Generation and analysis of transgenic mice expressing ovalbumin as a neo-self antigen under control of the myelin basic protein promoter

Dissertation zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

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Weil am Rhein, 2005

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I herewith declare that the following thesis „Generation and analysis of transgenic mice expressing ovalbumin as a neo-self antigen under the control of the myelin basic protein promoter" was independently carried out unless otherwise stated.

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The following thesis was completed from the 15th of August 1999 to the 30th of November 2003 at the Institute for Virology and Immunobiology under the supervision of Professors Drs rer. nat. Hünig, Schimpl and Nitschke.

Für Lina

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1. INTRODUCTION

1.1 The immune system

Constant external threat from infectious agents including viruses, bacteria, fungi, (microorganisms) protozoa and helminths (parasites) has led to the evolution of a highly complex immune system in vertebrates. Two main arms with extensive interaction define the system: the innate and the adaptive immune response. The non specific innate response serves the host with a first line of defence against invasion of pathogens while adaptive immunity provides the host with a repertoire of clonally selected lymphocytes bearing highly diverse antigen specific receptors enabling it to recognise a wide range of foreign antigens (Ags). The response to an initial infection is broadly defined into three phases, the immediate non-specific phase, the early-induced phase and the late specific adaptive phase.

Once pathogens have managed to overcome the first line of host defence, the epithelial barrier, the immediate innate immune response strives to eliminate them. Neutrophils and macrophages phagocytose microorganisms or destroy parasites by the release of granules from their lysosomal compartments. In addition, cytokines released by macrophages determine the form of the subsequent adaptive immune response. Recognition of invading pathogens is mediated by the expression of phagocytic receptors that recognise conserved microbial components. Phagocytosis and secretion of cytokines is further supported by soluble factors of the alternative complement pathway.

Before T and B effector lymphocytes are generated, the early innate immune response attempts to control the pathogens that have managed to establish an infection. Cellular components of this phase include the natural killer cells (NK), $\gamma\delta$ T cells and B-1 cells. These lymphocytes expressing receptors of limited diversity provide early protection by lysis of virus infected cells or the release of inflammatory mediators. However they can not provide a lasting immunity or immunological memory.

When an infection has eluded or overwhelmed innate defence mechanisms, the adaptive immune response is called upon to clear the infection and establish protective immunity. Adaptive immunity is predominantly mediated by lymphocytes. Generated in the bone marrow (BM) from pluripotent stem cells, two distinct types of lymphocytes complete maturation either in the thymus or in the spleen to become T or B lymphocytes respectively. T and B cells carry highly variable receptor molecules, which enable them to recognise and respond to a wide variety of pathogenic Ags. Once lymphocytes have encountered their specific Ag they undergo clonal expansion and differentiate into effector cells that aim to eliminate the pathogen. The most crucial features of the adaptive immune response are its ability to distinguish between foreign and self-Ag and the establishment of immunological memory. Memory is characterised by the ability of clonally expanded lymphocytes to respond rapidly and effectively to pathogens previously encountered and therefore prevent re-infection. Furthermore cytokines secreted during the early immune responses determine whether the ensuing adaptive immune response will be predominantly T or B cell mediated.

B lymphocytes are the mediators of the humoral immune response. B cells that survive negative selection in the BM exit at the transitional stage of development to preferentially home to the spleen. The ensuing mature B cells bear a highly diverse repertoire of surface immunoglobulins that function as the Ag receptor. Activation and differentiation of B cells is either T cell independent or T cell mediated. A T cell independent immune response is characterised by some microbial Ags being able to directly activate B cells via the Ag receptor. In contrast T cell dependent activation requires the antigen to be internalised via receptor mediated endocytosis prior to presentation on the B cell surface as peptide bound to major histocompatibility complex (MHC) II molecules. MHCI and MHCII molecules are cell-

surface glycoproteins containing a peptide-binding groove, which serves to present processed Ag on antigen presenting cells (APC). Recognition of the peptide: MHC II complex enables activated T helper cells to trigger B cell differentiation into an antibody (Ab) secreting cell. Soluble Abs can directly eliminate pathogenic molecules by neutralising them or alternatively serve as adaptor molecules in the complement fixation pathway which ultimately leads to cell lysis. Antibodies are comprised of two different polypeptide chains, the heavy and light chains joined by disulfide bonds. The Fc portion of the antibody engages the effector functions of the immune system. Different forms of the Fc portion classify the antibodies into five different isotypes; IgM, IgG, IgD, IgE and IgA. These isotypes are specialised in activating different effector cells leading to different effector mechanisms.

T lymphocytes are the central players of the cell-mediated immune response. They can be further classified into two main classes based on the different types of pathogen they recognise via their TCR in the context of a MHC molecule. Peptides derived from intravesicular, extracellular pathogens or toxins are presented by MHC II molecules and recognised by CD4+ T cells. CD4+ T cells can be further classified into two subsets based on their ability to activate additional effector cells. Th_1 CD4+ cells are important in cell mediated immunity. Specialised for the activation of macrophages they are also able to facilitate the differentiation of cytotoxic cells by secreting cytokines including IFN-γ and TNF-α. Th_2 CD4+ cells are important in the induction of the humoral immune response. Their release of IL-4 and IL-5 amongst other cytokines mediates B cell help during the thymus dependent immune response. Peptides derived from within the cytosol, including self proteins and viruses, are presented by MHC I molecules on APC and are recognised by cytotoxic CD8+ T cells which can kill the infected cell. In addition to their cytolytic function, CD8+ T cells also secrete cytokines typical of either Th_1 or Th_2 subsets, which are important in the recruitment of additional effector cells. Summarised by Janeway et al., (Janeway 2004).

1.2 T cell mediated immunity

1.2.1 Generation and function of T lymphocytes

Derived from pluripotent stem cells present in the foetal liver or adult bone marrow, the actual T cell development begins a few days after migration into the specialised microenvironment of the thymus. T cell selection processes in this central lymphoid organ enable mature T cells to distinguish between foreign and self-Ag. The thymus is divided into two main regions, the peripheral cortex and the central medulla. The principal developmental steps take place within the cortex in which progenitor T cell interaction with the thymic stroma triggers their differentiation, proliferation and expression of the T cell receptor (TCR) complex. As the T cell progenitors pass through their successive maturation stages they migrate from the cortex to the medulla from where they exit into the peripheral circulation as mature T cells.

Each lymphocyte expresses a specific surface membrane bound TCR. Two distinct sets of receptors characterise two different T cell lineages. About 95% of T cells carry the $\alpha\beta$ TCR while the remaining 5% express $\gamma\delta$ T cell receptors. $\gamma\delta$ T cells differ from $\alpha\beta$ T cells in their specificity and anatomical distribution. A functional TCR is generated when the Ag-receptor genes have undergone somatic rearrangement of the different sets of gene segments. This includes the productive rearrangement of the V (variable), D (diversity), J (joining) and C (constant) gene segments. The insertion of N-nucleotides at the gene segment junctions contributes to additional diversity. This process of somatic rearrangement generates the highly diverse TCR repertoire. The TCR association with the CD3 complex mediates intracellular signalling once Ag is bound.

Immature thymocytes pass through a number of maturation stages that can be defined by the expression of certain surface molecules. The three phases of development include the

double-negative, the double-positive and the single-positive stage. The most immature thymocytes are characterised by the absence of distinctive mature T cell makers including the CD3: TCR complex and the co-receptors CD4 or CD8. At this stage the rearrangement of the TCR β-chain gene occurs. Successful rearrangement of the β-chain leads to its pairing with a surrogate α-chain thus arresting further β-chain gene rearrangement. Subsequent expression of this pre-TCR signals the cell to proliferate, to express its co-receptor proteins and eventually to start transcribing the α-chain genes. Rearrangement of the α-chain genes continues until positive selection allows the maturation of a single positive thymocyte or until the thymocyte dies. Therefore TCR α-chain genes are not subject to allelic exclusion. The regulation of α-chain gene rearrangement by positive selection ensures that each T cell has one functional specificity even in the presence of two different α-chains.

The survival of the double positive thymocytes depends on positive and negative selection. Thymic epithelial cells mediate positive selection while dendritic cells and macrophages largely mediate negative selection. Double positive thymocytes are rescued from apoptosis when their TCR recognises self peptide in the context of self MHC molecules. Furthermore the specificity of the αβ TCR for either class I or class II MHC molecules determines the phenotype of the rescued cells as being either CD4-CD8+ killer or CD4+CD8- helper T cells. Therefore positive selection ensures that the selected T cells can recognise foreign Ag in the context of self MHC molecules (von Boehmer 1994). Elimination of potentially self-reactive T cells occurs during the negative selection process in which those thymocytes expressing a TCR of too high affinity for the self peptide: self MHC molecules undergo apoptosis (Nossal 1994). Nossal et al., were able to demonstrate that 'repertoire purging' of self-reactive T cells occurs early in the thymus. In particular newborn mice receiving semi-allogenic spleen cells had a permanent deficiency in CTL precursors. In this model two problems were considered. Firstly the newborn thymus contains functional T cells and the introduction of foreign T cells is not physiologically relevant. To circumvent this embryonic thymic rudiments were fused with foetal liver to provide the source of stem cells that could populate the rudiments and generated a T cell population. The resulting T cell population was devoid of CTL precursors specific for foetal MHC from either organ (Nossal and Pike 1981; Good, Pyke et al. 1983). Therefore functional inactivation by clonal deletion is an essential component of thymic function. Surviving mature single positive T cells expressing the αβ TCR-CD3 complex, exit the thymus and patrol the body as resting lymphocytes ready to exert effector functions.

The initial interaction of a naive T cell with its Ag occurs within the peripheral lymphoid organs to which the pathogen or its products have been transported by migrating APC. The priming event is mediated by professional APC which include specialised dendritic cells, macrophages and B cells. The dual requirement for both Ag recognition and costimulation prevents naive T cells responding to self-Ag in the absence of costimulation. B7.1 and B7.2 are the best characterised costimulatory molecules on APC. While microbes and cytokines can induce their upregulation, the use of adjuvants in experimental animal models provides an analogous stimulus. Ligation of CD28 by B7 molecules promotes the clonal expansion of naive T cells and the production of the autocrine growth factor IL-2. Once activated T cells express CTLA-4. Binding of CTLA-4 to B7 molecules delivers an inhibitory signal that makes the activated progeny of a naive T cell less sensitive to further costimulation by APC thereby hindering uncontrolled clonal expansion (Frauwirth and Thompson 2002).

The circulatory and migratory properties of naive T cells allows for efficient surveillance of tissues for infectious pathogens and the rapid accumulation at sites of infection. The selectin family of adhesion molecules provides the main trafficking signals. Consisting of P- E- and L-selectin, these adhesion molecules mediate leukocyte endothelial interactions. Constitutive expression of L-selectin, CD62L, on all leukocytes enables the initial binding or rolling of the lymphocyte on the capillary blood vessel wall in the direction of the blood flow. Additional cell surface molecules define the activation status of T lymphocytes. These include the adhesion molecule CD44 and the early activation Ag, CD69. CD44 enables migration into the inflammatory site by binding to hyaluronic acid containing extracellular matrix proteins, while

the upregulation of CD69 reflects the successful activation of the T cell. Following successful Ag recognition, mature T cells can survive in the periphery as long living effector or memory cells through constant Ag stimulation (Kirberg, Berns et al. 1997).

1.2.2 T cell tolerance

Tolerance is an essential requirement of the adaptive immune system in the avoidance of autoimmunity. Absence or loss of tolerance can lead to organ damage resulting in autoimmune diseases such as diabetes and multiple sclerosis (MS). Tolerance mechanisms exist to either delete or downregulate autoreactive T lymphocytes. A T cell repertoire contains over 25 million specificities (Arstila, Casrouge et al. 1999) enabling it to provide surveillance against a multitude of different foreign pathogens. In order to circumvent the danger of recognising self-Ag, a developing T cell is first tested for self reactivity within the thymus. Central tolerance is therefore carried out by negative selection of autoreactive thymocytes with those T cells bearing high affinity receptors being clonally deleted. Although this is the predominant mechanism for tolerance induction, receptor editing (Nemazee and Hogquist 2003), anergy induction (Lanoue, Bona et al. 1997) and positive selection of regulatory T cells (Annacker, Pimenta-Araujo et al. 2001) have also been described. T cells that have bypassed negative selection emigrate into the periphery where they are subjected to peripheral tolerance mechanisms. The parameters that determine whether individual self-reactive thymocytes escape, die or become regulatory T cells remain undefined. Importantly the intrinsic differences in TCR versus signals provided by specific APC during T cell development need to be considered.

Central tolerance is restricted by two requirements. Firstly, the relevant autoAg needs to be present within the thymus. Recently, it was shown that not only ubiquitously expressed Ags are represented in the thymus but also tissue specific Ags (TSAs) (Heath, Moore et al. 1998; Derbinski, Schulte et al. 2001). Expression of TSAs or better known as promiscuous gene expression is a physiological property of thymic epithelial cells. However, the represented repertoire of self-Ags is incomplete and some (eg the H^+/K^+-ATPase β-chain is not detectable in the human thymus (Gotter, Brors et al. 2004)), may be expressed at levels too low to induce T cell deletion. Secondly, negative selection can increase the risk of narrowing the T cell repertoire available to respond to foreign pathogens. Therefore it is an advantage to allow some thymocytes with a degree of self reactivity to be regulated in the periphery. Indeed healthy individuals have been shown to harbour autoreactive T cells in the periphery (Kuchroo, Anderson et al. 2002) (Antonia, Geiger et al. 1995) (Semana, Gausling et al. 1999) (Schluesener and Wekerle 1985). These T cells are more likely to bear low affinity TCR for self-Ags while those that carry high affinity TCR are deleted in the thymus.

As thymic deletion is incomplete and TCR recognition is promiscuous, it is important that peripheral tolerance can act at several different levels to prevent recognition of self-Ag and thereby increase the potential of an autoimmune response. Peripheral tolerance mechanisms can act either directly on the self reactive T cell (intrinsic) or indirectly via additional cells (extrinsic).

Intrinsic peripheral tolerance mechanisms include immunological ignorance, anergy, phenotype skewing and apoptosis.

Immunological ignorance results when the Ag is sequestered at a site not easily accessible to the immune system (Alferink, Tafuri et al. 1998) or if the amount presented doesn't reach the threshold required to trigger T cell activation (Kurts, Miller et al. 1998). Alternatively, encounter with a self-Ag may result in anergy, whereby the T cell is rendered partially or totally unresponsive. Originally it was observed that *in vitro* TCR ligation in the absence of costimulation results in anergy (Jenkins and Schwartz 1987). Anergic T cells are unable to produce or respond to proliferative signals such as IL-2. It appears that different levels of

anergy exist, corroborated by the fact that in some in vitro cell cultures anergy is reversed by exogenous IL-2 (Beverly, Kang et al. 1992) while in other systems this is not the case (Groux, Bigler et al. 1996). Although the physiological function and maintenance for the anergic T cell is still questionable, in vivo evidence supports their role as regulatory T cells (Buer, Lanoue et al. 1998). In the presence of fully activated T cells, tolerance can also be maintained by modulating the immune response by cytokine skewing. Another effective strategy to prevent autoimmunity is to delete a particular T cell clone specificity via activation induced cell death (AICD). This pathway of T cell suicide involves the upregulation of the Fas ligand that binds the death receptor Fas. However, passive cell death has also been observed in the absence of Fas (Bertolino, Trescol-Biemont et al. 1999) (Lenardo, Chan et al. 1999). Which signalling molecules are involved in this form of apoptosis is yet to be determined.

Extrinsic peripheral tolerance mechanisms include tolerogenic dendritic cells and regulatory T cells.

The principal APC, which is capable of initiating an immune response or inducing T cell tolerance, is the dendritic cell (DC). The phenotype of a tolerogenic versus a stimulatory DC has yet to be defined. However, two main theories attempt to explain how DCs regulate the decision between immunity and tolerance. The first proposes that DCs express pattern recognition receptors, (also known as Toll-like receptors) which recognise conserved motifs on invading pathogens, thereby either initiating an immune response upon pathogen encounter or in its absence anergy or deletion (Janeway 1992). The second suggests that only those parasites that cause damage to the host require an immune response. The subsequent release of self-molecules upon pathogen invasion, such as heat shock proteins, would serve as stimulatory signals activating DCs (Basu, Binder et al. 2000). Potentially both mechanisms cooperate with one another to detect pathogens and to detect host cell lysis.

A significant amount of evidence accumulated over the past 20 years has provided evidence for the active suppression of autoreactivity by a population of regulatory T cells (see the following recent reviews) (Shevach 2002) (Bach and Francois Bach 2003) (Bach and Francois Bach 2003) (Wood and Sakaguchi 2003) (Maloy and Powrie 2001) (Walker and Abbas 2002) (Bluestone and Abbas 2003). Two main subsets of regulatory T cells exist, which differ in terms of specificity and effector mechanism. Regulatory T cells continuously generated in the thymus during the normal T cell maturation process are known as the endogenous or ‚natural' Ag specific regulatory T cell (Apostolou, Sarukhan et al. 2002) (Bensinger, Bandeira et al. 2001) (Yamashiro, Hozumi et al. 2002). Expressing high levels of CD25 as well as the costimulatory molecules, CTLA-4 and GITR, they are a long lived peripheral T cell population that inhibit the actions of autoimmune T cells (Yamashiro, Hozumi et al. 2002) (Salomon, Lenschow et al. 2000) (Shimizu, Yamazaki et al. 2002). The ‚adaptive' Treg subset develops through the activation of peripheral mature T cells under conditions of suboptimal Ag exposure and/or costimulation. However, these two subsets are not mutually exclusive. Adaptive Tregs may develop from the natural CD4+CD25+ T cells or by altering the activity of classical T-cell subsets such as T helper cells. IL-2 promotes the development and function of both types of Treg cells based on studies showing complete absence of Treg cells in IL-2 and IL-2R deficient mice (Malek, Yu et al. 2002) (Furtado, Curotto de Lafaille et al. 2002) . In vitro studies have indicated that natural Tregs require T-cell-T-cell or APC interaction which is cytokine independent, to suppress the proliferation of other T cells, even those responding to unrelated Ag. It is debatable whether adaptive Treg cells mediate their suppressive functions by production of immunosuppressive cytokines, such as transforming growth factor$-\beta$ (TGF$-\beta$)(Singh, Read et al. 2001), IL-4 and IL-10 (Bach and Chatenoud 2001). Recently it was determined that the CD25+ T cells isolated from TGF-β-deficient mice were fully competent suppressors (Piccirillo, Letterio et al. 2002). It is likely that the adaptive Treg cell also functions via cell-cell contact to initiate the suppressive cascade.

Autoimmune diseases such as the human MS and Guillain-Barré syndrome (GBS) may be the result of tolerogenic mechanisms being undermined. The following parts will discuss disease pathogenicity mechanisms and corresponding animal models.

1.3 Multiple sclerosis

1.3.1 Clinical presentation

MS is a complex human inflammatory demyelinating disease of the central nervous system (CNS) white matter. Since the first clinical and pathological features were described more than a century ago, extensive studies have demonstrated that in most patients, the disease with acute onset is characterised by chronic inflammation and demyelination within the brain and spinal cord. With profound heterogeneity in its clinical course, neuroradiological presentation and response to therapy in an individual is largely unpredictable. However it is possible to categorise the disease into two distinct phases in which the one phase is dominated by acute relapses and the other by a steady progression of the disease. (Noseworthy, Lucchinetti et al. 2000).

In 80-90% of patients MS begins as a relapsing-remitting course (RR-MS). This form predominantly affecting females develops in the second or third decade of life. Patients are subjected to bouts of the disease, whereby the symptoms after a period of several days stabilise and either spontaneously or with the help of corticosteroids improve. Although after several years the relapses decrease, in 70% of patients the disease with neurological deficits can transform into secondary progressive MS (Weinshenker, Bass et al. 1989). The symptoms are diverse and range from tremors, disturbances in speech and vision to paralysis and neurogenic bladder and bowel symptoms (Noseworthy, Lucchinetti et al. 2000).

Ten to twenty percent of patients suffer from the primary progressive (PP-MS) form of disease. In the absence of remissions and relapses, the disease slowly evolves often with an upper-motor-neuron syndrome of the legs which gradually worsens developing amongst other symptoms, quadriparesis, cognitive decline, visual loss, bowel and bladder dysfunction (Noseworthy, Lucchinetti et al. 2000). Generally, the progression rate of the secondary phase of RR- MS is comparable to that of the PP-MS course (Kremenchutzky, Cottrell et al. 1999).

In a small proportion of MS patients the disease develops atypically leading to massive disability or even death within months after disease onset (Bjartmar, Wujek et al. 2003). Variants of MS include; Marburg's type of acute MS (a rapidly evolving form of chronic MS with more destructive lesions), Devic's type of neuromyelitis optica (predominantly involving spinal cord and optic nerves) and Balo's concentric sclerosis in which large demyelinated plaques with concentric layering of myelinated and demyelinated zones appear.

Disease diagnosis is based on established clinical and laboratory criteria (Poser, Paty et al. 1983). Cerebrospinal fluid analysis and MRI (Miller 1998) have significantly improved diagnosis. MRI imaging allows the expanded extracellular space within demyelinated areas and the number and size of lesions present within the periventricular white matter to be visualised. In addition the use of a paramagnetic tracer (Gd-DTPA), determines blood brain barrier damage which is an indication of the change in permeability of inflamed blood vessels within active lesions (Lassmann and Ransohoff 2004). Imaging studies have also helped reveal pathological differences in the brain during RR-MS and PP-MS (Miller, Grossman et al. 1998). Despite PP-MS patients experiencing a faster progression in the course of disability the prevalence and burden of CNS lesions in the white matter is less conspicuous compared with RR-MS (Filippi 2003).

1.3.2 Immunopathogenesis

The pathological hallmarks of MS are demyelinated plaques within the periventricular white matter and corpus callosum (cortex) of the brain and spinal column. Accompanying demyelination are activated microglia and inflammatory infiltrates containing T, B cells and macrophages.

Axonal injury and destruction in the demyelinated areas is the major correlate for a permanent neurological deficit. Recent work by Lassmann and co-workers has revealed a profound heterogeneity in the patterns of demyelination between different patients. They determined that the composition of the inflammatory infiltrates within the demyelinating lesions varied extensively between patients reflecting distinct pathogenic mechanisms. Different immune mediated mechanisms of CNS damage could be described with regard to four major types of MS lesions (Lassmann 2001). Despite the similarities in the inflammatory response, no specific association between the pattern of demyelination and clinical disease could be found.

PATTERN	MECHANISM OF DEMYELINATION
I **Macrophage mediated**	T cell mediated, activated microglia /macrophages macrophage toxins
II **Ab mediated**	T cell mediated, microglia/ macrophage activation complement mediated lysis of Ab-targeted myelin
III **Distal oligodendrogliopathy**	T cell mediated virus induced white matter injury
IV **Primary oligodendrocyte damage with secondary demyelination**	T cell mediated, microglia/ macrophage activation metabolic disturbance of oligodendrocytes genetic defect?

Table 1. Essential characteristics of 4 different lesions found in MS

All actively demyelinating lesions consist of inflammatory infiltrates composed mainly of T cells and macrophages. Although the inflammatory reaction is similar the lesions can be segregated into four different patterns of myelin destruction. Pattern I describes myelin sheath destruction due to toxic products (eg TNF-α) released by activated macrophages. Lesions similar with pattern I ascribe myelin damage to cooperation between encephalitogenic T cells and anti-myelin Abs in pattern II. Pattern III type lesions are found as a result of virus-induced white matter destruction while infrequent intrinsic metabolic disturbances to oligodendrocytes rendering them vulnerable to toxic actions of inflammatory mediators are mechanisms responsible for pattern IV. Adapted from Lassmann et al., (Lassmann 2001).

Recovery from symptoms during remission is mainly due to the restoration of axonal function, either by early remyelination, the resolution of inflammation, or the restoration of conduction to axons, which persist in the demyelinated state. In acute lesions oligodendrocyte progenitor cells are present and able to expand and develop into myelinating oligodendrocytes, remyelinating neurons and thereby repair local damage (Scolding and Franklin 1997). However, the ability to remyelinate is lost over time.

Although the exact immunological molecular mechanisms throughout the course of MS are still unknown, it is clear that the major damage to the CNS is a direct result of a coordinated immune attack. Immunological research has attempted to identify the immune cells that play either a causative or protective role in the inflammatory response accompanying deymyelination in the CNS of MS patients. Histopathological studies have clearly identified the accumulation of T & B lymphocytes and macrophages in acute MS lesions.

The presence of clonally expanded B cells and the elevation of oligoclonal IgG Abs, mainly involving the IgG1 and IgG3 isotypes, in the brain and cerebrospinal fluid argue for an Ag-driven immune response. However, even in the presence of high concentrations of Abs within the brain and cerebrospinal fluid of MS patients, the pathogenic role of Abs in MS remains unclear. The occurrence of elevated intrathecal Ab levels and oligoclonal IgG bands in the CSF of patients is a valuable diagnostic marker for disease pathogenesis. Whether the clonal B cell expansion is the result of an active ongoing immune response in the CNS or simply the result of resident memory B cells (Archelos, Storch et al. 2000) is still to be determined. Further studies need to be undertaken to determine the identity of resident CNS Ags which are the focus of the humoral immune response leading to demyelination (Burgoon, Owens et al. 2001).

Initial genetic studies showed an association of MS susceptibility to MHC II genes and analysis of cytokine and chemokine receptor expression within the active lesions propose MS as being a CD4+ Th1 mediated inflammatory disease (Sorensen, Tani et al. 1999). Individual MS patients show elevated levels of Th1 type cytokines as indicated by increased levels of IFNγ, TNFα or IL-2. In addition IL-12 and IL-18 both of which induce IFN-γ expression are also upregulated (Karni, Koldzic et al. 2002) (Balashov, Smith et al. 1997) (Comabella, Balashov et al. 1998).

The CD4+ T cell mediated disease hypothesis began to be disputed when further investigation of MS plaques revealed that CD8+ T cells outnumber CD4+T lymphocytes almost tenfold (Hauser, Bhan et al. 1986) (Booss, Esiri et al. 1983). Additional PCR analysis of rearranged TCRs has indicated the clonal expansion of CD8+ T cells at the site of actively demyelinating lesions is more prominent than in the CD4+ T cell population (Babbe, Roers et al. 2000). It is possible that the predominance of CD8+ T cells was overlooked for many years due to the inavailibility of anti-CD8 mAbs that bind to formalin fixed tissues.

The importance of the immune system in the pathogenesis of MS was initially demonstrated by accidentally immunising humans with rabbies vaccine containing myelin components which resulted in acute demyelination (Hemmer, Archelos et al. 2002). Based on these findings the prototypic animal model, known as experimental autoimmune encephalomyelitis (EAE), was established 70 years ago.

1.4 Experimental autoimmune encephalomyelitis

1.4.1 The EAE concept

The main immunological paradigms including therapy of MS are based on extensive data obtained from the EAE model. While MS is a relapsing and remitting disease, EAE in rodents is generally characterised by a monophasic clinical course of ascending paralysis, followed by spontaneous complete recovery. Ascending paralysis begins with tail and hind limb weakness commonly leading to paralysis of the hind and forelimbs. Electrophysiological and pathological features characterised by perivascular inflammatory lesions in the parenchyme of the CNS are very similar to MS. EAE can be actively induced in rodents by immunisation with either CNS tissue or with purified CNS myelin components emulsified in complete

Freund's adjuvant (CFA). Myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendroglial protein (MOG) are constituents of the CNS myelin sheath. Susceptibility, disease course and severity are strain dependent. Adoptive transfer of T cells from a diseased animal into a naive animal provided conclusive proof of an autoimmune response in this model. Subsequent identification of the disease inducing myelin Ags permitted the establishment of Ag specific, autoaggressive T cell lines. Separation of T cell subtypes prior to adoptive transfer have indicated that mainly CD4+ T cells polarised to the production of type I cytokines transmitted the disease (Sedgwick, MacPhee et al. 1989) (Vandenbark, Gill et al. 1985). Th1 cytokines such as IFNγ, TNFα and IL-2 and the pro-inflammatory cytokine TNFα were implicated in disease pathogenesis (Zamvil and Steinman 1990). In contrast blocking the TNFα pathway and polarising T cell responses towards a T helper type 2 phenotype, ie the secretion of IL-4, IL-5 IL-10 and IL-13 ameliorated EAE (Rocken, Racke et al. 1996). These and other supporting findings classified EAE as a prototypic T helper 1 type disorder.

No relevant experimental animal model exists which mimics either the different forms of MS or the complete spectrum of MS while existing models have reflected different aspects of the whole disease. The EAE model provides a tool with which to investigate the many different facets of pathogenesis such as disease induction and acute and recovery phases of MS.

Although extensive initial studies utilising the EAE Lewis rat model have offered significant insights into the immunopathogenesis of MS, its limitations include the lack of spontaneous relapses as well as the absence of primary demyelination. In contrast the mouse EAE model offers features of MS including a chronic relapsing course and histopathological evidence of demyelination (Skundric, Zakarian et al. 2003). Moreover, the murine EAE model enables genetically modified mutants to be crossbred providing the potential for investigation into the involvement of a single gene in the disease susceptibility, course and remyelination.

Initial studies of MBP-EAE actively induced by standard immunisation procedures divided various mouse strains into either susceptible or resistant phenotypes. Whereas susceptible mice include SJL/J and PL/J, C57BL/6, CBA and DBA/2 are considered as resistant strains. The basis of EAE resistance does not appear to be due to the lack of encephaltiogenic T cells recognising Ag in the context of MHC gene products, nor low cell frequency or affinity. Rather, selection of the suitable Ag and immunisation protocol can render a resistant strain susceptible. Modified protocols leading to EAE include a combination of active and passive immunisation (Shaw, Kim et al. 1992) (Shaw, Kim et al. 1996) and the use of high doses of encephalitogen or adjuvant. Furthermore use of the ancillary adjuvant pertussis toxin (PT) also influences disease induction by increasing the BBB permeability. Recently Linker et al., were able to induce MBP-EAE in C57BL/6 mice by immunising with rat MBP on days 0 and 17 followed by PT. While the course of EAE in the traditionally considered susceptible strains including the SJL/J mouse is relapsing-remitting the more resistant C57BL/6 strain presents a monophasic form of the disease.

1.4.2 Immunological mechanisms

The initial molecular events that ultimately lead to the clinical manifestations seen in EAE and MS have not been completely elucidated. Priming of the encephalitogenic T cells occurs within the peripheral lymphoid compartment. This has been elegantly shown in an adoptive transfer model of the rat. The authors were able to show that encephalitogenic MBP CD4+ T cells underwent activation in the lymph nodes and spleen prior to EAE induction. Upon EAE induction the GFP transfected MBP T cells migrated into the brain mediating inflammation in contrast to ovalbumin (OVA) specific T cells which proceeded into the draining lymph nodes (Flugel, Berkowicz et al. 2001).

Although potentially pathogenic CNS autoreactive CD4+ T cells are part of the normal immune repertoire of humans and rodents they can be controlled by CD4+CD25+ regulatory T cells (Kohm, Carpentier et al. 2002). Activated T cells specific for a non CNS Ag such as OVA (Zeine and Owens 1992), as well as naive CNS autoreactive T cells have been shown to survey the CNS (Krakowski and Owens 2000). It is hypothesised that these autoreactive T cells may cross react with viral proteins inducing pathogenesis (Wucherpfennig and Strominger 1995). Alternatively degenerative disorders that cause CNS tissue damage can lead to the release of CNS proteins which after APC presentation in the draining lymph nodes could promote an immune response.

Primed T cells must cross the relatively impermeable BBB before entry is gained to the CNS. The intercellular homing signals including cytokines and chemokines, which guide the T cells from the peripheral lymphoid compartment into the CNS are yet to be defined. Utilising an intravital microscopy model with blocking antibodies, Alt et al., were able to determine *in vivo* the importance of the chemokines CCL19 and CCL21 in mediating lymphocyte recruitment into the CNS during EAE (Alt, Laschinger et al. 2002). Subsequent adhesion to the endothelial lining and penetration of the BBB incorporate a highly coordinated interaction between adhesion molecules reciprocally expressed on invading leukocytes and endothelium (Piccio, Rossi et al. 2002). In addition activated T cells are capable of secreting matrix metalloproteinases leading to the destruction of the BBB. The secretion of pro-inflammatory cytokines such as IL-12, INF-γ and TNF-α elicit the production of chemokines ensuring further influx of monocytes and non-specific T cells into the CNS (Wekerle, Engelhardt et al. 1991; Merrill, Kono et al. 1992).

A synergistic interaction between autoimmune cellular and humoral immune responses to CNS Ag is most likely responsible for the demyelinating pathology observed not only in EAE but also in MS.

Primed CD4+ T cells entering the CNS recognise MHCII-Ag complexes on microglial cells or infiltrating activated macrophages. Reactivation of CD4+ T cells leads to a heightened production of inflammatory cytokines. One such example is the TNF-α mediated recruitment of macrophages, which direct a phagocytic attack on the myelin sheath and ultimately the underlying axon. Similarly clonally expanded CD8+ T cells identifying their respective MHCI-peptide complex on essentially all CNS cells including neuronal and glial cells can cause damage to the presenting cells.

The importance of B cells and their antibodies is reflected in B cell deficient mice in which the course of the disease is augmented (Wolf, Dittel et al. 1996). Furthermore co-administration of myelin specific Abs after disease induction increases EAE severity in some models (Linington, Bradl et al. 1988) (Iglesias, Bauer et al. 2001). Clonally expanded B cells mature to plasma cells on re-encountering their specific antigen and release large amounts of antibodies into the CNS. The antibodies may bind to soluble or membrane-bound antigen. Binding of IgG1 antibody to cell surfaces activates the complement cascade mediating damage to the myelin sheath via opsonisation. Although findings in the EAE model indicate that Abs are important in the chronic phase of the disease their role not only in the pathogenesis but also in the remyelination process remains to be elucidated (Morris-Downes, Smith et al. 2002).

