

**Université de Fribourg
Département de Médecine
Unité de Biochimie**

Dissecting the human CD4+T cell memory pool

THESE

**Présentée à la faculté des Sciences de l'Université de Fribourg (Suisse)
pour l'obtention du grade de
*Doctor rerum naturalium***

Par

**Laura Rivino
de
Milan, Italie**

Thèse N°:1540

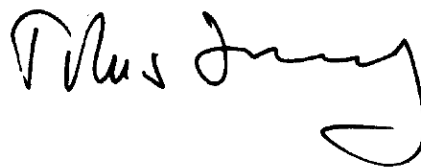
Thèse acceptée par la Faculté des Sciences de l'Université de Fribourg (Suisse) sur la proposition des professeurs:

- Jens Geginat, PhD (*IRB Bellinzona, local supervisor*)
- Anna Mondino, PhD (*San Raffaele Scientific Institute, expert externe*)
- Sandro Rusconi, Prof. Dr. (*UNIFR, Dép. de Medecine, Directeur de thèse*)
- Jean-Pierre Montani, Prof. MD. (*UNIFR, Dép. de Médecine, Président du jury*)

Fribourg, 11.10.06

Le Doyen:

Prof. Dr. Titus Jenny

A handwritten signature in black ink, appearing to read 'Titus Jenny', written in a cursive style.

Le Directeur de thèse

Prof. Dr. Sandro Rusconi

A handwritten signature in black ink, appearing to be a stylized 'SR', written in a cursive style.

A zia Mari

For aunt Mari

Table of contents

Abbreviations

Summary and aim of the study.....page 1

Riassunto e scopo dello studio.....page 3

I. Introduction.....page 6

1. Dendritic cells as bridges between innate and adaptive immunity

2. T cell maturation and induction of central tolerance

3. T cell activation

4. Generation of T cell memory

5. Heterogeneity of human memory T cells

5.1 *CD4⁺T cells: T_{H1}-T_{H2} versus Tr1*

5.2 *Migratory capacity reflecting effector function*

5.3 *T cell homing to skin versus gut and role of CCR6*

5.4 *Central memory and effector memory T cells*

5.4.1 *Heterogeneity of T_{CM} and T_{EM} cells*

6. Lineage relationship of memory T cell subsets

7. Mechanisms of peripheral tolerance

7.1 *Functional inactivation*

7.2 *Physical deletion*

7.3 *Ignorance*

7.4 *Suppression*

8. Regulatory T cells

8.1 *Naturally occurring T regulatory cells*

8.2 *Adaptive T regulatory cells*

References.....page 30

II. Resultspage 37

1. Comments on manuscripts

1.1 *Heterogeneity of the CD4⁺T central memory pool*

- 1.2 *Memory potential of antigen primed CD4⁺T cells*
- 1.3 *Characterization of IL-10 producing CD4⁺T memory cells*

2. Manuscripts

Manuscript 1page 45

Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4⁺ central memory T cells.

Laura Rivino, Mara Messi, David Jarrossay, Antonio Lanzavecchia, Federica Sallusto and Jens Geginat.

J.Exp.Med. 2004 200:725-735

Manuscript 2page 56

The strength of T cell stimulation determines IL-7 responsiveness, recall potential and lineage commitment of primed human CD4⁺IL-7R^{hi} T cells

Laura Lozza, Laura Rivino, Greta Guarda, David Jarrossay, Andrea Rinaldi, Francesco Bertoni, Federica Sallusto, Antonio Lanzavecchia and Jens Geginat.

(Submitted)

Manuscript 3page 108

CCR6 is induced upon tolerogenic priming and identifies Tr1 memory cells.

Laura Rivino, David Jarrossay, Antonio Lanzavecchia, Federica Sallusto and Jens Geginat.

(Manuscript in preparation)

III. Conclusions and perspectives.....page 130

Curriculum vitaepage 132

List of publicationspage 133

Acknowledgmentspage 134

Abbreviations

AICD	Activation induced cell death
APC	Antigen presenting cell
BrdU	Bromodeoxyuridine
CFSE	Carboxy-fluorescein succinimidyl ester
DC	Dendritic cell
IFN- γ	Interferon- γ
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
PdBu	Phorbol-12-13-dibutyrate
TCR	T cell receptor
IL-	Interleukin-
T _H 1	CD4 ⁺ T helper 1 cell
T _H 2	CD4 ⁺ T helper 2 cell
T _{CM}	Central memory T cell
T _{EM}	Effector memory T cell
TGF- β	Transforming growth factor- β
Treg	T regulatory cell
TREC	T cell receptor rearrangement excision circle
TSST	Toxic shock syndrome toxin
TT	Tetanus toxoid
VV	Vaccinia virus

Summary and aim of the study

Memory cells can persist for a whole lifetime. Consequently, these cells must possess characteristics which endows them with the capacity to survive in the absence of their cognate antigen, to self-renewal and to rapidly and efficiently respond upon secondary encounter of antigen. It has become increasingly clear that the memory pool can fulfil these diverse requirements because of its extreme heterogeneity which allows a division of labour among the different memory cell subsets. In my study I have investigated, together with my co-workers, the heterogeneity of the human CD4+T cell memory pool. These studies lead us to the identification of T cell memory subsets endowed with distinct immunological functions.

On the basis of expression of the chemokine receptors CXCR5, CXCR3 and CCR4, we identified cells among the CD4+T central memory (T_{CM}) pool, which represent unpolarized cells or precursors of T_H1 or T_H2 cells (pre- T_H1 , pre- T_H2), respectively. We show that in homeostatic conditions CCR7+CXCR3+ and CCR7+CCR4+ cells are pre-committed to give rise to T_H1 or T_H2 effector cells, respectively, whereas they maintain a certain degree of flexibility of cytokine gene expression upon secondary encounter of antigen in the presence of polarizing cytokines. Importantly, these results could explain how the quality of primary T cell responses is maintained in the absence of antigen, therefore allowing a rapid and efficient secondary response upon antigenic re-encounter (manuscript 1).

Furthermore, we investigated the signals that drive activated T cells to survive and generate long-lived memory T cells. We identified a subset of TCR-activated cells expressing CCR7 and the IL7R- α chain (CCR7+IL7R^{hi} cells), which possessed characteristics of memory cell precursors. However, we observed that CCR7+IL7R^{hi} cells were heterogeneous in their memory potential, depending on the signal strength they had received. In fact, CCR7+IL7R^{hi} cells generated at an intermediate strength of stimulation proliferated slowly with IL-7 and expanded efficiently upon secondary TCR stimulation, thus possessing characteristics of unpolarized T_{CM} cells. In contrast, strongly stimulated CCR7+IL7R^{hi} cells expanded poorly upon TCR engagement but displayed a high proliferative rate with homeostatic cytokines. In the latter conditions they differentiated spontaneously to T_H1 effector cells, a characteristic which is typical of circulating pre- T_H1 cells. We conclude that the memory potential of CD4+T

memory cells is dictated by the strength of stimulation they receive, and is not necessarily predictable by phenotypic markers. Our results are consistent with the view that T-cell memory is generated at an intermediate range of signal strength, and they suggest that T_{CM} and T_{EM} subsets could be derived from CCR7⁺ precursors that received different amounts of stimulation (manuscript 2).

Importantly, due to their antigen-experienced state, memory cells possess a lower threshold for proliferation compared to naïve cells and are thus more prone to autoreactivity. Autoreactivity may represent an important side effect of an anti-microbial immune response and mechanisms have evolved that maintain self-tolerance. In fact, in the peripheral blood of healthy individuals autoreactive T cells can be detected but are normally kept into check. We identified a subset of cells which seems to be involved in the maintenance of self-tolerance.

In manuscript 3 we describe a population of antigen-experienced CD4⁺Foxp3⁻ cells, distinct from natural Tregs, characterized by expression of the chemokine receptor CCR6. These cells secrete IL-10 in response to self-antigens and inhibit autoreactivity in an IL-10-dependent manner. Interestingly, we observed that in healthy donors, cells specific for MelanA, a self-antigen involved in the autoimmune disease vitiligo, are contained exclusively in the CCR6⁺ population, while in patients affected by vitiligo cells specific for MelanA can also be detected in the CCR6⁻ population. Surprisingly, the CCR6⁺ population also contains cells that proliferate and produce IL-2 in response to recall antigens. We show that single CCR6⁺T cell clones can respond to self upon neutralization of IL-10, but proliferate vigorously and produce IL-2 in response to tetanus toxoid. These results suggest a context dependent function of these CCR6⁺T memory cells, in that they exhibit a Tr1-like suppressive capacity when exposed to self-antigens, thus limiting autoimmune reactions, while in the presence of their specific antigen they behave as normal memory cells. The mechanism we describe may represent a strategy evolved by the immune system which allows the utilization of slightly autoreactive T cells, thus broadening the TCR repertoire available for pathogen recognition, while lowering the risk of autoimmune reactions.

Riassunto e scopo del lavoro

Le cellule della memoria possono persistere per tutta la vita. Pertanto queste cellule devono possedere caratteristiche che le rendano capaci di sopravvivere in assenza dell'antigene, di auto-rigenerarsi e di rispondere in modo rapido ed efficiente ad una successiva riesposizione all'antigene. Si e' ora compreso che l'insieme delle cellule della memoria puo' adempiere a compiti cosi' svariati grazie all'estrema eterogeneita' delle cellule che lo compongono, che permette una partizione dei compiti tra le diverse sottopopolazioni. In questo studio abbiamo analizzato l'eterogeneita' delle cellule T CD4+ umane e abbiamo identificato sottopopolazioni che sembrano essere destinate a particolari funzioni nell'ambito del sistema immunitario.

Abbiamo identificato diverse sottopopolazioni all'interno delle cellule T CD4+ "*central memory*" (T_{CM}) sulla base dell'espressione di diversi recettori di chemokine. Abbiamo osservato che sottopopolazioni di T_{CM} che esprimono CXCR5, CXCR3 e CCR4 rappresentano, rispettivamente, cellule non polarizzate o precursori di cellule T_H1 (pre- T_H1) o T_H2 (pre- T_H2). Qui mostriamo che in condizioni omeostatiche, cellule CCR7+ esprimenti CXCR3 e CCR4 sono pre-destinate a dare origine rispettivamente a cellule T_H1 o T_H2 , mentre mantengono un certo grado di flessibilita' in seguito ad un reincontro con l'antigene in presenza di citochine polarizzanti. La rilevanza di questo studio risiede nel fatto che potrebbe spiegare come viene mantenuta la qualita' della risposta primaria in assenza dell'antigene, permettendo in questo modo alle cellule della memoria di generare risposte secondarie rapide ed efficienti (manoscritto 1).

Abbiamo inoltre studiato i segnali che determinano se una cellula T attivata potra' sopravvivere e generare una cellula della memoria. Abbiamo identificato una sottopopolazione di cellule, attivate tramite il TCR, che esprimono CCR7 e la catena α del recettore dell'IL-7 (cellule CCR7+IL7-R^{hi}) e che possiede caratteristiche di precursori di cellule della memoria. Inoltre abbiamo osservato che la sottopopolazione di cellule CCR7+IL7-R^{hi} e' in realta' composta da cellule eterogenee che, in base alla forza dello stimolo che hanno ricevuto, possiedono una diversa potenzialita' a generare cellule della memoria. Infatti, cellule CCR7+IL7-R^{hi} che hanno ricevuto uno stimolo di forza intermedia, proliferano lentamente in presenza di IL-7 ma espandono

in maniera efficace in seguito a stimolazione antigenica, come osservato per cellule T_{CM} non polarizzate. Diversamente, cellule $CCR7+IL7-R^{hi}$ che hanno ricevuto uno stimolo più forte, proliferano poco in seguito ad una stimolazione antigenica ma si espandono rapidamente in presenza di citochine omeostatiche, differenziando spontaneamente in cellule T_H1 . Queste caratteristiche sono tipiche delle cellule pre- T_H1 . Possiamo concludere che la capacità di generare memoria di cellule $T\ CD4+$ è determinata dalla forza del segnale con cui queste cellule vengono stimulate e non è necessariamente prevedibile sulla base di marcatori fenotipici (manoscritto 2).

Le cellule della memoria, in quanto cellule che hanno precedentemente incontrato un antigene, possiedono una soglia di attivazione inferiore rispetto a cellule vergini o “naïve” e sono quindi maggiormente suscettibili a risposte autoimmunitarie. L’autoimmunità potrebbe costituire un importante e rischioso effetto collaterale di ogni risposta immunitaria anti-microbica e nel corso dell’evoluzione sono emersi diversi meccanismi atti a mantenere uno stato di non responsività verso componenti autologhi (tolleranza verso il *self*). In effetti, nel sangue periferico di individui sani si possono riscontrare numerose cellule autoreattive che in condizioni normali vengono mantenute inattive. In questo studio abbiamo identificato una sottopopolazione di cellule della memoria che sembra essere implicata in questo processo di tolleranza verso il *self*. Qui descriviamo una popolazione di cellule $CD4+Foxp3-$, diverse dalle cellule Treg $CD25+$, caratterizzate dall’espressione del recettore delle chemochine CCR6. Queste cellule secernono la citochina IL-10 in seguito al riconoscimento di un auto-antigene ed inibiscono l’autoreattività tramite un meccanismo dipendente dall’IL-10. Abbiamo osservato che in individui sani le cellule specifiche per MelanA, un autoantigene coinvolto nella malattia autoimmunitaria vitiligine, risiedono invariabilmente all’interno della sottopopolazione di cellule $CCR6+$. Diversamente, in pazienti affetti da vitiligine, le cellule specifiche per MelanA sono presenti anche nella sottopopolazione $CCR6-$.

La popolazione $CCR6+$ è inoltre costituita da cellule specifiche per antigeni estranei, incontrati precedentemente nel corso di una risposta primaria, le quali proliferano e producono IL-2 ad una riesposizione all’antigene. Qui mostriamo che cloni $CCR6+$ possono rispondere sia ad un antigene autologo, in seguito alla neutralizzazione dell’IL-10, sia ad un antigene estraneo come il tetano. I risultati qui riportati suggeriscono che la funzione delle cellule $CCR6+$ sia legata al particolare contesto. Infatti queste cellule si comportano in modo simile a cellule “soppressorie” quando

riconoscono un auto-antigene e quindi impediscono una risposta autoimmunitaria. Invece in presenza dell'antigene per le quali sono specifiche, si comportano da normali cellule della memoria. Il meccanismo che descriviamo potrebbe rappresentare una strategia che il sistema immunitario mette in atto per poter utilizzare cellule che possiedono una bassa affinità per i componenti autologhi. Questo permetterebbe una maggiore disponibilità di repertorio per le risposte verso i patogeni ma al contempo limiterebbe la risposta verso componenti autologhi (manoscritto 3).

I. Introduction

1. Dendritic cells as bridges between innate and adaptive immunity

Healthy individuals protect themselves against microbes by means of many different mechanisms. These include physical barriers, phagocytic cells and eosinophils in the blood and tissues, natural killer (NK) cells, and various blood-borne molecules, all of which participate in defending individuals from a potentially hostile environment. All of these defense mechanisms are present prior to exposure to infectious microbes or other foreign macromolecules, they are not enhanced by such exposures, and do not discriminate among most foreign substances. These are the components of the natural or innate immunity^{1, 2}. Differently, there are other defense mechanisms that are induced or stimulated by exposure to foreign substances. These mechanisms are extremely specific for distinct macromolecules, and increase in magnitude and defensive capabilities with each successive exposure to a particular macromolecule. They constitute the acquired or adaptive immunity, as they occur during the lifetime of an individual as an adaptation to infection with a pathogen. Foreign substances that induce specific immunity are called antigens. Adaptive immunity is a sophisticated antigen-specific defense system which is present only at higher levels of evolution and consists of T and B lymphocytes and their secreted products, such as antibodies. The particular feature of adaptive immune responses to “remember” the first encounter with a pathogen, is at the basis of the so-called “immunological memory”, and leads to an enhanced and more efficient response following reinfection with the same pathogen. The study of the complex mechanisms driving T and B lymphocyte memory generation is instrumental for the design of optimal vaccines which have already protected humans from many life-threatening diseases.

Dendritic cells (DCs) were first described more than thirty years ago and have been shown to be the only cell type which can initiate a primary immune response^{3, 4}. On one hand they possess the capacity to recognize pathogens, and on the other hand they are able to activate cells of the adaptive component of the immune system, thus constituting an important bridge between innate and adaptive immunity. DCs express

a broad repertoire of Toll-like receptors (TLRs), which are innate receptors that recognize distinct microbial products and trigger dendritic cell maturation and cytokine production⁵. After challenge with microbial or inflammatory stimuli, immature DCs undergo a complex process of maturation, resulting in their migration from tissues to secondary lymphoid organs and up-regulation of major histocompatibility complex (MHC) and co-stimulatory molecules, that are essential for the activation of cells of the adaptive immune system, such as T lymphocytes^{6, 7}.

Two subsets of DCs can be identified in human peripheral blood⁸. The first is represented by myeloid DC (mDC) which express CD1c, mature in response to a variety of stimuli, but produce IL-12 primarily in response to LPS or CD40L stimulation. The second subset is represented by plasmacytoid DC (pDC), which produce very high levels of IFN- α upon exposure to viruses. Furthermore, peripheral blood monocytes are considered as precursors of DCs⁹. Monocytes can be differentiated in vitro upon exposure to granulocyte/macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) to cells with characteristics similar to those of mDCs⁵. However, the situation in vivo appears to be more complex as recent work suggested that mDCs can derive from lymphoid or myeloid progenitors¹⁰.

2. T cell maturation and induction of central tolerance

T lymphocyte precursors arise in the bone marrow and subsequently migrate to the thymus where they undergo maturation. In the thymus, T cells learn to discriminate between self and non-self, in that on one side they are able to recognize foreign antigens in association to self-MHC molecules, but on the other side they will not attack self-components. This process is termed “central tolerance” and involves a phase of positive selection of T cells, which are selected for the ability to recognize through their T cell receptor (TCR), self-MHC/peptide complexes presented by thymic epithelial cells. Failure of such recognition within a certain window of affinity/avidity is interpreted as reflecting a useless specificity, i.e. lack of self-MHC restriction, and results in death by neglect of the respective T cell. T cells also undergo a phase of negative selection, where those cells that recognize peptide-MHC

complexes with too high affinity are deleted by apoptosis¹¹. As a result, the mature T cells which exit the thymus are self MHC-restricted and self-tolerant.

Recent data have pointed out an important role for thymic medullary promiscuous gene expression in the process of negative selection. In fact surprisingly, RNA transcripts encoding proteins previously considered to be synthesized only in particular peripheral tissues were detected in the thymus, in particular in medullary thymic epithelial cells (mTECs)¹². Several mouse models have revealed a direct link between ectopic synthesis of a designated protein in mTECs and the absence of peripheral lymphocyte reactivity to that protein. Many of the ectopically expressed antigens, (insulin, thyroglobulin, myelin basic protein, and retinal S-antigen) are associated with organ-specific autoimmune diseases (type 1 diabetes, thyroglobulin, multiple sclerosis and uveitis, respectively). Interestingly, there are some very suggestive correlations between antigen expression levels in the thymus and disease susceptibility in humans and rodents. An important clue towards understanding the regulation of this ectopic expression has been the finding that a protein with the features of a transcription factor, termed AIRE (autoimmune regulator), controls the expression of numerous genes in murine mTECs, with a predilection for tissue-restricted antigens. This important finding was further documented in humans with the observation that patients affected by the multiorgan autoimmune endocrine disease APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) presented mutations in the AIRE gene¹³. Two groups generated the AIRE-deficient mouse which presented a phenotype that was very similar to that of patients affected by APECED. AIRE deficient mice were characterized by a broad autoreactivity directed towards specific structures in many organs and analyses of these organs revealed lymphocytic infiltrates and presence of autoantibodies^{14, 15}.

3. T cell activation

Conventional T cells bear a T cell receptor composed of an α and β glycoprotein chain (TCR- $\alpha\beta$) and recognize antigen in association with MHC molecules displayed on the surface of APCs. Furthermore, a minor subset of T cells exists which possess a TCR composed of a γ and a δ chain (TCR- $\gamma\delta$). These cells are particularly abundant in the gut. Mature TCR- $\alpha\beta$ T cells can be subdivided into functionally distinct

populations expressing the membrane proteins CD4 or CD8. CD4+T cells are generally considered as “helper” T cells (T_H) and CD8+T cells as “cytotoxic” T cells (CTL), although additional features have now been identified for these populations.

When mature, antigen unexperienced, “naïve” T cells leave the thymus they continuously recirculate from the blood to secondary lymphoid organs and, within the T cell areas of these organs, they encounter DCs carrying antigen taken up locally or in peripheral tissues. The outcome of naïve T cell antigenic stimulation can lead to divergent responses that range from deletion of antigen-specific lymphocytes and tolerance to the generation of a large number of effector cells, followed by establishment of immunological memory.

The generation of such different T cell fates is dependent upon the strength of stimulation the T cell receives through its TCR interacting with peptide-MHC displayed on the surface of antigen-presenting cells (APCs), and the overall cytokine environment. The strength of T cell stimulation in turn is determined by different factors which all contribute and partially compensate each other: the concentration of and affinity for the antigen, which determines the rate of TCR triggering¹⁶⁻¹⁸, the presence or absence of co-stimulation, which regulates the extent of signal amplification¹⁹⁻²¹, and the duration of the interactions between T cells and DCs, which determines the duration of the signaling process^{22, 23}. Thus, T cells accumulate signals and gradually acquire “fitness”- defined as resistance to cell death and responsiveness to homeostatic cytokines- as a function of signal strength. These signals drive T cell differentiation by converging in a coordinated fashion in the regulation of transcriptional programmes that control the cell cycle, responses to cytokines, migratory capacity, effector function and susceptibility to activation-induced cell death (AICD). Consequently, the different T cell fates can be explained by a differential activation of some transcriptional programmes at a low strength of stimulation, whereas others require a higher strength of stimulation, as well as additional signals delivered by cytokines. This concept was proposed in the “progressive differentiation model”, which was also used to explain the generation of different memory subsets, and will be discussed further on.

4. Generation of T cell memory

In the course of a primary response three stages can be identified. The first stage, the “expansion” phase, is initiated in the lymphoid tissue where naïve T cells encounter their specific antigen presented by DCs, they clonally expand and differentiate into effector cells. Through the combined ability of CD4+T and CD8+T effector cells to secrete inflammatory cytokines and kill infected cells, a typical acute viral infection in mice can be cleared within days. Over the weeks that follow pathogen clearance, the majority (90%) of effector cells die, and this stage is often referred to as “contraction” period. The surviving T cells enter the third stage, the “memory” phase, in which the number of memory T cells stabilizes and can be maintained for a whole lifetime in the absence of antigen. Compared to naïve T cells, memory cells show more rapid and enhanced responses to antigenic stimulation, thus conferring better protection against secondary infections by the same pathogen.

Maintenance of memory T cells is achieved through a continuous and slow turnover of cells dependent upon the homeostatic cytokines IL-7 and IL-15, which belong to the common cytokine receptor γ -chain family (γ c or CD132 cytokines)^{24,25}. In particular, it was shown that IL-15 is important for maintaining CD8+ memory T cell numbers in vivo, while IL-7 seems to have a major role in the survival of naïve and memory CD4+T cells²⁶. It was shown recently that IL-7-R-derived signals play a non-redundant role for the generation of memory T cells in the mouse. Thus in the absence of IL-7 no memory T cells develop²⁷⁻²⁹. Furthermore, CD8+ T memory cell precursors can be identified by IL7-R α chain expression early at the peak of the proliferative response³⁰. These memory cell precursors showed enhanced survival, homeostatic proliferation and recall responses upon antigenic restimulation both in vivo and in vitro.

5. Heterogeneity of human memory T cells

5.1 CD4+T cells: T_H1 - T_H2 versus T_r1

Naïve CD4+ T helper (T_H) cells can undergo extremely different fates depending on the context in which they encounter their specific antigen. They can polarize towards T helper 1 (T_H1) or T_H2 cells which produce different sets of cytokines and mediate

protection from intracellular or extracellular pathogens respectively, or may be involved in B cell help^{31, 32}. T_H1 cells are characterized by secretion of interferon- γ (IFN- γ) and induction of cell-mediated responses against intracellular pathogens, including bacteria, parasites, yeast and viruses. T_H2 cells produce IL-4, IL-5, IL-10 and IL-13 and mediate protection against extracellular parasites and helminthes.

As well as their protective roles in host defense, both subsets of T_H cells have been implicated in pathological responses. T_H1 cells are involved in autoimmunity³³, and T_H2 cells have been implicated in the pathogenesis of asthma and allergy³⁴⁻³⁶. The balance between T_H1 and T_H2 cells is therefore critical in determining whether specific immunity against invading pathogens is successful and in avoiding pathological manifestations. T_H1 and T_H2 cells cross-regulate each other's function and development, in that IFN- γ produced by T_H1 cells inhibits the development of T_H2 cells, whereas production of IL-4 and IL-10 by T_H2 cells inhibits T_H1 development and activation.

It is widely accepted that the cytokines IL-12 and IL-4 are the key determinants in promoting T_H1 or T_H2 cells, respectively. Exposure of cells during TCR engagement to IL-12 or IL-4 leads to activation of specific transcription factors which are important in specifying the T_H1/ T_H2 phenotype, at least in part through regulation of chromatin structure and accessibility of cytokine genes. IL-12 and IL-4 act through signal transducer and activator of transcription (STAT4) or STAT6, respectively, which induce distinct signaling pathways. T_H1 cells express the transcription factor T-bet which induces remodeling of the endogenous *ifn- γ* locus and expression of the IL-12 receptor β 2 subunit (IL-12R β 2), thus increasing release of IFN- γ and response to IL-12. T_H2 cells express the transcription factor GATA-3 which plays an important role in inducing expression of T_H2 type cytokines³⁷.

T_H2 cells and T_H2 derived cytokines, in particular IL-10, have been shown to play a “regulatory” role in the process of cell tolerance through their capacity to inhibit immune responses. But now it is clear that specialized populations of regulatory T cells exist and can result from the stimulation of naïve CD4⁺ T cells in sub-optimal conditions. These cells have been named T regulatory 1 cells (Tr1) and will be discussed further on.

5.2 Migratory capacity reflecting effector function

Accumulating evidence indicates that during the T cell differentiation process, effector function and migratory capacity are coordinately regulated and as T cells polarize and acquire the capacity to produce a given cytokine they also acquire new migratory properties. The intimate connection between migratory capacity and function of a given T cell assures that the right cell will be at the right place at a given moment. The regulation of leukocyte migration is a complex process involving the sequential participation of adhesion molecules, such as selectins and integrins³⁸, as well as chemokines and chemokine receptors³⁹. The combined action of adhesion molecules and chemokines is thought to provide an address code for leukocyte migration to different sites⁴⁰. It is thus not surprising that T_H1 and T_H2 cells preferentially express different sets of chemokine receptors which guide them to sites where their function is needed.

Chemokines interact with G protein-coupled receptors possessing a seven transmembrane domain on their target cells, and receptor expression is a crucial determinant of the spectrum of action of chemokines⁴¹. Chemokines can be divided into different families (CC, CXC, CX₃C and C) according to the presence and the relative position of the NH₂-terminal cystein residues. Alternatively, they can be classified as homeostatic (constitutive) or inflammatory (inducible) according to their function. Homeostatic chemokines are expressed constitutively and appear to be responsible for trafficking of lymphocytes under conditions of homeostasis. In contrast, inflammatory chemokines are specifically up-regulated at sites of inflammation and are thought to play a role in the recruitment of lymphocytes to peripheral tissues in response to immunological challenge.

T_H1 cells preferentially express the chemokine receptors CCR5, CXCR3 and CCR1. In rheumatoid arthritis and multiple sclerosis, thought to be T_H1-associated diseases, virtually all T cells in the lesions express CCR5 and CXCR3, although usually only 5-15% of peripheral blood T cells have this phenotype. CCR1 and CCR5 are also expressed on monocytes and macrophages, and this explains their co-localization with T_H1 cells. T_H2 cells express CCR3 which is also expressed by eosinophils and basophils, and the shared expression may allow these cells to co-localize at sites of production of eotaxin, the ligand of CCR3. Besides CCR3, T_H2 cells also express CCR4, a receptor for TARC and MDC, important for DC-T interaction and also

expressed by basophils, CCR8, the receptor for I-309, and CrTh2, the ligand for prostaglandin D2. Of notice, some chemokine receptors are only preferentially expressed by T_H1 or T_H2 cells but may also be produced at lower levels by the opposite subset^{42, 43}.

5.3 T cell homing to skin versus gut and role of CCR6

The vast majority of antigens contact the body either at mucosal surfaces, in particular the intestine, or at the level of the skin. Increasing evidence shows that the microenvironment of these barriers has an influence on the immune response that develops locally. The gut mucosal environment is rich in the immunosuppressive cytokines IL-10 and TGF- β , which are believed to play a role in maintaining a state of unresponsiveness of lymphocytes to innocuous antigens deriving from food and commensal bacteria⁴⁴. Interestingly, it was shown that DCs from Peyer's patches produce high levels of IL-10 and constitutively express TGF- β mRNA, thus polarizing the immune response in an anti-inflammatory direction⁴⁵.

It has been proposed that during T cell priming, tissue-derived APCs within cutaneous versus intestinal secondary lymphoid organs imprint the corresponding tissue-specific homing phenotype to the T cell, such that the primed T cells have a predisposition to home back to the skin or gut, respectively^{46, 47}. Chemokine receptors expressed in combination with adhesion molecules were shown to allow tissue-specific homing of a particular T cell subset. Thus, the simultaneous expression of the cutaneous lymphocyte-associated antigen (CLA), binding to E-selectin, and CCR4 identifies skin-homing T cells⁴⁸. Conversely, expression of the integrin $\alpha_4\beta_7$ (binding to mucosal addressin cell-adhesion molecule-1, MADCAM-1) and CCR9 (binding to CCL25) is characteristic of gut-homing T cells⁴⁹. Not only do the skin-homing and gut-homing populations of cells expressing these markers home preferentially to these tissues, but also immunity to cutaneous and intestinal antigens, respectively, resides within them⁵⁰. The integrin $\alpha_E\beta_7$, which binds to E-cadherin, was initially described as a marker for T cells residing in the gut, but data from α_E deficient mice suggested an additional role of this molecule in the control of cutaneous inflammation^{51, 52}.

Another chemokine-receptor pair, CCR6 and its ligand CCL20/MIP-3 α /LARC/Exodus, seems to be involved in controlling migration to mucosal surfaces, in particular the gut, and to the skin⁵³. CCR6 is expressed on B cells, memory T cells, including skin-homing CLA⁺ and mucosa-homing $\alpha_4\beta_7$ ⁺ cells, and immature DCs, including immature Langerhans cells. Immature DCs were proposed to have a central role in inducing peripheral tolerance⁵⁴. Importantly, in humans, expression of CCR6 was also detected on a subset of suppressive DCs that over-express the enzyme indoleamine 2,3-dioxygenase (IDO)⁵⁵. It was reported that a “chemokine receptor switch” characterized by loss of CCR6 and gain of CCR7 expression occurs as DCs transition from immature cells, preferentially localized at epithelial sites to mature antigen-loaded cells, that home to T cell areas of draining lymphnodes⁵⁶.

CCL20 is thought to act both as an homeostatic and inflammatory chemokine, and it is expressed constitutively in several lymphoid and non lymphoid tissues (mainly tissues such as the skin and gut), although its expression is up-regulated with inflammation. Furthermore, human β -defensins, which are anti-microbial peptides released by epithelial cells at mucosal surfaces or in the skin following a pro-inflammatory stimuli, have been reported as non-chemokine ligands for CCR6⁵³.

Two research groups independently generated the CCR6 knock-out mouse and reported similar findings^{57, 58}. These mice were characterized by the absence of subsets of DCs expressing CD11c and CD11b from the sub-epithelial dome of Peyer’s patches and a 2-fold increase in the number of total lymphocytes and T cells within the mucosa. A closer analysis showed that this modest increase in total lymphocytes was due to much larger relative increases in several subpopulations of lymphocytes. The majority of the increased T cells were TCR- $\alpha\beta$ T cells, while there was little or no increase in TCR- $\gamma\delta$ T cells. Activated CD69⁺T cells, CD4⁺T and CD4⁺CD8⁺T were largely increased in the intestinal mucosa, but this was not accompanied by an increase in other sites. Furthermore, in T cell priming experiments CCR6 deficient mice presented altered T cell responses which varied considerably according to the experimental model of inflammation used^{55,57,58}. In fact, in 2,4-dinitrofluorobenzene (DNFB)-induced contact hypersensitivity studies these mice presented a more severe and more persistent inflammation than wild-type animals. The authors suggest a possible defect in the CD4⁺T cells responsible for down-regulating the DNFB-induced inflammation. In contrast, in a delayed-type

hypersensitivity model CCR6 defective mice developed no inflammatory response, which the authors suggest is due to an altered effector CD4⁺T cell response. These results seem to indicate that CCR6 identifies CD4⁺T cells possessing different functions, such that lack of these cells can impair either effector or “regulatory” T cell functions⁵⁸.

Several reports also suggest a role for CCR6/CCL20 in autoimmune diseases including psoriasis, inflammatory bowel disease and rheumatoid arthritis^{18, 59-63}. Indeed, CCL20 was expressed at higher levels in the synovial fluid of patients affected with rheumatoid arthritis, and both CCR6 and CCL20 were up-regulated within psoriatic lesions.

5.4 Central memory and effector memory T cells

A further heterogeneity of memory T cells has been recently proposed. Two subsets of CD4⁺ and CD8⁺ memory T cells have been identified in both humans and mice, based on functional and homing properties^{64, 65}.

Human memory T cells were defined according to expression of the lymph node homing receptors CCR7 and CD62L, which are characteristic of naïve T cells and are required for cell extravasation through high endothelial venules (HEV) and migration to T cell areas of secondary lymphoid organs (**Figure 1**)^{66, 67}. T central memory cells (T_{CM}) constitutively express CCR7 and CD62L and produce mainly IL-2 following TCR triggering, thus they lack immediate effector function but proliferate and become effector cells upon secondary stimulation. Conversely, T effector memory cells (T_{EM}) have lost the constitutive expression of CCR7, are heterogeneous for CD62L expression and express receptors involved in homing to inflamed tissues. Following TCR triggering, T_{EM} cells rapidly produce effector cytokines IFN- γ , IL-4 and IL-5, and CD8⁺ T_{EM} cells also release large amounts of perforin. Thus in humans, the T_{EM} pool contains *bona fide* T_H1, T_H2 and CTL.

The existence of two subsets of memory cells with distinct functions and migratory potential suggests a subdivision of tasks among memory cells. On one hand, T_{EM} cells represent a readily available pool of antigen-primed cells which can enter peripheral tissues due to expression of tissue-homing receptors and can give immediate protection. On the other hand, T_{CM} cells represent a “stem cell like” pool of antigen-

primed cells, which travel to secondary lymphoid organs due to expression of CCR7 and, upon a secondary challenge, can rapidly and efficiently generate a new wave of effector cells. In fact, data obtained in vitro with CD4⁺ human memory T cells show that T_{CM} cells expanded with homeostatic cytokines lose CCR7 expression and acquire CCR5 as well as the capacity to produce high levels of IFN- γ and IL-4, comparable to those produced by effector cells. Thus T_{CM} cells differentiate efficiently and generate cells with the characteristics of T_{EM} in an antigen-independent fashion. The behaviour of T_{CM} cells is different from naïve T cells which, expanded in the same condition, retain lymph node homing phenotype (CD45RA⁺CCR7⁺) and undergo only very limited differentiation⁶⁸. Similarly, cytokine-stimulated CD8⁺ T_{CM} cells differentiate and generate various types of effector cells expressing CCR7, perforin and CD45RA in different combinations. Single cell cloning of CD8⁺ T_{CM} cells with homeostatic cytokines revealed that this population is heterogeneous in that T_{CM} cells are programmed to generate different types of effector cells under homeostatic conditions⁶⁹.

The presence of memory cells with different migratory capacity and effector function was also documented in mice. Two populations of memory CD4⁺ T cells survive for months after immunization of antigen in adjuvant: a population of cells producing IL-2 was found primarily in the lymph nodes, while another larger population producing IFN- γ was found in non lymphoid tissues⁷⁰. Similarly, two populations of antigen-specific memory CD8⁺ T cells are detected following bacterial or viral infection⁶⁴. Whereas CD8⁺ memory T cells isolated from non lymphoid tissues exhibit lytic activity directly ex vivo, their splenic counterparts do not. These results allowed to extend the T_{EM}/ T_{CM} paradigm to the mouse system.

Since the first description of T_{CM} and T_{EM} it soon became evident that these two broad subsets of memory cells are heterogeneous in expression of chemokine receptors, adhesion and costimulatory molecules.

5.4.1 Heterogeneity of T_{CM} and T_{EM} cells

Primed and unprimed T cells can be distinguished on the basis of expression of the different isoforms of the leukocyte common antigen (CD45), a transmembrane tyrosinase phosphatase important for antigen receptor signal transduction⁷¹. Naïve T

cells express the isoform CD45RA which is progressively downregulated following TCR triggering and is accompanied by an increase in CD45RO expression⁷². Human memory T cells are mostly CD45RO+CD45RA-, except for a small subset of CD8+CCR7- cells (CD8+T_{EMRA}) which re-express CD45RA and are believed to represent the most differentiated type of memory cell. Human CD8+T_{EMRA}, specific for lytic but not latent Epstein-Barr virus antigens, have been detected after the acute phase of viral infection but were absent in persistent HIV infection. In vitro data suggests that CD45RA re-expression on antigen-experienced CD8+ T cells is inhibited by antigen and promoted by homeostatic cytokines, consistent with the selective and late appearance of T_{EMRA} cells in viral infections⁶⁹. More recently, a similar subset of cells was also described in the human CD4+ T cell compartment (CD4+ T_{EMRA})^{73, 74}. Human T_{CM} and T_{EM} can be subdivided into functional subsets on the basis of expression of the chemokine receptors described for T_H1 and T_H2 cells, and of other markers (**Figure 1**)⁷⁵.

A proportion of circulating T_{CM} express CXCR5, a chemokine receptor also expressed by B cells and whose ligand CXCL13 is expressed in B cell follicles. CXCR5+T cells are unpolarized and upon TCR triggering secrete mainly IL-2 and some IL-10, and were suggested to be involved in delivering help to B cells. Cells with this phenotype have also been identified in tonsils and were named follicular B helper T cells (T_{FH}) due to their capacity to potently induce antibody production during coculture with B cells. However, subsequent work demonstrated that the B cell helper activity was restricted to only a small subset of tonsillar CXCR5+ CD57+ cells⁷⁶⁻⁷⁸.

Chemokine receptors are expressed in combination with adhesion molecules, allowing tissue-specific homing of a particular T cell subset. For example as explained previously, the simultaneous expression of CLA and CCR4 identifies skin-homing T cells⁴⁸, whereas the expression of $\alpha_4\beta_7$ and CCR9 is characteristic of gut-homing T cells⁴⁹. Some skin and gut-homing T cells express CCR7, suggesting that they may be able to home to lymphoid as well as non-lymphoid tissues⁷⁹.

It should be noted that some of the described markers are rapidly and transiently modulated upon cell activation, thus the phenotypic characterization of T_{CM} and T_{EM} applies only to resting cells, i.e. those that are not engaged in an antigen-driven response.

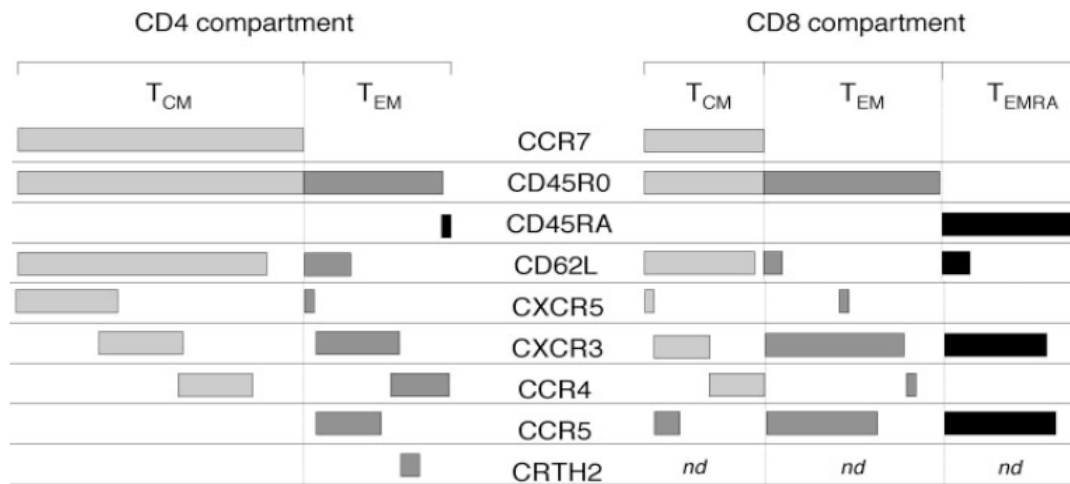


Figure 1. Phenotypic heterogeneity of human memory T cells (adapted from Sallusto et al. Annu. Rev. Immunol. 2004, 22:745-63).

6. Lineage relationship of memory T cell subsets

The lineage of memory T cells remains only partially understood and it is still controversial whether memory cells are direct descendants of effector cells or whether they arise independently from a separate lineage.

