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EXPRESSION OF APOPTOSIS AND SURVIVAL GENES IN HUMAN MEMORY T CELL POPULATIONS

THESE

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RÉSUMÉ

Introduction: Les cellules T mémoires humaines sont classées en trois sous-populations sur la base de l'expression d'un marqueur de surface cellulaire, CD45RA, et du récepteur aux chimiokines, CCR7. Ces sous-populations, nommées cellules mémoires centrales (T_{CM}), mémoires effectrices (T_{EM}) et mémoires effectrices terminales (T_{TEM}), ont des rôles fonctionnels distincts, ainsi que des capacités de prolifération et de régénération différentes. Cependant, la génération des ces différences reste encore mal comprise et on ignore les mécanismes moléculaires impliqués.

Matériaux et Méthodes: Des cellules mononucléaires humaines du sang périphérique ont été séparées par cytométrie de flux selon leur expression de CD4, CD8, CD45RA et CCR7 en sous-populations de cellules $CD4^+$ ou $CD8^+$ naïves, T_{CM}, T_{EM} ou T_{TEM}. Dans chacune des ces sous-populations, 14 gènes impliqués dans l'apoptose, la survie ou la capacité proliférative des cellules T ont été quantifiés par RT-PCR en temps réel, relativement à l'expression d'un gène de référence endogène. L'ARN provenant de 450 cellules T a été utilisé par gène et par sous-population. Les gènes analysés (cibles) comprenaient des gènes de survie (BAFF, APRIL, BAFF-R, BCMA, TACI, IL-15Rα, IL-7Rα), des gènes anti-apoptotiques (Bcl-2, Bcl-xL, FLIP), des gènes pro-apoptotiques (Bad, Bax, FasL) et le gène anti-prolifératif, Tob. A l'aide de la méthode comparative delta-delta-CT, le taux d'expression des gènes cibles de chaque sous-population des cellules T mémoires $CD4^+$ et $CD8^+$, à été comparée à leur taux d'expression dans les cellules T naïves $CD4^+$ et $CD8^+$.

Résultats: Dans les ***cellules CD8$^+$***, les gènes pro-apoptotiques Bax et FasL étaient surexprimés dans toutes les sous-populations mémoires, tandis que l'expression des facteurs anti-apoptotiques et de survie comme Bcl-2, APRIL et BAFF-R, étaient diminués. Ces deux tendances étaient particulièrement accentuées dans les sous-groupes des cellules mémoires T_{EM} et T_{TEM}. A noter que malgré le fait que leur expression était également diminuée dans les autres cellules mémoires, le facteur de survie IL-7Rα, était sélectivement surexprimé dans la sous-population de cellules T_{CM} et l'expression d'IL-15Rα était sélectivement augmentée dans les T_{EM}. Dans les ***cellules CD4$^+$***, le taux d'expression des gènes analysés était plus variable entre les sujets étudiés que dans les cellules $CD8^+$, ne permettant pas de définir un profil d'expression spécifique. L'expression du gène de survie BAFF par contre, a été significativement augmentée dans toutes les sous-populations mémoire $CD4^+$. Il en va de même pour l'expression d' APRIL et de BAFF-R, bien que dans moindre degré. A remarquer

que l'expression du facteur anti-apoptotique FasL a été observé uniquement dans la sous-population des T_{TEM}.

Discussion et Conclusions: Cette étude montre une nette différence entre les cellules $CD8^+$ et $CD4^+$, en ce qui concerne les profils d'expression des gènes impliqués dans la survie et l'apoptose des cellules T mémoires. Ceci pourrait impliquer une régulation cellulaire homéostatique distincte dans ces deux compartiments de cellules T mémoires. Dans les ***cellules CD8***$^+$ l'expression d'un nombre de gènes impliqués dans la survie et la protection de l'apoptose semblerait être diminuée dans les populations T_{EM} et T_{TEM} en comparaison à celle des sous-populations naïves et T_{CM}, tandis que l'expression des gènes pro-apoptotiques semblerait être augmentée. Comme ceci paraît être plus accentué dans les T_{TEM}, cela pourrait indiquer une plus grande disposition à l'apopotose dans les populations $CCR7^-$ (effectrices) et une perte de survie parallèlement à l'acquisition de capacités effectrices. Ceci parlerait en faveur d'un modèle de différentiation linéaire dans les cellules $CD8^+$. De plus, l'augmentation sélective de l'expression d'IL-7Rα observée dans le sous-groupe de cellules mémoires T_{CM}, et d'IL-15Rα dans celui des T_{EM}, pourrait indiquer un moyen de sélection pour des réponses immunitaires mémoires à long terme par une réponse distincte à ces cytokines. Dans les ***cellules CD4***$^+$ par contre, aucun profil d'expression n'a pu être déterminé; les résultats suggèrent même une résistance relative à l'apoptose de la part des cellules mémoires. Ceci pourrait favoriser l'existence d'un modèle de différentiation plus flexible avec des possibilités d'interaction multiples. Ainsi, la surexpression sélective de BAFF, APRIL et BAFF-R dans les sous-populations individuelles des cellules mémoires pourrait être un indice de l'interaction de ces sous-groupes avec des cellules B.

ABSTRACT

Introduction: Based on their surface expression of the CD45 isoform and of the CCR7 chemokine receptor, memory T cells have been divided into the following three subsets: central memory (T_{CM}), effector memory (T_{EM}) and terminal effector memory (T_{TEM}). Distinct functional roles and different proliferative and regenerative capacities have been attributed to each one of these subpopulations. The molecular mechanisms underlying these differences, however, remain poorly understood.

Materials and Methods: According to their expression of CD4, CD8, CD45RA and CCR7, human peripheral blood mononuclear cells were sorted by flow-cytometry into $CD4^+$ or $CD8^+$ naïve, T_{CM}, T_{EM} and T_{TEM} subsets. Using real-time PCR, the expression of 14 genes known to be involved in apoptosis, survival or proliferation of T cells was quantified separately in each individual subset, relative to an endogenous reference gene. The RNA equivalent of 450 T cells was used for each gene and subset. The target gene panel included the survival genes BAFF, APRIL, BAFF-R, BCMA, TACI, IL-15Rα and IL-7Rα, the anti-apoptotic genes Bcl-2, Bcl-xL and FLIP, the pro-apoptotic genes Bad, Bax and FasL, as well as the anti-proliferative gene Tob. Using the comparative C_T-method, the expression of the target genes in the three memory T cell subsets of both $CD4^+$ and $CD8^+$ T cell populations was compared to their expression in the naïve T cells.

Results: In ***$CD8^+$ cells***, the pro-apoptotic factors Bax and FasL were found to be upregulated in all memory T cell subsets, whereas the survival and anti-apoptotic factors Bcl-2, APRIL and BAFF-R were downregulated. These tendencies were most accentuated in T_{EM} and T_{TEM} subsets. Even though the survival factor IL-7Rα was also downregulated in these subsets, interestingly, it was selectively upregulated in the $CD8^+$ T_{CM} subset. Similarly, IL-15Rα-expression was shown to be selectively upregulated in the $CD8^+$ T_{EM} subset. In ***$CD4^+$ cells***, the expression levels of the analyzed genes showed a greater inter-individual variability than in $CD8^+$ cells, thus suggesting the absence of any particular expression pattern for $CD4^+$ memory T cells. However, the survival factor BAFF was found to be significantly upregulated in all $CD4^+$ memory T cell subsets, as was also the expression of APRIL and BAFF-R, although to a lesser extent. Furthermore, it was noted that the pro-apoptotic gene FasL was only expressed in the T_{TEM} $CD4^+$ subset.

Discussion and Conclusions: Genes involved in apoptosis and survival in human memory T cells have been shown to be expressed differently in $CD8^+$ cells as compared to $CD4^+$ cells, suggesting a distinct regulation of cell homeostasis in these two memory T cell compartments. The present study suggests that, in ***$CD8^+$ T cells***, the expression of various survival and anti-apoptotic genes is downregulated in T_{EM} and T_{TEM} subsets, while the expression of pro-apoptotic genes is upregulated in comparison to the naïve and the T_{CM} populations. These characteristics, potentially translating to a greater susceptibility to apoptosis in the $CCR7^-$ (effector) memory populations, are accentuated in the T_{TEM} population, suggesting a loss of survival in parallel to the acquisition of effector capacities. This speaks in favour of a linear differentiation model in $CD8^+$ T memory cells. Moreover, the observed selectively increased expression of IL-7Rα in $CD8^+$ T_{CM} cells – as that of IL-15Rα in $CD8^+$ T_{EM} cells – suggest that differential responsiveness to cytokines could confer a selection bias for distinct long-term memory cell responses. Relative to the results for $CD8^+$ T cells, those for ***$CD4^+$ T cells*** seem to indicate a certain resistance of the memory subsets to apoptosis, suggesting the possibility of a more flexible differentiation model with multiple checkpoints and potential interaction of $CD4^+$ memory cells with other cells. Thus, the selective upregulation of BAFF, APRIL and BAFF-R in individual memory subsets could imply an interaction of these subsets with B cells.

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1 GENERAL INTRODUCTION

1.1 THE IMMUNE SYSTEM

Humans and other organisms depend on their immune system to fight disease.[1-3] This is especially true when they are confronted with infection, caused by invasion of microorganisms such as bacteria, virus, fungi and parasites. Qualitatively, the immune system can be divided into two large entities - the innate immune system and the acquired immune system. The ***innate immune system*** mediates a non-specific response against a pathogen, and its efficiency does not depend upon the amount of times the organism encounters the pathogen. Its main components are the complement system, which consists of serum proteins and macrophages, phagocytes and natural killer (NK) cells, which destroy the pathogen or infected cells. The ***acquired immune system***, on the other hand, mounts an immune response, which is highly specific to the pathogen that it was elicited by. Moreover, the acquired immune system possesses an adaptive quality ensuring that, once the organism has survived an initial infection by a pathogen, it will generally present a more efficient defense and consequently be immune to further illness caused by that same pathogen.

In reality, this separation is not very accurate, and there are many cases where the innate and the acquired immune systems are intertwined. The protective action of the immune system, as a whole, depends on a variety of soluble factors (humoral immune response) and different types of white blood cells or leucocytes that are produced in the bone marrow (cellular immune response). In the acquired immune system, both these lines of defense are mostly based on the action of either B or T lymphocytes, which are subclasses of leucocytes. The ***acquired humoral immune system*** depends primarily on B lymphocytes that mature into plasma cells and produce soluble proteins called antibodies or immunoglobulins (Ig). Antibodies are highly pathogen-specific and act against extracellular microorganisms in the blood which are mostly bacteria. The ***acquired cellular immune system*** is mainly based on the action of different subtypes of T lymphocytes, such as regulatory T cells, helper T cells and cytolytic T cells (CTLs). One of its major functions is the destruction of cells invaded by intracellular pathogens such as viruses, but it also acts against other altered cells like tumor cells. The two systems coordinate with each other, thanks to helper T cells which provide important maturation signals to B lymphocytes.

1.2 T CELL MEDIATED IMMUNITY

Like B cells, T lymphocytes are also produced in the bone marrow; their maturation however, takes place in the thymus. They can be divided into many subpopulations according to their expression of surface antigens, which are called cluster of differentiation (CD) and have been classified by numbers. The two main categories of T cells are ***$CD4^+$ cells*** (also called helper T cells) and ***$CD8^+$ T cells*** (also called cytolytic T cells or CTLs).[1-3]

From the bone marrow, T cells migrate to the thymus where they mature from double positive (DP), $CD8^+CD4^+$ cells into single positive $CD8^+$ or $CD4^+$ cells. In parallel, they acquire specific T cell receptors (TCRs) which they express on their surface. Diverse TCRs are produced through a complex genetic rearrangement, so that in the end each T cell is unique and specific to a certain antigen. A selection process in the thymus ensures that self-reactive T cells and ineffective T cells are destroyed (positive and negative selection). From the thymus, T cells enter the bloodstream and recirculate between blood and secondary lymphoid organs. Secondary lymphoid organs include tonsils, lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT), such as Peyer's patches of the gut *(Figure 1.1)*. Even though these T cells are mature, they are known as ***naïve T cells***, because they have not yet encountered their antigen. The migration of naïve T cells into lymphoid tissues is mediated by chemokines, such as secondary lymphoid tissue chemokine (SLC), EB1 ligand chemokine (ELC) and addressins like PNAd.[4] The lymph-homing ***chemokine receptor CCR7,*** expressed on naïve T cells (but also on a subset of memory T cells), binds to SLC which is expressed by the high vascular endothelium (HEV), stromal cells and dendritic cells in lymphoid tissue. In this manner, CCR7 mediates T cell entry to lymph nodes *(Figure 1.1)*.

When there is infection, pathogens or their products are transported from the target organ to the lymphoid tissue by draining lymphatics or, more rarely, directly by the blood. Adaptive immune responses are therefore not initiated at the infection site, but rather in the organized peripheral lymphoid tissue, through which naïve T cells are continuously migrating. In order to initiate an adaptive immune response, T cells need to first recognize an infected cell and then eliminate it, by either killing it directly or indirectly through activation of macrophages and B cells. This is possible through a coordinated interaction between professional antigen-

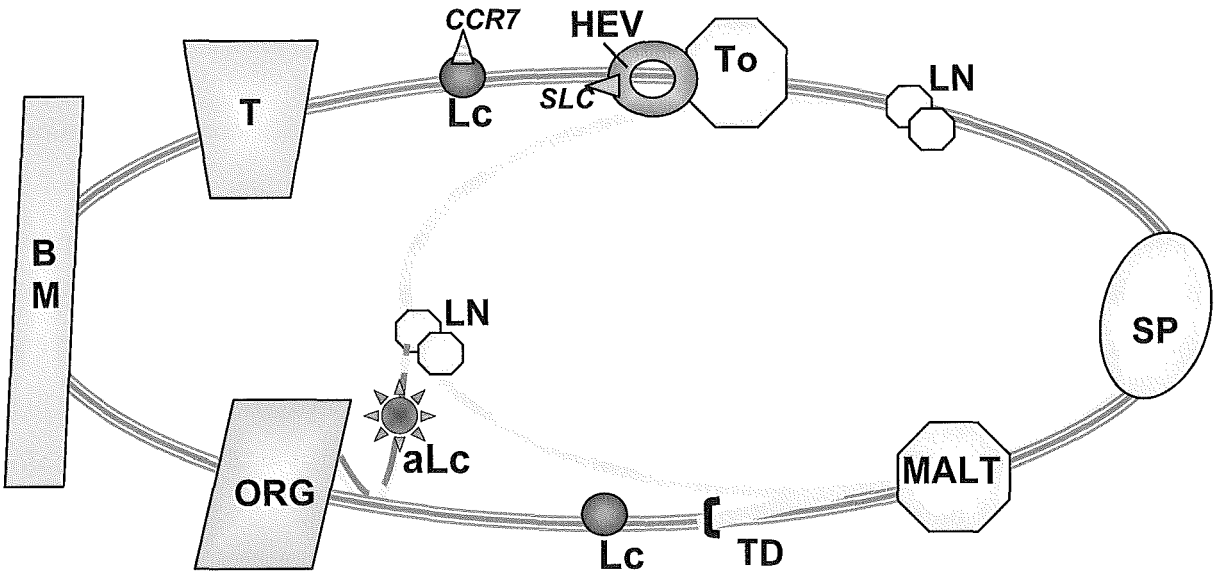


FIGURE 1.1 Physiological recirculation of T lymphocytes

T lymphocytes (Lc) are produced in the bone marrow (BM) and then migrate to the thymus (T), where they mature. Mature naïve T cells recirculate through blood *(red lines)* and lymphatic vessels *(yellow lines)*. They express the chemokine receptor CCR7, which enables them to interact with the secondary lymphoid tissue chemokine (SLC). SLC is expressed on the high endothelial venules (HEV) of secondary lymphoid organs, such as tonsils (To), lymph nodes (LN), spleen (SP) and mucosa-associated lymphoid tissue (MALT). T cells either re-enter the blood stream through the thoracic duct (TD) or leave the lymphatic circulation through the HEV once they have been activated by antigen encounter in a draining lymph node. Activated T cells (aLc) lose their expression of CCR7 and express other chemokine receptors, enabling them to migrate to target organs (ORG) such as the lung, liver and skin. *Primary lymphoid organs are shown in green; secondary lymphoid organs are shown in yellow.*

presenting cells (APCs), located in the secondary lymphoid tissues or in peripheral target organs, cytotoxic T cells (CTLs or $CD8^+$ T lymphocytes) and helper T cells ($CD4^+$ T lymphocytes). T cells encounter the pathogen products (antigen) by the help of APC, which carry different antigens in the form of peptide-MHC complexes on their surface. The most important APCs are dendritic cells (DCs) which are specialized in trapping antigen at the site of infection and migrating to a downstream lymph node. Once the antigen has been captured by the DC, it triggers a maturation process which results in the expression of co-stimulatory factors that are required, in addition to the antigen, for the activation of naïve T cells. Activated T cells lose the expression of CCR7 and instead express inflammation-specific receptors, such as CCR1, 2, 3, 4, 5 and CXCR3. These receptors bind to adhesion molecules, such as ICAM-1, VCAM-1, E-and P-selectins expressed on blood vessels and inflamed tissues.[5,6] This interaction enables the activated T cell to be recruited to the infection site *(Figure 1.1)*.

The antigen-specific clonal expansion of naïve T cells requires a second or co-stimulatory signal, which must be delivered by the same antigen-presenting cell on which T cells recognize the antigen. The best characterized co-stimulatory molecules are the structurally related glycoproteins B7.1 (CD80) and B7.2 (CD86), which are homodimeric members of the immunoglobulin superfamily and are exclusively found on the surface of cells that can stimulate T-cell proliferation. The receptors for B7 molecules on T cells are ***CD28*** and the cytotoxic T lymphocyte antigen 4 (CTLA-4). In vitro, an unspecific but efficient TCR-triggered response can be obtained by stimulating T cells with anti-CD3 antibodies (which bind to the TCR-associated molecule CD3) and anti-CD28 monoclonal antibodies. Interaction of CD28 with B7 molecules, or alternatively with anti-CD28 antibodies used in experimental conditions, co-stimulates the activation signal given to the naïve T cell by its TCR. This leads to a clonal expansion of the activated T cell. Antigen binding to the TCR in the absence of co-stimulation, not only fails to activate T cells, but even leads to a dysfunctional state termed anergy. Anergic T cells are refractory to activation by a specific antigen, even when the antigen is subsequently presented by an APC.

1.3 IMMUNOLOGICAL MEMORY

Immunological memory results in a faster and stronger immune response of an organism, following reexposure to the same antigen. In general, it leads to a better protection against an invading pathogen.[7] In what ways does this memory response differ from the primary response, so as to have a protective advantage over the latter?

In the case of the humoral response, these advantages are clearly identifiable. Not only is there a greater frequency of specific antigen reactive cells, the quality of the memory cells is also notably different from that of naïve B cells. The pathway of the memory B cell development occurs in the germinal centers of peripheral lymph nodes and involves isotype switching and hypermutation of immunoglobulin genes, allowing memory B cells to be easily identified. T cell memory is more difficult to define. No anatomical site has been identified where memory T cells develop, and there is no isotype switching or affinity-selected mutation of the TCR, making the identification of memory T cells difficult.[7] Thus, many aspects of T cell memory still remain an issue of debate. However, one can implicate three major mechanisms, leading to a greater protection of the organism during the memory response.

Firstly, clonal selection and expansion lead to increased numbers of antigen-specific cells, making it one of the earliest explanations[8] of the higher efficiency of the memory T cell response. A particular antigen will drive the expansion of the T cell with the most specific TCR, inducing it to clone itself – thus creating greater frequencies of highly antigen-specific cells. Due to this phenomenon, during the first days to weeks following exposure to a pathogen, there is an extensive expansion of antigen-specific T cells. Ideally this will culminate in the elimination of the pathogen. Then, however, follows a phase of contraction, during which the majority of the activated effector cells undergo apoptosis. Even so, the end result is a net increase in the numbers of pathogen-specific memory T cells,[9] and reencounter with the same antigen expands the population to a new, stable higher level *(Figure 1.2)*.

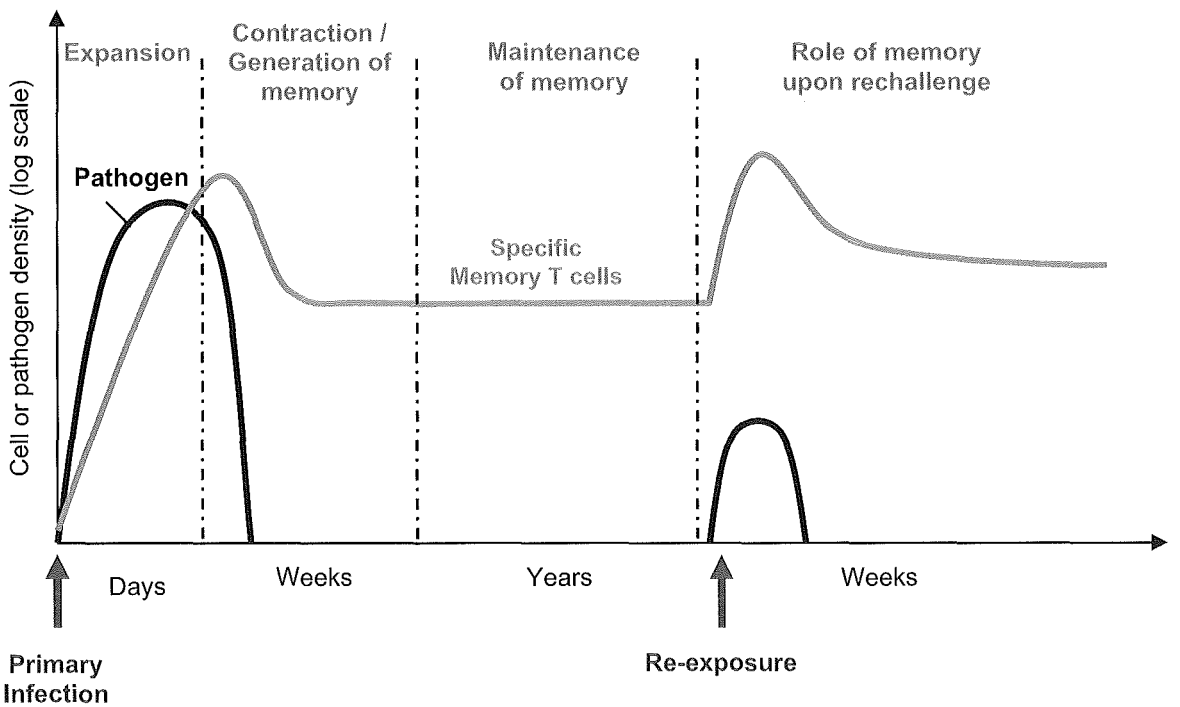


FIGURE 1.2 Phases of the immunological response in T-cell mediated immunity

Primary infection results in the clonal expansion of antigen-specific T cells that will eventually lead to eradication of the pathogen. Within a few weeks, the majority of the expanded effector T cells undergo apoptosis and the population of specific cells contracts. The surviving T cells develop into memory cells that can be maintained at constant levels for years in the absence of pathogen. Thus, even though these specific memory T cells represent just a fraction of the initially expanded effector population, their formation results in a net increase of antigen-specific cells after pathogen clearance. Besides, by being more efficient in the control of pathogen, this memory T cell population ensures a faster immune response upon re-infection, resulting in the absence or attenuation of disease. *(Figure adapted from Antia R et al).*[10]

Secondly, memory cells are more easily triggered than naïve cells, responding at lower antigen dose and with less stringent requirements for co-stimulation.[7] This results in a lower activation threshold and ensures higher and faster responsiveness to antigen. Finally, memory

cells can persist as non-dividing cells in the absence of antigen stimulation over extended periods of time.[10] The combination of these characteristic features are thought to be the basis of a more rapid and efficient immune response upon reexposure, which results in an enhanced protection from disease for the organism.

1.4 MEMORY T CELLS

As mentioned above, the development of T cell memory is complex and even though memory T cells are different from naïve cells, various criteria have to be combined in order to identify them. Firstly, memory T cells express activation markers and cell surface receptors distinct to those of naïve cells. Secondly, memory T cells can be distinguished by their capacity to secrete a wider range of cytokines. Notably, they can also be polarized to perform unique functions specified by the pattern of cytokines they secrete. On the basis of these characteristics, recent research has led to the development of different models of T cell memory, emphasizing the existence of distinct subpopulations within memory T cells. Their identification is based on:

- ***Phenotype***, characterized by differential expression pattern of cell surface markers.
- ***Effector functions***, such as cytokine secretion and cytotoxic potential.

The most widely used marker to distinguish between naïve and memory T lymphocytes in humans, is the CD45R isoform expressed on the cell surface in various combinations.[7] CD45R is a transmembrane phosphatase that regulates signaling through the TCR-CD3 complex. Distinct isoforms are generated by alternative splicing of amino-terminal A, B and C exons. The high-molecular-weight protein (CD45RA) contains A and B and/or C exons, whereas the low-molecular-weight protein (CD45RO) does not contain any of the variable exons. T-cell activation induces a shift from expression of CD45RA to CD45RO. Thus, human ***naïve T cells are defined as CD45RA$^+$ and CD45RO$^-$***. In contrast, human ***memory T cells express CD45RO and mostly lack CD45RA***. However, memory T lymphocytes are a heterogeneous group of cells, and it has been shown that some subgroups of memory T cells reexpress CD45RA.[11]

Another marker, now increasingly used, is the chemokine receptor CCR7. CCR7 enables T cells to leave the bloodstream and enter into lymphoid tissue via its interaction with SLC (Secondary Lymphoid tissue Chemokine). Thus, the advantage of this marker is not only in defining naïve and memory subsets, but also in that it enables a functional characterization. Most importantly, CCR7 has been found to define distinct subsets[12] of memory cells within the population of total memory T cells, depending on their homing and effector capacities:

- ***CCR7 $^-$ T cells*** express receptors for migration into inflamed tissues. Upon re-encounter with antigen, they can rapidly produce IFN-γ or IL-4 or release pre-stored perforin, thus mediating immediate protection by cytotoxicity.

- ***CCR7 $^+$ T cells*** home to secondary lymphoid organs such as lymph nodes and may have a high proliferative potential. However, they lack immediate effector functions such as cytokine secretion and cytotoxicity.

