UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Regulatory T cell control of anti-tumour responses

by

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ABSTRACT

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One of the main obstacles to immunotherapy of cancer in humans is the immunosuppressive environment that surrounds the tumour mass, preventing any effective immune response from halting or reversing the threat of the tumour. Thus the observations in Balb/c mice that CD25+ regulatory T cells (T regs) mediate suppression of antigen specific responses to CT26 and a number of tumours of distinct histological origin, was deemed worthy of investigation. The aim of this project was to examine in greater detail the immunosuppressive response generated by CT26, by using the irradiated tumour which we expected would represent an equivalent tumour challenge.

Overall the work described here indicates that, despite being essentially an equivalent antigen exposure, the response induced in the irradiated CT26 model is different to the live CT26 model. In the live CT26 model T reg depletion is critical to the survival of the tumour challenge, as well as the generation of the cross-protective response. In the irradiated CT26 model, the cross-protective response is not dependent on the T reg depletion, but the absence of T regs does boost the anti-CT26 response.

My second project sought to study tumour immunity in the context of the TAZ10 transgenic model of autoimmunity. The main conclusion from this project was that endogenous processing of the autoantigen TPO is dependent on the signal peptide, and at some point in the intracellular transport of TPO the pathway diverges into the pathway that allows the processing of TPO and the association of MHC class II molecules with TPO peptides, for recognition by CD4+ MHC class II restricted T cells.

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Author's declaration

I declare that this thesis represents entirely my work, except where acknowledged below.

This thesis has not been submitted for any other degree.

The work in chapters 3, 5 and 6 were done in collaboration with Catherine King.

The spectrotyping in chapter 6 was done in collaboration with Sue Harris (SUHT).

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Chapter 1. Introduction

1.1 The immune system overview

The world is full of microorganisms, many of them pathogens that are capable of killing other organisms. To deal with this threat all eukaryotes have developed anti-pathogen devices, the first line being the innate immune defences. The innate immune system recognises microorganisms via germline-encoded pattern-recognition receptors (PRRs), which recognise pathogen-associated molecular patterns (PAMPs). These PAMPs are found on microbial components that are evolutionary conserved, because they are essential to the survival of the microorganism and therefore not likely to be altered. These include bacterial products such as LPS, flagellin, LTA, PG and CpG DNA; and viral products such as dsRNA and ssRNA. (Akira, Uematsu et al. 2006).
In addition to these innate defences, jawed vertebrates have evolved an adaptive immune system mediated by lymphocytes. All jawed vertebrates, beginning with cartilaginous fish, have rearrangeable immunoglobulin (Ig) V, D, and J gene segments, which allow the generation of a diverse lymphocyte receptor repertoire, expressed by T and B lymphocytes, capable of recognising a nearly infinite antigenic world. Antigen-mediated triggering of T and B cells initiates specific cell-mediated and humoral immune responses (Cooper and Alder 2006). The birth of the adaptive immune system is thought to have occurred when a transposable element containing RAG1 and RAG2 invaded the Ig gene, allowing greatly increased receptor diversity (Flajnik and Du Pasquier 2004). RAG1 and RAG2 are recombination activation genes, which catalyse the process of TCR gene rearrangement when the V, D and J segments are brought together in a continuous V-J or V-D-J coding block, forming complete V domain exons that are responsible for antigen recognition by the TCR (see section 1.4). (Oettinger, Schatz et al. 1990)

This development of a complicated ‘anticipatory immunity’ must have given enormous fitness value on the early vertebrates that acquired it. This point of view is strengthened by the fact that the immunoglobulin (Ig) system of lymphocyte-based recombination is not the only such system present in vertebrates. In fact the only surviving jaw-less

vertebrates, the lamprey and hagfish, assemble receptors known as variable lymphocyte receptors (VLRs). Instead of immunoglobulin genes, the lymphocytes of these jaw-less fish rearrange modular leucine-rich repeat (LRR) cassettes to create functional mature VLR genes. A VLR of unique sequence is expressed by each lymphocte, the lymphocytes appear to undergo clonal amplification in response to stimuli, and can release their receptors into the plasma showing the potential for humoral immunity (Pancer, Amemiya et al. 2004).

The fact that two such similar systems arose, estimated to be approximately 500 million years ago (during the Cambrian period), suggest that the benefits may have been beyond simply recognising antigen. Fossil records do not indicate a massive eradication of species during the Cambrian period that would imply that a devastating new pathogen emerged that favoured a revolution in immunity (Cooper and Alder 2006).
It is now thought that the immediate selective pressure may instead have been facilitation of the developmental and morphological plasticity of the vertebrates. This plasticity allowed an explosion (known as the 'Cambrian explosion') of innovation in vertebrate development, morphology and function that led to the breadth and variety of vertebrate species we now witness. The development of the anticipatory immunity had the advantage that self-reactive lymphocytes were deleted before maturing to full immune status, which would have conferred a massive benefit to the organisms that first developed it. The removal of the risk of lethal autoimmunity freed them to diversify in morphology, creating anatomical structures that previously would have been targeted by the 'old' immune system. The wholly innate immune system of the early vertebrate and invertebrate ancestors had immune cells expressing a vast arsenal of LRR-containing receptors, engendering enormous binding versatility despite the receptors being germline-encoded. This system was at least in part used in order to allow many symbiotic relationships between host and microorganisms (Noverr and Huffnagle 2004). However, a system with such a vast array of receptors would have had the drawback of containing some self-reactive receptor variants that would have engaged in autoimmune type interactions with the newly evolving molecular determinants of early vertebrates. This interference may have been the crucial obstacle to the explosion of vertebrate

diversification seen during the Cambrian period, and one of the major factors why two forms of lymphocyte-based recombination immunity emerged more or less around the same time (Cooper and Alder 2006).

These ideas are by no means an interesting but irrelevant historical detail, as with such a perspective the way in which the innate and adaptive immune systems are arranged and regulated, and how the two spheres interact, are better understood.

This perspective lends a new insight on central tolerance, where the randomly generated receptors are assessed, with the self-reactive clones being deleted while potentially useful clones are spared (see section 1.5). The ability to select non-autoreactive clones becomes more central to the reason why the whole adaptive system exists, as the ability to delete self-reactive variants would have solved the problems arising from the arsenal-approach in vertebrate ancestors.

Furthermore, a possible vestige of this transition from an immunity that is entirely innate – the arsenal approach; to an immunity that has an adaptive component – the lymphocyte based receptor recombination approach; is that 'adaptive' lymphocytes retained 'innate' immune functionality.

For example Toll-like receptors (TLRs), which are LRR containing innate receptors that recognise specific PAMPs, and are present on innate cells like macrophages, are also present on B cells and some types of T cell (Akira, Uematsu et al. 2006). Plasmacytoid dendritic cells, derived from a common lymphoid progenitor, express TLR7 and TLR9 (Lund, Sato et al. 2003).

It also lends some perspective to the thought that the adaptive immune system was not simply grafted on to the innate system, when the ties between the two systems are more entwined than that. For example the cytotoxic killing mechanisms used by Natural killer (NK) cells and cytotoxic T cells (CTLs) are similar despite one cell coming from the innate arm, and the other from the adaptive (Flajnik and Du Pasquier 2004). On a similar theme, both NK cells and T cells produce IFN-γ (Hoebe, Janssen et al. 2004).

The innate system is defined by the germline-encoded, non-clonal, and constitutive pattern-recogntion receptors (PRRs), which serve as the early recognition receptors for

pathogen invaders. These receptors are expressed by antigen presenting cells (APCs), like macrophages and dendritic cells (DCs), and their triggering lead to phagocytosis of foreign-bodies or engagement of the complement-cascades that can eliminate the pathogens without necessarily needing to activate the adaptive immune response (Akira, Uematsu et al. 2006).

The adaptive system is defined by the clonal receptors expressed by its two main components B and T lymphocytes. These receptors form a diverse repertoire, capable of recognising and responding to a massive array of foreign peptides presented by APCs. Once activated, they release antibody and cytokines, and mediate cellular cytotoxicity in order to eliminate pathogens.

The bridge between the innate and adaptive immune components is the DC. Triggering of their PRRs by pathogen-associated molecules modulates their activation status, which in turn affects their induction of T lymphocytes that recognise the antigens that they present (Hoebe, Janssen et al. 2004).

These aspects will be described in detail in the following sections.

1.2 The innate immune system

Early recognition of microbes by components of the innate immune system is essential to the successful removal of pathogens. It serves as both a first line of defence in its own right, and secondly as a means of enlisting dendritic cells, and T and/or B cells into the overall response (Hoebe, Janssen et al. 2004).
The innate system has a number of means to recognise microbial pathogens. These means reside under the umbrella term of pattern-recognition receptors (PRRs), which recognise evolutionary conserved molecular patterns of microbial and viral origin, known as pathogen associated molecular patterns (PAMPs). PRRs possess other common characteristics: they are expressed constitutively in the host, are germline encoded, nonclonal, expressed on all cells of a particular type, and independent of immunological memory (Akira, Uematsu et al. 2006).

Much work of late has been focused of a family of PRRs that belong to the Toll-like receptors (TLRs), which were described just over ten years ago (Medzhitov, Preston-Hurlburt et al. 1997). TLRs are type I integral membrane glycoproteins characterised by the extracellular domains containing varying numbers of leucine-rich-repeat (LRR) motifs. The cytoplasmic signalling domain is homologous to the IL-1R, and is termed the Toll/IL-1R homology (TIR) domain (Bowie and O'Neill 2000).
The TLR receptors, of which there are 12 types in the mammalian genome, can be roughly divided into those that recognise viruses (TLR3, 7, 8, and 9) and those that recognise bacteria or protozoans (TLR1, 2, 4, 5, 6 and 11) (Lee and Iwasaki 2007). To serve such purposes those TLRs that seek to identify extracellular pathogens such as bacteria are located on the cell surface and generally recognise lipids, while those that seek to detect viruses are expressed in intracellular vesicles and generally recognise nucleic acids (Akira, Uematsu et al. 2006).
Signalling through TLRs activates the same signalling molecules used for IL-1 signalling, and thus it leads to the production of pro-inflammatory cytokines and chemokines (Akira and Takeda 2004). Cells that prominently express TLRs are APCs like macrophages and

DCs, and thus the production of such secreted mediators is important for the establishment of an adaptive response.

Although TLRs are receiving much recent attention, they are not the only receptors that are influential. Some receptors that have been known for some time include: C-type lectins, mannose receptors, scavenger receptors that enable the recognition, phagocytosis, and thus eventual elimination of foreign bodies (Lee and Iwasaki 2007). This is also the case with the complement system, which comprises a group of more than 30 plasma proteins. This system is often closely associated with an antibody response (the classic pathway) to facilitate the uptake of microbes by phagocytotic cells, but can also recognise and eliminate microbes independent of antibody (via the lectin pathway or the alternative pathway) (Hoebe, Janssen et al. 2004).

Another extremely significant part of the innate immune system is the natural killer (NK) cell. NK cells mediate cellular cytoxicity and produce chemokines and inflammatory cytokines such as IFNγ, and tumour necrosis factor (TNF) (Trinchieri 1989). They are important in attacking pathogen-infected cells, especially early in the immune response, and they also target tumour cells and are thought to be involved in tumour surveillance. They are also affected by, and can themselves regulate, the adaptive immune response, especially via interaction with dendritic cells (Raulet 2004).

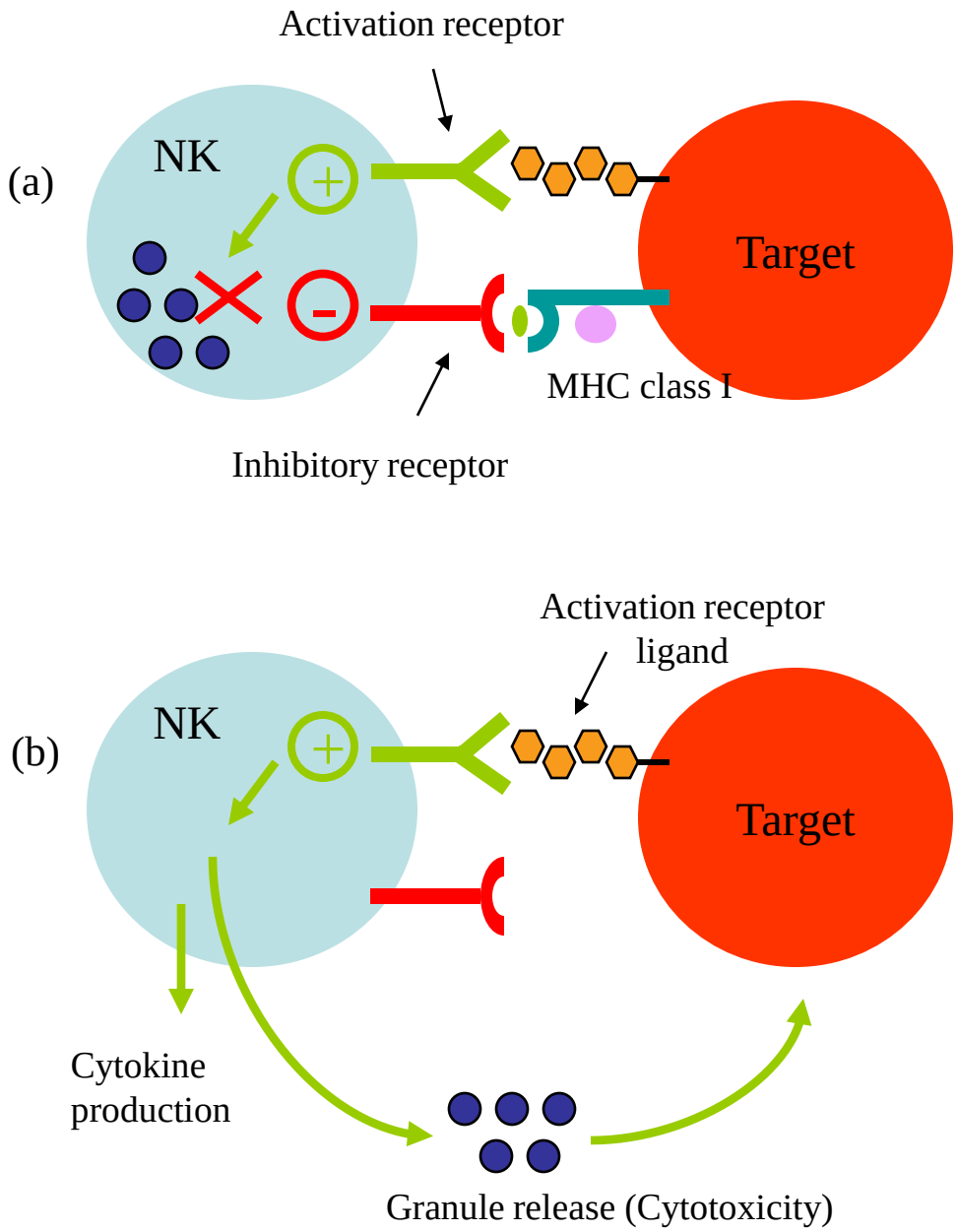
To recognise their targets NK cells use a multiple receptor strategy, whereby an individual NK cell can be triggered through various receptors independently or in combination, depending on the ligands presented by the target cell (Raulet, Vance et al. 2001). Although most of these receptors were first discovered in NK cells, and thus are called NK receptors (e.g NKG2D), many of them are also expressed on other cell types (like T cells) (Raulet 2004).

NK cells use three main recognition strategies. They can recognise pathogen-encoded molecules, the upregulation of self-protein in transformed and infected cells (induced self), or the absence of self-protein that is normally expressed but has been down-regulated by infected or transformed cells (missing-self) (Raulet, 2004).

An important NK receptor that recognises pathogen-encoded molecules is Ly49H, a stimulatory receptor on mouse NK cells which recognises a product of m157 of mouse cytomegalovirus (MCMV) (Arase, Mocarski et al. 2002). This receptor enables NK cells to limit early stage MCMV infections. Other examples of NK receptors specific for pathogens are NKp46 and NKp44 receptors which recognise influenza virus haemaglutinin (Mandelboim, Lieberman et al. 2001). Another strategy used by NK cells are receptors that recognise induced-self. An important one is the NKG2D receptor that recognises self-proteins that are upregulated on the surface of most tumours and many infected cells. Ligands for this receptor in humans include MHC class I chain-related A chain (MICA), MICB (Bauer, Groh et al. 1999), UL16-binding protein (ULBP), and Rae-1 (Cosman, Mullberg et al. 2001). Other receptors that have been linked to NK-mediated lysis of tumour cells are NKp46, NKp44 and NKp30 (Raulet 2004).

The other strategy that NK cells use, and actually was the first strategy that was discovered, is the recognition of missing-self (figure 1.1). The principle is that NK cells have receptors that send inhibitory signals when they bind their ligands, which are expressed on normal cells, but the loss of these ligands unleashes the NK cell to attack the target cell (Ljunggren and Karre 1990). This principle was first suggested when it was found that tumour cells lacking the MHC-class I molecule was the most sensitive to NK cell attack (Ljunggren and Karre 1985). Inhibitory recognition of classical MHC class I molecules (class Ia) is mediated mainly by Killer immunoglobulin-like receptors (KIR) in humans and Ly49 receptors in mice (Valiante, Lienert et al. 1997).

Figure 1.1. The control of NK cell activation. NK cell activation is controlled by the integration of signals from activation and inhibitory receptors. (a) Inhibitory NK cell receptors recognise self MHC class I and restrain NK cell activation. (b) When unimpeded by the inhibitory receptors, binding of NK cell activation receptors to their ligands on target cells results in NK cell stimulation. This stimulation results in cytokine production and granule release leading to cytoxicity.

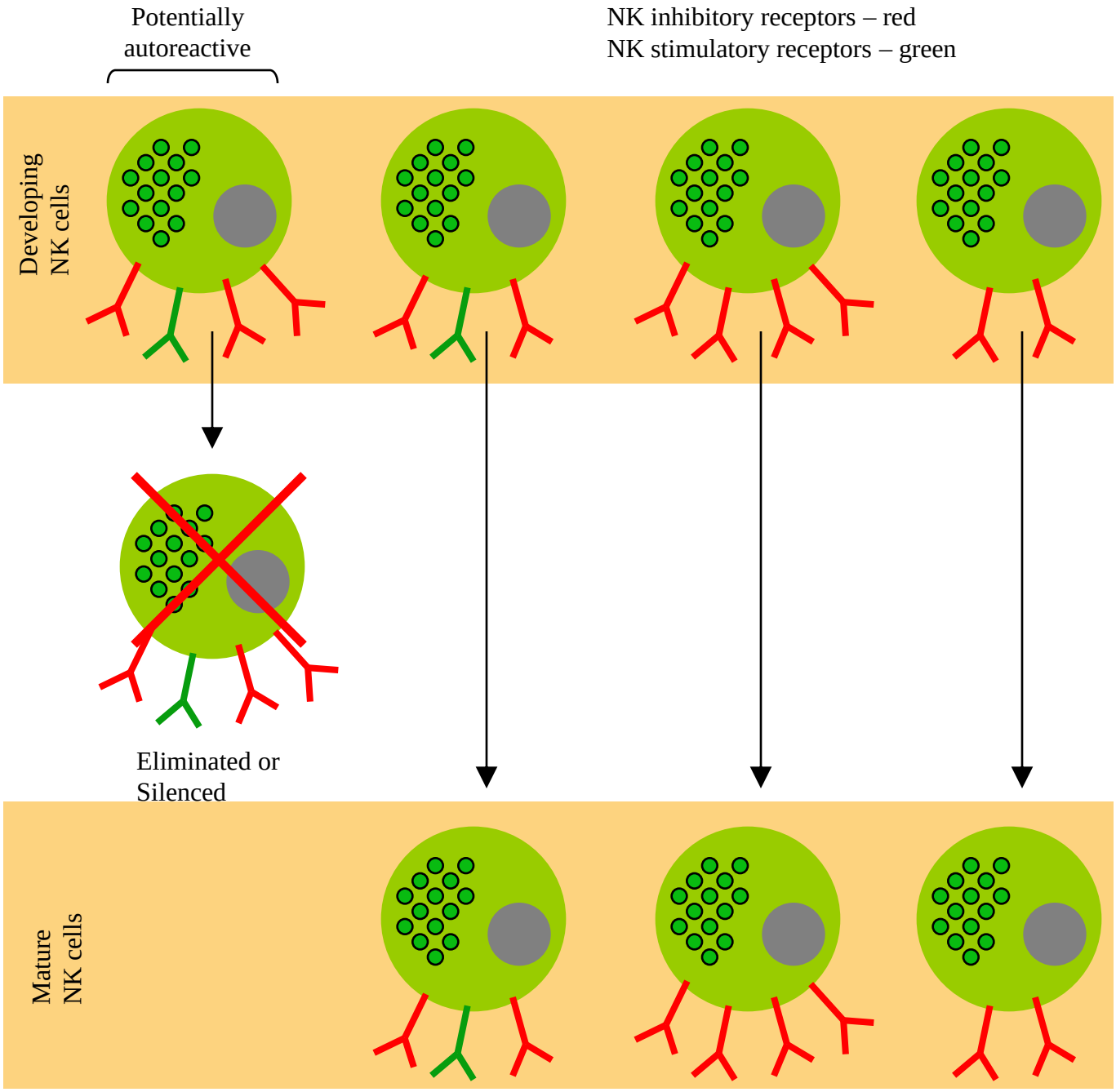


Although they are different in structure, KIRs and Ly49 receptors are very similar in their pattern of expression and function. Each family consists of approximately 10 genes that bind different subsets of MHC class I molecules (although not all of these are inhibitory). These receptors are expressed in a 'variegated' manner, meaning that each receptor is expressed on a subset of NK cells, and multiple receptors are expressed on each NK cell, so that a partially overlapping repertoire of NK specificities is generated (figure 1.2). This has the effect of allowing individual NK cells to discriminate among cells expressing different class I molecules. For example, a host cell that downregulates only one MHC class I molecule will elicit a response by the subset of NK cells whose only self-specific inhibitory receptor recognises that particular molecule. Cells that have completely lost class I expression will be even more sensitive to attack by most NK cells (Raulet, Vance et al. 2001).

When you examine the MHC-specific receptor system it begins to resemble adaptive immune receptor systems in some respects. Although there are many fewer NK receptors compared to T cell receptors, their repertoire is still relatively complex because of the random co-expression of many possible combinations of NK receptors. Unlike T cell receptors many of the NK cell receptors are inhibitory, although some are stimulatory. But like T cell receptors there is a possibility that the combination of NK cell receptors would make an autoreactive 'clone'. In the case of NK cells this may be because the cell expresses a stimulatory receptor for a self MHC molecule and/or lacks an inhibitory receptor for a self MHC molecule. In such a case these autoreactive 'clones' can be silenced, resembling the negative selection of T cell receptors (figure 1.2). As far as we know there is no positive selection of NK cells as there is for T cells (Raulet, Vance et al. 2001).

NK cells also show some clonal expansion in response to viral infections (Dokun, Kim et al. 2001), although this is considerably less than the 1,000-fold clonal expansion that can be seen by naïve T cells in response to viral antigen (Raulet 2004).

Figure 1.2. Generation of the NK cell repertoire. Many of the NK cell receptors are inhibitory (red), although some are stimulatory (green). Random expression of receptors can lead to the appearance of potentially autoreactive clones. This may occur because a clone expresses a stimulatory receptor specific for a self cells and/or because the clone lacks inhibitory receptors specific for self cells. Such potentially autoreactive clones are silenced.



1.3 The adaptive immune response

For the first five to six decades of the 20th century immunological research concentrated on examining the 'transferable' immunity represented by antibody. But by the 1960s it was becoming accepted that there was also 'cellular antibody', i.e. lymphocytes, and by the 1970s it was clear that thymus derived lymphocytes (T cells) were distinct from antibody-producing lymphocytes (B cells) (Masopust, Vezys et al. 2007). Further developments indicated that these T cells were cytotoxic, and expressed cloned receptors on their surfaces. This was demonstrated by experiments where lymphocytes were incubated on monolayers of allogeneic targets, resulting in their destruction. When non-absorbed cells were gently removed, they showed little specific cytotoxicity. However, when absorbed cells were eluted from the monolayer of target cells, these lymphocytes were both cytotoxic and specific (Golstein, Erik et al. 1971). In 1975 it was discovered that depletion of Ly-2 (CD8α) and Ly-3 (CD8β) bearing lymphocytes abolished the cell mediated cytotoxicity (Kisielow, Hirst et al. 1975). Thus the distinction between CD8 and CD4 T cells was established.

1.3a CD8+ T cells

It was in two papers in the mid 1970s by Zinkernagel and Doherty that established the MHC restriction of CD8 T cells (Zinkernagel and Doherty 1974). This was demonstrated by observing that T cells that were derived from a LCMV-infected mouse would only lyse targets that shared at least one set of H-2 molecules. They put forward two possible hypotheses to explain this observation. The first was called the intimacy (or two receptor) hypothesis, which suggested that MHC recognition occurred separately and in addition to viral protein recognition. The second was called the altered self hypothesis, which suggested that the infection induced a complex between the viral and H-2 antigens. Further experiments by the pair led to the rejection of the former hypothesis in favour of the latter (Zinkernagel and Doherty 1974). Concerns about this hypothesis, in particular questions as to how would the single MHC molecule structurally be able to bind the vast array of viral proteins, persisted for many years. But the altered self hypothesis gained

more support when, in the early 1980s, studies suggested that the MHC bound linear proteins rather than whole 3-dimensional proteins, which resolved many of these concerns (Townsend, Rothbard et al. 1986). Things became even clearer when the Bjorkman et al produced the crystal structure of MHC class I in 1987.

But before that discovery immunologists in the early 1980s were still concerning themselves with defining the putative T cell receptor (TCR). By this point the B cell receptor had been fully characterised, and the diversity of the receptor explained by the new and exciting idea of gene rearrangement (Masopust, Vezys et al. 2007). The T cell receptor was harder to define because it did not bind free antigen, it was not produced in high quantities, nor secreted. But by the early 1980s clonotypic antibodies, generated against T cell hybridomas, allowed immunologists to define the TCR as a heterodimeric receptor with variable and constant regions, quite like immunoglobulin (Samelson, Germain et al. 1983).

So by the mid 1980s the TCR had been defined and it seemed that MHC bound short peptides. But a lot of questions remained about how this TCR would see the 'altered self' MHC. These questions were answered when the crystal structure of class I MHC was shown in 1987 (Bjorkman, Saper et al. 1987). At the same time they interpreted this image as showing that the TCR engages peptide-binding groove of MHC along with the bound peptide (Bjorkman, Saper et al. 1987). Suddenly this previously clouded subject became clear. It was seen that most MHC-polymorphisms were situated at points that could contact peptide or TCR (so called functional positions), and also that allotypic differences affecting T cell reactivity were concentrated in the peptide-binding groove. Consequently this work simultaneously provided the mechanism for MHC restriction and allele specificity.

At the end of the 1980s the final connection between CD8 T cells and MHC class I was made when it was proposed that CD8 was not just a marker, but that it increases the avidity of T cells for their targets by directly binding to class I MHC. A range of experimental approaches confirmed this proposition, including co-immunoprecipitation studies, cell-cell binding assays, and the use of artificial vesicles expressing purified CD8 or HLA molecules (Rosenstein, Ratnofsky et al. 1989).

While the base understanding of CD8 T cells was now in place there were still several important discoveries to be made. In the early 1990s, work by Rammensee and colleagues sought to better characterise class I MHC-bound peptides. They found that the preferred length of bound peptides was shorter than was previously thought, at 8-10 amino acids, and also that each class I MHC had a preference for specific amino acids at specific 'anchor' positions (Falk, Rotzschke et al. 1991). This last observation allowed the characterisation of the binding motifs for different class I MHC molecules. Over the next decade and a half, incremental discoveries allowed the field to build up a much better picture of the 'life' of a CD8 T cell. In particular how a naïve T cell differentiates into effector T cells and then how effector T cells differentiate into memory T cells. For the first step major cellular programming is required to drive a quiescent naïve T cell through 10-15 divisions in just a week and convert these cells into effector T cells capable of robust cytokine production and cytoxicity. For the second step, in the absence of their stimulating antigen, effector cells differentiate into memory T cells that retain the capacity for rapid effector functions, and regain high proliferative potential and acquire the unique property of homeostatic proliferation. Homeostatic proliferation, a type of self-renewing division, is maintained by IL-7 and IL-15. Furthermore it was also found that CD4 T cells help CD8 T cells through the differentiation process and are essential for optimal memory CD8 T cell development (discussed in more detail below) (Williams and Bevan 2007).

As things stand today we know that CD8 T cells respond to short peptides produced in the cytosol, or via cross-presentation, allowing CD8 T cells to form the adaptive response against viruses, bacteria and protozoa. Activated CD8 T cells have two main pathways of inducing apoptosis in their target cell: via the granule exocytosis pathway, dependent on the pore-forming molecule perforin; or by upregulating FasL, which engages Fas on target cells. Both these pathways, initiated through stimulation of the TCR, induce cell death in the target cell via the caspase cascade (Harty, Tvinnereim et al. 2000).

1.3b. CD4+ T cells

CD4+ T cells establish and maximise the capabilities of the immune system, with most of their function concerned with activating and directing other cell types. This ability to activate and direct the response is generally encapsulated by the term 'CD4+ T cell help'. It is often the case that a lack of CD4+ T cell help does not prevent the initiation of immune responses by other cell types, but the absence limits the duration and the effectiveness of such responses.

Naïve CD4 T cells are maintained in a resting state as they recirculate from blood through lymphoid organs, surveying DCs for activating MHC-peptide complexes. Upon interaction with activated DCs, and with signals from the cytokine milieu, CD4+ T cells are driven through rapid rounds of division and acquire the ability to secrete effector cytokines. Eventually daughter cells become fully differentiated and fixed in their effector lineages and migrate to sites where their cytokines functionally organise the immune response. There are several T helper (Th) subsets that have been characterised, and most are defined by the major cytokine that it secretes. The original subsets of Th1 (IFNγ-secreting) and Th2 (IL4-secreting), have been joined more recently by Tregs (either thymically produced or peripherally induced suppressor cells) Tr1 (IL-10-secreting), Th3 (TGFβ-producing), ThFH (follicular helper cells), and Th17 (IL-17-producing) subsets (Reinhardt, Kang et al. 2006).
These subsets each have particular downstream effects. Th1 cells, through IFNγ, recruit NK cells and macrophages to mediate effector functions in the periphery. Th2 cells, through IL4, recruit eosinophils, basophils and alternatively activated macrophages for the same reason. Th17 cells, through IL-17, recruit PMNs to the periphery, and ThFH cells activate B cells at T cell-B cell follicular border (Hardtke, Ohl et al. 2005). Treg, Tr1 and Th3 cells perform distinct regulatory functions in the immune system.

In the absence of CD4+ T cell help B cell responses can be initiated, but the somatic hypermutation, isotype switching, and clonal selection necessary for production of high-affinity immunoglobulins is restricted (Mills and Cambier 2003). Furthermore, a lack of CD4+ T cell help will allow an acute but not a sustained or memory CD8+ T cell response. Early experiments investigating the role of CD4+ T cells in CD8+ T cell

responses, involving allograft rejection in vivo experiments and in vitro allogenic mixed lymphocyte reactions, concluded that MHC class II-specific CD4+ T cells were necessary for the generation of a cytotoxic CD8+ T cell responses and led to the original concept of CD4+ T cell help being essential for the clonal expansion of naïve CD8+ T cells (Keene and Forman 1982). This concept led to a simple hypothesis that the expansion of naïve CD8+ T cell precursors depended on CD4+ and CD8+ T cells being stimulated by antigen on the same APC, so that IL-2 secreted by CD4+ T cells can act on a neighbouring CD8+ T cell expressing high-affinity IL-2 receptors (Castellino and Germain 2006).

While this model has not been completely disproved, a series of recent studies have reached the conclusion that CD4+ T cells are very often dispensable for early clonal expansion and the generation of primary CD8+ cytotoxic effectors, but are critical in sustaining CD8+ effector response and are required for the generation of an optimal pool of functional memory CD8+ T cells (Janssen, Lemmens et al. 2003). Such studies showed that depletion of CD4+ T cells did not affect pathogen clearance mediated by CD8+ T cells, if mice were infected with low numbers of organisms, but led to persistent infection when higher doses of the same agent were used.

Once it became clear that CD4+ T cells aid in the formation of CD8+ T cell memory responses, the questions that remained were when, where and how this CD4+ T cell help was delivered. In terms of 'when', the bulk of evidence available indicates that CD4+ T cells must deliver one or more signals to the CD8+ T cells directly or indirectly at the time of, or shortly after, initial contact with antigen-bearing APCs (Masopust, Kaech et al. 2004). In terms of 'where' and 'how', there appears to be at least two alternate models to explain this. One is that antigen-stimulated CD4+ T cells activate DCs via CD40L-CD40 interaction, and the resultant 'licensed DCs' become fully competent to activate naïve CD8+ T cells, even in the absence of an associated CD4+ T cell. CD40 signalling on DCs increases MHC display, costimulatory molecule expression, cytokine secretion and chemokine production, which will coordinate to amplify or sustain CD8+ T cell responses during either the acute or memory phases (Ridge, Di Rosa et al. 1998).

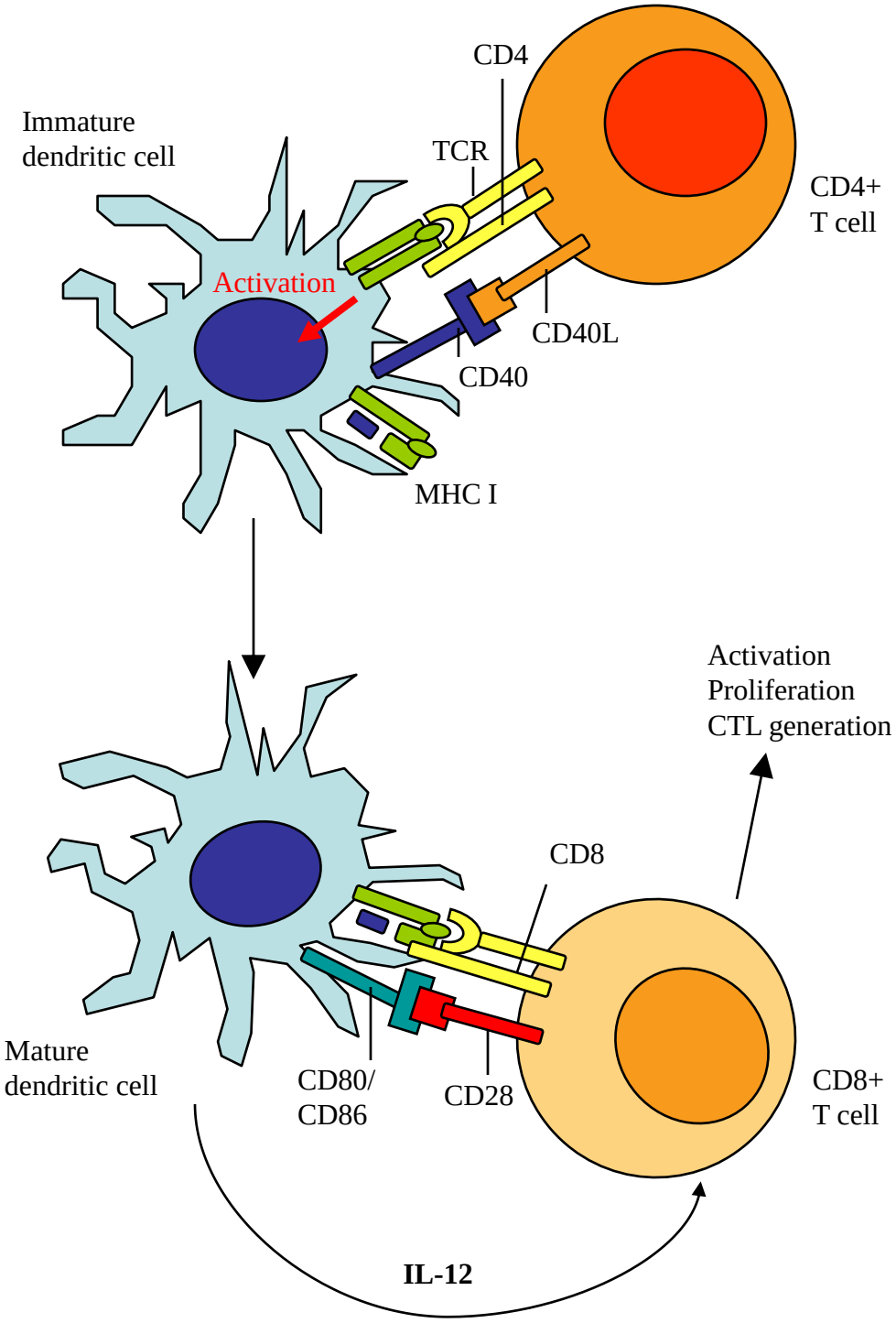
The other, known as the 'three-cell cluster' model, is similar to the early hypothesis regarding CD4+ and CD8+ T cell interactions in that the two cells need to recognise their

specific antigens simultaneously on the same APC, so that IL-2 secreted by the APC-bound CD4+ T cell can stimulate the CD8+ T cell (Keene and Forman 1982), or so that CD4+ T cells can directly stimulate the CD8+ T cell via CD40L (Bourgeois, Rocha et al. 2002). The problem with this latter model is that there is a low probability that these three rare cells, all bearing the right antigen or antigen-specific TCR, will find each other at the same time in the same place. More recent studies have addressed this improbability by showing that DC-CD4+ T cell associations can last for many hours (Shakhar, Lindquist et al. 2005), increasing the probability that the DC-CD4+ T cell couplet will encounter their relevant CD8+ T cell. Furthermore, studies showing that CD8+ T cells preferentially accumulate in lymph nodes in which CD4+ T cells were undergoing antigen-specific activation, suggest that some combination of chemokinesis and chemoattraction can further increase the probability of naïve CD8+ T cells of encountering DCs engaged in productive interactions with CD4+ T cells. Further blocking antibody experiments suggested that the inflammatory chemokines, CCL3 and CCL4, were responsible for the chemoattraction (Castellino and Germain 2006).

