Signalling mechanisms underlying priming and tolerance of T cells

Li-Heng Hsu

A thesis submitted to the College of Medicine, Veterinary and Life Science, University of Glasgow in fulfillment of the requirements for the degree of Doctor of Philosophy

May 2013

Division of Immunology, Infection and Inflammation
Glasgow Biomedical Research Centre
120 University Place
University of Glasgow
Glasgow
G12 8TA

Abstract

The primary mission of the immune system is to defend against invading pathogens. The normal healthy body can distinguish self from non-self antigens. When a new antigen is encountered, such discriminatory capacity would generate a productive immune response against invasive pathogens or exert antigen-specific tolerance, the latter to prevent harmful immune responses against self-components or non-dangerous food or environmental antigens. Peripheral tolerance plays an important role in preventing T cells response to self or harmless antigens. A breakdown in tolerance within an individual can result in the development of a variety of autoimmune disorders.

Full T cell activation requires at least two signals. The first one is provided by the TCR recognizing cognate peptides derived from antigen in the context of appropriate MHC molecules expressed by antigen presenting cells (APC). The second is mediated by “co-stimulation” via interaction of CD28 on the T cell with CD80/86 on the APC. The clonal anergy is induced when the TCR is ligated in the absence of co-stimulation, one of the proposed mechanisms of peripheral tolerance, describes a state of long lasting unresponsiveness to antigen, in the T cell. Despite widely studies in this area, however, the mechanisms of induction of anergy and the efficient markers for diagnosis of anergy are still not clear.

One of the mechanisms which contributes to forming tolerance is anergy, which can be defined as defect in cellular proliferation and IL-2 production. Furthermore, GTPase Rap1 has been reported to inhibit the generation of pERK signals and to accumulate in tolerant cells. However, most of previous studies have done by biochemical assessment of signaling in T cell lines or clones upon polyclonal stimulations in vitro, and thus has generated some conflicting data. For solving this

problem, our lab has developed the technique, laser scanning cytometry (LSC), for observation of responses in individual antigen-specific T cells within their environmental niche within primary or secondary lymphoid tissue. By LSC, it has reported that there are significant differences in the amplitude and cellular localization of phosphorylated ERK signals when naïve and in vitro-primed and tolerized T cells respond to Ag. To further investigate the role of Rap1 by LSC, it revealed that counter regulation in Rap1 and phosphor-ERK expression during the maintenance phase of tolerance and priming of antigen-specific $CD4^+$ T cells *in vitro* and *in vivo*. In T cells, the maintenance phase of anergy has been reported to reflect defective activation of transcription factor, such as c-Jun/c-Fos, that are involved in formation of the AP-1 complex, which is required for inducing transcription of the IL-2 gene and optimal activation and effector function of T cells. In turn, this appears to be determined by the lack of recruitment of the ERK, JNK and p38 MAPK signaling cascades. The small GTPase, Rap1, has long been implicated in such desensitisation of ERK, and the consequent reduced IL-2 production, observed in tolerised T cells. However, the most of these studies were processed with T cell lines or clones in vitro and as such are not necessarily representative of physiological responses of primary antigen-specific T cells. Consistent with the previous finding, we have extended these studies to investigate whether Rap1 plays a role in determining commitment to anergy and priming during induction and maintenance phases. As expected, analysis in the DNA synthesis during maintenance phase reported that the primed T cells exhibited a higher response than either naïve or anergic T cells, whilst the anergic T cells displayed an even lower DNA synthesis than naïve T cells undergoing a primary response. To further investigation in cytokine production of IL-2 and IFNγ at 24, 48 and 96 hour during the maintenance phase, consistent with previous studies, the primed T cells produced the highest levels of IL-2, relative to anergic cells with the lowest

levels, at the first 24 hours after challenge with antigen. However, the IL-2 production from primed and anergic T cells both drop down from 48 hours and to very low level at 96 hours but accompanying with gradual increase of IFNγ production. This implicates both anergic and primed cells consumed IL-2 secreted in the early stage of maintenance phase for supporting following cellular differentiation. The assessment of cellular proliferation also indicates that both primed and anergic cells had undergone several rounds of division. Whereas the primed cells proliferated more and faster than anergic cells over the first two days, after that anergic cells were able to catch up with primed cells. Consistent with above proliferative responses, the primed T cells showed higher levels of ERK activation than anergic cells at day 1 but lower levels of ERK activation than anergic cells at day 3. Surprisingly, there is no difference in Rap1 activation between primed and anergic T cells during maintenance phase.

The additional finding from cellular proliferation during maintenance phase revealed that both primed and anergic cells undergo clonal expansion during induction of priming and tolerance, which leads the further investigation in functional outcomes, MAPK signaling and mTOR pathways studies during induction phase. The primed cells exhibited higher levels of DNA synthesis than anergic cells at 48 hours whereas they had similar levels of DNA synthesis at 96 hours. The IL-2 and IFNγ production were only detectable within the first 48 hours but not 96 hour. Collaborating with the data from cellular proliferation indicates the IL-2 were consumed for promoting the cells survival and proliferation since both populations showed clear peaks representing differential numbers of cell division from day 2 (48 hour) onwards, whereas the primed cells proliferated more and faster than anergic cells during whole induction phase. Moreover, cyclic activation of ERK was seen in the primed T cells and at higher levels of activation than in the

anergic population, which did not exhibit these kinetics in western blotting. Interestingly, the primed T cells exhibited slightly higher levels of Rap1 than anergic cells from 48 hour until 96 hour during induction phase.

Consistent with data from in vitro, the proliferation response in mimicking physiological model also can be replicated. Additionally, the counter regulation in ERK and Rap1 activation also occurred during the induction of priming and tolerance, which is investigated by adenoviral gene transfer of Ad Rap1 S17N, an inactive mutant of Rap1. Furthermore, modulation of Rap1 expression with Ad Rap1 S17N in cells during induction of anergy, revealed that Rap1 activity acts to limit cellular proliferation and thus switching off Rap1 activity upregulates cellular proliferation to generate a phenotype more resembling priming of normal (or GFP-) T cells by antiCD3+anti-CD28, which showed higher proliferation that GFP- cells stimulated with anti-CD3 only. However, when these adenoviral transfer experiments were repeated in the more physiological model, the higher proliferation exhibited in anergic Ad Rap1 S17N transduced cells were not replicated, suggesting that the enhancing effect of Ad Rap1 S17N might be substituted by signals generated under these more physiological conditions. There did not appear any difference between anergic and primed cells in terms of ERK/Rap1 signalling during the induction phase and introduction of Ad Rap1 S17N did not modulate ERK activity in transduced cells treated with anti-CD3 or anti-CD3+anti-CD28, suggesting that Rap may target some other effector during the induction phase. To sum up these data, Rap signaling in anergy and priming as well as the use of the dominant negative construct suggested that Rap was not acting to suppress ERK activation during induction of anergy. The further investigation in the downstream targets, c-Myc, did not see any direct connection with ERK/Rap1 activation during induction of anergy and priming. Moreover, the

primed T cells tend to skew to catabolic rather anabolic metabolic pathways, when compared to anergic T cells during the induction phase, as evidenced by the primed cells exhibiting upregulation and phosphorylation of AMPK and Raptor to inhibit mTORC1 funtion and in turn, lower levels of pp70 S6K. However, the expression of phosphorylated Rictor in anergic t cells was higher than that of primed T cells, indicating inhibition of mTORC2 in anergic T cells resulting in downregulation of AKT activation during this induction phase.

Table of Contents

List of Figures

List of Tables

Acknowledgements

Firstly, I would like to thank my supervisor Professor Maggi Harnett for her unwavering support during the past 3 years of my studying in Glasgow. Maggie has been a scientific and life mentor, this thesis would not have been possible without her supervision and guidance.

A special thank to Dr. Alison Michie for sharing knowledge and providing suggestions especially on FACS analysis. Thank you to Professor Janet Allan for giving inspiring ideas and spending time to read through my thesis from her busy schedule. My warm thanks also to Dr. Alastair Gracie for his assistance in solving problems during my PhD student life and brought me to Maggie's lab.

This thesis would not have been possible without the help and support from my colleagues from laboratory. I am particularly grateful to Dr. Angela Morton for setting up experiment models in the beginning of this project. Many thanks to Dr. Miguel Pineda, the "FACS compensation magician", and Dr. Verica Paunovic for kindly providing all the technical helps. I am grateful to Dr. Theresa Thalhamer, Dr. Mairi McGrath, Dr. Jen Coltherd, Dr. Rusty Eason, Hatim Nada, Dimity Ball and David Rodgers for patiently answering my endless questions and sharing hilarious jokes with me. I will always keep the wonderful memories of room 428 with me.

I am thankful to my friends, Hsing-Hua Chang, Chuang-Chun Hsu and Kuan-Jen Wang for lovely company and laughter in many dinners we cooked together in Glasgow, Dr. Chia-Ling Yang for her help and moral support during my writing-up period in Edinburgh, and my family and friends in Taiwan for their kindness.

I wish to dedicate my thesis to my mother, who always supports me in all ways. Surely she will be very pleased to see the completion of my PhD.

Declaration

The work presented in this thesis represents original work carried out by the author.

This thesis has not been submitted in any form to any other University.

Li-Heng Hsu
University of Glasgow
May 2013

Chapter 1
Introduction

1 Introduction

1.1 The Immune system

The immune system is a complex defence system which provides protection to the host from a variety of pathogenic microorganisms, including bacteria and viruses. The immune system can be divided into innate and adaptive immunity. The innate immune system provides critical mechanisms for the rapid sensing and elimination of pathogens but is non-specific. Adaptive immunity provides a wider and specific recognition for self- and nonself-antigens. Moreover, whilst the innate immune response can detect the same pathogen on repeated exposure, adaptive immunity allows a memory response to develop so that on subsequent encounter with the same antigen, this secondary adaptive immune response is quicker and more efficient.

1.1.1 Innate immunity

Cells of the innate immune system provide critical mechanisms for the rapid sensing and elimination of pathogens. This kind of immunity occurs throughout life and is the initial response by the body to eliminate microbes and prevent infection. Phagocytes, macrophages and granulocytes, and natural killer (NK) cells provide the first line of defence. However, unlike adaptive immunity, innate immunity does not recognize individual antigens. Rather, the major mechanism for recognition of foreign antigens by these cells involves pattern recognition receptors (PRRs) which bind common pathogen-associated molecular patterns (PAMPs) leading to the initiation of an innate immune response. These common microbial molecules include LPS from gram-negative cell walls, peptidoglycan and lipotechoic acids from gram-positive cell walls, the sugar mannose, bacterial and viral unmethylated

CpG DNA, bacterial flagellin, the amino acid N-formylmethionine found in bacterial proteins, double-stranded and single-stranded RNA from viruses, and glucans from fungal cell walls. PAMPs are structures shared by many classes of pathogens ensuring that the innate immune response recognises and combats a broad spectrum of pathogens in the first few days of infection while the adaptive immune system becomes activated. Moreover, innate cells, via their role in antigen presentation are crucial for priming of the specific adaptive response.

1.1.2 Adaptive immunity

Adaptive immunity has evolved to provide a specific and more finely tuned repertoire of recognition of foreign antigen. The cells of the adaptive immune system are lymphocytes: T cells and B cells which broadly comprise the two branches of cellular and humoral (antibody-mediated) immunity. It is a self-regulated system because both the T cell and B cell receptor repertoires evolve by interaction with self-ligands to ensure self-tolerance. The activation of T cells requires antigen presenting cells (APC), like macrophages or in particular, dendritic cells for initiation of primary responses, and these cells present antigen that they capture during activation of the innate immune system. Additionally, the other significant functions of adaptive immunity are to develop class switched, affinity matured antibodies and immunological memory which collectively produces more rapidly and more effectively immune responses to the re-encountered antigen [1].

1.1.3 B cells

Humoral immunity is mediated by antibodies produced by plasma cells that develop from B cells under the direction of signals received from T cells and other cells, such as dendritic cells. Once the T cell response has been triggered, it can help B

cells to further differentiate into memory cells or plasma cells. B cells can mediate humoral immunity by producing a variety of antibodies such as IgM, IgD and IgG, IgA and IgE by class-switching. Depending on their isotype, the antibodies can function via neutralisation, opsonisation or complement activation to eliminate extracellular pathogens and toxic products. In addition to producing antibodies, B cells are able to act as APC for T cells because of their expression of MHC class II on cell surface but they are less effective at this than professional APC, like DCs and macrophages [2].

1.1.4 T cells

1.1.4.1 T cell subtypes

Cellular immunity is conducted by T cells which mature in the thymus: T cells can be divided into several subtypes depending on their expression of cell surface markers. Two distinct lineages exist in T cell development, αβ and γδ T cells, the majority being αβ T cells where the antigen-specific component of the TCR consists of two glycoprotein chains, α and β acting in concert with the signal transducing CD3 complex can be found on CD4 helper (Th) T cells or CD8 cytoxic (CTL) T lymphocytes whereas for γδ T cells which express CD3 but not CD4 or CD8, antigen recognition is mediated via γ and δ chains. Most γδ T cells are found in gut mucosa and the epidermis. Additionally, unlike αβ T cells which recognise MHC class I ($CD8^+$ T cells) or II ($CD4^+$ T cells), γδ T cells are not peptide-MHC-restricted and while the antigens recognised by γδ T cells have not been fully defined, it appears that they can recognize whole proteins rather than peptides presenting by MHC molecules on APCs [3].

As stated above, αβ T cells also express CD3 and can be divided into two main classes, $CD8^+$ and $CD4^+$ T cells that have different effector functions whilst a

further minor population of αβ T cells, called natural killer T cells (NKT), can be $CD4^+$ or $CD8^+$ or neither [4]. $CD8^+$ T cells, known as cytotoxic T cells (Tc) are MHC class I restricted and upon recognising their specific antigen in association with MHC class I, in the presence of appropriate cytokines, such cytotoxic T cells proliferate and differentiate into effector cells known as cytotoxic T lymphocytes (CTL). CTL are responsible for eliminating tumour cells, grafted cells or virally infected cells from the body. $CD4^+$ T cells are MHC class II restricted and are known as T helper (T_H) cells as they act to stimulate other cells, such as B cells and macrophages, of the immune system to participate in the immune response. Additionally, depending on the cytokine milieu in which an antigenic stimulus is received, Th1, Th2 and Th17 effector cells are generated.

1.1.4.2 T cell development

T cell development occurs in the thymus; the thymic microenvironment directs differentiation as well as positive and negative selection. Lymphoid progenitors which have developed from hematopoietic stem cells in the bone marrow migrate to the thymus to complete their antigen-independent maturation into functional T cells[5-7]. In the thymus, as T cells develop, they upregulate specific T cell markers, including TCR, CD3, CD4 or CD8, and CD2 and undergo thymic education through positive and negative selection of their TCR specificity to ensure self-tolerance. TCR gene-segment rearrangements are termed productive if they do not introduce stop codons and give rise to a gene encoding a full-length TCR protein. Sequential productive rearrangements of 2 TCR genes leading to surface expression of an αβ or γδ TCR in association with its signal transducing complex, CD3 (which comprises γ, δ, ε and ζ chains) marks the transition from a pre-T cell to a double-positive T cell which expresses both CD4 and CD8. Subsequently these double-positive cells which reside in the thymic cortex, differentiate to

single-positive T cells in the medulla [8]. This process, which is regulated by both positive and negative selection, is termed central tolerance. During positive selection, double-positive TCR T cells which bind with low affinity to self-MHC on thymic epithelium are selected while those that do not bind to self-MHC, are eliminated. In contrast to positive selection, the TCR of double-positive T cells which bind with high affinity to self-MHC/peptide are not permitted to mature and undergo apoptosis. This is termed negative selection and is the major mechanism of ensuring central self tolerance.

1.1.4.3 T cell activation

Full T cell activation for effector cell differentiation requires at least two signals. The first one is provided by the TCR recognising cognate peptides derived from antigen in the context of appropriate MHC molecules expressed by antigen presenting cells (APC). The second is mediated by “co-stimulation” via interaction of CD28 on the T cell with CD80/86 on the APC. When a T cell receives both signal-1 and signal-2, it proliferates, differentiates and has effector function (Figure 1.1A). However, the recent discovery that the novel co-stimulatory molecules such as inducible T cell co-stimulator (ICOS; aka CD278 [9]) participate to drive the optimal co-stimulation of T cells suggested that multiple signals are needed to fully activate T cells [10]. ICOS is a member of the CD28 family of co-stimulatory molecules that is not expressed on resting cells but can be induced on all activated T cells within 24-48 hour of T cell activation [11]. It binds to its ligand B7-related protein-1 (B7RP-1), which is constitutively expressed on DC and B cells [9]. Furthermore, ICOS plays a more important role in the activation of T_H2 cells as it preferentially induces secretion of IL-4 and IL-10 than IL-2 [12]. Conversely, when a T cell receives only signal-1, lack of co-stimulation, or only signal-2 it can undergo apoptosis or become anergic or tolerized (Figure 1.1B). The induction of anergy is an active process and

ligation of the TCR (signal 1), which occur T cell activation (up-regulation of CD69 [13]) and proliferation [14].

1.1.4.4 CD4 T cell differentiation

Naïve $CD4^+$ T cells stimulated with antigens differentiate in to Th1 cells in the presence of IL-12 and IFNγ, however, IL-4 plays an important role for Th2 cell differentiation. The activation of downstream signalling molecules of these cytokine receptors, such as STAT4/STAT1 or STAT6 are required for differentiation into either Th1 or Th2 cells, respectively.

After antigen recognition by TCR, naïve CD4 T cells undergo clonal expansion and differentiate into functionally polarized effector T_H cell subsets. Effector $CD4^+$ T_H cells are characterized phenotypically by a decrease in the expression of CD62L and an increase in expression of CD44. As stated above, there are at least four major distinct T_H subsets, i.e., Th1, Th2, Th17 and Treg cells that have been identified to date. The generation of different effector cells depends on the cytokine milieu in which antigenic stimuli is received. TH1 cells express the signature transcription factor T-bet and produce IL-2, IFNγ and TNF-α. IFNγ upregulates IL-12 expression by DC and macrophages and this IL-12, in turn, results in upregulation of IFNγ in TH cells and so promotes TH1 cell differentiation in a positive feedback loop. Additionally, IFNγ downregulates IL-4 production, therefore, further promoting the T_H1 phenotype which is involved in the cellular immune response and acts to improve phagocytosis of macrophages, proliferation of CTLs and production of opsonising antibodies from B cells [15].

By contrast to TH1 cells, the major function of TH2 cells is to contribute to humoral immunity. They express the transcription factor GATA-3 and can produce IL-4, IL-5, IL-6, IL-10 and IL-13. IL-4 stimulates T_H cells to differentiate to T_H2 cells and

contributes to induction of neutralising antibodies produced by B cells [15]. IL-2 and IFNγ production are inhibited by IL-10, as is the IL-12 production by DC and macrophages.

TH17 cells is another lineage of $CD4^+$ T cells that has been developed by the immune response to kill extracellular bacterial but their dysregulation appears to contribute to the induction of autoimmune and allergic inflammation [16]. Th17 cells can produce IL-17A, IL-17F, IL-22 as key cytokines but additionally, Th17 cells also secrete IL-21 in common with other Th cells. TGF-β and IL-6 are the two key cytokines responsible for TH17 differentiation in vitro [17-19] and IL-21 mediates many functions of IL-6 [20] as indicated by reports that IL-21 was able to replace IL-6: however, both TGF-β and IL-6 are required for promoting TH17 commitment [21, 22] IL-23 receptors are not expressed on naïve $CD4^+$ T cells, only being upregulated on Th that have partially completed differentiation towards the TH17 phenotype. Once IL-23 receptors appear, IL-23 is involved in the TH17 priming process apparently by promoting maintenance of the TH17 phenotype. Retinoic acid-related orphan receptors (ROR) are the signature transcription factors for TH17 differentiation [23] and TH17 express high level of RORγt with expression of RORγt leading to TH17 cells producing IL-17. RORγt is induced in naïve $CD4^+$ T cells within 8 hours of TCR stimulation in the presence of TGF-β and IL-6. Consistent with the key role of this transcription factor in Th17 differentiation, it has been observed that RORγt-deficient cells produce very low IL-17 and reflecting the role of IL-17 in driving autoimmune disease, RORγt-deficient mice are partially resistant to experimental autoimmune encephalomyelitis (EAE). However, RORγt-deficient cells can still produce low levels of IL-17 due to the activation of RORα which is also upregulated in TH17 cells. When both RORγt and RORα are deficient, production of IL-17 is completely eliminated in “TH17” cells.

The other subset of $CD4^+$ T cells, regulatory T cells (Tregs) can be divided into natural regulatory T cells (nTregs) and induced Tregs (iTregs). Although iTregs appear to perform similar functions as nTregs in mice, both in vivo and in vitro [24], iTregs in humans are not able to replicate the same behaviour as nTregs [25]. The major function of nTregs is to maintain immune system homeostasis and act to suppress pathological immune responses to self antigen as seen in autoimmune disorders or foreign antigens in transplantation and graft versus host disease [26].

1.2 Immune tolerance

The primary mission of the immune system is to defend against invading pathogens. The normal healthy body can distinguish self from non-self antigens. When a new antigen is encountered, such discriminatory capacity would generate a productive immune response against invasive pathogens or exert antigen-specific tolerance, the latter to prevent harmful immune responses against self components[27] or non-dangerous food or environmental antigens. A breakdown in tolerance within an individual can result in the development of a variety of autoimmune disorders. T cell tolerance is induced in two main sites: (1) in the thymus during early T cell differentiation, designated central tolerance and (2) in the secondary lymphoid tissues after T cells are exported from thymus, called peripheral tolerance.

1.2.1 Central tolerance

As described above, lymphoid progenitor cells are produced in the bone marrow or fetal liver before migrating to the thymus where they start out as double negative thymocytes, as they lack expression of CD4 and CD8. Subsequently, double negative thymocytes which possess a functional TCR-β chain are selected to

differentiate into double positive $CD4^+CD8^+$ cells. This process is termed β-selection. The double-positive thymocytes then undergo positive and negative selection, processes which promote the differentiation of single positive $CD4^+$ or $CD8^+$ αβ-T cells that are MHC-restricted and self-tolerant.

1.2.2 Peripheral tolerance

As central tolerance is not sufficient to prevent autoimmunity and allergy, therefore, peripheral tolerance is required to ensure that the tolerance is enforced for all self-antigens as well as food and environmental antigens which are not recognized in early life. There are several proposed mechanisms of peripheral tolerance.

1.2.2.1 Antigen ignorance

Antigen ignorance can arise if the antigen is not presented to the T cell or the TCR exhibits low affinity to antigen. For example, antigens can be hidden in immune-privileged sites such as the eye and so cannot be seen by circulating T cells [28].

1.2.2.2 Clonal anergy

Unresponsiveness of T cells to secondary antigenic stimulation is a mechanism of peripheral tolerance, known as anergy and which is the functional inactivation of T cells. Induction of anergy occurs when naïve T cell receive antigen presentation by immature DCs, which have not be exposed to inflammation or “danger” signals from pathogens and leads to such T cells being hyporesponsive to re-challenge with the same antigen. Anergy induction also occurs when the TCR is ligated upon recognition of its specific peptide in the context of MHC, but in the absence of co-stimulation, which again induces a state of long lasting unresponsiveness (anergy) in T cell [29, 30]. Some experimental methods have been developed to

mimic and induce such anergy *in vitro* [31-33], including exposure to immobilised anti-CD3 to ligate the TCR in the absence of co-stimulatory signals [32, 34]. Compared to priming conditions (TCR ligation + co-stimulation), under this condition re-stimulation with antigen result in downregulation of IL-2 production and thus reduction in proliferation of T cells. The state of anergy can be reversed by the addition of exogenous IL-2 to the T cells [35].

Induction of anergy is an active process. Although, anergic T cells fail to proliferate and produce decreased IL-2 production after re-challenge with antigen (maintenance phase of anergy), anergic T cells exhibit clonal expansion and upregulate the activation markerCD69 during induction phase of anergy [13, 36, 37]. As described above, anergy may result from lack of co-stimulation (CD28:CD80/86) as some studies have indicated that the low level of CD86 expressed on resting DCs [38, 39] is not sufficient to induce priming in T cells. However, although the interaction of CD80/86:CTLA-4 (cytotoxic T lymphocyte-associated antgen-4) may be involved in the maintenance of T cell tolerance [40-43] no specific marker for identification of anergic T cells has been found so far.

1.2.2.3 Active suppression

Active suppression or dominant tolerance on effector T cells is conducted by regulatory T cells. Early studies indicated both $CD8^+$ Treg and $CD4^+$ Treg are involved in active suppression by inhibit IL-2 production and cellular proliferation.

1.2.2.3.1 CD8+ suppressor T cells

Although early studies suggested that the involvement of $CD8^+$ T cells in active suppression, the effector mechanisms of these $CD8^+$ T cells were not fully elucidated [44, 45]. Nevertheless, it was clear that although systemic tolerance was

not affected, $CD8^{-/-}$ mice showed deficient local suppression of IgA responses in the gut after feeding Ag, indicating that $CD8^+$ T_{regs} may be important for the regulation of local mucosal immune responses [46]. More recently, it has emerged that in humans, there is a $CD8^+$ T_{regs} population that expresses the signature regulatory transcription factor. Foxp3 that inhibits T cell activation by interfering with TCR signalling by secretion of CC chemokine ligand 4 (CCL4) [47].

1.2.2.3.2 CD4$^+$ Tregs

Active suppression by $CD4^+$ Treg cells mediates downregulation of activation of $CD4^+$ T_H cells upon antigen challenge [48] and most of these T_{regs} cells express CD25, which is upregulated on antigen-specific T cells in periphery, for example, after feeding with tolerogenic doses of antigen [49, 50]. $CD4^+CD25^+$ T_{regs} are capable of “bystander” suppression of naïve T cells specific for an unrelated antigen [27, 51] and in addition, $CD4^+CD25^+$ T_{regs} can suppress the cytokine production and proliferation of both $CD4^+$ and $CD8^+$ T cells *in vitro* [52, 53]. Furthermore, the number of $CD4^+CD25^+$ T_{regs} in mice are decreased while the TGF-β signalling is disrupted since the TGF-β contributes to the generation of T_{regs} in periphery [54].

Natural regulatory T cells ($CD4^+CD25^+Foxp3^+$ T_{regs}) cells exist in the thymus and participate in central tolerance mechanisms, however, those cells can also migrate from the thymus and exhibit their regulatory functions in the periphery. Although the transcription factor Foxp3 is critically important for their differentiation other signals such as TCR/MHC class II interactions, IL-2 signalling, CD80/CD86:CD28 signalling are also essential [55].

1.2.2.3.3 Tr1

Some adaptive regulatory cells, like Tr1 and T_H3 can arise after contact with antigen. $CD4^+$ regulatory 1 (Tr1) cells were first identified, following multiple rounds of stimulation of naïve T cells with antigen, in the presence of high concentrations of IL-10 *in vitro*. Such Tr1 cells can suppress T_H2 responses by producing TGF-β instead of IL-4 in an Ag-specific manner [56], and OVA-specific Tr1 cells have been shown to prevent inflammatory bowel disease (IBD) when they are adoptively transferred into recipient mice subsequently fed OVA [57].

1.2.2.3.4 TH3

Another subset of $CD4^+$ T_{regs} has been described, which produce varying amounts of the T_H2 cytokines IL-4 and Il-10, but have been shown to be a population distinct from T_H2 cells since they produce TGF-β as well [57]. The IL-10 production suppresses TH1 activity via downregulation of expression of costimulatory molecules and IL-12 production by APCs [58]. Such cells were designated T_H3 cells and Ag-specific $CD4^+$ T_H3 cells have been found in the mesenteric lymph nodes (MLN) of DO11.10 Tg mice after feeding tolerogenic multiple low doses of antigen [59, 60]. Additionally, the induction of T_H3 phenotype was observed following co-stimulation mediated through CD86 but not CD80 [42].

1.3 T cell signaling

1.3.1 TCR-mediated signalling

1.3.1.1 Signalling proximal to the TCR

TCR ligation by the MHC-peptide complex initiates the signalling cascades and directing T cell fate. Within seconds of TCR ligation (Figure 1.2), the Src kinases,

Fyn and Lck are recruited and activated to phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAM) in the ζ chains associated with the TCR-CD3 complex [61], resulting in the subsequent recruitment of the tyrosine kinase, ζ-associated protein of 70 kDa (ZAP-70), via binding of its SH2 domains to the phosphorylated ζ-chains[62]. Following binding of ZAP-70 binds to the ITAMs and activation by Fyn/Lck, it phosphorylates a number of cytosolic proteins, triggering the assembly of an intracellular complex of scaffolding and activated signaling enzymes and adaptors, including linker of activated T cells (LAT) and SLP-76 and downstream kinases. For example, phosphorylated SLP-76 associates with the guanine nucleotide exchange factor (GEF), Vav, via its SH2 domain and binds the Tec family PTK, IL-2 tyrosine kinase (Itk).

1.3.1.2 Role of adaptor proteins in T cell signalling

Adaptor proteins play a role in linking to antigen receptor ligation to cellular signaling. For example, some of the signaling enzymes and adaptors recruited to LAT and phosphorylated by ZAP-70 are phospholipase Cγ1 (PLCγ1), growth factor receptor-bound protein 2 (Grb2) and the Grb2 family member, GADS. GADS is an adaptor for linking LAT and SLP-76 following TCR ligation [63]. Additionally, T cell activation and differentiation requires binding of PLC-γ1 and GADS [64] whilst Grb2 needs to complex with Son of Sevenless (SOS), which is a GEF for SOS to be able to convert Ras-GDP (inactive form) to Ras-GTP (active form) [65].

1.3.1.3 The PLC-γ1 pathway

Activation of PLC-γ1 is facilitated by recruitment to LAT and consequently PLCγ1 mediates hydrolysis of the membrane inositol phospholipid, phosphatidylinositol 4, 5 bisphosphate (PIP_2), generating inositol-trisphosphate (IP_3) and diacylglycerol (DAG). Inositol-trisphosphate (IP_3) binds to its receptor on the membrane of the ER

which induces a rapid increase in intracellular calcium (Ca^{2+}) levels by means of release of stores contained within the endoplasmic reticulum. This calcium flux activates a calcium release-activated calcium channel facilitating the influx of extracellular calcium [66]. Calcium entering the cytosol from the endoplasmic reticulum or extracellular space binds to the regulatory protein calmodulin, which in turn activates the phosphatase calcineurin (Cn). Cn is a calcium-dependent phosphatase which comprises a catalytic and calmodulin-binding subunit A (CnA) and a Ca^{2+}-binding regulatory subunit B (CnB). The activity of CnA is suppressed by binding to CnB in resting T cells but after Ca^{2+} binds to CnB, calmodulin is able to ligate to CnA, which causes the activation of enzymatic activity of Cn [67]. Activated Cn dephosphorylates nuclear factor of activated T cells (NF-AT) in the cytosol, revealing its nuclear localization sequence and generating the active form of this critical transcription factor, which then translocates to the nucleus, where it associates with activator protein-1 (AP-1) to direct the synthesis of genes like IL-2 [68]. At the same time, diacylglycerol (DAG), the other product of PLCγ1-mediated hydrolysis of phosphatidylinositol bisphosphate, triggers activation of a parallel pathway involving protein kinase C. This leads, through intermediates, to the activation of nuclear factor κB (NF-κB), another critical transcription factor in T cell activation. AP-1 itself is a complex of c-Fos and c-Jun transcription factors. Activation of AP-1 alone is not sufficient for transcription of the IL-2 gene. For this to occurs, signals from co-stimulatory receptors are necessary.

1.3.1.4 PKC-mediated signalling in T cells

The PKC family of serine/threonine kinases act as DAG binding proteins in lymphocyte activation that can stimulate accumulation of GTP-bound (activated) Ras, independent of SOS [69]. DAG can also bind the GEF Ras guanyl-releasing protein 1 (RasGRP1) through its DAG-binding domain. RasGRP1 also contains

Ca^{2+}-binding EF hands, and following increases in the levels of intracellular Ca^{2+} and DAG, RasGRP1 is recruited to the Golgi membrane [70, 71]whereupon it converts Ras-GDP to the GTP-bound form of Ras and consequently leads to activation of downstream mitogen-activated protein (MAP) kinases such as ERK [72, 73].