Using the markers CCR7 and CD45RA for flow cytometric studies, Sallusto and colleagues identified four subpopulations of CD4 memory T cells.[12] They established a lineage pattern for $CD4^+$ T cells by demonstrating a progressive differentiation from naïve to central memory, to effector memory T cells *(Figure 1.3)*. In their model, $CD45RA^+$ $CCR7^+$ T cells figure as precursors for the other subsets. They expand on antigen re-encounter to ensure continuous replenishment of the effector cell pools. $CD45RA^-$ $CCR7^+$ T cells are referred to as central memory T cells and have been shown to secrete smaller amounts of effector cytokines upon stimulation. One could thus imagine that they would not mediate effector-cell functions during the primary T cell response, but rather, serve as a reservoir, giving rise to secondary effector T cells upon antigen re-encounter.[13] $CD45RA^-$ $CCR7^-$ T cells, on the other hand, rapidly secrete effector cytokines upon restimulation with antigen. They are termed pre-terminally differentiated or effector memory T cells. The fourth subset reexpresses CD45RA and also homes to lymph nodes. Even though present in very small numbers in healthy individuals, this subset increases in response to chronic infection such as non-treated HIV infection.[14]

An analog model has been suggested for $CD8^+$ T cells. In $CD8^+$ T cells, the $CD45RA^+$ $CCR7^-$ cells were shown to rapidly secrete IFN-γ upon restimulation and presented the highest levels of perforin expression. They are ready to intervene immediately following antigen re-exposure and have accordingly been referred to as terminally differentiated memory T cells.[11]

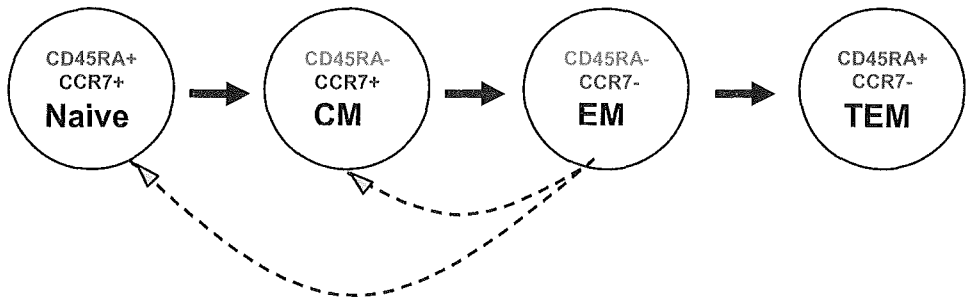


FIGURE 1.3 Lineage pattern of memory T cells (as proposed by Sallusto and Lanzavecchia [13])

Naïve T cells are CD45RA$^+$ CCR7 $^+$ and function as precursors for different subtypes of memory T cells. **CD45RA $^-$ CCR7 $^+$ or Central Memory T cells (CM)** do not have direct effector function and serve as a reservoir for secondary effector cells upon re-exposure. **CD45RA$^-$ CCR7 $^-$ Effector Memory T cells (EM)** are pre-terminally differentiated effector cells that will either develop into terminally differentiated effector cells or be directly used against antigen when re-exposure occurs. **CD45RA$^+$ CCR7 $^-$ or Terminal Effector Memory T cells (TEM)** are the most potent secondary effector cells and intervene rapidly upon re-encounter. Dashed arrows indicate that CD45RA$^-$ CCR7 $^-$ (EM) cells may revert to CD45RA$^-$ CCR7 $^+$ (CM), or even to a naïve (CD45RA$^+$ CCR7 $^+$) phenotype, upon antigen stimulation.

A functional implication of this model for memory T cell differentiation *(Figure 1.3)* would be an increased differentiation capacity and proliferation potential of the CD45RA$^-$ CCR7 $^+$ (CM) cell population, as compared to the CD45RA$^-$ CCR7 $^-$ (EM) and the CD45RA$^+$ CCR7 $^-$ (TEM) populations. This would enable the central memory subset to act as a reservoir for the effector memory T cell subsets. The latter, on the other hand, being at a more advanced state of differentiation should logically be more prone to cell death. Consequently, a linear loss of cell survival capacity and increase of cell death susceptibility from CM to TEM subsets could be indirect confirmations of the aforementioned differentiation model.

1.5 APOPTOSIS AND SURVIVAL

An organism is obliged to tightly regulate the balance of cell survival and cell death to maintain its structure and function. It is well known that the loss cell death control often leads to the formation of aberrantly long-lived, cancerous, cells – potentially dangerous for the organism. On the other hand, the positive side of the balance, survival, is important to allow correct development and differentiation of cells. This is crucial for the functional maturity of the organism. It is therefore not surprising that several pathways and genes have been found

to be implicated in the control of this balance.[15-22] Fundamentally, one can differentiate between two mechanisms of cell death. ***Necrosis***[23,24] normally results from a severe cellular insult and causes organelle swelling and loss of plasma membrane integrity. ***Apoptosis***,[25,26] on the other hand, is a programmed form of cell death, causing typical morphological changes in the cell in absence of an inflammatory response (apoptotic bodies).

Since immune responses are often characterized by large clonal expansions, the immune system is particularly vulnerable to the consequences of unchecked proliferation, such as autoimmunity and malignancy.[27] Apoptosis is a critical regulator of homeostasis in the immune system and permits to maintain constant cell numbers, once the antigenic stimulus has been cleared, thus avoiding the accumulation of a large number of cells. It is a complex, multifactorial process, tightly regulated by many genes and can be induced in two different pathways:

- The ***extrinsic pathway*** depends on external signals from death activators binding to receptors at the cell surface.
- The ***intrinsic pathway*** is initiated by death signals arising within the cell.

1.5.1 The extrinsic (death receptor) pathway

The extrinsic pathway[28-30] is induced by triggering death-receptors, all members of the TNF-receptor protein superfamily. The most important of these interactions is the binding of ***Fas*** to its ligand ***Fas ligand (FasL)*** *(Figure 1.4, A)*.

FasL is a transmembrane protein belonging to the TNF family and binds to its receptor Fas, which is an integral membrane protein with its receptor domain exposed at the surface of the cell. The interaction results in clustering of the death-receptor Fas on the cell surface and recruitment of the Fas-associated death domain protein (FADD), leading to the formation of the death-inducing signaling-complex (DISC). This complex recruits caspases 8 and 10 via the interaction of their death-effector proteins domains (DED) with the DED of the adaptor molecule FADD. Caspases are the main effectors of the extrinsic apoptotic pathway. They are a family of over a dozen cystein proteases that cleave at aspartic acid (Asp) residues. Caspases are expressed as latent zymogens and require cleavage (through each other mostly) for activation. The sequential activation of one caspase by another creates an expanding cascade of proteolytic activity culminating in the activation of Caspase-3. Caspase-3 is the

end-effector of the apoptotic process, which results in proteolytic cleavage of cellular substrates, digestion of structural proteins in the cytoplasm and degradation of chromosomal DNA. This is visible microscopically in the formation of apoptotic bodies, which represent a specific morphological change of the cell in response to apoptosis. Their formation leads to subsequential phagocytosis of the cell.

1.5.2 The intrinsic (mitochondrial) pathway

Stress conditions, such as starvation, or damage to a cell's DNA from toxicity or exposure to ionizing radiation such as ultraviolet or X-rays, induce an apoptotic process from within the cell via the mitochondrial pathway[31] *(Figure 1.4, B)*. This causes a change in the permeability of the mitochondrial membrane by triggering specific Bcl-2 family-regulated mechanisms, resulting in the release of cytochrome c from the mitochondria. Each of the Bcl-2 family members is either pro- (Bad, BAK, Bax, tBID, BIM, etc.) or anti-apoptotic (A1, Bcl-2, Bcl-w, Bcl-xL, etc.), and it is the balance between these factors that will determine cytochrome c release and therefore apoptosis.[32-34] In a healthy cell, the outer membranes of its mitochondria express the anti-apoptotic protein Bcl-2 on their surface. Bcl-2 is bound to a molecule of the protein APAF-1 ("apoptotic protease activating factor-1"). Internal damage to the cell causes Bcl-2 to release APAF-1 and a related protein, Bax, to penetrate mitochondrial membranes, causing cytochrome c to leak out. The released cytochrome c and Apaf-1 recruit procaspase-9 via its capase recruitment domain (CARD), and the resulting complex of cytochrome c, Apaf-1, caspase 9 and dATP is called the apoptosome.[35] Apoptosomes aggregate in the cytosol and lead to caspase-3 activation.

Caspase-3 is the common end-effector of both pathways. Under normal conditions, proteins of the inhibitor of apoptosis-family (IAP)[36] directly bind and inhibit capase-3, -6, -7, and -9. However, when a cell receives an apoptotic stimulus, the inhibitory IAP activity is itself inhibited by the release of SMAC (Second Mitochondria-derived Activator of Caspases, its mouse homolog being DIABLO). When SMAC binds to IAPs, it enables the apoptotic process to continue and conclude in cell death.[15, 37]

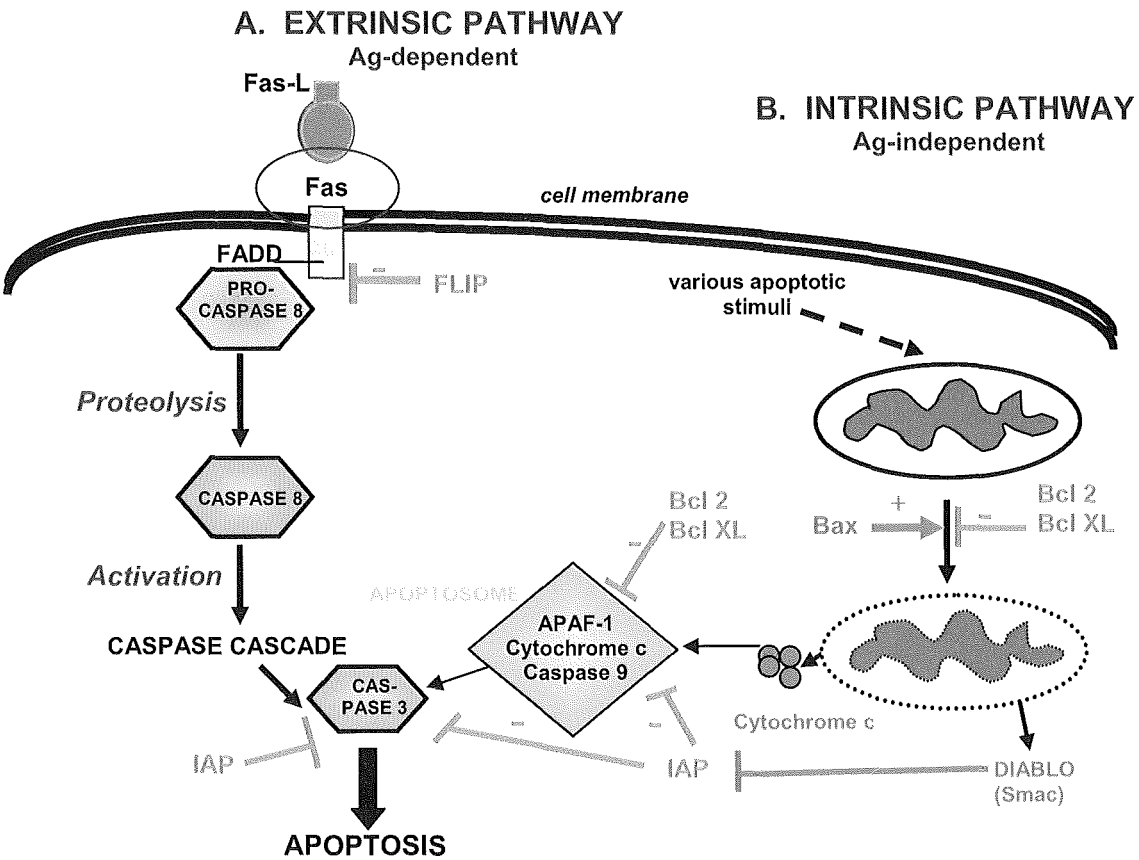


FIGURE 1.4 The two major apoptotic pathways in mammalian cells

A. The death –receptor pathway ***(extrinsic pathway)***
Binding of the ligand (FasL) to the death-receptor Fas, induces receptor clustering on the cell membrane and formation of a death-inducing signaling complex (DISC). This complex recruits multiple procaspase-8 molecules via the adaptor molecule FADD (Fas-associated death domain protein), leading to a caspase-caspase activation cascade beginning with Caspase-8 and ending with Caspase-3 activation and apoptosis. Caspase-8 activation can be blocked by FLIP.

B. The mitochondrial pathway ***(intrinsic pathway)***
This pathway is used in response to various apoptotic stimuli, such as DNA damage by radiation or reactive oxygen species. The lethalness of the stimuli is evaluated on the mitochondrial surface, where there is a competition between pro-apoptotic members of the Bcl-2 family, like Bax, and anti-apoptotic members, like Bcl-2 and Bcl-xL. If the death agonists prevail, cytochrome c is released from the mitochondria and associates with APAF-1 and Caspase 9 to form an apoptosome which enables Caspase-3 activation and subsequent apoptosis.

Both the pathways converge at the level of Caspase-3 activation which can be antagonized by IAP proteins, which in turn are inhibited by the SMAC/DIABLO protein released from mitochondria.

1.5.3 T cells and apoptosis

In T cells, the death receptor pathway is also often termed AICD (activation-induced cell death), as it results through repeated activation of the TcR. The importance of apoptosis in the human immune system is illustrated by the ***autoimmune lymphoproliferative syndrome (ALPS)***. ALPS is defined by functional analysis of lymphocyte sensitivity to Fas-induced apoptosis in vitro. The T cells of these patients are completely or partially resistant to AICD according to the severity of the genetic mutations of Fas, FasL or caspase-8.[38,39] Four criteria characterize the ALPS condition: a lymphoproliferative syndrome (splenomegaly and/or lymphadenopathies), autoimmune manifestations, hypergammaglobulinemia and detection of $CD4^-CD8^-$ T cells (double negative or DN cells) in the blood. Apart from rare exceptions, ALPS patients usually present at least three of these criteria.[40]

2 OBJECTIVE and APPROACH

As discussed in the introduction, a functional division of memory T cells into central and effector memory cells would logically imply a differential susceptibility of the distinct memory T cell subsets to cell death or to survival signals *(Figure 2)*. However, the mechanisms underlying the promotion of cell survival or cell death in memory cells are poorly understood, and various genes have been attributed pivotal roles in this regulation.

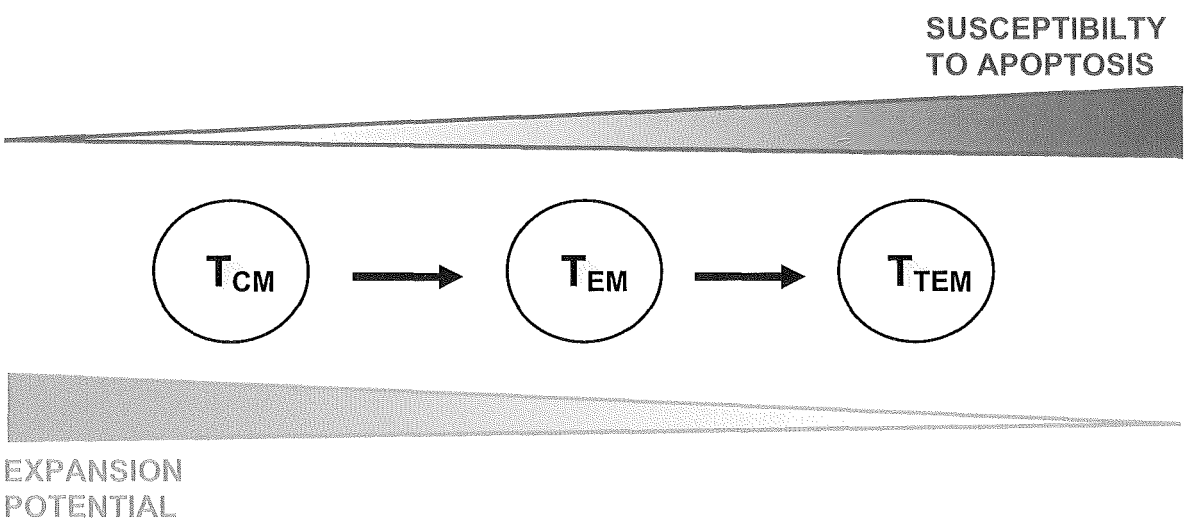


FIGURE 2 Working Hypothesis: Differential susceptibility of memory subsets to survival and death signals

In a linear differentiation model, the expansion potential and proliferative capacities of central memory T cells (T_{CM}) would have to be greater than those of effector memory (T_{EM}) and terminally differentiated effector memory T cells (T_{TEM}), in order to enable them to act as a reservoir for the more differentiated subsets. The effector memory populations, on the other hand, would show higher susceptibility to apoptosis than the central memory subset.

The aim of this study is to elucidate as to whether, in the case of memory T cell subsets, a difference of susceptibility to cell survival can be mirrored by a differential gene expression pattern in the individual subsets. For this purpose, a panel of 12 genes involved in apoptosis and survival was selected and studied in each memory subset separately. The various individual genes are discussed in the following chapter.

The approach has required a method allowing analysis of gene expression in a restricted number of cells and simultaneous comparison of the expression levels. Accordingly, the study can be divided into three steps:

1. Design of a real-time RT-PCR assay by which to analyze gene expression in a restricted number of human T lymphocytes.

2. Validation of a suitable endogenous reference gene for relative quantification.

3. Semi-quantitative analysis of the expression pattern of gene panel memory subpopulations of $CD8^+$ and $CD4^+$ peripheral human T cells and comparison to naïve $CD8^+$ and $CD4^+$ T cells, respectively.

3 GENE PANEL

3.1 SURVIVAL GENES

The generation and survival of memory T cell populations are crucial for prolonged protective immunity following infection. Memory lymphocytes are generated in the presence of antigen; their maintenance and persistence, however, is independent of TCR-mediated signals, as shown by studies demonstrating memory T cell survival in MHC-deficient mice.[41,42] Cytokine-induced homeostatic proliferation has been proposed as a mechanism to replenish the numbers of memory T cells in the absence of specific antigen, and various cytokines have been implicated in the long-term survival of memory T cells.[43-45]

3.1.1 IL-15

In humans, interleukin 15 (***IL-15***) was originally discovered as a cytokine produced by a human adult T cell leukemia cell line (HuT-102). It was shown to stimulate T cell proliferation and was designated IL-T.[46] IL-15 is a cytokine that shares many biological properties with IL-2. Apart from stimulating T cell proliferation,[47] it has been found to provide survival signals to support the growth of mature lymphocytes,[44] particularly activated peripheral blood T lymphocytes.[47-49] In addition, IL-15 has also been shown to be a chemoattractant for human blood T lymphocytes[50] and to be able to induce the generation of cytolytic effector cells.

Many types of cells secrete IL-15, but apparently this cytokine is not produced by T cells themselves;[51] IL-15 mRNAs have been detected in several human tissues and cell types, including heart, lung, liver, placenta, skeletal muscle, epithelial and fibroblast cell lines, but have not been detectable in activated peripheral blood T cells.[47]

3.1.1.1 IL-15-Receptor (IL-15Rα)

IL-15 signals through a trimeric receptor complex. IL-15-Receptor (IL-15R) consists of (1) the common γ-chain which is shared by IL-2, IL-4, IL-7, IL-9 and IL-21 for signal transduction, (2) the β-chain which is shared by IL-2 and also called IL-2Rβ, and (3) most importantly, a unique high affinity α-chain (***IL-15Rα***). These high-affinity cell surface

receptors for IL-15 have been detected on a variety of T cells and B cells, as well as non-lymphoid cells.[52]

3.1.1.2 IL-15 and memory T cells

IL-15 does not seem necessary to mount primary $CD8^+$ T cell responses. Thus, studies in IL-$15^{-/-}$ and IL-$15R\alpha^{-/-}$ mice have shown that IL-15 is not required to efficiently clear the pathogen and generate a pool of antigen-specific $CD8^+$ T cells. However, there is strong evidence showing that IL-15 can promote proliferation and long-term survival of memory phenotype $CD8^+$ T cells in an antigen-independent fashion.[20,27] These cells express the α-chain of the IL-15-Receptor and the shared IL-2-R β- and γ-chains, individually or together, to form various functional receptors with different affinities and signaling capabilities. The interaction of IL-15 with its receptors was shown to lead to a potent and selective proliferation of $CD8^+$ memory T cells.[43,44,53]

3.1.2 IL- 7

Interleukin 7 (***IL-7***)[54] is essentially a tissue-derived cytokine and is produced by several stromal tissues, including epithelial cells in thymus and bone marrow.[55] Additional sites of IL-7 production include intestinal epithelium, keratinocytes, fetal liver, adult liver, dendritic cells and follicular dendritic cells.[56-60] Interestingly, however, IL-7 mRNA has not been detected in normal lymphocytes. IL-7 has been shown to bind to extracellular matrix-associated glycoproteins, such as glycosaminoglycan, heparan sulfate and fibronectin. It is thought that this plays an important role in the regulation of IL-7-induced signaling within the cellular microenvironment.[61,62] All in all, several studies have shown the importance of IL-7 in the regulation of T cell survival.[43, 63-70]

3.1.2.1 Signal transduction

The IL-7-Receptor consists of two polypeptides: (1) a highly specific α-chain (***IL-7Rα*** or CD127), and (2) the common γ-chain, which is the same as for the IL-15-Receptor. Binding of IL-7 to the IL-7Rα-chain leads to dimerization of the α- and γ-chains and subsequent signal transduction. The α-chain is activated through phosphorylization by JAK-3 and the tyrosine kinase of the γ-chain. Once phosphorylated, the α-chain serves as a site for recruiting other

signaling molecules to the complex, to be phosphorylated and activated it turn. The most important targets of this signal transduction are STAT5, the src family kinases (lck and fyn), PI3 kinase, Pyk2 and Bcl2 proteins. The activation of these signaling molecules promotes different pathways in T cells. Bcl-2 and Pyk-2 have an enhancing effect on cell survival. PI3 kinase, src family kinases (lck and fyn) and STAT5, on the other hand, contribute to cellular proliferation. The transcription factor STAT5 migrates into the nucleus and leads to the activation of multiple downstream genes essential to proliferation and development in B and T cells (*Figure 3.1*).[71-73]

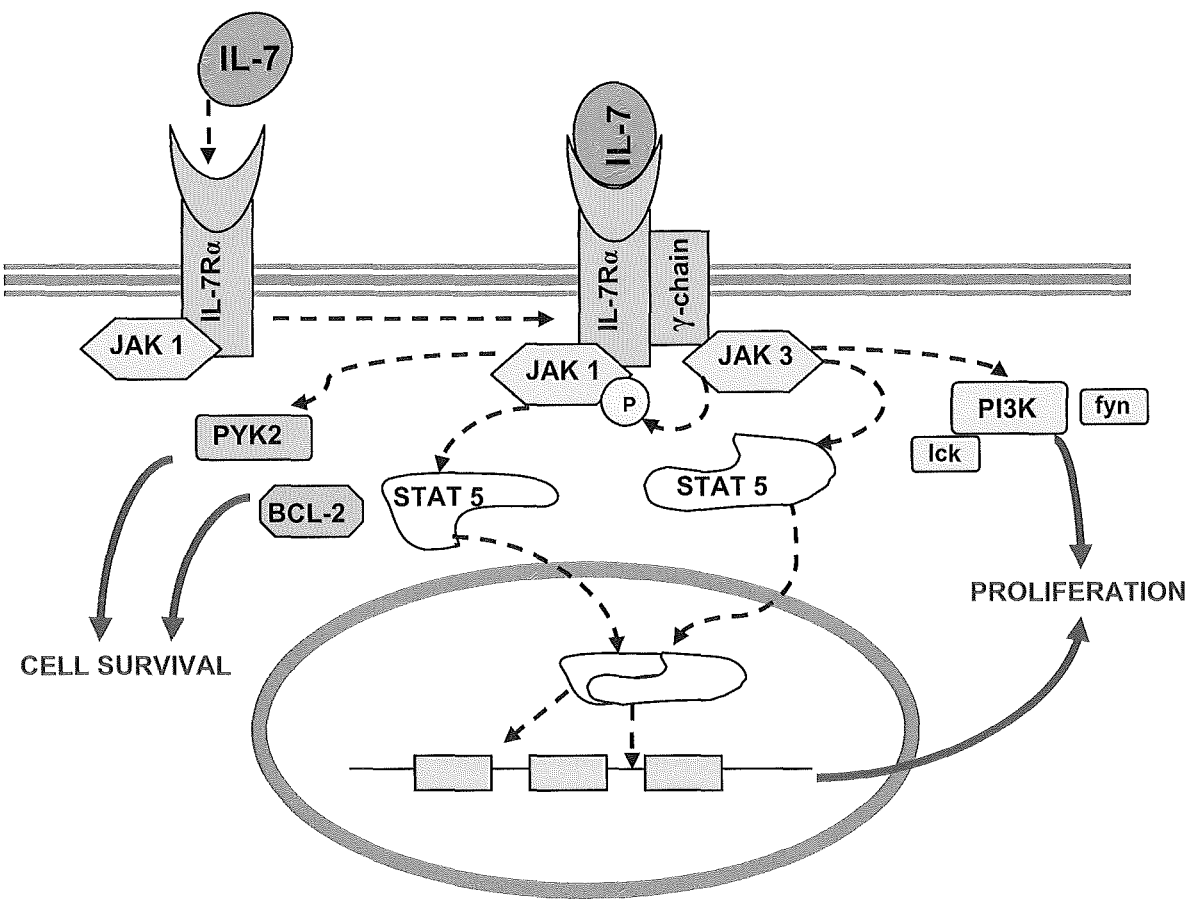


FIGURE 3.1 Overview of the IL-7 signaling pathway

See text for explanation.
(Note: Figure adapted from Croston G.[74]).

