UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Département de médecine, Service d'immunologie et d'allergie

Profiling of Signal Transduction in Human Memory T Cells

Thèse de doctorat ès science de la vie (PhD)

présentée à la
Faculté de biologie et de médecine de l'Université de Lausanne
par

Nicolò Costantino Brembilla

diplômé en Biotechnologie Medicale
de l'Université de Milan, Italie

Jury

Prof. Paolo Dotto
Prof. Giuseppe Pantaleo
Dr. Marie-Agnès Doucey
Prof. Oreste Acuto
Dr. Keith Harshman
Prof. Sanjiv Luther

Lausanne 2008

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur Prof.	Gian-Paolo **Dotto**
Directeur de thèse	Monsieur Prof.	Giuseppe **Pantaleo**
Experts	Madame Dr	Marie-Agnès **Doucey**
	Monsieur Dr	Keith **Harschman**
	Monsieur Dr	Sanjiv **Luther**
	Monsieur Prof.	Oreste **Acuto**

le Conseil de Faculté autorise l'impression de la thèse de

Monsieur Nicolo Constantino Brembilla

Diplômé en biotechnologie de l'Université de Milan

intitulée

Profiling of Signal Transduction in Human Memory T Cells

Lausanne, le 10 septembre 2008

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Gian-Paolo Dotto

Acknowledgments

First I would like to thank my thesis director, Prof. Giuseppe Pantaleo, who gave me the great opportunity to work in his lab and allowed me to bring my thesis up to the end.

I would like to express my special gratitude to Dr. Marie-Agnès Doucey, supervisor of my project, who always provided me with helpful advices and constructive critics increasing my scientific attitudes.

I would like also to show my appreciation to all the people I crossed during these years… for the technical and scientific support as well as the friendly working environment. A special thank goes to the members of the Laboratory of AIDS Immunopathogenesis at the CHUV and the Protein Analysis Facility (PAF) of the University of Lausanne, where I spent respectively the first and the second halves of my project.

I would like also to show my gratitude to the people of the DNA Array Facility (DAF) for their precious contribute to the setting of Reverse Phase Protein Array technology, the member of the Swiss Institute of Bioinformatics (SIB) for the really helpful advice in array data analysis and Dr. Donata Rimoldi who kindly hosted me in her lab for all the molecular biology experiments.

I would like to thank Prof. Paolo Dotto, Prof. Oreste Acuto, Prof. Sanjiv Luther and Dr. Keith Harshman for having accepted to be part of my "Jury de Thèse", for their constructive comments and their careful reading of the manuscript.

Last but not least, a special thank to my family who always supported me… from Italy
and
to my wife Marta who was always at my side when I most needed it and with whom this adventure began.

Résumé pour un large public

La vaccination a eu un impact énorme sur la santé mondiale. Mais, quel est le principe d'un vaccin? Il est basé sur la 'mémoire immunologique', qui est une particularité exclusive des systèmes immunitaires des organismes évolués. Suite à une infection par un pathogène, des cellules spécialisées de notre système immunitaire (les lymphocytes) le reconnaissent et initient une réaction immunitaire qui a pour but son élimination. Pendant cette réaction se développent aussi des cellules, appelées cellules lymphocytaires mémoire, qui persistent pour longue durée et qui ont la capacité de stimuler une réaction immunitaire très efficace immédiatement après une seconde exposition à ce même pathogène. Ce sont ces cellules mémoires (lymphocytes B et T) qui sont à la base de la 'mémoire immunologique' et qui sont stimulées lors de la vaccination. Chez l'homme, deux populations distinctes des lymphocytes T mémoires ont été identifiées: les cellules centrales (CM) et effectrices (EM) mémoires. Ces populations sont fonctionnellement hétérogènes et exercent des rôles distincts et essentiels dans l'immunité protectrice. Typiquement, les cellules effectrices mémoires sont capables de tuer immédiatement le pathogène tandis que les cellules centrales mémoires sont responsables d'initier une réponse immunitaire complète. Pourtant, les mécanismes biochimiques qui contrôlent les fonctions de ces cellules ont été jusqu'à présent peu étudiés à cause de la faible fréquence de ces cellules et de la quantité limitée de tissus humains disponibles pour les analyses. La compréhension de ces mécanismes est cruciale pour la réalisation de vaccins efficaces et pour le développement de nouveaux médicaments capables de moduler la réponse immunitaire lymphocytaire. Dans cette thèse, nous avons d'abord développé et amélioré une technologie appelée 'protéine array en phase inverse' qui possède un niveau de sensibilité beaucoup plus élevé par rapport aux technologies classiquement utilisées dans l'étude des protéines. Grâce à cette technique, nous avons pu comparer la composition protéique du système de transmission des signaux d'activation des cellules CM et EM humaines. L'analyse de 8 à 13 sujets sains a montré que ces populations des cellules mémoires possèdent un système de signalisation protéique différent. En effet, les cellules EM possèdent, par rapport aux cellules CM, des niveaux réduits d'une protéine régulatrice (appelée c-Cbl) que nous avons démontré comme étant responsable des fonctions spécifiques de ces cellules. En effet, en augmentant artificiellement l'expression de cette protéine régulatrice dans les cellules EM jusqu'au niveau de celui des cellules CM, nous avons induit dans les cellules EM des capacités fonctionnelles caractéristiques des cellules CM. En conclusion, notre étude a identifié, pour la première fois chez l'homme, un mécanisme biochimique qui contrôle les fonctions des populations des cellules mémoires.

Résumé en Français

Les cellules mémoires persistent inertes dans l'organisme et produisent des réactions immunitaires rapides et robustes contre les pathogènes précédemment rencontrés. Deux populations distinctes des cellules mémoires ont été identifiées chez l'homme: les cellules centrales (CM) et effectrices (EM) mémoires. Ces populations sont fonctionnellement hétérogènes et exercent des rôles distincts et critiques dans l'immunité protectrice. Les mécanismes biochimiques qui contrôlent leurs fonctions ont été jusqu'à présent peu étudiés, bien que leur compréhension soit cruciale pour le développement des vaccins et des nouveaux traitements/médicaments. Les limites majeures à ces études sont la faible fréquence de ces populations et la quantité limitée de tissus humains disponibles. Dans cette thèse nous avons d'abord développé et amélioré la technologie de 'protéine array en phase inverse' afin d'analyser les molécules de signalisation des cellules mémoires CD4 et CD8 humaines isolées *ex vivo*. L'excellente sensibilité, la reproductibilité et la linéarité de la détection, ont permis de quantifier des variations d'expression protéiques supérieures à 20% dans un lysat équivalent à 20 cellules. Ensuite, grâce à l'analyse de 8 à 13 sujets sains, nous avons prouvé que les cellules mémoires CD8 ont une composition homogène de leur système de signalisation tandis que les cellules CD4 EM expriment significativement de plus grandes quantités de SLP-76 et des niveaux réduits de c-Cbl, Syk, Fyn et LAT par rapport aux cellules CM. En outre, l'expression réduite du régulateur négatif c-Cbl est corrélée avec l'expression des SLP-76, PI3K et LAT uniquement dans les cellules EM. L'évaluation des propriétés fonctionnelles des cellules mémoires a permis de démontrer que l'expression réduite du c-Cbl dans les cellules EM est associé à une diminution de leur seuil d'activation. En effet, grâce a la technique de transduction cytosolique, nous avons augmenté la quantité de c-Cbl des cellules EM à un niveau comparable à celui des cellules CM et constaté une réduction de la capacité des cellules EM à proliférer et sécréter des cytokines. Ce mécanisme de régulation dépend principalement de l'activité d'ubiquitine ligase de c-Cbl comme démontré par l'impact réduit du mutant enzymatiquement déficient de c-Cbl sur les fonctions de cellules EM. En conclusion, cette thèse identifie c-Cbl comme un régulateur critique des réponses fonctionnelles des populations de cellules T mémoires et fournit, pour la première fois chez l'homme, un mécanisme contrôlant l'hétérogénéité fonctionnelle des ces cellules. De plus, elle valide l'utilisation combinée des 'RPP arrays' et de la transduction cytosolique comme outil puissant d'analyse quantitative et fonctionnel des protéines de signalisation.

Summary

Memory cells persist in a quiescent state in the body and mediate rapid and vigorous immune responses toward pathogens previously encountered. Two subsets of memory cells, namely central (CM) and effector (EM) memory cells, have been identified in humans. These subsets display high functional heterogeneity and assert critical and distinct roles in the control of protective immunity. The biochemical mechanisms controlling their functional properties remain so far poorly investigated, although their clarification is crucial for design of effective T-cell vaccine and drug development. Major limitations to these studies lie in the low frequency of memory T cell subsets and the limited amount of human specimen available.

In this thesis we first implemented the innovative reverse phase protein array approach to profile 15 signalling components in human CD8 and CD4 memory T cells isolated *ex vivo*. The high degree of sensitivity, reproducibility and linearity achieved, allowed an excellent quantification of variations in protein expression higher than 20% in as few as 20-cell equivalent per spot. Based on the analysis of 8 to 13 healthy subjects, we showed that CD8 memory cells have a homogeneous composition of their signaling machinery while CD4 EM cells express statistically significant increased amounts of SLP-76 and reduced levels of c-Cbl, Syk, Fyn and LAT as compared to CM cells. Moreover, in EM but not CM cells, reduced expression of negative regulator c-Cbl correlated with the expression of SLP-76, PI3K and LAT. Subsequently, we demonstrated that the higher functional properties and the lower functional threshold of EM cells is associated with reduced expression of c-Cbl. Indeed, by increasing c-Cbl content of EM cells to the same level of CM cells using cytosolic transduction, we impaired their proliferation and cytokine production. This regulatory mechanism was primarily dependent on c-Cbl E3 ubiquitin ligase activity as evidenced by the weaker impact of enzymatically deficient c-Cbl C381A mutant on EM cell functions. Together, these results identify c-Cbl as a critical regulator of the functional responses of memory T cell subsets and provides, for the first time in humans, a mechanism controlling the functional heterogeneity of memory CD4 cells. Moreover it validates the combined use of RPP arrays and cytosolic transduction approaches as a powerful tool to quantitatively analyze signalling proteins and functionally assess their roles.

Abbreviations used in the text

APC: antigen presenting cell

CARD: catalyzed reporter deposition amplification

CM: central memory

CV: coefficient of variation

DC: dendritic cell

E: effector

ELISA: emzyme-linked immunoadsorbent asssay

EM: effector memory

FPP: forward phase protein

GEM: glycolipid-enriched membrane microdomain

HCV: hepatitis C virus

HIV: human immunodeficiency virus

IL-: interleukin-

IFN-: interferon-

IS: immunological synapse

ITAM: immunoreceptor tyrosine activation motif

KO: knockout

LPS: lypopolysaccaride

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

N: naïve

NK: natural killer

OG: octylglucoside

PBMC: peripheral blood mononuclear cells

pMHC: peptide-MHC

PTPase: protein-tyrosine-phosphate phosphohydrolase

RCA: rolling circle amplification

RPP: reverse phase protein

SDS: sodium dodecyl sulfate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SH: Src homology

TCR: T cell receptor

T_H1: type 1 helper T cell

T_H2: type 2 helper T cell

TLR: Toll-like receptor

TX: triton X-100

Table of contents

INTRODUCTION *3*

1. Memory T cells **4**

1.1 Generation of memory T cells 5

1.1.1 Initiation of the immune response 5

1.1.2 T cell clonal expansion and contraction phase 6

1.2 Phenotypical and functional properties of memory T cells 7

1.2.General properties 7

1.2.2 Maintenance 8

1.2.3 Memory T cell subsets: central and effector memory cells 10

1.2.3.1 Phenotypical and functional properties 10

1.2.3.2 Generation 12

1.2.3.3 Gene expression profile 13

1.3 Memory T cell subsets and vaccines 14

2. Immunobiology of the T Cell Receptor (TCR) **15**

2.1 History and discovery of the TCR 15

2.2 Structure of the TCR 16

2.3 Activation and Signalling 19

2.3.1 MHC peptide-TCR interaction 19

2.3.2 From TCR ligation to intracellular signal propagation 20

2.3.3 Intracellular TCR signalling 20

2.3.3.1 Proximal events 21

2.3.3.2 Adaptor coupling and distal signalling events 23

2.3.4 CD28-mediated co-stimulation of TCR signalling 26

2.3.5 Regulators of TCR signalling 27

2.3.5.1 Cbl protein family as negative regulator of TCR signalling 27

2.3.6 Role of immunological synapse (IS) in TCR signalling 29

3. Rationale for using Reverse Phase Protein (RPP) arrays in this study **32**

4. Protein microarray technology **34**

4.1 History 34

4.2 Principle and features 34

4.3 Array formats and applications 36

4.3.1 Forward Phase Protein (FPP) arrays 37

4.3.1.1 Antibody arrays 37

4.3.1.2 Protein arrays 38

4.3.2 Reverse Phase Protein (RPP) arrays 39

PROJECT AIMS 42

RESULTS 44

5. Implementation of RPP arrays to profile signalling proteins in human memory T cell subsets **45**

5.1 Design and fabrication of RPP arrays 45

5.2 Octylglucoside-containing lysis buffer allows optimal protein retention on 3D nitrocellulose-coated slide 47

5.3 RPP arrays display excellent performance characteristics 49

5.4 Analysis of T cell populations using RPP arrays 51

5.5 RPP arrays as a tool to profile TCR signalling complex assembly 54

6. c-Cbl expression regulates the functional response of human central versus effector memory CD4 T cells **57**

6.1 Expression of signalling components in human CD8 and CD4 T cell subsets 58

6.2 Heterogeneity in the expression of the signalling components among subjects in CD4 and CD8 T cell subsets 61

6.3 In CD4 EM T cells, reduced expression of c-Cbl correlates with LAT, SLP-76 and PI3K content 62

6.4 EM CD4 T cells show a lower functional threshold and differentially regulate TCR-mediated signals as compared to CM cells 64

6.5 c-Cbl expression controls the functional threshold of EM CD4 T cells 66

6.5.1 Cytosolic transduction to alter c-Cbl content of memory CD4 T cells 66

6.5.2 Impact of c-Cbl on CD4 memory T cell functions 68

DISCUSSION & PERSPECTIVES *71*

7.1 General discussion **72**

7.2 Perspectives **80**

MATERIALS AND METHODS *82*

REFERENCES *89*

ANNEXES *103*

ANNEX I 104

INTRODUCTION

1. Memory T cells

"…Yet it was with those who had recovered from the disease…

…for the same man was never attacked twice- never at least fatally…"

(Thucydides, *The Peloponnesian Wars*)

Nearly 2500 years ago, the ancient Greek Thucydides noted that patients recovering from the plague of Athens did not contract it again (Retief and Cilliers 1998). That is what scientists call "Immunological Memory" or in other words, the fascinating capacity of our immune system to respond more rapidly and strongly to pathogens that were previously encountered. In 1846, the Danish physician Panum underlined in his study on measles epidemics on the Faeroe Islands how impressive could be the duration of immunological memory in humans. Indeed, survivors of the first epidemics in 1781 were still immune to a second epidemic that struck the island more than 65 years later (Panum 1939).

Immunological memory is based on long-lived antigen-specific memory B and T lymphocytes that arise during primary infection, persist for decades and are able to elicit a robust immune response when the antigen is re-encountered (**Fig.1**).

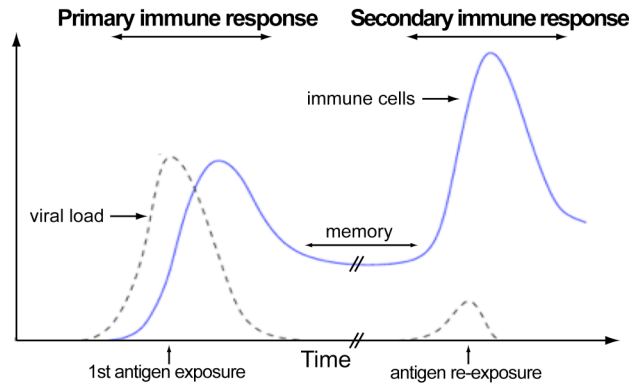


Fig.1 Immunological memory.
Long-lived memory cells arising from the primary immune response rapidly and strongly react to antigen previously encountered generating a robust secondary immune response.

The obvious application of this process is vaccination, a practice already common in China over 1000 years ago, but that took its name only by the late 1700 in honor of Edward Jenner who inoculated healthy subjects with cowpox exudates to protect them against antigenically related smallpox (Latin: cow = vacca) (Jenner 1798). Vaccination had an enormous impact on worldwide health, second only to the introduction of the modality of drinking clean water

(Wolman 1975). Therefore, it appears evident how important is the understanding of the mechanisms at the basis of immunological memory. I will focus the following discussion on memory T lymphocytes since these cells were studied in this thesis.

1.1 Generation of memory T cells

1.1.1 Initiation of the immune response

Prior to contact with antigen, quiescent naïve T cells (cells that have never encountered their cognate antigen) continuously migrate from the blood, through secondary lymphoid tissues, to the lymphatic vessels that collect in the thoracic duct to empty back into the blood at the left subclavian vein. Continuous blood-to-lymph recirculation is highly important for allowing T cells to make rapid contact in the secondary lymphoid tissues with antigens released from pathogens. Indeed, antigens entering the body are carried in afferent lymph to draining lymph nodes or taken up by professional antigen presenting cells (APCs) such as peripheral dendritic cells (DCs), which are stimulated and migrate to lymph nodes by shifting their inflammatory chemokine receptors (e.g. CCR1 and CCR5) to lymphoid-homing receptors (e.g CCR7) (Sprent and Surh 2002). Antigens are processed to short peptides by DCs and presented on major histocompatibility complex (MHC) class I and class II molecules to initiate the response of CD8 and CD4 T cells, respectively. However, simple recognition of MHC-peptide (p-MHC) by the T cell receptor (TCR) often produces tolerogenic responses (Goodnow 2001), and effective immune responses are only initiated in the presence of co-stimuli. Most infectious microorganisms contain “adjuvants” such as lipopolisaccarides (LPS), lipoproteins, unmethylated CpG DNA, and double-stranded RNA, that are recognized by a spectrum of highly conserved Toll-like receptors (TLRs) expressed on cells of the innate immune system and promote their activation. As a consequence, activated DCs up-regulate co-stimulatory molecules for T cells, such as CD40, B7-1 and B7-2, (ligands for CD40L, CD28 and CTLA-4, respectively, on T cell). In addition, other cells of the innate immune system (macrophages, natural killer (NK), mast cells) produce stimulatory cytokines and chemokines favoring T cell activation (Pasare and Medzhitov 2004).

1.1.2 T cell clonal expansion and contraction phase

Naïve T cells continue blood-to-lymph recirculation until their TCR recognizes the appropriate foreign antigen-MHC complex carried by activated APCs draining from the site of infection in the T cell zone of a secondary lymphoid organ (lymph nodes, spleen and Peyer's patches), resulting in T cell immobilization and activation. During the early phase of the response, rapid replication of the pathogen ensures large numbers of activated APCs into the T cell zone where responding T cells are stimulated to rapidly proliferate, synthesize cytokines and differentiate into effector T cells, i.e. cytotoxic and helper T cells (for CD8 and CD4 T cells respectively) (Sprent and Surh 2002). Both CD4 and CD8 T cells undergo an extensive clonal expansion, although CD4 T cell proliferation is slower and less pronounced than that of CD8 T cells (Homann, Teyton et al. 2001) (**Fig.2**).

Within 1-2 weeks, the peak of the proliferative response is reached and T cells undergo a contraction phase. Indeed, at later stages of the response, pathogen clearance reduces inflow of activated APCs into the T cell zone, and T cell interaction with diminishing numbers of antigen-bearing APCs exhausts the capacity of APCs to produce stimulatory cytokines, thus resulting in reduced formation of effector cells. The rapid disappearance of effector cells at the end of the primary response appears to be regulated by homing to non-lymphoid tissues and apoptosis (Sprent and Surh 2002). Of interest, CD4 T cells show a biphasic pattern of contraction whereas CD8 T cells display massive and rapid collapse after expansion in primary cellular immune response to lymphocytic choriomeningitis virus (De Boer, Homann et al. 2003). However, after the loss of short-lived effector cells, a small proportion of T cells survive to become long-lived memory cells (**Fig.2**).

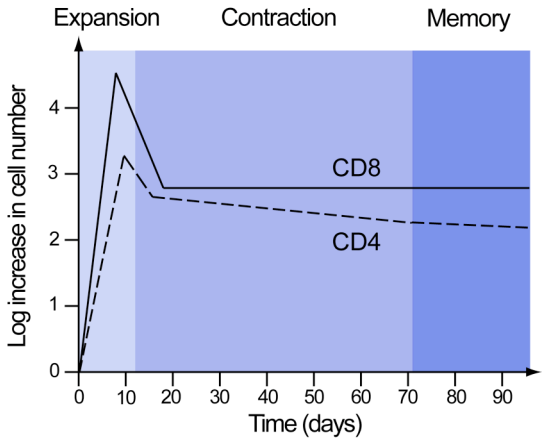


Fig.2 Kinetics of T-cell responses.
CD8 T-cell expansion and contraction is very rapid, so that memory is rapidly established. In contrast, CD4 T cells display a prolonged clonal biphasic contraction phase and memory is established only at later time point. (Beverley 2008)

1.2 Phenotypical and functional properties of memory T cells

1.2.General properties

Memory is the hallmark of the acquired immune system. Indeed memory cells confer protective immunity: they persist in a quiescent state and when reactivated by the cognate antigen mediate robust responses characterized by the rapid production of high levels of effector cytokines at low activation thresholds (Chandok and Farber 2004). Memory cells results from the differentiation of naïve cells during the primary immune response. However, how and at what stage of the immune response memory cells arise is still a matter of debate. It is possible that memory cells directly originate from cells undergoing an asymmetrical first division (Chang, Palanivel et al. 2007), or from cells that failed to differentiate towards effector cells following the initial contact with the APCs during primary response. However, evidence showing that precursors of memory cells undergo proliferation during primary response (Opferman, Ober et al. 1999; Rogers, Dubey et al. 2000) and, at least transiently, express effector functions (Jacob and Baltimore 1999; Opferman, Ober et al. 1999), supports the idea that memory cells might derive from effector cells (Sprent and Surh 2002). Of interest, clonal exhaustion caused from progressive shortening of telomere length is prevented in T lymphocytes during clonal expansion by up-regulation of telomerase (Maini, Soares et al. 1999). Unlike B cells, naïve T cells do not undergo somatic hypermutation-based affinity maturation during the primary immune response. It is instead believed that optimization of cellular immunity is mediated almost exclusively through selective expansion of T cells bearing receptors with the highest affinity for antigen. As a result of differentiation, memory cells display an optimization of the signal transduction machinery as well as of the expression of adhesion and effector molecules (Slifka and Whitton 2001) with respect to naïve cells. In addition, memory cells show more extensive lipid rafts (Viola 2001) and display higher degree of chromatin decondensation, with constitutive expression of mRNA of some effector proteins (including INFγ, RANTES..) (Kaech, Hemby et al. 2002). Together, when stimulated by antigen, memory cells show a shorter lag time (compared to naïve cells) for entering cell cycle, synthesizing cytokines, differentiating in effector cells, and migrating to non-lymphoid tissues while being less dependent on co-stimulation (Rogers, Dubey et al. 2000; Sprent and Surh 2002). Consistently, memory cells are metabolically more active than naïve cells and show higher rate of turnover *in vivo* (Tough and Sprent 1995). Thus, the efficacy of memory cells in mounting a rapid and robust secondary response is a direct consequence of their

enhanced responsiveness to antigen, as well as their ability to reside within (or migrate to) peripheral tissues (Sprent and Surh 2002). In addition, the precursor frequency of antigen-specific memory cells is far higher than for naïve cells, contributing to the generation of an intense immune response (Sprent and Surh 2002). Memory T cells therefore represent a highly effective and rapidly mobilizable "effector" system. As reported above, memory cells show distinct phenotypic differences (other than functional differences) from naïve cells, including alteration in the expression levels of adhesion (e.g. $CD62L^{lo}$, $CD44^{hi}$…) and activation molecules (e.g. $CD45RA/B/C^{hi}$, $CD122^{hi}$…) (Michie, McLean et al. 1992; Dutton, Bradley et al. 1998).

1.2.2 Maintenance

Naïve and memory T lymphocyte numbers are maintained constant in adult to ensure that the organism can mount an immune response to a variety of new antigens, while keeping appropriate levels of memory cells to previously encountered pathogens. As immunological space is finite, naïve (N) and memory cells must be subject to homeostasis and competition for survival within the T cell pool (Beverley 2008). Both signalling from TCR and cytokines receptor have been implicated in the maintenance and homeostatic expansion of T cell pools, but their relative contribution may vary under different conditions.

Long-term survival of CD4 and CD8 N T cells requires a combination of signals arising from joint contact with self-peptide-MCH ligands and interleukin-7 (IL-7). Abrogation of either of these signals reduced the life-spam of N T cells (usually years) to around 2-4 weeks. In contrast, overexpression of IL-7 leads to expansion of N T cell pool in absence of antigen. Thus IL-7 appears to be the primary cytokine for maintaining survival of N T cells, and the basal level of IL-7 appears to determine the overall size of naïve T-cell pool. In CD8 cells this survival is mediated through sustained high levels of the anti-apoptotic protein Bcl-2 and is inhibited by TGF-β (Boyman, Purton et al. 2007).

Survival of memory T cells appears to be independent of signalling through TCR while being dependent on signals received by cytokines. Indeed, memory cells are able to survive not only in the absence of antigen (Mullbacher 1994) but also in the absence of MHC molecules (Swain, Hu et al. 1999) or after conditional TCR ablation (Polic, Kunkel et al. 2001). Nonetheless, foreign antigens may play an indirect role through activation of the innate immune system or may provide an advantage in the competition for survival (Beverley 2008).

CD8 memory T cells were shown to be dependent primarily on the cytokines IL-15 for homeostatic proliferation and survival, although IL-7 did contribute to their long-term persistence, particularly in a competitive environment (Surh, Boyman et al. 2006). Notably, the majority of memory CD8 T cells express high levels of CD122 (IL-2 receptor β chain (IL-2Rβ) – common subunit for IL-2 and IL-15 receptor) and IL-7Rα. Recent evidence proved that transcription factor T-bet and eomesodermin are crucial for maintaining high levels of CD122 (Intlekofer, Takemoto et al. 2005) in memory cells.

Experiments with IL-$15^{-/-}$ mice demonstrated that IL-15 was not required for the generation of antigen-specific memory CD8 T cells, but was essential for their long-term survival (Becker, Wherry et al. 2002), as well as for maintenance of memory phenotype (MP) cells (Kennedy, Glaccum et al. 2000; Judge, Zhang et al. 2002). MP cells are spontaneously arising cells in normal animals that share many phenotypic and functional characteristic of antigen-specific memory cells (Sprent and Surh 2002). IL-15 probably supports survival of memory CD8 T cells by up-regulating anti-apoptotic molecules such as Bcl2 and Bcl-X_L. The signalling pathways triggered by IL-15 appear to be transmitted through STAT5 activation and are negatively modulated by SOCS-1 (Davey, Starr et al. 2005). Under normal condition, the basal level of IL-15 is established by the constitutive production of IL-15 by DCs. However, IL-15 production is efficiently enhanced by interferons (IFNs), especially IFN-I, produced following activation of the innate immune system by viral infection or LPS injection (Tough, Sun et al. 1997). The reduced numbers of MP CD8 cells found in IFN-I receptor-deficient mice suggest that background production of IFNs determine the basal level of IL-15 necessary for memory CD8 T cell maintenance. Apparently, the basal level of IL-15 (and IFNs) is induced solely through endogenous mechanism in the absence of signals from environmental or commensal antigens (Surh, Boyman et al. 2006). Despite the role of IL-15, other γc cytokines are known to affect memory CD8 T-cell homeostasis. In particular, increasing level of IL-7 can overcome the requirement for IL-15 in lymphopenic environment, as observed in IL-7 transgenic IL-$15^{-/-}$ mice that show increased number of MP CD8 T cells (Kieper, Tan et al. 2002). However, the level of IL-7 found in normal condition is probably too low to be used by memory cells while enough to support survival of N cells. The role of IL-2 in memory CD8 T cell survival is controversial. Injection of mAb that were thought to neutralize circulating IL-2 was causing a marked increase in basal homeostatic proliferation of MP CD8 T cells, underling a putative suppressing role for this cytokine (Ku, Murakami et al. 2000). However, it has been recently experimentally proved that anti IL-2

mAbs instead enhance the *in vivo* biological activity of pre-existing IL-2 on memory CD8 T cells (Boyman, Kovar et al. 2006).

The factors regulating homeostasis of memory CD4 T cells are less well understood than for memory CD8 T cells. CD4 memory cells were initially thought not requiring γc cytokines for survival, as CD4 memory cells deficient for CD132 (γc-chain) are effectively maintained *in vivo* (Lantz, Grandjean et al. 2000). However, subsequent studies revealed an essential role for IL-7 for survival of both MP and TCR transgenic CD4 memory cells (Li, Huston et al. 2003; Seddon, Tomlinson et al. 2003). Not surprising, memory CD4 cells express high levels of CD127 (Interleukin 7 receptor α chain) but low levels of CD122 (common chain to IL2 and IL15 receptor). Nonetheless, a long lasting boost in CD4 memory cells was observed following administration of IL-2 or IL-15 to rhesus macaques during the contraction phase following influenza virus (Villinger, Miller et al. 2004), underling a possible role for these cytokines in homeostasis of CD4 memory cells. The involvement of MHC ligands in memory CD4 cell homeostasis remains matter of debate. Recently, it has been proposed that signals from both TCR and cytokines (notably IL-7) could synergize to promote survival of CD4 memory cells (Riou, Yassine-Diab et al. 2007). Finally, in contrast to CD8 cells, the molecular mechanism governing memory CD4 cell survival appear to be based on up-regulation of Bcl-X_L but not Bcl2 (Sprent and Surh 2002).

1.2.3 Memory T cell subsets: central and effector memory cells

1.2.3.1 Phenotypical and functional properties

Human memory T lymphocytes (defined as phosphatase CD45RA negative) comprise two main distinct populations, termed central memory (CM) and effector memory (EM) cells. They were delineated based on the expression of chemokine receptor CCR7 that defines their distinct functions and homing capacities (Sallusto, Lenig et al. 1999)

CM cells (CD45RA-, CCR7+) constitutively express at their surface CCR7 and CD62L. These two receptors are also expressed by N cells and are required for homing to secondary lymphoid organs. While having limited effector functions, CM cells have higher sensitivity to antigenic stimulation as compared to N cells, are less dependent on CD28 co-stimulation and show high proliferative capacity. Moreover, CM cells up-regulate CD40L, providing more effective stimulatory feedback to DCs and B cells, and at least *in vitro,* can differentiate to EM cells upon secondary stimulation (Sallusto, Lenig et al. 1999)

EM cells (CD45RA$^-$, CCR7$^-$) lack the expression of CCR7, are heterogeneous for CD62L expression, and display adhesion molecules (e.g. high level of β1 and β2 integrins) and chemokine receptors (e.g. CCR1, CCR3, CCR5) that are required for homing to inflamed peripheral tissues. Compared to CM cells, EM cells display immediate effector functions (Sallusto, Geginat et al. 2004) and have an imprinted T_H1, T_H2 (for CD4 cells) or CTL (for CD8 cells) phenotype. Furthermore, in CD8 T cells, an additional subset lacking CCR7 and CD62L but keeping CD45RA expression has been identified. Since this subset displayed higher effector function, it was named "terminally differentiated" (Sallusto, Lenig et al. 1999).

The memory subset distribution in the blood of healthy individuals varies between CD4 and CD8 compartment, with CM more frequent in CD4 cells and EM predominant in CD8 cells (**Fig.3**). Within the tissues, CM cells are enriched in secondary lymphoid organs (lymph node and tonsils) while EM cells traffic mainly to peripheral tissues (especially lung, liver, gut) (Campbell, Murphy et al. 2001).

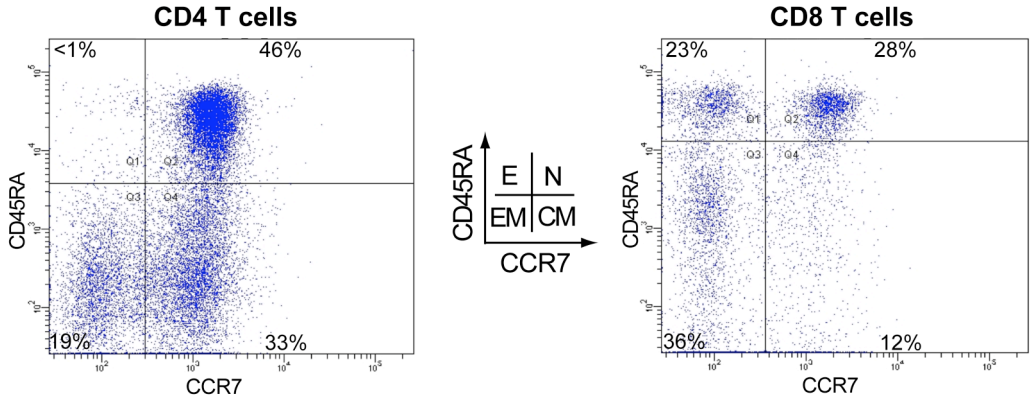


Fig.3 Memory T cell subset distribution.
Phenotypes of CD4 and CD8 T cell memory subsets in the peripheral blood of a representative healthy donor based on the expression of the chemokine receptor CCR7 and the phosphatase CD45. N (naïve), CM (central memory), EM (effector memory) and E (terminally differentiated) cells are shown with the corresponding frequency.

The existence of two subsets of memory cells with distinct functions and migratory capacities suggest a subdivision of tasks among memory cells. On one hand, EM cells represent a readily available pool of antigen-primed cells which can enter peripheral tissues and can give immediate protection. On the other hand, CM cells represent a "stem cell-like" pool of antigen-primed cells, which traffic through secondary lymphoid organs and, upon secondary challenge, can rapidly and efficiently generate a new wave of effector cells.

Using *in vivo* labeling with with deuterated glucose, it was shown that human CD4 EM cells have a proliferation rate of 4.7% per day compared to 1.5% of CM and only 0.2% of N cells. Thus, human memory subsets, and in particularly EM cells, constitute short-lived cell populations (average intermitotic times of 15 and 48 days for EM and CM, respectively) that require continuous replenishment *in vivo* (Macallan, Wallace et al. 2004).