These ideas can be simplified into one overall model of CD4+ T cell control of CD8+ T cell responses (figure 1.3). When CD8+ T cells recognise their antigen, generally presented by DCs, the T cell can make a productive response, or can be rendered anergic and possibly even die (Mescher, Agarwal et al. 2007). CD8+ T cell activation thus depends on three signals: antigen engagement by the TCR, costimulation via CD28 engagement of its ligands, and a third signal which is often IL-12 produced by the DC. Engagement on CD40 on DC stimulates the cells to produce IL-12, thus CD4+ T cells are in control of this first checkpoint for CD8+ T cell response. CD8+ T cells can proliferate and generate effector function in the absence of a third signal, but the death of the majority of the responding cells that occurs following the peak of clonal expansion is more rapid and profound in the absence of a third signal (Curtsinger, Lins et al. 2003). Even in the presence of a third signal, at around 72hr after first encountering antigen, and in the face of a persistent antigen, the CD8+ T cells can reach activation-induced non-responsiveness (AINR). This constitutes a second checkpoint, and if the effector CD8+ T cells are to continue to expand IL-2 must be provided by helper T cells, which works to

reverse the effects of AINR (Mescher, Agarwal et al. 2007). These checkpoints may be the major means by which self-reactive CD8+ T cells are prevented from productively responding to cause autoimmune disease or transplant rejection. This idea will be discussed further in section 1.5 on tolerance in the immune system.

Figure 1.3. The activation of dendritic cells by CD4+ T cells helps stimulate CTL responses. Phagocytosed antigen is presented to CD4+ T cells, which activate the DC through CD40-CD40L interactions. The activated DC can then promote the CD8+ T cell response via antigen engagement of the TCR, costimulation via CD28, and via the production of IL-12.



1.4 Generation, structure and function of the αβ T cell receptor (TCR)

The T cell receptor is a membrane bound heterodimer composed of two polypeptide chains (α and β) linked by a disulphide bond, that forms a complex with a peptide-MHC ligand (pMHC). Although TCR recognition of pMHC is functionally similar to antibody-antigen interaction in the humoral system (Davies and Metzger 1983), T cell recognition is a more complex process. Specificity in T cell responses arises from the extensive repertoire of TCRs coupled to polymorphism in the MHC that controls the size and diversity of the peptide repertoire presented (Garcia, Teyton et al. 1999). Furthermore, the TCR does not bind pMHC in isolation but does so in associated with a signalling complex that includes membrane-bound proteins including CD3 γ, δ, ε and ζ chains, and coreceptors CD8 or CD4 (Clevers, Alarcon et al. 1988).

The TCR is composed of constant (C) and variable (V) regions, which are assembled together during thymic ontogeny (Alt, Oltz et al. 1992). The diversity of the TCR is generated via gene rearrangement within the variable domains of the TCR, which is the (V) and junction (J) gene segments in the Vα chain, and the V, diversity (D) and J gene segments in the Vβ chain. The TCRα genes located at chromosome 14q 11-12 consist of 70 V segments and 61 J segments, while the TCRβ genes located at chromosome 7q 32-35 consist of 67 V segments, 2 D segments and 13 J segments. During TCR gene rearrangement the V and J, or V, D and J segments are brought together in a continuous V-J or V-D-J coding block, forming complete V domain exons that are responsible for antigen recognition. This process is catalysed by recombination activation genes, RAG1 and RAG2 (Oettinger, Schatz et al. 1990), and the enzyme terminal deoxynucleotidyl transferase (TdT) (Landau, Schatz et al. 1987). However the diversity of the TCR depends not only on the recombination of these genes but is also greatly increased by nucleotide insertion and deletion at the junctions between these genes. The greatest diversity is present at the third complementarity determining region (CDR3), which spans the V(D)J junction. The CDRs are regions of greatest sequence variability (CDR1 and CDR2 are located within the V domain) and constitute the binding site for the peptide-

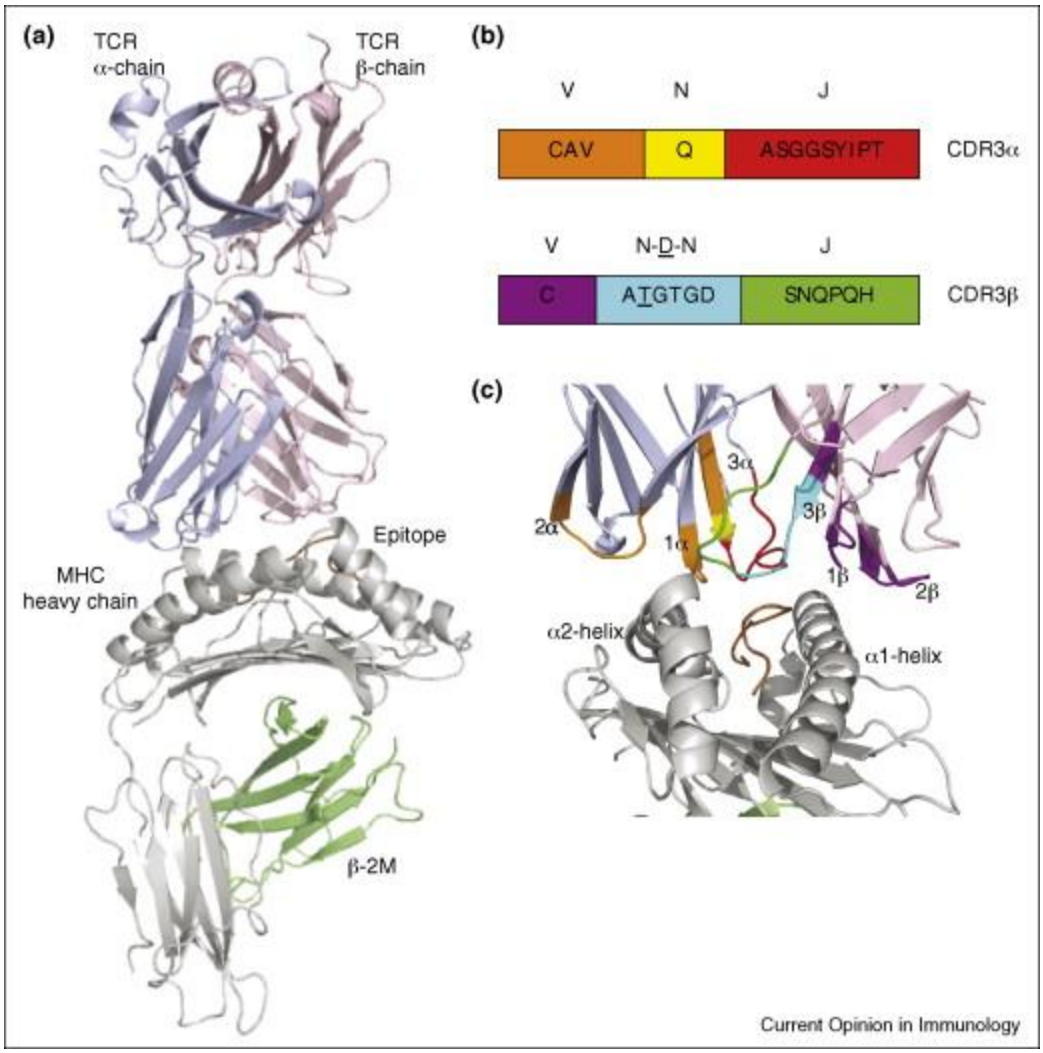
MHC complex, with the CDR3 positioned at the centre of the antigen binding site for direct contact with the MHC bound peptide (Jorgensen, Esser et al. 1992).

The generation of TCR-pMHC crystal structures has allowed us to visualise the interaction of these two molecules in more detail. The MHC molecule is also heterodimeric, with the class I molecule composed of a heavy chain and β2 microglobulin. Antigenic peptide resides within antigen-binding cleft, which is bound by two long α-helices (α1 and α2) (figure 6.2) (Gras, Kjer-Nielsen et al. 2008). The TCR and pMHC 'dock' together, so that the TCR Vα domain is positioned over the MHC α2-helix and the N-terminal end of the peptide, whilst the TCR Vβ domain contacts the MHC α1-helix and the C-terminal end of the peptide. Within this framework, either or both of the CDR3 loops can interact with the peptide and also with the MHC. Likewise whilst the CDR1 and CDR2 loops generally interact with the MHC, they have also been observed to interact with the peptide (Rudolph, Stanfield et al. 2006).

Once the nature of TCR recognition of MHC and peptide had been more or less resolved, the next question was what happens following TCR engagement by pMHC? The earliest biochemical events that have been detected after TCR engagement is the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of the TCR/CD3 complex, mediated by the Src family tyrosine kinases, Lck and Fyn (Weiss and Littman 1994). This step is believed to be essential for signal transduction by the TCR, and consequently there have been many models designed to explain how TCR binding to pMHC stimulates the ITAM phosphorylation. These models use one or more of three basic mechanisms to explain the transduction of the signal across the T cell membrane: aggregation, conformation change, and/or segregation (Choudhuri and van der Merwe 2007).

One of the more rigorously tested models is the kinetic-segregation model, which falls into the segregation category. The model depends on the fact that when a T cell and an APC come into close contact, they form 'close-contact' zones that exclude large molecules such as CD45 and CD148, which are inhibitory tyrosine phosphatases that keep the ITAM regions dephosphorylated and the T cell in a 'resting' state. TCRs that

Figure 1.4. The interaction of the T cell receptor with MHC class I. Panel (a) shows a ribbon schematic of the TCR α and β chains interacting with a ribbon schematic of a MHC class I molecule. Panel (b) gives a colour coded representation of the Variable, Diversity and Junctional regions that make up the parts of the TCR interface that interact with the surface of the MHC class I molecule and the peptide epitope in the peptide groove, as shown in panel (c). Reprinted from Current Opinion in Immunology (2008) February, volume 20(1), pages 119-25.



bind pMHC in these close-contact zones are able to be phosphorylated by tyrosine kinases like Lck, and the signal is transduced. If there are no TCR-pMHC associations then the TCRs will diffuse from the close-contact zone and come into contact with CD45 and CD148 again, and the T cell will remain in a resting state (Davis and van der Merwe 2006).

1.5 Tolerance in the immune system

Our knowledge of the how the TCR is assembled, and the mechanisms used to create TCR diversity, allows us to predict the potential number of TCRs that can be formed. Of the three CDR loops, which create the binding face that contacts antigen, CDR1 and CDR2 are encoded by the V gene segment, and have only the diversity provided by the number of germline V region gene segments, around 20-70 Vα and Vβ segements in mice and humans. If there were only 50 Vα and Vβ genes to encode the TCR repertoire, combinatorial pairing would provide only 2,500 TCRs (50 x 50). Fortunately the CDR3 loop, created by the juxtaposition of VJ or V(D)J segments, provides much more diversity, as a result of each V segment being able to rearrange to any (D)J segment compounded by the fact that the joining of these sequences is imprecise (Goldrath and Bevan 1999) (see figure 6.1). These factors boost the number of possible TCRs from the thousands to the billions, with a theoretical maximum approximated to be 10^{15} possible TCRs (Casrouge, Beaudoing et al. 2000). The actual number of TCR clones present in the periphery (around 10^7 in humans) is a fraction of this theoretical diversity maximum, partly due to structural limitations of harbouring that many T cells, but also due to the development and selection process in the thymus known as central tolerance.

1.5a. Central tolerance

Bone marrow stem cells enter the thymus and commit to the T cell lineage in response to signals from the microenvironment. The earliest precursors are CD4- CD8- double negative cells, at which point the TCRβ chain is assembled. These cells then proliferate extensively and become CD4+CD8+ double positive cells, at which point the TCRα chains rearrange, making these cells the targets for the TCR selective events. These selective events are severe, with the consequence that ultimately only about 5% of the double positive cells are allowed to emigrate (Goldrath and Bevan 1999). During their approximate 3-day lifespan the double positive cells will continue to rearrange their TCRα chain genes in an attempt to form a heterodimeric αβ TCR that can recognise MHC molecules expressed on thymic epithelial cells (TECs). Those cells that manage

this are ‘positively selected’, and are rescued from apoptic death by neglect of their receptor, allowing these cells to switch off further TCRα chain gene rearrangement. Depending on whether the double-positive cells recognise class I or class II MHC, cells then commit to either the CD8 or CD4 lineage respectively (Jameson, Hogquist et al. 1995). However, the selection of the TCR repertoire is not solely based on the recognition of self-MHC molecules, but on the recognition of those structures modified by the binding of numerous self-peptides. The number and nature of the self-peptides involved in positive selection has been controversial. The data accumulated to date suggests that relatively rare, low-affinity self-peptides promote positive selection, giving rise to mature T cells having high affinity for foreign peptides that are generally structurally related to the self-peptides involved in selection (Starr, Jameson et al. 2003). This ensures that a diverse repertoire is created able to bind strongly to pathogen-encoded peptides bound by the same MHC molecules in the periphery.

The weak TCR-self-peptide interactions extend beyond the thymus, as naïve T cells continue to depend on continuous survival signals supplied by these interactions in the periphery (Freitas and Rocha 1999). Only memory T cells are not dependent on recognition of self-antigen in the periphery.

However, positive selection is only half the story of central tolerance. Whereas low affinity interactions with self-peptide and MHC are necessary for the survival of double positive cells, high-affinity interactions lead to the death of those T cells through ‘negative selection’. Almost half of the cells reacting with self-MHC are lost though this mechanism (van Meerwijk, Marguerat et al. 1997). The purpose of negative selection is to prevent autoreactivity, as any high-affinity peptide-MHC interactions with TCRs in the periphery lead to rapid proliferation and generation of effector and memory T cells, irrespective of whether the peptide recognised is self or foreign (Goldrath and Bevan 1999). Positive selection peptides are generally not stimulatory for mature T cells, but stimulatory peptides for a given T cell cause clonal deletion if present in thymus during thymocyte development. However, a comprehensive negative selection of all potential autoreactive T cells would depend on presentation of stimulatory peptides beyond those that would be expected to be present during thymocyte development. To this end, TECs are able to constitutively synthesise and express many peripheral tissue-specific antigens

that would be otherwise be unavailable to induce thymic tolerance, a function which is dependent on expression of the autoimmune regulator (AIRE) gene. Consequently, AIRE-deficient humans and mice develop organ specific autoimmunity (Anderson, Venanzi et al. 2002). This TEC-mediated central tolerance can be extended by transfer of the antigens to bone marrow-derived APCs, which can also mediate negative selection in the thymus (Gallegos and Bevan 2006).

1.5b. Peripheral tolerance

Given the careful purging of autoreactive T cells from the repertoire in the thymus during central tolerance, it was thought for a time that additional peripheral tolerance mechanisms would not be necessary. As the affinity threshold of TCR-pMHC interaction that signals thymic deletion is lower than that for activation in the periphery, even T cells with low avidity for self-antigens will not be activated in the periphery and instead remain 'ignorant' of their cognate antigen.

For several reasons however, activation of auto-reactive T cells remains an ever present danger. One reason is that in the periphery the immune system is constantly exposed to the numerous innocuous environmental antigens, to which immune responses could be formed. Secondly, self-antigens that are restricted to immunological privileged sites, and are thus physically inaccessible to T cells, may be compromised through injury, exposing the immune system to potentially unseen autoreactive epitopes. Furthermore, the 'ignorance' of low affinity autoreactive T cells could be broken given the proper stimulatory milieu, leading to the formerly ignored antigens initiating autoimmune responses (Redmond and Sherman 2005). Indeed, it has been shown that viral priming can break CD8+ T cell ignorance and promote autoimmunity (Ohashi, Oehen et al. 1991). Finally, despite the activity of AIRE, not all self antigens are expressed in the thymus. For example one autoimmune disease is caused by the normal and tolerising version of a peptide being presented in the thymus, while a cryptic stimulatory version of the same peptide is presented in the periphery (Badami, Maiuri et al. 2005).

The tolerance mechanisms that exist to combat the persisting problem of autoreactivity in the periphery are numerous and varied. Perhaps the most important peripheral tolerance

mechanism is the requirement for multiple antigen-specific lymphocytes to interact, either directly with each other or through the intermediation of DCs (Castellino and Germain 2006). This in other words is the need to have DCs, CD8+ T cells and CD4+ T cells to initiate an autoreactive response. As examined in section 1.3, a lack of CD4+ T cell help will not allow a sustained or memory CD8+ T cell response. It is logical to assume that the chance of an autoreactive CD8+ T cell and an autoreactive CD4+ T cell firstly both escaping from the thymus and then secondly both meeting their cognate self-antigens together on the same APC, is much lower than the chances of only a CD8+ T cell doing this. Furthermore, T cells require that the DCs presenting their cognate antigen have acquired the capacity to effectively trigger T cell responses, and for this to happen DCs must be stimulated through receptors such as the TLRs with by-products of foreign invasion such as viral DNA (Lee and Iwasaki 2007). In the absence of pathogens however, DCs are quiescent and express low levels of costimulatory molecules such as CD80 and CD86, which interact with CD28 on T cells to enhance their responsiveness and survival. Consequently, T cells recognizing their antigen in the absence of costimulation only briefly proliferate and develop effector cell function only suboptimally (Redmond and Sherman 2005). This ultimately leads to either the death of the antigen-activated CD8+ T cells (deletion), or to the induction of a long-lived non-responsive state (anergy) (Redmond and Sherman 2005).
Some believe that the main reason behind the evolution of an immune system with critical requirements for cell-cell cooperation as detailed above, is to impose controls on the development of autoreactive responses (Bretscher and Cohn 1970). This idea also extends to the interaction between CD4+ T cells and B cells bearing potentially autoreactive antibody.

Studies that demonstrated that it was possible to develop an effector T cell response in the absence of adjuvant (Rocha, Grandien et al. 1995), found that the critical variable was the persistence of antigen. Later results also concluded that antigen localisation, dose and persistence are the critical factors that determine tolerance induction, rather than just the delivery of costimulating signals by APCs (Zinkernagel 2000).

The other main peripheral tolerance mechanism is the existence of suppressive cellular elements, the most well understood member being the CD25+ regulatory T cell (T reg) (detailed in section 1.6). It is clear that T regs play a significant role in maintaining peripheral tolerance, not least by the fact that numerous chronic and destructive autoimmune diseases that are unleashed by elimination of the T reg population in mice. These include gastritis, oophritis, thyroiditis, adrenalitis and insulitis, suggesting that the activation and expansion of such self-reactive T cells is normally kept in check by T regs. Furthermore, the appearance of various disease-specific autoantibodies in the T reg depleted animals implies that the breakdown of this mode of peripheral tolerance, and the development of autoimmune CD4+ helper T cells results in breakdown of B cell self-tolerance as well (Sakaguchi 2004).

1.6 Regulatory T cells

The theory of immune regulation via a network of suppressor T cells first gained popularity in the 1970s, but the theory experienced several set-backs and only relatively recently has the scientific community truly embraced it. Prior to recent key discoveries it was believed that the mechanism of negative selection in the thymus was sufficient to remove nearly all auto-reactive T cells, rendering the need for any peripheral suppressor mechanism redundant. However it is now abundantly clear that in the absence of a distinct population of regulatory cells, tolerance to self-tissues is lost and severe multi-system autoimmune disease results (Sakaguchi, Sakaguchi et al. 1995).

It is now clear that potentially very damaging auto-reactive T cells can escape deletion in the thymus and thus must be kept in check by one or more peripheral tolerance mechanisms. These include deletion, anergy and ignorance (Mackay 2000), plus the more 'active' mechanism of regulatory T cells.

The purpose of a regulatory T cell is to prevent damaging inflammatory responses in the periphery, playing a role in dampening not only autoimmune responses (Sakaguchi, Sakaguchi et al. 1995) but also responses to allergens, pathogens, and tumour cells (Grindebacke, Wing et al. 2004), (Lundgren, Suri-Payer et al. 2003), (Onizuka, Tawara et al. 1999). It is clear therefore, that regulatory T cells can be both beneficial and detrimental to the host organism.

To date there have been several types of regulatory T cell identified (see table 1.6.). Regulatory properties are found in gamma-delta cells, NKT cells, CD8+ T cells and CD4+ T cells (Bach 2003). Within CD4+ regulatory T cells there are further divisions: some such as Tr1 (Levings, Bacchetta et al. 2002) and Th3 cells (Weiner 2001) are induced to secrete suppressive cytokines such as IL-10 and TGF-beta; others occur naturally like the CD4+CD25+ regulatory T cell (T reg). Other additions to the regulatory cell family include double negative T cells (Zhang, Yang et al. 2000), and myeloid suppressor cells (Bronte, Apolloni et al. 2000). But the most extensively studied of all these cells is the CD4+CD25+ regulatory T cell (T reg).

Regulatory cell	Phenotype	Regulatory Mechanism
γδ T cells	γδ T cell receptor	Cytokines
NKT cells	NK1.1, αβTCR	IL-4, IL-10, TGFβ, IFNγ, cytoxicity
Tr1 cells	CD4+	IL-10, (TGFβ)
Th3 cells	CD4+	TGFβ, (CTLA-4)
T regs	CD4+ CD25+, Foxp3+	Cell-cell contact (bound TGFβ, CTLA-4), IL-2 sink, cytokines (TGFβ, IL-10)
DN T cells	CD3+, CD8-, CD4-	Cell-cell contact, soluble mechanism
MSCs	Gr1+, CD11b+	ARG, iNOS
CD8+ T regs	CD8+CD25+	Cell-cell contact
CD8 suppressors	CD8+CD28-	IFNγ, IL-6

Table 1.1. Table of common regulatory cell subsets.

1.6a. CD4+CD25+ regulatory T cells (T reg)

T regs are often referred to as 'naturally occurring' regulatory T cells as they exist as a distinct subset of T cells in every normal individual, comprising approximately 5-10% of the peripheral CD4+ T cells in mice and humans (Sakaguchi, Sakaguchi et al. 1995).

T regs were first identified when it was found that the high affinity IL-2 receptor alpha chain (CD25) could serve as a marker for a subset of CD4+ T cells with regulatory properties (Sakaguchi, Sakaguchi et al. 1995). Cell suspensions prepared from normal BALB/c mice were depleted of the peripheral $CD4^+$ cells that express CD25, and then inoculated into athymic nude mice. Subsequently the recipients spontaneously developed autoimmune diseases (throiditis, gastritis, insulitis, adrenalitis, oophoritis, glomerulonephritis, polyarthritsis), with some others also developing a graft-versus-host

like wasting disease. Prompt reconstitution of the $CD4^+CD25^+$ cells after transfer of $CD4^+CD25^-$ cells prevented the autoimmune developments.

Investigations into the phenotype of these cells found that they are generally $CTLA^+$, $CD45RB^{low}$, with GITR, CD62L, and membrane-bound TGF-β also sometimes present (Sakaguchi, Sakaguchi et al. 1995). However, the majority of the known markers for T regs, including CD25, are also upregulated on CD25- T cells after stimulation. Therefore no single surface marker is exclusively expressed by or needed for the development of functional T reg and such a marker remains to be identified.

Despite this, the idea that T regs are a lineage distinct from other T cells is supported by the T reg-specific forkhead/winged helix transcription factor *Foxp3*. The importance of *Foxp3* was discovered in scurfy mice, which have a spontaneous X-linked mutation in *Foxp3* causing a fatal lymphoproliferative disease (Brunkow, Jeffery et al. 2001). The human form of *Foxp3* is also mutated in patients with IPEX (Immunodysregulation, Polyendocrinopathy, and Enteropathy, X-linked), a severe and fatal autoimmune/allergic syndrome (Gambineri, Torgerson et al. 2003). Studies suggest that *Foxp3* is a master regulatory gene for T reg-lineage commitment, and is crucial in the differentiation of T regs in the thymus and the periphery (Hori, Nomura et al. 2003).

It is now generally accepted that T regs are selected during the process of T cell differentiation in the thymus. Thymectomy of mice at day 3 of life leads to multi-organ autoimmune disease, due to the fact that T regs do not emerge from the thymus until after day 3 (Asano, Toda et al. 1996). Studies suggest that T regs require a TCR-self-peptide/MHC-class II interaction stronger than what is required for normal positive selection, but lower than the threshold for negative selection. Where this interaction between the T reg and its self-antigen occurs is not certain, but the answer is probably either on medullary dendritic cells (Jordan, Boesteanu et al. 2001), or on thymic cortical epithelium (Bensinger, Bandeira et al. 2001).
However, it is also becoming clear that T regs can originate in the periphery. When T cells with known TCR specificities from TCR transgenic mice are transferred into wild-

type mice and immunized with low doses of the known peptide (but crucially with no adjuvant for costimulation), the transferred T cells develop into CD4+CD25+ T regs with regulatory properties (Thorstenson and Khoruts 2001).

Once in the periphery, it is very likely that T regs can and do respond to a number of different antigens via their TCR. Analysis of TCR αβ gene segments of $CD25^+$ T regs suggest that their TCR repertoire is as similarly diverse as $CD4^+CD25^-$ T cells (Kasow, Chen et al. 2004). Supporting this is the fact that $CD25^+$ T regs seem to play a role in balancing nearly all immune responses including chronic infection and allergy. For example, $CD25^+$ T regs can suppress responses to foreign antigens including *Heliobacter pylori* peptides (Lundgren, Suri-Payer et al. 2003) and pollen extract *in vitro* (Grindebacke, Wing et al. 2004). The reason behind such a broad TCR repertoire may be that T regs need to interact with their specific antigen in order to suppress immune responses.

Peripheral CD4+ T cells, from rats whose thyroids were ablated in utero, were unable to prevent autoimmune thyroiditis development upon adoptive transfer into thymectomized and irradiated recipients. However, the capacity of these regulatory T cells to protect against other autoimmune diseases, like diabetes, remained (Seddon and Mason 1999). Significantly, unlike the peripheral CD4+ T cells, CD4+ thymocytes from thyroid-ablated donors were still able to prevent thyroiditis upon adoptive transfer. This indicates that it is the peripheral autoantigen itself that stimulates the generation of the appropriate regulatory cells from thymic emigrant precursors.

Quite how $CD25^+$ T regs suppress other T cells is still poorly understood, but generally the mechanism seems to rely on the inhibition of IL-2 transcription in the effector populations, as suppression can be abrogated by the addition of exogenous IL-2 or by enhancing endogenous IL-2 production (Shimizu, Yamazaki et al. 1999). The *in vitro* data suggests that suppression by $CD25^+$Tregs relies on an unknown cytokine-independent cell-contact-dependent mechanism, involving CTLA-4 (Takahashi, Tagami et al. 2000), and/or cell surface TGF-β1. However, the *in vitro* data contrasts markedly with the *in vivo* data, which implicates several cytokines as mediators of inhibition,

including TGFβ, IL-10, and IL-4 (Asseman, Mauze et al. 1999), (Seddon and Mason 1999). The discrepancy between the *in vitro* and *in vivo* data may be explained by studies in which $CD25^{+}$Tregs induce suppressive properties in CD4+CD25- T cells when cultured *in vitro*. This 'infectious tolerance' causes the CD4+CD25- T cells to become anergic and produce IL-10 (Dieckmann, Bruett et al. 2002) or TGF-β (Jonuleit, Schmitt et al. 2002). The initial culture of CD4+CD25- T cells and $CD25^{+}$ Tregs required cell-contact for the induction of anergy, but if the anergic cells were transferred to fresh cultures, they suppressed naïve T cells in a cytokine-dependent cell-contact independent manner.

1.6b. Double negative T cells

Double negative (DN) T regs are a further suppressive subset of T cells, and are CD3+, CD4-, CD8- and NK1.1-. They represent a very small number of T lymphocytes in the periphery of mice and humans (1-5% in mice and 1-2% in humans). They have a unique set of cell surface markers, as they express neither CD4 or CD8 co-receptors, nor the costimulatory molecule CD28. They also produce a unique array of cytokines compared to other regulatory T cells, including predominantly IFN-γ, TNF-α and a low amount of TGF-β, but not IL-2, IL-4, IL-10 or IL-13 (Zhang, Yang et al. 2000). Also unlike other T reg cells, DN T regs do not express the activation markers CD44 or CD28 any time after activation, but Foxp3 mRNA has been detected in these cells. Furthermore, unlike CD4+ or CD8+ cells which are sensitive to activation induced cell death, DN T reg cells are resistant to apoptosis induction both in vitro and in vivo (Chen, Ford et al. 2004).

Numerous studies have shown these DN T regs to be a subset of potent immune regulatory cells. DN T cells were isolated from Donor Specific Transfusion (DST)-treated mice and used to suppress and kill CD8+ and CD4+ T cells in an antigen specific manner in vitro, and prolonged donor-specific allograft survival when adoptively transferred into naïve syngeneic mice (Zhang, Yang et al. 2000). Furthermore, mice infused with DN T reg cells were protected from the development of Graft Versus Host Disease (GVHD) (Young, DuTemple et al. 2003). The mechanism of this suppression has

shown to be antigen specific and cell-cell contact dependent. The DN T regs acquire MHC-peptide complexes from neighbouring antigen presenting cells (APCs), which remain expressed on the surface of the DN cells for several days, allowing suppression of other T cells by bringing the T cells that are able to recognize the acquired allo MHC-peptides into cell contact (Zhang, Yang et al. 2000). The suppression/elimination of these T cells is at least partially Fas-FasL dependent, because blocking of FasL on DN T reg cells using mAb significantly inhibits DN T cell mediated killing (Zhang, Yang et al. 2000), while DN T cells from gld mice that express mutant FasL showed a reduced ability to kill CD8 T cell targets when compared to wild-type FasL (Ford, Young et al. 2002). However there is also some evidence that DN T cells are able to partially suppress T cell responses in the absence of cell contact, suggesting some soluble factor could be involved in the suppression (Chen, Ford et al. 2004).

1.6c. Myeloid suppressor cells

Another potent suppressor of T cell responses is the myeloid suppressor cell (MSC), a cell that has been mostly defined in the context of facilitation of tumour growth. MSCs represent a heterogenous population of myeloid suppressor cells comprising immature macrophages, granulocytes, dendritic cells and other myeloid cells at an early stage of differentiation, and are identified in mice by the expression of CD11b and Gr-1. In healthy mice MSCs are only present in large numbers in the bone marrow, but they can be detected in small numbers in the blood and the spleen. These cells become suppressive cells only in the correct cytokine environment (with the Th2 cytokines IL4 and IL10), and will develop into functional APCs in other circumstances (Bronte, Apolloni et al. 2000).

Numerous findings have indicated the importance of tumour-derived factors (TDFs) in encouraging the suppressive aspects of MSCs, by both recruiting MSCs and promoting their maturation towards a suppressive phenotype. Such TDFs include: Colony stimulating factor 1 (CSF-1), IL-6, IL-10, VEGF, and GM-CSF (Serafini, Borrello et al. 2006). Once activated MSCs inhibit the immune system and promote tumour growth by

expressing ARG and iNOS. ARG is an enzyme that converts L-Arginine into L-Ornithine, which aids cell transformation and tumour proliferation (via neovascularisation) (Serafini, Borrello et al. 2006). Inducible Nitric oxide synthase (iNOS) expression increases super-oxide and NO production which inhibits the mitogenic and peptide-specific responses of the CTL response (Xia, Roman et al. 1998).

1.6d. CD8 suppressor T cells

A further group of regulatory cells are those that express the CD8 coreceptor. In particular, two distinct subpopulations of CD8+ T suppressor lymphocytes have been identified.

The first characterized CD8+ T suppressors induce an antigen-specific immune suppression through cell-to-cell contact with antigen presenting cells (APC) after antigen presentation (Liu, Tugulea et al. 1998). One report demonstrated that xenospecific suppressor CD8+ T cells can be generated by multiple in vitro stimulations of human T cells with pig PBMCs, which then specifically recognize xenogeneic MHC class I antigens and suppresses the proliferative response of CD4+ cells to MHC class II antigens expressed by the xenogeneic APCs (Ciubotariu, Colovai et al. 1998).

The second population of CD8+ Ts mediates a nonantigen specific suppression of T-cell proliferation via soluble factors, such as interferon-γ (IFNγ) and interleukin-6 (IL-6) (Balashov, Khoury et al. 1995). These non-antigen-specific CD8+ T suppressor lymphocytes originate from circulating CD8+CD28- T lymphocytes after stimulation with interleukin-2 and interleukin-10 (Filaci, Fravega et al. 2004). Interestingly, the nonantigen specific CD8+ Ts have been found functionally impaired in patients affected by relapsing phases of multiple sclerosis (Balashov, Khoury et al. 1995), and in patients with systemic lupus erythematosus in relapse (Filaci, Fravega et al. 2004), suggesting their possible direct involvement in the pathogenesis of autoimmune diseases.

CD8 suppressor T cells have also been implicated in being involved in tumor-induced-immunosuppression, as they have been found among tumour-infiltrating lymphocytes (Filaci, Fravega et al. 2004).

1.7 The current state of tumour "immune surveillance"

In Hanahan and Weinberg's landmark review in Cell (Hanahan and Weinberg 2000) the cell-intrinsic characteristics of cancer cells were articulated for the first time. They concluded that successful oncogenesis depended on six essential alterations, or "hallmarks", in cell physiology that collectively dictate malignant growth. These were: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these changes represents a successful breaching of an anticancer defense mechanism hardwired into cells and tissues. There is accumulating evidence that 'avoidance of immunosurveillance' might be the seventh hallmark of cancer. Avoidance of immunosurveillance takes the form of immunoselection, the selection of non-immunogenic tumour variants also known as immunoediting; or immunosubversion, which is the active suppression of the immune response (Zitvogel, Tesniere et al. 2006).

It is well established that mice that lack the essential components of the innate or adaptive immune system are more susceptible to the development of spontaneous or chemically induced tumours. From the current scientific literature it is possible to identify several cell types and a range of effector molecules that are involved in cancer immunosurveillance, including T and B cells, perforin, tumour necrosis factor-related apoptosis inducing ligand (TRAIL) (Takeda, Smyth et al. 2002) NK and NKT cells (Smyth, Wallace et al. 2005) and IFN-producing killer DCs (Taieb, Chaput et al. 2006).

But what is the evidence that immunosurveillance plays a role in suppressing human cancer? In patients the presence of immunosurveillance is indicated by responses to pre-malignant or early cancerous lesions. Examples include T cell responses to pre-malignant B cells in patients with monoclonal gammopathy (Dhodapkar, Krasovsky et al. 2003), and the presence of CD8+ T cells specific for peptides derived from breast cancer associated proteins in the bone marrow of patients with operable breast cancer (Beckhove, Feuerer et al. 2004). Notably however, whereas tumours may induce at least

transient immune responses, cancer can still develop. These immune responses fail to prevent the development of cancer either because tumour cells that evade the immune response are selected or because tumour-antigen-specific tolerance is induced (Willimsky and Blankenstein 2005). Despite this immune responses to tumours remain at the very least as useful diagnostic and prognostic factors. Antibodies specific to tumours (also known as antibody signatures) can be used detect cancers such as prostate cancer at early stages (Wang, Yu et al. 2005). In many cancers the presence of tumour-infiltrating lymphocytes (TILs) is a useful prognostic marker, especially in melanoma, ovarian carcinoma and colon carcinoma (Zitvogel, Casares et al. 2004).

