UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Vaccination and immunological memory

By

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I would like to dedicate this work to my family.

To the ones who in these recent and dramatic years have passed away. To my uncles Roberto and Ferdinando and to my mother Luciana, my father Dionisio and my beloved brother Giampiero. Your unconditioned love and support has sustained me throughout my life and continues to be a source of strength in all the difficult moments.

To my uncle Loreto, source of inspiration and vital reference. Your encouragement and guidance have made it possible for me to pursue this career and without you none of this work would have been done.

Finally to my beautiful wife Nati and daughter Lucia, source of love, light and life.

To all of you my immense love and gratitude

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ABSTRACT

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Human subjects maintain long-term immunological memory against infective organisms but the mechanism is unclear. $CD4^+$ T helper memory cells (Th_{mem}) are pivotal in controlling humoral and cellular responses, therefore their longevity and response to vaccination are critical for maintenance of protective immunity. To probe the dynamics of the Th_{mem} response to antigenic challenge, we investigated subjects following a booster injection with tetanus toxoid (TT). Expansion of TT-specific Th_{mem} cells, and cytokine production, showed complex kinetics. Strikingly, parallel expansion and cytokine production occurred in pre-existing Th_{mem} cells specific for two other common antigens, Purified Protein Derivative of tuberculin (PPD), and *Candida albicans* (C.al*b*). Bystander expansion occurred in Th_{mem} but not in Th_{naive} cells. Antibody production against TT peaked ~2 weeks post-vaccination and gradually declined. However, pre-existing antibody against the other antigens did not change. It appears that, although all Th_{mem} cells are readily stimulated to expand, antibody responses are controlled by antigen availability. These human findings which relate to the maintenance of memory and have consequences for assessments of specific T-cell responses to vaccination, have been further investigated in a mouse model.

A transgenic model (OT-II) where $CD4^+$ T cells express a TCR specific for an ovalbumin peptide (peptide 323-339, OVAp) was used first to ask the question as to whether naïve or antigen-activated T cells were influenced in a bystander manner during a secondary immune response directed against a protein antigen that was unrelated to their cognate one. For this, carboxyfluorescein diacetate succunimidyl ester (CFSE)-labeled OT-II cells were adoptively transferred, either as naïve or

following *in vitro* activation with OVAp, into C57/BL6 wild type recipient mice which were immune to TT. Recipient mice were then challenged with TT antigen and susceptibility of OT-II cells to bystander activation and proliferation was tested. Naïve T cells were found not to be influenced, but antigen-activated cells were responsive and underwent further activation and bystander proliferation, with accompanying phenotypic changes. Interestingly bystander proliferation appeared to be proportional to the strength of TT-specific cellular immune response.

The second question was whether the bystander influence on activated T cells was also evident during a primary immune response to TT. To address this question, antigen-activated OT-II cells and control naïve cells were adoptively transferred into wild type naïve recipient mice and their activation and proliferation was assessed after challenge with TT. In this case no bystander activation or proliferation of OT-II cells was observed.

These results underline the susceptibility to bystander activation and proliferation as a unique feature of antigen-activated OT-II cells as opposed to naïve OT-II cells. They mirror those obtained in our study on human subjects and add formal proof of bystander proliferation occurring *in vivo.* Furthermore this well defined mouse model paves the way for further investigations aimed at addressing the mechanisms responsible for the observed phenomenon.

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Declaration of Authorship

I, Gianfranco Di Genova, declare that this thesis and the work presented in it are my own. I confirm that:

- This work was done wholly while in candidature for a research degree at this University;
- Where I have consulted the published work of others, the source is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- Parts of this thesis are based on work done with others:
  Some of the experiments summarized in Figures 1.7 on subjects 4, 6, and 8, and in Figure 1.11 on subjects 1, 4, 6, 8 and 10 were performed by Joanne Roddick.
  The planning, performing and analysis of the experiments performed on the mouse model and reported in Chapter 2 were done jointly with Dr Natalia Savelyeva. Amy Suchacki helped setting up some of them.

Signed:…….....

Dated:…….

Publications

Publications directly related to work presented in this thesis (see Appendix I)

Stevenson F K, **Di Genova G**, Ottensmeier C H, and Savelyeva N. Cancer Vaccines. In Cancer Immunotharapy: Immune suppression and Tumor Growth, edited by Prendergast G. C. and Jaffee E. M. (Academic Press, London, 2007), pp. 183-204.

Di Genova G, Roddick J, McNicholl F, Stevenson FK. Vaccination of human subjects expands both specific and bystander memory T cells but antibody production remains vaccine specific. Blood. 2006;107:2806-2813.

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Abbreviations

α-GalCer	Alpha-galactosylceramide
AICD	Activation-induced cell death
APC	Antigen-presenting cell
BCG	Bacille Calmette-Guérin
BCR	B cell receptor
Blimp-1	B-lymphocyte-induced maturation protein-1
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C.alb	*Candida albicans*
CCR7	CC-chemokine receptor 7
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalovirus
CM	Complete medium
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
FI	Fold increase
FrC	Fragment C
GC	Germinal centre
γc	Gamma chain
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HepB	Hepatitis B
HEV	High endothelial venule
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
hTERT	Human telomerase reverse transcriptase
IL	Interleukin
IRB	Independent review board
IRF	Interferon regulatory factor
IFN	Interferon
LCMV	Lymphocytic choriomeningitis virus
LLPC	Long-lived plasma cell
LPS	Lipopolysaccharide
MBC	Memory B cell
MHC	Major histocompatibility complex
MITF	Microphthalmia-associated transcription factor
MoAlb	Mouse albumin
NK	Natural killer
OVA	Ovalbumin
OVAp	T helper epitope from ovalbumin (OVA 323-339, ISQAVHAAHAEINEAGR)

P30	Universal T helper epitope from tetanus toxin (tt 947-967, FNNFTVSFWLRVPKVSASHLE)
PAMP	Pathogen-associated molecular pattern
PAX5	paired box protein 5
PBMNC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Plasma cell
PD-1	Programmed death 1
PHA	Phytohaemagglutinin
Poly I:C	Polyinosinic-polycytidylic acid
PPD	Purified protein derivative of tuberculin
PRR	Pattern-recognition receptor
PTP	Protein tyrosine phosphatase
SI	Stimulation index
STAT	Signal transducer and activator of transcription
T_{CM}	Central memory T cells
T_{EM}	Effector memory T cells
TCR	T cell receptor
TGF	Tumour growth factor
Th	T helper
Th_{mem}	T helper memory cells
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	T regulatory cells
TT	Tetanus toxoid
XBP-1	X-box binding protein 1

Chapter 1. Bystander activation of $CD4^+$ memory T cells following protein vaccination in human healthy volunteers

1.1 Introduction

1.1.1 Innate and adaptive immunity

The immune system has the primary function of defending the body against microbial pathogens. Early lines of defence are provided by a combination of cellular and biochemical mechanisms already in place even before infection. They constitute the so called innate immunity and consist of (1) physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces; (2) circulating effector cells, such as phagocytic cells (e.g. neutrophils, macrophages) and natural killer (NK) cells; (3) circulating effector proteins, such as members of the complement system and so-called "natural" antibodies; (4) proteins, such as cytokines, that regulate and coordinate many activities of the cells of innate immunity. The components of innate immunity are there to prevent infection or, when infection occurs, to react rapidly (within hours) and eliminate the microbes. Specificity of action is assured by the recognition of structures that are shared by classes of microbes ('molecular patterns') and are not present on mammalian cells, and are often essential for survival of the microbes.

Together with the defence mechanisms of the innate immunity, there are immune responses that develop later (within days after infection) and are characterized by some key features: (1) an exquisite specificity, directed against pathogen-specific antigens, and which makes it possible to distinguish among different, even closely related, microbes and molecules; (2) an enormous diversity with the potential to recognize up to 10^9 distinct antigenic determinants; and (3) the unique ability to 'remember' and respond more effectively to repeated exposures to the same microbe, also known as immunological memory. This form of immunity develops in response to infection and adapts specifically to the infection and for these reasons is known as 'adaptive' or 'specific' immunity, or sometimes as 'acquired' immunity, to underline that it is acquired by experience. Adaptive immunity comprises two arms,

traditionally referred to as humoral immunity and cell-mediated or cellular immunity. The principal orchestrators of humoral and cell-mediated immunity are B and T lymphocytes respectively, and their products. These subpopulations of lymphocytes differ in the way they recognize antigens and in their functions. B lymphocytes use a surface-expressed B cell receptor (BCR) to recognize extracellular antigens. They then differentiate into antibody-producing cells or plasma cells. The secreted antibodies directly bind to and neutralize microbes and microbial toxins by blocking their binding to cellular receptors, or they coat (opsonize) invading pathogens making them visible targets for innate immunity.

T lymphocytes do not recognize free extracellular pathogens. Instead, they use their T cell receptor (TCR) to recognize (microbial and non-microbial) antigen-derived peptides associated and presented by major histocompatibility complex (MHC) molecules on the surface of infected cells or on the surface of specialized antigen presenting cell (APC). Among mature T lymphocytes two well defined subsets exist, which are easily identifiable by the surface expression of either the CD4 or the CD8 molecule. CD4 and CD8 are co-receptors that bind to non-polymorphic regions of MHC molecules and contribute to signal transduction at the time of antigen recognition [7]. $CD4^+$ and $CD8^+$ T cells recognize peptide presented by class II and class I MHC molecules, respectively. Upon antigen recognition and activation, T lymphocytes differentiate into functionally distinct populations of effectors such as cytotoxic T lymphocytes (CTLs) which can directly destroy infected cells, or helper T (Th) cells which secrete cytokines that regulate proliferation and differentiation of T and B cells or activation of other effector cells such as macrophages, and finally regulatory T cells which mainly function to inhibit immune responses.

While innate and adaptive immunity have traditionally been regarded as two distinct and temporally separated arms of the immune system, and the efficacy of one was thought to be independent of the other, there is now accumulating evidence to suggest that components of the innate and adaptive immunity interact and influence each other to assure an optimal outcome for the immune response. A relevant example is given by the bidirectional interactions between NK cells, a major effector cell population of the innate immune system, and dendritic cells (DCs), a class of highly specialized

APCs, which, as will be explained later in this introduction, are key initiators and regulators of the adaptive immune response [8].

1.1.2 **Immunological memory**

As discussed earlier, a key feature of the adaptive immune response is the ability to remember the first encounter with a pathogen, so that the severity of a second infection by the same pathogen is dramatically reduced. This phenomenon, of experiencing a milder form of a disease or being protected from a disease if previously exposed to it, is commonly known. People who have suffered from a disease like measles and mumps as children, if they survive the primary infection, are unlikely to succumb on re-exposure in their adult life. This phenomenon has been observed many times. In fact in 430 B.C. the Greek historian Thucydides, describing the plague of Athens, first reported that "The same man was never attacked twice" [9]. More recent studies on resistance to infections in individuals belonging to isolated human populations subjected to two consecutive outbreaks of the same infectious disease occurring decades apart have provided further scientific evidence for it [10]. From these studies two important conclusions can be drawn: first that protective immunity and therefore immune memory can be long-lived (between 40 and 75 years in these studies), and second that re-exposure to the pathogen is not necessary. Although this second principle, that is the maintenance of immunological memory by what we now call antigen-independent mechanisms, is still debated by the scientific community, recent elegant studies on long-term immunity to vaccinia virus in individuals who have received the smallpox vaccine which contains a live vaccinia virus [11], seem to offer further and robust evidence in its favour, and will be discussed in more detail later in this introduction.

Immunological memory and the principles behind it, also form the basis for vaccination. The origin of vaccination, defined as a deliberate attempt to protect against a severe disease by a direct exposure to a mild form of it, dates back to the ancient practice of variolation, popular in China and India as early as in the 10th century A.D. Healthy people were infected with dried pox material taken from someone recovering from smallpox, in the attempt of inducing a mild infection capable of protecting them from more virulent disease. This practice carried high

morbidity and although introduced also in Europe in the 18th century, it was soon discontinued [10]. With the pioneering work of Edward Jenner the era of modern vaccination and immunology begins. In 1798 he described how protection against smallpox could be obtained by vaccinating people with a related milder virus, cowpox, therefore without contracting the actual disease. The success of Jenner's approach is proven by the fact that in 1980 the World Health Organization declared smallpox eradicated worldwide and modern vaccines against common bacterial and viral diseases have reduced morbidity and mortality and improved the quality of life of millions of people.

What is the cellular and molecular basis of immunological memory and how is this immunological memory maintained, sometimes for the lifetime of an individual? These questions have fascinated immunologists for a long time and continue to do so. The answers are crucial to our basic understanding of the immune system and consequently to any conscious planning of intervention aimed at raising the immune system against pathogens. Parts of the answers are already known, others are beginning to be elucidated.

Humoral and cell-mediated immunity, both contribute to immunological memory, although their relative contributions to protective immunity can vary with the infection. Components of humoral immunity which contribute to immunological memory comprise pathogen-specific pre-existing neutralizing antibodies, memory B cells and long-lived plasma cells (LLPC). Components of cell-mediated immunity include pathogen-specific $CD4^+$ and $CD8^+$ memory T cells. Pre-existing neutralizing antibodies persisting in the serum constitute the first line of defence against re-infection. Memory B cells are populations of cells clonally expanded during the first encounter with the pathogen located mainly in secondary lymphoid organs where they are present at higher frequencies as compared to naïve B cells with the same antigen specificities. They do not actively secrete antibodies but possess a high affinity BCR and are able to mount a rapid recall response. In fact, upon re-encounter with the specific antigen, they quickly divide to replenish their pool and some of them differentiate into antibody-secreting plasma cells. LLPC are terminally differentiated effector cells which reside in the bone marrow and constitutively produce antibody in the absence of antigenic stimulation. In fact, they express minimal levels of BCR or

do not express it at all, and cannot be stimulated to divide or to boost the rate of antibody production. The ability to constitutively exert an effector function, in this case antibody production, independently of antigenic stimulation is a unique feature of plasma cells, and there is no cell type equivalent to the plasma cell in the T-cell compartment, where antigen is the main regulator of effector functions [12]. Similarly to memory B cells, memory $CD4^+$ and $CD8^+$ T cells are pathogen-specific cells clonally expanded during the primary response and enhanced in functions [12,13]. $CD4^+$ T cells can also provide help for antibody production and support for the generation and maintenance of $CD8^+$ memory T cells [14], therefore their role in the context of a pathogen-specific immune response is strategic.

How these populations of memory cells are maintained for long periods of time is still not clear but it is crucial to understand, especially in view of developing effective vaccines which require both arms of the immune response to be engaged and to remain active for a long time. In this introduction attention is focused mainly on memory T cells and among them on $CD4^+$ memory T cells. Their generation and maintenance together with their increasingly appreciated phenotypic and functional complex heterogeneity will be discussed.

1.1.3 **Phases of adaptive immune responses**

In the process leading to the generation of memory, T cells pass through three distinct kinetic stages: an initial 'expansion' phase, followed by a 'contraction' phase, which finally leads to a 'memory' phase [15]. The first stage begins when antigen-specific naïve T cells, normally present at low frequency in naïve hosts, following encounter with cognate antigen, are activated and undergo clonal expansion. Recognition of antigen can only take place in the specialized environment of secondary lymphoid organs where naïve $CD4^+$ and $CD8^+$ T cells recognize antigen-loaded MHC class II and I complexes respectively, on the surface of 'professional' APC, such as DCs, via their highly specific cell-surface TCR. DCs play a key role in this initial phase of naïve T cell activation, and also, as we shall see, in 'shaping' the following immune response.

A classical model proposes that DCs exist in two basic functional states [16]: an immature and a mature state. Immature DCs are characterized by very good antigen capture and antigen processing abilities, but by relatively poor immunogenicity. When located in peripheral organs and mucosal surfaces, they continually sample the environment for foreign antigens. They can 'sense' pathogens by recognizing pathogen-associated molecular patterns (PAMPs) via pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs), and signaling through TLRs influences many crucial aspects of DC biology. After antigen has been captured and processed, a maturation program begins, characterized by increased immunogenicity and decreased antigen acquisition and antigen processing ability [16]. Maturation can be accompanied by migration to secondary lymphoid tissues where 'mature' DCs are able to efficiently stimulate naïve T cells due to an increased surface expression of MHC class I and class II and costimulatory molecules, such as B7-1 and B7-2 (CD80 and CD86), which interact with their specific ligand CD28 on the T cell surface. When the combined intracellular biochemical signals provided by the TCR/MHC-peptide interaction (signal 1) and the costimulatory pathways (signal 2) reach a defined activation threshold, a program of activation, proliferation and differentiation begins for the naïve T cells. In the absence of signal 2 naïve T cells can become anergic, and this might lead to tolerance rather than immunity [7].

In Figure 1.1, the induction of the adaptive immune response is summarized. The key initial event is represented by the bidirectional interaction between a mature DC and a naïve $CD4^+$ T cell. The DC, after capturing and processing the antigen, displays antigen-derived peptides loaded on MHC class II molecules on the surface and, due to the high levels of costimulatory molecules, provides the $CD4^+$ T cell with all the signals it needs to become activated. The $CD4^+$ T cell, following activation, expresses the CD40L (CD154) molecule on the surface, which binds to CD40 on the surface of the DC and stimulates the DC. As a consequence, even higher levels of MHC molecule expression are induced on the DC. According to one prevailing model [17,18], this DC is now 'licensed' to activate a $CD8^+$ T cell which shares the same antigen specificity with the $CD4^+$ T cell. After initial activation by DCs, $CD4^+$ T cells can also interact with B lymphocytes that have previously captured native antigen by means of their BCR, and have internalized it and processed it. These B cells now act themselves as APCs and present antigen-derived peptides complexed with MHC class II

molecules to $CD4^+$ T cell which share the same antigen specificity. Again a cross talk takes place and reciprocal activation occurs and leads to the proliferation and differentiation of the B cell. Cytokines produced by the $CD4^+$ T cells further drive this process.

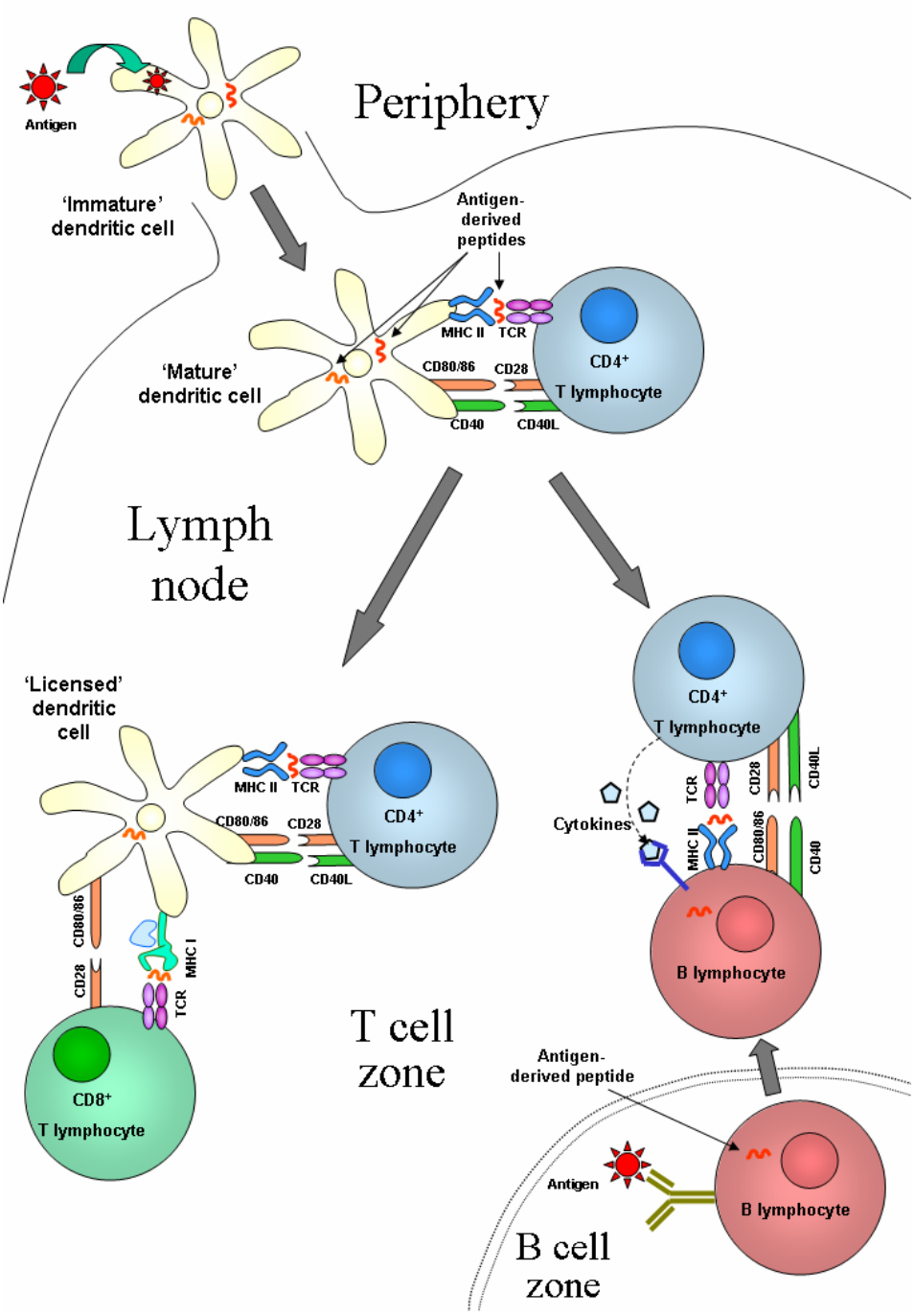


Figure 1.1 (Adapted from [1]). **The induction of the adaptive immune response**

Clonally expanded naïve T cells differentiate into effector T cells. Well characterized effector cells are T_h cells among the $CD4^+$, and CTLs among the $CD8^+$ T cells. Based on the pattern of cytokines secreted, $CD4^+$ T_h cells can be divided into functional subsets known as T_h1, which produce the signature cytokine interferon (IFN)-γ, and T_h2 cells, which typically produce interleukin 4 (IL-4), IL-5 and IL-13, but also other cytokines. T_h1 cells are essential for protection against a range of intracellular infections (and tumours). T_h2-like cytokines (e.g. IL-4) promote isotype switching in B cells to generate high affinity antibodies and can play a role in protection against extracellular infections, such as nematode parasitic infections [7]. More recently, a novel subset of $CD4^+$ T helper cells has been characterized and named T_h-17 based on the cytokine produced, IL-17 [19]. These are pro-inflammatory cells involved in several autoimmune disorders.

Among the $CD4^+$ T cells, there are also several subsets of cells with regulatory functions, capable of controlling effector T cell responses. Examples of T regulatory (Treg) cells are the well characterized 'naturally occurring' $CD4^+$ $CD25^+$ Tregs (nTreg), the IL-10-secreting T regulatory type 1 (Tr1) cells, the induced Treg (iTreg) and the T_H3 cells [20]. While nTreg develop directly from thymic precursors during the normal process of T cell maturation, the remaining subsets seem to differentiate from peripheral T helper precursors through the actions of different cytokines, and at least for Tr1 and T_H3 cells, in response to antigen[20,21].

Therefore, after antigenic stimulation, naïve $CD4^+$ T cells proliferate and differentiate into various effector subsets characterized by the production of distinct cytokines and by their distinct (sometime opposite) effector functions. This process of differentiation of $CD4^+$ T cell lineages from a common precursor is regulated by the cytokine-milieu present early during the immune response and by activation of specific transcription factors [2] as summarized in Figure 1.2.

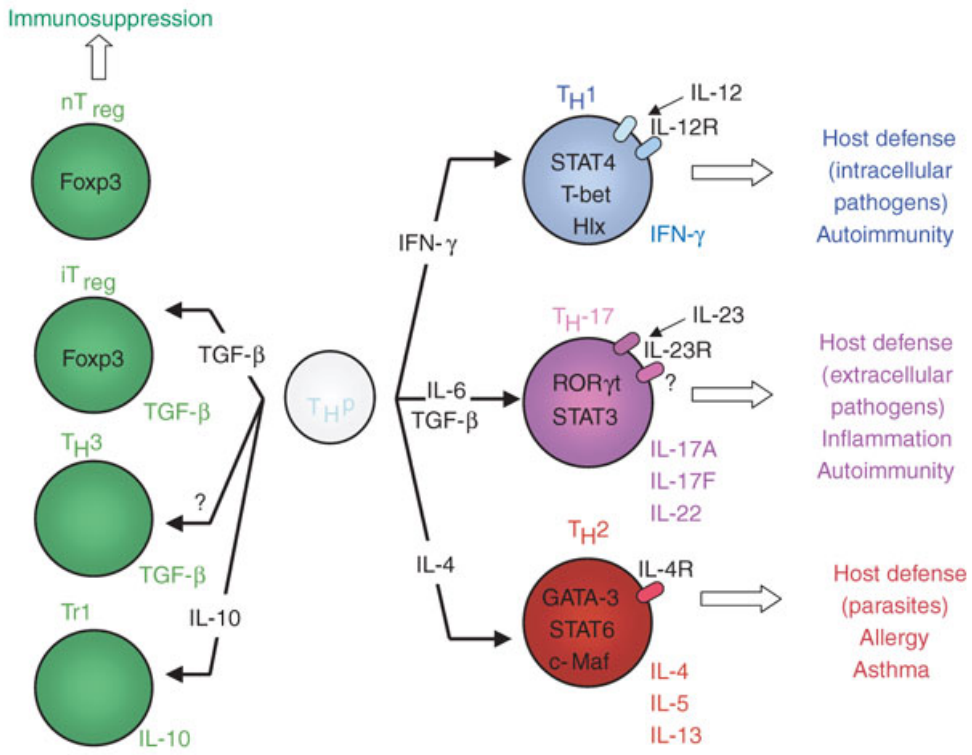


Figure 1.2 (from [2]). **Differentiation of CD4$^+$ T cell lineages**

For example, IL-12 produced by activated macrophages and DCs, and IFN-γ, in conjunction with signal transducer and activator of transcription 4 (STAT 4) and T-bet, promote differentiation of T_h1 effectors, while IL-4 and the transcription factors STAT 6 and GATA-3 favor induction of T_h2 cells. The differentiation of T_h-17 from naïve precursors depends on the presence of cytokines such as transforming growth factor (TGF-β) and IL-6 and requires blockade of T_h1 and T_h2 differentiation. Another cytokine, IL-23 is essential for their expansion and survival [19]. Once an immune response develops along one pathway, it becomes increasingly polarized in that direction because the cytokines produced by each subset (typically IFN-γ by T_h1 cells and IL-4 by T_h2 cells) tend to further promote differentiation of that subset and at the same time they inhibit the differentiation along the alternative pathway.

How can T cell responses be directed towards the most appropriate effector type in response to a given pathogen? Increasing evidence suggests that DCs play a pivotal role as an interface between the 'pathogen world' and the immune system [22]. Distinct pathogens activate phenotypically and functionally distinct subsets of effector DCs

(directly, via interaction of particular PRRs with PAMPS, and/or indirectly, through various inflammation-associated tissue factors released in response to pathogen invasion), which in turn, can polarize the adaptive immune response in order to produce the most appropriate effectors (T_h1 or T_h2 cells, or in some instances, regulatory T cells) [16,22].

Similarities and differences exist between $CD4^+$ and $CD8^+$ T cells in their requirements for efficient effector formation. Although for both a transient exposure to antigen is sufficient to induce an antigen-independent program of proliferation and differentiation, naïve $CD8^+$ T cells seem to require a shorter time of antigen stimulation and, in response to that, they also divide sooner and at a faster rate, as compared to naïve $CD4^+$ T cells, suggesting a higher proliferative potential [23]. While naïve $CD8^+$ T cells more readily develop into cytolytic and/or cytokine-producing effector cells, the differentiation of naïve $CD4^+$ T cells into terminally committed T_h1 or T_h2 cells is subject to more extensive regulatory mechanisms as described above. The response of naïve $CD8^+$ T cells is also less dependent on costimulatory signals, as shown in mouse models of viral or bacterial infections [15]. This suggests that either $CD4^+$ and $CD8^+$ T cells differ in their activation threshold (lower for $CD8^+$ T cells), or that factors extrinsic to the T cell (levels and type of activation signals) help $CD8^+$ T cells meet the activation threshold for proliferation and differentiation more rapidly [15]. A more efficient *in vivo* antigen presentation due to the ubiquitous expression of MHC class I molecules as compared to class II, could be one of those factors.