According to the “progressive differentiation model”, proposed by Lanzavecchia-Sallusto & co-workers, signal strength plays a major role in the differentiation of effector and memory cells, with the generation of memory cells occurring over a precise range of strength of stimulation (**Figure 2a**)³⁸. Stimulatory conditions which are below or above this productive range lead to cells which either die by neglect or are deleted by activation induced cell death, respectively. Within the productive range it is possible to generate cells which belong to a variety of differentiation stages ranging from effectors to cells that have been arrested at intermediate levels of differentiation, and possess distinct homing capacities. The latter cells represent precursors of T_{CM} cells and are maintained throughout the whole lifetime. Thus, within the same immune response T cells can receive different levels of signal strength either because the number and duration of T-APC interactions are stochastic events⁸⁰, or because T cells may be recruited at different times. In fact, at early stages of the immune response, there will be large numbers of mature DC carrying high doses of antigen and secreting large amounts of polarizing cytokines, while at later time points the stimulatory

conditions may change substantially with arrival of fewer DC, which carry low amounts of antigen and have exhausted their cytokine-producing capacity⁸¹. According to this model, precursors of T_{EM} would be preferentially generated early on, whereas precursors of T_{CM} would be preferentially generated at late stages of the immune response. The model proposed above is consistent with the observation that memory precursors are not present at the peak of the immune response but progressively appear during the contraction phase coincident with global changes in transcriptional profiling⁸². As mentioned previously, data obtained in vitro with human memory T cells indicate that T_{CM} can generate T_{EM} upon cytokine or antigen stimulation, whereas T_{EM} could either further differentiate (for instance acquire additional cytokine producing capacity) or retain the original imprinted phenotype^{68, 83}. In contrast with these observations, TCR repertoire analyses of human CD8+ T_{CM} and T_{EM} cells revealed that the two populations have a stable repertoire over a long period of time, but share few common clonotypes⁸⁴. The largely distinct repertoires between CD8+ T_{CM} and T_{EM} cells seem to suggest that T_{EM} cells may not have derived from T_{CM} cells, but that the two populations are distinct and are generated independently. Alternatively, the precursors of T_{EM} cells within the T_{CM} pool may have disappeared, do not re-circulate in blood, or have reached an undetectable frequency⁸⁵. Further studies will be required to distinguish between these possibilities. Recently, it was shown for human CD4+T memory cells that T_{EM} have a much more rapid in vivo turnover compared to T_{CM}, indicating that T_{EM} represent a short-lived cell population that requires continuous replenishment⁸⁶. This is compatible with a model in which long-lived T_{CM} cells continuously replenish the pool of T_{EM} cells, but could also suggest that the two populations are maintained as separate pools with distinct turnover rates.

Another model proposed by Ahmed and co-workers and called the “linear differentiation” model, was based on data obtained with CD8+T memory cells in the mouse system (**Figure 2b**). This model states that memory cells are direct descendents of effector cells, thus memory does not develop until antigen is cleared or greatly decreased in concentration, and the precursors of memory cells are true effector cells. According to this model, T_{EM} cells are only a transitory population representing an “intermediate” cell type in the effector to memory transition, and in the absence of antigen they convert directly into T_{CM}, which are the true memory cells, and only then gain the ability to undergo efficient homeostatic turnover. Indeed, it was shown that

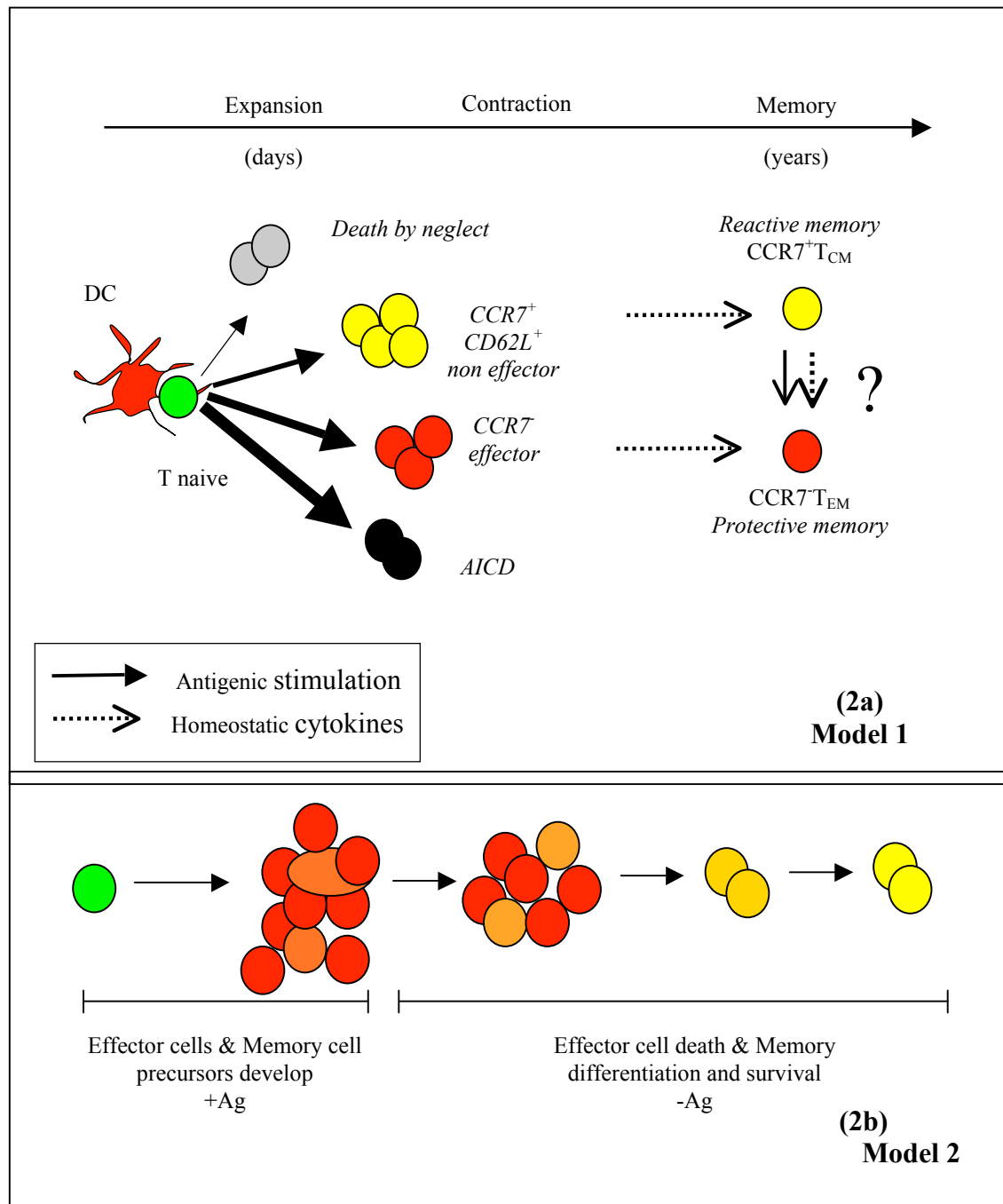


Figure 2. Models of memory T cell generation.

(2a) Model 1: Progressive differentiation model for T cell differentiation and memory T cell generation. The duration and intensity of antigenic stimulation is indicated by the length and thickness of 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 Sallusto et al. Ann.Rev.Immunol. 2004, 22:745-63) **(2b) Model 2: Linear progressive differentiation model of memory CD8 T cell precursors during the contraction phase following antigen clearance.** Memory T cells generate directly from effector cells during the expansion phase and gradually acquire memory cell properties (adapted from Kaech et al. Cell 2002, 111: 837-851).

cells with T_{EM} characteristics (cytotoxicity and low expression of CD62L) when adoptively transferred into naïve recipients, were able to “revert” to T_{CM}⁸⁷. As mentioned previously, further studies from the same group showed that at the peak of the CD8 response there is a small fraction of effector cells (typically CD62L negative) with high expression of CD127, the IL7R- α chain, which possess characteristics of memory cell precursors³⁰. Adoptive transfer in naïve recipients of IL-7R^{hi} and IL-7R^{lo} effector cells showed that IL-7R^{hi} cells preferentially gave rise to memory cells that could persist and confer protective immunity. Other groups described CD8+T cell expression of the IL7R- α chain as an early marker of memory T cells in vivo^{88, 89}. In particular they showed that IL7R expression combined with that of CD62L, could distinguish between functionally distinct memory T cell subsets. These cells possessed the characteristics of previously described central memory T cells (IL7R^{hi} CD62L^{hi}) and effector memory T cells (IL7R^{hi} CD62L^{lo}).

7. Mechanisms of peripheral tolerance

T cell tolerance is established early during T cell development in the thymus by deletion of autoreactive T cells (central tolerance). However, this deletion is not complete as there is evidence that potentially dangerous self-reactive T cells are present in the periphery of healthy individuals⁹⁰. These T cells are kept in check by additional mechanisms which go under the name of “peripheral tolerance” and act on mature T lymphocytes that have left the thymus. Peripheral tolerance is achieved by mechanisms which include functional inactivation, physical deletion of autoreactive T cells, ignorance and suppression by specialized T cell populations⁹¹.

7.1 Functional inactivation

At least two signals are required for optimal T cell activation. The first signal, which ensures specificity of the response, is delivered by TCR interaction with MHC and peptide, while the second signal involves ligation of co-stimulatory molecules and is

needed for enhancing cytokine production, augmenting cell proliferation and cell survival. Antigen recognition in the absence of co-stimulation may lead to a state of functional unresponsiveness, also called anergy. This state was first demonstrated in CD4⁺T cell clones in which antigen receptors were engaged without co-stimulation. In the absence of co-stimulation T cells were unable to proliferate, unable to produce IL-2 but were not deleted^{92, 93}. This situation could resemble the in vivo presentation of self-antigens in peripheral tissues, which are normally presented to the T cells in the absence of inflammation. Furthermore, it seems that anergy can also be induced by foreign antigens administered without adjuvant.

There is evidence that in vivo T cell anergy may be induced not only because of a lack of co-stimulation, but as a result of specific recognition of B7 molecules by CTLA-4. These evidences pointed out an important role for interaction of either CD28-B7 or CTLA-4-B7 on the outcome of antigen recognition by T cells⁹⁴.

7.2 Physical deletion

Autoreactive T cells can be physically deleted by apoptosis, which can be induced by opposing signals such as repeated antigenic stimulation, “activation induced cell death” (AICD), or inadequate survival stimuli, “death by neglect”. Although both pathways of apoptosis share the same terminal effector phase and show the same morphological and biochemical manifestations, their induction, molecular controls and physiological functions are largely distinct. AICD is induced by repeated stimulation, with high levels of IL-2 production and is not prevented by the anti-apoptotic molecules Bcl-2 or Bcl-X_L. Under these conditions, T cells co-express death cytokines such as FasL and TNF- α which interact with their respective receptors and recruit and activate caspases leading to apoptotic death of the T cell. In fact, autoreactive T cells in the periphery repeatedly encounter persistent self-antigen leading to co-expression of death cytokine-receptor pairs and apoptotic death of the T cell. Mice with defects in Fas, FasL, IL-2R- α or β chain exhibit defects in AICD and develop a lupus-like autoimmune disease, which is due to abnormally prolonged survival of autoreactive helper T cells and an inability to eliminate self-reactive B lymphocytes by apoptosis⁹⁵. Furthermore in humans, mutations in the *fas* gene are associated with a lymphoproliferative autoimmune syndrome⁹⁶.

At the other extreme, both naïve or activated T cells can die “by neglect” because of lack of survival stimuli, such as co-stimulators and cytokines. This form of apoptosis is distinct from that of AICD since it does not involve death cytokines and death receptors⁹⁷. Following growth factor withdrawal in fact, cells experience a metabolic arrest causing mitochondrial damage and release of cytochrome c into the cytoplasm, with consequent activation of caspases and death by apoptosis. Differently from AICD, over-expression of anti-apoptotic molecules Bcl-2 or Bcl-xL in lymphocytes prevents death by neglect^{98, 99}.

7.3 Ignorance

Some self-antigens fail to induce an immune response simply because they are ignored by the immune system. This occurs when antigens are expressed in immunoprivileged sites, where they are anatomically sequestered in a tissue and do not reach organized lymphatic tissues in sufficient amounts. Alternatively antigens could lack an appropriate presentation to lymphocytes in an inflammatory context by mature APCs. Potentially, these self-antigens could induce autoimmune pathology if an unrelated infection would cause their release and presentation in an immunogenic inflammatory context¹⁰⁰.

7.4 Suppression

There is now convincing evidence that, in addition to the mechanisms of self-tolerance described above, a specialized population of CD4⁺ T cells, called regulatory T cells (Tregs) exists. Tregs are involved in the maintenance of peripheral self-tolerance by actively suppressing the activation and expansion of self-reactive T cells. Two major populations of Tregs have been described so far, naturally occurring and adaptive or IL-10-secreting Tregs (**Table 1**).

8. Regulatory T cells

8.1. Naturally occurring T regulatory cells

Different findings, the first of which were made thirty years ago, suggested the existence within the CD4⁺T cell population, of a subset of CD4⁺T cells endowed with an autoimmune-inhibitory activity¹⁰¹. Furthermore, it was shown that in mice a population of cells contained within CD4⁺CD45Rb^{low} cells (which constitute the compartment of activated CD4⁺T cells found in normal mice) was able to control colitis induced by the transfer of CD4⁺CD45Rb^{high} (naïve) cells^{102, 103}. Finally, Sakaguchi and co-workers proposed CD25, the α -chain of the IL-2 receptor, as a cell surface marker which could identify this suppressor population¹⁰⁴. CD4⁺CD25⁺T cells also called “naturally occurring” Tregs, were initially described in mice as naturally anergic and suppressive cells, produced by the thymus as a functionally distinct subpopulation of T cells and involved in maintaining self-tolerance. Evidence is now accumulating that Tregs are also involved in controlling immune reactivity towards foreign antigens¹⁰⁵. Removal of CD25⁺ Tregs leads to a spontaneous development of various autoimmune diseases in otherwise normal mice. Furthermore, the removal of these cells also triggers excessive or misdirected immune responses to microbial antigens, causing immunopathologies such as inflammatory bowel disease (IBD) which is due to hyper-activation of the remaining T cells to commensal bacteria in the intestine¹⁰³.

At first, these cells were thought to be produced exclusively in the thymus but more recent data also suggested that, *in vivo*, they could be derived *de novo* from naïve T cells in the periphery after a prolonged exposure to low doses of antigen¹⁰⁶. These cells were phenotypically and functionally indistinguishable from intra-thymically derived Tregs. There is also evidence that mouse CD25⁺Tregs can be generated *in vitro* from peripheral CD25⁺CD4⁺T cells stimulated through their TCR in the presence of TGF- β . These “converted” Tregs inhibited CD4⁺T cell proliferation when transferred *in vivo*¹⁰⁷.

CD4⁺CD25⁺Tregs have also been identified in human peripheral blood and when assayed *in vitro* showed similar characteristics to mouse Tregs¹⁰⁸. In both rodents and humans, CD4⁺CD25⁺T cells represent 5-10% of the CD4⁺T lymphocytes.

Although CD25, the IL-2R α chain, was widely used as a marker for identifying Tregs in both humans and mice, it is not an optimal marker to distinguish these cells as it is also expressed by activated T cells. IL-2R α expression by Tregs correlates with the requirement of IL-2 for their survival and expansion in the periphery. In contrast, IL-2 could be dispensable for their generation. In fact, Tregs from IL2-/- mice are reduced in numbers but remain endowed with suppressive activity when transferred into wild-type recipients. Tregs do not themselves produce IL-2 and rely on IL-2 that is probably produced by effector cells. Thus, Tregs seem to be intimately linked to the effector immune response that they regulate¹⁰⁹⁻¹¹¹. In fact, it was recently shown that expression of the high affinity IL-2R α by Tregs endows these cells with the capacity to exploit the IL-2 resource and keeps Treg numbers tied to the number of activated IL-2 producing CD4+T cells¹¹⁰.

A milestone in the study of CD25+ Tregs was the description of Foxp3, a forkhead family of transcription factors specifically expressed by Tregs and considered to be the master control gene for the development and function of natural Tregs¹¹²⁻¹¹⁴. In mice Foxp3 is both necessary and sufficient for the development and function of Tregs. Mice lacking functional Foxp3 develop a fatal autoimmune lymphoproliferative disease and analyses of CD4+CD25+T cells from these mice revealed that they lack suppressive activity, indicating that they do not represent Tregs¹¹⁵. Foxp3 defective mice could be rescued by transferring wild-type CD4+CD25+T cells. Furthermore, ectopic expression of Foxp3 in conventional CD4+CD25-T cells by retro-virus mediated gene transfer conferred suppressor function to this T cell subset both in vitro and in vivo¹¹²⁻¹¹⁴. These data clearly indicated that in mice Foxp3 is an optimal marker for the identification of these cells¹¹⁶. However, the human system was found to be much more complex and analysis of Foxp3 expression showed several differences compared to the mouse system¹¹⁷. Human FOXP3 exists in two isoforms, one of which is ortholog to mouse Foxp3. It is unclear whether the 2 isoforms are expressed simultaneously and if there is a functional difference between them¹¹⁸. In humans, Foxp3 is expressed by CD25+ Tregs but differently from mice it is also induced in all CD4+ T cell populations following T cell activation. Whether Foxp3 expression in activated T cells correlates with a suppressive activity is still controversial^{118, 119}. Consistent with the idea that Foxp3 in humans is linked to TCR stimulation, human CD4+ T cell clones were

found to be Foxp3⁺, while expression of the protein was not detected in mouse T cell clones¹¹⁷. Several groups have attempted to ectopically express Foxp3 in human CD4⁺CD25⁺-T cells, as was done in mouse cells, and this resulted in T cell hypo-responsiveness and suppression of production of cytokines such as IL-2 and IFN- γ , but did not seem to lead to acquisition of significant suppressive activity in vitro¹¹⁸.

Furthermore, a human syndrome was identified, IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X-linked syndrome), which presents clinical features similar to those found in the Foxp3 deficient mice. Further analyses revealed that a large proportion of the patients carried mutations in the gene encoding for Foxp3.

How *foxp3* is controlled and which signals induce its expression still remains elusive, although some signalling pathways, including CD28, IL-2 and TGF- β are emerging that appear to have an effect on the expression of *foxp3*¹¹⁷.

It is known that an initial activation of natural Tregs via the TCR is required for suppression to occur, but the mechanism of suppression has been a controversial point. Studies utilizing different experimental models lead to contrasting results with major differences being observed between in vitro and in vivo models¹²⁰. In vitro suppression of Tregs seems to be totally dependent upon cell-contact mechanisms, as suppression does not occur when cells are separated by a permeable membrane. In contrast, in vivo experiments showed an essential role for cytokines such as IL-10 and TGF- β ¹²⁰. Recent data using a T cell transfer model of colitis revealed an essential role of TGF- β for Treg control of pathogenic effector cells, although surprisingly TGF- β did not necessarily have to be synthesized from Tregs themselves, as Tregs from TGF- β deficient mice were still functional and suppression remained TGF- β dependent, suggesting a different cellular source of TGF- β ¹²¹.

8.2. Adaptive T regulatory cells

An additional population of Tregs, called adaptive Tregs, was described to develop as a consequence of activation of mature T cells in the periphery under particular conditions of sub-optimal antigenic stimulation, such as with immature DCs or in the presence of immunosuppressive drugs or cytokines IL-10 and TGF- β .

A subset of adaptive T regulatory cells, designated T regulatory 1 cells, (Tr1), producing IL-10, was described in both humans and mice. This subset was derived by different groups by using different protocols, so it is still unclear whether and how they are related to each other¹²²⁻¹²⁵.

Tr1 cells were first described by Roncarolo and co-workers who showed that chronic activation of both human and murine CD4⁺T cells in the presence of IL-10, gave rise to CD4⁺ T cell clones characterized by low proliferative capacity, production of high levels of IL-10 and low levels of IL-2^{122, 123}. TGF- β was also produced by these cells, but at levels comparable to other T cell subsets. Human and murine Tr1 clones were capable of suppressing the proliferation of CD4⁺T cells in vitro, in an antigen specific manner. Furthermore, murine Tr1 cells, after stimulation in vivo, also prevented colitis induced in SCID mice by pathogenic CD4⁺CD45Rb^{high} splenic T cells. In both human and murine systems, suppression of T cell proliferation was dependent upon secreted IL-10 and TGF- β and was cell-contact independent.

Of note, Tr1 cells generated according to this protocol produce IL-10 but in addition inflammatory cytokines such as IL-5 and IFN- γ . Later reports by the same group showed that Tr1 cells secreting IL-10 and IFN- γ could be generated by using IL-10 in combination with IFN- α ¹²⁴.

By using a combination of the immunosuppressive drugs vitamin D3 (VitD3) and dexamethasone (Dex), O'Garra and co-workers succeeded in generating an homogeneous population of both human and mouse Tr1 cells, producing only IL-10 and no inflammatory cytokines such as IL-5 or IFN- γ ¹²⁵. Murine Tr1 cells generated according to this protocol were capable of suppressing, in an IL-10 dependent manner, experimental autoimmune encephalomyelitis (EAE) when activated at the site of disease. Importantly, it was reported that Tr1 cells do not express Foxp3, and are thus distinct from naturally occurring Tregs¹²⁶.

Another subset of adaptive T regulatory cells was described in both mice and humans, based on their ability to produce TGF- β and varying levels of IL-10 and IL-4. These cells were termed Th3 cells, although it still remains unclear whether they are distinct from Tr1 cells. Th3 cells were first described in mice after oral tolerance induction to myelin basic protein (MBP). These cells were able to suppress experimental autoimmune encephalomyelitis (EAE) via a TGF- β dependent mechanism¹²⁷. Generation of similar cells, endowed with the capacity to prevent autoimmune disease

in several animal models was further reported¹²⁷⁻¹²⁹. In vitro differentiation of Th3 cells was enhanced by TGF- β , IL-4 and IL-10¹³⁰. Furthermore, in patients suffering from multiple sclerosis, oral treatment with MBP and proteolipid protein (PLP) induced a significant increase in the frequency of MBP- or PLP-T cells that secreted TGF- β , suggesting that Th3 cells also exist in humans¹³¹. In this, as in many other studies, TGF- β and IL-10 were not evaluated in parallel, thus making it difficult to understand if Th3 and Tr1 cells represent distinct T regulatory subsets.

Several groups documented in the human system that adaptive T regulatory cells can also be induced in the periphery by CD25+ Tregs via a cell-contact dependent effect, mechanism which was termed “infectious tolerance”. The induced regulatory T cells, which much resemble the described Tr1 cells, produce immunosuppressive cytokines IL-10 and/or TGF- β and are able to suppress the activation of effector cells via a cytokine dependent, cell-contact independent mechanism^{132, 133}.

The existence of natural and adaptive T regulatory cells has been largely documented but many questions remain open regarding their origin, mechanism of action and the relations occurring between them.

The general characteristics of naturally occurring Tregs and adaptive Tregs are summarized in **Table 1**.

Treg type	Origin	Phenotype	Foxp3	Mode of suppression
Naturally occurring Tregs	Thymus (and periphery)	CD25+	Positive	In vitro: cell-contact In vivo: cell-contact IL-10, TGF- β
Adaptive Tregs (Tr1/Th3)	Periphery	Unknown	Negative	IL-10 and/or TGF- β

Table 1. General characteristics of naturally occurring and adaptive Tregs.

References

1. Medzhitov, R. & Janeway, C. A., Jr. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91, 295-8 (1997).
2. Fearon, D. T. & Locksley, R. M. The instructive role of innate immunity in the acquired immune response. *Science* 272, 50-3 (1996).
3. Steinman, R. M. & Cohn, Z. A. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137, 1142-62 (1973).
4. Hart, D. N. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* 90, 3245-87 (1997).
5. Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 179, 1109-18 (1994).
6. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5, 987-95 (2004).
7. Banchereau, J. & Steinman, R. M. Dendritic cells and the control of immunity. *Nature* 392, 245-52 (1998).
8. Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F. & Lanzavecchia, A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur J Immunol* 31, 3388-93 (2001).
9. Geissmann, F., Jung, S. & Littman, D. R. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19, 71-82 (2003).
10. Chicha, L., Jarrossay, D. & Manz, M. G. Clonal type I interferon-producing and dendritic cell precursors are contained in both human lymphoid and myeloid progenitor populations. *J Exp Med* 200, 1519-24 (2004).
11. Venzani, E. S., Benoist, C. & Mathis, D. Good riddance: Thymocyte clonal deletion prevents autoimmunity. *Curr Opin Immunol* 16, 197-202 (2004).
12. Gotter, J. & Kyewski, B. Regulating self-tolerance by deregulating gene expression. *Curr Opin Immunol* 16, 741-5 (2004).
13. Su, M. A. & Anderson, M. S. Aire: an update. *Curr Opin Immunol* 16, 746-52 (2004).
14. Anderson, M. S. et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298, 1395-401 (2002).
15. Ramsey, C. et al. Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. *Hum Mol Genet* 11, 397-409 (2002).
16. Viola, A. & Lanzavecchia, A. T cell activation determined by T cell receptor number and tunable thresholds. *Science* 273, 104-6 (1996).
17. Valitutti, S., Muller, S., Cella, M., Padovan, E. & Lanzavecchia, A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* 375, 148-51 (1995).
18. Wulfig, C. et al. Kinetics and extent of T cell activation as measured with the calcium signal. *J Exp Med* 185, 1815-25 (1997).

19. Viola, A., Schroeder, S., Sakakibara, Y. & Lanzavecchia, A. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* 283, 680-2 (1999).
20. Tuosto, L. & Acuto, O. CD28 affects the earliest signaling events generated by TCR engagement. *Eur J Immunol* 28, 2131-42 (1998).
21. Fraser, J. D., Irving, B. A., Crabtree, G. R. & Weiss, A. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* 251, 313-6 (1991).
22. Iezzi, G., Scotet, E., Scheidegger, D. & Lanzavecchia, A. The interplay between the duration of TCR and cytokine signaling determines T cell polarization. *Eur J Immunol* 29, 4092-101 (1999).
23. Iezzi, G., Karjalainen, K. & Lanzavecchia, A. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* 8, 89-95 (1998).
24. Jameson, S. C. Maintaining the norm: T-cell homeostasis. *Nat Rev Immunol* 2, 547-56 (2002).
25. Schluns, K. S. & Lefrancois, L. Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* 3, 269-79 (2003).
26. Surh, C. D., Boyman, O., Purton, J. F. & Sprent, J. Homeostasis of memory T cells. *Immunol Rev* 211, 154-63 (2006).
27. Li, J., Huston, G. & Swain, S. L. IL-7 promotes the transition of CD4 effectors to persistent memory cells. *J Exp Med* 198, 1807-15 (2003).
28. Lenz, D. C. et al. IL-7 regulates basal homeostatic proliferation of antiviral CD4⁺T cell memory. *Proc Natl Acad Sci U S A* 101, 9357-62 (2004).
29. Kondrack, R. M. et al. Interleukin 7 regulates the survival and generation of memory CD4 cells. *J Exp Med* 198, 1797-806 (2003).
30. Kaech, S. M. et al. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4, 1191-8 (2003).
31. Romagnani, S. Human TH1 and TH2 subsets: doubt no more. *Immunol Today* 12, 256-7 (1991).
32. Mosmann, T. R. & Coffman, R. L. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7, 145-73 (1989).
33. O'Garra, A., Steinman, L. & Gijbels, K. CD4⁺ T-cell subsets in autoimmunity. *Curr Opin Immunol* 9, 872-83 (1997).
34. Romagnani, S. Regulation of the development of type 2 T-helper cells in allergy. *Curr Opin Immunol* 6, 838-46 (1994).
35. Wills-Karp, M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol* 17, 255-81 (1999).
36. Romagnani, S. Lymphokine production by human T cells in disease states. *Annu Rev Immunol* 12, 227-57 (1994).
37. Murphy, K. M. & Reiner, S. L. The lineage decisions of helper T cells. *Nat Rev Immunol* 2, 933-44 (2002).
38. Butcher, E. C. & Picker, L. J. Lymphocyte homing and homeostasis. *Science* 272, 60-6 (1996).
39. Baggiolini, M., Dewald, B. & Moser, B. Human chemokines: an update. *Annu Rev Immunol* 15, 675-705 (1997).
40. Butcher, E. C. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* 67, 1033-6 (1991).

41. Mantovani, A. The chemokine system: redundancy for robust outputs. *Immunol Today* 20, 254-7 (1999).
42. Sallusto, F., Lenig, D., Mackay, C. R. & Lanzavecchia, A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 187, 875-83 (1998).
43. Sallusto, F., Mackay, C. R. & Lanzavecchia, A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol* 18, 593-620 (2000).
44. Nagler-Anderson, C. Man the barrier! Strategic defences in the intestinal mucosa. *Nat Rev Immunol* 1, 59-67 (2001).
45. Iwasaki, A. & Kelsall, B. L. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J Exp Med* 190, 229-39 (1999).
46. Campbell, D. J. & Butcher, E. C. Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. *J Exp Med* 195, 135-41 (2002).
47. Mora, J. R. et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* 424, 88-93 (2003).
48. Campbell, J. J. et al. The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature* 400, 776-80 (1999).
49. Zabel, B. A. et al. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J Exp Med* 190, 1241-56 (1999).
50. Campbell, J. J. & Butcher, E. C. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. *Curr Opin Immunol* 12, 336-41 (2000).
51. Schon, M. P. et al. Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. *J Immunol* 162, 6641-9 (1999).
52. Schon, M. P., Schon, M., Warren, H. B., Donohue, J. P. & Parker, C. M. Cutaneous inflammatory disorder in integrin alphaE (CD103)-deficient mice. *J Immunol* 165, 6583-9 (2000).
53. Schutysse, E., Struyf, S. & Van Damme, J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev* 14, 409-26 (2003).
54. Steinman, R. M. & Nussenzweig, M. C. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A* 99, 351-8 (2002).
55. Munn, D. H. et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science* 297, 1867-70 (2002).
56. Sallusto, F. et al. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* 28, 2760-9 (1998).
57. Varona, R. et al. CCR6-deficient mice have impaired leukocyte homeostasis and altered contact hypersensitivity and delayed-type hypersensitivity responses. *J Clin Invest* 107, R37-45 (2001).
58. Cook, D. N. et al. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity* 12, 495-503 (2000).
59. Homey, B. et al. Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis. *J Immunol* 164, 6621-32 (2000).

60. Varona, R., Cadenas, V., Flores, J., Martinez, A. C. & Marquez, G. CCR6 has a non-redundant role in the development of inflammatory bowel disease. *Eur J Immunol* 33, 2937-46 (2003).
61. Kaser, A. et al. Increased expression of CCL20 in human inflammatory bowel disease. *J Clin Immunol* 24, 74-85 (2004).
62. Ruth, J. H. et al. Role of macrophage inflammatory protein-3alpha and its ligand CCR6 in rheumatoid arthritis. *Lab Invest* 83, 579-88 (2003).
63. Matsui, T. et al. Selective recruitment of CCR6-expressing cells by increased production of MIP-3 alpha in rheumatoid arthritis. *Clin Exp Immunol* 125, 155-61 (2001).
64. Masopust, D., Vezys, V., Marzo, A. L. & Lefrancois, L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291, 2413-7 (2001).
65. Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401, 708-12 (1999).
66. Campbell, J. J. et al. 6-C-kine (SLC), a lymphocyte adhesion-triggering chemokine expressed by high endothelium, is an agonist for the MIP-3beta receptor CCR7. *J Cell Biol* 141, 1053-9 (1998).
67. Forster, R. et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99, 23-33 (1999).
68. Geginat, J., Sallusto, F. & Lanzavecchia, A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J Exp Med* 194, 1711-9 (2001).
69. Geginat, J., Lanzavecchia, A. & Sallusto, F. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* 101, 4260-6 (2003).
70. Reinhardt, R. L., Khoruts, A., Merica, R., Zell, T. & Jenkins, M. K. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* 410, 101-5 (2001).
71. Hermiston, M. L., Xu, Z. & Weiss, A. CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol* 21, 107-37 (2003).
72. Salmon, M. et al. The progressive differentiation of primed T cells is associated with an increasing susceptibility to apoptosis. *Eur J Immunol* 24, 892-9 (1994).
73. Harari, A., Valleliau, F., Meylan, P. R. & Pantaleo, G. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J Immunol* 174, 1037-45 (2005).
74. Harari, A., Valleliau, F. & Pantaleo, G. Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load. *Eur J Immunol* 34, 3525-33 (2004).
75. Sallusto, F., Geginat, J. & Lanzavecchia, A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 22, 745-63 (2004).
76. Schaerli, P. et al. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* 192, 1553-62 (2000).
77. Kim, C. H. et al. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med* 193, 1373-81 (2001).

78. Breitfeld, D. et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* 192, 1545-52 (2000).
79. Campbell, J. J. et al. CCR7 expression and memory T cell diversity in humans. *J Immunol* 166, 877-84 (2001).
80. Stoll, S., Delon, J., Brotz, T. M. & Germain, R. N. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* 296, 1873-6 (2002).
81. Langenkamp, A., Messi, M., Lanzavecchia, A. & Sallusto, F. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat Immunol* 1, 311-6 (2000).
82. Kaech, S. M., Hemby, S., Kersh, E. & Ahmed, R. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* 111, 837-51 (2002).
83. Messi, M. et al. Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. *Nat Immunol* 4, 78-86 (2003).
84. Baron, V. et al. The repertoires of circulating human CD8(+) central and effector memory T cell subsets are largely distinct. *Immunity* 18, 193-204 (2003).
85. Tough, D. F. Deciphering the relationship between central and effector memory CD8+ T cells. *Trends Immunol* 24, 404-7 (2003).
86. Macallan, D. C. et al. Rapid turnover of effector-memory CD4(+) T cells in healthy humans. *J Exp Med* 200, 255-60 (2004).
87. Wherry, E. J. et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4, 225-34 (2003).
88. Huster, K. M. et al. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc Natl Acad Sci U S A* 101, 5610-5 (2004).
89. Bachmann, M. F., Wolint, P., Schwarz, K., Jager, P. & Oxenius, A. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol* 175, 4686-96 (2005).
90. Danke, N. A., Koelle, D. M., Yee, C., Beheray, S. & Kwok, W. W. Autoreactive T cells in healthy individuals. *J Immunol* 172, 5967-72 (2004).
91. Van Parijs, L. & Abbas, A. K. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science* 280, 243-8 (1998).
92. Mueller, D. L., Jenkins, M. K. & Schwartz, R. H. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol* 7, 445-80 (1989).
93. DeSilva, D. R., Urdahl, K. B. & Jenkins, M. K. Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. *J Immunol* 147, 3261-7 (1991).
94. Perez, V. L. et al. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* 6, 411-7 (1997).
95. Van Parijs, L., Peterson, D. A. & Abbas, A. K. The Fas/Fas ligand pathway and Bcl-2 regulate T cell responses to model self and foreign antigens. *Immunity* 8, 265-74 (1998).
96. Rieux-Laucat, F. et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* 268, 1347-9 (1995).

97. Rathmell, J. C. & Thompson, C. B. The central effectors of cell death in the immune system. *Annu Rev Immunol* 17, 781-828 (1999).
98. Vander Heiden, M. G. & Thompson, C. B. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? *Nat Cell Biol* 1, E209-16 (1999).
99. Broome, H. E., Dargan, C. M., Krajewski, S. & Reed, J. C. Expression of Bcl-2, Bcl-x, and Bax after T cell activation and IL-2 withdrawal. *J Immunol* 155, 2311-7 (1995).
100. Zinkernagel, R. M. Antiinfection immunity and autoimmunity. *Ann N Y Acad Sci* 958, 3-6 (2002).
101. Sakaguchi, S. Naturally arising CD4⁺ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22, 531-62 (2004).
102. Powrie, F., Correa-Oliveira, R., Mauze, S. & Coffman, R. L. Regulatory interactions between CD45RB^{high} and CD45RB^{low} CD4⁺ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med* 179, 589-600 (1994).
103. Maloy, K. J. & Powrie, F. Regulatory T cells in the control of immune pathology. *Nat Immunol* 2, 816-22 (2001).
104. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155, 1151-64 (1995).
105. Shevach, E. M. CD4⁺ CD25⁺ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2, 389-400 (2002).
106. Apostolou, I. & von Boehmer, H. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med* 199, 1401-8 (2004).
107. Chen, W. et al. Conversion of peripheral CD4⁺CD25⁻ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198, 1875-86 (2003).
108. Shevach, E. M. Certified professionals: CD4(+)CD25(+) suppressor T cells. *J Exp Med* 193, F41-6 (2001).
109. Almeida, A. R., Legrand, N., Papiernik, M. & Freitas, A. A. Homeostasis of peripheral CD4⁺ T cells: IL-2R alpha and IL-2 shape a population of regulatory cells that controls CD4⁺ T cell numbers. *J Immunol* 169, 4850-60 (2002).
110. Almeida, A. R., Zaragoza, B. & Freitas, A. A. Indexation as a novel mechanism of lymphocyte homeostasis: the number of CD4⁺CD25⁺ regulatory T cells is indexed to the number of IL-2-producing cells. *J Immunol* 177, 192-200 (2006).
111. Maloy, K. J. & Powrie, F. Fueling regulation: IL-2 keeps CD4⁺ Treg cells fit. *Nat Immunol* 6, 1071-2 (2005).
112. Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat Immunol* 4, 330-6 (2003).
113. Khattri, R., Cox, T., Yasayko, S. A. & Ramsdell, F. An essential role for Scurfin in CD4⁺CD25⁺ T regulatory cells. *Nat Immunol* 4, 337-42 (2003).
114. Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299, 1057-61 (2003).

115. Brunkow, M. E. et al. Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27, 68-73 (2001).
116. Ramsdell, F. Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* 19, 165-8 (2003).
117. Ziegler, S. F. FOXP3: of mice and men. *Annu Rev Immunol* 24, 209-26 (2006).
118. Allan, S. E. et al. The role of 2 FOXP3 isoforms in the generation of human CD4⁺ Tregs. *J Clin Invest* 115, 3276-84 (2005).
119. Walker, M. R. et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4⁺CD25⁻ T cells. *J Clin Invest* 112, 1437-43 (2003).
120. von Boehmer, H. Mechanisms of suppression by suppressor T cells. *Nat Immunol* 6, 338-44 (2005).
121. Fahlen, L. et al. T cells that cannot respond to TGF-beta escape control by CD4⁽⁺⁾CD25⁽⁺⁾ regulatory T cells. *J Exp Med* 201, 737-46 (2005).
122. Groux, H. et al. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389, 737-42 (1997).
123. Levings, M. K. et al. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25⁺CD4⁺ Tr cells. *Blood* 105, 1162-9 (2005).
124. Levings, M. K. et al. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J Immunol* 166, 5530-9 (2001).
125. Barrat, F. J. et al. In vitro generation of interleukin 10-producing regulatory CD4⁽⁺⁾ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 195, 603-16 (2002).
126. Vieira, P. L. et al. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4⁺CD25⁺ regulatory T cells. *J Immunol* 172, 5986-93 (2004).
127. Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A. & Weiner, H. L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265, 1237-40 (1994).
128. Han, H. S., Jun, H. S., Utsugi, T. & Yoon, J. W. A new type of CD4⁺ suppressor T cell completely prevents spontaneous autoimmune diabetes and recurrent diabetes in syngeneic islet-transplanted NOD mice. *J Autoimmun* 9, 331-9 (1996).
129. Shi, F. D. et al. Mechanisms of nasal tolerance induction in experimental autoimmune myasthenia gravis: identification of regulatory cells. *J Immunol* 162, 5757-63 (1999).
130. Weiner, H. L. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* 182, 207-14 (2001).
131. Fukaura, H. et al. Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. *J Clin Invest* 98, 70-7 (1996).
132. Jonuleit, H. et al. Infectious tolerance: human CD25⁽⁺⁾ regulatory T cells convey suppressor activity to conventional CD4⁽⁺⁾ T helper cells. *J Exp Med* 196, 255-60 (2002).
133. Dieckmann, D., Bruett, C. H., Ploettner, H., Lutz, M. B. & Schuler, G. Human CD4⁽⁺⁾CD25⁽⁺⁾ regulatory, contact-dependent T cells induce interleukin 10-

producing, contact-independent type 1-like regulatory T cells [corrected]. *J Exp Med* 196, 247-53 (2002).

II. Results

1. Comments on manuscripts

1.1 Manuscript 1:

Heterogeneity of the CD4+T central memory pool

In this work we have dissected the human CD4+T central memory cell (T_{CM}) pool, and we have identified functionally distinct subsets of cells which are either unpolarized or pre-committed to become T_H1 (pre- T_H1) and T_H2 cells (pre- T_H2).

Previous studies from our group reported that T_{CM} cells, which constitutively express the lymph node homing receptors CCR7 and CD62L, are largely devoid of effector functions but acquire characteristics of effector memory cells (T_{EM}) after stimulation through the TCR or with homeostatic cytokines^{1, 2}. Conversely, T_{EM} cells have lost expression of lymph node receptors but express chemokine receptors involved in homing to inflamed tissues. Following TCR triggering, these cells rapidly produce the effector cytokines IFN- γ , IL-4 or IL-5. Thus, in humans the CD4+ T_{EM} pool contains *bona fide* T_H1 and T_H2 cells, which can be identified by their cytokine profile and chemokine receptor expression. It was proposed that T_{CM} cells represent a “stem cell like” pool of antigen-primed cells, which migrate to secondary lymphoid organs and, upon a secondary challenge, can rapidly generate new waves of antigen-specific effector cells. In contrast, T_{EM} cells can enter peripheral tissues and provide immediate protection against invading pathogens^{2,3}. Thus, understanding T_{CM} cell differentiation stage and potential is important for comprehending how T_H1 and T_H2 polarization is conserved from a primary response, and if it still maintains some degree of flexibility in secondary immune responses.

Here we show that three chemokine receptors identify functional subsets within the T_{CM} pool. T_{CM} cells expressing CXCR3 secrete low amounts of IFN- γ whereas CCR4+ T_{CM} cells produce low amounts of IL-4 but not IL-5. Following expansion in the presence of the homeostatic cytokines IL-7 and IL-15, these cells invariably differentiated to CCR7- cells that possessed the characteristics of T_H1 and T_H2 cells,

suggesting that they represent cells which are pre-committed to give rise to T_H1 (pre-T_H1) or T_H2 cells (pre-T_H2), respectively. Conversely, CXCR5⁺ T_{CM} cells lacking CXCR3 or CCR4 (CXCR5^{-/-}) expression remained unpolarized following expansion in homeostatic cytokines and retained lymph node homing potential (figure 3b).