1.7a The current state of cancer vaccines

The logical extension to the idea that the immune system could play a crucial role in controlling cancer is the development of tumour vaccines. To date the only clinically successful vaccine designed to combat cancer is the human papillomavirus (HPV) vaccine that has been shown to protect against the development of cervical cancer caused by HPV (Siddiqui and Perry 2006). Of course this has more in common with traditional vaccines, and a *bona fide* tumour vaccine has yet to be developed. However the advantages a tumour vaccine would have over traditional cancer treatments are clear. Their unique mechanism of action, recalibrating the patient's immune system to seek out and destroy cancer cells, overcomes the barrier of intrinsic drug resistance limiting current therapies. The immunisation would be relatively non-toxic when compared to the side effects of current therapies, presuming autoimmune reactions are not induced. The immune response induced by the vaccine would be very specific, further reducing toxicity and increasing efficacy. Perhaps most significantly a tumour vaccine would induce immunological memory, so that the immune system would be reactivated to clear any recurrence of disease. This would remove the need for the damaging repeated cycles of treatment typical of current therapies (Emens 2006).

The promise of tumour vaccines is somewhat undermined by the very different challenges that they face compared to traditional viral vaccines. Firstly cancers arise from

endogenous tissues that have acquired genetic mutations that disrupt the regulatory pathways governing cell division, and thus are viewed by the immune system as self rather than foreign. This also means that anti-tumour response is curtailed by the mechanism of self-tolerance that normally exists to prevent autoimmunity. Secondly tumours do not present foreign well-defined targets that vaccines are traditionally made against, and often present unaltered or only subtly altered endogenous molecules. Vaccine development is also complicated by the fact that traditional vaccines utilise antibodies as the main effector mechanism, whereas the key effector of anti-tumour responses are T cells (Emens 2006). Furthermore anti-tumour vaccines will be often faced with treating already established disease that may be too large a burden for the immune response to overcome (Perez-Diez, Spiess et al. 2002).

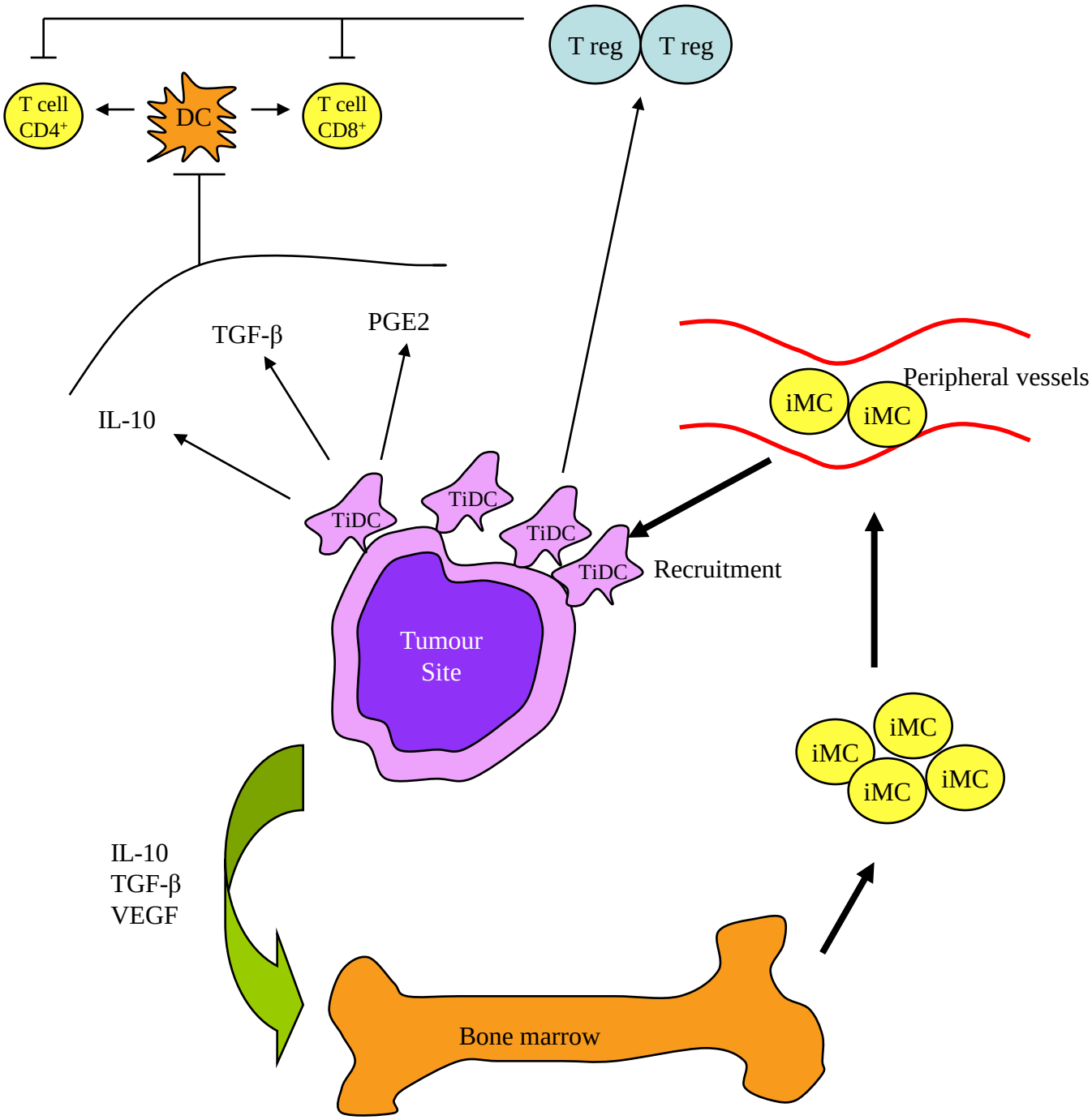
Thus the successful development of therapeutic vaccines depends on overcoming these challenges.

Perhaps the most simple is to use cancer vaccines at the same time as traditional treatment, the advantages being not only that disease burden would be reduced but also with the side-effect that chemotherapy-induced tumour cell death augments vaccine-mediated immune priming (Broomfield, Currie et al. 2005);(Lake and Robinson 2005). Besides this however there are many ways in which the host-tumour interaction can be tipped in favour of the immune response by increasing the effectiveness of the T cell response itself. This could be done by manipulating the events that regulate T cell priming, or by removing the regulatory systems that maintain tolerance to tumours.

1.7b. Tumour evasion of immune responses

However, it is not a simple task to simply ‘remove’ the tolerance to tumours, as it is now evident that tumours can create a multi-faceted immune suppression network to maintain tolerance. This is mediated by soluble factors derived from the tumours such as IL-10, TGF-β and VEGF, which induce immature myeloid cells and regulatory T cells to inhibit DC maturation and T cell activation that would otherwise have mediated an anti-tumour response (Kim, Emi et al. 2006) (see figure 1.4).

Figure 1.5. The cancer immunosuppressive network. Tumour derived soluble factors such as VEGF, IL-10 and TGFβ induce immature myeloid cells (iMCs) from the bone marrow. The iMCs are recruited to the tumour site where they are biochemically and functionally modulated to become tumour-associated immature DCs (TiDCs), which gain immunosuppressive activity and become resistant to apoptosis. TiDCs can secrete immunosuppressive factors such as prostaglandin E2 (PGE2), or recirculate to peripheral tissues where they can inhibit DCs or promote T regs.



Among their weapons is the fact that cancer cells can exploit the anti-inflammatory *and* pro-inflammatory response to escape the immune response. On the one hand, tumour-derived soluble factors (TDSFs) induce immature myeloid cells (iMCs), including immature dendritic cells (iDCs) and immature macrophages. These cells are recruited to the primary tumour site where they release the anti-inflammatory mediators IL-10, TGF-β and prostaglandin E2, which inhibit the activation of DCs and T cells. At the same time, clearance of apoptotic cells is impaired by tumour-derived soluble phosphatidylserine (sPS). This interacts with the PS receptor on DCs and macrophages, inhibiting engulfment by these cells. This defective clearance of apoptotic tumour cells induces auto-antibodies to be made against released self-antigens. This 'pseudo-autoimmune' status is pro-inflammatory, and thus in an effort to control this apparent self-reaction CD25+ T regs are induced that inhibit T cell function (Kim, Emi et al. 2005). Therefore both anti-imflammatory and pro-inflammatory responses can lead to reduced T cell activation against the tumour, due to the factors that the tumour can produce. In autoimmune diseases such as SLE, defective apoptotic cell clearance also causes a pro-inflammatory response involving autoantibodies, although the defect is due to a hereditary complement deficiency rather than sPS. The production of auto-antibodies does not induce an increase in CD25+ T regs in SLE, due to the reduced number and dysfunction of T regs in these patients (Wraith, Nicolson et al. 2004). This emphasises the key role of CD25+ T regs in deciding the outcome of immune responses in both autoimmunity and tumour immunity.

Immunological ignorance and tolerance of tumour cells is aided further by the fact that tumours are surrounded by non-tumour cells, including iMCs, fibroblasts, endothelium and extracellular matrix. These cells act as a barrier to sufficient tumour antigen reaching effector cells, either by binding tumour antigen (extracellular matrix) (Juprelle-Soret, Wattiaux-De Coninck et al. 1988), or by competing with DCs for the antigen (fibroblasts, endothelium) (Savinov, Wong et al. 2003). This reduced level of tumour antigen, coupled with the anti-inflammatory effects of iMCs leads to immunological tolerance of the tumour.

Tumour 'educated' anti-inflammatory iDCs and immature macrophages can also migrate to secondary lymphoid organs, such as the lymph nodes and the spleen, extending the

immunosuppressive network beyond the primary tumour site, assisting tumour progression and metastasis. Recent studies indicate that increased suppressive iMCs were observed in peripheral blood and LNs of patients with breast, head and neck, or lung cancer (Almand, Resser et al. 2000).

1.8 Autoimmunity: The other side of tumour immunology.

Classically, autoimmune diseases are characterised by the activity of autoreactive lymphocytes, which cause tissue or organ damage through the formation of antibodies that react against host tissues, or effector T cells, which are specific for endogenous self-peptides (Sinha, Lopez et al. 1990). These diseases occur when the central and peripheral tolerance mechanisms, detailed in section 1.5, break down. Although the reasons why this occurs are not always clear, phenomena such as molecular mimicry, where T and B cell responses to foreign antigens cross-react with self antigens, are implicated in some autoimmune diseases such as multiple sclerosis (Olson, Croxford et al. 2001). Autoimmune diseases are broadly classified as either systemic, such as systemic lupus erythromacytosis (SLE), or organ specific, such as multiple sclerosis (MS), type I diabetes, and Hashimoto's thyroiditis (Stassi and De Maria 2002).

In some respects immune responses to tumours are effectively autoimmune responses, not least because they are both responses to 'self'. Mutations in tumour cells may create alterations to self-proteins, but in all cases these alterations are against a background of normal gene products (Turk, Wolchok et al. 2002). Importantly the immune system is mostly tolerant of tumour cells, like it is of normal cells generally. Immunological tolerance in cancer and autoimmunity has opposite effects on the patient: in cancer patients tolerance stimulates the growth of tumours and is detrimental to the patient, and in autoimmune patients tolerance may stop the attack by the mediators of the disease and be beneficial to the patient. Thus it is perhaps inevitable that tumour immunology and autoimmunity have been generally viewed as separate subjects and have been investigated independently by separate groups. From the point of view of a biologist studying autoimmunity, self-reactive T cell responses are unfortunate aberrations in immune regulation that need to be suppressed. For tumour immunologists, the immune system appears full of potential autoreactive (but perhaps low-avidity) T and B cells that frustratingly ignore tumour cells. Understanding the basis of tumour immunity and autoimmunity need not be mutually exclusive pursuits, in fact there are often interesting insights gained when the two are compared. These two fields should always be kept close

together, not least because effective treatment in one area can have detrimental effects on the other. Inducing tumour immunity would be a more useful treatment if it did not also induce autoimmunity, and treatment of autoimmune disease would ideally not decrease the potency of tumour immunotherapy.

There are instances when the mechanisms that control tolerance in both tumour immunology and autoimmunity are very similar. As mentioned in section 1.7, the immunosuppressive environment that protects the tumour can be due to the induction of pseudo-autoimmunity. Clearance of apoptotic cells is impaired by tumour-derived soluble phosphatidylserine (sPS), which inhibits engulfment by DCs and macrophages. This defective clearance of apoptotic tumour cells induces auto-antibodies to be made against released self-antigens (that could also be classed as tumour antigens). This 'pseudo-autoimmune' status is pro-inflammatory, and thus in an effort to control this apparent self-reaction CD25+ T regs are induced that inhibit T cell function (Kim, Emi et al. 2005). In autoimmune diseases such as SLE, defective apoptotic cell clearance also causes a pro-inflammatory response involving autoantibodies, although the defect is due to a hereditary complement deficiency rather than sPS. The production of auto-antibodies does not induce an increase in CD25+ T regs in SLE, due to the reduced number and dysfunction of T regs in these patients (Wraith, Nicolson et al. 2004).

There are other instances where autoimmune responses and anti-tumour responses happen concurrently, even apparently in response to the same antigen. The melanocyte differentiation factor gp75 has been identified as an autoantigen of melanoma in both mice and humans (Vijayasaradhi, Bouchard et al. 1990). Immunisation with antibody that induced the development of gp75 autoantibodies in mice led to concurrent tumour immunity and autoimmunity (Hara, Takechi et al. 1995). The tumour immunity induced by immunisation dramatically reduced or even abrogated B16 melanoma metastases in the lung upon intravenous tumour challenge, and it was further demonstrated with depletion experiments that CD4+ T cells and NK cells were required for the tumour immunity. The vitiligo autoimmunity, manifested by depigmentation of hair, was different in that it was not dependent on NK cells or CD4+ T cells. Furthermore, the

immune threshold for depigmentation was substantially higher than for tumour rejection, shown by the fact that 5 times more antibody was required to achieve vitiligo than to prevent tumour growth.

Other studies have used xenogeneic immunisations of other melanoma differentiation antigens such as TYRP-2 to give further insights into concurrent autoimmune and tumour responses. In contrast to gp75, tumour immunity and autoimmunity elicited by human TYRP-2 vaccination of mice required CD8+ T cells (Bowne, Srinivasan et al. 1999). Interestingly tumour immunity, but not autoimmunity, could occur in the absence of perforin or fas ligand, suggesting a perforin-independent mechanism of tumour cell killing. This would probably be IFNγ production by CD8+ T cells, although both tumour and autoimmune responses required the presence of this cytokine.

These studies suggest the existence of overlapping, but alternative antigen-specific mechanisms that mediate tumour rejection and autoimmunity. Both responses can use either the cellular or humoral arm of the immune system in recognition and elimination of tumour or normal cell targets. Overall they suggest that active immunisation could lead to tumour immunity without necessarily evoking concurrent autoimmunity. There seems to be distinct pathways that lead to tumour immunity and autoimmunity, indicating that the immune system can react differently towards an antigen, depending on where that antigen is expressed. These differences could reflect qualitatively different responses to malignant vs. normal cell counterparts (Turk, Wolchok et al. 2002).

The information that is gathered about the similarities and differences between tumour immunology and autoimmunity can only assist the understanding of both fields. Mechanisms that are shared by both autoimmunity and tumour immunology can only help our further understanding of the fundamentals of tolerance in the immune system. The differences between these two spheres should indicate how these seemingly intertwined mechanisms may be uncoupled, benefiting our ability to translate our knowledge into effective treatments that do not cause damaging side effects.

1.8a. Autoimmune thyroiditis

Thyroid autoimmune diseases represent more than 30% of all organ-specific autoimmunity. Hashimoto's thyroiditis is the first described and most common organ-specific autoimmune disease, which affects about 3% of the population and represents the archetype for other T-cell-mediated degenerative diseases, such as type 1 diabetes and multiple sclerosis. Hashimoto's thyroiditis is characterized by an inflammatory infiltrate of immunocytes that replace the parenchyma and induce thyroid enlargement, which eventually leads to gland fibrosis. Progressive thyrocyte depletion results in impaired thyroid hormone production and clinical hypothyroidism, a condition that involves a marked reduction of metabolic activity in various cells and tissues (Weetman and McGregor 1994).

A relatively recently created animal model promises to shed new light on hypothyroidism. This new model is a humanised mouse model of spontaneously arising autoimmune thyroiditis, the TAZ10 mouse (Quaratino, Badami et al. 2004). The transgenic mice express the TCR of the autoreactive human T cell clone 37, isolated from a patient with autoimmune thyroidits. T cell clone 37 is specific for the dominant autoantigen thyroid peroxidise (TPO), $TPO_{535-551}$. Within this peptide, two contiguous epitopes are differentially recognised by T cell clone 37, $TPO_{536-547}$, an agonistic highly stimulatory epitope, and $TPO_{537-548}$, a naturally occurring antagonistic epitope. $TPO_{536-547}$ is a cryptic epitope preferentially displayed after endogenous processing during inflammation. Conversely, the antagonistic epitope induces in vitro anergy in clone 37 when presented by dendritic cells and preferentially displays when whole TPO is presented. There is a possibility that this T cell clone may be anergic and possibly regulatory in the patient, but in the mice it causes spontaneous histological, hormonal and clinical changes comparable to human destructive thyroiditis.
Clone 37 was a CD4+ T cell isolated from the thyroid infiltrate of an autoimmune patient specific for the cryptic $TPO_{536-547}$ epitope restricted by the histocompatibility leukocyte antigen (HLA) DQB1*0602-DQA1*0102 allele. As splenocytes from the CBA ($H\text{-}2^k$) strain of mice were able to present the $TPO_{536-547}$ to the T cell clone 37, the TAZ10 transgenic strain was established on the CBA ($H\text{-}2^k$) background. To exclude the

presence of endogenous TCR α chains, the TAZ10 strain was backcrossed onto the Rag1-/- $H2^k$ background. Experiments showed that TCR^+ $Rag1^{-/-}$ T cells, expressing either CD4 or CD8 co-receptors, are restricted by H2-A^k, and the cryptic epitope $TPO_{536-547}$ proved more efficient at inducing T cell proliferation than the $TPO_{535-551}$ epitope. Molecular modelling showed that this 'xenoreactivity' (i.e. that the TAZ10 TCR could be activated by human TPO peptides restricted by mouse H2-A^k molecules) is because the binding of human $TPO_{536-547}$ to HLA-DQB1*0602 and H2-A^k is similar, due to the structural homology of both molecules. Crucially the modelling also showed that the human $TPO_{536-547}$ epitope (N-DPLIRGLLARPA-C) and the homologous mouse $TPO_{524-535}$ epitope (N-DPIVRGLLARAA-C) presented by H2-A^k, would display a similar antigenic surface, despite the conserved residue differences. This explains why mouse TPO peptides presented by H2-A^k induce specific activation of the TAZ10 T cells, and cause spontaneous autoimmune thyroiditis.

1.9. Subject of the thesis

The goal of the project was to evaluate the immune response generated by the challenge of 'irradiated' CT26 tumour in Balb/c mice. This project would build upon the knowledge gained previously about the CT26 tumour model, and was based on two studies in particular. Huang et al (Huang, Gulden et al. 1996) immunised mice with the carcinogen-induced colorectal tumour, CT26, which was also engineered to secrete granulocyte/macrophage colony stimulating factor (GMCSF), generating CTL lines that were able to lyse the tumour in-vitro, and cure mice of established tumour in-vivo. The group went on to conduct experiments that demonstrated that virtually all the CT26-specific CTLs recognised a single peptide, which contrasted with other tumour systems where multiple bioactive peptide fractions have been detected. The bioactive peptide was identified as a non-mutated nonamer derived from the envelope protein (gp70) of an endogenous ecotropic murine leukaemia provirus, an epitope that became known as AH1. In subsequent work, Gogher et al (Golgher, Jones et al. 2002), showed that untransfected CT26 tumour cells are rejected in Balb/c mice following depletion of CD25+ regulatory T cells, and that this rejection led to the development of long-lived tumour immunity. They also suggested that this immunity was based on a shared-tumour antigen, as this long-lived tumour immunity also included tumours of distinct histological origin, such as A20, a Balb/c B cell lymphoma line derived from a spontaneous reticulum cell neoplasm. This antigen must be different from AH1 as immunisation with CT26-GMCSF tumour does not lead to protection from other tumours such as A20. They concluded that the selected expression of this shared antigen in multiple non-viral induced tumours provided evidence for a unique class of shared immuno-dominant tumour associated antigens as targets for anti-tumour immunity.
Thus my investigation seeks to build upon this work and so use an 'irradiated' CT26 model sought to define more precisely the mechanisms that govern both the anti-CT26 and 'cross-protective' responses.

Among the questions that I intended to address were whether the irradiation of the CT26 tumour altered the nature of the response compared to the live tumour; whether a robust

response to CT26 and cross-protective antigens can be seen in the presence of T regs in the irradiated model; and the relative contribution of CD8+ and CD4+ T cells in the irradiated model. Additionally I sought to evaluate the importance of NK cells in the live CT26 model, and to analyse the clonal composition of CD8+ T cell responses in the live tumour model.

Additional work was carried out with a model of autoimmune thyroiditis, which sought to investigate the spontaneous response to self-protein in the context of an anti-tumour response. In this humanised transgenic model thyroid peroxidise (TPO) peptides are recognised by every T cell, leading to destruction of the thyroid, despite the fact that the animals contain TPO-specific T regs. I sought to see whether this defective suppression of a self-response extends to tumour cells manipulated to express TPO protein.

Chapter 2. Materials and methods.

Table 2.1. All media and solutions

Media	Contents
R0	RPMI 1640 (Invitrogen)
R10	RPMI 1640 (Invitrogen) + 10% FCS
Phoenix media	IMDM medium (Invitrogen) + 10% HI FCS, 1% 10 mM Non Essential Amino Acids, 1% 100 mM Sodium Pyruvate, 1% 200 mM L-Glutamine, 1% Pen/Strep (10,000 U/ml)
DC media	RPMI 1640 (Invitrogen) with 10% F**B**S, 1% pen/strep (10,000 U/ml), 1% 10 mM Non Essential Amino Acids, 1% 100 mM Sodium Pyruvate, 1% 200 mM L-Glutamine, 0.1% 2-ME
Cold buffer	PBS with 0.5% BSA plus 2mM EDTA pH7.2
Complete DMEM	DMEM (Invitrogen) + 10% FCS
FACS buffer	PBS with 0.5% BSA
SOC medium	0.5% Yeast Extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$.

2.1 Depleting cell populations of Balb/c mice.

2.1a. Depleting CD25+ cells (PC61).

8-10 week old BALB/c mice were given two interperitoneal (i.p) injections of 1mg of PC61 in 200µl PBS to deplete the mice of $CD25^+$ regulatory T cells. Figure 5.1 shows that PC61 treatment reduced the percentage of T regs from 2.47% to 1.07%. These injections were given 3 days and 1 day prior to any tumour challenge at day 0. For the controls age-matched BALB/c mice were given two i.p. injections of 1mg of GL113 3 days and 1 day prior to any tumour challenge at day 0.

2.1b. Depleting NK cells (anti-GM1)

For the first experiment (5.3), BALB/c mice were injected i.p with 200ul of anti-GM1 serum, 1 and 3 days prior to subcutaneous inoculation of $5x10^4$ CT26 cells. Figure 5.1 shows that anti-GM1 antibody reduced the percentage of NK cells from 5.77% to 1.67%. For the rechallenge experiment (5.4), BALB/c mice were injected i.p with 200ul of anti-GM1 serum 1 and 3 days prior to the CT26 re-challenge and 3 and 7 days post re-challenge. As a control another group of BALB/c mice were injected i.p. with 200ul normal rabbit serum.

Table 2.2. Antibodies used

Antibody	Isotype	Source	Production
PC61	Rat IgG1	ATCC	Hybridoma cells were grown in tissue culture and antibody purified by precipitation in saturated ammonium sulphate
GL113	Rat IgG1	ATCC	See PC61 production
Anti-GM1	Rabbit	Wako Chemicals, GmbH, Germany	Ammonium sulphate precipitation of serum, followed by dialysis with PBS

2.2 Challenging with live and irradiated tumour.

2.2a. irradiated CT26.

Prior to irradiated CT26 challenge, mice were treated with PC61 or control antibody GL113 as detailed in 2.1a. For the tumour challenge at day 0 mice were injected subcutaneously in the flank with $1x10^6$ CT26 tumour cells suspended in 100µl PBS. The tumour was irradiated prior to immunization with 24 minutes in the cell irradiator (eq. 50 Gray). See figure 2.1 for schematic of immunisation and tumour challenge protocol.

2.2b. live CT26 and A20.

For the re-challenge experiments (4.2a and 4.2b) live tumour was administered 42 days after irradiated CT26 challenge at day 0. For both live CT26 and live A20 $5x10^4$ cells were administered subcutaneously to the flank. Mice were then carefully observed for over 100 days, with mice that succumbed to the tumours culled and recorded. See figure 2.2 for schematic of immunisation and tumour re-challenge protocol.
For the NK depletion experiments (5.3 and 5.4), $5x10^4$ cells of live CT26 were administered subcutaneously to the flank at day 0, and mice observed for over 60 days, with mice that succumbed to the tumours culled and recorded.

2.2c. irradiated CT26-GM

Irradiated CT26-GM is CT26 tumour stably transfected with the GMCSF gene, so that the tumour secretes GMCSF which acts as an adjuvant when tumour is injected into mice.
Mice were challenged subcutaneously in the flank with $1x10^6$ irradiated CT26-GM cells, with or without PC61 treatment, in the TCR repertoire experiments (6.2). Cells were irradiated prior to immunization with 24 minutes in the cell irradiator (eq. 50 Gray).

2.2d. live EL4 and EL4 TPO+.

In the tumour therapy experiment (Figure 7.14), 8 to 9 week old C57BL/6 mice and TAZ10 Rag1+ mice were challenged subcutaneously in the flank with $1x10^5$ EL4 or $1x10^5$ EL4 TPO+ cells suspended in 100ul PBS. Mice were observed for 3 to 4 weeks, recording the survival of mice, until all mice succumbed to tumour.

2.2e. Tumour end-points

Mice were sacrificed when mean tumour diameter was >15mm in accordance with humane end point guidelines (United Kingdom Coordinating Committee for Cancer Research). If mice were observed to be in distress, due to ulceration of the tumour or

Figure 2.1. Immunisation protocol in preparation of irrCT26 tumour challenge.
(a) 1mg of PC61 injected i.p. depletes T regs; (b) 1mg of GL113 is injected i.p. as an isotype control antibody. $1x10^6$ irrCT26 tumour cells are injected subcutaneously at day 0.

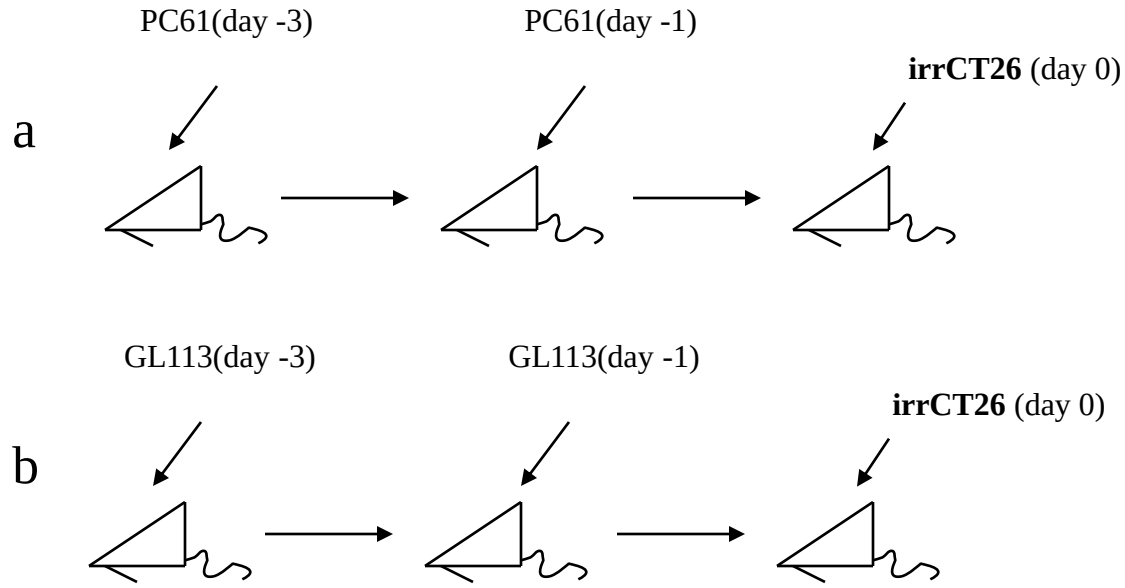
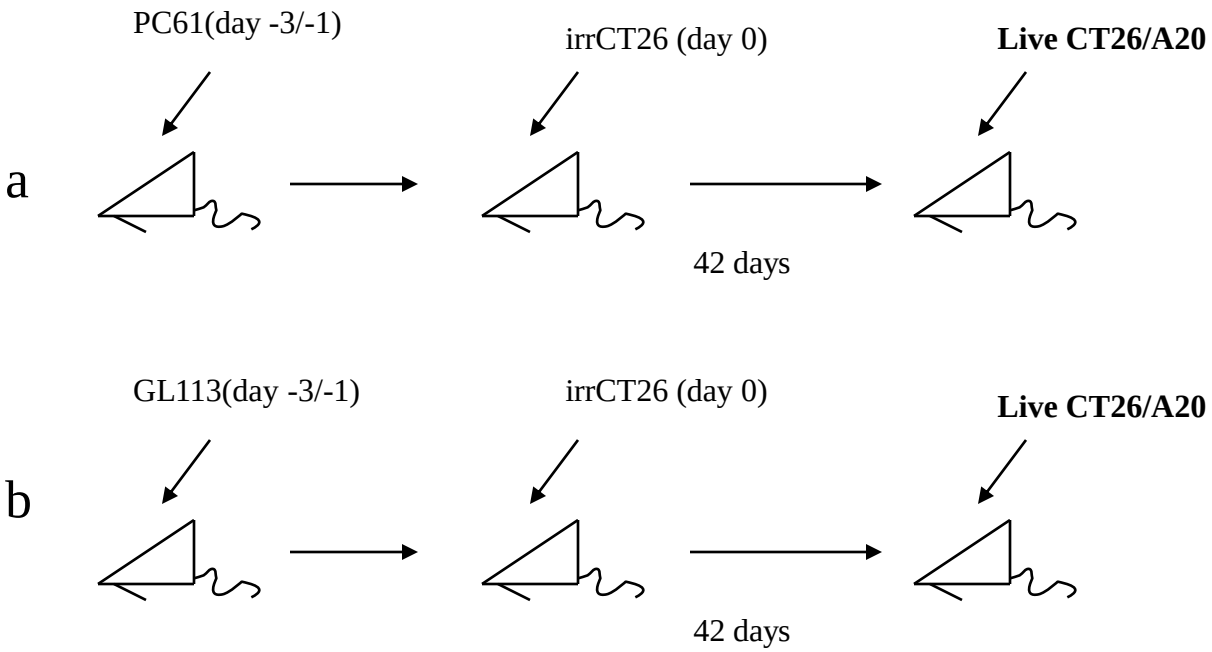


Figure 2.2. Immunisation protocol in preparation of CT26 or A20 tumour re-challenge. (a) PC61 depletes T regs; (b) GL113 is isotype control antibody. $1x10^6$ irrCT26 tumour cells are injected subcutaneously at day 0, followed 42 days later by subcutaneous injection of $5x10^4$ live CT26 or live A20 cells.



because of any other factor, before the tumour diameter was >15mm, they were culled by schedule 1 methods before the end point was reached.

2.3 Isolation of T cell populations from spleen using MACS.

2.3a. For ELISPOT experiments.

Mice were culled 8 days after challenge with irradiated CT26 (with or without CD25 cells). Surgically removed spleens were placed in R0 medium (RPMI 1640) and then mashed through a strainer to make a single cell suspension of splenocytes. The cells were then applied to ficoll solution and spun at 800 x g for 15 minutes to separate the lymphocytes from the red blood cells and other debris. The lymphocytes were retained and the remainder disposed of. If further purification of lymphocyte subsets was necessary then MACS beads were added and MACS columns were used as described in the MACS protocol provided in the kits. To summarise: Cells were suspended in 90µl of cold buffer and 10µl of CD8 (or CD4) Microbeads, and incubated at 4-8°C for 15 minutes. Cells were then washed with cold buffer, resuspended in 500µl per 10^8 cells, and applied to the appropriate MACS column. Unlabelled cells pass through the column as eluate, which allows magnetically labelled cells bound to the column to be collected by flushing out the column by applying a plunger. $CD8^+$, $CD4^+$ and $CD25^+$ regulatory T cells were isolated in this way using MACS.

2.3b. For adoptive transfer.

Balb/c mice were treated with PC61 antibody, and then challenged with $1x10^6$ irradiated CT26 cells. After 70 days mice were culled and spleens harvested. Either $3x10^6$ whole splenocytes; or $3x10^6$ CD4 or CD8 T cells, obtained by MACS enrichment (see above for details); were adoptively transferred into SCID mice, upon which these mice were re-challenged with $5x10^4$ of either live CT26 or live A20.

2.4 In vitro assays of cytokine production.

2.4a. ELISPOTs.

Millipore Multiscreen-IP plates (MAIP S45 10), were prepared by washing with 5% ethanol, washing with PBS, then applying IFNγ capture antibody. Plates were incubated at 37°C for 2 hours, then plates were blocked by adding R10 medium (RPMI + 10% FCS), R10 disposed after 2 hours. Cell suspensions plus antigen were added and plates were incubated for 36 hours at 37°C. After this cell suspensions are flicked off and the plate is washed with PBS-tween before applying IFNγ capture antibody. After 2 hours at room temperature plates were washed again with PBS-tween and streptavidin was added for 1 hour at RT. Spots were developed using the Zymed® BCIP/NBT substrate kit, and the plates were read on the Transtec 1300 ELISPOT reader (AID Diagnostika, Germany).

2.4b. IFNγ cytokine secretion assay.

(A) In vitro restimulation of the cells: $1x10^6$ of enriched CD8 T cells were suspended in 100ul R10 medium per well of a 96 well plate. Co-cultured with $3x10^5$ CT26 cells. Cells were incubated over night (37°C, 5% CO_2).

(B) Labelling with Cytokine Catch Reagent: Cells collected by careful pipetting, and wells washed with cold buffer (see table 2.1). Cells transferred to 2ml closable tube per sample. Cells washed twice with 1-2ml cold buffer, centrifuged at 300xg for 10 minutes at 4-8C, and then supernatant pipetted off completely. Pellet was re-suspended in 90µl of cold R10 medium. 10µl of Cytokine Catch Reagent was added to the cell mix, and incubated for 5 minutes on ice.

(C) Cytokine secretion period: 1ml of R10 medium warmed to 37°C was added to cell mix. Cells were incubated in closed tube for 45 minutes at 37°C, turning the tube every five minutes to re-suspend settled cells.

(D) Labelling cells with Cytokine Detection Antibody: The tube was put on ice and then washed twice with cold buffer, centrifuged at 300 x g for 10 minutes. The cell pellet was resuspended in 90µl cold buffer, to which was added 10µl

Cytokine Detection Antibody (IFNγ). This was mixed and incubated for 10 minutes on ice. Cells were washed in cold buffer, and then re-suspended in 500µl of cold buffer for FACS analysis.

2.5 FACS analysis.

Splenocytes were added at $1x10^6$ per FACS tube and were washed once with FACS buffer. The supernatant was discarded and the cellular pellet was resuspended in 200µl FACS buffer. Antibody for a particular surface marker and attached to a fluorochrome (CD4 APC/ CD8 PE/ CD25 FITC) was added and incubated on ice for 30 minutes. Excess antibody was washed off with FACS buffer and the pellet was resuspended in 200µl FACS fix. Samples were left overnight and then analysed on the FACS machine.

2.6 CDR3 spectotyping

2.6a. Selection of stimulated clones.