A reduced expression of lymph node-homing receptors, such as CC-chemokine receptor 7 (CCR7), a homing molecule required for T cells to cross high endothelial venules (HEV) and enter the lymph node from the bloodstream, and L-selectin (CD62L) together with an increased expression of chemokine receptors such as CCR5 and CCR2, allow migration of effector T cells to inflamed tissues where they can exert their primary task, which is clearance of the antigen [15]. They do this by various means, either by directly recognizing and killing infected cells, as CTL mainly do, or by producing inflammatory cytokines such as IFN-γ and tumour necrosis factor-α (TNF-α) which in turn can activate macrophages and enhance their microbicidal activity. These effector functions are displayed immediately by effector T cells,

thanks to their lower activation threshold and less stringent requirements for co-stimulation.

After the antigen is cleared the majority of effector T cells die by apoptosis during what it is known as the 'death' phase or contraction phase of the immune response. For $CD8^+$ effector T cells the contraction is quite remarkable. In various infection models, it accounts for the loss of up to 90-95 % of the effector cells, and this death rate seems to be independent of the magnitude of the initial expansion [24]. Information on the kinetics of $CD4^+$ T cell responses is more limited. However in one study on the response to smallpox vaccination in human volunteers, $CD4^+$ T cell responses showed less contraction and better persistence when compared to $CD8^+$ T cell responses, both after primary immunization and also after a boosting injection [25].

The extent of cell death during the contraction phase is influenced by the common γ chain family of cytokines (cytokines such as IL-2, IL-4, IL-7 and IL-15, which share the subunit γ of the cytokine receptor) [26], members of the TNF receptor superfamily (such as CD27, OX40, 4-1BB and CD154) [26,27] and effector cell molecules (such as perforin and IFN-γ) [23,26]. Effector T cell apoptosis can be the consequence of activation-induced cell death (AICD), which occurs when expanded T cells are re-stimulated via TCR in the absence of appropriate co-stimulatory signals. It can also result from the lack of appropriate survival signals (e.g. cytokine deprivation). In this case T cell death does not require re-stimulation of the TCR and it is known as activated cell autonomous death (ACAD) [28]. While AICD seems to be important for elimination of chronically activated and potentially autoreactive T cells, ACAD seems to play a major role during the contraction phase of the immune response [29].The pathways involved in effector T cell death appear to be activated by either external signals, such as the interaction of Fas expressed on the surface of activated T cells with its ligand FasL, via a caspase-dependent pathway, or by intrinsic signals via a pathway that leads to mitochondrial dysfunction. In this case the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family is critical [30].

The contraction phase is thought to be important for preserving immune system homeostasis, leaving space for T cell responses against new infections, and in

preventing possible immunopathological consequences often associated with an excessive effector T cell response. The surviving cells enter the third phase of the immune response and tend to persist for long periods of time as "memory" cells.

1.1.4 **The development of T cell memory**

Most of the information available on the development of memory T cells derives from mouse studies, in most cases using TCR transgenic T cells. The use of TCR transgenic animals makes it possible to obtain sufficient numbers of T cells of a given specificity which can be easily identified and monitored in their *in vitro* and/or *in vivo* response to the specific antigen usually by clonotypic antibodies directed against the transgenic TCR. Although these studies have been instrumental in deciphering some intriguing aspects of naïve, effector and memory T cell behaviour, there are limitations associated with the use of TCR transgenic T cells which cannot be ignored, such as for instance the high and usually non-physiological number of TCR transgenic T cells used. Also there is a dangerous tendency to extrapolate results obtained from mouse studies obtained in a particular setting (e.g. a particular model of viral or bacterial infection), and to draw general conclusions or rules which are then applied to humans. However, some data are available from studies in humans and in many cases they are revealing. An example is the identification of subsets of memory T cells with distinct phenotypic and functional properties initially described in humans [31], and then confirmed in mice. These studies have brought attention to the complexity of T cell memory and will be described in one of the following sections. In general a balance should be found, where mouse and human immunology complement each other with the communal aim of establishing robust and durable immunological principles.

1.1.5 **Pathways to T cell memory**

A central question in T cell memory development is the lineage relationship between naïve, effector and memory T cells. How early during an adaptive immune response is the commitment to memory T cell differentiation decided and what regulates it? The

answer is still unclear. Comparisons with B cells can be useful. After naïve B cells have been activated by antigen in the presence of CD4 T cell help (see above), they continue along one of two divergent pathways: they either differentiate into short-lived plasma cells (PC) (effectors) or they initiate a germinal center (GC) reaction. Somatic hypermutation, affinity maturation and selection take place in the GC and result in the generation of high affinity memory B cells (MBCs). MBCs face a similar choice when re-stimulated by antigen. In fact they divide rapidly and can differentiate into either PCs or give rise to more MBCs. The commitment of B cells to differentiate into PCs or to keep their B-cell 'identity' is finely regulated by the balance of transcription factors such as B-lymphocyte-induced maturation protein-1 (Blimp-1) and X-box binding protein 1 (XBP-1) and interferon regulatory factor 4 (IRF 4), which favour plasma cell differentiation, and those such as the paired box protein 5 (PAX5) and microphthalmia-associated transcription factor (MITF), which in turn help maintaining memory B cell identity [12]. Therefore for B cells, it is clearly a divergent pathway that leads to memory development, and the decision on which way to proceed, is taken very early by the differentiating B cell. It would also be difficult to envisage an alternative model, considering the irreversible changes B cells are subjected to when they differentiate into either effectors (loss of BCR) or memory cells (somatic hypermutation).

For naïve T cells the differentiation into effector cells is not accompanied by such radical changes. With some exceptions, effector T cells do not permanently lose TCR expression and TCR do not somatically mutate, therefore the B cell model of differentiation cannot be simply extrapolated to T cells. Historically memory T cells have been described as a minority of T cells which survive the contraction phase of the immune response. This implies that memory T cells derive from the expanded effector T cells, or in other words that a linear differentiation pathway exists leading from naïve to effector to memory. For $CD8^+$ T cells evidence to support this model comes from studies using TCR transgenic $CD8^+$ effector T cells generated either *in vitro* following stimulation with cognate peptide [32], or *in vivo* following infection with lymphocytic choriomeningitis virus (LCMV) [33], and adoptively transferred into naïve syngeneic recipients, where they gradually give rise to a population of long-lived memory T cells. These findings would suggest that precursors of CD8 memory T cells exist within the effector population. A similar linear differentiation pathway has also

been proposed for $CD4^+$ memory T cells [34]. Swain SL and colleagues (1994) were the first to demonstrate the emergence of $CD4^+$ memory T cells from a population of effector cells antigen-activated *in vitro* under polarizing (T_h1 or T_h2) conditions and transferred into naïve recipients[35]. Interestingly the polarized cytokine producing profile of the effector cells was maintained by the memory population, offering further evidence for their direct link.

If memory T cells derive from effectors, what regulates the effector → memory transition? This is still unclear. Attempts have been made to identify precursors within the effector population destined to become memory cells, looking, for instance, at differential expression of cytokine receptors. In a mouse model of LCMV infection, it has been proposed that among the $CD8^+$ effector T cells at peak of the response, expression of IL-7 receptor α-chain (IL-7Rα) identifies precursors that give rise to long-lived memory cells [36]. In this model, increased expression of IL-7Rα in the effector cells correlates with increased survival signals and reduced apoptosis [36]. Interestingly the degree of contraction and efficiency of CD8 T cell memory establishment seem to be linked to inflammatory events, and specifically to IFN-γ production, taking place very early during an infection as shown in a model of *Listeria monocytogenes* infection [37]. When bacterial load and inflammation (IFN-γ production) are decreased by pre-treating mice with antibiotics, an increased fraction of antigen-specific $CD8^+$ T cells express IL-7Rα at the peak of expansion and the development of functional antigen-specific memory $CD8^+$ T cells occurs in the absence of contraction [15].

A critical role in the establishment of $CD8^+$ T cell memory is performed by $CD4^+$ T cells. Several groups, using different experimental systems, have provided clear evidence in support of the idea that while $CD8^+$ effector T cells can be generated in the absence of $CD4^+$ T cell help, fully functional $CD8^+$ memory T cells can be induced only when $CD4^+$ T cells are present during the priming phase of the $CD8^+$ T cell response [38-41].

For $CD4^+$ T cells, factors, acting early during T cell activation, such as cytokines and costimulators, can contribute to memory formation. Signals provided by IL-2 and

CD28 during priming confer a long-lasting survival advantage in primed T cells, contributing to the persistence of a memory population [42]. IL-2 and CD28 signals do not act by promoting T-cell division and expansion, but by inducing survival proteins (e.g. anti-apoptotic molecules such as Bcl-2) and expression of receptors for survival factors such as IL-7 [43]. The role of IL-7 in the maintenance of $CD4^+$ memory T cells is well known and it will be discussed in more detail in one of the following sections. A lack of CD40L signals during priming did not seem to have an impact on subsequent memory development [42]. Other costimulatory molecules have been considered for their contribution to $CD4^+$ T cell memory, among them OX40. OX40 (CD134) is a member of the TNFR family and is induced on T cells after antigenic stimulation. It has been shown that OX40 promotes $CD4^+$ memory development by regulating primary clonal expansion [44] and by promoting expression of anti-apoptotic molecules Bcl-xL and Bcl-2 [45], and therefore by enhancing the overall survival and functionality of T cells that will persist as memory cells.

Differentiation into effectors is not always a requirement for memory development. Liu Y and colleagues demonstrated that activation of distinct costimulatory molecules induces either effector or memory CTLs [46]. Also, particular vaccine-formulations can induce priming of memory but not effector $CD8^+$ T cells [47], although the induced memory cells are non-protective against re-infections.

For $CD4^+$ T cells a branched differentiation model has been proposed as an alternative to the classic linear differentiation pathway [3]. The idea is that $CD4^+$ memory T cells can be generated from activated precursors in diverse differentiation states, including from cells which have not yet acquired effector functions [48] . A schematic diagram of these two alternative models is shown in Figure 1.3.

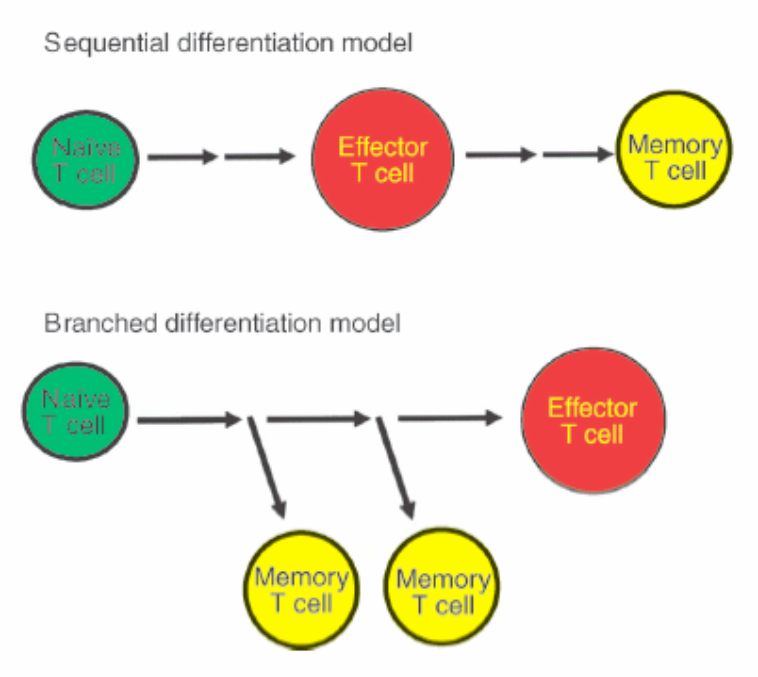


Figure 1.3 (from [3]). **Prevailing models for the differentiation of CD4$^+$ memory T cells**

If the differentiation into effectors is not a pre-requisite step for T cell memory development, how early during the transition from naïve to memory, does the divergent pathway branch off the naïve → effector pathway? Moulton VR and colleagues indicate that CD4$^+$ TCR transgenic naïve T cells stimulated with specific antigen *in vitro* for just one day, with no evident acquired effector functions, can develop into a stable population of memory cells when transferred into antigen-free mouse hosts [48]. In a recent breakthrough investigation Chang JT and colleagues have gone further, to show that the fate of a T cell is determined as early as during the very first division that follows antigen stimulation [49]. In fact, upon activation, naïve T cells undergo asymmetric cell division, with asymmetric redistribution of fate-determining cues (e.g. signaling molecules, phenotypic markers and effector gene products) among daughter cells. Data show that as a consequence, one daughter cell can become the precursor of the effector T cell progeny and the other can become the precursor of the memory T cell progeny. Interestingly, the daughter cell precursor of the memory T cell progeny shows greater expression of IL-7Rα mRNA (see above). These findings bring T cells and B cells closer in their developmental behaviour. Future research efforts might re-focus on the very early stages of naïve T cell activation, especially on

factors intrinsic to the cell and we might see rapid progress in our understanding of the pathway/s leading to T cell memory.

1.1.6 Phenotypic and functional characteristics of naïve, effector and memory T cells

Upon re-exposure to cognate antigen, memory T cells can mediate enhanced immune responses, which in association with antibody responses efficiently control secondary infections and therefore contribute to protective immunity. A direct comparison of naïve and memory T cells which share the same antigen specificity can explain the heightened recall response by memory T cells. In fact as a consequence of the expansion phase occurring during the primary immune response, memory T cells are present at a higher precursor frequency; they also maintain a feature typical of effector T cells, that is, the rapid display of effector functions at low activation thresholds.

For $CD8^+$ T cells, the rapid production of cytokines such as IFN-γ and cytotoxic molecules, such as perforin and granzyme B, can be achieved due to the constitutive expression of their respective genes which in naïve cells are not expressed. This leads to the presence of high levels of messenger RNA transcripts which, following encounter with antigen, can be promptly translated [15]. When compared to naïve cells, $CD4^+$ memory T cell responses to recall antigens are characterized by faster kinetics of cytokine secretion (effector cytokines IL-4, IL-5 and IFN-γ), cell division, and proliferation which occur at lower doses of antigen and are less dependent on costimulation by B7 molecules and are independent of costimulation by CD40 [50,51].

The transition from one stage to another in the development of memory T cells is accompanied by changes in the expression of phenotypic markers and there has always been an attempt to associate a particular phenotype with defined functional properties. In man, expression of CD45 isoforms distinguishes naïve ($CD45RA^+$ $CD45RO^-$) from memory T cells ($CD45RA^-$ $CD45RO^+$) [52], although this is not absolute since the expression of CD45RA has been demonstrated in subsets of antigen-experienced $CD8^+$ and $CD4^+$ T cells [53]. In mice, the search for definite phenotypic markers of memory T cells has been more problematic. High expression

of CD44 (a cell adhesion receptor whose principal ligand, hyaluronate, is a common component of extracellular matrices, and whose expression changes upon activation and differentiation of T cells) has widely been used as a marker of both $CD4^+$ and $CD8^+$ memory T cells, although it is also expressed on activated T cells. Less useful has been CD62L, because of its heterogeneous expression. For mouse $CD4^+$ T cells, it would appear that a consensus has now been reached, and the differential expression of three markers, CD25 (the α subunit of the IL-2 receptor complex, IL-2Rα), CD127 (IL-7Rα) and CD44, together with cell size, allow distinction of naïve, effector and memory T cells [6]. Some distinctive properties of mouse naïve, effector and memory $CD4^+$ T cells are summarized in Table 1.1

Property	Naive	Effector	Memory
Phenotype	Small $CD25^{lo}$ $CD44^{lo}$ $CD62L^{hi}$ $CCR7^+$ IL-$7R\alpha^{hi}$	Large $CD25^{hi}$ $CD44^{hi}$ $CD62L^{lo}$ $CCR7^-$ IL-$7R\alpha^{lo}$	Small $CD25^{lo}$ $CD44^{hi}$ $CD62L^{lo\ or\ hi}$ $CCR7^{+\ or\ -}$ IL-$7R\alpha^{hi}$
Lifespan	Long	Short	Long
Effector function	IL-2	Rapid effector cytokine production	Rapid effector cytokine production (IFNγ, TNF-α and IL-4)
Homing	Lymphoid tissue	Lymphoid and non-lymphoid	Lymphoid and non-lymphoid tissue
Heterogeneity	None	Functional and homing heterogeneity	Central memory ($CD62L^{hi}CCR7^+$) and effector memory ($CD62L^{lo}CCR7^-$)

Table 1.1 (from [6]). **Distinctive properties of mouse naïve, effector and memory $CD4^+$ T cells**

The co-stimulatory molecules CD27 and CD28, and the lymph node homing receptor CCR7 are expressed on naïve T cells and tend to be down-regulated in antigen-experienced effector T cells in relation to their less stringent requirements for additional costimulatory signals and their altered migratory capacity respectively. Down-regulation of CD27 in particular is irreversible. Based on the combined expression of these markers, a post-thymic linear development pathway has been proposed for $CD8^+$ T cells in man [53]. According to this, four main phenotypic subsets can be identified, starting from naïve ($CD28^+$ $CD27^+$ $CCR7^+$) T cells, progressing through early ($CD28^+CD27^+CCR7^{(+)\rightarrow(-)}$), intermediate ($CD28^-$ $CD27^+$ $CCR7^-$), and late ($CD28^-CD27^-CCR7^-$) differentiation stages for antigen-experienced T cells. The sequential down-regulation of CCR7, CD28 and CD27 is accompanied by up-regulation of cytotoxic factors. $CD4^+$ T cells go through similar phenotypic and functional changes with loss of expression of CD27 and CD28 and CCR7, gain of cytolytic functions and reduced capacity to produce IL-2 and to proliferate. Interestingly, the loss of CD27 precedes that of CD28 and the acquisition of lytic

capacity which begins early after priming of naïve $CD8^+$ T cells, occurs in $CD4^+$ T cells only when they reach a highly differentiated stage. Although the *in vivo* significance of each of these phenotypic subsets remains to be fully understood, one view is to see late differentiation stage T cells as cells approaching senescence, as suggested by their reduced proliferative capacity and shortened telomere length [53]. It still remains to be clarified what drives progressive differentiation of T cells and what is the potential of each subset in conferring protective immunity.

1.1.7 **T-cell differentiation in persistent human virus infections**

Phenotypic studies of virus-specific $CD8^+$ T cells in infections characterized by an acute phase followed by a persistent chronic phase, such those induced by human immunodeficiency virus (HIV), Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Hepatitis B virus (HBV) and Hepatitis C virus (HCV), have revealed interesting clues. Despite a similar phenotype of activated antigen-experienced cells, shown by virus-specific CD8 T cells during the acute phase of each of those infections, the degree of differentiation of CD8 T cells during the chronic phase seems to be virus-specific, with, for instance, HCV-specific $CD8^+$ T cells showing an early differentiation phenotype ($CD27^+CD28^+$), whereas CMV-specific $CD8^+$ T cells having a late differentiation phenotype ($CD27^-CD28^-$). Early intermediate and intermediate phenotypes characterize hepatitis B-specific or HIV-specific $CD8^+$ T cells respectively [53]. Furthermore, in EBV infection the pattern varies according to whether T cells recognize lytic or latent antigens. HIV and CMV chronic infections are also characterized by accumulation of oligoclonal antigen-experienced population with a restricted TCR usage, a phenomenon that leads to a reduced ability to respond to emerging viral variants or to other common and opportunistic pathogens.

The factors likely to influence the differentiation of virus-specific CD8+ T cells include the following: the antigen-load and the degree of antigen re-encounter [54]; persistence, anatomical location and presentation of antigen. Variable levels of activation and costimulation in the end are responsible for different levels of differentiation observed.

The relationship between the degree of differentiation and protective immunity is still controversial, although accumulating data suggest that protective immunity may better conferred by early differentiated T cells which maintain high proliferative potential [53]. When differentiation is far advanced, $CD8^+$ T cells become gradually impaired in effector functions, including cytokine production, cytolysis and proliferation, and this functional impairment has been called 'exhaustion'. Exhaustion ultimately leads to death of the $CD8^+$ T cells [55]. Whether exhaustion is a pre-step inevitably leading to death or is in some way reversible, is not clear. Interestingly, in a mouse model of LCMV infection, it has been shown that *in vivo* blockade of the interaction between the inhibitory protein programmed death 1 (PD-1), which is selectively up-regulated by the exhausted T cells, and its ligand PD-L1, can restore functionality in virus-specific $CD8^+$ T cells [56]. These results, although obtained in a mouse model, are promising and may pave the way for the development of therapies to improve T cell responses in patients with chronic viral infections.

1.1.8 **Central memory and effector memory T cells**

Recently, the expression of CD45RA and CCR7 together with that of another lymph node homing receptor CD62L has also been used to define two functionally distinct subsets of memory T cells, central and effector memory T cells, T_{CM} and T_{EM} respectively [31]. T_{CM} home to secondary lymphoid tissue, have little or no effector functions, but can rapidly proliferate and differentiate into effector cells upon antigenic stimulation. T_{EM} cells home to peripheral tissue (e.g. sites of inflammation), can promptly produce effector cytokines, such as IFN-γ, following antigenic stimulation, but have limited proliferative potential [31]. In the context of a secondary memory response, these two subsets of memory T cells would carry out distinct roles. While T_{EM} can offer immediate protection against invading pathogens in peripheral tissues, T_{CM} can offer long-term protection, being able to generate new waves of effector cells in antigen-draining lymph nodes.

A model has been proposed [4] (Figure 1.4) according to which T_{CM} and T_{EM} originate as intermediates in the process of differentiation from naïve to effector T cells. This process is controlled by the strength of stimulation received by TCR engagement and

cytokines. At increasing strength of stimulation activated T cells acquire the capability of responding to homeostatic cytokines; they acquire anti-apoptotic molecules, effector functions and tissue-homing receptors. Meanwhile they lose the capacity to produce IL-2 and to proliferate as well as lymph node homing receptors (CCR7 and CD62L). T cells receiving the optimal amount of stimulation survive as T_{CM} and T_{EM}, whereas T cells receiving a too weak or too high stimulation die.

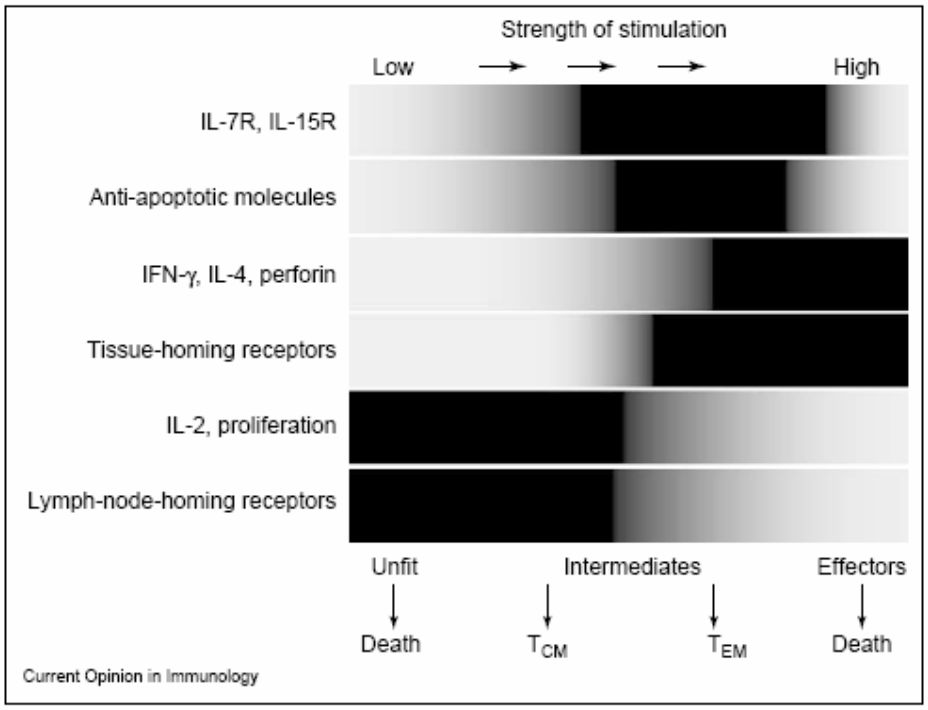


Figure 1.4 (from [4]). **A model of progressive T cell differentiation driven by signal strength**

Although the T_{CM} and T_{EM} model has provided us with a logical key for understanding the complexity of immune memory, proposing elegant correlations between phenotype, anatomic localization and distinct functions, and assigning distinct roles to subsets of memory T cells in the logic of an immune response, nevertheless recent data have highlighted many controversies on the differential function and role of T_{CM} and T_{EM} types and on their lineage relationship [57]. For instance, immediate effector functions can be exerted by human CMV- or EBV-specific CCR7+ CD8+ T cells [58], and in a mouse model of viral infection, LCMV-specific CCR7+ and CCR7- memory T cells do not differ in immediate effector cell function [59,60]. The role of T_{CM} and T_{EM} in mediating protection is also a matter of controversy. While in a secondary response

to LCMV T_{CM} maintained high expansion capacities and were responsible for protection [60], in a Sendai virus infection opposite results were observed, with T_{EM} showing potential to expand and to exert protective functions [61]. Also, the distinction between effector cells and effector memory cells based on phenotypic and functional characterization can become difficult when complete clearance of antigen is not fully guaranteed. In fact in theses cases an effector-like profile might reflect recent antigen re-stimulation rather than a resting memory subtype [19].

The lineage relationship between T_{CM} and T_{EM} has also been questioned and alternative models for the development of the two subsets have been proposed [62]. One of them describes a 'linear differentiation' pathway according to which CD8 T_{EM} develop directly from the pool of effector T cells generated from naïve T cells in response to antigen. The conversion of effector T cells to T_{EM} occurs after the primary infection is resolved. Interestingly, during the memory phase, and in the absence of antigen, T_{EM} gradually de-differentiate into T_{CM} [60]. So in this model and in contrast to what originally thought, T_{EM} convert to T_{CM}.

Some of the observed discrepancies can be explained by different criteria that various authors choose in defining the two subsets of memory T cells, either based on phenotype or anatomic location [57]. They can also reflect the heterogeneity that each of the two subsets itself can have [63] or differences existing among the models of infection chosen.

Although the T_{CM} and T_{EM} model is open to criticism and has led to some rather simplistic categorizations, overall it has had the merit of highlighting the complex heterogeneity of memory T cell populations [19].

1.1.9 **Maintenance of immunological memory**

Although observations on resistance to infection have indicated longevity of memory [64], the mechanism of maintenance has been unclear. Immunological memory can be maintained by several antigen-dependent mechanisms which include re-activation of latent/chronic infections or periodic re-exposures to a pathogen. Both are obvious and

effective ways to maintain high levels of immunity as shown by epidemiological studies carried out in areas where a given disease is endemic [10]. Immunological memory can also be maintained in the absence of repeated antigenic boosting events. Evidence in support of an antigen-independent maintenance of immunological memory will be discussed in the following sections together with the possible mechanisms responsible for it.

1.1.10 **Immunological memory following smallpox vaccination**

Elegant studies on immunity against vaccinia virus in human subjects vaccinated with smallpox vaccine, revealed that, although no exposure to smallpox (vaccinia) virus can have occurred for many years, survival of specific $CD4^+$ and $CD8^+$ T cells is evident decades after vaccination [65-67]. Similarly, plateau levels of functional memory B cells persist for >50 years [65]. In these studies, T cell responses declined slowly over time, with a half life of 8-12 years for $CD4^+$ and 8-15 years for $CD8^+$ vaccinia-specific T cells, nevertheless they were still detectable up to 75 years after vaccination [66]. Interestingly, CD4 and CD8 T cell responses seemed to be regulated independently over time and a direct comparison of CD4 and CD8 responses in individual vaccinees, revealed that in a significant proportion of cases there was a preferential loss of $CD8^+$ T cells while $CD4^+$ T cells were maintained long-term [66]. These findings were confirmed in an independent study by Amara et al.[25] who showed how the vaccinia-specific $CD8^+$ T cells displayed a higher contraction between peak effector and memory phases than $CD4^+$ T cells. Importantly those individuals who selectively lost $CD8^+$ T cells after primary immunization could still generate a strong CD8 T cell response after a booster vaccination with smallpox vaccine, demonstrating that their ability to mount a vaccinia-specific CD8 T cell response was intact.
Since vaccinia virus is believed neither to persist nor to become latent after the acute phase of infection, these studies support the idea that other antigen-independent mechanisms may contribute to the maintenance of immunological memory.
Strong evidence in support of this concept comes also from several mouse models showing persistence of $CD4^+$ and $CD8^+$ memory T cells in antigen-free MHC class II-, and class I-deficient mice, respectively [68,69].