Previous work has shown that CD4⁺T cells expressing CXCR5 comprise CCR7⁻CD57⁺ follicular helper T cells in the tonsils^{4, 5}, and the above mentioned, unpolarized circulating CXCR5⁺T cells of unknown function and specificity, that might have a recent activation history⁶. We show that CXCR5⁺ and CXCR5⁻ cells, lacking expression of CXCR3 or CCR4, display characteristics of cells that are at an earlier stage of memory cell differentiation compared to pre-T_H1 and pre-T_H2 cells.

Furthermore, we observed that CXCR3⁺ and CCR4⁺ T_{CM} cells maintained some degree of flexibility following TCR stimulation in T_H1 or T_H2 condition, as they were partially able to re-polarize to the opposite condition in the presence of the specific cytokines. T_H1 or T_H2 polarizing cytokines did not have a similar effect if they were added to cells stimulated with IL-7/IL-15 (figure 5). Similarly, CXCR5⁺ cells were able to polarize to the T_H1/T_H2 direction in the presence of the specific cytokine, following TCR triggering but not following IL-7/IL-15 stimulation. These results suggested that following TCR triggering with polarizing cytokines, T_{CM} cells maintain a certain degree of flexibility of cytokine gene expression.

Unlike naïve T cells, all memory subsets displayed low T cell receptor rearrangement excision circle content and spontaneously incorporated bromodeoxyuridine *ex vivo*, indicating that they had divided *in vivo* to a similar extent and were undergoing a slow turn over, under steady-state conditions. Furthermore, we observed that pathogen-specific CD4⁺T cells had characteristic distributions within T_{CM} and T_{EM} subsets, reflecting the T_H1/T_H2 polarization induced by the pathogen or vaccination. Memory cells specific for vaccinia virus or cytomegalovirus, which typically promote a T_H1 response, were largely detected among CXCR3⁺ T_{CM} and T_{EM} cells. In contrast, tetanus toxoid specific cells were detected among all subsets, consistent with the notion that vaccination against tetanus induces a mixed T_H1/T_H2 response⁷ (figure 6). Altogether, these results show that cells in the T_{CM} pool, identified by chemokine receptor expression, are pre-committed to give rise to T_H1 or T_H2 cells, in homeostatic conditions. Importantly, this could explain how the quality of primary T cell responses is maintained in the absence of antigen. In contrast, upon a secondary encounter with a given pathogen, memory cells may retain a certain degree of

flexibility and partially re-polarize to the opposite direction in the presence of the appropriate polarizing conditions.

1.2. Manuscript 2:

Memory potential of antigen-primed CD4+T cells

The signals involved in selecting an activated T cell to survive and to generate a long-lived memory T cell are still not completely understood. Previous work in mice showed that in the absence of IL-7 no memory cells develop⁸⁻¹¹, suggesting that IL-7 is required for the generation of memory cells. Furthermore, it was recently shown that CD8+T memory precursors can be identified by expression of the IL7 receptor α chain (IL-7R), early at the peak of the proliferative response^{11, 12}. Nevertheless, IL7R expression does not necessarily implicate that the T cell will survive and give rise to a memory cell^{11, 13}. We therefore analysed the signal strength requirements for IL7R expression on CD4+T memory cells and assessed their memory potential. We observed that CD4+T cells expressing CCR7 and IL7R possessed a high capacity to respond to IL-7 and to expand upon secondary TCR stimulation, therefore representing good candidates for memory precursors. Following antigen encounter, CCR7+IL7R^{hi} cells produced mainly IL-2, whereas CCR7-IL7R^{lo} identified effector cells with higher death rates. Furthermore, we observed that the CCR7+IL7R^{hi} subset included cells that varied in their memory potential, depending on the signal strength they had received. In fact, CCR7+IL7R^{hi} cells that had received a weak stimulation showed little or no responsiveness to IL-7 and possessed a high death rate. Cells generated at an intermediate strength of stimulation proliferated slowly with IL-7 and expanded efficiently upon secondary TCR stimulation, thus possessing characteristics of unpolarized T_{CM} cells. In contrast, strongly stimulated CCR7+IL7R^{hi} cells expanded poorly upon TCR-engagement but displayed a high proliferative rate in the presence of homeostatic cytokines (figure 3). In the latter conditions they differentiated spontaneously to T_{H1} effector cells, a characteristic which is typical of circulating CXCR3+ pre-T_{H1} cells (manuscript 1, figures 3 and 4). As IL7R^{hi} cells

generated in the different stimulatory conditions displayed such diverse responsiveness to IL-7, we analysed their capabilities of signalling through the IL7R^{hi}^{14, 15}. We observed that, while the activities of the JAK kinases were similar, those of s6-kinases correlated with IL-7 responsiveness and were dependent upon signal strength. Consistently, the expression levels of PTEN, a phosphatase that counteracts PI-3 kinase activity which in turn controls s6-kinase activity, was higher in weakly stimulated compared to strongly stimulated cells (figure 4). To better understand at a molecular level the observed differences in IL-7 responsiveness, we performed gene expression analyses of CCR7+IL7R^{hi} cells generated by weak or strong stimuli. We observed that cells that had received a strong stimulus were in a higher metabolic state, expressed pro-apoptotic molecules and possessed lower thresholds to cycle with mitogens. Furthermore, these cells expressed increased levels of genes involved in T_H1 development. These results are consistent with a T_H1 commitment of these cells and their reduced survival following antigen encounter (figure 5).

We conclude that the memory potential of CD4+T memory cells is dictated by the strength of stimulation they receive, and is not necessarily predictable by phenotypic markers. Our results are consistent with the view that T-cell memory is generated at an intermediate range of signal strength and suggest that T_{CM} and T_{EM} subsets could be derived from CCR7+ precursors that received different amounts of stimulation.

1.3 Manuscript 3:

Characterization of IL-10 producing CD4+T memory cells

In this study we have addressed another aspect of CD4+T cell memory concerning the maintenance of self-tolerance. We have identified a subset of IL-10 producing cells in the CD4+T cell memory compartment characterized by the expression of the chemokine receptor CCR6, and we investigated the mode of generation and maintenance of these cells and the role they may play in the context of an immune response.

Two major subsets of regulatory T cells have been described, naturally occurring

Foxp3+CD25+Tregs and Tr1 cells, or IL-10 producing Tregs. Tr1 cells are characterized by the capacity to produce IL-10 and inhibit immune responses in an IL-10-dependent, cell-contact independent manner¹⁶⁻¹⁸. It was shown that these cells can be generated in vitro by tolerogenic priming with immature DC, IL-10 or immune-suppressive drugs^{16, 19, 20}, but until now no surface markers that allow the identification of Tr1 cells have been described. Naturally occurring CD25+Tregs are also characterized by production of IL-10 although, at least in vitro, this cytokine does not seem to play a role in the suppressive activity of Tregs. In fact, Treg suppression of immune responses in vitro is dependent upon a still unclear cell-contact dependent mechanism. Accumulated evidence suggests that both T regulatory subsets play an important role in the control of autoimmune diseases¹⁶. Here we show that a population of antigen-experienced CD4+ Foxp3-cells, distinct from natural Tregs and characterized by expression of CCR6, secrete IL-10 in response to self-antigens and inhibit autoreactivity in an IL-10-dependent manner (figure 4). We show that CCR6 is induced in a tolerogenic environment, such as in the presence of IL-10 and TGF- β (figure 1). Interestingly, both cytokines have been implicated in maintenance of self-tolerance²¹⁻²³. Cells specific for self-antigens in healthy donors are contained exclusively within the CCR6+ population, while interestingly, cells with the same specificities are also detected among CCR6- cells in patients affected by an autoimmune pathology (figure 6). CCR6+T cells however, secrete IL-2 and proliferate also in response to recall antigens. Thus, to investigate whether IL-10 producing self-reactive and IL-2 producing recall-antigen specific cells represented distinct populations within CCR6+T cells, we analysed self-specific CCR6+T cells at a single cell level. We performed single-cell cloning of autoreactive CCR6+T cells which had undergone proliferation in the presence of autologous circulating mDCs and obtained a certain number of clones displaying a similar behaviour. These clones produced IL-10 and no IL-2 in the presence of autologous mDCs and proliferated upon neutralization of IL-10. Interestingly, the same clones also proliferated vigorously and produced IL-2 in the presence of the recall antigen tetanus toxoid (figure 7). We propose a context dependent function of these CCR6+T memory cells, in that they exhibit a Tr1-like suppressive capacity when exposed to self-antigens, thus limiting autoimmune reactions, while in the presence of their specific antigen

they behave as memory cells and confer protection.

The mechanism described by us may represent a strategy evolved by the immune system which allows the utilization of slightly autoreactive T cells, thus broadening the TCR repertoire available for pathogen recognition, while lowering the risk of autoimmune reactions. In fact, CCR6+T memory cells produce IL-10 upon encounter of self-antigen, and may in this way raise their threshold for an autoimmune response.

References

Manuscript 1

- 1 Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401, 708-12 (1999).
- 2 Sallusto, F., Geginat, J. & Lanzavecchia, A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 22, 745-63 (2004).
- 3 Lanzavecchia, A. & Sallusto, F. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* 290, 92-7 (2000).
- 4 Schaerli, P. et al. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* 192, 1553-62 (2000).
- 5 Kim, C. H. et al. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med* 193, 1373-81 (2001).
- 6 Schaerli, P., Loetscher, P. & Moser, B. Cutting edge: induction of follicular homing precedes effector Th cell development. *J Immunol* 167, 6082-6 (2001).
- 7 Rowe, J. et al. Heterogeneity in diphtheria-tetanus-acellular pertussis vaccine-specific cellular immunity during infancy: relationship to variations in the kinetics of postnatal maturation of systemic th1 function. *J Infect Dis* 184, 80-8 (2001).

Manuscript 2

- 8 Kondrack, R. M. et al. Interleukin 7 regulates the survival and generation of

memory CD4 cells. *J Exp Med* 198, 1797-806 (2003).

- 9 Li, J., Huston, G. & Swain, S. L. IL-7 promotes the transition of CD4 effectors to persistent memory cells. *J Exp Med* 198, 1807-15 (2003).
- 10 Lenz, D. C. et al. IL-7 regulates basal homeostatic proliferation of antiviral CD4+T cell memory. *Proc Natl Acad Sci U S A* 101, 9357-62 (2004).
- 11 Kaeck, S. M. et al. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4, 1191-8 (2003).
- 12 Huster, K. M. et al. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc Natl Acad Sci U S A* 101, 5610-5 (2004).
- 13 Lacombe, M. H., Hardy, M. P., Rooney, J. & Labrecque, N. IL-7 receptor expression levels do not identify CD8+ memory T lymphocyte precursors following peptide immunization. *J Immunol* 175, 4400-7 (2005).
- 14 Barata, J. T. et al. Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. *J Exp Med* 200, 659-69 (2004).
- 15 Abraham, R. T. Mammalian target of rapamycin: immunosuppressive drugs uncover a novel pathway of cytokine receptor signaling. *Curr Opin Immunol* 10, 330-6 (1998).

Manuscript 3

- 16 Groux, H. et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389, 737-742 (1997).
- 17 Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* 190, 995-1004 (1999).
- 18 Vieira, P. L. et al. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J Immunol* 172, 5986-93 (2004).
- 19 Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. & Enk, A. H. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* 192, 1213-1222. (2000).

- 20 Barrat, F. J. et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)-and Th2-inducing cytokines. *J Exp Med* 195, 603-16 (2002).
- 21 Gorelik, L. & Flavell, R. A. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 12, 171-81 (2000).
- 22 de Waal Malefyt, R., Yssel, H. & de Vries, J. E. Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation. *J Immunol* 150, 4754-65 (1993).
- 23 Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19, 683-765 (2001).

Manuscript 1

Chemokine receptor expression identifies pre-T_H1, pre-T_H2, and non polarized cells among human CD4⁺ central memory cells.

Laura Rivino, Mara Messi, David Jarrossay, Antonio Lanzavecchia,
Federica Sallusto and Jens Geginat

J.Exp. Med. 2004. 200, 725-735

Chemokine Receptor Expression Identifies Pre-T Helper (Th)1, Pre-Th2, and Nonpolarized Cells among Human CD4⁺ Central Memory T Cells

Laura Rivino, Mara Messi, David Jarrossay, Antonio Lanzavecchia, Federica Sallusto, and Jens Geginat

Institute for Research in Biomedicine, 6500 Bellinzona, Switzerland

Abstract

We previously reported that central-memory T cells (T_{CM} cells), which express lymph node homing receptors CCR7 and CD62L, are largely devoid of effector functions but acquire characteristics of effector-memory T cells (T_{EM} cells) (i.e., CCR7⁺ T helper [Th]1 or Th2 cells) after stimulation with T cell receptor agonists or homeostatic cytokines. Here we show that three chemokine receptors identify functional subsets within the human CD4⁺ T_{CM} cell pool. T_{CM} cells expressing CXCR3 secreted low amounts of interferon γ , whereas CCR4⁺ T_{CM} cells produced some interleukin (IL)-4, but not IL-5. In response to IL-7 and IL-15, CXCR3⁺ T_{CM} and CCR4⁺ T_{CM} cells invariably generated fully differentiated CCR7⁺ Th1 and Th2 cells, respectively, suggesting that they represent pre-Th1 and pre-Th2 cells. Conversely, CXCR5⁺ T_{CM} cells lacking CXCR3 and CCR4 remained nonpolarized and retained CCR7 and CD62L expression upon cytokine-driven expansion. Unlike naive cells, all memory subsets had a low T cell receptor rearrangement excision circle content, spontaneously incorporated bromodeoxyuridine *ex vivo*, and contained cells specific for tetanus toxoid. Conversely, recall responses to cytomegalovirus and vaccinia virus were largely restricted to CXCR3⁺ T_{CM} and T_{EM} cells. We conclude that antigen-specific memory T cells are distributed between T_{EM} cells and different subsets of T_{CM} cells. Our results also explain how the quality of primary T cell responses could be maintained by T_{CM} cells in the absence of antigen.

Key words: T cell subsets • memory maintenance • cytokines • differentiation • chemokine receptors

Introduction

Upon recognition of antigenic peptides on DCs, naive T lymphocytes proliferate and differentiate into a variety of effector cells depending on the stimulatory conditions and cytokine milieu (1, 2). Accumulating evidence indicates that during the T cell differentiation process, effector functions and homing potentials are coordinately regulated (3). For instance, developing Th1 cells acquire the capacity to produce IFN- γ and expression of chemokine receptors such as CCR5, CXCR3, and CXCR6 that drive them to sites of delayed-type hypersensitivity reactions. Conversely, developing Th2 cells acquire the capacity to produce IL-4 and express CCR3, CCR4, CCR8, and the prostaglandin D2 chemoattractant receptor CRTh2 (4–7), which are required to migrate at sites of allergic reactions (6, 8, 9).

Expression of the lymph node homing receptors CCR7 and CD62L (10, 11) has been used to define subsets of human memory T cells with distinct functional properties. T cells within the CCR7⁺ “central-memory” T cell (T_{CM} cell) cell subset show hypo-acetylated cytokine genes and have no or low effector functions, but efficiently differentiate to Th1 or Th2 effector cells after TCR stimulation in the presence of IL-12 or IL-4, respectively (12–14). In contrast, T cells of the CCR7⁺ “effector-memory” T cell (T_{EM} cell) subset show polarized cytokine gene acetylation patterns *in vivo* and rapidly produce high amounts of IFN- γ and IL-4 upon antigenic stimulation (12–14). It has been proposed that in secondary immune responses, T_{CM} cells generate

L. Rivino, M. Messi, and D. Jarrossay contributed equally to this work.

Address correspondence to Jens Geginat, Institute for Research in Biomedicine, Via Vela 6, 6500 Bellinzona, Switzerland. Phone: 41-91-8200313; Fax: 41-91-8200305; email: jens.geginat@irb.unisi.ch

Abbreviations used in this paper: BrdU, bromodeoxyuridine; CFSE, carboxy-fluorescein succinimidyl ester; PdBu, phorbol-12-13-dibutyrate; T_{CM} cell, central-memory T cell; T_{EM} cell, effector-memory T cell; TREC, T cell receptor rearrangement excision circle; TSST, toxic shock syndrome toxin; TT, tetanus toxoid; VV, vaccinia virus.

new waves of effector cells in antigen-draining lymph nodes, whereas T_{EM} cells provide immediate protection against invading pathogens in peripheral tissues (3, 14).

The maintenance of T cell memory is controlled by cytokines that promote cell survival and slow homeostatic proliferation (15). In particular, IL-7 and IL-15 have been shown to regulate mouse $CD8^+$ memory T cell survival and self-renewal in the absence of antigen (16, 17), whereas naive and $CD4^+$ memory cells require IL-7 and TCR ligands (16, 18, 19), but do not respond to IL-15 (17). Conversely, human $CD4^+$ memory T cells proliferate in response to IL-15 in a TCR-independent fashion and with slow kinetics (20, 21), suggesting different roles for IL-15 in mouse and human $CD4^+$ memory T cell homeostasis. Notably, T_{CM} cells proliferating in response to IL-7 and IL-15 differentiate and generate Th1 and Th2 effector cells (21), but how uncommitted T_{CM} cells proliferating in the absence of antigen could maintain the quality of the primary response remained unclear.

The existence of T_{CM} and T_{EM} cell subsets has also been documented in mice (22, 23). In this experimental system, it has been possible to directly examine the kinetics of memory cell generation and the capacity of effector and memory subsets to reconstitute long-term memory (24), and there is growing evidence that T_{CM} cells have higher reconstitution potential (24, 25). In particular, effector Th1 cells, defined by their secretion of IFN- γ , were found to be short-lived and unable to reconstitute T cell memory. In contrast, a population of activated Th1 lineage cells, which did not secrete IFN- γ after primary antigenic stimulation, persisted for several months in vivo and developed the capacity to secrete IFN- γ upon subsequent stimulation (26).

Since the first description of T_{CM} and T_{EM} cells, it was evident that other chemokine receptors, as well as adhesion and costimulatory molecules, are expressed on different fractions of T_{CM} cells (12, 14). Heterogeneity of human $CD4^+$ T_{CM} cells has further been documented using CXCR5, the receptor for CXCL13, a chemokine expressed in B cell follicles (27, 28). $CXCR5^+$ T_{CM} cells lacked effector functions and cells specific for tetanus toxoid (TT), but contained residual T cell receptor rearrangement excision circles (TRECs), suggesting that they represent recently activated cells (29–31). Conversely, other recent studies claimed that both T_{CM} and T_{EM} cells possess high levels of effector functions, and that consequently neither CCR7 nor CXCR5 expression identify nonpolarized $CD4^+$ memory T cells (9, 32–35). Understanding T_{CM} cell differentiation stage and potential is of importance for the homeostatic maintenance of memory T cells and for the conservation of T cell polarization in secondary responses.

Here we report that CXCR3 and CCR4 identify two novel subsets of pre-Th1 and pre-Th2 cells within T_{CM} cells. These cells possessed low IFN- γ - or IL-4-producing capacities when compared with $CXCR3^+$ and $CCR4^+$ T_{EM} cells and spontaneously differentiated to Th1 and Th2 effector cells in response to homeostatic cytokines IL-7 and IL-15 independently of conventional Th1 or Th2 cell-induc-

ing stimuli. In contrast, T_{CM} cells lacking CXCR3 or CCR4 and expressing CXCR5 were nonpolarized cells whose differentiation to Th1 or Th2 cells is dependent on TCR triggering and signaling by polarizing cytokines.

Materials and Methods

Cell Culture. PBMCs were isolated from buffy-coated blood from healthy donors. Monocytes were depleted by adhesion for 30 min and $CD4^+$ T cells were isolated by negative selection with magnetic beads using Automacs (Miltenyi Biotec). Memory T cells were isolated by further depletion of naive T cells with anti- $CD45RA$ beads (Miltenyi Biotec). Memory T cell subpopulations were purified to >95% by cell sorting after five-color staining as follows: anti-CXCR5 (R&D Systems) followed by anti-IgG2b PE (Biosystems), anti-CCR7 (R&D Systems) followed by anti-IgG2a FITC (Biosystems), and anti-CXCR3 Cy-Chrome, anti- $CD45RA$ APC, and anti-CCR4 biotin followed by streptavidin-APC-Cy7 (BD Biosciences). Labeling of T cells with carboxyfluorescein succinimidyl ester (CFSE) was performed as described previously (21). Monocytes were purified by positive selection with anti- $CD14$ beads (Miltenyi Biotec). For DC differentiation, $CD14^+$ cells were cultured for 4 d in complete medium (RPMI 1640 supplemented with 2 mM glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 50 μ g/ml kanamycin, 50 U/ml penicillin, and 50 μ g/ml streptomycin; GIBCO BRL) containing 10% FCS (Hyclone), 50 ng/ml granulocyte/macrophage colony-stimulating factor (Novartis), and 1,000 U/ml IL-4. The DCs obtained were stimulated for 24 h with 100 ng/ml lipopolysaccharide (*Salmonella abortus equi*; Sigma-Aldrich) and pulsed for 30 min with 100 ng/ml toxic shock syndrome toxin (TSST). CFSE-labeled 5×10^4 T cells were cultured with TSST-pulsed DCs in flat-bottom wells at a 5:1 ratio, and recombinant cytokines were used at either 25 ng/ml (IL-7 and IL-15; R&D Systems), 10 ng/ml (TNF, IL-6, IL-10, IL-4, and IL-12; BD Biosciences), or 1,000 U/ml (IL-2; Roche), whereas neutralizing antibodies to IL-4 and IL-12 (BD Biosciences) were used at 2 μ g/ml.

ELISA, Intracellular Cytokine Staining, and IFN- γ Secretion Assay. Cytokine-producing capacity of FACS-purified subsets was assessed after stimulation of purified cell populations at 5×10^4 /100 μ l for 24 h with 50 nM phorbol-12-13-dibutyrate (PdBu) and 0.5 μ g/ml ionomycin, or in wells coated with 2 μ g/ml each of anti- $CD3$ (clone TR66) and anti- $CD28$ antibodies (BD Biosciences). Cytokine concentrations of supernatants were then assessed by ELISA according to a standard protocol and analyzed with the Softmax program. Intracellular IFN- γ was detected after stimulating cells in the presence of 10 μ g/ml brefeldin A (Sigma-Aldrich) for the last 2 h and after fixation with paraformaldehyde and permeabilization with saponin. After saturation of nonspecific binding sites with 10% FCS, cells were incubated with APC-labeled antibody to IFN- γ and PE-labeled antibody to IL-2 or IL-4 (BD Biosciences), washed, and analyzed by flow cytometry on a FACSCalibur with CELLQuest software (Becton Dickinson). To sort live IFN- γ -producing cells, we stimulated cells for 60 h with 25 ng/ml cytokines (IL-7, IL-15, IL-12, TNF- α , and IL-18), and IFN- γ -producing cells were identified with an IFN- γ secretion assay kit (Miltenyi Biotec) and purified by cell sorting.

Recall Responses. PBMCs from 50 ml of fresh blood from healthy volunteers were prepared, monocytes were isolated and either incubated for 16 h with a replication-deficient vaccinia virus (VV; provided by G. Sutter, Institute for Virology, Munich,

Germany) or left untreated. 5×10^4 monocytes were then irradiated and incubated in the absence or presence of 1 $\mu\text{g}/\text{ml}$ TT or 2.5 μg of an extract of CMV-derived proteins (provided by R. Campanelli, University of Pavia, Pavia, Italy) with purified T cell subsets at a 1:1 ratio in U-bottom wells in complete medium containing 5% human serum. On days 5 and 7, cells were stained for CD14 and CD4, and CFSE dilution of $\text{CD4}^+ \text{CD14}^-$ viable cells was assessed by flow cytometry. On day 5, cells had not yet diluted CFSE completely, and the precursor frequency of antigen-specific cells could therefore be calculated as described previously (36). In some experiments, the presence of pathogen-specific cells was confirmed by restimulating cells with autologous monocytes treated as described above followed by assessment of cytokine production of proliferating T cells by intracellular staining.

Ex Vivo Bromodeoxyuridine (BrdU) Labeling. The assay was performed as described previously (25). In brief, fresh PBMCs were cultured with 10 $\mu\text{g}/\text{ml}$ BrdU (Sigma-Aldrich) for 16 h. CD4^+ cells were then positively selected with anti-CD4 magnetic beads and stained for CD45RA and chemokine receptor expression. Cells were then fixed, permeabilized, treated with DNase (Boehringer), stained with FITC-labeled anti-BrdU antibody (Becton Dickinson), and analyzed by flow cytometry.

Quantitative PCR of TRECs. Memory CD4^+ T cells were isolated by MACS as described above, stained for CD4, CD45RO, CCR7, and CXCR5, and sorted to >99.9% purity. Quantification of signal joint TRECs in sorted CD4^+ T cell subsets was performed by real-time quantitative PCR with the 5' nuclease (TaqMan) assay using an ABI 7700 sequence detector (Applied Biosystems). As described previously (37), $1-2 \times 10^5$ cells were lysed in 10 mM Tris, pH8, containing 100 $\mu\text{g}/\text{ml}$ of proteinase K (GIBCO BRL) for 2 h at 56°C , and then for 15 min at 95°C . PCR reaction of lysates was performed with 500 nM of primers (CACATCCCTTTCAACCATGCT and GCCAGCTGCAGGGTTTATAGG) and 125 nM of probe FAM-ACACCTCTGGTTTTTGTAAAGGTGCCCACT-TAMRA. PCR conditions were as follows: 1 cycle of 2 min at 50°C , 1 cycle of 10 min at 95°C , followed by 40 cycles of 30 s at 95°C , and 1 min

at 65°C . Levels of DNA were standardized by normalizing with 18S rRNA sequences.

Results

Subsets of Human CD4^+ T_{CM} Cells Identified by Expression of CXCR5, CXCR3, and CCR4. Purified human CD4^+ T cells were analyzed for chemokine receptor expression by five-color staining. CD45RA^+ cells expressed CCR7, but were largely negative for the other chemokine receptors, consistent with the view that they are predominantly antigen-inexperienced "naïve" T cells (not depicted). Conversely, the following three main subsets could be identified in CD45RA^- cells according to CCR7 and CXCR5 expression: $\text{CXCR5}^+ \text{CCR7}^+$ cells ($\text{CXCR5}^+ T_{\text{CM}}$), $\text{CXCR5}^- \text{CCR7}^+$ cells ($\text{CXCR5}^- T_{\text{CM}}$), and $\text{CXCR5}^- \text{CCR7}^-$ cells (T_{EM} ; Fig. 1 A). Within these main subsets, staining with antibodies to CXCR3 and CCR4 revealed further heterogeneity (Fig. 1 B). CXCR3 and CCR4 were expressed on different populations of T_{EM} cells, which contain Th1 and Th2 effector cells (4, 9, 38). However, CXCR3 and CCR4 were also expressed on some T_{CM} cells, especially within the CXCR5^- subset (Fig. 1 B). Thus, the following four major subsets of T_{CM} cells were identified: (a) $\text{CXCR3}^- \text{CCR4}^- \text{CXCR5}^+ T_{\text{CM}}$ cells (" $\text{CXCR5}^+ T_{\text{CM}}$ "), (b) $\text{CXCR3}^- \text{CCR4}^- \text{CXCR5}^- T_{\text{CM}}$ cells (" $-/- T_{\text{CM}}$ "), (c) $\text{CXCR3}^+ \text{CCR4}^- \text{CXCR5}^- T_{\text{CM}}$ cells (" $\text{CXCR3}^+ T_{\text{CM}}$ "), and (d) $\text{CXCR3}^- \text{CCR4}^+ \text{CXCR5}^- T_{\text{CM}}$ cells (" $\text{CCR4}^+ T_{\text{CM}}$ "). Mean values \pm standard deviations of the four subsets in four healthy donors were $11 \pm 5\%$, $18 \pm 10\%$, $17 \pm 8\%$, and $17 \pm 12\%$, respectively.

Because CXCR3 and CCR4 have been associated with differentiated Th1 and Th2 cells (7, 38), we analyzed expression of other surface markers that are acquired or lost

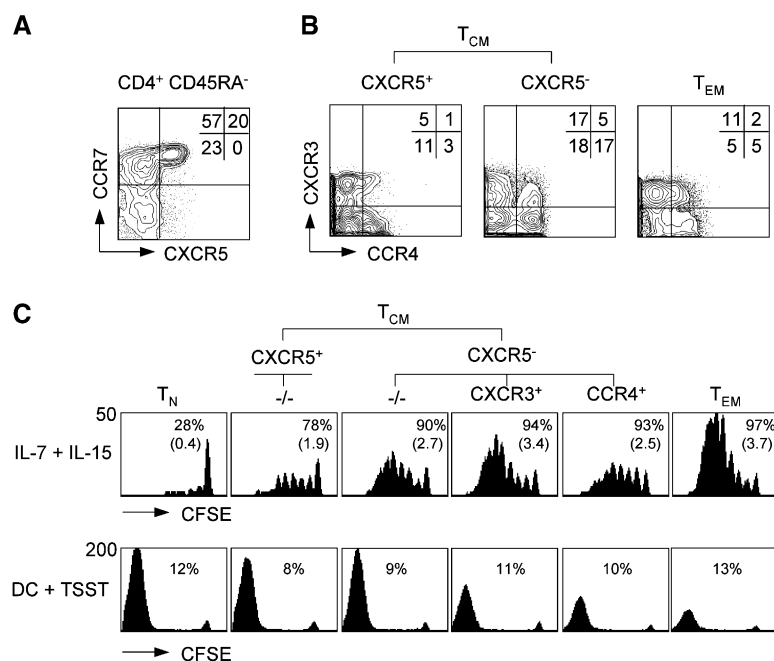


Figure 1. Expression of chemokine receptors defines CD4^+ T_{CM} cell subsets with distinct replicative potentials. Purified CD4^+ T cells were stained with antibodies specific for CXCR5, CCR7, CXCR3, CCR4, and CD45RA, and analyzed by five-color flow cytometry. (A) CCR7 and CXCR5 expression of CD45RA^- cells of one representative donor out of four. (B) $\text{CXCR5}^+ T_{\text{CM}}$, $\text{CXCR5}^- T_{\text{CM}}$, and T_{EM} cells were analyzed for CXCR3 and CCR4 expression. Percentages indicate the mean frequency of subsets in the memory pool of five healthy donors. (C) Purified, CFSE-labeled CD4^+ T cell subsets were stimulated with IL-7 and IL-15, or TSST-loaded DCs for 7 d. CFSE profiles of viable (propidium iodide⁻) cells were analyzed by flow cytometry. Numbers in the top row indicate the percentage of dividing cells and numbers in parenthesis indicate the mean division number. Numbers in the bottom row indicate the fraction of TSST-responsive TCR $\text{V}\beta 2^+$ cells. One representative donor out of four is shown.

Table I. Expression of CD27, CD62L, and IL-2/IL-15R β Chain (CD122) on Naive T Cells and Memory T Cell Subsets^a

	T _N	T _{CM}				T _{EM}
		CXCR5 ⁺	CXCR5 ⁻			
			-/-	CXCR3	-/-	
CD27 ⁺ (%) ^b	99 ± 1	98 ± 2	76 ± 9	83 ± 9	72 ± 17	40 ± 8
CD62L ^{hi} (%) ^b	99 ± 1	89 ± 5	55 ± 15	84 ± 8	79 ± 9	23 ± 12
CD122 (MFI) ^c	1 ± 1	3 ± 2	8 ± 3	6 ± 2	5 ± 2	10 ± 3
PI ⁺ (%) ^d	3 ± 2	7 ± 4	17 ± 9	9 ± 5	15 ± 8	25 ± 10

^aMean \pm standard deviation of four healthy donors.^bPercent of positive cells.^cMean fluorescence intensity.^dPropidium iodide⁺ cells after stimulation for 3 d with DC⁺ TSST.

with T cell differentiation (12, 17, 39; Table I). As expected, naive T cells expressed uniformly CD27 and CD62L, but not the IL-2/15R β chain (CD122), whereas most T_{EM} cells had lost CD27 and CD62L expression, but were CD122⁺. CXCR5⁺ T_{CM} cells had a phenotype similar to naive cells, whereas CXCR5⁻ T_{CM} cells expressed intermediate levels of CD27, CD62L, and CD122. In particular, CXCR3⁺ T_{CM} cells were CD122⁺ and had partially lost CD62L expression, consistent with a more differentiated phenotype. The differences in CD122 expression were functionally relevant because they closely correlated with proliferation in response to IL-7 and IL-15 (Fig. 1 C, top), being low in naive cells and CXCR5⁺ T_{CM} cells, intermediate in CCR4⁺ T_{CM} cells and -/- T_{CM} cells, and high in CXCR3⁺ T_{CM} and T_{EM} cells.

We then compared the expansion potential of purified CFSE-labeled CD4⁺ naive and memory T cell subsets after TCR stimulation with TSST-loaded DCs because replicative capacity diminishes with T cell differentiation (25, 40). Proliferation and accumulation was high in naive cells, CXCR5⁺ T_{CM} cells, and -/- T_{CM} cells, intermediate in CCR4⁺ and CXCR3⁺ T_{CM} cells, and low in T_{EM} cells (Fig. 1 C, bottom). As reported for the CD8 compartment (25), the reduced accumulation of T_{EM} cells was associated with a high rate of apoptosis (Table I). Similar results were obtained upon stimulation with anti-CD3 and anti-CD28 antibodies (not depicted).

Together, these results show that subsets of CD4⁺ T_{CM} cells identified by CXCR5, CXCR3, and CCR4 expression differ in their proliferative response to cytokines and TCR ligands, and suggest that CXCR5⁺ T_{CM} cells and -/- T_{CM} cells are at an early stage of memory cell differentiation, whereas CCR4⁺ and CXCR3⁺ T_{CM} cells have characteristics of more mature cell types.

CXCR3 and CCR4 Identify T_{CM} Cells with Low IFN- γ and IL-4-producing Capacities. Next, we analyzed effector cytokine-producing capacities of T_{CM} and T_{EM} cell subsets. Total T_{CM} and T_{EM} cell populations were sorted for

CXCR3 and CCR4 expression, stimulated with PdBu and ionomycin, and secreted cytokines were quantified by ELISA (Fig. 2 A). T_{CM} cells lacking CXCR3 and CCR4 failed to produce IFN- γ , IL-4, and IL-5, whereas double negative cells in the T_{EM} subset produced all three cytokines. Consistent with the role of CXCR3 and CCR4 as Th1 and Th2 cell markers, CXCR3-expressing cells produced predominantly IFN- γ , whereas CCR4⁺ cells pro-

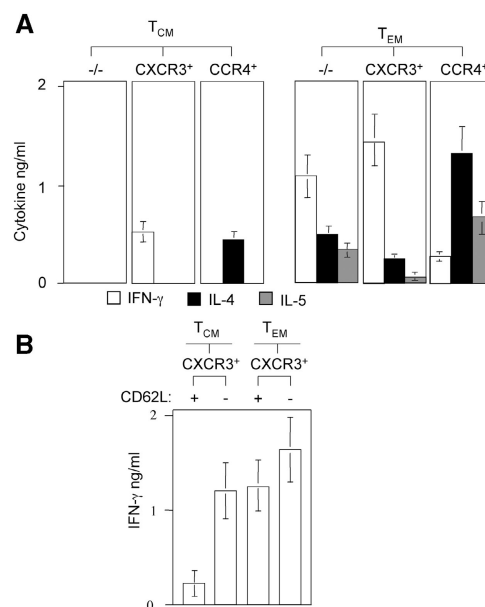


Figure 2. Ex vivo cytokine-producing capacities of CD4⁺ memory T cell subsets. (A) Purified CD4⁺ T_{CM} and T_{EM} cell subsets were stimulated with PdBu and ionomycin for 24 h and supernatants were analyzed for IFN- γ (diluted 1:4, white bars), IL-4 (black bars), and IL-5 (gray bars) by ELISA. Stimulation with anti-CD3 and anti-CD28 antibodies gave similar results (not depicted). Shown is the mean of four experiments with cells from different donors. (B) CXCR3⁺ CD4⁺ T cells were sorted for CCR7 and CD62L expression as indicated and IFN- γ production was assessed as described above. The mean of three independent experiments with three different donors is shown.

duced mainly type 2 cytokines. However, CXCR3⁺ T_{CM} and CCR4⁺ T_{CM} cells produced less effector cytokines than the corresponding T_{EM} cell subset, and IL-5 production was entirely restricted to T_{EM} cells. Similar results were obtained when CXCR5⁺ T_{CM} and CXCR5⁻ T_{CM} cell subsets were analyzed separately (not depicted). Because CXCR3⁺ T_{CM} cells contained a considerable fraction of CD62L⁻ cells (Table I), we further analyzed IFN- γ -producing capacity of CXCR3⁺ T_{CM} and T_{EM} cells according to CD62L expression (Fig. 2 B). CXCR3⁺ T_{CM} cells expressing CD62L produced only low amounts of IFN- γ , whereas CXCR3⁺ T_{CM} cells lacking CD62L produced high levels of IFN- γ comparable to T_{EM} cells. Thus, IFN- γ production among CCR7⁺ cells is largely restricted to a T_{EM} cell-like subset of CD62L⁻ CXCR3⁺ cells. Collectively, these results show that CXCR3 and CCR4 identify cells in the T_{CM} cell pool that are nonpolarized or produce low levels of IFN- γ or IL-4.

Cytokine-stimulated CXCR3⁺ and CCR4⁺ T_{CM} Cells Differentiate to Th1 and Th2 Cells. We previously showed that some cytokine-stimulated T_{CM} cells spontaneously differentiate to Th1 or Th2 cells, whereas naive cells require TCR ligands or inflammatory cytokines for differentiation (21, 41). To understand whether the subsets defined by CXCR5, CXCR3, and CCR4 could discriminate cells with predetermined fates, we induced proliferation of purified CD4⁺ T cell subsets by either TSST-loaded DCs or IL-7 and IL-15 (Fig. 3 A). Because memory subsets showed different proliferative responses to IL-7 and IL-15 (Fig. 1 C), analysis was performed by gating on cells that had performed the same number of divisions (cytokines: 4; TSST: >7). Under both conditions of stimulation, CXCR5⁺ T_{CM} cells that lacked CXCR3 and CCR4 expression remained nonpolarized, whereas ^{-/-}T_{CM} cells generated some Th1 and Th2 cells and acquired CXCR3 and CCR4 on a fraction of cells at the same time (Fig. 3 C). Cytokine-stimulated CXCR5⁺ T_{CM} cells progressively lost CXCR5 expression, but homogeneously maintained high levels of CCR7 and CD62L, whereas a fraction of CXCR5⁻ T_{CM} cells progressively lost CCR7 and CD62L expression, thus acquiring the phenotype of T_{EM} cells (Fig. 3 B). Notably, T_{EM} cells remained CCR7⁻ and maintained high effector functions under these conditions.

When CXCR3⁺ T_{CM} and CCR4⁺ T_{CM} cells were expanded with homeostatic cytokines, they maintained CXCR3 and CCR4 expression (Fig. 3 C) and spontaneously differentiated into Th1 and Th2 cells, respectively (Fig. 3 A). Thus, CCR4⁺ T_{CM} cells produced high levels of IL-4 and also secreted IL-5 (not depicted), a type 2 cytokine produced exclusively by T_{EM} cells (Fig. 2 A). Moreover, the amount of IFN- γ produced by CXCR3⁺ T_{CM} cells stimulated with IL-7 and IL-15 was comparable to that produced by CXCR3⁺ T_{EM} cells ex vivo, and a fraction of CXCR3⁺ T_{CM} cells acquired expression of CCR5 (Fig. 3 C), a receptor for inflammatory chemokines ex-

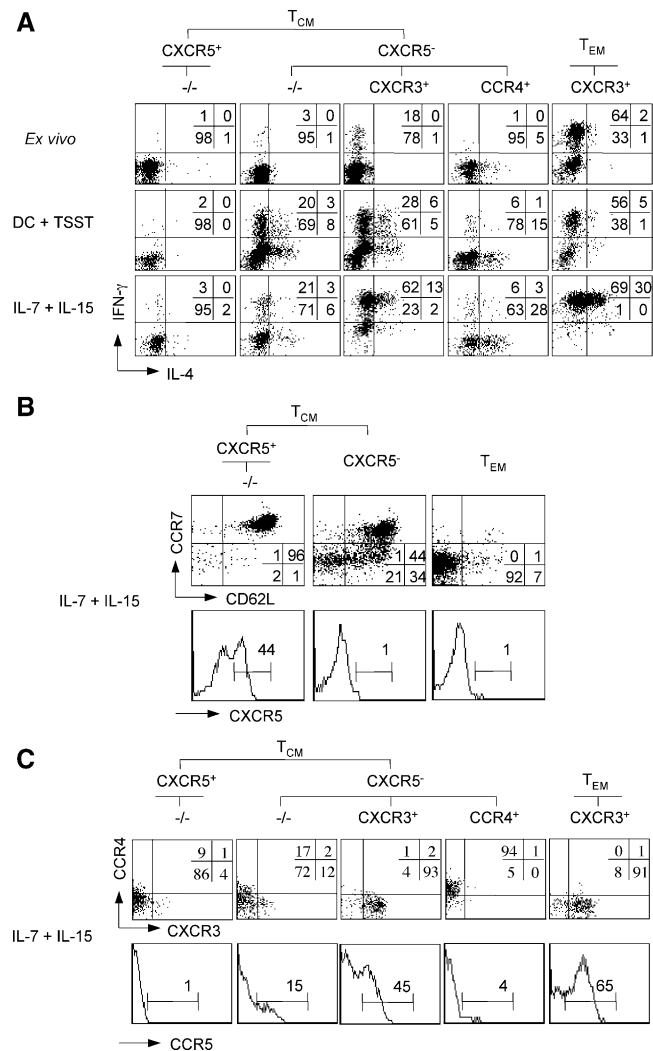


Figure 3. (A) Differentiation of T_{CM} cell subsets in response to TCR or cytokine stimulation. Purified CD4⁺ T cell subsets were CFSE labeled and stimulated with TSST-loaded DCs or with IL-7 and IL-15 in the presence of neutralizing anti-IL-4 and anti-IL-12 antibodies. After 7 d, cells were stimulated with PdBu and ionomycin, stained with APC-labeled anti-IFN- γ and PE-labeled anti-IL-4 antibodies, and cells of the same division number were analyzed by flow cytometry. Unstimulated T cell subsets were also analyzed ex vivo as control. Numbers indicate the percentage of cells producing IFN- γ or IL-4. One representative experiment out of five is shown. (B and C) Modulation of homing receptor expression by cytokine-stimulated CD4⁺ memory T cell subsets. Purified CFSE-labeled CD4⁺ T cell subsets were stimulated with IL-7 and IL-15. T_{CM} and T_{EM} cells in B were sorted for CXCR5, CCR7, and CD62L expression, whereas in C they were sorted for CXCR5, CCR7, CXCR3, and CCR4 expression as indicated. After 7 d, cells in division four were analyzed for the expression of CXCR5, CCR7, and CD62L (B), or CXCR3, CCR4, and CCR5 (C). One representative donor out of four is shown.

pressed on Th1 effector cells (5). Together, these results demonstrate that T_{CM} cells that lack CXCR3 and CCR4 expression are nonpolarized precursors, whereas CXCR3⁺ and CCR4⁺ T_{CM} cells represent pre-Th1 and pre-Th2 cells that become fully differentiated Th1 and Th2 effector cells in response to homeostatic cytokines.