1.3.1.5 MAPKinase pathways

The MAPKinases are a family of serine threonine protein kinases which are activated by a variety of extracellular stimuli and are capable of mediating an array of cellular functions, ranging from activation and proliferation to growth arrest and cell death [74]. The MAPK family includes kinases from three different signalling pathways, the classical extracellular signal-regulated kinases (ERKs), the p38 MAPK and the c-Jun N-terminal kinases (JNK). Each MAPK is activated by different upstream MAPK kinases (MEKs) and MAPK kinase kinases (MEKKs). MAPK are activated by dual phosphorylation on tyrosine and threonine residues, located in a T-X-Y motif [75], where X is different in each group of MAPK. MAPK activation results in phosphorylation and activation of distinct downstream transcription factors, depending on the group of MAPK activated. Thus, ERK activates Elk-1 and c-Myc [76], p38 activates c-Fos [77] and ATF-2 [78], and JNK activates c-Jun and ATF-2 [79]. The phosphorylation and activation of these transcription factors permits the MAPK family to regulate gene expression and hence, cellular fate and effector function.

The TCR ligation signals to the Ras-ERK MAPK pathway: Ras is a membrane-bound guanine nucleotide binding protein which can be activated at the plasma membrane (SOS-mediated) and also the Golgi membrane (RasGRP1-mediated), prior to recruitment to the plasma membrane. Ras-GTP

recruits the serine/threonine kinase Raf-1 to the plasma membrane, where Raf-1 is activated [80] following the interaction of Ras with the regulatory N-terminal region of Raf-1 the latter of which otherwise inhibit its kinase activity. Activated Raf-1 then phosphorylates the dual specificity (Thr/Tyr) mitogen-activated protein kinase kinase (MAPKK), MEK (MAPK extracellular signal-regulated kinase (ERK) kinase), which subsequently phosphorylates and activates ERK [81, 82]. ERK is a generic term that comprises several isoforms that includes the two well-characterised isoforms, ERK1 and ERK2. The activation of ERK1 and ERK2 are regulated by the dual phosphorylation of neighbouring threonine and tyrosine residues by MEK [83]. Additionally, unlike Raf-1, ERK 1 and ERK2 do not exhibit an autoinhibitory domain. Once the ERK is activated, in turn it translocates into the nucleus and thus it is able to phosphorylate and activates the downstream transcription factors such as AP-1. In addition, it can activate a number of cytosolic signalling enzyme effectors.

1.3.2 Co-stimulation-dependent signalling

The signalling described so far relates to that occurring following TCR ligation. However, co-stimulation is required for induction of IL-2 and consequent productive priming of a T cell (Figure 1.3). TCR-signalling alone causes a hypo-responsiveness status as in the absence of co-stimulation, there is only limited production of IL-2, which is required for autocrine and paracrine stimulation of T cell proliferation [84]. Co-stimulation-dependent (CD28-mediated) proliferative signals, independent of ERK activation, are crucial for activation of the full range of transcription factors required for transcription of the IL-2 gene, and hence proliferation [84].

T cell co-stimulation mediated by CD28 ligation contributes to TCR proximal signals via Lck and phosphatidylinositol 3-kinase (PI3K) activation with such PI3K

activation essential for optimal lymphocyte proliferation. For example, the production of IL-2 in T cells is decreased by pharmacological inhibition of PI3K, even upon stimulation of TCR and CD28 [85, 86]. PI3K is composed of a p85 regulatory subunit, which can bind with CD28 and a p110 catalytic subunit, which is activated by the regulatory subunit when this is recruited by CD28 [87]. Once PI3K is activated, in turn it will convert PIP2 into the second messenger phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3) which recruits cytoplasmic signalling enzymes containing Pleckstrin Homology (PH) domains such as, phosphoinositide-dependent kinase 1 (PDK1) and Akt [87]. Once located correctly at the plasma membrane, AKT is first phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) and subsequently activated by PDK1 [88]. By contrast, activation of AKT is negatively regulated by Tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor molecule that reverses PT3K action. AKT activation is observed in T cells upon TCR engagement and this is further upregulated by co-stimulatory signals [89, 90]. Activated AKT contributes to activation of several downstream effectors and importantly for IL-2 production, phosphorylated AKT in T cells correlates with upregulation of NF- κB activity [91, 92]. Additionally, PDK1 may contribute to phosphorylation of PKC which leads to more NF-κB nuclear translocation and activation [93]. PKC-θ, the predominant PKC isoform expressed in T cells promotes NK-κB activation induced by TCR/CD28-mediated co-stimulation [94] as indicated by studies using T cells from PKC-θ-deficient mice that have demonstrated a role for PKC-θ in the activation of NK-κB and AP-1 [95]. Moreover, such T cells from PKC-θ-deficient mice also exhibit impaired Ca^{2+} mobilization and NF-AT activation, and hence decreased IL-2 production and proliferation [96].

Co-stimulation via CD28 also leads to activation of two classes of MAPKs: JNK

(c-Jun N-terminal kinase) and p38 MAPK and such signaling is dependent on the key GEF, Vav1. Activation of Vav1 is conducted by two signals: The first one is mediated by Fyn after TCR ligation and the second one is by ZAP-70 after its recruitment to the immune synapse. Binding of Vav to PIP_3 (via its PH domain) increases its GEF activity and activation of Vav causes further cytoskeletal rearrangements, IS stabilization and signal transduction [97]. Vav1 also regulates the activity of PTK and ITK by PI3K-dependent and independent mechanisms [98], signals that are crucial to the positive regulation of PLC-γ1 and subsequent increase in intracellular calcium concentration, diacylglycerol-dependent activation of PKC and activation of RAS/RAF/ERK through RAS-GRP. Additionally, PTK and ITK regulate Wiskott-Aldrich syndrome protein (WASP) which is activated by Cdc42 resulting in actin polymerization and cytoskeletal rearrangement [99]. Cdc42 and another Rho family GTPases, Rac activate p21-activated kinase (PAK), which in turn activates the dual specificity kinase, SAPK/ERK1 kinase (MKK4) [100] to activate the stress activated protein kinase, c-Jun N-terminal kinase (JNK) by phosphorylation of its tyrosine threonine motif residues [101]. Activated JNK (c-Jun N-terminal kinase) can then phosphorylate c-Jun at serine 63/73 and hence contributes to the activation of AP-1. Similar to ERK, p38 can also activate c-Fos via activation of ternary complex factors (TCF) within the serum response element (SRE) of c-Fos to complete the activation of AP-1. ERK and p38 MAPK signalling coverage to act on c-Fos promoter by inducing members of the TCF family of E twenty-six (ETS)-domain proteins such as Elk-1 and SAP-1. Hence, all three ERK, JNK and p38 MAPK pathways are required for optimal activation of Elk and SAP-1 for transcription of c-Fos and resultant AP-1 activation.

Inducible T-cell co-stimulator (ICOS) is another member of CD28 superfamily that is expressed on activated T cells. Unlike CTLA-4, ICOS has its own ligand, ICOS-L

(also known as CD275, B7RP-1 and B7-H2). The induction of ICOS expression is regulated by both TCR and CD28 signalling. To date, the p85α subunit of PI3K is the only signalling molecule shown to interact with ICOS and ICOS stimulation appears to lead to greater recruitment, phosphorylation and activation of AKT. As with CD28, ICOS delivers positive signals and has been shown to play roles in T cell differentiation, cytokine secretion (increase in IL-4 production) and survival (namely by inhibiting AICD) [102].

To sum up, while ligation of the TCR alone results in activation of NF-AT and NF-κB, as well as ERK-mediated activation of c-Jun and c-Fos, these signals are not sufficient for full transcription of the IL-2 gene. For such transcription to occur, co-stimulation-dependent signals are required for further phosphorylation and activation of c-Jun and c-Fos by JNK and p38 MAPK respectively, as well as p38 MAPK-mediated activation of ATFs. Together, such signaling induces full transcription and hence, production, of IL-2 in T cells.

1.3.3 Inhibitory molecules

As mentioned above anergy may be caused by the absence of co-stimulation (CD28:CD80/86 interaction). Although resting DC or macrophages express low levels of CD86 [38, 39, 103], many studies have indicated that this default level of CD86 expression does not contribute to induction of priming but rather appears to be required for the induction of T cell tolerance and reflects that the CD80/86:CTLA-4 (cytotoxic T lymphocyte-associated antigen-4) interaction may actually be necessary for maintaining T cell tolerance [40-43, 104]. Although all the CD28 family of co-stimulators exhibit high homology with each other, some of the members exhibit inhibitory effects and include CTLA-4 (also known as CD152), inducible co-stimulator (ICOS) and programmed death-1 (PD-1). Additional T cell

stimulators and co-inhibitors belong to two other structural families of surface proteins, namely the immunoglobulin superfamily and the tumour necrosis factor receptor (TNFR) superfamily, with members of the TNFR superfamily including OX40, CD40, HVEM and others [105]

CTLA-4 shows high homology to CD28 and consistent with this is capable of binding with CD80 and CD86 [106, 107]. However, CTLA-4 is a major inhibitory regulator of the immune response, and acts to inhibit the activation of T cells [108]. It is expressed primarily by activated T cells being upregulated following antigen activation of T cells. Although CD28 is constitutively expressed on T cells, CTLA-4 binds CD80 and CD86 with higher affinity than CD28 and hence upregulation leads to homeostatic downregulation of immune responses. Indeed, deletion or blockage of CTLA-4 inhibits the induction of peripheral and oral tolerance, improves anti-tumour responses and aggravates autoimmune disorders. This regulation is reflected by the phenotype of CTLA-4 deficient mice, which develop a fatal lymphoproliferative disease with massive organ infiltration by immune cells [109]. Consistent with this, anti-CTLA-4 mAb treatment causes the promotion of cell cycle progression, expansion of antigen-specific T cells in the paracortex and follicle of draining lymph nodes and enhanced specific Ab production during what normally be the induction of tolerance [110]. In addition, the signalling mediated by CTLA-4 also appears to play an important role in regulating T cell differentiation, for instance, blocking of CTLA-4 appears to promote differentiation of $CD4^+$ T cells into IL-4 producing Th2 cells [111]. When CTLA-4 surface expression increases and out-competes CD28 for ligand (CD80/86) binding upon T cell activation, this results in cell cycle arrest at the G1 phase and decreased production of IL-2, due to reduction in activation of the transcription factors such as NF-κB, NFAT and AP-1 [112-115]. Although, the downstream inhibitory signals mediated by CTLA-4 are

still unclear, the upregulation of E3-ligase Cbl-b is reported to contribute to this inhibitory signal[116].

Programmed death 1 (PD-1) is another member of the CD28 superfamily that interacts with its ligands, PD-L1 (B7.H1; CD273) and PD-L2 (B7.H2; CD274) to cause T cell anergy [117] and is inducibly expressed on T cells, B cells, activated monocytes. PD-L1 is constitutively expressed on T cells, B cells, DC, macrophages, mesenchymal stem cells [118] and bone marrow-derived mast cells whereas, PD-L2 is inducibly expressed on DC, macrophages and bone marrow-derived mast cells [119]. Co-ligation of TCR or BcR with PD-1 results in the transduction of an inhibitory signal whereas no signal is transduced when PD-1 is cross-linked alone. PD-1 and its ligands are therefore thought to play important role in both central and peripheral tolerance with PD-1 being upregulated upon tolerogen recognition in lymphoid organs before they exit to the periphery. This upregulation of PD-1 is stimulated by the common gamma-chain cytokines IL-2, IL-7, IL-15 and IL-21 [120] and the role of PD-1 in regulation of T cell tolerance and autoimmunity in periphery is clearly illustrated by the phenotype of PD-1-deficient ($Pdcd1^{-/-}$) mice [121, 122]. Further evidence was provided more recently, by antibody blockade of PD-1 and PD-L1, PD-L2 which demonstrated a critical role for PD-1, but not CTLA-4, in maintaining established peripheral $CD4^+$ T cell tolerance. Moreover, disrupting the interaction between PD-1:PD-L1 (by antibody blockade) breaks CD8+ T cell tolerance to intestinal self-antigen, resulting in severe enteric autoimmunity [123]. In addition, PD-2 has been shown to have a role in oral tolerance, as PD-L2-deficient mice fed ovalbumin fail to induce tolerance of their $CD4^+$ or $CD8^+$ T cells.

1.3.4 Signalling in anergy

As described above primed T cells exhibit a characteristic pattern of signalling for transcription of the IL-2 gene and subsequent clonal expansion of T cells [124, 125]. In contrast to primed T cells, anergic T cells exhibit a state of antigenic unresponsiveness, which is accompanied by downregulation of IL-2 production and thus decreased proliferation. However, anergy induction requires T cell activation and new protein synthesis [126] and in fact the proximal TCR signalling machinery is still capable of being recruited to the TCR-CD3 complex under conditions of anergy although the levels of tyrosine phosphorylation of ZAP-70 were significantly reduced compared to primed cells [127]. Nevertheless, a number of signalling events have been postulated to be associated with the anergised T cells. For example, tyrosine phosphorylation of 39, 75 and 98 kDa proteins are decreased in an anergic T cell hybridoma compared with control cells [128]. In addition, expression of p56lck (Lck) is decreased while the level of p59fyn (Fyn) is increased in anergic Th1 clones induced by anti-CD3 signalling in vitro [129-131]. Restoration of Lck and Fyn expression to normal levels recovers these cells from anergy, suggesting modulated levels of those tyrosine kinases contribute to the maintenance of anergy [130]. Furthermore, anergy induced by oral administration of OVA has been shown to cause impaired phosphorylation of TCRζ, ZAP70, LAT and PLC-γ1 upon re-stimulation of purified splenic $CD4^+$ T cells with OVA and APC in vitro [132]. As mentioned above, activation of LAT leads to the recruitment of Grb2, GADS, SLP-76 and PLC-γ1 in activated T cells. However, recruitment and localisation of LAT to the lipid raft-rich immunological synapse has been shown to be defective in anergic T cells and such defects are believed to result from impaired palmitoylation of LAT [133].

PKC-θ signalling is believed to be important in the prevention of anergy by acting

as a positive regulator of NF-κB [134]. Moreover, T cells from PKC-θ-deficient mice exhibit impaired Ca^{2+} mobilisation and NF-AT activation, and hence decreased IL-2 production and proliferation [96]. An immediate downstream target of NF-AT is the early growth response (EGR) family of transcription factors. Expression of EGR2 and EGR3 is known to be upregulated in both in vitro and in vivo anergised T cells. EGR expression appears to be calcineurin and PKC dependent and transduction of T cells with EGR2 or EGR3 reduces transcription of the IL-2 gene. Moreover, expression of EGR2 and EGR3 also contributes to T cell anergy by upregulating expression of the E3 ligase Cbl-b [135]. Together, these results suggest a role for EGR proteins in the induction of anergy.

There is increasing evidence that JNK MAPK-mediated induction of c-Fos and activation of AP-1 and NF-AT complexes may also be defective in *in vitro* and *in vivo* anergised T cells. NF-AT and AP-1 associate to enhance transcription of the IL-2 gene. As it is well established that Ca^{2+}-mediated translocation of NF-AT into the nucleus is unaffected in anergic T cells [136, 137], such data implies that the defect in AP-1 binding to NF-AT likely lies at the level of the AP-1 subunits. Indeed, Mondino *et al* demonstrated that the induction of c-Fos and JunB was severely impaired in anergic Th1 cells while NF-AT activation was intact in these cells[138]. Consistent with this, Fields *et al* also reported that ERK1/2 MAPK activation was decreased in anergic T cells [139] and it has been shown that the reduced ERK MAPK activation and impaired IL-2-dependent proliferation observed in anergic T cells is due to downregulation of Ras activation [139, 140]. As mentioned earlier, Ras GRP1 promotes activation of Ras in a DAG-dependent manner and it has been hypothesized that defects in the Ras GRP1-mediated activation of Ras may also be involved in T cell anergy as RasGRP1-deficient thymocytes exhibit reduced Ras and ERK activation and proliferation [141]. Upon phosphorylation by

diacylglycerol kinases (DGKs), DAG is converted to phosphatidic acid and consequently, DAG signaling is downregulated. Decreased DAG signaling has been observed in multiple models of T cell anergy and is believed to reduce the recruitment of RasGRP1, and hence reduce RasGRP1-mediated Ras activation [142, 143].

1.3.4.1 The role of Rap1 in T cell anergy

Anergic anergic T cells exhibit downregulation in Ras-mediated ERK activation and it has been proposed that this is regulated by the small GTPase Rap1 (Ras-proximate-1) which is a small G protein in the Ras superfamily. Rap1 is expressed in cells as one of 2 isoforms, Rap1a and Rap1b and is most closely related to the 2 isoforms of Rap2: Rap2a and Rap2b, small G proteins with overlapping but distinct functions. Like all G proteins, Rap1 exists in an inactive guanine nucleotide diphosphate (GDP)-bound state and is activated when GDP is exchanged for guanine nucleotide triphosphate (GTP) and is targeted to lipid membranes by covalent attachment of lipid moieties to the carboxyl terminus. Rap and Ras proteins have similar effector-binding regions that interact predominantly with “Ras association” or the structurally similar “Ras-binding domain” present in a variety of different proteins. Ras is central in a network controlling cell proliferation and cell survival, whereas Rap1 predominantly controls cell adhesion, cell junction formation, cell secretion, and cell polarity [144]. These different functions are reflected in a largely different set of GEFs and GAPs required for activation and desensitisation of these G-proteins and by the finding that downstream effector proteins tend to operate in a selective manner in either one of the networks.

Lack of full ERK MAPK pathway activation has been associated with anergy [145]. Additionally, accumulation of active Rap1 has been reported to play a role in the

maintenance of anergy in human T cell clones where it has been associated with downregulation of ERK activation and IL-2 production, but not in primed T cells. Rap1 mediates its inhibitory effects on ERK activation by directly antagonising Ras-Raf-1 coupling and it has been suggested that the inability of Rap1 to activate such bound Raf-1 is because it is not localised in the plasma membrane [146, 147]. Consistent with these ideas, while anergic antigen-specific T cells exhibit less phosphorylated ERK than primed T cells following re-stimulation with antigen [13], anergic T cells displayed higher expression of Rap1 than primed T cells. Moreover, in primed T cells phosphorylated ERK was localised with the TCR and lipid raft structures whereas anergic T cells exhibited a more diffuse cellular distribution. By contrast, Rap1 localises to the TCR and lipid raft in anergic T cells, but not in primed T cells [14]. The details of the mechanisms which participate in this inhibitory signalling is still not fully understood, however, the Fyn-Cbl-CrkL-C3G-Rap1 signalling complex that accumulates in anergic cells is not found in primed T cells [30, 148]. Furthermore, there is an inverse relationship between ERK and Rap1 activation in various T cell lines [149] and TCR-coupled Rap1 activity is suppressed by CD28 signalling [150-152]. Perhaps reflected the inability of Rap1 to activate Raf-1, Rap1 has been reported to stimulate ERK via activation of B-Raf, however, peripheral T cells generally do not express B-Raf. Interestingly, therefore, data from a transgenic mouse model in which ectopic expression of B-Raf was used to examine how Rap1 acted to antagonise ERK activation in anergic cells, suggested that the suppression of ERK activity and anergy in Tg T cells [153]. Furthermore, defective ERK activation and progressive unresponsiveness, or anergy, of T cells occurred in mice that were deficient in GTPase-activating protein (Rap1GAP), the negative regulator of Rap1 [154]. C3G, which is a GEF, has also been reported to involved in activation of Rap1 via CrkL, a adaptor protein containing SH2 that interacts with C3G [155, 156]. Thus the

prediction is that Cbl contributes to the initiation of recruitment of CrkL although the precise mechanism is not fully understood [157].

1.3.5 The immunological synapse

In lymphocytes, many of the molecules, such as Src family tyrosine kinases, heterotrimeric G protein subunits, adaptor proteins, PIP_2 and lipid kinases and phosphatases, involved in cell signalling are associated with detergent-insoluble, sphingolipid and cholesterol-rich domains in the cellular membrane, known as lipid rafts [15]. These structures in lipid rafts are critical for T cell signalling as they function as specialised signalling compartments in the cell membrane where molecules are recruited, phosphorylated and activated [158]. For instance, the TCR-CD3 complex is found out of lipid rafts in resting T cells but the complex translocates into the lipid raft after ζ chain phosphorylation by Lck [159]. It is thought that lipid raft macrodomains the assemble to form the immunological synapse (IS) and a stable immunological synapse has been considered to be required for optimal activation and priming of T cells [160].

The IS is formed by several specific molecules which are located at the point of contact at the interface between a T cell and APC [15]. The proteins and intracellular molecules which are present at the IS are organised into distinct spatial domains known as supramolecular activation clusters (SMACs), including central supramolecular activation complex (cSMAC) and peripheral SMAC (pSMAC). The cSMAC is formed by smaller molecules such as the TCR, CD4 and CD28, which arise from small clusters of lipid rafts and combine together as a single, large molecular platform at the T cell-APC contact site. As the c-SMAC comprises the TCR-CD3 complex and PKC-θ, it is the site for TCR ligation. Within seconds of peptide-MHC ligation, the TCR initiates tyrosine phosphorylation signalling that

recruits many complex signalling cascades. Following this, within minutes following T cell contact with APC, the Lck and Fyn also localise to the cSMAC [161, 162]. Additionally, initiation of contact of between the T cell and APC drives rapid re-organisation of the cytoskeleton and T cell polarisation [163]. Signalling through heterotrimeric G proteins then triggers actin polymerisation and integrin activation and then large molecules such as leukocyte function-associated antigen-1 (LFA-1) and the cytoskeletal protein, talin form the peripheral SMAC (pSMAC) [160]. LFA: ICAM-1 binding has been proposed to contribute to the generation of membrane protrusions with TCR-enriched tips that allow the initial scanning of peptide-MHC complexes [164]. As mentioned before, anergic T cells fail to localise to the IS and furthermore, upon re-stimulation with antigen, translocation of CD3 into the cSMAC is defective and thus, assembly of the IS is arrested in human anergic T cells [165].

1.4 mTOR signalling

mTOR, mammalian target of rapamycin, is a serine/threonine protein kinase, which regulates cell survival, growth, proliferation, differentiation, migration and apoptosis. However, recently, it has become increasingly clear that mTOR comprises two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [166].

1.4.1 mTOR function

As described in Section 1.1.6, full activation of T cell requires two signals: TCR ligation and co-stimulation. When a T cell receives both signal-1 and signal-2, it proliferates, differentiates and exhibits effector function. Conversely, when a T cell receives only signal-1, lack of co-stimulation, or only signal-2 it can undergo apoptosis or become anergic or tolerized. In addition to the lack of IL-2 production

and resultant deficient cellular proliferation with blockage at the G1/S checkpoint of cell cycle, there is also deficiency of up-regulation in nutrient transporters, for instance, Glut1, CD71 and CD98 occurring in anergic T cells and this leads towards catabolic metabolism of the cell [167]. In contrast to anergic T cells, primed T cells signals by TCR engagement and ligation of co-stimulatory CD28 to undergo glycolysis by induction of PI3K-dependent activation of AKT [168, 169]. Since the paradigm of two signals has explained T cell activation and anergy, inhibition of mTOR was indicated in the induction of anergy in T cells by recent reports. For instance, inhibition of mTOR in T cells has been shown to lead to anergy, even when the T cell receives both signal I + signal II [170]. In addition, TCR stimulation in the absence of mTOR activation results in the generation of $Foxp3^+$ regulatory T cells [171]. However, mTOR is not only a downstream transducer of the CD28/IL-2 signalling pathway, it also participates in several energy and nutrient-sensing pathways [172]. For example, mTOR senses cellular nutrients and the levels of energy and thus glucose, amino acids and lipids are able to regulate mTOR signalling and, in turn, to control commitment to T cell activation or anergy. For instance, anergic T cells were induced following treatment of T cells with leucine or glucose antagonists whereas, in their absence, T cells that received signal I + II and secreted normal IL-2 production [167]. Thus, mTOR plays a critical role in controlling induction of anergy in T cells.

mTOR is also proposed to be an important regulator of in T cell differentiation, integrating the diverse signals deriving from the microenvironment in which the “second” signal is occurring [173, 174] as $CD4^+$ T cells fail to differentiate into effector T cells under appropriate stimulation conditions in the absence of mTOR signalling.

1.4.2 mTOR complex and signalling

In T cells, mTOR signalling is initiated by three instructive signals, TCR ligation + co-stimulation, growth factors such as insulin, and metabolic cues which are mainly derived from nutrients [175]. Once mTOR receives those environmental signals, it will be activated through the phosphoinositide3-kinase (PI3K)-AKT pathway. As described above, mTOR forms two distinctive signalling complexes, mTORC1 and mTORC2.

1.4.2.1 mTORC1

mTORC1 is composed of mTOR, Raptor (regulatory-associated protein of mTOR), mLST8/GβL (mammalian LST8/G-protein β-subunit like protein), PRAS40 (the proline-rich Akt substrate 40 kDa) and Deptor (DEP-domain-containing mTOR-interacting protein). In immune system cells, mTORC1 regulates cell growth and as a downstream molecule of PI3K-AKT, WNT-GSK3 and AMP-activated protein kinase (AMPK) signalling. mTORC1 is activated through PI3K-PDK1-AKT signalling and this, in turn, results in the downstream phosphorylation of S6K1 and 4E-BP-1. Thus, growth factors, cytokines, co-stimulatory molecules and antigen receptors, which all activate PI3K, in turn activates AKT by phosphorylation at threonine 308 [172], inducing the activation of mTORC1. Normally, tuberous sclerosis complex 1 (TSC1) and TSC2 form a functional complex which acts to inhibit mTORC1 with the activity of TSC1-TSC2 being upregulated by cellular stress and DNA damage and thus consequently to inhibiting mTORC1 [176, 177]. However, TSC2 is inhibited by fully activated AKT which phosphorylates TSC2 with consequent loss of TSC2 inhibitory effects on mTORC1 [178]. The TSC1-TSC2 complex is also inhibited by activation of Ras-MAPK signalling [176] and the mTORC1 complex is also upregulated by a crucial regulator, Ras homolog enriched in brain (Rheb), a small GTPase originally isolated from the central

nervous system. The mammalian Rheb family are comprised of Rheb1 and Rheb2. Both of Rheb1 and Rheb2, both of which are expressed in T cells, although their functions have not been clarified yet [179]. Rheb is regulated by the TSC complex which acts as a GTPase-activating protein (GAP) for Rheb. Thus, when the TSC complex is inhibited, the active, GTP-bound form of Rheb interacts with, and activates, mTORC1 [180]. Rheb is a significant regulator of the activation of mTORC1 and hence cell differentiation as Rheb-/- mice have shown the importance of mTORC1 signalling in T_H1 and T_H17 effector differentiation, T_H2 differentiation is preserved in its absence. Indeed, mice which harbour T cell-specific deletions of Rheb cannot mount T_H1 and T_H17 responses in vivo and hence, are also resistant to the development of classical experimental autoimmune encephalomyelitis (EAE) [181].

1.4.2.2 mTORC2

mTORC2 consists of mTOR, rapamycin-insensitive companion of mTOR (Rictor), Sin1, mLST8 and Protor [169]. In contrast to mTORC1, understanding of mTORC2 is limited because of the lack of specific mTORC2 inhibitors. Also unlike mTORC1, mTORC2 is not sensitive to nutrients or energy [182] but rather, it is an important regulator of the cytoskeleton [183]. For instance, mTORC2 regulates actin cytoskeletal rearrangements with mTORC2 being itself negatively regulated by phosphorylation of Rictor induced by signaling between small GTPase RAS homologue (RHO) and protein kinase C [176]. In contrast to mTORC1, the upstream signal which leads to activation of mTORC2 is still unclear although the activation of mTORC2 is also controlled by PI3K and leads to the phosphorylation of its downstream substrate, AKT at residue S473 [173]. Thus the activity of mTORC2 can be indirectly measured by this AKT-parameter. Furthermore, activation of mTORC1 is independent of mTORC2-mediated activation of AKT as

this reflects phosphorylation at S473 whereas activation of mTORC1 occurs by AKT phosphorylated at T308 [173]. Additionally, such activity of AKT is controlled by p70 S6 kinase as this negatively regulates mTORC2 by phosphorylation of Rictor at Thr1135 [184].

1.4.2.3 S6K1

Activated S6K1 contributes to upregulation of mRNA translation and cell growth by promoting the biosynthesis of the translational apparatus of the cell [176, 177]. mTORC1 activity can be measured by phosphorylation of p70 ribosomal S6 kinase 1 (S6K1) and phosphorylation of the translational inhibitor eukaryotic initiation factor 4E binding protein 1 (4EBP-1) [185] as activation of mTORC1 leads phosphorylation of S6K1. S6K1 can also phosphorylate and initiate the degradation of IRS1, an intermediary between the insulin receptor and PI3K, and thus IRS fails to transduce the signal from insulin receptor to PI3K [177, 186-190]. This explains the role of S6K1 in desensitizing tissues to insulin as S6K1-deficient mice appear hypersensitive to insulin and do not display the obesity-induced insulin resistance in wild-type mice which exhibit a dramatic increase in S6K1 activation that is accompanied by increasing in IRS1 phosphorylation and attenuation of insulin-induce AKT activation [191]. Furthermore, S6K1 can also negatively regulate GSK3, which is constitutively activated in the absence of growth factors and negatively regulates the mTOR pathway by activation of TSC complex, and hence in turn S6K1 activates mTOR signalling and promotes cell proliferation [192, 193]. However, whether S6K1 can also negatively regulate other input signals from other receptor systems that activate mTOR is still unknown.

1.4.2.4 AMPK

AMPK (5'-AMP-activated protein kinase) is a heterotrimeric serine/threonine kinase,

which is composed of a catalytic α subunit (63 kDa) and regulatory β and γ subunits. The α and γ subunits are connected by the β subunit with the γ regulatory subunit comprising the four cystathionine-β-synthase domains which can bind ATP or AMP interchangeably. With this structure, AMPK and liver kinase B1 (LKB1) act as cellular energy sensors and transducers regulated by various metabolic stresses. AMPK detects bioenergetic fluctuations in cells and synergises with LKB1 to maintain cellular energy homeostasis by promoting catabolic pathways of ATP production whilst limiting processes consuming ATP [194]. Thus AMPK is sensitive to changes of the AMP: ATP ratio in the cell and is activated by stresses that stimulate ATP consumption or inhibit ATP production [195]. For instance, while the ratio of AMP/ATP increase, AMP binds to the γ subunit and thus increases the kinase activity of the α subunit through allowing phosphorylation of AMPKα at threonine 172 by upstream kinases [196, 197].

Three upstream AMPK kinases have been identified so far: LKB1, calmodulin-dependent protein kinase kinase β (CamKKβ), and transforming growth factor-β (TGFβ)-activated kinase-1 (TAK1) [198-201]. LKB1 phosphorylates and activates AMPK subfamily members in response to bioenergetic stress, including nutrient withdrawal and disruption of mitochondrial energy production [201-203]. Additionally, LKB1-deficient T cells exhibit decreased TCR- and CD28- mediated AMPK activation [204]. This indicates that LKB1 play a role as intermediate linker between AMPK and antigen receptors. Deletion of LKB in T cells causes extensive apoptosis of T cells, impaired thymic selection and dysregulation in T cell metabolism and proliferation. Moreover, TCR stimulation contributes to increasing transient fluctuation in cellular energy, which leads LKB1-dependt AMPK activation. AMPK is phosphorylated immediately following receiving signals from TCR ligation and therefore results in the production of ATP to initiate T cell metabolism during

early stages of activation [205]. In addition, Ca^{2+} flux mediated by TCR ligation is also an activator of AMPK through CamKKβ, in lymphocytes [205]. However, whether TAK1 is able to mediate similar effects to LKB and CamKKβ in T cells is still unknown.

Recent data indicate that AMPK can influence diverse aspects of T-cell biology beyond metabolism, including T-cell development, peripheral T cell homeostasis, and T cell effector function [166]. For example, mRNA translation is a highly energy consuming process in proliferating cells, accounting for around 20% of cellular ATP consumption [206] and since AMPK is a cellular energy sensor, it thus plays an important role for controlling mRNA translation. AMPK appears to downregulate mRNA translation via negatively regulating mTORC1 signaling through activation of TSC1-TSC2 complex or inhibition of Raptor [207-210]. Consistent with this, T cells defective in LKB1 or AMPKα1 exhibit increased phosphorylation of mTORC1 and consequent upregulation of its downstream molecules, S6K and 4E-BP [204] indicating that LKB and AMPK act to antagonize mTORC1 effectors upon TCR stimulation. AMPK also acts as a metabolic cell cycle checkpoint occurring in mammalian cells in response to shortage of nutrients [208, 210, 211] via mediating phosphorylation of Raptor or activation of the cell cycle regulator p27kip1 [212].

