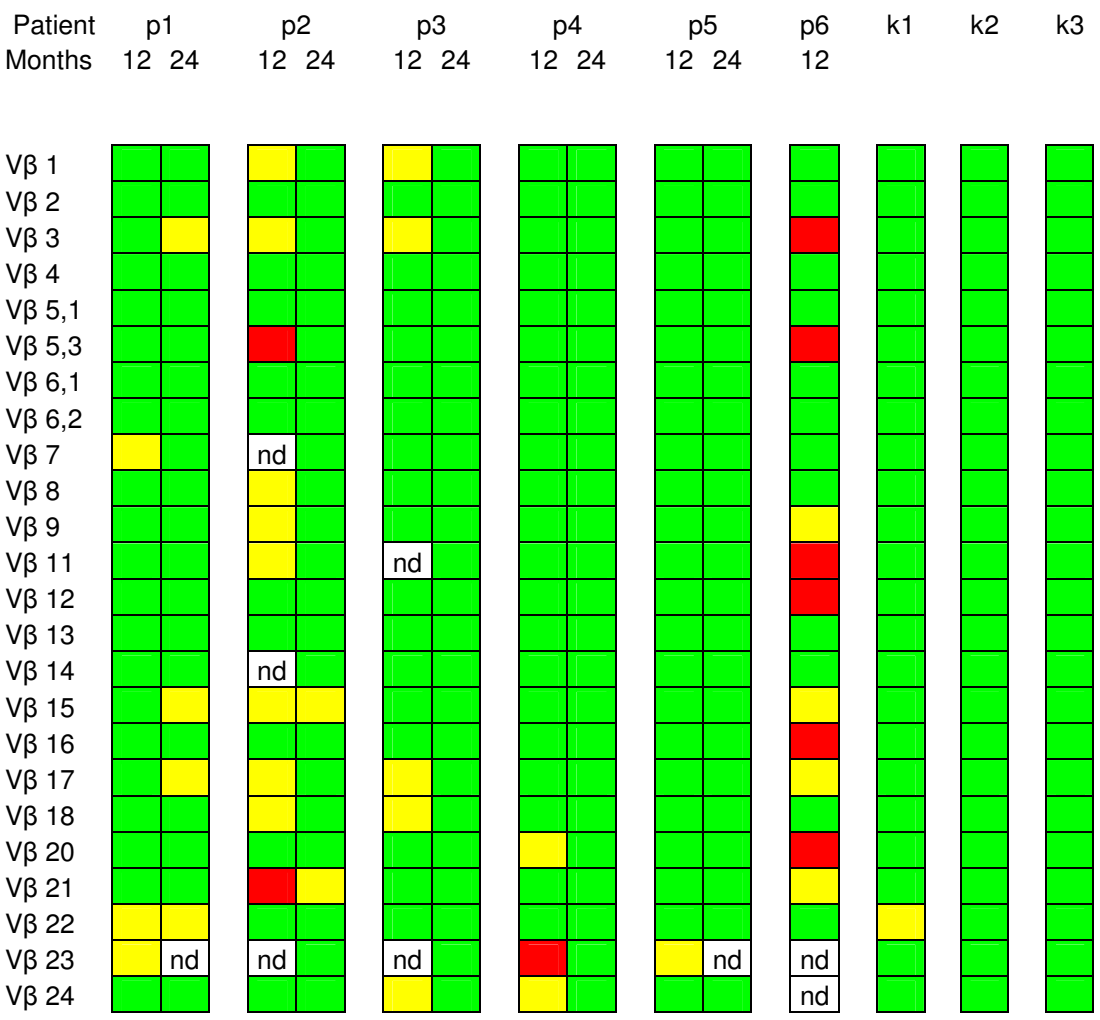
In vitro response of the patients' PBMC to polyclonal activators (PHA, PWM, and OKT3), generally low at 3-4 months after UCBT, improved progressively and reached normal levels in all patients within the first year follow-up as showed in Figure 3.1.

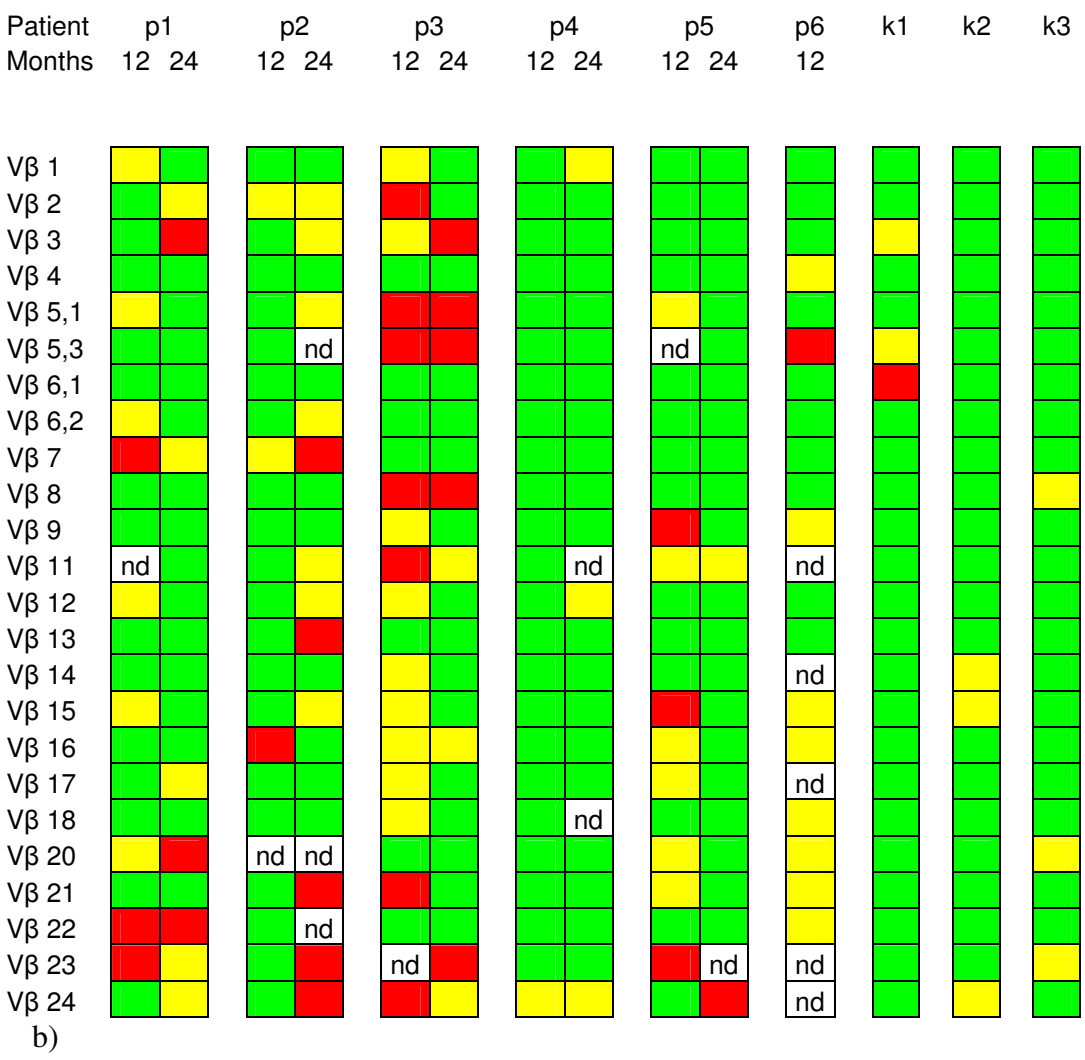
There was not an increment in the response to the antigens tested (Tetanus and Candida) (Data not shown).

TCRBV analyses

To evaluate the recovery of T-cell repertoire post UCBT, CDR3 length distribution of 24 TCRBV families and subfamilies in $CD4^+$ and $CD8^+$ T-cells of transplanted children were analysed by spectratyping at different time points. Data are presented in Figure 3.2

Spectratype analysis of the 5 surviving UCBT recipients at 12 months after transplantation showed a polyclonal Gaussian-like T-cell repertoire distribution on $CD4^+$ subset in most (80,9%) of total TCRBV families analysed, with 16,5% showing a polyclonal altered profile and only 2,6% showing skewed pattern. On the other hand $CD8^+$ subset still exhibited some perturbation in the T-cell repertoire at 12 months after UCBT, with 12,1% of the total TCRBV families showing a skewed pattern of distribution and 20,7% showing a polyclonal altered profile.


Patient p1 p2 p3 p4 p5 p6 k1 k2 k3
Months 12 24 12 24 12 24 12 24 12 24 12
Vβ 1
Vβ 2
Vβ 3
Vβ 4
Vβ 5,1
Vβ 5,3
Vβ 6,1
Vβ 6,2
Vβ 7
Vβ 8
Vβ 9
Vβ 11
Vβ 12
Vβ 13
Vβ 14
Vβ 15
Vβ 16
Vβ 17
Vβ 18
Vβ 20
Vβ 21
Vβ 22
Vβ 23
Vβ 24
nd
a)



b)

Figure 3.2: Kinetic of TCRBV repertoire patterns. *Analyses were performed in each patient and in three healthy control children. White plots: polyclonal; Yellow plots: polyclonal altered; Red plots: skewed; nd: not detected.* ***a)*** *$CD4^+$ TCRBV kinetics;* ***b)*** *$CD8^+$ TCRBV kinetic.*

Two years after transplantation, the reconstitution of TCR diversity appeared to be persistently improving. The majority of TCRBV families in the $CD4^+$ and $CD8^+$ subsets showed a polyclonal Gaussian-like profile (90,5% and 71,3% respectively) with a decrease in the percentage of polyclonal altered profile (9,5% and 16,5% respectively). The few skewed profiles were confirmed in $CD8^+$ subset (12,2%) although were no more present in $CD4^+$ subset. TCRBV repertoire analysis of patient p6 showed a marked altered profile in both $CD4^+$ and $CD8^+$ subsets.

Representative examples of T-cell repertoire analysis are shown in Figure 3.3.

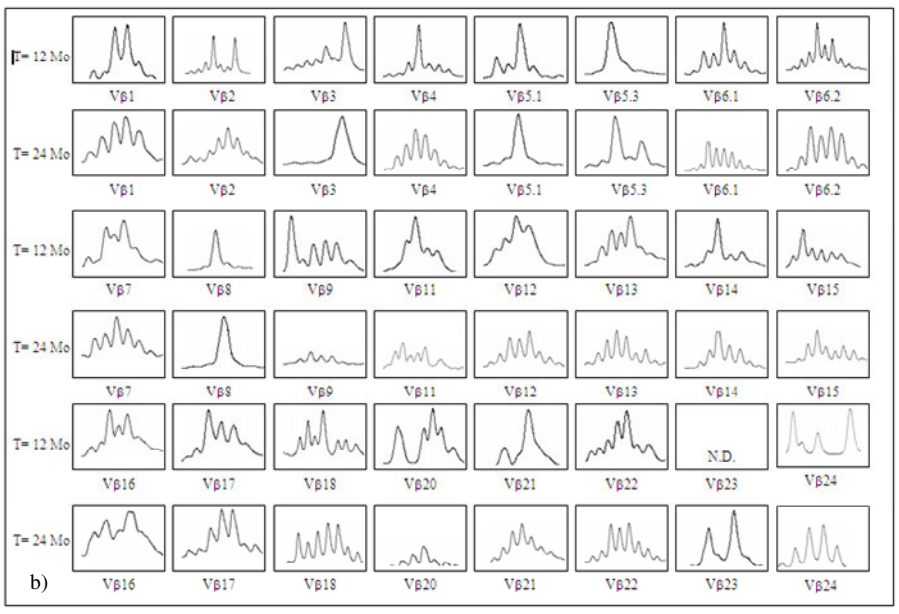


Figure 3.3 Representative examples of TCR CDR3 length distributions. *Data from patient p3 are shown.* ***a)*** *$CD4^+$ T-cell subset distribution.* ***b)*** *$CD8^+$ T-cell subset distributions.*

Discussion

In this 3rd study, we followed the immune reconstitution in a cohort of leukaemia children who underwent to UCBT that is a valid alternative source of haematopoietic stem cells especially for patients lacking bone marrow HLA-matched related donors (Gluckman 1989).

In our study, neutrophils and platelets reconstitute within 30 and 75 days respectively, whereas T, B and NK-cells attained relatively normal levels within 12 months, in accordance with literature data that showed a rapid recover of innate immunity and a slower reconstitution of acquired immunity (Locatelli 1996, Giraud 2000, Thompson 2000, Klein 2001, Crooks 2006, Fry 2005). The functional immune recovery, in terms of in vitro lymphoproliferative ability to mitogens, occurred between 10 and 12 months after transplantation. The absolute number of naïve $CD4^+CD45RA^+$ T cells reached normality within 12 months, while complete recovery of naïve $CD8^+CD45RA^+$ attained within 24 months. In the context of humoral immunity, B-cell reconstitution, assessed by immunoglobulin production, occurred within 3-4 months.

T-cell reconstitution occurs via two predominant pathway (Mackall 1993): a thymic-independent pathway termed homeostatic peripheral expansion that involves expansion of mature T cells which survive the preparative regimen and/or are contained within donor lymphocytes infusions and a thymic-dependent pathway that recapitulates ontogeny. Peripheral expansion is more rapid because thymic-independent, but cells generated through this first wave of immune reconstitution, followed by antigen or cytokine expansion, have a narrower and more skewed repertoire than T lymphocytes generated *de novo* in the thymus (Mackall 1996). Thymic-output is slower because of the

damaged thymic microenvironment by GVHD or exposure to cytotoxic drugs or radiation, but cells generated through this second wave of immune reconstitution have a more diverse T-cell repertoire (Peault 1991).

Useful tools to evaluate the thymic-dependent pathway for the immune reconstitution of T-cell compartment are represented by TREC and spectratype analyses. Previous few studies on TCRBV repertoire reconstitution showed a recovery in the TCR diversification only after two or more years (Klein 2001, Talvensaari 2002). Interestingly, already after one year from transplantation, we show an overall improvement of TCR repertoire diversity in all patient but one, who had persistence of recipient haematopoiesis and died later because of relapse. The faster immune reconstitution observed in our patients could be explained by their early age: indeed either in BMT transplanted children, either in HIV-infected children HAART treated, immune reconstitution resulted faster than in adult population, underlying a key role for the thymus (Mackall 1995, Douek 1998, Romiti 2001) It's important also to consider that we analysed the TCRBV repertoire distribution in isolated $CD4^+$ and $CD8^+$ populations, and not in the total CD3 T-cell population (Klein 2001 et al and Talvenssari et al 2002). This specificity, in our analysis, permits to distinguish cytotoxic and T-helper populations, which have different HLA restrictions and different spectratypes. Indeed we showed a faster normalization of the $CD4^+$ subpopulation toward a Gaussian-like distribution of the CDR3 fragment length, probably due to a preferential and more rapid reconstitution based on thymic function (Mackall 1997), whereas $CD8^+$ subset still exhibited some perturbations, possibly due to the expansion of $CD8^+$ cells resulting from peripheral expansion following antigen-specific exposure. Indeed, a clonal T-cell expansion, in particular within the $CD8^+$ populations was associated with HIV, CMV and EBV-infection (Pantaleo 1994, Callan 1996, Khan

2002,Crooks 2006), and a delayed and incomplete recovery of TCRBV repertoire was also observed in transplanted patients with CMV infection (Imamura 2003). In agreement with these data, our three patients who developed CMV reactivation, did not completely normalize their repertoire. However, the presence of oligoclonal $CD8^+$ T cells also in healthy subjects (Akolkar 1993), suggests that an effective T-cell immunity could be preserved in the presence of such expansions.

Another explanation for the persistence of alterations in $CD8^+$ subset compared to $CD4^+$ subset could be related to their different clonal expansion after Ag exposure. Indeed $CD4^+$ clonal burst is more constrained, so that clones only rarely reach the threshold of detection of current spectratyping methods. Furthermore it seems that in $CD4^+$, clonal expansion is followed by a more marked contraction or less efficient maintenance phase, compared to $CD8^+$ subset which show contraction in longer times (Maini 1999).

TCRBV repertoire data are reinforced by TREC analyses performed in 4 patients: TRECs are present, after 12 or 17 months, in 3 out of 4 patient analysed either in $CD4^+$ either in $CD8^+$ subsets (data not shown), underlying the important role of the thymus in the reconstitution of our patients.