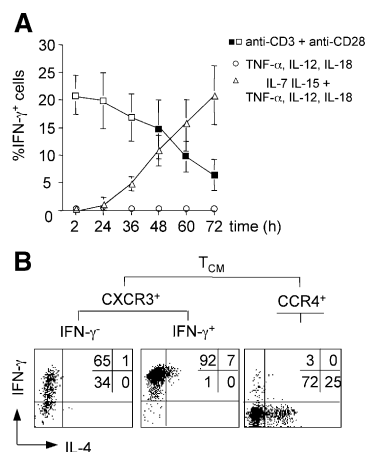


Figure 4. (A) Kinetics and requirements of TCR- and cytokine-induced IFN- γ production. CFSE-labeled CD4⁺ memory T cells were stimulated for the indicated times with either anti-CD3 and anti-CD28 antibodies (squares), or with TNF- α , IL-12, and IL-18 in the absence (circles) or presence (triangles) of IL-7 and IL-15. IFN- γ production was analyzed by intracellular staining. Empty symbols indicate conditions with undivided cells, whereas filled symbols indicate conditions with dividing cells. The mean percentage of IFN- γ ⁺ cells of three independent experiments is plotted. (B) Cytokine-stimulated CXCR3⁺ T_{CM} cells lacking IFN- γ -producing capacity become Th1 cell effector cells. Purified CFSE-labeled CXCR3⁺ T_{CM} and CCR4⁺ T_{CM} cells were stimulated with IL-7, IL-15, TNF- α , IL-12, and IL-18 for 60 h, and IFN- γ -secreting cells were purified by cell sorting. IFN- γ ⁺ and IFN- γ ⁻ cells were then expanded for an additional 5 d with IL-7 and IL-15, briefly stimulated with PdBu and ionomycin, and analyzed for IL-4 and IFN- γ production by intracellular staining. One representative donor out of three is shown.

To exclude a selective outgrowth of preexisting IFN- γ -producing cells from CXCR3⁺ T_{CM} cells, we wished to deplete cells with IFN- γ -producing capacity from cyto-

kine-stimulated cultures. To this aim it was necessary to induce IFN- γ production of Th1 cell-polarized memory cells without activating TCR-dependent signaling. It is well established that TCR-independent IFN- γ production of activated Th1 cells can be induced by inflammatory cytokines IL-12 and IL-18 (44, 45). We found that production of IFN- γ by resting CD4⁺ memory cells in response to IL-12 and IL-18 required activation by IL-7 and IL-15, was boosted by TNF- α , and occurred with delayed kinetics (Fig. 4 A). In contrast, IL-2 production was restricted to TCR-stimulated cells (not depicted), confirming that IFN- γ production by cytokine-stimulated cells is TCR independent. Notably, TCR and cytokine stimulation induced a similar fraction of cells to secrete IFN- γ before cell division (Fig. 4 A, note empty/filled symbols), and IFN- γ production was in both cases restricted to CXCR3⁺ T_{CM} and T_{EM} cells (Fig. 2 and not depicted). Thus, mature Th1 cell-polarized memory cells can be identified by IFN- γ secretion after either TCR or optimal cytokine activation.

Next, we induced IFN- γ production by purified CFSE-labeled CXCR3⁺ T_{CM} cells with cytokines, sorted undivided IFN- γ cells after 60 h, expanded them with IL-7 and IL-15, and analyzed effector cytokine-producing capacities of proliferating cells (Fig. 4 B). A large fraction of IFN- γ ⁻ CXCR3⁺ T_{CM} cells differentiated under these conditions and acquired the capacity to produce high levels of IFN- γ . Sorting IFN- γ cells after 72 h gave similar results (not depicted). In contrast, CCR4⁺ T_{CM} cells secreted IL-4 under the same conditions, whereas IFN- γ ⁺ CXCR3⁺ T_{CM} cells maintained high IFN- γ production. We conclude that CXCR3⁺ T_{CM} cells lacking IFN- γ -producing capacity become Th1 effector cells after TCR-independent proliferation induced by cytokines.

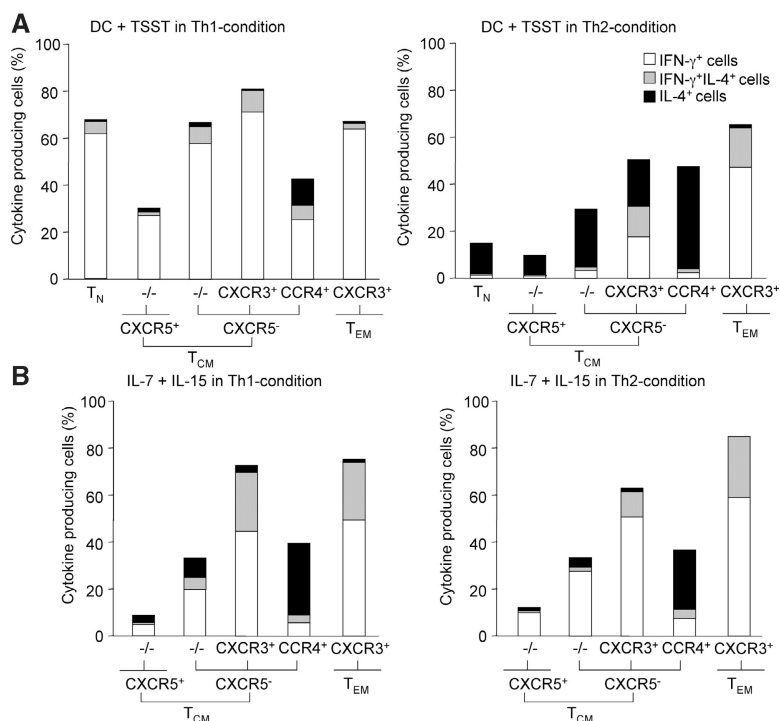


Figure 5. Effects of polarizing cytokines on TCR- and cytokine-induced differentiation. Purified CFSE-labeled CD4⁺ T cell subsets were stimulated with DC plus TSST or IL-7 plus IL-15 in the absence or presence of IL-12 and neutralizing anti-IL-4 antibody (Th1-condition) or IL-4 and neutralizing anti-IL-12 antibody (Th2-condition). After 7 d, cells were stimulated with PdBu and ionomycin and cells of the same division number were analyzed for IFN- γ and IL-4 production by intracellular staining. The percentages of IFN- γ ⁺ cells (white bars), IL-4⁺ cells (black bars), and of cells producing both cytokines (gray bars) are represented. One representative experiment out of five with different donors is shown.

We then analyzed the effects of polarizing cytokines on T cell differentiation induced by TCR agonists or homeostatic cytokines. IL-4 and IL-12 induced differentiation of TCR-stimulated CXCR5⁺ and especially $\gamma\gamma$ -T_{CM} cells into IL-4- and IFN- γ -producing cells, respectively, whereas they failed to modulate T cell differentiation in IL-7 plus IL-15-activated T cells (Fig. 5, A and B). Under the same conditions of TCR stimulation, IL-4 induced CXCR3⁺ T cells to produce the opposite cytokine IL-4 and promoted CCR4 expression, whereas IL-12 induced CCR4⁺ cells to produce IFN- γ (Fig. 5 A) and up-regulate

CXCR3 (not depicted). Again, polarizing cytokines had little effect on the extent of T cell differentiation in IL-7 plus IL-15-stimulated cells (compare Figs. 3 A and 5 B). Similar results were obtained when IL-2 substituted for IL-7 and IL-15, and in the absence or presence of TNF, IL-6 and IL-10, DC-derived cytokines that strongly boost proliferation of T_{CM} cells in response to IL-7 and IL-15 (not depicted; reference 21). Together, these findings suggest that flexibility of cytokine gene expression of human memory T cells requires TCR triggering and polarizing cytokines (13).

Proliferation History, In Vivo Turnover, and Recall Responses of CD4⁺ Memory T Cell Subsets. The proliferation history and in vivo turnover of the different memory T cell subsets was then assessed by measuring the amounts of TRECs and the spontaneous BrdU incorporation of ex vivo-isolated cells. TRECs carrying a particular signal joint sequence (37) were quantified by TaqMan PCR in T cell subsets from five healthy donors (Fig. 6 A). As expected, CD4⁺ CD45RA⁺ naive T cells contained high levels of TRECs, whereas B cells and T cell clones were negative (not depicted). Compared with naive T cells, CXCR5⁺ T_{CM} and CXCR5⁻ T_{CM} cells contained much lower amounts of TRECs, whereas T_{EM} cells contained the lowest amount. Although there were considerable quantitative differences among individual donors, these data indicate that the different subsets of T_{CM} cells have divided to a similar extent.

To measure the spontaneous BrdU incorporation, freshly isolated PBMCs were incubated with BrdU. CD4⁺ T cells were then purified and T cell subsets analyzed by intracellular staining with anti-BrdU antibodies. As shown in Fig. 6 B, CXCR5⁺ T_{CM}, CXCR3⁺ T_{CM}, and CCR4⁺ T_{CM} cells spontaneously incorporated BrdU to a similar extent, whereas T_{EM} and especially $\gamma\gamma$ -T_{CM} cells had a higher proliferation rate and naive cells were below the detection limit. These data indicate that memory T cells in different subsets slowly turn over under steady-state conditions in vivo.

To investigate whether the different memory subsets contained cells specific for recall antigens, T cell populations were isolated from smallpox- and/or tetanus-vaccinated donors and stimulated for 7 d with autologous monocytes that were incubated with TT or with an extract of CMV-derived proteins. To assess memory against smallpox, monocytes were infected with a replication-deficient VV. In these experiments, purified CFSE-labeled T cell subsets and CFSE dilution were used to read out proliferation of pathogen-specific T cells. From eight donors analyzed, seven responded strongly to TT, whereas one donor that had been boosted 20 yr ago had a low response (not depicted). Five donors responded strongly to CMV, and four of six donors that had also been vaccinated against smallpox had a detectable response to VV. Responses to autologous monocytes alone were undetectable or very low (not depicted).

We analyzed the distribution of TT-, CMV-, and VV-specific T cells among different memory subsets (one donor

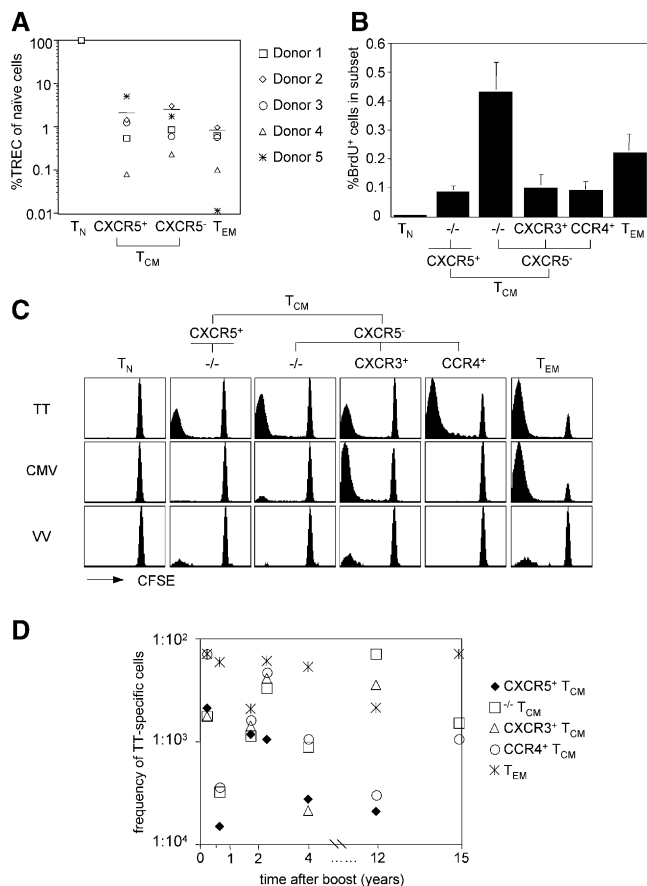


Figure 6. Proliferation history, in vivo turnover, and recall responses of CD4⁺ memory T cell subsets. (A) Total CXCR5⁺ T_{CM}, CXCR5⁻ T_{CM}, and T_{EM} cells were analyzed for their single joint TREC content and compared with naive cells from the same donor (naive cells: 100%). Bars indicate the mean TREC levels in memory subsets of five different donors. (B) Freshly isolated PBMCs were incubated with BrdU, CD4⁺ T cells were isolated, and BrdU incorporation was analyzed as a function of CD45RA and chemokine receptor expression by flow cytometry. The mean percentage of BrdU⁺ cells in a given subset of four donors is shown. (C) CD4⁺ naive and memory subsets were sorted, labeled with CFSE, and incubated with autologous monocytes that had either been infected with VV or incubated with TT or an extract of CMV-derived proteins. CFSE profiles of viable CD4⁺ CD14⁻ cells on day 7 of one representative donor are shown. (D) Recall responses of memory subsets to TT of eight different donors were assessed as described above, and the frequency of TT-specific cells was calculated after 5 d. The frequency of TT-specific cells in the indicated memory subsets of seven different TT-responsive donors was plotted against the time of the last boost.

responding to all three pathogens is shown in Fig. 6 C). In all cases, pathogen-specific cells were detected in both the T_{CM} and T_{EM} cell pools. TT-specific cells were undetectable among naive cells, but present in all memory subsets in six of seven responsive donors. Conversely, CMV-specific cells were largely restricted to $CXCR3^+ T_{CM}$ and T_{EM} cells in all five responsive donors, consistent with the notion that CMV infection promotes a Th1 cell response (46). Consistent with previous reports, VV-specific cells were less frequent (47, 48), but were detectable in $CXCR3^+ T_{CM}$ and T_{EM} cells and, interestingly, in three of four donors in $CXCR5^+ T_{CM}$ cells. Together, these results show that all memory subsets contain cells specific for recall antigens, and that the distribution of antigen-specific cells within T_{CM} cell subsets varies for different pathogens.

Next, we compared precursor frequencies of TT-specific cells in different subsets in donors that had been boosted recently or several years ago (Fig. 6 D). TT-specific cells were relatively frequent among T_{EM} cells ($>1:500$) in all donors. Conversely, $CXCR5^+ T_{CM}$ cells were less frequent ($<1:500$), especially in donors that had not been boosted for several years, possibly explaining the failure of previous studies to detect TT-specific cells in this subset using thymidine incorporation (29, 31). Interestingly, the relative distribution among $CXCR3^+ T_{CM}$ and $CCR4^+ T_{CM}$ cells was highly variable, with some donors having higher numbers of $CCR4^+ T_{CM}$ cells and others containing predominantly $CXCR3^+ T_{CM}$ cells. These results indicate that TT-specific T cells are present in high frequency in T_{EM} cells, even several years after vaccination, and are distributed in different subsets of T_{CM} cells.

Discussion

We have shown that the human $CD4^+ T_{CM}$ cell pool can be subdivided into subsets of nonpolarized cells and pre-Th1 and pre-Th2 cells based on chemokine receptor expression. These subsets have extensively divided in vivo and contain cells specific for recall antigens and with self-renewal capacity. Upon TCR-independent proliferation induced by homeostatic cytokines, T_{CM} cell subsets are committed for different fates and become Th1, Th2, or remain nonpolarized cells, explaining how the quality of the primary immune response could be maintained by T_{CM} cells in the absence of antigen.

Th cells expressing $CXCR5$ comprise $CCR7^- CD57^+$ follicular Th cells in tonsils (29, 30, 49), and nonpolarized circulating $CXCR5^+ T_{CM}$ cells of unknown function and specificity that might have a recent activation history (31). Our results show that nonpolarized cells are present in both $CXCR5^+$ and $CXCR5^- T_{CM}$ cell subsets and lack $CXCR3$ and $CCR4$ expression. Using CFSE dilution we were further able to show that $CXCR5^+ T_{CM}$ cells contained low numbers of TT-specific cells even several years after vaccination. Moreover, three of four smallpox-vaccinated, responsive donors contained VV-specific cells at low frequency in the $CXCR5^+ T_{CM}$ cell subset. Using quanti-

tative PCR, we found that $CXCR5^+ T_{CM}$ and $CXCR5^- T_{CM}$ cells contained comparable amounts of residual TRECs, whereas T_{EM} cells had slightly lower levels, suggesting that T_{CM} cell subsets had divided to a comparable extent (approximately seven times). However, because naive and memory cells were identified by CD45 isoform expression that is not a stable marker (25, 50), the number of divisions performed by memory cells might be underestimated by our analysis. Spontaneous BrdU uptake indicated that $CXCR5^+ T_{CM}$ cells have an in vivo proliferation rate that is comparable to that of other memory subsets. The relative small cell size and the absence of CD69 on BrdU⁺ cells suggest that this proliferation is driven by homeostatic mechanisms rather than by antigen. Why $^-/-T_{CM}$ cells have a higher turnover than other memory subsets is currently unclear. They might be particularly fit because they combine a relatively high cytokine responsiveness with a low susceptibility to apoptosis (51). Alternatively, they might be preferentially located in cytokine-rich microenvironments or in proximity to DCs that boost proliferation in response to IL-7 and IL-15 (21). In any case, these results show that all $CD45RA^-$ subsets, including $CXCR5^+ T_{CM}$ cells, are memory cells that have extensively divided, slowly turnover in vivo, and contain cells specific for recall antigens.

Different viruses are known to induce $CD8^+$ memory cells belonging preferentially to different subsets (52, 53). Here we showed that although pathogen-specific $CD4^+$ T cells are present in both the T_{CM} and T_{EM} cell pools, they have characteristic distributions in T_{CM} cell subsets, reflecting the Th1/Th2 cell polarization induced by the pathogens or vaccinations. Thus, TT-specific cells were detected in all subsets, consistent with the notion that vaccination against tetanus induces a mixed Th1/Th2 cell response (54). Conversely, CMV and VV promote Th1 cell polarization (46, 47), and virus-specific cells were consequently detected in $CXCR3^+ T_{CM}$ cells but not in $CCR4^+ T_{CM}$ cells. In one donor, we were able to show that VV-specific T_{EM} cells were also $CXCR3^+$ (not depicted). Collectively, these results suggest that immune responses generate heterogeneous populations of memory cells that belong to different subsets and comprise a broad spectrum of differentiation stages. The distribution between $CXCR3^+$ and $CCR4^+$ subsets in the T_{CM} and T_{EM} cell pools might be useful to monitor the quality of the memory response to different pathogens.

Human memory T cells can be subdivided into $CCR7^+ T_{CM}$ and $CCR7^- T_{EM}$ cells with different effector functions and homing potentials, suggesting a division of labor between these two subsets (12). However, several recent reports showed that antigen-experienced $CCR7^+$ cells possess immediate effector functions (9, 32–35). Although we identified here $CCR7^+$ memory cells with IFN- γ - and IL-4-producing capacities as $CXCR3^+ T_{CM}$ and $CCR4^+ T_{CM}$ cells, respectively, the following lines of evidence suggest that these cells are not fully differentiated effectors: (a) they had a higher expansion potential than T_{EM}

cells and most cells had retained CD27 and CD62L expression; (b) IFN- γ production by CXCR3⁺ T_{CM} cells was low and largely restricted to unconventional CCR7⁺ CD62L⁻ cells; (c) although CCR4⁺ T_{CM} cells produced some IL-4, production of IL-5, which acts on eosinophils at peripheral sites of allergic inflammation, was limited to T_{EM} cells; (d) many CXCR3⁺ T_{CM} and CCR4⁺ T_{CM} cells had retained flexibility to differentiate to Th2 and Th1 cells, respectively, upon antigenic stimulation in the presence of appropriate polarizing cytokines; and (e) upon cytokine stimulation, they further differentiated, losing CCR7 and CD62L and acquiring nonlymphoid homing potential and high levels of effector functions. Together, these findings are consistent with the notion that nonlymphoid tissue homing potential and effector cytokine-producing capacities are progressively acquired upon T cell differentiation and reside predominantly in the T_{EM} cell subset of the human CD4⁺ memory cell pool (3). However, because effector functions and nonlymphoid homing potentials are acquired in a stochastic manner (55), some cells have characteristics that are intermediate between T_{CM} and T_{EM} cells.

We previously proposed that cytokine-driven differentiation of T_{CM} cells might be a mechanism to replenish short-lived T_{EM} cells in the absence of antigen (21), but how nonpolarized T_{CM} cells could faithfully maintain polarized Th1 or Th2 effector cell populations remained unclear. A recent report showed that CD4 T cell priming upon viral infection generated both short-lived effector cells and long-lived precursors that lacked effector functions, but spontaneously acquired IFN- γ -producing capacity when transferred into antigen-free hosts (26). We showed here that CXCR3⁺ T_{CM} and CCR4⁺ T_{CM} cells invariably differentiated to CCR7⁻ Th1 or Th2 effector cells in an antigen-independent fashion, whereas CXCR5⁺ T_{CM} cells remained nonpolarized and CCR7⁺. CXCR3 and CCR4 are preferentially induced under type 1 and type 2 priming conditions, respectively (6, 7), and CXCR3⁺ T_{CM} and CCR4⁺ T_{CM} cells might therefore represent committed precursors of the Th1 and Th2 cell lineage with the capacity to generate effector cells for extended periods in the absence of antigen. Unlike cytokines, TCR ligands can still instruct T_{CM} cells to become Th1 or Th2 cells in the presence of appropriate polarizing cytokines (13). This differential flexibility might allow the human immune system to mount qualitatively different responses in the case of cross-reactive antigens (56), or alternatively, to maintain the quality of the primary response under homeostatic conditions.

We thank I. Giacchetto for technical assistance and Elisabetta Tragiai and Stefan Wirths for critical reading and comments.

This work has been supported in part by the European Community (contract no. QLK-CT-201-0105 to F. Sallusto), the NIH (grant no. U19AI057266-01 to A. Lanzavecchia), and by the Swiss National Science Foundation (grant no. 3100A0-104168 to J. Geginat and no. 3100A0-101962 to F. Sallusto). L. Rivino is supported by the Fondazione per la Ricerca sulla Trasfusione e i Trapianti.

The authors have no conflicting financial interests.

Submitted: 20 April 2004

Accepted: 5 August 2004

References

1. Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature*. 392:245–252.
2. Mosmann, T.R., and R.L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145–173.
3. Lanzavecchia, A., and F. Sallusto. 2000. Dynamics of T lymphocyte responses: intermediates, effectors and memory cells. *Science*. 290:92–97.
4. Sallusto, F., A. Lanzavecchia, and C.R. Mackay. 1998. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. *Immunol. Today*. 19:568–574.
5. Qin, S., J.B. Rottman, P. Myers, N. Kassam, M. Weinblatt, M. Loetscher, A.E. Koch, B. Moser, and C.R. Mackay. 1998. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J. Clin. Invest.* 101:746–754.
6. D'Ambrosio, D., A. Iellem, R. Bonecchi, D. Mazzeo, S. Sozzani, A. Mantovani, and F. Sinigaglia. 1998. Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. *J. Immunol.* 161:5111–5115.
7. Sallusto, F., D. Lenig, C.R. Mackay, and A. Lanzavecchia. 1998. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med.* 187:875–883.
8. Langenkamp, A., K. Nagata, K. Murphy, L. Wu, A. Lanzavecchia, and F. Sallusto. 2003. Kinetics and expression patterns of chemokine receptors in human CD4⁺ T lymphocytes primed by myeloid or plasmacytoid dendritic cells. *Eur. J. Immunol.* 33:474–482.
9. Kim, C.H., L. Rott, E.J. Kunkel, M.C. Genovese, D.P. Andrew, L. Wu, and E.C. Butcher. 2001. Rules of chemokine receptor association with T cell polarization in vivo. *J. Clin. Invest.* 108:1331–1339.
10. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. 99:23–33.
11. Bradley, L.M., S.R. Watson, and S.L. Swain. 1994. Entry of naive CD4 T cells into peripheral lymph nodes requires L-selectin. *J. Exp. Med.* 180:2401–2406.
12. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 401:708–712.
13. Messi, M., I. Giacchetto, K. Nagata, A. Lanzavecchia, G. Natoli, and F. Sallusto. 2003. Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. *Nat. Immunol.* 4:78–86.
14. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 22:745–763.
15. Sprent, J., D.F. Tough, and S. Sun. 1997. Factors controlling the turnover of T memory cells. *Immunol. Rev.* 156:79–85.
16. Schluns, K.S., W.C. Kieper, S.C. Jameson, and L. Lefrancois. 2000. Interleukin-7 mediates the homeostasis of naive and

- memory CD8 T cells in vivo. *Nat. Immunol.* 1:426–432.
17. Zhang, X., S. Sun, I. Hwang, D.F. Tough, and J. Sprent. 1998. Potent and selective stimulation of memory-phenotype CD8⁺ T cells in vivo by IL-15. *Immunity*. 8:591–599.
 18. Seddon, B., and R. Zamoyska. 2002. TCR and IL-7 receptor signals can operate independently or synergize to promote lymphopenia-induced expansion of naive T cells. *J. Immunol.* 169:3752–3759.
 19. Seddon, B., P. Tomlinson, and R. Zamoyska. 2003. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nat. Immunol.* 4:680–686.
 20. Kanegane, H., and G. Tosato. 1996. Activation of naive and memory T cells by interleukin-15. *Blood*. 88:230–235.
 21. Geginat, J., F. Sallusto, and A. Lanzavecchia. 2001. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4⁺ T cells. *J. Exp. Med.* 194:1711–1719.
 22. Reinhardt, R.L., A. Khoruts, R. Merica, T. Zell, and M.K. Jenkins. 2001. Visualizing the generation of memory CD4 T cells in the whole body. *Nature*. 410:101–105.
 23. Roman, E., E. Miller, A. Harmsen, J. Wiley, U.H. von Andrian, G. Huston, and S.L. Swain. 2002. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *J. Exp. Med.* 196:957–968.
 24. Wherry, E.J., V. Teichgraber, T.C. Becker, D. Masopust, S.M. Kaech, R. Antia, U.H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* 4:225–234.
 25. Geginat, J., A. Lanzavecchia, and F. Sallusto. 2003. Proliferation and differentiation potential of human CD8⁺ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood*. 101:4260–4266.
 26. Wu, C.Y., J.R. Kirman, M.J. Rotte, D.F. Davey, S.P. Perfetto, E.G. Rhee, B.L. Freidag, B.J. Hill, D.C. Douek, and R.A. Seder. 2002. Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. *Nat. Immunol.* 3:852–858.
 27. Cyster, J.G. 1999. Chemokines and cell migration in secondary lymphoid organs. *Science*. 286:2098–2102.
 28. Baggiolini, M. 1998. Chemokines and leukocyte traffic. *Nature*. 392:565–568.
 29. Breitfeld, D., L. Ohl, E. Kremmer, J. Ellwart, F. Sallusto, M. Lipp, and R. Forster. 2000. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* 192:1545–1552.
 30. Schaerli, P., K. Willmann, A.B. Lang, M. Lipp, P. Loetscher, and B. Moser. 2000. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J. Exp. Med.* 192:1553–1562.
 31. Schaerli, P., P. Loetscher, and B. Moser. 2001. Cutting edge: induction of follicular homing precedes effector Th cell development. *J. Immunol.* 167:6082–6086.
 32. Unsoeld, H., S. Krautwald, D. Voehringer, U. Kunzendorf, and H. Pircher. 2002. Cutting edge: CCR7⁺ and CCR7[−] memory T cells do not differ in immediate effector cell function. *J. Immunol.* 169:638–641.
 33. Debes, G.F., U.E. Hopken, and A. Hamann. 2002. In vivo differentiated cytokine-producing CD4(+) T cells express functional CCR7. *J. Immunol.* 168:5441–5447.
 34. Ravkov, E.V., C.M. Myrick, and J.D. Altman. 2003. Immediate early effector functions of virus-specific CD8⁺ CCR7⁺ memory cells in humans defined by HLA and CC chemokine ligand 19 tetramers. *J. Immunol.* 170:2461–2468.
 35. Wolint, P., M.R. Betts, R.A. Koup, and A. Oxenius. 2004. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8⁺ T cells. *J. Exp. Med.* 199:925–936.
 36. Gett, A.V., and P.D. Hodgkin. 2000. A cellular calculus for signal integration by T cells. *Nat. Immunol.* 1:239–244.
 37. Douek, D.C., R.D. McFarland, P.H. Keiser, E.A. Gage, J.M. Massey, B.F. Haynes, M.A. Polis, A.T. Haase, M.B. Feinberg, J.L. Sullivan, et al. 1998. Changes in thymic function with age and during the treatment of HIV infection. *Nature*. 396:690–695.
 38. Bonecchi, R., G. Bianchi, P.P. Bordinon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P.A. Gray, A. Mantovani, and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* 187:129–134.
 39. Hamann, D., P.A. Baars, M.H. Rep, B. Hooibrink, S.R. Kerkhof-Garde, M.R. Klein, and R.A. van Lier. 1997. Phenotypic and functional separation of memory and effector human CD8⁺ T cells. *J. Exp. Med.* 186:1407–1418.
 40. Weng, N.P., B.L. Levine, C.H. June, and R.J. Hodes. 1995. Human naive and memory T lymphocytes differ in telomeric length and replicative potential. *Proc. Natl. Acad. Sci. USA*. 92:11091–11094.
 41. Murali-Krishna, K., and R. Ahmed. 2000. Cutting edge: naive T cells masquerading as memory cells. *J. Immunol.* 165:1733–1737.
 42. Gett, A.V., and P.D. Hodgkin. 1998. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. *Proc. Natl. Acad. Sci. USA*. 95:9488–9493.
 43. Bird, J.J., D.R. Brown, A.C. Mullen, N.H. Moskowitz, M.A. Mahowald, J.R. Sider, T.F. Gajewski, C.R. Wang, and S.L. Reiner. 1998. Helper T cell differentiation is controlled by the cell cycle. *Immunity*. 9:229–237.
 44. Okamura, H., H. Tsutsi, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, et al. 1995. Cloning of a new cytokine that induces IFN- γ production by T cells. *Nature*. 378:88–91.
 45. Yang, J., T.L. Murphy, W. Ouyang, and K.M. Murphy. 1999. Induction of interferon- γ production in Th1 CD4⁺ T cells: evidence for two distinct pathways for promoter activation. *Eur. J. Immunol.* 29:548–555.
 46. Rentenaar, R.J., L.E. Gamadia, N. van DerHoek, F.N. van Diepen, R. Boom, J.F. Weel, P.M. Wertheim-van Dillen, R.A. van Lier, and I.J. ten Berge. 2000. Development of virus-specific CD4(+) T cells during primary cytomegalovirus infection. *J. Clin. Invest.* 105:541–548.
 47. Combadiere, B., A. Boissonnas, G. Carcelain, E. Lefranc, A. Samri, F. Bricaire, P. Debre, and B. Autran. 2004. Distinct time effects of vaccination on long-term proliferative and IFN- γ -producing T cell memory to smallpox in humans. *J. Exp. Med.* 199:1585–1593.
 48. Hammarlund, E., M.W. Lewis, S.G. Hansen, L.I. Strelow, J.A. Nelson, G.J. Sexton, J.M. Hanifin, and M.K. Slifka. 2003. Duration of antiviral immunity after smallpox vaccination. *Nat. Med.* 9:1131–1137.
 49. Kim, C.H., L.S. Rott, I. Clark Lewis, D.J. Campbell, L. Wu, and E.C. Butcher. 2001. Subspecialization of CXCR5⁺ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5⁺ T cells. *J. Exp. Med.* 193:1373–1381.

50. Bell, E.B., and S.M. Sparshott. 1990. Interconversion of CD45R subsets of CD4 T cells in vivo. *Nature*. 348:163–166.
51. Gett, A.V., F. Sallusto, A. Lanzavecchia, and J. Geginat. 2003. T cell fitness determined by signal strength. *Nat. Immunol.* 4:355–360.
52. Champagne, P., G.S. Ogg, A.S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G.P. Rizzardi, S. Fleury, M. Lipp, et al. 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature*. 410:106–111.
53. Appay, V., P.R. Dunbar, M. Callan, P. Klenerman, G.M. Gillespie, L. Papagno, G.S. Ogg, A. King, F. Lechner, C.A. Spina, et al. 2002. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat. Med.* 8:379–385.
54. Rowe, J., C. Macaubas, T. Monger, B.J. Holt, J. Harvey, J.T. Poolman, R. Loh, P.D. Sly, and P.G. Holt. 2001. Heterogeneity in diphtheria-tetanus-acellular pertussis vaccine-specific cellular immunity during infancy: relationship to variations in the kinetics of postnatal maturation of systemic th1 function. *J. Infect. Dis.* 184:80–88.
55. Reiner, S.L., and R.A. Seder. 1999. Dealing from the evolutionary pawnshop: how lymphocytes make decisions. *Immunity*. 11:1–10.
56. Welsh, R.M., and L.K. Selin. 2002. No one is naive: the significance of heterologous T-cell immunity. *Nat Rev. Immunol.* 2:417–426.

Manuscript 2

The strength of T cell stimulation determines IL-7 responsiveness, recall potential and lineage commitment of primed human CD4⁺IL-7R^{hi} T cells.

Laura Lozza, Laura Rivino, Greta Guarda, David Jarrossay, Andrea Rinaldi, Francesco Bertoni, Federica Sallusto, Antonio Lanzavecchia, and Jens Geginat

(Submitted)

The strength of T cell stimulation determines IL-7 responsiveness, recall potential and lineage commitment of primed human CD4⁺IL-7R^{hi} T cells

Laura Lozza*^o, Laura Rivino*^o, Greta Guarda*, David Jarrossay*, Andrea Rinaldi[^],
Francesco Bertoni[^], Federica Sallusto*, Antonio Lanzavecchia*, and Jens Geginat*^o

**Institute for Research in Biomedicine and [^]Laboratory of Experimental Oncology, Oncology
Institute of Southern Switzerland), Via Vincenzo Vela 6, 6500 Bellinzona, Switzerland*

*^oCurrent address: Charite University Medicine Berlin and German Arthritis Research
Center, Campus Charite Mitte c/o DRFZ, Chariteplatz 1, 10117 Berlin, Germany*

Running Title: Signal strength determines memory potential of IL7R⁺ cells

Keywords: T cell priming, memory generation, cytokines, differentiation, apoptosis

Address correspondence to:

Jens Geginat, Charite Universitaetsmedizin Berlin, Campus Mitte c/o DRFZ,
Chariteplatz 1, 10117 Berlin, Germany

Phone: ++49 30 28460708 Fax: ++49 30 28460656 E-mail: geginat@drfz.de

Abbreviations used in this paper: CCR, chemokine receptor; T_{CM}, T central memory; T_{EM}, T effector memory; PdBu, phorbol-12-13-dibutyrate; TSST, toxic shock syndrome toxin.

1007 characters on Title page

Mouse memory T cell precursors express IL-7R α , proliferate with homeostatic cytokines and undergo secondary expansions with antigen. Here we analyzed how the strength of antigenic stimulation - as determined by DC number, DC maturation state and antigen concentration - controls IL-7R α expression and responsiveness to IL-7, IL-15 and antigen in human CD4⁺ T cells. We found that T cells primed by different strengths of stimulation expressed IL-7R α in different proportions and preferentially on cells that maintained expression of the central memory marker CCR7. However, while CCR7⁺IL-7R^{hi} cells generated at high strength of stimulation proliferated vigorously in response to IL-7 or IL-15, CCR7⁺IL-7R^{hi} cells generated at low strength of stimulation responded poorly. High cytokine responsiveness was associated with reduced PTEN expression and enhanced s6-kinase activation, consistent with efficient receptor coupling to downstream signaling pathways. Interestingly, while intermediate-stimulated CCR7⁺IL-7R^{hi} cells were non-polarized, self-renewed with IL-7 and expanded with antigen, high-stimulated cells generated Th1 effector cells with cytokines but showed impaired IL-2 production and survival with antigen. Gene expression analysis suggested that high-stimulated cells represented pre-Th1 cells with low recall potential and high metabolic state. Taken together these results demonstrate that IL-7 receptor expression and coupling are instructed in T cells by the strength of stimulation and suggest that memory subsets may derive from CCR7⁺IL-7R^{hi} precursors that received different strengths of stimulation.

Introduction

Naïve T cell priming by antigen-carrying DC can result in different outcomes (1). Suboptimal T cell activation by immature DC induce an abortive proliferation that is thought to be important for maintenance of tolerance to tissue antigens (2, 3). Conversely, potent activation following infection with pathogens leads to effector and memory T cell generation (1), although persistent antigenic stimulation in chronic viral infections can lead to deletion of antigen-specific T cells due to clonal exhaustion (4, 5). The cell fate decisions are regulated by the conditions of T cell priming. Factors such as DC maturation stage, affinity of T cell receptor (TCR) for antigen, T cell competition for access to DC, and polarizing cytokines, all contribute to the strength of T cell stimulation and, consequently, determine the functional output (6). In particular, signal strength determines the “fitness” of primed T cells, i.e. the capacity to resist death by neglect, to proliferate in response to homeostatic cytokines and to persist in the absence of antigen *in vivo* (7).

It is still poorly understood how antigen-primed T cells are selected to survive and become memory cells. However, it was proposed that IL-7 receptor derived signals play a non-redundant role in mice since, in the absence of IL-7, memory T cells failed to develop (8-11). Following acute antigenic stimulation, mouse CD8⁺ memory cell precursors can be identified by IL-7 receptor α chain (IL-7R α) expression at the peak of the proliferative response (11, 12). These memory precursors show enhanced survival, homeostatic proliferation and powerful recall response both *in vitro* and *in vivo* (11, 12). Nevertheless, IL-15 might promote memory cell generation in the absence of IL-7 (13-15) and not all IL-7R^{hi} expressing cells do necessarily survive and become memory cells (11, 16). Moreover, some IL-7R^{lo} cells might persist in a functional inferior state both in mice and in humans (17, 18) and under conditions of chronic antigenic stimulation IL-7R^{hi} memory cells do not develop (5, 19, 20). In particular, whether IL-7R^{hi} cells can possess different fitness or potentials to become memory cells is currently unclear.

Human and mouse memory T cells are heterogeneous and can be subdivided into two broad subsets of central memory (T_{CM}) and effector memory (T_{EM}) cells based on CCR7 and CD62L expression (21). T_{CM} re-circulate through secondary lymphoid organs and rapidly proliferate upon antigenic stimulation, but possess only low levels of immediate effector function. Conversely, T_{EM} can migrate to inflamed non-lymphoid tissues where they exert immediate effector function, but are susceptible to apoptosis and proliferate poorly in response to antigen (22-24). The lineage relationship of T_{CM} and T_{EM} remains a matter of intense investigation. We previously showed that upon antigenic stimulation $CCR7^+$ T_{CM} differentiate to $CCR7^-$ effector cells and that committed precursors in the T_{CM} pool (pre-Th1, pre-Th2 and pre-CTL) can differentiate spontaneously to T_{EM} -like cells in response to homeostatic cytokines (23-25). Based on these results we proposed that T_{CM} are differentiation intermediates (21).

Here we investigated the stimulatory conditions that lead to the generation of human T cells with the characteristics of memory precursors, namely, high expression of $IL-7R\alpha$, responsiveness to IL-7 and IL-15 and proliferation in response to antigenic re-stimulation. We found that the expression of $IL-7R\alpha$ and especially its coupling to the signal transduction pathway are acquired as a function of increasing strength of stimulation. In contrast, the capacity to secrete IL-2 and expand in response to TCR stimulation decreases at high strength of stimulation. These results are consistent with the notion that T_{CM} and T_{EM} are generated at intermediate strength of stimulation.

Results

Modulation of IL-7R α expression in human CD4⁺ T cells as a function of signal strength

We assessed IL-7R α expression dynamics in human naïve CD4⁺ T cells that were primed by different strength of stimulation. This was achieved: i) by using low and high concentrations of TSST to vary the rate of TCR triggering (26); ii) by using low and high numbers of TSST-loaded DC to vary competition for antigen among T cells (27); and iii) by using immature DC (iDC) or LPS-matured DC (mDC) that offer different levels of co-stimulation (28) and might engage T cells in transient and sustained contacts, respectively (29). IL-7R α was expressed on virtually all naïve T cells (not shown), but was lost 16 hours after stimulation (i.e. prior to cell division) selectively on activated T cells that had up-regulated CD69 (Fig. 1a). Notably, although the different priming conditions resulted in large differences in the fraction of CD69⁺ T cell blasts, IL-7R α was efficiently down-regulated on all activated cells.

On day 7 following stimulation, TSST-responsive cells (TCR-V β 2⁺) were analyzed for CFSE dilution and IL-7R α expression (Fig. 1b). IL-7R α was re-expressed in all cases in cells that had diluted CFSE but the percentage of IL-7R α ⁺ cells varied widely according to the priming conditions. In particular, only a small fraction of cells primed by low or high strengths of stimulation were IL-7R α ⁺ (27% in culture stimulated by low numbers of immature DC and 14% in cultures stimulated by high numbers of mature DC pulsed with the highest TSST dose). In contrast, the percentage of IL-7R α ⁺ cells was high in cells that received intermediate strengths of stimulation (67% in cells primed by low numbers of mature DC pulsed with high TSST dose). Comparable results were obtained when cells were stimulated for different times with anti-CD3 antibodies alone or in combination with anti-CD28 antibodies in the absence of DC (not shown).

