The Role of Notch and TGF-β Pathways in $CD4^+$ T Cell Responses

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To my Parents

To my brothers,
Alberto & Gerardo

I would need a book and much more to

fill it with words of gratitude

Declaration

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All the work presented in this thesis, was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Marta Corsin-Jimenez

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Abbreviations

-μC	microCurie
-μg	microgram
-μl	microlitre
-^{3}H-TdR	Tritiated Thymidine
-Ab	Antibody
-AC	*Achaete Scute*
-Ag	Antigen
-AMV	*Avian Myeloblastosis Virus*
-APC	Antigen Presenting Cell
-BCR	B Cell Receptor
-bHLH	basic Helix Loop Helix
-BMP	Bone Morphogenetic Protein
-BM	Bone Marrow
-bp	base pairs
-BSA	Bovine Serum Albumin
-$CaCl_2$	Calcium Chloride
-CBF1	C promoter Binding Factor 1
-CD	Cluster of Differentiation
-cDNA	complementary DNA
-CFA	Complete Freunds Adjuvant
-CIA	Collagen Induced Arthritis
-ConA	Concanavalin A
-CO_2	Carbon Dioxide
-cpm	counts per minute
-CTLA-4	Cytotoxic T Lymphocyte associated Antigen-4
-Cy-C	Cy-Chrome
-DC	Dendritic Cell
-Der p 1	*Dermatophagoides pteronysinnus* allergen group 1
-DP	Double Positive
-dpp	*decaplentaplegic*
-EAE	Experimental Allergic Encephalomyelitis
-ELISA	Enzyme Linked ImmunoSorbant Assay
-FACS	Fluorescence Associated Cell Sorting
-FITC	Fluorescein Isothiocyanate
-FSH	Follicle Stimulating Hormone
-G-CSF	Granlulocyte-Colony Stimulating Factor
-GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
-HA	Haemagglutinin
-Hes	*Hairy enhancer of split*
-HLA	Human Leukocyte Antigen
-IFN	Interferon
-IκB	Inhibitory κB
-IL-	Interleukin
-i.n.	intranasal
-i.p.	intraperitoneal
-kb	kilobase
-LN	Lymph Node
-LPS	Lipopolysaccharide
-LT	*Eschericchia coli* heat labile enterotoxin

-M	Marker
-mAb	Monoclonal Antibody
-MACS	Magnetic Cell Sorting
-MALT	Mucosa-Associated Lymphoid Tissue
-MBP	Myelin Basic Protein
-$MgCl_2$	Magnesium Chloride
-MHC	Major Histocompatibility Complex
-ml	millilitres
-mins	minutes
-mRNA	messanger RNA
-NF-κB	Nuclear Factor-κB
-ng	nanograms
-NK	Natural Killer cells
-OVA	Ovalbumin
-PBMCs	Peripheral Blood Mononuclear Cells
-PBS	Phosphate Buffered Saline
-PE	Phycoerythrin
-PI	Propidium Iodide
-PMA	Phorbol 12-Myristate 13-Acetate
-pmol	picomoles
-RAG	*Recombination Activating Genes*
-rbc	Red Blood Cell
-rpm	revolutions per minute
-rRNA	ribosomal RNA
-RT	Room Temperature
-RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
-S	Spleen
-SCID	Severe Combined Immunodeficiency
-sog	*short gastrulation*
-SP	Single Positive
-Su(H)	*Suppressor of Hairless*
-T	Thymus
-TCR	T Cell Receptor
-*Tfl*	*Thermus Flavus*
-TGF-β	Transforming Growth Factor-β
-Th	T helper
-Th_1	T helper 1
-Th_2	T helper 2
-TLR	Toll-like receptor
-Tm	Annealing temperature
-TNF	Tumour Necrosis Factor
-Tr	Regulatory T cell
-U	Units
-wt	wild type

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Abstract

Notch receptors (1 to 4) are highly conserved cell surface molecules that bind two different families of ligands, Delta (1, 3 and 4) and Serrate (1 and 2, Jagged in vertebrates). Notch signalling plays a fundamental role in cell fate decisions during embryogenesis in both invertebrates and vertebrates, the best-characterised example of this is neurogenesis in *Drosophila*. This process involves the specification of neural versus epidermal precursors. In addition, other molecules have also been implicated in this decision making process, and are termed neural inducers (noggin, chordin, follistatin) and inhibitors (activin A, BMP-4, which are both transforming growth factor-β (TGF-β)-like molecules).

More recently, Notch signalling has been involved in lymphoid lineage commitment and thymocyte differentiation. In addition, work from our group has shown that antigen presented by murine dendritic cells over-expressing Jagged-1, can induce a population of antigen-specific regulatory T cells, which can transfer tolerance to naïve mice. These findings demonstrate the role Notch signalling in conjunction with T cell receptor (TCR) ligation, may play in the differentiation of $CD4^+$ T cells into regulatory T cells.

Furthermore, activin A and BMP-4, which like the Notch pathway are highly conserved and also involved in developmental processes, are TGF-β-like molecules which may share functional properties with the immune-regulatory cytokine, TGF-β.

The aim of this study was to investigate further the potential role of components of the Notch pathway and TGF-β superfamily members in $CD4^+$ T cell responses *in vitro* and *in vivo*.

The first question addressed if these developmentally related molecules were expressed in lymphoid organs and cells of adult mice. I used reverse transcriptase polymerase chain reaction (RT-PCR) to demonstrate that these molecules are expressed in adult lymphoid compartments, and real-time PCR revealed that activating $CD4^+$ T cells and B cells *in vitro* differentially regulated genes of the Notch signalling pathway and of the TGF-β superfamily.

Second I investigated if components of the Notch signalling pathway were modulated in murine splenic $CD4^+$ T cells in response to peptide delivered intranasally under conditions that induce tolerance or priming. Furthermore, I phenotyped by antibody staining the $CD4^+$ T cells in an attempt to characterise regulatory T cells generated during tolerance induction.

Finally, I carried out *in vitro* experiments to investigate the functional effects of the TGF-β-like molecules, activin A and BMP-4, on murine splenic $CD4^+$ T cells and how these effects correlated with expression of components of the Notch signalling pathway.

CHAPTER I

Introduction

1. Mucosal Tolerance

The mucosa of the respiratory, gastrointestinal and the urogenital tracts, as well as the eye conjunctiva, the inner ear and the ducts of all exocrine glands, are lined with epithelial cells that provide a physiochemical barrier to limit contact between environmental antigens and the immune system. In addition, they are protected by innate immune mechanisms and specialised adaptive immunity, that protects the mucosal surface against potential insults from the environment. In a healthy adult, this local immune system contributes almost 80% of all immunocytes. These cells have accumulated in or are in transit between various mucosal tissues and glands and together they form the mucosa-associated lymphoid tissue (MALT), the largest mammalian lymphoid organ (1). The mucosal immune system can discriminate between pathogenic microorganisms and innocuous matter such as dietary antigens, inhaled particles or commensal microorganisms, by promoting specific immunological unresponsiveness (tolerance). This form of tolerance is generally referred to as **mucosal tolerance**, and is an active process mediated by more than one mechanism including active suppression, clonal deletion, immune deviation, anergy and imunological ignorance (2, 3). Whether antigen induces tolerance or productive immunity is determined by a number of factors, including antigen dose, the nature of the antigen, the genetic background and immunological status of the host, and mucosal adjuvants.

Oral tolerance which is one form of mucosal tolerance can be achieved through the ingestion of soluble antigen. It takes place in the gut and is associated with a decrease in cell-mediated responses such as delayed-type hypersensitivity (DTH) reactions *in*

vivo and lymphocyte proliferation *in vitro*, and in humoral responses such as IgE and IgG production (2). The phenomenon of oral tolerance was first described by Well in 1911 (4), who found that anaphylactic reactions to ovalbumin (OVA) in guinea pigs could be inhibited by prior oral administration of OVA. This process was active and antigen specific, as repeated systemic immunisations failed to induce an OVA responsive state. An important feature of oral tolerance is **bystander suppression**. This involves the generation of **regulatory T cells** after feeding antigen, which are capable of dampening T cell responses to a different antigen provided both are present in the same anatomical microenvironment. This was first described *in vivo* by Miller *et al* (5) in the Lewis rat model of experimental allergic encephalomyelitis (EAE), induced by immunisation with myelin basic protein (MBP). They observed that in OVA-fed animals given aqueous OVA in the foodpad following immunisation in the footpad with MBP/complete Freund's adjuvant, EAE was suppressed. Suppression was mediated by OVA-specific regulatory T cells that migrated to the draining lymph node and secreted TGF-β on encountering OVA, thus inhibiting the generation of the MBP-specific immune responses in the lymph node. Other mechanisms have been attributed to oral tolerance including anergy and clonal deletion, however, description of these is beyond the scope of this study.
As well as through ingestion, mucosal tolerance can be induced via the respiratory tract through intranasal administration of antigen. This will be discussed in detail as it forms the basis of the *in vivo* work reported in this thesis.

1.1. Respiratory Tolerance

The respiratory tract is continually exposed to a wide variety of antigens. There are a number of innate mechanisms to limit contact between inhaled environmental allergens and the immune system. These include the nasal filter, tight gap junctions preventing diffusion of antigens across the epithelium, bronchial cilia, the muco-ciliary transport system and specific secretory antibodies and phagocytic cells contained in the fluid layer overlying the epithelium. Moreover, the bronchial-associated lymphoid tissue (BALT) is a well-developed mucosal surface in the respiratory tract. Pulmonary alveolar macrophages in the lower respiratory tract have

been shown to be efficient inhibitors of T cell activation both *in vivo* and *in vitro* (6) and lack co-stimulatory molecules CD80 and CD86 (7). In addition, pulmonary alveolar macrophages can prevent the maturation of dendritic cells which are the most potent antigen presenting cells (APCs) of the immune system, and this would have the effect of reducing T cell priming to inhaled antigen. In most instances, inhaled antigens provided they are not pathogen-derived, induce immunological tolerance. Tolerance induction to inhaled antigens via the nasal route was first reported in 1981 by Holt and co-workers (8). They found that brief exposure of mice to low-level nebulised OVA induced allergen-specific IgE responses, which spontaneously stopped after 3-4 weeks. When these mice were challenged parentally with OVA up to 6 months after the final exposure, IgE response was still suppressed along with IgG1 reactivity (Th2 cytokine-induced isotype, responsible for the allergic reaction). This was accompanied by a compensatory rise in IgG2a (Th1 cytokine-induced isotype, responsible for protection), and represents an example of immune deviation. Furthermore, this form of tolerance was an active process involving regulatory T cells (9, 10). Therefore, respiratory tolerance to non pathogen-derived antigens has been described in the context of **immune deviation** (from an allergic Th2 to a protective Th1 response), and in the context of **regulatory T cells** and the latter will be described below in more detail.

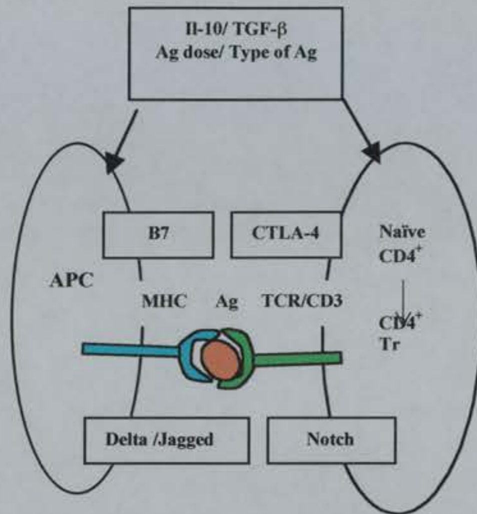
1.1.1. Establishment of Respiratory Tolerance by Regulatory CD4$^+$ T Cells

Much work in our group has been carried out on the role of CD4$^+$ T cells in response to protein antigens derived from the house dust mite (HDM), *Dermatophagoides pteronyssinus*, (Der p) a common source of aeroallergen in the environment. Hoyne *et al* (11) showed that in H-2^b mice, intranasal (i.n.) administration of the major T cell epitope of the Der p type 1 allergen (Der p 1), residues 111-139, followed by a rechallenge with the intact protein antigen, significantly down regulated T cell responses *in vitro*, when they were re-stimulated with the intact antigen, the major T cell epitope, or other minor epitopes. Therefore, treatment with a single immunogenic peptide could abrogate T cell responses to all the epitopes on the antigen. This phenomenon is known as **linked suppression**, and is only observed if

tolerant animals are rechallenged with the whole protein. This mechanism is similar to bystander suppression observed in oral tolerance in that both involve the generation of **regulatory T cells**. However, bystander suppression can rely on two different antigens, the one that generates the regulatory T cell population to suppress disease induced by the second antigen.

It was also reported that high-dose intranasal delivery of HDM peptide (epitope 111-139) elicited a strong but transient activation of $CD4^+$ T cells. This was followed by the down-regulation at day 14 of IL-2 and IFN-γ secretion, and the inability to provide B cell help for antibody production (a state of T cell unresponsiveness) *in vitro* (12). They proposed that following antigenic challenge and activation, $CD4^+$ T cells differentiated into a regulatory population capable of dampening other $CD4^+$ T cell responses specific for the same antigen. Furthermore, these regulatory T cells appeared to be maintained in the peripheral circulation for long periods, even 6 months after the original peptide treatment (13). From the work described in these studies, the following regulatory mechanism for tolerance induction is proposed (Figure 1).

A. Initial exposure to peptide



B. Re-challenge with whole protein

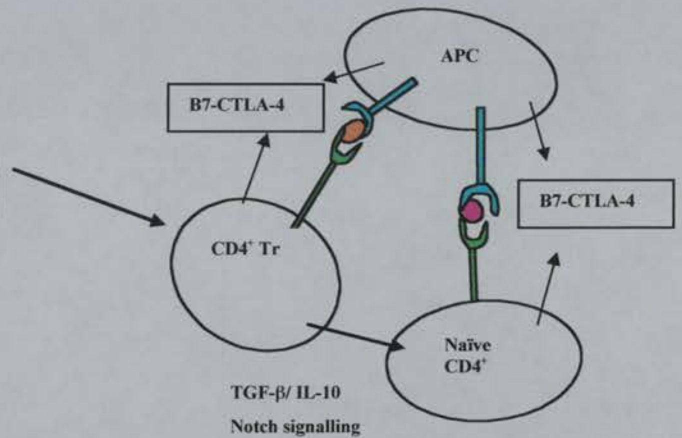


Figure 1. Proposed model for the generation of regulatory T cells after tolerance induction. Initial immunisation with a single immunodominant epitope generates a population of regulatory $CD4^+$ T cells (Tr cells). These T cells arise from an initial naive population, which upon recognition of the antigen (Ag) on the antigen presenting cell (APC) surface undergo clonal expansion and release cytokines. However, they receive an additional signal(s) that diverts them from a T helper to a regulatory T cell phenotype. This signal may result from a combination of factors including physiochemical properties of antigen and dose, site of antigen delivery, mode of antigen presentation, cytokine microenvironment (IL-10 and/or TGF-β), APC-T cell interactions through co-stimulatory molecules (B7-CTLA-4), and other cell-cell contact-mediated signals (MHC/Ag-TCR and/or Notch-Jagged/Delta). The combination of all or some of these factors will bias the functional differentiation of the $CD4^+$ T cell into the regulatory pathway. Subsequent immunisation with the whole protein will bring into close proximity the regulatory $CD4^+$ T cells specific for the immunodominat epitope, as well as naive $CD4^+$ T cells specific for all other epitopes presented by the same or adjacent APCs. In such an environment, the regulatory $CD4^+$ T cells are activated, and exert their effects on other $CD4^+$ T cells through the combined effect of inhibitory cytokines and cell-cell contact-mediated mechanisms that bias the naïve $CD4^+$ T cells into the regulatory pathway.

The key feature of this model is that naïve $CD4^+$ T cells after antigen recognition are presented with two choices. Thus, if the signal was stimulatory they would choose to differentiate into T helper cells, and this would lead to productive immunity. However, in this model, T cells choose to differentiate into the alternative cell fate, and become regulatory cells instead. The nature of the signal(s) that bias T cells into a regulatory phenotype still needs to be defined.

Cell fate choice is probably one of the most fundamental biological processes in development that directs the process of organogenesis and tissue patterning. This requires activation of tightly regulated spatial and temporal genetic pathways, which determine the outcome of cells. One pathway that is key in specifying cells is the Notch signalling pathway, which involves cell-mediated contact between a receptor on one cell and its ligands on a neighbouring cell, and subsequent intracellular signalling that affects transcription of target genes. This pathway is highly conserved from invertebrates (*Drosophila melanogaster)* to humans.

The hypothesis described here implicates **Notch signalling** in the specification of regulatory $CD4^+$ T cells. The choice between productive immunity or tolerance is prerequisite for immune homeostasis, therefore, it might involve a process that evolutionary is very stable, and thus also highly conserved. Based on the model of peripheral tolerance to HDM peptide given intranasally (11), I was prompted to investigate if Notch signalling is involved in the generation of regulatory T cells.

Below is a more extensive account on regulatory $CD4^+$ T cells and Notch signalling.

2. Regulatory CD4$^+$ T Cells

2.1. Induction of Regulatory CD4$^+$ T Cells

The induction of experimental autoimmunity and allergy-associated responses can be controlled in many cases by CD4$^+$ regulatory T cells that are generated after oral or intranasal exposure to specific antigen. Examples are found in experimental autoimmune myesthenia gravis (14), autoimmune diabetes (15), collagen-induced arthritis (16) or experimental autoimmune encephalomyelitis (17). In section 1.1.1 I have also described the generation of regulatory CD4$^+$T cells associated with intranasal administration of the house dust mite allergen, Der p 1.

2.2. Constitutive Regulatory CD4$^+$ T Cells

Increasing evidence suggests that a population of T cells called regulatory T cells (Tr cells), may be acting to down-regulate other T cell responses, including those that are directed against self, and help maintain peripheral tolerance. The earliest studies defined a population of specialised regulatory or suppressor CD4$^+$ T cells derived from the thymus (18), that were able to prevent organ-specific autoimmunity induced in mice that had been thymectomized on day 3 of life. The next major finding in support of regulatory T cells involved their characterisation on the basis of expression of the Lyt-1 (CD5) antigen (19). Lyt-1^{low} cells when transferred to athymic nude (nu/nu) mice resulted in autoimmunity in several organs. In contrast, transfer of the Lyt-1^{high} population or co-transfer with the Lyt-1^{low} population prevented the disease.

In the following account I give a description of some of the Tr cell populations that have been described. The markers that have been used for their identification were used in this thesis to characterise regulatory T cells in the spleen of unimmunised mice and in a model of intranasal peptide tolerance.

2.2.1. $CD4^+CD25^+$ Regulatory T Cells

Sakaguchi *et al* (20) further defined the regulatory T cell as a minor subset of $CD4^+$ T cells (10%) expressing the IL-2 receptor α-chain (CD25) in the spleen of normal mice. Studies using two different model systems have led to the suggestion that this $CD4^+CD25^+$ population can prevent certain autoimmune diseases. The first model involved mice that were thymectomized on day 3 of life and developed organ-specific autoimmune disease involving one or more organs. The disease, however, could be inhibited in the thymectomized animals receiving $CD4^+CD25^+$ T cells from normal mice, when transferred on day 10 of life (20). In a second model, when $CD4^+CD25^-$ cells from normal BALB/c mice were injected into athymic recipients, they all developed a high incidence of organ-specific autoimmune disease. This was prevented by co-transfer of populations enriched in $CD4^+CD25^+$ T cells (21).

Thornton and Shevach (22) showed *in vitro* that $CD4^+CD25^+$ T cells from BALB/c mice were unresponsive to stimulation with anti-CD3 antibody soluble or plate-bound, anti-CD3 and anti-CD28 antibodies, high IL-2 concentration, and con A in the presence of T cell-depleted spleen cells as a source of APCs. These cells could suppress proliferation of $CD4^+CD25^-$ cells cultured *in vitro* in the presence of APCs and soluble anti-CD3 antibody (not plate-bound), and inhibited induction of *IL-2* mRNA. Suppression was mediated by a cytokine-independent (neither IL-10 nor TGF-β), cell-cell contact-dependent mechanism.

2.2.2. $CD4^+CD45RB^{low}$ Regulatory T Cells

Powrie and Mason (23) also identified a sub-population of $CD4^+$ T cells in the rat, which displayed an immune regulatory function. These cells were characterised on the basis of CD45RB expression. $CD4^+CD45RB^{high}$ T cells when transferred to athymic rats led to severe wasting disease. However, no disease was observed when unseparated cells or $CD4^+CD45RB^{low}$ T cells were transferred.

The same $CD4^+$ sub-populations have also been found in the spleen of mice. The CD45RB antigen is expressed at high levels in 60-70% of the $CD4^+$ T cells, whilst the remainder express intermediate or low levels (24). It is widely thought that naïve T cells are contained within the $CD45RB^{high}$ whereas antigen-experienced T cells

reside largely within the CD45RB^{low} population. Studies on the function of the CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} subsets have shown that important regulatory interactions occur between these subsets *in vivo*. Transfer of small numbers of CD4$^+$CD45RB^{high} T cells from normal BALB/c to C.B-17 severe combined immunodeficient (SCID) mice led to a Th1 cell-mediated wasting disease and colitis in the recipients (25). In contrast, SCID mice restored with CD4$^+$CD45RB^{low} T cells alone did not develop disease, and this population when co-transferred with CD4$^+$CD45RB^{high} T cells, completely inhibited development of colitis. These results indicated that in addition to containing antigen-primed cells capable of mounting recall responses to antigen, the CD4$^+$CD45RB^{low} T cell subset also contained a population of regulatory T cells (Tr cells).

Takahashi *et al* (26) showed that purified BALB/c CD4$^+$CD45RB^{low} populations from the spleen and lymph nodes could be further sub-divided into CD25$^+$ and CD25$^-$ populations, consistent with Sakaguchi's original discovery (20). The CD4$^+$CD25$^+$CD45RB^{low} T cells were unresponsive to anti-CD3 antibody or con A-stimulation, and suppressed the CD4$^+$CD25$^-$CD45RB^{low} T cells in a dose-dependent fashion when the two populations were mixed in various ratios. In contrast, the CD4$^+$CD25$^-$CD45RB^{low} T cells were neither suppressive nor unresponsive, indicating that this could be attributed to the CD25$^+$ population.

This finding has also been demonstrated by Read *et al in vivo* (27). They showed that co-transfer of splenic CD4$^+$CD25$^+$CD45RB^{low} and the CD45RB^{high} populations, even at a ratio of 1:8, significantly inhibited colitis and wasting disease in SCID mice, compared to mice restored with a mixture containing the CD25$^-$CD45RB^{low} population, which remained unprotected. To further investigate the regulation of intestinal inflammation by the CD25$^+$CD45RB^{low} T cells, they examined the expression of the T cell co-stimulatory molecule, CTLA-4, as a potential candidate that may be involved in the signalling pathway that leads to immune suppression by Tr cells. CTLA-4 was expressed constitutively on CD4$^+$CD25$^+$CD45RB^{low} cells (46.4%) and at lower levels on the CD25$^-$ subset (12.5%). Very low levels were found on the CD45RB^{high} (0.8%).

If anti-CTLA-4 monoclonal antibody (mAb) was given to SCID mice which had the $CD25^{+}CD45RB^{low}$ and the $CD45RB^{high}$, the mice did not recover from colitis, suggesting that the $CD25^{+}$ cells were dependent on CTLA-4.

In vitro, Takahashi *et al* (28) also demonstrated that anti-CTLA-4 mAb, especially in the presence of anti-FcR mAb, or Fab-anti-CTLA-4 mAb, significantly neutralised $CD4^{+}CD25^{+}$ T cell-mediated suppression of the activation and proliferation of $CD4^{+}CD25^{-}$ T cells. In addition, the Fab-anti-CTLA-4 mAb also neutralised the $CD4^{+}CD25^{+}$ T cell-mediated control of antigen-specific activation and proliferation of OVA-specific T cells from D011.10 transgenic mice.

2.2.3. $CD4^{+}CD38^{+}$ Regulatory T Cells

The CD38 antigen which is expressed in 23% of BALB/c splenic $CD4^{+}$ T cells, has also been found to sub-divide the $CD45RB^{low}$ into $CD45RB^{low}CD38^{+}$ (40-50%) and $CD45RB^{low}CD38^{-}$ (15-20%) (29). Read *et al* found the $CD4^{+}CD38^{+}$ subset was resistant to proliferation with soluble anti-CD3 antibody, as opposed to the $CD38^{-}$ population. Furthermore, they tested the ability of the $CD38^{+}$ and $CD38^{-}$ populations to mount secondary responses to specific antigen. These $CD4^{+}$ T cell subsets were isolated from the spleens of *Leishmania major*-infected mice, and stimulated *in vitro* with antigen. $CD4^{+}CD38^{+}$ T cells showed no detectable cytokine production compared to $CD4^{+}CD38^{-}$ which secreted IL-4, IL-10 and IL-3. Further still, $CD4^{+}CD38^{+}$ T cells when transferred to *L. major*-infected SCID mice, were able to produce a healing Th1 response. They concluded that the $CD38^{+}$ subset of $CD4^{+}$ T cells contained regulatory T cells, which could inhibit T cell activation *in vitro*.

At present there is a discrepancy between the suppressive activity of the $CD4^{+}CD25^{+}CD45RB^{low}$ cells seen *in vivo* which appear to be TGF-β-dependent (27), and the activity of the $CD4^{+}CD25^{+}$ cells (22) and $CD4^{+}CD45RB^{low}CD38^{+}$ (29) seen *in vitro* which are TGF-β-independent.

3. Notch Signalling

3.1. The Notch Signalling Pathway

Multicellular development arises from the combinatorial and sequential activity of genetic pathways. One pathway that plays a central role in the specification of cell fates through local cell interactions in a wide variety of tissues and organisms is the Notch signalling pathway. The gene encoding the Notch receptor was discovered in *Drosophila melanogaster* more than 80 years ago. This evolutionary conserved pathway has also been identified in lower and higher vertebrates (30, 31), and related proteins have been found in the nematode, *Ceanorhabditis elegans* (32) (Table I and Figure 2).

Notch Receptors		Notch Ligands		Intracellular effectors	Target Genes
Drosophila	Notch	Delta	Serrate	[Su(H)]	[E(spl)]
<u>Vertebrates</u>					
Human	Notch 1-4	Delta-like 1, 3, 4	Jagged 1-2	CBF-1/RBP-Jκ	
Mouse	Notch 1-4	Delta-like 1, 3, 4	Jagged 1-2	CBF-1/RBP-Jκ	Hes 1, 3, 5
Rat :	Notch 1-3	Delta-like 3	Jagged-1		Hes 1-3, 5
Zebrafish	Notch 1-3, 5, 6	Delta A, B, D	SerrateB		
Xenopus	Xotch	X-Delta 1-2		XSu(H)1-2	
C.elegans	Glp-1 Lin-12	Apx-1	Lag-2	Lag-1	

Table I. Conservation of the Notch signalling pathway from invertebrates to higher vertebrates.

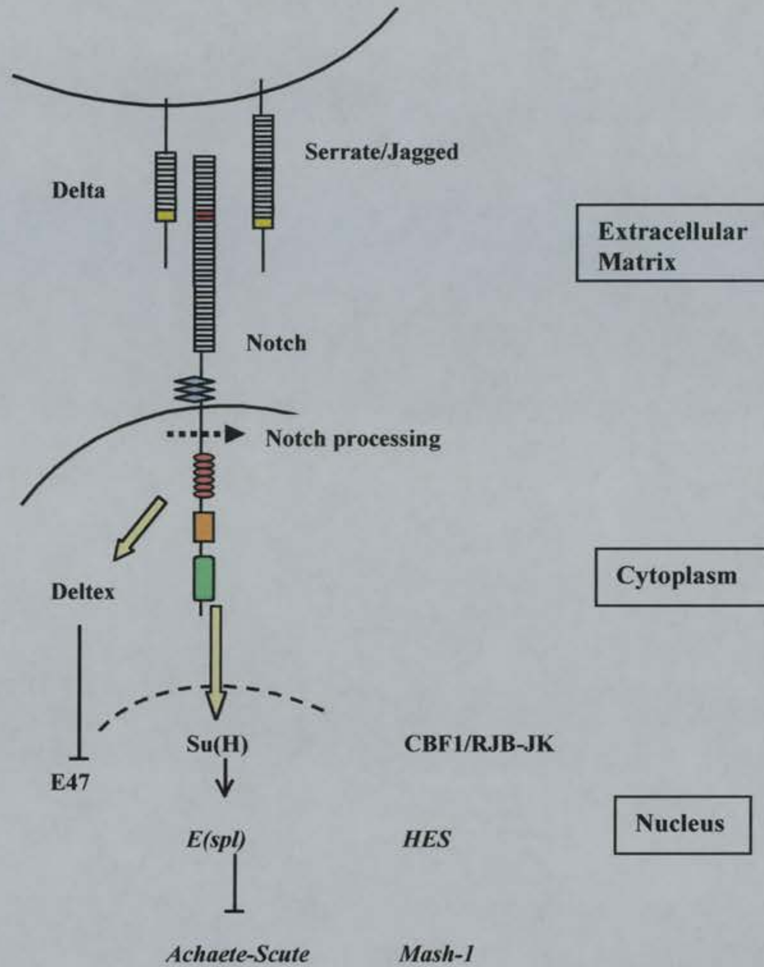


Figure 2. Conservation of the Notch Signalling pathway. Notch encodes for a 300-kD transmembrane receptor. The extracellular domain consists of 36 epidermal growth factor (EGF)-like repeats and three cysteine-rich Notch/Lin12 repeats (in blue). The intracellular domain is composed of 6 tandem ankyrin repeats (in red), a glutamine-rich domain (OPA, in orange) and a PEST sequence (proline-glutamine-serine-threonine-rich region, in green). The extracellular domain of the Notch receptor can bind to its two ligands; Delta and Serrate (or Jagged in vertebrates), which are membrane-bound extracellular proteins. This interaction occurs between EGF repeats 11 and 12 on the Notch receptor (in red) and a conserved cysteine-rich motif referred to as the Delta:Serrate:LAG-2 (DSL) domain (in yellow), on the Notch ligands. Ligand binding activates the Notch receptor which is proteolytically cleaved and translocates to the nucleus where it associates with the DNA-binding molecule Suppressor of Hairless, Su(H) (CBF1/RJB-Jk in mammals: C promoter Binding Factor/Recombination signal sequence Binding protein for Jk genes). Su(H) binds to regulatory sequences of the *Enhancer of split*, *E(spl)*, genes (*Hairy Enhancer of Split*, *HES*, in mammals) which up regulate expression of their encoded basic-Helix-Loop-Helix (bHLH) proteins. These, in turn, inhibit expression of downstream target genes, like the *Achaete-Scute* complex (*Mash-1* in mammals). Su(H)-independent events have also been documented and these involve the action of a protein called Deltex. Deltex is an evolutionary conserved cytoplasmic protein that binds to the ankyrin repeats of Notch and does not appear to localise to the nucleus after Notch activation. Signals through the Notch/Deltex pathway can result in repression of the bHLH protein E47 through a mechanism involving Ras and c-Jun N-terminal kinase (JNK) (33).

3.2. Types of Notch Signalling and Neural Specification

Cell fate determination requires signalling events that can take place either within different cell types or within an equivalent group of cells. In both instances, studies in *Drosophila* and *C.elegans,* have shown the involvement of the Notch signalling pathway (34). One type of regulative signalling, termed **inductive signalling** operates between non-equivalent cells. In this case intrinsic and extrinsic factors confer a bias to one of two neighbours, which is then consolidated by Notch-Notch ligand interactions. An example of the mechanism is the induction of the R4 cell by the R3 cell during *Drosophila* eye development (35). In the *Drosophila* imaginal eye disc a gradient of an unknown signal exists between the adjacent R3 and R4 photoreceptor precursor cells. This signal is capable of up regulating Delta expression on the R3 cell, and in turn R3 signals to R4 via its Notch receptor, so that it acquires the R4 fate.

In the second type of signalling event, there is a group of initially equivalent cells, and one cell from within the group of cells is singled to follow a different path of differentiation to the remaining cells. As signals will be transmitted back and forth between the two emerging cell types, this has been termed lateral specification or **lateral inhibition**. The molecular details of lateral specification are still largely undefined, however, it appears to require three regulatory steps (34). First, the interacting cells express both ligand and receptor. Over time, fluctuations occur in the expression of Notch receptor and ligand on the neighbouring cells, such that one cell will express more ligand compared to the other cells that express more receptor. The Notch ligand-expressing cell signals to the neighbouring cells, and these acquire alternate cell fates. The segregation of neural and epidermal precursor cells in the ventral ectoderm of the *Drosophila* embryo is considered a classic example of lateral specification. During normal development of the fly central nervous system, an ectodermal monolayer of equipotent cells gradually segregate into two distinct cell populations, namely neuroblasts which give rise to neurons, and dermoblasts which give rise to epidermal structures. A group of transcription factors that are encoded by the *Achaeta-Scute* (AS) gene complex (Figure 2) have been implicated in this process (36). All competent cells will be expressing Notch and Delta. A signal as yet unidentified will lead to up regulation of the Notch ligand, Delta, in one such cell. This cell is destined to become a neural precursor, and upon binding to Notch on a

neighbouring cell, will send an inhibitory signal along the Notch pathway to the receiving cell, that will lead to the suppression of the AS gene complex and therefore of its neural potential. As a result this cell will consequently down regulate Delta expression and adopt an epidermal fate. On the contrary, in the signalling cell the AS genes will maintain Delta expression, and this cell will adopt a neuronal fate. Therefore, Notch is the receptor for a signal that diverts cells from adopting a primary, neuronal fate (the default pathway) and directs them toward a secondary, epidermal fate.

Studies on the *Xenopus* homologues of *Drosophila* Delta suggest that the formation of neural precursors in vertebrate embryos may also be negatively regulated by **lateral inhibition** (37, 38).

In vertebrates, the process of generating a mature neuron can be divided into two distinct phases which take place during embryonic development: the specification of the neuroectoderm which takes place at the onset of gastrulation, and the specification of neural precursors, which takes place during neurula stages and after. The first process involves a series of molecules, which have been defined as **neural inducers** that specify neural ectoderm, and their **inhibitors** that antagonise their actions. The second process involves the **Notch signalling** pathway.

It is possible that a process involving Notch signalling may induce regulatory $CD4^+$ T cells. Thus, when Notch is activated, $CD4^+$ T cells are diverted from their primary (default) effector T helper fate, to their alternate regulatory T cell fate. Furthermore, I propose that the molecules that specify neuroectoderm (neural inducers and inhibitors) may also influence the differentiation of T cells in the periphery. Before describing the hypothesis on which this study is based, I will describe the process of neuroectoderm specification in vertebrates and the molecules involved.

4. Vertebrate Neural Inducers and Inhibitors

In all vertebrates, the development of the nervous system is directly linked to the establishment of the dorso-ventral axis, which in amphibians is initially established by a microtubule-directed rotation of the egg cortex. In *Xenopus* embryos about 9 hours after fertilisation, gastrulation (or cell rearrangement) begins. Cells located at the top of the embryo, or animal pole, are specified as ectoderm, and the cells at the bottom, or vegetal pole, are specified as the endoderm. When the embryo contains 16 or 32 cells, the specification of the third germ layer, the mesoderm, begins in the equatorial region, or marginal zone. Because dorso-ventral polarity is established during the first cell cycle, blastomeres of each germ layer at this stage also have a dorso-ventral identity, though it is not irreversibly fixed.

The ectodermal cells will make a choice between two possible fates; neural on the dorsal side of the embryo, and epidermal on the ventral side. Thus, the origin of the nervous system can be traced to ectodermal cells located on the prospective dorsal side of the embryo at gastrula stages (39).

Newly formed ectoderm thickens to form a flat neural plate, characterised by a central groove (neural groove) and thickened lateral edges (neural folds). The neural folds eventually fuse at the dorsal midline to form the neural tube, which will differentiate into brain at its anterior end and spinal cord at its posterior end (40). Primary neurones in lower vertebrates (*Xenopus*) arise from neuronal precursors in the posterior part of the neural plate, and this is regulated through a process of lateral inhibition by Notch-Delta signalling (37).

4.1. The Mechanism of Neural Induction

The concept of neural induction was first proposed in 1924 by Spemann and Goldman (41) in their studies of amphibian embryos. They identified a region in the dorsal side of the embryo, the Spemann Organiser, consisting of mesoderm, which contained factors that could divert cells in the ectoderm from an epidermal to a neural fate. Structures functionally equivalent to the amphibian organiser have been identified in other vertebrates, suggesting that the mechanism of neural induction is conserved.

However, this experiment was difficult to reconcile with cell dissociation experiments carried out in the late 1980's. Three groups (42, 43, 44) independently observed that ectodermal cells subjected to prolonged dissociation culture during gastrula stages, expressed neural markers or formed histologically recognisable neural tissue after reaggregation. These results were a considerable surprise because in all cases, the ectoderm had been isolated from contact with the organiser. It was proposed that neural inhibitors within the ectoderm had been lost on dissociation, allowing the ectoderm to acquire by default its neural fate.

The possibilities raised by the dissociation experiments were given new force by the discovery that ectodermal explants were neuralised by expression of a dominant-negative activin receptor (45, 46). Activin is a member of the transforming growth factor (TGF-β) superfamily. When truncated type II activin receptor mRNA was injected into embryos, the formation of mesoderm was blocked, demonstrating that signalling through this receptor was required for mesoderm induction *in vivo* (45). Furthermore, it was shown that upon injection of this receptor into ectodermal explants, the ectoderm adopted a neural fate in the absence of neural inducing signals from the mesoderm. Therefore, these studies proposed that neural fate represents the default state of ectodermal cells, and that signalling within the ectoderm (for example by activin and its receptors) inhibits neural specification. When this signalling is interrupted by cell dissociation or by molecular antagonists, neural tissue forms. Since these concepts were first proposed, a number of **neural inducing factors** and **neural inhibitors** have been cloned and identified, and some of them are listed below.

4.2. Neural Inducers

4.2.1. Noggin

Noggin was the first neural inducer identified (47). It can induce neural tissue directly in ectodermal explants (animal caps) both as an injected RNA and as a soluble protein (48). At gastrula stages noggin is expressed exclusively in the organiser region.

4.2.2. Follistatin

Follistatin is a direct inhibitor of activin (49), and ectopic expression of follistatin in embryonic ectodermal explants can turn on neural markers in the absence of mesoderm, suggesting that neural induction is direct. Follistatin is expressed in the organiser region in *Xenopus*, where it can act to block activin signalling in the dorsal ectoderm and permit neural tissue to form (50). As well as binding to activin, follistatin has been found in a complex with bone morphogenetic protein 4 (BMP-4), which is a neural inhibitor (51). Activin A and BMP-4 are both TGF-β-like molecules, and direct interaction of follistatin with these TGF-β-like molecules prevents them from reaching their receptors and initiating TGF-β-like signalling.

4.2.3. Chordin

It is a secreted factor localised in the organiser of the early *Xenopus* gastrula. It was later demonstrated that chordin is a vertebrate homologue of the *Drosophila* gene product, short gastrulation (sog) (52), and that the two proteins can in fact substitute for one another (53). Chordin has direct neural-inducing ability when provided as RNA or protein (54, 55), and it has been suggested that the neuralising activity of chordin is mediated through the inhibition of BMP-4 activity (54). This hypothesis parallels the genetic evidence demonstrating that sog inhibits the activity of the TGF-β protein decapentaplegic (dpp) (56, 57), which is the *Drosophila* homologue of BMP-2 and BMP-4, suggesting the molecular conservation of ectodermal specification from arthropods to chordates.

4.3. Neural Inhibitors

4.3.1. BMP-4

The role of BMP-4 has been assessed by adding the purified protein to dissociated cultures of gastrula ectoderm. BMP-4 not only inhibits neuralisation but can also induce an epidermal fate (58). Moreover, dominant-negative forms of BMP-4, which cannot be cleaved to give mature protein, as well as dominant-negative BMP-4 receptors, induce the formation of neural tissue in *Xenopus* ectoderm (58). These different studies suggested that cells of the gastrula animal cap are predisposed to form neural tissue in the absence of additional signals, and that an epidermal fate arises as a result of BMP signalling. This however, can be prevented by direct association of noggin, chordin and follistatin with BMP-4 (59, 54, 51) so it can no longer access its receptor.

4.3.2. Activin A

Activin A is a TGF-β-like molecule, and activin A signalling by binding to its receptor can inhibit the neural fate. However, unlike BMP-4, it cannot induce epidermis in the presence of dissociated ectodermal cells. Rather, these cells are shifted to a mesodermal fate.

The information presented above has attempted to describe the complex process of neural specification that takes place in vertebrates. Neural induction within the ectoderm is specified as early as gastrulation, by soluble neural inducers contained within the Spemann Organiser, such as **noggin, chordin** or **follistatin**. These molecules can bind to and antagonise the TGF-β-like neural inhibitors **BMP-4** and **activin A**, preventing their access to specific receptors so they cannot initiate signalling. Access of BMP-4 and activin A to their specific receptors prevents neural induction in favour of epidermis and mesoderm, respectively. Additionally, the functional similarities between the sog/dpp gene products in *Drosophila* and chordin/BMP-4 in *Xenopus*, suggest that the mechanism that determines cell fate during neural patterning is highly conserved.

The antagonism of TGF-β signalling appears to establish the domain in which neural precursors can be further specified. This later specification is mediated by intercellular

signalling between precursor cells involving the Notch signalling pathway. Interestingly, a degree of overlap between the Notch signalling pathway and the dpp/TGF-β signalling pathway has been found in different studies of *Drosophila* development (60, 61, 62), which may also take place in vertebrate development, although as yet there is no evidence for this.

Therefore, adding to the model of Notch signalling and regulatory $CD4^+$ T cell differentiation previously described, I propose that the BMP/activin signalling pathway may also be involved in regulating the differentiation of $CD4^+$ T cells in the periphery, by co-operating with or acting independently of the Notch signalling pathway.

From an immunological point of view it is of interest that activin A and BMP-4 are both TGF-β-like molecules, and TGF-β has been implicated in the regulation of mucosal tolerance *in vivo* (17, 63, 64), and in regulatory T cell effector function (65). Therefore, it is possible that activin A and/or BMP-4 might also contribute to tolerance induction by affecting the same downstream target genes as TGF-β.

Therefore, the main aim of this thesis was to determine the role of Notch and TGF-β signalling in the regulation of peripheral immune responses. The hypothesis was to determine if signals from Notch and/or TGF-β-like molecules might influence, together with other factors, the specification of $CD4^+$ T cells. Activation of these conserved signalling pathways may facilitate the cell fate decision making process that directs $CD4^+$ T cells to the helper (Th) or regulatory (Tr) pathway.

Before presenting the aims of this thesis I would like to review in more detail the biology of activin A and BMP-4, as well as to present evidence supporting a role for Notch signalling in the development and regulation of the immune system. The information described below reveal that Notch and TGF-β signalling are involved in similar biological processes.

5. Notch Signalling in the Immune System

5.1. Notch Signalling and Hematopoiesis

Hematopoesis is a continuous developmental process, in which pluripotent stem cells and their progeny make sequential cell fate decisions, producing mature blood cells of the various lineages. Given the extensive evolutionary conservation of Notch function and its role in cell fate determination, the Notch pathway has been implicated in the regulation of hematopoiesis.

5.1.1. Myeloid Differentiation

The myeloid progenitor in the bone marrow is the precursor of granulocytes (neutrophils, eosinophils, basophils and mast cells) and macrophages. The first evidence for Notch function in myelopoiesis came from studies in 32D cells, a progenitor cell line used as a model system for granulocytic differentiation (66). 32D cells can be induced to differentiate in the presence of granulocyte- and granulocyte-macrophage-colony stimulating factor (G-CSF and GM-CSF). Bigas *et al* (67) observed that constitutively active Notch-1 could inhibit 32D differentiation in response to G-CSF, whereas Notch-2 could inhibit 32D differentiation in response to GM-CSF. This finding suggests a potential link between Notch and cytokine signalling pathways in hematopoietic regulation.

More recently, however, two independent studies have reported that Notch signalling promotes, rather than inhibits, myeloid differentiation. In the first study Tan-Pertel *et al* (68) expressed constitutively active forms of the Notch-1 and Notch-2 proteins in 32D cells. During differentiation in the presence of G-CSF, 32D cells expressing either receptor had significantly higher numbers of viable cells compared with controls. They also displayed enhanced entry into granulopoeisis and exhibited postmitotic terminal differentiation. Furthermore, elevated numbers of viable cells were also observed in 32D cells over-expressing Hes-1, consistent with activation of the CBF1 pathway.

In the second study, Schroeder and Just (69) also expressed the constitutively active intracellular domain of Notch-1 in 32D cells. They found that Notch-1 promoted granulocytic differentiation of these cells. Furthermore, they tested whether ligand

binding to Notch would induce the same phenotype. For this, they used a fibroblast cell line engineered to express the Notch ligand, Jagged-1. Co-culture of Notch-1-expressing 32D cells with Jagged-1-expressing fibroblasts, resulted in an accelerated onset of differentiation compared to Notch-1-expressing cells co-cultured with the parental fibroblasts. They found that Notch signalling during granulocytic differentiation in 32D cells occurred via CBF1 signalling as a transcriptionally active form of CBF1 also increased granulocytic differentiation.

Human Notch-1 is expressed in bone marrow hematopoietic $CD34^{+}$ precursors (70), and Jagged-1 is expressed in human and murine stromal cell lines (71). This is consistent with a model in which Notch and Jagged mediate an interaction between hematopoietic cells and the bone marrow stroma, which could function in regulating cell type differentiation during hematopoiesis. Therefore, Walker *et al* (71) studied the role of Notch receptors and ligands in the lineage commitment and maturation of human $CD34^{+}$ cells. These cells were cultured in the presence or absence of recombinant cytokines on feeder layers that either did or did not express the Notch ligand, Jagged-1. They found that only in the absence of recombinant growth factors, the Jagged-1/Notch-1 pathway acts to preserve cells in an immature state and to prevent their proliferation. In the presence of colony-stimulating factor (CSF) which enhances the viability of hematopoietic progenitors but on its own does not drive proliferation and/or differentiation, Notch signalling favoured erythroid colony formation and survival.

5.1.2. Lymphoid Differentiation

The development of mature T cells from lymphoid progenitor cells involves a series of cell fate choices in the bone marrow and thymus that direct cells along one of several distinct developmental pathways (72). These include the choice of a common lymphoid progenitor to commit to the T cell rather than the B cell lineage, the choice of $CD4^-CD8^-$ double negative (DN) thymocytes to commit to the $\alpha\beta$ or $\gamma\delta$ lineage, and the choice of $CD4^+CD8^+$ double positive (DP) thymocytes to commit to the $CD4^+$ or $CD8^+$ single positive (SP) lineage (Figure 3).

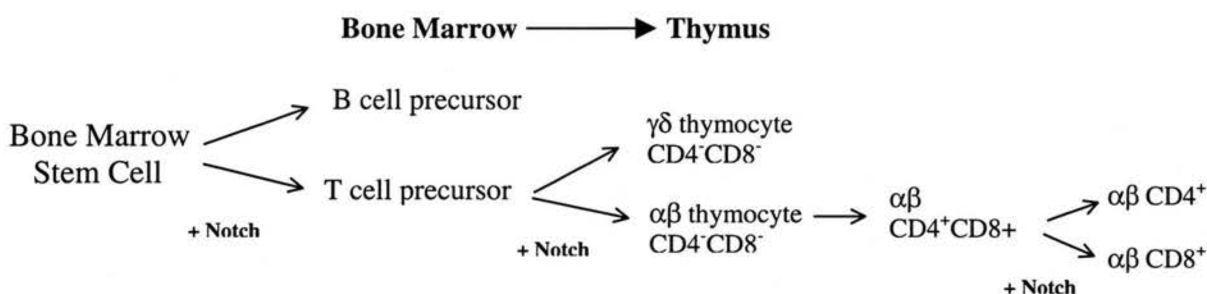


Figure 3. Schematic diagram to illustrate the differentiation check points in T cell development.

Felli *et al* (73) reported that *Notch-1* and *3* as well as *Jagged-2* and the Notch signalling downstream intracellular targets, *Hes-1* and *Hes-5*, were present in both lymphoid and stromal components of murine embryonic and adult thymus.

Recent experiments indicate that Notch signalling plays a critical role in the lineage choice between B and T cell fates. Radke *et al* (74) showed that following inducible deletion of Notch-1 using the *cre/lox* targeted recombination system in the bone marrow and thymus, the majority of DN cells in the thymus contained markers of the B cell lineage. They suggested that, in the absence of Notch-1 signalling, a common lymphoid progenitor cell entering the thymus differentiates into the B cell lineage. Conversely, retroviral transduction of constitutively active Notch-1 (NotchIC) in bone marrow stem cells transferred to irradiated hosts, resulted in the development of a thymus-independent population of cells in the bone marrow that expressed markers

of the T cell lineage (75). This suggested that Notch signalling blocked the differentiation of lymphoid progenitor cells into the B cell lineage.

Interestingly, Notch signalling via Deltex results in the repression of E47 activity (76), and this protein is necessary for B cell development (77, 78). E47 is one of the four mammalian bHLH transcription factors (E12, E47, E2-2 and HEB), collectively known as E proteins, which can bind to sites in DNA called E boxes. The E2A gene encodes both proteins E47 and E12, and Pui *et al* found that constitutively activated Notch-1 inhibited E2A-dependent transcription, which could explain why Notch signalling blocks differentiation of lymphoid progenitors into the B cell lineage. This has also been confirmed by Ordentlich *et a*l (79) who showed that constitutive Notch-1 and Notch-2 inhibited E47 activity in a murine fibroblast cell line, NIH 3T3.