B-cell compartment reconstitution, occurred within 3-4 months after UCBT, was faster in quantitative and qualitative terms, compared to B reconstitution after BMT, that normally occur at least after 1 year from transplantation. These data are in agreement with other UCBT studies (Shono Y 2006, Giraud et al., 2000, Niehues et al., 2001; Inoue et al., 2003, Locatelli 1996, Moretta 2001) and could be explained by a presence of more B-cell precursors in the umbilical cord blood (Hoyt 1999, Arakawa-Hoyt et al., 1999; Knutsen & Wall, 2000). Further studies on B cell subset Ag-specific functionality could be

important to the identification of new therapeutic protocols and to the identification of new prognostic markers.

In conclusion our data indicate a good immune reconstitution in terms of both B and T-cell population, with good lymphoproliferative responses and TCRBV repertoire distribution, confirming the applicability and the advantages of UCBT in spite of BMT especially for patients lacking bone marrow HLA-matched related donors.

CONCLUSIONS

In this study we evaluated the immune reconstitution on three paediatric populations:

1) HIV-infected children early treated with PI-based HAART delayed over the third month of age.
2) HIV-infected children switched from PI-based HAART to a 3NRTI regimen
3) Leukaemia children who underwent to UCBT

We showed as in children immune reconstitution is faster compared to adult population: this age-related immune restoration is a consequence of preferential thymic way of reconstitution in children, which lead to the production of naïve T cells with broad capacity to respond to antigens.

$CD4^+$ T cell subset reconstitution is more rapid compared to $CD8^+$ reconstitution probably due to the fact that $CD8^+$ T cells expansion prefer the extrathymic pathway, responding to specific antigens.

The studies on CTLs open future perspectives to enhance Ag-specific cytotoxicity by the use of engineered MDDCs.

Finally results on faster immune reconstitution of B cells on UCBT compared to BMT open new perspective on B studies and to the identification of new prognostic marker for the evaluation of transplantation success.

Future studies will be focused on the development of specific vaccine for HIV-patients and on the immunotherapeutic approaches to use in spite of, or in concomitance with the present therapies.

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Paper 1

Early highly active antiretroviral treatment delayed over 3 months of life is associated with good clinical outcome, long-term viral control and persistent antiviral T-cell response in HIV-1 vertically infected infants.

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Abstract
Limited data addressed the optimal timing of initiation of HAART treatment of HIV-1 vertically infected infants. Timing is important since the depletion of CD4 T cells and disease progression occur more rapidly in children than in adults. In the present observational study we evaluated the effects of HAART started between 3 and 8 months of life in a cohort of nine infants. The mean time of observation was 47.9 ±18.5 months. HIV-1-specific T-cell responses were detected by lymphoproliferative assay (SI>3) and by intracellular IFN gamma-production to HIV antigens. The T-cell Vβ repertoire (TCRBV) was analysed by spectratyping on CD4+ and CD8+ T-cell subsets. HIV-1-specific T-cell proliferation and complete viral suppression (mean time of persisting HIV RNA < 50 copies/ml: 42.4± 18.2 months) , were observed at multiple time points in all children studied. The TCRBV analysis showed a progressive normalization of the T cell repertoire in both CD8+ and CD4+ subsets. Moreover 2 out of 9 patients, who showed a persistent skewed repertoire on CD8-TCRBV, presented HIV-1- specific CD8+ T lymphocytes response.
Conclusions: Our data suggest that a delayed initiation of HAART after three months of age is associated with good clinical outcome, long-term viral control and a persistent detectable HIV-1-specific immune response.

Key words: HIV-1; infants, HAART, timing of treatment.

Manuscript

Abbreviations: E-HAART Early highly active antiretroviral treatment- HIV-1 Human immunodeficiency virus-1 - TCR T-cell receptor - *HAART* Highly active antiretroviral therapy – *SI* Stimulation index – CPM counts per minute - *PBMC* Peripheral blood mononuclear cells –TCRBV T-cell Vβ repertoire - PCR polymerase chain reaction – NRTI Nucleoside reverse transcriptase inhibitor - BID twice-daily – LPV/RTV lopinavir/ritonavir SD standard deviation – mAb monoclonal antibody- CTL cytotoxic T lymphocytes.

INTRODUCTION

Early highly active antiretroviral treatment (E-HAART) is conventionally defined as initiation of therapy in HIV-infected infants under three months of age [1,20]. E-HAART has recently been suggested in absence of definitive immunological markers recommending the optimal timing of treatment and for the higher risk of disease progression and death in the first year of life [9,11].

Furthermore, an early introduction of therapy with different drugs combination, was shown to induce a complete suppression of viral replication with a significant increase of CD4 counts and a normalization of the TCR repertoire [8,15,22]. However, an high rate of virological failure (over 50% at 48 weeks) has been observed, in infants treated with E-HAART in long-term follow-up studies [1,10,20].Whether to treat HIV-1 infected infants at the time of primari infection remains a crucial question for clinicians [2]. Some Authors suggested that a suppression of viral replication during, a period of relative immunological immaturity, might critically hamper the priming and expansion of virus-specific immune responses [25,28]. Moreover, humoral and cellular HIV-specific immune responses were not detected in the majority of HIV-1 infected infants treated within 3 months of age [4,15,19,25,28,29]. The paucity of HIV-1-specific immune responses in these children could represent an important limitation in periods of viral breakthrough secondary to poor medication adherence, drug resistance or in the prospective of a therapeutic vaccine [5,12,23]. This important issue forms the basis for the present observational study, in which we evaluated how delayed initiation of E-HAART after 3 months of age, influence viral control and the development of an HIV-1 specific T-cell response in a long term follow-up.

MATERIALS and METHODS

Patient population

HIV-1 vertically-infected infants, who started HAART over 3 months of age, were eligible for this observational study. All infants were diagnosed as HIV-1infected on the basis of at least two positive results for the HIV-1- DNA polymerase chain reaction (PCR) and one viral isolation. The institutional review ethical board approved the study and parents or legal guardians gave informed consent before inclusion of the children in the immunologic follow-up study.

Laboratory evaluation

Blood cell counts, lymphocyte phenotyping by flow cytometry and determination of plasma HIV-1 RNA level were performed at baseline and every 3 months. Lymphocyte proliferation assay, TCRBV repertoire analysis were performed at 0, 6-9 and 12 months from HAART initiation and subsequentely every 12 months; HIV-1-specific cytotoxic response was analyzed at the end of follow-up. ***Plasma HIV-RNA determination.*** Plasma HIV- RNA was determined using a quantitative b-DNA assay (Quantiplex HIVRNA 0 bDNA Assay, Chiron Diagnostic Corporation, Emerville, CA, USA) with a lower limit quantification of 50 copies/ml of plasma viral load: values yielding more than 500 000 copies/ml were confirmed by quantitative RT-PCR assay (Roche Amplicor,Roche Molecular Systems, Branchburg, NJ,

USA). The time to virologic suppression was defined as the interval between the start of therapy and the first viral load measurement that yielded undetectable levels (50 copies/ml); the viral load was confirmed by a second measurement. ***Flow cytometry.*** Flow cytometry was performed on peripheral blood mononuclear cells (PBMC) in accordance with standard protocols with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). Anti-human monoclonal murine antibodies conjugated to FITC or PE specific for CD3 (clone UCHT-1), CD4 (clone RPA-T4), CD8 (clone UCHT-2), CD45RO (clone UCHL1), CD45RA (clone HI 100), (Pharmingen, San Diego, CA, USA), were used for the staining. ***Lymphoproliferative assays.*** Patients' PBMC were obtained by Ficoll- Hypaque (Pharmacia,Uppsala, Sweden) density gradient centrifugation from heparinized blood samples. 2x105 PBMC in triplicate were cultured in microtiter plates for 72 hrs with mitogens phytohaemmagglutinin (PHA) 5µg/ml, pokeweed mitogen (PWM) 0.35 µg/ml (Sigma), OKT3 3µl (stock supernatant obtained from OKT3 cell line, ATCC-Sigma, MI, Italy) and 7 days with recall antigens Candida Albicans (7.5:100) Cytomegalovirus (CMV) (7.5:100) and Herpes Simplex Virus 1 (7.5:100) (Amplimedical, To, Italy) and HIV-1 antigens p24 (100 ng/ml) (Intracel, London, U.K.), gp120 (150 ng/ml), gp41 (50 ng/ml) and p17 (80 ng/ml) (NIBSC, Blanche Lane, U.K.). Finally, cultures were pulsed with 0.5 µCi/well 3H-thymidine for 6 hours, then harvested and analysed by B98 counter scintillator (Canberra Packard Instrument Company, Meriden, CT, U.S.A.). Data were valued as counts per minute (cpm) and expressed as Stimulation Index (SI), defined as the ratio between the average counts of stimulated and unstimulated PBMC. Assays were considered valid only for spontaneous proliferation well counts between 400 and 1500 cpm. Patients presenting a SI higher than 3 were considered responder**.** PBMCs were obtained from 10 HIV-negative healthy children and used as negative controls.

TCRBV repertoire analysis. Fresh PBMC from patients were fractionated into CD4+ and CD8+ subsets using anti- CD4 or anti- CD8 monoclonal antibody coupled magnetic beads (Dynal AS, Oslo, Norway). At the last point of follow up, CD8+ lymphocytes of patients #6 and #9 were fractionated into CD8+CD45RA+ and CD8+CD45RO+ subsets using antibody-coupled magnetic microbeads (Miltenyi, Bergisch Gladbach, Germany) according to the producer's protocol. From each cell subset obtained total RNA was extracted by TRIzol® (Gibco-BRL/Life Technologies, Milan, Italy) according to mildly modified manufacturing instructions from 2-3 x 106 cells. Briefly, cDNA was synthesized and amplified by a SuperScript One-Step RT-PCR kit (Invitrogen, Milan, Italy) using 24 different primers for unique 5'Vβ sequences in combination with a primer for the TCRBV region 3' Cβ primer labelled (Mmedical, Milan, Italy) [21]. Final mix were heat at 50°C for 20 min followed by 94°C for 5 min and cDNA were amplified by 32 cycles: 95°C 45 s, 55°C 45 s, 72°C 45 and finally extended at 72 ° C for 10 min in a GeneAmp 9600 DNA thermal cycler (Perkin Elmer Cetus). Products were run on a 6% polyacrylamide gel for 220 min on a DNA Automatic Fluorescence Sequencer (Pharmacia ALF DNA Sequencer) and analyzed by a specific software (Pharmacia DNA Fragment Manager 2.0). Three main patterns of distribution were observed and defined lightly changing our previous definitions [22]: polyclonal (p), polyclonal altered (pa) and skewed/perturbed (sk). Polyclonal profiles had 5-9 peaks per TCRBV family and showed a gaussian "bell shaped distribution"; polyclonal altered profiles showed 5 or more peaks with a non gaussian-like distribution pattern; skewed profiles consisted of 1 to 4 peaks or a multipeak pattern with one solitary peak >50% of the total area or one or more deleted peaks. ***Analysis of HIV-1-specific CD8+ T-lymphocytes.*** HIV-specific CD8+ T lymphocytes were analysed as previously described, with minor modifications [3]. Briefly,

HIV-1 clade independent and HLA-class I promiscuous peptides designed on HIV-1 GAG (EKIRLRPGGKKKYRL,PGGKKKYRLKHLVWA,PGGKKKYRMKHLVWA,KKYRLKHL VWASREL ,KKYRMKHLVWASREL,KHLVWASRELERFAV, RELERFAVNPGLLET, RELERFAVDPGLLET, SAPKTGTEELRSLYN, SALKTGTEELRSLYN, EELRSLYNTVATLYC, SLYNTVATLYCVHQR, SLYNTVAVLYCVHQR, SPRTLNAWVKVIEEK, KVIEEKAFSPEVIPM, EKAFSPEVIPMFSAL, SPEVIPMFSALSEGA, ATPQDLNMMLNIVGG, ATPQDLNTMLNIVGG, QDLNMMLNIVGGHQA, MMLNIVGGHQAAMQM, SNPPIPVGDIYKRWI, GDIYKRWIILGLNKI, KRWIILGLNKIVRMY, LGLNKIVRMYSPVSI, LGLNKIVRMYSPTSI, RMYSPVSILDIKQGP, RMYSPTSILDIKQGP), TAT (SQPKTACTKCYCKKC, SQPKTACTNCYCKKC, NCYCKKCCYHCQVCF, CCYHCQVCFLTKGLG, CCLHCQVCFLTKGLG, VCFLTKGLGISYGRK, VCFLNKGLGISYGRK, CFLTKGLGISYGRKK, CFLNKGLGISYGRKK, LGISYGRKKRRQRRG) and NEF (VPLRPMTYKAAL, AALDLSHFLKEK, DLSHFLKEKGGLDGL, DLWVYHTQGYFP, WVYHTQGYFPDWQ, FPDWQNYTPGPGIRY, NYTPGPGIRYPLT, GIRYPLTFGWCFKLVP, FKLVPVEPEKIEE, VEPEKIEEANKG, EANKGENNCLLHPM, ENNCLLHPMSQHGWM, SQHGWMTRREKC) proteins (Sigma-Genosys, Cambridge, UK) were used as specific stimuli for the patients' PBMC. 1x106 fresh PBMC in 1 ml of complete RPMI medium were incubated with 1 μg each of *anti*-CD28 and CD49d monoclonal antibodies and 1 μg pool of GAG, TAT and NEF peptides. The IFN-γ release induced by PMA (50 ng/ml) plus ionomycin (10 μg/ml) was used as a positive control. The cultures were incubated at 37 °C in a 5% CO2 incubator for 1 h, followed by additional 5 hr incubation with 10 μg/ml of the secretion inhibitor Brefeldin-A (Sigma, St. Louis, MO). Cells were washed and then stained with monoclonal antibodies specific for anti-human CD3 (IgG1, clone RPA-T3); anti-human CD8 (IgG1 clone MOPC-21) (all antibodies from Becton Dickinson Immunocytometry Systems, San Jose, CA) for 15 min at 4°C. Samples were fixed in 1% paraformaldehyde for 10 min at 4°C, incubated with anti-interferon (IFN)-γ mAb diluted in PBS 1X, BSA 1% and saponin 0.5%. Cells were acquired by FACScalibur (Becton Dickinson, Immunocytometry Systems). At least 200.000 live events were acquired, gated on small viable CD3+CD8+ lymphocytes. Data files were analysed using CellQuest software (Becton Dickinson). Results are expressed as frequency of IFN-γ producing CD8+ T cells per 106 CD8+ T cells. PBMCs were obtained from 5 HIV-negative healthy children and used as negative controls.

Statistics

Clinical data are presented as means ± standard deviation (SD) of the mean. The correlation between lymphoproliferative response and age was analysed by the non-parametric Spearman rank test. Data were analysed by using GraphPad Prism Software version 4.00.

RESULTS

Patient characteristics and Clinical outcome

Nine HIV vertically-infected infants, who started treatment between 3 and 8 months of age, with a combined antiretroviral therapy, were enrolled between January 1998 and December 2004 and studied for a mean follow-up period of 47.9 ±18.5 months. Their mean age at initiation of treatment was 4.9 ±1.8 months. The infants underwent HAART therapy with two or three NRTI plus Nelfinavir as a protease inhibitor at the dose of 75 mg/kg/BID or, for the last two patients enrolled under recent guidelines,[26] lopinavir/ritonavir at the dose of 12.5/3 mg/kg/BID. Children were followed at Paediatric Hospital "Bambino Gesù", in Rome. Individual patient characteristics and antiretroviral regimens at baseline are outlined in Table

1. During follow-up, all patients maintained a good clinical status with normal linear growth. No serious adverse events occurred, neither signs of lipodystrophy were clinically documented in any of the infants enrolled in this study. Good compliance to the antiretroviral treatment was reported in all patients by periodic questionnaires and pill counts.

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Virologic evaluations

The clearance of HIV-1 virions in plasma following the initiation of therapy was biphasic, as previously described [18]. Major part of the plasmatic virus was cleared during the firsts 4 weeks, with a slower second-phase decline, leading to complete viral suppression (<50 copies /ml) within a mean period of 5.4 ± 2.9 months of treatment. The HIV-1 viral load remained undetectable during the follow-up for a mean of 42.4± 18.2 months (Table 2).