1.4.2.5 c-Myc

The transcription factor c-Myc is a potent regulator in metabolic pathways and critical for cellular growth and proliferation, differentiation and apoptosis [213-215], Overexpression of c-Myc is accompanied by high proliferation both *in vivo* and also in cell culture, *in vitro.* Indeed, quiescent cells can be driven into cell cycle by ectopic expression of c-Myc [216] whereas by contrast, low expression of c-Myc causes accumulation of nondividing and differentiated cells [217]. Consistent with

this, deregulation of c-Myc expression plays a prominent role in human diseases with for instance, overexpression of c-Myc occurring in most cancer cells [218]. Thus, normally c-Myc expression is very unstable in quiescent cells that do not receive any stimuli. However, upon serum stimulation and cell cycle entry, c-Myc becomes transiently stabilised, with consequent accumulation of c-Myc to high levels. Activation of Ras has been indicated to contribute to the stabilisation of c-Myc, the downstream substrate of Ras, activated Raf, also appearing to stabilise c-Myc via the Raf-MEK-ERK kinase cascade, suggesting that regulation of c-Myc stability might be mediated by ERK phosphorylation.

c-Myc exhibits two phosphorylation sites, threonine 58 and serine 62, in its N-terminal, which are serum regulated. Serine 62 was indeed shown to be a downstream substrate of ERK whilst threonine 58 is a targeted of Glycogen Synthase Kinase (GSK-3β) [219-221]. Ras-mediated signalling pathways appear to regulate phosphorylation at both of these sites via the Raf/MEK/ERK kinase cascade and the phosphatidylinositol-3-OH kinase (PI3K)/AKT pathway, which negatively regulates GSK-3β. Serine 62 phosphorylation stabilises c-Myc whereas Threonine 58 phosphorylation destabilises c-Myc [222] although Threonine 58 phosphorylation requires prior phosphorylation of Serine 62 [220, 222]. Mitogen stimulation causes new c-Myc protein synthesis and Ras activation and thus contributes to stabilisation of c-Myc protein via ERK-mediated phosphorylation of Serine 62 and by prevention of c-Myc phosphorylation on Threonine 58 by AKT-mediated inhibition of GSK-3β, a dual-pronged mechanism of stabilising c-Myc and hence leading to increased c-Myc protein levels [223].

Induction of c-Myc expression occurs immediately after TCR stimulation and this in turn initiates the metabolic response for producing enough energy to support rapid proliferation [224]. This is because the kinase activity of mTORC1 appears to

facilitate c-Myc translation by directly phosphorylating the translational repressor 4E-BP, which results in the release of eIF-4e and upregulation of c-Myc protein levels. Moreover, as c-Myc is a transcriptional repressor of TSC2 gene expression, this suggests that high c-Myc expression will lead to less TSC2 protein and hence, higher mTOR activity [225]. Thus by directly regulating the expression of Myc protein, high levels of mTOR activity synergizes with Myc expression to cooperatively enhance protein translation and elongation [225].

Figure 1.1 Optimum activation of naïve T cells requires two signals.

Engagement of the TCR by peptide:MHC complex (signal 1) in the absence of co-stimulation can lead to T cell anergy and/ or apoptosis. In order for T cells to proliferate and begin the process of differentiation and production of effector cytokines, CD80/CD86 engagement of CD28 (signal 2) must take place alongside TCR engagement by peptide:MHC complex. APCs, namely DCs, upregulate CD80/CD86 expression after maturation, which occurs after activation by the pathogen's products, ensuring immune responses develop only when needed.

Figure 1.2 TCR-mediated signalling.

TCR binding by the MHC-peptide complex results in the activation of Fyn, associated with CD3, and Lck, associated with CD4. Activated Lck and Fyn then phosphorylate the ITAM of CD3, allowing the recruitment and activation of ZAP-70. ZAP-70 phosphorylates LAT, which delivers the activation signals through recruitment and assembly of a signalosome containing Grb2, GADS, SLP-76 and PLCγ-1. PLCγ-1 hydrolyses PIP_2 to yield IP_3 and DAG. IP_3 binds to its receptor on the membrane of the ER triggering Ca^{2+} release and subsequent Ca^{2+} influx through plasma membrane channels; this leads to an increased intracellular Ca^{2+} concentration which will lead to the activation of calcineurin. Activated calcineurin de-phosphorylates NFAT, allowing its translocation to the nucleus where it associates with other transcription factors, promoting transcription of many genes. DAG promotes membrane association and activation of PKCθ and RasGRP1. PKCθ mediates the phosphorylation of IKK complex phosphorylates IκB, releasing it from NF-κB, and thus allowing NF-κB to translocate to the nucleus, where it will promote gene transcription. RasGRP1 promotes the conversion of ras to its activated form and active Ras recruits Raf to the plasma membrane, allowing its activation. Raf phosphorylates MEK, which, in turn, phosphorylates Erk. Erk activation leads to the activation of c-Fos, allowing the formation of the heterodimeric transcription factor AP-1.

Figure 1.3 Co-stimulation-dependent signalling in T cells.

TCR binding by the MHC-peptide complex in the presence of CD28 co-stimulation allowing the full activation of the signalling events which lead to T cell priming. In this figure, signalling events induced by the TCR-mediated signalling are also depicted, but not described; see text and Figure 1.2. CD28 engagement enhances TCR proximal signals through Lck and PI3K. Activated PI3K converts PIP_2 into PIP_3 which allows the recruitment of cytoplasmic proteins containing pleckstrin homology domains to the plasma membrane, such as PDK1, Akt and Vav1. Akt is activated by PDK1 and will contribute to the activation of NF-κB by what is thought to be indirect activation of the IKK complex. PDK1 may also phosphorylate PKCθ thus contributing to its full activation and hence, leading to more NF-κB nuclear translocation. Vav1 requires Fyn, ZAP-70 and PIP_3 to become fully activated. Activated Vav1 associates with phosphorylated SLP-76 and then activates the plasma membrane-localised Cdc42 and Rac, which in turn activate Pak1 and Pak3. The Paks will activate MKK4, which in turn phosphorylates Jnk, thus activating it resulting in phosphorylation of Jun. The Paks also activate MKK3/6, which in turn phosphorylates p38 MAPK. Similarly to Erk, p38 MAPK contributes to the activation of c-Fos, and the activation of c-Fos and Jun resulting from Erk, p38 and Jnk MAPkinase recruitment allows formation of the heterodimeric transcription factor AP-1, which is necessary for IL-2 transcription.

Figure 1.4 mTOR signalling

mTOR is able to be activated by integration of insulin, growth factors and amino acids. It also senses cellular nutrient and energy levels. mTOR signals via two distinct complexes, mTORC1 and mTORC2. mTORC1 contains the scaffolding protein regulatory-associated protein of mTOR, Raptor. Activation of mTORC1 through the PI-3K-Akt promotes protein translation and regulates cell proliferation and growth by upregualtion of translational regulators S6K1 and inhibition of 4E-BP1. However, activation of PI3K-Akt signalling is negatively regulated by phosphatase and tensin homologue (PTEN). Moreover, activation of AKT and GSK3 lead to phosphorylate TSC2 with consequent loss of TSC2 inhibitory effects on mTORC1. The TSC1-TSC2 complex is also inhibited by activation of Ras-MAPK signalling. AMPK senses bioenergetic fluctuations in cells, thus while the ratio of AMP/ATP increased, AMPK phosphorylates TSC2 and Raptor and leads to inhibit mTORC1. Different from mTORC1, mTORC2 consists of Rictor which mainly regulates actin cytoskeletal rearrangements. mTORC2, activated by PI3K, phosphorylates Akt at a serine residue S473. The phosphorylation of AKT can be controlled by p70 S6 Kinase as this negatively regulates mTORC2 by phosphorylation of Rictor at Thr1135.

Chapter 2
MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Animals

Ovalbumin (OVA) TCR transgenic mice on the BALB/c background (DO.11.10 BALB/c) were used as donors of antigen-specific T cells for *in vitro* experiments. These Tg T cells recognise $OVA_{323-339}$ peptide in the context of $I\text{-}A^d$ and were detected by a clonotypic mAb KJ1.26 [226]. The hCARΔcyt Tg mice (on the BALB/c background) expressing the human coxsackie/adenovirus receptor (hCAR) with a truncated cytoplasmic domain (hCARΔcyt) on their thymocytes and T lymphocytes [227], doubly transgenic OVA-TCR hCARΔcyt mice were bred in house by crossing hCARΔcyt with OVA TCR mice and selected by phenotyping hCAR and KJ1.26 expression on $CD4^+$ T cells by FACs analysis. All mice were maintained at the Central Research Facility (CRF), University of Glasgow regarding to Home Office regulations.

2.2 Cell culture reagents

All cell culture reagents were purchased from Invitrogen (UK). All other reagents were from Sigma-Aldrich (UK) unless otherwise descripted. Cell culture was maintained under aseptic conditions at 37°C, 5% CO_2.

2.3 Preparation and purification of $CD4^+$ T cells

2.3.1 Generation of lymph node cells

Peripheral (axillary, brachial, inguinal, cervical and popliteal) lymph nodes (PLN) and mesenteric lymph nodes (MLN) were removed from OVA TCR Tg, hCAR Tg or

hCARΔcyt x OVA TCR double Tg mice, pooled and forced through Nitex (Cadisch Precision Meshes, UK) to generate single cell suspensions. LN cells were washed with sterile RPMI 1640 media and counted by trypan blue exclusion. The percentage of $CD4^+$ $KJ1.26^+$ T cells was calculated by flow cytometry.

2.3.2 Isolation of $CD4^+$ T cells

$CD4^+$ T cells were isolated by MACS $CD4^+$ Isolation kit according to the procedures of Miltenyi Biotec (UK) via an indirect magnetic labelling system. $CD4^-$ cells, such as cytotoxic T cells, B cells, NK cells, dendritic cells, macrophages, granulocytes and erythroid cells were indirectly magnetically labelled by conjugating with a cocktail of biotin-conjugated antibodies against CD8a (Ly-2), CD45R (B220), DX5, CD11b (Mac-1) and Ter-119, and sequential binding to anti-biotin MicroBeads. The magnetically labelled $CD4^-$ cells were depleted by retention within the MACS column while the unlabelled $CD4^+$ T cells flowed through the column and collected.

The details of the whole procedure were as follows: The LN cells were prepared as a single cell suspension as described in 2.3.1. The single cell suspension was centrifuged and the cell pellet re-suspended in 40 µl of MACS buffer (0.5% BSA/ 2 mM EDTA/PBS) and incubated with 10 µl of the biotin-conjugated antibodies per 10^7 total cells, at 4°C for 10 min. In turn, 30 µl of MACS buffer (per10^7 cells) and 20 µl of anti-biotin MicroBeads (per 10^7 total cells) were added in and incubated at 4°C for 15 min. Cells were washed with MACS buffer and centrifuged (300 g, 10 min). The cell pellet was re-suspended in 500 µl of MACS buffer per 10^8 total cells before being passed through the column.

2.3.3 Induction of anergy and priming of T cells in vitro

Tissue culture plates (6-well; Corning) were pre-coated with anti-mouse CD3ε Ab

(Clone 145-2C11; 1 µg/well) in the presence or absence of 1 µg/ml anti-mouse CD28 (Clone 37.51, BD Pharmingen, Oxford, UK) in PBS (1ml/well) and incubated at 4°C for 16 hours. Thereafter, the plates were washed with PBS before naïve $CD4^+$ T cells were plated out onto the plates and cultured in complete medium (RPMI 1640, 10% FCS, 2 mM $_L$-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 0.05 mM 2-ME) with anti-mouse CD3ε Ab in presence or absence of 1 µg/ml anti-mouse CD28 Ab to induce priming or anergy, respectively [32, 34, 228]. After 48 hours, the cells were washed twice with complete medium, re-seeded at a concentration of $1x10^6$ cells/ml/well in fresh plates and rested in complete RPMI medium for an additional 48 hours.

2.4 Functional analysis of T cells during the maintenance phse of anergy and priming in vitro.

2.4.1 Generation, maturation and antigen-loading of dendritic cells

Dendritic cells (DC) were generated from bone marrow of mice from the BALB/c background. Briefly, femurs and tibiae were asceptically removed and the bone marrow was harvested by using a syringe with 23 g needle by flushing RPMI 1640 medium through the bone. Single cell suspensions were prepared and washed with RPMI 1640 medium. Cells were counted by trypan blue exclusion and DC progenitors were plated at 2 x 10^5/ml in DC medium (RPMI 1640, 10% GM-CSF-conditioned media, 10% FBS, 2 mM $_L$-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 0.05 mM 2-ME) and incubated at 37°C for 7 days. Cells were supplemented by addition of fresh DC medium (5 ml per plate) at day 3 and days 5 before being matured by 1 µg/ml LPS (*Salmonella abortus*, Sigma) for 24 hours at day 7. Mature DCs were loaded with 1 µg/ml of $OVA_{323-339}$ at 37°C for 3 hours and

then washed.

2.4.2 Production of GM-CSF condition media

Conditioned medium (supernatant) from the GM-CSF-secreting X63 fibroblast cell line was used as the source of GM-CSF for the culture of DCs. X63 cells were quickly thawed at 37°C in a water bath, washed and cultured in 5 ml complete RPMI 1640 medium supplemented with 0.5 mg/ml of G418. After one week, cells were re-suspended in fresh complete media without G418 for further culture. One to two weeks later, cells were centrifuged, the supernatant were harvested and filter-sterilised before freezing.

2.4.3 Culture of T cells with DC

The anergic and primed T cells that were induced with anti-CD3 in absence or presence of anti-CD28, respectively, were harvested from tissue-culture plates. Naïve T cells were freshly isolated from LN cells of OVA TCR Tg or hCARx OVA TCR mice as a primary response control. The naïve, anergic and primed Tg T cells were analysed by flow cytometry to calculate the percentage of "live $CD4^+$ KJ^+ T cells" and then stimulated by culturing with LPS-matured, OVA-loaded DC at a ratio of 1:1 ($1x10^6$ DC+$1x10^6$T cells) in complete RPMI 1640 medium (2ml/well) in 12-well plates (Costar, Corning, NY). Alternatively, in some experiments cells, 2 x 10^6 DC + 2 x 10^6 T cells, were cultured in complete RPMI 1640 medium in 6-well plates (Costar, Corning, NY). T cells and DC were cultured together for up to 4 days at 37°C in 5% CO_2 incubator (Jencons, Leighton Buzzard, UK).

2.4.4 Assessment of antigen-specific proliferation by [3H] Thymidine

Naïve, anergic and primed T cells were cultured with DC ± $OVA_{323-339}$, as described

in section 2.4.1, at a concentration of $2x10^5$ DC + $2x10^5$ Tg T cells per well, in complete RPMI medium, in triplicate for 48 h in 96-well flat-bottomed plates (Corning) at 37°C in a 5% CO_2 incubator. DNA synthesis was measured in all samples by addition of 1μCi per well of [^{3}H] thymidine (Western Infirmary, Glasgow) for 16 hours. Cells were then harvested onto glass fibre filter mats (Wallac, Warrington, UK) using a Betaplate 96-well harvester (Amersham). [^{3}H] thymidine incorporation into DNA was assessed by a 1205 betaplate liquid scintillation counter (Amersham).

2.4.5 Assessment of antigen-specific cytokine production in vitro

The cytokine production of T cells were quantified by enzyme-linked immunosorbent assay (ELISA). To measure IL-2 and IFNγ in supernatants that were harvested at different time points (24, 48 and 96 hr), Immulon 4 plates (Costar) were coated with rat anti-mouse IL-2 or IFNγ capture Abs (1 or 1.5 μg/ml, respectively; 50μl/well; BD Pharmingen) for 16 hours at 4°C before being blocked with 10% FCS in PBS for 1 h at 37°C. The sample supernatants were added for 3 hours at 37°C and in turn following washing with 0.05% Tween®-20 in PBS, were subsequently incubated with biotinylated rat anti-mouse IL-2 or IFNγ detection Abs (0.5 or 1 μg/ml, respectively; 50 μl/well; BD PharMingen) for 1 h at 37°C. Plates were then incubated with 50 μl extravidin peroxidase per well (diluted 1:1000 in PBS/0.2% FCS/0.05% Tween®-20; Sigma-Aldrich) for 1hour at 37°C before being treated with TMB (3,3',5,5'-Tetramethylbenzidine) Microwell Peroxidase Substrate. Recombinant murine IL-2 or IFNγ preparations (BD Pharmingen) were used to produce standard curves from which cytokine levels in samples were calculated.

2.4.6 CFSE labelling of OVA TCR Tg lymphocytes

$CD4^+$ T cells were generated from PLN and MLN from OVA TCR or hCAR x OVA

TCR mice and isolated by MACS $CD4^+$ T cells isolation kit as described in Sections 2.3.1 & 2.3.2. $CD4^+$ T cells were stained with CFSE before or after the induction of priming or anergy. The naïve $CD4^+$ T cells (staining before induction phase), anergic and primed T cells (staining after induction phase) were washed twice in PBS via centrifugation at 450 g for 5 min and then cells were re-suspended at 1 $x10^7$/ml in cold PBS and labelled with 5 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA SE; CFSE; Invitrogen) for 10 min at 4°C. Next, cells were washed in blocking buffer (2% FCS/PBS) immediately. The CFSE-stained cells were washed two times more with blocking buffer before stimulation or co-culture with DC.

2.4.7 eFluor 670 labelling of Tg lymphocytes

$CD4^+$ T cells were generated from PLN and MLN from OVA TCR or hCAR x OVA TCR mice and isolated by MACS $CD4^+$ T cells isolation kit as described in Section 2.3.1 & 2.3.2. $CD4^+$ T cells were stained with eFluor 670 before or after induction of priming or tolerance. The naïve $CD4^+$ T cells (staining before induction phase), anergic and primed T cells (staining after induction phase) were washed twice in PBS via centrifugation at 450 g for 5 min before cells were re-suspended at 1 $x10^7$/ml in PBS and labelled with 5 μM eFluor 670 (eBioscience) for 10 min at 37°C in the dark. Labelling was then terminated by incubating in cold complete media (containing ≥ 10% FCS) for 5 min at 4°C. The eFluor 670-loaded cells were washed three times with complete media before induction of anergy and priming or alternatively, co-culture with DC.

2.5 Functional analysis of T cells during the induction phase of anergy and priming in vitro.

2.5.1 Assessment of DNA synthesis in anergic and primed T cells during induction phase

Naïve $CD4^+$ T cells were cultured with anti-CD3 in presence or absence of anti-CD28, as described in section 2.3.3, at a concentration of $2x10^5$ Tg T cells per well in complete RPMI medium, in triplicate, for 48 or 96 h, in 96-well flat bottomed plates (Corning) at 37°C in a 5% CO_2. DNA synthesis was measured in all samples by addition of 1 µCi per well of [^{3}H] thymidine (Western Infirmary, Glasgow) for 16 hours. Cells were then harvested onto glass fibre filter mats (Wallac, warrington, UK) using a Betaplate 96-well harvester (Amersham). [^{3}H] thymidine incorporation into DNA was assessed by a 1205 betaplate liquid scintillation counter (Amersham).

2.5.2 Assessment of cytokine production during induction of anergy and priming in T cells

The cytokine productions of T cells during induction of anergy and priming were quantified by ELISA. Naïve $CD4^+$ T cells were plated out onto 6-well plates coated with anti-CD3 in presence or absence of anti-CD28 at a concentration of $10x10^6$ /4ml/well. The supernatants were harvested at 48 h and the cells washed twice with complete medium, and rested in complete RPMI medium for an additional 48 h (96 h of induction) before supernatants were harvested again. The measurement of IL-2 and IFNγ in supernatants by ELISA was described in section 2.4.5.

2.5.3 Assessment of cellular proliferation by eFluor 670 during induction phase of T cell priming or anergy.

$CD4^+$ T cells were generated from PLN and MLN from OVA TCR or hCAR x OVA TCR mice and isolated by MACS $CD4^+$ T cells isolation kit as described in Section 2.3.1 & 2.3.2. Naïve $CD4^+$ T cells were washed twice in PBS via centrifugation at 450 g for 5 min and cells were re-suspended at 1 $x10^7$/ml in PBS and labelled with 5 µM eFluor 670 (eBioscience) for 10 min at 37°C in the dark. Next, labelling of cells was stopped labelling by incubating in cold complete media (containing ≥ 10% FCS) for 5 min at 4°C. The eFluor 670-loaded cells were washed three times with complete media before plating out onto 6-well plates pre-coated with anti-CD3 ± anti-CD28 for induction of priming and anergy for 4 days, respectively. The cells were harvested everyday and the profile of eFluor 670 fluorescence analysed by FACS (FACS Calibur, BD).

2.6 Transduction of Tg T cells with bicistronic Ad Rap1 WT and S17N adenoviral gene transfer construct

2.6.1 Preparation of Adenovirus

Ad Rap1 WT and Ad Rap1 S17N were amplified from the stock. High-titer stocks of Ad Rap1 WT and AdRap1 S17N were produced by large scale amplification of a plaque pure stock of adenovirus in a HEK293 cell line (ATCC, Rockville, MD, CRL 1573; Human Adenovirus 5-transformed Human Embryonic Kidney 293 cell line). Low passage 293A cells were sub-cultured in complete minimal essential medium (Dulbecco's modified Eagle's Medium (DMEM); 10% FCS, 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all Invitrogen)), in 75T Flask (Corning). Subsequently, HEK293 cells were sub-cultured 2-3 times in 150T flasks for large scale amplification. Cells were allowed to reach 80-90% confluence before being

infected with Ad Rap1 WT and S17N (approximately 1 plaque forming unit (pfu) per cell). The medium was replaced every 3 days until the cells started to detach from the flask. Fresh medium (10 ml) was added to the flasks until this cytopathic effect was complete. Cells were harvested immediately and centrifuged 1500 rpm for 10 minutes at room temperature. The cell pellet containing virus was resuspened in 500 µl sterile, fresh, complete culture medium. The cells were lysed with three consecutive freeze-thaw cycles: freeze cells in a dry ice/ethanol bath; then place the tube in a 37°C water bath until the ice is just thawed. Do not allow the suspension to reach 37°C. After the third cycle, briefly centrifuge to pellet debris and transfer the lysate to a clean, sterile centrifuge tube and store the lysate at -20°C. The purification of virus was using Adeno-X Virus Purificaiton Kit (Cat. No. 632248) and the titer of the lysate was assessed by Adeno-X Rapid Titer Kit (cat. No. 631028). All steps were following with the manual.

2.6.2 Adenoviral transduction of hCAR and hCAR X OVA TCR cells

LNs were generated from hCAR or hCAR x OVA TCR mice and single cell suspensions were prepared, as described in section 2.3.1. hCAR LN cells were then infected with adenovirus (Ad Rap1 WT or Ad Rap1 S17N) for 30 min at a variety of multiplicities of infection (MOI) before excess Ad was washed off and the cells were subsequently induced with anti-CD3 in absence or presence of anti-CD28 for induction of anergy and priming in T cells. In order to examine the optimal MOI for transduction of Tg T cells, LNs were infected with Ad at MOI 0, 3, 10, and 30. All cells were stained for CD4 expression and with 7-AAD to assess viability prior to induction experiments proceeding.

2.6.3 Assessment of cellular proliferation regulated by Rap1 during induction phase

LNs were generated from hCAR or hCAR x OVA TCR mice and single cell suspensions were prepared, as described in section 2.3.1. hCAR LN cells (10 x 10^6 /ml) were then infected with adenovirus (Ad Rap1 WT or Ad Rap1 S17N) for 30 min at MOI 30 in infection media (DMEM/10 mM HEPES) before excess Ad was washed off. Subsequently the infected cells were loaded with 5 μM eFluor 670 in (Section 2.4.7) before induction of anergy and priming in T cells for 4 days (Section 2.3.3). The cells were harvested everyday and analysed by FACS.

2.6.4 Induction of anergy and priming of T cells using immature and mature DC

2.6.4.1 Assessment of cellular proliferation during induction phase.

In order to investigate induction of priming and tolerance in T cells under more physiological conditions, LN cells (3 x 10^6/ml/well) generated from hCAR x OVA TCR Tg mice were infected, or not, with adenovirus (Ad Rap1 WT and S17N) for 30 min at MO 30 in infection media (DMEM/10 mM HEPES) before excess Ad was washed off and the cells were subsequently incubated for 24 h in complete media. The Ad-infected cells were analysed for GFP expression after transduction for 24 h and all cells were stained with 5 μM eFluor 670 before co-culturing with OVA-loaded, immature or LPS-matured DC in plates for 4 days. Cellular proliferation in terms of eFluor 670 fluorescence was analysed by FACS.

2.6.5 Analysis metabolism of induction of anergy and priming in T cells by metabolomics

Naïve $CD4^+$ T cells were generated from Tg mice and isolated by MACS $CD4^+$ T cell isolation kit. The $CD4^+$ T cells then stimulated with complete media alone,

anti-CD3 alone and anti-CD3 + anti-CD28, as described in section 2.3.3, at a concentration of $2x10^6$/4 ml/well Tg T cells per well in complete RPMI medium, for 48 hours, the cells were washed twice with complete medium, re-plate and rested in complete RPMI medium for an additional 48 h at 37°C in 5% CO_2. The incubation medium (secreted metabolites) and stimulated cells were harvested at 24, 48, 72 and 96 h. The sample preparation for metabolomics involved mixing of 0.2 ml of incubation medium with 0.8 ml of acetonitrile in Eppendorf tubes. The stimulated cells were washed with PBS three time at 37°C aspirating PBS completely before addition of 1 ml of Extraction solution (Methanol: Acetonitrile: H_2O = 50:30:20) (cooled to 0°C). The incubation medium samples were shaken by Thermo mixer at 1440 rpm for 12 min, at 4°C whilst lysed cell samples were centrifuged at 13000 rpm for 15 min at 0°C. The supernatant from both samples were transferred to fresh Eppendorf tubes and kept at -20°

2.7 Western Blotting

Naïve $CD4^+$ T cells ($1x10^7$) were stimulated with anti-CD3 in the presence or absence of anti-CD28 for 0, 1, 2, 4, 8, 24, 48 and 96 h during induction of priming and anergy as described in 2.3.3. The cells were harvested at each timepoint and lysed in lysis buffer (50mM Tris-HCl buffer pH 7.5 containing 150 mM NaCl, 2% (v/v) Nonidet P40, 0.25% (w/v) sodium deoxycholate, 1mM EDTA (pH 8.0), 1 mM PMSF, 10 mM Sodium orthovanadate, 10 µg/ml chymostatin, 10 µg/ml leupeptin, 10 µg/ml antipain, 10 µg/ml pepstatin A; all obtained from Sigma) for 30 min on ice before centrifugation of lysates at 16,000 g for 15 min at 4°C and the resulting supernatants (cell lysates) were stored at -20°C. The protein concentration of the cell lysates was determined by the Micro BCA protein assay reagent kit (Pierce, Rockford, IL). Samples (30 µg) were mixed with an equal volume of 2x SDS PAGE

gel loading buffer (20% (v/v) Glycerol, 4% (w/v) SDS, 100 mM Tris-HCl pH 6.8, 2 μg/ml Bromophenol Blue, 5% (w/v) β- mercaptoethanol), boiled for 5 min and then then separated (Precision Plus Protein Standard markers were added to one well) by 4-12% NuPAGE Bis-Tris gels with NuPAGE MOPS running buffer (supplemented with NuPAGE antioxidant) at 180 V for 1 hour following the manufacturers instructions. Proteins were then transferred onto nitrocellulose membranes (Amersham, UK) using NuPAGE transfer buffer with 20% (v/v) methanol at 30 V for 1 hour.

Following transfer, nitrocellulose membranes were washed once in TBS/Tween (0.5 M NaCl and 20 mM Tris pH7.5 with 0.1% (v/v) Tween-20) and blocked for 1 h in TBS/Tween containing 5% non-fat milk protein. The membranes were then incubated with the appropriate primary antibody (Table2.2) which is diluted in TBS/Tween/5% non-fat milk solution in 1:1000 in cold room (4°C) overnight. Following incubation with primary antibody nitrocellulose membranes were washed with TBS/Tween buffer 5 times (5 min each time) and incubated with HRP-conjugated secondary antibody (Table 2.2) within TBS/Tween/5% non-fat milk for 1 hour at room temperature. Again, the nitrocellulose membranes were then washed 5 times (5 min each time) with TBS/Tween buffer before incubation with a mixture of equal volumes of ECL solution A (2.5 mM luminol, 0.4 mM p-coumaric acid and 100 mM Tris buffer pH 8.5) and ECL solution B (0.002% hydrogen peroxide and 100 mM Tris buffer pH 8.5) for 1 min before exposing blots to Kodak X-Ray film. Nitrocellulose membranes were stripped and re-probed with other primary antibodies if necessary. The blots were stripped at room temperature for 1 h in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris buffer pH 7). Nitrocellulose membranes were washed thoroughly in TBS/Tween buffer for several times before adding alternative primary antibody.

2.8 FACS analysis

2.8.1 Staining of surface markers

Aliquots of cells (10^5-10^6 per sample) in 5 ml polystyrene tubes (Falcon, BD) were washed with 200 µl cold FACS buffer at 1400 rpm for 5 min at 4 °C. Cells were re-suspended in 100 µl of Fc receptor (FcR) blocking buffer (anti-CD16/32, clone 2.4G2, hybridoma supernatant, 10% mouse serum, 0.1% sodium azide) containing the appropriate fluorochrome-conjugated, biotinylated or purified primary Abs for 20 min in the dark at 4 °C. Anti-CD16/32 binds to FcγRII/III and the immunoglobulin in mouse serum binds to FcγRI, and so the FcR blocking buffer blocks non-specific binding of Ab to such FcR-bearing cells. Details of the antibody clones, their specificities and isotype controls used are provided in Table 2.1. Cells were washed with 1 ml of FACS buffer as before and, where appropriate, biotinylated or purified Abs were detected following incubation with fluorochrome-conjugated streptavidin for 10-20 min in the dark, at 4°C. Cells were washed again in FACS buffer and re-suspended in 200 µl FACS buffer before being analysed by FACS (FACS Calibur BD) and FlowJo software (TreeStar).

2.8.1.1 Phenotyping of DO11.10 TCR, hCAR, hCAR x DO11.10 transgenic T cells

Cells were incubated with PerCP-conjugated species anti- mouse CD4 antibody, biotinylated species anti-KJ1.26 antibody and purified species anti-CAR antibody, or their respective isotype controls, for 20 min at 4°C. Cells were then washed in FACS buffer and incubated with PE-conjugated Streptavidin and anti- species IgG1 FITC for 10 min at 4°C. Phenotyping was performed by two or three-colour flow cytometry (FACS Calibur) and analysis by FlowJo software (Figure 2.1 & Figure 2.2).

2.8.2 Detection of intracellular signalling molecules

For detection of intracellular signalling molecules, cells were firstly stained for surface markers, prior to fixation and permeabilisation, as described above. For staining intracellular proteins, cells were washed with 1 ml PBS before addition of 200 µl Cytofix/Cytoperm solution (BD Pharmingen) for 20 min at 4°C and further washing twice with 500 µl BD Perm/Wash solution (BD Pharmingen) (450 g, 5 min). The relevant anti-intracellular protein antibodies or their respective isotype controls were mixed properly with 50 µl Perm/Wash solution in the tube and incubated with the cells in the dark at RT for 30 min. After washing, cells were incubated with fluorochrome-conjugated secondary antibody, if applicable, in the dark at RT for 30 min. Finally, cells were washed in Perm/Wash twice and resuspended in 250 µl FACS buffer and analysed using FACS Calibur and Flowjo software.

2.8.3 Detection of intracellular signalling molecules in cells transduced with bicistronic adenoviral constructs

For detection of intracellular signalling molecules in cells which were transduced with virus, the cells were centrifuged and incubated in 0.5 ml of pre-warmed Fixation buffer (BioLegend) at 37°C for 10 min and then washed in cold FACS buffer. After treatment with Fixation buffer, the surface markers were stained at 4°C for 20 min. Cells were washed with 1 ml of PBS before addition of 1 ml diluted Permeabilisation Wash Buffer (BioLegend) and washed twice. The fixed and permeabiilsed cells were re-suspended in 50 µl of diluted Permeabilisation Wash Buffer and incubated with relevant anti-intracellular protein antibodies or appropriate isotype control in the dark at RT for 20 min. Cells were washed twice with 1 ml of Permeabilisation Wash Buffer and re-suspended in 250 µl FACS buffer and analysed using FACS Calibur and Flowjo software.

2.8.3.1 Measurement of ERK1/2 and Rap1 activation during induction phase of priming and tolerance

As described in 2.5.6.1, LN cells (3 x 10^6/ml/well) generated from hCAR x OVA TCR Tg mice were infected or not with adenovirus (Ad Rap1 WT and S17N) for 30 min at MOI 30 in infection media (DMEM/10 mM HEPES) before excess Ad was washed off and the cells were subsequently incubated for 24 h in complete media to allow for induction of expression of the gene of interest as indicated by GFP expression. All cells were co-cultured with OVA-loaded, immature or LPS-matured DC for 4 days. ERK and Rap1 activation during induction of anergy and priming in T cells was then assessed by FACS.