1.1.11 **Kinetics of naïve and memory T cells**

The long term persistence of memory T cells at a population level can result either from ongoing proliferation, from increased survival or from both. A comparison of the kinetics of naïve and memory T cell populations in mouse [70] and man [71], shows increased turnover of memory T cells compared with naïve T cells. In man, shortening of telomeres at each cell cycle limits the number of divisions the cell can go through, and this is thought to be an important control mechanism of cell growth, that finally leads to a non-dividing state known as replicative senescence. A higher division rate makes memory T cells more subject to telomere erosion and in fact $CD45R0^+$ memory T cells have shorter telomeres compared to $CD45RA^+$ naïve T cells [72]. The activity of the enzyme telomerase tends to compensate for the loss of telomeres; in fact ectopic expression of the human telomerase reverse transcriptase (hTERT) gene can prevent telomere erosion and extend the life span of human T cells [73,74]. Endogenous hTERT expression is up-regulated following activation, but T cells tend to lose the ability to up-regulate hTERT upon long-term culture [73]. Interestingly, although hTERT expression is up-regulated in both activated naïve and memory T cells freshly isolated from human subjects, the expression levels are lower in memory T cells, and this probably reflects the greater replicative history of memory T cells [73].

Overall these data suggest that long-term persistence of populations of memory T cells can only be assured by a combination of signals able to promote proliferation and some degree of telomerase activity. Furthermore, due to the limits imposed by replicative senescence, there must also be signals able to improve survival. The nature of these signals is not entirely understood, but a major role seems to be played by cytokines, as it will be described in the following sections.

1.1.12 **Role of cytokines: in vitro studies**

How an effective population of memory T cells can be maintained over the lifetime of the host in the apparent absence of antigen is a major question. One suggested route is via non-specific stimulation by cytokines, and this has been shown to occur in human Th_{mem} cells *in vitro* [63,75,76].

When highly purified naïve ($CD45RA^+$) and memory ($CD45R0^+$) resting human $CD4^+$ T cells are cultured *in vitro* with a combination of IL-2, tumor necrosis factor α (TNFα) and IL-6, in the absence of TCR engagement, they express activation markers, enter cell cycle and proliferate [75]. The cytokine-activated $CD45R0^+$ memory population can also exert effector functions (IFN-γ and IL-4 synthesis) and provide T cell help for antibody production by B cells [75]. The authors speculated that this antigen-independent pathway of T cell activation could play an important role *in vivo*, in boosting an antigen-specific immune response and at the same time in maintaining the peripheral pool of memory T cells. In fact, resting T cells at sites of an antigen-specific response could be activated by cytokines produced by antigen-specific T cells and antigen presenting cells. They would proliferate and secrete cytokines that in turn could further amplify the specific immune response. This model may perhaps explain the high proportion (40-80%) of activated T cells among the lymphocytes infiltrating the liver of patients with chronic viral hepatitis, despite the fact that only a small percentage of them are virus-specific [77]. In fact those T cells might have been activated in a bystander fashion. Similarly, high numbers of proliferating $CD8^+$ T cells have been reported in mouse models of viral infections such as influenza, and among the proliferating cells only a small percentage was virus-specific [78].

There is a group of cytokines whose members bind to multimeric receptors that share a common γ chain (γc). Among them, IL-2, IL-15, and IL-7 are critical for regulating lymphoid homeostasis [79]. Low-level signals provided by IL-7 and MHC molecules promote prolonged survival of naïve T cells without inducing proliferation [80]. By contrast, IL-7 and IL-15 induce background proliferation in $CD8^+$ memory T cells and also promote their survival. $CD4^+$ T cells rely mainly on signals provided by IL-7 for their slow turn-over and survival [80]. As already discussed in a previous paragraph, memory T cells do not require contact with peptide-MHC complexes for their long-term survival [68,69].

The ability of human $CD4^+$ naïve T cells, memory T cells, and T_{CM} and T_{EM} subsets of memory T cells to respond to IL-7 and IL-15 was tested *in vitro* [81]. When cultured with IL-7 and IL-15, naïve CD4+ T cells failed to respond, while memory T cells proliferated strongly. Addition of autologous dendritic cells (DCs) or supernatants

derived from cultures of LPS-activated DCs, enabled all naïve T cells to respond to the cytokine combination and enhanced the response of memory T cells. Among the memory subsets, T_{EM} cells proliferated extensively to IL-7 and IL-15, while T_{CM} cells were less responsive and could respond only when DC-derived cytokines, such as TNF-α, IL-6 and IL-10 were added to the culture. Interestingly, the boosting effect of DC-derived cytokines on IL-7 and IL-15 induced proliferation was linked to their ability of up-regulating the IL-15Rβ and the γc chains on target cells [81].

1.1.13 A role for cytokines in the bystander proliferation of memory T cells in vivo

Early studies by Sprent J and colleagues established that a significant bystander proliferation of T cells takes place during viral infections in mice [82]. Proliferation of $CD8^+$ T cells was more pronounced than proliferation of $CD4^+$ T cells, and the majority of the bystander proliferating $CD8^+$ T cells showed a memory-like phenotype ($CD44^{high}$) [82]. They could then demonstrate that activation of the innate immune system was involved. In fact injection of type I interferon (IFN I) or IFN I–inducing agents such as lipopolysaccharide (LPS) and the synthetic double strand RNA polyinosinic-polycytidylic acid (Poly I:C) could mimic the effect of viral infections in inducing bystander proliferation of $CD8^+$ memory T cells[82,83]. The effect of IFN-I was not direct, but was instead mediated by production of secondary cytokines, among which IL-15 appeared to be a good candidate. IL-15 was later shown to be produced by, and to activate dendritic cells in response to type I IFN, double-stranded RNA, or LPS [84]. Most importantly, IL-15 could induce strong and selective stimulation of memory-phenotype $CD8^+CD44^{high}$ (but not $CD4^+CD44^{high}$) T cells *in vivo* in an antigen-independent manner [85]. The high and selective sensitivity of $CD8^+$ $CD44^{high}$ memory T cells to the mitogenic effects of IL-15 could be possibly explained by the high expression of IL-2/IL-15Rβ (one of the components of the IL-15 receptor) on the surface of this subset of T cells as opposed to other subsets, including $CD4^+CD44^{high}$ cells [85].

The range of infection-induced cytokines able to cause bystander proliferation of memory T cells i*n vivo* remains to be elucidated. Among other cytokines tested, IL-

12, IL-18, and IFN-γ were found to exert effects similar to those induced by type I IFN, although all by an IFN-γ-dependent pathway [86].

In vivo, activation of NKT cells (a subset of CD1d-restricted T cells expressing a biased TCR repertoire and markers common to the NK cell lineage) by the synthetic ligand α-galactosylceramide (α-GalCer), and activation of T cells by a bacterial superantigen, also lead to extensive proliferation of both $CD4^+$ and $CD8^+$ memory ($CD44^{high}$) T cells which can be IFN-αβ-independent but IFN-γ- and IL-12-dependent [87].

Overall these studies, mainly focused on $CD8^+$ memory T cells, demonstrate that bystander or antigen-independent proliferation of memory T cells can be induced *in vivo* by several mechanisms which involve the activation of different cell types in response to viruses or bacterial products, and is driven by cytokines released during such activation. This bystander activation/proliferation of memory T cells could contribute to their long-term maintenance.

1.1.14 Bystander activation/proliferation of memory $CD4^+$ T cells in human subjects

One way to study antigen-independent bystander activation of memory T cells *in vivo* in human subjects is to evaluate the impact of infection or vaccination on resident memory cells with antigen specificities unrelated to the infectious agent or to the vaccine. Although there are few published studies, there have been some intriguing observations which point to bystander effects. In one study of vaccination with TT, limiting dilution analysis of proliferating cells revealed the expected increase in the frequency of TT-specific cells, but this was accompanied by a modest but significant increase in the frequency of herpes simplex virus-specific cells[88] . Similarly, after a TT boost, PBMNC from three healthy donors showed an increased proliferative response to PPD as compared to pre-boost responses[89] .

A part from these studies, not specifically designed to study bystander activation of memory T cells following protein vaccination, to our knowledge no other study has

systematically investigated the impact of conventional protein vaccination on the kinetics of vaccine-specific and bystander $CD4^+$ memory T cells.

1.1.15 **The maintenance of B cell memory**

In terms of B cells, maintenance of antibody levels can be partially explained by new estimates of the survival of plasma cells, which in mice can persist throughout life [90]. Non-dividing plasma cells are thought to occupy survival niches in bone marrow and spleen [91]. The bone marrow provides only a limited number of survival niches for long-lived plasma cells. Recent investigations [92,93] suggest that during a secondary immune response to tetanus vaccination, while newly produced tetanus-specific plasma blasts are released from secondary lymphoid organs into the blood and are then chemo-attracted to the bone marrow, long-lived plasma cells resident in the bone marrow and producing antibodies not specific for tetanus are mobilised and are detectable in the blood. These observations are suggestive of an active competition between newly released vaccine-induced plasma blasts and resident plasma cells for the limited number of survival niches available in the bone marrow. This could represent a mechanism by which humoral immunity adapts to the antigenic environment, by gradually lowering the titer of antibodies specific to antigens not seen for long time and by creating space for immunity against newly encountered antigens.

In long-lived human subjects, it is unlikely that long-lived plasma cells can be the only source of persisting antibody, and continuous maturation of memory B cells may be required. Memory B cells can certainly persist without antigen stimulation [94], and could be stimulated to differentiate via cross-reacting antigen, or possibly via an antigen-independent pathway [95].

1.2 Aims of the study

1.2.1 To verify whether, in healthy human subjects, bystander activation/proliferation of $CD4^+$ memory T cells occurs during an immune response against a protein antigen.

Our plan is to conduct a detailed analysis of the kinetics of cellular immune responses to TT recall vaccination in humans and, in parallel, to analyze responses against two un-related antigens, PPD and *C. alb*. Most individuals show various degrees of pre-existing immunity to these three antigens, either because they have been vaccinated (against TT and Bacille Calmette-Guérin (BCG) for PPD) or because they have been naturally exposed to them (*C. alb*). Therefore this represents an ideal setting for studying how perturbation of a population of memory T cells (in this case vaccine-induced), can impact on the behavior of surrounding memory T cells with unrelated antigen specificities.

We shall focus our attention on Th_{mem} because of their pivotal role in controlling humoral and cellular responses, therefore their longevity and response to vaccination are critical for maintenance of protective immunity. There is also relatively scarce information on the kinetics of CD4 T cell responses to conventional vaccination in humans. Gathering this information would help us in the interpretation of data derived from on-going clinical studies on cellular immune responses in patients with hematological malignancies who are receiving novel DNA fusion vaccines developed in our laboratory, which contain a tetanus toxin-derived sequence (fragment C) as immune-enhancer.

1.2.2 To verify whether bystander activation can occur in the B-cell compartment

Our plan is to analyze the kinetics of serum antibody responses again each of the three recall antigens: TT, PPD and *C. alb*.

1.3 **Materials and methods**

1.3.1 **Vaccination and sample collection**

After obtaining ethical approval for the study from the Independent Review Board (IRB) and informed consent, twelve healthy adults (8 males, 4 females), (31 – 44y), received a single dose of TT vaccine i.m. (Adsorbed Tetanus Vaccine BP, Aventis Pasteur MSD). All subjects had been vaccinated against TT but had not received a boost during the previous five years. All subjects had also received conventional vaccination with BCG. Blood samples were taken before vaccination (week 0) and then at weeks 1, according to availability, and 2. Subsequent samples were taken at 4-week intervals up to week 20. Serum was isolated by spinning clotted blood for 20 minutes at 2100 rpm. It was then aliquoted and stored at –20^{o}C. Peripheral blood mononuclear cells (PBMNC) were isolated by centrifugation of heparinized blood on a Lymphoprep^{TM} density gradient. Briefly, each 10 ml of blood were gently layered onto 10 ml of Lymphoprep in 25ml universal vials, and spun at 2100 rpm for 20 minutes without brake. PBMNC recovered at the interface using a plastic Pasteur pipette were washed twice in RPMI 1640 medium (Invitrogen) (2 x 1650 rpm for 7 minutes), resuspended in 50% human AB serum (Sigma), 40% RPMI 1640 and 10% DMSO, and frozen at -80 ^{o}C. The following day they were moved into liquid-N_2 for long-term storage. The viability of defrosted cells was always very good, overall 90.2 +/- 2.6 %. In one case (subject 13) PBMNC were isolated from blood and soon after used for immuno-assays.

1.3.2 **Antigens**

Antigens were: TT (not adsorbed) and *Candida albicans* whole extract, cytoplasmic protein (*C. alb*) (National Institute for Biological Standards and Control, NIBSC, UK); PPD prepared from human strains of *Mycobacterium tuberculosis* (Evans Vaccines Limited, UK); normal human immunoglobulin for intravenous administration (Vigam Liquid 5g, Bio Products Laboratory, UK) (control 1, Ctrl1) and an IgA paraprotein purified from the serum of a patient with multiple myeloma (control 2, Ctrl 2). These endotoxin-free proteins were selected as controls with no

pre-existing immunity. In cellular assays TT was used at 1μg/ml and all the remaining antigens at 10μg/ml.

1.3.3 **Proliferative responses**

Cryopreserved PBMNC were thawed in pre-warmed RPMI-1640, containing 1-2 % human AB serum (Sigma, Cat N^{o} H-4522) and washed for 10 minutes at 300g. Cells were counted and the number of viable cells was determined by Trypan blue exclusion. PBMNC were cultured in triplicates ($2x10^5$/well) in round-bottomed 96 well plates, in RPMI-1640, containing 10% human AB serum (Sigma, Cat N^{o} H-4522), 1X of Penicillin-Streptomycin-Glutamine (100X) (Invitrogen, Cat N^{o} 10378-016), 1mM Sodium Pyruvate (Invitrogen, Cat N^{o} 11360-039) hereafter referred to as complete medium (CM). PBMNC were cultured with either TT, PPD, *C. alb*, Ctrl1 or Ctrl2. PBMNC cultured without antigen were used as controls. At day 5, cells were pulsed with 1 μCi/well of [methyl-3H] thymidine (Amersham Biosciences, Cat N^{o} TRA120-1MCI) and proliferative activity was measured after 18h using a scintillation counter. The response is reported as stimulation index (SI), mean cpm of antigen-stimulated cultures/ mean cpm of control. A SI of 3 or higher was taken as a positive response. Comparisons between post-vaccine and pre-vaccine antigen-specific responses were made from the fold increase (FI) in the SI (post-vaccine SI/pre-vaccine SI) and SDs of the two compared triplicates. The symbol (-) indicates no response and was given when FI < 1; the symbol (=) indicates stable response and was given when FI=1 or FI is < one SD from 1; finally, the symbols (+), (++) and (+++) indicate different degrees of response and were given when $1 < FI < 2$, $2 \leq FI < 4$ and $FI \geq 4$ respectively.

1.3.4 **Cytokine measurement by Enzyme-linked immunosorbent spot (ELISPOT)**

ELISPOT plates (MAIPS4510; Millipore) were pre-treated with 70% ethanol 50μl/well for 2 minutes. They were than washed three times with sterile phosphate buffered saline (PBS) and coated overnight at 4°C with 10μg/ml of mAb against either human IFN-γ (1-D1K), IL-13 (IL-13-I) (both from Mabtech, Sweden, Cat N^{o}

3420-3-1000 and Cat N° 3470-3-1000 respectively), or IL-2 (from IL-2 ELISPOT pair Cat N° 551884, BD Biosciences). Antibodies were diluted in sterile PBS and 100µl were added per well. After washing and blocking for 1 hour with CM, $2x10^5$ viable PBMNC were added in triplicate with either antigen or medium alone. After 40h, cells were discarded, wells were washed 6 times with PBS, and the following biotinylated mAbs were then added: anti-human (α-h)-IFN-γ and α-h-IL-13 (both from Mabtech: Cat N° 7-B6-1 and IL-13-II respectively); α-h-IL-2 (from IL-2 ELISPOT pair Cat N° 551884, BD Biosciences). Antibodies were diluted in PBS/1% Bovine Serum Albumin (BSA) and used at the manufacturer's recommended concentrations. Plates were placed at 37°C and left for 1.5 hours. They were then washed again 6 times with PBS and 100µl of streptavidin-alkaline phosphatase (Mabtech, 40ng/well) diluted in PBS/1% BSA were added to each well. After one hour plates were washed with PBS (6 times) and a chromogenic substrate (BCIP/NBT substrate kit, Zymed Laboratories Inc.,USA) was added. Spot formation was inspected by eye and once spots developed the chromogenic reaction was blocked by washing the plates with distilled water. The number of spots was counted using a computer-assisted video image analyzer (Autoimmun Diagnostika GmbH, Germany). Antigen-specific responses are reported as the number of spots per $2x10^5$ PBMNC in antigen-stimulated cultures minus the number of spots in the corresponding control. The average number of spots in triplicate control wells was always <5. Responses were considered positive if: (mean number of spots in triplicates of antigen-stimulated cultures) – (mean number of spots in the control) ≥ 10 per well, and >the mean number of spots in the control +2S.D. Comparisons between post-vaccine and pre-vaccine responses were done applying criteria used to evaluate proliferative responses (see above).

1.3.5 **Functionality of cryopreserved PBMNC after thawing**

Although viability of defrosted cells was always very good (around 90%), an important issue was to check whether the functionality of cells was affected either by the processes of freezing and thawing or by the duration of storage. To address these points, antigen-specific proliferative responses and IFN-γ production (by ELISPOT) were studied using PBMNC freshly isolated from a healthy donor and were compared to the responses from the same PBMNC which had been cryopreserved and were

defrosted one, two and twelve weeks later. To check for intra-assay variability, in each case two frozen vials were defrosted and used in parallel in the assays. The results of these experiments are illustrated in Figure 1.5. It is clear that the process of freezing and thawing did not affect PBMNC in their potential to proliferate or to exert effector functions (IFN-γ production) in response to the recall antigens TT and PPD or to a mitogenic stimulus (phytohaemagglutinin, PHA). In fact the levels of proliferative activity and cytokine production of crypreserved PBMNC were comparable to those of fresh PBMNC. More importantly, they did not decrease over time in cells kept in liquid N_2 Figure 1.5.A. At each time point proliferative responses and IFN-γ production were comparable between the two samples assayed in parallel (Vial 1 versus Vial 2). The only exception was found at week 2, when proliferative activity was expressed as stimulation index (Figure 1.5.A). In this case a lower degree of background proliferation of the un-stimulated control resulted in a significantly higher S.I. for PBMNC from Vial 2, although the absolute CPMs were comparable. Cytokine production by fresh and cryopreserved PBMNC was studied further in two additional independent experiments (Figure 1.5.C). PBMNC isolated from two healthy volunteers (subject 2 and 3) were used to assess responses to TT by IFN-γ ELISPOT. Fresh PBMNC were compared to PBMNC cryopreserved for 1 week (subject 2) or for five weeks (subject 3). Confirming what was found in the previous experiment, no significant differences were found between fresh and cryopreserved PBMNC. Overall these results validate the use of cryopreserved PBMNC for the purposes of our study.

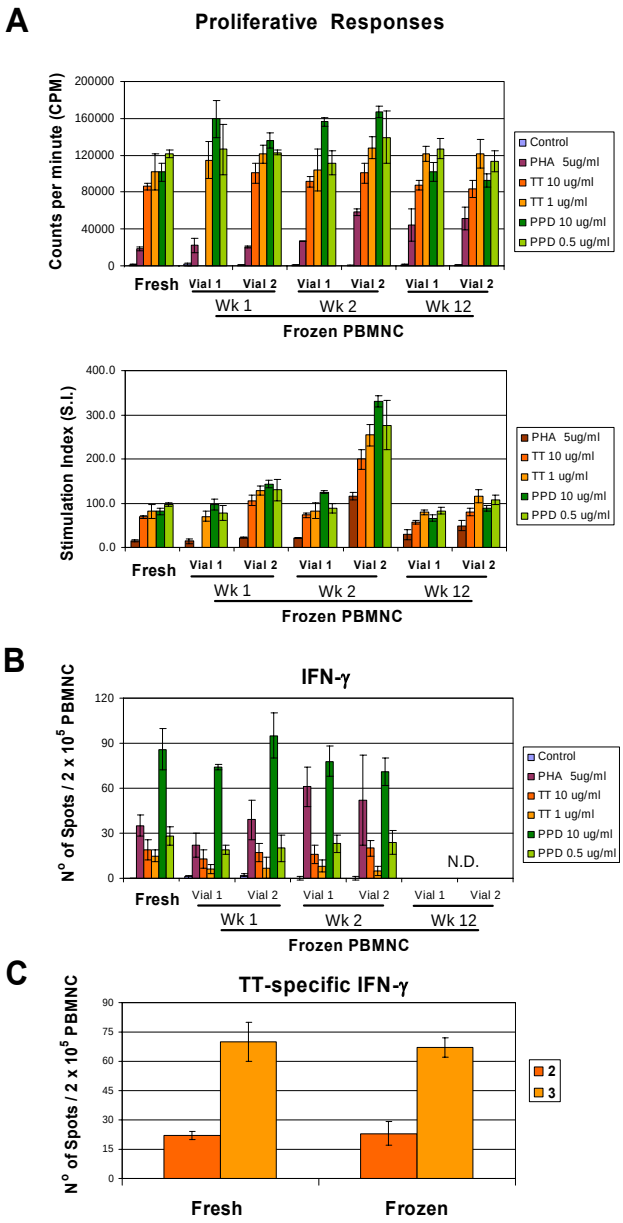


Figure 1.5. Functional comparisons between fresh and cryopreserved PBMNC.

Proliferation and IFN-γ ELISPOT assays (see above for details of the protocols) were used to compare functional behaviour of fresh and cryopreserved PBMNC isolated from healthy donors. Proliferative responses (**A**) and IFN-γ production (**B**) upon *in vitro* culture with medium only (control), with a mitogen (PHA) or with recall antigens TT and PPD were compared between PBMNC freshly isolated from one volunteer and the same PBMNC cryopreserved and defrosted one, two and twelve weeks later. To check for intra-assay variability, in each case two frozen vials were defrosted and used in parallel in the assays. (**C**) *In vitro* IFN-γ production in response to TT (10μg/ml) was compared between fresh PBMNC and PBMNC cryopreserved for 1 week (subject 2) or for five weeks (subject 3). Proliferative activity was measured by ^{3}H-thymidine incorporation after 6 days of *in vitro* culture and reported for each time point as the mean value of counts per minute (CPM) or as stimulation index (SI) in triplicate cultures ± SD. For IFN-γ ELISPOT data for each time point represent the mean number of spots ± SD of triplicate wells of antigen-stimulated cultures after subtracting the mean number of spots in the corresponding un-stimulated negative control. The mean number of spots in the controls was always <5 spots per 2 x 10^5 PBMNC. N.D. Not done

1.3.6 **Generation of antigen-specific T cell lines**

PBMNC were isolated from two healthy donors (D1 and D2) who, in a preliminary screening, showed strong proliferative responses against TT and PPD respectively (see Table 1.2 below).

	D-1				**D-2**			
	CPM		**S.I.**		**CPM**		**S.I.**	
	MEAN	STDEV	MEAN	STDEV	MEAN	STDEV	MEAN	STDEV
Unstimulated	647	239			863	44		
TT 1μg/ml	**60161**	3316	**93.0**	5.1	121797	22622	141	26.2
PPD 10μg/ml	30189	13083	46.7	20.2	**106310**	7219	**123.2**	8.4

Table 1.2. Pre-screening of proliferative responses to TT and PPD in two immune healthy subjects. Proliferative activity of PBMNC isolated from two healthy volunteers (D-1 and D-2 in response to TT or PPD stimulation, was measured by ^{3}H-thymidine incorporation after 6 days of *in vitro* culture and reported for each time point as the mean value of counts per minute (CPM) or as stimulation index (SI) in triplicate cultures ± SD.

To generate antigen-specific cell lines, PBMNC from D1 and D2 were cultured in CM in the presence of TT and PPD respectively (10μg/ml) at $2x10^{5}$/well in round-bottomed 96 well plates. Human recombinant IL-2 (20 U/ml) (R&D Systems, Cat N^{o} 202-IL-050) was added at day 7 and 9 and medium replaced if necessary. At day 14 cells were harvested, pooled and counted. Viable cells were then re-stimulated with antigen plus irradiated (40 Gy) autologous PBMNC as antigen presenting cells (APCs) at 1 to 3 ratio in 12 or 24 well plates. IL-2 was added at day 5. Lines were maintained by repeating this 14-day cycle of re-stimulation with antigen followed by culture in IL-2.

1.3.7 **Antigen specificity of T cell lines and identification of restricting HLA molecules**

The antigen specificity of the T cell lines was checked by a 3-day proliferative assay, performed as described above, using 2.5 x 10^{4} T cells plus irradiated (40 Gy)

autologous PBMNC as APCs at 1 to 3 ratio in round-bottomed 96 well plates. 10μg/ml of antigen was added in each well. PHA (10μg/ml) was used as positive control. To identify the restricting HLA isotype of the T cell lines, 10μg/ml of mAb directed against HLA-DR (L243), HLA- DP (B7/21.2) or HLA class I (W6/32) (all from Cancer Research UK) were added to the cell suspension one hour prior to addition of the antigen and kept in the medium for the duration of the proliferative assay.

1.3.8 **Flow cytometry analysis**

1.3.8.1 **CFSE labeling and cell division analysis.**

Cryopreserved PBMNC were thawed and counted as described above (see Proliferative responses). Then they were labeled *in vitro* with CFSE (Cell Trace, Molecular Probes, Ivitrogen, Cat N^{o}C34554) a modified protocol adapted from Lyons AB & Parish CR [96]. Briefly, cells were resuspended in RPMI 1640 at 10 x 10^6 cells/ml. CFSE was added to cell suspensions at a final concentration of 5μM, and tubes were transferred into a water bath at 37 ^{o}C. They were mixed occasionally and after 10 minutes ice cold FCS was added (1/2 of the initial volume) and tubes were left on ice for 5 minutes to quench the reaction. Two washes followed, one with RPMI and the second with PBS. Cells were finally resuspended in CM and cultured in six replicates ($2x10^5$/well) with CM alone or with antigen. At indicated times, cells from replicate wells were pooled, washed and stained with the following antibodies: anti-human CD3-PerCP (clone SK7), CD4-APC (clone RPA-T4) and CD25 PE (clone M-A251) (all BD Biosciences) or with their respective mouse immunoglobulin isotype controls. Samples were analyzed on a FACSCalibur flow cytometry using CELLQUEST software (both from BD Biosciences). An initial gating based on forward and side scatter characteristics included both resting lymphocytes and blasts. A minimum of 10,000 CD3-positive and CD4 positive gated events were acquired and analyzed for CFSE contents and CD25 surface expression.

1.3.8.2 **Phenotypic analysis**

White blood cell counts were done using an automated cell counter and analyzer at the Southampton General Hospital. For phenotype analysis, cryopreserved PBMNC were thawed, washed with PBS (5 minutes at 1600 RPM), resuspended in 50-100µl PBS and stained for 20 minutes on ice using the manufacturer's recommended concentrations of the following antibodies: anti-human CD3-PerCP (SK7), CD4-APC (RPA-T4), CD8-APC (RPA-T8), CD14-APC (M5E2), CD19-APC (HIB19), CD25 PE (M-A251), CD45RA (HI 100), CD45RO (UCHL1), CD56-APC (B159), CD80-FITC (L307.4), CD86-PE (2331, FUN-1) and HLA-DR-PE (G46-6, L243) (all BD Biosciences) or with their respective mouse immunoglobulin isotype controls. Cells were then washed once with PBS (5 minutes at 1600 RPM), fixed with PBS/1% formaldehyde and analyzed on a FACSCalibur flow cytometer using CELLQUEST software (both from BD Biosciences). Based on forward and side scatter characteristics, an initial gating was used to exclude dead cells and debris. A minimum of 20,000 viable cells was acquired.