The innate arm of the immune response may be important not only in providing an initial cytokine milieu necessary for the development of the adaptive immune response but also in regulating the course of the disease with the involvement of NKT cells. The initial systemic inflammatory response and tissue damage can lead to the activation of microglia which release inflammatory mediators leading to upregulation of immune receptors including MHC molecules on CNS cells (Aloisi 2001). Natural killer T cells have been found not only in the peripheral blood (Gausling, Trollmo et al. 2001) but also in the lesions of MS patients (Illes,

Kondo et al. 2000). However, the exact role of NKT cells as either enhancing or ameliorating the course of the EAE disease has yet to be determined (Jahng, Maricic et al. 2001).

1.4.3 Questioning the CD4+ Th1 model

The Th1 paradigm began to be questioned when Th1 cytokine knockout and transgenic animals displayed an unexpected phenotype with regard to the disease course of EAE. While severity of EAE is increased in IFN-γ knockout mice (Willenborg, Fordham et al. 1996) disruption of the IL-4 gene does not affect the course of EAE (Ferber, Brocke et al. 1996). Furthermore gene disruption of the Th2 type cytokine, IL-10 leads to a severe form of EAE in contrast to IL-10 transgenic mice which are resistant to the induction of EAE (Bettelli, Das et al. 1998). These findings strongly support the idea that the suppressive activity of resident CD4+CD25+ T cells in EAE is hampered in the absence of IL-10 (Zhang, Koldzic et al. 2004). The overexpression in the CNS or disruption of TNF-α leads to a series of heterogeneous results. TNF-α transgenic mice develop spontaneous demyelination or at least a more severe form of EAE in most models (Liu, Marino et al. 1998). Depending on the genetic background TNF-α knockout mice still develop EAE with differing grades of severity (Kassiotis, Pasparakis et al. 1999) (Frei, Eugster et al. 1997). Of course the information gathered from the overexpression or disruption of certain genes has to be viewed with caution in light of the impact this can have on the lymphoid and nervous system.

Quantitative PCR demonstrated the upregulation of both Th1 and Th2 type cykokines in MS brain lesions (Baranzini, Elfstrom et al. 2000). In parallel several laboratories reported on the encephalitogenic potential of Th0 cells which secrete both Th1 and Th2 type cytokines (Brocke, Gijbels et al. 1996). Furthermore Lafaille was able to demonstrate the ability to induce EAE by transfer of Th2 myelin specific T cells into immunodeficient mice (Lafaille, Keere et al. 1997).

The role of CD8+ T cells in the pathogenesis of EAE remains controversial. Initial studies have provided conclusive evidence for the encephalitogenic potential of myelin specific CD8+ T cells (Sun, Whitaker et al. 2001) (Huseby, Liggitt et al. 2001). Progressive and destructive EAE was induced in irradiated hosts upon adoptive transfer of purified MBP specific CD8+ T cells. Interestingly the lesions which featured little inflammation but massive perivascular cell death and demyelination, were located primarily in the brain and not in the spinal cord as is observed in most CD4+ induced EAE models. These results may support the notion that CD8+ T cells play an essential role in the early loss of oligodendrocytes and neurons. The Theiler's virus model of demyelination with similarities to MS, provides evidence for an important role for cytotoxic mechanisms in inducing inflammatory tissue damage by CD8+ CTLs (Murray, Pavelko et al. 1998). MHC-I light chain β2 microglobulin deficient mice infected with Theiler's virus show preservation of axons despite extensive demyelination indicating a contribution of CD8+ CTLs to neuronal injury (Rivera-Quinones, McGavern et al. 1998). Additionally it has been shown that CD8+ T cells can lyse oligodendrocytes and neurons *in vitro* (Jurewicz, Biddison et al. 1998; Medana, Gallimore et al. 2000). However, CD8+ T cells may also have a regulatory function and be involved in the remission of EAE as PJL/J CD8+ deficient mice displayed decreased mortality but more relapses (Jiang, Zhang et al. 1992; Koh, Fung-Leung et al. 1992).

Autoreactive CD4+ T cells may not be purely pathogenic by initiating the inflammatory process but also provide protection from inflammation induced nerve injury by secretion of neurotrophins such as brain derived neurotrophic factor (BDNF) (Kerschensteiner, Gallmeier et al. 1999) which can support myelin regeneration. Moreover CD4+CD25+ regulatory T cells have been shown to inhibit encephalitogenic inflammation of the CNS (Lafaille, Nagashima et al. 1994). Recently, EAE induced in CD4 and CD8 DBA/1 deficient mice, (Abdul-Majid,

Wefer et al. 2003) support the results of (Sun, Whitaker et al. 2001) which suggest that CD8+ T cells orchestrate the extent of demyelination while CD4+ T cells appear to be critical for disease induction.

All these observations provide evidence for a more complex pathogenesis than a simple CD4+ Th1 driven neuroautoimmune disease. In addition it helps to explain the failure of certain human MS trials. Treatments that were either inefficient or worsened the disease course included global depletion of CD4+ T cells (van Oosten, Lai et al. 1997), a Th2 induced shift in myelin specific T cells by altered peptide ligands or oral administration of myelin (Bielekova, Goodwin et al. 2000; Kappos, Comi et al. 2000) and the inhibition of TNF-α by a blocking Ab (van Oosten, Barkhof et al. 1996).

Finally it is important to consider variabilities in the EAE mouse model which include the use of different genetic strains and the diverse combinations of adjuvants used. Importantly the use of CFA in the disease induction regime may bias the T cell response. Th1 type cells are most likely to expand in response to the CpG motifs found in the killed mycobacteria tuberculosis DNA containing adjuvant (Steinman 2001).

1.5 Guillain-Barré Syndrome

1.5.1 Clinical course

In 1916, Guillain, Barré and Strohl redefined a disease affecting the peripheral nervous system (PNS) without involvement of the CNS. The symptoms included ascending weakness and areflexia with early recovery (Ho and Griffin 1999). The Guillain-Barré Syndrome (GBS) has been classified amongst the broad rubric of Acute Inflammatory Demyelinating Polyneuropathies (AIDP) as the most common form of an acute immune-mediated paralytic disease caused by inflammatory nerve demyelination.

Manifestations of GBS may be acute or chronic and temporary or permanent depending on the extent of neuronal demyelination. The onset of symptoms in this acute monophasic disease include symmetrical limb weakness, loss of tendon reflexes, sensory disturbances with the potential of total paralysis of the extremities and frequent involvement of the respiratory muscles lasting up to 4 weeks. Recovery is spontaneous and accelerated by immunomodulating therapies, such as plasma exchange and intravenous immunoglobulins. From 80 percent of patients which recover completely within a few weeks to two years, 65 percent have persistent neurological problems (Sulton 2002).

The incidence of GBS is highest in males in the fifth to seventh decade of life with the annual incidence being 1-2 cases per 100 000 throughout the world (Sulton 2002). Rarely diagnosed in Caucasians but more commonly in Asians, diagnostic criteria include electrophysiological abnormalities consistent with demyelination, progressive motor weakness of more than one limb as well as elevated protein levels in the CSF in the absence of increased lymphocytes. In contrast to other autoimmune diseases GBS does not strongly correlate with MHC haplotypes but instead may be related to the FcγRIIa-H131 allotype (Hartung, Kieseier et al. 2001). Fc-γ receptors are involved in antibody dependent cellular cytotoxicity including myelin directed Abs.

1.5.2 Immunopathogenesis

Strong epidemiological evidence for the association between pathogens and GBS include Campylobacter jejuni (4-66%), cytomegalovirus (5-15%), Epstein-Barr virus (2-10%), and Mycoplasma pneumoniae (1-5%) infections (Jacobs, Rothbarth et al. 1998; Hughes, Hadden et al. 1999). Lymphocyte infiltration and macrophage mediated demyelination are features of the early pathology (Ho and Griffin 1999). Severe axonal degeneration is most common following C. jejuni infection. During the acute phase of the disease high Ab titres against gangliosides are found. Expressed abundantly in the nervous system, gangliosides are sialic acid-containing glycolipids. Implicated in cell growth and differentiation they also serve as receptors for bacterial toxins and function in signal transduction. The existence of ganglioside-like epitopes in the bacterial wall of the enteritic bacterium, C. jejuni and its high association with GBS propose a role for molecular mimicry in which cross-reactive T and B cells recognise microbial structures in addition to the host leading to the symptoms of an autoimmune disease. In addition to IgM GBS patient serum also contains high titres of IgG Abs including the IgG1 and IgG3 subclasses, pointing to an isotype switch involving T cell help. However, anti-ganglioside Abs only peak in the sera of patients after the acute phase of GBS suggesting their production is secondary to nerve damage (Press, Mata et al. 2001).

Therapeutic responses to plasma exchange and intravenous Ig as well as the presence of activated T cell and macrophage derived cytokines in peripheral nerve biopsies, provide evidence for an important role for humoral factors as well as cellular immunity. Histopathological analysis shows the presence of extensive cellular infiltrates in the nerve biopsies of patients with inflammatory demyelinating neuropathies, supporting a role for cell mediated immunity against myelin Ags (Hughes, Hadden et al. 1999). While the majority of infiltrating cells are macrophages, CD4+ and CD8+ T cells have also been identified in sural nerve biopsies (Schmidt, Toyka et al. 1996). Further indirect evidence pointing to heightened levels of activated circulating T cells are increased levels of soluble IL-2 and its receptor in the serum of patients with GBS (Hartung, Reiners et al. 1991). Although activated $\alpha\beta$ (Taylor and Hughes 1989; Hartung, Hughes et al. 1990) and a small population of $\gamma\delta$ T cells (Cooper, Ben-Smith et al. 2000), are a significant component of the nerve lesion, their exact specificity and function in the development of GBS remains unknown. It is conceivable that a small population of Ag specific T cells can control the action of non-specific inflammatory cells. This might include the function of CD4+ helper/inducer T cells in directing CD8+ CTLs as well as stimulating plasma cells to secrete peripheral myelin specific Abs that mediate the recruitment of macrophages.

1.6 Experimental autoimmune neuritis

1.6.1 The prototypic animal model for GBS

Valuable insights into the pathogenesis, immunregulation and therapy of GBS have been gained from animal models including Lewis rat EAN and chronic EAN of rabbits. Many of the clinical, electrophysiological and immunopathological features of this animal model are similar to the human GBS disease. EAN is therefore the animal model that is widely used to investigate the molecular mechanisms involved in acute inflammatory demyelinating diseases of the PNS.

The initial successful EAN induction was carried out in rabbits by immunisation with whole peripheral nerve homogenate in adjuvant (Waksman and Adams 1955) demonstrating an involvement of the immune response. In particular immunisation with neuritogenic components of the PNS myelin such as the purified myelin proteins P2 (Kadlubowski and

Hughes 1979), P0 (Milner, Lovelidge et al. 1987) and synthetic peptides of P2 containing the neuritogenic amino acid sequences 53-78 and 61-70 (Olee, Powell et al. 1990) emulsified in CFA can induce the inflammatory demyelinating disease in rodents. Acute EAN is characterised by the infiltration of macrophages and lymphocytes as well as primary demyelination of the nerve roots and peripheral nerves. Other PNS autoAgs which are shared with the CNS, include myelin basic protein (MBP) (Abromson-Leeman, Bronson et al. 1995), peripheral myelin protein (PMP) 22 (Gabriel, Hughes et al. 1998) and myelin associated glycoprotein (MAG) (Weerth, Berger et al. 1999). Immunisation of rats with these autoAgs induce inflammation predominantly in the spinal cord while focal cellular infiltration is present in the spinal roots as well as in the sciatic nerves (Weerth, Berger et al. 1999).

Comparative with the EAE model, the decisive role of T lymphocytes in the initiation of an immune mediated neuropathy has been established by adoptive transfer of T cells. Studies involving adoptive transfer of P2- or P0 CD4+ specific T cells into naive Lewis rats and MBP-specific CD4+ T cells into syngenic naive Balb/c mice induced EAN whereby cellular infiltration and demyelination began shortly before Ab responses were likely to have developed (Linington, Izumo et al. 1984) (Izumo, Linington et al. 1985). Simultaneous administration of Abs to glycoproteins can enhance the severity and extent of demyelination (Hahn, Feasby et al. 1993; Spies, Pollard et al. 1995).

Similar to the EAE Lewis rat model, EAN is an acute monophasic disease in which mice develop a progressive limb paralysis that gradually resolves. The severity of EAN correlates with the dose of neuritogenic Ag administrated. Similar observations are made with the adoptive transfer model of EAN in which disease severity and pathological changes correlate with the number of autoreactive T cells transferred.

The exact molecular pathogenesis of EAN remains undefined. Although EAN is generally regarded as CD4+ T cell mediated, growing evidence indicates that the integrated attack does not solely involve CD4+ T cells but also CD8+ T cells, macrophages, B cells, $\gamma\delta$ T cells as well as NK T cells.

1.6.2 Immune mechanisms within the PNS

The proposed immunological mechanisms occurring in the EAN model which ultimately lead to the inflammatory mediated demyelination of the PNS are to a great extent analogous to the mechanisms described in the EAE model.

The peripheral nervous system is jeopardised by an immunoinflammatory response from the systemic immune system due to the lack of an anatomical barrier most proximally at the root exit zone and most distally at the motor terminals. While the initial priming events and specificity of the autoAg in the periphery are unknown, activated T cells must transverse the blood nerve barrier (BNB) before being able to enter the PNS to initiate an inflammatory attack. In the PNS it is not clear whether T cells are primed upon encountering their target Ag in the PNS or whether this occurs in the lymphoid organs. T cell activation as reflected by BrdU incorporation has indicated that activation of encephalitogenic T cells occurs primarily within lymphoid organs before disease onset (Zetti, Gold et al. 1996). The fate of the activated T cell is also relatively poorly understood. Encephalitogenic T cells either leave their target tissue and accumulate in the peripheral immune organs or are eliminated in situ. Physiological defence mechanisms such as apoptosis appear to be important in curtailing inflammatory responses in immune protected sites such as the PNS. Further BrdU incorporation studies in sciatic nerve have shown activated T cells to exhibit morphological signs of apoptosis shortly after disease onset. However, whether only Ag-specific or also recruited T cells are destroyed within the nervous system remains to be determined.

Adhesion molecules such as VLA-4 (Enders, Lobb et al. 1998), MMPs (Kieseier, Clements et al. 1998) as well as chemokines including MIP1a and MCP-1 (Zou, Pelidou et al. 1999) have

all been shown to be involved in the transmigration process of activated T cells across the BNB into the PNS. Immigrated T cells can recognise peripheral nerve autoAg: MHC II complexes expressed on resident macrophages lying adjacent to blood vessels (Kiefer, Kieseier et al. 2001) and most likely Schwann cells (Bergsteinsdottir, Kingston et al. 1992) leading to their re-activation and a subsequent immune response. This immune attack directed against the peripheral nerve myelin sheath results in the recruitment of B cells and macrophages which in turn can cause demyelination directly by phagocytosis or indirectly by secretion of inflammatory mediators such as TNF-α (Spies, Westland et al. 1995). Damage to the PNS is also the result of Ab-mediated and complement-dependent phagocytosis of myelin, cytotoxic T cell lysis in addition to membrane perturbation from cytokines and free radicals released by inflammatory cells (Maurer, Toyka et al. 2002).

The central role of T cells in the pathogenesis of EAN was shown by adoptive transfer of a P2 T cell line capable of inducing EAN in Lewis rats (Linington, Izumo et al. 1984) and also BN rats (Linington, Mann et al. 1986), which are resistant to active EAN. The importance of T cells is further exemplified by the inability of T cell deficient rats to develop EAN (Brosnan, Craggs et al. 1987) as well as the prevention of EAN by administration of an anti $\alpha\beta$ TCR Ab (Jung, Kramer et al. 1992). However, the relative contributions of CD4 or CD8 T cells during the induction or effector phases of EAN are relatively unknown. Predominantly lymphocyte infiltrates of the nerve contain CD4+ T cells with a T helper phenotype based on the secretion of IFN-γ (Zhu, Mix et al. 1998). Further FACS analysis studies have determined that $\alpha\beta$ T cells of a CD45R+CD8+ (activated cytotoxic lymphocytes) and CD45R- CD4+ (memory Th cells) phenotype are a major population infiltrating peripheral nerves during EAN (Fujioka, Purev et al. 2000). While most CD4+ Th cells were present at the onset of clinical signs, CD8+ T cells prevailed after the peak of disease and during recovery suggesting a role as suppressor cells in the PNS (Rosen, Brown et al. 1992). In a recent study by Zhu et al., a pivotal but not exclusive role for CD4+ T cell mediated EAN induction was confirmed. CD4-/-C57BL/6 mice displayed milder symptoms and reduced levels of IFN-γ post active EAN induction. Interestingly a protective role for CD8+ T cells in the pathogenesis of EAN was also verified by the induction of EAN in CD8-/- mice. In the absence of CD8+ T cells the duration of EAN was shorter and milder in addition to a delayed disease onset (Zhu, Bao et al. 2002). A pathogenic role in the induction of EAN is proposed for CD4-CD8-TCR $\alpha\beta$+ T cells as CD4-CD8- mice develop heightened clinical symptoms when compared with wild type or CD4 or CD8 deficient mice (Zhu, Nennesmo et al. 1999).
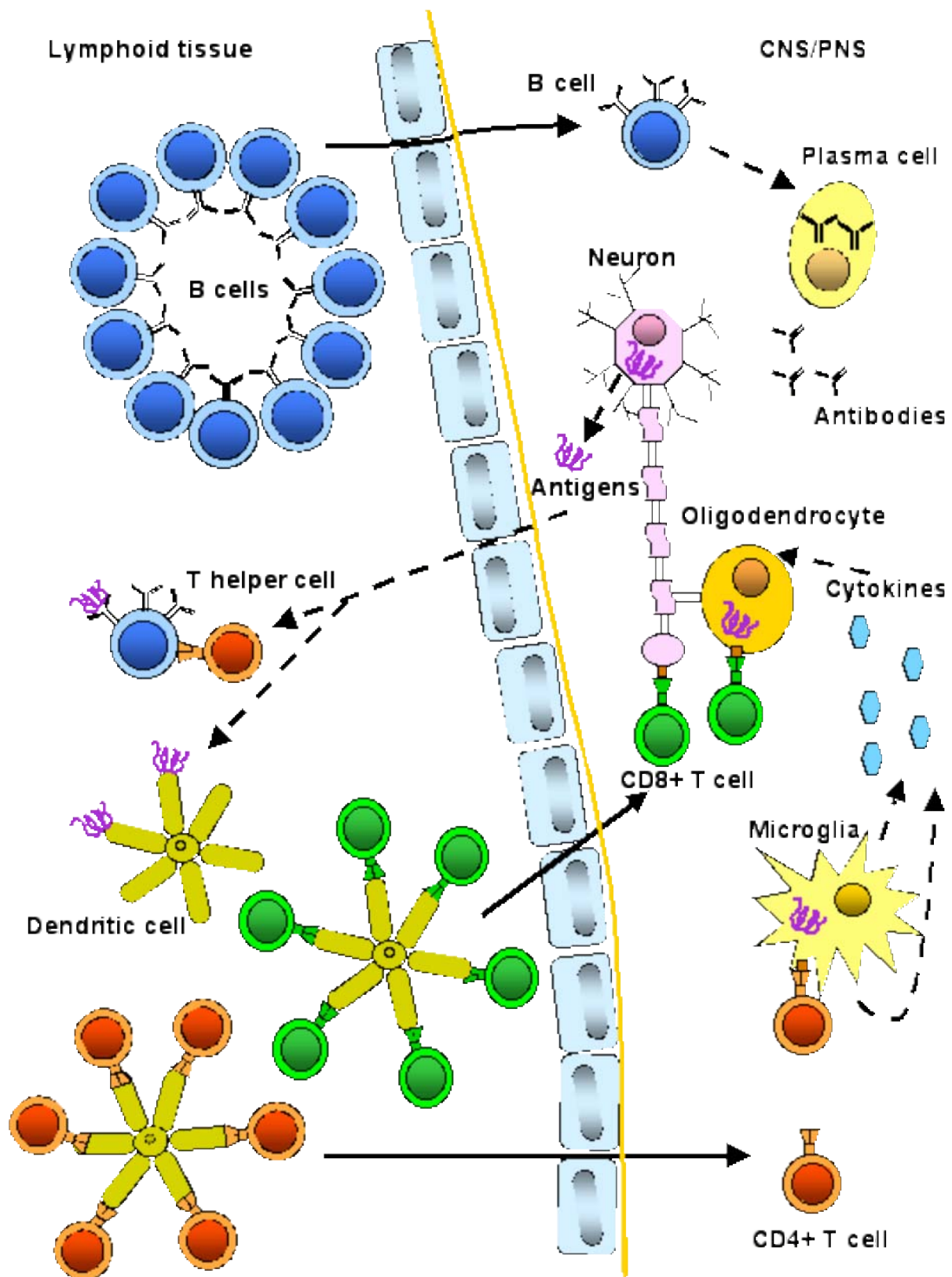
Fig. 1 Proposed cellular immune mechanisms within the CNS and PNS during an autoimmune response.

The hypothesised CNS or PNS immune response proposes that two steps are required for induction. A pro-inflammatory milieu in the CNS/PNS, leading to upregulation of MHC

molecules, co-stimulatory receptors and inflammatory cytokines and an Ag driven acquired immune response. Independent of the causative event, T and B cells are primed in the lymphoid tissues by DCs presenting either Ags released from the nervous system tissue or cross-reactive foreign Ag. After clonal expansion, T and B cells can cross the BBB/BNB and infiltrate the CNS/PNS. Clonally expanded B cells re-encounter their specific Ag and mature into plasma cells, which release large amounts of immunoglobulin-γ (IgG) Abs. These Abs bind soluble or membrane-bound Ag on expressing cells. CD8+T cells invading nervous system tissue encounter specific peptide ligand presented by glial or neuronal cells on MHCI molecules prompting direct cell damage. Conversely CD4+ T cells encounter Ags presented by microglial cells on MHCII molecules. Reactivation of CD4+ T cells results in the secretion of inflammatory cytokines and chemokines, which recruit other immune cells such as macrophages. The process of inflammation is then promoted further by the release of injurious immune mediators and direct phagocytic attack on the myelin sheath. The resulting breakdown of the BBB/ BNB allows the passage of circulating autoAbs, which can synergistically act with T cells to induce demyelination. Adapted from Hemmer et al., (Hemmer, Archelos et al. 2002).

1.7 The myelin basic protein promoter

Myelin basic proteins (MBPs) are a major constituent of the myelin sheath. They belong to a family of polypeptides that are predominantly expressed within the nervous system ie oligodendrocytes and Schwann cells where they play a major role in myelination. Myelinating oligodendrocytes and Schwann cells form myelin sheaths surrounding axons in the central nervous system (CNS) and in the peripheral nervous system (PNS) respectively. The myelinating glial cells form the myelin sheath by extensive spiral wrapping and compaction of the cell plasma membrane around the associated axon. These cells are also involved in regulation processes during development, repair and regeneration of the myelin sheath. Myelination allows for a dramatic increase in the conduction velocities of electrical impulses along the axon. Conversely glial cells are also involved in demyelination which can have deleterious effects on nervous activity leading to the human demyelinating diseases MS or GBS.

By investigating the expression pattern of certain reporter genes such as lacZ, the DNA regulatory elements that confer expression to myelin genes have to a great extent been identified. While certain constructs containing different lengths of MBP 5' flanking sequence conferred specific expression to oligodendrocytes, the location of the MBP-related Schwann cell targeting elements remained until recently undefined (Foran and Peterson 1992) (Gow, Friedrich et al. 1992) (Goujet-Zalc, Babinet et al. 1993) (Miskimins, Knapp et al. 1992) (Stankoff, Demerens et al. 1996). Forghani et al., were able to locate and characterise the DNA regulatory domain that exclusively controls MBP expression in Schwann cells. The domain was defined as a 0.6kb Schwann cell enhancer 1 (SCE1) sequence that when combined with the heterologous promoter hsp68 was able to drive specific and exclusive expression of the lacZ reporter gene to Schwann cells. Conversely transgenic mice bearing a -6.5kb 5' MBP promoter construct, lacking the SCE, targeted lacZ expression to oligodendrocytes but not Schwann cells (Forghani, Garofalo et al. 2001).

Further detailed analysis of the -6.5kb 5' MBP promoter revealed it to be comprised of 4 evolutionary conserved modules. While module 1 and 2 exhibit oligodendrocyte specific enhancer activity, the most proximal module 3, considered as being the core of the promoter, is important in the post weaning period and in remylinating oligodendrocytes (Farhadi, Lepage et al. 2003). Although module 4 has been demonstrated to provide robust Schwann cell specific expression it appears that this enhancer activity is primarily active in myelinating SC. This was illustrated by positive β-galactosidase staining in juvenile mice containing the SCE-hspLacZ promoter but lack of staining when the same mice reached sexual maturity (Forghani, Garofalo et al. 2001).

A

SCHWANN CELLS

B

OLIGODENDROCYTES
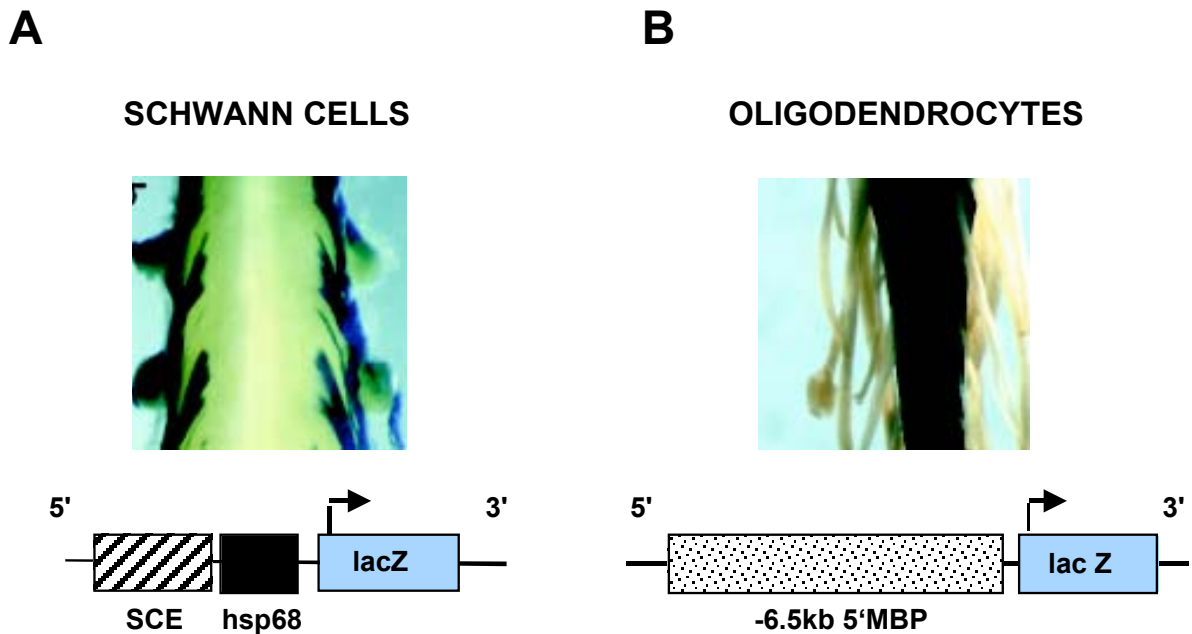
Fig. 2 Specific PNS and CNS regulatory elements are found within the MBP promoter.

Whole mount β-galactosidase labelling of lumber spinal cord and attached roots. LacZ reporter constructs bearing either the SCE1-hsp68 promoter or the –6.5kb 5' MBP promoter target expression exclusively either to Schwann cells (A) or oligodendrocytes (B) respectively. The isolation of these two specific DNA regulatory elements from within the 12kb of MBP 5' flanking sequence were important for the foundation of this project. Adapted from Forghani et al., (Forghani, Garofalo et al. 2001).

1.8 Anti-OVA TCR transgenic mice

As the frequency of T cells that are specific for a nominal Ag is normally very low, the availability of T cells with a single TCR specificity enables insights into the fate of a T cell during an immune response to be studied. Of particular interest for this project was the use of the two anti- chicken OVA TCR Tg mice; OT-I and OT-II.

OVA-specific, class I restricted TCR transgenic or simply OT-I mice were derived from the CD8+ OVA-specific T cell clone 149.42 (Kelly, Sterry et al. 1993). This T cell clone expresses the Vα2 and Vβ5 variable regions of the TCR specific for an octamer peptide from the chicken OVA (OVA $_{257-264}$; SIINFKEL) in the context of the MHC I H-2K^b molecule. Successful generation of TCR Tg mice occurs when Tg rearranged Vα and Vβ genes are integrated into the germline (Hogquist, Jameson et al. 1994). Inhibition of further endogenous β-chain rearrangement by the integration of the transgene and positive selection on MHC I molecules, results in a pronounced skewing to the CD8+ T cell subset, not only in the thymus but also in the peripheral lymphoid compartment (Clarke, Barnden et al. 2000). In contrast to the TCR β-chain, rearrangement of the TCR α-chain locus is inefficiently blocked by allelic exclusion resulting in the generation of a small population of T cells carrying a Tg TCR β-chain and an endogenous TCR α-chain. Backcrossing OT-I mice onto recombination activating gene (RAG)-1-deficient mice reduces this population in the thymus and lymph nodes (Clarke, Barnden et al. 2000). Additionally, a small population of CD4+ T cell exists in the peripheral lymph node compartment. Although they carry the Tg TCR they are unresponsive to the OVAp *in vivo*.

The TCR genes that were used to construct the OT-II mice originated from the CD4+ MHCII I-A^b restricted T cell clone 1.1 which is specific for the chicken OVA peptide (OVA $_{323-339}$; ISQAVHAAHAEINEAGR) (Barnden, Allison et al. 1998). Similar to the OT-I mice, OT-II mice contain T cells expressing the Vα2 and Vβ5 variable regions of the TCR. T cells in OT-II mice expressing the Tg TCR are positively selected on the MHC II molecule resulting in the skewing of the T cell compartment to the CD4+ T cell subset (Barnden, Allison et al. 1998). As seen in the OT-I mice, OT-II mice not only generate T cells carrying a heterogenous TCR consisting of an endogenous α-chain and a Tg β-chain but also a small population of non-OVAp specific CD8+ T cells carrying the Tg TCR.

1.9 Overall aims

The overall aim of this project was to generate and characterise two novel OVA Tg mice to study in detail the mechanisms involved in neuroautoimmune inflammatory diseases such as MS and GBS. The novel murine disease models would serve to deliver additional insights into the induction, propagation and termination of a CNS and PNS autoimmune disease. Generation of OVA Tg mice on a C57BL/6 background would enable further investigation into disease regulation at the single gene level by permitting the employment of a wide range of congenic Tg and knockout animals.

The well characterised whole chicken OVA protein was to be utilised as the model autoAg expressed constitutively and exclusively in either the CNS or the PNS. OVA has been successfully applied as a neo-Ag in autoimmune disease models of the kidney (Kurts, Heath et al. 1996), heart (Grabie, Delfs et al. 2003) and intestine (Shastri and Gonzalez 1993). The recently characterised regulatory domains of the MBP promoter were to be employed to drive specific tissue expression of OVA. The use of the SCE-hsp68 promoter would express the OVA transgene specifically in spinal roots and sciatic nerve (Schwann cells) and therefore the PNS while the –6.5kb 5' MBP promoter would drive spinal cord and brain (oligodendrocytes) or CNS specific expression. The murine disease model would be complemented with the adoptive transfer of anti-OVA TCR Tg T cells enabling potential encephalitogenic T cells to be tracked in vivo. This feature would lead to the elucidation of several so far uncertain roles of T cell substs in CNS and PNS autoimmune neuropathies.

The following questions were to be answered;

- will the OVA transgene construct containing either the SCE-hsp68 or –6.5kb 5' MBP promoter direct exclusive *in vivo* expression of the neo-Ag, to either the PNS or CNS respectively?
- can a PNS or CNS neuroinflammatory disease be induced in peripheral nerve OVA Tg (PNO) or central nerve OVA Tg (CNO) mice?
- would the disease mirror that seen in common EAN or EAE models in its clinical and subclinical symptoms?
- which immunological roles do CD4+ and CD8+ T cells play in the initiation, propagation and termination of a PNS and CNS autoimmune disease?

These questions were attempted to be answered during the following phases of the project;

In the initial phase of the project two transgene constructs were to be cloned. Each would contain the complete chicken OVA cDNA, a rabbit poly A tail and either a CNS or PNS specific promoter. The –6,5kb 5' MBP promoter would serve for the CNS construct while the 0,6kb SCE combined with the hsp68 was to be employed for the PNS construct. Initial transient transfection studies in various cell lines including Schwann cells would test for the functionality of each of the promoters by analysing the expression of OVA. Both OVA

transgenic constructs were to be microinjected into C57BL/6 oocytes by an experienced microinjector.

In the second phase of the project the generated founder CNO and PNO mice were to be characterised for the transgene expression at the genomic and protein level. Firstly a genomic PCR was to be established that would enable identification of positive founders and screening of future generations. Secondly the genomic expression of the transgene in founder mice was to be confirmed via Southern blot. Once positive founders had been identified they were to be paired with C57BL/6 mice for germline transmission. The expression of OVA at the mRNA and protein level was to be determined via RT-PCR, immunocytochemistry and immunohistochemistry. In parallel the breeding pairs of OT-I and OT-II mice were to be established.

In the third phase of the project the induction of a neuroautoimmune disease was to be attempted. Two different methods of disease induction were to be tested. Firstly active immunisation with the autoAg emulsified in CFA and secondly passive induction via transfer of activated anti-OVA Tg TCR T cells ie OT-I CD8+ and OT-II CD4+ T cells. Each regime would include the use of PT. Clinical signs of a nervous system disease were to be scored on an adapted graded scale. Subclinical disease symptoms were to be detected via histological analysis of the PNS and CNS tissues at different time points. Furthermore, LNC and splenocytes would be analysed by FACS to determine the presence and activation status of transferred clonotype+ T cells.