Immunologic evaluations

The percentage of CD4+ T-cells increased from a mean value of 29±13.2% to 36±5.9% during the long-term follow-up (Table 2). A rapid and consistent increase in CD4+ T-cell counts was observed within the first months even in the three infants (patients #6, #7, #9) with a severe immunodeficiency at baseline. The subset distribution of CD4+ and CD8+ naïve and memory (CD45RA+ and CD45RO+ respectively) was maintained in the normal range values for age [27] (data not shown).

Proliferation to mitogens and recall antigens showed normal conservation during the follow-up compared with age-matched healthy controls. In all children studied, an *in vitro* T-cell proliferation (SI >3) to at least one of the HIV-1 antigens tested (p24, p17, g120 or gp41) was detected at multiple time points, The capability of responding to HIV-1- specific antigens is not related with age (Fig.1).

TCRBV repertoire analysis

Normal polyclonal profiles of the CD4+TCRBV repertoire were observed in five study subjects (patients #1, #2, #3, #6 and #7) at baseline. In the remaining subjects (patients #4, #5, #8, #9) the CDR3 profiles of the CD4+ subset demonstrated a skewed pattern (Table 1). During the follow-up, a complete normalization of the CDR3 patterns was observed in three out of four patients (patients #4, #5 and #8), with only a partial improvement in patient #9 (Table 2). The CD8+ TCR CDR3 length distribution, as expected, was significantly altered at baseline in all infants analysed (Fig. 2). At the end of follow-up , the TCR analysis on CD8+ T-cell subset showed Gaussian-like distributions of CDR3 length in almost all patients (Fig. 2). Only patients #6 and #9 presented persisting skewed distributions in over 50% of TCRBV families during 5-6 years of therapy (Fig.2, Table 2). The CD8+/CD45RA+ and CD8+/CD45RO+ TCRBV repertoires analysed in these two children at the end of follow-up period showed a higher prevalence of altered TCRBV in the CD45RO+ subset (skewed: 57.14%) compared to CD45RA+ (skewed: 12.50%).

Analysis of HIV-specific CD8+ T-lymphocytes

Intracellular staining to evaluate the frequency of IFN-γ positive CD8 T-cells after stimulation with HIV-1 -specific peptide pools for GAG, TAT and NEF protein was performed in 7 out of the 9 subjects in the study at the end of follow-up period. In all patients, CD8+ T lymphocytes had the capacity to respond to non-specific stimuli such as PMA/ionomycin (34674±31388 CD8+/IFN- γ + T cells/106 CD8+ cells). Two of these patients (patients #6 and #9) presented a higher number of HIV-1-specific CD8+/IFN-γ + T-cells in response to HIV-1 peptides (1200/106 CD8+ cells and 3800/106 CD8+ cells, respectively). The other study subjects (patients #1, #3, #4, #7 and #8) did not show specific CD8+ reactivity (all <100/106 CD8+ cells) (Table 2), similarly to HIV-1-negative control (data not shown).

DISCUSSION

The exact timing of initiation of antiretroviral therapy in vertically infected infants has recently been debated [1,2,8-11,26,29].To date no robust immunological markers in the first year of life were available to indicate when to initiate therapy [9]. Timing of treatment is important since the depletion of CD4 T cells and disease progression occur more rapidly in children than in adults [11].

In this context, we report that the delayed initiation of early antiretroviral treatment over 3 months of life is associated with good clinical and immunological outcome, with a persistent detectable HIV-1-specific cellular response. in all the infants enrolled in this long-term observational study. Anti-HIV-T-cell responses appear to be crucial in the control of viral replication during periods of viral breakthrough or in a prospective of a therapeutical vaccine [5,12,23]. Several Authors [4,19,25,28,29] argue that, a robust antiviral therapy initiated in the first weeks of life may reduce the availability of viral antigen for processing and presentation to the T-cells compartment, thus impairing the generation and expansion of HIV-1 specific memory response. As timing of treatment appears to be crucial; a longer exposure to HIV-1 after birth could initiate and maintain a persistent memory response [6,16]. Supporting this hypotesis is the observation of immunological data arising from untreated HIV-1 - vertically infected infants, which show that an HIV-1- specific T-cell response is most commonly detected after 6 months of age [25]. In this cohort of infants, the virus was completely cleared from the plasma only after 6 months of age in all patients. After this period, a complete viral control, with values of viral load below 50 copies/ml was maintained over time. This long-term viral control contrasts to previous studies in which, similar antiretroviral treatments, started early after birth were associated with a high rate of viral failure (over 50% after 48 weeks) in the long term [1,10].The development of the HIV-1-specific immune response might have contributed to achieve the long-term viral control observed in this cohort. In fact, we detected, at multiple time points, a robust specific lymphoproliferative response (SI > 3) to HIV antigens , in all infants. Several Authors have outlined that the ability of CD4+ cells to proliferate upon re-stimulation with HIV-1- antigens seems to uniquely correlate with viral control and long-term non-progressor status [12,23]. Fenney et al. [12]. suggested that the development of specific HIV-1-immune responses may be linked either to age or to insufficient antigenic stimulation during a critical period such as the first year of life. Data reported in this study support this latter hypothesis. (see also Fig.1). The functional data herein reported are further supported by the analysis of TCRBV T-cell repertoire distribution. Although marked alterations were observed at the baseline, the TCRBV T-cell repertoire distribution improved during the long-term follow-up, thus mirroring an intact T-cell population with the potential to recognize a wide range of immunogens. [14,22]. However, a partially skewed repertoire characterized by a clonal dominance in the CD8+ T-cell subset persisted at multiple time points in some TCRBV families in two patients. Interestingly, this oligoclonal distribution was restricted to the CD8+ memory subset (CD8CD45RO+) [17]. It is noteworthy that, these two patients (#6 and #9) showing a persistent altered CD8+ memory TCRBV repertoire were the ones presenting an anti-HIV specific CTL response at the end of the follow-up period (Table 2). The lack of HIV- specific CD8+ response in the other five children could be explained by the long-term viral suppression and the young age of the patients at the time of the analysis. In this context, recent data indicate that CTL responses against HIV-1 in infants are mostly detectable only after 3 years in those patients with a partial viral control [6,24]. Interestingly, an HIV-specific CD8+ response was further detected in these five patients once switched to a simplified therapy regimen (data not shown) [7].Our

study lacks the statistical potency necessary to yield definitive conclusions. Particularly limitations are the small sample size and single-arm design . However, these data show that a delayed initiation of HAART after three months of age is associated with good clinical outcome, long-term viral control and a persistent detectable HIV-1-specific immune response. Additional studies are necessarily to determine the optimal timing of treatment in vertically infected infants and the mechanisms by which early therapy may influence the development of HIV-1 specific response. Understanding how initial antigen exposure, virus-specific T-cell response and viral control interact will have important implications for clinical management of HIV-1 vertically infected newborns, and would be mandatory in a prospective of therapeutical vaccine.

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Figure legends

Fig.1 Correlation of lymphoproliferative response to HIV-1 p24 (panel a), p17 (b), gp120 (c) and gp41 (d) proteins with age during patients follow-up. The stimulation index for each test performed during follow-up related to the age of the child when the test was performed is represented respectively on y-axis and x-axis. Spearman rank test and regression curves are shown.

Fig.2. Percentage of skewed/polyclonal altered TCRBV families in CD8+ subset analysed at baseline and at the end of follow-up.

Table 1. Patients baseline characteristics

Patient ID	Race	Neonatal prophylaxys	Mode of delivery	CDC classification at baseline	Age at initiation of HAART (baseline months)	Plasma HIV RNA (Log copies/ml)	Baseline % CD4	Distribution of CD4 TCRBV Repertoire (percentage of skewed and alterated TCRBV family)	Antiretroviral treatment
1	Caucasian	AZTx 42 days	Elec Ces.Section	N1	3.1	5.1	54	Polyclonal	ABC+3TC+LPV/RTV
2	Caucasian	None	Vaginal	N1	3.2	7.1	23	Polyclonal	AZT+3TC+NFV
3	African	None	Emerg.Ces.Section	A1	3.3	5.8	40	Polyclonal	3TC+d4T+NFV
4	Caucasian	AZT x 42 days	Vaginal	N1	3.3	5.1	31	Skewed (85%)	AZT+3TC+LPV/RTV
5	Caucasian	None	Vaginal	A1	4.2	5.7	29	Skewed (78%)	3TC+d4T+NFV
6	Caucasian	None	Emerg.Ces.Section	B2	5.9	5.7	27	Polyclonal	AZT+3TC+NFV
7	Caucasian	None	Vaginal	C3	6.6	5.7	5	Polyclonal	ABC+AZT+3TC+NFV
8	African	None	Vaginal	B1	7.0	5.6	30	Skewed (30%)	3TC+d4T+NFV
9	African	None	Emerg.Ces.Section	C3	7.6	5.6	24	Skewed (25%)	3TC+d4T+NFV

Table 2. Patients data at the end of follow-up

Patient ID	Age (months)	Duration of Viral Load <50(cps/ml) (months)*	CD4%	Nadir CD4	Lymphoproliferative response to specific HIV antigens tested (p24;p17;gp120;gp41)*	Distribution of CD4 TCRBV Repertoire (percentage of skewed and alterated TCRBV family)	Distribution of CD8 TCRBV Repertoire (percentage of skewed and alterated TCRBV family)	HIV-specific CD8+ IFN-γ+ cells/10^6 CD8+cells
1	26	22	39	37	4/5	Polyclonal	Polyclonal alterated (32%)	<100
2	45	34	42	38	4/5	Polyclonal	Polyclonal alterated (21%)	n.d.
3	66	63	42	35	7/9	Polyclonal	Polyclonal	<100
4	23	20	29	25	2/3	Polyclonal alterated (22%)	Polyclonal alterated (37%)	<100
5	61	58	34	28	4/5	Polyclonal	Polyclonal	n.d.
6	55	49	34	32	7/9	Polyclonal	Skewed (54%)	1200
7	59	50	44	37	4/7	Polyclonal	Polyclonal	<100
8	26	22	32	26	2/2	Polyclonal.	Polyclonal alterated (39%)	<100
9	70	64	28	25	7/9	Polyclonal. Alterated(19%)	Skewed (61%)	3800

*Number of positive lymphoproliferative responses to at least one HIV-antigen tested detected at different time points (approximately every 6 months)

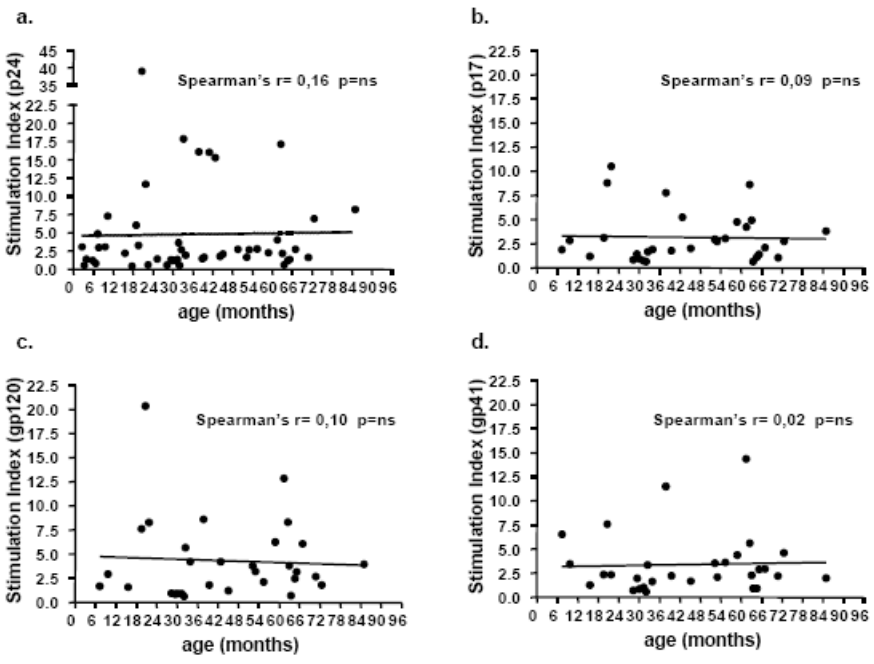

Figure 1.

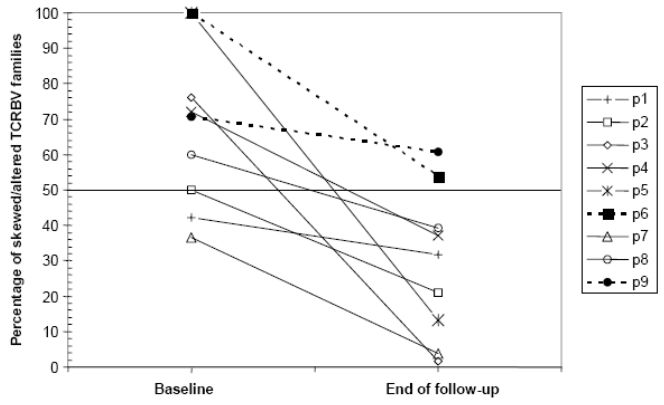

Figure 2.

Paper 2

Successful simplification of protease inhibitor-based HAART with a triple nucleosides regimen in HIV-1 vertically infected children.

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Short title: Triple NRTI therapy as simplification of PI-based HAART in children

Abstract

Objective: To assess the virologic, immunologic and metabolic effects of switching from an efficacious first line protease inhibitor (PI) -based HAART to a simplified triple nucleoside regimen in HIV-vertically infected children

Design: Prospective, open-label, before-after study .

Methods: HIV-1 vertically infected children with at least 12 consecutive months of undetectable viral load under a PI- based HAART and no previous history of NRTI treatment were included in the study.

At study entry, HAART was shifted to a 3-NRTI regimen.

Results: Twenty patients age 2 to 18 years (median 7.9y) were enrolled in the study and followed for 96 weeks. All children were receiving a PI-based regimen for an average duration of 4 years before the enrolment. At study entry 12 patients (60%) switched to ABC; 5 (25%) to 3TC; 2 (10%) to AZT and 2 to ddI (10%). All but one patient maintained undetectable viral load (HIV-RNA <50 cps/mL) during the entire follow-up. No immunological failure was observed at week 96.A trend of normalization (P<0,001) of T-cell receptor V beta (TCRBV) families of $CD8^+$ subset was detected in 19/20 (95%) with an increased HIV-specific CD8+ T-cell response (P<0,01) in 17/20 (85%) of the children. Dyslipidemia significantly (P<0,001) improved during the follow up. No new cases of lipodystrophy were detected.

Conclusions: Switching to 3-NRTI regimens in selected HIV-infected children with an extremely low likelihood of harbouring nucleoside associated mutations maintains viral suppression and immunologic function, improving metabolic abnormalities and the effort to take medication for up to 96 weeks.

Key words: simplification, HAART, HIV-1 infected children

Introduction

Highly active antiviral therapy (HAART) has changed the clinical profile of HIV infection. [Gortmaker; de Martino] However, the chronic nature of HIV-infection usually requires lifelong therapy, and the efficacy of treatment is strictly related to treatment adherence. High daily pill burden associated with dietary restrictions and HAART-related toxicities are factors compromising long-term adherence [Giacomet; O'brien, gibb]. Furthermore, the potentially increased risk of lipodistrophy related with long-term PI exposure has attracted particular concern [Taylor pediatrcs 2004; Viganò JAIDS 2003,Rhoads 2006]. Noteworthy, PI-based regimens have been linked with a significant increase in dyslipidaemia and insulin resistance in children [McComsey AIDS 2004, Carter RJ JAIDS 2006] As a result, numerous strategies of simplified treatment have been explored in order to improve patient quality of life and adherence to treatment, as well as to manage drug-related toxicities while maintaining viral suppression. [Negredo 2006]

Switching to 3 nucleoside reverse transcriptase inhibitors (NRTI) has been shown to be an effective strategy of simplification in adult patients with no previous therapeutic history of suboptimal NRTI exposure or NRTI-related mutations.