Figure 2.1 Idedntification of DO11.10 OVA TCR transgenic T cells by flow cytometry

Lymphocytes were firstly distinguished via forward scatter (FSC), which correlates with size, and side scatter (SSC), which correlates with granularity; events gated within region 1 (R1) were lymphocytes. These events were then analysed for the (B) expression of CD4 and DO11.10 (gated within R2). (A) Isotype control for KJ1.26 antibody was mouse IgG2a.

Figure 2.2 Identification of hCAR x DO11.10 TCR transgenic T cells by flow cytometry

Lymphocytes were firstly distinguished via forward scatter (FSC), which correlates with size, and side scatter (SSC), which correlates with granularity; events gated within region 1 (R1) were lymphocytes. Firstly the $hCAR^+$ and $KJ1.26^+$ populations were discriminated by setting appropriate gates on isotype controls, mouse IgG2a and mouse IgG1 for (A) hCAR and (B) KJ1.26 respectively. (C) PerCP-conjugated anti-CD4 was used to identify $CD4^+$ T cells (R2). (D) Biotinylated KJ1.26 plus SA-PE and hCAR-FITC were used to stain the Ag-specific $hCAR^+$ T cell population (G1).

Figure 2.3 Identification of hCAR x DO11.10 TCR transgenic T cells by flow cytometry

Lymph node cultures from hCARΔcyt.DO11.10 mice were cultured alone or infected with Ad Rap1 S17N, which encodes a bicistronic GFP reporter gene, for 30 minutes at MOI of 3, 10 and 30. Following infection, anergy or priming was induced in the T lymphocytes, as described in Section 2.3.3, and the cells were cultured for the other 24 hours. Subsequently, cells were stained with $CD4^+$ PerCP for assessing CD4 expression. Lymphocytes were identified on the basis of size and granularity (A). R1 was gated as “live cells population” and assessed for CD4 and GFP expression (B). The analysis of $CD4^+$ population showed that by comparison to mock infection of cells (MOI 0) that resulted in no GFP^+CD4^+ T cells, naïve lymphocytes infected with Ad Rap1 S17N at MOI 3, 10 and 30 produced about 5%, 14% and 30%, respectively, of the $CD4^+$ T cells expressing GFP.

Table 2.1 Antibodies used for flow cytometry.

Table 2.2 Antibodies used in Western blotting.

Chapter 3
Results

3 Results

Introduction

Previous studies from this lab have reported that the distinct functional outcomes of T cell priming and tolerance are associated with marked differences in the amplitude, kinetics and cellular localisation of activated, phosphorylated ERK signals with primed Ag-specific T cells showing enhanced activation of ERK relative to tolerised Ag-specific cells at the single cell level [13]. Consistent with this, it has also previously been shown that anergic T cells exhibit a deficiency in IL-2 production which is the outcome of a lack of ERK and AP-1 activation [229]. These earlier studies suggested that such defective ERK activation is accompanied by accumulation of the GTPase, Rap1 [150, 230]. Such accumulation of Rap1 is able to disrupt TCR coupling to ERK activation by sequestering Raf-1 and thus directly antagonising the Ras-Raf-ERK signalling cascade [148]. Supporting this, accumulation of active Rap1 has been reported to play a role in the maintenance of anergy in human T cell clones [30, 148], with tolerized cells displaying decreased ERK activation because of recruitment of a Fyn-Cbl-CrkL-C3G-Rap1 signalling complex not found in their primed counterparts [148]. Furthermore, an inverse relationship between ERK and Rap1 activation has been shown in various T cells lines and also that CD28 signalling abolishes TCR-coupled Rap1 activity [150-152]. Our lab also reported that inverse relationship between Rap1 and phosphorylated ERK expression occurs during maintenance phase of anergy and priming of antigen-specific $CD4^+$ T cells *in vitro* and *in vivo* [14]. However, these latter findings only related to the initial 24 h period of the maintenance phase and were not able to probe the role of Rap activity, rather than Rap expression because of lack of suitable reagents to investigate this in situ. Thus, it was the aim of this project to

assess the functional importance of ERK and Rap1 activation throughout the maintenance phase.

Maintenance of Ag-specific priming and tolerance was examined by exploiting the capability of the OVA TCR Tg mouse to provide antigen-specific $CD4^+$ T cells which can be induced to be anergic and primed T cells by anti-CD3 or anti-CD3 plus anti-CD28 antibodies, respectively before stimulation with antigen. In particular, it was planned to focus on the associations between ERK and Rap1 activation and cellular proliferation and cytokine production. In addition to examining proliferation by DNA synthesis by [^{3}H] thymidine administration, the two cell tracking dyes, CFSE and eFluor 670 can be utilized to monitor long term cellular proliferation and cell division to reveal more detail than that provided by DNA synthesis analysis during the maintenance phase. For instance, the percentage and numbers of antigen-specific $CD4^+$ T cells in each division are could be analysed by FACS. Furthermore, the observation of ERK and Rap1 activation in Ag-specific cells at the single cell level can also be assessed during the maintenance phase using antibodies that recognise the active form of these enzymes by FACS analysis. With these reagents and techniques, the profile of cellular proliferation and MAPK molecular signalling in antigen-specific anergic and primed cells it is now possible to investigate the functional relationship of these signals in priming and tolerance.

3.1 Characterisation of the Maintenance phase of anergy and priming in $CD4^+$ T cells

The core aim of the project is to investigate the differential signalling mechanisms underpinning the induction and maintenance of anergy and priming of Antigen (Ag)-specific T cells. Thus, it was first planned to build on our previously published

work [14] indicating that counter-regulatory Erk and Rap-1 signalling occurred during the maintenance phase of such priming and tolerance by investigating the functional role of these potentially antagonistic signals. To do this, the *in vitro* model of induction and maintenance of priming and tolerance was further characterised and optimised. In this model, $CD4^+$ T cells from OVA-specific TCR Tg DO11.10 Tg mice (purified as described in Chapter 2) in which 80% of the T_H cells are specific for OVA and preferentially differentiate into Th1 cells upon priming with DC and high levels of Ag in vitro, were induced into an anergic or primed state by TCR ligation with or without appropriate co-stimulation (via CD28), respectively, by culture for 2 days on plate bound anti-CD3 [32, 34]. These cells were then washed and rested for an additional 2 days in fresh medium (4-day induction phase) before being re-stimulated with LPS-matured DC that have been loaded or not with or without OVA $peptide_{323-339}$ (1μg/ml), the peptide Ag which the T cells generated from DO11.10 TCR Tg mice can recognise (Figure 3.1). LPS-matured DC are used to mimic DC that have been exposed to inflammation and/or danger signals and have therefore upregulated the costimulatory antigens necessary for “priming” signals as “immature” DC are widely reported to induce tolerance [231].

3.1.1 Assessment of DNA synthesis during the maintenance phase of anergy and priming in $CD4^+$ T cells in vitro

To establish the optimal concentration of antigen in the functional study during maintenance phase, the naïve T cells loaded with the proliferation-tracking, fluorescent dye CFSE (Chapter 2.4.6) were co-cultured with LPS-matured DC loaded with 0.1 μg/ml or 1 μg/ml of OVA $peptide_{323-339}$ and cellular proliferation assessed from day 1-4. CFSE is a cell permeable fluorescent dye that is retained within cells and covalently couples to intracellular molecules. During each cell division, the daughter cells inherit half the amount of cellular dye from the original

cell population. Thus CFSE is a valuable fluorescent dye for investigating lymphocyte proliferation. Such analysis showed that cells treated with either of these two concentrations of OVA peptide both exhibited clear profiles of proliferation and cell division from day 3 although it was obvious that a substantial population in both cases did not undergo division. Nevertheless, it was also clear that the cells treated with 1 μg/ml of OVA proliferated more than 0.1 μg/ml one (Figure 3.2). Regarding this result, 1 μg/ml of OVA was therefore used in the following experiments to produce sufficient intensity of stimulation for the cells.

Analysis of the proliferative responses of naïve cells to OVA-loaded LPS-matured DC versus immature DC showed that whilst the OVA-loaded immature DC induced some proliferation, the mature DC induced higher levels of proliferation, both in proportions of cells undergoing more rounds of division but also in terms of absolute numbers of cells generated (Figure 3.3). Although the dogma suggests that immature DC should not induce proliferation we and others have observed this [232] and this in part probably reflects the difficulty in maintaining DC in an “Immature” state where there is no costimulation: indeed, analysis of costimulatory molecule expression by such populations of DC shows that matured DC expressed higher levels of CD80 expression than immature DC, although this increase was not to a great extent (Figure 4.6B). By contrast, the matured DC expressed substantially higher levels of CD86 than immature DCs (Figure 4.6C). Collectively, these data suggested the DC treated with LPS were more matured than immature DC but the “immature DC” had been partially matured during derivation. However, it also more likely reflects that it is becoming increasingly evident that clonal expansion of T cells occurs during the induction phase of tolerance, albeit at a lower level than observed in response to priming signals [14]. The first functional assessment of the primed and anergic populations was of the proliferative

response and this was first analysed in terms of DNA synthesis (by [^{3}H] uptake into DNA) as an indication of cell cycle progression (Figure 3.4). Firstly, it was shown that naïve CD4$^+$ T cells were activated and proliferated well in response to the challenge with peptide-loaded DC, and this response was substantially higher than that of the naïve CD4$^+$ T cells which were challenged with or without $OVA_{323-339}$ alone, in the absence of DC. Neither DC, nor unstimulated naïve T cells displayed DNA synthesis (Figure 3.4A). Whilst the strong response of naïve T cells stimulated with OVA-DC underlined the crucial role of DC in priming naïve T cell responses, it was somewhat unexpected that DNA synthesis of naïve T cells in response to OVA was observed, although this was much lower than the response of naïve T cells co-cultured with antigen loaded, matured DC. This high basal activation might simply reflect the presence of some “activated” or “memory” T cells in the “naïve” population (due to the non-SPF status of the animal house) that could respond to the high levels of peptide in vitro, or alternatively non-specific activation of these antigen-specific cells during isolation from lymph nodes. That allowed them to respond to the high levels of Ag present in the assay. Nevertheless, these data indicated that this assay was suitable for corroborating cell cycle progression and proliferation by primed and anergic populations.

Thus, to assess the DNA synthesis of T cells to antigen during the maintenance phase of priming and tolerance, anergic and primed populations of CD4$^+$ KJ$^+$ T cells were re-challenged with OVA-loaded matured DCs (as described in 2.4.1). As expected, the primed T cells (induction of CD4$^+$ T cells with anti-CD3 and anti-CD28) exhibited a higher response than either naïve or anergic T cells (induction of CD4$^+$ T cells with anti-CD3), which were challenged with antigen-pulsed DCs, whilst the anergic T cell displayed an even lower DNA synthesis than the naïve T cells undergoing a primary response (Figure 3.4B). In

addition, and consistent with the dogma that for full T cell activation and proliferation, APC-derived signals in addition to antigen are required, the primed T cells exhibited similar basal levels of proliferation as the OVA-treated naïve T cells (Figure 3.4A).

Analysis of DNA synthesis provides a snapshot of the cells transiting S phase of the cell cycle when [^{3}H] thymidine has been administered. Hence, although it is an indicator of cellular proliferation, it provides no information relating to the actual numbers of cell division undergone.

3.1.2 Assessment of proliferation of populations of primed and anergic T cells when challenged with immature or mature DC during maintenance phase.

To further examine the differential proliferative responses among naïve, anergic and primed populations following challenge with antigen, the responses of these populations to immature and LPS-matured DC loaded were investigated. Firstly, the cells in the anergic and primed T cells populations were stained with proliferation dye, CFSE, before the induction phase (Figure 3.3A) and this showed that whilst the cells undergoing induction of anergy proliferated, this was to a lower level that observed for the primed group both in terms of proportions and absolute numbers. There was however, a much larger population of the “anergic” cells that did not appear to undergo division at all when compared to the primed group. Nevertheless, collectively these data provide further support to previous reports of clonal expansion of cells during the induction of anergy. Replicate group of these cells were then further analysed at day 4 following challenge with immature or LPS-matured DC loaded with OVA and this showed that whilst immature DC did not induce substantial proliferation of either primed or anergic T cells, the LPS-matured DC did, with primed cells proliferating more than anergic cells, both in

terms of proportions and absolute numbers (Figure 3.3B). The final parameter optimised was the ratio of DC to T cells utilised for priming. The anergic and primed cells co-cultured with LPS-matured, OVA-loaded DC in ratio of 10:1 (Figure 3.5A) and 1:1 (Figure 3.5B) for 4 days. The primed T cells exhibited faster and more obvious proliferation than anergic cells while co-culture with LPS-matured, OVA-loaded DC in ratio of 1:1 (Figure 3.5).

Following this optimisation of the protocols, the differential proliferation responses of naïve, anergic and primed populations in response to OVA-loaded, LPS-matured DC were explored in more detail. Firstly, analysis of the naïve population showed that T cells alone, or cultured in the absence of antigen, respectively, did not proliferate as indicated by the single peak of high CFSE fluorescence. By contrast, naïve T cells stimulated with OVA-loaded LPS-matured DC exhibited a clear cellular division peak profile, thus indicating cell proliferation (Figure 3.6A). Furthermore, similar proliferation peaks were observed in the anergic and primed T cells upon re-stimulation, with both anergic and primed populations appearing to have undergone several rounds of division. As predicted, relative to the primed population, anergic $CD4^+KJ^+$ T cells had higher percentage of non-dividing cells even at the end of the maintenance phase: 17.8% versus 11.7% on day 1 and 18.5% versus 9.68% on day 2. By contrast, the primed T cells obtained higher percentage of cells that had divided: 27.2% versus 14.7% on day 1 and 19.2% versus 8.13% on day 2 (Figure 3.6B & C). Although individual peaks representing higher numbers of divisions, due to resulting low levels of CFSE fluorescence, at day 3 and day 4 cannot be seen, the progress of cellular proliferation from these anergic and primed $CD4^+KJ^+$ T cells still can be assessed by comparing the percentage of cells from the fifth peak (near the Y-axis). This again indicated that the primed population proliferated more than anergic group: 68.4% versus 50.6%

on day 3 and 66.7% versus 53.2% on day 4. Briefly summarized, the primed T cells proliferated faster than anergic T cells over the first two days with, as seen in Figure 3.6 D, primed T cells showing less non-dividing cells and more proliferating cells on day 1. The difference between these two populations was most obvious on day 2, after which the proliferation of anergic T cells started to catch up with the primed T cells (Figure 3.6E), possibly partly due to exhaustion of the media for primed cells and/or loss of sensitivity of CFSE-tracked division as the levels of CFSE fall below detection.

3.1.3 Assessment of cytokine secretion during the maintenance phase of anergy and priming in CD4$^+$ T cells in vitro

As defective IL-2 production has been reported to be the signature defect associated with anergy, the secretion of IL-2 by naïve, primed and anergic cells in response to antigen-pulsed DCs was examined at the following timepoints: 0-24, 24-48 and 48-96 hours during the maintenance phase. Perhaps as predicted, during the first 24 hours, primed T cells produced the highest levels of IL-2, relative to anergic and naïve T cells, after challenge with antigen (Figure 3.7A), data consistent with the idea that the primed cells make faster, stronger responses than naïve cells. However, the secretion of IL-2 from the primed cell population dropped between 24-48 h and particularly dramatically, 48-96 h following stimulation with antigen, although it cannot be ruled out that this loss of production reflects that the produced IL-2 is being consumed by the primed cells. Nevertheless, these data indicate that this rapid but transient production of primed T cells fuels the faster (secondary) response observed in primed cells. Consistent with this anergic T cells produce the lowest levels of IL-2 overall with similar levels of IL-2 production at 24 and 48 hours but as with the primed cells, the quantity of IL-2 also diminished by 96 hours perhaps also indicating exhaustion and/or death of these cells. By contrast,

the level of IL-2 produced from naïve T cells increased gradually with time in keeping with the slower kinetics of primary responses. Interestingly, the levels of IFNγ production by primed T cells continued to rise strongly over the first 48 h and was maintained for the further period to 96 h indicating that the loss of IL-2 production by these cells did not reflect cell death. Moreover, such IFNγ production was much stronger than that of naïve of anergic cells, neither of which was detectable before the 24-48 h time point and reflected the slower kinetics of Th1 differentiation from naïve cells and the desensitized responses of anergic (Th1) cells (Figure 3.7B).

Collectively, therefore in terms of cell cycle progression (DNA synthesis) proliferation (CFSE), IL-2 production and Th1 (IFNγ) differentiation and maintenance, these data indicate that primed cells make faster and stronger responses to Ag presented by APC expressing costimulatory molecules, for instance those matured under inflammatory or “danger” conditions. Similarly, they indicate that such responses are desensitized when T cells are anergised by exposure to Ag alone in the primary response. Indeed, these data probably underestimate the full nature of differential responses of the primed and anergic populations as not all the cells in the former will be primed nor will all be anergised in the latter group. Thus, this system appears to constitute a good model for studying both the induction and maintenance phases of priming and tolerance *in vitro*.

3.1.4 Assessment of expression of inhibitory co-stimulatory molecules on T cells during maintenance phase of priming and tolerance

As stated above, the analysis probably underestimates the differences between anergic and primed cells as not all cells in either populations will be completely

functionally polarised. Indeed much effort has been spent on trying to identify markers of anergy by our group and others [25, 29, 117] but to date, a robust marker has proved elusive. Thus it was decided to further characterise the surface expression of “anergic” and “primed” T cells (induced with anti-CD3 alone or anti-CD3+anti-CD28) following their challenge with immature or LPS-matured DC loaded with OVA in terms of costimularory molecules and Treg markers such as CD25 as Tregs have been reported to exhibit some of the attributes of anergic cells [50, 233]. Moreover, it has been reported that after feeding with tolerogenic doses of antigen, antigen-specific T cells in the periphery showed upregulation of CD25 expression [49, 50]. Furthermore, CD25 is thought to expressed constitutively on a subset of antigen-specific cells, $CD4^+$ $CD25^+$, that are capable of suppressing the cytokine production and proliferation of $CD4^+$ and $CD8^+$ T cells *in vitro* [233, 234]. Therefore, the expression of CD25 was examined on anergic and primed T cells which were treated with antigen-loaded, LPS-matured DC and immature DC. When anergic and primed T cells were co-cultured with OVA-loaded, LPS-matured DC, the expression of CD25 on both these two populations increased over the first 48 hours before returning to below the level on day 1 (Figure 3.8A). A similar pattern was also seen following treatment with immature DC but the expression of CD25 was lower than that observed when the cells co-cultured with matured DC (Figure 3.8D). Interestingly, however, although the patterns were similar the anergic cells (in both cases) showed higher levels of CD25 relative to primed cells perhaps consistent with them displaying a more Treg/anergic phenotype. However, these cells are not the same as Tregs as analysis of Foxp3 expression showed that those cells exhibited very low Foxp3 expression (Figure 3.8G).

As described earlier, when CD80/86 on the APC interacts with CD28 on T cells, this provides costimulatory signals essential for full T cell activation. CD28 is a

member of the CD28 family of co-stimulators which exhibits high homology amongst members including those with inhibitory effects. Here, we phenotyped the expression of CTLA-4 (also known as CD152) and programmed death-1 (PD-1; also known as CD279) on anergic and primed T cells co-cultured with OVA-loaded LPS-matured DC to see if expression of either correlated with an “anergic” functional response. The expression of CTLA-4 on anergic and primed T cells that co-cultured with matured DC+OVA had very similar level from day 1 to day 4 (Figure 3.8B) and comparing the MFI of CTLA-4 on anergic and primed populations which treated with LPS-matured DC and immature DC, revealed no difference in the value of MFI of these populations (Figure 3.8B&E). Furthermore, the levels of PD-1 which expressed by anergic and primed T cells were very similar and decreased by time (Figure 3.8C), irrespective of whether in anergic and primed populations were co-cultured with immature DC or LPS-matrued DC (Figure 3.8F). Collectively, these data indicated that anergic and primed cells could not be discriminated of any of these markers as although CD25 was elevated in anergic cells, it is not a reliable marker due to its upregulation during activation in the primary response and also in Tregs.

3.1.5 Assessment of cellular proliferation of during maintenance phase of anergy and priming in CD4$^+$ T cells in vitro.

The CFSE proliferation data resulting after stimulation of naïve and primed populations with OVA-loaded LPS-matured DC presented so far relate to the cumulative number of divisions by T cells during both the induction and maintenance phases of priming and tolerance and do not give an indication of the proliferative responses occurring in the two individual phases. Although analysis of an aliquot of the anergic and primed cells prior to challenge with antigen would allow analysis of the induction phase, interpretation of maintenance phase

responses from the cumulative analysis would be difficult due to the technical inability to dissect whether the extra proliferation reflected that of the high or low dividing populations. Moreover, our data suggest that the loss of CFSE detection resulting from multiple rounds of division might cause artefacts that lead to an underestimation of the levels of proliferation in primed cells.

Thus to address analysing the proliferative responses of the maintenance phase, freshly isolated naïve $CD4^+$ T cells and $CD4^+$ T cells from the anergic and primed populations were labelled with CFSE after the induction phase and prior to challenge with OVA-loaded LPS-matured DC. As expected the naïve $CD4^+$ T cells showed clear evidence of multiple rounds of division over 4 days following challenge with Ag (Figure 3.9A). However, the primed and anergic cells did not appear to reproducibly load/retain dye in the same manner as naïve cells as evidenced by a broad peak profile and trailing edge (Figure 3.9B&C) that made interpretation of cell status difficult: in particular, rather than showing individual peaks indicating different numbers of cell division, this broad peak simply widened and shifted to a lower fluorescence intensity making meaningful analysis impossible. The reasons for this are not clear but may reflect that following induction of priming and tolerance the T cell membrane may differ from that of naïve cells and hence not allow equivalent loading or indeed, may permit leakage of the dye.

It was therefore planned to address the possible impact of using a different fluorescent dye, in particular one which could be optimised for used in later experiments utilising bicistronic GFP adenoviral vector constructs for which CFSE which is detected in the GFP channel (FL1) would not be suitable. We therefore tested another cell tracker dye, CMTPX (detected in FL2) which like CFSE passes through cell membranes and is converted to cell-impermeant reaction products and

subsequently passed to at half the intensity of cellular dye to daughter cells. In order to optimise the system, LN cells were stained with CMTPX in different concentrations, the titration initially involving a series concentration of CMTPX from 5 μM to 1 μM and subsequently stained for CD4 expression prior to Flow Cytometric analysis (Figure 3.10B). Again, lymphocytes were identified on the basis of their known size (forward scatter) and granularity (side scatter) and only the viable cells in the lymphocyte population was subsequently analysed. These live lymphocytes were then examined for expression of CD4 (Figure 3.10A) before assessing the optimal CMTPX staining concentration. Unfortunately, as shown in Figure 3.10B, these concentrations (5, 4, 3, 2 and 1 μM) are too high as the intensity of CMTPX is too strong causing detection of the dye to leak through FL-3 parameter into FL-4 channel as indicated by the "loss" of the $CD4^+$ (APC-FL4) population, thereby inducing autofluorescent artefacts. So following the same protocol, the LNs were stained with lower concentrations of CMTPX (0.2 to 0.8 μM) and this demonstrated that the LNs stained with CMTPX (≤ 0.8 μM) and biotinylated anti-CD4/streptavidin-APC obtained the best resolution, with two clear separate populations evident (Figure 3.10C). In order to have efficient staining and better cell populations, a CMTPX concentration of 0.75 μM was selected as the optimal concentration since staining in 0.7 μM had more clear separation of the two populations relative to 0.8 μM.

After optimizing the staining concentration, CMTPX was used to observe proliferation of LN cells transduced with a bicistronic Adenoviral construct expressing GFP to determine whether it would be possible to analyse proliferation of the GFP+ and GFP- cells using this dye. Thus, as we were particularly interested in the counter-regulatory signalling of Rap and Erk, LN cells were transduced with a construct expressing a dominant negative (DN) version of Rap1 upstream of GFP

(Ad Rap1 S17N) at various MOI (0, 3,10,30 and 50) for 30 minutes prior to staining with CMTPX (0.75 μM). Anergy or priming of the transduced and stained LN cells was induced by culturing with anti-CD3 in absence or presence of anti-CD28 for 4 days during which period, the LN cells were harvested everyday for monitoring cellular proliferation. As mentioned above, all LN cells were subsequently stained for CD4 expression prior to Flow Cytometric analysis the Th cells.

As expected, mock infection (MOI 0) resulted in no GFP-expressing $CD4^+$ cells, and so only analysis of the cellular proliferation of the GFP- population was possible for this group. This showed that, as suggested above, although primed cells proliferate more, during the induction phase both the anergic and primed cells proliferated similarly and in this particular experiment this appeared almost to an identical degree with only a more pronounced shift to lower fluorescence and loss of a higher fluorescence trailing edge being evident at day 3 by the primed population (Figure 3.10 F&G). By contrast, at each MOI (3, 10, 30 and 50) the cellular proliferation of GFP- and GFP+ of the adenovirally-infected LN cell populations could be compared and this analysis appeared to indicate that, for both primed and anergic cells, the LN transduced with Ad Rap1 S17N (GFP+) proliferated more than LN without construct (GFP-) irrespective of whether they were treated with anti-CD3 or anti-CD3+anti-CD28 on both day 1 but particularly on day 3. Moreover, although the enhancement was similar throughout there did appear to be a positive association with MOI. Reassuringly, the GFP- populations exhibited responses similar to the MOI 0 control (Figure 3.10 E-H).

However, to try to further investigate the potential link of increasing MOI inducing stronger enhancement of proliferation and hence examine the effect of Rap signal strength on limitation of CD4 T cell proliferation, the analysis at day 1 was also performed at a higher voltage setting (860 vs 620) to amplify the fluorescence and

hence try to circumvent quantitation artefacts arising out of loss of CMTPX signal as exemplified the pile-up of cells of the y-axis which prevents determination of differential numbers of cell division. However, this approach had an unexpected result in that the completely opposite result was obtained, for example the DN Rap construct appeared to inhibit proliferation although this appeared artefactual as the fluorescence was higher than that being observed in the cells at day 0 suggesting that CMTPX was again induce autofluorescence artefacts at high signal strength, this time occurring in conjunction with GFP of the construct, and this idea appeared to be validated by an additional experiment on day where the responses an intermediate voltage (720) was compared with the standard 620 level and this showed no difference between the GFP ± populations and indicating a titration effect on the autofluorescence. Thus, although the data, obtained at the lowest voltage where there appeared to be negligible autofluorescence, suggested that blocking Rap activity promoted proliferation of both anergic and primed cells during their induction, these conclusions were not considered convincing and hence it was proposed to optimise a more robust system using a different dye.

3.1.6 Comparing the assessment of cellular proliferation by CFSE and eFluor 670 during maintenance phase.

Thus, although an optimal staining concentration of CMTPX was achieved by titration, CMTPX was difficult to apply in the following experiments because of its strong intensity and hence, difficult compensation when used in conjunction with multiple fluorochromes. Thus, another “red” cell tracking dye, eFluor 670 which had been developed at this time to circumvent these problems was trialled in order to assess its exploitability in future adenovirus experiments. To illustrate any impact of using this different dye, which possibly influences the proliferation result, the first assessment of eFluor 670 was to compare proliferation responses of anergic and

primed cells with those previously shown above, obtained with CFSE. The first assessment was to examine the proliferation of $CD4^+$ T cells at the end of the maintenance phase (day 8) which were stained with eFluor 670 before induction of anergy or priming (day 0). Analysis of staining at day 4 immediately prior to challenge with OVA-loaded LPS-matured DCs revealed that, as previously shown with CFSE (Figure 3.3B), $CD4^+$ T cells undergoing induction of priming proliferated more than those undergoing anergy (Figure 3.11A). Moreover, although the difference were slight, this was also clear day 1 post challenge with antigen although the ability to track this was lost on following days due to loss of detectable signal (Figure 3.11A). Importantly however, optimisation experiments revealed that unlike the situation with CFSE, it was possible to efficiently load cells with eFluor 670 after the induction of anergy and priming and hence track subsequent proliferation during the challenge/maintenance phase: this is illustrated in panel B which shows that although similar in this experiment, there is increased proliferation of the primed population.

Thus these data indicated that eFluor 670 was competent for investigation of proliferation during both the induction and maintenance phases of priming and tolerance.

3.1.7 Assessment of cellular proliferation $CD4^+$ T cells in vitro during maintenance phase of priming and tolerance.

Since it was possible to efficiently stain and track proliferation of $CD4^+$ T cells after induction phase using eFluor 670, it was now possible to compare the cellular proliferations of the different groups (naïve, anergic and primed). Analysis at day post-stimulation with OVA-loaded LPS-mature DC showed that, as expected, the primed cells have started first and although the naïve cells start to catch up by day 2 (Figure 3.12A) the responses of anergic cells remain lower than that of the

primed cells, especially in terms of absolute numbers of T cells given that they were seeded at equivalent levels. These results are consistent with the belief that secondary responses are faster and stronger than primary responses and that anergic responses reflect desensitisation (Figure 3.12 B&C). This was reinforced when, to further understand what the proliferation result reflected in terms of T cell clonal expansion, the percentage of live cells was examined in both populations from day 1 to day 4 (Figure 3.12D&E). This showed that the primed T cells exhibited a higher percentage of live cells than the anergic T cells population after challenge with OVA. Thus, clonal expansion and survival of these two populations correlated with the cellular proliferation data since the primed T cells showed more proliferation than anergic cells throughout days 1-4 (Figure 3.12F).

3.1.8 Assessment of pERK and active Rap expression in Ag-specific T cells during maintenance phase of priming and tolerance.

We had previously shown that primed cells showed enhanced levels of active Erk (dually phosphorylated pErk) and reduced expression of Rap1 relative to anergic cells both in vitro and in vivo [14], consistent with the idea that Rap1 antagonised TCR-mediated Ras-dependent signaling by sequestering Raf-1 and disrupting coupling to Erk. However, at that time due to a lack of in situ reagents, we were not able to determine if Rap activity was required for this, but with the advent of antibodies that recognize the active GTP-bound form of Rap, this was now possible.

Consistent with our previous data, there is a higher percentage of live cells, particularly in the blasting gate, in the primed, relative to anergic, group following challenge of $CD4^+$ T cells with OVA-loaded LPS-matured DC (Figure 3.13B). Also, consistent with our previous findings, analysis of ERK activation by $CD4^+$ T cells in

the blasting gate, as determined by the ratio of pERK/ERK expression, indicated that primed T cells expressed higher level of ERK activation than anergic T cells and anergic cells expressed lower levels than those of naïve cells undergoing a primary response, on day 1 following challenge with Ag in this maintenance phase. Perhaps surprisingly, given the increase in Rap1 expression in anergic relative to primed cells [14], there was no real apparent change in Rap1 activity detected amongst the 3 blasting groups on this day, although the level in the primed group was very slightly decreased (Figure 3.13C). A similar pattern was observed with resting cells, although here Rap1 activation was suppressed below those of naïve cells in both primed and anergic groups relative to naïve cells (Figure 3.13D). In order to address the potential for counter-regulation of these signals analysis of the ratio of ERK: Rap1 activation showed that this was highest in primed cells at day 1 (Figure 3.13G), consistent with our previous proposal that Erk activation was associated with priming whilst that of Rap promoted desensitization and anergy [14, 235]. Moreover, the elevated levels of Erk and Rap1 in naïve, relative to anergic, cells is consistent with the proposal by Cantrell's group [236] that Rap was important in primary responses.

Here we extended our analysis to day 3 and rather surprisingly we found Erk activation to be higher in anergic, and to a lesser extent, primed proliferating cells relative to those undergoing a primary response (Figure 3.13E), whilst Rap1 activation was equivalent in all populations. Moreover, analysis of the resting cells indicated that the anergic cells exhibited the highest the Erk activation whilst, and consistent with counter-regulatory signaling, primed cells displayed the lowest levels of Erk and the highest levels of Rap1 activation, kinetics that were supported by analysis of the ratios of Erk:Rap activation (Figure 3.13F&H). These data were therefore consistent with our previous findings that the anergic population tended to

“catch-up” with the primed populations in terms of proliferation at days 3-4 and may indicate that the maintenance of tolerance may be “leaky” in this in vitro system. Alternatively, it may indicate that not all the “anergic” cells are anergic and hence are responding to Ag normally whilst not all of the “primed” cells are primed. Moreover, the data also suggest that in primed cells Erk:Rap counter-regulation may occur to terminate responses homeostatically. Alternatively, this switch in signaling may reflect cessation of proliferation of primed cells due to media exhaustion (Figure 3.13C&E).

Conclusion

Previous published work from this laboratory indicated that counter-regulatory ERK and Rap1 signalling occurred during the maintenance phase of Ag-specific priming and tolerance. This study further investigated the functional role of these potentially antagonistic signals in the functional outcomes of anergy and priming in throughout the maintenance phase.