2. MATERIALS AND METHODS

2.1 Mice

Genotype	Source
C57BL/6	Institute for Virology and Immunbiology, Wuerzburg
OT-I	Charles River, (Sulzfeld) (Hogquist et al., 1994)
OT-II	Charles River, (Sulzfeld) (Barnden et al., 1998)

All mice used were bred and maintained on a C57BL/6 background in the pathogen-free animal facility at the Institute for Virology and Immunbiology, in accordance with animal health guidelines. Mice were used in experiments at an age of 5-8 weeks unless otherwise stated. Permission to use the OT-I and OT-II TCR Tg mice strains was kindly provided by Drs W.R. Heath, M.J. Bevan and F. Carbone.

2.2 Materials

2.2.1 Bacterial strains

***E.coli*DH5α:** F- delta(lacZYA-argF)U169 phi80delta(lacZ)M15 endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1 rpsL lambda served as the standard cloning strain.

2.2.2 Cell lines

Name	Description	Morphology	Growth property	Tissue
293T	Human	epithelial	adherent	kidney
L929	Mouse	fibroblast	adherent/ suspension	fibrosarcoma
Schwann cell (SCL 519L 14SG)	Neonatal rat	neuron	adherent	nerve

2.2.3 Oligonucleotides

Oligonucleotides were synthesised by BIG-Biotech (Freiburg) NAP^{TM} purified and dissolved in ddH$_2$O.

Name	Sequence
Bgl linker A	5' GAT CTT GCG GCC GCG GAT CC 3'
Bgl linker B	5' GAT CGG ATC CGC GGC CGC AA 5'
hspova	5' CCT GCC GGG AGC GGA ACT TT 3'
NotAse	5' GGC CGC AAC CAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATT GAA AT 3'
AseNot	5' TAA TTT CAA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT GGT TGC 3'
XhoHind	5' TCG AGG TTC ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TGT TAC CGG GGA 3'
HindXho	5' AGC TTC CCG GGT AAC ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA TGA ACC 3'
ovahspreverse	5' GCT GTC TTT TGC ACC CAG GTA TAC 3'
psp72SCE	5' ATA TTG TCG TTA GAA CGC GGC TAC 3'
SCEhsp	5' GCT GGG TCG CAC TTA ACC CTG A 3'
SalKpnHindA	5' GTG TCG ACG GTA CCA AGC TTG T 3'
HindKpnSalB	5' ACA AGC TTG GTA CCG TCG ACA CGT AC 3'
ovaPA	5' CCT TGT ACC CAT ATG TAA TGG GTC 3'
PApsp72	5' GCC AGA TTT TTC CTC CTC TCC TG 3'
b-actin sense	5' CCA GGT CAT CAC TAT TGG CAA CGA 3'
b-actin antisense	5' GAG CAG TAA TCT CCT TCT GCA TCC 3'
ova	5' TGA GGA GAT GCC AGA CAG AT 3'
ova reverse	5' TTC CAG GAT TCG GAG ACA GT 3'

2.2.4 Peptides and Proteins

Anti-OVA TCR Tg mice were stimulated with the following chicken OVA peptides:

Amino acid sequences:

OT-II; OVAp$_{323-339}$; ISQAVHAAHAEINEAGR
OT-I; OVAp$_{254-267}$; SIINFEKL

In addition, whole OVA grade II (crude dried chicken egg white) and later in the course of the project whole OVA grade V, min. 98% agarose gel electrophoresis; purified by crystallisation, lypholised powder, was used. (Sigma, Deisenhofen, Germany).

2.2.5 Interleukins

Interleukin	Form	Source
murine IL-2	P30-IL-2 supernatant	F Melchers group
murine IL-12	supernatant	E Schmidt group, Mainz

2.2.6 Monoclonal antibodies

All antibodies used were directed against mouse if not otherwise stated.

Name	Clone	Isotype	Source
CD16/CD32 (anti-Fc Receptor)	2.4G2	Rat IgG2b	Lamers MC et al., 1982
CD3	145-2C11		Leo O et al., 1987
CD28	37.51		Harding FA et al., 1992
CD4 (L3T4)	GK1.5	Rat(Lewis)IgG2b,k	BD
CD8a (Ly-2)	53-6.7	Rat(LOU/Ws1/M) IgG2a,k	BD
CD8b (Ly3.2)	53-5.8	Rat (LOU/W1)IgG2,k	BD
Va2	B20.1	Rat (LOU/W1)IgG1a,l	BD
Vb5	MR9.4	mouse IgG1,k	BD
CD44 (Pgp-1, Ly-24)	IM7	Rat IgG2b,k	BD
CD62L (L-selectin, LECAM, Ly-22)	Mel-14	Rat (Fischer) IgG2a,k	BD
CD69 (Very early activating antigen)	H1.2F3	Armenian Ha IgG1	BD
CD25 (IL-2 Rc a chain, p55)	7D4	Rat (Lewis) IgM,k	BD
CD45RB	RA3-6B2	Rat IgG2a	BD
CD5 (Ly-1)	53,7.3	Rat(LOU/Ws1/M) IgG2a,k	BD
HSA	30-F1	rat IgG2c,k	BD
IL-4	11B11	Rat IgG1	BD
IFNg	XMG1.2	Rat IgG1	BD
CD3 (anti-human)	MCA 1477	Rat IgG1	Serotec
Mac-3	M3/84	Rat (LewisxBN)F1 IgG1k	BD
OT-1 tetramer			Skinner PJ et al., 2000
mouse monoclonal anti-chicken egg albumin	OVA-14		Sigma, Deisenhofen, Germany

2.2.7 Second step reagents

Name	Source
Affinity purified goat anti-rabbit IgG (H+L) polyclonal, biotinylated	Linaris, Vector, Wertheim, Germany
Affinity purified mouse adsorbed rabbit anti-rat IgG(H+L) biotinylated	Linaris, Vector
Human adsorbed goat anti-mouse IgG1 alkaline phosphatase conjugated	Southern Biotechnology Associates, Birmingham, Alabama, USA
Affinity purified goat anti-mouse IgM alkaline phosphatase conjugated	Dianova, Hamburg, Germany
Sheep anti-mouse IgG horse raddish peroxidase linked whole antibody	Amersham Life Science, Freiburg, Germany

2.2.8 Polyclonal antibodies, sera, adjuvants

Product	Source
Mouse serum	own lab
rabbit anti-OVA antiserum	Sigma
CFA	Sigma
PT	Sigma

2.2.9 Enzymes and substrates

Product	Source
PanScript Taq-DNA-Polymerase	Biotech GmbH, Aidenbach, Germany
Proteinase K	Gibco BRL, Eggenstein, Germany
Deoxyribonuclease I	Sigma
Restriction enzymes	MBI Fermentas, Heidelberg, Germany
KF (Klenow Fragment of DNA polymerase 1)	MBI Fermentas
CIP (calf intestinal alkaline phosphatase)	MBI Fermentas
SAP (shrimp alkaline phosphatase)	Boehringer-Mannheim, Germany
T4 DNA ligase highly concentrated	Gibco-Invitrogen, Karlsruhe, Germany
DAKO StreptABComplex	DAKO, Hamburg, Germany
DAB tablet (10mg)	Kem-En-Tec Diagnositics, Copenhagen, Denmark
p-Nitrophenylphosphate tablet (20mg)	Sigma
Chemiluminescence Reagent	NEN Life Science Products, Boston, Massachusetts, USA

2.2.10 Chemicals

Laboratory chemicals were if not otherwise mentioned, from Roth (Karlsruhe) or Merck (Darmstadt).

2.2.11 Frequently used buffers and solutions

Solution	Recipe
Alsevers	4.2g NaCl 8.0g $C_6N_5Na_3O_7.2H_2O$ 22.5g D(+) Glucose ad. 1L ddH_2O; adjust to pH 7.2 with 10% citric acid (0.6g)
BSS/BSA **(Balanced Salt Solution/** **Bovine Serum Albumin)** **BSSI**	10g Glucose 0.6g KH_2PO_4 2.38g $NaHPO_4.H_2O$ 0.1g Phenol red ad. 1L ddH_2O
BSSII	1.86g $CaCl_2.2H_2O$ 4,0g KCl 80g NaCl 2.0g $MgCl_2.6H_2O$ 2.0g $MgSO_4.7H_2O$ ad. 1L ddH_2O
Working solution **(100mL)**	0.11L BSSI 0.11L BSSII

	2g BSA ad. 1L ddH$_2$O
Coating buffer	0.05M Tris-HCl pH9,5
Coomassie Blue Stain	0.125% w/v Coomassie Blue R250 45% v/v Methanol 9% v/v glacial acetic acid in ddH$_2$O
Coomassie Blue Destain	5% v/v Methanol 7.5% glacial acetic acid in ddH$_2$O
Detergent Solution (X-gal stain)	10x PBS 2mM MgCl2 0.01% NaDoc 0.02% NP40
1M Diethanolamine buffer	26,28mL 1M diethanolamine pH9.8 (Merck) 125uL 0.5mM MgCl$_2$ ad. 250mL ddH$_2$O
Electrophoresis running buffer 10x	100mL 1% SDS 144.13g Glycine 30.3gTris base ad. 4L ddH$_2$O
Electrophoresis running buffer 1x	10mL (10x electrophoresis buffer) 90mL ddH$_2$O
ELISA block	0.2% Gelatine dissolved in 1x PBS (-CaCl2 -MgCl2) in the microwave cooled down for 1h at 4ºC.
Ethidium Bromide	10mg/mL in ddH$_2$O; (Roth)
Gel loading dye (10x)	0.5% w/v Bromophenol blue 0.5% v/v Xylenecyanol 0.5% v/v Glycerine 10mM EDTA
Gey's Solution **Stock A**	35.0g NH$_4$Cl 1.85g KCl 1.5g Na$_2$HPO$_4$.2H$_2$O 0.12g KH$_2$PO$_4$ 5.0g Glucose ad. 1L ddH$_2$O, filter sterile
Stock B	0.42g MgCl$_2$.6H$_2$O 0.14g MgSO$_4$.7H$_2$O

	0.34g $CaCl_2.2H_2O$ ad. 100mL ddH_2O, filter sterile
Stock C	2.25g $NaHCO_3$ ad. 100mL ddH_2O, filter sterile
Working solution (100mL)	20mL StockA 5mL StockB 5mL StockC ad. 100mL ddH_2O
FACS Buffer	PBS/BSA 0.1% 0.02% v/v NaN_3
Fixation Buffer (Histology; 4%) **(FACS staining; 1%)** **(X-gal staining; 4%)**	40g Paraformaldehyde dissolved in 500mL ddH_2O at 60ºC under constant stirring O/N ad. 500mL 1x PBS adjust to pH 7.3-7.5 with HCl, store RT Formaldehyde (37%) v/v in 1x PBS 4% paraformaldehyde 0.1M PIPES (pH6.9) 2mM $MgCl_2$ 5mM EGTA (pH 8.0)
Injection Buffer	10mM Tris-HCl 0.15 mM EDTA in ddH_2O, pH7.5; filtered sterile
LB (Luria Bertani) Medium **LB Medium + Ampicilin** **LB Agarose plates**	20g LB ad. 1L ddH_2O; autoclaved LB Medium + 100ug/mL (stock 0,1g/mL) Ampicilin added prior to use from -20ºC stored aliquots LB Medium + 15g Agar after autoclaving poured onto 200mL petri dishes
Lysis buffer	20mM Hepes buffer pH 7.4 2mM EGTA pH 7.9 50mM beta-glycerophosphate 1% TritonX100 10% Glycerol 50mM NaF 0.04% Na Azide prior to use add the following protease inhibitors: 1mM dTT 1mM Na-ortho-Vanadate 2uM Leupeptine 0.4mM PMSF

PBS (Phosphate Buffered Saline)	8.0g NaCl 0.2g KCl 1.15g $Na_2HPO_4.2H_2O$ 0.2g $KH_2.PO_4$ 1.67g $CaCl_2.4H_2O$ 0.1g $MgCl_2.6H_2O$ 0.1g BSA ad. 1L ddH_2O; adjust to pH 7.2 with HCl
Resolving gel 10% (10mL)	3.3mL Bisacrylamide 30:0,08(%w/v) 2.5mL 1M Tris (pH6,8) 0.1mL 10%SDS 4mL ddH_2O addition directly before use 0.004mL TEMED 0.1mL 10% APS
Saponin (permeabilisation) Buffer	0.1% w/v Saponin 0.02% v/v NaN_3 1% v/v FCS in 1x PBS; pH 7.4
20x SSC	3M NaCl 0.3M Sodium citrate, pH 7.4
Stacking gel 5% (3mL)	0.5 mL Bisacrylamide 30:0,08(%w/v) 0.38 mL 1,5M Tris(pH8,8) 0.03mL 10%SDS 2.1 mL ddH_2O addition directly before use 0.003mL TEMED 0.03 mL 10% APS
TAE (50x)	2,42g Tris 57,1mL Citric acid 100mL 0,5M EDTA ad. 1L ddH_2O; pH 8.0
TBE (10x)	108g Tris 55g H_3BO_3 3,7g EDTA pH 8.0 ad. 1L ddH_2O
TE Buffer	10mM Tris-HCl 1mM EDTA in ddH_2O; pH 8.0
TfBI	30mM KOAc 50mM $MnCl_2$ 100mM $CaCl_2$ filter sterile before adding 15% glycerol

TfBII	10mM Na-MOPS pH 7.0 75mM $CaCl_2$ 10mM KCl filter sterile before adding 15% glycerol
TENS (1x)	50mM Tris-HCl pH 8.0 100mM EDTA pH 8.0 100mM NaCl 1% SDS
Transfer buffer 10x	116g Tris base 580g Glycine ad. 4L ddH_2O
Transfer buffer 1x	100mL (10x transfer buffer) 200mL Methanol ad. 700mL ddH_2O
Trypan Blue	1% v/v in PBS/NaN_3 (Sigma)
WASHING BUFFERS:	
ELISA	0.05% Tween20 in 1x PBS (-$CaCl_2$ -$MgCl_2$)
Southern blot	0.1x SSC 0.1% SDS
Western blot	0.1% Tween20 (Polyoxyethylene, Sorbitan, Monolaurate) in 1x TBS (100mM Tris-HCl pH 7.5, 0.9% NaCl)
X-gal stain	10x PBS 2mM $MgCl_2$
YT/Mg	20g bacto-tryptone 5g yeast extract 5g NaCl 2.5g $MgSO_4$ ad. 1L ddH_2O and autoclave
X-gal staining solution	10x PBS (-$MgCl_2$, -$CaCl_2$) 5mM $K_3Fe(CN)_6$ 5mM $K_4Fe(CN)_6.3H_2O$ 2mM $MgCl_2$ 0.01% NaDoc 0.02% NP40 1mg/mL X-gal
X-gal stock	40mg/mL in Dimethylformamide make up in glass and store at 4ºC, protected from light

2.2.12 Media and solutions for cell culture

Medium	Recipe
ATV	80g NaCl 4g KCl 10g D(+) Glucose 5.8g NaHCO$_3$ 5g Trypsin Difco 250 2g EDTA Diatriumsalt Dihydrate ad. 10L ddH$_2$O
293T **(DMEM+)**	500mL DMEM (Gibco) 1 aliquot SC filtered sterile
L929 **(RPMI+)**	500mL RPMI1640 (Gibco) 1 aliquot SC filtered sterile
Freezing medium	10% v/v DMSO (Roth) 40% v/v FCS 50% v/v complete cell culture medium filtered sterile; stored at -20 C
Schwann cell	RPMI 1640 (Gibco) supplemented with; 10% v/v FCS 100U/mL penicillin 100ug/mL streptomycin 2mM $_L$-Glutamine 5ug/mL insulin (Sigma) (stock 5mg/mL in PBS) 0.1mM β-Mercaptoethanol (Gibco) filtered sterile
SC **(Supplement complete)**	25mL FCS (heat inactivated at 56 C for 45min) 5mL 100mM Na Pyruvate (Gibco) 5mL 100x non essential amino acids (Gibco) 1.2mL 10^{4}U/mL penicillin (Grünenthal) 1.2mL 0.1g/mL streptomycinsulphate (Sanavita) 0,5 mL 50mM β-Mercaptoethanol (Gibco) 7mL 342mM $_L$-Glutamine (Roth) add 1 aliquot to 500mL cell culture medium
Thawing medium	10% v/v FCS in complete cell culture medium filtered sterile
X-VIVO-15 **complete medium**	1L X-VIVO-15 (Bio Whittaker, Vervier Belgium) 2 aliquots SC filtered sterile

2.3 Methods

2.3.1 Molecular cloning

2.3.1.1 Filter paper plasmid recovery

Plasmids that were received on filter paper were recovered by cutting out the indicated circle and placed into an Eppendorf tube. 50μL of ddH$_2$O was added, vortexed and left stand for 5 min at RT to rehydrate the plasmid. After brief centrifugation the supernatant was used to transform competent bacteria.

2.3.1.2 Digestion of DNA with Restriction Endonucleases

The selection criteria for the use of specific restriction enzymes was based on the following; desired fragment size, methylation sensitivity, generation of blunt-ended/sticky ended fragments and compatibility of reaction conditions when more than one enzyme was used. Restriction enzymes were used in the appropriate reactions containing water, buffer, DNA and enzyme according to the manufacture's guidelines. Incubation times varied according to the type of digest. Incubations at 37°C were performed for approx. 2 h for analytical digests while a minimum of 4h to overnight was required for preparative digests. It was important that the restriction enzyme added to the reaction did not exceed 10% of the total reaction volume as the glycerol in which the enzyme is supplied can inhibit the digestion.

2.3.1.3 Gel electrophoresis

Agarose gels (0,6-1,5%) in different sizes were used for the analysis of either genomic or plasmid DNA. For plasmid DNA they were useful in determining the size and conformation of nucleic acids, quantification of the amount of DNA loaded and allowing separation and extraction of DNA fragments. Agarose gels were prepared with TBE or TAE buffer and ethidium bromide at an end concentration of 0,5ug/mL. Before running the gel, DNA probes were prepared in 1/5 volumes of 6x loading buffer. Molecular weight markers were always included in the run for DNA fragment size analysis. Standard markers included; 100bp gene ruler (MBI), 1kb gene ruler (MBI) and genomic marker II (MBI) Hind III (AG Nitschke). Electrophoresis times were generally 1-2 h at 100-140V while Southern blot gels were run overnight at 30-40 V. Ethidium bromide-DNA complexes were visualised with a transiluminator and gels were photographed with a digital gel documentation system from Cybertech.

2.3.1.4 Gel extraction

The desired DNA fragment was quickly extracted from an agarose gel using a scapel under low strength UV light (λ = 300nm) to limit DNA damage. The gel slice was then purified using the Qiagen Gel Extraction kit (Qiagen, Hilden). The gel slice was accordingly solublised for 10min, or until completely dissolved, at 50ºC in a buffer containing chaotropic salts. Further purification steps were carried out as in 2.3.1.5.

2.3.1.5 Purification of DNA using an ion-exchange column (Qiagen/Genomed)

For the purification of DNA fragments such as those generated in restriction digests, the Qiagen Nucleotide Removal Kit (Qiagen, Hilden) was used. The principal of the kit is based on the binding of DNA to a silica-gel covered membrane in a spin-column format, after solublisation in the buffer containing chaotropic salts. Subsequent wash steps remove RNA and other impurities after which the DNA is eluted in ddH_2O or TE buffer. All steps were carried out according to the manufacture's instructions and the exact protocol is to be found in the QIAquick handbook.

Alternatively the JETSORB gel extraction kit (Genomed, Bad Oeynhausen) which is also based on the ‚bind-wash-elute' principal, was used. Essentially dsDNA was extracted from agarose gels and bound to a matrix surface consisting of a modified silica resin from which the DNA was eluted in either ddH_2O or TE buffer. One advantage of this protocol is the absence of an ethanol/isopropanol precipitation step.

2.3.1.6 Purification of PCR products (Qiagen)

Purification of PCR products, such as those produced in the preparation of sequencing reactions, was carried out using the Qiagen PCR purification kit (Qiagen, Hilden). As in 2.3.1.5, the purification principal is based on the adherence of the DNA to a silica-gel covered membrane in a spin-column format. The washing steps removed nucleotides, primers and short stranded DNA molecules ensuring that only longer stranded DNA molecules were retained in the eluate.

2.3.1.7 Precipitation of DNA

Alcohol precipitation was commonly carried out for recovering or concentrating DNA. Soluble DNA was precipitated by addition of 2,5 volumes ethanol or 0,7 volumes isopropanol at RT and mixed thoroughly. The DNA sample was pelleted by centrifugation at 13000 rpm, 4ºC for 10-20 min. After decanting the supernatant, the pellet was washed in RT 70% ethanol, at 13000rpm, 4ºC for 5-15min. Subsequently the pellet was air dried for 5-20 min before being dissolved in ddH_2O.

2.3.1.8 Quantification of DNA

DNA quantification by spectrophotometry

DNA samples were diluted 1:25-1:50 depending on estimated concentrations. DNA concentration was determined by measuring the absorbance at λ =260nm (A260) in a spectrophotometer using a quartz cuvette. The concentration was calculated based on the DNA coefficient of 1 unit at 260nm being equal to 50μg/mL DNA. The purity of the DNA was determined by analysis of the A260/A280 ratio. Ratios around 1.8 were regarded as being free of protein contamination.

DNA quantification by agarose gel analysis

Small amounts of DNA were sometimes more effectively quantitated using agarose gel analysis. A DNA sample was run alongside known concentrations of DNA of the same or similar size. The amount of sample loaded was estimated visually based on band intensity with the standard. The standard generally used was the lambda III DNA marker.

2.3.1.9 Filling of protruding 5' termini with Klenow fragment and removal of 3' termini with T4 DNA polymerase

To enable DNA fragments with incompatible 5' overhangs to be ligated with one another, the overhangs were filled with the Klenow Fragment (KF) of *E. coli* DNA polymerase I. The resulting blunt ends then enabled ligation.

A 40μL reaction mixture contained;

5-10μg DNA fragment (isolated from an agarose gel)
4μL 10x KP buffer
1μL dNTP mix (2mM each)
5-10 U KF
ad. 40μL ddH$_2$O

and was incubated for 15min at 37ºC. KF was inactivated by heating the reaction mixture at 75ºC for 10 min. The DNA was recovered either by using the Qiagen Nucleotide Removal kit (Qiagen, Hilden) or by agarose gel extraction.

The 3' exonuclease activity of the bacteriophage T4 DNA polymerase enabled removal of 3' protruding termini.

A 30μL reaction mixture contained;

5-10μg DNA fragment in 20μL (isolated from an agarose gel)
3μL 10x T4 Polymerase buffer
1.5μL dNTP mix (2mM each)
5-10U T4 Polymerase
ad. 30μL ddH$_2$O

The reaction mixture was incubated for 15min at 37ºC. Enzymatic activity was inactivated by heating at 75ºC for 10min. Prior to ligation the DNA was recovered as above.

2.3.1.10 Dephosphorylation of DNA ends with alkaline phosphatase

Prior to ligation restriction endonuclease digested plasmids were dephosphorylated with alkaline phosphatase. Removal of 5' phosphates from linearised vector DNA helped prevent re-ligation in the case of incomplete digestion and improved ligation efficiency. CIP was directly added to the restriction digest reaction when buffers were compatible for both the CIP and the restriction enzyme.

A 50µL reaction mixture contained;

1,5-2,5µg DNA
10µL 5x calf intestine phosphatase (CIP) buffer
10 U CIP

and was incubated for 30-40min at 37ºC and inactivated by heating to 75ºC for 20 min. The DNA was recovered with the Qiagen Nucleotide removal kit (Qiagen, Hilden). Alternatively the schrimp alkaline phosphatase (SAP), was used in an equivalent reaction. In contrast to CIP, SAP can be heat inactivated by incubating for 20min at 80ºC.

2.3.1.11 Hybridisation of oligonucleotides

For the generation of a double stranded oligonucleotide or linker, complementary single stranded oligonucleotides were incubated in a water bath at 70ºC for 2 min. After switching the water bath off, the reaction mixture remained in the water until it cooled down to approx. RT. Double stranded oligonucleotides were designed in such a way that after hybridisation sticky ends were generated which were important for subsequent ligation with a cut vector. After cooling down, the synthetic linker was immediately used in a ligation reaction.

2.3.1.12 Ligation of DNA fragments

In order to construct new DNA molecules the desired fragments were ligated with the highly concentrated T4-DNA ligase. Ligation of blunt ended or sticky ended DNA fragments were mixed at a molar ratio of vector to insert 1:4- 1:10 respectively.

a 20 µL reaction mixture contained;

10ng – 1µg vector
5x Ligase buffer (1/10 volume + 1/10 ATP 10mM final concentration)
5 U T4 DNA Ligase
ad. 20µL ddH$_2$O

Incubation times varied:
sticky end: 1-1.5h RT to 24h 4ºC
blunt end: 24h 4ºC

It was important to include a mock ligation control, containing only vector and no insert, to check for religation of vector. A part of the mix was directly used for the transformation of competent bacteria.

2.3.1.13 Generation of competent bacteria

Competent bacteria have the ability to take up DNA. Before beginning with the protocol it was important to pre chill everything that came into contact with the bacteria. This included the centrifuge bottle, rotor and pipettes. 1mL of LB/Mg was innoculated with a fresh bacterial colony or glycerol stock and incubated at 37ºC for 1h with constant shaking. The bacteria

were transferred to 40mL warm LB/Mg and grown to mid-log for approx. 5h. Bacteria were added to 200mL warm YT/Mg and grown to OD600=0.6. After removal from the incubator, bacteria were chilled in a water/ice bath for 1/2h to ensure complete shut down of their metabolism. Bacteria were then centrifuged at 3500rpm for 15 min at 2ºC in a pre chilled rotor bottle before being resuspended in 80mL of ice cold TfBI followed by centrifugation. This was followed by resuspension in 10mL ice cold TfBII before aliquoting 200μL into cryotubes prechilled in an ethanol/dry ice bath. Cryotubes containing competent bacteria were stored in liquid nitrogen. Freshly made competent bacteria were always tested with old competent bacteria to compare transformation efficiency.

2.3.1.14 Transformation of competent bacteria

Plasmid DNA (60-200ng) was pipetted into a prechilled Eppendorf tube before being mixed with 100-200μL freshly thawed competent bacteria. The reaction was incubated for 30 min on ice before being heat shocked for 90 sec at 42ºC in a water bath. The transformed bacteria were returned to ice for 2 min before addition of 800μL of prewarmed LB medium in a 15mL tube. The transformed bacteria were then incubated for 40 min at 37ºC under constant shaking before different volumes of the transformed bacteria were plated out onto LB-Amp plates. The LB-Amp plates were incubated overnight at 37ºC to support colony growth.

2.3.1.15 Preparation of glycerol stocks

To ensure long term storage of bacteria containing important plasmids, glycerol stocks were prepared. 100μL of 100% glycerol was added to 900μL of a logarithmic-phase *E. coli* culture and vortexed vigorously to ensure even mixing. Vials were then stored at –80ºC.

2.3.1.16 Plasmid mini purification (analytical)

Alkaline lysis is a common method for the lysis of bacteria prior to plasmid DNA purification. The protocol involves four essential steps; resuspension, lysis, neutralisation and clearing of lysates. The mini purification protocol is designed for the high throughput analysis of a number of bacterial colonies for the correct plasmid. The protocol was essentially carried out according to the Qiagen Plasmid Mini Handbook (Qiagen, Hilden). Bacteria from an overnight 3mL LB-Amp starter culture were centrifuged 13000rpm for 10 min at 4ºC and resuspended in ice cold buffer P1 (50mM Tris-HCl, pH 8.0, 10mM EDTA, 100μg/mL RNaseA). Lysis of bacteria followed upon addition of buffer P2 (200mM NaOH, 1% SDS) and incubation at RT for 5 min. Cell debris, proteins and genomic DNA were precipitated by addition of ice cold 300μL buffer P3 (3M KAc, pH 5.5) and incubation on ice for 15 min. The lysates were cleared by centrifugation at 13000 rpm for 15 min, 4ºC. The plasmid DNA from the supernatant was precipitated upon addition of 0.7 volumes of isopropanol and pelleted by centrifugation at 13000 rpm, 4ºC for 15 min. After carefully decanting the supernatant the pellet was washed once with 70% ethanol at RT before being briefly air dried and resuspended in an appropriate volume of ddH$_2$O, (20μL). Plasmid DNA was stored at –20ºC until identification of the correct plasmid via restriction digests was carried out.

2.3.1.17 Plasmid maxi purification (preparative)

Plasmid maxi purification allowed for the production of up to 500μg of plasmid DNA after positive plasmid identification in section 2.3.1.16. The protocol was essentially carried out

according to the Qiagen Plasmid Purification Handbook (Qiagen, Hilden). A single colony was picked from a freshly streaked LB-Amp plate and used to inoculate a 3mL LB-Amp starter culture, which was incubated for approx. 8h at 37ºC. The starter culture was diluted 1/100-1/500 into LB-Amp medium grown overnight. On the following day, the bacterial culture was harvested by centrifugation at 6000rpm, 15min at 4ºC. As in section 2.3.1.16, the pellet was resuspended in Buffer P1followed by bacterial lysis and cell debris precipitation by incubating in buffer P2 and buffer P3 respectively. The lysate was efficiently cleared by centrifugation twice at 1300rpm for 30 at 4ºC or filtration using a QIAfilter Maxi cartridge. The plasmid DNA was eluted from the supernatant using a QIAGEN-tip (DEAE column). Refer to the Qiagen handbook for further details. Essentially the supernatant was applied to an equilibrated column and emptied via gravity flow. The column was washed twice before being eluted. Plasmid DNA was precipitated out of the elute by addition of 0.7 volumes isopropanol and centrifugation at 13000rpm for 30 min at 4ºC. After carefully decanting the supernatant the pellet was washed once with 70% ethanol and centrifuged for 10 min, before being briefly air dried. The pellet was redissolved in an appropriate volume of ddH$_2$O. Concentration of the plasmid DNA was determined via spectrophotometry and gel agarose analysis. Plasmid DNA was stored at –20ºC prior to analysis.

2.3.1.18 DNA sequencing

To sequence DNA the AbiMed Prism 310 Sequencer (Perkin-Elmer) was used. Essentially four differently fluorescently labelled dNTPs are added to a thermocycle sequencing reaction whereby the products are separated via capillary electrophoresis before being passed through an analytical unit.

A 10μL sequencing reaction contained the following:

0.5 – 1μg plasmid DNA
70ng primer
2.5-5% DMSO
2.5μL „Abi Prism Big Dye DNA Sequencing Kit" (Perkin-Elmer)
ad. 10μL ddH$_2$O

The reaction was briefly mixed before being placed into a T3 thermocyler, (Biometra) using the following programme:

1. 30 sec 96ºC
2. 15 sec 50ºC
3. 4 min 60ºC

Steps 1-3 for 25 cycles

The reaction product was precipitated in a 1.5μL Eppendorf reaction tube by the addition of

90μL ddH$_2$O
10μL 3M NaAc pH5.2
250μL EtOH

followed by centrifugation at 14000 rpm for 20 min. The resulting pellet was washed twice with 70%EtOH, resuspended in 15μL TSR (template suppressive reagent, Perkin-Elmer), denatured at 94ºC for 2 min and immediately chilled on ice. The reaction was transferred to a Genetic Analyser Tube, Perkin-Elmer, and analysed in the DNA sequencer, AbiMed Prism 310.

2.3.2 Southern blot

2.3.2.1 Denaturation of double stranded DNA

To confirm the insertion of the transgene, Southern blots were carried out using PNO founder mice tail genomic DNA. 10-15μg tail genomic DNA was digested with the respective restriction enyzme overnight. A highly concentrated form of the restriction enzyme was used to digest genomic DNA at an excess of 25U/μg of DNA. On the following day, the digest was loaded onto a 0.8% TBE gel containing ethidium bromide and run overnight at 30-40V. The resulting bands were photographed using a ruler.

2.3.2.2 Transfer of DNA onto membrane

To transfer the genomic DNA onto nitrocellulose membrane an alkaline blot was conducted. The gel was incubated for 20 min in 0.4M NaOH at RT on a shaker. Subsequently the blot was built up in a tray containing a reservoir of 0.4M NaOH in the following sequence; ontop of an upside down gel tray one sheet of Whatmann 3MM paper which reached into the reservoir, the upside down gel, the nitrocellulose membrane (Hybond X, Amersham, Braunschweig), 3 sheets of Whatmann 3MM and a stack of paper towelling. For the overnight transfer the blot was held down with a glass plate on which stood a light weight (150-200g). Overnight capillary action forces soaked the paper towelling with the NaOH reservoir. As a result the DNA was transferred from the gel and associated with the charged nitrocellulose membrane. Following overnight transfer, the membrane was washed once in Southern blot washing buffer and dried briefly on a piece of Whatmann paper. An UV crosslinker (Stratagene, Amsterdam, Netherlands) crosslinked the DNA to the membrane. The 13x15cm membrane was prehybridised as well as hybridised in 20mL QuickHyb-hybridisation solution (Stratagene). Hybridisation took place in a glass roller bottle, cleaned thoroughly with ddH$_2$O. Forceps were used to roll the membrane ends before being placed into the roller bottle in the absence of air bubbles. Prehybridisation took place in a hybridisation oven for 1h at 68ºC prior to addition of the radioactively labelled probe.

2.3.2.3 Radioactive labelling and hybridisation of probe

To radioactively label the probe, the Oligo labelling kit (Gibco/Invitrogen, Karlsruhe) was used. The kit is based on the random priming method according to Feinburg and Vogelstein. Essentially, the procedure relies on the ability of random hexanucleotides to anneal nonspecifically to a DNA template. 50ng of the DNA probe was denatured at 95ºC for 5 min followed by 2min on ice. The primer-template provides a substrate for the Klenow fragment of DNA polymerase I which synthesises new DNA. This feature of the enzyme is used to substitute a radioactively labelled nucelotide ($\alpha[^{32}P]$-dATP or ($\alpha[^{32}P]$-dCTP) in place of a non-radioactively labelled one. Radioactively labelled dNTPs (10μCi/μL) were from Amersham-Pharmacia, Buckinghamshire, England). The radioactively labelled probe was purified using the Nucleotide removal kit (Qiagen) and added to the prehybridisation reaction for 2h.