In addition, Hes-1 also affects the expansion of early thymocyte precursors. Hes-$1^{-/-}$ mice (80) do not survive past birth, and most embryos completely lack a thymus or have a significantly smaller one, but expression of TCRβ and TCR$\gamma\delta$ is not detected suggesting that mature T cells are virtually absent. Examination of chimeras in which Hes-$1^{-/-}$ fetal liver was injected into irradiated RAG-$2^{-/-}$ mice revealed that B cells developed normally while T cells were arrested at the DN stage of development. This provided evidence that Hes-1 transcription factor is required for thymocyte expansion.

Notch signalling has also been implicated in later stages of thymocyte development involving the differentiation of DN thymocytes into the $\alpha\beta$ or $\gamma\delta$ lineage. Washburn *et al* (81) used mice reconstituted with bone marrow stem cells derived from mice containing two copies of a functional *Notch-1* gene (Notch$^{+/+}$) or just one (Notch $^{+/-}$). They found that the Notch$^{+/-}$ stem cells contributed less than the Notch$^{+/+}$ stem cells to the $\alpha\beta$ T cell lineage, but only when the Notch$^{+/-}$ and Notch$^{+/+}$ cells developed next to each other. They proposed that Notch signalling was favouring $\alpha\beta$ over $\gamma\delta$ T cell lineage commitment, by a process akin to **lateral inhibition**. Thus, cells expressing less Notch adopted the primary $\gamma\delta$ fate, whereas thymocytes expressing more Notch adopted the alternate $\alpha\beta$ fate.

Other studies (82) have also revealed the importance of the Notch ligands in $\gamma\delta$ T cell lineage commitment. Jagged-2 mutant mice, with a deletion in the portion of Jagged-

2 that interacts with Notch, died perinatally. Examination of E18 (18th day of gestation) embryos, revealed impaired differentiation of the γδ T cell lineage.

At a later stage in thymocyte development, Notch signalling has been shown to bias $CD8^+$ SP thymocyte commitment over $CD4^+$ (83). Robey *et al* showed that transgenic mice with NotchIC under control of the proximal *lck* promoter expressed in DP thymocytes, had a 10-fold increase in the number of $CD8^+$ SP thymocytes and a slight decrease in the number of $CD4^+$ SP thymocytes. Expression of the *Notch-1* transgene in major histocompatibility complex class I (MHC class I)-deficient mice permitted the development of $CD8^+$ T cells in the absence of MHC class I molecules, which are normally required for differentiation to this lineage. However, expression of Notch-1 was not sufficient to promote the generation of $CD8^+$ T cells in mice lacking both MHC class I and II, suggesting that MHC ligation is required for the developing thymocyte to commit to the $CD8^+$ T cell fate, and is not just reliant upon a Notch signal. They proposed that DP thymocytes that are selected on MHC class I also receive a Notch-1 signal that directs them to the $CD8^+$ lineage, whereas DP thymocytes that interact with MHC class II do not receive a Notch-1 signal and develop along the $CD4^+$ lineage. In the presence of constitutive Notch-1 signalling, DP thymocytes selected on MHC class II are diverted from the $CD4^+$ to the $CD8^+$ lineage. However, an intriguing finding was that these transgenic mice had normal ratios of CD4:CD8 in peripheral lymphoid tissues, such as lymph nodes.
These results were consistent with the work of Kim *et al* (84) who examined the effects of Hes-1 on CD4 expression. This is developmentally regulated, in part, by a silencer element that prevents its expression in DN and $CD8^+$ SP thymocytes. They found that the CD4 silencer contained a Hes-1-binding site to which Hes-1 was binding *in vitro*, and over-expression of Hes-1 inhibited endogenous CD4 expression.
The findings of Robey *et al* (83), however, have been challenged by Deftos *et al* (85). They retrovirally transduced a DP thymoma cell line (AKR1010) with NotchIC. Their data revealed that active Notch signalling in this cell line conferred phenotypic changes associated with the transition from DP to both SP thymocytes, including resistance to glucocorticoid-induced apoptosis, up-regulation of TCR and Bcl-2.

Furthermore, they found that *Deltex* was expressed at higher levels in DN and both $CD4^+$ and $CD8^+$ SP thymocytes, compared to DP thymocytes. In a more recent study (86), they generated trangenic mice expressing NotchIC under the control of the *lck*-proximal promoter in thymocytes. They found that, contrary to Robey's *et al* data (83), NotchIC promoted the differentiation of both $CD4^+$ and $CD8^+$ SP thymocytes, and this happened even in the absence of MHC molecules, when bone marrow cells from NotchIC were transferred to irradiated MHC-deficient hosts. Retroviral transfection of a DP thymoma cell line (AKR1010) with NotchIC also promoted SP thymocytes in the absence of MHC molecules. Despite the finding that ectopic Notch signalling can overcome the normal requirement for TCR-MHC interactions for SP thymocyte maturation, they did not exclude the necessity of TCR-dependent signals. Rather, they favoured a model in which the strength of the initial TCR signal influences CD4 versus CD8 cell fate choice (87, 88). Thus, thymocytes that receive a weak TCR signal are induced by Notch to mature into the $CD8^+$ SP lineage, and those that receive a strong TCR signal are induced by Notch to the $CD4^+$ SP lineage. Therefore, the majority of unselected DP thymocytes expressing TCRs with a low affinity for MHC molecules, which would normally die by neglect are rescued by Notch to the $CD8^+$ lineage. This would explain the earlier findings of Robey *et al.* They also proposed that DP thymocytes from NotchIC mice that had not seen MHC may have received a Notch signal in the absence of a TCR signal, and the CD4 versus CD8 cell fate choice may have been made stochastically.

In a very recent study Wilson *et al* (89) analysed the role Notch-1 plays in the development of extrathymic T cells that are present in the epithelium of the intestinal mucosa. These cells differ phenotypically from conventional mature T cells, and in their TCR-associated signalling components which also affects their requirements for positive and negative selection. The gut epithelium itself might provide the inductive microenvironment necessary for their development (90, 91). Wilson *et al* used a competitive mixed bone marrow (BM) chimera, where BM from Notch-1-deleted mice was mixed with wild type BM and injected into irradiated hosts. They found that these mice had virtually no thymus-dependent or independent intraepithelium lymphocytes, and proposed that Notch-1 receptor mediated an essential inductive

signal for T cell development in both the thymus and the gut. Importantly, though, the T cell progenitors in the gut did not differentiate as B cells, unlike those in the thymus, suggesting that the intestinal epithelium cannot support the differentiation of B cell progenitors.

5.2. Notch Signalling and Apoptosis

Two groups have independently reported that Notch-1 has anti-apoptotic properties in T cells. The observations reported suggest that Notch-1 may regulate 'death by neglect' and/or negative selection.

As described previously, Deftos *et al* (85) reported that retrovirally transduced NotchIC inhibited glucocorticoid-induced cell cycle arrest and apoptosis in a thymic lymphoma line (AKR1010) and a T-cell hybridoma (2B4.11). They found that this was CBF1-dependent and Deltex expression was up regulated suggesting both pathways might be activated in this event. Furthermore, the anti-apoptotic protein Bcl-2 was up regulated in AKR1010 cells but not in 2B4.11 cells. In addition, they found that thymocytes from transgenic mice expressing NotchIC showed some resistance to dexamethasone-induced apoptosis.

Jehn *et al* (92) showed that in a T cell hybridoma (DO11.10), NotchIC inhibited Nur-77-dependent apoptosis, which is required for TCR-mediated apoptosis during negative selection. This occurred through a direct interaction between Nur-77 and the intracellular portion of Notch-1.

Taken together, data from Deftos *et al* and Jehn *et al* suggest that Notch signalling may regulate apoptosis during thymocyte maturation by preventing death by neglect and/or negative selection in cells destined to die.

Very different, however, is the effect Notch-1 has been reported to have on the viability of B cells and monocytes. Morimura *et al* (93) found that Notch-1 is expressed in the follicles of the bursa of Fabricius, which is the central organ for chicken B cell development. To examine the function of Notch-1 in B cells, a constitutively active form of chicken Notch-1 was expressed in a chicken cell line, DT40, by a Cre/loxP mediated inducible expression system. The active Notch-1

caused growth suppression of the cells, accompanied by a cell-cycle inhibition at the G1 phase and apoptosis. The expression of Hes-1 also induced apoptosis, although no cell-cycle inhibition. They suggested that Notch-1 signalling induced apoptosis of the B cells through Hes-1, and the G1 cell-cycle arrest through other pathways.
Ohishi *et al* (94) investigated the effects of Notch signalling in monocyte survival. They observed that expression levels of Notch-1 and Notch-2 were increased as bone marrow-derived $CD34^+$ cells matured into monocytes, but decreased as they differentiated into granulocytes. They tested the role of the Notch ligand, Delta-1, in monocyte survival. An immobilised, truncated form of Delta-1 was incubated with monocytes, in the presence of the monocyte survival/differentiation factor, M-CSF, and this lead to a significant increase in monocyte apoptosis.

The interpretation of selected experiments described in this section on Notch should be viewed with some caution. For example, in studies showing the anti-apoptotic effects of Notch or its role in thymocyte differentiation, constitutively active forms of Notch were used. These constructs encode Notch-1 forms that lack all or most of the extracellular portion of the receptor, and they have biological properties, such as transforming activity (95), that have not been observed with intact Notch-1 receptor. Additionally, these truncated forms of Notch proteins localise to the nucleus in readily detectable amounts (95). This is different to what has been observed with intact Notch receptors, where extensive immunocytochemical analyses have consistently failed to detect Notch in the nucleus, and evidence for its nuclear translocations comes indirectly from its effects on gene transcription (33).

5.3. Notch Signalling and Oncogenesis

Notch-1 was first identified in vertebrates as a gene involved in chromosomal translocations with the *TCRβ* gene in a subset of cases of human T cell acute lymphoblastic leukemia (96). These translocations result in the expression of truncated Notch-1 polypeptides that lack most of the extracellular domain and constitutively activate the Notch signalling pathway (97).

More recently, Bellavia *et al* (98) investigated the capacity of Notch-3 to regulate thymocyte development, by generating transgenic mice in which expression of the constitutively active Notch-3 intracellular domain (Notch3IC) was driven by the proximal *lck* promoter. These mice had dysregulated thymocyte development, and after 6-8 weeks developed T cell lymphomas in the spleen and lymph nodes, implicating Notch-3 in T-cell tumorigenesis.

5.4. Notch Signalling and Peripheral Tolerance

The role of Notch signalling in cell fate decisions and the increasing evidence in support of its contribution to thymocyte differentiation prompted us to investigate its potential role in cell fate decisions during an immune response. The role of Notch signalling in the differentiation of $CD4^+$ T cells into regulatory T cells was initially reported by Hoyne and colleagues (99). Murine dendritic cells (DCs) in which human Jagged-1 was over-expressed, were pulsed with the immunodominant peptide of house dust mite, p1 110-131, and then transferred to naïve recipients which were subsequently challenged to induce productive immunity. When the T cells from these mice were cultured *in vitro* with the whole protein, the major epitope p110-131 or a minor T cell epitope, the cells were hyporesponsive. Furthermore, the transfer of $CD4^+$ T cells from mice injected with the pulsed DCs into recipient mice, induced tolerance in the latter when they were re-challenged with the intact house dust mite protein, Der p 1. These studies suggest that Notch signalling at the time of antigen priming may directly influence the path of cellular differentiation in favour of regulatory $CD4^+$ T cells.

6. TGF-β Superfamily

6.1. TGF-β Signalling

TGF-β superfamily members are multifunctional cell-cell signalling proteins that play pivotal roles in tissue development and homeostasis in multicellular animals.
They mediate their pleitropic effects from membrane to nucleus through distinct combinations of **type I and type II** serine/threonine kinase receptors and their downstream effectors, known as Smad proteins (Table II). These signal transducers are highly conserved, and the first protein was identified in *Drosophila* and termed Mothers against dpp (Mad) (100). Subsequently, three Mad homologues called Sma-2, Sma-3 and Sma-4 were found in *C.elegans* (101). At present, eight different Sma- and Mad-related proteins have been identified in mammals and are termed **Smads**. In vertebrates, they can be subdivided into three distinct subclasses: receptor-activated (R-Smads, Smad 1, 2, 3, 5 and 8), common-partner Smads (Co-Smads, Smad 4) and inhibitory Smads (I-Smads, Smad 6 and 7). TGF-β1 and activins initially bind to their corresponding type II receptors with high affinity, after which the type I receptors are recruited into the signalling complex, and phosphorylated by the type II receptor kinase (102). BMPs, however, have low affinity for type II or type I receptors individually, and high-affinity binding requires formation of a heteromeric type I/ typeII receptor complex (103, 104). Specificity of signal propagation to Smad molecules is determined by type I receptors (105, 106) (Figure 4).

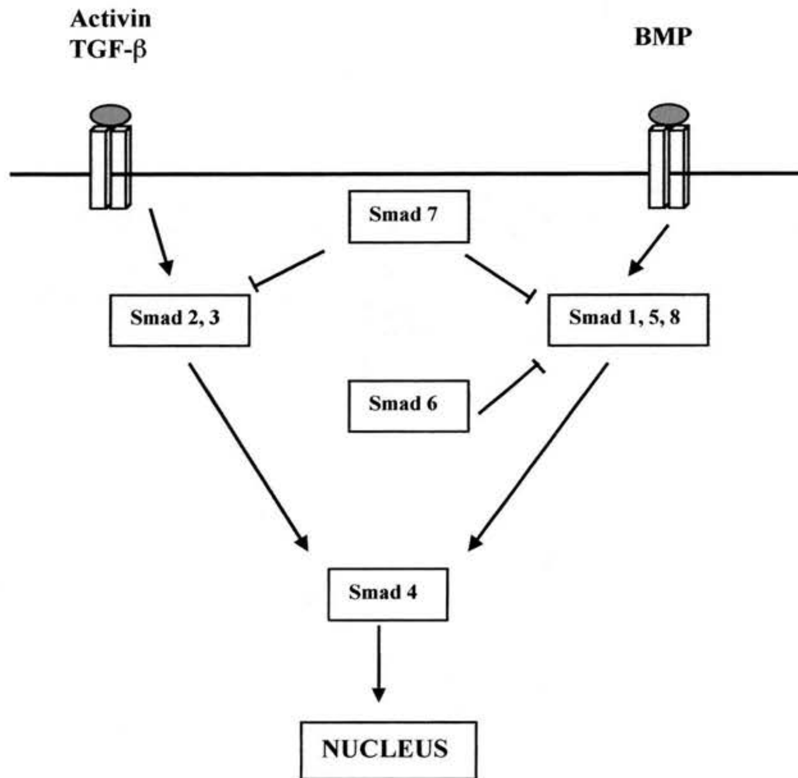


Figure 4. TGF-β signalling pathway. The type I receptors for TGF-β and activin A (in red) recognise and phosphorylate the R-Smads 2 and 3, whereas the BMP type I receptors (in blue) recognise and phosphorylate the R-Smads 1, 5 and 8. Phosphorylation of the R-Smads triggers their dimerization with Co-Smad 4 or other R-Smads, and this complex translocates to the nucleus, where it is involved in transcriptional regulation of target genes. The principal Smads in the TGF-β/Activin pathway lead to target genes different from those controlled by the Smads in the BMP pathway. In addition, inhibitory Smads (Smad 6 and 7 in mammals, Dad in *Drosophila*) can suppress TGF-β signal transduction by associating with type I receptors and interfering with receptor binding and activation of R-Smads. The expression of the inhibitory Smads is quickly induced upon stimulation by TGF-β superfamily members, providing an autoinhibitory mechanism in TGF-β signalling. Whereas Smad 7 inhibits both TGF-β and BMP-mediated signalling, Smad 6 appears to play a more pronounced role in inhibition of BMP signalling.

Analysis of *Smad* genes by targeted mutagenesis is beginning to provide insight into Smad function during vertebrate development and tumorigenesis. For example, Smad 4 null mice die around embryonic day 6.5 (107) and Smad 2 null mice show defects in axis formation and mesoderm induction (108). More recently, Yang *et al* (109) disrupted exon 8 of the *Smad 3* gene in mice. In contrast to the early effects of Smad 2 deletion, Smad 3 disruption generated viable mutant mice that had extensive inflammation at mucosal surfaces. T cell receptor-induced activation of thymocytes and T cells in these mutant mice was completely resistant to inhibition by TGFβ.

Ligand	**Type I Receptor**	**Type II Receptor**	**R-Smad**	**Co-Smad**	**I-Smad**
TGF-β	TβRII	TβRI	2, 3	4	7
Activins	ActR-II ActR-IIB	ActRI ActRIB	2, 3	4	7
BMPs	ActR-II ActR-IIB BMPR-II	ActR-I BMPR-IA BMPR-IB	1, 5, 8	4	6, 7

Table II. Summary of the different type I / type II receptors and Smad molecules that TGF-β, activin A and BMP-4 interact with for signal propagation.

6.2. TGF-β

The prototype of this family, transforming growth factor 1 (TGF-β1) was isolated and characterised from the supernatants of transformed fibroblasts, and it was described as a growth-stimulating polypeptide. The further nomenclature 'transforming growth factor' was adopted because of its ability to confer on untransformed indicator fibroblasts functional properties associated with neoplastic transformation (110). At present, the generic term TGF-β refers to the three isoforms TGF-β1, 2, and 3, which have been identified in mammalian species. Two other isoforms TGF-β4 and TGF-β5 have been cloned in the chicken (111) and *Xenopus* (112), respectively. TGF-β1 is a predominant TGF-β isoform in lymphoid organs and is the major TGF-β species

constitutively present in serum. Conversely, TGF-β2 and 3 are predominantly expressed in mesenchymal tissues and bones. Differential tissue distribution of the TGF-β isoforms and their differential affinity for the TGF-β receptors may account for the wide range of physiological activities of the TGF-β isoforms *in vivo*.

Numerous studies have revealed the significance of TGF-β1 in immune regulation. Thus, TGF-β1-deficient mice die about 2 weeks after birth from a wasting syndrome associated with a multifocal infiltration of lymphocytes and macrophages into targeted organs, especially the heart, lungs and salivary glands (113). More recently, Gorelik and Flavell (114) have generated transgenic mice expressing a dominant-negative form of the TGF-β receptor type II in $CD4^+$and $CD8^+$ T cells. These mice had impaired TGF-β signalling exclusively in T cells and developed autoimmune disease characterised by inflammatory infiltration in several organs, and the presence of circulating autoimmune autoantibodies.

TGF-β1 is produced by every leukocyte lineage, including lymphocytes, macrophages and dendritic cells. Its expression serves in both autocrine and paracrine modes to exert a positive or negative effect on the differentiation, proliferation and state of activation of these immune cells, depending on their developmental stage, the *in vivo* environment or the medium used for *in vitro* studies.

TGF-β1 has an inhibitory effect on B-cell maturation and differentiation, and can inhibit B cell proliferation and induce apoptosis (115). It also enhances the maturation of DCs (116) and induces CD8 expression in the triple negative, $TCR^-CD4^-CD8^-$, thymocytes during T cell development.

Many lines of evidence have implicated TGF-β in the pathogenesis of autoimmune diseases. Studies of experimental allergic encephalomyelitis (EAE) and collagen-induced arthritis (CIA) in mice and rats demonstrated that systemic administration of TGF-β suppressed the symptoms of the disease, whereas antibodies to TGF-β enhanced the disease process, demonstrating that endogenous TGF-β has an effect on disease progression (117, 118).

Furthermore, TGF-β plays a critical role in oral tolerance. In the EAE model, oral tolerisation with myelin basic protein induces peripheral tolerance by generating both a population of $CD8^+$ T cells that secrete active TGF-β and a regulatory population of

$CD4^+$ Th2-like cells producing IL-4, IL-10, and secreting TGF-β in an antigen-specific manner (17, 63, 64). As well, secretion of TGF-β after oral administration of a MBP peptide that does not induce EAE, can suppress EAE induced in rats after feeding them with a encephalitogenic peptide. This phenomenon is known as bystander supression (5) (described in section 1) and in this EAE model, it may be mediated by TGF-β-producing $CD4^+$ T cells or Th3 cells, as they have been referred to. TGF-β is also a critical effector molecule of $CD4^+CD45RB^{low}$ regulatory T cells, which can suppress colitis in SCID mice (65). Paradoxically, TGF-β1 is also the most abundant isoform at sites of inflammation/injury, and is the major isoform secreted by circulating monocytes and tissue macrophages (119, 120). Other biological processes in which TGF-β has been implicated are listed in Table III.

6.3. Activin A

The activins are a family of proteins that consist of disulphide-linked homodimers and heterodimers of the β subunits of inhibin termed βA and βB. These three proteins are known as activin AB (βA-βB), activin A (βA-βA) and activin B (βB-Bβ) (121). More recently, three additional members of this family have been identified, called βC, βD and βE, but as yet their actions remain to be defined (122, 123, 124).

Activin A was originally purified from ovarian follicular fluid, which stimulates the synthesis and release of pituitary follicle-stimulating hormone (FSH) (125). Molecular analysis has revealed that the structure of activin A is the same as that of erythroid differentiation factor (126). Since its original discovery, activin A has been implicated in a wide spectrum of biological activities. The gonads, liver and kidney are also able to synthesise activin A (127), as are bone marrow stromal cells (128) and monocytes (129). Expression of activin A has also been detected in a number of breast cancer cell lines and prostate carcinoma (Table III). Furthermore, activin A has been implicated in inflammatory processes, and is present at sites of tissue inflammation, fracture repair and wound healing (Table III).

Activin A can induce differentiation of a number of cell types including hematopoietic cells, osteoblasts (130), endocrine cells (131) and lung fibroblasts

(132). As well, it can inhibit proliferation of gonadal cell lines (133), hepatocytes (134) and endothelial cells (135). Furthermore, it modulates proliferation in fibroblasts (136) and vascular smooth muscle (137), and induces apoptosis of B cell hybridoma cell lines (Table III).

In addition, activin A constitutes an inhibitor of neuronal cell differentiation and plays a role in mesoderm induction during amphibian development (section 4.3.2.), and in mammalian organogenesis. Activin βA-deficient mice die within 24 hours of birth, and morphological and histological analysis reveals that activin A is required for the normal development of whiskers and teeth (138).

Activins can be functionally regulated by follistatin (section 4.2.2.), which is a monomeric glycoprotein structurally unrelated to activins (139). It binds to activin A and B with high affinity, neutralising their biological activities (140). It is not known whether follistatin binds the other activin members. mRNA transcripts for activin βA and βB subunits and for follistatin were shown to be co-expressed in mid-gestational human fetal tissues (141), and mice lacking the activin βA chain (138) or follistatin (142) both showed similar patterns of disturbed whisker development, suggesting the activin/follistatin system is involved in this biological process. As well, this system has been implicated in fracture healing (143) and inflammation (144).

6.4. BMP-4

Bone morphogenetic proteins (BMPs) were first described as constituents of bone extracts that induce ectopic bone formation when injected into animals (145). They comprise an ever-growing number of homologues, representing almost one third of the TGF-β superfamily, with more than 30 members already described (146). BMPs are synthesised as large precursor proteins. Upon dimerisation, they are proteolytically cleaved to yield carboxy-terminal mature dimers, which once secreted fulfil their signalling function by binding to their specific type I/II receptors (147).

It is now clear that the name BMP is misleading because there is strong genetic and experimental evidence indicating that these molecules regulate biological processes as diverse as cell proliferation, apoptosis, differentiation, cell-fate determination and

morphogenesis (Table III). Moreover, the vertebrate BMPs are involved in the development of nearly all organs and tissues (147). Studies in *Xenopus* embryos have revealed that BMP-4 is crucial for specifying ventral mesoderm during gastrulation, and in inhibiting neural induction in favour of epidermal specification in the ectoderm (section 4.3.1.), and in contributing to erythropoiesis (148). In mammals, studies in BMP-4 homozygous null mice have revealed that the majority die at or around the time of gastrulation without making embryonic mesoderm (149). Studies of BMP-4 expression in embryonic mice have revealed its involvement in limb patterning, kidney, tooth, lung and gut development (147). In chick embryos it has been reported that BMP-4 is involved in dorsal-ventral patterning of the neural tube (150), in regional neural development by inducing apoptosis (151) and in somite patterning that specifies muscles in the limbs and body wall (152). In addition BMP-4 is expressed at sites of fracture repair (153) and *in vitro* studies using rat osteoblasts have revealed its importance in bone formation (154). More recently, BMP-4 has been shown to be involved in human hematopoietic stem cell development (155). All these roles have been demonstrated by examining BMP-4 expression patterns *in vivo* and by direct functional tests, including treating embryonic cells and tissue with BMP-4 *in vitro*, over-expressing BMP-4 *in vivo*, and inhibiting BMP signalling *in vivo* using dominant negative mutant BMP receptors.

	TGF-β	ACTIVIN A	BMP-4	NOTCH
HAEMATOPOIESIS	• Affects **erythropoiesis** and granulopoiesis (156). • Favours CD8 expression during **thymocyte** development (116). • Involved in maturation and differentiation of **B** cells and DCs (116).	• Involved in **erythropoiesis** (162, 163).	• Involved in the differentiation of early human hematopoietic cells (155) • Involved in **erythropoiesis** (148).	• Affects **granulopoiesis** (68, 69). • Affects **erythropoiesis** (71). • **Thymocyte** maturation (83, 85). • Affects **B** and **T** cell lineage commitment (74, 75).
CANCER	• Increased expression in the majority of primary human **breast** cancers, in gastric, colorectal, bladder, cervical and **prostate** cancers (157).	• Increased expression in **prostate cancer** cell lines and numerous **breast** cancer cell lines (164).		• Identified in human T cell leukemia (96). • Constitutive Notch-3 causes T cell lymphomas (98).
T CELL FUNCTION	• Can promote Th2 or Th1 differentiation depending on cytokine milieu (158). • Produced by the immune-regulatory Th3 subset (158). • Can promote or **inhibit T cell proliferation** depending on cytokine milieu and activation state of T cells (158).	• Can **inhibit T cell proliferation** (165).		• Over-expression of the ligand Jagged-1 in DCs induces regulatory T cells *in vivo* (99). • Over-expression of the ligand Delta-1 in a T cell hybridoma **inhibits** its **proliferation** (Gerard Hoyne, personal communication).
B CELL FUNCTION	• Stimulates **apoptosis** of resting **B** cells (115). • Inhibits activated B cell proliferation (115).	• Stimulates **apoptosis** of **B** cell hybridoma cells (166).		• Rescues thymocytes from **apoptosis** (85). • Stimulates **B** cell **apoptosis** (93).
INFLAMMATION	• Secreted by circulating **monocytes** and tissue macrophages (159). • Induces monocyte **IL-6** and **IL-1** production (159). • Present at sites of **inflammation** and tissue damage (159). • Down-regulates **nitric oxide** (NO) production (159).	• Secreted by human **monocytes** (129). • Induces monocyte **IL-6** production (167). • Can inhibit monocyte (168) and promote macrophage-derived **IL-1** production (169) and induce monocyte IL-1 receptor antagonist production (168). • Present at sites of **inflammation** (167, 170). • Induces macrophage-derived **NO** (169).		
DISEASE **TREATMENT**	• Involved in **wound healing** and **fracture repair** (160). • Systemic administration of TGF-β suppresses the symptoms of EAE and CIA (161). • Involved in oral **tolerance** (2).	• Involved in **wound healing** (171). • Involved in **fracture repair** (143).	• Involved in **fracture repair** (173).	• Involved in nasal **tolerance** (99).
DEVELOPMENT		• Induces dorsal **mesoderm** in *Xenopus* (172). • Inhibits *Xenopus* **neural tissue** (46).	• Induces ventral **mesoderm** in *Xenopus* (174). • Inhibits *Xenopus* **neural tissue** (54).	• Inhibits *Drososphila* **neural** induction (36).

Table III. Summary of some of the biological activities of TGF-β, activin A, BMP-4 and Notch. The text in bold highlights functional similarities between the different molecules.

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7. Aims

The Notch and the TGF-β superfamily signalling pathways, which exhibit a high degree of conservation across species, are involved in numerous developmental processes, being neurogenesis the one I have described in this account. The experimental findings summarised in Table III indicate that both pathways are also implicated in a number of similar biological processes including those associated with functions of the immune system. Furthermore, there is a degree of overlap between the two pathways, as studies in *Drosophila* development have demonstrated (61, 62, 63).

Both pathways are involved in directing cell fate decisions, and both operate by an inhibitory mode of action. Thus, activation of Notch signalling inhibits cells from their neural potential, and activation of the activin or BMP signalling pathway inhibits tissue from its neural potential. Thus, they ensure that one phenotype is adopted at the expense of another. From an immunological point of view Notch signalling has been implicated in lymphoid and myeloid differentiation, and activin A and BMP-4 have both been found to affect erythropoiesis. Furthermore, Activin A shares a lot of the immune regulatory features of TGF-β. Both have been found at sites of tissue inflammation and wound repair. As well, both can induce apoptosis in B cells and inhibit T cell proliferation.

In the murine model of mucosal tolerance described previously, I propose that Notch and/or activin/BMP signalling may influence cell fate decisions of $CD4^+$ T cells, by promoting their differentiation into a regulatory phenotype. Evidence for the involvement of Notch signalling is based on our studies in which we demonstrate that Jagged-1 retrovirally-transfected DCs are capable of inducing regulatory T cells that can transfer tolerance in an antigen-dependent manner to other T cells.

The purpose of the work presented in this thesis was to gain further insight into the involvement of the Notch and TGF-β signalling pathways during $CD4^+$ T cell responses *in vivo* and *in vitro*.

The initial aim was to determine if components of the Notch and TGF-β pathways were expressed in lymphoid organs and cells of unimmunised mice, and if these genes were modulated upon lymphocyte activation *in vitro*.

Next, I wanted to investigate the regulation of Notch signalling genes during peripheral immune responses *in vivo*. To do this, I measured the expression of gene transcripts from components of the Notch pathway in $CD4^+$ T cells from mice that had been tolerised or primed to intranasal administration of peptide. In addition, I characterised by flow cytometry the splenic $CD4^+$ T cells from these mice, in an attempt to describe the regulatory T cells generated following intranasal-induced peptide tolerance.

Finally, I wanted to investigate *in vitro* the effects of the TGF-β superfamily members, activin A and BMP-4, on $CD4^+$ T cell responses, in an attempt to describe similar effects to those attributed to the immune-regulatory cytokine, TGF-β. Furthermore, I wished to investigate if the effects of TGF-β, activin A and/or BMP-4 on $CD4^+$ T cells could be attributed to modulation of genes of the Notch signalling pathway, and thus establish an interaction between the two pathways during $CD4^+$ T cell responses.

CHAPTER II

Materials and Methods

1. Immunisation Protocols

6-8 week old C57BL/6 mice were slightly anaesthetised with halothane. The priming protocol consisted in administering mice intranasally with 25µl of 0.03µg of *E.coli* heat labile enterotoxin (LT) alone as the control group, or in the presence of 100µg of HDM p110-130, as the experimental group. The mice were sacrificed at days 2, 4 or 7 after treatment and the spleens were removed.

For the tolerance protocol, the mice were administered on three consecutive days with 25µl of phosphate buffered saline without $CaCl_2$ and $MgCl_2$ (PBS) (SIGMA, UK) alone for the control group, or in the presence of 100µg of p110-130. The mice were sacrificed at days 2, 4 or 7 after the last peptide treatment and the spleens were removed. For all the experiments two mice were set up per group.

2. Cell Biology Techniques

2.1. Organ Preparations

Spleen, lymph nodes and thymus were removed from C57BL/6 (H-2^b) or BALB/c (H-2^d) female mice, aged 6-8 weeks. Lymph nodes (LNs) and thymus were removed for total RNA extraction. The organs were placed in a 70µm cell-strainer (Becton Dickinson, USA) sitting on a 6cm petri-dish containing 5ml of PBS (SIGMA, UK). The tissues were ground using the end of a 1ml syringe plunger. Cell suspensions were passed through 30µm pre-separation filters (Miltenyi Biotech, Germany) and

washed by centrifuging at 1200 revolutions per minute (rpm) for 7 minutes (mins) at room temperature (RT). The supernatant was discarded and the LN and thymus pellets were resuspended in an appropriate volume of PBS for cell counting, using a haemocytometer chamber with 0.4% trypan blue exclusion (SIGMA). The cells were washed and used for total RNA extraction.

For spleen preparations, the supernatant was discarded and the pellet was resuspended in 1ml of PBS and 4 ml of cold red blood cell (RBC) lysis buffer, and left on ice for 5 minutes. Cell suspensions were then diluted down in cold PBS to 20ml and washed as before. The pellet was resuspended in 10ml of PBS, and the spleen cells were counted. Typically 70-100x10^6 cells were obtained per spleen.

For removal of dead cells, the spleen suspension were spun down, diluted in 1ml of RT PBS and gently pipetted over 2ml of RT lympholyte-M (Cedarlane, UK) per spleen, and centrifuged at 2200rpm, RT for 20 mins. The interface was carefully removed and washed 2 times with RT PBS. The cells were counted and used for cell isolation or RNA extraction.

2.2. Cell Isolations

Cell populations were isolated from the spleens of BALB/c or C57BL/6 mice using a magnetic cell sorting method (MACS) (Miltenyi Biotech). This technique relies on antibodies conjugated to magnetic microbeads that recognise cell-specific surface markers, which will bind to a magnetic column when placed in a magnetic field. Therefore, the cells that are recognised by the antibodies will bind to the column and will be eluted out as a highly pure population, and all other cell types are collected in the negative fraction.

The following antibodies were used:

- CD19 microbeads-antibody for separation of B cells.
- CD11c microbeads-antibody for separation of DCs.
- CD4 (LT34) microbeads-antibody for separation of $CD4^+$ T cells.
- CD8 (Ly-2) microbeads-antibody for the separation of $CD8^+$ T cells.

For the separation of DCs, the spleens were removed from C57BL/6 or BALB/c mice placed in a 6cm petri-dish and covered with 5ml of 1mg/ml collagenase D

(Boehringer Mannheim, Germany). Each spleen was injected with 500μl of collagenase D, cut into smaller pieces and incubated at 37°C for 1 hour. Afterwards, spleens were prepared as described in section 2.1.

For separation of $CD4^+$, $CD8^+$ and B cells, spleens were processed as described in section 2.1. After cell counting, the spleen suspension was washed and the pellet was resuspended in $400\mu l/10^8$ total cells of cold MACS buffer for DCs, or $900\mu l/10^8$ total cells for all other cell types. $100\mu l/10^8$ cells of specific microbeads-antibody were added, and the cell suspension was mixed and incubated for 15 mins at 4°C. The cells were washed and the pellet resuspended in 500μl of cold MACS buffer before loading into an LS (for $CD4^+$ and B cell isolation) or MS (for $CD8^+$ and DC isolation) MACS column sitting on a VarioMACS or MidiMACS separator, respectively. The column was washed with cold MACS buffer (3x3ml for LS columns and 3x500μl for MS column) and the positive fraction eluted out using the supplied plunger in 5ml (for LS columns) or 1ml (for MS columns) of cold MACS buffer. The cells were counted. A fraction was kept for flow cytometry, and the rest were kept for use in proliferation assays or RNA extraction.

$CD4^+$ T cells from mice that had been primed or tolerised using the house dust mite peptide p110-130, were isolated by negative selection using the MACS $CD4^+$ T cell isolation kit. This relies on a cocktail of magnetically labelled antibodies specific for all cell types other than $CD4^+$ T cells. Briefly, the spleens were red blood cell lysed and passed over lympholyte as described in section 2.1. The cells were incubated in $400\mu l/10^8$ total cells of MACS buffer and 100μl of the supplied biotinylated antibody cocktail for 10 mins at 4°C. Following on, another $300\mu l/10^8$ cells of MACS buffer were added together with 200μl of anti-biotin microbeads. The cells were incubated for 15 mins at 4°C, after which they were washed with 15ml of MACS buffer, and resuspended in $500\mu l/10^8$ cells of MACS buffer, before loading into an LS column as described previously. The negative fraction was eluted out in 9 ml of MACS buffer and the $CD4^+$ T cells were counted. A fraction was kept for flow cytometry, and the rest were kept for RNA extraction.

2.3. Flow Cytometry (Flourescence Associated Cell Sorting-FACS)

Fluorescence analysis of cells for cell surface expression of proteins was carried out in a FACScan flow cytometer (Becton Dickinson, USA). All the antibodies used were purchased from Pharmingen/BD (USA) or e-Bioscience (UK). Phycoerythrin (PE)-conjugated antibodies were used for single staining. Fluorescein isothiocyanate (FITC)- and PE-conjugated antibodies for double staining and Cy-Chrome-(CyC), FITC- and PE-conjugated antibodies for triple staining. The appropriate isotype controls were included for each antibody. $2.5x10^5$ cells were used per FACS sample. For surface antibody labelling, $2x10^5$ splenocytes or MACS-separated cells were added to flexible 96 round-bottom well plates (BD, USA), and washed for 3 mins at 1200rpm, 4°C. The cells were pre-incubated in PBS + 0.5% BSA + 10% mouse serum for 15 mins at 4°C, to decrease non-specific binding of the antibodies. The cells were washed and resuspended in 50 µl of FACS buffer containing 0.5µg of FITC-labelled and/or 0.2µg of PE- and/or CyC-labelled antibody. The cells were incubated in the dark at 4°C for 15 mins, and washed 2 times in cold FACS buffer. Cells were resuspended in 300µl of cold FACS buffer and were ready for FACS analysis. Cells that were not analysed immediately after were fixed with 300µl of PBS and 2% paraformaldehyde final concentration, and stored at 4°C for a maximum of 2 days.

Cell sorting by flow cytometry was performed for MACS-separated splenic $CD4^+$ T cells, from 8 wild-type C57BL/6 mice. The cells were double stained with $15\mu g/10^8$ cells of PE-CD4 and FITC-CD25 antibodies in $4ml/10^8$ cells of PBS + 0.5% BSA + 10% mouse serum, and incubated for 30 mins at 4°C in the dark. The cells were washed 2 times in FACS wash, and resuspended in PBS + 0.5% BSA at $5x10^6$ cells/ml ready for FACS sorting.

For intracellular labelling, the cells were first surface stained as previously described. They were then fixed in 100µl of 4% paraformaldehyde and washed. They were pre-incubated for 5 mins at RT with permeabilisation solution + 10% mouse serum to reduce non-specific binding of the antibody. The appropriate PE-

labelled antibody was added at 0.2μg to the sample and incubated in the dark for 20mins at RT. The cells were washed in permeablisation buffer, and resuspended in 300μl of PBS + 0.5% BSA before proceeding to FACS analysis.

2.4. Apoptosis Assay (AnnexinV-FITC staining)

Annexin-V conjugated to FITC was used to detect early apoptotic cells. Propidium iodide (PI), on its own or together with Annexin-V, was used to detect late apoptotic cells or necrotic cells.

AnnexinV-FITC staining of cells was performed using an apoptosis detection kit following the manufacturer's instructions (Pharmingen, BD, USA). Briefly, freshly isolated cells or cells that had been in culture were washed twice in cold PBS (1200rpm, 7mins, 4°C), resuspended at 10^5 cells/100μl of the supplied 1x binding buffer, and added to flexible 96 round-bottom well plates (BD). 5μl of AnnexinV-FITC were added per 10^5 cells and incubated at RT in the dark. Cells were washed once in 200μl of cold PBS and resuspended in 400μl 1x Binding Buffer. Immediately before flow cytometry, 1μl of PI was added per sample. Cells were analysed with a FACScan flow cytometer (BD).

2.5. Enzyme Linked ImmunoSorbant Assay (ELISA)

Cytokine secretion was analysed by detecting soluble protein in supernatants collected from cultures at 48 hours. Similar methods were used for murine and human cytokine detection. The capture and detection antibodies as well as the standards were purchased from Pharmingen/BD.

Briefly, anti-cytokine capture antibodies were diluted to 2μg/ml (human cytokines) or 1μg/ml (murine cytokines) in ELISA binding buffer. 50μl/well of diluted capture antibody were added to 96 flat-bottomed well plates, with enhanced protein binding capacity (Corning, USA). The plates were sealed and incubated overnight at 4°C. Next day, the plates were washed 4 times with RT ELISA wash buffer and blotted dry in tissue. 200μl/well of blocking buffer were added and left at RT for 30 mins,

and the plates were washed as before. 50μl/well of the standards (8 double dilutions in blocking buffer) and of the supernatants were added, and the plates were incubated overnight at 4°C. The standards were, recombinant human IL-4 and IL-5 at 4ng/ml, IL-10 at 2ng/ml and IFN-γ at 66ng/ml, or recombinant mouse IL-4 at 20ng/ml, IL-10 at 30ng/ml and IFN-γ at 40ng/ml.

Next day, the plates were washed 6 times, and 50μl/well of the biotinylated detection antibody was added at 1μg/ml (diluted in blocking buffer) for 2 hours at RT. The plates were washed 8 times before the addition of 50μl/well of Streptavidin-alkaline phosphate conjugate (Amersham Life Sciences, UK) diluted 1:2000 in blocking buffer. The plates were left at RT for 30 mins and washed 8 times. Finally, 100μl/well of substrate solution were added and the plates left to develop for 20-60 minutes in the dark, at RT. The plates were read in a Microplate Reader 450 (BioRad Laboratories, U.K).

2.6. Human PBMC Isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated by centrifugal separation over Histopaque 1077. Briefly, blood was obtained from adult volunteers by intravenous puncture into heparinised syringes (5000 units Heparin sodium salt/ml of blood). The blood was diluted in a 1:1 ratio with RT PBS and carefully layered onto RT Histopaque 1077, and centrifuged at 2200rpm, 20 mins, RT. The mononuclear cell layer was gently aspirated and washed 3 times with 3 volumes of PBS, and centrifuged at 1200rpm, 7 mins, RT. Cells were resuspended in complete RPMI-1640 medium and counted using a haemocytometer chamber with 0.4% trypan blue exclusion. PBMC numbers typically recovered using this method ranged from 1-2.5x10^6 cells/ml of blood.

2.7. Maintenance of Human $CD4^+$ T Cell Clone

The HA1.7 $CD4^+$ human T cell clone is specific for the haemagglutinin peptide, HA 306-318, and restricted by HLA-DRB1*0101. PBMCs were isolated from histocompatible HLA-DRB1*0101 donors and γ-irradiated with 2000 rad. Irradiated PBMCs were washed, and added to 24-well plates (Costar, USA) in complete RPMI medium at 10^6/ml/well. HA1.7 $CD4^+$T cells were added at 10^6/ml/well, and HA peptide 306-318 at 1μg/ml. The cultures were supplemented with 10% final concentration of recombinant IL-2 (BIOGEN, Switzerland). 1ml of medium was replaced with 1ml of fresh medium containing 10% IL-2, every 3 days. When the cells were confluent, they were split 1 in 2. Every week the cells were pooled, washed in complete RPMI medium, counted and re-stimulated with fresh PBMCs and peptide.

2.8. Human $CD4^+$ T Cell Clone Proliferation Assay

Irradiated PBMCs were washed and added to 96 round-bottom well plates at $2.5x10^4$ cells/well in complete RPMI medium. HA peptide 306-318 was added to appropriate wells at 1μg/ml. Confluent HA1.7 $CD4^+$ T cells were washed once in RT PBS, and added to feeder cells at $2x10^4$ cells/well in complete RPMI medium. Alternatively, HA1.7 T cells were grown in 10% IL-2 (Lymphocult) or with 1μg/ml HA peptide final concentration, in the absence of feeder cells. Recombinant proteins were added to the appropriate wells at the following concentrations: Activin A was added at 1, 10 and/or 100ng/ml, BMP-4 at 1, 10 and/or 100ng/ml and TGF-β at 0.1, 1 and/or 10ng/ml. Cells were incubated at 37°C in a humidified incubator 5% CO_2 for 3 days. 50μl aliquots of supernatants were removed after 48 hours of culture to measure cytokine production. Proliferation was assessed by incorporation of 2.5μCi/well of tritiated thymidine, ^{3}[H]-TdR (Amersham Life Sciences, UK), 16 hours before harvesting. All samples were tested in triplicate.

2.9. Polyclonal Stimulation of Lymphocytes

Stimulation of human HA1.7 T cells or MACS-purified murine $CD4^+$ T cells was performed using immobilised monoclonal antibodies against CD3ε and soluble monoclonal antibodies directed against CD28 (Pharmingen, USA). Briefly, anti-CD3ε antibody was immobilised onto 96 flat-bottom well plates (for proliferation assays) or 24 flat-bottomed well plates (For RNA extraction or FACS analysis), by adding at 1μg/ml/well (murine cells) or 5μg/ml/well (human cells) in RT PBS. The plates were incubated for 2 hours at 37°C. Wells were washed 3 times with RT PBS to remove excess unbound antibody. Murine $CD4^+$ T cells were added in complete DMEM medium at $2.5x10^5$/well (96-well plates) or $2x10^6$/well (24-well plates), and human HA1.7 T cells at $2x10^4$/well (96-well plates) in complete RPMI medium. Soluble anti-CD28 antibody was added at 5μg/ml/well. 96-well plates were incubated at 37°C in a humidified incubator with 5% CO_2 for 72 hours and proliferation was measured by incorporation of ^{3}[H]-TdR, 16 hours before harvesting. Each sample was tested in triplicate.
24-well plates were incubated over different time points and the cells were used for RNA extraction or FACS analysis.

Stimulation of MACS-purified C57BL/6 splenic B cells was performed by culturing $2x10^6$ cells/well in 24-well plates in complete DMEM medium, and adding 100μg final concentration of *E. coli* lipopolysaccharide (LPS) (serotype 055:B5, SIGMA). The cells were incubated at 37°C in a humidified incubator with 5% CO_2 over different time points, and used for RNA extraction.

2.10. Murine $CD4^+$ T Cell Proliferation Assay

$CD4^+$ T cells were isolated by MACS from the spleens of wild type C57BL/6 or BALB/c mice and they were plated at $2.5x10^5$/well to 96 flat-bottomed well plates, that had been pre-coated with 1μg/ml of anti-CD3ε antibody. Soluble anti-CD28 antibody was added at 5μg/ml. Recombinant activin A and BMP-4 were added at 1, 10 and/or 100ng/ml to the appropriate wells, and TGF-β was added at 0.1, 1 and/or

10ng/ml. The cells were incubated in a humidified incubator with 5% CO_2 for 72 hours. 50μl aliquots of supernatants were removed after 48 hours of culture to measure cytokine production. Proliferation was assessed by incorporation of 2.5μCi/well of ^{3}[H]-TdR, 16 hours before harvesting. All samples were tested in triplicate.

Statistics

Statistics were performed using GraphPad InStat.

The Bonferroni Multiple Comparison Test was used to compare the significance of the data obtained from the test samples against the data of the control.

When $p<0.05$ it was represented as * and regarded as significant.

When $p<0.01$ it was represented as ** and regarded as highly significant.

When $p<0.001$ it was represented as *** and regarded as very significant.

3. Molecular Biology Techniques

3.1. Extraction of Total RNA

Extraction of total RNA from cells was done using QIAGEN's RNeasy Mini Kit, according to the manufacturer's instructions. Briefly, cells were resuspended in an appropriate volume of the supplied LRT lysis buffer, and the samples homogenised using QIAGEN's QIAshredder spin columns. The homogenised lysate was mixed with one volume of 70% ethanol, which provides the appropriate binding conditions, and applied to an RNeasy spin column. This was centrifuged for 15 secs, 10000 rpm at RT, as the total RNA would bind to the column. The spin column was washed briefly in the supplied RW1 buffer, and any contaminating DNA was digested and washed out using QIAGEN's RNAse-Free DNase according to the manufacturer's instructions. The spin column was washed again twice in the supplied RPE buffer. Finally, the RNA was eluted out in 20-30μl of RNAse/DNAse-free water, and quantified.

3.2. Quantification of Nucleic Acids

To determine the concentration of nucleic acids in solution, absorbance (A) at 260 and 280 nanometers (nm) was measured in a spectrophotometer (Ultrospec 200, Pharmacia Biotech).

The readings were converted to concentrations using the following formulae (175).

1 A_{260} unit of double-stranded deoxy ribonucleic acid (dsDNA) = 50µg/ml

1 A_{260} unit of oligonucleotide = 20µg/ml

1 A_{260} unit of RNA = 40µg/ml

To determine the purity of solutions of DNA and RNA, the ratio of absorbance at 260nm and 280nm ($OD_{260/280}$) was taken. This value should ideally be about 1.8 for DNA and 2.0 for RNA.

3.3. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

3.3.1. Designing Gene-Specific Primers

DNA sequences of interest were obtained from PubMed's DNA nucleotide sequence program (Internet Navigator), and primers were designed using Holyrood's gcg Prime program, or MacVector program. They were tested for binding specificity using DNA Strider program.

All PCR primer pairs met the following criteria:

- They were not GC rich (as this creates high annealing temperatures).
- They did not form primer-dimers (primer pairs could not have more than 3 consecutive bases complementing each other).
- The primer pairs had similar annealing temperatures.
- They were at least 16 bases long, optimum 20-24.
- They did not form hairpin-loop folding with energy less than 1.0, and had a free 3' tail.