3.1.2.2 IL-7 in developing and mature naïve T cells

IL-7 is best known for its effects on developing B-cell and T-cell populations, e.g. its crucial role in thymopoiesis,[75-77] but it also potently modulates mature, naïve T-cell function.[78] One of the major effects of IL-7 on naïve T cells is the inhibition of programmed cell death. In this manner, IL-7 acts as a trophic factor for mature T cells, similar to the effects observed on developing B and T lymphocytes - partly through the upregulation of Bcl-2 family molecules[63-65] and potentially through the upregulation of the T-cell survival factor, lung Kruppel-like factor.[67] Not surprisingly then, IL-7 enhances T-cell survival in long-term cell cultures and, in some studies, IL-7 was shown to be superior to IL-2 in this regard.[69] The survival of T-cells in general is increased by IL-7 inhibiting apoptosis. Furthermore, IL-7 plays an important role in the case of naïve T cells. Thus, in animals, naïve cells seem to be kept alive in absence of antigen by continuous interaction of self-peptides bound to MHC and exposure to IL-7.[75,76] In particular, IL-7 has been found to be present in secondary lymphoid tissue, a location that is part of naïve $CD4^+$ T-cell circulation. When naïve $CD4^+$ T-cells are removed from their micro-environment, they quickly die. $CD4^+$ T cells bear the IL-7R on their cell surface and have been shown to be maintained in vitro for up to 15 days in the presence of IL-7, suggesting that it is a survival factor for these cells.[78]

IL-7 has also been shown to drive the activation and proliferation of resting $CD4^+$ and $CD8^+$ T-cells along a pathway that is independent of IL-2.[79] It also directly enhances cytolytic function of mature $CD8^+$ T-cells. Moreover, recent in vitro studies in mice indicate that IL-7 restores immune competence by a thymus-independent pathway.[65] It increases peripheral expansion of T lymphocytes by inhibiting apoptosis and enhancing the survival of T cells, as well as increasing the antigenic capacity of APCs. Thus, in patients with AIDS, the plasmatic level of IL-7 is higher than in healthy donors and has been shown to be inversely correlated with the level of $CD4^+$ T cells. This possibly reflects an attempt to restore normal T cell levels, since these high levels of IL-7 decrease under ART when T cell counts increase.[80]

3.1.2.3 IL-7 and memory T cells

The combination of enhanced costimulation and programmed cell death inhibition by IL-7 is probably responsible for the role of IL-7 in facilitating memory T-cell differentiation in vivo. Unlike IL-15, which is absolutely required for the development of memory T-cell populations, the absence of physiologic levels of IL-7 leads to a significant reduction in the number of

memory T cells generated following a primary antigenic stimulus in vivo.[43,68] It is not known as to whether supra-physiologic doses administered at the time of primary antigen exposure can actually increase the number of long-term memory cells generated in vivo, but such studies will be important in determining the potential role of IL-7 as a vaccine adjuvant. By similar mechanisms as in the case of naïve T cells, IL-7 might promote cell survival of memory cells as well. For example, increased survival was found to be concomitant with the upregulation of anti-apoptotic protein Bcl-2 expression and memory cell markers in total $CD4^+$ cells. The requirement of IL-7 for $CD4^+$ memory T-cell generation and persistence has been confirmed in recipient mice which were rendered deficient in IL-7 through a blocking IL-7 antibody.[81,82]

3.1.3 BAFF, APRIL and their receptors BAFF-R, TACI and BCMA

BAFF (B cell-activating factor of the TNF-family; also termed B-lymphocyte stimulator, BLyS; TALL-1, THANK, zTNF4) and ***APRIL*** (A PRoliferation-Inducing Ligand, also termed TRDL-1, TALL-2) are recently discovered members of a new subfamily of TNF-like ligands that are expressed in haematopoietic cells *(Figure 3.2).*[83-90] They have two common receptors: Transmembrane activator and calcium modulator cyclophilin ligand interactor (***TACI***) and B-cell maturation antigen (***BCMA***). The third receptor, ***BAFF-R*** (or BR3) is a specific receptor to BAFF only. The existence of a specific receptor to APRIL is suspected, but has yet to be proven. Until now, these proteins have been extensively, but almost exclusively, studied in B cells. They have been shown to provide essential survival signals to activated B cells by inducing upregulation of antiapoptotic proteins such as Bcl-2, by down-regulation of proapoptotic proteins such as Bim, and possibly through blockade of the Fas-dependent apoptosis pathway.[92]

3.1.3.1 BAFF

BAFF is a homotrimeric protein. It is either expressed as a type II transmembrane protein on the cell surface, or is released in a soluble form after cleavage by furin-type proteases. It is produced by macrophages, monocytes and dendritic cells.[93,94] BAFF is not expressed by B cells and its expression on T cells remains controversial. Indeed, very little is known about BAFF in T cells. However, one group has demonstrated efficient TcR stimulation through BAFF and anti-CD3 in the absence of anti-CD28. They concluded that BAFF might thus have

a co-stimulatory role in T cells.[95,96] In some cases, BAFF may act in an autocrine manner.[93,94] It has also been reported that BAFF and APRIL form biologically active heterotrimers.[97]

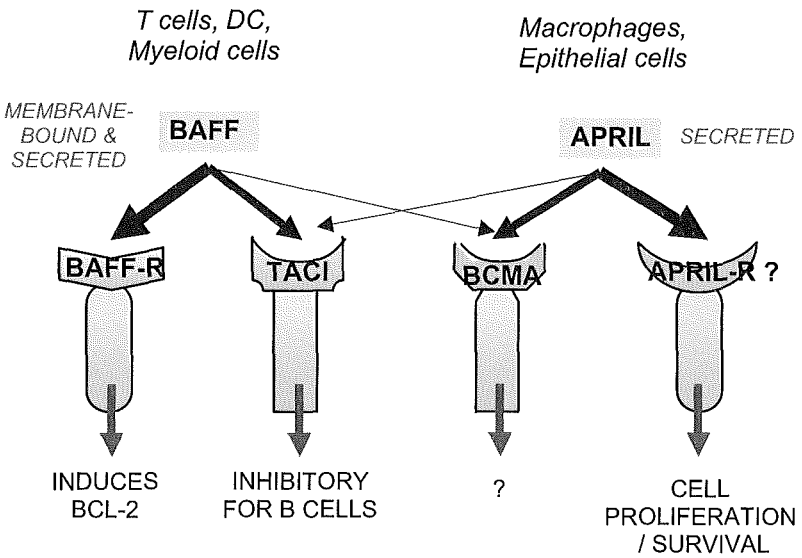


FIGURE 3.2 Overview of the TNF-like-ligands BAFF and APRIL and their receptors.

The TNF-like ligand BAFF binds specifically to it receptor BAFF-R, thereby inducing Bcl-2 and peripheral B-cell survival. BAFF also interacts with the receptor TACI and, to a lesser extent, with BCMA, both of which bind APRIL as well. APRIL-R may be a specific, hypothetical receptor to APRIL.
(Note: Figure adapted from Vaux, D.L.[91])

3.1.3.2 APRIL

APRIL shares a very similar amino-acid sequence with BAFF, making it probable that the two ligands share biological activities. APRIL transcript levels are reported to be low in normal tissues, among which the highest levels are found in peripheral blood leucocytes.[98] In contrast, much higher mRNA levels have been detected in tumor cell lines and in a variety of primary tumor tissues.[98-101]

It has been shown that APRIL promotes proliferation in human T cell lines, such as Jurkat cells and also in NIH-3T3 cell lines.[98] In normal T cells, several reports propose a role for APRIL as a T-cell stimulator. For instance, anti-APRIL antibodies have been shown to prevent T-cell stimulation in vitro.[102-104] T cells derived from APRIL transgenic mice show significantly increased proliferation after in vitro activation; this was especially evident after stimulation with anti-CD3anti-CD28.[105] The proliferative effect induced by APRIL could be mediated by TACI, whose cell surface expression is upregulated following T-cell activation.[106]

Apart from having an effect on proliferation, transgenic APRIL-expressing T cells have a highly significant increase in in vitro survival under conditions in which cells are deprived of survival factors and receive suboptimal or no activation signals. This coincides with increased Bcl-2 expression in the same cells, which would explain the protection against growth factor deprivation-induced apoptosis.[107] APRIL also seems to induce resistance to apoptosis by upregulating X-linked inhibitor of apoptosis (XIAP), a member of the inhibitor of apoptosis (IAP) family[101] *(Figure 1.4, General Introduction).*

3.1.3.3 BCMA, TACI and BAFF-R

The signal transduction pathways used by BCMA and TACI have not been fully characterized. BCMA binds TNF receptor-associated factors TRAF 1, 2 and 3, which appear to mediate NF-κB, p38, mitogen-activating protein kinase (MAPK) and c-Jun NH2-terminal kinase (JNK) pathway activation. TACI engagement, on the other hand, results in activation of NF-κB, AP-1 and NF-AT via TRAF 2, 5 and 6.[106]

3.2 GENES OF THE APOPTOSIS PATHWAY

The apoptosis pathways contain many control points regulated by either anti- or proapoptotic proteins. The mitochondrial pathway is closely regulated by proteins belonging to the ***Bcl-2 protein family***. They have been classified into three functional groups. Members of the first group, such as ***Bcl-2*** and ***Bcl-xL***, possess anti-apoptotic activity and confer protection from cell death. In contrast, groups II and III consist mainly of pro-apoptotic proteins, such as ***Bax***, Bak and Bim and promote cell death. The ratio of death antagonists to agonists determines whether a cell will respond to an apoptotic signal. In part, this ratio is determined by competitive dimerization between selected antagonists and agonist molecules which form heterodimers. In this manner, Bcl-2 protein family members can intimately regulate cytochrome c release from the mitochondria and either induce or inhibit apoptosis[32,108-110] *(Figure 1.4, General Introduction).*

3.2.1 Anti-apoptotic genes: Bcl-2, Bcl-xL and FLIP

3.2.1.1 Bcl-2

The Bcl-2 proto-oncogene is a cytoplasmic protein that contains two evolutionarily conserved domains, termed BH1 and BH2. It is the founding member of the large Bcl-2 protein family and is one of the most well characterized survival proteins in the immune system. It is essential for the survival of both B and T cells.[108] Thus, it has been shown that overexpression of Bcl-2 in lymphocytes leads to an increased resistance to irradiation-induced death in vitro.[111] Bcl-2 prevents apoptosis by inhibiting the release of mitochondric proteins into the cytosol.[112] It can act either directly on its own as a suppressor of programmed cell death, or indirectly by interacting with the pro-apoptotic protein Bax. By forming heterodimers with its homologue, Bcl-2 binds and inhibits Bax, thereby indirectly promoting cell survival.

3.2.1.2 Bcl-xL

Similarly to Bcl-2, Bcl-xL also protects cells from multiple apoptotic stimuli.[113-114] Apart from its role in thymic development,[115-116] where it protects $CD4^+$ $CD8^+$ DP cells from apoptosis, it has been documented to increase the half-life of peripheral mature T cells.[117]

3.2.1.3 FLIP

FLIP belongs to a novel family of apoptosis inhibitors,[118] also termed FLICE/caspase-8 inhibitory proteins. v-FLIP was first discovered as an anti-death receptor-induced apoptosis factor in γ-herpesvirus. The cellular homolog, c-FLIP (also CASH, Casper, CLARP, FLAME, I-FLICE, MRIT or usurpin) has at least two splice variants, the long 55-kDa form (c-$FLIP_L$) and the short 26-kDa form (c-$FLIP_S$). $FLIP_L$ is expressed in many tissues, most abundantly in heart, skeletal muscle, lymphoid tissue and kidney; $FLIP_S$ is additionally detectable in lymphatic tissue.

FLIP inhibits apoptosis by interacting with caspase-8. Caspase-8 is the key initiator caspase downstream of the extrinsic apoptosis pathway induced by Fas and other death receptors *(Figure1.4, General Introduction)* and interacts with FADD through homologous death effector domains. Aggregation and proximity result in the activation of caspase-8 by processing the caspase domain into two active subunits. FLIP is structurally similar to

caspase-8 and also possesses death effector domains (DED), as well as a caspase-like domain. However, it lacks caspase enzymatic activity. Thanks to its structural homology with caspase-8, FLIP competes with capase-8 at the level of the DISC (death-inducing signaling complex). Its DED-terminal enables FLIP to interact with the Fas-associated adaptor molecule FADD and be recruited to the Fas/FasL complex in the place of caspase-8. By these means, FLIP impedes the progression of apoptotic signals and blocks activation-induced cell death (AICD). AICD is an important mechanism for T cell homeostasis, because it intervenes after the expansion phase triggered by antigen-recognition and induces a phase of increased cell-death which counterbalances increased T cell numbers. Additionally, FLIP enables the recruitment of multiple signaling molecules to the DISC, leading to the activation on NF-κB and ERK pathways, thus also accounting for a pro-survival effect.

Besides its anti-apoptotic function, FLIP is suspected to play an important role in embryonic development. FLIP knock-out mice do not survive past day 11 of embryogenesis and show signs of cardiac failure associated with impaired heart development.[119]

3.2.2 Pro-apoptotic genes: Bax and Bad

3.2.2.1 Bax

Bax is a protein with a molecular weight of 21 kDa and plays an important role in the regulation of cell death in a number of eukaryotic cells. The over-expression of Bax has been shown to accelerate cell death. The ratio of Bax to other Bcl-2 family members and its subcellular distribution are thought to help regulate the process of programmed cell death. It can be directly inhibited by Bcl-2 with which it forms heterodimers.[108,120]

3.2.2.2 Bad

Bad is a promoter of apoptosis that has been shown to dimerize with the anti-apoptotic proteins Bcl-2 and Bcl-xL. It is supposed to bind these anti-apoptotic members of the Bcl-2 family through its BH3-domain. In this manner, Bad inhibits the survival promoting functions of these proteins and promotes cell death.[32,108]

3.2.2.3 *Fas-Ligand*

Fas-ligand (FasL) is the ligand to the death-receptor Fas (CD95). FasL-Fas interaction is the first step in the extrinsic pathway *(Figure 1.4, General Introduction)* and induces the clustering of several death-receptors on the cell membrane and recruitment of the Fas-associated death domain adapter protein (FADD). FADD is a bipartite molecule with a death-effector-domain (DED) N-terminal and a death-domain (DD) C-terminal. It binds to Fas by means of homophilic DD-DD interactions and recruits caspase-8 by the means of DED-DED interactions. This results in the formation of a death-inducing signaling complex (DISC), enabling neighboring caspase-8 molecules within this newly formed complex to proteolytically activate each other. Subsequently, apoptosis is initiated by cleavage and activation of the downstream effector caspases, such as caspase-7, -6 and -3.[121]

Stimulation of the TcR in conjunction with appropriate co-stimulation, not only results in the activation and differentiation of T cells, but also renders them susceptible to Fas-mediated apoptosis. In fact, T-cell activation leads to the increased expression of Fas and FasL,[122] permitting the activated T cells to eliminate neighboring Fas-positive cells. By this mechanism, the expression of Fas and FasL is crucial to T cell homeostasis. Indeed, mice deficient in Fas or FasL show massive lymphoadenopathy and disruption of lymphocyte homeostasis due to a lack of programmed cell death. Interestingly, however, $CD4^+$ memory T cells are more resistant to Fas-induced death in vitro.[123,124]

3.3 ANTI-PROLIFERATIVE GENE: TOB

Proliferating cells undergo mitosis, e.g. a parent cell replicates its DNA to form genetically identical daughter cells. Mitogenic signaling allows cells to progress from a non-dividing, quiescent state (G_0) through the G_1/S phase of the cell cycle. Once they have crossed this restriction point and have entered the S phase, which corresponds to the DNA replication phase, cells are programmed to divide and undergo mitosis. Many genes control cell cycle progression, and abnormalities in this control contribute to the development of cancer. Thus, cellular homeostasis is tightly regulated by systematic activation and/or inactivation of proliferative and antiproliferative genes.

Tob is a member of the BTG anti-proliferative protein family, which also includes B cell translocation gene (BTG1 and BTG2), abundant in neuroepithelial area (ANA) and Tob2.

These proteins all share structural and functional characteristics. Members of this family have been isolated from libraries of various cell types, including malignant cells, NIH3T3 and PC12 cells. In these cell types, BTG-family proteins are rapidly induced in response to growth factors and tumor promoters.[125] On the functional level, however, these gene products, when overexpressed, suppress cell growth by inhibiting cell cycle progression.[126] Another important common feature is that the proteins of this family are associated with transcription factors such as Caf1, Hoxb9 and Smad.

Mice lacking the Tob gene frequently develop tumors and show an osteopetrotic phenotype that is caused by the facilitated growth and differentiation of osteoblasts.[127] All this strongly suggests the importance of Tob in cell growth regulation. The antiproliferative action of Tob has been analyzed by exogenously expressing Tob in NIH3T3 cells. In this case, Tob expression has been shown to inhibit cell cycle progression in the G_1 phase by suppressing cyclin D1 expression. Alternatively, Tob can be inactivated by growth-factor-induced phosphorylation, canceling cyclin D1 suppression. This phosphorylation of Tob is catalyzed by Erk1/2, thus linking MAPK activity to cyclin D1 expression.[128-129]

3.3.1 Tob and T cells

Peripheral tolerance mechanisms play an indispensable role in the maintenance of natural self-tolerance: autoimmune responses are averted and inflammatory reactions are controlled. One of the mechanisms of peripheral tolerance is induction of **anergy**. One way of achieving this state is through suboptimal TCR-triggering. The state of anergy has been shown to correspond to a blockade of the cell cycle in the G_1 phase.

In the effort to identify genes preferentially linked to an anergic state, Tob was recently shown to be selectively expressed in anergic clones. When further examining Tob expression in T cells, Tob was found to be constitutively expressed in quiescent human peripheral blood $CD4^+$ T lymphocytes. Upon stimulation with anti-CD3 and anti-CD28 antibodies, as well as after IL-2 stimulation, however, Tob mRNA was found to be downregulated to almost undetectable amounts. By expressing human Tob as a fusion protein, Tzachanis D et al.[126] could induce inhibition of anti-CD3-induced T cell proliferation. This inhibition was directly related to the levels of Tob expression. In the same manner, the fusion protein also inhibited the production of IL-2 and other cytokines.

When examining the role of Tob on a molecular level, Tob was shown to allow cells to enter into the G_1 phase of the cell cycle (as determined by Cdk4 expression). The lack of cyclin A expression, however, demonstrated that Tob concomitantly inhibited progression into the S phase. Thus, it can be concluded that Tob actively blocks the cell cycle in the G_1 phase and before the $G_1 \rightarrow S$ restriction point. Immunoprecipitation assays showed that Tob associates preferentially with Smad2 and Smad4. Smads are known to function as negative regulators of T cell activation. They mediate signals induced by TGF-β superfamily members, resulting in inhibition of anti-CD3- and anti-CD28-mediated stimulation of T cells. Tob and Smad interaction may enhance Smad binding to the negative-regulatory element of the IL-2 promotor, thus blocking IL-2 transcription *(Figure 3.3)*. Hence, Tob may actively maintain the resting state of T cells by preventing cell cycle progression. If this is the case, the repression of Tob would be mandatory for T cell activation.

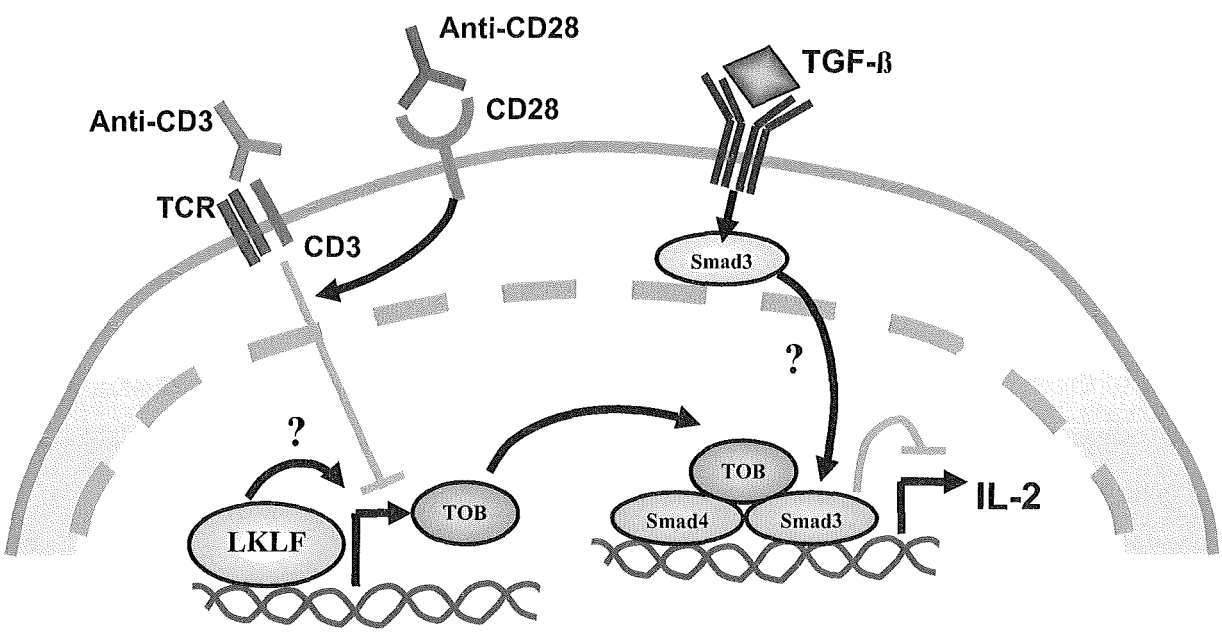


FIGURE 3.3 Hypothetical role of Tob in maintenance of T cell quiescence and possible connection of Tob to the TGF-β signaling pathway.

Tob has been shown to be selectively expressed in quiescent T cells and to interact preferentially with Smad2 and Smad4. The Smad proteins bind the IL-2 promoter and may therefore also recruit Tob to the promoter, enhancing the inhibition of IL-2 transcription. Thus, Tob may actively maintain the quiescent state of T lymphocytes. Anti-CD3 and anti-CD28 stimulation, however, could turn off the expression Tob. This would relieve the repression of IL-2 transcription and promote T cell activation. It remains to be determined whether lung-Krüppel-like-factor (LKLF) induces the expression of Tob.
(Note: Figure adapted from Tzachanis D et al.[126]).

4 MATERIALS AND METHODS

4.1 SUBJECTS

Buffy coats from healthy blood donors were obtained from the Lausanne Blood Transfusion Centre. Each healthy donor was identified by the prefix LHD, followed by a number. Samples were screened by flow cytometry for the percentages of $CD4^+$ and $CD8^+$ T cells prior to use. Subjects with adequate cell numbers and subpopulation percentages for sorting were selected. Thus, four different subjects (LHD 216, 234, 283 and 288) were used for the analysis of gene expression in $CD8^+$ cells, and three subjects (LHD 216, 283 and 288) were chosen for analysis of $CD4^+$ T cells.

4.2 MATERIALS AND SOLUTIONS USED

Ficoll-PAQUE Plus *(Amersham, Pharmacia Biotech AB)*

PBS:	Phosphate-buffered saline 1x	*(CHUV Pharmacy)*
EDTA:	Ethylenediaminetetraacetic acid	*(SIGMA)*
FBS:	Fetal Bovine Serum	*(GIBCO BRL, Life Technologies)*
Cell Culture Medium:		
	-RPMI 1640 Glutamax-1 medium	*(GIBCO BRL, Life Technologies)*
	-Penicillin 0.1%	*(GIBCO)*
DMSO:	Dimethyl-sulphoxide	*(SIGMA)*

Freezing solution: 52.5% Cell culture medium, 40% FBS, 7.5% DMSO

P2: PBS 1x, 2% FBS

R10: Cell culture medium 1x, 10% FBS

R20: Cell culture medium 1x, 20% FBS

4.3 ISOLATION, PRESERVATION AND THAWING OF CELLS

Peripheral blood mononuclear cells (PBMC) from healthy donors were separated by density gradient centrifugation. For this, the buffy coats were washed with 100 mL of PBS containing EDTA at a 2 Mm concentration. 25 ml of the solution was gently added over 15 ml of Ficoll-

Paque Plus and centrifuged for 20 min at 836 g without the brake, so as not to disturb the layering. Differential sedimentation during centrifugation resulted in the formation of layers containing different blood cell types. Because of their low density, lymphocytes gather at the interface between the plasma (lowest density) and the Ficoll-Paque (highest density) along with other slowly sedimenting cells, such as platelets and monocytes. The white disk corresponding to the PBMC was recuperated with a Pasteur pipette and cells were washed 2 times with PBS-FBS 2% to remove any platelets. Cells were counted and re-suspended in freezing solution, containing medium, FBS and DMSO. Aliquots of approximately 10 million cells per vial were preserved at -80 °C in liquid nitrogen. Each buffy coat delivered between 120-250 million cells.

Before use, the cryo-preserved PBMC were thawed by putting them in a water bath at 37.5°C and adding R20 solution drop-by-drop, while swirling the cells continuously. Cells were spun down at 677 g and the supernatant was discarded. Subsequently, the cells were washed with R10 and P2 solutions. The recuperation rates after thawing were between 60-100%.

4.4 STAINING AND SORTING OF MEMORY T CELL SUBPOPULATIONS

4.4.1 Staining

PBMC were thawed, washed and re-suspended in PBS-FBS 2% before staining. Cells were stained with the required fluorochrome-labelled primary monoclonal antibodies *(Table 1)*.