2.3.2.4 Development of blot

The membrane was washed once with 60-70mL Southern blot washing solution followed by 3x 30min washes with 35mL washing buffer in the hybridisation oven. Subsequently the membrane was sealed in plastic and exposed to a Kodak x-ray film at –80ºC overnight to a week depending on the strength of signal developed.

2.3.3 RNA analysis

2.3.3.1 Homogenisation of organs

It was important throughout the complete protocol to work quickly and in an RNase free environment to prevent RNA degradation. A homogeniser with twin blades was used to destroy organ tissue prior to RNA extraction. The RNeasy mini kit (Qiagen) was used to extract RNA. Organs were removed from mice, weighed and immediately frozen away in liquid nitrogen to be stored at -70°C until further use. To remove excess tissue the tip of the homogeniser was rinsed once in 70% ethanol and once in RLT (a highly denaturing guanidine isothiocyanate containing buffer) before use and between organs. 5mL screw cap tubes were prefilled with 600μL RLT buffer before adding 0.03g of frozen organ and homogenising it for 40 sec at 22 000U. Foaming was kept to a minimum by keeping the tip of the homogeniser submerged. Samples were either kept on dry ice ready for further processing or stored at -70°C until further use.

2.3.3.2 Extraction of RNA

The protocol was essentially carried out according to the Qiagen RNeasy mini kit handbook (Qiagen). Essentially, ethanol is added to the disrupted tissue lysates promoting selective binding of RNA to a RNeasy silica-gel membrane. Contaminants are washed away before being eluted in RNase-free water.

The lysate was transferred to an Eppendorf and centrifuged at maximum speed for 3 min. One times volume 70% ethanol was added to the supernatant and mixed immediately by pipetting up and down before applying 700μL of the lysate and precipitate to an RNeasy mini spin column before centrifuging for 15 sec at full speed. The flow through was discarded and the spin column was washed with 700μL RW1 buffer and centrifugation at full speed for 15 sec. The RNeasy column was transferred to a new 2mL collection tube before being washed a second time with 500μL RPE buffer and centrifugation for 15 sec at full speed. Flow through was discarded before addition of another 500μL of RPE buffer and centrifugation at full speed for 2 min to dry the RNeasy silica-gel membrane. RNA was eluted by placing the spin column into a 1.5mL Eppendorf tube and pipetting 20-40μL of nuclease free H_2O (Ambion) directly onto the membrane before centrifugation at full speed for 1 min. The elution step was repeated by using the first eluate. The sample was stored at -20°C before further use.

2.3.3.3 Spectrophotometric determination of RNA concentration

RNA samples were diluted 1:20-1:50 in a total volume of 60μL of nuclease free water. RNA concentration was determined by measuring the absorbance at $\lambda = 260$nm (A_{260}) in a spectrophotometer using a quartz cuvette. The concentration was calculated based on the RNA coefficient of 1 unit at 260nm being equal to 40μg/mL RNA. The purity of the RNA was determined by analysis of the A_{260}/A_{280} ratio. RNA samples with an absorbency ratio of 1.8 were considered to be free of protein contamination.

2.3.3.4 DNase I treatment of RNA and transcription to cDNA

DNase I treatment

DNaseI treatment was carried out using the DNaseI kit (Sigma). Duplicate reaction tubes were prepared for the treatment with and without RT. To 5µg RNA in 8 µL nuclease free water, 1µL 10x reaction mix and 1µL DNaseI was added before incubation at RT for 15min. To bind calcium and magnesium ions and to inactivate the DNaseI, 1µL of Stop solution was added. The solution was heated at 70ºC for 10 min and chilled on ice to denature both the DNaseI and the RNA before continuing with first strand cDNA synthesis.

Synthesis of First Strand cDNA

The MBI cDNA first strand synthesis kit was used to generate cDNA. The reaction was carried out on fresh ice. A control RNA was incorporated.

A following 20 µL reaction mixture contained:

5µg template RNA in	8µL H_2O
oligo(dT)18 primer	1µL
ad. nuclease free H_2O	11µL

The reaction mixture was mixed gently before being spun down for 3-5 sec in a microcentrifuge. The mix was incubated at 70ºC for 5 min, chilled on ice and drops were collected by brief centrifugation.

The following components were added to the reaction:

5 x reaction buffer	4µL
Ribonuclease inhibitor (20U/µL)	1µL
10mM dNTP mix	2µL

mixed gently, drops collected by brief centrifugation and incubated at 37ºC 5 min.

Finally 2µL (20U/µL) M-MuLV RT was added to all tubes except the controls containing no RT before being incubated at 37ºC for an hour. RT was inactivated by heating at 70ºC for 10 min and chilled on ice before being stored at –20ºC until further use.

2.3.4 Transfection

2.3.4.1 Liposome mediated transfection

Transient expression in different cell lines was achieved by complexing liposome suspensions (Roche) in serum-free medium with linearised plasmid DNA.

Cells were subcultured one day before transfection. Adherent cells were plated out at $1 \cdot 10^6$ cells per 100mm cell culture dish. On the following day the DOTAP/DNA mixture was freshly prepared in 24 well polystyrene plates. To 60µL DOTAP diluted in 190µL serum free cell culture medium, 10µg plasmid DNA in 100µL serum free cell culture medium was added. The DOTAP/DNA mixture was incubated for 10-15 min at RT. Fresh serum free cell culture medium was added to the adherent cells to which the DOTAP/DNA mixture was added and

gently mixed by rocking the cell culture dish to ensure equal distribution of the transfection mixture. Cells were incubated with the lipsosome DNA complex for 3-10h at 37ºC. Culture medium was replaced with complete fresh medium and cells were incubated an additional 24-48h before transfection efficiency was determined by performing a β-galactosidase assay or Western blot.

2.3.4.2 Electroporation

Transient transfection of plasmid DNA was also attempted via electroporation. Cells were placed into a cuvette, connected to a power supply and subjected to a high-voltage pulse of defined magnitude and length.

Prior to electroporation for transient transfection, $1\text{-}4\cdot10^7$ cells, were grown to 80% confluency in complete medium without antibiotics. For electroporation, $8\cdot10^7$cells/mL were resuspended in 0.5-0.8mL ice-cold PBS in an electroporation cuvette (width 4mm) to which 10-20μg plasmid DNA was added followed by incubation on ice for 10min. Electroporation was carried out using the Biorad Gene-Pulser II (Biorad, Munich), at a range of different voltages, 100-350V and a capacitance of 950-1000μF. The electroporated cells were returned to ice for another 10min. Transfected cells were rinsed from the cuvette and diluted 10-20 fold in cell culture medium without antibiotics prior to plating out for 24h. Cells were cultured for an additional 24h in complete cell culture medium before being subjected to a β-galactosidase assay.

2.3.5 Beta galactosidase assay

Cells lines growing on cell culture plates (100mm) were washed once with 1x PBS followed by fixing for 20 min in (5mL) 4% paraformaldehyde at RT. The cells were washed twice with washing solution before incubating in (5mL) detergent solution for 5 min on ice or in the fridge. Washing solution was aspirated and cells were incubated in (5mL) X-gal staining solution for 10-60min or 24h at 37ºC. X-gal staining solution was aspirated and cells were washed twice in washing solution before examining blue cells under a light. Stained and unstained cells were counted in randomly selected fields. The percentage of stained cells from the total cell population was calculated. Non-transfected or cells transfected with a blank plasmid were used as controls to determine the level of background activity caused by endogenous β-galactosidase activity.

2.3.6 Genotyping of mice

2.3.6.1 Preparation of genomic tail DNA

Mouse tail tips or ear tips were cut with sharp scissors and digested overnight at 56ºC in 700μL TENS buffer containing 35μL Proteinase K (10mg/mL). On the following day proteins were precipitated by adding 250μL 6M NaCl and incubated at RT for 10 min. To precipitate genomic DNA, solutions were first centrifuged at maximum speed followed by transfer of 500μL supernatant to 500μL isopropanol. Using a hooked glass pipette the DNA was carefully wrapped out of the interphase, dipped briefly in 70% EtOH and left to evaporate isopropanol at RT for 5-10 min before being dissolved in 150μL ddH$_2$O. The tip of the glass pipette in an Eppendorf, carrying the wrapped DNA was incubated overnight at 56ºC on a heat block in preparation for PCR.

2.3.6.2 Blood typing

Phenotyping of the TCR Tg mice was carried out by three colour FACS analysis of the peripheral tail blood to determine the presence of the complete Tg TCR, including the $V\alpha 2$ and $V\beta 5$ chains as well as the corresponding costimulatory molecules CD4 or CD8.

Mice tails were prewarmed with an infrared lamp to enable milking of $50\mu L$-$100\mu L$ of tail vein blood into Eppendorfs containing 1mL Alsevers. Frequently, blood samples were stored overnight at 4ºC. Subsequent steps were carried out on ice. Excess heparin was washed away by addition of $500\mu L$ PBS and centrifugation at 1150rpm, 5min, 4ºC. Supernatant was aspirated, the cell pellet was resuspended in remaining fluid before being incubated for 5 min in $500\mu L$ Gey's solution to lyse erythrocytes. Lysed erthrocytes were separated from lymphocytes by centrifugation at 1150rpm, 5min, 4ºC. Supernatant was aspirated before resuspension of the pellet in $500\mu L$ FACS buffer and transfer into FACS tubes. Cells were washed once more at 1300rpm, 7min, 4ºC prior to immunofluorescence staining.

2.3.7 PCR

All steps were carried out on ice. All reagents were freshly thawed and frozen away at -20ºC when the protocol was completed. Reaction tubes, pipette tips, ddH_2O, $MgCl_2$ and PCR buffer were exposed to UV light for 15-30min. The components of the PCR mix were added in the given order and 48-49μL or 45μL of the PCR mix were added to the genomic or cDNA respectively prior to addition of 1-2 μL genomic DNA or 5μL cDNA.

The OVA PCR mix for one reaction tube contained:

Components	Volume (uL)	End concentration
ddH_2O	34,9	-
$MgCl_2$	1,45	1.5mM
DMSO	2,5	-
PCR buffer	5	1x
ova forward primer	1,67	0.3uM
ova reverse primer	1,67	0.3uM
dNTPs	1,67	0.2mM each
Taq polymerase	0,5	2.5U
End Volume	50	

The following OVA PCR programme was used:

Temperature	Time	Cycles
94˚C	4 min	1
94˚C	1 min	35
57˚C	1 min	35
72˚C	1 min	35
72˚C	5 min	0
4˚C	∞	∞

The β-actin PCR mix for one reaction tube contained:

Components	Volume (uL)	End concentration
ddH$_2$O	15	-
2x PCR Master Mix	25	1x
β-actin sense	5	10pmol
β-actin antisense	5	10pmol
End Volume	50	

The 2x PCR Master Mix from MBI Fermentas contains:

Taq DNA Polymerase (recombinant) in reaction buffer,	0.05U/μL
MgCl$_2$,	4mM
dNTPs (dATP, dCTP, dGTP, dTTP),	0.4mM of each

The following β-actin PCR programme was used:

Temperature	Time	Cycles
94˚C	2 min	0
94˚C	30sec	25
62˚C	30sec	25
72˚C	30 sec	25
72˚C	5 min	0
4˚C	∞	∞

TBE agarose gels were loaded with the PCR products and run as described in 2.3.1.3.

2.3.8 Cell culture

2.3.8.1 Cultivation of cell lines

All three adherent cell lines were cultivated according to standard sterile cell culturing techniques. Culture medium was aspirated and discarded before briefly rinsing the cell layer with PBS to remove traces of FCS, which can inhibit trypsin activity. To disperse and detach cells ATV was added to the cell layer and detachment progression, which usually occurred within 5-15min, was observed under an inverted microscope. To avoid clumping of cells the flask was not agitated by hitting or shaking. If cells were difficult to detach they were placed in the tissue culture incubator at 37ºC until complete detachment. Trypsin activity was inhibited by addition of the respective complete growth medium and washed off by centrifugation in a 50mL tissue culture falcon tube at 1300rpm for 5min at 4ºC. Supernatant was removed, discarded and the cell pellet was resuspended in the desired volume of complete cell medium and aliquoted into new tissue culture flasks. Flasks were returned to the incubator until the next splitting. Cells were incubated in a humidified 37ºC, 5% CO$_2$ incubator.

2.3.8.2 Cryopreservation of cells

Cell lines were frozen away in liquid nitrogen for long term storage. DMSO served as the cryopreservative. Cells were pelleted as in 2.3.8.1 and resuspended in cryotubes, in a small volume (0.5-0.7mL) of freezing medium containing the respective growth medium for the particular cell line. Cryotubes were slowly cooled down at $-80^{\circ}C$ for 24h before being transferred to liquid nitrogen.

2.3.9 Isolation of thymus, lymph node and spleen cells

Mice were sacrificed by CO_2 inhalation. After disinfection with Rotisol, the peritoneal cavity was opened using sterile forceps and scissors. Organs were removed, separated from fatty and connective tissue and placed into a 6 well plate containing BSS/0.1%BSA on ice. The organs were mashed through a cell strainer using the plunger of a 1mL syringe, rinsed twice with BSS/0.1%BSA and then centrifuged at 1300 rpm, $4^{\circ}C$, 5 min. To lyse the erthrocytes within the splenic cell suspension, the cell pellet was responded in 5mL ice cold hypotonic Gey's solution and incubated for 5 min on ice, followed by two washes with BSS/0.1% BSA. Cells were counted in a Neubauer Heamatocytometer using trypan blue to exclude the dead cells and then washed once more with BSS/0.1%BSA before being resuspended and adjusted to the desired cell concentration in X-VIVO-15 complete medium.

2.3.10 In vitro activation of T cells

Various polyclonal activators can be used *in vitro* to induce T cell activation and ultimately proliferation of activated T cells. These include phorbal 12-myristate 13-acetate (PMA) together with calcium inophore, Staphylococcus enterotoxin B (SEB) and mAbs directed against subunits of the TCR/CD3 complex with or without Abs directed against costimulatory receptors such as CD28. Alternatively, when TCR Tg T cells are being utilised a specific response can be elicited by addition of the cognate peptide or protein.

Mouse lymphocytes or splenocytes were prepared as described in 2.3.9. Cells were plated at a density of $2 \cdot 10^5$cells/well in round bottom 96-well tissue culture plates in a final volume of $200\mu L$ of X-VIVO-15 complete medium. Cells were stimulated with various concentrations of whole OVA or cognate peptide or controls including anti-CD3 mAb for polyclonal activation or left unstimulated in only medium, for 72hours. Cell cultures were pulsed with $[^3H]$-thymidine ($1\mu Ci$/well) for the final 16 h of incubation and the DNA incorporated isotope was quantitated by liquid scintillation using a Betaplate scintillation counter.

2.3.10.1 Skewing of in vitro activated T cells towards Th1 and Tc1 T cell subtypes

Single cell suspensions of LNC were prepared as in 2.3.9 and plated out at $1 \cdot 10^6$cells/mL in 24 well plates. OT-I and OT-II LNC were stimulated with SIINFEKL (1ug/mL) and OVAp (1ug/mL) respectively in X-VIVO-15 complete medium. To polarise cells towards a Th1 or Tc1 phenotype, 200U/mL of IL-12 was added. OT-I LNC additionally received 10U/mL IL-2. Cells were stimulated for 3 days after which medium was aspirated and fresh X-VIVO-15 complete medium containing 10U/mL IL-2 was added for another 3 day incubation prior to harvesting for intracellular cytokine analysis and adoptive transfer.

2.3.11 Fluorescence activated cell sorting (FACS)

Flow cytometry is an analytical tool that allows cells within a heterogeneous population to be discriminated based on their size and different structural features including cell surface molecules. Cell surface antigens can be labelled with its specific mAb coupled to a flurorochrome. The cell suspension containing the labelled cells is directed into a thin stream enabling them to pass single-file through a laser beam. Each cell scatters some of the laser light, and also emits fluorescent light at a particular wavelength excited by the laser. This is detected by a photocell and subsequently analysed.

2.3.11.1 Extracellular staining

Single cell suspensions were prepared from organs as described in 2.3.9. $2 \cdot 10^5$ -$1 \cdot 10^6$ cells were labelled with mAbs per staining. The cell pellet was resuspended in 50uL FACS buffer diluted Ab mix and incubated on ice in the dark for 20-30min. Excess Ab was always washed off with 3mL FACS buffer and cells were centrifuged at 1300 rpm for 5min after which the supernatant was aspirated. To detect biotin labelled primary Ab, cells were resuspended in 50μL diluted secondary reagent which was streptavidin fluorochrome conjugated, and incubated for 20min in the dark on ice.

Before being acquired by a FACScan™ or FACScalibur™ (Becton Dickinson), the cells were washed once more and resuspended in an appropriate volume of FACS buffer ie 200-350μL per $5 \cdot 10^5$ cells. Analysis of data was carried out using the CellQuest™ software (Becton Dickinson) Data were collected on all events until 100 000 cells were counted in the live gate. The number of cells for each lymphocyte population was obtained as follows: number of cells in population/mL = number of lymphocytes/mL x percentage of the population.

To reduce FcγII/IIR-mediated non specific Ab binding which could contribute to background staining, anti-FcRc block (2.4G2/ 145-2C11) mAb was added to the cell suspension with the primary reagents. Negative controls included the same cell population exposed to isotype.

Flurochromes used included fluorescein isothiocyanate (FITC), R-phycoerythrin (R-PE), Cy-Chrome (Cy5-PE), allophycocyanin (APC) and Peridinin chlorophyll protein (PerCP)

2.3.11.2 Intracellular cytokine staining

Stimulation of cells

Cell suspensions were prepared from LN as described in 2.3.9.

Various biological agents can specifically or nonspecifically induce T cell activation resulting in cytokine production. It has been reported that cell activation with PMA alone or in combination with calcium ionophore can cause transient loss of CD4 or CD8 expression respectively on mouse peripheral T lymphocytes (Anderson and Coleclough 1993).

As the analysis of the expression of CD4 and CD8 was important in the following protocol, the cells were stimulated by direct crosslinking the TCR with plate bound anti-CD3 mAb and soluble anti-CD28 mAb. 24well plates were coated overnight at 4ºC with 500μL anti-CD3 mAb at 10μg/mL in coating buffer. On the following day wells were washed 3 times with 1-2mL BSS/BSA followed by an incubation step of 5 min with BSS/BSA. After aspiration of

BSS/BSA, cells previously resuspended in X-VIVO-15 complete medium were plated out at a density of $2 \cdot 10^6$ cells/mL. Additionally soluble anti-CD28 mAb was added at a concentration of 5ug/mL as well as the intracellular protein transport inhibitor Brefeldin A in EtOH at a final concentration of $5\mu g$/mL.

Protein transport inhibitors block intracellular transport processes. As a result most cytokine proteins accumulate in the rough endoplasmic reticulum or Golgi complex thereby enhancing the ability to detect cytokine producing cells.

After culturing for 5-6hrs at 37ºC cells were harvested and washed once with BSS/BSA at 1300 rpm for 5min prior to immunofluorescence staining.

Multicolour staining for intracellular cytokines and cell surface antigens

Stimulated cells ($2 \cdot 10^5$ cells per FACS tube) were washed once in 3mL ice-cold FACS buffer, centrifuged at 1300 rpm for 5min and resuspended in $50\mu L$ FACS buffer. FcγII/IIRc were blocked by incubation with the anti-FcRc mAb for 10 min on ice. Cell surface antigens were stained as in 2.3.11.1. Subsequently cells were fixed in $50\mu L$ 4% Paraformaldehyde for 20 min at RT in the dark.

Fixation with paraformaldehyde allows for the preservation of cell morphology and intracellular antigenicity while enabling the cells to withstand the permeabilisation by detergent.

After washing off excess fixative with 3 mL FACS buffer the supernatant was aspirated. To permeabilise the cell membrane, cells were incubated in 1mL of 0.1% Saponin buffer for 10 min at RT.

Membrane permeabilisation by saponin allows cytokine specific Abs to penetrate the cell membrane, cytosol, and gain access to the membranes of the endoplasmic reticulum and the Golgi apparatus.

After centrifugation, the supernatant was aspirated and $20\mu L$ of the primary cytokine antibody as well as isotype control previously diluted in saponin buffer at 1:10 were added. Cell suspension was vortexed and incubated for 15 min at RT in the dark.

To remove excess Ab, cells were washed once with 1mL 0.1% Saponin buffer. To close the membrane pores; cells were washed once with 3mL FACS buffer ready for FACScan™ or FACScalibur™ acquisition.

2.3.12 Histology

2.3.12.1 Perfusion and fixation of organs

Mice were aneasethised by an injection of 3.5mL 0.01mL/g of body weight of Narcoren in NaCl 1:40. The chest cavity was opened and the left heart chamber was punctured while the right heart tip was cut using scissors. Firstly a rinse with approx. 20-50mL 10% HES was performed prior to perfusion with 4% PFA whereby the pump was set at 20% output. The perfusion was successful when the tail moved upright and then became stiff.

Alternatively organs were fixed overnight – 1 week in 4% PFA followed by rinsing in 3 changes of PBS for 20 min and 10 min running tap water.

2.3.12.2 Embedding

The following embedder programme of the automated embedder in the Neurology department was used for the preparation of organs for histological analysis:

Programme D

20 min 50% EtOH
20 min 70% EtOH
20 min 80% EtOH
20 min 96 % EtOH
20 min 100% EtOH
30 min 100% EtOH
30 min 100% EtOH + Chloroform 1:1
30 min 100% EtOH + Chloroform 1:1
50 min Paraffin I
30 min Paraffin II

2.3.12.3 Coating of superfrost slides with poly-L-lysine

To ensure long term adherence of the tissue section on the slide, super frost slides were left standing in detergent overnight at RT. The next day slides were rinsed for 10 min with running tap water followed by 3 changes of 30 min in ddH$_2$O. Thereafter slides were left to dry overnight at 60ºC. Slides were then placed in the poly-L-lysine hydrobromide (Sigma) suspension for 30 min at RT, followed by another round of drying at 60ºC overnight.

2.3.13 Immunohistochemistry

2.3.13.1 Heamatoxylin and Eosin stain

Paraffin sections were deparafinised in the following manner:

2x 15 min Xylene
2x 2 min 100% EtOH
2x 2 min 96% EtOH
1x 2min 80% EtOH
1x 2min 75% EtOH
1x 2min 65% EtOH
2x 2min 50% EtOH

Slides were rinsed in Aqua dest. and then stained in heamatoxylin for about 5 to 10min. It was important to check the intensity of the stain under the microscope. To develop the stain the slides were placed under warm running tap water for 10 min. Subsequently slides were counterstained in eosin 1% citric acid for about 5min. Again it was important to check the intensity of the stain with the eye. Slides were then dehydrated in the following manner:

2x 2min 70% EtOH
1x 2min 80% EtOH
2x 2min 100% EtOH

Slides were cleared in Xylene and then mounted with a coverslip with a drop of Vitro Clud (Langenbrinck).

2.3.13.2 anti-OVA and anti-CD3 antibody stain

Paraffin sections were deparafinised as in section 2.3.13.1 and subsequently washed in TBS. The area around tissue sections was carefully dried with paper towelling before encirling with a pap pen to hinder loss of added antibody. Non-specific binding sites were then blocked with 10%BSA/TBS for 20 min at RT. Blocking solution was tipped off before addition of the primary antibody. The primary Abs for each stain were added as follows:

anti-OVA stain	anti-CD3 stain
rabbit anti-OVA serum 1:800 in 1% BSA/TBS incubated for 1h at 37ºC	rat anti-human CD3 mAb 1:200 in 1% BSA/TBS incubated overnight at 4ºC

Tissue sections were washed in 3-4 changes of TBS before addition of the blocking reagent. To block endogenous peroxidase activity 3% H_2O_2 in methanol was applied to the sections for 12-15 min at RT followed by 4 changes in TBS. The secondary reagents for each stain were added as follows:

anti-OVA stain	anti-CD3 stain
biotinylated goat anti-rabbit IgG preabsorbed 1:1 with mouse serum for 15 min at 37ºC 1:50 in 1% BSA/TBS incubated for 45 min at RT	biotinylated rabbit anti-rat IgG Ab 1:200 in 1% BSA/TBS incubated for 45 min at RT

Slides were washed in 3 changes of TBS. Bound antibody was then detected using the preformed avidin and biotinylated horseradish peroxidase macromolecular complex. The avidin-biotin complex was mixed 1:100 in TBS for 15-30 min at RT prior to incubating slides for 35 min at RT. Sections were washed in 3 changes of TBS. Diaminobenzydine (DAB) substrate was used for development. One DAB substrate tablet was equilibrated to RT before being dissolved in 10mL of ddH$_2$O, followed by filtration through a 0,45μm filter. The DAB solution was activated by addition of 3% H_2O_2 before addition to the slides for 5-10 min at RT in the dark. The enzymatic reaction was then stopped in ddH$_2$O followed by rinsing in 3 changes of ddH$_2$O. Slides were counterstained in heamatoxylin for 40'' to 5 min, developed in running tap water for 10 min before being dehydrated as in 2.3.13.1.

2.3.14 Protein biochemical methods

2.3.14.1 Cell lysis

To remove albumin, which is similar in size to OVA, cells were harvested and washed twice in PBS. $1 \cdot 10^6$ cells were resuspended in approx. 1mL PBS and centrifuged until 5000rpm at 4ºC. Supernatant was aspirated and 30μL lysis buffer was added. The mixture was vortexed and incubated on ice for 30 min before vortexing again. This was followed by centrifugation for 10 min at full speed at 4ºC. The supernatant containing cytosolic protein extracts was transferred to a newly chilled Eppendorf tube. 1x reducing loading buffer (4x Rotiload, Roth,

Karlsruhe) was added and samples were heated at 95ºC for 5 min before being stored at –20ºC until further analysis.

2.3.14.2 SDS-Polyacrylamide Gel Electrophoresis of Proteins (SDS-PAGE)

General Introduction

SDS-PAGE enables uniformly negatively charged proteins to be separated based on their molecular weight. The strongly anionic detergent SDS in combination with the β-mercaptoethanol reducing agent and heat breaks down the protein tertiary structure by reducing disulfide bonds in addition to coating proteins with a negative charge before they are loaded onto the gel. Consequently the amount of SDS bound is proportional to the molecular weight and independent of the polypeptide sequence

To separate the proteins, chemically inert polyacrylamide gels within a discontinuous buffer system are used, across which an electric current is passed. For optimal resolution, the SDS-polypeptide complexes first migrate through the stacking gel. This gel builds a thin zone on the surface of the resolving gel onto which the protein complexes, based on their different pH and ionic charge are collected, before migrating through the resolving gel in a zone of uniform voltage and pH and are separated according to size.

Use of markers of known molecular weight enabled the molecular weight of unknown proteins to be estimated.

Preparation of SDS-Polyacryalmide gels

The glass and aluminium plates were cleaned with 70% Ethanol, the T spacers placed between and mounted in the gel pouring chamber. To check for leaks, ddH$_2$O was pipetted between plates. First the resolving gel was cast and pipetted into the gap between the plates, leaving sufficient space for the stacking gel (about the length of the teeth of the comb plus 1 cm). The gel was overlayed with 70% EtOH to prevent oxygen from diffusing into the gel and therefore inhibiting polymerisation. After complete polymerisation (15-30min, RT), the overlay was poured off and the top of the gel washed with ddH$_2$O to remove unpolymerised acrylamide. The staking gel was prepared and poured on top of the resolving gel. Without delay the teflon comb was inserted, being careful to avoid trapping air bubbles. After complete polymerisation the gel was removed from the pouring chamber, the comb carefully removed from the gel, the gel pockets rinsed with ddH$_2$O and the gel mounted vertically into the gel electrophoresis apparatus. Electrophoresis running buffer 1x was added to the top and bottom reservoirs before loading the preheated samples in loading buffer (Laemmli).

Samples in one gel were run for approx. 30 min at 80V followed by 2hrs at 120V or for 60min at 30mAmp. The molecular weight markers ‚Rainbow-Marker‘ (Pharmacia) and Bench marker (Amersham) were used.

Staining and drying SDS-Polyacrylamide gels with Coomassie brilliant blue

Directly after electrophoresis, gels were analysed for the presence of migrated polypeptides by immersion in 5 volumes of Coomassie blue stain for 4h at RT on a slow rocking platform. The stain was removed by incubating the gel in destaining solution overnight at 4ºC with three changes of destain solution. A sponge was added to absorb the stain as it leached from the gel.

To make a permanent record of the gel, it was placed between a piece of Whatmann filter paper and clingwrap before being vacuum dried at 80ºC for 2h.

2.3.14.3 Western Blot (transfer and detection of proteins onto nitrocellulose membranes)

The Western blot technique enables SDS-PAGE separated proteins to be electrophphoretically transferred onto a solid support i.e. a nitrocellulose membrane, which can then be probed with specific antibodies. The unlabelled primary antibodies can subsequently be detected with an enzyme labelled secondary reagent that converts a substrate into a brown precipitate.

The Western blot was set up in a wet chamber. The blotting sandwich was built up in the following order; a blotting sponge, 2 pieces of 3MM Whatman paper (9x10cm), one nitrocellulose membrane (8x9cm), the SDS-PAGE gel, 2 pieces of 3MM Whatman paper and another blotting sponge. All components, except the nitrocellulose, which was soaked in dH$_2$O, were soaked in transfer buffer. The transfer was carried out overnight at 4ºC at 40V (150mAmp). It was important to ensure that no air bubbles were captured between the different layers and that the proteins were transferred from the gel onto the membrane in the direction of the positively charged electrode.

Staining proteins immobilised on nitrocellulose membranes with Ponceau S

Transient staining of the membrane with Ponceau S allows visual evidence of the success of the electrophoretic transfer of proteins. Staining with Ponceau S does not interfere with subsequent detection of Ags with chromatogenic reactions.

The nitrocellulose membrane was incubated in Ponceau S working solution for 5-10 min on a rocker after which excess stain was washed off with several changes of dH$_2$O. The stained proteins were permanently recorded by photocopying the nitrocellulose membrane held between two overhead sheets.

Immunobiological detection

Excess Ponceau S stain was washed off by incubating the membrane in three changes of TBS 0.1% Tween 20 and gentle agitation for 1/2h. All following incubations were carried out on a shaker with gentle agitation. Nonspecific binding sites were blocked by incubation of the membrane overnight at 4ºC in 10mL of 5% w/v skimmilk TBS 0.1%Tween20. On the following day, 10mL of the primary Ab, diluted in 5% skimmilk TBS 0.1%Tween20 was added and incubated overnight at 4ºC. Excess primary Ab was washed off by incubating the membrane in 3 changes of TBS 0.1%Tween20 for 15 min. This was followed by incubation of the membrane in 10mL of the secondary HRP conjugated Ab, in TBS 0.1%Tween20 for 2h at RT. Subsequently the membrane was washed in five changes of TBS 0.1%Tween20 each for 15 min. The secondary enzyme coupled Ab was detected by incubating the nitrocellulose in 10mL of freshly prepared chromatogenic substrate ECL (Amersham), (1 Solution A: 1 Solution B) for 1 min at RT. The membrane was drained of excess substrate before being placed between two overhead sheets in a film cassette and exposed onto a Kodak x-ray film for different time points (ranging from 10 sec to 10 min). The x-ray film was fixed (2min) and then developed (2min) prior to analysis.

2.3.15 Acquisition of blood and serum from mice

Two methods were employed to obtain blood from mice. In the first, tails of mice were prewarmed with an infrared lamp. Two small incisions were made with a scapel on the tail underside and blood droplets were collected in an Eppendorf tube. Alternatively the mice were prepared as for section 2.3.9 and blood was removed directly from the heart using a 20

gauge needle and a 1mL syringe. After coagulation at 1h at 37ºC or at 4ºC overnight, serum was separated from the coagulated blood components by centrifugation at 4000rpm, 5 min. The serum was stored at -20ºC until further use.

2.3.16 ELISA

A sandwich ELISA (Enzyme Linked Immunosorbent Assay) determined the presence and amount of isotype specific immunoglobulins from immunised mice sera. Binding of the specific Ab to the immobilised cognate Ag, under conditions in which non-specific adsorption was blocked and where unbound antibody and other proteins were washed away, was detected using an enzyme labelled secondary Ab.

ELISA plates with an enhanced surface from Becton Dickinson (Falcon 3912 Micro TestIII flexible assay plates) with 96 flat-bottom wells, were coated with 20μg/mL of whole OVA grade V in PBS at 100μL/well, overnight at 4ºC in a moist chamber. On the following day excess unbound antigen was washed away once with 200μL/well of PBS. Additional unbound well sites were blocked with 200μL/ well of 0.2% gelatine in PBS for 1h at RT in the moist chamber. Excess gelatine was removed with one wash of PBS. Dilutions of sera from immunised mice ranging from 1:50-1:500 were plated out in duplicates. In addition a serial 5 fold dilution of the positive control, mouse anti-OVA mAb, was added to the same plate. The prepared samples were added to the wells at 100μL/well and incubated for 1h at RT in the moist chamber. Excess Ab was removed with four washes of PBS/0.05% Tween 20. The alkaline phosphatase conjugated secondary antibodies, anti-mouse IgG and anti-mouse IgM were added at 100μL/well. After 30 min incubation at RT in the moist chamber, excess secondary Ab was removed with 4 washes of PBS/0.05% Tween 20. To quantitate specifically bound Ab, 100uL/well of substrate solution containing 1mg/mL p-Nitrophenylphosphate (1 Tablet; 20mg) in Diethanolaminebuffer was added. The absorption values of the resulting yellow substrate product were measured at 405nm in an ELISA Vmax reader. Analysis of the raw data was carried out using the Softmax Pro 3.0 programme.