In vitro restimulation of the cells:

Three months after the CT26-GM challenge described in 2.2c, spleens were removed from mice, homogenised in vitro, and then depleted of CD4+ cells and B cells using Dynabeads. To summarise: Splenic cells were suspended in 100µl of cold buffer, 20µl of FCS and 20µl of antibody mix per 10^7 cells, and incubated at 2-8°C for 20 minutes. Cells were washed in cold buffer and resuspended in 800µl of cold buffer and 200µl of Dynabeads per 10^7 cells, and incubated at RT for 15 minutes. Then a further 1ml of cold buffer was added per 10^7 cells and the tube was placed in the magnet for 2 minutes. The supernatant can then be removed which contains the isolated CD8+ T cells. 10^6 of these enriched CD8 T cells were suspended in 100µl medium (5% serum) per well of a 96 well plate. The T cells were co-cultured with $3x10^5$ CT26 cells, or no tumour as a control. Cells were incubated over night (37°C, 5% CO_2). The following day an IFNγ capture

assay (see 2.4b) was performed in order to distinguish wells from which there were T cells that had sufficiently responded to the CT26 cells.

2.6b. mRNA extraction (from CD8 T cells and EL4/B6-SJ003 cells)

Cells remaining from the wells deemed positive from the IFNgamma capture assay were used as the source of mRNA. Cells were pelleted with centrifugation and were lysed in TRIzol reagent (Invitrogen) by repetitive pippeting. 1ml of TRIzol reagent was used per 5-10 x 10^6 cells. Cell-TRIzol mix was incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2ml of chloroform was added per 1ml TRIzol reagent. Tubes were vigorously shaken for 15 seconds and incubated at room temperature for 3 minutes, and then centrifuged at 12,000 x g for 15 minutes at 4°C. The colourless upper aqueous phase (containing the RNA) was transferred into a fresh tube, and the RNA precipitated using 0.5ml of isopropanol per 1ml of TRIzol used in the original mix. The samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet washed with 75% ethanol (vortexed and centrifuged at 8,900 x g for 5 minutes). The pellet was left to partially air-dry, and then dissolved in 20µl depc RNase-free water.

2.6c. cDNA synthesis.

Promega RT buffer 5x	4 µl
Promega oligodT	1 µl
dNTPs (10mM)	2.5 µl
RNA inhibitor	1 µl
Promega Reverse Transcriptase	1 µl
DPTC water	5.5 µl
RNA	5 µl
	20 µl

This mix was incubated in a 37°C water bath for 2 hours. The enzyme was denatured by heating to 72°C for 2 minutes. The mix was then made up to 100 µl with water.

2.6d. PCR using V-, J- and C-beta primers.

First the cDNA was used as a template for PCR using multiple Vβ primers with a single Cβ primer. Primers were used from the following Vβs: 1, 3, 4, 5, 12.1, 13.1, 14, 15, 16, 26, and 29. Primer concentration was 100pmol/µl. Q-solution is an additive that improves suboptimal PCRs caused by templates that have a high degree of secondary structure or that are GC-rich.

This PCR was set up as follows:

cDNA	1µl
Cβ primer	1µl
Vβ primer	1µl
dNTP	5µl
10x buffer	5µl
Q solution	10µl
Water	26µl
Taq	1µl
	50 µl

The PCR cycle was a follows: 94˚C for 10 mins, 35 cycles of 94˚C for 1 minute, 68˚C for 1 minute and 72˚C for 1 minute, then a final 10 minutes at 72˚C.

These Vβ-Cβ PCR products were then used as templates themselves for the next round of PCR, where dye-labelled-Jβ primers were used with the relevant Jβ primer for each Vβ-Cβ PCR product. The J primers have dyes attached to them so that the DNA fragments produced from the PCR can be visualised. Three different dyes were used: HEX (green), FAM (blue) and NED (black). Primers were used for the following Jβs: 1.1 (NED), 1.2

(NED), 1.3 (NED), 1.4 (NED), 1.5 (FAM), 1.6 (FAM), 1.7 (FAM), 2.1 (FAM), 2.2 (HEX), 2.3 (HEX), 2.4 (HEX), 2.5 (HEX), 2.7 (HEX).

The PCRs were set up as follows:

Vβ-Cβ PCR product	1µl
Vβ primer	1µl
Jβ primer	1µl
dNTP	2µl
10x buffer	2µl
Q solution	4µl
Water	8µl
Taq	1µl
	20µl

The PCR cycle was as before. 5µl of each PCR product was used for analysis with Genescan.

2.6e. GeneScan

The GeneScan Analysis Software analyzes the data collected by the ABI PRISM 310 Genetic Analyzer to size and quantitate DNA fragments. The results were displayed as electropherograms that show fluorescence intensity as a function of fragment size. Each electropherogram represents a single injection and provided precise sizing and quantitative information.

2.7 TPO work

2.7a PCR and primers for mTPO cloning

5' end of A segment of mTPO primer (containing **XhoI** restriction site):
CA**CTCGAG**ATGAGAACACTTGGAGCTATGGC

3'end of A segment of mTPO primer:
TCACTATCGGATCCAAACCAC

5' end of B segment of mTPO primer:
CAGTCCATCCACTGGTGAGAC

3' end of B segment of mTPO primer (containing **EcoRI** restriction site):
CG**GAATTC**TCTATTCGCACAGGAGGAC

PCR was set up as follows:

Mouse thyroid cDNA	4µl
dNTPs (1.25mM)	4µl
10x NH_4	5µl
$MgCl_2$ (25mM)	1.5µl
BioTaq	1µl
DMSO	2.5µl
5'primer (25µM)	2µl
3'primer (25µM)	2µl
Water	27µl
	50µl

PCR cycle: 92˚C for 2 minutes, 35 cycles of 92˚C for 20 seconds, 55˚C for 70 seconds, and 72˚C for 3 minutes, followed by a further 72˚C for 5 minutes.

2.7b XhoI, EcoRI, Sac I and BglII endonuclease digestion

17µl of the PCR product of portion A of mTPO was cut with 0.5µl XhoI and 0.5µl SacI enzymes in a 25µl solution containing 2.5µl NE buffer 4 and 2.5µl 10xBSA.

17µl of the PCR product of portion B of mTPO was cut with 0.5µl EcoRI and 0.5µl SacI enzymes in a 25µl solution containing 2.5µl NE buffer 1 and 2.5µl 10xBSA.
17µl of the MIGR1 vector (100ng/µl) was cut with 0.5µl EcoRI and 0.5µl XhoI enzymes in a 25µl solution containing 2.5µl NE buffer 2 and 2.5µl 10xBSA.
The final construct of MIGR1-TPO plasmid DNA was cut with 1µl BglII in a 10µl solution containing 1µl NE buffer 3 with 1µl 10xBSA.
All reactions were incubated at 37˚C for 2 hours.

2.7c T4 ligase

The T4 ligase reaction combined 4 moles of each insert (A and B) with 1 mole of MIGR1 vector, all previously cut with their relevant restriction enzymes. The final reaction mix contained 10.5µl of DNA with 1.5µl T4 buffer, and 1µl of T4 ligase in a total volume of 15µl. This was incubated overnight at 4˚C.

2.7d Transformation of DH5α cells

To 50µl of DH5α competent bacteria solution was added 5µl of the ligation reaction mix in a 1ml tube. This tube was put on ice for 30 minutes, then it was kept at 42˚C for 30 seconds. 250µl of warm SOC medium was then added to the tube, and then kept on ice for 2 minutes, followed by 1 hour at 37˚C. The mix was then spread on a Petri dish of agar infused with ampicilin. The dish was then left overnight at 37˚C. The colonies that grow on the dish have taken up the retroviral plasmid containing ampicilin resistance. These colonies can be picked and used to make bacterial culture solutions.

2.7e Caesium chloride maxiprep and Phenol:cloroform miniprep.

Phenol miniprep: To 0.5ml of bacterial culture in a microfuge tube was added 0.5ml of phenol:chloroform:isoamylalcohol. The mix was vortexed at maximum speed for 1 minute. It was then centrifuged at 12,000 x g for 5 minutes. After this the upper aqueous phase was removed (approximately 400µl) and added to 0.5ml of isopropanol in a fresh

tube. This was mixed well and centrifuged at 12,000 x g for 5 minutes. The supernatant was poured off, and the pellet washed by slowly pipetting 70% ethanol to the side of the tube and pouring off. The pellet was air-dried and was suspended in 100µl of water.

Caesium chloride maxiprep: 500ml of bacterial culture was centrifuged at 3,000 x g for 30 minutes. The pellet was resuspended in 30mls resuspension buffer (25mM Tris pH 8.0, 50mM glucose, 20mM EDTA), and placed into 3 sorvall tubes, 10ml per tube. To each tube was added 10mls of freshly made 0.2M NaOH + 1% SDS, and the tubes were mixed by inversion and left in ice for 10 minutes. Then 10mls of cold sodium acetate was added and the tubes were mixed and left on ice for 20 minutes. Then the tubes were centrifuged at 13000RPM (SS-34 rotor in a Sorval RC6) for 30 minutes. The 3 tubes were then emptied into 3 separate falcon tubes via a 0.7µM filter and filled up to 50mls with isopropanol and mixed by inversion. Tubes were then left at -20˚C for 20 minutes and centrifuged at 3,000 x g for 10 minutes at 4˚C. The supernatant was poured away and the pellet mixed with 50mls of 100% ethanol per tube, and centrifuged again at 3,000 x g for 10 minutes at 4˚C. The pellet was then dissolved in 3mls of T.E. solution per tube, and then all 9mls was collected and put into two small sorvall tubes. 18g of CsCl was added to 12mls of T.E. until dissolved and added to each sorvall tube. 315µl of Ethidium Bromide was then added to each sorvall tube without mixing. Both tubes were then centrifuged at 42000RPM (T-865 rotor in a Sorvall Discovery 100S) for 42 hours. After 42 hours the tubes were removed and the supercoiled plasmid DNA, present as a pink layer in the middle of the tube, was removed using a micro-tube attached to a peristalsis pump. The recovered DNA was mixed with 10ml of AnalaR water and 40ml of 100% ethanol in a falcon tube. The tube was mixed by inversion and stored at -20˚C for 20 minutes. The tube was centrifuged at 3,000 x g and the pellet resuspended in 300µl of water. This DNA solution was mixed with 40µl sodium acetate and 1ml of 100% ethanol in a 1.5ml eppendorf tube. This tube was left at -20˚C for 20 minutes and then centrifuged at 1,800 x g for 15 minutes. The pellet was then resuspended in enough water to resuspend it, maintaining a high concentration of final plasmid DNA.

2.7f Phoenix cell transfection

Before the transfection the phoenix cells were plated in a 6 well plate: $3x10^5$ cells in 2ml phoenix medium per well. This plating was done when phoenix cells were at 70-80% confluence in its flask, and once plated cells are left overnight to achieve 70-80% confluence in the 6 well-plate for effective transfection. The transfection mix consisted of 94µl of DMEM, 6µl of fugene-6 reagent (obtained from Roche), 2µg of retroviral construct DNA and 2µg pCleco (all per well). This mix was vortexed in a small eppendorf, left at room temperature for 15 minutes, then applied dropwise to the plated phoenix cells and incubated at 37˚C for 48 hours.

2.7g Harvesting of retroviral and target cell infection

Once transfection of the phoenix cells was confirmed by fluorescence microscopy, the supernatant was pooled from all the transfected wells, centrifuged at 200 x g for 2 minutes to remove cell debris, and then hexadimethrine bromide (5mg/ml) was added (1µl per ml of S/N). The supernatant was then added dropwise to wells containing the target cells. The plates were then centrifuged at 800 x g for 90 minutes at 37˚C. The HBr added to the supernatant should bind and add weight to the viral particles so that this centrifuge step brings the viral particles and target cells into close contact (this is called 'spinoculation'). The target cells were then incubated at 37˚C for 48 hours. After this the target cells were collected and analysed on the flow cytometer, and positively infected cells fluoresced in the FL1 channel

2.7h Production of DCs from bone marrow

Leg bones of C57BL/6 mice were removed and purified from surrounding muscle tissue. Intact bones were left in 70% ethanol for 2-5 minutes for disinfection and washed with PBS. Both ends were cut with scissors and the bone marrow flushed with PBS using a syringe with a 0.45 mm diameter needle. Washed with PBS and resuspended pellet in DC medium – counted and seeded bacteriological Petri dishes at day 0 at a concentration of 2

x 10^6 per dish in 10 ml DC medium containing 200 U/ml rGM-CSF. At day 3 another 10 ml DC medium containing 200U/ml rGM-CSF were added to plates. At days 6 and 8 half of the culture supernatant was collected, centrifuged, and the cell pellet resuspended in 10 ml fresh medium containing 200 U/ml rGM-CSF, and given back to the original plate. At day 10 cells can be used or continued to reduce granulocyte contamination. Then plates were fed as day 6/8 but with 30-100 U/ml. For complete maturation at day 10 non-adherent cells were collected with gently pipetting, centrifuged at 300 x g for 5 min at RT, and resuspended in fresh medium in a fresh dish containing 100U/ml rGM-CSF and LPS at 1μg/ml. Cells were cultured for 1 more day.

2.7i Lysis of tumour cells and feeding of DCs

$2x10^6$ DCs were fed the lysate of either $4x10^5$ mock-transfected EL4 cells or $4x10^5$ TPO+EL4 cells. To lyse the tumour cells, the small eppendorf containing the cells was dipped into liquid nitrogen for 30 seconds, then allowed to thaw in a 37˚C water bath for 3 minutes. This was repeated two more times to ensure effective lysing of the cells. This lysate was then mixed with the DCs in 5ml RPMI, and incubated in a Petri dish for 2 hours at 37˚C.

2.7j CFSE labeling and analysis

Lymph node cells, harvested from TAZ10 and C57BL/6 mice in the animal house, were homogenized and washed in complete DMEM, then pelleted and resuspended in 1ml PBS. To this was added 1ml of FCS-free medium containing 2ul of CFSE solution, making CFSE at a final concentration of 1:1000 in the cell mix (5μM final concentration). This was incubated at 37˚C for 10 minutes, then the CFSE washed from the cells using complete RPMI. The assay was then set up with LN cells mixed with DCs fed with tumour lysate. At day 3 of the assay, cells were harvested and analysed for CFSE dilution on the flow cytometer.

Results Chapter 3

Irradiated CT26 induces immune responses similar to live CT26.

3.1 Introduction

The original observations that preceded this project were made in the investigation of tumour responses to live CT26 in Balb/c mice (Golgher, Jones et al. 2002). A challenge of live CT26 cells overcame all the mice inoculated. However it was observed that Balb/c mice that were initially depleted of CD25+ cells (using PC61 antibody) were capable of rejecting the live tumour challenge. Furthermore surviving mice were able to subsequently reject live tumour challenges of different histological origin (A20, RENCA, C26, and BCL1). This "cross-protective" response was not seen if the mice were challenged with live CT26 tumour transfected with GM-CSF (known as CT26-GM) subsequent to CD25+ cell depletion. Thus it was hypothesised that the depletion of the CD25+ cells (assumed to be primarily CD25+ regulatory T cells) and subsequent challenge of live CT26 cells revealed a "new" response that not only recognised CT26 tumour cells, but also the cells of the tumours of different histological origin. This response must be different to the anti-AH1 response induced by the CT26-GM tumour challenge, which was capable of rejecting CT26 tumour but not the tumours of distinct histological origin.

This study suggested that normal mice challenged with CT26 were able to mount an immunosuppressive response to the tumour, resulting in its unchecked proliferation. Furthermore, amelioration of this situation by the depleting antibody PC61 suggested that the mechanism was via antigen specific T regs. In order to study the regulatory mechanisms inhibiting an anti-tumour response to CT26, it was necessary to seek a model system in which the CT26 tumour could be transplanted without it subsequently proliferating to a lethal level. To this end, I examined the response to irradiated CT26 in the presence and absence of T regs. Among the questions that the irradiated CT26 model would answer is whether the "cross-protective" response requires the depletion of T regs,

or whether it is induced by the tumour challenge alone. Another advantage of the irradiated CT26 model is that the variability of response in the live CT26 model, which meant that survival rates of mice depleted of T regs and challenged with live CT26 was also unpredictable, would be reduced by the non-fatal irradiated CT26 challenge. This would remove concerns over whether some of the live challenges were sub-optimal, and also allow in-vitro analysis of the response generated in the days immediately following the challenge.

The irradiated CT26 model will inform on the regulatory mechanisms of the live CT26 model only if the basic features of the response induced by both tumours are similar. It is possible that this might not be the case, either in the protection against a subsequent live CT26 challenge or in the cross-protection against an unrelated tumour. Furthermore the effect of depleting T regs prior to the irradiated challenge may be different to the effect on the live challenge. As the two models should represent an identical antigenic exposure, differences in the responses generated by these two challenges would indicate a greater importance for the context of the tumour delivery. In this case we would have to expand our current hypothesis to differentiate the irradiated CT26 model from the live CT26 model. Such a conclusion could be relevant to the design of vaccines based upon killed autologous tumours or autologous tumour lysates.

3.2 Adoptive transfer experiments

To compare the live and irradiated CT26 challenges adoptive transfer experiments were performed. In this procedure both the ability to protect against a subsequent live CT26 challenge and a live A20 (cross-protective) challenge would be tested. It was only possible to do these adoptive transfers in the absence of T regs, as the initial live CT26 always proliferates to a lethal level in the presence of T regs so that undepleted mice would not be able to provide cells for transfer.

To compare these two tumour models SCID mice were adoptively transferred with whole, CD8 or CD4 fractions of splenocytes obtained from mice depleted of CD25+

regulatory T cells and challenged with either irradiated or live CT26, and then the SCID mice challenged themselves with live CT26 and live A20. Significantly the whole spleen and CD4 fractions would include T regs that had recovered from the original depletion, but the antigen-specific T regs that would have been generated at the point of initial challenge should still be absent. Severe Combined Immunodeficiency (SCID) mice are mice that possess a genetic mutation in chromosome 16, which confers a deficiency that impairs rearrangement of separate gene elements of the immunoglobulin and T cell receptor genes. This disrupts the differentiation of B and T-lymphocyte progenitor cells, with the consequence that SCID mice are born lacking all their B and T cells. This allows us to reinstate either wholly or partially the immune system in the recipient SCID mice, informing on the efficacy of separate parts of the immune system without worry of 'contamination' from the host B and T cells.

To make the initial tumour challenges as comparable as possible, $1x10^6$ irradiated CT26 cells were injected compared to $5x10^4$ live CT26 cells, as the irradiated cells would not be able to divide in the mice whereas the live cells would divide very rapidly. Furthermore the inflammatory environment that would normally be caused by an aggressive tumour like CT26 growing rapidly in the mouse, with the associated turnover of the tumour cells, would be at least partially mirrored by the $1x10^6$ cells that were irradiated and would be therefore dead or dying when injected subcutaneously into the mice.

Before adoptive transfer of T cell fractions into SCID mice, the purity of the MACS isolated CD4 and CD8 fractions were analysed on FACS. The average CD8 purity after MACS separation was 89%, and the average CD4 purity after MACS separation was 95%. The results of the challenges of SCID mice receiving whole spleen, CD4 or CD8 fractions from mice treated with PC61 and challenged with *irradiated* CT26 are shown in figure 3.1. The results show that the SCID mice survived the live CT26 or live A20 tumour challenges whether they had received CD4 cells, CD8 cells or whole spleen cells. This indicates that the anti-CT26 response and 'cross-protective' response induced by the irradiated CT26 was transferable by either CD4 or CD8 T cells.

The results of the challenges of SCID mice receiving whole spleen, CD4 or CD8 fractions from mice treated with PC61 and challenged with *live* CT26 are shown in figure 3.2. As in figure 3.1 all SCID mice that had received CD4 cells, CD8 cells or whole spleen cells remained tumour free after either the live CT26 or the live A20 challenges. Consequently as with the irradiated CT26 challenged adoptive transfer experiment, it appears that either the anti-CT26 or the 'cross-protective' responses can be transferred either in the CD8 or the CD4 populations.

Overall these results indicate that it is likely that the challenge of $1x10^6$ irradiated CT26 cells is comparable to $5x10^4$ live CT26 cells (at least in the absence of T regs). It suggests that the increased number of irradiated cells compared to the live cells satisfactorily ensures that the antigen load of the two challenges is broadly equivalent once the division of the live tumour is taken into account. Furthermore it suggests that the inflammatory context that the two antigen loads are presented is broadly similar.

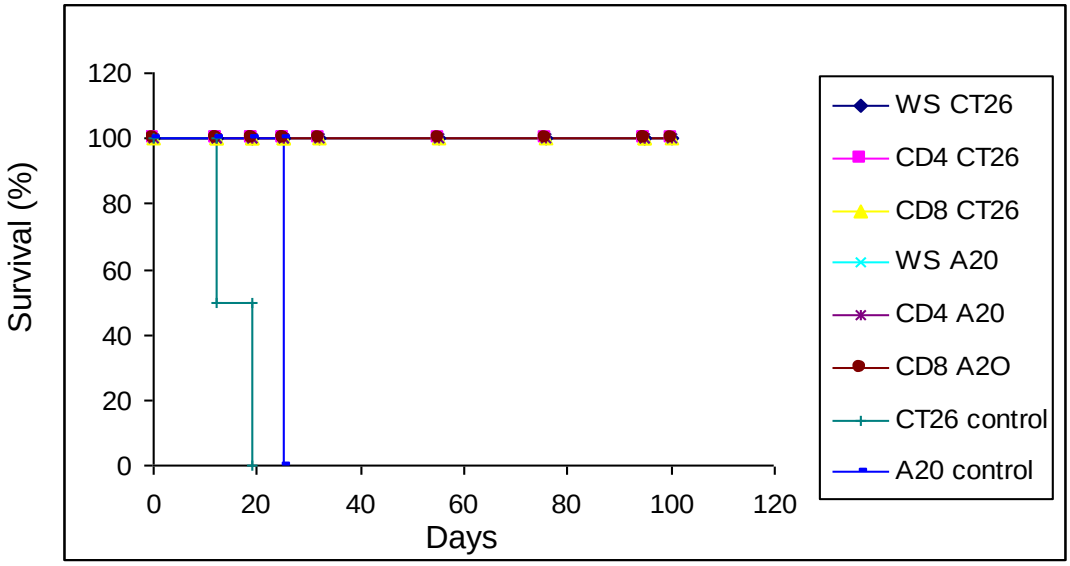


Figure 3.1. Survival of SCID mice to CT26 or A20 challenge after adoptive transfer of cells from irrCT26 challenged mice. 3x106 CD4+ T cells, CD8+ T cells,or a mixed population of CD4+ and CD8+ T cells (WS), purified from mice that had been vaccinated with 1x106 irradiated CT26 T cells after PC61 treatment, were adoptively transferred into SCID mice. All groups are made up of 3 mice, except CT26 control and A20 control, which have 2 mice per group. The percentage of tumour-free SCID mice after an A20 or CT26 challenge (5x104 cells) is shown.

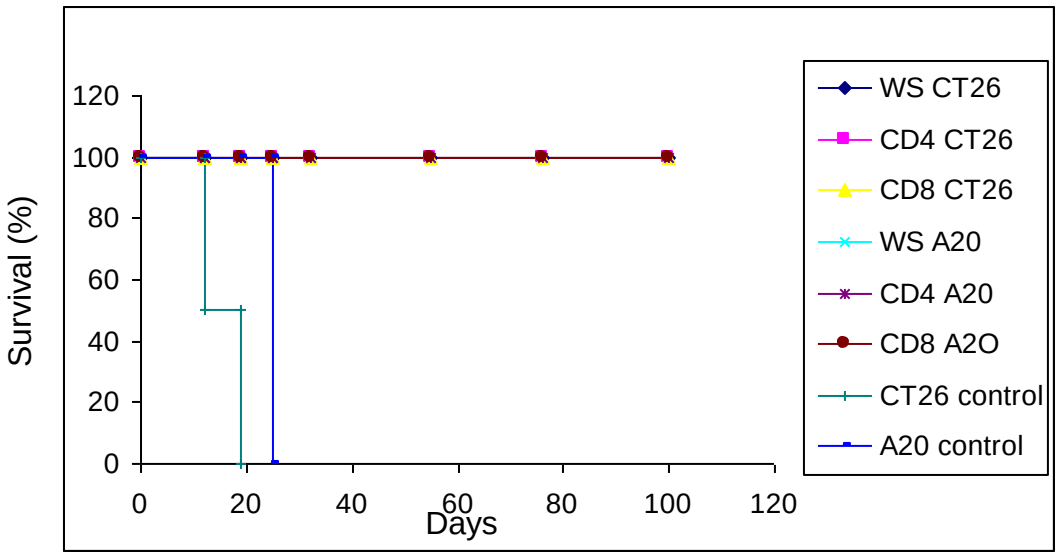


Figure 3.2: Survival of SCID mice to CT26 or A20 challenge after adoptive transfer of cells from live CT26 challenged mice. 3x106 CD4+ T cells, CD8+ T cells, or a mixed population of CD4+ and CD8+ T cells (WS), purified from mice that had been vaccinated with 5x104 live CT26 T cells after PC61 treatment, were adoptively transferred into SCID mice. CD4 CT26 and CD4 A20 groups have 4 mice per group, the control groups have 2 mice per group, and all other groups have 3 mice per group. The percentage of tumour-free SCID mice after an A20 or CT26 challenge (5x104 cells) is shown.

3.3 Discussion

Now that it seems that irradiated CT26 does induce a similar response to live CT26 it points towards me being able to use irradiated CT26 to answer a number of the questions that arose from the original live CT26 experiments. Such questions include whether the absence of T regs is essential to the generation of a cross-protective response. This conclusion also removes some of the technical concerns that accompanied the use of live tumour as a vaccine.

Beyond the overall conclusion that the irradiated and live challenges seem to be comparable, these results are interesting in that they are a slight departure from the work done by Golgher et al, upon which this project was based. These results suggest that the responses generated by the tumour challenges are more robust than thought previously, such that CD4 cells or CD8 cells alone can confer cross-protection (Golgher, Jones et al. 2002). Previously only weak cross-protection was seen with individual subsets, with optimal cross-protection when the subsets were co-transferred. To account for this we could speculate that perhaps the course of time has changed the phenotype of the CT26 and A20 tumours sufficiently so that CD4 rejection antigens are more prominent, allowing rejection of the tumours with CD4 T cells alone. Or maybe there are technical differences between the way the old and more recent experiments were carried out, which are subtle enough to be not easily accounted for.

Results Chapter 4:

The effect of CD25 depletion on the immune responses induced by irradiated CT26 challenge

4.1 Introduction

The previous chapter indicated that the response generated to irradiated CT26 tumour cells by subcutaneous challenge of $1x10^6$ cells is comparable to the response generated by a live CT26 challenge of $5x10^4$ cells. However, by further investigation using both in vivo and in vitro experiments, I aimed to get a more precise idea of the nature of the response generated by irradiated CT26 challenge. In particular the previous chapter lacked an experiment in which splenocytes were adoptively transferred from mice challenged with irradiated CT26 without depleting T regs with PC61 antibody. Without it I couldn't conclude whether the absence of T regs is essential to the generation of a cross-protective response by the irradiated CT26 challenge. This was one of the main questions that I sought to address in this chapter.

In the in vivo setting, the aim was to do re-challenge experiments where the initial irradiated CT26 exposure (in the presence or absence of CD25+ T regs) is followed by live CT26 or A20 challenges. The CT26 rechallenge assesses whether the irradiated CT26 challenge can evoke a response that will reject the live CT26 tumour, and whether this response requires the absence of T regs at the point of irradiated CT26 challenge. The A20 rechallenge assesses whether the irradiated CT26 challenge can evoke a response that is cross-protective against tumours of different histological origin, and whether this cross-protective response requires the absence of T regs at the point of the original irradiated CT26 challenge.

The hypothesis formed from previous work with live CT26 was that although a potentially cross-protective CD4+ and CD8+ T cells can develop in live CT26 challenge with T regs, it develops into a robust anti-tumour response only in the absence of T regs. This is due to the suppressive effect of T regs, which keeps these responses below the level of activation which would be needed to be exceeded to see an effector response. It

remained to be seen whether this hypothesis could be carried over to irradiated CT26 or whether a new hypothesis was needed. If there was an expectation either way it was to see cross-protection induced by the irradiated CT26 challenge when T regs have been initially been depleted by PC61 antibody.

4.2 In vivo re-challenge experiments

In order to evaluate the level of anti-tumour immunity evoked by irradiated CT26 in the presence or absence of T regs, mice were immunized with irradiated CT26 after being depleted of CD25+ cells (PC61), or not depleted (GL113), and were challenged with liveCT26 subcutaneously. A group of control mice that were not immunized were also challenged with live CT26 tumour.

As seen in figure 4.1, the *in vivo* response to live CT26 challenge indicates that depletion of CD25+ cells prior to irradiated CT26 challenge offers complete protection from the subsequent live CT26 challenge. However, approximately 65% of the mice that were challenged with irradiated CT26 but without CD25+ cell depletion also survives the live CT26 challenge. The difference between the PC61 and GL113 groups was significant with a p value < 0.01.

The same experiment was performed with the exception that mice immunized with irradiated CT26 after being depleted of CD25+ cells (PC61), or not depleted (GL113), were challenged with live A20 to assess the cross-protective *in vivo* response that the irradiated CT26 exposure evokes. A group of control mice that were not immunized were also challenged with live A20 tumour.

As seen in figure 4.2, the *in vivo* response to live A20 challenge indicates that depletion of CD25 cells prior to irradiated CT26 subcutaneous challenge makes a small difference to the survival to the live A20 challenge, with the survival being almost identical for the first 130 or so days, but by the end of the experiment the mice receiving PC61 had a 20% better survival rate than the mice that received GL113. A T-test showed that this difference was significant, with a p value < 0.05. However compared to the naive control, even the irradiated CT26 challenge itself (without PC61) offers significant protection

from the A20 challenge, with the mice taking significantly longer to succumb to the A20 challenge, and 20% surviving the A20 challenge completely.

This data suggests that as a vaccine irradiated CT26 performs well in protecting mice against subsequent tumour rechallenges. For live CT26 challenge, irradiated CT26 offers good protection, with its efficacy enhanced by CD25 depletion. The attractiveness of irradiated CT26 as a vaccine is increased because it is partially cross-protective, and the cross-protective response is modestly enhanced by T reg depletion.
These observations also show that irradiated CT26 provokes a different response to live CT26. In the case of irradiated CT26 the generation of a cross-protective (A20) response is not absolutely dependent on the absence of T regs at the time of initial challenge, although survival is slightly improved by depletion.
Thus the hypothesis formed from previous results, that the checks that the regulatory cells hold over the cross-protective response remain intact when immunising with the live CT26 tumour; checks that are only removed when the regulatory T cells are depleted (Golgher et al, 2001), may not extend to the response induced by irradiated CT26, where the tumour challenge itself seems to be sufficient to generate a cross-protective response in at least some of the mice.

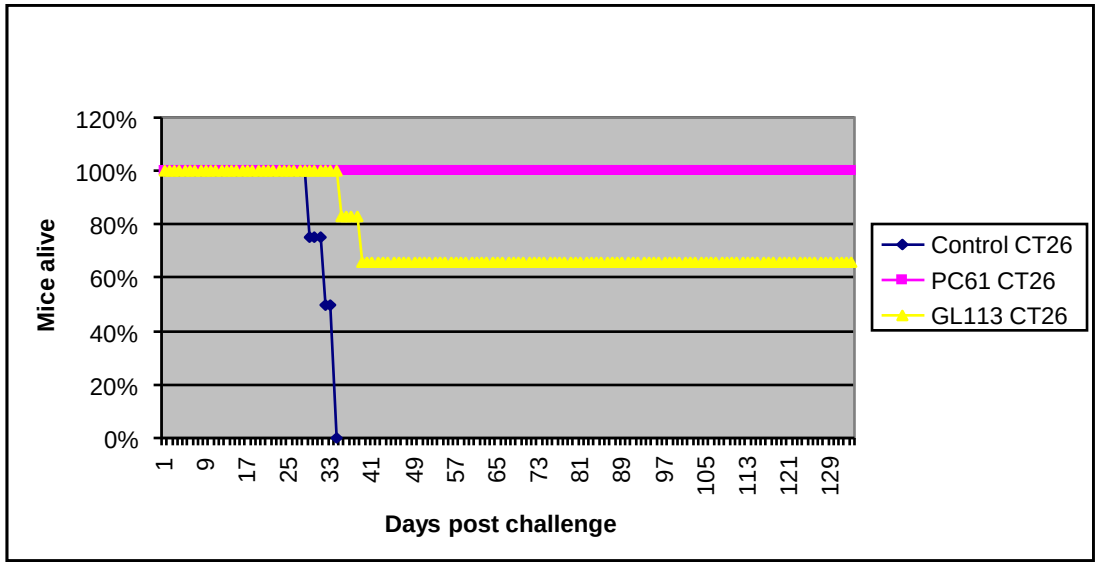


Figure 4.1. Survival of mice challenged with live CT26, 42 days after vaccination. Balb/c mice were challenged with 5x104 live CT26 tumour cells 42 days after an irradiated 1x106 CT26 challenge either in the presence (GL113) or absence (PC61) of T regs. A control group of naïve mice were also challenged with 5x104 live CT26 tumour cells. PC61 and GL113 treated groups consist of 6 mice per group, with the control group consisting of 4 mice. The development of tumours was observed for 130 days and mice culled when tumours were judged to be terminal. A t-test shows that the difference between the PC61 group and the GL113 group was significant with a p value < 0.01.

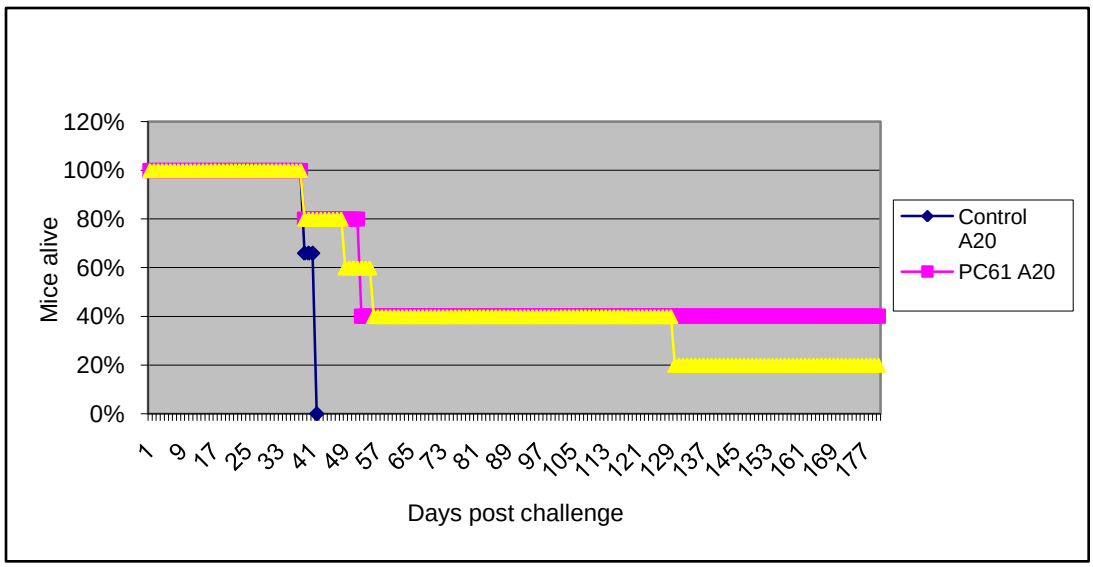


Figure 4.2. Survival of mice challenged with live A20, 42 days after vaccination. Balb/c mice were challenged with 5x104 live A20 tumour cells 42 days after an irradiated 1x106 CT26 challenge either in the presence (GL113) or absence (PC61) of T regs. A control group of naïve mice were also challenged with 5x104 live A20 tumour cells. PC61 and GL113 treated groups consist of 5 mice per group, with the control group consisting of 3 mice. The development of tumours was observed for 130 days and mice culled when tumours were judged to be terminal. A t-test shows that the difference between the PC61 group and the GL113 group was significant with a p value < 0.05.

4.3 In vitro IFNγ assays (ELISPOTS).

To gain further understanding of the irradiated CT26 model and the regulatory mechanisms that control the anti-tumour response, I examined the early effector response. The aim was to see whether there was a difference in the early responses made to an irradiated CT26 challenge in the absence of T regs compared to in the presence of T regs. I also wanted to see whether the IFNγ produced by the early responses would broadly correspond to the full effector response seen in the protection experiments in section 4.2.