1.3.9 **Antibody measurement by Enzyme-linked immunosorbent assay (ELISA)**

Ninety-six well flat-bottomed plates (ImmunoSorp plates, Nunc, Denmark.Distributed by Invitrogen) were coated overnight at 4°C with either 2.5 µg/ml of TT, 10µg/ml of PPD or 10µg/ml of *C. alb*. Antigens were diluted in ELISA coating buffer (Na_2CO3 (anhydrous) 1.58g, $NaHCO_3$ 2.92g, dH2O 1litre. pH 9.5) and 100µl were distributed into each well. Plates were then washed once with 200ml/well PBS-tween (0.01%) and blocked for 90' at 37^{o}C with 200ml/well PBS/1% BSA. After washing once with PBS-tween (0.01%), serial dilutions of serum samples (100µl/well) or appropriate standards in at least four replicate wells were added. Antibody standards were: TT, tetanus antitoxin human immunoglobulin (Tetanus Antitoxin Human Serum NIBSC code: 76/589, NIBSC); PPD, serum from a healthy individual showing relatively high anti-PPD antibody titers; *C. alb*, serum from a known antibody-positive subject (kindly provided by Dr R.Hobson, Mycology Reference Centre, University of Leeds, UK). Plates were kept 90 minutes at 37^{o}C and then washed 4 times with PBS-tween

(0.01%) prior to adding a 1/2000 dilution (100µl/well) of a horse-radish peroxidase (HRP)-conjugated monoclonal anti-human IgG (Fc) (The Binding Site, Birmingham, UK) which was used for detection. Following 1h incubation at 37^{o}C, plates were washed 4 times and incubated with 100µl/well substrate (citric acid (anhydrous) 4.68g, Na_2HPO_4 7.30g, dH2O 1litre. pH 5.5) until a colour change was detected. The reaction was then stopped by adding 80µl/well 2.5M H_2SO4. Plates were read using a Dynex MRX Platereader (Dynatech Instruments Inc., Santa Monica, USA) set at 490nm wavelength. For TT, results are expressed in I.U./ml. For PPD and *C. alb*, results are expressed in arbitrary units (A.U.)/ml. Comparisons between post-vaccine and pre-vaccine antigen-specific responses were done using fold increase in titers (post-vaccine response/pre-vaccine response) and SDs of the two compared replicates. Symbols indicating no response (-), stable (=) or different degrees (+ to +++) of positive response, were assigned using the same criteria as for the cellular responses.

1.4 Results

1.4.1 Vaccine-specific and bystander proliferative responses of T_{mem} cells following booster vaccination with TT

The kinetics of the TT-specific proliferative responses over 16 weeks following booster vaccination with TT are shown for six representative cases (Figure 1.6.A). Importantly, results were reproducible using samples thawed from stocks on two different occasions, as shown for a representative case in Figure 1.6.B.
Pre-existing immunity, as indicated by a positive response at week 0, was evident in all, but at different levels (Figure 1.6.A). Increased responses were detected after vaccination but the time to peak responses varied from 2-12 weeks and the dynamic complexity of the responses was striking. Surprisingly, parallel kinetics were observed for proliferative responses against two unrelated antigens for which pre-existing immunity was also evident, PPD and *C. alb* (Figure 1.6.A). Parallel kinetics were seen in individuals with early (cases 5, 3,10) or late (cases 6, 8, 9) responses to TT. In patient 8 who lacked pre-existing immunity against *C. alb*, bystander proliferative responses did not develop arguing that T_{mem} cells are required. In support of that, no responses against control antigens (Ctrl 1 and Ctrl 2) without pre-existing immunity were detected. It appears therefore that vaccination expands both specific and non-specific T_{mem} coordinately, and that contractions also occur in parallel. These contractions were observed in various individuals (e.g. case 5 at wk 4, case 3 at wk 8, case 10 at wk 8 etc.) (Figure 1.6.A) and were not due to low viability of thawed cells, which was always ~ 90%. They were observed also when the assay was done using fresh PBMNC (Figure 1.6.C), for which viability exceeded 95%, excluding any other differential loss of functionality during cryopreservation. Finally they were still observed when higher (super-optimal) doses of antigen were used (Figure 1.6.C, time point wk 4), arguing against a drop in assay sensitivity at those particular time points. Overall these contractions seem to represent a genuine phenomenon, which at least in some individuals can been seen as the natural decline of the immune response following the peak.

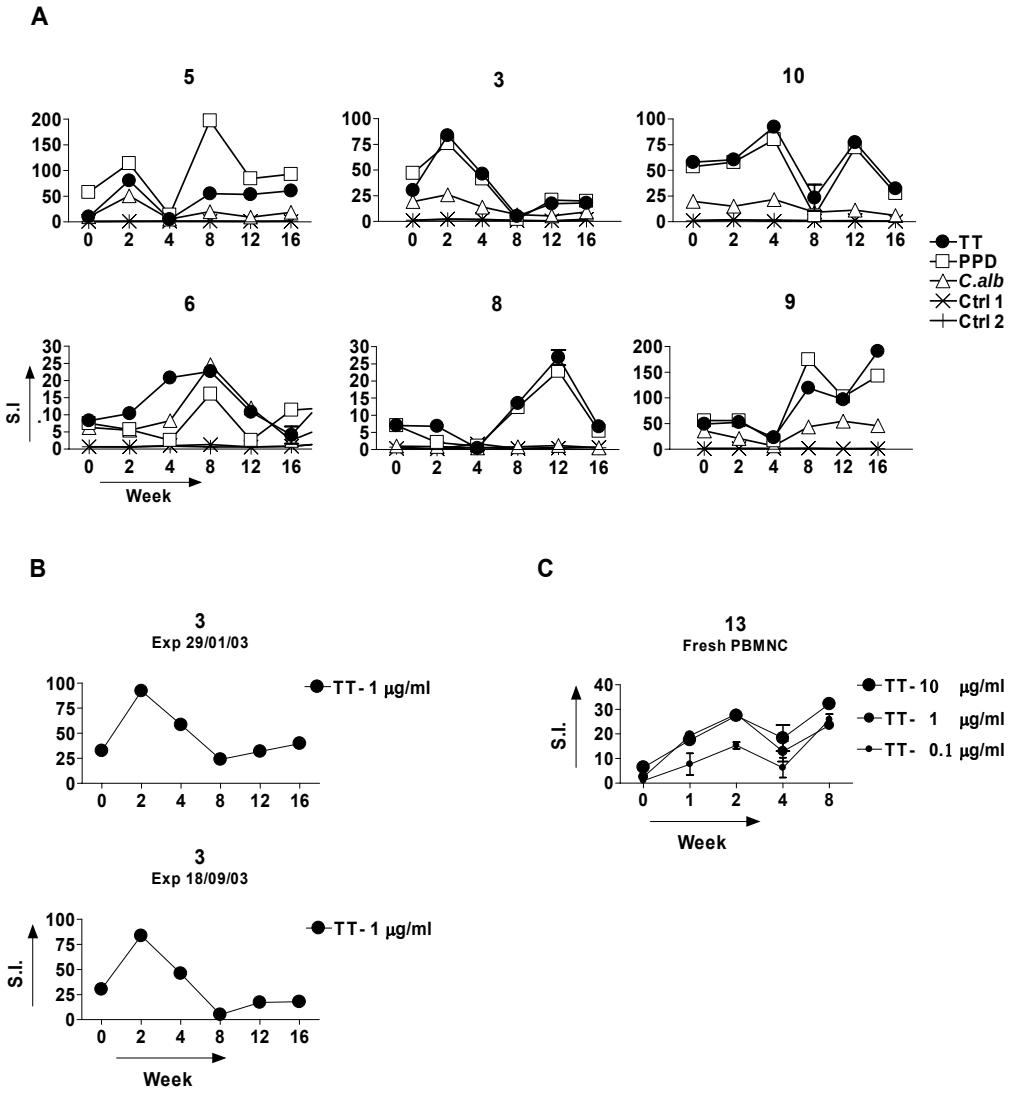


Figure 1.6 Kinetics of vaccine-specific and bystander proliferative responses of T_{mem} cells following booster vaccination with TT.

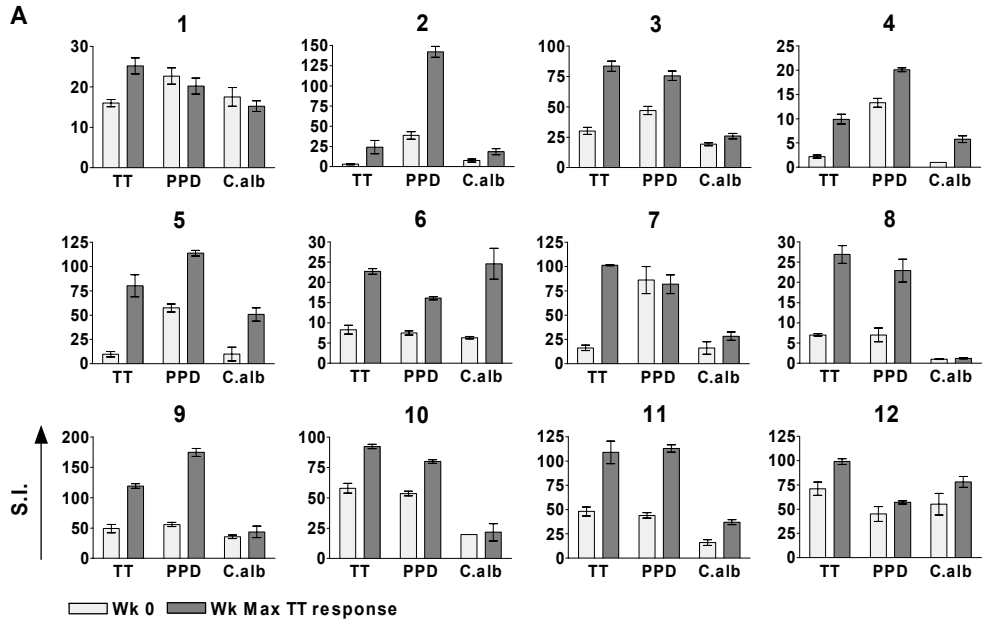
(A) From [5] . Six representative examples are shown. Vaccination (week 0) induces early (subjects 5, 3 and 10) and late (subjects 6, 8, and 9) TT-specific responses. Parallel kinetics were observed in the responses against two unrelated antigens (PPD and *C. alb*) for which pre-existing immunity was evident, but no responses were detected against control antigens (Ctrl 1 and Ctrl 2) with no evident pre-existing immunity.

(B) Proliferative response to TT in individual 3, measured in two different occasions.

(C) Proliferative responses of freshly isolated PBMNC to super-optimal (10µg/ml) and sub-optimal (0.1µg/ml) concentrations of TT in individual 13.

Proliferative activity was measured by ^{3}H-thymidine incorporation after 6 days of *in vitro* culture and reported for each time point as the mean value of stimulation index (SI) in triplicate cultures ± SD.

Proliferative responses of the 12 subjects to the 3 antigens TT, PPD and *C.alb* are shown in Figure 1.7.A and 1.7.B. Due to the variable kinetics, the time of maximum response against TT varied from 1-12 weeks, and, in order to condense the data, responses against the other antigens are shown at this time. Responses have been summarized and graded (+ to +++) in terms of fold increases over pre-existing levels, as defined in Section 1.3.3 of Methods (Figure 1.7.B). Although this grading documents the significant changes occurring, it minimizes the apparent responses of subjects, such as case 10, with a high initial level (SI ± SD = 57.9 ± 4) which clearly increased to an SI = 92.2 ± 1.8 (Figure 1.7.A). In contrast, it maximizes the apparent responses of subjects, such as case 4, with a very low pre-existing response (SI = 2.2 ± 0.3) but with a clear increase (SI = 9.9 ± 1.0). Overall, 12/12 subjects showed significantly increased responses to the TT vaccine, and the majority (11/12) had increased responses to at least one of the other non-vaccine antigens. For example, cases 2, 5 and 11, with strong responses to TT and pre-existing immunity to PPD and *C. alb*, showed high bystander effects. The single subject (case 1) who failed to expand the pre-existing response to PPD or *C. alb* had responded relatively weakly to the TT vaccine (Figure 1.7.A and B). 7/12 subjects responded to all 3 antigens.



B

Subject	Wk	TT	PPD	*C. alb*
1	8	+	=	=
2	1	+++	++	++
3	2	++	+	+
4	2	+++	+	+++
5	2	+++	++	+++
6	8	++	++	++
7	2	+++	=	+
8	12	++	++	-
9	8	++	++	=
10	4	+	+	=
11	8	++	++	++
12	1	+	+	+

Figure 1.7. From [5] **.Vaccine-specific and bystander proliferative responses of T_{mem} cells in twelve individuals following booster vaccination with TT.**

(A) Responses are shown at the week of maximal TT response, and are compared to the pre-vaccine (Wk 0) response. Proliferative activity was measured by ^{3}H-thymidine incorporation after 6 days of *in vitro* culture and is reported as the mean value of stimulation index (SI) in triplicate cultures ± SD.

(B) Summary of proliferative responses against PPD and *C. alb* at the week of maximal TT response. The week of maximal TT response is reported for each individual. Symbols indicating no response (-), stable (=) or different degrees (+ to +++) of a positive response, were given according to the criteria stated in Materials and methods.

1.4.2 **Lack of cross-reactivity among the antigens**

To exclude the possibility that the observed proliferative responses against the unrelated antigens PPD and *C. alb* were due simply to cross-reactivity, two antigen-specific T cell lines, D1-TT and D2-PPD, were generated by repeated stimulation *in vitro* of PBMNC with TT and PPD respectively. Antigen specificity was then tested in a short term proliferative assay. As shown in Figure 1.8.A, the D1-TT cell line specifically responded only to TT but not to PPD or *C. alb* while the D2-PPD cell line specifically responded to PPD but not to TT or *C. alb*. Both T cell lines strongly proliferated in the presence of PHA, used as positive control. Taken together these data indicate lack of cross reactivity among the antigens used in the present study.

1.4.3 **Nature of the responding T cells**

To identify the restricting HLA element and the phenotype of the responding cells, the proliferation assay was repeated for D1-TT in the presence of monoclonal antibodies (mAbs) directed against human MHC antigens (Figure 1.8.B). L243 and B7/21.2 recognize two class II molecules, HLA-DR and HLA- DP respectively. W6/32 recognizes HLA class I molecules. The proliferative response to TT was strongly inhibited (>95%) only in the presence of L243. This identifies HLA-DR as the restricting class II element involved in the presentation of antigen and $CD4^+$ helper T cells as the population of lymphocytes responding *in vitro* to TT.

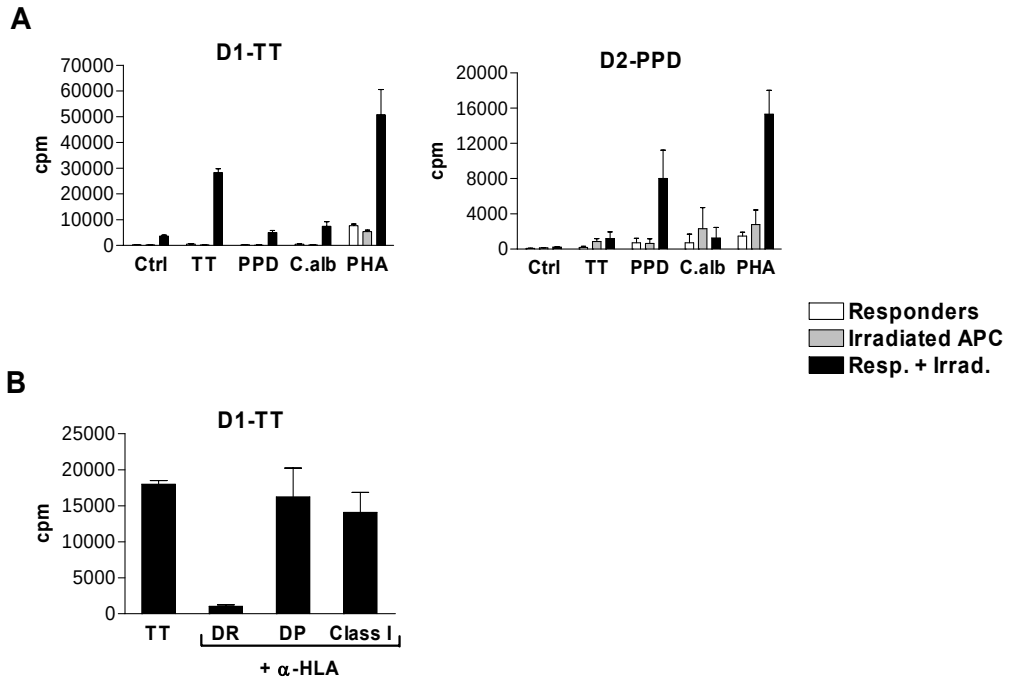


Figure 1.8 From [5].

(**A**). **Lack of cross-reactivity among TT, PPD and *C. alb* antigens.** To exclude cross-reactivity among the antigens used, two different T cell lines D1-TT and D2-PPD were generated from PBMNCs of two healthy donors, by repeated cycles of *in vitro* stimulation with TT and PPD respectively, as described in Materials and methods. Their antigen specificity was then tested in a three-day *in vitro* proliferative assay. Proliferative activity was measured by ^{3}H-thymidine incorporation and is reported as counts per minute (cpm) in triplicate cultures ± SD.

(**B**). **Identification of HLA-DR as the restricting MHC molecule of the T cell lines**. To identify the restricting HLA isotype of the T cell lines, antigen presentation by autologous APCs was assayed by repeating the proliferative assay after addition of mAbs against HLA-DR (L243), -DP (B7/21.2) or HLA class I (W6/32) at the beginning of the culture time. The proliferative response of the D1-TT to TT was strongly inhibited only when the HLA-DR-specific mAb L243 was added to the culture.

1.4.4 Vaccine-specific and bystander cytokine production by Th_{mem} cells following booster vaccination with TT

IFNγ, IL-2, IL-5 and IL-13 ELISPOTs were used to study the effect of vaccination with TT on the ability of the T-cell population to produce cytokines in response to antigen *in vitro*. IFNγ and IL-2 are generally considered to indicate Th1 responses, while IL-13 mainly reflects Th2 activation [97]. In initial experiments, we used both IL-

5 and IL-13 ELISPOT assays to analyse responses to vaccination in healthy volunteers boosted with TT. In four subjects, we confirmed that production of IL-13 correlated closely with that of IL-5, a classical Th2-associated cytokine (Figure 1.9). However, while in one subject (subject 2) IL-5 and IL-13 responses were comparable, in all three remaining subjects (4, 7 and 9) IL-13 ELISPOT showed higher sensitivity, especially when low levels of cytokine production were detected (subject 4). For this reason, and also to reduce the number of cells required to set up these assays, IL-13 ELISPOT only, was thereafter used.

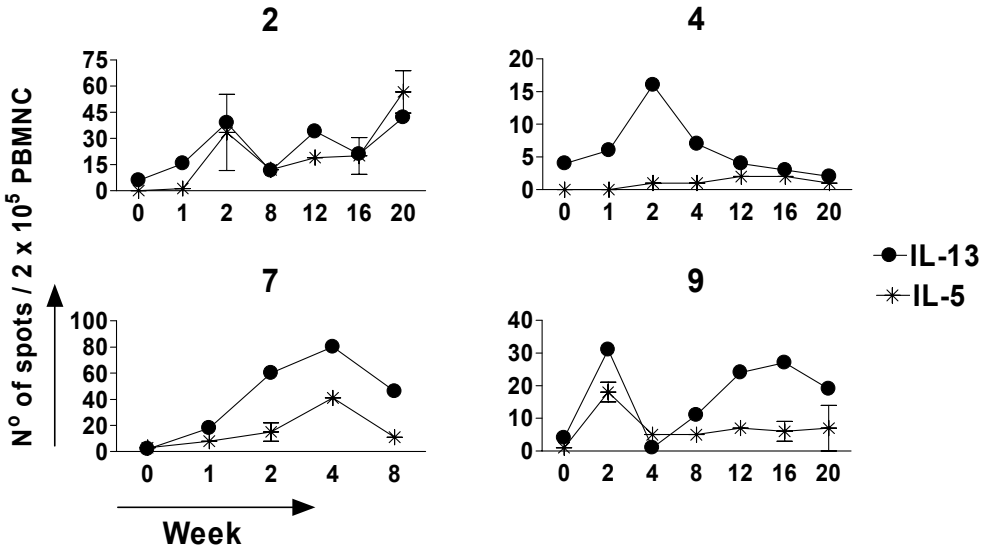


Figure 1.9. Kinetics of TT-specific IL-5 and IL-13 production by Th_{mem} cells following booster vaccination (week 0) with TT.

Both IL-5 and IL-13 are produced and detected by ELISPOT assay following *in vitro* re-stimulation with TT of PBMNC taken from four healthy individuals boosted with TT. These responses show parallel kinetics which reflect a Th2 component in the vaccine-specific immune response. However IL-13 ELISPOT appears to offer higher sensitivity especially when low responses to the vaccine are detected (see subject 4). To simplify the graphs, responses to protein Ctrl1 or Ctrl2 are not shown, but they were always below 5 spots / 2 x 10^5 PBMNC. Data for each time point represent the mean number of spots ± SD of triplicate wells of antigen-stimulated cultures after subtracting the mean number of spots in the corresponding negative control. The mean number of spots in the controls was always < 5 spots per 2 x 10^5 PBMNC.

The effect of vaccination with TT on the ability of the T-cell population to produce cytokines in response to TT and to unrelated antigens *in vitro*, also revealed complex dynamics. Kinetic data from 2 representative subjects (cases 3 and 9) showed

parallel induction of IFNγ, IL-2 and IL-13 (Figure 1.10) in response to TT. Production of all three cytokines is consistent with a mixed Th1 and Th2 response (Figure 1.10). Cytokine production generally mirrored proliferative responses (Figure 1.6), with early and late peaks. However, in case 9, there was more evidence of cytokine-producing T cells at week 2, without a significantly increased proliferative response above the relatively high pre-existing levels.

For both cases, bystander cytokine production against PPD was also observed, with a similar pattern to the proliferative responses (Figures 1.6, 1.7 and 1.10). PPD induced high IFNγ levels, as reported previously [98], but also IL-13, indicating a mixed Th1/Th2 response. Cytokine production against *C.alb* in these subjects showed less consistent changes, reflecting the more modest proliferative responses (Figure 1.6). However, a significantly increased IL-2 response was detected in case 3, at 2 weeks post vaccination, the time of maximum response to TT (Figures 1.6, 1.7 and 1.10). In case 9, a significant IFNγ response was observed against *C. alb* at week 12, again in parallel with the high responses against TT and PPD (Figure 1.10).

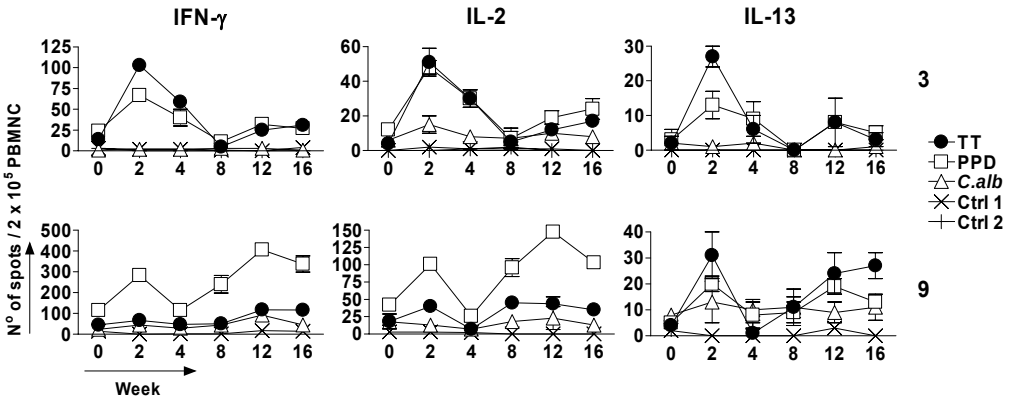
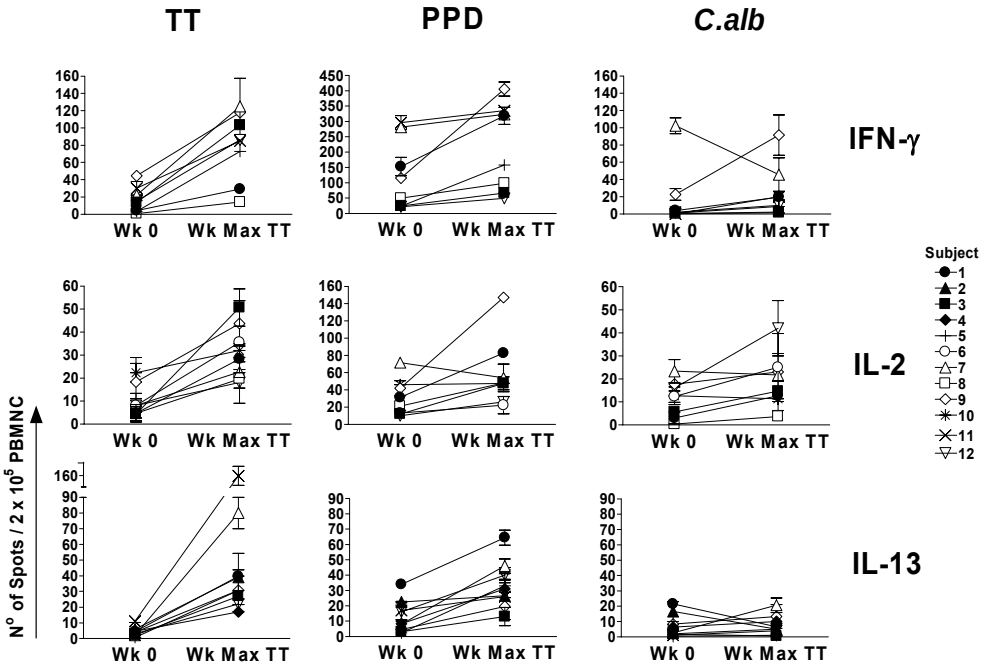


Figure 1.10. From [5]. Kinetics of vaccine-specific and bystander cytokine production by Th_{mem} cells following booster vaccination (week 0) with TT.

Two representative examples are shown. Characteristic Th1 (IFN-γ and IL-2) and Th2 (IL-13) cytokines are produced following *in vitro* re-stimulation with TT and detected by ELISPOT assay. Parallel changes in the number of cytokine-producing cells are seen against PPD and (to a lesser extent) against *C. alb*, but not against Ctrl1 or Ctrl2. Data for each time point represent the mean number of spots ± SD of triplicate wells of antigen-stimulated cultures after subtracting the mean number of spots in the corresponding negative control. The mean number of spots in the controls was always <5 spots per 2 x 10^5 PBMNC.

Cytokine profiles against PPD and *C. alb*, for all subjects showing a response to TT, were measured at the point of maximum response to TT, which generally matched that of the proliferative response. Data are shown in Figure 1.11.A as the number of spots/ 2 x 10^5 cells. In Figure 1.11.B responses have been summarized and graded (+ to +++) in terms of fold increases over pre-vaccine levels, following the same criteria applied for the evaluation of proliferative responses, and the week of maximum response to TT is reported for each cytokine. The pattern of cytokine production against PPD tended to mirror that against TT, with production of IFNγ, IL-2 and IL-13 (Figure 1.11.A and B). Cytokine responses against *C. alb* were less common, probably due to a lower degree of pre-existing immunity. For example, subject 8 with no apparent pre-existing or post-TT vaccination proliferative response to *C. alb* (Figure 1.6), failed to produce IL-2, while this was readily produced in response to TT and PPD (Figure 1.11.A and B). The largest increase in the IFNγ response against *C. alb* was in subject 9 at week 12 (see above), which paralleled responses to TT and PPD. However, in contrast to TT and PPD, there was no detectable increase in IL-2 and IL-13-producing T cells against *C. alb* at this time point (Figure 1.11.A and B).

A



B

Subject	IFN-γ				IL-2				IL-13			
	Wk	TT	PPD	*C.alb*	Wk	TT	PPD	*C.alb*	Wk	TT	PPD	*C.alb*
1	2	+++	++	+++	2	+++	++	+++	2	+++	++	-
2		=				-			2	+++	=	-
3	2	+++	++	-	2	+++	+++	++	2	+++	+++	-
4		-				-			2	++	++	=
5	2	+++	+++	-		ND	ND	ND	2	+++	+++	-
6		-			8	+++	=	++		-		
7	9	+++	+	-	2	++	-	=	4	+++	+++	+++
8	1	+	+	-	12	+++	++	-		-		
9	12	++	++	+++	12	++	++	=	2	+++	+++	=
10		-			2	+	=	=		-		
11	1	++	+	+++		ND	ND	ND	1	+++	=	-
12	1	+++	++	-		=			1	+++	++	-

Figure 1.11. From [5]. Vaccine-specific and bystander cytokine production in twelve individuals following booster vaccination with TT.
(A) Responses are shown at the week of maximal TT response and are compared to the pre-vaccine (Wk 0) response. Cytokine responses were measured by ELISPOT assay after 40h of *in vitro* culture and are reported as the mean number of spots ± SD of triplicate wells of antigen-stimulated cultures after subtracting the corresponding negative control. The mean numbers of spots in the controls were always <5 spots per 2 x 10^5 PBMNC. (B) Summary of cytokine responses against PPD and *C alb* at the week of maximal TT response. The week of maximal TT response is reported for each individual and for each cytokine. Symbols indicating no response (-), stable (=) or different degrees (+ to +++) of a positive response, were given according to the criteria stated in Materials and methods.
ND, not done.