2.3.17 Disease induction

2.3.17.1 Active induction

To actively induce a PNS like autoimmune disease, PNO mice were injected with the autoAg in combination with an adjuvant. Whole chicken OVA protein, grade V was solubulised in PBS and emulsified 1:1 in CFA containing 1mg/mL, *Mycobacterium tuberculosis* (H37Ra; ATCC 25177). Each mouse was sc injected with 200μg OVA and 100μg *Mycobacterium tuberculosis* in a total volume of 400μL. This was distributed over three different sites on the dorsal flank. OVA sensitised mice received 24 and 72h later, unless otherwise stated, a single ip injection of 400ng PT in 200μL PBS.

2.3.17.2 Passive induction

Alternatively, disease induction was attempted via adoptive transfer of autoAg activated T lymphocytes (Pape, Kearney et al. 1997). Tc1 and Th1 polarised OT-I and OT-II LNC respectively, were harvested and resuspended in an appropriate volume of BSS prior to counting viable cells. Mice were iv injected with the indicated total viable cell number in 200-500μL BSS at RT. If the cell concentration was high, cell clumps, which can be fatal to mice, were removed from suspensions by passing through a cell strainer prior to injection. Unless otherwise stated, 400ng PT was also injected on the same day and 72h later following transfer.

2.3.17.3 Irradiation of mice

One day prior to adoptive transfer, mice were placed in a rectangular plexi glass chamber containing air holes and sublethally gamma irradiated at 500rad (5.0 Gy) for about 1/2 h using a Cs137 source. Afterwards mice were transferred back into the pathogen free animal facility and allowed to recover until further experimental treatment the following day.

2.3.17.4 Clinical evaluation of disease

Following immunisation or adoptive transfer of anti-OVA TCR Tg T cells, Tg positive mice and their WT counterparts were examined every other day for clinical signs of disease which included ruffled fur, incontinence and weight loss. Additionally a long term inability to socially interact with fellow cage members was considered as a sign of illness. Mice were weighed by placing them one at a time in a tarred plastic beaker on a laboratory digital scale.

In addition to being weighed every other day mice were also assessed for disease severity using a 5-point scale (Baron, Madri et al. 1993) (Zou, Ljunggren et al. 2000);

1: limp tail
2: partial hind leg paralysis, difficulty turning over
3: total hind leg paralysis
4: hind and front limb paralysis
5: moribund or dead

3. RESULTS

3.1 Generation of transgenic mice expressing OVA either in the CNS or PNS

3.1.1 Overview

In order to investigate in detail the mechanisms of the neuroautoimmune inflammatory diseases MS and GBS, two novel murine models were to be generated. It was intended that these murine models would deliver additional insights into cellular and molecular mechanisms of disease initiation, propagation and termination already alluded to in existing models of EAE and EAN. To this end, the complete chicken OVA cDNA (genebank accession no. V00383.1) encoding for a soluble cytoplasmic form of OVA was to be implemented and would serve as the neuroAg. OVA has been successfully used as an autoAg in autoimmune disease models of the pancreas (Kurts, Heath et al. 1996), heart (Grabie, Delfs et al. 2003) and intestine (Shastri and Gonzalez 1993). Employment of the different regulatory elements within the MBP promoter would permit exclusive expression of the target Ag in either the CNS or PNS. The adoptive transfer of anti-OVA TCR Tg T cells would compliment the OVA Tg mouse model by allowing encephalitogenic T cells to be tracked in vivo and thereby help reveal in more detail the dynamic role of T cells in neuroinflammatory diseases.

As MBP is a major constituent of the myelin sheath in the CNS and in the PNS (see also introduction) it was decided to target the neoAg to the above mentioned tissues using the MBP promoter. The MBP promoter was to be exploited for its distinct regulatory elements to provide exclusive expression of OVA either in the CNS or PNS. The –6.5' 5'MBP promoter has been shown to be active exclusively in oligodendrocytes of the CNS. In contrast the SCE1 confers specific expression to Schwann cells of the PNS. The following diagram depicts the origin of the two different promoter regions used to generate the CNS or PNS specific OVA expression plasmids. A number of different cloning strategies were attempted for the PNS and CNS constructs before successful cloning of the final version as presented here. The correctness of all constructs was confirmed via sequencing (data not shown).
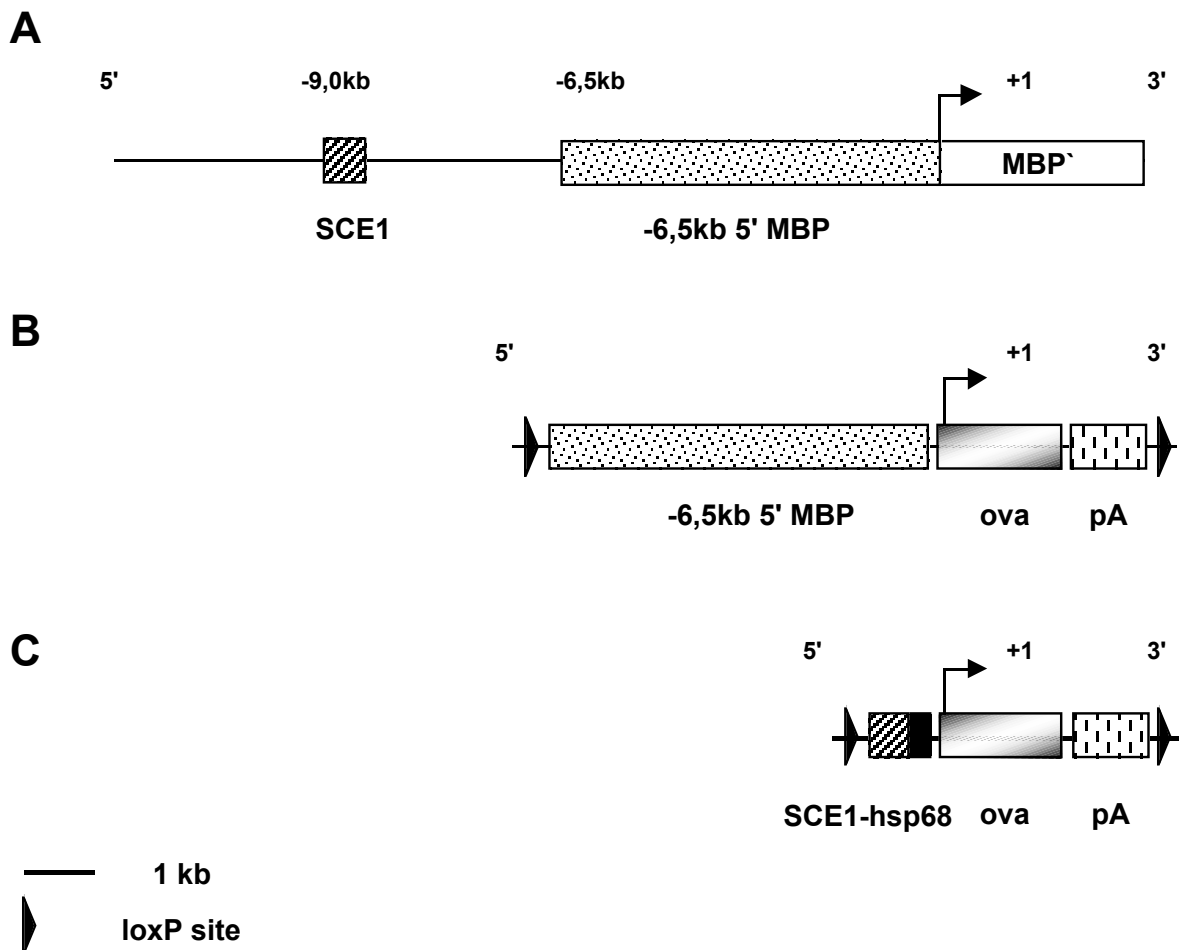
A

B

C

— 1 kb

loxP site

Fig. 3 Two different MBP promoter regions confer either CNS or PNS specific expression.

The two OVA constructs used to generate the CNO and PNO Tg mice were based on two different MBP 5' flanking sequences. A) Depiction of the 12kb 5' MBP flanking sequence indicating the location of the 5,5kb MBP promoter and the 0.6kb Schwann cell enhancer (SCE1) used to generate B) and C) respectively. B) The pMBPova construct used to generate the CNO mice contained the –6.5kb 5' MBP promoter, the complete chicken OVA cDNA encoding amino acids 1-386 and a rabbit poly A tail. C) The pSCova construct used to generate the PNO mice included the SCE-hsp68 promoter, in addition to the complete chicken OVA cDNA and the rabbit polyA tail used for the construction of pMBPova. Two loxP sites in the same orientation were additionally added to both constructs. OVA; ovalbumin cDNA, pA; poly A tail, hsp68; minimal promoter, black triangle; loxP site.

3.1.2 Construction of the Schwann cell specific OVA expression plasmid: pSCova

To generate the pSCova construct, the standard cloning vector pSP72 2.5kb (Promega, genebank accession number X65332) was used as the starting vector. For future cloning strategies two extra restriction enzyme sites were inserted. An additional NotI restriction site was inserted into the BglII site with a twentytwo nucleotide long linker generating pSP72L. Two directly repeated loxP sites were included to allow future deletion of the transgene by Cre recombinase. To this end a second oligonucleotide, fiftyfour nucleotides in length, containing the second restriction enzyme site SmaI and a loxP site were cloned into XhoI/HindIII of pSP72L generating pSP72LX/H. To enable ligation of the OVA cDNA together with the SCE1-hsp68 promoter the EcoRI site outside of the OVA cDNA reading frame was destroyed. The OVA cDNA was therefore released from pAc-neo-OVA (R. Bogatzki, University of Washington, Washington, USA; (Moore, Carbone et al. 1988) by partial digestion with BamHI and EcoRI before being subcloned into the pKSII vector to generate pKSIIova. Partial digestion of pKSIIova with EcoRI followed by blunting with Klenow polymerase and religation with T4 ligase destroyed the extra EcoRI site and generated pKSIIova(E). In parallel the minimal 0.3kb hsp68 promoter (HindIII/NcoI; clone p610ZA; J. Rossant, Mount Sinai Research Institute, Toronto, Ontario, Canada) ligated to the 0.6kb SCE1 (SacII/ SacI; clone SCE-hsp2G; kind gift of R. Forghani, Laboratory of Developmental Biology, McGill University, Montreal, Quebec, Canada) was isolated with SmaI and BamHI. The SCE1-hsp68 promoter (SmaI/BamHI) in conjunction with the OVA cDNA (BamHI/EcoRI) was subsequently cloned into SmaI and EcoRI sites of pSP72LX/H generating pSChspova. For the final cloning steps, the rabbit 1.1kb polyA tail was released from the pBI-5 plasmid (U. Baron/ S. Freundlieb, ZMBH, Heidelberg, Germany) with ClaI and AseI and cloned with a third NotI/AseI oligonucleotide containing an AseI overhang and the second loxP site into pSChspova. The resulting pSCova construct was released with NotI/XhoI from prokaryote sequences and eluted from a 0.8% TBE agarose gel using the Qiagen Qiaquick kit and the TE based (pH 7.5) injection buffer. The linearised pSCova construct was kindly microinjected into C57BL/6 oocytes by B. Kanzler, in Freiburg, Germany.

Subcloning of ovalbumin into pKSII
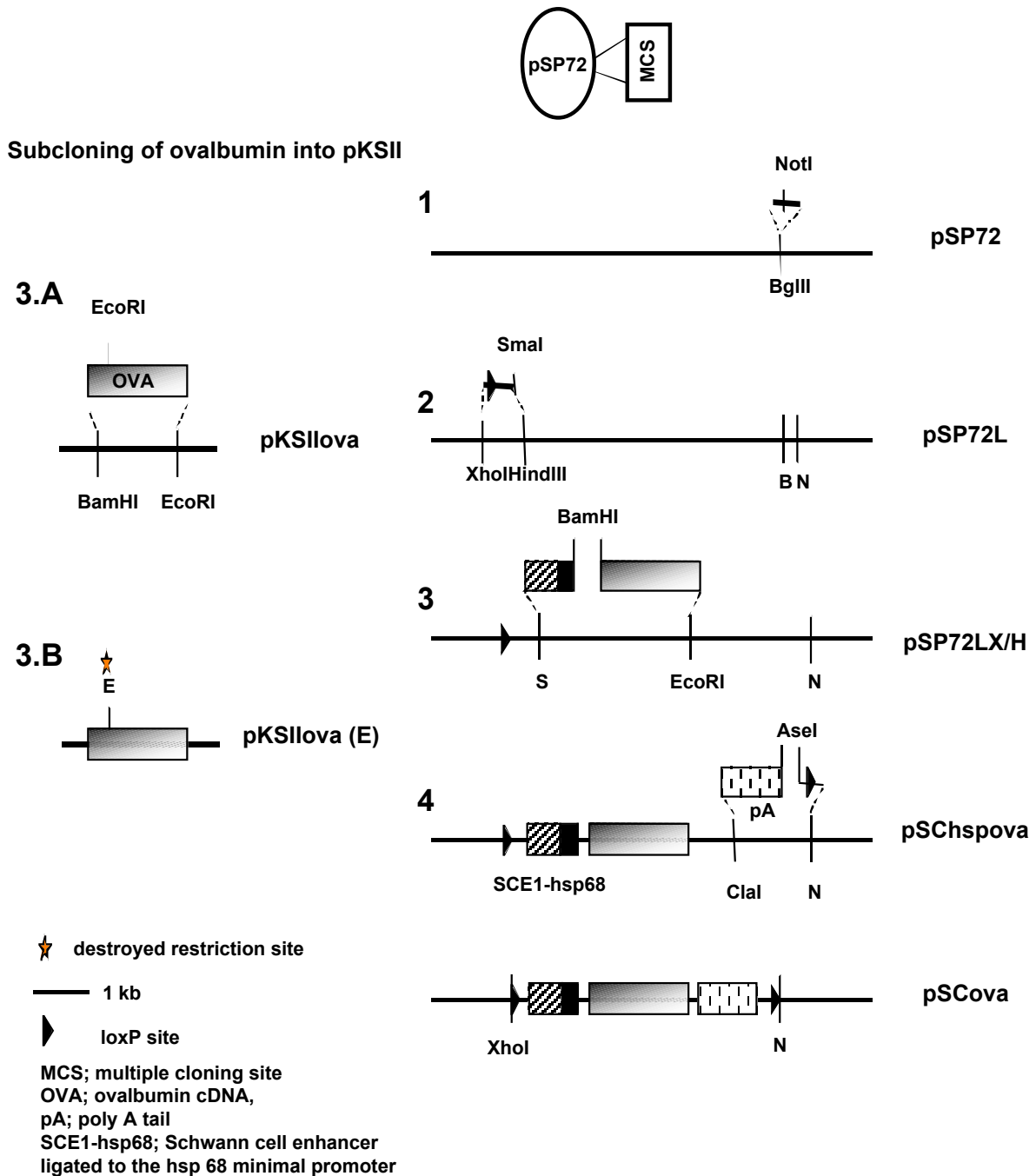
Fig. 4 Cloning strategy employed for the generation of the pSCova vector.
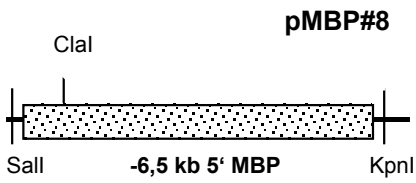
OVA was cloned behind the SCE-hsp68 promoter to direct specific expression to Schwann cells of the PNS. Complete OVA cDNA containing the destroyed EcoRI site and the SCE1-hsp68 promoter were cloned into pSP72LX/H. Subsequently the polyA tail as well as the second loxP site was inserted generating pSCova.

3.1.3 Generation of the oligodendrocyte specific OVA expression plasmid: pMBPova
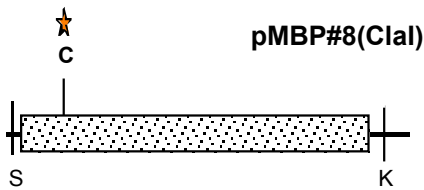
The cloning strategy used to generate the pMBPova vector is depicted in Fig 5. The starting vector, pSP72LX/H already contained one loxP site. Before inserting the second loxP site the 1.8kb OVA cDNA was released from pAc-neo-OVA and cloned into the EcoRI site in pSP72LX/H. Prior to insertion of the 6.5kb MBP promoter, an oligonucleotide linker containing KpnI and SalI restriction sites was cloned into the respective sites in pSP72LX/HOVA. To retain the ClaI site in pSP72LX/H for the insertion of the polyA tail, an additional ClaI site in the 5' flanking sequence of the −6.5 5' MBP promoter had to be destroyed. To this end the ClaI site in the pMBP clone #8 plasmid (a kind gift of Drs. Petersen and Forghani, Laboratory of Developmental Biology, McGill University, Montreal, Quebec, Canada) was destroyed by cutting with ClaI, blunting with Klenow before religating with T4 ligase. In parallel a SalKpnHind linker containing Hind, KpnI and SalI restriction sites in addition to a disrupted KpnI overhang was inserted into pSP72LX/H generating pSP72LX/Hova. The modified 6,5kb MBP promoter was subsequently released with KpnI and SalI and ligated 5' upstream of the OVA cDNA into the respective sites in pSPovaHKS generating pMBPovaHKS. The second loxP site contained within an oligonucleotide and supporting an AseI overhang was cloned together with the 0,68kb polyA tail (ClaI/ AseI) into ClaI/NotI sites in pMBPovaHKS, which resulted in pMBPova. The MBPova construct was released with NotI /XhoI from prokaryote sequences and eluted from a 0.8% TAE agarose gel using the Qiagen Qiaquick kit and a TE based (pH7.5) injection buffer. B. Kanzler (Freiburg, Germany) again kindly carried out microinjection of the linearised pMBPova construct into C57BL/6 oocytes.
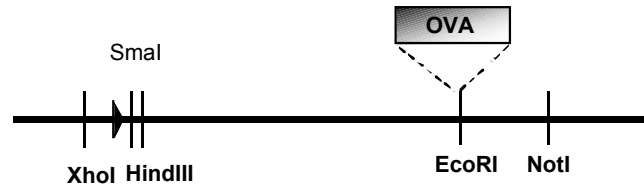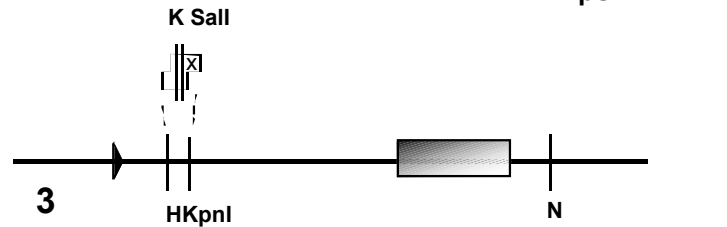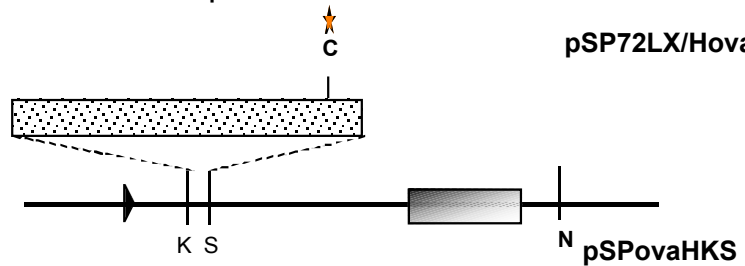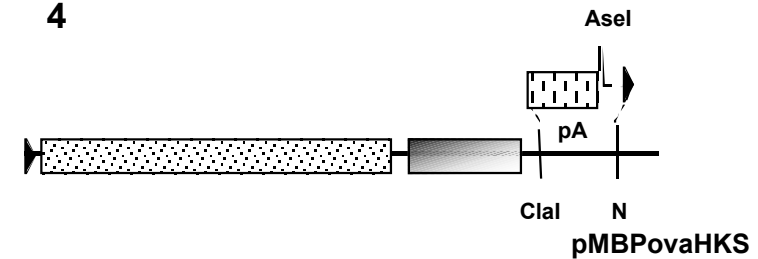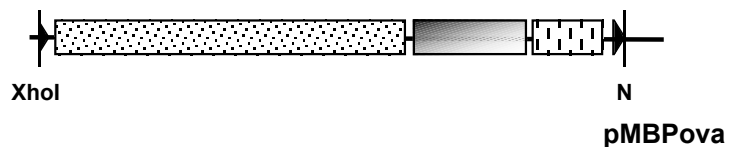
Fig. 5 Cloning strategy used for the generation of the pMBPova vector.

OVA cDNA was cloned behind the −6,5kb 5' MBP promoter to direct expression to oligodendrocytes of the CNS. The complete OVA cDNA was inserted into pSP72LX/H followed by ligation of the polyA tail and the second loxP site resulting in pMBPova.

3.1.4 The Schwann cell specific construct transiently expresses OVA

Preliminary in vitro experiments attempted to assess whether the MBP or SCE-hsp68 promoter could successfully drive OVA expression. Initial experiments attempted to define the transfection efficiency of both constructs using a lacZ reporter gene assay. Subsequent transfection studies demonstrated the ability of both promoters to drive OVA expression within the below mentioned cell lines.

Transient transfections using the pSCova and pMBPova constructs were carried out in the fibroblast cell line L929, the human kidney epithelial cell line 293T/17 (both from within the institute), and the neonatal rat Schwann cell (SC) line (kind gift of Prof. Gold).

Transfection efficiency studies included the test vectors pSCova and pMBPova and the following control vectors; pSCE-hsp clone 2G vector (kind gift of R. Forghani, Canada) which contains the lacZ reporter gene under control of the SCE-hsp68 promoter, pMBP·d10lacZ clone 5 (kind gift of R. Forghani, Canada) which expressed lacZ under control of the MBP promoter and pSVZt (kind gift of I. Berberich) in which the CMV promoter regulates lacZ expression. Two different transfection methods including electroporation and DOTAP liposome mediated transfection were conducted. Liposome mediated transfection was determined to be more efficient than electroporation. The transfection experiments were repeated 5 times in all three cell lines. X-gal staining of test vector transfected L929 and 293T/17 cells determined 20-50% more blue colonies compared to controls. Unfortunately the SC line could never be transfected successfully using either method of transfection or the two different promoters (results not shown).

Additional transfection studies using the 293T/17 cell line and the Schwann cell specific construct and the positive control plasmid, pAc-neo-OVA were carried out. Western blot analysis determined successful transfection as the 45kDa OVA protein was detected in cell lysates containing the test vector; pSCova or the control vector; pAc-neo-OVA.
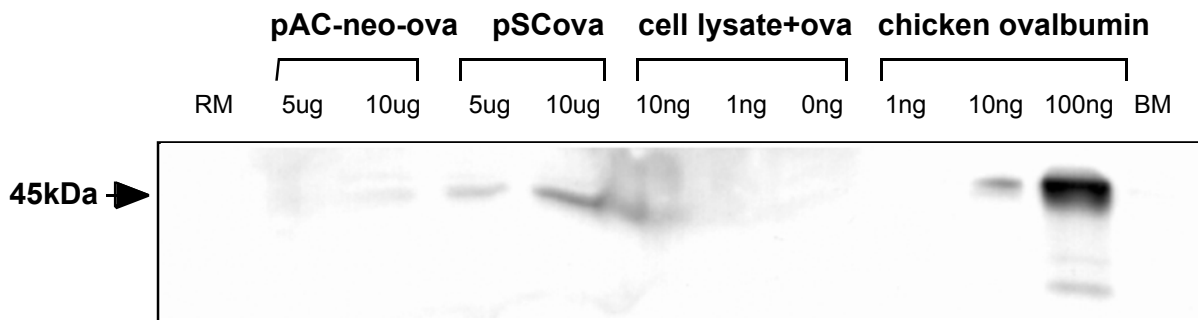
Fig. 6 The SCE-hsp68 promoter directs transient OVA expression in 293T/17 cells

293T/17 cells were DOTAP mediated transfected with test vector, pSCova and the control pAC-neo-ova vector.48h post transfection $1·10^6$ lysed cells were subjected to a Western blot. Whole OVA alone and cell lysates containing whole OVA added exogenously served as positive controls. Cell lysate without OVA served as the negative control. OVA was detected by probing with mouse anti-chicken OVA mAb followed by sheep anti-mouse HRP. The Bench marker determined the protein band to be at 45kDa, the size of OVA. One representative experiment out of three is shown. pAc-neo-ova; control vector, pSCova; test vector, chicken ovalbumin/OVA; solubulised whole ovalbumin, BM; bench marker.

3.1.5 Identification of OVA transgene integration within the PNO and CNO mouse genome

Dr Benoit Kanzler, MPI Freiburg, Germany carried out pronuclear injections of the two linearised OVA plasmids. All live offspring were received in the institute at about 2-12 weeks of age. To determine the presence of the OVA transgene within the mouse genome tail biopsies were analysed by PCR followed by Southern blot. An OVA PCR was established by optimising Mg2+ concentration, annealing temperature and dNTP concentration on a gradient thermocycler. The OVA specific primers were designed to lie within the OVA cDNA sequence generating a 733bp PCR fragment. The PCR was always carried out on proteinase K digested genomic tail DNA in parallel with either an β-actin or IL-2 PCR serving as an internal control.

A

Generation of PNO mice			No. of positive founders	
Pronuclear injection	Date of birth	No. of live offspring	PCR	Southern Blot
04.2001	05.2001	0	0	0
05.2001	06.2001	4	0	0
08.2001	09.2001	9	3	3
08.2001	09.2001	0	0	0
Total		**13**	**3**	**3**

B

Generation of CNO mice			No. of positive founders	
Pronuclear injection	Date of birth	No. of live offspring	PCR	Southern Blot
11.2001	12.2001	0	0	nd
12.2001	01.2002	20	5	nd
12.2001	01.2002	14	3	nd
Total		**34**	**8**	**nd**

Table 2. List of pronuclear injections with pSCova and MBPova.

B. Kanzler carried out pronuclear injections of both OVA constructs. Subsequent tail biopsy analysis via OVA specific PCR and Southern blot determined the presence of the transgene. A) Four microinjections were carried out with the pSCova construct. From 13 live offspring 3 were determined to be positive for the transgene. Hereafter the three peripheral nerve OVA Tg mouse lines were each assigned the names; Peripheral Nerve OVA; PNO3, PNO8 and PNO9. B) Three pronuclear injections were undertaken with the pMBPova plasmid. Amongst 34 live offspring 8 were positively identified via PCR as carrying the OVA transgene. However, only two mouse lines expressed the OVA protein at levels detectable by Western blot and were assigned the names; Central Nerve OVA; CNO2 and CNO7.

Southern blot analysis of genomic DNA from OVA PCR positive mice confirmed the integration of the OVA transgene. For identification of the OVA transgene, a 0,871kb radioactively labelled probe generated by digestion of pSCova with PvuII and XbaI was applied. The Southern blot showed differences in OVA band intensities. The PNO8 band appeared lightest while the PNO9 band appeared brightest (see Fig. 7).

A
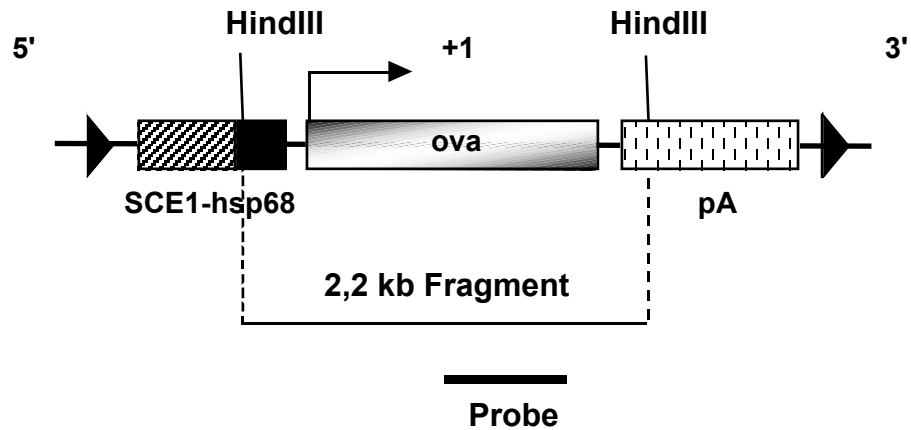
B
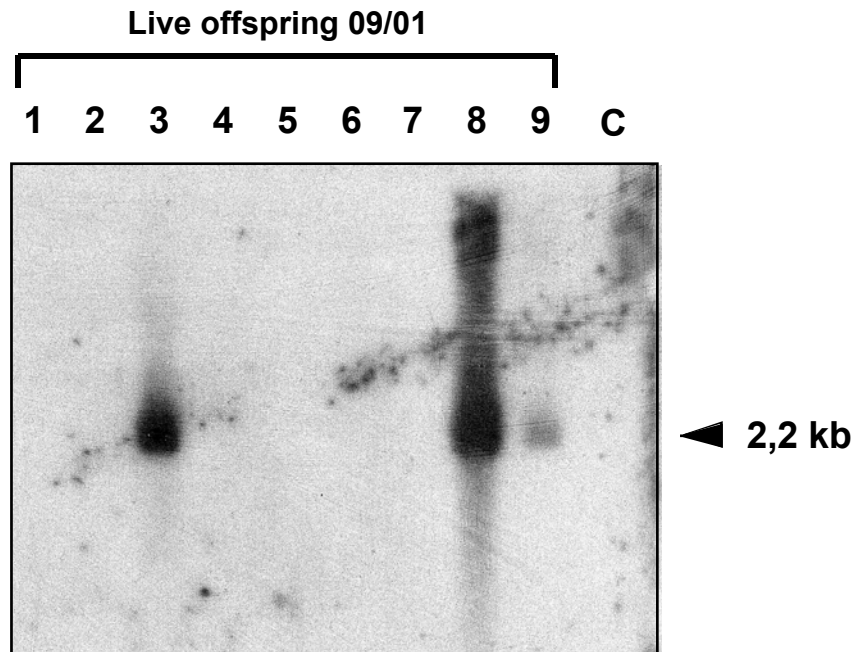
Fig. 7 Southern blot shows the presence of the OVA transgene within PNO founder mice.

A) HindIII digestion of pSCova generated a 2.2kb sequence between the SCE-hsp68 promoter and the OVA cDNA. Location of the HindIII restriction sites and the OVA specific probe is shown. B) Recovered genomic DNA from proteinase K digested PNO tail biopsies was first digested with HindIII before being run on a 0.8% TBE agarose gel. Three positive PNO founder mice were confirmed via Southern blot in which a 0,8kb OVA specific radioactively labelled probe probed genomic DNA. Depicted is genomic DNA from 3 OVA PCR positive PNO founder mice and the C57BL/6 negative control. SCE; Schwann cell enhancer, P; hsp68 minimal promoter, pA; poly A site, C; control C57BL/6 genomic DNA.

The positive Tg founders were mated with C57BL/6 mice. After three generations it was determined that the PNO3 mouse line carried the OVA transgene exclusively on the Y

chromosome. Therefore succeeding generations of PNO3 mice could only be bred to hemizygosity in contrast to PNO8 and PNO9 mice.

F1 generation PNO OVA PCR positive mice were also confirmed via Southern blot. Identification of the OVA transgene was accomplished by probing with a radioactively labelled 733bp OVA PCR probe. This confirmed the presence of the OVA transgene within the genome of 3 PNO founders.
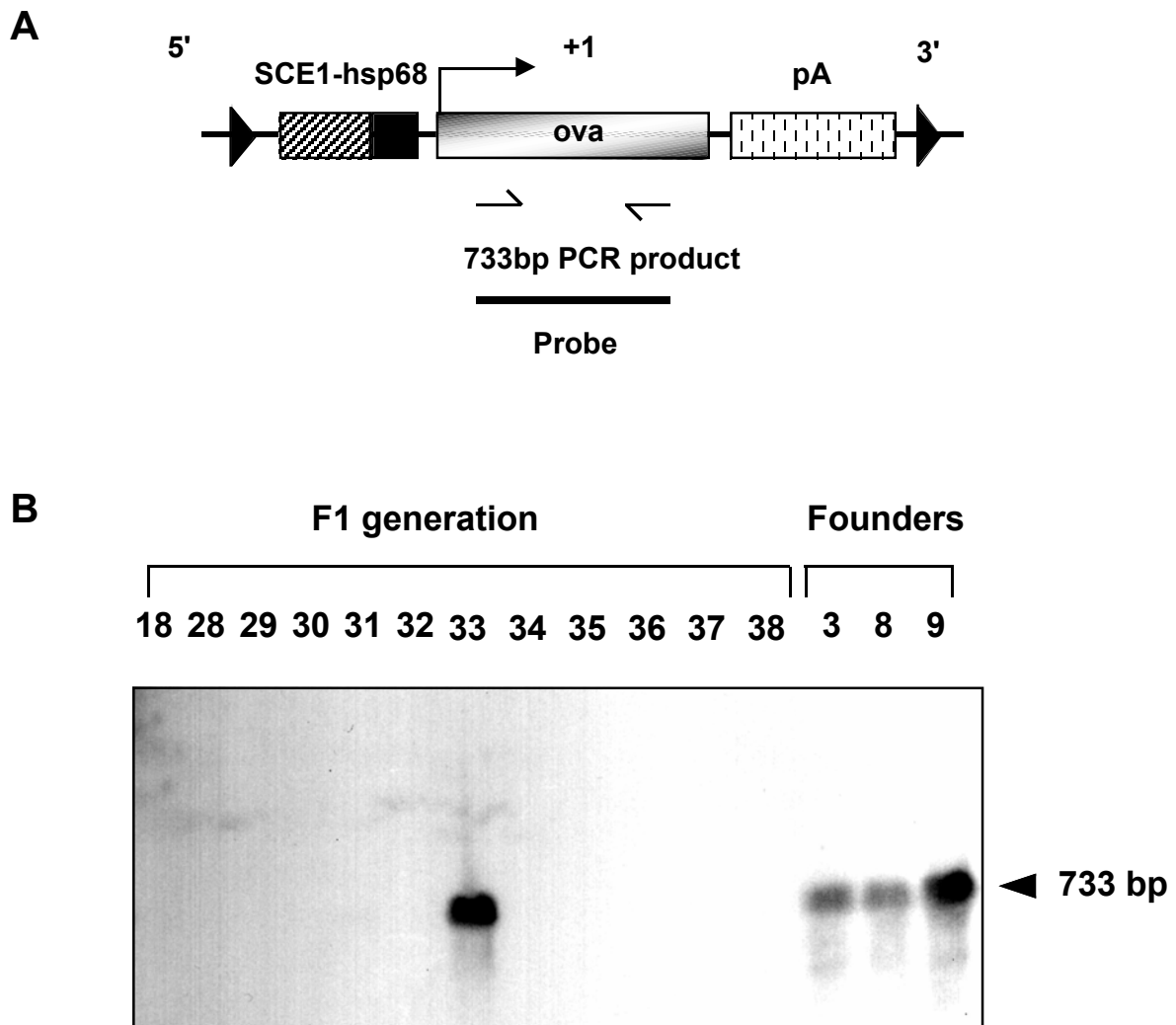
Fig. 8 F1 generation PNO mice carry the OVA transgene within their genome as shown by Southern blot.

