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STRATEGIES FOR T-CELL RECONSTITUTION: INSIGHTS FROM HUMAN CLINICAL MODELS

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Aos meus pais
Às minhas irmãs

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γ_c	Common gamma chain
ADA	Adenosine deaminase
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cells
APECED	Atoimmune polyendocrinopathycandidiasis-ectodermal dystrophy
AIRE	Autoimmune regulator element
ART	Antiretroviral therapy
BCG	Bacillus Calmette-Guérin
CGD	Chronic granulomatous disease
cj	Coding-joint
CMV	Cytomegalovirus
cTEC	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T lymphocyte associated protein 4
DC	Dendritic cell
DGS	DiGeorge syndrome
DN	Double-negative
DP	Double-positive
EBV	Epstein-Barr virus
EDP	Early double positive
FOXN1	Forkhead box protein N1
GC	Germinal center
HEV	High endothelial venule

HIV	Human immunodeficiency virus
HSCT	Hematopoietic stem cell transplantation
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IL-7Rα	Interleukin 7 receptor α
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy X-linked
ISP	Immature single positive
iTreg	Induced regulatory T cell
JAK–STAT	Janus kinase–signal transducer and activator of transcription
LTR	Long terminal repeat
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cells
NADPH	Nicotinamide adenine dinucleotide phosphate
nTreg	Natural regulatory T cell
OS	Omenn syndrome
PCD	Programmed cell death
PI3K	Phosphoinositide 3-kinase
PID	Primary immunodeficiency
PTK7	Protein tyrosine kinase 7
RAG	Recombination-activating gene
RTE	Recent thymic emigrants
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SIV	Simian Immunodeficiency Virus
sj	Signal-joint

SP	Single positive
T_{CM}	Central memory T cells
TCR	T cell receptor
TEC	Thymic epithelial cells
T_{EM}	Effector-memory T cells
TGF	Transforming growth factor
Th	T helper
TN	Triple negative
TREC	T cell receptor excision circles
Treg	Regulatory T cell
WHN	Winged–helix–nude
X-SCID	X-linked severe combined immunodeficiency

RESUMO

O timo é o orgão linfóide primário que tem por função a produção de células T. A actividade tímica decresce progressivamente com a idade, mas, apesar de ocorrer uma clara involução do timo na puberdade, está claramente demonstrada a produção "de novo" de células T até depois dos 60 anos. Esta é considerada essencial para assegurar um repertório do receptor de células T (TCR) suficientemente diverso para responder a qualquer novo agente patogénico e capaz de prevenir a emergência de estirpes resistentes em infecções crónicas como a infecção HIV/SIDA. O compartimento periférico de células T é assim mantido não só pela proliferação linfocitária nos órgãos linfoides secundários mas também pela entrada contínua de novas células produzidas no timo.

Este trabalho tem por objectivo investigar o contributo relativo do timo e da periferia para a homeostasia e reconstituição do compartimento de células T através do estudo de imunodeficiências.

O FOXN1 é um factor de transcrição expresso pelo epitélio do timo essencial para o seu desenvolvimento e também recentemente implicado na prevenção da involução tímica. Mutações no gene *FOXN1* no ratinho associam-se a ausência de timo e a alopécia total, devido ao papel adicional deste na diferenciação dos folículos pilosos ("nude-SCID mice"). Os primeiros casos de mutação do *FOXN1* foram descritos por Pignata *et al* em duas irmãs da Sicília que apesar da evidência de atímia, apresentavam um número significativo de células T circulantes. Identificámos a mesma mutação

homozigótica R255X numa criança Portuguesa com alopécia total a quem foi diagnosticada, aos 5 meses de idade, disseminação do Bacillus Calmette-Guérin (BCG) após vacinação perinatal de rotina com BCG. Nesta altura, apresentava um número de células T circulantes próximo do normal que não tinham origem materna. Assim, o primeiro objectivo do trabalho consistiu no estudo da população de células T gerada na presença de deficiência de *FOXN1*.

As células T circulantes distribuíam-se igualmente entre as subpopulações CD4, CD8 e, aberrantemente, a subpopulação T de células com TCR αβ que não exprimem nem CD4 nem CD8 (duplas-negativas, DNαβ), que são geralmente inferiores a 1%. As células T apresentavam marcadores de memória e activação; eram oligoclonais e não proliferavam após estimulação *in vitro*. O timo produz também uma população de células T reguladoras (Treg) que representam cerca de 5% da subpopulação CD4, com propriedades supressoras, fundamental para a prevenção de autoimunidade, identificadas pela expressão do factor de transcrição "forkhead box P3" (FoxP3). No presente caso, mais de 40% das células CD4 circulantes expressavam altos níveis de FoxP3 e um fenótipo claramente supressor. Assim, a deficiência de *FOXN1* devida à mutação R255X associa-se à presença de células T oligoclonais sugerindo a existência de manutenção de desenvolvimento de linfócitos T, embora com alterações da selecção positiva/negativa ilustrada pela expansão aberrante de células FoxP3+ e DNαβ.

Uma vez que o defeito se restringia ao epitélio tímico, era plausível que o transplante tímico pudesse ser uma estratégia curativa apesar de ser actualmente uma terapia experimental nunca anteriormente usada neste contexto. Observou-se uma progressiva recuperação imunológica após o transplante tímico realizado aos 14 meses

de idade, ilustrada pela associação temporal entre a documentação de respostas T específicas ao BCG e a resolução da adenite persistente.

O estudo da cinética de estabelecimento do compartimento de células T após transplante de timo com um grau elevado de incompatibilidade HLA representa um contributo único para a compreensão da dinâmica de reconstituição do sistema imunitário. Observou-se uma reconstituição progressiva das células T *naïve* exibindo uma diversidade do repertório conservada apesar da incompatibilidade HLA do epitélio tímico. O estabelecimento da população Treg teve uma cinética semelhante às células CD4 mantendo níveis normais. Dados recentes sugerem que o desenvolvimento da população Treg no timo está dependente de um pequeno "nicho" que controla o número de células produzidas. É plausível especular que o FOXN1 desempenhe um papel nestes "nichos" e contribua para a regulação do número de Treg. Em contraste, observou-se a persistência da população DNαβ ao longo dos 5 anos de seguimento, sugerindo uma produção mantida eventualmente num possível rudimento tímico associado à mutação *FOXN1*.

A função do enxerto tímico alogénico foi ainda estimada pela quantificação do sj/βTREC, um rácio entre produtos precoces e tardios do rearranjo do TCR no timo, que representa uma medida indirecta do número de divisões intra-tímicas e que se correlaciona directamente com a produção tímica. O rácio sj/βTREC atingiu níveis comparáveis aos observados em crianças saudáveis. No entanto, 4 anos pós-transplante, documentou-se um declínio marcado do rácio sj/βTREC suportando uma diminuição da actividade do enxerto. Apesar disso, observou-se apenas uma pequena redução seguida de estabilização das células T *naive*, sugerindo que após reconstituição do sistema imunitário a homeostasia periférica consegue assegurar a manutenção da população T *naive*.

Assim, os nosso dados demonstram que é possível adquirir competência imunológica com transplante tímico independentemente da compatibilidade HLA, mesmo na ausência de sustentabilidade do enxerto sugerida pela diminuição do rácio sj/βTREC, o que tem implicações para o desenvolvimento de novas estratégias de reconstituição imunológica noutros contextos clínicos.

Estudos recentes sugerem um aumento compensatório da actividade tímica em resposta a uma diminuição do compartimento de linfócitos T. Tem sido atribuído um papel à citocina IL-7 neste processo para além dos seus efeitos na proliferação homeostática das células T na periferia. A infecção pelo HIV está associada a uma perda progressiva de células T CD4, sendo ainda controversa a capacidade do timo de compensar esta perda bem como de promover a reconstituição desta população após terapêutica antiretroviral (ART) em doentes infectados pelo HIV-1. Por outro lado, os efeitos periféricos da IL-7 podem minimizar a possível redução da actividade tímica quer devida à infecção directa dos timócitos pelo HIV quer mediada pela hiper-activação generalizada associada à infecção. De facto, a activação persistente do sistema imunitário, com a consequente anergia e aumento da susceptibilidade à apoptose, é considerada determinante na progressão para SIDA.

O segundo objectivo deste trabalho foi investigar a relação destas vias na infecção HIV/SIDA. A originalidade deste estudo residiu na comparação de doentes infectados pelo HIV-2, que mesmo na ausência de terapêutica antiretroviral têm virémia reduzida, com indivíduos infectados pelo HIV-1 com supressão terapêutica da virémia e diferentes graus de recuperação das células T CD4. A comparação com doentes infectados pelo HIV-1 não tratados e, portanto, com elevada virémia, permitiu concluir

que a linfopénia CD4 se associa a uma perda de células T *naive,* quer CD4 quer CD8, independentemente do tipo de infecção ou exposição à ART. Nos doentes HIV-1+ tratados com ausência de recuperação das contagens CD4 apesar de resposta virológica, várias estratégias parecem contribuir para suster o declínio das células T CD4 e proteger de infecções oportunistas, nomeadamente: menores níveis de activação imunológica e uma melhor utilização da IL-7 atestada pelo aumento dos níveis intracelulares de Bcl-2, uma molécula anti-apoptótica induzida pela IL-7.

O terceiro objectivo deste trabalho baseou-se no estudo de um doente com Doença Granulomatosa Crónica (CGD) e uma linfopénia CD4 persistente na ausência de infecção pelo HIV. A CGD constitui a deficiência fagocitária primária mais frequente (1:200000 nascimentos) e é causada por defeitos na capacidade oxidativa fagocitária. Este doente de 32 anos, com uma clínica de infecções tipicamente associadas à CGD, apresentou contagens de células T CD4 inferiores 200 células/μl durante mais de 16 anos. O estudo efectuado revelou uma activação linfocitária generalizada, com diferenciação efectora terminal dos linfócitos T CD8 e uma redução da dimensão dos telómeros das subpopulações T, sugerindo um *turnover* celular aumentado devido a uma estimulação persistente do sistema imunitário. Além disso, observou-se uma depleção marcada das células T *naive,* com evidência de diminuição da timopoeise estimada pelo rácio sj/βTREC, apesar dos níveis elevados de IL-7 sérica. Este perfil imunológico tem sido associado a um risco elevado de infecções e sido considerado um factor preditivo de morte em indivíduos com idade avançada. O aparente esgotamento dos recursos imunológicos é particularmente relevante tendo em conta o aumento de esperança de vida dos doentes com CGD, devendo ser considerado na definição de indicações terapêuticas, incluindo transplante de células hematopoiéticas.

Em conclusão, este trabalho demonstra que diferentes vias podem ser exploradas para atingir competência imunológica mesmo na presença de profundas alterações linfocitárias. O seu estudo baseado em modelos humanos é fundamental para o desenvolvimento de novas estratégias de reconstituição imunológica.

Palavras chave: Células T, Timo, Imunodeficiência, FOXN1, HIV/SIDA, Doença Granulomatosa Crónica, Reconstituição Imunológica.

SUMMARY

The thymus is essential for both the establishment of the peripheral T cell pool and the generation of the diverse T cell receptor (TCR) repertoire capable of dealing with new pathogens and controlling the escape of persistent infections. In humans, the thymus is almost fully developed at birth, with the rate of T cell production markedly decreasing after puberty. However, it is now clear that this central lymphoid organ plays an essential role in the lifetime "*de novo*" generation of T cells. The maintenance of naïve T cells is currently thought to depend upon a combination of peripheral T cell proliferation as well as to an age-dependent contribution of recent thymic emigrants.

The overall aim of this work was to investigate the relative roles of the thymus and the "periphery" in the maintenance/recovery of the human T cell compartment through the study of specific clinical models.

FOXN1 is a transcription factor, expressed by thymic epithelium, crucial for both the development of the thymus and prevention of its involution. Defects in *FOXN1* in mice lead to athymia in association with total alopecia, due to its additional role in hair follicle differentiation ("nude-SCID mice"). Human *FOXN1* deficiency was first reported by Pignata *et al.* in two sisters from Italy that, despite the evidence of athymia, exhibited a significant number of circulating T cells. We identified the same homozygous R255X mutation in a Portuguese child, who presented at 5 months of age with alopecia, respiratory failure due to Bacillus Calmette-Guérin (BCG) dissemination following routine neonatal BCG vaccination, and circulating T cells of non-maternal

origin at close to normal numbers. The first aim of this work was to investigate the T cell population generated in the presence of *FOXN1* deficiency.

Circulating T cells were equally distributed between CD4, CD8 and, strikingly, an abnormally increased population of TCR αβ+ cells that expressed neither CD4 nor CD8 (double-negative, DNαβ), which usually represent less than 1% of the peripheral T cell pool. T cells were non-naïve, oligoclonal, activated, and unable to proliferate *in vitro*. The thymus is known to produce a regulatory CD4 T cell subset (Treg), with suppressive properties, fundamental for preventing autoimmunity. Currently they are best identified by expression of the forkhead box P3 transcription factor FoxP3. Notably, more than 40% of the CD4 subset expressed high levels of FoxP3 and had a clear regulatory-like T cell (Treg) phenotype. Thus, human *FOXN1*-deficiency due to R255X mutation was associated with significant numbers of oligoclonal T cells suggesting that, to a certain extent, T cell development still occured, albeit with altered positive/negative selection, as illustrated by the aberrant expansion of FoxP3+ and DN subsets.

As *FOXN1* mutations impact on thymic epithelium rather than hematopoietic precursors, we predicted that thymic transplantation, although never performed before in this setting, could be a curative strategy. This was confirmed by the documentation of the clinical efficacy of HLA-mismatched thymic transplantation, as attested by the temporal association between the clearance of the ongoing BCG adenitis and the development of specific responses against mycobacterium antigens.

Our study of the kinetics of establishment of the T cell pool after HLA mismatched thymic transplantation provides unique data regarding the dynamics of replenishment of the immune system.

A progressive increase of naive (antigen non-experienced cells) T cells was also observed, resulting in the generation of a fully diverse CD4 T cell repertoire in spite of the HLA-mismatched thymic epithelia.

Reconstitution of the Treg pool occurred in parallel to that of the CD4 subset, leading to stable frequencies within the normal range. Thymic Treg development is currently thought to be dependent upon a small developmental niche that tightly controls Treg output. It is thus possible that FOXN1 plays a role in such niches, contributing to the thymic regulation of Treg numbers.

In contrast, a significant population of circulating DNαβ persisted, at relatively stable frequencies (17% of αβ T cells) and phenotype, throughout 5yrs of follow-up. Although the possibility that DNαβ T cells are long-lived cells generated pre-transplant cannot be excluded, it is also plausible that their persistence may reflect a continuous production of this population by a putative thymic rudiment.

The functionality of the allogeneic thymic graft was further estimated by sj/βTREC quantification; a ratio between early and late products of TCR rearrangements that was shown to represent an indirect measurement of thymocyte division-rate, and a direct correlate of thymic output. A progressive increase of the sj/βTREC ratio was observed, reaching levels comparable to those found in healthy children. Importantly, a sharp decline of sj/βTREC, accompanied by a decrease in the proportion of naïve cells was observed 4yrs post-transplant. Notably, these values plateaued thereafter, suggesting that steady-state equilibrium could be established after replenishment of the immune

system.

Importantly, our data showed that immunocompetence can be achieved through HLA-mismatched thymic transplantation, despite the lack of a sustained thymocyte-division rate (as evidenced by sj/βTREC). These novel data regarding the long-term sustainability of allogeneic thymic tissue and the immunological reconstitution achieved have implications for the design of immune-based therapeutic strategies to be used in other clinical settings.

Recent studies suggested the possibility of a thymic rebound as a compensatory feedback loop triggered by emptiness of the peripheral T cell pool, particularly in HIV infection. The cytokine IL-7 has been highlighted as a possible factor in this process. IL-7 has also a central role in peripheral T cell homeostasis. The IL-7 driven peripheral T cell proliferation/survival is thought to be able to compensate any putative thymic impairment resulting either directly from HIV infection, or from the heightened state of immune-activation that characterizes HIV disease. In fact, the persistent immune stimulation, and the consequent T cell anergy and susceptibility to apoptosis, are considered key determinants of CD4 decline and AIDS progression.

The second specific aim of this work was to investigate the interplay of these pathways during HIV/AIDS. The originality of this study mainly resulted from the definition of the cohorts under investigation. HIV-1+ patients with discordant responses to ART (ART-Discordants, poor CD4 recovery despite suppression of viremia) were compared with HIV-2+ patients that exhibited a similar degree of CD4-depletion and reduced circulating virus in the absence of ART. Untreated HIV-1+

patients who are expected to have high viremia, as well as HIV-1+ patients under ART with successful virological and immunological responses were studied in parallel.

Low CD4-counts were associated with major naïve CD4 and CD8 depletion, irrespective of type of infection or ART-exposure. Several pathways were likely to contribute to the CD4-count stability and low rate of opportunistic infections documented in ART-Discordants in spite of their presumed thymic impairment, namely lower levels of T cell activation and a better ability to use IL-7 as indicated by the expression levels of the IL-7 induced, anti-apoptotic molecule, Bcl-2.

Our third specific aim was based on the study of an individual with chronic granulomatous disease (CGD), a primary defect in the phagocytic oxidative burst, who, despite being HIV negative, presented CD4 T cell depletion at levels similar to those found in advanced AIDS patients. CGD represents the most prevalent (1:200000 live births) primary phagocytic defect. This 32-year-old patient, presenting with typical CGD-associated infections, had a CD4 lymphopenia of less than 200 cells/μl, for more than 16 years. Although there are previous reports of diminished T cell numbers in CGD patients, these studies did not include phenotypic and functional T cell analysis. We found a generalized immune activation, in conjunction with markers of increased cell-turnover, including a reduced telomere length of both the CD4 and CD8 subsets, and expansion of terminally-differentiated effector CD8 T cells. Additionally a marked loss of naïve T cells was found, with evidence of impaired thymic production as assessed by sj/βTREC ratio, despite the increased IL-7 levels. This immunological profile has been considered a risk for infections, and was shown to be an independent predictor of death in aged subjects. Therefore, it is worth considering this putative exhaustion of immune resources in the evaluation of long-term therapeutic strategies,

including stem-cell transplantation, given the increasing life-expectancy of CGD patients.

Overall, our data provide evidence that, although immunodeficiency may be associated with profound lymphocyte disturbances, different pathways can be exploited to achieve immunological competence. The characterization of these pathways in human models is of importance for the definition and design of new strategies for immune reconstitution.

Keywords: T cells, Thymus, Immunodeficiency, FOXN1, HIV/AIDS, Chronic Granulomatous Disease, Immunological Reconstitution.

Introduction

The main function of the immune system is to protect the individual from the continual exposure to potentially pathogenic microorganisms. Another fundamental property is to discriminate these foreign antigens from self-antigens and thus, to maintain immune tolerance.

T lymphocytes play a central role in this process, in two ways. Firstly, they act as orchestrators of the many cell types involved in both innate (non-specific) and adaptive (specific) immune responses. With regard to the latter, they provide the help necessary for B cell differentiation and antibody production. Secondly, they have key effector functions, as clearly illustrated by the contribution of cytotoxic T lymphocytes to the effective control of intracellular pathogens, particularly viruses. In order to carry out these diverse roles, it is necessary for T lymphocytes to possess a broadly reactive TCR repertoire able to react against foreign antigens whilst maintaining self-tolerance.

Potential strategies for T cell reconstitution have become a critical research area for clinical practice. The newly developed, more aggressive therapeutic interventions in the areas of oncology, auto-immune disease, and transplantation are frequently associated with a major side-effect: T cell depletion. It has been shown that the reconstitution of cellular immunity after hematopoietic stem cell transplantation is a critical determinant of its long-term success. On the other hand, in spite of the major

impact of antiretroviral therapy (ART) on the survival and morbidity of Human Immunodeficiency Virus (HIV)-1 infected patients, it is now clear that immune-based complementary therapies are needed to achieve the immunological recovery required to allow the discontinuation of the antiretroviral drugs. Moreover, although primary T cell immunodeficiencies are rare, they are frequently life-threatening conditions that require prompt diagnosis and therapeutic intervention.

The overall aim of this work is to investigate mechanisms involved in the preservation and recovery of the human T cell compartment. Therefore, the introduction has been divided into three main sections.

The first section comprises an overview of T cell physiology, focusing on the central role of the thymus in the establishment and maintenance of a diverse T cell population. Moreover, the main mechanisms thought to be involved in T cell homeostasis and in the regulation of T cell responses will be discussed.

The second part addresses the main causes of T cell deficiencies, with particular focus on the clinical settings that were explored in this work as a strategy to address the relative roles of the thymus and periphery in the maintenance/recovery of the T cell pool.

The final section provides a review of the most important, currently available, therapeutic strategies for achieving T cell recovery.

1.1 T cell physiology

1.1.1 T cell generation

T lymphocytes are produced in the thymus. This primary lymphoid organ provides a unique environment that enables the differentiation of bone-marrow derived T cell precursors into mature T cells. The key feature of this process is the generation of a diverse repertoire of functional T cell receptors (TCR), with a reduced reactivity to self-antigens. As a primary site for T cell lymphopoiesis, the thymus is active, not only during the foetal stages of development, but also throughout postnatal life.

In the mouse, the thymic rudiment is first evident on day 11 of gestation, having evolved from the endoderm of the third pharyngeal pouch (1). The development of the thymus in humans closely follows that observed in the murine model of thymic development. The growth of the thymus primordium from the third pouch coincides with the onset of *FOXN1* (*Forkhead box protein N1*) gene expression (2). The forkhead transcription factor FOXN1 is the best-known regulator of foetal thymus development (3). Its expression has been shown to be required for differentiation of thymic epithelial cells (4) and the induction of cortical and medullary differentiation in the thymus (5). Before undergoing the differentiation into the medullary and cortex compartmentalization, the thymus is colonized by hematopoietic precursors (1, 6, 7).

The thymic architecture consists of distinct anatomical compartments which include the subcapsular area, the cortex, the cortical medullary junction and the medulla (8). Thymic epithelial cells (TECs) constitute the major component of the stromal population. They can be subdivided according to their functional,

morphological, and antigen presentation capacity, into two main subpopulations, cortical (cTEC) and medullary (mTEC) epithelial cells. In addition to the TEC subsets described above, the thymus is composed of other cell types, including populations of haematopoietic (dendritic cells, macrophages, B cells) and nonhaematopoietic (fibroblasts, endothelial cells and others) origin. This stromal scaffold provides the specialized microenvironment necessary for the life-long attraction of haematopoietic precursor cells; the signalling infrastructure required to instruct early thymocyte differentiation; the factors to guide precursor cells to the different anatomical sub-compartments; the molecular constraints that are needed for the selection of immature T cells; and the molecules necessary for the functionally mature T cells to exit to the periphery. The hallmark of T cell development is the generation of T cells that express a functional TCR, whether it be TCRαβ or TCRγδ, able to recognize antigenic peptides bound to major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APC).

The CD34+ precursor cells, which originate from bone-marrow stem cells, migrate to the thymus to undergo T cell development (9). These precursor cells enter the human thymus at the cortico-medullary junction. From this area they migrate towards the cortical region where proliferation and differentiation are initiated via interactions with the thymic stroma. The distinct stages of T cell development are defined by the sequential expression of cell-surface antigens (Figure 1) (10). Rearrangement of the TCR β, γ and δ loci, as well as expression of the CD1a molecule mark the commitment to the T cell lineage. The definition of thymocyte differentiation steps is typically based on the cell-surface expression of CD4 and CD8, with undifferentiated populations

being classified as CD4-CD8- double negative (DN). In humans, three distinct DN stages can be recognized: a CD34+CD38-CD1a- stage that represents the most immature thymic subset, and the consecutive CD34+CD38+CD1a- and CD34+CD38+CD1a+ (Pre-T1) stages. Human DN thymocytes mature via a CD4 immature single positive (CD4ISP) stage (Pre-T2), which express CD4 in the absence of CD8. This population contains precursors for both the αβ and γδ T cell lineages. The CD4ISP stage is followed by an early double-positive (EDP) stage composed of cells expressing CD4 and the α chain of CD8. EDP cells are the immediate precursors of double-positive (DP) cells, which are characterized by the co-expression of CD4 and the α and β chains of CD8. Following this DP stage, cells differentiate into CD4+ or CD8+ SP T cells that express a functional TCR and exit the thymus (8).

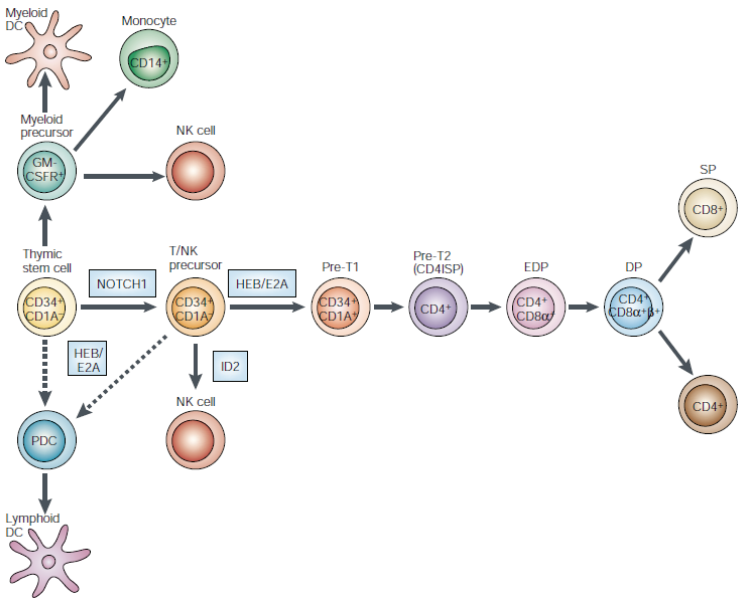

Figure 1: Early stages of T cell development in the thymus. From Spits *et al* (10).

The DN thymocytes mature in distinct areas within the cortex, as well as in the subcapsular region. Their proliferation, survival and differentiation are controlled by a combination of cell-autonomous factors and a number of stromal cell-derived signals

(including interleukin-7 (IL-7) and stem cell factor (SCF)) secreted by TEC; Wnt molecules, Hedgehog (an essential positive regulator of T cell progenitor differentiation); and Notch, amongst others (11-13).

TCR gene rearrangement plays a crucial role in thymocyte fate. Thymocytes can only survive to maturity if they successfully carry out combinations of gene rearrangements that will generate α and β, or γ and δ, chains, that can support the assembly of TCRαβ/CD3, or TCRγδ/CD3 complexes, respectively. The TCR diversity that can be generated within the thymocyte pool has been estimated to be as large as 10^{20} α/β-chain combinations**.**

The antigen-recognising, variable domains of TCRα and β chains are encoded by combinations of variable (V), diversity (D), and joining (J) gene segments (TCRβ chains), or V and J gene segments (TCRα chains).

D-to-J recombination occurs first in the β chain of the TCR. This process can involve either the joining of the Dβ1 gene segment to one of six Jβ1 segments, or the joining of the Dβ2 gene segment to one of seven Jβ2 segments. D-to-J recombination is followed by Vβ-to-DβJβ rearrangements leading to the formation of the TCR Cβ chain.

T cell commitment is thought to occur with the initiation of TCRβ gene rearrangement. β-selection is the process by which precursor T cells with a productive rearrangement of the TCRβ locus are selected to undergo further differentiation, in the form of TCRα rearrangements. The developmental stage at which β-selection occurs in humans is still the subject of controversy (10). However, it was recently shown to occur as early as the CD34+CD38+CD1a+ stage (14). Thymocytes harbouring a rearranged

TCRβ locus generate a TCRβ molecule that pairs covalently with the invariant pre-TCRα (pTα), and noncovalently with CD3 signal-transducing molecules, resulting in formation of a pre-TCR complex at the cell surface.

The rearrangement of the β chain of the TCR is followed by α chain rearrangement. The subsequent assembly of the β and α chains results in formation of the αβ-TCR that is expressed on a majority of T cells. As the TCRδ gene segments are embedded within the TCRα locus, the V-to-Jα rearrangements lead to deletion of the δ locus from the chromosome (15).

After completion of TCRα rearrangements, αβ T cells are selected by a low-affinity interaction of the TCRαβ heterodimer with self-peptides complexed with MHC antigens, a process termed positive selection. Thymocytes differentiate into single positive (SP) CD4 or SP CD8 thymocytes whose TCR recognises MHC class II or class I molecules, respectively. Death by neglect of thymocytes whose TCR cannot recognise antigen in the context of either MHC class I or II molecules ensures that only those thymocytes with appropriate TCR specificities survive and differentiate into functionally mature T cells (16). Thymocytes that express high-affinity receptors for self-peptide–MHC complexes expressed on thymic dendritc cell (DCs) are deleted through a process known as negative selection.

The usual end result of these combined selection mechanisms is the generation of a naïve T cell pool composed of cells with a stringently selected TCR repertoire, able to respond to foreign non-self antigens. They are exported to the periphery where the majority of them remain tolerant to the host's tissues (self).

A variety of T cell receptor excision circles (TRECs) are formed from the excised DNA generated by the rearrangements of the TCRα, β and δ loci described above.

During TCRα rearrangement, the end-to-end ligation of the recombination signal sequences flanking the δ-rec locus and the ψ-Jα locus generates a single TREC containing a signal joint (sj) sequence (sjTREC), as shown in Figure 2. Coding-joint (cj)TRECs are produced during the TCRα rearrangement of V to J gene segments.

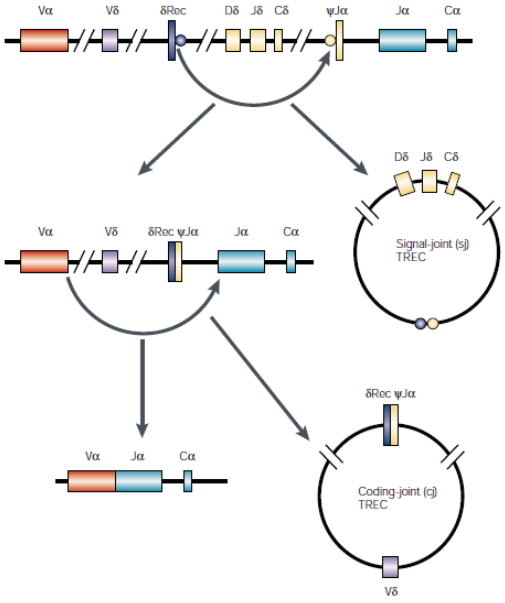

Figure 2: T cell receptor excision circles. From Spits *et al* (10).

TRECs are not duplicated during mitosis, and are therefore diluted out with each cellular division (17, 18). Since recent thymic emigrants (RTE) are enriched in these molecules, TREC levels have been used to assess thymic function, through their quantification in peripheral blood using real time PCR technology.