3.3.2. Murine Gene-Specific Primers for PCR

GENE	PRIMER SEQUENCE: Forward Primer Reverse primer	Length of PCR Product (bp)	T_m (°C)
Notch-1	5'-TGTTAATGAGTGCATCTCCAACCC-3' 5'-CATTCGTAGCCATCAATCTTGTCC-3'	638	58
Notch-2	5'-CAGAGGAATAGCAAGACGTGCAAG-3' 5'-GATGAAGAACAGGATGATGACAACAG-3'	599	58
Notch-4	5'- TTCTACTGCGAGTGTCTCCC -3' 5'- ACAAGCTGTCACCTCCTCACTAC -3'	183	58
Jagged-1	5'- CAAAAATCAGGACACACAAC -3' 5'- CAGCCAACCACAGAAACTAC -3'	289	58
Jagged-2	5'- CCGTGCCTTAATGCTTTTTC -3' 5'- CAGTTCTTGCCACCAAAGTC -3'	282	58
Delta-1	5'- GACTCTCCCGATGACCTC -3' 5'- GATGCACTCATCGCAGTAG -3'	389	60
Hes-1	AATGGAGAAAAATTCCTCCTCC TCACCTCGTTCATGCACTCG	350	56
Noggin	5'-TTGGTGGACCTCATCGAACATC-3' 5'-CAGACTTGGATGGCTTACACACC-3'	308	50
BMP-4	5'-ACGAAGAACATCTGGAGAAC-3' 5'-GAGTCTGATGGAGGTGAGTC-3'	369	58
Activin A	5'-GAGAGTGGTGCCAGTCTAGT-3' 5'-GCCACACTCCTCCACAATCA-3'	522	58
Activin A Receptor TypeIIβ	5'-GAAGGCTCAGCTCATGAAC-3' 5'-CGCTCTTCAGCAGTACATTC-3'	380	58
TGF-β	5'-ACCCACAACGAAATCTATGAC-3' 5'-GCGAAAGCCCTCAATTTCC-3'	300	58
β-Actin	5'-CCACCAACTGGGACACATG-3' 5'-GTCTCAAACATGTGGGTCATC-3'	153	58

3.3.3. Setting up an RT-PCR

The reverse transcription of total RNA into first strand DNA, and the synthesis of second strand complementary DNA (cDNA) and its amplification, was performed using Promega's Access RT-PCR kit (USA), which allows the two reactions to take place in a single tube. The reaction was set up according to the manufacturer's instructions.

Prior to study expression of specific genes, different RNAs were adjusted to equal levels, by performing an RT-PCR on the house keeping gene, β-actin.

Each reaction tube consisted of 25μl of the following reagents:

Reagent	Volume/Tube (μl)	Final Concentration
5x Reaction Buffer	5.0	1x
25mM Magnesium Sulphate ($MgSO_4$)	1.0	1 mM
dNTP mix	0.5	200 μM of each dNTP
Primer mix	1.0	25 pmol of each primer
Avian Myeloblastosis Virus (*AMV*) RT (5 units/μl)	0.5	0.1 U/μl
Thermus flavus (*Tfl*) DNA polymerase (5u/μl)	0.5	0.1 U/μl
RNA 50 ng	16.5	

To test for DNA contamination in the RNA samples, the reaction was set up with all the reagents except AMV reverse transcriptase.

RNAse/DNAse-free water was included as a negative control for each gene tested, to ensure there was no DNA contamination in the reagents.

The thermocycler was programmed as follows:

- First strand synthesis:

1 cycle   48°C, 45 mins (RT)
1 cycle   94°C, 2 mins (RT inactivation and RNA/cDNA/primer denaturation).

- Second strand cDNA synthesis and PCR amplification:

35 cycles   94°C, 30 seconds (denaturation)
primer-specific Tm, 1 min.
68°C, 2 mins (amplification).
1 cycle   68°C, 7 mins.

3.4. Preparation of Agarose Gels

2% agarose gels (SeaKem LE agarose, FMC) were prepared in 1xTAE buffer (GibcoBRL UK), containing 10μg/μl of ethidium bromide (SIGMA) per ml of agarose. The gel was transferred to a gel tank containing 1xTAE buffer. 10μl of the PCR reaction were mixed with 2μl 6x PCR loading buffer and loaded into each well. A 50ml gel was run at 80 volts for 1 hour. The UVGrab program connected to a white/ultraviolet transilluminator (UVP) was used to detect the expression of PCR products. SIGMA's PCR marker or Gibco's 1kb DNA ladder were used as molecular weight markers.

Alternatively, to visualise the quality of RNA, 10μl of 1μg RNA were mixed with one volume of RNA loading buffer (SIGMA), and the mixture was heated at 65°C for 10 mins and cooled on ice for 3 mins, before loading into the agarose gel. SIGMA's RNA marker was used to visualise the 28S and 18S rRNA bands.

3.5. Real-Time PCR

Higuchi R *et al* (176) pioneered the analysis of PCR kinetics by constructing a system that detects PCR products as they accumulate in 'real time'. Two chemistries are available to detect PCR product accumulation. One is the use of a DNA-binding dye, which has the disadvantage of binding to all double-stranded DNA, including non-specific reaction products.

The other relies on the 5'nuclease activity of *Taq* DNA polymerase (177), which cleaves a probe that binds specifically to its target DNA sequence. Only when the PCR product is amplified is the probe cleaved, therefore the signal emitted by the probe corresponds to the amount of product amplified in real-time (Figure 1).

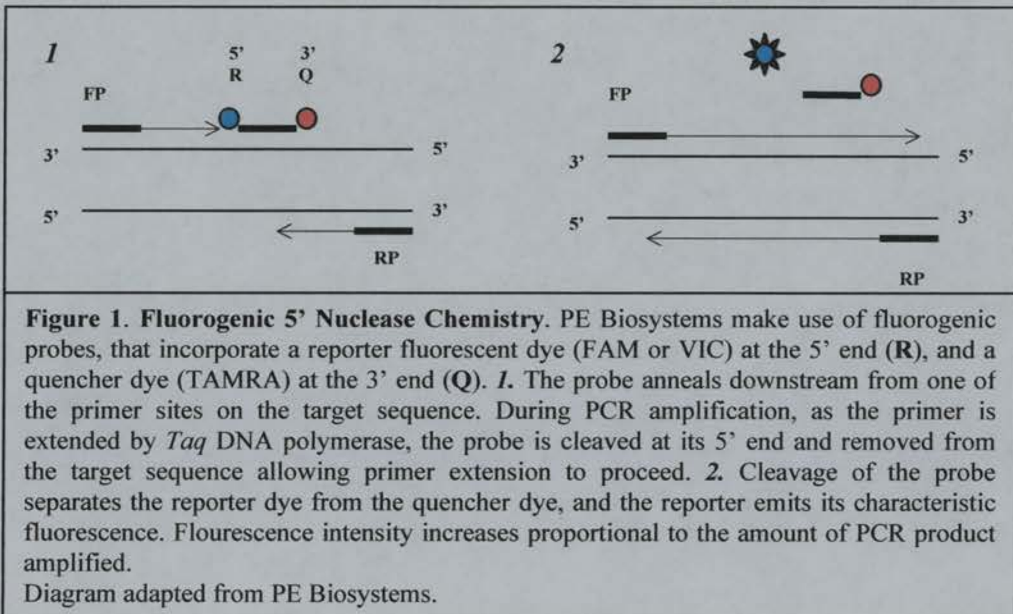


Figure 1. Fluorogenic 5' Nuclease Chemistry. PE Biosystems make use of fluorogenic probes, that incorporate a reporter fluorescent dye (FAM or VIC) at the 5' end (**R**), and a quencher dye (TAMRA) at the 3' end (**Q**). *1.* The probe anneals downstream from one of the primer sites on the target sequence. During PCR amplification, as the primer is extended by *Taq* DNA polymerase, the probe is cleaved at its 5' end and removed from the target sequence allowing primer extension to proceed. *2.* Cleavage of the probe separates the reporter dye from the quencher dye, and the reporter emits its characteristic fluorescence. Flourescence intensity increases proportional to the amount of PCR product amplified.
Diagram adapted from PE Biosystems.

3.5.1. Real-Time PCR Amplification

Reactions are characterised by the point in time during cycling when amplification of a PCR product is first detected. Figure 2 shows a representative amplification plot.

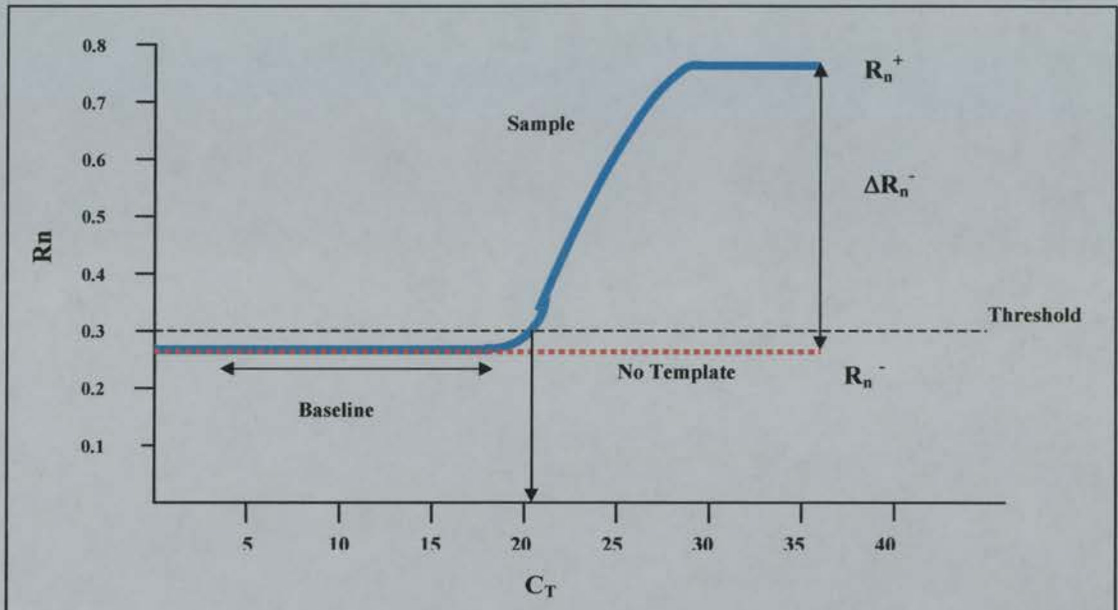


Figure 2. Model of a single amplification plot. An amplification plot is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR there is little change in fluorescence signal. This defines the **baseline** for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated product. A fixed fluorescence **threshold** can be set above the baseline.

- C_T (threshold cycle) is defined as the cycle number at which the fluorescence passes the fixed threshold.
- $\mathbf{R_n}$ is the value obtained by dividing the emission intensity of each reporter dye signal with that of an internal reference dye (ROX), which normalises for non-PCR related fluorescence fluctuations occurring well-to-well or over time.
- $\mathbf{R_n^+}$ is the $\mathbf{R_n}$ value of a reaction containing all components including the template.
- $\mathbf{R_n^-}$ is the $\mathbf{R_n}$ value obtained from either the early cycles of a real-time run (prior to detectable increase in fluorescence) or from a reaction not containing template.
- $\mathbf{\Delta R_n}$ is the difference between the $\mathbf{R_n^+}$ and the $\mathbf{R_n^-}$ value. It reliably indicates the magnitude of the signal generated by the given set of conditions.

Diagram adapted from PE Biosystems.

3.5.2. Real-Time PCR Optimisation

The following parameters need to be optimised for efficient real-time amplification of target cDNA:

A) Primer and Probe Design and Optimisation

Ideally, the primers should be designed to span at least an intron to distinguish between mRNA and genomic sequences, and ensure only the cDNA is amplified. However, all the sequences obtained from PubMed's nucleotide sequence program corresponded to mRNA sequences, and therefore this distinction was not possible. For this reason I treated all the RNA samples with DNAse (section 3.1) to remove any genomic DNA contaminants, and I always ran a PCR on the RNA samples using β-actin to test for contamination.

Primers and probes are designed using Primer Express® software, supplied by PE Biosystems. The primers are optimised by independently varying forward and reverse primer concentrations (50, 300, 900 nM). Optimal performance is achieved by the primer concentration that provides the lowest $\mathbf{C_T}$ and the highest $\mathbf{\Delta R_n}$.

The probe can also be optimised by varying the concentration 50-250 nM. Optimal performance is obtained at the concentration that provides the lowest $\mathbf{C_T}$ and the highest $\mathbf{\Delta R_n}$. However, a probe limiting concentration should be avoided when wishing to detect low copy numbers of a target sequence.

In real-time PCR assays examined here, primer concentrations of 300 nM for both the forward and reverse primer, and probe concentrations of 200 nM per reaction, were used.

B) Choosing a Method of Quantitation

Relative quantitation of gene expression can be performed using the **standard curve** method or the **comparative** ($\Delta\Delta C_T$) method. Either method needs:

- A **standard**, which is the sample to be tested.
- A **reference**. This can be an endogenous control with its own set of primers and probe. It is used to standardise all the cDNA samples used in a reaction, and as a control of the PCR reaction itself.

- A **calibrator,** is the positive control used in the reaction, which all the standards are made relative to. Thus, the calibrator becomes the 1x sample, and all other quantities are expressed as an *n*-fold difference relative to the calibrator.

The **standard curve** method relies on a known stock of RNA or DNA which is diluted down to get the standard curve. Standard curves for the gene of interest and for an endogenous control can be obtained, by running the stock sample in separate reactions. The equation on the standard curves is then used to find the amount of gene of interest and endogenous control in the target sample tested. Dividing these two values allows quantitation normalised to the endogenous reference. This value can then be further divided by the value of the normalised calibrator (x1) to generate relative expression levels.

The **comparative method** is similar to the standard curve method, except it uses arithmetic formulas to achieve the same result.

The amount of target, normalised to an endogenous reference and relative to a calibrator, is given by $\mathbf{2}^{-\Delta\Delta C_T}$, where

- ΔC_T is the C_T of the gene of interest - C_T of the endogenous control (normalises the amount of target to the endogenous control).
- $\Delta\Delta\ C_T$ is the ΔC_T - C_T of the calibrator (makes the amount of target relative to the calibrator).

For the $\Delta\Delta\ C_T$ to be valid, the efficiency of the target amplification and of the reference amplification must be approximately equal. This can be assessed by running dilutions of a stock cDNA and plotting the standard curves for the gene of interest and for the endogenous control, like was described earlier for the standard curve method.

3.5.3. Multiplex PCR

Multiplex PCR is the use of more than one primer pair in the same tube. This method can be used in relative quantitation where one primer pair amplifies the target and another primer pair amplifies the endogenous reference in the same tube.
For accurate quantitation it is important that the two reactions do not compete. This can be avoided by limiting the concentration of primers of the most abundant species. The desired concentration is that which shows a reduction in ΔR_n but little effect on C_T.
It can be performed for both the standard curve method and the comparative method.

For the real-time PCR studies I used the comparative method of quantitation in multiplex PCR reactions.
The following components were used throughout these studies:

- Endogenous control: PE Biosystems 18srRNA. This is already primer limited to avoid competition for the amplification of the target sample. It contains specific primers and a probe that is labelled with a fluorogenic dye called VIC.
- Calibrator: this depends on the type of experiment that is set up. In this study I used the following calibrators:
- Wild type spleen, if the test samples where lymphocytes or dendritic cells (DCs).
- Wild type $CD4^+$ T cells, if the test samples were $CD4^+$ T cells that had been treated *in vitro* or isolated from mice that had been treated *in vivo*.
- Wild type DCs, as above.
- Wild type B cells, as above.
- No template control for each specific gene tested, to ensure that there was no genomic DNA contaminating the samples.

3.5.4. Reverse Transcription of Total RNA

RNA samples for real-time PCR were reverse transcribed into cDNA using TaqMan MultiScribe Reverse Transcriptase kit (PE Biosystems, UK). A 20μl reaction was set up as follows:

Reagent	Volume/Tube (μl)	Final Concentration
10x TaqMan RT buffer	2.0	1x
25mM Magnesium Chloride ($MgCl_2$)	4.4	5.5 mM
dNTP mix	4	500 μM of each dNTP
Random hexamer	1	2.5 μM
RNase inhibitor	0.4	0.4 U/μl
MultiScribe RT (50 U/μl)	0.5	1.25 U/μl
RNA 400ng	7.7	

The thermocycler (Perkin Elmer) was programmed as follows:

1 cycle 25°C, 10 mins. (incubation)
1 cycle 48°C, 30 mins. (reverse transcription)
1 cycle 95°C, 5 mins. (RT inactivation)

3.5.5. Preparing the Plates for Real-Time PCR

cDNA synthesised by reverse transcription was used for real-time PCR. The method uses a fluorogenic probe that specifically anneals the template between the PCR primers. The use of a sequence detector (ABI Prism 7700, PE Biosystems, USA) measures amplification of the product, in direct proportion to the increase in fluorescence emitted by the probe. Each sample was run in duplicates in 96-well optical reaction plates (PE Biosystems). 25μl of the PCR reaction were added per well.

A 25μl PCR reaction consisted of the following reagents:

Reagent	Volume/Well (μl)	Final Concentration
10x Universal Master Mix	2.5	1x
20x Pre-Developed 18S ribosomal RNA (rRNA)	1.25	1x
Primer/Probe Mix	7	300nM for both primers 200nM for probe
Water	1.75	

The thermal cycler conditions were standard for all the genes tested. These were

40 cycles 2 min, 50°C,
10 min, 95°C
15 secs, 95°C
1 min, 60°C.

The comparative method of quantitation was used for data analysis. All the values obtained were normalised to 18S rRNA, which was included in the PCR reaction as an internal endogenous control. Furthermore, each sample was made relative to a relevant positive control, which always had a value of 1. The data was plotted relative to the positive control in a column-graph format (Microsoft Excel).

3.5.6. Murine Gene-Specific Primers for Real-Time PCR

PRIMERS	PRIMER SEQUENCE Forward Primer (5'-3') Reverse Primer (5'-3')	PROBE
Notch-1	TCCAGAGTGCCACCGATGT TCCACCGGCTCACTCTTCAC	CTGCCTTCCTAGGTGCTCTTGCGTCA
Notch-2	ACCCTCCGCCGAGACTCT TCCCAGAACCAATCAGGTTAGC	CCTGTCCCACAGGTTCACGGCG
Notch-3	GGCTGCAACACTGAGGAATGT TCAGGAGGCAGAAGAACTGTGA	CCGGGACCTCGCTGGCACA
Notch-4	TGTCTCCCCCATAGAGTATGCA CTCGAAATCAACTTTGTCCTCTTG	CCGGACATCCTAAACCCTCTTCCCA TTG
Delta-1	TTCTTTCGCGTATGCCTCAA CATCAGGCAGGCTGAAGGA	ACTACCAGGCCAGCGTGTCACCG
Delta-3	GGCGGTGAAGATCCTGACTCT CGGTCCACCCTCTTCTCACA	ATTGCCCACCTGGTTTCCAAGGCTC
Jagged-1	CCCGCACCCAGGATGTGT CACCCCAGTTGGTCTCACAGA	CACCTGCAATGAACCCTGGCAGTG
Jagged-2	CAGCTGGACGCCAATGAGT GCCAATCAGGTTTTTGCAAGA	AGCATTAAGGCACGGCTTCCCTTCA
Deltex	GCCTCTAGCCTGGCACATG CCAGATCCCCTTAGCGCTTCT	CTCCCTCCGCTCTCGGCGG
Hes-1	GCTTCAGCGAGTGCATGAAC TTGATCTGGGTCATGCAGTTG	TGACCCGCTTCCTGTCCACGTG
IL-10	CCACAAAGCAGCCTTGCA AGTAAGAGCAGGCAGCATAGCA	AGAGCTCCATCATGCCTGGCTCAGC
TGF-β	GAGCCCGAAGCGGACTACT GGTTTTCTCATAGATGGCGTTGT	TGCTAAAGAGGTCACCCGCGTGC
Activin A	TGGCAAGTTGCTGGATTATAGTGA TACCATCTCTGGCTGAGAGTTAGGT	AGTTCCCCCACCCCAGGATCCG
Noggin	CGAGATCAAAGGGCTGG TGCTCAGGCGCTGTTT	CCTTGGGCCAAGCCCTCGGA
BMP-4	CGAGCCATGCTAGTTTG CCGCGTGGCCCTGAA	TCGGCGACTTTTTCTTCCC

Materials and Buffers

All chemicals were purchased from SIGMA (Dorset, U.K.) unless otherwise stated.

- **Red Blood Cell Lysis Buffer**

1mM ammonium hydrogen carbonate (NH_4HCO_3) (BDH, UK) and 114mM ammonium chloride (NH_4Cl) (A-0171) in distilled water. Kept at 4°C.

- **MACS Buffer**

0.5% BSA (A-9418) in PBS (D-5652). Kept at 4°C.

- **Collagenase D**

At 1mg/ml in 10mM Hepes-NaOH pH 7.4, 150mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1.8mM $CaCl_2$. Freshly prepared.

- **FACS Buffer**

10% normal mouse serum (Scottish Antibody Production Unit-SAPU, Scotland, U.K.-NMS) and 0.5% BSA (A-9418) in PBS (D-5652). Kept at 4°C.

- **FACS Fixing Buffer**

4% paraformaldehyde (P-6148) in PBS (D-5652). Kept at 4°C.

- **Permeabilisation Buffer**

0.1% sodium azide (S-2002), 0.2% BSA (A-9418) and 0.1% saponin (S7900) in PBS (D-5652). Kept at 4°C.

- **ELISA Binding Buffer**

Carbonate-Bicarbonate buffer (C-3041). 1 capsule dissolved in 100ml of distilled water yields a 50mM Carbonate-Bicarbonate buffer, pH 9.6. Kept at 4°C.

- **ELISA Wash Buffer**

PBS tablets diluted in water (1 tablet/100ml distilled water) containing 0.05% Tween-20 (P-7949). Kept at room temperature.

- **ELISA Blocking Buffer**

ELISA washing buffer containing 1% BSA (A-9418). Freshly prepared.

- **ELISA Substrate Solution**

ELISA ethanolamine buffer (Don Whitley Scientific, Shipton, U.K. E-016) containing 1mg/ml pNPP (N-2770). Made fresh.

- **Complete Human RPMI Culture Media**

500mls RPMI 1640 (R-0883) with 5% human AB serum [heat inactivated] (H-1513), 100IU/ml penicillin/streptomycin (Gibco, Paisley, U.K. 15070-071), 2mM L-Glutamine (Gibco, Paisley, U.K., 25033-010). Kept at 4°C.

- **Complete Murine DMEM Culture Media**

500mls DMEM (D-5921) with 10% foetal calf serum (FCS) (F-9665), 5×10^{-5}M mercaptoethanol (M-7522), 100IU/ml penicillin/streptomycin (Gibco, Paisley, U.K. 15070-071), 2mM L-Glutamine (Gibco, Paisley, U.K., 25033-010). Kept at 4°C.

- **Preparation of LPS**

E. coli LPS (serotype 055:B5) powder was reconstituted to 1mg/ml in complete DMEM medium. Aliquots were prepared and stored at -20°C.

- **Recombinant Proteins**

Recombinant human activin A (R&D Systems, UK) and recombinant human BMP-4 (mature protein identical to mouse) were reconstituted in filter-sterilised PBS (D-5652) containing 0.1% BSA (A-2153), to a final concentration of 10μg/ml. Aliquots were prepared and stored at -20°C for up to 3 months.

Recombinant human TGF-β (mature protein identical to mouse) was reconstituted in filter-sterilised 4mM HCl containing 0.1% BSA, to a final concentration of 1μg/ml. Aliquots were prepared and stored at -20°C for up to 3 months.

- **Synthetic Peptides and Mucosal Adjuvant**

The Der p 1 peptide p110-130 and the HA1.7 T cell-specific peptide 306-318 are synthesised as lyophilised pellets by the Advanced Biotechnology Centre (Imperial College School of Medicine, Charing Cross Hospital, London). Der p 1 p110-130 was reconstituted in PBS to the desired concentration for use in immunisations. HA p306-318 was reconstituted to 1mg/ml in complete RPMI medium and aliquots stored at -20°C. The mucosal adjuvant, *Escherichia coli* (*E.coli*) heat labile enterotoxin was a gift from Dr. G. Douce (University of Glasgow). It originated from the periplasm of recombinant *E. coli* strains (179). It was made up to the appropriate concentration in PBS.

- **PCR Loading Buffer**

0.25% weight/volume of bromophenol blue (BDH, UK) and 40% weight/volume of sucrose in distilled water. Stored at 4°C.

CHAPTER III

Expression of Developmental Genes in Lymphoid Organs and Cells

1. Background

The Notch signalling pathway plays a central role in the specification of cell fates through local cell interactions in a wide variety of tissues. Notch is a transmembrane receptor that can bind to its ligands Delta and Jagged. This evolutionary conserved pathway was originally identified in *Drosophila* and it has also been identified in lower and higher vertebrates, and related proteins have been found in the nematode, *Caenorhabditis elegans*. Activation of the pathway leads to cleavage of the intracellular portion of Notch, which is translocated to the nucleus and associates with DNA binding proteins to initiate transcription of target genes (33).

Studies over the last 5 years suggest that components of the pathway are expressed differentially in the different thymic compartments (73), and Notch signalling plays a critical role at multiple steps during T cell development. Notch-1 signalling is required to commit precursor cells to the T cell rather than B cell lineage (74, 75) and for $\alpha\beta$ but not $\gamma\delta$ T cell specification (81).

The ability of Notch signalling to direct cell growth and differentiation during embryonic development prompted to investigate its involvement in the regulation of peripheral immunity. Overexpression of the Notch ligand, Jagged-1, in murine DCs pulsed with the immunodominant epitope of house dust mite, could induce long-lived antigen specific tolerance (99). These studies revealed that the recognition of antigen by $CD4^+$ T cells in the presence of a Notch ligand, could direct T cells to differentiate as Tr cells.

Additionally, I was also interested in another group of developmental-related proteins, which are known as the neural inducers and inhibitors, because of their capacity to

induce neural tissue or inhibit it. These molecules have been described in *Drosophila* as well as lower and higher vertebrates (39). In this study I have concentrated on the neural inducer, noggin, and the neural inhibitors and TGF-β superfamily members, activin A and BMP-4. These molecules, like components of the Notch pathway, are highly conserved and are involved in neurogenesis. As well, activin A and BMP-4 are both TGF-β-like molecules, and TGF-β-is a well known immune-regulatory cytokine which has been implicated in regulatory T cell function and oral tolerance induction (17, 63, 64, 65). Therefore, I propose that signals derived from the Notch and the TGF-β signalling pathways (TGF-β, activn A and /or BMP-4) may co-operate to bias the differentiation of naïve $CD4^+$ T cells into regulatory T cells in a situation that favours tolerance induction such as intranasal peptide treatment.

At the time of starting this work for my PhD there was little information regarding the expression pattern of components of the Notch pathway, as well as the neural inducers and inhibitors in cells of the immune system. This information is necessary if we are to understand their potential relevance in the adult immune system. It is worth mentioning that expression could only be studied at the gene level, because at the time this work was initiated there were no antibodies available for many of the molecules here involved.

Therefore, in this study I investigated the expression of genes associated with the Notch signalling pathway (*Notch-1, -2* and *-4*, *Jagged-1* and *-2*, *Delta-1* and the downstream target gene, *Hes-1*). I also examined the expression of neural inducers/inhibitors (*noggin*, *activin A* and *BMP-4*) in lymphoid tissues and cells of adult mice by reverse-transcription polymerase chain reaction (RT-PCR). Furthermore, to gain some insight into the potential relevance of these genes in immune responses, I investigated by real-time PCR their differential regulation in $CD4^+$ T cells and B cells that had been activated *in vitro*.

2. Results

2.1. Gene Expression in Lymphoid Organs

The spleen, LNs and thymus of adult (6-8 weeks-old) unimmunised, wild type C57BL/6 (C57) and BALB/c mice were removed and processed for total RNA extraction. The integrity of the RNA was confirmed using agarose gel electrophoresis and all the samples were normalised to the house-keeping gene, β-actin, by reverse-transcriptase polymerase chain reaction (RT-PCR). In addition, each sample was checked for genomic DNA contamination using PCR (Figure 1).

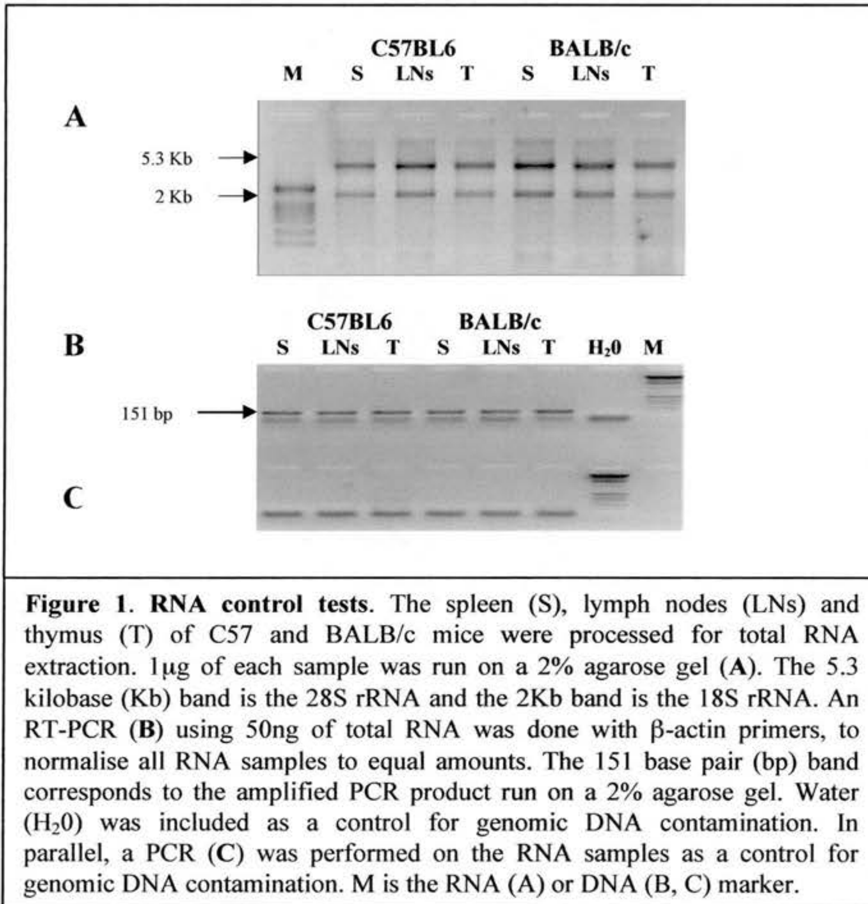


Figure 1. RNA control tests. The spleen (S), lymph nodes (LNs) and thymus (T) of C57 and BALB/c mice were processed for total RNA extraction. 1μg of each sample was run on a 2% agarose gel (**A**). The 5.3 kilobase (Kb) band is the 28S rRNA and the 2Kb band is the 18S rRNA. An RT-PCR (**B**) using 50ng of total RNA was done with β-actin primers, to normalise all RNA samples to equal amounts. The 151 base pair (bp) band corresponds to the amplified PCR product run on a 2% agarose gel. Water (H_20) was included as a control for genomic DNA contamination. In parallel, a PCR (**C**) was performed on the RNA samples as a control for genomic DNA contamination. M is the RNA (A) or DNA (B, C) marker.

Tissue RNA from spleen, thymus and LNs of C57 and BALB/c mice were examined for expression of component genes of the Notch signalling pathway, including *Notch-1, 2* and *4*, the Notch ligands, *Jagged-1, 2* and *Delta-1* and the donwstream signalling target, *Hes-1*, by RT-PCR. (Figure 2). They were also checked for expression of the neural inducer *noggin*, and neural inhibitors *activin A* and *BMP-4* (Figure 3).

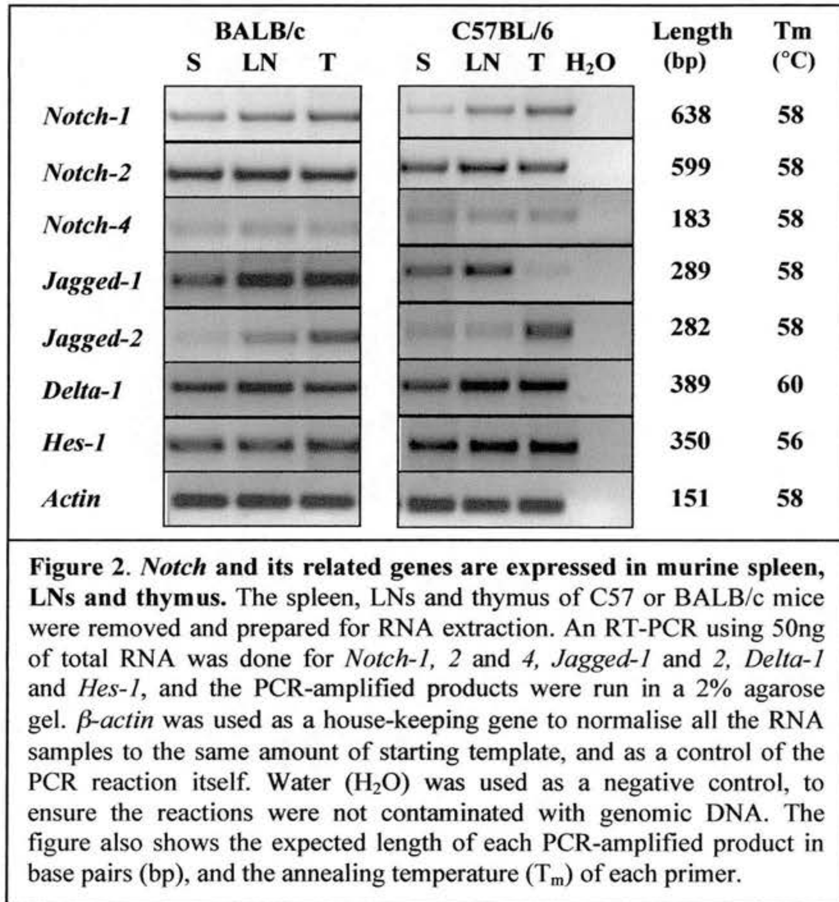


Figure 2. *Notch* and its related genes are expressed in murine spleen, LNs and thymus. The spleen, LNs and thymus of C57 or BALB/c mice were removed and prepared for RNA extraction. An RT-PCR using 50ng of total RNA was done for *Notch-1, 2* and *4, Jagged-1* and *2, Delta-1* and *Hes-1*, and the PCR-amplified products were run in a 2% agarose gel. *β-actin* was used as a house-keeping gene to normalise all the RNA samples to the same amount of starting template, and as a control of the PCR reaction itself. Water (H_2O) was used as a negative control, to ensure the reactions were not contaminated with genomic DNA. The figure also shows the expected length of each PCR-amplified product in base pairs (bp), and the annealing temperature (T_m) of each primer.

Figure 2 shows that all the genes tested were expressed at varying levels in the spleen, LNs and thymus of both C57 and BALB/c mice. The Notch receptors tested, *1, 2* and *4*, were expressed in all the organs, although *Notch-2* was the most abundant in both mouse strains. The Notch ligands, *Jagged-1, 2* and *Delta-1* were also expressed in these organs. *Jagged-2* was more abundant in the thymus. Finally, *Hes-1* was also expressed in all the tissues tested. I cannot however, exclude the role

Deltex might be playing in signalling (Introduction, section 3.1), as this gene was not included in this analysis.

Figure 3 reveals that the TGF-β-like molecule, *activin A*, was expressed in all three lymphoid tissues in both mouse strains. In C57s it was strongly expressed in the spleen. The *activin receptor type II B* (ActRIIB), to which activin A binds, was expressed weakly in lymphoid tissues. However, it should be noted that the type II receptor tested here is not the only receptor involved in activin signalling. There are two type II receptors (ActRII and the closely related ActRIIB) that bind activin with high affinity, after which the newly formed complex interacts with the type I activin receptor, ActRIB (178). Activins have also been shown to interact with ActRI, although this receptor appears to play a minor role in activin signalling.

Follistatin was not found in any of the organs tested (not shown), but this could relate more to inefficient primers than to levels of expression, as no message was ever found in any sample tested. Finally, *Noggin* and *BMP-4* were only weakly expressed in the lymphoid tissues tested in C57 and BALB/c mice.

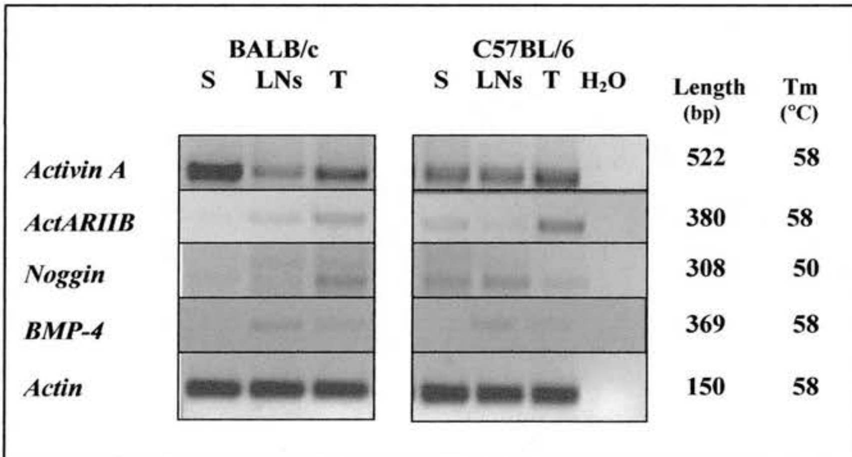


Figure 3. Neural inducers and inhibitors are expressed in murine spleen, LNs and thymus. The spleen, LNs and thymus of C57 or BALB/c mice were removed and prepared for RNA extraction. An RT-PCR using 50ng of total RNA was done for *Activin A, activin type II B receptor (ActARII), Noggin* and *BMP-4*, and the PCR-amplified products were run in a 2% agarose gel. *β-actin* was used as a house-keeping gene to normalise all the RNA samples to the same amount of starting template, and as a control of the PCR reaction itself. Water (H_2O) was used as a negative control, to ensure the reactions were not contaminated with genomic DNA. The figure also shows the expected length of each PCR-amplified product in base pairs (bp), and the annealing temperature (T_m) of each primer.

2.2. Gene Expression in Lymphoid Cells

Having confirmed the expression of genes associated with Notch and TGF-β-like signalling in various lymphoid organs, I next focused on the expression of these genes within purified cell populations isolated from the spleen.

2.2.1. Cell Separation

Cell isolation was performed using the MACS technique (described in Chapter II, section 2.2). This relies on magnetically labelled antibodies specific for each cell type. Mouse anti-CD4 and anti-CD8 antibodies were used for CD4 and CD8 isolation, respectively. Anti-CD19 and anti-CD11c antibodies were used for the isolation of B cells and DCs, respectively. High purities were expected after cell separation with the anti-CD4, anti-CD8 and anti-CD19 antibodies, as they are specific for their cell type. CD11c was the only antibody available for DC separation, and this is not DC-exclusive (it is weakly expressed on NK cells, B cells and T cell subsets), therefore, purities in the range of 50-70% were expected after isolation.

On average, a normal C57 or BALB/c spleen contained about 60-75 million cells after lysing the red blood cells, of which about 15-20% were $CD4^+$ T cells, 7-10% $CD8^+$ T cells, 25-30% B cells and 2-5% $CD11c^+$ cells. After isolation, some of the cells were kept for antibody staining and flow cytometry. The remaining cells were lysed in RNA lysis buffer, and the cells stored at -70°C until the RNA was required for extraction.

2.2.2. Phenotyping Lymphoid Cells

Flow cytometry was used to determine the purity of the cells isolated by MACS and the cell viability (Figure 4). Typically, $CD4^+$ T cells and B cells were >95% pure. $CD8^+$ T about 90% pure and the CD11c positives ranged from 50-70% pure. Propidium iodide (PI) staining (Figure 4) showed that the isolation procedure was not disruptive to cells as the percentage of total live cells after MACS was still high (82% $CD4^+$ T cells, 88% $CD8^+$ T cells, 90% B cells and 83% DCs).

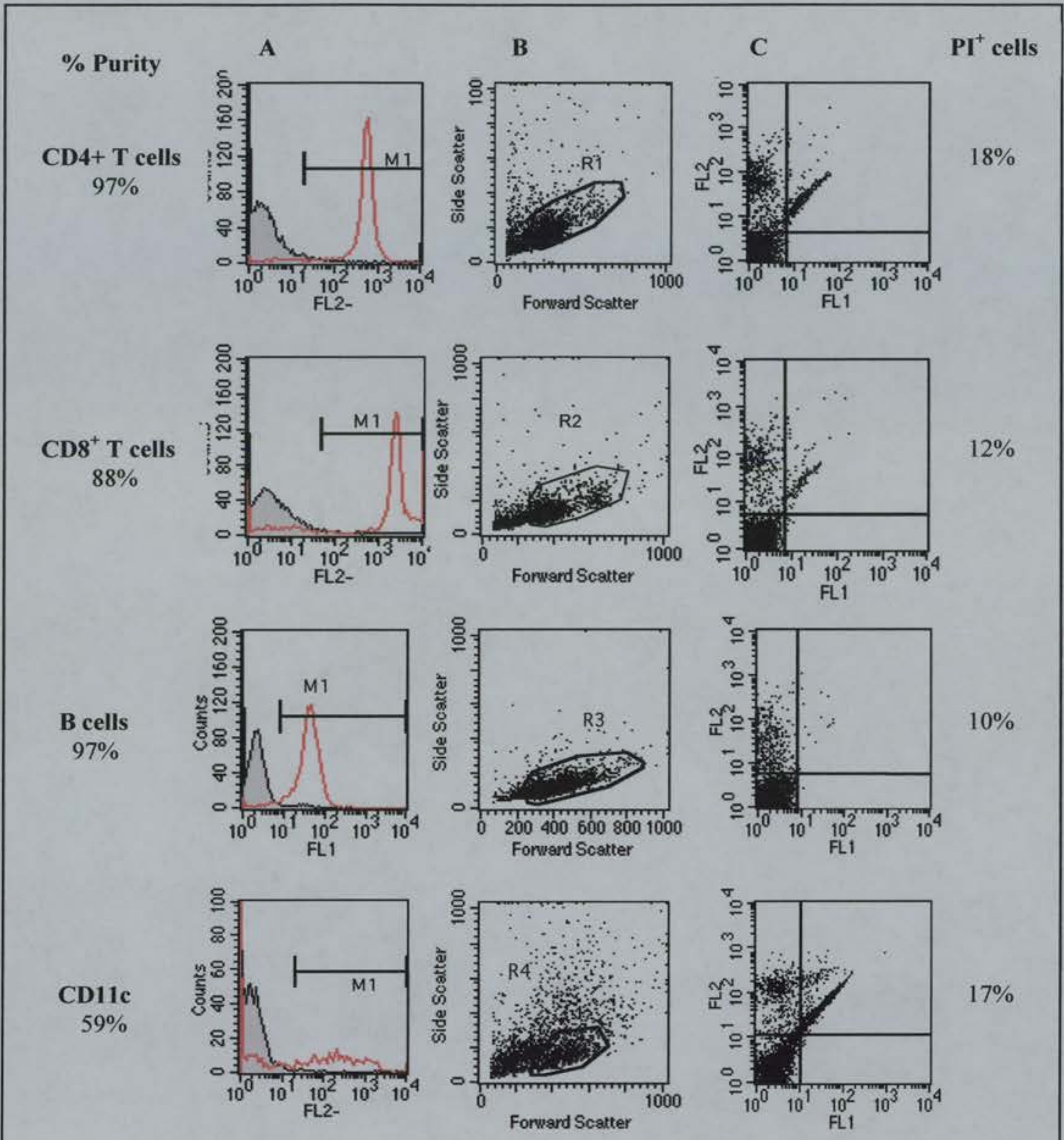


Figure 4. Purity and viability of MACS-separated murine lymphoid cells. Splenic $CD4^+$ T cells, $CD8^+$ T cells, B cells and enriched DCs were isolated from C57BL/6 female mice by MACS, and the purity assessed by antibody staining with PE-CD4, CD8, CD19 and CD11c, respectively (red lines). Each marker was accompanied by its corresponding isotype control (shaded grey) (**A**). The percentage values correspond to the purity of each cell preparation gated on the live population (**B**). Cell viability was tested after each cell separation by addition of PI (**C**). The values indicate the percentage of dead cells in the total, ungated population.

2.2.3. RT-PCR on Lymphoid Cell Populations

The isolated splenic lymphoid populations were processed for RNA, checked for genomic DNA contamination and prepared for RT-PCR (Figure 5). The *Notch-1* receptor was expressed in all the lymphoid populations tested, and more so *Notch-2*, whereas *Notch-4* was weakly expressed. The Notch ligands *Jagged-1* and *2* were barely detected, except for *Jagged-1* in DCs, but this could have been a problem with the primers as the agarose gel revealed that they were annealing non-specifically to other products. *Delta-1* was expressed in all the cell types although more strongly in the APC populations. *TGF-β* and *Activin A* were strongly expressed in all the cells. Transcripts for *Noggin* were found in all the cells but at low levels and no *BMP-4* was detected in any of the samples tested (not shown), confirming the finding that *BMP-4* was not present in the spleen either (Figure 2).

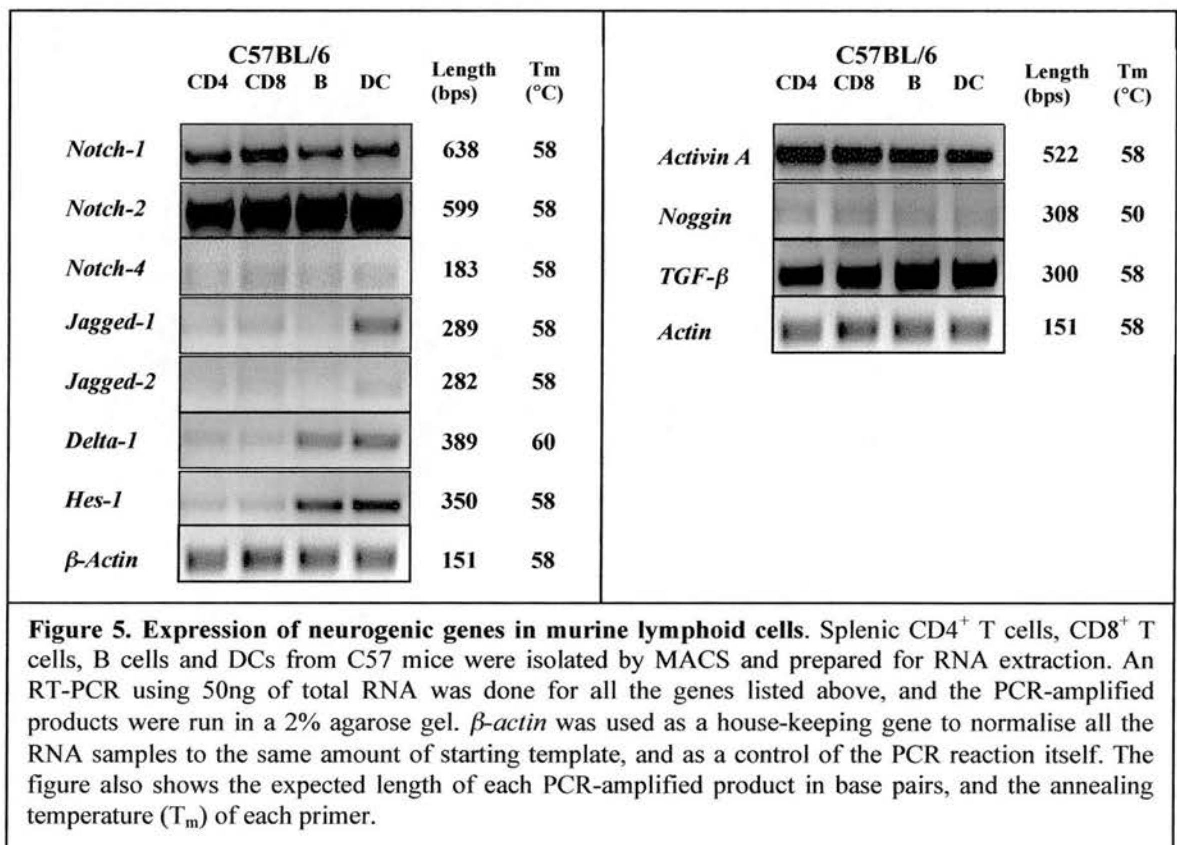


Figure 5. Expression of neurogenic genes in murine lymphoid cells. Splenic $CD4^+$ T cells, $CD8^+$ T cells, B cells and DCs from C57 mice were isolated by MACS and prepared for RNA extraction. An RT-PCR using 50ng of total RNA was done for all the genes listed above, and the PCR-amplified products were run in a 2% agarose gel. *β-actin* was used as a house-keeping gene to normalise all the RNA samples to the same amount of starting template, and as a control of the PCR reaction itself. The figure also shows the expected length of each PCR-amplified product in base pairs, and the annealing temperature (T_m) of each primer.

2.2.4. Real-Time PCR of *in vitro*-activated Lymphocyte Populations

A limitation to RT-PCR analysis of gene expression is that it is difficult to make quantitative comparisons of genes between different samples. Therefore, to overcome this I used real-time PCR technology. This was pioneered by Higuchi *et al* (176), and involves a system that detects PCR products as they accumulate in 'real-time' making quantitation possible. For a more detailed account of real-time PCR technology see Chapter II, section 3.5.

The use of technologies that measure gene expression raises a point of concern, as an increase in the expression of a specific gene does not always equate to an increase in the amount of protein that is being synthesised, and questions its functional relevance. However, at the time of conducting these experiments there were no antibodies available to facilitate the study of protein expression in either lymphoid tissues or cells of components of the Notch pathway.

