
# UNIVERSITY OF LONDON THESIS

Degree PhD Year 2007 Name of Author ALESSANDRA DE RIVA

# Role of Major Histocompatibility Complex Class II molecules in the maintenance of CD4 memory T cell function

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A thesis presented for the degree of Doctor of Philosophy

in the University College London, 2007





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I, Alessandra De Riva, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

**ACKNOWLEDGEMENTS**

I am very grateful to my supervisor, Brigitta Stockinger, for the chance she gave me to start this PhD project and for her support during these three years.

I also want to thank all the members of the lab, past and present, for the help and advice they gave me, as well as the other members of the division of Molecular Immunology and NIMR. In particular I would like to express my gratitude to Christine Bourgeois for her help during the second and the third year of my PhD.

Finally, I want to thank my family for their support, in particular my husband Guy Nicholson, my sister Valentina De Riva and Arto.

**ABSTRACT**

The inheritable modifications that occur during activation enable memory T cells to mount a faster and stronger immune response than naïve cells upon re-encounter with the same antigen. Memory CD4 T cells do not require MHC class II contact for survival, but previous analyses from our lab have underlined important differences between CD4 T cells transferred in MHC class II competent or deficient hosts that question the complete

independency of memory cells from signals derived from MHC class II contact in terms of functionality.

In this study, resting memory CD4 T cells generated in MHC class II competent or deficient hosts were characterised at the molecular level by analysis of gene expression with the aim to identify potential mechanisms to explain how MHC class II molecules preserve memory CD4 T cell function. Candidate molecules highlighted by analysis of gene expression were further investigated using FACS analysis and *in vitro* and *in vivo* experiments. As the phenotypic and functional characteristics of memory CD4 T cells generated in MHC class II deficient hosts were found established already in the early stages of the memory phase, the analysis was extended to the effector phase. The results obtained indicated that CD4 T cells may not achieve an optimal differentiation into effector cells in MHC class II deficient hosts suggesting that CD4 T cells require non-cognate interactions with MHC class II molecules during activation. Therefore, a universal role of MHC contact with non-antigen presenting MHC molecules during the initial

activation step may be instrumental in shaping the functional competence of memory T cells.

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**ABBREVIATIONS**

| | |
|---|---|
| AB medium | Air Buffered medium |
| AICD | Activation Induced Cell Death |
| Akt | murine thymoma viral (v-akt) oncogene homolog |
| AP | Adaptor Proteins |
| AP-1 | Activating Protein-1 |
| APC | AlloPhycoCyanin |
| APCs | Antigen Presenting Cells |
| *a*RNA | anti-sense RNA |
| Bcl-2 | B Cell Leukemia-2 |
| BCR | B Cell surface Receptor |
| Bio | Biotin |
| BM | Bone Marrow |
| BrDU | BromoDeoxyUridine |
| Cbl | Casitas B-lineage Lymphoma |
| CCR | Chemokine (CC) motif Receptor |
| CD | Cluster of Differentiation |
| *c*DNA | complementary DNA |
| CFSE | 5(-6) CarboxyFluorescein diacetate Succinidyl Ester |
| CIITA | Class II TransActivator |
| CLIP | Class II-associated Ii Peptide |
| c-Rel | C-terminal to the Rel homology domain |
| *c*RNA | complementary RNA |
| CSK | COOH-terminal Scr kinase |

| | |
|---|---|
| $C_t$ | Threshold Cycle |
| CTLA-4 | Cytotoxic T-cell Lymphocyte-associated Antigen 4 |
| DCs | Dendritic Cells |
| DNA | DeoxyriboNucleic Acid |
| EOMES | EOMESodermin |
| ER | Endoplasmatic Reticulum |
| ESTs | Expressed Sequence Tags |
| FACS | Fluorescence Activated Cell Sorter |
| FCS | Fetal Calf Serum |
| FITC | Fluorescein IsoThioCynate |
| FLICE | Fas-associated death domain-like IL-1 Converting Enzyme |
| FOXO | FOrkhead boX O |
| Foxp3 | Forkhead/winged-helix p3 |
| Gadd-45a | Growth Arrest and DNA-Damage-inducible 45 alpha |
| GATA-3 | GATA binding protein 3 |
| GCOS | GeneChip® Operating Software |
| GDP | Guanosine DiPhosphate |
| Glut-1 | Glucose transporter 1 |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| Grb2 | Growth factor Receptor-Bound protein 2 |
| GSK-3 | Glycogen Synthase Kinase-3 |
| HGMP | Human Genome Mapping Project |

| | |
|---|---|
| HIV | Human Immundeficiency Virus |
| HPRT | Hypoxanthine guanine PhosphoRibosyl Transferase |
| IFN | Interferon |
| i.v. | Intra Venously |
| ICOS | Inducible T-cell co-stimulator |
| Ifit | Interferon-induced protein with tetratricopeptide repeats |
| Ifitm | Interferon induced transmembrane protein |
| Ifrd | Interferon-related developmental regulator |
| Ig | ImmunoGlobulin |
| Ii | Invariant chain |
| IL | InterLeukin |
| IMDM | Iscove's Modified Dulbecco's Medium |
| IS | Immunological Synapse |
| ITAMs | Immunoreceptor Tyrosine-based Activation Motifs |
| Itga4 | Integrin α4 |
| IVT | *In Vitro* Transcription |
| Jak | Janus Kinase |
| JNK | c-Jun $NH_2$-terminal Kinase |
| ko | Knocked Out |
| Klr | Killer cell Lectin-like Receptors |
| LAT | Linker for Activation of T cells |
| Lck | Lymphocyte cell-specific protein-tyrosine kinase |
| LOWESS | Locally weighted Least Squares |
| LPS | LipoPoliSaccaride |

| | |
|---|---|
| mAb | monoclonal Antibody |
| MAC | Membrane Attack Complex |
| MALT | Mucosal-Associated Lymphoid Tissue |
| MAPK | Mitogen-Activated Protein Kinase |
| MFI | Mean Fluorescence Intensity |
| MHC | Major Histocompatibility Complex |
| MMR | Macrophage Mannose Receptor |
| Morf4l2 | Mortality Factor 4 like 2 |
| MRG | MORF4 Related Genes |
| *m*RNA | messenger RiboNucleic Acid |
| mTOR | mammalian Target Of Rapamycin |
| NF-AT | Nuclear Factor of Activated T cells |
| NF-kB | Nuclear Factor – kappa B |
| NIA | National Institute for Aging |
| NKs | Natural Killer cells |
| NP40 | Nonidet P40 |
| PALS | PeriArteriolar Lymphoid Sheath |
| PAMPs | Pathogen Associated Molecular Patterns |
| PBS | Phophate-Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PD-1 | Programmed Death-1 |
| PdBU | 4-beta-phorbol-12,13-dibutyrate |
| PE | PhycoErythrine |
| PerCP | Peridinin Chlorophyll Protein |
| PH | Pleckstrin Homology |
| PI | Propidium Iodide |

| | |
|---|---|
| PI3K | Phosphatidyl-Inositol-3-Kinase |
| PIC | Pre-Initiation Complex |
| PKB | Protein Kinase B |
| PKC | Protein Kinase C |
| PLCγ-1 | PhosphoLipase Cγ-1 |
| PM | Perfect Match |
| PRR | Pattern Recognition Receptors |
| PtdInsP2 | Phosphatidyl-Inositol 4,5-bisphosphate |
| PTEN | Phosphatase and Tensin homologue |
| PTKs | Protein Tyrosine Kinases |
| PTPases | Protein Tyrosine Phosphatases |
| RAG | Recombination Activating Genes |
| RBC | Red Blood Cell |
| RNA | RiboNucleic Acid |
| ROS | Reactive Oxygen Species |
| RSS | Recombination Signal Sequences |
| RT | Room Temperature |
| S6K1 | Single 6 Kinase 1 |
| SAPE | StreptAvidin R-PhycoErythrin |
| SCID | Severe Combined ImmunoDeficiency |
| SDS | Sodium Dodecyl Solfate |
| SH2 domains | Src Homology 2 domains |
| SHP-2 | Src Homology 2-domain-contining tyrosine Phosphatase 2 |
| SMAC | SupraMolecular Activation Cluster |
| SMAD | Similar to Mothers Against Decapentaplegic |

| | |
|---|---|
| SPF | Specified Pathogen Free |
| SSC | Sodium cloride Sodium Citrate |
| STAT | Signal Transducer and Activator of Transcription |
| TAP | Transporters associated with Antigen Processing |
| t-Bet | T-Box Expressed in T cells |
| TCR | T Cell surface Receptor |
| Tdt | Terminal-deoxynucleotide transferase |
| TECs | Thymic Epithelial Cells |
| TGF-β | Transforming Growth Factor β |
| TGN | Trans-Golgi Network |
| Th | T helper cells |
| TLRs | Toll-Like Receptors |
| TM | TransMembrane |
| TNF | Tumor Necrosis Factor |
| TRIM | T cell Receptor Interacting Molecule |
| UTR | UnTranslated Region |
| vs | versus |
| XLP | X-linked LymphoProliferative |
| X-SCID | X-linked SCID |
| ZAP-70 | Zeta-chain (TCR) Associated Protein Kinase 70 kDa |

# 1. INTRODUCTION

## 1.1 Overview of the immune system

Multicellular organisms have evolved an immune system to protect themselves from pathogens. The immune system has two major components: innate immunity and adaptive immunity.

### 1.1.1 Innate immune system

The innate immune system is composed of various cell types of myeloid and lymphoid lineage. Eosinophils, neutrophils, mast cells, Natural Killer cells (NKs), macrophages and Dendriitc Cells (DCs) are the first line of defence of the organism and they rely on a limited number of germline encoded receptors, Pattern Recognition Receptors (PRR), to recognise conserved molecular patterns on the surface of infectious agents, Pathogen Associated Molecular Patterns (PAMPs). This mechanism allows the discrimination between the infectious non-self from the non-infectious self [1].

For example, macrophages are able to recognise a variety of bacteria and fungi through their Macrophage Mannose Receptor (MMR) [2], while murine DCs can express up to 10 Toll-Like Receptors (TLRs), each of them able to bind in a specific and non redundant manner bacterial proteins. Recognition of the cognate ligand alerts the cells of the innate immune system to danger [3] and initiates various mechanisms such as opsonisation, triggering of the complement and coagulation cascade, phagocytosis and inflammation. The

goal of this response is to kill the infectious agents, to contain the damage and to induce maturation of the cells specialised in antigen presentation.

The direct recognition of PAMPs induces the maturation and migration DCs. The maturation enables DCs to secrete inflammatory cytokines and chemokines that recruit circulating DC precursors and other immune cells to the site of infection. Consequently, the maturation of the DCs leads to the loss of tissue adhesive molecules and the expression of receptors for lymphoid chemokines, like the Chemokine (CC) motif Receptor 7 (CCR7) and co-stimulatory molecules [4].

Although very efficient, the innate immune system is often not sufficient to clear the host from infectious agents.

### 1.1.2 Adaptive immune system

The adaptive immune system adds to the immune system the plasticity needed to respond to new and evolving pathogens and gives the advantage of being able to select and maintain a repertoire of new antigen specificities. At the same time, the cells of the adaptive immune system can switch on the innate immune system whenever pathogens manage to evade detection.

The two major classes of lymphocytes of the adaptive immune system are B cells and T cells.

B lymphocytes, first identified in the Bursa Fabricii in birds, develop in the bone marrow and are the cells responsible for humoral immunity. Each B cell is programmed to make a unique cell surface receptor able to recognise antigens. Antigens that bind to the receptor are internalised into endosomes that can fuse with vesicles carrying Major Histocompatibility Complex (MHC)

class II directed to the cell surface. B cells complete maturation through hypermutation, isotype switching and affinity maturation, which is carried out in the lymph nodes [5]. Activated B cells undergo proliferation, which originates a clone that differentiates into an antibody secreting plasma cell. Circulating antibodies bind to microbes and synergise with the complement cascade, activating the classical pathway which culminates in the production of the Membrane Attack Complex (MAC). Moreover, small amounts of antibody added to complement-coated organisms enhance their phagocytosis.

T lymphocytes differentiate in the thymus from bone marrow-derived progenitor cells, reviewed in [6]. The TCR is a membrane-bound molecule composed of two chains. There are three classes of TCRs. T cells resident in the intestinal tract, as well as in the skin, bear a receptor formed of the rearrangement of γ and δ genes (TCR1). An additional lineage of lymphocytes is represented by the NKT cells, which express a restricted αβ TCR repertoire in association with markers characteristic of NK cells, such as Cluster of Differentiation (CD)161 (NK1.1) and CD122 (InterLeukin-2Receptor β, IL-2Rβ) [7].

The vast majority of the T lymphocytes bear a TCR that is formed from the rearrangement of α and β genes (TCR2).

T cells and B cells are able to generate different receptors that allow them to recognise a vast number of antigens. This is achieved through the T Cell surface Receptor (TCR) and B Cell surface Receptor (BCR) which provide an immense polyclonal repertoire [8]. TCR and BCR have a similar structure with constant and hypervariable regions. The random rearrangement of the variable (V), jointing (J) and diversity (D) regions by the enzymes produced

by the Recombination Activating Genes (RAG-1 and RAG-2) generates the somatic variations that enable T and B cells to bind different antigens [9] [10].

The TCR recombination is initiated when a complex of RAG-1 and RAG-2 binds to specific recognition sequences, called Recombination Signal Sequences (RSS) in the TCRβ gene locus (an analogous mechanism operates in B cells) [11] [12] [13] [14] [15]. The recombinases mediate the excision of DeoxyriboNucleic Acid (DNA) fragments at the RSS sequences, while DNA-repair enzymes rejoin the fragments. Lack of functional RAG-1 or RAG-2 genes results in the absence of T and B cells. Additional diversity is created by the addition of nucleotides in a template-independent manner to the N regions at the V-D and D-J junction by the Terminal-deoxynucleotide transferase (Tdt) [16]. Successful rearrangement of the TCR β-chain transiently turns off the recombination process, thus allowing the presence of a single TCR β-chain in each T cell. This process is called allelic exclusion [17]. TCR α-chain rearrangement can commence only after successful TCR β-chain rearrangement.

T cells that have successfully rearranged the TCR undergo two selection steps [18]. The selection is made on the basis of the interaction with MHC molecules. Positive selection ensures that only T cells bearing a receptor able to recognise self-MHC differentiate to mature T cells, while cells that bear receptors that recognise self-MHC fail the positive selection and die by apoptosis [19]. In negative selection, cells bearing high affinity receptors for self-peptides are deleted [20]. The result of this process is the selection of a mature T cells repertoire with an intermediate affinity for self-peptide: MHC complexes.

### 1.1.3 MHC molecules

MHC molecules are highly polymorphic transmembrane glycoproteins encoded by a cluster of genes localised in chromosome 17 of the mouse genome [21]. Two major classes of MHC molecules can be found in an organism: MHC class I and MHC class II. MHC class I molecules are present in the surface of all cells of the body, while MHC class II molecules are expressed on Thymic Epithelial Cells (TECs) and professional Antigen Presenting Cells (APCs) such as DCs, macrophages, and B cells. In addition fibroblasts, astrocytes, endothelial and epithelial cells express MHC class II molecules if they are exposed to specific stimuli (InterFeroN (IFN)-γ, signals of inflammation, infection, and trauma).

The peptide: MHC complex is recognised by the TCR and its co-receptor. The co-receptor CD8 recognises invariable regions of MHC class I molecules, while the co-receptor CD4 recognises invariable regions of MHC class II molecules [22]. The mechanisms that regulate the choice to express one or other co-receptor during T cell development seem to be related to the "strength of signal", such as the intensity and/or the duration of the stimuli received by the TCR with or without one of the co-receptor [23 24 25].

MHC molecules play a crucial role not only in thymic selection, but also in delivering signals for survival and activation to peripheral T cells. In the periphery, CD8 T cells are programmed to kill infected cells upon engagement of the TCR with antigen loaded MHC class I. MHC class I molecules present peptides that have been synthesised by the cellular ribosomes and that reach the Endoplasmatic Reticulum (ER). Transporters associated with Antigen Processing (TAP) proteins guide the synthesised

peptide to the antigen-binding groove during MHC class I synthesis [26] [27]. As a consequence, the vast majority of peptides presented in this way are endogenous peptides derived from defective nascent proteins [28] and viral products.

CD4 T cells have the role of secreting cytokines to help B lymphocytes to produce antibody and macrophages to release bactericidal molecules upon engagement of the TCR with antigen loaded MHC class II. In the mouse, MHC class II expression is regulated by a single regulatory factor, the Class II TransActivator (CIITA), a non-DNA-binding co-activator which regulates the SXY module (S, X, X2 and Y boxes) up-stream of the encoding region of the α-chain and β-chain of class II H2-A and H2-E isotypes. CIITA is encoded by the *C2ta* gene, under the control of 3 different promoters, pI, pIII and pIV. The use of various knocked out murine transgenic lines have indicated that TECs rely on pIV for MHC class II transcription, while B cells and plasmacytoid DCs need the expression of pIII. In conventional DCs and macrophages, CIITA expression is regulated by pIV promoter.

Differentiation of B cells into plasma cells or maturation of DCs is concomitant with shut down of *de novo* MHC class II synthesis, by inactivation of the *C2ta* gene by a global repression mechanism which involves histone deacetylation of the domain that spans the regulatory region of the gene [29]. The presentation of only the antigens that have specifically induced APC maturation is promoted by their prolonged surface expression by MHC class II molecules, the up-regulation of co-stimulatory molecules and the reduction of further endocytosis.

MHC class II molecules present foreign antigens that APCs acquire by endocytosis. The degradation of foreign antigens into peptide fragments is

carried out in lysosomes by means of acidification and activation of proteases. The loading of processed antigens into the MHC class II peptide binding groove, requires the fusion of lysosomes with MHC class II-rich vesicles (Fig. 1).

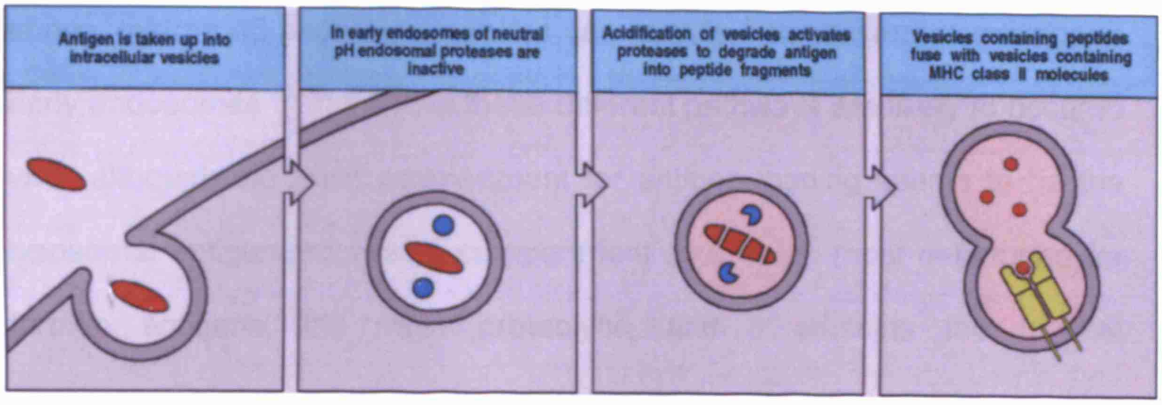



Figure 1: antigen acquisition and degradation by proteases.

The antigen is endocytosed and loaded into MHC class II molecules upon degradation into peptides by proteases. From Immunobiology, the immune system in health and disease, 6th edition by Janeway, Travers, Walport, Shlomchik (Chapter 5, page 177)

αβ class II molecules newly synthesised in the ER are escorted through the biosynthetic pathway by the Invariant chain (Ii), resulting in αβIi complexes. Due to its short amino terminal cytoplasmatic domain which occupies the peptide-binding groove of class II (Class II-associated Ii peptide, CLIP), Ii blocks the loading of any peptides into the peptide-binding groove of the MHC class II molecule during its synthesis. As shown in Fig. 2, four pathways of antigen loading have so far been hypothesised [30]: 1. from the Trans-Golgi Network (TGN), class II αβIi complexes traffic direct to lysosomes and do not pass through endosomes or pre-lysosomal intermediates [31, 32]; 2. from the TGN, class II αβIi complexes traffic to the

plasma membrane and are then internalised into protease-rich lysosomes [33], process regulated by the two di-leucine endocytosis signals present in the cytosolic domain of the Ii [34]; 3. from the TGN, class II αβI complexes traffic to early endosome and then to lysosome where the peptides are loaded into the groove of αβ dimers [35, 36]; 4. in addition to the three pathway described above, mature αβ peptide complexes can recycle from plasma membrane to early endosomes [37 38, 39 30]. All these different pathways are likely to occur *in vivo*, although the main compartment for antigen loading seems to be the lysosomal antigen-processing compartment as it is the most denaturing for foreign antigens, the most proteolytic, and it contains the highest concentration of the CLIP-removing proteases.

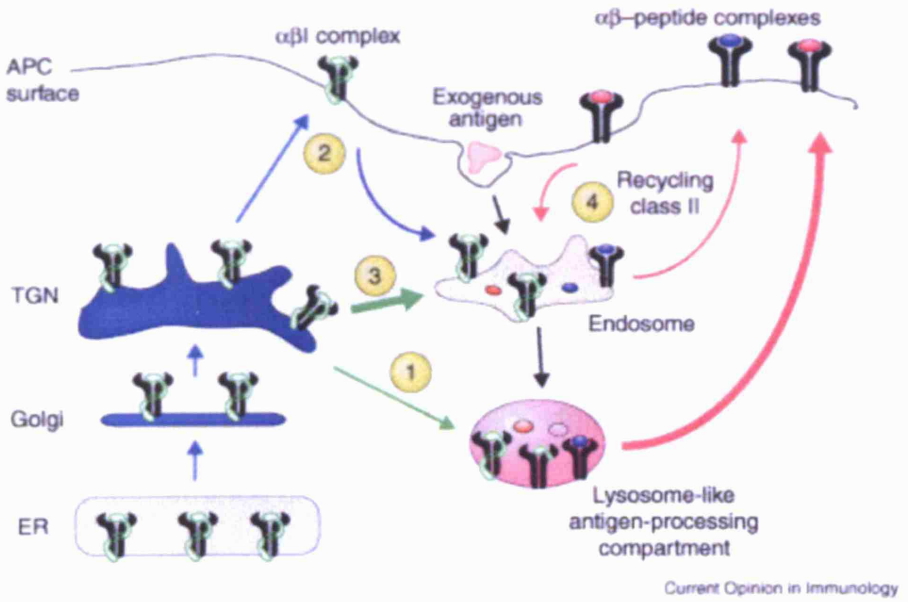



Figure 2: Pathways of antigen loading into MHC class II molecules.

Newly synthesised MHC class II molecules are: 1. directed to lysosome where the Ii is degraded and antigen is loaded; 2. exposed to the cell surfaces and secondarily internalised to merge with antigen containing endosomes; 3. directed to endosomes that then fuse with

lysosomes; 4 recycled from the cell surface to the endosomal compartment and re-exposed on the cell surface [30].

Various cell types can deliver antigenic stimulation to T cells, but the main cell type responsible for T cell priming are DCs. DCs represent the critical link between innate and adaptive immunity. In their immature phase, DCs reside in peripheral tissues (skin and mucosal surfaces) where they act as sentinel cells ready to capture antigens by means of macropinocytosis, phagocytosis or through receptors such as mannose receptors (C-type lectins) or Fcγ receptors type I and III. Antigen loaded DCs migrate from the periphery to the secondary lymphoid organs where they instruct T cells and probably B cells and NK cells to induce an effective immune response [4]. The role of APCs other than DCs in T cell priming is controversial [40]. For instance, B cells can act as APCs, but it is believed that their secondary role *in vivo* is due to the low frequency of antigen specific B cells (1 in $10^4$-$10^5$ cells) [41]. In addition, B cells do not reside in any of the physical barriers of the body and they depend on help from activated CD4 T cells [42]. Nevertheless, it has recently been published that T cells require antigen specific cognate interaction with B cells during activation for optimal induction and maintenance of T cell division and differentiation [43]. Recently, it has been suggested that regulatory B cells may function as a second line APC that can support or dampen ongoing T cell responses initiated by DCs [44].

### 1.1.4 Organs of the immune system

During the life span of an organism, various organs are of immunological relevance.

As far as haematopoiesis is concerned, during embryogenesis the generation of the blood cells occurs in the yolk sac and then in the fetal liver, while in the adult this function is carried out by the bone marrow [45]. The bone marrow is in fact the organ responsible for the generation of the vast majority of the mature cells of the immune system, with the exception of T cells which mature in the thymus [46].

The lymphoid system, together with the circulatory system, constitutes the network that allows the co-operation between the different cell types of the immune system. Vast surfaces of the body are exposed to antigens, for example, the lungs or the digestive tract. These vulnerable areas are monitored and protected by the Mucosal-Associated Lymphoid Tissue (MALT), which forms a net of cells dedicated mainly to antibody secretion. Antibodies and cells continuously re-circulate between blood, tissues, lymphatic ducts and secondary lymphoid organs, such as lymph nodes and the spleen. The structure of lymph nodes and spleen allows the drainage of lymph and blood. Pathogens as well as dead cells are trapped in the meshwork of draining lymph nodes and spleen, where they are promptly disposed by phagocytic cells. In both lymph nodes and spleen, B cells and T cells reside in different areas. In the lymph node, B cells are found in the cortex, while T cells are found in the paracortical areas. In the spleen, T cells are present in the PeriArteriolar Lymphoid Sheath (PALS) of the white pulp, where interaction with DCs and passing B cells can be made. Activated B cells move from the white pulp to the red pulp, where plasmablasts and plasma cells secrete antibodies into the blood stream [47].

## 1.2 Peripheral T cells

CD4 and CD8 T cells that have passed positive and negative selection checkpoints exit the thymus. In the periphery, T cells can further differentiate if exposed to infectious agents. Peripheral T cells are organised in two pools of small resting populations: naïve and memory cells. T cells that have not encountered an antigen are defined as naïve, while antigen experienced cells that persist in a resting state after antigen clearance are called memory cells. In normal conditions, i.e. in the absence of chronic inflammation or autoimmunity, the formation of memory is preceded by a short lived effector phase. Effector cells are not a stable population, but are normally eliminated as soon as the pathogen is cleared.

### 1.2.1 Naïve T cells

CD4 or CD8 T cells expressing adhesion and homing molecules, such as CD62L and CCR7, exit the thymus and reach the secondary lymphoid organs. Naïve T cells continuously re-circulate between blood and secondary lymphoid organs maintaining a quiescent metabolic state [48]. Long term survival of naïve T cells requires two factors: TCR interaction with self-peptide-MHC complexes (sp:MHC) and interleukin-7 (IL-7). The question if naïve T cells require sp:MHC interaction for survival still remains controversial, but experiments carried out in murine models strongly suggest that CD4 and CD8 naïve T cells survive for long periods of time when transferred into MHC class II or class I deficient hosts, respectively [49 50 51 52 53].

As most of the experiments carried out to investigate the role of sp:MHC have been conducted by adoptive transfer of T cells into lymphopenic hosts, it has been difficult to discriminate between survival and sp:MHC driven homeostatic proliferation of naïve T cells. In fact, higher number of cells recovered from adoptive transfers in MHC competent hosts could originate from proliferative signals delivered by MHC [54] [55]. Recently it appears possible that, depending on the environment, different answers can be obtained, as survival of naïve CD4 T cells seems to be independent from MHC class II derived signals in a lymphopenic environment, but requires those signals in a full host [56]. One group studying CD4 T cells reported that naïve CD4 T cells showed a substantial reduction in the level of TCR ζ chain phosphorylation when losing MHC class II contact [57]. The reduction observed in the level of TCR ζ chain phosphorylation was further correlated to reduced responsiveness of these cells to antigen stimulation measured by proliferation, IL-2 production and cell size determination [58]. These results suggested that MHC class II continuous contact may be necessary not only for naive CD4 T cells survival but also to preserve their functionality.

Adoptive transfers of naïve T cells into IL-7 deficient mice [59] as well as into normal mice treated with anti-IL-7 antibody [60] have shown that IL-7 signalling is essential in the maintenance of this pool. In the absence of Lymphocyte cell-specific protein-tyrosine kinase (Lck) and protein-tyrosine kinase fyn (Fyn), two molecules downstream of TCR signalling, IL-7 was shown to sustain the half-life of naïve T cells up to 26 days in CD4 T cells and 18 days in CD8 T cells [61], in contrast with ~ 6 months in the presence of TCR signalling [62].

### 1.2.2 Effector T cells

The stages that follow the recognition of antigen are clonal expansion, during which naïve T cells proliferate and acquire those effector functions that allow them to clear the organism from the pathogen, and clonal contraction, during which 90-95% of the cells generated in the previous phase die, leaving behind a resting memory population [63]. These two mechanisms regulate the effector phase and aim to limit the persistence of the infection on one side and immune pathology on the other.

#### 1.2.2.1 Expansion phase

The idea that a few antigen responding T cells can generate a large number of antigen specific T cells through proliferation was introduced by Burnet [64]. T cell activation starts with the recognition of an antigen presented in the context of MHC molecules on the cell surface of APCs. Various correlations between antigen dose, number of T cells recruited as well as proliferation rate have been found depending on the model used. While some groups have indicated that the dose of antigen presented by DCs positively correlates with both the number of CD4 T cells recruited and their level of proliferation [65], others have shown that there is no such correlation in CD8 T cell expansion *in vivo* [66, 67]. In particular, considering the duration of the antigenic stimulus required to initiate the differentiation toward the effector phenotype, naïve CD4 T cells seem to require prolonged interactions with antigen loaded APCs to start division and differentiation [65]. As indicated by the examples above, differences in the kinetics of the commitment to effector

functions have been observed between CD4 and CD8 T populations [68] [69]. CD8 T cells seem to follow an 'autopilot model' [70] [71]. In this model, a short antigen interaction (up to 2 h) has been shown to be sufficient to induce a complete transcriptional program for CD8 T cells differentiation, without further need for antigenic stimulation [72].

*In vitro* activation of naïve CD4 T cells follows a biphasic pattern. In the antigen dependent phase, activated CD4 T cells prepare to enter into cell cycle. This process requires up to 10 hours and it is followed by a second phase, which is cytokine dependent and during which activated CD4 T cells divide at a slightly higher pace (6-8 hours per division) [73].

Persistence of antigen, like in chronic viral infections, leads activated T cells to exhaustion and to the inability to eliminate the pathogen. In this respect, it has been recently shown that CD8 T cells do not become irreversibly dysfunctional, but can revert back to functional T cells by the blockade of the Programmed Death-1 (PD-1)/PD-L1 inhibitory pathway [74].

With activation, naïve T cells modify the expression of various surface molecules. The down-regulation of CCR7 and CD62L enables activated T cells to leave the secondary lymphoid organs and to reach peripheral tissues. CD25 (IL-2Rα) synthesis occurs within a few hours of activation and regulates the consumption of IL-2, the growth factor necessary for the initiation of many effector functions. CD25 as well as CD69 are early markers of activation and their up-regulation is transient, while other markers, like CD44, remain stable throughout activation and are retained in the subsequent memory phase.

#### 1.2.2.2 Contraction phase

The mechanism that limits the extensive effector cell proliferation triggered by antigen recognition is the induction of apoptosis via the Tumor Necrosis Factor (TNF), B Cell Leukemia-2 (Bcl-2) families, and Transforming Growth Factor β (TGF-β), reviewed in [75] [76].

As mentioned above, activated T cells up-regulate IL-2R, which sustains proliferation and survival during activation, but also sensitises the cells to Fas mediated Activation Induced Cell Death (AICD). Fas (CD95) is expressed on the surface of activated cells and initiates cell death through the caspase pathway [77]. Mutations of Fas (*lpr* mice) or FasL (*gld* mice) lead to accumulation of abnormal numbers of T cells in the periphery by week eight of age [78]. Other reports have shown that activated T cells die normally in the absence of Fas or its ligand and may indicate that multiple pathways can result in the death of activated cells [79].

For example, the Bcl-2 family also has a role in the contraction phase. There is evidence that cell death depends on the ratio of pro-apoptotic and anti-apoptotic members of this family. A key role in maintaining the balance seems to be attributed to Bcl-2, whose expression is down-regulated during activation at the messenger RiboNucleic Acid (*m*RNA) and protein levels [80]. It has been proposed that Bcl-2 reduction is controlled by the levels of Reactive Oxygen Species (ROS) produced during T cell activation [81] [82], but also by IL-2 and IL-7 [83] [84]. In the mitochondria, Bcl-2 is associated with Bim, one of the pro-apoptotic members of the Bcl-2 family. Following activation, low Bcl-2 levels free Bim, which can either associate with Bcl-$x_L$, reducing the cellular availability of this anti-apoptotic molecule [85], or activate pro-apoptotic proteins Bax and Bad [86]. The ultimate consequence is metabolic dysfunction of the mitochondrion and consequent cell death [75].

Another important molecule that plays a role in the contraction phase is TGF-β. In fact, TGF-β has been reported to have a role in restoring normal T cell numbers in response to activation, probably by controlling the transcription of pro-apoptotic molecules via SMAD proteins [87]. Interestingly, the release of TGF-β from apoptotic cells may ensure the removal of such cells avoiding the release of inflammatory signals.

### 1.2.2.3 Subsets of effector T cells

TCR and co-stimulatory signals drive T cells to synthesise and secrete IL-2, to divide extensively and to undergo a series of epigenetic changes, such as DNA de-methylation [88] [89], reduce histone acetylation [90] and enhance the ability to bind transcription factors [91]. While effector CD8 T cells exert mainly cytolytic functions, CD4 T cells can differentiate into a variety of effector cells.

#### Cytolytic CD8 T cells

It is not clear which is the master transcription factor for acquisition of effector function of CD8 T cells upon antigen encounter, but some have reported that naïve CD8 T cells undergo a genetic remodelling program that starts with T-Box Expressed in T cells (T-bet) and EOMESodermin (EOMES) expression which is followed by IFN-γ production and acquisition of lytic activity [92] [93].

## T helper (Th) 1, Th2, Th17, Tregs, and Tr1

Two transcription factors, T-bet and GATA binding protein 3 (GATA-3), define the commitment of naive CD4 T cells to Th1 or Th2 lineage respectively [94] [95]. Th1 or Th2 differentiation is induced by various factors, such as cytokine environment and TCR signalling as well as the cell type that delivers the stimulus. Type I and II IFNs, IL-12 and IL-27 are cytokines secreted by the cells of the innate immune system and they can signal through Signal Transducer and Activator of Transcription 1 (STAT1)-associated cytokine receptors which, together with TCR signalling, promotes T-bet up-regulation. T-bet enhances *Ifnγ* gene expression and the up-regulation of the inducible chain of the IL-12R. The signalling through the IL-12 receptor activates STAT4 and drives effector T cells to sustain the production of IFN-γ and therefore Th1 differentiation [96] [97]. In addition, it has been proposed that Th1 responses are also favoured by strong interactions between MHC class II and TCR. Therefore, MHC class II enhanced level of expression [98], as well as increase in antigenic peptide availability and binding [99] [100] determine a Th1 type response, while a weaker interaction would favour a Th2 type response. Moreover, the differentiation into Th1 or Th2 effectors has been shown to depend on the APC presenting the antigen. In fact, B cells have been shown to directly influence the differentiation of T cells into Th2 response [101] while different DC subsets have been reported to direct naïve T cells towards Th1 or Th2 effectors [102]. Moreover, different DCs, like classical and plasmacytoid DCs, have been shown to elicit the same CD4 T effector population once activated via the same TLR [103]. Interestingly some have suggested that memory CD4 T cells may shape the

activation of naïve CD4 T cells by means of educating DCs, which in this scenario would act as 'temporal bridges' between memory and naïve T cells [104].

Th1 differentiated cells produce pro-inflammatory cytokines, in particular IFN-γ, IL-2 and TNF which induce macrophage activation and provide protection against intracellular pathogens. TCR signalling together with IL-4 receptor signalling induces up-regulation of GATA-3 via STAT6 and expression of the Th2 cytokines cluster (*Il4, 5, 13*), which suppress Th1 differentiation [96 97]. Th2 cells are characterised by secretion of IL-4, IL-5 and IL-3, cytokines essential for promoting high affinity antibody responses against extra-cellular pathogens.

In addition to the Th1 and Th2 lineages, Th17 is another population of CD4 T cells that has recently been described. There are six IL-17s (IL-17A to IL-17F) and of these only IL-17A (IL-17), IL-17E (IL-25) and IL-17F have been reported to be produced by CD4 T cells [105]. Naïve CD4 T cells can be polarised to Th17 in the presence of IL-1, IL-6, TNF-α and TGF-β [106]. IL-23, previously reported to induce Th17 differentiation, is now thought to have a role in the maintenance of this population [96 107]. The transcription factor that characterise this lineage has not been identified yet although the activation of STAT3 by IL-6 and its ability to bind IL-17 and IL-17F promoter indicate its possible involvement in Th17 differentiation [107].

IL-25 is another cytokine of the IL-17 family that is now receiving some attention. Although there is very little known about it, the Th25 population seems to have more features in common with the Th2 population as it limits Th1 induced inflammation and supplies a protective role in host defence to pathogens like *N. brasiliensis* [105].

In addition to these new subsets of pro-inflammatory CD4 T cells, regulatory CD4 T cells have the role of suppressing T cell responses and maintaining tolerance. Two main classes of regulatory T cells have been described: the naturally occurring CD25+CD4 suppressor T cells (or Tregs) and the Tr1 (or Th3). Tregs were first described as 'small CD4CD25+ cells' to discriminate them from the newly activated, and therefore blasting, large CD25+ effector CD4 T cells [108] [109]. The vast majority of Tregs are produced in the thymus as a distinct and mature population. This unique population constitutes 5-10% of the peripheral CD4 T cells in normal naïve mice. Phenotypically, Tregs share with activated CD4 T cells the expression of high levels of Cytotoxic T-cell Lymphocyte-associated Antigen 4 (CTLA-4 or CD152) in the cell surface, but distinctively, only Tregs express the transcription factor Forkhead/winged-helix p3 (Foxp3 or Scurfin) [110] [111] [112]. Tregs act as immunoregulators in a variety of immune processes from responses to pathogens to the induction of tolerance. Although not yet characterised, the mechanism by which Tregs suppress the immune response is thought to rely on cell contact *in vitro* [113], but it seems to be mediated by cytokines such as IL-10 and/or TGF-β *in vivo* [114] [115]. Interestingly, Tregs express high levels of TGF-β on their cell surface and this cytokine has a well known role in the inhibition of T cell differentiation affecting processes such as IL-2 synthesis and cell proliferation [116]. Other models of suppression suggest that Tregs may act as competitors for specific MHC/peptide complex binding or as 'sink' for IL-2 due to their high IL-2R expression. Tregs are crucial in maintaining self-tolerance as demonstrated by the fact that animals that lack this T cell compartment (due to a mutation in the transcription factor Foxp3) develop autoimmune diseases and die in the early stages of life [110] [117].

Contrary to the thymic origin of Tregs, Tr1 are thought to originate in the periphery from naïve T cells. This 'adaptive' regulatory T cell population derives from naïve T cells activated in low co-stimulatory conditions (e.g. by immature DCs) or naïve T cells activated in the presence of cytokines such as IL-10, or immunosuppressive drugs such as vitamin D or dexamethasone *in vitro*. Another difference between Tr1 and Tregs is that Tr1 are negative for Foxp3. Tr1 suppress the immune response by secretion of IL-10 and TGF-β and are thought to represent an important response to those pathogens that promote their differentiation [118] [119].

Like the protection to different infectious agents that has given rise to dichotomy between Th1 and Th2, Tregs and Th17 may represent the dichotomy the immune system has developed to cope with inflammation. This hypothesis is supported by the fact that Tregs and Th17 both can differentiate in the context of inflammation in the presence of TGF-β alone or TGF-β plus IL-6 respectively [120].

### 1.2.3 Memory T cells

Immunological memory can be defined as a faster and stronger immune response upon re-encounter with the same pathogen. Memory T cells achieve this goal through various mechanisms briefly summarised here.

Memory T cells are present in an elevated precursor frequency in immunised animals compared to non immunised animals. One of the intrinsic characteristics of memory T cells is to have undergone a process of maturation that increases their affinity and functional avidity for antigen. The consequence of this selection process is that only highly relevant clones

from an antigen-selected repertoire are retained in the memory pool. Moreover, memory T cells have increased sensitivity to low antigen doses and need low or no co-stimulatory signals to become effectors. One of the most relevant characteristics of memory T cells is to gain immediate effector function, such as the quick entrance into the cell cycle and fast cytokine production [121] [69] [122]. Memory T cells acquire the expression of surface molecules that enable them to reach non-lymphoid organs [123].

Memory T cells are small and metabolically inactive cells which cycle at a low rate. Despite their resting status, analysis of the gene expression of memory T cells has demonstrated that they express similar numbers of genes compared to activated cells. However, the spectrum of the genes expressed is very different [124] [125].

Phenotypically, most of the murine memory CD4 T cells are $CD62L^{lo}$ and $CD44^{hi}$. This phenotype is not unique to CD4 T memory cells but it identifies also effector cells. In CD8 T cells the expression of CD62L (L-selectin receptor) is only transiently down-regulated during the effector phase and it is re-expressed in the memory phase. In humans, the kinetic of expression of this adhesion molecule in combination with the CCR7 has been used to distinguish 'effector memory' cells ($T_{EM}$), which are $CD62L^{lo}$ $CCR7^{-}$ and are found preferentially in non-lymphoid organs, from 'central memory' cells ($T_{CM}$), which are $CD62L^{hi}$ $CCR7^{+}$ and reside in lymph nodes. Along with different homing properties, the two cohorts have been shown to differ in their functional properties, with $T_{EM}$ being more efficient than $T_{CM}$ in the secretion of effector cytokines upon stimulation [126]. Although this model has been repeatedly challenged [127], this view has highlighted the heterogeneous and dynamic nature of memory T cells.

In terms of survival factors, memory T cells do not require interaction with MHC molecules [128]. Nevertheless, the absence of MHC class II interaction compromises the quality of memory CD4 T cell responses upon TCR stimulation and therefore indicates that memory CD4 T cells are not completely independent of MHC class II signalling for the maintenance of their intrinsic properties [129].

Survival of memory T cells depends on cytokines signalling. For CD8 memory T cells, it has been established that IL-15 and IL-7 are both required for their survival and homeostatic proliferation [130]. The key cytokines for CD4 memory T cell survival remain to be defined [121]. While IL-15 has been ruled out as possible candidate [131] [132], the data in favour of a role for IL-7 as the cytokine for memory CD4 T cells survival are not clear as some groups have found data in support of such a role for IL-7 [133] [134] while others could not confirm it [135].

In the absence of clone specific antigenic stimulation, memory T cells persist in a quiescent state throughout the life span of an organism, although in contrast to CD8 memory T cells, memory CD4 T cells numbers have been described as declining in certain experimental models of viral infection, as reviewed in [121]. These observations could be biased by the fact that memory CD4 T cell may be more heterogeneous and composed of smaller clone sizes than memory CD8 T cells. Moreover, low numbers of memory CD4 T cells could be enough to activate and direct the responses of CD8 T cells and B cells, reviewed in [136].

### 1.2.3.1 Theories on generation of memory T cells

There are various hypotheses that describe the mechanism by which a population of memory T cells emerge from the contraction phase of an immune response. As a universal mechanism of memory generation be excluded, it is also possible that the differentiation of effector CD4 and CD8 T cells into a resting memory population follow different patterns. The current models proposed for generation of memory T cells are summarised in Fig. 3.

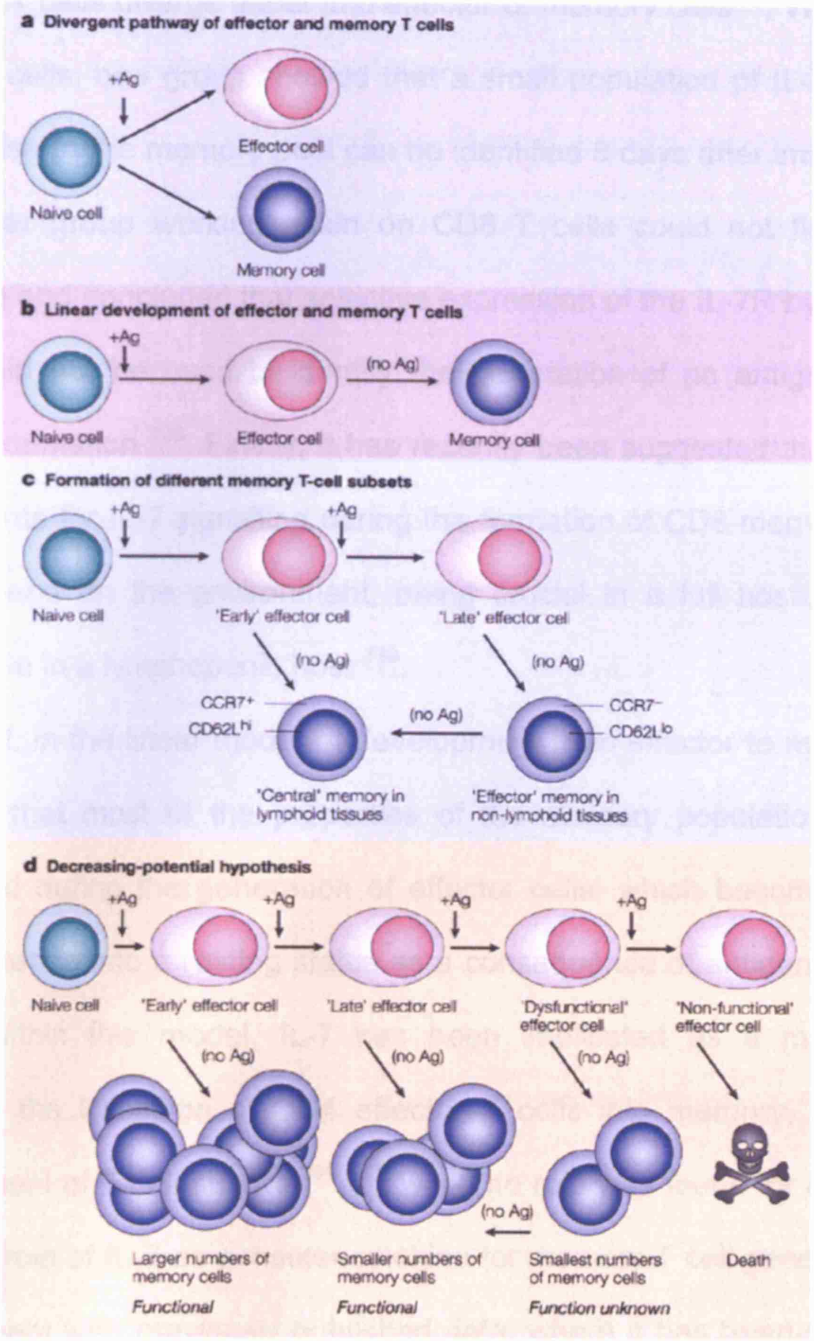



Figure 3: Proposed pathways of memory differentiation.

Upon antigen encounter, naïve T cells differentiate either into effector or memory cells (a); effector cells become resting following antigen clearance (b); the duration of antigen exposure either determines the generation of different subsets of memory T cells (c) or causes a reduction of the quality of the memory T cells generated (d) [69].