When activated *in vitro* both memory subsets show low-activation threshold and vigorous proliferation. However, the expansion potential decreases from CM to EM cells, with a corresponding decrease in telomere length (Sallusto, Lenig et al. 1999; Geginat, Sallusto et al. 2003). Moreover, CD4 CM cells stimulated *in vitro* appear heterogeneous in their ability to differentiate: some cells appear uncommitted and can be induced to differentiate to T helper 1 (T_H1, which mainly secrete IFNγ/TNF and lead to cell-mediated immunity) and T helper 2 (T_H2, which mainly secrete IL-4, IL-5, IL-6 and promote humoral immunity) by IL12 and IL4 stimulation, respectively. Other cells spontaneously differentiate to INF-γ or IL-4 producing cells, representing pre-T_H1 and pre-T_H2 cells (defined by CXCR3 and CCR4 expression, respectively) (Sallusto, Geginat et al. 2004).

In contrast to CM cells, EM cells stimulated under non-polarizing conditions, retain their T_H1 and T_H2 imprinted phenotype. However, when stimulated under opposite polarizing conditions, EM cells acquire in addition the ability to secrete the alternative cytokines (Sallusto, Geginat et al. 2004). Of interest, upon antigenic stimulation, CM cells loose the expression of CCR7 whereas EM cells, following a rapid and transient CCR7 upregulation, maintain their CCR7- phenotype (Sallusto, Geginat et al. 2004).

1.2.3.2 Generation

While the heterogeneity in humans of memory T cells is now well accepted, the signals regulating the generation of the different populations are not fully understood. However, increasing evidence (Lanzavecchia and Sallusto 2002; Gett, Sallusto et al. 2003; Sallusto, Geginat et al. 2004; Stemberger, Neuenhahn et al. 2007; Kalia, Sarkar et al. 2008) suggests that the strength and the quality of the signals delivered by TCR and cytokine receptors drive CD4 and CD8 naive T cells through hierarchical thresholds of differentiation and lead to the generation of effector as well as memory populations. Thus, in increasing order of strength of stimulation, the fate of a naïve T cell range from unfit cells that undergo abortive

differentiation or apoptosis, to fit cells that give rise to CM, EM and terminal differentiated cells (progressive differentiation model, proposed by Lanzavecchia and co-workers) (**Fig.4**). Alternatively, studies on mouse CD8 T cells proposed that memory cells could directly origin from effector cells. According to this model, EM cells are a transitory population representing an intermediate cell type in the effector to memory transition that, in the absence of antigen, convert directly to CM cells (linear differentiation model, proposed by Ahmed and coworkers) (Wherry, Teichgraber et al. 2003). Finally, another model based on the idea that CM and EM cells are independent population has been recently proposed (Baron, Bouneaud et al. 2003).

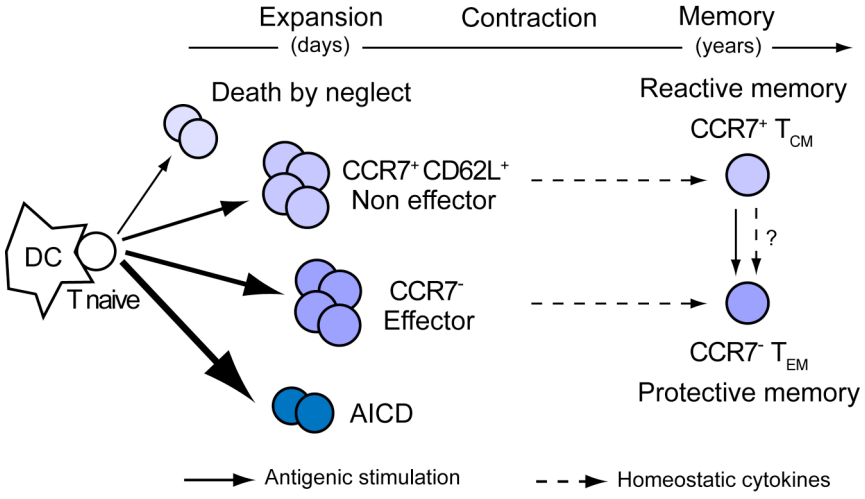


Fig.4 Signal strength model of memory T cell generation.
The duration and intensity of antigenic stimulation is indicated by the length and thickness of the solid arrows. Antigen-independent events leading to T cell proliferation and differentiation are indicated by the dotted lines. AICD, activation-induced cell death. (Adapted from Gett, Sallusto et al. 2003)

1.2.3.3 Gene expression profile

Despite these phenotypical and functional differences, only few studies have conducted comparative gene expression profiling of human memory CD4 (Liu, Li et al. 2001; Riou, Yassine-Diab et al. 2007) and CD8 T cells (Willinger, Freeman et al. 2005). Few differences were reported in gene expression between N and memory CD4 T cells (Liu, Li et al. 2001), while analysis of CD8 T cells revealed a clear dichotomy between CM and EM cells. However, a very small number of truly CM- and EM-specific genes were identified in spite of the fact that CM cells possess a gene transcription signature intermediate between that of N and effector (E) cells (Willinger, Freeman et al. 2005). More recently CD4 CM cells

(CD45RA-, CCR7+, CD27+) have been reported to express lower levels of proapoptotic and cell cycle-related genes described as FOXO3a target genes, relative to their EM counterparts. The convergence of TCR and cytokine-induced signals causes FOXO3a phosphorylation and seems to drive the survival of CM cells (Riou, Yassine-Diab et al. 2007). Moreover, both CD4 and CD8 CM cells display a high basal and induced STAT5 phosphorylation in response to IL-2-related cytokines (Willinger, Freeman et al. 2005; Riou, Yassine-Diab et al. 2007).

1.3 Memory T cell subsets and vaccines

Most currently successful vaccines induce antibody responses against antigenically stable pathogens that cause lytic or acute infection (Hilleman 2000). However, many chronic diseases such as malaria, human immunodeficiency virus (HIV) and hepatitis C virus (HCV) require strong cellular immune response for pathogen control or elimination. The recognition of this aspect has recently encouraged many researchers to develop vaccine strategies able to induce an effective cell-mediated immunity. CM and EM T cells play different roles in the control of viral diseases (Appay, Dunbar et al. 2002; Harari, Vallelian et al. 2004) (Peretz, Ndongala et al. 2007; Mason, De Rose et al. 2008). In mice, CM T cells are more efficient in conferring disease protection (Wherry, Teichgraber et al. 2003; Castiglioni, Gerloni et al. 2004; Zaph, Uzonna et al. 2004). Moreover, murine (Lauvau, Vijh et al. 2001; Seaman, Peyerl et al. 2004) and macaque (Vaccari, Trindade et al. 2005) models of vaccination suggest that the type of memory T cells elicited is a key element for the clinical effectiveness of the vaccine. Of interest, in a model of SIV infection in rhesus macaque, vaccine-induced protection correlated with expansion of CM but not EM CD8 T cells (Vaccari, Trindade et al. 2005). Therefore, defining the different types of memory T cells and determining which subset correlates with protective immunity is a basis for rational design of effective T-cell vaccines. Recently, it has been shown that the levels of viral load correlates with distinct functional signatures of T-cell responses (Pantaleo and Harari 2006). In particular, antiviral protective immunity in chronic virus infections is characterized by a polyfunctional (IL-2 and IFN-γ secretion and proliferation) but not monofunctional signature (Harari, Dutoit et al. 2006). Therefore, the ability of a T-cell vaccine to induce polyfunctional T-cell responses may be an important parameter for its evaluation and immunological improvement.

2. Immunobiology of the T Cell Receptor (TCR)

2.1 History and discovery of the TCR

March 8, 1984. This date marked the end of a long and frustrating search and the beginning of a new chapter in immunology: two *Nature* back-to-back articles finally described the genes encoding the β-chains of the human and mouse TCR (Hedrick, Cohen et al. 1984; Yanagi, Yoshikai et al. 1984). Since that date, more that 30000 scientific papers have been published on TCR-related subjects and many questions that accumulated in the two decades prior this discovery could be answered. As far back as the 1960s, it was clear that the functions of T lymphocytes were important for immunity. However, it was largely unclear how these cells recognize and respond to non-self antigens. It proved to be much more difficult to define the moleculare nature of the TCR than that of the B cell receptor (BCR) (Mak 2007). Indeed, the Ig proteins that function as antigen recognition component of the BCR are not only expressed on the B cell surface but are also secreted in large quantities as antibodies. The latter were already familiar to immunologists before the characterization of the BCR (Porter 1963). This information, coupled with the fact that B cells contain large amount of mRNA encoding Ig proteins (5% of total mRNA), paved the way for the cloning of the genes encoding the BCR and the discovery of Ig gene rearrangement (Hozumi and Tonegawa 1976). In contrast, as TCRs are not secreted like antibodies, it was difficult to purify sufficient protein for gene cloning and structural analysis. In addition, many researchers believed that TCR recognized antigen in much the same way as B cells did and therefore they unsuccessfully devoted a great deal of time to prove this. Between 1960 and 1983, immunologists concentrated their efforts in establishing reagents able to unambiguously identify and characterize TCR proteins. Unfortunately, these intense efforts went largely un-rewarded. Finally, in 1982, Allison and colleagues generated a mAb recognizing a particular structure on mouse T cell lymphoma cells which had the unusual property of being clone-specific (Allison, McIntyre et al. 1982). Within a year, other laboratories succeed in isolating mAbs with similar properties. The clone-specific structures recognized by the different mAbs were thought to be the TCR. Biochemical analysis of these surface structures revealed heterodimeric proteins of about 45-50 KDa that were composed of a α and a β chain (Acuto, Hussey et al. 1983; Kappler, Kubo et al. 1983). The cloning of the genes encoding the putative TCR β-chain both in human and mouse was accomplished one year later by applying a differential hybridization approach designed to isolate cDNA that were specific for T cells and not expressed in the related B

cells (Hedrick, Cohen et al. 1984; Yanagi, Yoshikai et al. 1984). Within one year the α-chain was also cloned and the complete structure of TCR was revealed (Chien, Becker et al. 1984; Yanagi, Chan et al. 1985).

2.2 Structure of the TCR

The TCR delivers signals that are crucial both for T cell development in the thymus and antigen-induced cell activation in the periphery. In its smaller form, it is a genetically recombined receptor, similar to a Fab antibody fragment, and it is composed of two disulphide-bonded chains: either α and β chains in αβ T cells or γ and δ chains in γδ T cells. αβ TCRs are expressed by most T cells (>95% in adult donor (Lanier, Ruitenberg et al. 1988)) and typically detect antigens presented on the surface of APCs in the context of appropriate MHC proteins (**Fig.5A**). A small subset of T cells, that I will not further describe, express γδ TCRs which bind directly to pathogen-derived glycoproteins or non-classical MHC molecules (Adams, Chien et al. 2005). Each polypeptide chain of the heterodimers is, for the most part, exposed to the extracellular side of the membrane (variable and constant domains), with a transmembrane domain located toward the C terminus and a short cytoplasmic tail of 2-7 amino acids (Bentley and Mariuzza 1996). The constant domain of the β chain adopts a standard Ig fold (sandwich like structure formed by two disulphide-bonded antiparallel β-sheets) (Adams, Chien et al. 2005), while that of the α chain adopts an atypical Ig fold due to the absence of a top β sheet. Clonotypically rearranged variable (V), diversity (D), and joining (J) gene segments encode the variable domain which is responsible for antigen recognition. The p-MHC binding site is comprised of six loops at the end of the variable domain called complementarity-determining regions (CDRs), with each chain contributing with three loops, named CDR1, CDR2 and CDR3. The CDR1 and CDR2 loop sequences are referred as "germline derived" because they are constant for each type of chain, whereas the CD3 loops vary and largely dictate the specificity for the peptide (Garcia and Adams 2005) (**Fig5B**).

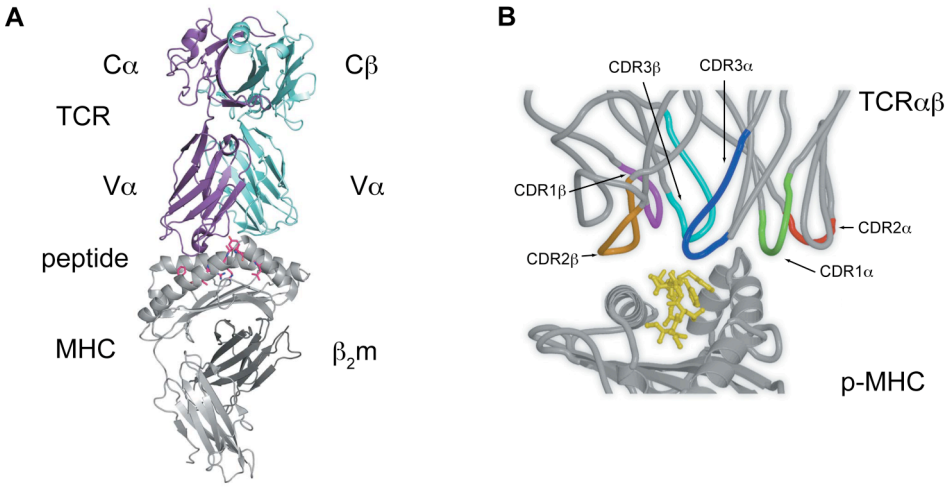


Fig.5 High resolution crystal structure of αβTCR-pMHC-I complex.
(A) TCR–pMHC-I complex (PDB accession code 1MI5). MHC, grey; peptide, magenta; TCR α- and β-chain, purple and cyan respectively. **(B)** Diagram highlighting the CDR loops in αβTCR-pMHC-I complex. The CDR loops are colored as follows: Vα CDR1, green; Vα CDR2, red; Vα CDR3, blue; Vβ CDR1, magenta; Vβ CDR2, orange; Vβ CDR3, cyan. The view is along the peptide-binding groove. (Stewart-Jones, McMichael et al. 2003).

The TCR itself lacks a significant intracellular domain and is coupled to downstream signalling *via* association with CD3 molecules. Indeed, at the cell surface, αβ TCR is non-covalently associated with the CD3δε, CD3γε, and CD3ζζ dimers (Wegener, Hou et al. 1995; Call and Wucherpfennig 2007). The extracellular domains of the CD3δε and CD3γε heterodimers consists of side-by-side paired Ig folds, whereas those of the CD3ζζ homodimer are only nine residues long and are unstructured. In contrast to the long connecting peptides of the TCR chains (19-26 aa), CD3 heterodimers have short conserved cysteine-rich (RxCxxCxE) stalk regions (Sun, Kim et al. 2004). The intracellular domain of the CD3 δ, ε and γ chains contains a single immunoreceptor tyrosine-based activation motif (ITAM), while CD3ζ possesses a longer intracellular domain holding 3 ITAMs (Love and Shores 2000) **(Fig.6).**

The association of each of the three dimeric signalling modules with the TCR is based on specific interactions between two conserved acid residues in their transmenbrane domains and one basic residue on one of the TCR chains (**Fig.6**) (Call, Pyrdol et al. 2002). The mutation of any one of these residues to alanine abrogates formation of the TCR/CD3 complex (Kearse, Roberts et al. 1995; Call and Wucherpfennig 2004).

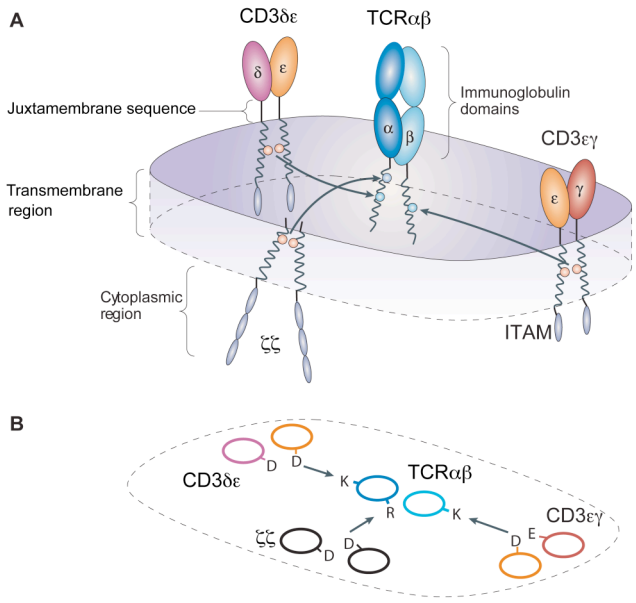


Fig.6 Organization of the TCR–CD3 complex.
The T-cell receptor (TCR) αβ heterodimer recognizes peptides in the context of MHC proteins and signals through the non-covalently associated CD3δε, CD3γε and ζζ dimers. **(A)** The association of each of the CD3 dimeric signalling modules with the TCR is dependent on a specific basic residue (shown in blue) in the TCR transmembrane domains and a pair of acidic residues (shown in orange) in the transmembrane domains of the respective signalling module. **(B)** A projection of the three-dimensional TCR–CD3 assembly illustration is shown in two dimensions. Each circle represents the view down the axis of the respective transmembrane helix projecting into the membrane. (Call and Wucherpfennig 2004).

Assembly of a single copy of TCR heterodimer (that carries only 3 positive charges versus 6 negative ones in the CD3 dimers) produces a potential charge imbalance in the TCR/CD3 complex. Starting from this considerations, investigators proposed two models for TCR/CD3 complex: the first holding a stoichiometry of 1:1:1:1 ratio of TCRαβ:CD3δε:CD3γε:CD3ζζ (Kearse, Roberts et al. 1995; Call, Pyrdol et al. 2002), the second proposing that 2 or more TCR heterodimers are present per complex to balance the charges (San Jose, Sahuquillo et al. 1998; Fernandez-Miguel, Alarcon et al. 1999). Using immunogold labeling and electron microscopy, Alarcon and colleagues observed co-existence of monovalent and multivalent forms of TCR at the cell membrane of resting T cells (Schamel, Arechaga et al. 2005). Currently, it is widely accepted that monovalent complexes are composed of 1:1:1:1 ratio between their subunits. However, it remains unknown if the multivalent receptor clusters are multimers of the monovalent form or contain different ratio between the individual subunits (Alarcon, Swamy et al. 2006).

In αβ TCR, the assembly of the CD3 heterodimers with the receptor is based on similar electrostatic interaction, however, the CD3δε dimer associates strictly with TCRα and CD3γε with TCRβ. This specificity is most likely dictated by the extracellular regions of the different subunits rather than their transmembrane domains (Wegener, Hou et al. 1995; Call, Pyrdol et al. 2002). Xu et al. identified the TCR contact point for the conserved stalk region of the CD3δε subunits to be most probably the connecting peptides of the TCRα chain. Indeed, truncation of the TCRα immunoglobulin domains had no effect on assembly, whereas shortening of the TCRα connecting peptide significantly reduced CD3δε association (Xu, Call et al. 2006). Moreover, interaction of CD3δε with the αβ TCR enables the subsequent association of CD3γε dimers, although the molecular mechanisms are still unclear (Call, Pyrdol et al. 2002). Finally, point mutations (Bolliger, Johansson et al. 1997) or artificially enlongation (Minguet, Swamy et al. 2008) of the membrane-proximal nine amino-acid extracellular domain of CD3ζζ have been shown to impair TCR/CD3ζζ assembly.

2.3 Activation and Signalling

2.3.1 MHC peptide-TCR interaction

TCR paradoxically shows high sensitivity and specificity, while having a low affinity for its ligand (Karjalainen 1994). Increasing evidence shows that T cells can be activated by as few as 1–10 antigenic p-MHCs among a huge number of self-pMHCs on APCs (Irvine, Purbhoo et al. 2002). In addition, T cells exhibit an unsaturated response to a large range of p-MHC concentrations (up to 10^3-10^6 fold) (Alarcon, Swamy et al. 2006). Experimental (Schamel, Arechaga et al. 2005) and computational (Schamel, Risueno et al. 2006) evidence supports a recently proposed model, in which the multivalent TCRs are important for the detection of low-antigen concentrations, therefore conferring sensitivity, whereas the co-expressed monovalent TCRs could be responsible for dose-response effects at antigen concentrations that would saturate multivalent TCRs (Alarcon, Swamy et al. 2006). The model accommodates also recent finding suggesting that combination of antigenic p-MHC/self p-MHC could modulate the sensitivity of T cell activation (Krogsgaard, Li et al. 2005).

During antigen recognition, the co-receptor molecules CD4 or CD8 associate on the T cell surface with the TCR (either directly or indirectly *via* CD3 molecules), and bind invariant sites on antigen-loaded MHC class II or MCH class I molecules on the APC, respectively

(Gao, Rao et al. 2002). The binding of co-receptor to the cognate MHC molecule is thought to stabilize the p-MHC-TCR interaction and to bring the kinase Lck (physically associated to the cytoplasmic tail of the co-receptor) in contact with the TCR to initiate phosphorylation events (Zamoyska 1998). Therefore, co-receptor binding greatly increases the sensitivity of a T cell to antigenic peptide presented on the MHC molecule. However, activation of CD4 T cells is dependent on the co-receptor engagement only at low antigen density whereas CD8 T cell activation depends on co-receptor engagements regardless of antigen density (Krogsgaard, Juang et al. 2007)

2.3.2 From TCR ligation to intracellular signal propagation

The immediate outcome of TCR ligation by appropriate p-MHC complex is a rapid increase in phosphorylation of ITAMs in the CD3 subunits, leading to the initiation of the signalling cascade. How these events are generated as a consequence of TCR ligation remains partly understood. Multiple models have been put forward, including TCR clustering, co-receptor (CD4/CD8) recruitment and/or conformational changes as possible mechanisms (Kuhns, Davis et al. 2006; Krogsgaard, Juang et al. 2007). Structural comparisons between p-MHC bound and free soluble αβ TCRs have not revealed conformational changes that propagate further than the interface with p-MHC (particularly at CDR3 loop) (Rudolph, Stanfield et al. 2006). Nevertheless, TCR ligation induces a conformational change in the cytoplasmic tails of CD3ε that results in exposure of a prolin-rich sequence able to recruit the adaptor protein Nck independently from protein tyrosine kinase (PTK) activation (Gil, Schamel et al. 2002). In addition, upon TCR/CD3 triggering the cytoplasmic tail of CD3ζ undergoes a conformational change from a lipid-bound helical structure to an unfolded structure (Aivazian and Stern 2000). Recently, it has been demonstrated that full activation of the TCR requires both clustering and conformational changes at the CD3ε subunit (Minguet, Swamy et al. 2007).

2.3.3 Intracellular TCR signalling

The sequence of intracellular signalling events coupled to TCR triggering has been elucidated by studies of T cell lines, clones and mouse knockout models. In the simplest sense, TCR signalling proceeds from the cytoplasm to the nucleus *via* a series of phosphorylation events that culminate in the activation of transcription factors and reorganization of the cytoskeleton. From a functional side, TCR signalling is a complex

dynamic event that can elicit a variety of T-cell responses (from immune response to unresponsiveness) depending on the cell subsets, the strength of TCR engagement by specific p-MHC ligand and the co-stimulatory/accessory molecules presented by the APC at the immunological synapse *in vivo*. According to the two-signal hypothesis model for lymphocyte activation proposed in the middle 1970s (Lafferty and Cunningham 1975), delivering of antigen-specific "signal 1" alone (TCR) promotes tolerance whereas development of an effective immune response requires the presence of an additional "signal 2" derived from a co-stimulatory factor (e.g. CD28). Because of the complexity of the TCR signalling networks, I will simplify the following discussion by emphasizing the general architecture of the signalling and the few known events belonging to a differential signalling pathway in memory cells. Indeed, the role of many specific signalling molecules, such as Lck, ZAP-70, SLP-76, LAT and ERK, in memory T cell function *in vivo* has been difficult to establish due to the lack of peripheral T cells in the corresponding knockout (KO) mice (Straus and Weiss 1992; Negishi, Motoyama et al. 1995; Clements, Yang et al. 1998; Zhang, Sommers et al. 1999).

2.3.3.1 Proximal events

A key initiating event in TCR signalling is tyrosine phosphorylation of the consensus sequence (D/E)xxYxxL$(x)_{6-8}$YxxL present in the ITAMs of the CD3 subunits by the Src kinase Lck and Fyn. This leads to recruitment of the cytosolic tyrosine kinases Syk and ZAP-70 through their tandem SH2 domains to the CD3 subunits (Kane, Lin et al. 2000) (**Fig.7**). ITAMs appear as a single copy on the CD3γ, δ, and ε chains and as a triplicate repeats on the ζ chains, thus each TCR complex contributes 10 ITAM motifs (Love and Shores 2000). While the precise mechanism of Src kinase activation remains unknown, the critical role of CD45 tyrosine phosphatase in CD4 cells is well established. Indeed, CD45 participates in a multimeric complex with the CD4 co-receptor (which is physically associated with Lck) and promotes the removal of the inhibitory C-terminal tyrosine phosphate residue of Lck (Y505), leading to a conformational change and activation. This conformational change also frees up the autologous SH2 domain, allowing Lck to interact with new partners (Weil and Veillette 1994). CD45 exists in multiple alternatively spliced isoforms (CD45RO in memory cells; CD45RA and CD45RB in naïve and effector cells of human and mice, respectively) that differentially associate with TCR and/or co-receptor molecule. In mice CD45RO-TCR association is able to more efficiently promote Ras activation than CD45RA-TCR (Czyzyk,

Leitenberg et al. 2000). In humans, CD45RA is used to delineate memory T cells, although the correlation between CD45 isoforms and their functions remains yet unknown. After its docking to ITAM motifs, ZAP-70 is phosphorylated by Lck and therefore activated. In addition, Lck interacts directly with ZAP-70 through binding of its SH2 domain to the ZAP-70 pY^{319} residue, leading to recruitment of additional ZAP-70 molecules and Lck substrates, such as the Tec kinase Itk (Pelosi, Di Bartolo et al. 1999). The stoichiometry of ITAM phosphorylation and the number of ZAP-70 molecules recruited is dictated by the affinity of the TCR for its peptide ligand (Madrenas, Wange et al. 1995).

Both ZAP-70 and Lck are essential for T cell development and activation (Straus and Weiss 1992; Negishi, Motoyama et al. 1995). However some evidence suggests that memory and effector cells may undergo different signalling events at this proximal level. Indeed, increased expression of Fyn (Guillaume, Tuosto et al. 2003) and membrane and co-receptor associated Lck in mouse memory and effector CD8 T cells differentiated *in vitro* (Bachmann, Gallimore et al. 1999) have been associated with a decreased activation threshold. With respect to naive cells, human (Hall, Heffernan et al. 1999) and mouse (Farber, Acuto et al. 1997) memory CD4 T cells display reduced ZAP-70 tyrosine phosphorylation following TCR-stimulation, although CD3ζ is equally phosphorylated in both cell subsets (Farber, Acuto et al. 1997). In mice, this reduced ZAP-70 phosphorylation is coincident with reduced phosphorylation of downstream linker/adaptor molecules, such as SLP76 (Hussain, Anderson et al. 2002). In mice, ligation of MHC class II and of CD4 was reported to inhibit memory CD4 cell activation (Farber, Luqman et al. 1995). Moreover, human CD4 effector T cells differentiated *in vitro* reorganize their TCR signalling machinery by up-regulating the expression of FcRγ protein that belong to an altered TCR/CD3 signalling complex lacking CD3ζ. The TCR/CD3ε/FcRγ complex recruits and activates the Syk kinase in place of ZAP-70 (Krishnan, Warke et al. 2003). Since ZAP-70, but not Syk, can trigger apoptosis in T cell (Zhong, Wu et al. 2004), preferential use of Syk may be a mechanism to promote human effector T cell survival. Finding of FcRγ/Syk^{+} T cells in the peripheral blood of systemic lupus erythematosus (autoimmune disease SLE) patients suggest that this proximal switch can occur also *in vivo* (Krishnan, Farber et al. 2003).

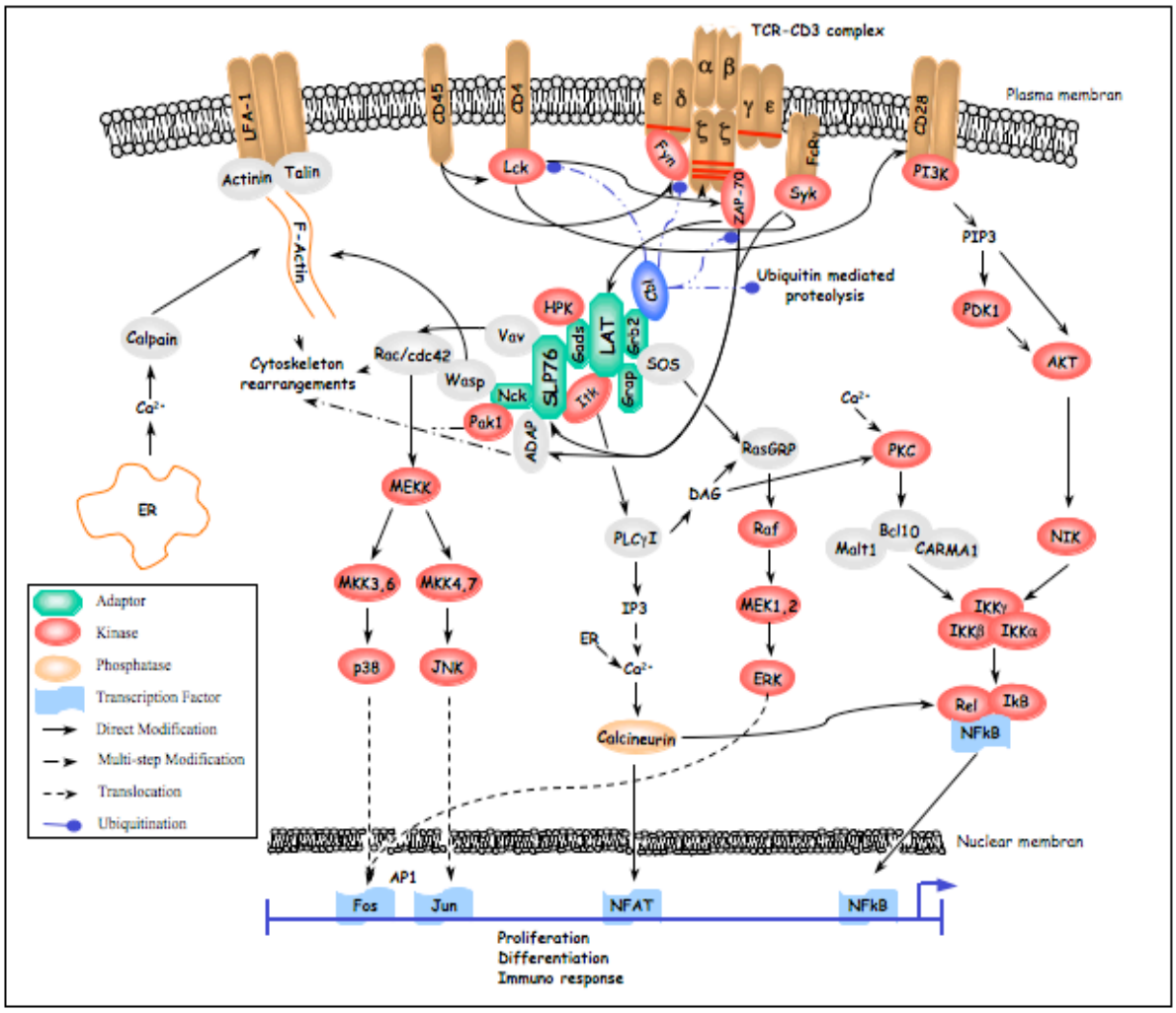


Fig.7 Schematic representation of TCR signalling pathways.
TCR engagement by p-MHC and co-stimulatory molecules (CD28) results in T cell activation and differentiation. Activation of MAP Kinases and Ca^{2+} signalling leading to nuclear translocation and mobilization of transcription factors NFAT, AP1 (Fos/Jun) and NF-κB are shown.

2.3.3.2 Adaptor coupling and distal signalling events

The direct substrates of ZAP-70 kinase are two critical adaptor proteins: linker for activated T cells (LAT) and SH2 domain-containing leukocyte protein of 76 KDa (SLP-76) (Chandok and Farber 2004). While lacking intrinsic enzymatic activity, these molecules function as molecular connection between proximal and distal signalling events by recruiting additional proteins to the signalling complex (**Fig.7**). Although LAT carries a transmembrane domain sufficient for membrane localization, palmitoylation at C26 and C29 is necessary for its efficient partitioning into glycolipid-enriched microdomains (GEMs, lipid raft) and for its tyrosine phosphorylation (Zhang, Sloan-Lancaster et al. 1998). When phosphorylated by ZAP-70, the 9 tyrosine-based motifs of LAT serve as docking sites for specific SH2-domain

containing proteins, including PLC-γI, Grb2, PI3 Kinase and Gads (Wange 2000). Moreover, through the interposition of the adaptor Gads, LAT recruits and stabilizes SLP-76 binding to the signalling complex (Nel 2002). SLP-76 recruits additional signalling molecules that bind to distinct structural regions: the N-terminal phosphorylated tyrosine-based motifs binds the guanine-exchange factor Vav, the proline rich region recruits the SH3 of Gads and the SH2 domain binds to phosphorylated tyrosines of other adaptor proteins such as ADAP that promotes integrin-mediated T cell adhesion to APC (Chandok and Farber 2004; Burbach, Medeiros et al. 2007). The signalling complex resulting from these interactions represents a molecular crossroad from where key TCR signalling pathways arise, leading to intracellular Ca^{2+} mobilization, activation of MAPK cascades (ERK1/2, p38 and JNK), as well as cytoskeleton reorganization.

PLC-γI tyrosine phosphorylation is one of the major determinants for Ca^{2+} signalling. As mentioned above, PLC-γI SH2 domain is recruited to LAT at the vicinity of the cell surface membrane (Wange 2000). SLP-76, which is bound *via* Gads to LAT, recruits a Tec kinase, Itk, which ultimately phosphorylates PLC-γI leading to its catalytic activation and cleavage of phosphatidyl inositol-4,5 bisphosphate (PI-4,5-P_2). The resulting cleavage products, IP_3 and DAG, are responsible, respectively, for sustained elevation of $[Ca^{2+}]_I$ (which promotes NFAT translocation to the nucleus *via* calcineurin activation) and activation of PKCθ (which is mainly involved in activation of NF-κB pathways) (Nel 2002).