[Opravil 2004 Markowitz 2005bojonch JAIDS 2005, Negredo JAC 2006]

Triple-NRTI regimens potentially present important advantages for long-term treatment of HIV infected children which are: good tolerability, decreased metabolic disturbances, low pill burden and reduced economic cost of antiretroviral treatment [Aribas JAC 2004]. Overall, NRTIs have relatively few serious drug–drug interactions and dosing can be independent from food intake. In addition, a triple NRTI strategy allows other antiretroviral drug classes to be kept in reserve for further regimens. [Negredo JAC 2006]

Data on efficacy and tolerability of triple nucleoside regimens as simplified therapy in HIV infected children are not available thus far. In the present study we report the extended follow-up (96 weeks) of a group of HIV infected children switched from successful PI-based HAART to a triple NRTI regimen .

Material and method

Study design and patients enrolment criteria

This is a prospective, open-label, before-after study, approved by the Ethical Commission of Children Hospital "Bambino Gesù**".** Inclusion criteria were: 1) HIV-1 infection as confirmed by at least 2 HIV-1 polymerase chain reaction (PCR) assays; 2) age between 2 and 18 years; 3) first-line treatment with a stable PI-based HAART regimen for at least 12 months; 4) plasma HIV-1 RNA <50 copies/mL for at least 12 consecutive months; 5) stable CD4 values over 25 % in the last year before study entry; 6) no previous treatment with NNRTI or NRTI or history of virologic failure with other regimens. Patients with known opportunistic infections must have had no acute symptoms of infection within the last 24 months before study entry and must have been receiving a stable approved antimicrobial therapy.

At study entry, the PIs were switched, preferentially to a fixed-dose combination of zidovudine (AZT) + lamivudine (3TC) + abacavir (ABC) or to other NRTIs , didanosine (ddI) or stavudine (d4T) at weight-dependent doses, as recommended by the HIV paediatric international guidelines [Sharland 2004].Patients were maintained on their pre-entry NRTI backbone throughout the duration of the study. Older children assuming AZT+3TC or AZT+3TC+ABC used Combivir^{R} or Trizivir^{R} tablets, respectively. Younger children used

broken capsules, weighted to the prescribed dose, and packed tiny pieces in special capsules. Written informed consent was obtained from each patient's legal guardian before study entry.

Endpoints
We defined the followings as primary endpoints: HIV-RNA load >1000 cp/mL at 2 consecutive evaluations and a new AIDS- progressing event. A "blip" was defined as an intermittent plasma viremia with plasma HIV-1 RNA levels between 50 and 1000 copies/mL and a return to an undetectable level at the next determination. When a "blip" was detected a new HIV-RNA quantification was performed within 3 weeks. Secondary efficacy outcomes included immunological failure (considered as changing of CDC immunological classification) and the development of adverse events of grade 3-4. The severity of adverse clinical events and laboratory abnormalities were evaluated according to the standard paediatric AIDS Clinical Trial Group (PACTG) toxicity grading scale.

Laboratory evaluation. Evaluations were performed at baseline and at weeks 0, 2, 12, 24, 36, 48, 60, 72 and 96. At each evaluation, patients underwent a complete medical history and a physical examination that included weight, height, and blood pressure measurements. Laboratory evaluations obtained after at least 8 hours of fasting included complete blood count , blood chemistries (glucose, BUN, creatine, electrolytes, total proteins, albumin, total and direct bilirubin, aspartate transaminase, , LDH, creatin kinase, amylase, lipase) and metabolic evaluations (total cholesterol, HDL and LDL fraction, triglycerides, insulin and lactic acidemia). Dyslipidaemia, was defined as cholesterolaemia and triglyceridaemia over the 95th percentile for age, race and gender, [Christensen et al. Ped. Research. 1980]
In addition, T lymphocytes phenotyping, TCR analysis , HIV-1-specific cytotoxic and lymphoproliferative response and determination of plasma HIV-1 RNA level were obtained at study entry and every 12 weeks thereafter. HIV-1 DNA quantification was performed at baseline and at week 48 in 12 out of 20 patients. ***Flow Cytometry.*** Flow cytometry was performed on peripheral blood mononuclear cells (PBMC) in accordance with standard protocols with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). Anti-human monoclonal murine antibodies conjugated to FITC or PE specific for CD3 (clone UCHT-1), CD4 (clone RPA-T4), CD8 (clone UCHT-2), CD45RO (clone UCHL1), CD45RA (clone HI 100), (Pharmingen, San Diego, CA, USA), were used for the staining. ***T-cell Vβ repertoire*** (***TCRBV)repertoire analysis.*** To analyse the TCRBV repertoire PBMC from patients were fractionated into CD4+ and CD8+, and cDNA was synthesized and amplified from RNA extracted as previously described [Romiti AIDS 2001] by 24 different primers for unique 5'Vβ sequences in combination with a primer for the TCRBV region 3' Cβ primer labeled (Mmedical, Milan, Italy) . RT-PCR products were run on a 6% polyacrylamide gel on a DNA AutomaticFluorescence Sequencer (Pharmacia ALF DNA Sequencer) and analysed by a specific software (Pharmacia DNA Fragment Manager 2.0). Two main patterns of distribution were observed: polyclonal profiles "p" (5 or more peaks) and skewed/perturbed profiles "sk" (from 1 to 4 peaks or a multipeak pattern with one solitary peak >50% of the total area or one or more deleted peaks)..***Analysis of HIV-1-specific CD8+ T-lymphocytes.*** HIV-specific CD8+ T-lymphocytes were analysed as previously described, with minor modifications [Amicosante M., C. Mol. Med. 8:798-807]. Briefly, HIV-1 clade independent and HLA-class I promiscuous peptides designed on HIV-1 GAG, TAT and NEF proteins (Sigma-Genosys, Cambridge, UK) were used as specific stimuli for the patients' PBMC. 1x10^{6} fresh PBMC in 1 ml of complete RPMI medium were incubated with 1 µg each of anti-CD28

and anti-CD49d monoclonal antibodies and 1 µg pool of GAG, and TAT and NEF peptides. The IFN-γ release induced by PMA (50 ng/ml) plus ionomycin (10 µg/ml) was used as a positive control. The cultures were incubated at 37 °C in a 5% CO_2 incubator for 1 h, followed by an additional 5 hr incubation with 10 µg/ml of the secretion inhibitor Brefeldin-A (Sigma, St. Louis, MO). Cells were washed and then stained with monoclonal antibodies specific for anti-human CD3 (IgG1, clone RPA-T3); anti-human CD8 (IgG1 clone MOPC-21) (all antibodies from Becton Dickinson Immunocytometry Systems, San Jose, CA) for 15 min at 4°C. Samples were fixed in 1% paraformaldehyde for 10 min at 4°C, incubated with anti-interferon (IFN)-γ mAb diluted in PBS 1X, BSA 1% and saponin 0.5%. Cells were acquired by FACScalibur (Becton Dickinson, Immunocytometry Systems). PBMCs were obtained from 10 HIV-negative healthy children and used as negative controls At least 200.000 live events were acquired, gated on small viable CD3+CD8+ lymphocytes. Data files were analysed using CellQuest software (Becton Dickinson). An intracellular cytokine staining is to be considered positive when the percentage of cytokine-secreting cells is above 0.01%. Results are expressed as percentage of CD3+ CD8+ IFN-γ producing cells. A two fold increase of HIV-1-specific CD8+ T lymphocytes frequencies during the follow-up was considered significant. ***Plasma HIV-1 RNA determinations.*** Viral control was evaluated by plasma RNA determination, using a quantitative b-DNA assay (Quantiplex HIV-RNA 2.0 bDNA Assay, Chiron Diagnostic Corporation, Emerville, CA, USA) with a detection limit of 50 copies/ml of plasma viral load. Viral values between 50 and 999 copies/ml, preceded and followed by measurements less than 50 copies/ml were defined as "blips". Rate of blips was evaluated, as previously described [Pensieroso AIDS 2006], as the ratio between the number of detected blips and the number of determinations: Rate= n°of blips/ n° of determinations. ***Quantification of HIV-1 proviral DNA.*** To quantify the proviral HIV-DNA copy number in PBMC, we adapted the Real Time TaqMan protocol to the Light Cycler (Roche Molecular Biochemicals, Indianapolis, IN) as previously described [Sarmati J.Clini. Microb. 2005] . We have utilized a standard curve built by seven dilutions of 8E5 cells, known to contain one copy of HIV-DNA integrated for each cell (75.000-37.500-3.750-375-37.5-3.75-1.87 copy/cells). The sensibility of PCR is one copy of HIV-DNA reaction (1 cp reaction = 13.3 cps X 10^6 PBMC). Nevertheless replicates of HIV-DNA sample scoring negative is considered undetectable or has an HIV-DNA load below 20 copies/10^6 PBMC. The HIV-DNA target was hybridized with TaqMan probe and read on channel F1/F2 of the LightCycler.

Adherence and difficulty of taking medications.

Adherence to treatment and difficulty of taking medications were assessed from week 0 onwards by the PENTA (Paediatric European Network for Treatment of Aids) questionnaire as previously described. [Giacomet V Ped Infet Diseas. Volume 22(1), January 2003, pp 56-62].

Questionnaires were filled in at the time of scheduled clinic visits for the trial by caregivers (with children where appropriate) and with the help of the nurse or doctor if required.

Statistical analysis .

To calculate the patients' *z*-scores of lipids and T-cell subsets at each time interval we used normal means and standard deviations for age [for lipids Christiensen J. Paediatr.1980,SHerarer JACI] after controlling that all variables had a normal distribution. Average measures at multiple time points were compared to reference (i.e. initial values) during follow up. Student T-test for paired samples was used for assessing statistical significance . Qualitative variables were analysed trough the χ^2 test.

Results

Study Population

Between January 2003 and June 2005, 20 vertically HIV-infected children, age 2 to 18 years (median 7.9 years), were enrolled in the study and were followed for 96 weeks. Baseline characteristics of the cohort are summarized in Table 1. All patients were stably receiving a PI-containing regimen for a median duration of 4.4 years (range 2 -5years), with a median period of 2.8 years (range 1-4.4 years) of undetectable viral load before study entry.

Immunological evaluations

No immunological failure was observed at week 96. The mean CD4 and CD8 values at baseline were respectively 35,4±7,1 % (*z*-score 0,11±0,99) and 30,3±7,7% (*z*-score -0,1±1,2). The percentage of CD4 slightly increased during the period of follow-up reaching a mean value of 36,2% (*z*-score 0,4±1,8) at weeks 48, and 36,6 ± 5,4 % (*z*-score 0,81±1,7) at weeks 96. The nadir of CD4 percentage for each patient, under the simplified regimen is reported in Table 2. The percentage of CD8% T cells did not vary significantly over time with a mean value of 30,2 ±6,5% (*z*-score -0,03±1,4) at weeks 48, and 28,9 ±5,6% (*z*-score -0,14±1,8) at week 96. Proportions of memory and naïve CD4 and CD8 subsets were normal for age at study entry [ShearerJ.Allergy Cli. Immun] and remained stable over time (data not shown).

At study entry T-cell Vβ (TCRBV) repertoire distribution revealed relevant (≥ 25%) perturbed patterns of CD8+ TCRBV families in 11/20 patients (patient #3, #5, #6, #7, #9, #11, #12, #13, #16, #19, #20). As shown in Table 2 in all but one patient (patient#4 who experienced virologic failure at week 84) a significant (P<0.001) trend toward a polyclonal pattern in TCRBV families of the $CD8^+$ subset was observed.

In addition, compared to the baseline, an increased percentage (mean at baseline 0,12±0,1 versus 0,78±0,77) (P<0.001) of HIV-specific IFN-γ producing CD8+ T-cells was detected in 17/20 (85%) children (Figure 3).

Virological outcome

All but one patients (patient #4) maintained virologic control till the end of the study. The median blips ratio observed during triple NRTI treatment did not increase over time compared with previous PI- based HAART regimen (0,1 [range 0-0,4] versus 0,05 [range 0-0,3]). Ten patients did not present blips during simplified regimen (patient #1, #2, #5, #9, #11, #12, #14, #15, #17, #18). (Table 2) Patient #4 experienced virological failure at week 84 after autonomous interruption of treatment. Genotypic analysis at failure yielded mutations M184V, and other secondary mutations such as: V35M, S68GS, W88C, D123E, I178L, R211K.

Quantification of proviral DNA-load was performed in 12 out of 20 (60%) children (Table 1).The median proviral DNA-load remained stable between baseline and week 48 (respectivel 2.5 $\log_{10}$ copies/10^6 PBMC [range 1.6-3 $\log_{10}$ copies/10^6 PBMC] and 2.6 $\log_{10}$ copies/10^6 PBMC [range 1.8-2.9 $\log_{10}$ copies/10^6 PBMC]).

Safety and Metabolic Profile.

The 3NRTI regimen was well tolerated. A hypersensitivity reaction to ABC was reported after 2 weeks of switching in one patient (p#13). The discontinuation of ABC and shifting to ddI resulted in the resolution of symptoms.

No clinical, laboratory signs of hyperlactatemia or other side effects were detected.

Levels of cholesterol and triglycerides over the 95th percentile for age, race and gender were present respectively in 7/20 (35%) ad 5/20 (25%) of patients at baseline. The proportion of patients with high levels of cholesterol declined over time from 35% at baseline to 0% at week 96. Mean total cholesterol decreased from 189 ± 51 mg/dl (*z*-score: 1,18± 2,08) at baseline to 157 ± 27,5 mg/dl (*z*-score: -0,07± 1,19) at week 48 (*P*<0.01), and to 152 ± 29,5 mg/dl (*z*-score: 0,11± 0,99) at week 96 (Figure 1a). Low-density lipoprotein (LDL) cholesterol significantly decreased from a mean of 114 ± 37 mg/dl at baseline to 84 ±26 mg/dl at week 48, and to 76 ± 23 mg/dl at 96 weeks (*P*<0.01) while high-density lipoprotein (HDL) cholesterol did not change significantly over time (Figure 2). The cholesterol:HDL ratio significantly (*P*<0.01) decreased from a mean baseline of 3.4 ±0.6 to 2.2 ±0.5 at 96 weeks (Figure 2).
Fasting triglycerides mean value declined from 93 ± 52 mg/dl (*z*-score: 1,18 ± 2,2) to 72 ± 38 mg/dl (*z*-score 0,4± 1,2) at week 48, and to 55 ±25 mg/dl (*z*-score 0,06± 1,03) at week 96 (*P*<0.01). Furthermore, the percentage of patients with high levels of triglycerides decreased from 25% to 0% at week 96 (Figure 1b).
Detailed dietary history revealed no significant changes during the study period. None of the patients initiated therapy with lipid-lowering agents.
Lipodystrophy syndrome clinically diagnosed at baseline in 2/20 children as lipoatrophy (patient #5,#11) and in 1/20 (patient #8) as lipo-accumulation remained stable overtime. No new cases of lipodystrophy were clinically detected .

Adherence and difficulty of taking medications.
The mean pills burden per patient decreased from 11,4 ± 2,8 to 2,5 ±1,2 capsules after switching to the 3-NRTI regimen. Compared with the previous PI-based HAART regimen, adherence remained high during the study follow-up. Full adherence was reported at baseline in 99 ± 3% of the children enrolled and in 98 ± 4% and 99 ± 2% at week 48 and at week 96 respectively.
The difficulty of taking medications significantly decreased (*P*< 0.001) at week 12 and remained stable till week 96 .

Discussion
This prospective open-label trial in HIV-1 infected children receiving successful PI- first-line HAART combinations originally shows the efficacy of a simplified treatment with 3 NRTI regimens in terms of maintenance of viral suppression (<50 copies/mL) and immunological function for up to 2 years.
Strategies of HAART simplification with 3NRTI-regimens have been recently suggested for the management of paediatric HIV. [Sharland M. PENTA guidelines 2004, Sharland M. Pediatrics Drugs 2004] In adults an effective and safe simplification of standard PI-based HAART regimen can be achieved with 3-NRTI if this approach specifically targets patients with a low likelihood of harboring nucleoside analogue-associated resistance mutations (NAMs). [OPravil,Bonjoch, Markowitz and Negredo] . In accordance with these studies, our data suggest to consider this strategy in previously antiretroviral-naive HIV infected children, reaching a permanent and durable suppression of plasma viral replication (<50 copie/ml) under their first PI-based HAART regimen, who have low likelihood to develop new viral mutations. [CT Ruff Jour. Virology 2002]
In this cohort the ratio of intermittent episodes of detectable viremia (“blips”) did not increase during the 3-NRTI regimen compared to the previous PI-based HAART treatment. [Pensieroso] Furthermore, proviral DNA , reported to be an informative marker to explore viral

reservoirs and to assess the long-term impact of antiviral treatment[Pellegrin I JID2003, Re MC New Microb. 2006], did not vary significantly during the fist year of follow up. HIV-specific cellular immune response, in addition to the stable good adherence reported in this group, might have contributed to achieve long-term viral control.