In the model for a divergent pathway of effector and memory, after antigen

encounter T cells diverge either into effector or memory cells [72]. With respect to CD8 T cells, one group showed that a small population of IL-7Rα$^{hi}$ cells that give rise to the memory pool can be identified 8 days after immunisation [137]. Another group working again on CD8 T cells could not find such a correlation and concluded that selective expression of the IL-7R by effector T cells should not be used to identify the generation of an antigen specific memory population [138]. Finally, it has recently been suggested that different requirements for IL-7 signalling during the formation of CD8 memory T cells could depend on the environment, being crucial in a full host and being dispensable in a lymphopenic host [139].

In contrast, in the linear model of development from effector to memory it is proposed that most of the properties of the memory population are pre-determined during the generation of effector cells, which become memory just by entering into a resting status as a consequence of antigen clearance [140] [122]. Within this model, IL-7 has been implicated as a major factor promoting the transition of CD4 effector T cells into memory, mainly via enhancement of T cell survival [84] [133], while no role was found for MHC class II [141]. The role of IL-7 as a master cytokine for memory T cell generation is in disagreement with previously published data, where it has been shown that none of the cytokines of the IL-2 family seem to have a relevant effect in memory cells survival [135].

From the linear model of memory development stems the model of formation of different memory T cell subsets and the decreasing-potential hypothesis [69]. In the model of formation of different memory T-cell subsets, a short duration of antigenic stimulation would give rise to 'early effector' cells that would differentiate into central memory cells without requiring more contact

with antigen. Long antigenic stimulation would induce the differentiation of the 'early effector' cells into 'late effector' cells that, in the absence of antigen, could become effector memory cells and ultimately acquire central memory properties [69]. Also for the decreasing potential-hypothesis the length of the exposure to the antigen is the discriminating factor that determines the cell fate, but in this case the prolonged exposure to antigen could gradually reduce the potential of effector cells to generate a functional memory population till the complete loss of such memory forming potential, review in [142]. In line with this hypothesis, it has been reported that in adoptive transfer experiments, the degree of differentiation of Th1 CD4 T cells into effectors inversely correlates with the potential of such cells to develop a resting memory population [143].

## 1.3 T cell signalling

### 1.3.1 The Immunological Synapse

The need for cell-cell interaction during an immune response was first highlighted in delayed hypersensitivity reactions [144] and transplant rejection [145]. Antigen recognition and signalling events that lead to T cell activation take place at the APCs-T cell interface, called the Immunological Synapse (IS) [146] or SupraMolecular Activation Cluster (SMAC) [147]. In the course of an immune response, activated DCs migrate to paracortical areas of secondary lymphoid organs. Here T cells are recruited to form large clusters of T cells interacting with DCs [148]. As shown in Fig. 4, T cells scan the APC surface, docking on the peptide:MHC complex when an antigen is recognised and

adjusting the TCR conformation to the peptide:MHC complex. At this point the signalling cascade is triggered and the initial activation promotes the formation of the IS with the TCR-CD3-CD4 complexes in the centre of the T cell-APC interface. In addition to the cognate peptide:MHC and TCR complexes recruited in this area it has also been shown that an estimated 20% of endogenous peptide:MHC complexes participate to the IS [149] [150]. TCRs are clustered in a central supra-molecular activation cluster facing the peptide:MHC on the APC. This phase is followed by a consolidation phase, during which the CD4 co-receptor moves out of the centre of the IS. In the consolidation phase, the activation signals continue for hours shaping the amount of cytokine secretion and the proliferation of T cells [151].

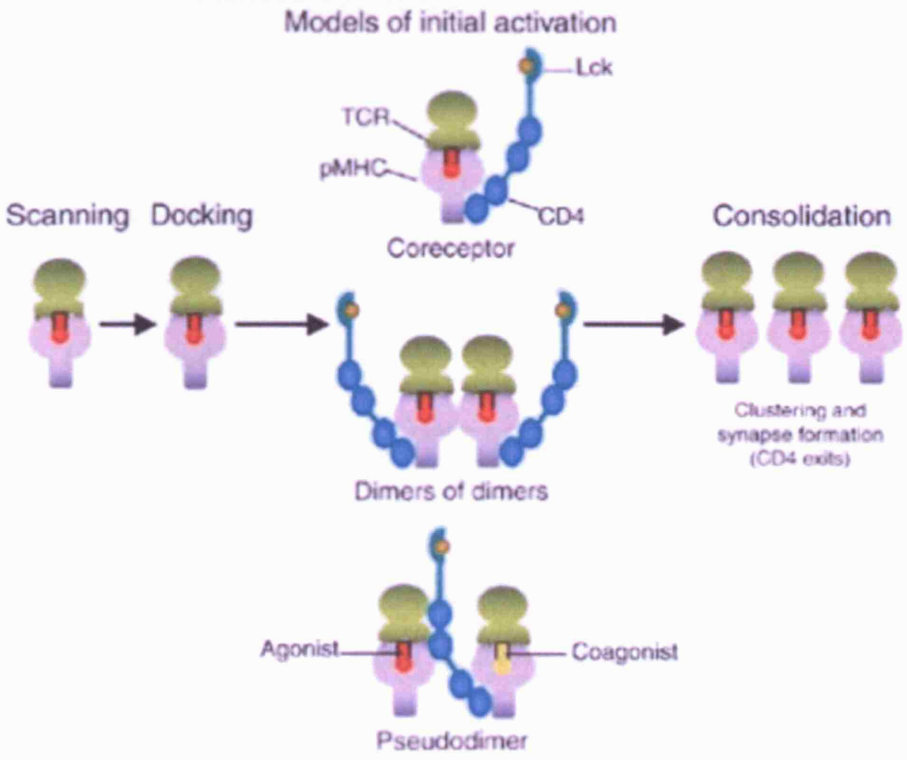



Figure 4: Models of CD4 T cell activation.

CD4 T cells scan the DC surface and dock on cognate:MHC complexes, initiating the activation which promotes the formation of the IS. This process, called consolidation phase, leads to cognate:MHC/TCR clustering and the exclusion of CD4 [151].

Each DC engages a number of T cells via peptide:MHC and TCR. The cognate:MHC and TCR interaction is highly specific although it can have a very low affinity (in the order of micromolar Kd), therefore various molecules are necessary to stabilise and prolong the contact between T cell and APCs. In addition to peptide:MHC interaction with the TCR, co-stimulatory and adhesion molecules participate in the IS, and enable APC and T cells to exchange information through the TCR and through the release of cytokines [152].

### 1.3.2 Co-stimulatory molecules

TCR signalling is enhanced by the interaction with co-stimulatory molecules up-regulated on the activated APC, reviewed in [4].

The two signal hypothesis proposes that T cells need a signal through the TCR (signal one) followed by a second wave of stimulation through soluble or membrane bound molecules, such as cytokines or co-stimulatory molecules (signal two) [153 154].

CD4 T cells rely more than CD8 T cells on co-stimulatory molecules such as CD28, Inducible T-cell COStimulator (ICOS), CD40L and OX40 to provide the second signal, as indicated by impaired activation observed in the responses of CD4 T cells from mice deficient for these molecules [155].

The B7 family and their receptors represent the most important group of proteins necessary for co-stimulatory signals [156 157]. B7-1/B7-2:CD28 is one of the most studied interactions because CD28 engagement allows IL-2 transcription, CD25 expression, cell cycle progression and survival of activated cells through Bcl-$x_L$ [158]. CD28 is constitutively expressed on T cells,

while the expression of CTLA-4, the inhibitory counterpart, is up-regulated after activation. CTLA-4 acts as negative regulator of T cell co-stimulation and has a higher affinity receptor for B7.1/B7.2 than CD28 [157].

ICOS is another co-stimulatory molecule, which is up-regulated on activated cells [159]. Ligation of both CD28 and ICOS can induce expression of CD40L (CD154), a co-stimulatory molecule particularly important for the interaction of CD4 T cells with CD40 expressing B cells.

Moreover, sustained co-stimulation through OX40 provides survival signals to the antigen primed population via Akt signalling as demonstrated by impaired survival observed in activated CD4 T cells from OX40 deficient mice [160].

### 1.3.3 TCR mediated signalling

#### 1.3.3.1 TCR-CD3 complex structure

The TCR is formed of two highly polymorphic heterodimeric chains, the α- and β-chains and the four components of the CD3 invariant chain, CD3γ,CD3δ, CD3ε and ζ which bear Immunoreceptor Tyrosine-based Activation Motifs (ITAM) in the cytoplasmatic domains and are phosphorylated following receptor activation [161] [162] [163] [164] [165]. The assembly of the TCR-CD3 complex takes place in the ER. Invariant subunits of the CD3 heterodimers are assembled in a specific order CD3δε, CD3γε and ζ-ζ. This is followed by the association of the α- and β-chains, which prevents their degradation. Unassembled TCR α- and β-chain are retained in the ER and rapidly degradated following synthesis [166] [167]. The TCR-CD3 complex is

most likely formed of one copy of each of the four dimers (monovalent model) [168] [169], although some have reported that the receptor is composed of 2 copies of the αβTCR (bivalent model) [170].

The TCR-CD3 complex is held together by nine basic/acidic residues present in the TransMembrane (TM) domains. If the TCR chains fail to assemble the basic residues are exposed and promote degradation. Degradation is avoided by dynamic equilibration of the TM domain of a nascent polypeptide between the lipid and the aqueous phases depending on the hydrophobicity of its sequence [171] [172]. The correct assembly of the TCR-CD3 complex is necessary to direct the complex to the cell surface. This information depends on the masking by the ζ chain of a di-leucine motif in the CD3γ cytoplasmatic tail. The di-leucine motif in the CD3γ cytoplasmatic tail also directs the internalisation of the TCR-CD3 complex from the cell surface into early endosomes via interaction binding of Adaptor Proteins-1 (AP-1) and AP-2 which direct clathrin-coated sorting vesicles. The TCR-CD3 complex degradation depends on ubiquitination of the CD3 and ζ cytoplasmatic tails by Casitas B-lineage Lymphoma (Cbl) proteins recruited during TCR signalling [173]. The half-life of assembled TCR complex is ~ 10-12 hours in steady state T cells [174]. In addition to *de novo* synthesis, TCR complexes are dynamically internalised and re-expressed [175]. The synthesis and recycling processes maintain ~ 30.000 TCR complexes on the cell surface of resting T cells.

### 1.3.3.2 Proximal signal transduction

Following TCR triggering by cognate peptide:MHC complex, Lck, a member of the Src-family protein tyrosine kinases, is recruited in the proximity of the CD3-associated ζ chain. Perhaps reflecting the presence of mature T cell populations such as CD8 and CD4, two main models have been proposed to explain the mechanism by which the CD3 invariant chain is phosphorylated by Lck [176].

The heterodimerisation model has been formulated to explain the data obtained from studies on CD8 T cells. In this model, TCR signalling is initiated by the heterodimerisation of the TCR and the co-receptor CD8. As Lck is associated with the co-receptor, the heterodimerisation brings Lck in the proximity of CD3 allowing its phosphorylation [177]. Therefore in this model, the 'activating' unit is represented by one TCR interacting with one antigen loaded MHC class I molecule.

To explain the data on CD4 T cells, the pseudodimer model suggests that the CD3 of the TCR engaged with a single MHC molecule loaded with an antigen could be cross-linked by a CD4 co-receptor from another TCR engaged with an MHC molecule loaded with a self-peptide [150 178].

In addition to the two models proposed for CD8 and CD4 T cell activation, the 'T cell priming by self-peptide', the 'spatial and temporal summation model', and the 'kinase promisquity model' have been proposed. Non-cognate interactions seem to play a role not only in CD4 T cell during activation, as proposed in the pseudodimer model, but also in maintaining naïve CD4 T cell responsiveness to antigen encounter, as suggested by the 'T cell priming by self-peptide' theory [176 179]. In the experiment that inspired the 'T cell priming by self-peptide' model, it was shown that naïve CD4 T cells that loose non-cognate MHC class II interaction by experimental

conditions, such as resting in culture, as well as physiological conditions by travelling into the blood stream, are less responsive than naïve CD4 T cells freshly isolated from secondary lymphoid organs that maintain their interaction with MHC class II [58]. Therefore merging the pseudodimer model and the 'T cell priming by self-peptide' theory, it may be possible to consider that weak signals generated by low affinity ligands, such as endogenous peptides, are necessary to maintain naïve CD4 T cell responsiveness to cognate peptide and can enhance the signal delivered by the cognate peptide during activation. From the observation that the process of T cell commitment to cytokine production and proliferation requires several hours of TCR signalling, the model of 'temporal and spatial summation' has been proposed which states that cells reach full activation by counting the serial engagements of the TCR [180]. In addition, the 'kinase promiscuity' model proposes that co-stimulation through CD28 could biochemically supply additional activated SRC kinases that amplify TCR signalling [154].

The signal transduction pathway downstream of the TCR is tightly regulated by phosphorylation and dephosphorylation reactions carried out by Protein Tyrosine Kinases (PTKs) and Protein Tyrosine Phosphatases (PTPases). Minor changes in the PTK/PTPase balance can have a major impact on tyrosine phosphorylation and thereby on the activation and proliferation of T cells [181]. For example, Lck activation is positively regulated by CD45 and negatively regulated by COOH-terminal Scr kinase (CSK). Positive regulation by CD45 is achieved by the dephosphorylation of the COOH-terminal tyrosine, while the phosphorylation of this tyrosine by CSK keeps Lck in an inactive conformation. The over-expression of CSK in T cells

causes reduced TCR-induced tyrosine phosphorylation and IL-2 production [182].

Lck phosphorylates the ITAMs on the ζ chain of the TCR which, once phosphorylated, can function as docking sites for the tandem Src Homology 2 (SH2) domains of the PTK Zeta-chain (TCR) Associated Protein Kinase 70 kDa
(ZAP-70). The important role of Lck during activation is demonstrated by Lck Knocked Out (ko) cell lines as well as primary T cells from mice lacking Lck. In this condition T cells fail to become activated as indicated by inhibition of calcium flux, reduced up-regulation of activation markers, reduced proliferation and IL-2 production [183]. Paradoxically, in human CD4 T cells, it has been reported that memory T cells show decreased levels of phosphorylated ZAP-70 and downstream molecules compared to naïve T cells, perhaps suggesting that memory T cells have developed a short-cut where fewer biochemical events are required to trigger activation [184]

### 1.3.3.3 Distal signal transduction

The phosphorylation of ZAP-70 propagates the signal through linker/adapter molecules like Linker for Activation of T cells (LAT) [185] towards second messengers such as Mitogen-Activated Protein Kinase (MAPK) [186], Vav [187] and PhosphoLipase Cγ-1 (PLCγ-1)[187], which activates Protein Kinase C (PKC), leading to cell proliferation and differentiation, reviewed in [188].
TCR engagement also results in the phosphorylation of the T cell Receptor Interacting Molecule (TRIM) [189] which recruits Phosphatidyl-Inositol-3-Kinase (PI3K) to the TCR [190].

Distal signalling pathways can be initiated by linker/adapter molecules and PI3K such as Erk1/2 MAP kinases, which are activated via the Ras pathway [191] [192], and the c-Jun $NH_2$-terminal Kinase (JNK), which are activated via the Rac pathway [193] [194]. In mammals, three members of the JNK have so far been identified. JNK 3 is expressed only in the brain, testis and heart. Both JNK1 and JNK2 have been implicated in Th1/Th2 differentiation [195]. In addition, the disruption of the genes *Mapk8* and *Mapk9*, the genes coding for JNK1 and JNK2 respectively, have suggested that deregulation of these two kinases may play a role in development of autoimmunity [196] [197]. JNK1 and JNK2 are ubiquitously expressed proteins whose function is regulated by phosphorylation. The observation that the level of expression of these molecules is undetectable in naïve CD4 T cells has suggested the possibility that a transcriptional regulation of JNK expression could represent a novel mechanism to control JNK activity in addition to phosphorylation [198]. Both Erk1/2 and the JNK pathways lead to IL-2 production. In addition, p38 MAP kinases are activated by small Guanosine DiPhosphate (GDP)-binding proteins [199].

### 1.3.3.4 PI3K and Akt pathway

As mentioned above, antigen recognition through the TCR results in the activation of the PI3K regulated pathway. The regulation of cell growth, differentiation, survival, proliferation and metabolism through this pathway is evolutionary conserved throughout organisms and cell types [200].

The PI3K family is divided into several classes depending on their structural characteristics. In particular the members of PI3K Class Ia are involved in

signalling by antigen and co-stimulatory receptors as well as cytokine receptors via tyrosine-kinase-associated receptors. Four isoforms belong to class IA and they are formed by a catalytic subunit (p110α, β, γ, δ) and a regulatory subunit (p85 α, β and p55 γ), reviewed in [201]. Functionally, PI3K is activated by receptors with intrinsic or associated tyrosine kinase activity therefore this pathway can be initiated by phosphorylation of cytokine receptors (as discussed in the next section) as well as by kinase activities downstream the TCR signalling.

It is not completely clear how the signal through the MHC:TCR complex is propagated to reach PI3K [190], but it is likely that adaptor molecules could transduce the signal. The importance of this pathway has been shown in T cells from transgenic mice. Cells from mice deficient in the regulatory subunit p85 of the PI3K have been shown to have impaired proliferation and survival [201]. The catalytic subunit p110δ is expressed only in T cells and mice carrying a mutation in this subunit exhibit a higher proportion of cells with a naïve phenotype compared to wild type mice, reduced proliferation as well as reduced IL-2 production in response to anti-CD3 stimulation. Interestingly, full recovery of function was achieved when cells were activated in the presence of anti-CD28, indicating that the needs for PI3K activation during T cell stimulation may be dependent on the strength of the stimulus [202]. PI3K I catalyses the enzymatic reaction to convert Phosphatidyl-Inositol 4,5-bisphosphate (PtdInsP2) to PtdInsP3 and its action is inhibited by Phosphatase and Tensin homologue (PTEN) [76]. PtdInsP3 acts as binding site for numerous intracellular enzymes; one of the most important is Akt/PKB [203].

By using different fluorescent proteins associated with the CD3ζ chain and the Pleckstrin Homology (PH) domain of Protein Kinase B or cellular homology to the retroviral oncogene viral Akt (PKB or AKT) to monitor early signalling activity and TCR-derived signal respectively, it was possible to establish the causal relationship between TCR engagement and PI3K activation [204]. Thanks to the technology used in these experiments, it has been possible to follow in detail the kinetic of APC T cell interaction that leads to T cell activation. Following the early burst in signalling, 70% of the TCR was internalised within 30 minutes. Despite TCR internalisation, PI3K activity was sustained up to 10 hours, but ceased within 2 minutes from the addition of monoclonal antibodies to block MHC:TCR interaction. Lower levels of IL-2 production were observed when the blocking antibody was added early during activation, corroborating the notion that acquisition of optimal effector function requires prolonged T cell:APC contact. In line with the requirement for sustained PI3K/Akt signalling for full T cell activation and differentiation another study showed that active signalling through Akt is crucial for commitment to long term survival and proliferation of activated T cells [160].

Studies carried out using the PI3K pharmacological inhibitor LY294002 have shown that the Akt and the ribosomal Single 6 Kinase 1 (S6K1), two serine kinases with a crucial role during T cell activation, are phosphorylated upon TCR engagement in a PI3K dependent manner [205].

Akt is a serine/threonine protein kinase with three highly homologous members found in mammals: PKBα/Akt1, PKBβ/Akt2, PKBγ/Akt3 [206]. Akt is a well established downstream molecule of PI3K and it is often used as the surrogate read-out of PI3K activity [207].

Akt has been demonstrated to have an important role in the signal transduction pathway in response to growth factors and insulin and to play a role in metabolism, cell growth, transcriptional regulation and cell survival [206]. Growth factors withdrawal result in a catabolic state characterised by atrophy and decline of mitochondrion potential due to loss of surface transporters for glucose, amino acids, low-density lipoprotein and iron. It has been demonstrated that a constitutively active form of Akt can prevent the down-regulation of glucose, amino acids and iron transporters in a mammalian Target Of Rapamycin (mTOR)-dependent manner [208]. TOR was first identified in yeast where it controls cell growth in response to nutrients availability. The molecules upstream of TOR are not yet known in the yeast, but in mammalian cells there are indications that PI3K and Akt are the upstream molecules that regulated mTOR [209]. mTOR has various downstream of molecules such as STAT3 and S6K [207].

The role of S6K is related to cell metabolism as it concerns protein synthesis, cell size and glucose homeostasis [210]. There are two isoforms for S6K, S6K1 and S6K2. S6K1 ko and S6K1 ko / S6K2 ko mice are smaller and have a mild glucose intolerance [211] [212]. In T cells, the phosphoryation of S6K1 occurs within 10 minutes of TCR triggering and it is essential to allow small, metabolically inactive T cells to increase protein synthesis and reach the size required to perform optimal effector function and to proliferate [205]. Once phosphorylated, S6K activates the small ribosomal protein S6, one of the members of the Pre-Initiation Complex (PIC) [213] [214]. Although S6 seem to be mainly phosphorylated by S6K an alternative activating pathway catalysed by a MAPK-dependent kinase has been described in S6K1 ko / S6K2 ko cells [215].

Another important role of Akt in T cell activation is to inactivate the Glycogen Synthase Kinase-3 (GSK-3) by phosphorylation. The inactivation of this enzyme allows the nuclear retention of the Nuclear Factor of Activated T cells (NFAT), which promotes cytokine production [216]. Akt has also been demonstrated to promote glucose uptake upon activation [217]. Glucose metabolism is very important not only in maintaining cellular ATP levels necessary for cell survival but also for cell growth and activation [218]. Resting lymphocytes do not have large internal glycogen stores, therefore they have to up-regulate the expression of transporters to import glucose into the cell, when the demand arises. T cell expression of *m*RNA coding for the Glucose transporter 1 (Glut1), the predominant glucose transporter in T cells, has been demonstrated to be increased by 6 hours following TCR mediated stimulation *in vitro* [219]. The level of expression of Glut1 is synergistically enhanced by engagement of TCR and CD28 [218].

During the resting state PI3K and Akt pathway may control T cell quiescence by targeting the members of the FOrkhead boX O (FOXO) family that regulate cell cycle arrest at the transcriptional level [220]. The PI3K/Akt/FOXO signalling module appears to be highly conserved during evolution [201]. In activated lymphocytes FOXO nuclear exclusion following its phosphorylation by Akt is crucial to allow cells progression into cell cycle [207].

It is important to underline that most of the effects described above are obtained also through cytokine signalling and that different stimuli can result in overlapping responses. For example, during T cell activation the succession of temporal events would point to the TCR signalling to initiate cell growth, protein synthesis and the metabolic changes that could then be sustained by IL-2 signalling during later stages of the antigenic response.

### 1.3.4 Cytokines

Cytokines are low molecular weight proteins that are made by one cell and act on another cell [221] and play a critical role in T cell development, proliferation, activation and survival. Cytokines transmit intracellular signals in autocrine fashion (one cell produces it and responds to it) and/or paracrine fashion (one cell produces it, but it acts on another). Cytokines are distinguished into two major groups, type I and type II, on the basis of their structure. Type I cytokines are defined by a four α-helical bundle structure and by the fact that they signal through the type I cytokine receptors. IL-2, IL-3, IL-4, IL-5, IL6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) together with other molecules such as growth hormone, prolactin, leptin, thrombopoietin belong to type I cytokines [222, 223, 224]. Type II cytokines include IFN-α, IFN-β, IFN-γ and IL10.

#### 1.3.4.1 Common γ-chain

Type I cytokines are divided into long chain cytokines, which have helices of 25 amino acids, and short chain cytokines, which have helices of 15 amino acids. An important group of cytokines belonging to the type I short chain is the 'IL-2 family' of cytokines, reviewed in [223]. IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are all members of the IL-2 family. The receptors for these cytokines are formed by the common γ chain, which is shared by all of them, and one or two distinctive chains. Mutations in the common γ chain result in Severe Combined ImmunoDeficiency (SCID). The most common form of SCID is

caused by a mutation in the chromosome X, and therefore it is called X-linked SCID (XSCID). Humans and mice affected by this condition have defects in the generation or function of T, B and NK cells and generally die of infection in the early stages of life [225], unless treated with bone marrow transplantition or gene therapy. Signalling through the receptor of the IL-2 family activates the Jak/STAT, PI3K/AKT and Ras-MAPK pathways [225]. This section will concentrate on the Jak/STAT pathway and on the parts of the PI3K/AKT and Ras-MAPK pathways that have not been treated in the discussion of the TCR signalling.

### 1.3.4.2 Jak/STAT

Janus Kinase (Jak) 1 is constitutively associated with the IL-2Rβ and IL-7Rα chains of the IL-2 and IL-7 receptors respectively, while Jak3 is constitutively associated with the common γ chain [225]. With the ligation of IL-2 or IL-7, the chains that form the cytokine receptors are brought together. Due to this close proximity, Jak1 and Jak3 increase their enzymatic activity (through cross-phosphorylation) and phosphorylate the tyrosine residues in the intracellular domain of the cytokine receptors creating a docking site for STAT5. The binding of STAT5 to these sites followed by its phosphorylation by Jak1/Jack 3 allows STAT5 dimerisation, its translocation into the nucleus and DNA binding to target genes, such as cyclins D1, D2, p21WAF/Cip1 and anti-apoptotic molecules such as Bcl-2 and Bcl-$x_L$ [226]. Although STAT5 phosphorylation is mainly described as the outcome of cytokine ligation [227], one report suggested that also TCR signalling can induce STAT5 phosphorylation [228]. There are two STAT5 isoforms: STAT5a and STAT5b.

Due to the high degree of homology, single knocked out mice do not show a clear impairment in lymphoid development and T cell activation, but the disruption of the genes for both isoforms has highlighted that STAT5a and STAT5b play a key role in these processes [229, 230].

In addition to STAT5, other adaptor signalling proteins containing the SH2 domain such as Shc, Growth factor Receptor-Bound protein 2 (Grb2) and Cbl, can dock on the cytoplasmatic tail of the cytokine receptors and transduce the signal to diverse pathways [231].

#### 1.3.4.3 PI3K/Akt

There are indications that cytokine signalling is propagated to the PI3K/Akt pathway via the recruitment of the catalytic subunit p110 to the cytoplasmatic tail of the cytokine receptor via high affinity binding of the SH2 domain of the regulatory subunit p85 to the receptor [232 233]. Alternatively, engagement of the cytokines receptor could result in the activation of PI3K via the Jak3 phosphorylation. Jak3 has been demonstrated to associate with the regulatory subunit p85 of PI3K, inducing its activation [234]. Therefore cytokine signalling could result in the activation of PI3K via multiple pathways.

#### 1.3.4.4 Ras-MAPK

The activation of the Ras/MAP pathway seems to be elicited by only some members of the IL-2 family such as IL-2 and IL-15 [235 236]. Adapter molecules such as Shc, Grb2, and SHP-2 have been shown to link the cytokine receptors to Ras, and therefore to activate the Raf-MEK-MAP kinase

pathway [237], that culminate with the activation of transcription factors such as c-myc or c-fos in the nucleus [238]

### 1.3.4.5 Role of IL-2 and IL-7 in T cell signalling

IL-2 and IL-7 are two very important cytokines during T cell activation and for T cell survival and for this reason they are going to be discussed here.
The IL-2 receptor is formed of three chains: IL-2Rα (CD25), IL-2Rβ (CD122) and the γ chain (CD132) [239]; IL-7R is composed of IL-7Rα chain (CD127) and the γ chain (CD132).

#### IL-2

IL-2 is a growth factor [240] produced mainly by CD4 activated T cells and to a lesser extent by activated CD8 T cells. The simultaneous engagement of TCR and co-stimulatory molecules on T cells promotes the synthesis and secretion of IL-2 and the expression of IL-2R. Optimal TCR stimulation in the presence of un-limiting antigen and APC availability induces biallelic expression of the IL-2 genes, while in suboptimal conditions the expression is monoallelic, with fewer cells expressing IL-2 gene [241]. Therefore, the gradient of TCR successful engagment correlates with the gradient of IL-2 production and shapes the immune response in the periphery.
The IL-2 promoter is regulated by 3 factors: Activating Protein-1 (AP-1), Nuclear Factor of Activated T cells (NF-AT) and Nuclear Factor – kappa B (NF-kB), which associate with c-Rel promoted by CD28 and which are necessary for continuous expression of the IL-2 gene. The transcriptional

program driven by IL-2 enables T cells to enter into the cell cycle and to proliferate [242]. Addition of recombinant IL-2 to *in vitro* activated T cells increases their rate of proliferation of ~1000 fold [243]. Surprisingly, mice deficient of IL-2 or of its receptor do not show a major impairment in T cell clonal expansion upon antigen challenge questioning the role of IL-2 as master of T cells proliferation *in vivo* [130]. A recent study that may offer an explanation to this observation has shown that clonal expansion can be sustained by prolonged CD3 and CD28 stimulation, bypassing the need for IL-2 [244]. Alternatively, other factors could be responsible for CD4 T cell proliferation. IL-2 has been implicated in sensitising activated T cells to AICD by preventing the activation of Fas-associated death domain-like IL-1 Converting Enzyme (FLICE), a protein that inhibits Fas signalling [245]. Mice deficient of IL-2 develop autoimmunity due to the lack of functional Tregs. Tregs do not need IL-2 for their generation in the thymus, but they depend on IL-2 for their maintenance in the periphery [246]. For this reason IL-2 is considered a non redundant cytokine with an essential role in the maintenance of T cell tolerance [243] as well as activation of NK cells, B cells, cytotoxic T cells and macrophages [238].

**IL-7**

IL-7 is secreted by various cell types, such as thymic stromal cells [247] [248] and bone marrow [249]. IL-7 is essential for T and B cell development [250]. In mature T cells, IL-7 plays a role in cell survival, proliferation, and metabolism [251]. IL-7 signalling has been demonstrated to regulate various members of the Bcl-2 family to promote T cell survival. In fact, IL-7 induces *Bcl-2 m*RNA synthesis

as well as Bax phosphorylation and Bad cytoplasmatic retention [252]. All these events account for T cell survival because they maintain intact the function of the mitochondrion [75].

IL-7 maintains metabolism through activation of PI3K which phosphorylates Akt [253] [206]. The proposed binding site for PI3K to the IL-7R is the Tyr449 on the cytoplasmatic tail of the α-chain of the receptor [251].

IL-7 also maintains glucose metabolism [219] [254]. In one study, naïve peripheral T cells were cultured *in vitro* in the presence or absence of IL-7 and the effect on survival, cell size and metabolism were assessed. As far as survival was concerned, lack of IL-7 signalling did not show to have an immediate effect on the viability of cultured cells as assessed by Bcl-2 and Bax expression, but cells cultured in the absence of IL-7 showed a decrease in size and eventually died in a caspase-independent manner. Neglect and atrophy affected T cell physiology and delayed response to mitogenic stimuli. Although addition of IL-7 restored cell size, this did not recover function of neglected cells as measured by anti-CD3 and anti-CD28 induced proliferation. It is possible that neglected cells need additional growth requirements before they can proliferate and acquire effector functions [255].

### 1.4 T cell homeostasis

The term 'homoestasis' was introduced by W. Cannon to describe the tendency of an organism to restore its original status in the face of unexpected disturbances, and homeostasis is now a concept which is considered of major importance in immunology, as infection and lymphopenia are disturbances that often challenge the original composition

of the immune system and to which the immune system respond in order to restore its original status [256].

In the periphery, the number of cells of the immune system remains constant although perturbations of various nature occur all the time. For example, in the mouse the thymus exports ~ 1 x $10^6$ T cells per day [257]. As the number of T cells remains constant, it is logical to conclude that an equal number of T cells die daily in the periphery. A major and relatively frequent perturbance is represented by the response of the immune system to pathogens. As during the clonal expansion of the effector phase antigen responding T cells proliferate extensively - up to five-fold in CD8 T cells during infection [256] - homeostatic control of such proliferation has to guarantee that the numbers of peripheral T cells regain a sustainable size.

This is achieved through T cell competition for space. T cells are aware of the space available for their survival and expansion and are able to adapt to it. As a consequence of their sense for space, T cells proliferate when transferred into the 'empty' space represented by a lymphopenic animal, where survival factors are largely available and there are not other competitor cells, while in a 'full' host, competition with other cells for survival factors does not allow an expansion of the transferred cells [121].

Different pools of cells perceive the space in different ways. In fact, naïve and memory T cells are thought to occupy different 'niches' on the basis of their requirements for survival. The two main hallmarks that define the space are cytokines and MHC availability [256]. For naïve T cells, signals from both these components, self peptide/MHC complexes on the surface of APC and the cytokine IL-7, are important for their survival [50 53]. While naïve CD4 and CD8 cells do not compete for the same MHC molecules, they do compete for

the same cytokine and thus occupy the same 'niche'. An 'altruistic' mechanism has been proposed to explain how the largest number of naïve T cells could share the limited amount of IL-7 available [258]. According to this study, the transcription of IL-7Rα would be down-regulated after cytokine ligation. In this way, signalled cells will not compete with cells that have not received the cytokine mediated survival signal.

Homeostatic maintenance of memory T cells is thought to depend on cytokines. CD8 memory T cells are positively regulated by IL-15, but it is still to be clearly determined which is the cytokine fulfilling this role for CD4 memory T cells. IL-7 has been proposed to be the survival factor for CD4 memory T cells, but the joint usage by naïve and memory CD4 T cells, as well as naïve and memory CD8 T cells [60] seems to indicate in IL-7 a general lymphocyte survival factor more than the specific factor responsible for maintaining CD4 memory T cells. IL-7 as possible sole regulator of the naïve and memory T cells compartments is not compatible with the idea that different pools occupy different 'niches' and are therefore regulated independently.

The CD4/CD8 ratio in peripheral T cells is maintained constantly at about 2/1, even though CD8 T cells expand to a greater extent in the presence of antigen than CD4 T cells. Since activated CD4 T cells can amplify the immune response due to their strategic helper function that induces expansion of B cells, CD8 T cells or macrophages, it is conceivable that smaller numbers suffice for their function. Possibly due to the vigorous expansion following activation, memory CD8 T cells are more subjected to 'attrition' than CD4 T cells. 'Attrition' can be defined as the decrease in frequency of memory T cell specific clones for one antigen due to

competition with newly generated memory T cells [259]. On the other hand, attrition could be compensated by the higher plasticity shown by CD8 T cells in response to a diverse range of pathogens [121].

### 1.5 The role of MHC class II in maintaining immunological memory

A series of experiments was previously carried out in our lab to analyse the role of MHC class II molecules in the maintenance of the quality of memory CD4 T cells. A summary of the main findings is reported here, as that work is at the basis of the research developed in this project. To analyse the role of MHC class II in maintaining memory CD4 T cells, H-Y specific memory CD4 T cells were generated by transferring naïve CD4 T cells isolated from lymph nodes of transgenic TCR A1 Rag1 ko female mice and H-Y peptide pulsed syngeneic DCs into adoptive hosts expressing either an allogeneic MHC class II or lacking MHC class II molecules. In both systems, antigen presentation depended exclusively on the syngeneic DCs co-injected with A1 naïve CD4 T cells, which have been shown to disappear within 3 weeks of transfer [80]. In the absence of antigenic stimulation CD4 T cells became resting memory cells. The experiments, performed on memory CD4 T cells recovered 6 weeks after transfer, confirmed that memory CD4 T cells survive in the absence of MHC class II because similar number of cells were recovered from both hosts, although in MHC class II deficient hosts memory T cells were shown to divide at a higher rate *in vivo*. Further tests showed that memory CD4 T cells surviving in the absence of MHC class II signal had compromised function. Comparable levels of IL-2 production were found in response to optimal stimuli such as peptide pulsed DCs or anti-CD3 and

anti-CD28, but stimulation with only anti-CD3 failed to fully activate memory T cells from MHC II deficient hosts indicating a reduced signalling capability in the absence of co-stimulation in memory CD4 T cells generated in MHC class II deficient hosts. The difference was further confirmed using non-professional APC such as naïve B cells. Naïve B cells can act as APC for memory T cells, while naïve T cells do not respond to such activation. Memory CD4 T cell proliferation, IL-2 production and MHC class II up-regulation on B cells as read-out of T cell activation indicated that cells recovered from MHC class II deficient hosts were functionally impaired. The loss of function in memory cells surviving in MHC class II deficient hosts was observed also in *in vivo* experiments where only cells from MHC class II competent hosts were able to expand and help B cells for the production of class-switched antibody. Finally, memory CD4 T cells generated in MHC class II competent or deficient hosts were secondarily transferred into MHC class II competent recipients, which were grafted with syngeneic skin grafts from male and female mice 24 hours before receiving the memory CD4 T cells. In this case graft rejection depended on recognition of H-Y antigen expressed in the male skin graft presented by the donor DCs. While recipients that received memory CD4 T cells from MHC class II competent hosts rejected the male skin graft by day 14, hosts that received memory CD4 T cells from MHC class II deficient hosts were unable to reject the male graft. The differences in memory cells described here indicate that MHC class II signals are important for memory CD4 T cells to maintain effective immunocompetence.

### 1.6 Aim of the project

The aim of the project was to define the underlying differences between memory cells maintained in the presence or absence of MHC class II contact at the molecular level and, using the information obtained at the molecular level, identify a potential mechanism to explain how MHC class II molecules preserve memory CD4 T cell function. To achieve this goal, the gene expression profile of resting memory CD4 T cells generated in MHC class II competent and deficient hosts were analysed using microarray analysis.

The study then focused on the verification of the involvement of the candidate molecules identified by gene expression analysis in the process of memory generation using FACS analysis, *in vitro* and *in vivo* experiments.

By means of FACS analyses and *in vitro* experiment aiming to activate CD4 T cells recovered from MHC class II competent or deficient hosts, it has been established that the defect in functionality of CD4 T cells is an event that occurs in the early phases of the memory generation.

On the other hand, the *in vivo* experiments aimed to rescue the functionality of memory CD4 T cells generated in MHC class II deficient hosts making use of bone marrow chimeric hosts as well as providing MHC class II contact by continuous injection of cells expressing non-cognate MHC class II molecules, such as DCs. These experiments have suggested that the contact with non-cognate MHC class II molecules is required by effector cells to completely acquire effector function and to be able to differentiate into functional memory cells.

## 2. MATERIALS AND METHODS

### 2.1 Mice

The following mouse strains were used in this thesis: as donor of naïve CD4 T cells were used either A1 Rag1 ko (H-$2^k$) [260] or A1 Ly5.1 Rag1 ko (H-$2^k$) mice, which are specific for a peptide from the *Dby*-encoded H-Y epitope antigen [261]; allogeneic adoptive hosts were either Rag2 ko Il-2 rγ ko (H-$2^b$), MHC class II competent hosts, or Rag2 ko Il-2 rγ ko I-$A^\beta$ ko (H-$2^b$), MHC class II deficient hosts. For the purpose of simplification, from now on MHC class II competent hosts will be referred to as MHC+ hosts and MHC class II deficient hosts will be referred to as MHC- hosts. CBA (H-$2^k$) were used to generate bone marrow DCs and RAG1 ko B6 (H-$2^b$), RAG1 ko (H-$2^k$), and TCR α ko (H-$2^b$) as donors for bone marrow transplants in the chimera experiments. In appendix 1 are shown the mice used in the thesis.

All mice were bred in Specified Pathogen Free (SPF) conditions. Experiments were conducted in conventional, but pathogen-free, animal facilities at the National Institute for Medical Research (London, U.K.). All mouse experiments were done according to institutional guidelines and Home Office regulations.

#### 2.1.1 Generation of bone marrow chimeras

Chimeric animals were generated by intra-venous (i.v.) injection of 10 x $10^6$ bone marrow donor cells per mouse into MHC+ and MHC- hosts. Rag1 ko (H-$2^k$) bone marrow cells were injected into MHC+ hosts, referred to as Rag1

ko → MHC+, and MHC- hosts, referred to as Rag1 ko → MHC-, 28 days prior to co-transfer of A1 naïve CD4 T cells and H-Y peptide pulsed DCs. Rag1 ko B6 (H-$2^b$) bone marrow cells were injected into irradiated MHC+ hosts, referred to as Rag1 ko → MHC+, and irradiated MHC- hosts, referred to as Rag1 ko → MHC-, 30 days prior to co-transfer of A1 naïve CD4 T cells and H-Y peptide pulsed DCs. The adoptive hosts were sub-lethally irradiated at 800 rads and let to rest for 8 hours before the bone marrow transplant. TCR α ko (H-$2^b$) bone marrow cells were injected into MHC+ hosts, referred to as α ko → MHC+, and MHC- hosts, referred to as α ko → MHC-, 4 days after co-transfer of A1 naïve CD4 T cells and H-Y peptide pulsed DCs.

Table 1. Bone marrow chimeras used in this study.

| Chimera | Abbreviation | Irradiation | Day of transfer |
|---|---|---|---|
| Rag1 ko (H-$2^k$) | Rag1 ko → MHC+ or MHC- | no | - 28 |
| Rag1 ko B6 (H-$2^b$) | Rag1 ko → MHC+ or MHC- | 800 rads | - 30 |
| TCR α ko (H-$2^b$) | α ko → MHC+ orMHC- | no | +4 |

## 2.2 Culture media

The culture medium used was Iscove's Modified Dulbecco's Medium (IMDM, Gibco BRL) supplemented with 5% heat inactivated Fetal Calf Serum (FCS,

Gibco BRL), 2 x $10^{-3}$ M L-glutamine, 100 U/ml penicillin, 100 µg/ ml streptomycin and 5 x $10^{-5}$ M β-mercaptoethanol (all Sigma).

Medium for washing cells was Air Buffered IMDM (AB medium) containing 25 mM HEPES and L-glutamine and supplemented with 0.21% NaCl, 100 U/ml penicillin, 100 µg/ ml streptomycin and 12.5 mM NaOH.

**2.3 Single cell suspension**

Spleens were harvested and gently pressed through a 70 µm strainer (BD Falcon™) using AB medium for resuspension. Alternatively, spleens were infused with 2 ml of Liberase CI Purified Enzyme Blend (Roche) at 0.4 mg/ml final concentration in AB medium and incubated at 37°C for 30 minutes. Reaction was stopped by adding 5 ml FCS 5%. Cells were washed once, and erythrocytes were removed by resuspending in 1 ml/spleen Red Blood Cell (RBC) lysis buffer (0.15 $NH_4Cl$, 1mM $KHCO_3$, 0.1 mM EDTA, pH 7.2-7.4) for 5 minutes at Room Temperature (RT). Cells were washed, clumps were removed by passing the cells through a 70 µm strainer and viable cells were counted.

**2.4 Determination of cell viability and numbers**

Trypan blue (Sigma) at a final concentration of 0.08% in Phosphate Buffered Saline (PBS) (10.1 g NaCl, 0.362 g KCl, 0.362 g $KH_2PO_4$, 1.449 g $Na_2HPO_4$ in 1 l $H_2O$) was used to determine the viability of cells. Cells were counted in a 1:1 mixture of Trypan blue using a Neubauer counting chamber (BDH Ltd., UK) under the light microscope. Dead cells, stained blue, were excluded.

## 2.5 Generation of Bone Marrow derived DC

As in [262], femurs and tibia from female donors were flushed with culture medium, and 3 x $10^6$ bone marrow cells were cultured for 7-8 days at 37° C in 9 cm Petri-dishes (NUNC, Denmark) in 10 ml culture medium containing 10% supernatant of Ag 8653 myeloma cells transfected with murine Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) *c*DNA (25 U/ml).

## 2.6 *In vitro* T cell activation

Anti-CD3 antibody (clone 145-2C11) and 10 µg/ml of plate-bound anti-CD28 antibody (clone 37-51) were coated onto U-bottom 96-well plates (Becton Dickinson) in PBS by overnight incubation at 4°C. 1 x $10^4$/well memory CD4 T cells were stimulated with the indicated concentrations of plate-bound anti-CD3 alone or in the presence of 10 µg/ml of plate-bound anti-CD28. IL-2 production was assessed by a CTLL-2 assay at day 2.

## 2.7 B cell depletion

Splenocytes were labelled with anti-B220 (RA3-6B2) biotinylated antibody at 1 x $10^7$/ml total cells. Cells were washed in PBS and 10 µl of Dynalbeads® M-280 streptavidin (Dynal) were added per 1 x $10^7$ cells and incubated for 30 minutes at 4°C on a shaker. B cells were depleted using magnets (Dynal).

## 2.8 CTLL-2 Assay

50 µl of supernatant from *in vitro* activated T cells were transferred to fresh flat-bottom 96-well plates together with 5 x $10^3$/well IL-2-dependent CTLL cells (ATCC cat No TIB 214) to assess IL-2 production. An AlamarBlue™ (Biosource) assay was used to measure cell proliferation based on detection of metabolic activity [263]. The system incorporates an oxidation-reduction indicator that changes in colour in response to chemical reduction of growth medium resulting from cell proliferation. Data were collected by monitoring fluorescence at 590 nm using a Luminescence Spectrometer LS50B (Perkin Elmer Life Sciences, UK) and analysed with FL WinLab software (Perkin Elmer). Results were calculated from a standard curve of human recombinant IL-2 (Sigma) to give units of IL-2/ml produced. One unit of IL-2 biological activity is defined as the value at which 50% of maximum incorporation of [$^3$H]-tritiate-thymidine is achieved by CTLL cells in a period of 24 h [264].

**2.9 FACS analysis and cell sorting**

Cell suspensions were pre-incubated at a concentration of 2 x $10^6$ cells/ml on ice for 30 minutes with unlabelled monoclonal Antibody (mAb) to FcγRII/III to minimise unspecific staining. Stainings were performed with fluorescein isothiocynate- (FITC-), phycoerythrine- (PE-), Peridinin chlorophyll protein- (PerCP-) and PE-Texas Red, allophycocyanin- (APC-) or biotin (Bio)-conjugated mAbs, followed by a second staining with streptadivin APC or FITC. All stainings were performed on ice and cells were washed

with FACS washing buffer (PBS, 2% FCS, 0.1% azide) in between staining. Cells were stained for isotype control whenever possible.

For determination of intracellular proteins, cells were fixed on ice in 100 µl 3% paraformaldehyde in PBS for 15 mins, and permeabilised with 0.1% Nonidet-P40 (NP40) in PBS for 3 mins, followed by staining with specific antibodies.

For determination of intracellular IL-2 production, cells were stimulated with 50 ng/ml 4-beta-phorbol-12,13-dibutyrate (PdBU) (Sigma), 50 ng/ml ionomycin (Sigma), and 10 µg/ml Brefeldin A (Sigma, Poole, UK) for 4 h at 37°C in the presence of labelling anti-CD4 (GK1.5) and anti-TCR antibodies (H57-597). For determination of intracellular levels of phosphorylated S6, cells were stimulated with 50 ng/ml PdBU (Sigma), 50 ng/ml ionomycin (Sigma) or with anti-CD3 (2C11) for 30 minutes at 37°C in the presence of labelling anti-CD4 (GK1.5) and anti-TCR antibodies (H57-597). Cells were washed, fixed and permeabilised on ice before staininig them with the specific antibody.

Analytical flow cytometry was conducted using a FACSCalibur (Becton Dikinson), and the data were processed using FlowJo software (Tree Star Inc.). Cell sorting was done on a MoFlo cell sorter (Cytomation, Fort Collins, CO).

### 2.9.1 Antibodies

All antibodies were purchased from e-Bioscience with the exception of PeTexasRed anti-CD4 from Caltag Laboratories (Burlingame, Ca); biotin anti-mouse H-2K$^k$, PE anti-IL-2, PE anti-DO.11.10, Bio anti-CD19 from BD

Pharmingen™; Phospho-S6 ribosomal protein (Ser235/236), Phospho-Stat5 (Tyr694), and Phospho-Akt1 (Ser473) primary antibodies from Cell Signaling Technology, FITC labelled goat anti-rabbit Ig from BD Pharmingen™ as secondary antibody; Streptadivin, allophycocyanin, crosslinked, conjugated from Molecular probes, Invitrogen. FcγIII/II R, MHC II I-E $^{k,d}$, MHC II I-A/I-E were purified and labelled here.