The formation of the signalling complex is also crucial for MAPK activation. Through the interposition of Grb2, LAT can indirectly recruit to the cell membrane the guanidine exchange factor Sos, which promotes the transition of Ras to his active GTP-bound form. Once activated, Ras interacts and activates the serine-threonine kinase Raf-1, leading to the activation of the ERK cascade. Ultimately, ERK plays an essential role in the expression of transcription factors c-Fos and c-Myc (Nel 2002).

TCR engagement also leads to the activation of PI3K, that gets recruited to the signalling complex by directly binding LAT or indirectly binding the TCR *via* the phosphorylated adaptor TRIM. PI3K is comprised of a regulatory (p85) and a catalytic (p110) subunits and serves to anchor PLC-γI, Tec and Vav proteins to the plasma membrane (Blomberg, Baraldi et al. 1999). Of interest Vav, which is also recruited *via* SLP-76, is a guanidine exchange factor for Rac-1/Cdc42, which ultimately plays a crucial role in cytoskeleton reorganization as well as in activation of the JNK/p38 cascade, leading to expression of Jun and Fos

transcription factors, respectively. (Crespo, Schuebel et al. 1997; Acuto and Cantrell 2000). PI3K is also responsible for the recruitment and activation of the serine-threonine kinase Akt, which has critical role for promoting cell survival (Alessi and Cohen 1998). Moreover, PI3K is involved in CD28 signalling (see INTRODUCTION section 2.3.4), underlying a possible role in setting the threshold for T cell activation and differentiation (Michel, Attal-Bonnefoy et al. 2001).

Together, the sequence of signalling events coupled to TCR engagement results in the combined and regulated activation of MAP Kinases and Ca^{2+} signalling, leading to nuclear translocation and mobilization of transcription factors NFAT, AP1 (Fos/Jun) and NF-κB as exemplified by IL-2 gene transcription (**Fig.8**) (Northrop, Ho et al. 1994; Nel 2002). The type of MAP kinase activated during T cell stimulation may have a key role in driving effector and memory T cell differentiation and functions. For instance, p38 MAPK cascade regulates IFN-γ gene expression in T_H1 cells, but does not affect T_H2 cytokines (Rincon, Conze et al. 2000). Furthermore, it has been shown that JNK, ERK and p38 MAP kinase may differentially couple to apoptosis or survival (Xia, Dickens et al. 1995).

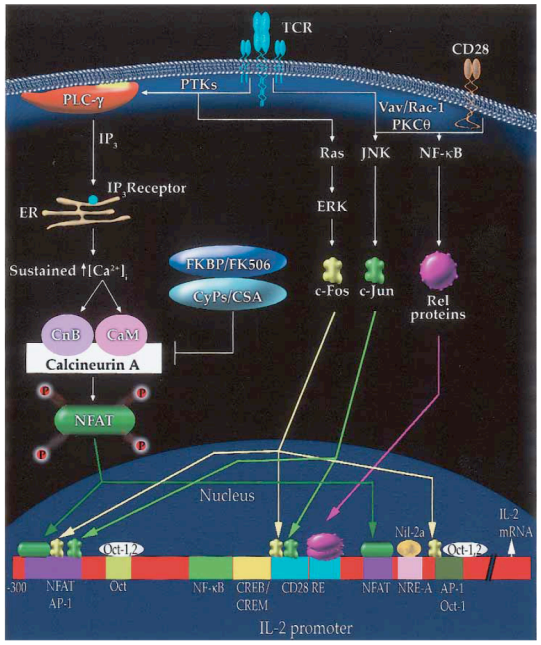


Fig.8 Signalling cascade leading to IL-2 gene transcription. Synergistic activation of IL-2 promoter by Ca^{2+}/cacineurin, Ras/MAP, and NF-κB cascades. The CD28 response element (CD28R) is a combinatorial element that requires activation of both the NF-κB and JNK cascade. Both cascades are dependent on CD28 costimulation. (Nel 2002)

2.3.4 CD28-mediated co-stimulation of TCR signalling

The membrane receptor CD28 is expressed on T cells at 3-fold higher frequency than the TCR and asserts key roles in enhancing TCR-mediated T cell activation. The ligands for CD28 are the structurally homologous proteins B7.1 (CD80) and B7.2 (CD86), which are expressed at high levels on activated professional APCs. The importance of the signals delivered by CD28-ligand interaction in T cell activation is underlined by the phenotype of both CD28- and CD80/CD86-deficient mice that display marked impairment of T cell responses (Lenschow, Walunas et al. 1996; Sharpe and Freeman 2002). However, CD28 triggering alone results in the transient expression of only few genes (Diehn, Alizadeh et al. 2002) and has no obvious biological consequences. Indeed, CD28-mediated signals are integrated with those that are induced by the TCR at a membrane proximal level and quantitatively act on TCR signalling networks enabling functional threshold to be overcome (Acuto and Michel 2003).

Of note, the cytoplasmic tail of CD28 contains 4 phosphorylated tyrosines and 2 proline-rich motifs that are involved in PI3K, Grb2 binding as well as Lck, Itk and Vav1 activation (Nel 2002). CD28-mediated activation of PI3K involves activation of AKT by upstream PDK1. Activation of AKT links CD28 to two crucial pathways for controlling gene transcription during activation. Indeed, AKT cooperation with the TCR-induced activation of PKCθ promotes formation of active NF-kB and AKT-induced activation of GSK3α/GSK3β inhibits the nuclear export of NFAT. CD28 triggering induces also the activation of Itk and Tec kinases through binding *via* its proline-rich regions. These kinases are positive regulators of PLCγI pathways (increasing iCa^{2+}, DAG-mediated activation of PKC, RAS/RAF/ERK activation through RASGRP activation) and appears to regulate WASP, a CDC42 activated protein involved in actin-cytoskeleton reorganization (Acuto and Michel 2003). Vav1 phosphorylation also results from CD28 triggering, although the mechanism regulating CD28-mediated recruitment of Vav1 to the membrane remains unclear. CD28-activated Vav1 affect many TCR signalling pathways (NFAT, NF-kB, AP1 as well as cytoskeleton changes) by synergizing with phosphorylated SLP76 provided by the ligated TCR (Michel and Acuto 2002).

Together, CD28 seems to quantitatively act on the pre-formed TCR-dependent signalling block by supplying activated signalling elements (such as AKT, TEK kinases and Vav1) that have pleiotropic effects and sustain gene-transcription and TCR-induced activation.

2.3.5 Regulators of TCR signalling

Tyrosine phosphatases (PTPase) are key regulators of TCR signalling by directly antagonizing kinase activity. PTPases have been shown to be important in maintaining T cells in resting state (Secrist, Burns et al. 1993) other than being involved in modulation of activation threshold and T cell signalling (Mustelin, Rahmouni et al. 2003). In addition to CD45, which plays a positive role by antagonizing the kinase Csk (phosphorylates inhibitory Y505 of Lck), a number of cytoplasmic PTPase contribute to negatively regulate T cell responses at distinct differentiation stages. For instance, SHP1 directly dephosphorylates ZAP-70 and Syk kinases, leading to decreased downstream signalling during T cell maturation in the thymus, as demonstrated by a general hyper-responsiveness to TCR stimulation of both thymocytes and peripheral T cells from SHP1-deficient mice (Brockdorff, Williams et al. 1999). Differently, the PTPase PEP, which contains a PEST domain for protein degradation, has been shown to specifically dampen responses of effector/memory T cell by regulating Lck activity while leaving normal naïve T cell functions (Hasegawa, Martin et al. 2004).

In addition to PTPase, others signalling molecules such as ADAP (Burbach, Medeiros et al. 2007), Sprouty (Choi, Cho et al. 2006), Gab2 (Yamasaki, Nishida et al. 2001), Sts-1/2 (Carpino, Turner et al. 2004) and Cbl (Thien and Langdon 2005) have been shown to modulate TCR signalling *via* processes distinct from direct dephosphorylation. Of interest, in mice the regulator Sprouty can exert different effects on TCR signalling depending on the differentiation state of the T cell. Indeed, overexpressed Sprouty inhibited TCR signalling and decreased IL-2 in Th1 clones, while enhancing TCR signalling and IL-2 production in naïve T cells. Sprouty influences both the NFAT and MAPK pathways and may have positive functions by sequestrating the inhibitory protein c-Cbl (Choi, Cho et al. 2006). Cbl proteins are also critical regulators of T cell development and signalling (Thien and Langdon 2005). Since in this thesis I focused on the role of c-Cbl, a member of Cbl family, in regulating the functional response of human memory T cells (see RESULT section 6), I will describe this protein in more details in a separate section (see following section).

2.3.5.1 Cbl protein family as negative regulator of TCR signalling

The multifunctional adaptor Cbl acts as a negative regulator of TCR signalling mainly by catalyzing ubiquitylation and targeting of critical signalling molecules to lysosomal or proteosomal degradation (Dikic, Szymkiewicz et al. 2003). In addition, Cbl can modulate

activity of signalling proteins and receptors via a yet unknown proteolysis-independent mechanism (Naramura, Jang et al. 2002; Dikic, Szymkiewicz et al. 2003; Duan, Reddi et al. 2004). Intensive research on Cbl proteins originated from the identification of the v-Cbl oncogene (Casitas B-lineage lymphoma), which is transduced by the mouse Cas NS-1 retrovirus and induces pre- and pro-B lymphomas. v-Cbl encodes the amino terminal portion (human c-Cbl residues 1-357) of the mammalian c-Cbl protein (Blake, Shapiro et al. 1991), which does not possess transforming capacities. In mammalian cells three members of the Cbl family have been reported; c-Cbl, Cbl-b and Cbl-3 (Swaminathan and Tsygankov 2006). All three homologues share highly conserved regions in their N-terminal halves, which include their tyrosine-kinase-binding (TBK) domain, the linker and the RING finger domains. In contrast, the C-terminal regions of these family members are less well conserved but they have common roles with abilities to interact with a range of SH2- and SH3- domain-containing proteins (Swaminathan and Tsygankov 2006). The RING finger domain has a E3 ubiquitin ligase activity that catalyses ubiquitination, thus targeting critical signalling molecules to proteosomal degradation (**Fig.9**) (Naramura, Jang et al. 2002; Duan, Reddi et al. 2004).

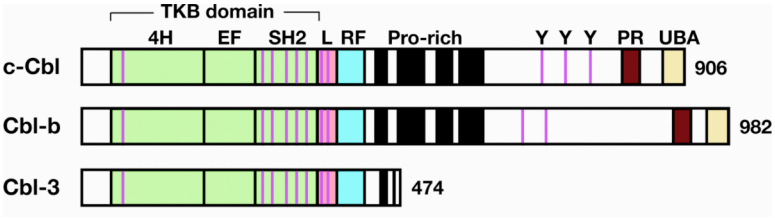


Fig.9 Cbl protein family in mammals.
All Cbl proteins share a high level of sequence conservation between their TKB, linker (L) and RING finger (RF) domains. c-Cbl and Cbl-b have extensive proline-rich regions (black) in their C-terminal halves that mediate interactions with numerous SH3-domain-containing proteins. The TKB domain is composed of three interacting regions comprising a four-helix bundle (4H), a calcium-binding EF hand and a variant SH2 domain that is connected to the RING finger by a short linker domain. The PR domain refers to a PX(P/A)XXR motif that binding SH3 domains of regulator of ubiquitin kinase family of proteins. UBA: ubiquitin-associate domain. Conserved tyrosine residues are shown in purple. (Thien and Langdon 2005)

Cbl-b is preferentially expressed in peripheral mature T cells while c-Cbl is highly expressed in thymocytes (Naramura, Kole et al. 1998). Not surprisingly, c-Cbl was reported to play a critical role during T cell development, while Cbl-b was shown to be more important in negatively regulating peripheral T cell activation. In contrast, Cbl-3, a shorter homologue

of c-Cbl, is enriched in endodermally derived organs, but expressed at very low levels in hematopoietic tissues (Fiore, Ollendorff et al. 2001).

In Jurkat cells, Cbl-b impairs the recruitment of PI3K to CD28 and indirectly regulates Vav activation by inducing ubiquitylation of PI3K activity (Fang, Wang et al. 2001). Cbl-b knock out mice show hyperproliferation (Bachmaier, Krawczyk et al. 2000), increased production of IL-2 uncoupled from the requirement for CD28 costimulation (Chiang, Kole et al. 2000; Krawczyk, Jones et al. 2005) and display auto-immune disorders (Bachmaier, Krawczyk et al. 2000). In addition, Cbl-b -/- T cells are resistant to induction of anergy (Jeon, Atfield et al. 2004).

In thymocytes, c-Cbl modulates TCR expression, inhibits ZAP-70 activation by an unknown mechanism and co-operates with SLP76 to target TCR/CD3ζ to degradation (Myers, Sosinowski et al. 2006). Accordingly, c-Cbl KO thymocytes exhibit hyperphosphorylation of ZAP-70 and ERK1/2, and TCR signalling uncoupled from CD4 co-stimulation requirements leading to increased proliferation (Murphy, Schnall et al. 1998) and intracellular calcium mobilization (Joazeiro, Wing et al. 1999). Of interest, CD4 peripheral T cells from c-Cbl KO mice show a higher functional threshold whereas cells from double Cbl KO mice show the phenotype and functions of EM cells (Naramura, Jang et al. 2002). However, in these KO mice, Cbl proteins are absent throughout development, therefore their role on TCR functions in normally developed T cells remains unclear. Importantly, adenovirus-mediated gene expression of dominant negative Cbl (355aa N-terminal residues of c-Cbl) in T cells from CAR Tg mice (mice rendered amenable to adenovirus-mediated gene expression without the need for T cell proliferation) enhanced cytokine production and phosphorylation of key signalling proteins (Vav, Akt, ERK and p38 MAPK) in response to CD3 stimulation, thus indicating that inhibition of Cbl function lowers the activation threshold of (post-thymic) peripheral T cells (Zha and Gajewski 2007). Finally, phosphorylation of Y731, which is unique of c-Cbl, provides a docking site for the SH2 domains of the p85 regulatory subunit of PI3K, thus enabling c-Cbl to function as a positive regulator by recruiting PI3K to the cell membrane (Thien and Langdon 2005)

2.3.6 Role of immunological synapse (IS) in TCR signalling

Recognition of pMHC complex by the TCR is strengthened by co-receptors (i.e. CD4 or CD8) and co-stimulatory receptor (e.g. CD28) expressed on T cell surface that pair their cognate ligands on the APC. The initial TCR-APC adhesion mediated by integrin (e.g. LFA-1

/ ligand ICAM-1) and accessory molecule (e.g. CD2 / ligand LFA-3) interactions is followed by the formation of a stable but dynamic molecular interface between the two cells which has been named the immunological synapse (IS) (also known as supramolecular activation cluster – SMAC) (Bromley, Burack et al. 2001). In the central region of the mature IS (named cSMAC) are concentrated the TCR, CD4 and CD28 receptors, with associated signalling molecules such as Lck or PKCθ. Larger molecules, including LFA-1, CD2 and the phosphatase CD45 are excluded from the central region and form a peripheral ring around cSMAC known as pSMAC (kinetic segregation model) (Bromley, Burack et al. 2001; Nel 2002).

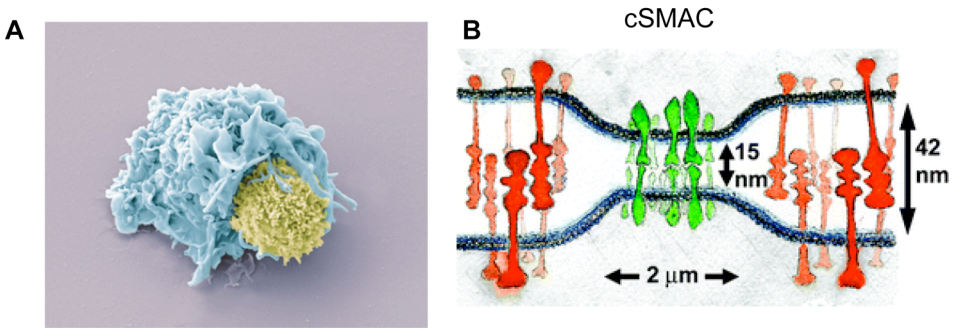


Fig.10 The immunological synapse.
(A) Contact between a T cell (yellow) and an antigen-presenting cell (APC, cyan) (http://www.science.gouv.fr)
(B) Large-sized tyrosine phosphatases (i.e CD45 – approximately 28-50 nm) are excluded from the cSMAC where concentrate small-sized TCR, p-MHC (<15 nm) and co-receptor molecules (http://www.rsc.org/ej/SM/2007/b608631d)

The actin cytoskeleton has an important structural role in shaping the IS (Samstag, Eibert et al. 2003) and plays active functions in signal transduction, as exemplified by the fact that drugs inhibiting actin polymerization interfere with T cell activation (de Mello Coelho, Nguyen et al. 2004). Staining experiments with colera toxin B subunit, which binds Ganglioside M1, a marker for membrane rafts, clearly demonstrated the presence of lipid raft in the IS (Bromley, Burack et al. 2001). Lipid rafts are cholesterol- and glycosphingolipid-enriched membrane microdomains insoluble in low concentrations of nonionic detergents (i.e. Triton X-100) that provide an anchoring site for many key acylated signalling molecules (such as Lck, Fyn, LAT…) (Nel 2002). The molecular compartmentalization promoted by lipid rafts favors some protein-protein interactions crucial for kinase activity and TCR signalling while preventing others, such as the association of CD45 with TCR (Zhang, Trible et al. 1998).

TCR activation and early tyrosine phosphorylation precedes IS formation and occurs in membrane TCR-microclusters containing activated Lck, ZAP-70 and LAT (Yokosuka, Sakata-Sogawa et al. 2005). Subsequent IS formation may contribute not only to the stabilization of the signalling complex, but also to the eventual cessation of the signal. The precise role of lipid rafts and IS in TCR signalling in naïve, effector and memory T cells are not yet known. However, recent evidence suggests that memory cells may have specific proteins associated in rafts that may direct differential signalling (Li, Liu et al. 2001). Indeed, Li et al. demonstrated that cellular prion protein (PrP^c) associates with membrane raft and is highly expressed in memory cells following TCR activation (Li, Liu et al. 2001). In addition, the increased expression of IS components such as LFA-1 in memory cells, may results in formation of stable IS at lower pMHC-TCR affinity.

3. Rationale for using Reverse Phase Protein (RPP) arrays in this study

CM and EM T cells represent low frequency cell populations in humans (2.5-13% and 0.8-6% of PBMC for CD4 and CD8 memory T cell subsets respectively). Moreover, only limited amounts of peripheral blood are available for analysis from each donor (400-450 ml blood or 100-250x10^6 PBMC per subject). These limitations represent real technical challenges to the study of memory T cell signalling (see PROJECT AIMS) that, therefore, cannot be achieved by means of traditional approaches such as western blotting or immunoprecipitation. In the last years, new emerging strategies, such as regular/phosphoFACS and protein arrays, have been successfully applied for the study of signalling pathways in rare populations of cells (Paweletz, Charboneau et al. 2001; Perez and Nolan 2002). Both approaches have unique sets of advantages/disadvantages that highlight their complementary nature.

PhosphoFACS originated as a refinement of regular flow cytometry applications (cell-surface marker analysis) and represents an extremely powerful multiparameter method for detection of phospho-specific epitopes at single-cell level in heterogeneous cell population (Perez and Nolan 2006). However, measuring intracellular antigens by flow cytometry requires extensive optimization of both protocols and reagents used (i.e. fixation/permeabilization) in order to maintain phospho-epitopes and fluorochrome stability, antigen accessibility, cell surface staining and light scatter properties. Moreover, only few antibodies work in this format and in many cases phospho-epitope analysis of a particular protein are simply not possible because of intracellular localization, low affinity antibodies or buried epitopes (Krutzik, Irish et al. 2004). Indeed, upon cell activation and signal transduction, phospho-specific epitopes are often buried by protein-protein or protein-substrate interactions (Schlessinger 2000) and therefore masked after fixation, leading to biased results. However, recent experimental evidence suggests that with proper fixation/permeabilization reagents, some antigens can be measured efficiently (Krutzik and Nolan 2003). Finally, a major limitation in FACS analysis lies in the small signal-to-noise ratio when detecting low-abundance signalling proteins, especially generated by the lack of signal amplification (Krutzik, Irish et al. 2004).

An interesting different approach to study signalling pathways in rare cell populations is represented by protein arrays, and in particular the recently described reverse phase protein (RPP) arrays (Paweletz, Charboneau et al. 2001). This format (described in details in

INTRODUCTION section 4.3.2) allows multiple samples to be analyzed simultaneously on the same substrate, greatly increasing throughput and simplifying quantitative comparisons between samples. Compared with FACS, RPP arrays use denatured protein/cell lysates and, although requiring the handling of homogeneous cell populations, need fewer cells and smaller amounts of reagents in parallel experiments. Importantly, most commercially available antibodies suitable for western blotting work in this format, provided they are not cross-reactive with other proteins (Balboni, Chan et al. 2006). Moreover, signal amplification strategies can be applied during array detection and small changes (as little as 20%) in protein levels or protein phosphorylation status can be efficiently quantified (See Results) (Chan, Ermann et al. 2004).

In addition to phosphoFACS and RPP arrays, beads-suspension arrays have been recently applied to the multiplex analysis of intracellular signalling pathways (Khan, Mendoza et al. 2006). However, to date, the limited availability of antibody pairs that function in this format, greatly reduces the potential of this approach to well defined applications, such as analysis of soluble proteins like cytokines (Szodoray, Alex et al. 2007).

Finally, approaches based on mass spectrometry coupled to immunoprecipitation, have become powerful tools to identify signalling proteins in a protein complex following activation. However, current sensitivity still make this approach unsuitable for the characterization of signalling pathways in rare cell populations (Berggard, Linse et al. 2007).

Taken together, more meaningful data would result from the combined use of these different approaches, but inevitably, at least at the current state of the art, this would result in too high costs. Therefore, the choice of the technology platform used in this study was dictated by the hypothesis that we wanted to test (study of membrane receptor signalling) and the availability of specific reagents and equipment. At the end of 2004 when this project started, RPP arrays emerged as the most solid and robust platform for profiling signalling protein in rare populations of cells as compared to regular/phosphoFACS and beads-suspension arrays (Chan, Ermann et al. 2004). To date, RPP arrays still represent the methodology that better adapts to our experimental purpose. Indeed, with respect to the other approaches, this strategy allows the analysis of a larger panel of signalling proteins, requires fewer cells, smaller amount of reagents and has the unique ability to efficiently and reliably quantify small changes in protein abundance or phosphorylation state.

4. Protein microarray technology

4.1 History

Historically, the roots of protein detecting-microarrays lie in the development of immunoassays. As early as 1929, antibodies were used in serology to precipitate antigens for subsequent quantification (Heidelberger, M., and F.E. Kendall. 1929. J. Exp. Med. 50:809–823). Analytical immunoassay technology was greatly advanced with the introduction of the radioimmunoassay (RIA) by Yarlow and Berson in 1959 (Yalow and Berson 1959) and the enzyme-linked immunosorbant assay (ELISA) by Engvall and Perlman in 1971 (Engvall and Perlman 1971). However, the basic concept of microarray technology was initiated only in 1989 by the ambient analyte model of Ekins and colleagues (Ekins, Chu et al. 1989; Ekins, Chu et al. 1990), which states that immunoassay "arrays" (that rely on the immobilization of interacting elements on a few square microns) should, in principle, be capable of measuring large numbers of different analytes in small samples with a higher sensitivity than conventional macroscopic immunoassays. Early biochemical experiments on solid surfaces (Fodor, Read et al. 1991; Maskos and Southern 1992) and filter-based assays (Lennon and Lehrach 1991), boosted by the completion of the whole-genome sequencing projects, culminated with the development of DNA microarrays, the first concrete application of the Ekins's model. Gene expression profiling experiments have generated an unprecedented amount of data regarding concomitant genetic events during disease. However, the functional consequence of disease is manifested ultimately in the deregulation of protein products and protein networks. The realization of this important aspect, coupled with the success of DNA-based array technologies (platforms and software tools), has finally encouraged the transition to protein microarrays (Macbeath and Schreiber, 2000).

4.2 Principle and features

Protein microarrays consist of protein spots arrayed at high spatial density on a solid support (MacBeath 2002; Zhu and Snyder 2003). The spots contain a bait molecule (such as antibody, cell or phage lysate, drug, recombinant protein or peptide) that capture corresponding binding molecules from a sample (antibody, ligand or serum/cell lysate). Readout systems based on fluorescence, chemiluminescence, radioactivity, electrochemistry

or label-free methods can be used to detect specific interactions within each microspot. Microarray-based assays are crucial to large scale and high throughput biological analysis, as they allow true parallelism, miniaturization, multiplexing and automation (Schena, Heller et al. 1998). These key features provide a set of performance specifications such as rapidity and low cost that can not be obtained with other technologies. Moreover, protein microarrays enable profiling of protein levels and their post-translational modifications at a scale that is beyond what traditional techniques such as western blot or ELISA can achieve (Balboni, Chan et al. 2006).

The main current limitation of protein microarrays lies in the limited availability of high quality, specific antibodies or suitable protein binding ligands. This necessity has strengthened the development of new high-throughput methods for the generation of antibodies (Cahill 2001; Zhu and Snyder 2003) and pure functional proteins (Phizicky, Bastiaens et al. 2003; Hall, Ptacek et al. 2007). Prior to use on any microarray format, antibody specificity and sensitivity must be thoroughly validated (Liotta, Espina et al. 2003). A second limitation is the lack of established set of reference standard and reagents for each of the various components of protein microarray platforms. Major efforts should be taken in this direction since without the establishment of reference standards, it will be difficult to interpret results across time and between laboratories and platforms. Currently, several pilot studies have been carried out by the International Human Proteome Organization (HUPO: http://www.hupo.org) and standards for proteomics are under discussion in different national initiatives, such as the German National Genome Research Network (http://www.ngnf.de) and the US National Institute of Health Roadmap ('Standard in Proteomics'- http://nihroadmap.nih.gov/buildingblocks/proteomics/).

Protein microarrays pose a significant set of analytical challenges not faced by gene microarrays (Espina, Mehta et al. 2003). The initial challenge is developing a system capable of detecting a vast range of analyte concentrations (up to a factor of 10^{10}). The second challenge is identifying bait molecules with adequate sensitivity (femtomolar range) and specificity for detecting low abundance proteins, since low abundance analyte always exists in a complex biological mixture containing a vast excess of contaminating proteins. The third challenge concerns the amplification strategy that must be linear and reproducible. Moreover, the amplification chemistry must be tolerant to the complexity of the biologic samples that can naturally contain molecules, i.e. biotin, peroxidase, etc interfering with the amplification process (Hunyady, Krempels et al. 1996). Finally, the last challenge lies in the clinical power

of protein microarrays that can only be achieved if the technology is able to handle very small amounts of input material.

4.3 Array formats and applications

Protein arrays have great potential not only in proteomics, but also in many other fields, as broad as food industry, commercial diagnosis, quality control and many research disciplines. Within this large window of applications, I will focus my discussion on relevant aspect of protein arrays in immunology and, in addition, I will mention selected references regarding general applications. Particularly, I have organized this chapter into two main sections, corresponding to the two current format of protein array: forward and reverse phase (**Fig.11**).

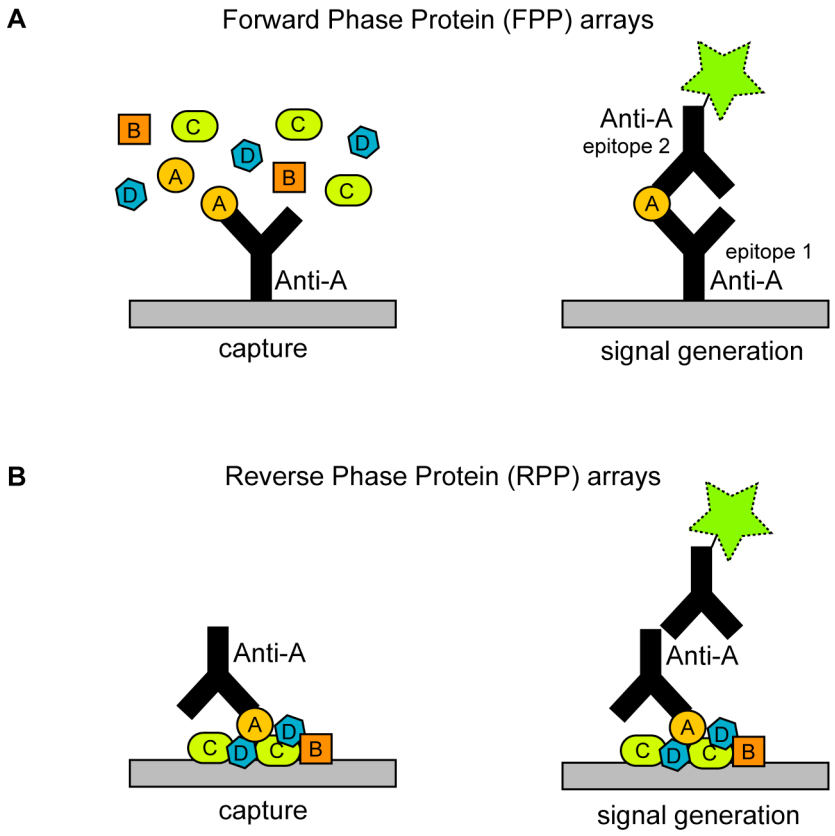


Fig.11 Protein array formats.
(A) Forward phase protein arrays: in this format, a bait molecule (typically an antibody) is directly immobilized onto the slide support. The array is subsequently incubated with a test sample containing different analytes and the captured molecule detected using label-based method (such as sandwich assay). **(B)** Reverse phase protein arrays: in this format, the whole test sample containing multiple analytes is immobilized onto the slide support. The targeted molecules is then detected by incubating the array with specific antibodies.

4.3.1 Forward Phase Protein (FPP) arrays

In this format a bait molecule, typically an antibody (antibody microarray) or a peptide/functional protein (antigen/protein array) is immobilized as individual microspot on the substratum. The array is incubated with only one test sample that contains different analytes and the captured molecule is subsequently detected using labeled-based or labeled-free methods (**Fig.11A**). The use of FPP arrays in immunology has largely been limited to the profiling of secreted factors (cytokines, chemokines, growth factors) and autoantibodies.

4.3.1.1 Antibody arrays

In immunology, antibody arrays are traditionally used to quantify serum proteins, such as cytokines and chemokines in physiological and pathological conditions, for instance in autoimmune disease. Identification and quantification of cytokines contribute to the understanding of pathogenesis (e.g. disease classification) (Kader, Tchernev et al. 2005) and discovery of new therapeutic targets and novel markers for therapy monitoring. Planar antibody arrays were first developed as an extension of the sandwich ELISA platform (Tam, Wiese et al. 2002). A capture antibody is spotted on a slide surface, a sample is applied and washed, and then a cocktail of labeled detection antibodies is added to bind available epitopes on the captured species. Detection is typically achieved with fluorescent- or chemioluminescent-based methods giving sensitivity at least equivalent to traditional ELISA (Huang, Huang et al. 2001). To further increase sensitivity, multicolor rolling-circle amplification (RCA) has emerged as a well-suited detection method. Arrays using RCA amplification operate like other multiplex sandwich ELISAs, with the exception than the final step involves an oligonucleotide elongation reaction from a circular DNA template. Schweitzer *et al.* first applied this strategy demonstrating simultaneous detection of 75 human cytokines with femtomolar sensitivity, 3 log quantitative range and reporting the feasibility of time-course analysis of cytokine secretion by mature dendritic cells (DCs) (Schweitzer, Roberts et al. 2002). Further optimization of this protocol allowed simultaneous measurement of up to 150 cytokines (Shao, Zhou et al. 2003) and analysis of two serum samples on the same arrays using two-color RCA detection (Zhou, Bouwman et al. 2004).

Besides planar arrays, beads-suspension arrays have been recently developed. In this format, optically encoded micron-sized beads serve as surface binder of capture antibodies. Since each particle has unique optical properties, multiplexing is possible and beads array technologies can easily handle 100 analytes simultaneously. Compared with planar arrays,

this approach enables lower cost, higher flexibility and faster analysis of each sample (Nolan and Sklar 2002). Clinical applications of this approach include the study of circulating cytokines in Sjogren's syndrome (Szodoray, Alex et al. 2004) and in psoriatic arthritis (Szodoray, Alex et al. 2007) versus healthy controls. Both studies revealed the presence of disease-state-specific cytokine pattern and suggested biomarkers for diagnosis and disease management.

Other applications of antibody arrays include study of tissue-specific protein expression in cancer versus healthy donors (Usui-Aoki, Shimada et al. 2005) and cell-based arrays (Ko, Kato et al. 2005).

Compared to the existing single analyte ELISA platform all of the above technologies allow multiplexing, high-throughput, at least comparable if not higher sensitivity and require smaller sample volume. Nevertheless, these technologies are dependent on highly specific, high-affinity pairs of binders. Therefore, despite the growing number of successful applications, their use is currently limited to specific investigations definable with a relatively small set of validated antibodies.

4.3.1.2 Protein arrays

The general concept of protein microarray lies in the immobilization of an antigen or pure functional protein/peptide as an individual spot. The array is then probed with a cell/serum lysate and the captured molecules are detected. Despite early application to determine antibody specificities (Lueking, Horn et al. 1999), protein arrays were first used in immunology to characterize the humoral immune response of autoimmune diseases. Indeed, autoantibodies are generally directed against evolutionary conserved molecules and have very high-affinity and relative high-titer (von Muhlen and Tan 1995).

Although traditional methods like ELISA, immunoprecipitation and western blot are readily available and easy to perform for profiling of a single given autoantigen, they become costly, time and labor consuming when analyzing multiple autoantibodies. Moreover, the amount of serum needed to test multiple antigens can be limiting, especially in seriously ill or pediatric patients. Protein microarrays have the advantage of allowing comprehensive analysis of autoantibodies directed against hundreds to thousands of antigens (including proteins, peptide, nucleic acids, lipids, ecc) in microliter volume of sera (Robinson, DiGennaro et al. 2002). Importantly, the amount of serum, time and cost to run the assay remain the same regardless of the number of analytes.

Joos and colleagues first demonstrated an antigen-microarray based approach to be sufficiently sensitive and specific for detecting 18 different serological markers in serum samples obtained from human patients with systemic rheumatic diseases (Joos, Schrenk et al. 2000). In 2002 the first large-scale autoantigen microarray was produced, designed specifically for large-scale multiplexed characterization of autoantibody responses. This 1152-feature connective tissue disease (CTD) array was subsequently tested with either monoclonal antibodies or highly characterized serum samples from eight distinct human autoimmune diseases (Robinson, DiGennaro et al. 2002). These studies reported sensitivity 4 to 8 times higher than regular ELISAs over 1000-fold linear range. Using immunoglobulin subclass-specific secondary antibodies, these arrays can also be used to characterize autoantibody subclasses that may be important in disease pathogenesis.