Indeed most of the work that has been done involving the differential expression of Notch receptors or ligands or intracellular mediators in the pathway, has relied on gene expression at the RNA level. Felli *et al* (73) investigated the expression of *Notch-1*, *-2* and *-3*, *Jagged-1* and *-2*, and *Hes-1* and *-5* in lymphoid and stromal components of murine thymus, by Northern blot analysis. Similarly, Kaneta *et al* (180) investigated by RT-PCR the expression of Notch receptors, ligands and Hes molecules in individual thymocyte populations (double negative, double positive and single positive) and thymic stroma. As well, Deftos *et al* (85) used Northern blot analysis to demonstrate that constitutive intracellular Notch in a double positive thymoma cell line provided a continuos source of Notch activity, that led to an up regulation of the intracellular Notch mediator *Deltex*. They also analysed by RT-PCR expression levels of *Deltex* (85) and *Hes-1* (86) in double positive, double negative and single positive thymocytes.

Therefore, having confirmed by RT-PCR that lymphoid tissues and organs express genes associated with the Notch pathway and TGF-β-related genes, I wanted to characterise by real-time PCR if these genes were modulated during the activation of $CD4^+$ T cells or APC populations *in vitro*.

I choose to look at $CD4^+$ T cells based on our previous findings that DCs over-expressing Jagged-1 could induce a population of antigen-specific regulatory $CD4^+$ T cells (99), therefore, I was interested in investigating if Notch signalling was active in other type of $CD4^+$ T cell responses. B cells were chosen as the source of APCs, for practical reasons. They are very abundant in the spleen, their isolation by magnetic beads (unlike dendritic cells) yields a very pure population, and they can be easily activated in *vitro*. C57BL/6 $CD4^+$ T cells were activated using anti-CD3 and anti-CD28 antibodies for 24 and 48 hours, and C57/BL6 B cells were stimulated with LPS for 24, 48 and 72 hours. The RNA was extracted from these cell populations, checked for genomic DNA contamination and reverse transcribed into complementary DNA (cDNA). Real-time PCR using fluorogenic probes was used to measure expression levels of *Notch-1* and *2*, *Jagged-1* and *2*, *Delta-1* and *3*, *Hes-1*, *Deltex*, *Activin A*, and *TGF-β*. *Noggin* and *BMP-4* were also included, however, only the data for *Noggin* in B cells is shown, as no message was detected in any of the other samples tested.
18S rRNA was used as the endogenous control to which all the samples were normalised. Wild type (wt) untreated $CD4^+$ T cells or B cells from the spleens of 6-8 weeks old C57BL/6 mice were used as the positive control (calibrator), against which the data from the activated $CD4^+$ T cell or B cell samples were made relative to. Therefore, the calibrator was assigned a value of 1. The values obtained after real-time PCR analysis for each gene were plotted as individual graphs (Figures 6 and 7).

An important point to address is what fold-increase in the level of expression of a specific gene relative to the calibrator is regarded as significant. The work of Chtanova *et al* (181) described the differential expression of genes in Th1 and Th2 as well as $CD8^+$ T cells (Tc1) and Tc2, by using microarray analysis. They found the same patterns of gene expression when they compared their results to real-time PCR, and established that genes were differentially expressed if a change of at least two-fold or greater was observed. Similarly, Granucci *et al* (182) used microarray analysis to investigate differential effects of TNF-α versus LPS on DC priming. Again, they only considered differential regulation of gene expression where at least

a two-fold change in the level of mRNA expression was observed compared to the control.

There, is however, no specific consensus as to what is considered a significant fold increase. Other groups include statistical analysis (error bars) where the samples have been run in triplicates (183, 184), or where the values represent the means of more than three independent experiments (185), to address significant changes in gene expression. Further still, other groups do not comment on what they consider significant or not (186, 187).

In view of the lack of consensus, I relied on the work of Chtanova *et al* (181) and Granucci *et al* (182) to consider a fold-increase in gene expression of 2.5 or greater above the calibrator as significant.

It is important to mention that the data displayed for *Hes-1* prior to the review of this thesis showed that *Hes-1* was up regulated in $CD4^+$ T cells by more than 200-fold after 48 hours of activation. However, this data was included by mistake in Figure 6 and corresponded to Hes-1 primers that were found to anneal to genomic sequences. This data has been replaced in Figure 6 by the data obtained from a different set of Hes-1 primers that was tested in all the samples analysed for the work presented in this thesis.

Figure 6 reveals that upon TCR-mediated $CD4^+$ T cell activation, levels of expression for the *Notch* receptors *-1* and *-2* are similar to the untreated, wild type (wt) $CD4^+$ population. However, a very different pattern emerges for the Notch ligands. *Jagged 1* and *Jagged 2* are significantly up regulated (20-fold and 7-fold, respectively) 24 hours after activation, although by 48 hours the levels drop. More so, *Delta-1* is up regulated by about 50-fold relative to wt $CD4^+$ T cells, as well 24 hours after TCR-mediated activation.

Interestingly, the increased expression of the Notch ligands correlates with a significant up regulation at the same time point of *Hes-1* (7-fold), suggesting Notch signalling may have occurred in the activated population by 24 hours. Furthermore, the observation that *Deltex* levels go down upon activation suggests that $CD4^+$ T cells may not be using *Deltex* as a downstream target of Notch signalling. The TGF-

β-like molecule *activin A* is significantly up regulated by 24 hours following activation (50-fold), whereas transcripts for *TGF-β* do not change.

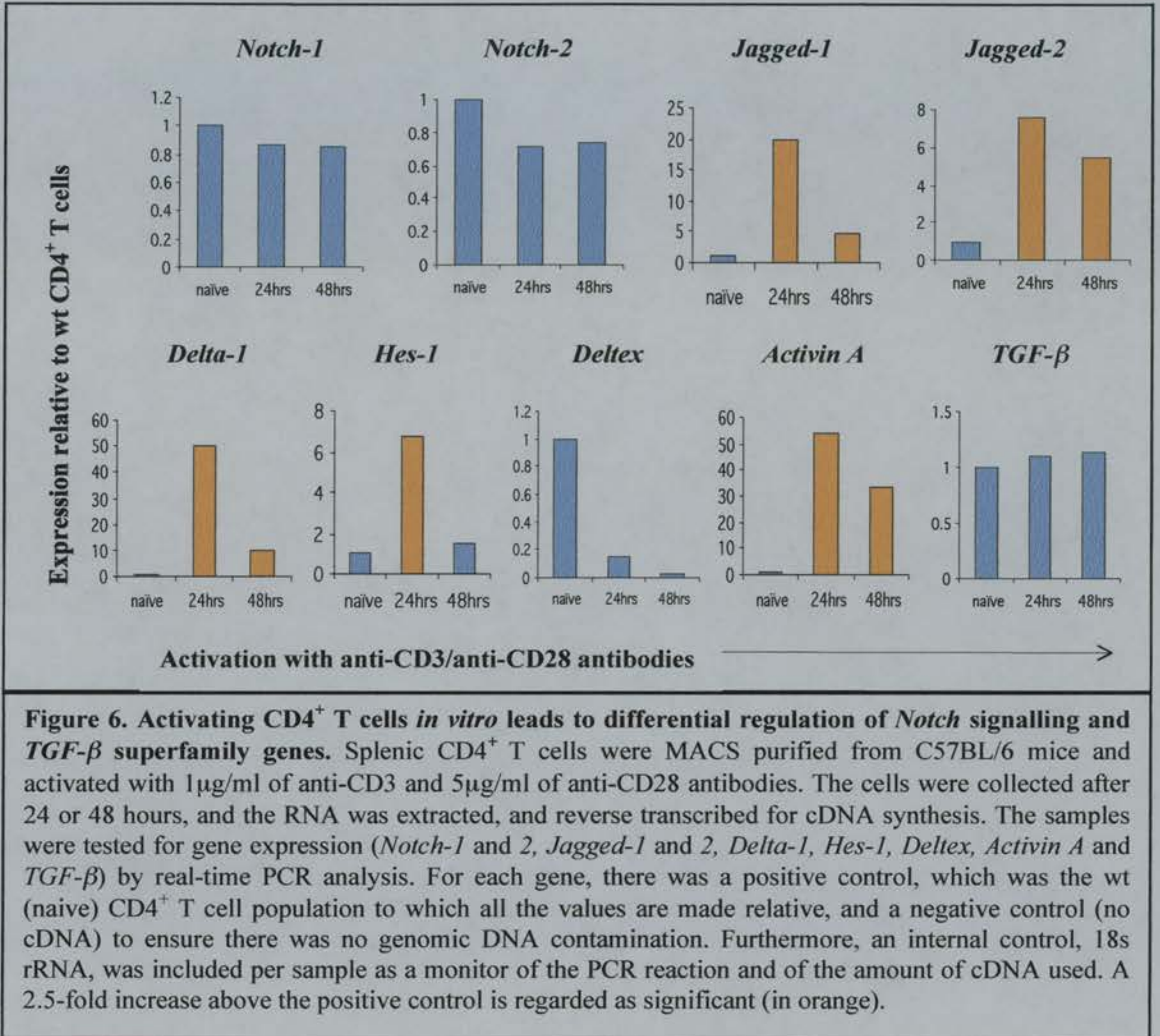


Figure 6. Activating CD4$^+$ T cells *in vitro* leads to differential regulation of *Notch* signalling and *TGF-β* superfamily genes. Splenic CD4$^+$ T cells were MACS purified from C57BL/6 mice and activated with 1μg/ml of anti-CD3 and 5μg/ml of anti-CD28 antibodies. The cells were collected after 24 or 48 hours, and the RNA was extracted, and reverse transcribed for cDNA synthesis. The samples were tested for gene expression (*Notch-1* and *2, Jagged-1* and *2, Delta-1, Hes-1, Deltex, Activin A* and *TGF-β*) by real-time PCR analysis. For each gene, there was a positive control, which was the wt (naive) CD4$^+$ T cell population to which all the values are made relative, and a negative control (no cDNA) to ensure there was no genomic DNA contamination. Furthermore, an internal control, 18s rRNA, was included per sample as a monitor of the PCR reaction and of the amount of cDNA used. A 2.5-fold increase above the positive control is regarded as significant (in orange).

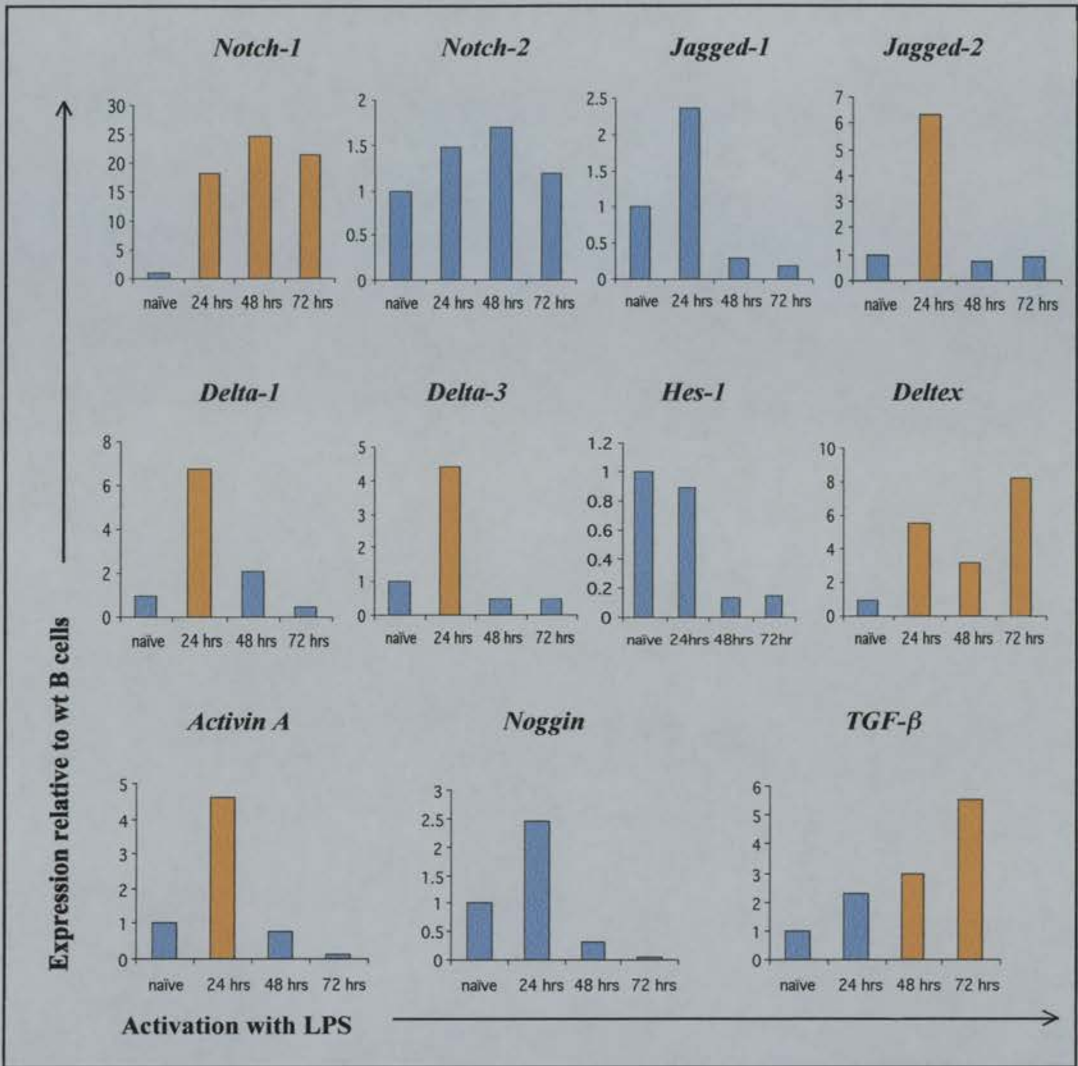


Figure 7. Activating B cells *in vitro* leads to differential regulation of *Notch* signalling and *TGF-β* superfamily genes. Splenic B cells were MACS purified from C57BL/6 mice and activated with 100μg/ml of LPS. The cells were collected after 24, 48 or 72 hours, and the RNA was extracted, and reverse transcribed for cDNA synthesis. The samples were tested for gene expression (*Notch-1* and *2, Jagged-1* and *2, Delta-1* and *3, Hes-1, Deltex, Activin A, Noggin* and *TGF-β*) by real-time PCR analysis. For each gene, there was a positive control, which was the wt (naïve) B cell population to which all the values are made relative, and a negative control (no cDNA) to ensure there was no genomic DNA contamination. Furthermore, an internal control, 18s rRNA, was included per sample as a monitor of the PCR reaction and of the amount of cDNA used. A 2.5-fold increase above the positive control is regarded as significant (in orange).

Interestingly, Figure 7 reveals a very different pattern of gene expression. Activated B cells significantly up regulate *Notch 1*, which by 24 hours has more than a 15-fold increase in expression relative to the control. In addition, levels of *Notch-1* are maintained even 72 hours after activation. Similar to $CD4^+$ T cells, the Notch ligands *Jagged-2* and *Delta-1*, and in addition *Delta-3*, are also significantly up regulated by 24 hours following activation (6-fold, 7-fold and 4.5-fold, respectively), but by 48 hours levels drop to and below the calibrator. *Activin A* shows a similar patterns of gene up-regulation to the Notch ligands, a significant (4.5-fold) induction by 24 hours that drops sharply by 48 hours, whereas *TGF-β* message steadily increases, peaking at 72 hours (more than 5-fold). Interestingly, *Hes-1* expression in B cells drops at 48 hours, whereas *Deltex* expression increases steadily, and peaks at 72 hours (8-fold), suggesting that *Deltex* may be the downstream transducer of Notch signalling in B cells.

3. Discussion

At the time of starting this work for my PhD, little information was available regarding the expression of Notch signalling genes as well as the neural inducers and TGF-β-like inhibitors, in peripheral lymphoid organs and cells. I was prompted to study their expression in the spleen, thymus and lymph nodes of adult wild type mice (C57BL/6 and BALB/c), as well as in splenic lymphocytes and DCs. This would provide us with a better understanding of their potential role in peripheral immunity.
By RT-PCR I studied the expression of *Notch-1 -2* and *-4* receptors, the Notch ligands, *Jagged-1*, *-2* and *Delta-1*, and the downstream target gene, *Hes-1* (Figure 2). *Notch 2* was strongly expressed in all the organs tested of both mouse strains, consistent with the finding that *Notch-2* is highly expressed in the spleen (188). *Notch-4* was weakly expressed and *Notch-1* was also found in all the tissues, including the thymus, confirming the findings of previous reports (73). The ligand *Delta-1* and the downstream intracellular effector, *Hes-1*, were expressed in all the organs of both mouse strains, and *Jagged-1* and *Jagged-2* showed differential expression in the various organs, but were both present in the thymus consistent with the work of other groups (73, 189, 190). As well, the findings of *Jagged-1* expression in spleen and lymph nodes agreed with the work of Bash *et al* (191).
Isolation of the different splenic lymphoid populations, namely $CD4^+$, $CD8^+$, B and DCs, gave further insight into the differential regulation of the Notch signalling genes in cells of the immune system (Figure 5). Consistent with the previous data, *Notch-2* was strongly expressed in all cell types, whereas *Notch-4* and *Jagged-2* were weakly expressed. *Jagged-1* was preferentially expressed in DCs, and *Delta-1* and *Hes-1* in B cells and DCs.

By RT-PCR I also examined the expression of the neural inducer, *noggin*, and the TGF-β-like neural inhibitors *activin A* and *BMP-4* in lymphoid organs (spleen, thymus and lymph nodes) and lymphoid cells (Figure 3). I found that *noggin* and *BMP-4* were only weakly expressed in lymphoid tissues, whereas *activin A* was highly expressed. A similar pattern of expression of these genes was observed within individual splenic $CD4^+$, $CD8^+$, B cells and enriched DC populations (Figure 5). This study reports for the first time the expression of Notch signalling genes and neural

inducers/inhibitors in lymphoid tissues and cells of adult mice, and provides valuable information as to their possible involvement in the immune system.

$CD4^+$ T cells and B cells were also activated *in vitro* to study the changes in gene expression of the Notch signalling and TGF-β-like signalling genes.
The cells were isolated by positive selection using antibody-labelled magnetic microbeads specific for each cell type. These remain attach to the cells throughout the isolation procedure and therefore, I cannot exclude the possibility that binding of the antibody may trigger signalling pathways that interfere with the expression of components of the Notch pathway. Nevertheless, it has to be considered that all the cells were isolated by the same procedure. Therefore, as far as real-time PCR gene analysis is concerned, any effects triggered by the MACS antibodies contribute equally to all the samples. In effect, as untreated cells become the calibrator, any changes within this population constitute baseline levels, and therefore, the changes observed for all the other samples relative to the calibrator represent real differences in gene expression.

$CD4^+$ T cells were activated via their T cell receptor, TCR, with anti-CD3 antibody and the co-stimulatory molecule, CD28, and B cells were activated via their Toll-like receptor, TLR-4, using LPS (192). The expression of genes was monitored by real-time PCR. I observed (Figure 6 and 7) that the expression of the Notch ligand genes, *Jagged-2* and *Delta-1* were both significantly up-regulated in the lymphocyte populations following activation. From this observation I could make the assumption that activation of lymphocytes affects a common downstream signalling pathway that results in transcriptional activation of the Notch ligand genes. Indeed, one possible candidate is nuclear factor κB (NF-κB), which is found in all cell types and is activated in response to a variety of extracellular stimuli, including LPS, microbial and viral pathogens, cytokines and growth factors (193, 194). Mammalian cells contain five NF-κB subunits; RelA (p65), c-Rel, RelB, p50 and p52, which form various hetero-and homo-dimers. Activation of NF-κB in the cytoplasm involves liberation of the dimers from a family of inhibitory proteins (IκBs), and their translocation to the nucleus where they activate transcription of target genes,

including cytokines, chemokines, immunoreceptors, growth factors, acute phase proteins and adhesion molecules (195).

NF-κB is activated in B cells after mitogenic stimulation (196), and in T cells after anti-CD3/anti-CD28 antibody-mediated activation (197, 198), suggesting that NF-κB might be the common transcription factor that affects Notch ligand gene expression after lymphocyte activation. Evidence for this was provided by the studies of Bash *et al* (191) who used the CCR43 cell line, which conditionally expresses c-Rel under the control of a tetracyclin-regulated transactivator. c-Rel expression in these cells led to induction of *Jagged-1* transcripts. Similarly, they used phorbol 12-myristate 13-acetate (PMA) plus ionomycin to activate endogenous NF-κB factors in human Jurkat T cells. They found that under these conditions, there was a 4-fold increase in *Jagged-1* mRNA levels compared to unstimulated cells. Furthermore, when they co-cultured the c-Rel expressing CCR43 cell line with Jurkat T cells, there was a 2- to 3-fold increase in *Hes-1* levels in the Jurkat T cells. This would suggest that NF-κB up-regulates Jagged-1 expression in the CCR43 cells, which in turn activates Notch signalling in the neighbouring Jurkat T cells. Furthermore, other groups have also shown an association between Notch signalling and the NF-κB pathway (98, 199, 200).

Therefore, the *in vitro* real-time data support a model whereby activation of lymphocytes may lead to activation of NF-κB, which in turn induces transcription of the Notch ligands, *Jagged-2* and/or *Delta-1*.

This investigation can be carried out by performing electrophoretic mobility shift assays (EMSAs). This technique can identify the DNA binding activity of transcription factors in the nuclear extracts of cells, as they anneal to radioactively labelled oligonucleotides containing their specific DNA binding sites. In addition, the DNA binding capacity of individual components within the transcription factors can be tested by the use of specific antibodies. The assumption that NFκB activation leads to *Jagged-2* and/or *Delta-1* transcription, could be investigated in two different ways. One way could involve the over-expression of IκB, which upon cellular activation would inhibit NFκB translocating to the nucleus and binding to its specific consensus sequence on the DNA. Therefore, it would prevent the transcription of target genes, such as *Jagged-2* and/or *Delta-1*. EMSA would detect the inhibition of

NF-κB from binding to its probe. An alternative approach would be the use of dominant negative mutants of NF-κB, which retain their DNA binding capacity but contain a mutation in the p65 subunit which blocks transcription. In this way NFκB binding would still be visualised by EMSA, but its function as a transcriptional activator would be inhibited, thus affecting expression of target genes.

Expression of *Hes-1* and *Deltex* were also assessed in this study. Both are intracellular mediators of the Notch pathway. Interestingly, both were up regulated in $CD4^+$ T cells and B cells, respectively, suggesting that Notch signalling was active in these cells.

Hes-1 up-regulation results from the binding of the Notch receptor to its ligand, Jagged or Delta. After ligand binding, translocation to the nucleus of the intracellular portion of Notch promotes Hes-1 transcription. In developmental studies, Hes-1 has been defined as a transcriptional repressor of Mash-1 activity (32). The mechanism of repression of Hes-1 involves three proteins, the bHLH and Orange domains which repress specific transcriptional activators, and the WRPW domain which binds to corepressors that inhibit other activators (84). In development it has been defined as a negative regulator of neurogenesis. Ishibashi *et al* (201) found that in the developing brains of Hes-$1^{-/-}$ embryos expression of the neural differentiation factor Mash-1 was up-regulated and post-mitotic neurons appeared prematurely. Therefore, they concluded that by limiting the expression of Mash-1, Hes-1 was participating in mechanisms that control the timing of neurogenesis. In other studies they have demonstrated that over-expression of Hes-1 in precursor cells represses Mash-1 activity and leads to a block in neuronal differentiation in the brain and retina (202).

Hes-1 has also been implicated as a transcriptional repressor during thymocyte development (84). Kim *et al* determined by EMSA that Hes-1 was binding a functional site in the CD4 silencer, which repressed the CD4 promoter, and led to a down regulation of endogenous CD4 expression in $CD4^+$ Th cells.

In similar experiments I could use EMSAs to confirm the up-regulation of *Hes-1* in activated T cells, as they reveal or not its DNA binding activity.

Another gene, Deltex, is also involved in regulating Notch signalling, through a Hes-1-independent way (79, 203). Deltex encodes a protein that physically interacts with

the Notch ankyrin repeats in the intracellular portion (203, 204). Matsuno *et al* (204) defined Deltex as a positive regulator of the Notch pathway. Additionally Deftos *et al* (85) have proposed that Deltex is a target of Notch signalling in T cell development. They found that over expression of NotchIC in a double positive thymoma cell line, led to an up regulation of *Deltex*.

Although *Hes-1* was up regulated in $CD4^+$ T cells and *Deltex* in B cells, it is unknown how their effects on transcription of other target genes would participate in the events that follow activation of these cells, such as differentiation, cytokine secretion or cell survival.

In this study I have shown that genes of the Notch and TGF-β superfamily signalling pathways are differentially expressed in lymphoid tissues and cells. Furthermore, the finding that activation of lymphocytes up regulates Notch ligand expression suggests that a common downstream effector molecule such as NF-κB may affect transcription of Notch pathway genes.

CHAPTER IV

SECTION 1

Expression of Notch Signalling Genes in CD4$^+$ T Cells during Priming or Tolerance Induction

1.1 Background

Notch is a highly conserved transmembrane protein that can bind to two separate families of ligands, Delta and Jagged (32). Activation of the Notch receptor after ligand binding leads to a series of intracellular events that affect transcription of certain target genes, involved in the specification of cell and tissue fates during development (33). The signalling pathway is evolutionarily highly conserved and has been described in *Drosophila* and vertebrates, including man (31). Its involvement in the immune system is known from studies that have associated Notch signalling with different stages of thymocyte and myeloid differentiation (69, 73, 74). We have also demonstrated that the Notch signalling pathway may be important in regulating peripheral immune responses, as over-expression of the Notch ligand, Jagged-1, in DCs, induced a population of antigen-specific regulatory T cells that was capable of transferring tolerance to recipient mice (99).

Additionally, in Chapter III I have provided evidence to show that the Notch ligands (Figure 6) are differentially regulated when CD4$^+$ T cells are activated *in vitro* with anti-CD3 and anti-CD28 antibodies. Although the mechanism of Notch ligand up regulation after CD4$^+$ T cell activation is still unknown, I was prompted to investigate whether the same effect would be seen *in vivo*, and more importantly if two different modes of CD4$^+$ T cell activation would result in differential regulation of the Notch pathway genes. One mode results in the differentiation of effector CD4$^+$ T cells and the other in the differentiation of regulatory CD4$^+$ T cells. For this

purpose I used a murine model of $CD4^+$ T cell recognition of the immunodominant peptide (p110-130) derived from the group 1 allergen of house dust mite (HDM).
Initially, the experimental set up for these *in vivo* studies entailed intranasal administration of p110-130 on three consecutive days which induces tolerance, or when injected intraperitoneally in the presence of incomplete Freund's adjuvant, primes mice for a Th1 response. However, results from these experimental protocols are compromised by the fact that the route of antigen administration is different, and therefore, I cannot control for the differences in antigen delivery to the spleen, which was the chosen organ of study. Thus the two modes cannot be compared directly which restricts the analysis of gene expression of components of the Notch pathway. In order to control for this limitation, further experiments were performed in which p110-130 was administered via the intranasal route to induce either tolerance or productive immunity. The latter can be achieved with the use of mucosal adjuvants. In this study I have used *Eschericchia coli* heat labile enterotoxin (LT), which when administered via the respiratory mucosa acts as a powerful adjuvant in combination with soluble antigen.
This section is divided into two parts. In the first part I report on the experimental conditions that had to be optimised for the priming protocol using LT, as this information was not available at the time of carrying out this investigation. In the second part I report on the patterns of gene expression of components of the Notch signalling pathway, in splenic $CD4^+$ T cells from mice that had been tolerised or primed to house dust mite p110-130 via the respiratory mucosa.

1.2. The immunisation protocols

Mucosal surfaces provide a physical barrier between the external environment and the body, and substances that come into contact with the respiratory mucosa usually do not interact with them. However, those that do will encounter the nasal associated lymphoid tissue (NALT) and the bronchial associated lymphoid tissue (BALT). Both structures are important for the induction of either immunity or tolerance. Encounter of harmless environmentally derived antigens will lead to tolerance, whereas the

presence of pathogenic substances can trigger a powerful immune response. Experimentally, tolerance can be induced when soluble antigen is administered intranasally (11, 12, 13), whereas in combination with bacterial-derived toxins such as cholera toxin or *E. coli* heat labile toxin (LT), a strong immune response is elicited.

LT is a powerful mucosal immunogen when administered at a high dose on its own, and in combination with antigen at lower doses acts as a mucosal adjuvant (205). LT belongs to a family of AB_5 bacterial toxins consisting of a pentameric B oligomer that binds the receptor(s) on the surface of eukaryotic cells, and an enzymatically active A subunit that is responsible for the toxicity. The B subunit can act as a mucosal adjuvant following intranasal delivery. This has been shown in studies looking at responses to a range of antigens including herpes simplex virus, glycoproteins, influenza virus haemagglutinin, *Streptococcus pneumoniae* pneumolysin and diptheria toxoid (206).

LT is a weak toxin for mice, but highly toxic for humans, therefore, in attempts to use LT as a vaccine or an immunomodulator in the treatment of diseases, mutants of the protein have been developed that retain adjuvant effect but lose toxicity.

Takahashi *et al* (207) assessed LT both as an immunogen and as an adjuvant for tetanus toxoid (TT). They showed that oral administration of 25μg of LT alone to BALB/c mice could induce high levels of IgA in faecal extracts and IgG1, IgG2a and IgG2b in serum. Additionally, cells from Peyer's patches and spleen, re-stimulated *in vitro* with LT produced both Th1 and Th2 cytokines. Additionally, LT had adjuvant properties when administered orally at lower doses (10μg) in the presence of tetanus toxoid. The antibody isotype response and the expression of cytokine mRNA was identical to that seen when mice were orally immunised with LT alone, but not when TT was given alone.

The first, and to date only study to report on the effects of a HDM peptide given intranasally on the response to subsequent respiratory immunisation has come from Jarnicki *et al* (208). Conventionally, intranasal exposure of mice to house dust mite peptides can induce tolerance which is not reversed by injection of the whole protein in complete Freund's adjuvant (11). Jarnicki *et al* investigated the effects of

respiratory, rather than parenteral sensitisation following intranasal peptide exposure. For this they used a mutated subunit of *E.coli* LT which has little toxicity and enhanced adjuvant activity. The adjuvant in the presence of Der p 1 protein induced a rise in IgE titers as well as Th2-associated cytokines. However, prior exposure to intranasal p114-128 or Der p 1 protein did not alter IgE titers but led to a reduction in the levels of the Th-2-associated cytokines IL-4 and IL-13 (but not IL-2, IL-5, IL-10 or IFN-γ).

The basis of this study was to determine the concentration of LT required as a mucosal adjuvant in the presence of one of the major $CD4^+$ T cell epitopes of house dust mite, peptide 110-130. Different groups have used different amounts of LT as adjuvants for their experiments. Bowman and Clements (209) used 10μg of tetanus toxoid and 5μg of LT to immunise mice intranasally once per week for three weeks. Alternatively, Jarnicki *et al* (208) treated mice intranasally with 100μg of house dust mite peptide 118-126 in combination with 10μg of a mutated form of LT. In contrast, Millar *et al* (210) used LT at a range of concentrations (0.6, 6 and 30μg) in combination with 1μg of hen egg lysozyme to treat mice intranasally on two occasions, seven days apart. Therefore, in the context of each experimental model, it is very important to establish the right concentration at which LT on its own will not induce an immune response, but in combination with antigen will act as an adjuvant.

Figure 1 shows that 5μg of LT on its own given intranasally (which is within the concentration range of what other groups have used as an adjuvant-references 208, 209, 210) was enough to prime mice to house dust mite. Jarnicki *et al* (208) administered intranasally 10μg of a mutated form of LT with 100μg of p114-128, however, they did not report on the responses to HDM peptide after intranasal admisnitration of LT alone.

The splenic $CD4^+$ T cells proliferated vigorously when stimulated *in vitro* with the major $CD4^+$ T cell epitope p110-130, at day 7 after the intranasal treatment (Figure 1). As the mice were housed in conventional cages, small amounts of HDM antigens would be expected to be present in their environment. Thus if given at too high a dose, LT can induce a response to traces of foreign bystander antigen present simultaneously at the mucosal surface.

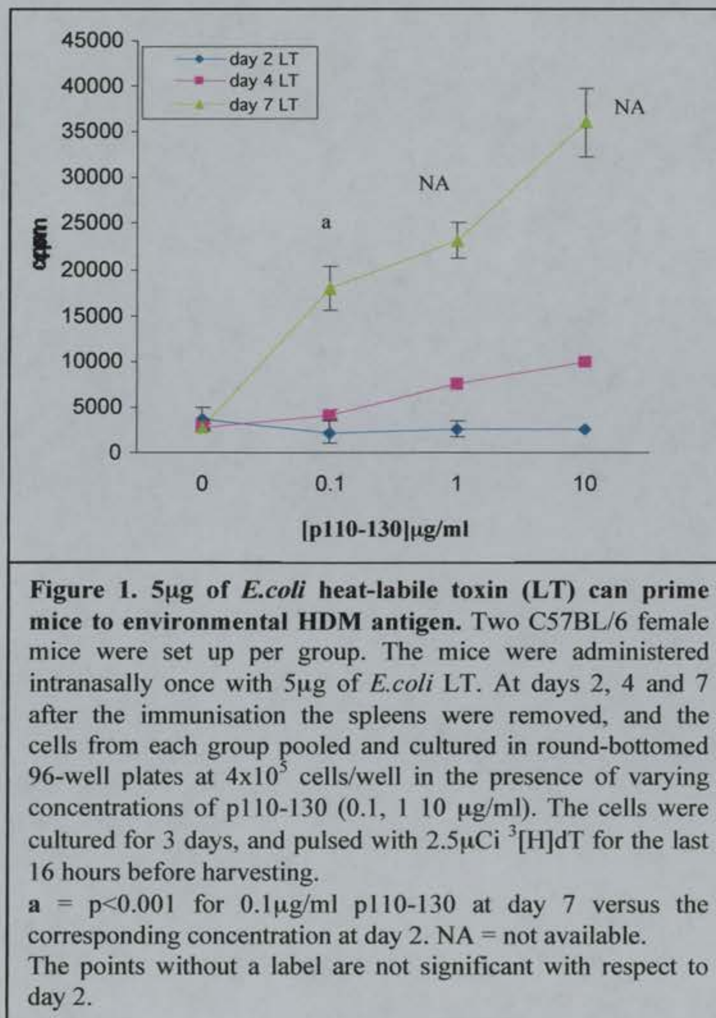


Figure 1. 5μg of *E.coli* heat-labile toxin (LT) can prime mice to environmental HDM antigen. Two C57BL/6 female mice were set up per group. The mice were administered intranasally once with 5μg of *E.coli* LT. At days 2, 4 and 7 after the immunisation the spleens were removed, and the cells from each group pooled and cultured in round-bottomed 96-well plates at $4x10^5$ cells/well in the presence of varying concentrations of p110-130 (0.1, 1 10 μg/ml). The cells were cultured for 3 days, and pulsed with 2.5μCi ^{3}[H]dT for the last 16 hours before harvesting.

a = $p<0.001$ for 0.1μg/ml p110-130 at day 7 versus the corresponding concentration at day 2. NA = not available.

The points without a label are not significant with respect to day 2.

Therefore, to determine the concentration of LT to use, where no response to environmental antigen was detectable, mice were immunised intranasally with LT at a range of concentrations (Figure 2). The spleens were removed at day 7 after treatment, when the $CD4^+$ T cells were found to respond maximally to p110-130 (Figure 1).

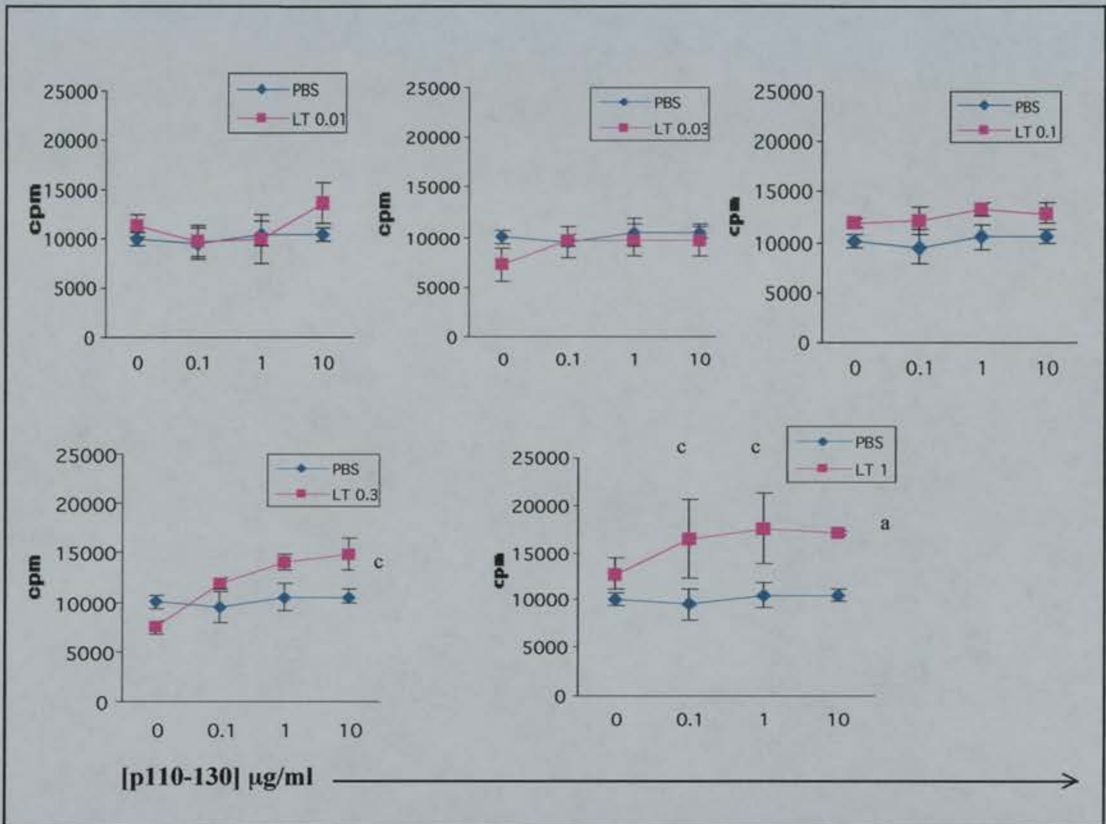


Figure 2. High doses of *E.coli* heat-labile toxin (LT) alone can prime mice to environmental HDM antigen. Two C57BL/6 female mice were set up per group. The mice were administered intranasally once with varying concentrations of *E.coli* LT (0.01, 0.03, 0.1, 0.3, 1μg) or with saline (PBS) alone as a control group. At day 7 after LT administration the spleens were removed and the cells from each group pooled, and cultured in round-bottomed 96-well plates at $4x10^5$ cells/well in the presence of varying concentrations of p110-130 (0.1, 1 10 μg/ml). The cells were cultured for 3 days, and pulsed with 2.5μCi ^{3}[H]dT for the last 16 hours before harvesting.
a = $p<0.001$ at 10μg/ml p110-130 for 1μg LT versus its corresponding concentration in the PBS group.
c = $p<0.05$ at 0.1, 1μg/ml or 10μg/ml p110-130 for 0.3μg or 1μg LT groups versus their corresponding concentrations in the PBS group. The points without a label are not significant with respect to the PBS group.

Low doses of LT administered intranasally did not prime mice to p110-130, and the proliferative responses of splenic CD4$^+$ T cells *in vitro* were comparable to mice given saline alone. However, a dose as low as 0.1μg of LT already showed a subtle difference with respect to the control group, and when the dose was increased to 0.3 or 1μg of LT, the CD4$^+$ T cells responded in a dose-dependent manner to p110-130. This experiment demonstrates the powerful adjuvant effect of LT, and shows the importance of titering the concentration of this molecule for the experimental context in which it is being used.

At this point another experiment was necessary to determine whether a low dose of LT was sufficient to use as a mucosal adjuvant in combination with p110-130. The effect of 0.03μg of LT, which had no effect on HDM peptide CD4$^+$ T cell responses *in vitro* (Figure 2), was compared against higher doses (0.1 and 0.3μg) in the presence of 100μg of p110-130 given intranasally (Figure 3).

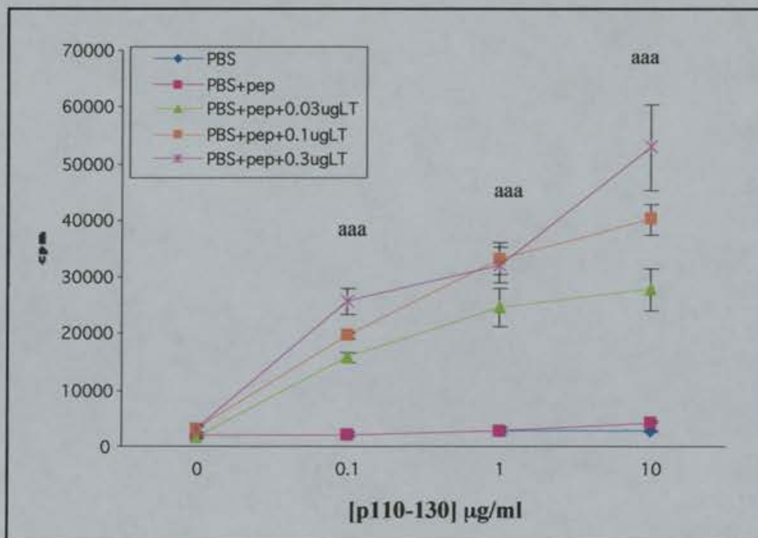


Figure 3. Low doses of *E.coli* LT in the presence of HDM p110-130 can prime mice to the peptide. Two C57BL/6 female mice were set up per group. The mice were immunised intranasally once with 100μg of p110-130 alone or in the presence of varying concentrations of *E.coli* LT (0.03, 0.1 and 0.3μg). Mice were administered saline (PBS) intranasally as a control group. At day 7 after the immunisation the spleens were removed and the cells from each group pooled, and cultured in flat-bottomed 96-well plates at $4x10^5$ cells/well in the presence of varying concentrations of p110-130 (0.1, 1 10 μg/ml). The cells were cultured for 3 days, and pulsed with 2.5μCi ^{3}[H]dT for the last 16 hours before harvesting.
a = $p<0.001$ for each LT group at 0.1, 1 or 10μg/ml of p110-130 versus the PBS group alone.

Figure 3 shows that by day 7 after the immunisation, 100μg of peptide alone was not enough to trigger a $CD4^+$ T cell response to the peptide *in vitro*, and the response induced was the same as that observed in the control group of mice which received saline alone. However, when the peptide was given in combination with as little as 0.03μg of LT, the splenic $CD4^+$ T cells displayed proliferation responses to p110-130 in a dose-dependent manner. These increased accordingly if the concentration of LT was raised to 0.1 or 0.3 μg, however, these had proved to have weak adjuvant activity when given alone (Figure 2). Therefore, from this experiment the optimal concentration of LT to use as a mucosal adjuvant was established at 0.03μg. It is worth mentioning that LT was not used in these experiments to break tolerance, since 100μg of peptide given intranasally were insufficient to establish a tolerogenic response (data not shown).

Mice were, therefore, primed with 100μg of p110-130 and 0.03μg of LT and the response of splenic $CD4^+$ T cells to the peptide *in vitro* monitored over a seven-day period (Figure 4).

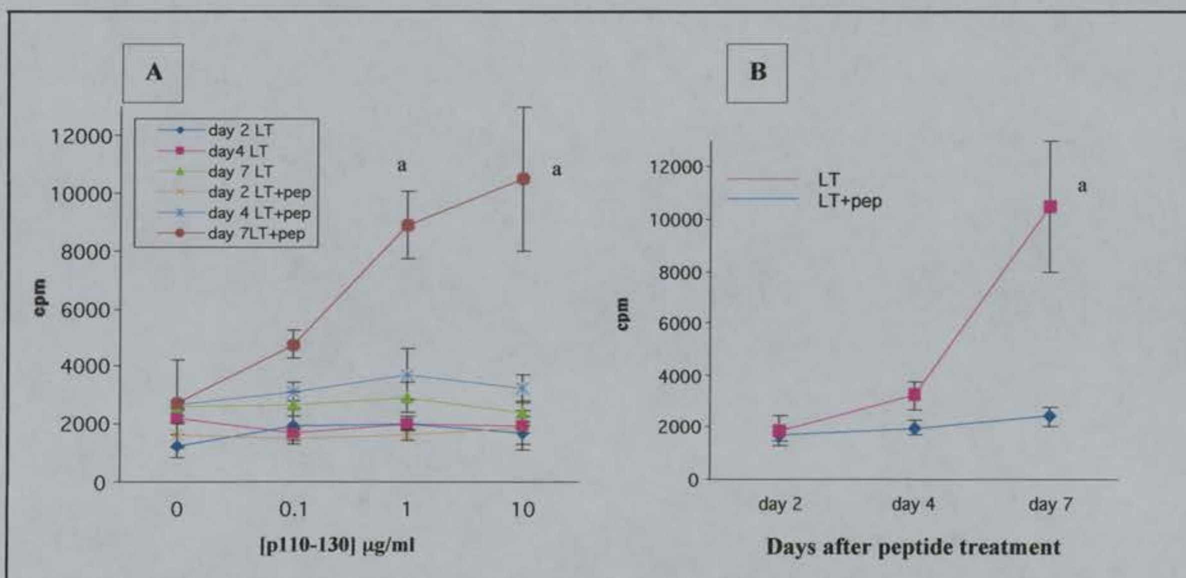


Figure 4. Priming C57BL/6 mice to HDM p110-130 using *E.coli* LT as a mucosal adjuvant.
A. Two C57BL/6 female mice were set up per group. The mice were treated intranasally once with 0.03μg of *E.coli* LT alone or in the presence of 100μg of p110-130. At days 2, 4 and 7 after the immunisation the spleens were removed, and the cells from each group pooled and cultured in flat-bottomed 96-well plates at $4x10^5$ cells/well in the presence of varying concentrations of p110-130 (0.1, 1 10 μg/ml). The cells were cultured for 3 days, and pulsed with 2.5μCi ^{3}[H]dT for the last 16 hours before harvesting. **B** The same experiment as (A) but represented is the proliferative response of $CD4^+$ T cells from the LT and LT+peptide-treated groups to p110-130 at 10μg/ml over the seven day period.
a = $p<0.001$ for day 7 LT+pep group at 1 or 10μg/ml of p110-130 versus the day 7 LT group alone. The points without a label are not significant with respect to the LT groups.

Mice primed intranasally with 0.03μg of LT alone did not respond to p110-130 when splenic $CD4^+$ T cells were re-stimulated *in vitro* with the peptide (Figure 4A). However, when given intranasally in the presence of 100μg of p110-130, $CD4^+$ T cells proliferated maximally in a dose-dependent manner by day seven after the immunisation (Figure 4B).

Having optimised the conditions of priming, mice were challenged with peptide alone for tolerance induction. The observation of Hoyne *et al* (11, 12) demonstrated that mice given 100μg of p111-139 in saline intranasally on three consecutive days became unresponsive to immunogenic challenge with the whole Der p 1 protein in complete Freund's adjuvant. The lymph node T cells from these mice were unresponsive to whole Der p 1 protein and Der p 1 peptides *in vitro* (11). Furthermore, the cervical lymph node and splenic T cells from tolerised mice secreted high levels of IL-2, IL-3 and IFN-γ at day 4 after intranasal challenge, but levels dropped by day 8, suggesting that $CD4^+$ T cells underwent transient activation before the induction of tolerance (12). This same immunisation protocol was used for the induction of tolerance using house dust mite peptide 110-130. Spleen T cells were re-stimulated *in vitro* with the same peptide at days 2, 4 and 7 after the last antigenic challenge (Figure 5).

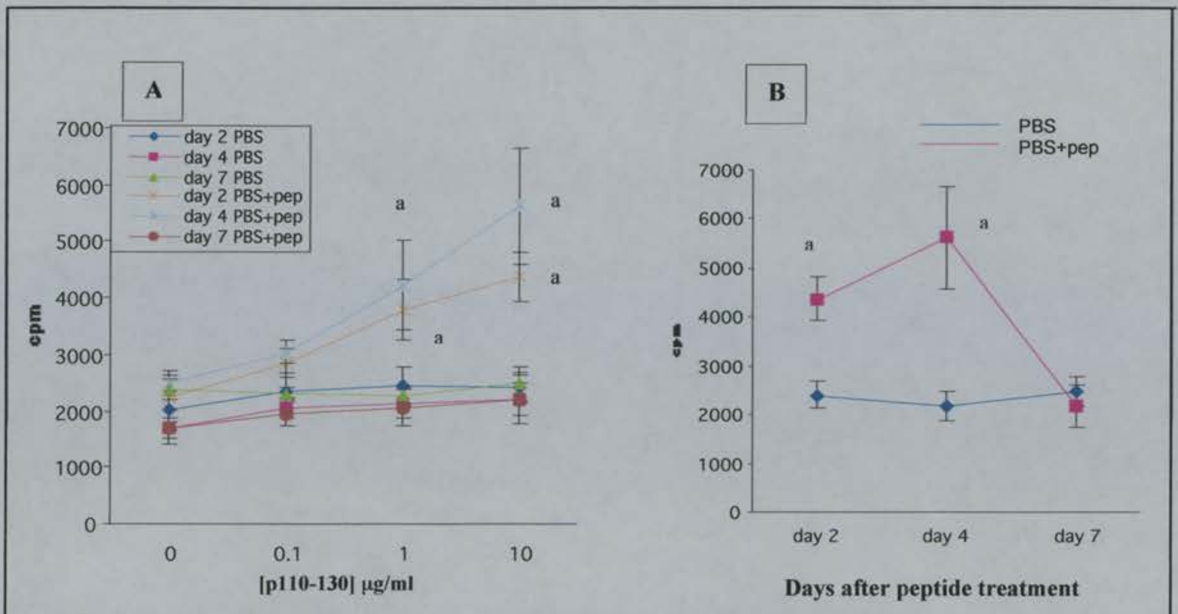


Figure 5. Intranasal administration of HDM p110-130 induces tolerance. Two C57BL/6 female mice were set up per group. The mice were treated intranasally on three consecutive days with 100µg of HDM p110-130 or saline alone. At days 2, 4 and 7 after the last immunisation the spleens were removed, and the cells from each group pooled, and cultured in flat-bottomed 96-well plates at $4x10^5$ cells/well in the presence of varying concentrations of p110-130 (0.1, 1 10 µg/ml). The cells were cultured for 3 days, and pulsed with 2.5µCi ^{3}[H]dT for the last 16 hours before harvesting.
B. The same experiment as (A) but represented is the proliferative response of $CD4^+$ T cells from the PBS and PBS+peptide-treated groups to p110-130 at 10µg/ml over the seven day period.
a = $p<0.001$ for day 2 and 4 PBS+pep groups at 1 or 10µg/ml p110-130 versus day 2 and 4 PBS groups alone, respectively. The points without a label are not significant with respect to the PBS groups.