Table 2. Clone name, specificity and labelling of the Abs used in this study.

| **Clone name** | **Specificity** | **Conjugated** |
|---|---|---|
| 14.4.4 | anti-MHC II I-E $^{k,d}$ | FITC |
| A19-3 | anti-Bcl-2 isotype (Ar Ham IgG1, $_{k}$) | FITC |
| 3F11 | anti-Bcl-2 | FITC |
| polyclonal (goat anti-serum) | Goat anti-rabbit Ig | FITC |
| KJ1-26 | anti-DO11.10 | PE |
| A7R34 | anti-IL-7Rα | PE |
| SG31 | anti-CD49d | PE |
| H1.2F3 | anti-CD69 | PE |
| N418 | anti-CD11c | PE |
| JES6-5H4 | anti-IL-2 | PE |
| RM4-5 | anti-CD4 | PerCP |

| | | |
|---|---|---|
| GK1.5 | anti-CD4 | PE-Texas Red |
| H57-597 | anti-TCR | APC |
| 36-7-5 | anti-MHC I H-2$^{k}$ ($K^{k}$) | Bio |
| R17217 | anti-CD71 | Bio |
| IM7 | anti-CD44 | Bio |
| 1D3 | anti-CD19 | Bio |
| M5/114.15.2 | anti-MHC II I-A/I-$E^{b, d, q}$ | Bio/FITC |
| RA3-6B2 | anti-B220 | Bio/PE |
| 2.4G2 | FcγRII/III | --- |
| --- | Anti-phospho-Stat5 Tyr694 | --- |
| --- | Anti-phospho-Akt Ser473 | --- |
| --- | Anti-phospho-S6 Ser235/236 | --- |

### 2.9.2 CFSE labelling

5(-6) CarboxyFluorescein diacetate Succinidyl Ester (CFSE) is a fluorescein based dye that allows the analysis of the history of individual cells that have undergone multiple rounds of division [265]. CFSE, a fluorescein molecule containing a succinimidyl ester functional group and two acetate moieties, diffuses freely into cells where intracellular esterases cleave the acetate

groups converting it to a fluorescent and membrane impermeant dye. CFSE is retained by the cell in the cytoplasm and does not compromise cellular functions. During each round of cell division, the relative intensity of the dye is decreased by half. For determination of *in vivo* proliferation, single cell suspensions of memory CD4 T cells were washed and resuspended at a concentration of 1 x $10^7$ cells/ml and incubated with CFSE (Molecular Probes, Eugene, OR) at a final concentration of 2.5 µM for 10 minutes at 37°C. Cells were then washed three times with ice cold 5% FCS medium, resuspended in AB medium and injected i.v. into adoptive hosts.

### 2.9.3 PI staining

Propidium Iodide (PI) is a dye that intercalates into cellular DNA. The intensity of the PI signal is directly proportional to the DNA content [266]. Memory CD4 T cells were purified using MoFlow to a level of purity of 95%. Sorted cells were washed and resuspended in PBS at a concentration of 1 x $10^7$ cells/ml. Cells were fixed with ice cold 70% EtOH and left at RT for 20 min. The reaction was stopped adding ice cold PBS. Cells were washed twice and finally resuspended in PBS. PI was added at a final concentration of 10 µg/ml and cells were incubated on ice for 3 minutes before FACS analysis.

## 2.10 Generation of effector and memory T cells

Naïve CD4 T cells were isolated from lymph nodes of A1 RAG1 ko (H-$2^k$) or A1 Ly5.1 RAG1 ko (H-$2^k$) female mice. Syngeneic bone marrow derived DCs

were pulsed *in vitro* for 3 hours at 37°C with an H-Y peptide at a final concentration of 10 μM. After incubation, DCs were washed in AB medium and mixed to naïve CD4 T cells in a 1:4 ratio. The mix was co-injected i.v. into allogeneic adoptive hosts, either MHC+ hosts or MHC- hosts.

### 2.11 Analysis of gene expression

#### 2.11.1 RNA isolation and *In Vitro* Transcription (IVT)

Spleens from mice of the same experimental group were pooled, gently mashed and the resulting single cell suspension was treated with RBC lysis buffer. Cells were stained with anti-CD4 and FACS sorted obtaining > 97% purity. Total RNA was extracted from FACS sorted CD4 T cells using Triazol (Invitrogen) or RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. For glass arrays, linear amplification was performed using MessageAmp$^{TM}$ *a*RNA Kit (Ambion). Briefly, *c*DNA was generated from 400-700 ng of total RNA obtained from 1-2 x $10^6$ FACS purified CD4 T cells, using T7 Oligo (dT) primers. After purification, the *c*DNA was incubated overnight with T7 polymerase at 37°C, DNase I treated, purified using columns and eluted in RNAse free water (Eppendorf). For Affymetrix, 600 ng of total RNA were *in vitro* amplified using GeneChip® Two-Cycle *c*DNA Synthesis Kit (manufactured by Invitrogen for Affymetrix). RNA quantity and quality was assessed by 2100 BioAnalyzer (Agilent Technologies) using RNA 6000 Nano LabChip® Kit (Agilent) following manufacturer's instructions or by spectrophotometry using a Bio Spec-Mini (Shimadzu) and RNase free cuvette (UVette 220-1600nm, Eppendorf). Alternatively, samples were run

on a 1% agarose gel (Sigma) in 1 x Tris-Acetate-EDTA buffer (TAE: Tris 0.04 M, $Na_2EDTA$ 0.001 M, acetic acid 0.02 M). Size was assessed comparing RNA with RNA Markers (Promega).

### 2.11.2 Glass arrays

2 µg of *a*RNA and Random primers (Perkin Elmer) were used to generate *c*DNA using LabelStar Array Kit (Qiagen). Direct dye incorporation was performed using dCTP-Cy3 or dCTP-Cy5 (Amersham Bioscience). For each experiment a dye swap was performed. Briefly, 5 µg of *c*DNA from each of the two samples labelled with Cy3 and Cy5 were combined and denaturated at 95°C for 10 minutes. After adding 10 µl of mouse Cot1DNA (10 µg/ µl Invitrogen), 12 µl 20x SSC, 3.6 µl 5% Sodium Dodecyl Sulfate (SDS) (BioRad) and 15 µl of ArrayHyb Microarray Hybridisation Buffer (Sigma) to the denaturated *c*DNA, the samples were loaded onto the glass array and covered with a coverslip (Lifter Slips, VWR). Hybridisation was performed at 62°C overnight. Glass arrays were washed in 1x SSC/ 0.25% SDS at 60 °C for 5 minutes and twice with 1x SSC for 5 minutes. Images were acquired at the wavelength of 535 nm and 632 nm using the software GenePix 5.0 in a GenePix 4000A Scanner (Axon Instruments).

The set of glass arrays used was: National Institute for Aging (NIA) Clone Set Arrays version 2 (~15k *c*DNAs on 2 slides) from Human Genome Mapping Project (HGMP). Data, analysed with GeneSpring 6.2 (Silicon Genetics), were normalised (Locally WEighted Least Squares (LOWESS) 40% and cut off 0.1, dye swap applied), filtered on flags (presence of

signal/hybridisation), error (error type Standard Deviation 0-1.4), and -fold expression of 1.5 was set as the cut-off value.

### 2.11.3 Affymetrix

GeneChip analyses were performed using the facility at the NIMR. Briefly, complementary RNA (*c*RNA) was hybridised on Mouse 430 A plus chip (Affymetrix) overnight at 45° C and 60 rpm in a GeneChip® Hybridization Oven 640 (Affymetrix). Chips were then washed using GeneChip® Fluidics Station 450 (Affymetrix). Images were acquired using Affymetrix® GeneChip® Scanner 3000 and GeneChip® Operating Software (GCOS) software and the quality of the arrays was assessed using BioConductor. The results were analysed with GeneSpring 7.1 (Silicongenetics). Data were normalised (per chip: normalised to $50^{th}$ percentile, and per gene: normalised to median cut-off value 0.01) filtered on flags (presence of signal/hybridisation) and -fold expression of 1.5 was set as the cut-off value.

### 2.11.4 Real Time Polymerase Chain Reaction (PCR)

RNA was extracted from FACS sorted memory CD4 T cells obtained from pooled spleen from > 10 mice per experimental group using Triazol (Invitrogen). Similarly, RNA was obtained also from FACS sorted A1 naïve CD4 T cells from pooled spleen from 3 mice as a reference. Reverse Transcription from total RNA was performed using GeneAmp® RNA PCR Core Kit (Perkin Elmer) according to the manifacturer's instructions. The *c*DNA served as template for the amplification of genes of interest and the

houskeeping gene Hypoxanthine guanine phosphoribosyl transferase *(Hprt1)*. *Il2* (Mm434256_A1), *Mapk9* (Mm00444231_A1), *FoxO1* (Mm00490672_m1), *Glut1* (Mm00441473_A1) and *Hprt1* (Mm00446968_A1) gene expression was assessed using Assays-on-Demand™ Gene Expression Products (Applied Biosystems) on the ABI-PRISM 7000 Sequence detection system (Applied Biosystems, Foster Dity, CA). Level of expression of target gene was calculated using the $2^{-\Delta\Delta Ct}$ method [267]. Briefly, a fluorogenic probe enable the detection for a specific transcript as it accumulates during the PCR. The fluorescence is low at the beginning of the reaction and it sets the baseline. Threshold Cycle, $C_t$ value, is defined as the number of cycle at which the fluorescence of amplified target reaches a fixed threshold. The threshold was set in the region of exponential growth of the PCR products. $C_t$ values from target genes were normalised to *Hprt* gene expression as endogenous reference, $\Delta C_t$ = ($C_t$ target gene - $C_t$ *Hprt*). For *Il2*, *Mapk9*, and *FoxO1*, the $\Delta C_t$ values were then normalised to A1 naïve CD4 T cells $\Delta C_t$ values, $\Delta\Delta C_t$ = ($\Delta C_t$ memory - $\Delta C_t$ naive). Given that the efficiency of the amplification is comparable over the primers used and that the size of the amplicons is < 150 base pairs, the amount of input is calculated as $2^{-\Delta\Delta Ct}$ . The data are presented as -fold change in gene expression relative to gene expression of A1 naïve CD4 T cells.

### 2.12 Statistical analyses

Means and Standard Deviations were calculated using Excel 2003.

*p*-values, obtained using Mann-Whitney's two tailed T test, were calculated in experiments where > 3 mice per group were used.

# 3. RESULTS

## 3.1 Molecular and phenotypic characterisation of memory T cells maintained in the absence of MHC class II molecules

### 3.1.1 Microarrays

Microarray analysis of gene expression represents a powerful tool for the elucidation of functional differences between cells at various stages of differentiation. One of the main advantages of this technology is that from a relatively small amount of RNA it is possible to obtain the gene expression profile of a cell population. Microarray analysis was used with the aim to identify genes differentially expressed in antigen specific memory CD4 T cells that survived in different environments. As *in vitro* and *in vivo* experiments previously done had shown that memory CD4 T cells maintained in the absence of MHC class II were functionally impaired [129], we decided to perform microarray analysis to identify candidate molecules that may be involved in the functional impairment.

#### 3.1.1.1 Experimental design and Memory CD4 T cell recovery

Memory CD4 T cells were generated by adoptive transfer of naïve CD4 T cells from the A1 TCR transgenic strain [260], specific for a peptide from the *Dby*-encoded H-Y epitope antigen [261], with syngeneic DCs into two different hosts. Both hosts lacked the cytokine common gamma chain and RAG-2 genes (RAG2 ko γc ko), but one of them also lacked MHC class II gene

expression. For the purpose of simplification, the MHC class II competent hosts will be referred to as MHC+ and MHC class II deficient hosts as MHC-. DCs disappear about 2 weeks after transfer into the adoptive hosts [80] and at this time CD4 T cells, having gone through the effector phase, enter into a resting memory state (Fig. 5a). For all microarray experiments memory CD4 T cells were recovered 6 weeks after the co-transfer of naïve CD4 T cells and H-Y antigen pulsed syngeneic DCs.

Fig. 5b shows an example of memory CD4 T cell recovery. In these experiments, A1 naïve CD4 T cells and H-Y peptide pulsed bone marrow derived DCs per mouse in a ratio 4:1 were injected i.v. into 16 MHC+ hosts and 12 MHC- hosts. Confirming previous data [129], the average number of memory CD4 T cells recovered from MHC+ and MHC- hosts per mouse was not different. Total splenocytes from individual mice were pooled and memory CD4 T cells were isolated by FACS sorting. The purity of sorted memory CD4 T cells was >97%. High level of purity was necessary to guarantee that the gene expression profile truly reflected the memory CD4 T population. Nevertheless, as illustrated in Fig. 5b right, the rigorous FACS sorting necessary to obtain such pure populations drastically reduced the yield of viable memory CD4 T cells.

Four microarray experiments on memory CD4 T cells from MHC+ and MHC- hosts were performed using 2 different platforms. Three biological replicates were analysed on glass arrays using the Mouse NIA Clone Set Arrays from the HGMP, while the RNA from one biological experiment was hybridised to the Mouse Genome 430A 2.0 chip from Affymetrix.

In this experimental set up, RNA quantity was a limiting factor. This was expected considering the number and the resting state of the memory CD4 T cells recovered. In the periphery, memory T cells persist in a quiescent state, characterised by low cycling and metabolic rate. Although the maintenance of quiescence requires the active expression of a similar number of genes as are expressed during activation, the total amount of RNA in resting cells is half the amount of RNA synthesised by activated cells [124]. The *m*RNA isolated from memory CD4 T cells recovered from MHC+ and MHC- hosts was not sufficient to perform the chip hybridisation and therefore it was necessary to amplify the starting material *in vitro* as described in the following parts.

### 3.1.1.2 Glass Arrays: RNA and IVT

For the glass arrays, the *m*RNA was amplified by reverse transcription performed on total RNA using a modified poly(dT) primer coupled with the T7 promoter. After first-strand synthesis, the reaction was treated with RNase H to cleave the *m*RNA into small fragments, which served as primers during a second-strand synthesis reaction that produced a double-stranded *c*DNA, the template for transcription. Using the T7 polymerase, *c*DNA was *in vitro* transcribed into *a*RNA. The linear amplification of RNA first described in [268] permitted the synthesis of enough *a*RNA to complete microarray analysis.

RNA quality is a critical parameter in obtaining reliable gene expression data. RNA quality was assessed by spectrophotometer, gel electrophoresis and Bioanalyser. Fig. 6a reports an electropherogram as example of the RNA

quality used in microarray experiments. In this assay, fluorescently labelled RNA is electrophoretically separated through a microchannel. Plotting fluorescence in function of time, the software creates an electropherogram. When the RNA quality is poor, high levels of degraded RNA are registered between the ribosomal 28S and 18S peaks and the 28S:18S rRNA ratio is less than 1. Total RNA from memory CD4 T cells recovered from MHC+ and MHC- hosts tested with this technique showed clear 28S and 18S peaks with low levels of noise between the two peaks. Following *in vitro* amplification, *a*RNA quality was assessed on 1% agarose gels. The average size of *in vitro* amplified RNA was ~ 1 kb, indicative of the good quality of the starting material as well as efficient *in vitro* amplification (Fig. 6b).

#### 3.1.1.3 Glass arrays

The microarray platform initially used to analyse the transcriptome of memory CD4 T cells maintained in the presence or absence of MHC class II molecules was the Mouse NIA Clone Set Arrays from the HGMP. The Mouse NIA Clone Set Arrays consisted of two glass arrays that contained 15,000 PCR products generated from a *c*DNA clone set amplified from the NIA. The PCR products had a 5' C6 modification that allowed covalent binding to the glass array. The clones were obtained from a mouse *c*DNA library from pre- and peri-implantation embryos, E12.5 female gonads/mesonephros, and new born ovaries. An estimated 50% of these transcripts represented novel genes.

Three biological replicates were analysed with the Mouse NIA Clone Set Arrays version 2. Equal amounts of *a*RNA from memory CD4 T cells from

MHC+ and MHC- hosts were used for *c*DNA synthesis. The single stranded *c*DNA used for the hybridisation was generated with random primers. During this process, one *c*DNA sample was labelled with Cy5 dye, and the other with Cy3. Equal amounts of both samples were loaded on the array, where they competitively hybridised to the set of genes spotted on the glass. Different intensities of the dyes were registered in cases of differentially expressed genes, while equal intensity was registered for genes equally expressed. Due to its bigger size, Cy5 is less efficiently incorporated into *c*DNA than Cy3, a fact that can potentially introduce bias (false negative). To overcome this problem, for each biological replicate the hybridisation was repeated swapping the dyes (technical replicate). Images were acquired using a GenePix 4000A Scanner and the slides were edited to guarantee optimal overlapping of the grid that represents the array within each slide. Editing the slide was an important step because the software was not always able to discriminate real spots from background. After image acquisition, the raw data from the 3 biological replicates were loaded into Genespring version 6.2. Here samples were subjected to LOWESS normalisation and a series of filtering steps. Spots which hybridised poorly, or which had significant variations in intensity values between replicates were eliminated. The remaining genes were filtered on -fold change. The genes differentially expressed with a cut-off of 1.5 -fold represented only 2% of the sequences printed on the glass array.

Within the 272 sequences differentially expressed in the Mouse NIA Clone Set Arrays, more than 53% were sequences with unknown function (Expressed Tag Sequences (ESTs) RIKEN and *c*DNAs from various libraries). These sequences are likely to be revealed to be the coding region

of known or putative proteins in the future, so they represent useful information when considering new molecules as candidates for further investigation.

10% of the differentially expressed sequences seemed interesting in an immunological context and were listed in Table 3. They are listed in descending order according to -fold change in memory CD4 T cells recovered from MHC- hosts compared to memory CD4 T cells from MHC+ hosts. Genes with -fold change ≥ 1.5 were over-expressed in CD4 T cells from MHC- hosts, while values ≤ 0.6 were down-regulated in the same group. The Gene name refers to the number of the clone in the NIA library. The common name, Genebank accession number and description are reported as well.

Table 3: Glass arrays: gene differentially expressed.

The 30 differentially expressed sequences are listed in descending order according to -fold change of CD4 T memory cells recovered from MHC - hosts. Genes with -fold change ≥ 1.5 are over-expressed in CD4 T cells recovered from MHC - hosts, while values ≤ 0.6 are down-regulated in the same group. In the case of down-regulation the fold change can also be expressed as (1/reported value).

| **Gene Name** | **Fold Change** | **Common Name** | **Genbank** | **Description** |
|---|---|---|---|---|
| H3059F12 | 3.3273 | Tgtp | BG067921 | T-cell specific GTPase |
| H3023B11 | 2.5623 | Hspd1 | BG064728 | heat shock protein 1 (chaperonin) |

| | | | | |
|---|---|---|---|---|
| hhhhhZ46663 | 2.5340 | Gh | Z46663 | M.musculus DNA for growth hormone gene and promoter. |
| H3020C05 | 2.4044 | Rab18 | BG064483 | RAB18, member RAS oncogene family |
| H3053E12 | 2.3707 | Atf3 | BG067364 | activating transcription factor 3 |
| H3005H12 | 2.3140 | Donson | BG063337 | downstream neighbor of SON |
| H3031E05 | 2.2617 | Catnb | BG078748 | catenin beta |
| H3117F06 | 2.2501 | Rbbp7 | BG073015 | retinoblastoma binding protein 7 |
| H3088G10 | 2.0501 | Mina | BG070573 | myc induced nuclear antigen |
| H3088C04 | 2.0370 | Mllt3 | BG083516 | myeloid/lymphoid or mixed lineage-leukemia translocation to 3 homolog (Drosophila) |
| H3008H04 | 1.8071 | Sp3 | BG076933 | trans-acting transcription factor 3 |
| H3131E06 | 1.6820 | Rab6 | BG074166 | RAB6, member RAS oncogene family |
| H3071C06 | 1.6248 | Gbas | BG068968 | glioblastoma amplified sequence |
| H3018D04 | 1.6057 | Rap1b | BG064333 | RAS related protein 1b |
| H3139E03 | 0.5288 | Kai1 | BG087428 | kangai 1 (suppression of tumorigenicity 6, prostate) |
| H3057G10 | 0.5233 | Pscd2 | BG080814 | pleckstrin homology, Sec7 and coiled-coil domains 2 |
| H3022E01 | 0.4780 | Mbc2 | BG064664 | membrane bound C2 domain containing protein |
| H3007C09 | 0.4699 | Sh3bgrl3 | BG063442 | SH3 domain binding glutamic acid-rich |

| | | | | |
|---|---|---|---|---|
| | | | | protein-like 3 |
| H3017F01 | 0.3984 | Mel | BG077618 | cell line NK14 derived transforming oncogene |
| H3004A05 | 0.3815 | Tbrg4 | BG063180 | transforming growth factor beta regulated gene 4 |
| H3073G07 | 0.3771 | Gzmm | BG082217 | granzyme M (lymphocyte met-ase 1) |
| H3033A07 | 0.3432 | Mkrn1 | BG078870 | makorin, ring finger protein, 1 |
| H3073E02 | 0.3417 | Pttg1 | BG069168 | pituitary tumor-transforming 1 |
| H3072H12 | 0.2235 | E2f5 | BG082143 | E2F transcription factor 5 |
| H3035F09 | 0.1017 | Rce1 | BG065824 | Ras and a-factor-converting enzyme 1 homolog (S. cerevisiae) |
| H3001A02 | 0.0650 | Sct | BG062929 | secretin |
| H3083A04 | 0.0219 | Crk | BG069956 | v-crk sarcoma virus CT10 oncogene homolog (avian) |

Within this list, 30% of the genes can be related to biological processes such as signaling, stress and cell cycle. Ultimately, we could not find in these results suitable candidate genes or networks that would have potentially explained the loss of function of memory CD4 T cells recovered from MHC-hosts.

For this reason we decided to analyse the transcriptome of resting memory CD4 T cells using the Mouse Genome 430A 2.0 chip from Affymetrix, which offered a comprehensive coverage of the mouse genome.

### 3.1.1.4 Affymetrix: RNA and IVT

As previously discussed, RNA quantity was a limiting factor in our experimental setting up. For this experiment, RNA was isolated from FACS sorted CD4 T cells from 12 MHC+ hosts and 16 pooled MHC-. 0.7 µg of total RNA were isolated from memory CD4 T cells from MHC+ hosts and 0.5 µg from MHC- hosts. RNA quality was assessed by a Bioanalyser (Fig. 7a). The amount of RNA isolated was not sufficient to perform the hybridisation directly, therefore it was necessary to introduce two amplification steps in order to carry out microarray analysis. The RNA *in vitro* transcription generated 85 µg of *c*RNA from memory CD4 T cells from MHC+ hosts and 81 µg from MHC- hosts. The electropherograms in Fig. 7b show that the biotin-labelled *c*RNA from memory CD4 T cells from MHC+ hosts (left) and MHC- hosts (centre) have similar size distribution. The average size for both samples is approximately 850 bases as shown in the gel image generated from the assay (Fig. 7b, right), as expected from good quality *m*RNA. As the probes for the transcripts on the chip have a size of 21 nucleotides, *c*RNA has to be fragmented to obtain optimal chip hybridisation. RNA fragmentation is obtained by metal-induced hydrolysis carried out at 94°C for 35 minutes. Fig. 7c shows that the RNA from memory CD4 T cells recovered from MHC+ (left) and MHC- (centre) hosts was successfully fragmented into small RNA molecules with a size distribution from 35 to 200 bases, as represented by the virtual gel generated by the program (Fig. 7c, right).

### 3.1.1.5 Affymetrix: array quality controls

To obtain a brighter signal, two cycles of IVT amplification were performed. RNA was biotin-labelled during the second round of amplification by adding a synthetic biotinylated nucleotide. The biotin-labelled anti-sense RNA was hybridised to the Mouse Genome 430A 2.0 chip, which contains more than 22,000 probes, representing 15,000 transcripts of which 10,000 are well characterised genes. The array therefore is representative of approximately 1/3 of the mouse genome.

After staining the biotinylated *c*RNA in a 3 step process using StreptAvidin R-PhycoErythrin (SAPE), which binds to biotin and anti-streptavidin antibody, images were acquired using a Gene Array Scanner and the gene expression was calculated as function of the fluorescence intensity for each probe. The quality of the chip hybridisation was assessed using BioConductor. This program generated a representation of the overall intensity for each array. The graphical comparison allows the identification of possible differences in the distribution of the intensities and densities between arrays. Fig. 8a shows the kernel density estimates of Perfect Match (PM) intensities for the arrays hybridised with RNA from memory CD4 T cells from MHC+ hosts (1, black line) and MHC- hosts (2, red dashed line). Both chips present a good distribution of intensity and importantly, their density profiles have overlapping shapes. Any arrays with low average intensity or significantly different shaped density should not be considered for comparisons, as this indicates that technical problems have occurred at some stages during chip hybridisation or staining. We have generated images of the arrays hybridised with RNA isolated from memory CD4 T cells from MHC+ and MHC- hosts respectively using the log transformed intensities. These images are very useful to detect spatial artefacts such as areas of uneven stains or spots. If

not noticed, such artefacts could be wrongly interpreted as differentially expressed genes and cost a lot of time and effort to be rectified. As illustrated in Fig. 8b, arrays from memory CD4 T cells generated in MHC+ hosts (left) and MHC- hosts (right) show a homogeneous hybridisation and there is no evidence of artefacts.

#### 3.1.1.6 Affymetrix: differentially expressed genes

The aim of the microarray experiment was to identify, at the molecular level, genes that were differentially expressed in memory CD4 T cells as a consequence of their maintenance in the presence or absence of MHC class II. Due to the fact that the 3 glass array experiments previously performed did not provide the output of information we had hoped for, the Affymetrix experiment was performed in the hope that this would give us candidate molecules for follow-up studies. Due to technical factors (numbers of mice needed for each replicate) as well as cost considerations (Affymetrix chips exceeded consumable budget available), only one Affymetrix experiment was performed. The data were used as starting point for further functional analysis of parameters that were likely to affect memory function.

Affymetrix data were normalised as described in Materials and Methods. All normalised transcripts are shown in Fig. 9a. Here, as well as in the following graphs, normalised intensities of genes expressed by memory CD4 T cells from MHC+ hosts are plotted along the x axis, while that from memory CD4 T cells from MHC- hosts are plotted along the y axis. Each dot represents one transcript and its expression in relation to the other memory population. When the expression of one gene is the same in the two cell populations, the

transcript is located on the regression line (MHC-=MHC+, dark blue and thick line). Genes over-expressed by memory CD4 T cells from MHC- hosts fall on the left of the regression line (MHC->MHC+), while genes over-expressed in memory CD4 T cells from MHC+ hosts are found on the right of the regression line (MHC-<MHC+). Transcripts that are differentially expressed at values greater than 1.5-fold are represented outside the thin blue lines. Additional information is given by the color of the dots. Precisely, the level of the expression of the transcripts correlates with the color bar on the side of the graph. Therefore, the green dots indicate that those transcripts are expressed at low level, while red dots correspond to transcripts with high levels of expression.

A cut-off of 1.5-fold difference between the two cell populations was set. Although this was an arbitrary choice, it was justified by the observation that such a cut-off included the over-expression of the transcript for CD5 in memory CD4 T cells from MHC- hosts compared to MHC+ hosts, which was previously described as one of the phenotypic characteristics of memory CD4 T cells from MHC- hosts at the protein level using FACS analysis [129].

At a cut-off of 1.5-fold difference, 360 sequences were found differentially expressed (Fig. 9b and Appendix 1). We decided to concentrate only on the transcripts which coded for known proteins. Within this group, most of the transcripts, approximately 80%, were differentially expressed with a range of variability between 1.5- and 3-fold difference. The remaining 20% of the genes showed more pronounced differences up to 21-fold up-regulation and 14.9-fold down-regulation in memory CD4 T cells from MHC- hosts compared to MHC+ hosts. None of these genes were T cell specific, being involved in general biological processes. The small difference in the level of

expression found for the majority of genes may be explained by the fact that the two cell populations were in a quiescent state. 10% of the genes differentially expressed seemed particularly relevant in an immunological context and therefore appeared to be a good and justifible starting point for further analyses. In addition, as will become clearer later, some of these genes are part of a common signalling pathway, and for this reason we valued their difference in expression as a strong indication that the signalling pathway in itself was impared in one of the two memory populations. These genes were organised in categories according to their biological function in immunological processes as discussed in the following sections.

**Surface molecules**

This category contains the transcripts that code for molecules expressed on the cell surface (Fig. 10a). At the RNA level, 2 chemokine receptors, *Cxcr4* and *Cxcr6* showed 1.97 and 1.88-fold increase in memory CD4 T cells from MHC- hosts compared to MHC+ hosts. In addition, 3 probes for *Ccr2* were over-expressed at similar levels of -fold difference in memory CD4 T cells from MHC- hosts: 2.11, 1.94 and 1.84. These 3 transcripts are likely to be different splicing variants. Two isoforms of CCR2 which differ only in the untranslated region were recently described [269]. CCR2 is not expressed on naïve T cells, but it has been shown to be expressed on effector and memory T cells [270] where it may play a role in T cell differentiation [271]. *CD5,* a negative regulator of TCR signalling, was 1.92-fold over-expressed in memory CD4 T cells from MHC- hosts compared to MHC+ hosts in agreement with its known under-expression as seen by FACS analysis [129]. In

addition, *Cd224* and *Cd24a* were 3.56 and 3.01-fold up-regulated respectively in the memory CD4 T cell population from MHC- hosts compared to MHC+ hosts. CD224 is an N-glycosylated protein which so far has not been reported expressed on CD4 T cells, but is expressed on NK cells, γδ cells and a subset of CD8 T cells [272], while CD24a (also known as heat stable antigen) is a cell surface glycosyl-phosphatidylinositol-anchored protein which is down-regulated when T cells reach maturity [273], but it is rapidly up-regulated upon TCR engagement [274 275]. The function of CD24 in T cells is not completely clear [276 277]. Three genes coding for surface molecules were down-regulated in CD4 T cells from MHC- hosts compared to MHC+ hosts: *Cd151* and *Cd5l* were 2.09-fold and 1.93-fold down-regulated in CD4 T cells from MHC- hosts compared to MHC+ hosts respectively, while *Integrin α4*, *Itga4* also known as *Cd49d*, was 1.92-fold down-regulated in CD4 T cells from MHC- hosts compared to MHC+ hosts. Gene name, Common name, Genbank accession number and gene description are listed in Fig. 10b, where data are sorted in a descending order according to -fold change in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells from MHC+ hosts.

**Genes potentially involved in cellular senescence**

The term 'cellular or replicative senescence' was introduced to describe the characteristic of somatic cells to enter a non-replicative stage by arresting irreversibly at the G1 stage of the cell cycle, after a defined number of divisions [278]. We identified 3 transcripts potentially involved in cellular senescence (Fig. 11a). In particular, Killer cell Lectin-like Receptors, *Klrk1*

(NKG2D) and *Klrd1* (CD94) were up-regulated 2.6 and 2-fold respectively in memory CD4 T cells from MHC- hosts compared to MHC+ hosts. In addition to Klrs we found that *Mortality Factor 4 like 2* (*Morf4l2*) transcript was 2.6-fold over-expressed in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells from MHC+ hosts. Gene name, Common name, Genbank accession number and gene description are listed in Fig. 11b, where data are sorted in a descending order according to -fold change in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells from MHC+ hosts. Klrs molecules are a family of glycoproteins expressed on NK cells and bind MHC class I. On NK cells, Klrs are involved in recognition of abnormal cells [279] and their engagement can result in NK activation as well as in inhibition of activation [280]. In particular, *Klrd1* (CD94) belongs to the inhibitory MHC class I receptors, which are involved in maintaining NK cell tolerance [281]. CD94 forms heterodimers with NKG2 molecules and provides an inhibitory signal to NK activation [282]. Due to the lack of the common γ chain in both our hosts, we can exclude that the expression of Klrs is due to NK cell contamination in the cell preparate. Along with the described function on NK cells, the complex CD94/NKG2C has been shown to represent an alternative pathway for the activation of a CD8 T cells subset [283]]. Of interest is the observation that viral infections induce abundant numbers of senescent CD8 T cells. Senescent CD8T cells could be identified by their high levels of expression of KLRG1 [284], another member of the Klr family. Therefore, although there are no data suggesting that a similar mechanism could be found in CD4 T cells, it is possible that Klrs may be expressed also on CD4 T cells. Unfortunately, no antibodies are available for these molecules and therefore the data have not been validated by FACS analysis.

Moreover, *Morf4l2* was 2.6-fold over-expressed in memory CD4 T cells from MHC- hosts. MORF4 molecules are members of a new family of transcription factors, the MORF4 Related Genes (MRG) family, that is thought to play an important role in senescence pathways or/and cell growth control [285]. The involvement of these molecules in CD4 T cell function has not been reported so far.

**Cytokines and cytokine receptors**

Fig. 12a shows the transcripts for cytokines and cytokine related genes differentially expressed in memory CD4 T cells from MHC+ and MHC- hosts. Four transcripts were over-expressed in memory CD4 T cells from MHC- hosts: 3 transcripts controlled by/related to interferon, Interferon-related developmental regulator 1 (*Ifrd1*), Interferon induced transmembrane protein 1 (*Ifitm1*) and *Ifitm2* were 1.76, 4.99 and 1.83-fold up-regulated respectively; *Growth Arrest and DNA-Damage-inducible 45 alpha* (*Gadd45a*) was 3.5-fold over-expressed in memory CD4 T cells from MHC- hosts compared to MHC+ ones. Interferon-induced protein with tetratricopeptide repeats 1 (*Ifit1*), another protein regulated by interferon, was 2.17-fold down-regulated in memory CD4 T cells from MHC- hosts compared to MHC+ hosts. The transcript for the cytokine receptor *Il17rb* was 2.96-fold down-regulated in memory CD4 T cells from MHC- hosts versus (vs) MHC+ hosts. In addition, the transcript for *Il2* was 3.17-fold under-expressed in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells from MHC+ hosts. This result suggested that memory CD4 T cells from MHC- hosts do not express large amounts of pre-stored transcripts for IL-2 compared to memory CD4 T

cells from MHC+ hosts. Gene name, Common name, Genbank accession number and gene description are listed in Fig. 12b, where data are sorted in a descending order according to -fold change in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells from MHC+ hosts.

**Signalling and survival**

In this category we grouped the transcripts that belong to signalling cascades reported to be relevant for T cells (Fig. 13a). By investigating the role of the genes reported in this group, we realised that a considerable number of these genes belong to the same pathway. Among the over-expressed transcripts in memory CD4 T cells from MHC- hosts, *Stat4* and *Map3k1* were 2.02 and 2.09-fold up-regulated. Ten genes were down-regulated in memory CD4 T cells from MHC- hosts. *Stat3*, *5a* and *5b* were 2.03, 2.11 and 1.99-fold down-regulated in the CD4 T cells from MHC- hosts compared to MHC+ ones. *Akt1* and *Bcl-2* were 2.4 and 1.93-fold down respectively. Two transcription factors, *FoxO1* and *Gsk3b* were 1.94 and 1.84-fold down-regulated in CD4 T cells from MHC- hosts compared to MHC+ hosts. In addition, *Mapk9* and *Map4k4* were 2.05 and 1.82-fold down-regulated in memory CD4 T cells from MHC- hosts vs MHC+ hosts. Gene name, Common name, Genbank accession number and gene description are listed in Fig. 13b, where data are sorted in a descending order according to -fold change in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells from MHC+ hosts.

**3. 2 Validation of Affymetrix results**

Validation of differentially expressed genes identified by Affymetrix analysis was carried out by real time PCR and by FACS analysis. Using real time PCR we tested the reliability of the data generated by Affymetrix at the RNA level. Once the reproducibility of the information obtained by Affymetrix was assured, wherever possible the validation was continued at the protein level. Some of the transcripts identified by microarray and further verified by FACS turned out to be particular relevant in the biological context of our experiments.

### 3.1.2.1 Validation by Real Time PCR (Quantitative PCR)

*c*DNA was generated from FACS sorted resting memory CD4 T cells from MHC+ hosts, MHC- hosts and from naïve CD4 T cells. The level of expression of each gene was calculated relative to the expression value of A1 naïve CD4 T cells, to which the value of 1 was arbitrarily assigned. By real time PCR, *Il2* was 3-fold under-expressed in memory CD4 T cells from MHC- hosts compared to MHC+ hosts (Fig. 14a), data consistent with the 3.5-fold under-expression obtained by Affymetrix. Memory CD4 T cells from MHC- hosts under-expressed *Mapk9* (Fig. 14b) and *FoxO1* (Fig. 14c) compared to MHC+ hosts. The difference in the level of expression of the 3 genes tested fully supported the results previously obtained by Affymetrix.

### 3.1.2.2 Validation by FACS analysis *ex vivo*

FACS analyses were performed on memory CD4 T cells from MHC+ hosts, MHC- hosts and A1 naïve CD4 T cells. Memory CD4 T cells were generated as previously described and recovered by week 4 after transfer.

The expression of CD24a and Itga4 on memory CD4 T cells from MHC+ and MHC- hosts was further validated by FACS analysis. In contrast with microarray data, resting memory CD4 T cells from MHC+ hosts and MHC- hosts did not express different amounts of CD24a on the cell surface (Fig.15a, left). Instead both resting memory populations showed a down-regulation of this molecule compared to A1 naïve CD4 T cells. The level of expression of Itga4, CD49d, was higher in memory CD4 T cells recovered from MHC+ hosts compared to memory CD4 T cells recovered from MHC- hosts (Fig.15a, right). This result confirmed the data obtained by analysis of gene expression. Fig. 15b shows the Mean Fluorescence Intensity (MFI) for CD24a and Itga4 on memory CD4 T cells from MHC+ and MHC- hosts. Values for A1 naïve CD4 T cells are reported as reference.

By microarray analysis Stat5 and Akt1 were indicated as genes under-expressed in memory CD4 T cells from MHC- hosts vs MHC+ hosts. Their under-expression at *m*RNA level suggested that their activity could be reduced, but differences in the *m*RNA are not necessarily meaningful when protein activity is regulated by phosphorylation as it is in the case of these two proteins. However, the availability of antibodies that can discriminate between phosphorylated and unphosphorylated molecules made it possible to validate the array results. Akt1 was 2.4-fold down-regulated in memory CD4 T cells from MHC- hosts vs MHC+ host at the *m*RNA level. Similarly, *ex*

*vivo* the level of Akt1 phosphorylated at serine 473 was lower in memory CD4 T cells from MHC- hosts than in memory CD4 T cells from MHC+ hosts (Fig. 16a, right), as assessed by intracellular staining. According to the microarray data, the two isoforms for Stat5, Stat5a and Stat5b, were both down-regulated in memory CD4 T cells recovered from MHC- hosts vs MHC+ hosts. The level of phosphorylated Stat5 from freshly isolated memory CD4 T cells was assessed by FACS analysis using an antibody able to identify both phosphorylated isoforms. Memory CD4 T cells from MHC- hosts showed reduced levels of phosphorylated Stat5 compared to the levels observed on memory CD4 T cells recovered from MHC+ hosts (Fig. 16a, left and Fig.16b). Therefore, along with a reduction in the level of transcription, memory CD4 T cells generated in MHC- hosts showed a decrease in the level of phosphorylation of Stat5 and Akt1 compared to memory CD4 T cells recovered from MHC+ hosts and displayed an intermediate phenotype between the level of expression observed in naïve A1 CD4 T cells and the level of expression observed in memory CD4 T cells generated in MHC+ hosts.

The analysis of Bcl-2 by FACS showed that memory CD4 T cells from MHC- hosts express reduced amount of Bcl-2 compared to memory CD4 T cells from MHC+ hosts (Fig. 17a, left). Akt1 and Bcl-2 are two important molecules for cell survival. The differential level of expression of these two molecules suggested a subtle but important reduction in survival signals in resting memory CD4 T cells from MHC- hosts compared to resting memory CD4 T cells from MHC+ hosts.

Another molecule that is connected with T cell survival is IL-7Rα, CD127, which together with the common γ chain forms the IL-7 receptor. As IL-7 is constitutively expressed by various cell types, the use of this cytokine by T cells is regulated by the expression of its receptor. IL-7Rα up-regulation has been proposed as the selective factor for memory CD8 T cells differentiation and survival [137], while an 'altruistic' down-regulation of the receptor after IL-7 exposure has been proposed for naïve T cells in order to maximise the number of cells receiving the survival signal through the IL-7 receptor [258]. Although there were no direct indications from microarray data that IL-7Rα was differentially expressed on the two resting memory CD4 T cell populations, the level of expression of this molecule was analysed by FACS analysis because both the Akt and the Stat5 pathways are down-stream of the IL-7 receptor [251] and because IL-7Rα level of expression on T cells has been reported to parallel the level of expression of Bcl-2 [286]. In line with these observations, resting memory CD4 T cells recovered from MHC- hosts expressed a reduced level of IL-7Rα compared to memory CD4 T cells recovered from MHC+ hosts (Fig. 17a, right), showing again an intermediate phenotype between that of A1 naïve and memory CD4 T cells from MHC+ hosts. Fig. 17b shows the MFI for Bcl-2 (left) and IL-7Rα (right) on memory CD4 T cells from MHC+ and MHC- hosts. Values for A1 naïve CD4 T cells are reported as reference.

Microarray analysis of gene expression highlighted several transcripts differentially expressed in memory CD4 T cells recovered from MHC+ and MHC- hosts. Among all the interesting genes, some candidates appeared to be particularly relevant as they were likely to be involved, directly or indirectly, in processes that could be linked to the functional impairment

observed in memory CD4 T cells generated in the absence of MHC class II. Whenever possible, those candidates were further validated by an alternative method, such as real time PCR or FACS analysis. The results obtained by real time PCR and FACS analysis supported in general the data generated by microarray (with the exception of CD24), as summarised on table 4.

Table 4: summary of the validation of the data obtained from analysis of gene expression.

Genes differentially expressed by analysis of mRNA levels were tested either at mRNA levels by real time PCR or at protein levels by FACS analyses on ex vivo cells or upon in vitro activation. The accordance of results between different methods of analysis is reported.

| Gene Affymetrix | Validation method | Accordance |
|---|---|---|
| IL-2 | Real time PCR *ex vivo*<br>FACS upon *in vitro* stimulation | ✓<br>✓ |
| Mapk9 | Real time PCR *ex vivo* | ✓ |
| CD24a | FACS *ex vivo* | × |
| Itga4 | FACS *ex vivo* | ✓ |
| Stat5a/b | FACS *ex vivo*<br>(on phosphorylated form) | ✓ |
| Akt1 | FACS *ex vivo*<br>(on phosphorylated form) | ✓ |

| | | |
|---|---|---|
| Bcl-2 | FACS *ex vivo* | ✓ |

Overall, memory CD4 T cells generated in MHC- hosts appear to have reduced expression of several key molecules implicated in T cell survival compared to memory CD4 T cells recovered from MHC+ hosts. In respect of all the molecules evaluated so far memory CD4 T cells from MHC- hosts exhibit an intermediate phenotype between the A1 naïve CD4 T cell population and the memory CD4 T cell population recovered from MHC+ hosts.

### 3.1.2.3 Functional analysis of memory T cell responses with respect to candidate molecules.

In accordance with the data presented above, in steady-state conditions, memory CD4 T cells generated in MHC- hosts compared to memory CD4 T cells generated in MHC+ hosts showed reduced levels of molecules known to affect memory T cell survival, such as Bcl-2 and IL-7Rα, reduced levels of molecules crucial during T cell activation, such as IL-2, and reduced levels of molecules with central roles in T cell survival as well as in T cell activation, such as Akt1 and Stat5.

The differential expression of molecules likely to shape T cell responses upon activation suggested a link between the resting and the activation status which might explain the functional differences observed in the two cell populations of this study.

The following experiments were devised to evaluate the functional impairment of memory CD4 T cells generated in MHC- hosts upon re-stimulation focussing on IL-2 expression and Akt1 activity as read-outs of memory T cell responses.

One of the characteristics that distinguishes memory T cells from naïve T cells is their ability to produce a faster and stronger immune response upon antigen encounter. IL-2 production is a good example to illustrate this property of memory T cells as the memory T cell population, in contrast to the naïve one, rapidly makes elevated IL-2 upon TCR triggering. Memory CD4 T cells recovered from MHC- hosts expressed reduced amounts of IL-2 at the *m*RNA level according to analysis of gene expression. To evaluate if the difference observed in steady-state at the RNA level was still present upon cells stimulation, IL-2 synthesis and secretion were analysed upon *in vitro* activation. Memory CD4 T cells recovered from MHC+ and MHC- hosts and A1 naïve CD4 T cells were stimulated *in vitro* for 6 hours using PdBU and ionomycin in the presence of brefeldin A and IL-2 synthesis was assessed by intracellular staining (Fig. 18a). In line with the result obtained at the *m*RNA level, memory CD4 T cells from MHC- hosts showed reduced percentages of IL-2 producing cells compared to those detected on memory CD4 T cells from MHC+ hosts. As PdBU and ionomycin activate CD4 T cells bypassing the TCR, this strong stimulation highlighted an intrinsic difference between the two cell populations, but not necessarily reflected different properties in a physiological context. In fact, the higher percentage of IL-2 producers in the CD4 T cell population recovered from MHC+ hosts could well reflect their higher amount of *m*RNA. To verify if memory CD4 T cells from MHC- hosts showed an impairment in IL-2 production upon TCR

triggering or whether the two memory CD4 T cell populations simply followed different kinetics, memory CD4 T cells from both hosts were stimulated *in vitro* with plate-bound anti-CD3 (Fig. 18b, left) in the presence or absence of plate-bound anti-CD28 (Fig. 18b, right) in a 48 hours assay. In line with the previous results, memory CD4 T recovered from MHC- hosts secreted lower amounts of IL-2 than memory CD4 T recovered from MHC+ hosts. The data indicated that the ability to secrete IL-2 was truly compromised in memory CD4 T cells from MHC- hosts, confirming the data already published [129].

According to the microarray results, the Akt pathway was down-regulated in memory CD4 T cells recovered from MHC- hosts compared to memory CD4 T cells recovered from MHC+ hosts. Akt activity affects multiple aspects of T cell survival and activation. We decided to focus directly on the study of some of the molecules that, being down-stream Akt can be used as read-out of successful signalling through the pathway itself. For this purpose, the level of phosphorylation of the small ribosomal protein S6 and the *m*RNA levels of *Glut1* were analysed during *in vitro* re-stimulation of memory CD4 T cells generated in MHC+ and MHC- hosts. S6 is one of the members required for the assembly of the ribosomal complex necessary for protein synthesis [213]. In T cells, S6 phosphorylation is mainly due to S6K1 activation which is induced by the TCR in an Akt dependent manner. As a result, small and metabolically inactive T cells increase protein synthesis and reach the size required for proliferation and for optimal effector functions [205]. Glut1 is the main glucose transporter in peripheral T cells [287] and its up-regulation is induced upon TCR activation [219]. Akt has a major role in glucose metabolism as it not only regulates *Glut1* gene transcription [288], but also Glut1 cellular

localisation [289]. By FACS analysis, memory CD4 T cells generated in MHC- hosts showed reduced S6 phosphorylation after a short (30 minutes) *in vitro* activation period (Fig. 19a) with PdBU and ionomycin (left) or anti-CD3 (right) compared to memory CD4 T cells generated in MHC+ hosts. Moreover, the analysis of *Glut1 m*RNA expression from FACS sorted memory CD4 T cells recovered from MHC+ and MHC- during a 3 day *in vitro* stimulation with anti-CD3 and anti-CD28 showed that memory CD4 T cells from MHC- hosts were not able to sustain the same level of expression of *Glut1* expression than memory CD4 T cells from MHC+ hosts (Fig. 19b).