In addition to diagnosis of autoimmune diseases, protein arrays have been used to determine IgE reactivity profiles in patients with seasonal allergies (Jahn-Schmid, Harwanegg et al. 2003), to perform serodiagnosis of infectious disease (Bacarese-Hamilton, Mezzasoma et al. 2004) and to monitor disease progression and response to therapy (Balboni, Chan et al. 2006). Antigen arrays have also been used for the identification of new diagnostic and therapeutic targets. Qiu et al. investigated the severe acute respiratory syndrome (SARS) using a protein arrayed composed of 13 recombinant proteins of the virus and identified a unique immunogenic protein as a promising vaccine candidate (Qiu, Shi et al. 2005).

Beside the immunological analytical studies reported above, protein arrays have emerged as a technology to assess a wide range of functional biochemical activities in many field of research (Hall, Ptacek et al. 2007). Indeed, early proof-of-concept studies (MacBeath and Schreiber 2000) demonstrated that native proteins immobilized on an array remain enzymatically active. Proteome arrays have been used to study protein-protein and protein-lipid interactions (Zhu, Bilgin et al. 2001), protein-DNA interactions (Hall, Zhu et al. 2004), protein-receptor interactions (Jones, Gordus et al. 2006), protein-drug interactions (Huang, Zhu et al. 2004).

4.3.2 Reverse Phase Protein (RPP) arrays

In contrast to the FPP arrays, which involves immobilizing proteins or antibodies on the array surface, the reverse phase approach is based on the immobilization of whole cell lysates as distinct microspots (**Fig.11B**) (Charboneau, Tory et al. 2002). The arrays are then probed with highly specific antibodies that recognize a given protein either in a phosphorylation

dependent (for detecting activation states) or independent fashion (for measuring protein abundance). Bound antibodies are subsequently detected using a secondary antibody directly conjugated to a fluorophore (dyes, quantum dots) or enzymes for signal amplification (HRP). The preferred amplification strategy for the detection of low abundance signalling protein involves HRP-mediated biotin-tyramide deposition, followed by detection by a fluorophore-conjugated streptavidin (Bobrow, Litt et al. 1992). Since multiple samples (each spot represents a different sample) on one array are simultaneously analyzed under the same experimental conditions, quantitative comparison between samples is extremely simplified. Moreover, only minute amounts of protein extracts are required for the generation of hundred of arrays, thus permitting the comprehensive analysis of rare cell types and valuable patient samples. In addition, multiple replica and dilution series can be included on the microarray, increasing the robustness of protein quantification (Mircean, Shmulevich et al. 2005).

The concept of RPP arrays was originally described in 2001 by Paweletz *et al.*, in a study showing increased phosphorylation of the prosurvival signalling protein Akt and decreased phosphorylation of ERK in the transition from normal prostate epithelium to prostate intraepithelial neoplasia and invasive prostate cancer (Paweletz, Charboneau et al. 2001). The authors could also demonstrate femtomolar sensitivity and a high degree of precision and linearity, allowing accurate quantification of signalling proteins.

The majority of the work accomplished so far using RPP arrays was carry out by Liotta and Petricoin's research groups in the field of oncology. Their studies especially underlined the utility of RPP arrays, coupled with laser capture microdissection (LCS) approach, to i) discover unexpected signalling defects in tumor specimens relevant for therapy (Petricoin, Espina et al. 2007) and ii) identify tumor subtypes based on specific phosphorylation-signalling signatures (Sheehan, Calvert et al. 2005). For instance, in a recent study, Petricoin *et al*. showed that interlinked components of the Akt/mTOR pathway exhibited increased levels of phosphorylation in rhabdomyosarcoma (the most common soft tissue sarcoma in children) of patients with short-term survival. The functional significance of this finding was verified using CCI-779 inhibitor (mTOR downstream protein inhibitor), that suppressed tumor growth in a beige SCID rhabdomyosarcoma xenograft model (Petricoin, Espina et al. 2007).

In addition to these studies that use tumor specimens frozen immediately after resection, Chan *et al.* first applied in December 2004 RPP arrays to profile signalling events in stimulated living cells (Chan, Ermann et al. 2004). Indeed, they profiled the phosphorylation state of 62

signalling components in Jurkat T cells stimulated through TCR/CD3 and CD28 receptors, identifying a previously unknown link between TCR/CD3 crosslinking and dephosphorylation of Raf-1 protein. Moreover, as a feasibility study, they used RPP array to profile the phosphorylation state of 23 signalling proteins in a rare population of T cells. Their analysis revealed a differential STAT protein phosphorylation in CD4+CD25+ regulatory T cells (Treg) and T cell blasts isolated from naïve mice in response to IL-2 stimulation. Importantly, they showed a two-log linear range and a detection limit of one protein in 10^5 to 10^6 lysate protein.

In this thesis work this strategy has been applied for the first time to perform an analysis of signalling pathways in rare human cell populations isolated *ex-vivo*, allowing us to accurately quantify signalling proteins from as little as 20 cell-equivalents per spot. We profiled 16 signalling parameters in human memory CD4 and CD8 T cell subsets. Among the differences identified, we demonstrated that decreased content of the negative regulator c-Cbl in effector versus central memory CD4 T cell subsets is responsible for their different functional responsiveness (see RESULTS).

The main limit of RPP arrays lies in its dependency on commercially-available highly-specific antibodies. Nevertheless, compared with other technologies (i.e. FPP arrays and phosphoFACS), RPP arrays is the approach that suffers the least from this drawback. Indeed, RPP arrays do not require chemical modification of the antibodies and, in addition, their validation can be carried out by regular western blotting immunodetection. Moreover, most of the commercially available antibodies suitable for western blotting work in this format, provided that they have no cross-reactivity with other proteins in the sample analysed.

PROJECT AIMS

Two subsets of human memory T cells (central and effector memory) have been described based on the surface expression of the phosphatase CD45 and the chemokine receptor CCR7 (see INTRODUCTION section 1.2.2). These subsets display a high functional heterogeneity and have critical and distinct roles in the control of protective immunity. However, the biochemical mechanisms controlling the functional properties of memory T cell subsets are still unknown and remain poorly investigated. Their clarification is crucial for a rational design of effective T-cell vaccines and for the identification of new molecular targets for drug development. Major limitations to these studies lie in the technical challenges imposed by the low frequency of memory T cell subsets and the limited amount of human specimens available for analysis that make traditional protein profiling approaches inadequate.

The aim of this is the identification of the mechanisms responsible for the functional heterogeneity that characterizes human central and effector memory T cell subsets. In particular, we hypothesized that this heterogeneity derives from a different composition/regulation of the signalling machinery and/or a differential assembly/recruitment of the TCR signalling complex components in these cells.

Our first hypothesis has been successfully investigated in this thesis thanks to the implementation of reverse phase protein arrays, which represent a highly sensitive and quantitative protein profiling approach for the study of rare cell populations. Critical differences in the global composition of the signalling machinery of memory T cell subsets were identified and correlated with the functional properties of these cells.

Our second hypothesis remains currently under investigation. However, a technical approach has been developed and validated to achieve this aim.

The results obtained from the sets of experiments presented in this thesis are part of the following manuscripts:

1) Brembilla NC, Weber J, Rimoldi D, Pradervand S, Schutz F, Pantaleo G, Ruegg C, Quadroni M, Harshman K, Doucey MA. c-Cbl expression regulates the functional response of human central versus effector memory CD4 T cells. Blood. 2008 Aug 1;112(3):652-60.

2) Brembilla NC, Cohen-Salmon I, Weber J, Rüegg C, Quadroni M, Harshman K and Doucey MA. Profiling of T cell receptor signaling complex assembly in human CD4 T lymphocytes using reverse phase protein arrays. Proteomics. (in press)

RESULTS

5. Implementation of RPP arrays to profile signalling proteins in human memory T cell subsets

The first objective of this thesis lies in the comprehensive analysis of the composition of the signalling machinery of human central and effector memory T cells isolated *ex-vivo*. However, the low frequency of memory T cell subsets and the limited amount of human specimen available for analysis make traditional protein profiling approaches inadequate. To address these issues, we established and implemented a technological platform able to accurately quantify signalling proteins and their modifications in minute amount of cells.
The technology we selected and implemented was Reverse Phase Protein (RPP) arrays. The rationale for using this approach is discussed in details in INTRODUCTION section 3.

RPP arrays were initially applied in 2001 by Paweletz *et al.* for the characterization of signalling defects in prostate cancer biopsies (Paweletz, Charboneau et al. 2001). Subsequently, at the end of 2004 and concomitantly with the beginning of this thesis project, Chan *et al*. adapted this technology to monitor signalling events in living cells (Chan, Ermann et al. 2004).

In this chapter, I report the implementation of RPP arrays for the profiling of signalling proteins in highly purified subsets of human memory T cells isolated *ex-vivo*. This work was conducted in collaboration with the DNA Array Facility (DAF, CIG, Lausanne) of the University of Lausanne and led to the set-up of RPP array technology for the first time in Lausanne (http://www.unil.ch/dafl/page44650_en.html).
The results described in this section are also part of a manuscript accepted for publication in **PROTEOMICS** journal (www.proteomics-journal.com) on August 2008 (see PROJECT AIMS).

5.1 Design and fabrication of RPP arrays

To fabricate our RPP arrays we used the contact-printing robotic microarrayer Omnigrid 300 fitted with SMP3 solid pins that deliver approximately 0,7 nl of whole cell lysate per spot onto a slide containing 16 distinct 3D-nitrocellulose pads. One pad accommodates 21 different samples printed in triplicate. A 21 pad-specific series of samples is then replicated

along the 16 pads of a slide. Each pad can be treated independently by using a 16 well leak-proof incubation chamber, thus allowing 16 simultaneous detections on 21 different samples (**Fig.12A**) on the same slide.

After blocking, pads were probed with specific primary antibodies, followed by HRP-secondary antibody incubation. To ensure detection of low-abundance signalling molecules, a Catalyzed Reporter Deposition (CARD) Amplification step was introduced (Bobrow, Litt et al. 1992). Briefly, HRP catalyzes the formation of reactive biotin-tyramide intermediates that covalently attach to tyrosine residues of surrounding proteins. The half life of the intermediates is so short that deposition occurs only close to the HRP. Bound biotin was detected with streptavidin-Cy3 and signal intensities measured using a microarray scanner (**Fig.12B**). Gain in signal intensity after the amplification step was in the range of 8 to 30 fold (**Fig.12C**).

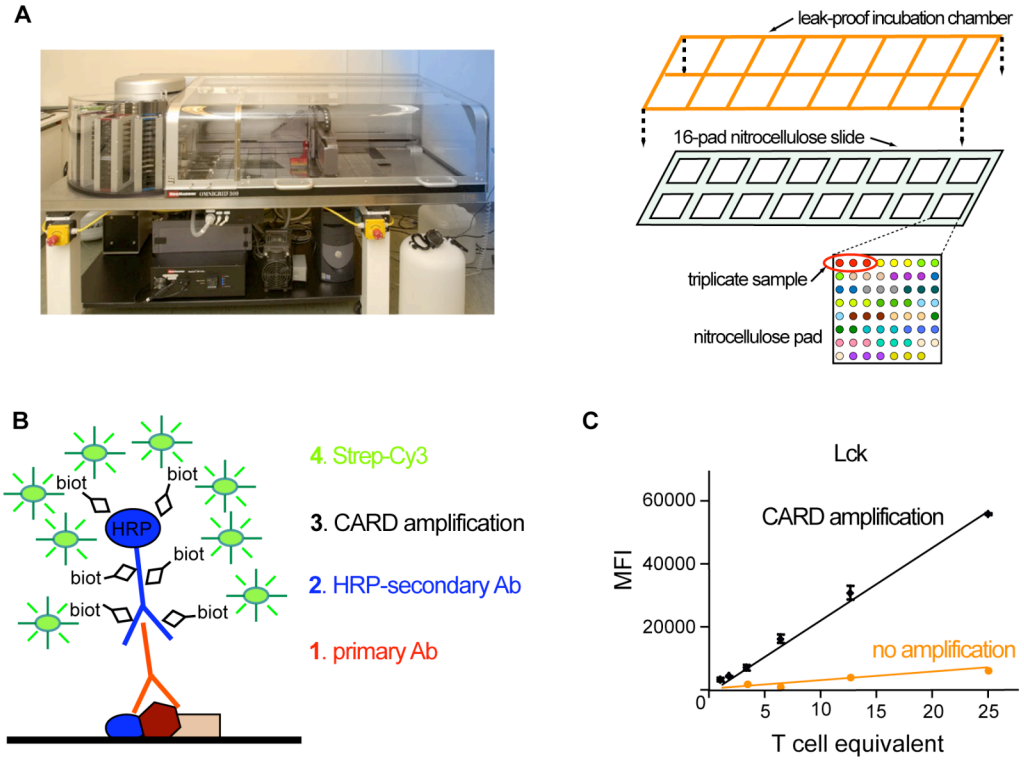


Fig.12 RPP arrays: equipment and detection strategy.
(A) Contact-printing robotic microarrayer OMNIGRID 300 (left panel). Representation of a 16-pad nitrocellulose-coated glass slide. Each pad contains 21 different samples printed in triplicate and can be treated independently by means of a leak-proof chamber (right panel). **(B)** RPP arrays detection strategy: 1. primary Ab (red), 2. HRP-linked secondary Ab (blue), 3. CARD amplification step (black), 4. Streptavidin-Cy3 (green). **(C)** gain in signal intensity after CARD amplification.

5.2 Octylglucoside-containing lysis buffer allows optimal protein retention on 3D nitrocellulose-coated slide

Cell lysate quality is of critical importance to the reliability and outcome of RPP arrays (Winters, Dabir et al. 2007). Indeed, different detergent-containing lysis buffers have unique solubilization properties and behavior with respect to protein solubilization and retention on 3D nitrocellulose. Protein extraction buffers for RPP arrays usually contain SDS as detergent and have been mainly applied to the solubilization of tissue (Sheehan, Calvert et al. 2005) or cell lines (Nishizuka, Charboneau et al. 2003; Chan, Ermann et al. 2004). However, the impact of the detergent on lysate retention on 3D nitrocellulose remains still unknown. This thesis is the first study that investigates signalling proteins in *ex-vivo* isolated human T cells using RPP arrays, and therefore no established and validated lysis conditions for these cells were available.

An optimal extraction buffer has to meet the three following criteria: i) it contributes to protein denaturation, ii) it allows efficient protein solubilization including proteins associated to lipid rafts (often the case for signalling protein) and iii) it shows minimal interference with protein retention on the RPP array surface (i.e 3D nitrocellulose).

To allow a direct comparison of the detergent effects, *ex vivo* isolated human CD4 T cells from the same subject were lyzed using Triton X-100 (TX), Triton X-100/SDS (TX/SDS) and octylglucoside (OG)-containing buffers. Importantly, lipid rafts are resistant to TX solubilization while efficiently solubilized by OG or SDS-containing buffers (Doucey, Legler et al. 2001). CD4 T cell lysates obtained with the three different buffers were serially diluted and each condition arrayed as triplicate spots on the same pad, which was subsequently detected with antibodies specific for the T cell adaptor protein c-Cbl. The impact of the different detergents on protein retention on 3D nitrocellulose was evaluated based on signal intensity, sensitivity, dynamic range and reproducibility. The buffer containing TX, a detergent unable to solubilize lipid raft, displayed a marked loss in signal intensity and dynamic range compared to TX/SDS- or OG-containing buffers which, in contrast, produced strong and comparable signal intensities. However, while TX/SDS-based buffer repeatedly resulted in a loss of dynamic range at higher lysate concentration, OG-based buffer produced a reproducible linear regression between signal intensity and lysate concentration covering two orders of magnitude (**Fig.13A**).

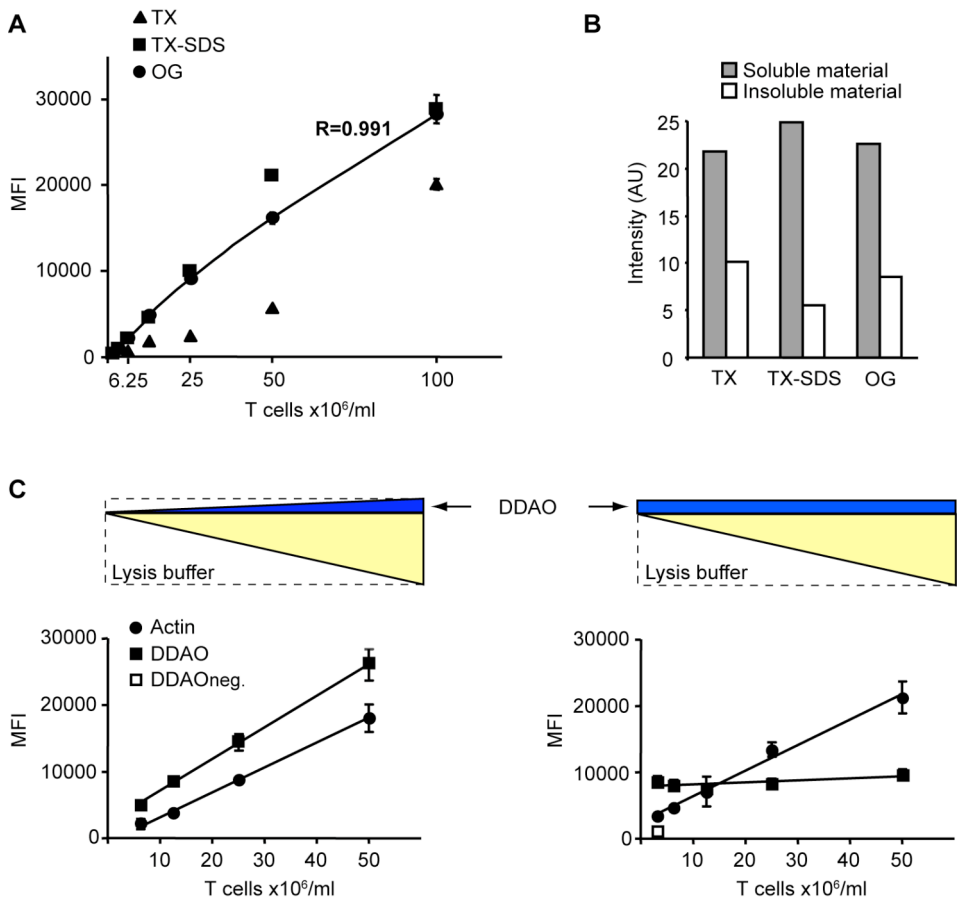


Fig.13 Octylglucoside containing CD4 T cell lysates are optimally retained on 3D nitrocellulose.
(A) T cells were lysed in TX-100, TX-100/SDS mixture or octylglucoside (OG) containing buffers. Increasing dilutions of protein cell lysates were printed on 3D nitrocellulose slide. Shown is the mean fluorescence intensity (MFI) of c-Cbl detected in triplicate spots with specific antibodies. **(B)** Solubilization efficiency of the different detergent-containing buffers as measured by total protein content of soluble (S) or insoluble (INS) fractions. Following fractionation of the lysates by SDS-PAGE, total proteins from the gel were stained with Coomassie Blue and quantified with Image J software. **(C)** Protein retention efficiency on 3D nitrocellulose-coated slide is not affected by protein to OG ratio. T cell proteins were labelled with the fluorescent cell tracker DDAO, lyzed, mixed with unlabelled T cell lysate at a 3/97 ratio and serially diluted in lysis buffer (left panel, increasing DDAO /detergent ratio). Conversely, increasing amounts of unlabelled lysate were added to a constant amount (1.5% of total proteins) of DDAO-labelled T cell lysate (right panel, DDAO/detergent ratio constant). Actin and DDAO signals were detected in triplicate spots. One representative experiment out of three is shown.

The efficiency of protein solubilization produced by these buffers was assessed by SDS-PAGE using samples obtained under the same experimental conditions. Surprisingly, signal intensities obtained after RPP arrays did not directly reflected the efficiency of protein solubilization as assessed by SDS-PAGE. Indeed, while TX-SDS and OG signal intensities obtained by RPP arrays were comparable and higher than the one obtained with TX (**Fig.13A**), protein solubilization achieved with TX-SDS was only slightly higher (+15-20%)

as compared with the other buffers (**Fig.13B**). Thus, linear and robust spot signal intensities from crude T cell lysate results from a complex process involving the efficiency of protein solubilization and the interference of the detergent on protein retention on 3D nitrocellulose. For further experiments, OG- was preferred to TX/SDS-containing buffers due to its lager dynamic range and better linearity (**Fig.13A**).

We next examined whether the protein content of the lysate affected the efficiency of protein retention on 3D-nitrocellulose when printing is performed at constant OG concentration (thus changing protein/detergent ratio). T cells were treated with DDAO-ester to fluorescently label membrane and cytosolic proteins, lyzed, mixed with unlabelled T cell lysate and serially diluted in lysis buffer. As expected, in this condition where DDAO and total protein/detergent ratio were increased, both actin and DDAO signals increased in a linear and parallel fashion (**Fig.13C, left panel**). In contrast, when a constant amount of DDAO lysate was supplemented with increasing amount of unlabelled lysates (thus increasing protein/detergent ratio but keeping constant DDAO/detergent ratio), DDAO signals remained unchanged while actin signal linearly increased (**Fig.13C, right panel**). Similar results were obtained when cytosolic (Syk) or membrane-associated proteins (Lck, FcRγ) were detected (data not shown), thus confirming no significant impact of OG on protein retention over the range of lysate dilutions examined. Together, OG-containing lysis buffer displays comparable solubilization efficiency with respect to TX and TX-SDS based buffers while allowing better protein retention on 3D nitrocellulose and larger dynamic range.

5.3 RPP arrays display excellent performance characteristics

In order to enable statistical analysis and to increase the robustness of the data, each sample used in this thesis project was printed as triplicate spots. A single spot contained 2 to 5 ng of proteins from total T cell lysate, which correspond to the protein equivalent of roughly 20 to 50 T cells.

Qualitative analysis of the spots revealed an average diameter of 209±15 μm per spot and a circularity of 93,5 % (circular shape=100 %). Coefficient of variation (CV) intra-triplicate and inter-sample were respectively 2,9% and 6,9% for diameter and 2,2% and 1,9% for circularity, revealing that diameter, but not circularity, was slightly sample-dependent. Good signal reproducibility was achieved, with CV within triplicate spots from 200 samples less than

5.6%. Similarly, inter-pad variability of replicated samples (re-print of 21 samples on 16 pads of the same slide) was ranging from 3 to 6% in low to high protein lysate concentrations, respectively (**Table 1**).

	Geometry		Intensity
	Diameter	Circularity	
Mean	209±15 µm	93.5 %	-
CV intra-triplicate (%)	2.9	2.2	5.6
CV inter-triplicate (%)	6.9	1.9	3-6 *
nb of spots	249	249	600

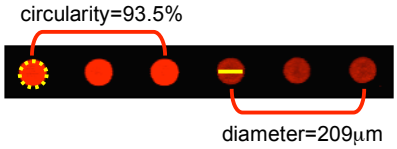


Table 1. Qualitative analysis of the spots.
Geometry and intensity of the spots were evaluated by calculating the Coefficient of Variation (CV) intra-triplicate or inter-samples. * refers to inter-pad variability (right panel). Representative spots are shown (left panel).

Specificity of the signal was assessed by measuring SLP-76 expression in peripheral blood mononuclear cells (PBMC) and colon carcinoma Caco-2 cells, where it is expressed and not expressed, respectively. Signal specificity was further demonstrated by detecting RNase-2 in PBMC lysates supplemented or not with exogenous RNase-2 (**Fig.14A**).

Since each antibody is unique in its binding affinity for its cognate antigen, we determined its specific dynamic range by including a lysate serial dilution in the same pad containing the samples to analyze. For most of the antibodies used, signal linearity was demonstrated in spots containing 5 to 200 cell equivalent (**Fig.14B** and data not shown). An antibody was used for RPP arrays only if it detected by immunoblotting a single band of the expected molecular weight in a biological sample similar to that analyzed on the arrays (**Fig.14C**).

Together, these data show that RPP array approach possess performance characteristics that are largely beyond what traditional technologies can achieve and therefore can be suitable for the profiling of protein levels in minute amount of cells. Of note, the amount of cells required for the quantification of a single signalling protein by immunodetection of Western Blot (e.g. c-Cbl ~300000 cells) is sufficient to print more than 4000 spots on RPP arrays.

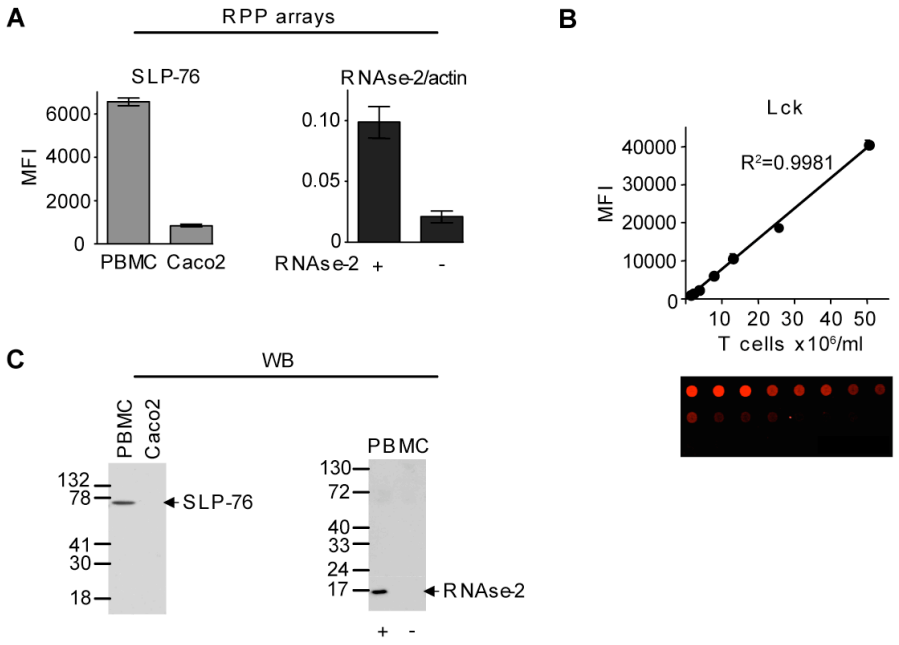


Fig.14 Performance characteristics of RPP arrays.
(A) Mean Fluorescence Intensity (MFI) of SLP-76 specific signals detected in spots containing equal protein amount of PBMC or intestinal Caco-2 cell lysates (left panel). Alternatively, exogenous RNase-2 was added to PBMC lysate and specific RNase-2 signals were normalized to β-actin content (right panel). **(B)** Quantitative analysis of Lck detection in serially diluted PBMC lysates using specific antibodies. Corresponding spots printed in triplicate are shown. **(C)** Immunodetection of Western Blots with specific antibodies for SLP-76 and RNAse-2 in the lysates as shown in A.

5.4 Analysis of T cell populations using RPP arrays

The RPP array technology described above has been validated and used in this thesis to profile the abundance of 15 relevant signalling proteins in *ex vivo* isolated human CD4 and CD8 memory T cell subsets in 8 to 13 healthy subjects (see RESULTS section 6.1). As described in details in RESULTS section 6.1, highly purified CD8 and CD4 T cell subsets (naïve -N-, central memory -CM-, effector memory -EM- and effector -E- cells) were isolated by cytofluorimetry, lyzed and arrayed as triplicate spots on a nitrocellulose-coated slide that was subsequently detected with antibodies against the targeted signalling proteins. Since *ex-vivo* isolated T cell populations do not display the homogeneity of T cell lines or clones, combined analysis of distinct subjects was needed to reach statistical robustness. Thus, in this study three sources of variability can be identified: variability in the amounts of total protein printed per spot, inter-subject variability, and experimental variation (i.e. technical error). It is

crucial to take these variabilities into account when comparing the relative abundance of a signalling protein among the different T cell subsets.

Variation in spotting and sample preparation were adjusted by normalizing the mean signal intensity of each triplicate spot (one sample) to the corresponding mean intensity of β-actin signal, which serves as an internal marker for total protein deposition. Indeed, we show that the amount of actin per mg of protein lysate was constant in the different T cell subsets, while β2-microglobulin (**Fig.15**) or tubulin levels (data not shown) varied considerably. Signalling protein/actin ratios were calculated by probing identical pads on the same slide with actin-specific antibodies. Since normalization is introduced, accurate quantification of protein abundance was possible only when signalling protein and actin signals were included in their dynamic range. The dynamic range, defined as the linear range of detection of a specific protein, was determined by detecting a serial dilution of a CD4 T cell lysate in the same pad of the samples analyzed.

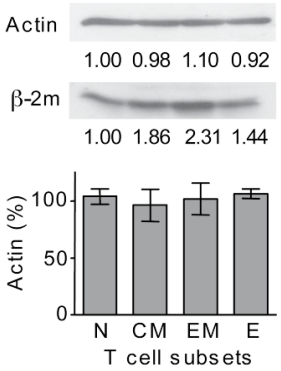


Fig.15 β-actin as protein for normalization.
β-actin and β2-microglobulin contents were measured in lysates of sorted N, CM, EM and E cells following SDS-PAGE and immunoblotting with specific antibodies. Shown are representative immunodetection and quantitative measurements of β-actin signals from WB in three cumulated subjects

The relative error in signal intensity ratio (signalling protein/actin) represents an estimation of the technical error in the quantification of the signalling components. It was assessed by analyzing the CV within triplicate spots of the parameters under study (n=15) in 200 distinct cell extracts from 10 distinct subjects and ranged from 10.3 to 11.4% in the different T cell subsets (**table 2**). Based on the technical error (~11%), changes higher than 20% in the quantification of signalling protein were considered as potentially significant.

T cell subsets	N	CM	EM	E
CV mean (%)	10.6	10.3	11.4	11.0

Table 2. Evaluation of the coefficient of variation in the spot intensity in the different T cell subsets.
A total of 200 triplicate spots and 15 different signalling components were examined for each T cell subset. The means of the coefficient of variation in signal to β-actin ratio ($CV \sim CV_{protein}+CV_{\beta\text{-}actin}$) within triplicate spots were calculated from 10 subjects.

To avoid biases generated by the inter-subject variability, each subject was considered as independent from the others when cumulated analyses were performed. Significant changes in the expression levels of signalling protein between two subsets were calculated within each subject using paired t-test (see RESULTS section 6.1, Fig.18 – individual graphs). Changes were considered as significant when higher than 20% and calculated p-value of two-tailed paired *t*-test was less than 0.05.

Alternatively, in order to represent only the differences identified among the different T cell subsets (having no interference of the inter-subject variability) the data were plotted using a heat-map (see RESULTS section 6.1, Fig.18 – heat-maps). Relative fold increase in signalling protein for each subject was calculated by internally normalizing the measurements obtained in the different T cell subsets to a cell subset used as "normalization subset". In this study, we selected *a priori* as subset for normalization N cells because they have never encounter their cognate antigen and can be considered as precursor of the other cell subsets. Consistently, analysis of our data revealed that N cells displayed the highest inter-subject homogeneity for the majority of signalling component analyzed (see RESULTS section 6.2). Data were subsequently logged in base 2, in order to compensate for the asymmetry introduced by the ratio used in the normalization to N cells (e.g quantification CM / quantification N). $\log_2$ ratios derived from the analysis of different subjects were finally combined by calculating the mean $\log_2$ ratio for each signalling protein and displayed in continuous colour scale on a heat-map (see RESULTS section 6.1, Fig.18 – heat-maps).

5.5 RPP arrays as a tool to profile TCR signalling complex assembly

The functional heterogeneity of memory T cell subsets may result from a different composition of their signalling machinery as well as from a different organization of their TCR signalling complex. Indeed, signalling complexes are highly dynamic cluster of interacting proteins essential for efficient signal propagation from the cell membrane to the nucleus. While the profiling of signalling components in memory T cell subsets can be assessed using RPP arrays as demonstrated in RESULTS section 6.1, the analysis of TCR complex assembly in this cells remains an issue. To date, the most widely used experimental methods to study protein interactions are two hybrid screens (2HS) and mass spectrometry (MS)-based approaches combined to affinity capture. However, MS-based approaches require relatively large amounts of cells (10^8 to 10^9) due to the low abundance and high breakdown of molecular complexes during isolation and purification procedures (Yang, Steen et al. 2008). Since such amounts of cells are impossible to obtain working with *ex-vivo* isolated human memory T cell subsets (e.g. highly purified CM CD8 cells <6 x10^6 cells per donor; EM CD4 cells <1,4 x10^7 cells per donor), we developed an alternative strategy based on RPP arrays to overcome this limitation. By combining small-scale affinity capture of TCR and RPP arrays we reported specific recruitment of signalling proteins to the TCR complex upon T cell activation in as few as 0.5 x10^6 cells. Since this technology has not been used in this thesis project to compare TCR signalling complex assembly in CM and EM cells (see DISCUSSION and PERPECTIVES), I will report in this section only a brief description of the strategy and the main results obtained.

The strategy developed in the laboratory consists of a two-steps procedure. TCR complexes are first captured from a non-denaturing T cell lysate by TCR/CD3ε antibodies covalently immobilized at the bottom of micro-wells (2 mm in diameter) built at the surface of an aldehyde-derivatized glass slides. Following washing out of the contaminants, isolated complexes are then eluted under denaturing condition and arrayed on nitrocellulose-coated slide to allow efficient profiling of their components by RPP arrays (**Fig.16**)

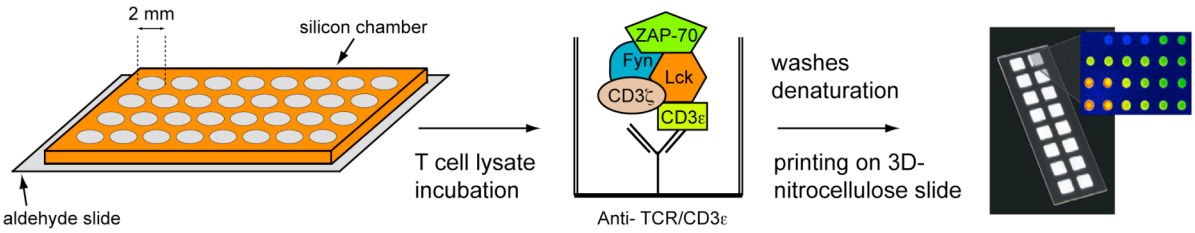


Fig.16 Analysis of TCR-signalling complex assembly.
Silicone rubber manifold was used to generate chambers of 2 millimeters in diameter at the surface of an aldehyde glass slide. Anti-TCR/CD3ε antibodies were covalently linked to the glass surface to capture TCR/CD3 signalling complexes from human CD4 T cell lysates. Immuno-captured TCR complexes were washed free of contaminants, recovered under denaturing conditions and their composition analyzed by RPP arrays.