Indeed, we recently reported, in this cohort of children, an enhancement of HIV specific limphoproliferative response after switching to 3-NRTI therapy [Pensieroso S et al. AIDS 2006]. Moreover, in this paper we describe an increased HIV-specific CD8+ T cell response. We argue that the further improvement observed is possibly due to the PI removal. In fact, PI may cause immunesuppression by interfering with antigen presentation.[Andre P PNAS 1998] Specifically the PIs nelfinavir and ritonavir [Piccinini Antiviral 2005] , which were mostly given to these patients, have been shown to modulate proteasome peptidase activity, and cause intracellular accumulation of ubiquitin-tagged proteins, a hallmark of proteasome proteolytic inhibition, in vivo[Piccinini Aids 2002].Supporting this hypothesis, *Legrand et al.* recently reported an increased T-cell HIV-specific immune response in terms of intracellular IFN-γ and TNF-α production, in a group of HIV-infected children who underwent to o PI-sparing therapy due to viral failure [le grand AIDS 2005] . The enhancement of HIV specific CD8+ T cells response in our cohort of children is associated with a progressive trend to a polyclonal distribution of TCRVB families in the CD8+ T-subset . This broad T-cell receptor repertoire may supply a multipotential ability to develop specific T cell responses against a variety of antigens including HIV. [Pantaleo 2007 Sader 2003]

Generally, triple nucleoside regimen was well tolerated. The incidence of ABC-related hypsensitivity (5%) observed was similar to the frequency reported in most other paediatric studies (3-5%) [Saez-Llorens X,Paediatrics 2001, PENTA 5 Lancet 2002]. Neither clinical nor laboratory signs of hyperlactatemia were detected in our cohort, confirming the data that this event is more rare in paediatric age. [Leonard EG, .Pediatr Infect Dis J. 2003]. Conversely, nearly 40% of the patients enrolled presented signs of dyslipidaemia at the baseline. Lipid abnormalities improved early and in sustained fashion, in agreement with previous studies showing benefits of PI-sparing treatment on lipid metabolism in children and adults. [Mc Comsey J. Paedi 2003, Viganò A. Antiviral Ther 2005, Keiser P.H. BMC infct. Disease 2005, Katlama 2003 Bonjoch JAIDS 2005] Noteworthy, we have observed a significant decrease of the cholesterol:HDL ratio, an excellent predictor of future ischemic heart disease. [Lemiux I. Archiv. Intern. Medic. 2001]. This improvement is clinically important and implies a decrease in dietary restrictions, lipid lowering agents, and probably in the overall cardiovascular risk of the patient. [European Pediatric lipo group AIDS 2004 and Farley J 2005]. No remarkable changes in body fat mal-distribution were observed in children studied. This data can be partially explained by the perpetuation of mitochondrial toxicity through the continuation of NRTI exposure, particularly d4T and AZT [Viganò A. Antiviral Ther 2005, Nolan D. Antiv Ther 2003]. Moreover, more sensitive measurements such as dual-energy x-ray absorptiometry (DEXA) or MRI scan, should be performed in order to assess changes in body fat distribution. [Viganò A. JAIDS 2003 and Brambilla P. AIDS 2001]

Finally, although our data lacks the statistical potency necessary to yield definitive conclusions, this pilot study demonstrate that simplification to 3-NRTI regimens in selected HIV-infected children is able to maintain viral suppression and immunological function, improving metabolic abnormalities and the effort to take medication in the long term.

Larger randomized trials are necessary to investigate simplifications strategies in HIV-infected children.

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Figure 1. Variations from the baseline of means ± SD of a) Cholesterol z-score and b) Triglycerides z-score. Points indicate mean and whiskers SD. ***P < 0.001, ** P < 0.01, *P <0,05

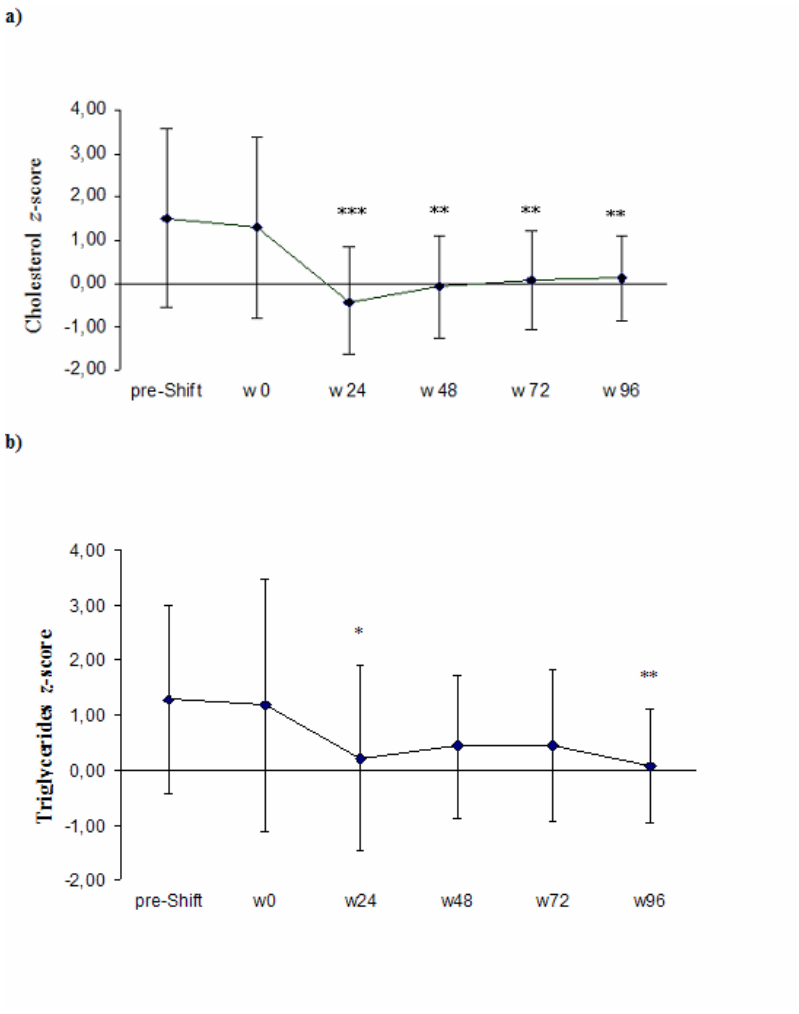
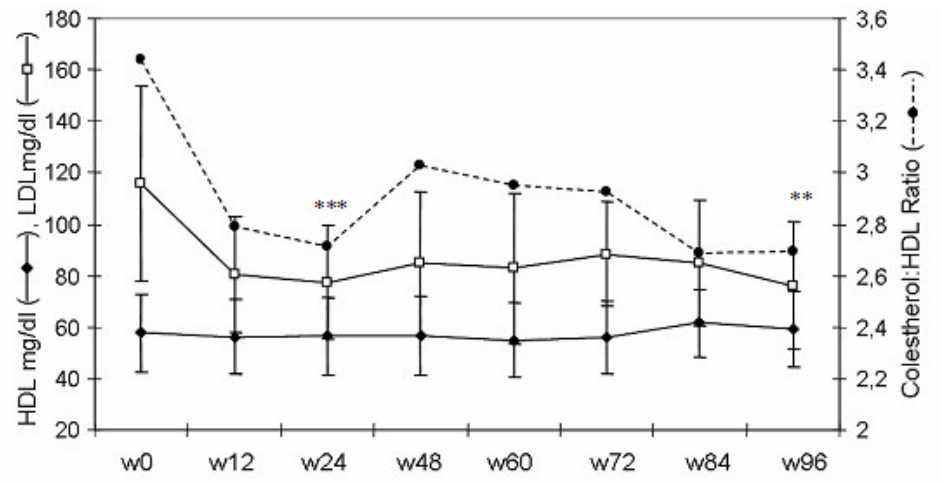


Figure 2. Variations from the baseline of HDL, LDL and the cholesterol:HDL ratio Points indicate mean and whiskers SD. ***P < 0.001, ** P < 0.01 refer to the cholesterol:HDL ratio.



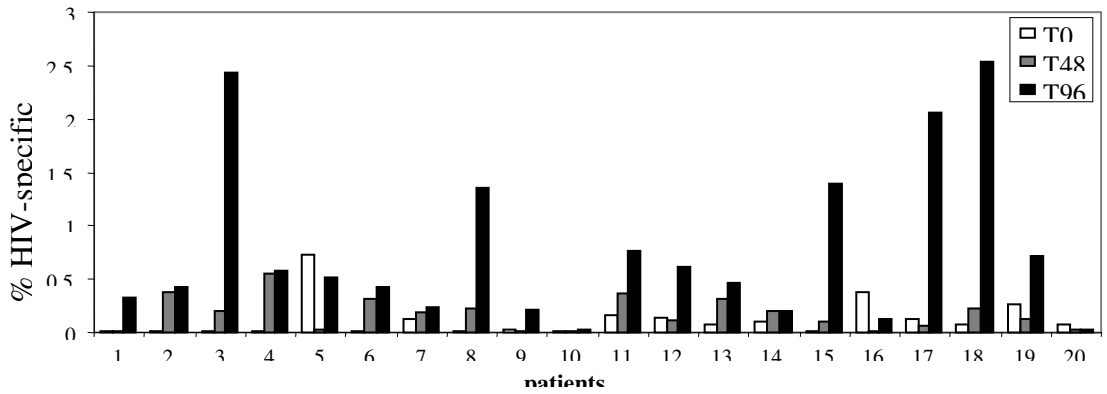


Figure 3. HIV-specific CD8+ T lymphocytes during follow-up. White grey and black columns represent percentages of HIV-specific CD8+ T lymphocytes for each patient. at week 0, 48 and 96 respectively.

Tab.1 Baseline characteristics of patients

Pat. ID	Race	Gender	CDC Classification	Age (years)	Time on PI-based HAART (years)	CD4 nadir % (cell / µl)	Time on consecutive HIV-RNA <50copies/l (years)	DNA load log_{10} copies/10^6 PBMC	Previous HAART treatment	Simplified regimen
1	W	F	A2	6.8	4.5	19 (827)	3.3	2.5	AZT+3TC+**NLF**	AZT+3TC+**ABC**
2	W	F	B3	14.5	5.0	28 (673)	3.1	2.3	AZT+3TC+**RTV**	AZT+3TC+**ABC**
3	W	M	A3	10.6	4.7	14 (506)	3.5	n.d	AZT+3TC+**NLF**	AZT+3TC+**ABC**
4	W	M	N3	13.5	4.1	2 (36)	1	2.2	AZT+ABC+**NLF**	AZT+3TC+**ABC**
5	W	F	A2	7.4	4.6	18 (682)	3.2	n.d	3TC+d4T+**NLF**	3TC+d4T+**ABC**
6	W	M	B2	9.8	4.6	14(523)	1.9	n.d	AZT+ABC+**NLF**	AZT+3TC+**ABC**
7	W	F	A1	9.3	4.6	30(1334)	2.3	3	3TC+d4T+**NLF**	3TC+d4t+**ABC**
8	W	F	N1	7.9	4.4	29 (770)	1.9	n.d	AZT+ABC+**NLF**	AZT+**3TC**+ABC
9	B	F	B2	18	2.7	11 (208)	2.3	2.4	AZT+3TC+**IDV**	AZT+**3TC**+ABC
10	W	F	A2	8.3	4.7	30(980)	3.5	n.d	AZT+ABC+**NLF**	AZT+**3TC**+ABC
11	W	F	B2	14.2	2.6	36(329)	2.5	3	3TC+d4T+**LPV/r**	3TC+d4T+**ddI**
12	W	M	A2	6.7	3.5	28(1262)	2.8	2.9	AZT+ddI+**NLF**	AZT +ddI +**3TC**
13	W	F	B3	12.9	4.4	3 (41)	2	1.6	AZT+3TC+**NLF**	AZT+3TC+**ABC*** /AZT+3TC+**ddI**
14	W	M	N1	11.5	4.6	34 (809)	3.7	n.d	3TC+ABC+**NLF**	**AZT**+3TC+ABC
15	W	F	N1	5	4.9	36(2840)	4.4	1.6	3TC+ABC+**NLF**	**AZT**+3TC+ABC
16	B	M	C3	5.1	4.5	24(555)	3.5	2.5	3TC+d4T+**NLF**	3TC+d4T+**ABC**
17	W	M	B2	5.9	2.1	23(995)	1.7	3	AZT+3TC+**NLF**	AZT+3TC+**ABC**
18	W	F	C3	2.4	2	5(40)	1.3	2.8	AZT+3TC+ABC+**NLF**	AZT+3TC+ABC
19	W	F	A1	4.5	4.2	38(1357)	2. 8	n.d	AZT+ABC+**NLF**	AZT+**3TC**+ABC
20	W	M	N1	3.2	2.9	23 (3195)	1.7	n.d	AZT+3TC+**NLF**	AZT+3TC+**ABC**

Table 1. AZT denote zidovudine, 3TC lamivudine, ddI didanosine, ABC abacavir, d4T stavudine, NLF nelfinavir, IDV indinavir, RTV ritonavir, LPV/r lopinavir/ritonavir, n.d not done. PIs previously used and replacing NRTIs are highlighted. *Patient #13 shifted ABC to ddI due to hypersensitivity reaction.

Pat. ID	Rate of blips during PI-based HAART	CD4 counts % (cells / µl)	CD8 counts % (cells / µl)	Percentage of polyclonal CD8 TCRBV families*	Rate of blips during 3-NRTi regimen	CD4 counts % (cells / µl)	CD4 nadir % (cell / µl)	CD8 counts % (cells / µl)	Percentge of polyclonal CD8 TCRBV families*
		TO					**T96**		
1	0.1	35 (1268)	33 (1182)	91.7	0	41 (1168)	39 (906)	29 (792)	**95.8**
2	0	34 (594)	39 (730)	81.8	0	38 (676)	35 (529)	38 (676)	**95.8**
3	0	25 (400)	36 (529)	75	0.1	29 (524)	24 (406)	38 (505)	**75**
4*	0.4	32 (698)	38 (765)	100	0.2	33 (565)	29 (464)	37 (677)	91.8
5	0.1	27 (412)	26 (319)	66.7	0	29 (389)	26 (443)	26 (348)	**95.7**
6	0.2	29 (980)	29 (1300)	66.7	0.3	35 (1106)	27 (540)	36 (1137)	**100**
7	0.3	33 (833)	26 (770)	61.9	0.1	34 (1159)	28 (1113)	27 (920)	**100**
8	0.1	36 (1172)	27 (935)	90.9	0.3	37 (1043)	35 (940)	30 (846)	**100**
9	0.2	25 (583)	23 (1201)	50	0	26 (654)	27 (505)	26 (1076)	**90.9**
10	0	37 (754)	22 (422)	95.8	0.3	42 (926)	38 (764)	19 (367)	**100**
11	0	46 (906)	29 (552)	72.2	0	38 (1181)	35 (412)	28 (870)	**87.5**
12	0.1	40 (708)	29 (557)	38.9	0	32 (499)	31 (531)	36 (561)	**83.4**
13	0.1	28 (423)	32 (452)	73.9	0.2	30 (513)	31 (498)	34 (581)	**86.4**
14	0	46 (1217)	27 (754)	87.5	0	44 (1007)	44 (994)	26 (595)	**100**
15	0.2	37 (1360)	21 (772)	100	0	41 (1115)	38 (360)	20 (544)	**100**
16	0.1	27 (812)	29 (933)	57.1	0.1	28 (935)	21 (809)	27 (955)	**91.7**
17	0.2	34 (2237)	31 (1956)	82.6	0	32 (1171)	31 (987)	26 (951)	**95.8**
18	0.1	41 (994)	28 (564)	95.7	0	41 (832)	36 (768)	25 (507)	**100**
19	0.2	45 (999)	22 (460)	38.9	0.1	44 (836)	38 (651)	26 (494)	**100**
20	0.1	42 (1332)	25 (629)	60.4	0.1	41 (1025)	40 (1284)	25 (625)	**90**

Table 2 *Percentage of TCRBV families with polyclonal patterns CD8+repertoire (by spectratyping) are reported; the maintaining and improving percentages are highlighted.