In order to assess the early response to irradiated CT26 in the presence or absence of T regs, mice were immunized with irradiated CT26 after being depleted of CD25 cells (PC61), or not depleted (GL113), and were culled eight days after immunization and the lymphocytes were purified from the spleen. 100,000 lymphocytes were mixed with 25,000 tumour cells in each well of the ELISPOT plate. The number of spots counted at the end of the procedure reflected IFNγ production by the lymphocytes in response to the tumours. Figure 4.3 indicates that depleting of CD25+ cells prior to immunization boosts the subsequent *in vitro* response of splenocytes to both CT26 and the unrelated tumour A20, with a t-test indicating that these differences are significant.
This matches results from the protection experiments, where T reg depletion improved survival to CT26 and A20 challenges. The higher IFNγ levels in response to CT26 compared to A20 also mirrors the protection data, where survival to CT26 challenge was also greater than survival to A20 challenge.

In order to understand these results more fully, I next evaluated the sensitivity of the CD4+ and CD8+ anti-tumour response to the presence of T regs independently in order to determine the main correlate with the protection data in section 4.2.
In order to assess the CD8 T cell anti-tumour response, mice were immunized with irradiated CT26 after being depleted of CD25 cells (PC61), or not depleted (GL113), and were culled eight days after immunization and the lymphocytes were purified from the spleen. Then the whole spleen lymphocytes were further purified by MACS columns to

isolate the $CD8^+$ T cells. In the wells 50,000 $CD8^+$ T cells were mixed with either 25,000 CT26 cells or 25,000 A20 cells and the IFNγ production measured by counting the spots (average per well shown). Figure 4.4 shows that depleting CD25+ cells prior to immunization boosts the *in vitro* $CD8^+$ T cell response to CT26 (with a very clear significance) but much less significantly to A20. An identical experiment was carried out on purified CD4+ T cells and figure 4.5 indicates that depleting CD25+ cells prior to immunization boosts the *in vitro* $CD4^+$ T cell response to A20 (with a high significance) but not significantly to CT26.

The data shows that the CD8+ T cell data broadly correlates with the protection data as it indicates that the anti-CT26 response is made more efficacious by the depletion of T regs, whereas the cross-protective response isn't enhanced nearly as much by the depletion. The CD4+ T cell data partially correlates with the protection data as it indicates that the depletion of the T regs makes a difference to the efficacy of the cross-protective response, but no difference to the efficacy of the anti-CT26 response.

Another conclusion that this data suggests is that the best protection to tumour challenge is supplied by a strong CD8+ T cell response. The protection data (section 4.2) indicates that $CD25^+$ depletion prior to irradiated CT26 challenge increases survival to a subsequent live CT26 challenge (figure 4.1), which fits with the IFNγ data (section 4.3) showing $CD25^+$ depletion increases both total lymphocyte (figure 4.3) and $CD8^+$ T cell (figure 4.4) response to CT26. The protection data also indicates that $CD25^+$ depletion prior to irradiated CT26 challenge enhances protection against A20 above that of the mice not depleted but still immunized, but to a lesser extent than to CT26 (figure 4.2). Seeing as $CD25^+$ depletion doesn't enhance the IFNγ response of $CD8^+$ T cells to A20 (figure 4.4) it would seem that a good $CD8^+$ T cell response is preferable for full cross-protection, and that a good $CD4^+$ T cell response to A20 (figure 4.5) is less able to cross-protect.

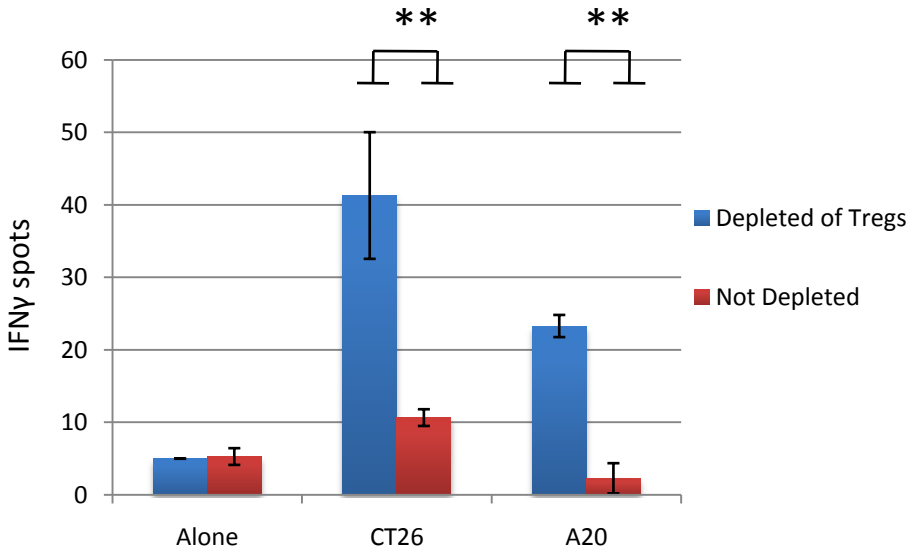


Figure 4.3. IFNγ response of splenocytes to tumour challenge in ELISPOT assays. CT26 or A20 tumour cells were mixed with whole spleen cells obtained 8 days after Balb/c mice were challenged with irradiated $1x10^6$ CT26 cells and either depleted or not depleted of T regs. 100,000 splenocytes were mixed with 25,000 tumour cells in each well of the ELISPOT plate and the number of spots counted at the end of the procedure. The error bars indicate the standard deviation (SD) from the mean (n=3). This is a single experiment representative of a trend. Significance: * = $p<0.05$; **= $p<0.01$; ***= $p<0.001$.

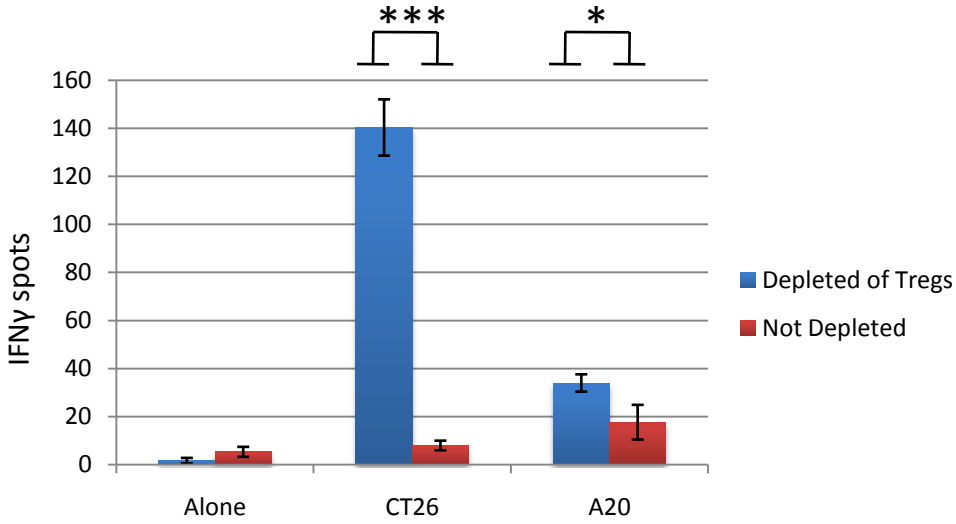


Figure 4.4. IFNγ response of CD8+ T cells to tumour challenge in ELISPOT assays. CT26 or A20 tumour cells were mixed with CD8 T cells purified from splenocytes obtained 8 days after Balb/c mice were challenged with irradiated 1x106 CT26 cells and either depleted or not depleted of T regs. 50,000 CD8 T cells were mixed with 25,000 tumour cells in each well of the ELISPOT plate and the number of spots counted at the end of the procedure. The error bars indicate the standard deviation (SD) from the mean (n=3). This is a single experiment representative of a trend. Significance: * = $p<0.05$; **= $p<0.01$; ***= $p<0.001$.

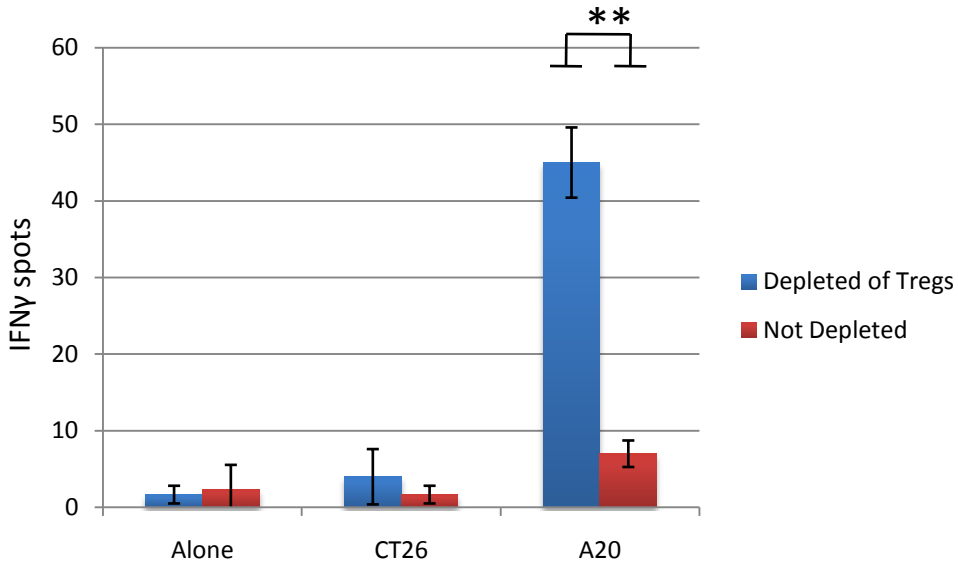


Figure 4.5. IFNγ response of CD4+ T cells to tumour challenge in ELISPOT assays
CT26 or A20 tumour cells were mixed with CD4 T cells purified from splenocytes obtained 8 days after Balb/c mice were challenged with irradiated $1x10^6$ CT26 cells and either depleted or not depleted of T regs. 50,000 CD4 T cells were mixed with 25,000 tumour cells in each well of the ELISPOT plate and the number of spots counted at the end of the procedure. The error bars indicate the standard deviation (SD) from the mean (n=3). This is a single experiment representative of a trend. Significance: * = $p<0.05$; **= $p<0.01$; ***= $p<0.001$.

4.4 Discussion

Our previous comparisons of the responses generated from live CT26 with and without T regs suggested that antigen-specific T regs were responsible for creating an immunosuppressive environment that allowed the unchecked proliferation of the live tumour. Only when the T regs were depleted were the mice able to survive the tumour challenge. Comparisons with the response induced by CT26-GM also indicated that in the absence of T regs the response to CT26 broadens to include cross-reactive antigens, allowing rejection of unrelated tumours. However, the data collected in this chapter complicates the conclusions that we can draw from this CT26 model.
It indicates that both anti-CT26 and cross-reactive T cells are generated in response to irradiated CT26 with T regs, but with the difference that both responses are partially protective to both live CT26 challenge and a challenge of an unrelated tumour. Furthermore, the cross-protective response is only modestly improved by first depleting T regs, although the anti-CT26 response is significantly boosted by the depletion.
It may be that the anti-CT26 and cross-reactive CD4+ and CD8+ T cells develop in response to the live CT26 with T regs, but only in the absence of T regs are these responses robust enough to be protective. This kind of conclusion can not be completely certain when none of the immunotherapy experiments described here using irradiated CT26 have direct live CT26 controls. This is due to the fact that with the live tumour challenge in order for the mice to survive, and thus allow re-challenge experiments, mice must be depleted of T regs.

The fact that the anti-CT26 response is boosted by the depletion of T regs in the irradiated CT26 model may prove to be the most valuable of all these conclusions. In the field of whole-cell tumour vaccines the aim is to develop a vaccine that presents multiple tumour epitopes to the immune response in the hope that a much more potent response is generated. Other cancer immunotherapy research has concentrated on a ‘single target’ approach in which a tumour antigen or epitope is identified, which is either uniquely expressed or overexpressed in tumour tissue, and targeted. This approach is limited by the chosen antigen continually being expressed and not down-regulated, and that this

antigen being expressed on all the tumour cells in the patient, some of which may have metastasised and differentiated from the primary tumour. The whole-cell tumour vaccine should in theory induce responses to multiple antigens and even unknown antigens, and also should provide the necessary determinants for CD4+ T cell help. A recent study where patients with renal-cell carcinoma received autologous renal tumour cell vaccines indicated that some clinical benefit from the procedure (Jocham, Richter et al. 2004). Despite this and a few other successes it is probable that the future of tumour-cell vaccines may lie in their combination with other modes of treatment. The data in this chapter indicates that depletion of T regs may increase the effectiveness of tumour cell vaccines. Thus the depletion of T regs (or some other means of immune-modulation) used in conjunction with a tumour-cell vaccine, along with perhaps soluble cytokines or chemotherapy drugs may lead to us being able to vaccinate patients to effectively cure them of existing tumours.

Another interesting conclusion from this data is that T regs have only partial control over the cross-protective response when challenging with irradiated CT26. It is a possibility that the irradiated CT26 exposure, at approximately $1x10^6$ cells, is so large as to overcome the checks that regulatory T cells hold over any response made to cross-protective epitopes. When faced with such an overwhelming load in an inflammatory context (as it would be with irradiated and thus a largely dying or dead tumour mass), the antigen exposure and positive feedback signals the immune response would receive may be so strong that, what ever control regulatory T cells held over the cross-protective T cells, it would be overcome. However, the live CT26 tumour challenge would also, given the passage of time and the aggressive nature of the tumour, lead to a substantial tumour load, which would also be in an inflammatory context.
Therefore, it may be due to the kinetics of the exposure as well as the overall load that is the crucial difference. With the irradiated CT26 challenge the tumour exposure is overwhelming right from the outset, whereas the with the live CT26 challenge the tumour exposure is comparatively low at the outset. While the live CT26 load is relatively small it would be able to recruit regulatory cells to the tumour site that would offer the tumour some protection as it establishes itself, with the result that the response made to the

tumour is relatively weak. Therefore when the tumour load is as large as the irradiated load is at the outset, the tumour response is insufficient to reject the tumour. It would seem that in the face of an overwhelming irradiated CT26 exposure the T regs are less able to affect the strength of the anti-tumour response, explaining why the responses, particularly the cross-protective response, is unaffected by T reg depletion. A way of further investigating this model, while avoiding these problems, may be to reduce the irradiated exposure at the outset, perhaps even to the same level as the live CT26 challenge. At first one could try several separate irradiated CT26 challenges of different number, a 'titration of immunogenicity' in other words, and investigate the result of that on subsequent tumour responses. Alternatively, in a more subtle approach, the growth of the live CT26 tumour could be mirrored by repeated subcutaneous injections of irradiated CT26 cells over time, with perhaps incremental increases in cell number. In fact several experiments could run co-currently, one with the same level of irradiated CT26 cells injected at equal increments, and one where the number of irradiated CT26 cells in each challenge continuously increases over time.

Another possible explanation for why the large irradiated CT26 challenge evokes a different response to the live CT26 challenge is that the large irradiated CT26 load could activate the innate immune response. Thus the role of the innate immune response in this CT26 model, in particular the role of NK cells, is addressed in the next chapter.

Results Chapter 5:

The effect of NK cells on priming the anti-CT26 response.

5.1 Introduction

An important bridge between the innate and adaptive immune system is the natural killer (NK) cell. Their main function is to defend the host against foreign invaders such as viruses, parasites, bacteria and transformed cells, via the production of immunostimulatory cytokines, like IFNγ and TNFα, and cytotoxicity against particular target cells using the perforin/granzyme pathway (Trinchieri 1995). NK cells identify their targets through a set of activating or inhibitory receptors that recognize foreign antigens encoded by pathogens, the increased expression of target molecules (induced self), or the decreased expression of target molecules (missing self), such as MHC class I molecules (Hoebe, Janssen et al. 2004). Depending on the balance between inhibitory and activating signals NK cells are triggered to kill or ignore target cells.
Although NK cells are characterised as innate cells, they also participate directly in adaptive immune responses. In fact, according to the literature, NK cells' interaction with the adaptive immune system is extensive and multi-faceted. Although NK cells may act directly on T cells via the secretion of cytokines like IFNγ (Kelly, Darcy et al. 2002), the main way that NK cells might influence the adaptive response is by interacting with DCs. This interaction can be positive or negative (either by maturing immature DCs or killing them), and this interaction is bidirectional with DCs also acting on NK cells (Raulet 2004). We wanted to assess therefore whether NK cells played a significant role in the rejection of a live CT26 challenge, especially in the primary response to the tumour.

5.2 FACS analysis of the effectiveness of PC61 and anti-GM1 depleting antibodies.

In order to assess whether NK cells are playing a role in the rejection of tumours in our CT26 model we repeated the normal vaccination procedure with live CT26 after NK cell depletion. NKs are depleted with antibody against GM1, which is a glycolipid on the

surface of mouse natural killer cells. GM1 is expressed on mouse NK cells in high concentration, and it has been shown that the GM1 antiserum specifically eliminates NK cells, but not other lymphocytes (Kasai, Iwamori et al. 1980). Firstly however, I needed to assess the efficacy of the two depleting antibodies I was to use (PC61 and anti-GM1) and whether these antibodies could be used effectively in conjunction. Figure 5.1 shows the effect of the depleting antibodies PC61 and GM1 on Balb/c splenocyte populations. PC61 antibody reduced the proportion of CD4+ CD25+ cells in the spleen from 2.5% to 1%, and the anti-GM1 antibody reduced the proportion of GM1+ cells in the spleen from nearly 5.8% to 1.7%. While these are not complete depletions, experience with this model suggests that depletions to this extent are sufficient to see significant changes when it comes to tumour challenge experiments. It also appears clear that these antibodies do not hinder each others function, allowing me to use these antibodies in conjunction.

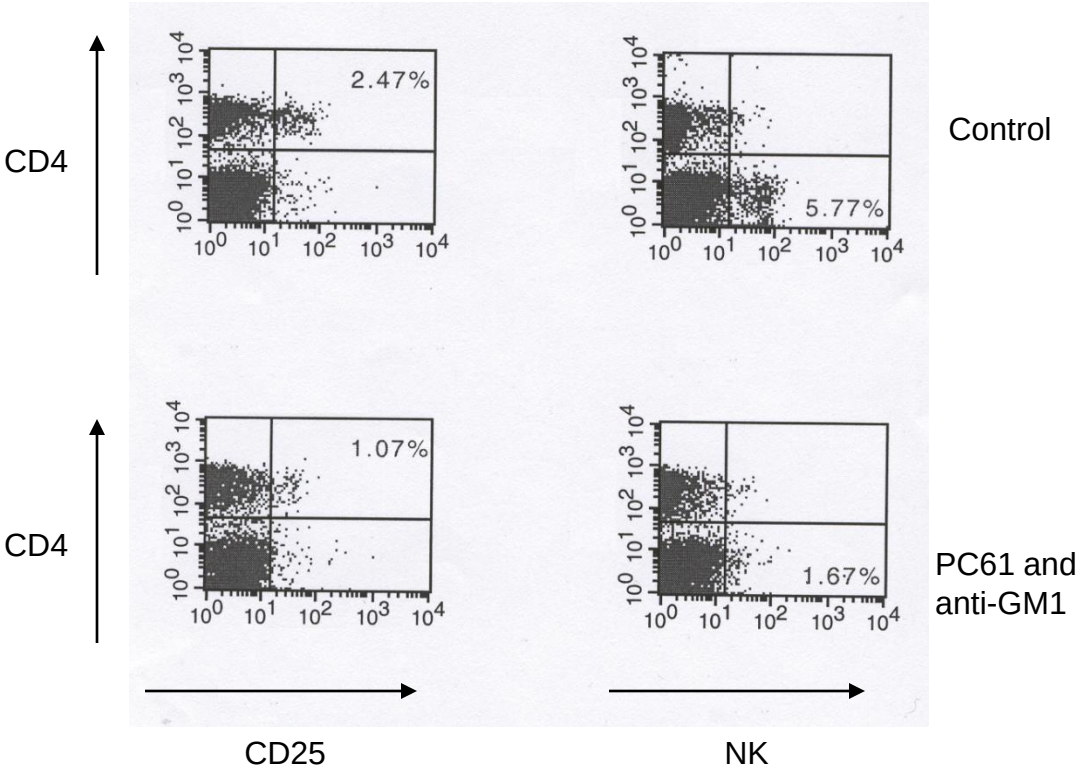


Figure 5.1. Depletion of cell subsets with antibody. Treatment of Balb/c mice with PC61 and anti-GM1 depletes both CD4+CD25+ T cells and NK cells from splenocyte populations.

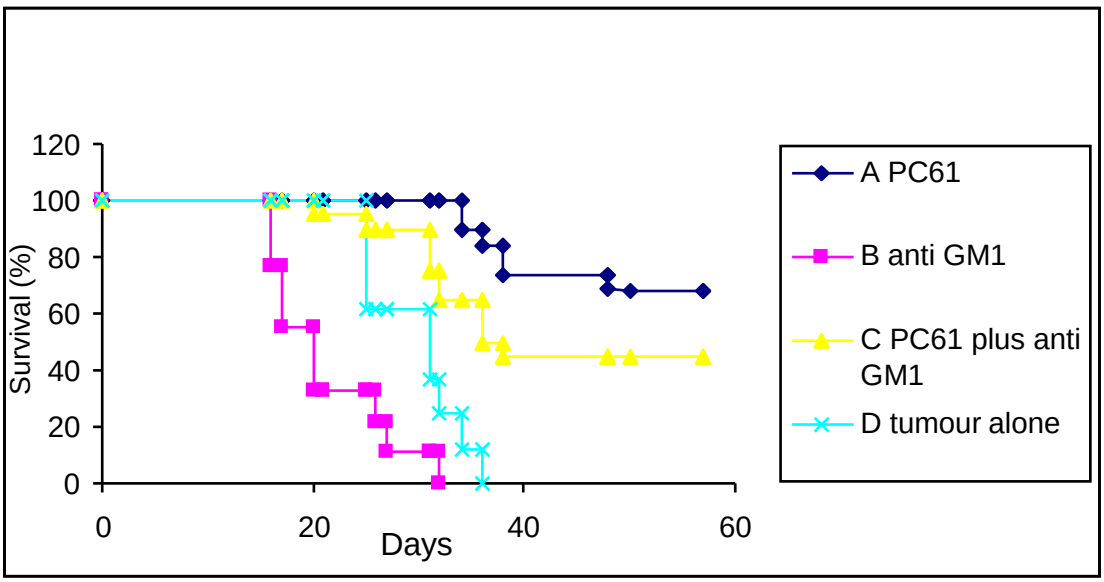


Figure 5.2. Survival to live CT26 with or without NK cells and T regs at the point of initial challenge. Mice were injected i.p. with 1mg of PC61 (which depletes T regs) and i.p. with 200ul of anti-GM1 serum (which depletes NK cells), 1 and 3 days prior to sc inoculation of $5x10^4$ CT26 cells. Some mice (pink) were depleted of only NK cells (B), others were depleted only of T regs (dark blue) (A) and others were depleted of both (yellow) (C). One group were left undepleted (light blue) (D). The percentage of tumour-free mice in each group is shown over time (days).

5.3 Effect of depleting NK cells (with/without CD25 cells) prior to inoculation with live CT26 tumour cells.

Firstly I wanted to test the role of NK cells in the primary response to CT26. NK cells would be depleted prior to the initial challenge with live CT26, and thus the NK cells would be absent at the point at which the immune response is first primed with the tumour antigens. This depletion of NK cells prior to initial challenge was done in conjunction with T reg depletion. Thus, Balb/c mice were challenged with $5x10^4$ CT26 cells subsequent to four different depletion combinations: no depletion; anti-PC61 only; anti-GM1 only; and both PC61 and anti-GM1. Depletions were carried out 1 and 3 days prior to live CT26 challenge, and survival was followed over the next 60 days. Therefore as well as assessing the effect NK cells have on the response, we could also assess the effect T regs have on NK cells. A few studies have indicated that NK cell function may be modulated by T regs, including one where anti-CD25 antibody administration before tumour inoculation abolished tumour growth and promoted the generation of cytotoxic cells including NK cells (Shimizu, Yamazaki et al. 1999), and we wanted to see whether this was the case in this CT26 model.

As shown in figure 5.2, the mice depleted of NK cells but with regulatory T cells had the shortest survival time, approximately 32 days. This was followed with the group without any cell depletion, with all mice dying by approximately 36 days. Mice that had been depleted of both regulatory T cells and NK cells had approximately 45% of mice surviving the tumour challenge. But the mice that had been depleted of regulatory T cells but who still had NK cells had the best survival, with approximately 70% completely rejecting the tumour challenge.

These results show that in this model, the efficacy of the anti-tumour response is decreased if NK cells are depleted prior to the initial live CT26 challenge. This occurs whether CD25+ cells are depleted as well or not. The conclusion is that NK cells are important in the anti-tumour response in the presence or absence of regulatory T cells. In the presence of regulatory T cells the effect of the absence of NK cells is shown by the difference in survival of the light blue and pink lines (figure 5.2). Although both groups of mice die, the survival rate is poorer in the group of mice without NK cells (pink). The

effect of CD25+ cells on the anti-tumour response is shown by the difference in survival between the dark blue and light blue lines (figure 5.2). Mice challenged in the absence of CD25+ cells have a much greater survival rate (70%), compared to the mice challenged in the presence of CD25+ cells (0%). However, if NK cells are also depleted prior to challenge, as shown in the yellow line, the positive effect of the CD25+ cell depletion is reduced, with only 45% of mice surviving the challenge (see table 5.1). Thus not only are the NK cells important in the anti-tumour response in the presence of regulatory T cells, but also in the absence of regulatory T cells.
Clearly the effect of removing the T regs does not solely affect the action of the NK cell population as even without NK cells the survival rate of 45% compares well with mice that have T regs and NK cells (0%). This particular positive effect must be mainly down to the adaptive immune response in these mice.

5.4 Effect of depleting NK cells before and after rechallenge with live CT26.

While it was apparent that NK cells have a role in the primary response to tumour, it was not thought that NK cells would be as influential once the response had been formed. Therefore I assessed the role of NK cells in the secondary response to CT26, and thus whether NK cells affect the memory T response or not. To do this, Balb/c mice that had been injected with PC61 antibody and survived a $5x10^4$ live CT26 cell challenge were re-challenged 42 days later with $5x10^4$ live CT26 cells. 1 and 3 days prior to and 3 and 7 days post the CT26 re-challenge, mice were treated with anti-GM1 antibody or a control rabbit serum. As a control mice naïve to CT26 tumour were also challenged with $5x10^4$ live CT26 cells. Thus NK cells would be absent at the point when the memory cells, formed during the primary response to the tumour, are attempting to eliminate the subsequent tumour challenge. As figure 5.3 shows, whereas all control mice had died approximately 35 days after challenge, all the mice that were being re-challenged survived, whether they had been treated with anti-GM1 antibody or not.
These results show that in terms of survival rates the importance of NK cells in the anti-tumour response diminishes to nothing once a good adaptive immune response has been generated. In the rejection of the CT26 rechallenge, the tumour growth is visible for a

time then it recedes, and it may be worth considering for future experiments that the rate of either the initial growth or the recession might be affected by the absence of NK cells. However, overall the inference is, once a primary response has been made to a tumour, the memory T cells produced during that primary response are sufficient to eliminate the tumour in a secondary response even in the absence of NK cells.

NK cells	T regs	Survival
+	+	0% (36 days)
+	-	70%
-	+	0% (32 days)
-	-	45%

Table 5.1: Survival data from figure 5.2, in tabulate form.

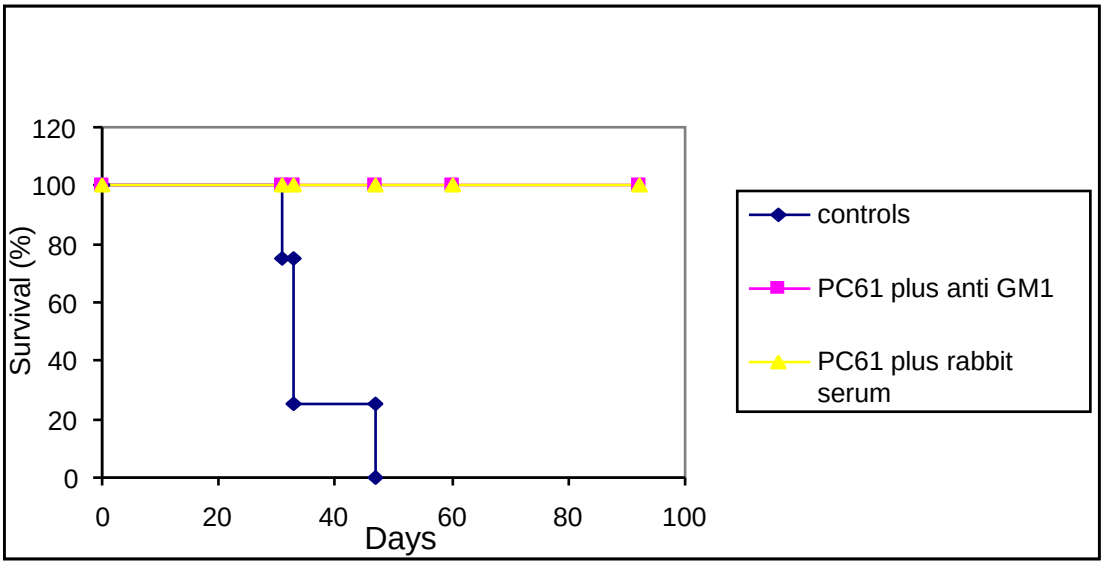


Figure 5.3. Survival to live CT26 with or without NK cells at the point of rechallenge. Mice injected with PC61 and that had been challenged with $5x10^4$ live CT26 cells were re-challenged 42 days later with $5x10^4$ live CT26 cells. 1 and 3 days prior to the CT26 re-challenge and 3 and 7 days post CT26 re-challenge, mice were injected i.p. with 200ul anti-GM1 rabbit serum or control normal rabbit serum. Mice were not depleted of T regs a second time. A group of tumour-naïve mice were also challenged with $5x10^4$ live CT26 cells as a control. The percentage of tumour-free mice each group is shown over time (days).

5.5 Discussion

From these results it is clear that the absence of NK cells, as well as the absence of T regs, impacts on the primary response to CT26 tumour challenge. These data, together with the literature that already exists on this subject, means that I can suggest several models of cell interaction that could be at work during priming in this CT26 model. These models must encompass the conclusions that depletion of NK cells, either combined with T reg depletion or not, impacts negatively on Balb/c survival to CT26 challenge, and that depletion of T regs, either combined with NK depletion or not, impacts positively on Balb/c survival to CT26 challenge.

NK model A (T regs suppress effector T cells only), shown in figure 5.4, suggests that depletion of NK cells deprives the anti-tumour response of a cell type that would target tumour cells directly, but also of cells that would act to directly positively influence the response to the tumour made by T cell effectors. To support this, NK anti-tumour activity and the consequent production of IFNγ by the NK cells has be shown in mice to evoke the subsequent development of a specific cytotoxic T lymphocyte (CTL) and T helper type 1 (Th1) responses against RMA tumour cells (Kelly, Darcy et al. 2002). Some evidence also exists to show that human NK cells express MHC class II and TCR co-stimulatory molecules, thus enabling them to act as APCs and present antigens directly to T cells, a phenomenon that may extend to mice (Hanna, Gonen-Gross et al. 2004). NK model A also suggests that the depletion of T regs would affect the anti-tumour response by removing suppression of the effector T cell response, which leads then to the increased survival of mice to CT26 challenge.

NK model B (T regs suppress NKs and T cells) differs from model A only in one respect, which is that the depletion of T regs also releases suppression of NK cells. This would release the NK cells to target more tumour cells and have a greater positive effect on the effector T cell response. There is good evidence that the removal of T regs might positively impact NK cell responses. In one of the early studies of the effect of T reg depletion on tumour responses, anti-CD25 mAb administration before tumour inoculation

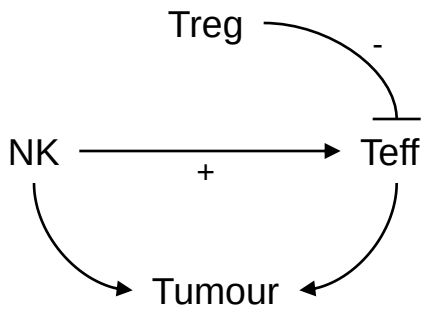
abolished tumour growth and promoted the generation of cytotoxic cells including NK cells. Furthermore, tumour-naïve splenic cell suspensions, depleted of regulatory T cells, contained NK cells capable of killing a broad spectrum of tumours. This may be explained by the fact that the removal of regulatory T cells might be activating self-reactive CD4 cells that then secreted IL-2, which would then activate NK cells to kill tumour cells. The subsequent release of tumour antigens, coupled with the IL2 from the CD4 cells, might then aid the development of tumour-specific CD8+ CTLs (Shimizu, Yamazaki et al. 1999). More recent studies have shown that NK cell proliferation was significantly enhanced in the absence of T regs, and that this suppression was TGFβ dependent (Ghiringhelli, Menard et al. 2005). Furthermore, in the murine model of 3LL lung carcinoma, depletion of T regs before tumour inoculation reduced the number of lung metastases, yet co-depletion of NK1.1+ cells restored the establishment of metastases (Smyth, Teng et al. 2006).

NK model C (NKs act indirectly on T cells) suggests that the DC functions as an interface between the NK and the effector T cell, and implies that the NK cell influences the state of the DC which in turn positively affects the T cell effectors. There is significant support for the existence of NK – DC cross-talk in the literature. Firstly activation of NK cells in vivo may be in large part due to interactions with DCs. DCs prestimulated with IFN-α upregulate the MICA and MICB NKG2D ligands, which contribute to activating NK cells in coculture (Dokun, Kim et al. 2001). Furthermore, in mice infected with MCMV, CD8α+ DCs are necessary for the expansion of Ly49H+ NK cell populations and blocking Ly49H prevents NK population expansion (Andrews, Scalzo et al. 2003). In the other direction the maturation of DCs stimulated by NK cells represents a key mechanism to bridge the NK response to the stimulation of T cell responses. Studies have shown that in co-culture with NK cells, immature DCs undergo maturation, produce TNF and interleukin 12, and upregulate costimulatory ligands such as CD86 (Gerosa, Baldani-Guerra et al. 2002). Other studies also conclude that efficient DC activation in cell culture requires contact with NK cells, with the NKp30 receptor being important in this interaction (Ferlazzo, Morandi et al. 2003). Furthermore, an in vivo study showed that NK cells activated by encounters with MHC class I low tumour

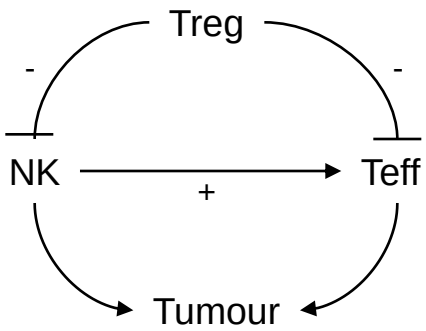
cells stimulate DCs to produce interleukin 12 and enhance the induction of CD8+ T cell responses (Mocikat, Braumuller et al. 2003). Overall, there is ample evidence that NK cells, via the DC interface, are effective at inducing T cell responses. NK model C also expands the role of the T reg to include the suppression of DCs as well as NK cells and T cell effectors.

These data alone cannot for certain determine which model is the correct one, although further experiments could reveal this. One way to further investigate the immunological interactions in this model would be to perform a series of in-vitro co-culture experiments with purified cell subsets. Purified T regs co-cultured with NK cells or T cells and assessing the effect on CT26 killing could elucidate the suppression mechanisms in this model. Furthermore T cells co-cultured with DCs or DCs previously co-cultured with NK cells, possibly using trans-well cultures, could elucidate the contribution of NK cells to the anti-CT26 T cell response.

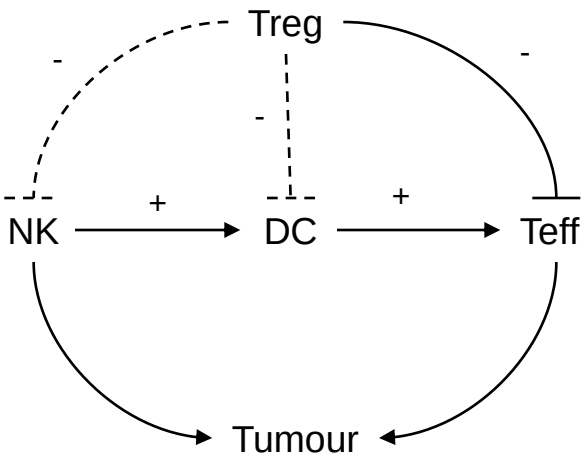
Figure 5.4. The potential role of NK cells in anti-CT26 responses. Below are three potential models of the effects NK cells have on the anti-CT26 response. In model A, NK cells and effector T cells both target the tumour, but NK cells also positively affect effector T cells directly. T regs suppress effector T cells but do not affect NK cells. Model B shares the features of model A but the T regs also suppress NK cells as well as effector T cells. In model C, NK cells positively affect effector T cells indirectly via DCs. T regs suppress effector T cells, and may negatively affect NK cells or DCs (indicated by dashed lines).