This approach was validated by profiling the changes in TCR signalling complex composition upon activation in CD4 T cells. To this end, $0.5x10^6$ *ex vivo* isolated CD4 cells from human peripheral blood were subjected to polyclonal stimulation using anti-CD3 antibodies over a period of 20 min. TCR complexes were immuno-isolated and their content in CD3ζ, Src kinases Lck and Fyn, and ZAP-70 kinase was determined by RPP arrays. CD3ε and Hsp-90 were used for normalization and as negative control, respectively. The recruitment of signalling molecules to the TCR complex was measured as the percentage of increase relative to the resting state (time zero, **Fig.17A**). In agreement with the T cell signalling pathway (see INTRODUCTION section 2.3.3), we observed a specific and strong recruitment to TCR/CD3ε of Src (Lck and Fyn) and ZAP-70 kinases. Notably, Hsp-90 spot intensities remained close to background level. Finally, CD3ζ dissociation from CD3ε subsequent to ZAP-70 and Src kinase recruitment (**Fig.17A**) was consistent with the reported ubiquitylation-mediated degradation of TCR/CD3ζ induced by Lck and ZAP-70 activation (Call and Wucherpfennig 2005). Importantly, the whole kinetic analysis required only 2 millions of CD4 T cells. Cumulated data from three distinct subjects showed homogenous results, confirming the reliability of this approach (**Fig.17B**)

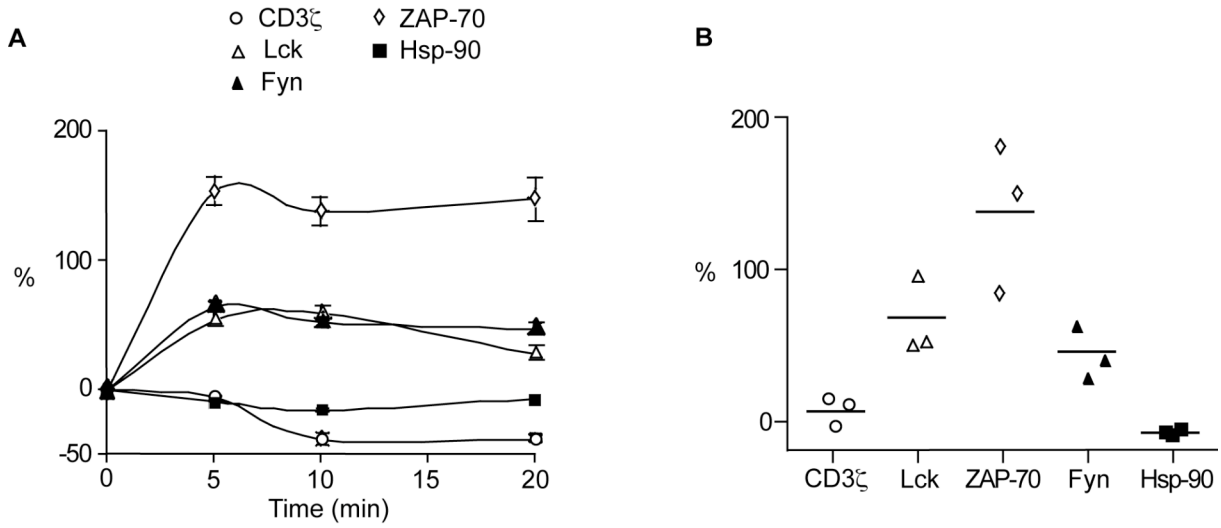


Fig17. Kinetic of TCR-signalling complex assembly upon T cell activation.
(A) Following activation with anti-TCR/CD3ε and -CD28 antibodies over 20 min, CD4 T cells were lysed and their TCR/CD3 complexes captured and analyzed as described in Fig.16. The recruitment of TCR/CD3ζ, Lck, Fyn, ZAP-70 and Hsp-90 to TCR/CD3ε at different time post activation was measured by RPP arrays. Shown is the percentage of increase in intensities normalized to TCR/CD3ε and relative to unstimulated cells (time zero). One representative kinetic out of three is presented. **(B)** Recruitment of signalling molecules to TCR/CD3ε following 5 min of activation as shown in A). Shown are data obtained from three distinct subjects

6. c-Cbl expression regulates the functional response of human central versus effector memory CD4 T cells

The aim of this thesis is to identify the signalling patterns that define the functional heterogeneity of *ex-vivo* isolated human central and effector memory T cells. This functional heterogeneity may derive from a differential regulation of the signalling pathways downstream of the TCR. In order to assess this hypothesis, we first profiled by RPP arrays the expression levels of the main signalling components in these cells. Understanding the mechanisms at the basis of this functional heterogeneity is critically important to better characterize protective immunity and to design efficient vaccine strategies.

At present, few and contrasting studies (mainly done in mouse) have investigated the expression of signalling proteins (Farber, Acuto et al. 1997; Bachmann, Gallimore et al. 1999; Hall, Heffernan et al. 1999; Hussain, Anderson et al. 2002; Krishnan, Warke et al. 2003) and gene transcription (Chtanova, Newton et al. 2005; Willinger, Freeman et al. 2005; Riou, Yassine-Diab et al. 2007) in memory T cell. In humans at the beginning of 2007, lower transcription of proapoptotic FOXO3a target genes have been correlated with enhanced survival of central versus effector memory T cells (Riou, Yassine-Diab et al. 2007). However, no study has yet investigated the expression of signalling proteins in these subsets, mainly due to the limited amount of cells available in human specimens.

Thanks to the implementation of the RPP array technology described in detail in RESULTS section 5, we performed analysis of 15 main signalling components in *ex-vivo* isolated human CD8 and CD4 memory T cell subsets. In contrast to CD8 cells, we showed that the composition of signalling machinery among the CD4 cell subsets differed remarkably. Moreover, we identified for the first time that the functional response of CD4 memory T cell subsets depends on specific variations of critical proteins downstream of the TCR. Indeed, we demonstrated that the adaptor/regulator protein c-Cbl plays a crucial role in modulating the functional responses of effector memory cells through a mechanism mainly dependent on its E3 ubiquitin ligase activity.

The results described in this section are part of a manuscript that has been accepted for publication in **BLOOD**, Journal of the American Society of Hematology (http://bloodjournal.hematologylibrary.org) on April 2008 (see PROJECT AIMS).

6.1 Expression of signalling components in human CD8 and CD4 T cell subsets

To understand how TCR triggering directs different functional outcomes in memory T cell subsets, we first profiled 15 signalling components using RPP arrays. Highly purified (purity >98%) CD8 and CD4 T cell subsets were isolated by cytofluorimetry based on their surface expression of the phosphatase CD45RA and the CC chemokine receptor CCR7, molecules which define their distinct functional and homing capacities (Sallusto, Lenig et al. 1999): naïve (N; $CD45RA^+$, $CCR7^+$) central memory (CM; $CD45RA^-$, $CCR7^+$), effector memory (EM; $CD45RA^-$, $CCR7^-$) and terminally differentiated cells (E; $CD45RA^+$, $CCR7^-$). Cells were lyzed and arrayed as triplicate spots on a slide pad containing a serial dilution of CD4 T cell lysate used to determine the amplitude of the linear range. The number of E CD4 T cells in peripheral blood of healthy donor is extremely limited (0.5 to 1% of CD4 T cells) and thus this population was not investigated. Data processing and statistical analysis of the slide was performed as described in RESULTS section 5.4. Changes in the expression of a signalling component between two cell subsets were considered as significant when higher than 20% and calculated p-value of two-tailed paired *t*-test was less than 0.05. As a control of efficient protein denaturation, we used antibodies recognizing N- and C- terminal end of the Src kinase Lck. Comparable results were obtained in both cases, thus indicating fully denaturing conditions.

The examination of different CD8 T cell subsets in 8 to 13 healthy subjects revealed a comparable and homogenous composition of the signalling machinery with EM and E cells, relatively to CM cells, expressing significantly increased amounts of p38MAP ($p<0.05$) but not of the functionally related ERK1/2 ($p>0.05$, **Fig.18A**). Although PLCγ and Fyn expression increased consistently from CM to EM cell subsets ($p<0.05$), the amplitudes of these increases were close to 20% (19.5 to 20.1%) and therefore, based on our criteria, not further investigated (**Fig.18A**, left panel and data not shown). Taken together, our data showed that CM cells were significantly more closely related to N cells than EM or E cells (**Fig.18A**), in agreement with their gene expression and cytokine signalling profiles (Willinger, Freeman et al. 2005).

In contrast, CD4 memory T cell subsets showed more profound changes in the expression levels of specific signalling components. Indeed EM cells expressed significantly increased amount of SLP-76 ($p<0.05$) and reduced levels of c-Cbl ($p<0.001$), Syk ($p<0.001$), Fyn ($p<0.01$) and LAT ($p<0.05$) as compared to CM cells (**Fig.18B**). Importantly, three out of the five proteins showing significant changes in their expression levels are adaptor molecules: c-Cbl, SLP-76 and LAT. The largest differences were observed for Syk (-46%) and these adaptor molecules (-33 to -35%, **Fig.18B**). Finally, the changes in the expression of Lck and PI3K were lower than 20% and not significant, respectively.

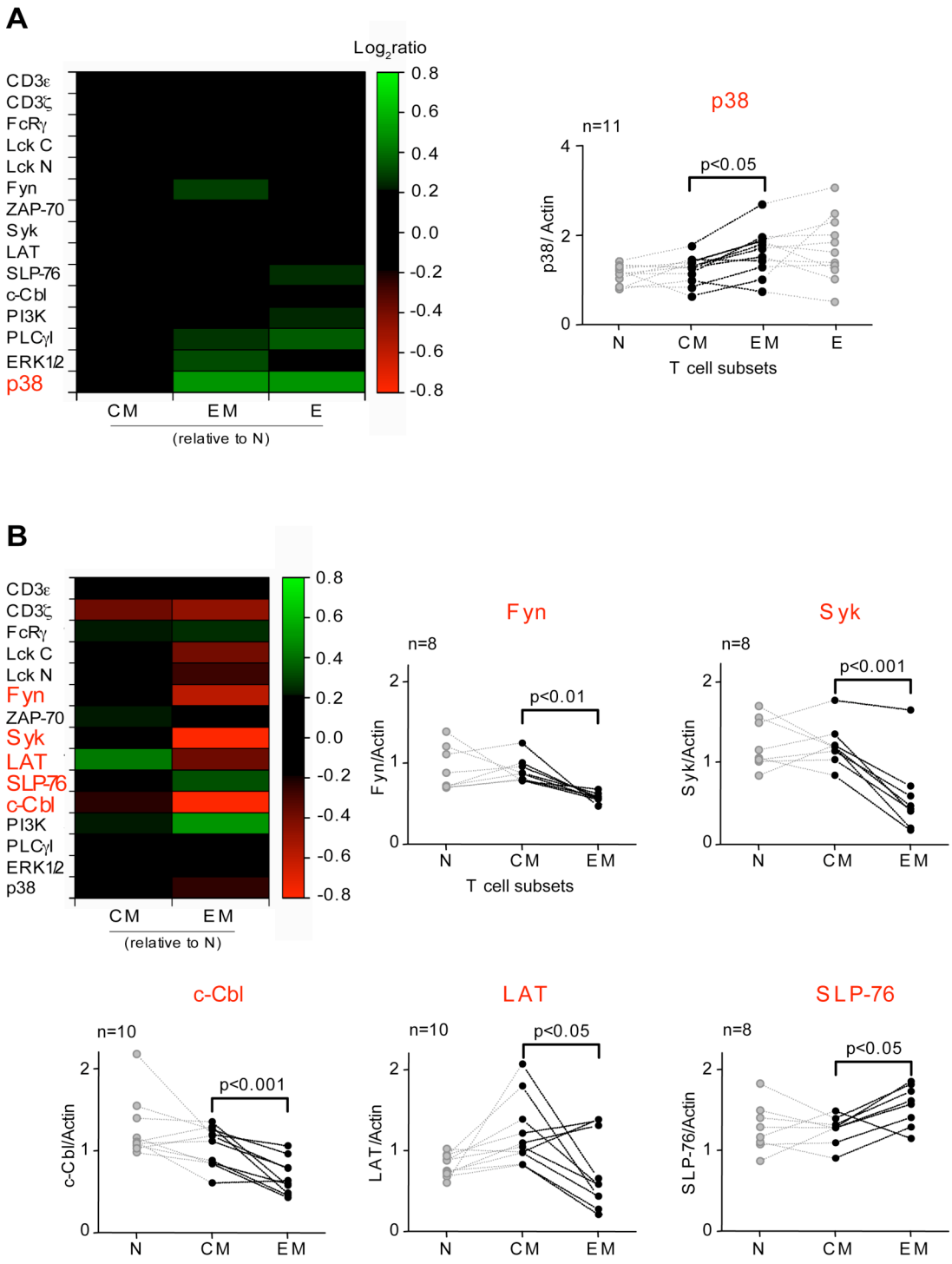


Fig.18 Analysis of the signalling components in CD8 (A) and CD4 (B) T cell subsets.
Signalling components were quantified by RPP arrays and normalized to β-actin in the different T cell subsets. The colours indicate the mean $\log_2$ ratios of signalling components relative to N cells from 8 to 13 subjects. Signalling components showing significant differences (two-tailed paired t-test $p<0.05$, shown in red) between CM and EM cells are represented as row ratios on individual graphs. CM and EM compartments are shown in black. Each set of linked dots correspond to a distinct subject and n indicates the total number of subjects examined. Lck was detected using specific antibodies recognizing epitopes located at the N- and C-terminal ends of the protein (Lck N and Lck C, respectively) to ensure fully denaturing conditions.

6.2 Heterogeneity in the expression of the signalling components among subjects in CD4 and CD8 T cell subsets

We observed that the variations in the expression of signalling components among the subjects were higher than the technical error (Figure 3, median 21.5-28.5% > technical error 11%) and are thus likely to represent inter-subject heterogeneity. Furthermore, a limited number of signalling components showed drastically high inter-subjects heterogeneity (up to 63%): LAT, Syk and CD3ζ in memory CD4 T cells (**Fig.19A**); ZAP-70, SLP-76 and PLCγI in memory CD8 T cells (**Fig.19B**). These variations were independent of the signalling parameter examined and moreover restricted to specific T cell subsets such as observed for CD3ζ and Syk in EM, but not CM CD4 T cells (**Fig.19A**). In memory CD4 cells, subject heterogeneity increased from CM to EM cells along CD3ζ/Syk/LAT pathway (**Fig.19A**). In CD8 T cells, overall subject heterogeneity increased with cell differentiation with signalling components being significantly ($p<0.05$) more homogenous in N than in memory and effector cells (see median lines in **Fig.19B**). The expression of SLP-76 and PLCγ among the subjects was highly heterogenous in both memory CD8 T cell subsets while heterogeneity in ZAP-70 expression increased from CM to EM cells (**Fig.19B**). Finally, Lck was the most homogeneously expressed protein among subjects in all CD4 and CD8 T cell subsets (data not shown).

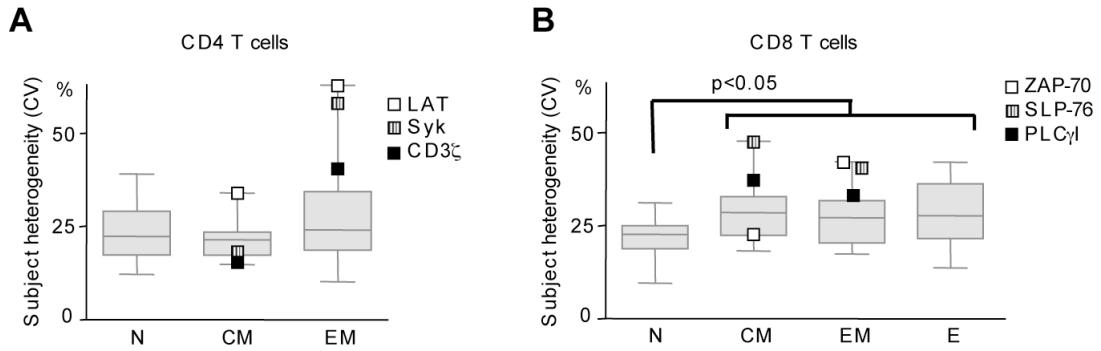


Fig.19. Subject heterogeneity in the expression of signalling components in CD4 and CD8 T cell subsets. Subject heterogeneity as expressed by CV of the 15 signalling components (n=15) under study in 8-13 cumulated subjects in CD4 **(A)** and CD8 **(B)** T cell subsets. Box plots were automatically generated using GraphPad, the box represents values between 25th and 75th percentile with a line at the median (50th percentile). The whiskers extend above and below the box to show the highest and the lowest values. Shown are signalling components with highest subject heterogeneity in CM or EM cells.

6.3 In CD4 EM T cells, reduced expression of c-Cbl correlates with LAT, SLP-76 and PI3K content

Examination of c-Cbl expression levels in 10 subjects by RPP arrays revealed a 34% reduction in EM as compared to CM CD4 T cells ($p<0.001$, **Fig.18B**, **20A**). c-Cbl immunoblots of T cell lysates from four different subjects confirmed the result obtained by RPP arrays (**Fig.20A** and **C**). Importantly, in contrast to c-Cbl, CM and EM cells showed similar levels of Cbl-b expression (homologue of c-Cbl) (**Fig.20D**). Cbl proteins are multifunctional adaptor proteins acting as key negative regulators of T cell signalling mainly through an ubiquitin-dependent mechanism (see INTRODUCTION section 2.3.5.1).

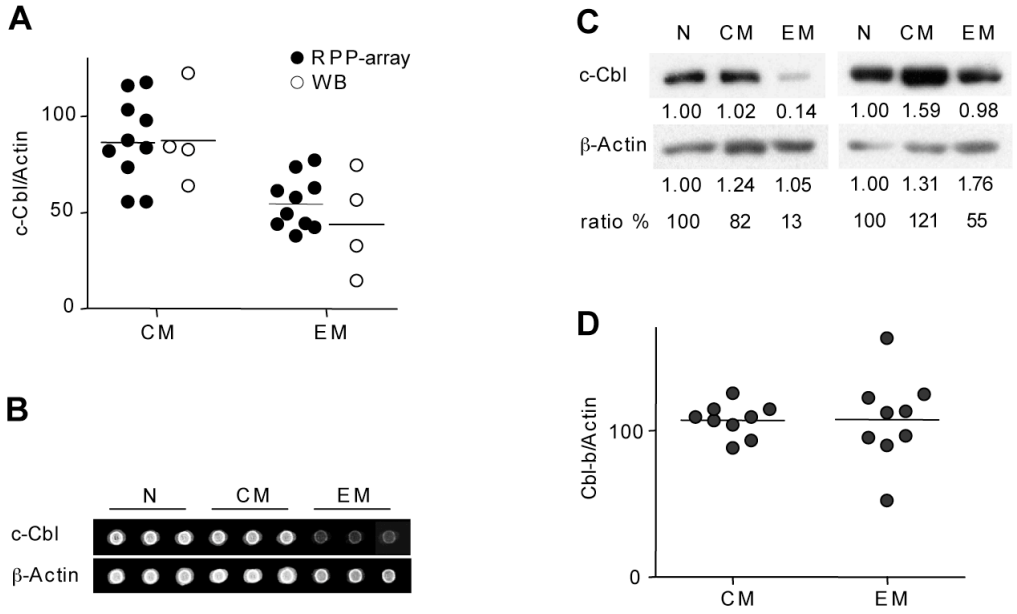


Fig.20 c-Cbl, but not Cbl-b expression is reduced in EM CD4 T cells.
(A) Quantification of c-Cbl expression levels in CM and EM cells by RPP arrays and immunodetection of Western Blots with antibodies specific for c-Cbl and actin. Shown are c-Cbl to actin ratios in 10 (RPP arrays (●), 30 cells equivalent/spot) or 4 (WB (O), 300 000 cells equivalent/lane) distinct subjects**.** Examples of detected spots **(B)** and quantified immunodetections **(C)** are shown. **(D)** Quantification of Cbl-b by RPP arrays in nine subjects as described in A.

To understand how c-Cbl impacts memory T cell signalling, we compared its expression to the other signalling proteins. We observed a linear correlation of the expression of c-Cbl with the adaptors SLP-76 and LAT (Pearson r=0.78, p=0.022 and r=0.92, p=0.0001 respectively) and PI3K (r=0.84, p=0.0084), suggesting that these proteins might be co-regulated (**Fig.21A**). Interestingly, these correlations were specific to EM cells and restricted to c-Cbl and SLP-76 in CM cells (r=0.89, p=0.0028, **Fig.21A**, first panel). Among the signalling parameters examined, only one additional significant correlation was observed between CD3ε and ζ in CM (r=0.83, p=0.0056, **Fig.21B**) and N cells (r=0.95, p=0.000019, data not shown), consistent with their dimerization in the TCR complex. This was not the case in EM cells (**Fig.21B**) that, as has been reported for effector cells (Krishnan, Warke et al. 2001), may regulate their ζ chain independently.

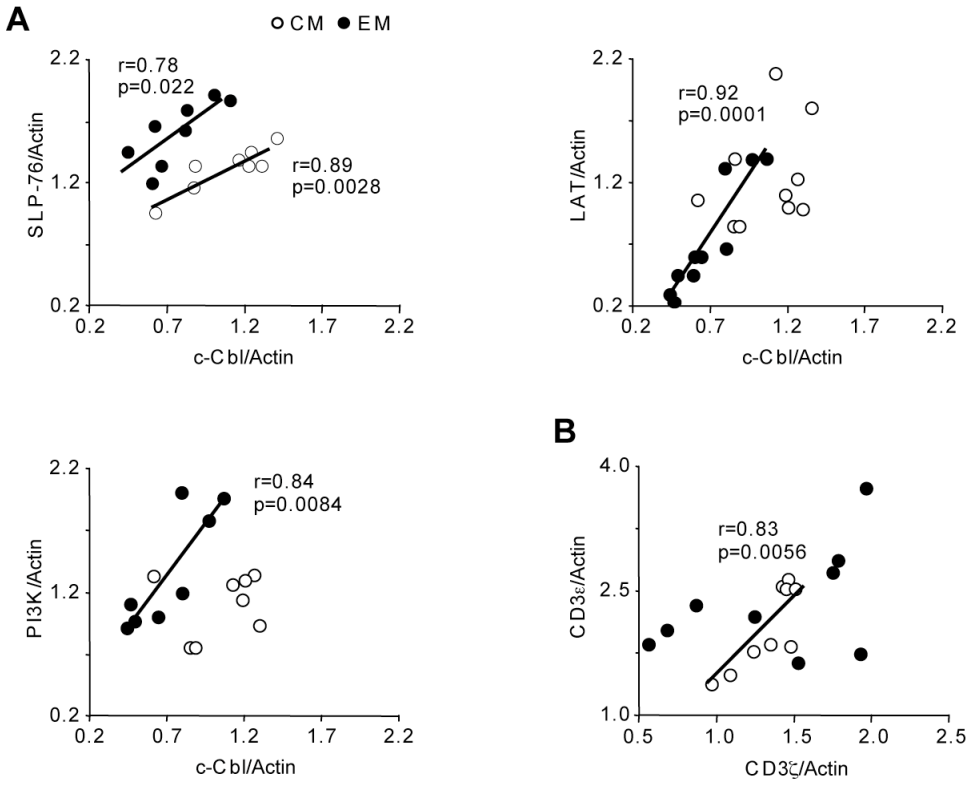


Fig.21 In EM cells, SLP-76, LAT, PI3K and c-Cbl expression are co-regulated.
(A) Correlation of the expression of SLP-76, LAT, PI3K with c-Cbl in 8 to 10 distinct subjects. The significance of their linear correlation is shown (Pearson r and p value<0.05). **(B)** Correlation of the expression of TCR/CD3 ε and ζ components as described in A.

6.4 EM CD4 T cells show a lower functional threshold and differentially regulate TCR-mediated signals as compared to CM cells

EM CD4 T cells display immediate effector functions (Sallusto, Lenig et al. 1999) and produce IL-2 and IFN-γ in response to polyclonal stimulation with TCR/CD3 and CD28-specific antibodies. In contrast, CM cells lack immediate effector functions and produce limited amounts of these cytokines, detectable only at high levels of TCR triggering (**Fig.22A, left panel**). In EM cells, CD28 co-stimulation enhanced significantly IL-2, but not IFN-γ production, while CD4 cross-linking had little effect (**Fig.22A, right panel**). Highly purified memory CD4 T cell subsets (purity>98%) were analyzed for their ability to proliferate following six days of polyclonal stimulation. EM cells were consistently more responsive and showed a lower threshold for proliferation than CM cells (**Fig.22B, left panel**). High level of TCR triggering (10 μg/ml of anti-CD3 antibodies) in the presence of co-stimulation (1 μg/ml of anti-CD28 antibodies) induced proliferation of 20% of CM cells after six days (**Fig.22B, left panel**) consistent with low but detectable number of IL-2 producing cells following one night stimulation (**Fig.22A, left panel**). Under these conditions of stimulation (10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 antibodies) roughly four times more EM cells were proliferating and producing IL-2 (**Fig.22A and B, left panels).** Proliferation of both cell subsets was highly dependent on CD28 co-stimulation. However, CD28 co-stimulation and IL-2 impacted significantly stronger CM than EM cells (**Fig.22B, right panel**). At lower level of TCR triggering, this effect was even stronger with IL-2 inducing a 9.3 and 1.8 fold increase in CM and EM cell proliferation, respectively (data not shown). In contrast, the analysis of 5 subjects revealed no significantly different intracellular calcium mobilization in CM versus EM cells (**Fig.22C**) upon TCR and CD28 crosslinking (CM: 47.8% +/- 11.5 and EM: 38.8%+/-9.5, n=5, p=0.102).

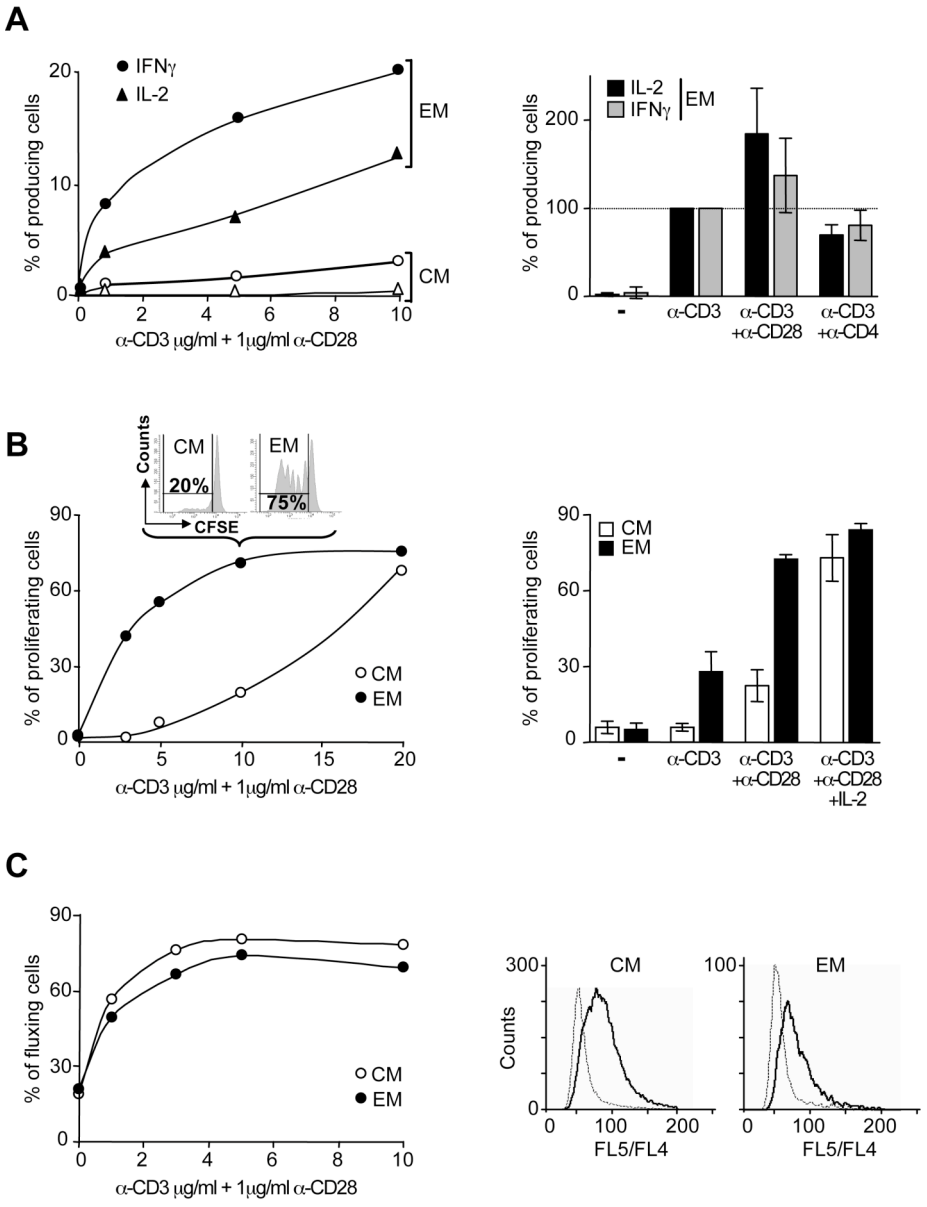


Fig.22 Functional threshold in CM and EM CD4 T cells.
(A) Detection by FACS of intracellular production of IFN-γ and IL-2 in memory cells exposed for one night to graduated concentrations of immobilized anti-CD3 and 1μg/ml soluble anti-CD28 antibodies. Shown is one representative response out of four (left panel). Cumulated data of EM cells from four subjects in response to 5 μg/ml of immobilized anti-CD3 antibody supplemented with 1 μg/ml soluble anti-CD28 or -CD4 antibodies are shown (right panel). **(B)** Proliferative potential of memory T cells measured by CFSE dilution in sorted CM and EM cells stimulated for six days as described in A (left panel). Shown are cumulated data of four subjects in response to 5μg/ml of immobilized anti-CD3 antibody supplemented with 1 μg/ml soluble anti-CD28 antibody in the presence or absence of IL-2 (right panel). **(C)** Intracellular calcium mobilization in CM and EM cells in response to graduated concentrations of soluble anti-CD3 and 1μg/ml soluble anti-CD28 antibodies (left panel). The percentage of cell showing changes in intracellular calcium concentration was assessed by measuring variations of FL5/FL4 ratio (right panel).

Finally, in marked contrast to double positive thymocytes (Thien and Langdon 2005; Myers, Sosinowski et al. 2006) and consistent with observations in peripheral CD4 T cells from c-Cbl KO mice (Thien and Langdon 2005), the expression of TCR/CD3, CD4 or CD28 at the surface of EM cells was not increased relative to CM cells (**Fig.23**). These observations indicate that CM and EM cells differentially regulate signalling pathways downstream the TCR.

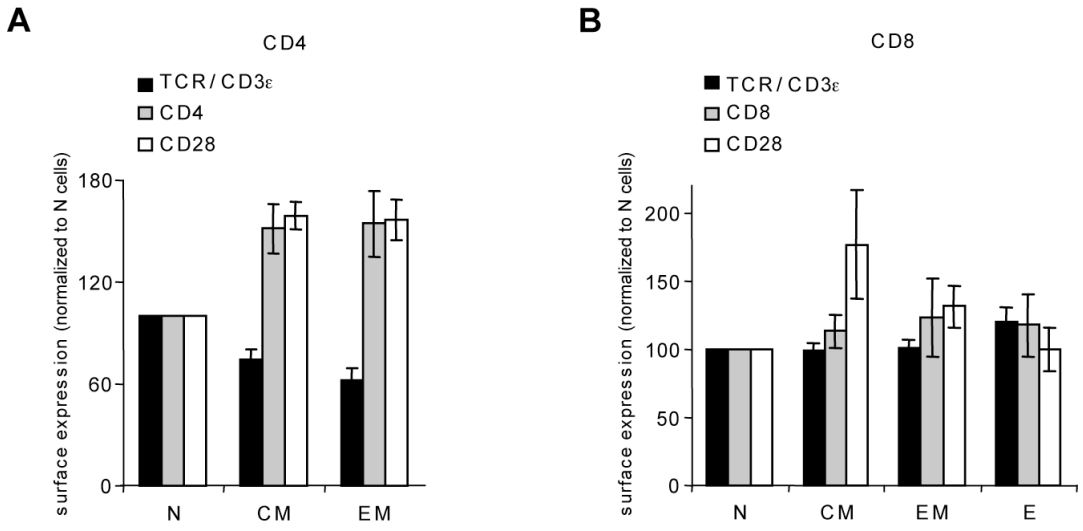


Fig.23 Surface expression of TCR/CD3ε, CD28 and co-receptors in memory T cell subsets.
PBMC were stained with CCR7 and CD45RA and the expression of co-receptors, CD28 and TCR/CD3ε measured by FACS at saturating concentrations of antibodies in the different CD4 **(A)** or CD8 **(B)** T cell subsets. Shown are cumulated data of seven subjects relative to N cells.

6.5 c-Cbl expression controls the functional threshold of EM CD4 T cells

6.5.1 Cytosolic transduction to alter c-Cbl content of memory CD4 T cells

Consistent with their reduced expression of the negative regulator c-Cbl (**Fig.18B and 20A**), EM cells show a lower threshold for activation (**Fig.22A and 22B, left panels**) relative to CM cells. These observations suggest that c-Cbl expression may control the functional response of memory cells. In order to verify this hypothesis, we increased c-Cbl content of EM cells and measured their functional responses. *Ex vivo* isolated T cells are refractory to

transfection and modulation of gene expression through viral infection remains suboptimal. Furthermore, the strong and sustained cell activation required for viral infection interferes with memory T cell differentiation and is incompatible with our study. To circumvent these limitations, we took advantage of the recently described cytosolic transduction peptide (CTP:YGRRARRRRRR) (Kim, Jeon et al. 2006). In particular, CTP peptide is derived by specific mutations of the protein transduction domain (PTD) of HIV TAT protein in order to induce a conformational change that inhibits nuclear transport without affecting the membrane transduction properties specific of TAT peptide (coil-structure responsible for importin-α binding converted in a more rigid α-helix unable to bind importin-α). Resulting CTP peptide promotes the delivery of CTP-fused protein selectively to the cytosol (Kim, Jeon et al. 2006).