Altogether these results show that IL-7R α is rapidly lost upon T cell priming and that its re-expression on proliferating cells is optimal at intermediate strengths of stimulation, reduced in cells that receive low strengths of stimulation and impaired at high signal strength.

IL-7R α and CCR7 expression define functional subsets of activated T cells

We previously showed that CCR7 expression in primed T cells is regulated by the strength of stimulation (30). We therefore investigated the expression of CCR7 and IL-7R α on proliferating T cells that received low (10^2 iDC pulsed with 10 ng/ml TSST), intermediate (10^2 mDC pulsed with 10 ng/ml TSST) and high (10^4 mDC pulsed with 10 ng/ml TSST) strengths of stimulation (Fig. 2a). As expected, the expression of CCR7 was retained in cells receiving low strength of stimulation and was progressively lost with increasing signal strength. At low and high signal strength only few cells expressed IL-7R α and in both cases they were mainly CCR7 $^+$. Conversely, at intermediate signal strength a large fraction of cells (58%) expressed IL-7R α and most cells co-expressed CCR7, although some were CCR7 $^-$.

The above results suggest that at an intermediate strength of stimulation different T cell phenotypes could be generated. We therefore asked whether these T cells have distinct functional properties. T cells primed at intermediate strength of stimulation were sorted according to the expression of IL-7R α and CCR7 and immediately analyzed for cytokine production. Cells were furthermore either starved overnight or stimulated with IL-7 or TSST-pulsed DC following re-labeling with CFSE.

Single cell analysis of cytokine production revealed that IFN- γ was secreted by CCR7 $^-$ cells whereas IL-2 was more efficiently produced by IL-7R $^{\text{hi}}$ cells (Fig. 2b). Consequently, most CCR7 $^+$ IL-7R $^{\text{hi}}$ cells secreted IL-2 only, while CCR7 $^-$ IL-7R $^{\text{lo}}$ cells produced high amounts of IFN- γ (Fig. 2b).

Resistance to overnight starvation was higher in CCR7⁺ cells (either IL-7R^{lo} or IL-7R^{hi}) as compared to CCR7⁻ cells (Fig. 2c, percentage of viable cells in parentheses). Interestingly, upon overnight culture a considerable fraction of purified IL-7R^{lo} and CCR7⁻ cells spontaneously re-acquired IL-7R α and CCR7. Consistent with their different survival and IL-2 producing capacities, subsets purified according to IL-7R α and CCR7 expression showed different responses to IL-7 and antigenic stimulation (Fig. 2c). CCR7⁺IL-7R^{hi} cells proliferated vigorously in response to IL-7, IL-15 (not shown) or TSST-pulsed DC. These responses were decreased in the two single-positive subsets (CCR7⁺IL-7R^{lo} and CCR7⁻IL-7R^{hi}) and were impaired in the CCR7⁻IL-7R^{lo} subset. Notably, all cells proliferating in response to IL-7 were CCR7⁺, independently of whether they were derived from CCR7⁺ or CCR7⁻ subsets (Fig. 2c). A similar hierarchy of survival and proliferation in response to cytokines and antigen was observed in subsets displaying the same phenotype but generated under low or high strength of stimulation (data not shown).

Altogether the above findings show that primed human CD4⁺ T cells with a CCR7⁻IL-7R^{lo} phenotype are effector cells that are highly susceptible to apoptosis while CCR7⁺IL-7R^{hi} cells possess characteristics of memory precursors.

Different properties of CCR7⁺IL-7R^{hi} cells generated at different strength of stimulation

The results obtained so far suggest that memory precursors are present within the CCR7⁺IL-7R^{hi} subset. However, cells with this phenotype can be generated in all stimulatory conditions, although at different frequencies. It was therefore important to investigate whether CCR7⁺IL-7R^{hi} T cells generated under different strength of stimulation have the same or different capacities to respond to cytokines and antigenic stimulation. CFSE⁻CCR7⁺IL-7R^{hi} cells that proliferated following low, intermediate or high strength of stimulation were sorted 7 days after priming and re-labeled with CFSE. Low-stimulated CCR7⁺IL-7R^{hi} cells showed little or no proliferation in response to IL-7 or IL-15 and had a high death rate (Fig. 3a and 3b, left

panels). Intermediate-stimulated CCR7⁺IL-7R^{hi} cells proliferated slowly in response to IL-7 and IL-15, survived better and retained expression of CCR7 under these conditions (Fig. 3a and 3b, central panels). Finally, only high-stimulated CCR7⁺IL-7R^{hi} cells proliferated extensively in response to IL-7 and IL-15, and some lost CCR7 expression, especially in IL-15-supplemented cultures (Fig. 3 a and 3b, right panels). Furthermore, CCR7⁺ T cells arising in these cytokine-driven cultures acquired the capacity to produce high amounts of IFN- γ as compared to cells that in the same culture maintained CCR7 expression (Fig. 3c). Notably, unlike intermediate-stimulated cells, high-stimulated CCR7⁺IL-7R^{hi} cells already displayed a low level IFN- γ producing capacity after priming (data not shown).

In striking contrast with cytokine-induced proliferation, TCR-induced proliferation was maximal in cells that received an intermediate strength of stimulation and was severely reduced in high-stimulated T cells (Fig. 3a). The low expansion potential of high-stimulated cells was associated with high death rate (Fig. 3b) and impaired IL-2 production, and could be partially reverted by addition of IL-2, but not by neutralizing antibodies to TNF- α , FasL or TRAIL (data not shown). CCR7⁺IL-7R^{hi} cells that received a low strength of stimulation in the primary culture also showed suboptimal secondary expansions with antigen.

In summary, high strength of stimulation promotes cytokine responsiveness but inhibits secondary antigen-driven expansions of primed CCR7⁺IL-7R^{hi} T cells. Thus, putative memory T cell precursors, i.e. cells that combine both cytokine responsiveness and recall potential, possess a CCR7⁺IL-7R^{hi} phenotype and are optimally generated at intermediate strengths of stimulation.

Signal transduction capacities of the IL-7 receptor on activated human T cells

The different IL-7 responsiveness' of cells expressing IL-7 receptor α raised the question whether the IL-7R might have different signaling capacities in cells that had received different strengths of stimulation. We first used a panel of specific inhibitors to address the signal

requirements for IL-7 receptor-dependent proliferation of TCR-activated human T cells. Inhibitors specific for JAK3 (JAK3 Inhibitor 2), PI 3-kinase (LY294002) or TOR (Rapamycin) blocked IL-7-dependent proliferation (31), while inhibitors specific for src tyrosine kinases (PP2) and p38 Map kinase (SB202190) did not (not shown). We then assessed s6 ribosomal protein phosphorylation at Ser 235/236 by immuno-blotting as a read-out of s6-kinase activity (Fig. 4a), which is controlled by PI-3 kinase and TOR (32-34). High-stimulated CCR7⁺IL-7R^{hi} cells showed increasing phosphorylation of s6 ribosomal protein following culture with IL-7, IL-15 and anti-CD3 plus anti-CD28 antibodies, respectively. Strikingly, in intermediate-stimulated cells phosphorylation of s6 ribosomal protein was efficient following stimulation with anti-CD3/CD28 antibodies and IL-15, but not with IL-7. Similar results were obtained with antibodies specific for phospho-Ser240/44 (not shown).

We then monitored the expression levels of IL-7 and IL-15 receptor components in purified CCR7⁺IL-7R^{hi} cells generated upon low, intermediate or high strength of stimulation (Fig. 4b). Surface expression of IL-15R β and common γ chain was found to be lower in low-stimulated cells than in high-stimulated cells, but the differences were small and the receptors were clearly detectable under all conditions. IL-15R α could not be detected in these conditions (not shown). Interestingly, expression of PTEN, a phosphatase that counteracts PI-3 kinase activity, was low in high-stimulated cells (Fig. 4b), thus inversely correlating with IL-7 induced s6-kinase activity and proliferation.

In order to understand if the observed signaling defect in weakly stimulated cells was specific for PI 3-kinase/PTEN-dependent signaling pathways, we compared phosphorylation of STAT5 and s6 ribosomal protein at the single cell level by intracellular staining (Fig. 4c). Consistent with the immuno-blotting results, IL-7 or IL-15 efficiently triggered s6-kinase activity in high-stimulated cells, but failed to do so in low-stimulated cells and only IL-15 induced efficient s6 ribosomal protein phosphorylation in cells that had received an intermediate level of stimulation. Interestingly, s6 ribosomal protein phosphorylation occurred

exclusively in phospho-STAT5^{hi} cells, suggesting a higher activation threshold for activation of s6-kinase than for JAK. Consistently, STAT5 phosphorylation was readily detectable upon cytokine stimulation under all conditions, indicating the presence of functional IL-7 and IL-15 receptors. As expected, phosphorylation of s6 ribosomal protein was blocked by Rapamycin or by a PI-3 kinase inhibitor (not shown).

In summary, a strong T cell stimulation down-regulates PTEN expression and effectively couples the IL-7R to s6-kinase activation.

Gene expression profile of activated CCR7⁺IL-7R^{hi} T cells

In order to further understand how the differential responses to IL-7 and TCR stimulation could be regulated in primed T cells, we performed global gene expression analysis of FACS-purified CCR7⁺IL-7R^{hi} cells derived from cultures at intermediate and high strength of stimulation. Microarray gene expression profiles were obtained using Affymetrix GeneChip Human Genome U133 2.0 Plus arrays, which target over 47,000 transcripts, including 38,500 well-characterized genes.

More than 24,000 genes were expressed in at least one of the experimental conditions. Differentially expressed genes were selected as those showing a statistically significant change of at least two-fold in the two replicates. Using this criterion, 408 unique transcripts (266 increased and 141 decreased) were identified (Supplementary Table 1). To have a broad picture of the changes in gene expression, we performed a functional annotation analysis of the 408 genes using the Gene Ontology and the Panther Classification, and Biocarta and Kegg pathways. There was no group of functionally related genes over-represented among the 141 down-regulated transcripts. Conversely, functional annotation of the up-regulated genes showed an over-representation of genes involved in immunity and defense, cell cycle, apoptosis, regulation of transcription and metabolism such as glykolysis (Table 1 and supplementary Tables 2 and 3).

Figure 5 shows the behavior of transcripts that code for gene products with a known role in immune responses, signaling, cell cycle, regulation of transcription and apoptosis, and that were up- or down-regulated in CCR7⁺IL-7R^{hi} cells from high- as compared to intermediate-stimulated cultures of at least 4-fold in both experiments. The Figure contains also some genes that varied less than 4-fold, but that were arbitrarily chosen because of interesting functions. Among strongly up-regulated genes involved in immune responses there were several genes involved in Th1 development, including IFN- γ , STAT1, IL-12R β 2 and JAK2 (35). The selective expression of IL-12R β 2 in high-stimulated cells was confirmed by cell surface staining (not shown). Surprisingly, strongly stimulated cells expressed high levels of SOCS3, which inhibits JAK activation by several cytokines, including IL-2 and IL-12 (36). Several genes involved in inhibition of IL-2 production following TCR stimulation, including CTLA-4 (37), MAP kinase phosphatase 1 (38) and the transcription factors CREM (39) and SNFT (40) were also expressed at higher levels in high-stimulated cells than in intermediate-stimulated cells. Conversely, expression of β 2 integrin and LAT, which positively regulate IL-2 production upon TCR stimulation (41-43), were reduced. Moreover, high-stimulated cells expressed significantly higher levels of crucial cell cycle regulators including CDK2, CDK6, CDC6 and cyclin E2 as well as the Ki67 antigen (the latter also at the protein level, not shown). They also expressed moderately higher levels of several pro-apoptotic molecules, including TRAIL, while an apoptotic inhibitory molecule was reduced.

Overall these results suggest that high-stimulated T cells can sustain a high metabolic rate and have a low threshold to cycle with mitogens. Moreover, the data are consistent with Th1 commitment of high-stimulated cells and provide a plausible explanation for their reduced IL-2 production and survival following antigenic re-stimulation.

Discussion

The signals that regulate the generation of memory T cells from antigen-activated precursors are a field of intense research. We showed here that the strength of stimulation of human CD4⁺ naïve T cells upon priming regulates the generation of CCR7⁺IL-7R^{hi} cells, their capacity to proliferate and differentiate with IL-7 and their expansion potential following antigenic re-stimulation, i. e. their potential to become long-lived memory cells. In particular we found that CCR7⁺IL-7R^{hi} cells differ in their capacity to signal via the IL-7 receptor and in the expression of genes that regulate survival, proliferation and differentiation.

IL-7 is a key cytokine that regulates naïve and memory T cell survival and homeostatic proliferation as well as memory cell generation from activated precursors (8-10, 44-47). The expression of its receptor is known to be modulated upon T cell activation and is down-regulated by IL-2 (48), IL-7 (49) and chronic antigenic stimulation as it occurs in persistent virus infections (5, 17, 19, 20). We showed here that the early loss of IL-7R α on activated T cells is largely independent of the priming conditions, while the later appearance of dividing IL-7R α ⁺ cells is regulated by signal strength and is optimal at intermediate strength of stimulation. We mimicked tolerogenic priming that occurs under steady state conditions and results in abortive T cell proliferation (2) by weak stimulation with low numbers of immature DC and were surprised to find that a considerable fraction of proliferating cells re-acquired IL-7R α under this condition. However, these IL-7R^{hi} cells survived poorly and did not proliferate with IL-7 or IL-15 and were thus unfit to become memory cells (7). These findings are consistent with the notion that IL-7R α expression is not sufficient *per se* to predict the memory potential of activated T cells (13, 16).

Activated CD8⁺ mouse T cells that perform immediate effector functions or that become long-lived memory cells and mediate protective immunity can be identified by expression of IL-7R α and lymph node homing receptors, such as CCR7 and CD62L (11, 12, 50, 51). We found that in human CD4⁺ T cells IL-7R α and CCR7 expression can be used to

identify subsets with characteristics of memory or effector cells. Remarkably however, cells with a putative memory precursor phenotype, i.e. CCR7⁺IL-7R^{hi} had strikingly different responses to IL-7 and TCR ligands that were dependent on the strength of stimulation received during priming. In particular, CCR7⁺IL-7R^{hi} cells generated upon intermediate signal strength were non-polarized, proliferated slowly with IL-7 but expanded efficiently upon TCR re-stimulation and therefore possessed characteristics of circulating non-polarized T_{CM} cells (24). Conversely, high-stimulated CCR7⁺IL-7R^{hi} cells showed poor capacity to proliferate upon TCR re-engagement, but proliferated efficiently with homeostatic cytokines and differentiated spontaneously to Th1 effector cells, and therefore have characteristics of pre-Th1 central memory cells that have been described both in the mouse and in human blood (24, 52, 53). Interestingly, efficient Th1 lineage commitment of high-stimulated cells required high concentrations of TSST, but was not prevented by neutralizing anti-IL-12 antibodies (data not shown).

CCR7⁺IL-7R^{hi} can be generated upon antigenic stimulation in low numbers. However, these cells had a high death rate and spontaneously re-acquired CCR7 upon withdrawal of TCR agonists. This phenotypic reversion has been originally described for *in vivo* primed TCR-transgenic CD8⁺ mouse T cells following adoptive transfer (51), although it was later shown not to occur at physiological precursor frequency (54). TCR-Vβ2⁺ cells also have a high frequency among human T cells and we also detected a reduced phenotypic reversion and enhanced survival of CCR7⁺IL-7R^{hi} cells when the competition for antigen was reduced. It is therefore possible that CCR7⁺IL-7R^{hi} generated upon infections or vaccinations *in vivo* contribute to a higher extent to the T_{EM} pool than suggested by our results.

The observed striking differences in IL-7 responsiveness of CCR7⁺IL-7R^{hi} T cells derived from low, intermediate and high-stimulated cultures are unlikely to be caused by cytokine receptor expression levels. Indeed, common γ chain expression and IL-7-induced STAT5 phosphorylation were comparable in cells receiving low or high stimulation. These

results suggest that all IL-7R^{hi} cells possess a functional IL-7R, but that the cellular context in which IL-7 receptor-derived signals act is different. IL-7 and other common γ cytokines are known to maintain the metabolic rate of T cells and to alter the expression of critical survival factors and cell cycle regulators (31, 32, 55). Gene expression analysis suggested that the enhanced IL-7 responsiveness and fitness of strongly stimulated cells reflected a higher metabolic rate and partially pre-build cell cycle machinery (56). Moreover, we found that protein expression levels of the critical inhibitory phosphatase PTEN (57) were reduced in high-stimulated cells. Consistent with more efficient signaling via PTEN-regulated pathways, we found that s6 ribosomal protein phosphorylation, which is important for translation of ribosomal components and cell cycle regulators, required strong TCR stimulation. Thus, the strength of stimulation can license the IL-7 receptor to signal for proliferation by altering a key component of the signal transduction machinery that couples membrane-proximal signalling to cell cycle progression. Interestingly, high PTEN levels also correlated with low IL-2 responsiveness of CD25⁺ T regulatory cells in the mouse (58), suggesting that PTEN expression levels in T cells might be a conserved mechanism regulating responsiveness to common γ cytokines. Consistently, low-stimulated cells failed to phosphorylate s6 ribosomal protein in response to either IL-7 or IL-15. Surprisingly however, IL-15, but not IL-7 induced s6 kinase activity in T cells primed with an intermediate level of stimulation (Fig. 4a). A possible explanation for this finding is that IL-7, unlike other common γ cytokines, down-regulates its own receptor (49) and therefore inhibits its own signaling.

Despite their high cytokine responsiveness high-stimulated T cells failed to undergo efficient secondary expansion following antigenic re-stimulation. This defect was associated with cell death and reduced IL-2 production in response to stimulation with TSST, but not with phorbol esters and ionomycin (data not shown), suggesting an early signaling defect. Gene expression analysis revealed that several critical molecules affecting TCR-dependent IL-2 production were altered, including LFA-1, CTLA-4 and LAT. Moreover, the

unexpectedly high SOCS3 expression in high-stimulated cells did not lead to reduced STAT5 phosphorylation (Fig. 4c), but might inhibit IL-2 production following TCR stimulation (59). Exogenous IL-2, but not antibodies neutralizing death-inducing cytokines, enhanced accumulation of TCR re-stimulated cells (not shown), suggesting that death receptor-induced apoptosis was not limiting cellular expansion under these conditions (60).

In summary we have shown that the strength of stimulation of CD4⁺ T cells determines the memory potential and lineage commitment of activated T cells that is not predictable by phenotypic markers. Our work might be helpful to identify conditions for the *in vitro* generation of human antigen-specific T cells with desired properties to improve vaccination and adoptive immunotherapy.

Materials and Methods

Cell culture. PBMC were isolated from buffy-coated blood from healthy donors. Monocytes were purified by positive selection with anti-CD14 beads (Miltenyi). For DC differentiation, CD14⁺ cells were cultured for 4 days in complete medium (RPMI 1640 supplemented, 2 mM glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 50 µg/ml kanamycin, 50U/ml Penicillin and 50 µg/ml streptomycin, Gibco) containing 10% FCS (Hyclone), 50 ng/ml GM-CSF (Novartis) and 1000 Units (U)/ml IL-4. The DC obtained were stimulated for 24 hours with 100ng/ml LPS (*Salmonella abortus equi*, Sigma) and pulsed for 30 minutes with TSST. Total CD4⁺ T cells were isolated from the CD14⁺ fraction by negative selection with a CD4⁺ T cell isolation kit on an Automacs (Miltenyi). CD4⁺ T cells were stained with anti-CCR7 mAb (R&D systems) followed by a biotin-labeled anti-γ2a antibody and labeled streptavidin, FITC-labeled anti-CD45RA and PC5-labeled anti-CD8, anti-CD16 (Beckmann Coulter) and anti-CD19 (Becton Dickinson) and naïve CD4⁺ T cells (CD45RA⁺CCR7⁺) were purified by cell sorting to >99%. Labeling of naïve or activated T cells with CFSE was performed as described previously (25). 10⁴ CFSE-labeled naïve T cells were co-cultured with TSST-pulsed DC in round bottom wells, and stained after 16 hours with CD69-APC and IL-7Rα-PE. After 7 days CFSE^{lo} cells were purified on a FACS Aria (Becton Dickinson) according to CCR7 and IL-7Rα expression (IL-7Rα mAbs, Beckmann coulter and R&D system), cells were relabeled with CFSE and 10⁴ cells cultured in the absence or presence of recombinant cytokines IL-7, IL-15 (25ng/ml, R&D systems) or autologous TSST-pulsed DC. Cell viability was assessed by exclusion of propidium iodide at 1 µg/ml. Protein kinase inhibitors were used at optimal concentrations after a pre-incubation for 30 minutes at 37C as described (25): the src tyrosine kinase inhibitor PP2 at 1µM (Alexis), the JAK3 2 Inhibitor at 25 ng/ml (Alexis), the PI3-kinase inhibitor LY294002 at 10µM (Cell Signaling), Rapamycin at 100nM (Cell Signaling) and the p38 inhibitor SB202190 at 10 µM (Alexis).

Intracellular staining. Cytokine producing capacity of FACS-purified subsets was assessed after stimulation of purified cell populations at $10^4/100\mu\text{l}$ for 24h with 50 nM Phorboldibutyrate (PdBu, Sigma) and 0.5 $\mu\text{g/ml}$ Ionomycin (Sigma) or in wells coated with anti-CD3 (clone TR66) and anti-CD28 antibodies (Pharmingen BD, 2 $\mu\text{g/ml}$ each). Intracellular IL-2 and IFN- γ was detected after stimulating cells for 6 hours in the presence of 10 $\mu\text{g/ml}$ Brefeldin A (Sigma) for the last 2 hours and after fixation with paraformaldehyde 4% and permeabilization with saponin (Sigma). After saturation of non-specific binding sites with 10% FCS, cells were incubated with labeled antibodies to IFN- γ and IL-2 (Pharmingen), washed and analyzed by flow cytometry on a FACSCalibur with the CellQuest software (Becton Dickinson). For intracellular staining of phospho-proteins or PTEN (Cellsignalling), purified cells were cultured in the absence or presence of recombinant cytokines or with immobilized anti-CD3 plus anti-CD28, and cells were treated following the manufacturer's instructions. Briefly, cells were washed, fixed for 10 minutes at 37C with 1% paraformaldehyde followed by incubation in Metahnlol on ice for 30 minutes, non-specific sites blocked with 0.5% BSA for 30 minutes at room temperature and cells were stained for intracellular phospho-STAT5 with a labeled mouse antibody (Beckton Dickinson) and phospho-s6 ribosomal protein or PTEN was detected with a specific rabbit antibody (Cell Signaling) followed by incubation with a labeled multiple-adsorbed anti-rabbit antibody (Jackson lab).

Immunoblotting. Cell lysates (Tris-Cl 50mM pH 7.4, NP-40 0.5%, 0.15M NaCl, 2 mM EDTA, 10 mM NaF, 10 mM P207, 0.5 mM Na_3VO_4 , 100 $\mu\text{g/ml}$ PMSF, 1mg/ml Aprotinin and Leupeptin) were cleared by centrifugation, boiled in sample buffer and resolved on a 10% SDS-PAGE. Proteins were transferred on a nitrocellulose membrane (Amersham). Incubations with the primary anti-phospho-s6 ribosomal protein antibodies (Cellsignalling) and secondary, horseradish peroxidase-conjugated antibodies were performed in blocking

buffer (5% dry non-fat milk in Tris buffered saline pH 7.4 containing 0.05% Tween 20 (TBST). Blots were developed with an ECL kit (Amersham).

Microarray and data analysis. FACS-purified CFSE^{lo}CCR7⁺IL-7R^{hi} cells (>10⁶) generated upon stimulation with 10² or 10⁴ TSST-loaded mature DC were obtained from two different donors and analyzed separately. RNA was extracted with the TRIzol method (Invitrogen). RNA concentration was determined using a Nanodrop spectrophotometer (Witec). RNA quality was verified using the 2100 BioAnalyzer (Agilent Technologies). Total RNA (2μg) was labeled and hybridized with the GeneChip Expression 3' Amplification One-Cycle target labeling kit on Affymetrix GeneChip Human Genome U133 2.0 Plus arrays (Affymetrix) according to the manufacturer's protocol. Washes and scanning were done according to the Affymetrix protocols with a Fluidics Station 400 and a GeneChip Scanner 3000 (Affymetrix). Data analysis was carried out using the GeneChip Operating System (GCOS) 1.4 (Affymetrix). For normalization, the output of each experiment was multiplied by a scaling factor to bring its average to a target intensity of 200. The standard GCOS Detection algorithm was used to determine the call Present (P), Absent (A) or Marginal (M) for each probe set in each experimental condition. The standard GCOS Change and Signal Log Ratio (SLR) algorithms were used to identify differentially expressed genes comparing the CCR7⁺IL-7R^{hi} cells stimulated with 10⁴ TSST-loaded mature DC against the CCR7⁺IL-7R^{hi} cells stimulated with 10² TSST-loaded mature DC as baselines. Based upon the p-values, the Affymetrix default settings define Increase (I) Change, Marginal Increase (MI) Change, No Change (NC), Marginal Decrease (MD) Change and Decrease (D) Change calls. The outputs of the SLR algorithm are values in log scale of base 2, thus SLR values of 1 and -1 indicate a 2-fold increase or a 2-fold decrease, respectively.

Genes showing an Increase Change call plus SLR ≥ 1 in both replicates or showing a Decrease Change call plus SLR ≤ -1 in both replicates were considered as differentially expressed between

the CCR7⁺IL-7R^{hi} cells stimulated with 10⁴ TSST-loaded mature DC and the CCR7⁺IL-7R^{hi} cells stimulated with 10² TSST-loaded mature DC. Probe sets defined as absent in all the experiment conditions were discarded; probe sets were also discarded when having an Increase Change call plus SLR ≥ 1 but with a call Absent in the CCR7⁺IL-7R^{hi} cells stimulated with 10⁴ TSST-loaded mature DC, or when having a Decrease Change call plus SLR ≤ -1 but with a call Absent in the CCR7⁺IL-7R^{hi} cells stimulated with 10² TSST-loaded mature DC. Functional characterization was performed to identify groups of functionally similar genes over-represented among the differentially expressed genes in comparison with the whole population of human genes. The Database for Annotation Visualization and Integrated Discovery 2.0 tool (National Institute of Allergy and Infectious Disease, Frederick, MD, USA) was used for identification of Gene Ontology classes, Kegg and Biocarta pathways (61). The Panther Gene Expression Data Analysis tool (Applied Biosystems) was used for Panther ontology classes and pathways (62). A p-value lower than 0.01 was used as a cut-off to identify statistically significant over-represented functional classes. Raw microarrays expression data will be made publicly available through Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>).

Acknowledgements

We thank F. Grassi and S. Wirths for critical reading and discussion. J.G. is supported by the Swiss National Science Foundation (Grant n. 3100A0-104168) and the Deutsche Forschungsgemeinschaft (SFB650 Junior Group). This work was also supported in part by the Swiss National Science Foundation (Grants n. 3100-112678 and 3100-101962), by the European Community (6th Framework, Contract n. LSHP-CT-2003-503367) and by the National Institute of Health (Grant n. U19AI057266-01). The Institute for Research in Biomedicine is supported by the Helmut Horten Foundation. The authors have no conflicting financial interests.

References

1. Sprent, J., and C.D. Surh. 2002. T cell memory. *Annu Rev Immunol* 20:551-579.
2. Kurts, C., H. Kosaka, F.R. Carbone, J.F. Miller, and W.R. Heath. 1997. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *J. Exp. Med.* 186:239-245.
3. Hernandez, J., S. Aung, W.L. Redmond, and L.A. Sherman. 2001. Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *J Exp Med* 194:707-717.
4. Moskophidis, D., F. Lechner, H. Pircher, and R.M. Zinkernagel. 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 362:758-761.
5. Lang, K.S., M. Recher, A.A. Navarini, N.L. Harris, M. Lohning, T. Junt, H.C. Probst, H. Hengartner, and R.M. Zinkernagel. 2005. Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation. *Eur J Immunol* 35:738-745.
6. Lanzavecchia, A., and F. Sallusto. 2002. Progressive differentiation and selection of the fittest in the immune response. *Nat Rev Immunol* 2:982-987.
7. Gett, A., F. Sallusto, A. Lanzavecchia, and J. Geginat. 2003. T lymphocyte fitness determined by signal strength. *Nat Immunol* 4:355-360.
8. Kondrack, R.M., J. Harbertson, J.T. Tan, M.E. McBreen, C.D. Surh, and L.M. Bradley. 2003. Interleukin 7 regulates the survival and generation of memory CD4 cells. *J Exp Med* 198:1797-1806.
9. Li, J., G. Huston, and S.L. Swain. 2003. IL-7 promotes the transition of CD4 effectors to persistent memory cells. *J Exp Med* 198:1807-1815.

10. Lenz, D.C., S.K. Kurz, E. Lemmens, S.P. Schoenberger, J. Sprent, M.B. Oldstone, and D. Homann. 2004. IL-7 regulates basal homeostatic proliferation of antiviral CD4+T cell memory. *Proc Natl Acad Sci U S A* 101:9357-9362.
11. Kaech, S.M., J.T. Tan, E.J. Wherry, B.T. Konieczny, C.D. Surh, and R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4:1191-1198.
12. Huster, K.M., V. Busch, M. Schiemann, K. Linkemann, K.M. Kerksiek, H. Wagner, and D.H. Busch. 2004. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc Natl Acad Sci U S A* 101:5610-5615.
13. Klonowski, K.D., K.J. Williams, A.L. Marzo, and L. Lefrancois. 2006. Cutting edge: IL-7-independent regulation of IL-7 receptor alpha expression and memory CD8 T cell development. *J Immunol* 177:4247-4251.
14. Schluns, K.S., K. Williams, A. Ma, X.X. Zheng, and L. Lefrancois. 2002. Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J Immunol* 168:4827-4831.
15. Buentke, E., A. Mathiot, M. Tolaini, J. Di Santo, R. Zamoyska, and B. Seddon. 2006. Do CD8 effector cells need IL-7R expression to become resting memory cells? *Blood* 108:1949-1956.
16. Lacombe, M.H., M.P. Hardy, J. Rooney, and N. Labrecque. 2005. IL-7 receptor expression levels do not identify CD8+ memory T lymphocyte precursors following peptide immunization. *J Immunol* 175:4400-4407.
17. van Leeuwen, E.M., G.J. de Bree, E.B. Remmerswaal, S.L. Yong, K. Tesselaar, I.J. ten Berge, and R.A. van Lier. 2005. IL-7 receptor alpha chain expression distinguishes functional subsets of virus-specific human CD8+ T cells. *Blood* 106:2091-2098.

18. Fuller, M.J., D.A. Hildeman, S. Sabbaj, D.E. Gaddis, A.E. Tebo, L. Shang, P.A. Goepfert, and A.J. Zajac. 2005. Cutting edge: emergence of CD127^{high} functionally competent memory T cells is compromised by high viral loads and inadequate T cell help. *J Immunol* 174:5926-5930.
19. Paiardini, M., B. Cervasi, H. Albrecht, A. Muthukumar, R. Dunham, S. Gordon, H. Radziewicz, G. Piedimonte, M. Magnani, M. Montroni, S.M. Kaech, A. Weintrob, J.D. Altman, D.L. Sodora, M.B. Feinberg, and G. Silvestri. 2005. Loss of CD127 expression defines an expansion of effector CD8⁺ T cells in HIV-infected individuals. *J Immunol* 174:2900-2909.
20. Wherry, E.J., D.L. Barber, S.M. Kaech, J.N. Blattman, and R. Ahmed. 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A* 101:16004-16009.
21. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 22:745-763.
22. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.
23. Geginat, J., A. Lanzavecchia, and F. Sallusto. 2003. Proliferation and differentiation potential of human CD8⁺ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* 101:4260-4266.
24. Rivino, L., M. Messi, D. Jarrossay, A. Lanzavecchia, F. Sallusto, and J. Geginat. 2004. Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4⁺ central memory T cells. *J Exp Med* 200:725-735.

25. Geginat, J., F. Sallusto, and A. Lanzavecchia. 2001. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J Exp Med* 194:1711-1719.
26. Valitutti, S., and A. Lanzavecchia. 1997. Serial triggering of T-cell receptors: a basis for the sensitivity and specificity of T cell antigen recognition. *Immunol. Today* 18:299-304.
27. Langenkamp, A., G. Casorati, C. Garavaglia, P. Dellabona, A. Lanzavecchia, and F. Sallusto. 2002. T cell priming by dendritic cells: thresholds for proliferation, differentiation and death and intraclonal functional diversification. *Eur J Immunol* 32:2046-2054.
28. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* 179:1109-1118.
29. Hugues, S., L. Fetler, L. Bonifaz, J. Helft, F. Amblard, and S. Amigorena. 2004. Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity. *Nat Immunol* 5:1235-1242.
30. Langenkamp, A., M. Messi, A. Lanzavecchia, and F. Sallusto. 2000. Kinetics of dendritic cell activation: impact on priming of Th1, Th2 and nonpolarized T cells. *Nat. Immunol.* 1:311-316.
31. Barata, J.T., A. Silva, J.G. Brandao, L.M. Nadler, A.A. Cardoso, and V.A. Boussiotis. 2004. Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. *J Exp Med* 200:659-669.
32. Cornish, G.H., L.V. Sinclair, and D.A. Cantrell. 2006. Differential regulation of T-cell growth by IL-2 and IL-15. *Blood* 108:600-608.

33. Abraham, R.T. 1998. Mammalian target of rapamycin: immunosuppressive drugs uncover a novel pathway of cytokine receptor signaling. *Curr Opin Immunol* 10:330-336.
34. Ward, S.G., and D.A. Cantrell. 2001. Phosphoinositide 3-kinases in T lymphocyte activation. *Curr Opin Immunol* 13:332-338.
35. Szabo, S.J., A.S. Dighe, U. Gubler, and K.M. Murphy. 1997. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* 185:817-824.
36. Krebs, D.L., and D.J. Hilton. 2001. SOCS proteins: negative regulators of cytokine signaling. *Stem Cells* 19:378-387.
37. Thompson, C.B., and J.P. Allison. 1997. The emerging role of CTLA-4 as an immune attenuator. *Immunity* 7:445-450.
38. Whitehurst, C.E., and T.D. Geppert. 1996. MEK1 and the extracellular signal-regulated kinases are required for the stimulation of IL-2 gene transcription in T cells. *J Immunol* 156:1020-1029.
39. Tenbrock, K., Y.T. Juang, M. Tolnay, and G.C. Tsokos. 2003. The cyclic adenosine 5'-monophosphate response element modulator suppresses IL-2 production in stimulated T cells by a chromatin-dependent mechanism. *J Immunol* 170:2971-2976.
40. Iacobelli, M., W. Wachsman, and K.L. McGuire. 2000. Repression of IL-2 promoter activity by the novel basic leucine zipper p21SNFT protein. *J Immunol* 165:860-868.
41. Geginat, J., G. Bossi, J.R. Bender, and R. Pardi. 1999. Anchorage dependence of mitogen-induced G1 to S transition in primary T lymphocytes. *J Immunol* 162:5085-5093.
42. Geginat, J., B. Clissi, M. Moro, P. Dellabona, J.R. Bender, and R. Pardi. 2000. CD28 and LFA-1 contribute to cyclosporin A-resistant T cell growth by stabilizing the IL-2 mRNA through distinct signaling pathways. *Eur J Immunol* 30:1136-1144.

43. Finco, T.S., T. Kadlecsek, W. Zhang, L.E. Samelson, and A. Weiss. 1998. LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. *Immunity* 9:617-626.
44. Kieper, W.C., J.T. Tan, B. Bondi-Boyd, L. Gapin, J. Sprent, R. Ceredig, and C.D. Surh. 2002. Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8⁺ T cells. *J Exp Med* 195:1533-1539.
45. Tan, J.T., B. Ernst, W.C. Kieper, E. LeRoy, J. Sprent, and C.D. Surh. 2002. Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8⁺ cells but are not required for memory phenotype CD4⁺ cells. *J Exp Med* 195:1523-1532.
46. Seddon B, T.P., Zamoyska R. 2003. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nat Immunol* 4:680-686.
47. Schluns, K.S., W.C. Kieper, S.C. Jameson, and L. Lefrancois. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* 1:426-432.
48. Xue, H.H., P.E. Kovanen, C.A. Pise-Masison, M. Berg, M.F. Radovich, J.N. Brady, and W.J. Leonard. 2002. IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. *Proc Natl Acad Sci U S A* 99:13759-13764.
49. Park, J.H., Q. Yu, B. Erman, J.S. Appelbaum, D. Montoya-Durango, H.L. Grimes, and A. Singer. 2004. Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. *Immunity* 21:289-302.
50. Bachmann, M.F., P. Wolint, K. Schwarz, P. Jager, and A. Oxenius. 2005. Functional properties and lineage relationship of CD8⁺ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol* 175:4686-4696.

51. Wherry, E.J., V. Teichgraber, T.C. Becker, D. Masopust, S.M. Kaech, R. Antia, U.H. von_Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4:225-234.
52. Divekar, A.A., D.M. Zaiss, F.E. Lee, D. Liu, D.J. Topham, A.J. Sijts, and T.R. Mosmann. 2006. Protein vaccines induce uncommitted IL-2-secreting human and mouse CD4 T cells, whereas infections induce more IFN-gamma-secreting cells. *J Immunol* 176:1465-1473.
53. Wu, C.Y., J.R. Kirman, M.J. Rotte, D.F. Davey, S.P. Perfetto, E.G. Rhee, B.L. Freidag, B.J. Hill, D.C. Douek, and R.A. Seder. 2002. Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. *Nat Immunol* 3:852-858.
54. Marzo, A.L., K.D. Klonowski, A. Le Bon, P. Borrow, D.F. Tough, and L. Lefrancois. 2005. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat Immunol* 6:793-799.
55. Rathmell, J.C., E.A. Farkash, W. Gao, and C.B. Thompson. 2001. IL-7 enhances the survival and maintains the size of naive T cells. *J Immunol* 167:6869-6876.
56. Veiga-Fernandes, H., and B. Rocha. 2004. High expression of active CDK6 in the cytoplasm of CD8 memory cells favors rapid division. *Nat Immunol* 5:31-37.
57. Krystal, G. 2000. Lipid phosphatases in the immune system. *Semin Immunol* 12:397-403.
58. Bensinger, S.J., P.T. Walsh, J. Zhang, M. Carroll, R. Parsons, J.C. Rathmell, C.B. Thompson, M.A. Burchill, M.A. Farrar, and L.A. Turka. 2004. Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. *J Immunol* 172:5287-5296.
59. Banerjee, A., A.S. Banks, M.C. Nawijn, X.P. Chen, and P.B. Rothman. 2002. Cutting edge: Suppressor of cytokine signaling 3 inhibits activation of NFATp. *J Immunol* 168:4277-4281.

60. Lenardo, M., K.M. Chan, F. Hornung, H. McFarland, R. Siegel, J. Wang, and L. Zheng. 1999. Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment. *Annu Rev Immunol* 17:221-253.
61. Dennis, G., Jr., B.T. Sherman, D.A. Hosack, J. Yang, W. Gao, H.C. Lane, and R.A. Lempicki. 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4:P3.
62. Mi, H., B. Lazareva-Ulitsky, R. Loo, A. Kejariwal, J. Vandergriff, S. Rabkin, N. Guo, A. Muruganujan, O. Doremiex, M.J. Campbell, H. Kitano, and P.D. Thomas. 2005. The PANTHER database of protein families, subfamilies, functions and pathways. *Nucleic Acids Res* 33:D284-288.

Figure legends

Figure 1. Expression of IL-7R α on primed T cells is regulated by the strength of antigenic stimulation. Purified human CD4⁺ naïve T cells were stimulated as indicated with 10² or 10⁴ immature (iDC) or LPS-matured monocyte-derived DCs (mDC) loaded with 0.1 or 10 ng/ml TSST. **a)** After 16 hours T cells were stained for CD69 and IL-7R α expression and analyzed by flow cytometry. Grey dots represent small cells and black dots show blasts. Upper numbers indicates the fraction of activated CD69⁺ blasts, and lower numbers in parenthesis the percentage among CD69⁺ blasts that have lost IL-7R α expression. Results are representative of three separate experiments. **b)** After 7 days IL-7R α expression and CFSE dilution were analyzed by gating on TCRV β 2⁺ cells. Numbers indicate the fractions of CFSE^{lo} cells that express IL-7R α . Results are representative of four separate experiments.

Figure 2. CCR7 and IL-7R α expression identify subsets of primed CD4⁺ T cells. a) Expression of CCR7 and IL-7R α on CFSE^{lo} T cells on day 7 following priming with low (10² iDC, 10ng/ml TSST), intermediate (10² mDC, 10ng/ml TSST) or high (10⁴ mDC, 10ng/ml TSST) strength of stimulation. **b)** Secretion of IL-2 and IFN- γ by cells primed at intermediate strength of stimulation and sorted according to IL-7R α and CCR7 expression following re-stimulation with PdBu and ionomycin for 6 hours. **c)** Phenotypes and proliferation of intermediate-stimulated cells sorted for CCR7 and IL-7R α expression immediately after sorting, after overnight culture in medium alone, or after 7 days culture in the presence of 25ng/ml IL-7 or TSST-pulsed mDCs (DC-T cell ratio, 1:5). Numbers in parenthesis indicate cell viability following overnight rest. Results are representative of four separate experiments.

Figure 3. Expansion of CCR7⁺IL-7R^{hi} cells in response to IL-7 or antigenic stimulation has different requirements. Purified naïve CD4⁺ CFSE-labelled T cells were stimulated with low, intermediate, or high strength of stimulation (as specified in Figure 2) and CFSE^{lo}CCR7⁺IL-7R^{hi} subsets purified by cell sorting. Cells were re-labelled with CFSE and

cultured with IL-7, IL-15 or TSST-pulsed DCs for 7 days. **a)** CFSE dilution and CCR7 modulation of viable TCRV β 2⁺ cells. Numbers indicate -fold expansion **b)** Cell viability assessed by PI staining. Numbers indicate percentages of PI⁺ cells **c)** After culture with IL-7 for 7 days T cells were stimulated for 6 hours with phorbol ester and ionomycin and IFN- γ versus CFSE dilution was analyzed according to CCR7 expression. Numbers indicate percentage of IFN- γ secreting cells. Results are representative of three separate experiments.