Antibody-Fluorochrome	Q
CD4-PE	10
CD8-PE	10
CD45RA-CyChrome	15
CD45RO-FITC	15
CD4-FITC	15
CD8-FITC	12
Pure CCR7	0.5
Goat anti-rat IgG-PE	5

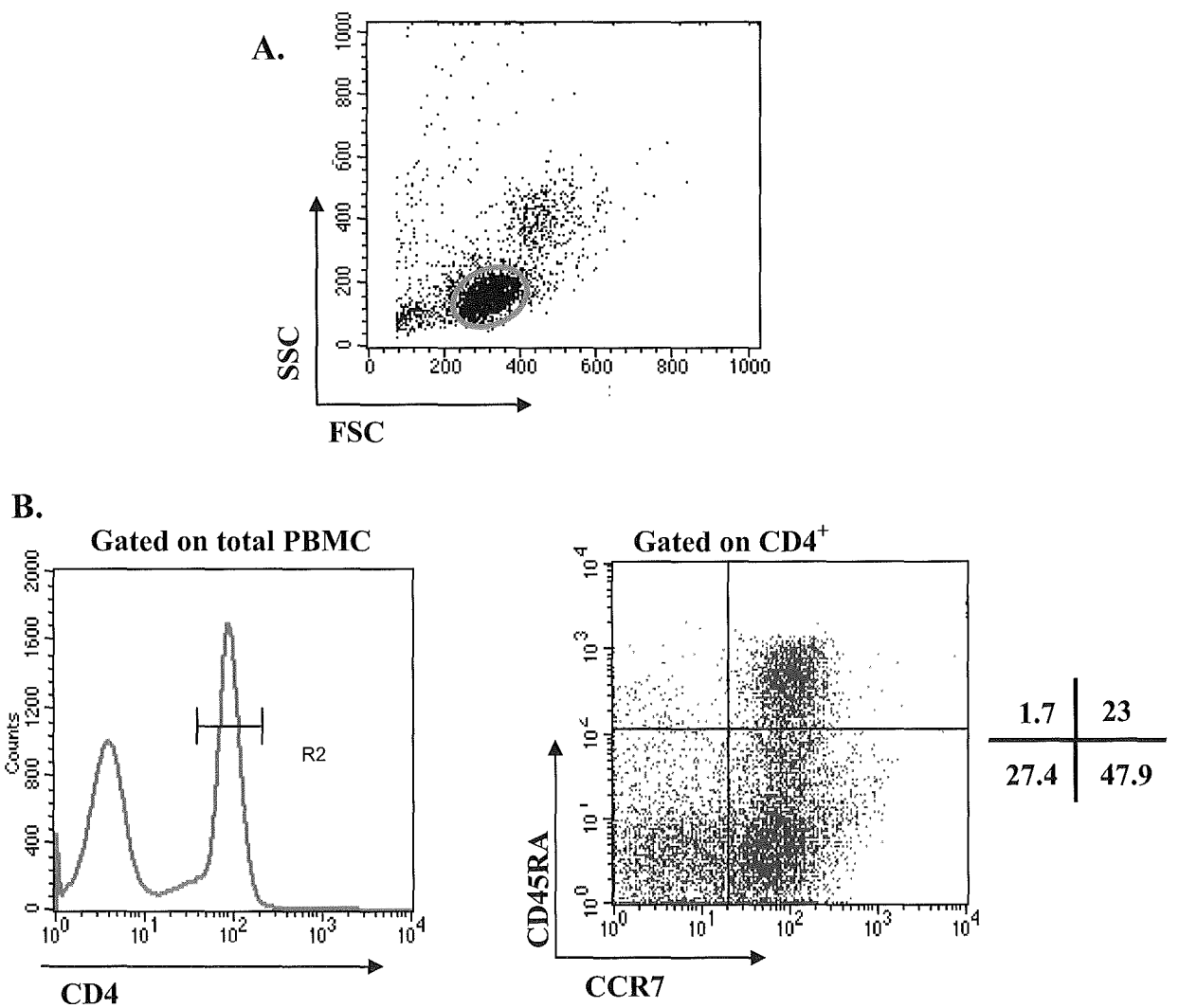
TABLE 1 Antibodies used in this study for the FACS-Vantage

All antibodies were rat anti-human (if not indicated otherwise) and purchased from BD.
(Q: quantity in µl per million cells)

Each layer was incubated for 30 minutes at 4°C in the dark, washed and then re-suspended in P2 solution at a concentration of approximately 3 million cells per ml after staining and sorted immediately. The anti-CCR7 staining was indirect and revealed with PE-conjugated goat anti-rat IgG.

4.4.2 Sorting and FACS analysis

Cells were sorted on a FACS-Vantage flow cytometer *(Becton Dickinson Immunocytometer Systems)* using FITC, PE and CyChrome as fluorescent colors. Screening of donors was performed by 6-parameter flow cytometric analysis on a FACS-Calibur flow cytometer *(Becton Dickinson Immunocytometer Systems)*, using FITC, PE, CyChrome or CyChrome Cy5.5 and APC as fluorescent parameters *(Figure 4.1)*. BD CellQuest Pro Software was used for data acquisition and analysis.



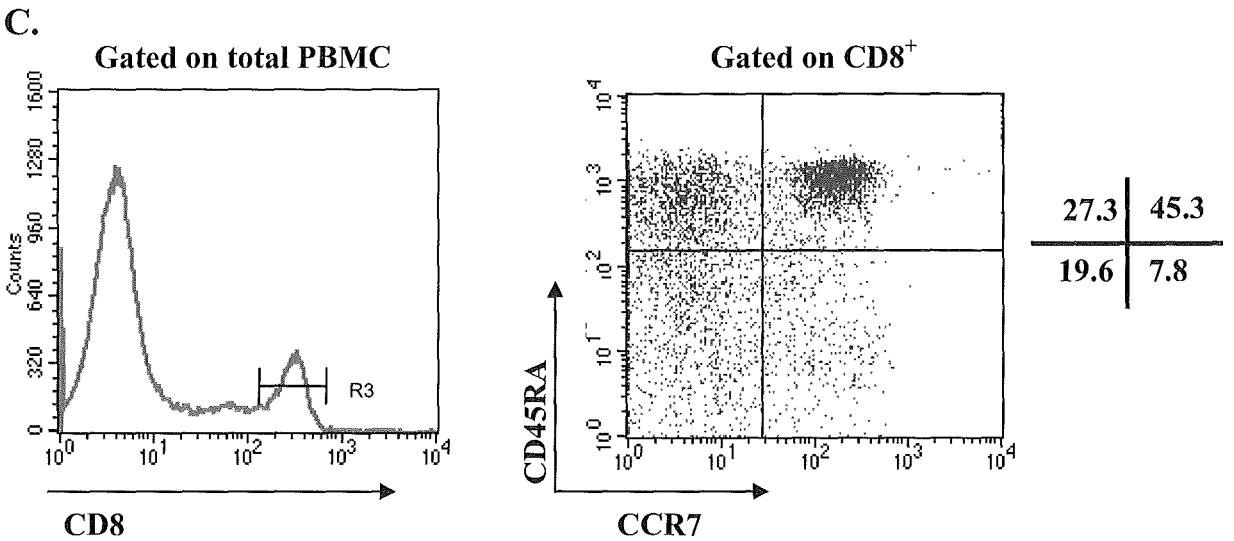


FIGURE 4.1 Sorting of human T cell populations by flow-cytometry.

A: The encircled population of PBMC corresponds to lymphocytes

B and C: $CD4^+$ and $CD8^+$ T cells are separated into 4 populations consisting of: (1) **Naïve, $CD45RA^+$ $CCR7^+$** T cells (upper right quadrant); (2) **Central memory, $CD45RA^-$ $CCR7^+$** T cells (lower right quadrant); (3) **Effector memory $CD45RA^-$ $CCR7^-$** T cells (lower left quadrant) and (4) **Terminal effector memory $CD45RA^+$ $CCR7^-$** T cells (upper left quadrant). The percentages of each population are noted in the grid.

4.5 REVERSE TRANSCRIPTION AND REAL-TIME PCR

Reverse transcription-polymerase chain reaction (RT-PCR) is an in vitro method for enzymatically amplifying defined sequences of RNA. It is one of the most sensitive and flexible methods to quantify transcription levels of genes and capable of detecting mRNA from as little as a single cell. It can be used to compare levels of mRNAs in different sample populations and to characterize patterns of mRNA expression. Total RNA is used as a template for a reverse transcription reaction using an enzyme (reverse transcriptase, RT) that transcribes RNA into cDNA (complementary DNA). The cDNA is subsequently used in a polymerase chain reaction (PCR), together with primers which will specifically hybridize with the sequence of interest and amplify it. The amplified sequence can then be detected by either using a non-quantitative method (e.g. gel electrophoresis) or in a quantitative analysis.

4.5.1 RNA extraction

RNA extraction was performed using the MiniRNEasy Kit *(QIAGEN)* according to the manufacturer's instructions. To be sure to have the same number of cells per extraction, 15'000 cells of each T cell subpopulation were sorted separately by the FACS Vantage into Eppendorffs containing 200 µl of FBS and 300 µl of cell culture medium. Cells were spun down, the supernatant was discarded and the remaining cell-pellet was re-suspended in PBS-FCS 2% previous to RNA extraction. Extracted total RNA was eluted in 12 µl of nuclease-free water and stored at minus 56°C (-56°C).

4.5.2 Reverse Transcription (RT Reaction)

First strand complementary DNA was synthesized in a final volume of 50 µl using the TaqMan® RT-kit (*Applied Biosystems, TaqMan Reverse Transcription Reagents – P/N N808-0234).* Cryo-preserved RNA was heated for 1 minute at 65°C, put on ice and immediately added to the RT-mixture. The RT-mixture contained RT-Buffer, $MgCl_2$, deoxyribonucleoside triphosphate mixture (dNTPs), Random Hexamers, Ribonuclease Inhibitor, MultiScribe Reverse Transcriptase and nuclease-free water and was aliquoted under a PCR hood previous to adding RNA. Samples were incubated at 25°C for 10 minutes, elongation was performed at 48°C for 30 minutes and enzyme was deactivated at 95°C for 5 minutes. Each sample contained total RNA from 15'000 sorted cells. A stock RNA from total $CD4^+$ T cells, with subsequent C-alpha PCR amplification, was used as a control for the RT step.

4.5.3 Principles of Real-Time PCR

A basic PCR run can be split into three phases. In the beginning, assuming 100% reaction efficiency, there is exact doubling of the product at every cycle (exponential phase). In the linear phase, the reaction components are consumed and the reaction slows down. In the plateau phase, the reaction has stopped; no more products are being made and, if left long enough, the PCR products will degrade.[130] Traditional PCR measures the product at the end-point (plateau phase) by gel detection. However, quantification at this point is inaccurate because amplification is only exponential in the initial phase, when all the reagents are fresh and available *(Figure 4.2).*

For accurate quantitation, it is necessary to detect the PCR product in the initial phase of the reaction. This was achieved by incorporating fluorescence techniques into the PCR reaction, such as with the TaqMan Probe *(Figure 4.3)*. The increase in fluorescent signal is directly proportional to the number of amplicons generated. Thus, it is possible to monitor the real-time progress of the the PCR and collect data in the exponential growth phase. The fluorescent signal, proportional to the amplification, is captured by a Sequence Detection instrument and displayed as a plot of signal versus cycle number (amplification plot). In the initial cycles of PCR, there is little change in the fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold *(Figure 4.4)*. The CT number is inversely proportional to the log of initial copy number. Therefore plotting C_T values versus the log of serial dilutions of a sample of known concentration gives a straight line. This is used as a standard curve from which the quantity of an unknown sample can be derived. For relative quantitation the C_T number can be used in an arithmetic formula (Comparitive C_T method). [131]

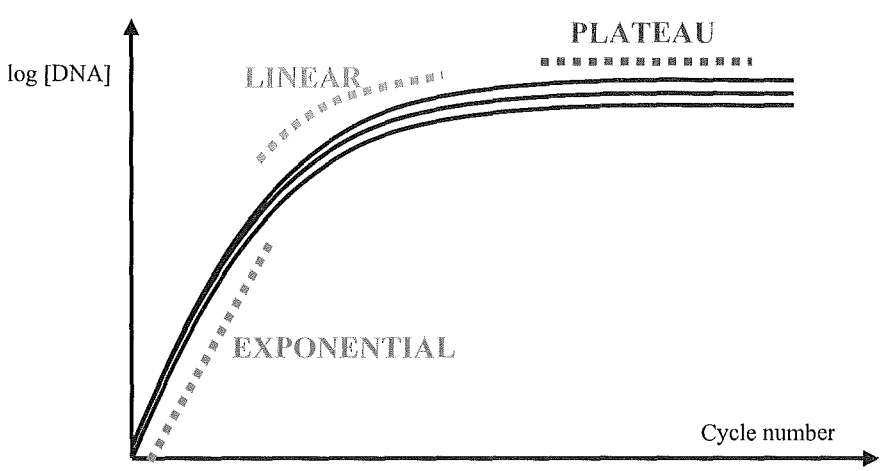


FIGURE 4.2 PCR – Phases

Exponential Phase: Exact doubling of the product at every cycle (assuming 100% reaction efficiency).
Linear Phase: The reaction components are consumed, the reaction slows down and products start to degrade.
Plateau Phase: The reaction has stopped, no more products are being made and, if left long enough, the PCR products will degrade.

Three samples, identical in terms of the initial amount of DNA, are considered to be amplified separately. Fluctuations in the quantification of the amplified DNA will be considerably larger at the end of the reaction than in the initial phase in which the amplification is exponential.

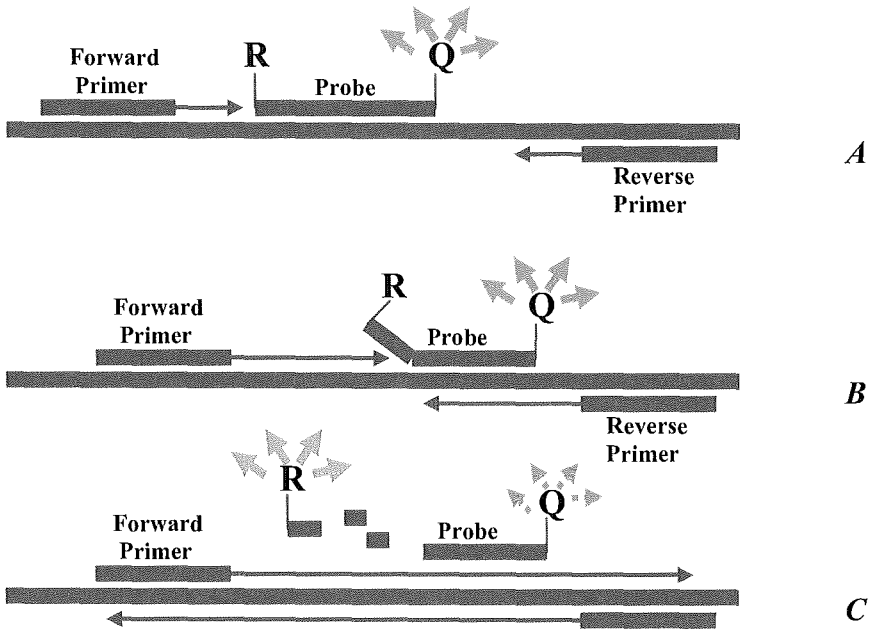


FIGURE 4.3 Cleavage of the TaqMan Probe

The TaqMan probe consists of a high-energy reporter dye at the 5' end (R) and a low-energy molecule termed quencher (Q) at the 3' end; it is designed to anneal to the template between the primers. When the probe is intact and excited by a light source, the reporter dye's emission is suppressed by the quencher dye as a result of the close proximity of the dyes *(A)*. The probe sits on the path of the Taq polymerase as it starts to replicate the cDNA *(B)*. The probe is cleaved by the 5' exonuclease activity of the Taq Polymerase, so that the distance between the reporter and the quencher increases, causing the transfer of energy to stop. The fluorescent emission of the reporter dye increases and that of the quencher decreases *(C)*.

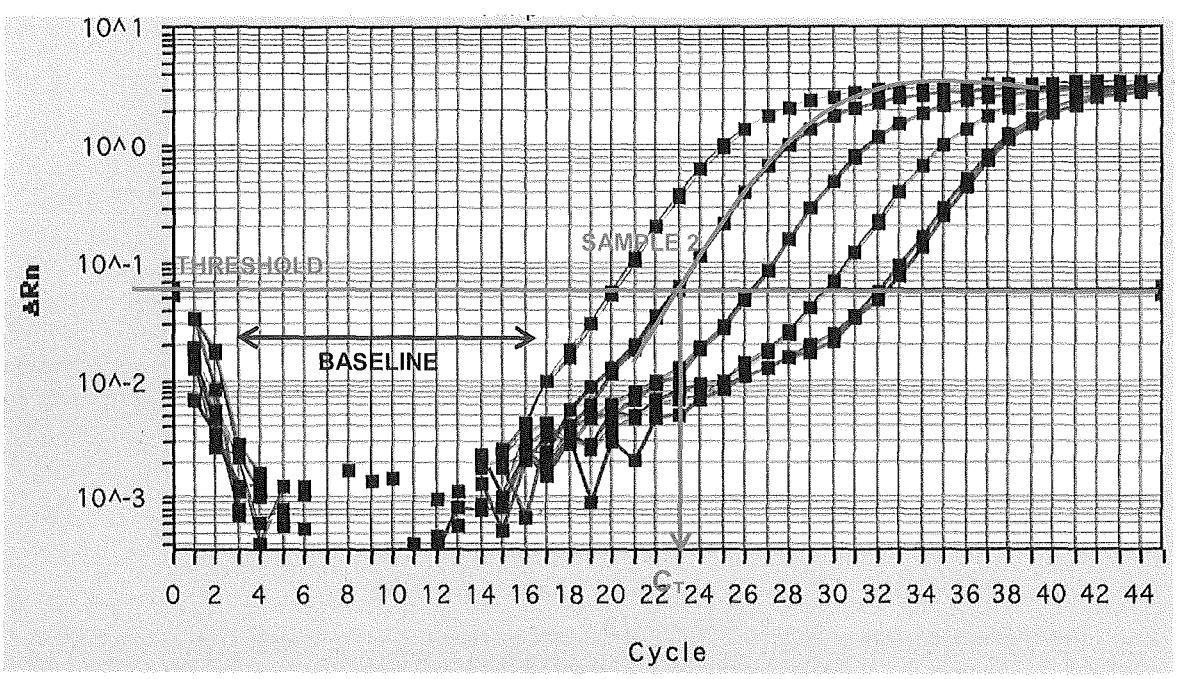


FIGURE 4.4 Amplification plot

The example depicted is merely illustrative and shows a threshold at 0.06. The threshold is set above the background signals and in such a way as to clearly distinguish the distinct sample amplifications, i.e. it has to cross the amplification plots in the exponential phase. In the actual experimental conditions the threshold was set at 0.02 for all experiments.

4.5.4 Materials and Methods for the Real-Time PCR

DNA amplification was carried out in a final volume of 25 µl using the TaqMan® Universal PCR Master Mix *(Applied Biosystems, P/N 4304437)* according to the manufacturer's instructions. Primers and FAM- and TAMRA-labeled probes were purchased from Applied Biosystems and either chosen from the Assay by Demand catalog, with the sequences chosen by the manufacturer, or designed and ordered as an Assay by Design *(Table 2)*. The master mix and primers were seeded into MicroAmp® Optical 96-well plates *(Applied Biosystems, P/N 403012)* under a PCR hood in the dark. 1.5 µl of cDNA (equivalent to the RNA of 450 cells) was added per well, and duplicates of each gene per population *(Figure 4.5)* were performed. The plate was sealed with MicroAmp® Optical Caps. The following Thermal Cycler conditions were used on a Taqman ABI Prism 7700 Sequence Detector: 50°C for 2 minutes and 95°C for 10 minutes (incubation and AmpliTaq Gold enzyme activation). Each run consisted of 45 cycles (of denaturing at 95°C for 15 seconds and annealing at 60°C for 1 minute).

GENE (human)	1) 5' - forward primer - 3' 2) 5' - reverse primer – 3' 3) 5' - (FAM)-probe-(TAMRA) - 3'
C-α	1) ATGTGCAAACGCCTTCAACAA 2) GCTTGACATCACAGGAACTTTCTG 3) CAGCATTATTCCAGAAGACAC
APRIL	1) TGCACCTGGTTCCCATTAACG 2) GTTGCCACATCACCTCTGTCA 3) CATCGGAGTCATCCTTG
IL15-Rα	1) TGACCCCACAGCCAGAGA 2) TGTTGTTTGAGCTGGGAGATGAAG 3) CTGCGGGCTCTTTT
IL7-Rα	1) GTGTGAAGGTTGGAGAAAAGAGTCT 2) GACGACACTCAGGTCAAAAGGA 3) ACCTAACCACTATAGTTAAACCT
BAFF-R	1) CCCCGACGGAGACAAGGA 2) TCAGAGATTCCCGGAGACAGAA 3) CCCCAGAGCCCCTGGACA
EF1-α	1) CAATTTCTGGTTGGAATGGTGACAA 2) GGGTGACTTTCCATCCCTTGAA 3) CCAAGGCATGTTAGCACTT

TABLE 2 Sequences of the sets of the primers and probes ordered as Assay by Design

C-α: gene for the constant part of the TCR-chain. **APRIL:** TNF-like ligand. **IL-7Rα:** Interleukin-7 Receptor chain alpha. **IL-15Rα:** Interleukin-15 receptor chain alpha. **BAFF-R:** BAFF-Receptor, TNF-like ligand

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	**Posit. Contr.**	**Posit. Contr.**			NTC **T 7**	NTC **T 7**	Naïve **T 7**	Naïve **T 7**	CM **T 7**	CM **T 7**
B	NTC **C α**	NTC **C α**	Naïve **C α**	Naïve **C α**	CM **C α**	CM **C α**	NTC **T 8**	NTC **T 8**	Naïve **T 8**	Naïve **T 8**	CM **T 8**	CM **T 8**
C	NTC **T 1**	NTC **T 1**	Naïve **T 1**	Naïve **T 1**	CM **T 1**	CM **T 1**	NTC **T 9**	NTC **T 9**	Naïve **T 9**	Naïve **T 9**	CM **T 9**	CM **T 9**
D	NTC **T 2**	NTC **T 2**	Naïve **T 2**	Naïve **T 2**	CM **T 2**	CM **T 2**	NTC **T 10**	NTC **T 10**	Naïve **T 10**	Naïve **T 10**	CM **T 10**	CM **T 10**
E	NTC **T 3**	NTC **T 3**	Naïve **T 3**	Naïve **T 3**	CM **T 3**	CM **T 3**	NTC **T 11**	NTC **T 11**	Naïve **T 11**	Naïve **T 11**	CM **T 11**	CM **T 11**
F	NTC **T 4**	NTC **T 4**	Naïve **T 4**	Naïve **T 4**	CM **T 4**	CM **T 4**	NTC **T 12**	NTC **T 12**	Naïve **T 12**	Naïve **T 12**	CM **T 12**	CM **T 12**
G	NTC **T 5**	NTC **T 5**	Naïve **T 5**	Naïve **T 5**	CM **T 5**	CM **T 5**	NTC **T 13**	NTC **T 13**	Naïve **T 13**	Naïve **T 13**	CM **T 13**	CM **T 13**
H	NTC **T 6**	NTC **T 6**	Naïve **T 6**	Naïve **T 6**	CM **T 6**	CM **T 6**	NTC **T 14**	NTC **T 14**	Naïve **T 14**	Naïve **T 14**	CM **T 14**	CM **T 14**

FIGURE 4.5 Example of a plate setup

NTC: no template control (no cDNA); **Posit. Contr.:** Positive Control; **Cα:** Endogenous control; **T1-T14:** target genes; **Naïve:** Naïve T cells **CM:** central memory subset

4.6 QUANTIFICATION OF GENE EXPRESSION

A semi-quantitative approach using the Comparative Threshold-Cycle (C_T)-Method[131] was used for the quantification of target gene expression. Cα was chosen as an endogenous control to normalize the amount of target messenger RNA for differences in the amount of total RNA added to each reaction. The naïve T cell population was used as the basis for comparative results (calibrator). Each target, plus a negative control, was amplified in a separate well on a 96-well plate. Each plate contained the amplification of the endogenous control in separate wells, with a negative as well as a positive control *(Figure 4.4)*. Amplification plots were generated by the system, and C_T-values for each well were read at a threshold level of 0.02 for the sample plates and at an arbitrary threshold for the dilution curves.

Calculations were done by transcribing the C_T-values on an excel-sheet. C_T-values were averaged for each duplicate. Differences between the mean C_T-values of the target gene and those of the endogenous control were calculated as $\Delta C_T = C_{T\ \text{target gene}} - C_{T\ \text{C-alpha}}$ for each T cell subpopulation *(Table 3)*. Differences between the ΔC_T-values of calibrator and memory cell populations were calculated as $\Delta\Delta C_T = \Delta C_{T\ \text{naïve}} - C_{T\ \text{memory}}$ for each memory cell subpopulation. Final results, expressed as n-fold differences of target gene expression in memory subpopulations relative to the naïve T cell population, were determined as $2^{-(\Delta\Delta CT)}$.

T cell population	Average C_T of target gene	Average C_T of Cα gene	ΔC_T ($C_{T\ target\ gene}$ - $C_{T\ C\alpha}$)[a]	$\Delta\Delta C_T$ ($\Delta C_{T\ memory}$ - $\Delta C_{T\ naive}$)[b]	Target gene relative to Naïve T cells[c] $2^{-(\Delta\Delta CT)}$
Naïve	30.49 ± 0.15	23.63 ± 0.09	6.86 ± 0.17	0.00 ± 0.17	1.0 (0.9 - 1.1)
CM	27.03 ± 0.06	22.66 ± 0.08	4.37 ± 0.10	-2.50 ± 0.10	5.6 (5.3 - 6.0)
EM	26.25 ± 0.07	26.60 ± 0.07	1.65 ± 0.10	-5.21 ± 0.10	37.0 (34.5 - 39.7)
TEM	25.83 ± 0.07	23.01 ± 0.07	2.81 ± 0.10	-4.05 ± 0.10	16.5 (15.4 – 17.7)

TABLE 3 Example of Relative Quantification using the Comparative C_T Method

a: *The standard deviation from the ΔCT-value is calculated as* $s = \sqrt{(s_1^2 + s_2^2)}$. ***b:*** *The calculation of the ΔΔCT-value involves subtraction of an arbitrary constant (calibrator), so that the standard deviation of ΔΔCT is the same as that of ΔCT.* ***c:*** *The range for the final result is determined by evaluating* $2^{-(\Delta\Delta CT)}$ *with ΔΔCT + s and ΔΔCT – s.*

Note: *The numbers are taken from the User Bulletin no.2 (Applied Biosystems) and used as an illustration.*

4.7 STIMULATION EXPERIMENTS

4.7.1 Polyclonal stimulation with anti-CD3 and anti-CD28

A 96-well flat bottom culture plate was first coated with anti-CD3 diluted in PBS at 1µg/ml (100 µl per well) and incubated at 4°C overnight. Excess antibody was removed by washing the plate twice with sterile PBS. Cells were stained, sorted, counted and re-suspended in cell culture medium containing 2% AB-Serum and 1µg/ml of anti-CD28 *(unconjugated mouse-anti-human CD28, 0.5mg/ml, BD Pharmingen)* at a concentration of 100'000 cells per 100 µl. 200'000 cells per well were incubated at 37°C with 5% CO_2. At each time-point (0h, 24h, 48 h, 72h), cells were collected and resuspended in PBS-2% FBS. Viable cells were gated, and 15'000 cells were sorted by FACS Vantage into Eppendorffs. RNA was extracted immediately.

4.7.2 Homeostatic stimulation assay

A mix of the five cytokines, IL-7, IL-15, IL-10, IL- 6 and TNFα, is known to induce homeostatic proliferation in human T cells. We used the same conditions as previously published by Sallusto et al.[141]

The cytokine mix *(IL- 6, IL-7, IL-15, IL-10 and TNFα: recombinant human cytokines, R&D Systems)* was prepared at a concentration of 50 ng per ml for each cytokine in RPMI 2% AB-Serum and used to seed a 96-U-bottom culture plate with 100 µl per well. Cells were stained, sorted, counted and re-suspended in RPMI 2% AB-Serum at 500'000 cells per ml. 100 µl of cells were added to each well, thus obtaining 100'000 cells per well in a culture medium containing 25 ng per ml of each cytokine. Cells were incubated at 37°C with 5% CO_2, collected at 4 time points (day 5, 7, 9, 12) and re-suspended in PBS-2% FBS. Viable cells were gated by flow cytometry, and 15'000 cells were sorted by FACS Vantage into Eppendorffs. RNA was extracted immediately.