CMV promoter-based expression vectors coding for CTP-GFP, CTP-c-Cbl (wt and C381A mutant) and a control CTP-fusion proteins (CTP-mock) were constructed in the laboratory (see MATERIALS AND METHODS for details). The C381A substitution in the RING domain of c-Cbl abolishes its interaction with E2 conjugating enzymes, thus preventing c-Cbl from functioning as an E3 ubiquitin ligase without affecting its association with signalling molecules (Joazeiro, Wing et al. 1999). Large-scale expression of CTP-fusion proteins in Hek 293 cells was performed by the protein expression facility of the EPFL (Lausanne, Switzerland). CTP-proteins were purified from $3.2x10^8$ cells/prep by nickel-affinity chromatography through recombinantly added N terminal 6xHis (CTP-mock and -GFP) or endogenously present 7xHis (aa36-42 in c-Cbl proteins, **Fig.24**). Both memory T cell subsets were transduced with comparable efficiency as assessed by transduction of low amounts of CTP-GFP (1.25 and 1.3 fold increase respectively, **Fig.26A**) or CTP-c-Cbl constructs (data not shown).

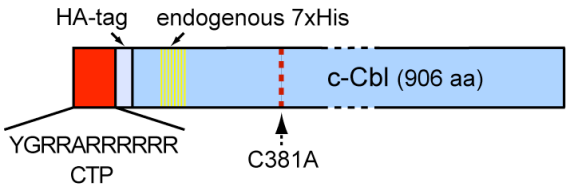


Fig.24 Structure of recombinant CTP-c-Cbls. Positions of CTP peptide, HA-tag, endogenous 7xHis-tag and C381A mutation are shown.

6.5.2 Impact of c-Cbl on CD4 memory T cell functions

To examine the impact of c-Cbl on the functional responses of EM cells, memory cells were transduced with wt and C381A mutant CTP-c-Cbls and stimulated with anti-CD3 and -CD28 antibodies at concentrations (5μg/ml anti-CD3 and 1μg/ml anti-CD28) inducing EM but only limited CM cell functional responses (**Fig.22A, 22B and 26C**). Transduced wt and C381A mutant CTP-c-Cbls remained intact over the duration of T cell activation (**Fig.26B**) and showed comparable kinetics of degradation (data not shown). Furthermore while in EM cells the expression levels of Syk, LAT, SLP-76 and PI3K was not altered by CTP-c-Cbl transduction, TCR and CD28 triggering increased SLP-76 expression by 45% (**Fig.25**).

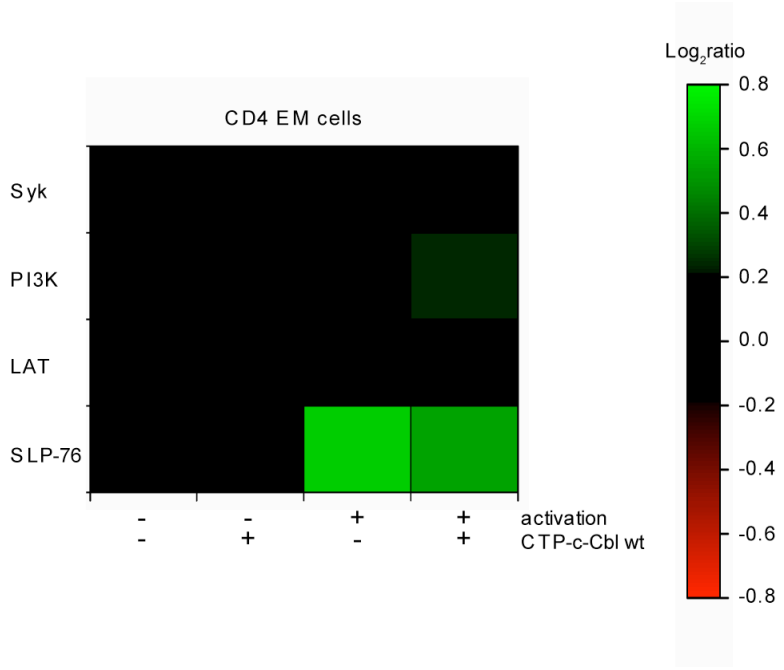


Fig.25 Expression of signalling molecules in presence of CTP-c-Cbl transduction.
Following transduction with CTP-c-Cbl, purified EM cells were stimulated with α-CD3 (5 μg/ml) and α-CD28 (1 μg/ml) antibodies for 16 hours. Signalling components were quantified by RPP arrays and normalized to β-actin. Colours indicate the mean log2 ratios of signalling components relative to resting non-transduced EM cells in 3 distinct subjects.

Transduction of EM cells with CTP-c-Cbls impaired significantly their proliferation in a dose dependent manner whereas CTP-mock had no effect (**Fig.26C**). However, wt CTP-c-Cbl was 3-fold more potent ($p<0.05$) than C381A mutant (**Fig.26C**). Importantly, transduction of EM cells with CTP-c-Cbl increased their threshold for proliferation (**Fig.26E**) and their sensitivity to IL-2 (**Fig.26F**) thus restoring, at least partially, the functional phenotype of CM cells (**Fig.22B**, right panel).

In addition, wt CTP-c-Cbl specifically and significantly impaired IFN-γ and IL-2 production by 46% and 40%, respectively, while C381A mutant was significantly less efficient ($p<0.05$, **Fig.26D**). In contrast to cell proliferation, cytokine production was not further lowered by increasing the amount of wt CTP-c-Cbl transduced in memory cells (data not shown). Remarkably, these effects were obtained by increasing c-Cbl content of EM cells using CTP-c-Cbl transduction (+55% in wt or C381A mutant CTP-c-Cbls, **fig.26G**) to that of CM cells (+34% average increase in CM cells as compared to EM cells, **fig.18B and 20A**) thus matching c-Cbl content in CM and transduced EM cells (data not shown). These data demonstrate that c-Cbl expression controls the functional phenotype of memory T cells through a mechanism mainly dependent of its E3 ubiquitin ligase activity, as evidenced by the weaker impact of enzymatically deficient c-Cbl C381A on EM cell functions.

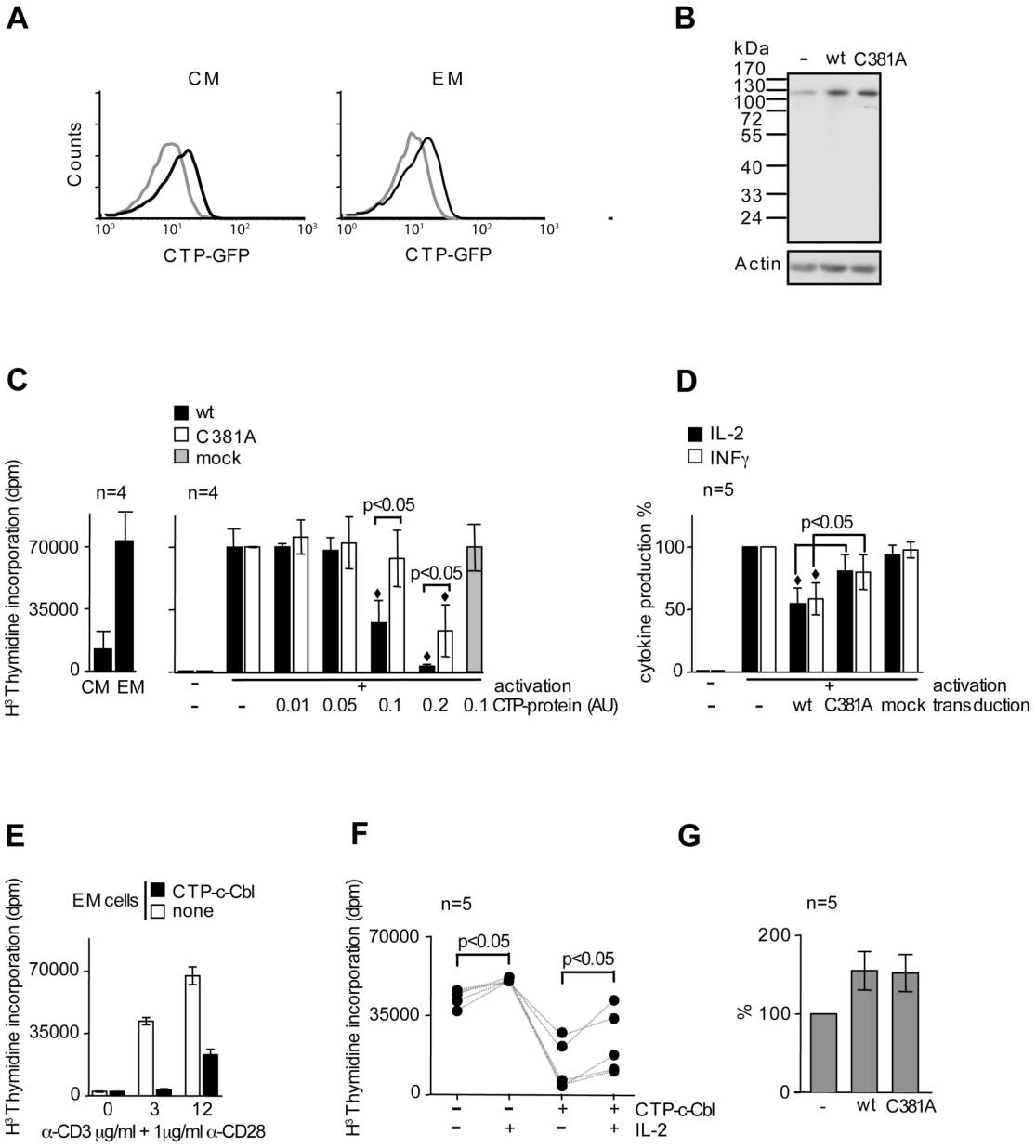


Fig.26 Transduction of CTP-c-Cbl in EM cells results in lower functional response.
(A) Limited amounts of CTP-GFP were transduced in memory cells and the transduction efficiency in CM and EM cells assessed by FACS. Thin and bold lines depict non-transduced and transduced cells, respectively. **(B)** Detection of c-Cbl following SDS-PAGE and immunoblotting of lysates of memory CD4 T cells previously transduced with wt or C381A CTP-c-Cbls and activated over night with anti-CD3 (5 μg/ml) and -CD28 (1 μg/ml) antibodies. **(C)** Proliferative capacities of CM and EM cells as measured by H^3 thymidine incorporation (left panel). Memory cells were transduced with increasing amount of wt and C381A CTP-c-Cbls fusion proteins (AU: arbitrary units), stimulated for 16 hours with anti-CD3 (5 μg/ml) and -CD28 (1 μg/ml) antibodies and their proliferation measured after three days. CTP-mock transduction was used as negative control (grey bar). Shown are data from four cumulated subjects (right panel). **(D)** Effect of CTP-c-Cbls transduction on memory T cell cytokine production in response to the stimulation described in C. Cytokine production was normalized to non-transduced cells and CTP-mock transduction used as negative control. Significant changes ($p<0.05$) in the functions of transduced cells relative to control and mock-transduced cells are indicated (✦) as well as p values for significant differences between wt and C381A mutant c-Cbls. **(E)** Effect of CTP-c-Cbl on the proliferation threshold of purified EM cells stimulated as described in C. One representative example out of three is shown. **(F)** Impact of CTP-c-Cbl transduction on the proliferation of EM cells in response to IL-2, anti-CD3 (3 μg/ml) and -CD28 (1 μg/ml) antibodies. Each set of linked dots correspond to a distinct subject and significant changes ($p<0.05$) between IL-2 treated and untreated cells are shown. **(G)** Quantification of the amounts of CTP-c-Cbls transduced in memory T cells relative to endogenous expression of c-Cbl following 16 hours of activation.

DISCUSSION & PERSPECTIVES

7.1 General discussion

The aim of this is the identification of the mechanisms responsible for the functional heterogeneity that characterizes human central (CM) and effector (EM) memory T cell subsets (Sallusto, Lenig et al. 1999). Since the surface expression of TCR/CD3 and co-stimulatory receptor CD28 is comparable in the two cell subsets (**Fig.23**), we hypothesized that this functional heterogeneity derives from a differential use/regulation of the TCR signalling pathway. In particular, a different composition of their signalling machinery and/or a different assembly of their TCR-associated signalling complexes could be the basis of their heterogeneity. While the first aspect has been successfully addressed during this thesis, the second one remains under investigation. Indeed, although we developed a highly sensitive and quantitative system for profiling TCR signalling complex assembly (small scale affinity capture-RPP arrays, RESULTS section 5.5), we could not yet apply this methodology to CM and EM cells due to technical limitations related to their isolation procedure. Since FACS sorting is a long isolation procedure that is likely to interfere with memory T cell signalling (data not shown), cell isolation strategies based on beads were preferred for signalling assembly studies. However, to date no commercial systems (not interfering with cell activation and RPP arrays) reached a satisfactory level of purification. Alternative approaches are currently under investigation in collaboration with the EPFL (Lausanne, Switzerland).

In this study we report for the first time accurate *ex vivo* quantification of signalling components in human memory T cell subsets and demonstrate that the functional response of these subsets depends on specific variations in the expression levels of signalling proteins downstream of the TCR. Indeed, we demonstrate that the adaptor/regulator c-Cbl plays crucial roles in modulating the functional responses of EM cells through a mechanism mainly dependent on its E3 ubiquitin ligase activity.

RPP arrays: a quantitative approach for profiling signalling proteins in memory T cell subsets

Efficient protein profiling of *ex-vivo* isolated human memory T cells has been possible due to the implementation of RPP array approach. Indeed, this technology posses sensitivity and performance characteristics that are largely beyond what traditional technologies (WB, Beads

suspension array) can reach. Moreover, the high degree of sensitivity, reproducibility and linearity achieved (**Fig.14 and table 1**), allowed excellent quantification of variations greater than 20% in protein modification or expression in as few as 20 cell equivalent per spot. The technical error, reflected by the normalized CV (normalized data $CV_{signalling\ protein}$+CV_{actin}), was in the range of 11%. RPP array format displays the following technical advantages: i) it enables multiple sample to be analyzed under the same experimental conditions, thus simplifying quantitative comparison, ii) it allows inclusion of triplicate (or more) samples and dilution series in the same pad increasing robustness, accuracy and reproducibility of protein quantification and iii) it drastically reduces amounts of biological material, reagents, time and costs.

Compared to previous studies (Paweletz, Charboneau et al. 2001; Chan, Ermann et al. 2004; Tibes, Qiu et al. 2006), we improved the overall quality of the spots (smaller size, better circularity and higher reproducibility in spot intensity) without affecting the sensitivity, the dynamic range and the linearity of the detection. These improvements resulted in higher throughput potential and more accurate quantification.

In addition, we implemented RPP array approach with respect to solubilization of lipid raft (Doucey, Legler et al. 2001) and efficient protein retention on 3D nitrocellulose in presence of detergent (**Fig.13**). The inconsistency observed between solubilization efficiency (**Fig.13B**) and RPP spot signal intensity (**Fig.13A**) using three distinct buffers (TX-, TX/SDS- and OG-containing buffers) confirmed that protein retention on 3D nitrocellulose substrate was heavily biased towards the detergent properties. OG-containing buffer was not affecting protein retention in the range of working protein concentration and resulted in better linearity and reproducibility relative to TX and TX-SDS buffers (**Fig.13**). The inclusion of TX in TX/SDS-containing buffer was necessary to decrease lysate viscosity and to prevent precipitation of SDS protein lysate at the temperature used for printing (<23˚C). Moreover, the implementations here discussed apply for the first time to human T cells isolated *ex-vivo*.

Only 0.7 nl of crude protein cell lysate was printed per spot, corresponding to approximately 2-20 ng of proteins. However, the printing robot requires handling of much larger volume (order of µl) due to limiting compatibility between the pin and the geometry of the sample-containing plate. Using pyramidal 384-well plate, we have already reduced the minimal workable volume to 3 µl. New types of plate are currently under investigation in the

laboratory. Remarkably, the sample amount required for the quantification a typical signalling protein by immunodetection of Western Blot (~300000 cells) was sufficient to print more than 4000 spots.

In order to profile proteins in such limited amounts of biological samples (2-20 ng/spot), a CARD amplification reaction has been introduced. Although this strategy is considered state of the art for RPP array detection, the amplification chemistry makes dual detection on the same spot difficult (data not shown) and limits multiplexing. Indeed interaction of biotinylated residues with fluorescent streptavidin (**Fig.12B**) produces a protein scaffold epitope masking of the lysate. Direct detection based on quantum dots (Geho, Killian et al. 2007) have been applied to overcome this limitation. Nevertheless, arrays can be printed in a multisector format (many pads identically printed in the same slide), allowing up to 16 analytes to be analyzed in the same samples at the same time. Remarkably, the inter-pad variability was in the same range of the inter-spot variability, making replicated pads almost identical (considering the technical error).

Housekeeping proteins are commonly used to normalize protein spot content, although some evidence supports that their expression *in vivo* are influenced by physiological and pathological factors (Ferguson, Carroll et al. 2005; Dittmer and Dittmer 2006). For that reason, quantification of total protein is often preferred for protein normalization (Winters, Dabir et al. 2007). The choice of actin as normalization protein in this study was justified by the absence of total protein quantification method compatible with the RPP array equipment available at the time when this project started. Furthermore, the use of actin but not β2-microglobulin (**Fig.15**) nor tubulin (data not shown), has been validated by WB and RPP arrays in the cell subsets under study. Future effort will be addressed to validate total protein staining method such as colloidal gold (Winters, Dabir et al. 2007) and Sypro Ruby (Grubb, Calvert et al. 2003) as possible normalization means. However, activation-induced degradation of specific signalling components such as observed for ZAP-70 (Penna, Muller et al. 1999) represents a severe limitation to these total protein normalization approaches when phosphorylation state are profiled. In these studies, normalization to the pan-specific form of the phosphorylated target signalling protein are preferred. Ideally, a normalization strategy would allow reversible stain of the normalization protein coupled to detection of target signalling protein on the same spots, thus further reducing the technical error.

The major limitation of RPP array technology lies in its dependency on antibody availability. However, most of the antibodies suitable for western blotting immunodetection work in this format, provided that they have no cross-reactivity with other protein in the sample analyzed (50-70% of the Abs). Moreover this technology requires the handling of highly purified cell populations.

c-Cbl plays critical roles in controlling the functional heterogeneity of human memory CD4 T cell subsets

RPP arrays have been used in this thesis to profile the abundance of 15 relevant signalling proteins downstream of the TCR in human CD4 and CD8 memory T cell subsets (see RESULT section 6.1). Although CD8 memory subsets had a homogenous composition of their signalling machinery, it is worth emphasizing that CM cells appeared more closely related to N than EM or E cells (**Fig.18A**), in agreement with their gene expression and cytokine signalling profiles (Willinger, Freeman et al. 2005). In contrast to CD8 cells, CD4 memory T cell subsets showed more profound changes in the expression levels of specific signalling components.

The lineage relationship between the different T cell subsets remains controversial. Our study could not directly address this relationship since was not focussed on a precise antigen-specific population but examined the whole (heterogeneous) repertoire of central and effector memory cells. However, despite the relatively small number of molecules analyzed, our findings for both CD8 and CD4 cells suggest that CM cells may represent an intermediate population in the transition from N to EM cells. Indeed, the expression levels of the majority of molecules analyzed were gradually regulated along the N-CM-EM differentiation pathways while none was truly cell subsets-specific. These observations could be consistent with the progressive differentiation model proposed by Lanzavecchia and colleagues (low Ag dose: N-non effector-CM, high Ag dose: N-effector-EM, see INTRODUCTION section 1.2.3.2) (Lanzavecchia and Sallusto 2002).

In CD4 EM cells, reduced expression of c-Cbl was significantly associated with lower expression of c-Cbl kinases (Fyn and Syk) and altered expression of the TCR key adaptors LAT and SLP-76 (**Fig.18B**). Of note, the expression levels of the related kinases Lck and ZAP-70 (functionally homologous to Fyn and Syk, respectively) that do not phosphorylate c-Cbl, remained unchanged. This selective regulation may be a mechanism to further impairs c-Cbl functions in EM cells by decreasing its phosphorylation and its E3 ligase activity.

However, this hypothesis remains to be investigated (see DISCUSSION and PERSPECTIVES section 7.2). Of note, increased expression of Syk in human CD4 effector cells differentiated *in vitro* was found to be associated with TCR signalling through FcRγ/Syk complex in place of CD3ζ/ZAP-70 in naïve cells (Krishnan, Warke et al. 2003). Along the same lines, ZAP-70 deficient patients expressing high level of Syk show impaired CD3-mediated proliferation and IL-2 secretion (Noraz, Schwarz et al. 2000).

In addition, our data showed that exclusively on EM cells the expression level of c-Cbl correlates with that of PI3K, SLP-76 and LAT (**Fig.21A**), thus suggesting that the two memory subsets may possess a different organization of their signalling machinery. Of interest, inactivation of c-Cbl was reported to alter the functions of PI3K (Jeon, Atfield et al. 2004), SLP-76 (Carpino, Turner et al. 2004) and LAT (Noraz, Schwarz et al. 2000; Carpino, Turner et al. 2004) and consequently to interfere with T cell development and activation.
Importantly, the differences identified in the expression levels of these signalling proteins are likely to derive from a post-transcriptional regulation. Indeed, the transcription of the corresponding genes was not reported as significantly altered in CD4 memory T cell subsets (Chtanova, Newton et al. 2005; Riou, Yassine-Diab et al. 2007).

Cumulated analysis of the data revealed that the inter-subjects heterogeneity was increasing in both CD4 and CD8 cells with cell differentiation. Moreover, we observed a marked high inter-subject heterogeneity which was dependent on precise signalling component/T cell subset combination (**Fig.19**). This is likely to reflect inaccurate cell subset delineation based on partial phenotypical and functional correlates. The inclusion of CCR7 as a subset marker is justified due to the very clear correlation with cytokine secretion (Sallusto, Lenig et al. 1999; Geginat, Sallusto et al. 2001; Geginat, Sallusto et al. 2003). However, more recently CD4 cells were shown to be distributed among six major compartments with EM cells ($CD28^+$, $CCR7^-$, $CD45RA^-$) having the greatest capacity to produce IL-2, IFN-γ and TNF-α in response to Staphylococcal Enterotoxin B (SEB) stimulation and showing proliferation when co-cultured with autologous monocytes and exposed to PHA (Amyes, McMichael et al. 2005).

In keeping with previous studies (Sallusto, Lenig et al. 1999; Geginat, Sallusto et al. 2001), our data showed that in response to CD3 and CD28 stimulation, EM cells have a higher capacity for cytokine production and a lower threshold for proliferation than CM cells (**Fig.22**). The latter observation is not in contrast with the higher proliferative capacity (i.e. expansion potential) of CM as compared to EM cells (Sallusto, Geginat et al. 2004). Moreover, CM cells produced IL-2 in response to EBV or CMV antigens (Amyes,

McMichael et al. 2005; Harari, Vallelian et al. 2005) or when triggered with anti-CD3 antibodies and PHA (Sallusto, Lenig et al. 1999), but not in response to anti-CD3 and anti-CD28 antibodies (**Fig.22A**). These results indicate that IL-2 production in CM cells may require additional triggering to TCR and CD28. The significance of the increased SLP-76 expression following CD3/CD28 stimulation in EM cells (Fig.24) remains to be investigated, but underline the dynamicity and plasticity of the TCR signalling pathway in activated cells.

Our results are in marked contrast with CD4 peripheral T cells from c-Cbl KO mice, which show a higher functional threshold and are likely to arise from altered thymic selection and to reflect unbalanced c-Cbl and Cbl-b expression (Naramura, Jang et al. 2002). While c-Cbl and Cbl-b have been reported to be functionally redundant (Naramura, Jang et al. 2002), their specific substrates are cell type dependent and their regulatory effects controlled by their relative abundance (Dikic, Szymkiewicz et al. 2003). Indeed, the functional phenotype of c-Cbl KO thymocytes and peripheral cells differed dramatically, consistent with high expression of c-Cbl in thymocytes and preferential expression of Cbl-b in mature CD4 T cells. c-Cbl KO thymocytes exhibit hyperphosphorylation of ZAP-70 and ERK1/2, and TCR signalling uncoupled from CD4 co-stimulation requirements leading to increased proliferation (Murphy, Schnall et al. 1998), intracellular calcium mobilization (Joazeiro, Wing et al. 1999) and TCR/CD3ζ degradation (Myers, Sosinowski et al. 2006). Finally, positive selection is enhanced in CD4 c-Cbl KO thymocytes that express higher levels of TCR, CD3, CD4, CD5 and CD69 at their surface (Murphy, Schnall et al. 1998; Joazeiro, Wing et al. 1999). We reported similar expression of Cbl-b in CM and EM cells and reduced expression of c-Cbl in EM cells, leading to lower proliferation threshold, increased cytokine production but comparable intracellular calcium mobilization and expression of TCR/CD3, CD4 and CD28 (**Fig.18, 20, 22, and 23**). Consistent with our data, cells from double Cbl KO mice show phenotype and functions of EM cells (Naramura, Jang et al. 2002), underling that the balance between c-Cbl and Cbl-b expression might be fundamental in controlling the functional phenotype of human memory cells. Of interest, c-Cbl acts as negative regulator of TCR-mediated signals while functioning as positive regulator of PI3K by recruiting this protein to the cell membrane (Ueno, Sasaki et al. 1998; Lazaar, Krymskaya et al. 2001). Of note, this property is mediated by Tyr731 (docking site for SH2 domain of p85 regulatory subunits of PI3K), that is unique of c-Cbl but not Cbl-b (Thien and Langdon 2005). In line with this observation, CD28 stimulation (which activate PI3K pathways) impacted more strongly CM (which display higher expression levels of c-Cbl) than EM cells (**Fig.22B**).

Our data showed that the expression levels of c-Cbl decrease from N to EM cell. Conversely, the proliferation rate of these cells decrease from EM to N cells (4.7%, 1.5% and 0.2% per day in EM, CM and N, respectively) (Macallan, Wallace et al. 2004). In addition, the enhanced survival of CM versus EM cells has been associated to lower transcription of anti-apoptotic genes by inactivation of FOXO3a transcription factor. Complete inactivation of FOXO3a is optimally achieved through the convergence of signals from engagement of γc cytokine (IL-2 or IL-7) and antigen-specific TCR via activation of the common mediator PI3K (Riou, Yassine-Diab et al. 2007). Since c-Cbl may act as positive modulator of PI3K (Ueno, Sasaki et al. 1998; Lazaar, Krymskaya et al. 2001), its regulation during the differentiation from N to EM cells may represent a key point to modulate the responsiveness to survival/homeostatic signals of these cells. Consistently, c-Cbl was shown to enhance mitogenic and survival signals triggered through IL-4 (Ueno, Sasaki et al. 1998) and IL-2 (Gesbert, Garbay et al. 1998) receptors (in B and T cell systems, respectively) by linking the PI3K pathway.

Together, our data indicate that CM and EM cells differentially regulate the signalling pathways downstream their TCR and that c-Cbl may play central role in controlling the functional response of these cells. In order to verify this hypothesis, we increased c-Cbl content of EM cells as endogenously expressed in CM cells by cytosolic-mediated protein transduction (CTP) to measure the impact on their functional responses. CTP approach represents an attractive alternative strategy to virus-mediated and interfering RNA-based alteration of gene expression that are inappropriate to the study of memory T cell activation (see RESULTS section 6.5.1). Moreover, the time window necessary to obtain an efficient protein regulation through these approaches is probably too long to avoid phenotypical and functional differentiation of the memory subsets analysed. In addition, CTP peptide derives from the protein transduction domain (PTD) of HIV TAT protein which has been extensively documented with regard to its membrane transduction potential in T cells, as well as its efficient delivery of biomolecules in vivo (Morris, Depollier et al. 2001).

Increased expression levels of c-Cbl in EM cells by CTP-c-Cbl transduction correlated with increased threshold for proliferation and impairment of cytokine production, thus confirming the important role of c-Cbl in controlling the functional phenotype of central and effector memory cells. Importantly, we reported that IL-2 impacted more strongly the proliferation of CM cells than EM cells (**Fig.22B**). In agreement with this observation, IL-2 treatment reversed the proliferation defect of EM cells induced by CTP-c-Cbl transduction (**Fig.26F**). Finally, the weaker impact of enzymatically deficient CTP-c-Cbl C381A mutant on EM cell

proliferation and cytokine production (**Fig.26**) is supportive of a mechanism mainly mediated by the RING finger domain and likely to involve multiple targets. Transduction of larger amounts of c-Cbl in memory cells failed to further reduce their cytokine production, suggesting that besides c-Cbl, TCR/CD28 engagement triggers additional negative regulatory pathways for the fine-tuning of TCR signalling. Possible additional regulators include the phosphatases SHP-1, SHP-2 and PEP (Hasegawa, Martin et al. 2004) or the C-terminal Src Kinase Csk (Brdicka, Pavlistova et al. 2000), the antagonist of receptor tyrosine kinase Sprouty 1 (Choi, Cho et al. 2006), the inhibitory adaptor Gab2 (Yamasaki, Nishida et al. 2001) and the suppressor of T cell signalling Sts (Carpino, Turner et al. 2004).

Together, our data support an important role for adaptor/regulator c-Cbl in the biology and function of memory cells. Indeed, regulation of c-Cbl expression in memory cells may be a key control point for balancing TCR and CD28/cytokines mediated signals towards enhanced effector functions (EM cells) or long term survival (CM cells). Consistently, increased expression levels of c-Cbl in CM cells may determine decrease sensitivity to TCR stimulation while enhancing the capacity of these cells to respond to survival signals. Latter signals may include both co-stimulatory signals through CD28 and signals from cytokines involved in long-term maintenance of memory cells, such as IL-7 and IL-15. Moreover, the progressive regulation of c-Cbl expression levels (N-CM-EM) may reflect a differentiation program that naïve cells undergo during the primary immune response, leading to the generation of the different memory subsets. In order to validate this model, however, the role of c-Cbl in modulating the responsiveness of the memory cell subsets to survival signals should be addressed (see DISCUSSION and PERSPECTIVES section 7.2).

In conclusion, our work demonstrates for the first time the pivotal role of c-Cbl in the control of the heterogeneity and the plasticity of the functional phenotype of human memory CD4 T cell subsets. Moreover, it validates the combined use of RPP arrays and cytosolic transduction approaches as a powerful tool to quantitatively analyze signalling proteins and functionally assess their roles.

7.2 Perspectives

The work reported in this thesis highlighted the pivotal role of c-Cbl in generating the functional heterogeneity of human central and effector memory CD4 T cells. However, although we demonstrated a clear correlation between the expression level of this adaptor/regulator and the functional responses of CD4 memory cells, further efforts are necessary to clarify the signalling events involved and the precise impact of c-Cbl on the TCR signalling pathway. To this end, we plan to characterize the specific usage of the signalling pathways downstream the TCR in CM and EM cells, as well as in EM cells where the content of c-Cbl has been modulated using CTP-c-Cbl transduction. This analysis will include the use of specific kinase inhibitors, the profiling of the phosphorylation state of critical signalling proteins by RPP arrays (resting state and following TCR and/or CD28 triggering), and the profiling of TCR signalling complex assembly by using the approach that we recently developed (small scale TCR affinity capture-RPP arrays, see RESULTS section 5.5).

In addition, our data suggest that c-Cbl may represent a key control point for balancing TCR and CD28 mediated signals in memory cells, leading to their specific functional phenotype. Indeed, in CM cells, increased c-Cbl content promotes higher negative regulation of TCR signalling pathway, dampening their functional properties, while possibly enhancing CD28-mediated signals, leading to increased survival potential (see DISCUSSION and PERSPECTIVES section 7.1). Consistently, CM cells appear significantly more sensitive to CD28 than EM cells (see RESULTS section 6.4).

Moreover, experimental evidence proved that c-Cbl could enhance survival-mediated signals triggered by cytokines receptors (Gesbert, Garbay et al. 1998; Ueno, Sasaki et al. 1998). Therefore, differential c-Cbl expression level in memory cells may be implicated in the control of their long-term maintenance.

In order to test this hypothesis, we plan to characterize by taking advantage of the cytosolic transduction approach described in this thesis the role of c-Cbl as possible enhancer of CD28 and cytokine signals. Of particular interest is to understand how c-Cbl may regulate a differential responsiveness to homeostatic cytokines (such as IL-7 and IL-15) in central and effector memory cells.

Finally, we would like to investigate if the different expression levels of c-Cbl in the memory subsets could reflect a step in the differentiation process. To achieve this aim, we plan to

follow by RPP array the regulation of c-Cbl following *in vitro* differentiation of CM to EM cells.

In addition, it would be interesting to assess if the differential composition/organization of the TCR signalling machinery in the memory T cell subsets may drive (or result from) a differential association of TCR and CD28 molecules at the cell membrane. Indeed, preliminary data from our laboratory suggests that TCR/CD28 are mainly pre-associated in EM but not CM cells and that this pre-association in EM cells is enhanced following cell activation.

Together, defining the mechanisms at the basis of the functional phenotype of memory T cells is crucial to better design efficient T-cell vaccine strategies. Moreover, such knowledge might favor the development of pharmacological treatments, concomitant to vaccination, able to promote following vaccination the generation and time-life maintenance of the memory cell subset that correlate with efficient protective immunity. Finally, since c-Cbl mutations observed in acute myeloid leukaemia were associated with increased proliferation (Caligiuri, Briesewitz et al. 2007; Sargin, Choudhary et al. 2007) and reduced expression of Cbl occurred in HIV-positive patients (Leng, Borkow et al. 2002), a better understanding of Cbl expression and action in memory cells might also contribute to the development of novel therapeutic approaches to control lymphocyte functions.