Therefore, along with the functional impairment observed in IL-2 synthesis in response to stimulation, the reduction in the metabolic rate assessed by the level of S6 phosphorylation and *Glut1* up-regulation upon activation suggested that the quality of the response of memory CD4 T recovered from MHC- hosts was compromised at several levels.

### 3.1.3 Survival of memory T cells

The reduction of the expression of molecules involved in T cells survival such as Akt1, Bcl-2 and IL-7Rα suggested that memory CD4 T cells maintained in the absence of MHC class II molecules might have a survival defect *in vivo*. This at first glance contradicted the finding that similar numbers of memory cells are recovered from both hosts. However, previous analysis of BromoDeoxyUridine (BrDU) incorporation showed that memory CD4 T cells divided at a higher rate in the absence of MHC class II interaction *in vivo* [129]. This suggested that increased cell proliferation compensates for increased death in the MHC- hosts. Two experiments were set up to further investigate these results. In one experiment memory CD4 T cells in MHC+ and MHC- hosts were compared in respect to their turnover *in vivo* in the memory phase. In another experiment, memory CD4 T cells from MHC- hosts were challenged to compete with memory CD4 T cells from MHC+ in the same environment to analyse the extent of their survival defect.

#### 3.1.3.1 Decreased survival of memory CD4 T cells in the absence of MHC class II molecules

To study the turnover of memory CD4 T cells generated in MHC+ and MHC- hosts, we decided to assess their DNA content by PI staining (Fig. 20). PI is a dye that intercalates into cellular DNA. The intensity of the PI signal directly correlates with the DNA content, such that cells that are actively dividing show twice the intensity of the PI signal as result of DNA duplication than resting cells, while cells that are in apoptosis show lower DNA content as

result of active DNA fragmentation and degradation. In steady-state condition, the majority of the CD4 T cells in the periphery are found arrested in the $G_0/G_1$ phase of the cell cycle. Nevertheless, a small fraction of memory CD4 T cells cycle to maintain the normal homeostasis. While the percentage of dividing naïve CD4 T cells is negligible in the periphery, the percentage of cycling T cells in the thymus is reported to be ~ 10%. The analysis of the DNA content of sorted naïve CD4 T cells and total thymocytes showed as expected, a very low percentage of cell with high DNA content (cycling cells) within the naïve CD4 T cell population and a high proportion of cycling cells within the thymocytes (Fig. 20a and b). Memory CD4 T recovered from MHC- hosts showed a slightly higher percentage of cycling cells compared to memory CD4 T cells from MHC+ hosts (Fig. 20c and d). Interestingly, memory CD4 T cells from MHC- hosts showed a substantial percentage of cells with decreased DNA content indicative of apoptosis which was not seen in memory CD4 T recovered from MHC+ hosts. This result indicated that memory CD4 T cells in MHC- hosts die at a higher rate compared to memory CD4 T cells in MHC+ hosts. Increased cell death is compensated by accelerated division and thus recovery of cells appears to be the same for both populations.

### 3.1.3.2 Memory CD4 T cells from MHC+ hosts do not have a competitive advantage in co-transfer experiments

According to the analysis of various parameters such as expression of several survival molecules and DNA fragmentation, memory CD4 T cells from MHC- hosts showed a reduced survival capacity compared to memory CD4 T cell from MHC+ hosts. In order to assess whether memory CD4 T cells from MHC+ hosts would have a competitive advantage over memory CD4 T cells from MHC- hosts with respect to homeostatic equilibrium, memory CD4 T cells from both hosts were challenged in co-transfer experiments. The percentage and absolute numbers of both cell populations were assessed after transfer into lymphopenic hosts either alone or together. For this experiment, naïve A1 (Ly5.2) CD4 T cells were used to generate memory CD4 T cells in MHC+ hosts, while naïve A1 (Ly5.1) CD4 T cells were used to generate memory CD4 T cells in MHC- hosts. The use of Ly5.2 and Ly5.1 as allotypic markers allowed the identification of each memory CD4 T cell subset in the co-transfer. Memory CD4 T cells were recovered 6 weeks after immunisation and cells were transferred into syngeneic MHC+ H-$2^k$ secondary hosts. We decided to use syngeneic MHC+ mice as secondary adoptive hosts in order to enhance possible difference in T cell homeostatic expansion and survival. In fact, homeostatic proliferation of memory CD4 T cells has been shown to be higher in syngeneic selecting MHC hosts than non selecting allogeneic MHC hosts [290]. The homeostatic proliferation was assessed by CFSE staining. CFSE is a dye that binds to cytoplasmatic proteins. Every time a labelled cell divides, the CFSE content of the daughter cells is halved. Following CFSE dilution it is therefore

possible to establish how many divisions a population of cells has gone through. One group of MHC+ hosts received CFSE labelled memory CD4 T cells from MHC+ hosts (Ly 5.2), another group received CFSE labelled memory CD4 T cells from MHC- hosts (Ly 5.1) while a third group was injected with CFSE labelled memory CD4 T cells from MHC+ hosts (Ly5.2) and from MHC- hosts (Ly 5.1) mixed in a 3:1 ratio (Fig. 21a and b upper line). The ratio chosen for the co-transfer aimed to enhance a possible disadvantage of memory CD4 T cells generated in MHC- hosts. Two mice per group were killed 7 and 49 days after the secondary transfer and the T cell recovery as well as the ratio memory CD4 T cells from MHC+: MHC- hosts was analysed by FACS.

Seven days after secondary transfer, both memory CD4 T cell populations recovered from syngeneic MHC+ secondary hosts had divided at least 7 times as demonstrated by the fact that it was not possible to detect the CFSE dye by FACS analysis (data not shown). As shown by numbers of CD4 T cell recovered (Fig. 21a, middle panel), no differences were observed between memory CD4 T cells from MHC+ and MHC- hosts transferred into MHC+ hosts whether they were transferred alone or co-transferred. Memory CD4 T cells from MHC+ hosts expanded to a larger extent than memory CD4 T cells from MHC- hosts either when secondarily transferred into MHC+ hosts alone or co-transferred with memory CD4 T cells from MHC- hosts as judged by the CD4 T cell recovery 7 days after transfer. In contrast, memory CD4 T cells from MHC- hosts did not expand significantly when transferred alone into MHC+ hosts, but seemed to proliferate to a larger extent when co-transferred with memory CD4 T cells from MHC+ hosts. At a later time point (49 days, Fig. 21a lower panel) the total number of cells recovered from the

recipients that received memory CD4 T cells from MHC+ hosts and those that received the co-transfer were comparable, while a slightly reduced number of CD4 T cells was recovered from MHC+ hosts that received CD4 T cells from MHC- deficient hosts. In the co-transfer, the maintenance of the ratio MHC+:MHC- as 3:1 from the initial transfer to the recovery 7 and 49 days later suggests that memory cells from MHC+ hosts did not out-compete those from MHC- hosts during equilibration for homeostasis (Fig. 21, right).

A similar experiment was repeated, but using MHC+ H-$2^b$ hosts as secondary adoptive hosts and analysed the number of T cells recovered and CFSE profile 7 days after transfer. One group of MHC+ hosts received CFSE labelled memory CD4 T cells from MHC+ hosts (Ly 5.2), another group received CFSE labelled memory CD4 T cells from MHC- hosts (Ly 5.1) while a third group was injected with CFSE labelled memory CD4 T cells from MHC+ hosts (Ly5.2) and from MHC- hosts (Ly 5.1) mixed in 3:1 ratio (Fig. 22a, left). Seven days after the secondary transfer, memory CD4 T cells from MHC+ hosts expanded to a larger extent than memory CD4 T cells from MHC- hosts either when secondarily transferred into MHC+ hosts alone or co-transferred with memory CD4 T cells from MHC- hosts (Fig. 23a). In contrast, memory CD4 T cells from MHC- hosts did not expand significantly when transferred alone into MHC+ hosts or when co-transferred with memory CD4 T cells from MHC+ hosts. In this experiment cell divisions were still detectable by CFSE dilutions because the avidity for H-$2^b$ is lower than that for syngeneic H-$2^k$ and as a consequence cell proliferation is reduced [290]. Memory CD4 T cells from MHC- hosts divided at a higher rate than memory CD4 T cells from MHC+ hosts when transferred on their own into

lymphopenic hosts (Fig. 22b, left). In co-transfer the division rate was only marginally higher in memory CD4 T cells from MHC- hosts than memory CD4 T cells from MHC+ hosts (Fig. 22b, right). The higher rate of cell division observed in memory CD4 T cells from MHC- hosts vs MHC+ hosts did not correlate with an increase cell recovery, pointing again at impaired survival of memory CD4 T cells generated in MHC- hosts.

Although these experiments were limited by the small number of mice assessed for each time point and were only done once, we concluded that the preservation of the ratio of the two memory subsets from initial transfer to recovery 49 days later suggests that memory cells from MHC+ hosts do not have a competitive advantage over those from MHC- hosts during equilibration for homeostasis.

### 3.2 Early establishment of phenotypic and functional defects in memory CD4 T cells in the absence of non cognate MHC class II interactions

To assess at what stage in memory formation MHC class II absence accounted for the functional defect observed in memory CD4 T cells from MHC- hosts, A1 naïve CD4 T cells were co-injected with H-Y pulsed syngeneic DCs as previously described and recovered 14, 28 and 42 days after transfer. At each time point, CD4 T cell recovery was assessed along with phenotypic and functional analysis. Again the number of CD4 T cells recovered from MHC- hosts at all three time points was not significantly different from the numbers of CD4 T cells recovered from MHC+ ones (data

not shown). Already 14 days after immunisation and throughout the duration of this time course experiment, CD4 T cells transferred into MHC- hosts showed an intermediate phenotype in terms of Bcl-2 expression between the level of expression observed in A1 naïve CD4 T cells and the level of expression observed in CD4 T cells generated in MHC+ hosts (Fig. 23, left). As discussed above, IL-7Rα expression on T cells parallels the level of expression of Bcl-2 [286] and occupies a central role in the debate on memory T cell generation. Moreover, the level of expression of IL-7Rα provides additional information in terms of the activation status of T cells, as activated cells typically down-regulate the surface expression of this molecule. At day 14 after immunisation, the level of IL-7Rα expression in CD4 T cells from MHC- hosts was slightly lower than the level of expression of this molecule observed in naïve CD4 T cells and CD4 T cells transferred into MHC+ hosts, but gained an intermediate position between naïve and memory from MHC+ hosts at later time points (Fig. 23, right).

To assess the quality of the memory generated in the two hosts, memory CD4 T cells recovered from MHC+ and MHC- hosts 14 days after immunisation were re-stimulated *in vitro* and their response in terms of pS6 and IL-2 synthesis were analysed by intracellular staining. Memory CD4 T cells generated in MHC- hosts showed reduced S6 phosphorylation after a short (30 minutes) *in vitro* activation period (Fig. 24a) with PdBU and ionomycin (left) or anti-CD3 (right) compared to memory CD4 T cells generated in MHC+ hosts. Similarly to what observed at later time points, memory CD4 T cells from MHC- hosts were not able to equal the percentage IL-2 producing cells reach by memory CD4 T cells from MHC+ hosts after 6

hours using PdBU and ionomycin in the presence of brefeldinA (Fig. 24b). Memory cells were also activated *in vitro* with anti-CD3 with or without anti-CD28. Reproducing the results observed at later time points, 14 days after immunisation memory CD4 T recovered from MHC- hosts showed an impairment in IL-2 production upon plate-bound anti-CD3 stimulation alone (Fig. 24c, left) or in the presence of plate-bound anti-CD28 (Fig. 24c, right).

Taken together these results showed that since the early stages of the memory phase, CD4 T cells from MHC- hosts exhibited compromised phenotypic and functional properties which persisted in later time points. The difference observed already at the early stages of the memory phase suggested that CD4 T cells injected into MHC- hosts followed a different pattern of differentiation that precluded the acquisition of full memory properties. To verify this hypothesis, the development of the effector phase in the two hosts was analysed.

### 3.3 Effector phase in MHC+ and MHC- hosts

In order to evaluate events in the effector phase, A1 naïve CD4 T cells were co-injected with H-Y peptide pulsed DCs into either MHC+ or MHC- hosts and mice were analysed 4, 8, and 14 days after.

As shown in Fig. 25, similar numbers of CD4 T cells were recovered from both hosts at day 4, but at day 8 CD4 T cells seemed to have expanded to a lesser extent in MHC- hosts. At day 14 after transfer into adoptive hosts, the numbers of CD4 T cells recovered reflected the numbers usually recovered in the memory phase, in agreement with what was previously observed.

The activation status achieved by transferred T cells was assessed by measuring the expression of various activation molecules. There was no significant difference in the level of expression of the early activation marker CD69 at any of the time points tested (Fig. 26). In both hosts, CD4 T cells up-regulated the expression of CD44 throughout the effector phase, although CD4 T cells from MHC- hosts followed a slower kinetic than those from MHC+ hosts (Fig. 27). IL-7Rα expression was down-regulated to a similar degree in CD4 T cells from both hosts during the acute effector stage of the immune response in accordance with reduced expression of this marker following activation (Fig. 28). CD71, the transferrin receptor and CD98 (4F2 antigen or Ly-10), the common heavy chain subunit component of the aminoacid transporter, were expressed at lower levels in CD4 T cells from MHC- hosts vs MHC+ hosts (Fig. 29 and 30). These data indicated that metabolic activity, which is necessary to sustain activation (and possibly proliferation) was reduced in these cells. However CD71 and CD98 up-regulation were transient since the expression of both molecules was down-regulated at day 8 and 14. Stat5 phosphorylation on day 4 of the effector phase was only evident in a subset of CD4 T cells transferred into MHC- hosts whereas virtually all CD4 T cells transferred into MHC+ hosts displayed phosphorylated Stat5 at this stage (Fig. 31). Again Stat5 phosphorylation was only detected early after activation.

At each time point, CD4 T cells recovered from MHC+ and MHC- hosts were analysed for IL-2 production upon a short re-stimulation *in vitro* with PdBU and ionomycin in the presence of brefeldinA (Fig. 32). Effector cells recovered at day 4 of the immune response were refractory to re-stimulation

and no intracellular IL-2 was detected. By day 8 and 14 after transfer, CD4 T cells from MHC+ hosts showed a high proportion of IL-2 producers comparable with that seen in established memory CD4 T cells. In contrast, CD4 T cells from MHC- hosts showed impairment in IL-2 production already on day 8 after activation. As expected, this reduced capability to synthesise IL-2 was sustained at day 14 and it was similar to that observed at later time points in the memory phase. The reduced levels of activation observed in CD4 T cells recovered from MHC- hosts compared to CD4 T cells from MHC+ hosts at day 4 after activation was surprising, since at that stage co-transferred antigen presenting cells were still present.

The survival of the transferred DCs population was assessed using the H-2K$^k$ antibody which is specific for the haplotype of the donor DCs (Fig. 33a). The number of adoptively transferred DCs recovered from MHC+ and MHC- hosts was similar at all three time points (Fig. 33b) indicating that there was no difference in their survival in MHC+ and MHC- hosts.

Thus, it appeared that another factor than antigen presentation was responsible for the reduced level of activation observed in CD4 T cells transferred into MHC- hosts. We hypothesised that the presence of non-cognate MHC class II, which is not directly involved in antigen presentation, may play an important role in the functional differentiation of activated CD4 T cells.

### 3.4 Rescue experiments

To address the question whether the loss of function in memory CD4 T cells residing in MHC- hosts was reversible or could be prevented, a series of experiments using bone marrow chimeras was started with the aim of reconstitute class II expression in MHC- hosts. MHC- and MHC+ hosts were reconstituting with bone marrow cells from donor mice of H-$2^k$ as well as H-$2^b$ backgrounds as both MHC class II molecules equally maintain memory CD4 T cell functionality [129]. RAG1 ko mouse transgenic lines were used as bone marrow donors to provide MHC class II expressing DCs. Due to a modification in the RAG-1 gene, these mice do not develop T nor B cells. To increase the amount of MHC class II available as well as the diversity of the cell subsets supplying MHC class II molecules, chimeras were also generated using TCRα ko transgenic strains as bone marrow donors. In this case, the reconstituted hosts were deficient in T cells, due to a modification on the gene encoding the α chain of the TCR, but developed normal B cells expressing additional MHC class II to the MHC class II supplied by DCs.

### 3.4.1 Bone marrow chimeras: reconstitution with DCs from MHC + donors

In these experiments, we considered that a period of time of 10 days was necessary for the bone marrow to establish and provide MHC class II expressing peripheral cells.

To determine whether MHC class II interactions provided by DCs were sufficient to prevent CD4 T cell loss of function in memory T cells generated in MHC- hosts an experiment was set up, which allowed time for the bone marrow transplant to establish before the co-injection of 2 x $10^6$ naïve CD4 T

cells and H-Y pulsed DCs. 10 x $10^6$ bone marrow cells from Rag1 ko H-$2^k$ mice were transferred into MHC+ and MHC- hosts 28 days before immunisation and mice were analysed 35 days after immunisation (Fig. 34a). Matching control MHC+ and MHC- hosts that only received the adoptive co-transfer of naïve CD4 T cells and H-Y peptide pulsed DCs were set up as well. Similar numbers of memory CD4 T cells were recovered from MHC+ and MHC- hosts that received the bone marrow chimeras and the controls MHC+ and MHC- hosts (Fig. 34b).

Memory CD4 T cells transferred into MHC- hosts reconstituted with bone marrow chimeras from RAG1 ko mice resumed the phenotype characteristic of memory CD4 generated in MHC- hosts in terms of IL-7Rα (Fig. 35). When analysed for function by *in vitro* activation with anti-CD3 in the absence or presence of anti-CD28 (Fig. 36, left and right respectively), memory CD4 T cells from MHC- hosts that received the bone marrow cells from RAG1 ko donors did not produce levels of IL-2 comparable to memory CD4 T cells from MHC+ hosts or MHC+ hosts that received the bone marrow transplant from RAG1 ko mice. This result showed that the function of memory CD4 T cells transferred into MHC- hosts was not rescued in the chimeras.

As the animals that received the bone marrow transplant from MHC class II positive donors were not irradiated prior to the transfer, it was possible that the establishment of the DCs population from the transplanted bone marrow had to compete with the endogenous DCs precursors for space (survival and maturation factors). In this scenario, a partial reconstitution of the host could have failed to provide enough MHC class II molecules to rescue memory

CD4 T cell function. For this reason a new experiment was set up where mice were irradiated prior to bone marrow transplant.

MHC+ and MHC- hosts were sub-lethally irradiated (800 rads) prior to the injection of 15 x $10^6$ bone marrow cells from Rag1 ko (Fig. 37). As the background of the donors was H-$2^b$, the reconstitution of irradiated MHC+ and MHC- hosts resulted in the generation of hosts expressing MHC class II of the H-$2^b$ background.

Thirty days after the irradiation and injection of bone marrow cells, one mouse per group was killed and analysed for DC chimerism. As the donor bone marrow had successfully engrafted and produced mature DCs detectable in the spleen of the adoptive hosts (Fig. 38), the mice were immunised by co-injection of 2 x $10^6$ naïve CD4 T cells with H-Y peptide pulsed bone marrow derived DCs and the kinetics of the establishment of the CD4 T cells population in the bone marrow chimeras was followed.

Only one mouse per group was analysed at day 4 of the effector phase. At this time point, the number of CD4 T cells recovered from the MHC+ and MHC- chimeras was similar to the number of CD4 T cells recovered from MHC+ and MHC- hosts (Fig. 39, upper line), but at day 8 CD4 T cells recovered from both MHC+ and MHC- chimeras were strongly reduced compared to recovery at day 4 (Fig. 39, lower line). Both MHC+ and MHC- hosts are on a γ chain knockout background, which means that they lack NK cells. For this reason, the rejection of H-$2^k$ cells injected into H-$2^b$ adoptive hosts has never been a matter of concern in our experimental setting up.

Nevertheless, the same principle did not apply in this case where a clear drop in the recovery of CD4 T cells from both RAG1 ko H-$2^b$ chimeras compared to the recovery from MHC+ and MHC- hosts was observed 8 days after the adoptive transfer. The loss of more than 90% of the cells in a period of 4 days suggested that NK cells derived from both Rag1 ko H-$2^b$ chimeras had rejected the CD4 T cells adoptively transferred, due to their differences in the MHC haplotypes.

### 3.4.2 Bone marrow chimeras: reconstitution with B cells and DCs from MHC+ donors

In the previous experiments MHC+ and MHC- hosts were reconstituted with bone marrow cells from RAG1 ko donors, which provided expression of MHC class II molecules from DCs only. To determine whether MHC class II expression on B cells in addition to DCs would rescue the function in memory CD4 T cell generated in MHC- hosts, we used TCRα ko H-$2^b$ mice as bone marrow donors which provided the establishment of a normal B cells compartment in the MHC+ and MHC- recipients. As the hosts lacked an endogenous B cell population, it was not necessary to create space for the B cells from the bone marrow transfer and therefore the hosts were not irradiated. On the other hand, the DCs from the transplanted bone marrow would have had to compete with the endogenous precursors cells for DCs possibly diluting the strength of MHC class II reconstitution in this compartment. To prevent NK rejection of H-$2^k$ cells by H-$2^b$ NK from the bone marrow graft, ~ 6 x $10^6$ bone marrow cells from α ko H-$2^b$ donors were

injected into MHC- and MHC+ hosts 4 days after the adoptive co-transfer of A1 naïve CD4 T cells and H-Y peptide pulsed DCs (Fig. 40a). Because of the presence of some mature B cells in the donor bone marrow at the moment of the transfer, a certain amount of MHC class II could already be provided to the CD4 T cells at the moment of transplantation. Matching control MHC+ and MHC- hosts that only received the adoptive transfer of naïve CD4 T cells and H-Y peptide pulsed DCs were set up as well. To check for the establishment of the bone marrow transplant, mice were bled 14 days after the injection of bone marrow cells from α ko and analysed by FACS for presence of circulating B cells. Similar percentages of mature B cells were detected in blood samples from MHC+ and MHC- hosts, indicating that the bone marrow transfer had successfully engrafted (Fig. 40b). Memory CD4 T cells from the 4 groups of adoptive hosts were analysed 5 weeks after transfer. As expected, due to the bone marrow transplant, the spleen cellularity was significantly increased to similar levels in both hosts that received the bone marrow compared to the hosts that did not (Fig. 40c, left). The numbers of CD4 T cells recovered were similar in all groups (Fig. 40c, right). As shown by the FACS histograms (Fig. 41a) and by the MFI (Fig. 41b), memory CD4 T cells from both α ko H-$2^b$ chimeras showed a reduction of the expression of Bcl-2 (left, lower line) and IL-7Rα (right, lower line) compared to memory CD4 T cells from MHC+ (upper line).

Memory CD4 T cell functionality was checked by *in vitro* activation. To assess memory CD4 T cells function upon activation, it was necessary to deplete the B cells from the total splenocytes of the bone marrow chimeras as, in their presence, CD4 T cells from both bone marrow reconstituted hosts

showed an enhanced IL-2 secretion upon *in vitro* stimulation with anti-CD3 in the absence or presence of anti-CD28 (Fig. 42a), presumably due to increased antibody binding via B cell Fc receptors. When the experiment was repeated depleting the B cells, the level of IL-2 secreted by memory CD4 T cells recovered from the MHC- α ko bone marrow chimeras were similar to those secreted from memory CD4 T cells from MHC- hosts (Fig. 42b), indicating that the experiment did not succeed in its purpose to prevent the loss of function in MHC- reconstituted hosts.

### 3.4.3 Continuous injection of bone marrow derived DCs

Since all rescue experiments with bone marrow chimeras were complicated by various technical problems, a final set of experiments aimed to assess the potential rescue by MHC class II in a more direct manner. Effector CD4 T cell showed reduced levels of activation in MHC- hosts compared to MHC+ hosts already 4 days after the start of the immune response, when the co-transferred antigen presenting cells were still present in similar numbers. As previously mentioned, one factor that may have been responsible for the difference in T cell activation observed in the two hosts was that in addition to the H-Y peptide loaded MHC class II molecules, non-cognate interactions with endogenous MHC class II molecules were available in the MHC+ hosts but not in the MHC- hosts. It has been proposed that the basic unit for CD4 T cell activation is an heterodimer of cognate peptide-MHC and endogenous peptide-MHC complexes [149] [291]. These results well fitted with data suggesting a role for endogenous (non-cognate) MHC class II interactions in maintaining naïve CD4 T cell antigenic sensitivity [58] and indicate that such

interaction is important also for successful CD4 T cell activation. While there are no reasons to question CD4 T cell accessibility to such complexes in MHC+ hosts, CD4 T cells interactions with non-cognate MHC class II molecules would be completely absent in the MHC- hosts, where most if not all the syngeneic MHC class II molecules on transferred DCs would be occupied by H-Y peptide and where the endogenous DCs would not express any MHC class II molecules.

Therefore, 2-4 x $10^6$ bone marrow derived DCs from the MHC class II competent mice were injected every three days into both MHC+ and MHC- hosts. This protocol offered the advantage not only to test if the interaction required by CD4 T cells was dependent on non cognate MHC class II signalling but also whether DCs were sufficient to provide such signals. Three days prior to immunisation, 5 x $10^6$ bone marrow derived DCs were injected into MHC+ and MHC- hosts. 2 x $10^6$ CD4 T cells, 0.5 x $10^6$ H-Y peptide pulsed syngeneic bone marrow derived DCs and 2.5 x $10^6$ allogeneic bone marrow derived DCs were co-transferred into MHC+ and MHC- hosts. Control MHC+ and MHC- hosts received only the co-transfer of CD4 T cells with H-Y peptide pulsed bone marrow derived syngeneic DCs. All mice were analysed 14 days after immunisation.

As illustrated in Fig. 43, the numbers of CD4 T cells recovered from MHC+ and MHC- hosts that received fresh bone marrow derived DCs every three days were slightly lower than the numbers of CD4 T cells recovered from control MHC+ and MHC- hosts.

CD4 T cells recovered from all adoptive hosts up-regulated CD44 to a similar extent (Fig. 44, left). IL-7Rα was down-regulated in MHC- hosts compared to

MHC+ hosts, whether they had received fresh bone marrow derived DCs every 3 days or not (Fig. 44, right). CD4 T cells were challenged in a 2 days *in vitro* activation with anti-CD3 in the presence or absence of anti-CD28 (Fig. 45). Memory CD4 T cells from MHC+ hosts that received fresh bone marrow derived DCs every 3 days were not as efficient as memory CD4 T cells from MHC+ hosts, as if the continuous injection may had worsened their functionality. Memory CD4 T cells from MHC- hosts that received fresh bone marrow derived DCs every three days secreted IL-2 to a similar level than memory CD4 T cells from MHC- hosts. In summary, CD4 T cells from MHC+ and MHC- hosts did not benefit from the supply of fresh bone marrow derived DCs. Once again, the failure of this experiment maybe due to technical difficulty to control the various parameters involved in such complicated issue, such as the co-localisation of the injected un-pulsed DCs in the areas occupied by the T cells [148] and the timing of DCs and T cells interactions.

## FIGURES

Fig. 5 a) and b)

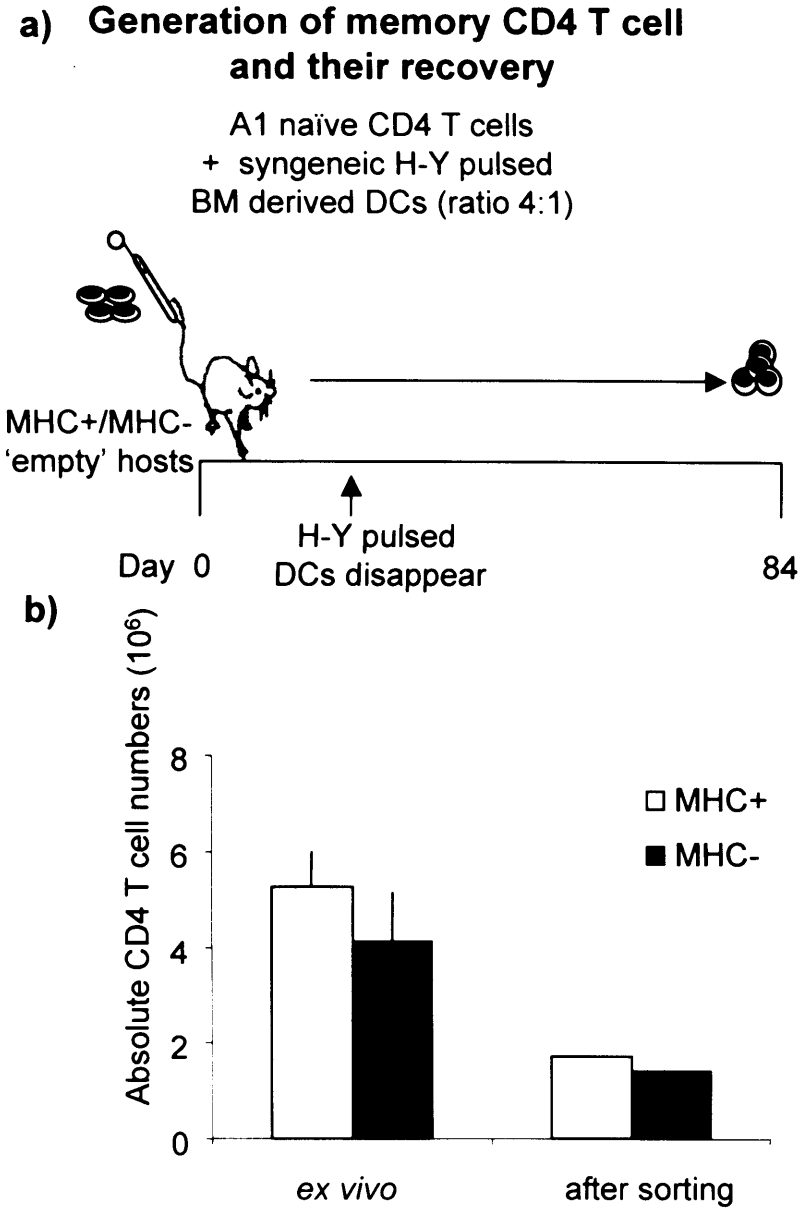



Figure 5 a) and b)

a) Experimental design; b) CD4 T cells recovery: T cell recovery was calculated from percentages of CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation of CD4 T cell per mouse recovered ex vivo (left) and total number of memory CD4 T cells recovered after FACS sorting (right) from 16 pooled MHC+ hosts (white bars) and 12 pooled MHC- hosts (grey bars). Data representative of 5 independent experiments.

Fig. 6 a) and b)

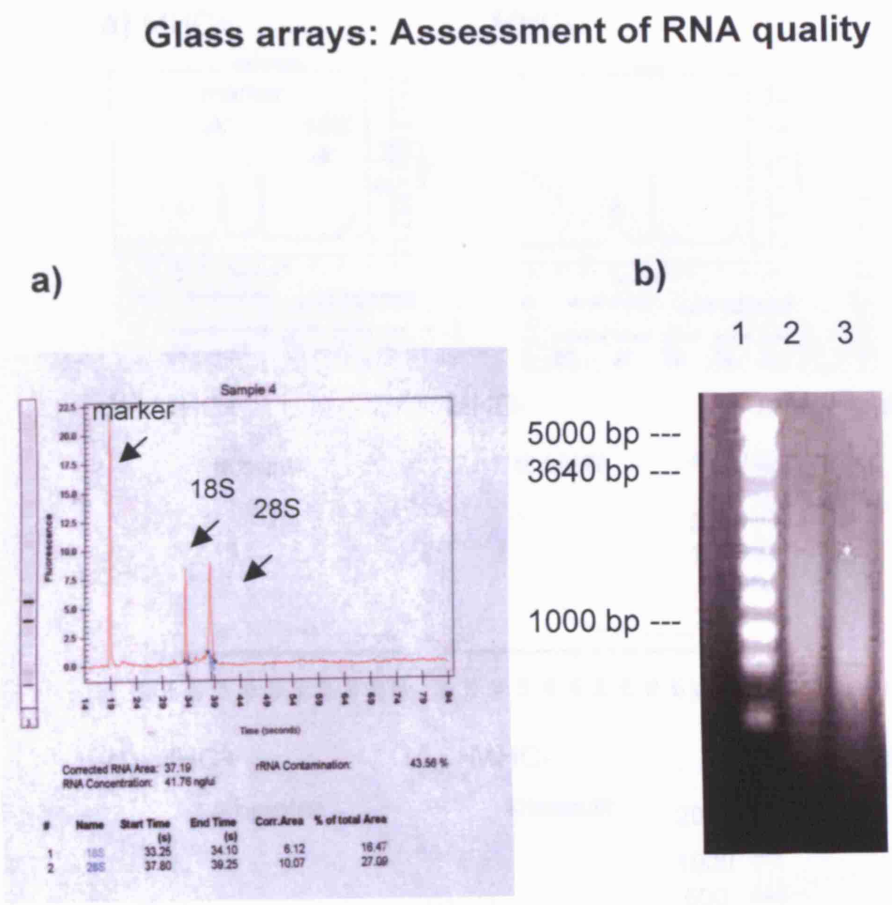



Figure 6 a) and b)

a) Example of electropherogram of total RNA from FACS sorted memory CD4 T cells isolated from MHC+ hosts. b) Efficiency of in vitro transcription amplification assessed on 1% agarose gel. RNA Markers (lane 1), aRNA generated from FACS sorted memory CD4T cells from MHC+ hosts (lane 2) and aRNA generated from FACS sorted memory CD4 T cells from MHC- hosts (lane 3).

Fig. 7 a), b) and c)

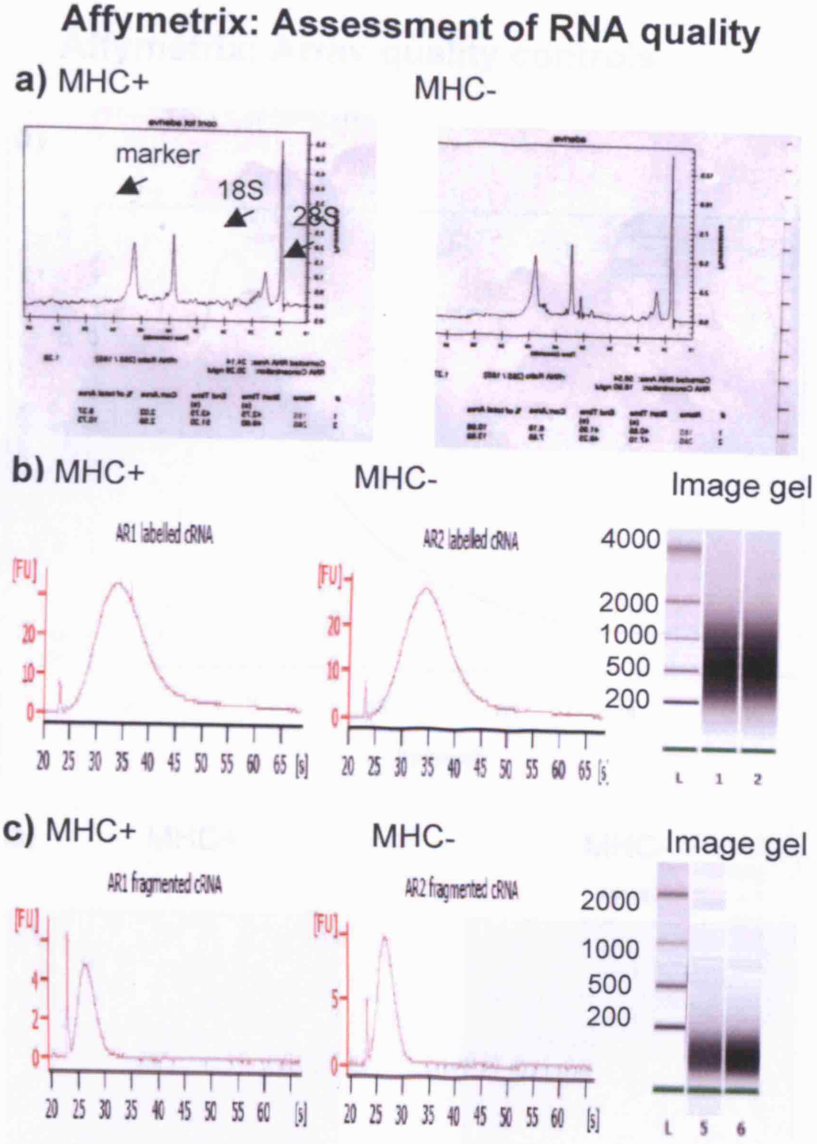



Figure 7 a), b) and c)

a) Electropherograms of total RNA from memory CD4 T cells from MHC+ (left) and MHC- (right) hosts; b) electropherograms of cRNA generated from FACS sorted memory CD4 T cells from MHC+ hosts (left) and MHC- hosts (centre); image gel (right) of cRNAs generated from FACS sorted memory CD4 T cells from MHC+ hosts (lane 1) and MHC- hosts (lane 2) and RNA 6000 Ladder (L). c) electropherograms of fragmented cRNA generated from FACS sorted memory CD4 T cells from MHC+ hosts (left) and MHC- hosts (centre); image gel (right) of cRNAs generated from FACS sorted memory CD4 T cells from MHC+ (lane 5) and MHC- (lane 6) hosts and RNA 6000 Ladder (L).

Fig. 8 a) and b)

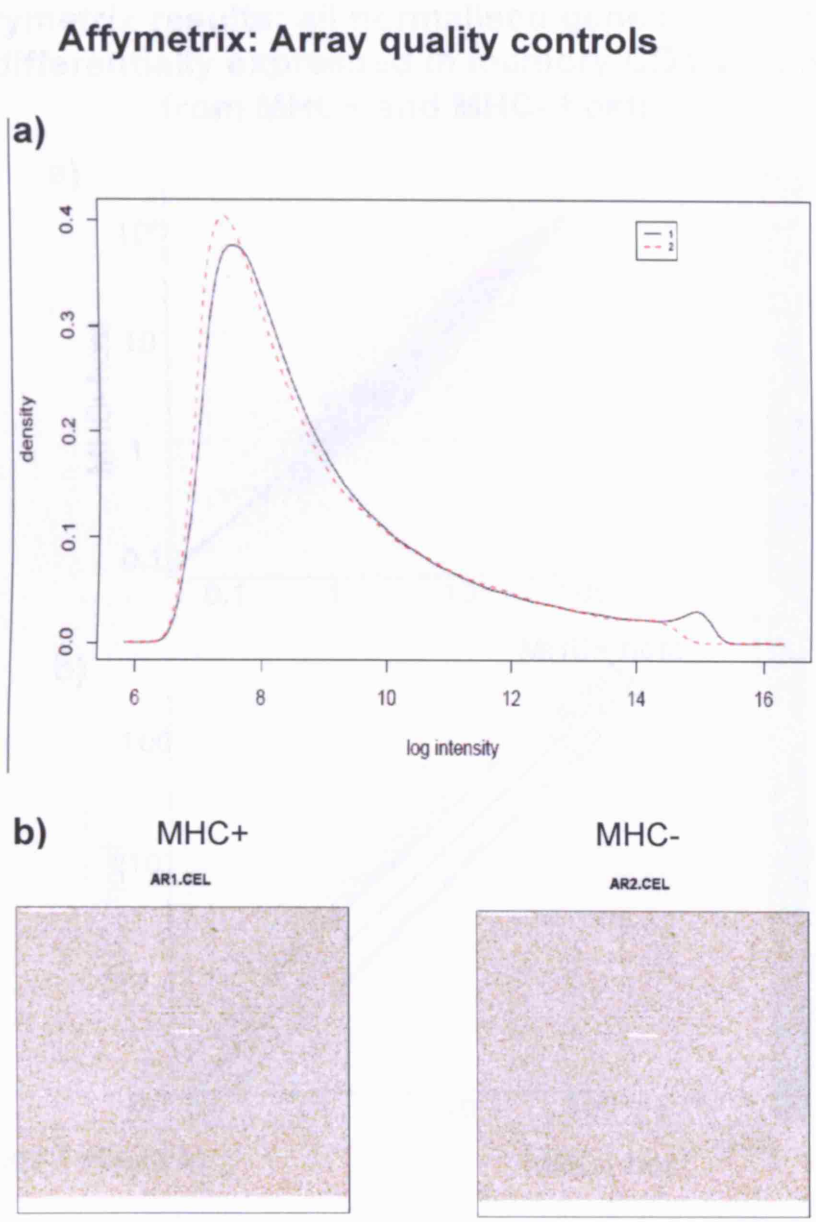



Figure 8 a) and b)

a) Kernel density estimates of Perfect Match (PM) intensities of arrays hybridised with cRNA generated from FACS sorted memory CD4 T cells from MHC+ hosts (1, open histogram black line) and MHC- hosts (2, open histogram red dash line). b) Image of the log intensities of array hybridised with cRNA generated from FACS sorted memory CD4 T cells from MHC+ hosts (left) and MHC- hosts (right). Data generated using BioConductor.

Fig. 9 a) and b)

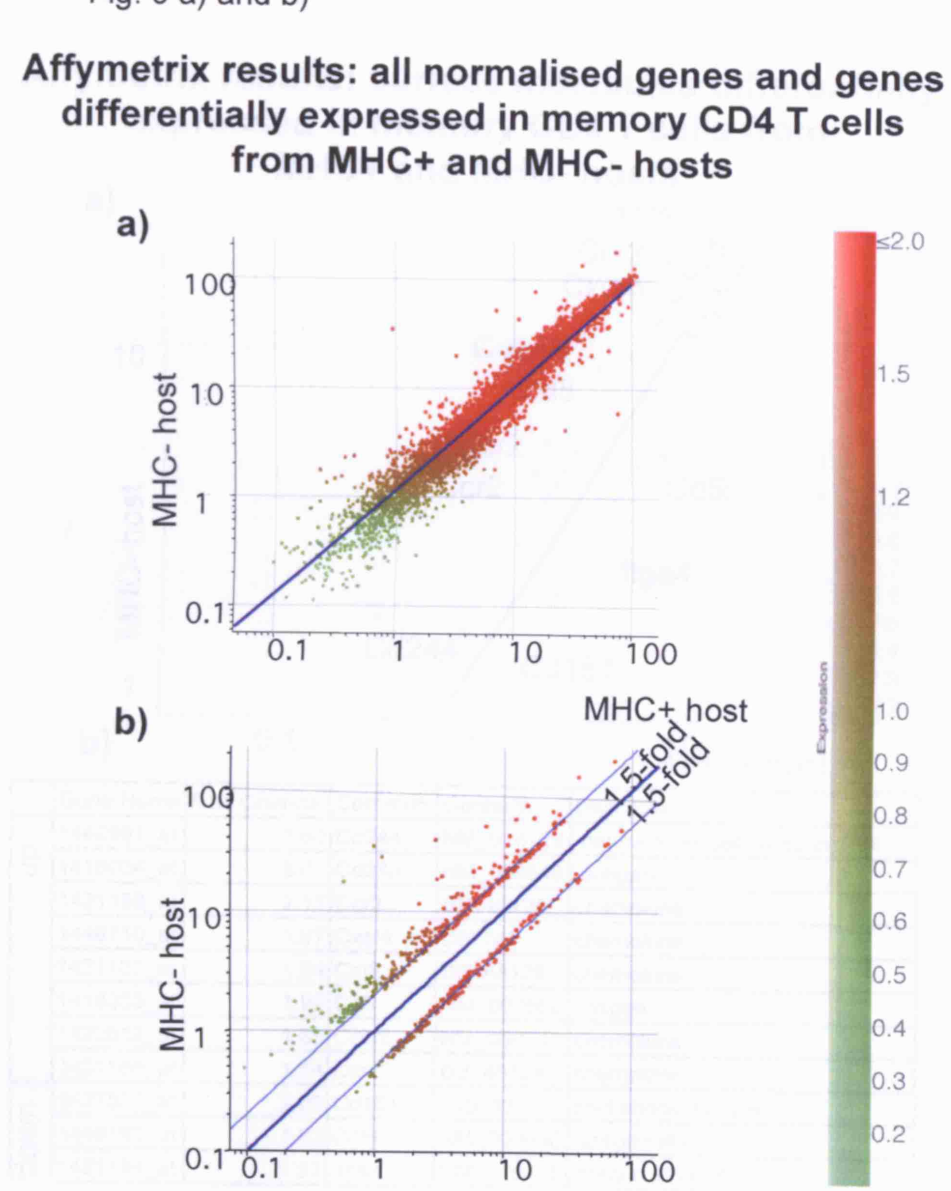
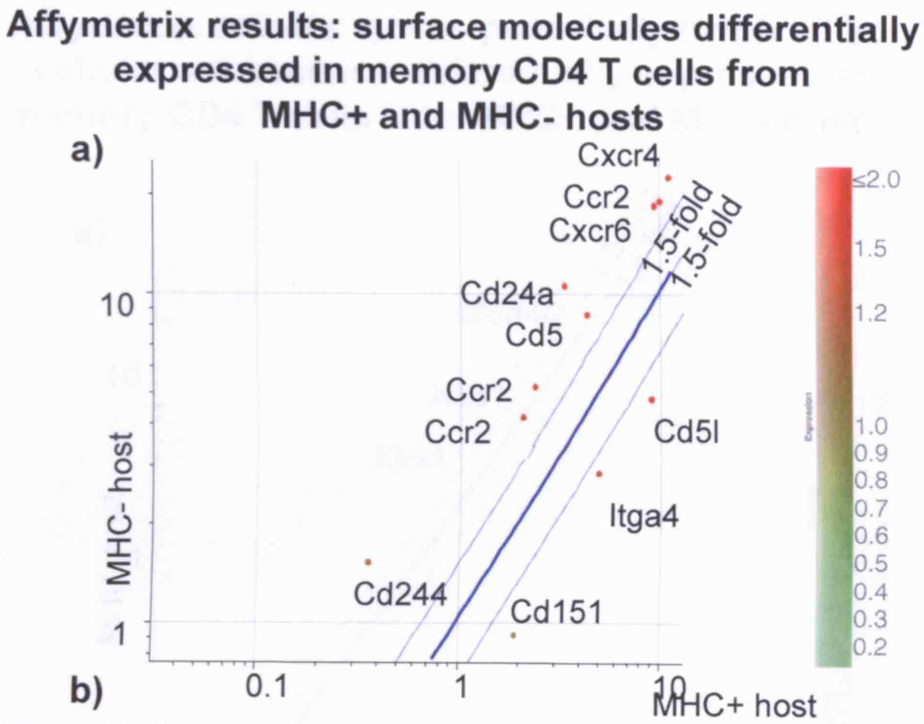



Figure 9 a) and b)

Normalised intensities of genes expressed by memory CD4 T cells from MHC+ hosts (x axis) and by memory CD4 T cells from MHC- hosts (y axis). Genes expressed equivalently in both memory populations fall on the diagonal regression line (MHC-=MHC+). Genes over-expressed by memory CD4 T cells from MHC- hosts fall on the left of the regression line (MHC->MHC+), while genes over-expressed in memory CD4 T cells from MHC+ hosts fall on the right of the regression line (MHC-<MHC+). The level of the expression of the transcripts correlates with the colour bar on the right side of the graph. Light blue lines represent 1.5-fold cut-off point. a) All genes, normalised data; b) differentially expressed genes.