5 VALIDATION OF THE ASSAY

5.1 VALIDATION OF THE ENDOGENOUS REFERENCE GENE

Real-time reverse transcription (RT)-PCR[131-133] is currently one of the most sensitive methods for the detection of low-abundance mRNAs and quantification of genes with low expression levels. Quantification can either be absolute or semi-quantitative. In the semi-quantitative approach, the mRNA level of a standard in the sample can be used to normalize the expression level of a selected target gene. This normalization is often performed by measuring the nucleic acid level of an external reference molecule. However, the preparation and inclusion of synthetic DNA or RNA standards does not account for manipulations of the sample before addition of the standard. Therefore, an endogenous reference gene exposed to the same manipulations as the target gene prior to quantification, is a more natural choice. For this purpose, the expression of a constitutively expressed endogenous gene (or housekeeping gene) in the same sample is often monitored.

The ideal internal standard, or endogenous control,[134] has the following characteristics:

- When analyzing different donors, the control presents an intra- and inter-donor consistency in its expression.
- The standard should be expressed at constant levels within the cell subsets under analysis (target population consistency).
- It should be expressed at roughly the same level as the RNA under study (target gene equivalency).
- Its expression should remain unaffected by the experimental treatment (preservation of expression).

Three possible internal controls have been examined:

1) **Cα:** Cα designates the constant domain of the α-chain of the αβ-T-cell-receptor and is expressed specifically on virtually all peripheral human T cells *(Figure 5.1)*.

2) **Beta-2-microglobulin (β2m):** β2m is the small subunit of the MHC class I molecule and is associated with the outer membrane of many cells of the body,

including lymphocytes. It is a house-keeping gene that is commonly used as an internal control in semi-quantitative gene analysis.

3) **Elongation factor-1α (EF-1α):** EF-1α has been shown to be stable during human CD4+ T cell differentiation by comparing its expression in naïve human $CD4^+$ T cells to that of in vitro activated T cells.[134]

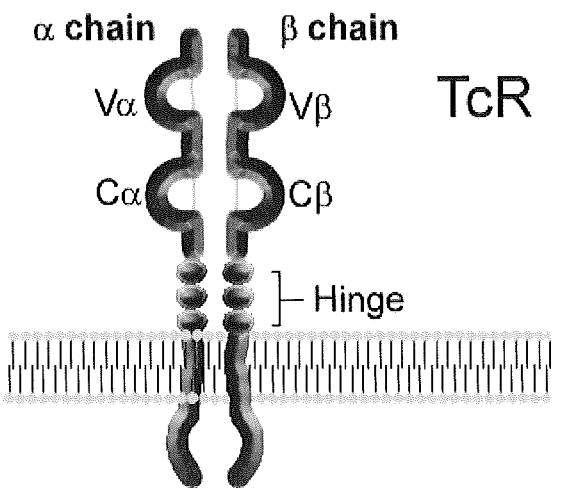


FIGURE 5.1 The T-cell-Receptor (TcR)

The TcR is a heterodimer with a molecular weight of ~ 85,000 - 90,000 D. It is composed of either alpha and beta, or gamma and delta, polypeptide chains. The alpha/beta TcR is present on more than 95% of peripheral T cells. Each chain has a variable region domain and a constant region domain (designated Vα and C α, Vβ and Cβ, respectively). The variable region domains of the alpha and beta chains come together to form the antigen binding cleft. *(Figure and text adapted from the Western Kentucky University Biology course).*

To test the consistency of the expression of the control genes within a single donor ***(intra-donor consistency)***, RNA from an equivalent amount of cells was extracted seven times from three different donors and tested for the expression level of the control gene. The Cα-gene was found to present highly consistent intra-donor expression levels, as only very slight variations of the C_T-levels were found in all three cases *(Figure 5.2)*.

Further, the consistency of the control gene expression between different donors was analyzed ***(inter-donor consistency)***. For this purpose, ***dilution curves*** were created for different individuals. Total RNA was extracted for each individual on a separate mini-column and quantified by a spectrometer. 100 ng of RNA was diluted 4 times. Individual cDNA was prepared with each dilution, which was subsequently analyzed for the control gene

expression. Expression levels of the Cα-gene were found to be significantly consistent amongst different donors as the dilution curves were virtually identical *(Figure 5.3)*.

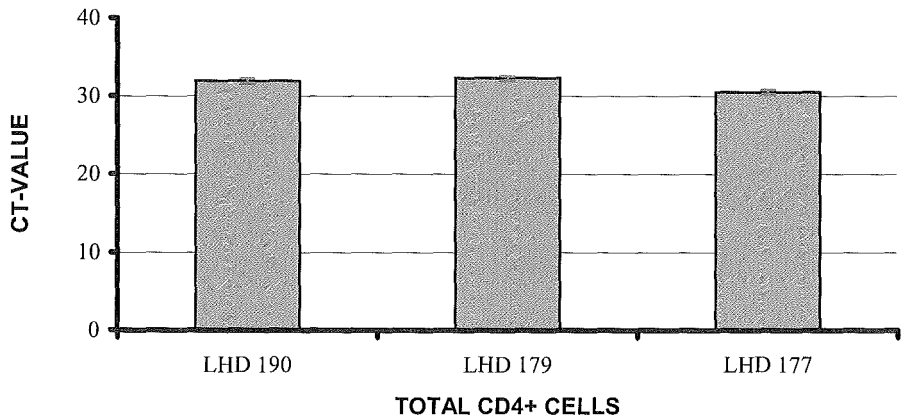


FIGURE 5.2 Intra-donor consistency of the endogenous control gene

Cα expression on 100 $CD4^+$ T cells in 3 different individuals (LHD 190, LHD 179 and LHD 177). In each case, the depicted C_T value corresponds to the average value of 7 duplicates, the red error bar indicating the corresponding standard deviation.

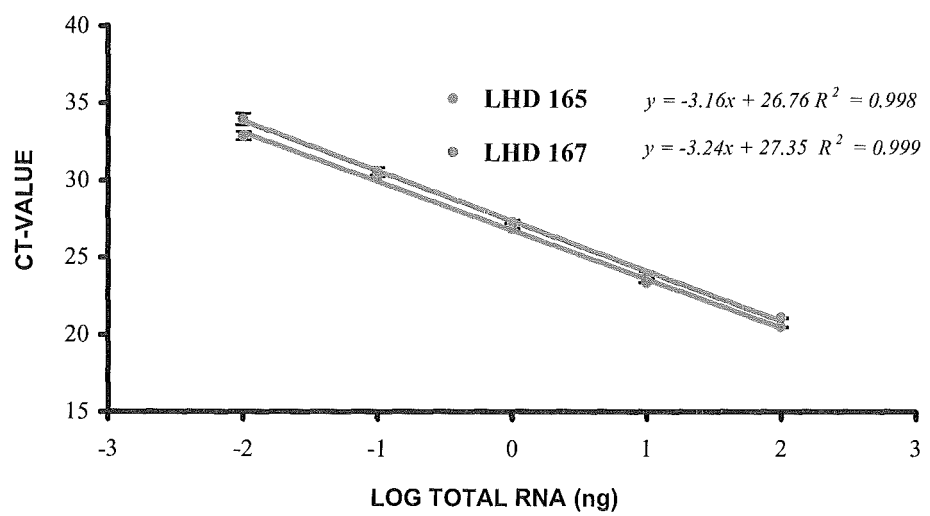


FIGURE 5.3 Inter-donor consistency of the endogenous control gene

Cα RNA dilution curves for 2 different individuals (LHD 165 and LHD 167). The dilution curves of the two individuals are nearly identical.

To test whether the endogenous control was expressed constantly amongst the cell subsets to be compared ***(target population consistency)***, the different memory T cell subsets were sorted individually and analyzed for the expression of the control gene. The C_T values of each subpopulation for each control gene were plotted as a trend line. The consistency of the internal standard was evaluated by measuring the slope of this trend line. The more the slope approaches zero, the more consistent is the expression levels of the control gene amongst the different subsets *(Figure 5.4)*. The slope of the trend line for Cα was closer to zero (0.075) in the $CD8^+$ memory subsets, as compared to that for β2m (0.188).

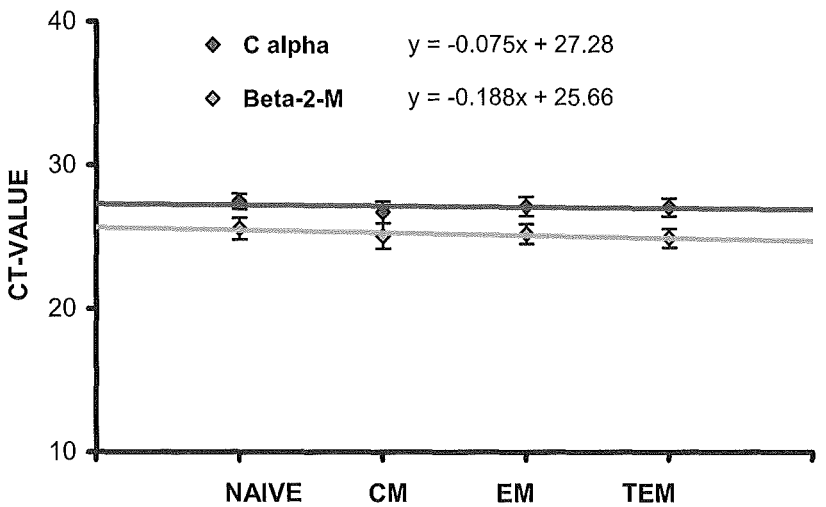


FIGURE 5.4 Target population consistency

Expression levels of Cα and Beta-2-microglobulin in $CD8^+$ T memory cell populations. The consistency of the gene expression amongst the 4 subpopulations is reflected by the slope of the trend line being close to zero.
Blue: Trend line for Cα, slope: 0.075. **Orange**: Trend-line for β2m, slope: 0.188. The value for each subpopulation corresponds to the average of 4 different individuals with 2 duplicates per gene.
Naïve: $CD8^+$ $CD45RA^+$ $CCR7^+$ cells. **CM** (central memory subset): $CD8^+$ $45RA^-$ $CCR7^+$. **EM** (effector memory subset): $CD8^+$ $45RA^-$ $CCR7^-$. **TEM** (terminal effector memory subset): $CD8^+$ $45RA^+$ $CCR7^-$.

Ideally, the internal standard should express mRNA levels similar to those of the target gene ***(target gene equivalency)***. In the case of this study, the target genes were all expressed at low levels. The tested references, EF-1α and β2m, however, were both expressed at significantly higher levels than the target genes. The Cα-gene, on the other hand, expressed approximately the same amount of mRNA as the target genes, thus being the only of the three tested references to fulfill the required condition. This was tested experimentally by analyzing the expression of the three reference genes from equivalent RNA amounts of a given donor.

C_T values of each endogenous control gene were compared to the expression of the target gene on the same sample of the same donor. These results were confirmed in three different donors *(Figure 5.5)*.

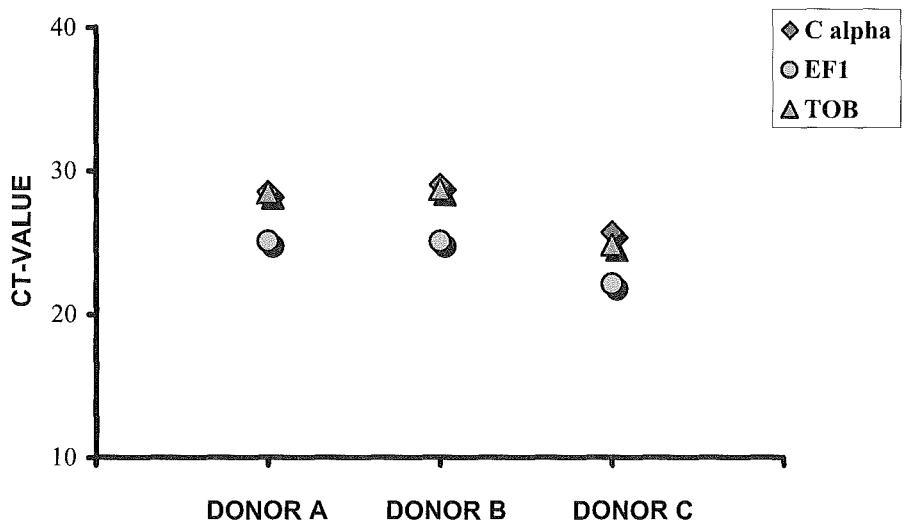


FIGURE 5.5 Expression levels of endogenous control genes compared to target gene

Cα *(blue diamonds)* is expressed at similar levels as the target gene Tob *(orange triangles)*. EF-1α *(green circles)* amplified at lower C_T values than the target gene. Duplicates for each gene were performed in each donor. Tob is depicted as an example for the target genes. The RNA content in this particular experiment was not the same for the individual donors

Another important aspect of the internal standard is its constant expression when subjected to different experimental conditions ***(preservation of expression)***. The following two experimental conditions were tested:

a) homeostatic proliferation in T cells *(Figure 5.6)*

b) polyclonal stimulation by anti-CD3 and anti-CD28 antibodies *(Figure 5.7)*

For this purpose, total $CD4^+$ T cells were incubated either (a) with a cytokine mix consisting of IL-6, IL-7, IL-10, IL-15 and TNFα for a total of nine days, or (b) with anti-CD28 antibody in anti-CD3 coated culture plates for a total of 6 days. Samples with equivalent cell numbers were collected from 4 time-points for each experiment and analyzed for the reference gene expression. The C_T values of the time-points of each experiment were plotted as a trend line for each control. The consistency of expression was determined by analyzing the slope of the trend line. The trend line for Cα demonstrated a slope close to zero in both the experimental conditions for two different individuals tested *(Figure 5.6 and Figure 5.7)*.

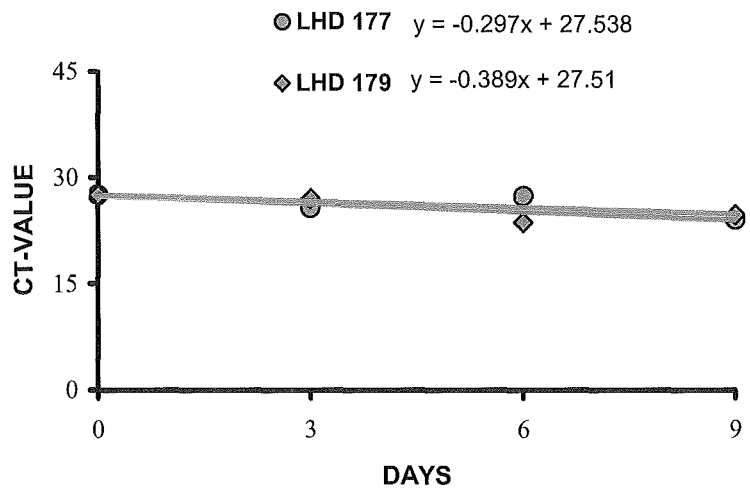


FIGURE 5.6 Cα expression during homeostatic stimulation of total CD4$^+$ T cells

Cα expression in total CD4$^+$ of two different individuals (LHD 177 and 179) was analyzed at day 0, 3, 6 and 9 of homeostatic proliferation induced by cytokines. The slope of the trend lines of both the subjects tested was close to zero. (**Green**: LHD 177; slope: 0.297. **Orange**: LHD 179; slope 0.389).

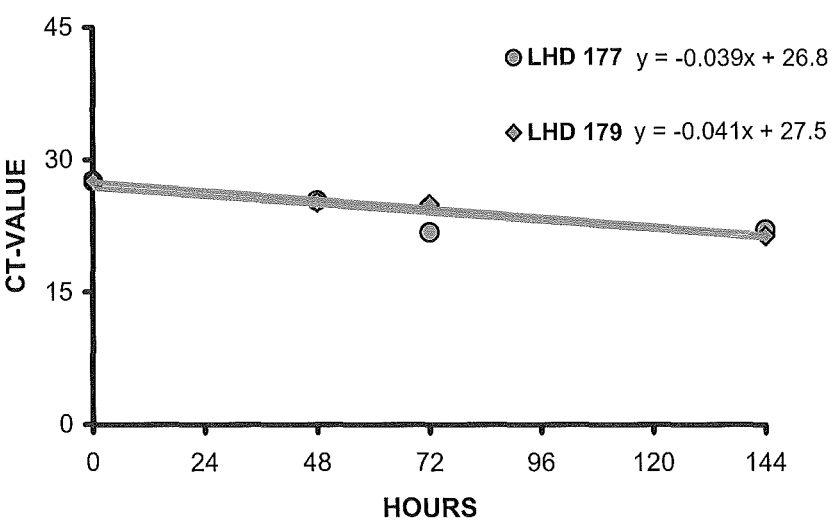


FIGURE 5.7 C alpha expression during polyclonal stimulation of total CD4$^+$ T cells

Cα expression in total CD4$^+$ of two different individuals (LHD 177 and 179) was analyzed at 0, 48, 72 and 144 hours of polyclonal proliferation induced by anti-CD3 and anti-CD28 antibodies. The slope of the trend lines of both the subjects tested was close to zero. (**Green**: LHD 177; slope: 0.039. **Orange**: LHD 179; slope 0.041).

5.2 VALIDATION OF THE PCR REACTIONS

In an ideal ***PCR reaction***,[135] a two-fold doubling of amplicon occurs at each cycle, which corresponds to a 100% efficiency of the reaction. The kinetics of a PCR reaction can be expressed by the following equation:

$$R_n = R_0 \times (1 + E)^n \quad \ldots (1)$$

where R_n is the number of template molecules at cycle n and R_0 corresponds to the initial number of template molecules. E is the PCR efficiency ($0 \leq E \leq 1$; if $E = 1$, the efficiency of the reaction is 100%).

The efficiency of the real-time PCR reaction can be calculated by the following relation:

$$E = 10^{(-1/m)} - 1 \quad \ldots (2)$$

where m is the slope of a dilution curve established by tenfold dilutions. In order to obtain accurate and reproducible results, reactions should have an efficiency as close to 100% as possible ($m = 3.32$). Thus, the ***efficiency*** of the PCR should be 90-100% ($3.6 > m > 3.1$). A number of variables can affect the efficiency of the PCR. These factors include length of the amplicon, presence of inhibitors, secondary structure and primer design. Although valid data can be obtained that fall outside of the above efficiency range, the qRT-PCR– reaction should be further optimised, or alternative primers designed, in case $E < 0.90$.

To test the amplification efficiencies for the individual primers, dilution curves were prepared using stock RNA extracted from total PBMC. The total RNA was quantified by a spectrometer, and tenfold dilutions ranging from 0.001 ng to 100 ng were used for the subsequent cDNA preparations. Individual cDNA preparations for each target gene were subsequently used for amplification of all the target genes and the reference gene. C_T values were plotted against log RNA amount *(Figures 5.8 and 5.9)*. The slopes of these dilution curves were given by the curve equation. All the primers could be amplified with an efficiency superior to 90%, since the values for all the slopes were $3.6 > m > 3.1$ *(Table 4)*.

Since each of the dilutions was prepared prior to cDNA preparation, possible variations occurring during the reverse transcription step, as well as during manipulation (pipetting), could be considered negligible, the ***rectitude*** values of the dilution curves being all superior to 0.99 *(Table 4)*.

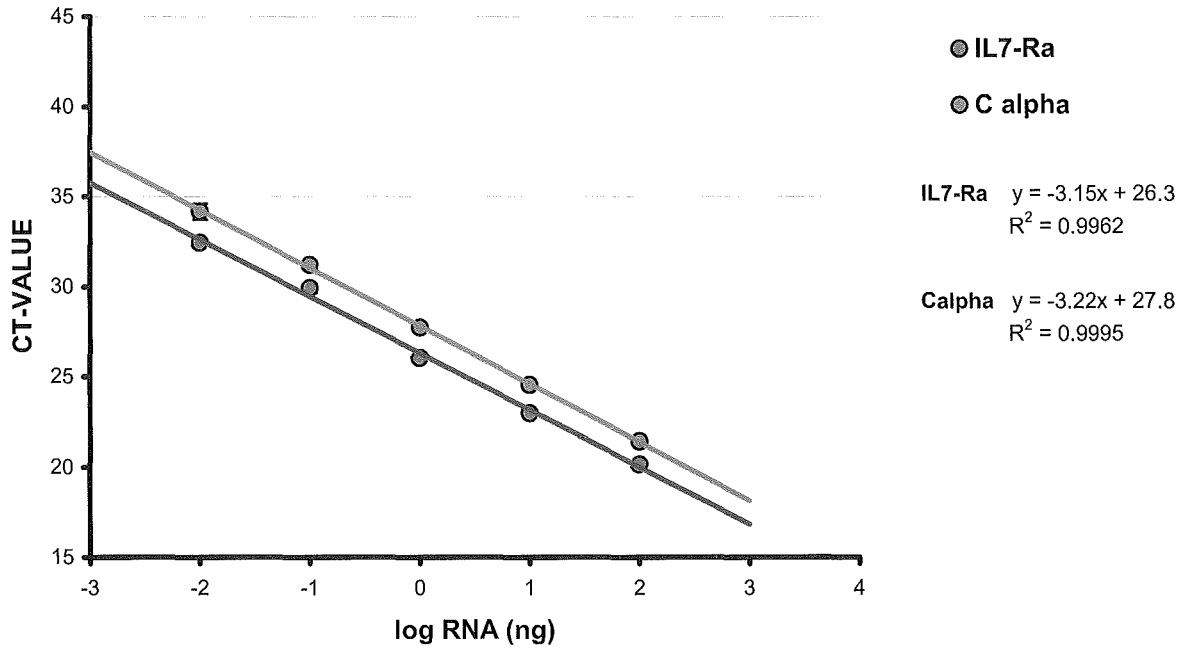


FIGURE 5.8 RNA dilution curve for IL-7-Rα and C alpha

IL7-Rα is depicted as an example of the various primers designed and purchased as "Assays by Design" from Applied Biosystems. These include: Baff-R, Cα, IL15-Rα, IL7-Rα, APRIL and EF-1α.

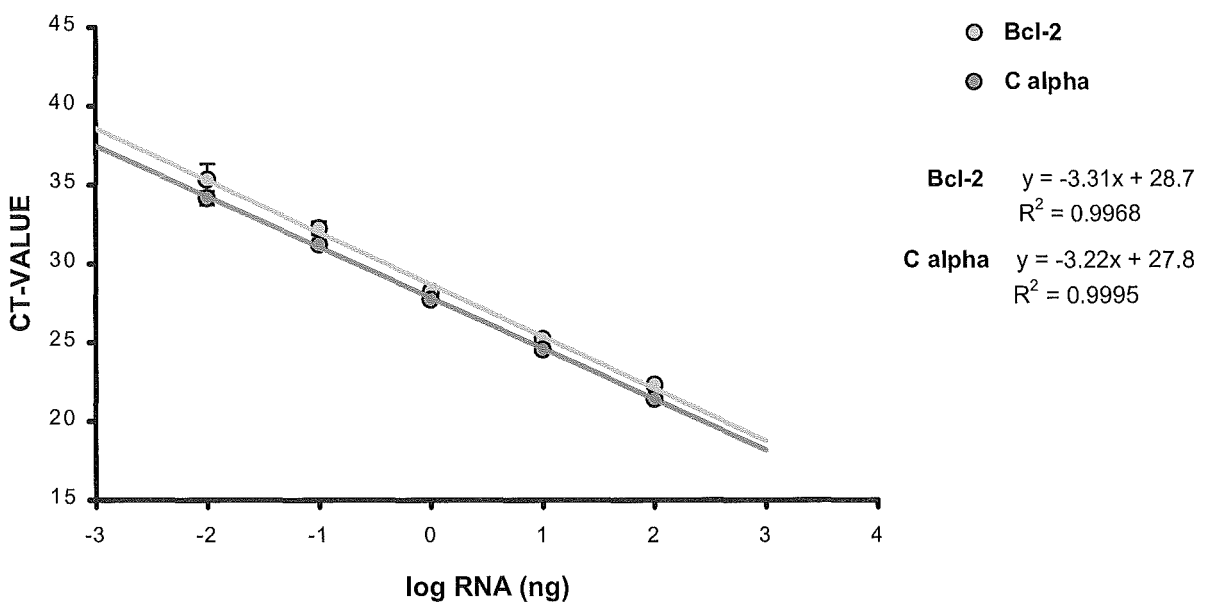


FIGURE 5.9 RNA dilution curve for Bcl-2 and C alpha

Bcl-2 is depicted as an example of the various pre-designed primers, purchased as "Assays on Demand" from Applied Biosystems. These include: Bcl-2, Bcl-xL, Bad, Bax, Flip, Tob, Baff, Taci, FasL and β2m.

GENE	SLOPE (m)	RECTITUDE (R^2)
C ALPHA	-3.216	0.9995
BCL-2	-3.303	0.9968
FLIP	-3.263	0.9927
BAD	-3.248	0.9950
TOB	-3.532	0.9958
APRIL	-3.358	0.9992
IL15-Ra	-3.420	0.9997
IL7-Ra	-3.152	0.9962
TACI	-3.392	0.9978
BAFF	-3.126	0.9975
BCMA	-3.506	0.9995
FAS-L	-3.414	0.9909
BCL-XL	-3.348	0.9969
BAX	-3.488	0.9985
BAFF-R	-3.119	0.9976
EF-1a	-3.269	0.9941

TABLE 4 PCR efficiency

RNA dilution curves were established for each primer pair. The slope of each individual curve allowed estimation of the PCR efficiency. All the primers could be amplified with an efficiency superior to 90%, since their slope values were between 3.6 and 3.1.

In order to test the purity of the primer pairs, a ***RT-minus control*** was performed for all the target genes and the reference gene. The RT-minus control consists of a sample cDNA which is synthesized without the presence of RTase, e.g. the enzyme that enables reverse-transcription of RNA to DNA. This control cDNA can then be used in the normal semi-nested PCR. If the RT-minus control is negative, there is no contaminating DNA in the sample used *(Figure 5.10)*.