MATERIALS AND METHODS

Antibodies and reagents. Mouse anti-TCR/CD3 Ab (OKT3) was affinity purified from hybridoma supernatant (American Type Culture Collection, ATCC, Rockville). Goat anti-CD3ε chain (M20), rabbit anti-Lck (aa 475-505), goat anti-Cbl-b (C-20), rabbit anti-PLCγ Abs were purchased from Santa Cruz Biotechnology. The following rabbit Abs were obtained from Cell Signaling Technology: anti-ZAP-70 (99F2), anti-p38 MAP Kinase, anti-p44/42 MAP Kinase, anti-Syk. Rabbit Abs against Lck (aa 1-58) and FcεRI γ-subunit (aa 80-86) were purchased from Upstate Biotechnology (Millipore). Mouse monoclonal Abs against CD28 (CD28.2), CD3ζ chain (8D3), TCR/CD3 (UCHT1), PI3 Kinase p85α subunit (4), c-Cbl (17), SLP76 (8), LAT (45), Actin (C4), CD45RA-PE (HI100), INFγ-APC (B27), and rat Abs against CCR7 (clone 3D12) and IL2-PE (MQ1-17H12) were purchased from BD PharMingen. Rabbit anti-actin (AC-40), anti-Hsp-90 Abs were obtained from Sigma-Aldrich and anti-beta2-microglobulin Ab was from BioVendor Laboratory Medicine. Purified rabbit anti-RNAse2, rabbit anti-Fyn and mouse anti-CD4 (B66-6) Abs were kindly provided by Drs. J. Hofsteenge (FMI, Basel, Switzerland), M.F. White and D. Rimoldi (LICR, Epalinges, Switzerland), respectively. Goat anti-rat IgG(H+L)-FITC Ab was purchased from SouthernBiotech and streptavidin-Cy3 was from Jackson ImmunoResearch Laboratories. The following horseradish peroxidase (HRP)-conjugated Abs were purchased by BD PharMingen: goat anti-mouse IgG and goat anti-rabbit IgG. Goat anti-mouse IgG_1 Ab was from Santa Cruz Biotechnology and rabbit anti-goat IgG-HRP Ab was purchased from DAKO or Zymed laboratories.

All chemicals unless indicated otherwise were from Sigma-Aldrich. 3% Blocking solution and Tyramide Amplification Reagent were purchased from Bio-Rad Laboratories. RPMI1640+GlutamaxTMI, Dulbecco's Modified Eagles Medium (DMEM), penicillin/streptomycin antibiotics and Fetal Bovine Serum (FBS) were from Gibco BRL (Life Technologies). FACS-Perm2 solution and GlogiPlugTM were purchased from BD Bioscience. SuperSignal® West Pico Chemiluminescent substrate was from Pierce and carboxyfluorescein diacetate succinimidyl ester (CFSE), Indo1 AM, red-fluorescent dimethylacridinone (DDAO), magnetic protein A beads and CD4 negative immunomagnetic depletion kit were from Invitrogen. Human recombinant interleukin 2 (IL-2) was purchased by RnD Systems and LymphoPrep™ was from Axis-Shield. Restriction enzymes were from Roche Applied Science and ligation Kit was from Takara Bio Inc. DNA purification kits were purchased from Macherey-Nagel GmbH and PfuTurbo® DNA-Polymerase was from Stratagene.

Cells, T cell isolation and lysis. HEK 293 cells were obtained from ATCC and maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin antibiotics. Peripheral blood mononuclear (PBMC) specimens from healthy donors were obtained from the local blood bank and lymphocytes were enriched by LymphoPrep™ (Axis-Shield) gradient centrifugation. Following CD4 negative immunomagnetic depletion (Invitrogen), CD4 T cells were lysed (80×10^6/ml) for 2h on ice in the following buffers supplemented with 1 mM Na_3VO_4, 15 mM NaF and EDTA-free complete protease inhibitor cocktail (Roche): i) Octyl-β-D-glucopyranoside-containing buffer: 100 mM Na_2HCO_3 pH 8.0, 80 mM Octyl-β-D-glucopyranoside, 5 mM EDTA, 1 % (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, ii) Triton X-100-containing buffer: 100 mM Na_2HCO_3 pH 8.0, 0.3% TX-100, 300 mM NaCl, 5mM EDTA, 5mM EGTA, 5% glycerol, 1% 2-mercaptoethanol, iii) Triton/SDS-containing buffer: 100 mM Na_2HCO_3 pH 8.0, 0.3% TX-100, 2% SDS, 100 mM NaCl, 5mM EDTA, 5mM EGTA, 5% glycerol, 2% 2-mercaptoethanol. Lysates were clarified by centrigugation (10000g, 10 min) and the supernatants heated for 10 min at 95°C. Alternatively, lysates (soluble fractions) and pellets (insoluble fractions) were boiled in SDS-PAGE protein loading buffer and analyzed by SDS-PAGE followed by gel staining with colloidal Coomassie Brilliant Blue. Proteins were quantified using Image J 1.34s image processing software (National Institute of Health, USA).

For protein profiling experiments, CD8 and CD4 T cell subsets were isolated at 4°C by positive immunomagnetic selection (Miltenyi Biotech and BD Biosciences) followed by staining with mAbs specific for rat anti-CCR7, anti rat-FITC and CD45RA-PE and sorting into N (CCR7+, CD45RA+), CM (CCR7+, CD45RA-), EM (CCR7-, CD45RA-) and E (CCR7-, CD45RA+) populations using a Facs Aria cell sorter (BD Biosciences). Purity of the isolated populations was 98% as assessed by FACS analysis. Cell were pelleted and lysed in Octyl-β-D-glucopyranoside-containing buffer as previously described. Lysates were stored at –20°C for up to 6 months. For functional assays, CD4 memory cells were isolated by CD4 negative immunomagnetic depletion (Invitrogen) supplemented with anti-CD45RA Ab (HI100).

CD4 T cell activation and TCR complex capture/denaturation An aldehyde-derivatized glass microscope slide (Nexterion® Slide AL, Schott Nexterion) was sub-divided into 24 wells of 2 mm in diameter using a silicone rubber manifold (Electron Microscopy Sciences).

All steps were performed in a humid chamber. Anti-TCR/CD3ε antibodies (OKT3 at 3mg/ml in 150 mM Na_2HCO_3 pH 8.0, 5% glycerol, 0.005% CHAPS) were covalently immobilized on aldehyde-derivatized glass surface by incubation for one hour at RT. Following 3 washes with PBS containing 0.1% Tween-20 (PBST), remaining reactive aldehyde groups were blocked in ethanolamine buffer (50 mM ethanolamine, 50mM Sodium Borate; pH 8.0) for 90 min at RT. The slide was subsequently washed 3 times with PBS-T, once with water and used immediatly.

Ex vivo isolated CD4 T cells were stimulated for 20 min at 37˚C using anti-CD3ε/CD28 coated beads. 6µl of protein A beads (Invitrogen) were incubated with 1 µg of anti-CD3ε (UCHT1) and 5µg of anti-CD28 (CD28.2) antibodies for 40 min at RT, washed twice with PBS and resuspended in 40µl of PBS. CD4 T cells (0.3 million) were incubated with 6 µl of UCHT1-coated beads at 37˚C. The stimulation was stopped at different times by adding 0.5 ml of ice-cold PBS containing 1mM Na_3VO_4 to the bead-cell mixture. Following centrifugation (5 min, 1200g), the cells (50 M/ml) were lyzed for 4 h on ice in lysis buffer (1% Brij 78 in 50 mM Tris pH 7.5, 0.5 mM Na_3VO_4, 10 mM NaF and protease inhibitors) that solubilizes lipid rafts and keeps protein-protein interactions intact (Legler, Micheau et al. 2003). Lysates were subsequently clarified by centrifugation (10 min, 10000g). Coated beads were removed from the lysate with a magnet and a lysate equivalent to 0.5 M cells (10µl) distributed in three wells of the glass slide. The wells were covered with parafilm and following o/n incubation in a humid chamber, the wells were washed twice with ice-cold lysis buffer containing 0.3% Brij78. The captured complexes were collected in denaturing buffer (2% SDS, 2.5% 2-mercaptoethanol, 10% glycerol, 50 mM NaCl, 1.5 mM $MgCl_2$, 1mM EDTA) heated at 96˚C. The content of 3 wells was successively collected with 4µl of denaturing buffer and stored at -20˚C. Ovalbumin (GE Healthcare Bio-Sciences AB) at the final concentration of 0.1 mg/ml was added to the mixture before printing.

Array printing and staining. 3 to 5µl of T cell lysates (80×106 cells/ml) or TCR isolated complexes were transferred into a polypropylene pyramidal bottom 384-well plate (Laubsher Labs, Miecourt, Switzerland) and 0.7 nl of sample were printed with Omnigrid 300 contact-printing robotic microarrayer (Genomic Solutions, Ann Arbor, MI) using a 75 µm SMP3 pin (TeleChem International Inc.) onto a 16-pad 3D nitrocellulose-coated glass slides (Fast slide) (Schleicher and Schuell). Spots were obtained by a single pin hit and all samples were printed in triplicate. Leak-proof incubation chambers allowed detection of each pad independently. A

T cell lysate serial dilution was printed on each pad to assess the dynamic range of detection (~1 nl of a lysate at a concentration of 2-100x106 T cells/ml equivalent to 2-100 T cells/spot and 200 pg-100 ng protein/spot). Before staining, slides were dried o/n at room temperature (RT), rinsed 3 times with PBS 0.1% tween20 (PBST) and blocked for two hours at RT in PBST containing 3% BioRad blocking solution. Staining was carried out using a catalyzed signal amplification system followed by fluorescent detection. Briefly, arrays were incubated with primary antibody diluted in PBST containing 3% fish gelatine at a concentration ranging from 1:300 to 1:1000 for 14 hours at 4°C, followed by HRP-linked secondary antibody at a concentration of 1:1000 for anti-mouse and 1:3000 for anti-rabbit/anti-goat for 60 min at RT. Between each following steps, arrays were rinsed 3 times and washed 3 times 5 min each in PBST. Signals were amplified by Tyramide Signal Amplification reaction according to manufacturer instructions (BLAST reagent, PerkinElmer, Boston, MA) for 10 min at RT, followed by staining with streptavidin-Cy3 for 45 min in PBST containing 1% BSA and 5% PEG-8000. Secondary antibodies did not cross-reacted with cell lysates or TCR-complexes as assessed by amplification and detection with secondary antibodies alone. An antibody was accepted and used for RPP arrays detection only if it detected by immunoblotting a single predominant band at the expected molecular weight in lymphocyte lysates (Fig 3D).

Array detection and analysis. Cy3 fluorescent-stained slides were scanned on GenePix 4000B microarray scanner (Axon Instruments, Molecular Devices, Sunnywale, CA) at 10 μm resolution and saved as 16-bit TIFF files. Images were analyzed with GenePix Pro 6.0 (Axon Instruments) and Excel software (Microsoft, Redmond, USA). Local background-subtracted average pixel intensities of individual spot were used to determine the mean value of triplicate spots (each sample printed in triplicate spots). Data were considered when signal intensities were included in the linear range of detection of the lysate serial dilution present in the same array. Mean values were then normalized to the mean values of corresponding actin signal and used for subsequent cumulative analysis of the subjects. Changes between two subsets were considered as statistically significant when i) higher than 20% (technical error <11%) ii) calculated p-value of two-tails paired *t*-test was less than 0.05. In heat-map graph, normalized data were logged in base 2, in order to compensate for the asymmetry introduced by the ratio used in the normalization to N cells (e.g quantification CM / quantification N). log2 ratios derived from the analysis of different subjects were cumulated by calculating the mean log2 ratio for each signalling protein and displayed in continuous colour scale. In TCR complex

analysis, mean values of each TCR complex sample were normalized to the mean signal intensity of corresponding bait protein (CD3ε) signals. All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA). The coefficient of variation (CV, defined as the standard deviation divided by the mean) data represent the mean CV value obtained for low to high protein concentrations of T cell lysates.

Functional assay. Cells were stimulated in 96-well plates coated with anti-TCR/CD3 (OKT3) Ab and indicated soluble antibodies at 1 μg/ml. Cytokine secretion was blocked with GlogiPlug™ transport inhibitor and intracellular IL-2 and INF-γ production were monitored by flow cytometry using FACSCalibur™ and FACSDiva software (BD Biosciences). Memory cells were surface stained for CCR7 (using anti-CCR7 and anti rat-FITC Abs), fixed/permeabilized with PERM2 solution according to the manufacturer's instructions and stained with PE-conjugated IL-2 and APC-conjugated INF-γ Abs.

For proliferation assays, cells (40 millions/ml) were reacted with 6 μM CFSE for 7 min at 37°C and the reaction quenched with serum. CFSE-labelled cells were surface stained for CCR7 (anti-CCR7 and anti-rat A647 Abs) and sorted in CM and EM cell populations. After 6 days of stimulation the percentage of proliferating cells were determined by cytoflorimetry based on their CFSE content. Alternatively, incorporation of H^3 thymidine (MP Biomedicals) was measured over 24 hours 3 days post-activation.

For calcium analysis, negatively selected memory cells were loaded with 1 μM Indo1-AM for one hour at 37°C and incubated with OKT3 and anti-CD28 (1 μg/ml) Abs on ice following surface staining of CCR7 as described above. Immediately after crosslinking with anti-mouse Ab, intracellular changes in calcium were monitored at 37°C with a LSRII cytometer (BD Biosciences) by measuring the FL5/FL4 ratio over 5 min.

Plasmids. Cytoplasmic Transduction Peptide 512 (CTP) sequence and corresponding plasmid were kindly provided by Dr. Y-S. Bae (Sungkyunkwan Univerity, Suwon, Korea). Complementary forward (5'TATCGGATCCGCCACCATGTACGGACGCCGCGCACGCC GCCGCCGCCGCCGCGCTAGCTACCCTTATGATGTGCCAGATTATGC3') and reverse (5'GGCCGCATAATCTGGCACATCATAAGGGTAGCTAGCGCGGCGGCGGCGGCGG CGTGCGCGCGTCCGTACATGGTGGCGGATCCGA3') primers coding for CTP-HA peptide were annealed by heating 5 minutes at 98°C, followed by cooling at 20 °C. After

phosphorylation of 5'ends, the fragment was inserted into pET21a vector (Novagen) digested with NdeI/NotI and the recombinant construct (pET21a-CTP-HA) transformed in *E.coli* DH5α (Invitrogen). Positive clones were identified by restriction analysis. Wt and C381A mutant c-Cbl cDNA were amplified by polymerase chain reaction (PCR) from plasmids kindly provided by Dr. S. Lipkowitz (Ettenberg, Keane et al. 1999) and Dr. W. Langdon (Thien, Blystad et al. 2005) and His-tagged GFP was obtained by PCR from pEGFP-CI vector (clontech). The following primer pairs were used: Fw_c-Cbl: GCGGCCGCCGGCAACGTGAAGAAGAG / Rev_c-Cbl: GCGCCTCGAGCTACTAGGTA GCTACATGGGCAGG and Fw_GFP: GCGCGGCCGCGCACCATCACCACCATCATGT GAG / Rev_GFP: GCCTCGAGCTACTACTTGTACAGCTCGTCCATGCCG. Amplified fragments were inserted into NotI/XhoI restriction sites of pET21a-CTP-HA vector and CTP constructs subcloned into pCR^{TM}3 vector (Invitrogen) digested BamH1/XhoI. A pCR^{TM}3 derived vector containing only the sequences corresponding to CTP-HA was also built by subcloning the CTP-HA cassette of pET21a(+)-CTP-HA into pCR^{TM}3 vector digested BamH1/XhoI (pCR3-CTP-HA). Sequences of all constructs were confirmed by DNA sequencing. A frameshift mutation of pCR3-CTP-HA-GFP construct (keeping intact only the CTP sequence while altering the frame of the GFP sequence) was used as CTP-mock and resulted in a CTP-tagged unrelated protein of ~33 KDa. Maps and details of the recombinant vectors built in this thesis are reported in Annex I.

Purification of CTP-recombinant proteins and cell transduction. Large scale transient expression of CTP-proteins in HEK293 cells was performed at the Protein Expression Core Facility (http://pecf.epfl.ch/, EPFL, Lausanne, Switzerland). Cells ($3.2x10^8$/preparation) were lysed in 50 mM phosphate buffer, pH 7.6 containing 0.1 % Tween-20 and 0.2 M NaCl. CTP-recombinant proteins were purified from cell lysate through binding to endogenous (N terminal 7xHis (aa 36-42) in c-Cbl wt and C381A) or recombinant (N terminal 6xHis in CTP-mock and CTP-GFP) His-tag by nickel-agarose chromatography. CTP proteins were enriched 100-fold by a step elution in 0.5 M imidazole and dialyzed o/n against PBS. Their sequences were confirmed by nano liquid chromatography mass spectrometry (data not shown). CTP-proteins were transduced into T cells by incubation for 30 min at 37°C in RPMI containing 0.5% gelatine. For exact determination of amount of transduced proteins, transduced cells were treated with trypsin (3 min, 37°C) to remove any CTP-Cbl potentially interacting with the cell membrane.

REFERENCES

Acuto, O. and D. Cantrell (2000). "T cell activation and the cytoskeleton." Annu Rev Immunol **18**: 165-84.
Acuto, O., R. E. Hussey, et al. (1983). "The human T cell receptor: appearance in ontogeny and biochemical relationship of alpha and beta subunits on IL-2 dependent clones and T cell tumors." Cell **34**(3): 717-26.
Acuto, O. and F. Michel (2003). "CD28-mediated co-stimulation: a quantitative support for TCR signalling." Nat Rev Immunol **3**(12): 939-51.
Adams, E. J., Y. H. Chien, et al. (2005). "Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22." Science **308**(5719): 227-31.
Aivazian, D. and L. J. Stern (2000). "Phosphorylation of T cell receptor zeta is regulated by a lipid dependent folding transition." Nature Structural Biology **7**(11): 1023-6.
Alarcon, B., M. Swamy, et al. (2006). "T-cell antigen-receptor stoichiometry: pre-clustering for sensitivity." EMBO Rep **7**(5): 490-5.
Alessi, D. R. and P. Cohen (1998). "Mechanism of activation and function of protein kinase B." Curr Opin Genet Dev **8**(1): 55-62.
Allison, J. P., B. W. McIntyre, et al. (1982). "Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody." J Immunol **129**(5): 2293-300.
Amyes, E., A. J. McMichael, et al. (2005). "Human CD4+ T cells are predominantly distributed among six phenotypically and functionally distinct subsets." J Immunol **175**(9): 5765-73.
Appay, V., P. R. Dunbar, et al. (2002). "Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections." Nat Med **8**(4): 379-85.
Bacarese-Hamilton, T., L. Mezzasoma, et al. (2004). "Serodiagnosis of infectious diseases with antigen microarrays." Journal of Applied Microbiology **96**(1): 10-7.
Bachmaier, K., C. Krawczyk, et al. (2000). "Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b." Nature **403**(6766): 211-6.
Bachmann, M. F., A. Gallimore, et al. (1999). "Developmental regulation of Lck targeting to the CD8 coreceptor controls signaling in naive and memory T cells." J Exp Med **189**(10): 1521-30.
Balboni, I., S. M. Chan, et al. (2006). "Multiplexed protein array platforms for analysis of autoimmune diseases." Annual Review of Immunology **24**: 391-418.
Baron, V., C. Bouneaud, et al. (2003). "The repertoires of circulating human CD8(+) central and effector memory T cell subsets are largely distinct." Immunity **18**(2): 193-204.
Becker, T. C., E. J. Wherry, et al. (2002). "Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells." J Exp Med **195**(12): 1541-8.
Bentley, G. A. and R. A. Mariuzza (1996). "The structure of the T cell antigen receptor." Annu Rev Immunol **14**: 563-90.
Berggard, T., S. Linse, et al. (2007). "Methods for the detection and analysis of protein-protein interactions." Proteomics **7**(16): 2833-42.
Beverley, P. C. (2008). "Primer: making sense of T-cell memory." Nat Clin Pract Rheumatol **4**(1): 43-9.
Blake, T. J., M. Shapiro, et al. (1991). "The sequences of the human and mouse c-cbl proto-oncogenes show v-cbl was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif." Oncogene **6**(4): 653-7.
Blomberg, N., E. Baraldi, et al. (1999). "The PH superfold: a structural scaffold for multiple functions." Trends Biochem Sci **24**(11): 441-5.
Bobrow, M. N., G. J. Litt, et al. (1992). "The use of catalyzed reporter deposition as a means of signal amplification in a variety of formats." Journal of Immunological Methods **150**(1-2): 145-9.

Bolliger, L., B. Johansson, et al. (1997). "The short extracellular domain of the T cell receptor zeta chain is involved in assembly and signal transduction." Mol Immunol **34**(12-13): 819-27.
Boyman, O., M. Kovar, et al. (2006). "Selective stimulation of T cell subsets with antibody-cytokine immune complexes." Science **311**(5769): 1924-7.
Boyman, O., J. F. Purton, et al. (2007). "Cytokines and T-cell homeostasis." Curr Opin Immunol **19**(3): 320-6.
Brdicka, T., D. Pavlistova, et al. (2000). "Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation." J Exp Med **191**(9): 1591-604.
Brockdorff, J., S. Williams, et al. (1999). "Dephosphorylation of ZAP-70 and inhibition of T cell activation by activated SHP1." Eur J Immunol **29**(8): 2539-50.
Bromley, S. K., W. R. Burack, et al. (2001). "The immunological synapse." Annu Rev Immunol **19**: 375-96.
Burbach, B. J., R. B. Medeiros, et al. (2007). "T-cell receptor signaling to integrins." Immunol Rev **218**: 65-81.
Cahill, D. J. (2001). "Protein and antibody arrays and their medical applications." Journal of Immunological Methods **250**(1-2): 81-91.
Caligiuri, M. A., R. Briesewitz, et al. (2007). "Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia." Blood **110**(3): 1022-4.
Call, M. E., J. Pyrdol, et al. (2002). "The organizing principle in the formation of the T cell receptor-CD3 complex." Cell **111**(7): 967-79.
Call, M. E. and K. W. Wucherpfennig (2004). "Molecular mechanisms for the assembly of the T cell receptor-CD3 complex." Mol Immunol **40**(18): 1295-305.
Call, M. E. and K. W. Wucherpfennig (2005). "The T cell receptor: critical role of the membrane environment in receptor assembly and function." Annu Rev Immunol **23**: 101-25.
Call, M. E. and K. W. Wucherpfennig (2007). "Common themes in the assembly and architecture of activating immune receptors." Nat Rev Immunol **7**(11): 841-50.
Campbell, J. J., K. E. Murphy, et al. (2001). "CCR7 expression and memory T cell diversity in humans." J Immunol **166**(2): 877-84.
Carpino, N., S. Turner, et al. (2004). "Regulation of ZAP-70 activation and TCR signaling by two related proteins, Sts-1 and Sts-2." Immunity **20**(1): 37-46.
Castiglioni, P., M. Gerloni, et al. (2004). "Genetically programmed B lymphocytes are highly efficient in inducing anti-virus protective immunity mediated by central memory CD8 T cells." Vaccine **23**(5): 699-708.
Chan, S. M., J. Ermann, et al. (2004). "Protein microarrays for multiplex analysis of signal transduction pathways." Nature Medicine **10**(12): 1390-6.
Chandok, M. R. and D. L. Farber (2004). "Signaling control of memory T cell generation and function." Semin Immunol **16**(5): 285-93.
Chang, J. T., V. R. Palanivel, et al. (2007). "Asymmetric T lymphocyte division in the initiation of adaptive immune responses." Science **315**(5819): 1687-91.
Charboneau, L., H. Tory, et al. (2002). "Utility of reverse phase protein arrays: applications to signalling pathways and human body arrays.[erratum appears in Brief Funct Genomic Proteomic. 2007 Jun;6(2):159 Note: Scott, Heather [corrected to Tory, Heather]]." Briefings in Functional Genomics & Proteomics **1**(3): 305-15.
Chiang, Y. J., H. K. Kole, et al. (2000). "Cbl-b regulates the CD28 dependence of T-cell activation." Nature **403**(6766): 216-20.

Chien, Y., D. M. Becker, et al. (1984). "A third type of murine T-cell receptor gene." Nature **312**(5989): 31-5.
Choi, H., S. Y. Cho, et al. (2006). "Dual effects of Sprouty1 on TCR signaling depending on the differentiation state of the T cell." J Immunol **176**(10): 6034-45.
Chtanova, T., R. Newton, et al. (2005). "Identification of T cell-restricted genes, and signatures for different T cell responses, using a comprehensive collection of microarray datasets." J Immunol **175**(12): 7837-47.
Clements, J. L., B. Yang, et al. (1998). "Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development." Science **281**(5375): 416-9.
Crespo, P., K. E. Schuebel, et al. (1997). "Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product." Nature **385**(6612): 169-72.
Czyzyk, J., D. Leitenberg, et al. (2000). "Combinatorial effect of T-cell receptor ligation and CD45 isoform expression on the signaling contribution of the small GTPases Ras and Rap1." Mol Cell Biol **20**(23): 8740-7.
Davey, G. M., R. Starr, et al. (2005). "SOCS-1 regulates IL-15-driven homeostatic proliferation of antigen-naive CD8 T cells, limiting their autoimmune potential." J Exp Med **202**(8): 1099-108.
De Boer, R. J., D. Homann, et al. (2003). "Different dynamics of CD4+ and CD8+ T cell responses during and after acute lymphocytic choriomeningitis virus infection." J Immunol **171**(8): 3928-35.
de Mello Coelho, V., D. Nguyen, et al. (2004). "Quantitative differences in lipid raft components between murine CD4+ and CD8+ T cells." BMC Immunol **5**: 2.
Diehn, M., A. A. Alizadeh, et al. (2002). "Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation." Proc Natl Acad Sci U S A **99**(18): 11796-801.
Dikic, I., I. Szymkiewicz, et al. (2003). "Cbl signaling networks in the regulation of cell function." Cell Mol Life Sci **60**(9): 1805-27.
Dittmer, A. and J. Dittmer (2006). "Beta-actin is not a reliable loading control in Western blot analysis." Electrophoresis **27**(14): 2844-5.
Doucey, M. A., D. F. Legler, et al. (2001). "CTL activation is induced by cross-linking of TCR/MHC-peptide-CD8/p56lck adducts in rafts." Eur J Immunol **31**(5): 1561-70.
Duan, L., A. L. Reddi, et al. (2004). "The Cbl family and other ubiquitin ligases: destructive forces in control of antigen receptor signaling." Immunity **21**(1): 7-17.
Dutton, R. W., L. M. Bradley, et al. (1998). "T cell memory." Annu Rev Immunol **16**: 201-23.
Ekins, R., F. Chu, et al. (1990). "Multispot, multianalyte, immunoassay." Annales de Biologie Clinique **48**(9): 655-66.
Ekins, R., F. Chu, et al. (1989). "High specific activity chemiluminescent and fluorescent markers: their potential application to high sensitivity and 'multi-analyte' immunoassays." Journal of Bioluminescence & Chemiluminescence **4**(1): 59-78.
Engvall, E. and P. Perlman (1971). "Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G." Immunochemistry **8**(9): 871-4.
Espina, V., A. I. Mehta, et al. (2003). "Protein microarrays: molecular profiling technologies for clinical specimens." Proteomics **3**(11): 2091-100.
Ettenberg, S. A., M. M. Keane, et al. (1999). "cbl-b inhibits epidermal growth factor receptor signaling." Oncogene **18**(10): 1855-66.
Fang, D., H. Y. Wang, et al. (2001). "Cbl-b, a RING-type E3 ubiquitin ligase, targets phosphatidylinositol 3-kinase for ubiquitination in T cells." J Biol Chem **276**(7): 4872-8.
Farber, D. L., O. Acuto, et al. (1997). "Differential T cell receptor-mediated signaling in naive and memory CD4 T cells." Eur J Immunol **27**(8): 2094-101.

Farber, D. L., M. Luqman, et al. (1995). "Control of memory CD4 T cell activation: MHC class II molecules on APCs and CD4 ligation inhibit memory but not naive CD4 T cells." Immunity **2**(3): 249-59.
Ferguson, R. E., H. P. Carroll, et al. (2005). "Housekeeping proteins: a preliminary study illustrating some limitations as useful references in protein expression studies." Proteomics **5**(2): 566-71.
Fernandez-Miguel, G., B. Alarcon, et al. (1999). "Multivalent structure of an alphabetaT cell receptor." Proc Natl Acad Sci U S A **96**(4): 1547-52.
Fiore, F., V. Ollendorff, et al. (2001). "Characterization of the mouse Cblc/Cbl3 gene." Biochem Biophys Res Commun **280**(1): 182-7.
Fodor, S. P., J. L. Read, et al. (1991). "Light-directed, spatially addressable parallel chemical synthesis." Science **251**(4995): 767-73.
Gao, G. F., Z. Rao, et al. (2002). "Molecular coordination of alphabeta T-cell receptors and coreceptors CD8 and CD4 in their recognition of peptide-MHC ligands." Trends Immunol **23**(8): 408-13.
Garcia, K. C. and E. J. Adams (2005). "How the T cell receptor sees antigen--a structural view." Cell **122**(3): 333-6.
Geginat, J., F. Sallusto, et al. (2001). "Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells." J Exp Med **194**(12): 1711-9.
Geginat, J., F. Sallusto, et al. (2003). "Cytokine-driven proliferation and differentiation of human naive, central memory and effector memory CD4+ T cells." Pathol Biol (Paris) **51**(2): 64-6.
Geho, D. H., J. K. Killian, et al. (2007). "Fluorescence-based analysis of cellular protein lysate arrays using quantum dots." Methods Mol Biol **374**: 229-37.
Gesbert, F., C. Garbay, et al. (1998). "Interleukin-2 stimulation induces tyrosine phosphorylation of p120-Cbl and CrkL and formation of multimolecular signaling complexes in T lymphocytes and natural killer cells." J Biol Chem **273**(7): 3986-93.
Gett, A. V., F. Sallusto, et al. (2003). "T cell fitness determined by signal strength." Nat Immunol **4**(4): 355-60.
Gil, D., W. W. Schamel, et al. (2002). "Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation." Cell **109**(7): 901-12.
Goodnow, C. C. (2001). "Pathways for self-tolerance and the treatment of autoimmune diseases." Lancet **357**(9274): 2115-21.
Grubb, R. L., V. S. Calvert, et al. (2003). "Signal pathway profiling of prostate cancer using reverse phase protein arrays." Proteomics **3**(11): 2142-6.
Guillaume, S., L. Tuosto, et al. (2003). "Proximal changes in signal transduction that modify CD8+ T cell responsiveness in vivo." Eur J Immunol **33**(9): 2551-6.
Hall, D. A., J. Ptacek, et al. (2007). "Protein microarray technology." Mechanisms of Ageing & Development **128**(1): 161-7.
Hall, D. A., H. Zhu, et al. (2004). "Regulation of gene expression by a metabolic enzyme." Science **306**(5695): 482-4.
Hall, S. R., B. M. Heffernan, et al. (1999). "CD4+ CD45RA+ and CD4+ CD45RO+ T cells differ in their TCR-associated signaling responses." Eur J Immunol **29**(7): 2098-106.
Harari, A., V. Dutoit, et al. (2006). "Functional signatures of protective antiviral T-cell immunity in human virus infections." Immunol Rev **211**: 236-54.
Harari, A., F. Vallelian, et al. (2005). "Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence." J Immunol **174**(2): 1037-45.