Fig. 10 a) and b)




| | Gene Name | Fold Change | Common | Genbank | Description |
|---|---|---|---|---|---|
| up | 1449991_at | 3.60 | Cd244 | NM_018729 | natural killer cell receptor 2B4 |
| | 1416034_at | 3.01 | Cd24a | NM_009846 | antigen |
| | 1421188_at | 2.11 | Ccr2 | BB148128 | chemokine |
| | 1448710_at | 1.97 | Cxcr4 | D87747 | chemokine |
| | 1421187_at | 1.94 | Ccr2 | BB148128 | chemokine |
| | 1418353_at | 1.92 | Cd5 | NM_007650 | antigen |
| | 1422812_at | 1.88 | Cxcr6 | NM_030712 | chemokine |
| | 1421186_at | 1.84 | Ccr2 | BB148128 | chemokine |
| down | 1427525_at | 2.09 | Cd151 | L35549 | cold shock domain |
| | 1449193_at | 1.93 | Cd5l | NM_009690 | antigen-like |
| | 1421194_at | 1.92 | Itga4 | NM_010576 | integrin alpha 4 |

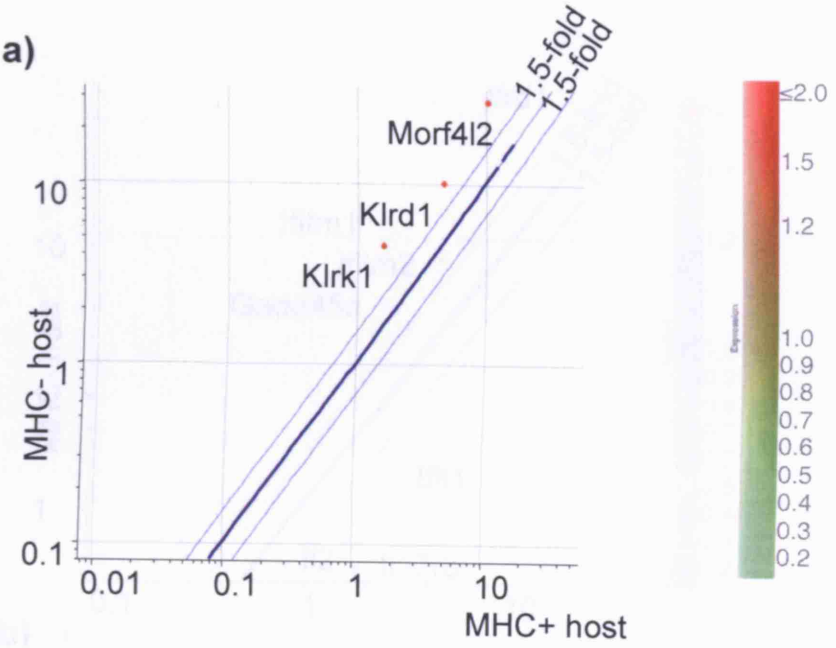
Figure 10 a) and b)

Surface molecules differentially expressed by memory CD4 T cells from MHC+ hosts (x axis) and by memory CD4 T cells from MHC- hosts (y axis). The level of the expression of the transcripts correlates with the colour bar on the right side of the graph. a) The plot shows normalised intensities of 11 differentially expressed transcripts coding for surface molecules; b) table showing Gene name, Common name, Genbank accession number and gene description listed in a descending order according to -fold change in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells from MHC+ hosts.

Fig. 11 a) and b)

**Affymetrix results: genes potentially involved in cellular senescence differentially expressed in memory CD4 T cells from MHC+ and MHC- hosts**

a)




b)

| | Gene Name | Fold Change | Common | Genbank | Description |
|---|---|---|---|---|---|
| up | 1450495_a_ | 2.68 | Klrk1 | AF039026 | killer cell lectin-like receptor |
| | 1415778_at | 2.66 | Morf4l2 | NM_019768 | mortality factor 4 like 2 |
| | 1460245_at | 2.07 | Klrd1 | NM_010654 | killer cell lectin-like receptor |

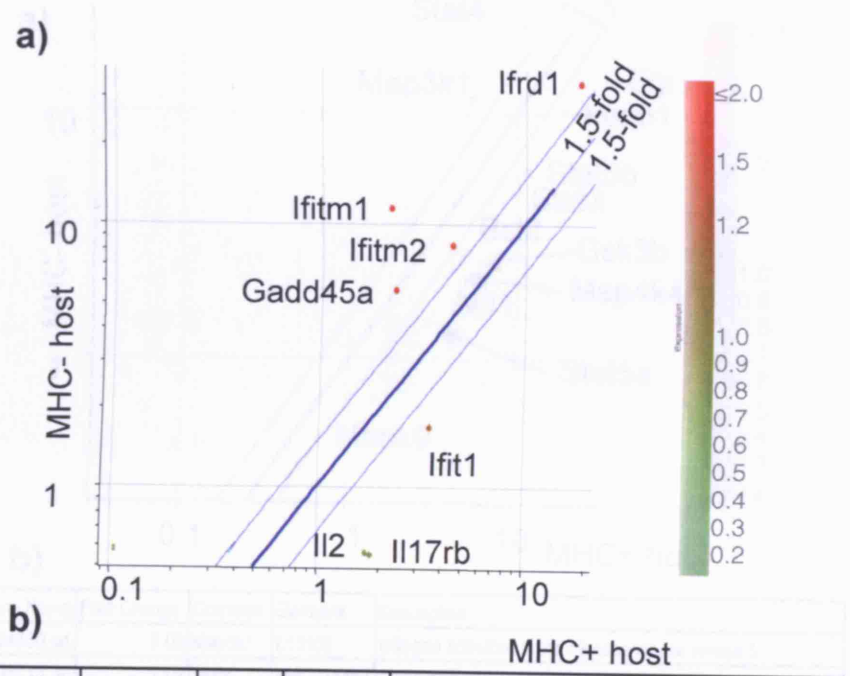
Figure 11 a) and b)

Genes potentially involved in cellular senescence differentially expressed by memory CD4 T cells from MHC+ hosts (x axis) and by memory CD4 T cells from MHC- hosts (y axis). The level of the expression of the transcripts correlates with the colour bar on the right side of the graph. a) The plot shows normalised intensities of 3 differentially expressed transcripts coding for molecules potentially involved in senescence; b) table showing Gene name, Common name, Genbank accession number and gene description listed in a descending order according to -fold change in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells from MHC+ hosts.

Fig. 12 a) and b)

## Affymetrix results: cytokines and cytokine related genes differentially expressed in memory CD4 T cells from MHC+ and MHC- hosts

a)



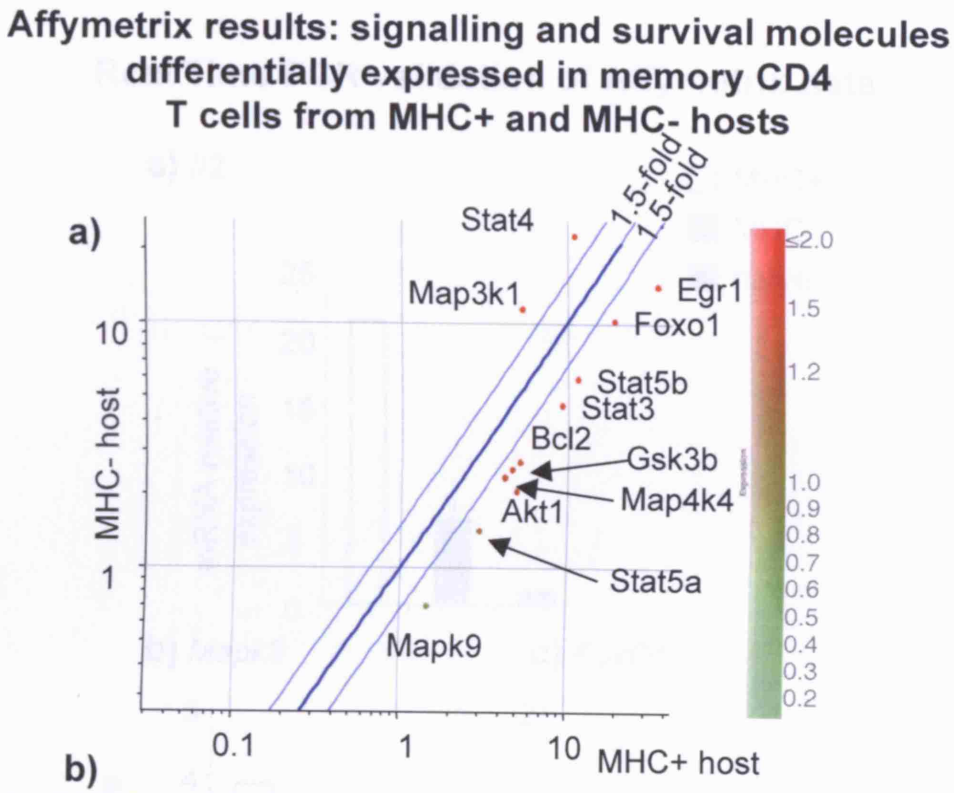

b)

| | Gene Name | Fold Change | Common | Genbank | Description |
|---|---|---|---|---|---|
| up | 1424254_at | 4.99 | Ifitm1 | BC027285 | interferon induced protein 1 |
| | 1449519_at | 2.31 | Gadd45a | NM_007836 | growth arrest and DNA-damage-inducible 45 alpha |
| | 1417460_at | 1.83 | Ifitm2 | NM_030694 | IFN induced transmembrane protein 2 |
| | 1416067_at | 1.76 | Ifrd1 | NM_013562 | IFN-related developmental regulator 1 |
| down | 1449990_at | 3.17 | Il2 | AF065914 | interleukin 2 |
| | 1420678_a_ | 2.95 | Il17rb | NM_019583 | interleukin 17 receptor B |
| | 1450783_at | 2.17 | Ifit1 | NM_008331 | IFN-induced protein with tetratricopeptide repeats 1 |

Figure 12 a) and b)

Cytokines and cytokine related genes differentially expressed by memory CD4 T cells from MHC+ hosts (x axis) and by memory CD4 T cells from MHC- hosts (y axis). The level of the expression of the transcripts correlates with the colour bar on the right side of the graph. a) The plot shows normalised intensities of 7 differentially expressed transcripts coding for cytokines or cytokine related genes; b) table showing Gene name, Common name, Genbank accession number and gene description listed in a descending order according to -fold change in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells from MHC+ hosts.

Fig. 13 a) and b)




b)

| | Gene Name | Fold Change | Common | Genbank | Description |
|---|---|---|---|---|---|
| up | 1424850_at | 2.09 | Map3k1 | L13103 | mitogen activated protein kinase kinase kinase 1 |
| up | 1448713_at | 2.02 | Stat4 | NM_011487 | signal transducer and activator of transcription 4 |
| down | 1416657_at | 2.40 | Akt1 | NM_009652 | thymoma viral proto-oncogene 1 |
| down | 1421469_a_ | 2.11 | Stat5a | U36502 | signal transducer and activator of transcription 5A |
| down | 1421878_at | 2.04 | Mapk9 | BC024514 | mitogen activated protein kinase 9 |
| down | 1460700_at | 2.03 | Stat3 | AK004083 | signal transducer and activator of transcription 3 |
| down | 1422103_a_ | 1.99 | Stat5b | BC024319 | signal transducer and activator of transcription 5B |
| down | 1416982_at | 1.94 | Foxo1 | AI462296 | forkhead box O1 |
| down | 1422938_at | 1.93 | Bcl2 | NM_009741 | B-cell leukemia/lymphoma 2 |
| down | 1448050_s_ | 1.82 | Map4k4 | BF450398 | mitogen-activated protein kinase kinase kinase kinase 4 |

Figure 13 a) and b)

Signalling and survival molecules differentially expressed by memory CD4 T cells from MHC+ hosts (x axis) and by memory CD4 T cells from MHC- hosts (y axis). The level of the expression of the transcripts correlates with the colour bar on the right side of the graph. a) The plot shows normalised intensities of 12 differentially expressed transcripts coding for molecules involved in signalling and survival; b) table showing Gene name, Common name, Genbank accession number and gene description listed in a descending order according to -fold change in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells from MHC+ hosts.

Fig. 14 a), b) and c)

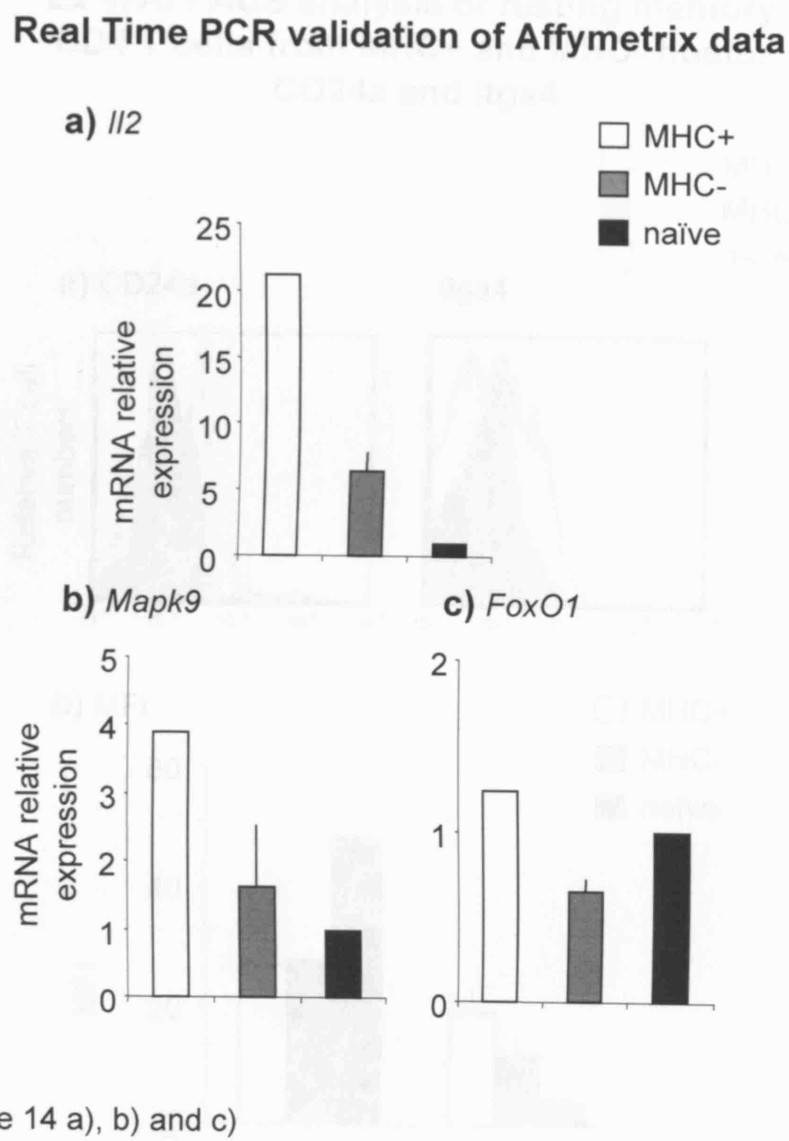



Figure 14 a), b) and c)

mRNA from FACS sorted memory CD4 T cells of > 10 pooled mice per biological replicate recovered from MHC+ hosts (2 biological replicates), MHC- hosts (3 biological replicates) and A1 naïve CD4 T cells from 3 pooled mice (1 biological sample) was reverse transcribed and the message for the genes of interest was amplified and analysed as described in Material and Methods. The mRNA relative expression of memory CD4 T cells from MHC+ hosts (open bars) and MHC- hosts (grey bars) was normalised to the level of expression of naïve CD4 T cells set to 1 (black bars). Mean values ± standard deviation are shown. a) Il2, b) Mapk9 and c) FoxO1.

Fig. 15 a) and b)

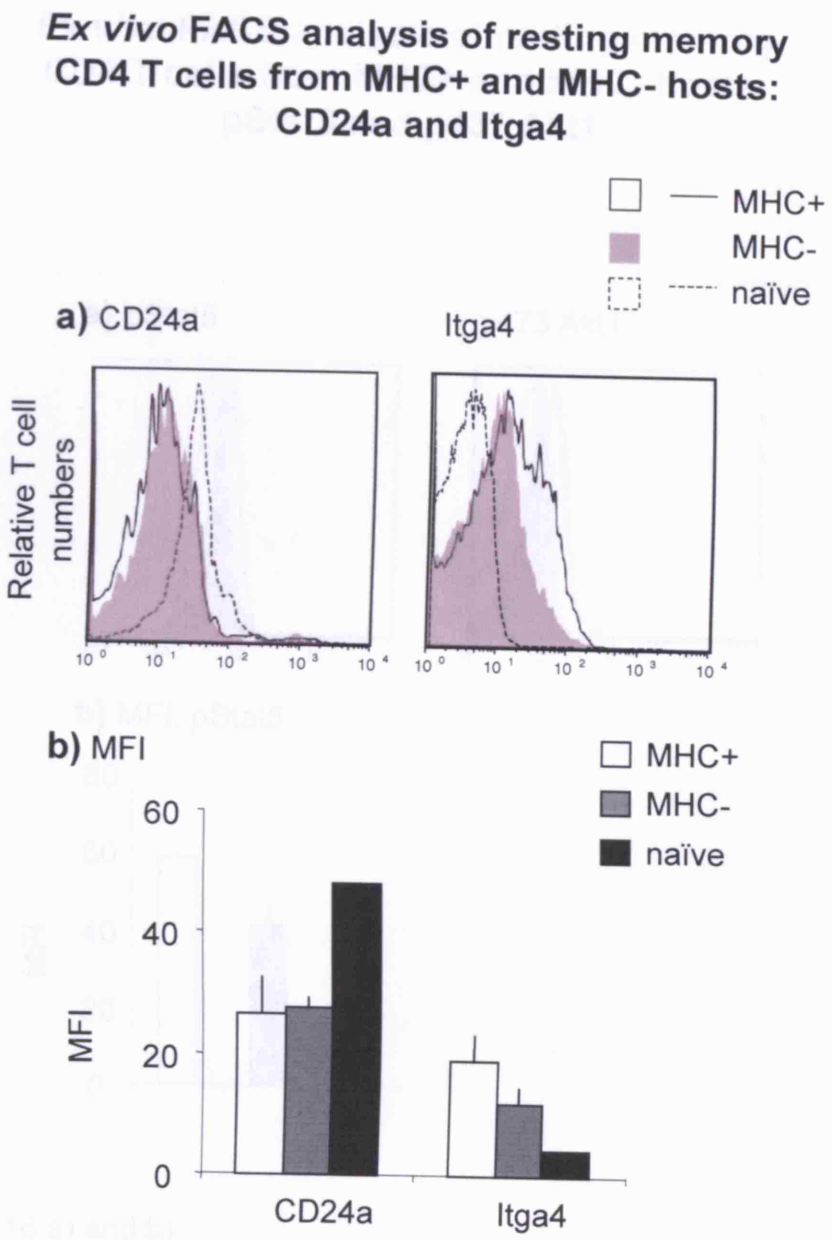



Figure 15 a) and b)

White histograms and bars show memory CD4 T cells from MHC+ hosts. Grey histograms and bars show memory CD4 T cells from MHC- hosts. Stippled lines histograms and black bars show A1 naïve CD4 T cells as reference (one mouse). a) FACS histograms and (b) MFI for CD24a (left), 3 mice per group, and Itga4 (right), 4 mice per group. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation are shown.

Fig. 16 a) and b)

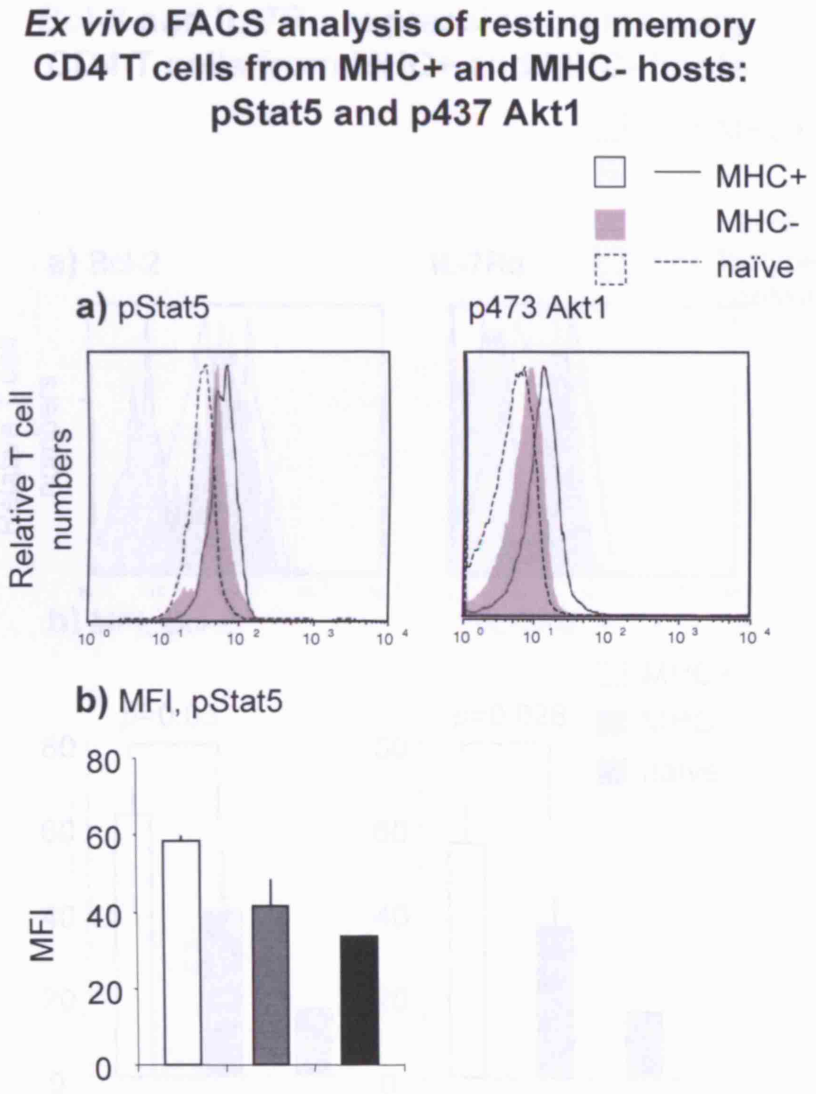



Figure 16 a) and b)

White histograms and bars show memory CD4 T cells from MHC+ hosts. Grey histograms and bars show memory CD4 T cells from MHC- hosts. Stippled lines histograms and black bars show levels of expression on naïve CD4 T cells as reference (one mouse). a) FACS histograms and (b) MFI for pStat5 (left), 3 mice per group, and p473 Akt (right), 3 pooled mice per group. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation are shown.

Fig. 17 a) and b)

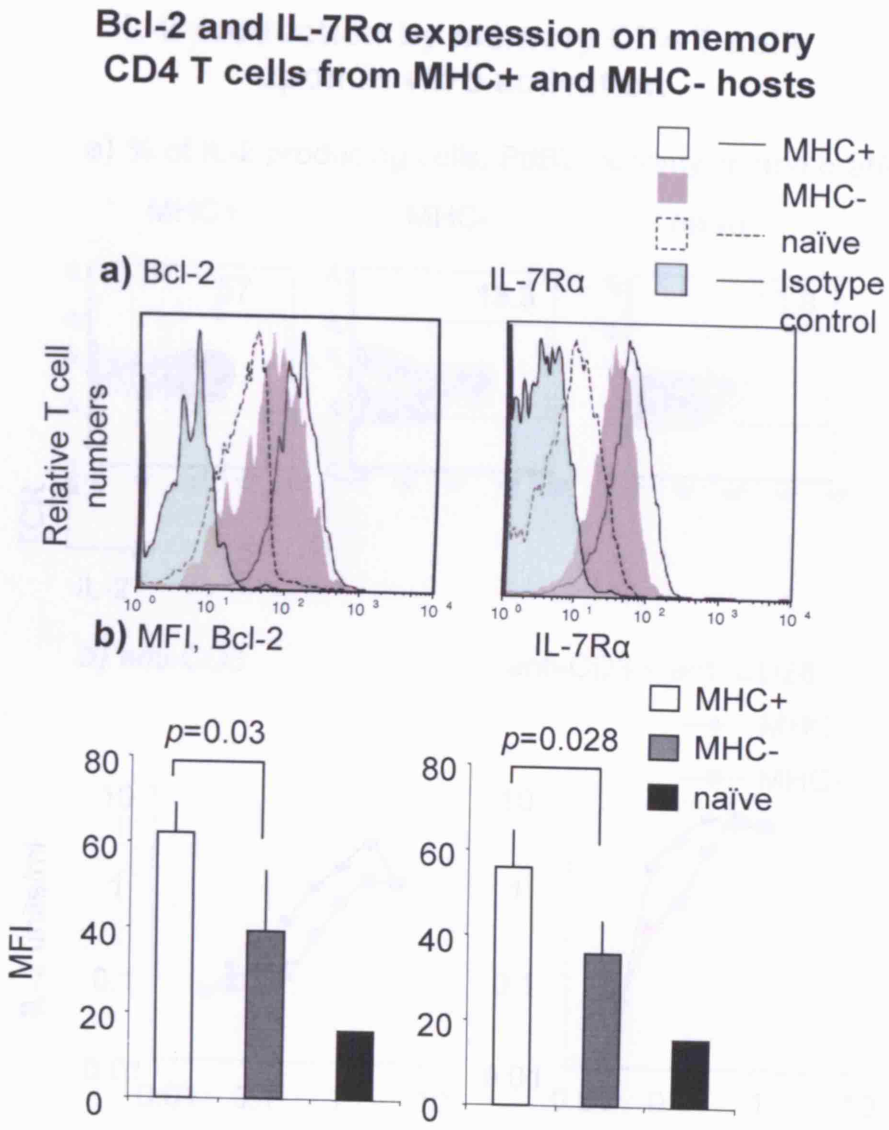



Figure 17 a) and b)

White histograms and bars show memory CD4 T cells from MHC+ hosts. Grey histograms and bars show memory CD4 T cells from MHC- hosts. Stippled lines histograms and black bars show levels of expression on naïve CD4 T cells (one mouse) as reference as well as isotype control antibody (light green histograms). a) FACS histograms and (b) MFI for Bcl-2 (left), 4 mice per group, and IL-7Rα (right), 4 mice per group. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation and p-values, obtained with two-tail Mann-Whitney test, are shown. Similar results were obtained in 3 independent experiments.

Fig. 18 a) and b)

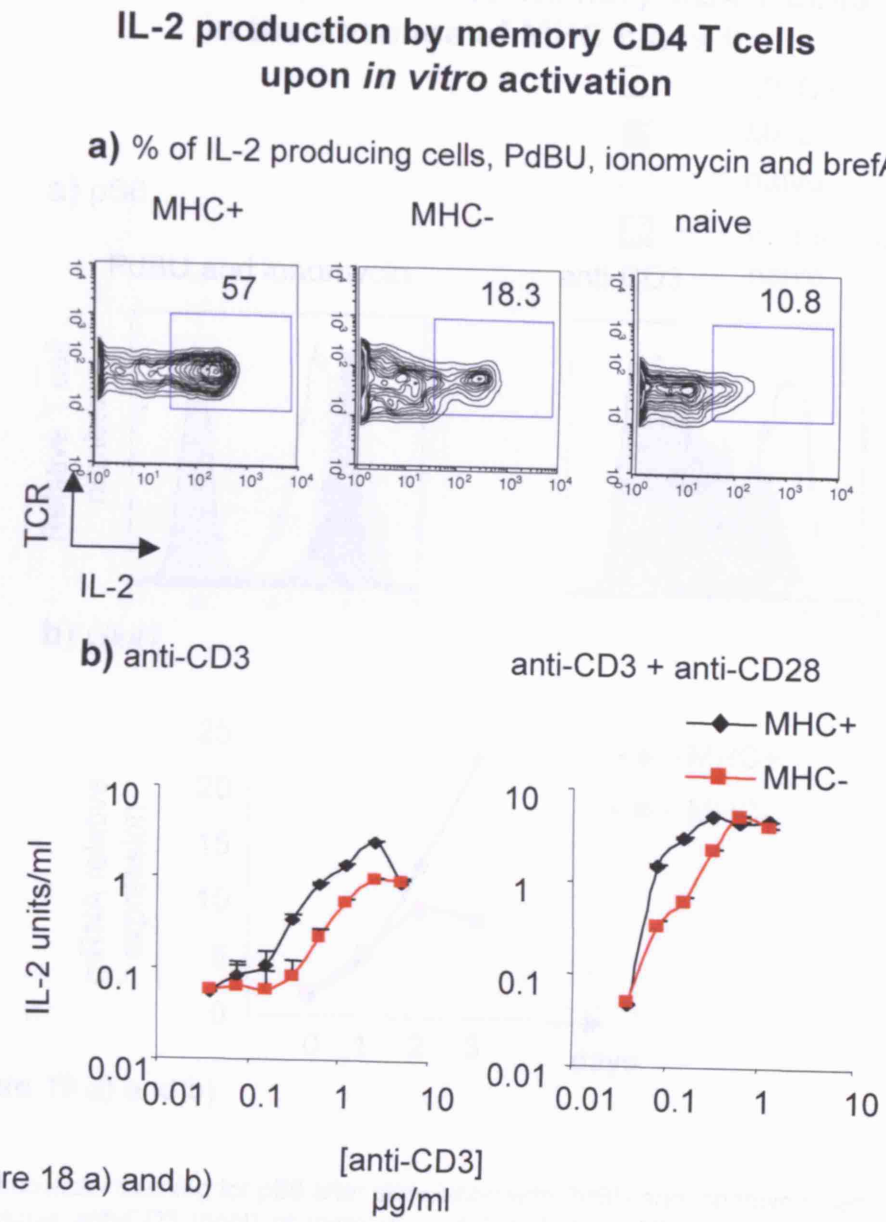



Figure 18 a) and b)

a) Percentage of IL-2 producing cells on memory CD4 T cells from MHC+ hosts (left), MHC- hosts (centre) and A1 naïve CD4 T cells (right) upon in vitro stimulation with PdBU and ionomycin in the presence of Brefeldin A assessed by intracellular staining. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. b) IL-2 secretion by memory CD4 T cells from MHC+ hosts (black diamonds) and MHC- hosts (red squares) upon stimulation with plate-bound anti-CD3 (left) and anti-CD3 + anti-CD28 (right). IL-2 was detected as proliferation of the IL-2 dependent cell line CTLL-2 and results were calculated from a standard curve of recombinant IL-2 to give units of IL-2/ml produced.

Fig. 19 a) and b)

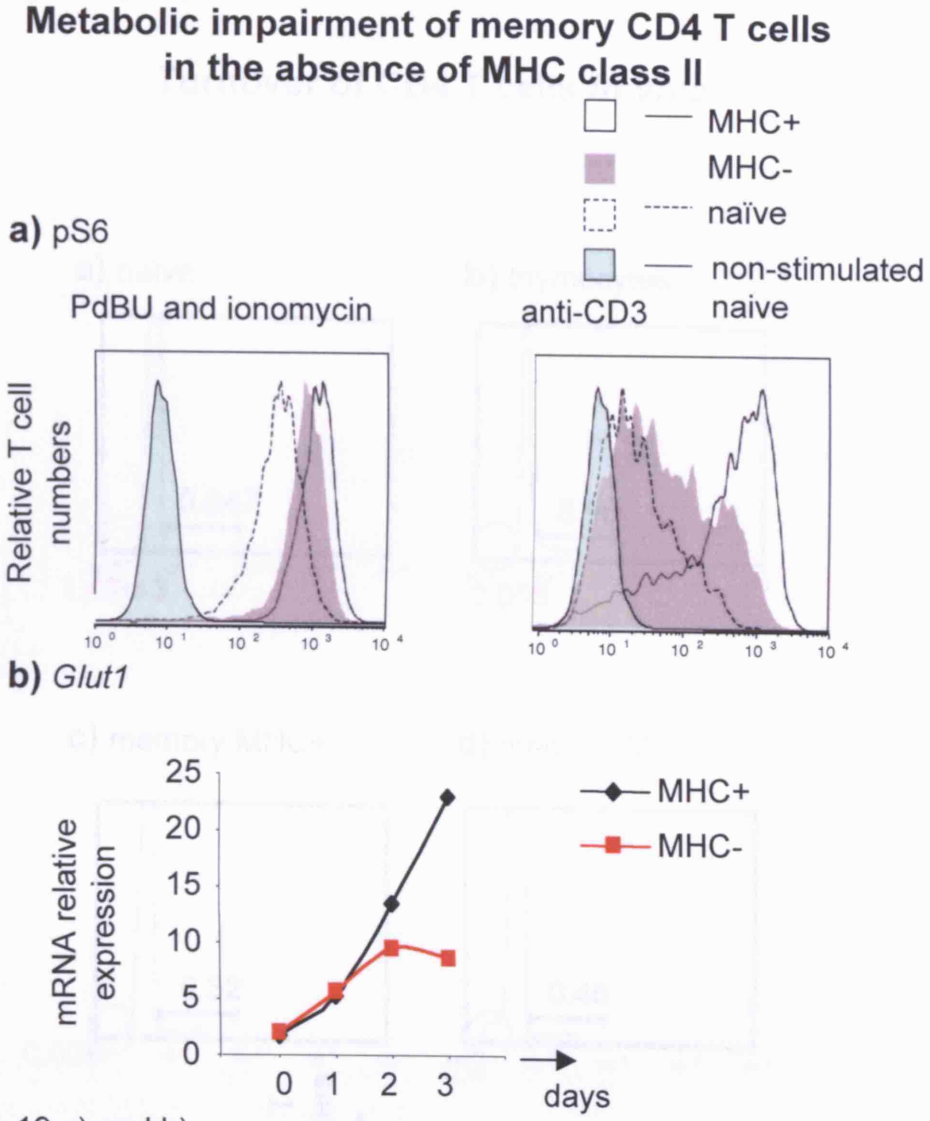



Figure 19 a) and b)

a) Intracellular staining for pS6 after stimulation with PdBU and ionomycin (left) or soluble anti-CD3 (right) of memory CD4 T cells from MHC+ hosts (white histograms) and memory CD4 T cells from MHC- hosts (grey histograms). Stimulated A1 naïve CD4 T cells (stippled lines histograms) and non-stimulated A1 naïve CD4 T cells (green histograms) are shown as reference. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Data representative of 3 independent experiments. b) Glut1 mRNA relative expression (y axis) assessed over the course of 3 days (x axis) of in vitro stimulation with anti-CD3 and anti-CD28. Expression was assessed on FACS sorted memory CD4 T cells pooled from 3 MHC+ hosts (black diamonds) and 3 MHC- hosts (red squares). Glut1 mRNA expression was normalised to hprt house keeping gene.

Fig. 20 a) - d)

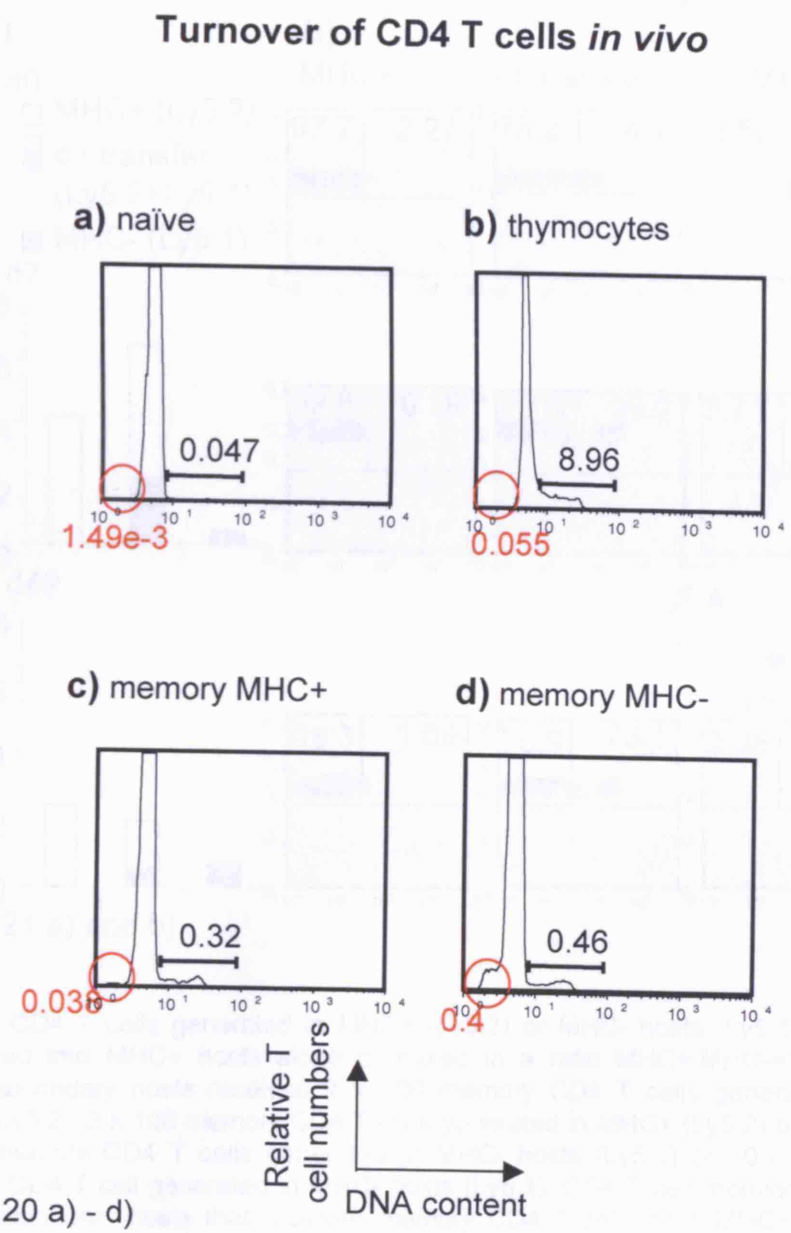



Figure 20 a) - d)

FACS histograms of CD4 T cells recovered 6 weeks after memory generation. Cells were FACS sorted, fixed and PI stained. The figure shows percentages of cells in G0/G1 (middle peak), S phase (right of G0/G1 peak, bar percentages) and apoptotic cells (left of G0/G1 peak, circle percentages). a) Naïve CD4 T cells and b) thymocytes from 2 pooled A1 naive mice; c) memory CD4 cells from 8 pooled MHC+ hosts; d) memory cells from 9 pooled MHC- hosts. Data representative of 2 independent experiments.

Fig. 21 a) and b)

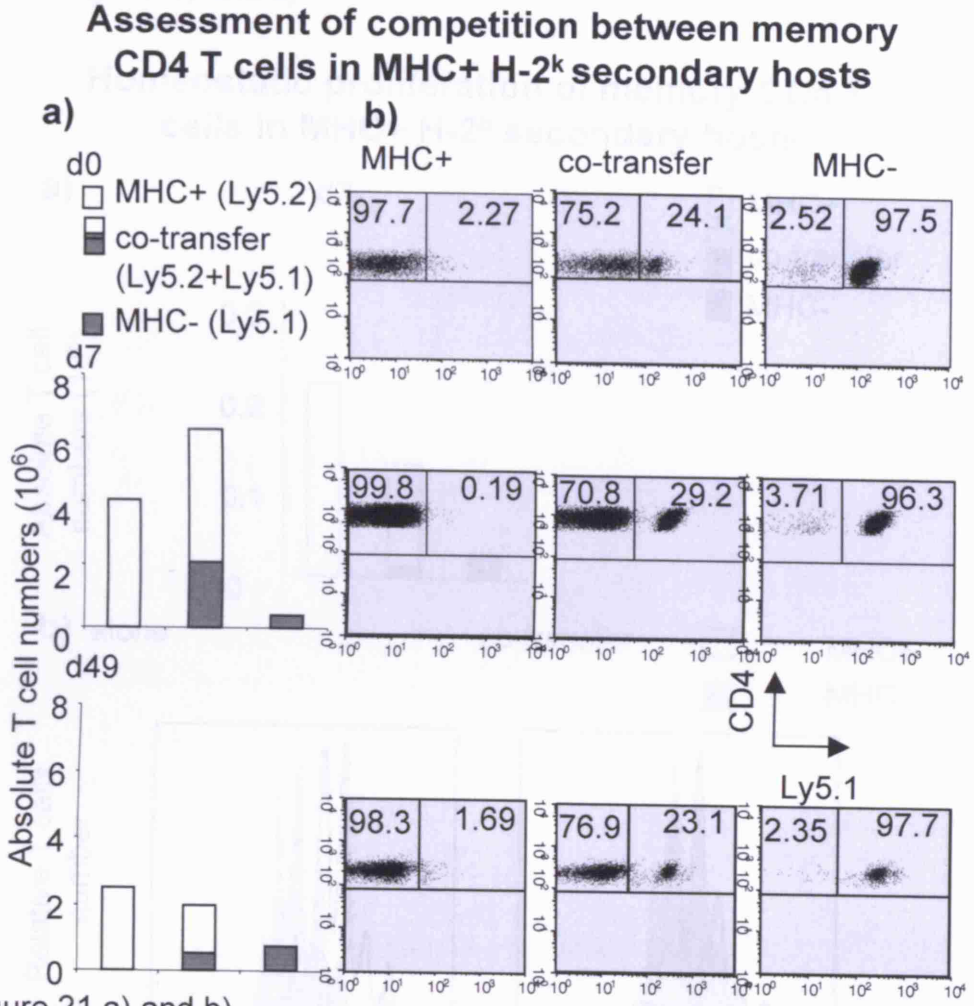



Figure 21 a) and b)

Memory CD4 T cells generated in MHC+ (Ly5.2) or MHC- hosts (Ly5.1) were transferred into MHC+ hosts alone or mixed in a ratio MHC+:MHC-=3:1. a) MHC+ secondary hosts received 2 x 106 memory CD4 T cells generated in MHC+ (Ly5.2), 2 x 106 memory CD4 T cells generated in MHC+ (Ly5.2) plus 0.7 X 106 memory CD4 T cells generated in MHC- hosts (Ly5.1) or 0.7 X 106 memory CD4 T cell generated in MHC- hosts (Ly5.1). CD4 T cell recovery from MHC+ secondary hosts that received memory CD4 T cells from MHC+ hosts (white bars), mix of memory CD4 T cells from MHC+ hosts (white bars) and MHC- hosts (grey bars) or memory CD4 T cells from MHC- hosts only (grey bars) (top panel) was assessed at day 7 (middle panel) and day 49 (lower panel) after transfer. b) Top panel: FACS plots show percentages of Ly5.1+ CD4+ H57+ cells injected into MHC+ hosts that received memory cells from MHC+ hosts (left), the mix MHC+:MHC-=3:1 (centre) and memory cells from MHC- hosts (right). FACS plots show percentages of Ly5.1+ CD4+ H57+ recovered from MHC+ hosts that received memory cells from MHC+ hosts (left), the mix MHC+:MHC-=3:1 (centre) and memory cells from MHC- hosts (right) at day 7 (middle panel) and at day 49 (lower panel). Percentages of Ly5.2+ CD4+ H57+ cells were calculated from percentages of Ly5.1- CD4+ H57+. Data from 2 mice per group are shown at each time point.

Fig. 22 a) and b)

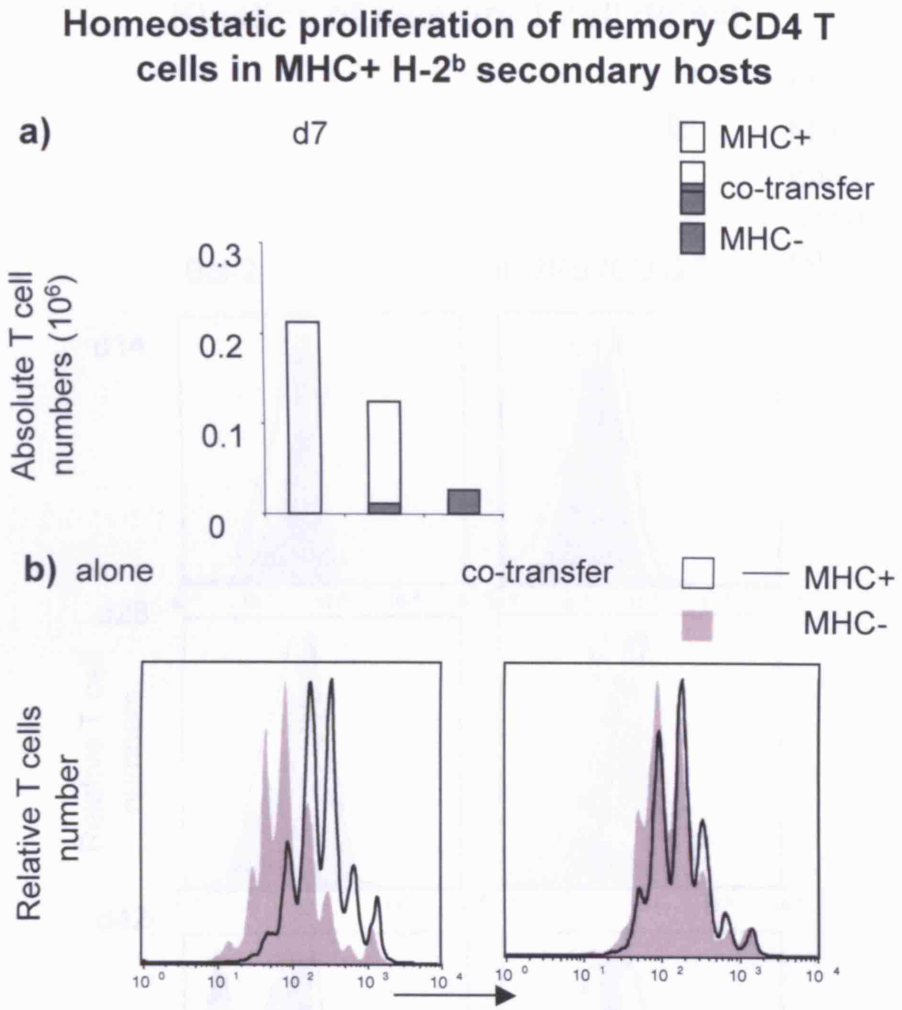



Figure 22 a) and b)

Memory CD4 T cells from MHC+ or MHC- hosts transferred into MHC+ hosts alone or mixed in a ratio MHC+:MHC-=3:1. a) MHC+ secondary hosts received 0.3 x 106 memory CD4 T cell generated in MHC+, 0.3 x 106 memory CD4 T cell generated in MHC+ plus 0.1 X 106 memory CD4 T cell generated in MHC- hosts or 0.1 X 106 memory CD4 T cell generated in MHC- hosts. CD4 T cell recovery from MHC+ secondary hosts that received memory CD4 T cells from MHC+ hosts (white bars), mix of memory CD4 T cells from MHC+ hosts (white bars) and MHC- hosts (grey bars) or memory CD4 T cells from MHC- hosts only (grey bars) was assessed at day 7. b) Overlay of the CFSE profiles of memory CD4 T cells from MHC+ hosts (open histograms) and from MHC- hosts (grey histograms) transferred alone (left) or co-transferred (right) assessed 7 days after secondary transfer. Data representative of 2 mice per group are shown.