1.4.5 **The increased numbers of vaccine-specific and bystander memory T cells detectable by ELISPOT after TT-vaccination, seem to reflect an *in vivo* expansion**

One question is whether the increased number of cytokine-producing cells observed in post-vaccine samples after 40h incubation *in vitro* with PPD or *C. alb* was by T cells expanded *in vivo.* An alternative explanation might be that Th_{mem} cells against bystander antigens might not have divided *in vivo*, but could have been rendered more sensitive to specific antigenic proliferative signals *in vitro.* To investigate this, we used CFSE labeling [96] to track cell division occurring *in vitro* in $CD3^+CD4^+$ T cells from the blood of case 3, at Day 0 and 2 weeks post TT injection (the time of maximum response against TT, see Figures 1.6 and 1.10). We found that cell division did not occur during incubation with antigen for 40h *in vitro*, up to the point where cytokine production was measured (Figure 1.12), although cells could be all activated ($CD25^+$) and induced to divide if stimulated with PHA. The number of cytokine-producing cells, as measured by ELISPOT, therefore reflects cells already existing *in vivo*. Booster vaccination has apparently increased functional antigen-specific and bystander T_{mem} cells *in vivo*, able to respond *in vitro* to TT or to PPD/*C. alb* respectively by proliferation and cytokine production.

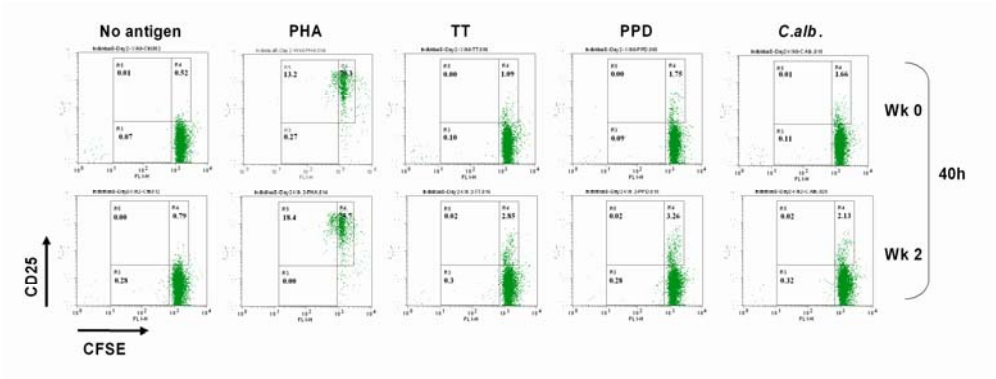


Figure 1.12 CFSE-based cell division analysis.

PBMNC from subject 3 pre-vaccination (Wk 0) and after a TT boost at the peak TT-specific proliferative and cytokine responses (Wk 2) (see Fig 1.6 and Fig 1.10), were labeled with CFSE and stimulated i*n vitro* with either TT, PPD or *C. alb*. After 40h, cells were harvested and surface-stained for CD3, CD4 and CD25. All dot plots are gated on $CD3^+CD4^+$ cells in the lymphocytes and blasts region. Cells are analyzed for CFSE content and CD25 expression and % of positive cells are indicated. No indication of cell division is evident at 40h in either Wk 0 or Wk 2 antigen-stimulated cultures.

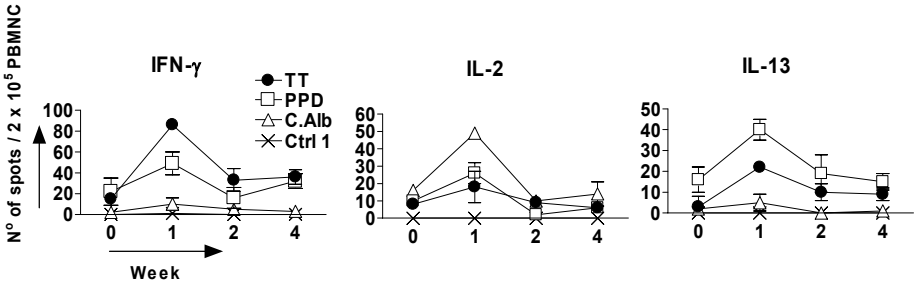
1.4.6 Stable immunophenotypic profile of blood cells during the antigen-specific responses to vaccination with TT

To investigate any relationship between the observed kinetic pattern in the antigen-specific responses and possible changes in the number and/or phenotype of immunologically relevant cell subsets in the peripheral blood, a detailed phenotypic analysis was carried out at each time point in one individual (case 12) and analyzed in parallel with IFN-γ, IL-2 and IL-13 antigen-specific responses. Results are shown (Figure 1.13) up to wk 4 (the remaining time points are not shown for clarity). While a clear expansion in the number of TT-specific and bystander T_{mem} cells measured by ELISPOT is visible at week 1, followed by a down-regulation to pre-vaccine levels at week 2 (Figure 1.13.A), a parallel remarkable stability was found in the number and phenotype of all the analyzed cell subsets (Figure 1.13.B). In fact, no significant changes were found in the number of total white cells, or in the number of lymphocytes and monocytes in the blood (Figure 1.13.B). No significant changes were found in the percentages of $CD3^+CD4^+$ T lymphocytes, known to be the major population of cells responding in vitro to TT and PPD[21,22] and in the proportion of

$CD14^+$ monocytes which constitute together with $CD19^+$ B cells the major populations of antigen presenting cells within the PBMNC.

Expression of the activation marker CD25 within the total PBMNC and within the $CD3^+$ lymphocyte population was checked and found to be unchanged (Figure 1.13.B). The level of MHC and co-stimulatory molecules was also measured and no change in the total percentages of cells expressing HLA-DR or the co-stimulatory molecule CD86 was detected (Figure 1.13.B). Finally, no significant changes were found in the percentages of T regulatory cells defined here as $CD3^+$, $CD4^+$, $CD25^{High}$, $CD45R0^{High}$ (Figure 1.13B).

A



B

	0	Wk 1	Wk 2	Wk 4
White Blood Cells (n x 10^6/ml)				
Total	6.7	6.9	6.3	6.3
LYM	2.0	1.8	1.7	1.8
MON	0.5	0.6	0.7	0.4
Phenotype (%)				
CD3+	58.7	57.5	53.5	40.9
CD3+/CD4+	47.8	45.5	43.4	32.3
CD3+/CD8+	10.9	11.8	10.5	9.3
CD3-/CD56+	6.9	10.3	11.9	12.3
CD3+/CD56+	1.8	2.1	2.9	1.7
CD19+	6.4	3.6	6.0	5.3
CD14+	20.1	20.1	20.7	30.1
CD86+	24.0	24.4	25.3	36.1
HLA-DR+	34.3	33.2	34.1	44.8
CD25+	21.6	22.1	19.9	16.7
CD25+CD3+	11.5	11.5	10.3	9.1
Treg	2.7	2.6	2.5	2.0

Figure 1.13. From [5]. Immunophenotypic profile of blood cells during the antigen-specific responses to vaccination with TT in subject 12.

(A) Kinetics of IFN-γ, IL-2 and IL-13 antigen-specific responses against TT, PPD and *C. alb* in subject 12 vaccinated with TT as measured by ELISPOT. Data for each time point (0, 1, 2 and 4 weeks) represent the mean number of spots ± SD of triplicate wells of antigen-stimulated cultures after subtracting the mean number of spots in the corresponding negative control. The mean numbers of spots in the controls were always lower then 5 spots per 2 x 10^5 PBMNC.

(B) Blood counts and phenotype of immunologically relevant cell subsets at the corresponding time points.

This analysis points to no gross changes in relative proportions, composition or activation status of cell populations during vaccination. An analysis of phenotypic changes in activation markers within selected populations of $CD4^+$ memory T cells or subtypes of T_{CM}, T_{EM} cells using multi-color flow cytometry might be more revealing and is now planned on a new cohort of vaccines.

1.4.7 **Antibody responses**

Measurement of the antibody responses against TT showed the expected boost after vaccination, with a maximum at or near week 2 (Figure 1.14). Levels were variable but all subjects showed a significant increase to a plateau followed by a very slow decline over 20 weeks (Figure 1.14), confirming other studies [88,95]. The most dramatic change was observed in two individuals (7 and 11) showing pre-vaccination TT-specific antibody titers below the level of 0.1 I.U. /ml, known to be required for full protection (fold increase at wk 2 = 162 and 135, respectively). Interestingly, the same individuals had shown the strongest IL-13 responses in the ELISPOT assay (see Figure 1.11). In contrast to TT, there was no significant change in antibody levels against PPD or *C. alb* which remained remarkably stable. These findings indicate a dependence of antibody formation by B cells on specific antigen, and argue for restriction of a functional bystander effect to T cells.

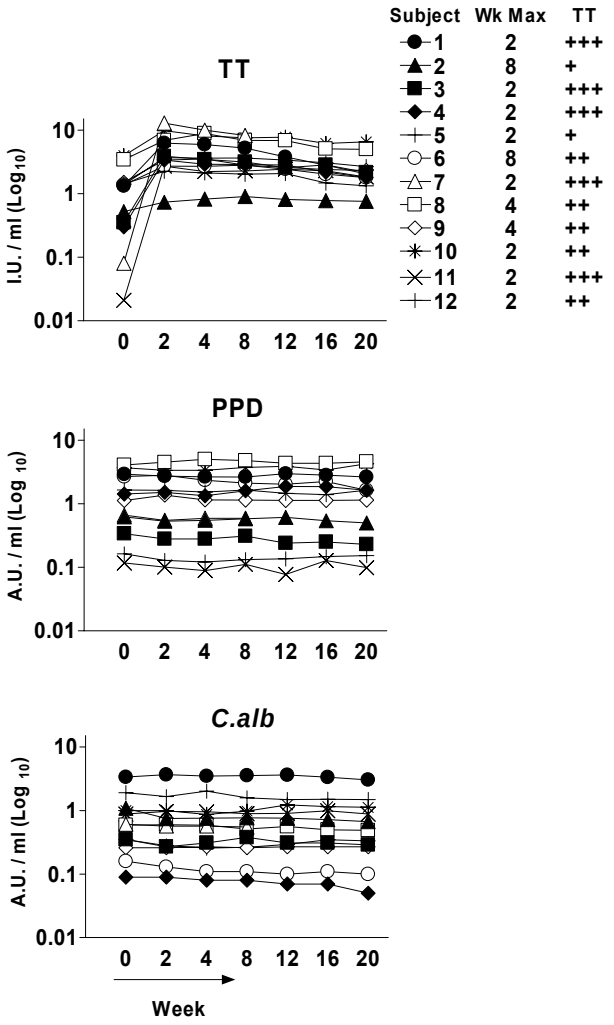


Figure 1.14. From [5]. Kinetics of antigen-specific antibody responses as measured by ELISA in twelve individuals following booster vaccination (week 0) with TT.

A boost in the levels of TT-specific serum IgG was evident in all the vaccinated subjects, with a maximum at the indicated week, but no significant changes were detected in either PPD- or *C.alb*-specific antibody responses. Data for each time point represents the mean value of at least four replicate wells. SD bars are not shown for clarity. Symbols (+ to +++) indicate different degrees of positive responses analyzed at the week of maximal TT response (indicated), as compared to the pre-vaccine (week 0) response, and were given according to the criteria stated in Materials and methods.

1.5 **Discussion**

The control of memory T cells has obvious implications for natural and vaccine-induced immunity. The mechanisms of induction and, above all, of maintenance of T cell memory, although widely investigated, remain unclear. Whether or not antigen is required for the maintenance of memory T cells is still debated, although in mice long-term persistence of $CD4^+$ and $CD8^+$ memory T cells can occur in antigen-free MHC class II-, and class I-deficient mice, respectively [68,69]. In humans, vaccinia-specific humoral and cellular responses can be detected for decades after smallpox vaccination in the absence of re-exposure to the virus [65-67], supporting the idea that antigen-independent mechanisms may contribute to the maintenance of immunological memory.

To study antigen-independent activation of memory T cells *in vivo* in human subjects, we have assessed the impact of TT boost vaccination on the kinetics of populations of memory T cells specific for TT, and also on the kinetics of memory T cells specific for two common un-related recall antigens, PPD and *C.alb*. We have focused our analysis on Th_{mem} cells which are known to be the population responding to TT and to PPD *in vitro* [99,100]. Our data support the view that human Th_{mem} cells are susceptible *in vivo* to stimulation from surrounding Th_{mem} cells which are responding to specific antigen. This bystander response has parallel kinetics to the specific response, and is likely to be mediated by cytokines. In this respect the immune system uses Th_{mem} cells to add an innate "infectious" element to the specific response. Although there is a tempting parallel with infectious tolerance [101], this cannot be simply drawn. Infectious tolerance has been studied mainly at the point of priming and appears to require presentation of both specific and bystander antigens by the same DC [102]. For Th_{mem} stimulation, the bystander antigens apparently need not be present. A hypothetical model describing the bystander activation of Th_{mem} cells is depicted in Figure 1.15.

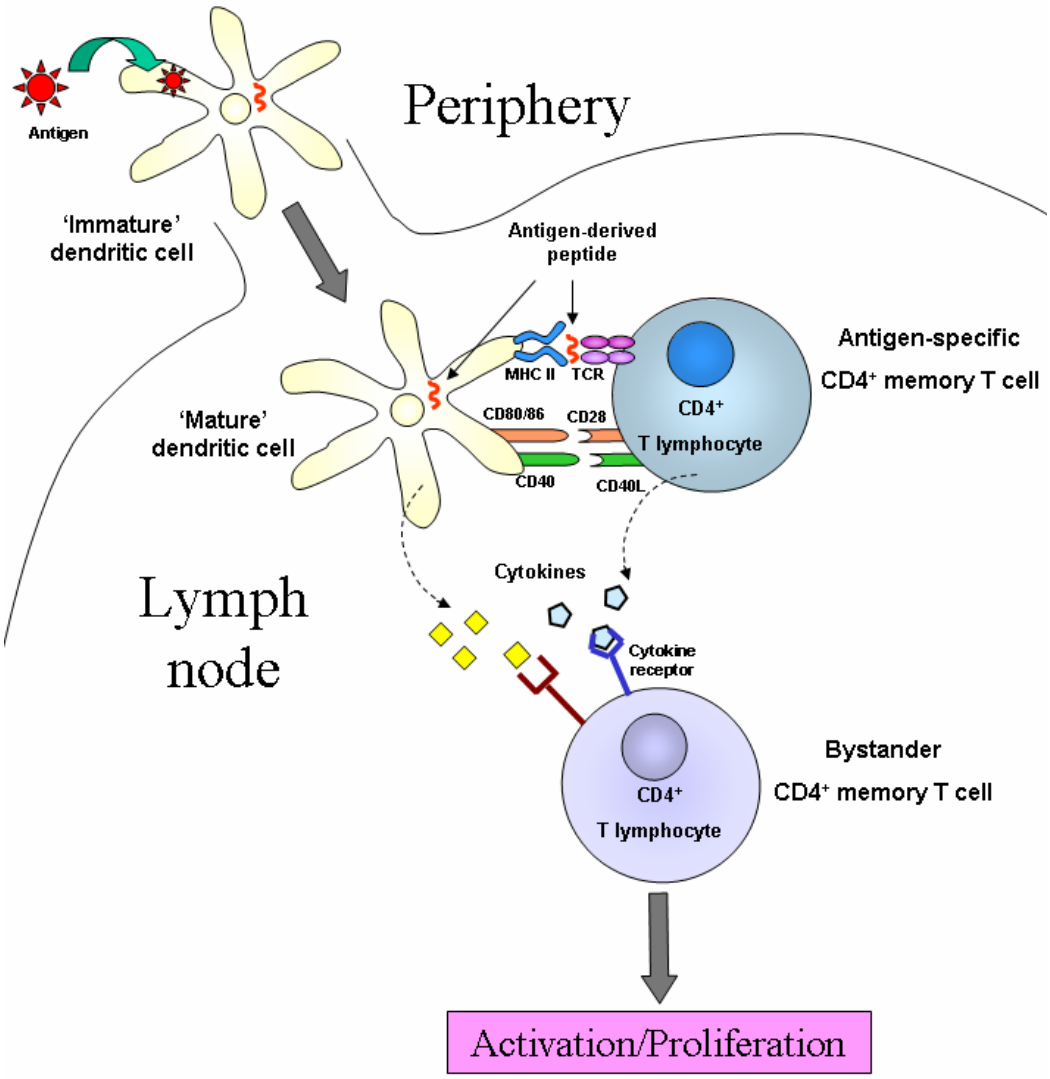


Figure 1.15. Bystander activation/proliferation of CD4$^+$ memory T cells: a hypothetical model
(see text for a description)

In vitro proliferative responses against TT and PPD were detected at various degrees in all subjects prior to vaccination with TT, indicating pre-existing immunity probably generated by the prophylactic TT- and BCG-vaccinations that all the individuals had received. Pre-existing immunity against *C. alb* would depend on natural exposure, and initial proliferative responses were more heterogeneous (Figure 1.7).

In terms of immune protection, the ability of the wider bystander population to secrete pro-inflammatory cytokines at the site of infection would enhance the effectiveness of the specific response. Clearly it is now necessary to dissect the CM and EM components of these Th_{mem} populations in human subjects (see Section 1.6.1).

With regard to the maintenance of Th_{mem} cells, a speculative hypothesis could be that periodic bystander expansions of Th_{mem} cells ultimately contribute to their long-term persistence. This point would be probably difficult to address in humans, and in this case a well defined mouse model could provide more definitive answers (see Chapter 2).

The ability of human $CD4^+$ T_{mem} cells to respond directly to cytokines has been suspected for some time from *in vitro* data [75]. However, the focus in mouse models has been mainly on $CD8^+$ T_{mem} cells. The latter are clearly susceptible to cytokine stimulation, with the main mediators being IL-15 and IL-7 [34,103]. In fact, "bystander" activation of human $CD8^+$ T cells has been suggested in a recent study of responses to two different latent herpes viruses where contemporaneous co-fluctuations of virus-specific $CD8^+$ T cells were observed without perturbation of the total number or phenotype of $CD3^+$ T cells [104]. This stability in the $CD3^+$ T-cell population seen during spontaneous activation of immunity against latent herpes viruses was also evident in our study of the effects of vaccination (Figure 1.13).

In contrast to $CD8^+$ T cells, it has been proposed that $CD4^+$ T_{mem} do not need cytokine signals for survival or expansion [105]. However, $CD4^+$ T_{mem} cells do express IL-7 receptors, and it has been reported that they are regulated by signaling via IL-7, with an overlapping influence of TCR signals [106]. Dependence on IL-7 for generation and survival of transgenic $CD4^+$ T_{mem} cells has also been observed [107], with evidence for involvement in the transition from effector to memory T cells [108].

A close parallel to our observations in human subjects was described recently in a mouse model where infection with *Leishmania donovani* led to expansion of adoptively transferred transgenic OVA-specific memory $CD4^+$ T cells [109]. In this case, OVA antigen was required, possibly to maintain the transferred cells, whereas in our case it is unlikely, but not impossible, that PPD or *C alb* antigens or cross-reactive antigens persist *in vivo*. While definitive data on generation and survival of T_{mem} cells can be obtained from transgenic and knock out mice, it is difficult to mimic the situation in human subjects who have a normal T_{mem} repertoire under continuous exposure to environmental antigens. Perhaps in this case, data gleaned from human subjects, although more difficult to manipulate, can be illuminating.

Our data on the behavior of human Th_{mem} tie together several previous observations on T-cell responses following vaccination with TT [110], including the fact that transient expansion of TT-specific T cells occurred during a flu-like illness [99]. It presents a problem for evaluation of specific memory responses to vaccines, since cells could be expanding simply as bystanders. It will be necessary next to determine if there are phenotypic or functional features which distinguish the T cell population responding to specific antigen from the bystander population, by analyzing isolated subpopulations. One particular question will be the balance between T_{EM} and T_{CM} in the two responses.

While the bystander Th_{mem} can secrete cytokines, and could potentially influence B cells, no increase in bystander antibody was evident (Figure 1.14). This would appear to argue against the proposal, again derived from subjects vaccinated with TT, that antigen-independent serological memory can be maintained by bystander activation of memory B cells [95]. Our data are more consistent with a study on the effects of a booster injection of diphtheria vaccine on diphtheria- and tetanus toxoid-specific B cell responses, which showed a rise in the antibody response only against the immunizing antigen[111]. However, it remains possible that activation of B cells is occurring, which could contribute to survival, but that no significant antibody results. Our data indicate that B cells are dependent on engagement of antigen by the B-cell receptor before they can receive the help apparently on offer from stimulated Th_{mem}. Without this control, the antibody response would perhaps be too easily induced, with possible autoimmune consequences.

1.6 Future studies in humans

1.6.1 Further phenotypic and functional characterization of vaccine-specific and vaccine-stimulated bystander CD4$^+$ memory T cells in human healthy volunteers following booster vaccination with tetanus toxoid

Our observation that CD4$^+$ memory T cells are susceptible to bystander activation during a recall immune response raises a crucial question about the nature of these cells and the role that they might play *in vivo*. Of particular interest are stage of differentiation, functionality and potential life-span of vaccine-stimulated bystander CD4$^+$ memory T cells as opposed to vaccine-specific CD4$^+$ memory T cells.

As discussed in the Introduction (see Sections 1.1.6, 1.1.7, 1.1.8) expression or lack of expression of a number of surface markers has been used to phenotypically define distinct populations of CD4$^+$ and CD8$^+$ T cells at different stages of differentiation [112]. Markers can also delineate distinct functional subtypes of memory T cells, such as T_{CM} and T_{EM}. For CD4$^+$ T cells, functionality is generally assessed by the ability to proliferate and/or to produce various cytokines. A correlation has been shown between proliferation capacity and pattern of cytokine secretion, such as IL-2 secretion. In viral infections, an emerging concept is that T cells able to simultaneously produce more that one cytokine (multifunctional or polyfunctional) can play a major role in vaccine-induced immune protection. This has been demonstrated for CD8$^+$ T cells in a study of vaccinia virus immunization against smallpox [113], and for CD4$^+$ T cells in a mouse model of *Leishmania major* infection [114]. There is also considerable interest in defining the contribution of T_{CM} and T_{EM} to vaccine-induced long term immune protection.

To gather information about the potential life span of antigen-specific T cells, two markers are often considered: the subunit α of the interleukin 7 receptor (IL-7Rα) and the anti-apoptotic molecule Bcl-2. Expression of IL-7Rα identifies cells able to respond to IL-7, important in sustaining long-term viability of memory T cells [80]. The usefulness of IL-7Rα as marker to identify among CD8$^+$ effector T cells precursors

that give rise to long-lived memory cells has already been mentioned (See Section 1.5.1 and [36]).

In a recent paper, Pantaleo and colleagues have addressed phenotype, function and potential life span of TT-specific $CD4^+$ T cells in healthy subjects who received booster TT vaccination [115]. They report that while during the resting state the majority of TT-specific $CD4^+$ T cells have the phenotype of T_{CM} ($CD45RA^- CCR7^+$), produce mainly IL-2 and express high levels of IL-7Rα and BCL-2, upon TT re-immunization, however, most of the TT-specific $CD4^+$ T cells acquire the phenotypic and functional characteristics of T_{EM}, down-regulating CCR7 and secreting IFN-γ. Expression of IL-7Rα and BCL-2 is also down-regulated, but interestingly a fraction of TT-specific $CD4^+$ T cells remain IL-7Rα^{high} and Bcl-2^{high} at the peak of the response to TT, and the authors speculate that these cells might be the precursors of TT-specific memory $CD4^+$ T cells present later [115].

In our study on TT vaccination we have partially addressed the point regarding functionality of vaccine-induced TT-specific and bystander $CD4^+$ memory T cells. In fact we showed that both could proliferate and produce Th1 and Th2-related cytokines upon *in vitro* re-stimulation with antigen . We used 3H-thymidine incorporation and ELISPOT assays to measure proliferation and cytokine production respectively. Although ELISPOT method allows analysis of cytokine production at single cell level, it does not allow analysis of production of multiple cytokines by the same cell; neither allows a phenotypic characterization of cytokine-producing cells. For these reasons, we are planning to use multiparameter flow cytometry to study the phenotype of TT-specific and vaccine-induced bystander $CD4^+$ memory T cells and to test their ability to produce multiple cytokines. Intracellular expression of the marker Ki67 will help identify within the population of antigen-specific $CD4^+$ T cells, those which were actually proliferating *in vivo*.

A panel of phenotypic markers will be used to characterize the stage of differentiation of antigen-specific $CD4^+$ T cells and their belonging to well characterized subtypes of $CD4^+$ memory T cells. Among them, expression of the co-stimulatory molecules CD27 and CD28, whose loss is associated with late-effector stages of differentiation,

will be analysed. CD45RA in combination with CCR7 will be used to define subpopulations of $CD4^+$ T_{CM} ($CD45RA^-CCR7^+$), T_{EM} ($CD45RA^-CCR7^-$), and terminally differentiated effectors ($CD45RA^+CCR7^-$, T_{ET}).

To gather information about the potential life span of vaccine-stimulated bystander $CD4^+$ memory T cells as opposed to vaccine-specific $CD4^+$ memory T cells, two markers will be studied: the subunit α of the interleukin 7 receptor (IL-7Rα) and the anti-apoptotic molecule Bcl-2.

As a consequence of bystander activation, it could be predicted that quantitative and/or qualitative changes in $CD4^+$ memory T cells may become visible at a population level during the TT-induced immune response. In other words, if $CD4^+$ memory T cells are all sensitive to bystander activation during TT recall immune response, we should be able to monitor expansion and activation in phenotypically defined population or subpopulations of $CD4^+$ memory T cells in the blood. Furthermore, if the phenotype of antigen-specific bystander $CD4^+$ memory T cells is that of T_{EM} cells as it is that of TT-specific $CD4^+$ memory T cells after TT immunization as described by Pantaleo and colleagues [115](we have to verify this in our setting, see above), it might be predicted that changes in the relative proportions of T_{CM} and T_{EM} within the $CD3^+CD4^+$ T lymphocyte population should also become visible.

An interesting hypothesis that we also would like to explore is that the recall TT vaccination might enhance the basal reactivity of $CD4^+$ memory T cells by lowering the activation threshold required for recall effector functions, such as cytokine production. This would offer an additional mechanistic explanation for the increased number of bystander cytokine-producing cells that we detected after TT recall vaccination. In fact the increased number could result from an *in vivo* expansion but also from a higher number of antigen-specific cells with lower activation threshold for cytokine production. It is known that $CD4^+$ memory T cell display heterogeneity in activation threshold for cytokine synthesis. Regulation of activation thresholds can be independent of TCR specificity, and signals such as those provided by co-stimulatory molecules reduce activation thresholds by lowering the TCR-mediated signals needed

to achieve triggering [116,117]. Recently, in a mouse model, the rapid recall effector function which characterizes memory as opposed to naïve $CD4^+$ T cells has been associated with an elevated protein expression of the proximal tyrosine kinase Zap 70 [118]. The authors propose a "signal threshold model, in which the level of Zap 70 controls effector responses in $CD4^+$ T cells, with increased Zap 70 expression leading to more efficient cellular responses. In our setting of TT recall vaccination, we are planning to investigate basal levels of ZAP 70, but also of other proteins and phosphorylated proteins known to play a role in intracellular signaling pathways leading to activation of human lymphocytes, in $CD4^+$ memory T cells and in T_{CM} and T_{EM} subpopulations. To our knowledge no study has yet investigated the effect of vaccination on the reactivity of $CD4^+$ memory T cells from this particular angle.

To address all these issues, a new cohort of eleven healthy human volunteers has already been recruited. They have received a booster vaccination with TT and their PBMNC and sera have been stored for future analysis.

1.6.2 Study of bystander activation/proliferation of $CD4^+$ memory T cells following primary hepatitis B immunization

An interesting question is whether bystander activation/proliferation of $CD4^+$ memory T cells can also occur during priming of immune responses. To try to answer this question we are planning to analyze vaccine-specific and bystander memory T cell responses in healthy human subjects who receive a primary hepatitis B (HepB) immunization course. In its standard schedule, this consists of three-doses of vaccine (recombinant HepB surface antigen). The first dose (day 0) is followed by a second dose after one month and a third dose after six months. Therefore the analysis of immune responses in individuals who are receiving the vaccine for the first time, offers the unique opportunity to look at bystander activation of T_{mem} cells (we are planning to analyze PPD- and TT-specific $CD4^+$ T cell responses) while the establishment of immune memory against the immunizing antigen is taking place. Hep B vaccine is also given to some individuals already immunized against Hep B. In this case one dose of vaccine is given to boost pre-existing immunity. These individuals would constitute a useful control group in our study.