Figure 4. A strong TCR stimulation licenses the IL-7R to trigger s6 kinase activity.

a) Naive T cells were primed with intermediate (10² mDCs, 10 ng/ml TSST) or high (10⁴ mDCs, 10 ng/ml TSST) strength of stimulation. After 7 days CFSE^{lo}CCR7⁺IL-7R^{hi} cells were purified and rested or stimulated with IL-7, IL-15 or anti-CD3/CD28 antibodies. Phosphorylation of s6 ribosomal protein at Ser 230/5 was analysed by immunoblotting. One experiment out of three. **b)** Purified CFSE^{lo}CCR7⁺IL-7R^{hi} cells from low-, intermediate- or high-stimulated cultures were purified and analyzed for cytokine receptor or PTEN expression by surface and intracellular staining, respectively. Jurkat cells stained negative for PTEN (not shown). Numbers indicate mean fluorescence intensities. **c)** Cells primed under the indicated conditions were cultured in the absence or presence of IL-7 or IL-15 and phosphorylation of STAT5 and s6 ribosomal protein were assessed by intracellular staining. Results are representative of five separate experiments.

Figure 5. Gene expression analysis of CFSE^{lo}CCR7⁺IL-7R^{hi} cells. The relative changes of genes with known roles in immune response, signalling, cell cycle, transcription and apoptosis that were strongly up- or downregulated (>4 times) in high-stimulated cells are shown. Some genes that were only moderately altered (2-4 fold), but codified for interesting gene products are also shown. Genes of particular interest are in bold. Numbers indicate the fold-increase when exceeding 30-fold or the fold-reduction when exceeding 12-fold.

Table 1. Functional annotation according to Panther classification of 266 genes up-regulated with at least a two-fold change in CCR7⁺IL-7R^{hi} cells stimulated with 10⁴ TSST-loaded mature DCs (high strength of stimulation) when compared with CCR7⁺IL-7R^{hi} cells stimulated with 10² TSST-loaded mature DCs (intermediate strength of stimulation).

Panther Ontology	Panther Ontology Term	Number of	P-value
		genes	
<u>Biological Process</u>	Interferon-mediated immunity	11	2.70E-08
	Glycolysis	9	1.75E-07
	Cell cycle	31	4.65E-07
	Immunity and defense	37	1.79E-06
	Chromosome segregation	9	7.16E-04
	Cell cycle control	15	3.73E-03
	Stress response	10	6.72E-03
<u>Molecular Function</u>	Kinase	19	4.06E-03

File: Lozza et al. supl.Table 1.xls

Supplementary Table 1. List of 408 differentially expressed genes with at least a two-fold change in CCR7⁺IL-7R^{hi} cells stimulated with 10⁴ TSST-loaded mature DCs when compared with CCR7⁺IL-7R^{hi} cells stimulated with 10² TSST-loaded mature DCs.

Gene Ontology		Number of	
Classification	Gene Ontology Term	genes	P-value
<u>Biologic Process</u>	Cell Proliferation	52	7.40E-17
	Cell Cycle	41	7.49E-15
	Mitotic Cell Cycle	19	3.65E-11
	Nuclear Division	16	1.61E-09
	Cellular Physiological Process	93	3.19E-09
	M Phase	16	3.60E-09
	Regulation Of Cell Cycle	23	4.09E-09
	Mitosis	13	5.87E-08
	M Phase Of Mitotic Cell Cycle	13	8.20E-08
	Cell Growth And/Or Maintenance	81	9.44E-08
	Alcohol Metabolism	16	4.85E-07
	Response To Stress	30	5.87E-07
	Regulation Of Cellular Process	28	1.59E-06
	Cell Cycle Checkpoint	7	4.04E-06
	Glycolysis	8	9.18E-06
	Physiological Process	173	1.90E-05
	Regulation Of Cell Proliferation	14	2.14E-05
	Glucose Catabolism	8	2.48E-05
	Gluconeogenesis	6	3.15E-05
	Macromolecule Metabolism	66	5.38E-05
	Hexose Catabolism	8	5.80E-05
	Alcohol Catabolism	8	6.27E-05
	Monosaccharide Catabolism	8	6.27E-05
	Alcohol Biosynthesis	6	7.63E-05
	Hexose Biosynthesis	6	7.63E-05
	Monosaccharide Biosynthesis	6	7.63E-05
	Response To DNA Damage Stimulus	12	1.31E-04

Sterol Metabolism	7	1.40E-04
Chromosome Segregation	5	1.79E-04
Response To Endogenous Stimulus	12	2.03E-04
Glucose Metabolism	8	2.30E-04
Carbohydrate Catabolism	8	2.59E-04
Sterol Biosynthesis	5	2.87E-04
Cellular Process	110	2.91E-04
Hexose Metabolism	9	3.07E-04
Monosaccharide Metabolism	9	3.53E-04
Metabolism	117	4.46E-04
DNA Repair	10	7.48E-04
Cholesterol Metabolism	6	8.25E-04
Carbohydrate Biosynthesis	7	8.98E-04
Apoptosis	15	9.55E-04
Programmed Cell Death	15	1.02E-03
Negative Regulation Of Transcription From		
Pol II Promoter	6	1.03E-03
Chromosome Condensation	4	1.03E-03
Regulation Of Biological Process	54	1.12E-03
Negative Regulation Of Cell Proliferation	8	1.24E-03
Transcription From Pol II Promoter	15	1.26E-03
Steroid Metabolism	8	1.41E-03
Cell Death	15	1.70E-03
Death	15	1.88E-03
Meiosis	5	1.89E-03
G2/M Transition Of Mitotic Cell Cycle	5	2.06E-03
Cholesterol Biosynthesis	4	2.54E-03
Regulation Of Transcription From Pol II		
Promoter	10	2.82E-03
Meiosis I	4	2.88E-03

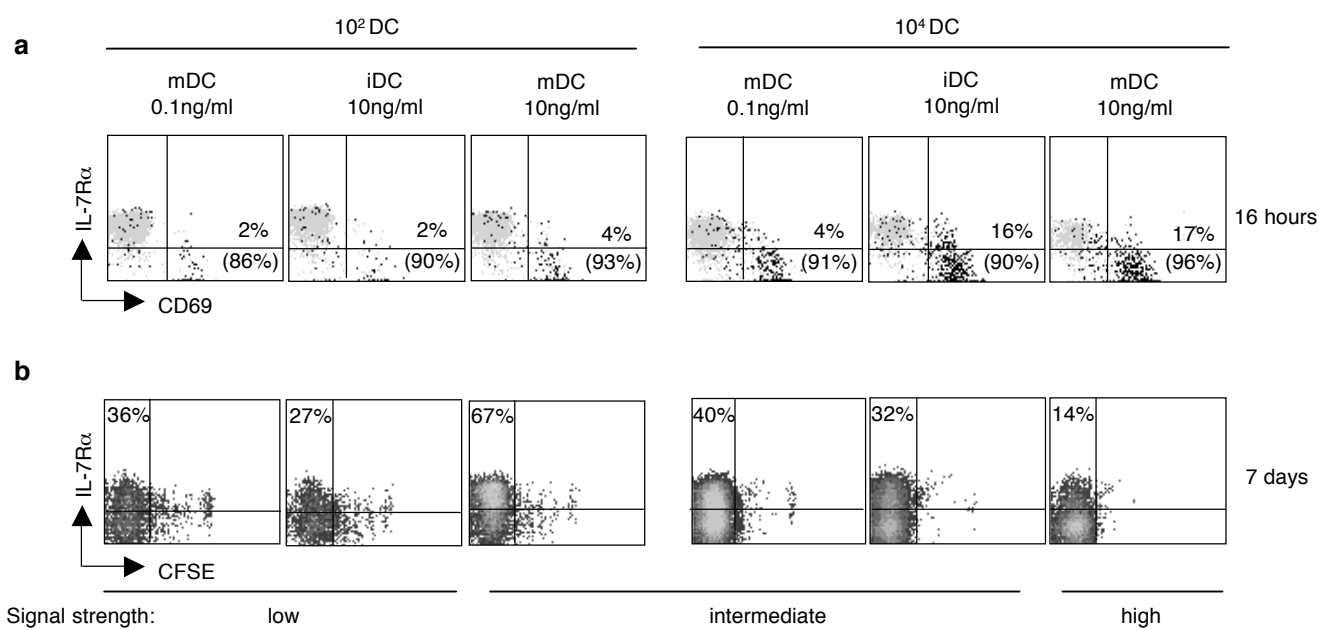
	Meiotic Prophase I	4	2.88E-03
	DNA Recombination	6	3.61E-03
	Mitotic Chromosome Condensation	3	3.85E-03
	Mitotic Prophase	3	3.85E-03
	Negative Regulation Of Transcription, DNA - Dependent	6	3.99E-03
	G1/S Transition Of Mitotic Cell Cycle	5	4.30E-03
	Negative Regulation Of Nucleobase, Nucleoside, Nucleotide And Nucleic Acid Metabolism	7	4.88E-03
	Protein Amino Acid Phosphorylation	17	5.72E-03
	Biosynthesis	25	5.98E-03
	Lipid Biosynthesis	8	5.99E-03
	Steroid Biosynthesis	5	8.20E-03
	DNA Metabolism	16	8.49E-03
	Regulation Of Mitosis	4	9.15E-03
	Macromolecule Biosynthesis	19	9.37E-03
	Response To Stimulus	54	9.89E-03
<u>Cellular Component</u>	Intracellular	114	1.08E-06
	Cytoplasm	70	4.16E-06
	Spindle	6	2.14E-04
	Nucleus	60	4.72E-04
	Spindle Pole	5	6.61E-04
	Endoplasmic Reticulum	14	8.28E-04
	Condensed Chromosome	4	1.25E-03
	Chromosome	10	1.56E-03
	Microtubule Cytoskeleton	9	2.03E-03
	Microtubule Organizing Center	4	7.41E-03
<u>Molecular Function</u>	ATP Binding	39	2.64E-09
	Purine Nucleotide Binding	45	3.07E-09

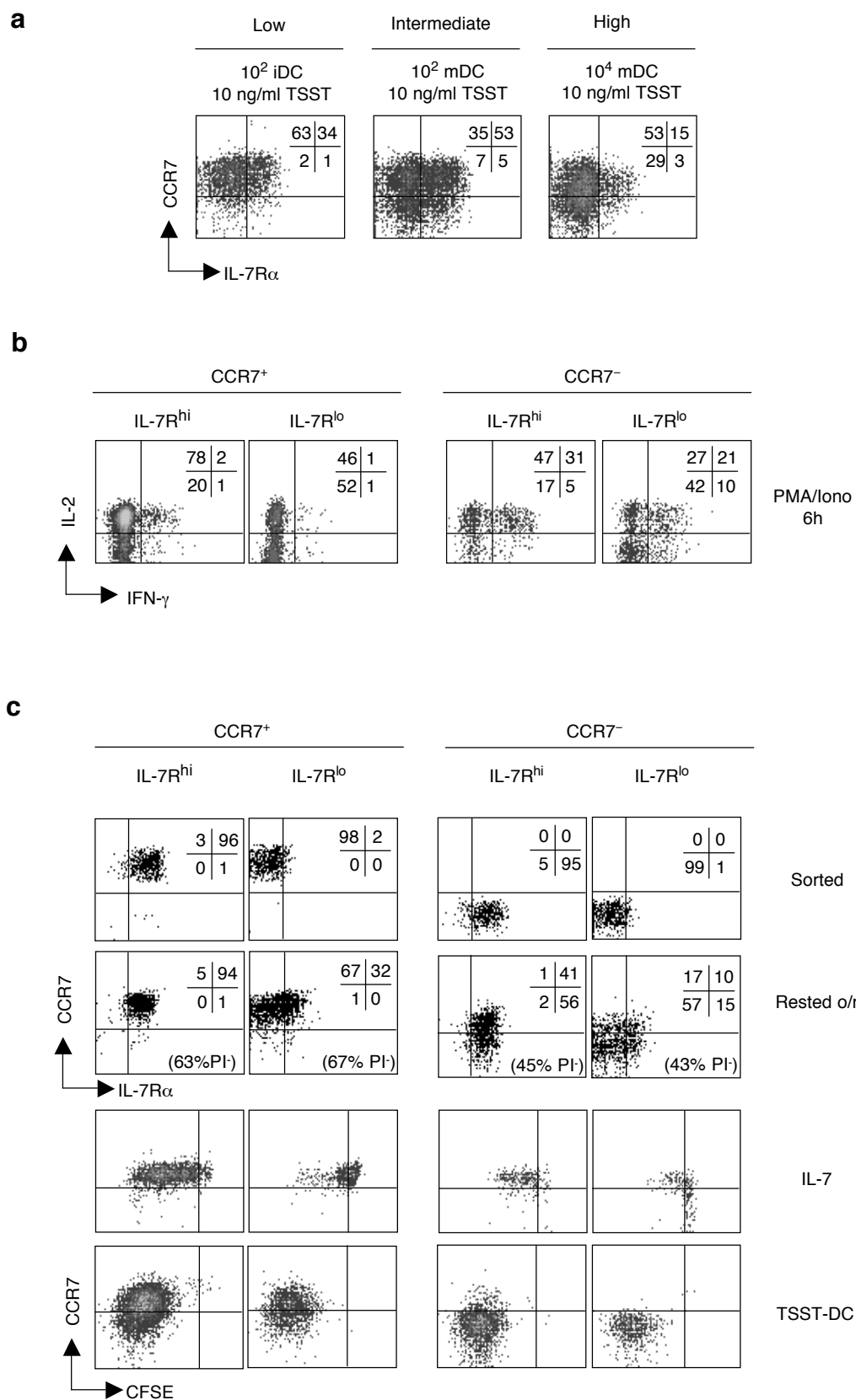
Adenyl Nucleotide Binding	39	3.78E-09
Nucleotide Binding	45	5.97E-09
Transcription Factor Binding	12	8.70E-05
Catalytic Activity	85	1.29E-04
Transferase Activity	36	1.47E-04
Transferase Activity, Transferring		
Phosphorus-Containing Groups	25	1.84E-04
Transcription Cofactor Activity	11	1.96E-04
Protein Serine/Threonine Kinase Activity	13	1.68E-03
Transcription Corepressor Activity	6	1.94E-03
Magnesium Ion Binding	8	2.55E-03
Phosphotransferase Activity, Alcohol Group		
As Acceptor	18	3.23E-03
Map Kinase Phosphatase Activity	3	4.39E-03
Specific RNA Polymerase II Transcription		
Factor Activity	4	4.51E-03
Protein Serine/Threonine Phosphatase		
Activity	5	5.35E-03
Kinase Activity	20	5.68E-03
Protein Kinase Ck2 Activity	8	6.52E-03
Camp-Dependent Protein Kinase Activity	8	7.17E-03
Heat Shock Protein Activity	4	8.88E-03

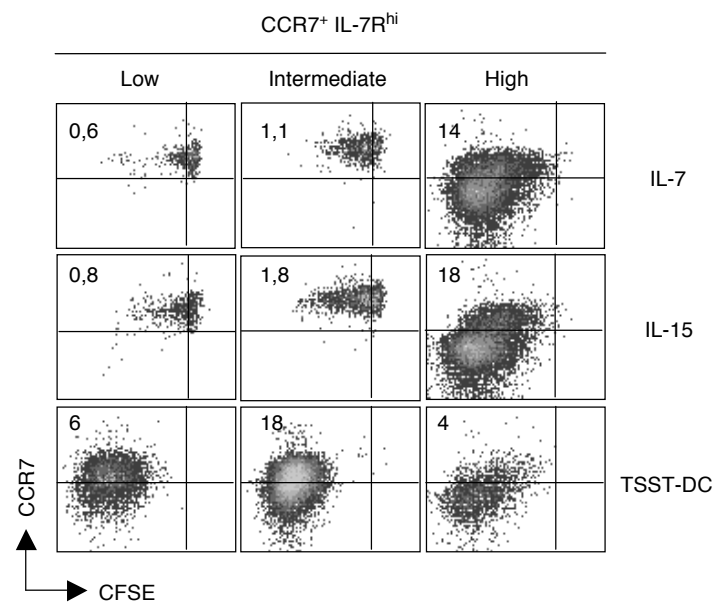
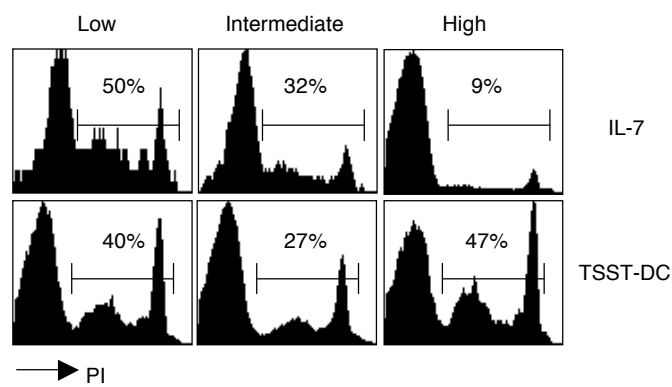
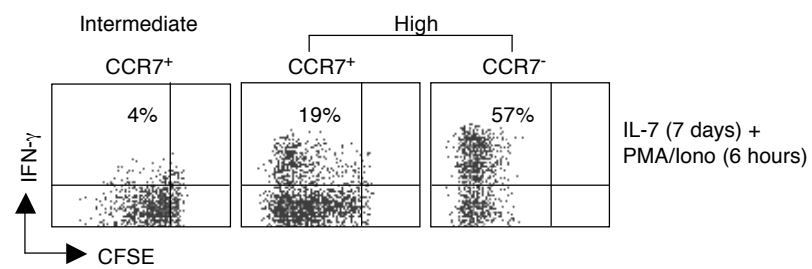
Supplementary Table 2. Functional annotation according to the Gene Ontology of 266 genes up-regulated with at least a two-fold change in CCR7⁺IL-7R^{hi} cells stimulated with 10⁴ TSST-loaded mature DCs when compared with CCR7⁺IL-7R^{hi} cells stimulated with 10² TSST-loaded mature DCs.

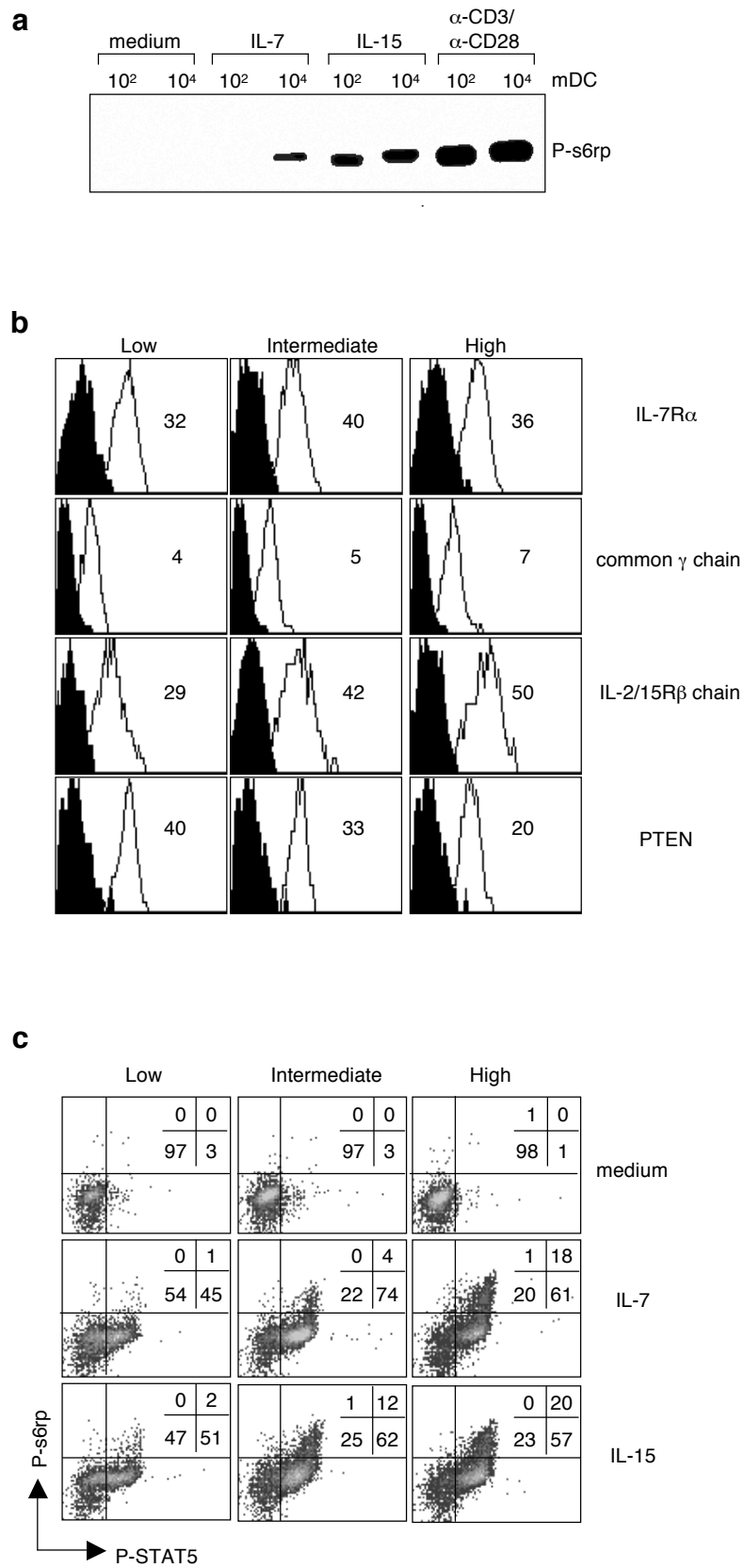
Database	Pathways	Genes	
		Observed	P-value
<u>Biocarta</u>	Epo signaling pathway	5	3.03E-03
	Tpo signaling pathway	5	3.74E-03
	Glycolysis pathway	4	4.30E-03
	IL12 and Stat4 Dependent Signaling Pathway in Th1 Development	5	4.55E-03
	Inhibition of Cellular Proliferation by Gleevec	5	4.55E-03
	NO2-dependent IL 12 Pathway in NK cells	4	9.50E-03
<u>Kegg</u>	Glycolysis / gluconeogenesis	8	2.05E-04
	Cell cycle	8	4.01E-03
<u>Panther</u>	Glycolysis	7	2.33E-06
	Apoptosis signaling pathway	10	2.44E-04

Supplementary Table 3. Over-represented Biocarta, Kegg and Panther pathways among 266 genes up-regulated with at least a two-fold change in CCR7⁺IL-7R^{hi} cells stimulated with 10⁴ TSST-loaded mature DCs when compared with CCR7⁺IL-7R^{hi} cells stimulated with 10² TSST-loaded mature DCs.

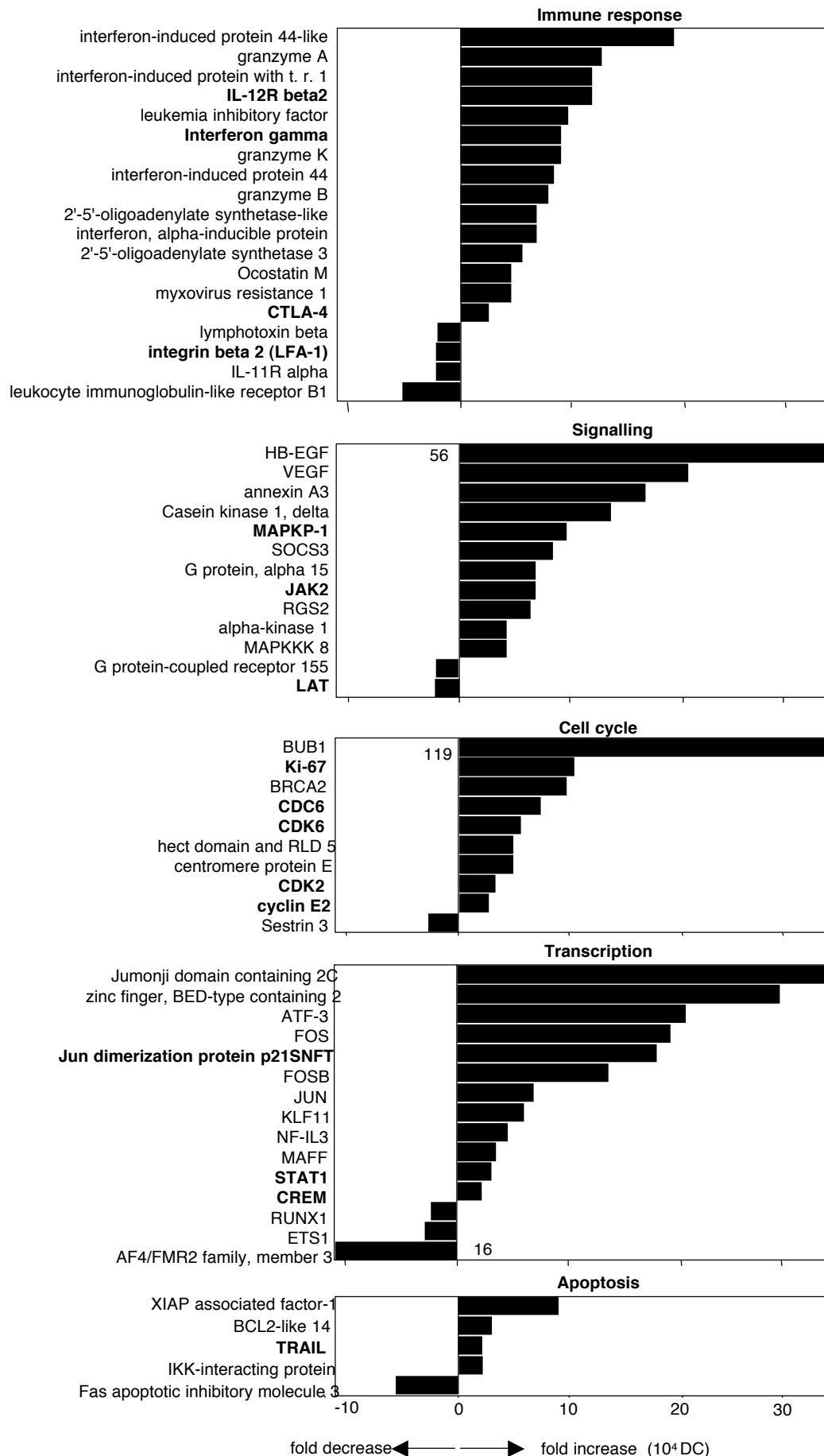




a**b****c**



Lozza et al. Figure 4



Lozza et al. Figure 5

Probe Set ID	Gene Title	Gene Symbol	Signal Log Ratio1	Signal Log Ratio2
215509_s_at	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	BUB1	6,9	1
229610_at	hypothetical protein FLJ40629	FLJ40629	6,5	1,2
223721_s_at	DnaJ (Hsp40) homolog, subfamily C, member 12	DNAJC12	6,4	1
208962_s_at	fatty acid desaturase 1	FADS1	6,3	1,1
204347_at	adenylate kinase 3-like 1 /// adenylate kinase 3-like 2	AK3L1/2	6,2	5,3
201560_at	chloride intracellular channel 4	CLIC4	6,2	1,1
208964_s_at	fatty acid desaturase 1	FADS1	6,1	2,2
203821_at	heparin-binding EGF-like growth factor	HBEGF	5,8	1,9
213281_at	---	---	5,7	1,5
204068_at	serine/threonine kinase 3 (STE20 homolog, yeast)	STK3	5,3	1
218976_at	DnaJ (Hsp40) homolog, subfamily C, member 12	DNAJC12	5,2	3,3
244385_at	Jumonji domain containing 2C	JMJD2C	5,1	1,9
200831_s_at	stearoyl-CoA desaturase (delta-9-desaturase)	SCD	5	1,6
232278_s_at	DEP domain containing 1	DEPDC1	4,9	1,4
238015_at	hypothetical protein LOC201725	LOC201725	4,9	1,1
219836_at	zinc finger, BED-type containing 2	ZBED2	4,9	1,2
218792_s_at	B-box and SPRY domain containing	BSPRY	4,8	1,7
202672_s_at	activating transcription factor 3	ATF3	4,4	2
223839_s_at	Stearoyl-CoA desaturase (delta-9-desaturase)	SCD	4,4	1,2
210512_s_at	vascular endothelial growth factor	VEGF	4,4	1,5
209189_at	v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	4,3	1,5
204439_at	interferon-induced protein 44-like	IFI44L	4,3	1,2
218507_at	hypoxia-inducible protein 2	HIG2	4,2	3,2
219510_at	polymerase (DNA directed), theta	POLQ	4,2	1,2
220358_at	Jun dimerization protein p21SNFT	SNFT	4,2	1,9
209369_at	annexin A3	ANXA3	4,1	1,1
219073_s_at	oxysterol binding protein-like 10	OSBPL10	4,1	1,1
200832_s_at	stearoyl-CoA desaturase (delta-9-desaturase)	SCD	4,1	1,1
220158_at	lectin, galactoside-binding, soluble, 14	LGALS14	4	3,2
204348_s_at	adenylate kinase 3-like 1	AK3L1	3,9	4,5
202411_at	interferon, alpha-inducible protein 27	IFI27	3,9	1,3
1552309_a_at	nexilin (F actin binding protein)	NEXN	3,9	2,1
225342_at	adenylate kinase 3-like 1	AK3L1	3,8	1,2

1569263_at	Casein kinase 1, delta	CSNK1D	3,8	1,2
202768_at	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	3,8	2,1
205488_at	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3) /// granzyme	GZMA	3,7	1,3
210050_at	triosephosphate isomerase 1	TPI1	3,7	1,8
1554452_a_at	hypoxia-inducible protein 2	HIG2	3,6	3,1
203153_at	interferon-induced protein with tetratricopeptide repeats 1 /// interferon-induced protein	IFIT1	3,6	3,3
206999_at	interleukin 12 receptor, beta 2	IL12RB2	3,6	2
205769_at	solute carrier family 27 (fatty acid transporter), member 2	SLC27A2	3,6	3,6
1552619_a_at	anillin, actin binding protein (scraps homolog, Drosophila)	ANLN	3,5	1,3
202581_at	heat shock 70kDa protein 1B	HSPA1B	3,5	1,2
225710_at	CDNA FLJ34013 fis, clone FCBBF2002111	---	3,4	1,9
202887_s_at	DNA-damage-inducible transcript 4	DDIT4	3,4	2
241716_at	heat shock 60kDa protein 1 (chaperonin)	HSPD1	3,4	3,1
212022_s_at	antigen identified by monoclonal antibody Ki-67	MKI67	3,4	1,1
205552_s_at	2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	3,4	1,6
209822_s_at	very low density lipoprotein receptor	VLDLR	3,4	1,2
208368_s_at	breast cancer 2, early onset	BRCA2	3,3	1,5
201041_s_at	dual specificity phosphatase 1	DUSP1	3,3	1,3
204709_s_at	kinesin family member 23	KIF23	3,3	1
205266_at	leukemia inhibitory factor (cholinergic differentiation factor)	LIF	3,3	3,8
212021_s_at	antigen identified by monoclonal antibody Ki-67	MKI67	3,3	3,9
242234_at	XIAP associated factor-1	BIRC4BP	3,2	3,7
238075_at	CHK1 checkpoint homolog (S. pombe)	CHEK1	3,2	1,8
208963_x_at	fatty acid desaturase 1	FADS1	3,2	1,6
206666_at	granzyme K (granzyme 3; tryptase II) /// granzyme K (granzyme 3; tryptase II)	GZMK	3,2	2,1
218662_s_at	chromosome condensation protein G	HCAP-G	3,2	1,9
210354_at	interferon, gamma	IFNG	3,2	1,3
243109_at	Multiple C2 domains, transmembrane 2	MCTP2	3,2	2,3
227697_at	suppressor of cytokine signaling 3	SOCS3	3,2	1,3
225496_s_at	synaptotagmin-like 2	SYTL2	3,2	1
220118_at	zinc finger and BTB domain containing 32	ZBTB32	3,2	1,4
214453_s_at	interferon-induced protein 44	IFI44	3,1	1,5
206359_at	suppressor of cytokine signaling 3	SOCS3	3,1	1,3
202308_at	sterol regulatory element binding transcription factor 1	SREBF1	3,1	2,3

227337_at	ankyrin repeat domain 37	ANKRD37	3	1,5
210164_at	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) /// granz	GZMB	3	1
218883_s_at	MLF1 interacting protein	MLF1IP	3	1,4
207746_at	polymerase (DNA directed), theta	POLQ	3	1,2
219622_at	RAB20, member RAS oncogene family	RAB20	3	5,6
203967_at	CDC6 cell division cycle 6 homolog (S. cerevisiae)	CDC6	2,9	5
228499_at	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	PFKFB4	2,9	1,4
202856_s_at	solute carrier family 16 (monocarboxylic acid transporters), member 3	SLC16A3	2,9	1,2
204415_at	interferon, alpha-inducible protein (clone IFI-6-16)	GIP3	2,8	2
205349_at	guanine nucleotide binding protein (G protein), alpha 15 (Gq class)	GNAI5	2,8	3
218663_at	chromosome condensation protein G	HCAP-G	2,8	1,1
228266_s_at	hepatoma-derived growth factor, related protein 3	HDGFRP3	2,8	1,4
1562031_at	Janus kinase 2 (a protein tyrosine kinase)	JAK2	2,8	3,5
201466_s_at	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	2,8	1,1
205660_at	2'-5'-oligoadenylate synthetase-like	OASL	2,8	1,7
234377_at	T-cell receptor rearranged beta-chain V-region (V-D-J) mRNA, clone ph5	---	2,7	1,9
219918_s_at	asp (abnormal spindle)-like, microcephaly associated (Drosophila)	ASPM	2,7	1,1
222680_s_at	denticleless homolog (Drosophila)	DTL	2,7	1,5
1553642_at	hypothetical protein FLJ36779	FLJ36779	2,7	3,3
201464_x_at	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	2,7	2,5
202388_at	regulator of G-protein signalling 2, 24kDa	RGS2	2,7	4
235643_at	sterile alpha motif domain containing 9-like	SAMD9L	2,7	1,2
219670_at	chromosome 1 open reading frame 165	C1orf165	2,6	1,6
218486_at	Kruppel-like factor 11	KLF11	2,6	1,6
235088_at	hypothetical protein LOC201725	LOC201725	2,6	3,4
229391_s_at	hypothetical protein LOC441168	LOC441168	2,6	5
200737_at	phosphoglycerate kinase 1	PGK1	2,6	1,3
210567_s_at	S-phase kinase-associated protein 2 (p45)	SKP2	2,6	1,2
235287_at	cyclin-dependent kinase 6	CDK6	2,5	4,4
200862_at	24-dehydrocholesterol reductase	DHCR24	2,5	1
213008_at	hypothetical protein FLJ10719	FLJ10719	2,5	2,9
214804_at	FSH primary response (LRPR1 homolog, rat) 1	FSHPRH1	2,5	1,1
205822_s_at	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	HMGCS1	2,5	3
229390_at	hypothetical protein LOC441168	LOC441168	2,5	3,3

218400_at	2'-5'-oligoadenylate synthetase 3, 100kDa	OAS3	2,5	3,6
205768_s_at	solute carrier family 27 (fatty acid transporter), member 2	SLC27A2	2,5	2,4
209218_at	squalene epoxidase	SQLE	2,5	1,1
206513_at	absent in melanoma 2	AIM2	2,4	1,3
232094_at	chromosome 15 open reading frame 29	C15orf29	2,4	1,9
215501_s_at	dual specificity phosphatase 10	DUSP10	2,4	3,7
209709_s_at	hyaluronan-mediated motility receptor (RHAMM)	HMMR	2,4	1,2
214059_at	Interferon-induced protein 44	IFI44	2,4	1,3
236180_at	Transcribed locus, strongly similar to NP_598827.1 aldo-keto reductase family 1, memb	---	2,3	3,1
205046_at	centromere protein E, 312kDa	CENPE	2,3	1,5
213060_s_at	chitinase 3-like 2 /// chitinase 3-like 2	CHI3L2	2,3	1,1
235458_at	hepatitis A virus cellular receptor 2	HAVCR2	2,3	1,1
219863_at	hect domain and RLD 5	HERC5	2,3	1,6
204747_at	interferon-induced protein with tetratricopeptide repeats 3	IFIT3	2,3	2,6
221258_s_at	kinesin family member 18A /// kinesin family member 18A	KIF18A	2,3	1,1
205569_at	lysosomal-associated membrane protein 3	LAMP3	2,3	2,2
228280_at	similar to RIKEN cDNA 1200014N16 gene	MGC14289	2,3	1,3
207543_s_at	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polype	P4HA1	2,3	1,4
230165_at	shugoshin-like 2 (S. pombe)	SGOL2	2,3	1,2
219211_at	ubiquitin specific peptidase 18	USP18	2,3	1,5
204603_at	exonuclease 1	EXO1	2,2	1,8
38037_at	heparin-binding EGF-like growth factor	HBEGF	2,2	1,8
212570_at	KIAA0830 protein	KIAA0830	2,2	1,4
239893_at	Multiple C2 domains, transmembrane 2	MCTP2	2,2	1,3
202086_at	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) /// my	MX1	2,2	1,9
203574_at	nuclear factor, interleukin 3 regulated	NFIL3	2,2	2,5
230170_at	oncostatin M	OSM	2,2	1,1
204286_s_at	phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	2,2	1
223274_at	transcription factor 19 (SC1)	TCF19	2,2	3,3
243352_at	alpha-kinase 1	ALPK1	2,1	2,3
218782_s_at	ATPase family, AAA domain containing 2	ATAD2	2,1	1,7
1560999_a_at	Interleukin 12 receptor, beta 2	IL12RB2	2,1	1,1
205027_s_at	mitogen-activated protein kinase kinase kinase 8	MAP3K8	2,1	1,8
220651_s_at	MCM10 minichromosome maintenance deficient 10 (S. cerevisiae)	MCM10	2,1	4,9

202869_at	2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	2,1	1,2
204285_s_at	phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	2,1	1,1
1562255_at	synaptotagmin-like 3	SYTL3	2,1	4,8
210844_x_at	catenin (cadherin-associated protein), alpha 1, 102kDa	CTNNA1	2	1,6
201231_s_at	enolase 1, (alpha)	ENO1	2	1
202862_at	fumarylacetoacetate hydrolase (fumarylacetoacetase)	FAH	2	2,1
222843_at	fidgetin-like 1	FIGNL1	2	2,2
1554285_at	hepatitis A virus cellular receptor 2	HAVCR2	2	4
201626_at	insulin induced gene 1	INSIG1	2	1
202068_s_at	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	2	1
222962_s_at	MCM10 minichromosome maintenance deficient 10 (<i>S. cerevisiae</i>)	MCM10	2	1
201251_at	pyruvate kinase, muscle	PKM2	2	2,8
202446_s_at	phospholipid scramblase 1	PLSCR1	2	1,9
202022_at	aldolase C, fructose-bisphosphate	ALDOC	1,9	2
209526_s_at	hepatoma-derived growth factor, related protein 3	HDGFRP3	1,9	2,1
200800_s_at	heat shock 70kDa protein 1A /// heat shock 70kDa protein 1B	HSPA1A /// HSF	1,9	1
206316_s_at	kinetochore associated 1	KNTC1	1,9	1,6
202145_at	lymphocyte antigen 6 complex, locus E	LY6E	1,9	1,6
201136_at	proteolipid protein 2 (colonic epithelium-enriched)	PLP2	1,9	2,6
1553713_a_at	Ras homolog enriched in brain like 1	RHEBL1	1,9	1,7
204240_s_at	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	SMC2L1	1,9	1,2
213011_s_at	triosephosphate isomerase 1	TPI1	1,9	2
242904_x_at	---	---	1,8	1,9
230630_at	Adenylate kinase 3-like 1	AK3L1	1,8	2,3
222740_at	ATPase family, AAA domain containing 2	ATAD2	1,8	4,2
206133_at	XIAP associated factor-1	BIRC4BP	1,8	1
201848_s_at	BCL2/adenovirus E1B 19kDa interacting protein 3	BNIP3	1,8	2,1
210571_s_at	cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMP-N-acetylneuraminic acid)	CMAH	1,8	1,4
216607_s_at	cytochrome P450, family 51, subfamily A, polypeptide 1	CYP51A1	1,8	1,9
208308_s_at	glucose phosphate isomerase	GPI	1,8	1,3
1555464_at	interferon induced with helicase C domain 1	IFIH1	1,8	1,5
225128_at	KDEL (Lys-Asp-Glu-Leu) containing 2	KDELC2	1,8	1,3
226901_at	hypothetical protein LOC284018	LOC284018	1,8	1,5
36711_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	MAFF	1,8	1,6