Fig. 23

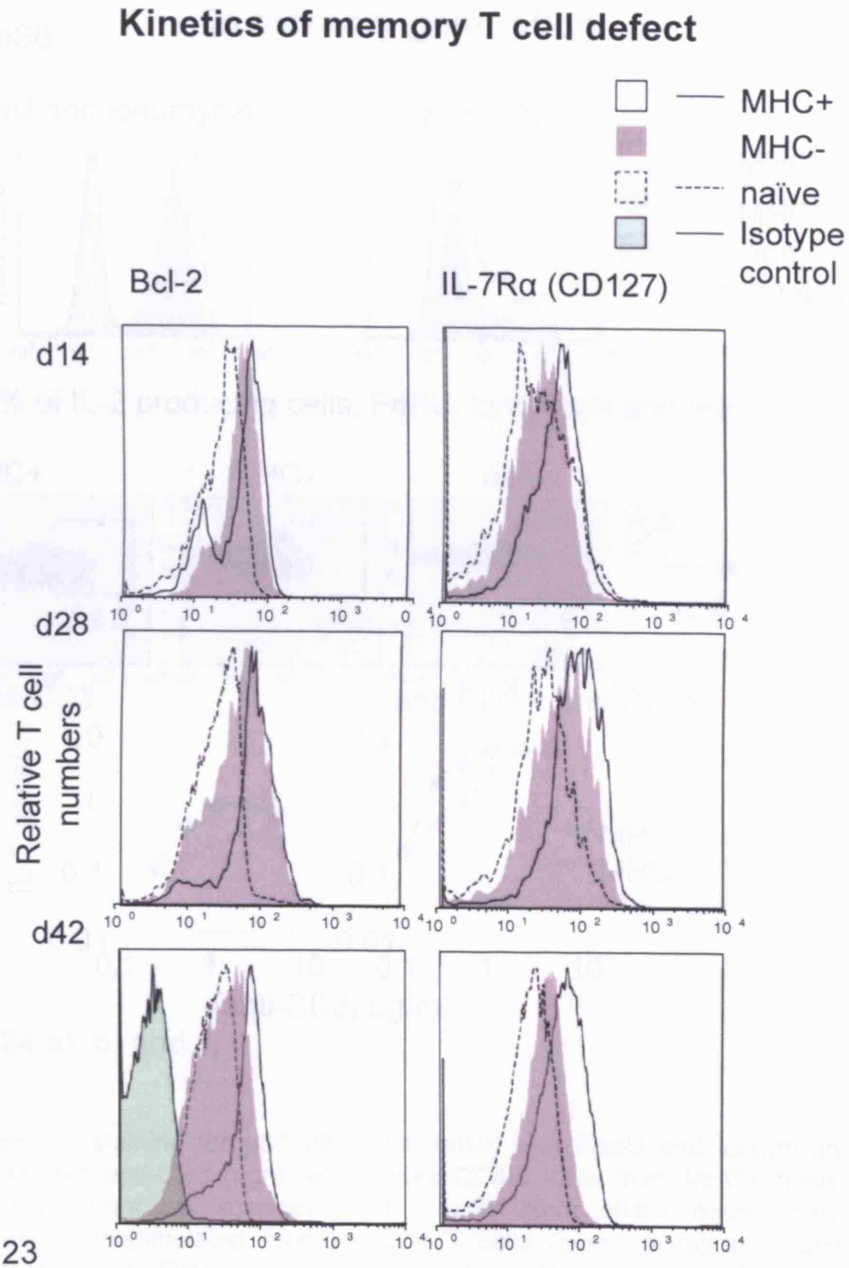



Figure 23

FACS histograms for Bcl-2 (left) and IL-7Rα (right) expression on memory CD4 T cells generated in MHC+ hosts (open histogram) and MHC- hosts (grey histogram) recovered 14, 28 and 42 days after immunisation. A1 naïve CD4 T cells (stippled lines histograms) and isotype control antibody (light green histogram) are shown as reference in some plots. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. 3-5 pooled mice per group were used.

Fig. 24 a), b) and c)

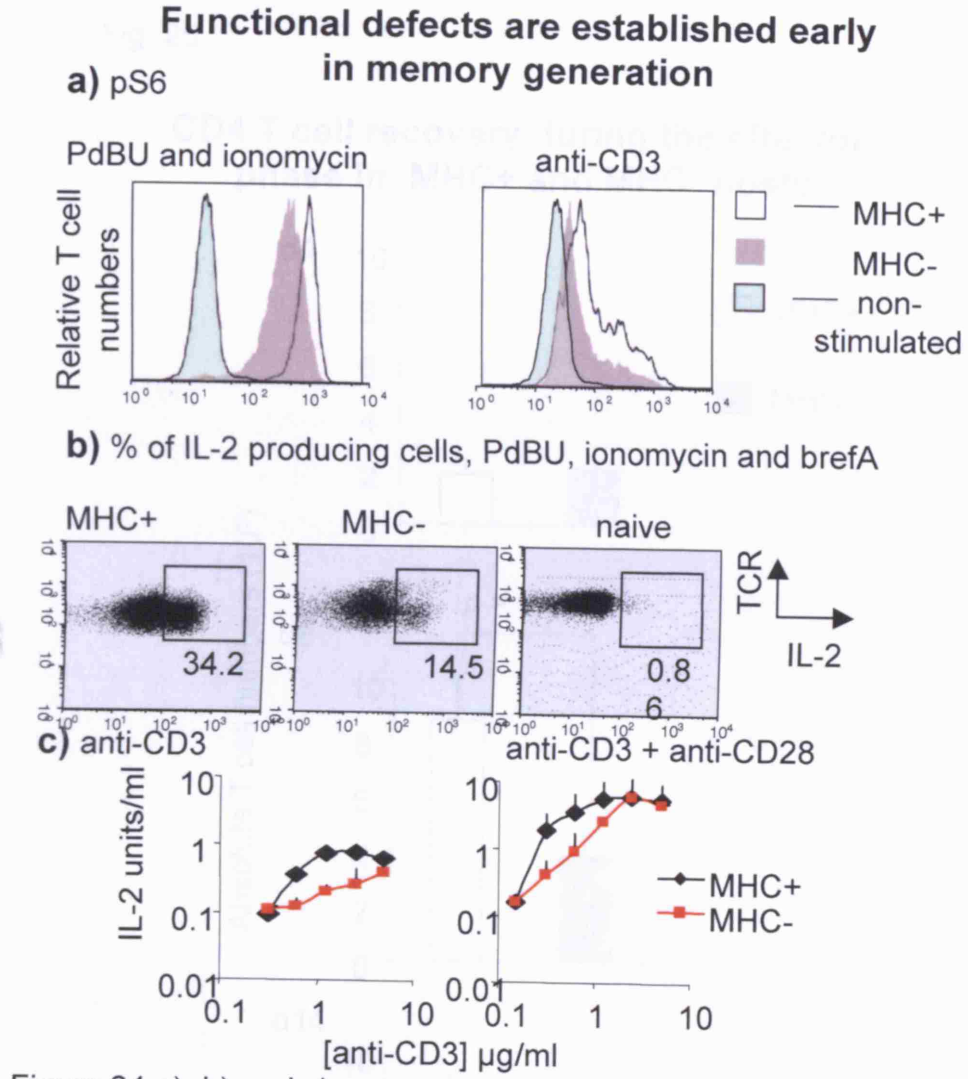



Figure 24 a), b) and c)

a) Intracellular staining for pS6 after stimulation with PdBU and ionomycin (left) or soluble anti-CD3 (right) of memory CD4 T cells from MHC+ hosts (white histograms) and memory CD4 T cells from MHC- hosts (grey histograms). Non-stimulated memory CD4 T cells (green histograms) are shown as reference. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. b) Percentages of IL-2 producing cells detected by intracellular staining for memory CD4 T cells from MHC+ (left), MHC- (centre) and naïve CD4 T cells (right). Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. c) IL-2 secretion by memory CD4 T cells from MHC+ hosts (black diamonds) and MHC- hosts (red squares) upon stimulation with plate-bound anti-CD3 (left) and anti-CD3 in the presence of anti-CD28 (right). IL-2 was detected as proliferation of the IL-2 dependent cell line CTLL-2 and results were calculated from a standard curve of recombinant IL-2 to give units of IL-2/ml produced. Mean values ± standard deviation are shown.

Fig. 25

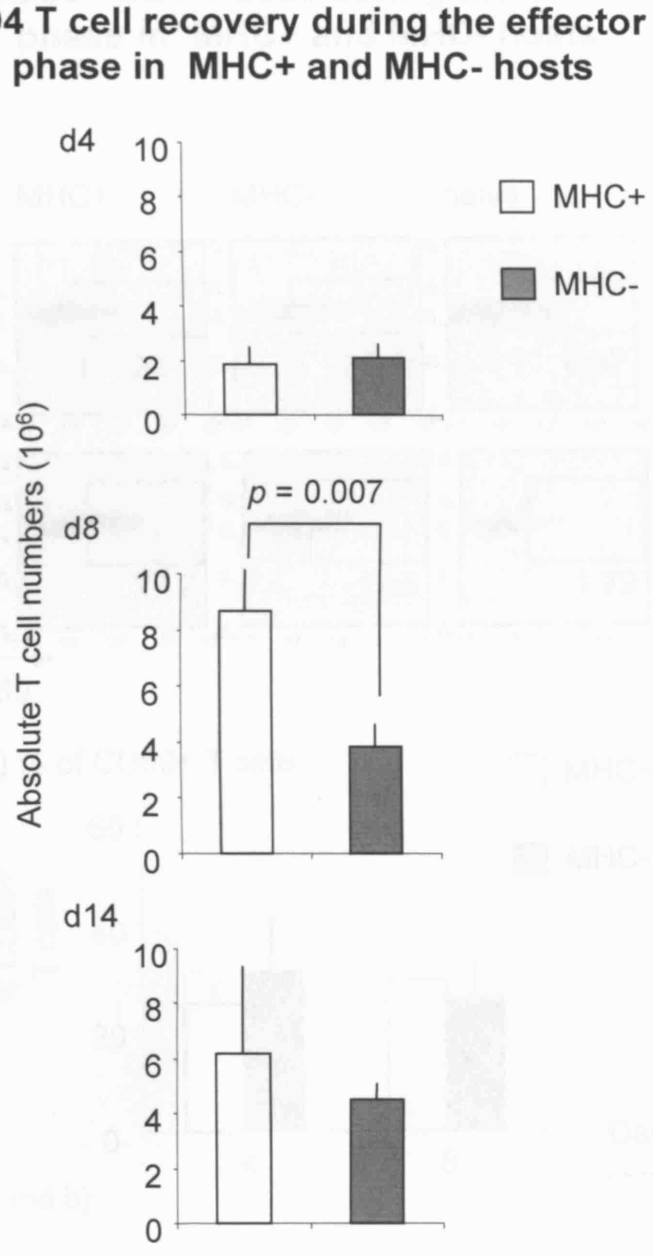



Figure 25

3 x $10^6$ A1 naïve cells were co-injected with 0.7 x 106 H-Y peptide pulsed DCs into MHC+ hosts (white bars) and MHC- hosts (grey bars). CD4 T cell recovery was assessed at day 4, 8 and 14 of the effector phase. T cell recovery was calculated from percentages of CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation and p-value, obtained with two-tail Mann-Whitney test, are shown. 3-5 mice per group were used.

Fig. 26 a) and b)

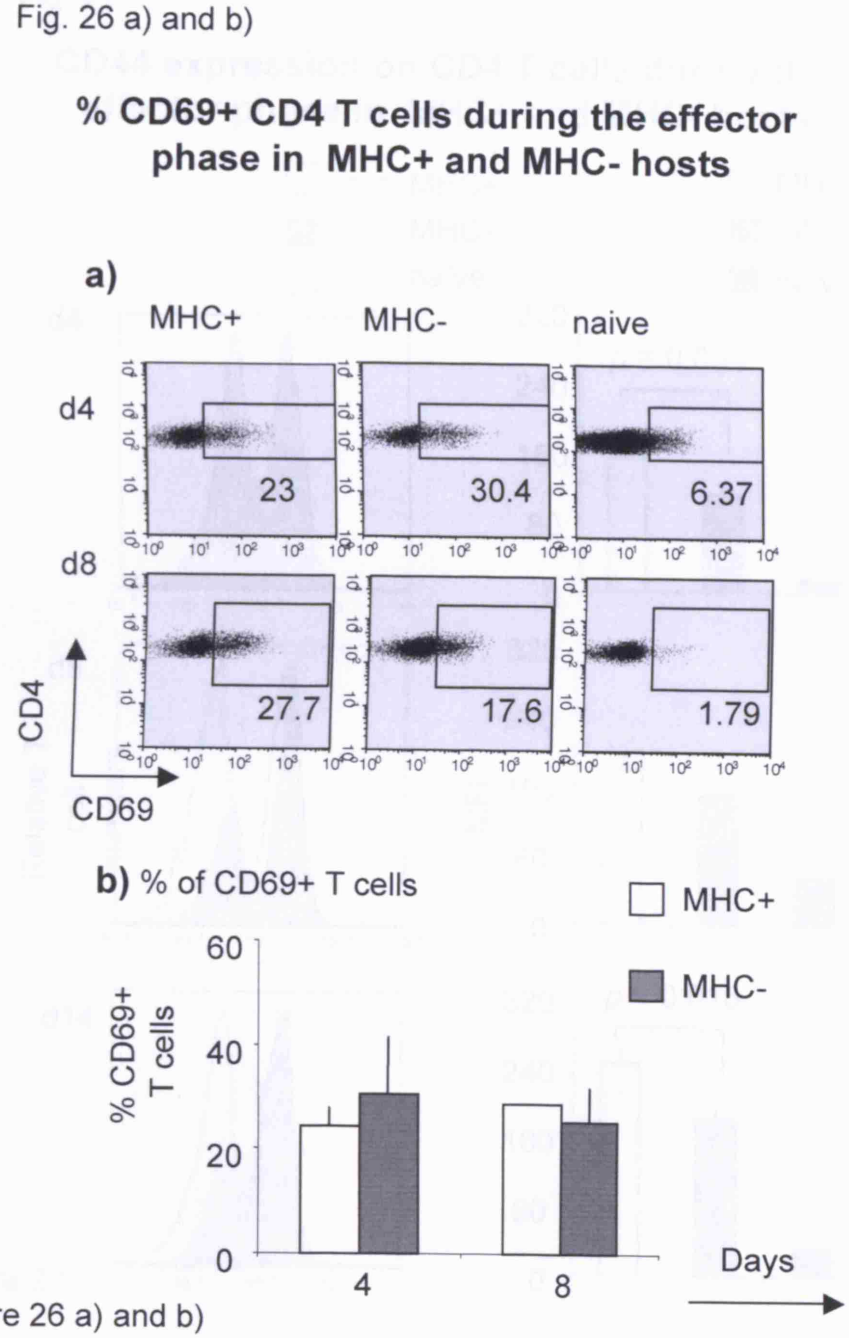



Figure 26 a) and b)

a) Representative FACS plot showing percentages of CD69+ cells of CD4 T cells assessed at day 4 and 8 of the effector phase in MHC+ hosts (left) and MHC- hosts (centre). Percentages of CD69+ cells of A1 naïve CD4 T cell (right) are shown as reference. b) Percentages CD69+ CD4 T cells recovered from 3 MHC+ hosts (white bars) and 3 MHC- hosts (grey bars) at day 4 and 8 of the effector phase. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation are shown.

Fig. 27

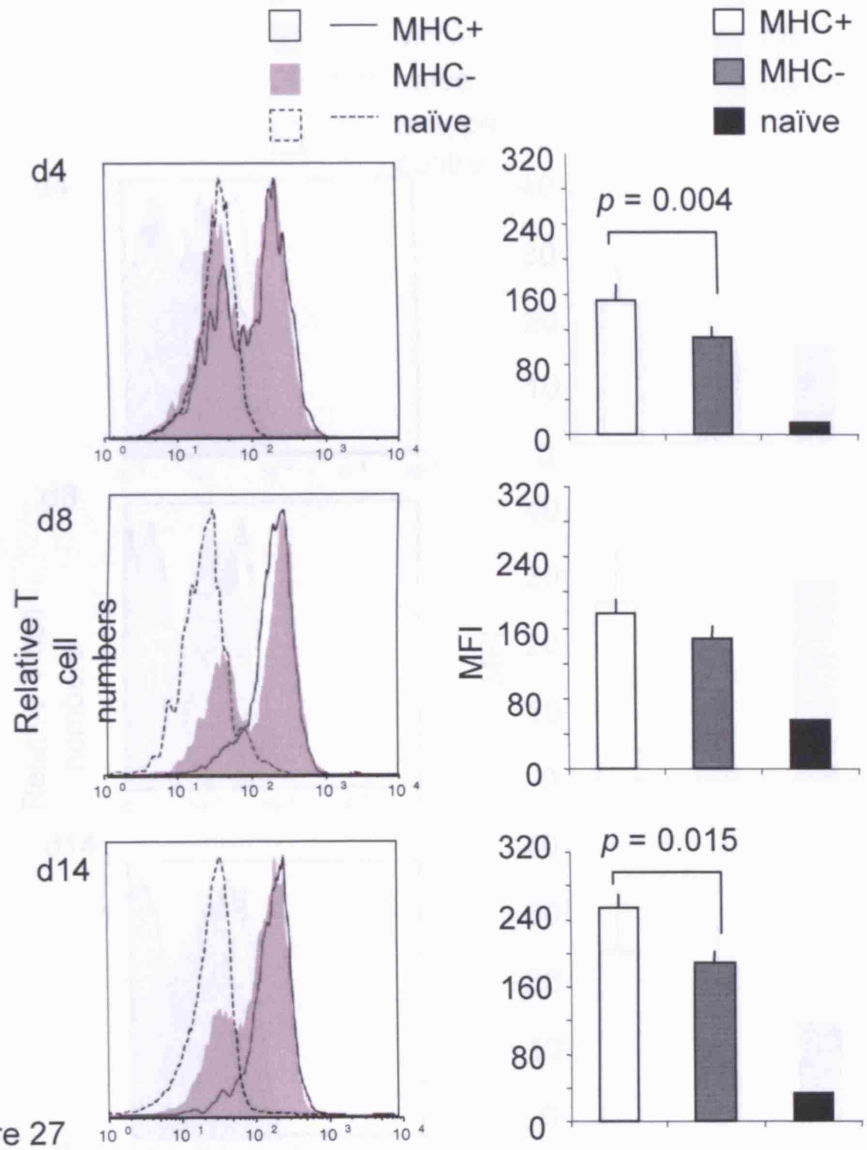



Figure 27

FACS histograms (left) and MFI (right) of CD44 expression on CD4 T cells at day 4, 8 and 14 of the effector phase in MHC+ hosts (open histograms and bars) and MHC- hosts (grey histograms and bars). A1 naïve CD4 T cells (stippled lines histograms, black bars) are shown as reference (one mouse). Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation and p-values, obtained with two-tail Mann-Whitney test, are shown. 3-5 mice per group were used. Similar results were obtained in 3 independent experiments.

Fig. 28

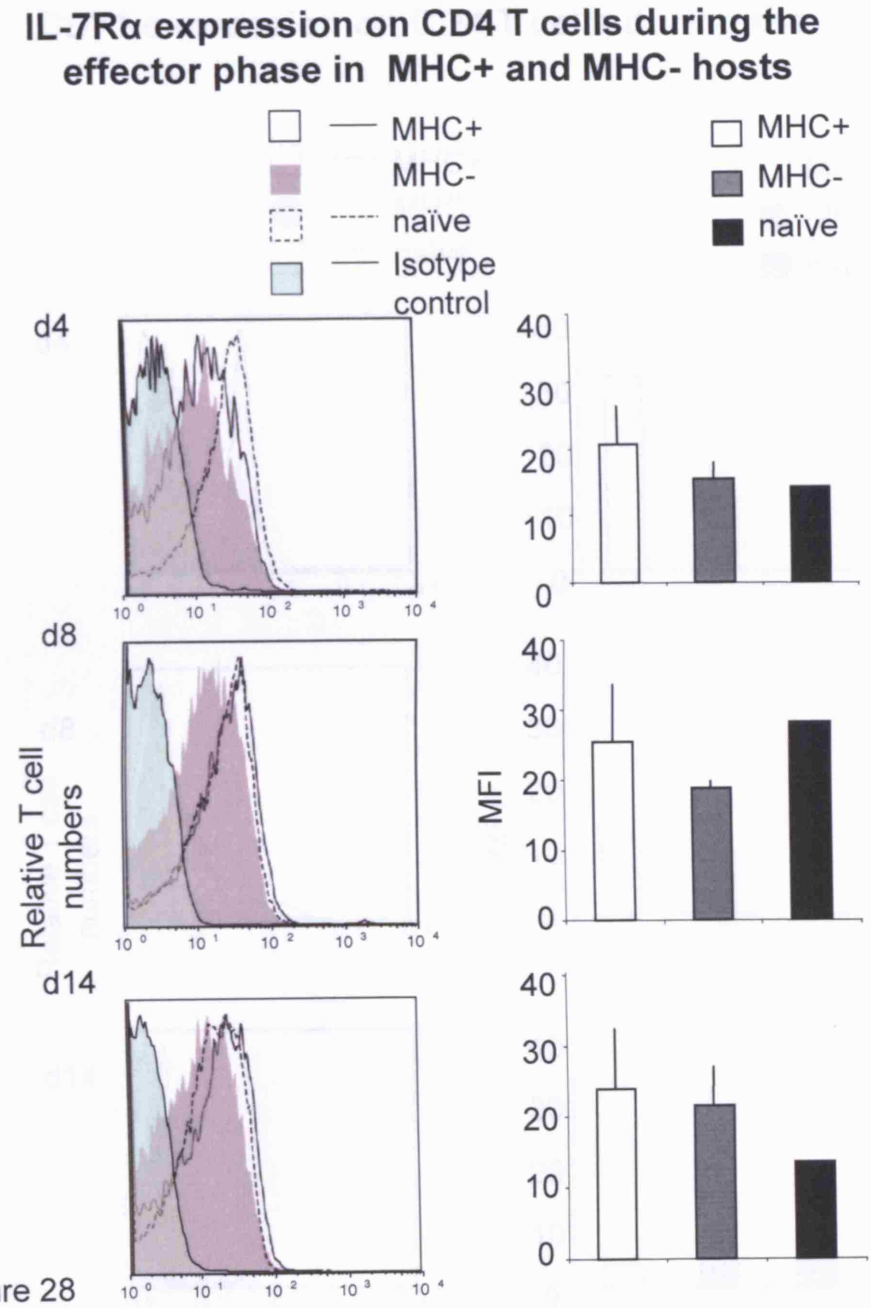



Figure 28

FACS histograms (left) and MFI (right) of IL-7Rα expression on CD4 T cells at day 4, 8 and 14 of the effector phase in MHC+ hosts (open histograms and bars) and MHC- hosts (grey histograms and bars). A1 naïve CD4 T cells (stippled lines histograms, black bars; one mouse) and isotype control antibody (light green histogram) are shown as reference. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation are shown. 3-5 mice per group were used. Similar results were obtained in 3 independent experiments.

Fig. 29

## CD71 expression on CD4 T cells during the effector phase in MHC+ and MHC- hosts

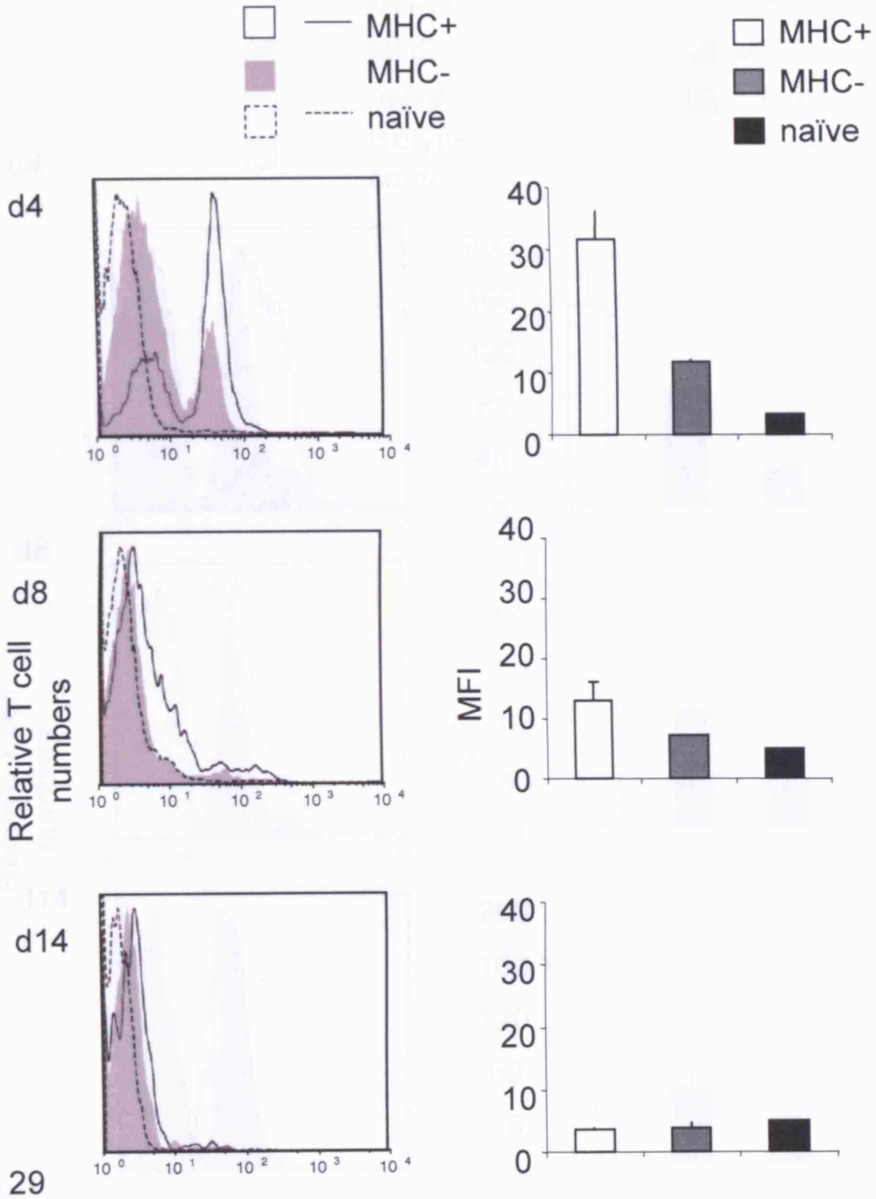



Figure 29

FACS histograms (left) and MFI (right) of CD71 expression on CD4 T cells at day 4, 8 and 14 of the effector phase in MHC+ hosts (open histograms and bars) and MHC- hosts (grey histograms and bars). A1 naïve CD4 T cells (stippled lines histograms, black bars) are shown as reference (one mouse). Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation are shown. 3 mice per group were used. Similar results were obtained in 2 independent experiments.

Fig. 30

**CD98 expression on CD4 T cells during the effector phase in MHC+ and MHC- hosts**

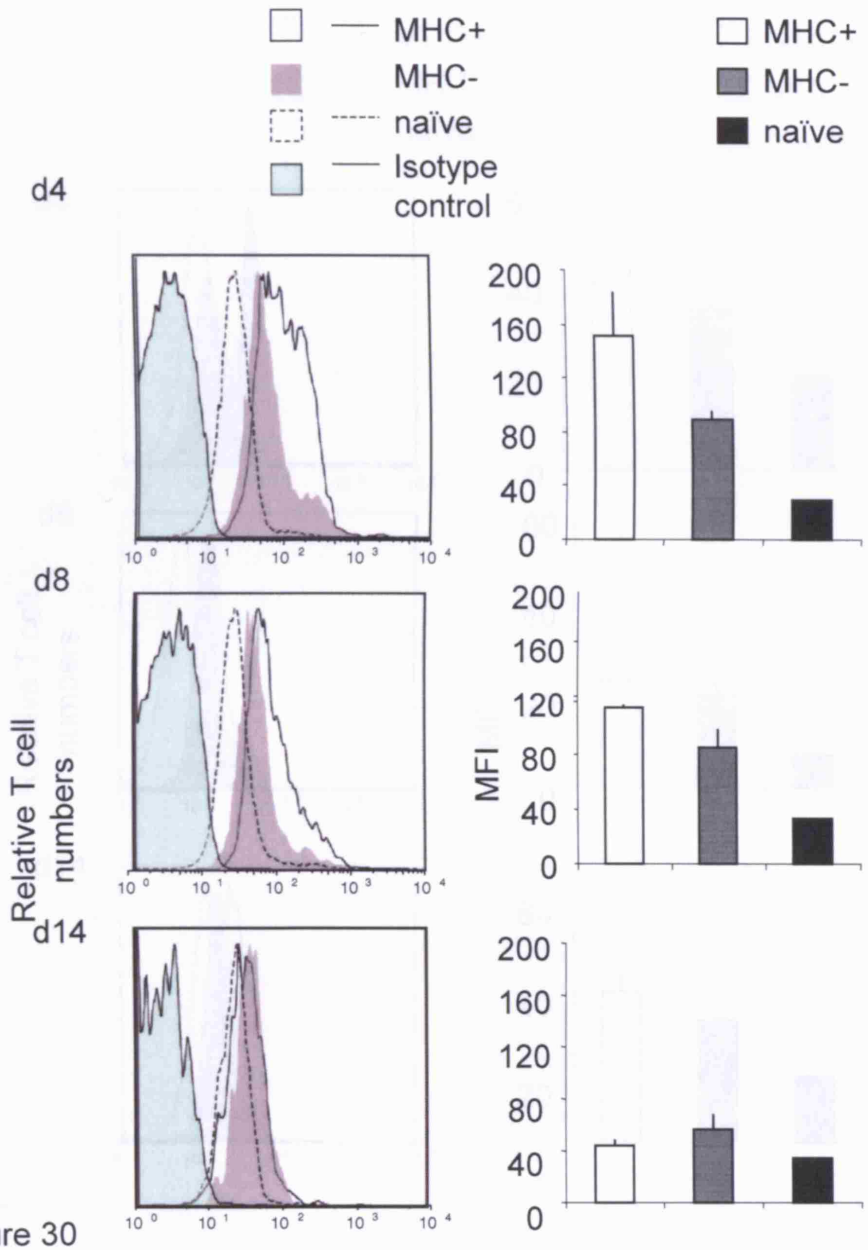



Figure 30

FACS histograms (left) and MFI (right) of CD98 expression on CD4 T cells at day 4, 8 and 14 of the effector phase in MHC+ hosts (open histograms and bars) and MHC- hosts (grey histograms and bars). A1 naïve CD4 T cells (stippled lines histograms, black bars; one mouse) and isotype control antibody (light green histogram) are shown as reference. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation are shown. 3-4 mice per group were used. Similar results were obtained in 2 independent experiments.

Fig. 31

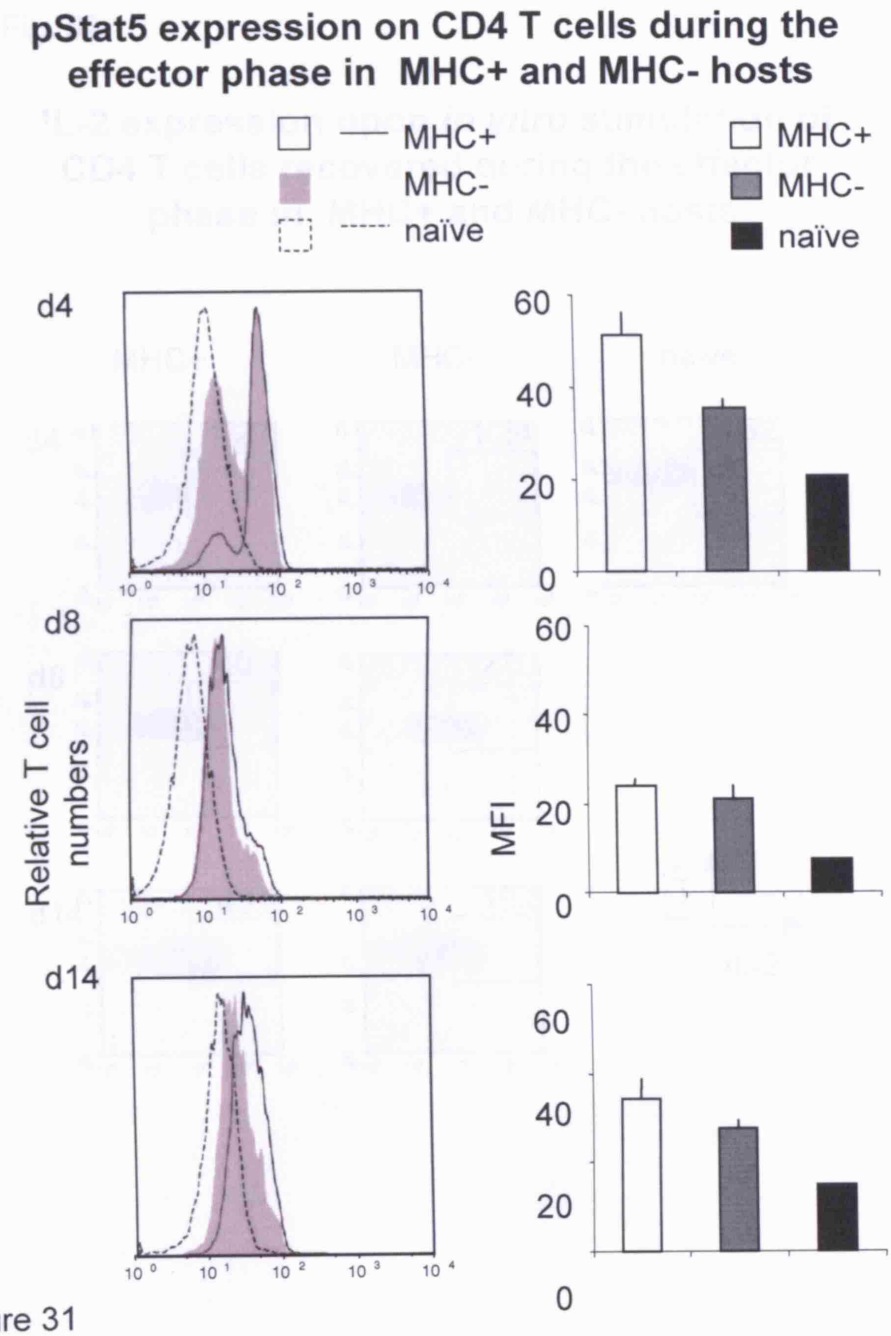



Figure 31

FACS histograms (left) and MFI (right) of pStat5 expression on CD4 T cells at day 4, 8 and 14 of the effector phase in MHC+ hosts (open histograms and bars) and MHC- hosts (grey histograms and bars). A1 naïve CD4 T cells (stippled lines histograms, black bars) are shown as reference (one mouse). Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation are shown. 3-4 mice per group were used. Similar results were obtained in 2 independent experiments.

Fig. 32

**IL-2 expression upon *in vitro* stimulation of CD4 T cells recovered during the effector phase in MHC+ and MHC- hosts**

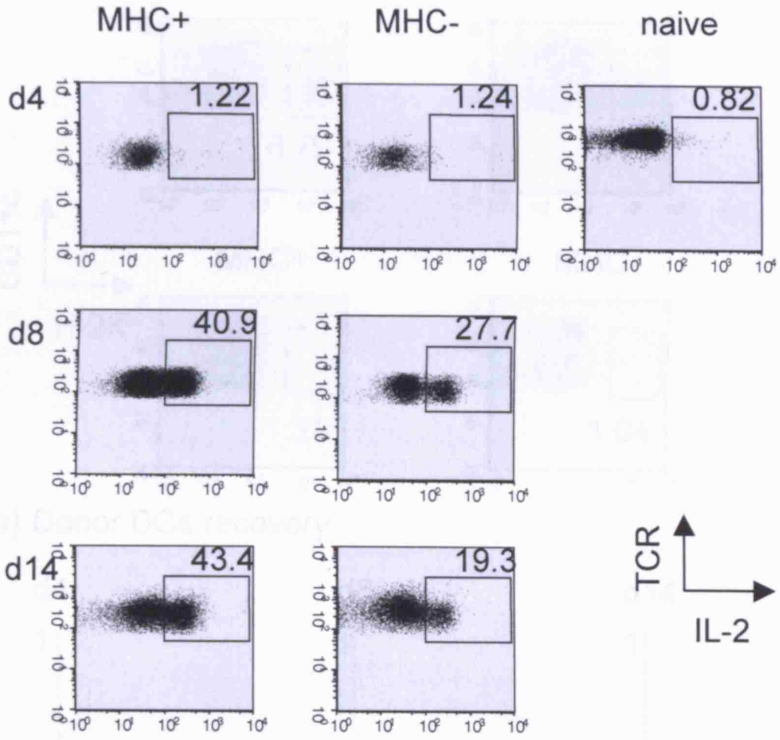



Figure 32

Percentage of IL-2 producing cells on CD4 T cells upon in vitro stimulation with PdBU and ionomycin in the presence of brefeldinA assessed by intracellular staining at day 4 (upper row), 8 (middle row) and 14 (lower row) of the effector phase in MHC+ hosts (left), MHC- hosts (centre) and A1 naïve CD4 T cells (right, one mouse). Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Pooled cells from 3-5 mice per group were used.

Fig. 33 a) and b)

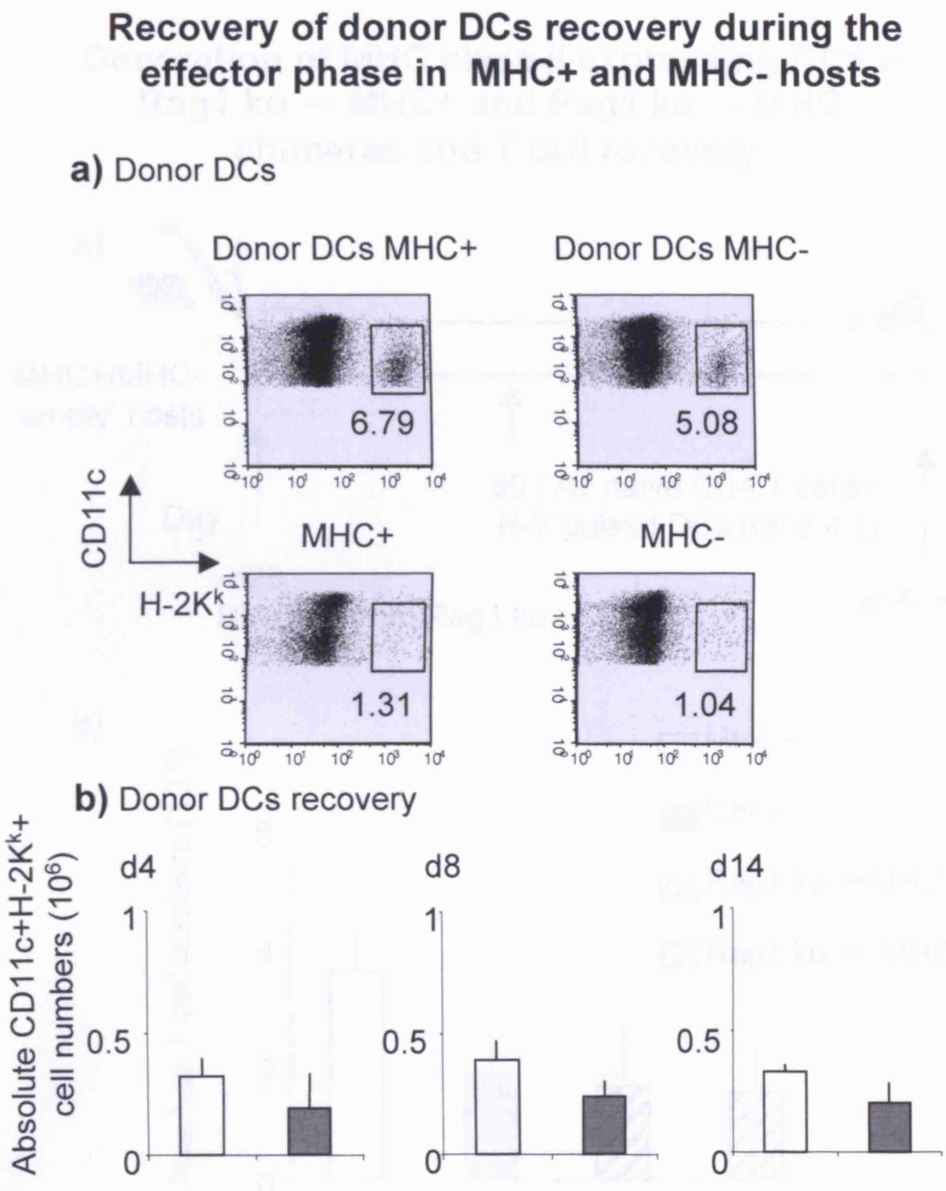



Figure 33 a) and b)

a) FACS plot show percentages of donor DCs (CD11c+ H-2k+) recovered 4 days after transfer into MHC+ hosts (upper line, left) and MHC- (upper line, right). Negative control staining for H-2k DCs from MHC+ (lower line, left) and MHC- (lower line, right) hosts are shown as reference. b) Absolute numbers of donor DCs recovered at day 4 (left), 8 (centre) and 14 (right) of the effector phase from MHC+ hosts (white bars) and MHC- hosts (grey bars). Mean values ± standard deviation are shown. 4 mice per group were used.

Fig. 34 a) and b)

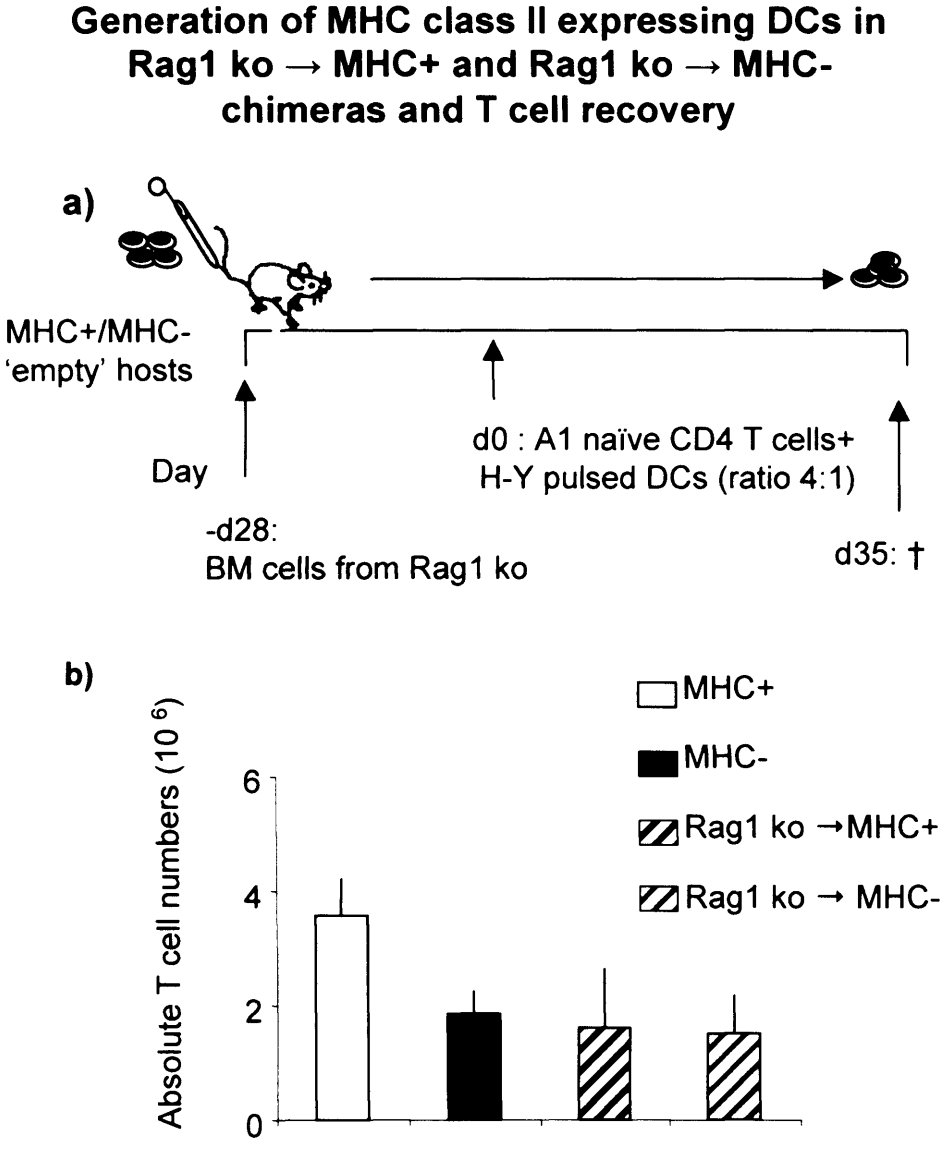



Figure 34 a) and b)

a) Experimental design; b) CD4 T cells recovery from 3 MHC+ hosts (white bars), 3 MHC- hosts (grey bars), 6 Rag1 ko → MHC+ chimeras (black diagonal bars) and 6 Rag1 ko → MHC- chimeras (grey diagonal bars) 35 days after transfer.

Fig. 35 b) and c)

**IL-7Rα expression on memory CD4 T cells generated in MHC+ and MHC- hosts and Rag1 ko → MHC+ and Rag1 ko → MHC- chimeras**

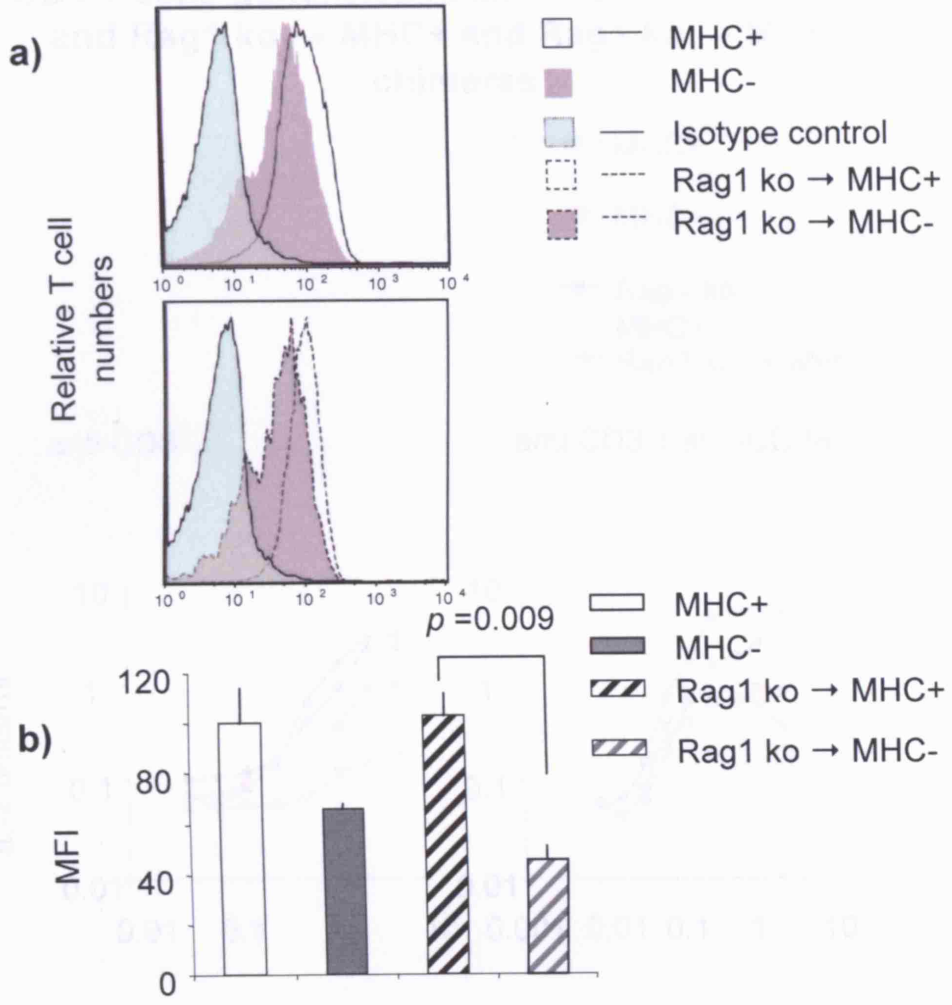



Figure 35 a) and b)

a) FACS histograms of IL-7Rα expression on memory CD4 T cells generated in MHC+ hosts (open histogram), MHC- hosts (grey histogram) upper line, and from memory CD4 T cells generated in Rag1 ko → MHC+ chimeras (open histograms dashed line) and in Rag1 ko → MHC- chimeras (grey histograms dashed line). Isotype control antibody (light green histogram) is shown as reference. b) MFI for IL-7Rα from 3 MHC+ hosts (white bars), 3 MHC- hosts (grey bars), 6 Rag1 ko → MHC+ chimeras (black diagonal bars) and 6 Rag1 ko → MHC- chimeras (grey diagonal bars). Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation and p-values, obtained with two-tail Mann-Whitney test, are shown.