Mice that received saline alone did not respond to p110-130 when splenic $CD4^+$ T cells were re-stimulated *in vitro* with the peptide (Figure 5A). However, splenic $CD4^+$ T cells from mice that received the peptide intranasally could already respond by day 2 after the last antigenic challenge, and by day 4 there was maximal T cell response (Figure 5B). By day 7 proliferation dropped to levels of the control group. Therefore, these results are in agreement with those of Hoyne *et al* (12).

Since tolerance can be transferred by $CD4^+$ T cells isolated from mice that have received peptide intranasally, it seems that Tr cells may have been induced. The inhibitory cytokine IL-10 is associated with their induction (211) and effector function (212, 213) therefore, I was prompted to analyse by real-time PCR

expression of IL-10 in $CD4^+$ T cells during the induction of intranasal peptide tolerance or priming. In the original experiments presented in this thesis, where mice had been tolerised or primed by peptide given intranasally or intraperitoneally, respectively, the real-time data had revealed an up regulation of *IL-10* in the $CD4^+$ T cells of tolerised mice by day 2 after the last peptide treatment. However, this pattern of gene regulation was not observed in the $CD4^+$ T cells of primed mice. I concluded that perhaps the up-regulation of *IL-10* was associated with the differentiation of Tr cells in this model of mucosal tolerance. However, it is possible that this difference in gene expression may have resulted from variability in the kinetics of antigen delivery to the spleen and activation of the $CD4^+$ T cells, as the routes of antigen administration differed for both experimental groups. In this set of experiments expression of *IL-10* was also assessed by real-time PCR to determine if the gene was modulated when the mice were either tolerised or primed by intranasal delivery of peptide (Figure 6).

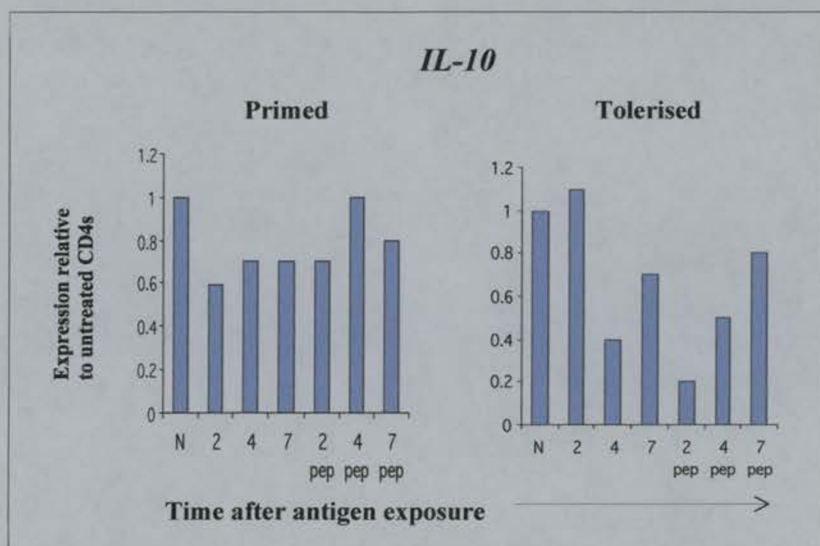


Figure 6. *IL-10* expression is not modulated during intranasal tolerance or priming induction. Mice were tolerised or primed to p110-130 as described in Figures 4 and 5. The spleens from each group of mice were removed after the last intranasal treatment and $CD4^+$ T cells were isolated by negative selection. The RNA was extracted and reversed transcribed for cDNA synthesis. The samples were tested for gene expression (*IL-10*) by real-time PCR analysis. The gene was run with a positive control, which was the untreated $CD4^+$ T cell population (N) to which all the samples are relative, and with a negative control (no cDNA) to ensure there was no genomic DNA contamination. Furthermore, an internal control, 18srRNA, was included per sample as a monitor of the PCR reaction and of the amount of cDNA used. A 2.5-fold increase above the positive control is regarded as significant.

The data presented in Figure 6 show that priming mice with LT and peptide did not affect the expression of *IL-10* compared to the control or untreated $CD4^+$ T cells. Additionally, treating the mice intranasally with saline or peptide in saline did not up regulate expression of *IL-10*.

The data so far presented show the effects on systemic $CD4^+$ T cell activation of two different experimental protocols that rely on the $CD4^+$ T cell epitope of house dust mite peptide 110-130. Both use the same route of antigen delivery (nasal route) but the outcome is very different. Giving *E.coli* LT once in combination with 100μg of p110-130 primes mice to this peptide by day 7. In contrast, when 100μg of the peptide are given in saline alone on three consecutive days the mice undergo an initial phase of $CD4^+$ T cell activation which peaks at day 4, before becoming unresponsive by day 7.

1.3. Expression of Notch signalling in $CD4^+$ T cells during peripheral immune responses

The role of Notch in lymphocyte development has been studied over the last 5 years. Gene expression studies have revealed expression of the four *Notch* receptors, as well as the Notch ligands *Jagged-2* and *Delta-1* in double negative, double positive and CD4/CD8 single positive thymocytes from adult mice (73, 180). Functional studies to address the role of Notch signalling in lymphocyte development have relied mainly on the use of two independent experimental systems. One involves **gain-of-function** approaches, mainly over-expressing the intracellular portion of Notch (NotchIC) with cell or tissue-specific promoters, or by retrovirally transducing NotchIC into bone marrow precursors (to study B versus T lineage fate), thymocytes or thymoma cell lines (to study CD4 versus CD8 and αβ versus γδ commitment). The other is a **loss-of-function** approach that relies on the Cre/loxP recombination system in bone marrow precursors or thymocytes, where lox P sites are introduced to delete a portion of the Notch-1 gene by the Cre-recombinase enzyme. Using these experimental systems different groups have demonstrated that Notch signalling

favours T cell over B cell specification (74, 75), $\alpha\beta$ over $\gamma\delta$ lineage commitment (81, 82) and may play a role in CD4/CD8 T cell differentiation (83, 85, 86), although the data for the latter is still very conflicting. A more detailed account of the role of Notch in lymphopoiesis is given in the Introduction (section 5.1.2).

Very few studies have addressed the role of Notch signalling in the adult immune system. The first report came from our group (99). Murine DCs were transfected with Jagged-1 and pulsed with the house dust mite peptide 110-131 before injecting into mice, which were primed with whole Der p 1 protein two weeks later. Lymph node cells from these mice were unresponsive to re-stimulation *in vitro* with the whole protein or HDM peptides. Additionally, when $CD4^+$ T cells from mice that had received the Jagged-1-infected DCs were transferred to recipient mice and the latter primed with whole Der p 1, the mice showed reduced LN responses to the protein *in vitro*. This study demonstrated the ability of Jagged-1 on DCs to induce a population of regulatory T cells which could also transfer tolerance.

More recently Wan Fai Ng *et al* (187) have shown by real-time PCR analysis that transcripts for *Delta-1*, *Notch-4* and *Hes-1* were up-regulated in human $CD4^+CD25^+$ T cells after activation with anti-CD3/anti-CD28 antibodies compared to $CD4^+CD25^-$ T cells, whereas levels of *Deltex* were down-regulated.

Having established the immunisation protocols to induce tolerance or productive immunity via the nasal route with the major $CD4^+$ T cell epitope of HDM, peptide 110-130, I addressed the question as to whether or not functionally different outcomes of antigen recognition also affected expression of components of the Notch signalling pathway. Mucosal tolerance induced by p110-130 delivered intranasally induces a population of regulatory $CD4^+$ T cells, (11, 12, 13) which remain unresponsive to re-stimulation *in vitro* with whole Der p 1 protein or peptides, whereas nasal priming results in the generation of effector Th2-like $CD4^+$ T cells (208). Therefore, I selected to analyse whether or not the functional difference in $CD4^+$ T cells in p110-130 primed and tolerised mice, correlated with a difference in gene expression of components of the Notch pathway. The results of this study would provide with further information on the involvement of the Notch pathway in peripheral immune responses, and more specifically in the induction of tolerance

and/or productive immunity. At the present time there is a lack of antibody reagents that can be used to specifically identify expression of the various Notch receptors and ligands. Therefore, to circumvent this problem I examined the expression of genes associated with the Notch signalling pathway using real-time PCR. It is worth mentioning that in this study I am not comparing the primed and tolerised groups directly as the antigen dose and frequency varied for the two experimental groups. Rather, both groups are compared back to untreated $CD4^+$ T cells. Therefore, untreated $CD4^+$ T cells, which serve as the calibrator, always have a value of one and the other samples are relative to that value.

Based on the experimental protocols described previously, mice were primed or tolerised by the intranasal route with HDM p110-130, the $CD4^+$ T cells were isolated by negative selection and the purity determined (Figure 7). This method of isolation ensures that the $CD4^+$ T cells are unmanipulated, and therefore, prevents the risk of activation by a positive selection method, which may modulate Notch signalling. The purity of $CD4^+$ T cell isolation was always in the region of 87-89% (Figure 7). The cells were directly processed for RNA extraction to study gene expression by real-time PCR.

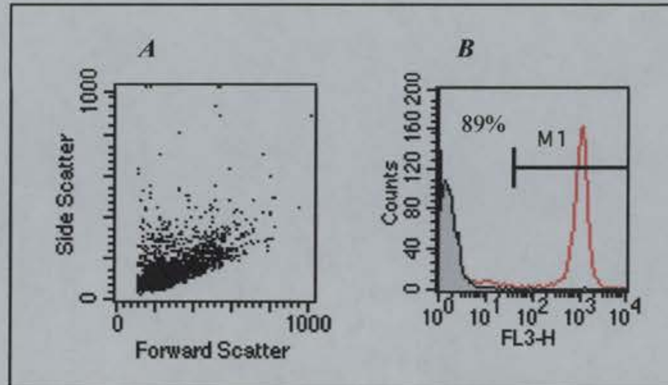


Figure 7. Purity of negatively selected $CD4^+$ T cells. Splenic $CD4^+$ T cells (**A**) were isolated from untreated, control or experimental C56BL/6 mice by negative selection. The cells were stained with Cy-Chrome-CD4 (**B**) to determine the purity of the isolation procedure (red bar) or with the corresponding isotype control (grey shaded bar). The purity (89%) is representative of all the $CD4^+$ T cell isolations carried out.

Priming mice to p110-130 in the presence of LT (Figure 8), did not result in changes in gene expression of the Notch receptors (*Notch-1* and *2*) or ligands (*Jagged-1*, *-2* and *Delta-1*) analysed at days 2 and 4 following antigen challenge. Additionally, at day 7 when the peak of the $CD4^+$ T cell response to p110-130 was detected (Figure 4), there were no changes in gene expression.

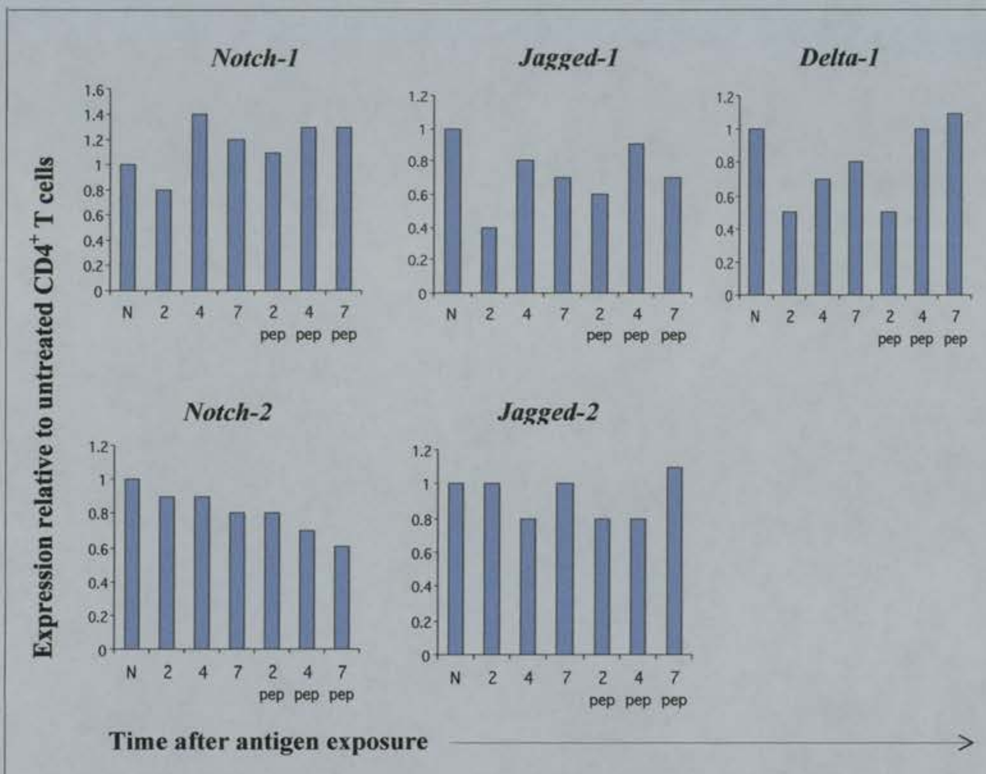


Figure 8. The effect of priming mice to HDM p110-130 in gene expression of *Notch* receptors and ligands. Splenic $CD4^+$ T cells were isolated by negative selection from mice that had been immunised intranasally with 0.03μg of *E.coli* LT alone, or in the presence of 100μg of p110-130 for 2, 4 or 7 days. The RNA was extracted and reversed transcribed for cDNA synthesis. The samples were tested for gene expression (*Notch-1* and *Notch-2*, *Jagged-1* and *Jagged-2*, *Delta-1*) by real-time PCR analysis. Each gene was run with a positive control, which was the untreated $CD4^+$ T cell population (N) to which all the samples are relative, and with a negative control (no cDNA) to ensure there was no genomic DNA contamination. Furthermore, an internal control, 18srRNA, was included per sample as a monitor of the PCR reaction and of the amount of cDNA used. A 2.5-fold increase above the positive control is regarded as significant.

A number of events follow binding of the Notch receptor to its ligands, Delta or Jagged. One of the consequences results in translocation of the intracellular portion of Notch (NotchIC) to the nucleus where it interacts with the DNA-binding protein Su(H)/CBF-1 which promote transcription of target genes (32) (Introduction, section 3.1). Amongst these target genes are E(spl)/Hes genes. Hes-1 is a bHLH protein that functions primarily as a transcriptional repressor of the transcription factor Mash-1, and is involved in many cell-fate decisions. Therefore, Notch signalling results in *Hes-1* up-regulation.

Another gene, Deltex, is also involved in regulating Notch signalling, through a Su(H)/CBF-1-independent way (79, 203). Deltex encodes a protein that physically interacts with the Notch ankyrin repeats in the intracellular portion (203, 204). Matsuno *et al* (204) found that the functional phenotypes observed in *Drosophila* by over-expression of Deltex were similar to those induced by over-expression of an activated Notch receptor, and these phenotypes were modulated in identical fashion by changing the Notch activity in the genetic background. They proposed that Deltex was acting as a positive regulator of the Notch pathway.

Additionally Deftos *et al* (85) have proposed that Deltex is a target of Notch signalling in T cell development. They found that over expression of NotchIC in a double positive thymoma cell line, led to an up regulation of *Deltex*.

An increase in *Hes-1* is associated with Notch signalling (32, 214), therefore, an up-regulation of the gene in $CD4^+$ T cells during the course of tolerance or priming would suggest that Notch signalling in these cells has taken place. Figure 9 shows that there were no changes in expression of *Hes-1* in $CD4^+$ T cells from the LT-control or LT-primed groups of mice compared to untreated $CD4^+$ T cells, implying that the Hes-1-dependent Notch signalling may not be important in this type of immune response.

Similarly, an increase in *Deltex* is associated with Notch signalling (85) therefore, I was also interested in investigating if levels of expression of *Deltex* were affected in $CD4^+$ T cells after priming or tolerising mice with HDM p110-130.

In Figure 9 data is presented that shows *Deltex* was up regulated in the control group of mice that received LT alone at day 4 after peptide treatment by about 2.5-fold with

respect to the calibrator, but it was not significantly greater than the value of the LT + peptide group at day 4.

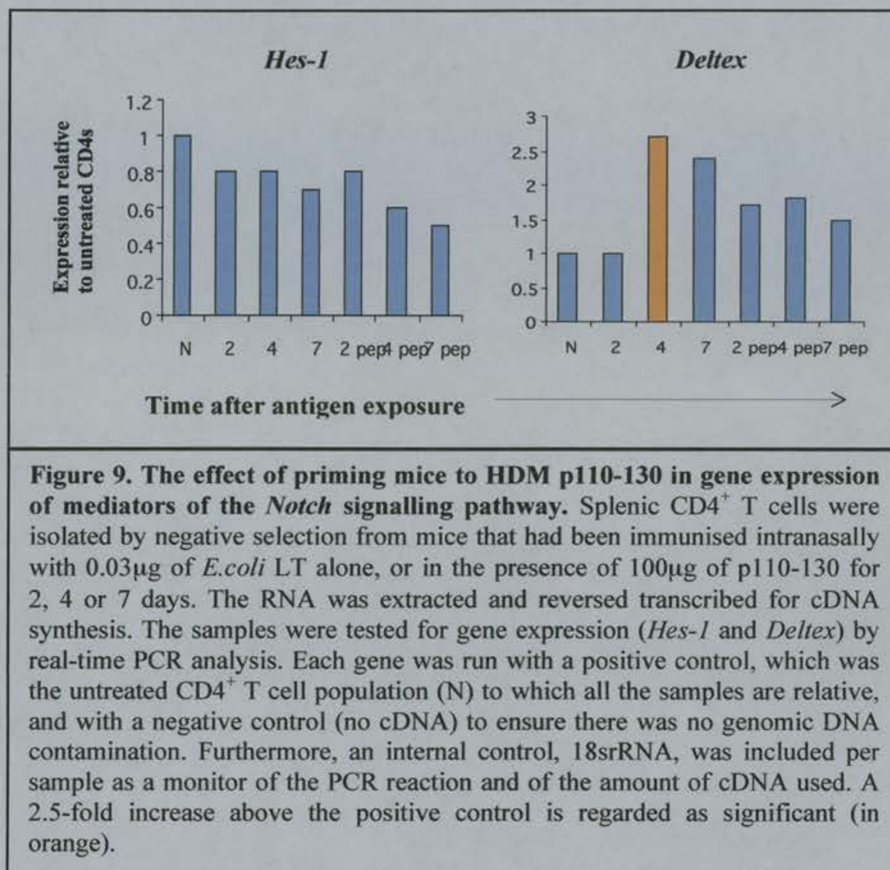


Figure 9. The effect of priming mice to HDM p110-130 in gene expression of mediators of the *Notch* signalling pathway. Splenic CD4^{+} T cells were isolated by negative selection from mice that had been immunised intranasally with 0.03μg of *E.coli* LT alone, or in the presence of 100μg of p110-130 for 2, 4 or 7 days. The RNA was extracted and reversed transcribed for cDNA synthesis. The samples were tested for gene expression (*Hes-1* and *Deltex*) by real-time PCR analysis. Each gene was run with a positive control, which was the untreated CD4^{+} T cell population (N) to which all the samples are relative, and with a negative control (no cDNA) to ensure there was no genomic DNA contamination. Furthermore, an internal control, 18srRNA, was included per sample as a monitor of the PCR reaction and of the amount of cDNA used. A 2.5-fold increase above the positive control is regarded as significant (in orange).

Identical gene expression studies were carried out in mice that had been tolerised with HDM p110-130 (Figure 10).

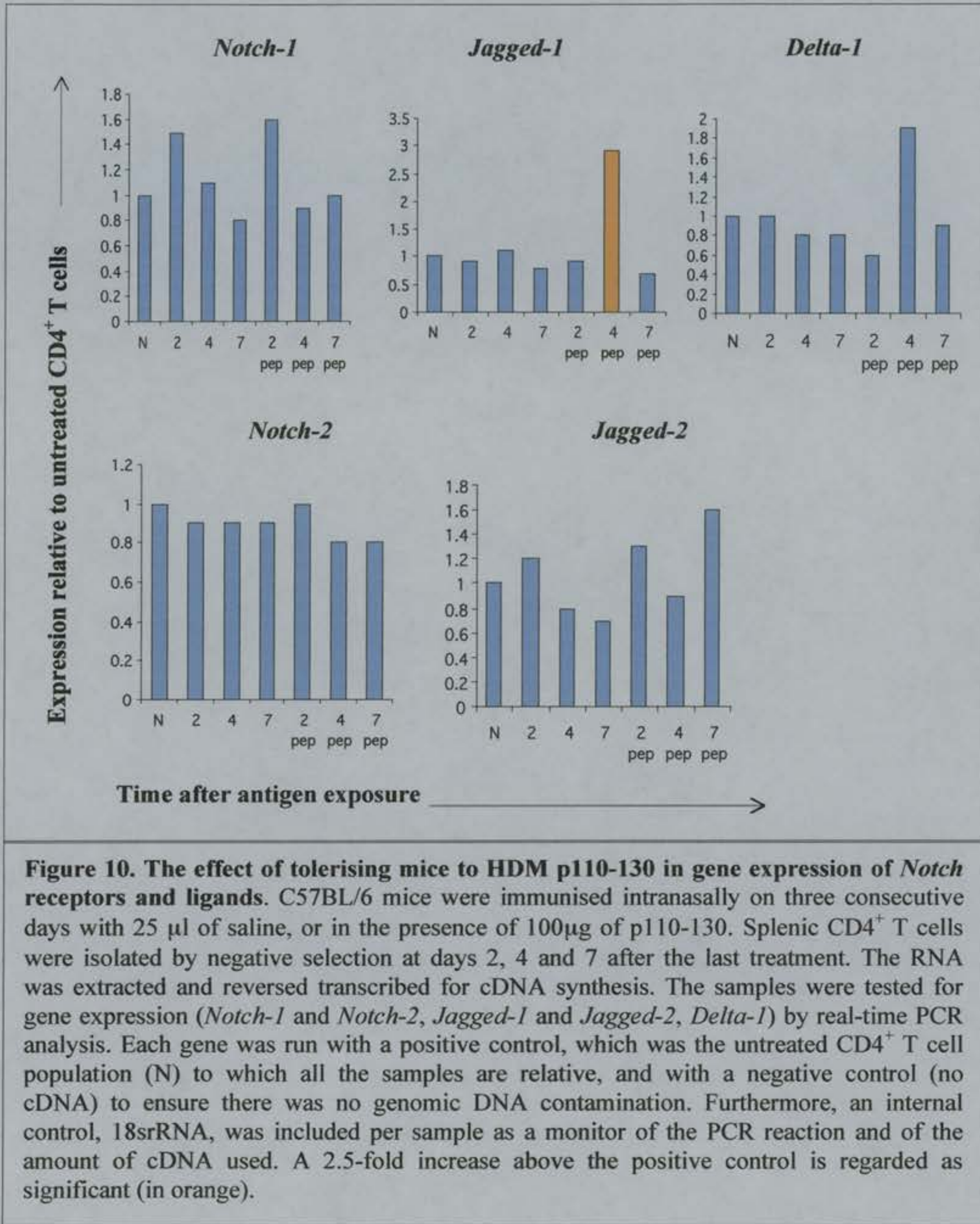


Figure 10. The effect of tolerising mice to HDM p110-130 in gene expression of *Notch* receptors and ligands. C57BL/6 mice were immunised intranasally on three consecutive days with 25 μl of saline, or in the presence of 100μg of p110-130. Splenic $CD4^+$ T cells were isolated by negative selection at days 2, 4 and 7 after the last treatment. The RNA was extracted and reversed transcribed for cDNA synthesis. The samples were tested for gene expression (*Notch-1* and *Notch-2*, *Jagged-1* and *Jagged-2*, *Delta-1*) by real-time PCR analysis. Each gene was run with a positive control, which was the untreated $CD4^+$ T cell population (N) to which all the samples are relative, and with a negative control (no cDNA) to ensure there was no genomic DNA contamination. Furthermore, an internal control, 18srRNA, was included per sample as a monitor of the PCR reaction and of the amount of cDNA used. A 2.5-fold increase above the positive control is regarded as significant (in orange).

Figure 10 shows that the Notch receptors, *Notch-1* and-*2* were not modulated during the induction of peptide tolerance, however, the Notch ligand *Jagged-1* was up-regulated by more than 2.5-fold compared to the untreated $CD4^+$ T cells and also compared to the $CD4^+$ T cells from the saline-treated group. The rise in *Jagged-1*

level correlates with the peak of $CD4^+$ T cell activation in response to p110-130 *in vitro*, at day 4 after the last peptide treatment (Figure 5). This rise in *Jagged-1* possibly corresponds to the time when the $CD4^+$ T cells are differentiating into regulatory T cells before becoming unresponsive to peptide stimulation *in vitro* by day 7 (Figure 5). Additionally there is a trend towards an up-regulation of *Delta-1* in $CD4^+$ T cells at day 4 following intranasal peptide treatment.

Analysis of expression of *Hes-1* and *Deltex* (Figure 11) reveal that only *Deltex* is up-regulated by more than 2.5 fold at day 2 following the last peptide treatment in comparison to untreated $CD4^+$ T cells. However, the rise is not significant in comparison to day 2 $CD4^+$ T cells from mice that received saline alone, and therefore, the contribution of peptide administration to changes in *Deltex* levels becomes minimal.

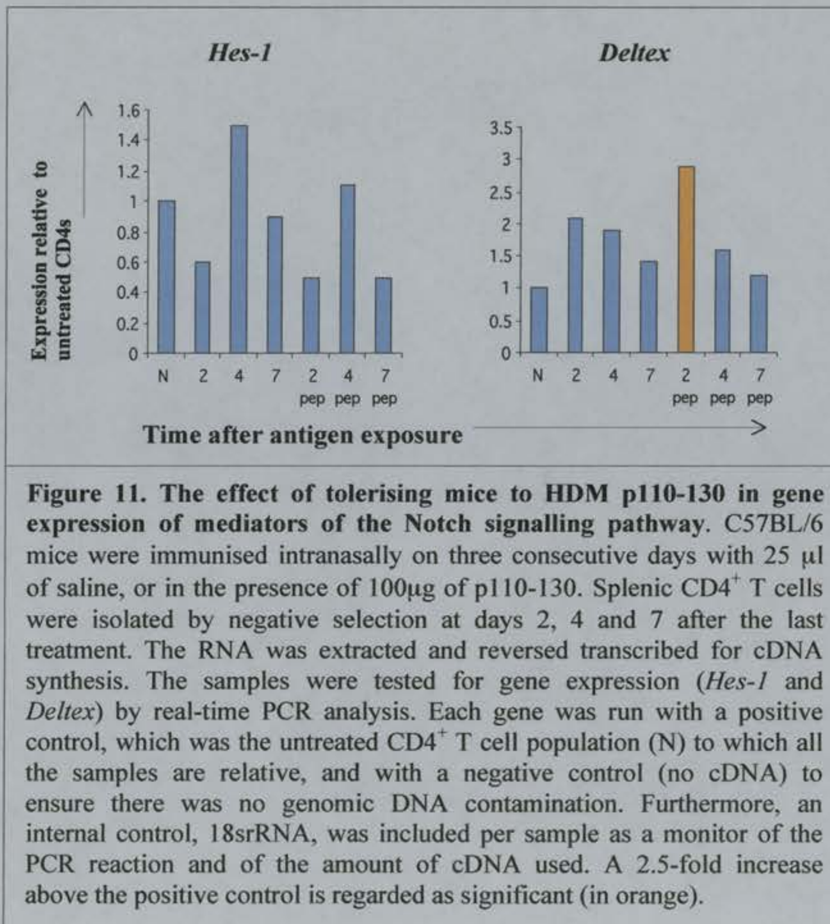


Figure 11. The effect of tolerising mice to HDM p110-130 in gene expression of mediators of the Notch signalling pathway. C57BL/6 mice were immunised intranasally on three consecutive days with 25 μl of saline, or in the presence of 100μg of p110-130. Splenic $CD4^+$ T cells were isolated by negative selection at days 2, 4 and 7 after the last treatment. The RNA was extracted and reversed transcribed for cDNA synthesis. The samples were tested for gene expression (*Hes-1* and *Deltex*) by real-time PCR analysis. Each gene was run with a positive control, which was the untreated $CD4^+$ T cell population (N) to which all the samples are relative, and with a negative control (no cDNA) to ensure there was no genomic DNA contamination. Furthermore, an internal control, 18srRNA, was included per sample as a monitor of the PCR reaction and of the amount of cDNA used. A 2.5-fold increase above the positive control is regarded as significant (in orange).

Thus, by examining Notch signalling in $CD4^+$ T cells isolated from mice either tolerised or primed to HDM p110-130 via the intranasal route, the following observations can be made:

- The Notch receptors, *-1* and *-2* are not modulated during the induction of priming (Figure 8) or tolerance (Figure 10).
- The Notch ligands, *Jagged-1* and *-2*, and *Delta-1* are not modulated under conditions that induce priming (Figure 8).
- *Jagged-1* and to a lesser extent *Delta-1* appear to be up regulated in tolerance at day 4 (Figure 10).
- *Deltex* is up regulated by LT alone (Figure 9) and by the presence of peptide in saline (Figure 11) at days 4 and 2, respectively.

SECTION 2

Phenotypic Characterisation of $CD4^+$ T Cells during Priming or Tolerance Induction

2.1. Background

Increasing evidence suggests that a population of T cells called regulatory T cells (Tr cells), may act to down-regulate other T cell responses, including those that are directed against self, and help maintain peripheral tolerance and immune homeostasis.

Sakaguchi *et al* (20) defined Tr cells as a minor subset of $CD4^+$ T cells (10%) expressing the IL-2 receptor α-chain (CD25) that are present in the spleen, lymph nodes and peripheral blood of normal adult mice. $CD4^+CD25^+$ T cells are capable of preventing certain autoimmune diseases (20, 21), remain unresponsive to TCR stimulation and can inhibit the response of $CD4^+CD25^-$ T cell in co-culture experiments *in vitro*.

Other Tr cells have also been defined on the basis of different cell surface markers, such as CD38. Splenic $CD4^+CD38^+$ T cells do not secrete any cytokines upon antigen-specific stimulation compared to the $CD4^+CD38^-$ subset, and can induce a Th1 healing response in mice infected with *Leishmania major* (29). Another marker that has been used to identify Tr cells is CD45RB. $CD4^+CD45RB^{low}$ cells are capable of inhibiting $CD4^+CD45RB^{high}$ cells from inducing colitis in SCID mice (25). Interestingly, these cells have also been further sub-divided into $CD25^+$ and $CD25^-$ populations (26), consistent with Sakaguchi's original observation (20), and have been found to suppress $CD4^+CD25^-CD45RB^{low}$ T cells *in vitro*, and to inhibit $CD45RB^{high}$-mediated colitis in SCID mice (27).

One question that remains unresolved is what is the mode of action of Tr cells. CTLA-4 is important in down regulating $CD4^+$ T cell activation (215, 216) and has been studied as a potential candidate that may be involved in the signalling pathway

that leads to immune suppression by Tr cells. Read *et al* (27) showed that anti-CTLA-4 monoclonal antibody inhibited the $CD4^{+}CD45RB^{low}$ T cells from suppressing colitis in SCID mice, suggesting that the $CD25^{+}$ cells were dependent on CTLA-4. More recently, Nakamura *et al* (217) showed that $CD4^{+}CD25^{+}$ T cells express cell-surface bound TGF-β and this may regulate the cell-cell contact-mediated suppression of proliferation of the $CD4^{+}CD25^{-}$ T cells.

The Tr cells so far described have the potential to suppress autoreactive T cells that can lead to autoimmune disorders. However, the host also needs to suppress or prevent the induction of unwanted immune responses to non-pathogenic airborne or dietary antigens, and this phenomenon is known as mucosal tolerance. Experimentally, this can be induced by intranasal administration of the house dust mite allergen, Der p 1, peptide 110-131 (section 1, Figure 5). This form of tolerance induces a population of long-lived $CD4^{+}$ Tr cells that are able to transfer tolerance to recipient mice.
Therefore, the aim of this study was to gain further insight into the phenotype of $CD4^{+}$ T cells after induction of intranasal peptide tolerance or priming compared to untreated $CD4^{+}$ T cells. In an attempt to define the characteristics of the Tr cells that are generated during intranasal peptide tolerance but not during intranasal priming, the cells from all the groups were stained with antibodies to markers that define Tr cells (CD25, CD38 and CD45RB) or Tr cell function (CTLA-4), and analysed by flow cytometry.

2.2. Charaterisation of untreated CD4^{+} T cells

2.2.1. Characterisation of CD4^{+}CD25^{+} and CD4^{+}CD25^{-} T cells

The initial part of this study consisted in characterising phenotypically CD4^{+} T cells from untreated mice, so they could serve as a baseline against which all other CD4^{+} T cells from treated mice would be compared to. Since it has been previously described that CD4^{+}CD25^{+} T cells are a constitutive regulatory T cell population (20, 22), splenocytes from untreated mice were stained with CD4-CyChrome and CD25-FITC to gate on CD25^{+} and CD25^{-} populations. A third PE-labelled marker, characteristic of Tr cells was used to determine the percentage of cells positive for each of the PE-labelled markers tested, and the values used as the baseline which to compare the samples from the peptide-treated groups. The cells were also stained with CD4-CyC, and the appropriate isotype controls for CD25-FITC and each of the PE-labelled markers tested (Figure 12). Table 1 and Figure 13 show the percentage information from a typical experiment. It is important to mention at this point that for all the flow data shown, the quadrant labels on the dot plots for each of the markers examined were set according to the corresponding isotype controls, however, for convenience the isotypes are not shown again in the thesis.

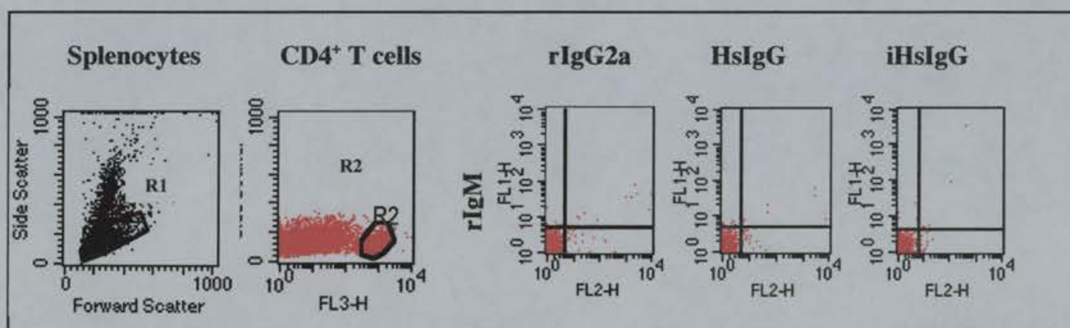
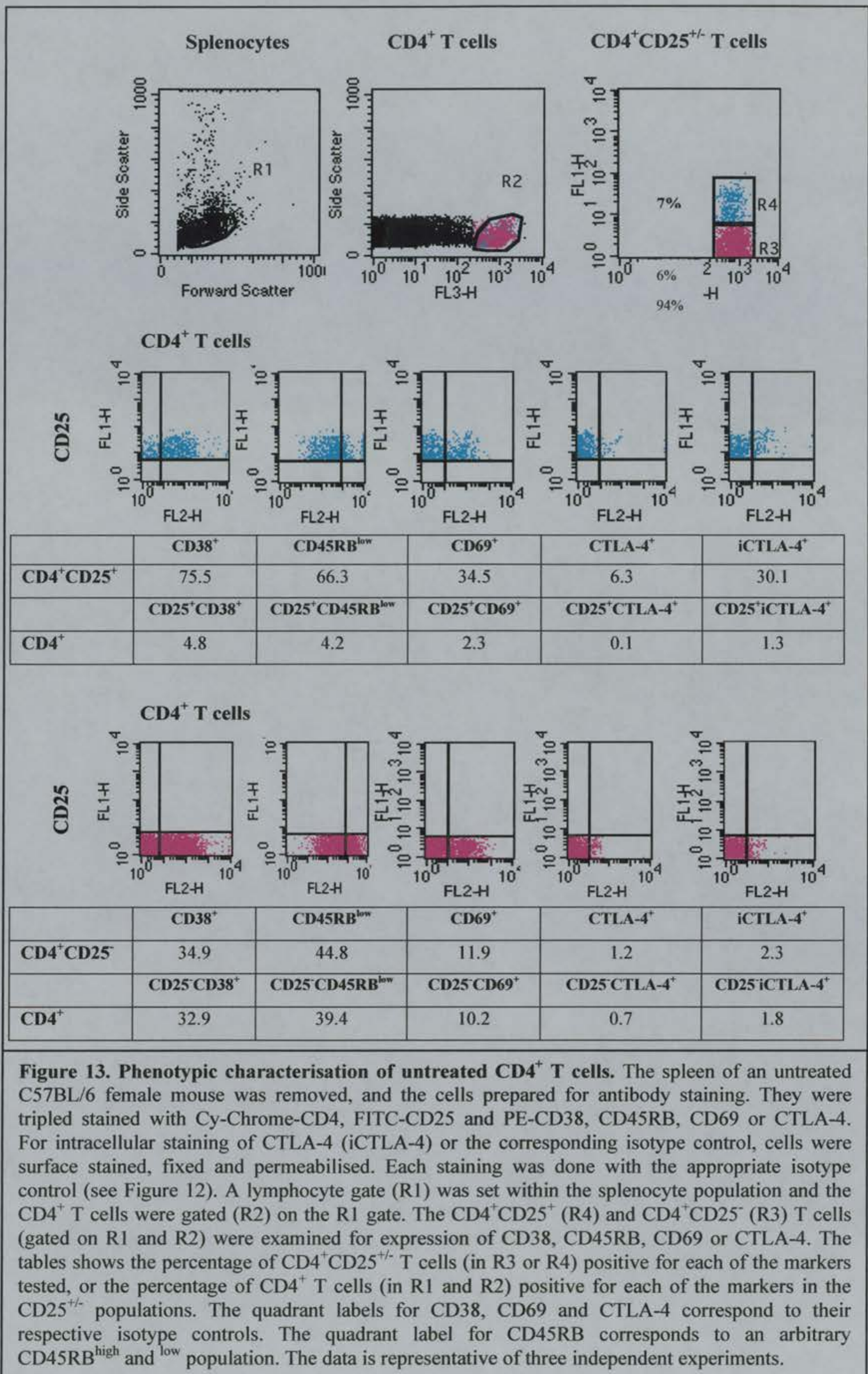


Figure 12. Phenotypic characterisation of untreated CD4^{+} T cells: a look at the isotype controls. The spleens from C57BL/6 female mice were removed, and the spleen cells prepared for flow cytometry by antibody staining using the markers of interest and the corresponding isotype controls. For the isotype controls splenocytes were triple stained with Cy-Chrome-CD4, rat FITC-IgM (rIgM, the isotype for CD25) and rat PE-IgG2a (rIgG2a, the isotype for CD38 and CD45RB) or hamster PE-IgG (HsIgG, the isotype for CD69 and CTLA-4). For intracellular staining, the cells were surface stained first (with Cy-Chrome CD4 and FITC-rIgM), then fixed and permeabilised with the antibody of interest (iHsIgG for the isotype). For data analysis, a lymphocyte gate (R1) was drawn in the splenocyte population, and the CD4^{+} T cells (R2) were gated on R1. To determine the percentage of positive cells for each marker of interest the cells were gated on R1 and R2, and quadrant labels set according to the isotype controls.



	$CD38^+$	$CD45RB^{low}$	$CD69^+$	CTLA-4^+	iCTLA-4^+
$CD4^+CD25^+$	75.5	66.3	34.5	6.3	30.1
	$CD25^+CD38^+$	$CD25^+CD45RB^{low}$	$CD25^+CD69^+$	$CD25^+$CTLA-4^+	$CD25^+$iCTLA-4^+
$CD4^+$	4.8	4.2	2.3	0.1	1.3

	$CD38^+$	$CD45RB^{low}$	$CD69^+$	CTLA-4^+	iCTLA-4^+
$CD4^+CD25^-$	34.9	44.8	11.9	1.2	2.3
	$CD25^-CD38^+$	$CD25^-CD45RB^{low}$	$CD25^-CD69^+$	$CD25^-$CTLA-4^+	$CD25^-$iCTLA-4^+
$CD4^+$	32.9	39.4	10.2	0.7	1.8

Figure 13. Phenotypic characterisation of untreated $CD4^+$ T cells. The spleen of an untreated C57BL/6 female mouse was removed, and the cells prepared for antibody staining. They were tripled stained with Cy-Chrome-CD4, FITC-CD25 and PE-CD38, CD45RB, CD69 or CTLA-4. For intracellular staining of CTLA-4 (iCTLA-4) or the corresponding isotype control, cells were surface stained, fixed and permeabilised. Each staining was done with the appropriate isotype control (see Figure 12). A lymphocyte gate (R1) was set within the splenocyte population and the $CD4^+$ T cells were gated (R2) on the R1 gate. The $CD4^+CD25^+$ (R4) and $CD4^+CD25^-$ (R3) T cells (gated on R1 and R2) were examined for expression of CD38, CD45RB, CD69 or CTLA-4. The tables shows the percentage of $CD4^+CD25^{+/-}$ T cells (in R3 or R4) positive for each of the markers tested, or the percentage of $CD4^+$ T cells (in R1 and R2) positive for each of the markers in the $CD25^{+/-}$ populations. The quadrant labels for CD38, CD69 and CTLA-4 correspond to their respective isotype controls. The quadrant label for CD45RB corresponds to an arbitrary $CD45RB^{high}$ and ^{low} population. The data is representative of three independent experiments.

Cell-surface marker	$CD4^+CD25^+$ (%) (expected)	$CD4^+CD25^+$ (%) (observed)	$CD4^+CD25^-$ (%) (observed)
$CD25^+$	10 (1)	7	
$CD38^+$	60 (2)	75.5	34.9
$CD45RB^{low/int}$	Majority (2)	66.3	44.8
$sCTLA\text{-}4^+$	Yes (3)	6.3	1.2
$iCTLA\text{-}4^+$	47.4 (4)	30.1	2.3
$CD69^+$	25 (1)	34.5	11.9

Table 1. Expression levels of T cell markers on $CD4^+$ T cells from untreated mice. Expression is shown as a percentage of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells positive for all the antibodies tested (based on Figure 13). The first column shows the values published by other groups.
sCTLA-4=surface CTLA-4. iCTLA-4=intracellular CTLA-4.
1= Reference 20
2= Reference 22
3= Reference 28
4= Reference 27

The data presented in Figure 13 and Table 1 show that about 7% of splenic $CD4^+$ T cells from untreated mice are $CD25^+$. Compared to the $CD25^-$ population more of them are $CD45RB^{low/int}$ (66.3% versus 44.8%), and $CD38^+$ (75.5% versus 34.9%). Additionally a higher number of cells are $CD69^+$ (34.5% versus 11.9%) which has been described as an early activation marker. I consistently found $CD4^+$ T cells to be CD69 positive, irrespective of the immunological status of the mice, and this may reflect on the health status of the animals kept in our Animal House facilities. Furthermore, surface CTLA-4 was also expressed at a higher level on the $CD25^+$ T cells compared to the $CD25^-$ (6.3% versus 1.2%), and when the cells were permeabilised to detect intracellular CTLA-4 the percentage was further increased (30.1% versus 2.3%). Typically expression of CTLA-4 is measured intracellularly as this molecule has been primarily localised in intracellular vesicles and may cycle between intracellular stores and the cell surface (218).

Therefore, the data presented in Table 1 show that the splenic $CD4^+CD25^+$ T cells analysed express a range of cell surface markers that are consistent with those reported by other groups.

2.2.2. Expression of Notch signalling genes in $CD4^{+}CD25^{+/-}$ T cells

The finding that *Jagged-1* was up-regulated in a murine model of intranasal peptide tolerance (section 1, Figure 10), a treatment that generates Tr cells, prompted to investigate if the constitutive $CD4^{+}CD25^{+}$ Tr cells also displayed increased levels of *Jagged-1* or other Notch ligand genes. For this, $CD4^{+}$ T cells were isolated from the spleens of untreated C57BL/6 mice by positive selection using MACS, and the cells prepared for FACS sorting by double staining with CD4-PE and CD25-FITC (Figure 14). Having sorted the $CD4^{+}$ T cells into $CD25^{+}$ and $CD25^{-}$, the total RNA was extracted and the samples were prepared for real-time PCR analysis (Figure 15).

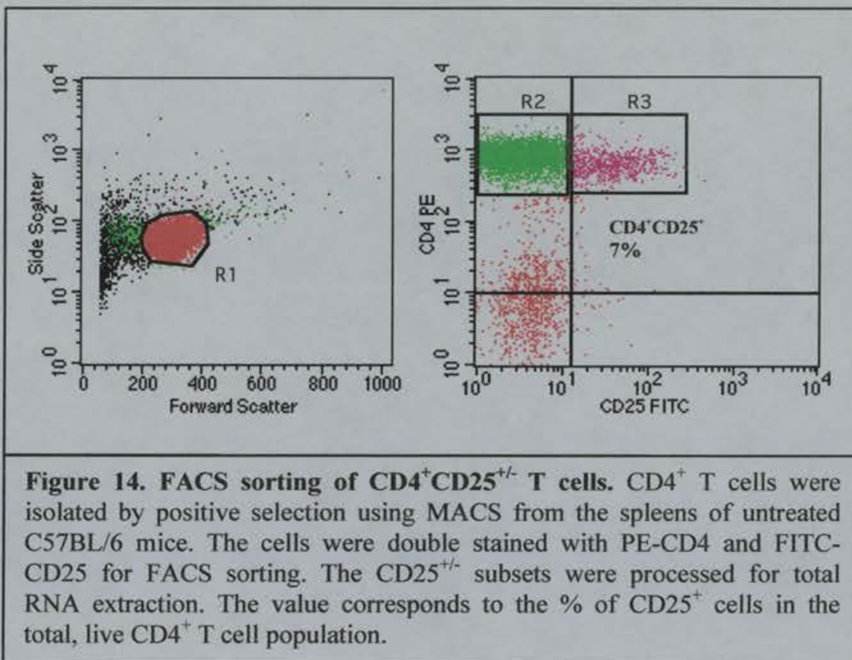


Figure 14. FACS sorting of $CD4^{+}CD25^{+/-}$ T cells. $CD4^{+}$ T cells were isolated by positive selection using MACS from the spleens of untreated C57BL/6 mice. The cells were double stained with PE-CD4 and FITC-CD25 for FACS sorting. The $CD25^{+/-}$ subsets were processed for total RNA extraction. The value corresponds to the % of $CD25^{+}$ cells in the total, live $CD4^{+}$ T cell population.