The quantification of sjTREC as a marker of RTE, and measure of thymic output was first utilized by Douek *et al* (19). As expected, given the gradual loss of thymic function during aging, they showed a decreasing number of sjTRECs with age in

healthy individuals. Moreover, they also reported that sjTREC levels were decreased in HIV infected patients, but rapidly increased during antiretroviral therapy (19).

Since sjTRECs are not replicated during cell division, their levels can also be influenced by events occurring in the periphery, such as cell proliferation and differentiation, redistribution, or alterations in cell survival. All of these can lead to a dilution of TREC-bearing cells in the periphery. Thus, TREC quantification data requires cautious interpretation; particularly in those conditions, such as the process of immune reconstitution following bone marrow transplantation or during HIV infection, that are associated with the previously described confounding factors.

The level of thymic output has been shown to be primarily determined by the intrathymic proliferation of precursor T cells (20). Thus, a new assay was developed to estimate the relative changes in intrathymic proliferation occurring between the TN and early DP stages (21), as represented in Figure 3. This approach is based not only in the quantification of sjTRECs, generated at the DP stage, but also the DβJβTREC created during the previous DβJβ rearrangement and organization of the TCRβ locus. The proliferation occurring between these stages can then be estimated by the measurement of the ratio of βTREC to sjTREC (sj/βTREC ratio) (21). It was also shown that this parameter was not influenced at the periphery, given that both types of TREC were equally affected by the rates of proliferation and death of peripheral T lymphocytes (21).

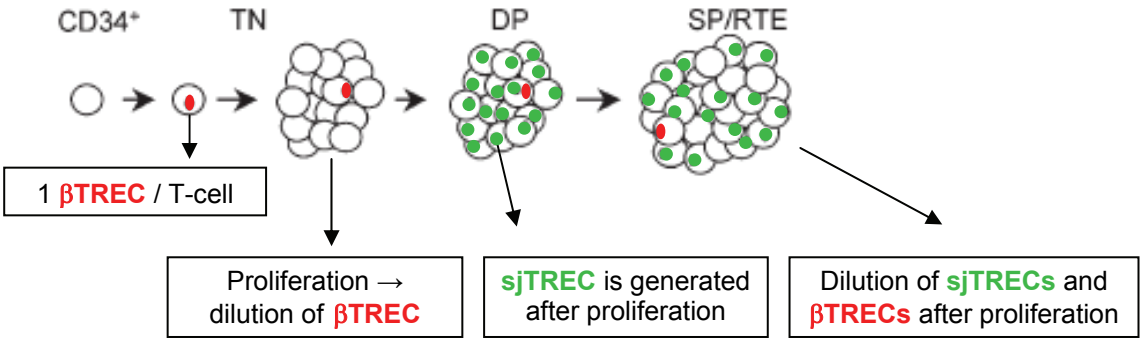

Figure 3: Schematic representation of intrathymic proliferation occurring between late TN and early DP thymocyte differentiation stages and its effects on βTREC and sjTREC levels. TN: Triple Negative stage; DP: Double Positive stage; SP/RTE: Single Positive/ Recent Thymic Emigrant stage. Adapted from Dion, *et al* (21).

1.1.2 The naïve T cell compartment

RTEs emigrating from the thymus are incorporated into the pool of naïve T cells. The size of this pool remains relatively constant throughout adult life despite continuous antigenic stimulation and the reduction of naïve T cell production by the thymus. The maintenance of the naïve T cell pool is thought to mainly depend upon survival signals, such as those provided through TCR engagement of self-peptide-MHC complexes and by IL-7 (22-24). IL-7 has been shown to be a key cytokine involved in controlling the survival and homeostatic turnover of peripheral T cells (25-27). Its effects on T cells appear to be multifactorial and are regulated by the expression of its specific receptor, a heterodimer consisting of the IL-7 receptor α chain (IL-7Rα) and common gamma chain (γc). The binding of IL-7 to its receptor induces several signalling cascades, such as the JAK–STAT (Janus kinase–signal transducer and activator of transcription) and the PI3K (phosphoinositide 3-kinase) pathways, that promote lymphocyte survival (28, 29). Furthermore, IL-7 has been shown to inhibit

programmed cell death by up-regulating the expression of the anti-apoptotic molecule Bcl-2 (30).

Prior to contact with antigen, naive T cells continuously recirculate between blood and secondary lymphoid tissues (spleen, lymph nodes, Peyer's patches and mucosal-associated lymphoid tissues) (31, 32). Entry into secondary lymphoid tissues is a highly regulated process. In the case of lymph nodes and Peyer's patches, specialized blood vessels called high endothelial venules (HEVs) serve as the entry point into the tissue. HEVs express a unique set of ligands that are recognized by homing receptors expressed on the naïve T cells' surfaces. Amongst the most critical of these are the chemokine CCL21, that engages CCR7, and vascular addressins, that interact with CD62L (L selectin) (31). Thus, naïve T cells moving along HEV's encounter IL-7, self-peptide–MHC complexes and CCR7 ligands, all of which cooperate to produce homeostatic survival signals (33). Continuous migration of T cells through the secondary lymphoid tissues is a key mechanism in providing antigenic surveillance. Within lymphoid tissues, these antigens are presented to T cells in the form of peptide fragments bound to MHC molecules expressed upon specialized APC, in particular DC. These cells are strategically positioned within a dense network in the T cell zones and are continuously surveyed by recirculating T cells for their expression of foreign peptide/MHC complexes.

Several surface molecules have been shown to be expressed by, and thus identify, naïve T cells. The co-expression of CD45RA and CD62L on CD4+ T cells is frequently used, and is currently thought to identify the majority of this population. With respect to CD8 T cells, the surface co-expression of CD45RA and CD27 is commonly used to

identify the naïve subset (34). However, the combination of these markers with other molecules, such as CCR7 or CD28, has enabled a more detailed definition of CD8 T cell differentiation states and/or functional capacity (35-37).

Some groups have shown that the CD31 (PECAM-1) molecule is expressed preferentially on the cell surface of naïve, TREC-rich T cells that have undergone a low number of cell divisions (38, 39). On this basis, Kimmig *et al* were able to distinguish two populations of naive T cells: "true" recent thymic emigrants (^{thymic}naive CD4 cells, that co-expressed CD45RA and CD31 and have high TREC content), and peripherally expanded naive CD4 cells (^{central}naive CD4 cells) (38), as illustrated in Figure 4.

As previously discussed, aging is associated with decreased thymic activity that results in reduced numbers of ^{thymic}naive CD4 cells (38). Triggering by self peptide/MHC complexes is thought to induce ^{thymic}naive CD4 cells to proliferate and differentiate into ^{central}naive CD4 cells (38). This population featured lower TREC levels, expressed CD45RA and lacked CD31 expression. Of note, both ^{thymic}naive and ^{central}naive CD4 cells are able to differentiate into CD31-CD45RA-CD45RO+ memory-effector cells upon antigen encounter (38).

Importantly, results from our lab suggest that IL-7 may play a role in the maintenance of CD31+ naïve CD4 T cells during adult life (40). In adults, this cytokine was shown to preferentially drive the proliferation of the CD31+ naive CD4 subset, and to increase or sustain the expression of CD31.

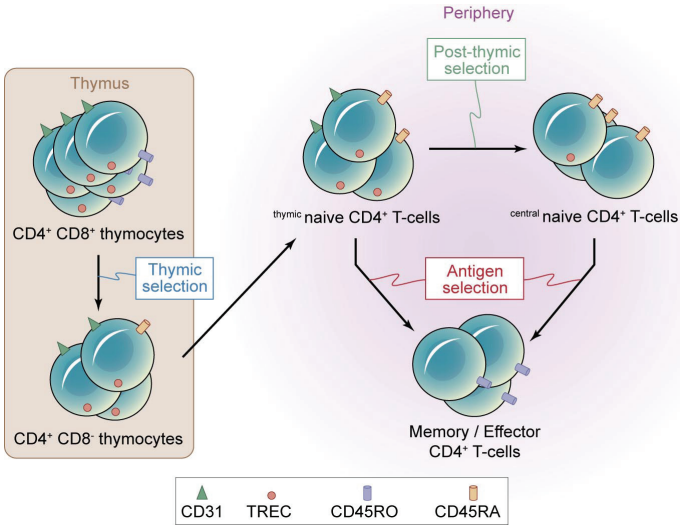

Figure 4: Post-thymic proliferation of human naive CD4 T cells. From Kohler *et al* (39).

Recently, protein tyrosine kinase 7 (PTK7) was described as a novel marker of human CD4 RTEs (41). A fraction of the naïve CD31+ CD4 subset was shown to express PTK7 and to contain higher levels of sjTREC as compared with the PTK7-CD31+ counterpart. Additionally, patients that underwent complete thymectomy were shown to have a more pronounced and persistent loss of PTK7+CD31+ than PTK7-CD31+ naive CD4+ T cells, suggesting that PTK7, unlike CD31, may identify those RTEs most recently produced by the thymus (41).

Upon antigen stimulation, naïve T cells differentiate into distinct cell types that play important functions during the development of an immune response.

1.1.3 The mounting of specific immune responses

Specific immune responses are mostly initiated in the T cell areas of secondary lymphoid organs where naïve T lymphocytes encounter antigen-loaded DC. T lymphocytes recognize antigen through the interaction of their TCR with foreign peptide-MHC complexes displayed on the surface of APCs, through which a tight synapse can form (34, 42). Activation of the T cell follows synapse formation and is associated with rapid clustering of TCR molecules, bound to peptide/MHC complexes upon the APC, and a consequent, local accumulation of intracellular signalling molecules. The intensity of signal that a T cell receives is dependent both on the number TCRs triggered by the peptide–MHC complexes, and the level of costimulatory signals that regulate the activation process (43). A large number of costimulatory/adhesion molecules expressed on T cells (CD28, LFA-1, CD40L, ICOS, OX40, CD2, CD27, and 41BB) (44, 45) bind to their receptors on the APC. Some costimulatory/adhesion molecules provide essential second signals for T cell activation, whilst others act by enhancing TCR triggering via stabilization of the synapse, and/or through the recruitment of intracellular signalling molecules (46, 47). A further level of co-stimulation is provided by the release of various cytokines that can act in an autocrine and/or paracrine fashion to enhance the process of T cell activation and subsequent downstream events.

The continuous TCR and cytokine stimulation induces T cell to divide and progressively differentiate into effector and memory subsets, during which they acquire the capacity to produce effector molecules, such as cytokines and cytolytic mediators (48, 49).

At later stages of the immune response, the clearance of pathogen from the site of infection, by effector cells, reduces the influx of antigen-loaded APC into the T cell zones.

After antigen withdrawal, the survival of activated T cells becomes dependent upon the expression of anti-apoptotic molecules such as Bcl-2 and the expression of receptors for homeostatic cytokines such as IL-7 and IL-15, as discussed below.

1.1.4 Memory-effector T cell subsets

During an immune response, antigen-specific T cells proliferate, generating a large number of effector cells that migrate to the distal site of infection to fight the invading pathogen. Some of these primed T cells develop into memory cells, which confer protection in peripheral tissues, through their ability to mount a more rapid and effective response to their cognate antigen. This process is known as a secondary immune response. Of note, memory T cells increase progressively with age as a consequence of T cell responses to diverse foreign and self-antigens.

Understanding the pathways of memory T cell differentiation in humans has been a central issue in immunology. Distinct memory T cell subsets have been defined on the basis of homing capacity and effector function (34, 50). These definitions, based on the expression of several differentiation markers, have failed to identify a clear phenotype for each putative subset. Nevertheless, both in humans and mice, memory cells have been shown to comprise of populations of "central" memory (T_{CM}) and effector-memory (T_{EM}) T cells. T_{CM} and T_{EM} cells were initially defined in the human immune system based on the absence or presence of immediate effector function, and

on the expression of homing receptors that allow cells to migrate to secondary lymphoid organs *vs.* nonlymphoid tissues (34, 50).

Human T_{CM} are CD45RO+ memory cells that recirculate between blood and the secondary lymphoid organs, entering the latter via high expression of CCR7 and CD62L (51). These cells also feature high surface expression of CD127 and CD122, which allow them to readily respond to IL-7 and IL-15, promoting survival and homeostatic proliferation. Central memory T cells have little or no immediate effector function but readily proliferate and differentiate into effector cells in response to antigenic stimulation (51).

Effector memory T cells (T_{EM}) migrate to inflamed peripheral tissues and display immediate effector function, as evidenced by their rapid production of effector cytokines, such as IFN-γ, and contain large amounts of cytolytic mediators, such as perforin. Human TEM are cells that have lost CCR7 expression, have a heterogeneous CD62L expression profile, and display characteristic sets of chemokine receptors and adhesion molecules that are necessary for homing to inflamed tissues (51).

Memory and effector CD8 T cells have been shown to play an important role in viral infections throught their cytotoxic activity and also because of their ability to produce various factors involved in suppression of viral replication, including cytokines and chemokines (51-53).

The surface expression of the costimulatory molecules CD27 and CD28 together with CD45RA have been used by several authors to discriminate between distinct stages of human CD8 T cell differentiation (36, 37, 54). A T cell differentiation pathway

has been proposed in which CD27+CD28+CD45RA+ naive cells progress through a CD27+/-CD28+/-CD45RA- to a CD27-CD28-CD45RA+ terminally differentiated effector phenotype with increased cytotoxic potential and reduced ability to proliferate (35, 51, 53).

Both IL-7 and IL-15 have been described as playing a role in the maintenance of memory T cells (55). In particular, IL-15 has been shown to have a crucial role in memory CD8 T cell generation and/or maintenance, as illustrated by the lack of memory CD8 T cells in IL-15Rα- and IL15-deficient mice (56, 57). Although IL-15 has been reported to have only a minimal role in the homeostasis of memory CD4 T cells (58), it has been recently published that antigen-specific CD4 memory cells are also dependent upon this cytokine for their basal homeostatic proliferation and long-term survival (59).

Effector CD4 T cells, also known as CD4 T helper (Th) cells, can be polarized into distinct subsets characterized by the acquisition of cytokine production and other specialized functions. Polarization of lymphocytes to T helper 1 (Th1) or Th2 cells is promoted by IL-12 and IL-4, respectively (60), as well as by the strength and duration of TCR stimulation (61). The differentiation processes involves upregulation of specific transcription factors (62, 63) and activation of STAT proteins (64).

As part of their differentiation programme, Th1 and Th2 cells down-regulate lymph-node homing receptors, and up-regulate the expression of those receptors necessary to enable their migration to inflamed non-lymphoid tissues, where they exert effector functions. Each lineage also expresses unique cytokine receptors, enabling them to respond to cytokines produced by accessory cells. Th1 cells are characterized

by their secretion of IFN-γ and are important activators of macrophages, NK cells, and CD8 T cells (65). They are thought to be involved in the defense against intracellular pathogens. Th2 cells secrete IL-4, IL-13, IL-15, IL-10 and IL-25 and are important for barrier defense at mucosal and epithelial surfaces, as well as in the control against parasites. These cells mobilize and activate eosinophils, basophils, mast cells and, alternatively, activated macrophages (66).

A third subset of IL-17-producing effector T helper cells, referred to as Th17 cells, has recently been characterized. The differentiation of Th17 cells is initiated by the activation of naive T cells in the presence of IL-6 plus transforming growth factor (TGF)-β. This leads to the expression of the transcription factor ROR-γt, and production of IL-17 (67). Th17 cells have been suggested to play a role in the induction of autoimmunity and inflammation. They act in concert with neutrophils and are important for defense against extracellular bacteria and fungi (68, 69).

Recently, IL-9 producing CD4 T cells have been described as a novel Th subset (TH9) (70, 71). Th9 differentiation has been shown, both in humans and mice, to require exposure to the cytokines TGF-β and IL-4, and to be mediated through the expression of the transcription factor GATA-3. Of note, several inflammatory cytokines, such as IL-1β, IL-6, IL-10, IL-21 and type I IFNs, enhance IL-9 production, suggesting a complex regulation of Th9 differentiation (72). The exact role of Th9 cells in the immune system has yet to be fully defined. Studies in mice have suggested this subset plays a role in tissue inflammation (70), immunity against helminth infections, as well as in allergy (71).

Follicular helper T cells, or ThF cells, are memory-effector CD4 T cells found in lymph nodes and identified by high expression of CXCR5 (73, 74). They are found at the periphery of B cell follicles and in germinal centers (GC). They are thought to mediate naïve B cell activation and GC formation, probably through the expression of CD40 ligand (CD40L) and secretion of IL-21 and IL-10.

1.1.5 Regulatory T cells

An important feature of the immune system is its capacity to discriminate between self and non-self, whilst establishing and maintaining a lack self-responsiveness. The maintenance of immunological self-tolerance is a tightly regulated process. As previously discussed, the deletion of self-reactive cells in the thymus plays a key role in this process. As this process is not perfect, autoreactive clones may occasionally escape into the periphery, hence the need for other mechanisms to maintain a state of tolerance in the periphery. So-called "peripheral tolerance" is maintained through a variety of mechanisms, including the presence of a population of regulatory T cells (Treg) that actively suppress autoreactive T cells (75-78).

Treg form a subset of CD4 T cells that either develop in the thymus (naturally occurring Treg; nTreg) or in the periphery (induced Treg; iTreg). The latter can be generated in a variety of circumstances; such as direct differentiation from naive T cells that have undergone TCR-stimulation in the presence of TGF-β, or via the conversion of other pre-existing T cell subsets.

Treg constitute 5% to 10% of the peripheral CD4 T cell compartment (79). In both mice and humans, this population was first defined by their expression of the IL-2

receptor α-chain (CD25), and their capacity to suppress the proliferation of other T cells. CD4+CD25+ Treg were first defined by Sakaguchi *et al.* (75) in the murine system. They showed that the transfer, into athymic nude mice, of lymphoid-cell populations depleted of CD4+ T cells expressing CD25 caused the spontaneous development of autoimmune disease. Additionally, reconstitution with CD4+CD25+ T cells prevented the development of autoimmunity.

In humans the CD4+ T cell subset expressing the highest levels of CD25 (termed $CD25^{bright}$) was shown to be enriched in cells with regulatory properties (76).

The transcription factor FoxP3 has been shown to be specifically expressed by Treg, and is currently considered the most specific marker of these cells. This was further supported by the discovery that mutations in the *FOXP3* gene in humans cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). This syndrome is characterized by a high incidence of autoimmune disease, including type 1 diabetes and thyroiditis, inflammatory bowel disease, and allergic diseases (80, 81). The *FOXP3* gene has been shown to be a key regulator of Treg development and functional activity (82-84). However, due to its nuclear expression, FoxP3 cannot be used to purify Tregs (83, 85).

Importantly, FoxP3 expression can be up-regulated as a result of T cell activation, at least in humans (86). The TCR stimulation of human naïve FoxP3-CD4+ cells was shown to induce FoxP3 expression without conferring suppressive activity on these cells (86). Thus, functional characterization of FoxP3+ cell is of major importance in order to definitively confirm their true identity, especially in the context of pathological conditions associated with hyperimmune T cell activation.

One of the current challenges in the field of regulatory T cell research remains the determination of the relative *in vivo* contributions of nTreg versus iTreg to the Treg pool as a whole. nTreg develop in the thymus and exit into the periphery.

In younger adults, around 10% of Foxp3+ Treg express CD45RA and are considered an unprimed, or *naïve* Treg population (87, 88). In addition, a large proportion of CD45RA+ Treg in adults express CD31, however its frequency rapidly declines with age (89). However, naïve CD45RA+ Treg have been shown to proliferate significantly less than their CD45RO+ counterparts (89, 90). Thus, the contribution of different Treg sub-populations to the maintenance of the total Treg pool in adults is yet not clear.

Regarding iTregs, it was recently suggested that human Tregs may be generated from rapidly dividing, differentiated memory CD4 T cells (91). Although these memory Tregs were highly proliferative, they were also highly susceptible to apoptosis and replicative senescence, suggesting they possessed a limited capacity for self-renewal (91).

In an attempt to clarify the dynamics of Treg cell differentiation, Sakaguchi *et al.* have recently reported that human FoxP3+CD4+ T cells can be separated into three functionally and phenotypically distinct subpopulations based on the expression of FoxP3 (90, 92). These included CD45RA+FoxP3^{low} resting Treg cells (rTreg cells); CD45RA-FoxP3^{high} activated Treg cells (aTreg cells); and cytokine-secreting CD45RA-FoxP3^{low} non-Treg cells. Both rTeg and aTreg cells where shown to have *in vitro* suppressive capacity, whereas CD45RA-FoxP3^{low} non-Treg cells did not. Importantly, they were able to distinguish the differentiation pathways of these subpopulations. FoxP3^{high} aTreg cells were shown to originate from rTreg cells, although some FoxP3^{high} Treg cells may also have arisen from FoxP3-CD4+ non-Treg cells (91). Moreover, a

large proportion of the FoxP3^{high} aTreg cells proliferated *in vivo* and appeared to be recently activated, whilst nearly all rTreg cells did not express the cycling marker Ki-67. On stimulation, rTreg cells upregulated FoxP3 expression, and differentiated into aTreg cells, a process associated with a concomitant gain of proliferative capacity (90). These newly differentiated aTreg were also shown to be highly susceptibility to apoptosis. Thus, the identification of the inter-relationship between the variously described Treg subpopulations, and the unique and/or common functions they may serve, is fundamental to the understanding of their role in both disease states and the maintenance of peripheral tolerance.

Several other molecules, that may be potentially important for Treg function, have been shown to be expressed by this T cell subset. In particular, cytotoxic T lymphocyte associated protein 4 (CTLA-4) was shown to be constitutively expressed by the majority of Treg (93). It is not clear whether CTLA-4 expression is absolutely required for Treg generation, regulatory capacity or both. CTLA-4-deficient mice have been shown to be able to generate CD4+FoxP3+ Tregs that maintain their suppressive capacity *in vitro* and *in vivo* (94, 95), supporting the idea that CTLA-4 expression is not an absolute requirement for the development or suppressive function of Treg. Another molecule, CD39, has been shown to be expressed by immunosupressive Treg (96). However, in contrast to mice, where CD39 is ubiquitously expressed upon Treg cells, expression of this molecule in humans was shown to be confined to a subset of FoxP3+ cells of an activated effector/memory-like suppressor cell phenotype (96). Other groups have also shown that FoxP3 expression and suppressive capacity are enriched in CD4 T cells that express low levels of IL-7Rα (CD127), suggesting that the low CD127 expression can be used to isolate Treg (97).

The expression of the integrin $\alpha_E\beta_7$ (CD103) has been shown to potentially distinguishes a unique functional subset of Foxp3+ T cells with suppressive properties in mice (98, 99). CD103 is expressed on CD4+ and CD8+ T cells in the intestine and other epithelial compartments. Treg cells expressing this marker were shown to display an effector/memory phenotype, possess more potent suppressor activity *in vitro*, and selectively home to inflamed tissue sites *in vivo* (98-100). However, in humans, this markers has been described to be expressed by only a minority of the Treg population (101, 102)

Several other surface markers have been put forward as candidates to identify Treg. Overall it is not known whether the apparent degeneracy of Treg marker expression reflects the existence of discrete *in vivo* Treg subsets, levels of Treg activation or even if it is potentially an artefact of experimental design.

1.1.6 Maintenance of T cell homeostasis

Claude Bernard was the first to realize that the body has control mechanisms able to maintain its internal equilibrium (in terms of body temperature and the levels of nutrients and waste products) in spite of changes in the environment (103). Later, this type of control was given the name homeostasis (104). The immune system is under homeostatic control, such that it can react to changes in the environment, enabling the maintenance of a relatively constant number of cells throughout the life of an individual (105, 106). Therefore, homeostasis of both naïve and memory T cell pool is a highly dynamic and tightly regulated process depending on a balance between generation, proliferation, differentiation, survival, and death.

Cytokines that signal through receptor complexes containing the common γ (γc or CD132) chain receptor subunit are central regulators of lymphocyte homeostasis. These include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Despite the shared usage of the γc chain, individual γc cytokine family members have diverse roles in lymphocyte development and homeostasis. Loss of γc signaling in humans result in X-linked severe combined immunodeficiency (X-SCID) characterized by functionally deficient B cells and absent T and NK cells (107).

IL-7 is considered a key cytokine in T cell homeostasis, acting both during thymopoiesis and in the periphery; where it promotes naive T cell proliferation and survival, and maintains memory T cells (25-27, 108). It is produced by stromal tissues, including the bone marrow and lymph nodes, as well as thymic and intestinal epithelial cells (109-111). IL-7 functions by binding to its receptor (IL-7R), a heterodimer consisting of the IL-7 receptor alpha chain (IL-7Rα or CD127) and the γc. IL-7Rα has been shown to play an important role in the regulation of IL-7 biology (112, 113). Transient downregulation of this chain after IL-7 signalling was shown to be an important homeostatic mechanism for maximizing IL-7 availability. Thus, cells that have received cytokine-mediated survival signals do not compete with unstimulated T cells for the remaining IL-7 (112). Moreover, although the biological role of soluble IL-7Rα receptor is not yet known, it has been suggested that it could bind circulating IL-7, thereby decreasing its bioavailability (114). IL-7Rα is expressed by naive T cells but is downregulated following their activation and subsequent transition to effector cells (108, 115). However, IL-7Rα is re-expressed by a proportion of effector cells and is thought to be important for the development and survival of memory T cells (116).

Terminally-differentiated effector cells, particularly CD8 T cells were shown to not express IL-7Rα (116).

Circulating levels of IL-7 increase in clinical settings associated with T cell depletion. A marked inverse correlation was observed between the level of circulating IL-7 and peripheral CD4 T cell numbers in both children and adults infected with HIV (25, 117-119). Following effective antiretroviral therapy (ART), recovery of CD4 numbers was accompanied by a decline in the level of circulating IL-7 (120, 121). Inverse relationships were also described in children and young adults treated with cytotoxic chemotherapy for cancer, and in patients with idiopathic CD4 T cell lymphopenia (25). Two main mechanisms could underlie the increases in circulating IL-7 levels observed in states of T cell lymphopenia. Firstly it could result from increased IL-7 production as part of a compensatory feedback loop designed to enhance T cell differentiation, survival and/or expansion. Alternatively, it could also result from an increased availability of the cytokine due to decreased T cell numbers expressing IL-7 receptor (25).

It is expected that increased levels of IL-7 enhance both thymopoiesis and peripheral homeostatic expansion of T cell populations, and thus represents an important mechanism contributing to the restoration of immune competence in lymphopenic individuals (25-27, 108).

Although naive T cells are generally thought to turnover relatively slowly, peripheral T cell proliferation could make an important contribution to the maintenance of this T cell pool in human adults (122). The contribution of recent thymic emigrants and peripheral T cell proliferation to the maintenance of the naive T

cell pool has been an issue of investigation both in children and adults, given its potential importance to the understanding of human T cell dynamics (122).

Hazenberg *et al.* have shown that in healthy children, an age-related increase in total body numbers of naive and memory T cells was not accompanied by an increase in absolute numbers of TRECs, suggesting a proliferation-dependent expansion of the naïve T cell pool (122). Indeed, they found that the proportion of dividing CD4 T cells, as assessed by Ki-67 expression, was highest in infants and declined with increasing age. This was observed in both naive and memory T cell subsets (122). Their data suggest that peripheral expansion may be an important factor in the establishment of both the naive and memory T cell pools.

Regarding human adults, Vrisekoop *et al.* have used *in vivo* labelling with stable isotopes, in combination with mathematical analysis, to study both T cell decay and production rates, and follow the fate of recently produced T cells (recent thymic emigrants, RTE). By measuring of the deuterium enrichment in DNA, following deuterated water-labelling, they showed that newly produced naive T cells tended to live longer than the average cell in the naive T cell population (123). This would suggest that the few, newly-produced naive T cells are preferentially incorporated into the peripheral naïve T cell pool. This model argues that the preferential incorporation of RTEs into the resident naive T cell pool provides an efficient way to continuously rejuvenate the naive T cell pool, and thus the T cell repertoire (123).

Important questions regarding lymphocyte kinetics/turnover remain to be addressed in human diseases such as HIV infection, other clinical conditions associated with immunodeficiency, and in clinical settings requiring immune reconstitution.

All of these conditions are often associated with high levels of immune activation (124-126). In particular, chronic hyper-immune activation has been well characterized in the context of HIV infection, and has been suggested as the driving force behind CD4 depletion in this disease (127, 128). With respect to HIV infection, it has been reported to be associated with ineffective control of virus replication (129, 130); accelerated apoptosis and turnover of T and B lymphocytes (131-133); increased frequency of T cells expressing markers of activation and proliferation (CD38, HLA-DR, Ki-67) (128, 134); and elevated serum levels of proinflammatory cytokines (135). Chronic immune activation can also lead to severe immune dysfunctions such as anergy and enhanced activation-induced cell death. Any and all of these mechanisms can contribute to alterations in T cell homeostasis, which is of particular importance in those clinical settings associated with immunodeficiency.

1.2 T cell deficiencies

1.2.1 Overview of immunodeficiencies

Immunodeficiency usually results in increased susceptibility to life-threatening infections, but may also be associated with other immunological manifestations such as allergy, autoimmune disease and lymphoproliferation.

Immunodeficiency diseases may affect any part of the immune system and have been traditionally grouped in defects in cell-mediated immunity and/or humoral immunity. Qualitative and/or quantitative defects of the innate immune system also fall within this category, such as impairments in phagocytic cell function, or damage to anatomical barriers such as the skin and the mucosal surfaces.

An immunodeficiency may be caused either by an inborn defect in the cells of the immune system, or by extrinsic factors or agents. In the case of an inborn, or congenital defect, the disease is designated a primary immunodeficiency (PID). Immunodeficiencies caused by extrinsic factors are referred as a secondary or acquired immunodeficiency. PIDs were originally considered to be very rare, but there is evidence now showing that they are more common than previously thought. Secondary immunodeficiencies are a much more common occurrence with the most frequent cause being that associated with bad nutrition. Another prevailing secondary immunodeficiency is the one caused by infection with HIV.

1.2.2 Primary immunodeficiency

1.2.2.1 Classification

PIDs comprise a large number of disorders that affect the development and/or function of the immune system (136). The estimated overall prevalence of PIDs is of approximately 1:10000 live births. However, this rate is much higher amongst populations with high consanguinity, and/or in populations considered to be genetically isolated (137).

Patients with PIDs provide unique models to study the consequences of various immune defects that may underlie other clinical conditions such as autoimmunity, lymphoproliferation, allergy and cancer. They are often referred to as "experiments of nature".

Infections are the hallmark of PIDs (138). The type of infection/pathogen involved provides a guide to which part of the immune system is deficient. Thus, recurrent

infection with extracellular encapsulated bacteria is suggestive of a defect in antibody, complement or phagocyte function, reflecting the role of these components of the immune system in host defence against this type of pathogen. By contrast, a history of recurrent viral infections is more suggestive of a defect in host defence mediated by T lymphocytes.