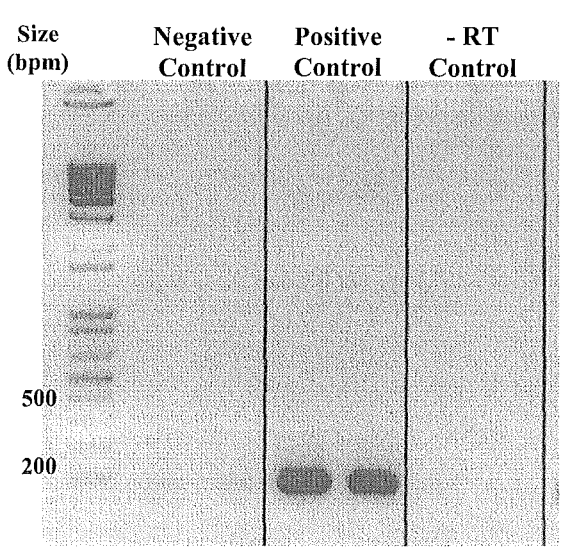


FIGURE 5.10 RT-minus control for Cα visualized on an agarose–gel

5.3 VALIDATION OF THE COMPARATIVE C_T METHOD

In the case of a semi-quantitative analysis,[136-139] both target and reference (endogenous control) should have similar efficiencies. The comparative C_T method used in this study, and previously described by Livak,[136] detects the relative gene expression with the formula $2^{-\Delta\Delta CT}$. This formula is based on the following two assumptions:

(1) Amplification efficiency should be close to 100%

(2) Amplification efficiencies of the target gene and of the reference gene must be approximately equal.

Thus, the comparative C_T method used in this study can only be applied when both these requirements are fulfilled. As discussed in the previous chapter, all the primers presented amplification efficiencies between 90-100% *(Table 4)*. To test whether the second requirement was valid for the internal standard selected for this study (Cα), efficiencies of all the target genes of the gene panel had to be compared to the amplification efficiency of the Cα primers.

A sensitive method for assessing whether two amplicons have the same efficiency is to examine how their ΔC_T varies with template dilution ***(relative efficiency plot)***. For this purpose, the ΔC_T of the target gene and Cα can be plotted against the log template amount and the relative efficiency estimated by analyzing the slope of the thus generated tendency curve. Ideally, the slope should equal 0, implying that the target gene and Cα have identical amplification efficiencies. Thus, if the absolute value of the slope (c) is < 0.1, the $2^{-\Delta\Delta CT}$ formula can be applied *(Figure 5.11)*.

In practical terms, this also means that the $\Delta\Delta C_T$ calculation can be used for a given target gene without running standard curves on the same plate. The validation experiment was performed for all the primer pairs. Since the efficiencies of all the target genes were shown to be approximately equal to that of Cα ($c < 0.1$; *Table 5*) the $\Delta\Delta C_T$ calculation could be applied to the entire gene panel.

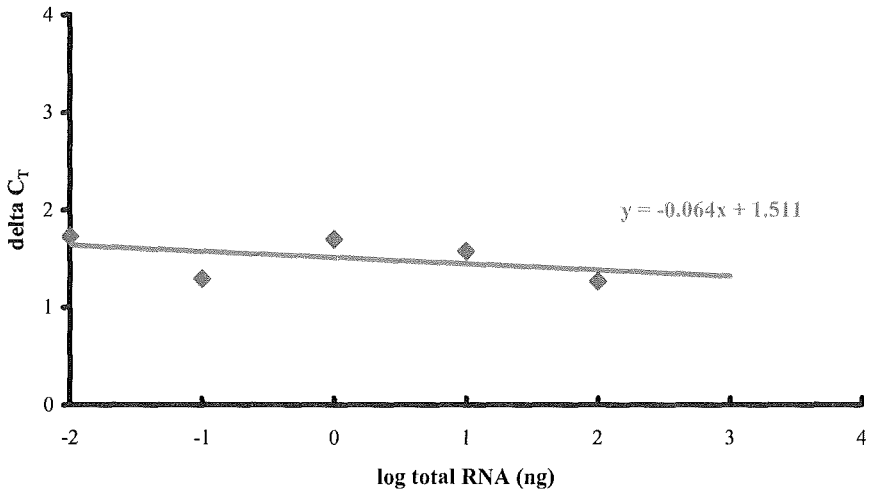


FIGURE 5.11 Relative efficiency plot for IL7-Rα and Cα.

The values from the RNA dilution curves of Cα and IL7-Rα were used to obtain the delta C_T value: $\Delta C_T = \Delta C_T$ (Cα) - ΔC_T (IL7-Rα). The slope is < 0.1 ($c = 0.064$), indicating that the amplification efficiencies of Cα and IL7-Rα are sufficiently close to allow the use of the comparative C_T method.

GENE	EQUATION	SLOPE (Delta C_T gene- Cα)
BCL-2	y = 0.087x – 0.86	0.08
BCL-xL	y = -0.0865x + 0.062	-0.08
BAD	y = 0.0315x - 4.596	0.03
BAX	y = 0.053x - 0.621	0.05
APRIL	y = 0.0505x - 2.446	0.05
BAFF	y = -0.0075x - 1.1133	-0.007
BAFF-R	y = -0.0975x + 0.624	-0.09
TACI	y = 0.06x - 2.8383	0.06
IL15-Ra	y = 0.0975x - 4.9825	0.09
IL7-Ra	y = -0.064x + 1.511	-0.06
FAS-L	y = 0.06x - 4.735	0.06
FLIP	y = -0.035x + 0.17	-0.03
TOB	y = 0.0635x + 2.1845	0.06

TABLE 5 Relative efficiencies of target genes versus Cα.

The values of the RNA dilution curves were used to plot the delta C_T values of each target gene minus the internal control (Cα), versus log RNA (template). The equation for the straight-line fit obtained and the corresponding slope value are listed for each individual gene. The slopes are seen to be all < 0.1, implying that the comparative C_T method can be applied reliably to calculate relative mRNA expression as compared to Cα expression.

6 RESULTS

The leucocyte filters of healthy blood donors from the regional blood bank were used to obtain total PBMC, which were then screened for the expression of CD4 and CD8. The total PBMC of 4 donors (LHD 234, LHD 275, LHD 283 and LHD 288) were sorted according to their surface expression of CD8, CD45RA and CCR7; 3 donors were sorted for CD4, CD45RA and CCR7 (LHD 216, LHD 283 and LHD 288). The expression of a total of 15 genes, including the endogenous control gene (Cα), was analyzed separately in naïve $CD4^+$ and $CD8^+$ T cells, as well as in the three corresponding, individually sorted memory subsets.

- "**CM**" correspond to central memory cells characterized by a $CD45RA^-CCR7^+$ phenotype.
- "**EM**" are effector memory cells with a $CD45RA^-CCR7^-$ phenotype.
- "**TEM**" are terminally differentiated effector memory cells with a $CD45RA^+CCR7^-$ phenotype.

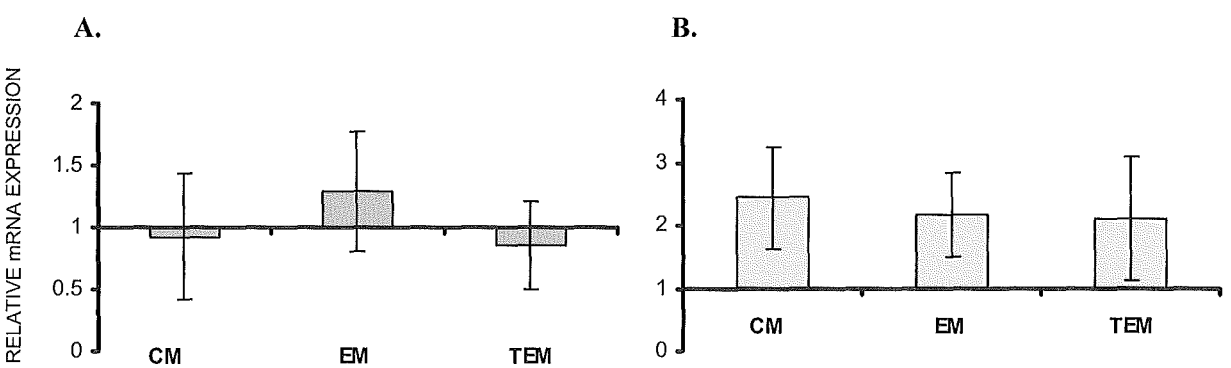
Gene expression was assessed using cDNA corresponding to total mRNA from 450 cells per population and gene. Semi-quantitative analysis was based on the comparative C_T Method, using Cα as an endogenous control and the naïve population as the calibrator. Cα amplification was performed on the same cDNA and in parallel to target gene amplification, on the same plate. The calculation as a whole was first performed for each individual. The relative expression of each gene was then averaged for each population and presented graphically.[140] The results are presented as gene expression in a memory subset, relative to gene expression in the corresponding naïve T cell population. This implies that:

- *Results superior to 1* mean that the gene expression levels in that particular memory subpopulation are higher than expression levels in naïve cells.
- *Results equal to 1* mean that the gene is expressed in equal amounts in the analyzed memory subset and in naïve cells.
- *Results inferior to 1* mean that the expression levels of that gene are lower in the particular memory subset than in the respective naïve cell population.

6.1 Survival genes: BAFF, APRIL and their RECEPTORS

In the $CD8^+$ memory subsets, ***BAFF*** expression levels were quite variable from one individual to another, as indicated by the substantial standard deviations *(Figure 6.1A)*. However, on average, expression levels vary moderately from the expression level in naïve $CD8^+$ T cells. In contrast, the $CD4^+$ memory subsets were all shown to upregulate BAFF expression – reaching up to three times the level of expression of naïve $CD4^+$ cells in the central memory population *(Figure 6.1B)*.

Figure 6.1

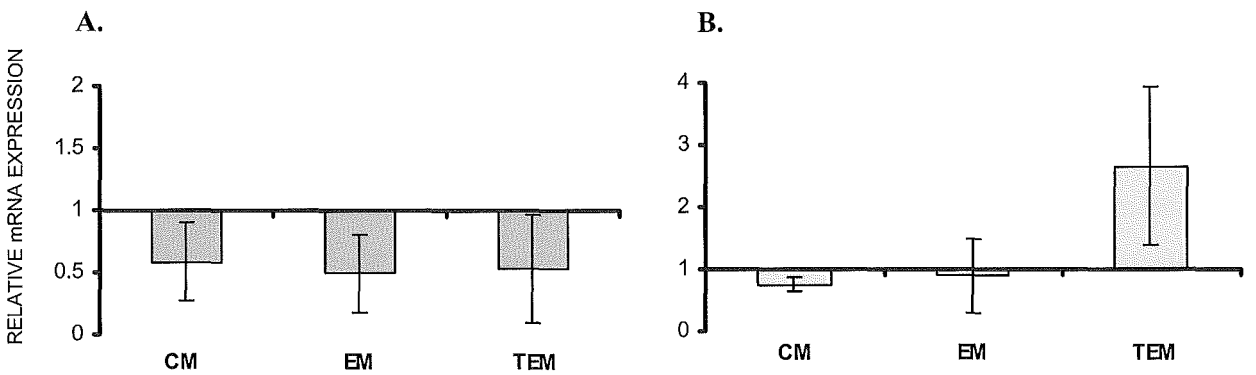


6.1A. BAFF expression in $CD8^+$ memory T cell subsets. The CD45RA-CCR7- (EM) subset expresses the highest levels of BAFF mRNA, with levels up to 1.8 times the levels of expression in naïve cells (LHD 288).

6.1B. BAFF expression in $CD4^+$ memory T cell subsets. All 3 individuals expressed higher levels of BAFF mRNA the memory subsets, as compared to naïve CD4+ cells. The highest upregulation was in the CM subset with up to 3.4 times the levels (LHD 283).

The expression of the other ligand, ***APRIL***, on the other hand, is clearly downregulated in all $CD8^+$ memory cell subsets *(Figure 6.2A)*. In CD4, on the other hand, the $CD45RA^-CCR7^+$ (T_{CM}) and $CD45RA^-CCR7^-$ (T_{EM}) subsets express levels of APRIL that are more or less equivalent to those of the naïve population. The $CD4^+$ $CD45RA^+CCR7^-$ (T_{TEM}) population however, seems to upregulate APRIL mRNA *(Figure 6.2B)*.

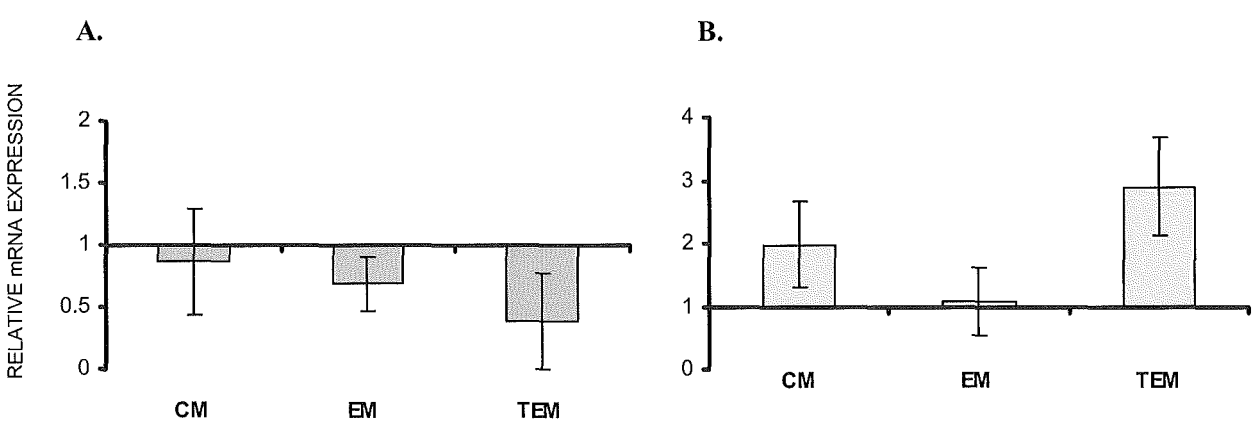
Figure 6.2



6.2A. APRIL expression in CD8$^+$ memory T cell subsets. APRIL mRNA was present in lower levels in all the three memory CD8 subsets. Lowest levels were found in the CD45RA-CCR7- (EM) and CD45RA+CCR7- (TEM) subsets. Expression levels were as low as 23% of mRNA levels in naïve cells.

6.2B. APRIL expression in CD4$^+$ memory T cell subsets. The CD45RA+CCR7- (TEM) subset was the only subset to express higher levels of mRNA than naïve cells. This was consistent in the 3 individuals tested. The highest levels of APRIL mRNA were 3.5 times higher than levels in naïve cells (LHD 283).

Figure 6.3



6.3A. BAFF-R expression in CD8$^+$ memory T cell subsets. Relative Baff-R expression was lower in the memory subsets for all individuals, except for LHD 234 who expressed 1.4 times higher levels of mRNA in the CD45RA-CCR7+ (CM) subset only. The weakest expression was within the CD45RA+CCR7- (TEM) subset, with levels as low as 4.5% (LHD 288).

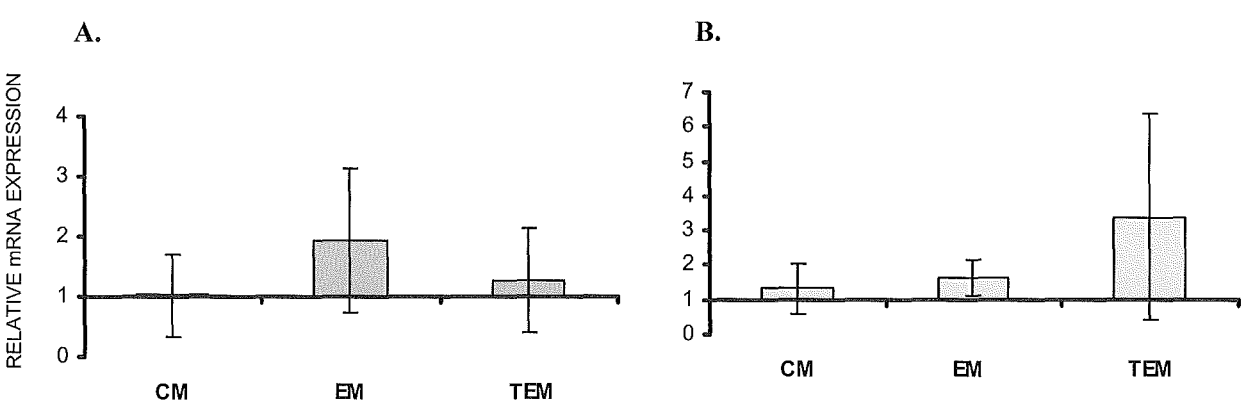
6.3B. BAFF-R expression in CD4$^+$ memory T cell subsets. In all the 3 individuals, Baff-R mRNA least expressed in the CD45RA-CCR7- (EM) subset and most expressed in the CD45RA+CCR7- (TEM) subset, in which levels were as high as 3.4 times as high as in naïve CD4+ cells (LHD 216).

When considering the expression of the BAFF and APRIL receptors, it should be noted that ***TACI*** and ***BCMA*** were never detected in naïve or memory CD4, or in any CD8 T cells during this study *(data not shown)*. Therefore, importantly, the only receptor found to be expressed consistently in our experimental conditions, is ***BAFF-R*** which is known to be specific to BAFF. In $CD8^+$ T cells, BAFF-R mRNA is clearly expressed at lower levels in memory cells than in naïve cells. This downregulation seems to increase with the differentiation to a more effector phenotype memory cell *(Figure 6.3A)*. Contrarily, in $CD4^+$ memory subpopulations, BAFF-R expression levels are higher than in the naïve population, except for the $CD4^+$ $CD45RA^-CCR7^-$ (T_{EM}) subset *(Figure 6.3B)*.

6.2 Survival genes: IL-15-RECEPTOR and IL-7-RECEPTOR

The ***IL-15Rα*** expression in $CD8^+$ T cells was shown to be quite variable from one individual to another in all the memory subsets. The $CD8^+CD45RA^-CCR7^-$ (T_{EM}) subset, however, seems to preferentially upregulate IL-15Rα mRNA levels in CD8 T cells *(Figure 6.4A)*. In $CD4^+$ memory T cells, the general expression levels of IL-15Rα seem to be slightly higher than expression in naïve $CD4^+$ cells. The $CD4^+CD45RA^+CCR7^-$ (T_{TEM}) subpopulation showed a very variable expression amongst the individuals tested with sometimes very high levels *(Figure 6.4B)*.

Figure 6.4

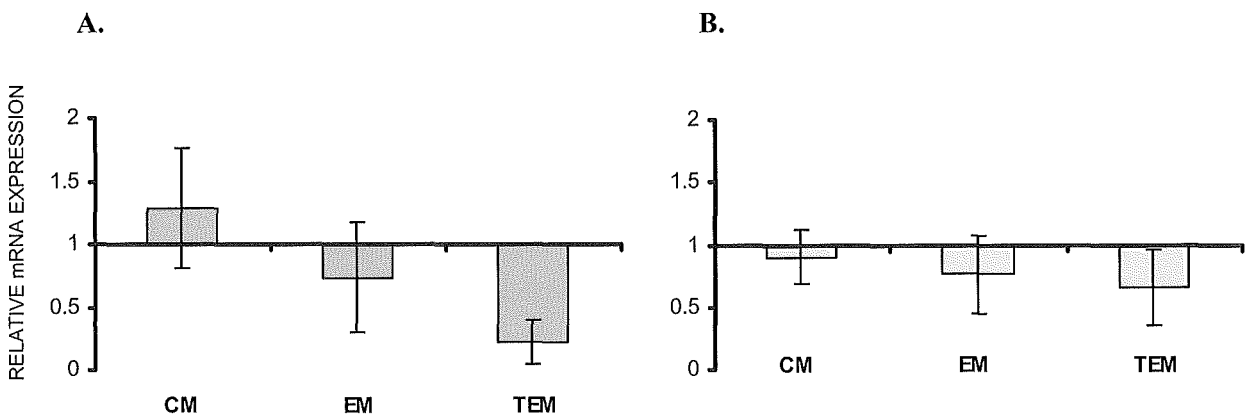


6.4A. IL-15Rα expression in $CD8^+$ memory T cell subsets. The CD45RA-CCR7- (EM) subset expressed the highest levels IL-15Rα, with levels up till 3.7 times the levels of expression in naïve CD8+ cells (LHD 283).

6.4B. IL-15Rα expression in $CD4^+$ memory T cell subsets. IL-15Rα expression was generally higher in the memory subsets in all individuals, but expression in the CD45RA+CCR7- (TEM) subset varied extensively between the individuals. LHD 283 expressed 5.5 times higher levels in the TEM subset.

As regards the expression of the ***IL-7Rα*** gene, only the $CD45RA^-CCR7^+$ (T_{CM}) subset of memory $CD8^+$ T cells was found to express higher levels of IL-7Rα transcripts than naïve $CD8^+$ cells. In both the effector memory subsets, IL-7Rα mRNA was downregulated, moderately in $CD8^+CD45RA^-CCR7^-$ (T_{EM}) cells and much more strongly in the $CD8^+CD45RA^+CCR7^-$ (T_{TEM}) subpopulation *(Figure 6.5A)*. In $CD4^+$ T cells, all the memory subsets expressed lower levels of IL-7Rα mRNA as compared to naïve $CD4^+$ T cells, with a tendency to greater downregulation in the most terminally differentiated subtype *(Figure 6.5B)*.

Figure 6.5



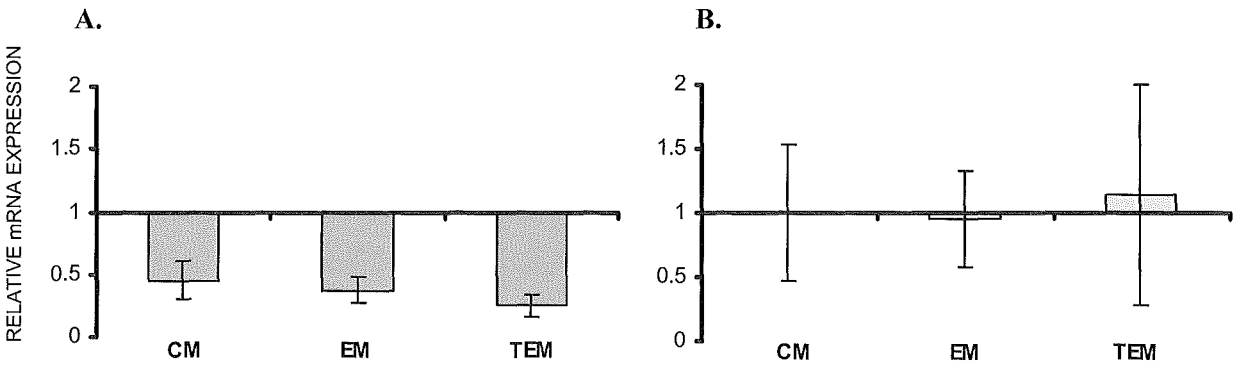
6.5A. IL-7Rα expression in $CD8^+$ memory T cell subsets. In 3 out of the 4 individuals, IL-7Rα levels were higher in the CM population (up to 1.8 times) and lower in the EM subset. All 4 individuals expressed the lowest levels in the TEM subset (up to 0.05 times).

6.5B. IL-7Rα expression in $CD4^+$ memory T cell subsets. IL-7Rα levels were virtually lower in all the memory CD4 subsets for the 3 individuals. The TEM subset expressed the lowest levels of IL-7Rα mRNA (up to 0.3 times lower than naïve CD4 cells).

6.3 ANTI-APOPTOTIC GENES

The expression of the ***Bcl-2*** gene was significantly downregulated in all the three $CD8^+$ T cell memory subsets. The levels decreased with further differentiation towards an effector phenotype *(Figure 6.6A)*. In the $CD4^+$ cells, the Bcl-2 mRNA levels varied substantially from one individual to another, and no tendency could be demonstrated for any of the subsets *(Figure 6.6B)*.

Figure 6.6



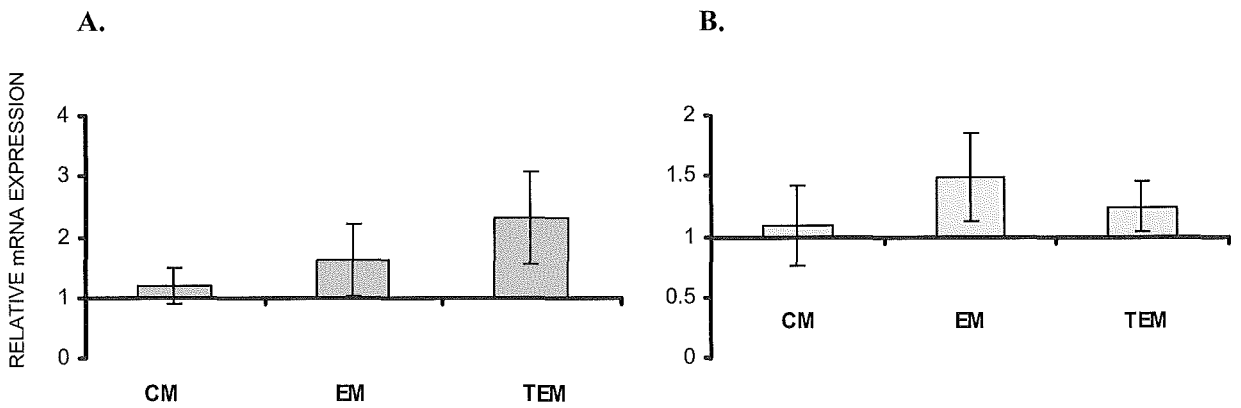
6.6A. Bcl-2 expression in $CD8^+$ memory T cell subsets. Bcl-2 levels were clearly lower in all the CD8 memory subsets than expression in naïve CD8. This was very consistent in all the 4 individuals. Lowest relative mRNA levels (minimum 0.13 for LHD288) were present in the TEM population.

6.6B. Bcl-2 expression in $CD4^+$ memory T cell subsets. Bcl-2 expression was variable amongst the 3 individuals. The greatest variations were observed for the TEM subset.

Bcl-xL mRNA was present in greater amounts in all subsets of memory T cells, both for $CD8^+$ and $CD4^+$ populations. Especially in $CD8^+$ memory T cells, the expression levels were clearly increased in the four individuals examined. The more effector phenotype subsets (T_{EM} and T_{TEM}) had significantly higher expression levels as compared to the central memory, $CD8^+CD45RA^-CCR7^+$ (T_{CM}) subset *(Figure 6.7A)*. In $CD4^+$ cells, the T_{EM} consistently showed the highest level of expression in all the individuals examined *(Figure 6.7B)*.

The expression of the ***FLIP*** gene was found to be upregulated in all the three memory subsets of $CD8^+$ T cells. The terminally differentiated effector subset ($CD45RA^+CCR7^-$, T_{TEM}), however, showed the highest levels of FLIP mRNA *(Figure 6.8A)*. In the $CD4^+$ population, on the other hand, FLIP expression levels were downregulated in all the memory subsets *(Figure 6.8B)*.