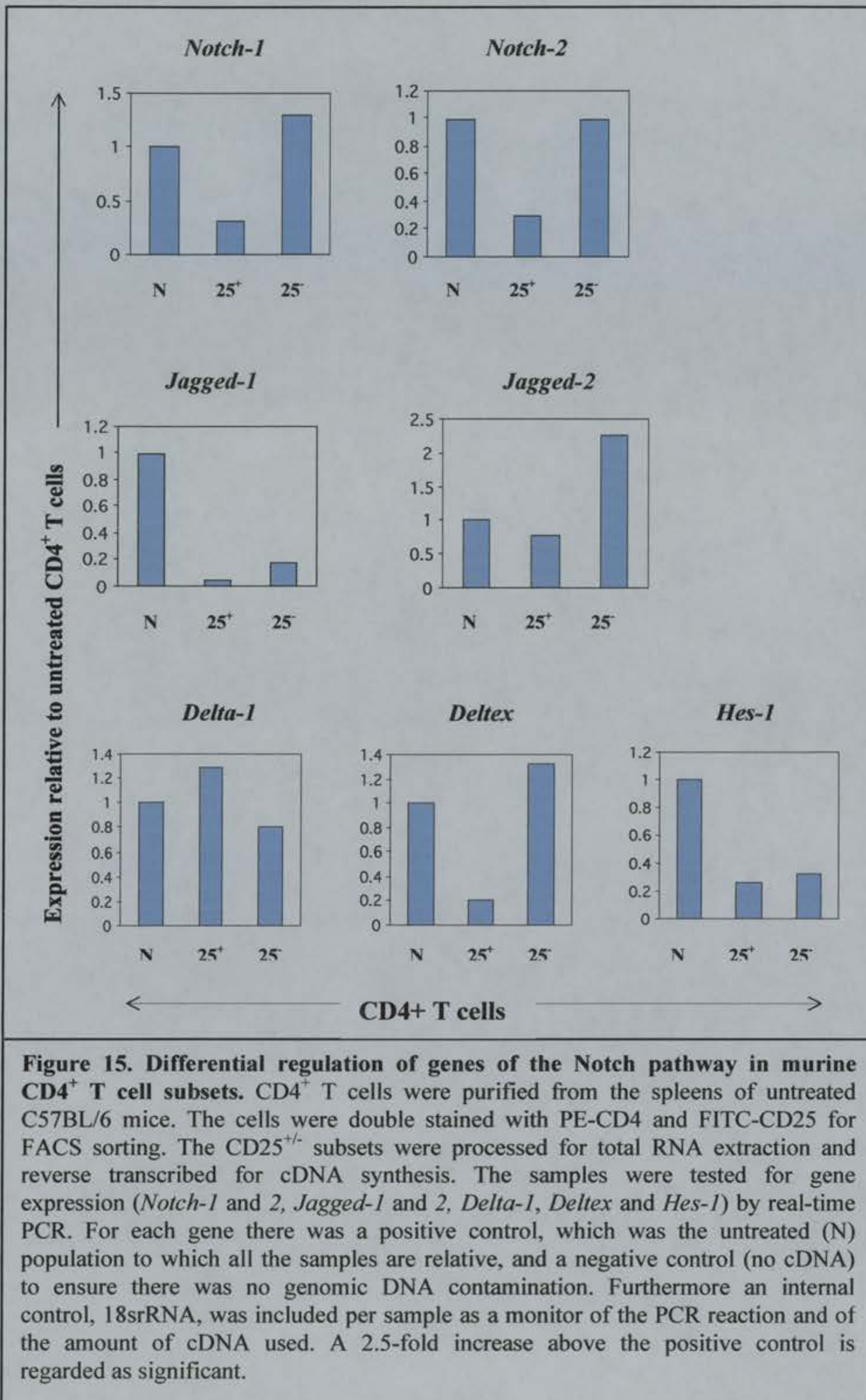
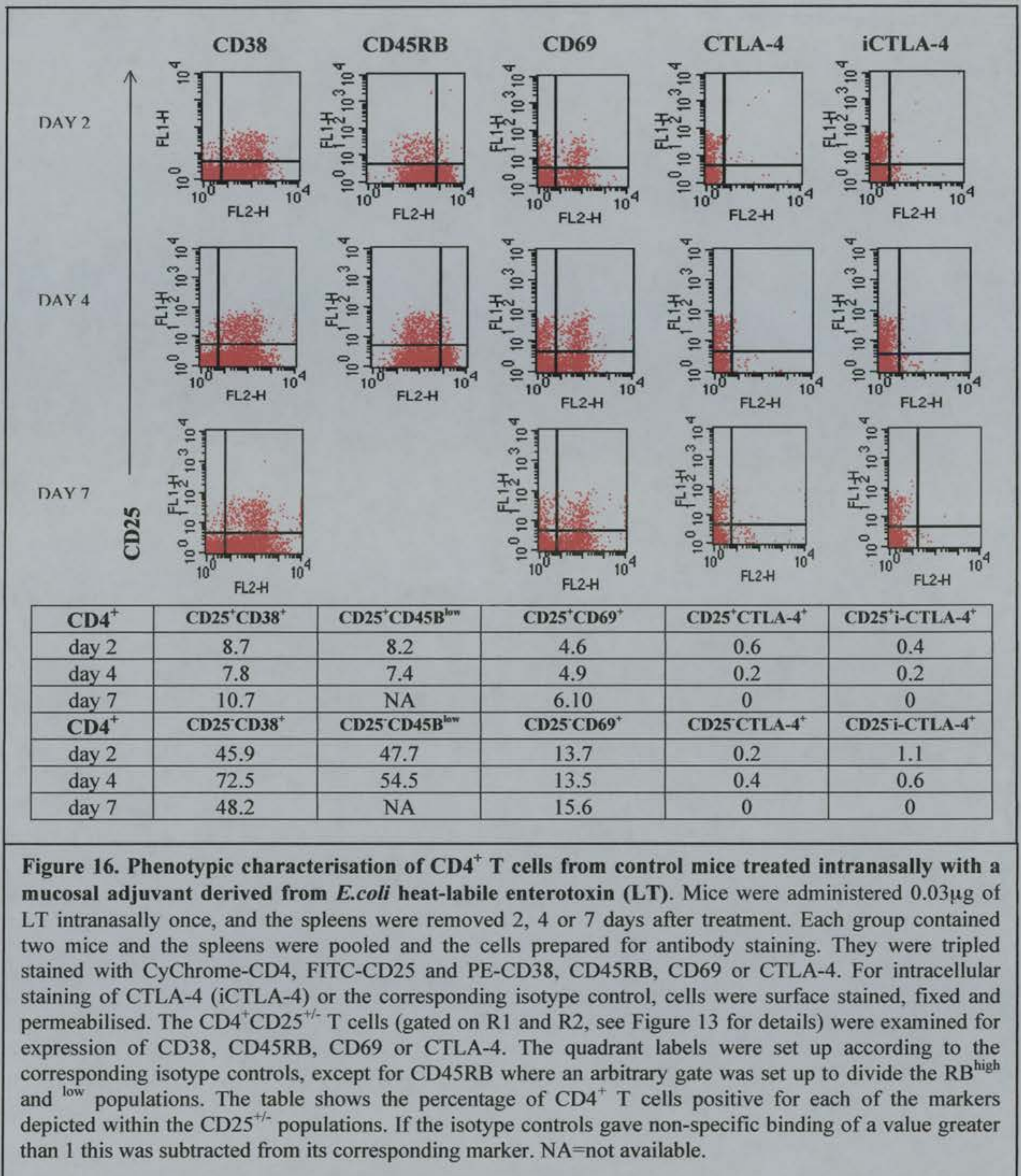


Figure 15. Differential regulation of genes of the Notch pathway in murine CD4$^+$ T cell subsets. CD4$^+$ T cells were purified from the spleens of untreated C57BL/6 mice. The cells were double stained with PE-CD4 and FITC-CD25 for FACS sorting. The CD25$^{+/-}$ subsets were processed for total RNA extraction and reverse transcribed for cDNA synthesis. The samples were tested for gene expression (*Notch-1* and *2, Jagged-1* and *2, Delta-1*, *Deltex* and *Hes-1*) by real-time PCR. For each gene there was a positive control, which was the untreated (N) population to which all the samples are relative, and a negative control (no cDNA) to ensure there was no genomic DNA contamination. Furthermore an internal control, 18srRNA, was included per sample as a monitor of the PCR reaction and of the amount of cDNA used. A 2.5-fold increase above the positive control is regarded as significant.

The data in Figure 15 show that for the $CD4^+CD25^+$ subset either the genes were expressed at lower levels (*Notch-1, Notch-2, Jagged-1, Jagged-2* and *Deltex*) or at the same levels (*Delta-1* and *Hes-1*) as the $CD25^-$ cells. This could be explained by the fact that the regulatory $CD4^+CD25^+$ T cells need TCR triggering to exert their suppressive function (22, 26). Therefore, if the $CD4^+CD25^+$ T cells were activated may be they would up-regulate expression of these genes as was seen for *Jagged-1* in $CD4^+$ T cells during tolerance induction (section 1, Figure 10). Interestingly the only other study to date where they have analysed by real-time PCR changes in the expression of components of the Notch pathway in $CD25^{+/-}$ T cells, (187) showed that unstimulated $CD25^+$ and $CD25^-$ T cells displayed very subtle differences in the expression of *Delta-1, Notch-4* or *Hes-1* genes. However, when the cells were stimulated with anti-CD3/anti-CD28 antibodies, transcripts for *Notch-4*, *Delta-1* and *Hes-1* increased considerably in the $CD25^+$ compared to the $CD25^-CD4^+$ T cells. They also found that transcripts for *Notch-1*, *Jagged-1* and *Jagged-2* were similar amongst the $CD25^{+/-}$ of unstimulated and stimulated cells. Therefore, this study demonstrates the need to activate the $CD25^+$ T cells to appreciate significant changes in components of the Notch pathway.

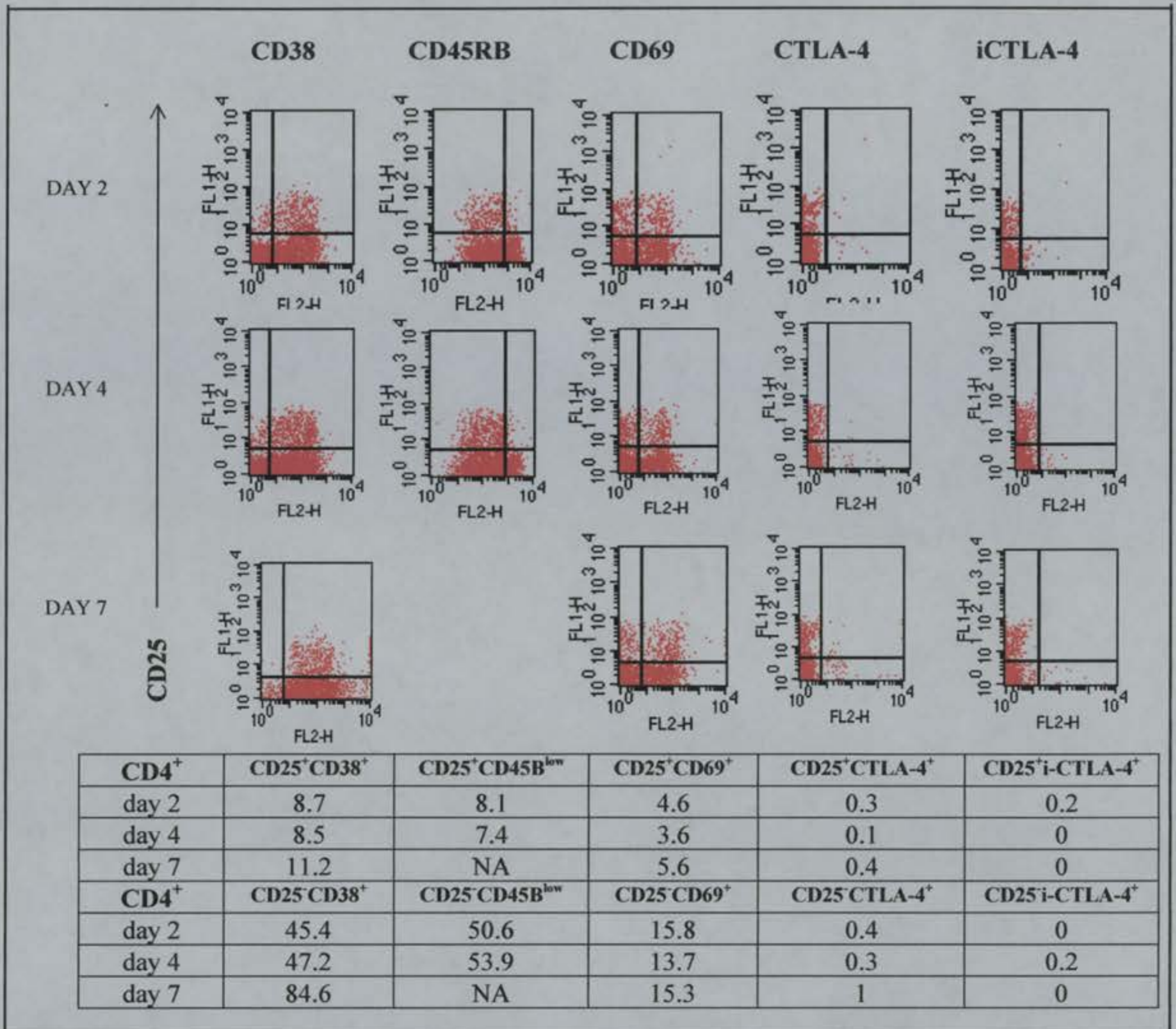
2.3. Charaterisation of $CD4^+$ T cells from mice tolerised or primed by intranasal peptide administration

In the experiments that follow, I addressed the question of whether the regulatory $CD4^+$ T cells induced in the model of intranasal peptide tolerance expand their $CD25^+$ T cell population as well as increasing markers of Tr cells (CD38, CD45RB, CTLA-4), compared to primed or untreated mice. For this the following groups of mice were set up. One group received LT alone intranasally (Figure 16), and another group was primed with LT in the presence of p110-130 to induce a T helper response (Figure 17). A third group received saline alone (Figure 18) and another group was given p110-130 in saline to induce tolerance, and generate Tr cells (Figure 19).
All the experimental groups were compared to the untreated $CD4^+$ T cells described in Figure 13.
Isolated splenocytes were stained with CD4-CyC and CD25-FITC to gate on $CD25^{+/-}$ cells. Thus, if the regulatory $CD4^+$ T cells generated during intranasal peptide tolerance were $CD25^+$, I would be able to detect an increase in numbers by flow cytometry compared to the primed and control groups (Table 2). Additionally, the cells were stained with other markers, which have been described for Tr populations, like CD38 and CD45RB (25, 27, 29), or with CTLA-4 which is important in Tr function (27, 28). Gating on the $CD4^+CD25^{+/-}$ would allow detecting changes in the other Tr markers, and help explain if they belong to the same regulatory T cell or may represent distinct subsets of regulatory T cells. The flow data is represented as dot plots. It should be stressed that each sample was accompanied by its corresponding isotype control. Figure 12 shows a representative example of the isotypes used in this study. The quadrant labels in the dot plots of the flow data, therefore, were set according to the corresponding isotypes. However, for convenience the isotypes controls for each of the samples in all the experimental groups are not shown in the rest of the thesis.



$CD4^+$	$CD25^+CD38^+$	$CD25^+CD45B^{low}$	$CD25^+CD69^+$	$CD25^+CTLA\text{-}4^+$	$CD25^+i\text{-}CTLA\text{-}4^+$
day 2	8.7	8.2	4.6	0.6	0.4
day 4	7.8	7.4	4.9	0.2	0.2
day 7	10.7	NA	6.10	0	0
$CD4^+$	$CD25^-CD38^+$	$CD25^-CD45B^{low}$	$CD25^-CD69^+$	$CD25^-CTLA\text{-}4^+$	$CD25^-i\text{-}CTLA\text{-}4^+$
day 2	45.9	47.7	13.7	0.2	1.1
day 4	72.5	54.5	13.5	0.4	0.6
day 7	48.2	NA	15.6	0	0

Figure 16. Phenotypic characterisation of $CD4^+$ T cells from control mice treated intranasally with a mucosal adjuvant derived from *E.coli* heat-labile enterotoxin (LT). Mice were administered 0.03μg of LT intranasally once, and the spleens were removed 2, 4 or 7 days after treatment. Each group contained two mice and the spleens were pooled and the cells prepared for antibody staining. They were tripled stained with CyChrome-CD4, FITC-CD25 and PE-CD38, CD45RB, CD69 or CTLA-4. For intracellular staining of CTLA-4 (iCTLA-4) or the corresponding isotype control, cells were surface stained, fixed and permeabilised. The $CD4^+CD25^{+/-}$ T cells (gated on R1 and R2, see Figure 13 for details) were examined for expression of CD38, CD45RB, CD69 or CTLA-4. The quadrant labels were set up according to the corresponding isotype controls, except for CD45RB where an arbitrary gate was set up to divide the RB^{high} and ^{low} populations. The table shows the percentage of $CD4^+$ T cells positive for each of the markers depicted within the $CD25^{+/-}$ populations. If the isotype controls gave non-specific binding of a value greater than 1 this was subtracted from its corresponding marker. NA=not available.



$CD4^+$	$CD25^+CD38^+$	$CD25^+CD45B^{low}$	$CD25^+CD69^+$	$CD25^+CTLA\text{-}4^+$	$CD25^+i\text{-}CTLA\text{-}4^+$
day 2	8.7	8.1	4.6	0.3	0.2
day 4	8.5	7.4	3.6	0.1	0
day 7	11.2	NA	5.6	0.4	0
$CD4^+$	$CD25^-CD38^+$	$CD25^-CD45B^{low}$	$CD25^-CD69^+$	$CD25^-CTLA\text{-}4^+$	$CD25^-i\text{-}CTLA\text{-}4^+$
day 2	45.4	50.6	15.8	0.4	0
day 4	47.2	53.9	13.7	0.3	0.2
day 7	84.6	NA	15.3	1	0

Figure 17. Phenotypic characterisation of $CD4^+$ T cells from experimental mice primed intranasally with HDM peptide 110-130 in the presence of *E.coli* LT. Mice were immunised once intranasally with 0.03μg of LT and 100μg of p110-130, and the spleens were removed 2, 4 or 7 days after treatment. Each group contained two mice and the spleens were pooled and the cells prepared for antibody staining. They were tripled stained with Cy-Chrome-CD4, FITC-CD25 and PE-CD38, CD45RB, CD69 or CTLA-4. For intracellular staining of CTLA-4 (iCTLA-4) or the corresponding isotype control, cells were surface stained, fixed and permeabilised. The $CD4^+CD25^{+/-}$ T cells (gated on R1 and R2, see Figure 13 for details) were examined for expression of CD38, CD45RB, CD69 or CTLA-4. The quadrant labels were set up according to the corresponding isotype controls, except for CD45RB where an arbitrary gate was set up to divide the RB^{high} and ^{low} populations. The table shows the percentage of $CD4^+$ T cells positive for each of the markers depicted within the $CD25^+$ and $CD25^-$ populations. If the isotype controls gave non-specific binding of a value greater than 1 this was subtracted from its corresponding marker. NA=not available.

Untreated		Day 2	Day 4	Day 7
7%	**LT**	10.8	10.3	10.5
	LT + p110-130	10.2	9.9	10.3
	PBS	8.4	8	10.2
	PBS + p110-130	9.7	8.6	9

Table 2. Percentage of CD4$^+$CD25$^+$ T cells. The values were taken from untreated mice, or mice that had been intranasally primed (LT plus p110-130) or tolerised (PBS plus p110-130), or had received LT alone or PBS alone. Splenocytes from each group were stained with CD4-Cyc and CD25-FITC. To determine the percentage values gates were drawn (R1 and R2 to gate on the CD4$^+$ T cells and R3 and R4 to gate on the CD25$^{+/-}$ populations) as represented in Figure 13.

Table 3: CD4$^+$CD25$^+$ T cells

CD4$^+$	CD25$^+$CD38$^+$	CD25$^+$CD45RB^{low}	CD25$^+$CD69$^+$	CD25$^+$CTLA-4$^+$	CD25$^+$iCTLA-4$^+$
Untreated	4.8	4.2	2.3	0.1	1.3
day 2 - LT	8.7	8.2	4.6	0.6	0.4
day 4 - LT	7.8	7.4	4.9	0.2	0.2
day 7 - LT	10.7	NA	6.10	0	0
day 2 - LT + pep	8.7	8.1	4.6	0.3	0.2
day 4 - LT + pep	8.5	7.4	3.6	0.1	0
day 7 - LT + pep	11.2	NA	5.6	0.4	0

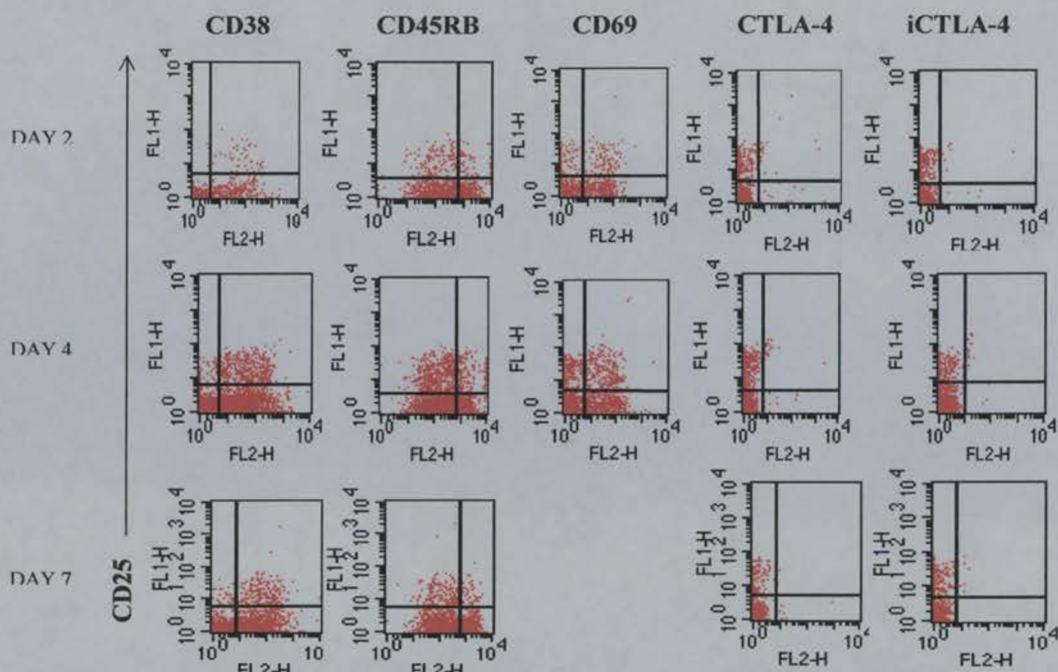
Table 4. CD4$^+$CD25$^-$ T cells

CD4$^+$	CD25$^-$CD38$^+$	CD25$^-$CD45RB^{low}	CD25$^-$CD69$^+$	CD25$^-$CTLA-4$^+$	CD25$^-$iCTLA-4$^+$
Untreated	32.9	39.4	10.2	0.7	1.8
day 2 - LT	45.9	47.7	13.7	0.2	1.1
day 4 - LT	72.5	54.5	13.5	0.4	0.6
day 7 - LT	48.2	NA	15.6	0	0
day 2 - LT + pep	45.4	50.6	15.8	0.4	0
day 4 - LT + pep	47.2	53.9	13.7	0.3	0.2
day 7 - LT + pep	84.6	NA	15.3	1	0

Table 3 and 4. Summary of the percentage of CD4$^+$CD25$^{+/-}$ T cells positive for CD38, CD45RB^{low}, CD69 and CTLA-4 (surface or intracellular) in untreated, LT and LT+peptide-treated mice (taken from Figures 13, 16 and 17).

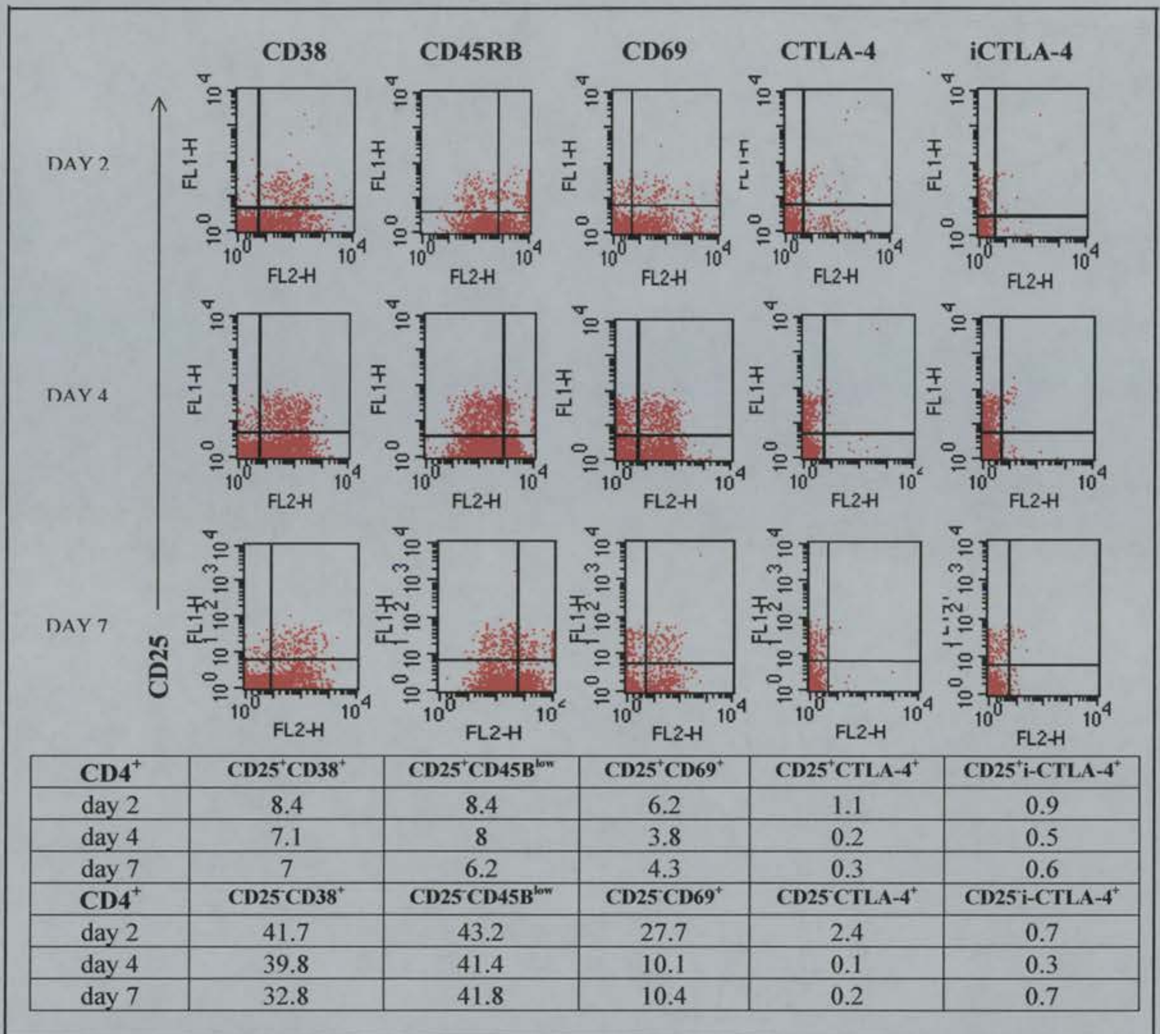
A point to mention is that by looking at the dot plots some seem to have less events than others for a given marker, but the percentage of positive cells is higher. This reflects on the number of events that were picked up by the flow cytometer, as sometimes not enough cells were available to collect 10,000 events in the $CD4^+$ gate. Table 2 shows that the percentage of $CD25^+$ T cells increased from 7% in untreated mice to about 10% in both the LT and LT/peptide groups by day 2, through to day 7. Additionally the percentage of $CD25^+CD38^+$, $CD45RB^{low/int}$ and $CD69^+$ approximately doubled in both experimental groups compared to untreated mice over the 7 day period (Table 3), and this corresponded to the increase in $CD25^+$ numbers observed. There were no detectable changes in CTLA-4 expression (surface or intracellular) between the groups, other than intracellular CTLA-4 being higher in the untreated group. A comparison of the LT plus peptide group and LT group revealed that there are no major differences in the percentage of $CD4^+$ T cells that were $CD25^+CD38^+$, $CD45RB^{low/int}$, $CD69^+$ and CTLA-4^+ (surface and intracellular) over the time course followed. These findings suggest that the increase in numbers of the $CD25^+$ population and the equivalent rise in the other surface markers may be due to the activating effect of the mucosal adjuvant and not the peptide, as CD38, $CD45RB^{low}$ and CD69 are also markers of T cell activation. LT when used as an adjuvant may be activating $CD4^+$ T cells in a non-specific manner, as Figure 4 (section 1) shows that cells from mice receiving LT alone did not respond to p110-130 *in vitro.*

The numbers of cells that were $CD25^-$ and expressed $CD38^+$, $CD45RB^{low/int}$ $CD69^+$ also increased (Table 4). However, similar to the $CD25^+$ findings this increase was no different in the LT and LT plus peptide groups. Therefore, these findings reinforce the capacity of LT on its own to activate $CD4^+$ T cells. The only striking difference came from the $CD25^-CD38^+$ numbers in the control group which increased from 45.9% at day 2 after LT administration, to 72.5% at day 4, and dropped to 48.2% by day 7. In contrast, the LT plus peptide treated group showed an increase in the $CD4^+CD25^-CD38^+$ population at day 7 instead (84.6% versus 45.4% at day 2 and 47.2% at day 4). This may represent a difference in the kinetics of expression of CD38 as a result of LT being or not in the presence of peptide.



$CD4^+$	$CD25^+CD38^+$	$CD25^+CD45B^{low}$	$CD25^+CD69^+$	$CD25^+CTLA\text{-}4^+$	$CD25^+i\text{-}CTLA\text{-}4^+$
day 2	6.1	7.7	3.9	0.4	0.2
day 4	5.9	6.7	3.6	0.5	0.5
day 7	8.1	6.9	NA	0.2	0.6
$CD4^+$	$CD25^-CD38^+$	$CD25^-CD45B^{low}$	$CD25^-CD69^+$	$CD25^-CTLA\text{-}4^+$	$CD25^-i\text{-}CTLA\text{-}4^+$
day 2	43.7	45.3	11.9	0.3	0.2
day 4	39.3	40.2	8.5	0.2	0.1
day 7	41.8	49.2	NA	0.3	0.3

Figure 18. Phenotypic characterisation of $CD4^+$ T cells from control mice treated intranasally with saline (PBS) alone. Mice were administered 25μl of saline intranasally on three consecutive days, and the spleens were removed 2, 4 or 7 days after treatment. Each group contained two mice and the spleens were pooled and the cells prepared for antibody staining. They were tripled stained with CyChrome-CD4, FITC-CD25 and PE-CD38, CD45RB, CD69 or CTLA-4. For intracellular staining of CTLA-4 (iCTLA-4) or the corresponding isotype control, cells were surface stained first with the other antibodies, fixed and permeabilised. The $CD4^+CD25^{+/-}$ T cells (gated on R1 and R2, see Figure 13 for details) were examined for expression of CD38, CD45RB, CD69 or CTLA-4. The quadrant labels were set up according to the corresponding isotype controls, except for CD45RB where an arbitrary gate was set up to divide the RB^{high} and ^{low} populations. The table shows the percentage of $CD4^+$ T cells positive for each of the markers depicted within the $CD25^+$ and $CD25^-$ populations. If the isotype controls gave non-specific binding of a value greater than 1 this was subtracted from its corresponding marker. NA=not available.



$CD4^+$	$CD25^+CD38^+$	$CD25^+CD45B^{low}$	$CD25^+CD69^+$	$CD25^+CTLA\text{-}4^+$	$CD25^+i\text{-}CTLA\text{-}4^+$
day 2	8.4	8.4	6.2	1.1	0.9
day 4	7.1	8	3.8	0.2	0.5
day 7	7	6.2	4.3	0.3	0.6
$CD4^+$	$CD25^-CD38^+$	$CD25^-CD45B^{low}$	$CD25^-CD69^+$	$CD25^-CTLA\text{-}4^+$	$CD25^-i\text{-}CTLA\text{-}4^+$
day 2	41.7	43.2	27.7	2.4	0.7
day 4	39.8	41.4	10.1	0.1	0.3
day 7	32.8	41.8	10.4	0.2	0.7

Figure 19. Phenotypic characterisation of $CD4^+$ T cells from experimental mice treated intranasally with p110-130 in saline. Mice were administered 100μg of p110-130 in saline intranasally on three consecutive days, and the spleens were removed 2, 4 or 7 days after the last treatment. Each group contained two mice and the spleens were pooled and the cells prepared for antibody staining. They were tripled stained with CyChrome-CD4, FITC-CD25 and PE-CD38, CD45RB, CD69 or CTLA-4. For intracellular staining of CTLA-4 (iCTLA-4) or the corresponding isotype control, cells were surface stained first with the other antibodies, fixed and permeabilised. The $CD4^+CD25^{+/-}$ T cells (gated on R1 and R2, see Figure 13 for details) were examined for expression of CD38, CD45RB, CD69 or CTLA-4. The quadrant labels were set up according to the corresponding isotype controls, except for CD45RB where an arbitrary gate was set up to divide the RB^{high} and ^{low} populations. The table shows the percentage of $CD4^+$ T cells positive for each of the markers depicted within the $CD25^+$ and $CD25^-$ populations. If the isotype controls gave non-specific binding of a value greater than 1 this was subtracted from its corresponding marker.

Table 5. $CD4^+CD25^+$ T cells

$CD4^+$	$CD25^+CD38^+$	$CD25^+CD45RB^{low}$	$CD25^+CD69^+$	$CD25^+CTLA\text{-}4^+$	$CD25^+iCTLA\text{-}4^+$
Untreated	4.8	4.2	2.3	0.1	1.3
day 2 - PBS	6.1	7.7	3.9	0.4	0.2
day 4 - PBS	5.9	6.7	3.6	0.5	0.5
day 7 - PBS	8.1	6.9	NA	0.2	0.6
day 2 - PBS+pep	8.4	8.4	6.2	1.1	0.9
day 4 - PBS+pep	7.1	8	3.8	0.2	0.5
day 7 - PBS+pep	7	6.2	4.3	0.3	0.6

Table 6. $CD4^+CD25^-$ T cells

$CD4^+$	$CD25^-CD38^+$	$CD25^-CD45RB^{low}$	$CD25^-CD69^+$	$CD25^-CTLA\text{-}4^+$	$CD25^-iCTLA\text{-}4^+$
Untreated	32.9	39.4	10.2	0.7	1.8
day 2 - PBS	43.7	45.3	11.9	0.3	0.2
day 4 - PBS	39.3	40.2	8.5	0.2	0.1
day 7 - PBS	41.8	49.2	NA	0.3	0.3
day 2 - PBS+pep	41.7	43.2	27.7	2.4	0.7
day 4 - PBS+pep	39.8	41.4	10.1	0.1	0.3
day 7 - PBS+pep	32.8	41.8	10.4	0.2	0.7

Table 5 and 6. Summary of the percentage of $CD4^+CD25^{+/-}$ T cells positive for CD38, $CD45RB^{low}$, CD69 and CTLA-4 (surface or intracellular) in untreated, PBS and PBS+ peptide-treated mice (taken from Figures 13, 18 and 19).

The data in Table 2 also show that the $CD25^+$ numbers in the tolerised and control groups increased modestly by equivalent numbers (about 9%) over the three time points followed, compared to the numbers in the untreated mice (7%). This rise also corresponded to a small increase in $CD38^+$, $CD45RB^{low}$ and $CD69^+$ numbers within the $CD25^+$ population of both experimental groups (Table 5). Thus saline alone seems to have an effect on the expansion of the $CD25^+$ population. The peptide-treated group showed almost a two-fold increase in $CD25^+CD69^+$ cells and more than a two-fold increase in $CD25^+CTLA\text{-}4^+$ cells (surface and intracellular) at day 2

after the last peptide treatment compared to the control group (Table 5). However, this may not be of biological relevance as the numbers drop to normal by day 4. Giving mice saline alone or with p110-130 intranasally also increased the numbers of $CD25^{-}CD38^{+}$, $CD45RB^{low}$ and $CD69^{+}$ cells compared to untreated mice, although not to the extent that LT did (Table 6). The numbers of $CD25^{-}CD69^{+}$ and CTLA-4^{+} more than doubled in the peptide-treated group compared to the saline group at day 2 but dropped to levels of the untreated mice by day 4, which corresponds to the finding in the $CD25^{+}$ population.

In summary, priming mice with peptide and a mucosal adujvant (*E. coli* LT) led to an increase in $CD4^{+}CD25^{+}$ T cell numbers by day 2 after intranasal treatment through to day 7, which seemed to be associated with the presence of LT rather than peptide. This rise was also accompanied by an increase in $CD25^{+}CD38^{+}$, $CD45RB^{low}$ and $CD69^{+}$ by day 2, which may reflect on the capacity of LT to activate these cells. Therefore, the antigen-driven response of $CD4^{+}$ T cells to p110-130 *in vitro* at day 7 (section 1, Figure 4), did not correlate to any distinct phenotypic changes, as far as the markers that were chosen in this study are concerned.

Giving mice saline or peptide in saline to induce tolerance, also led to a modest increase in $CD4^{+}CD25^{+}$ numbers, which were seen in both experimental groups, therefore, the rise was again not specific to the presence of peptide. Within the $CD25^{+}$ and $CD25^{-}$ populations, the peptide-treated group showed an increase in the percentage of $CD69^{+}$ (about two-fold) and CTLA-4^{+} cells (more than two-fold) at day 2 only compared to the saline group.

In general, the increase in markers detected for both the primed and tolerised groups of mice correspond more to an activated phenotype of the $CD4^{+}$ T cells rather than to cells that have become helper or regulatory.

SECTION 3

Discussion

Notch signalling is a highly conserved developmental pathway, which has been described in many cell fate decisions. It is involved in the differentiation of cell lineages during thymocyte development (73, 74, 75), and work in the group has demonstrated that it may also play a part in the differentiation of regulatory $CD4^+$ T cells during the induction of peripheral tolerance (99). Therefore, the question I wished to address was if Notch signalling was involved in $CD4^+$ T cell differentiation during ongoing peripheral immune responses. To study this I relied on a model of $CD4^+$ T cell recognition of the house dust mite peptide 110-131. If the peptide is administered intranasally in saline it generates a population of Tr cells, which are unresponsive to re-stimulation *in vitro* with the whole protein or peptide derivatives (11, 12, 13) and can transfer tolerance to recipients. However, if the peptide is given intraperitoneally with CFA, it leads to a Th-1-like response and productive immunity. Initially this model was used to induce Tr cells or Th cells, and study expression of genes associated with the Notch pathway. Additionally, in an attempt to characterise the $CD4^+$ T cells from the two experimental groups the cells were analysed for expression of CD25, CD38, CD45RB and CTLA-4, all of which are markers that describe Tr cells (25, 26, 27, 28, 29).
Furthermore, the requirement for antigen presentation in the induction of helper or regulatory $CD4^+$ T cells, prompted an investigation of the gene expression of Notch receptors and ligands in APC populations from tolerised or primed mice. Therefore, splenic B cells and enriched DCs were positively selected from both experimental groups and analysed.

However, this approach is limited in that different routes of antigen administration are used and arguably, by giving peptide either intranasally or intraperitonally this may affect the kinetics of antigen uptake, delivery to the spleen and activation of the resident $CD4^+$ T cells differently.

As well it raises the question of where the APCs come from. Splenic APCs may have a very different pattern of expression of Notch signalling genes to those that have migrated from the draining lymph nodes, where they will have encountered antigen. Additionally, the APC populations in the spleen of tolerant or primed mice may differ functionally. The former will have encountered antigen in the cervical lymph nodes, possibly in an environment which already favours tolerance by default (from a continuous exposure to inhaled antigens), and the latter encounters antigen in the mesenteric lymph nodes and spleen in the presence of complete Freund's adjuvant.
Therefore, the concern raised by the route of antigen administration and the added complexity of studying gene expression in APC populations prompted to change the experimental design and procedures.
First I decided to concentrate only on $CD4^+$ T cells, and therefore I did not analyse gene expression of APC populations in the experiments reported in this thesis.
Second, the experiments were repeated keeping the route of antigen administration for both tolerance and priming the same, namely the nasal route. Priming mice intranasally with antigen has been achieved with mucosal adjuvants, one of which is *E.coli* heat labile toxin (LT). LT when used at a high concentration has powerful immunogenic effects and on its own can induce antibody secretion and modulate cytokine profiles. It can also induce a selective increase in the cell surface expression of B7.1, but not B7.2, MHC class II or CD40 on mouse bone marrow-derived DCs (206), and it can activate B cells (219). These are reasons that add to the complexity of studying expression of Notch signalling genes in APC populations.

Therefore, it was important to determine the optimal concentration of LT, which on its own would not be immunogenic, but in combination with peptide would act as an adjuvant. When used alone at 5μg, it could prime mice to environmental house dust mite present in the Animal House facilities where the mice were kept. Splenocytes responded *in vitro* to HDM p110-130 in a dose-dependent manner at day 7 after LT administration (section 1, Figure 1). Therefore, lower doses of LT were given to mice and it was found that as low as 0.3μg of LT could still prime mice to environmental HDM (Figure 2). From these experiments it was found that LT given intranasally at 0.03μg did not prime mice to HDM, but in combination with p110-

130 retained its adjuvant effect (Figures 2 and 3). Therefore, the mice were primed with 0.03μg of LT and 100μg of peptide once, and the spleens removed at days 2, 4 and 7 after treatment. $CD4^+$ T cell responses to p110-130 *in vitro* were measured at each time point and the data revealed that the antigen-driven response took place at day 7 (Figure 4).

The tolerance protocol was based on the work of Hoyne *et al* (11, 12 13). Mice were given 100μg of p110-130 in saline on three consecutive days and the spleens were removed at days 2, 4 and 7 after the last peptide treatment. The $CD4^+$ T cells from this mice were initially activated in response to p110-130 *in vitro* by day 2 and displayed maximal proliferative responses to the peptide at day 4, before becoming unresponsive at day 7 (Figure 5).
The priming and tolerance protocols clearly differ in the dose and frequency of antigen administration, which it could still be argued, may have differential effects on the expression of Notch signalling genes. However in experiments that are not shown in this thesis if I kept the antigen dose and frequency of administration the same as for the priming experiments (100μg of p110-130), this did not induce tolerance. The $CD4^+$ T cells proliferated to a similar extent at days 2, 4 or 7 after the intranasal treatment when re-stimulated *in vitro* with p110-130. Similarly, if I gave a higher concentration of peptide (300μg) once, to keep the frequency of antigen administration constant, the $CD4^+$ T cells re-stimulated *in vitro* to p110-130 did not respond as well to the peptide as they had when 100μg were given on 3 consecutive days. Therefore, for these reasons the dose and frequency of antigen administration differ in both experimental protocols, and this is why the two groups are not compared to each other, but to an untreated group of mice.

The $CD4^+$ T cells were isolated by negative selection (Figure 7) to avoid the risk that a positive selection method may introduce in activating signalling pathways that interfere with expression of components of the Notch pathway.
Analysis of gene expression by real-time PCR revealed that $CD4^+$ T cells from mice primed with LT and peptide (Figure 8), showed no significant differences in levels of expression of the Notch receptors (*Notch-1,* and *-2*) or ligands *(Jagged-1, -2* and

Delta-1), compared to $CD4^+$ T cells from mice receiving LT alone or from untreated mice. Additionally the expression of the signalling molecules, *Hes-1* and *Deltex* (Figure 9), which are both up regulated as a result of Notch signalling (32, 85, 214), did not vary much amongst the different groups. Only *Deltex* was up regulated by about 2.5-fold in the $CD4^+$ T cells of the LT-treated mice at day 4 compared to untreated $CD4^+$ T cells. However, this was not significant compared to the LT/peptide-treated group at day 4.
A note of caution should be introduced. Arguably, the LT on its own may be affecting the expression of Notch signalling genes in $CD4^+$ T cells to such an extent that even in the presence of antigen, and after the cells have been activated by day 7, the effects are not appreciated. And it could be for this reason that the pattern of expression for all the different genes is not modulated to a great extent for any of the time points examined, between the LT and LT plus peptide groups of mice.

The $CD4^+$ T cells from mice that were tolerised did not show significant modulation of the Notch receptors *Notch-1* and *-2* compared to mice that received saline alone (Figure 10). However, interestingly *Jagged-1* was significantly up regulated by more than 2.5-fold at day 4 after the last peptide treatment, with respect to untreated or saline-treated mice. As well, *Delta-1* was up regulated at this time point, although not to the same extent. Interestingly, day 4 is the time point at which the cells maximally proliferate in response to peptide *in vitro* before becoming regulatory and unresponsive by day 7. The observation that this feature is not seen at day 7 after peptide priming, would suggest it is not simply a result of $CD4^+$ T cell activation. Therefore, it could be a feature unique to the differentiation of Tr cells in this model of peptide tolerance.
Having said that some points need to be addressed. The finding that *Jagged-1* was up regulated is not indicative of Notch signalling. Other signalling pathways that are activated at the time of Tr cell differentiation may be affecting the expression of *Jagged-1*. Indeed the finding that *Hes-1* and *Deltex* (Figure 11), which would have been up regulated following Notch activity, did not display significant differences in their patterns of expression compared to the saline-treated groups, supports this observation.

Sakaguchi *et al* (20) defined a susbset of $CD4^+$ T cells that expressed the IL-2 receptor α-chain, CD25, which constituted 10% of the total splenic $CD4^+$ T cell population, and that could prevent organ-induced autoimmunity in mice. I was interested in investigating the pattern of expression of Notch signalling genes in this population, as any similarities to the $CD4^+$ T cells previously described in the model of intranasal peptide tolerance would indicate that may be these Tr cells are also $CD25^+$. Therefore, the $CD4^+$ T cells were sorted into $CD25^+$ and $CD25^-$ and basal levels of gene expression were analysed by real-time PCR. The data revealed that $CD4^+CD25^+$ T cells did not up-regulate any of the Notch receptors (*Notch-1*, and *-2*) or ligands studied (*Jagged-1*, *-2* and *Delta-1*) with respect to unsorted $CD4^+$ T cells or $CD25^-$ T cells. As well, expression of *Hes-1* and *Deltex* was not modulated (Figure 15). Therefore, this would indicate that Notch activity is not important in the $CD25^+$ T cells. However, it should be mentioned that the cells in this study had not been activated, and it has been reported that $CD25^+$ T cells require TCR triggering to exert their suppressive function (22, 26). Therefore, I would need to re-address this question by activating the $CD25^+$ and $CD25^-$ subsets and analyse expression of Notch signalling genes. Interestingly, the only other study to date which has looked at $CD4^+CD25^+$ and $CD25^-$ populations in the context of Notch signalling, reported that unstimulated $CD25^+$ and $CD25^-$ cells did not differ greatly in the expression of *Notch-4*, *Delta-1* and *Hes-1* (187). However, when the cells were activated with anti-CD3/anti-CD28 antibodies they greatly up regulated these three genes in the $CD25^+$ compared to the $CD25^-$ population.

I was also interested in studying the $CD25^+$ population after peptide priming or tolerance. I would hope that if the Tr cells generated in peptide tolerance were $CD25^+$ they would expand, whereas numbers would remain the same in the primed group. Additionally I wanted to study the expression of other markers that have described Tr cell populations, [CD38 (29), CD45RB (25, 26)] or Tr function [CTLA-4 (27, 28)]. An increase in the number of cells expressing these markers within the tolerised but not the primed group would be indicative of Tr cells. If they were also confined to the $CD25^+$ population this would also suggest that they belong to the same Tr lineage.

At first I characterised $CD25^+$ T cells from untreated mice which would serve as the control group to which all other experimental groups would be compared. They constituted about 7% of the whole $CD4^+$ T cell population (Table 2) and more of them were $CD38^+$, $CD45RB^{low}$ and CTLA-4^+ compared to their $CD25^-$ counterparts (Figure 13). These findings are consistent with the work of other groups (22, 28).
When the mice were given LT and LT plus peptide the numbers of $CD25^+$ T cells increased to about 10% from day 2 through to day 7 (Table 2). This suggests that the expansion of the $CD25^+$ T cells is more the effect of the LT rather than the peptide, and may be due to the activating properties of LT on T cells, as CD25 is up-regulated on activated $CD4^+$ T cells. Indeed Nashar *et al* (219) found that mice injected intraperitoneally with 30μg of LT in CFA caused an increase in the numbers of $CD4^+CD25^+$ T cells in the mesenteric lymph nodes compared to mice that received the same dose of a mutated form of LT.
The increase in $CD25^+$ T cells also correlated with an increase within this population of $CD38^+$, $CD45RB^{low}$ and $CD69^+$ T cells, in the LT and LT/peptide-treated mice (Figures 16 and 17). As these markers are also specific to $CD4^+$ T cell activation, it appears as if the increase in expression of all the surface markers examined are a result of the activating properties of LT on $CD4^+$ T cells. This observation raises a point of concern for investigating expression of Notch signalling genes. Therefore, the finding that no modulation in the expression of these genes was observed for the LT and LT/peptide groups (Figures 8 and 9) could be attributable to the effects of the LT, as has been previously discussed.

When mice were treated with saline or saline plus peptide, there was also an increase from day 2 through to day 7 of $CD4^+CD25^+$ numbers (Table 2), which also correlated with a rise in $CD25^+CD38^+$, $CD45RB^{low}$ and $CD69^+$ cells. This suggests that this general increase was also a result of injecting saline into the mice. The only main difference observed between the saline and the saline/peptide-treated groups was that $CD25^+CD69^+$ and $CD25^+$CTLA-4^+ numbers about doubled in the peptide-treated group compared to the control group (Figures 18 and 19). However, this was not peptide-specific as the numbers dropped to levels in the control group at day 4,

when there was maximal proliferation of the $CD4^+$ T cells in response to the peptide *in vitro* (Figure 5).

Clearly, it has proven difficult to characterise the Tr cells generated in the model of intranasal peptide tolerance used in this study. However, there was a significant up-regulation of *Jagged-1* message in the $CD4^+$ T cells of these mice prior to the cells becoming unresponsive to the peptide *in vitro*, and this could be a feature unique to the differentiation of Tr cells.

In the discussion to the experiments conducted prior to the review of this thesis I discussed how new experiments would help determine if the $CD4^+CD25^+$ T cells are responsible for inducing tolerance in the model of intranasal peptide tolerance used throughout this work. This is what I wrote:

"An experiment I can conduct to expand our knowledge on the regulatory T cell population is to isolate lymphoid $CD4^+CD25^+$ and $CD4^-CD25^-$ T cells from mice immunised intranasally with the house dust mite peptide. As a control group I can use the same $CD4^+$ subsets from unimmunised mice. I could culture them on their own, or at varying ratios with unsorted $CD4^+$ T cells from unimmunised mice, and stimulate them with the house dust mite peptide, an irrelevant antigen such as OVA to test for antigen-specificity, a self-peptide such as MBP to test for self-reactivity, and via the TCR using anti-CD3/anti-CD28 antibodies. These experiments would tell us if the $CD25^+$ and $CD25^-$ T cells from the unimmunised mice behave the same or differently to those derived from the peptide-treated mice. This would provide us with clues relating to the regulatory properties of the $CD25^+$ versus $CD25^-$ subsets, and answer if the Tr cells are generated upon specific antigenic challenge or they derive from the constitutive population found in the spleen, and which can regulate potentially autoreactive T cells."