Paper 3

Switching from protease inhibitor-based-HAART to a protease inhibitor-sparing regimen is associated with improved specific HIV-immune responses in HIV-infected children

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To evaluate the effects of switching from successful long-term protease inhibitor (PI)-based HAART to a three nucleoside reverse transcriptase inhibitor PI-sparing regimen, viral load quantification, HIV-specific lymphoproliferative assay and T-cell receptor (TCR) spectratyping were performed during 96 weeks of simplification follow-up in 19 HIV-infected children. Our data showed that simplification of therapeutic strategies acts positively on immune competence in HIV paediatric patients. Our children maintained viral suppression, increased lymphoproliferative responses and normalized TCRBV repertoire on the CD8 subset.

Protease inhibitor (PI)-based HAART controls viral replication, augments CD4 cell counts and restores the polyclonality of the T-cell receptor Vβ (TCRBV) repertoire distribution leading to a significant reduction in morbidity and mortality in HIV infection [1–3]. However, long-term exposure to PI-based HAART is associated with a significant incidence of adverse events [4]. Furthermore, a greater risk of viral failure, because of poor compliance, and a limitation of the degree of immune restoration with respect to HIV-specific immune responses were reported in long-term HAART-treated adult patients [4,5].

These limitations have prompted the search for alternative treatment approaches either with more simplified regimens or combined immunotherapy.

Our aim was to evaluate, in a prospective study starting in June 2003 until January 2006, the immunological effects after the switch from a first-line long-term (2–5.5 years, median 4.4) successful PI-based HAART (< 50 HIV copies/ml) to a three nucleoside reverse transcriptase inhibitor (NRTI) PI-sparing regimen in a cohort of 19 HIV-1 vertically infected children (aged 2.4–18.9 years, median 5) for a 96-week follow-up.

Children were enrolled in the study when they fulfilled the following criteria: naive patients between 2 and 18 years of age, receiving a stable PI-containing antiretroviral regimen for at least 2 years, with a normal value of CD4 cell percentage for age before study entry, no previous NRTI therapy nor a history of viral failure. Patients were followed every 3 months for clinical and laboratory investigations. Parents or legal guardians were informed of the therapeutic choices and they gave written informed consent.

Viral control was evaluated by plasma RNA determination, using a quantitative branched DNA assay (Quantiplex HIV-RNA 2.0 bDNA assay; Chiron Diagnostic Corporation, Emeryville, California, USA) with a detection limit of 50 copies/ml of plasma viral load. Viral values between 50 and 999 copies/ml preceded and followed by measurements less than 50 copies/ml were defined as 'blips'. The rate of blips was evaluated as the ratio between the number of blips detected and the number of determinations:

$$\text{Rate} = \frac{\text{no. of blips}}{\text{no. of determinations}}$$

To evaluate lymphoproliferation, 2×10^5 peripheral blood mononuclear cells (PBMC) in triplicate from patients were cultured in microtitre plates for 72 h with mitogens phytohaemmagglutinin (PHA) 5 μg/ml, pokeweed mitogen (PWM) 0.35 μg/ml (Sigma, Milan, Italy), OKT3 3 μl (stock supernatant obtained from OKT3 cell line; ATCC-Sigma) and 7 days with recall antigens *Candida albicans* (7.5 : 100) and Herpes Simplex Virus 1 (HSV-1) (7.5 : 100; Amplimedical, Turin, Italy) and HIV-1 antigens p24 (0.1 μg/ml; Intracel, London, UK), gp120 (0.15 μg/ml), gp41 (0.05 μg/ml) and p17 (0.08 μg/ml) (NIBSC, Potters Bar, UK). Finally, cultures were pulsed with 0.5 μCi/well ^{3}H-thymidine for 6 hours, then harvested and analysed by B-counter scintillator (Canberra Packard Instrument Company, Meriden, Connecticut, USA). Data were valued as counts per minute (cpm) and expressed as stimulation index (SI), defined as the ratio between the average counts of stimulated and unstimulated PBMC:

$$\text{SI} = \frac{\text{proliferation of PBMC with mitogen or antigen}}{\text{spontaneous proliferation of PBMC}}$$

Assays were considered valid only for spontaneous proliferation well counts between 400 and 1500 cpm. For analyses, an SI of 3 or greater was considered respondent.

To analyse TCRBV repertoire PBMC from patients were fractionated into CD4 and CD8 cells, and complimentary DNA was synthesized and amplified from RNA extracted as previously described [2] by 24 different primers for unique 5′Vβ sequences in combination with a primer for the TCRBV region 3′Cβ primer labeled (M-Medical, Milan, Italy) [6].

Reverse transcriptase polymerase chain reaction products were run on a 6% polyacrylamide gel on a DNA Automatic Fluorescence Sequencer (Pharmacia ALF DNA Sequencer) and analysed using specific software (Pharmacia DNA Fragment Manager 2.0).

Two main patterns of distribution were observed: polyclonal profiles 'p' (five or more peaks) and

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Table 1. Individual patient data.

	Years on HAART	PI- based HAART	Rate of blips	CD4 cell counts % (cells/μl)	CD4 cell nadir % (cell/μl)	k	Lymphoproliferation SI (cpm) p 24	p 17	gp 120	gp 41	CD8 cell counts % (cells/μl)	TCR CD8 polyclonality (%)	3 NRTI HAART	Rate of blips	CD4 cell counts % (cells/μl)	k	Lymphoproliferation SI (cpm) p 24	p 17	gp 120	gp 41	CD8 cell counts % (cells/μl)	TCR CD8 polyclonality (%)
Patients	Presimplif						T = 0 weeks										T = 96 weeks					
P1	4.5	3TC ZDV NFV	0.1	35 (1268)	39 (906)	1150	1.67 (1924)	1.36 (1565)	1.90 (2194)	1.08 (1243)	33 (1182)	91.7	3TC ZDV ABC	0	41 (1168)	688	**15.26** (10502)	**3.34** (2299)	**3.00** (2064)	**4.22** (2902)	29 (792)	95.8
P2	5.0	3TC ZDV Rit	0	34 (594)	35 (529)	1074	**4.23** (4550)	2.17 (2329)	0.86 (919)	2.11 (2269)	39 (730)	81.8	3TC ZDV ABC	0	38 (626)	567	5.49 (3118)	4.46 (2532)	4.20 (2387)	4.22 (2395)	38 (676)	95.8
P3	4.7	3TC ZDV NFV	0	25 (400)	24 (406)	1108	1.03 (1143)	1.70 (1887)	1.34 (1484)	0.56 (621)	36 (529)	75	3TC ZDV ABC	0.1	29 (524)	453	3.74 (1696)	2.29 (1038)	3.79 (1717)	2.34 (1062)	38 (505)	75
P4	4.1	ZDV ABC NFV	0.4	32 (698)	29 (464)	1365	2.74 (3743)	0.99 (1349)	6.9 (8580)	1.79 (2438)	38 (765)	100	ZDV ABC 3TC	0.2	33 (565)*	435*	4.87 (2121)*	5.60 (2438)*	5.68 (2472)*	2.20 (955)*	37 (677)*	**91.8***
P5	4.6	3TC d4T NFV	0.1	27 (412)	26 (443)	1185	1.16 (1372)	0.80 (950)	0.92 (1096)	1.01 (1202)	26 (319)	66.7	3TC d4T ABC	0	29 (389)	400	2.73 (1093)	7.78 (3112)	2.14 (857)	3.42 (1366)	26 (348)	95.7
P6	4.6	ZDV ABC NFV	0.2	29 (980)	27 (540)	1180	7.48 (8827)	2.61 (3080)	2.12 (2504)	1.81 (2131)	29 (1300)	66.7	ZDV ABC 3TC	0.3	35 (1106)	436	**4.01** (1752)	**8.29** (3617)	**3.80** (1660)	**12.22** (5329)	36 (1137)	**100**
P7	4.6	3TC d4T NFV	0.3	33 (833)	28 (1113)	489	**11.23** (5493)	2.02 (990)	8.94 (4371)	2.29 (1120)	26 (770)	61.9	3TC d4T ABC	0.1	34 (1159)	1157	0.90 (1043)	1.68 (1945)	1.13 (1310)	1.13 (1308)	27 (920)	**100**
P8	4.4	ZDV ABC NFV	0.1	36 (1172)	35 (940)	1478	**3.96** (5865)	1.30 (1923)	3.75 (5554)	1.91 (2836)	27 (935)	90.9	ZDV ABC 3TC	0.3	37 (1043)	451	7.39 (3334)	**15.10** (6808)	**9.46** (4267)	**11.98** (5402)	30 (846)	**100**
P9	2.7	ZDV 3TC IND	0.2	23 (583)*	27 (505)*	574*	1.07 (614)*	2.03 (1167)*	1.91 (1098)*	**3.05** (1750)*	23 (1201)*	50*	ZDV 3TC ABC	0	26 (654)	708	5.97 (4227)	5.39 (3818)	3.73 (2642)	**13.14** (9304)	26 (1076)	**90.9**
P10	4.7	ZDV ABC NFV	0	37 (754)	38 (764)	729	1.39 (1010)	1.44 (1051)	4.85 (3535)	1.73 (1261)	22 (422)	95.8	ZDV ABC 3TC	0.3	42 (926)	494	7.64 (3776)	**4.61** (2276)	**8.32** (4111)	**8.32** (4112)	19 (367)	**100**
P11	2.6	3TC d4T Lop/Rit	0	46 (906)*	35 (412)*	798*	1.52 (1214)*	4.69 (3740)*	2.08 (1662)*	0.87 (694)*	29 (552)*	72.2*	3TC d4T ddI	0	38 (1181)	1051	9.31 (9785)	**23.88** (25101)	7.39 (7777)	**22.26** (23397)	28 (870)	87.5
P12	3.5	ZDV ddI NFV	0.1	40 (708)	31 (531)	911	**8.12** (7405)	**9.32** (8488)	6.67 (6072)	**5.20** (4736)	29 (557)	38.9	ZDV ddI 3TC	0	32 (499)	865	**5.88** (5089)	**3.06** (2648)	**3.18** (2755)	**9.01** (7794)	36 (561)	83.4
P13	4.4	3TC ZDV NFV	0.1	28 (423)	31 (498)	680	0.73 (496)	0.64 (437)	0.88 (596)	0.95 (648)	32 (452)	73.9	3TC ZDV ABC	0.2	30 (513)	459	7.96 (3653)	**4.14** (1902)	2.63 (1210)	2.53 (1161)	34 (581)	**86.4**
P14	4.6	3TC ZDV NFV	0	46 (1217)	44 (994)	790	**3.54** (2793)	1.14 (902)	4.71 (3722)	1.08 (851)	27 (754)	87.5	3TC ZDV ABC	0	44 (1007)	1126	**14.69** (16538)	**12.70** (14298)	**8.01** (9019)	**9.11** (10262)	26 (595)	**100**
P15	4.9	3TC ABC NFV	0.2	37 (1360)	38 (360)	1286	1.22 (1570)	1.10 (1419)	2.45 (3153)	0.91 (1166)	21 (772)	100	3TC ABC ZDV	0	41 (1115)	553	**3.30** (1825)	4.74 (2623)	2.88 (1592)	0.99 (545)	20 (544)	**100**
P16	4.5	3TC d4T NFV	0.1	27 (812)	21 (809)	603	**8.64** (5210)	**8.42** (5007)	8.76 (5287)	**9.81** (5917)	29 (933)	57.1	3TC d4T ABC	0.1	28 (935)	782	**19.47** (15225)	4.39 (3438)	1.74 (1357)	6.77 (5295)	27 (955)	**91.7**
P17	2.1	3TC ZDV NFV	0.2	34 (2237)	31 (987)	926	1.04 (965)	0.64 (597)	5.52 (5110)	5.55 (5140)	31 (1956)	82.6	3TC ZDV ABC	0	32 (1171)	470	9.94 (4670)	**9.31** (4377)	**8.14** (3828)	**6.27** (2946)	26 (951)	**95.8**
P18	2	3TC ABC ZDV NFV	0.1	41 (994)	36 (768)	413	**4.03** (1665)	2.14 (883)	2.38 (984)	2.11 (871)	28 (564)	95.7	3TC ZDV ABC	0	41 (832)	766	2.73 (2097)	2.66 (2042)	**3.21** (2464)	2.14 (1646)	25 (507)	**100**
P19	4.2	ZDV ABC NFV	0.2	45 (999)	38 (651)	597	1.14 (680)	1.50 (897)	1.26 (751)	1.34 (799)	22 (460)	38.9	ZDV ABC 3TC	0.1	44 (836)	542	**3.83** (2075)	**9.28** (5032)	**8.24** (4464	**3.53** (1914)	26 (494)	**100**

ABC, Abacavir; ddI, didanosine; d4T, stavudine; IND, indinavir; 3TC, lamivudine; NFV, nelfinavir; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; Rit, ritonavir; SI, stimulation index; TCR, T-cell receptor; ZDV, zidovudine. PI previously used and replacing NRTI are highlighted. The percentage of viral blips detected are reported. CD4 cell count nadirs and at study baseline and completion and CD8 cell counts at baseline and completion are also reported as percentages and absolute numbers. Lymphoproliferation: SI and cpm responses are showed for each patient, highlighted characters represents HIV-specific antigen response of 3 or greater. K, Unstimulated background in medium alone. The percentage of CD8 TCR repertoire polyclonal patterns (by spectratyping) are reported; the maintaining and improving percentages are highlighted.
*As samples were missing at the right time point, data reported were those at 48 weeks.

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skewed/perturbed profiles 'sk' (from one to four peaks or a multipeak pattern with one solitary peak > 50% of the total area or one or more deleted peaks).

P values less than 0.01 obtained using the Student's *t*-test were considered significant either for lymphoproliferation or for TCRBV repertoire analyses. The results are reported in Table 1.

Viral suppression was maintained over time in 18 out of 19 patients (child p4 had a viral failure at 48 weeks as a result of poor adherence). Intermittent episodes of detectable viremia (between 50 and 999 copies/ml) did not increase during the three NRTI regimen.

The lymphoproliferative T-cell response to HIV-specific antigens (p24, p17, gp120, gp41) increased significantly ($P < 0.01$) during follow-up, and a significant augmentation ($P < 0.01$) in the total number of patients responding to at least two out of four HIV-specific antigens was observed; lymphoproliferation towards PHA, PWM, OKT3 mitogens and towards *C. albicans* and herpes simplex virus 1 recall antigens was maintained within laboratory values comparable with healthy children.

TCRBV repertoire distributions showed a normalization trend in the CD8 cell subset: the percentage number of polyclonal patterns significantly increased ($P < 0.01$) during follow-up. Interestingly, in patient p4, who failed the protocol, there was an increase in alterations.

The immunological improvement observed after the switch is in accordance with data obtained in adult studies [7]. In HIV-infected children placed on interrupted PI-based therapy as a result of viral failure, Legrand *et al.* [8] recently reported an increased HIV-specific immune response in terms of intracellular IFN-γ and TNF-α production, underlining the necessity of other immunological assays.

In our paediatric cohort, we observed a significant enhancement of HIV-specific lymphoproliferative response to HIV antigens and a progressive normalization of TCRVB families in the CD8 cell subset during 96 weeks.

Our patients showed good compliance, stable viral control and immune reconstitution already in previous PI-based HAART. Despite the switching, they maintained previous immunological parameters and some even showed an improvement.

These observations at enrolment were not correlated with the different duration of previous regimens (range 2–5 years), thus we hypothesized that the further improvement observed was possibly a result of the PI elimination.

A potential reason for the immunological benefits observed may be the immunomodulatory role of PI in the degradation of proteins [9]; these drugs could alter lymphoproliferative cytokine production (TNF-α, IL-2, IFN-γ) or cause immunosuppression by hampering antigen presentation [10]. As an enhancement in specific lymphoproliferative responses and the normalization of TCR distribution were not associated with a detectable increase in viral replication, as demonstrated by the maintenance of viral control, we hypothesized that the elimination of PI could play a role in the improvement in HIV-specific cell-mediated immune responses.