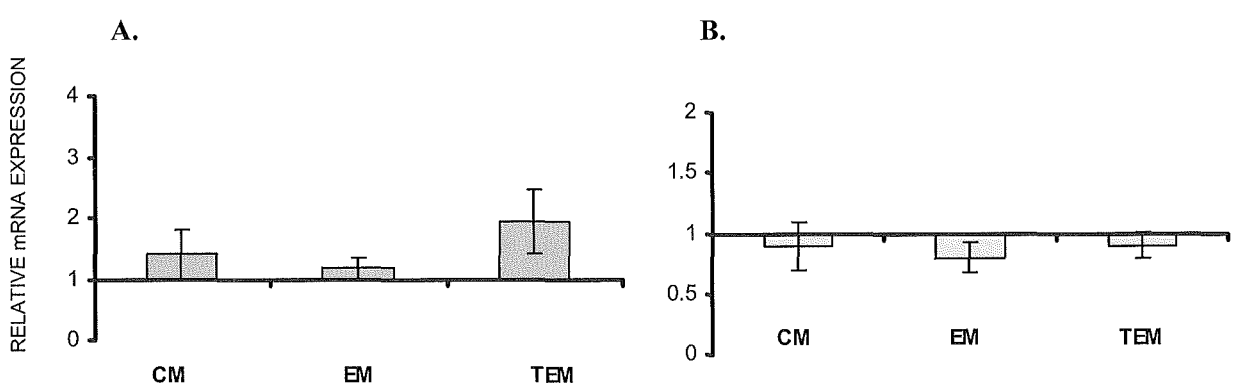
Figure 6.7



6.7A. Bcl-xL expression in CD8$^+$ memory T cell subsets. Bcl-xL mRNA levels were generally higher in all the CD8 memory subsets as compared to naïve CD8 cells. Significantly high levels, with up to 3.3-fold increase, were observed in the CD45RA+CCR7- (TEM) subset.

6.7B. Bcl-xL expression in CD4$^+$ memory T cell subsets. In CD4 cells, Bcl-xL expression was moderately upregulated in all subsets, but consistently highest in the CD45RA-CCR7- (EM) subset.

Figure 6.8



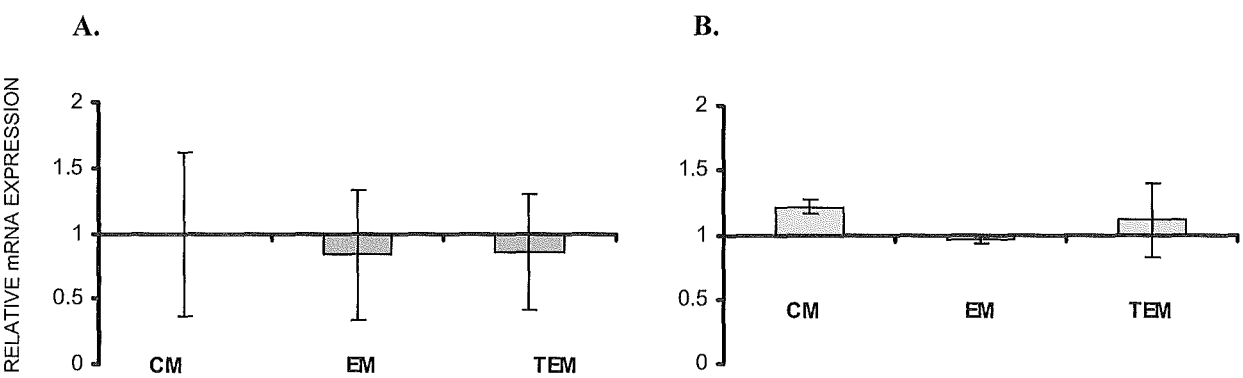
6.8A. FLIP expression in CD8$^+$ memory T cell subsets. Even though all the CD8 memory subsets expressed more FLIP mRNA than naïve cells, the highest levels were clearly present in the CD45RA+CCR7- (TEM) subset, with relative levels as high as 2.7 times expression levels in naïve cells.

6.8B. FLIP expression in CD4$^+$ memory T cell subsets. In the three individuals analyzed, FLIP mRNA levels were slightly lower in all the three memory subpopulations of CD4$^+$ T cells as compared to expression in naïve CD4$^+$ cells.

6.4 PRO-APOPTOTIC GENES

Expression of the pro-apoptotic gene ***Bad*** within the $CD8^+$ memory T cell compartment was extremely heterogeneous in the four individuals analyzed *(Figure 6.9A)*. In $CD4^+$ memory T cells, Bad expression showed much less inter-individual variability *(Figure 6.9B)*. In both $CD4^+$ and $CD8^+$ memory cells, average mRNA levels were quite close to those of naïve T cells.

Figure 6.9



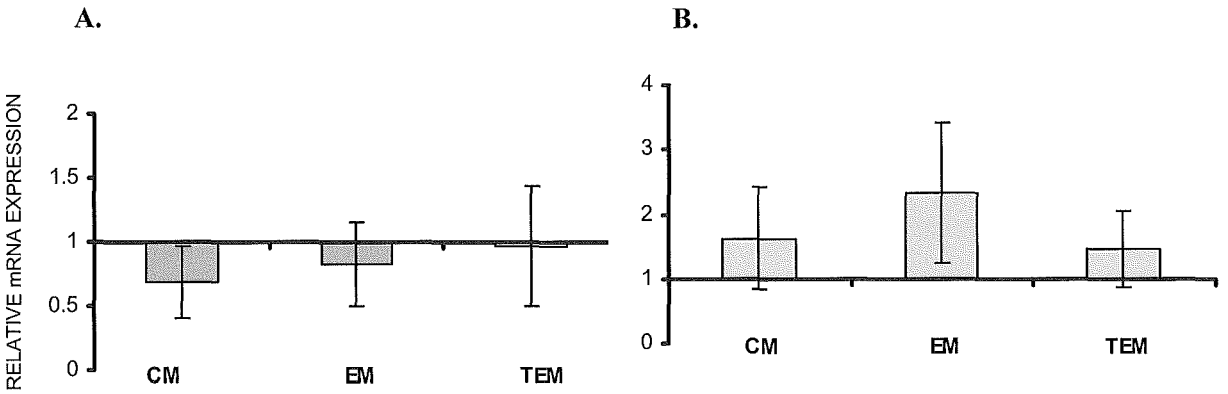
6.9A. Bad expression in $CD8^+$ memory T cell subsets. Bad expression in CD8 memory T cells was variable amongst the four individuals tested. The lowest levels were found in the effector memory subsets.

6.9B. Bad expression in $CD4^+$ memory T cell subsets. Expression in memory $CD4^+$ T cells varied only slightly from expression in naïve $CD4^+$ cells. The CD45RA-CCR7+ (CM) population, however, consistently presented moderately higher Bad mRNA levels than naïve $CD4^+$ cells.

Bax mRNA levels were shown to be heterogeneously expressed amongst the donors analyzed, resulting in a high inter-individual variability in both $CD8^+$ and $CD4^+$ memory T cells. In the $CD8^+$ T cell compartment, Bax expression was generally downregulated in all the memory subsets *(Figure 6.10A)*. In contrast, Bax mRNA levels were clearly upregulated in all the $CD4^+$ memory T cell subsets *(Figure 6.10B)*.

FasL gene expression was found to be absent in naïve cells of both the CD4 and CD8 T cell compartments. In memory T cells, FasL mRNA levels were highly upregulated in increasingly differentiated effector subpopulations of $CD8^+$ memory T cells *(Figure 6.11A)*. In $CD4^+$ cells, however, FasL expression was only found in the terminally differentiated $CD45RA^+CCR7^-$ subset *(Figure 6.11B)*.

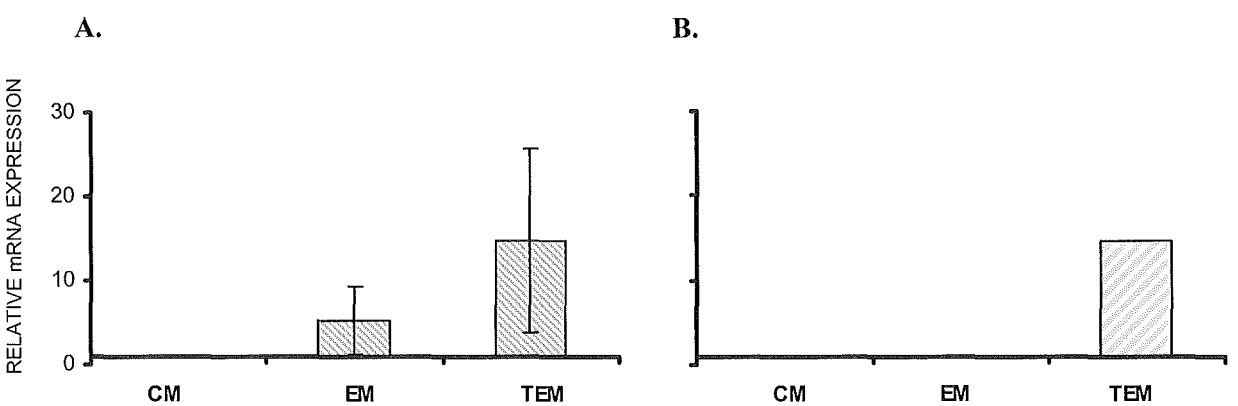
Figure 6.10



6.10A. Bax expression in CD8$^+$ memory T cell subsets. Bax expression was generally lower in all the CD8 memory subsets. This was the most consistent in the CD45RA-CCR7+ (CM) subset, where all the 4 individuals expressed the lowest levels of Bax mRNA.

6.10B. Bax expression in CD4$^+$ memory T cell subsets. For CD4$^+$ T cells, Bax mRNA was present in higher levels in all the memory subsets. The greatest expression was observed in the CD45RA-CCR7- (EM) subset, with levels as high as 3.4 times the expression levels in naïve CD4 cells.

Figure 6.11



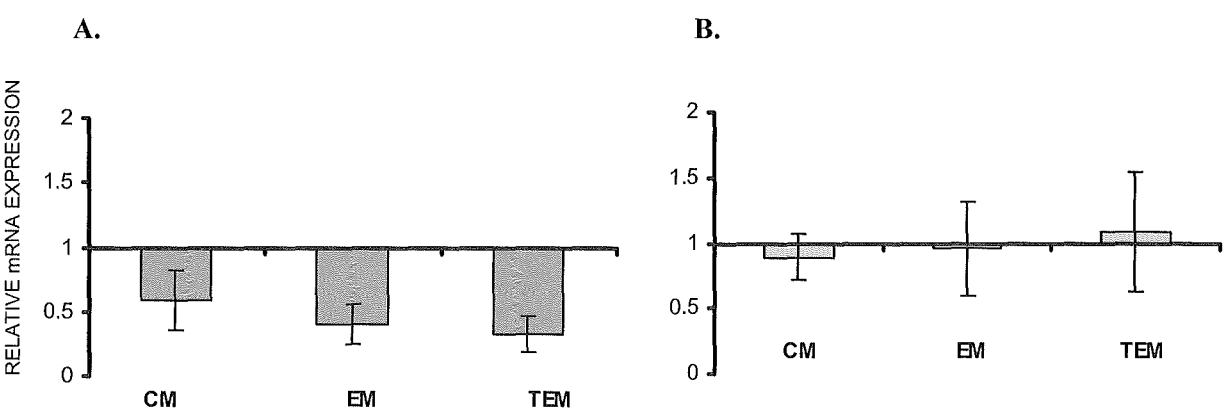
6.11A. FasL expression in CD8$^+$ memory T cell subsets. FasL expression was not observed in the naïve CD8 cells of any of the 4 individuals. FasL mRNA was highly upregulated from one subset to another, with the highest amounts in the TEM subset. LHD 288, for example, expressed approximately 11 times more FasL in the EM subset and 28 times more in the TEM subset than in the CM cells.

6.11B. FasL expression in CD4$^+$ memory T cell subsets. FasL mRNA was only detected in the CD45RA+CCR7- (TEM) subset of CD4+ T cells in the 3 individuals tested. A semi-quantitative analysis could therefore not be performed. In the figure, the depiction of the presence of FasL in the TEM population is purely symbolic.

6.5 Anti-proliferation gene: TOB

In the $CD8^+$ T cell compartment, ***Tob*** mRNA levels were found to be constantly downregulated in all the memory subpopulations. Moreover, downregulation correlated with a differentiation towards a more effector-type phenotype *(Figure 6.12A)*. Contrarily, in $CD4^+$ cells, expression levels were variable amongst the individuals analyzed, and average mRNA levels in the different memory cells remained close to mRNA levels of naïve $CD4^+$ T cells *(Figure 6.12B)*.

Figure 6.12



6.12A. Tob expression in $CD8^+$ memory T cell subsets. Tob mRNA was clearly present in lower levels for the 3 subpopulations of memory CD8 T cells as compared to naïve cells, in all the 4 individuals analyzed. Relative expression was as low as 0.16 in the TEM subset (LHD 288).

6.12B. Tob expression in $CD4^+$ memory T cell subsets. In the $CD4^+$ T cells, Tob expression amongst the 3 individuals was quite variable for the EM and TEM subsets. Generally, expression levels in all the memory subsets were only moderately different from expression levels in naïve cells.

7 DISCUSSION

Memory T cells are generally considered to be more resistant to apoptosis than naïve T cells. For example, the reduction of naïve cells is 8-fold greater than that of memory cells after whole body irradiation.[142] However, recent research has emphasized the heterogeneity of memory T cells and the existence of distinct subpopulations which differ not only in their effector functions, but also in their ability to survive. Many genes have been attributed roles in the survival and death pathways of human T cells, but their individual importance is poorly understood.

This study aims at helping identify the genes that play pivotal roles in the maintenance of long-lived human T cell populations and at increasing the understanding of regulation of survival and apoptosis in memory T cells. In this context, gene expression patterns been differentially studied in the individual memory cell subsets. Additionally, expression patterns have been compared between CD4 and CD8 memory T cells. Technically, the approach has consisted of (a) flow-cytometric sorting of three memory subpopulations separately in CD4 and CD8 positive T cells (according to their expression of CD45RA isoform and the chemokine receptor CCR7) and (b) study of gene-expression by real-time PCR. A panel of 14 genes potentially involved in survival and apoptosis of T cells has been analyzed. The obtained gene expression results, as well as possible implications of the expression pattern in the immune response, are discussed separately below for each gene.

It should be mentioned that the reported results, as well as the conclusions drawn, need confirmation through corroborative protein or functional data. Such experimental validation, however, falls beyond the scope of the current research.

BAFF, APRIL and their receptors

As mentioned earlier, the expression of these TNF-like-ligands and their receptors has been poorly studied in T cells. In fact, their expression in T cells has been quite controversial[83,85,143] and previous studies have actually refrained from showing expression of BAFF or APRIL in human T cells. The results obtained here, however, clearly demonstrate the presence of BAFF and APRIL mRNA in human T cells. The presence of BAFF and APRIL gene expression has not only been confirmed in total T cells (preliminary experiments, data not shown), but also

separately in naïve as well as memory $CD4^+$ and $CD8^+$ T cells. For the first time, the mRNA expression of these ligands is now documented in the individual T_{CM}, T_{EM}, and T_{TEM} memory subpopulations. Even though no absolute quantitative analysis has been performed, the high C_T numbers indicate that the expression level of the two ligands is probably quite low in resting human T cells. This may have been one of the reasons why other studies have failed to show any expression at all, possibly due to experimental limitations with techniques such as Northern Blots that are less sensitive than RT-PCR.

From the obtained results, one can draw the following conclusions concerning the expression of ***BAFF and its receptors*** in memory human T cells:

- $CD4^+$ memory T cells express higher levels of BAFF mRNA than naïve $CD4^+$ cells; the expression in $CD8^+$ memory cells however is variable.
- The receptors TACI and BCMA are not expressed in naïve or memory human T cells.
- BAFF-R expression is more differentially regulated in $CD8^+$ cells as compared to $CD4^+$ cells. In $CD8^+$ memory T cells, BAFF-R mRNA levels decrease with increasing differentiation towards an effector memory phenotype, whereas in $CD4^+$ only the effector-memory subset shows a slightly downregulated expression.

The previously unreported expression of BAFF-R transcripts in human T cells indicates a potential new role of BAFF-BAFF-R signaling specific to T cells. This has to be confirmed by showing BAFF-R surface expression on T cells. Since expression levels of BAFF-R have been found to be downregulated in effector phenotype memory cells, BAFF-BAFF-R signaling may be implicated in T cell survival and memory maintenance independent of antigen. This can be considered as quite striking, since the binding of BAFF to BAFF-R has been attributed a crucial and somewhat exclusive role in B-cell maturation and homeostasis.[93] But in any case, the expression of this receptor in T cells suggests other implications and functions of BAFF / BAFF-R interaction, yet to be elucidated.

Concerning the expression of ***APRIL and its receptors***, this study shows that in memory human T cells:

- All $CD8^+$ memory T cell subsets downregulate APRIL mRNA.
- In $CD4^+$ memory T cells, only the $CD4^+$ $CD45RA^+CCR7^-$ (T_{TEM}) population upregulates APRIL gene expression.

- The genes of the receptors TACI and BCMA are not expressed.

In general, naïve cells apparently express higher levels of APRIL mRNA than memory T cells. Since even the naïve $CD45RA^{+}CCR7^{+}$ population wasn't found to express either TACI or BCMA, one can conclude that either APRIL expressed by T cells doesn't influence normal human T cell populations in an autocrine or paracrine manner, or that T cells express a yet to be discovered specific receptor to APRIL. Supporting the existence of an additional APRIL receptor is the fact that the Jurkat human leukemia T cell line, which is highly susceptible to APRIL stimulation, does not contain detectable levels of TACI or BCMA mRNA.[99]

Considering, however, that APRIL expression is most clearly downregulated in the CD8 memory population (which is the "executor" arm of cell-mediated immunity), it is reasonable to argue that APRIL probably doesn't influence the T cell mediated immune response directly. The upregulation of APRIL in the most differentiated phenotype of CD4 memory cell populations, however, could point to a role for this molecule in T cell–dependent B cell responses, such as the elicitation of an efficient humoral immune response. Similarly, a recently published paper reported APRIL expression in activated Th1 or Th2 cells, but not in naive cells.[99] One should note that another study also demonstrated the importance of APRIL in T-cell-dependent and independent antibody production in human B cells.[105]

Both BAFF and APRIL have previously been shown to stimulate human T cell proliferation in presence of suboptimal amounts of anti-CD3 and blocked by TACI-Fc. This has suggested that these ligands may act as a costimulatory factor in TCR-mediated activation mediated by TACI binding.[95,96,89,103,105] However, neither BCMA nor TACI expression was detected in any of the naïve or memory subpopulations in this study, making it difficult to explain a costimulatory function for either of the TNF-like-ligands. A possible explanation would be if TACI or BCMA were transiently expressed upon recent anti-CD3 stimulation and then rapidly downregulated, thus escaping detection in the experimental conditions of this study.

The highest levels of APRIL and BAFF transcripts have been found in monocytes and macrophages and other APCs. The receptors to these ligands, on the other hand, have preferentially been found to be expressed in B cells. This recently discovered ligand-receptor pathway has been profoundly implicated in survival, proliferation and differentiation of B cells.[93,102] The results of this study provide new insights into other possible roles of this signaling system. Additionally, the differential gene expression of the ligands BAFF and APRIL on subsets of CD4 T cells indicates that these ligands may be involved in T cell help

as well. Further studies are needed to clarify the role of this TNF-like-ligand-receptor system in human T cells.

IL-15Rα

The results obtained show considerable inter-individual variability in IL-15Rα gene expression. Still, certain tendencies emerge:

- The IL-15Rα gene seems to be generally expressed at slightly higher levels in memory T cells than in naïve T cells.
- The IL-15Rα-mRNA levels are most upregulated in the $CD8^+$ T_{EM} and in the $CD4^+$ T_{TEM} subsets.

One can consequently expect both $CD4^+$ and $CD8^+$ memory T cells to express more IL-15Rα on their surfaces and generally be more responsive to stimulation through IL-15. This would indeed be consistent with numerous studies reporting the requirement of IL-15 for long-term maintenance of $CD8^+$ cell memory, in both humans and mice.[53,144].

Interestingly, in both $CD4^+$ and $CD8^+$ cells, the effector populations (T_{EM} in $CD8^+$ and T_{TEM} in $CD4^+$) seem to express the highest transcript levels of IL-15Rα, suggesting that these subsets would react most sensitively to the presence of IL-15. This is of importance, because it could implicate a different role of IL-15 in humans as compared to mice, where IL-15 has been shown to play a crucial role in the expansion potential of central memory populations.[144] In humans, on the other hand, IL-15 could possibly play a selective role on the effector memory populations. This is in agreement with the study of Geginat et al.,[141,146] showing that the expression of a functional IL-15 receptor and concomitant IL-15 responsiveness are associated with differentiation. These characteristics increase from naive subsets to central memory and effector memory T cells, without however increasing the expansion potential of memory cells as a whole.

Which role could the IL-15 / IL-15Rα interaction have in effector memory T cells? To explain this, it is useful to imagine a situation when the survival of effector memory cells is essential, such as in persistent viral infections. In healthy individuals, the subset of $CD4^+$ T_{TEM} is very small. In the case of progressive HIV-infection, however, this population is expressed in higher numbers and is apparently capable of persisting as long as antigen loads are high.

Antigen-specific $CD4^+$ T_{TEM} cells are considered to be the most differentiated cells and have been shown to consist mostly of IL-2^- / IFNγ^+ cells, the cells that predominate in the immune response to progressive HIV-infection.[14,147,148] Since these cells don't produce the IL-2 necessary for self-renewal, their higher responsiveness to IL-15 could allow them to survive during acute viral infection. On the other hand, in $CD8^+$ cells, the presence of high levels of antigen seems to promote the accumulation of the greatest number of antigen-specific cells in the T_{EM} population. The skewing of the T_{TEM} subset towards the pre-terminally differentiated T_{EM} subset has been proposed as one of the mechanisms of HIV-induced impairment of proper $CD8^+$ T cell function.[11] Another possible explanation for the greater number of antigen-specific cells within the effector $CD8^+$ population during persisting HIV infection is that this could simply occur because of the kinetics in progressive disease which is characterized by an extremely high turnover of $CD8^+$ T cells. Consequently, T_{EM} cells would accumulate in their effort to replenish the terminally differentiated effector cells - but also because of their higher responsiveness to IL-15, giving them an edge over the T_{TEM} subset. Indeed, IL-15 is known to be produced endogenously by fibroblasts, keratinocytes, endothelial cells, and macrophages. Recalling that effector memory populations lack expression of CCR7 and home preferentially to non-lymphoid tissue, one can speculate that they might also more readily have access to IL-15 produced by fibroblast-like tissue stroma than central memory populations.

One can thus conclude that, in humans, IL-15 might have a role in driving an efficient effector response in viral infections, for both CD8 and Th1 responses, by acting as a specific growth factor for effector cell populations. If this is the case, the use of IL-15 may be interesting in boosting the immune response after vaccination. Quite a few studies support a preferential role of IL-15 on effector memory cells. Just recently, the selective expansion of effector memory $CD8^+$ T cells by IL-15 has been shown in simian immunodeficiency virus-infected macaques.[149] Similarly, the in vitro treatment of PBMCs with IL-15 has been shown to enhance IFN-γ production of HIV-specific $CD8^+$ T cells,[150] further supporting IL-15's possible therapeutic use in persisting viral infections such as chronic HIV-infection.

One should note that $CD4^+$ memory T cells have previously been reported to be less sensitive to IL-15 stimulation.[151] Our results do not contradict this observation, since transcription levels between $CD4^+$ and $CD8^+$ cells were not compared at baseline. Also, one might argue that the mRNA expression of IL-15Rα may not reflect the effective functional expression of the receptor on the cell surface. Either functional or flow-cytometric studies of IL-15Rα

surface expression would be required to further confirm the interpretation of the gene expression patterns.

IL-7Rα

The data on IL-7Rα gene expression can be briefly interpreted as follows:

- IL-7Rα-mRNA is generally present in smaller amounts in memory T cells than in naïve T cells – except for in the $CD8^+CD45RA^-CCR7^+$ (T_{CM}) subset where IL-7Rα-mRNA levels were slightly upregulated.

Presuming again that gene expression correlates with the expression of a functional receptor, these results indicate that, even though memory cells express IL-7Rα and would respond to stimulation by IL-7, this cytokine probably preferentially influences naïve T cells - reflected by the higher levels of IL-7Rα mRNA in naïve T cells as compared to memory T cells. This is consistent with the fact that IL-7 is known to be an important growth factor for naïve T cells in both humans and animals. Indeed, naïve T cells have been shown to require the presence of IL-7 in an absolute manner for their survival and long-term persistence through peripheral homeostatic proliferation, as well as for their development.[70,78,152]

Especially $CD8^+$ memory cells seem to downregulate IL-7Rα mRNA levels to a greater degree while acquiring a more differentiated effector phenotype. In line with this, recent studies have shown a positive correlation between the loss of IL-7R surface expression in $CD8^+$ T cells and the activity of HIV-disease in untreated HIV-seropositive patients. After treatment by antiretroviral drugs, IL-7R expression in $CD8^+$ cells was found to be restored.[153,154] Since our results show that IL-7Rα is preferentially upregulated in the central memory subset, the loss of its expression in total $CD8^+$ memory cells might actually reflect a loss of central memory type $CD8^+$ cells during untreated HIV disease and a shift towards effector memory phenotype cells. Several studies have shown an accumulation of antigen-specific $CD8^+$ cells within the effector memory subsets ($CD45RA^-CCR7^-$ and $CD45RA^+CCR7^-$ phenotypes) during chronic viral infections such as CMV and HIV infection. It is noteworthy that the central memory subset of $CD8^+CD45RA^-CCR7^+$ (T_{CM}) cells stands out by showing nearly twice as high IL-7Rα mRNA levels relative to naive $CD8^+$ cells. In fact, Ahmed et al.[155] have recently identified a subpopulation of $CD8^+$ cells that highly and selectively expresses IL-7Rα as potential memory cell precursors. By adoptively

transferring IL-7Rα-high and -low $CD8^+$ cells into mice, they could demonstrate that the IL-7Rα-high cells gave rise to memory cells that could persist and confer long-term protective immunity. One could therefore speculate that, even though IL-7 responsiveness doesn't characterize survival of memory cells in general, it may play an important role in selecting a defined subset of CD8 memory cells to survive over long periods of time. The fact that these IL-7 responsive cells are found to be in the central memory subset makes the suggestion even more plausible.