Firstly however, it was important to optimize the *in vitro* assay system: thus to optimize Ag-presentation, LPS-matured DC were loaded with 0.1 and 1 μg/ml of OVA peptide$_{323-339}$ and priming of naïve T cells assessed, by using, proliferation-tracking fluorescent dye CFSE, were for the optimal concentration of antigen for proliferative responses. According to the cellular proliferation, although cells treated with either of these two concentrations of OVA peptide both exhibited clear profiles of proliferation and cell division from day 3, the cells treated with 1μg/ml of OVA proliferated more than those with 0.1 μg/ml. Thus 1 μg/ml of OVA was used in the all the experiments investigating maintenance phase. Meanwhile, the analysis of the proliferative responses of naïve cells to OVA-loaded LPS-matured DC versus immature DC showed that whilst the OVA-loaded immature DC induced some proliferation, the mature DC induced higher levels of proliferation, both in proportions of cells undergoing more rounds of division but also in terms of absolute numbers of cells generated. However, in theory the immature DC should not induce proliferation, thus the “maturation” status of DC as in both immature and LPS-matured DC was further investigated. Analysis of CD11c-expressing DC were therefore examined for expression of CD80 and CD86, and as expected, LPS-matured DC expressed higher levels of CD80 expression than immature DC, although this increase was not to a great extent. In contrast to

CD80, the LPS-matured DC expressed substantially higher levels of CD86 than the immature DC.

The first functional assessment of the primed and anergic populations was of their proliferative response and this was first analysed in terms of DNA synthesis by thymidine uptake. As expected, the primed T cells (induction of $CD4^+$ T cells with anti-CD3+anti-CD28) exhibited a higher response than either naïve or anergic T cells (induction of $CD4^+$ T cells with anti-CD3), when challenged with antigen-pulsed DC, whilst the anergic T cell displayed an even lower DNA synthesis than the naïve T cells undergoing a primary response. To further investigate the functional outcomes of anergic and primed T cells, the IL-2 and IFNγ responses were also assessed at 24, 48 and 96 hours during the maintenance phase. During the first 24 hours, primed T cells produced the highest levels of IL-2, relative to anergic and naïve T cells, after challenge with antigen. However, the secretion of IL-2 from the primed cell population dropped between 24-48 hour and particularly dramatically, 48-96 hour following stimulation with antigen. Consistent with this, anergic T cells produce the lowest levels of IL-2 at 24 hours and similar levels of IL-2 production at 48 hours but as with the primed cells, the quantity of IL-2 also diminished by 96 hours. On the other hand, the levels of IFNγ production by primed T cells continued to rise strongly over the first 48 hours and was maintained for the further period to 96 hour indicating that the loss of IL-2 production by these cells did not reflect cell death. Additionally, the levels of IFNγ production by anegic T cells increased gradually from 24 to 96 hour. However, it was not as rapid and much less than primed T cells. These data illustrated that both anergic and primed T cells utilize the IL-2 secreted in the early stage of induction phase to sustain the consequent differentiation responses.

Compared to the snapshot information provided by DNA synthesis assessed by [^{3}H]

thymidine of the cells transiting S phase of the cell cycle, cell tracking dyes, CFSE and eFluoro provide more details of primed and anergic cell proliferative responses and this approach indicated that both anergic and primed cells had undergone several rounds of division. Although, the primed T cells proliferated faster than anergic T cells over the first two days, after that the proliferation of anergic T cells was able to catch up with primed T cells, a point, that had not been revealed from our previous studies. Notably, this additional information revealed that whilst the cells undergoing induction of anergy proliferated, this was to a lower level that observed for the primed group both in terms of proportions and absolute numbers. With this lead, further investigation was conducted and assessment of expression of inhibitory co-stimulatory molecules illustrated that both anergic and primed exhibited upregulation of CD25 following treatment with immature and mature DC, although the cell culture with mature DC resulted in expression of higher levels of CD25 on both populations. Moreover, anergic cells showed higher levels of CD25 relative to primed cells but further investigation also indicated those cells are not Treg ($CD4^+CD25^+Foxp3^+$) since they expressed very low Foxp3. Neither anergic nor primed cells exhibited any difference in expression of CTLA-4 and PD-1 following co-culture with immature and mature DC. Furthermore, the expression of CTLA-4 and PD-1 on anergic and primed T cells had very similar level during maintenance phase as well.

Consistent with the observed proliferative responses, the primed T cells expressed higher levels of ERK activation than anergic T cells and indeed, anergic T cells expressed lower levels than those of naïve cells undergoing a primary response on day 1 following challenge with Ag in this maintenance phase. However, there was no real change in Rap1 activity amongst naïve, anergic and primed cells on day 1. The ratio of ERK: Rap1 activation, which addresses the potential for

counter-regulation of these signals, exhibited that the primed cells had the highest ratio at day 1. However, when these studies were extended to day 3, ERK activation was at higher levels in anergic T cells than primed T cells whilst Rap1 activation was equivalent in all populations. The ratio of ERK: Rap1 activation showed that the primed cells had lower ratio than anergic cells at day 3 and interestingly, these data were consistent with previous finding that the anergic population tended to “catch-up” with the primed populations in terms of proliferation at day 3-4.

To sum up, the primed T cells exhibited a higher percentage of live cells than the anergic T cells population after challenge with OVA. Thus, clonal expansion and survival of these two populations correlated with the cellular proliferation data since the primed T cells showed more proliferation than anergic cells throughout day 1 to day 4 during maintenance phase. Additionally, ERK activation correlated with the differential cellular proliferation observed between anergic and primed T cells whereas Rap activation presented no difference between these two populations during maintenance phase.

Figure 3.1 Induction of anergy and priming in Ag-specific TCR Tg T cells.

Lymph node cells were generated from OVA TCR or hCAR x OVA TCR mice and prepared as single cells suspension. $CD4^+$ T cells were isolated from suspension solution by $CD4^+$ T cell isolation MACS and stimulated with anti-CD3 in presence or absence of anti-CD 28 for 2 days as anergic or primed T cells, respectively. After 2 days induction, the anergic and primed T cells were washed and rested for the other 2 days before co-cultured day. On the co-culture day, the naïve $CD4^+$ T cells were freshly extracted from OVA TCR mice and isolated by $CD4^+$ T cell isolation MACS. The naïve, anergic and primed T cells were co-cultured with OVA loaded LPS matured dendritic cells for 4 days as maintenance phase.

Figure 3.2 Assessment of the cellular proliferation in naïve TCR Tg T cells with different concentration (0.1&1µg/ml) of antigen OVA.

The naïve $CD4^+$ T cells were generated from lymph node cells of OVA TCR mice and freshly isolated by $CD4^+$ T cell isolation MACS. The $CD4^+$ T cells were stained with CFSE in 5 µM. The CFSE-stained $CD4^+$ T cells ($4x10^5$) were co-cultured with LPS-matured, OVA (0.1 and 1µg/ml) loaded DCs ($4x10^5$) in 2 ml of media for 4 days. The cellular proliferation was analysed by FACS.

Figure 3.3 Assessment of cellular proliferation of anergic and primed T cells cocultured with immature and mature DC during maintenance phase.

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hcAR x OVA TCR mice and isolated by MACS $CD4^+$ T cell isolation kit. The $CD4^+$ T cells were stained with CFSE in 5 μM before induction of anergy and priming. As mentioned in Figure 3.1, briefly, the CFSE stained- $CD4^+$ T cells were stimulated with anti-CD3 in presence or absence of anti-CD28 for induction of anergy and priming, respectively, for 2 days and rested for the other 2 days. The DC were cultured in conditional media for 7 days before pulsed with LPS or culture with complete media alone for 24 hours as matured and immature DC, respectively. The naïve $CD4^+$ T cells (freshly prepared and stained with CFSE on co-culture day), CFSE stained-anergic and CFSE stained-primed $CD4^+$ T cells ($4x10^5$/ml) were co-cultured with immature or mature, OVA-loaded DC ($4x10^5$/ml) for 4 days. The cellular proliferation were analysed by FACS (A) (B). Data are representative of at least three independent experiments.

Figure 3.4 Analysis of DNA synthesis in anergy and primed T cells by Thymidine [^{3}H] during maintenance phase.

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hCAR x OVA T cR mice and isolated by MACS $CD4^+$ T cell isolation kit. As mentioned in Figure 3.1, briefly, the naïve $CD4^+$ T cells (freshly prepared on co-culture day), anergic and primed $CD4^+$ T cells ($2x10^5$/200µl) were stimulated with anti-CD3 ± anti-CD28 for 2 day and rested for 2 days before co-cultured with LSP matured, antigen loaded DC ($2x10^5$/200µl) for 48 hours. Sequentially the thymidine were added into naïve, anergic and primed T cells culture for extra 16 hours culture. (A) The APC and antigen were essential for T cell fully activation (B) Primed T cells exhibited higher DNA synthesis than anergic T cells. Data are representative of at least three independent experiments.

Figure 3.5 Assessment of cellular proliferation of anegic and primed T cells cultured with matured DC in ration 10:1 and 1:1 in maintenance phase.

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hCAR x OVA TCR mice and isolated by MACS $CD4^+$ T cell isolation kit. As mentioned in Figure 3.1, briefly, $CD4^+$ T cells were stained with CFSE in 5μM before stimulated with anti-CD3 in presence or absence of anti-CD28 for induction of anergy and priming, respectively, for 2 days and rested for the other 2 days. The CFSE-stained anergic and CFSE-stained primed T cells ($4x10^5$/2ml) were co-cultured with LPS-matured, OVA loaded DC (A) ($4x10^4$/2ml) or (B) ($4x10^5$/2ml) for 4 days.

Figure 3.6 Assessment of cellular proliferation by CFSE in maintenance phase (CFSE staining from induction phase)

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hCAR x OVA TCR mice and isolated by MACS $CD4^+$ T cell isolation kit. As mentioned in Figure 3.1, briefly, $CD4^+$ T cells were stained with CFSE in 5μM before stimulated with anti-CD3 in presence or absence of anti-CD28 for induction of anergy and priming, respectively, for 2 days and rested for the other 2 days. The naïve $CD4^+$ T cells were freshly prepared on co-culture day and stained with CFSE in 5μM. CFSE-stained naïve, CFSE-stained anergic and CFSE-stained primed T cells ($4x10^5$/2ml) were co-cultured with LPS-matured, OVA loaded DC ($4x10^5$/2ml) for 4 days. The cells were harvested from day 1 to day 4 and the cellular proliferation of (A) naïve, (B) anergic and (C) primed T cells were analysed by FACS. (D-G) The percentage of cells in each division were calculated from day 1 to day 4.

Figure 3.7 Analysis of cytokine secretion by anergic and primed T cells during maintenance phase

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hCAR x OVA T cR mice and isolated by MACS $CD4^+$ T cell isolation kit. As mentioned in Figure 3.1, briefly, the naïve $CD4^+$ T cells (freshly prepared on co-culture day) anergic and primed $CD4^+$ T cells were stimulated with anti-CD3 in presence or absence of anti-CD28, respectively, for 2 day and rested for 2 days before co-cultured with LSP matured, antigen loaded DC. The supernatant were harvested at 24, 48 and 96 hours after naïve, anergic and primed T cells re-challenged with OVA $peptide_{323-339}$. The levels of IL-2 (A)and IFN-γ (B) were measured by ELISA.

Figure 3.8 Assessment of inhibitory molecules expression in anergic and primed T cells cocultured with immature and mature DC during maintenance phase.

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hcAR x OVA TCR mice and isolated by MACS $CD4^+$ T cell isolation kit. The $CD4^+$ T cells were stained with CFSE in 5 μM before induction of anergy and priming. As mentioned in Figure 3.1, briefly, the CFSE stained-$CD4^+$ T cells were stimulated with anti-CD3 in presence or absence of anti-CD28 for induction of anergy and priming, respectively, for 2 days and rested for the other 2 days. The DC were cultured in conditional media for 7 days before pulsed with LPS or culture with complete media alone for 24 hours as matured and immature DC, respectively. The naïve $CD4^+$ T cells (freshly prepared and stained with CFSE on co-culture day), CFSE stained-anergic and CFSE stained-primed $CD4^+$ T cells ($4x10^5$/ml) were co-cultured with immature or mature, OVA-loaded DC ($4x10^5$/ml) for 4 days. The cells were harvested everyday and stained with KJ-bio-PerCP, CD25 APC, CTLA-4 APC, PD-1 APC and Foxp3 PE. The expression of these inhibitory molecules were analysed by FACS. Data are representative of two independent experiments.

Figure 3.9 Assessment of cellular proliferation by CFSE in maintenance phase (CFSE staining from maintenance phase)

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hcAR x OVA TCR mice and isolated by MACS $CD4^+$ T cell isolation kit. As mentioned in Figure 3.1, briefly, $CD4^+$ T cells were stimulated with anti-CD3 in presence or absence of anti-CD28 for induction of anergy and priming, respectively, for 2 days and rested for the other 2 days. The naïve $CD4^+$ T cells (freshly prepared on co-culture day), anergic and primed T cells ($4x10^5$/2ml) were stained with CFSE in 5μM before co-cultured with LPS-matured, OVA loaded DC ($4x10^5$/2ml) for 4 days. The cells were harvested on day 4 and the cellular proliferation of (A) naïve, (B) anergic and (C) primed T cells were analysed by FACS. Data are representative of at least three independent experiments.

Figure 3.10 Assessment of cellular proliferation by regulation of Rap1 S17N during induction of anergy and priming by CMPTX

In order to observe the how the Rap1 S17N regulate the cellular proliferation of induction of anergy and priming during induction phase. The LNs extracted from hCAR mice were transduced with Ad Rap1 S17N which carry GFP (FL-1). For observation of the cellular proliferation in virus transduced LNs, the new cell track dye, CMPTX (FL-2) was used to stain to transduced LNs. Before the CMPTX was applied to the following Adenovirus experiments, the CMPTX was titrated for optimal concentration. The LNs were stained with (A) CD4-bio-PerCP antibody for population gating. (B) The titration of CMPTX was testing from high concentration 5μM, 4μM, 3μM, 2μM and 1μM, however, the fluorescence was too strong to distinguish the CD4 population. Thus the LNs were stained with (C) lower concentration series, 0.8μM, 0.7μM, 0.6μM, 0.5μM and 0.4μM.

Regarding to the titration of CMPTX, 0.75μM was chosen as the optimal staining concentration. The LN extracted from hCAR mice were transduced with Ad Rap1 S17N in MOI 0, 3, 10, 30 and 50 before staining with CMPTX in 0.75μM. The virus-transduced, CMPTX-stained LNs were stimulated with anti-CD3 in presence or absence of anti-CD28 for 2 days and rested for the other 2 days. The cellular proliferation were analysed by FACS under voltage 620 on day1 (D) and day 3 (E), voltage 860 on day1 (F) and 720 on day 3 (G).

Figure 3.11 Assessment of cellular proliferation of anergic and primed T cells which were stained with track dye, eFluor 670 from induction phase and maintenance phase.

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hCAR x OVA TCR mice and isolated by MACS $CD4^+$ T cell isolation kit. The half of $CD4^+$ T cells were stained with eFluor 670 in 5μ before stimulating with anti-CD3 in presence or absence of anti-CD28 for induction of anergy and priming, respectively, for 2 days and rested for the other 2 days. The eFluor 670 stained-anergic and eFluor 670 stained-primed T cells were co-cultured with LPS-matured, OVA loaded DC for 4 days. (A) The cellular proliferation of anergic and primed T cells which were stained with eFluor 670 from induction phase were analysed by FACS. (in Figure 3.10A, Day 4 is co-culture day since the time was counted from the cells were stained with track dye)

The other half of $CD4^+$ T cells were treated with anti-CD3 or anti-CD3+anti-CD28 for induction of anergy and priming for 2 days and resting for the other 2 days. The anergic and primed T cells were harvested on co-culture day and stained with eFluor 670 in 5μM before co-cultured with LPS-matured, OVA loaded DC for 4 days. (B) The cellular proliferation of anergic and primed T cells that were stained with eFluor 670 from maintenance phase (on co-culture day).

Figure 3.12 Assessment of cellular proliferation and percentage of clonal expansion in anergic and primed T cells during maintenance phase by eFluor 670.

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hCAR x OVA TCR mice and isolated by MACS $CD4^+$ T cell isolation kit. The $CD4^+$ T cells were stimulated with anti-CD3 in presence or absence of anti-CD28 for induction of anergy and priming, respectively, for 2 days and rested for the other 2 days. The naïve $CD4^+$ T cells were isolated and stained with eFluor 670 on co-culture day. The naïve, anergic and primed T cells ($1x10^6$) were co-cultured with LPS-matured, OVA loaded DC ($1x10^6$) for 4 days. The cellular proliferation of (A) naïve, (B) anergic and (C) primed T cells were analysed by FACS. The percentage of clonal expansion of (D) anergic and (E) primed T cell within large gate were plot in (F). Data are representative of two independent experiments.

Figure 3.13 Analysis of activation of ERK1/2 expression during the maintenance phase by FACS.

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hCAR x OVA TCR mice and isolated by MACS $CD4^+$ T cell isolation kit. The $CD4^+$ T cells were stimulated with anti-CD3 in presence or absence of anti-CD28 for induction of anergy and priming, respectively, for 2 days and rested for the other 2 days. The naïve $CD4^+$ T cells were isolated by MACS $CD4^+$ T cell isolation kit on co-culture day. Naïve, nergic and primed Ag-specific T cells ($1x10^6$) were co-cultured with LPS matured OVA loaded DC ($1x10^6$) for 4 days. The cells were harvested from each group on day 1 and day 3 during maintenance phase. The cells were sequentially stained with CD4, KJ1.26 and ERK, pERK, Rap1, active Rap1 antibodies for analysis by FACS. (A) The expression profile of ERK activation and Rap1 activation assessed in both blasting and resting gates within large gate. (B) The percentage of clonal expansion of anergic and primed T cells within blasting and resting gates on day 1 and day 3. (C) The activation of ERK and Rap1 in naïve, anergic and primed T cells within blasting gate on day 1. (D) The activation of ERK and Rap1 in naïve, anergic and primed T cells within resting gate on day 1. (E) The activation of ERK and Rap1 in naïve, anergic and primed T cells within blasting gate on day 3. (F) The activation of ERK and Rap1 in naïve, anergic and primed T cells within resting gate on day 3.

Chapter 4
Results

4 Results

Introduction

As discovered in Chapter 3, both primed and anergic cells exhibited clonal expansion before and during the maintenance phase: thus, the widely held view that only cells receiving costimulation proliferate is a misconception that more likely reflects the situation during the maintenance phase. Additionally, an inverse relationship between Rap1 activation and phospho-ERK has been shown during the maintenance phase of tolerance and priming of antigen-specific $CD4^+$ T cells in both in vitro and in vivo. Thus, it was decided to investigate the functional outcomes and regulation of ERK/Rap signalling during induction phase, to determine whether these signals also play counter regulatory roles in the induction of priming and anergy.

In addition to the assays of DNA synthesis, cellular proliferation, cytokine production and Erk and Rap1 activation used in the previous maintenance phase experiments, to investigate the role of Rap1 activity during induction of anergy and priming, viral transduction offers a method of gene delivery into primary resting cells. Here we used a bicistronic GFP-system which by allowing instant transgenesis does not induce adaptation artefacts and also allows direct analysis of the modification (GFP+ cells) of the gene-of-interest relative to control cells (GFP-) within the same population of cells.

In addition to examining ERK and Rap1 activation by FACS, those signalling molecules were also investigated by Western Blot analysis. Moreover, many studies have reported that regulation of metabolism in T cells, which is a downstream target of Erk signalling, is significant in the control of T cell activation

and differentiation, and so it was also planned to investigate the role of the mTOR signalling pathways in the induction of anergy and priming in T cells.

4.1 The induction phase of anergy and priming in $CD4^+$ T cells

To investigate the differential signalling of anergic and primed T cells during the induction phase *in vitro*, OVA-specific $CD4^+$ T cells were freshly isolated and purified from DO11.10 Tg mice as described in Chapter 2. These cells were induced into a primed or anergic state by TCR ligation with or without appropriate co-stimulation (via CD28), respectively, for 2 days on plate bound anti-CD3 [32, 34]. These cells were then washed and rested for an additional 2 days in fresh medium to allow full induction.

4.2 Characterisation of the induction phase of anergy and priming in $CD4^+$ T cells

4.2.1 Assessment of DNA synthesis during the induction phase of anergy and priming in $CD4^+$ T cells in vitro

As discussed above, it is increasingly clear that cells undergoing induction of priming or tolerance both undergo clonal expansion, albeit to a lesser extent during anergy. Thus the widely held view that only cells receiving costimulation undergo proliferation is a misconception that more likely reflects the situation during the maintenance phase although as we have shown above such cells also undergo proliferation in this phase, at least in this *in vitro* model. To address this, we analysed the functional responses of Ag-specific T cells during the induction phase of priming and tolerance. Here we have also used the OVA TCR Tg cells as we wished to address developing a more physiological system that anti-CD3 ± anti-CD28 but also because, by using the Tg TCR cells, essentially all the $CD4^+$ T cells would be expressing the Tg TCR and hence should all have receptors of the

same affinity and avidity and hence we would be controlling for clonal artefacts.

Thus, firstly we examined the proliferative capacity of the cells during induction of anergy and priming as indicated by DNA synthesis ([^{3}H] thymidine uptake) at 48 and 96 h following stimulation with anti-CD3 or anti-CD3+anti-CD28, respectively. Alternative treatments have previously been used in the literature for the induction of anergy and priming, for example, Ionomycin or Ionomycin plus PMA, respectively. PMA plus Ionomycin is considered mitogenic since Ionomycin is a calcium Ionophore which raises the intracellular level of calcium and activates Ca^{2+}/calmodulin-dependent signalling pathways leading to activation of NFAT whilst PMA is able to activate Protein kinase C and consequently, NFκB and AP-1 [32]. Thus, in addition to our normal protocols of induction of priming and tolerance by anti-CD3 and anti-CD28 antibodies, the $CD4^+$ T cells were also stimulated with other mitogenic treatments such as ConA or PMA plus Ionomycin as positive priming controls whilst stimulation with Ionomycin alone was used to induce anergy as a mimetic of the unbalanced calcium signalling proposed to underly anergy [237]. In addition, cells were treated with anti-CD28 alone as a control for responses to costimulation occurring in the absence of antigen eg during sterile inflammation: thus these combinations provide a range of positive and negative controls for comparing the induction of anergy and priming via Ag-specific receptors.

As expected, the primed T cells (all “priming” stimuli) exhibited higher levels of DNA synthesis than anergic T cells (both anti-CD3 and particularly Ionomycin) at 48 hours although the antibody-primed or anergic cells presented similar levels of DNA synthesis at 96 hours (Figure 4.1A&B). As shown in Figure 4.1A, the $CD4^+$ T cells treated with Ionomycin had the lowest levels of DNA synthesis whilst PMA together with Ionomycin, induced the highest responses.

To further investigate these responses, the profile of IL-2 secretion, a widely accepted defective function of anergic cells was measured during this induction phase, with the cell culture media were harvested at 48 and 96 hours. Both IL-2 and IFN-γ released from anergic and primed T cells were only detectable at the first 48 hours (Figure 4.1C&D), indicating that production ceased during the resting period or was consumed to promote survival/proliferation of the cells at this point. Surprisingly, the significant difference shown in DNA synthesis (Figure 4.1A) between anergic and primed T cells did not appear here, although the level of IL-2 produced from primed T cells was very slightly higher than that from the anergic T cells (Figure 4.1C).

4.2.2 Assessment of cellular proliferation during induction of anergy and priming in CD4$^+$ T cells by eFluor 670, in vitro

$CD4^+$ T cells were isolated from OVA TCR/hCARxOVA TCR mice as described in the Materials and Methods and stained with eFluor 670. The stained $CD4^+$ T cells were stimulated with immobilised anti-CD3 or anti-CD3+anti-CD28 antibodies to induce anergy and priming, respectively (Figure 3.1). Cellular proliferation was measured by FACS analysis of eFluor 670 fluorescence from day 1 to day 4 during this induction phase. As expected the cells cultured with media alone did not proliferate over the 4 days (Figure 4.2A). In contrast to non-treated cells, the $CD4^+$ T cells that were primed with antibodies exhibited clear peaks representing differential numbers of cell divisions (Figure 4.2B&C) for anergic and primed cells from day2. For example, on day 3 whilst for the primed T cells, 29.5% of cells had not proliferated, the anergic T cells retained a much higher proportion of non-proliferated cells at 41.5%. The difference between these two groups was even more obvious at day 4 when many more of the primed T cells (66.9%) proliferated relative to the anergic T cells (27.3%) during this induction phase.

4.2.3 Functional assessment of the role of Rap1 in the priming and tolerance of CD4$^+$ T cells in vitro.

Thus, whilst studies using Tg mice expressing constitutively active constructs of Rap (Cantrell vs Bouyssiotis) resulted in conflicting conclusions as to the role of Rap1 in priming and tolerance, it was decided to investigate the potential role of Rap1 in the induction of anergy and priming, by adenoviral gene transfer using the bicistronic GFP-system which by allowing instant transgenesis does not induce adaptation artefacts and also allows direct analysis of the modification (GFP$^+$ cells) of the gene-of-interest to control cells (GFP$^-$) within the same population of cells. Moreover, this adenoviral approach is tightly regulated in that the cells express normal, not overexpressed levels of the constructs and hence are less likely to induce unforeseen artefacts due to disruption of unknown interacting pathways. Thus, following preliminary optimization experiments, LN cells from hCAR or hCARxOVA TCR mice were first transduced with Ad Rap1 S17N at MOI 30 for 30 minutes and stained with eFluor 670 before plated out onto plates pre-coated with anti-CD3 in presence or absence of anti-CD28 for 4 days to test the effect of this dominant negative Rap1 construct on the induction of priming and anergy respectively (Figure 4.3B). GFP$^+$ cells started to appear 24 hours after the LN cells were transduced with the adenoviral construct and both groups of cells started to proliferate from day 2. Analysis of the GFP$^-$ and GFP$^+$ CD4$^+$ T cells which had been stimulated with anti-CD3 in presence or absence of anti-CD28 was therefore used to investigate how the Rap1 S17N dominant construct regulated cellular proliferation during the induction phase.

Both of GFP$^-$ and GFP$^+$ CD4$^+$ T cells which were treated with anti-CD3 exhibited clear proliferation peaks by day 3, however, it was clear, especially when analyzing absolute numbers of cells, that the GFP$^+$ cells expressing Rap1 S17N were

undergoing more division that the control GFP^- cells, both in terms of numbers of divisions and also in terms of numbers of cells produced (Figure 4.3C). Since Rap1 S17N is the inactive mutant of Rap1, this suggested, as we have suggested previously for the maintenance phase [14], that Rap1 activity acts to limit cellular proliferation during anergy and that switching off Rap1 activity upregulates cellular proliferation. As predicted, GFP^- cells stimulated with anti-CD3 plus anti-CD28, showed higher proliferation than GFP^- anti-CD3-treated cells: indeed the GFP^- anti-CD3/CD28-treated cells somewhat resembled the GFP^+ anti-CD3 cells (Figure 4.3D) suggesting that CD28 signalling may in part act to switch off Rap1 activation. Analysis of the GFP^+ anti-CD3/CD28 population also showed enhanced proliferative capacity in this case also but there was less evidence of a further increase in the number of divisions but rather a decrease in the number of cells not undergoing any proliferation and an increase in the absolute numbers of cells being produced and/or surviving (Figure 4.3D-F).

4.2.4 Assessment of ERK activation following adenoviral regulation of Rap1 activity during induction of anergy and priming, in vitro.

The data presented above suggest that T_H cell proliferation is upregulated when Rap 1 is switched off during induction of anergy and priming. To further investigate this, in particular to determine whether this reflects counter-regulation of the activation of ERK, the LN cells were transduced with wild type (WT) Rap1 and dominant negative (DN) Rap1 S17N and then cultured in media alone or stimulated with anti-CD3 in presence or absence of anti-CD28 for 4 days.

Analysis of the GFP- populations indicated that although the levels of Erk activation tended to oscillate with peaks around 1 and 24 h as demonstrated previously [14], there was only low levels of Erk activation generally and these did not differ

dramatically between the anergic and primed groups. Interestingly, therefore, given the evidence for counter-regulation of ERK and Rap1 during the maintenance phase of priming and tolerance and the fact that switching off Rap1 promotes proliferation of cells undergoing both priming or tolerance, that whilst as expected there was no significant difference in ERK activation among the cells which had been transduced with Ad Rap1 WT (Figure 4.4 A&B), there was also no difference in terms of ERK activation when the cells were transduced with Ad Rap1 S17N (Figure 4.5 A&B).

To determine the impact of Rap1 activation during the induction of anergy and priming, active Rap1 (Rap1-GTP) was also assessed and again, the GFP^- data (Figure 4.4C&D) (Figure 4.5C&D) suggested that during induction of priming and anergy the cells were signalling similarly in terms of Rap1 activation. Moreover, although the GFP^+ data at the early time points somewhat surprisingly suggested an increase in Rap1 activation in cells transduced with either the WT and S17N constructs (Figure 4.4C&D) (Figure 4.5 C&D), these were considered artefacts of the very low numbers and percentage of cells that were GFP^+ at these time points. Also, it should be noted here that only active Rap1 could be detected here, with detection of total Rap1 not being possible in these experiments and as the absolute levels of Rap1 could not be determined here, it is possible that simply analysing the levels of active Rap1 expression do not give a true picture of the relative Rap1 activation in that a small pool of Rap1 could be highly activated under these conditions.

Collectively, these data suggested that under these priming and tolerance induction protocols, Erk and Rap1 signalling was very similar and Rap1 counter-regulation of Erk signalling could not account for the enhanced proliferation of Ad Rap1 S17N-GFP^+ cells.

4.2.5 Functional assessment of the induction of anergic and primed $CD4^+$ T cells using immature and LPS-matured DC, in vitro.

We next investigated whether a more physiological, antigen-specific system of inducing priming and anergy using immature and LPS-matured DC could be developed. However, before the proliferation assay was assessed, the DCs were firstly examined for the expression of maturation markers to determine how "immature" or "mature" these cells were. DCs were derived from the bone marrow progenitors (Balb/c mice) by culture with GM-CSF conditioned complete media onto low adhesion 6-well plates (to prevent maturation) for 7 days. The "immature DC" were maintained in complete media alone whilst DCs were treated with LPS for 24 hours on day 7 to induce "maturation". Following harvesting, the matured and immature DCs were phenotyped in terms of CD11c, CD80 and CD86 expression. Analysis of CD11c-expressing DC (Figure 4.6A) were examined for expression of CD80 and CD86 and, as expected, matured DC expressed higher levels of CD80 expression than immature DC, although this increase was not to a great extent (Figure 4.6B). By contrast, the matured DC expressed substantially higher levels of CD86 than immature DCs (Figure 4.6C). Collectively, these data suggested the DC treated with LPS were more matured than immature DC but the "immature DC" had been partially matured during derivation.

4.2.6 Functional assessment of the induction of anergy and priming in $CD4^+$ T cells in response to OVA-loaded immature and LPS-matured DC, in vitro.

Immature and LPS-matured DC were loaded with OVA peptide and co-cultured with eFluor 670-labelled naïve $CD4^+$ T cells for induction of anergy and priming for 4 days (Figure 4.7A). The cells were harvested daily and cellular proliferation was assessed by FACS. The cells co-cultured with mature DC exhibited proliferation

from day 2 whereas the cells cultured with immature DC were still not showing any evidence of division at this time point. The cells treated with immature DC started to proliferate on day 3, however, those cells still showed higher percentage and absolute number of cells that had not undergone on day 3 and even on day 4, albeit to a much lesser extent. These data are consistent with the LPS-matured DC driving priming whilst as the immature DC were not so effective at driving proliferation it is possible that they are inducing anergy.

In addition, the cytokine secretions of both groups of cells were also examined for further functional assessment. Thus, the supernatants were harvested from each group everyday and the levels of cytokines were analysed for IL-2 and IFN-γ by ELISA (Figure 4.7 B&C). Generally, this revealed that as expected, T cells stimulated by the LPS-matured DC group exhibited higher concentration of IL-2 and IFN-γ release than those in the immature DC group throughout the whole period of induction.

Thus, although maintenance phase experiments have not been performed to corroborate this, it appears that this is a promising protocol for inducing Ag-specific priming and tolerance, particularly if the levels of costimulatory molecules such as CD80 and CD86 can kept to an absolute minimum in the “immature” DC.