NK Model A: T regs suppress effector T cells only



NK Model B: T regs suppress NK and T cells



NK Model C: NK act indirectly on T cells

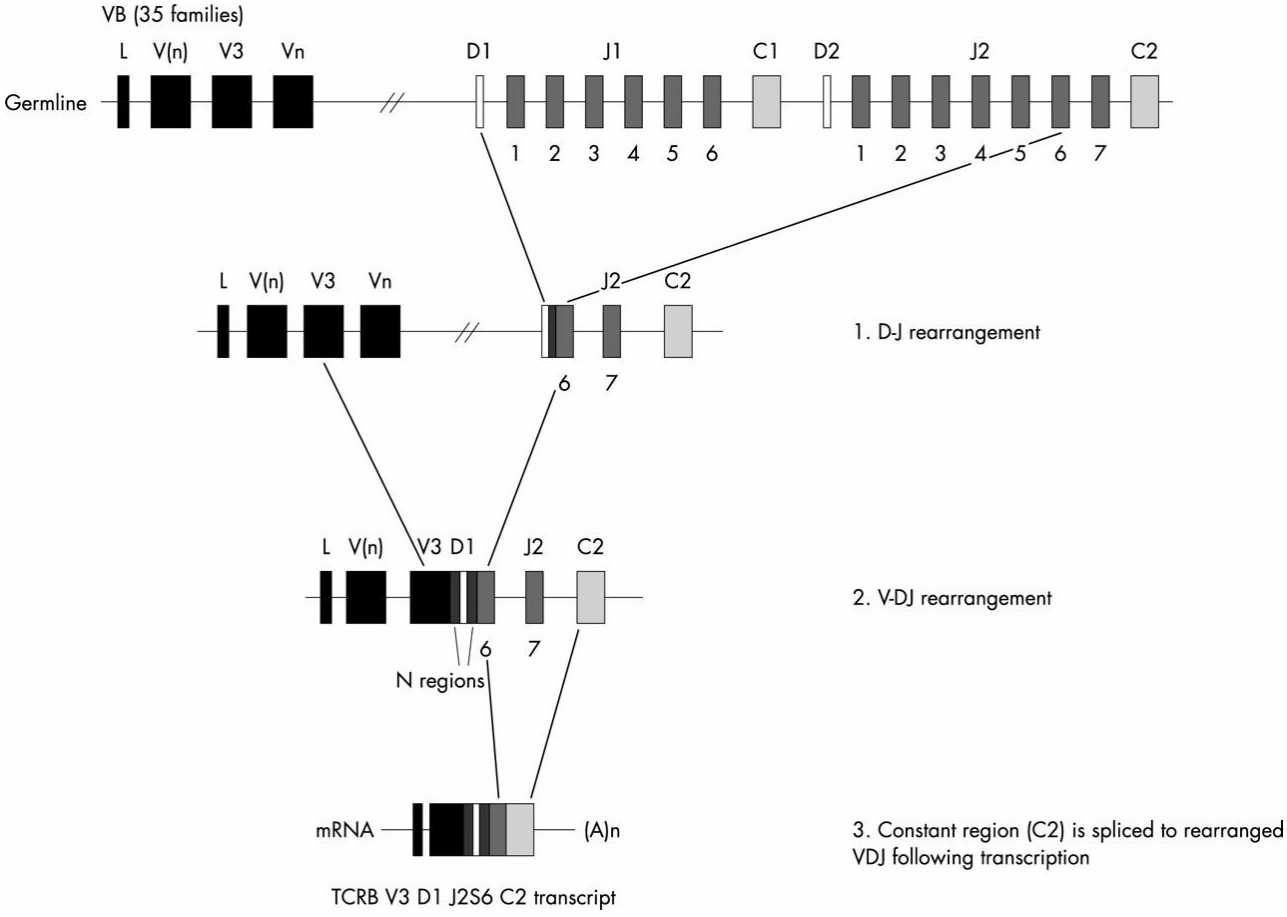
Results Chapter 6:

Is the number of T cell clones induced by CT26 different in the absence and presence of regulatory T cells?

6.1 Introduction

The TCR is composed of constant (C) and variable (V) regions, which are assembled together during thymic ontogeny (Alt, Oltz et al. 1992). The diversity of the TCR is generated via gene rearrangement within the variable domains of the TCR, which is the (V) and junction (J) gene segments in the Vα chain, and the V, diversity (D) and J gene segments in the Vβ chain. During TCR gene rearrangement the V and J, or V, D and J segments are brought together in a continuous V-J or V-D-J coding block, forming complete V domain exons that are responsible for antigen recognition (shown in figure 6.1). However the diversity of the TCR depends not only on the recombination of these genes but is also greatly increased by nucleotide insertion and deletion at the junctions between these genes. The greatest diversity is present at the third complementarity determining region (CDR3), which spans the V(D)J junction. The CDRs are regions of greatest sequence variability (CDR1 and CDR2 are located within the V domain) and constitute the binding site for the peptide-MHC complex, with the CDR3 positioned at the centre of the antigen binding site for direct contact with the MHC bound peptide (Jorgensen, Esser et al. 1992). The generation of TCR-pMHC crystal structures has allowed us to visualise the interaction of these two molecules in more detail (see figure 6.2) (Gras, Kjer-Nielsen et al. 2008). The TCR and pMHC 'dock' together, so that the TCR Vα domain is positioned over the MHC α2-helix and the N-terminal end of the peptide, whilst the TCR Vβ domain contacts the MHC α1-helix and the C-terminal end of the peptide (Rudolph, Stanfield et al. 2006).

Figure 6.1. The rearrangement of Variable, Diversity and Junctional gene regions to form the T cell receptor gene complex. Reprinted from the Journal of Clinical Pathology (2003) Volume 56, pages 1-11 (BMJ publishing group).



In every immune response the T cell repertoire will skew towards TCRs that recognise the peptides that are presented by the particular infection. Depending on the breadth of the peptides presented to the immune response, and the strength of the response to these peptides, the diversity of TCRs will be decreased to a greater or lesser extent. The clonal composition of the CD8+ T cell response has been evaluated for viral infections such as human cytomegalovirus (HCMV). Peripheral blood lymphocytes (PBLs) from HCMV-seropositive donors were cultured for two weeks with autologous DCs and exogenously added CMV antigen (Peggs, Verfuerth et al. 2002), which increased the percentage of HCMV-specific CD8+ T cells from 0.3% to 8.0%. TCR CDR3 spectratypic analysis was carried out on the cultured CD8+ T cells, using 22 TCRβ chain V gene family primers and the β chain C region primer. The PCR product lengths thus reflect the CDR3 lengths of the input TCR RNA, being dependent on the Jβ and Dβ usage as well as the variation in the junctional regions. This analysis showed that while there was a restriction in the CDR3 length repertoire postculture, the repertoire of Vβ usuage was not particularly restricted postculture.

6.2 Analysis of Vβ-Jβ DNA lengths from CD8 T cells stimulated by CT26GM tumour.

We wanted to assess the clonal composition of CD8+ T cells present post CT26 tumour challenge either in the presence or absence of T regs to further test the idea that in the absence of T regs the activation threshold is lowered which allows the broadening of the response against the tumour, including presumably the cross-protective antigen among others. As well as CT26, CT26GM was also used at the point of initial challenge, as we wanted to look at unfocussing of the response to CT26GM in the absence of T regs as well.

To start this procedure, Balb/c mice were challenged with CT26-GM tumour at day 0, prior to which the mice were depleted of T regs with PC61 antibody, or not (by using a control antibody GL113). Spleens from these mice were removed 3 months later, depleted of CD4 T cells and B cells with dynabeads to leave a principally CD8 T cell population, and these cells then either re-stimulated with $3x10^5$ irradiated CT26 tumour cells per well, or left unstimulated. Of those cells restimulated, positive responders were

identified using an intracellular IFN-gamma assay, and the mRNA extracted from these cells. Otherwise mRNA was extracted from the unstimulated splenocytes. This meant there were four groups (plus a group of naïve unstimulated splenocytes) that were analysed for the number of T cell clones:

1. Challenge of CT26-GM with T regs and CD8 T cells unstimulated in vitro.
2. Challenge of CT26-GM with T regs and CD8 T cells restimulated with CT26 in vitro.
3. Challenge of CT26-GM without T regs and CD8 T cells unstimulated in vitro.
4. Challenge of CT26-GM without T regs and CD8 T cells restimulated with CT26 in vitro.

In the next step of the procedure the cDNA made from the mRNA extracts was used as a template for a particular Vβ primer and several Cβ primers. Primers were used from the following Vβs: 1, 3, 4, 5, 12.1, 13.1, 14, 15, 16, 26, and 29. Of those PCR runs that were positive for a Vβ-Cβ product, those sequences were then used as templates for another set of PCRs that used the Vβ primer in conjunction with several Jβ primers. This reaction would produce various lengths of DNA that would indicate the amount of N-terminal addition between the Vβ region and the various Jβ regions. The J primers have dyes attached to them so that the DNA fragments produced from the PCR can be visualised. Three different dyes were used: HEX (green), FAM (blue) and NED (black). Primers were used for the following Jβs: 1.1 (NED), 1.2 (NED), 1.3 (NED), 1.4 (NED), 1.5 (FAM), 1.6 (FAM), 1.7 (FAM), 2.1 (FAM), 2.2 (HEX), 2.3 (HEX), 2.4 (HEX), 2.5 (HEX), 2.7 (HEX). The GeneScan Analysis Software analyses the data collected by the ABI PRISM 310 Genetic Analyser to size and quantitate DNA fragments automatically, allowing faster and more accurate analysis than traditional methods such as radiolabeling. The software displays the results as profiles (as seen in figure 6.2), which show fluorescence intensity as a function of fragment size. Each profile represents a single injection. Figure 6.2 shows examples of such profiles produced by the software, and importantly the examples are separated into their various interpretations of the breadth of the response that these Vβ-Jβ fragments represent: polyclonal, oligoclonal, clonal and not interpretable. Each peak corresponds to an additional nucleotide present at the junction

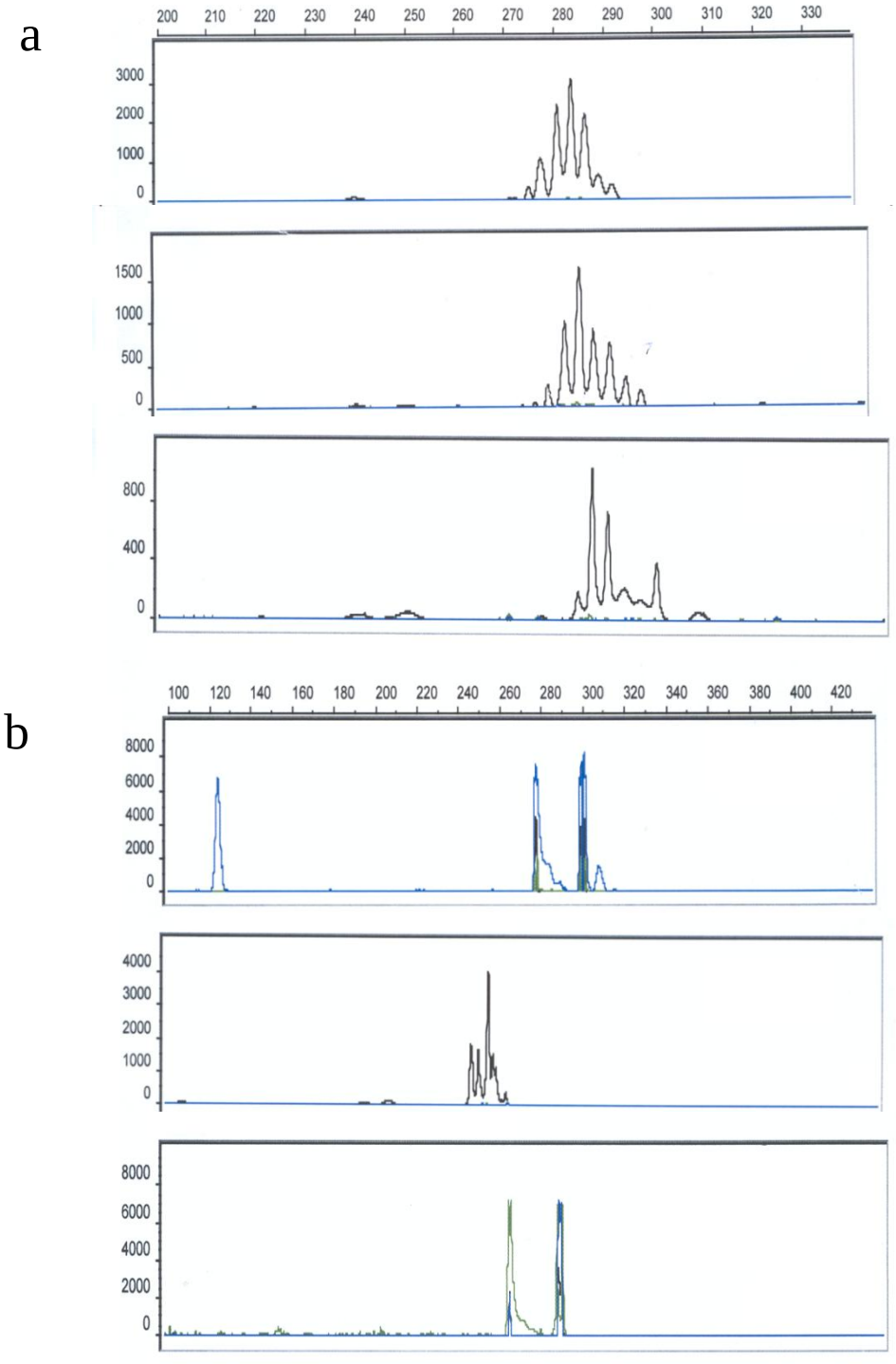
between the V and J regions that form the CDR3, and so more peaks indicates a greater variability at the CDR3, ultimately indicating more TCR variants are present. The results shown in tables 6.1, 6.2 and 6.3 were interpreted with the help of skilled and experienced technical assistance from SUHT Wessex Regional Immunology service who specialize in analyzing cancer patients with diseases such as T cell lymphoma.

The diversity of the response can be interpreted at several levels. The first is at the level of Vβ usage, as a greatly focused response might reduce the presence of certain Vβ subfamilies to an extent that they no longer show up on the profiles. It is unlikely that in the case of these experiments that this will be an instructive level of analysis, as even in the analysis of the HCMV response mentioned above, where you would expect a greater focusing compared to a tumour response, there was almost no cases where Vβ subfamilies were lost as a result of the HCMV stimulation of CD8+ T cell responses.
In any case, of all the Vβ primers used in these experiments time constraints meant that only the Vβ1, Vβ13.1 and Vβ5 regions were analysed and all were positive. Tables 6.1, 6.2 and 6.3 show the tabulated results of the interpretations from the profiles, with Vβ1 (6.1) being the most complete of these data sets.
The next level of interpretation is at the level of Jβ usuage, and here there is a much greater chance that certain combinations of Jβ and Vβ primers will no longer amplify sequences and thus indicate a focusing of the response. This is due to the fact that instead of one highly variable region (the V region) being paired with the single C region, you have the V region paired with the variable J region. However, as figure 6.4 shows, of the 65 profiles only 10 are negative, and of those, half were in Vβ1-Jβ1.7 which is negative in the naïve control. Furthermore negative results are hard to interpret as they can either be evidence of a loss of a Jβ subfamily, or just a bad assay. A series of repeat experiments would be necessary to differentiate, and again time constraints prevented those.
The final level of interpretation, and the one which is the most instructive here, is at the level of junctional diversity, or in other words the variability in the CDR3 region. This is indicated by the number of peaks in each of the profiles, which allows each profile to be judged to be either polyclonal, oligoclonal or clonal. Table 6.1 shows that at this level of junctional diversity there appears to be a trend of polyclonal profiles in the naïve control

(1st column), being replaced by oligoclonal profiles in the group where T regs were present (3rd column), and then reverting back to polyclonal profiles in the group where T regs were depleted (5th column). Notably the 3rd and 5th columns represented groups primed in the presence or absence of T regs which were then restimulated in vitro with CT26 tumour cells. This trend is present in Vβ1 –Jβ1.6, –Jβ2.3, and –Jβ2.7. This trend is evidence that depletion of T regs at the point of CT26GM challenge leads to the broadening of the immune response, as indicated by an increased polyclonality of the CD8+ T cell response. This trend is only countered in –Jβ1.3, where a polyclonal response reverts to an oligoclonal one. Otherwise, for the remaining Jβ subfamiles the profiles for the T reg depleted and non-depleted groups are the same. This trend can be articulated in a different way, which is to look at the percentage of recorded profiles in the 1st, 3rd and 5th columns that are polyclonal or oligoclonal. In the naïve control, 100% of positive profiles are polyclonal, which is not surprising given as this represents the clonal composition background of the naïve Balb/c mouse. In the CT26GM challenged mice (3rd column), 66% of the positive profiles are oligoclonal, with 33% polyclonal, representing a significant focusing of the T cell response from the background. Finally in the CT26GM challenged, T reg depleted mice (5th column), 27% of the positive profiles are oligoclonal, and 73% polyclonal, representing a significant shift back towards the 'unfocused' response.

Table 6.2 and 6.3 are incomplete data sets and so are more difficult to interpret. Table 6.2 lacks any data where T regs were depleted, and there are a number of 'No result' entries in the second column, which further hampers analysis of this data. The only thing to note in this table is that CT26 challenged mice show a much more focused response than the naïve control, with 8/13 profiles being oligoclonal and 2/13 profiles clonal, compared to 12/13 polyclonal profiles in the naïve control. Table 6.3 has more data than table 6.2, but there appears no difference between any of the conditions, which makes this dataset much less interesting than table 6.1. There are exclusively polyclonal profiles in table 6.3, whether T regs were present or depleted, or whether mice were challenged with CT26 or left naïve. An explanation may be that this particular Vβ region, Vβ 5, is not a region involved in producing TCRs that make a prominent response to CT26 antigens, and so are not more focused in response to CT26 challenge.

Figure 6.2. Examples of the different types TCR variability. (a) Polyclonal; (b) Oligoclonal; (c) Clonal; (d) Not interpretable. Each of the graphs represents a particular Vβ gene segment, with all the differences between the unique TCRs restricted to the CDR3 region, where there are differences in length due to the imprecision of the rearrangement process. Using primers specific for an individual V gene segment at one end and for a conserved part of the C region at the other, it is possible to generate a set of DNA fragments that span the CDR3 region. These fragments can be labelled with fluorochromes, and analysed by automated gel readers, so they can be displayed as a series of peaks corresponding to the different length fragments (this is a spectratype). More peaks on the spectratype indicates an expansion of the number of clones generated in response to a particular antigenic challenge. Three or more peaks = polyclonal; two peaks = oligoclonal; one peak = clonal; not interpretable = no clear distribution.



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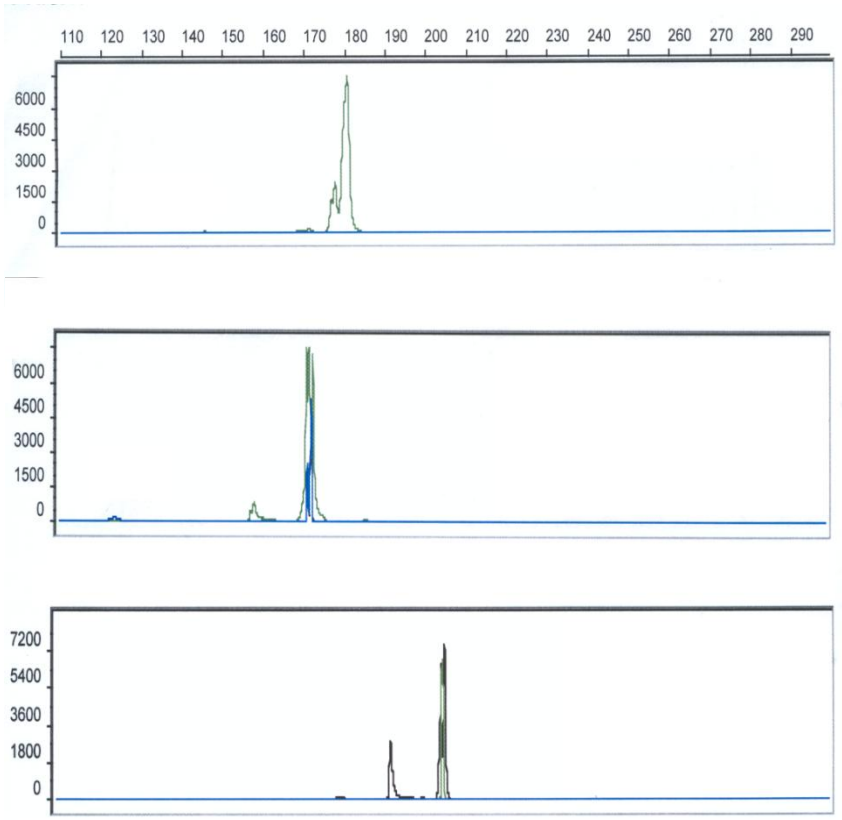
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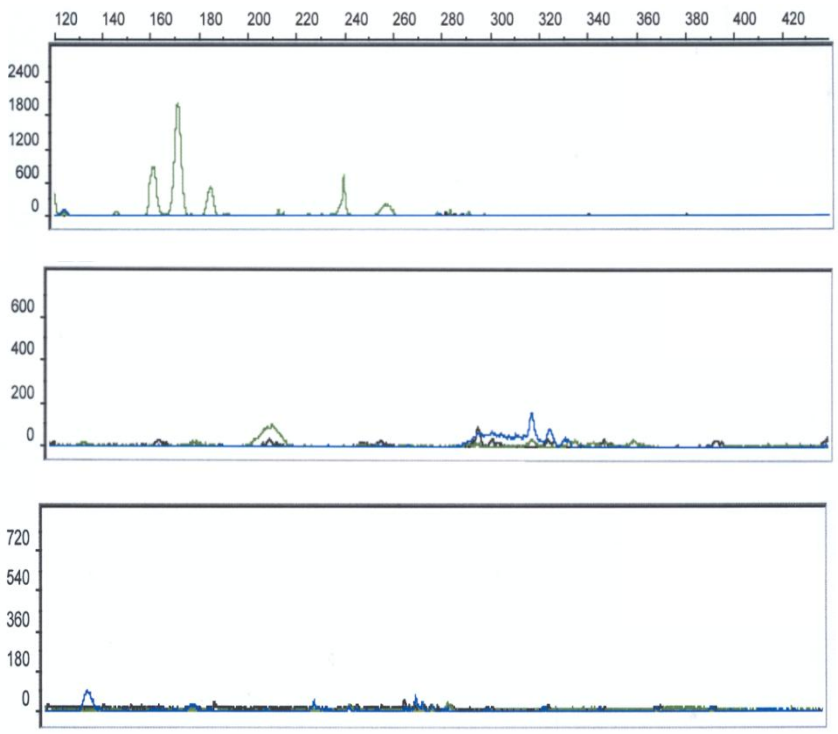
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Table 6.1. Results of the Vβ1 region

Vβ 1 - Jβ	Naïve control	CT26GM with T regs (Unstim)	CT26GM with Tregs (Restim)	CT26GM w/o Tregs (Unstim)	CT26GM w/o T regs (Restim)
1.1	Polyclonal	Polyclonal	Oligoclonal	Clonal	Oligoclonal
1.2	Polyclonal	Polyclonal	Oligoclonal	Polyclonal	Oligoclonal
1.3	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Oligoclonal
1.4	Polyclonal	Polyclonal	No result	Polyclonal	Polyclonal
1.5	Polyclonal	Oligoclonal	No result	Polyclonal	Polyclonal
1.6	Polyclonal	Oligoclonal	Oligoclonal	Polyclonal	Polyclonal
1.7	No result	No result	No result	No result	No result
2.1	Polyclonal	Oligoclonal	Oligoclonal	No result	No result
2.2	Polyclonal	No result	Polyclonal	Polyclonal	Polyclonal
2.3	Polyclonal	Oligoclonal	Oligoclonal	Oligoclonal	Polyclonal
2.4	Polyclonal	Polyclonal	No result	Polyclonal	Polyclonal
2.5	Polyclonal	Polyclonal	Polyclonal	Oligoclonal	Polyclonal
2.7	Polyclonal	Oligoclonal	Oligoclonal	Polyclonal	Polyclonal

Table 6.2. Results for the Vβ 13.1 region

Vβ 13.1 – Jβ	Naïve control	CT26GM with T regs (unstim)	CT26GM with T regs (restim)
1.1	Polyclonal	polyclonal	clonal
1.2	Polyclonal	No result	oliogclonal
1.3	Polyclonal	No result	oligoclonal
1.4	Polyclonal	No result	oligoclonal
1.5	Polyclonal	No result	polyclonal
1.6	Polyclonal	polyclonal	Polyclonal
1.7	No result	No result	No result
2.1	Polyclonal	No result	Oligoclonal
2.2	Polyclonal	Clonal	Oligoclonal
2.3	Polyclonal	Polyclonal	Oligoclonal
2.4	Polyclonal	Clonal	Clonal
2.5	Polyclonal	No result	Oligoclonal
2.7	Polyclonal	Clonal	Oligoclonal

Table 6.3. Results for the Vβ 5 region

Vβ5 - Jβ	Naïve control	CT26GM with Tregs (unstim)	CT26GM with Tregs (restim)	CT26GM w/o T regs (unstim)
1.1	Polyclonal	polyclonal	polyclonal	No result
1.2	Polyclonal	polyclonal	polyclonal	Polyclonal
1.3	Polyclonal	No result	No result	No result
1.4	Polyclonal	Polyclonal	Polyclonal	Polyclonal
1.5	No result	Polyclonal	polyclonal	Polyclonal
1.6	Polyclonal	Polyclonal	Polyclonal	Polyclonal
1.7	No result	No result	No result	No result
2.1	Polyclonal	Polyclonal	Polyclonal	Polyclonal
2.2	Polyclonal	Polyclonal	Polyclonal	Polyclonal
2.3	Polyclonal	Polyclonal	Polyclonal	Polyclonal
2.4	Polyclonal	Polyclonal	No result	No result
2.5	Polyclonal	No result	Polyclonal	No result
2.7	Polyclonal	polyclonal	polyclonal	Polyclonal

6.3 Discussion

There are several variables in this procedure that may or may not affect the validity of the conclusions made.

One variable is whether this method is accurately reflecting the number of T cell clones. It may be that analysing the clonal composition of CD8+ T cells derived from the spleen may not give an accurate impression of the specificity of T cell clones induced by tumour cells injected subcutaneously in the flank.

Another variable is the elapse of time from the point of CT26-GM challenge to the extraction of splenocytes. This was a consequence of one lab operator starting the procedure and it being revived by another operator. This could have the effect of lessening the skewing of the response of the T cells to the original CT26GM challenge. However against that the memory cells produced during that primary response should be expanded by the CT26 restimulation, and the fact that the T regs would have regained their normal levels by the time of the restimulation shouldn't distort the effect the original depletion had on the preference of the T cell response to the tumour challenge.

Ultimately it is likely that the conclusions from these results would have been strengthened by more data. Originally mice were challenged with CT26-GM or CT26 in the absence or presence of T regs. The plan was to use numerous primers corresponding to many of the Vβ and Jβ regions present in the mouse genome, in order to create a more complete picture of the T cell response to these tumours and the effect T reg depletion had on these responses. Time constraints meant that only a fraction of the possible data that could have been collected was collected, and of that only one table had analysis for all the conditions including the controls. It happened that this was the response to CT26-GM, which although important, hasn't been the focus of the rest of the experiments that I have carried out. However, the fact that these results have indicated that the clonal composition in response to CT26GM is less focused in the absence of T regs, in a model where survival to challenge is not dependent on T reg depletion, it could be reasonably extrapolated that the clonal composition in response to CT26, where T reg depletion does provide protection, would also be broadened by the absence of T regs.

The original hypothesis, formed in relation to the live CT26 model, stated that it was the breadth of the T cell response that is changed by the depletion of T regs, and that certain T cell responses (like the cross-protective response) would be invisible in the presence of T regs. Data from this chapter seems to back this up as it indicates that the response induced by CT26GM is able to recognise a greater variety of epitopes in the absence of T regs (although a broader repertoire does not necessarily mean that there will be an increase in cross-reactivity with other tumours). Interestingly, chapter 4 also shows that the anti-CT26 response, induced in the presence of T regs, is actually boosted in their absence. These data taken together may suggest that the in addition to the effect that T regs have on broadening the response, T regs may also be significant in controlling the quality of the anti-tumour T cell responses. It is quite possible that cross-reactive T cells are induced by live CT26 challenge, but that to make them effective *cross-protective* T cells would probably need the absence of T regs. The fact that the T reg control of responses in the live CT26 model appears to be different to the T reg control of responses in the irradiated model is an intriguing conclusion, but it also means that it is difficult to see how I could use these models to further investigate the T reg control of T cell responses to tumour cells. To successfully do this may require the use of a different model.

Results Chapter 7:

Investigating regulatory control of T cell tumour responses in a model of autoimmune thyroiditis.

7.1 Introduction

The CT26 model was chosen in an attempt to better define the role of regulatory T cells in the control of anti-tumour responses, in particular the suppression of the cross-reactive response. During the course of the investigation, it became clear that the response to the irradiated CT26 was qualitatively different to the response to live CT26. While this provided some mechanistic insight into the value of immunotherapeutic approaches involving irradiation of autologous tumour and T reg depletion, it did not permit a mechanistic study of the immune response to the transplanted live tumour. Some cancer vaccine candidates are overexpressed self-antigens (eg. Tyrosinase, hTERT etc) and a concern over their use in immunotherapy is the induction of autoimmune responses. Also T cell responses to these and others may be under peripheral tolerance so a special problem with cancer vaccines is breaking tolerance without inducing autoimmunity. I sought an informative model in which a spontaneous immune response to a self protein could be studied in the context of both autoimmune pathology and anti-tumour response.

This new model was a humanised mouse model of spontaneously arising autoimmune thyroiditis, the TAZ10 mouse (Quaratino, Badami et al. 2004). The transgenic mice express the TCR of the autoreactive human T cell clone 37, isolated from a patient with autoimmune thyroidits. T cell clone 37 is specific for the dominant autoantigen thyroid peroxidise (TPO), $TPO_{535-551}$. Within this peptide, two contiguous epitopes are differentially recognised by T cell clone 37, $TPO_{536-547}$, an agonistic highly stimulatory epitope, and $TPO_{537-548}$, a naturally occurring antagonistic epitope. $TPO_{536-547}$ is a cryptic epitope preferentially displayed after endogenous processing during inflammation. Conversely, the antagonistic epitope induces in vitro anergy in clone 37 when presented by dendritic cells and preferentially displays when whole TPO is presented. There is a

possibility that this T cell clone may be anergic and possibly regulatory in the patient, but in the mice it causes spontaneous histological, hormonal and clinical changes comparable to human destructive thyroiditis.

Clone 37 was a CD4+ T cell isolated from the thyroid infiltrate of an autoimmune patient specific for the cryptic $TPO_{536-547}$ epitope restricted by the histocompatibility leukocyte antigen (HLA) DQB1*0602-DQA1*0102 allele. As splenocytes from the CBA ($H\text{-}2^k$) strain of mice were able to present the $TPO_{536-547}$ to the T cell clone 37, the TAZ10 transgenic strain was established on the CBA ($H\text{-}2^k$) background. To exclude the presence of endogenous TCR α chains, the TAZ10 strain was backcrossed onto the Rag1-/- $H2^k$ background. Experiments showed that TCR^+ $Rag1^{-/-}$ T cells, expressing either CD4 or CD8 co-receptors, are restricted by H2-IA^k, and the cryptic epitope $TPO_{536-547}$ proved more efficient at inducing T cell proliferation than the $TPO_{535-551}$ epitope. Molecular modelling showed that this 'xenoreactivity' (i.e. that the TAZ10 TCR could be activated by human TPO peptides restricted by mouse H2-IA^k molecules) is because the binding of human $TPO_{536-547}$ to HLA-DQB1*0602 and H2-IA^k is similar, due to the structural homology of both molecules. Crucially the modelling also showed that the human $TPO_{536-547}$ epitope (N-DPLIRGLLARPA-C) and the homologous mouse $TPO_{524-535}$ epitope (N-DPIVRGLLARAA-C) presented by H2-IA^k, would display a similar antigenic surface, despite the conserved residue differences. This explains why mouse TPO peptides presented by H2-IA^k induce specific activation of the TAZ10 T cells, and cause spontaneous autoimmune thyroiditis.

TAZ10 mice were found to develop disease spontaneously as early as 12 weeks after birth, with many of the histological and hormonal changes very similar to the human disease. A contributing factor to the spontaneous nature of this disease is the fact that (TPO specific) regulatory T cells are recruited to the lymph nodes draining the thyroid, and then subsequently die, probably via activation induced cell death (AICD) (Badami, Maiuri et al. 2005). This means that over time the self-antigen specific T reg population that is able to suppress the anti-thyroid response is gradually depleted, and the TPO antigen becomes gradually more immunogenic. All these factors make this model a good one for investigating anti-tumour responses. This is because tumour antigens are

effectively self-antigens in the same way TPO is in autoimmune thyroidits, and consequently anti-tumour responses could be considered to anti-self-responses just like autoimmune responses. We could use this situation to study tumour responses by manipulating tumour cells to produce TPO protein, thus making the cells a target for the anti-TPO T cells that make up the immune system of the mouse. Furthermore TPO could be introduced both into a MHC class II^+ tumour cell line to study direct effector mechanisms, and also into a MHC class II^- tumour to look at indirect effector mechanisms. The TPO+ tumour cell could then be injected subcutaneously into the mouse and the result studied in the same way as other tumour challenge experiments. If the same mechanism that caused the death of the self antigen-specific T regs in the draining lymph nodes of the thyroid causes the death of the T regs in the draining lymph node of the tumour, then the anti-tumour response could resemble the anti-thyroid response (where the thyroid is extensively damaged). This would add support to the idea that local or global depletion of T regs would be an effective treatment of tumours in humans.

7.2 Cloning TPO into a retroviral vector

The first goal in this work was to clone the TPO gene and ligate the gene into the MIGR1 retroviral vector. There were no restriction sites present in the TPO gene that matched the ones present in the multiple cloning site of the retroviral vector (figure 7.1), thus necessitating that restriction sites be added the 5' and 3' end of the cloned TPO gene. It was decided that a XhoI restriction site be added at the 5' end of the TPO gene, and an EcoRI restriction site at the 3' end. At the outset of this work I had attempted to clone TPO in one chunk from human TPO in an existing plasmid, and then in multiple parts from that plasmid. All those attempts failed, probably due to primers annealing to the plasmid instead of the gene, which no amount of primer variation seemed to eradicate. To solve this problem I decided to try and clone the mouse TPO gene. One drawback with the use of mouse rather than human TPO is that there is not a readily available antibody that recognises mouse TPO, whereas there is one for human TPO. This would

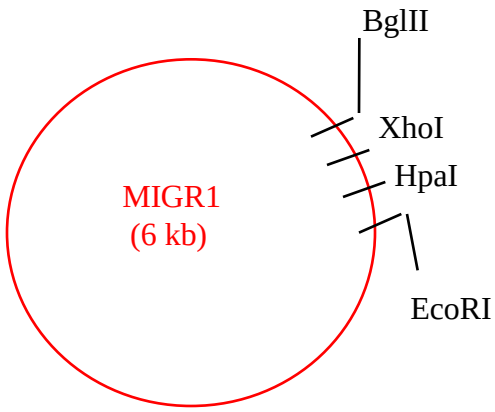


Figure 7.1. Map of the MIGR1 vector, showing the multiple cloning site.