In the last few years, advances in molecular genetics have resulted in the identification of a growing number of gene defects causing PIDs in human subjects and a better understanding of the pathophysiology of these disorders (139-143). Identification of the molecular defect in the various forms of PIDs is important, given the observation that the same immunological and clinical phenotype can result from distinct gene defects. This information not only provides basic clues as to how the immune system functions, but also has important medical applications for diagnosis, genetic counselling, prognosis, and potential therapeutic intervention strategies that can be made available to affected patients.

PIDs are classified according to which component of the immune system is primarily involved. Every two years, an update of the classification system is carried out by The International Union of Immunological Societies Expert Committee on Primary Immunodeficiencies to allow for the inclusion of newly described PIDs (144). As shown in Table I, this classification groups PIDs into 6 major categories, that cover a large number of already identified immunodeficiencies.

In the following sections of the introduction, a summary of the PIDs investigated in this work, as well as those other major PIDs associated with deficiencies or alterations in the T cell compartment will be provided.

Table I – Classification of primary immunodeficiencies. Adapated from Notarangelo *et al.* (144)

Combined T and B-cell immunodeficiencies
Predominantly antibody defects
Other well defined immunodeficiency syndromes
Diseases of immune dysregulation
Congenital defects of phagocyte (number, function, or both)
Defects in innate immunity
Autoinflammatory disorders
Complement deficiencies

1.2.2.2 Primary immunodeficiencies investigated in this work

1.2.2.2.1 Defects mainly targeting T cells

Combined Immunodeficiencies comprise a heterogeneous group of disorders characterized by impaired development and/or function of T and/or B cells that may or may not be accompanied by NK cell impairments (145). Some of the most frequent defects affect T cell development (see Figure 5). Severe combined immunodeficiencies (SCID) represent the most severe form of combined immunodeficiencies, featuring a lack of functional peripheral lymphocytes due to a profound block in lymphocyte differentiation (107, 145). Usually, SCID is associated with thymic hypoplasia. Studies of patterns of inheritance, immunological characteristics, and, more recently, genotypes have led to the identification of several distinct SCID conditions (146).

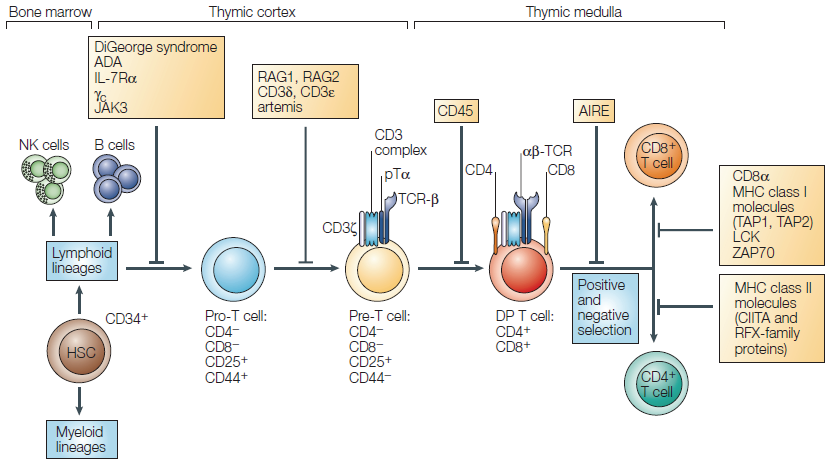

Figure 5: Protein and gene defects in T cell development ad function.
From *Cunningham-Rundles* et al. (147).

Affected infants present in the first few months of life with frequent episodes of diarrhea, pneumonia, otitis, sepsis, and cutaneous infections. Persistent infections with opportunistic organisms such as *Candida albicans*, *Pneumocystis jiroveci*, Varicella-zoster virus, Parainfluenza 3 virus, Respiratory syncytial virus, Adenovirus, Cytomegalovirus (CMV), and Epstein-Barr virus (EBV) are common. Infections caused by live vaccines are also frequent, especially Bacillus Calmette-Guérin (BCG) in countries, such as Portugal, where BCG is part of the vaccination plan. Skin rash may reflect graft-versus-host disease caused by maternal T cell engraftment, or tissue damage caused by infiltration of activated autologous T lymphocytes (148).

SCID defects are classified, according to the immunologic phenotype, into 2 main groups: SCID with absence of T lymphocytes but presence of B lymphocytes (T-B+ SCID) or SCID with absence of both T and B lymphocytes (T-B- SCID) (144). Both groups include forms with or without NK lymphocytes (144). SCIDs have a prevalence of approximately 1:50000 live births. Examples include γc deficiency, adenosine deaminase (ADA) deficiency, Janus kinase 3 (Jak3) deficiency, IL-7 receptor α-chain

deficiency (IL-7Rα), recombination-activating gene (RAG-1 or RAG-2) deficiencies, Artemis deficiency, CD3 α-, ε-, and ζ-chain deficiencies, and CD45 deficiency (107, 144).

The severity of the clinical manifestations makes SCID a medical emergency that, in the absence of treatment, leads to death within the first year of life. Approaches to achieve immune reconstitution in these patients include bone marrow transplantation, and gene therapy; and will be discussed on sections 1.3.3 and 1.3.5 of the introduction, respectively.

Hypomorphic mutations in the RAG genes, which impair, but not completely abolish V(D)J recombination process are associated with a severe primary immunodeficiency known as Omenn Syndrome (OS) . This leads to the generation of only a few productive antigen receptor gene rearrangements (149, 150). Defects in other genes involved in lymphocyte differentiation have also recently been described to account for OS (151). As a consequence of this defect, the T cell repertoire of effected patients is greatly restricted (152) resulting in activated oligoclonal T cells (152). These cells infiltrate and expand in peripheral tissues, including the skin and the gastrointestinal tract, causing profound tissue damage (153, 154). Circulating B cells are usually low or absent.

The thymus from patients with OS is markedly abnormal (155). It was recently shown that the few autoreactive T cells that differentiate in the thymus of these patients likely escape negative selection and expand in the periphery, thereby leading to the autoimmune manifestations characteristic of OS (156). This loss of central tolerance was suggested to be associated with a deficiency in Autoimmune regulator element (AIRE) expression in these patients. The analysis of AIRE expression in the

thymi of two Omenn syndrome patients, by real-time RT-PCR and immunohistochemistry, demonstrated a profound reduction in the levels of AIRE mRNA and protein as compared with a normal control subjects (155). AIRE is a transcription factor expressed by medullary thymic epithelial cells. It regulates the ectopic expression of tissue-specific proteins in the thymus, normally restricted to the periphery, suggesting that it has a key role in the maintenance of central tolerance (157). Mutations in the *AIRE* gene result in decreased expression of the transcription factor, and, consequently, impaired presentation of self-antigens by mTEC and dendritic cells to developing T cells (158). As a result, central tolerance to a number of self-antigens is lost, thereby inducing multiple autoimmune disorders as autoreactive T cells escape into the periphery. Mutations in *AIRE* have been shown to be the cause of autoimmune polyendocrinopathycandidiasis-ectodermal dystrophy (APECED), a monogenic disease characterized by autoimmune manifestations (159, 160).

Anther gene defect associated with thymic impairments is *FOXN1* deficiency. The human *FOXN1* deficiency is caused by mutations of the winged–helix–nude (*WHN*) gene, also known as *FOXN1* gene. *FOXN1* gene encodes a forkhead-winged helix transcription factor, which is absolutely required for the early development of the thymus. *FOXN1* expression is restricted to thymic epithelium, epidermis and hair follicles (161, 162). Mutations in the highly conserved *FOXN1* gene are associated with severe immunodeficiency due to athymia, total absence of hair, and nail dystrophy (163). It is considered the human homologue of the *nude* mouse and it is the first example of a human PID caused by defects in a gene not expressed in hematopoietic cells (163).

The phenotype associated with *FOXN1* deficiency was originally described in two siblings from consanguineous parents originating from a small community in southern Italy (163). The sequencing of the *FOXN1* gene revealed a homozygous C-to-T transition at nucleotide position 792 in exon 5 that led to a nonsense mutation at residue 255 (R255X) (GenBank accession no. Y11739) (164). In the same community, four additional children from previous generations were affected with congenital alopecia and died in early childhood because of severe infections (165). Geographically, this community is located in an isolated village lying in the mountains between Naples and Salerno, and presents an elevated rate of endogamy. A genealogical study of 55 heterozygous carriers of the R255X mutation in this village (6.52% of the inhabitants) allowed the identification of the ancestral origin of the mutation, to one couple, born at the beginning of the 19th century (165).

Both Italian siblings lacked a thymic shadow on radiologic examination and presented with a similar immunological phenotype (163). Although circulating T cells of non-maternal origin were present in both sisters, their frequency was below the normal range for the age. In agreement with the absence of a functional thymus, no naive CD4 lymphocytes were detected in the periphery. Severe functional lymphocyte impairment was demonstrated characterized by a lack of proliferative responses to mitogens and failure to upregulate activation markers following mitogen stimulation (163).

One of the children died at 12 months of age as a result of recurrent infections (163). The other child was submitted to an allogeneic, HLA-identical bone marrow transplantation, from her unaffected brother before the diagnosis of *FOXN1* deficiency was performed (166). Given that the genetic alteration does not affect the

hematopoietic system but primarily the thymus, it was not surprising that the bone marrow transplantation was not associated with recovery of the naive T cell pool in this patient (166).

DiGeorge syndrome (DGS) is caused by developmental defects in the third and fourth pharyngeal pouches during early embryogenesis. It is characterized by variable degrees of alterations of the thymus (hypoplasia, aplasia or unaffected) and parathyroid glands, heart abnormalities and facial dysmorphisms (167). The majority of the patients show microdeletions of specific DNA sequences in chromosome 22q11.2 (168).

In most cases, referred as partial DGS, there is mild-to-moderate T cell deficiency, reflecting residual thymic development. In less than 1% of cases, there is profound immunodeficiency due to a total absence of thymus (athymia) (169). Athymic patients are categorized as having complete DGS (170) and have been shown to have less than 50 naïve T cells/μl, and low TREC levels (171). They usually die within the first 2 years of life due to severe, recurrent infections. Patients with complete DGS can present with either a "typical" or "atypical" phenotype (172). Infants with complete DGS are usually born with the “typical” phenotype (very low T cell numbers and absence of rash) (172). At some point, they switch to an “atypical” phenotype characterized by the presence of oligoclonal T cell populations, associated with rash and lymphadenopathy (173). These oligoclonal T cells undergo extensive *in vivo* activation and infiltrate the skin and other organs (173).

Reconstitution of the T cell pool in patients with complete DGS has been successfully achieved by thymus transplantation as shown by the 75% survival after a mean three years of follow-up (172).

In recent years, autoimmune manifestations have emerged as important symptoms of many types of PID (174-176). This provides strong evidence that the immune sytem's ability to maintain tolerance to self is of equal importance to the host as its ability to provide protection from pathogenic organisms. Regulatory T cells (Treg) play a key role in this context, as illustrated by the association between autoimmunity and PIDs that result from defects in the development, and/or function of these cells.

This is best exemplified by Immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome. This syndrome is most often caused by mutations in the *FOXP3* gene that encodes a transcription factor critical for the development and peripheral function of Treg (177-179).

Several mutations in the *FOXP3* gene have been identified in IPEX patients (180). However, sequencing of the *FOXP3* gene in a large cohort of individuals diagnosed with IPEX revealed that not all of these patients had *FOXP3* mutations (180). IPEX in those individuals lacking *FOXP3* mutations has been proposed to result from mutations in genes whose products interact with FOXP3. Neutrophil and lymphocyte numbers were normal in these patients, but eosinophilia was frequently observed (180). Despite the severe disease clinical phenotype associated with IPEX, T lymphocyte numbers, including CD4 and CD8 subsets, are usually within the normal range. Proliferative responses to mitogens, and specific antigens, are also normal, or only slightly decreased (181). Depletion of naïve CD4 and CD8 T cell subsets is, however, frequently observed (182, 183).

IPEX is usually lethal in infancy unless treated with hematopoietic stem cell transplantation. It's extremely severe clinical manifestations highlight the critical importance of regulatory T cells in preventing autoimmunity (184).

1.2.2.2.2 Defects mainly targeting other immune populations but also leading to T cell alterations

Defects of T cells can also be found in immunodeficiencies mainly involving other compartments of the immune system.

Chronic granulomatous disease (CGD) (185, 186) is a PID associated with phagocyte defects. Normally, these cells play a key role in defence against bacteria and fungi. Thus, individuals who have deficits in phagocytic cell number and/or function usually experience recurrent and severe infections caused by these types of organism. Chronic CGD is the classic example of a disorder related to phagocyte dysfunction. It is caused by defects in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which is required for the generation of reactive oxygen species (187). During phagocytosis, the activation of the NADPH oxidase complex results in production of microbicidal compounds (superoxide radicals and hydrogen peroxide) and activation of lytic enzymes (cathepsin G, elastase and myeloperoxidase), leading to intracellular killing of bacteria and fungi. Thus, the main types of infections observed in CGD patients reflect this inability to kill phagocytosed bacteria and fungi. The defective respiratory burst characteristic of CGD patients renders them susceptible to infections with catalase-positive organisms such as *Staphylococcus aureus.* Other common bacterial infections associated with this immunodeficiency include *Serratia marcescens*, *Burkholderia cepacia*, and *Nocardia* species whilst *Candida* and *Aspergillus*

species cause most of the associated fungal infections (188). Granulomatous manifestations of the disease are also very common. Given the anti-inflammatory effects of oxygen species (189), the defects in the NADPH system, and defective reactive oxygen species generation, have been recently suggested to underlie the inflammatory phenotype observed in CGD patients (190).

The most common form of CGD is X-linked, and is caused by mutations in the gene encoding the gp91phox subunit of NADPH oxidase (*CYBB* gene). The second most common form is autosomal recessive and is due to a mutation in the gene coding for p47phox. Mutations in genes expressing p22phox and p67phox subunits have also been described (187, 191).

The diagnosis of CGD is usually made by flow-cytometric measurement of dihydrorhodamine oxidation. The X-linked carriers of gp91phox have two populations of phagocytes due to the random inactivation of one of the X chromossomes: one able to produce superoxide and another that does not, giving carriers a characteristic mosaic pattern on oxidative testing. Lupus erythematosus-like symptoms has been reported to be common in CGD carriers (192, 193). The mechanisms underlying this association are not known. It is possible that impairments in the regulation of T and B lymphocytes are implicated (194, 195).

In addition to their important microbicidal role, reactive oxygen species have been shown to play a role in the regulation of transcription factor function, proliferation, apoptosis, and cytokine production (196-199). A defective NADPH oxidase function has been shown to be associated with other immunological disturbances in CGD patients (185, 186, 200). These patients were shown to have a profound depletion of B cells expressing the memory B cell marker CD27 in conjunction with increased CD5

expressing B cells (186). In addition, X-linked carriers of CGD revealed a significant correlation between the percentage of CD27-positive B cells and the percentage of neutrophils with normal NADPH activity, suggesting a role for NADPH in the process of memory B cell generation (186). Alterations in T lymphocytes have also been described (185), with the authors showing that a cohort of CGD patients of more than 3 years of age had decreased numbers of CD3, CD4 and CD8 T cells suggesting that quantitative and qualitative defects in T cells may occur in the context of CGD.

1.2.3 Acquired immunodeficiency

1.2.3.1 Classification

Human immunodeficiencies can develop as a result of factors extrinsic to the immune system. The main cause of secondary immunodeficiency world-wide is protein–calorie malnutrition due to poverty and poor intake. Other causes include malabsorption or excessive loss of nutrients. Protein–calorie malnutrition is associated with a progressive loss of T cell production and function due to atrophy of primary and secondary lymphoid organs, together with an impaired antibody production capacity and neutrophil function (201, 202). The second most frequent cause is HIV/AIDS immunodeficiency.

Secondary immunodeficiencies can also develop as a consequence of surgery or trauma, or following administration of immunosuppressive drugs such as corticosteroids and cytostatics. Immunodeficiency can also be present in premature children or with aging due to senescence of the immune system.

Environmental factors have also been reported to lead to immunodeficiency and include ultraviolet light (203), ionizing radiation (204), and cold, heat or hypoxia (205).

Importantly, several infectious agents can lead to secondary immunodeficiency. These include, amongst others, EBV, CMV and Influenza virus. As previously stated, the most frequent infectious agent causing immunodeficiency is the human immunodeficiency virus (HIV).

1.2.3.2 HIV/AIDS

The first cases of AIDS (acquired immunodeficiency syndrome) were reported in the early 1980's (206-208). The etiologic agents of AIDS, HIV type 1 (HIV-1) and HIV-2, were first described in 1983 and 1986, respectively (209-211). The AIDS pandemic represents one of the most important health-care issues world-wide. It is estimated that in 2010, around 33.4 million people are infected by HIV (212). The number of new infections per year is around 2.7 million and over 2 million people die each year from AIDS (212).

HIV-2 was first identified in West Africa and the infection has been largely confined to this area (211). However, infected patients can be identified in European countries, mostly in Portugal where the prevalence is significant due to its connections with ex-colonies in this region.

HIV is a lentivirus, belonging to the *Retroviridae* family, which makes use of the reverse transcriptase enzyme to convert viral genomic RNA into double-stranded proviral DNA. This proviral DNA enters the cell nucleus and integrates into the host cell's genome using the virally encoded integrase enzyme. The integrated provirus constitutes the primary template for the subsequent transcription of the virus' structural, regulatory, and accessory genes.

The HIV genome consists of three main structural genes, *gag, pol* and *env*, flanked by long terminal repeat (LTRs) sequences. The *gag* gene encodes the structural proteins of the virion capsid, the *pol* gene encodes precursors for several viral enzymes required for its replication and integration (protease, reverse transcriptase, RNase, and integrase) and the *env* gene, which encodes the viral envelope glycoproteins. The viral genome also contains a number of genes that encode a series of small regulatory (Tat and Rev) and accessory (Nef, Vif and Vpr, and Vpu in HIV-1 or Vpx in HIV-2) proteins (213).

Viral entry into cells usually requires the interaction of the envelope glycoprotein of the virus with the CD4 molecule and one of several chemokine receptors that function as a co-receptor (214). CCR5 and CXCR4 were identified as the main co-receptors for HIV-1, whilst HIV-2 is able to use a broader range of chemokine receptors (215).

The acute phase of HIV infection is characterized by viral dissemination throughout the lymphoid tissue. At this stage, a peak in viral replication occurs and is accompanied by a marked depletion of circulating and mucosal memory CD4 T cells (216). HIV replication occurs very efficiently in a very small number of activated CD4 T cells, whilst the majority of infected lymphocytes harbour the latent proviral genome (217). Thus, a cellular reservoir of the virus is most probably established soon after primary infection. Following initial infection, the viremia decreases, mainly due to the response of virus specific CD8 T lymphocytes (218). An expansion of CD8 cells, both HIV-specific and non-specific, occurs leading to the inversion of the CD4/CD8 ratio.

Following this stage, a chronic and persistent infection is established that is associated with an asymptomatic period named clinical latency. Ongoing virus replication can be detected in the plasma and in lymphoid tissue during this phase of infection. Despite the immune response against the virus, the infection eventually leads to a progressive immune dysregulation and CD4 T cell loss.

A CD4 T cell count of less than 200 CD4+ T cells/µl is usually associated with the development of characteristic opportunistic infections and tumours, leading to the diagnosis of AIDS.

The hallmark of HIV infection is progressive CD4 depletion (219). Given that CD4 T cells are the main cell target for HIV infection, it was initially thought that the depletion of CD4 lymphocytes was a direct consequence of viral replication, and of its cytopathic effect upon infected cells (220). However, it is now clear that HIV infection induces profound qualitative changes in various components of the immune system, which also contributes to the characteristic CD4 depletion.

Several lines of evidence suggest that chronic immune activation is the driving force for CD4 depletion in HIV infection. Studies of Simian Immunodeficiency Virus (SIV)-infected primates showed, that upon SIV infection, Rhesus macaques suffered a progressive CD4 depletion and progression to AIDS that is associated with marked T cell activation (221). In contrast, SIV-infected sooty mangabeys and African green monkeys, which are natural hosts of SIV, do not develop AIDS and exhibit minimal T cell activation despite high levels of viral replication (222).

In humans, the levels of T cell activation, as measured by the expression of CD38 on CD8 T cells, were reported to predict an adverse prognosis in infected patients (223,

224). Several authors subsequently confirmed a direct correlation between disease progression and CD8 T cell activation levels (225-227). In addition, results from our laboratory suggest that CD4 T cell depletion is directly linked to immune activation, both in HIV-2 and HIV-1 infections, and only indirectly to viral load (127, 128).

The development of ART has been one of the most dramatic achievements in the history of medicine. In the majority of the cases, ART is associated with a marked decrease in the plasma viral load followed by an increase in CD4 T cell counts and gradual reconstitution of the immune system (228). The introduction of ART has been associated with a decrease in the morbidity and mortality of HIV-infected patients (229, 230). However, following its introduction, it has become clear that antiretroviral agents fail to eliminate the virus entirely, despite prolonged suppression of viremia, as the provirus can persist in a latent, integrated form in CD4 T cells (231-233). Furthermore, other data suggests ongoing low-level of HIV replication occurs in both these latent viral pools and in sequestered sites, neither of which are effectively targeted by current ART modalities (234).

Antiretroviral drugs are broadly classified by the phase of the retrovirus life-cycle that they inhibit. The first drugs made available for clinical use were inhibitors of the HIV reverse transcriptase. These drugs are nucleoside analogues that act by terminating DNA elongation. They are competitive inhibitors that serve as substrates for the reverse transcriptase, which incorporates them into the elongating HIV proviral DNA, resulting in premature termination of proviral genome replication. The non-nucleoside reverse transcriptase inhibitors are a group of structurally diverse agents which bind to reverse transcriptase at a site distant to the active site. This results

in conformational changes at the active site and inhibition of enzyme activity. The protease inhibitors bind competitively to the substrate binding site of the viral protease resulting in the production of immature virus particles (235, 236). Integrase inhibitors are a recently discovered class of antiretroviral drugs designed to block the action of the integrase enzyme. The first approved integrase inhibitor was Raltegravir. It has been shown to be virologically effective in phase II and phase III clinical trials. Finally, entry inhibitors, also known as fusion inhibitors, are a new class of drugs that interfere with the binding, fusion and entry of the virus into the host cell.

The main goal of ART is to reduce, and maintain plasma viral load levels to below the threshold of detection of the current viral load assays (< 40 copies/ml).

The development of resistance to therapy by the virus, especially in regimens that are only partially suppressive, is one of the main reasons for ART failure. If there is resistance to several drug classes, the number of alternative treatment regimens is limited and the virological success of subsequent therapies may be only short-lived. The rapid development of resistant variants is due to the high turnover of HIV and the exceptionally high error rate of reverse transcriptase. This leads to a high mutation rate and constant production of new viral strains, even in the absence of treatment. In the presence of antiretroviral drugs, resistant strains are selected as the dominant species (237).

Successful ART results in an increase in the number of circulating CD4 T cells and the functional reconstitution of the immune system. After ART initiation, peripheral CD4 T cell counts start and continue to rise for at least 3-5 years (238). The initial increase in CD4 cell count is very rapid and is usually observed in the first 3-6 months

(239). This phenomenon is associated with a reduction in T cell activation, and primarily results from a release of memory CD4 T cells trapped in the lymphoid tissue (240). A second phase, characterized by a slower increase in CD4 T cell numbers, follows, until this parameter stabilizes at 4–6 years of therapy (241). During this second phase, naïve, as well as memory CD4 T lymphocytes, contribute to the reconstitution of the CD4 T cell pool. The increase in the proportion of naïve cells has been shown to be accompanied by an increase in TREC levels (19, 242).

Achieving a CD4 cell count over a specific threshold has been shown to depend on baseline CD4 cell count and to take substantially longer in patients who initiate antiretroviral therapy at a lower CD4 T cell frequency (243, 244). The factors that determine CD4 T cell rebound during therapy are only partially understood, and most likely depend upon both the host and the viral factors.

Virological, immunological and clinical parameters can be evaluated as indicators of successful or failed ART. Virological treatment success is defined by the achievement of suppression of viral load to below the level of detection of 40 copies/ml. Definitions of immunological responses to treatment have varied in different studies. Achievement of a CD4 T cell count higher than 300 cells/μl or increases of 50 or 100 CD4+ cells/μl after at least one year upon ART have been used (245-247). Both virological and immunological therapeutic success contributes to an overall clinical treatment success. Failure to achieve immunological or virological therapeutic goals is referred as a discordant response. Several studies have reported patients with discordant immunological or virological responses (245, 248, 249).

Around 20% of patients on ART maintain a high CD4 T cell count in the face of sustained viral replication under ART (238, 249). Mechanisms shown to be involved

include modifications in viral fitness and acquisition of resistance mutations to drugs, decreased viral cytopathicity, and increased half-life of CD4 cells related to decreased T cell apoptosis (250, 251).

In 5 to 27% of HIV-1-infected patients, a failure to recover circulating CD4 T cells despite apparently complete suppression of viral replication is observed (252-254). Poor CD4 reconstitution under ART has been shown to be associated with older age and/or a more advanced disease stage at the beginning of treatment (252-257). Clinical data are limited but these patients appear to have low rates of opportunistic infections (245, 246, 252).

Thus, the ability of ART alone to restore immunecompetence in HIV-1 infected patients is far from absolute. As a result, new strategies to improve T cell reconstitution have been devised, best illustrated by cytokine-based therapies.

1.3. Strategies for T cell reconstitution

1.3.1 Overview

Severe T cell deficiency in humans can develop in the context of several clinical settings such as PIDs or infection with HIV. Strategies to improve the recovery and function of T cells currently include cytokine-based therapies, hematopoietic stem cell transplantation, thymic transplantation and gene therapy.

1.3.2 Cytokine based therapies

The common γ-chain cytokines, IL-2, IL-7 and IL-15, have crucial roles in the development, proliferation, survival and differentiation of T cells. Cytokine-based therapies with γ-chain cytokines have been used concomitantly with ART in the context of HIV infection to help reconstitute the T cell compartment.

IL-2 has been tested experimentally in HIV infection in phase I and II studies since the early years of the AIDS epidemic. Two large clinical trials were conducted in ART treated individuals to assess whether the effects of IL-2 therapy on CD4 T cell restoration in HIV-1-infected patients would translate into clinical benefit: the Evaluation of Subcutaneous Proleukin in a Randomized International Trial (ESPRIT) and the Subcutaneous, Recombinant, Human IL-2 in HIV-Infected Patients with Low CD4 Counts under Active Antiretroviral Therapy (SILCAAT) studies (258, 259). The ESPRIT study (258) compared IL-2 plus antiretroviral therapy with antiretroviral therapy alone in patients with CD4+ T cell counts >350 cells/μl, whilst the SILCAAT (259) study was focused on patients with CD4+ T cell counts between 50 and 299 cells/μl. Although the average CD4 T cell count over time was significantly greater in the IL-2 arms of both studies than in the respective control arms, no statistically significant differences were found in the clinical endpoints. Thus, the increase in CD4 T cells did not translate into a reduced risk of HIV-1-associated opportunistic diseases or death, as compared with volunteers who were on ART alone (260).

Interestingly, administration of IL-2 to HIV-infected patients on ART has been reported to increase the numbers of T cells with a classical Treg phenotype (261). IL-2-

expanded Tregs suppressed proliferation of effector cells *in vitro* and therefore shared functional characteristics of Treg cells (261). The authors reported that patients with a marked expansion of this population had a higher relative risk of clinical progression to AIDS, suggesting a link between Treg expansion in these individuals and their clinical outcome.

However, these cells were not strongly suppressive in *ex vivo* assays, thus their function *in vivo* remains the subject of debate (262).

As previously detailed, IL-7 is a key player in T cell homeostasis, and is essential for T cell development in humans (263). This cytokine modulates thymic output and mediates the expansion and survival of naive and memory T cells (108, 264). It also inhibits the apoptosis of CD4 and CD8 T cells from HIV-infected patients *in vitro* (265). These findings provided a rationale for the consideration of IL-7 as a therapeutic agent for immune reconstitution.

The first clinical trial of IL-7 initiated in humans involved patients with nonlymphoid cancer that was refractory to standard therapy (266). The therapy was associated with an expansion of CD4 and CD8 T cells bearing a diverse TCR repertoire. This was shown to be primarily mediated by increased peripheral cell cycling and augmented cell survival (266).

Prior to the IL-7 trials in humans, it was reported that IL-7 therapy in SIV-infected macaques was associated with a consistent but transient increase in peripheral blood T cells, both CD4 and CD8 T cells (267, 268).

The safety and efficacy of IL-7 administration to HIV-1-infected subjects has recently been evaluated in a phase I/IIa trial (269). IL-7 was administrated to HIV-1 infected patients presenting persistently low CD4 T cell counts despite virological suppression under ART (ART-Discordants). IL-7 therapy was associated with significant increases in circulating naive and memory CD4 and CD8 T cells. The expanded CD4 and CD8 T cells were shown to respond *in vitro* to TCR stimulation, and to produce intracellular cytokines after polyclonal and antigen-specific stimulation (269). In addition, another phase I study, from the AIDS Clinical Trials group (ACTG) 5214, also reported increased numbers of circulating CD4 and CD8 T cells after IL-7 therapy, predominantly with a central memory phenotype (270). Thus, these initial human trials suggest that IL-7 therapy may improve T cell recovery and function in the context of ART-treated HIV-1 infection.

Of note, exogenous IL7 has been reported to up-regulate the *ex vivo* expression of HIV-1 in latently infected cells from HIV-1-infected individuals on suppressive ART (271). Accordingly, it has been suggested that IL-7 therapy, besides having beneficial immunological effects, could potentially purge the latent reservoirs of HIV-1, formed by the pool of latently infected CD4 T lymphocytes, promoting viral clearance.

1.3.3 Hematopoietic stem cell transplantation

Developments in the field of transplantation have accelerated remarkably since the discovery of human MHC. Currently, hematopoietic stem cell transplantation (HSCT) is the treatment of choice for various haematological diseases. The main aim of this procedure is the reconstitution of all blood cell lineages following administration of hematopoietic stem cells. The potential sources of hematopoietic stem cells include

bone marrow, peripheral blood or cord blood. Stem cell transplantation may be performed with cells from a family member or an unrelated volunteer (allogeneic transplantation) or with stem cells previously collected from the patient (autologous transplantation).

This transplant technique involves donor and recipient matching for HLA antigens. The ideal donor is an HLA-identical sibling, although transplants with only partially matched HLA antigens can be performed. Generally, the recipient undergoes a conditioning regimen. This involves dosing with immunosuppressive agents to ablate the recipient's immune system in order to prevent it rejecting the donor's stem cells, and to facilitate engraftment (272).