4.2.7 Assessment of the role of Rap1 during induction phase of priming and tolerance mimicking physiological condition

In order to investigate whether the effects shown in Figure 4.3, Figure 4.4&Figure 4.5 were replicated under more physiological conditions, priming of T cells, transduced with Ad Rap1 WT and S17N was induced by co-culture with LPS-matured, OVA pulsed DC for 4 days. As expected, since adenoviral transduction does not result in overexpression of the gene of interest, the GFP^- and

GFP^+ T cells transduced with Ad Rap1 WT exhibited similar proliferation progress after inducing by mature DC+OVA (Figure 4.8A). Moreover, and similarly to what was observed with cells primed with anti-CD3/CD28, but not those induced with anti-CD3 alone, the cells transduced with Ad Rap1 S17N did not undergo higher number of cell division than the GFP^- population from the same treatment (Figure 4.8B). Furthermore, in this case, there did not appear to be higher absolute numbers of cells recovered suggesting that perhaps the more physiological conditions substituted for the “survival” effects of Ad Rap1 S17N observed when cells were primed with anti-CD3 plus anti-CD28.

Although the proliferation outcomes of mimicking more physiological conditions did not replicate the increased cellular proliferation of T cells stimulated with anti-CD3/28 resulting from transduction with Ad Rap1 S17N, the effects of the constructs on ERK and Rap1 activation were also examined under these conditions. As shown previously, the cell populations were analyzed within blasting and resting gates with the GFP^- and GFP^+ populations being investigated for ERK and Rap activation expression. This revealed that, as expected due to the low levels of GFP expression, that there was essentially no difference in ERK or Rap1 activation between the GFP^+ and GFP^- populations on day 0. Again ERK activation did not appear to change much on day 1 between the GFP^- and GFP^+ populations in either gate, but was decreased below the level observed on day 0 suggesting that for this model of T cell activation, Erk activation is at or below basal levels in the cyclic pattern observed previously [14] and consistent with this, the level in GFP^- and GFP^+ cells is very slightly upregulated from this at day 3 in both cellular gates. Similarly, these was no difference in Rap1 activation between GFP^- and GFP^+ cells in either gate at day 0 although there was a reduction in the GFP^+ cells at day 1, particularly clear in those in the blasting gate as there was also a partial reduction

in the GFP^- cells in the resting gate at this time point suggesting that in these control cells stimulation with OVA+DC also acted to switch off Rap1 activation. This enhanced reduction of Rap1 activation in the GFP^+ cells was lost at day 3 in both the resting and blasting gates but as the GFP^- cells showed essentially identical levels of active Rap1 expression, this suggested that at this time point we were detected only the basal level of Rap1 activity in these cells, a proposal consistent with the gradual decline in Rap 1 activity observed in GFP^- and GFP^+ populations following stimulation with anti-CD3 or anti-CD3+anti-CD28. The divergent nature of ERK and Rap1 signalling, and its modulation by the Ad Rap1 S17N construct was amplified when the data were analyzed as ERK/Rap1 activity ratios (Figure 4.10A) (Figure 4.10B). Thus analysis of the ratio of pERK/active Rap showed a clear cycling pattern of increased ERK relative to Rap1 activation in the blasting, but not resting, cells perhaps consistent with them progressing through the cell cycle. This was reflected by an enhanced relative level of ERK activation in the day 1 samples of GFP^+ relative to GFP^- cells, perhaps suggesting some degree of counteregulation that did not translate to enhanced proliferation at this time point. Rather counterintuitively, therefore, whilst the cells transduced with Rap1 WT exhibited lower levels of ERK at day 1 in blasting cells, they expressed higher levels in both cell populations at day 3 (and also day 1 for resting cells) suggesting here that transduction of Rap1 promoted ERK activation (Figure 4.9A & D). Although the expression of active Rap did not appear to differ between GFP^- and GFP^+ populations in resting cells (Figure 4.9E), there did appear to be an increase at day 0 and day 1 in the blasting cells suggesting that the ectopic Rap1 resulted in Rap1 activation (Figure 4.9B). This was not predicted because the Rap mutant constructs carried by Adenovirus do not appear to overexpress in the bicistronic system, and so it was expected that the cells transduced with Ad Rap1 WT would exhibit similar behaviour as GFP^- cells. When the relation between phosphorylated

ERK and active Rap was further analyzed, a similar but more dramatic pattern of enhanced ERK signaling (relative to Rap1) was seen in GFP^+ blasting cells at day 3, relative to those transduced with the Ad Rap1 S17N construct (Figure 4.10C), although the pattern seen in the control GFP^- cells was the inverse of that seen with Ad Rap1 S17N experiment, perhaps indicating that these cells are at a different stage in the kinetics of their response. In any case, it appeared that transduction with either WT or DN Rap1 appeared to disrupt Rap1 activation and lead to a higher relative level of ERK activation. This suggested that the activity of Rap1 perhaps was not the key factor in this modulation but rather perhaps reflected differential localization/processing or Rap1 or alternatively its differential interaction with signaling partners to that of endogeneous. Whatever, the molecular mechanism, this shift in relative ERK/Rap1 activation did not appear to exert any substantial effect on the proliferative responses of the cells.

4.2.8 Assessment of active Rap1 and ERK MAP kinase expression during induction of anergy and priming by Western Blot

Since the ERK MAPKinase is critically important for many aspects of T cell biology, especially in IL-2 production, cell cycle and proliferation [137], the potential for counter-regulatory Rap1/ERK signaling during the induction of priming and tolerance was further explored. Overall, both anergic and primed T cells exhibited steady expression of active Rap1 after priming with antibodies for at least 48 hours after which this increased dramatically until 96 hours, with the primed T cells expressing slightly higher levels of active Rap1 than anergic T cells (Figure 4.11A) and this higher expression was also reflected at the level of Rap1 protein (Figure 4.11B). In contrast to the early static expression of active Rap, the expression of pERK during induction phase was more dynamic. Reflecting our previous studies of the maintenance phase [14], cyclic activation of ERK was seen in the primed T

cells but not the anergic population, with overall the primed T cells exhibiting higher levels of activation of ERK than in anergic T cells until 48-96 hours (Figure 4.11C). These western blot experiments did not completely reflect the patterns observed in the FACS analysis of intracellular staining of these signaling elements in cells treated with the adenoviral constructs but this could reflect the differential culture conditions required for the FACS and Western Blotting experiments, differential efficacy of the antibodies with respect to intracellular staining and Western Blotting, or else a difference in kinetics induced by exposure to the virus as it has previously been reported that exposure of hCAR-expressing T cells to virus results in some activation, perhaps suggesting earlier expression of the ERK peaks in the intracellular staining experiments. Although this would not appear to be the case for the Rap1 activation, this may reflect our failure to measure total Rap1 expression in the FACS experiments as it can be seen here that Rap1 expression falls 24-96 h, consistent with expression of a residual pool of highly activated Rap1. Analysis of the relative ratio of active pERK/active Rap1 showed that a clear cycling pattern of increased ERK relative to Rap1 activaion during 0-8 hr in primed T cells. However, anergic T cells exhibited dramatically increased ERK during 24-48 hr, which is consistent with proliferative response during induction of anergy. Both populations drop down to similar level after 48 hr, this may suggest that after anergic T cells catch up the proliferative progress of primed T cells, both anergic and primed T cells had similar level of proliferation (Figure 4.11D).

4.2.9 Investigation of potential differential c-Myc signaling during induction of priming and tolerance

The transcription factor, c-Myc plays important roles in the regulation of cell growth, proliferation, differentiation and apoptosis [213-215]. The previous studies [216] have indicated that high proliferation rates in vivo and in cell culture experiments

were related to c-Myc overexpression. By contrast, low expression of c-Myc is associated with nondividing cells and defects in cellular differentiation [217]. Expression of c-Myc in quiescent cells is not stable but once cell cycle progression occurs, c-Myc becomes transiently stabilized and accumulates to high levels. Such stabilization requires activation of the Ras-Raf-MEK-ERK kinase cascade and hence activation of ERK MAP kinase impacts on cellular proliferation and cell survival, at least in part via this effector transcription factor. c-Myc contains two phosphorylation sites, Threonine 58 (T58) and Serine 62 (S62), in the N-terminal region and these two phosphorylation sites play opposite roles in controlling stability of c-Myc, with Serine 62 being targeted by ERK and Threonine 58 is targeted by define GSK-3β. The phosphorylation of Ser62 participates in the stabilization of c-Myc whereas phosphorylation of Thr58 targets c-Myc towards to degradation through the ubiquitin-proteasome pathway [222]. Thus, because of the potential conter-regulatory ERK/Rap1 signalling, we decided to investigate c-Myc expression and its stabilization/proteasome targeting status during induction of priming and tolerance of T cells to determine whether these signaling events could be linked. As shown in Figure 4.11C, ERK activation exhibited an oscillating pattern in both anergic and primed T cells and consistent with it being downstream of the MAP kinase pathway, the kinetics of pS62 c-Myc expression were consistent with following activation of pERK in both primed and tolerised cells at 24-96 h (Figure 4.12A). However, although the primed T cells showed higher levels of activation of ERK than the anergic cells before 24 hours, generally the anergic T cells exhibited higher levels of pS62 c-Myc than primed T cells during induction phase. Surprisingly, the pT58 c-Myc expression more similarly reflected the pattern of ERK activation which had not been predicted as phosphorylation at T58 has the opposite role to that at S62, as it targets c-Myc for degradation (Figure 4.12C). However, and perhaps consistent with the stabilization data, the total expression of

cMyc was strongly upregulated accumulating at later times in both anergic and primed T cells with the anergic T cells expressing higher levels of c-Myc at all the time points tested (Figure 4.12B&D). Moreover, the delay in such accumulation and the apparent downregulation at the end of the timecourse occurred following he peaks in the proteasome targeting pT58 form. Although, this accumulation of c-Myc in anergic cells was surprising as c-Myc upregulation is typically associated with proliferation it may reflect its involvement [213, 225] in other cellular processes such as apoptosis.

4.2.10 Assessment of mTOR signaling during induction of anergy and priming

mTOR is a Serine/Threonine protein kinase that regulates cell survival, growth, proliferation, differentiation and cell migration. It forms two distinct signaling complexes: mTORC1 and mTORC2 [166]. The mTORC1 is composed of Raptor, mLST8, PRAS40 and DEPTOR, whilst mTORC2 is consisted of mTOR, Rictor, Sin1, mLST8 and Protor [166] (Figure 1.4). The PI-3K-PDK1-AKT signaling is involved in activation of mTORC1 and leads to phosphorylation of p70 ribosomal S6 kinase 1 (S6K1) and 4E-BP-1, thus promoting translation and protein synthesis [185]. Ras/Raf/MEK/ERK signaling has been indicated to positively regulate mTORC1. Indeed, ERK1/2 phosphorylate tuber sclerosis protein 2 (TSC2) and suppress the inhibitory function of TSC2 [238, 239]. In contrast to mTORC1, the upstream signals which lead to activation of mTORC2 are still unclear although the activity of mTORC2 can be monitored by the downstream phosphorylation of AKT at its hydrophobic motif, serine 473 [240]. In brief therefore, AKT phosphorylation occurs both upstream and downstream of mTOR although the phosphorylation of AKT at threonine 308 which leads to activation of mTORC1 is independent from that mTORC2-mediated phosphorylation of AKT at serine 473 [173].

Initial studies using rapamycin, the mTOR inhibitor, had shown that blocking mTOR activation was sufficient to induce anergy in T cells following full activation with anti-CD3 and anti-CD28 and leads to inhibit cell proliferation [170]. The further investigation showed that full anergy induction required the activation of the CaN/NFAT pathway with concomitant repression of mTOR activation. Thus as the downstream of the IL-2R pathway, mTOR was indicated to be a major regulator of anergy [241]. mTOR is not only downstream of CD28 and IL-2 signalling pathways but is also participates several energy and nutrient-sensing pathways in eukaryotic cells [172]. Regarding to Warbur effect, during TCR stimulation, signals from growth factor like IL-2, and the ligation of co-stimulatory CD28, lead to an increase in glycolysis by inducing the PI3K-dependent activation of Akt. While T cells undergo clonal expansion, they preferentially ferment glucose to obtain their mitochondrial oxidative phosphorylation [242-244]. With this line, in the presence of a leucine or glucose antagonist could actively induce anergy in T cells, even T cells receive the signals 1 and 2 from TCR ligation and co-stimulation, as well as normal IL-2 production. Thus, the investigation of induction of anergy was extent to energy and nutrition homeostasis regulated by mTOR.

AMPK is a cellular energy sensor and signal transducer which is regulated by various metabolic stresses. AMPK is activated under conditions of stress and acts to inhibit energy consumption. Indeed, activation of AMPK by AICAR, activator of AMPK, has been reported to induce anergy in T cells. Raptor, the downstream target of negative regulation by AMPK, plays an important role in activation of mTORC1 which in turn leads to activation of p70 S6 kinase to promote protein synthesis. However, when under conditions of energy stress, the ratio of AMP: ATP increases, the AMPK is phosphorylated at serine 772 and 792 and this activation results in AMPK phosphorylating TSC2 and Raptor, with consequent inhibition of

mTORC1 and its downstream effector, p70 S6 kinase.

We therefore investigated mTOR activation during the induction phase of priming and tolerance of Th cells, firstly by examining AMPK activation. Upregulation of phosphorylation of AMPK was observed during the induction of anergy (24-48 h) and, more rapidly, priming of (8-48h) T cells, the latter perhaps reflecting that during productive immune responses T cells undergo catabolic metabolism (Figure 4.13A) [245]. These data suggest that mTORC1 might be being inhibited to conserve energy and consistent with this, the same pattern was reflected by phosphorylated Raptor at these time points (Figure 4.13C). Interestingly, and perhaps reflecting their quiescient state levels of phosphor-Raptor were high in both groups at time zero and were maintained so for the first 4 hours of stimulation before dropping dramatically presumably as the cells began to enter and progress through cell cycle and this was reflected by a peak in pp70S6K between 8-24 h when protein synthesis would be required for this process. S6K activity was then dramatically switched off corresponding to the activation of AMPK and phosphorylation of Raptor resulting in inhibition of mTORC1 and, in turn, the phosphorylation of p70 S6 kinase (Figure 4.13E). Notably, the primed cells exhibited higher activation of AMPK than anergic T cells (Figure 4.13A) and correspondingly lower levels of S6K, perhaps reflecting induction of anergy-specific genes. Additionally, and unlike ERK expression, the total expression of AMPK, raptor and p70 S6 kinase also changed (Figure 4.13B, D&F) in response to the different stimulations. For instance, the anergic cells expressed more AMPK and less S6K than primed cells during the induction phase. Although as mentioned above, Raptor was highly phosphorylated in the early stages of induction of priming and tolerance, this did not reflect AMPK activation and hence must involve some other regulatory mechanism, as yet not identified.

By comparison to mTORC1, the precise mechanisms of activation of mTORC2 are still not fully understood because of lack of an mTORC2 specific inhibitor. However previous studies have shown that mTORC2 is activated by costimulation and cytokines in T cells [90] and that PI3K controls the activity of mTORC2 to phosphorylate its downstream substrate, AKT at residue S473. The phosphorylation of AKT can be controlled by p70 S6 Kinase as this negatively regulates mTORC2 by phosphorylation of Rictor at Thr1135 [184]. Consistent with this, the pattern of phosphorylation of Rictor in both anergic and primed T cells mirrored that of pp70S6K in that it peaked at 24 h when it started to decline till 48 hours before being upregulated again in primed, but not anergic T cells (Figure 4.14A). Interestingly the expression of phosphorylated Rictor in anergic T cells was higher than that of primed T cells (Figure 4.14A), which again represented the same pattern as the activation of p70 S6 kinase in both populations (Figure 4.13E). This indicated that anergic T cells exhibited higher level of phosphorylated p70 S6 kinas and thus induced higher expression of phosphorylated Rictor to inhibit the AKT activation (Figure 4.14C), with the peaks in pS437AKT (24 and 96h) corresponding to the decline in pRictor. It is notably that whilst the anergic T cells continued to exhibit decreased expression of pRictor after 48 hours, primed T cells did not and this was reflected by a switch in the relative levels of pS473AKT in primed and tolerised cells with much higher levels in the anergic cells relative to primed T cells after 48 hours. This may suggest that in anergic T cells, activation of the mTORC2 pathway by be triggered to promote cell survival after 48 hours.

Conclusion

Following the findings that during the maintenance phase cells undergoing induction of priming and tolerance both undergo clonal expansion, the functional outcomes, ERK/Rap1 activation and influence of modulation of Rap1 expression within anergic and primed cells were assessed during induction phase.

As expected, during the induction phase T cells undergoing priming exhibited higher levels of DNA synthesis than those becoming anergic at 48 hours, although the primed and anergic T cells presented similar levels at 96 hours. In the aspect of cytokine secretion, IL-2 and IFNγ released from anergic and primed T cells was only detectable within the first 48 hours but not 96 hours, indicating that early production was then consumed to promote their survival and proliferation. As regarding to the proliferation of anergic and primed T cells during induction phase, both populations exhibited clear peaks representing differential numbers of cell divisions for anergic and primed cells from day2 (48 hour) onwards and proliferated strongly and rapidly from day 3 to day 4. In order to try and develop a more physiological model with which to induce priming and tolerance, immature and LPS-matured DC were loaded with OVA and co-culture with antigen-specific naïve T cells. As predicted, T cells stimulated by the LPS-matured DC group exhibited proliferation from day 2, whilst the cells cultured with immature DC still showed relatively high percentages and absolute number of cells that had not undergone any proliferation on day 3 and even on day 4. Consistent with this proliferation data, as expected, the T cells stimulated by the LPS-matured DC group exhibited higher IL-2 and IFNγ release than those stimulated with the immature DC group.

Regarding whether the inverse Rap1 and ERK signalling observed during maintenance phase also occurred during the induction of priming and tolerance,

the role of Rap1 in the induction of anergy and priming was investigated by adenoviral gene transfer of Ad Rap1 S17N, an inactive mutant of Rap1. This revealed that both normal (GFP^-) and dominant negative Rap1-expressing $CD4^+$ T cells (GFP^+) which were treated with anti-CD3 exhibited clear proliferation peaks by day 3. However, the GFP^+ cells expressing Rap1 S17N were undergoing more division than the control GFP^- cells. This suggested, Rap1 activity acts to limit cellular proliferation during anergy and thus switching off Rap1 activity upregulates cellular proliferation to generate a phenotype more resembling priming of normal (or GFP^-) T cells by anti-CD3 plus anti-CD28, which showed higher proliferation that GFP^- cells stimulated with anti-CD3 only. Indeed, as the GFP^- cells treated with anti-CD3+anti-CD28 somewhat resembled the GFP^+ cells treated with anti-CD3, this suggested that CD28 signalling may in part act to switch off Rap1 activation. GFP^+ cells treated with anti-CD3+anti-CD28 also showed enhanced proliferative capacity, however, this is not as obvious as with comparison of GFP^-/GFP^+ cells treated with anti-CD3. However, there did not appear to be any difference between anergy and primed cells in terms of Erk/Rap signalling during the induction phase and the introduction of Ad Rap1 S17N did not appear to modulate Erk activity in transduced cells treated with anti-CD3 or anti-CD3+anti-CD28, suggesting that Rap was targeting some other effector during the induction phase. When these adenoviral transfer experiments were repeated in the more physiological model, the higher proliferation exhibited in anergic Ad Rap1 S17N transduced cells were not replicated suggests that the enhancing effects of Ad Rap1 S17N might be substituted by other signals generated under these more physiological conditions.

Nevertheless, as indicated above, the activation of Rap1 and ERK as assessed by western blotting revealed that both anergic and primed T cells exhibited steady expression of active Rap1 after priming with antibodies for at least 48 hours after

which this increased dramatically until 96 hours, with the primed T cells expressing slightly higher levels of Rap1 than anergic T cells. Cyclic activation of ERK was seen in the primed T cells and at higher levels of activation than in the anergic population, which did not exhibit these kinetics. However, as these data showing similarities in Rap signaling in anergy and priming as well as the use of the dominant negative construct suggested that Rap was not acting to suppress ERK activation during induction of anergy other potential downstream targets such as the key transcription factor, c-Myc were also examined. The phosphorylation of Ser62 (by ERK) participates in stabilization of c-Myc whereas phosphorylation of Thr58 targets c-Myc towards to degradation. The kinetic of pS62 c-Myc expression were consistent with following activation of pERK in both primed and tolerised cells at 24-96 hour during induction phase. Although the primed T cells exhibited higher levels of ERK activation than anergic T cells, the anergic T cells showed higher levels of pS62 c-Myc than primed T cells during induction phase. Surprisingly, the pT58 c-Myc expression more similarly reflected the pattern of ERK activation.

Additionally, the primed T cells tend to skew to catabolic, rather anabolic metabolic pathways, when compared to anergic T cells during the induction phase, as evidenced by the primed cells exhibiting upregulation and phosphorylation of AMPK and Raptor to inhibit mTORC1 function and in turn, lower levels of pp70 S6 kinase. However, the expression of phosphorylated Rictor in anergic T cells was higher than that of primed T cells, indicating inhibition of mTORC2 in anergic T cells resulting in downregulation of AKT activation during this induction phase.

Figure 4.1 Analysis of DNA synthesis and cytokine secretion in anergic and primed T cells by Thymidine [^{3}H] and ELISA during induction phase.

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hCAR x OVA T cR mice and isolated by MACS $CD4^+$ T cell isolation kit. As mentioned in Figure 3.1, briefly, the naïve $CD4^+$ T cells ($2x10^5$/200µl) were stimulated with anti-CD3 ± anti-CD28 for 48 and 96 hours. Sequentially the thymidine were added into the $CD4^+$ T cells which were treated in different conditions and cultured for extra 16 hours culture. (A) The DNA synthesis at 48 hours (B) The DNA synthesis at 96 hours.

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hCAR x OVA T cR mice and isolated by MACS $CD4^+$ T cell isolation kit. As mentioned in Figure 3.1, briefly, the naïve $CD4^+$ T cells ($10x10^6$/4ml) were stimulated with anti-CD3 ± anti-CD28 in 6-well tissue culture plate for 48 and 96 hours. The supernatant were firstly collected at 48 hours meanwhile the cells were washed and rested until 96 hours when the supernatant were harvested. (C) The IL-2 production at 48 and 96 hour during induction phase (D) IFN-γ production at 48 and 96 hour during induction phase.

Figure 4.2 Assessment of cellular proliferation of anergic and primed T cell during induction phase.

The $CD4^+$ T cells were extracted from lymph nodes of OVA TCR or hCAR x OVA T cR mice and isolated by MACS $CD4^+$ T cell isolation kit. The naïve $CD4^+$ T cells were stained with eFluor 670 in 5μM before induction of anergy and priming with anti-CD3 ± anti-CD28 for 4 days. The cells were harvested everyday and the cellular proliferation was analysed by FACS. (A) The cellular proliferation of naïve $CD4^+$ T cells which were cultured with media alone (B) The cellular proliferation of $CD4^+$ T cells which were induced with anti-CD3. (C) The cellular proliferation of $CD4^+$ T cells which were induced with anti-CD3/anti-CD28. Data are representative of at least three independent experiments.

Figure 4.3 Assessment of cellular proliferation by regulation of Rap1 S17N during induction of anergy and priming in Ag specific $CD4^+$ T cells by eFluor 670.

The LNs were generated from hCAR or hCARxOVA TCR mice and transduced with Ad Rap1 S17N at MOI 30 before staining with eFluor 670 in 5μM. The transduced and stained LNs were cultured with anti-CD3 in presence or absence of anti-CD28 for 2 days and rested for the other 2 days. The cellular proliferation were analysed by FACS on day 1 and day 3. (A) Uninfected cells (B) LNs were transduced with Ad Rap1 S17N at MOI 30 (uninfected cell (GFP-) was 16.9% and infected cells (GFP+) was 54.5%). (C) The cellular proliferation of GFP- and GFP+ LNs during induction of anergy. (D) The cellular proliferation of GFP- and GFP+ LNs during induction of priming (E) The percentage of clonal expansion among each cell division in GFP- and GFP+ populations during induction of anergy (F) The percentage of clonal expansion among each cell division in GFP- and gFP+ populations during induction of priming. Data are representative of at least three independent experiments.

Figure 4.4 Assessment of ERK and Rap1 activation in Ag specific $CD4^+$ T cells regulated by Ad Rap1 WT during induction of anergy and priming.

The LNs were generated from hCAR or hCARxOVA TCR mice and transduced with Ad Rap1 WT at MOI 30. The LNs transduced with Ad Rap1 WT were cultured with anti-CD3 and anti-CD3+anti-CD28 for 0, 10, 30, 60 and 180 minutes and 6 to 96 hours. ERK activation (A) (B) and active Rap1 (C) (D) were assessed by FACS.

Figure 4.5 Assessment of ERK and Rap1 activation in Ag specific $CD4^+$ T cells regulated by Ad Rap1 S17N during induction of anergy and priming.

The LNs were generated from hCAR or hCARxOVA TCR mice and transduced with Ad Rap1 S17N at MOI 30. The LNs transduced with Ad Rap1 S17N were cultured with anti-CD3 and anti-CD3+anti-CD28 for 0, 10, 30, 60 and 180 minutes and 6 to 96 hours. ERK activation (A) (B) and active Rap1 (C) (D) were assessed by FACS

Figure 4.6 Assessment of maturation markers expressed on immature and mature DC *in vitro*.

The DC were generated from bone marrow of OVA TCR mice and cultured in the low adhesion plate with conditional media for 6 days. On the day 7 the DC were scrap off from plate and washed with complete media. Half of DC were cultured with complete media alone as immature DC. The other half of DC were treated with LPS for 24 hours as mature DC. On the day 8, the DC were harvested from low-adhesion plate and (A) stained with CD11c and its Isotype control. 22% of cells within in R1 gate were $CD11c^+$ cells. (B) The immature and mature DC were stained with CD80 antibody and (C) CD86 antibody. Data are representative of three independent experiments.

Figure 4.7 Assessment of cellular proliferation in $CD4^+$ T cells induced by immature and mature DC.

The lymph nodes were extracted from hCAR x OVA TCR mice and prepared as single cell suspension. The immature and mature DC generation and preparation was same as described in Figure 4.3. The immature and mature DCs were treated with OVA pepetide before they were co-cultured with lymph nodes. The lymph nodes ($1x10^7$) were induced with OVA-loaded immature and OVA-loaded, LPS-matured DC ($1x10^6$) for 4 days. The LN cells and supernatant were harvested everyday for (A) analysis of proliferation and the production of (B) IL-2 and (C) IFN-γ.

Figure 4.8 Assessment of cellular proliferation regulated by Ad Rap1 WT and S17N underlying more physiological condition.

The LNs ($3x10^6$) were transduced with (A) Ad Rap1 WT and (B) S17N at MOI 30 for 24 hours before co-culture day. The transduced LNs were stained with eFluor 670 in 5μM before co-cultured with OVA-loaded, mature DC ($1.5x10^6$) for 4 days. The cells were harvested on day 2 and day 4 and analysed by FACS.

Figure 4.9 Assessment of ERK and Rap1activation in Ag specific $CD4^+$ T cells regulated by Ad Rap1 WT underlying more physiological condition.

The LNs ($3x10^6$) were transduced with Ad Rap1 WT at MOI 30 for 24 hours before co-culture day. The transduced LNs were co-cultured with OVA-loaded, mature DC ($1.5x10^6$) for 4 days. The cells were harvested on day 2 and day 4 and analysed by FACS. To investigate more than previous experiment, the ERK and Rap1 activation were analysed in GFP- and GFP+ populations within (A) (B) blasting gate and (D) (E) resting gate. Regarding to the difference between the ratio of pERK/ERK and active Rap1/Rap1 was not very obvious, the ratio was calculated as pERK/active Rap1 (C) (F) to amplify the bias towards to ERK activation between GFP- and GFP+ populations.

Figure 4.10 Assessment of ERK and Rap1activation in Ag specific $CD4^+$ T cells regulated by Ad Rap1 S17N underlying more physiological condition.

The LNs ($3x10^6$) were transduced with Ad Rap1 S17N at MOI 30 for 24 hours before co-culture day. The transduced LNs were co-cultured with OVA-loaded, mature DC ($1.5x10^6$) for 4 days. The cells were harvested on day 2 and day 4 and analysed by FACS. To investigate more than previous experiment, the ERK and Rap1 activation were analysed in GFP- and GFP+ populations within (A) (B) blasting gate and (D) (E) resting gate. Regarding to the difference between the ratio of pERK/ERK and active Rap1/Rap1 was not very obvious, the ratio was calculated as pERK/active Rap1 (C) (F) to amplify the bias towards to ERK activation between GFP- and GFP+ populations.

Figure 4.11 Analysis of Rap1 and ERK1/2 activation during induction of anergy and priming by Western Blotting.

The naïve $CD4^+$ T cells were stimulated with anti-CD3 in presence or absence of anti-CD28 for 0, 1, 2, 4, 8, 24, 48 and 96 hours. The levels of active Rap1, total Rap1, phosphorylated ERK1/2 (pERK1/2), total ERK1/2 and loading control (GAPDH) in whole cell lysates of each population were measured at each timepoint using Western Blot analysis. The activation of Rap1 (A), expression of Rap1 (B), activation of ERK (C) and ERK/Rap1 (D) were quantitated by ImageJ64.

Figure 4.12 Analysis of c-Myc activation during induction of anergy and priming by Western Blotting.

The naïve $CD4^+$ T cells were stimulated with anti-CD3 in presence or absence of anti-CD28 for 0, 1, 2, 4, 8, 24, 48 and 96 hours. The levels of phosphorylated c-Myc (S62), phosphorylated c-Myc (T58), c-Myc and loading control (GAPDH) in whole cell lysates of each population were measured at each timepoint using Western Blot analysis. The activation of c-Myc S62 (A) and c-Myc T58 (C), and expression of c-Myc (B) (D) were quantitated by ImageJ64.

Figure 4.13 Analysis of mTORC1 activation during induction of anergy and priming by Western Blotting.

The naïve $CD4^+$ T cells were stimulated with anti-CD3 in presence or absence of anti-CD28 for 0, 1, 2, 4, 8, 24, 48 and 96 hours. The levels of phosphorylated AMPK, AMPK, phosphorylated Raptor, Raptor, phosphorylated p70 S6 kinase, p70 S6 kinase and loading control (GAPDH) in whole cell lysates of each population were measured at each timepoint using Western Blot analysis. The activation of AMPK (A) expression of AMPK (B), activation of Raptor (C), expression of Raptor (D), activation of p70 S6 kinase (E) and expression of p70 S6 kinase (F) were quantitated by ImageJ64.

Figure 4.14 Analysis of mTORC2 activation during induction of anergy and priming by Western Blotting.

The naïve $CD4^+$ T cells were stimulated with anti-CD3 in presence or absence of anti-CD28 for 0, 1, 2, 4, 8, 24, 48 and 96 hours. The levels of phosphorylated Rictor, Rictor, phosphorylated AKT, AKT and loading control (GAPDH) in whole cell lysates of each population were measured at each timepoint using Western Blot analysis. The activation of Rictor (A) expression of Rictor (B), activation of AKT (C) and expression of AKT (D) were quantitated by ImageJ64.

Chapter 5
Discussion

5 Discussion

5.1 Comparison of the levels of DNA synthesis, cellular proliferation and cytokine secretion during the induction and maintenance phase of anergy and priming in $CD4^+$ T cells in vitro

As the DNA synthesis (measured by [^{3}H] thymidine uptake into DNA) is an indication of the S phase of cell cycle progression, it is thus referred as an indicator of cellular proliferation. Thus, as a first step to investigating the differential proliferative responses of primed and anergised T cells, DNA synthesis in primed and anergic antigen-specific T cells was first investigated during the induction and maintenance phases. Previous studies had shown that the anergic T cells exhibited reduced proliferation after re-stimulation with $OVA_{323-339}$ *in vitro [14]* and here, a similar result was replicated in these optimised models as assessment of [^{3}H] thymidine uptake during maintenance phase of anergy and priming in $CD4^+$ T cells (Figure 3.3) showed that anergic T cells also undergo Ag-driven DNA synthesis, they do to a lesser extent than primed cells (Figure 3.3A). This finding goes against the concept that only cells receiving costimulation are able to proliferate during maintenance phase and thus it was investigated further by examining if this also occurred during the induction phase. Thus, firstly we examined the proliferative capacity of the cell during induction of anergy and priming as indicated by DNA synthesis at 48 and 96 h following stimulation with anti-CD3 or anti-CD3+anti-CD28, respectively. Again, the primed T cells exhibited higher levels of DNA synthesis than anergic T cells at 48 hours although both of primed and anergic cells presented similar levels of DNA synthesis at 96 hours. Nevertheless, there is no

doubt that the primed cells shows higher level of DNA synthesis after re-stimulation of with Ag. Moreover, although both groups of cells had similar levels of DNA synthesis at 96 hours after induction of anergy and priming, this could reflect limitations of the culture system, particularly with respect to the primed group. Further, since analysis of DNA synthesis only provides a snapshot of the cells transiting S phase of the cell cycle, it provides no information relating to the actual numbers of cell division undergone.