Fig. 36

**IL-2 secretion upon *in vitro* activation by memory CD4 T cells generated in MHC+ and MHC- hosts and Rag1 ko → MHC+ and Rag1 ko → MHC- chimeras**

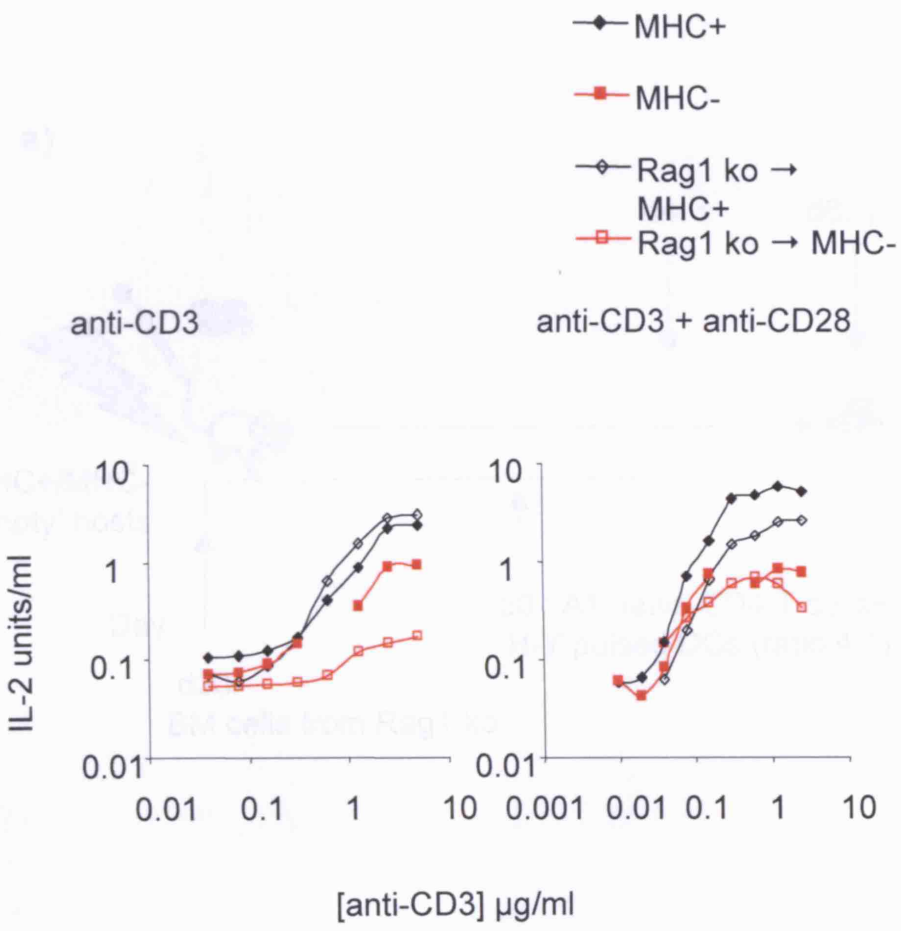



Figure 36

IL-2 secretion by memory CD4 T cells from 3 pooled MHC+ hosts (black diamonds), 3 pooled MHC- hosts (red squares), 6 pooled Rag1 ko → MHC+ chimeras (open black diamonds) and 6 pooled Rag1 ko → MHC- chimeras (open red squares) upon stimulation with plate-bound anti-CD3 (left) and anti-CD3 in the presence of anti-CD28 (right). IL-2 was detected as proliferation of the IL-2 dependent cell line CTLL-2 and results were calculated from a standard curve of recombinant IL-2 to give units of IL-2/ml produced.

Fig. 37

**Generation of bone marrow chimerasRag1 ko → MHC+ and Rag1 ko → MHC- chimeras in sub-lethally irradiated hosts**

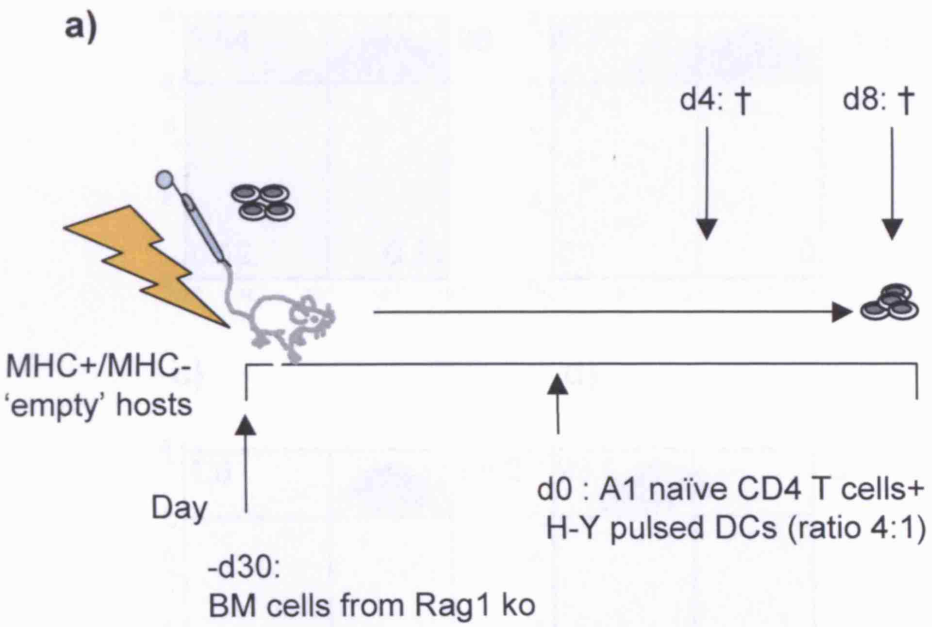



Figure 37

Experimental design of generation of Rag1 ko → MHC+ and Rag1 ko → MHC- bone marrow chimeras.

Fig. 38 a) – d)

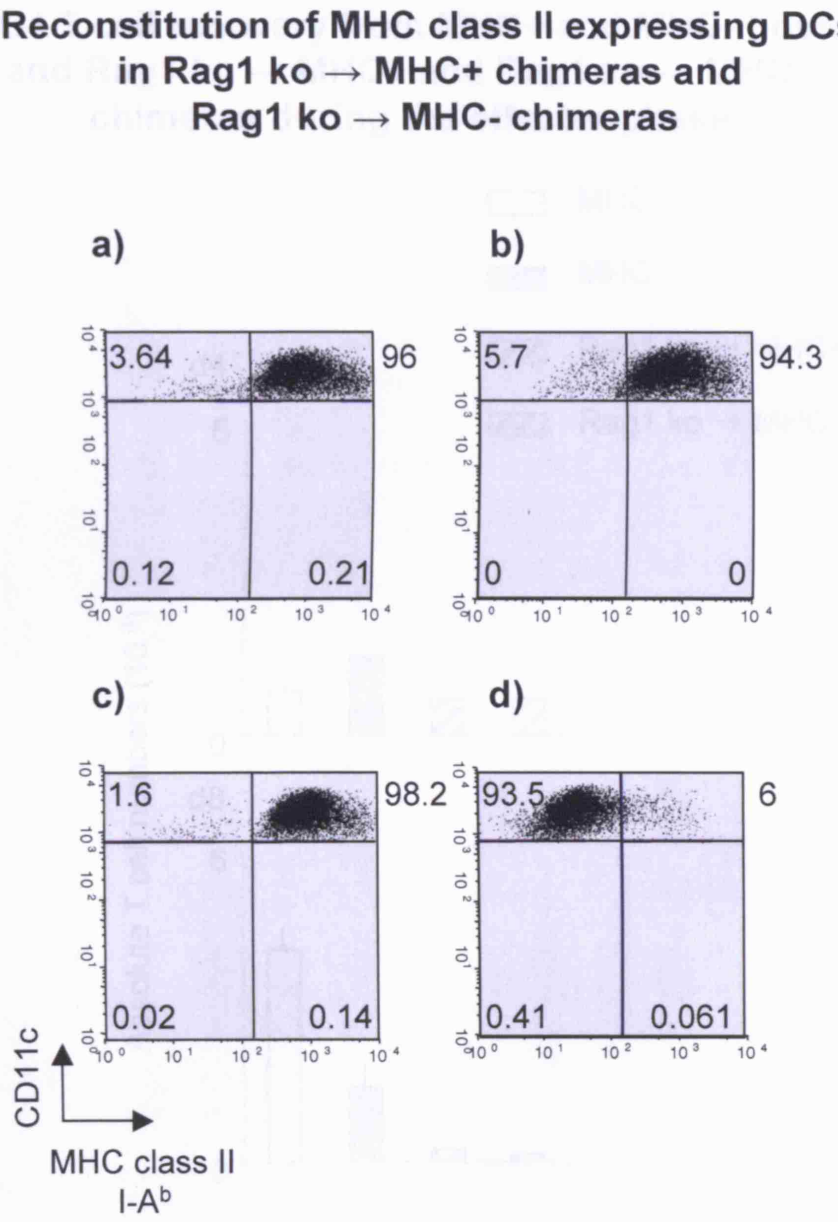



Figure 38 a) - d)

FACS plot show percentages of DCs expressing MHC class II molecules (CD11c+ I-Ab+) from Rag1 ko → MHC+ chimeras (a) and Rag1 ko → MHC- chimeras (b). DCs from MHC+ hosts (c) and MHC- hosts (d) are shown as reference.

Fig. 39

**CD4 T cell recovery from MHC+ and MHC- hosts and Rag1 ko → MHC+ and Rag1 ko → MHC- chimeras during the effector phase**

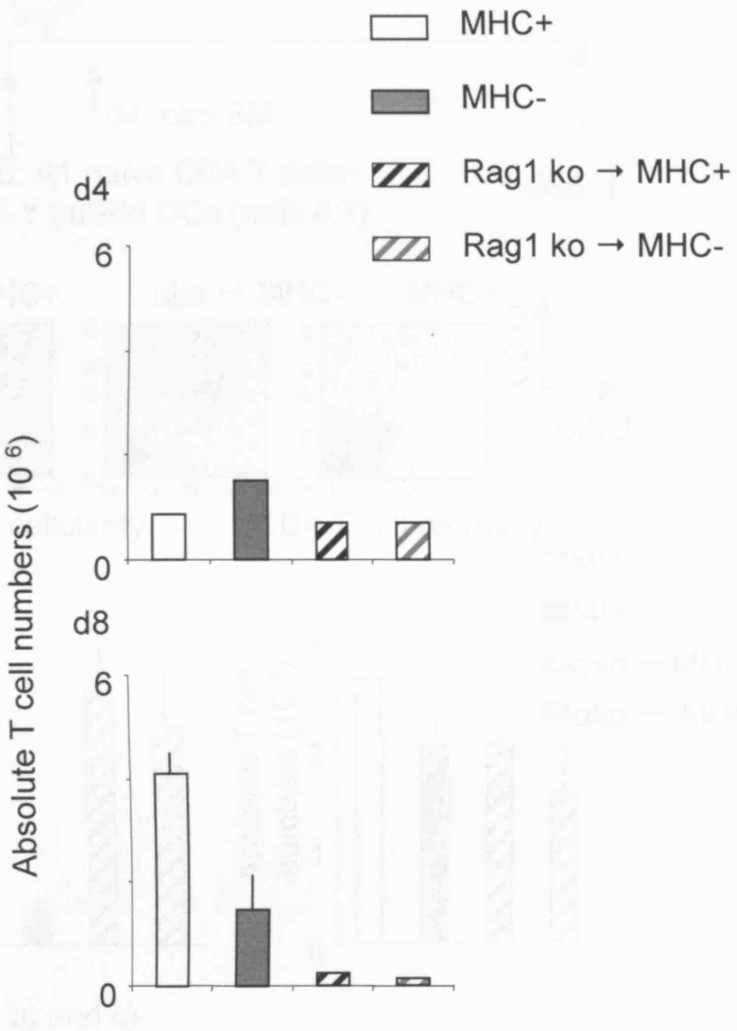



Figure 39

CD4 T cells recovery from MHC+ hosts (white bars), MHC- hosts (grey bars), Rag1 ko → MHC+ chimeras (black diagonal bars) and Rag1 ko → MHC- chimeras (grey diagonal bars) at day 4 (1 mouse per group) and day 8 (3 mice per group) of the effector phase. T cell recovery was calculated from percentages of CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation are shown.

Fig. 40 a), b) and c)

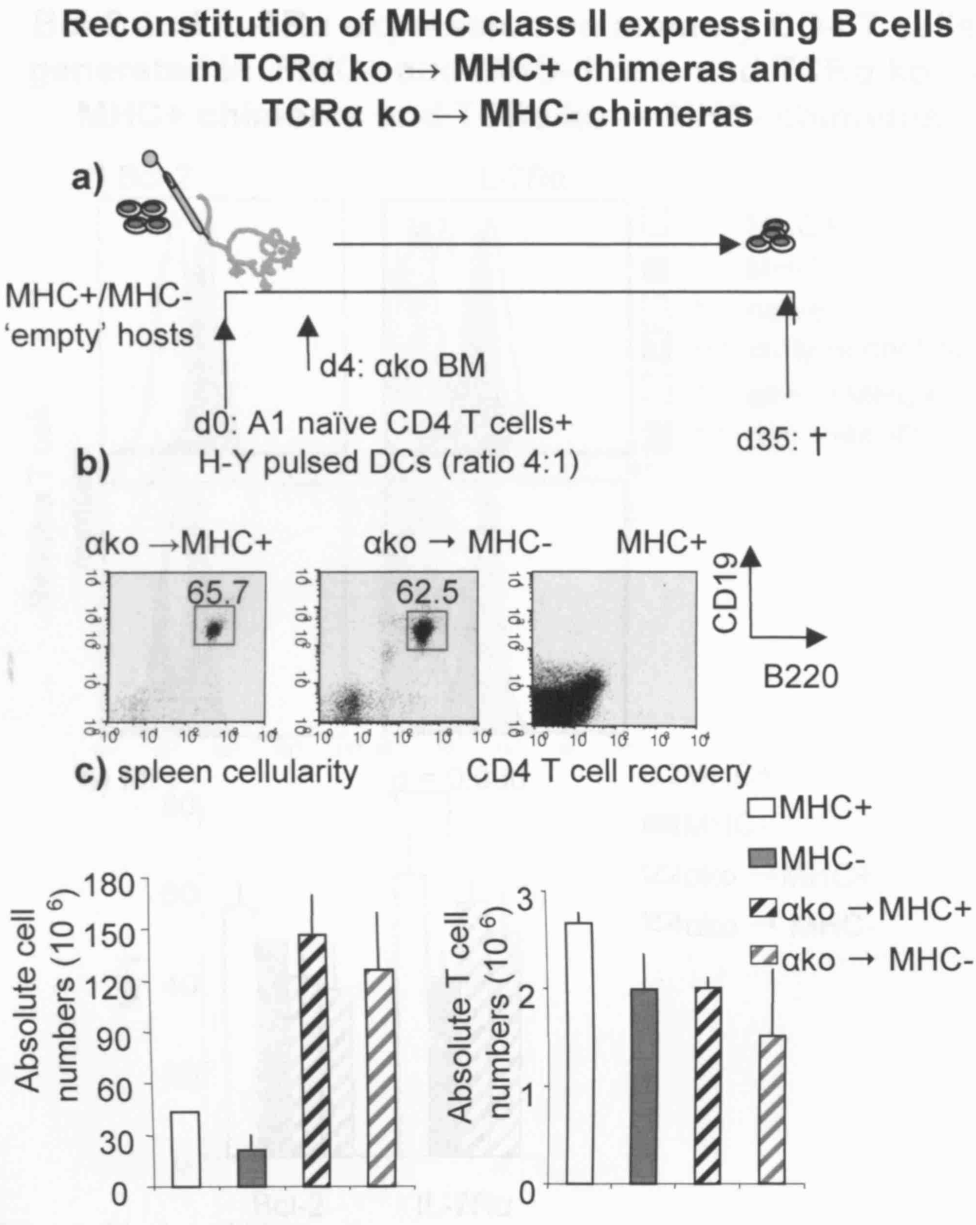



Figure 40 a), b) and c)

a) Experimental design. b) Percentages of B cells in αko →MHC+ chimeras (left) and αko → MHC- chimeras (centre). An example of splenocytes from MHC+ hosts (right) stained for B cells is reported as a negative control. Percentages of B cells were obtained from B220+ CD19+ cells from the lymphocyte gate of blood sample. c) Spleen cellularity (left) and CD4 T cell recovery (right) from 3 MHC+ hosts (white bars), 3 MHC- hosts (grey bars), 5 αko →MHC+ chimeras (black diagonal bars) and 5 αko → MHC- chimeras (grey diagonal bars). Mean values ± standard deviation are shown.

Fig. 41 a) and b)

**Bcl-2 and IL-7Rα expression on memory CD4 T cells generated in MHC+ and MHC- hosts and TCRα ko → MHC+ chimeras and TCRα ko → MHC- chimeras**

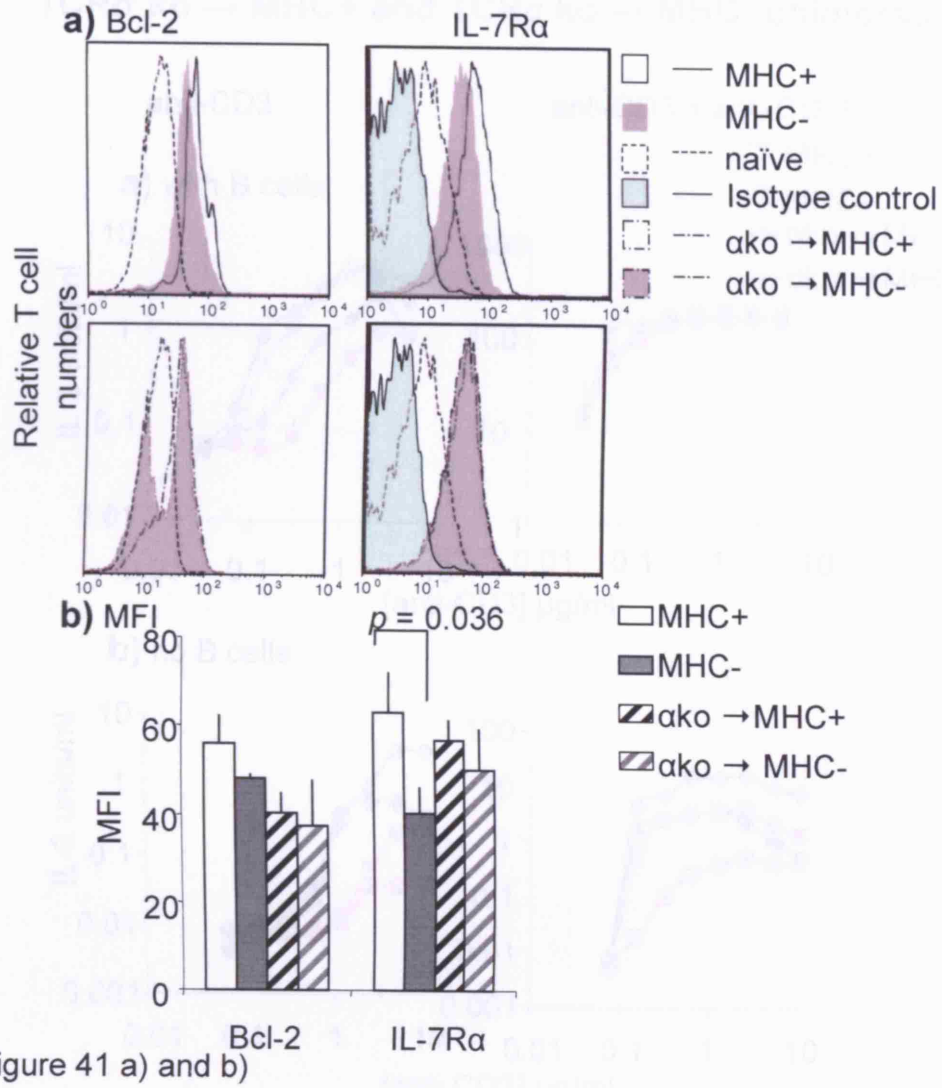



Figure 41 a) and b)

a) FACS histograms of Bcl-2 (left) and IL-7Rα (right) expression on memory CD4 T cells generated in MHC+ hosts (open histogram), MHC- hosts (grey histogram) upper line, and in αko →MHC+ chimeras (open histograms dashed line) and αko →MHC- chimeras (grey histograms dashed line). A1 naïve CD4 T cells (stippled lines histograms) and isotype control antibody (light green histogram) are shown as reference. b) MFI for Bcl-2 (left) and IL-7Rα (right) from 3 MHC+ hosts (white bars), 3 MHC- hosts (grey bars), 5 αko →MHC+ chimeras (black diagonal bars) and 5 αko →MHC- chimeras (grey diagonal bars). Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation are shown.

Fig. 42 a) and b)

## IL-2 secretion upon *in vitro* activation by memory CD4 T cells generated in MHC+ and MHC- hosts and TCRα ko → MHC+ and TCRα ko → MHC- chimeras

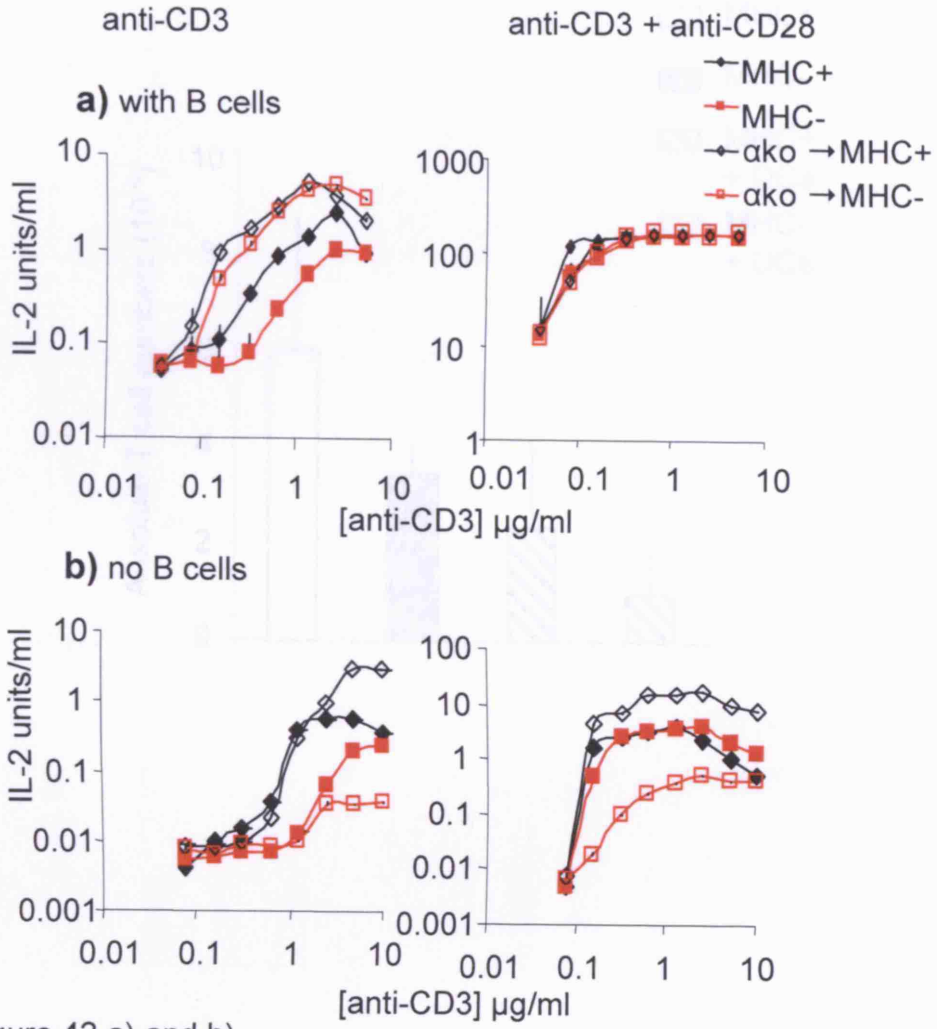



Figure 42 a) and b)

IL-2 secretion by memory CD4 T cells from MHC+ hosts (black diamonds), MHC- hosts (red squares), αko →MHC+ chimeras (open black diamonds) and αko →MHC- chimeras (open red squares) upon stimulation with plate-bound anti-CD3 (left) and anti-CD3 in the presence of anti-CD28 (right) either in co-culture with B cells (a) or after B cell depletion (b). IL-2 was detected as proliferation of the IL-2 dependent cell line CTLL-2 and results were calculated from a standard curve of recombinant IL-2 to give units of IL-2/ml produced.

Fig. 43

## CD4 T cell recovery from DC injected hosts

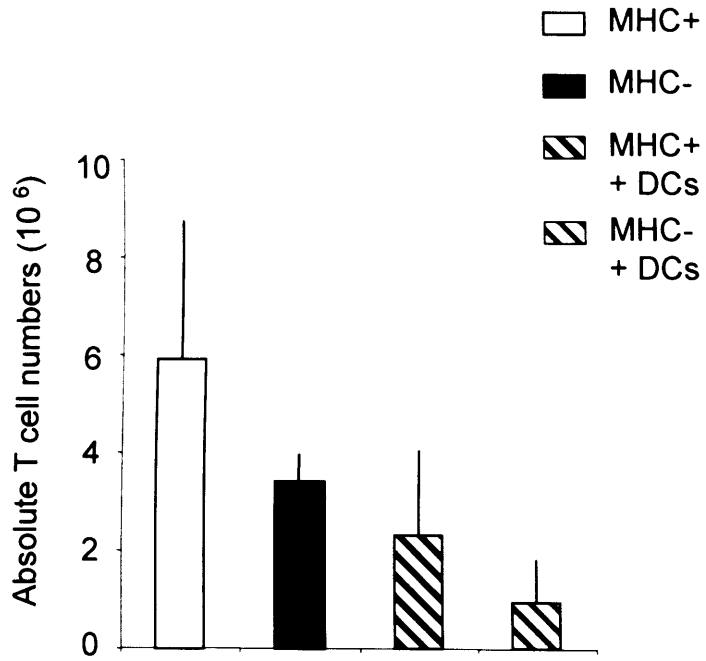



Figure 43

CD4 T cell recovery at day 14 from 4 MHC+ hosts (open bars) and 3 MHC- hosts (grey bars) as well as 4 MHC+ hosts (black diagonal bars) and the 4 MHC- hosts (grey diagonal bars) that received additional DCs every 3 days until the experiment was concluded. T cell recovery was calculated from percentages of CD4+ H57+ cells from the lymphocyte gate. Mean values ± standard deviation are shown.

Fig. 44 a) and b)

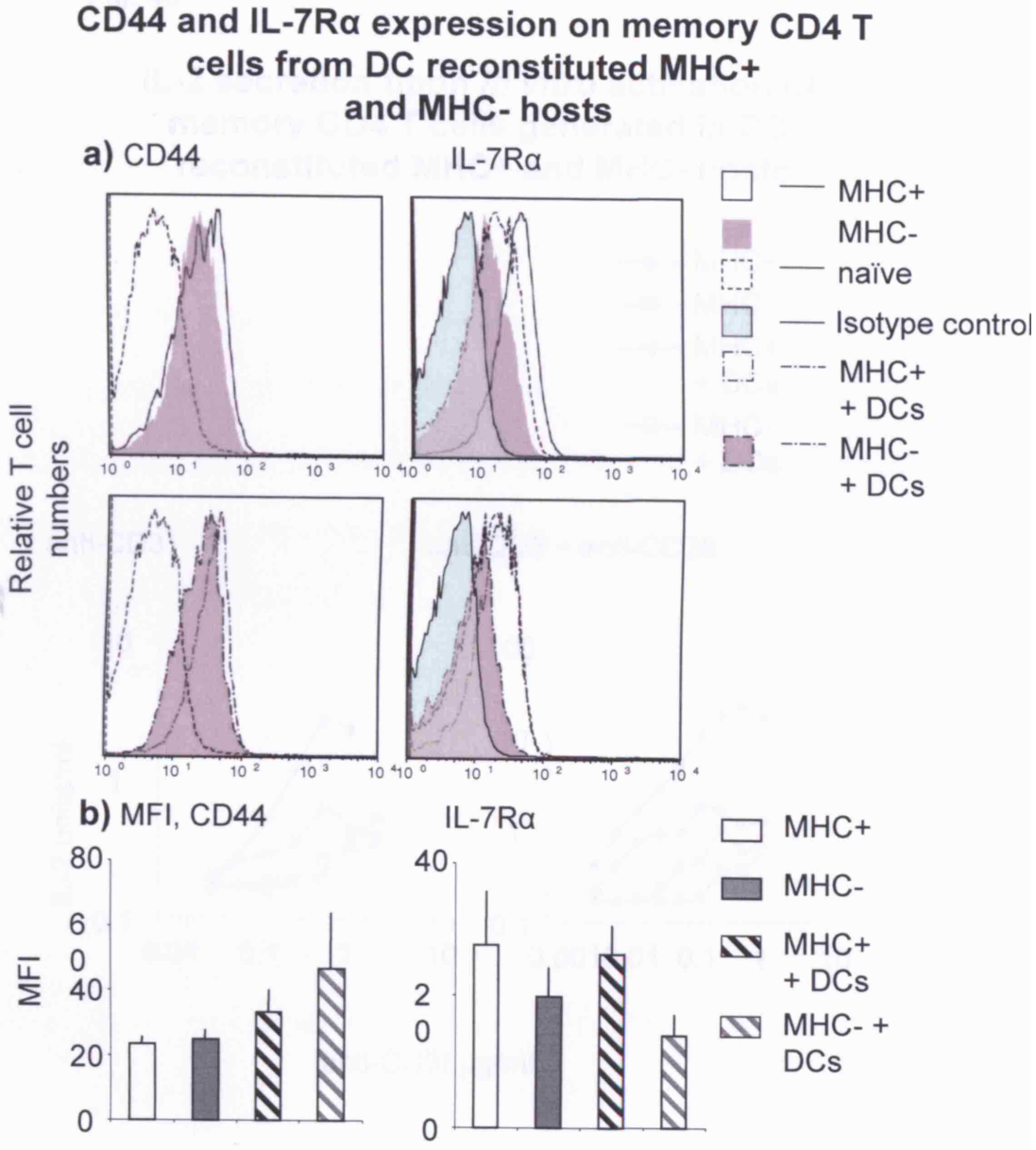



Figure 44 a) and b)

a) FACS histograms and (b) MFI of CD44 (left) and IL-7Rα (right) expression on CD4 T cell 14 days after immunisation from 4 MHC+ hosts (open histograms), 3 MHC- hosts (grey histograms) as well as 4 MHC+ hosts (black diagonal bars) and 4 MHC- hosts (grey diagonal bars) that received additional DCs every 3 days until the experiment was concluded. A1 naïve CD4 T cells (stippled lines histograms) and isotype control antibody (light green histogram) are shown as reference. Expression was assessed on CD4+ H57+ cells from the lymphocyte gate. Mean values ± Standard deviation are shown.

Fig. 45

**IL-2 secretion upon *in vitro* activation of memory CD4 T cells generated in DC reconstituted MHC+ and MHC- hosts**

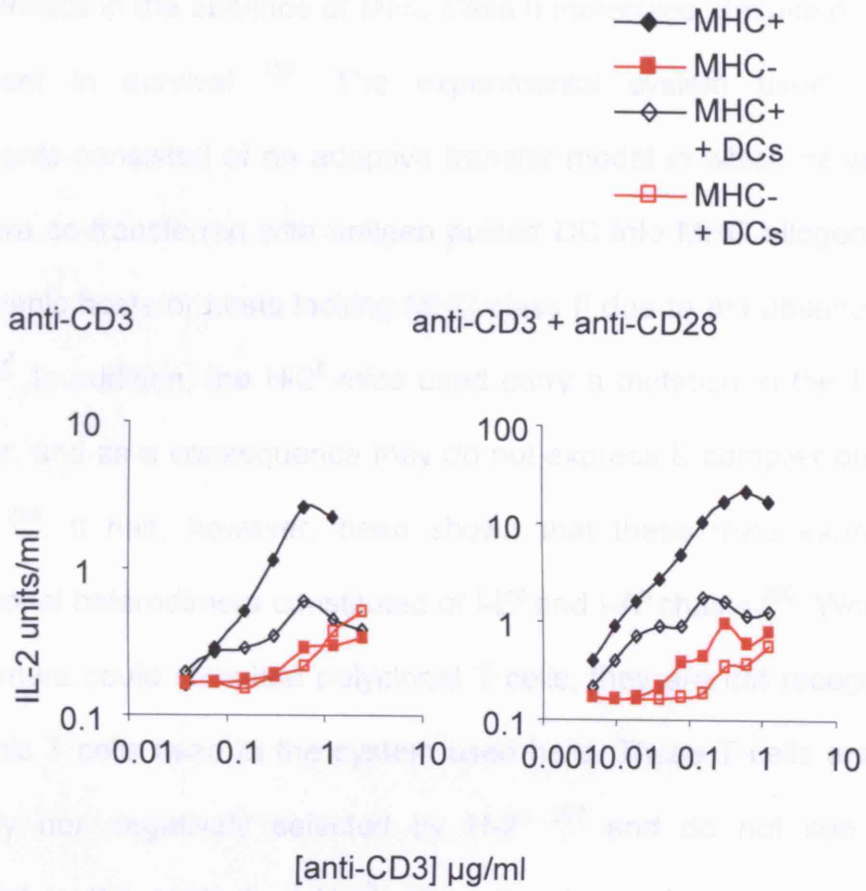



Figure 45

IL-2 secretion upon stimulation with plate-bound anti-CD3 (left) and anti-CD3 in the presence of anti-CD28 (right) by memory CD4 T cells from 4 pooled MHC+ hosts (black diamonds) and 3 pooled MHC- hosts (red squares) as well as 4 MHC+ hosts (open black diamonds) and 4 MHC- hosts (open red squares) that received additional DCs every 3 days until the experiment was concluded. IL-2 was detected as proliferation of the IL-2 dependent cell line CTLL-2 and results were calculated from a standard curve of recombinant IL-2 to give units of IL-2/ml produced.

## DISCUSSION

MHC contact with TCR plays a crucial role in the survival of naïve T cells [49] [50] [51] [52] [53]. Memory T cells have been shown to be less reliant on MHC contact for survival, although some exceptions have been reported [292] [293]. Our lab previously showed that CD4 T cells lose their functional memory characteristics in the absence of MHC class II molecules, despite no obvious impairment in survival [129]. The experimental system used in those experiments consisted of an adoptive transfer model in which naïve CD4 T cells were co-transferred with antigen pulsed DC into MHC allogeneic H-$2^b$ lymphopenic hosts or hosts lacking MHC class II due to the absence of I-$A^\beta$ chain [294]. In addition, the H-$2^b$ mice used carry a mutation in the I-$E^\alpha$ gene promoter, and as a consequence they do not express E complex on the cell surface [295]. It has, however, been shown that these mice express non conventional heterodimers constituted of I-$E^\beta$ and I-$A^\alpha$ chains [296]. While these heterodimers could stimulate polyclonal T cells, they are not recognised by transgenic T cells used in the system used here. These T cells are neither positively nor negatively selected by H-$2^b$ [297] and do not see antigen presented in the context of H-$2^b$. The advantage of using an allogeneic system was the generation of a pure population of memory T cells which was not contaminated by naïve T cells that escaped stimulation, as naïve T cells do not survive in the absence of syngeneic MHC [80]. Moreover, as the hosts lacked NK cells due to the absence of the cytokine common γ chain [298], no rejection was possible on the basis of differential MHC haplotype expression on the donor cells. A potential disadvantage of this system was the transfer of cells in lymphopenic hosts, which is thought to cause artificially high cell

expansion. However, it should be noted that T cell expansion in response to antigen is far higher and faster in kinetics than the expansion observed in response to lymphopenia alone [299].

The experimental system described and used here therefore allowed us to study the influence of MHC interactions on a pure CD4 T cell memory population.

In order to define underlying mechanisms that caused the functional defects of memory CD4 T cells described before [129], memory CD4 T cells generated in MHC+ hosts and memory CD4 T cells generated in MHC- hosts were compared by means of microarray analysis. The rationale was not to achieve necessarily a complete gene comparison, but to pinpoint candidate molecules that could be involved in the maintenance of memory function.

Analysis of gene expression by microarray is a powerful tool which generates an enormous amount of information. Despite the numerous advantages, the technique has some limitations which are briefly discussed here. For example, the amount and the relevance of the information retrievable by analysis of gene expression is restricted to the probes represented on the chip. This became clear during the analysis of the gene expression profiles from memory populations using glass arrays, as the genes displayed on those arrays turned out to be not relevant to the biological question posed. In addition, only in the case of molecules that have an active biological function as RNA molecules, differences in gene expression can directly correlate with their biological significance. In all the other cases, differences in gene expression do not necessarily correlate with biological relevance, as often cellular mechanisms are tightly regulated at many different stages in addition to gene transcription (i.e. proteins post-

translational modifications). Consequently, once it has been established by real time PCR that the Affymetrix data were a reliable representation of the molecular profiles of the two memory populations, the efforts and resources were concentrated in verifying and developing those data with other techniques and experiments instead of repeating the Affymetrix experiments the numbers of times necessary to achieve statistical significance. Moreover, as the cell populations under investigation were *ex vivo* and not synchronised *in vitro* cell cultures, the number of replicates necessary to overcome the biological variability would have been unaffordable.

Microarray analysis was performed using *in vitro* amplified RNA. The choice was dictated by the limited number of cells recovered and the extensive cell sorting required to achieve the purity needed to perform analysis of gene expression. This decision implied the possibility of artefacts introduced by preferential amplification of some transcripts rather than others. For this reason, some of the transcripts indicated as differentially expressed in the two cell populations by the microarray analysis were validated on total RNA without *in vitro* amplification. For the genes that were validated by real time PCR, the results obtained were consistent with those obtained by chip analysis. Clearly this does not allow to conclude on the level of expression of all the genes in the array, but nevertheless it improved the confidence in the data generated.

Basic data analysis was applied which aimed to identify genes that were differentially expressed in the two populations in one condition only (resting status). Even in this simple scenario, arbitrary choices were made in the data analysis and an example is given by cut-offs. The cut-off for the difference in the level of expression between the two cell populations was set at 1.5-fold.

As there are no right or wrong choices in this respect, this value was selected as relevant for the dataset based on the fact that such a cut-off included the over-expression of the transcript for CD5 in memory CD4 T cells from MHC- hosts compared to MHC+ hosts, which was previously described as one of the phenotypic characteristics of memory CD4 T cells from MHC- hosts at the protein level [129].

At the stage of data management, the differentially expressed genes were classified into categories relevant for T cells. This 'tailored' grouping was preferred to the 'already made' categories offered by the software for analysis of gene expression, as, at the moment, 'T cells' specific gene ontologies are not available. The organisation of the genes differentially expressed according to the categories proposed here offered the advantage of bringing together all the information generated and putting it into context. Some of the genes differentially expressed were involved in common pathways or similar biological functions that were relevant for the physiology of the memory response. Interestingly, the transcripts that were involved in common processes all pointed in the same direction, enhancing the strength of the biological significance of their differential expression.

Relevant and interesting genes highlighted by microarray analysis are discussed in the following section.

From the gene expression profiles generated, transcripts for various surface molecules were differentially expressed in memory CD4 T cells from MHC- hosts vs MHC+ hosts. For example, the over-expression of CD224 and the under-expression of CD151 in memory CD4 T cells from MHC- hosts suggest once again a higher rate of proliferation in memory CD4 T cells from MHC- hosts. Although these molecules were not further analysed using other

techniques, it is interesting to report that they were both associated with lymphoproliferation. In fact, signal transduction through CD224 has been implicated in the human X-linked LymphoProliferative (XLP) disease [300], although so far CD224 has not been reported to be expressed on CD4 T cells [272]. In addition, the analysis of T cells from CD151-null mice showed that lack of CD151 induces a hyperproliferative phenotype in response to TCR stimulation [301].

An interesting set of molecules are the 3 chemokine receptors *Ccr2*, *Cxcr4* and *Cxcr6*, although more questions than answers arise when looking into the intricate world of chemokines. There is an increasing body of evidence indicating that specific patterns of chemokine receptors characterise T cells subsets [302]. In this context, the over-expression of CCR2 in memory CD4 T cells from MHC- hosts seems curious as CCR2 has been associated with a Th2 phenotype [271] and CD4 T cells from the A1 strain tend to be 'skewed' towards a Th1 phenotype. In addition, memory CD4 T cells from MHC- hosts also show an over-expression of *Cxcr6* (also known as Bonzo), which characterise a small subset of activated CD4 T cells [303], preferentially secreting IFN-γ [304]. CXCR6 expression has been reported to inversely correlate with the expression of CXCR4 [305], another molecule expressed on activated memory T cells (and one of the co-receptors for Human Immundeficiency Virus (HIV) [306]), which was again over-expressed in memory CD4 T cells. It is possible that the apparent contradictions could be explained by further studies, and therefore these data are still worth mentioning. As from our data there was no indication of a different anatomical distribution or migratory pattern between the two memory CD4 T

cell populations, this set of data generated by Affymetrix was not further analysed.

The over-expression of CD5 in the memory CD4 T cells from MHC- hosts vs MHC+ hosts could be seen as the validation at the RNA level of the FACS data already described for this cell population [129]. Similarly, we analysed the surface expression of CD49d, another molecule in which our lab had previously shown an interest in the context of the characterisation of different memory CD4 T cell subsets [307]. In that study, memory CD4 T cells were found to express CD49d in correlation to their anatomical localisation, rather than in correlation to functional differentiation. FACS profiles confirmed the down-regulation of CD49d in the memory population recovered from MHC- hosts reported by microarray analysis, but in the context of our experiment we could not find an interesting angle to pursue the study of this molecule.

By microarray analysis, 3 genes involved in cellular senescence were over-expressed in memory CD4 T cells from MHC- hosts compared to memory CD4 T cells recovered from MHC+ hosts. The terms 'cellular or replicative senescence' was introduced to describe the characteristic of somatic cells to enter a non-replicative stage by arresting irreversibly at the G1 stage of the cell cycle, after a defined number of divisions [278]. It has been reported that T cells can divide up to 56 times before becoming senescent [308]. Senescent T cells are metabolically active, but are unable to proliferate or to generate Ca2+ fluxes upon stimulation [309] [310]. It is interesting to note that T cell senescence is a phenomenon that increases with aging and that larger numbers of senescent T cells are found in the elderly compared to the young [311]. The role of cellular senescence was evaluated as a potential explanation for the functional impairment upon activation observed in memory CD4 T

cells maintained in the absence of MHC class II interaction. Considering that memory CD4 T cells divide at a higher rate in the absence of MHC class II compared to memory CD4 T cells that maintain contact with MHC class II and that memory CD4 T cells from MHC- hosts show a functional impairment, it is possible to hypothesise that the loss of function could be the consequence of extensive proliferation in the absence of MHC class II interaction. A point that would need clarification in this case is the lack of accumulation of senescent cells, unless it is assumed that T cells that reach the senescent status may have a disadvantage in terms of survival compared to those that are still cycling. This assumption is not in line with the concept of senescence, which so far has pointed to an inability to produce daughter cells and not to an impairment in survival. Nevertheless, it is not possible to exclude that different homeostatic mechanisms regulate the turnover of memory T cells in the absence of MHC class II, which could somehow limit the accumulation of senescent T cells. In addition, the molecules identified by analysis of gene expression can only provide a possible indication that senescence could affect memory CD4 T cells in the absence of MHC class II. The lack of good antibodies and limited knowledge about the functional mechanisms of these molecules did not allow further investigation of these results.

Some of the genes differentially expressed in the two memory populations were validated by real time PCR. The choice to validate *Mapk9*, the genes coding for JNK2, by a second method based on RNA expression, was related to a recent report in which JNK was shown to be undetectable in naïve CD4 T cells prior to activation. The interpretations that were proposed for this observation suggested that the absence of the JNK pathway may

protect cells from cell death induced by activation or alternatively may prevent the expression of cytokines by naïve cells before proliferation in response to antigen, reviewed in [198]. JNK2 is a ubiquitously expressed protein whose function is regulated by phosphorylation, but these new findings indicate that this molecule is also regulated at the transcriptional level. In the context of our experiments, the down-regulation of the *m*RNA expression by memory cells may be indicative of an incomplete differentiation status achieved by memory CD4 T cells from MHC- hosts. In fact, the amount of *m*RNA for *Mapk9* expressed by memory CD4 T cells from MHC- hosts was reduced compared to memory CD4 T cells from MHC+ hosts and very similar to the level expressed by A1 naïve CD4 T cells.

Differential amounts of *m*RNA for IL-2 were detected between the two memory CD4 T cell populations. From these data, one can conclude that memory CD4 T cells from MHC- hosts store lower amounts of the transcript for *Il2* than memory CD4 T cells from MHC+ hosts, but higher levels than those observed in A1 naïve CD4 T cells. These findings paralleled the results obtained by a short *in vitro* stimulation of the three populations, suggesting a direct correlation between the amount of *m*RNA detected and the percentage of memory CD4 T cells able to synthesise IL-2 when challenged with PdBU and ionomycin. Using PdBU and ionomycin T cells were activated in a 'un-physiological' way, bypassing the TCR and acting directly on PKC and inducing calcium fluxes. Therefore, in this assay the potential of T cells to respond to the stimulus was tested rather than the TCR signalling capacity. The findings showed that a decreasing gradient in the efficiency of IL-2 response existed between memory CD4 T cells from MHC+ hosts, memory CD4 T cells from MHC- hosts and A1 naïve CD4 T cells,

which reflected the *m*RNA results. The same effect was observed upon *in vitro* activation by TCR triggering using anti-CD3 with or without anti-CD28, as already described [129]. The data on *m*RNA and short *in vitro* stimulation indicate that the impairment in memory CD4 T cells from MHC- hosts in IL-2 production is not only related to a defect in the signalling upon activation, but also to an intrinsic defect that is already present and defined at the *m*RNA level during the resting memory phase. It is not possible to discriminate whether the differential *m*RNA content for IL-2 was due to a difference in the basal synthesis of this cytokine in the memory phase or to a difference in the stability of the *m*RNA in the two hosts. Interestingly, it has recently been shown that signalling through the PI3K/Akt pathway sustains IL-2 synthesis by promoting IL-2 *m*RNA stabilisation at the 3' UnTranslated Region (UTR), specifically in the late stage of T cell activation [312]. In light of these findings, it can be consider that CD4 T cells from MHC- hosts may fail to undergo a complete program of differentiation due to the lack of signals that sustain and prolong IL-2 production during the development of the effector phase. Such sustained signals through the Akt signalling pathway could affect IL-2 production as well as the survival of activated T cells, as recently suggested [160], compromising both effector and memory differentiation. To further define the possible involvement of a deficient Akt signalling pathway during activation, two molecules down-stream Akt were monitored in *in vitro* activation of memory CD4 T cells. S6 and *Glut1* are two molecules that can be used as a read-out of Akt activity. S6 phosphorylation is required to allow the assembly of the ribosomal machinery and therefore to initiate protein synthesis necessary to sustain cell growth and effector functions [213] [205]. Using the TCR induced phosphorylation of S6 to verify the impairment in the

PI3K/Akt pathway, memory CD4 T cells from MHC- hosts were not as efficient as memory CD4 T cells from MHC+ hosts in starting protein synthesis upon TCR triggering. The expression of the glucose transporter, Glut1, is also controlled by Akt [288], a master regulator of glucose metabolism [289]. Memory CD4 T cells from MHC- hosts showed impaired Akt signalling as they synthesised lower levels of *Glut1 m*RNA during activation compared to memory CD4 T cells from MHC+ hosts. The defect in glucose metabolism in response to activation may indicate that a metabolic impairment could account for the inability of memory CD4 T cells from MHC- hosts to fully respond to stimulation.