A) Location of the 733bp radioactively labelled OVA PCR probe within the OVA transgene is shown. B) Amongst nine F1 PNO3 generation mice, one male mouse was confirmed OVA positive by probing with the OVA PCR product. Furthermore genomic DNA from the three PNO founders again displayed the OVA transgene. SCE; Schwann cell enhancer, P; hsp68 minimal promoter, pA; poly A tail.

The presence of the OVA transgene within genomic DNA from CNO mice was assessed solely via OVA PCR. After CNO founder and F1 generation mice had been identified via PCR, Dr Yi Cao (from within our group) characterised protein expression via immunocytochemical and immunohistochemical methods.

A

B

◄ 733bp

◄ 733bp

Fig. 9 CNO founder mice carry the OVA transgene within their genome as shown by OVA PCR.

A) Location of the OVA specific primers within the OVA transgene used to generate the 733bp PCR product is shown. B) Recovered genomic DNA from proteinase K digested CNO mouse tail biopsies was subjected to the OVA PCR. Depicted are 5 PCR positive CNO founders from 19 tail biopsies as well as a C57BL/6 negative control. MBP; myelin basic promoter, pA; poly A tail, C; control C57BL/6 genomic DNA.

All CNO founder mice were mated with C57BL/6 mice. Both PNO and CNO non-manipulated mice were healthy throughout their normal lifespan. OVA Tg mice did not show demyelination or any other signs of a PNS or CNS disorder. Furthermore neither PNO nor CNO mice displayed histopathological hallmarks of inflammation in either PNS or CNS tissues respectively.

3.1.6 Detection of OVA protein expressed in PNO mice

Immunohistochemical and immunocytochemical approaches including in situ immunohistochemistry and Western blot, were undertaken to identify the OVA transgene product in the CNS (brain, spinal cord) and PNS (sciatic nerve) of PNO mice. Polyclonal anti-OVA Ab staining of sciatic nerve was unsuccessful in detecting specific OVA expression within Schwann cells (Fig. 11B). Nor did the CNS show OVA expression (data not shown). However thymic epithelial cells appeared brown implying OVA expression (Fig. 11D).

In the RIPmOVA Tg mouse, the rat insulin promoter (RIP) has been used to confer specific expression of OVA to the β-islet cells of the pancreas. Therefore pancreas sections from RIPmOVA Tg mice were always included in immunohistochemical stainings as positive controls (Fig. 10). Immunological relevant organs from PNO mice including heart, lungs, liver, kidneys, stomach, small intestine, large intestine, lymph nodes, spleen, pancreas, bladder, testis, ovaries were negative for OVA (data not shown). For certain tissues such as sciatic nerve staining with the polyclonal Ab led to high background (Fig. 11) that could not be removed with azide or in OVA blocking studies (data not shown).

A   **B**
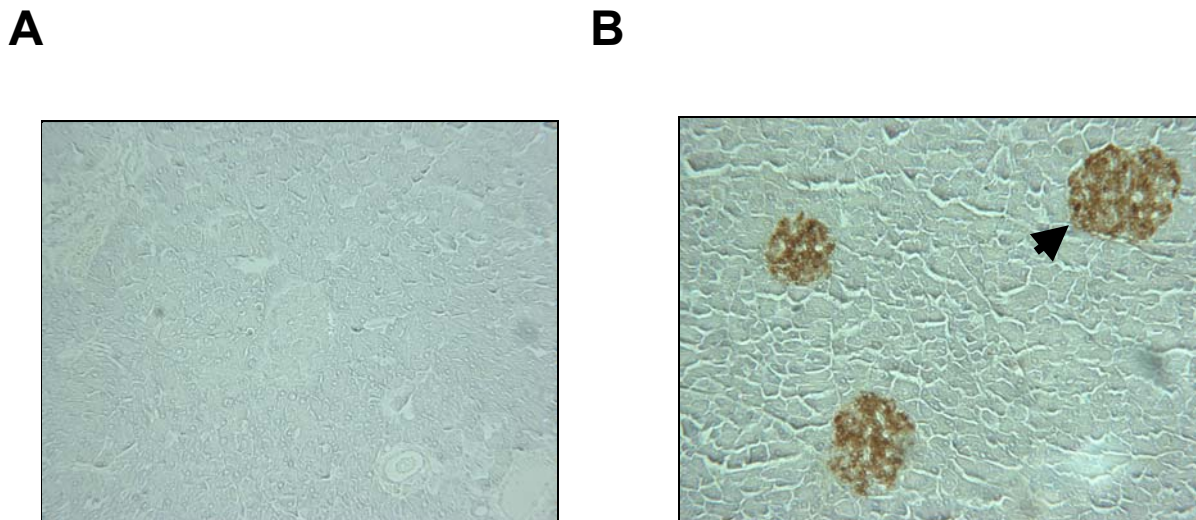
Fig. 10 Detection of OVA expression within pancreatic islets of pancreas sections from RIPmOVATg mice served as positive controls.

5μm pancreas paraffin sections from RIPmOVA Tg mice were stained with polyclonal anti-OVA Ab and counterstained with haematoxylin. The arrow in the photomicrographs shows β-islet cells remaining unstained in the absence of the polyclonal Ab A) and stained brown in its presence B). Original magnification; X400.

C57BL/6   **PNO**

A   B

C   D
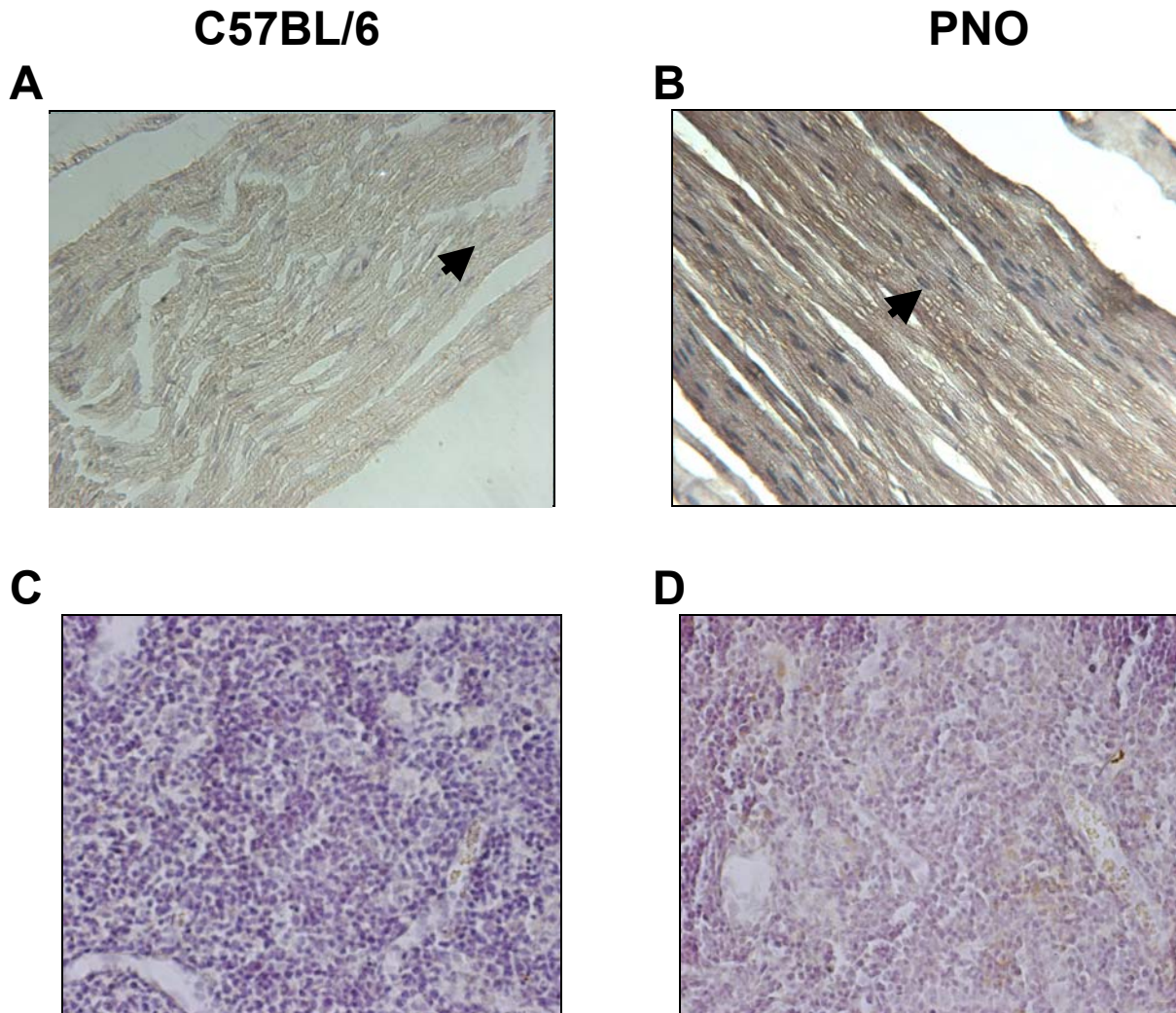
Fig. 11 OVA expression was detected in the thymus, but not in sciatic nerve, of PNO mice

5μm paraffin sciatic nerve and thymus sections were stained with polyclonal anti-OVA Ab and counterstained with haematoxylin. A) and B) show photomicrographs of sciatic nerve while C) and D) show photomicrographs of thymi from C57BL/6 and PNO mice respectively. Sciatic nerve stained with the polyclonal anti-OVA Ab led to non-specific staining. Thymus sections incubated with anti-OVA Ab identified OVA expression in PNO but not C57BL/6 mice. Arrows point to Schwann cells in sciatic nerve. Original magnification; X400.

Immunoprecipitation, western blotting, and ELISA assays, using anti-OVA mAb, were unsuccessful in detecting immunoreactive OVA protein in PNS tissue extracts and serum of PNO mice (data not shown). This suggested that Tg OVA protein in PNO mice is expressed at levels below the assay detection limit or absent. Dr Cao reported contrasting results for the CNO mice. Western blot and immunoprecipitation results confirmed the presence of OVA protein within the brain but not in the spinal cord and sciatic nerve. Further histological analysis determined OVA protein to be present within brain sections of two PCR positive CNO mouse lines. Expression was restricted to the CNS and at the cellular level to oligodendrocytes.

To further determine the presence of potential OVA protein in PNS, CNS and other immunological relevant organs a more sensitive approach was undertaken by investigating

mRNA expression. Total RNA was extracted from PNO and WT mice. The Qiagen RNeasy kit was used to transcribe mRNA to cDNA before the OVA specific PCR could amplify any OVA transgene. In parallel the β-actin PCR served as the internal control. Although OVA mRNA could not be detected in sciatic nerve it was present in lungs and kidney of PNO mice. Again, it is possible that minute amounts of OVA mRNA expressed within sciatic nerve were below assay detection limit or simply absent.
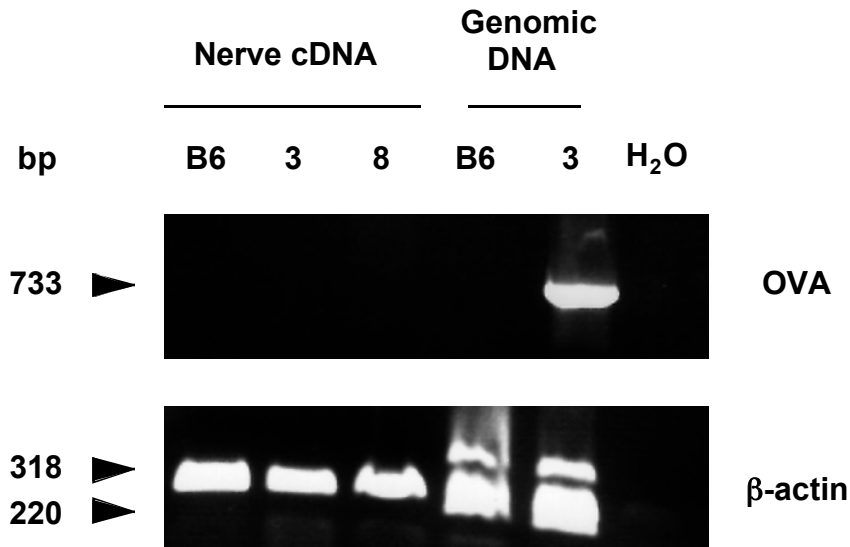
Fig. 12 OVA mRNA is not detectable from sciatic nerve of PNO mice.

Total RNA was extracted from sciatic nerve and reverse transcribed to cDNA. The OVA specific PCR was unable to detect OVA transgene expression within PNO3 and PNO8 mice. The β-actin PCR served as the internal control. Genomic tail DNA from PNO3 and C57BL/6 mice served as the positive and negative OVA PCR control respectively. bp; base pairs, B6; C57BL/6, 3; PNO3, 8; PNO8, H_2O; water control
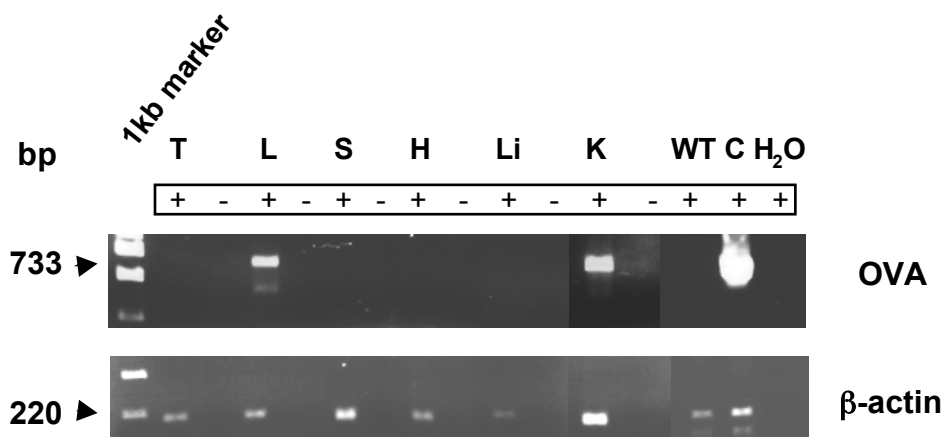
Fig. 13 OVA mRNA is ectopically expressed within lungs and kidneys of PNO mice.

Total mRNA was extracted from thymus, lungs, spleen, heart, liver, kidney and reverse transcribed to cDNA. OVA specific PCR determined transgene expression within lungs and kidney of PNO mice. The β-actin PCR served as the internal control. bp; base pairs, WT; wild type/C57BL/6 genomic tail DNA, C; control/ PNO3 genomic tail DNA, H2O; water control, +;

added reverse transcriptase, -; no added reverse transcriptase, T; thymus, L; lungs, S; spleen, H; heart, Li; liver, K; kidney.

3.2 Attempts to induce disease in PNO mice by active immunisation with OVA

Previous Tg models have shown that although the transgene protein product could not be detected via immuno -cytochemistry or -histochemistry, disease induction proved successful. Whitton et al., showed that CTL cell recognition of autoAg expressed on pancreatic β cells is more sensitive than Ab detection methods (Whitton and Oldstone 1989). As the OVA protein may have been expressed within the PNS of PNO mice at very low levels active PNS disease induction by immunising mice with whole OVA was attempted. The following flow chart depicts the procedure used to induce and analyse disease induction in PNO and also in CNO mice. Dr Yi Cao conducted all immunisations with the CNO mice.

Day 0
sc   injection of 200ug whole ovalbumin/PBS emulsified 1:1 in CFA

PNO mouse

Day 1&3
ip injection of 400ng pertusis toxin

Parameters analysed

• **Clinical symptoms including weight loss assessed**

• **Anti-ova IgM and IgG serum analysis**

• **In vitro ovalbumin proliferative recall response measured**

• **Histological analysis via H&E stainings of nerve, brain & spinal cord**
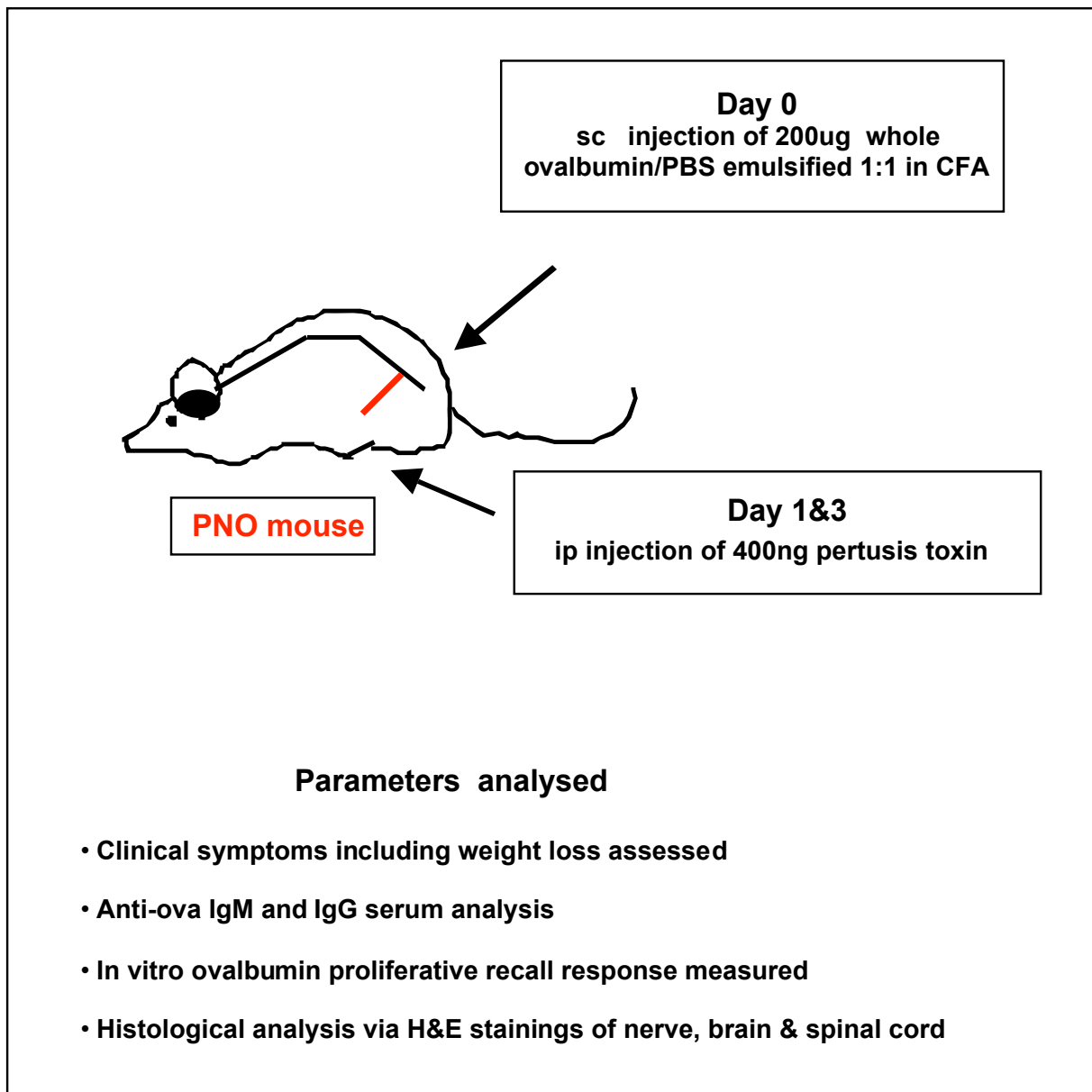
Fig. 14 Flow chart illustrating the protocol for active disease induction of PNO mice and the subsequent analysis.

On day 0 mice were sc immunised with whole OVA in CFA, followed by ip injection of PT on days 1 and 3. At time points mentioned mice were analysed for weight loss, anti-OVA IgM and IgG, in vitro OVA proliferative recall response and the presence of infiltrating mononuclear infiltrates in the PNS and CNS.

Each of the three different PNO mouse lines, 3, 8 and 9 were immunised with whole OVA. After monitoring the degree of paralysis according to the adapted scale from 0-5 (mentioned in materials and methods) it was determined that no PNO mouse succumbed to a clinical form of a PNS disease. In previous EAE and EAN models weight loss has been shown to occur in parallel to the developing subclinical disease symptoms (Slavin, Bucher et al. 1996) (Pollak, Ovadia et al. 2000). PNO mice did not significantly lose weight compared with NTg counterparts nor was there any significant difference between males or females. In fact, the PNO immunised group contained one female and one male mouse that gained nearly double the initial weight. Interestingly a slight decrease of about 10% of the initial weight was observed in both groups post immunisation day 1 to 3 suggesting that the immunisation procedure itself influenced the well being of the mice.
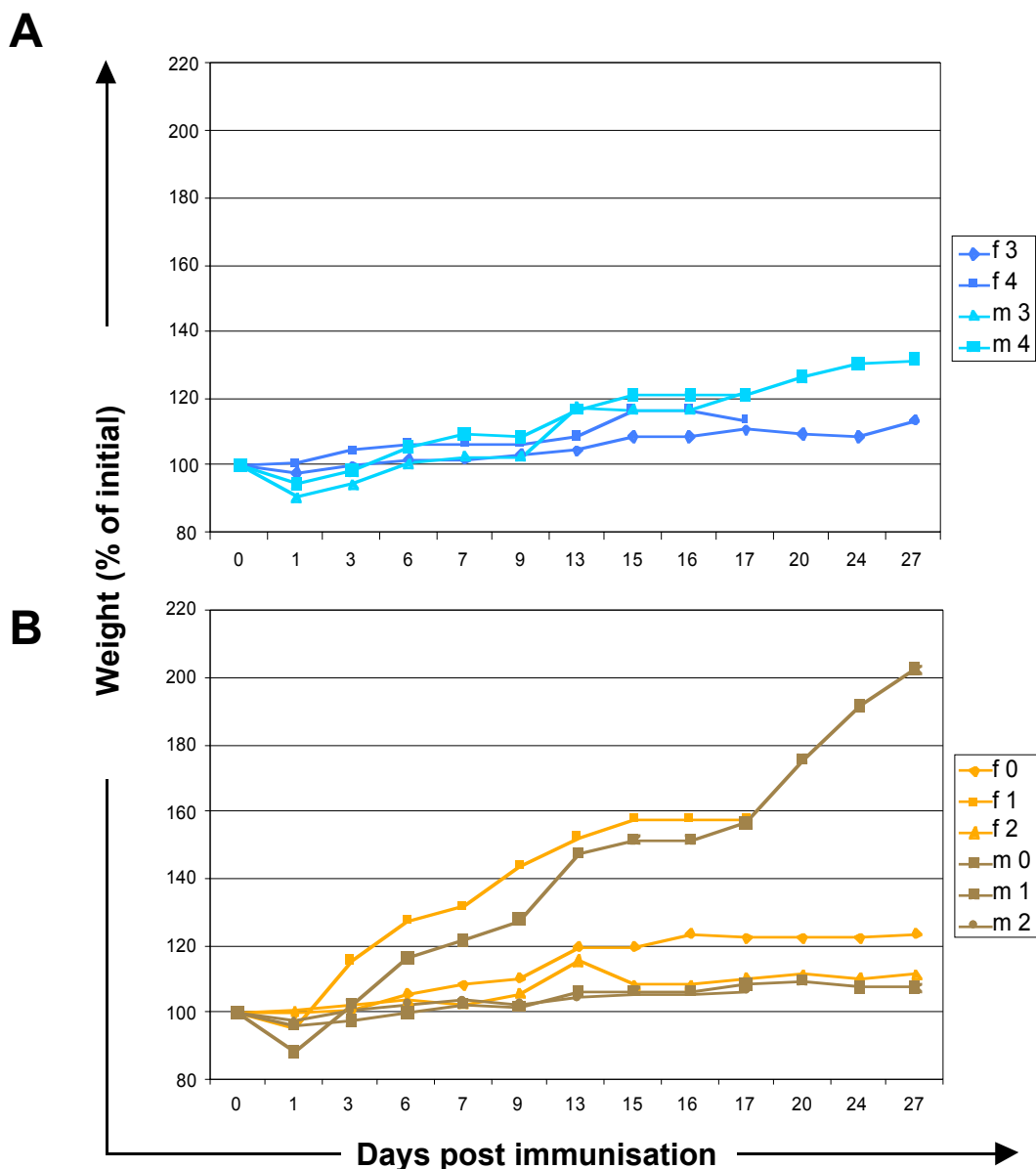
Fig. 15 PNO9 mice do not show significant weight loss when compared with NTg controls post immunisation with whole OVA.

PNO9 mice and their NTg counterparts were immunised sc day 0 with 200ug whole OVA emulsified 1:1 in CFA. 400ng PT was administered ip on days 1 and 3. PNO9 mice were monitored for a total of 27 days in which time mice were weighed at different time points. A representative experiment is shown which was also conducted with PNO3 and PNO8 mice. The weight analysis is shown as the percentage of initial weight. The average age of the immunised mice was 9-12 weeks. A) depicts the NTg mice while B) shows the PNO9 mice. m; male, f; female

The success of the immunisations was also judged by anti-OVA IgM and IgG serum levels as determined by ELISA on day 14-16 post immunisation. In contrast to C57BL/6 mice, immunised PNO9 mice displayed almost a 2 fold decrease in anti-OVA IgM and a 3 fold decrease in anti-OVA IgG levels suggesting at least partial tolerance to OVA in the T helper cell and/or B cell compartment in PNO mice.

A

B
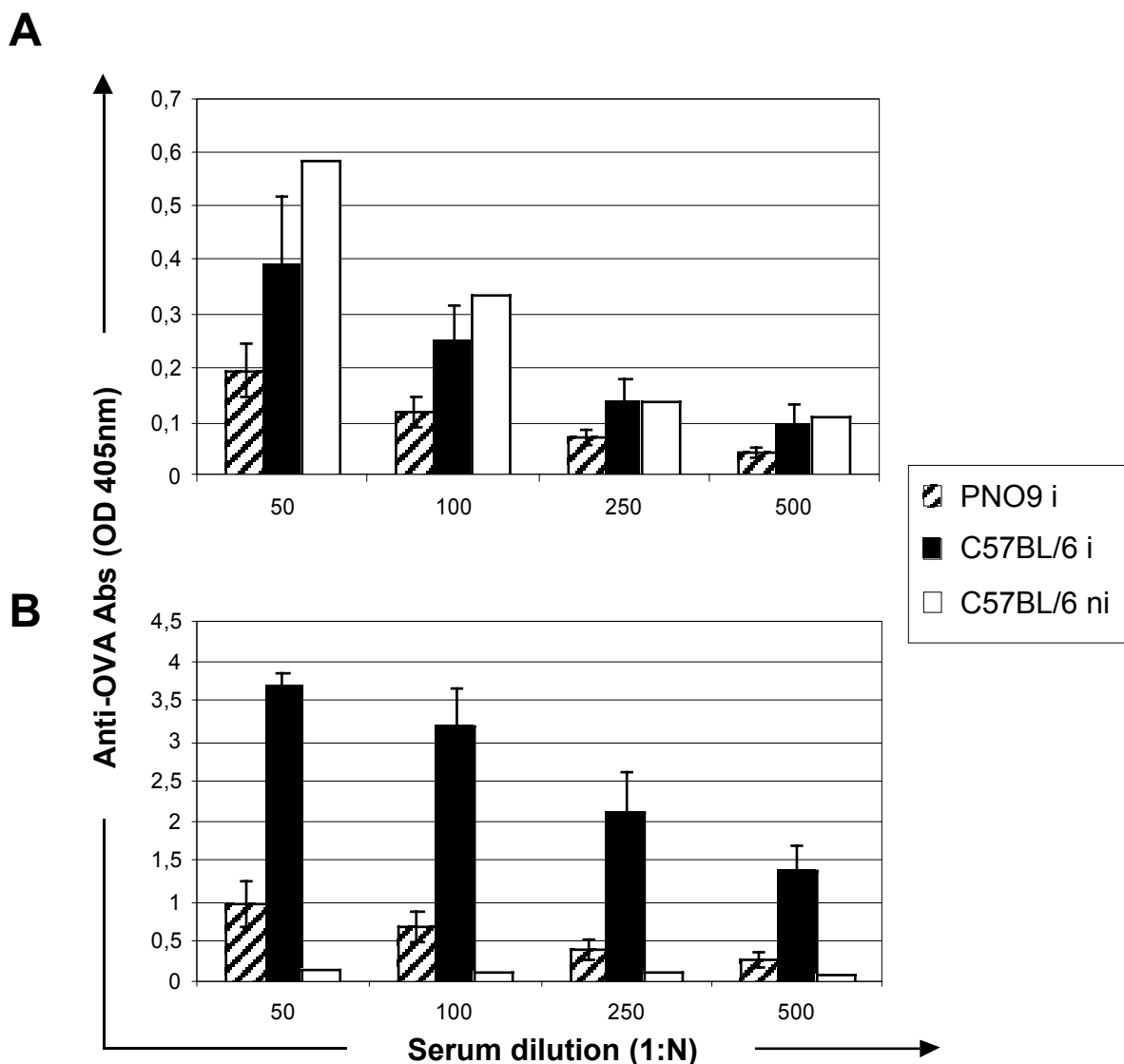
Fig. 16 PNO9 mice generated a lower anti-OVA Ab response when compared to their NTg counterparts.

PNO9 mice and their NTg counterparts were treated as in Fig 15. Sera were collected on day 16 post immunisation. Anti-OVA A) IgM and B) IgG antibodies in diluted triplicate samples were determined by sandwich ELISA. One representative experiment is shown. The same experiment was conducted with PNO3 and PNO8 mice with a similar outcome. The column graphs show the mean and SEM of 6 PNO9 and 5 C57BL/6 immunised mice and one C57BL/6 non immunised mouse. Enzymatic activity was measured at the absorbency wavelength of 405nm. i; immunised, ni; non immunised

Sixteen days post immunisation LN T cell responses were assessed. PNO9 LNC showed a reduced *in vitro* proliferative recall response to whole OVA by about 2.5 fold in comparison to WT, which again suggested tolerance induction.

Fig. 17 the in vitro proliferative recall response to OVA is significantly reduced in PNO LNC when compared with NTg.

PNO9 mice and their NTg counterparts were treated as in Fig 15. Single LNC suspensions prepared from mice 16 days post immunisation were stimulated in vitro with either A) whole OVA and B) medium alone or ConA for 72h. During the last 16h of culture, incorporation of [^{3}H]-Thymidine by proliferating LNC was measured. The column graphs are representative of the mean [^{3}H]-Thymidine incorporation of 2 PNO9, C57BL/6 immunised mice and one non immunised C57BL/6 mouse. i; immunised, ni; non immunised

The overall proliferative recall response of splenocytes when compared with LNC was lower accounting for the presence of fewer T cells. The individual recall response of immunised PNO splenocytes was also reduced by about 1/2 fold when compared with WT. As the overall recall response in both LNC and splenocytes of immunised PNO mice was as low as the background obtained with non-immunised WT mice, it became clear that substantial OVA specific priming did not occur in PNO mice. Both PNO and WT LNC and splenocytes were equally responsive to the polyclonal activator ConA indicating no intrinsic defect in proliferation potential.