In our previous study on healthy subjects who received TT booster vaccination, we did not observe bystander antibody production (Figure 1.14), in keeping with a previous study after diphtheria vaccination. These data appear to argue against a previously published set of data in subjects vaccinated with TT, where some evidence for antigen-independent maintenance of serological memory was seen [95]. Recently Odendahl et al. [92] and Gonzalez-Garcia et al.[93] reported that in normal volunteers following TT booster vaccination there is an increase of TT-specific plasma blasts in the blood, identified by intracellular staining and flow cytometry analysis using a fluorescently-labelled fragment C (FrC) of TT, which peak at day 6 after vaccination. Interestingly, they also showed a parallel increase in antibody-secreting plasma cells of unidentified (non-TT) specificity. They proposed that these may represent long-lived plasma cells mobilized from survival niches in bone marrow as a result of competition with the newly generated TT-specific plasma blasts. In both papers serum antibodies against TT or unrelated antigens were not studied. All together our study and these reports show that TT-recall vaccination induces a detectable increase in the number of blood plasma cells non TT-specific but does not result in increased levels of antibodies with unrelated specificities. So mobilization of resident plasma cells but not an increase in number seems to be a correct interpretation.

To further dissect the humoral immune response to vaccination and monitor a possible bystander effect in the plasma cell compartment in the setting of hepatitis B vaccination, we can take advantage of the fact that most of the recruited healthy individuals would be immune to TT. We can therefore monitor the frequency of TT-specific blood plasma cells, using the techniques described by Odendahl and Gonzalez-Garcia , during the primary and the booster HB vaccination, and relate it to the levels of TT-specific antibodies in the serum and to vaccine-specific humoral responses.

Ethical approval for this study has been obtained (REC Ref: 06/Q1702/155) and ten healthy volunteers have already been recruited. We have started the analysis of the immune responses with some encouraging preliminary results.

2 Chapter 2. Bystander activation of $CD4^+$ memory T cells: further studies using the transgenic OT-II mouse model

2.1 Introduction

In the study carried out on human healthy volunteers and described in Chapter 1, we have evaluated the impact of TT booster vaccination on the functional behaviour of populations of human Th_{mem} cells either vaccine-specific or with unrelated antigen specificities. The data are indicative of an *in vivo* bystander expansion of Th_{mem} cells specific for unrelated antigens, which occurs possibly as a result of the cytokine-milieu induced in the lymphoid organs by the ongoing vaccine-specific recall immune response.

The techniques used to analyse the kinetics of vaccine-specific and bystander Th_{mem} responses, that is the proliferative assay and the ELISPOT assay for cytokine production, are both functional assays which require *in vitro* restimulation of T cells with antigen to reveal the presence of antigen-specific T cells. The frequency of antigen-specific cells as measured by ELISPOT is generally accepted to closely mirror the frequency of those cells in the blood. Although this seems to be the case in our study, since we have excluded antigen-induced divisions of cells during the short *in vitro* stimulation period used for the assay, a formal proof of divisions occurring *in vivo* in cells with antigen specificities unrelated to that of the vaccine antigen, is still missing. Also the mechanisms responsible for this phenomenon still need to be elucidated. Clarifying these issues would require sources of material and manipulations which are clearly difficult to obtain in humans.

For this reasons we have decided to develop a mouse model which should help us analyze in a more systematic and quantitative way the bystander activation and/or proliferation of Th_{mem} cells which follows protein booster vaccination. This is achievable by increasing the size of the bystander cell population and its traceability.

An obvious choice is that of using $CD4^+$ T-cell receptor (TCR) transgenic T cells. These are isolated from (transgenic) mice in which the rearranged α and β chain genes of a single TCR of known specificity are expressed as a transgene, so that the majority of mature T cells in the mice will express that TCR. TCR transgenic T cells can be activated *in vitro* or *in vivo* with a single peptide antigen and they can be identified by antibodies specific for the transgenic TCR.

$CD4^+$ TCR transgenic T cells have been widely used to study generation of CD4 T cell memory. A well established protocol involves activation of TCR transgenic T cells *in vitro* and subsequent transfer of these cells into syngeneic, non-transgenic recipients [35]. Upon *in vitro* activation, TCR transgenic T cells acquire the ability of surviving for long periods when transferred *in vivo*, in the absence of re-exposure to cognate antigen and without requiring interactions with MHC class II molecules [68]. After adoptive transfer, cells also acquire the phenotype (small resting cells , $CD25^{low}$, $CD44^{high}$, IL-7Rα^+) and functional characteristics of 'bona fide' $CD4^+$ memory T cells [6]. In fact, when compared to naïve cells, $CD4^+$ memory T cell responses to recall antigens are characterized by faster kinetics of cytokine secretion, cell division, and proliferation which occur at lower doses of antigen and are less dependent on co-stimulation [50,51]. Furthermore, Löhning at al. have recently shown that long-lived functional virus-reactive memory T cells can be generated from purified cytokine-secreting Th1 and Th2 effectors primed *in vitro* or *in vivo* with similar results [119], providing further evidence for the existence, within the population of $CD4^+$ effector T cells (including *in vitro*-derived effectors), of precursors destined to become memory T cells. Interestingly, up-regulation of IL-7Rα, the expression of which is instrumental for the survival and homeostatic proliferation of $CD4^+$ memory T cells [19,42], occurs very rapidly after adoptive transfer (by day 5) and it correlates positively with the number of divisions that the cells had undergone [119].

For our purposes, we have chosen a well characterized model of $CD4^+$ transgenic T cells (OT-II) specific for a T helper epitope of ovalbumin (peptide 323-339, OVAp). Our initial experimental approach has been to compare the susceptibility to bystander proliferation of naïve OT-II to that of antigen-activated OT-II cells. We have labeled naïve and antigen-activated OT-II cells with CFSE and then transferred them into

wild type C57/B6 mice in which a population of TT-specific $CD4^+$ memory T cells had already been established. Recipient mice were then challenged with TT and during the strong recall response against TT, the behavior of the transgenic population of OVA-specific memory T cells was monitored (see Figure 2.1). Control experiments included the adoptive transfer of naïve and activated OT-II cells into naïve recipient mice, as described in more details in Methods. Our results show that antigen-activated but not naïve OT-II cells are prone to further activation and bystander proliferation when exposed to the particular microenvironment created *in vivo* by an ongoing recall immune response directed against an antigen which is unrelated to the one they are specific to. The extent of this OT-II bystander activation and proliferation appears proportional to the strength of the secondary immune response directed against the unrelated antigen.

2.2 **Methods**

2.2.1 **Antigens**

TT, not adsorbed (National Institute for Biological Standards and Control, NIBSC, UK). Albumin, chicken egg (ovalbumin, OVA) was purchased by several companies and levels of endotoxin were tested by a Limulus Amebocyte Lysate Endochrome-based assay (Endosafe, Charles River). Ovalbumins from SIGMA (Cat N A 5378-10G) and from Fluka BioChemika (Cat N 05438) contained very high levels of endotoxin and were therefore not used. Ovalbumin from Calbiochem (Cat N 32467) contained lower levels of endotoxin which were further reduced to < 0.1 IU/ML by two passages through a polimixin B column (Detoxi-Gel, Endotoxin Removing Gel, Pierce Cat 20344) according to the manufacturer's recommended protocol and subsequently used for *in vivo* and *in vitro* studies. Mouse albumin (MoAlb) also from Calbiochem (Cat N 126674), at the concentrations used for *in vitro* or *in vivo* assays contained ≤ 0.1 IU/ML of endotoxin, and was not further purified. The peptides: p30, a universal T-helper epitope from tetanus toxin [120](tt 947-967, FNNFTVSFWLRVPKVSASHLE) and OVAp, a T helper epitope from ovalbumin (OVA 323-339, ISQAVHAAHAEINEAGR) were synthesized commercially and supplied at >95% purity by Peptide Protein Research, Southampton, U.K.

2.2.2 **Mice**

C57/BL6 mice were aged between 6 and 10 weeks at the start of all experiments. OT-II transgenic mice were up to 12 weeks old. Mice were kept in pathogen free conditions and in accordance with Home Office Guidelines. Experiments were conducted under Project Licence number PPL 70/6401 and Personal Licence PIL 30/5860.

2.2.3 **Generation of TT-specific T cell immunity in CD57/BL6 mice.**

To generate TT-specific T cell immunity, C57BL/6 mice were primed sub-cutaneously (s.c.) in the flank, at day 0 with 50μg of TT in Complete Freund's Adjuvant (CFA). Four weeks later they were boosted with the same amount of protein without adjuvant in the same region where the primary injection was delivered. IL-2 and IFN-γ ELISPOT assays was used to check efficacy of priming and boosting at week 2 and 6, respectively. After boosting, mice were left for a period of 8 weeks. This period of time is believed to be long enough for T cell memory to be established (Prof. Eric Bell, University of Manchester, personal communication). At the end of this period, levels of TT-specific T cell responses were checked again and used as baseline reference for the final experiment.

2.2.4 **IL-2 and IFN-γ ELISPOTs**

For IL-2 and IFN-γ ELISPOTs, kits purchased from BD biosciences (IL-2 and IFN-γ ELISPOT sets) were used, following the manufacturer's recommended protocols. Spleens were isolated from C57/BL6 mice at weeks 2, 4 and 12 after priming with TT and then 1 week after receiving adoptively transferred OT-II cells (Figure 2.1), and also from two un-primed naïve mice. Single cell suspensions were obtained by passing spleens through a 70μm cell strainer (BD Falcon^{TM}). Splenocytes were subjected to a density gradient centrifugation (Lymphoprep) followed by one wash in RPMI 1640 medium (5 minutes at 1600 RPM) before re-suspending them in complete medium (RPMI 1640, 1mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids (1X of 100X stock), 50 μM 2-mercaptoethanol, 100 U/mL penicillin, 100

μg/mL streptomycin (all from Invitrogen) with 10% heat-inactivated foetal calf-serum, FCS). 2.5 x 10^5 cells were added to each well of a 96 well ELISPOT plate and cultured for 40hours at 37°C 5% CO_2, in triplicates, in the absence (Control) or in the presence of TT (20 and 2 μg/ml), MoAlb (100μg/ml), OVA (100μg/ml) and OVAp (1μM). Antigen-specific responses were calculated by subtracting the average number of spots in Control wells to the number of spots in antigen-stimulated cultures. The average number of spots in Control wells was always ≤ 10.

2.2.5 *In vitro* generation of antigen-activated OT-II T cells

Spleens and lymph nodes (cervical, axillary, inguinal and mesenteric) were isolated from naïve OT-II mice and processed as described above (see IL-2 and IFN-γ ELISPOTs), with the difference that cell suspensions from lymph nodes were washed once in RPMI (5' at 1600 RPM) without the need for density gradient centrifugation. Both cell suspensions were then counted and the number of viable cells was determined by Trypan blue exclusion. The percentage of viable cells always exceeded 95% among splenocytes and 80% among lymph node cells. Viable cells were pooled and incubated in complete medium in the presence of 1μM or, in a repeated experiment, 0.1μM OVAp. Cells were distributed into 6-well tissue culture plates at 1 x 10^6 cells/ml , 4-5 ml/well. At day 4, cultures were split 1/2 and 2ml of fresh complete medium was added to each well. All this was done very gently in order not to disrupt the cell clusters present in high numbers. At day 11 cells were harvested and separated by Lymphoprep to remove dead cells and debris. Cells were then washed, resuspended in RPMI, counted and labeled with CFSE as described below. The phenotype of OT-II cells was checked at day 0 (*ex-vivo*), day 4 and at day 11 just before adoptive transfer (see below 2.2.8 **Flow cytometry analysis**).

2.2.6 CFSE labeling of OT-II cells

Naïve OT II cells or OT-II cells cultured *in vitro* with OVAp were washed, resuspended in RPMI and counted. The concentration was adjusted to 20 x 10^6 cells/ml. Carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Invitrogen) was added to cell suspensions at a final concentration of 2.5μM, and tubes

was transferred into a water bath at 37 ^{o}C. They were mixed occasionally and after 10 minutes, ice cold FCS was added (1/2 of the initial volume) and tubes were left on ice for 5 minutes to quench the reaction. Two washes followed, one with RPMI and the second with PBS. After the final wash the number of viable cells was determined by Trypan blue exclusion. A small aliquot was used to verify CFSE staining by flow cytometry analysis and the remaining cells were used for adoptive transfer experiments.

2.2.7 **Adoptive transfers of CFSE-labeled OT-II cells**

Naïve or antigen-activated OT-II cells were CFSE-labeled and adoptively transferred intravenously (i.v.) into the tail vein (1-2 x 10^6 cells in a final volume of 200μL of sterile PBS) into either naïve recipient mice or into mice that had been primed and boosted with TT. The following day (Day 0) recipient mice were split into three groups and received s.c. injections in the flank of either saline, TT or the control protein mouse albumin. This control was used to exclude that the simple injection of a protein (in this case an autologous protein) could itself induce activation of the OT-II cells. A week later, mice were sacrificed. Spleens were isolated and processed (see above) and splenocytes were used to study division history and phenotype of adoptively transferred OT-II cells by flow cytometry (see below **Flow Cytometry Analysis**) and to quantify endogenous T cell responses against TT by IL-2 and by IFN-γ ELISPOTs. This experimental design is summarized in Figure 2.1. A total of four different types of adoptive transfer experiments were done as summarized in the Table 2.1 below.

Adoptively-transferred cells	**C57/BL6 Recipients**
Naïve OT-II	Naïve
Naïve OT-II	TT-immune
Antigen-activated OT-II	Naïve
Antigen-activated OT-II	TT-immune

Table 2.1. Summary of adoptive transfer experiments.

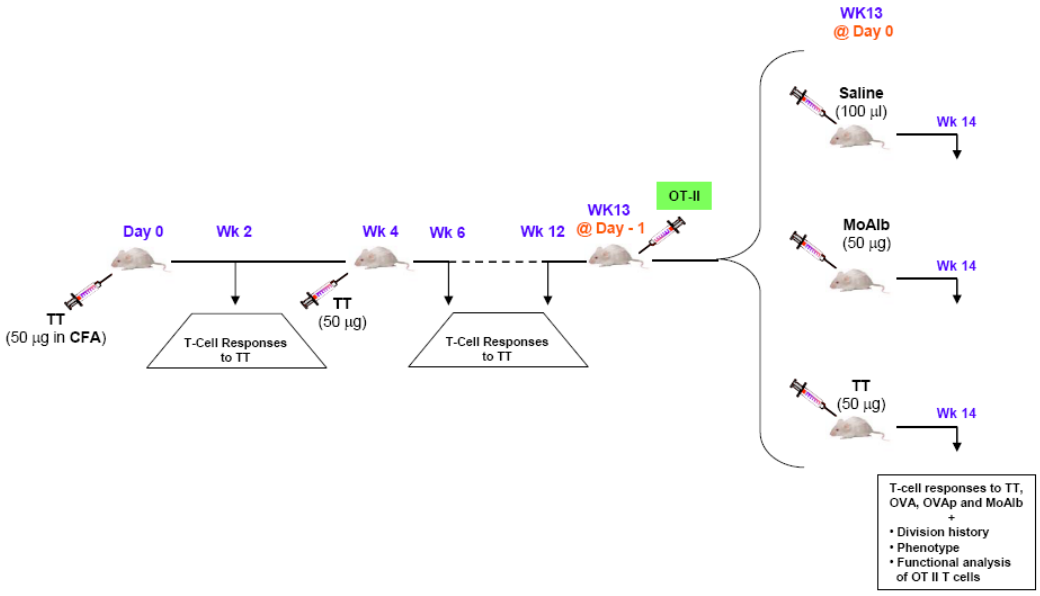


Figure 2.1. A transgenic mouse model to study the bystander activation of CD4$^+$ T cells (see text above for a detailed description)

2.2.8 **Flow cytometry analysis**

Phenotypic characterization of OT-II cells was carried out on naïve cells, on cells cultured *in-vitro* with OVAp at day 4 and at day 11 (just before adoptive transfer) and, finally, on cells rescued from adoptively transferred recipient mice. In the latter case phenotypic analysis was combined with analysis of the cell division history by CFSE. Cells were washed in PBS, resuspended in 50-100µl of PBS and stained on ice for 10 minutes with the appropriate fluorochorome-labeled antibodies as describe below. They were then washed once in PBS, fixed with PBS/1% formaldehyde and analysed on a FACSCalibur flow cytometry using CELLQUEST software (both from BD Biosciences). OT-II cells were identified as being CD4$^+$ and by the unique combination of a T cell receptor (TCR)-V alpha (α) and TCR V beta (β) chain. In fact OT-II cells are TCR Vα2$^+$ and V β5.1, 5.2$^+$. An example of the gating strategy

applied to cells derived from the spleen and lymph nodes of a naïve OT-II transgenic mouse is shown below in Figure 2.2.

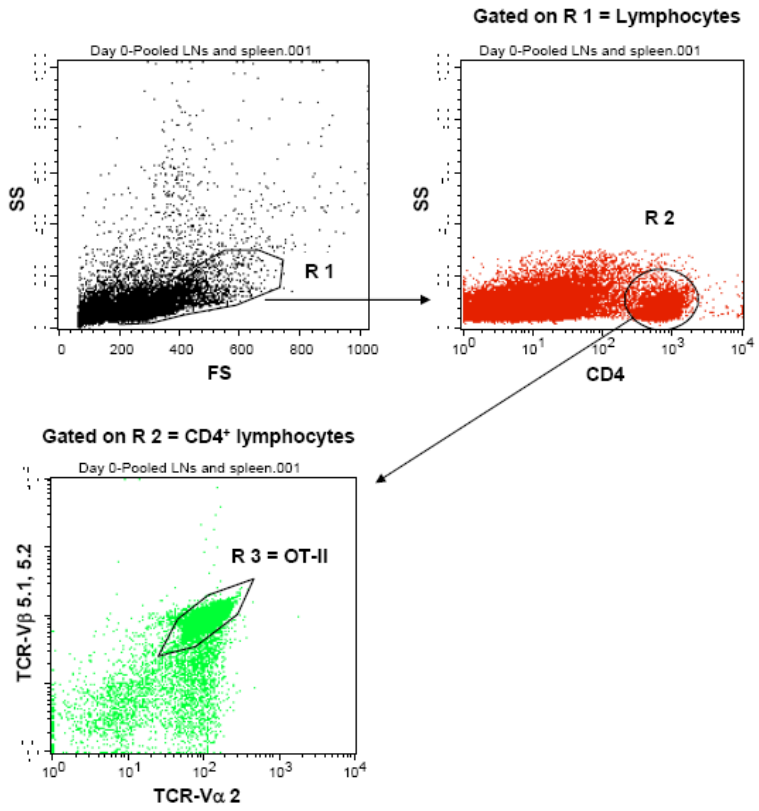


Figure 2.2. Gating strategy for the identification of OT-II cells. OT-II cells from splenocyte and lymph node cell suspensions were identified as $CD4^+$ lymphocytes co-expressing TCR-Vβ 5.1, 5.2 and TCR-Vα2 chains.

OT-II cells identified in this way were further characterized for surface expression of a panel of phenotypic markers which included early and late activation markers and lineage markers. Among them: CD25 (PC61) and CD122 (TM-β1) (the subunits α and β respectively of the IL-2 receptor), CD127 (SB-199) (the subunit α of the IL-7 receptor), CD44 (IM7), CD45RB (16A), CD62L (L selectin) (MEL-14) and CD69 (H1.2F3). CD4 (RM4-5) was conjugated to PerCP, while TCR-Vβ 5.1, 5.2 (MR9-4) and TCR Vα 2 (B20.1) were FITC and APC respectively. All the other phenotypic markers were PE. All fluorochrome-conjugated antibodies were purchased from BD Biosciences, except for CD62L, CD122 and TCR Vα 2, which were purchased from

E-Bioscience. OT-II cells rescued from adoptively transferred recipient mice were identified among the $CD4^+$ lymphocytes, as $CFSE^+$ and TCR-$V\alpha2^+$ cells as shown in the representative example depicted below (Figure 2.3.A). The proportion of cells which had been through one or more divisions was then analysed in details (Figure 2.3.B) using appropriate gates (M1-M4 in this example) and their phenotype was studied using the same combination of markers described above.

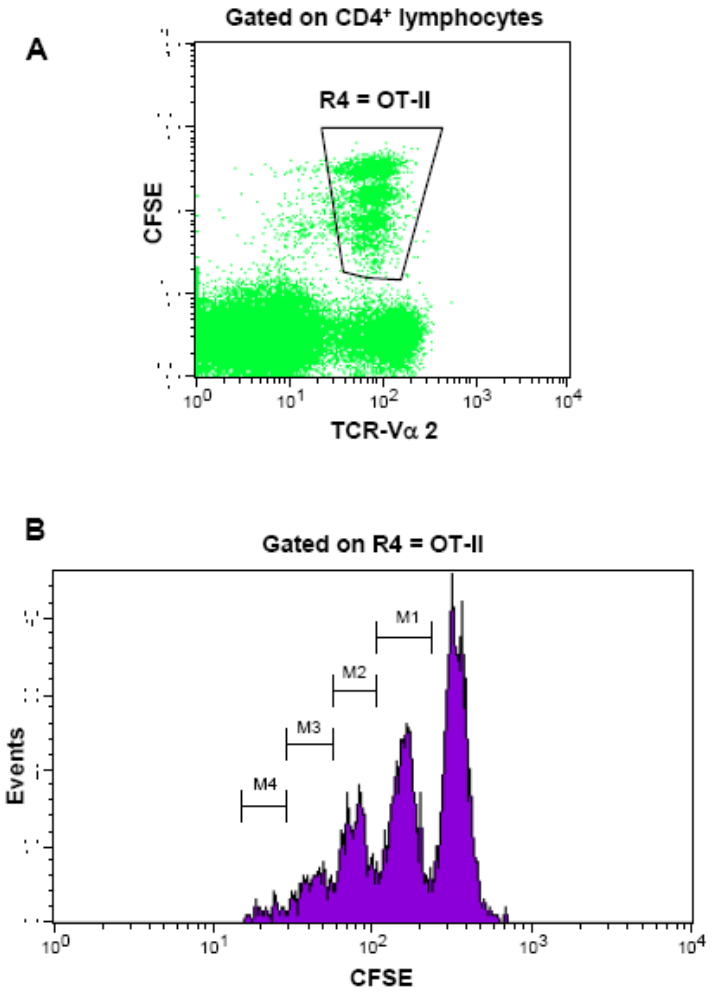


Figure 2.3. Gating strategy for the identification of OT-II cells rescued from adoptively-transferred recipient mice. OT-II cells from splenocytes of adoptively transferred recipient mice were identified as $CD4^+$ $CFSE^+$ TCR-$V\alpha2^+$ lymphocytes. In order to get enough cells for an accurate analysis, between 1 and 2 x 10^6 events were collected in the live-lymphocytes gate

2.3 **Results**

Susceptibility of $CD4^+$ T cells to bystander activation and proliferation was studied by adoptively transferring CFSE-labeled $CD4^+$ transgenic T cells (OT-II) specific for OVAp into recipient mice which had been previously primed and boosted with TT, and by analyzing their division history and phenotype after inducing a recall immune response against TT (see Figure 2.1). In the next two paragraphs the induction of TT-specific T cell immunity in recipient mice and the generation and phenotypic/functional characterization of activated OT-II cells, are described. Finally the results from adoptive transfer experiments will be described. From these results it emerges that antigen-activated but not naïve OT-II cells are susceptible to bystander proliferation which occurs during a secondary immune response directed against the unrelated antigen TT.

2.3.1 **Generation of a TT-specific T cell memory in mice to be used as recipients of adoptively transferred OT-II cells**

In order to generate TT-specific immunity in mice which were to be used as recipients of OT-II cells in the adoptive transfer experiments, a prime and boost approach was used. C56/BL6 mice were primed in the flank with 50μg of TT in CFA and boosted four weeks later in the same region with 50μg TT without adjuvant. Levels of TT-specific T cell immunity were assessed by IL-2 and IFN-γ ELISPOTs two weeks after priming and then 2 and 8 weeks after boosting and the results are shown in Figure 2.4. Splenocytes were restimulated *in vitro* for 40h with either optimal or suboptimal concentrations of TT (20 and 2 μg/ml) or with p30 (a universal T-helper epitope from tetanus toxin). The specificity of cellular immune response was verified by restimulating splenocytes with OVA (an antigen which mice had not been primed with) and OVAp (a T helper epitope from OVA, which is also the cognate antigen for OT-II cells).

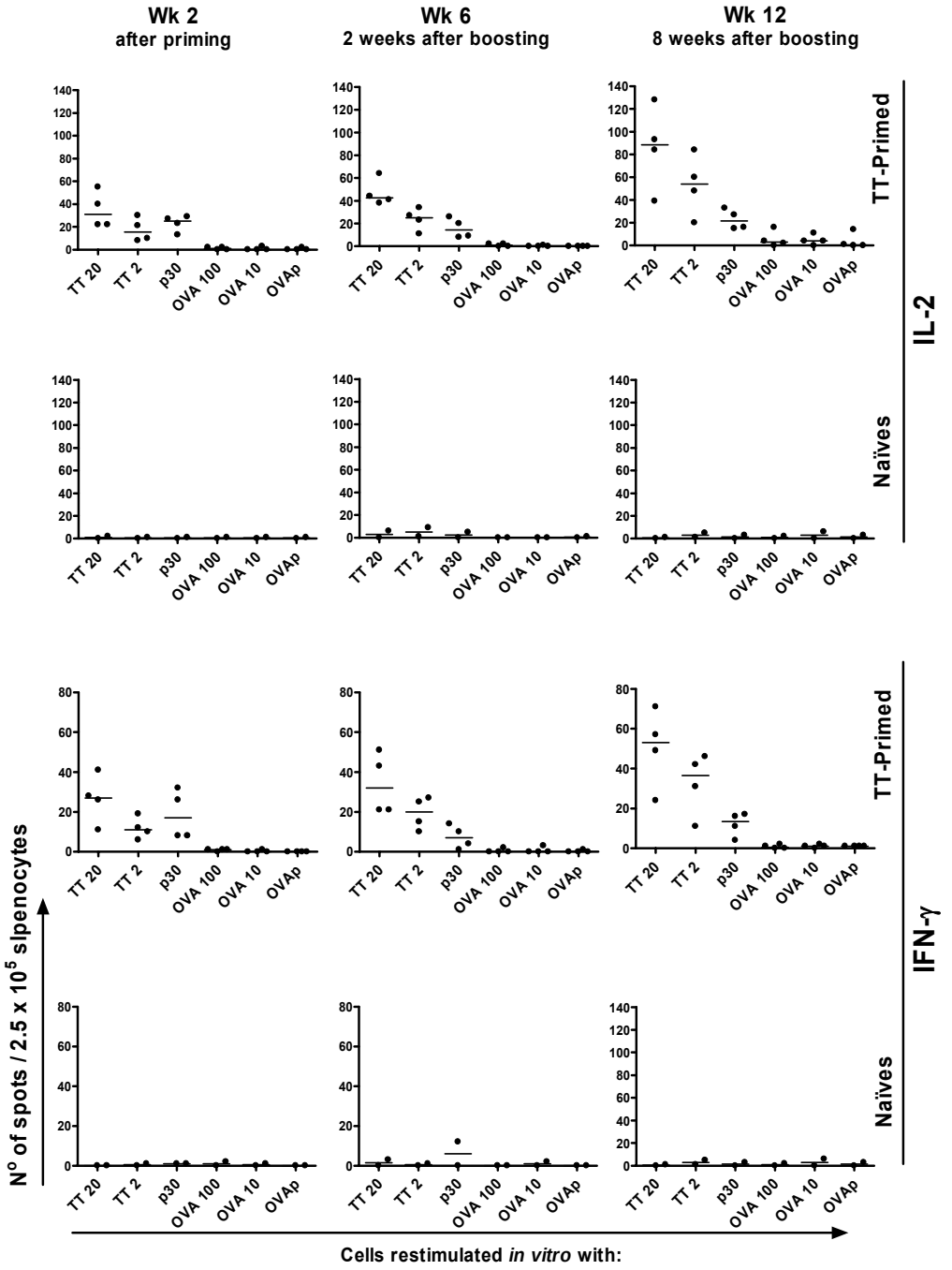


Figure 2.4. Generation of TT-specific immunity in C57/BL6 mice. In order to induce TT-specific immunity, mice were primed subcutaneously with 50μg of TT in complete Freund's adjuvant (CFA) and boosted 4 weeks later with 50μg of TT without adjuvant. T cell responses were checked 2 weeks after priming and 2 and 8 weeks after boosting by IL-2 and IFN-γ ELISPOTS following the protocol described in Methods (Section 2.2.4). Splenocytes from TT-primed or unprimed naïve control mice (2.5×10^5 / well, in triplicates) were cultured for 40h in the absence (Control) or in the presence of TT (20 and 2 μg/ml), p30 (1μM), OVA (100 and 10 μg/ml) or OVAp (1μM). Antigen-specific responses were calculated by subtracting the average number of spots in Control wells to the number of spots in antigen-stimulated cultures. The average number of spots in Control wells was always ≤ 10. Cytokine responses are reported as the mean number of spots in individual mice and the median value of group of mice is indicated by the line across.