In the context of PIDs, HSCT was first reported for the treatment of patients with SCID (273). SCID is a medical emergency and is usually fatal unless diagnosed promptly and treated successfully. With few exceptions, in which alternative strategies can be used (gene therapy, enzyme replacement therapy), HSCT represents the single most effective form of treatment, and potential cure for these disorders, since most SCIDs are related to hematopoietic defects. In addition, SCID patients presenting with lack of T cells represent a unique situation since immunosuppression is usually not required.

HLA-identical or haploidentical transplantation of SCID patients have reported survival rates close to 80%, for more than 20 years post-transplantation (107, 274). The effectiveness of HSCT in SCID is best illustrated by the normalization of the number and function of T lymphocytes achieved after transplantation. Post-transplant T cell reconstitution involves two main mechanisms. Firstly, clonal expansion of mature

donor T lymphocytes co-infused, along with donor HSC, into the recipient; and secondly, *de novo* thymic generation of donor T lymphocytes (275). The development of immune function in patients transplanted with a graft containing mature donor T lymphocytes can be detected around 2 weeks and is due to the expansion of donor cells. In contrast, newly generated, naïve T lymphocytes do not appear in circulation until 3-4 months after HSCT, irrespective of the type of transplant (107, 145). The thymic contribution to post-transplant T cell recovery is dependent upon several factors including the degree of age-associated thymic involution, the engraftment of donor-derived haematopoietic stem cells, the occurrence of GvHD, the extent of thymic damage caused pre-transplant by treatments, and by the conditioning regimen utilized (275).

Several studies have now been published that analyze long-term immune reconstitution in patients who underwent transplantation for PID and survived more than 10 years (275-277). Importantly, the long-term analysis of the outcome of these patients demonstrates, in many cases, cure with a relatively good quality of life.

1.3.4 Thymic transplantation

Thymic transplantation has been used as an investigational treatment for pediatic patients with severe PID with athymia due to non-hematopoietic defects, specifically DGS (171, 172). Donors of the thymic tissue are usually infants less than 6 months of age who underwent corrective cardiac surgery, during which thymus tissue requires excision to expose the operative field. The harvested thymic tissue is then aseptically

sliced into small pieces and cultured *ex vivo* in the presence of nutrient medium and deoxyguanosine to deplete the hematopoietic cells, for around 12-21 days (278).

The allogeneic thymus tissue slices are then transplanted for example in the quadriceps of the athymic patient. Bone marrow stem cells migrate to the allograft where they develop into naïve T cells (279). Since patients with typical complete DGS completely lack T cells, immunosuppression is only necessary in patients with atypical complete DGS, as rejection of the thymic allograft by the few oligoclonal T cells these individuals have may occur before immune reconstitution is achieved (280).

Results from thymic transplantation performed in 60 patients with DGS reported by Markert *et al.* showed evidence of effective thymopoiesis in biopsies of the transplanted tissues 2 months after transplantation with naïve T cells appearing in peripheral blood approximately 3-5 months post-transplantation in 20 of the patients (171). This T cell reconstitution was also associated with development of a diverse T cell repertoire.

Thymic transplantation is usually performed without HLA-matching between the thymus donor and the recipient (171). How T cell selection occurs in this setting is not yet clear. Murine studies have suggested that recipient bone marrow-derived cells such as antigen presenting cells or thymocytes may play a role in positive selection in the thymus (281-283). Alternatively, circulating host-derived epithelial progenitors may migrate to the thymus and provide signal for positive selection of the developing thymocytes (284). With respect to negative selection, it is likely that recipient bone marrow derived dendritic cells that are able to colonize the thymic graft may play a role in this process.

Nevertheless, thymic transplantation performed in the context of complete DGS is associated with reconstitution of the T cell compartment, leading to increased survival of a significant proportion of treated patients.

1.3.5 Gene therapy

Gene therapy is a therapeutic strategy that involves the genetic modification of a target cell by the transfer of an exogenous gene. Since the mid 1980s, gene therapy has been attempted for a wide range of conditions, including SCIDs, cystic fibrosis, hemophilia A and B, and type I diabetes. However, some issues require careful consideration prior to the selection of a gene therapy approach, such as the pathogenesis of the disease, the target cell intended for modification, the therapeutic gene to be used, and the vector used to transfer this gene into the target cell (285). Several vectors have been designed with the capacity to deliver the corrective gene. Those based upon retroviruses have long been preferred because of their ability to integrate permanently into the genome of the target cells. Retroviral vectors can be engineered, through recombinant DNA technology, to carry the cDNA of the desired human gene, with high efficiency into target cells, such as hematopoietic stem cells, and integrate the gene into the chromosomal DNA of these cells. The integrated provirus behaves as a gene transcription unit, with the therapeutic gene copied into daughter cells during cell replication. Stable persistence of the corrective gene is essential when targeting cells such as hematopoietic stem cells, as they have to undergo massive proliferation to produce mature progeny cells. Although integration offers the advantage of maintaining the foreign gene in dividing cells, it carries the risk of leukemia generation due to insertional mutagenesis (286).

Gene therapy, as a therapeutic treatment option, has proved successful for treatment of inherited immunodeficiencies including γc-deficiency (287-289), adenosine deaminase (ADA)-deficient SCID (290, 291), and, more recently, X-linked chronic granulomatous disease (292).

The first clinical trial for gene therapy was designed at the National Institutes of Health, targeting peripheral blood T lymphocytes from patients with ADA-deficient SCID (293), the first human SCID to be characterized in terms of biochemical and genetic etiology (294). ADA-deficiency is an autosomal recessive SCID caused by defects of the enzyme adenosine deaminase (ADA). This is a purine enzyme, expressed in all tissues of the body that catalyzes the deamination of deoxydenosine. Deficiency of this enzyme results in the accumulation of metabolic substrates, leading to impairments in lymphocyte development and function. Although several patients were initially treated, the early trials were unsuccessful, mainly because patients treated with gene therapy continued to receive ADA enzyme replacement therapy (293). This was later proven to blunt the putative selective advantage conferred to ADA-corrected lymphocytes (286). However, second generation trials, utilising improved methods for gene transfer combined with more efficient vectors, and ADA-deficient SCID patients not receiving ADA enzyme replacement therapy were associated with immunological and metabolic reconstitution in a large number of patients (295, 296).

Two gene therapy trials for γc deficiency have been conducted in Europe, one at the Necker Hospital in Paris, and the second at Great Ormond Street Hospital in London (146, 288). In both protocols CD34+ cells were isolated from bone marrow, activated

with cytokines, and exposed to a supernatant containing retroviral vectors carrying the gene of interest. The results in the first two subjects were reported in 2000, and demonstrated a rapid and robust production of T lymphocytes, with lesser improvements in the numbers of circulating B and NK cells (287). Subsequent reports demonstrated similar responses in 9 of 10 subjects proving that gene therapy for γc deficiency is associated with significant immune reconstitution (289). Another trial at UCL Institute of Child Health using similar techniques achieved immune reconstitution in 10 subjects (288). However, 2–5 years after the treatment, leukemia developed in 5 of 20 treated subjects in these trials (297, 298). The leukemias were found to result from an outgrowth of a clonal population of T cells containing the retroviral vector integrated adjacent to one or more cellular proto-oncogenes (299, 300).

Phase I gene therapy trials were carried out for CGD due to mutations in *p47phox* or *gp91phox* genes. The initial trials for CGD also had modest results, as indicated by the reported low frequencies of corrected granulocytes in the peripheral blood. (301, 302).

Subsequently, a trial performed in Germany reported relatively high levels of corrected leukocytes in peripheral blood (~20%) of two patients in the first months after the gene therapy procedure and these rose to as high as 80% over the first year (292). The vector integration sites were studied in the CGD patients and revealed a highly restricted pattern, with the majority of vector integrants in the engrafted stem cells being near one of a few genes known to be involved in myeloid cell proliferation. However, these two patients developed myelodysplasia, a preleukemic condition. One subject subsequently had a bone marrow transplant and the other died of an acute infection, associated with a loss of the restored neutrophil function (292). Recently, a

study reported successful results in two patients, who demonstrated a fully sustained correction of neutrophil oxidase activity with a concomitant resolution of infections (303).

Gene therapy is a promising therapeutic option for the treatment of PIDs. Continuous technological progress in gene targeting, manipulation of viral vectors, and stem cell manipulation should improve the safety and efficacy of this type of therapy in the future.

Advances in the investigation of the biology of immune reconstitution have elucidated the pathways that lead to the recovery of T cells. Particularly, remarkable progress has been achieved in relation to understanding the biology of lymphoid precursors, thymic development and peripheral T cell homeostasis, coupled with an improved understanding of the molecules involved in these processes. This progress will in turn allow the development of new strategies for immune reconstitution that will improve the clinical outcome in patients experiencing a variety of T cell deficiencies.

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Aim and Work Plan

The overall aim of this work is to investigate the relative roles of the thymus and the "periphery" in the maintenance/recovery of the human T cell compartment, through the study of particular clinical models.

Specific Aim 1

The thymus is essential for both the establishment of the peripheral T cell pool and the generation of the diverse T cell receptor repertoire capable of dealing with new pathogens and controlling the escape of persistent infections. In humans, the thymus is almost fully developed at birth, with the rate of T cell production markedly decreasing after puberty. However, it is now clear that this central lymphoid organ plays an essential role in the lifetime "de novo" generation of T cells.

FOXN1 is a transcription factor, expressed by thymic epithelium, crucial for both development of the thymus and prevention of its involution.

Although FOXN1 deficiency in humans and mice has been associated with athymia, significant number of circulating T cells were found in a Portuguese patient, followed by us, and in two patients, reported by Pignata *et al.*, that had the same homozygous R255X mutation in the *FOXN1* gene.

These data raised the possibility that either *FOXN1* mutations may allow the development of a thymic rudiment capable of supporting T cell differentiation, or that extrathymic lymphopoiesis may occur in the context of athymia.

Thus, our first specific aim was to investigate the phenotype and function of the T cells we observed in our patient described above.

Since this patient had a primary defect in the thymic epithelium, without evidence of hematopoietic defects, we speculated that transplantion could provide a curative strategy, although this procedure had never been performed in this clinical setting. Thus, the patient was submitted to a thymic transplantation, fully mismatched for HLA class I and matched for only one class II allele. Another important objective of this work was the sequential study of the immunological recovery and of the potential acquisition of immunecompetence post-thymic transplantation.

Importantly, this study could also provide unique data on the kinetics of establishment of the T cell pool after HLA mismatched thymic transplantation, and insights regarding the dynamics of replenishment of the immune system after establishment of the T cell pool following T cell depletion.

Chapter 3.1. features the results of the clinical and immunological data pre and post thymic transplantation of two patients with *FOXN1* deficiency. The data on the French patient (subject 2) included in this work results from collaboration with University Paris René Descartes - Necker School and Duke University Medical Center, and we did not contribute to the related experiments or results generated. The

immunological studies of the patient with R255X mutation are further detailed in the chapter 3.2 of the results.

Specific Aim 2

Despite the progressive replacement of the perivascular spaces with fat, the remaining cortical and medullary tissue in the aging thymus is histologically normal. The maintenance of naïve T cells (antigen non-experienced cells) is currently thought to depend upon proliferation of peripheral T cells as well as to an age-dependent contribution of recent thymic emigrants, as demonstrated through the measurement of excision circles generated by intrathymic TCR gene rearrangement (TRECs). Moreover, recent studies suggested the possibility of a thymic rebound as a compensatory feedback loop triggered by emptiness of the peripheral T cell pool. The cytokine IL-7 has been highlighted as a possible factor in this process, and has also been shown to play an important role in the peripheral homeostatic proliferation of naïve and central memory T cells.

There is significant debate regarding the contribution of maintained thymic activity in the context of HIV infection; both with respect to the rate of disease progression and successful immunological reconstitution under antiretroviral therapy (ART).

Peripheral mechanisms such as IL-7 driven T cell proliferation/survival have been suggested to be able to compensate any potential thymic impairment resulting from HIV infection. On the other hand, the persistent immune stimulation that leads to a

heightened state of activation, and the associated T cell anergy and susceptibility to apoptosis, may also play a significant role.

The second specific aim of this work was to investigate the interplay of these pathways during HIV/AIDS using cohorts of patients with different degrees of CD4 depletion, possible thymic impairment, and viral load.

To this end, both untreated and ART-treated HIV-1 infected patients who achieved suppression of viral replication under therapy were studied. Of note, these treated individuals included a cohort lacking CD4 recovery despite their adequate virological response to ART, usually referred to as an immunologically discordant response to ART (ART-Discordants), and thought to be in part related to thymic impairment.

The originality of the study was further enhanced by including a comparison with HIV-2 infected patients. HIV-2 infection represents an attenuated form of HIV/AIDS and is associated with very low levels of circulating virus in the absence of ART. Nevertheless, CD4 depletion does occur, although at a much slower rate than in HIV-1 infected patients. Our laboratory has previously shown that this may be in part related to a better preservation of thymic function.

The inclusion of HIV-2 infected patients also allowed us to compare ART-Discordant patients to HIV infected individuals with the same levels of CD4 depletion and viremia. To better understand the relative influence of viremia, CD4 depletion and ART, we also included in the study a cohort of untreated HIV-1 infected patients with similarly low CD4 counts and expected high viremia, together with ART-treated HIV-1 infected patients with successful virological and immunological responses.

The results generated from these cohorts are detailed and discussed in Chapter 4 of this thesis.

Specific Aim 3

Interestingly, we have been able to follow, through our primary immunodeficiency centre, a HIV negative individual with marked CD4 depletion that reaches levels similar to those found in advanced AIDS patients. This patient has a defect in the phagocytic oxidative burst, namely a mutation in the *CYBB* gene encoding the gp91phox subunit of NADPH oxidase. We hypothesised that the investigation of the immunological profile of this patient could provide unique insights into mechanisms of T cell homeostasis.

Thus, our third specific aim was to perform a parallel investigation of the T cell perturbations in the context of this oxidative defect, and in untreated HIV-1 infected patients with a similar degree of CD4 depletion.

These data are presented in chapter 5 of this thesis.

The personal contribution of the candidate Adriana S. Albuquerque for chapters 3.2, 4 and 5 of this Thesis consisted in performing research, analysing the data, discussing the results and participating in the writing of the manuscripts for submission to publication. Regarding the chapter 3.1, her contribution was performing research and analysing the data related to subject 1.

In agreement with the Decreto-Lei 388/70, art. 8o, parágrafo 2, the results presented here were published or are currently being prepared for publication in the following scientific journals:

Low CD4 T cell counts despite low viremia: insights from the comparison of HIV-1 infected patients with discordant response to antiretroviral therapy and untreated advanced HIV-2 disease.

Adriana S. Albuquerque, Russell B. Foxall, Catarina S. Cortesão, Rui S. Soares, Manuela Doroana, Alice Ribeiro, Margarida Lucas, Francisco Antunes, Rui M.M. Victorino, Ana E. Sousa.

Clinical Immunology 2007, 125: 67–75.

First use of thymus transplantation therapy for Foxn1 deficiency (nude/SCID): A report of two cases.

M. Louise Markert, José G. Marques, Bénédicte Neven, Blythe H. Devlin, Elizabeth A. McCarthy, Ivan K. Chinn, **Adriana S. Albuquerque**, Susana L. Silva, Claudio Pignata, Geneviève de Saint Basile, Rui M. Victorino, Capucine Picard, Marianne Debre, Nizar Mahlaoui, Alain Fischer, Ana E. Sousa.

Blood; in press.

Deciphering the human FOXN1-deficiency phenotype through thymic transplantation.

Adriana S. Albuquerque, José G. Marques, Susana L. Silva, Dario Ligeiro, Blythe H. Devlin, Jacques Dutrieux, Rémi Cheynier, Claudio Pignata, Rui M. Victorino, M. Louise Markert, Ana E Sousa.

Manuscript in preparation.

An AIDS-like immunological profile in a phagocytic immunodeficiency: Chronic Granulomatous Disease.

Adriana S. Albuquerque, Margarida Lucas, Catarina S. Cortesão, Alcinda Melo, Dirk Roos, Rémi Cheynier, Rui M.M. Victorino, Ana E. Sousa

Manuscript in preparation.

T cells in FOXN1 deficiency and kinetics of their recovery upon thymic transplantation

We present in this chapter, two manuscripts reporting the *FOXN1* deficiency phenotype and the follow-up post-thymic transplantation. The first manuscript describes the clinical and immunological presentation and the follow-up after thymic transplantation of a Portuguese and a French patient born with *FOXN1* mutations. The patients have different mutations in the *FOXN1* gene. The mutation found in the French patient was associated with an absence of circulating T lymphocytes. In the Portuguese patient there is a significant number of circulating T cells. In the second manuscript we aim to investigate the T cell compartment associated with the mutation that we found in the Portuguese patient associated with the absence of thymus and following thymic transplantation.

Publication:

3.1. First use of thymus transplantation therapy for *FOXN1* deficiency (nude/SCID): A report of two cases

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Blood; *in press.*

Abstract

FOXN1 deficiency is a primary immunodeficiency characterized by athymia, alopecia totalis, and nail dystrophy. Two infants with FOXN1 deficiency were transplanted with cultured postnatal thymus tissue. Subject 1 presented with disseminated Bacillus Calmette-Guérin infection and oligoclonal T cells with no naïve markers. Subject 2 had respiratory failure, Human Herpes Virus 6 infection, cytopenias, and no circulating T cells. The subjects were given thymus transplants at 14 and 9 months of life, respectively. Subject 1 received immunosuppression before and for 10 months after transplantation. With follow up of 4.9 and 2.9 years, subjects 1 and 2 are well without infectious complications. The pretransplantation mycobacterial disease in subject 1 and cytopenias in subject 2 resolved. Subject 2 developed autoimmune thyroid disease 1.6 years posttransplantation. Both subjects developed functional immunity. Subjects 1 and 2 have 1,053/mm3 and 1,232/mm3 CD3+ cells, 647/mm3 and 868/mm3 CD4+ T cells, 213/mm3 and 425/ mm3 naïve CD4+ T cells, and 10,200 and 5,700 TRECs per 100,000 CD3+ cells, respectively. They have normal CD4 T cell receptor beta variable repertoires. Both subjects developed antigen specific proliferative responses and have discontinued immunoglobulin replacement. In summary, thymus transplantation led to T cell reconstitution and function in these FOXN1 deficient infants.

Introduction

The nude/severe combined immunodeficiency (SCID) phenotype due to deficiency of the transcription factor FOXN1 was first described in mice by Flanagan in 1966, who noted an absence of hair, poor growth, early mortality, and susceptibility to infection.[1] Subsequent investigations revealed that the immunodeficiency resulted from athymia caused by mutations in the *WHN* gene, since renamed *FOXN1*.[2-4] The first human cases of FOXN1 deficiency were reported by Pignata in two children with athymia, reduced T cell numbers, absence of hair, and nail dysplasia.[5] One child died and the other received bone marrow transplantation without reconstitution of the naïve T cell pool.[6] In the same community, four other children with alopecia had died early in life from severe infections, which suggested that they, too, had the same *FOXN1* mutation.[7]

Here we report 2 unrelated infants who presented with congenital athymia due to the human nude/SCID phenotype resulting from mutations in *FOXN1*. We treated the 2 infants with FOXN1 deficiency with thymus transplantation, taking advantage of the experience using this therapy to achieve immunoreconstitution in infants with athymia secondary to complete DiGeorge anomaly.[8] In this report, we describe the presentation of the research subjects and their clinical and immune outcomes.

Materials and Methods

Research subjects:

Both subjects were enrolled in protocols that were approved by the Duke University Health System Institutional Review Board (IRB) and were reviewed by the Food and Drug Administration under an Investigational New Drug (IND) application.[8] The parents of each subject provided written informed consent. The clinical trial registration numbers are NCT00579709 "Thymus Transplantation with Immunosuppression" for subject 1 and NCT00576407 "Thymus Transplantation in DiGeorge Syndrome" for subject 2.

Thymus transplantation:

Unrelated allogeneic thymus tissue, routinely discarded during cardiac surgery, was collected from infants less than 9 months of age. The tissue was used for transplantation after informed consent was obtained under protocols approved by the Duke IRB and reviewed by the FDA. Detailed descriptions of the procedure are published.[8-10]

Immune testing:

Standard flow cytometry and proliferation assays were performed as previously described. In brief, antibodies for flow cytometry included CD3, CD4, CD8, CD14, CD16, CD19, CD45, CD45RA, CD56 and CD62L (all from BD Biosciences, San Jose, CA). The TCRBV analysis by flow cytometry used the Beta Mark TCR Vβ repertoire kit (#IM3497, Immunotech, Beckman Coulter, Marseille, France). The proliferative response to phytohemagglutinin (PHA) was performed in triplicate using 100,000 cells

per well with 3 concentrations of mitogen. Tritiated thymidine incorporation was measured on days 3 and 4. Cultures with purified protein derivative (PPD, 5 µg/ml, Statens Serum Institut), *Candida albicans* (40 µg/ml, Greer Laboratories), varicella zoster virus (VZV, 1 µg/ml, Virusys Corp.), and tetanus toxoid (20 µg/ml, Virusys Corp.) were performed in quadruplicate and then pulse labeled and harvested on day 6.

Spectratyping was performed using RNA isolated from cells separated magnetically with CD3, CD4, or CD8 microbeads (Miltenyi Biotec, Auburn, CA). After capillary gel electrophoresis, the data (Gene Scan Software, Applied Biosystems, Foster City, CA) were uploaded onto a web accessible analysis program, SpA.[11-13] The result was reported as the Kullback-Leibler divergence (D_{KL}) score. High scores reflect oligoclonal repertoires (highly divergent from normal) whereas low scores reflect polyclonal repertoires.[13]

Signal joint (sj) T cell receptor rearrangement excision circle (TREC) analyses were performed as described.[11]

Immunohistochemistry was performed on allograft biopsies as described.[14, 15]

To evaluate for maternal engraftment, DNA was obtained from isolated circulating T cells in the subject, from maternal peripheral blood, and from the subject's own buccal swab. The hospital laboratory compared the samples using multiplex PCR amplification for 8 microsatellite markers followed by electrophoretic separation of each sample. The limit of detection was 2%.

Results

Subject 1

Subject 1 was born at term to Portuguese parents who were distant cousins. The female infant had nail dystrophy and no hair. Genetic analysis revealed a homozygous nonsense mutation at residue 255 (R255X) in exon 4 (formerly exon 5) in *FOXN1.*

Slowly progressive Bacillus Calmette-Guérin (BCG) adenitis and mild erythroderma had been apparent at 3 months of life. At day 157 of life, the subject was admitted with respiratory failure and noted to have marked posterior cervical adenitis and inguinal adenitis. Gastric and bronchoalveolar lavage secretions grew *Mycobacterium bovis* resistant to isoniazid; treatment included streptomycin, rifampicin, ethambutol, itraconazole, trimethoprim/sulfamethoxazole and intravenous immunoglobulin.

The initial immune evaluations were performed when the subject presented with respiratory failure and the subsequent pre-transplantation evaluations are included in Table 1. Although circulating T cells were present, naïve T cells were profoundly low. The striking feature of the immune profiles in the first year of life was the expansion of double negative ($CD4^-CD8^-$) T cells. The TCRγδ population composed approximately one third of the double negative population (unpublished data); the remainder of the double negative T cells were thus TCRαβ positive.

The T cell receptor beta variable (TCRBV) repertoire was evaluated before thymus transplantation by flow cytometry and spectratyping. The CD4 TCRBV repertoire assessment of subject 1 by flow cytometry on day 297 of life (127 days prior to thymus transplantation) showed expansions of T cells expressing BV3 and BV22 (Figure 1A); the spectratyping analysis of CD4 RNA was markedly oligoclonal (Figure 2A).[13] Additional flow cytometry evaluations of TCRBV repertoire are shown in Figures 1A and 1B. Lastly, sjTREC analysis on day 409 of life (15 days prior to transplantation) revealed <100 TRECs per 100,000 $CD3^+$ T cells. Overall, these data were consistent with a lack of thymic function in subject 1.

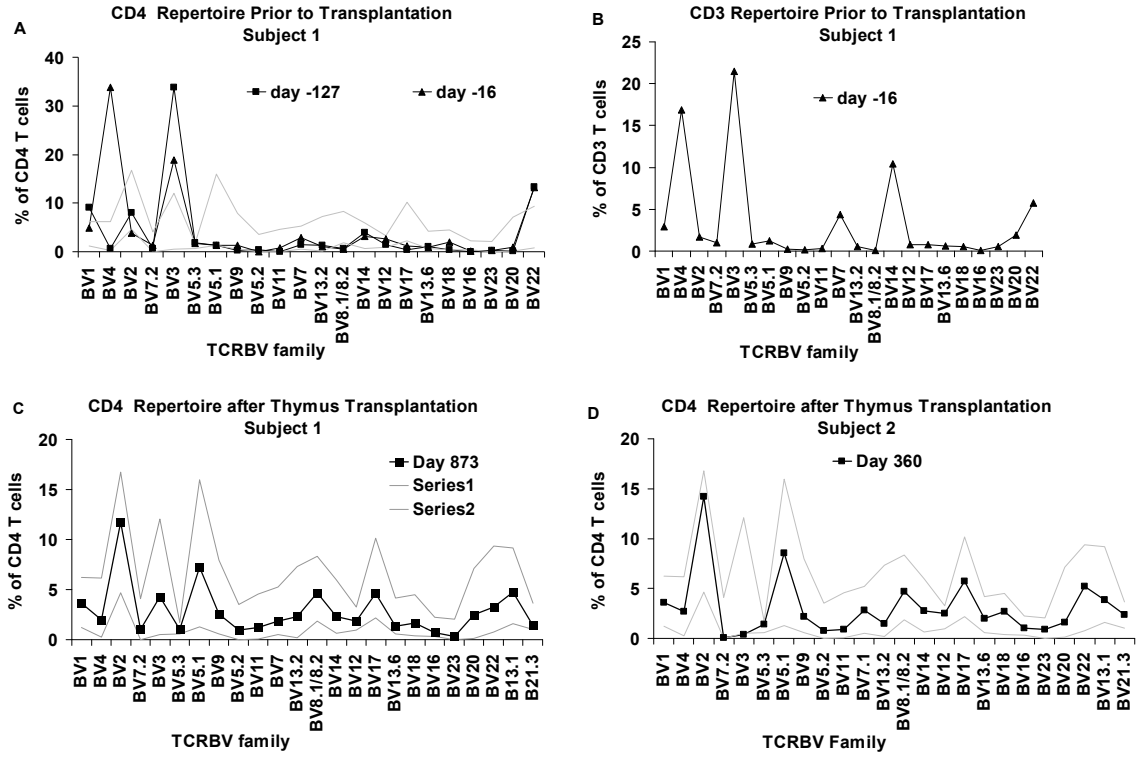

Figure 1: T cells are oligoclonal in subject 1 prior to transplantation but are polyclonal after transplantation in both subjects by flow cytometry. T cell receptor diversity for CD4 (A) and CD3 (B) T cells was assessed for subject 1 prior to transplantation. Panel C shows the latest diversity assessment in subject 1; panel D shows the latest assessment in subject 2. Note that the Y axis in the CD4 panel A differs from the Y axis in CD4 panels C and D. The shaded area in panels A, C, and D represents the normal range ± 3 SD based on data from 19 healthy adults.

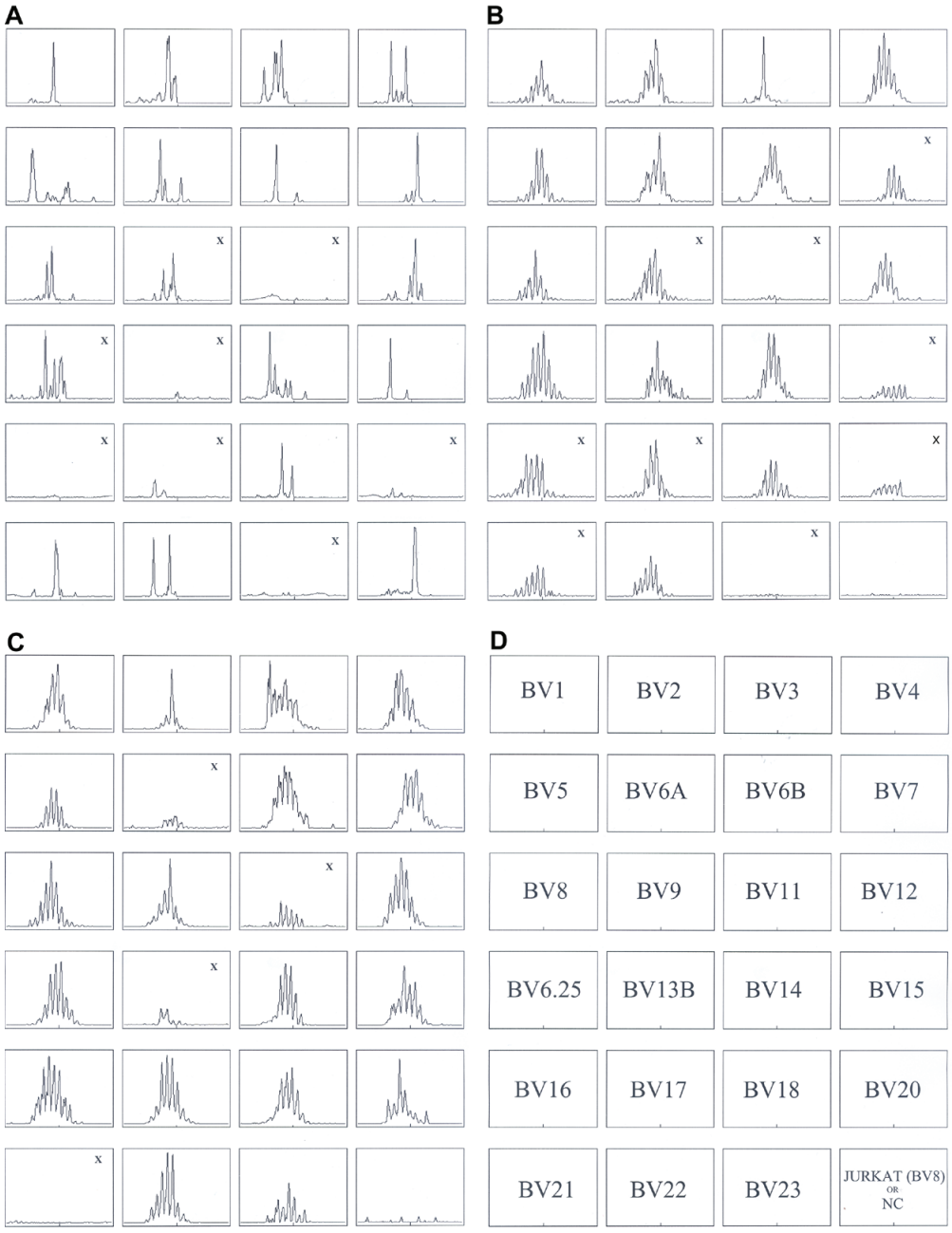

Figure 2: CD4 RNA spectratyping shows oligoclonality in subject 1 prior to transplantation and polyclonality in both subjects after transplantation. Subject 1 at A) day -127 and B) day 873 after transplantation; Subject 2 at C) day 368 after transplantation. The D_{KL} score for subject 1 pre-transplantation (panel A) is 1.38 compared with the D_{KL} score of 0.19 after transplantation (panel B). For subject 2, the post transplantation D_{KL} was 0.08 (panel C). Lower D_{KL} scores reflect greater diversity in the T cell receptor repertoire. The "X" indicates panels with insufficient RNA concentrations. These panels were not included in the calculation of the D_{KL} score.