200822_x_at	triosephosphate isomerase 1	TPI1	1,8	1,4
238677_at	WD repeat domain 36	WDR36	1,8	3
201294_s_at	WD repeat and SOCS box-containing 1	WSB1	1,8	1,4
1564063_a_at	ATPase, Class VI, type 11B	ATP11B	1,7	1
239504_at	BCL2-associated transcription factor 1	BCLAF1	1,7	2
204252_at	cyclin-dependent kinase 2	CDK2	1,7	2,2
200764_s_at	catenin (cadherin-associated protein), alpha 1, 102kDa	CTNNA1	1,7	1
229450_at	Interferon-induced protein with tetratricopeptide repeats 3	IFIT3	1,7	1,2
203595_s_at	interferon-induced protein with tetratricopeptide repeats 5	IFIT5	1,7	1,4
201508_at	insulin-like growth factor binding protein 4	IGFBP4	1,7	1,2
204057_at	interferon regulatory factor 8 /// interferon regulatory factor 8	IRF8	1,7	1,9
206553_at	2'-5'-oligoadenylate synthetase 2, 69/71kDa	OAS2	1,7	1,8
227068_at	phosphoglycerate kinase 1	PGK1	1,7	1,7
222088_s_at	solute carrier family 2 (facilitated glucose transporter), member 3 /// solute carrier family SLC2A3 /// SLC	SLC2A3	1,7	2,5
201663_s_at	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	SMC4L1	1,7	2,4
203827_at	WD40 repeat protein Interacting with phosphoinositides of 49kDa	WIP149	1,7	1,5
222816_s_at	zinc finger, CCHC domain containing 2	ZCCHC2	1,7	1
221241_s_at	BCL2-like 14 (apoptosis facilitator) /// BCL2-like 14 (apoptosis facilitator)	BCL2L14	1,6	1,6
236979_at	chromosome 1 open reading frame 178	C1orf178	1,6	1,4
200765_x_at	catenin (cadherin-associated protein), alpha 1, 102kDa	CTNNA1	1,6	3,1
204794_at	dual specificity phosphatase 2	DUSP2	1,6	1,2
205483_s_at	interferon, alpha-inducible protein (clone IFI-15K)	G1P2	1,6	1,7
202934_at	hexokinase 2	HK2	1,6	1,5
203971_at	solute carrier family 31 (copper transporters), member 1	SLC31A1	1,6	1
201195_s_at	solute carrier family 7 (cationic amino acid transporter, y ⁺ system), member 5	SLC7A5	1,6	1,1
AFFX-HUMISC	signal transducer and activator of transcription 1, 91kDa	STAT1	1,6	1,6
238423_at	synaptotagmin-like 3	SYTL3	1,6	1,2
202391_at	brain abundant, membrane attached signal protein 1	BASP1	1,5	4,7
221479_s_at	BCL2/adenovirus E1B 19kDa interacting protein 3-like /// BCL2/adenovirus E1B 19kDa	BNIP3L	1,5	1,7
222746_s_at	B-box and SPRY domain containing	BSPRY	1,5	1,1
242167_at	Chromosome 10 open reading frame 119	C10orf119	1,5	1,5
223513_at	centromere protein J	CENPJ	1,5	1,2
202314_at	cytochrome P450, family 51, subfamily A, polypeptide 1	CYP51A1	1,5	1,1
208436_s_at	interferon regulatory factor 7	IRF7	1,5	1,7

212573_at	KIAA0830 protein	KIAA0830	1,5	1,3
216565_x_at	similar to Interferon-induced transmembrane protein 3 (Interferon-inducible protein 1-8U)	LOC391020	1,5	1,5
211450_s_at	mutS homolog 6 (E. coli)	MSH6	1,5	1
240960_at	Moesin	MSN	1,5	5,4
233479_at	---	---	1,4	1,9
220840_s_at	chromosome 1 open reading frame 112	C1orf112	1,4	1,9
208853_s_at	calnexin	CANX	1,4	1,1
217881_s_at	cell division cycle 27	CDC27	1,4	5,6
203716_s_at	dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	DPP4	1,4	2,1
223785_at	hypothetical protein FLJ10719	FLJ10719	1,4	1,1
238551_at	fucosyltransferase 11 (alpha (1,3) fucosyltransferase)	FUT11	1,4	2,4
212203_x_at	interferon induced transmembrane protein 3 (1-8U)	IFITM3	1,4	1,5
217933_s_at	leucine aminopeptidase 3	LAP3	1,4	5,5
202364_at	MAX interactor 1 /// MAX interactor 1	MXI1	1,4	1,5
222330_at	Phosphodiesterase 3B, cGMP-inhibited	PDE3B	1,4	1,1
236571_at	Solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	1,4	1,5
212290_at	solute carrier family 7 (cationic amino acid transporter, y ⁺ system), member 1	SLC7A1	1,4	1,9
219753_at	stromal antigen 3	STAG3	1,4	3
AFFX-HUMISC	signal transducer and activator of transcription 1, 91kDa	STAT1	1,4	1
205594_at	zinc finger protein 652	ZNF652	1,4	4,5
205733_at	Bloom syndrome	BLM	1,3	1,5
201849_at	BCL2/adenovirus E1B 19kDa interacting protein 3	BNIP3	1,3	1,1
222154_s_at	DNA polymerase-transactivated protein 6	DNAPTP6	1,3	1,3
231769_at	F-box protein 6	FBXO6	1,3	2,6
204972_at	2'-5'-oligoadenylate synthetase 2, 69/71kDa	OAS2	1,3	1
210797_s_at	2'-5'-oligoadenylate synthetase-like	OASL	1,3	1,2
243296_at	Pre-B-cell colony enhancing factor 1	PBEF1	1,3	2,5
202430_s_at	phospholipid scramblase 1	PLSCR1	1,3	1,6
213988_s_at	spermidine/spermine N1-acetyltransferase	SAT	1,3	1,1
202855_s_at	solute carrier family 16 (monocarboxylic acid transporters), member 3	SLC16A3	1,3	2,5
202499_s_at	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	1,3	1
216236_s_at	solute carrier family 2 (facilitated glucose transporter), member 3 /// solute carrier family SLC2A3 /// SLC		1,3	1,1
241879_at	Transcribed locus, moderately similar to XP_517655.1 PREDICTED: similar to KIAA08---		1,2	1
208852_s_at	calnexin	CANX	1,2	1,3

1555827_at	Cyclin L1	CCNL1	1,2	4,5
213379_at	coenzyme Q2 homolog, prenyltransferase (yeast)	COQ2	1,2	1,1
225965_at	DDHD domain containing 1	DDHD1	1,2	1,4
220942_x_at	growth and transformation-dependent protein	E2IG5	1,2	1,4
224345_x_at	growth and transformation-dependent protein /// growth and transformation-dependent pi	E2IG5	1,2	1,1
201313_at	enolase 2 (gamma, neuronal)	ENO2	1,2	1,8
1553750_a_at	family with sequence similarity 76, member B	FAM76B	1,2	4,1
218986_s_at	hypothetical protein FLJ20035	FLJ20035	1,2	1
209524_at	hepatoma-derived growth factor, related protein 3	HDGFRP3	1,2	1,2
1564776_at	leukocyte receptor cluster (LRC) member 10	LENG10	1,2	4
214181_x_at	leukocyte specific transcript 1	LST1	1,2	1,2
228468_at	microtubule associated serine/threonine kinase-like	MASTL	1,2	1,6
225520_at	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	MTHFD1L	1,2	1,3
202733_at	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polype	P4HA2	1,2	1
212902_at	SEC24 related gene family, member A (<i>S. cerevisiae</i>)	SEC24A	1,2	1,9
201689_s_at	tumor protein D52	TPD52	1,2	2,2
201399_s_at	translocation associated membrane protein 1	TRAM1	1,2	1,1
210561_s_at	WD repeat and SOCS box-containing 1	WSB1	1,2	2,5
233425_at	zinc finger, CCHC domain containing 2	ZCCHC2	1,2	2,6
208442_s_at	ataxia telangiectasia mutated (includes complementation groups A, C and D)	ATM	1,1	3,5
228155_at	chromosome 10 open reading frame 58	C10orf58	1,1	2,6
213294_at	Coiled-coil domain containing 75	CCDC75	1,1	1,3
214508_x_at	cAMP responsive element modulator	CREM	1,1	1,3
204211_x_at	eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK2	1,1	1
227609_at	epithelial stromal interaction 1 (breast)	EPSTI1	1,1	1,1
227295_at	IKK interacting protein	IKIP	1,1	1,3
215018_at	KIAA1731	KIAA1731	1,1	1
244321_at	GPI deacylase	PGAP1	1,1	1,4
202497_x_at	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	1,1	2,2
219869_s_at	solute carrier family 39 (zinc transporter), member 8	SLC39A8	1,1	1,6
AFFX-HUMISC	signal transducer and activator of transcription 1, 91kDa	STAT1	1,1	1,3
201061_s_at	stomatin	STOM	1,1	1
202687_s_at	tumor necrosis factor (ligand) superfamily, member 10 /// tumor necrosis factor (ligand) : TNFSF10	TNFSF10	1,1	1,5
212038_s_at	voltage-dependent anion channel 1	VDAC1	1,1	3,7

213836_s_at	WD40 repeat protein Interacting with phosphoinositides of 49kDa	WIP149	1,1	1,1
231241_at	Transcribed locus, moderately similar to XP_517655.1 PREDICTED: similar to KIAA08---	---	1	1,5
214844_s_at	docking protein 5	DOK5	1	1,1
213056_at	FERM domain containing 4B	FRMD4B	1	1,2
216693_x_at	hepatoma-derived growth factor, related protein 3	HDGFRP3	1	4,3
200799_at	heat shock 70kDa protein 1A	HSPA1A	1	2,1
205842_s_at	Janus kinase 2 (a protein tyrosine kinase)	JAK2	1	1,2
1558093_s_at	matrin 3	MATR3	1	5,3
223220_s_at	poly (ADP-ribose) polymerase family, member 9	PARP9	1	1,4
203664_s_at	polymerase (RNA) II (DNA directed) polypeptide D	POLR2D	1	1
203344_s_at	retinoblastoma binding protein 8	RBBP8	1	1
236620_at	RAP1 interacting factor homolog (yeast)	RIF1	1	1,1
202498_s_at	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	1	2,2
209267_s_at	solute carrier family 39 (zinc transporter), member 8	SLC39A8	1	1,7
208097_s_at	thioredoxin domain containing /// thioredoxin domain containing	TXNDC	1	1
231837_at	ubiquitin specific peptidase 28	USP28	1	1,6
217140_s_at	voltage-dependent anion channel 1	VDAC1	1	1,9
229670_at	5.5 kb mRNA upregulated in retinoic acid treated HL-60 neutrophilic cells	---	-1	-1,1
213039_at	rho/rac guanine nucleotide exchange factor (GEF) 18	ARHGEF18	-1	-1,4
228381_at	Activating transcription factor 7 interacting protein 2	ATF7IP2	-1	-1,6
203633_at	carnitine palmitoyltransferase 1A (liver)	CPT1A	-1	-1,7
227056_at	KIAA0141	KIAA0141	-1	-1
226158_at	kelch-like 24 (Drosophila)	KLHL24	-1	-1,1
229101_at	Hypothetical protein LOC150166	LOC150166	-1	-1,1
207339_s_at	lymphotoxin beta (TNF superfamily, member 3)	LTB	-1	-1
221756_at	HGFL gene /// HGFL gene	MGC17330	-1	-1,1
205006_s_at	N-myristoyltransferase 2	NMT2	-1	-1,3
238935_at	Ribosomal protein S27-like	RPS27L	-1	-1
201569_s_at	sorting and assembly machinery component 50 homolog (S. cerevisiae)	SAMM50	-1	-1,1
226652_at	ubiquitin specific peptidase 3	USP3	-1	-1,3
212774_at	zinc finger protein 238	ZNF238	-1	-1
226993_at	CDNA FLJ46626 fis, clone TRACH2001612	---	-1,1	-1,1
213702_x_at	N-acylsphingosine amidohydrolase (acid ceramidase) 1	ASAH1	-1,1	-1,4
210980_s_at	N-acylsphingosine amidohydrolase (acid ceramidase) 1	ASAH1	-1,1	-1,3

203232_s_at	ataxin 1	ATXN1	-1,1	-1
234983_at	Chromosome 12 open reading frame 49	C12orf49	-1,1	-1,5
213398_s_at	chromosome 14 open reading frame 124	C14orf124	-1,1	-1
217967_s_at	chromosome 1 open reading frame 24	C1orf24	-1,1	-1,8
235964_x_at	Chromosome 20 open reading frame 118	C20orf118	-1,1	-1
219452_at	dipeptidase 2	DPEP2	-1,1	-2
229041_s_at	Homo sapiens, clone IMAGE:5205388, mRNA /// Integrin, beta 2 (antigen CD18 (p95),	ITGB2	-1,1	-1,7
226352_at	Junction-mediating and regulatory protein	JMY	-1,1	-1,5
1562698_x_at	hypothetical protein LOC339988	LOC339988	-1,1	-1,7
229429_x_at	LOC440669	LOC440669	-1,1	-1,1
226538_at	Mannosidase, alpha, class 2A, member 1	MAN2A1	-1,1	-1,2
221593_s_at	ribosomal protein L31	RPL31	-1,1	-1
224763_at	ribosomal protein L37	RPL37	-1,1	-2,2
201811_x_at	SH3-domain binding protein 5 (BTK-associated)	SH3BP5	-1,1	-1,2
225037_at	solute carrier family 35, member C2	SLC35C2	-1,1	-1,4
235174_s_at	CDNA clone IMAGE:5286843	---	-1,2	-1,9
238604_at	---	---	-1,2	-1,5
242289_at	Transcribed locus, weakly similar to XP_527300.1 PREDICTED: similar to zinc finger f	---	-1,2	-1,4
230466_s_at	Mesenchymal stem cell protein DSC96	---	-1,2	-1,3
226272_at	Full length insert cDNA clone ZD79H10	---	-1,2	-1
219315_s_at	chromosome 16 open reading frame 30	C16orf30	-1,2	-1,4
227580_s_at	DKFZP434B0335 protein	DKFZP434B0335	-1,2	-1,1
231771_at	gap junction protein, beta 6 (connexin 30)	GJB6	-1,2	-1,5
231166_at	G protein-coupled receptor 155	GPR155	-1,2	-1
225732_at	kelch domain containing 5	KLHDC5	-1,2	-1
229872_s_at	LOC440667 /// LOC440669 /// LOC440688	LOC440667 /// 1	-1,2	-1,1
242239_at	NOL1/NOP2/Sun domain family, member 6	NSUN6	-1,2	-1,2
213888_s_at	TRAF3 interacting protein 3	TRAF3IP3	-1,2	-1,1
213326_at	vesicle-associated membrane protein 1 (synaptobrevin 1)	VAMP1	-1,2	-1,3
243729_at	CDNA FLJ37931 fis, clone CTONG2004397	---	-1,3	-1,1
226558_at	Full-length cDNA clone CS0DI062YC15 of Placenta Cot 25-normalized of Homo sapien	---	-1,3	-1
228710_at	---	---	-1,3	-1
217969_at	chromosome 11 open reading frame2	C11orf2	-1,3	-1
241435_at	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	ETS1	-1,3	-1,6

221602_s_at	Fas apoptotic inhibitory molecule 3 /// Fas apoptotic inhibitory molecule 3	FAIM3	-1,3	-2,5
224688_at	Hypothetical protein FLJ10099	FLJ10099	-1,3	-1,1
211005_at	linker for activation of T cells	LAT	-1,3	-1,1
228046_at	Hypothetical protein LOC152485	LOC152485	-1,3	-1,8
229787_s_at	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide OGT	OGT	-1,3	-1
214097_at	ribosomal protein S21	RPS21	-1,3	-1,2
205922_at	vanin 2 /// vanin 2	VNN2	-1,3	-1
227020_at	yippe-like 2 (Drosophila)	YPEL2	-1,3	-1,4
213256_at	membrane-associated ring finger (C3HC4) 3	03. Mär	-1,4	-1,2
243366_s_at	Transcribed locus	---	-1,4	-1,1
244798_at	---	---	-1,4	-1
201855_s_at	ATM/ATR-Substrate Chk2-Interacting Zn2+-finger protein	ASCIZ	-1,4	-1,2
218421_at	ceramide kinase	CERK	-1,4	-1,1
222490_at	polymerase (RNA) III (DNA directed) polypeptide E (80kD)	POLR3E	-1,4	-1,3
225123_at	Sestrin 3	SESN3	-1,4	-1
228760_at	Splicing factor, arginine/serine-rich, 46kD	SRP46	-1,4	-1,4
221218_s_at	thiamin pyrophosphokinase 1	TPK1	-1,4	-1,1
223741_s_at	tweety homolog 2 (Drosophila)	TTYH2	-1,4	-1,9
231940_at	zinc finger protein 529	ZNF529	-1,4	-1,4
229544_at	MRNA; cDNA DKFZp564C0762 (from clone DKFZp564C0762)	---	-1,5	-1,5
235352_at	CDNA FLJ31593 fis, clone NT2RI2002481	---	-1,5	-1
239449_at	Ankylosis, progressive homolog (mouse)	ANKH	-1,5	-1,5
221601_s_at	Fas apoptotic inhibitory molecule 3 /// Fas apoptotic inhibitory molecule 3	FAIM3	-1,5	-2,1
203413_at	NEL-like 2 (chicken) /// NEL-like 2 (chicken)	NELL2	-1,5	-1,6
204019_s_at	SH3 domain containing, Ysc84-like 1 (S. cerevisiae)	SH3YL1	-1,5	-1,2
65630_at	transmembrane protein 80	TMEM80	-1,5	-1,1
1570143_at	Homo sapiens, clone IMAGE:3932570, mRNA	---	-1,6	-1
208636_at	Actinin, alpha 1	ACTN1	-1,6	-1,8
207996_s_at	chromosome 18 open reading frame 1	C18orf1	-1,6	-1,7
224870_at	KIAA0114 gene product	KIAA0114	-1,6	-1,2
227172_at	hypothetical protein BC000282	LOC89894	-1,6	-1,3
227626_at	progesterin and adipoQ receptor family member VIII	PAQR8	-1,6	-1,4
225725_at	CDNA FLJ31683 fis, clone NT2RI2005353	---	-1,7	-1,6
219049_at	chondroitin beta1,4 N-acetylgalactosaminyltransferase	ChGn	-1,7	-2,2

214735_at	phosphoinositide-binding protein PIP3-E	PIP3-E	-1,7	-1,2
228049_x_at	Transcribed locus	---	-1,8	-3,3
204773_at	interleukin 11 receptor, alpha	IL11RA	-1,8	-1,1
215743_at	N-myristoyltransferase 2	NMT2	-1,8	-1,4
226587_at	Small nuclear ribonucleoprotein polypeptide N /// CDNA FLJ33569 fis, clone BRAMY2 SNRPN	---	-1,8	-1
236016_at	CDNA FLJ38419 fis, clone FEBRA2009846	---	-1,9	-1,7
209160_at	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type I AKR1C3	HIST1H2AC	-1,9	-4,7
215071_s_at	histone 1, H2ac	HIST1H2BK	-1,9	-1,9
209806_at	histone 1, H2bk	SLC35D2	-1,9	-2,2
213083_at	solute carrier family 35, member D2	ABAT	-1,9	-1,3
209459_s_at	4-aminobutyrate aminotransferase	SPON1	-2	-2
213994_s_at	spondin 1, extracellular matrix protein	MGC16044	-2	-3,1
228298_at	hypothetical protein MGC16044	RUNX1	-2,1	-1
209360_s_at	runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	---	-2,1	-1,2
228826_at	CDNA clone IMAGE:5576786	---	-2,2	-1,7
236301_at	Putative ORB3 gene, 5'	---	-2,2	-1,7
227030_at	---	---	-2,2	-1,1
1562697_at	hypothetical protein LOC339988	LOC339988	-2,2	-2
204684_at	neuronal pentraxin I	NPTX1	-2,2	-2,5
213975_s_at	lysozyme (renal amyloidosis) /// leukocyte immunoglobulin-like receptor, subfamily B (v LYZ /// LILRB1	LOC285628	-2,4	-3,1
232504_at	hypothetical protein LOC285628	---	-2,6	-1,6
235526_at	CDNA FLJ42171 fis, clone THYMU2029578	---	-2,7	-1,1
227198_at	AF4/FMR2 family, member 3	AFF3	-2,8	-4
202087_s_at	cathepsin L	CTSL	-3,3	-1,8
214470_at	killer cell lectin-like receptor subfamily B, member 1 /// killer cell lectin-like receptor sul	KLRB1	-3,5	-1,8
230570_at	Transcribed locus	---	-4	-4,7
206942_s_at	pro-melanin-concentrating hormone	PMCH	-4	-2,4

Manuscript 3

CCR6 is induced upon tolerogenic priming and identifies human Tr1 memory cells.

Laura Rivino, David Jarrossay, Antonio Lanzavecchia, Federica Sallusto and Jens Geginat

(Manuscript in preparation)

CCR6 is induced upon tolerogenic priming and identifies human Tr1 memory cells

Laura Rivino*, David Jarrossay*, Antonio Lanzavecchia*, Federica Sallusto* and Jens Geginat[°].

**Institute for Research in Biomedicine (IRB), Via Vela 6, 6500 Bellinzona, Switzerland*

[°]German Arthritis Research Center (DRFZ), Charite-Campus Mitte, Schuhmannstr.20/1, 10117 Berlin, Germany

The authors have no conflicting financial interests

Running Title: CCR6 identifies human Tr1 memory cells

Keywords: T cell subsets, memory maintenance, cytokines, differentiation, chemokine receptors

Address correspondence to:

Jens Geginat, Charite-Campus Mitte, DRFZ, Schuhmannstr. 20/1, 10117 Berlin, Germany

Phone: ++49-3028460708 E-mail: geginat@drfz.de

Abbreviations used in this paper: DC, dendritic cell; TT, tetanus toxoid; Treg, regulatory T cell

Abstract

Two types of regulatory T cells have been described: anergic CD25⁺ Foxp3⁺ “natural” Tregs and IL-10 secreting “adaptive” Tr1 cells with unknown phenotype. Here we show that CCR6 expression on antigen-experienced T cells requires a tolerogenic cytokine environment, but is not necessarily associated with Foxp3 induction. Circulating human CCR6⁺ CD25⁻ memory T cells are largely Foxp3⁻, they secrete IL-10 in response to self-antigens and inhibit autoreactive T cell proliferation in an IL-10-dependent manner. Cells specific for self-antigens in healthy donors are contained exclusively within the CCR6⁺ population, while cells with the same specificities are also detected among the CCR6⁺ cells in patients affected by an autoimmune pathology. Unlike naturally occurring CD25⁺ Tregs, CCR6⁺ memory T cells secrete IL-2 and proliferate in response to recall antigens. Single cell analyses of self-specific CCR6⁺ T cells showed that they proliferate and produce IL-10, but not IL-2, in response to cross-reactive self-antigens. We propose that CCR6 expression marks memory cells that act as Tr1-like cells when encountering innocuous antigens but behave as memory cells in response to recall antigens.

Introduction

It is now widely accepted that regulatory T cells exist and that they are important for the control of autoimmune diseases and for limiting immune responses

¹. “Natural” Tregs can be identified by CD25 surface expression in both mice ^{2, 3} and humans ⁴⁻⁹. They mature in the thymus ¹⁰⁻¹² or in the periphery following tolerogenic priming ¹³⁻¹⁷, are anergic *in vitro* but not *in vivo* ^{18, 19} and lack IL-2 producing capacity ^{1, 2, 4-6}. Their maintenance and function relies on IL-2 that is produced probably by activated T cells ²⁰⁻²⁴. They inhibit T cell priming *in vitro* by a not fully understood cell contact-dependent mechanism ^{4-6, 25}, while *in vivo* tolerogenic cytokines also seem to play a role ²⁶⁻²⁸. It is believed that these cells represent an independent T cell lineage that is under the control of the transcription factor Foxp3 ²⁹⁻³². Their T cell receptor repertoire is biased towards autoreactivity ^{7, 12, 33, 34} and they have been shown to prevent autoimmune diseases in several different models ^{35, 36}. On the other hand, ‘adaptive’ or Tr1 cells have also been shown to be important for control of autoimmune diseases ³⁷ and they are believed not to belong to the natural Treg lineage ^{35, 36, 38-40}. They are characterized by their capacity to produce IL-10 and to inhibit immune responses in an IL-10-dependent manner ^{37, 40, 41}. These cells can be generated *in vitro* by tolerogenic priming with immature DC, IL-10 or immune-suppressive drugs ^{37, 42, 43}, but until now no surface markers which allow the identification of Tr1 cells have been described and it is currently not clear if they are stably maintained. CCR6 is a chemokine receptor expressed on B cells, a fraction of memory T cells ^{44, 45} and immature DC ⁴⁶⁻⁴⁹. The ligand of CCR6, CCL20 is widely expressed constitutively in lymphoid and non-lymphoid tissues and up-regulated upon inflammation, but the CCR6-CCL20 axis appears to be particular important for migration of immune cells to the gut and the skin ^{49, 50}. Studies on CCR6 deficient mice indicated a non-redundant role for CCR6 in gut lymphoid tissue homeostasis ⁵⁰.

⁵¹. Furthermore, CCR6 deficient mice have altered CD4⁺ T cell responses, including reduced contact hypersensitivity and enhanced delayed type hypersensitivity responses ⁵¹⁻⁵³. CCL20/CCR6 are further involved in several autoimmune diseases including psoriasis ⁵⁴, inflammatory bowel disease ^{55, 56} and rheumatoid arthritis ^{57, 58}.

Here we show that CCR6 induction and maintenance on human peripheral blood T cells requires tolerogenic priming. CCR6⁺ T cells represent a broad subset of human Foxp3⁺ CD25⁺ antigen-experienced T cells that secrete IL-10 in response to self-antigens and inhibit autoreactivity in an IL-10-dependent manner. This Tr1-like suppressive capacity is however

context-dependent, since single-cell analyses revealed that autoreactive CCR6⁺T cells also secrete IL-2 and proliferate in response to recall antigens.

Materials and Methods

Cell culture. Peripheral blood mononuclear cells (PBMC) were isolated from buffy-coated blood from healthy or autoimmune patients. CD4⁺ T cells were isolated by negative selection with magnetic beads using a CD4⁺ T cell isolation kit and Automacs (Miltenyi). T cell subpopulations were purified by cell sorting based on expression of CD45RA, CD25 and CCR6 at a purity of >95%. Cells were cultured in complete medium (RPMI 1640 supplemented, 2 mM glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 50 µg/ml kanamycin, 50U/ml penicillin and 50 µg/ml streptomycin, Gibco) containing 5% pooled human serum or autologous plasma. Labelling of T cells with CFSE was performed as described previously. Monocytes were purified by positive selection with anti-CD14 beads (Miltenyi). CD14⁺ cells were directly used for antigen presentation or were cultured with IL-4 and GM-CSF for 5-7 days, as described previously. Mature monocyte-derived DCs were obtained by stimulating cells at day 5-6 for 24h with LPS (used 100ng/ml, Invivogen, ultra-pure LPS). CFSE labelled T cells were co-cultured with autologous DCs in the presence of TSST (100ng/ml), in 96 round-bottom wells. Circulating myeloid DC were isolated by cell sorting following enrichment for CD11c⁺ cells with magnetic beads. 2x10⁴ CFSE-labelled CD4⁺T cells were cultured with autologous or allogenic mDCs in 96-round bottom wells at a 5:1 ratio or with autologous monocytes at a 1:1 ratio. Recombinant cytokines were used at the concentrations of 25ng/ml (TGF-β purchased from R&D, IL-2, IL-10, IL-4, IL-12, purchased from Pharmingen BD), while neutralizing antibodies to IL-10 and IL-2 or MHC class II (Pharmingen BD) were used at the concentration of 10µg/ml. Recombinant antigens were used at

the following concentrations: MelanA 0.1 and 1 µg/ml (Prospec), tetanus toxoid (kindly provided by Chiron, Siena) 1 µg/ml.

ELISA and intracellular staining. Cytokine producing capacity of T cell subsets purified by cell-sorting was assessed after stimulation of cells at the density of 25x10³/100µl for 24h in wells coated with 2 µg/ml of anti-CD3 (clone TR66) and anti-CD28 antibodies (Pharmingen BD). Cell culture supernatants were then assessed by ELISA for presence of cytokines

following a standard protocol. Analyses was performed with the Softmax program. Intracellular cytokines were detected after stimulating cells in the presence of 10 µg/ml Brefeldin A (Sigma) for the last 2 hours of culture and after fixation with 4% formaldehyde and permeabilization with saponin. After saturation of non-specific binding sites with 10% FCS, cells were incubated with FITC-labelled antibody to TNF-α and PE-labeled antibody to IL-2, washed and analysed by flow cytometry on a FACSCalibur with the CellQuest software (Becton Dickinson). Intracellular Foxp3 was detected with a staining kit by following the manufacturer's instructions (e-bioscience).

Recall responses and T cell clones. Antigen specificity of cell-sorted memory CD4⁺T cell subsets was assessed by co-culturing purified CFSE labeled T cell subsets with irradiated autologous monocytes, at a 1:1 ratio, in the presence or absence of recombinant antigens, in 96 U-bottom wells. On days 5 and 7, cells were stained for CD14 and CD25, and CFSE dilution of CD14⁺ cells was assessed and analysed for CD25 expression. In some experiments, the presence of antigen-specific cells was confirmed by restimulating cells with autologous monocytes with or without antigen followed by assessment of cytokine production of proliferating T cells by intracellular staining, as described above. Isolation of autoreactive T cells was performed by stimulating CFSE labeled T cells with autologous mDCs and by sorting the population of divided cells at day 7. Single cell cloning of the autoreactive population was performed by limiting dilution by plating cells in 96 U-bottom wells at a concentration of 0.5 cells/well in the presence of 1×10^6 /ml irradiated allogeneic PBMCs, 1µg/ml PHA (Sigma) and IL-2 (1000U/ml). Clones were then expanded and maintained in medium containing IL-2.

Real time quantitative RT-PCR. CD25⁻CCR6⁺ T cells and autologous mDCs were isolated as described above and incubated together or separately for 24 hours. Cells were then re-sorted to separate T cells from mDC populations. Total RNA was extracted with the TRIzol method (Invitrogen Life Technologies) according to the manufacturer's instructions. cDNA synthesis was performed by RT-PCR by using random hexamers and an MMLV transcriptase kit (Stratagene). IL-10 transcripts were quantified by real time quantitative PCR on an ABI PRISM 7700 Sequence detector (Perkin-Elmer Applied Biosystems), with applied biosystems predesigned Taqman Gene Expression Assays and reagents according to the manufacturer's instructions. For each sample, the mRNA abundance was normalized to the amount of 18S rRNA and is expressed in arbitrary units.

Results

CCR6 is induced upon naïve T cell priming with tolerogenic cytokines

Tolerogenic T cell priming is thought to result in the induction of regulatory T cells. We searched for phenotypic markers that were induced upon T cell receptor (TCR) activation in the presence of tolerogenic cytokines and observed that TGF- β induces expression of CCR6 on proliferating T cells. We therefore analysed CCR6 expression requirements of human CD4⁺ naïve (CD45RA⁺, CD25⁻) T cells following TCR stimulation in the absence or presence of TGF- β alone or combined with other immunomodulatory cytokines (IL-4, IL-12, IL-10). We also assessed Foxp3 expression in these cells to address if cells were directed to the natural Treg lineage. As shown in Figure 1 CCR6 was poorly induced in naïve T cells following activation with anti-CD3 and anti-CD28 antibodies or allogeneic DCs (not shown). In contrast, CCR6 expression was specifically induced upon addition of exogenous TGF- β . As previously described, TGF- β also induced Foxp3 expression, but importantly some CCR6⁺ cells remained Foxp3⁻, suggesting that at least a fraction of CCR6⁺ cells is not directed to the natural Treg lineage. Importantly, both CCR6 and Foxp3 expression induced by TGF- β were prevented by the addition of polarizing cytokines IL-4, IL-12 or IFN- γ (data not shown). Conversely, addition of IL-10 did not affect CCR6 induction by TGF- β , but partially inhibited Foxp3 up-regulation. Thus, CCR6 expression following naïve CD4⁺ T cell priming requires TGF- β and is inhibited by TH1/TH2 polarizing cytokines but not by IL-10, but is not necessarily linked to a commitment to the natural Treg lineage.

CCR6 is expressed on subsets of memory T cells and CD25⁺ Tregs

We then analyzed CCR6 and Foxp3 expression *ex vivo* on human peripheral blood CD4⁺ naïve, memory (CD45RA⁻ CD25⁺) and regulatory T cells (CD25⁺). As expected, naïve CD4⁺ T cells were largely negative for both CCR6 and Foxp3 expression (Figure 2a). Memory cells were also largely Foxp3⁻, but a consistent fraction of these cells expressed CCR6. Finally, CD25⁺ cells were enriched for both Foxp3⁺ and CCR6⁺ cells. Both CCR6⁺ and CCR6⁻ memory cells were heterogeneous and contained TCM, TEM, gut and skin-homing cells (Figure 2b). Conversely, CD103, the α E integrin subunit, that is also induced by TGF- β and mediates adhesion to epithelia, was expressed only by a fraction of CCR6⁺ cells. In conclusion, a large fraction of antigen-experienced cells expresses CCR6 but not Foxp3 indicating that they do not belong to the natural Treg lineage. Interestingly, circulating myeloid DC also expressed CCR6 when analyzed *ex vivo*, suggesting that they might encounter CCR6⁺ T cells *in vivo* when attracted by CCL20 (data not shown). Consistent with previous reports, CCR6 was

slightly down-regulated, upon *in vitro* maturation of DCs with LPS (data not shown).

CCR6 is lost following memory T cell priming with polarizing cytokines

We next addressed the stability of CCR6 expression of purified CCR6⁺ memory cells following proliferation in the presence of IL-7 and IL-15, homeostatic cytokines that drive antigen-independent turnover, or following priming in the presence of either tolerogenic or polarizing cytokines (Figure 2c). CCR6 was maintained upon expansion with homeostatic cytokines or following priming in the presence of TGF-β, but was down-regulated upon antigenic activation in the presence of polarizing cytokines. Reciprocally, CCR6⁻ memory cells remained CCR6⁻ following cytokine-driven expansion or priming in the presence of polarizing cytokines, but acquired CCR6 following priming with TGF-β with a similar efficiency as CD4⁺ naïve T cells (data not shown). Altogether, these results suggest that CCR6 expression on T cells may be an indication that their previous antigenic contact has occurred in a tolerogenic cytokine environment.

CCR6⁺ T cells produce IL-2 and IL-10

We then compared the cytokine producing capacity of CCR6⁺ and CCR6⁻ memory cells to that of naturally occurring CD25⁺ Tregs (Figure 3a). CCR6⁺, CCR6⁻ and CD25⁺ Tregs were purified by cell sorting and were stimulated for 24 hours with plate-bound anti-CD3 and anti-CD28 antibodies. Cell-culture supernatants were then analysed for the presence of cytokines by ELISA. IL-2 and IFN-γ were produced at high levels by both CCR6⁺ and CCR6⁻ memory cells, but not by Tregs. Conversely, high levels of IL-10 were produced by CCR6⁺ cells, while CCR6⁻ cells were poor IL-10 producers and, in contrast to CCR6⁺ cells, also secreted IL-4. As reported previously, Tregs produced IL-10, although perhaps at lower levels compared to CCR6⁺ T cells. Thus CCR6⁺ T cells secrete cytokines associated with both memory (IL-2, IFN-γ) and regulatory cells (IL-10). Furthermore, it was possible to isolate a subset of CCR6⁺ T cells, co-expressing CCR4, which were characterized by expression of IL-10 but not of IFN-γ, thus constituting a population of cells distinct from TH1 cells (Figure 3b). In contrast, CCR6⁻ CCR4⁻ cells were characterized by expression of IL-10 and IFN-γ.

CCR6⁺ memory T cells proliferate with autologous DCs upon IL-10 neutralization

In order to understand if the CCR6⁺ memory population contained cells that were autoreactive, we incubated purified CFSE-labelled T cell subsets with *ex vivo* isolated, autologous myeloid DC as a physiologically relevant source of self-antigens. After 5 and 7

days T cell proliferation was assessed by analyzing CFSE dilution by flow cytometry. CCR6⁺ memory cells proliferated very poorly or not at all in the presence of autologous mDCs, but they proliferated extensively upon allogeneic stimulation (Figure 4a). In contrast, CCR6⁺ T cells were able to proliferate in the presence of both types of mDCs, but proliferation with autologous mDCs required addition of neutralizing anti-IL-10 antibody. Thus, IL-10 produced by either mDCs or T cells inhibited proliferation of autoreactive T cells contained within the CCR6⁺ population. Analyses of IL-10 mRNA indicated that IL-10 was induced in T cells but not in myeloid DCs following T-DC co-culture, thus indicating that IL-10 was derived from activated CCR6⁺ T cells (Figure 4b). Interestingly, the presence of anti-IL-10 antibody was not required for proliferation of CCR6⁺ T cells induced by allogeneic DC (Figure 4a). This different requirement for IL-10 neutralization was associated with low IL-2 but not with higher IL-10 production in autologous compared to allogeneic cultures (Figure 4c), suggesting that IL-2 produced in autologous cultures is insufficient to bypass the inhibitory effects of IL-10. Consistently, addition of exogenous IL-2 led to vigorous proliferation in autologous DC-T cell cultures in the absence of anti-IL-10 antibody (Figure 4a). Although requirements for IL-10 neutralization differed for T cell proliferation induced by allogeneic or autologous DC, both types of proliferation were MHC class II-restricted, IL-2-dependent and were under the control of natural Tregs (Figure 4a). We conclude that CCR6⁺ T cells produce IL-10 in response to self-antigens presented by autologous DCs and inhibit autoreactive T cell proliferation in an IL-10 dependent manner.

Treg and CCR6⁺ T cell proliferation differ in requirements for exogenous IL-2

Natural Tregs are believed to be biased towards autoreactivity, to lack secretion of IL-2 and to be anergic. We compared proliferation requirements of CCR6⁺ memory T cells and CD25⁺ Tregs in response to autologous and allogeneic DCs (Figure 4a). Unlike CCR6⁺ T cells, proliferation of CD25⁺ Tregs in response to both autologous and allogeneic mDCs required addition of exogenous IL-2, consistent with previous work reporting their anergic state *in vitro*. In order to rule out the possibility that CCR6⁺ T cells proliferating with autologous mDCs were due to Foxp3⁺ CD25⁺ Tregs (Figure 2), we analyzed Foxp3 expression of proliferating CCR6⁺ T cells and CD25⁺ Tregs following expansion with autologous mDCs. We observed that autoreactive and alloreactive proliferating CCR6⁺ T cells were largely Foxp3⁻ (data not shown), while the fraction of Foxp3⁺ Treg proliferating *in vitro* with autologous or allogeneic DC was similar to the fraction expressing Foxp3 *ex vivo* (data not shown). Altogether these results show that both CCR6⁺ memory T cells and CD25⁺ Tregs

contain autoreactive cells but possess different requirements for proliferation for exogenous IL-2.

Antigen-specificity of CCR6⁺ and CCR6⁻ memory T cells in health and disease

In order to confirm that CCR6⁺ T cells can react to innocuous and self-antigens, we assessed T cell proliferation induced by recombinant antigens presented by non-professional APCs. We stimulated CFSE-labelled purified T cell subsets with purified autologous monocytes that, unlike myeloid DCs, are poor inducers of autoreactive T cell proliferation, but are able to present purified antigens at high concentrations to induce proliferation of antigen-specific T cells (Figure 5a). Responses to recall antigens such as tetanus toxoid were generally detected in both CCR6⁺ and CCR6⁻ T cell subsets. In contrast, responses to the melanocyte-derived self-antigen MelanA were detected exclusively among CCR6⁺ T cells in all normal donors tested. Interestingly, in patients affected by the autoimmune disorder Vitiligo, both CCR6⁺ and CCR6⁻ T cells responded to MelanA (Fig. 5b). Thus, cells specific for innocuous antigens are contained within the CCR6⁺ population in healthy donors but may become partially CCR6⁻ in an autoimmune pathology.

Recall antigen-specific CCR6⁺ T cells cross-react with self-antigens

We next investigated whether autoreactive and recall-specific T cells represented two distinct populations within CCR6⁺ T cell, or if recall antigen-specific CCR6⁺ T cells could cross-react with self-antigens. We therefore re-stimulated with tetanus toxoid cells that expanded upon stimulation with autologous DC. Response to MelanA is also shown as a control (Figure 6a). A small fraction of auto-reactive T cells secreted cytokines upon re-stimulation with tetanus toxoid, but not with autologous monocytes alone, showing that some auto-reactive CCR6⁺ T cells are specific for recall antigens. Single cell cloning of these auto-reactive, TT-specific cells was performed and clones were analyzed for proliferation and cytokine production following stimulation with self-ligands or tetanus toxoid. Two representative clones are shown in Figure 6b-d. These clones secreted mainly IL-10 following re-stimulation with autologous mDCs, and in these conditions showed low proliferative capacity, evaluated as ³H incorporation, only upon addition of an anti-IL-10 antibody. In contrast the same clone displayed high proliferative capacity following stimulation with monocytes in the presence of tetanus toxoid and secreted IL-2 in these conditions. We conclude that CCR6⁺ memory T cells contain cells specific for self-antigens that can cross-react with recall antigens. The

behaviour of these cells differs greatly when in the presence of self versus recall antigens, suggesting that they might possess distinct roles in these different contexts.