In conclusion, our data add more evidence on the immunological benefits of simplified PI-sparing regimens, after successful first-line PI-based HAART, without increasing the risk of viral failure. This could represent a capable therapeutic strategy to enhance HIV-specific immune responses in HIV-infected children in view of alternative immunotherapeutic approaches.

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Paper 4

ORIGINAL ARTICLE

Rapid T-cell Receptor CD4$^+$ Repertoire Reconstitution and Immune Recovery in Unrelated Umbilical Cord Blood Transplanted Pediatric Leukemia Patients

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Summary: Umbilical cord blood transplantation has been successfully employed for treatment of many immune and hematologic disorders. The aim of this study was to evaluate the quality of immune reconstitution after umbilical cord blood transplantation in 6 leukemia children. T-cell receptor Vβ third complementary region spectratyping was used for monitoring the contribution of the thymic pathway in patients' immune reconstitution. Absolute numbers of lymphocyte subsets (T, B, and natural killer), and lymphoproliferative in vitro response to mitogens, recovered within 12 months after transplantation. Furthermore, an overall diversification of T-cell receptor complexity in the repopulating T cells, with a polyclonal Gaussian profiles in most (74%) of total families was observed. Noteworthy, we showed a wider and more rapid reconstitution of T-cell receptor CD4$^+$ T cell families compared with T-cell receptor CD8$^+$ T ones still exhibiting some perturbations at 24 months. These data show that umbilical cord blood transplantation allows immune reconstitution already within 12 months with generation of newly diversified CD4$^+$ T lymphocyte subsets.

Key Words: T-cell repertoire, immune reconstitution, umbilical cord blood, spectratyping, leukemia

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Umbilical cord blood transplantation (UCBT), particularly from unrelated donors, has progressively become a widely employed treatment for patients with both malignant and nonmalignant hematologic disorders.[1–7] In comparison to bone marrow transplantation (BMT), UCBT offers several advantages including simplicity to be obtained, rapid availability of biologic product, absence of risk for donors, reduced likelihood of transmitting infections, less stringent criteria for HLA matching for donor/recipient selection, and reduced incidence and severity of graft-versus–host disease (GVHD).[7,8] Cord blood lymphocytes seem functionally and phenotypically naïve, demonstrating a higher threshold for cytokine stimulation, have a low T-cell mediated cytotoxic capacity, and show a widely polyclonal T-cell repertoire.[8–11]

Immaturity and unique immunologic properties of cord blood cells could explain the lower risk of inflammatory states such as GVHD observed after UCBT.[12,13] On the other hand, cell immaturity as well as lower number of lymphocytes and cord blood stem cells could represent a potential problem for a complete immune recovery and T-cell reconstitution after UCBT. Indeed, a prompt immune recovery and T-cell reconstitution are of basic importance after transplant, because delayed immune competence contributes to engraftment failure, susceptibility to latent viruses and relapse of hematologic malignancies.[14,15]

Previous published reports have shown that, after UCBT, recovery of lymphocyte subsets, as well as of their proliferative response to mitogens and recovery of natural killer (NK) function, are prompt and at least as efficient as those observed after BMT.[16–21]

Furthermore, reconstitution of a functional T-cell compartment requires the restoration of a diverse T-cell receptor vβ (TCRBV) repertoire. Several studies of third complementary region (CDR3) fragment length distribution have demonstrated a normalization of T-cell repertoire diversity after BMT from human lymphocyte antigen (HLA) identical related donors,[22,23] whereas transplantation from unrelated or HLA-mismatched related donors resulted in severe skewing during the first posttransplantation year.[22–25] Only few data, however, are available on T-cell receptor (TCR) repertoire reconstitution after unrelated UCBT.[21,26]

In our study, we analyzed T-cell repertoire reconstitution of the 2 main subsets CD4$^+$ and CD8$^+$ T cells in

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6 pediatric leukemia patients who underwent unrelated UCBT. Recovery of T, B, and NK-lymphocyte subsets and proliferative in vitro response to mitogens were also investigated.

PATIENTS AND METHODS

Patients

Six children who underwent UCBT from unrelated donors with a 24 months observation time were enrolled in this study. All children were transplanted at the Hematology and Bone Marrow Transplantation Unit, Pediatric Hospital Bambino Gesù (Rome, Italy) since November 2000. Written informed consent was obtained from the patients' parents. Cord blood units for unrelated transplants were provided by the cord blood banks in Milan, Padua, and Düsseldorf.

Details on the patients' clinical characteristics are reported in Table 1.

HLA typing was performed by serology for HLA-A and B, and by high-resolution molecular typing for HLA-DRB1. Five patients were matched for 4 out of 6 antigens, whereas 1 patient was matched for 5 out of 6 antigens. Peripheral blood samples were analyzed at multiple time points after UCBT as routine posttransplant clinical care. Chimerism was analyzed by fluorescence in situ hybridization for sex chromosomes, or restriction analysis fragment length polymorphisms analysis on peripheral blood leukocytes and mononuclear cells.

The conditioning regimen consisted in chemotherapy in 2 patients and chemotherapy with total body irradiation in 4 patients. All patients received horse antilymphocytes globulin (Lymphoglobuline, Sangstat, Lyon, France) (600 IU/kg once daily by IV infusion over 16 hours for 4 consecutive days starting on day –6, total 4 doses) during the conditioning regimen. GVHD prophylaxis included a combination of cyclosporin A and steroids. Patient no. 1 intolerant of cyclosporin A received tacrolimus (FK506). Recombinant human granulocyte colony-stimulating factor was administered after transplantation to all patients to accelerate myeloid recovery. No routine intravenous immunoglobulin (IVIG) was used unless serum Immunoglobulin G (IgG) level was less than 400 mg/dL and the patients had developed an infectious complication. Only 1 of the 5 patients, received IVIG for low IgG level and concurrent infections.

Flow Cytometry

Flow cytometry was performed on peripheral blood mononuclear cells (PBMCs) according to standard protocols with a FACSscan flow cytometer (Becton Dickinson, San Jose, CA). Monoclonal murine antibodies conjugated to FITC or PE specific for CD3, CD4, CD8, CD16, CD19, CD45RO, and CD45RA (Pharmingen, San Diego, CA) were used for staining.

TABLE 1. Patients Characteristics

Patients	Age at Transplant (y)	Diagnosis	HLA Compatibility	NC	CFU-GM	$CD34^+$	Conditioning	GVHD Prophylaxis	GVHD Acute	GVHD Chronic	Chimerism	CMV Reactivation	Current Status
1	13	ALL	4/6	4.7×10^7	7.7×10^4	1.7×10^6	TBI/CY/VPI6/ATG	CSA/FK506	I	II	Full donor	Yes	Alive
2	6	ALL	4/6	3.9×10^7	2.9×10^4	0.12×10^6	TBI/CY/VPI6/ATG	CSA/PDN	I	N	Full donor	Yes	Alive
3	5	ALL	4/6	7.9×10^7	25×10^4	0.44×10^6	TBI/CY/VPI6/ATG	CSA/PDN	II	I	Full donor	Yes	Alive
4	4	ALL	4/6	6.7×10^7	5.7×10^4	0.49×10^6	BU/CY/ATG	CSA/PDN	N	N	Full donor	No	Alive
5	1	AML	5/6	6.7×10^7	15×10^4	0.15×10^6	BU/CY/ATG	CSA/PDN	III	N	Full donor	No	Alive
6*	14	ALL	4/6	0.3×10^7	4.8×10^4	0.20×10^6	TBI/CY/ATG	CSA/PDN	N	N	Mixed	No	Death

*Dead patient.
ALL, indicates acute lymphoblastic leukaemia; AML, acute myeloblastic leukaemia; ATG, antithymocyte globulins; BU, busulfan; $CD34^+$, $CD34^+$ cell/kg infused; CFU-G, granulocyte colony forming units/kg infuse; CSA, cyclosporin A; CY, cyclophosphamide; FK506, tacrolimus; FLU, fludarabine; NC, nuclear cell/kg infused; PDN, prednisolone; TBI, total body irradiation; VP16, etoposide.

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Proliferation Assay

2×10^5 PBMC were cultured in triplicates in flat bottom 96 well microtitre sterile plates with one of the following mitogens: phytohaemmaglutinin (PHA) 5 μg/mL, pokeweed mitogen (PWM) 0.35 μg/mL (Sigma Chemical, Milan, Italy), and anti-CD3 monoclonal antibody (OKT3) 3 μL (stock supernatant obtained from OKT3 cell line, ATCC-Sigma, MI, Italy). PBMC were then incubated 72 hours at 37°C before harvesting (Canberra Packard Instrument Company, Meriden, CT). ^{3}H-thymidine (Amersham, Milan, Italy) was added to medium (2.5 μCi/mL) during the last 6 hours of incubation.

Immunoglobulins

Serum immunoglobulin levels (IgG, IgA, and IgM) were assessed with a nephelometer. IgG subclasses were determined by means of radial immunodiffusion.

TCR Repertoire Analysis

Cells

Fresh PBMC, obtained from 6 patients' and 3 healthy controls (range: 12 to 14 mo) were fractionated into $CD4^+$ and $CD8^+$ subsets by the use of anti-CD4 or anti-CD8 monoclonal antibody-coupled magnetic beads (Dynal AS, Oslo, Norway) according to standard protocol.

RNA Extraction cDNA Synthesis and PCR

Total RNA from 2×10^6 cells was extracted by TRIzol (Gibco-BRL/Life Technologies) according to manufacturing instructions and suspended in 25 μL deionized "RNAse-free" H_2O. A Superscript One-Step reverse transcriptase-polymerase chain reaction (RT-PCR) with Platinum Taq kit (Invitrogen, Milan, Italy) according to mildly modified standard protocol and 24 5′Vβ primers coupled with one 3′Cβ (y5 labeled in 5′)[27] were used. The temperature profile in a GeneAmp 9600 DNA thermal cycler (Perkin Elmer Cetus) included the following steps: a 50°C cycle for 20 minutes (cDNA synthesis) followed by "denaturation" at 94°C 45 seconds, "annealing" at 55°C 45 seconds, "extension" at 72°C 45 seconds, and "final extention" at 72°C for 10 minutes.

PCR products analysis. PCR products were kept at 95°C for 4 minutes, loaded in wells of a 6% polyacrylamide gel (Pharmacia Ready Mix Gel), run in electrophoresis 1500 V, 220 minutes in a DNA Automatic Fluorescence Sequencer (Pharmacia ALF DNA Sequencer), and finally analyzed by a specific software (Pharmacia DNA Fragment Manager 2.0). Peaks' patterns were defined lightly changing our previous definitions[28]: a CDR3 fragments profile having 5 to 9 peaks/vβ families with a Gaussian (bell shaped) distribution, was called polyclonal (p). Alteration of the polyclonal profile of 5 to 9 peaks consisted in a disruption of the Gaussian distribution or appearance of one or more clonal expansions was defined polyclonal altered (pa). Finally, oligoclonal profile resulted in a pattern of no more than 4 peaks per vβ family or presence of a peak which area was >50% of the total area for that family was defined skewed (sk).

RESULTS

Transplant Outcome

Successful engraftment was observed in all children enrolled in this study. Five out of 6 children survived with a median follow-up duration of 36 months (range 31 to 48 mo). One patient died from relapse of the original disease 18 months posttransplantation. Acute GVHD was observed in 4 children (grade I in 2 of them, grade II in 1, and grade III in the forth). Limited chronic GVHD was detected in 2 children.

All children analyzed had sustained recovery of both neutrophil and platelet counts. The median time to neutrophil engraftment was 26 days (range 20 to 40 d). The median time to reach sustained platelet count of more than 20,000 was 65 days (range 33 to 184 d). All but 1 (patient no. 6) of the 6 patients were documented to be fully donor chimeric after transplantation.

Three children, positive for cytomegalovirus (CMV) before transplantation, developed recurrent CMV reactivations after UCBT and were successfully treated with ganciclovir.

Lymphocyte Subsets and In Vitro Response to Mitogens

Changes in absolute values of total lymphocytes, T-cell subsets, B-cells, and NK-cells are shown at 3, 12, and 24 months posttransplant (Table 2). The absolute count of lymphocytes increased gradually during the first year posttransplantation and reached a normal count[30] within 12 months in 4 out of 6 patients. The absolute numbers of $CD3^+$ T cells at this time point was also normal in 3 out of 6 patients. Similar to $CD3^+$ T cells, $CD4^+$ recovery was relatively rapid and 4 out of 6 patients restored $CD4^+$ T-cell counts within the normal range by 12 months post-UCBT. $CD8^+$ T-cell reconstitution was less rapid than $CD4^+$ T-cell recovery with 5 out of 6 patients having $CD8^+$ T-cell count below the normal range until 12 months post-UCBT. At 12 months post UCBT the absolute numbers of $CD4^+$ T-cells expressing the CD45 RA+ were within the normal range in the majority of patients, whereas the absolute numbers of $CD8^+$ CD45RA+T cells were much lower. Similar analysis at 24 months post-UCBT showed a normalization of the naive compartment in all patients as reported in Table 3. In vitro response of the patients' PBMC to the polyclonal activators (PHA, PWM, and OKT3) was generally low at 3 to 4 months after UCBT but improved progressively and reached normal levels in all patients within the first year follow-up (Figs. 1A, B). The NK cells on the other hand, recover quickly and were within the normal range at 3 months after UCBT in the majority of the recipients (Table 2). The B-cell recovery rose to normal levels by the first 12 months of follow-up

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TABLE 2. Immunological Recovery of Patients Treated With CBT: Laboratory Findings at Different Time Points After Transplantation

Patients	Age at CBT (y)	No. Lymphocytes			No. CD3+			No. CD4+			No. CD8+			No. CD16+/CD56+(NK)			No. CD19+		
		3–4 mo	12 mo	24 mo	3–4 mo	12 mo	24 mo	3–4 mo	12 mo	24 mo	3–4 mo	12 mo	24 mo	3–4 mo	12 mo	24 mo	3–4 mo	12 mo	24 mo
1	13	1020	2000	2310	184	1040	1317	163	680	485	10	240	462	704	540		163	360	
2	6	640	3120	2870	205	1872	1837	90	1248	1119	109	562	631	358	218		64	998	
3	5	590	1930	3500	159	1177	1995	65	733	1400	83	290	525	83	232		136	502	
4	4	910	2600	2660	ND	1664	1383	ND	1248	851	ND	338	293	ND	78		ND	832	
5	1	920	1360	6040	368	272	1872	220	217	1872	129	54	785	460	285		10	557	
6†	14	1010	1110	—	50	288	—	40	200	—	10	78	—	848	200		151	544	
Normal values*	12–18	2200 (1400–3300)			1480 (1000–2200)			840 (530–1300)			530 (330–920)			190 (70–480)			300 (110–570)		
	6–12	2700 (1900–3700)			1820 (1200–2600)			980 (650–1500)			680 (370–110)			230 (100–480)			480 (270–860)		
	2–6	3600 (2300–5400)			2390 (1400–3700)			1380 (700–2200)			840 (490–1300)			300 (130–720)			750 (390–1400)		
	1–2	5500 (3600–8900)			3550 (2100–6200)			2160 (1300–3400)			1040 (620–2000)			360 (180–920)			1310 (720–2600)		

Values are reported as absolute number ($\times 10^6$/L).
*Age-matched reference values according to Shearer[29]; values are the medians, with ranges from 5 to 95 percentiles in parentheses.
†Dead patient.
ND indicates not determined.

TABLE 3. Absolute Numbers of CD4+CD45RA+ and CD8+CD45RA+ T-cell Subsets

Patients	Age at CBT (y)	No. CD4+ CD45RA+			No. CD8+ CD45RA+		
		3–4 mo	12 mo	24 mo	3–4 mo	12 mo	24 mo
1	13	34	238	243	83	144	328
2	6	9	1023	705	30	478	505
3	5	10	542	1190	29	209	404
4	4	ND	890	ND	ND	281	ND
5	1	23	80	1741	77	40	746
6†	14	ND	73	—	ND	55	—
*Normal values	12–18	400 (230–770)			400 (240–710)		
	6–12	570 (320–1000)			540 (310–900)		
	2–6	980 (430–1500)			670 (380–1100)		
	1–2	1650 (1000–2900)			940 (490–1700)		

Values are reported as absolute number ($\times 10^6$/L).
*Age-matched reference values according to Shearer[29]; values are the medians, with ranges from 5 to 95 percentiles in parentheses.
†Dead patient.
ND indicates not determined.

and was associated with a normalization of serum Ig levels. For all patients except one, who received IVIG, IgG, IgA, IgM, and the subclasses of IgG began to normalize within 3 to 4 months and were in the normal range at 12 months after UCBT (Table 4).