Harari, A., F. Vallelian, et al. (2004). "Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load." Eur J Immunol **34**(12): 3525-33.
Hasegawa, K., F. Martin, et al. (2004). "PEST domain-enriched tyrosine phosphatase (PEP) regulation of effector/memory T cells." Science **303**(5658): 685-9.
Hedrick, S. M., D. I. Cohen, et al. (1984). "Isolation of cDNA clones encoding T cell-specific membrane-associated proteins." Nature **308**(5955): 149-53.
Hilleman, M. R. (2000). "Vaccines in historic evolution and perspective: a narrative of vaccine discoveries." Vaccine **18**(15): 1436-47.
Homann, D., L. Teyton, et al. (2001). "Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory." Nat Med **7**(8): 913-9.
Hozumi, N. and S. Tonegawa (1976). "Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions." Proc Natl Acad Sci U S A **73**(10): 3628-32.
Huang, J., H. Zhu, et al. (2004). "Finding new components of the target of rapamycin (TOR) signaling network through chemical genetics and proteome chips." Proceedings of the National Academy of Sciences of the United States of America **101**(47): 16594-9.
Huang, R. P., R. Huang, et al. (2001). "Simultaneous detection of multiple cytokines from conditioned media and patient's sera by an antibody-based protein array system." Analytical Biochemistry **294**(1): 55-62.
Hunyady, B., K. Krempels, et al. (1996). "Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostaining." Journal of Histochemistry & Cytochemistry **44**(12): 1353-62.
Hussain, S. F., C. F. Anderson, et al. (2002). "Differential SLP-76 expression and TCR-mediated signaling in effector and memory CD4 T cells." J Immunol **168**(4): 1557-65.
Intlekofer, A. M., N. Takemoto, et al. (2005). "Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin." Nat Immunol **6**(12): 1236-44.
Irvine, D. J., M. A. Purbhoo, et al. (2002). "Direct observation of ligand recognition by T cells." Nature **419**(6909): 845-9.
Jacob, J. and D. Baltimore (1999). "Modelling T-cell memory by genetic marking of memory T cells in vivo." Nature **399**(6736): 593-7.
Jahn-Schmid, B., C. Harwanegg, et al. (2003). "Allergen microarray: comparison of microarray using recombinant allergens with conventional diagnostic methods to detect allergen-specific serum immunoglobulin E." Clinical & Experimental Allergy **33**(10): 1443-9.
Jenner, E., Ed. (1798). An inquiry into the causes and effects of the *Variolae Vaccinae*. London: Low.
Jeon, M. S., A. Atfield, et al. (2004). "Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction." Immunity **21**(2): 167-77.
Joazeiro, C. A., S. S. Wing, et al. (1999). "The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase." Science **286**(5438): 309-12.
Jones, R. B., A. Gordus, et al. (2006). "A quantitative protein interaction network for the ErbB receptors using protein microarrays." Nature **439**(7073): 168-74.
Joos, T. O., M. Schrenk, et al. (2000). "A microarray enzyme-linked immunosorbent assay for autoimmune diagnostics." Electrophoresis **21**(13): 2641-50.
Judge, A. D., X. Zhang, et al. (2002). "Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells." J Exp Med **196**(7): 935-46.
Kader, H. A., V. T. Tchernev, et al. (2005). "Protein microarray analysis of disease activity in pediatric inflammatory bowel disease demonstrates elevated serum PLGF, IL-7, TGF-beta1, and IL-12p40 levels in Crohn's disease and ulcerative colitis patients in

remission versus active disease." American Journal of Gastroenterology **100**(2): 414-23.
Kaech, S. M., S. Hemby, et al. (2002). "Molecular and functional profiling of memory CD8 T cell differentiation." Cell **111**(6): 837-51.
Kalia, V., S. Sarkar, et al. (2008). "Fine-tuning CD4+ central memory T cell heterogeneity by strength of stimulation." Eur J Immunol **38**(1): 15-9.
Kane, L. P., J. Lin, et al. (2000). "Signal transduction by the TCR for antigen." Curr Opin Immunol **12**(3): 242-9.
Kappler, J., R. Kubo, et al. (1983). "The mouse T cell receptor: comparison of MHC-restricted receptors on two T cell hybridomas." Cell **34**(3): 727-37.
Karjalainen, K. (1994). "High sensitivity, low affinity--paradox of T-cell receptor recognition." Curr Opin Immunol **6**(1): 9-12.
Kearse, K. P., J. L. Roberts, et al. (1995). "TCR alpha-CD3 delta epsilon association is the initial step in alpha beta dimer formation in murine T cells and is limiting in immature CD4+ CD8+ thymocytes." Immunity **2**(4): 391-9.
Kennedy, M. K., M. Glaccum, et al. (2000). "Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice." J Exp Med **191**(5): 771-80.
Khan, I. H., S. Mendoza, et al. (2006). "Multiplex analysis of intracellular signaling pathways in lymphoid cells by microbead suspension arrays." Molecular & Cellular Proteomics **5**(4): 758-68.
Kieper, W. C., J. T. Tan, et al. (2002). "Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells." J Exp Med **195**(12): 1533-9.
Kim, D., C. Jeon, et al. (2006). "Cytoplasmic transduction peptide (CTP): new approach for the delivery of biomolecules into cytoplasm in vitro and in vivo." Exp Cell Res **312**(8): 1277-88.
Ko, I. K., K. Kato, et al. (2005). "Parallel analysis of multiple surface markers expressed on rat neural stem cells using antibody microarrays." Biomaterials **26**(23): 4882-91.
Krawczyk, C. M., R. G. Jones, et al. (2005). "Differential control of CD28-regulated in vivo immunity by the E3 ligase Cbl-b." J Immunol **174**(3): 1472-8.
Krishnan, S., D. L. Farber, et al. (2003). "T cell rewiring in differentiation and disease." J Immunol **171**(7): 3325-31.
Krishnan, S., V. G. Warke, et al. (2003). "The FcR gamma subunit and Syk kinase replace the CD3 zeta-chain and ZAP-70 kinase in the TCR signaling complex of human effector CD4 T cells." J Immunol **170**(8): 4189-95.
Krishnan, S., V. G. Warke, et al. (2001). "Generation and biochemical analysis of human effector CD4 T cells: alterations in tyrosine phosphorylation and loss of CD3zeta expression." Blood **97**(12): 3851-9.
Krogsgaard, M., J. Juang, et al. (2007). "A role for "self" in T-cell activation." Semin Immunol **19**(4): 236-44.
Krogsgaard, M., Q. J. Li, et al. (2005). "Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity." Nature **434**(7030): 238-43.
Krutzik, P. O., J. M. Irish, et al. (2004). "Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications." Clinical Immunology **110**(3): 206-21.
Krutzik, P. O. and G. P. Nolan (2003). "Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events." Cytometry Part A: The Journal of the International Society for Analytical Cytology **55**(2): 61-70.
Ku, C. C., M. Murakami, et al. (2000). "Control of homeostasis of CD8+ memory T cells by opposing cytokines." Science **288**(5466): 675-8.

Kuhns, M. S., M. M. Davis, et al. (2006). "Deconstructing the form and function of the TCR/CD3 complex." Immunity **24**(2): 133-9.
Lafferty, K. J. and A. J. Cunningham (1975). "A new analysis of allogeneic interactions." Aust J Exp Biol Med Sci **53**(1): 27-42.
Lanier, L. L., J. Ruitenberg, et al. (1988). "Structural and serological heterogeneity of gamma/delta T cell antigen receptor expression in thymus and peripheral blood." Eur J Immunol **18**(12): 1985-92.
Lantz, O., I. Grandjean, et al. (2000). "Gamma chain required for naive CD4+ T cell survival but not for antigen proliferation." Nat Immunol **1**(1): 54-8.
Lanzavecchia, A. and F. Sallusto (2002). "Progressive differentiation and selection of the fittest in the immune response." Nat Rev Immunol **2**(12): 982-7.
Lauvau, G., S. Vijh, et al. (2001). "Priming of memory but not effector CD8 T cells by a killed bacterial vaccine." Science **294**(5547): 1735-9.
Lazaar, A. L., V. P. Krymskaya, et al. (2001). "VCAM-1 activates phosphatidylinositol 3-kinase and induces p120Cbl phosphorylation in human airway smooth muscle cells." J Immunol **166**(1): 155-61.
Legler, D. F., O. Micheau, et al. (2003). "Recruitment of TNF receptor 1 to lipid rafts is essential for TNFalpha-mediated NF-kappaB activation." Immunity **18**(5): 655-64.
Leng, Q., G. Borkow, et al. (2002). "Attenuated signaling associated with immune activation in HIV-1-infected individuals." Biochem Biophys Res Commun **298**(4): 464-7.
Lennon, G. G. and H. Lehrach (1991). "Hybridization analyses of arrayed cDNA libraries." Trends in Genetics **7**(10): 314-7.
Lenschow, D. J., T. L. Walunas, et al. (1996). "CD28/B7 system of T cell costimulation." Annu Rev Immunol **14**: 233-58.
Li, J., G. Huston, et al. (2003). "IL-7 promotes the transition of CD4 effectors to persistent memory cells." J Exp Med **198**(12): 1807-15.
Li, R., D. Liu, et al. (2001). "The expression and potential function of cellular prion protein in human lymphocytes." Cell Immunol **207**(1): 49-58.
Liotta, L. A., V. Espina, et al. (2003). "Protein microarrays: meeting analytical challenges for clinical applications." Cancer Cell **3**(4): 317-25.
Liu, K., Y. Li, et al. (2001). "Augmentation in expression of activation-induced genes differentiates memory from naive CD4+ T cells and is a molecular mechanism for enhanced cellular response of memory CD4+ T cells." J Immunol **166**(12): 7335-44.
Love, P. E. and E. W. Shores (2000). "ITAM multiplicity and thymocyte selection: how low can you go?" Immunity **12**(6): 591-7.
Lueking, A., M. Horn, et al. (1999). "Protein microarrays for gene expression and antibody screening." Analytical Biochemistry **270**(1): 103-11.
Macallan, D. C., D. Wallace, et al. (2004). "Rapid turnover of effector-memory CD4(+) T cells in healthy humans." J Exp Med **200**(2): 255-60.
MacBeath, G. (2002). "Protein microarrays and proteomics." Nature Genetics **32 Suppl**: 526-32.
MacBeath, G. and S. L. Schreiber (2000). "Printing proteins as microarrays for high-throughput function determination.[see comment]." Science **289**(5485): 1760-3.
Madrenas, J., R. L. Wange, et al. (1995). "Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists." Science **267**(5197): 515-8.
Maini, M. K., M. V. Soares, et al. (1999). "Virus-induced CD8+ T cell clonal expansion is associated with telomerase up-regulation and telomere length preservation: a mechanism for rescue from replicative senescence." J Immunol **162**(8): 4521-6.
Mak, T. W. (2007). "The T cell antigen receptor: "The Hunting of the Snark"." Eur J Immunol **37 Suppl 1**: S83-93.

Maskos, U. and E. M. Southern (1992). "Oligonucleotide hybridizations on glass supports: a novel linker for oligonucleotide synthesis and hybridization properties of oligonucleotides synthesised in situ." Nucleic Acids Research **20**(7): 1679-84.
Mason, R. D., R. De Rose, et al. (2008). "CD4+ T-cell subsets: what really counts in preventing HIV disease?" Expert Rev Vaccines **7**(2): 155-8.
Michel, F. and O. Acuto (2002). "CD28 costimulation: a source of Vav-1 for TCR signaling with the help of SLP-76?" Sci STKE **2002**(144): PE35.
Michel, F., G. Attal-Bonnefoy, et al. (2001). "CD28 as a molecular amplifier extending TCR ligation and signaling capabilities." Immunity **15**(6): 935-45.
Michie, C. A., A. McLean, et al. (1992). "Lifespan of human lymphocyte subsets defined by CD45 isoforms." Nature **360**(6401): 264-5.
Minguet, S., M. Swamy, et al. (2007). "Full activation of the T cell receptor requires both clustering and conformational changes at CD3." Immunity **26**(1): 43-54.
Minguet, S., M. Swamy, et al. (2008). "The extracellular part of zeta is buried in the T cell antigen receptor complex." Immunol Lett **116**(2): 203-10.
Mircean, C., I. Shmulevich, et al. (2005). "Robust estimation of protein expression ratios with lysate microarray technology." Bioinformatics **21**(9): 1935-42.
Morris, M. C., J. Depollier, et al. (2001). "A peptide carrier for the delivery of biologically active proteins into mammalian cells." Nat Biotechnol **19**(12): 1173-6.
Mullbacher, A. (1994). "The long-term maintenance of cytotoxic T cell memory does not require persistence of antigen." J Exp Med **179**(1): 317-21.
Murphy, M. A., R. G. Schnall, et al. (1998). "Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice." Mol Cell Biol **18**(8): 4872-82.
Mustelin, T., S. Rahmouni, et al. (2003). "Role of protein tyrosine phosphatases in T cell activation." Immunol Rev **191**: 139-47.
Myers, M. D., T. Sosinowski, et al. (2006). "Src-like adaptor protein regulates TCR expression on thymocytes by linking the ubiquitin ligase c-Cbl to the TCR complex." Nat Immunol **7**(1): 57-66.
Naramura, M., I. K. Jang, et al. (2002). "c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation." Nat Immunol **3**(12): 1192-9.
Naramura, M., H. K. Kole, et al. (1998). "Altered thymic positive selection and intracellular signals in Cbl-deficient mice." Proc Natl Acad Sci U S A **95**(26): 15547-52.
Negishi, I., N. Motoyama, et al. (1995). "Essential role for ZAP-70 in both positive and negative selection of thymocytes." Nature **376**(6539): 435-8.
Nel, A. E. (2002). "T-cell activation through the antigen receptor. Part 1: signaling components, signaling pathways, and signal integration at the T-cell antigen receptor synapse." J Allergy Clin Immunol **109**(5): 758-70.
Nishizuka, S., L. Charboneau, et al. (2003). "Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays." Proc Natl Acad Sci U S A **100**(24): 14229-34.
Nolan, J. P. and L. A. Sklar (2002). "Suspension array technology: evolution of the flat-array paradigm." Trends in Biotechnology **20**(1): 9-12.
Noraz, N., K. Schwarz, et al. (2000). "Alternative antigen receptor (TCR) signaling in T cells derived from ZAP-70-deficient patients expressing high levels of Syk." J Biol Chem **275**(21): 15832-8.
Northrop, J. P., S. N. Ho, et al. (1994). "NF-AT components define a family of transcription factors targeted in T-cell activation." Nature **369**(6480): 497-502.
Opferman, J. T., B. T. Ober, et al. (1999). "Linear differentiation of cytotoxic effectors into memory T lymphocytes." Science **283**(5408): 1745-8.

Pantaleo, G. and A. Harari (2006). "Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases." Nat Rev Immunol **6**(5): 417-23.
Panum, P. L. (1939). "Observations made during the epidemic of measles on the Faroe Islans in the year 1846." Med Classics **3**: 830-86.
Pasare, C. and R. Medzhitov (2004). "Toll-like receptors: linking innate and adaptive immunity." Microbes Infect **6**(15): 1382-7.
Paweletz, C. P., L. Charboneau, et al. (2001). "Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front." Oncogene **20**(16): 1981-9.
Pelosi, M., V. Di Bartolo, et al. (1999). "Tyrosine 319 in the interdomain B of ZAP-70 is a binding site for the Src homology 2 domain of Lck." J Biol Chem **274**(20): 14229-37.
Penna, D., S. Muller, et al. (1999). "Degradation of ZAP-70 following antigenic stimulation in human T lymphocytes: role of calpain proteolytic pathway." J Immunol **163**(1): 50-6.
Peretz, Y., M. L. Ndongala, et al. (2007). "Functional T cell subsets contribute differentially to HIV peptide-specific responses within infected individuals: correlation of these functional T cell subsets with markers of disease progression." Clin Immunol **124**(1): 57-68.
Perez, O. D. and G. P. Nolan (2002). "Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry." Nature Biotechnology **20**(2): 155-62.
Perez, O. D. and G. P. Nolan (2006). "Phospho-proteomic immune analysis by flow cytometry: from mechanism to translational medicine at the single-cell level." Immunological Reviews **210**: 208-28.
Petricoin, E. F., 3rd, V. Espina, et al. (2007). "Phosphoprotein pathway mapping: Akt/mammalian target of rapamycin activation is negatively associated with childhood rhabdomyosarcoma survival." Cancer Research **67**(7): 3431-40.
Phizicky, E., P. I. Bastiaens, et al. (2003). "Protein analysis on a proteomic scale." Nature **422**(6928): 208-15.
Polic, B., D. Kunkel, et al. (2001). "How alpha beta T cells deal with induced TCR alpha ablation." Proc Natl Acad Sci U S A **98**(15): 8744-9.
Porter, R. R. (1963). "Chemical Structure of Gamma-Globulin and Antibodies." Br Med Bull **19**: 197-201.
Qiu, M., Y. Shi, et al. (2005). "Antibody responses to individual proteins of SARS coronavirus and their neutralization activities." Microbes & Infection **7**(5-6): 882-9.
Retief, F. P. and L. Cilliers (1998). "The epidemic of Athens, 430-426 BC." S Afr Med J **88**(1): 50-3.
Rincon, M., D. Conze, et al. (2000). "Conference highlight: do T cells care about the mitogen-activated protein kinase signalling pathways?" Immunol Cell Biol **78**(2): 166-75.
Riou, C., B. Yassine-Diab, et al. (2007). "Convergence of TCR and cytokine signaling leads to FOXO3a phosphorylation and drives the survival of CD4+ central memory T cells." J Exp Med **204**(1): 79-91.
Robinson, W. H., C. DiGennaro, et al. (2002). "Autoantigen microarrays for multiplex characterization of autoantibody responses." Nature Medicine **8**(3): 295-301.
Rogers, P. R., C. Dubey, et al. (2000). "Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen." J Immunol **164**(5): 2338-46.
Rudolph, M. G., R. L. Stanfield, et al. (2006). "How TCRs bind MHCs, peptides, and coreceptors." Annual Review of Immunology **24**: 419-66.
Sallusto, F., J. Geginat, et al. (2004). "Central memory and effector memory T cell subsets: function, generation, and maintenance." Annu Rev Immunol **22**: 745-63.

Sallusto, F., D. Lenig, et al. (1999). "Two subsets of memory T lymphocytes with distinct homing potentials and effector functions." Nature **401**(6754): 708-12.
Samstag, Y., S. M. Eibert, et al. (2003). "Actin cytoskeletal dynamics in T lymphocyte activation and migration." J Leukoc Biol **73**(1): 30-48.
San Jose, E., A. G. Sahuquillo, et al. (1998). "Assembly of the TCR/CD3 complex: CD3 epsilon/delta and CD3 epsilon/gamma dimers associate indistinctly with both TCR alpha and TCR beta chains. Evidence for a double TCR heterodimer model." Eur J Immunol **28**(1): 12-21.
Sargin, B., C. Choudhary, et al. (2007). "Flt3-dependent transformation by inactivating c-Cbl mutations in AML." Blood **110**(3): 1004-12.
Schamel, W. W., I. Arechaga, et al. (2005). "Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response." J Exp Med **202**(4): 493-503.
Schamel, W. W., R. M. Risueno, et al. (2006). "A conformation- and avidity-based proofreading mechanism for the TCR-CD3 complex." Trends Immunol **27**(4): 176-82.
Schena, M., R. A. Heller, et al. (1998). "Microarrays: biotechnology's discovery platform for functional genomics.[see comment]." Trends in Biotechnology **16**(7): 301-6.
Schlessinger, J. (2000). "Cell signaling by receptor tyrosine kinases.[see comment]." Cell **103**(2): 211-25.
Schweitzer, B., S. Roberts, et al. (2002). "Multiplexed protein profiling on microarrays by rolling-circle amplification." Nature Biotechnology **20**(4): 359-65.
Seaman, M. S., F. W. Peyerl, et al. (2004). "Subsets of memory cytotoxic T lymphocytes elicited by vaccination influence the efficiency of secondary expansion in vivo." J Virol **78**(1): 206-15.
Secrist, J. P., L. A. Burns, et al. (1993). "Stimulatory effects of the protein tyrosine phosphatase inhibitor, pervanadate, on T-cell activation events." J Biol Chem **268**(8): 5886-93.
Seddon, B., P. Tomlinson, et al. (2003). "Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells." Nat Immunol **4**(7): 680-6.
Shao, W., Z. Zhou, et al. (2003). "Optimization of Rolling-Circle Amplified Protein Microarrays for Multiplexed Protein Profiling." J Biomed Biotechnol **2003**(5): 299-307.
Sharpe, A. H. and G. J. Freeman (2002). "The B7-CD28 superfamily." Nat Rev Immunol **2**(2): 116-26.
Sheehan, K. M., V. S. Calvert, et al. (2005). "Use of reverse phase protein microarrays and reference standard development for molecular network analysis of metastatic ovarian carcinoma." Molecular & Cellular Proteomics **4**(4): 346-55.
Slifka, M. K. and J. L. Whitton (2001). "Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR." Nat Immunol **2**(8): 711-7.
Sprent, J. and C. D. Surh (2002). "T cell memory." Annu Rev Immunol **20**: 551-79.
Stemberger, C., M. Neuenhahn, et al. (2007). "Origin of CD8+ effector and memory T cell subsets." Cell Mol Immunol **4**(6): 399-405.
Stewart-Jones, G. B., A. J. McMichael, et al. (2003). "A structural basis for immunodominant human T cell receptor recognition." Nat Immunol **4**(7): 657-63.
Straus, D. B. and A. Weiss (1992). "Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor." Cell **70**(4): 585-93.
Sun, Z. Y., S. T. Kim, et al. (2004). "Solution structure of the CD3epsilondelta ectodomain and comparison with CD3epsilongamma as a basis for modeling T cell receptor topology and signaling." Proc Natl Acad Sci U S A **101**(48): 16867-72.

Surh, C. D., O. Boyman, et al. (2006). "Homeostasis of memory T cells." Immunol Rev **211**: 154-63.

Swain, S. L., H. Hu, et al. (1999). "Class II-independent generation of CD4 memory T cells from effectors." Science **286**(5443): 1381-3.

Swaminathan, G. and A. Y. Tsygankov (2006). "The Cbl family proteins: ring leaders in regulation of cell signaling." J Cell Physiol **209**(1): 21-43.

Szodoray, P., P. Alex, et al. (2004). "Circulating cytokines in primary Sjogren's syndrome determined by a multiplex cytokine array system." Scandinavian Journal of Immunology **59**(6): 592-9.

Szodoray, P., P. Alex, et al. (2007). "Circulating cytokines in Norwegian patients with psoriatic arthritis determined by a multiplex cytokine array system." Rheumatology **46**(3): 417-25.

Tam, S. W., R. Wiese, et al. (2002). "Simultaneous analysis of eight human Th1/Th2 cytokines using microarrays." Journal of Immunological Methods **261**(1-2): 157-65.

Thien, C. B., F. D. Blystad, et al. (2005). "Loss of c-Cbl RING finger function results in high-intensity TCR signaling and thymic deletion." Embo J **24**(21): 3807-19.

Thien, C. B. and W. Y. Langdon (2005). "c-Cbl and Cbl-b ubiquitin ligases: substrate diversity and the negative regulation of signalling responses." Biochem J **391**(Pt 2): 153-66.

Tibes, R., Y. Qiu, et al. (2006). "Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells." Mol Cancer Ther **5**(10): 2512-21.

Tough, D. F. and J. Sprent (1995). "Life span of naive and memory T cells." Stem Cells **13**(3): 242-9.

Tough, D. F., S. Sun, et al. (1997). "T cell stimulation in vivo by lipopolysaccharide (LPS)." J Exp Med **185**(12): 2089-94.

Ueno, H., K. Sasaki, et al. (1998). "c-Cbl is tyrosine-phosphorylated by interleukin-4 and enhances mitogenic and survival signals of interleukin-4 receptor by linking with the phosphatidylinositol 3'-kinase pathway." Blood **91**(1): 46-53.

Usui-Aoki, K., K. Shimada, et al. (2005). "A novel approach to protein expression profiling using antibody microarrays combined with surface plasmon resonance technology." Proteomics **5**(9): 2396-401.

Vaccari, M., C. J. Trindade, et al. (2005). "Vaccine-induced CD8+ central memory T cells in protection from simian AIDS." J Immunol **175**(6): 3502-7.

Villinger, F., R. Miller, et al. (2004). "IL-15 is superior to IL-2 in the generation of long-lived antigen specific memory CD4 and CD8 T cells in rhesus macaques." Vaccine **22**(25-26): 3510-21.

Viola, A. (2001). "The amplification of TCR signaling by dynamic membrane microdomains." Trends Immunol **22**(6): 322-7.

von Muhlen, C. A. and E. M. Tan (1995). "Autoantibodies in the diagnosis of systemic rheumatic diseases." Seminars in Arthritis & Rheumatism **24**(5): 323-58.

Wange, R. L. (2000). "LAT, the linker for activation of T cells: a bridge between T cell-specific and general signaling pathways." Sci STKE **2000**(63): RE1.

Wegener, A. M., X. Hou, et al. (1995). "Distinct domains of the CD3-gamma chain are involved in surface expression and function of the T cell antigen receptor." J Biol Chem **270**(9): 4675-80.

Weil, R. and A. Veillette (1994). "Intramolecular and extramolecular mechanisms repress the catalytic function of p56lck in resting T-lymphocytes." J Biol Chem **269**(36): 22830-8.

Wherry, E. J., V. Teichgraber, et al. (2003). "Lineage relationship and protective immunity of memory CD8 T cell subsets." Nat Immunol **4**(3): 225-34.
Willinger, T., T. Freeman, et al. (2005). "Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets." J Immunol **175**(9): 5895-903.
Winters, M., B. Dabir, et al. (2007). "Constitution and quantity of lysis buffer alters outcome of reverse phase protein microarrays." Proteomics **7**(22): 4066-8.
Wolman, A. (1975). "[Importance of environmental sanitation in urban and rural areas for the control of intestinal infections]." Bol Oficina Sanit Panam **78**(4): 343-5.
Xia, Z., M. Dickens, et al. (1995). "Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis." Science **270**(5240): 1326-31.
Xu, C., M. E. Call, et al. (2006). "A membrane-proximal tetracysteine motif contributes to assembly of CD3deltaepsilon and CD3gammaepsilon dimers with the T cell receptor." J Biol Chem **281**(48): 36977-84.
Yalow, R. S. and S. A. Berson (1959). "Assay of plasma insulin in human subjects by immunological methods." Nature **184**(Suppl 21): 1648-9.
Yamasaki, S., K. Nishida, et al. (2001). "Docking protein Gab2 is phosphorylated by ZAP-70 and negatively regulates T cell receptor signaling by recruitment of inhibitory molecules." J Biol Chem **276**(48): 45175-83.
Yanagi, Y., A. Chan, et al. (1985). "Analysis of cDNA clones specific for human T cells and the alpha and beta chains of the T-cell receptor heterodimer from a human T-cell line." Proc Natl Acad Sci U S A **82**(10): 3430-4.
Yanagi, Y., Y. Yoshikai, et al. (1984). "A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains." Nature **308**(5955): 145-9.
Yang, W., H. Steen, et al. (2008). "Proteomic approaches to the analysis of multiprotein signaling complexes." Proteomics **8**(4): 832-51.
Yokosuka, T., K. Sakata-Sogawa, et al. (2005). "Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76." Nat Immunol **6**(12): 1253-62.
Zamoyska, R. (1998). "CD4 and CD8: modulators of T-cell receptor recognition of antigen and of immune responses?" Curr Opin Immunol **10**(1): 82-7.
Zaph, C., J. Uzonna, et al. (2004). "Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites." Nat Med **10**(10): 1104-10.
Zha, Y. and T. F. Gajewski (2007). "An adenoviral vector encoding dominant negative Cbl lowers the threshold for T cell activation in post-thymic T cells." Cell Immunol **247**(2): 95-102.
Zhang, W., J. Sloan-Lancaster, et al. (1998). "LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation." Cell **92**(1): 83-92.
Zhang, W., C. L. Sommers, et al. (1999). "Essential role of LAT in T cell development." Immunity **10**(3): 323-32.
Zhang, W., R. P. Trible, et al. (1998). "LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation." Immunity **9**(2): 239-46.
Zhong, L., C. H. Wu, et al. (2004). "Zeta-associated protein of 70 kDa (ZAP-70), but not Syk, tyrosine kinase can mediate apoptosis of T cells through the Fas/Fas ligand, caspase-8 and caspase-3 pathways." J Immunol **172**(3): 1472-82.
Zhou, H., K. Bouwman, et al. (2004). "Two-color, rolling-circle amplification on antibody microarrays for sensitive, multiplexed serum-protein measurements." Genome Biology **5**(4): R28.

Zhu, H., M. Bilgin, et al. (2001). "Global analysis of protein activities using proteome chips." Science **293**(5537): 2101-5.
Zhu, H. and M. Snyder (2003). "Protein chip technology." Current Opinion in Chemical Biology **7**(1): 55-63.

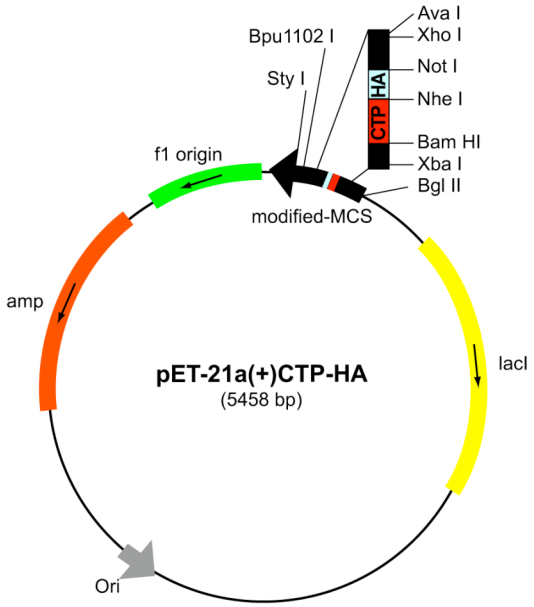
ANNEXES

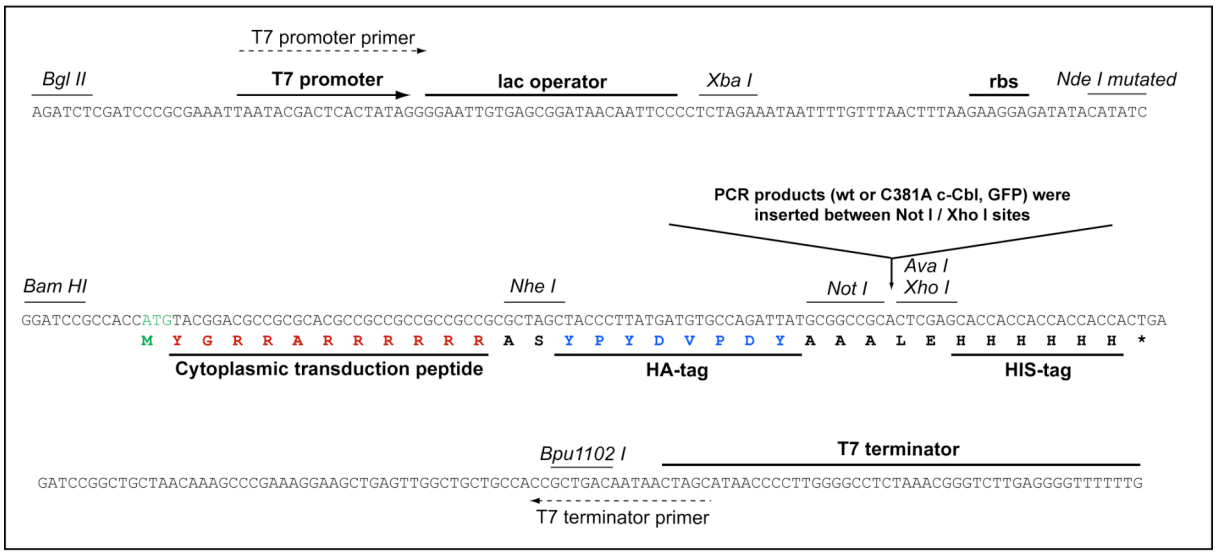
ANNEX I

1) Map of **bacterial expression vector** pET21a(+)-CTP-HA.

pET21a(+)-CTP-HA vector is derived from pET21A(+) vector (Novagen) and carries a cytoplasmic transduction peptide (CTP) sequence right after the starting metionine. Optional HA- and His-tag sequences are present at the N- and C-terminal of the cloning site, respectively. The cloning/expression region of the cloning strand transcribed by T7 RNA polymerase is shown below.

Wt or C381A c-Cbls or N-terminal His-tagged GFP obtained by PCR have been inserted into NotI / XhoI restriction sites. A stop codon was added at the end of the inserted genes, resulting in a construct not transcribing the C-terminal His-tag sequence.



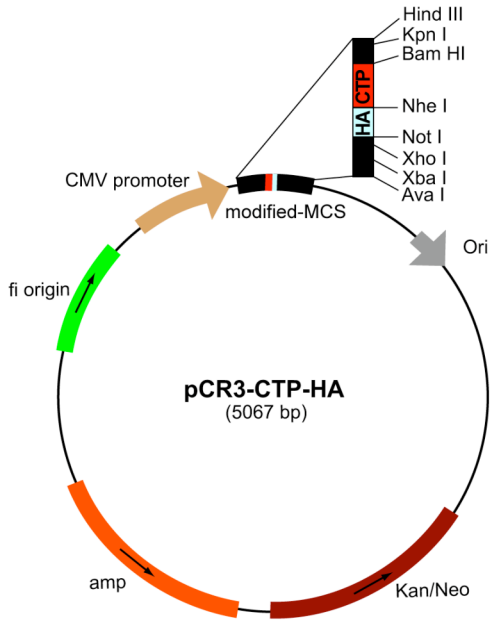


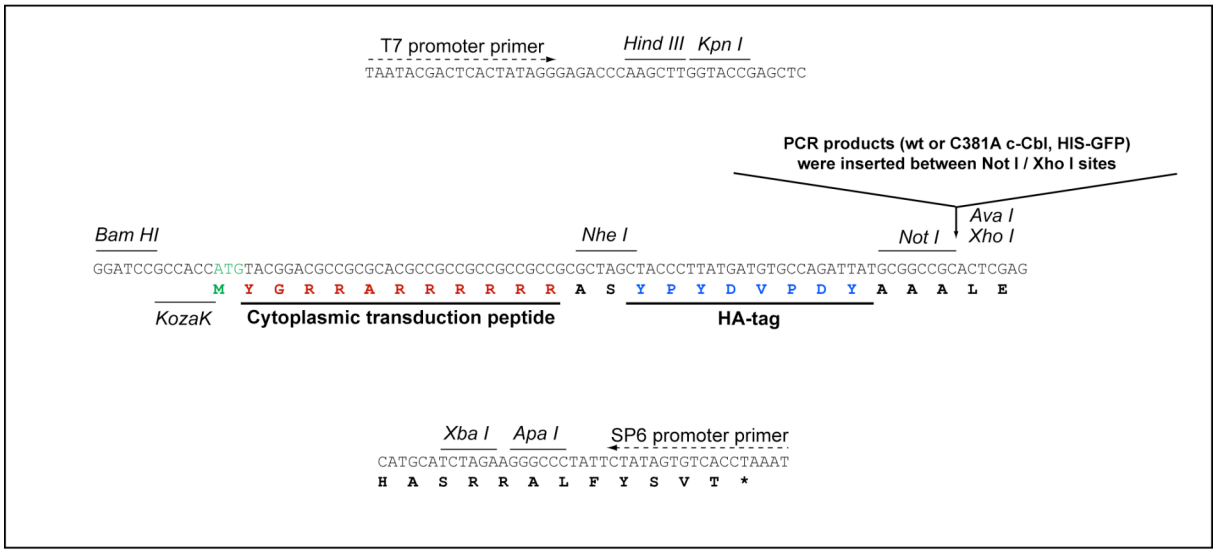
pET-21a(+)CTP-HA cloning/expression region

2) Map of **mammalian expression vector** pCR3-CTP-HA.

pCR3-CTP-HA vector is derived from pCR^{TM}3 vector (Invitrogen) and carries a cytoplasmic transduction peptide (CTP) sequence right after the starting metionine. Optional HA-tag sequence is present at the N-terminal of the cloning site. The cloning/expression region of the cloning strand transcribed by CMV promoter is shown below.

Wt or C381A c-Cbls or N-terminal His-tagged GFP obtained by PCR have been inserted into NotI / XhoI restriction sites.





pCR3-CTP-HA cloning/expression region

List of recombinant vectors built during this thesis.

Bacterial (pET21a-CTP-HA) and mammalian (pCR3-CTP-HA) expression plasmids carrying the cytoplasmic transduction peptide (CTP) at the N-terminal of the multiple cloning site were build and used as backbone for the insertion of the various genes. Details regarding the expression products obtained are provided.

Vector name	derived from	insert	comments
pET21a-CTP-HA	pET21a(+) (Novagen)	CTP-HA	bacterial expression vector
pCR3-CTP-HA	pCR™3 (Invitrogen)	CTP-HA	mammalian expression vector

Vector name	derived from	insert	expression host	comments
pET21a-CTP-HA c-Cbl wt	pET21a-CTP-HA	c-Cbl wt	*E.Coli* BL21(DE3)	multiple cleavage products
pET21a-CTP-HA c-Cbl C381A		c-Cbl C381A		
pCR3-CTP-HA c-Cbl wt	pCR3-CTP-HA	c-Cbl wt	Hek 293 cells	main product of 130 KDa as endogenously expressed by PBMC
pCR3-CTP-HA c-Cbl C381A		c-Cbl C381A		
pCR3-CTP-HA GFP		His-GFP		single fluorescent product of ~30 KDa
pMock		His-GFP (frameshift)		single product of ~33 KDa