Interestingly, these experiments have since been conducted in the ovalbumin (OVA) TCR transgenic mice, clone DO11.10, where all or the majority of the $CD4^+$ T cells are specific for the OVA peptide 323-339 (220). The OVA-specific $CD4^+$ T cells can be identified by use of the clonotypic antibody KJ 1-26. The use of a transgenic mouse model has obvious advantages over using a polyclonal TCR mouse model, as

in the former all, or the majority, of the $CD4^+$ T cells are specific for one peptide and the antigen-reactive cells can be identified by the KJ 1-26 antibody. This facilitates the characterisation of the antigen-specific cells and makes gene expression studies a lot more accurate as most of the $CD4^+$ T cells are antigen-specific, therefore the signals detected by real-time PCR after antigenic challenge would derive from the majority of $CD4^+$ T cells.

In this OVA TCR transgenic study, Zhang *et al* (220) investigated if oral administration of OVA at a dose that would induce tolerance, resulted in the activation of the $CD4^+CD25^+$ T cells *in vivo*. They found a significant increase in the percentage of KJ $1\text{-}26^+CD25^+CD4^+$ T cells in inguinal, mesenteric lymph nodes and spleen from OVA-fed mice compared to unfed or BSA-fed mice. Furthermore the $CD25^+CD4^+$ T cells were better at suppressing the proliferation of the $CD25^-$ T cells from fed or unfed mice *in vitro* in response to OVA, than the $CD25^+CD4^+$ T cells from unfed mice. The $CD25^+$ T cells from fed mice secreted more TGF-β in response to anti-CD3 stimulation *in vitro*, compared to those of unfed mice. They also found an up-regulation of CTLA-4 in the $CD25^+$ from fed mice compared to unfed mice, but inhibiting CTLA-4 signalling *in vitro* or TGF-β signalling, only partially abrogated the suppressive activity of $CD25^+$ T cells from fed mice, suggesting other factors also come into play.

In addition, in the same discussion I also mentioned adoptive transfer studies to gain further insight into the immune regulatory properties of the $CD25^+$ population. This is what I wrote:

"*In vivo* I could adoptively transfer the $CD4^+CD25^+$ or $CD4^+CD25^-$ from peptide-treated mice into recipient mice, and immunise them with an immunogenic dose of Der p 1 protein. *In vitro* stimulation of the recipient $CD4^+$ T cells with house dust peptide or protein, or an irrelevant antigen, would reveal information regarding the immune-regulatory properties of the $CD25^+$ population compared to the $CD25^-$ subset."

Zhang *et al* also addressed this issue in their OVA TCR transgenic model. They found that in co-culture experiments the $CD25^+$ T cells from OVA-fed mice were

better at inhibiting the antigen-specific responses of $CD25^-$ T cells, compared to $CD25^+$ T cells from unfed mice. However, when $CD25^+$ T cells from unfed and fed mice were transferred intravenously to BALB/c mice they both suppressed delayed type hypersensitivity responses to immunisation with OVA to a similar extent.

In another recent study, Thorstenson and Khoruts (221) also conducted adoptive transfer experiments to investigate the role of $CD4^+CD25^+$ T cells in the induction of peripheral tolerance to antigen administered intravenously or orally. They transferred $CD4^+$ T cells from DO11.10 mice on a
RAG-$2^{-/-}$ background into BALB/c mice. The RAG-$2^{-/-}$ mice lack T cells and B cells, and the DO11.10 RAG-$2^{-/-}$ mice contain less than 1% of $CD4^+CD25^+$ T cells as opposed to DO11.10 BALB/c, which contain about 5% $CD4^+CD25^+$ T cells. This difference results from the contribution of the endogenous TCR-α chain during TCR rearrangement in $CD4^+$ T cell development. Therefore, the use of these mice ensures that the differentiation of the $CD4^+CD25^+$ T cells is exclusive to peripheral antigen exposure. Typically, intravenous administration of antigen results in an antigen-specific expansion of the $CD4^+$ T cells followed by clonal deletion and functional inactivation of the remaining antigen-specific $CD4^+$ T cells. Thorstenson *et al* found that low-dose intravenous injection of OVA peptide led to an increase in KJ 1-$26^+CD25^+CD4^+$ T cells between days 3 and 8 after injection compared to mice primed with peptide and LPS. As well they found that antigen administration had an inverse correlation to the percentage of KJ 1-26^+CD25^+ T cells. Thus low-dose antigen resulted in a higher percentage of KJ 1-26^+CD25^+ T cells than high-dose antigen, and these cells went through less cycles of cell division than the high-dose antigen exposed cells. This observation was interesting as dividing cells are associated with IL-2 production, and the KJ 1-26^+CD25^+ T cells were found not to produce IL-2 *in vivo*. They also found that low-dose intravenous and oral administration of antigen both had similar effects on the cycling of KJ 1-$26^+CD25^+CD4^+$ T cells.
Finally they demonstrated that $CD25^+$ T cells generated following peripheral tolerance induction by low dose intravenous antigen exposure, or $CD25^+$ T cells from

transgenic DO11.10 mice could inhibit the IL-2 secretion of DO11.10 responder cells to specific OVA peptide, to a similar extent.

Therefore, it appears that to re-address the question of $CD4^+CD25^+$ Tr cell involvement during intranasal peptide tolerance, the DO11.10 TCR transgenic mouse system can be effectively relied upon. Preliminary experiments would be needed to determine the right concentration and frequency of OVA peptide administration to induce tolerance. After optimising the protocol the antigen-specific cells from these mice would be phenotyped as described previously, and the $CD4^+$ T cells would be analysed for expression of components of the Notch signalling pathway.
To test if the Tr cells generated in the DO11.10 mice are $CD25^+$ T cells these cells could be re-stimulated with OVA peptide *in vitro*, and compared to the proliferative responses of $CD25^+$ T cells from mice that received saline, or to their $CD25^-$ counterparts. Co-culture of the $CD25^+$ with the $CD25^-$ population from the peptide-treated and saline-treated mice would also address the issue of their immune suppressive capacity in response to antigen recognition.
$CD25^+$ cells could also be depleted from peptide or saline-treated DO11.10 $CD4^+$ T cells, transferred into BALB/c and mice then primed with OVA peptide. If the $CD25^+$ T cells were required for inducing and transferring tolerance, mice receiving $CD25^+$-depleted $CD4^+$ T cells would respond to the peptide in *vitro* upon re-stimulation of the antigen-specific $CD4^+$ T cells.
If the antigen-specific cells during tolerance induction were $CD25^+$ DO11.10 RAG-$2^{-/-}$ mice could be used to test if the generation of $CD25^+$ T cells is specific to a tolerogenic exposure of antigen or results from the expansion of the constitutive $CD4^+CD25^+$ T cells. Adoptive transfer experiments could also be carried out to ensure that only the antigen-specific $CD4^+$ T cells are taken into account. $CD4^+$ T cells from DO11.10 RAG-$2^{-/-}$ mice can be adoptively transferred into BALB/c mice and these mice tolerised by intranasal administration of antigen. If the $CD4^+CD25^+$ T cells are the Tr cells in this system, their expansion in the different lymphoid compartments can be followed over time, to study if the initial expansion of the cells correlates with the site of antigen administration. Other experiments similar to the ones conducted by Zhang *et al* and Thorstenson *et al* could also be conducted.

This study has demonstrated that using a polyclonal TCR murine model of recognition of the house dust mite $CD4^+$ T cell epitope, peptide 110-130 has proven difficult in an attempt to characterise the $CD4^+$ T cells that are generated under conditions that induce tolerance or priming. Still, expression of components of the Notch pathway revealed that *Jagged-1* was up regulated in $CD4^+$ T cells after day 4 of peptide tolerance as opposed to peptide priming. The observation that it did not seem to correlate merely with activation of the $CD4^+$ T cells suggests that this finding may be unique to the differentiation of Tr cells in this model of intranasal peptide tolerance. Therefore, repeating these experiments in a transgenic mouse model such as the DO11.10 TCR transgenic system would give clearer answers to the characterisation of $CD4^+$ T cells generated after intranasal peptide administration. Additionally, it would provide with more valuable and significant information regarding the expression of components of the Notch signalling pathway.

CHAPTER V

Role of TGF-β, Activin A and BMP-4 in $CD4^+$ T Cell Responses and Effects on Notch Signalling Genes

1. Background

The involvement of Notch signalling during development in cell fate decisions is well documented (31, 32, 33, 34). We have also demonstrated (99) that Notch signalling may play a role during peripheral immune responses in the differentiation pathway that generates $CD4^+$ Tr cells from naïve cells after antigenic encounter.
However, the potential role of Notch signalling in $CD4^+$ T cell differentiation during antigen-specific responses is a novel area of study. Rather, the contribution of inhibitory cytokines such as TGF-β in the generation of Tr cells *in vivo*, has been investigated more thoroughly (63, 64, 222, 223).
Data from Chapter III (Figures 2, 3 and 5) demonstrate that genes associated with the Notch and TGF-β signalling pathways are differentially expressed in peripheral lymphoid organs and cells. Furthermore, the Notch ligands, the downstream signalling gene, *Hes-1*, and the TGF-β-like molecule, *Activin A,* were up-regulated as early as 24 hours after *in vitro* activation of $CD4^+$ T cells (Figure 6).
Due to its immune-regulatory capacity I went on to investigate the effect of TGF-β and the TGF-β superfamily members, activin A and BMP-4, on $CD4^+$ T cell activation. Apart from belonging to the TGF-β superfamily, evidence has implicated them all in similar biological processes, such as erythropoiesis and fracture repair (Chapter I, Table III). Furthermore, activin A uses the same downstream effector molecules as TGF-β, and has been found to affect proliferation of several cell types. Therefore, the prediction is that functionally the different TGF-β-like molecules, such as activin A and BMP-4, may display similar immune-regulatory properties to those attributed to TGF-β *in vitro* (224, 225, 226).

As well, I wished to investigate interactions between the TGF-β and the Notch pathway. In development, activin A/BMP-4 and Notch/Notch ligands are highly conserved and both can act to inhibit neurogenesis in favour of alternate fates. Additionally, both have been implicated in similar biological processes such as haematopoiesis, cancer, apoptosis and cell growth (Chapter I, Table III).

My hypothesis proposes that signals derived from Notch and TGF-β superfamily genes may contribute to the outcome of $CD4^+$ T cell function *in vivo* and *in vitro*. Therefore, the question I wished to address was twofold. First, how do activin A and BMP-4 affect $CD4^+$ T cell responses *in vitro*. Second, if any effect on $CD4^+$ T cell responses mediated by these molecules may correlate with changes in expression of genes associated with the Notch pathway. To carry out this work I used an antigen-specific $CD4^+$ human T cell clone (section 2.1), and murine splenic $CD4^+$ T cells activated with anti-CD3 and anti-CD28 antibodies (section 2.2).

2. Results

2.1. Effect of TGF-β-like Molecules on a Human $CD4^+$ T cell clone

2.1.1. Effect of TGF-β-like Molecules on HA1.7 T Cell Proliferation

The HA1.7 is a human $CD4^+$ T cell clone reactive with the influenza haemagglutinin (HA) peptide (HA306-318) and restricted by HLA-DRB1*0101. Murine recombinant TGF-β, activin A and BMP-4 were used to study the effects of these proteins on the proliferation of HA1.7 T cells *in vitro* (Figure 1).

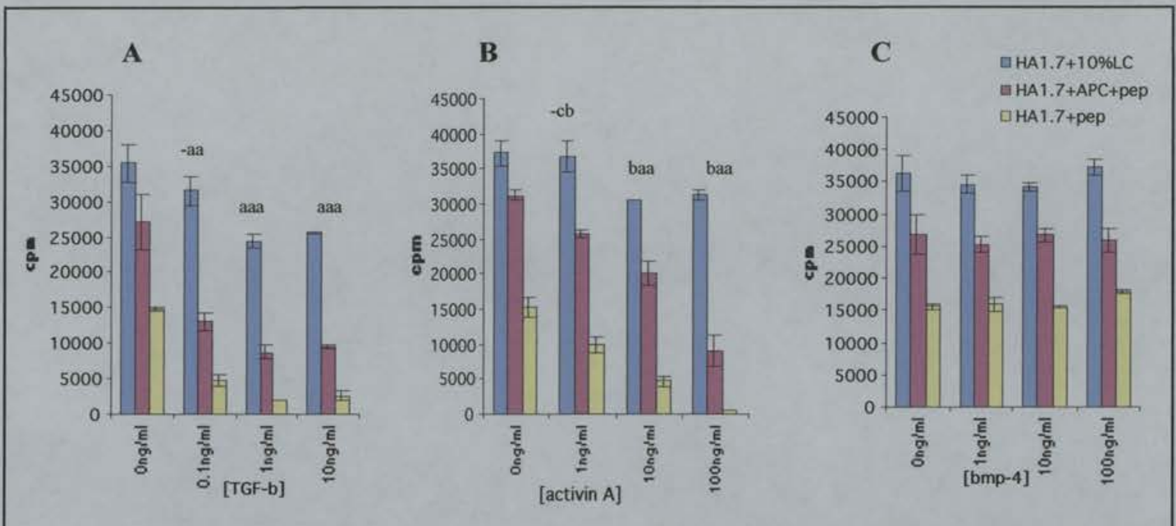


Figure 1. TGF-β and activin A inhibit HA1.7 T cell proliferation. $2x10^4$ HA1.7 T cells/well were cultured in the presence of 10% IL-2, (lymphocult, blue bars), or $2.5x10^4$ irradiated human peripheral blood mononuclear cell (PBMCs) and 1μg/ml of HA peptide (purple bars), or in 1μg/ml of peptide alone (yellow bars). Human recombinant TGF-β (A), activin A (B) or BMP-4 (C) were added at varying concentrations. Cells were cultured for 3 days, and the plates were pulsed with 2.5μCi of ^{3}[H]-TdR for 18 hours before harvesting. The cells were cultured in triplicates in round-bottomed 96-well plates.
- = not significant, **a** = $p<0.001$, **b** = $p<0.01$, **c** = $p<0.05$ for each bar versus the media alone bar of the same colour.

Figure 1 reveals that BMP-4 had no effect on HA1.7 T cell proliferation regardless of the concentration used, and the method of T cell activation. On the contrary, TGF-β and activin A strongly inhibited proliferation of HA1.7 in a dose-dependent manner. This effect was achieved when the cells were stimulated with IL-2 alone (lymphocult) or with the peptide, in the presence or absence of APCs.

The ability of TGF-β to inhibit human T cell responses is well documented (224, 225, 226), and therefore my findings (Figure 1) are consistent with the published literature. Interestingly, activin A had the same effect. This molecule has been shown to modulate the proliferation of a number of cell types. For example, it has been found to inhibit proliferation of mid-gestation human foetal adrenal cells *in vitro* in a dose-dependent manner, and the effect is additive when TGF-β is also added (227). Similarly, it inhibits proliferation of the BALB/c fibroblast cell line, 3T3 (136) and the rat intestinal epithelial cell line, IEC-6 (228) in a concentration-dependent manner. Other groups have also reported activin A and TGF-β to have similar inhibitory effects in different cell types. They can both inhibit proliferation of rat hepatocytes (134), murine primordial germ cells (229) and porcine thyrocyte cell growth (230). Interestingly, in the latter study the authors investigated the effects of TGF-β, activin A and BMP-7. Like Figure 1 reveals, they found that the growth inhibitory effect of activin A was not as pronounced as that of TGF-β, and BMP-7 did not significantly inhibit cell growth.

Furthermore, activin A has been found to inhibit rat testicular $CD4^+$ and $CD8^+$ T cell proliferation (165) and proliferation of rat T cells isolated from peripheral blood (231).

2.1.2. Effect of TGF-β-like Molecules on HA1.7 T Cell Cytokine Production

Having determined the effects of TGF-β-like molecules on T cell proliferation, I analysed if they had differential effects on cytokine production. The supernatants from cultures of HA1.7 T cells incubated with APCs and HA peptide, in the presence of increasing doses of TGF-β, activin A or BMP-4 were tested for cytokine secretion of IL-4, IL-5, IL-10 and IFN-γ (Figure 2). Figure 2 shows that addition of BMP-4 had little effect on cytokine production. On the contrary, activin A and TGF-β inhibited Th2 cytokine secretion (IL-4, IL-5 and IL-10) in a dose-dependent manner. In contrast, the levels of IFN-γ secretion (Th1 cytokine) remained constant regardless of the protein concentration added.

not be done. Although this limits the interpretation of these results, a trend towards inhibition of Th-2 cytokine production is still observed.

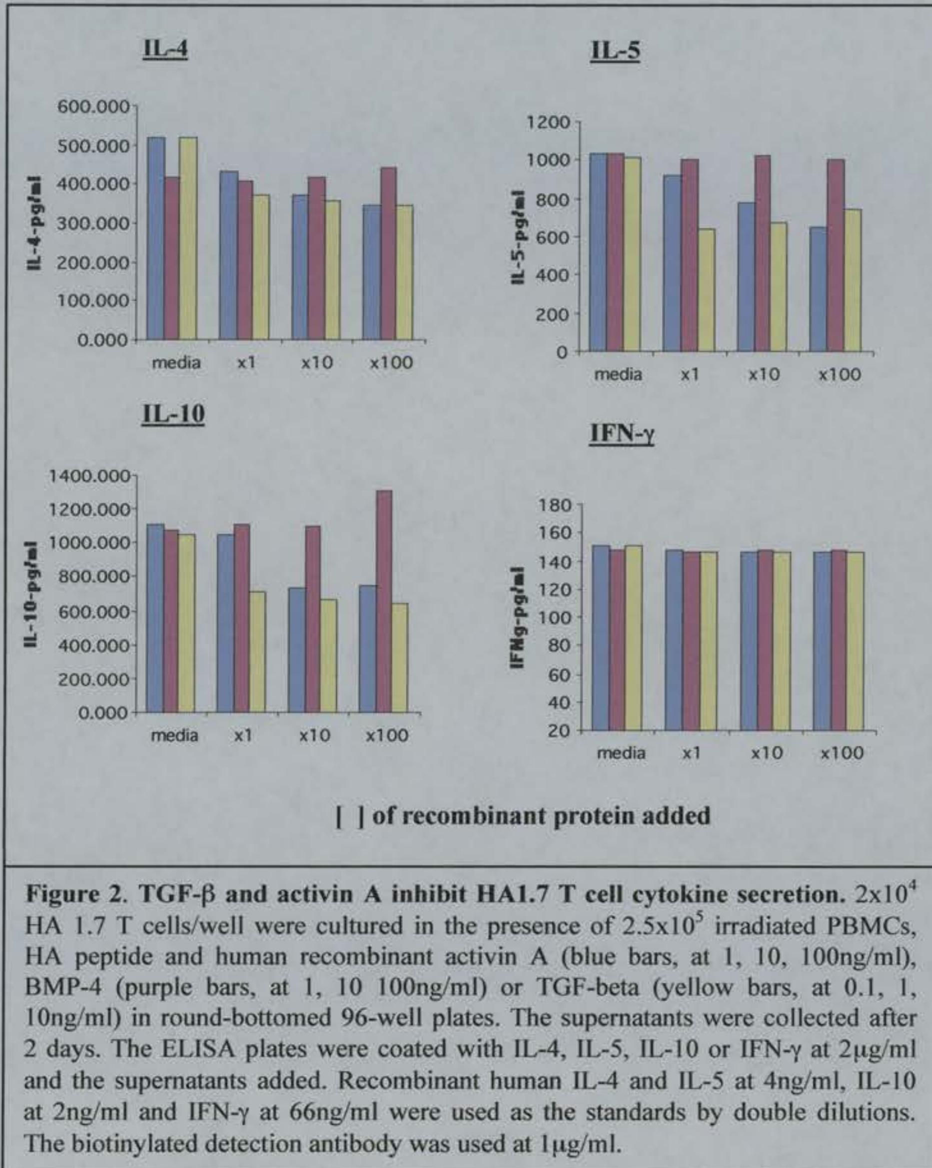


Figure 2. TGF-β and activin A inhibit HA1.7 T cell cytokine secretion. $2x10^4$ HA 1.7 T cells/well were cultured in the presence of $2.5x10^5$ irradiated PBMCs, HA peptide and human recombinant activin A (blue bars, at 1, 10, 100ng/ml), BMP-4 (purple bars, at 1, 10 100ng/ml) or TGF-beta (yellow bars, at 0.1, 1, 10ng/ml) in round-bottomed 96-well plates. The supernatants were collected after 2 days. The ELISA plates were coated with IL-4, IL-5, IL-10 or IFN-γ at 2μg/ml and the supernatants added. Recombinant human IL-4 and IL-5 at 4ng/ml, IL-10 at 2ng/ml and IFN-γ at 66ng/ml were used as the standards by double dilutions. The biotinylated detection antibody was used at 1μg/ml.

Therefore, it appears from these data that TGF-β and activin A may be capable of down regulating Th2-type cytokines, but have only a minimal effect on Th1 cytokine production. In agreement with this observation is the work of Fargeas *et al* (232) who showed that human T cells purified from peripheral blood and stimulated *in vitro* with anti-CD3 antibody and TGF-β, suppressed the production of IL-4 whereas IFN-

showed that human T cells purified from peripheral blood and stimulated *in vitro* with anti-CD3 antibody and TGF-β, suppressed the production of IL-4 whereas IFN-γ secretion was unaffected. Furthermore, recent work by different groups has reported the same finding in the mouse. Ludviksson *et al* (233) showed that addition of TGF-β during primary or secondary antigen-driven responses *in vitro*, significantly decreased Th1 (IFN-γ) and Th2 (IL-4) cytokine production, although the effect was more profound on the Th2 cytokines. Similarly, Heath *et al* (234) showed that antigen-specific primary responses in the presence of TGF-β also led to a marked decrease in IL-4, IL-5 and IL-10 production.

Little is known about the effects of activin A on cytokine production by $CD4^+$ T cells, and the only studies that have been carried have been using monocytes and macrophages. Ohguchi *et al* (168) found that activin A could inhibit IL-1 production by different human monocytic cell lines, whereas others (169) showed that activin A caused release of TNF-α and IL-1β in rat bone marrow-derived macrophages. Further still, activin A has been found to suppress IL-6-mediated biological activities (235). Therefore, most of the work that has been carried out using activin A has been in connection with pro-inflammatory cytokines. In this study I report for the first time how activin A may have the potential to inhibit Th2 cytokine production by a human $CD4^+$ T cell clone, although its significance *in vivo* is still unknown.

2.1.3. Effect of Combining TGF-β-like Molecules on HA1.7 T Cell Proliferation

The observation that activin A and TGF-β had an inihibitory effect on the proliferation of HA1.7 T cells prompted me to investigate whether these two proteins in combination might have a synergistic effect on T cell growth. Therefore, I stimulated cells with IL-2 (or lymphocult) in the presence of a sub-optimal dose of TGF-β, activin A or BMP-4, and added TGF-β or activin A in a dose-dependent manner (Figure 3).

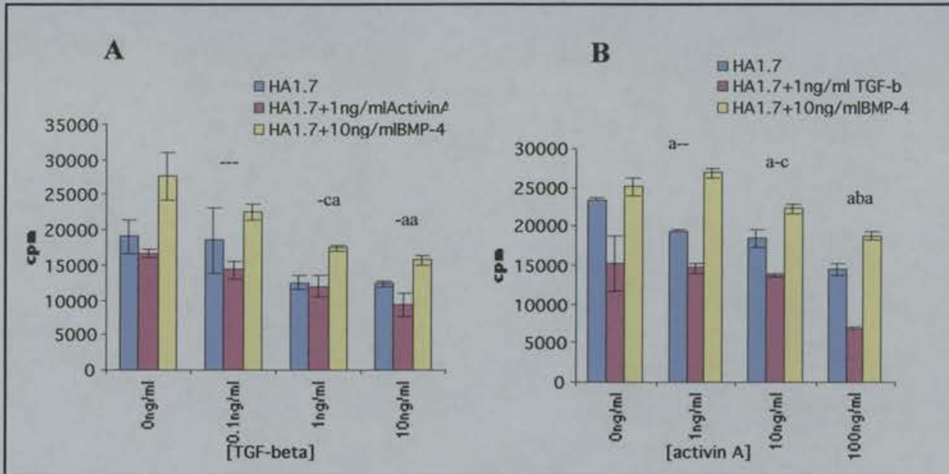


Figure 3. TGF-β and activin A act in synergy to inhibit HA1.7 T cell proliferation.
A. $2x10^4$ HA 1.7 T cells/well were cultured in the presence of 10% lymphocult and increasing concentrations of human recombinant TGF-β (A, blue bars) plus 10ng/ml of activin A (purple bars) or 10ng/ml of BMP-4 (yellow bars). The cultures were set up in triplicates in round-bottomed 96-well plates.
B. Alternatively, HA 1.7 T cells were cultured under the same conditions in the presence of increasing concentrations of activin A (B, blue bars) plus 1ng/ml of TGF-β (purple bars) or 10ng/ml of BMP-4 (yellow bars).
The plates were left in the incubator for 3 days, pulsed with 2.5 μCi of ^{3}[H]-TdR and harvested after 18 hours.
- = not significant, **a** = $p<0.001$, **b** = $p<0.01$, **c** = $p<0.05$ for each bar versus the media alone bar of the same colour.

Figure 3B reveals that addition of 1ng/ml of TGF-β to increasing amounts of activin A has a synergistic dose-dependent effect. The same outcome is observed when 10ng/ml of activin A are added to increasing concentrations of TGF-β (Figure 3A), but the effect is not as pronounced, suggesting TGF-β is a more potent inhibitor of T cell proliferation. This result suggests that activin A and TGF-β are having the same effect but are not competing for the same receptors.

When BMP-4 was added at 10ng/ml to increasing concentrations of activin A or TGF-β, it promoted more HA1.7 T cell proliferation than cells grown in its absence. However, the stimulatory capacity of BMP-4 did not override the inhibitory signals of TGF-β and activin A which were still capable of suppressing proliferation of HA1.7 T cells in a dose-dependent manner.

The finding that activin A and TGF-β have similar inhibitory effects on HA1.7 T cells, and in combination their effects are additive, whereas BMP-4 alone has no effect on T cell proliferation, could be explained in terms of the receptors they bind to and the signalling pathways they activate.

The members of the TGF-β superfamily bind type I and type II serine/threonine kinase receptors (Chapter I, section 6.1). Initially, the ligand (TGF-β, activin A or BMP-4) binds its type II receptor and this results in recruitment of the type I receptor. The type II receptor then phosphorylates the type I, and this stimulates intracellular interactions with distinct signal transducers called Smads. Smad 1, 5 and 8 are involved in BMP signalling and Smad 2 and 3 are mediators of TGF-β and activin signalling. The fact that activin A and TGF-β bind to different receptors but share a common signalling pathway could explain why they both have similar inhibitory effects on T cell proliferation, and why when added in combination have an additive effect. In contrast, the observation that BMP-4 had no effect on T cell growth could be explained by the fact that this molecule signals through a different pathway to TGF-β and activin A.

2.1.4. The Effect of TGF-β-like Molecules is Direct on HA1.7 T cells

Having demonstrated that TGF-β and activin A can inhibit the proliferation and Th2-type cytokine production of HA1.7 T cells, I wanted to study whether this effect was mediated by signals derived from the APCs, or by direct interaction on the HA1.7 T cells. This question was addressed in two ways. First, the APCs were incubated for three hours with TGF-β, activin A or BMP-4. After the incubation period they were washed and added to cultures containing HA1.7 T cells and HA peptide (Figure 4).

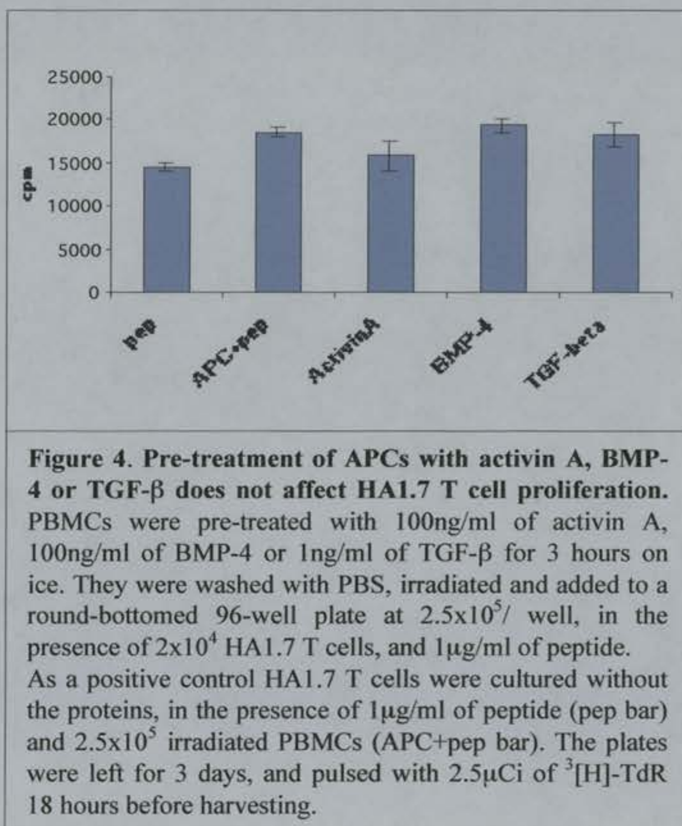


Figure 4. Pre-treatment of APCs with activin A, BMP-4 or TGF-β does not affect HA1.7 T cell proliferation. PBMCs were pre-treated with 100ng/ml of activin A, 100ng/ml of BMP-4 or 1ng/ml of TGF-β for 3 hours on ice. They were washed with PBS, irradiated and added to a round-bottomed 96-well plate at $2.5x10^5$/ well, in the presence of $2x10^4$ HA1.7 T cells, and 1μg/ml of peptide. As a positive control HA1.7 T cells were cultured without the proteins, in the presence of 1μg/ml of peptide (pep bar) and $2.5x10^5$ irradiated PBMCs (APC+pep bar). The plates were left for 3 days, and pulsed with 2.5μCi of ^{3}[H]-TdR 18 hours before harvesting.

Figure 4 reveals that pre-treating the APCs with any of the recombinant proteins did not affect the proliferation of the HA1.7 T cells, suggesting that these cytokines are not inducing changes in the APCs which could affect T cell growth.

The second experiment was set up to assess if the TGF-β-like molecules were acting directly on the T cells. This involved activating the HA1.7 T cells with plate-bound

anti-CD3 and soluble anti-CD28 antibodies, in the absence of signals delivered by APCs (Figure 5).

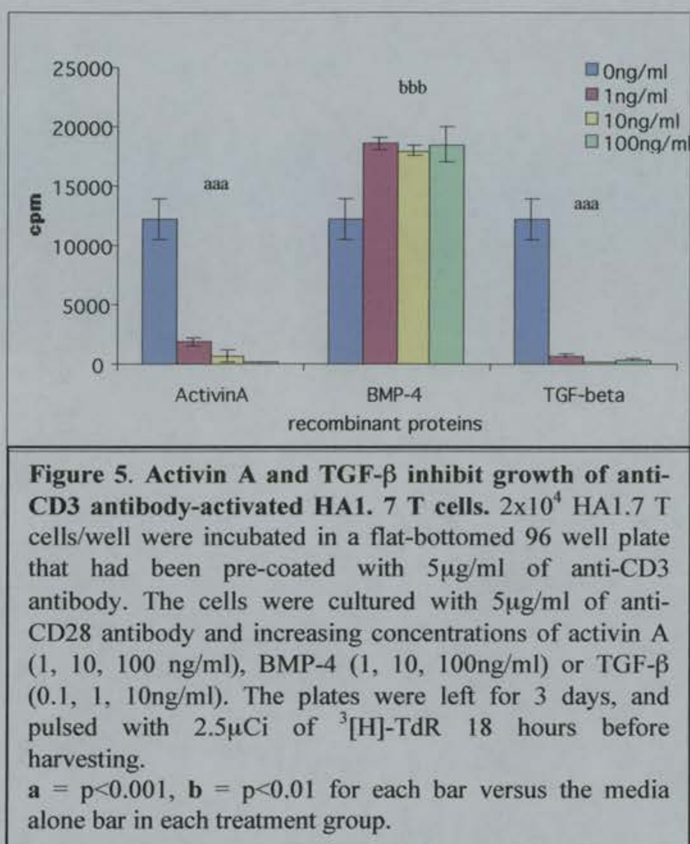


Figure 5. Activin A and TGF-β inhibit growth of anti-CD3 antibody-activated HA1. 7 T cells. $2x10^4$ HA1.7 T cells/well were incubated in a flat-bottomed 96 well plate that had been pre-coated with 5μg/ml of anti-CD3 antibody. The cells were cultured with 5μg/ml of anti-CD28 antibody and increasing concentrations of activin A (1, 10, 100 ng/ml), BMP-4 (1, 10, 100ng/ml) or TGF-β (0.1, 1, 10ng/ml). The plates were left for 3 days, and pulsed with 2.5μCi of ^{3}[H]-TdR 18 hours before harvesting.
a = $p<0.001$, **b** = $p<0.01$ for each bar versus the media alone bar in each treatment group.

Figure 5 reveals that activin A and TGF-β can markedly inhibit HA1.7 T cell proliferation in the absence of APC and peptide, indicating that these molecules must mediate their effects directly on the T cell, via recruitment of their type I and II receptors. BMP-4 has the opposite effect, rather, it increases HA1.7 T cell proliferation even at the lowest concentration.

In summary, the data revealed that activin A like TGF-β, inhibited HA1.7 T cell responses to specific antigen and to stimulation via antibody-mediated TCR triggering. Furthermore, this was associated with a decrease primarily in Th2-type cytokine production. If activin A and TGF-β were combined, their suppressive effect was additive. BMP-4 on the contrary, had no effect on HA1.7 T cell proliferation

when it was added on its own to antigen-stimulated cultures, and similarly it did not modify cytokine production. However, when it was added in combination with activin A or TGF-β to antigen-stimulated cells or on its own to anti-CD3-activated cells, it stimulated HA1.7 T cell growth above controls. The most likely explanation for the results observed is that all the molecules tested bind to different type I and type II receptors. However, only TGF-β and activin A signal through the same pathway and, therefore, have the same effect on T cell growth.

2.2. Effect of TGF-β-like Molecules on Murine $CD4^+$ T cells

2.2.1. Effect of TGF-β-like Molecules on Murine $CD4^+$ T Cell Proliferation

To determine if TGF-β-like molecules had the same effect on murine $CD4^+$ T cell proliferation as they had on the human HA1.7 $CD4^+$ T cell line, the cells were isolated by MACS from the spleens of 6-8 week old wild type (wt) BALB/c or C57BL/6 mice, and the purity checked by flow cytometry. Freshly isolated $CD4^+$ T cells were stimulated with increasing concentrations of plate-bound anti-CD3 antibody and 5μg/ml of soluble anti-CD28 antibody (Figure 6), and increasing concentrations of the recombinant proteins, to determine the optimum conditions of the assay. Consistent with the HA1.7 results (Figure 5), activin A and TGF-β could inhibit proliferation of BALB/c and C57BL/6 $CD4^+$ T cells. For BALB/c $CD4^+$ T cells, if the concentration of anti-CD3 antibody exceeded 1μg/ml, the inhibitory effects of activin A or TGF-β were masked. At 1μg/ml of anti-CD3 antibody, the effect of TGF-β was dose-dependent, whereas activin A was only effective at 100ng/ml, suggesting that TGF-β is a more potent inhibitory cytokine like in the HA1.7 T cell clone. Interestingly, anti-CD3 antibody used at 5 or 10μg/ml reversed the inhibitory effects of TGF-β, and instead the cells proliferated above controls. BMP-4 on the contrary, did not inhibit proliferation regardless of the concentration used.

For C57BL/6-derived $CD4^+$ T cells, the concentration of anti-CD3 antibody did not affect the inhibitory capacity of TGF-β and activin A, although at 1μg/ml both suppressed $CD4^+$ T cell proliferation in a dose-dependent manner. BMP-4 also had an inhibitory effect. The data reveal that the inhibitory capacity of TGF-β and activin A are as effective on human as on murine $CD4^+$ T cells and, furthermore, this is not mouse-strain related. However, in this study the BALB/c assays gave more consistent results than the C57BL/6 assays, therefore the work described here was carried out using BALB/c $CD4^+$ T cells.

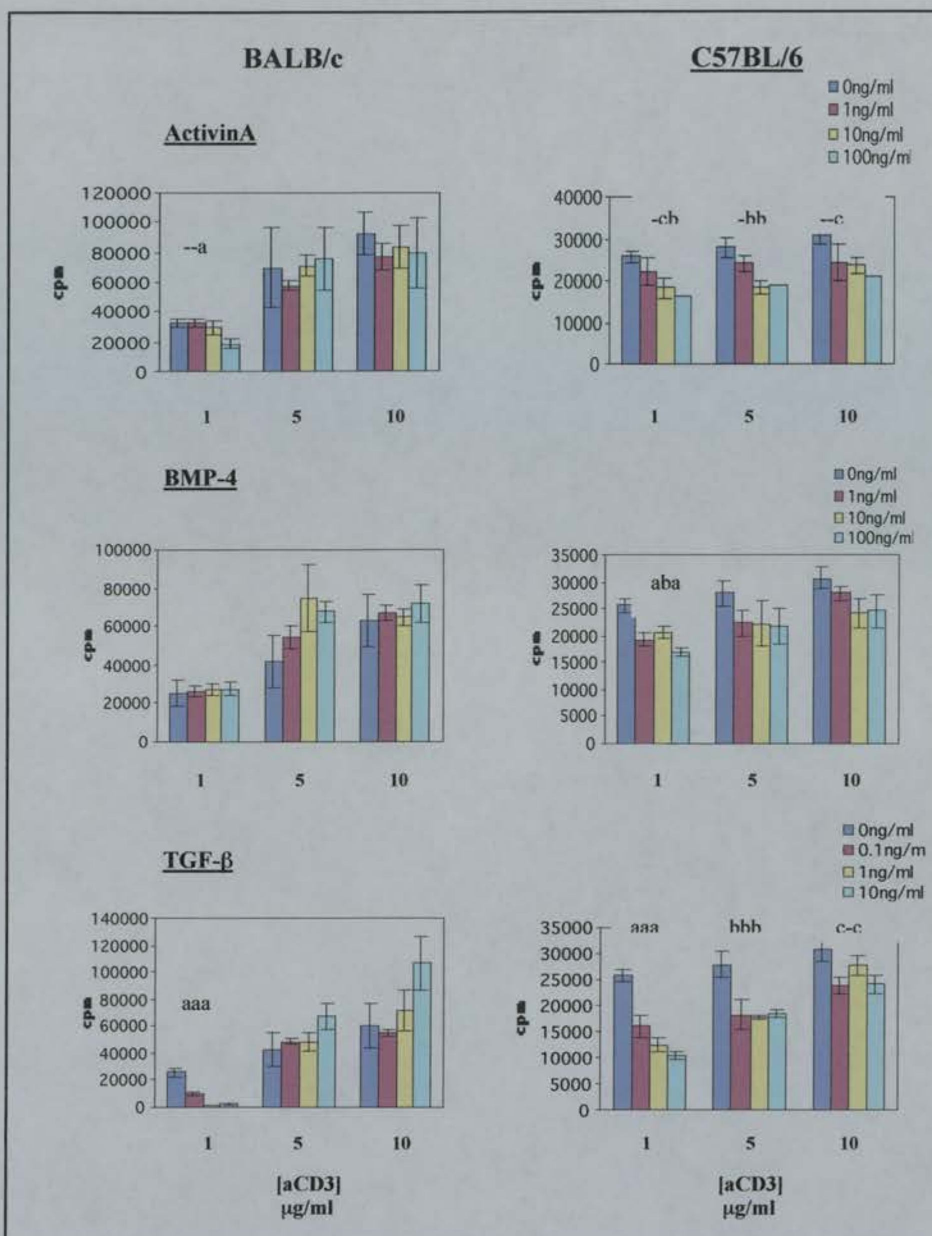


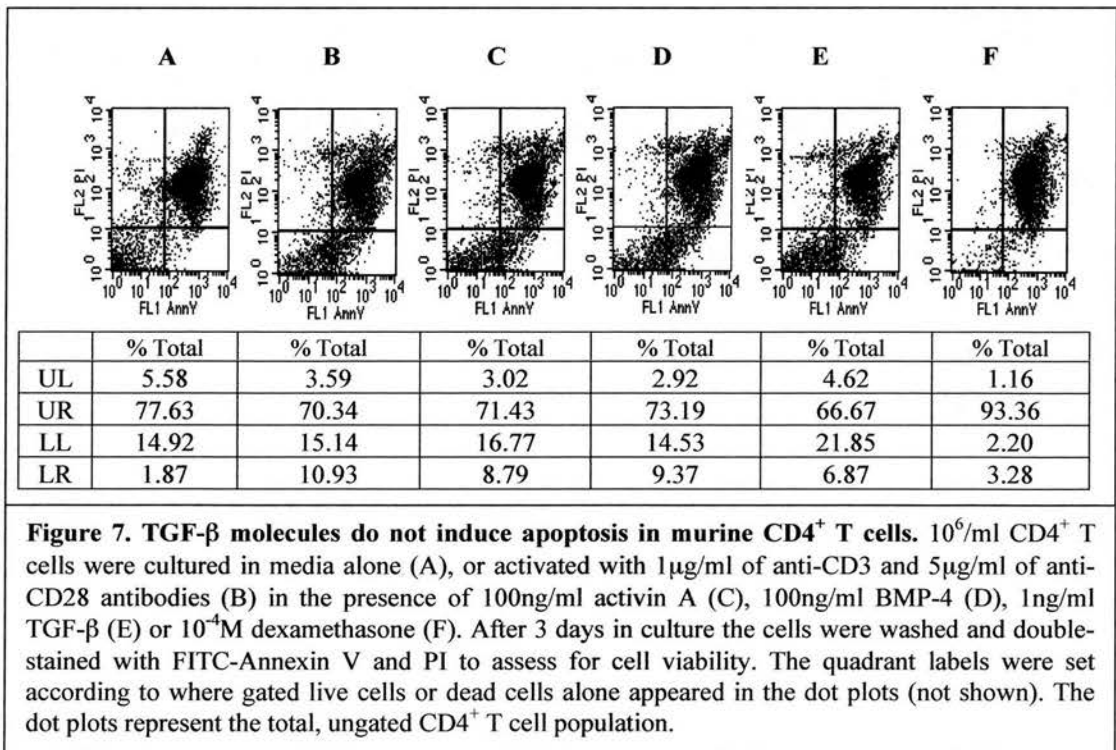
Figure 6. Activin A and TGF-β inhibit growth of anti-CD3/anti-CD28 antibody-activated murine CD4^{+} T cells. CD4^{+} T cells were isolated by magnetic beads from the spleens of wt BALB/c or C57BL/6 mice. 2.5×10^{5} cells/well were added to flat-bottomed 96 well-plates that had been pre-coated with varying concentrations of anti-CD3 antibody (1, 5, 10μg/ml). At each anti-CD3 antibody concentration, anti-CD28 antibody was added at 5μg/ml along with 1, 10 and 100ng/ml of activin A or BMP-4, or 0.1, 1 and 10ng/ml of TGF-β. The cells were cultured for 3 days, and pulsed for 18 hours with 2.5μCi of ^{3}[H]-TdR before harvesting.

- = not significant, **a** = $p<0.001$, **b** = <0.01, **c** = $p<0.05$ of a specific bar versus the media alone bar in its treatment group. Significant values are only plotted for responses that were inhibited.

2.2.2. Effect of TGF-β-like Molecules on BALB/c CD4$^+$ T Cell Viability

The optimum conditions for the BALB/c CD4$^+$ T cell proliferation assay were set up at 1μg/ml of anti-CD3 antibody and 5μg/ml of anti-CD28 antibody for stimulating the cells, and 100ng/ml of activin A, 1ng/ml of TGF-β and 100ng/ml of BMP-4 to determine their effects on T cell growth. It was important to determine if the suppressive effects of activin A and TGF-β were a consequence of apoptosis, therefore, the cultures were set up as described above. In addition, cells were also grown in the presence of dexamethasone as a positive control, as this reagent induces apoptosis in lymphocytes. Three days later the percentage of total live cells was assessed by FACS analysis with Annexin V and PI staining (Figure 7).

Annexin V binds to the membrane phospholipid phosphatidylserine (PS), which is translocated from the inner to the outer leaflet of the plasma membrane during the early stages of apoptosis. PI is permeable to the membrane of dead or damaged cells and, therefore, distinguishes viable from nonviable cells. Cells that stain positive for Annexin V and negative for PI are in the early stages of apoptosis. Cells that stain positive for both Annexin V and PI are either in the later stages of apoptosis, are undergoing necrosis, or are already dead. Cells that are negative for both Annexin V and PI staining are viable.



	% Total	% Total	% Total	% Total	% Total	% Total
UL	5.58	3.59	3.02	2.92	4.62	1.16
UR	77.63	70.34	71.43	73.19	66.67	93.36
LL	14.92	15.14	16.77	14.53	21.85	2.20
LR	1.87	10.93	8.79	9.37	6.87	3.28

Figure 7. TGF-β molecules do not induce apoptosis in murine CD4$^+$ T cells. 10^6/ml CD4$^+$ T cells were cultured in media alone (A), or activated with 1μg/ml of anti-CD3 and 5μg/ml of anti-CD28 antibodies (B) in the presence of 100ng/ml activin A (C), 100ng/ml BMP-4 (D), 1ng/ml TGF-β (E) or 10^{-4}M dexamethasone (F). After 3 days in culture the cells were washed and double-stained with FITC-Annexin V and PI to assess for cell viability. The quadrant labels were set according to where gated live cells or dead cells alone appeared in the dot plots (not shown). The dot plots represent the total, ungated CD4$^+$ T cell population.

In Figure 7, the UR quadrant staining positive for Annexin V and PI are late stage apoptotic cells, or necrotic cells. Cells in the LR quadrant are early apoptotic cells, staining positive for Annexin V but not for PI. Activated cells (dot plots B to E) have a higher percentage of early apoptotic cells than unstimulated cells (dot plot A), and this may represent the effects of activation-induced cell death. Cells in the LL quadrant represent the viable cells. Figure 7 and 8 show that the addition of activin A, BMP-4 or TGF-β to CD4^{+} T cells is not affecting their viability over a three-day period in culture, whereas treating cells with dexamethasone clearly induces cell death.

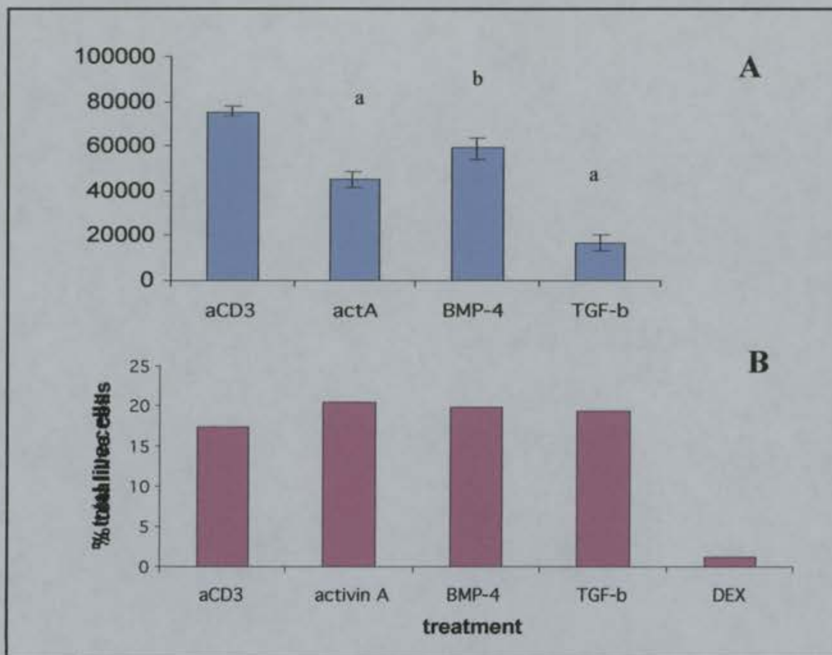


Figure 8. TGF-β and TGF-β-like molecules do not induce apoptosis in murine CD4^{+} T cells. *A*. 2.5x10^{5} CD4^{+} T cells/well were activated with 1μg/ml of anti-CD3 and 5μg/ml of anti-CD28 antibodies (aCD3) plus 100ng/ml of Activin A (ActA) 100ng/ml of BMP-4 (BMP-4) or 1ng/ml TGF-β (TGF-b). The cells were cultured for 3 days, and pulsed for 18 hours with 2.5μCi of ^{3}[H]-TdR before harvesting. ***B.*** 10^{6}/ml CD4^{+} T cells were stimulated as described for *A* including 10^{-4}M dexamethasone (DEX). The percentage of total live cells in each treatment was assessed by Annexin V and PI staining after 3 days in culture, and represents the mean from three different experiments.
a = p<0.001, **b** = p<0.01 for each bar versus the aCD3 bar.

2.2.3. Effect of TGF-β-like Molecules on BALB/c $CD4^+$ T Cell Cytokine Production

To determine if TGF-β and activin A had the same effect on $CD4^+$ T cell cytokine production as on the HA1.7 T cell line (Figure 2), cells were cultured as described earlier, and two days later the supernatants were collected to assess levels of IL-4, IL-10 and IFN-γ secretion. Unlike the data from the HA1.7 $CD4^+$ T cells (Figure 2), TGF-β or the TGF-β-like molecules had negligible effects on $CD4^+$ Th1 or Th2-type cytokine production (Figure 9).