Thus for further investigation of cellular proliferation during induction of anergy and priming, cell division tracking dyes were used to provide more information on differential proliferative responses between anergic and primed populations. Interestingly, during the induction phase, both anergic and primed T cells exhibited clear peaks representing differential numbers of cell divisions for anergic and primed cells from day 2, the same time point (48 hours) that the cells treated with anti-CD3/anti-CD28 exhibited much higher levels of DNA synthesis. Additionally, the difference between these two groups was even more obvious at day 4 when many more of the primed T cells (66.9%) proliferated relative to the anergic T cells (27.3%) during induction phase. By contrast, the DNA synthesis data presented no significant difference between these two groups at 96 hours (day 4) in the induction phase. Again, this suggests that cellular proliferation analysed with a cellular track dye provides more detail than the [^{3}H] thymidine assay. These results did not reflect an artefact of CFSE staining as regarding the proliferation of anergic and primed cells stained with eFluor 670, although the primed T cells had 29.5% of cells had not proliferated, the anergic T cells retained a much higher proportion of non-proliferated cells at 41.5% on day 3. The difference between two groups was even more obvious at day 4 at the end of the induction period of anergy and priming, where 66.9% of primed T cells.

Similar patterns were shown during the maintenance phase as well. Consistent with the above data for the induction phase studies, anergic $CD4^{+}KJ^{+}$ T cells had a higher percentage of non-dividing cells than primed T cells at the beginning of the maintenance phase: 17.8% versus 11.7% on day 1 and 18.5% versus 9.68% on day 2. By contrast, the primed T cells obtained higher percentage of cells that had divided: 27.2% versus 14.7% on day 1; and 19.2% versus 8.13% on day 2; 68.4% versus 50.6% on day 3 and 66.7% versus 53.2% on day 4. Overall, the primed T cells proliferated faster than anergic T cells in the first two days with primed T cells showing less non-dividing cells and more proliferating cells on day 1. The difference between the proliferation of anergic T cells was most obvious on day 2, after which the proliferation of anergic T cells started to catch up with primed T cells, which possibly partly due to exhaustion of the media for primed cells and loss of sensitivity of CFSE-tracked division since the levels of CFSE was too low to detect.

In addition to assessing cellular proliferation, the cytokine secretion during induction and maintenance phase were further investigated, as the lack of IL-2 production is the key indicator for identification of T cell tolerance [137]. IL-2 and IFNγ released from anergic and primed T cells was only detectable at the first 48 hours of the induction phase, indicating that production ceased during the resting period or was consumed to promote survival or proliferation of the cells at this point. Notably, the dramatic difference exhibited in DNA synthesis between anergic and primed T cells was not reflected here, as the level of IL-2 produced from primed T cells was only slightly higher than anergic T cells and appeared to correlate better with the observed patterns of cellular proliferation. For example, the primed T cells proliferated slightly faster and underwent more than rounds of division than anergic T cells at day 2, reflecting the relative IL-2 production at 48 hours. Meanwhile the anergic T cells released higher IFNγ than primed T cells at 48 hours, which

suggested that more anergic T cells may have been differentiated to Th1 cells. However, both IL-2 and IFNγ were diminished at 96 hours, which suggested that the IL-2 might be being consumed for proliferation since both anergic and primed T cells proliferated much faster and more on day 3 and day 4 than day 2. The reduction in IFNγ may reflect lack of production on establishment of anergy but this as also observed with the primed cells and so may reflect some homeostatic regulatory mechanism.

Distinct from the induction phase, primed T cells produced the highest levels of IL-2 during the first 24 hours after challenge with antigen. However, the secretion of Il-2 from the primed cell population dropped between 24-48 h and particularly dramatically, 48-96 h following stimulation with antigen, findings which were reflected functionally by the cellular proliferation data, since the primed T cells exhibited strong proliferation, relative to anergic T cells. Additionally, the reduced levels of IL-2 at 96 hours for both anergic and primed T cells reflected their strong proliferation by day 4. The levels of IFNγ production also supports the evidence of cell survival and differentiation at 48 and 96 hours. To sum up, it is interesting that there were no differences in IL-2 and IFNγ production during the first 48 hours and release of these two cytokines were diminished at 96 hours during the induction phase. Unlike induction phase, however, higher levels of IL-2 were produced from primed T cells during the first 24 hours after stimulation with antigen. Although the secretion of IL-2 from both the primed and anergic cell populations dropped at 48 and 96 hours, this was also reflected in the cellular proliferation responses. However, the levels of IFNγ generated from these two populations supports that both populations are surviving and d9fferentiating. Thus contrary to what is widely portrayed and believed, T cells proliferate during the induction and maintenance phases of both priming and tolerance. However, anergic cells exhibited slower and

less proliferation than primed cells. Interestingly, whilst the anergic and primed cells had produced similar levels of IL-2 and IFNγ during induction phase, primed cells produce higher IL-2 and IFNγ during maintenance phase, findings more consistent with the dogma of functional defects in anergy suggesting that the precise phases and models of priming and tolerance in the literature need to be carefully evaluated.

5.2 pERK and active Rap expression during induction and maintenance phase

The molecular signalling pathway in anergic cells responsible for the differential functional outcomes outlined above remain to be fully explored in order to explain these phenomena. The first step was to build on data from this lab showing counter-regulatory Erk/Rap1 signalling during the first 24 h of the maintenance phase of priming and tolerance, especially as these data could only account for Rap1 expression, not activity. The levels of ERK activation presented here show it to oscillate with peaks during 1 to 24 hours in induction phase (Figure 4.7B-F), which is similar as what has been demonstrated previously for survival and proliferation of B cells [246]. However, only low levels of ERK activation were generated and no significant difference shown between anergic and primed populations during this time period whereas the primed T cells exhibited more and faster proliferation than anergic cells during induction phase. The similar pattern also showed that the active Rap1 did not exhibit significant difference between anergic and primed cells during induction of priming and anergy, however, the assessment of total Rap1 was not available at the time, thus the relative Rap1 activation was not able to provide here, indicating the comparison of Rap1 activation in cells during priming and tolerance is still not available from this FACS approach. However, the information of ERK and Rap1 activation is available from

the cells assessed by western blot where the primed T cells exhibited cyclic activation of ERK and expressed higher levels of activation of ERK than anergic from 0- 48 hour, whereas the anergic T cell exhibited increasing ERK activation from 48 -96 hour (Figure 4.11C), consistent with their relative patterns of cellular proliferation during induction of priming and anergy (Figure 4.2). The further analysis of Rap1 activation by western blot, both anergic and primed T cells exhibited steady expression of active Rap1 from 0- 48 hour after priming with antibodies, after which the Rap1 activation increased dramatically in both anergic and primed T cells until 96 hours (Figure 4.11A). This higher expression was also reflected at the level of Rap1 protein (Figure 4.11B). Notably, these western blot experiments perform different pattern of ERK and Rap1 activation from these signals detected by FACS analysis of intracellular staining. These differences between two different detection system may reflect the differential culture conditions required for the FACS and Western blotting experiments, differential efficacy of the antibodies with respect to intracellular staining and Western Blotting or else a difference in kinetics induced by exposure to the virus (in the GFP± experiments) as it has previously been reported that exposure of hCAR-expressing T cells to virus results in some activation [227], perhaps suggesting earlier expression of the ERK peaks in the intracellular staining experiments. Although this would not appear to be the case for the Rap1 activation, this may reflect the failure to measure total Rap expression in the FACS experiments as it can be seen here that Rap1 expression falls 24-96 h (Figure 4.12B), consistent with expression of a residual pool of highly activated Rap1 (Figure 4.12A).

The previous study from our lab shown that primed cells exhibited enhanced levels of active ERK (dually phosphorylated pERK) and reduced expression of Rap1 relative to anergic cells in the first 24 h of the maintenance phase both *in vitro* and

in vivo, consistent with the idea that Rap1 antagonised TCR-mediated Ras-dependent signalling by sequestering Raf-1 and disrupting coupling to ERK [144]. Following this line, the Ras-Raf-MEK-ERK signalling cascade has now been examined during induction and the later stages of the maintenance phases with respect to Rap1 activation rather than just Rap1 expression. Consistent with our previous findings, the inverse relationship between Rap1 and phosphorylated ERK expression occurred during maintenance phase of induction of anergy and priming [14], the ERK activation by $CD4^+$ T cells in the blasting and resting gate indicated that primed T cells expressed higher levels than anergic T cells and indeed, anergic T cells expressed lower levels than naïve cells which undergo a primary response on day 1 following challenge with antigen during maintenance phase. However, there was no real apparent change in Rap activity detected amongst the 3 blasting groups on day 1 although, analysis of ERK and Rap1 activation by ratio of ERK: Rap1 activation representing counter-regulation emphasised this inverse relationship. As shown in Figure 3.13G, the primed cells exhibited highest ratio of ERK: Rap1 activation in both blasting and resting cells on day 1. Additionally, the elevated levels of ERK and Rap1 in naïve, relative to anergic, cells is consistent with the proposal by Cantrell's group [236] that Rap is important in primary responses. However, perhaps surprisingly, the anergic cells exhibited highest ERK activation but consistent with counter-regulatory signaling, primed cells displayed the lowest levels of ERK and the highest levels of Rap1 activation on day 3. Nevertheless, these data were consistent with the cellular proliferation which showed that the primed cells proliferated much faster and more than anergic cells at the first two days but anergic cells are able to catch up at day 3 and day 4 and may indicate that the maintenance of tolerance may be "leaky" in this in vitro system. Alternatively, the other possibility is that not all "anergic" and "primed" cells which induced with antibodies are anergic and primed since no efficient markers or

methods to ensure the cells treated with anti-CD3 or anti-CD3/anti-CD28 are “all” induced to anergic and primed cells. Moreover, it may suggest that counter-regulatory Erk-Rap1 signalling functions to homeostatically regulate proliferation in the antigen-rechallenge maintenance phase with high ERK/Rap1 signalling for proliferation whilst a switch to low Erk/Rap1 could function as a negative feedback signal and is reminiscent of Boussiotis proposal that high Rap1 activity may be representative of T cell unresponsiveness in general, for example in Tregs or anergy cells [26, 31, 237], or in this case, during the termination of primed responses.

5.3 Modulation of Rap1 in regulation of MAPK during induction of anergy and priming

As mentioned above, Th cell proliferation is upregulated when Rap 1 activation is switched off during induction of anergy and priming and thus, it was further investigated whether this reflected counter-regulation of ERK and Rap1 activation. As expected, there was no difference in ERK and Rap1 activation between GFP^- and GFP^+ cells transduced with Ad Rap1 WT during induction of anergy and priming (Figure 4.6 B &C) as the adenoviral system does not generally lead to overexpression of the gene of interest [227, 247]. Perhaps surprisingly, there was also no difference in the levels of ERK activation and active Rap1 in cells transduced with Ad Rap S17N during induction of anergy and priming as well despite the cells transduced with Ad Rap1 S17N proliferating more than the cells without Rap S17N during induction of anergy and priming. Although the GFP^+ data at the early time points indicates an increase in active Rap1 in cells transduced with either the WT and S17N constructs, these were considered artefacts of the very low numbers and percentage of cells that were GFP^+ at these time points.

Additionally, due to data for total Rap1 expression not being available in these studies, the absolute levels of Rap1 activation could not be determined here as analysing the levels of active Rap1 expression only does not give the true picture of the relative cellular Rap1 activation. Nevertheless, overall, the anti-CD3 treated Ad Rap1 S17N transduced cells (the cells switching off Rap and under induction of anergy) despite exhibiting more proliferation than GFP^- cells do not show increased ERK activation, suggesting that other pathways may contribute for proliferation here and Rap1 may target other its downstream proteins such as RAPL and Mst1 (Figure 5.1A). RAPL, a critical Rap1 effector that regulates lymphocyte adhesion, negatively controls lymphocyte proliferation via modulation of lymphocyte function-associated antigen-1 (LFA-1) adhesiveness [248]. Alternatively, Rap1 has been reported to be an essential modulator of NF-κB-mediated pathways. As NF-κB is induced by ectopic expression of Rap1, whereas its activity is inhibited by Rap1 depletion [249].

Although the proliferation outcomes of mimicking more physiological conditions did not replicate the increased cellular proliferation of T cells stimulated with anti-CD3 or anti-CD3/anti-CD28 resulting from transduction with Ad Rap1 S17N, the effects of constructs on ERK and Rap1 activation were also examined under these conditions. The ERK activation exhibited the cyclic pattern in both GFP+ (S17N) and GFP- populations within either blasting or resting gates, similar the pattern observed previously [14] (Figure 4.11A &D). Furthermore, the level of Rap1 activation decreased in GFP^+ cells on day 2 but not in GFP^- cells, indicating the efficacy of the construct. This enhanced reduction of Rap1 activation in the GFP^+ cells was lost at day 3, with both GFP^- and GFP^+ cells showing similar levels at day 2, perhaps indicating that this is the basal level of Rap1 activity in these cells. Additionally, the gradual decline in Rap1 activity observed in GFP^- and GFP^+

populations following stimulation with anti-CD3 or anti-CD28 (Figure 3.12) is consistent with the kinetic pattern showed here (Figure 4.11B). Analysis of the ratio of pERK/active Rap showed a clear cycling pattern of increased ERK relative to Rap1 activation in the blasting but not resting cells, perhaps consistent with them progressing through the cell cycle. This was reflected by an enhanced relative level of ERK activation in the day 1 samples of GFP+ relative to GFP- cells, perhaps suggesting some degree of counter regulation that did not translate to enhanced proliferation at this time point. Alternatively, it may reflect that such counter-regulation was a requirement for induction of “late” priming signals.

Rather surprisingly, analysis of the levels and pattern of ERK and Rap1 activation in the cells transduced with Ad Rap1 WT in this model revealed that transduction of Rap1 promoted ERK activation (Figure 4.9C&D). Since cells transduced with Ad Rap1 WT did not exhibit different levels of Rap1 activation between GFP^- and GFP^+ populations but increased ERK activation, indicating the ectopic Rap1 somehow results in ERK activation independently of Rap1 activation (Figure 4.9F). Combining these two factors, the changes in the ratio of pERK/active Rap1 display with more dramatic patterns. Additionally, whilst the Rap1 activation in GFP^+ (S17N) cells drops at day 1 but not in GFP^- cells, indicating the dominant negative effects of this construct, both GFP^- and GFP^+ cells exhibited similar level of Rap1 activation at day3 (Figure 4.11B). By contrast, the level of Rap1 activation in both GFP^- and GFP^+ (WT) decrease and display similar levels at day 1, reflecting that the cells transduced with Ad Rap1 WT and S17N are at different stage in the kinetics of their response and also perhaps that the WT construct is exerting some Rap1 activation-independent effects that could reflect, for example, signalsome scaffolding and/or subcellular localisation effects relative to the endogenous element, possible due to defects in post-translational modification of the ectopic

Rap1 construct.

5.4 c-Myc activation during induction phase

The kinetic of pS62 c-Myc expression were consistent with that predicted following activation of pERK in both primed and tolerised cells at 24-96 h during induction phase. However, although the primed T cells showed higher levels of activation of ERK than anergic cells before 24 hours, generally the anergic T cells exhibited higher levels of pS62 c-Myc than primed T cells during induction phase. Surprisingly, the pT58 c-Myc expression more similarly reflected the pattern of ERK activation, a finding which had not been predicted as phosphorylation at T58 has the opposite role to that at S62, as it targets c-Myc for degradation (Figure 4.12C & 3.26C). Additionally, the total expression of c-Myc was strongly upregulated accumulating at later times in both anergic and primed T cells with anergic T cells expression higher levels of c-Myc at all the time points tested (Figure 4.12B&D). However, the delay in such accumulation and the apparent downregulation at the end of the timecourse occurred following the peaks in the proteasome targeting pT58 form. Although, this accumulation of c-Myc in anergic cells was surprising as c-Myc upregulation is typically associated with proliferation it may reflect its involvement [224] in other cellular processes such as apoptosis and may reflect homeostatic cell death in the end of induction of anergy and priming. Tracking c-Myc activation in a prolonged induction phase and its relative downstream targets may reveal however that this apparently similar signal may have very different functional outcomes for example in terms of gene induction and cell survival and apoptosis.

5.5 mTOR

5.5.1 mTORC1

Naïve cells exhibited high levels of phosphorylated Raptor at time zero and these were maintained for the first 4 hours of stimulation, perhaps indicating their emergence from a quiescient state and entry into cell cycle. Consistent with this, levels of pp70S6K largely increased between 8-24 h corresponding with the decline in pRaptor. S6K activity was then dramatically switched off (48-96h) which is coherent with the activation of AMPK (Figure 4.13A, C &E). This pattern illustrates the energy status and metabolism of the cells following the stimuli, from quiescient state to catabolic metabolism associated with proliferation, resulting in a low energy status in the cells that therefore leads to AMPK activation to inhibit mTORC1 pathway. Notably, the primed cells exhibited higher activation of AMPK than anergic T cells and correspondingly lower levels of S6K, the latter finding perhaps reflecting induction of anergy-specific genes. Additionally, unlike ERK expression in the induction phase, the total expression of AMPK, Raptor and p70 S6 kinase changed in response to the different stimulations. During induction of anergy and priming, the anergic cells expressed more AMPK and less S6K than primed cells, perhaps due to the low level of glucose within cells or other associated regulatory mechanisms. Thus further investigation of the energy and nutrition status of those cells are necessary and although initial metabolomics studies have been performed, at this stage the analysis is still awaited.

5.5.2 mTORC2

The pattern in phosphorylation of Rictor is consistent with pp70 S6 kinase in both anergic and primed cells. As seen in Figure 4.13E & 3.27A, the anergic cells exhibited higher proportion of pp70 S6 kinase than primed cells most of the time,

this reflects in the phosphorylation of Rictor as well. Thus the anergic cell exhibited higher phosphorylation of Rictor than primed cells from beginning of culture until 48 hour at which point the primed cells displayed increasing pRictor. This suggests that the anergic cells exhibited lower activation of mTORC2 than primed for the first 48 hours after receiving stimulation. It is also clear, the primed cells showed higher pRictor than anergic cells after 48 hour, which consistent with the pAKT expression between these two populations. This may suggest that in anergic T cells, activation of the mTORC2 pathway was triggered to promote cell survival after 48 hours.

To sum up, both mTORC1 and mTORC2 delivery details about energy status and metabolism in anergic and primed cells. The cellular proliferations occurred in both populations are accompany with low evergy and lead to AMPK activation. Surprisingly, the primed cells exhibited lower activation of mTORC1 than anergic. This may due to low energy consuming by rapid proliferation during induction of priming. On the other hand, the anergic T cells exhibited higher activity of mTORC1 during 8-24 h, which reflected in higher expression in pp70S6K, may also explain that the proliferation progress of anergic T cells were able to catch up primed t cells after 48 h during induction of anergy and priming. Consistent with this, the anergic T cells exhibited higher activation of mTORC2 than primed T cell at 48 h after receiving stimulation, which also refer to similar behavior pattern occurred in anergic T cells. However, the further investigation in downstream molecules are required, because upregulation of mTORC1 activity during 8-24 h in induction of anergy occurred earlier than the time point of increasing the activity of mTORC2. It is more likely that mTORC1 contributes to the signaling which involve in cellular rescue and survival and mTORC2 participates after mTORC1 been activated.

5.6 Potential mechanism for Rap1 S17N in enhanced proliferation under *in vitro* and physiological model

Rap1, a molecule originally identified as an integrin regulator, also plays an inhibitory role in cell proliferation and provides a molecular link with cell adhesion and growth. Rap1 induces cell adhesion via RAPL-Mst1-LFA-1 signaling upon TCR stimulation (Figure 5.1A) [250, 251]. Interestingly, several studies have shown that proliferative responses were enhanced in RAPL- and Mst1-deficient T cells (Figure 5.1B) [252, 253]. RAPL-deficient T cells exhibited increasing DNA synthesis in response to stimulation of anti-CD3 and anti-CD3/CD28, accompanying with similar levels in activation of proliferative signaling pathways, MAPK, NF-κB and PI-3K [253]. The evidence revealed that the enhanced proliferation in RAPL-deficient T cell during stimulation of anti-CD3 in presence or absence of anti-CD28 was due to mislocalization of p27kip1, inhibitor for Cdk2. This occurred via kinase interacting stathmin (KIS) mediated-upregulation of Serine 10 phosphorylation in p27 that inhibited nuclear localization of p27 in T cell, leading to an increased Cdk2 activity in nucleus, which contributes G_1-S phase transition in lymphocytes. As partial inhibition of hyperproliferation was observed in S10 mutation of p27 in RAPL-deficient T cell, it suggests that other mechanisms like Mst1 may involve in the enhanced proliferation in RAPL-deficient T cell (Figure 5.2). Although the hyperproliferative response was also observed in Mst1-deficient T cell, the enhanced proliferation was regulated independently of p27 [253]. The work described in my study indicated that both anti-CD3 and anti-CD3/CD28-treated cells transduced with Ad Rap1 S17N exhibited enhanced proliferation, and no difference in activation of ERK, which might be able to be explained by Katagiri's paper [253]. The hypothesis would be that Rap1 S17N impairs the

signaling in RAP1-1RAPL pathway, the cellular adhesion is abolished and cellular proliferation is enhanced via upregulated Serine 10 phosphorylation in p27 via increased KIS activity, which leads an increase of nuclear Cdk2 activity and promotes cell cycle (Figure 5.2). Alternatively, as mentioned above, abrogating the signaling pathway in RAPL by Rap1 S17N, which in turn disrupts Mst1 signaling, might offer another possible explanation on enhanced proliferation result. Although the proliferation outcomes of mimicking more physiological conditions did not replicate the increased cellular proliferation of T cells stimulated with anti-CD3 or anti-CD3/CD28 resulting from transduction with Ad Rap1 S17N, this did not mean that the model showed in Figure 5.2 was wrong. Since the Ad Rap1 S17N transduced T cells co-cultured with OVA-loaded, LPS-matured DC received not only signal I and signal II, other molecules, e.g. integrin, triggering other signaling pathways also involved while DCs present antigen to T cells. As Rap1 can play dual functions in inhibition of cell proliferation and induction of cell adhesion in T cell, with this line, the hypothesis would be that Ad Rap1 S17N transduced T cell received “positive” signal promoting cell proliferation from TCR ligation and co-stimulation (Figure 5.3B), which replicated the antibody treatment model (anti-CD3/CD28) (Figure 5.3A) and “negative” signal inhibiting cell proliferation via integrin signaling pathway (Figure 5.3B). Overall, Ad Rap1 S17N transduced T cell received both “positive” and “negative” signals from DC, no more “enhanced” signal appeared in cellular proliferation between GFP- and GFP+ (Rap1 S17N) cells underlying this physiological model.

Figure 5.1 Rap1 induced cell adhesion pathway and its downstream effectors.

(A) Rap1 mediates cell adhesion via its effector RAPL associating with Mst1 and leads to LFA-1 clustering in turns promoting cell adhesion and migration. (B) Both RAPL-deficient and Mst1-deficient lymphocytes exhibit enhanced cellular proliferation.

Figure 5.2 Potential mechanism for Rap1 S17N in *in vitro* model.

Figure 5.3 Potential mechanism for Rap1 S17N regulating cellular proliferation underlying in vitro and physiological models.

(A) *In vitro* model: Ad Rap1 S17N transduced LN were stimulated with antibodies: anti-CD3+anti-CD28. (B) Mimicking physiological model: Ad Rap1 S17N transduced LN were co-culture with OVA-loaded, LPS-matured DC. In this more physiological model, Ad Rap1 S17N transduced LN received “positive” signal for enhancing cellular proliferation from TCR ligation and co-stimulation, and “negative” signal for integrin mediated-cell adhesion from DC.

Bibliography

1. Abbas, A.K. and C.A. Janeway, Jr., *Immunology: improving on nature in the twenty-first century.* Cell, 2000. 100(1): p. 129-38.
2. Lund, F.E., et al., *Regulatory roles for cytokine-producing B cells in infection and autoimmune disease.* Curr Dir Autoimmun, 2005. 8: p. 25-54.
3. Pennington, D.J., B. Silva-Santos, and A.C. Hayday, *Gammadelta T cell development--having the strength to get there.* Curr Opin Immunol, 2005. 17(2): p. 108-15.
4. Godfrey, D.I., S. Stankovic, and A.G. Baxter, *Raising the NKT cell family.* Nat Immunol, 2010. 11(3): p. 197-206.
5. Hedrick, S.M., *Thymus lineage commitment: a single switch.* Immunity, 2008. 28(3): p. 297-9.
6. Jenkinson, E.J., et al., *The thymus and T-cell commitment: the right niche for Notch?* Nat Rev Immunol, 2006. 6(7): p. 551-5.
7. Takahama, Y., *Journey through the thymus: stromal guides for T-cell development and selection.* Nat Rev Immunol, 2006. 6(2): p. 127-35.
8. Bonilla, F.A. and H.C. Oettgen, *Adaptive immunity.* J Allergy Clin Immunol, 2010. 125(2 Suppl 2): p. S33-40.
9. Hutloff, A., et al., *ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28.* Nature, 1999. 397(6716): p. 263-6.
10. Coyle, A.J., et al., *The CD28-related molecule ICOS is required for effective T cell-dependent immune responses.* Immunity, 2000. 13(1): p. 95-105.
11. McAdam, A.J., et al., *Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells.* J Immunol, 2000. 165(9): p. 5035-40.
12. Smith, K.M., et al., *Inducible costimulatory molecule-B7-related protein 1 interactions are important for the clonal expansion and B cell helper functions of naive, Th1, and Th2 T cells.* J Immunol, 2003. 170(5): p. 2310-5.
13. Adams, C.L., et al., *Differences in the kinetics, amplitude, and localization of ERK activation in anergy and priming revealed at the level of individual primary T cells by laser scanning cytometry.* J Immunol, 2004. 173(3): p. 1579-86.
14. Morton, A.M., et al., *Inverse Rap1 and phospho-ERK expression discriminate the maintenance phase of tolerance and priming of antigen-specific CD4+ T cells in vitro and in vivo.* J Immunol, 2007. 179(12): p. 8026-34.
15. Cronin, S.J. and J.M. Penninger, *From T-cell activation signals to signaling control of anti-cancer immunity.* Immunol Rev, 2007. 220: p. 151-68.
16. Louten, J., K. Boniface, and R. de Waal Malefyt, *Development and function of TH17 cells in health and disease.* J Allergy Clin Immunol, 2009. 123(5): p.

1004-11.

17. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. 441(7090): p. 235-8.
18. Mangan, P.R., et al., *Transforming growth factor-beta induces development of the T(H)17 lineage.* Nature, 2006. 441(7090): p. 231-4.
19. Veldhoen, M., et al., *TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells.* Immunity, 2006. 24(2): p. 179-89.
20. Zhou, L., et al., *IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways.* Nat Immunol, 2007. 8(9): p. 967-74.
21. Korn, T., et al., *IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells.* Nature, 2007. 448(7152): p. 484-7.
22. Nurieva, R., et al., *Essential autocrine regulation by IL-21 in the generation of inflammatory T cells.* Nature, 2007. 448(7152): p. 480-3.
23. Steinman, L., *A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage.* Nat Med, 2007. 13(2): p. 139-45.
24. Davidson, T.S., et al., *Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells.* J Immunol, 2007. 178(7): p. 4022-6.
25. Tran, D.Q., H. Ramsey, and E.M. Shevach, *Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype.* Blood, 2007. 110(8): p. 2983-90.
26. Schwartz, R.H., *Natural regulatory T cells and self-tolerance.* Nat Immunol, 2005. **6**(4): p. 327-30.
27. Matzinger, P., *Tolerance, danger, and the extended family.* Annu Rev Immunol, 1994. 12: p. 991-1045.
28. Rocha, G., M.G. Baines, and J. Deschenes, *The immunology of the eye and its systemic interactions.* Crit Rev Immunol, 1992. 12(3-4): p. 81-100.
29. Alegre, M.L., K.A. Frauwirth, and C.B. Thompson, *T-cell regulation by CD28 and CTLA-4.* Nat Rev Immunol, 2001. 1(3): p. 220-8.
30. Lechler, R., et al., *T-cell anergy and peripheral T-cell tolerance.* Philos Trans R Soc Lond B Biol Sci, 2001. 356(1409): p. 625-37.
31. Boussiotis, V.A., et al., *Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1.* Science, 1997. 278(5335): p. 124-8.
32. Jenkins, M.K., et al., *Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody.* J Immunol, 1990. 144(1): p. 16-22.
33. Quill, H. and R.H. Schwartz, *Stimulation of normal inducer T cell clones with*

antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. J Immunol, 1987. 138(11): p. 3704-12.

34. Chai, J.G. and R.I. Lechler, *Immobilized anti-CD3 mAb induces anergy in murine naive and memory CD4+ T cells in vitro.* Int Immunol, 1997. 9(7): p. 935-44.
35. DeSilva, D.R., K.B. Urdahl, and M.K. Jenkins, *Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation.* J Immunol, 1991. 147(10): p. 3261-7.
36. Smith, K.M., J.M. Davidson, and P. Garside, *T-cell activation occurs simultaneously in local and peripheral lymphoid tissue following oral administration of a range of doses of immunogenic or tolerogenic antigen although tolerized T cells display a defect in cell division.* Immunology, 2002. 106(2): p. 144-58.
37. Van Houten, N. and S.F. Blake, *Direct measurement of anergy of antigen-specific T cells following oral tolerance induction.* J Immunol, 1996. 157(4): p. 1337-41.
38. Hathcock, K.S., et al., *Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function.* J Exp Med, 1994. 180(2): p. 631-40.
39. Kawamura, T. and M. Furue, *Comparative analysis of B7-1 and B7-2 expression in Langerhans cells: differential regulation by T helper type 1 and T helper type 2 cytokines.* Eur J Immunol, 1995. 25(7): p. 1913-7.
40. Bluestone, J.A., *Is CTLA-4 a master switch for peripheral T cell tolerance?* J Immunol, 1997. 158(5): p. 1989-93.
41. Lane, P., C. Haller, and F. McConnell, *Evidence that induction of tolerance in vivo involves active signaling via a B7 ligand-dependent mechanism: CTLA4-Ig protects V beta 8+ T cells from tolerance induction by the superantigen staphylococcal enterotoxin B.* Eur J Immunol, 1996. 26(4): p. 858-62.
42. Liu, L., V.K. Kuchroo, and H.L. Weiner, *B7.2 (CD86) but not B7.1 (CD80) costimulation is required for the induction of low dose oral tolerance.* J Immunol, 1999. 163(4): p. 2284-90.
43. Perez, V.L., et al., *Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement.* Immunity, 1997. 6(4): p. 411-7.
44. Miller, A., et al., *Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering.* Proc Natl Acad Sci U S A, 1992. 89(1): p. 421-5.
45. Lider, O., et al., *Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. II. Suppression of disease and in vitro immune responses is mediated by antigen-specific CD8+ T lymphocytes.* J Immunol, 1989. 142(3): p. 748-52.

46. Grdic, D., et al., *Lack of local suppression in orally tolerant CD8-deficient mice reveals a critical regulatory role of CD8+ T cells in the normal gut mucosa.* J Immunol, 1998. 160(2): p. 754-62.
47. Joosten, S.A., et al., *Identification of a human CD8+ regulatory T cell subset that mediates suppression through the chemokine CC chemokine ligand 4.* Proc Natl Acad Sci U S A, 2007. 104(19): p. 8029-34.
48. Garside, P., et al., *CD4+ but not CD8+ T cells are required for the induction of oral tolerance.* Int Immunol, 1995. 7(3): p. 501-4.
49. Fowler, S. and F. Powrie, *CTLA-4 expression on antigen-specific cells but not IL-10 secretion is required for oral tolerance.* Eur J Immunol, 2002. 32(10): p. 2997-3006.
50. Thorstenson, K.M. and A. Khoruts, *Generation of anergic and potentially immunoregulatory CD25+CD4 T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen.* J Immunol, 2001. 167(1): p. 188-95.
51. Shortman, K. and L. Wu, *Early T lymphocyte progenitors.* Annu Rev Immunol, 1996. 14: p. 29-47.
52. Zuniga-Pflucker, J.C. and M.J. Lenardo, *Regulation of thymocyte development from immature progenitors.* Curr Opin Immunol, 1996. 8(2): p. 215-24.
53. Dudley, E.C., et al., *T cell receptor beta chain gene rearrangement and selection during thymocyte development in adult mice.* Immunity, 1994. 1(2): p. 83-93.
54. von Boehmer, H., et al., *Pleiotropic changes controlled by the pre-T-cell receptor.* Curr Opin Immunol, 1999. 11(2): p. 135-42.
55. McGeachy, M.J. and D.J. Cua, *T cells doing it for themselves: TGF-beta regulation of Th1 and Th17 cells.* Immunity, 2007. 26(5): p. 547-9.
56. Kyewski, B. and J. Derbinski, *Self-representation in the thymus: an extended view.* Nat Rev Immunol, 2004. 4(9): p. 688-98.
57. Derbinski, J., et al., *Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels.* J Exp Med, 2005. 202(1): p. 33-45.
58. Smith, K.M., et al., *Pancreatic gene expression in rare cells of thymic medulla: evidence for functional contribution to T cell tolerance.* Int Immunol, 1997. 9(9): p. 1355-65.
59. Chen, Y., J. Inobe, and H.L. Weiner, *Inductive events in oral tolerance in the TCR transgenic adoptive transfer model.* Cell Immunol, 1997. 178(1): p. 62-8.
60. Gonnella, P.A., et al., *In situ immune response in gut-associated lymphoid tissue (GALT) following oral antigen in TCR-transgenic mice.* J Immunol, 1998. 160(10): p. 4708-18.
61. Zamoyska, R., et al., *The influence of the src-family kinases, Lck and Fyn, on T cell differentiation, survival and activation.* Immunol Rev, 2003. 191: p. 107-18.