As regards the expression of the IL-7Rα gene in $CD4^+$ cells, it generally seems to be less tightly regulated than in $CD8^+$ cells. Moreover, unlike their $CD8^+$ counterparts, $CD4^+$ $CD45RA^-CCR7^+$ cells do not selectively upregulate IL-7Rα mRNA levels. Thus, there seems to be a differential regulation of responsiveness to IL-7 in the central memory subsets of $CD4^+$ and $CD8^+$ T cells.

Bcl-2

The results obtained indicate that mRNA levels of the Bcl-2 gene:

- are clearly downregulated in all memory $CD8^+$ T cell subsets, relative to the expression in naïve $CD8^+$ cells. Lowest levels were present in the $CD45RA^+CCR7^-$ population.
- are expressed in a more variable manner in memory $CD4^+$ cells with expression levels closer to levels in naïve $CD4^+$ cells.

In this study, Bcl-2 gene expression has been shown to be very consistently downregulated in $CD8^+$ memory T cells, as compared to the naïve $CD8^+$ cell population. Additionally, the data not only suggests that Bcl-2 expression is generally lower in the memory $CD8^+$ population, but also that it is progressively lost from naïve to central memory to effector and terminally differentiated effector subsets. These results are in agreement with other studies showing decreased Bcl-2 protein expression in memory CD8 subsets.[146] There remains, however, the apparently confusing fact that, as a whole, the memory population has to contain long-lived cells, yet expresses lower levels of Bcl-2, thus apparently being more susceptible to apoptosis. Hence, one could speculate that memory T cells have to be actively "rescued" from cell death by extrinsic factors, such as cytokines and growth factors. This is quite clearly the case in vitro, where cell death of primed T cells is inhibited by adding exogenous IL-2. One can

hypothesize that similar mechanisms may exist in vivo where, amongst possible cytokines such as IL-2 and interferon-alpha/beta (IFN-alpha/beta), IL-15 could play an important role in protecting effector cells from premature cell death. This is supported by the data of this study concerning the expression of IL-15Rα. Indeed, higher levels of specific IL-15 receptor chain (IL-15Rα) mRNA were found in the $CD8^+CD45RA^-CCR7^-$ subset, suggesting a higher responsiveness of the $CD8^+$ effector memory population to IL-15.

Another important observation is that the $CD8^+$ $CD45RA^-CCR7^+$ subset, corresponding to the central memory subset, expresses nearly twice as much Bcl-2 than the effector subpopulations, suggesting them to be the least apoptosis-susceptible subset out of the memory cells. Previous studies have demonstrated the unique ability of the central memory population to convert into other phenotypes upon cytokine stimulation, designating them a role as precursors of other memory subsets.[141,146] A higher resistance to apoptosis could enhance the potential role of T_{CM} cells as a reservoir for other memory cell subsets.

Surprisingly, the results in the $CD4^+$ population are quite different from the results in $CD8^+$ T cells. Besides being only moderately different from expression levels in naïve $CD4^+$ cells, the inter-individual expression levels of Bcl-2 in $CD4^+$ memory cells have been found to be very variable in the individuals examined in this study. Of course, a larger study examining a greater number of individuals might result in demonstrating certain tendencies of gene expression between the $CD4^+$ memory subsets as well. It is nevertheless interesting to note that the increased variability in $CD4^+$ memory cells was also observed for the expression of the IL-15Rα gene. This was especially apparent in the $CD4^+$ $CD45RA^-CCR7^+$ subset, as opposed to the concordant expression of Bcl-2 and IL-15Rα genes in the $CD8^+$ $CD45RA^-$ $CCR7^+$ cell population. It is therefore plausible that the susceptibility of $CD4^+$ memory cells to cell death is controlled by different pathways than in $CD8^+$ cells.

Bcl-xL

The results obtained indicate that:

- Bcl-xL mRNA levels are upregulated in memory $CD8^+$ cells. Expression levels increase with differentiation to a more effector phenotype.

- In $CD4^+$ cells, Bcl-xL has been found to be moderately higher in all memory $CD4^+$ cells, as compared to naïve $CD4^+$ cells. However, only the T_{EM} population showed significant upregulation.

Thus, oppositely to Bcl-2 expression, Bcl-xL gene expression is upregulated in $CD8^+$ memory T cells and increases progressively from naïve to central memory to effector and terminally differentiated effector subsets. In this context, it is important to emphasize that especially in the $CD8^+$ compartment, T_{EM} and T_{TEM} subsets often consist of highly expanded populations, resulting from repetitive TCR stimulation through antigen. CD28 ligation, which simulates adequate antigen stimulation in vitro, has been shown to induce Bcl-xL production.[156,157] It has been postulated that expanding cells may be protected from apoptosis by this mechanism. Hence, effector memory $CD8^+$ cells may be protected against apoptosis, not only through enhanced cytokine responsiveness, as discussed above, but also by increased expression of anti-apoptotic Bcl-xL. On the other hand, the increase in Bcl-xL may also just reflect the greater amount of stimulation and the degree of expansion that effector memory subsets have undergone.

The Bcl-xL expression in $CD4^+$ T cells seems to be similar, even though the results indicate a more moderate relative upregulation of Bcl-xL levels in memory $CD4^+$. Thus, it would seem that Bcl-xL plays a less important role in memory $CD4^+$ T cell subsets than in the $CD8^+$ T cell compartment. One needs to note that, during the development in the thymus, Bcl-xL is known to selectively promote maturation of the single positive CD8 lineage and that $CD8^+$ cells seem to be more Bcl-xL dependant.[117] In contrast to the case of $CD8^+$ cells, however, the highest expression levels within $CD4^+$ cells have been observed in the $CD45RA^-CCR7^-$ subset (T_{EM}). Therefore, one could argue that the most expanded populations in the $CD4^+$ T cells of healthy donors might be localized in the T_{EM} subset. Possibly, this ratio could change in clinical settings of high antigen presence, where a shift towards the terminally differentiated subset ($CD45RA^+CCR7^-$ population, T_{TEM}) has been observed.

$FLIP_S$

The results obtained indicate that, relative to expression in naïve cells, the $FLIP_S$ gene expression:

- is upregulated in all memory $CD8^+$ T cell subsets.

- is slightly lower in all memory $CD4^+$ cells, as compared to expression in naïve $CD4^+$ cells.

Within the $CD8^+$ T cell population, $FLIP_S$ gene expression has been found to be globally upregulated in all the memory subsets; increased expression levels, however, predominated in the T_{TEM} ($CD45RA^+CCR7^-$) population. The anti-apoptotic role of $FLIP_S$ mainly consists in protecting T cells from activation-induced-cell-death (AICD). It does so by intervening in the extrinsic, predominantly Fas-mediated pathway of apoptosis by inhibiting caspase-8 activation and consequent apoptosis. TCR-mediated upregulation of $FLIP_S$ has also been reported to correlate with FasL-resistance in a proportion of restimulated T cells, indicating it may contribute to T-cell survival and memory development in this manner.[124,158] Indeed, in vivo, highly activated cells are placed in the terminally differentiated subset (TEM). This population consequently requires the most active protection from AICD. Thus, the upregulation of $FLIP_S$ found in the TEM subset of $CD8^+$ cells seems to reflect this necessity. Why however, were the lowest $FLIP_S$ levels found in the EM population? Why should the EM subset be more sensitive to AICD? The cells of the EM population reside in the target organ of the infection, so that they are likely to receive the most antigen stimulation during the immune response. The low levels of $FLIP_S$ could enhance their sensitivity to AICD, thus controlling their expansion and limiting the number of EM cells. The reduced survival of the EM population would then be proportional to the antigen load, allowing the EM population to act as an accurate sensor of antigen load. By this mechanism, the EM cell number could drive the replenishment of the effector pool from the CM population.

The situation in $CD4^+$ T cells seems to be quite different since, in contrast to $CD8^+$ cells, $FLIP_S$ mRNA levels have been found to be preferentially downregulated in all the $CD4^+$ memory subsets. This suggests that $CD4^+$ memory T cells might be intrinsically less susceptible to Fas-mediated death than $CD8^+$ memory T cells. One should note that, analogous to the situation in $CD8^+$ T cells, the EM population seems to express the least amount of FLIP, making it the most AICD-sensitive population. The results obtained, however, are not as clearly interpretable as in $CD8^+$ cells, since the differences in expression levels of naïve and memory cells are quite small, making it difficult to judge the significance of the changes.

Bad and Bax

The data for ***Bad*** expression in $CD8^+$ T cells demonstrates a high inter-individual variability of the expression of this gene in the different memory subsets. Even though mRNA levels are present in a more consistent way amongst the $CD4^+$ population of the donors tested, $CD4^+$ memory T cells showed only very slight differences of Bad expression levels in comparison to naïve $CD4^+$ cells. Thus, it would seem that Bad does not play an important and obvious role in regulating the sensitivity to cell death in either $CD8^+$ or $CD4^+$ memory cells.

Bax gene expression on the other hand, even though also quite variable amongst the individual donors tested, seems to follow clearer tendencies. It is apparently preferentially upregulated in $CD4^+$ memory cells, in contrast to a discrete downregulation observed in $CD8^+$ memory cells. $CD4^+$ memory cells might therefore be more susceptible to apoptosis mediated by Bax than their $CD8^+$ counterparts.

FasL

The interactions between Fas and its ligand FasL are supposedly the major mechanisms involved in AICD. Naïve human T lymphocytes, however, are known to be insensitive to death receptor-induced apoptosis or to AICD. Indeed, activation of naïve T cells results in proliferation, cytokine secretion, and effector function. In fact, T cell blasts have been shown to start to be sensitive to AICD induction only at day 6 post-activation.[159-161] In this study, the resistance of naïve T cells to AICD is reflected by the total absence of FasL expression in naive T cells, both for $CD8^+$ T cells and $CD4^+$ T cells, in all the individuals tested.

In the memory cells of the CD8+ compartment, FasL expression has been shown to increase progressively from central memory to terminally differentiated effector cells. Restimulated T cells are known to be more sensitive to Fas-mediated apopoptosis.[162] The progressive increase of FasL expression in $CD8^+$ memory T cell subsets could thus be interpreted as the result of a greater replication history of the effector subsets. Alternatively, one should take into consideration another possible interpretation: $CD8^+$ cells are also known as cytotoxic cells CTLs) or killer lymphocytes. Their cytotoxic activity is mediated directly by either granzymes or perforin, but they can also induce cell death via FasL. The interaction of FasL with Fas, expressed on cells other than T cells, enables the induction of immediate cell death in these cells through the membrane-bound FasL, expressed on CTLs. The extremely high

upregulation of FasL expression could therefore simply be the reflection of the increased cytotoxic potential in increasingly effector memory populations.

The interpretation of the results is less obvious in $CD4^+$ memory T cells, since FasL expression was absent in the first two memory subsets (CM and EM). It seems that circulating memory $CD4^+$ cells are less susceptible to AICD than their $CD8^+$ counterparts.

Tob

The results obtained indicate that Tob expression in $CD8^+$ T cells seems to be lost in the process of acquiring a more differentiated state. Since forced Tob expression has previously[126] been shown to inhibit anti-CD3 / anti-CD28-mediated proliferation, the downregulation of Tob in vivo should, oppositely, enhance proliferation in response to antigen and co-stimulation. It is therefore perceivable that activation threshold levels are lowered in memory $CD8^+$ T cells by the means of Tob downregulation. This makes sense, as one would expect memory $CD8^+$ T cells to react faster and to lower levels of antigen than naïve cells. Inversely, constitutive expression of Tob in naïve $CD8^+$ cells could confer peripheral tolerance to circulating naïve $CD8^+$ T cells by increasing their activation threshold. This may be a possible explanation of the fact that naïve T cells are known to have more restrictive requirements for co-stimulation than memory T cells. It is also consistent with the results of Tzachanis et al.,[126] who showed that only TCR-stimulation coupled with appropriate co-stimulation would induce a downregulation of Tob, enabling progression of the cell cycle and cell division.

In this study, as opposed to Tob expression in $CD8^+$ T cells, $CD4^+$ memory cells in general and the $CD4^+$ effector-memory populations in particular, have not been shown to downregulate Tob mRNA levels. This seems to be in contradiction to the results obtained in the above cited study,[126] which had shown a greater downregulation of Tob mRNA in total $CD4^+$ cells as compared to $CD8^+$ cells upon stimulation. In their experimental system however, the authors measured expression levels between 3 and 24 hours after stimulation. It could thus be possible that, upon stimulation, $CD4^+$ T cells transiently downregulate Tob expression, which is then regained in circulating memory cells. This would implicate that $CD4^+$ T cells require efficient co-stimulation at any stage of differentiation, showing no significant difference of the activation thresholds between subsets of memory $CD4^+$ T cells, or between memory and naïve $CD4^+$ T cells. Again, this does not correspond well with the known fact that memory $CD4^+CCR7^+$ and $CD4^+CCR7^-$, to an even greater extent, show

enhanced responsiveness to T-cell receptor triggering.[7] Hence, how can activation thresholds be modulated without affecting Tob mRNA levels? The inhibitory effect of Tob has been shown to be inactivated by phosphorylation through Erk1 and Erk2.[129] One could therefore also imagine that in $CD4^+$ cells, in contrast to the case of $CD8^+$ cells, Tob activity may be regulated not by lowering expression levels, but by phosphorylation instead. This is supported by the fact that ERK MAP kinases are essential for TCR-induced responses.[163] Of course, an alternative pathway, not implicating Tob at all, may also be involved.

Results in $CD8^+$ memory cells

When looking at the gene expression pattern in the $CD8^+$ T cell compartment as a whole, the more cytotoxic, directly effector $CD8^+$ memory cells, e.g. the T_{EM} and T_{TEM} subsets, seem to be much more susceptible to cell death than the central memory subset. This is reflected not only in their extremely prominent upregulation of FasL, but also in the downregulation of protective factors such as Bcl-2, IL-7Rα, BAFF-R, and APRIL. Their decreased expression of Tob also indirectly increases the AICD susceptibility by lowering the activation threshold. On the other hand, they are actively protected against cell death by higher levels of the anti-apoptotic factors Bcl-xL and FLIP, as well as possibly through a higher responsiveness to IL-15.

Thus, generally speaking, the results of this study indicate that in $CD8^+$ T cells there is a greater sensitivity to cell death in the $CCR7^-$ effector memory subsets (T_{EM} and T_{TEM}), as compared to the $CCR7^+$ population consisting of naïve and central memory (T_{CM}) $CD8^+$ T cells. In the $CCR7^-$ cells, re-encounter with antigen would therefore not only induce execution of effector functions, but also rapidly cause apoptosis. $CD8^+$ $CCR7^-$ cells have been shown to mediate particularly effective effector function through IFNγ and also express high levels of perforin. Why then should the most effective cells die? One possible explanation is that, in viral infections, the cells that accumulate in the target organ of the infection are mostly of the T_{TEM} cell-type, reflecting the capacity of $CCR7^-$ cells to preferentially home to non-lymphoïd tissue. Upon re-exposure, one can imagine that the already present T_{TEM} cells would rapidly intervene and their frequency in situ might be sufficiently elevated to mediate an effective immune response without requiring cell proliferation. Additionally, AICD in T cells in vivo has been proposed to limit the expansion of an immune response by eliminating effector cells that are no longer needed. Indeed, after antigen or pathogens have been eliminated from the

organism, these T cells are potentially dangerous because of their potent effector functions and low activation requirements. Hence, their increased susceptibility to death is also extremely important to counterbalance these traits, which would otherwise lead to uncontrolled cytotoxicity, harmful to the host. Another influence on the results may be the fact that the terminally differentiated effector population (T_{TEM}) might be the population containing the most in vivo expanded clones in any individual. The sensitivity to cell death would then simply be a reflection of the cell age, e.g. the number of prior cell divisions. This was shown in previous reports stating that, in the $CD8^+$ compartment, the loss of CD28 identifies a subset of antigen-experienced T cells with high cytotoxic potential and reduced proliferative capacity, and which frequently contains in vivo-expanded clones in the elderly or in human immunodeficiency virus (HIV-) infected individuals.[164-166]

Apart from showing a possible linear differentiation pattern in $CD8^+$ T cells with progressive susceptibility to apoptosis in the $CCR7^-$ memory subsets, there seems to be a differential responsiveness to cytokines amongst the memory subsets. Cytokines may therefore play a crucial role in modulating the function of the distinct memory subsets. Thus, the upregulation of IL-7Rα on the central memory subset of $CD8^+$ T cells may confer a selective survival signal to this population, making them the true memory cells of the immune response.

Results in $CD4^+$ memory cells

One of the most notable features of the gene expression patterns observed in $CD4^+$ cells has been the greater inter-individual variability, as compared to $CD8^+$ T cells. Secondly, the differences in expression levels of memory and naïve cells were found to be much less contrasted than in the $CD8^+$ compartment. One can conclude that gene expression levels between circulating memory cells and naïve cells are quite stable. One might argue that $CD4^+$ cells possibly do not control their survival pathways at the gene expression level, but that they regulate it though activation and inactivation at a protein level. This may of course be true for individual pathways, but it does not explain the variability in expression between the donors. Another possible explanation is that $CD4^+$ cells modify their gene expression profiles in a much more transient manner, regaining stable expression levels in a resting, circulating state. This option would need further clarification by analyzing gene expression in recently primed CD4 cells and combining it with a chronologically progressive follow-up of the gene profile.

$CD4^+$ memory T cells have been shown to present less downregulation of the survival factor IL-7Rα and the anti-apoptotic factors Bcl-2, as also lower levels of FasL mRNA, suggesting them to be generally less sensitive to cell-death than their $CD8^+$ counterparts

Differences between $CD4^+$ and $CD8^+$ memory cells

Firstly, the point to be emphasized is that there seems to be a considerable difference between the $CD4^+$ and $CD8^+$ compartments of memory T cells. This is interesting in itself since even though $CD4^+$ and $CD8^+$ T cells have different roles in the immune response, one could have imagined that the conditions allowing them to maintain memory traits would be quite similar.

On the whole, $CD4^+$ memory T cells have been shown to be less sensitive to activation-induced cell death (less downregulation of Bcl-2, lower levels of FasL mRNA). This mirrors the inherent difference in the functional roles of $CD8^+$ and $CD4^+$ cells. $CD4^+$ T cells have a "helper" role, as opposed to the "killer" role of $CD8^+$ lymphocytes. One could imagine that the heterogeneity in survival and apoptosis gene expression patterns observed between $CD8^+$and $CD4^+$ T cells may also reoccur on the level of their differentiation pathways. In the $CD8^+$ compartment, a linear differentiation pattern makes sense. It associates replication history to differentiation, and differentiation to increased cell death susceptibility. The rather straight-forward role of $CD8^+$ T cells is compatible with this; they differentiate to kill and, once they have executed their mission, their function is superfluous. The role of $CD4^+$ memory T cells is more complex. Their effector role consists in communicating with other components of the immune system and enhancing the overall effectiveness of the immune response. Moreover, the effector $CD4^+$ T cells are quite heterogeneous, consisting of Th-1 cells with an IFNγ-secreting profile, IL-4 secreting Th-2 cells and the immunosuppressive $CD4^+CD25^{high}$ T_{reg} cells amongst others. Only a more complex differentiation pattern with multiple checkpoints would enable the generation of such a heterogeneous population. The overall increased relative resistance to activation-induced cell death could thus guarantee a higher plasticity in the differentiation of memory $CD4^+$ cells.

8 CONCLUSIONS

By separating T cells into different subsets according to the expression of CD45RA and their lymph-homing ability as mediated by expression of CCR7, human T cells can be separated into 4 subsets compromised of naïve cells, central memory cells, effector memory and terminally differentiated effector memory cells. In this study, the differential sensitivity to survival and cell death of these subsets has been studied by analysis of the gene expression pattern of a selection of pertinent genes. The data collected shows that $CD4^+$ memory T cells and $CD8^+$ human memory T cells regulate genes implicated in survival and apoptosis in different ways. The $CD8^+$ T cell compartment preferentially showed progressive susceptibility to cell death from naïve and central memory ($CCR7^+$) subsets to effector and terminally differentiated effector ($CCR7^-$) subsets. The expression of genes involved in protection from apoptosis (Bcl-2), as well as of genes implicated in survival pathways (IL-7R, APRIL, BAFF-R), is progressively lost. In contrast, expression of pro-apoptotic factors such as Bax and FasL is gradually increased *(Figure 7)*.

Importantly, the $CD8^+$ $CD45RA^-$ $CCR7^+$ central memory subset was shown to selectively upregulate the expression of the functional IL-7 receptor (IL-7Rα), proposing the enhanced responsiveness to IL-7 as a selection bias for long-term memory. On the other hand, IL-15Rα expression was selectively increased in the $CD8^+$ $CD45RA^-CCR7^-$ effector memory subset, suggesting a role for IL-15 in enhancing long-term effector responses.

From this study, it is clear that human T cell memory subsets depend on many factors that influence their ability to survive, on the one hand, and their susceptibility to cell death on the other. The balance of these interactions seems to influence their long-term survival. The differences observed between the gene expression patterns of $CD4^+$ and $CD8^+$ cells speak in favour of distinct memory cell differentiation patterns in these two T lymphocyte compartments.

To further unravel the interactions of survival and death signals in memory T cell subsets is crucial to our understanding of the secondary immune response and of adaptive cell-mediated immunity in general. It is thus central to our comprehension of the natural evolution of infectious diseases, such as acute infection and resolution versus establishment of chronic disease. As such, it will help discovering mechanisms of inducing and enhancing long-term immunological memory, which is essential to the field of vaccine development.

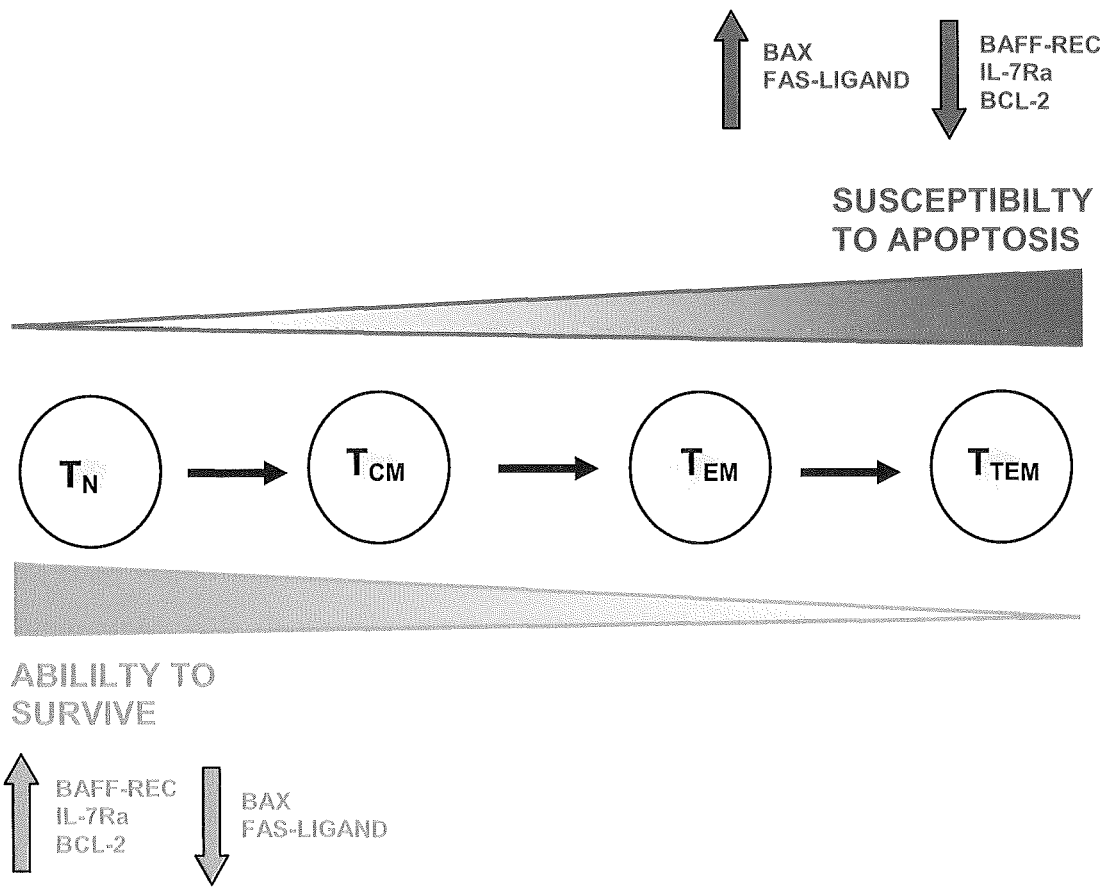


FIGURE 7 **Expression of survival and pro-apoptotic genes in CD8 memory subsets**. Genes potentially mediating survival enhancing mechanisms (e.g. BAFF-Receptor, IL-7Rα and Bcl-2) are expressed in higher levels in central memory T cells (T_{CM}). Pro-apoptotic genes (e.g. Bax and Fas-Ligand) are suppressed in this population, but expressed at higher levels in the effector populations (T_{EM} and T_{TEM}), implying a higher susceptibility to apoptosis.

9 PERSPECTIVES

The present study has reported tendencies in gene expression patterns of memory T cells of healthy individuals. Considering the great inter-individual heterogeneity in gene expression, a larger study including more individuals should be performed to evaluate the significance of the differences observed.

Additionally, it would be of great interest to follow this up with studies on antigen-specific memory T cells. Extremely little is known of the relative sensitivity to apoptosis in vivo of these cells. This would be possible using the same experimental design which combines flow-cytometric sorting with a sensitive RT-PCR assay. Indeed, this methodology has allowed the use of extremely few cells per subset and to evaluate a fairly large gene panel. By further confining the gene panel to maybe just four genes per run, this approach could allow the investigation of gene expression in even more restricted subsets, such as tetramer positive or cytokine positive T cells from CMV, EBV or HIV-infected individuals. This could provide insights into the specific genetic pattern of "real" antigen-specific memory T cells. It would be interesting to know, for example, as to whether in HIV (where the antigen-specific CD8 memory compartment seems to be skewed) a different expression of apoptosis genes could account for the skewing.

As mentioned earlier, experimental evidence at the protein level is required in order to investigate the possible functional importance of the genetic pattern observed. In this context, it would be particularly interesting to further examine the expression of transcripts of BAFF, APRIL and their receptors in human T cells. Do T cells secrete APRIL and BAFF, or do they express them as membrane-bound proteins on their surface? Do BAFF-R mRNA transcript levels in T cells correlate with a surface expression of BAFF-R? If so, how is the expression induced, which are the cells which will provide the stimulus, what are the consequences of ligand-receptor interactions, and which are the downstream signaling pathways implicated?

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