The prime and boost protocol induced highly specific IL-2 and IFN-γ responses, which were still detectable at high levels 8 weeks after the TT boost, suggesting effective induction of TT-specific T cell memory. No cytokine production was detected in non-primed naïve mice at any time point (Figure 2.4).

2.3.2 *In vitro* activation of naïve OT-II cells with cognate peptide: phenotypic and functional characterization.

To generate activated OT-II cells, splenocytes and lymph node cells were isolated from naïve OT-II mice. Typically between 80 and 90% of the $CD4^+$ T cells were identified as OT-II (TCR $V\alpha2^+$ and $V\beta5.1, 5.2^+$). OT-II cells were cultured *in vitro* for 11 days with cognate peptide (OVAp). Flow cytometric analysis was used before stimulation and then at day 4 and 11, to study their activation and differentiation status. Results of this analysis are shown below in Figure 2.5. *In vitro* stimulation of OT-II cells with OVAp (1μM) resulted in strong cell activation which peaked at day 4 and was maintained, although to a lower degree, until day 11. In Figure 2.5.A forward and side scatter characteristics are shown and a region has been placed on viable lymphocytes and their percentage is shown. Cells went through an initial phase of expansion, followed by a contraction phase characterized by extensive cell death (Figure 2.5.A). At day 4 a large proportion of cells displayed forward and side scatter properties (large size and complexity) typical of fully activated and replicating blasts, and good viability (78%). At day 11, cultured cells displayed reduced size and their viability was around 35% in this experiment (Figure 2.5.A). This trend towards a reduced viability with longer culture times is one of the reasons for choosing activated OT-II cells at this stage of *in vitro* culture for subsequent adoptive transfer experiments (see paragraph 2.3.4).

Figure 2.5.B shows a detailed analysis, focused now on OT-II cells, of side scatter characteristics and levels of surface expression of a panel of markers, which includes activation and differentiation markers, and cytokine receptors. Among them, we analysed expression of CD69 and CD44. CD69 is an early activation marker. CD44 is a cell adhesion receptor whose principal ligand, hyaluronate, is a common component of extracellular matrices. In the mouse, recently activated and memory T cells express

higher levels of CD44 than do naïve T cells [6]. We also analysed CD45RB. CD45 is a member of the protein tyrosine phosphatase (PTP) family which comprises signaling molecules that regulate a variety of cellular processes including cell growth and differentiation [121]. Various isoforms of CD45 exist. In the mouse, the CD45RB isoform has been used by some investigators to distinguish naïve T cells ($CD45RB^{high}$) and memory T cells ($CD45RB^{low}$) [122]. Another molecule which was included in the panel of phenotypic markers is CD62L or L-selectin, a molecule which mediates the binding of T cells to high endothelial venules and therefore serves as a homing receptor to lymph nodes, for naïve and some subtypes on memory T cells[6]. Among the cytokine receptors, expression of CD25, CD122 and CD127 was also studied. CD25 is the subunit α of IL-2 receptor (IL-2Rα) and CD122 is the subunit β, which is common to IL-2 and IL-15 receptors (IL-2/IL-15Rβ). IL-2Rα and IL-2/IL-15Rβ together with the common γ chain (γ_c), form a high affinity receptor for IL-2 [79]. IL-2/IL-15Rβ with a unique IL-15Rα and the γ_c form a high affinity receptor for IL-15 [79]. Finally CD127 is the specific receptor α chain for IL-7 (IL-7Rα), which together with the γ_c forms the heterodimeric receptor for IL-7 [79].

The phenotype of OT-II cells at day 0 was typical of resting naïve T cells; in fact they were $CD69^{-}$ $CD25^{-/low}$ $CD44^{low}$ $CD45RB^{high}$ $CD62L^{high}$. After 4 days *in vitro* culture in the presence of OVAp, the majority of OT-II cells expressed CD69 and, as expected, higher levels of CD44 as compared to day 0 naïve cells. CD62L was down-regulated. Both CD25 and CD122, not expressed on naïve OT-II cells, were strongly up-regulated. These phenotypic changes from a $CD25^{lo}$ $CD44^{lo}$ $CD62L^{hi}$ to a $CD25^{hi}$ $CD44^{hi}$ $CD62L^{lo}$ phenotype marked the transition from a typical naïve phenotype to an effector-like phenotype [6] (Figure 2.5.B).

At day 11, the percentages of $CD69^{+}$, $CD25^{+}$ and $CD122^{+}$ OT-II cells were reduced but still a vast proportion of cells expressed these markers, and CD44 was expressed at a level which was intermediate between naïve and fully activated day 4 blasts (Figure 2.5.B). Naïve OT-II cells did not express IL-7Rα (CD127). This is difficult to explain, given the major role played by IL-7 as survival factor for $CD4^{+}$ naïve T cells [79] and given that expression of CD127 by other $CD4^{+}$ TCR transgenic naïve T cells with different [48] or same antigen specificity as the OT-II cells [43] has been reported.

Interestingly CD127 appeared to be up-regulated during *in vitro* culture, and at day 11 a minor proportion of OT-II cells expressed CD127 at relatively low level (Figure 2.5.B). Overall these results demonstrate that naïve OT-II cells cultured *in vitro* with cognate peptide undergo extensive cell activation which is still maintained when at day 11 they are adoptively transferred into recipient mice.

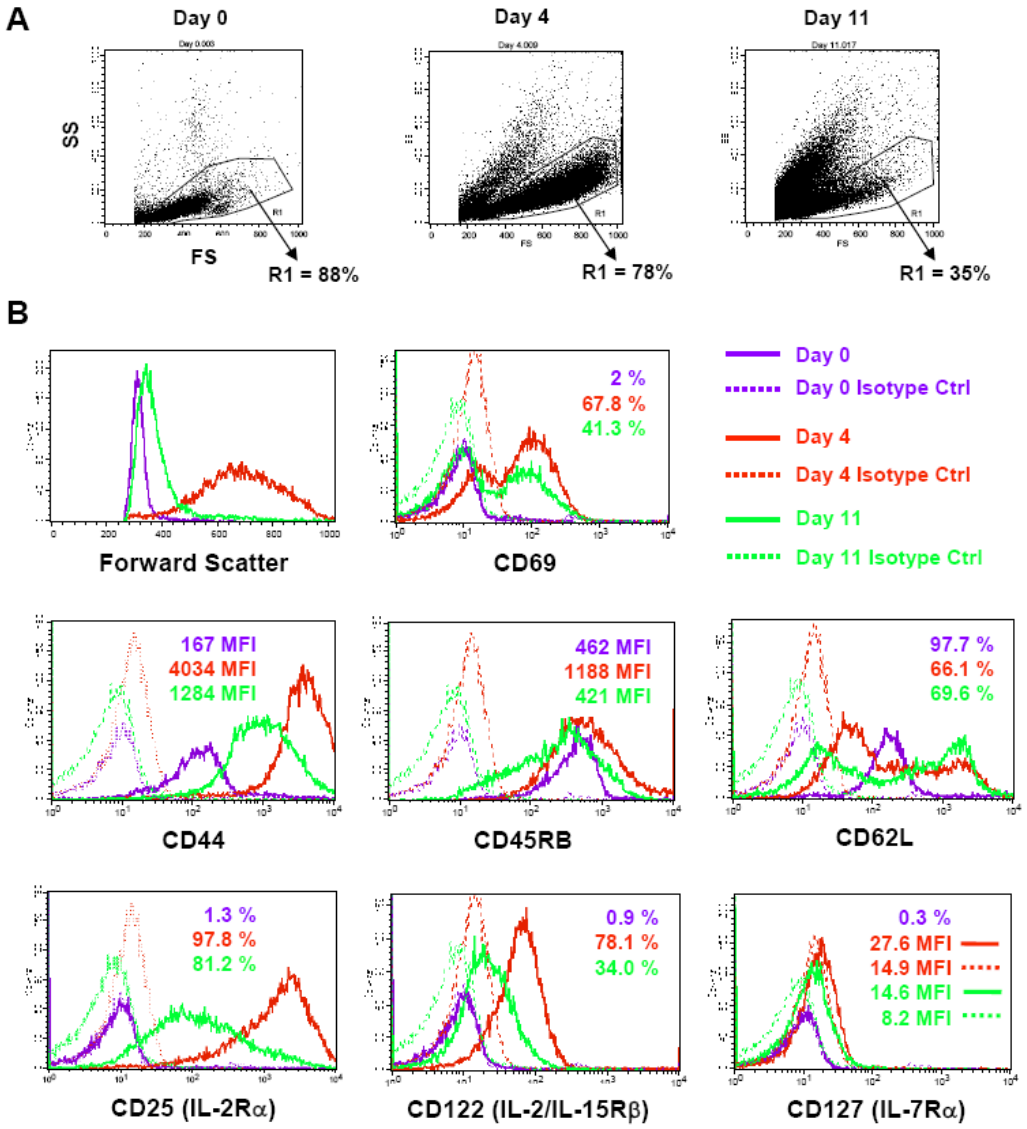


Figure 2.5. Phenotypic characterization of *in vitro*-activated OT-II cells. Splenocytes and lymph node cells from naïve OT-II transgenic mice were cultured for 11 days *in vitro* in the presence of 1μM OVAp. Freshly isolated cells and cells harvested at day 4 and 11 from *in vitro* cultures, were stained with fluorescent-antibodies specific for a panel of phenotypic markers or with their correspondent isotype control and analysed on a flow cytometer (see Methods, Section 2.2.8). **(A).** Forward (FS) and side scatter (SC) dot plot illustrating the size (FS) and complexity (SS) of cells. The gate (R1) has been drown around live lymphocytes and lymphoblasts and the percentage of gated cells is indicated. **(B).** Size and phenotype of OT-II cells gated as described in Figure 2.2. In each histogram, the percentages of positive cells or, where it is more indicated, the mean fluorescence intensity (MFI) are shown for day 0 (purple), day 4 (red) and day 11 (green) OT-II cells. Similar results were obtained in a separate experiment.

2.3.3 **Unresponsiveness of OT-II cells activated *in vitro* with OVAp to *in vivo* priming with cognate antigen (OVA)**

OT-II cells which had been activated *in vitro* with OVAp were further characterized and compared to naïve OT-II cells for their ability to respond to cognate antigen *in vivo* after they were adoptively transferred into naïve recipient mice. Naïve or antigen-activated day 11 OT-II cells were CFSE-stained and adoptively transferred into C57/Bl6 naïve recipient mice. The following day mice were challenged s.c. with either 50μg of OVA or with saline, and a week after, splenocytes were isolated and the division history of adoptively transferred OT-II cells was studied by flow cytometric analysis as described in Methods. Naïve OT-II cells, as expected, responded strongly to the challenge with OVA by proliferating extensively as documented by reduced CFSE staining (Figure 2.6). This suggested that *in vivo* OVA antigen was processed and OVAp could be successfully presented to peptide–specific OT-II cells. On the contrary, OT-II cells previously activated *in vitro* with OVAp, failed to respond when recipient mice were challenged with OVA. Only a small proportion of them had divided at the time of analysis, but similar levels of divisions were found also in control mice challenged with saline and they were probably due to residual activation and divisions already occurring at time of adoptive transfer.

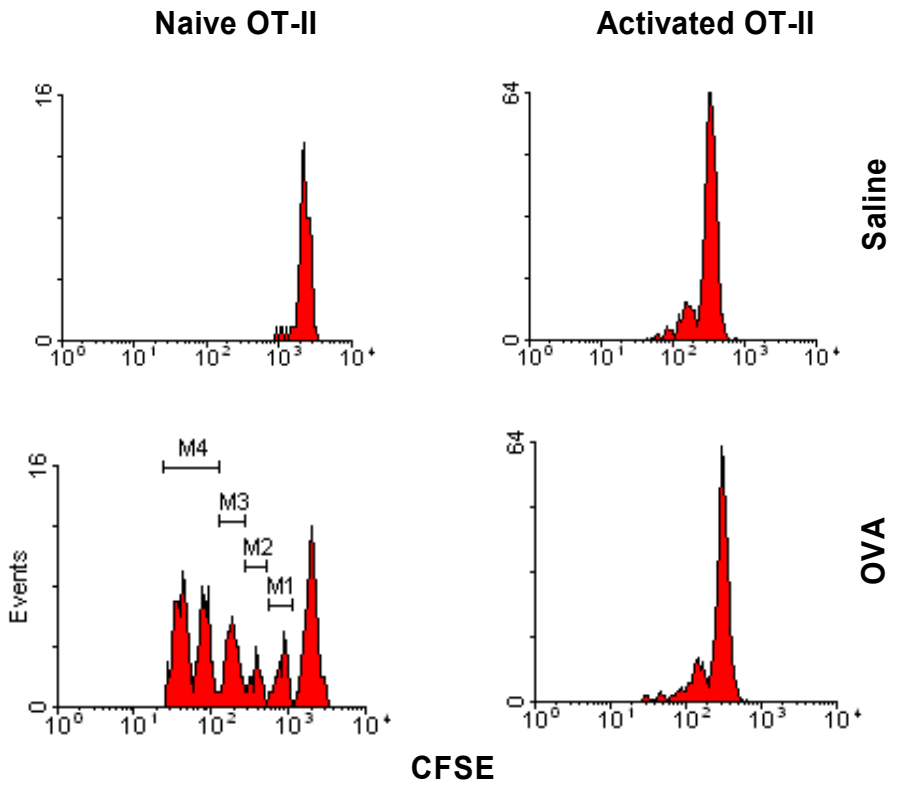


Figure 2.6. Unresponsiveness of *in vitro*-activated OT-II cells to *in vivo* priming with cognate antigen. Naive or *in vitro*-activated OT-II cells were labeled with CFSE and adoptively transferred into naïve C57/B6 wild type recipients. Recipient mice were then challenged with 50µg OVA or with control saline, and a week later the cell division history of OT-II cells was analyzed by flow cytometry using the gating strategy described in Figure 2.3.

2.3.4 Bystander proliferation of antigen-activated OT-II during a secondary immune response to TT.

After 11 days of in vitro culture with cognate peptide, the susceptibility of OT-II cells to bystander activation and proliferation was tested and compared to that of naïve OT-II cells. The choice of this particular time point (day 11) was dictated by two factors. Firstly, to give antigen-activated OT-II cells enough time to start resting and to initiate a program of differentiation predicted to lead towards a memory-like phenotype once the cells are transferred into recipient mice. Secondly, to enable us rescue enough cells from *in vitro* cultures as viability decreases dramatically over time.

Naïve or antigen-activated OT-II cells were adoptively transferred either into naïve recipient mice or into mice immune to TT, and the effect of a primary or a booster injection of TT on OT-II cells was investigated. Recipient mice were also challenged with an autologous protein MoAlb, to exclude the possibility that any observed effect on OT-II cells could be due to the injection of a protein itself. This experimental design is described in more details in Methods and summarized in Figure 2.1. Here in Figure 2.7, the results from the analysis of OT-II divisions by CFSE from four representative mice, one from each group of recipient animals, are shown. A comprehensive analysis of responses in all animals from all groups is shown in Figure 2.8. Analysis was done on splenocytes isolated from recipient mice one week after they received a s.c. injection of either saline, the control protein MoAlb (50μg) or TT (50μg). The gating strategy described in Figure 2.3 was used.

A clear distinction emerged in the behavior of naïve OT-II cells when compared with antigen-activated OT-II cells, with respect to their susceptibility to bystander activation and proliferation during an immune response directed against an unrelated antigen. In fact the results show that naïve OT-II cells were not sensitive to the particular environment created *in vivo* by either a primary injection of TT (Figure 2.7. A), or a booster injection of TT (Figure 2.7.B) and did not divide. A similar result was obtained when TT-vaccinated recipient mice where challenged with MoAlb (Figure 2.7.B). On the contrary, OT-II cells previously activated *in vitro* with cognate antigen, were indeed prone to bystander activation and they divided several times during the recall response to TT (Figure 2.7.D, black arrow). This bystander proliferation did not occur when recipient mice were challenged with the control autologous protein MoAlb, excluding in this way that it could be simply induced by the sub-cutaneous injection of a protein (Figure 2.7.D). Also and more importantly, it only occurred during a recall response to TT, but not after a primary injection of TT (Figure 2.7.C).

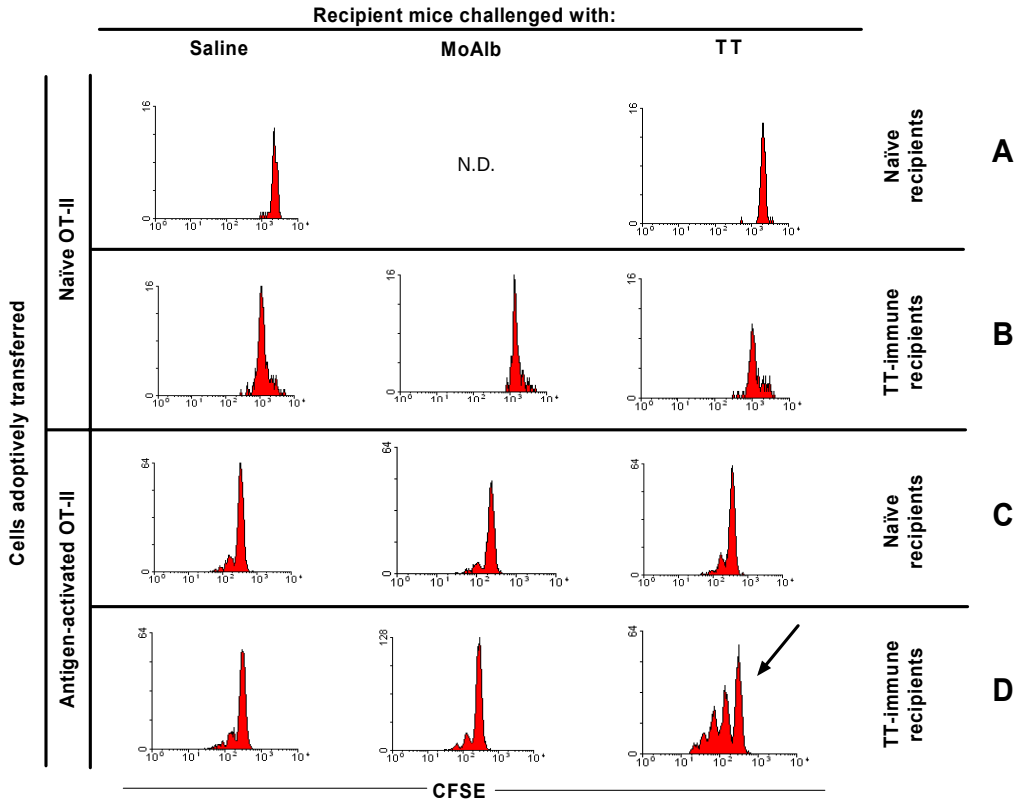


Figure 2.7. Bystander activation and proliferation of antigen-activated OT-II TCR transgenic T cells during a recall response against TT: representative examples. Naive or antigen-activated OT-II cells were labeled with CFSE and adoptively transferred either into naïve C57/B6 wild type recipients or into recipients which had been previously vaccinated with TT. Recipient mice were then challenged with either TT, the control protein MoAlb or saline (see Figure 2.1), and the cell division history of OT-II cells was analyzed by flow cytometry using the gating strategy described in Figure 2.3.

A detailed analysis of cell divisions of OT-II cells from all mice from the four different adoptive transfer experiments is shown in Figure 2.8. In each diagram, the percentage of dividing OT-II cells is shown on the Y axis. On the X axis, for mice challenged either with saline (S), mouse albumin (MA), TT or with OVA, the percentages of OT-II cells which divided once or more than once (≥1), or more specifically once (1), twice (2) or three or more than three times (≥3) are indicated. The results of the adoptive transfers of naïve OT-II cells into either naïve recipients or into TT-immune recipients are illustrated in Figure 2.8.A and Figure 2.8.B respectively. As anticipated in Figure 2.7, naïve OT-II cells are not susceptible to bystander proliferation and they did not proliferate during a primary (Figure 2.8.A) or a secondary (Figure 2.8.B) immune response to TT; however they strongly responded

to the challenge with OVA, with extensive proliferation (Figure 2.8.A and B). Therefore naïve OT-II maintained fully functionality after adoptive transfer and they responded to the cognate antigen which was presented to them upon *in vivo* processing of OVA protein.

The results of the adoptive transfers of antigen-activated OT-II cells into either naïve recipients or into TT-immune recipients are shown in Figure 2.8.C and Figure 2.8.D respectively. It is clear that OT-II cells primed *in vitro* with OVAp still show evidence of residual activation at the time of adoptive transfer, in fact around 15% and 20% of them divided once or more than once during the week after the transfer into naïve or TT-immune recipients which were then injected with saline (Figure 2.8.C and D respectively). The percentage of OT-II cells that have undergone one or more divisions is slightly but not significantly higher than the saline control in naïve recipients which were challenged with TT (primary injection) (Figure 2.8.C). The most dramatic change in the percentage of antigen-activated OT-II that had undergone one or more that one division, as anticipated from Figure 2.7, occurred when TT-immune recipient mice received a booster injection of TT which induced a strong secondary immune response (Figure 2.8.D). In this case an average of 42.3 % of OT-II cells (33.1-58.9 %) divided once or more than once compared to an average of around 20% in the control saline group. Furthermore only in this case OT-II cells were found which had undergone three or more divisions. As anticipated this bystander proliferation of *in vitro*-primed OT-II cells was consequence of the particular environment created by the on going secondary immune response to TT and was not simply due to the injection of a protein as proved by the lack of bystander proliferation which followed the injection of MoAlb.

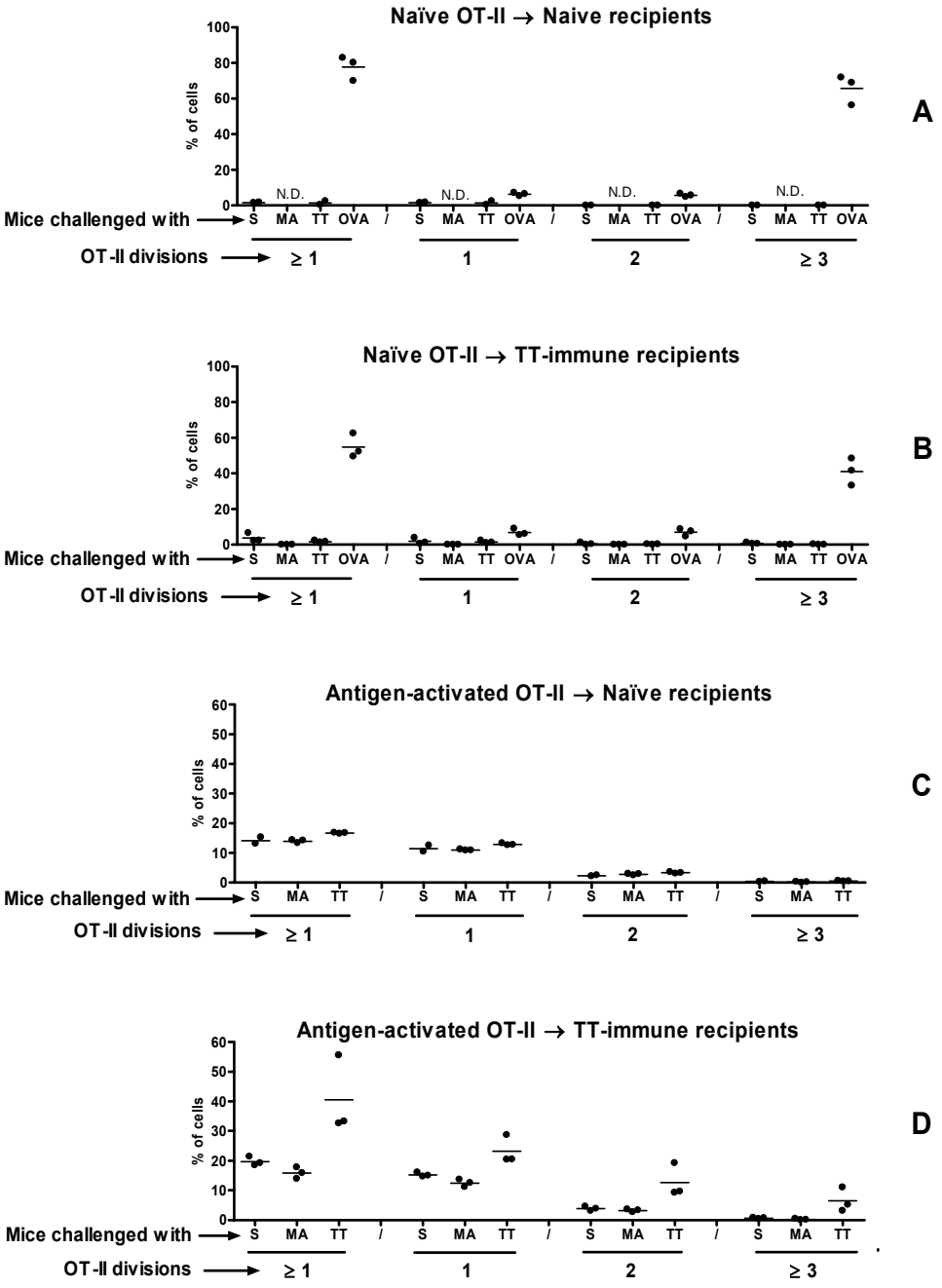


Figure 2.8. Bystander activation and proliferation of OT-II TCR transgenic T cells during a recall response against TT: summary. Naive or in vitro-activated OT-II cells were labeled with CFSE and adoptively transferred either into naïve C57/B6 wild type recipients or into recipients which had been previously vaccinated with TT. Recipient mice were then challenged with either TT, the control protein MoAlb or saline (see Figure 2.1), and the cell division history of OT-II cells was analyzed by flow cytometry using the gating strategy described in Figure 2.3. Results from the four different adoptive transfer experiments are show here. In each graph, the percentage of dividing OT-II cells is indicated on the Y axis. On the X axis, a detailed analysis of cell divisions is shown for group of mice challenge with saline (S), mouse albumin (MA) or tetanus toxoid (TT). Percentages are indicated for OT-II cells which divided once or more than once (≥1), or more specifically once (1), twice (2) or three or more than three times (≥3). ≥1 = 1 + 2 + ≥3

2.3.5 **Bystander proliferation of activated OT-II cells in TT-immune recipient mice appears to correlate with the levels of endogenous TT-specific T cell responses induced by TT booster injection.**

Some suggestive evidence for a link between the ongoing TT-specific secondary immune response in TT-immune recipient mice boosted with TT and the bystander proliferation of adoptively transferred OT-II cells was noted. By measuring the level of response to TT in recipient mice, using cytokine-specific ELISPOTs, and then assessing the percentages of OT-II cells undergoing one or more cell divisions, it was possible to see a correlation (Figure 2.9). In Figure 2.9.A the percentage of OT-II cells undergone one or more divisions after adoptive transfer into TT-immune recipients challenged with TT is displayed. In Figure 9B this percentage is shown in relation to the percentage of divisions occurring in control recipient mice challenged with saline. IL-2 and IFN-γ ELISPOT responses by splenocytes from TT-immune mice, recipients of antigen-activated OT-II cells, and isolated one week after challenge with TT, are shown Figure 2.9.C.

Splenocytes restimulated *in vitro* with TT showed strong IL-2 and IFN-γ *in vitro* production, confirming high effector precursor frequency and therefore successful TT booster challenge. The proportion of OT-II cells undergone one or more division was higher in one of the three recipient mice (TT-1) which showed the highest immune response to TT boost, suggesting a correlation between the levels of TT-specific cellular responses and the extent of OT-II bystander proliferation. Evidence for a correlation was also found in a second independent experiment (see Figure 2.10).