The T cell proliferative responses to PHA were initially low but unexpectedly increased to over 100,000 counts per minute (cpm) prior to transplantation (Table 1). Evaluation for possible maternal engraftment was performed at the time of diagnosis and 9 days prior to transplantation. No evidence of circulating maternal T cells was found.

Table 1: Presenting immunophenotypes prior to transplantation

Subject	Day of life (day prior to transplant)	$CD3^+$ /mm^3	$CD4^+$ /mm^3	$CD8^+$ /mm^3	$CD3^+CD4^-CD8^-$ /mm^3	Naïve $CD4^{+*}$ %	CD19 /mm^3	$CD16^+$ and/or $CD56^+$, $CD3^-$ /mm^3	PHA (bkg)† cpm‡	Eosinophils /mm^3
1	166 (-258)	2,219	660	612	678	<1%	3,940	51		53
	215 (-209)	6,250	1,950	1,737	1,507	<1%	1,507	886	4,189 (654)	4,155
	254 (-170)	2,296	745	576	749	<1%	1,307	487	2,582 (584)	2,436
	297 (-127)	1,463	446	246	752	<1%	1,946	1,667	18,912 (103)	1,613
	408 (-16)	964	4043	146	411	<2%	3632	582	63,733 (413)	468
	418 (-6)	1,010	473	155	381	<1%	2,734	409	104,246 (154)	510
2	140 (-126)	2§					315	273		
	252 (-14)	1§					638	167		36
	255 (-11)								635 (205)	76

* For subject 1, naïve T cells were defined by the phenotype $CD4^+CD45RA^+CD27^+$ for the first 3 values done in the referring center and by the phenotype $CD4^+CD45RA^+CD62L^+$ in the transplant center. Naïve CD8 cells (unpublished) were similarly very low.
† Abbreviation: bkg, medium plus cells background.
‡ The first two PHA assays for subject 1 were performed in the referring center ; the lower limit of normal is 20,000 cpm. The remaining PHA assays in this table were performed at the transplantation center laboratory in which the lower limit of normal is 75,000 cpm.
§ The T cell numbers were too low for accurate determination of the naïve percentages.

Beginning prior to transplantation, the subject was treated with cyclosporine, steroids, and rabbit anti thymocyte globulin as described previously.[8] The immunosuppression was initiated because of the increased T cell proliferation in response to PHA, the large numbers of oligoclonal double negative T cells, and the increased levels of T cell activation markers (unpublished data). One dose of daclizumab, 1 mg/kg, was also given shortly prior to transplantation. The female O+ blood type subject was transplanted with cultured thymus tissue from an unrelated

female A- infant who was under 1 month of age. The HLA types for the subject and thymus donor are shown in Table 2.

Table 2. HLA typing of subjects and thymus donors

	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1
Subject 1	2601, 3101	3503, 3801	1203	0701, 1201	0202, **0301***
Thymus 1	0101, 2301	0801, 4901	0701	1101, 1301	**0301**, 0603
Subject 2	2402, 2501	1501, 4402	0303, 0501	0404, 1301	0302,0603
Thymus 2	0101, 0201	0801, 4002	0202, 0701	0301, 0408	0201, 0301

* The bold specificities indicate sharing between the recipient and the thymus donor.

Clinical course after transplantation:

Subject 1 was weaned off steroids and cyclosporine by 10 months after transplantation. Pneumocystis pneumonia prophylaxis and immunoglobulin replacement were stopped at 33 months after transplantation.

The subject had several serious infections in addition to *M. bovis*. A severe rotavirus infection was present from the time of admission for transplantation until discharge. After transplantation, she developed a *Klebsiella pneumoniae* urine infection on day 19; pneumocystis pneumonia requiring oxygen therapy on day 53 (despite pentamidine prophylaxis); a central venous catheter infection with blood cultures positive for *K. oxytoca* and *Enterococcus faecalis* at 6 months; and varicella at 8 months that was treated with intravenous acyclovir with an uneventful course. By 18 months after transplantation, adenopathy from BCG had resolved. The anti-mycobacterial medications were stopped 33 months after transplantation.

Immune results following thymus transplantation:

A biopsy of the allograft was performed on day 99 after thymus transplantation. Figure 3 (panels A and B) shows evidence of thymopoiesis with a Hassall body and medullary thymocytes.

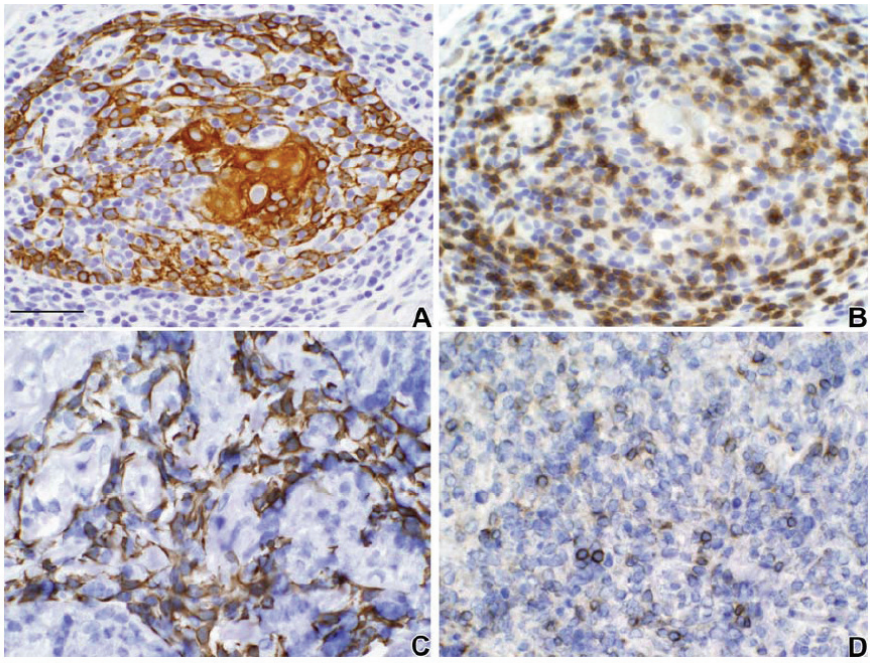

Figure 3: Biopsy evaluation of thymus allografts shows thymopoiesis. Subject 1 in panels A and B, subject 2 in panels C and D. Cytokeratin reactivity in A and C, CD3 reactivity in B and D. Bar is 50 µm. The microscope was an Olympus VANOX AHBS3. The magnification was 40x using a 40x numerical aperture objective lens (Olympus SPLAN 40X). The photomicrograph was taken at room temperature. Neither imaging media nor fluorochromes were used. The camera used was an Olympus DP70 digital imaging camera. Acquisition software was Olympus DP Controller. Subsequently Adobe Photoshop 6.0 was used to compose this figure.

Subject 1 had presented with circulating T cells that were predominantly $CD4^{-}CD8^{-}$ (Table 1). By 6 months after transplantation, the CD4 T cells outnumbered the double negative T cells (Figure 4A). Naïve T cells began increasing 1 year after transplantation (Figure 4B). The most recent naïve CD4 count at 4.9 years after transplantation is 213 cells/mm^3 (normal 420-1500, 10^{th} – 90^{th} percentile).[16] No thymus

donor T cells nor maternal T cells were detected when tested at day 101 after transplantation.

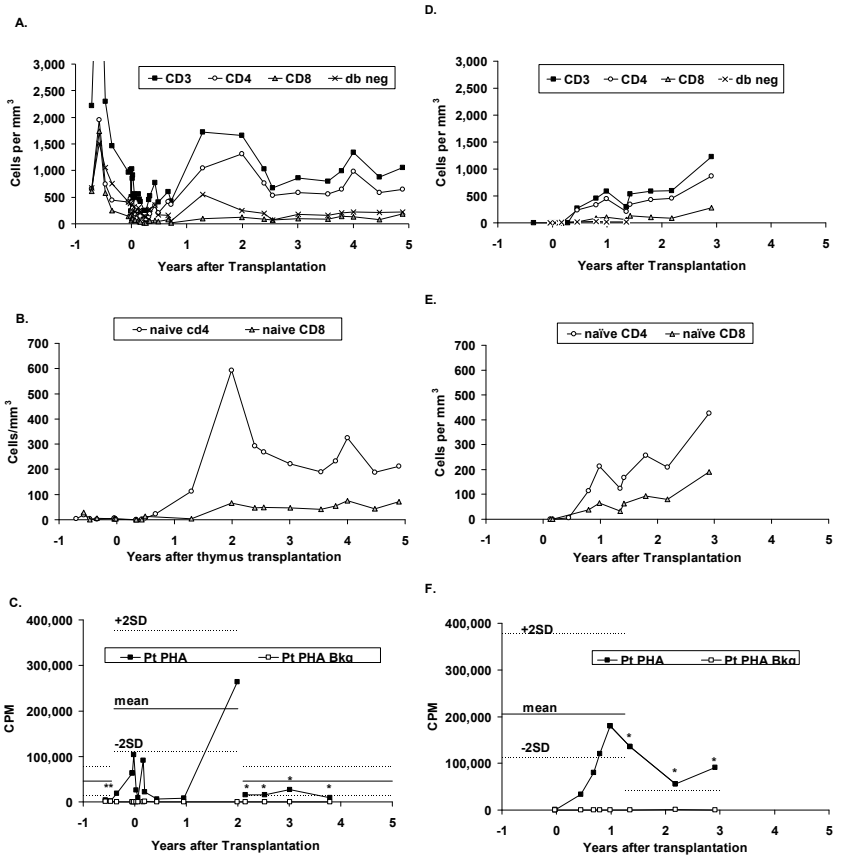

Figure 4: T cell subtype populations and PHA responses before and after thymus transplantation. Subject 1 is shown in panels A, B, and C; and subject 2 in panels D, E, and F. Panels A and D show T cell phenotypes; panels B and E show naïve T cells, and panels C and F show proliferative responses to PHA. In panels A and B, the data points starting at 2.1 years were obtained by the referring hospital laboratory. In panels D and E, the first 3 data points and the data points starting at 1.4 years after transplantation were obtained by the referring hospital laboratory. The phenotypes of the naïve $CD4^+$ and $CD8^+$ T cells are as described in Table 1. In panels C and F, the asterisks indicate the values obtained in the referring hospital laboratories. The mean (solid line) and ± 2 SD (dotted lines) for healthy adult data are shown in Panel C for the referring and transplant center laboratories. In Panel F, the lower limits of the normal PHA response observed in the referring hospital laboratory is indicated by the single dotted line.

Antigen specific T cell responses developed after transplantation. At 17 months after transplantation, subject 1 developed a proliferative response to PPD (Table 3). An antigen specific proliferative response to VZV was detected when assayed at 45

months after transplantation (Table 3). The proliferative response to PHA is normal (Figure 4C).

Table 3: T cell proliferative responses to antigens

Antigen	Month after transplantation	Antigen stimulated cells (cpm)	Unstimulated cells (cpm)	Stimulation index
Subject 1				
PPD	7	686	700	1
	17	16,167	630	26
	36	31,063	243	128
	45	22,019	430	34
C. albicans	17	645	630	1
	45	6,732	430	10
Tetanus toxoid	45	15,240	430	24
VZV	45	17,938	430	28
Subject 2				
Tetanus toxoid	15	18,500	3,000	6
	35.4	27,000	700	39
C. albicans	15	20,500	3,000	7
	35.4	74,500	700	106

The CD4 TCRBV repertoire as assessed by spectratyping improved significantly after transplantation (Figure 2B) compared to the previous analysis (Figure 2A). The spectratyping results correlated with an improvement in the flow cytometry assessment of the repertoire, which no longer contained expansions of VB3, VB4, VB14 or VB22 (Fig 1C). The sjTREC analysis at 2 years after transplantation showed 10,220 TRECs per 100,000 $CD3^+$ T cells, a value within the normal range for age.[17]

Subject 1 stopped immunoglobulin replacement approximately 2.7 years after transplantation. By this time, subject 1 had developed normal serum IgA and IgM levels, both of which had been undetectable early in life (Table 4). Serum IgG levels were within the normal range 4 of 5 times when tested after discontinuation of immunoglobulin therapy (Table 4). At 3.2 years after transplantation, the subject demonstrated an excellent response to tetanus toxoid immunization (Table 4).

Subject 2

Subject 2 was born at term to unrelated parents of mixed French/African origin who came from nearby communities. The male infant had no hair and dystrophic nails. Genetic analysis revealed a homozygous missense mutation in exon 6 of *FOXN1*, C987T (R320W). The subject was not given BCG vaccination at birth. At 3 months of life, the infant presented in respiratory distress and was mechanically ventilated for 15 days. No microorganism was recovered by bronchoalveolar lavage, but he was treated with antibiotics including trimethoprim/sulfamethoxazole and liposomal amphotericin. One month later, after transfer to a tertiary hospital, Human Herpes Virus 6 (HHV6) infection, was detected (140,000 copies/ml) associated with mild anemia and neutropenia. Thrombocytopenia, felt likely to be related to HHV6 infection, developed at 8 months of life. The initial immune evaluation at 4 months of life revealed no T cells and no proliferative response to PHA (Table 1). Flow cytometry evaluations of TCRBV repertoire and sjTREC analysis were not performed in subject 2 due to lack of T cells prior to transplantation.

Table 4. B cell function in subjects after stopping immunoglobulin replacement

Age (years)	Year after tx*	Serum IgG (mg/dl)	Serum IgA (mg/dl)	Serum IgM (mg/dl)	Serum IgE (IU/ml)	Other titers
Subject 1 - stopped immunoglobulin replacement at 3.9 years of life, 2.7 years after transplantation						
0.4	-0.7	126	<22	<17	409	
4.0	2.8	761	68	104		
4.2	3.0	775	93	80		
4.3	3.1	686	93	103	13	Tetanus titer (pre vaccine) 1 µg/ml
4.4	3.2					Tetanus titer (post vaccine) 135 µg/ml
4.7	3.5	568	82	116		
5.5	4.4	831	110	162		
Normal, age 4 - 6 years		640 – 1,420	52 - 220	40 - 180	<52	
Subject 2 - stopped immunoglobulin replacement at 1.8 years of life, 1.1 years after transplantation						
0.4	-0.3	292	15	55		
2.1	1.4	477	26	99		
2.1	1.4					Tetanus titer: 1.24 IU/ml† ; Hemophilus titer: 17.4 µg/ml (>1†) ; Polio #1: 15 (<5‡) Polio #2: >160 (<5‡); Polio #3: 80 (<5‡)
2.3	1.6	432	32	103		
2.5	1.8	408	39	87		
2.9	2.2	504	39	110		
3.6	2.9	506	96	112		HBs: 70 mIU/ml (<10‡) Rubella: 154 IU/ml (<10‡)
3.6	2.9					Anti-A: 1:32 (IgM)†; Anti-B: 1:16 (IgM)†
3.7	2.9					Mumps: 351 (<50‡); Measles: 265 (<70‡)
3.7	2.9				7.7 IU/ml	
Normal, age 2 years		582 -1200	46 -157	54 -155	0-126	
Normal, age 3 years					3-135	

* Abbreviations: tx, transplantation; † This is a normal response. ‡ This control value indicates absence of immunity

Subject 2 did not receive any immunosuppression. Thymus transplantation was performed at 9 months of life. The male O+ blood type subject received thymus tissue from a type O+ female who was less than 9 months old. The HLA types for the subject and thymus donor are shown in Table 2.

Clinical course after transplantation:

The HHV6 viral load was 110,000 copies/ml at 1 month after transplantation and dropped to 600 copies/ml 18 months later. The thrombocytopenia resolved 3 months after transplantation. At 4 months after transplantation, the subject developed a polymicrobial central line infection. At month 11 after transplantation, he developed a mild but chronic urticaria. Immunoglobulin replacement and

trimethoprim/sulfamethoxazole prophylaxis were stopped at 13 and 16 months after transplantation, respectively. At 1.6 years after transplantation, subject 2 developed autoimmune hypothyroidism with positive anti-thyroglobulin, anti-thyroid peroxidase, and anti-thyroid stimulating hormone (TSH) receptor antibodies. Three years post thymus transplantation, the subject is well without recurrent or chronic infection. He has a normal life and normal growth with thyroid hormone replacement.

Immune results following thymus transplantation:

Subject 2 underwent a biopsy of the allograft on day 53 after thymus transplantation. The biopsy showed lacy cytokeratin and the presence of $CD3^+$ thymocytes (Figure 3, panels C and D). Scattered thymocytes were Ki-67 (nuclear proliferation marker) and CD1a positive (not shown). These markers are characteristic of cortical thymocytes.

T cell numbers began to increase at 5.5 months after thymus transplantation (Figure 4D). Naïve T cells appeared by 9 months (Figure 4E). At 16.5 months after transplantation, all T cells were shown to be genetically host. The most recent total $CD4^+$ T cell and naïve $CD4^+$ T cell numbers (obtained at 2.9 years after transplantation) are in the normal range for the age of the subject (3.6 years of life).[16] The total $CD8^+$ T cell and naïve $CD8^+$ T cell numbers remain below the 10^{th} percentile for age.[16]

The CD4 TCRBV repertoire spectratype analysis was polyclonal when tested at one year after thymus transplantation, comparable to those of healthy controls (Figure 2C). The flow cytometry evaluation of the CD4 TCRBV repertoire was similarly polyclonal (Figure 1D). An sjTREC assessment at one year revealed 5,700 TRECs per 100,000 $CD3^+$ cells.

The subject demonstrated a normal T cell response to PHA response by 9 months after transplantation (Figure 4F) and antigen proliferative responses by 15 months after transplantation (tetanus toxoid and *Candida albicans*) (Table 3). Both responses remained normal over time. The subject was given a live measles, mumps, and rubella (MMR) vaccine 33 months after transplantation without any adverse sequelae.

Antibody function was tested after immunoglobulin replacement was stopped 1.1 years after transplantation. Table 4 shows the serum immunoglobulin levels and several antibody responses to immunizations. Although the serum IgG levels are slightly low compared to the age matched range, all specific antibody titres tested were within the protective range.

Discussion

We report here for the first time the use of allogeneic thymus transplantation for the treatment of athymia and its associated lack of naïve T cells in two human subjects with the nude/SCID phenotype due to *FOXN1* mutations. The two subjects are well 5 and 3 years post transplantation. They both developed functional T and B cell immune reconstitution.

The subjects were diagnosed after severe infections (disseminated BCG in subject 1, and a severe respiratory infection of unknown etiology in subject 2). They presented with absence of naïve T cells, total alopecia and nail dystrophy. FOXN1 deficiency was suspected and genetically confirmed in both subjects. Subject 1 was homozygous for the same mutation previously described in southern Italy[7] and present in the first FOXN1 deficient human described.[7, 18] Subject 2 was homozygous for a novel missense

mutation, C987T (R320W) in exon 6. This mutation is in the middle of the forkhead domain that is involved in DNA binding and is highly conserved among species.[3, 19] This homozygous mutation would likely abolish FOXN1 activity, although protein function was not tested.

The presentation of subject 1 to the transplantation center bore a striking resemblance to the presentation of infant patients with atypical complete DiGeorge anomaly. [20] Infants with complete DiGeorge anomaly characteristically present with a heart defect, hypoparathyroidism and athymia. These athymic infants with complete DiGeorge anomaly represent less than 5% of all infants with DiGeorge anomaly.[21-23] The diagnosis of athymia is based on the absence of naïve T cells. Some patients with complete DiGeorge anomaly develop a rash and circulating oligoclonal T cells after birth. [20] They are said to have "atypical" complete DiGeorge anomaly. [20] In occasional patients, the oligoclonal T cells infiltrate the liver or the gut and lead to graft versus host like-disease in these organs. This presentation resembles that of Omenn syndrome. [20, 24]

Similar to patients with atypical complete DiGeorge anomaly, subject 1 presented to the transplant center at 13.7 months of life with oligoclonal T cells (which were predominantly double negative T cells), absence of naïve T cells, and a T cell proliferative response to PHA within the normal range (although the PHA response had initially been low per the laboratory standards at the referring institution). This subject had lymphadenopathy, although this finding was likely related to the underlying *M. bovis* infection, and eosinophilia. The skin manifestations in subject 1 were different from those in atypical complete DiGeorge anomaly since subject 1

presented only with mild erythroderma. The proliferative response to PHA observed in subject 1 was unusually high compared to other infants with athymia. Only 3 patients with complete DiGeorge anomaly (out of 60 transplanted with thymus tissue) have developed PHA responses greater than 100,000 cpm prior to transplantation (data not shown).

We find it interesting that the first subject reported with FOXN1 deficiency had an Omenn syndrome-like appearance with erythroderma and lymphadenopathy associated with circulating T cells that did not proliferate to mitogens, including anti-CD3.[5] That subject's FOXN1-deficient sibling also had erythroderma and circulating T cells that did not proliferate in culture. These features suggest that these two subjects, who were reported previously, had circulating oligoclonal T cells. Subject 1, who had the same mutation as the previously reported patients, also presented with erythroderma. This phenotype contrasts with subject 2, who carries a different mutation and had no circulating T cells.

The oligoclonal T cells of atypical complete DiGeorge anomaly and Omenn/atypical FOXN1 deficiency may have an extra-thymic origin or may arise secondary to a nest of thymus epithelium able to support atypical development of T cells. Studies of nude mice have also demonstrated the presence of oligoclonally expanded T cells.[25, 26] The mechanisms for the proliferation and lack of homeostasis by these oligoclonal T cells are poorly understood.[20]

Because of athymia in FOXN1 deficiency, thymus transplant was chosen as the appropriate treatment, although bone marrow transplantation had been performed in

one child with the nude/SCID phenotype due to FOXN1 deficiency.[6] That child did not develop naïve T cells, as might be expected given the absence of a thymus.[6]

In determining the strategy to use for thymus transplantation in the 2 subjects presented in this report, we drew on our experience with infant patients who have complete DiGeorge anomaly. Immunosuppression has not been necessary in patients with typical complete DiGeorge anomaly who have few if any T cells.[8, 11] Thus, immunosuppression was not used for subject 2. Atypical complete DiGeorge anomaly patients, who have oligoclonal T cell expansions, have required immunosuppression to prevent graft rejection.[8, 14] The same immunosuppression regimen was used for subject 1.

Just as seen in thymus transplantation for complete DiGeorge anomaly patients, both FOXN1 deficient subjects developed naïve T cells, T cell function, and diverse TCR repertoires after thymus transplantation. The development of an *in vitro* proliferative T cell response to PPD in subject 1 was temporally associated with the clearance of BCG infection (Table 3). Subject 2 also developed *in vitro* proliferative T cell responses against antigens, namely tetanus toxoid and *C.albicans* (Table 3).

The kinetics of appearance of T cells and the ultimate T cell numbers of the two FOXN1 deficient subjects fall within the ranges seen for infants with complete DiGeorge anomaly who receive postnatal allogeneic thymus transplants.[8, 10] Naïve T cells in subject 1 developed later than in most patients with complete DiGeorge anomaly who are given immunosuppression.[10] A slower development of naïve T cells in subject 1 was expected given the presence of *M. bovis* infection.[27, 28] Subject 2 also showed slightly delayed development of naïve T cells compared to most subjects with

typical complete DiGeorge anomaly[10] who usually develop naïve T cells before 6 months after transplantation.[8] Of note, in these subjects and the infants with complete DiGeorge anomaly who are given thymus transplantation, the CD8+ T cell numbers are substantially below the 10th percentile for age in the first years after thymus transplantation.[8] As in the infants with complete DiGeorge anomaly, the low CD8 numbers have not resulted in clinical infection.

In subject 1, concern arose that the preexisting infection with *M. bovis* would suppress thymopoiesis.[27, 28] The most recent T cell count for this subject (1,053 cells/mm^3 at 58 months after transplantation) indicates that the thymus transplantation has been successful in restoring relatively normal T cell numbers. The success in this subject gives hope for future athymic subjects who have mycobacterial infection.

Our data indicate that B cell function was restored after thymus transplantation. Both subjects were able to discontinue immunoglobulin replacement, maintain normal serum immunoglobulin levels, and generate protective antigen specific titers. Of particular note, both subjects received the MMR vaccine without any adverse events. Normal post-vaccine antibody responses to these three viruses were confirmed in subject 2 (Table 4).

It is remarkable that functional immunity developed in subject 1 with only one HLA match (HLA-DQB1) and in subject 2 without any HLA matches. This is similar to the findings in infants with complete DiGeorge anomaly for whom matching for HLA Class I and Class II has not been found to affect CD4 or CD8 T cell counts after thymus transplantation.[9]

The mechanisms involved in positive and negative thymic selection after unmatched thymus transplantation are not clear. Classically, it has been hypothesized that cortical thymic epithelium is necessary for positive selection to occur.[29] Murine studies suggest that recipient bone marrow-derived cells such as antigen presenting cells[30, 31] or thymocytes[32, 33] may also play a role in positive selection in the thymus. Alternatively, circulating host-derived epithelial progenitors[34-37] may migrate to the thymus. These recipient epithelial cells could then provide signals for positive selection of the developing thymocytes. In our subjects, even though the thymus graft is unmatched to the recipient, the recipients develop T cells that proliferate in response to antigens presented by recipient antigen presenting cells (Table 3) and provide help for B cell antibody production leading to protective antibody titers after vaccination (Table 4).

Regarding negative selection, dendritic cells have been shown to be involved.[38, 39] Thus, it is likely that recipient bone marrow derived dendritic cells that colonize the thymic graft may play a role. This putative mechanism for negative selection appears to be able to prevent the development of a graft versus host disease like syndrome mediated by the genetically host T cells that develop in the thymus.

Negative selection in the thymus does not prevent all autoimmune disease. Subject 2 developed autoimmune thyroid disease at 1.6 years after transplantation. The urticaria seen in subject 2 may have been related to the presence of anti-thyroid antibodies, as observed in approximately 30% of patients with chronic urticaria.[40, 41] Thyroid disease (Hashimoto or Graves) has occurred in 16 of 60 patients with complete DiGeorge anomaly after thymus transplantation (unpublished, [8, 10]). The mechanism for

the increased prevalence of thyroid disease remains unclear. These data further emphasize the importance of continuing surveillance of these subjects for autoimmune disease.

In summary, after thymus transplantation in two FOXN1-deficient subjects, naïve T cells and diverse T cell receptor repertoires developed in parallel with normalization of T cell proliferative responses and immunoglobulin levels. More importantly, the associated clearance of the ongoing disseminated infections raises the expectation that this therapeutic approach may have long-term clinical benefit for subjects with athymia secondary to FOXN1 deficiency. Overall, thymus transplantation offers a promising treatment for FOXN1 deficiency (nude/SCID).

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Publication:

3.2. Deciphering the human *FOXN1*-deficiency phenotype through thymic transplantation

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Abstract

How T-cell progenitors and thymic epithelium interact to generate T-cells is incompletely understood. FOXN1 is a transcription factor expressed by thymic epithelium crucial for both development of the thymus[1,2] and prevention of its involution[3]. Investigation of the phenotype of *FOXN1*-deficiency in a patient with homozygous R255X mutation[4,5], which causes alopecia universalis, absence of thymus and consequent T-cell immunodeficiency unexpectedly revealed a high number of circulating T-cells displaying a regulatory T-cell-like phenotype which was normalized following HLA-mismatched thymic transplantation. Conversely, a large population of αβ T-cells expressing neither CD4 nor CD8 (double-negative, DN) persisted 5 years post-transplant despite the evidence that functional immune-competence had been achieved. Our data suggest that *FOXN1* mutations may allow the development of a thymic rudiment that supports T-cell development albeit with disturbances of positive/negative selection, as indicated by the expansion of DN and FOXP3+ subsets.

Results and Discussion

The unique ability of the thymic microenvironment to generate and select T-cells requires specialized epithelium that is regulated by FOXN1[1-3]. Defects in *FOXN1* lead to athymia in association with total alopecia ("nude-SCID"), due to its additional role in hair follicle differentiation[1,2,6].

Human *FOXN1* deficiency was first reported by Pignata *et al.* in two sisters from Campania[4,7]. Notwithstanding the evidence of athymia, these children had a significant number of circulating T-cells[5]. We identified the same homozygous R255X mutation[4,5,8], in a Portuguese child, who presented at 5 months of age with alopecia universalis and respiratory failure due to Bacillus Calmette-Guérin (BCG) dissemination following routine neonatal vaccination with this live-attenuated mycobacterium. Circulating T-cells of non-maternal origin were observed at close to normal numbers (2219 cells/μl), with similar proportions of CD4+, CD8+ and, strikingly, also of αβ-cells that expressed neither CD4 nor CD8 (double-negative, DNαβ) which usually are less than 1%. Athymia was diagnosed based on absent thymus-shadow on x-ray, lack of naïve T-cells, undetectable single-joint T-cell receptor excision circles (sjTREC), and an oligoclonal repertoire, according to spectratyping (submitted manuscript). We report here that CD4+ T-cells exhibited an activated memory-effector phenotype with preserved IL-2, IFN-γ and IL-4 production (Fig.1a). CD8+ T-cells showed a similar activated phenotype with no terminal-effector differentiation, as illustrated by the maintenance of CD45RO/CD28/CD27 expression (Fig.1b) and the low frequency of perforin-producing cells (3%). The aberrantly expanded DNαβ T-cells also expressed CD45RO and were mostly CD27+ and IL-2-producing cells in agreement with lack of

terminal-effector differentiation (Fig.1c). A significant proportion of T-cells were cycling (Ki-67+, Fig.1d), and it is likely that IL-7 played a role in their maintenance/expansion given the preserved expression of IL-7 receptor α-chain (CD127, see ahead Fig.2d) and evidence of IL-7 used based on the lack of increased IL-7 serum levels associated with lymphopenic settings. In agreement, IL-7 serum levels increased transitorily from 6.3pg/ml to 44.3pg/ml during the lymphopenic peri-transplant period.