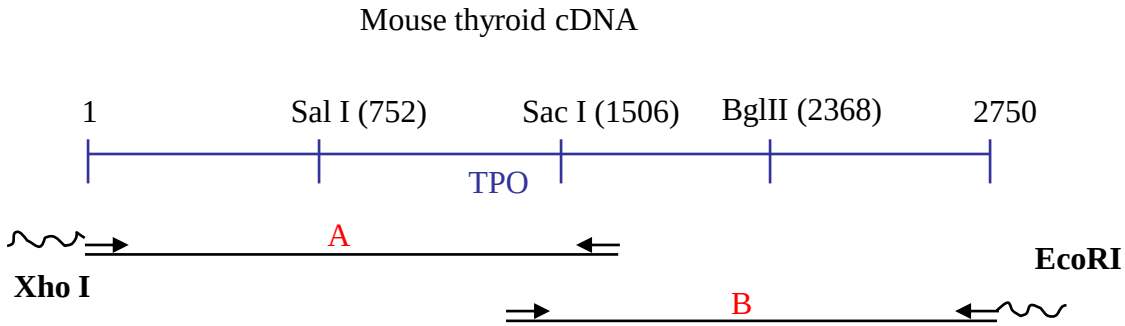


Figure 7.2. Schematic showing how the TPO gene was cloned. Mouse TPO was cloned using PCR from mouse thyroid cDNA in two parts, adding the restriction sites XhoI and EcoRI. The overlapping fragments were ligated together using the shared SacI restriction site.

make it more difficult to test whether the transfected tumour cells are producing TPO protein in later experiments.

The source of the mouse TPO gene sequence was mouse thyroid cDNA, made from mRNA extracted from lysed mouse thyroid cells. Although thyroid cDNA would contain many gene sequences, PCR should be able to easily amplify the TPO sequence from the mix. As initial attempts to clone the gene from mouse thyroid cDNA in one 3kb fragment failed (data not shown), I had to clone TPO in two parts (figure 7.2). This necessitated that the two parts overlap at a unique restriction site at the centre of the TPO gene, which turned out to be a SacI restriction site, so that the two parts could be ligated together. Figure 7.3 shows an agarose gel of the result of the PCR reaction to clone the TPO gene in two parts, which indicated that the reaction had been successful. These two products, along with the MIGR1 retroviral vector were then cut with the relevant restriction enzymes and ligated with T4 ligase. The ligation mix was then used to transform DH5α bacterial cells, which are particularly receptive of plasmid DNA. By virtue of the ampicillin resistance gene contained in the MIGR1 vector any bacteria that took up the ligated MIGR1-TPO plasmid would become resistant to ampicillin, while those that did not should remain susceptible. Consequently transformed bacteria were grown on ampicillin-enriched agar overnight, and the surviving colonies picked and grown in separate ampicillin-enriched medium 5ml tubes, again overnight. Minipreps were made of the separate tubes, and the resulting DNA run on an agarose gel. Figure 7.4 shows the result of this gel, with one lane showing a plasmid running at 9kb, which corresponds to the size of the MIGR1 vector plus the two parts of TPO. This could be confirmed by cutting the MIGR1-TPO plasmid with BglII restriction enzyme, which would cut a site in the MIGR1 vector and a site in the TPO gene (Figure 7.5). A correctly ligated plasmid would be predicted to be cut into two parts by BglII. Figure 7.5 also shows the impurities that were removed by the caesium chloride maxiprep that was done on the original DNA seen in lane 6 of figure 7.4. This maxiprep produced sufficient amounts of the MIGR1-TPO plasmid to move on to the next step, the transfection of a packaging cell line.

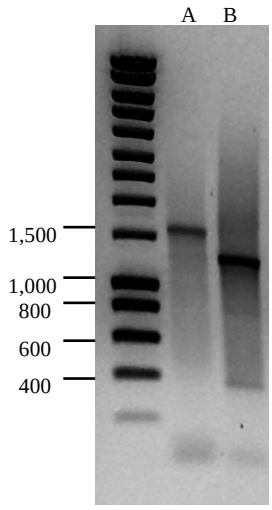


Figure 7.3. Cloning mouse TPO in two parts from mouse thyroid cDNA. Part A has a predicted size of 1582 bases, while part B has a predicted size of 1295 bases. The primer at the 5' end of part A adds the XhoI restriction site to the sequence, and the primer at the 3' end of the part B adds the EcoRI restriction site to the sequence. Part A and part B overlap at a SacI restriction site.

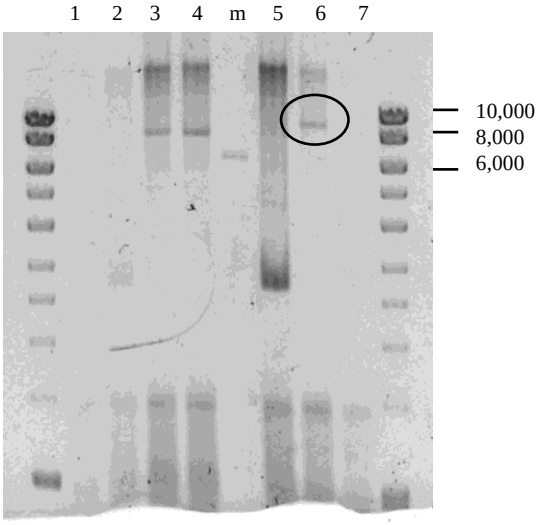


Figure 7.4. Miniprep of the MIGR1-TPO construct. Part A and part B are cut with SacI, EcoRI and XhoI restriction enzymes, allowing ligation of the two fragments to each other, and to the MIGR1 retroviral plasmid which was also cut with EcoRI and XhoI enzymes. This ligation was catalysed by T4 ligase. The ligation mixture was used to transform bacteria grown on ampicillin enriched agar. As uptake of the MIGR1-TPO construct conferred ampicillin resistance, transformed bacteria could be picked and grown in medium overnight. Minipreps were made of the transformed bacteria medium, and lane 6 shows a plasmid running at approximately 9kb, corresponding to 3kb TPO + 6kb MIGR1. 'm' refers to the empty MIGR1 vector which runs at approximately 6kb. Lanes 1-4, 5 and 7, show bands of incorrect size to be the ligated construct or no discernable band at all. (All plasmids were cut with EcoRI).

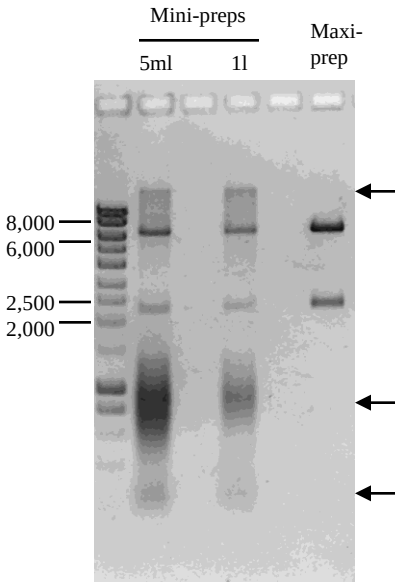


Figure 7.5. Maxiprep of the MIGR1-TPO construct. The bacteria that carried the 9kb construct were grown in 2 litres of medium, and used to make a maxiprep using caesium chloride. The maxiprep greatly increased DNA yield purity. The resulting maxiprep was cut with BglII enzyme to produce fragments of approximately 6.5kb and 2.5 kb, these were run alongside the original miniprep to ensure the construct had not been lost. The arrows show where impurities have been removed. This maxiprep DNA was then used to transfect the Phoenix packaging cell line.

7.3 The transfection of the phoenix packaging cell line with the MIGR1-TPO vector to generate TPO carrying virus.

Retrovirus vectors are used to integrate genes into the genome of the host cell, and effect long-term expression through cell division. To do this though the viral vector with the gene of interest must be processed through a packaging cell line. Packaging cell lines produce all the necessary trans-proteins – gag, pol and env – that are required for packaging, processing, reverse transcription and integration of recombinant genomes. The Phoenix packaging cell line was designed to remove the potential of replication competency that was present in the early packaging cell lines. This is where recombination events in the packaging cell line leads to the production of replication competent virus. Notably high titre production of virus by the Phoenix cell line can be enhanced by co-transfecting retroviral constructs with the pCLeco helper plasmid, which also contains cDNA encoding the viral structural proteins "gag, pol and env", but without these genes being packaged into retroviral particles. In my experiments Phoenix cells were transfected with the TPO-retrovirus DNA using the fugene-6 method. The successful transfection of these cells was shown by the expression of GFP, a fluorescent protein that can be seen under the microscope, which would co-express with any gene present in the retrovirus. Thus because expression of the TPO gene is also driven from the same viral promoter in the MIGR1 construct that expression of GFP protein is driven from, one can be confident that if GFP is being expressed, so is TPO protein. Figure 7.6 shows a successful transfection as scored by fluorescence microscopy. As a control experiment, phoenix cells were transfected with the empty vector, in which only GFP is expression is driven from the viral promoter. The transfection efficiency was better assessed by measuring GFP expression in the FL1 channel of a flow cytometer. Figure 7.7 shows that a typical transfection with the transfection efficiency at approximately 70% of the total cells.

Successfully transfected phoenix cells should produce retrovirus into the medium that the cells reside in. The retroviral particles were harvested from the supernatant of the phoenix cell culture, and used to infect splenocytes, very susceptible cells that served as a test of

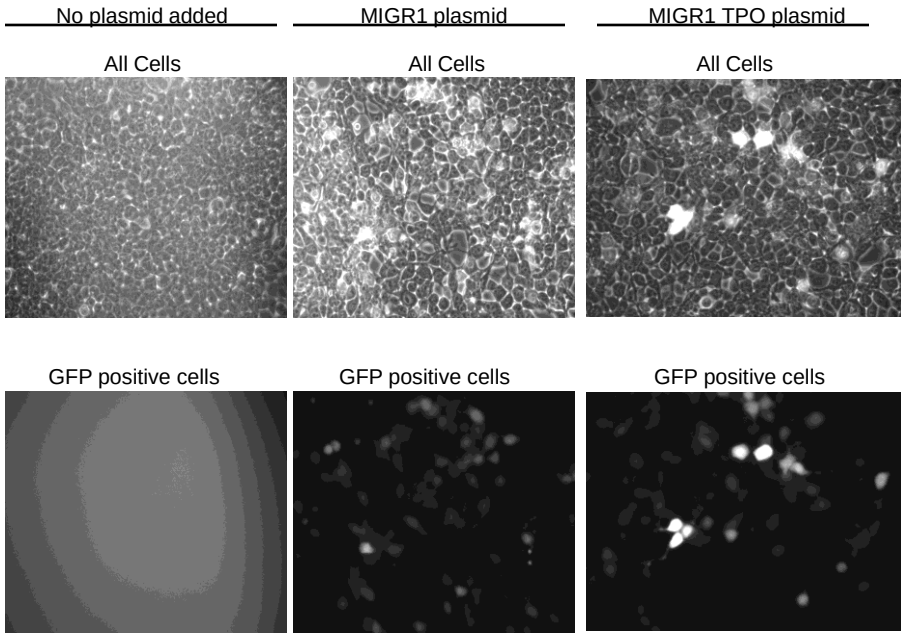


Figure 7.6. The transfection of the Phoenix cell line with the MIGR1 and MIGR1-TPO plasmids (fluorescent microscope). Plasmid DNA mixed with fugene-6 reagent to mediate the transfection and pCLeco plasmid to aid production of retrovirus. Successful transfection indicated by GFP expression of cells, visualised under a fluorescent microscope.

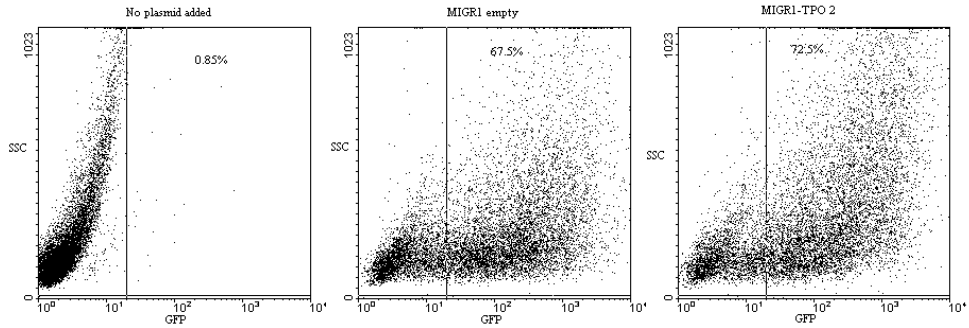


Figure 7.7. The transfection of the Phoenix cell line with the MIGR1 and MIGR1-TPO plasmids (flow cytometer). As in figure 7.6., but the GFP expression recorded as fluorescence in the FL1 channel of a flow cytometer. This indicates that approximately 70% of phoenix cells have been transfected.

the procedure. The average infection efficiency of splenocytes, as measured by flow cytometry, was approximately 20%.

7.4 The infection of susceptible tumour cell lines with virus, to generate tumour cells stably expressing the TPO gene.

The first tumour cell line that was the target of infection with harvested MIGR1-TPO retrovirus was B16, a mouse melanoma. This was a well characterised tumour that grows predictably in vivo, just below the skin making evaluation of growth relatively easy. However, this tumour line was unexpectedly resistant to infection with the retrovirus I generated. The next two tumour cell lines that I attempted to infect, EL4 (a mouse T cell lymphoma), and B6-SJ003 (a mouse B cell lymphoma), were found to be susceptible to infection however. These tumours were characterised according to their cell surface markers (figure 7.8). Crucially EL4 was confirmed as being a class II negative tumour, and B6-SJ003 was confirmed as being a class II positive tumour. The class II-restricted TAZ10 TCR will consequently only be able to interact directly with the B6-SJ003 tumour, and will rely on antigen presenting cells for indirect interaction with the EL4 tumour.

Figure 7.9 shows the infection efficiency of the two tumour cell lines with the MIGR1 retrovirus alone, and with the MIGR1 retrovirus carrying the TPO gene. Again using flow cytometry to evaluate GFP expression, figure 7.9 shows that varying levels of infection were achieved. In order to make the GFP expression, and by association the TPO gene expression, as high and as equal as possible in the tumour cell lines I used a flow cytometer to sort for GFP+ cells. Figure 7.10 shows that six weeks post the sort, high levels of GFP expression is present in all cell lines. This suggests that the retrovirus, either with TPO or without, is stably introduced into the genomes of both tumour cell lines. Figure 7.11 shows the evaluation of mRNA expression in infected EL4 tumour cells showed that TPO mRNA was being produced in the cells infected with MIGR1-TPO retrovirus, but not in the cells infected with retrovirus alone. This was done by producing cDNA from the mRNA extracts from the EL4 cells and mouse thyroid cells,

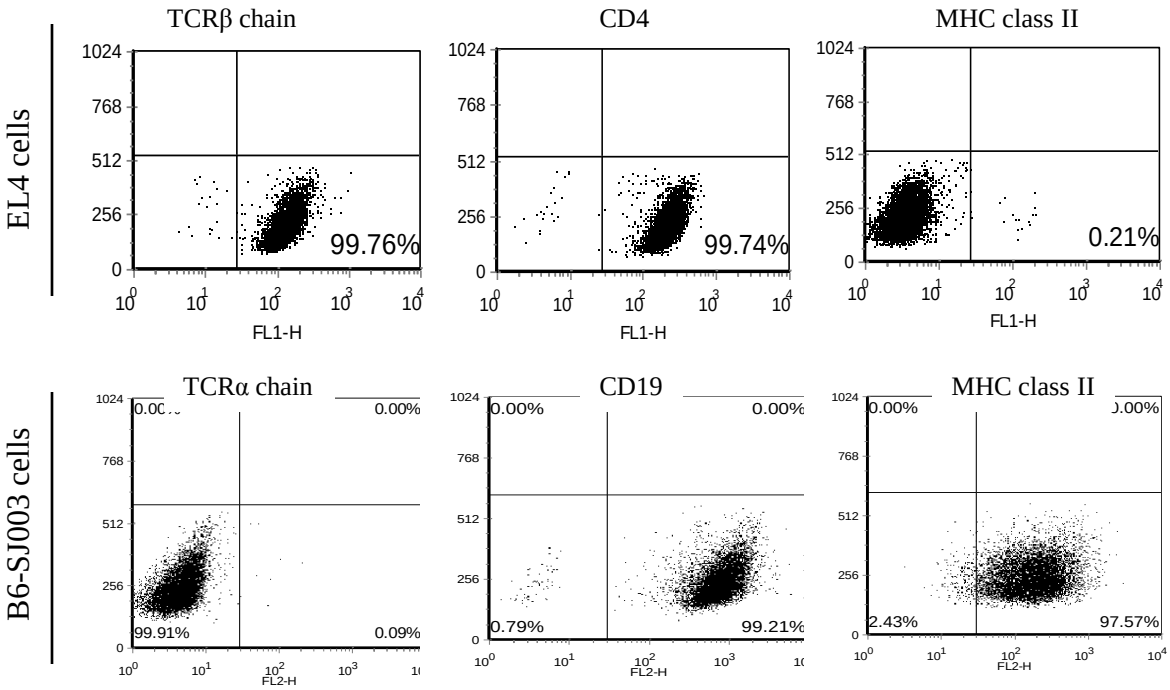


Figure 7.8: The characterisation of the tumour cells EL4, a mouse T cell lymphoma; and B6-SJ003, a mouse B cell lymphoma. EL4 cells are positive for a TCR and CD4, but negative for class II. B6-SJ003 cells are negative for a TCR, but positive for CD19 and class II.

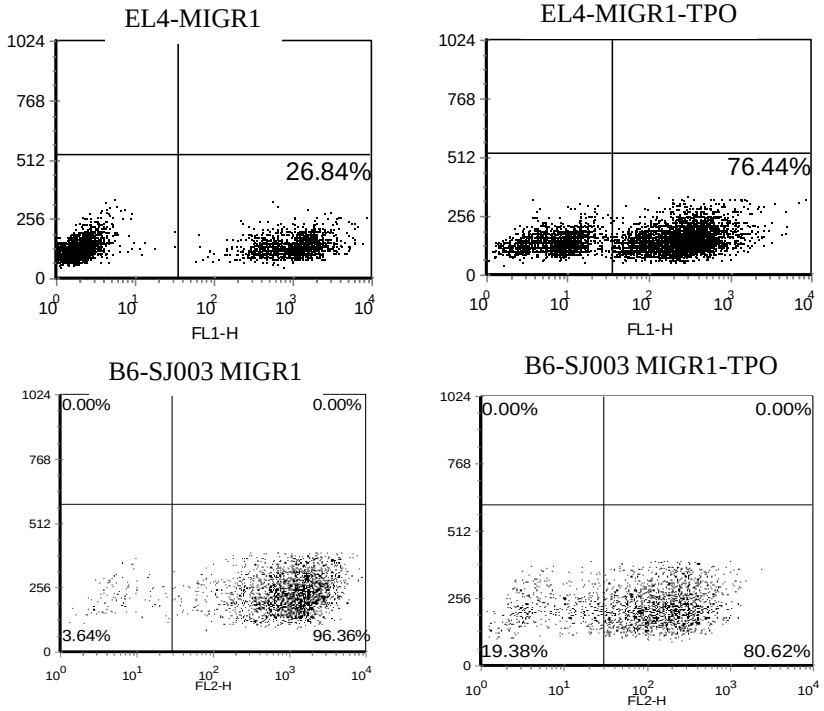


Figure 7.9: The flow cytometry analysis of EL4 cells and B6-SJ003 cells transduced either with retrovirus alone (MIGR1) or retrovirus containing TPO (MIGR1-TPO). GFP fluoresces in the FL1 channel. These populations would subsequently be sorted to enrich for GFP+ cells.

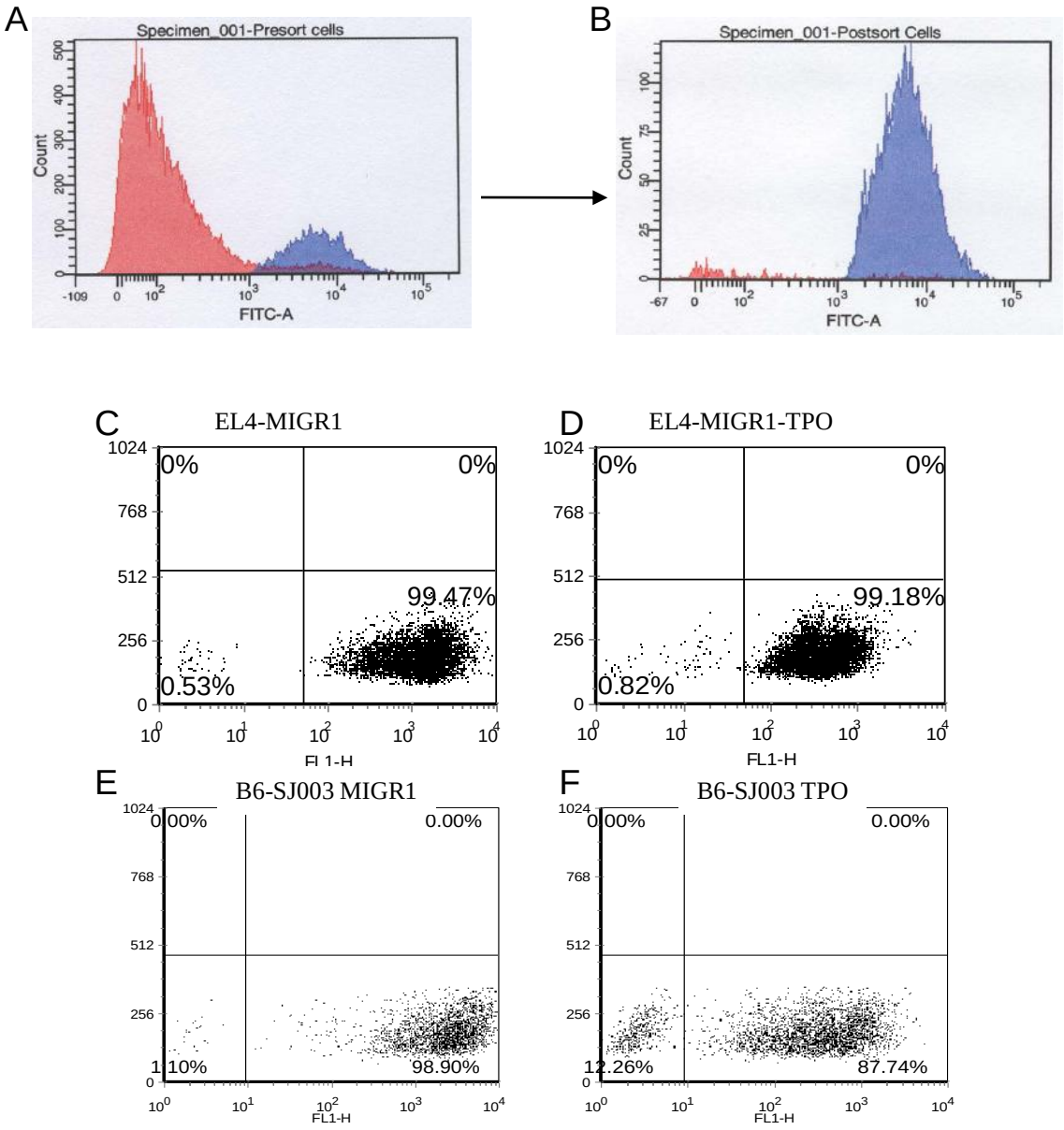


Figure 7.10. Sorting tumour cells for high GFP expression. Panels A and B are an example of a FACSAria sort, pre-sort on the left (A), and post-sort on the right (B), with the blue population being greatly enriched. The same was done to the transduced EL4 cells and B6-SJ003 cells, with the GFP+ population enriched. Panels C-F show flow cytometry plots indicating that this enrichment was maintained post the sort (6 weeks), and further analysis (not shown) indicated that GFP expression never dropped from this level.

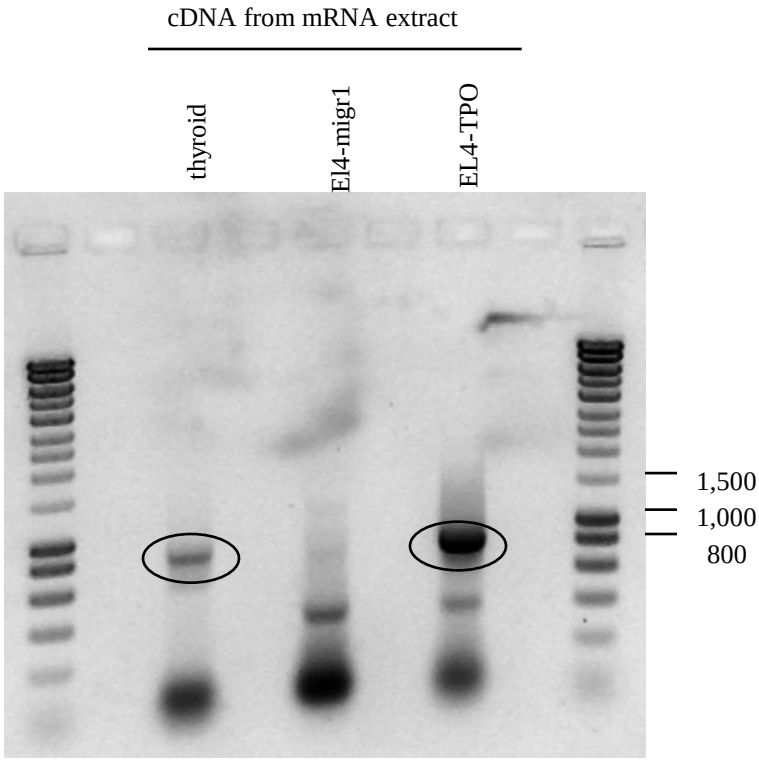


Figure 7.11: PCR of cDNA made from mRNA extracted from EL4 cells infected with TPO containing retrovirus, compared to EL4 cells infected with retrovirus alone, and mouse thyroid tissue (the native cell of TPO). Circled products indicate the presence of TPO mRNA, corresponding to the predicted size (757 bp) of two primers amplifying a short portion of the TPO sequence.

and then using PCR to amplify a short sequence from the TPO sequence. This was also done for B6-SJ003 cells and the cell line that was infected with MIGR1-TPO retrovirus was also positive for TPO mRNA.

7.5 Assessing whether the TAZ10 T cells can recognise the TPO+ tumours, in vitro and in vivo.

Once it was clear that TPO mRNA was being made in the cell lines infected with TPO carrying retrovirus, I sought to assess whether the TAZ10 TCR could now recognise either of these TPO+ tumour cell lines. In the first instance this assessment involved mostly in vitro experiments, where $Rag1^{-/-}$ TAZ10 lymph node (LN) cells were used in proliferation experiments (CFSE dilution), either directly against TPO+ tumour cells, or indirectly via dendritic cells (DCs) fed with tumour lysate. The B6-SJ003 tumour, which is class II+, was used for the direct experiments, whereas the EL4 tumour, which is class II-, was used with the DCs. This is important because the TAZ10 T cells are exclusively IAb restricted.

From the first experiment, in figure 7.12, it seemed that the DCs fed with TPO+ EL4 lysate induced proliferation of TAZ10 LN cells above DCs fed with mock infected EL4 lysate. The positive control, where DCs were mixed with the TAZ10 TCR agonist peptide p3, stimulated approximately 29% of the LN cells to divide above the background of 9%. The DCs fed with TPO negative EL4 lysate only stimulated 9% of the LN cells, whereas the DCs fed with TPO positive EL4 lysate stimulated 19% of the LN cells.

As shown in figure 7.13, the direct presentation with TPO+ B6-SJ003 cells failed to induce proliferation of TAZ10 LN cells above that of B6-SJ003 cells infected with retrovirus alone. The positive control, where p3 peptide was mixed with the antigen presenting cell CX81, stimulated 33% of the LN cells to divide above the background of 1%. The B6-SJ003 cells negative for TPO stimulated only 4% of the LN cells, and the B6-SJ003 cells positive for TPO stimulate only 1% of the LN cells.

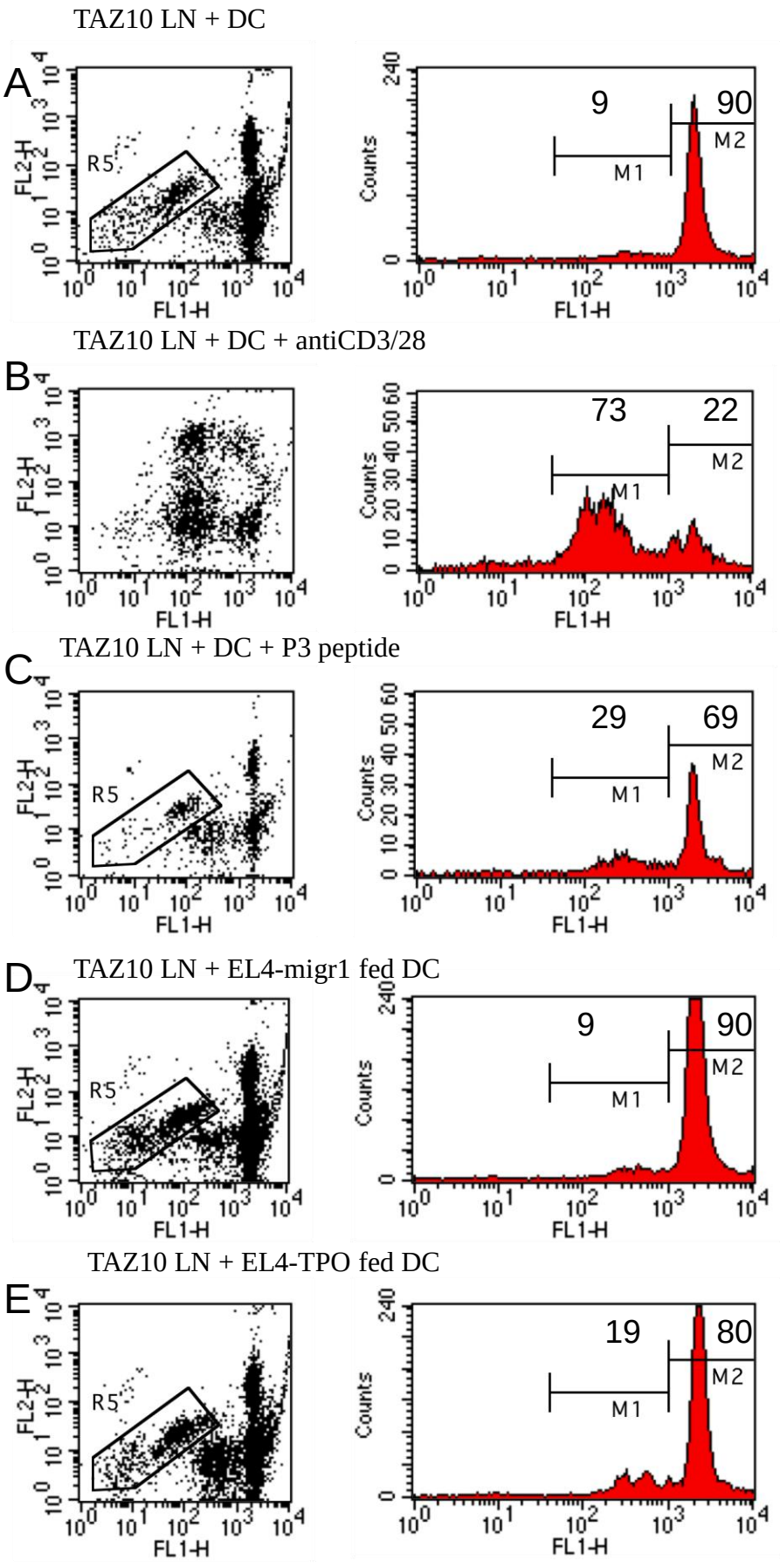


Figure 7.12: The in-vitro analyses of the responses of TAZ10 lymph node (LN) cells to DCs, fed with EL4 tumour lysate and then matured, using CFSE dilution. Panel A shows the background response of TAZ10 LN to unfed DCs. Panel B shows the positive control where anti-CD3 and anti-CD28 is added to the LN and DC mix. Panel C shows the response of TAZ10 LN if DCs are mixed with p3 peptide, which is the peptide that the TAZ10 TCR recognises. Panel D shows the response with DCs fed with lysate from EL4 tumour cells that had been transduced with retrovirus alone. Panel E shows the response with DCs fed with lysate from EL4 tumour cells that had been transduced with the retrovirus-TPO construct. The response is measured by the reduction of the CFSE signal, which would be diluted as the TAZ10 LN cells divide. The R5 gate in the far left panels exclude some unidentified background, perhaps unlabelled dendritic cells, from the CFSE panels on the right. The panel at the bottom right shows a typical SSC - FSC plot, the cells in the R4 gate represent the lymphocytes.

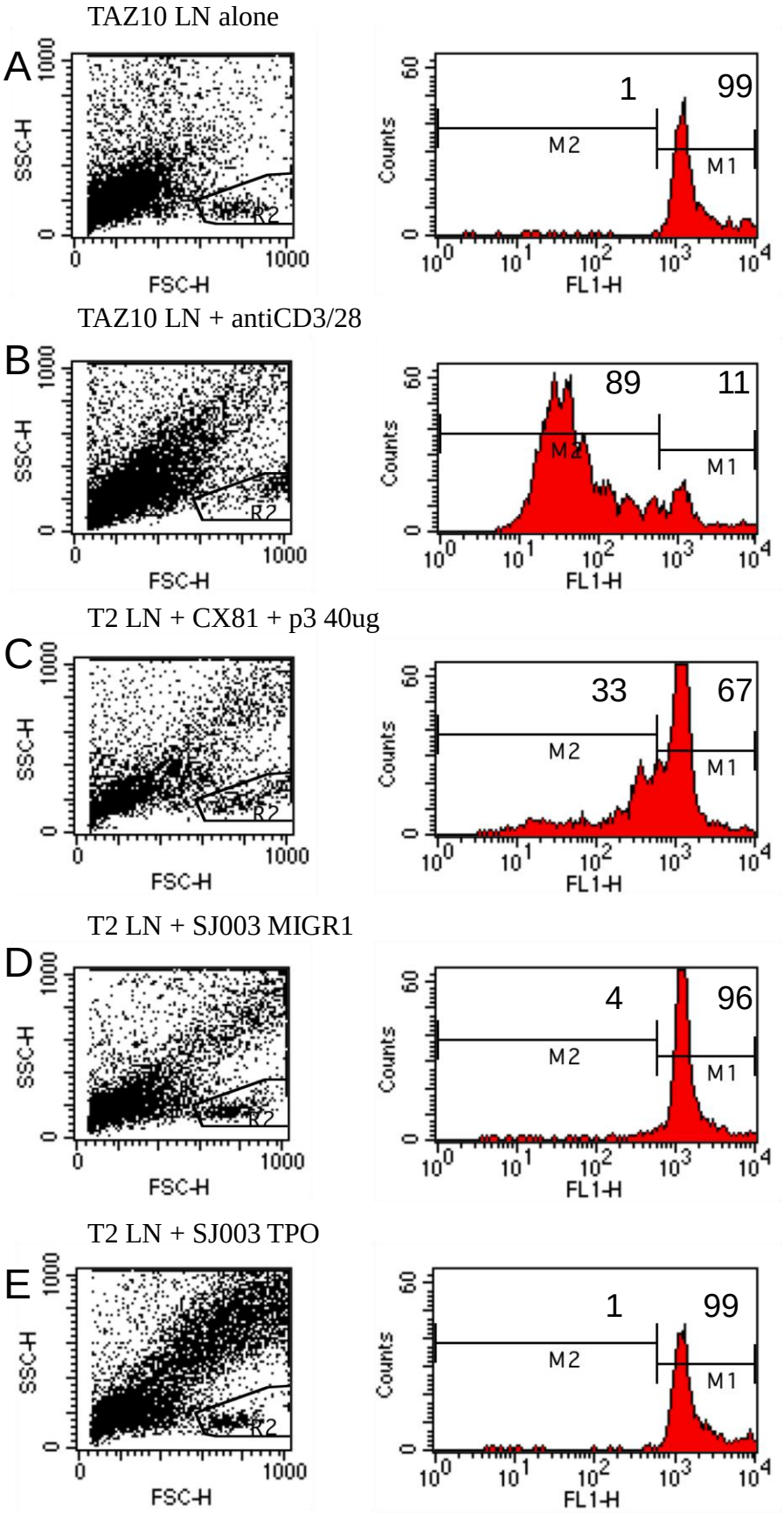


Figure 7.13: The in-vitro analyses of the responses of TAZ10 lymph node (LN) cells to B6-SJ003 cells directly, using CFSE dilution. Panel A shows the background response of TAZ10 LN alone. Panel B shows the positive control where anti-CD3 and anti-CD28 is added to the LN cells. Panel C shows the response of TAZ10 LN if p3 peptide is added with the CX81 APC to present the peptide. Panel D shows the response when LN cells were mixed with B6-SJ003 cells that had been transduced with retrovirus alone. Finally panel E shows the response when LN cells were mixed with B6-SJ003 cells that had been transduced with the retrovirus-TPO construct. The response is measured by the reduction of the CFSE signal, which would be diluted as the TAZ10 LN cells divide.