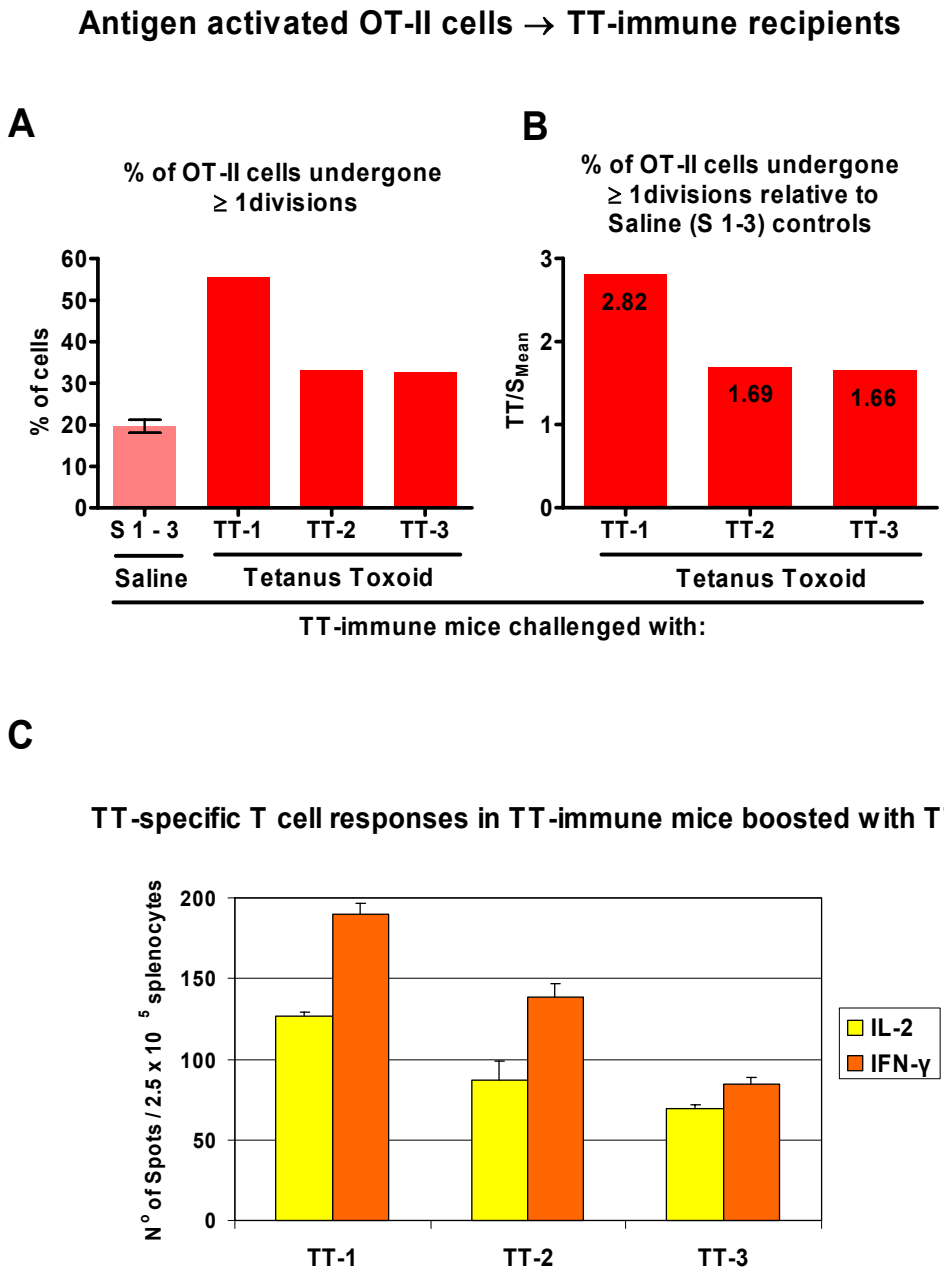


Figure 2.9. The extent of bystander proliferation of antigen-activated OT-II cells appears to correlate with the levels of the endogenous TT recall response (measured by IL-2 and IFN-γ ELISPOTs) in TT-boosted animals.
(A) Percentages of activated OT-II cells undergone one or more divisions when adoptively transferred into TT-Immune recipient mice which received either a booster injection of TT (TT-1, TT-2 and TT-3) or saline (S 1-3, mean and Standard Deviation of S-1, S-2 and S-3).
(B) Divisions of OT-II cells in TT-boosted mice relative to OT-II divisions in control (S-1-3) mice.
(C) TT-specific T cell immune responses measured by IL-2 and IFN-γ ELISPOTS in TT-immune mice boosted with TT.

In order to confirm these results, the adoptive transfer of antigen-activated OT-II cells into either naïve or TT-immune recipient mice, was repeated in an independent experiment. A second aim of this experiment was also to reduce the residual activation of OT-II cells at the time of adoptive transfer. In order to do so, a possibility was to stimulate OT-II cells *in vitro* with lower doses of OVAp. OT-II cells were stimulated *in vitro* with 0.1μM OVAp, a concentration which was ten times lower than that used in the initial experiment (1μM). The results of this second experiment are summarized in Figure 2.10. The use of a ten times lower concentration of OVAp to stimulate naïve OT-II *in vitro*, reduced the residual activation of OT-II cells at the time of adoptive transfer, so that only around 10% of them divided more than once during their persistence into control recipient hosts (Figure 2.10.A and B: Saline, S). As previously observed (Figure 2.8.C), when naïve recipients were challenged with TT no bystander proliferation of OT-II cells was evident (Figure 2.10. A). In contrast, and in line with the results of the previous experiment, when TT-immune recipients were challenged with a booster dose of TT, the average percentage of OT-II cells undergone more than one division doubled reaching an average of around 20% (Figure 2.10.B). Furthermore, while virtually no OT-II cell divided twice in the control group (saline, S), more than 5% of them divided twice during the secondary response to TT (Figure 2.10.B). When the divisions of antigen-activated OT-II cells in individual TT-immune recipient mice boosted with TT (Figure 2.10.C and D) were correlated to the endogenous TT-specific cellular immune responses induced by the booster injection of TT (Figure 2.10.E), again it was found that bystander proliferation of OT-II cells was more pronounced in two mice (TT-1 and TT-3) which had higher IL-2 and IFN-γ cytokine responses to TT. Overall these results mirror those obtained in the previous experiment and support the idea that antigen-activated OT-II cells are susceptible to bystander proliferation when exposed *in vivo* to the particular microenvironment created by a secondary immune response to an antigen unrelated to the one they are specific to.

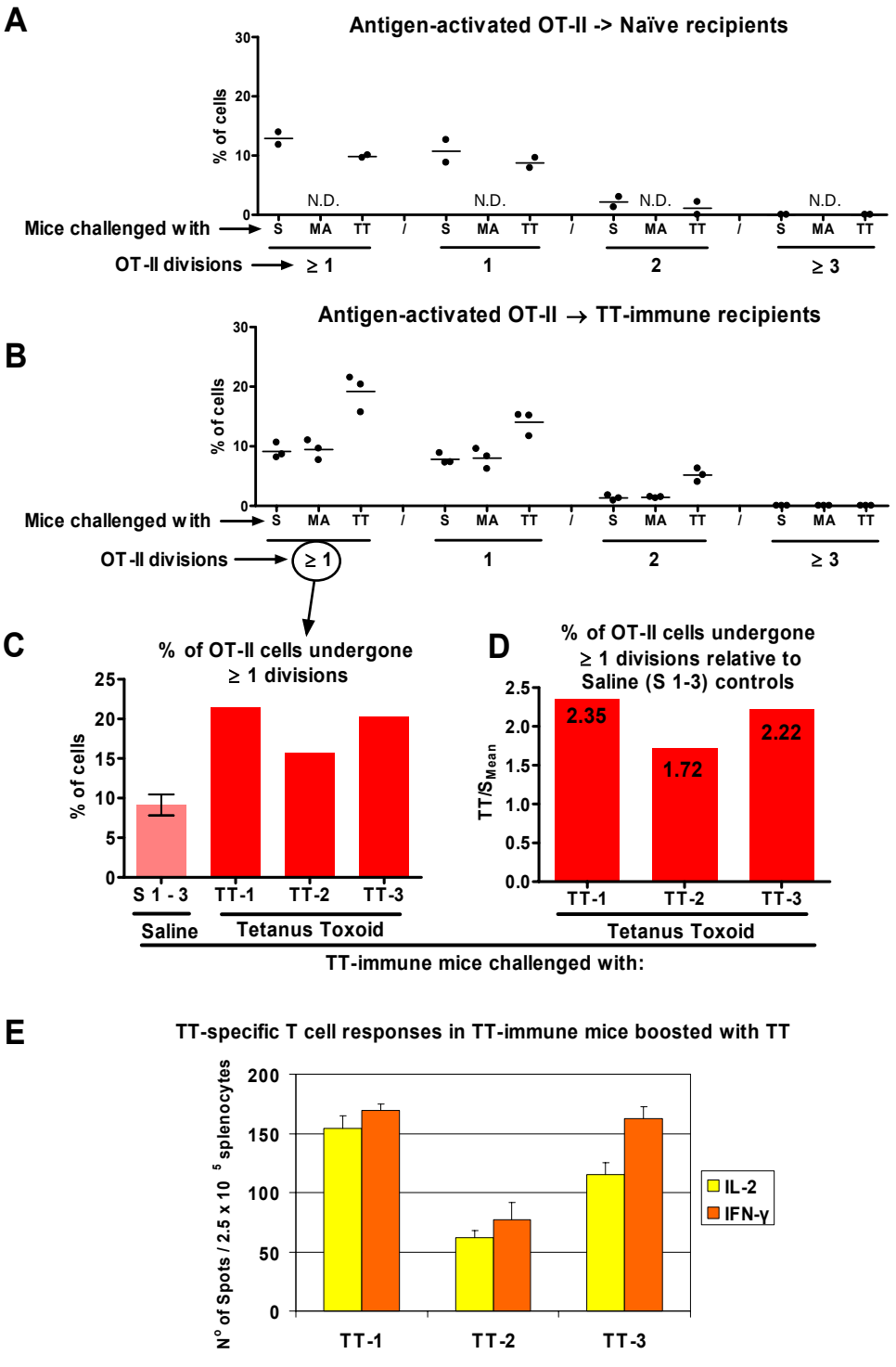


Figure 2.10. Bystander proliferation of antigen-activated OT-II cells during a secondary immune response to TT. Summary of the results obtained in a second independent experiment.
Analysis of OT-II divisions in: (**A**) naïve recipients and (**B**) TT-Immune recipients. **(C)** Analysis of OT-II divisions in individual TT-Immune mice challenged with TT (TT-1, TT-2 and TT-3) or with saline (S 1-3, mean and Standard Deviation of S-1, S-2 and S-3). **(D)** Divisions of OT-II cells in TT-boosted mice relative to OT-II divisions in control (S 1-3) mice. **(E)** TT-specific T cell immune responses measured by IL-2 and IFN-γ ELISPOTS in TT-immune mice boosted with TT.

2.3.6 **Phenotype of antigen-activated OT-II cells before and after adoptive transfer into TT-immune recipient mice**

The phenotype of OT-II cells activated *in vitro* with OVAp was studied at day 0 (naïve), at day 11 before adoptive transfer (baseline) and then on cells rescued from TT-immune recipient mice one week after challenge with either saline, MoAlb or TT. Results are shown below in Figure 2.11.A. The phenotype of naïve cells is indicated by the dotted purple line and the baseline phenotype is indicated by the solid green line. As previously shown in Figure 2.5, also in this experiment, day 11 OT-II cells before adoptive transfer still display high expression of activation markers such as CD69 and CD44, and CD25 (IL-2Rα). Expression of CD122 (IL-2/IL-15Rβ) on day 11 OT-II cells, is around 9 %. This value probably under-estimates the real percentage of CD122 positive cells as suggested by the significant shift in the fluorescence intensity of CD122 as compared with the isotype control (Figure 2.11B). A higher expression of CD122 at day 11 would also be consistent with what was previously observed during the phenotypic characterization of antigen-activated OT-II (see Figure 2.5). In fact in that experiment the percentage of CD122 positive cells reached 78.1% at day 4 and than decreased, but at day 11 still 34% of OT-II cells expressed this marker. Studying co-expression of CD25 and CD122 by the same cell might help discriminate the OT-II CD122 positive cells from the negative ones, and it is planned for future experiments.

Interestingly, day 11 OT-II cells express CD127 (IL-7Rα) which was not expressed by naïve cells. Furthermore they have considerably down-regulated CD45RB which was highly expressed on naïve cells (Figure 2.11.A). Overall and in line with what was previously found (Figure 2.5), OT-II cells cultured *in vitro* with cognate peptide, before adoptive transfer, show a phenotype which indicate still residual cell activation but it also suggests the beginning of a program of differentiation, as indicated by the down-regulation of CD45RB and the expression of CD127.

After one week of persistence in recipient control mice (mice challenged with saline), a clear down-regulation of activation markers (CD44 and CD69) and of IL-2 receptors α (CD25) was visible on OT-II cells. Interestingly CD44 was maintained at levels

which were intermediate between the low levels expressed on naïve OT-II cells and those expressed on day 11 OT-II cells, and this was accompanied by a parallel further up-regulation of IL-7Rα and a further down-regulation of CD45RB (Figure 2.11.A). Taken together these changes would suggest that following the initial *in vitro* activation and expansion, OT-II cells adoptively transferred into recipient mice were differentiating towards a "resting-like" phenotype with some features typical of memory-like cells.

No significant changes in the phenotype of OT-II were detected between the three experimental groups, although there was a trend for OT-II cells isolated from TT-immune recipient mice boosted with TT, to further up-regulate CD44 (Figure 2.11.A. Black arrow). This is shown by the same cells which were prone to bystander proliferation during the recall response to TT (see Figure 2.8.D). Interestingly, CD44 expression was the highest (MFI 1088.9) on OT-II cells which had also shown the highest bystander proliferation (TT-1 in Figure 2.9.A) and which had been rescued from the mouse showing the highest T cell response against TT (TT-1 in Figure 2.9.C).

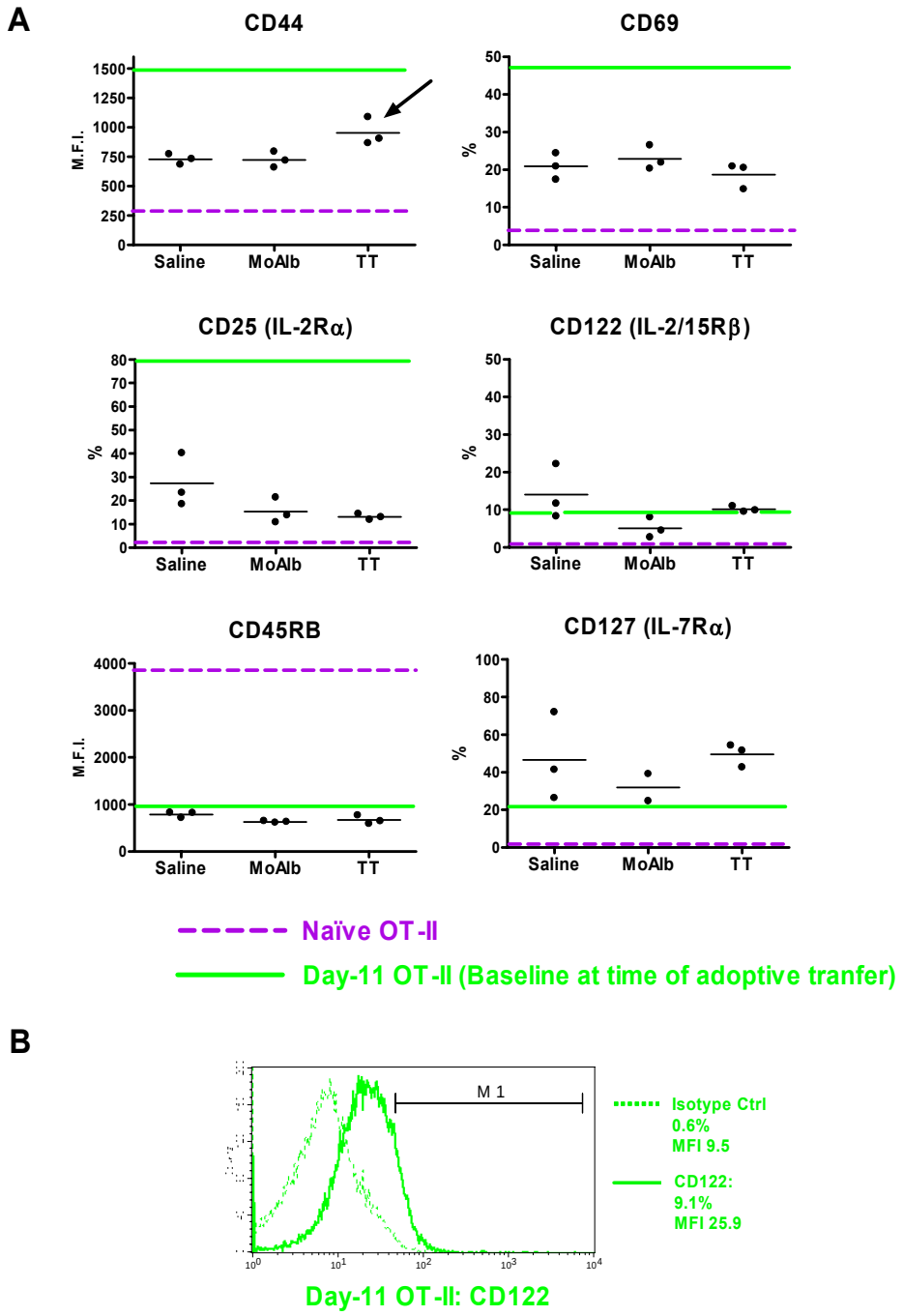


Figure 2.11. Phenotype of activated OT-II cells before and after adoptive transfer into TT-immune recipient mice. **(A)**. Phenotype of antigen-activated OT-II cells was studied at day 0 on naïve cells (dotted purple line), on cells cultured *in vitro* with OVAp at day 11 before adoptive transfer (baseline, solid green line), and then on OT-II cells rescued from TT-immune recipient mice one week after challenge with either saline, mouse albumin (MoAlb) or tetanus toxoid (TT). The method used is described in Section 2.2.8 and the gating strategy is illustrated in Figure 2.3. For CD69, CD25, CD122 and CD127 the percentage of positive cells in relation to the isotype control is indicated. For CD44 and CD45RB the mean fluorescence intensity (MFI) is indicated. **(B)** Expression of CD122 on day 11 OT-II cells.

2.4 **Discussion**

In this chapter we have described the early development of a mouse model aimed at studying in more depth the phenomenon of bystander activation of $CD4^+$ memory T cells observed in humans and described in Chapter 1. Our object was to provide formal evidence of bystander proliferation of $CD4^+$ memory T cells occurring *in vivo* during a secondary immune response, and to lay the foundation for addressing the possible mechanisms responsible for it in a systematic and quantitative way.

The initial requirements were therefore:

1. Possibility of tracking a particular population of antigen-specific $CD4^+$ (memory) T cells during an immune response directed against an unrelated antigen;
2. Possibility of quantifying activation status and cell divisions in this population.

In our experimental approach, we met those requirements by using $CD4^+$ TCR transgenic T cells (OT-II) specific for a given epitope of ovalbumin (OVAp), which were available in sufficient numbers and easily traceable thanks to a particular combination of fluorochrome-labelled TCR-specific monoclonal antibodies. Once identified in this way their activation status could be assessed by a panel of phenotypic markers and flow cytometric analysis. CFSE-labelling could provide crucial evidence of divisions occurring *in vivo*. Finally we used an adoptive transfer system where CFSE-labelled OT-II cells were transferred into recipient mice in which a polyclonal population of TT-specific memory T cells had already been established. Therefore when these mice received a booster injection of TT, a strong recall immune response was induced. In this context, we initially compared the behaviour of naïve OT-II cells to that of OT-II cells activated *in vitro* with OVAp and then adoptively transferred, at day 11, into recipient mice.

It has been shown that, following *in vitro* activation with cognate antigen and subsequent adoptive transfer into wild type recipient mice, $CD4^+$ TCR transgenic naïve T cells differentiate into memory cells and this approach has been widely used to study phenotypic, functional and biochemical properties of $CD4^+$ memory T cells

in the mouse [35,50,51,123]. In our hands the *in vitro* stimulation of naïve OT-II with OVAp led to their strong activation and to an initial phase of clonal expansion followed by a contraction phase characterized by extensive cell death (Figure 2.5.A). The choice of day 11 as the day for the adoptive transfer was therefore justified by the necessity of giving cells enough time to rest and at same time to allow us rescue enough viable cells to be used in the adoptive transfer experiments. Nevertheless, residual activation was still present at day 11, as shown by the phenotypic profile (Figure 2.5.B). The phenotype also shows some features, such as the reduced expression of CD45RB and the appearance of IL-7Rα which suggests the initiation of a process of differentiation predicted to lead towards a memory-like phenotype once the cells are transferred into recipient mice. Clearly the lack of response against cognate antigen when recipient mice were challenged with ovalbumin (Figure 2.6) suggests that these cells, from the functional point of view, were still far from being at a memory differentiation stage. They were probably more in need of cytokines to sustain their viability after the recent antigen-stimulation.

Bearing in mind that clear differences exist between $CD4^+$ T cells at a memory differentiation stage and the antigen-activated OT-II cells used in the adoptive transfer experiments described here, this study has demonstrated a fundamental feature of antigen-activated OT-II cells, which characterize them and distinguish them from naïve cells. Specifically this is their susceptibility to bystander proliferation which occurs during a secondary immune response directed against an antigen unrelated to their cognate one (Figures 2.7 and 2.8). The CFSE analysis done after adoptive transfer shows that divisions of antigen-activated OT-II cells occurred *in vivo*, and they were clearly linked to the endogenous recall response to TT. In fact they were not a consequence of the injection of a protein itself, as demonstrated by the lack of division after challenge with the autologous mouse albumin, neither were they present after a primary injection of TT (Figures 2.7 and 2.8). The lack of bystander proliferation of antigen-activated OT-II T cells after primary injection with TT, also excludes a remote possibility for the bystander proliferation to be a consequence of cross-reactivity between the OT-II cognate peptide OVAp, and any possible peptide presented to the OT-II cells upon processing of TT *in vivo*.

The mechanisms behind this bystander proliferation still need to be clarified. In discussing the study carried out in humans, we pointed to a possible role of cytokines secreted during the secondary immune response to TT and to the local microenvironment created as a consequence in the lymphoid tissues. Sources of cytokines could be the vaccine-specific $CD4^+$ memory T cells themselves, although secretion of cytokine by other immune cells has also to be considered. A cytokine-driven proliferation is still a possible explanation for the bystander proliferation of antigen-activated OT-II cells we reported here. It is supported by the apparent correlation between the degree of bystander proliferation of OT-II cells and the magnitude of the TT-specific T-cell response. This was shown by measuring the frequency of TT-specific cytokine (IL-2 and IFN-γ)-producing T cells present within the splenocyte population isolated from recipient mice after the booster injection of TT and comparing this to the bystander activity of adoptively transferred antigen-activated OT-II cells (Figures 2.9 and 2.10).

If cytokines induced by stimulated TT-specific memory $CD4^+$ T cells are driving the bystander proliferation of antigen-activated OT-II cells, it is still difficult for us at this point to come to a definitive conclusion as to which cytokine is involved. Certainly, the expression of IL-2 receptors α and β on day 11 antigen-activated OT-II cells would render them susceptible to the activity of IL-2 produced *in vivo* in recipient mice during the secondary response to TT. IL-2 is also produced during the priming of an immune response, and this is presumably happening during the challenge with TT in naïve recipients. In this case the concentrations reached *in vivo* in the lymphoid tissues are probably not high enough for inducing proliferation of adoptively transferred antigen-activated OT-II cells. Furthermore it cannot be excluded that at day 11, before adoptive transfer, the *in vitro*-cultured OT-II cells are still producing IL-2 and use it in an autocrine loop. If so, this IL-2 is probably sustaining the residual proliferation of antigen-activated OT-II cells, which is clearly visible in control mice challenged with saline only (Figures 2.8.C and D, 2.10.A and B). However there is no reason to believe that the *in-vitro* activated OT-II cells are induced to produce more IL-2 during the TT-specific secondary immune response and that this drives the bystander proliferation of OT-II cells.

Together with IL-2, other cytokines might also be playing a role in inducing bystander proliferation of antigen-activated OT-II cells. Among them IL-7 and IL-15 are good candidates, given the role that they play in sustaining long-term maintenance of memory T cells [80]. Day 11 antigen-activated OT-II cells express CD122 which is the subunit β common to the IL-2 receptor but also to the IL-15 receptor. They also express CD127, the subunit α of IL-7R which is not expressed on naïve OT-II (Figures 2.5 and 2.11). Overall whether one or more than one of these three cytokines has an effect on antigen-activated OT-II cells remains to be proved. In any case a putative effect is not accompanied by a further up-regulation of the specific receptor. In fact no significant differences were detected between TT-Immune recipient mice challenged with saline or MoAlb, and mice challenged with TT in the levels of CD25, CD122 and CD127 (Figure 2.11). Therefore if the bystander proliferation of OT-II cells is driven by these cytokines, it can only be explained by the fact that these cytokine are present, or their concentration *in vivo* is sufficiently high, only during the TT-specific recall immune response. Although this might well be the case for IL-2, as discussed above, we don't have any supportive evidence to suggest that during the secondary immune response to TT there is a rise *in vivo* in the levels of IL-7 or IL-15 above those which might be found in control mice. Getting this evidence might also be technically challenging considering that many cytokines have short half-lives and do not accumulate to detectable levels as measured in serum samples.

Overall these results are very encouraging, since they reproduce in a defined mouse model the observations made in human subjects. They show how OT-II cells activated *in vitro* with cognate antigen and with the capacity to differentiate into memory T cells are susceptible *in vivo* to bystander activation. Bystander stimulation leads to proliferation presumably due to changes in the local micro-environment (cytokine milieu), occurring during a recall response directed against an unrelated antigen.

2.5 **Further investigations**

Two aspects of the bystander activation phenomenon require further investigation: first the effect of bystander stimulation on "true" memory $CD4^+$ T cells; second the molecular pathway involved in the effect.

To allow full differentiation of antigen-activated OT-II cells into memory cells, we are planning to repeat the adoptive transfer experiments of antigen-activated OT-II cells into TT-immune recipient mice, but to leave a longer period of time (6-8 weeks) between adoptive transfer and induction of the TT recall response. Establishment of memory from *in vitro*-activated precursors will be judged by the phenotype of putative memory OT-II cells and by their ability to exert rapid effector functions and to proliferate upon re-stimulation with cognate peptide (OVAp). Differentiation of antigen-activated OT-II cells into memory cells could take place in an intermediate host (if necessary Rag $2^{-/-}$), and in this case after rescuing them, it would still be possible to label them with CFSE before the adoptive transfer into the final TT-immune host, and to study by this means their cell division history. As an alternative we could use a congenic system (Thy1.1/Thy 1.2) to discriminate donor and recipient cells. To identify proliferating cells within the OT-II population in this setting, we could assess DNA incorporation of bromodeoxyuridine (BrdU, an analogue of thymidine). BrdU would be administered daily by intraperitoneal injection for the last seven days during the TT-specific recall response.

Having established that memory OT-II cells, as well as their antigen-activated precursors, are susceptible to bystander activation and proliferation, additional questions could be asked. For instance, a crucial issue would be to study the effect of repeated TT booster injections, on the long-term persistence of adoptively-transferred memory OT-II cells in TT-immune hosts. In other words, if as a consequence of the bystander proliferation occurring during secondary immune responses directed against unrelated antigens (TT in this case), the lifespan of a population of $CD4^+$ memory T cells (OT-II) is increased, we would have proven in a defined mouse model that our speculative hypothesis formulated in the discussion to the study carried out in healthy human subjects vaccinated with TT, is fundamentally correct. This would contribute

significantly to our understanding of the maintenance of $CD4^+$ memory T cells with important repercussions on the study of natural and vaccine-induced immunity.

Appendix

Publications

Stevenson F K, **Di Genova G**, Ottensmeier C H, and Savelyeva N. Cancer Vaccines. In Cancer Immunotharapy: Immune suppression and Tumor Growth, edited by Prendergast G. C. and Jaffee E. M. (Academic Press, London, 2007), pp. 183-204.

Di Genova G, Roddick J, McNicholl F, Stevenson FK. Vaccination of human subjects expands both specific and bystander memory T cells but antibody production remains vaccine specific. Blood. 2006;107:2806-2813.

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