T-cell Repertoire

To evaluate the recovery of T-cell repertoire post UCBT, CDR3 length distribution of 24 TCRBV families and subfamilies in $CD4^+$ and $CD8^+$ T cells of transplanted children were analyzed by spectratyping at different time points. Data are presented in Figures 2A, B and representative examples of T-cell repertoire analysis are shown in Figure 3. Spectratype analysis of the 5 surviving UCBT recipients at 12 months after transplantation showed a polyclonal Gaussian T-cell repertoire distribution in both $CD4^+$ and $CD8^+$ T-cell subsets in most (74%) of total TCRBV families. Indeed, CDR3 length distribution of $CD4^+$ T-cell subsets revealed a polyclonal Gaussian profile in 80.9% of TCRBV families with 16.5% showing a polyclonal altered profile and only 2.6% of TCRBV families showing a skewed pattern. Noteworthy, in contrast with $CD4^+$ T-cell subsets, $CD8^+$ T-cell subsets still exhibited some perturbations in the T-cell repertoire at 12 months after UCBT, with 12.1% of the total TCRBV families showing a skewed pattern of distribution and 20.7% showing a polyclonal altered profile. Two years after transplantation, the reconstitution of TCR diversity appeared overall to be persistently improving (83.7%). The majority of TCRBV families in the $CD4^+$ and $CD8^+$ TCRBV families showed a polyclonal Gaussian profile (90.5% and 71.3%, respectively) with a decrease in the percentage of polyclonal altered profile (9.5% and 16.5%, respectively). The few skewed profiles were confirmed in $CD8^+$ T (12.2%) subset although were no more present in $CD4^+$ T subset. Spectratype analysis of patient no. 6 showed a

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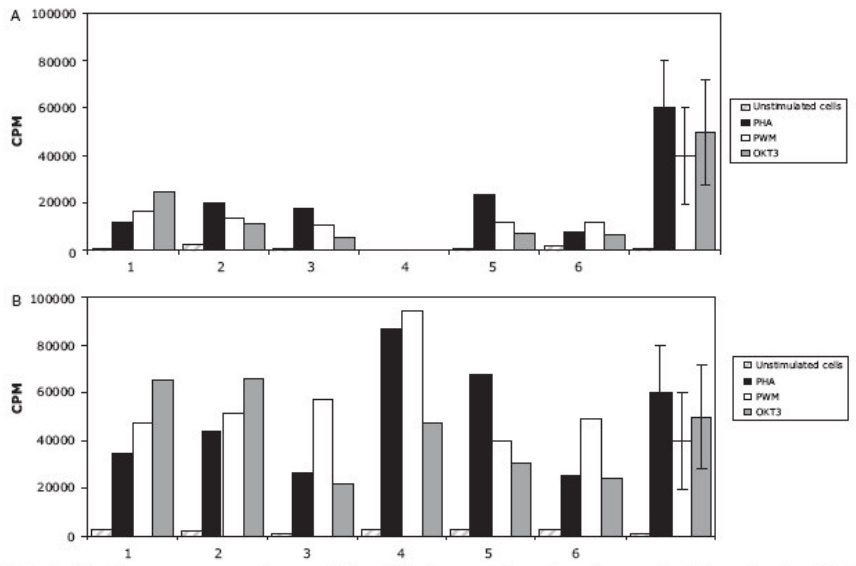

FIGURE 1. Proliferative responses to mitogens. ^{3}H-thymidine incorporation after mitogen stimulation of patients' PBMC in response to PHA, PWM, and OKT3 was evaluated 3 to 4 months (A) and 10 to 12 months after transplantation (B). Values are expressed as cpm, control proliferations are reported in mean ± SD.

marked altered profile in both CD4+ and CD8+ T-cell subsets with 19.5% of the total TCRBV families showing a skewed pattern, 29.2% showing a polyclonal altered profile and only 51% of TCRBV families showing a polyclonal Gaussian T-cell repertoire distribution.

DISCUSSION

Since 1988, umbilical cord blood has been proven to be an alternative source of hematopoietic stem cells mainly for those patients lacking bone marrow HLA-matched related donors.[1] Several studies reported that

TABLE 4. Serum Immunoglobulin Levels and IgG Subclasses

Patients	Age at CBT (y)	IgG (mg/dL)			IgA (mg/dL)			IgM (mg/dL)			IgG1 (mg/dL)		IgG2 (mg/dL)		IgG3 (mg/dL)		IgG4 (mg/dL)	
		3–4 mo	12 mo	24 mo	3–4 mo	12 mo	24 mo	3–4 mo	12 mo	24 mo	12 mo	24 mo	12 mo	24 mo	12 mo	24 mo	12 mo	24 mo
1	13	707	925	1228	26	65	97	116	261	268	689	943	181	191	27	31	33	10
2	6	383	561	741	38	41	98	35	72	126	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	5	240	620	765	44	63	63	65	101	234	534	665	84	160	30	29	9	9
4	4	n.d.	518	577	n.d.	35	58	n.d.	117	83	385	433	84	113	23	22	14	9
5	1	IVIG	IVIG	IVIG	31	39	31	48	50	28	IVIG	IVIG	IVIG	IVIG	IVIG	IVIG	IVIG	IVIG
6†	14	460	1470	n.d.	25	99	n.d.	21	182	n.d.	1030	n.d.	251	n.d.	113	n.d.	115	n.d.
Normal	1–2		801			54			128			500		84		23		—
Values Ref. 31*			(264–1509)			(17–178)			(48–337)			(260–970)		(31–214)		(7–63)		
	4–5		1117			98			119			630		125		30		—
			(528–1959)			(37–257)			(49–292)			(340–1230)		(47–313)		(10–84)		
	12–16		1105			136			132			690		250		43		—
			(640–1909)			(61–301)			(59–297)			(380–1290)		(91–654)		(12–134)		

*Age-matched reference values according to Shearer[29]; values are the medians, with ranges from 5 to 95 percentiles in parentheses.
†Dead patient.
n.d. indicates not done.

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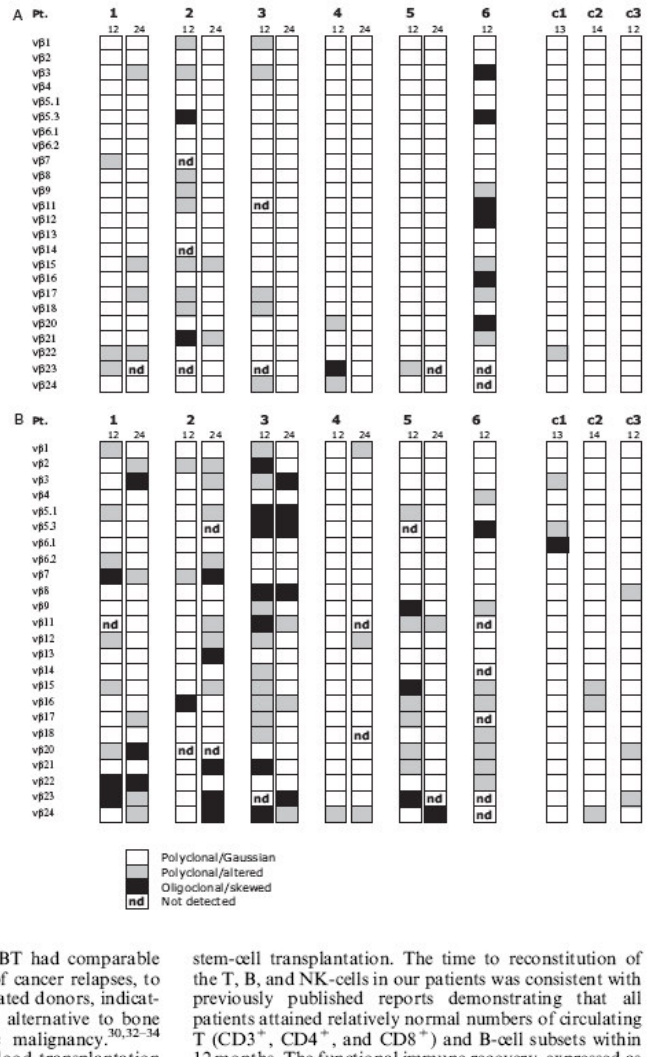

FIGURE 2. TCRBV repertoire patterns. A, Kinetic of $CD4^+$ TCRBV repertoire patterns in each patient and in 3 healthy control children. B, Kinetic of $CD8^+$ TCRBV repertoire patterns in each patient and in 3 healthy control children.

children treated with unrelated UCBT had comparable rates of survival, and similar rates of cancer relapses, to those who received BMT from unrelated donors, indicating that unrelated UCBT is a valid alternative to bone marrow in treatment of pediatric malignancy.[30,32–34] Immune reconstitution after cord blood transplantation has been recently addressed by few studies.[16–21] Innate immunity is generally assumed to recover within the first 2 months posttransplantation, whereas reconstitution of antigen specific immunity takes longer time. Successful reconstitution of T-cell–mediated immunity is of particular relevance to reduce morbidity and mortality after stem-cell transplantation. The time to reconstitution of the T, B, and NK-cells in our patients was consistent with previously published reports demonstrating that all patients attained relatively normal numbers of circulating T ($CD3^+$, $CD4^+$, and $CD8^+$) and B-cell subsets within 12 months. The functional immune recovery, expressed as in vitro lymphocyte proliferation in response to mitogens occurred between 10 and 12 months after transplantation, whereas B-cell function, assessed by immunoglobulin production, began to recover since 3 to 4 months posttransplant. The absolute numbers of naive $CD4^+CD45\ RA+$ T cells was normal in the majority

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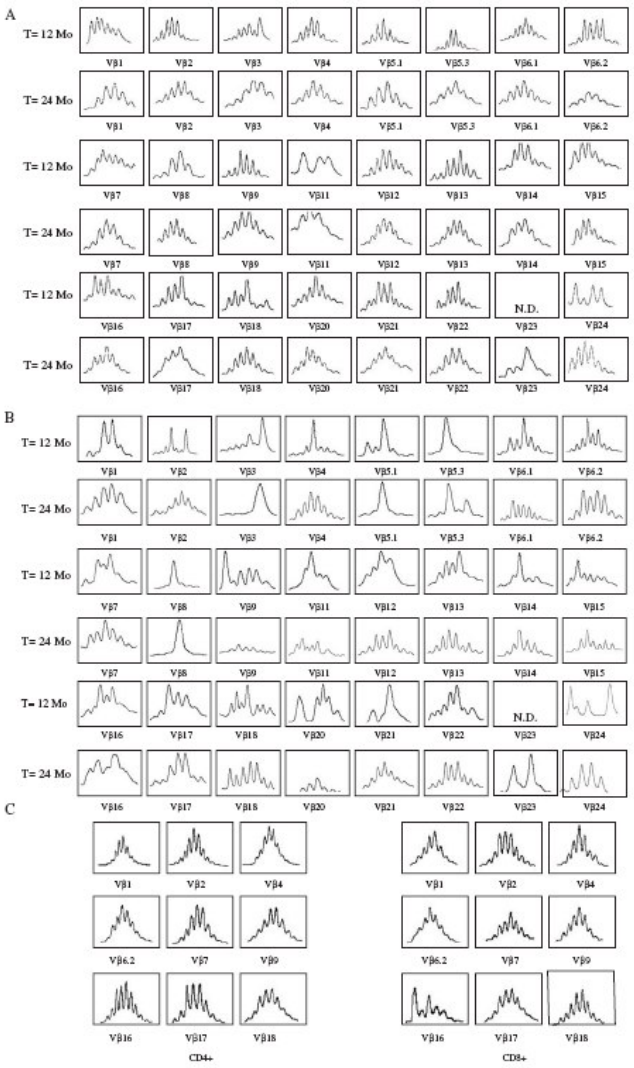

FIGURE 3. Representative examples of TCR CDR3 length distributions. Patterns in peripheral blood CD4+ T cells (A) and CD8+ T cells (B) at 12 and 24 months after CBT. Data from 1 patient (no. 3) are shown. Some demonstrative CD4+ and CD8+ CDR3 length distributions in a healthy control child (c2) are reported (C).

of patients at 12 months post-UCBT, whereas the complete recovery of naive $CD8^+CD45RA+$ T cells was delayed until 24 months. Immune reconstitution after hematopoietic stem-cells transplantation has been reported to be dependent on 2 different pathways.[35–38] The first wave of immunoreconstitution is consisting in thymic independent transfer of graft-derived mature donor's T cells to the periphery followed by antigen or cytokine expansion. The resulting circulating T cells have a limited T-cell repertoire diversity and are unable to respond to a broad range of pathogens.[39,40] The second wave of immunoreconstitution consists in a thymic-dependent

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pathway, which involves selection of graft-derived precursor cells in the host's thymus and/or possibly in the periphery.[41–44] This process accounts for a more durable reconstitution of the T-cell compartment with a more diverse T-cell repertoire. In this context, there are some limitations in estimating thymic function on the basis of naive phenotype alone. In fact, evaluation of CD45RA expression is not capable of fully discriminating naive from memory T cells. In particular, some CD45RO+ T cells can reexpress CD45RA+ remaining functional memory T cells,[45] and naive and memory cells may have overlapping phenotypes, especially among $CD8^+$ subset.[46,47] Therefore, analysis of TCR repertoire diversity represents a useful tool to evaluate the contribution of the thymic-dependent pathway among peripheral blood T cells. Previous longitudinal study of the T-cell repertoire diversity in adolescent/adult patients with various forms of malignancy after UCBT reported recovery of TCR diversification only after 2 or more years.[21,26] Interestingly, we show an overall improvement of TCR repertoire diversity in all surviving patients we studied already 1 year after UCBT with the exception of patient no. 6. Of note, this patient had persistence of recipient hematopoiesis and died later because of relapse. In addition, it is important to consider that, contrary to previous studies where the TCRBV analysis has been performed using total T-cell populations,[21,26] we analyzed the TCRBV in isolated $CD4^+$ and $CD8^+$ populations. This specific analysis is more rationale because CDR3 spectra of cytotoxic and helper T lymphocyte would have different HLA restriction and differences between the 2 populations will have been missed by the spectra overlapping if only total T cells are assessed. Indeed, interestingly we found differences between $CD4^+$ and $CD8^+$ T-cell compartments. $CD4^+$ T cells showed a faster normalization toward a Gaussian distribution of CDR3 fragment length, whereas $CD8^+$ subset still exhibited some perturbations of the repertoire. The reason why the oligoclonality appears mainly in the $CD8^+$ cells is unclear at the moment, although it has been also reported in normal subjects.[48,49] One possible mechanism could be due to the expansion of $CD8^+$ cells resulting from antigen-specific immune response to viral antigens. Indeed, the presence of clonal T-cell expansions in particular within the $CD8^+$ populations was associated with viral infections including CMV and Epstein-Barr virus,[50–52] and a delayed and incomplete recovery of TCRBV repertoire was also observed in transplanted patients with CMV infection.[15] In agreement with these data, none of the 3 patients studied who developed CMV reactivation was among those showing $CD8^+$ TCRBV repertoire widely normalized. Another possibility is that peripheral mechanisms or extrathymic maturation may have had a role to the reconstitution of the $CD8^+$ TCR repertoire. The long-term consequences of oligoclonality in the $CD8^+$ T cells are unclear. However the presence of oligoclonal $CD8^+$ T cells in healthy subjects suggests that effective T-cell immunity can be preserved in the presence of such expansions.[53]

Our results, nevertheless the limited number of patients, indicate that unrelated cord blood transplantation of children with hematologic malignancies allows a successful immune reconstitution. In addition, our data on the TCR repertoire suggest that reconstitution is faster in $CD4^+$ than $CD8^+$ T cells. Larger and prospective studies are necessary to confirm these results and to determine the immune system evolution overtime.

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