Freshly isolated resting memory CD4 T cells from MHC- hosts expressed reduced amounts of phosphorylated Akt1 as well as reduced amounts of Bcl-2, corroborating the results from microarray analysis and strengthening the data in favour of a reduced survival potential of memory CD4 T cells in the absence of MHC class II interactions. In addition, by FACS analysis, a consistent down-regulation of IL-7Rα expression in the memory CD4 T cell population from MHC- hosts vs MHC+ hosts was detected, which correlates with Bcl-2 down-regulation as well as Stat5 down-regulation. The lower expression of IL-7Rα in memory CD4 T cells from MHC- hosts could not only point to a reduced survival capacity of these cells in the absence of MHC class II, but perhaps also to an incomplete process of memory differentiation of which IL-7Rα could count as an indicator. In this view, full IL-7Rα up-regulation following activation could be considered a marker of CD4 memory T cell differentiation that parallels the ability of cells to promptly acquire effector function upon activation. The indication of an incomplete differentiation towards a memory phenotype is supported also by the fact

that IL-7Rα expression in memory CD4 T cells recovered from MHC- hosts was lower than that observed in memory CD4 T cells recovered from MHC+ hosts but higher than that detected in effector or A1 naïve cells. This may indicate that in the MHC- hosts, CD4 T cells fail to acquire all the characteristics of memory cells. Alternatively, memory CD4 T cells in MHC- hosts may regulate IL-7Rα in an 'altruistic' way to enable the largest number of cells to receive survival signals as shown in naïve CD4 T cells [258], due to an increase in the reliance in this survival signal in the absence of MHC class II.

The latter observation does not fit with the down-regulation of Stat5 in memory CD4 T cells in MHC- hosts compared to MHC+ hosts. In fact, resting memory CD4 T cells from MHC- hosts did not show an increase in Stat5 phosphorylation due to an increase in the signalling through the IL-7 receptor, but on the contrary, showed a reduction of the phosphorylated protein compared to memory CD4 T cells from MHC+ hosts. In this respect, the impairment in cytokine signalling in the quiescent state may suggest a reduction in survival potential in memory CD4 T cells recovered from MHC- hosts compared to MHC+ hosts that find further evidence in the down-regulation of other crucial molecules that promote T cell survival such as Bcl-2 and Akt1.

These findings can explain why, despite a higher rate of cell division, memory CD4 T cells from MHC- hosts do not accumulate in comparison to those from MHC+ hosts. The turnover of memory CD4 T cells generated in MHC+ and MHC- hosts was analysed by PI staining. Complementing previous data showing a higher level of BrDU incorporation during a period of time of 12 days *in vivo* in memory CD4 T cells from MHC- hosts compared

to MHC+ hosts [129], a higher proportion of memory CD4 T cells from MHC- hosts was found in the G2/M-S phase of the cell cycle than in the memory CD4 T cells from MHC+ hosts. Moreover, 10 times more apoptotic cells were detected in the memory CD4 T cell population from MHC- hosts than from MHC+ hosts. The higher rate of cell cycling seemed to be an acquired characteristic of memory CD4 T cells generated in MHC- hosts, which was retained when cells were secondarily transferred into MHC+ lymphopenic adoptive hosts. Seven days after the secondary transfer, memory cells from MHC- hosts underwent 7 divisions compared to the 4 divisions showed by memory CD4 T cells from MHC+ hosts, as assessed by CFSE dilution. Similarly to what was previously observed, higher rates of cell division observed in memory CD4 T cells from MHC- hosts did not correlate with expansion of the cell population, implying that higher cell division was again balanced by higher cell death. Nevertheless, the higher proliferation and cell death of memory CD4 T cells from MHC- hosts did not result in a competitive disadvantage when memory cells from MHC- and MHC+ hosts were secondarily co-transferred into the same recipients. In these secondary hosts, the ratio between the two cell populations remained constant throughout a period of 49 days showing that, despite the fact that memory CD4 T cells from MHC- hosts showed an impairment in survival over memory CD4 T cells from MHC+ hosts, they were not out-competed.

During a time course experiment that aimed to follow the development of memory in the two hosts, it became clear that the differences observed in CD4 T cells recovered from late stages of the memory phase were already detectable 14 days after immunisation. This result indicates an early

establishment of a survival and functional impairment in CD4 T cells recovered from MHC- hosts. In light of this result, earlier time points were analysed. CD4 T cells from MHC- hosts showed an impaired activation *in vivo* compared to CD4 T cells transferred into MHC+ hosts during the effector phase. This result was particular evident in terms of CD71 and CD98 up-regulation and Stat5 phosphorylation at day 4 of the effector phase. The reduced ability of CD4 T cells to metabolically sustain their activation, and a possible impaired capacity to synthesise IL-2 suggested by the reduced percentages of Stat5 phosphorylation, may explain the decreased expansion in terms of CD4 T cell recovery observed at day 8 in MHC- hosts vs MHC+ ones.

This suggests that normal memory may never be established in MHC- hosts due to an impaired primary response in the first instance.

Data previously generated in our lab have shown that DCs transferred into adoptive hosts persisted for a period of 2-3 weeks [80]. In those experiments, the C5 antigen was continuously presented by the DCs, as it was present in the serum of the hosts [80]. In the experiments described here, antigen presentation was achieved by pulsing the DCs with the H-Y peptide. However, a limitation in antigen presentation seems an unlikely explanation because donor DCs were recovered in similar number from both hosts.

Therefore, one has to consider the hypothesis that the presence of allogeneic MHC class II, unable to provide any antigen presentation, may offer a 'tonic' signal that actively promotes the development of a full immune response.

Although there was no difference in the availability of the interaction with the syngeneic antigen presenting MHC class II molecules in the two hosts, CD4 T cells transferred into an MHC class II competent environment appear to receive additional signals from the allogeneic MHC class II molecules provided by endogenous host APCs. Non-cognate interactions may be required by effector CD4 T cells to fulfil their programme of differentiation during the immune response. In this case, the delivery of a 'tonic signal' in the form of self-peptide: MHC would be necessary for successful effector and memory generation. As the syngeneic DCs were antigen pulsed before injection, we can imagine that the vast majority of the MHC class II molecules exposed on the DCs surface would have been loaded with H-Y peptide. In this case, a 'tonic signal' could have been delivered by the endogenous DCs only in MHC class II competent hosts. It has been demonstrated that such 'tickling' of the TCR by self-peptide:MHC complex is important in naive CD4 T cells to maintain their sensitivity to antigen [58]. Non-cognate interactions have also been suggested to participate to CD4 T cell activation in the pseudodimer model where cognate and non-cognate:MHC molecules form the basic unit for T cell activation [291] [149].

Chimeric animals were used to try to address the role of non-cognate interactions. In these experiments, antigen presentation was restricted to the H-Y peptide pulsed DCs co-transferred with A1 naïve CD4 T cells, and non-cognate interaction was offered by the reconstitution of the adoptive hosts with bone marrow cells from MHC competent donors.

MHC- hosts, as well as MHC+ ones, were reconstituted with bone marrow cells from donors that provided MHC class II interaction on DCs or DCs and B cells at various stages of effector or memory generation. None of these

experiments was able to prevent loss of function in memory CD4 cells from MHC- hosts. In the case of non irradiated hosts transplanted with bone marrow cells to provide DCs, the competition for space with the endogenous DCs population may have reduced the efficiency of the reconstitution of the MHC class II signal. In the case of non irradiated hosts transplanted with bone marrow cells to provide DCs and B cells, the timing of the reconstitution may not have been optimal to supply a significant number of mature B cells expressing MHC class II during the effector phase, compromising the following stages. Also host irradiation prior to bone marrow transplantation did not allow us to answer our question as it resulted in the rejection of the CD4 T cells during the effector phase. The alternative approach used to provide additional MHC class II was to inject every three days bone marrow derived DCs. The failure of this experiment highlighted the complexity and difficulty in controlling all the factors involved, such as the migration and homing of the delivered DCs in relation to their co-localisation and interactions with CD4 T cells [148].

Overall, the results presented in this thesis suggest a universal role of MHC contact with non-antigen presenting MHC molecules during the initial activation step, which is instrumental in shaping the functional competence of memory T cells.

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**Appendix 1:** Mice used in the thesis and their characteristics

| Mice strain/line | Usage | MHC haplotype | T cell compartment | B cell compartment | NK cell compartment |
|---|---|---|---|---|---|
| A1 Rag1 ko | T cell donor | $H\text{-}2^K$ | present | absent | present |
| Ly5.1 A1 Rag1 ko | T cell donor | $H\text{-}2^K$ | present | absent | present |
| Rag2 ko Il-2 rγ ko | host | $H\text{-}2^b$ | absent | absent | absent |
| Rag2 ko Il-2 rγ ko $I\text{-}A^\beta$ ko | host | $H\text{-}2^b$ | absent | absent | absent |
| CBA | bone marrow derived DCs donor | $H\text{-}2^K$ | present | present | present |
| Rag1 ko B6 | donor for bone marrow chimera | $H\text{-}2^b$ | absent | absent | present |
| Rag1 ko | donor for bone marrow chimera | $H\text{-}2^K$ | absent | absent | present |
| TCR α ko | donor for bone marrow chimera | $H\text{-}2^b$ | absent | present | present |

**Appendix 2:** Affymetrix: genes differentially expressed

The 360 differentially expressed sequences are listed in ascending order according to -fold change of CD4 T memory cells recovered from MHC + hosts. Genes with -fold change ≥ 1.5 are over-expressed in CD4 T cells recovered from MHC $^{+}$ hosts, while values ≤ 0.6 are down-regulated in the same group. In the case of down-regulation the fold change can also be expressed as (1/reported value).

| Gene Name | Fold Change | Common | Genbank | Description |
|---|---|---|---|---|
| 1451263_a_at | 0.131 | Fabp4 | BC002148 | fatty acid binding protein 4, adipocyte |
| 1419691_at | 0.16 | Camp | NM_009921 | cathelicidin antimicrobial peptide |
| 1433693_x_at | 0.163 | Vamp3 | BB491065 | vesicle-associated membrane protein 3 |
| 1448756_at | 0.164 | S100a9 | NM_009114 | S100 calcium binding protein A9 (calgranulin B) |
| 1449984_at | 0.188 | Cxcl2 | NM_009140 | chemokine (C-X-C motif) ligand 2 |
| 1424254_at | 0.188 | Ifitm1 | BC027285 | interferon induced transmembrane protein 1 |
| 1416886_at | 0.189 | C1d | NM_020558 | nuclear DNA binding protein |
| 1438358_x_at | 0.196 | Pfdn5 | AV124256 | prefoldin 5 |
| 1459884_at | 0.209 | D13Ertd332e | AA190297 | cytochrome c oxidase, subunit VIIc |
| 1419394_s_at | 0.211 | S100a8 | NM_013650 | S100 calcium binding protein A8 (calgranulin A) |
| 1418934_at | 0.217 | Mab21l2 | NM_011839 | mab-21-like 2 (C. elegans) |
| 1449991_at | 0.231 | Cd244 | NM_018729 | CD244 natural killer cell receptor 2B4 |

| | | | | |
|---|---|---|---|---|
| 1419024_at | 0.236 | Ptp4a1 | BC003761 | protein tyrosine phosphatase 4a1 |
| 1448792_a_at | 0.25 | Cyp2f2 | NM_007817 | cytochrome P450, family 2, subfamily f, polypeptide 2 |
| 1450322_s_at | 0.253 | Slfn4 | NM_011409 | schlafen 3 |
| 1435172_at | 0.261 | Eomes | BB128925 | eomesodermin homolog (Xenopus laevis) |
| 1450009_at | 0.27 | Ltf | NM_008522 | lactotransferrin |
| 1420572_at | 0.27 | Ms4a3 | NM_133246 | membrane-spanning 4-domains, subfamily A, member 3 |
| 1415806_at | 0.276 | Plat | NM_008872 | plasminogen activator, tissue |
| 1427747_a_at | 0.277 | Lcn2 | X14607 | lipocalin 2 |
| 1420398_at | 0.279 | Rgs18 | BB139986 | regulator of G-protein signaling 18 |
| 1448801_a_at | 0.287 | Timm44 | NM_011592 | translocator of inner mitochondrial membrane 44 |
| 1448881_at | 0.288 | Hp | NM_017370 | haptoglobin |
| 1416267_at | 0.292 | Scoc | NM_019708 | short coiled-coil protein |
| 1418990_at | 0.295 | Ms4a4d | NM_025658 | membrane-spanning 4-domains, subfamily A, member 4D |
| 1424057_at | 0.297 | Gdap2 | BC025070 | ganglioside-induced differentiation-associated-protein 2 |
| 1418901_at | 0.298 | Cebpb | NM_009883 | CCAAT/enhancer binding protein (C/EBP), beta |
| 1418497_at | 0.308 | Fgf13 | AF020737 | fibroblast growth factor 13 |
| 1423233_at | 0.309 | Cebpd | BB831146 | CCAAT/enhancer binding protein (C/EBP), delta |
| 1416034_at | 0.315 | Cd24a | NM_009846 | CD24a antigen |
| 1415960_at | 0.315 | Mpo | NM_010824 | myeloperoxidase |
| 1425466_at | 0.325 | Senp2 | AV317107 | SUMO/sentrin specific protease 2 |
| 1422598_at | 0.326 | Casq1 | NM_009813 | calsequestrin 1 |
| 1422873_at | 0.333 | Prg2 | NM_008920 | proteoglycan 2, bone marrow |
| 1426968_a_at | 0.34 | Rdh10 | BG073496 | retinol dehydrogenase 10 (all-trans) |
| 1426260_a_at | 0.342 | Ugt1a2 | D87867 | UDP glycosyltransferase 1 family, polypeptide A6 |
| 1448261_at | 0.343 | Cdh1 | NM_009864 | cadherin 1 |
| 1423547_at | 0.344 | Lyzs | AW208566 | lysozyme |
| 1454699_at | 0.346 | Sesn1 | BG076140 | sestrin 1 |
| 1450495_a_at | 0.349 | Klrk1 | AF039026 | killer cell lectin-like receptor subfamily K, member 1 |
| 1422280_at | 0.353 | Gzmk | AB032200 | granzyme K |
| 1418722_at | 0.354 | Ngp | NM_008694 | neutrophilic granule protein |
| 1448485_at | 0.357 | Ggt1 | NM_008116 | gamma-glutamyltransferase 1 |
| 1449903_at | 0.359 | Crtam | NM_019465 | cytotoxic and regulatory T cell molecule |

| | | | | |
|---|---|---|---|---|
| 1417458_s_at | 0.361 | Cks2 | NM_025415 | CDC28 protein kinase regulatory subunit 2 |
| 1415778_at | 0.361 | Morf4l2 | NM_019768 | mortality factor 4 like 2 |
| 1436986_at | 0.361 | Sntb2 | BB219478 | syntrophin, basic 2 |
| 1448147_at | 0.371 | Tnfrsf19 | NM_013869 | tumor necrosis factor receptor superfamily, member 19 |
| 1448179_at | 0.377 | Usmg5 | BC024355 | upregulated during skeletal muscle growth 5 |
| 1422976_x_at | 0.384 | Ndufa7 | NM_023202 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7 (B14.5a) |
| 1451335_at | 0.387 | Plac8 | AF263458 | placenta-specific 8 |
| 1427843_at | 0.391 | Kua | AB012278 | CCAAT/enhancer binding protein (C/EBP), beta |
| 1417626_at | 0.391 | Usmg4 | NM_031401 | upregulated during skeletal muscle growth 4 |
| 1417652_a_at | 0.393 | Tbca | NM_009321 | tubulin cofactor a |
| 1453722_s_at | 0.395 | Sfrs1 | BF682801 | splicing factor, arginine/serine-rich 1 (ASF/SF2) |
| 1417164_at | 0.397 | Dusp10 | NM_022019 | dual specificity phosphatase 10 |
| 1437302_at | 0.399 | Adrb2 | AV083350 | adrenergic receptor, beta 2 |
| 1436454_x_at | 0.405 | Fen1 | BB393998 | flap structure specific endonuclease 1 |
| 1428570_at | 0.406 | Ccnc | AK009615 | cyclin C |
| 1449519_at | 0.408 | Gadd45a | NM_007836 | growth arrest and DNA-damage-inducible 45 alpha |
| 1455002_at | 0.411 | Ptp4a1 | AV331223 | protein tyrosine phosphatase 4a1 |
| 1417103_at | 0.413 | Ddt | NM_010027 | D-dopachrome tautomerase |
| 1459885_s_at | 0.414 | D13Ertd332e | AA190297 | cytochrome c oxidase, subunit VIIc |
| 1435323_a_at | 0.414 | Oact1 | AV366860 | O-acyltransferase (membrane bound) domain containing 1 |
| 1460277_at | 0.421 | Otx3 | NM_130865 | orthodenticle homolog 3 (Drosophila) |
| 1417769_at | 0.421 | Psmc6 | AW208944 | proteasome (prosome, macropain) 26S subunit, ATPase, 6 |
| 1417936_at | 0.422 | Ccl9 | AF128196 | chemokine (C-C motif) ligand 9 |
| 1435446_a_at | 0.425 | Chpt1 | BF180212 | choline phosphotransferase 1 |
| 1454875_a_at | 0.425 | Rbbp4 | BF011461 | retinoblastoma binding protein 4 |
| 1416020_a_at | 0.426 | Atp5g1 | NM_007506 | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1 |
| 1437164_x_at | 0.426 | Atp5o | AV066932 | ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit |
| 1417457_at | 0.427 | Cks2 | NM_025415 | CDC28 protein kinase regulatory subunit 2 |
| 1422425_at | 0.427 | Sprr2k | NM_011477 | small proline-rich protein 2K |
| 1423470_at | 0.428 | Ptbp2 | BB076855 | polypyrimidine tract binding protein 2 |
| 1425521_at | 0.43 | Paip1 | BC019726 | polyadenylate binding protein-interacting protein 1 |

| | | | | |
|---|---|---|---|---|
| 1419594_at | 0.431 | Ctsg | NM_007800 | cathepsin G |
| 1416484_at | 0.431 | Ttc3 | BB833716 | tetratricopeptide repeat domain 3 |
| 1417395_at | 0.432 | Klf4 | BG069413 | Kruppel-like factor 4 (gut) |
| 1416838_at | 0.432 | Mut | NM_008650 | methylmalonyl-Coenzyme A mutase |
| 1452007_at | 0.432 | Sybl1 | BC003764 | synaptobrevin like 1 |
| 1422045_a_at | 0.433 | Ptpn12 | X63440 | protein tyrosine phosphatase, non-receptor type 12 |
| 1423112_at | 0.435 | Ube2d3 | AK009276 | ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) |
| 1460223_a_at | 0.437 | Epb4.9 | NM_013514 | erythrocyte protein band 4.9 |
| 1424443_at | 0.443 | Tm6sf1 | AV378394 | transmembrane 6 superfamily member 1 |
| 1452333_at | 0.444 | Smarca2 | BM230202 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2 |
| 1416384_a_at | 0.447 | Cope | NM_021538 | coatomer protein complex, subunit epsilon |
| 1421188_at | 0.449 | Ccr2 | BB148128 | chemokine (C-C) receptor 2 |
| 1417441_at | 0.45 | Dnajc12 | NM_013888 | J domain protein 1 |
| 1423590_at | 0.45 | Kdap | AW105774 | kidney-derived aspartic protease-like protein |
| 1421615_at | 0.451 | Myo15 | NM_010862 | myosin XV |
| 1439426_x_at | 0.452 | Lzp-s | AV058500 | lysozyme |
| 1425551_at | 0.457 | Hip1r | AA590970 | huntingtin interacting protein 1 related |
| 1424850_at | 0.457 | Map3k1 | L13103 | mitogen activated protein kinase kinase kinase 1 |
| 1421817_at | 0.458 | Gsr | AK019177 | glutathione reductase 1 |
| 1451006_at | 0.459 | Xdh | AV286265 | xanthine dehydrogenase |
| 1422659_at | 0.46 | Camk2d | NM_023813 | calcium/calmodulin-dependent protein kinase II, delta |
| 1460245_at | 0.461 | Klrd1 | NM_010654 | killer cell lectin-like receptor, subfamily D, member 1 |
| 1418656_at | 0.464 | Lsm5 | NM_025520 | LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae) |
| 1452037_at | 0.464 | Mgat2 | AI481328 | mannoside acetylglucosaminyltransferase 2 |
| 1419368_a_at | 0.464 | Rnf138 | NM_019706 | ring finger protein 138 |
| 1460701_a_at | 0.466 | Mrpl52 | AV021593 | mitochondrial ribosomal protein L52 |
| 1416317_a_at | 0.466 | Stambp | AA289490 | Stam binding protein |
| 1420994_at | 0.466 | B3gnt5 | BM214359 | UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5 |
| 1416702_at | 0.467 | Serpini1 | NM_009250 | serine (or cysteine) proteinase inhibitor, clade I, member 1 |
| 1416567_s_at | 0.468 | Atp5e | NM_025983 | ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit |

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|---|---|---|---|---|
| 1448858_at | 0.469 | Ulk2 | NM_013881 | Unc-51 like kinase 2 (C. elegans) |
| 1417091_at | 0.471 | Chuk | NM_007700 | conserved helix-loop-helix ubiquitous kinase |
| 1448561_at | 0.472 | Ncf2 | NM_010877 | neutrophil cytosolic factor 2 |
| 1416464_at | 0.473 | Slc4a1 | NM_011403 | solute carrier family 4 (anion exchanger), member 1 |
| 1427504_s_at | 0.473 | Sfrs2 | AF250133 | splicing factor, arginine/serine-rich 2 (SC-35) |
| 1426742_at | 0.474 | Atp5f1 | AK019459 | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1 |
| 1455089_at | 0.475 | Gng12 | AW228775 | guanine nucleotide binding protein (G protein), gamma 12 |
| 1448713_at | 0.475 | Stat4 | NM_011487 | signal transducer and activator of transcription 4 |
| 1421930_at | 0.476 | Icos | AB023132 | inducible T-cell co-stimulator |
| 1416086_at | 0.476 | Tpst2 | NM_009419 | protein-tyrosine sulfotransferase 2 |
| 1452439_s_at | 0.476 | Sfrs2 | AF250135 | splicing factor, arginine/serine-rich 2 (SC-35) |
| 1454663_at | 0.478 | Eif5 | BQ176989 | eukaryotic translation initiation factor 5 |
| 1415836_at | 0.478 | Pycs | NM_019698 | pyrroline-5-carboxylate synthetase (glutamate gamma-semialdehyde synthetase) |
| 1449077_at | 0.479 | Eraf | NM_133245 | erythroid associated factor |
| 1422614_s_at | 0.479 | Bloc1s1 | NM_015740 | GCN5 general control of amino acid synthesis-like 1 (yeast) |
| 1418989_at | 0.481 | Ctse | NM_007799 | cathepsin E |
| 1417042_at | 0.481 | Slc37a4 | NM_008063 | solute carrier family 37 (glycerol-6-phosphate transporter), member 4 |
| 1418332_a_at | 0.482 | Agtpbp1 | NM_023328 | ATP/GTP binding protein 1 |
| 1421187_at | 0.486 | Ccr2 | BB148128 | chemokine (C-C) receptor 2 |
| 1448710_at | 0.487 | Cxcr4 | D87747 | chemokine (C-X-C motif) receptor 4 |
| 1450698_at | 0.488 | Dusp2 | L11330 | dual specificity phosphatase 2 |
| 1437683_x_at | 0.488 | Serf2 | AV047585 | small EDRK-rich factor 2 |
| 1449855_s_at | 0.488 | Uchl3 | AB033370 | ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase) |
| 1423608_at | 0.489 | Itm2a | BI966443 | integral membrane protein 2A |
| 1460168_at | 0.489 | Slbp | NM_009193 | stem-loop binding protein |
| 1425844_a_at | 0.49 | Rngtt | AF034568 | RNA guanylyltransferase and 5'-phosphatase |
| 1449079_s_at | 0.49 | Siat10 | NM_018784 | sialyltransferase 10 (alpha-2,3-sialyltransferase VI) |
| 1449393_at | 0.492 | Sh2d1a | NM_011364 | SH2 domain protein 1A |
| 1452352_at | 0.493 | Ctla2b | BG064656 | trophoblast specific protein beta |
| 1421923_at | 0.495 | Sh3bp5 | BQ179335 | calpain 7 |

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|---|---|---|---|---|
| 1436871_at | 0.495 | Sfrs7 | BE825013 | splicing factor, arginine/serine-rich 7 |
| 1433942_at | 0.496 | Myo6 | BE133806 | myosin VI |
| 1433548_at | 0.497 | Mare | BB137740 | alpha globin regulatory element containing gene |
| 1418353_at | 0.497 | Cd5 | NM_007650 | CD5 antigen |
| 1423384_s_at | 0.498 | Tex261 | BF181445 | testis expressed gene 261 |
| 1448483_a_at | 0.499 | Ndufb2 | NM_026612 | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2 |
| 1415904_at | 0.5 | Lpl | BC003305 | lipoprotein lipase |
| 1416008_at | 0.5 | Satb1 | AV172776 | special AT-rich sequence binding protein 1 |
| 1426304_x_at | 0.5 | B4galt7 | BC027195 | xylosylprotein beta1,4-galactosyltransferase, polypeptide 7 (galactosyltransferase I) |
| 1416868_at | 0.501 | Cdkn2c | BC027026 | cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) |
| 1423465_at | 0.503 | Sdfr2 | BB032852 | stromal cell derived factor receptor 2 |
| 1448784_at | 0.503 | Taf10 | NM_020024 | TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor |
| 1455719_at | 0.506 | Tubb5 | BG064086 | tubulin, beta 5 |
| 1418233_a_at | 0.507 | Trappc5 | AV226526 | trafficking protein particle complex 5 |
| 1452016_at | 0.509 | Alox5ap | BC026209 | arachidonate 5-lipoxygenase activating protein |
| 1422812_at | 0.509 | Cxcr6 | NM_030712 | chemokine (C-X-C motif) receptor 6 |
| 1415897_a_at | 0.51 | Mgst1 | BI150149 | microsomal glutathione S-transferase 1 |
| 1433711_s_at | 0.511 | Sesn1 | BG076140 | sestrin 1 |
| 1450021_at | 0.511 | Ubqln2 | AV171029 | ubiquilin 2 |
| 1426269_at | 0.514 | Sybl1 | BC003764 | synaptobrevin like 1 |
| 1420052_x_at | 0.516 | Psmb1 | C81484 | proteasome (prosome, macropain) subunit, beta type 1 |
| 1451862_a_at | 0.517 | Prf1 | M23182 | perforin 1 (pore forming protein) |
| 1442745_x_at | 0.517 | C79248 | C79248 | RNA-binding region (RNP1, RRM) containing 2 |
| 1418432_at | 0.519 | Cab39 | AK005226 | calcium binding protein 39 |
| 1423080_at | 0.519 | Tomm20 | AK002902 | translocase of outer mitochondrial membrane 20 homolog (yeast) |
| 1422490_at | 0.52 | Bnip2 | AV144704 | BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP2 |
| 1438546_x_at | 0.52 | Slc25a5 | C81442 | solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5 |
| 1450890_a_at | 0.521 | Abi1 | AW912678 | abl-interactor 1 |
| 1438761_a_at | 0.521 | Odc | C81193 | Similar to Odc protein (LOC231462), mRNA |

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|---|---|---|---|---|
| 1421186_at | 0.522 | Ccr2 | BB148128 | chemokine (C-C) receptor 2 |
| 1417460_at | 0.522 | Ifitm2 | NM_030694 | interferon induced transmembrane protein 2 |
| 1460271_at | 0.523 | Trem3 | NM_021407 | triggering receptor expressed on myeloid cells 3 |
| 1426205_at | 0.524 | Ppp1cb | M27073 | protein phosphatase 1, catalytic subunit, beta isoform |
| 1416085_s_at | 0.527 | Zfp216 | AA124553 | zinc finger protein 216 |
| 1434790_a_at | 0.529 | Lta4h | BI696719 | leukotriene A4 hydrolase |
| 1450777_at | 0.532 | Xrn2 | NM_011917 | 5'-3' exoribonuclease 2 |
| 1430982_at | 0.532 | Sfrs1 | BF682801 | splicing factor, arginine/serine-rich 1 (ASF/SF2) |
| 1423159_at | 0.533 | Dld | AI647805 | dihydrolipoamide dehydrogenase |
| 1418693_at | 0.534 | Hnrpc | NM_016884 | heterogeneous nuclear ribonucleoprotein C |
| 1417394_at | 0.534 | Klf4 | BG069413 | Kruppel-like factor 4 (gut) |
| 1415948_at | 0.535 | Creg | BC027426 | cellular repressor of E1A-stimulated genes |
| 1419194_s_at | 0.536 | Gmfg | NM_022024 | glia maturation factor, gamma |
| 1417741_at | 0.536 | Pygl | NM_133198 | liver glycogen phosphorylase |
| 1450865_s_at | 0.536 | Mrps24 | BF167852 | mitochondrial ribosomal protein S24 |
| 1424210_at | 0.537 | Keo4 | BC011220 | similar to Caenorhabditis elegans protein C42C1.9 |
| 1425937_a_at | 0.538 | Clp1 | BC022111 | cardiac lineage protein 1 |
| 1419193_a_at | 0.54 | Gmfg | NM_022024 | glia maturation factor, gamma |
| 1416807_at | 0.542 | Rpl36a | NM_019865 | ribosomal protein L36a |
| 1422128_at | 0.543 | Rpl14 | NM_025974 | ribosomal protein L14 |
| 1417090_at | 0.544 | Rcn | NM_009037 | reticulocalbin |
| 1442744_at | 0.544 | C79248 | C79248 | RNA-binding region (RNP1, RRM) containing 2 |
| 1424923_at | 0.544 | Serpina3g | BC002065 | serine (or cysteine) proteinase inhibitor, clade A, member 3G |
| 1416067_at | 0.546 | Ifrd1 | NM_013562 | interferon-related developmental regulator 1 |
| 1451369_at | 1.703 | Commd5 | BC025891 | COMM domain containing 5 |
| 1433723_s_at | 1.713 | Serf2 | BB119541 | small EDRK-rich factor 2 |
| 1434987_at | 1.717 | Aldh2 | AI462635 | aldehyde dehydrogenase 2, mitochondrial |
| 1456309_x_at | 1.718 | Lasp1 | BG146595 | LIM and SH3 protein 1 |
| 1427168_a_at | 1.72 | Col14a1 | AJ131395 | procollagen, type XIV, alpha 1 |
| 1451745_a_at | 1.723 | Znhit1 | BC026751 | zinc finger, HIT domain containing 1 |
| 1421504_at | 1.734 | Sp4 | NM_009239 | trans-acting transcription factor 4 |
| 1435304_at | 1.736 | Sod1 | BM240246 | superoxide dismutase 1, soluble |

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|---|---|---|---|---|
| 1435288_at | 1.74 | Coro1a | BB740218 | coronin, actin binding protein 1A |
| 1423785_at | 1.762 | Egln1 | BE995700 | EGL nine homolog 1 (C. elegans) |
| 1424527_at | 1.763 | Ppp2r2d | AF366393 | protein phosphatase 2, regulatory subunit B, delta isoform |
| 1451898_a_at | 1.763 | Sema6c | AF363972 | sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6C |
| 1448050_s_at | 1.766 | Map4k4 | BF450398 | mitogen-activated protein kinase kinase kinase kinase 4 |
| 1449237_at | 1.77 | Aloxe3 | NM_011786 | arachidonate lipoxygenase 3 |
| 1434190_at | 1.77 | Sms | BG071956 | spermine synthase |
| 1420375_at | 1.776 | Kif3a | NM_008443 | kinesin family member 3A |
| 1451020_at | 1.792 | Gsk3b | BB831420 | glycogen synthase kinase 3 beta |
| 1419716_a_at | 1.797 | Pou2f1 | NM_011137 | POU domain, class 2, transcription factor 1 |
| 1433966_x_at | 1.802 | Asns | AV212753 | asparagine synthetase |
| 1424407_s_at | 1.802 | Nptxr | BC019942 | neuronal pentraxin receptor |
| 1451396_at | 1.802 | Pomt2 | AF246235 | protein-O-mannosyltransferase 2 |
| 1432372_a_at | 1.805 | Spr | AK004941 | sepiapterin reductase |
| 1422000_at | 1.82 | Akr1c12 | AF177041 | aldo-keto reductase family 1, member C12 |
| 1452653_at | 1.822 | Slc25a22 | AK018760 | solute carrier family 25 (mitochondrial carrier: glutamate), member 22 |
| 1455021_at | 1.824 | Gabbr1 | BE688087 | gamma-aminobutyric acid (GABA-B) receptor, 1 |
| 1421910_at | 1.825 | Tcf20 | AW552808 | transcription factor 20 |
| 1448957_at | 1.83 | Rbpsuh | NM_009035 | recombining binding protein suppressor of hairless (Drosophila) |
| 1436079_s_at | 1.833 | Vapb | BB308907 | vesicle-associated membrane protein, associated protein B and C |
| 1454670_at | 1.835 | Rere | AW556696 | arginine glutamic acid dipeptide (RE) repeats |
| 1449200_at | 1.843 | Nup155 | BG073833 | nucleoporin 155 |
| 1434004_at | 1.848 | Dhps | AV033768 | deoxyhypusine synthase |
| 1424619_at | 1.848 | Sf3b4 | BC024418 | splicing factor 3b, subunit 4 |
| 1450870_at | 1.848 | Rala | BG073338 | v-ral simian leukemia viral oncogene homolog A (ras related) |
| 1454268_a_at | 1.855 | Cyba | AK018713 | cytochrome b-245, alpha polypeptide |
| 1419402_at | 1.858 | Mns1 | NM_008613 | meiosis-specific nuclear structural protein 1 |
| 1424287_at | 1.859 | Prkx | BC006875 | protein kinase, X-linked |
| 1416568_a_at | 1.864 | Acin1 | AW228855 | apoptotic chromatin condensation inducer in the nucleus |
| 1420947_at | 1.865 | Atrx | BB825830 | alpha thalassemia/mental retardation syndrome X-linked homolog (human) |

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|---|---|---|---|---|
| 1424671_at | 1.865 | Plekhf1 | BC002120 | pleckstrin homology domain containing, family F (with FYVE domain) member 1 |
| 1420663_at | 1.867 | Zfp67 | NM_009565 | zinc finger protein 67 |
| 1421194_at | 1.868 | Itga4 | NM_010576 | integrin alpha 4 |
| 1448518_at | 1.868 | Timm22 | AK012130 | translocase of inner mitochondrial membrane 22 homolog (yeast) |
| 1417578_a_at | 1.869 | Gmppa | NM_133708 | GDP-mannose pyrophosphorylase A |
| 1449193_at | 1.873 | Cd5l | NM_009690 | CD5 antigen-like |
| 1455959_s_at | 1.875 | D9Wsu168e | AW825835 | glutamate-cysteine ligase, catalytic subunit |
| 1416982_at | 1.876 | Foxo1 | AI462296 | forkhead box O1 |
| 1422938_at | 1.877 | Bcl2 | NM_009741 | B-cell leukemia/lymphoma 2 |
| 1419256_at | 1.877 | Spnb2 | BM213516 | spectrin beta 2 |
| 1460280_at | 1.878 | Mona | NM_010815 | monocytic adaptor |
| 1422679_s_at | 1.878 | Sh2bp1 | BB459423 | SH2 domain binding protein 1 (tetratricopeptide repeat containing) |
| 1420483_at | 1.884 | Cnnm3 | NM_053186 | cyclin M3 |
| 1426779_x_at | 1.886 | Dag1 | BG094386 | dystroglycan 1 |
| 1450298_at | 1.886 | Tnfsf14 | NM_019418 | tumor necrosis factor (ligand) superfamily, member 14 |
| 1436714_at | 1.894 | Lpp | BB297498 | LIM domain containing preferred translocation partner in lipoma |
| 1450965_at | 1.894 | Tex261 | BF181445 | testis expressed gene 261 |
| 1428133_at | 1.901 | Snip1 | BF135932 | Smad nuclear interacting protein 1 |
| 1418809_at | 1.91 | Pira1 | NM_011087 | synonyms: 6M21, Ly89, PIR-A1; Mus musculus paired-Ig-like receptor A1 (Pira1), mRNA. |
| 1422103_a_at | 1.922 | Stat5b | BC024319 | signal transducer and activator of transcription 5B |
| 1418649_at | 1.923 | Egln3 | BB284358 | EGL nine homolog 3 (C. elegans) |
| 1421861_at | 1.925 | Clstn1 | BG065300 | calsyntenin 1 |
| 1423646_at | 1.926 | Zdhhc3 | BB815190 | zinc finger, DHHC domain containing 3 |
| 1424907_a_at | 1.937 | Farsla | BC013533 | phenylalanine-tRNA synthetase-like, alpha subunit |
| 1455009_at | 1.939 | Cpd | AW550842 | carboxypeptidase D |
| 1434610_at | 1.943 | Plec1 | BM210485 | plectin 1 |
| 1416681_at | 1.944 | Ube3a | AK018443 | ubiquitin protein ligase E3A |
| 1456193_x_at | 1.945 | Gpx4 | AV116973 | glutathione peroxidase 4 |
| 1427408_a_at | 1.945 | Thrap3 | BC012655 | thyroid hormone receptor associated protein 3 |
| 1418893_at | 1.953 | Pbx2 | NM_017463 | pre B-cell leukemia transcription factor 2 |

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|---|---|---|---|---|
| 1452173_at | 1.955 | Hadha | AW107842 | hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit |
| 1448579_at | 1.957 | Glg1 | BG074127 | golgi apparatus protein 1 |
| 1453427_at | 1.961 | Csnk2a1 | AK011501 | casein kinase II, alpha 1 polypeptide |
| 1419289_a_at | 1.961 | Syngr1 | NM_009303 | synaptogyrin 1 |
| 1451938_a_at | 1.964 | Sntb1 | BC003748 | syntrophin, basic 1 |
| 1460700_at | 1.968 | Stat3 | AK004083 | signal transducer and activator of transcription 3 |
| 1427604_a_at | 1.969 | Atp9a | AF011336 | ATPase, class II, type 9A |
| 1454754_a_at | 1.973 | Aamp | BF468097 | angio-associated migratory protein |
| 1451695_a_at | 1.977 | Gpx4 | AF274027 | glutathione peroxidase 4 |
| 1426615_s_at | 1.99 | Ndrg4 | AV006122 | N-myc downstream regulated 4 |
| 1423236_at | 1.991 | Galnt1 | BM217066 | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 |
| 1416331_a_at | 1.998 | Nfe2l1 | NM_008686 | nuclear factor, erythroid derived 2,-like 1 |
| 1436802_at | 2.001 | Ilf3 | BG069530 | interleukin enhancer binding factor 3 |
| 1424656_s_at | 2.006 | Usp19 | BC006916 | ubiquitin specific protease 19 |
| 1417326_a_at | 2.009 | Anapc11 | NM_025389 | anaphase promoting complex subunit 11 homolog (yeast) |
| 1418018_at | 2.011 | Cpd | NM_007754 | carboxypeptidase D |
| 1422533_at | 2.012 | Cyp51 | NM_020010 | cytochrome P450, 51 |
| 1453307_a_at | 2.019 | Anapc5 | AK003821 | anaphase-promoting complex subunit 5 |
| 1427885_at | 2.02 | Pold4 | AK010477 | polymerase (DNA-directed), delta 4 |
| 1416689_at | 2.026 | Tuft1 | NM_011656 | tuftelin 1 |
| 1450083_at | 2.028 | Cnot4 | AI448404 | CCR4-NOT transcription complex, subunit 4 |
| 1423917_a_at | 2.03 | Cttn | BC011434 | cortactin |
| 1423609_a_at | 2.031 | Mgat1 | BB205495 | mannoside acetylglucosaminyltransferase 1 |
| 1428510_at | 2.039 | Lphn1 | AA987131 | latrophilin 1 |
| 1452137_at | 2.041 | Acbd3 | BB704602 | acyl-Coenzyme A binding domain containing 3 |
| 1421878_at | 2.042 | Mapk9 | BC024514 | mitogen activated protein kinase 9 |
| 1421265_a_at | 2.047 | Rnpc1 | NM_019547 | RNA-binding region (RNP1, RRM) containing 1 |
| 1416863_at | 2.049 | Abhd8 | NM_022419 | abhydrolase domain containing 8 |
| 1420911_a_at | 2.058 | Mfge8 | NM_008594 | milk fat globule-EGF factor 8 protein |

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|---|---|---|---|---|
| 1422006_at | 2.064 | Prkr | BE911144 | protein kinase, interferon-inducible double stranded RNA dependent |
| 1426801_at | 2.064 | Sep-08 | BB756379 | septin 8 |
| 1417181_a_at | 2.065 | Kifap3 | AV213603 | kinesin-associated protein 3 |
| 1427525_at | 2.068 | Cd151 | L35549 | cold shock domain; Mus musculus Y-box binding protein (oxyR) mRNA, partial cds. |
| 1421469_a_at | 2.076 | Stat5a | U36502 | signal transducer and activator of transcription 5A |
| 1449705_x_at | 2.086 | Mcm3 | C80350 | minichromosome maintenance deficient 3 (S. cerevisiae) |
| 1421827_at | 2.09 | Dll4 | AK004739 | delta-like 4 (Drosophila) |
| 1421299_a_at | 2.105 | Lef1 | NM_010703 | lymphoid enhancer binding factor 1 |
| 1451618_at | 2.119 | Rho | BI738491 | rhodopsin |
| 1423430_at | 2.12 | Mybbp1a | AW228043 | cDNA sequence BC011467 |
| 1426248_at | 2.124 | Stk24 | BG060677 | FERM, RhoGEF (Arhgef) and pleckstrin domain protein 1 (chondrocyte-derived) |
| 1417069_a_at | 2.131 | Gmfb | BG228815 | glia maturation factor, beta |
| 1450227_at | 2.137 | Ankrd6 | BM199504 | ankyrin repeat domain 6 |
| 1450783_at | 2.148 | Ifit1 | NM_008331 | interferon-induced protein with tetratricopeptide repeats 1 |
| 1423965_at | 2.151 | Mic2l1 | BB038546 | MIC2 (monoclonal Imperial Cancer Research Fund 2)-like 1 |
| 1455205_a_at | 2.153 | Usp19 | BB252881 | ubiquitin specific protease 19 |
| 1435372_a_at | 2.165 | Pa2g4 | AI152156 | proliferation-associated 2G4 |
| 1448350_at | 2.167 | Asl | NM_133768 | argininosuccinate lyase |
| 1434813_x_at | 2.168 | Wars | AI528863 | tryptophanyl-tRNA synthetase |
| 1451496_at | 2.178 | Mtss1 | BC024131 | metastasis suppressor 1 |
| 1448225_at | 2.186 | Gpaa1 | NM_010331 | GPI anchor attachment protein 1 |
| 1450128_at | 2.201 | Pla2g2a | NM_011108 | phospholipase A2, group IIA (platelets, synovial fluid) |
| 1425021_a_at | 2.204 | Pex16 | BC010822 | peroxisome biogenesis factor 16 |
| 1423978_at | 2.204 | Sbk | BC025837 | SH3-binding kinase |
| 1454675_at | 2.216 | Thra | BI076689 | nuclear receptor subfamily 1, group D, member 1 |
| 1416218_x_at | 2.216 | Rpl37a | NM_009084 | ribosomal protein L37a |
| 1418856_a_at | 2.224 | Fanca | NM_016925 | Fanconi anemia, complementation group A |
| 1428585_at | 2.228 | Actn1 | BE853286 | striamin |
| 1436784_x_at | 2.243 | Sf3b4 | AU021081 | splicing factor 3b, subunit 4 |
| 1420954_a_at | 2.256 | Add1 | BF140063 | adducin 1 (alpha) |

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|---|---|---|---|---|
| 1428942_at | 2.256 | Mt2 | AA796766 | metallothionein 2 |
| 1419928_at | 2.259 | Lrig1 | AW557176 | leucine-rich repeats and immunoglobulin-like domains 1 |
| 1416736_at | 2.261 | Casc3 | NM_138660 | cancer susceptibility candidate 3 |
| 1418110_a_at | 2.288 | Inpp5d | U39203 | inositol polyphosphate-5-phosphatase D |
| 1418627_at | 2.298 | Gclm | NM_008129 | glutamate-cysteine ligase , modifier subunit |
| 1416410_at | 2.305 | Pafah1b3 | NM_008776 | platelet-activating factor acetylhydrolase, isoform 1b, alpha1 subunit |
| 1460246_at | 2.315 | Mecp2 | BC027153 | methyl CpG binding protein 2 |
| 1422864_at | 2.327 | Runx1 | NM_009821 | runt related transcription factor 1 |
| 1422799_at | 2.347 | Bat2 | AK019427 | HLA-B associated transcript 2 |
| 1416657_at | 2.354 | Akt1 | NM_009652 | thymoma viral proto-oncogene 1 |
| 1422865_at | 2.36 | Runx1 | NM_009821 | runt related transcription factor 1 |
| 1448056_at | 2.401 | C80292 | AI607291 | upstream regulatory element binding protein 1e |
| 1421168_at | 2.43 | Abcg3 | NM_030239 | ATP-binding cassette, sub-family G (WHITE), member 3 |
| 1460294_at | 2.433 | Atp8a2 | NM_015803 | ATPase, aminophospholipid transporter-like, class I, type 8A, member 2 |
| 1435139_at | 2.447 | Narg1 | AV275156 | NMDA receptor-regulated gene 1 |
| 1415787_at | 2.453 | G2an | NM_008060 | alpha glucosidase 2, alpha neutral subunit |
| 1430681_at | 2.461 | Cryl1 | AI482548 | crystallin, lamda 1 |
| 1417544_a_at | 2.482 | Flot2 | NM_008028 | flotillin 2 |
| 1419204_at | 2.484 | Dll1 | NM_007865 | delta-like 1 (Drosophila) |
| 1417065_at | 2.489 | Egr1 | NM_007913 | early growth response 1 |
| 1430092_at | 2.497 | Serac1 | BB785152 | serine active site containing 1 |
| 1450699_at | 2.51 | Selenbp1 | NM_009150 | selenium binding protein 1 |
| 1427036_a_at | 2.521 | Eif4g1 | BF227830 | eukaryotic translation initiation factor 4, gamma 1 |
| 1448883_at | 2.581 | Lgmn | NM_011175 | legumain |
| 1423141_at | 2.585 | Lip1 | AI596237 | lysosomal acid lipase 1 |
| 1450596_at | 2.726 | Olfr66 | NM_013618 | hemoglobin Y, beta-like embryonic chain |
| 1438248_at | 2.735 | Pcsk5 | BB041089 | proprotein convertase subtilisin/kexin type 5 |
| 1418260_at | 2.798 | Hunk | NM_015755 | hormonally upregulated Neu-associated kinase |
| 1417210_at | 2.808 | Eif2s3y | NM_012011 | eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked |
| 1416239_at | 2.818 | Ass1 | NM_007494 | argininosuccinate synthetase 1 |