A

B
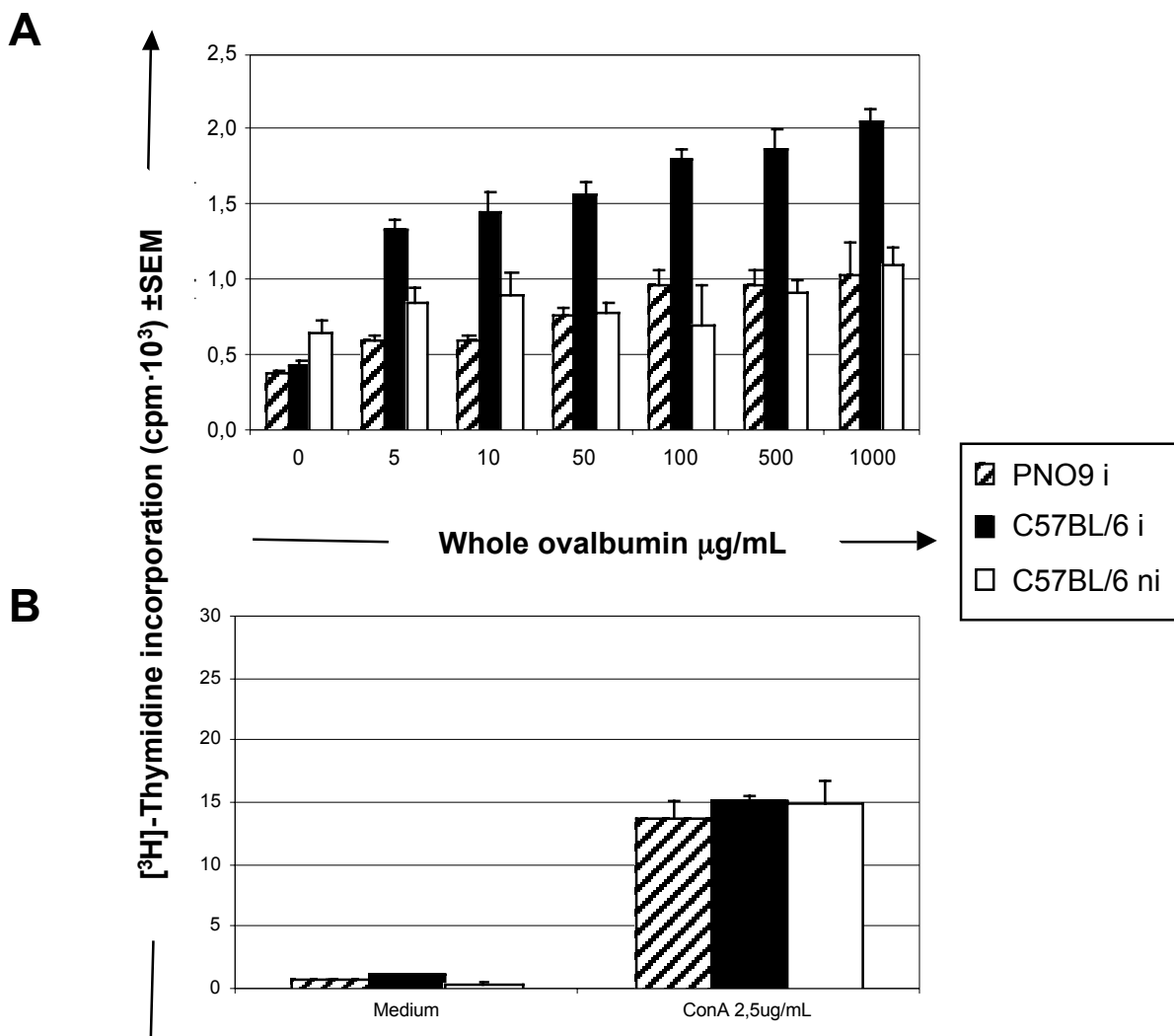
Fig. 18 the in vitro proliferative recall response to OVA is significantly reduced in PNO splenocytes when compared with NTg.
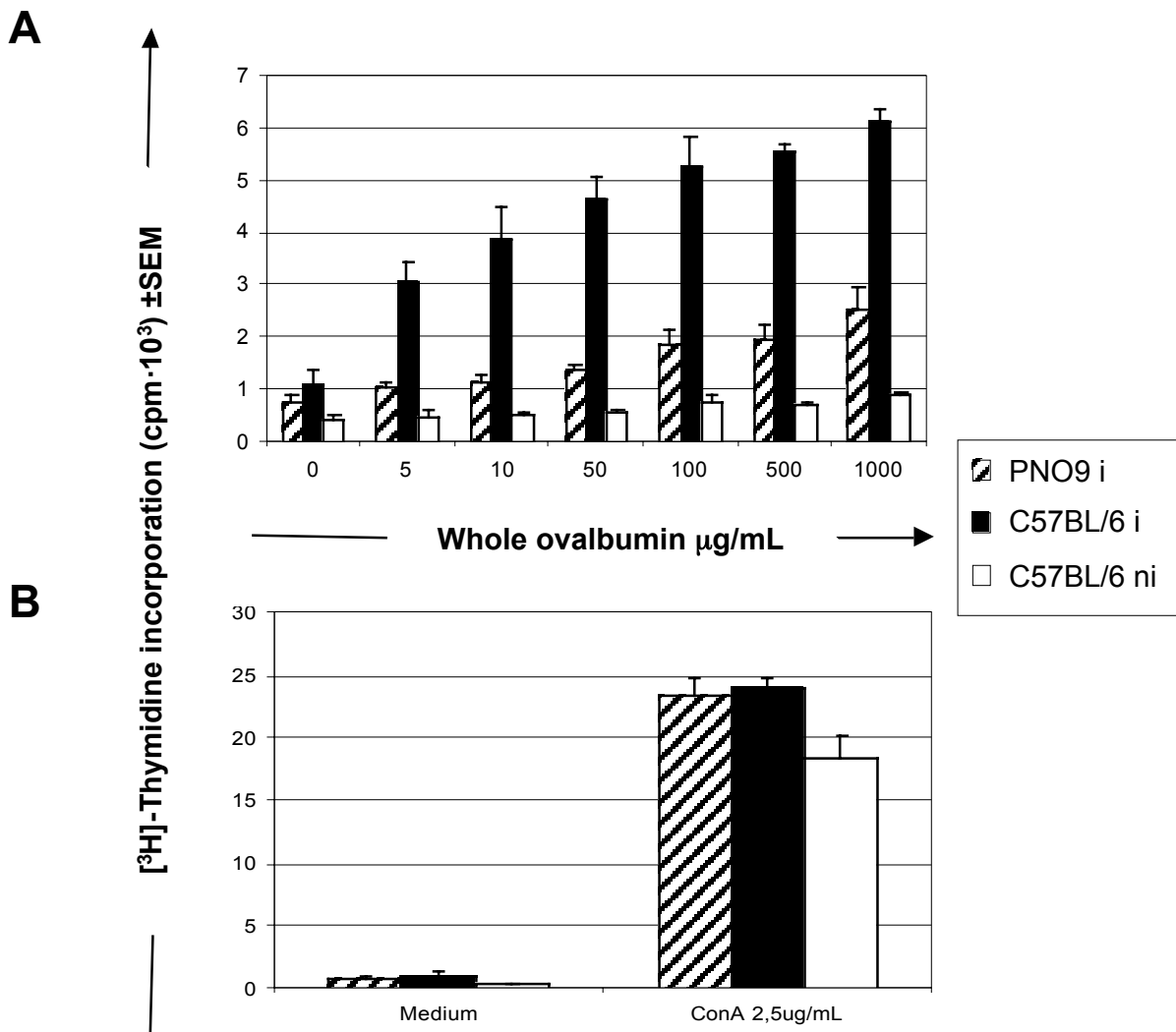
PNO9 mice and their NTg counterparts were treated as in Fig 15. Single cell splenocyte suspensions prepared from mice 16 days post immunisation were stimulated in vitro with either A) whole OVA and B) medium alone or ConA for 72h. During the last 16h of culture, incorporation of [^{3}H]-Thymidine by proliferating splenocytes was measured. The column graphs are representative of the mean [^{3}H]-Thymidine incorporation of 2 PNO9, C57BL/6 immunised mice and one non immunised C57BL/6 mouse. i; immunised, ni; non immunised

Histological investigation of the PNS and CNS organs for subclinical signs of disease proved negative. Perivascular mononuclear cell infiltrates were absent from H&E stainings of paraffin sections from brain, spinal cord, sciatic nerve.

A

B

C
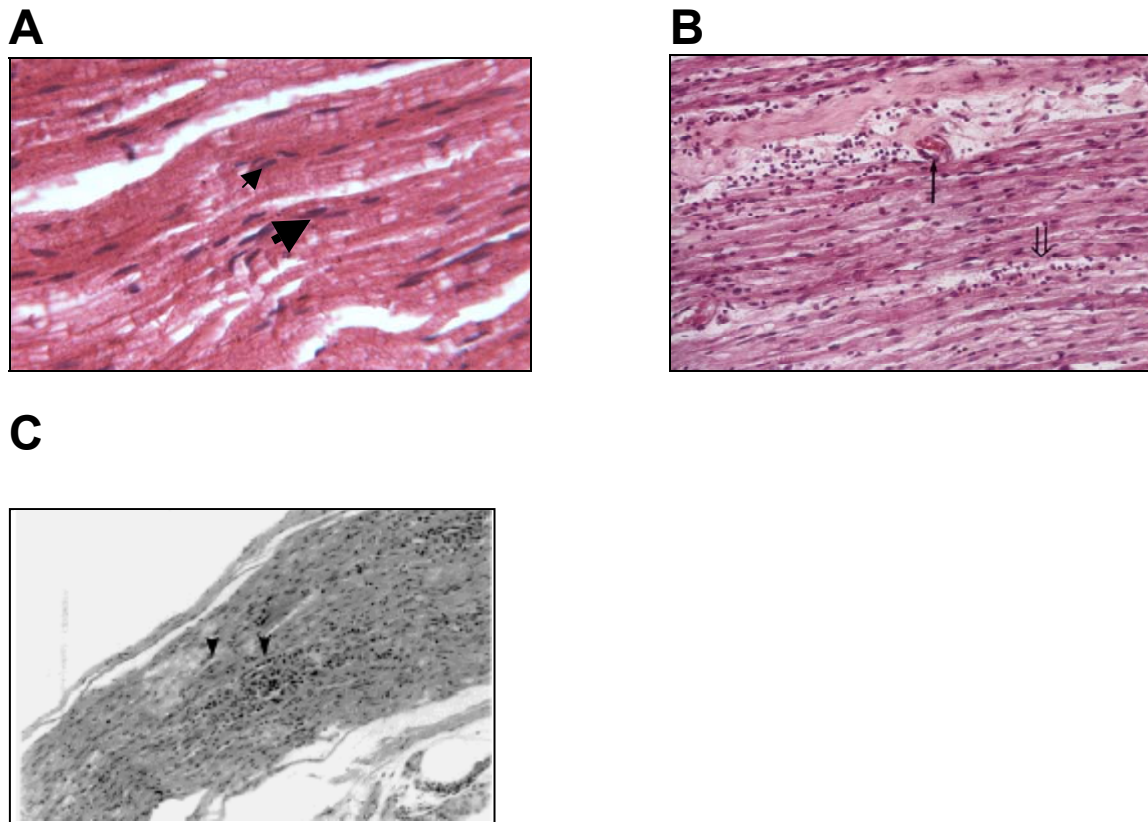
Fig. 19 perivascular mononuclear infiltrates are absent from sciatic nerve of immunised PNO mice.

5μm paraffin sciatic nerve sections from PNO9 and their NTg counterparts were H&E stained. A) Photomicrograph of paraffin sections taken from sciatic nerve of PNO mice did not contain mononuclear infiltrates. Arrows indicate the nuclei of Schwann cells. Original magnification X400. In contrast inflammatory infiltrates can be seen in photomicrographs of sural nerve taken from a GBS patient B) (original magnification X230; adapted from Rosalind King (1999) In Atlas of Peripheral Nerve Pathology, Great Britain: Arnold, pp141, original magnification x230)) and sciatic nerve from a PO-EAN diseased C57BL/6 mouse (original magnification X200; adapted from Zou et al., (Zou, Ljunggren et al. 2000). Arrows indicate the mononuclear infiltrates in both cases.

Contrasting results were reported for the CNO mice. In approximately half of the animals, active EAE was successfully induced by immunisation with OVA. In most cases EAE symptoms and weight loss were mild. However, some animals responded with a maximum score of becoming moribund and had to be sacrificed. Furthermore, perivascular mononuclear infiltrates were observed in brain sections of diseased mice (Dr. Yi Cao, personal communication).

3.3 Attempts to induce disease by adoptive transfer of OVA specific TCR Tg T cells

Previous reports have shown that iv transfer of encephalitogenic CD8+ T cell clones results in a more severe nervous disease (Sun, Whitaker et al. 2001) than immunisation with an encephalitogenic peptide. Furthermore, in an experimental autoimmune uveitis model induction of disease was unsuccessful via immunisation with the retinal Ag but successful by adoptive transfer of the autoAg specific T cell clone (Xu, Wawrousek et al. 2000). It was therefore attempted to induce a PNS or CNS autoimmune disease in CNO and PNO mice respectively via adoptive transfer of *in vitro* activated OT-I and OT-II cells either concomitantly or sequentially.

Day0
iv injection of in vitro activated and Tc1/Th1 polarised OT-I and/ or OT-II cells

ip injection of 400ng pertusis toxin

PNO /CNO mouse

Parameters analysed

• **Clinical symptoms including weight loss assessed**

• **FACS analysis for the recovery and activation status of clonotypic+ T cells from LN and spleen**

• **Histological analysis via H&E stainings of nerve, brain & spinal cord**
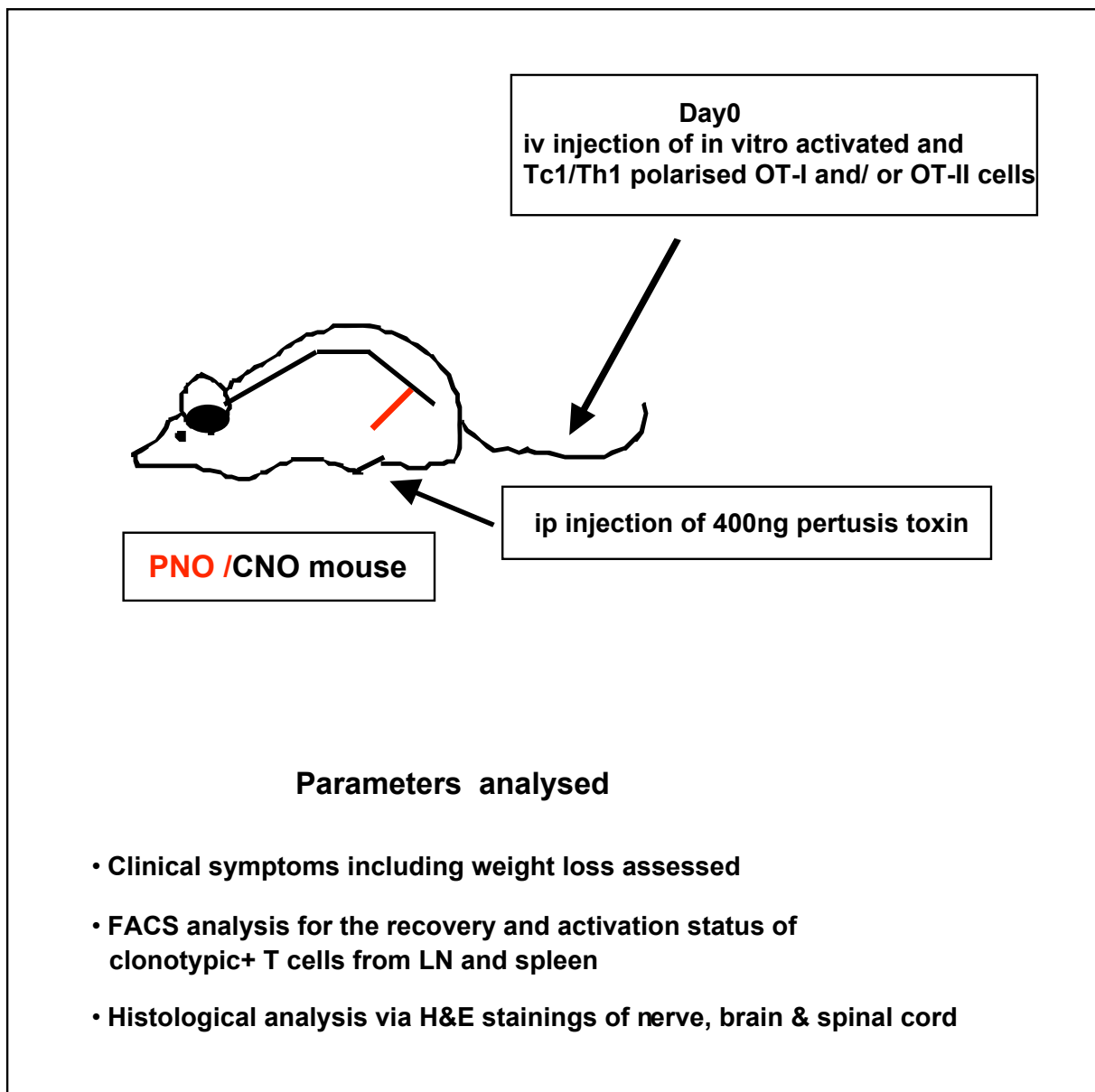
Fig. 20 Flow chart illustrates passive disease induction of PNO and CNO mice and the subsequent analysis.

On day 0 mice were iv injected with in vitro activated Tc1 OT-I and/or Th2 OT-II T cells. Mice were ip injected with PT on the days indicated in table 4. At time points indicated, mice were analysed for weight loss. In addition LNC and splenocytes were analysed via FACS for the

recovery and activation status of clonotype+ T cells. Furthermore, PNS and CNS tissues were histologically analysed for the presence of infiltrating mononuclear infiltrates.

Based on established adoptive transfer models of EAE or EAN a number of different parameters for disease induction in CNO and PNO mice had to be considered. These included the following parameters:

- polarisation of *in vitro* activated T cells to the Th1 and Tc1 subtype prior to transfer
- the number of cells to be injected
- concomitant or sequential injection of the two different clonotype+ T cells
- time point for PT injection
- time point for the retrieval and examination of transferred OVA specific T cells *ex vivo*

No. of Expt.	Genotype	Mice/group	Total cells transferred (x10^6)	Time pts. (dpi)	PT (d)	Irradiated
1	PNO & BL/6	3	5	11, 15, 17	0 & 2	-
2	PNO & BL/6	10	10	10, 15, 16, 22, 23, 30, 36, 43, 56	1&3	-
3a	PNO & BL/6	6	10	10, 21, 35	-2 &0	+
3b	CNO	8	10	11, 23, 43	0& 2	-
4	CNO	5	30	3, 7, 12	1&3	+

Table 4. Features of the adoptive transfers carried out on PNO and CNO mice.

The following table depicts the different parameters that were altered in the numbered experiments. These included the total number of OT-I/ OT-II cells transferred, time points of analysis, day of PT injection and irradiation of recipients. dpi; days post injection, d; day, PT; pertussis toxin, Time pts; time points.

OT-I and OT-II LN single cell suspensions were stimulated *in vitro* with cognate peptide in the presence of soluble IL-12 and IL-2. Six days *in vitro* stimulation instead of only three guaranteed nearly a 100% yield in OT-I clonotype+ T cells and a 90% yield for clonotype+ OT-II T cells. A small fraction of clonotype- T cells diminished from OT-II cell cultures between days 3-6 of stimulation indicating that they were not responsive to antigenic peptide. This again determined that stimulation of OT-I or OT-II LNC with peptide was specific and led to the expansion of clonotype+ T cells.

A

0 days — 33,62 — 36,12

3 days — Vα2 — 83,69 — Vβ5 — 89,35

6 days — 99,95 — 99,88

CD8 → — Vα2 →

B

0 days — 36,53 — 37,81

3 days — Vα2 — 68,88 — Vβ5 — 75,39
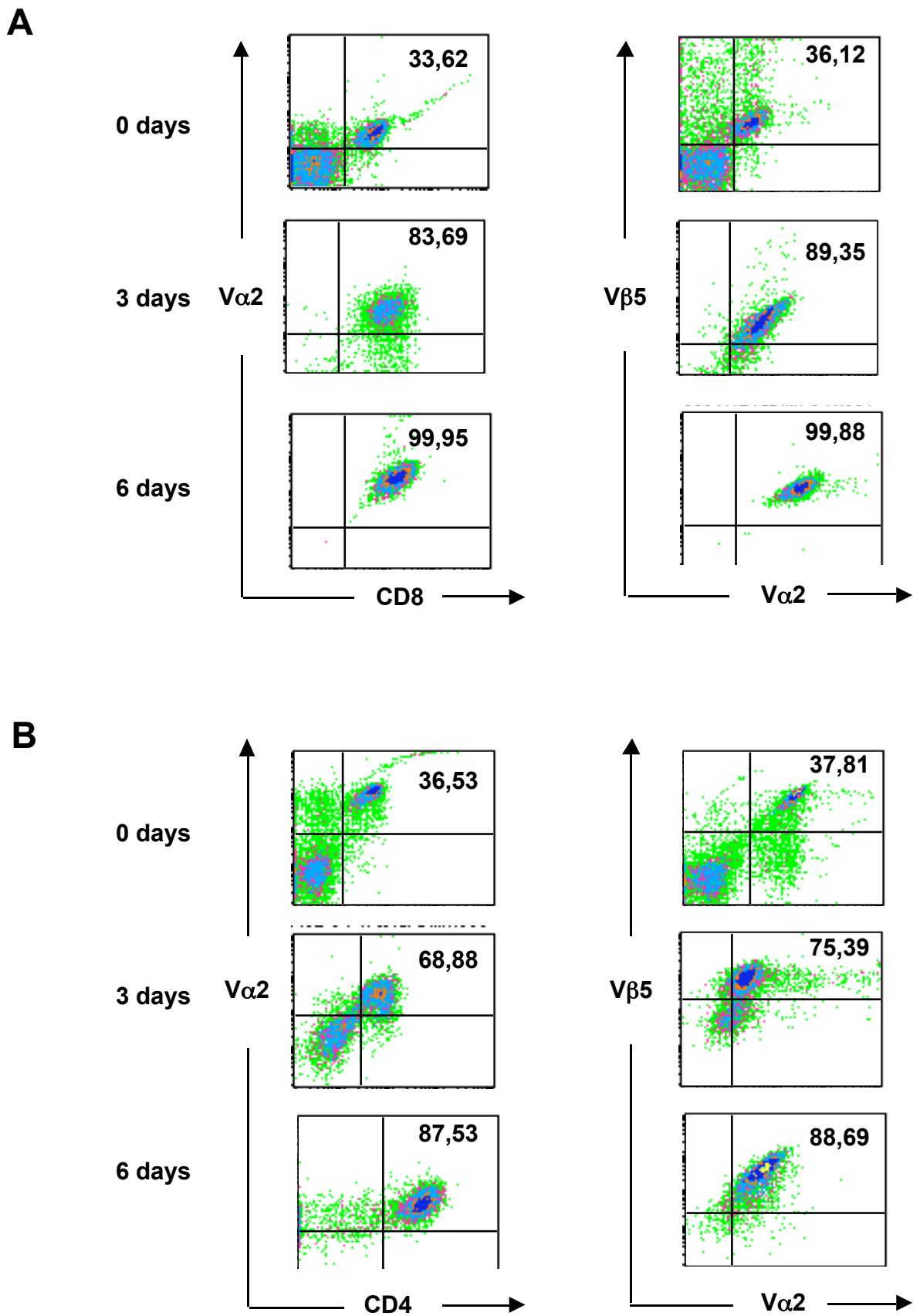
6 days — 87,53 — 88,69

CD4 → — Vα2 →

Fig. 21 Six instead of three days in vitro stimulation with the respective OVA peptide provided a higher yield of clonotype+ T cells.

LNC were cultured in vitro for 6 days in the presence of the antigenic peptide. IL-12 was added for polarisation to the Tc1 or Th1 subtype. The density plots depict the percentage of A) OT-I or B) OT-II clonotype+ T cells obtained on days 0, 3 and 6 days post stimulation with the respective antigenic peptide. Four colour stainings with anti- CD4, CD8, Vα2 and Vβ5 mAbs identified clonotype+ T cells within the LNC population. One staining out of three is shown.

Furthermore secretion of IFNγ by antigenic peptide stimulated OT-I or OT-II clontoype+ T cells provided further evidence of successful *in vitro* polarisation to a Tc1 or Th1 T cell subtype. To exclude polarisation to the Tc2 or Th2 subtype, IL-4 secretion also analysed.
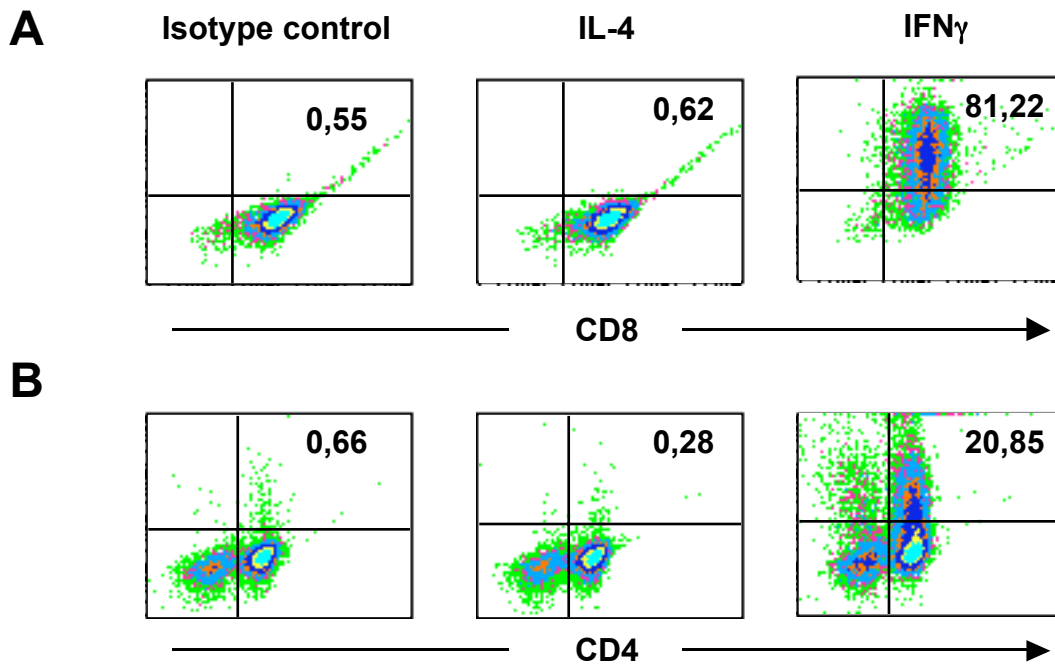
Fig. 22 OT-I Tc1 and OT-II Th1 LNC secrete IFN-γ and not IL-4 after 6 days in vitro stimulation with cognate peptide and soluble IL-12.

OT-I A) and OT-II B) cognate Ag stimulated LNC were polarised to Tc1 and Th1 subtypes in vitro in the presence of IL-12. Intracellular anti-IFN-γ and anti-IL-4 mAb cytokine stainings were conducted whereby density plots indicate the percentage of clonotype+ T cells secreting the respective cytokine.

In all adoptive transfer experiments mice were asymptomatic for a neuroautoimmune disease. Furthermore PNO and CNO mice did not display a subclinical form of disease based on histological analysis of CNS and PNS tissues.

In a preliminary experiment $2.5 \cdot 10^6$ *in vitro* OT-II Th1 and OT-I Tc1 skewed T cells were adoptively transferred into PNO recipients. PT was ip injected on day 0 and day 2. There were neither clinical nor subclinical signs of an inflammatory nervous disorder based on the graded clinical scoring and H&E stainings of CNS and PNS tissues on days 11, 15 or 17 post transfer (data not shown).

The second experiment was also conducted on PNO mice. $5 \cdot 10^6$ *in vitro* OT-I Tc1 and OT-II Th1 polarised cells were sequentially transferred. The sequential transfer was based on the premise that CD4+ T cells are important in providing a conducive environment for a CD8+ CTL response (Lu, Yuan et al. 2000). PT was injected on days 1 and 3. Again histological

evidence for a disease was not found nor were any clinical symptoms observed at any of the time points shown in Table 4 (data not shown).

Based on the unsuccessful attempts to induce a peripheral neuroautoimmune disease in PNO mice by immunising with OVA or upon transfer of anti-OVA TCR Tg T cells it was speculated that peripheral tolerance mechanisms that inhibited an immune response might include the effector function of regulatory T cells. It was therefore decided to sublethally irradiate the PNO mice in an attempt to override all peripheral immune regulatory mechanisms including those exerted by regulatory T cells.

PNO mice were sublethally irradiated (500rad) on day –1, followed by the simultaneous adoptive transfer of $5 \cdot 10^6$ OT-I and OT-II Ag activated and Tc1 and Th1 polarised T cells. In addition PT was administrated on day –2 and day 0. It has been reported that the administration of PT prior to transfer of cells might be an advantage in the induction of disease.

As already mentioned weight loss is a common feature in neuroinflammatory diseases and therefore mice were weighed every other day in parallel to scoring for clinical signs of disease. Mice lost approximately 5% of their initial weight following sublethal irradiation suggesting that this treatment affected the general physical state (Fig. 23). Initial weight was almost recovered by day 2. Although PNO mice displayed a 1-5% reduction in initial weight this was also observed for NTg counterparts. At 3-4 months of age weight gain plateaued.
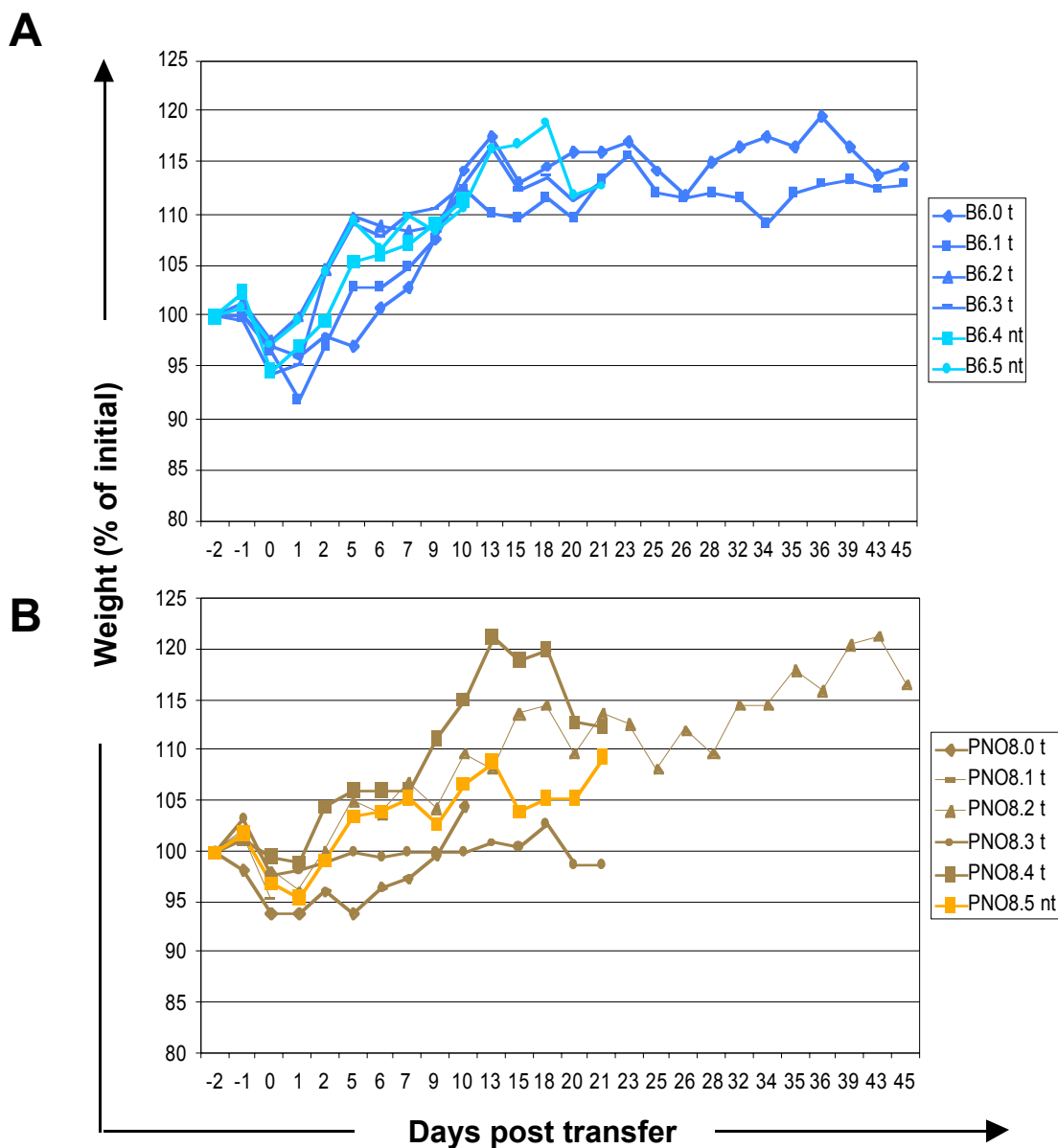
Fig. 23 After transfer of OVA specific T cells sublethally irradiated PNO recipient mice show a comparative weight gain to C57BL/6 control mice.

C57BL/6 A) and PNO B) mice were sublethally irradiated, 500rad on day-1 and ip injected with 400ng PT on days-2 and 0. A total of $10 \cdot 10^6$ Tc1 OT-I and Th2 OT-II in vitro stimulated T cells were adoptively transferred on day 0. Weight is shown as a percentage of the initial weight. t; transfer, nt; no transfer

LN and spleen were analysed at the given time points for the presence of clonotype+ T cells. Four colour FACS stainings with anti- CD4, CD8, Vα2, and Vβ5 mAbs were conducted on LNC and splenocytes. The percentage of recovered Vα2+Vβ5+ T cells from within the gated CD4+ or CD8+ T cell population appeared indistinguishable between PNO and C57BL/6 recipients. This was reflected in the spleen. The relative number of CD8+ T cells recovered from both C57BL/6 and CNO recipients was greater than that of CD4+ T cells at day 10 post transfer suggesting either an intrinsic longer survival ability or homeostatic mechanisms that preferentially prolonged the survival of transferred CD8+ T cells in irradiated mice.