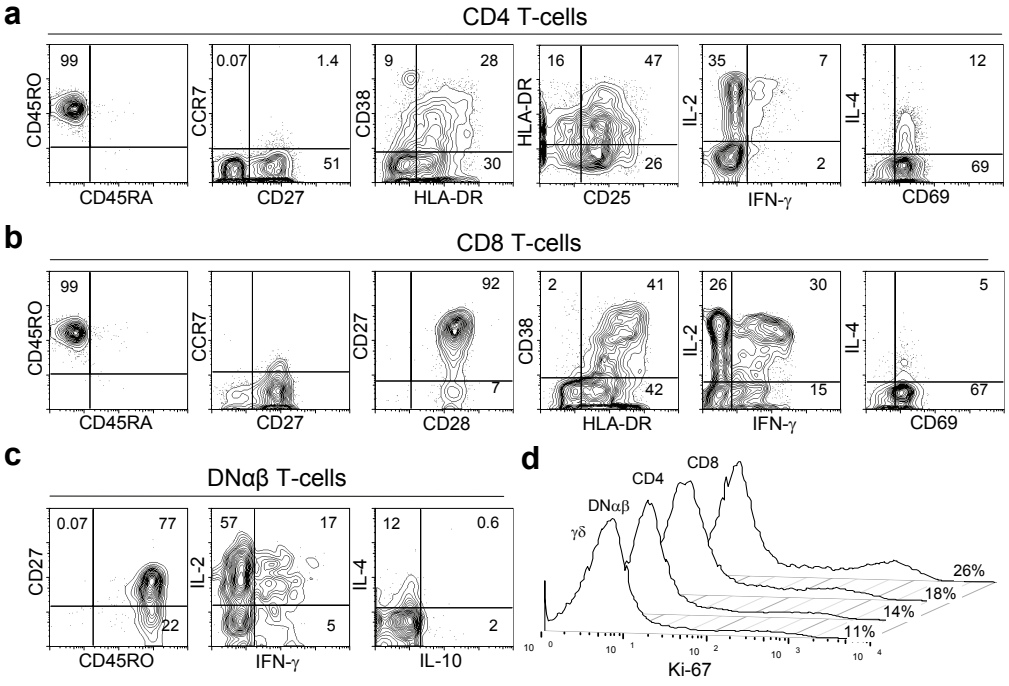


Figure 1: Peripheral T-cells in a patient with R255X *FOXN1* mutation. CD4+ (**a**), CD8+ (**b**), and DNαβ T-cells (**c**) exhibited a memory-effector activated phenotype and were able to produce significant amounts of IL-2, IFN-γ and IL-4. Dot-plots show analysis after gating on the respective populations; numbers within each quadrant represent the proportion of cells expressing the respective molecules. Cytokine production was assessed after 4h stimulation of PBMC with PMA plus Ionomycin in the presence of Brefeldin A. Intracellular CD69 staining was used as a control for T-cell activation. (**d**) Proportion of cycling cells within CD8+, CD4+, DNαβ and γδ T-cells. Histograms show levels of intracellular Ki-67 expression within these populations; percentages of Ki-67+ cells are shown. Proportions within PBMC and absolute counts at day 166 of life were: 10.4% (660 cells/μl) for CD4+ T-cells; 9.7% (612 cells/μl) for CD8+ T-cells; and 10.7% (676 cells/μl) for DNαβ T-cells.

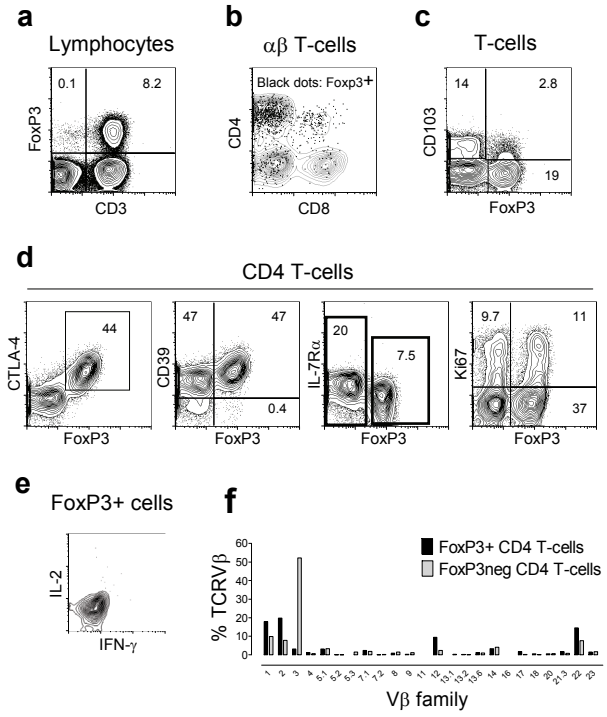


Figure 2: Expansion of circulating Treg-like (FoxP3+) cells in a patient with R255X *FOXN1* mutation. (**a**) Analysis of FoxP3 expression within total lymphocytes showing that FoxP3 is restricted to CD3+ cells. (**b**) Expression of CD4 and CD8 within total αβ cells (grey) and within FoxP3+ cells (black). Within total FoxP3+ cells there were 67% CD4+, 17% DNαβ and 12% cells co-expressing CD4 and CD8 (DP). DP represented 3% of total αβ cells and 26% of them were FoxP3+. FoxP3+ cell count was 328 cells/μl. (**c**) Expression of the mucosal homing molecule CD103 within T-cells according to FoxP3 expression. (**d**) Analysis of the concomitant expression of FoxP3 with other Treg markers namely: CTLA-4, CD39, CD127, or Ki-67 within total CD4+ T-cells. (**e**) Lack of cytokine production by FoxP3+ cells. Analysis performed upon short-term PMA and Ionomycin stimulation in the presence of Brefeldin A. Dot-plots show analysis after gating on the populations mentioned on the top; numbers inside quadrants represent the proportion of cells expressing the respective molecules. (**f**) Vβ distribution within FoxP3+ and FoxP3- CD4+ T-cells. Graph show the proportion of FoxP3+ CD4+ T-cells (black) and FoxP3- CD4+ T-cells (grey) belonging to a given Vβ family as assessed by flow cytometry.

The thymus is known to produce a regulatory CD4+ T-cell subset (Treg), fundamental for preventing autoimmunity, currently best identified by expression of the forkhead transcription factor FoxP3[9-11]. We found that up to 40% of the CD4 subset (328 cells/μl) expressed high levels of FoxP3, and also observed atypical populations of FoxP3+ DNαβ and double-positive T-cells (Fig.2a-b). FoxP3 can also be up-regulated in non-Treg T-cells upon activation[11,12]. Nonetheless, several findings support these cells

being *bona fide* Treg. In agreement with human Treg phenotype[11], these cells expressed FoxP3 at high intensity concomitantly with other Treg-associated markers (CTLA-4, CD25 and CD39) and reduced levels of CD127 (Fig.2c-d). Moreover, in contrast to activated T-cells, they did not produce IL-2 or IFN-γ (Fig.2e). Importantly, comparison of the relative representation of different Vβ families within the FoxP3+ and FoxP3- CD4 sub-populations revealed distinct oligoclonal expansions, further supporting that FoxP3+ cells are a separate CD4 lineage (Fig.2f).

The Treg compartment has been recently investigated in other clinical settings associated with peripheral oligoclonal T-cell proliferation following thymic impairment either due to hypomorphic mutations in hematopoietic precursors (Omenn syndrome)[13-15], or due to developmental defects associated with variable degrees of thymic hypoplasia (DiGeorge syndrome)[16,17]. In these settings, Treg frequencies were found to be unaltered or reduced[13-17], emphasizing the particularity of the *FOXN1* mutation.

Our data raise important questions regarding T-cell origin in the context of athymia. It is possible that a thymic rudiment persisted, facilitating the limited production of T-cells that subsequently expanded in the periphery. FoxP3 induction can occur in early stages of both murine and human T-cell differentiation[18]. In mice, the *Foxn1* gene was shown not to be required for the initial formation of the thymic primordium[19], and there is evidence of functional T-cells in nude-SCID mice[20]. However, at least some of these T-cells seem to be generated extra-thymically, mainly in mesenteric lymph nodes[21]. CD4+ and CD8+ αβ T-cells accumulate with aging in nude-SCID mice[22], however, characterization of their putative Treg compartment has not been conducted. Of note, significant DNαβ as well as FoxP3+ cells were found in a

mouse model of extra-thymic lymphopoiesis induced by Oncostatin M, a cytokine that induces thymic atrophy and lymph node alterations that support T-cell differentiation[23].

Since all patients reported with R255X *FOXN1* mutation presented circulating T-cells, it is plausible that these patients retained a dysplastic thymic rudiment capable of supporting T-cell differentiation, albeit with a narrow TCR repertoire and impaired T-cell selection, allowing the emergence of atypical DNαβ and Treg. Alternatively, this thymic rudiment could allow T-cell commitment of precursors for subsequent extra-thymic development. Progenitor T-cell commitment was shown to occur in the thymus prior to their extra-thymic development in mouse models[24]. Notably, no circulating T-cells were recently found in a patient with a different *FOXN1* mutation (submitted manuscript).

As *FOXN1* mutations impact on thymic epithelium rather than hematopoietic precursors, we predicted that thymic transplantation, although never performed before in this setting, could be a curative strategy. This was confirmed by the documentation of the clinical efficacy of HLA-mismatched thymic transplantation, as attested by the temporal association between the clearance of ongoing BCG adenitis and development of PPD-specific proliferative responses (submitted manuscript). The child remains free of significant infections 3yrs after having stopped all prophylaxis therapies.

The post-transplant kinetics of T-cell recovery and the fate of the expanded pre-transplant Treg-like and DNαβ T-cell populations were investigated. A slow progressive increase in the proportion of circulating naïve T-cells was observed (Fig.3a), accompanied, as expected, by increasing sjTREC levels (Fig.3b). Notably, in

spite of the HLA-mismatch of the thymic epithelia, naïve CD4+ T-cells showed a fully diverse TCR repertoire 5yrs post-thymic transplant (Fig.3c). The functionality of the allogeneic thymic graft was further estimated by sj/βTREC quantification; a ratio between early and late products of TCR rearrangements representing an indirect measurement of thymocyte division-rate and a direct correlate of thymic output[25,26]. A progressive sj/βTREC increase was observed (Fig.3b), reaching levels comparable to those observed in healthy children. Importantly, a sharp decline of sj/βTREC, accompanied by a decrease in sjTREC levels and proportion of naïve cells was observed 4yrs post-transplant (Fig.3a-b). Notably, these values plateau thereafter (Fig.3a-b), suggesting that a steady-state equilibrium could be established after replenishment of the immune system. These data provide novel evidence regarding the long-term sustainability of allogeneic thymic tissue, with implications for other clinical settings aimed at immunological reconstitution.

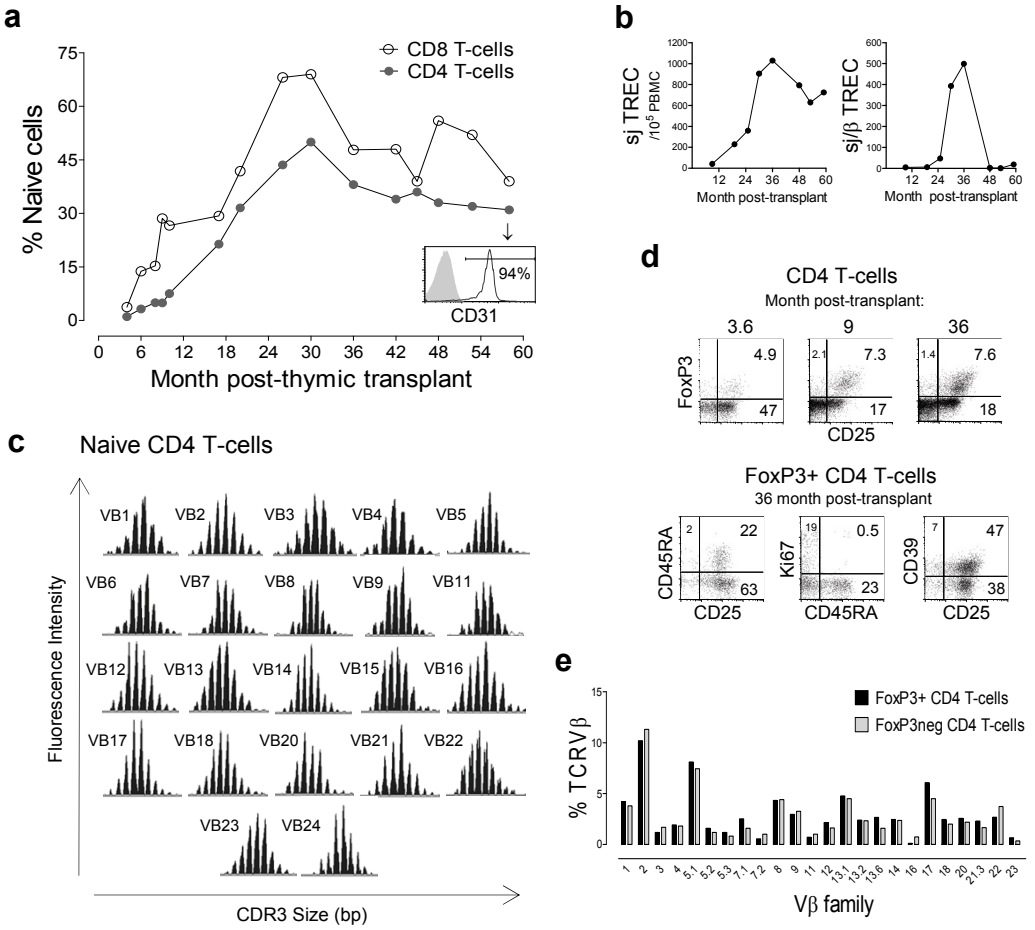
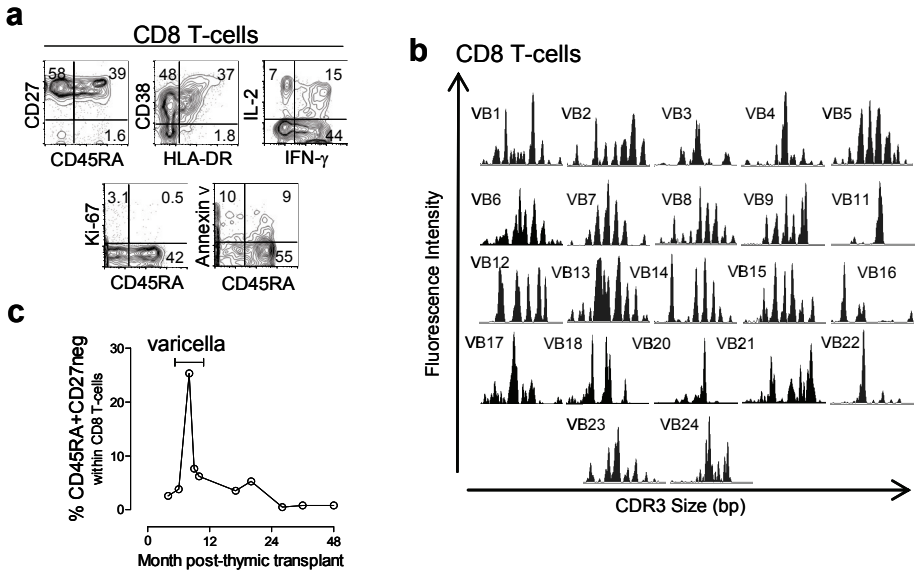


Figure 3: Immunological reconstitution and recovery of the Treg compartment upon HLA-mismatched thymic transplantation in a patient with R255X *FOXN1* mutation. (**a**) Kinetics of the frequency of cells with a naïve phenotype (CD45RA+CD27+) within CD4+ and CD8+ T-cells; histogram shows CD31 expression within naïve CD4+ T-cells at 58 month post-transplant, a marker associated with recent thymic emigrants. (b) Longitudinal quantification of sjTREC (left) and sj/βTREC ratio (right). (c) Assessment of TCR repertoire by spectratyping analysis of the CDR3 Vβ regions of purified naïve CD4+ T-cells at 59 month post-transplant. (d) Longitudinal analysis of the frequency of cells expressing FoxP3 and/or CD25 within total CD4+ T-cells. (e) Phenotype of circulating FoxP3+ cells at 36 month post-transplant; analysis was performed after successive gates on CD4+ and FoxP3+ T-cells; numbers inside quadrants represent the frequency of cells expressing the mentioned molecules. (f) Graph shows the proportion of FoxP3+ (black bars) and FoxP3- (grey bars) CD4+ T-cells belonging to each of the Vβ families assessed by flow cytometry at 48 month post-transplant.

CD8+ T-cell recovery was disproportionally low (92 cells/µl, 9% of T-cells), as described following HLA-mismatched thymic transplantation in DiGeorge syndrome[27,28]. Importantly, the kinetics of naïve cell expansion within CD8+ subset paralleled that observed for their CD4+ counterparts (Fig.3a), and, in agreement,

similar sjTREC levels were found in purified CD4+ and CD8+ T-cells (7155 and 7540 sjTREC/10^5 cells; respectively, 30 months post-transplant). Moreover, CD8+ T-cells were apparently functional and able to differentiate, as illustrated during varicella-zoster virus infection (Supplementary Fig.1).



Supplementary Figure 1: Reduced but functional CD8+ T-cell compartment after fully-mismatched HLA class I thymus transplantation. 58 month post-transplant the frequency of circulating CD8+ αβ T-cells was 5% (83 cells/μl). CD8+ T-cells were analysed in terms of: (**a**) naïve/memory/effector phenotype assessed by CD45RA and CD27 expression, activation markers expression (CD38 and HLA-DR), IFN-γ or IL-2 production upon PMA+ionomycin stimulation, *ex vivo* frequency of cycling cells (Ki67+), and *ex vivo* frequency of apoptotic cells assessed by annexin V staining evaluated in gated CD8+ T-cells by flow cytometry; (**b**) TCR repertoire diversity evaluated by spectratype of CDR3 Vβ regions of purified CD8+ T-cells. (**c**) Graph shows the timely increase in the proportion of terminally differentiated cells defined as CD45RA+CD27neg cells within total CD8+ T-cells during Varicella and its decrease in parallel with the clinical resolution of the infection.

A parallel reconstitution of the Treg pool and CD4 subset was observed, leading to stable frequencies within the normal range (Fig.3d). Of note, distribution of Vβ-families within FoxP3+ and FoxP3- CD4+ T-cells was strikingly similar despite the HLA-mismatch between thymic epithelia and host (Fig.3e). Thymic Treg development is currently thought to be dependent on a small developmental niche that tightly controls Treg output[29,30]. It is possible that FOXN1 plays a role in such niches, contributing to

the thymic regulation of Treg numbers.

By contrast, a significant population of circulating DNαβ persisted, at relatively stable frequencies, throughout the 5yrs of follow-up (Fig.4a). These cells were not previously observed following HLA-mismatched thymus transplant [27] (submitted manuscript), and were shown to disappear after effective naïve reconstitution in complete DiGeorge patients that presented with this atypical phenotype before thymic transplant[27]. Remarkably, DNαβ cells maintained a similar memory phenotype (Fig.4b) and skewed repertoire, as assessed by spectratyping (Fig.4c), despite the lack of terminal-effector differentiation (Fig.4d-e), and ability to produce IL-2 (Fig.4f). Thus, it is unlikely that they are activated terminally-differentiated CD8+ T-cells that lost CD8 expression, as has been suggested in other clinical settings associated with abnormal expansions of circulating DNαβ cells[31]. Moreover, they expressed high levels of IL-2 and IL-7 receptors as well as high levels of Bcl-2, suggesting *in vivo* responsiveness to IL-7 (Fig.4g); further supported by their ability to phosphorylate STAT-5 upon IL-7 or IL-2 stimulation (Fig.4h). On the other hand, the low *ex vivo* frequency of cycling cells (Fig.4i), and their reduced proliferative response to these cytokines (Fig.4j), suggest that the DNαβ cell maintenance may be largely dependent on cytokine-induced survival. Notwithstanding the possibility of DNαβ T-cells being long-lived cells generated pre-transplant, their persistence 5yrs post-transplant favors a thymic rather than an extra-thymic origin for these cells, since any putative extra-thymic lymphopoiesis is likely to be shut-down upon thymic transplantation[21].

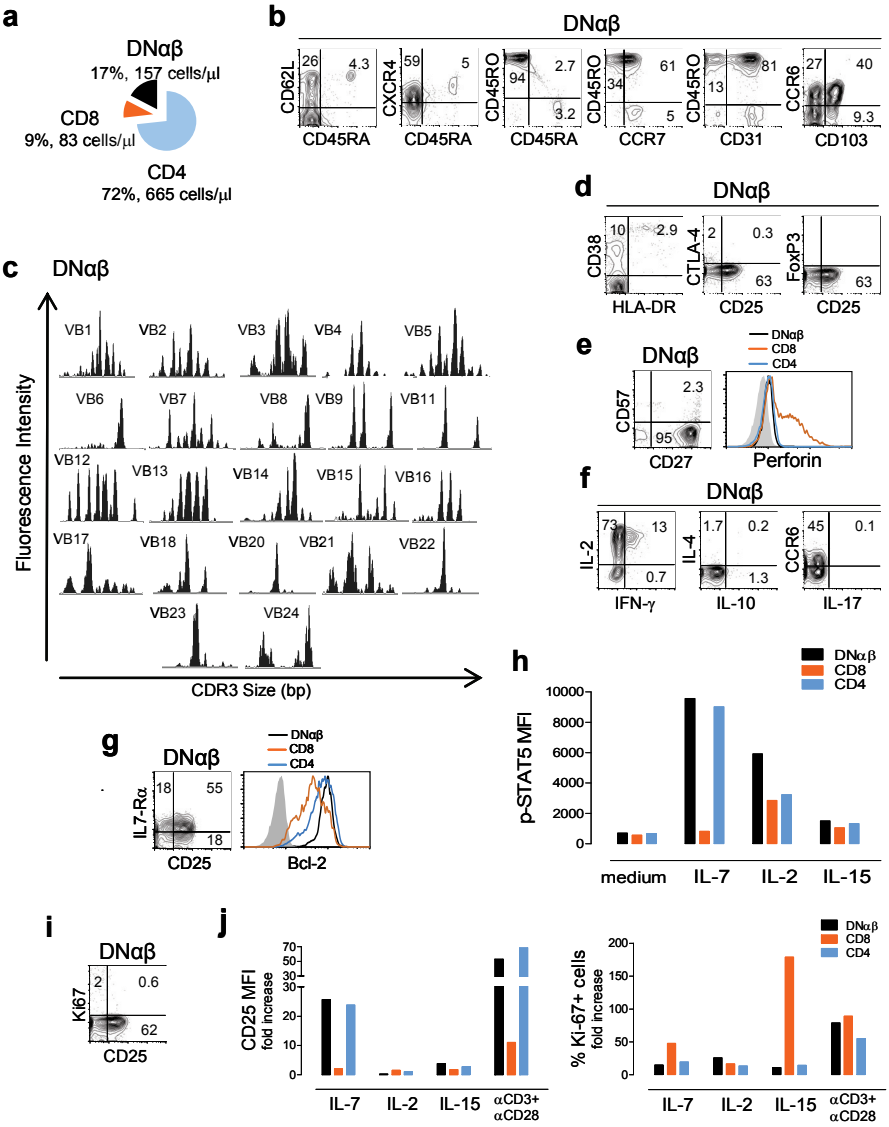


Figure 4: Persistence of DNαβ T-cells in a patient with R255X *FOXN1* mutation upon thymic transplantation. (**a**) Absolute counts and proportion of DN, CD8+, CD4+ cells within circulating αβ+ T-cells. Analysis of DNαβ T-cells revealed: (**b**) a relatively undifferentiated memory phenotype with increased expression of mucosal homing molecules; (**c**) a skewed repertoire as assessed by spectratype of CDR3 Vβ regions; (**d**) increased CD25 expression in the absence of other activation or Treg-associated markers; (**e**) no terminal-effector differentiation accordingly to CD27, CD57 and perforin expression (histogram compares perforin levels within DNαβ, CD8+ and CD4+ T-cells); (**f**) low ability to produce IFN-γ, IL-4, or IL-17 but high IL-2 production upon PMA+ionomycin; (**g**) preserved expression of IL-7Rα with high levels of Bcl-2 (histogram compares Bcl-2 expression within DNαβ, CD8+ and CD4+ T-cells); (**h**) up-regulation of p-STAT5 upon 15min stimulation with IL-7 (50ng/ml), IL-2 (100U/mL) or IL-15 (25ng/ml), bars represent p-STAT5 MFI within gated DNαβ, CD8+ and CD4+ T-cells; (**i**) low levels of circulating cycling cells (Ki67+) despite the increased CD25 expression; (**j**) marked up-regulation of CD25 levels but no preferential proliferation of DNαβ upon 5-day culture in the presence of IL-7 (10ng/ml), IL-2 (10U/mL), or IL-15 (12.5ng/ml) or anti-CD3 plus anti-CD28 stimulation, graphs represent the fold change of CD25MFI and frequency of Ki67+ cells with respect to medium, within gated DNαβ, CD8+ and CD4+ T-cells. 59 month post-transplant data are shown. Numbers inside dot-plots represent frequency of cells expressing the mentioned molecules acquired in a FACSCanto (p-STAT5 and 5-day cultures) and FACSCalibur flow cytometers.

Overall, human *FOXN1*-deficiency due to R255X mutation was associated with significant numbers of oligoclonal T-cells suggesting that T-cell development to a certain extent still occurs, albeit with altered positive/negative selection, as illustrated by the aberrant expansion of FoxP3+ and DN subsets. Importantly, immune-competence can be achieved through HLA-mismatched thymic transplantation, despite the lack of a sustained thymocyte-division rate (as evidenced by sj/βTREC), and this has implications for the design of immunological reconstitution strategies to be used in other clinical settings.

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M. L. Markert is a member of the Duke Comprehensive Cancer Center. The authors have no conflicting financial interests.

Methods

Patient

Female child, born at term to consanguineous Portuguese parents, admitted at day 157 of life with respiratory failure due to Bacillus Calmette-Guérin (BCG) dissemination, following routine neonatal BCG vaccination. The *FOXN1* mutation identified is a homozygous C-to-T transition at nucleotide position 792 (GenBank accession no. Y11739) leading to nonsense mutation at residue 255 (R255X) in exon 4 (formerly exon 5)[8]. Maternal chimerism was assessed using AmpFISTR Identifiler PCR Amplification Kit (Applied Biosystems, detection limit 1/100). Patient's clinical data was the subject of another manuscript (submitted manuscript). Failure to thrive and progressive nutritional status deterioration were observed despite antibiotic/tuberculostatic therapy and intravenous immunoglobulin G. Thymus transplantation was performed (day 424 of life), under protocols approved by the Duke Institutional Review Board (IRB) and reviewed by the Food and Drug Administration under an Investigational New Drug (IND) application, as described (submitted manuscript). Unrelated allogeneic thymus tissue, routinely discarded from infants with less than 9 months of age undergoing cardiac surgery, was used for transplantation after informed consent[27,28,32,33]. Studies were performed with the parent informed consent under the ethical guidelines of the respective institutions.

Cell isolation and cell sorting

Peripheral blood mononuclear cells (PBMC) were isolated immediately after collection by Ficoll-Hypaque; naïve CD4+ T-cells using the EasySep CD4+ naïve T-cell enrichment magnetic kit (StemCell Technologies); and DNαβ and CD8 T-cells using the

BD FACSAria High Speed Cell Sorter (BD Biosciences) after surface staining for CD3, CD4, CD8 and TCRαβ; population purity higher than 98%.

Flow Cytometry

Lymphocyte subsets were characterized using fresh whole blood after acquiring at least 100,000 events within a lymphogate using a FACSCalibur flow cytometer (BD Biosciences). TCR Vβ family frequency was quantified in whole blood using IOTest Beta Mark (Beckman Coulter). PBMC were stained intracellularly for CTLA-4 (clone BNI3) and/or Ki-67 (clone MOPC-21) both from BD Biosciences, and FoxP3 (clone PCH101) using eBiosciences's kit after surface staining, as described[18]. FoxP3 expression post-transplant was assessed in fresh PBMC, whereas cryopreserved PBMC were used for time-points prior to thymic transplantation. Apoptosis was assessed in fresh PBMC using Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) and propidium iodide (PI) staining. Analysis was done using FlowJo software (TreeStar). Results were expressed as median intensity of fluorescence (MFI) of a molecule or percentage of positive cells, and absolute numbers calculated by multiplying their percentage by the absolute lymphocyte count.

Assessment of cytokine production at the single-cell level

Cytokine production was assessed at the single-cell level after 4h PBMC culture with PMA/ionomycin in the presence of brefeldin A, as described[34], with mAb against IFN-γ (clone 4S.B3), IL-2 (clone MQ1-17H12), and IL-4 (clone MP4-25D2) from BD Biosciences; IL-10 (clone JES3-9D7); and IL-17 (clone eBio64DEC17) from eBiosciences.

STAT5 tyrosine phosphorylation analysis

STAT-5 phosphorylation (p-STAT5) was evaluated by flow cytometry on fresh PBMC stimulated for 15min with IL-7 (50ng/ml), IL-2 (100U/mL) or IL-15 (25ng/ml) or medium alone as described[35].

Proliferative response to cytokines

Fresh PBMC were stimulated with IL-7 (10ng/ml), IL-2 (10U/mL), IL-15 (12.5ng/ml, immobilized anti-CD3 (1µg/ml) plus anti-CD28 (1µg/ml) or medium for 5d. The fold change of the percentage of Ki-67+ cells and CD25 MFI with respect to medium alone were evaluated by flow cytometry on gated DNαβ, CD4+ and CD8+ T-cells.

TCR - chain CDR3 spectratyping

Total RNA was extracted from 10^5 to 10^6 cells with RNeasy kit (Qiagen) and first strand cDNA synthesized from 1-2µg of RNA with the Superscript III kit (Invitrogen) using an equivolume mixture of random hexamers and oligo (dT). Amplification of the TCRVB CDR3 was performed using primers specific for each V family[36] except for VB6 and VB21[37] and a common CB reverse primer[36]; followed by a run-off reaction that extends each different PCR product with a constant CB FAM labelled primer; and a third step, in which each different VB PCR labelled fragment is separated on a capillary electrophoresis based DNA automated sequencer. Data was collected and analyzed with GeneMapper v4.0 (Applied Biosystems) for size and fluorescence intensity determination.