62. Iwasaki, A. and B.L. Kelsall, *Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells.* J Exp Med, 1999. 190(2): p. 229-39.
63. Buday, L., et al., *A complex of Grb2 adaptor protein, Sos exchange factor, and a 36-kDa membrane-bound tyrosine phosphoprotein is implicated in ras activation in T cells.* J Biol Chem, 1994. 269(12): p. 9019-23.
64. Chan, A.C., et al., *ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain.* Cell, 1992. 71(4): p. 649-62.
65. Sommers, C.L., et al., *Knock-in mutation of the distal four tyrosines of linker for activation of T cells blocks murine T cell development.* J Exp Med, 2001. 194(2): p. 135-42.
66. Feske, S., *Calcium signalling in lymphocyte activation and disease.* Nat Rev Immunol, 2007. 7(9): p. 690-702.
67. Manicassamy, S., et al., *Requirement of calcineurin a beta for the survival of naive T cells.* J Immunol, 2008. 180(1): p. 106-12.
68. Crabtree, G.R. and E.N. Olson, *NFAT signaling: choreographing the social lives of cells.* Cell, 2002. 109 Suppl: p. S67-79.
69. Chen, L., et al., *Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA.* Nature, 1998. 392(6671): p. 42-8.
70. Guerrero, C., et al., *Dopamine activates ERKs in alveolar epithelial cells via Ras-PKC-dependent and Grb2/Sos-independent mechanisms.* Am J Physiol Lung Cell Mol Physiol, 2002. 282(5): p. L1099-107.
71. Ebinu, J.O., et al., *RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs.* Science, 1998. 280(5366): p. 1082-6.
72. Bivona, T.G., et al., *Phospholipase Cgamma activates Ras on the Golgi apparatus by means of RasGRP1.* Nature, 2003. 424(6949): p. 694-8.
73. Cullen, P.J. and P.J. Lockyer, *Integration of calcium and Ras signalling.* Nat Rev Mol Cell Biol, 2002. 3(5): p. 339-48.
74. Ebinu, J.O., et al., *RasGRP links T-cell receptor signaling to Ras.* Blood, 2000. 95(10): p. 3199-203.
75. Elion, E.A., *Routing MAP kinase cascades.* Science, 1998. 281(5383): p. 1625-6.
76. Ward, Y., et al., *Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1.* Nature, 1994. 367(6464): p. 651-4.
77. Gille, H., A.D. Sharrocks, and P.E. Shaw, *Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter.* Nature, 1992. 358(6385): p. 414-7.
78. Price, M.A., F.H. Cruzalegui, and R. Treisman, *The p38 and ERK MAP kinase pathways cooperate to activate Ternary Complex Factors and c-fos transcription in response to UV light.* EMBO J, 1996. 15(23): p. 6552-63.

79. Raingeaud, J., et al., *Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine.* J Biol Chem, 1995. 270(13): p. 7420-6.
80. Perez de Castro, I., et al., *Ras activation in Jurkat T cells following low-grade stimulation of the T-cell receptor is specific to N-Ras and occurs only on the Golgi apparatus.* Mol Cell Biol, 2004. 24(8): p. 3485-96.
81. Smith, E.R., et al., *Disassociation of MAPK activation and c-Fos expression in F9 embryonic carcinoma cells following retinoic acid-induced endoderm differentiation.* J Biol Chem, 2001. 276(34): p. 32094-100.
82. Isakov, N. and A. Altman, *Protein kinase C(theta) in T cell activation.* Annu Rev Immunol, 2002. 20: p. 761-94.
83. Tran, N.H. and J.A. Frost, *Phosphorylation of Raf-1 by p21-activated kinase 1 and Src regulates Raf-1 autoinhibition.* J Biol Chem, 2003. 278(13): p. 11221-6.
84. Fathman, C.G. and N.B. Lineberry, *Molecular mechanisms of CD4+ T-cell anergy.* Nat Rev Immunol, 2007. 7(8): p. 599-609.
85. Janknecht, R., et al., *Activation of ternary complex factor Elk-1 by MAP kinases.* EMBO J, 1993. 12(13): p. 5097-104.
86. Fruman, D.A., et al., *Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha.* Science, 1999. 283(5400): p. 393-7.
87. Fruman, D.A. and G. Bismuth, *Fine tuning the immune response with PI3K.* Immunol Rev, 2009. 228(1): p. 253-72.
88. Park, S.G., et al., *The kinase PDK1 integrates T cell antigen receptor and CD28 coreceptor signaling to induce NF-kappaB and activate T cells.* Nat Immunol, 2009. 10(2): p. 158-66.
89. Frias, M.A., et al., *mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s.* Curr Biol, 2006. 16(18): p. 1865-70.
90. Cantrell, D., *Protein kinase B (Akt) regulation and function in T lymphocytes.* Semin Immunol, 2002. 14(1): p. 19-26.
91. Otero, D.C., S.A. Omori, and R.C. Rickert, *Cd19-dependent activation of Akt kinase in B-lymphocytes.* J Biol Chem, 2001. 276(2): p. 1474-8.
92. Jones, R.G., et al., *Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo.* J Exp Med, 2000. 191(10): p. 1721-34.
93. Mora, A., et al., *PDK1, the master regulator of AGC kinase signal transduction.* Semin Cell Dev Biol, 2004. 15(2): p. 161-70.
94. Coudronniere, N., et al., *NF-kappa B activation induced by T cell receptor/CD28 costimulation is mediated by protein kinase C-theta.* Proc Natl Acad Sci U S A, 2000. 97(7): p. 3394-9.
95. Sun, Z., et al., *PKC-theta is required for TCR-induced NF-kappaB activation in*

mature but not immature T lymphocytes. Nature, 2000. 404(6776): p. 402-7.

96. Pfeifhofer, C., et al., *Protein kinase C theta affects Ca2+ mobilization and NFAT cell activation in primary mouse T cells.* J Exp Med, 2003. 197(11): p. 1525-35.
97. Valensin, S., et al., *F-actin dynamics control segregation of the TCR signaling cascade to clustered lipid rafts.* Eur J Immunol, 2002. 32(2): p. 435-46.
98. Reynolds, L.F., et al., *Vav1 transduces T cell receptor signals to the activation of phospholipase C-gamma1 via phosphoinositide 3-kinase-dependent and -independent pathways.* J Exp Med, 2002. 195(9): p. 1103-14.
99. Zeng, R., et al., *SLP-76 coordinates Nck-dependent Wiskott-Aldrich syndrome protein recruitment with Vav-1/Cdc42-dependent Wiskott-Aldrich syndrome protein activation at the T cell-APC contact site.* J Immunol, 2003. 171(3): p. 1360-8.
100. Lenormand, P., et al., *Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts.* J Cell Biol, 1993. 122(5): p. 1079-88.
101. Mor, A., M.L. Dustin, and M.R. Philips, *Small GTPases and LFA-1 reciprocally modulate adhesion and signaling.* Immunol Rev, 2007. 218: p. 114-25.
102. Yong, P.F., U. Salzer, and B. Grimbacher, *The role of costimulation in antibody deficiencies: ICOS and common variable immunodeficiency.* Immunol Rev, 2009. 229(1): p. 101-13.
103. Inaba, K., et al., *The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro.* J Exp Med, 1994. 180(5): p. 1849-60.
104. Samoilova, E.B., et al., *CTLA-4 is required for the induction of high dose oral tolerance.* Int Immunol, 1998. 10(4): p. 491-8.
105. Murphy, T.L. and K.M. Murphy, *Slow down and survive: Enigmatic immunoregulation by BTLA and HVEM.* Annu Rev Immunol, 2010. 28: p. 389-411.
106. Brunet, J.F., et al., *A new member of the immunoglobulin superfamily--CTLA-4.* Nature, 1987. 328(6127): p. 267-70.
107. Linsley, P.S., et al., *CTLA-4 is a second receptor for the B cell activation antigen B7.* J Exp Med, 1991. 174(3): p. 561-9.
108. Brunner-Weinzierl, M.C., H. Hoff, and G.R. Burmester, *Multiple functions for CD28 and cytotoxic T lymphocyte antigen-4 during different phases of T cell responses: implications for arthritis and autoimmune diseases.* Arthritis Res Ther, 2004. 6(2): p. 45-54.
109. Tivol, E.A., et al., *Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4.* Immunity, 1995. 3(5): p. 541-7.
110. Perrin, P.J., et al., *Role of B7:CD28/CTLA-4 in the induction of chronic*

relapsing experimental allergic encephalomyelitis. J Immunol, 1995. 154(3): p. 1481-90.

111. Chen, Y., Y. Ma, and Y. Chen, *Roles of cytotoxic T-lymphocyte-associated antigen-4 in the inductive phase of oral tolerance.* Immunology, 2002. 105(2): p. 171-80.
112. Walunas, T.L., C.Y. Bakker, and J.A. Bluestone, *CTLA-4 ligation blocks CD28-dependent T cell activation.* J Exp Med, 1996. 183(6): p. 2541-50.
113. Brunner, M.C., et al., *CTLA-4-Mediated inhibition of early events of T cell proliferation.* J Immunol, 1999. 162(10): p. 5813-20.
114. Krummel, M.F. and J.P. Allison, *CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells.* J Exp Med, 1996. 183(6): p. 2533-40.
115. Olsson, C., et al., *CTLA-4 ligation suppresses CD28-induced NF-kappaB and AP-1 activity in mouse T cell blasts.* J Biol Chem, 1999. 274(20): p. 14400-5.
116. Li, D., et al., *Cutting edge: Cbl-b: one of the key molecules tuning CD28- and CTLA-4-mediated T cell costimulation.* J Immunol, 2004. 173(12): p. 7135-9.
117. Keir, M.E., et al., *PD-1 and its ligands in tolerance and immunity.* Annu Rev Immunol, 2008. 26: p. 677-704.
118. Okazaki, T. and T. Honjo, *The PD-1-PD-L pathway in immunological tolerance.* Trends Immunol, 2006. 27(4): p. 195-201.
119. Augello, A., et al., *Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway.* Eur J Immunol, 2005. 35(5): p. 1482-90.
120. Kinter, A.L., et al., *The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands.* J Immunol, 2008. 181(10): p. 6738-46.
121. Nakae, S., et al., *Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF.* J Immunol, 2006. 176(4): p. 2238-48.
122. Nishimura, H., et al., *Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor.* Immunity, 1999. 11(2): p. 141-51.
123. Reynoso, E.D., et al., *Intestinal tolerance is converted to autoimmune enteritis upon PD-1 ligand blockade.* J Immunol, 2009. 182(4): p. 2102-12.
124. Stoka, V., et al., *Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route.* J Biol Chem, 2001. 276(5): p. 3149-57.
125. Alberola-Ila, J., et al., *Differential signaling by lymphocyte antigen receptors.* Annu Rev Immunol, 1997. 15: p. 125-54.
126. Huang, C.T., et al., *CD4+ T cells pass through an effector phase during the process of in vivo tolerance induction.* J Immunol, 2003. 170(8): p. 3945-53.

127. Smith, J.A., et al., *Nonmitogenic anti-CD3 monoclonal antibodies deliver a partial T cell receptor signal and induce clonal anergy.* J Exp Med, 1997. 185(8): p. 1413-22.
128. Dubois, P.M., et al., *T cell long-term hyporesponsiveness follows antigen receptor engagement and results from defective signal transduction.* Eur J Immunol, 1994. 24(2): p. 348-54.
129. Gajewski, T.F., et al., *Anergic T-lymphocyte clones have altered inositol phosphate, calcium, and tyrosine kinase signaling pathways.* Proc Natl Acad Sci U S A, 1994. 91(1): p. 38-42.
130. Quill, H., et al., *Anergic Th1 cells express altered levels of the protein tyrosine kinases p56lck and p59fyn.* J Immunol, 1992. 149(9): p. 2887-93.
131. Yu, S.C. and B. Nag, *Differential expression of protein tyrosine kinases and their phosphorylation in murine Th1 cells anergized with class II MHC-peptide complexes.* Immunol Cell Biol, 1997. 75(3): p. 295-302.
132. Asai, K., et al., *T cell hyporesponsiveness induced by oral administration of ovalbumin is associated with impaired NFAT nuclear translocation and p27kip1 degradation.* J Immunol, 2002. 169(9): p. 4723-31.
133. Hundt, M., et al., *Impaired activation and localization of LAT in anergic T cells as a consequence of a selective palmitoylation defect.* Immunity, 2006. 24(5): p. 513-22.
134. Berg-Brown, N.N., et al., *PKCtheta signals activation versus tolerance in vivo.* J Exp Med, 2004. 199(6): p. 743-52.
135. Safford, M., et al., *Egr-2 and Egr-3 are negative regulators of T cell activation.* Nat Immunol, 2005. 6(5): p. 472-80.
136. Li, W., et al., *Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells.* Science, 1996. 271(5253): p. 1272-6.
137. Mondino, A., et al., *Defective transcription of the IL-2 gene is associated with impaired expression of c-Fos, FosB, and JunB in anergic T helper 1 cells.* J Immunol, 1996. 157(5): p. 2048-57.
138. DeSilva, D.R., et al., *Anergic T cells are defective in both jun NH2-terminal kinase and mitogen-activated protein kinase signaling pathways.* J Exp Med, 1996. 183(5): p. 2017-23.
139. Fields, P.E., T.F. Gajewski, and F.W. Fitch, *Blocked Ras activation in anergic CD4+ T cells.* Science, 1996. 271(5253): p. 1276-8.
140. Kang, S.M., et al., *Transactivation by AP-1 is a molecular target of T cell clonal anergy.* Science, 1992. 257(5073): p. 1134-8.
141. Dower, N.A., et al., *RasGRP is essential for mouse thymocyte differentiation and TCR signaling.* Nat Immunol, 2000. 1(4): p. 317-21.
142. Olenchock, B.A., et al., *Disruption of diacylglycerol metabolism impairs the induction of T cell anergy.* Nat Immunol, 2006. 7(11): p. 1174-81.
143. Zha, Y., et al., *T cell anergy is reversed by active Ras and is regulated by*

diacylglycerol kinase-alpha. Nat Immunol, 2006. 7(11): p. 1166-73.

144. Raaijmakers, J.H. and J.L. Bos, *Specificity in Ras and Rap signaling.* J Biol Chem, 2009. 284(17): p. 10995-9.
145. Liu, L.M. and G.G. MacPherson, *Antigen acquisition by dendritic cells: intestinal dendritic cells acquire antigen administered orally and can prime naive T cells in vivo.* J Exp Med, 1993. 177(5): p. 1299-307.
146. Abraham, R.T., *Antigen receptors rap to integrin receptors.* Nat Immunol, 2002. 3(3): p. 212-3.
147. Frech, M., et al., *Inhibition of GTPase activating protein stimulation of Ras-p21 GTPase by the Krev-1 gene product.* Science, 1990. 249(4965): p. 169-71.
148. Kitayama, H., et al., *A ras-related gene with transformation suppressor activity.* Cell, 1989. 56(1): p. 77-84.
149. Hata, Y., et al., *Inhibition of the ras p21 GTPase-activating protein-stimulated GTPase activity of c-Ha-ras p21 by smg p21 having the same putative effector domain as ras p21s.* J Biol Chem, 1990. 265(13): p. 7104-7.
150. Czyzyk, J., et al., *Combinatorial effect of T-cell receptor ligation and CD45 isoform expression on the signaling contribution of the small GTPases Ras and Rap1.* Mol Cell Biol, 2000. 20(23): p. 8740-7.
151. Appleman, L.J. and V.A. Boussiotis, *T cell anergy and costimulation.* Immunol Rev, 2003. 192: p. 161-80.
152. Carey, K.D., et al., *CD28 and the tyrosine kinase lck stimulate mitogen-activated protein kinase activity in T cells via inhibition of the small G protein Rap1.* Mol Cell Biol, 2000. 20(22): p. 8409-19.
153. Reedquist, K.A. and J.L. Bos, *Costimulation through CD28 suppresses T cell receptor-dependent activation of the Ras-like small GTPase Rap1 in human T lymphocytes.* J Biol Chem, 1998. 273(9): p. 4944-9.
154. Dillon, T.J., et al., *Ectopic B-Raf expression enhances extracellular signal-regulated kinase (ERK) signaling in T cells and prevents antigen-presenting cell-induced anergy.* J Biol Chem, 2003. 278(38): p. 35940-9.
155. Tanaka, S., et al., *C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of CRK and GRB2/ASH proteins.* Proc Natl Acad Sci U S A, 1994. 91(8): p. 3443-7.
156. Hattori, S. and M. Matsuda, *[Activation of Rap1, antagonist to ras, by Crk-C3G].* Gan To Kagaku Ryoho, 1997. 24(11): p. 1414-21.
157. Swaminathan, G. and A.Y. Tsygankov, *The Cbl family proteins: ring leaders in regulation of cell signaling.* J Cell Physiol, 2006. 209(1): p. 21-43.
158. Simons, K. and D. Toomre, *Lipid rafts and signal transduction.* Nat Rev Mol Cell Biol, 2000. 1(1): p. 31-9.
159. Horejsi, V., *The roles of membrane microdomains (rafts) in T cell activation.*

Immunol Rev, 2003. 191: p. 148-64.

160. Monks, C.R., et al., *Three-dimensional segregation of supramolecular activation clusters in T cells.* Nature, 1998. 395(6697): p. 82-6.
161. Lee, K.H., et al., *T cell receptor signaling precedes immunological synapse formation.* Science, 2002. 295(5559): p. 1539-42.
162. Freiberg, B.A., et al., *Staging and resetting T cell activation in SMACs.* Nat Immunol, 2002. 3(10): p. 911-7.
163. Stone, J.D., et al., *Aberrant TCR-mediated signaling in CD45-null thymocytes involves dysfunctional regulation of Lck, Fyn, TCR-zeta, and ZAP-70.* J Immunol, 1997. 158(12): p. 5773-82.
164. Sanchez-Madrid, F. and M.A. del Pozo, *Leukocyte polarization in cell migration and immune interactions.* EMBO J, 1999. 18(3): p. 501-11.
165. Carlin, L.M., et al., *Secretion of IFN-gamma and not IL-2 by anergic human T cells correlates with assembly of an immature immune synapse.* Blood, 2005. 106(12): p. 3874-9.
166. Laplante, M. and D.M. Sabatini, *mTOR signaling at a glance.* J Cell Sci, 2009. 122(Pt 20): p. 3589-94.
167. Zheng, Y., et al., *Anergic T cells are metabolically anergic.* J Immunol, 2009. 183(10): p. 6095-101.
168. Frauwirth, K.A., et al., *The CD28 signaling pathway regulates glucose metabolism.* Immunity, 2002. 16(6): p. 769-77.
169. Wieman, H.L., J.A. Wofford, and J.C. Rathmell, *Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking.* Mol Biol Cell, 2007. 18(4): p. 1437-46.
170. Powell, J.D., C.G. Lerner, and R.H. Schwartz, *Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation.* J Immunol, 1999. 162(5): p. 2775-84.
171. Delgoffe, G.M., et al., *The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment.* Immunity, 2009. 30(6): p. 832-44.
172. Thomson, A.W., H.R. Turnquist, and G. Raimondi, *Immunoregulatory functions of mTOR inhibition.* Nat Rev Immunol, 2009. 9(5): p. 324-37.
173. Powell, J.D. and G.M. Delgoffe, *The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism.* Immunity, 2010. 33(3): p. 301-11.
174. Delgoffe, G.M. and J.D. Powell, *mTOR: taking cues from the immune microenvironment.* Immunology, 2009. 127(4): p. 459-65.
175. Chi, H., *Regulation and function of mTOR signalling in T cell fate decisions.* Nat Rev Immunol, 2012. 12(5): p. 325-38.
176. Wullschleger, S., R. Loewith, and M.N. Hall, *TOR signaling in growth and metabolism.* Cell, 2006. 124(3): p. 471-84.
177. Yang, Q. and K.L. Guan, *Expanding mTOR signaling.* Cell Res, 2007. 17(8): p.

666-81.

178. Potter, C.J., L.G. Pedraza, and T. Xu, *Akt regulates growth by directly phosphorylating Tsc2.* Nat Cell Biol, 2002. 4(9): p. 658-65.
179. Yamagata, K., et al., *rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein.* J Biol Chem, 1994. 269(23): p. 16333-9.
180. Manning, B.D. and L.C. Cantley, *Rheb fills a GAP between TSC and TOR.* Trends Biochem Sci, 2003. 28(11): p. 573-6.
181. Delgoffe, G.M., et al., *The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2.* Nat Immunol, 2011. 12(4): p. 295-303.
182. Alessi, D.R., L.R. Pearce, and J.M. Garcia-Martinez, *New insights into mTOR signaling: mTORC2 and beyond.* Sci Signal, 2009. 2(67): p. pe27.
183. Sarbassov, D.D., et al., *Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton.* Curr Biol, 2004. 14(14): p. 1296-302.
184. Dibble, C.C., J.M. Asara, and B.D. Manning, *Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by S6K1.* Mol Cell Biol, 2009. 29(21): p. 5657-70.
185. Beugnet, A., et al., *Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability.* Biochem J, 2003. 372(Pt 2): p. 555-66.
186. O'Reilly, K.E., et al., *mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt.* Cancer Res, 2006. 66(3): p. 1500-8.
187. Tremblay, F., et al., *Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes.* Endocrinology, 2005. 146(3): p. 1328-37.
188. Sun, S.Y., et al., *Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition.* Cancer Res, 2005. 65(16): p. 7052-8.
189. Greene, M.W., et al., *Modulation of insulin-stimulated degradation of human insulin receptor substrate-1 by Serine 312 phosphorylation.* J Biol Chem, 2003. 278(10): p. 8199-211.
190. Harrington, L.S., et al., *The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins.* J Cell Biol, 2004. 166(2): p. 213-23.
191. Um, S.H., et al., *Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity.* Nature, 2004. 431(7005): p. 200-5.
192. Armstrong, J.L., et al., *Regulation of glycogen synthesis by amino acids in cultured human muscle cells.* J Biol Chem, 2001. 276(2): p. 952-6.
193. Zhang, Q., et al., *Lymphoid sequestration of alloreactive memory CD4 T cells promotes cardiac allograft survival.* J Immunol, 2006. 176(2): p. 770-7.

194. Blagih, J., C.M. Krawczyk, and R.G. Jones, *LKB1 and AMPK: central regulators of lymphocyte metabolism and function.* Immunol Rev, 2012. 249(1): p. 59-71.
195. Hardie, D.G., *Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status.* Endocrinology, 2003. 144(12): p. 5179-83.
196. Sanders, M.J., et al., *Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade.* Biochem J, 2007. 403(1): p. 139-48.
197. Suter, M., et al., *Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase.* J Biol Chem, 2006. 281(43): p. 32207-16.
198. Hawley, S.A., et al., *Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase.* Cell Metab, 2005. 2(1): p. 9-19.
199. Hurley, R.L., et al., *The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases.* J Biol Chem, 2005. 280(32): p. 29060-6.
200. Woods, A., et al., *Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells.* Cell Metab, 2005. 2(1): p. 21-33.
201. Hawley, S.A., et al., *Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade.* J Biol, 2003. 2(4): p. 28.
202. Shaw, R.J., et al., *The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress.* Proc Natl Acad Sci U S A, 2004. 101(10): p. 3329-35.
203. Woods, A., et al., *LKB1 is the upstream kinase in the AMP-activated protein kinase cascade.* Curr Biol, 2003. 13(22): p. 2004-8.
204. MacIver, N.J., et al., *The liver kinase B1 is a central regulator of T cell development, activation, and metabolism.* J Immunol, 2011. 187(8): p. 4187-98.
205. Tamas, P., et al., *Regulation of the energy sensor AMP-activated protein kinase by antigen receptor and Ca2+ in T lymphocytes.* J Exp Med, 2006. 203(7): p. 1665-70.
206. Buttgereit, F. and M.D. Brand, *A hierarchy of ATP-consuming processes in mammalian cells.* Biochem J, 1995. 312 (Pt 1): p. 163-7.
207. Corradetti, M.N., et al., *Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome.* Genes Dev, 2004. 18(13): p. 1533-8.
208. Inoki, K., T. Zhu, and K.L. Guan, *TSC2 mediates cellular energy response to control cell growth and survival.* Cell, 2003. 115(5): p. 577-90.
209. Shaw, R.J., et al., *The LKB1 tumor suppressor negatively regulates mTOR signaling.* Cancer Cell, 2004. 6(1): p. 91-9.

210. Gwinn, D.M., et al., *AMPK phosphorylation of raptor mediates a metabolic checkpoint.* Mol Cell, 2008. 30(2): p. 214-26.
211. Jones, R.G., et al., *AMP-activated protein kinase induces a p53-dependent metabolic checkpoint.* Mol Cell, 2005. 18(3): p. 283-93.
212. Liang, J., et al., *The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis.* Nat Cell Biol, 2007. 9(2): p. 218-24.
213. Cole, M.D., *The myc oncogene: its role in transformation and differentiation.* Annu Rev Genet, 1986. 20: p. 361-84.
214. Luscher, B. and R.N. Eisenman, *New light on Myc and Myb. Part I. Myc.* Genes Dev, 1990. 4(12A): p. 2025-35.
215. Prendergast, G.C., *Mechanisms of apoptosis by c-Myc.* Oncogene, 1999. 18(19): p. 2967-87.
216. Oster, S.K., et al., *The myc oncogene: MarvelouslY Complex.* Adv Cancer Res, 2002. 84: p. 81-154.
217. Spotts, G.D. and S.R. Hann, *Enhanced translation and increased turnover of c-myc proteins occur during differentiation of murine erythroleukemia cells.* Mol Cell Biol, 1990. 10(8): p. 3952-64.
218. Nesbit, C.E., J.M. Tersak, and E.V. Prochownik, *MYC oncogenes and human neoplastic disease.* Oncogene, 1999. 18(19): p. 3004-16.
219. Henriksson, M., et al., *Phosphorylation sites mapping in the N-terminal domain of c-myc modulate its transforming potential.* Oncogene, 1993. 8(12): p. 3199-209.
220. Lutterbach, B. and S.R. Hann, *Hierarchical phosphorylation at N-terminal transformation-sensitive sites in c-Myc protein is regulated by mitogens and in mitosis.* Mol Cell Biol, 1994. 14(8): p. 5510-22.
221. Pulverer, B.J., et al., *Site-specific modulation of c-Myc cotransformation by residues phosphorylated in vivo.* Oncogene, 1994. 9(1): p. 59-70.
222. Sears, R., et al., *Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability.* Genes Dev, 2000. 14(19): p. 2501-14.
223. Sears, R.C., *The life cycle of C-myc: from synthesis to degradation.* Cell Cycle, 2004. 3(9): p. 1133-7.
224. Wang, R., et al., *The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation.* Immunity, 2011. 35(6): p. 871-82.
225. Schmidt, E.V., et al., *Growth controls connect: interactions between c-myc and the tuberous sclerosis complex-mTOR pathway.* Cell Cycle, 2009. 8(9): p. 1344-51.
226. Haskins, K., et al., *The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody.* J Exp Med, 1983. 157(4): p. 1149-69.

227. Hurez, V., et al., *Efficient adenovirus-mediated gene transfer into primary T cells and thymocytes in a new coxsackie/adenovirus receptor transgenic model.* BMC Immunol, 2002. 3: p. 4.
228. Williams, M.E., et al., *Antigen receptor-mediated anergy in resting T lymphocytes and T cell clones. Correlation with lymphokine secretion patterns.* J Immunol, 1992. 149(6): p. 1921-6.
229. Croft, M. and C. Dubey, *Accessory molecule and costimulation requirements for CD4 T cell response.* Crit Rev Immunol, 1997. 17(1): p. 89-118.
230. Nel, A.E. and N. Slaughter, *T-cell activation through the antigen receptor. Part 2: role of signaling cascades in T-cell differentiation, anergy, immune senescence, and development of immunotherapy.* J Allergy Clin Immunol, 2002. 109(6): p. 901-15.
231. Kleindienst, P., et al., *Simultaneous induction of CD4 T cell tolerance and CD8 T cell immunity by semimature dendritic cells.* J Immunol, 2005. 174(7): p. 3941-7.
232. Whelan, M., et al., *A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells.* J Immunol, 2000. 164(12): p. 6453-60.
233. Takahashi, T., et al., *Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state.* Int Immunol, 1998. 10(12): p. 1969-80.
234. Piccirillo, C.A. and E.M. Shevach, *Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells.* J Immunol, 2001. 167(3): p. 1137-40.
235. Li, L., et al., *Rap1-GTP is a negative regulator of Th cell function and promotes the generation of CD4+CD103+ regulatory T cells in vivo.* J Immunol, 2005. 175(5): p. 3133-9.
236. Sebzda, E., et al., *Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling.* Nat Immunol, 2002. 3(3): p. 251-8.
237. Zheng, Y., Y. Zha, and T.F. Gajewski, *Molecular regulation of T-cell anergy.* EMBO Rep, 2008. 9(1): p. 50-5.
238. Martelli, A.M., et al., *The phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin signaling network and the control of normal myelopoiesis.* Histol Histopathol, 2010. 25(5): p. 669-80.
239. Martelli, A.M., et al., *The emerging role of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling network in normal myelopoiesis and leukemogenesis.* Biochim Biophys Acta, 2010. 1803(9): p. 991-1002.
240. Bhaskar, P.T. and N. Hay, *The two TORCs and Akt.* Dev Cell, 2007. 12(4): p.

487-502.

241. Zheng, Y., et al., *A role for mammalian target of rapamycin in regulating T cell activation versus anergy.* J Immunol, 2007. 178(4): p. 2163-70.
242. Wang, T., C. Marquardt, and J. Foker, *Aerobic glycolysis during lymphocyte proliferation.* Nature, 1976. 261(5562): p. 702-5.
243. Brand, K.A. and U. Hermfisse, *Aerobic glycolysis by proliferating cells: a protective strategy against reactive oxygen species.* FASEB J, 1997. 11(5): p. 388-95.
244. Greiner, E.F., M. Guppy, and K. Brand, *Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production.* J Biol Chem, 1994. 269(50): p. 31484-90.
245. Pearce, E.L., *Metabolism in T cell activation and differentiation.* Curr Opin Immunol, 2010. 22(3): p. 314-20.
246. Gauld, S.B., et al., *Differential roles for extracellularly regulated kinase-mitogen-activated protein kinase in B cell antigen receptor-induced apoptosis and CD40-mediated rescue of WEHI-231 immature B cells.* J Immunol, 2002. 168(8): p. 3855-64.
247. Wan, Y.Y., et al., *Transgenic expression of the coxsackie/adenovirus receptor enables adenoviral-mediated gene delivery in naive T cells.* Proc Natl Acad Sci U S A, 2000. 97(25): p. 13784-9.
248. Katagiri, K., et al., *RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1.* Nat Immunol, 2003. 4(8): p. 741-8.
249. Teo, H., et al., *Telomere-independent Rap1 is an IKK adaptor and regulates NF-kappaB-dependent gene expression.* Nat Cell Biol, 2010. 12(8): p. 758-67.
250. Katagiri, K., M. Imamura, and T. Kinashi, *Spatiotemporal regulation of the kinase Mst1 by binding protein RAPL is critical for lymphocyte polarity and adhesion.* Nat Immunol, 2006. 7(9): p. 919-28.
251. Katagiri, K., et al., *Mst1 controls lymphocyte trafficking and interstitial motility within lymph nodes.* EMBO J, 2009. 28(9): p. 1319-31.
252. Avruch, J., et al., *Rassf family of tumor suppressor polypeptides.* J Biol Chem, 2009. 284(17): p. 11001-5.
253. Katagiri, K., et al., *Deficiency of Rap1-binding protein RAPL causes lymphoproliferative disorders through mislocalization of p27kip1.* Immunity, 2011. 34(1): p. 24-38.