Figure 7.14 shows an in vivo experiment where wild-type C57BL/6 mice or Rag+ TAZ10 mice were challenged either with 10^5 TPO+ or TPO- EL4 cells. The hypothesis was that the EL4 tumour cells would die due to natural turnover of cells, releasing antigen that would be presented to the class II-restricted T cells by APCs. In the case of the TPO+ EL4 cells in the TAZ10 mice, this would lead to activation of TPO-specific CD4+ T cells by the TPO peptides presented by the APCs in these mice, leading to killing of the TPO+ EL4 cells, presumably by a cytokine-mediated method of killing. The result showed that there was no difference in the rejection of TPO+ or TPO- EL4 tumours the wild-type, indicating that the addition of the TPO gene did not affect the normal response to the EL4 tumour. However the result also showed that there was no advantage to the survival of the TAZ10 mice if the EL4 tumour they were challenged with was TPO+. Overall, mice of all groups began succumbing to tumour after 17 days, and all were dead after 22 days.

This short survival time, and lack of difference between the TPO+ and TPO- EL4 rejection, might be explained by the relatively large dose that the mice received of the tumour. However, a repeat of this experiment with a dose of 10^4 EL4 cells, proved non-fatal to the mice. This may have been due to the disparate nature of this T cell lymphoma upon sub-cutaneous injection. A B16 mouse melanoma tumour forms a small dense lump of cells at the point of injection, whereas the 10^5 dose of EL4 cells forms a larger, flatter subcutaneous mass as it is not a melanoma and so does not share the cell adhesion molecules that are expressed by B16 tumour cells. It is possible that with a lower dose, the tumour is disparate enough to be disseminated to various parts of the animal, making rejection easier for the host immune system. Ultimately this in vivo result suggests that TPO+ EL4 cells do not evoke a similar response to the tumour as seen in vitro, or that this response is not sufficient to reject an aggressive and large tumour dose.

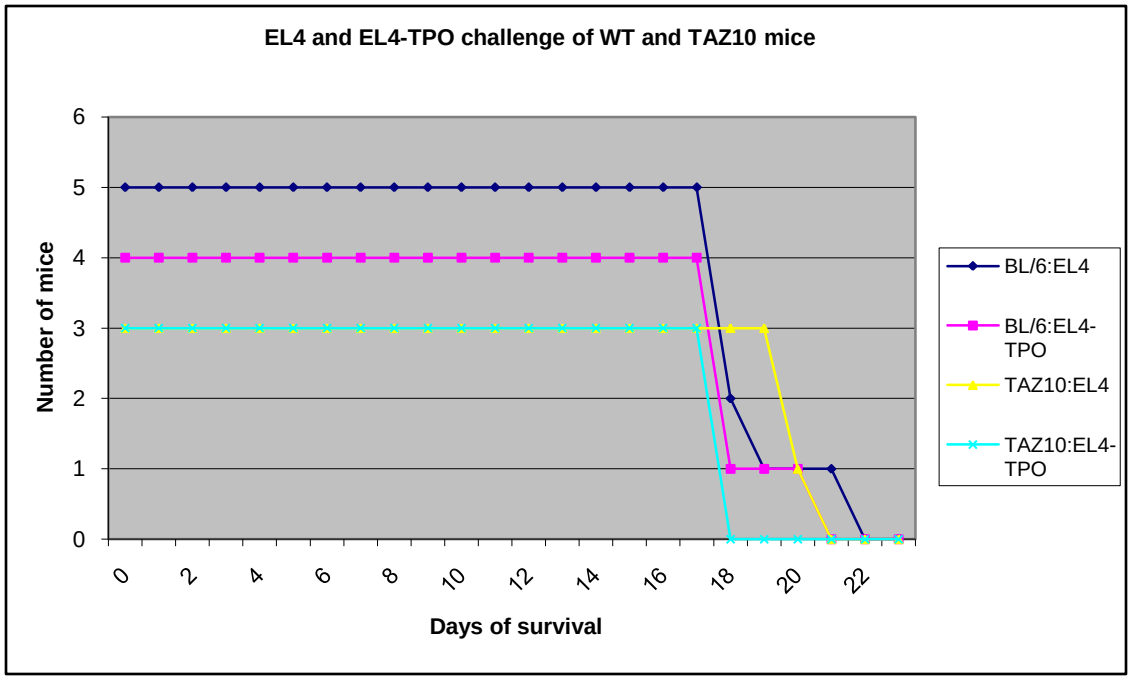


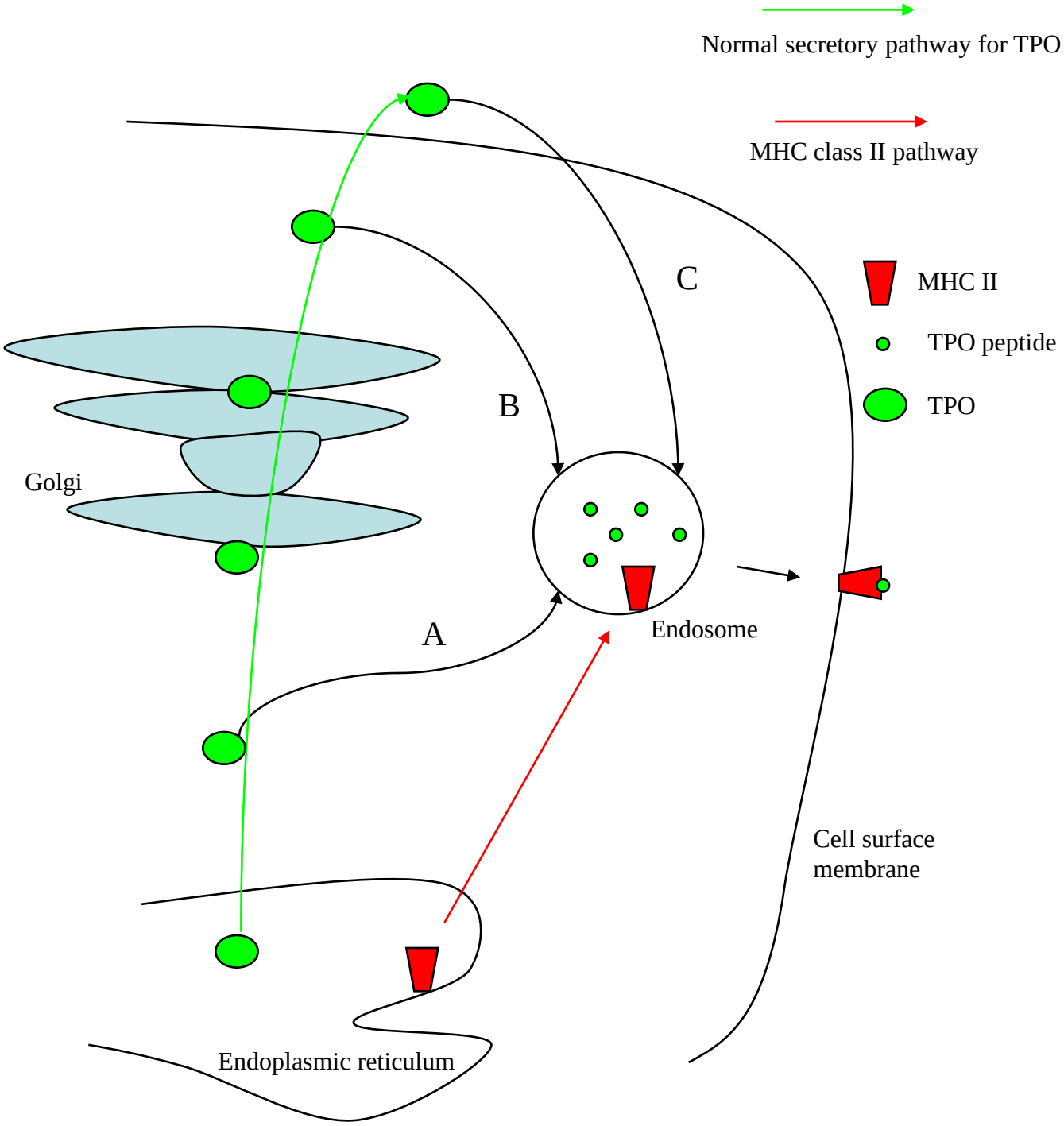
Figure 7.14. The in-vivo response TAZ10 mice make to TPO+ EL4 cells. Four groups of mice were challenged with 10^5 EL4 cells. Wild type black mice were challenged with normal EL4 tumour cells (dark blue); wild type black mice were challenged with EL4 tumour cells transduced with the TPO carrying retrovirus (pink); TAZ10 mice were challenged with normal EL4 tumour cells (yellow); and TAZ10 mice were challenge with EL4 tumour cells transduced with the TPO carrying retrovirus. Mice of all groups began succumbing to tumour after 17 days, and all were dead after 22 days.

7.6 Discussion

After all this work had been completed it was discovered that the TPO gene that was cloned into the retrovirus lacked a signal sequence at the 5' end of the gene, which alters the conclusions that can be made from this data. A lack of signal sequence would mean that the TPO polypeptide would not be exported to the cell surface, as occurs in the thyroid cell, the native home of this protein. This would explain the absence of proliferation induced by the direct presentation experiments with B6-SJ003 tumour cells, and why the tumour lysate fed to DCs is antigenic. It also shows that TPO is not processed and TPO peptide is not associated with MHC class II molecules via an endogenous (cytosolic) pathway, shedding light on previous work that shows that 'endogenous' TPO can be presented and is processed differently to 'exogenous' TPO. Previous studies had indicated that if whole TPO was endocytosed by an APC then the antagonist peptide $TPO_{537\text{-}548}$ was presented, but endogenous processing of TPO that was made by thyroid epithelial cells produced the stimulatory peptide $TPO_{536\text{-}547}$.

Like other transmembrane proteins, TPO is first synthesised in the endoplasmic reticulum (ER). After folding to the native state within the ER, intracellular transport of TPO to the cell surface occurs via the Golgi complex, a compartment typically associated with N-glycan processing of many cell surface glycoproteins. The data in this chapter indicates that the endogenous processing is dependent on the signal peptide, suggesting that at some point in the intracellular transport of TPO the pathway diverges into the pathway that allows the processing of TPO and the association of MHC class II molecules with TPO peptides. This divergence could be at several points along the intracellular transport pathway, for example before the trans-Golgi, pre-secretion, or post secretion. What the new model of endogenous TPO processing may look like is shown in figure 7.15.

Figure 7.15. Simple schematic of the possible models of how secreted TPO enters the endosomal pathway, allowing TPO peptides to bind to MHC class II molecules. The secreted TPO could enter the endosomal pathway at three points: (A) Pre-transgolgi. (B) Pre-secretion. (C) Post-secretion.



Despite the lack of the signal peptide there are some additional conclusions that can be made from this work. In vitro, DCs fed with TPO+ EL4 cells are capable of inducing proliferation of TAZ10 T cells as measured by CFSE dilution. However in vivo, challenging TAZ10 mice with TPO+ EL4 cells does not lead to tumour rejection, despite the fact that most of the T cells in that mouse express TCR that recognises TPO peptide. This lack of a rejection might be explained by the context of autoimmune disease pathology at the point of challenge. In the tumour therapy experiment the TAZ10 mice were challenged at 8-9 weeks old, and the experiment was concluded when the mice were 11-12 weeks old. All TAZ10 Rag+ mice develop spontaneously autoimmune thyroiditis by the age of 4-5 months, but the cellular changes can be seen before then. In a paper by Badami et al, the activation levels of CD4 T cells in TAZ10 Rag+ mice were shown to be comparable to wildtype at 3 weeks of age, but by 20 weeks the CD4 T cells of TAZ10 Rag+ mice show signs of activation, characterised by upregulation of PD1 and CD69, and down regulation of CD62L and CD45RB. The levels of CD4+CD25+ T regs were reduced in TAZ10 mice at 3 weeks of age compared to wildtype, and decreased even further by the age of 20 weeks (Badami, Maiuri et al. 2005). Figure 7.16, shows representations of data from another paper on TAZ10 mice, but this time on the Rag-/- version (Quaratino, Badami et al. 2004). This paper indicates the clinical and hormonal signs of thyroiditis in the TAZ10 Rag -/- mouse, but as the disease progression and weight gain in TAZ10 Rag-/- model is similar to the Rag+ model, it is fair to apply this data to the Rag+ TAZ10 model as well. The data indicates that in the TAZ10 mouse hormonal signs, T4 and TSH levels, are altered from the wild-type levels steadily from birth to beyond 18 weeks, and that weight gain in the TAZ10 mice relative to the wild-type mice really only becomes apparent after 12 weeks of age.

The clinical, hormonal and cellular changes are indicators of the progression of the autoimmune disease in these transgenic mice, and therefore also the activation state of the anti-TPO T cells in these mice. The cellular indicators lead me to conclude that at 8 weeks the anti-TPO response is becoming more active but it is by no means at the limit of its activation. The continued deterioration of the clinical and hormonal signs well after 8 weeks also backs up this conclusion. Overall, whereas there must be a balance so that the mice are not challenged with tumour when they are very sick, it is likely that the in vivo

response to a TPO+ tumour may have been more robust if the mice were challenged at an older age, perhaps approximately 12-16 weeks.

The lack of a response in the tumour therapy experiment could be due to the presence of the numerous regulatory cells that are present in these mice, although subsequent work would be needed to fully explore that possibility. The TAZ10 model has been found to contain a functioning immunoregulatory network beyond just CD25+ Foxp3+ regulatory T cells, with suppressive CD8-CD4- T cells and myeloid suppressor cells also present. All three subtypes have been found to be capable of suppressing T cell responses in vitro, and yet in vivo the disease still spontaneously occurs.

These discoveries are one of the reasons why there are several ways that this model could be further exploited in the future. The presence of these different regulatory elements presents an opportunity to investigate the role of these cells in a model that effectively mirrors a human autoimmune disease. There has already been novel discoveries made in this model concerning double negative (CD4-CD8-) suppressor cells and myeloid suppressor cells, and there can only be further discoveries made in the future.

Additionally this model still represents a good opportunity to investigate tumour immunity in a model of autoimmunity. Perhaps the manipulation of various tumours to synthesise smaller portions of the TPO protein, in particular the portion that carries the immunostimulatory peptide, may be a more efficient means of generating host immune responses to the tumour. Once this is done in vivo experiments could include challenging TAZ10 mice with TPO+ tumour either simultaneously with or followed by a challenge of untransfected (TPO-) tumour to test whether an anti-tumour response can broaden from being focused on a single antigen to reject a tumour based on multiple antigens. Or wildtype mice could be challenged with TPO+ tumour and then adoptively transferred with TAZ10 T cells to test the significance of the anti-TPO response in rejecting the tumour challenge.

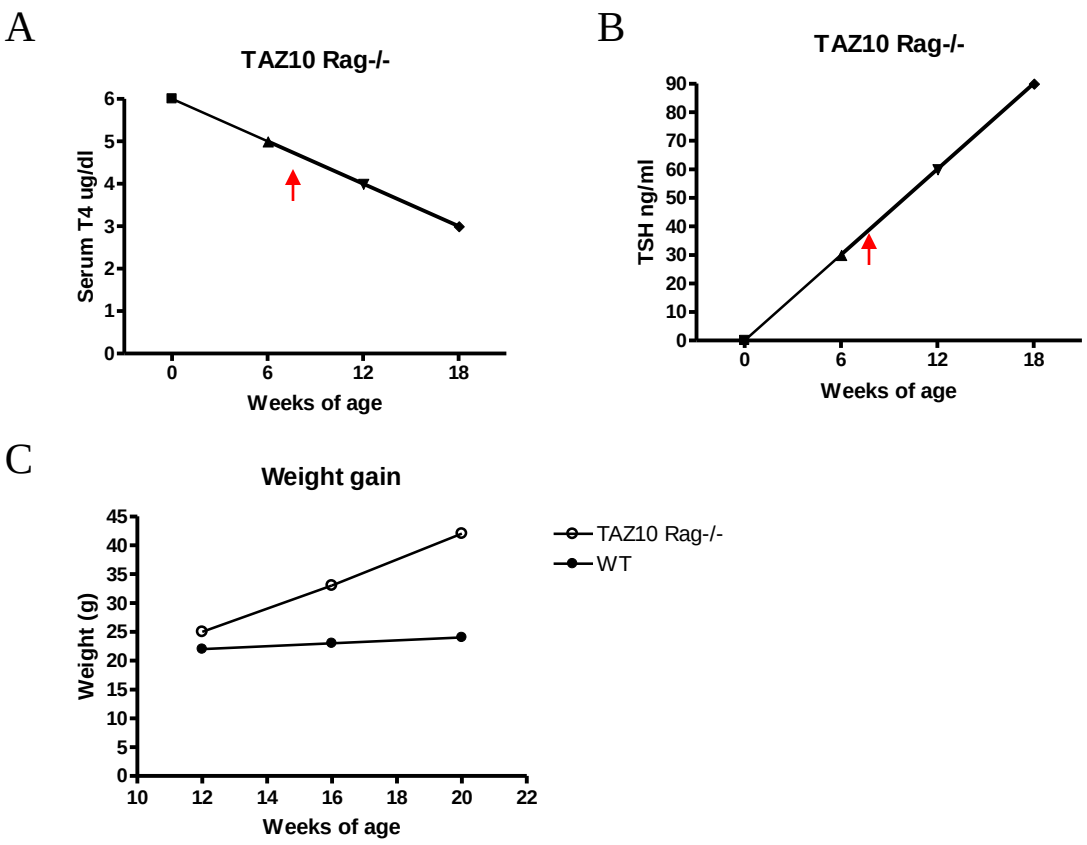


Figure 7.16. The clinical and hormonal signs of thyroiditis. These figures approximate data in a paper by Quaratino et al (Nature medicine, vol 10, no. 9, p920), that indicates the clinical and hormonal signs of thyroiditis in the TAZ10 Rag -/- mouse, though these can be approximately applied to the Rag+ TAZ10 mouse too. Panel **A** shows the level of T4 in the serum in TAZ10 Rag-/- mice, showing a steady decrease of T4 over time (WT levels do not decrease). Panel **B** shows the level of TSH in TAZ10 Rag-/- mice, showing a steady increase over time (WT levels do not increase). Panel **C** shows the weight in grams of TAZ10 mice (open circles) compared to wildtype mice (closed circles). After 12 or so weeks the weights of the two mice are beginning to diverge and by 20 or so weeks the weights are radically different. Red arrows indicate 8 weeks, which is the age at which TAZ10 Rag+ mice were challenged with EL4 tumour. As this data was collected in Rag-/- mice this can only be an approximate guide to the hormonal levels at that point. Rag+ mice were challenged before weight gain changes were evident.

Chapter 8: General Discussion

8.1 Defining the irradiated CT26 model

One of the main obstacles to immunotherapy of cancer in humans is the immunosuppressive environment that surrounds the tumour mass, preventing any effective immune response from halting or reversing the threat of the tumour. Thus much research has been focused on understanding the anti-tumour response, and the ways in which it is controlled. Early experiments with the carcinogen-induced colorectal tumour, CT26, indicated that all CT26-specific CTLs induced by CT26 engineered to produce GMCSF, recognised a single peptide, and these CTLs could lyse the tumour in-vitro, and cure mice of established tumour in vivo (Huang, Gulden et al. 1996). This peptide was identified as a non-mutated nonamer derived from the envelope protein (gp70) of an endogenous ecotropic murine leukaemia provirus, an epitope that became known as AH1. In subsequent work it was shown that untransfected CT26 tumour cells are rejected in Balb/c mice following depletion of CD25+ regulatory T cells, and that this rejection led to the development of long-lived tumour immunity (Golgher, Jones et al. 2002). It was suggested that this immunity was based on a shared-tumour antigen, as this long-lived tumour immunity also included tumours of distinct histological origin, such as A20, a Balb/c B cell lymphoma line derived from a spontaneous reticulum cell neoplasm. This antigen must be different from AH1 as immunisation with CT26-GMCSF tumour does not lead to protection from other tumours such as A20.

The original aim of this project was to investigate further this apparent shared immune-dominant tumour associated antigen and to examine in greater detail the immunosuppressive response generated by CT26, following immunisation with the irradiated tumour thereby allowing the measurement of T cell responses in the absence of overwhelming tumour growth. Initial adoptive transfer experiments, shown in chapter 3, suggested that irradiated CT26 induces a similar response to live CT26 in the absence of T regs.

One question that was not addressed by these initial experiments however, was whether the ‘cross-protective’ response requires the depletion of T regs. This was investigated in

further experiments shown in chapter 4, which indicated that both anti-CT26 and cross-reactive T cells are generated by irradiated CT26 challenge in the presence of T regs, with the anti-CT26 response being significantly boosted by depletion of T regs, and the A20 response boosted more modestly by the depletion of T regs. Our previous hypothesis for the CT26 model suggested that although potentially cross-protective CD4 or CD8 T cells can develop from live CT26 challenge with T regs, it develops into a robust anti-tumour response only in the absence of T regs. In contrast the irradiated CT26 challenge generates partial CT26-protection and partial cross-protection even in the presence of T regs. This suggested that the irradiated CT26 challenge is inducing a different response to the live CT26 challenge, possibly because the irradiated tumour exposure delivers a large antigen bolus in an inflammatory context soon after injection. The difference may also be due to differences in the activation of the innate response, and to get a clearer picture of such activation the role of NK cells was investigated in the live CT26 model. The data in chapter 5 indicates that the absence of NK cells impacts on the primary response to live CT26 tumour challenge, adversely affecting survival rates, but the situation is ameliorated if the NK depletion is accompanied by the depletion of T regs. This suggested that the live CT26 challenge activates the innate response to a significant extent, and that the innate response plays an important role in live tumour rejection. From this data I could speculate that the irradiated CT26 challenge may activate the innate response to an even greater extent than the live challenge, due to the fact that the large amount of dead or dying tumour cells would release factors that would recruit and activate many cells of the innate immune response.

Finally, chapter 6 investigated the idea that in the CT26 model, cross-protection is due to a broadening of reactivity leading to a more diverse TCR response. To accomplish this, the clonal diversity of the response to the tumour, as shown by the CDR3 lengths of the TCR, was investigated in the absence and presence of T regs. The conclusion was that the response induced by CT26GM is broader in the absence of T regs (compared to the presence of T regs), as indicated by the replacement of several oligoclonal responses in the presence of T regs with polyclonal responses in their absence. Although these data support the idea that T regs are necessary for the broadening of the response in this

model, by itself it can not prove that more diverse TCR structures leads to broader reactivity or that this indicates cross-reactivity with other tumours.

Overall these studies indicate that, despite being essentially an equivalent antigen exposure, the response induced in the irradiated CT26 model is different to the live CT26 model. In the live CT26 model T reg depletion is critical to the survival of the tumour challenge, as well as the generation of the cross-protective response. In the irradiated CT26 model, the cross-protective response is not dependent on the T reg depletion, but the absence of T regs does boost the anti-CT26 response. Ultimately these discrepancies are difficult to resolve, due to the problem that live CT26 proliferates to a lethal level in the presence of T regs. The live CT26 challenge may be generating cross-reactive T cells in the presence of T regs, as irradiated CT26 is able to do, but the lack of a sufficient anti-CT26 response makes it difficult to assess whether this potential cross-reactive response would be cross-protective in vivo.

Answers to these questions may yet be found in work that has run alongside mine in this lab, which has been able to shed additional light on this CT26 tumour model. The critical advance has been the elucidation of a candidate epitope for a dominant cross-protective response revealed by T reg depletion, the shared tumour antigen as mentioned before. This epitope, GSW11, which also resides in MuLV gp90, has allowed the comparison of the CD8 T cell responses to AH1, as the dominant epitope in the anti-CT26 response in the presence of T regs, with the response to GSW11, as an epitope of the cross-protective response in the absence of T regs. These experiments compared the AH1 and GSW11 CD8 T cell responses with either live CT26 or live CT26GM tumours, in the absence or presence of T regs in both instances, and the results have led to some interesting conclusions:

Firstly, the absence of T regs is absolutely essential to see a response to the GSW11 epitope, but surprisingly this response is revealed both in response to CT26 and CT26GM (see figure 8.1). The AH1 response is seen with both tumours, with more CD8 T cells specific for AH1 in response to CT26GM compared to CT26, but in both cases the removal of T regs increased the number AH1-specific CD8 T cells, though the number of

AH1-specific CD8 T cells remained higher with CT26GM. Another interesting observation was that in the absence of T regs the number of GSW11 CD8 T cells was equivalent to the number of AH1 CD8 T cells in the CT26 model. This is despite the fact that AH1 CD8 T cells are there in the presence of T regs, when GSW11 CD8 T cells are absent, indicating the aggressiveness of the GSW11 clone when released from T reg suppression.

More recently performed in vivo experiments have also been instructive. Survival to CT26 depends on the depletion of T regs, whereas CT26GM is rejected in all conditions, a rejection that is more robust in the absence of T regs (i.e. both AH1 responses and GSW11 responses increase). Although AH1-specific T cells are the only means of tumour rejection in the CT26GM challenged mice in the presence of T regs (see figure 8.1), both AH1 and GSW11 responses are seen in the absence of T regs with CT26 challenge, making it more difficult to ascertain which is the most important when it comes to CT26 rejection. As GSW11 responses are necessary to see cross-protection it might be that the CT26 rejection is primarily the focus of AH1 specific T cells but that is not definite.

Further discoveries could be made by investigating the CD4+ T cell dependence of the CD8+ anti-GSW11 response. Work by Golgher et al, showed that tumour rejection by CD4+ T cells, but not CD8+ T cells, was a specific feature of T reg depletion (Golgher, Jones et al. 2002); leading one to expect that CD4s will have a crucial role in the GSW11 response. It is known that there is a helper epitope also in MuLV gp90, but it is unknown how much help the GSW11 CD8+ T cell response requires. If there is little requirement for help it would suggest that the GSW11 specific T cells are quite potent, whereas if there is a lot of requirement for help it would lead one to expect the response to have slower kinetics.

Furthermore antigen-specific T regs, presumably activated by the gp90 helper epitope, are able to completely suppress the GSW11 response, suggesting that the GSW11 CD8+ T cell response is more sensitive to T reg suppression that the AH1 response.

One possibility is that the avidity of GSW11 specific T cells for their antigen is low enough for them to be close to death by neglect when being selected in the thymus, and

Figure 8.1. Summary of the in-vitro CD8+ T cell responses to AH1 and GSW11. The relative strength of the responses are indicated by the number of + signs: (+) = weak; (++) = modest; (+++) = strong; (++++) = very strong; (-) = absent.

Pre-treatment of the mice	**Tumour challenge**	**CD8+ T cell response to AH1**	**CD8+ T cell response to GSW11**
Untreated	CT26	+	-
Untreated	CT26GM	++	-
T reg depleted	CT26	+++	+++
T reg depleted	CT26GM	++++	++

as a consequence need a lot of stimulus in the periphery to form a response. Thus the response would take more time to build up, giving peripheral tolerance mechanisms such as T regs ample time to prevent the 'self' response fully forming. More experiments would be needed to be certain of the requirement for CD4+ T cell help, and the mechanisms behind the T reg suppression, of the GSW11 response.

These additional observations can be used to inform the data I have collected in this thesis. The observations in chapter 6 that the clonality of the response to CT26GM is broadened in the absence of T regs, backs up the observation that a CD8 T cell response is revealed to GSW11 in the CT26GM model in the absence of T regs. Furthermore, the observation that the response to GSW11 is only revealed in the absence of T regs in the CT26 model adds weight to the supposition that only in the irradiated CT26 model are cross-protective responses generated in the presence of T regs. It is likely that further experiments into the cross-protective epitope will continue to yield insights into the impact of T regs on induction of cross-protective tumour immunity. For example, as mentioned above, it will be interesting to discern the impact T regs have on the helper T cell response in particular, or experiments may yield answers to whether T regs are truly antigen-specific in suppression of tumour responses.

8.2 The state of immunotherapeutic strategies to tackle cancer

Despite the potential of the immune system to become a key member of the therapies we have to combat cancer, clinical trials employing a range of immunotherapeutic strategies have had somewhat limited success in inducing immune sensitisation against tumour antigens. However there has recently been revived interest in the possibility of combining radiation and immune-based therapies to achieve better local and systemic tumour control. It had been thought that radiation therapy would be immunosuppressive, due to the fact that lymphocytes are sensitive to radiation (Roses, Xu et al. 2008). It now seems that radiation therapy might be immunomodulatory rather than immunosuppressive, with a potential role for radiation in enhancing anti-tumour immunity. It seems radiation therapy is effective at signalling 'danger' via the increased expression of

proinflammatory cytokines, and in the activation of antigen presenting cells (McBride, Chiang et al. 2004). This is supported by the observations that radiation therapy enhances the expression of tumour-associated antigens, induces immune-mediated targeting of tumour stroma, and diminishes regulatory T cell activity. Radiation therapy may also activate effectors of innate immunity through TLR-dependent mechanisms, thereby augmenting the adaptive immune response to cancer (Roses, Xu et al. 2008). Radiation induced upregulation of Fas on tumour cells has also been shown, which would enhance immune recognition of antigen-expressing tumour cells (Chakraborty, Abrams et al. 2004).

Building upon the hypothesis that radiation can enhance anti-tumour immunity, investigators have begun to combine radiation therapy with immunotherapies. Generally the radiation is used to induce tumour cell apoptosis or necrosis, releasing tumour antigens for subsequent presentation by DCs. There have been promising results with irradiation combined with intra-tumoural or peri-tumoural DC administration (Nikitina and Gabrilovich 2001), or administration of Flt-3L, a growth factor that stimulates production of dendritic cells (Chakravarty, Guha et al. 2006). There have also been explorations of combining irradiation with cytokine therapy; studied cytokines include IL-3 (Chiang, Hong et al. 2000), IL-12 (Seetharam, Staba et al. 1999) and TNF-α (Weichselbaum, Hallahan et al. 1994). Local radiation therapy in combination with CTLA-4 blockade has also been demonstrated to induce CD8 T cells in a poorly immunogenic murine adenocarcinoma model, whereas CTLA-4 blockade alone did not (Demaria, Kawashima et al. 2005). These studies overall are encouraging for the future of combined radiotherapy and immunotherapy.

The work presented here involving the effect of irradiating CT26 would also back up the fact that radiation of tumours can enhance anti-tumour immunity. Furthermore my work suggests that the depletion of T regs might be an immunotherapy that would combine with radiotherapy to further boost the anti-tumour response.

Another area of promise in the quest to induce effective anti-tumour immunity are the tumour cell lysate vaccines, which to date have had some success in the clinic. In one instance hundreds of patients with advanced stage melanoma, many with metastatic

disease having failed chemotherapy, participated in a study of the vaccine Melacine. Melacine is composed of two allogeneic cell lines, derived from biopsies of subcutaneous nodules, which is administered as mechanically disrupted cell lysate in the presence of DETOX adjuvant (Sondak and Sosman 2003). In phase I and II trials of Melacine in patients with especially advanced disease (stage IV), 10-20% of patients showed clearing of some metastatic sites, and in another 10-20% of patients the disease was stabilised. In a phase III study, Melacine was compared with a four-drug chemotherapy regimen and the response rates and survival were the same, with the advantage that Melacine was non-toxic compared to the chemotherapy (Mitchell 1998). A similar vaccine preparation, Canvaxin, was evaluated in ~1,000 stage IV melanoma patients and compared with an equal number of patients who were treated with surgery and chemotherapy during the same time period, but did not receive the vaccine. The result was a small but significant increase in the overall survival of the vaccinated group (Morton, Hsueh et al. 2002). In a more recent radomised phase III study, Jocham et al. used an autologous tumour-cell lysate, which had been pretreated with IFNγ, to vaccinate renal cell carcinoma patients after radical nephrectomy. The results indicated that the vaccine was beneficial, with 5-year and 70-month progression-free survival rates at 77.4% and 72%, respectively, in the vaccine group and 67.8% and 59.3%, respectively in the control group (surgery only) (Jocham, Richter et al. 2004).

Despite these small successes however, no trial of tumour-cell vaccines has been successful enough for routine use in the clinic. Like other immunotherapies therefore, the future of tumour-cell vaccines may lie in their combination with other forms of treatment. The data collected here adds support to that notion, with the irradiation of the tumour cell seemingly pushing T cell priming in favour of forming a productive response, as well as the removal of T regs proving to be sufficient to boost the response still further.

8.3 The use of autoimmunity to investigate tumour immunity.

The original hypothesis that predicted that the immune response would respond to tumours, thus giving us the potential of tumour vaccines, was the ‘tumour surveillance’ hypothesis, put forward by Thomas and Burnet (Burnet 1957). The problem with the

hypothesis is that if there is spontaneous and successful tumour immunity then it would never become apparent to observers. This makes testing the validity of this hypothesis very difficult. Fortunately, recent studies have offered the first direct evidence of naturally occurring, successful tumour immunity in humans, evident only because it is linked to a second phenomenon – autoimmune neurologic disease (Darnell and Posner 2003). These diseases are the rare paraneoplastic neurologic degenerations (PNDs), in which the patients develop degeneration in discrete regions of the nervous system. Clinical examinations reveal cancers in all these patients, which can be breast or ovarian carcinoma or small cell lung cancer. These tumours are malignant, but show unusually limited spread and the patients respond well to treatment. Sometimes only microscopic foci of the tumours can be found, accompanied by inflammatory infiltrates, and occasionally spontaneous tumour regression is observed (Darnell and DeAngelis 1993). It is clear that this naturally occurring tumour immunity is directly linked to the autoimmunity, as the tumours have been shown to express neuronal proteins, and the PND patients harbour high titre antibodies in their blood and spinal fluid directed to neuronal antigens (Musunuru and Darnell 2001). This example demonstrates that autoimmunity and tumour immunity are both naturally occurring and spontaneous immune responses, which can occur simultaneously and can use the same mechanism of tissue destruction. This strengthens the argument that autoimmunity models can, and should, be used to study tumour immunity.

Another example of a concurrent autoimmune response and anti-tumour response is in mice with vitiligo and melanoma. As mentioned in the introduction (section 1.8) not only do the responses happen concurrently but also in response to the same antigen. These antigens are the melanocyte differentiation factors, such as gp75 (Vijayasaradhi, Bouchard et al. 1990), or TYRP-2 (Bowne, Srinivasan et al. 1999). In these models B16 mouse melanoma rejection and depigmentation of the skin were the two manifestations of the tumour immunity and autoimmunity respectively. The conclusions of these studies were that these two responses overlapped, but that they used alternative antigen-specific mechanisms: The tumour response was perforin independent, but required CD4+ T cells and NK cells; while the autoimmune response did not require CD4+ T cells or NK cells, but was perforin dependent. This insight means that while these two responses are to the

same antigens, the responses can be uncoupled, lending further reassurance that in the future we will be able to induce better anti-tumour responses without autoimmunity side effects.

Overall there is much knowledge to be gained from studying tumour immunity and autoimmunity responses together, which was the rationale behind my work with TPO+ tumours in the TAZ10 transgenic models. The main conclusion from this project was that endogenous processing of TPO is dependent on the signal peptide, and at some point in the intracellular transport of TPO the pathway diverges into the pathway that allows the processing of TPO and the association of MHC class II molecules with TPO peptides, for recognition by CD4+ MHC class II restricted T cells. This was shown by the fact that the absence of the signal peptide meant that no TAZ10 lymphocyte response was seen to tumour cells engineered to produce TPO protein. Exogenous processing of tumour lysate by dendritic cells did produce a response however, suggesting that stimulatory TPO epitopes are present in these engineered tumours. These interesting conclusions could be the first of many to come out of this study of tumour immunity in the TAZ10 autoimmunity model, were this work to be continued further.

8.4 Final comments

My work has been concerned with evaluating anti-tumour responses, both in the context of T reg control of those responses, and in the context of how autoimmunity models may be used to understand them. My general conclusion is that immunotherapies that are designed to tackle cancer must either be multi-faceted or combined with other cancer treatments if we are going to see the best results in the clinic. Despite the severe potential side effects of manipulating regulatory T cells in humans, my work has shown that there still is a case for removing the influence of these cells in order to boost anti-tumour responses. Additionally the severest of these side-effects may be averted if we can more thoroughly understand the similarities and differences between anti-tumour and autoimmune responses, and crucially learn to uncouple those responses.

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