TREC analysis

Signal joint (sj) and DJ T-cell receptor rearrangement excision circle (TREC) analyses were conducted as described[25]. Briefly, PBMC were lysed in Tween-20 (0.05%), NP-40 (0.05%) and proteinase K (100µg/mL) for 30min at 56°C, and then 15min at 98°C. Multiplex PCR amplification was performed for sjTREC together with the CD3 chain, in a final volume of 100µl (10min at 95°C, then 22 cycles of 30s at 95°C, 30s at 60°C, 2min at 72°C) using outer 3′/5′ primer pairs. PCR conditions in the LightCycler™ experiments, performed on 1/100th of the initial PCR products, were: 1 min at 95°C, then 40 cycles of 1s at 95°C, 10s at 60°C, 15s at 72°C. Measurements of the fluorescent signals were performed at the end of annealing steps. TREC and CD3 LightCycler™ quantifications were performed in independent experiments, using the same first-round serial dilution standard curve. Similarly, DJ1TRECs (DJ1.1 to 1.6) and DJ2TRECs (DJ2.1 to 2.7) were quantified in multiplex quantitative PCR assays. This highly sensitive nested quantitative PCR assay made it possible to detect one copy in 10^5 cells for any excision circle. The sjTREC, DJ1TRECs and DJ2TRECs were quantified in triplicate for each sample. The sj/βTREC ratio, sj/βTREC=sjTREC/10^5cells / (DJ1TRECs/10^5cells+DJ2TRECs/10^5cells), was calculated as described[25].

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T cell loss and recovery in HIV/AIDS

Publication:

Low CD4 T cell counts despite low viremia: insights from the comparison of HIV-1 infected patients with discordant response to antiretroviral therapy and untreated advanced HIV-2 disease.

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Abstract

A significant proportion of HIV-1+ patients with suppression of viremia under antiretroviral therapy fail to recover $CD4^+$ T-cell counts (ART-Discordants). Similarly, untreated HIV-2+ patients can also exhibit major CD4-depletion in spite of undetectable viremia. We characterize here the immunological disturbances associated with major CD4-lymphopenia in these two scenarios as compared to untreated viremic HIV1+ patients with similar CD4-lymphopenia and HIV1+ patients with successful immunological and virological responses under ART. Low CD4-counts were associated with major naïve CD4 and CD8 depletion, irrespective of type of infection or ART-exposure. However, ART-Discordants exhibited lower levels of T-cell activation as compared to both untreated HIV-2 and HIV-1 cohorts, and a less marked increase in circulating IL-7 despite similar CD4-depletion. Nevertheless, ART-Discordants showed a preserved Bcl-2 expression, suggesting increased IL-7 consumption, which in conjunction with the relatively lower T-cell activation may contribute to their CD4-count stability and low rate of opportunistic infections.

Introduction

Antiretroviral therapy (ART) is associated in 5 to 27% of HIV-1+ patients with failure to recover circulating $CD4^+$ T-cells despite apparently complete suppression of viral replication [1-3]. Low-level CD4 reconstitution under ART has been shown to be associated with older age and an advanced disease stage at the beginning of treatment [1-7]. Although clinical data are limited, these patients appear to have low rates of opportunistic infections [1, 8, 9].

HIV-2 infection is also associated with major CD4-depletion despite low to undetectable viremia and a favourable outcome with limited impact on the survival of the majority of infected adults [10]. As in HIV-1 infection, CD4-counts decline progressively, but at a much slower rate [11, 12]. HIV-2+ patients maintain low viremia in the absence of ART irrespective of the degree of CD4-depletion [13-18], in agreement with the reduced frequency of successful virus isolation from the peripheral blood [19] and the reduced rates of both horizontal and vertical transmissions [20, 21]. Of note, HIV-2 immunodeficiency has been shown to be associated with the same clinical spectrum as HIV-1 disease [12]. Given the past connections of Portugal with West Africa where HIV-2 infection is endemic, HIV-2 infection has reached a significant prevalence in the autochthon Portuguese population, a situation that is unique within the countries with access to all antiretroviral drugs [15]. Therefore, we were able to study a Portuguese cohort of untreated HIV-2 patients that exhibit low CD4 counts and reduced viremia like the HIV-1+ patients with discordant response to ART.

CD4 lymphopenia is known to have significant impact per se upon the immune system. Therefore, the comparison of discordant patients with cohorts with similar degrees of CD4 depletion may add significantly to the previous studies that were essentially based on the comparison with patients with good immunological ART responses as well as seronegative individuals.

Here, we compared HIV-1+ patients with discordant responses to ART with HIV-2+ patients that exhibited a similar degree of CD4-depletion and reduced circulating virus in the absence of ART, with respect to fundamental determinants of HIV-associated immunodeficiency namely, loss of naïve CD4 and CD8 T-cells, immune activation, IL-7Rα expression and circulating IL-7 levels. Untreated HIV-1+ patients with similarly low CD4-counts who are expected to have high viremia, as well as HIV-1+ patients under ART with successful virological and immunological responses were studied in parallel, in order to evaluate the relative contributions of viremia, CD4-depletion and ART in relation to these parameters.

Patients and Methods

Study population

A cross-sectional study was performed involving 65 HIV-1+ and 18 HIV-2+ patients with no evidence of ongoing opportunistic infections or tumours, currently living in Portugal and attending outpatient's clinics in Lisbon. The following cohorts were studied: a) HIV-1+ patients with discordant responses to ART (ART-Discordants): defined as viremia below 50 RNA copies/ml and less than 300 $CD4^+$ cells/μl after at least one year of triple ART; b) HIV-1+ patients with virological and immunological responses to ART (ART-Responders) defined as viremia below 50 copies/ml, and more than 100 $CD4^+$ cells/μl increase reaching levels above 300 cells/μl; c) ART-naïve HIV-1+ patients with less than 300 $CD4^+$ cells/μl (Untreated HIV-1); d) ART-naïve HIV-2+ patients with less than 300 $CD4^+$ cells/μl (Untreated HIV-2); and, finally, e) seronegative age-matched controls. Their epidemiological and clinical features are detailed in Tables 1 and 2. The study was approved by the Ethical Board of the Faculty of Medicine of Lisbon.

Table 1: Clinical and epidemiological features of the cohorts

	Healthy Controls	ART Responders	ART Discordants	Untreated HIV-1 CD4<300	Untreated HIV-2 CD4<300
Number (males/females)	16 (7/9)	11 (8/3)	11 (5/6)	27 (15/12)	18 (7/11)
Age, years	46±4 (26-84)	47±4 (35-79)	52±5 (32-77)	42±2 (21-63)	48±3 (21-63)
Race					
White	13	10	10	15	7
Other	3	1	1	12	11
HIV transmission category					
Heterosexual	n.a.	7	5	20	15
Homosexual/bisexual	n.a.	4	4	3	1
Injection drug user	n.a.	0	2	3	0
Blood transfusion	n.a.	0	0	0	2
Unknown	n.a.	0	0	1	0
Lymphocyte count ($x10^6$ cells/l)	2327±174 (1323-3381)	2162±183 (1180-3140)	1665±210 (847-2880)	1647±213 (294-5913)	1214±110 (533-2080)
CD4 count, cells/μL	1050±105 (567-2163)	630±57 (398-981)	206±25 (20-300)	161±21 (1-300)	182±22 (28-292)
CD8 count, cells/μL	594±76 (234-1236)	722±71 (320-999)	775±144 (94-1605)	730±95 (32-2210)	646±92 (126-1456)
Viremia, RNA copies/ml	n.a.	<50	<50	287489±119625 (1915-3072371)	518±300[a] (200-3607)
Proviral DNA[b], copies/10^6 PBMC	n.a.	185±105 (5-1161)	1002±334 (92-3210)	773±291 (5-3695)	2264±1415 (5-8895)

NOTE: Data are mean±SEM (range) unless indicated otherwise. ART, highly active antiretroviral therapy. n.a., not applicable.

[a] HIV-2 viral load was quantified in 16 HIV-2 patients and 13 had viremia below 200 RNA copies/ml. In these cases the cut-off value of the test was considered for the calculation of the mean.

[b] The sensitivity of the HIV-1 and HIV-2 Proviral DNA real-time assays were 5 copies/10^6PBMC. Quantifications were below this value in 6/11 ART-Responder, 1/14 Untreated HIV-1, and 2/7 Untreated HIV-2 patients tested and in these cases it was considered for the calculation of the mean. Proviral DNA was quantified in 10/11 ART-Discordants.

Table 2: Characterization of the HIV-1 Cohorts under ART

	ART Responders	ART Discordants
Length of HIV-1 diagnosis, months	118±17 (65-217)	82±15 (18-148)
CD4 T-cells/μL at the beginning of ART	222±53[a]	111±29
Plasma HIV-1 RNA copies/ml at the beginning of	235276±83446[b]	193234±81638
Length of ART, months	68±7 (18-98)	45±6 (16-77)
Drugs used:		
NRTI, n	1	2
NRTI + PI, n	6	6
NRTI + NNRTI, n	4	3
ZDV containing, n	5	6
Mean CD4 count increase, cells/μL	400±57	95±30
Length of previous double or mono therapy,	3 (78±28)	3 (38±17)
Positive HCV antibody, n	1	1[c]
Positive HBs Ag, n	0	0[c]

NOTE: Data are presented as mean±SEM with limits in brackets. ART, highly active antiretroviral therapy; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; ZDV, zidovudine; HBV, hepatitis B virus; HCV, hepatitis C virus.
[a] Data from 10 ART-Responder patients.
[b] Data from 9 ART-Responder patients.
[c] HCV antibody and HBs Ag were unknown in 2/11 ART-Discordant patients.

Cell preparation and flow cytometric studies

Freshly isolated peripheral blood mononuclear cells (PBMC) were stained and analysed for cytokine production at the single cell level using a FACSCalibur flow cytometer and Cellquest software (BD Biosciences, San Jose, CA) as described [10, 22]. Briefly, PBMC were resuspended in PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO) and 0.1% sodium azide (AZ; Sigma-Aldrich), stained for 20 min with monoclonal antibodies at room temperature, washed three times with PBS/BSA/AZ and fixed with 1% formaldehyde. Intracellular staining of Bcl-2 was performed after fixation of PBMC with 2% formaldehyde and permeabilization with

PBS containing 1% bovine serum albumin and 0.5% saponin (Sigma-Aldrich). Cytokine production was assessed at the single-cell level after short-term culture of PBMC with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml; Sigma-Aldrich) plus ionomycin (500 ng/ml; Calbiochem, Merck Biosciences, Nottingham, UK) in the presence of brefeldin A (10 μg/ml; Sigma). After fixation with 2% formaldehyde, PBMC were surface-stained, permeabilized and intracellular stained as described above. Absolute numbers of lymphocyte subsets were found by multiplying their representation by the absolute lymphocyte count obtained at the clinical laboratory.

IL-7 quantification

Serum IL-7 levels were measured using Quantikine HS ELISA kit (R&D Systems, Minneapolis, MN), as previously reported [23].

Proviral load quantification

HIV-2 and HIV-1 cell-associated proviral load was quantified by real-time PCR, as described [17]. Briefly, genomic DNA was extracted from 10^6 PBMC using an AbiPrism 6100 Nucleic Acid Extractor (Applied Biosystems, Foster City, CA) and quantified using a NanoDrop ND-10 spectrophotometer (NanoDrop technologies, Wilmington, DE). Standard curves were generated by serially diluting plasmids containing HIV-2 gag, HIV-1 gag (10^6 copies-5 copies), or albumin (10^6-10 copies) sequences. Samples were run in duplicate and the input level of DNA was normalized relative to the albumin copy number. Data were expressed as number of HIV DNA copies per 10^6 PBMC.

Plasma viral load assessment

HIV-1 viremia was quantified by RT-PCR (Ultrasensitive Test; detection threshold: 50 RNA copies/ml; Roche, Branchburg, NJ) and HIV-2 viremia by a RT-PCR assay (detection threshold: 200 RNA copies/ml) [15].

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA). Data are presented as arithmetic mean±SEM, assessed for distribution and compared using variance analysis ANOVA or Kruskal-Wallis test, and unpaired *t* test or Mann-Whitney test, as well as Pearson's coefficient. $p<0.05$ was considered significant.

Results

Naïve/memory/effector T-cell subsets

Similar levels of lymphopenia were observed in the cohorts with less than 300 $CD4^+$ T-cells/μl, irrespective of plasma viral load, type of infection or ART-exposure (Table 1). Regarding the CD4 subset, a similar decrease in the proportion of naïve cells, was documented in ART-Discordants and untreated HIV-1+ or HIV-2+ patients (Fig. 1A). An equivalent expansion of the $CD8^+$ T-cell subset accompanied by a major reduction of the proportion of naïve $CD8^+$ T-cells were also observed in these cohorts (Table 1 and Fig. 1B).

ART-Discordants, as previously described for HIV-2+ patients [22], had a less marked expansion of cells with an intermediate-stage of differentiation, as defined by

the expression of CD27 in the absence of CD45RA, than the untreated HIV-1 cohort (Fig. 1B), suggesting, as others have proposed, a failure to fully differentiate $CD8^+$ T-cells in viremic patients [24]. Moreover, the untreated HIV-1 patients also demonstrated a diminished frequency of IL-2 producing $CD8^+$ T-cells that is apparently recovered in ART-Discordants (19.0±3.2% IL-2^+ cells within the CD8 subset for ART-Discordants, p= 0.3220, 10.6±1.4% for Untreated HIV-1, p<0.0001, in comparison with 23.3±2.7% for healthy controls). As shown in Table 1, the high levels of viremia observed in the untreated HIV-1 cohort contrast with the low to undetectable ones found in untreated HIV-2+ patients (maximum: 3607 RNA copies/ml), but similar levels of proviral DNA were observed in the two infections, as previously reported [14, 17, 18, 25, 26]. Worth noting, there were no statistical differences between the cell-associated viral burden for all the cohorts, which could be due to the slow kinetics of proviral DNA decline under ART [7, 27].

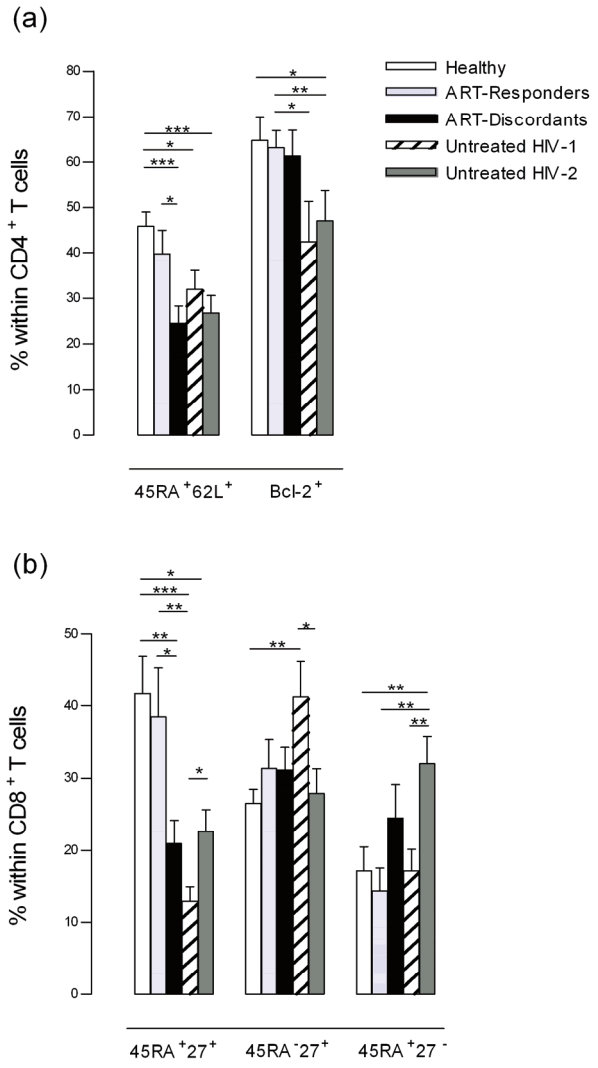

Figure 1: T-cell differentiation. Comparison of treated HIV-1 patients without significant CD4 recovery despite undetectable viremia (ART-Discordants), with therapy-naive HIV-2+ (Untreated HIV-2) and HIV-1+ (Untreated HIV-1) patients with the same degree of CD4 depletion, as well as with HIV-1+ patients with successful immunological response under ART (ART-Responders), and healthy controls with respect to: (a) Frequency of naïve ($CD45RA^+CD62L^+$) and $Bcl\text{-}2^+$ cells within the $CD4^+$ subset; (b) $CD8^+$ T-cell differentiation - proportion of the following populations within $CD8^+$ T-cells: $CD45RA^+CD27^+$, $CD45RA^-CD27^+$ and $CD45RA^+CD27^-$. The numbers of untreated HIV-1 and HIV-2 patients investigated were respectively: 25 and 18 for naïve CD4 T-cells; 6 and 8 for Bcl-2+; and 9 and 10 for CD8+ T-cell subsets. These groups are representative of the respective cohorts in terms of the data showed in Table 1. Bars represent mean±SEM. Analysis of variance was significant (1-way ANOVA) and p values of two-group comparisons (t test) are shown as follows: * $p<0.05$; ** $p<0.01$ and *** $p<0.001$.

Circulating IL-7 levels and expression of the IL-7Rα

IL-7 is a non-redundant cytokine with critical roles in thymopoiesis and maintenance of the peripheral T-cell pools [28]. An inverse correlation between circulating IL-7 and CD4-counts has been shown in untreated HIV-1 and HIV-2 infections [23, 29, 30], as well as in other CD4-lymphopenic clinical settings [28]. As shown in Fig. 2, serum IL-7 levels were significantly increased in ART-Discordants as compared to healthy subjects and ART-Responders. However, the increase observed in ART-Discordants was significantly lower than that found in untreated HIV-1+ and HIV-2+ patients.

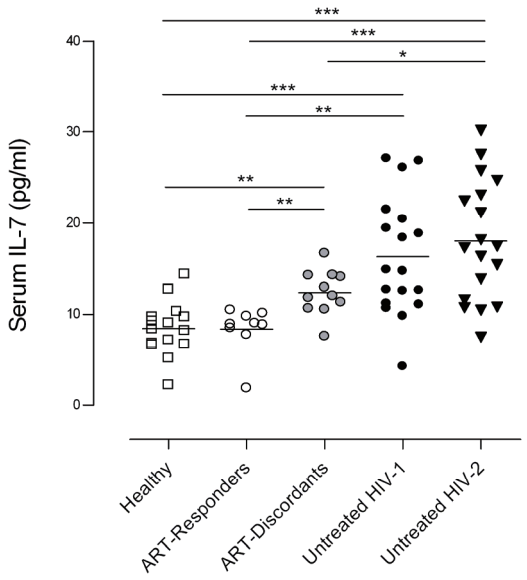

Figure 2: Circulating IL-7 levels. Comparison of serum IL-7 levels as quantified by ELISA, in treated HIV-1 patients without significant CD4 recovery despite undetectable viremia (ART-Discordants), with therapy-naive HIV-2+ (Untreated HIV-2) and HIV-1+ (Untreated HIV-1) patients with the same degree of CD4 depletion, as well as with HIV-1+ patients with successful immunological response under ART (ART-Responders), and healthy controls. Each dot represents one individual, bars represent means. Analysis of variance was significant (1-way ANOVA) and p values of two-group comparisons (t test) are shown as follows: * $p<0.05$; ** $p<0.01$ and *** $p<0.001$.

Since IL-7 effects are modulated by its receptor expression on T-cells [31], we evaluated IL-7Rα levels on naïve and memory/effector $CD4^+$ and $CD8^+$ T-cell subsets (Fig. 3). The frequency of IL-$7R\alpha^+$ cells was decreased in ART-Discordant as compared to ART-Responder and healthy cohorts within both the naïve and the memory $CD4^+$ subsets, as well as within the naïve $CD8^+$ subset. Progressive differentiation of $CD8^+$ T-cells, as assessed by CD45RA and CD27 expression, was associated with reduced levels of IL-$7R\alpha^+$ expression in all cohorts, as expected [23]. ART-Discordants exhibited a lower level, but the significance of the differences became progressively weaker as compared to the healthy cohort and lost significance as compared to ART-Responders. These data are in agreement with previous reports [2]. Of note, the levels documented in ART-Discordant patients do not differ significantly from the ones observed in the limited number of untreated HIV-1 and HIV-2 patients that we were able to assess for IL-7Rα, except for the $CD8^+CD45RA^-CD27^+$ subset (Fig. 3).

IL-7 mediates its effects in part by the up-regulation of the anti-apoptotic protein Bcl-2 [32]. When we analysed intracellular Bcl-2 expression, the ART-Discordants exhibited levels similar to ART-Responders as well as to healthy subjects, in contrast to the reduced levels documented in untreated HIV-1 and HIV-2 patients in spite of the higher circulating IL-7 levels (Fig. 1A).

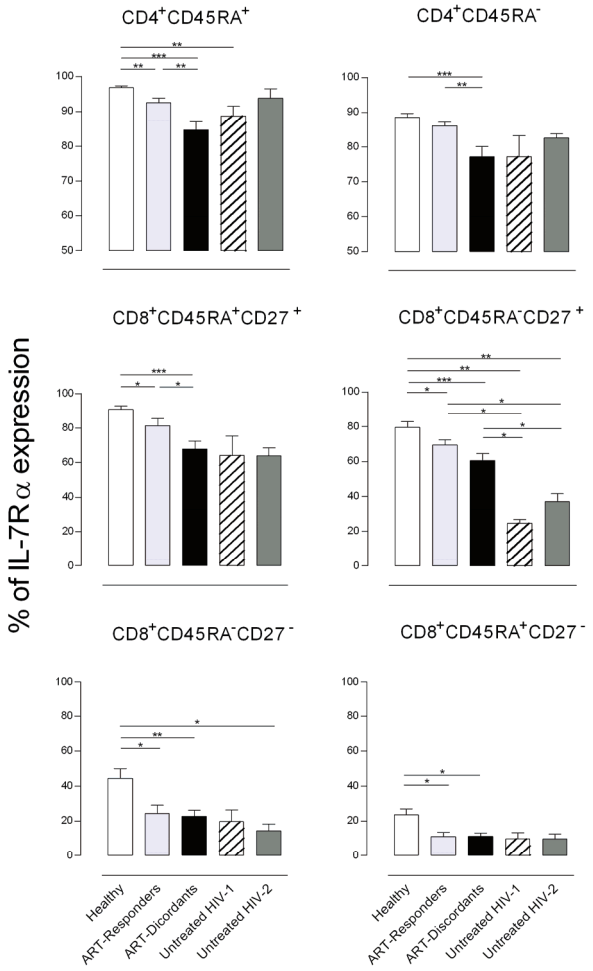

Figure 3: IL-7Rα expression. Percentage of IL-7Rα+ cells within the CD4+ naïve (CD4+CD45RA+) and memory (CD4+CD45RA-) subsets, as well as within CD8+ T-cell subpopulations defined according to their degree of differentiation as assessed by CD45RA and CD27 expression (CD8+CD45RA+CD27+, CD8+CD45RA-CD27+, CD8+CD45RA-CD27- and CD8+CD45RA+CD27-), as described at the top of each graph. Analysis was performed on freshly isolated PBMC. Treated HIV-1 patients without significant CD4 recovery despite undetectable viremia (ART-Discordants) were compared with HIV-1+ patients with successful immunological response under ART (ART-Responders), and healthy controls, as well as with a subgroup of the therapy-naive HIV-2+ (Untreated HIV-2, n=3) and HIV-1+ (Untreated HIV-1, n=3) cohorts. Bars represent mean±SEM. Analysis of variance was significant (Kruskal-Wallis test) and *p* values of two-group comparisons (Mann-Whitney test) are shown as follows: * $p<0.05$; ** $p<0.01$ and *** $p<0.001$.

CD4⁺ and CD8⁺ T-cell activation

There is now increasing evidence that persistent immune activation is directly related to CD4-depletion in HIV/AIDS and to reduced CD4-gains under ART [7, 10, 33-36].

We found a significant increase in the proportion of $CD4^+$ T-cells expressing the MHC class II molecule, HLA-DR, in ART-Discordants compared to ART-Responders and to controls (Fig. 4a). However, ART-Discordants exhibited significantly lower levels than untreated HIV-1 or HIV-2 patients. Of note, both the proportion of $CD8^+$ T-cells that express CD38 and its mean intensity of fluorescence, which has been shown to be an independent prognostic factor for disease progression [37], were increased in untreated HIV-1 and HIV-2 cohorts, whereas ART-Discordants had levels similar to that seen in ART-Responders and healthy controls (Fig. 4b and 4c). Moreover, the ART-Discordants exhibit a trend to a higher proportion $CD8^+$ T-cells that express HLA-DR in the absence of CD38, a population that has been associated with a favourable prognosis in HIV-1 infection [38], as shown in Fig. 4b.

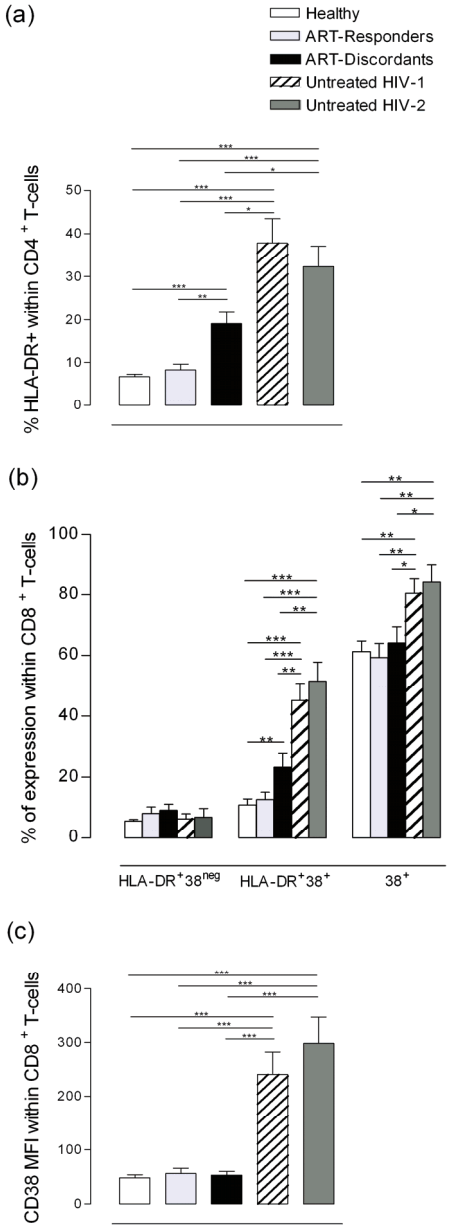

Figure 4: T-cell activation. T-cell activation was compared in treated HIV-1 patients without significant CD4 recovery despite undetectable viremia (ART-Discordants), with therapy-naive HIV-2+ (Untreated HIV-2, n=8) and HIV-1+ (Untreated HIV-1, n=15) patients with the same degree of CD4 depletion, as well as with HIV-1+ patients with successful immunological response under ART (ART-Responders), and healthy controls. The phenotypic studies were performed in freshly isolated PBMC by flow cytometry. (a) Proportion of $CD4^+$ T-cells that express HLA-DR. (b) Frequency of $HLA\text{-}DR^+CD38^{neg}$, $HLA\text{-}DR^+CD38^+$ as well as total $CD38^+$ cells within the $CD8^+$ T-cell subset. (c) Mean fluorescence intensity (MFI) of CD38 expression within $CD8^+$ T-cells. The Untreated HIV-2 and HIV-1 groups are representative of the respective cohorts in terms of the data showed in Table 1. Bars represent mean±SEM. In the cases where analysis of variance was significant (1-way ANOVA), the p values of two-group comparisons (t test) are shown as follows: * $p<0.05$; ** $p<0.01$ and *** $p<0.001$.

Discussion

The study of a natural "attenuated" form of HIV disease, HIV-2, in parallel with ART induced aviremic HIV-1 infection represents a novel approach to gain insights into the mechanisms of CD4 depletion and recovery in HIV/AIDS.

Correlations between the degree of CD4-depletion and the loss of both naïve $CD4^+$ and $CD8^+$ T-cells have been reported in HIV-1 infection by Roederer et al., and we have reported similar findings in HIV-2 disease [10, 39]. The observation of comparative imbalances in naïve T-cell compartments in ART-Discordants and untreated HIV-1+ and HIV-2+ patients strengthens the possibility that a compromise in naïve T-cell production or survival ultimately determines $CD4^+$ T-cell counts.

Although a similar degree of expansion of the $CD8^+$ T-cell subset was observed in the three cohorts with major CD4-depletion, the analysis of the patterns of $CD8^+$ differentiation suggest that viremia may impair achievement of a fully-differentiated effector phenotype, in agreement with previous reports [24].

IL-7 is a key cytokine for T-cell development and homeostasis. The increase in circulating IL-7 in lymphopenic clinical settings has been viewed as an increase in production to enable enhanced thymopoiesis and/or peripheral T-cell survival [29]. We found, as expected, augmented IL-7 levels for all cohorts with CD4-depletion. However, ART-Discordants had a significantly lower increase than untreated HIV-1+ and HIV-2+ patients, suggesting a defective compensatory response that could limit

immune reconstitution. Previous reports of circulating IL-7 quantification in treated HIV-1+ patients without CD4 recovery generated conflicting data due to variations in patient selection or in the definition of immunological failure [1, 2, 40, 41]. Although, IL-7 was reported to be increased in ART-Discordants as compared to seronegatives in the majority of the studies, some reports did not find statistically significant differences in comparison with patients with good immunological plus virological response under ART [1, 40].

Rather than a deficit in IL-7 production, the reduced IL-7 levels that we observed in ART-Discordants may reflect higher IL-7 consumption. This is in agreement with the hypothesis suggested by Fry and Mackall as well as other authors, that explains the increase in circulating IL-7 in lymphopenic settings as a result of diminished usage due to the reduction of cellular targets [23, 28, 31]. The high Bcl-2 levels documented in ART-Discordants would fit this alternative interpretation, suggesting a maintained IL-7 consumption that could account for the reduced circulating IL-7 levels in this cohort as compared to the untreated HIV-2 and HIV-1 cohorts with similar CD4 depletion. Our data is in agreement with previous reports showing over-expression of Bcl-2 within the CD4 subset in ART-discordant cohorts [40]. The preserved Bcl-2 levels within the CD4 subset and the possible maintained ability to use IL-7 may contribute to the sustained CD4-counts and low rate of opportunistic infections that is usually observed in HIV-1+ ART-Discordant patients [1].

Persistent generalized immune activation is a main determinant of the low CD4 T-cell counts in HIV infection and of its recovery under ART [7, 10, 33-36]. Of note, although $CD4^+$ and $CD8^+$ T-cell activation was increased in ART-Discordants, it was significantly lower than that observed in both untreated HIV-1 and HIV-2 cohorts.