Discussion

Here we show that CCR6 expression is induced in TCR activated naïve CD4⁺T cells in the presence of TGF- β alone or combined with IL-10. In contrast, addition of TH1 or TH2 polarizing cytokines completely abolished CCR6 induction by TGF- β . These in vitro generated CCR6⁺ T cells were heterogeneous for Foxp3 expression, indicating that CCR6 induction by TGF- β does not necessarily correlate with Foxp3 expression and thus with generation of Tregs. Analyses of CCR6⁺ T memory cells from human peripheral blood showed that, upon ex vivo TCR stimulation, these cells produce high levels of IL-10 and IFN- γ and are largely Foxp3⁻. Furthermore, it was possible to identify a sub-population of CCR6⁺ T cells, co-expressing CCR4, that secreted IL-10 but not IFN- γ CCR6⁺ CCR4⁺ cells thus constitute a subset of IL-10 producing cells distinct from TH1 or TH2 cells. In fact, IL-10 was first described as a TH2-associated cytokine which could limit TH1 development by inhibiting the activation and cytokine production of TH1 cells. It was further shown shown that TH1 cells themselves can produce some levels of IL-10 and some time later, IL-10 production was associated to regulatory T cells and to maintenance of self-tolerance. However, the exact role of IL-10 in Treg function is still not completely understood. Adaptive, IL-10 producing Tregs were shown to develop following TCR activation in the presence of an immunosuppressive environment, with reports suggesting an involvement of IL-10 and TGF- β . It is known that TGF- β is a potent regulatory cytokine which inhibits differentiation of T cells to the TH1/ TH2 lineage⁵⁹. In fact, mice with a T cell specific blockage of TGF- β signalling develop an autoimmune inflammatory phenotype associated with CD4⁺ T cell differentiation to TH1 or TH2 cells⁶⁰. Interestingly it was also proposed that IL-10 treatment enhances TGF- β RII expression on activated T cells, thus augmenting responsivity to TGF- β ⁶¹. However, in our experiments we generally did not observe an enhancement of TGF- β induced CCR6 expression upon addition of IL-10. As the generation conditions of CCR6⁺ T cells in vitro resemble those reported for the generation of Tr1 cells, we investigated whether cells expressing CCR6 possessed a regulatory function and if they represented cells biased towards autoreactivity. Interestingly, CCR6⁺ T cells proliferated vigorously with circulating autologous myeloid DCs upon neutralization of IL-10. This suggested the presence of autoreactive T cells among the CCR6⁺T cell population which were normally blocked in their proliferation by T cell-derived IL-10. This phenomenon was

generally observed for both IL-10 producing CCR4⁺CCR6⁺ and IL-10/IFN- γ producing CCR4⁻CCR6⁺ T cells, with some variability between different donors (data not shown). Of note, as APCs we used circulating myeloid DCs sorted directly from human peripheral blood, as we can envisage that these cells have sampled antigens in vivo, and thus they presumably constitute a physiologically relevant source of self-antigens. Compared to naturally occurring CD25⁺ Tregs, which are considered to be biased towards autoreactivity, the autoreactive cells contained within the CCR6⁺ T cell population do not require addition of exogenous IL-2 for proliferation to self-antigens. Instead, they require neutralization of IL-10, which in contrast has no effect on CD25⁺ Tregs. In these particular experiments we cannot exclude that the autoreactive CCR6⁺ T cells, which constitute only a fraction of the total CCR6⁺ T cells, are unable to produce IL-2 themselves and utilize IL-2 produced by cells in the remaining fraction. Furthermore, we show that in healthy donors, cells specific for the melanocyte differentiation antigen MelanA are contained invariably within the CCR6⁺ T cell pool, whereas in patients affected by vitiligo MelanA specific cells are also present among the CCR6⁻ population. T cell mediated pathologies have been associated with a breakdown in the critical balance between TH1, TH2 cells and regulatory populations. We show that, in vitro, CCR6 expression on CCR6⁺ T memory cells from peripheral blood of healthy individuals is maintained in the presence of a tolerogenic environment such as IL-10 and TGF- β , while it is down-regulated in these cells upon stimulation in the presence of TH1 or TH2 polarizing cytokines. Reciprocally, CCR6⁻ T cells up-regulate CCR6 to some extent in the presence of IL-10 and TGF- β while they remain CCR6⁻ when stimulated in TH1 or TH2 polarizing conditions. These results are consistent with our previous observations regarding the requirements for the generation of CCR6⁺ T cells in vitro from stimulated naïve cells. On the basis of our observations, we speculate that in vivo, in healthy individuals, self-specific CCR6⁺ T cells may behave as in vitro and be blocked in their proliferation to self by production of IL-10. Conversely, in autoimmune patients, the inflammatory environment may cause differentiation of the self-specific cells towards TH1 or TH2 cells. These self-specific cells may produce pro-inflammatory cytokines and be involved in the autoimmune pathology.

Furthermore, we show that in donors which have previously been vaccinated against tetanus, cells specific for the recall-antigen tetanus toxoid were detected among both CCR6⁺ and CCR6⁻ memory pools. This is consistent with our previous reports showing that vaccination against tetanus induces a mixed TH1/TH2 response detectable in all memory subsets⁶². Thus, we investigated at a single cell level whether CCR6⁺ autoreactive or recall antigen specific T

cells represented distinct populations. Interestingly, we obtained clones that presented a dual specificity. On one hand they behaved as classical memory cells upon encounter of tetanus toxoid they proliferated and produced IL-2, in addition to IL-10. On the other hand, when stimulated with autologous mDCs, they produced IL-10 but not detectable levels of IL-2 and proliferated upon neutralization of IL-10, although at low levels. Surprisingly, the levels of IL-10 produced by these cells in the presence of self or recall antigens was similar. It must be considered that upon encounter of self-antigens IL-10 is also consumed by the T cell, as demonstrated by its proliferative block. Furthermore, we speculate that upon stimulation with tetanus toxoid production of IL-2 by these cells may overcome the effect of IL-10. Of note, these clones do not represent classical autoreactive cells as they do not proliferate with autologous monocytes or with mDCs in the absence of IL-10 neutralization. The lack of proliferation with autologous monocytes could be due to the presentation of different self-peptides by monocytes compared to mDCs. In fact, it was previously reported that distinct types of APCs may possess different proteases, leading to a differential expression of self-peptides bound to their MHC molecules (ref). Alternatively, lack of recognition could be due to the poor antigen presenting capacity of monocytes compared to mDCs. We generally observed that neutralization of IL-10 has no effect on the proliferation of CCR6⁺ T cells with autologous monocytes (unpublished observation). We propose a context dependent function of these CCR6⁺ T memory cells. Upon encounter of a self-antigen, they exhibit a Tr1-like suppressive capacity, thus limiting autoimmune reactions. Presumably, T cells will possess a low affinity for self-antigens and this could explain the production of IL-10 and the lack of production of detectable amounts of IL-2 in these conditions. In contrast, when exposed to their specific antigen these cells will behave as memory cells and confer protection. Thus, we propose that CCR6 expression on memory T cells may indicate that their previous antigenic encounter has occurred in a tolerogenic environment. We speculate that this mechanism may represent a strategy evolved by the immune system, which allows the utilization of slightly autoreactive T cells, thus broadening the TCR repertoire available for pathogen recognition, while lowering the risk of autoimmune reactions. In fact, CCR6⁺ T cell memory production of IL-10 upon encounter of self-antigen may raise their threshold for an autoimmune response.

Figures

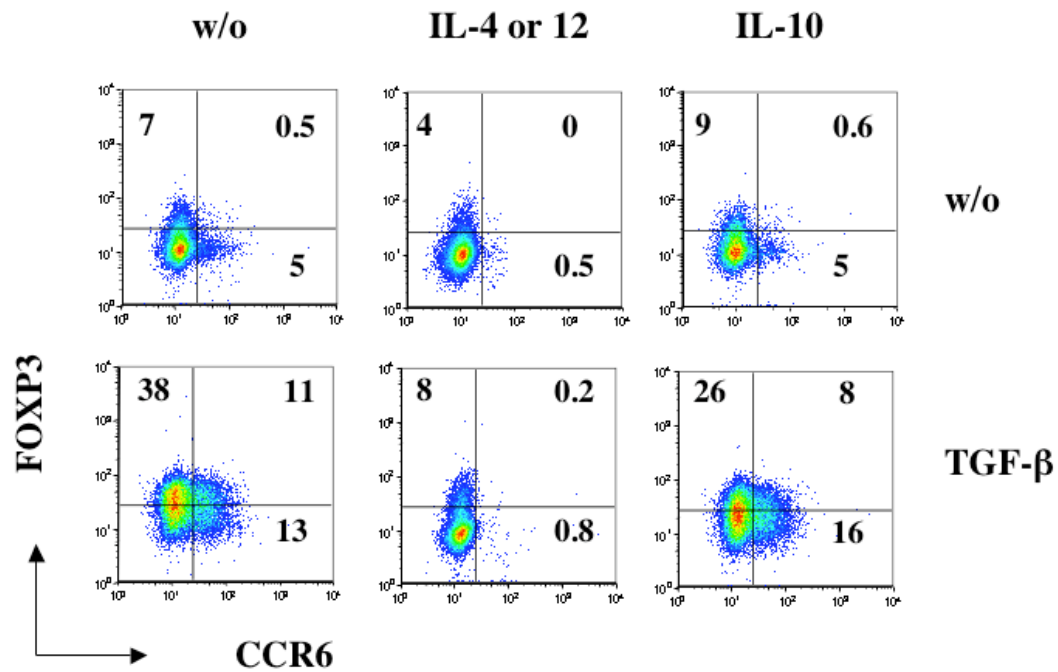


Figure 1. CCR6 expression upon CD4⁺ naïve T cell priming requires a tolerogenic cytokine environment.

CFSE labelled peripheral blood naïve CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 for 4 days in the presence or absence of the indicated cytokines. Cells were stained at d5 and analysed for CCR6 and Foxp3 expression by flow cytometry. Results are representative of three experiments.

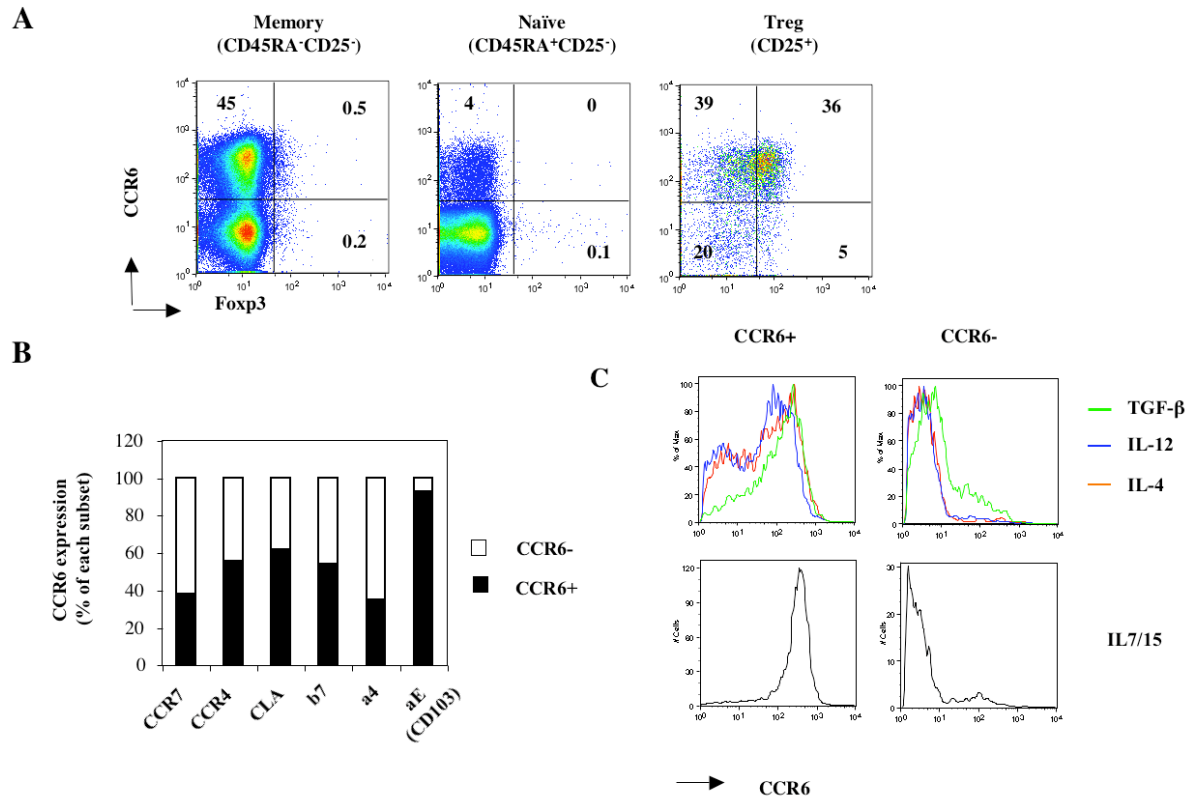


Figure 2. CCR6 is expressed on CD4⁺ T memory cells and CD25⁺Tregs and is lost upon T cell priming with polarizing cytokines.

(a). Peripheral blood CD4⁺ T cell subsets were analysed for CCR6 and Foxp3 expression by flow cytometry. **(b).** Peripheral blood CD4⁺ T memory cells were stained with antibodies specific for CCR6, CCR7, CLA, b7, a4 and aE (CD103), and analysed by flow cytometry. Shown is the percentage of CCR6⁺ or CCR6⁻ cells present in each subset. One representative donor out of three. **(c).** Purified CCR6⁺ T memory cells were assessed for CCR6 expression at day 7 after stimulation with autologous DCs and TSST in the presence of TGF- β (black line), IL-12 (grey line), IL-4 (dotted line), or following expansion in IL-7 and IL-15. Shown is one representative experiment out of three.

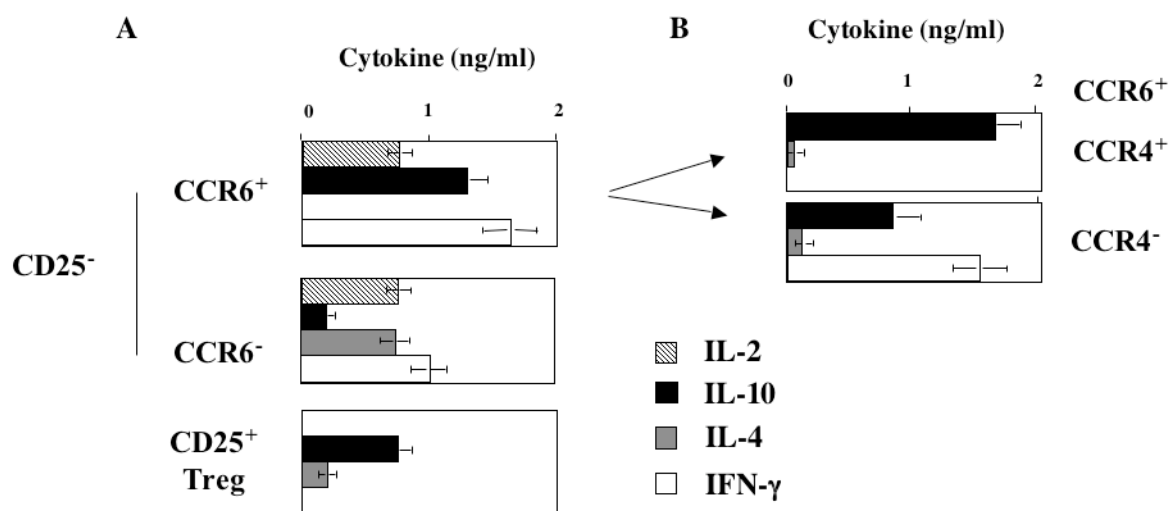
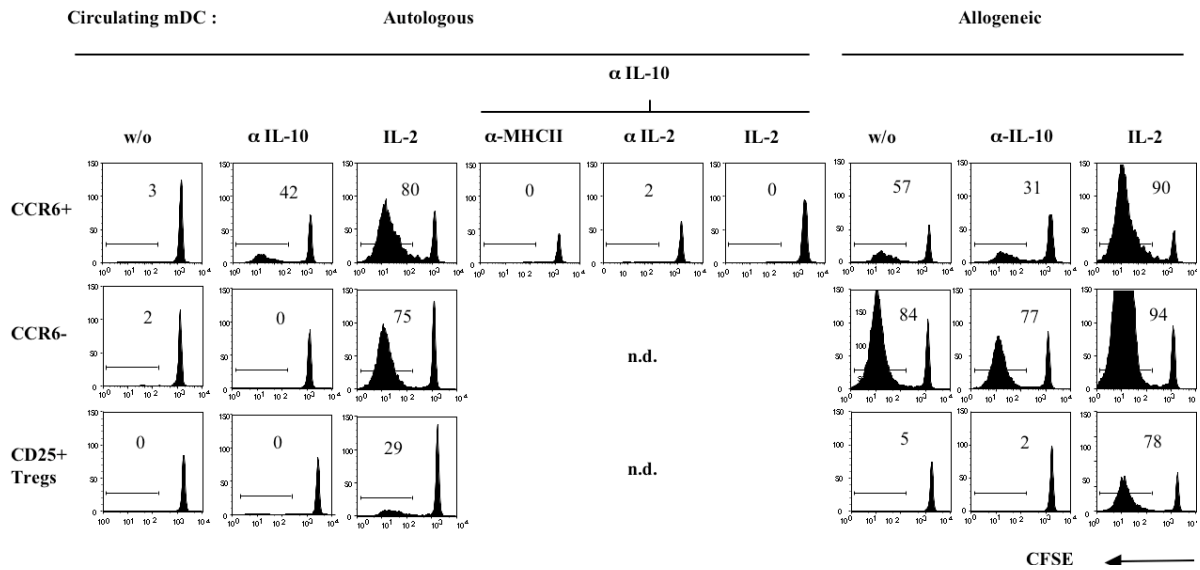


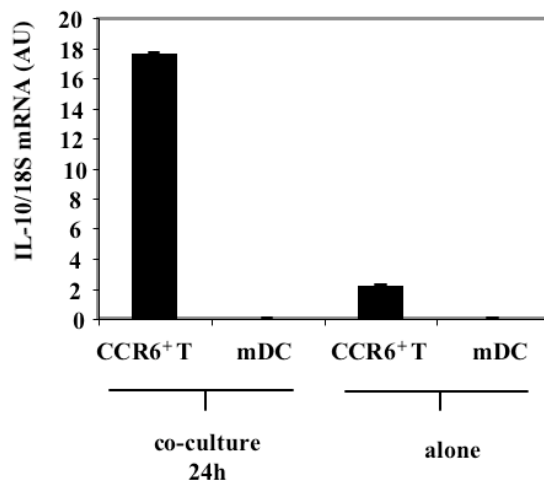
Figure 3. Ex vivo cytokine producing capacities of CCR6⁺ and CCR6⁻ memory cells and CD25⁺Tregs.

(a) Purified CCR6⁺ T memory cells and CD25⁺ Tregs were stimulated for 24h with plate-bound anti-CD3 and anti-CD28 and culture supernatants were analysed for presence of the indicated cytokines by ELISA. (b) CCR6⁺ T cells were furthermore subdivided according to CCR4 expression. Purified CCR6⁺ CCR4⁺ and CCR6⁺ CCR4⁻ cells were analysed for secretion of the indicated cytokines as described above. Indicated is one representative experiment of at least three.

A



B



C

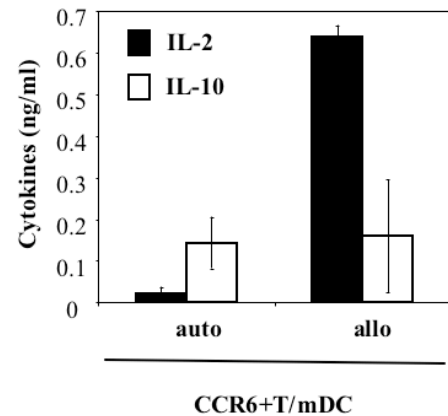


Figure 4. CCR6⁺ cells proliferate with autologous circulating myeloid DCs upon neutralization IL-10. (a) Purified CCR6⁺, CCR6⁻ and CD25⁺ T cells were incubated with ex vivo isolated autologous or allogeneic myeloid DCs in the presence or absence of the indicated antibodies or upon addition of IL-2. On day 7, CFSE profiles of T cells were analysed by flow cytometry. (b) CCR6⁺ T cells and autologous mDCs were purified after 24h of co-culture or of culture alone. IL-10 mRNA levels relative to 18S rRNA were quantified by quantitative real-time RT-PCR. Results are expressed in arbitrary units (AU) of mRNA/rRNA. Single experiments representative of at least three are indicated. (c) Culture supernatants of CCR6⁺ T cells co-cultured with autologous or allogeneic DCs and were assessed by ELISA for presence of IL-2 and IL-10. All experiments are representative of three.

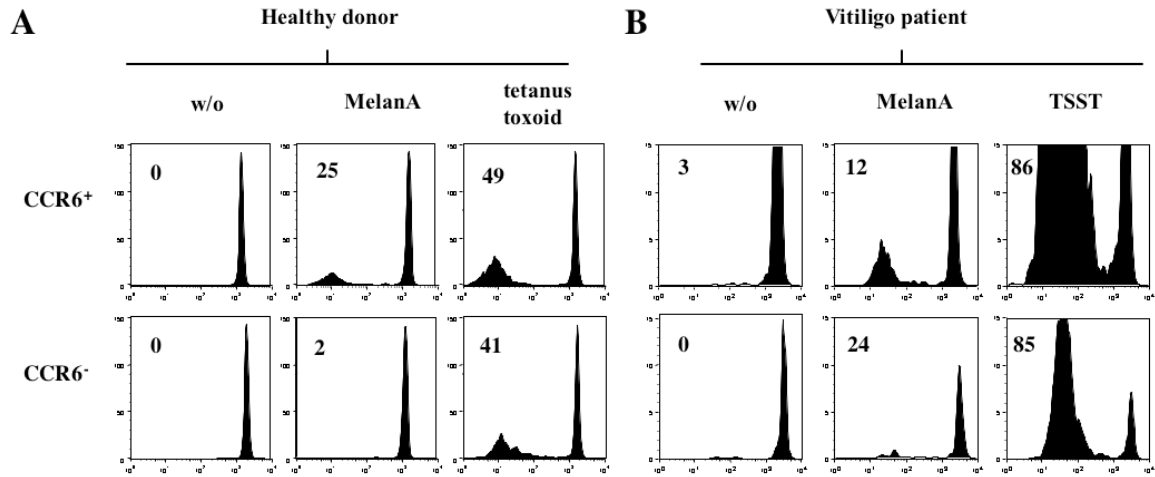


Figure 5. MelanA specific cells are contained in the CCR6⁺ population in healthy individuals, whereas they are present also among CCR6⁻ cells in vitiligo patients. Sorted CFSE labelled CCR6⁺ and CCR6⁻ T cells from healthy individuals (a) or vitiligo patients (b) were cultured with autologous monocytes with or without recombinant human MelanA, tetanus toxoid (a) or TSST (b). CFSE profiles of CD3⁺CD14⁻ cells were analysed on day 7 by flow cytometry. Shown are representative experiments of 10 healthy donors and 4 vitiligo patients.

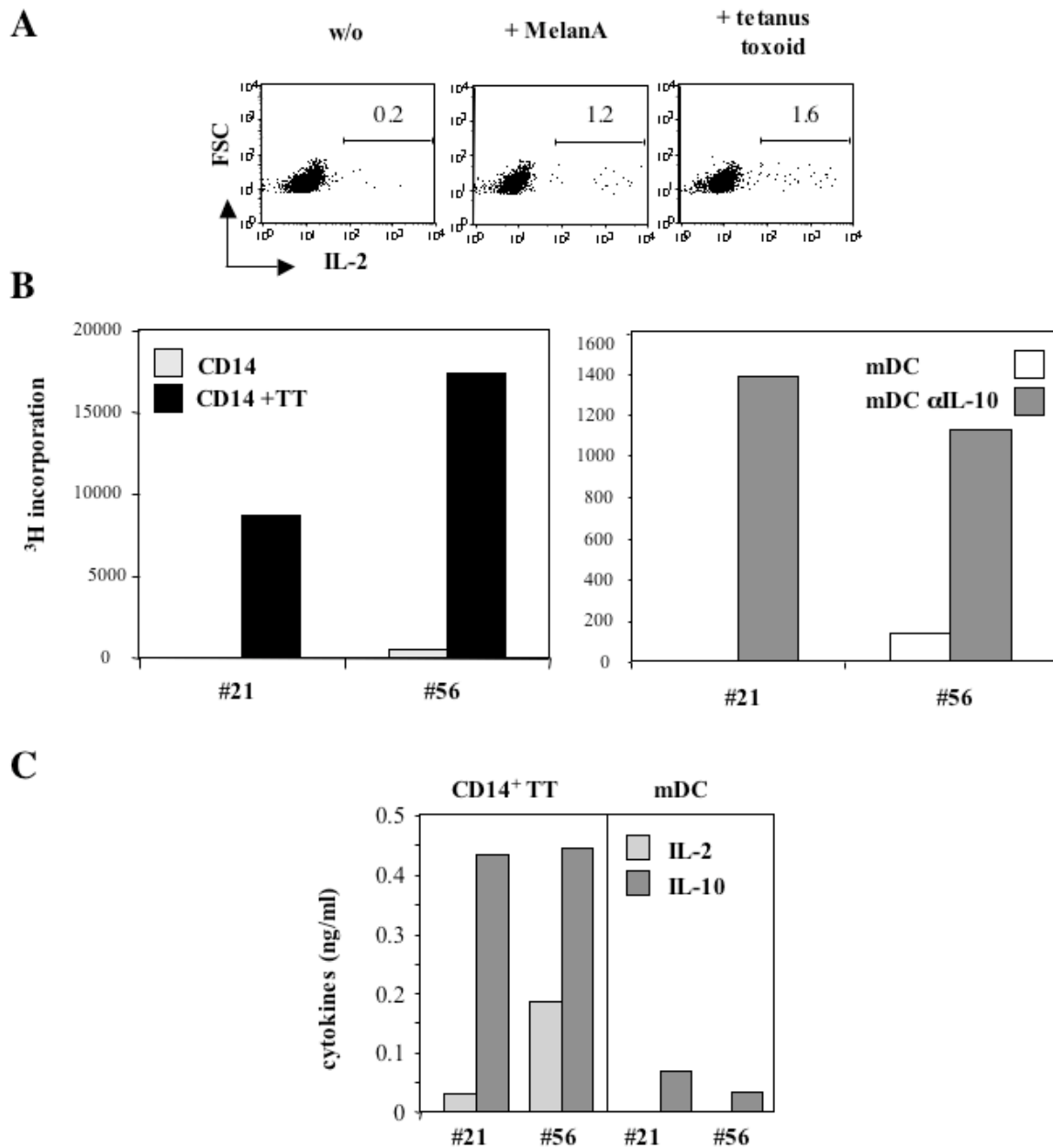


Figure 6. Autoreactive CCR6⁺ cells contain tetanus toxoid-specific cells. (a) Autoreactive CCR6⁺ T cells were incubated with autologous monocytes with or without Melan A or tetanus toxoid for 24h, in the presence of Brefeldin A. Intracellular staining was performed using an anti-IL-2 antibody. Forward scatter (FSC) and staining for IL-2 of autoreactive cells is shown. (b) Proliferation evaluated as ^3H incorporation of two representative clones is shown. Clones were stimulated with either myeloid DCs with or without anti-IL-10 antibody, or with autologous monocytes with or without TT. (c) Culture supernatants were assessed for presence of IL-2 and IL-10 by ELISA

References

- 1 Sakaguchi, S. Naturally arising CD4⁺ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22, 531-62 (2004).
- 2 Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155, 1151-64 (1995).
- 3 Suri-Payer, E., Amar, A. Z., Thornton, A. M. & Shevach, E. M. CD4⁺CD25⁺ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J Immunol* 160, 1212-8 (1998).
- 4 Jonuleit, H. et al. Identification and functional characterization of human CD4⁽⁺⁾CD25⁽⁺⁾ T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 193, 1285-94 (2001).
- 5 Dieckmann, D., Plottner, H., Berchtold, S., Berger, T. & Schuler, G. Ex vivo isolation and characterization of CD4⁽⁺⁾CD25⁽⁺⁾ T cells with regulatory properties from human blood. *J Exp Med* 193, 1303-10 (2001).
- 6 Levings, M. K., Sangregorio, R. & Roncarolo, M. G. Human cd25⁽⁺⁾cd4⁽⁺⁾ t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 193, 1295-302 (2001).
- 7 Taams, L. S. et al. Human anergic/suppressive CD4⁽⁺⁾CD25⁽⁺⁾ T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol* 31, 1122-31 (2001).
- 8 Baecher-Allan, C., Brown, J. A., Freeman, G. J. & Hafler, D. A. CD4⁺CD25^{high} regulatory cells in human peripheral blood. *J Immunol* 167, 1245-53 (2001).
- 9 Ng, W. F. et al. Human CD4⁽⁺⁾CD25⁽⁺⁾ cells: a naturally occurring population of regulatory T cells. *Blood* 98, 2736-44 (2001).
- 10 Itoh, M. et al. Thymus and autoimmunity: production of CD25⁺CD4⁺ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 162, 5317-26 (1999).
- 11 Stephens, L. A., Mottet, C., Mason, D. & Powrie, F. Human CD4⁽⁺⁾CD25⁽⁺⁾ thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol* 31, 1247-54 (2001).
- 12 Jordan, M. S. et al. Thymic selection of CD4⁺CD25⁺ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2, 301-6 (2001).
- 13 Apostolou, I. & von Boehmer, H. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med* 199, 1401-8 (2004).
- 14 Chen, W. et al. Conversion of peripheral CD4⁺CD25⁻ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198, 1875-86 (2003).
- 15 Walker, M. R. et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4⁺CD25⁻ T cells. *J Clin Invest* 112, 1437-43 (2003).
- 16 Kretschmer, K. et al. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6, 1219-27 (2005).
- 17 Seddon, B. & Mason, D. Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. *J Exp Med* 189, 877-82 (1999).
- 18 Walker, L. S., Chodos, A., Eggena, M., Dooks, H. & Abbas, A. K. Antigen-dependent proliferation of CD4⁺ CD25⁺ regulatory T cells in vivo. *J Exp Med* 198, 249-58 (2003).
- 19 Yamazaki, S. et al. Direct expansion of functional CD25⁺ CD4⁺ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* 198, 235-47 (2003).
- 20 Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. & Rudensky, A. Y. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6, 1142-51 (2005).

- 21 Almeida, A. R., Legrand, N., Papiernik, M. & Freitas, A. A. Homeostasis of peripheral CD4⁺ T cells: IL-2R alpha and IL-2 shape a population of regulatory cells that controls CD4⁺ T cell numbers. *J Immunol* 169, 4850-60 (2002).
- 22 D'Cruz, L. M. & Klein, L. Development and function of agonist-induced CD25⁺Foxp3⁺ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol* 6, 1152-9 (2005).
- 23 Furtado, G. C., de_Lafaille, M. A., Kutchukhidze, N. & Lafaille, J. J. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med* 196, 851-7 (2002).
- 24 de la Rosa, M., Rutz, S., Dorninger, H. & Scheffold, A. Interleukin-2 is essential for CD4⁺CD25⁺ regulatory T cell function. *Eur J Immunol* 34, 2480-8 (2004).
- 25 Nakamura, K., Kitani, A. & Strober, W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *Journal of Experimental Medicine* 194, 629-44 (2001).
- 26 Fahlen, L. et al. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 201, 737-46 (2005).
- 27 Hara, M. et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol* 166, 3789-96 (2001).
- 28 Green, E. A., Gorelik, L., McGregor, C. M., Tran, E. H. & Flavell, R. A. CD4⁺CD25⁺ T regulatory cells control anti-islet CD8⁺ T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes. *Proc Natl Acad Sci U S A* 100, 10878-83 (2003).
- 29 Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299, 1057-61 (2003).
- 30 Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat Immunol* 4, 330-6 (2003).
- 31 Allan, S. E. et al. The role of 2 FOXP3 isoforms in the generation of human CD4⁺ Tregs. *J Clin Invest* 115, 3276-84 (2005).
- 32 Khattry, R., Cox, T., Yasayko, S. A. & Ramsdell, F. An essential role for Scurfin in CD4⁺CD25⁺ T regulatory cells. *Nat Immunol* 4, 337-42 (2003).
- 33 Hsieh, C. S. et al. Recognition of the peripheral self by naturally arising CD25⁺CD4⁺ T cell receptors. *Immunity* 21, 267-77 (2004).
- 34 Fisson, S. et al. Continuous activation of autoreactive CD4⁺ CD25⁺ regulatory T cells in the steady state. *J Exp Med* 198, 737-46 (2003).
- 35 Maloy, K. J. & Powrie, F. Regulatory T cells in the control of immune pathology. *Nat Immunol* 2, 816-22 (2001).
- 36 Roncarolo, M. G. & Levings, M. K. The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr Opin Immunol* 12, 676-83 (2000).
- 37 Groux, H. et al. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389, 737-742 (1997).
- 38 Levings, M. K. et al. Human CD25⁺CD4⁺ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J Exp Med* 196, 1335-46 (2002).
- 39 Bluestone, J. A. & Abbas, A. K. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 3, 253-7 (2003).
- 40 Vieira, P. L. et al. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4⁺CD25⁺ regulatory T cells. *J Immunol* 172, 5986-93 (2004).
- 41 Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* 190, 995-1004 (1999).
- 42 Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. & Enk, A. H. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive

- stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* 192, 1213-1222. (2000).
- 43 Barrat, F. J. et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 195, 603-16 (2002).
 - 44 Liao, F. et al. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. *J. Immunol.* 162, 186-194 (1999).
 - 45 Fitzhugh, D. J., Naik, S., Caughman, S. W. & Hwang, S. T. Cutting edge: C-C chemokine receptor 6 is essential for arrest of a subset of memory T cells on activated dermal microvascular endothelial cells under physiologic flow conditions in vitro. *J Immunol* 165, 6677-81 (2000).
 - 46 Vanbervliet, B. et al. Sequential involvement of CCR2 and CCR6 ligands for immature dendritic cell recruitment: possible role at inflamed epithelial surfaces. *Eur J Immunol* 32, 231-42 (2002).
 - 47 Greaves, D. R. et al. CCR6, a CC chemokine receptor that interacts with macrophage inflammatory protein 3alpha and is highly expressed in human dendritic cells. *J Exp Med* 186, 837-44 (1997).
 - 48 Dieu, M.-C. et al. Selective Recruitment of Immature and Mature Dendritic Cells by Distinct Chemokines Expressed in Different Anatomic Sites. *J. Exp. Med.* 188, 373-386 (1998).
 - 49 Schutyser, E., Struyf, S. & Van Damme, J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev* 14, 409-26 (2003).
 - 50 Cook, D. N. et al. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity* 12, 495-503. (2000).
 - 51 Varona, R. et al. CCR6-deficient mice have impaired leukocyte homeostasis and altered contact hypersensitivity and delayed-type hypersensitivity responses. *J Clin Invest* 107, R37-45 (2001).
 - 52 Varona, R., Cadenas, V., Gomez, L., Martinez, A. C. & Marquez, G. CCR6 regulates CD4+ T-cell-mediated acute graft-versus-host disease responses. *Blood* 106, 18-26 (2005).
 - 53 Lukacs, N. W., Prosser, D. M., Wiekowski, M., Lira, S. A. & Cook, D. N. Requirement for the chemokine receptor CCR6 in allergic pulmonary inflammation. *J Exp Med* 194, 551-5 (2001).
 - 54 Homey, B. et al. Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis. *J Immunol* 164, 6621-32 (2000).
 - 55 Kaser, A. et al. Increased expression of CCL20 in human inflammatory bowel disease. *J Clin Immunol* 24, 74-85 (2004).
 - 56 Varona, R., Cadenas, V., Flores, J., Martinez, A. C. & Marquez, G. CCR6 has a non-redundant role in the development of inflammatory bowel disease. *Eur J Immunol* 33, 2937-46 (2003).
 - 57 Ruth, J. H. et al. Role of macrophage inflammatory protein-3alpha and its ligand CCR6 in rheumatoid arthritis. *Lab Invest* 83, 579-88 (2003).
 - 58 Matsui, T. et al. Selective recruitment of CCR6-expressing cells by increased production of MIP-3 alpha in rheumatoid arthritis. *Clin Exp Immunol* 125, 155-61 (2001).
 - 59 Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K. & Flavell, R. A. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* 24, 99-146 (2006).
 - 60 Gorelik, L. & Flavell, R. A. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 12, 171-81 (2000).
 - 61 Cottrez, F. & Groux, H. Regulation of TGF-beta response during T cell activation is modulated by IL-10. *J Immunol* 167, 773-8 (2001).
 - 62 Rivino, L. et al. Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-

Th2, and nonpolarized cells among human CD4⁺ central memory T cells. *J Exp Med* 200, 725-35 (2004).

63 Harari, A., Vallelian, F., Meylan, P. R. & Pantaleo, G. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J Immunol* 174, 1037-45 (2005).

64 Harari, A., Vallelian, F. & Pantaleo, G. Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load. *Eur J Immunol* 34, 3525-33 (2004).

III. Conclusions and perspectives

Human CD4⁺T memory cells are extremely heterogeneous and can be subdivided into different subsets endowed with distinct immunological functions.

In the first two manuscripts we identify distinct subsets based on expression of chemokine receptors and the IL-7 receptor. Among the T_{CM} cells we distinguished between cells expressing CXCR3 or CCR4 which represent cells that are pre-committed to give rise to T_H1 (pre-T_H1) or T_H2 cells (pre-T_H2), respectively. These cells are capable of generating either T_H1 or T_H2 cells spontaneously in steady state conditions while they retain a certain degree of flexibility and can re-polarize to the opposite subset in the presence of antigen and of the appropriate polarizing conditions. Cells lacking expression of these chemokine receptors are largely unpolarized. These results further support the model previously proposed by Lanzavecchia & Sallusto stating that central memory cells represent a “stem cell pool” of antigen-primed cells which can rapidly generate new waves of effector cells. Furthermore our results shed light on the mechanisms allowing T_H1 or T_H2 polarization to be maintained from primary to secondary responses (manuscript 1). The model mentioned above also suggests that the generation of memory T cells occurs under a precise range of signal strength. We further analysed the stimulatory conditions driving the generation of memory cells and the phenotypic markers induced under these conditions. We observed that cells expressing CCR7 and the IL-7R possessed characteristics typical of memory cells. Furthermore, cells with this phenotype could be generated at both intermediate and strong stimulatory conditions and differed in their memory potential (Manuscript 2). Interestingly, cells receiving a strong or intermediate level of stimulation possessed characteristics of respectively pre-T_H1 or unpolarized T_{CM} cells, described by us in manuscript 1.

In manuscript 3 we consider another important aspect of immunological memory such as the maintenance of self-tolerance of CD4⁺T memory cells. Among these cells we identified a population of cells expressing CCR6 which are enriched in autoreactive T

cells. CCR6⁺T cells produce IL-10 in the presence of autologous myeloid DCs thus inhibiting autoreactivity in an IL-10 dependent manner. In healthy donors cells specific for the self antigen MelanA were detected exclusively among the CCR6⁺ subset while in patients affected by vitiligo these cells are also present among the CCR6⁻ subset. Furthermore, we observed that autoreactive CCR6⁺T cells could cross-react to recall antigens. We identified autoreactive CCR6⁺T cell clones which respond to tetanus toxoid. These clones secrete IL-2 and proliferate vigorously in the presence of tetanus toxoid while they secrete IL-10 and inhibit their own proliferation in the presence of self DCs. These results suggest a context dependent role of CCR6⁺T cells, in that they exhibit a Tr1-like suppressive capacity when exposed to autologous DCs, while in the presence of their specific antigen they behave as memory cells.

We would like to further address the context dependent behaviour of these cells. The results obtained so far seem to point out a role for IL-2 versus IL-10 production. We plan to investigate the regulation and the mode of action of these two cytokines, especially that of IL-10 which is still largely unknown.

Production of IL-10 but not IL-2 could also be a hallmark of the classical Tr1 cells described in the mouse system. In fact there are still no known surface markers which identify human Tr1 cells. Discovery of these surface markers would allow the isolation of Tr1 cells from human peripheral blood. Expansion of these cells in vitro and re-infusion in patients may prove to be a useful tool in immunotherapy for suppressing undesired immune reactions.

Furthermore, we wish to address the role of circulating myeloid dendritic cells in tolerance. We observed that self-reactive CCR6⁺ T cells proliferated in response to autologous mDCs but not to autologous monocytes upon neutralization of IL-10. The reason for this is currently unknown but we hypothesize that this may be due either to the poor antigen presenting capacity of monocytes compared to mDCs or the two cell types may differ in expression of self-antigens. In the latter situation there could be a role for the transcription factor AIRE in driving expression of self-antigens.

Curriculum vitae

Laura Rivino

Born in Milan, Italy on the 12th January 1975

E-mail: laura.rivino@irb.unisi.ch

Education

Since 2003

PhD student

University of Fribourg, Switzerland, under the supervision of Prof. Sandro Rusconi. PhD program in Natural Sciences.

Experimental work was carried out at the Institute for Research in Biomedicine, Bellinzona, Switzerland.

2001

“Laurea” graduation in Biological Sciences (110/110 cum laude), University of Milan, Italy.

Thesis title: “Development of a murine tumor model for the study of CD4⁺ T lymphocytes specific for a tumor associated antigen”.

Professional qualification

2003- present

Institute for Research in Biomedicine, Bellinzona, Switzerland

PhD student in the laboratory of Prof. Antonio Lanzavecchia, under the supervision of Dr Jens Geginat.

2001-2003:

DIBIT, San Raffaele Scientific Institute, Milan, Italy

Fellowship in the Cancer Immunotherapy and Gene Therapy Program (CIGTP), in the laboratory of Dr Vincenzo Russo.

Research concerned the study of tumor specific responses in patients undergoing active and specific tumor vaccination treatments.

1999-2001:

DIBIT, San Raffaele Scientific Institute, Milan, Italy

Under-graduate research training at the Unit of Molecular Genetics, in the laboratory of Dr Anna Mondino.

List of publications

Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4⁺ central memory T cells.

Rivino L., Messi M., Jarrossay D., Lanzavecchia A., Sallusto F. and Geginat J.
J.Exp.Med. 200:725-735 (2004).

Phenotype and homing of CD4 Tumor-specific T cells is modulated by tumor bulk.

Benigni F., Zimmermann V., Huges S., Caserta S., Basso V., Rivino L., Ingulli E., Malherbe L., Glaichenhaus N. and Mondino A.
J.Immunol. 175: 739-748 (2005).

The strength of TCR stimulation determines IL-7 responsiveness, recall potential and lineage commitment of primed human CD4⁺IL-7R^{hi} T cells.

Lozza L, Rivino L., Rinaldi A., Jarrossay D., Guarda G., Bertoni F., Sallusto F., Lanzavecchia A, and Geginat J.
(Submitted)

CCR6 is induced upon tolerogenic priming and identifies Tr1 memory cells.

Rivino L., Jarrossay D., Sallusto F., Lanzavecchia A, and Geginat J.
(Manuscript in preparation)

Acknowledgments

I would like to acknowledge many people for making this work possible.

First of all Antonio Lanzavecchia and Federica Sallusto for allowing me to be part of their research group and of course my supervisor Jens Geginat for his continuous presence and precious guidance. Every minute of the work together in these last years has been intense and gratifying.

Many thanks to Sandro Rusconi for his help and generosity and to Anna Mondino for the critical reading of my thesis.

Thank you to David Jarrossay for all the hard work together and for making it so fun! Thanks to many past and present members of the IRB, for the stimulating working environment but also for their friendship and the good times out of the lab.

Last but not least I would like to thank many people who were not directly involved in this work but whose daily support was fundamental. My wonderful family, in particular my parents and my sisters, and my precious friends scattered throughout the world. Thank you for being with me always in the good and the bad times!