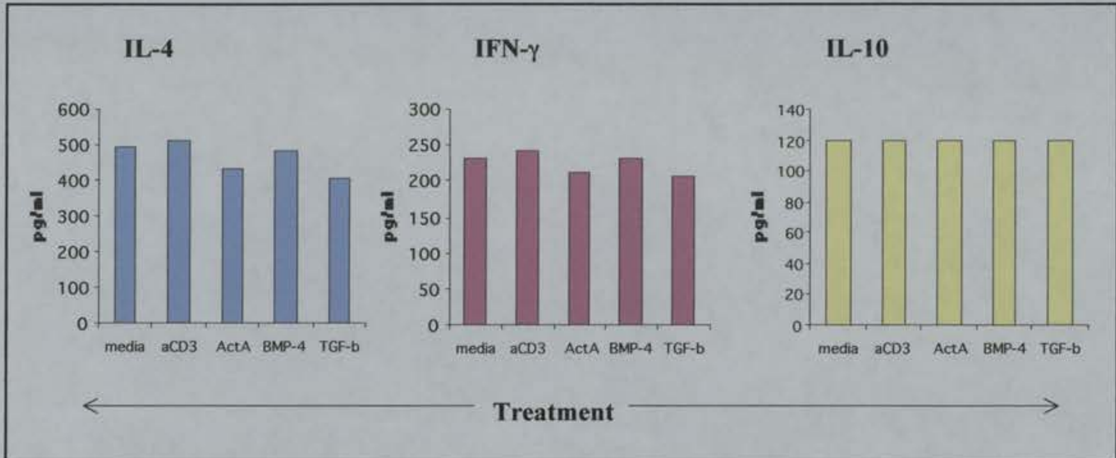


Figure 9. TGF-β and TGF-β-like molecules do not affect cytokine production of anti-CD3/anti-CD28 antibody-activated BALB/c $CD4^+$ T cells. 2.5×10^5 BALB/c $CD4^+$ T cells/well were cultured in media only (media bar), or with 1μg/ml of anti-CD3 and 5μg/ml of anti-CD28 antibodies (aCD3 bar) plus 100ng/ml of activin A (ActA bar), 100ng/ml BMP-4 (BMP-4 bar) or 1ng/ml of TGF-β (TGF-b bar). The supernatants were collected after 2 days. The ELISA plates were coated with IL-4, IL-10 or IFN-γ at 1μg/ml and the supernatants added. Recombinant mouse IL-4 at 20ng/ml, IL-10 at 30ng/ml and IFN-γ at 40ng/ml were used as the standards by double dilutions. The biotinylated detection antibody was used at 1μg/ml.

2.2.4. TGF-β and activin A Induce CD4⁺ T Cell Unresponsiveness

Previous reports have demonstrated that IL-10 and TGF-β can induce long-lasting unresponsiveness in $CD4^+$ T cells. The first study on IL-10 was carried out by Groux *et al* (213) and they found that human $CD4^+$ T cells activated by anti-CD3 antibody in the presence of IL-10 for 10 days, remained unresponsive to re-stimulation with anti-CD3 antibody and IL-2, or anti-CD3 and anti-CD28 antibodies. Similarly, Ludviksson *et al* (233) showed that OVA-TCR $CD4^+$ T cells stimulated with APC and OVA peptide in the presence of TGF-β, remained unresponsive to subsequent re-stimulation with anti-CD3 and anti-CD28 antibodies, or exogenous IL-2. These observations prompted to investigate if activin A and TGF-β were also able to induce long-lasting unresponsiveness in anti-CD3-antibody-activated $CD4^+$ T cells (Figure 10).

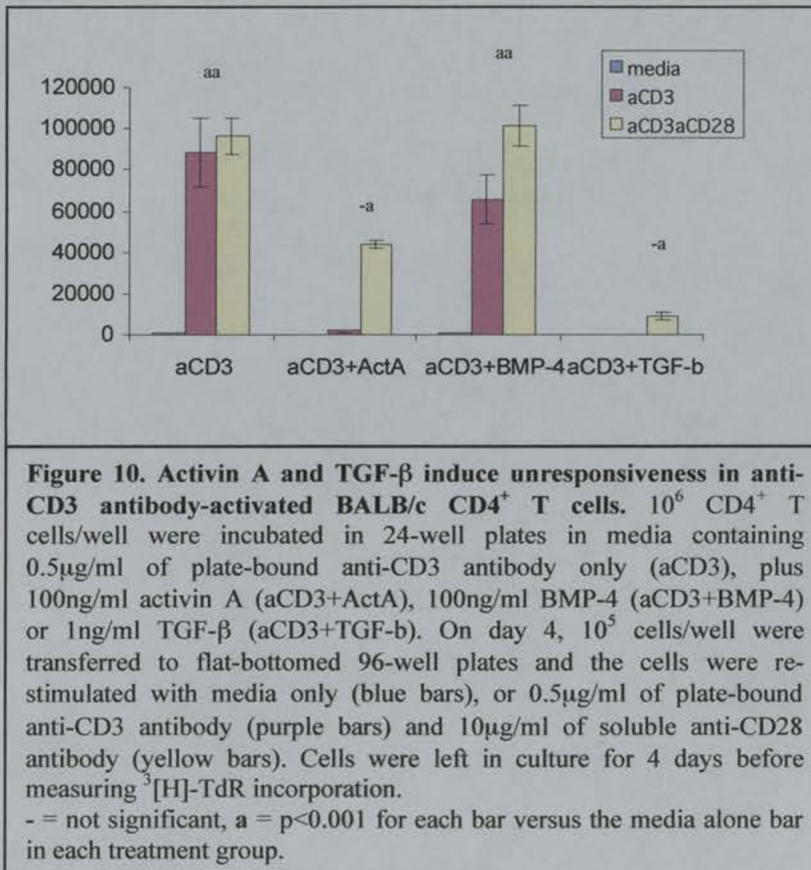
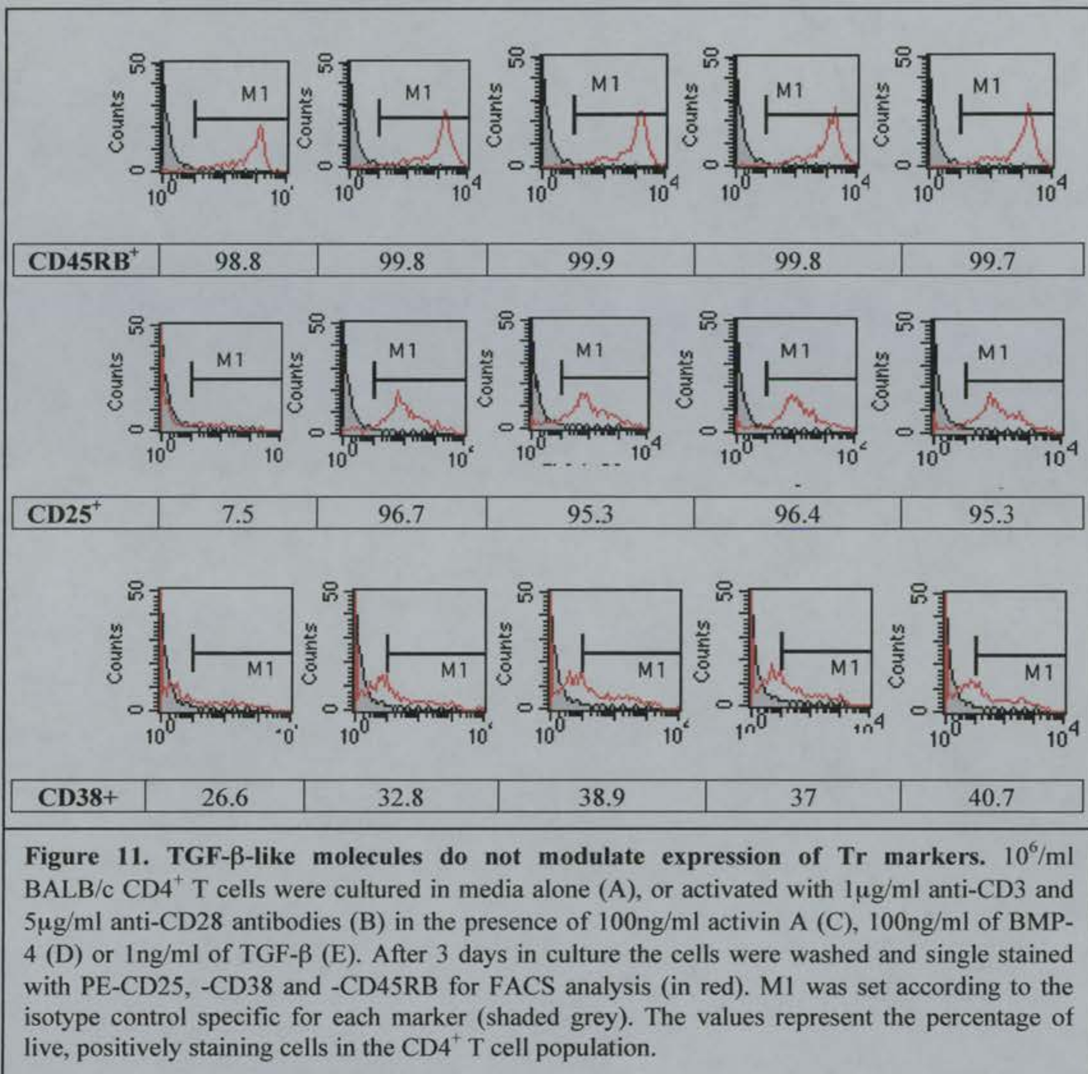


Figure 10. Activin A and TGF-β induce unresponsiveness in anti-CD3 antibody-activated BALB/c $CD4^+$ T cells. 10^6 $CD4^+$ T cells/well were incubated in 24-well plates in media containing 0.5μg/ml of plate-bound anti-CD3 antibody only (aCD3), plus 100ng/ml activin A (aCD3+ActA), 100ng/ml BMP-4 (aCD3+BMP-4) or 1ng/ml TGF-β (aCD3+TGF-b). On day 4, 10^5 cells/well were transferred to flat-bottomed 96-well plates and the cells were re-stimulated with media only (blue bars), or 0.5μg/ml of plate-bound anti-CD3 antibody (purple bars) and 10μg/ml of soluble anti-CD28 antibody (yellow bars). Cells were left in culture for 4 days before measuring ^{3}[H]-TdR incorporation.
- = not significant, **a** = $p<0.001$ for each bar versus the media alone bar in each treatment group.

Figure 10 shows that $CD4^+$ T cells treated with activin A and TGF-β failed to proliferate in response to re-stimulation with anti-CD3 antibody. In the presence of anti-CD3 and anti-CD28 antibodies the inhibition was partly reversed in activin-A treated cells and to a lesser extent in TGF-β-treated cells, although the cells did not proliferate as vigorously as activated cells grown in media only or in the presence of BMP-4. Therefore, TGF-β and activin A can induce unresponsiveness in $CD4^+$ T cells to further stimulation via the TCR, in a similar manner as previously reported by Groux *et al* (213).

2.2.5. Phenotypic Analysis of $CD4^+$ T Cells After Treatment with TGF-β-like Molecules

$CD4^+CD25^+$, $CD4^+CD45RB^{low}$ cells and $CD4^+CD38^+$ T cells have all been described as Tr cells (20, 22, 25, 29). From the findings that activin A and TGF-β induce $CD4^+$ T cell unresponsiveness, I was prompted to investigate if this effect was the result of TGF-β signalling directing cells into a Tr pathway. Therefore, I cultured $CD4^+$ T cells as previously described, and after 3 days the cells were stained for surface expression of CD25, CD38 and CD45RB, and analysed by FACS (Figure 11).



CD45RB^{+}	98.8	99.8	99.9	99.8	99.7
CD25^{+}	7.5	96.7	95.3	96.4	95.3
CD38+	26.6	32.8	38.9	37	40.7

Figure 11. TGF-β-like molecules do not modulate expression of Tr markers. 10^6/ml BALB/c CD4^{+} T cells were cultured in media alone (A), or activated with 1μg/ml anti-CD3 and 5μg/ml anti-CD28 antibodies (B) in the presence of 100ng/ml activin A (C), 100ng/ml of BMP-4 (D) or 1ng/ml of TGF-β (E). After 3 days in culture the cells were washed and single stained with PE-CD25, -CD38 and -CD45RB for FACS analysis (in red). M1 was set according to the isotype control specific for each marker (shaded grey). The values represent the percentage of live, positively staining cells in the CD4^{+} T cell population.

The high affinity IL-2 receptor (IL-2R) consists of 2 polypeptide chains, each containing IL-2 binding sites. One is constitutively expressed, IL-2Rβ chain and the other is induced upon T cell activation, IL-2Rα chain or CD25 (236). The FACS data (Figure 11) show that upon activation, CD4^{+} T cells up-regulate CD25 as would be expected. However, treatment with any of the proteins does not modulate CD25 expression, suggesting that inhibition of CD4^{+} T cell proliferation is not the result of down-regulated IL-2R making cells less responsive to IL-2. Our results are similar to what other groups have reported. Ahujas *et al* (226) found that pre-treating human T cells with TGF-β prior to activation did not affect their IL-2Rα chain expression.

However, they did observe that TGF-β could inhibit phosphorylation of the IL-2Rβ chain, and thus, of the IL-2 signalling pathway. In a more recent report Sudarshan *et al* (237) found that TGF-β did not inhibit IL-2-induced phosphorylation of Jak-1 and Stat-5 in activated human T cells, and they proposed that inhibition of IL-2-induced activities by TGF-β may result from IL-2 signalling-independent events.

Activation of $CD4^+$ T cells also led to a slight up-regulation of CD38 compared to resting T cells, which is consistent with the role it is thought to play in lymphocyte activation (238). Adding TGF-β, activin A or BMP-4 slightly up-regulated expression of this marker but to the same extent in all three treatments, and no changes were observed in the expression of CD45RB. Therefore, activating $CD4^+$ T cells *in vitro* and adding activin A or TGF-β led to a state of T cell unresponsiveness, which was not affecting expression of Tr cell surface markers, only to the extent that may be expected following $CD4^+$ T cell activation.

2.2.6. Effect of TGF-β and TGF-β-like Molecules on Notch Signalling Genes

I have focused my interest on the role that highly conserved molecules, such as Notch and its ligands may be playing in specifying Tr cells during the establishment of peripheral tolerance. In addition, it is well-known that TGF-β is required for Tr cell function (65), and the data provided in this study show that TGF-β and the TGF-β-like molecule, activin A, can induce $CD4^+$ T cell unresponsiveness. Therefore, I wanted to investigate if the Notch signalling genes were differentially regulated by treatment of $CD4^+$ T cells *in vitro* with TGF-β and the TGF-β-like molecules, activin A and BMP-4. For this, $CD4^+$ T cells were cultured in the presence of either molecule and after 3 days in culture, the cells were washed and prepared for extraction of total RNA and real-time PCR analysis (Figure 12).

The cells in Figure 12 were stimulated with 0.5 μg of anti-CD3 only like in Figure 10, as at this concentration the cells still retained their proliferative capacity if re-stimulated with anti-CD3, but remained unresponsive if they had been pre-treated with activin A or TGF-β.

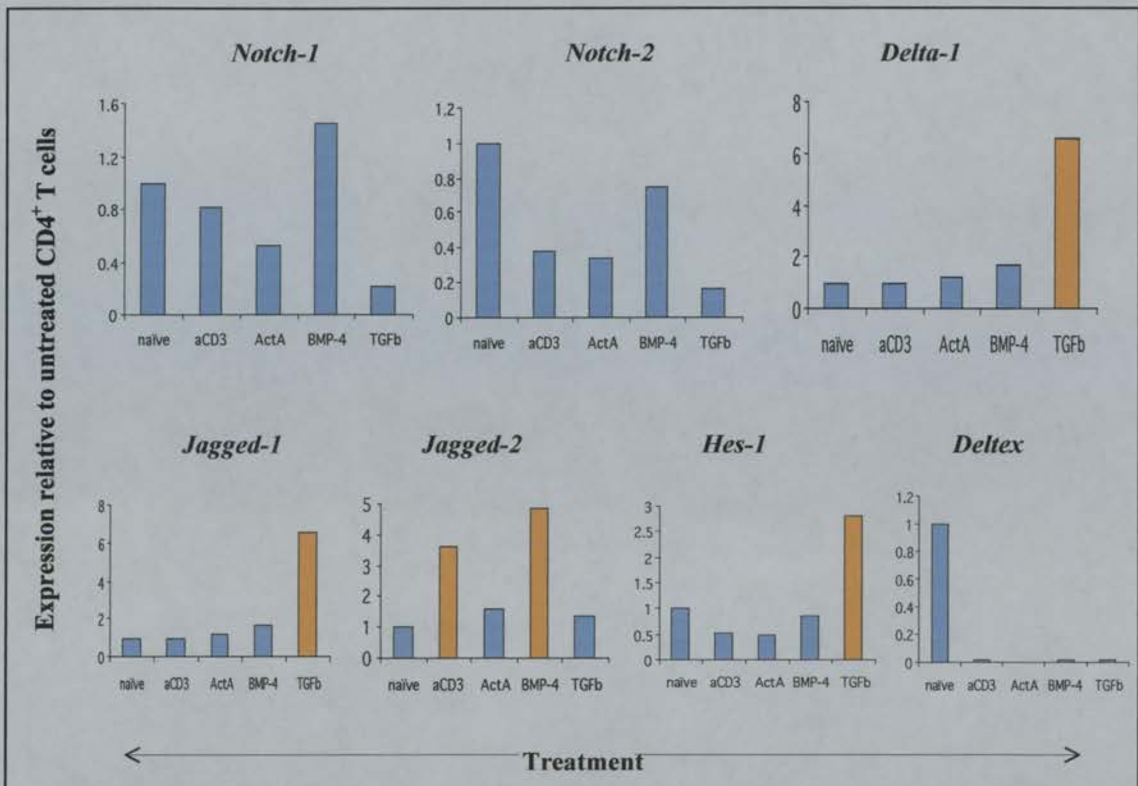


Figure 12. Differential regulation of Notch and its related genes in anti-CD3 antibody-activated BALB/c CD4⁺ T cells. $2x10^6$ BALB/c $CD4^+$ T cells/well were cultured in media only (naive bar), or with 0.5μg/ml of anti-CD3 (aCD3 bar) plus 100ng/ml of activin A (ActA bar), 100ng/ml BMP-4 (BMP-4 bar) or 1ng/ml of TGF-β (TGFb bar). Three days later the cells were processed for total RNA extraction and reverse transcribed for cDNA synthesis. The samples were tested for gene expression (*Notch-1* and *2*, *Jagged-1* and *2*, *Delta-1*, *Hes-1* and *Deltex*) by real-time PCR. For each gene, the samples were run with a positive control, which was the untreated (naïve) population to which all the values are relative, and with a negative control (no cDNA) to ensure there was no genomic DNA contamination. Furthermore an internal control, 18srRNA, was included per sample as a monitor of the PCR reaction and of the amount of cDNA used. A 2.5-fold increase above the positive control is regarded as significant (in orange).

The real-time PCR data show that the Notch receptors, *Notch-1* and *-2* were not modulated significantly with any of the treatments. Activating the cells with anti-CD3 led to a marked drop in the expression of *Deltex*, which was unaffected by addition of TGF-β, activin A or BMP-4. Interestingly, only in the presence of TGF-β there was a significant up-regulation of *Delta-1*, *Jagged-1* (about seven-fold), and *Hes-1* (2.5-fold) relative to untreated or anti-CD3-activated cells by 72 hours. In contrast, activating the cells with anti-CD3 led to an up regulation of *Jagged-2* (3.5-fold), which was not modulated by BMP-4, but was down regulated in the presence of activin A and TGF-β.

Therefore, although activin A and TGF-β were both capable of inducing $CD4^+$ T cell unresponsiveness and both signal through the same intracellular mediators, they displayed differential regulation of Notch ligand *(Jagged-1* and *Delta-1*) and *Hes-1* expression. BMP-4, which did not affect $CD4^+$ T proliferation, did not modulate the expression of the genes studied to any significant extent compared to untreated cells, or anti-CD3 activated cells.

The data presented in Figure 12 can be compared to the data from $CD4^+$ T cells stimulated with 1μg of anti-CD3 and 5μg of anti-CD28 (Chapter III, Figure 6).
It is apparent that the Notch receptors are not greatly modulated by activating the $CD4^+$ T cells via the TCR alone (anti-CD3), or in the presence of co-stimulation (anti-CD28). *Delta-1* is significantly up regulated with anti-CD3 and anti-CD28 antibodies by 24 hours (about 50-fold) and transcripts are still high after 48 hours of activation (about 10-fold). Although treatment with anti-CD3 did not seem to affect *Delta-1* expression after 72 hours, this could reflect differences in kinetics.
Levels of *Jagged-1* in the anti-CD3 and anti-CD28-activated $CD4^+$ T cells drop to about 5-fold with respect to untreated cells by 48 hours, suggesting that by 72 hours levels may be closer to those in Figure 12. Similarly, *Jagged-2* also shows similar kinetics of expression in anti-CD3 and anti-CD3/anti-CD28 activated $CD4^+$ T cells.
As far as the intracellular mediators are concerned, *Deltex* is down-modulated irrespective of the mode of activation, and *Hes-1* in anti-CD3/anti-CD28-activated $CD4^+$ T cells is down-modulated to levels in untreated cells after 48 hours, identical to 72 hours of activation in anti-CD3 alone. Therefore, from these observations it emerges that different modes and length of activation may tightly regulate the kinetics and levels of expression of Notch signalling genes.

3. Discussion

In this study I have compared the effects of the TGF-β-like molecules, activin A and BMP-4, on CD4$^+$ T cell responses *in vitro*, with respect to TGF-β, which is a well known inhibitory cytokine of T cells (224, 225, 226). The RT-PCR and real-time data from Chapter III had revealed that BMP-4 was not expressed in lymphoid tissues or lymphocyte populations of untreated mice. However, Activin A and TGF-β, were both highly expressed. The availability of activin A and BMP-4 allowed us to study their effects on a human T cell clone, and on murine splenic CD4$^+$ T cells *in vitro*, as this had not been investigated before.

Interestingly, activin A and TGF-β inhibited human and murine T cells activated via the TCR, and their effects were additive when they were added in combination. These data agrees with other reports on the inhibitory actions of activin A and TGF-β (134, 229, 230). BMP-4, on the contrary, did not affect T cell proliferation. These results suggest that TGF-β/activin A and BMP-4 mediate their effects differently. Indeed, activin A and TGF-β signal through different intracellular mediators compared to BMP-4. The latter binds to type I and II receptors that activate intracellular Smads 1, 5 and 8, whereas activin A and TGF-β activate Smads 2 and 3. Furthermore, the CD4$^+$ T cells that had been activated in the presence of activin A or TGF-β remained unresponsive to further re-stimulation with anti-CD3 and this was only partly reversed by anti-CD3/anti-CD28 antibodies. However, there was no difference in the expression of various cell surface markers (CD25, CD38, CD45RB) when the cells were cultured in the presence of activin A or TGF-β. Therefore, the inhibitory effects exerted by TGF-β and activin A did not lead to modulation of cell surface markers that are normally expressed by Tr cells. Nevertheless, it would be interesting to characterise this population further. Other necessary experiments involve re-stimulating the cells with IL-2 or mitogens (con A and PMA/ionomycin), to study if the cells regain their proliferative capacity.

Our belief that the TGF-β and Notch signalling pathways may co-operate in the induction of Tr cells, prompted to investigate the effect of activin A, BMP-4 or TGF-β-treated CD4$^+$ T cells in the expression of Notch signalling genes. BMP-4 did not modulate expression of any of the genes studied compared to untreated cells or anti-

CD3 activated $CD4^+$ T cells. Surprisingly, activin A and TGF-β did not affect gene expression in the same way. TGF-β induced a significant up-regulation of the Notch ligands, *Jagged-1* and *Delta-1*, and the transcription factor *Hes-1*, which was not found in the activin A-treated cells. Interestingly, however, both activin A and TGF-β down-regulated the expression of *Jagged-2* induced upon anti-CD3 activation. Therefore, the similar inhibitory effects observed *in vitro* did not always correlate to gene expression profiles of the Notch signalling genes tested, suggesting these molecules might signal through the same intracellular mediators, but activate different target genes.

Although it is not known how TGF-β may modulate expression of these genes, this the first time an association is made between both signalling pathways, and this finding may be of biological relevance in the role TGF-β plays as an immunosuppressive cytokine.

Conclusions

1. The role of the Notch pathway in the adult immune system

The mucosal immune system can discriminate between pathogenic microorganisms and innocuous matter, such as dietary antigens, inhaled particles or commensal microorganisms (1, 2, 3) by promoting specific immunological unresponsiveness. This form of tolerance is referred to as mucosal tolerance and some mechanisms that have been associated with it include clonal deletion, anergy or regulatory T cells (Tr cells). Breakdown of tolerance and the recognition of pathogenic substances leads to the differentiation of $CD4^+$ T helper cells, which induce productive immunity.
These opposing outcomes of the immune response are controlled by many factors, which include the nature of the antigen (pathogenic or harmless), its route of entry, the microenvironment at the site of antigen presentation (including cytokines, APCs, and co-stimulatory molecules), as well as signals derived from the innate immune system. In addition, I propose that the tightly regulated genetic pathway, the Notch signalling pathway may also contribute to whether the immune system responds to antigen or becomes unresponsive. This statement is supported by our observations that DCs which over-express the Notch ligand, Jagged-1, are capable of inducing a population of Tr cells which can transfer tolerance (99).

Over the last 5 years intense research has focused on elucidating the role of Notch signalling during thymocyte development. Interestingly, the Notch receptors and ligands are expressed in thymocyte subsets, as well as in thymic stroma (73, 180), and numerous reports have reported on the contribution of Notch signalling in T cell differentiation and lineage commitment (74, 75, 81, 83).
The involvement of Notch signalling in cell fate decisions during development raises the question of whether or not the pathway may also be involved in decision making processes in the adult, such as its participation in determining the outcome of peripheral immune responses.
In the work presented in this thesis I have investigated if components of the Notch signalling pathway are differentially regulated during $CD4^+$ T cell responses *in vitro*

and *in vivo*, and if this may be associated to the functional differentiation of helper or regulatory $CD4^+$ T cells.

The experiments conducted in this thesis have demonstrated that

1. Components of the Notch pathway are differentially expressed in adult lymphoid tissues (spleen and lymph nodes) and cells (splenic $CD4^+$ T cells, $CD8^+$ T cells, B cells and enriched DCs).
2. Activating $CD4^+$ T cells and B cells *in vitro* with anti-CD3/anti-CD28 antibodies and LPS, respectively, up-regulate significantly expression of *Jagged-2* and *Delta-1*.
3. Activating $CD4^+$ T cells *in vivo* by intranasal administration of HDM peptide under conditions that induce T helper cells does not modulate expression of components of the Notch pathway. However, under conditions that induce regulatory T cells there is a significant up-regulation of *Jagged-1*.
4. It is difficult to rely on a polyclonal $CD4^+$ TCR murine model for recognition of a HDM peptide to define the cell surface phenotype of $CD4^+$ T cells under conditions that induce productive immunity or tolerance via the respiratory mucosa.

1. Components of the Notch pathway are differentially expressed in adult lymphoid tissues (spleen and lymph nodes) and cells (splenic $CD4^+$ T cells, $CD8^+$ T cells, B cells and enriched DCs).

Initially expression of genes of the Notch pathway was measured by RT-PCR, as there are only very limited availability of antibody reagents. I noted that the Notch receptors, *Notch-1*, *-2* and *-4*, and the Notch ligands, *Jagged-1*, *-2* and *Delta-1*, were differentially expressed in all the different lymphoid tissues (spleen, thymus and LNs) and cells (lymphocytes and DCs) studied (Chapter III, Figures 2, 3 and 5).
Activation of the pathway is associated with an up-regulation of two signalling components, which operate independently of each other. One is the transcriptional repressor, Hes-1 (32), and the other is Deltex (85), a protein which interacts with the intracellular domain of the Notch receptor, and positively regulates Notch activity

(204). I observed that *Hes-1* is expressed in the different tissues and cell populations of the immune system, suggesting that the pathway may contribute to the control of peripheral immunity. Deltex was not analysed in this study.

2. **Activating CD4$^+$ T cells and B cells *in vitro* with anti-CD3/anti-CD28 antibodies and LPS, respectively, up-regulate significantly expression of *Jagged-2* and *Delta-1*.**

In order to obtain a more quantitative measure of gene expression I opted to use real-time PCR (Section 3.5). Regulation of Notch signalling genes during the activation of B cells and CD4$^+$ T cells *in vitro* was determined. B cells were activated with LPS, and CD4$^+$ T cells by ligation of the TCR using anti-CD3 and anti-CD28 antibodies. The data (Chapter III, Figures 6 and 7) showed that only *Notch-1* was significantly up regulated in B cells (more than 15-fold) throughout the 72 hour period of activation. However, *Jagged-2* and *Delta-1* were significantly up regulated in both populations after 24 hours of activation. An interesting finding was that *Hes-1* was only increased in CD4$^+$ T cells but not B cells, and *Deltex* in B cells but not CD4$^+$ T cells. This could result from the intracellular signalling events specific to B cells or CD4$^+$ T cells after LPS or antibody-mediated activation, respectively.
The marked up-regulation of some of these genes after 24 hours (50-fold for *Delta-1* and 20-fold for *Jagged-1* in CD4$^+$ T cells) suggests that these genes may have earlier kinetics which show a more gradual induction, but their expression was only measured in this study after 24 hours.
Based on these observations I suggest that activation of lymphocytes affects a common downstream signalling pathway that results in transcriptional activation of the Notch ligand genes, *Jagged-2* and *Delta-1*. Indeed one possible candidate is NF-κB, which is activated in B cells after mitogenic stimulation (196) and in T cells after anti CD3/anti-CD28 activation (197, 198). Evidence for this has been provided by the studies of Bash *et al* (191) who used the CCR43 cell line, which conditionally expresses the NF-κB subunit, c-Rel, under the control of a tetracycline-regulated transactivator. c-Rel expression in these cells led to induction of *Jagged-1* transcripts. Similarly, they used PMA plus ionomycin to activate endogenous NF-κB factors in human Jurkat T cells. They found that under these conditions, there was a

4-fold increase in *Jagged-1* mRNA levels compared to unstimulated cells. In addition, a more recent study (239) has demonstrated that B cells from Notch-1 deficient mice had a reduced capacity to proliferate in response to LPS, supporting an interaction between NF-κB and Notch signalling.

The biological significance of the cross talk between the NF-κB and Notch signalling pathways remain to be fully elucidated. There are several conflicting reports about possible interactions between Notch and NF-κB. One group demonstrated an ability of Notch-1 to mimic IκB and to sequester NF-κB dimers, thus preventing NF-κB-dependent activation of transcription (199). More recently (240) this inhibitory interaction has been shown to require a domain of Notch-1 in the intracellular portion that interacts directly with the p50 subunit of NF-κB, preventing it from binding to its DNA binding site and initiating transcription of specific genes. In contrast, a different group reported that constitutive Notch-1 mediated stimulation of the p52 promoter (200), and Bellavia *et al* (98) reported that Notch-3 induced phosphorylation and degradation of IκB, which in turn resulted in nuclear translocation of NF-κB dimers and activation of transcription. A more recent study has demostrated that Notch-1 deficient hematopoeitic progenitor cells have reduced NF-κB activity (239).

Despite the discrepancies, the published observations have demonstrated a physical interaction between Notch and NF-κB components, and this may be important in the regulation of cellular differentiation, activation, proliferation and survival.

3. Activating $CD4^+$ T cells *in vivo* by intranasal administration of HDM peptide under conditions that induce T helper cells does not modulate expression of components of the Notch pathway. However, under conditions that induce regulatory T cells there is a significant up-regulation of *Jagged-1*.

I also investigated the regulation of components of the Notch pathway in $CD4^+$ T cells during *in vivo* immune responses. I used a murine model of responsiveness to the HDM peptide 110-130 in which productive immunity or tolerance can be generated through the induction of $CD4^+$ T helper cells and regulatory $CD4^+$ T cells, respectively. With this model I was able to investigate if the functional outcome of

$CD4^+$ T cells after antigen encounter was associated with a differential regulation of Notch signalling genes.

In the initial experiments, the mice were primed and tolerised by different routes of antigen administration, which restricts the interpretation of the gene analysis studies. Therefore, the experiments were extended using the intranasal route for antigen delivery. To induce tolerance the mice were given 100μg of p110-130 on three consecutive days, following the protocol established by Hoyne *et al* (11).
The protocol for intranasal priming relied on the use of the mucosal adjuvant *E.coli* heat labile enterotoxin (LT). The concentration of LT required modifications to what other studies had reported. Initially I used LT at a concentration of 5μg, which is within the range of what other groups have used as a mucosal adjuvant (208, 209, 210). However, I found that this concentration on its own was sufficient to prime mice to the HDM, which is presumably present in the Animal House (Chapter IV, Figure 1). In control experiments, I established the concentration of LT required to prime mice in the presence of p110-130 but not on its own (Chapter IV, Figures 2 and 3) namely priming with 100μg of p110-130 in the presence of 0.03μg of LT.
Thus, mice were primed or tolerised to p110-130 and the $CD4^+$ T cells were isolated at 2, 4 or 7 days after the last peptide treatment to study expression of Notch signalling genes.

The kinetics of the response in priming and tolerance induction differed. In the presence of LT, the $CD4^+$ T cells responded to peptide re-stimulation *in vitro* at day 7 after peptide treatment (Chapter IV, Figure 4). On the contrary, $CD4^+$ T cells from tolerised mice responded to peptide re-stimulation by day 2, which peaked at day 4, before becoming unresponsive on day 7 (Chapter IV, Figure 5). This difference may question the interpretation of the real-time data if it is argued that the difference in kinetics of $CD4^+$ T cell activation may also reflect differences in gene expression. However, given that the experiments were adequately controlled and that the route of antigen administration was kept constant, the $CD4^+$ T cells from both experimental groups were not compared to each other in terms of kinetics. Rather, they were compared to $CD4^+$ T cells from untreated mice. Therefore, the gene expression of

untreated $CD4^+$ T cells was taken as the baseline, to which the expression of genes in the other samples was related.

The real-time PCR data revealed that the Notch receptors (*Notch-1* and *-2*) and ligands (*Jagged-1* and*-2*, and *Delta-1*) were not modulated over the time points investigated in $CD4^+$ T cells under conditions that primed the mice (Chapter IV, Figure 7). In contrast, tolerising mice to the peptide resulted in a significant increase in *Jagged-1* at day 4 after the last peptide treatment, and to a lesser extent, in *Delta-1* (Chapter IV, Figure 9). The observation that up-regulation of *Jagged-1* was not observed in priming suggests that it is not a consequence of $CD4^+$ T cell activation after antigen presentation, but rather it may be a feature unique to the induction of peptide tolerance. Furthermore, an interesting observation was that the up regulation of *Jagged-1* occurred at a time prior to the cells becoming unresponsive, when the decision is made to become Tr cells.
The increase in ligand expression was not accompanied by an increase in *Hes-1* or *Deltex* up regulation in the peptide treated group compared to the saline alone group (Chapter IV, Figure 10). These two signalling mediators have been associated with Notch activity, and therefore, the finding that they were not significantly modulated under conditions that induce tolerance would suggest that Notch signalling has not occurred. Therefore, it may be more plausible to speculate that the up regulation of *Jagged-1* in $CD4^+$ T cells during tolerance induction may result from signals derived from the APCs and the site of antigen presentation under conditions that favour tolerance induction.

4. **It is difficult to rely on a polyclonal $CD4^+$ TCR murine model for recognition of a HDM peptide to define the cell surface phenotype of $CD4^+$ T cells under conditions that induce productive immunity or tolerance via the respiratory mucosa.**

The relevance of Tr cells in controlling immune responses both to self and non-pathogenic foreign antigens highlights their importance as a potential candidate for immunotherapy, in individuals where tolerance breakdown leads to disease progression. The attention focused in this thesis in the generation of Tr cells, led to

an attempt to characterise the Tr cells generated in the model of intranasal peptide tolerance discussed previously.

Peripheral lymphoid compartments contain a population of $CD4^+CD25^+$ T cells that are unresponsive to numerous stimuli *in vitro* (22), and can suppress the autoreactive potential of $CD4^+CD25^-$ T cells *in vitro* and *in vivo* (19, 20, 21, 22). In addition $CD25^+$ T cells have been found to be predominantly $CD45RB^{low}$ (26, 27), and to express constitutive CTLA-4 at higher levels than their $CD25^-$ counterparts (28), similar to the data I have presented. Recent studies have also attributed a role to the $CD25^+$ population in the induction of oral tolerance in OVA TCR transgenic mice (220, 221).

Therefore, I investigated if $CD4^+$ T cells in mice that were primed or tolerised intranasally to HDM p110-130 expressed the same surface phenotype reported for Tr cells. Splenocytes were isolated from mice that had been primed or tolerised at days 2, 4 or 7 after the last peptide treatment, and stained with antibodies to CD4 and CD25, to gate on $CD4^+CD25^{+/-}$ populations, and with a third marker, being CD38, CD45RB, CTLA-4 or CD69. CD38 has also been associated with Tr cells (29) and CD69 is an early activation marker.

Characterisation of these cells was hampered by the low frequency of antigen-reactive cells (about 1 in10^5) and therefore, it is very difficult to detect changes in the phenotype of these cells. The flow cytometry data revealed that intranasal administration of the mucosal adjuvant LT on its own led to an increase in the numbers of $CD4^+CD25^+$ T cells (10%) compared to untreated $CD4^+$ T cells (7%) (Chapter IV, Table 2). This increase also corresponded to a rise in the numbers of $CD25^+CD38^+$, $CD69^+$ and $CD45RB^{low}$ cells (Chapter IV, Table 3). However, there were no differences between the control group and the primed group that received LT and peptide, which suggests that the increase was a feature of the activating properties of the adjuvant on the $CD4^+$ T cells, rather than of the antigen-driven response. Indeed Nashar *et al* (219) found that mice injected intraperitoneally with 30μg of LT in CFA caused an increase in the numbers of $CD4^+CD25^+$ T cells in the mesenteric lymph nodes compared to mice that received the same dose of a mutated form of LT.

$CD4^+$ T cells from mice that received saline alone also had an increased number of $CD25^+$ T cells (9%) compared to $CD25^+$ T cells from untreated mice (7%), which corresponded to an increase in the numbers of $CD25^+CD38^+$, $CD45RB^{low}$, $CD69^+$ cells (Chapter IV, Tables 2 and 5). Similar increases were also observed in $CD4^+$ T cells from mice that were tolerised with p110-130 in saline. Only CD69 and CTLA-4 were increased by two-fold or more, respectively, at day 2 after the last peptide treatment, but it is difficult to ascertain the biological relevance of this finding.

Therefore, these results demonstrate the limitation in using a polyclonal TCR murine model to characterise Tr cells that are generating during the induction of peptide tolerance. It also demonstrates that these markers are characteristic of activated cells and treatments that would not normally be considered as modulators of $CD4^+$ T cell phenotype, were capable of inducing changes in the expression of cell surface markers that were not driven by antigen.
To gain further insight into the characterisation of $CD4^+$ T cells and the involvement of Notch signalling during peripheral immune responses, I will address this question in the OVA transgenic TCR system, clone DO11.10. The majority of $CD4^+$ T cells from these mice express TCRs specific for the OVA peptide 323-329, which can be recognised by the clonotypic antibody KJ 1-26. This facilitates the characterisation of the antigen-specific cells and allows gene expression to be linked to antigen driven events.

2. The role of TGF-β signalling in the adult immune system

TGF-β is a well known immune-regulatory cytokine and numerous studies have reported that its secretion by regulatory T cells has important implications in the control of autoimmune disorders. Hence, Powrie *et al* (65) showed that TGF-β was prerequisite for the function of the regulatory $CD4^+CD45RB^{low}$ T cells in inhibiting colitis. As well, TGF-β production is associated with the protective effects seen in animal models of mucosal tolerance of both collagen-induced arthritis (CIA) and experimental allergic encephalomyelitis (EAE) (16, 223). Additionally, TGF-β can

inhibit $CD4^+$ T cell-mediated immune responses, both *in vitro* (224, 225, 226) and *in vivo* (63, 64, 222).

Other members of the TGF-beta superfamily include activin A and BMP-4. Specifically, I have analysed their effects on $CD4^+$ T cell function because, like members of the Notch signalling pathway, they are highly conserved and also regulate developmental processes (45, 46, 54, 55). Additionally, they also participate in numerous biological processes in adult life (127, 153). From an immunological point of view they were interesting because of their potential to function like TGF-β. Activin A uses the same intracellular mediators (Smads 2 and 3) as TGF-β for signal propagation (105), which suggests that functionally both may function in a similar manner. Indeed, studies on targeted disruption of Smad 3 (109) have shown that T cells display an activated phenotype and are not inhibited by TGF-β1 *in vitro*, and therefore, activin A which signals through Smad 3, may have similar effects. As far as BMP-4 is concerned, it mediates signalling via different Smads to activin A and TGF-β. Activation of the pathway recruits Smads 1, 5 and 8, and therefore it is possible that signalling through BMP-4 might have different effects on the outcome of immune responses compared to activin A or TGF-β.

In the work presented in this thesis I have investigated if members of the TGF-β superfamily, like components of the Notch pathway, are differentially regulated during $CD4^+$ T cell responses *in vitro* and *in vivo*, and if this may contribute to the functional differentiation of $CD4^+$ T cells.
In addition in an attempt to establish a link between the Notch and TGF-β signalling pathways during $CD4^+$ T cell responses *in vitro*, I have investigated how TGF-β superfamily members may be regulating expression of genes of the Notch pathway.

The experiments conducted in this thesis have demonstrated that

1. ***TGF-β* and *activin A* are highly expressed in splenic $CD4^+$ T cells, $CD8^+$ T cells, B cells and enriched DCs. However, *BMP-4* is weakly expressed in these cells.**

2. ***Activin A* is significantly up-regulated by 24 hours in CD4$^+$ T cells (50-fold) and B cells (4.5-fold) following anti-CD3/anti-CD28 and LPS activation, respectively. *TGF-β* is only gradually up regulated in B cells.**

3. **Activin A and TGF-β can inhibit proliferation of a human CD4$^+$ T cell clone and of primary splenic CD4$^+$ T cells. BMP-4 has no effect.**

Analysis of their effects in T cell responses *in vitro* revealed that activin A and TGF-β could inhibit proliferation of the human CD4$^+$ T cell clone, HA1.7, in response to specific antigen, and of murine CD4$^+$ T cells in response to antibody-mediated activation (Chapter V, Figures 1 and 8). Furthermore, both proteins could induce a state of CD4$^+$ T cell unresponsiveness, which could not be reversed by anti-CD3 re-stimulation, and only partially by both anti-CD3 and anti-CD28 re-stimulation (Chapter V, Figure 10). BMP-4, in contrast, had no effect on CD4$^+$ T cell proliferation. Thus the differential effects of activin A and TGF-β versus BMP-4 may be attributed to signalling via different intracellular mediators. Activin A and TGF-β signal via Smad 2 and 3, whereas BMP-4 signals through Smad 1, 5 and 8.
In an attempt to describe the cell surface phenotype of the CD4$^+$ T cells treated with the different TGFβ superfamily members, the cells were surface stained with antibodies characteristic of Tr cells. The data (Chapter V, Figure 11) demonstrated that these treatments did not affect the increase in expression of CD25, CD38 or CD45RB triggered by antibody-mediated CD4$^+$ T cell activation. This could indicate that the unresponsiveness induced by the activin-A or TGF-β-treated cells might not be attributable to changes in the expression of the markers examined.

4. **TGF-β, but not activin A or BMP-4, can up-regulate expression of *Jagged-1*, *Delta-1* and *Hes-1* in CD4$^+$ T cells.**

Activin A and TGF-β had differential effects on the expression of Notch signalling genes, which may suggest that these molecules might signal through the same intracellular mediators, but activate different target genes (Chapter V, Figure 12). The experiments demonstrated that TGF-β was a more potent inhibitor of CD4$^+$ T cell activation, as 1ng/ml was sufficient to suppress activation, whereas 100ng/ml were required for activin A. This concentration dependent difference may interfere

with the type of intracellular mediators activated, or the effect to which they affect transcription of target genes.

Both molecules down regulated the induction of *Jagged-2* mediated by TCR triggering in $CD4^+$ T cells. However, only TGF-β significantly up regulated *Jagged-1* and *Deta-1*, and this was also accompanied by a rise in *Hes-1*. Hes-1 is known to act as a transcriptional repressor and has been studied for its ability to block neuronal differentiation in the brain and retina (202). An interesting parallel can be drawn to the rise in *Hes-1* observed by TGF-β treatment, as inducing $CD4^+$ T cell unresponsiveness inhibits the cells from their capacity to differentiate into effector cells.

In trying to unify the results presented in this thesis one consistent finding is that the expression of the Notch receptors in $CD4^+$ T cells (*Notch-1* and *-2*) was not modulated by any form of activation *in vivo* or *in vitro*.

In contrast, Notch ligand expression was differentially modulated in response to different $CD4^+$ T cell treatments. An interesting observation is that peptide-induced tolerance *in vivo* and TGF-β treatment of $CD4^+$ T cells *in vitro*, both lead to an up-regulation of the Notch ligands *Jagged-1* and *Delta-1*, although the latter was only significant in the *in vitro* treatment.

The common feature in these two very different experiments is that they both lead to $CD4^+$ T cell unresponsiveness and both are involved in the generation of Tr cells. It is difficult to make a direct comparison between these two experimental protocols. Although there were some functional similarities, the cells induced *in vivo* and *in vitro* may differ in their phenotype and this may explain why there is a differential regulation of *Hes-1*. Furthermore, in the model of peptide tolerance used in this study Hoyne *et al* reported that TGF-β was not involved in Tr cell induction (12), which indicates that the $CD4^+$ T cells from the *in vivo* and *in vitro* studies are different, and therefore the genes they express may differ too.

Nevertheless, despite the differences, an interesting finding is that *Jagged-1,* and to a lesser extent *Delta-1,* were up regulated during conditions *in vivo* and *in vitro* that favour Tr cell differentiation or $CD4^+$ T cell unresponsiveness.

However, there are additional discrepancies between the data sets. *In vitro* activation of $CD4^+$ T cells with anti-CD3 and anti-CD28 antibodies led to a significant up regulation of *Delta-1* and *Jagged-1*, and of the transcriptional repressor, *Hes-1*, by 24 hours of activation. This would argue against these genes being up regulated under conditions that favour generation of Tr cells.

However, I should stress the importance of kinetics in the expression of these genes. The $CD4^+$ T cells proliferate in respond to TCR-mediated activation after about 3 days in culture, and the effect in gene expression seen for TGF-β occurs at a time when $CD4^+$ T cell proliferation is inhibited. Therefore, the pattern of gene regulation noted for $CD4^+$ T cell activation after 24 hours may not directly relate to the functional state of the cells, but rather to the early effects that activation of intracellular pathways have, on the transcription of Notch ligand genes and *Hes-1*. These pathways may involve NF-κB as it has been previously discussed.

Additionally, the threshold of $CD4^+$ T cell activation in an antibody-mediated response is possibly much lower than that driven by antigen, and this may also affect the type and levels of genes transcribed.

In summary, the work in this thesis has provided evidence to demonstrate that components of the Notch signalling pathway and members of the TGF-β superfamily are expressed in different lymphocyte and APC populations and in peripheral lymphoid organs. Whereas the Notch receptors are highly expressed in cells and organs of the immune system, they are not differentially modulated in $CD4^+$ T cells during the course of different treatments *in vivo* and *in vitro*. In contrast, activation of lymphoytes ($CD4^+$ T cells with anti-CD3/anti CD28 antibodies and B cells with LPS) via pathways that activate NF-κB, lead to the up regulation of the Notch ligands *Jagged-2* and *Delta-1*, as well as of *activin A*. The intracellular mediator *Hes-1* is only up regulated in $CD4^+$ T cells, and *Deltex* in B cells.

I have also established an experimental protocol of intranasal priming to the HDM p110-130 using *E.coli* heat labile enterotoxin as a mucosal adjuvant and have primed or tolerised mice to p110-130 to induce $CD4^+$ T helper or Tr cells, respectively. This allowed me to study how Notch signalling genes may be modulated.

This work was extended to include an *in vitro* model of $CD4^+$ T cell unresponsiveness by treating cells with TGF-β. In an attempt to define the immune regulatory properties of other TGF-β-like molecules, I have made the novel observation that activin A, like TGF-β, can inhibit splenic $CD4^+$ T cell proliferation and induce $CD4^+$ T cell unresponsiveness, whereas BMP-4 had no effect.
The induction of peptide tolerance *in vivo* or the induction of $CD4^+$ T cell unresponsiveness *in vitro* by TGF-β, led to the up regulation of the Notch ligands *Jagged-1* and *Delta-1*, as was observed by CD3/CD28 mediated activation. The up regulation of the Notch ligands upon antibody-mediated activation may reflect early effects in $CD4^+$ T cell activation.

A final personal note to add is that overall the work reported in this thesis has attempted to gain further insight into the regulation of Notch signalling genes during the outcome of different $CD4^+$ T cell responses. I have shown that lymphocyte activation differentially regulates components of the Notch signalling pathway. However, this may have been a direct consequence of gene transcription following activation, and the early kinetics of gene expression may not reflect protein expression and function.
Additionally I have also shown that the Notch ligands are up regulated during antigen-driven responses that induce tolerance *in vivo*, or after treatment of $CD4^+$ T cell with TGF-β *in vitro*. However, it is difficult to ascertain the biological relevance of these demonstrations as they only represent gene analysis studies, and a link between the *in vivo* and *in vitro* data is highly speculative. I believe future experiments using a TCR transgenic murine model will provide a clearer understanding of the questions I have tried to address.

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