Several factors could contribute to this observation, namely, re-setting of the levels of activation through the rapid reduction of circulating virus during the first weeks of ART [36, 42], a direct effect of the antiretroviral drugs *per se* [43], or differences in the amount of ongoing viral replication. The latter case would imply unexpectedly high viral replication rates in the untreated HIV-2+ patients despite low level viremia, a possibility that remains so far unsupported [18].

Most studies addressing mechanisms involved in low CD4 recovery under ART in patients with an effective suppression of viremia compare these individuals with full ART-Responders and healthy controls. However, given the likely impact of lymphopenia per se upon the immune system, it is important to evaluate the homeostatic responses in this context through the comparison with cohorts of patients with similar degrees of CD4 depletion. In this respect, our study generates unique data through the inclusion of an HIV-2 cohort that shares some features of the ART-Discordants namely CD4 depletion in the absence of viremia.

In summary, we found a similar loss of CD4 and CD8 naïve T-cells in patients with low CD4 counts irrespective of type of infection, viremia or ART-exposure. However, in spite of similar levels of CD4-depletion and absence of viremia, HIV-1+ patients with discordant responses to ART exhibited lower circulating IL-7 in association with preserved Bcl-2 expression as well as lower T-cell activation levels when compared to advanced untreated HIV-2 infected patients, suggesting distinct immunological disturbances associated with major CD4-lymphopenia in these two scenarios. Longitudinal studies will be important to address their relevance, but it is reasonable to speculate that the relatively lower immune activation in conjunction with an

apparent better ability to use IL-7 may play a role in the stability of CD4 counts and the low rate of opportunistic infections that are features of ART-Discordants.

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An AIDS-like immunological picture in a primary defect of the phagocytic oxidative burst

Publication:

An AIDS-like immunological profile in a phagocytic immunodeficiency: Chronic Granulomatous Disease

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Abstract

Chronic Granulomatous Disease (CGD) results from primary defects in phagocytic oxidative-capacity. A 32-year-old X-linked CGD patient, with typical infections, also presented long-lasting CD4-lymphopenia, associated with persistent immune-activation and impaired T-cell production. The occurrence of immunological-exhaustion is clinically relevant given increasing CGD life-expectancy and the ongoing debate regarding early aggressive intervention.

Introduction

Chronic Granulomatous Disease (CGD) results from genetic defects in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase leading to an impaired oxidative capacity of monocytes and neutrophils [1]. CGD represents the most frequent phagocytic defect, with an estimated prevalence of 1:200000 live births [1]. Several mutations in the four genes coding for NADPH oxidase subunits have been reported, but the most frequent, accounting for approximately 65% of cases, occur in the *CYBB* (gp91^{phox}) gene on the X chromosome (X-linked), while the remainder are autossomal recessive mutations. The generation of reactive oxygen species (ROS), namely superoxide anion, hydroxyl radical and hydrogen peroxide, by NADPH oxidase is essential for the efficient clearance of microorganisms. Thus, CGD patients typically present with prolonged infections, usually due to fungi, or catalase-positive bacteria that are able to further degrade the little hydrogen peroxide produced. In accordance with its name, CGD is also associated with granuloma and chronic inflammation although the mechanisms involved remain poorly defined [1], as well as with an increased incidence of autoimmune manifestations in patients and relatives [2].

Although CGD is a primary phagocytic defect, a decreased frequency of memory B cells [3] and diminished T-cell numbers have been reported in a large American CGD cohort [4] [5]. However, these studies did not include phenotypic and functional T-cell analysis. We report here a clinical case of a 32-year-old patient with X-linked CGD with persistent CD4 lymphopenia below 200cells/µl for more than 16 years. We investigated here the pathways involved in T cell production and peripheral homeostasis, given the clinical relevance of a possible immunological exhaustion in

view of the increased life-expectancy of CGD patients. The immunological disturbances we described are remarkably similar to the ones found in untreated HIV-1 infected patients, with a similar degree of CD4 depletion, studied in parallel.

Case report

A 32-year-old male with X-linked CGD (mutation at the extreme end of exon 8 of *CYBB* gene causing a G-897 to A change resulting in mRNA missplicing) presented with sustained depletion of CD4 T-cells with levels below 200cells/μl, since 15-year-old (Figure 1A). At the time of the CGD diagnosis, at the age of 13, a significant T-cell depletion already existed (48% T-cells), mainly related to CD4+ T-cells (13%, 338cells/μl). It is important to stress the delay in CGD diagnosis, which would have been easily achieved by an oxidative burst test. In fact, this patient had a past history typical of CGD, namely: neonatal *Staphylococcus aureus* sepsis; *Salmonella enteritidis* sepsis at 7-year-old; multiple liver abscesses due to *Staphylococcus aureus* requiring partial hepatectomy at 10-year-old; and persistent axilar and cervical suppurative lymphadenitis and subcutaneous abscesses. Following CGD diagnosis, prophylactic cotrimoxazole and itraconazole were prescribed. No major infections have been reported since, except *Pseudomonas aeruginosa* keratoconjunctivitis at 16-year-old. A tendency for clinical improvement with aging has been previously reported in CGD patients [1], and this has been linked with the ongoing development of acquired immunity [1]. However, it is notable that the patient presented a long-lasting CD4 T-cell lymphopenia at levels usually associated with major opportunistic infections in HIV infected patients [6] and in patients with idiopathic CD4 lymphopenia [7]. HIV-1, HIV-2, HTLV-1 and HTLV-2 infections were excluded by PCR-based assays.

As a strategy to better understand the mechanisms involved, we combined the longitudinal study of the patient with parallel evaluation of untreated HIV-1+ patients with similar levels of CD4 depletion (mean CD4 counts=73±23 cells/μl; n=9) and healthy subjects (mean CD4 counts=933±53 cells/μl; n=25). All subjects gave written informed consent under the approval of Ethical Committee of the Faculdade de Medicina da Universidade de Lisboa. The CGD patient, similarly to untreated HIV-1+ patients, showed: marked loss of naive CD4 (Figure 1B) and CD8 (Figure 1C) T-cells; an inverse CD4/CD8 ratio (0.17, at 30-year-old), with expansion of terminally differentiated effector CD8 T-cells illustrated by the marked high levels of production of IFNγ and perforin (Figure 1C-D); increased levels of expression of activation markers, exemplified by HLA-DR and CD38, within both the CD4 (Figure 1E) and CD8 (Figure 1C) subsets; as well as reduced *in vitro* lymphoproliferative responses to mitogens and recall antigens, revealing impaired T-cell function (Figure 1F). The pro-inflammatory cytokine IL-17 has been shown to have a role on the pathogenesis of chronic inflammatory and granulomatous disorders as well as in a mouse model of CGD [8]. Likewise, an increased frequency of CD4 T-cells producing IL-17 was observed in the CGD patient (Figure 1F). The control of the immunopathology associated with these hyper-activated states is thought to be in part achieved by a population of regulatory CD4 T-cells with suppressive properties (Treg), best identified by the expression of high levels of the α-chain of the IL-2 receptor, CD25, and the fork-head transcription factor FoxP3. The CGD patient had preserved circulating Treg levels (Figure 1F). The past-replicative history of cells can be estimated by telomere length. These repeat regions at the chromosome's ends are progressively lost upon cell-division [9]. Both CD4 and CD8 T cells showed reduced telomere length (Figure 1G), as assessed by flowFISH [9], further supporting persistent immune

stimulation and increased cell turnover. The low telomere length is also in agreement with an impaired replenishment of the T-cell pool by recently produced cells. Indeed we found evidence of impaired thymopoiesis based on the quantification of products of T-cell receptor (TCR) rearrangements generated during thymic T-cell development (sj and β TCR rearrangement circles, TREC) that progressively decline as the thymus involutes with aging [10]. The CGD patient showed a remarkable reduction of the sj/βTREC ratio for his age (Figure1H). This ratio is considered to estimate intrathymic precursor T-cell proliferation and to directly correlate with thymic output [10]. Thymic activity declined in spite of the levels of IL-7, a crucial cytokine for T-cell development and homeostasis [11, 12], being markedly enhanced (Figure1I). An inverse correlation between circulating IL-7 levels and CD4 T cell counts has been reported in lymphopenic clinical settings [11-13] . The CGD patient has circulating IL-7 levels much above those observed in untreated HIV-1 infection (Figure 1), measured by ELISA, as we have previously reported [11]. In agreement with existing data [3], a reduced proportion of memory cells (6%; CD27+) within B lymphocytes was observed. Overall, this CGD patient presented B-cell and T-cell disturbances usually associated with AIDS progression in HIV-1+ individuals. Nevertheless, the patient's clinical profile featured no major opportunistic infections in the last 20 years, just episodic suppurative lymphadenitis and pustulous acne.

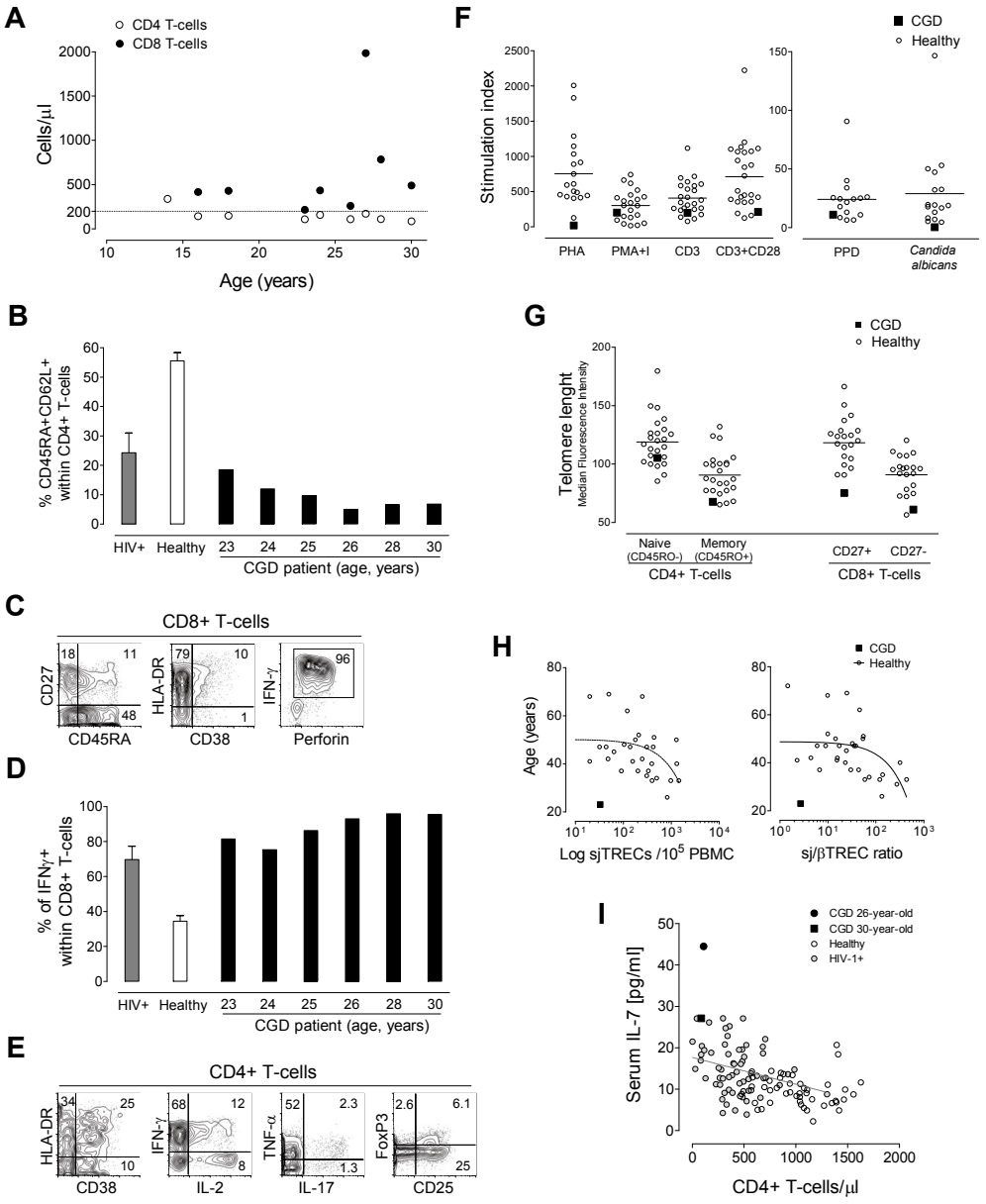

Figure 1. Immunological profile. (A) CGD patient longitudinal CD4 and CD8 T-cell absolute counts. (B) Frequency of naive cells within the CD4 subset in the CGD patient at different ages and in HIV-1+ patients with less than 200 CD4 cells/µl (n=9) and healthy subjects (n=25). (C) Expression of T-cell differentiation and activation markers, as well as of intracellular perforin and IFNγ within the CD8 subset of the CGD patient at 30-year-old. (D) Frequency of IFNγ-producing cells within the CD8 subset in the subjects described in (B). (E) Expression of T-cell differentiation and activation markers, as well as of intracellular IL-2, IFNγ, TNFα, IL-17 and FoxP3 within the CD4 subset of the CGD patient at 30-year-old. (continue, next page)

Discussion

We report here a case of a patient with X-linked CGD presenting infections typically linked to this NADPH oxidase defect, in association with a sustained long-term CD4 T cell lymphopenia and an AIDS-like immunological profile.

Reduced T cell numbers have been previously reported as a feature of CGD [4, 5]. It is expected that the number of CGD patients with severe CD4 lymphopenia will increase given the impact on their life-expectancy of the currently available therapies. Therefore, much effort should be devoted to the understanding of the mechanisms underlying this process and their clinical implications.

The most striking immunological alterations relate to the inflammatory and hyper-activated state. Of note, persistent immune activation is currently considered a major driving force of the CD4 decline in HIV/AIDS [6].

Figure 1 (continued)
(F) Peripheral blood mononuclear cells (PBMC) proliferation to mitogens (PHA 20 μg/ml, anti-CD3 1μg/ml, anti-CD3 1μg/ml+anti-CD28 1 μg/ml, and PMA 50 ng/ml + I 500 ng/ml) and antigens (*Candida albicans* 40 μg/ml and Purified Protein Derivative, PPD 5 μg/ml) were assessed by ^{3}H-Thy incorporation, as previously described [6]. Results are expressed as stimulation indexes (ratio of mean counts per minute (cpm) of replicates in the presence of a given stimulus over mean cpm in its absence). (G) Telomere length of gated CD4 and CD8 subpopulations from the CGD patient at 30-year-old and healthy subjects, estimated by flowFISH. (H) sjTREC levels and sj/βTREC ratio from PBMC of the CGD patient at 23-year-old and healthy subjects assessed by nested PCR, as previously reported [10], in relation to age. (I) Serum IL-7 levels assessed by ELISA, in the CGD patient, HIV-1+ patients and healthy subjects in relation to CD4 count. Each symbol represents one individual. Bars represent mean±SEM. Flow cytometric analysis was performed after acquisition of at least 100,000 events using a FACSCalibur. Cytokine production at the single-cell level was assessed after short-term culture of PBMC with PMA plus ionomycin in the presence of brefeldin A, as previously described [12].

In CGD mouse models, inflammation has been linked to a defective production of the immunossupressive molecule L-kynurenine [8], an intermediate of tryptophan catabolism generated by the enzyme indoleamine 2,3-dioxygenase (IDO), as this reaction requires superoxide that is limited by the defective NADPH oxidase function. Although, this represents an attractive hypothesis in view of the key regulatory role of IDO in the immune system [14], recent data revealed that CGD patients had no alterations in the IDO pathway [15].

The high frequency of CD4 T-cells producing the pro-inflammatory cytokine IL-17 may contribute to the hyper-activated state [8], though, given its putative role in controlling fungal infections, it may also occur in order to counter-act the increased susceptibility to fungal infections observed in CGD patients [1; 16].

The levels of circulating IL-7 are much higher than those found in individuals with similar levels of T-cell depletion [11, 13]. This lead us to speculate that they may be in part related to an increased liver production of IL-7 as an acute-phase response due to persistent microbial products, as has been recently reported upon TLR-signalling in mouse models [17]. The massive IL-7 levels may contribute to the exaggerated immune stimulation. Nevertheless, impaired thymopoiesis and a reduced replenishment of the naive compartment with *de novo* produced T-cells also seem to significantly contribute to the T-cell disturbances.

Remarkably, the patient's immunological alterations also resemble the profile of immune-senescence documented in aged individuals, as well as in patients that have been thymectomized early in life as part of the surgical protocols for correction of congenital cardiac defects [18]. This profile has been considered of immunological risk of infections, and was shown to be an independent predictor of death in aged subjects

[18]. Therefore, it is worth considering this putative exhaustion of immune resources in the evaluation of long-term therapeutic strategies for CGD, including stem-cell transplantation [19].

In conclusion, we report here a case of long-lasting severe CD4 lymphopenia in CGD, probably due to persistent immune-activation and impaired T-cell production. Although the impaired adaptive immunity did not translate into major infections, its putative long-term implications favour an aggressive early therapeutic intervention in CGD.

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Conclusion and Future Perspectives

The maintenance and/or recovery of T lymphocytes are currently thought to depend on a combination of "peripheral mechanisms" and an age-dependent contribution of the thymus. We addressed the role of these mechanisms by studying discrete human clinical cohorts, all of which are associated with lymphopenia.

The *FOXN1* gene is expressed in thymic epithelial cells and has been shown to regulate the differentiation and proliferation of these cells (1-2). Mutations in the *FOXN1* gene lead to athymia. Nevertheless, we found significant numbers of circulating T cells in a Portuguese patient with a R255X mutation in the *FOXN1* gene. Notably, naïve T cells represented less than 1% of these cells.

The investigation of the phenotype of the T cells present in this individual revealed an expansion of circulating DNαβ T cells to proportions similar to those observed for the CD4 and CD8 subsets. In healthy individuals, DNαβT cells only represent a small subpopulation, usually less than 1% of the circulating lymphocytes.

In addition, we also found that up to 40% of the CD4 subset expressed high levels of FoxP3. Although FoxP3 expression can be up-regulated in non-Treg cells upon activation (3-4), our data suggest that these cells represent a Treg-like population. In particular, the co-expression of other Treg markers, such as CD25, CTLA-4 and CD39, in parallel with FoxP3 supports this hypothesis. Moreover, in contrast to activated T cells, they did not produce IL-2 or IFN-γ. Interestingly, alterations in the development

and function of Treg cells were recently reported in Omenn Syndrome, an immunodeficiency associated with thymic impairment due to hypomorphic mutations in hematopoietic precursors (5). A severe reduction of FoxP3+ cells was documented in the lymph nodes and thymus of these patients. However, the frequency of FoxP3+ cells in peripheral blood was within normal range in 3 patients and markedly increased in another patient. In all of these subjects, isolated CD4+CD25^{high} T cells failed to suppress proliferation of allogenic activated CD4 T cells (7). The disturbances in the Treg pool in this clinical setting were suggested to be related to abnormal TCR rearrangement and compensatory peripheral homeostatic proliferation (5).

Our findings raise important questions regarding T cell origin in the context of athymia. Althought it was reported that the R255X mutation at *FOXN1* gene would lead to a complete absence of functional protein (6), it is possible that technical constrains did not allowed the measurement of small amounts of FOXN1 expression. This hypothetic low level of expression of FOXN1 might allow the development of a thymic rudiment. Alternatively, a thymic rudiment could develop even in the absence of FOXN1 that support T cell differentiation, albeit with a skewed TCR repertoire, and altered T cell selection that generated the DNαβ and FoxP3+ subsets. Interestingly, the first two cases of *FOXN1* deficiency reported by Pignata *et al*. had the same R255X mutation and also presented with T cells in circulation (7). In contrast, no circulating T cells were found in a French patient presenting a different *FOXN1* mutation.

However, we cannot exclude the possibility that T cells were generated by extra-thymic lymphopoiesis. Significant numbers of FoxP3+ and DNαβ T cells were also found in a mouse model of extra-thymic lymphopoiesis induced by Oncostatin M. This cytokine induces thymic atrophy and drives alterations in the lymph nodes, rendering

them able to support T cell development similar to that normally observed in the thymus (8).

Since *FOXN1* deficiency is associated with defects in thymic epithelial cells rather than hematopoietic precursors, thymic transplantation was proposed, for the first time in this clinical setting, as a treatment strategy. Thymic transplantation, in this context, was associated with a progressive increase in the frequency of naïve T cells in parallel with increasing TREC levels. In addition, despite the HLA-mismatch between the thymic epithelia and the patient, we showed that the pool of naïve CD4 T cells had a fully diverse TCR repertoire. However, around 4 years after the transplant, we documented a marked decline in TREC levels accompanied by a decrease in the proportion of naïve cells that eventually stabilised. This finding may suggest the establishment of a steady-state equilibrium following the replenishment of the immune system. In this regard, we think that a PET-computed tomography scan would be interesting to perform in order to assess any remaining thymic activity in both the transplanted tissue and/or any putative thymic rudiment.

Following thymic transplantation, the FoxP3+ population recovered in parallel with the CD4 subset, reaching frequencies within the normal range. In contrast, the DNαβ T cells persisted throughout 5 years of post-transplant follow-up, at a fairly stable frequency. The maintenance of this population was observed, despite a 10 month post-transplant period of immunosuppression. The population of DNαβ T cells also maintained a similar pre-transplant memory phenotype. Moreover, they had a skewed TCR repertoire despite the lack of terminal-effector differentiation. We were able to show that they expressed high levels of CD25, CD127 and Bcl-2, and were also able to phosphorylate STAT-5 upon IL-2 or IL-7 stimulation. Moreover, there was no

evidence of significant *ex vivo* turnover or proliferative responses to either IL-2 or IL-7 which could suggest that this population may be maintained by these cytokines. In the future, the assessment of telomere length, as an indicator of this population's replicative history, could provide a better understanding of the processes underlying its persistence.

Importantly, after the transplant, the patient was able to mount adequate antigen-specific responses, and develop T cell memory. This is illustrated by the resolution of the Bacillus Calmette-Guérin (BCG) adenitis that had been present since the initial diagnosis, as a result of the BCG vaccination this individual received, that occurred in temporal association with the development of specific *in vitro* proliferative responses to PPD. Moreover, despite the low post-transplant CD8 recovery, a varicella infection was associated with increased frequency of terminally differentiated CD8 T cells and the development of *in vitro* specific proliferative responses to the varicella antigen. The child remains free of significant infections 3 years after having stopped all prophylactic therapies and is now attending school.

Although we did not assess the expression of the gene at the protein level, we can speculate that the *FOXN1* mutation in our patient could be associated with a certain degree of protein expression. In this regard, we think that the study of different *FOXN1* gene mutations' effects upon protein expression, and their associated phenotype in a mouse model could provide a greater understanding of the human *FOXN1* deficiency phenotype.

Importantly, our data provide evidence that despite the HLA-mismatch, thymic transplantation was associated with achievement of immunocompetence and futher

supporting its use as a strategy for immune reconstitution in specific clinical settings associated with thymic defects.

Although thymic activity dramatically decreases during adulthood, it is now clear that generation of new T cells in the human thymus continues throughout life. In HIV/AIDS infection, the thymus has been shown to have a role in the maintenance of T cells and the immune reconstitution that can occur under antiretroviral therapy (ART) (9-10). In addition, T cells are also maintained by a variety of peripheral homeostatic mechanisms such as the IL-7/IL-7R network. Notably, an inverse correlation between circulating IL-7 levels and CD4 T cell counts has been demonstrated in HIV infection (11). The increased circulating levels of IL-7 in this, and other lymphopenic clinical settings, have been suggested to result from increased production of this cytokine, aimed at enhancing thymopoiesis and/or peripheral T cell survival (11, 12).

On the other hand, chronic immune activation has also been suggested to play a major role in the CD4 depletion associated with HIV infection (13). We studied the contribution of both T cell activation and circulating IL-7 levels in HIV infected patients in the context of differing CD4 depletion, thymic impairment and viral load.

Effective ART is usually associated with a decline in plasma viral load in parallel with an increase in CD4 T cell count, allowing a gradual quantitative and qualitative recovery of the immune system (14, 16). However, around 5 to 27% of HIV-1 infected patients fail to recover circulating CD4 T cells despite apparently complete suppression of viral replication under ART (ART-Discordants) (15-17). Several mechanisms have been reported to be involved in low CD4 recovery under ART. An impairment in thymic function seems likely to contribute, at least in part, to the failure of these

patients to restore circulating CD4 T cell numbers after suppression of HIV-1 replication. This is supported by the decrease naïve CD4 T cells and low TREC levels (18, 19) observed in these individuals. Chronic T cell activation, and its association with high levels of apoptosis and increased T cell proliferation, has also been suggested to have a role in the low CD4 recovery in these patients (19, 20). Mavigner *et al.* showed that the levels of CD4 and CD8 activation, as assessed by HLA-DR and CD38 expression, were positively correlated with the residual viremia in poor immunological responders (21). These authors developed an ultrasensitive assay with a limit of detection of 2.5 copies/ml, able to measure residual viremia. Using this assay, they demonstrated that the plasma of poor immunological responders contained more residual HIV-1 RNA than that of good immunological responders, suggesting that ongoing low-level viral production that occurs despite ART, might also contribute to persistent immune activation, and thus impact upon the reconstitution of the CD4 T cell pool.

HIV-2 infection is also associated with a major CD4 depletion and causes AIDS in the majority of infected patients (22, 23). As in HIV-1 infection, CD4 counts decline progressively, but at a much slower rate (24, 25). HIV-2 infection is also characterized by low viremia in the absence of ART, irrespective of the degree of CD4 depletion (26), 30]. Thus, we thought to compare cohorts of patients with a similar degree of CD4 depletion and reduced circulating virus, namely ART-Discordants and untreated HIV-2 infected patients. We also studied in parallel, cohorts of untreated HIV-1 patients with similarly low CD4 counts, who are expected to have high viremia, and ART-treated HIV-1 patients with successful virological and immunological responses. The

combined study of the above cohorts allowed the evaluation of the relative impact of viremia, CD4 depletion and ART upon the process of immune reconstitution.

Specifically, we investigated whether T cell activation, and/or IL7, could play a role in the relatively stable CD4 T cells counts and low rate of opportunistic infections observed in ART-Discordants (17, 27, 31). In this respect, we noted that, although T cell activation was increased in ART-Discordants, it was significantly lower than that observed in both untreated HIV-1 and HIV-2 cohorts. Moreover, although increased IL-7 levels were found in all cohorts with CD4 lymphopenia, the ART-Discordants showed a less marked increase in comparison to the untreated HIV cohorts. The reduced IL-7 levels observed in ART-Discordants may reflect a deficit in IL-7 production or higher IL-7 consumption. One of the mechanisms by which IL-7 exerts its effects is via the up-regulation of Bcl-2, which, through its anti-apoptotic properties, is thought to increase cell survival (28). We found that ART-Discordants featured a preserved Bcl-2 expression which could suggest increased IL-7 consumption that, in conjunction with the relatively lower T cell activation, could contribute to the stability their CD4 count and low rate of opportunistic infections.

Finally, we investigated impairments in the T cell compartment of a patient with Chronic Granulomatous Disease (CGD) and marked CD4 lymphopenia. CGD is a gene defect of phagocytic cells that fail to generate a respiratory burst in response to stimulation, rendering patients highly susceptible to infections.

Besides having a defect in phagocytic cells, our patient also presented with a persistent (more than 15 years) CD4 depletion. The magnitude of reduction of his CD4 T cell pool is similar to that observed in untreated HIV-1 infected patients at an advanced stage of disease. Thus, we compared the long-term clinical impact and the

immunological disturbances associated with the CD4 depletion in this individual with those observed in untreated HIV-1 infected patients matched for the degree of CD4 depletion.

We found that the patient's CD4 lymphopenia was associated with a marked and persistent depletion of naïve T cells at levels comparable to those found in the untreated HIV-1 cohort. Moreover, sj/βTREC levels were also significantly decreased in the CGD patient suggesting an impairment of thymopoiesis in this individual. Also, as in the case of AIDS patients, the patient's T cells showed an effector-memory and activated phenotype. In particular, a high proportion of the CD8 lymphocytes were IFN-γ producing cells and expressed increased levels of perforin. This chronic immune activation, possibly related to the patient's past history of persistent infections, could contribute to the CD4 lymphopenia. The fact that we observed a decreased telomere length in T cells from the CGD patient further support an increased T cell turnover and the idea of an exhaustion of the immune system, due to persistent antigen challenge. Of note, a recent report suggested that impaired activation of the immunoregulatory indoleamine 2,3-dioxygenase (IDO) pathway contributed to the activated and inflammatory phenotype of the mouse model of CGD (29). IDO is an enzyme that uses superoxide as a "cofactor" for oxidative cleavage of tryptophan, yielding the intermediate L-kynurenine. It is considered a key regulator of immune responses, amongst its other effects, suppressing T cell responses and inflammation (30). This enzyme is upregulated during inflammation by proinflammatory stimuli, most notably IFN-γ (29). However, the IDO pathway was recently reported to be functional in monocyte-derived DCs from peripheral blood of CGD patients, despite their defect in NADPH oxidase function (31). Although this suggests that the hyperinflammatory and

activated phenotype seen in these patients could not be atributed to disabled IDO activity (31), a possible impairment of IDO function in the tissues was not excluded.

A strong correlation between circulating IL-7 levels and CD4 T cell counts has been described in lymphopenic clinical settings as a homeostatic response to T cell depletion (11). Accordingly, we found a major increase in circulating IL-7 levels, much above those we observed in the untreated HIV-1 cohort. Nevertheless, we cannot exclude the possibility that this marked increase in circulating IL-7 was due to its production by the liver, as part of an acute-phase response, a phenomenon recently reported to occur as a result of TLR-signalling (37).

Our observations regarding the CGD patient suggest an AIDS-like immunological picture. However, the patient's clinical history was typical of CGD, with no evidence of the opportunistic infections normally seen in AIDS patients.

Reduced T cell numbers were also demonstrated in a large American CGD cohort (32). Current improved care and treatment of the infections in CGD patients are associated with an increased longevity. Thus, it is plausible to speculate that an increasing number of CGD patients with CD4 lymphopenia will be identified, emphasizing the importance of clarifying the main mechanisms involved.

Bone marrow transplantation has been performed as an attractive option for the definitive cure of CGD patients and is associated with resolution of infections (33). However, the improved outcome of the patients and their high clinical variability in the disease course make selection of eligible patients for HSCT difficult (33).

The immune system is composed of a network, both pleiotropic and redundant, that is capable of protecting the individual against disease. The data collected in this thesis illustrates possible pathways and strategies to overcome immunodeficiency and to achieve clinical and immunological competence, even in situations characterized by major impairments of multiple components of the immune system, as exemplified by patients with primary immunodeficiency or HIV infection.

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