Day 10
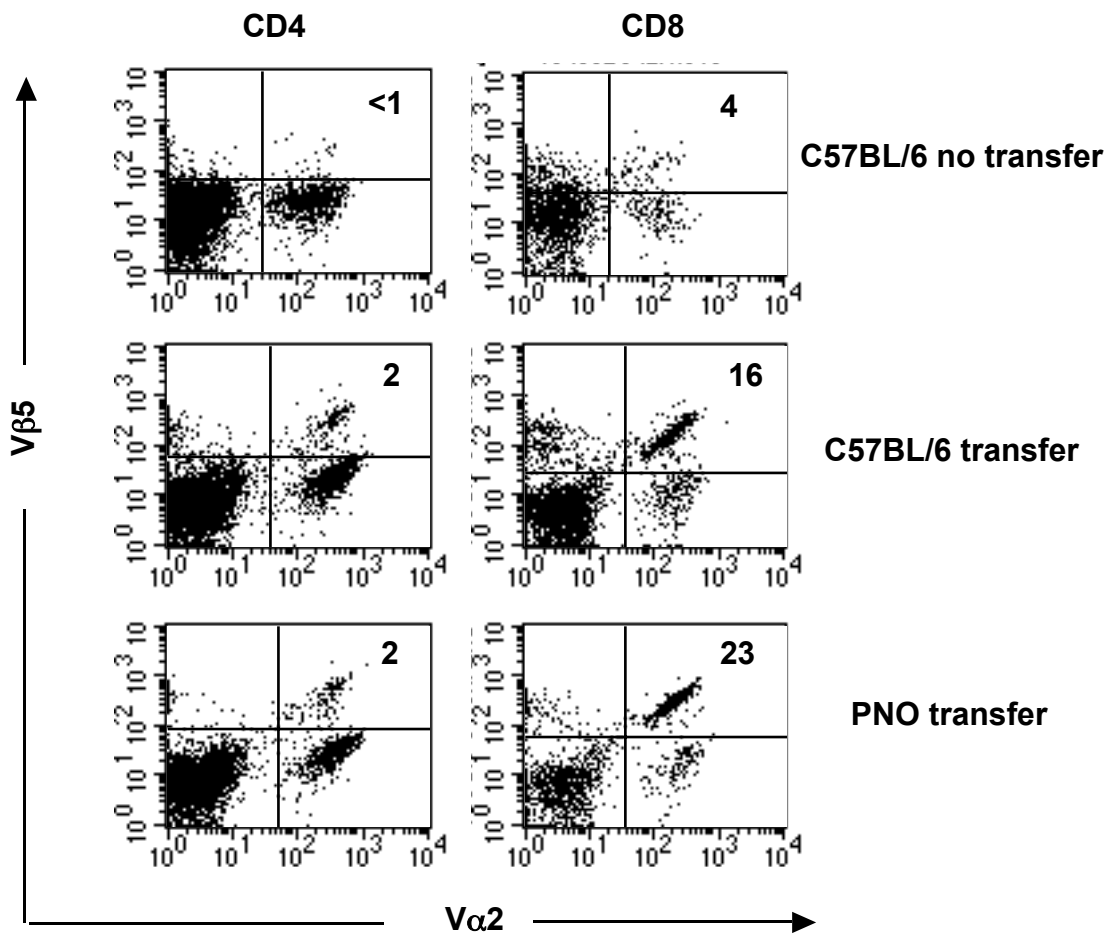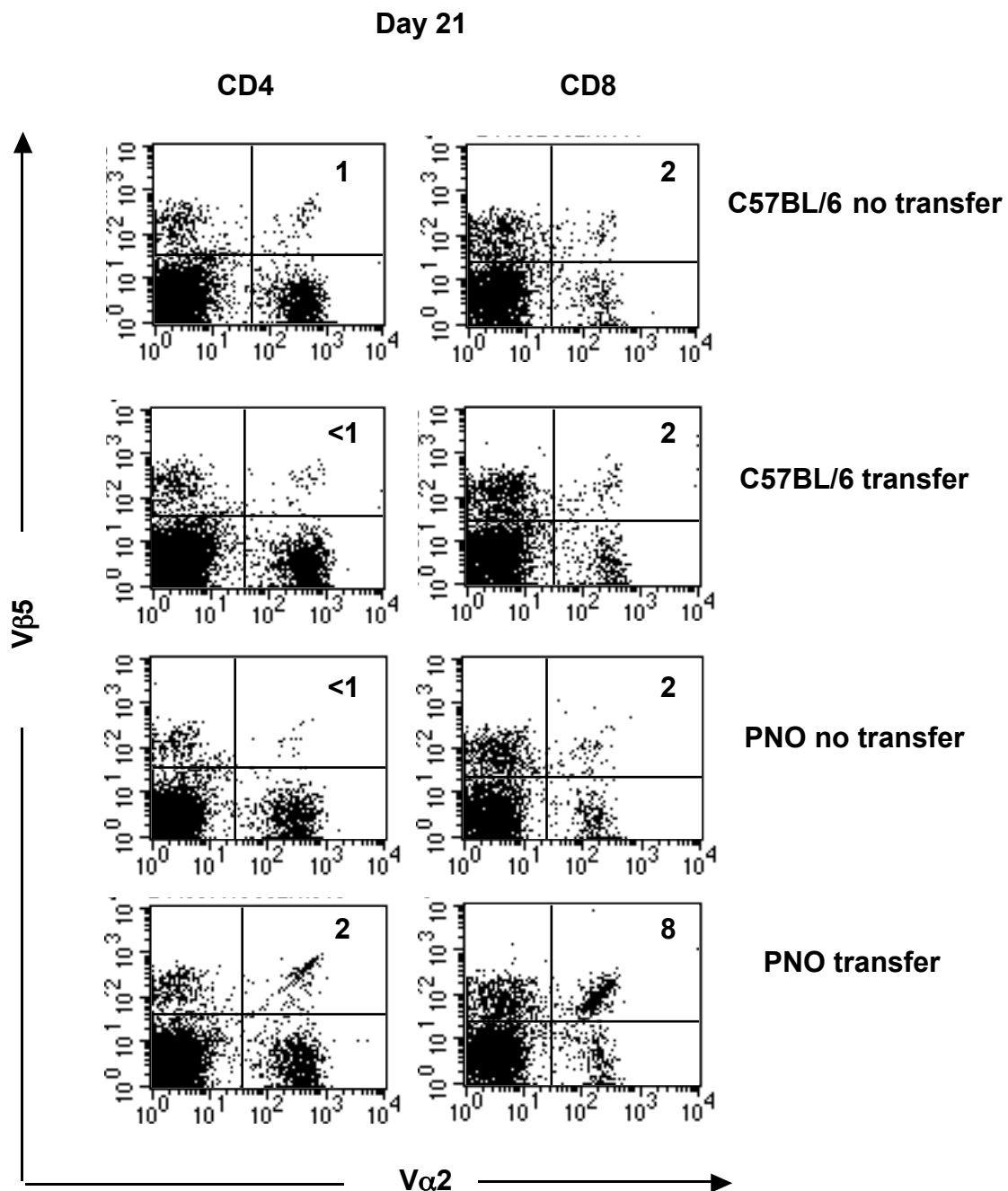
Day 21

Fig. 24 The relative number of transferred clonotype+ CD4+ T cells are already abated at day 10 post transfer while CD8+ T cells decreased at day 21 in LN from sublethally irradiated PNO recipient mice.

Total LNC were removed from PNO mice on days 10 and 21 and analysed by FACS staining with anti-CD4, CD8, Vα2 and Vβ5 mAbs. Dot plots show the percentage of Vα2+Vβ5+ T cells present within gated live CD4+ or CD8+ T cells.

Approximately 2-16% of transferred clonotype+ T cells were recovered from irradiated PNO or C57BL/6 mice on days 10 and 21. This indicated that transferred T cells could be recovered from recipient mice and in the case of CD8+ clonotype+ T cells even at day 10 post transfer. Although the fraction of CD8+ T cells increased four fold at day 21 in PNO recipients, the total number of CD8+ or CD4+ clonotype+ T cells recovered from both PNO and C57BL/6 mice was similar indicating that OVA specific restimulation was not occurring

(data not shown). Furthermore activation marker expression remained unchanged on transferred cells from both C57BL/6 and PNO recipient mice (data not shown).

Although PNO mice remained asymptomatic throughout the course of the experiments it was postulated that perhaps mice were undergoing a subclinical form of an inflammatory disease which could only be determined via H&E stainings. However, perivascular mononuclear infiltrates could not be found in H&E stainings of paraffin embedded brain, spinal cord and sciatic nerve sections (data not shown).

Clonotype+ CD4+ or CD8+ T cells did not display signs of restimulation in vivo as based on diminished cell number and unaltered activation markers when recovered from PNO mice. In conclusion these results support the notion that OVA specific TCR Tg T cells could not be stimulated in the absence of significant target Ag expression.

Based on the successful active disease induction reports in CNO mice (personal communication Dr Cao) it was predicted that the presence of high numbers of target Ag specific T cells could induce a CNS disease. Therefore initial adoptive transfers were carried out on unirradiated CNO mice. A total of $5 \cdot 10^6$ OT-I and OT-II *in vitro* stimulated Tc1 and Th1 polarised T cells were transferred in addition to PT being injected on days 0 and 2. Between days 11 and 27 post transfer OT-I and OT-II transferred T cells were reduced almost 2 fold in both LN and spleen. Transferred T cell numbers were therefore reduced to background levels as found in non transferred CNO recipient mice (data not shown). Histological analysis of either brain or spinal cord determined no mononuclear infiltrates (data not shown). This suggested that either the peak response time point at which restimulated clonotype+ T cells would be present in greater numbers was overlooked or that the number of transferred T cells was too small to recognise presented OVA.

Peripheral tolerance mechanisms including the action of regulatory T cells may have also suppressed any signs of a CNS disease. To address this issue CNO recipient mice were sublethally irradiated in the following preliminary experiment. Furthermore the total number of OT-I and OT-II cells injected was increased 3 fold ie from 5 to $15 \cdot 10^6$ each. PT was injected ip on days 1 and 3 post transfer.

Irradiated CNO mice did not significantly lose weight compared to WT counterparts. However, Fig. 25 suggests that CNO mice gained weight more slowly relative to C57BL/6 control mice. However, CNO recipient mice did not display any outward signs of a CNS type disorder.
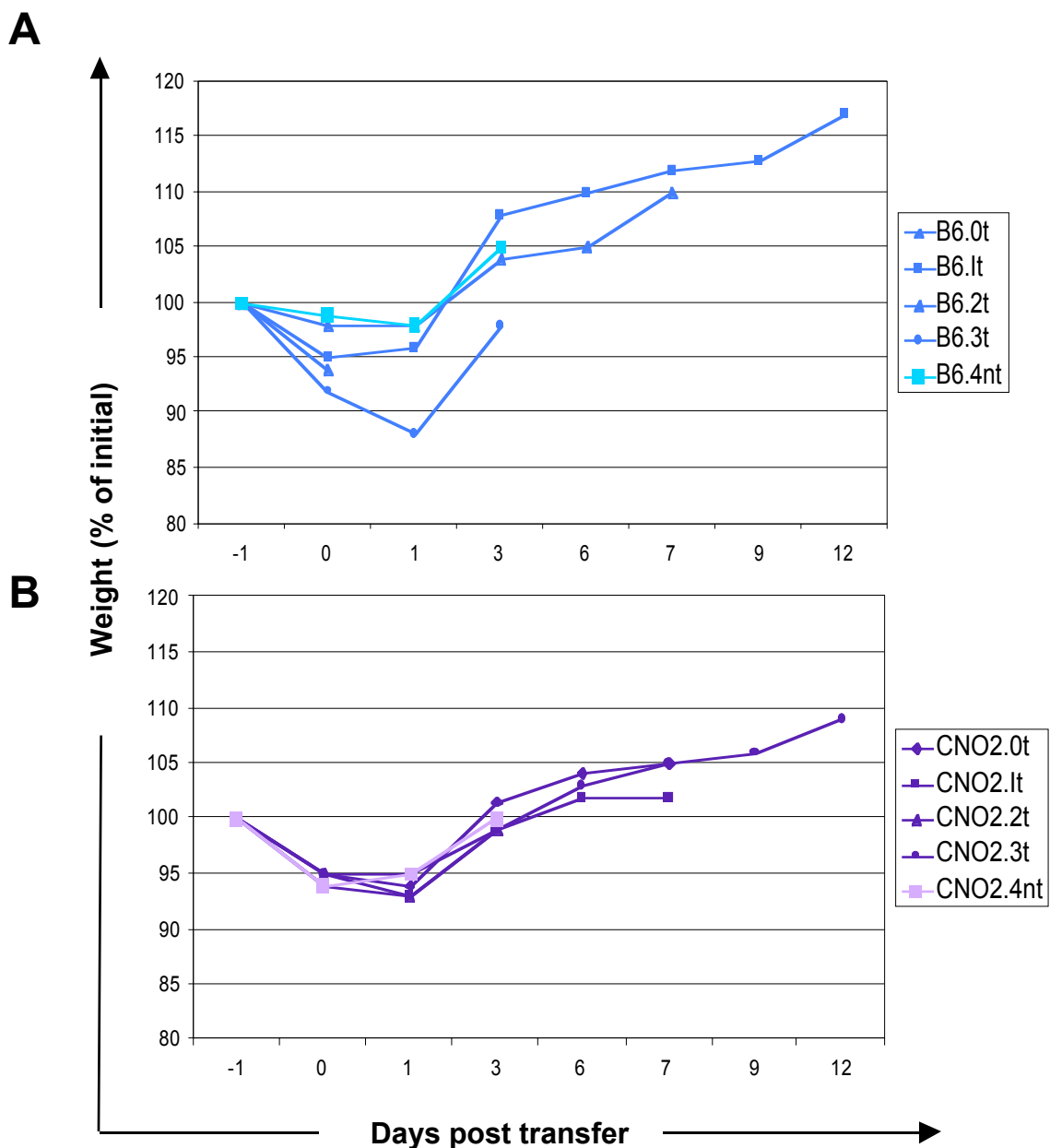
Fig. 25 Sublethally irradiated CNO recipient mice show a comparative weight gain to C57BL/6 control mice.

C57BL/6 A) and CNO B) mice were sublethally irradiated 500rad on day-1 and ip injected with 400ng PT on days 1 and 3. A total of 30 million OT-I and OT-II in vitro stimulated Tc1 and Th1 polarised T cells were adoptively transferred day 0. Mice were weighed every other day and assessed for clinical signs of a nervous system disorder based on the graded clinical score. t; transfer, nt; no transfer

Transferred clonotype+ T cells were identified in LN and spleen from CNO and C57BL/6 mice by FACS analysis. As shown in the following figures, the fraction of OT-II T cells recovered from of CNO and C57BL/6 recipient mice was comparable. However, the fraction of OT-I cells recovered from CNO recipients was decreased 2-5 fold when compared with control mice between days 7 to 12 post transfer.

Day 3

CD4 CD8

Vβ5

Vα2

C57BL/6 no transfer

C57BL/6 transfer

CNO no transfer
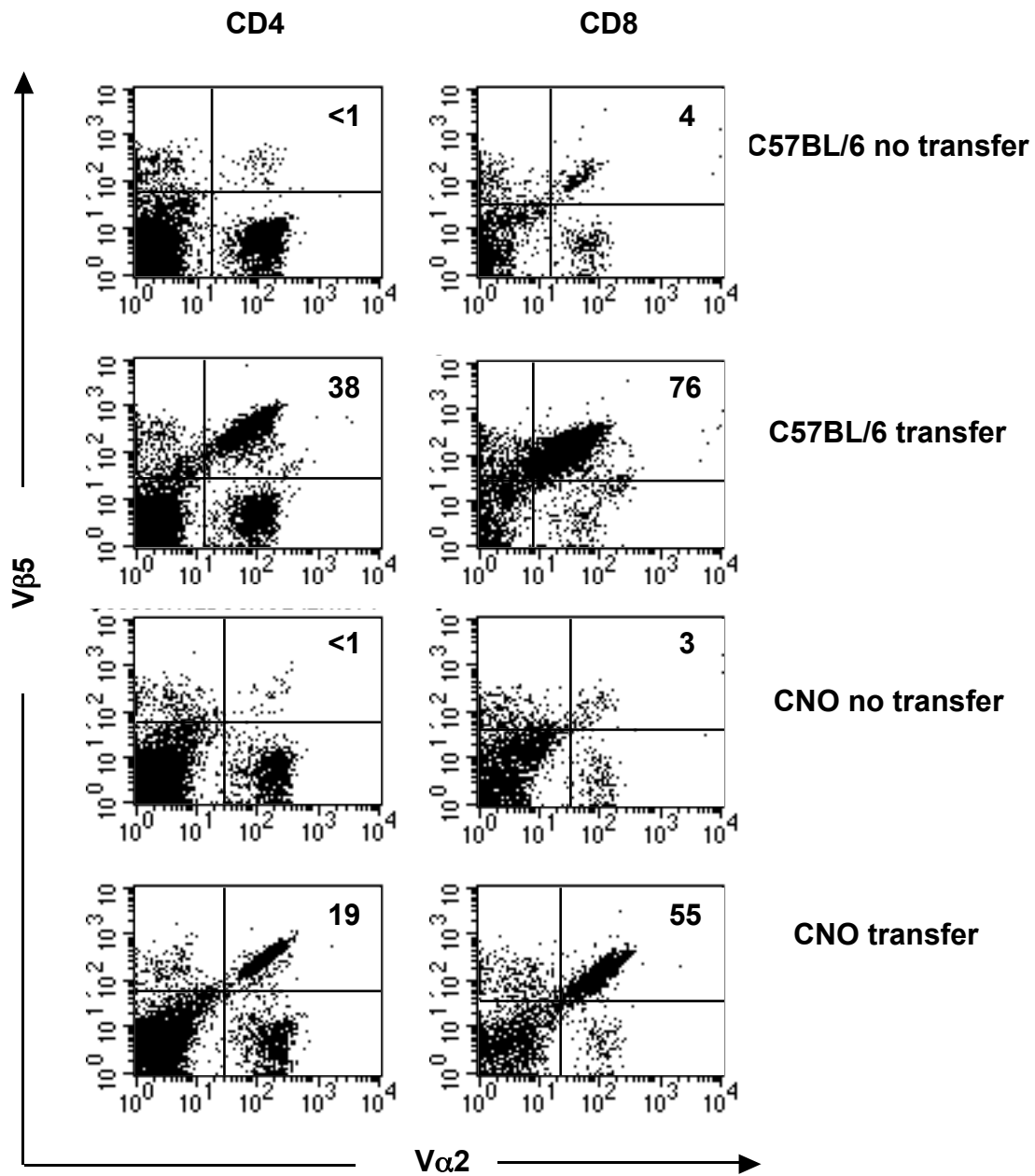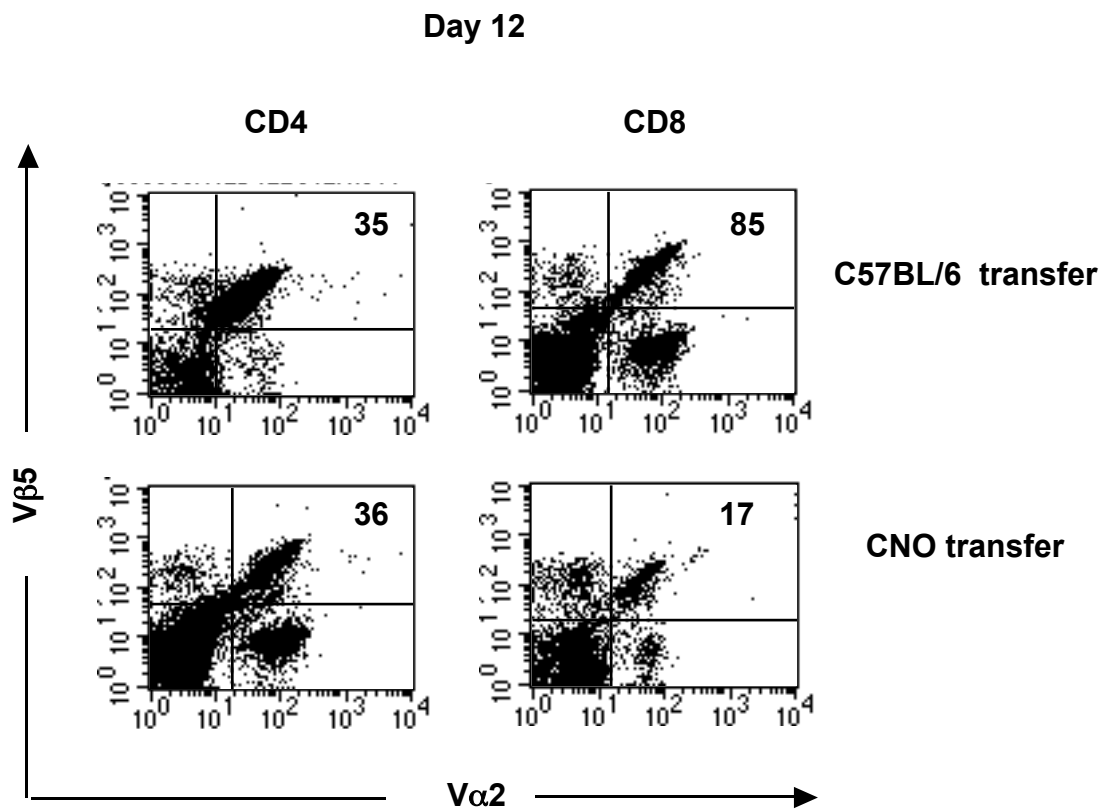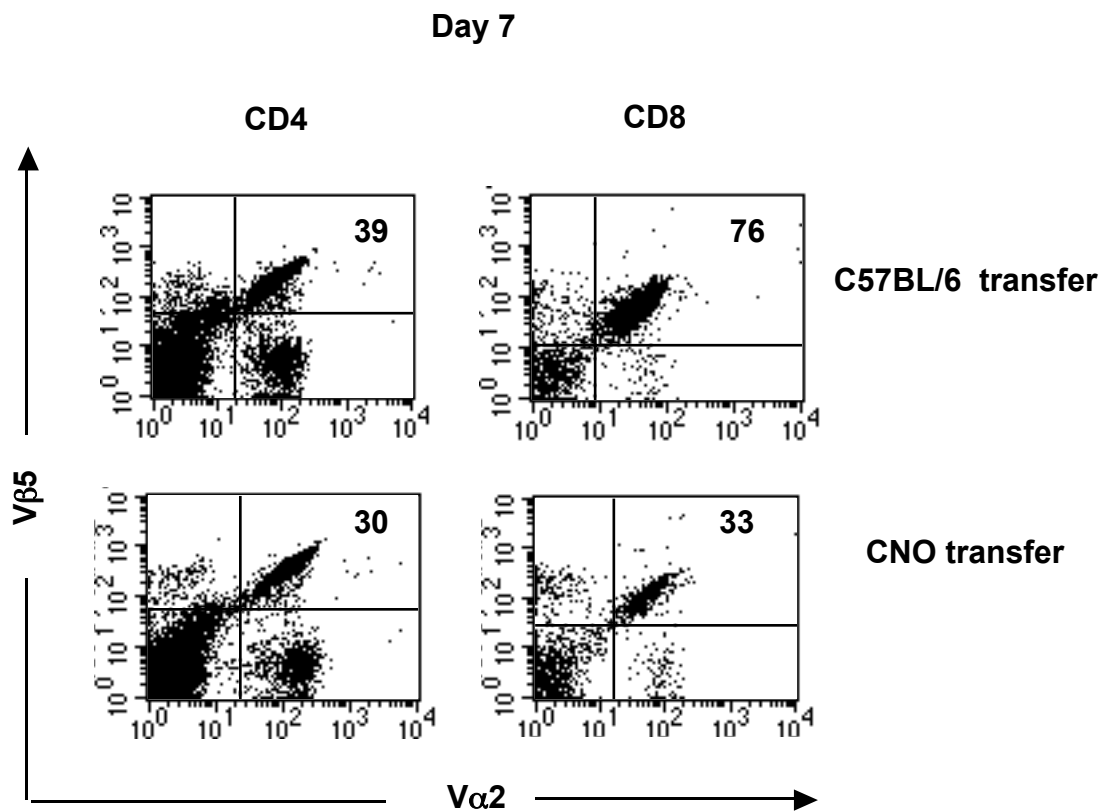
CNO transfer

Day 7

Fig. 26 The relative number of transferred CD4+ clonotype+ T cells remained constant while CD8+ clonotype+ T cells were diminished at day 7 and 12 post transfer in LN from sublethally irradiated CNO recipient mice.

Total LNC were removed on days 3, 7 and 12 from irradiated CNO and C57BL/6 mice before being stained with anti- CD4, CD8, Vα2 and Vβ5 mAbs and analysed by FACS. Dot plots show the percentage of Vα2+Vβ5+ T cells present within gated live CD4+ or CD8+ T cells.

Further analysis of the total number of OT-II T cells recovered from LN determined an increase in both CNO and C57BL/6 recipient mice between days 3 and 12 post transfer. In contrast the total number of OT-I T cells recovered from LN were 3-8 fold diminished in CNO mice compared with control mice.
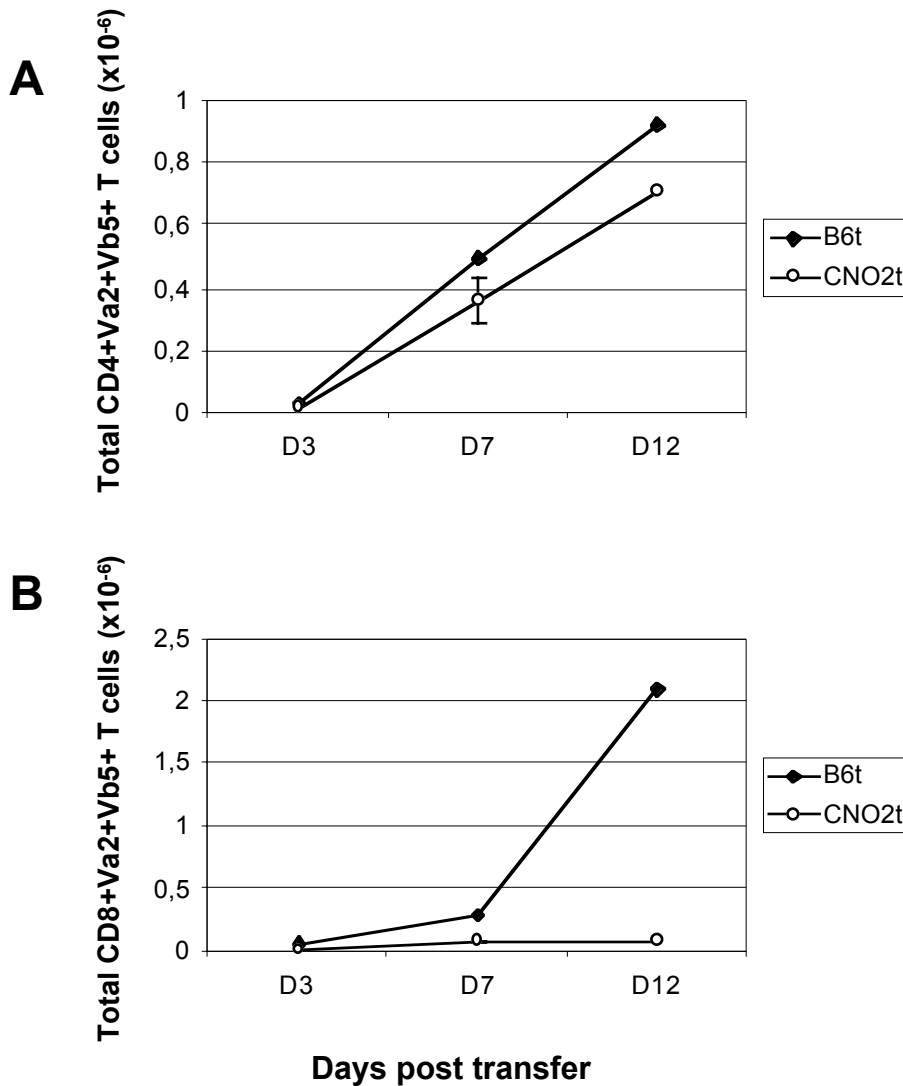
Fig. 27 Total number of recovered CD8+Vα2+Vβ5+ T cells are diminished in LN from CNO recipient mice days 7 and 12 post transfer when compared with WT counterparts.

The total number of OT-I and OT-II T cells recovered from CNO and C57BL/6 LN on days 3, 7 and 12 post transfer were subjected to the four colour staining already mentioned. Total A) OT-II (CD4+Vα2+Vβ5+) or B) OT-I (CD8+Vα2+Vβ5+) T cells were calculated by multiplying the percentage of clontoype+ T cells by the total number of LNC recovered at the given time points. t; transfer

Subsequent analysis of the spleen determined the total number of OT-II CD4+ T cells to be decreased in both CNO and C57BL/6 recipient mice between days 7 and 12 post transfer. In

contrast to LNs the total number of OT-II T cells from spleen declined more than 2 fold on day 12 in both CNO and C57BL/6 mice. The total number of OT-I T cells recovered from CNO mice was 1-4 fold reduced at days 7-12 post transfer, when compared with C57BL/6 mice.
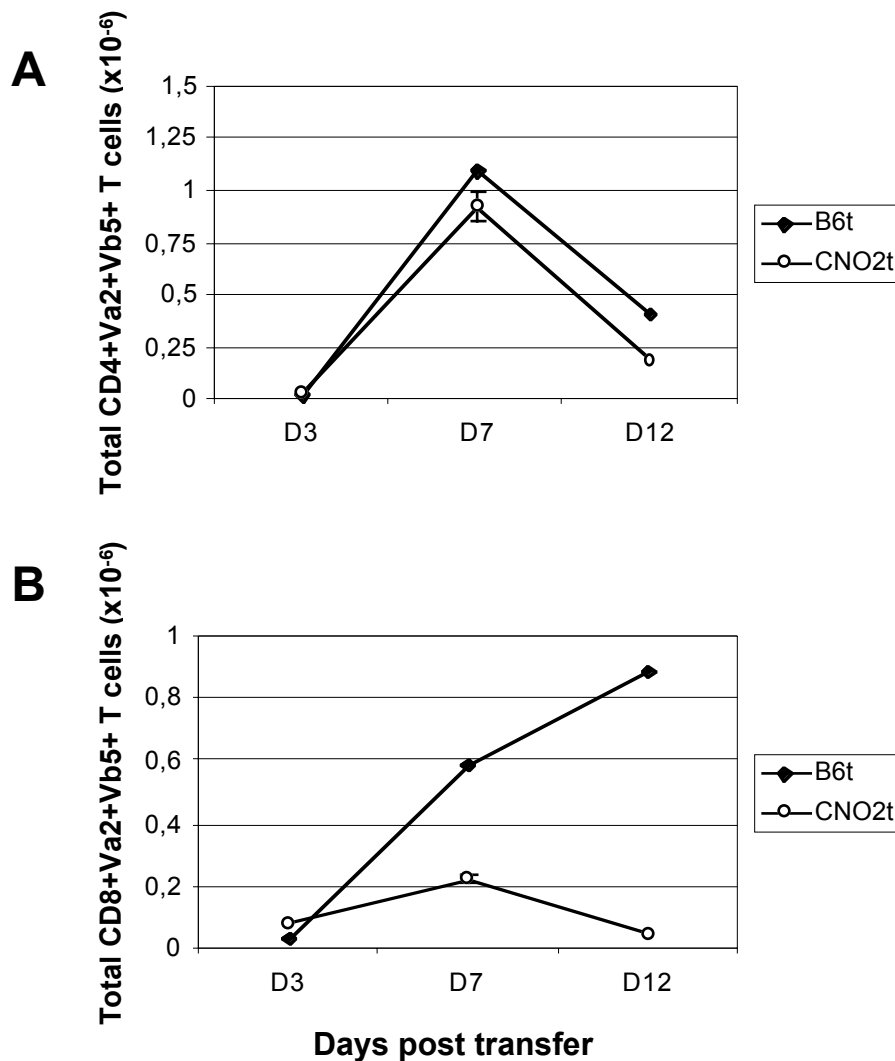
Fig. 28 Total number of recovered CD8+Vα2+Vβ5+ T cells are diminished in spleen from CNO recipient mice at days 7 and 12 post transfer when compared with WT counterparts.

The total number of OT-I and OT-II T cells recovered from CNO and C57BL/6 recipient spleen on days 3, 7 and 12 post transfer were subjected to the four colour staining already mentioned. Total A) OT-II (CD4+Vα2+Vβ5+) or B) OT-I (CD8+Vα2+Vβ5+) T cells were calculated by multiplying the percentage of clonotype+ T cells by the total number of splenocytes recovered at the given time points. t; transfer

Further FACS analysis showed an increase in the fraction of CD62L^{lo} and CD69+ OT-I cells recovered at day 7 and 12 from LNs of CNO when compared with C57BL/6 recipient mice (data not shown) suggesting that circulating OT-I T cells were restimulated by the target Ag.

Additional histological analysis brain and spinal cord sections stained with H&E or anti-CD3 mAb did not identify any perivascular infiltration of mononuclear cells.

The above results showed that at early time points post sublethal irradiation transferred CD4+ and CD8+ T cells in C57BL/6 and CNO recipient mice could still be recovered. However, clonotype+ CD4+ total T cell numbers diminished earlier in spleen than in LN from both C57BL/6 and CNO mice at day 12 post transfer. In contrast only CNO LNs and spleen showed a dramatic decrease in CD8+ clonotype+ T cells at day 12 post transfer. This again implied that OT-I T cells carry an anti-OVA TCR with a higher avidity for OVA than OT-II T cells enabling them to recognise presented OVA in CNO mice more readily than OT-II cells. Previous published experiments have also shown that OT-I cells were more responsive than OT-II cells to peptide stimulation, highlighting the limitation when comparing these two T cell subsets (Li, Davey et al. 2001). Furthermore class I restricted Ag presentation occurs constitutively on many different cell types whereas the presentation of class II Ags requires transfer of Ag to MHC II positive cells prior to CD4+ T cell activation. The resulting demise in restimulated OT-I T cell numbers suggested migration into the CNS or Ag induced apoptosis. However, in contrast to immunisation with whole OVA adoptive transfer of OVA specific TCR Tg T cells did not induce a CNS autoimmune disease suggesting additional disease inducing mechanisms are needed.

3.4 Characterisation of OVA specific TCR Tg T cells generated in PNO and CNO mice

3.4.1 Overview

In an attempt to better understand the tolerance mechanisms operating centrally and peripherally as well as confirming the histological findings of OVA expression within the thymus, PNO mice were crossed with anti-OVA TCR Tg mice; OT-I or OT-II mice. The presence of minute amounts of a self-Ag can be confirmed in vivo by thymic clonal T cell deletion or peripheral T cell activation. Therefore double Tg mice containing an increased number of OVA specific T cells would enable tolerance mechanisms operating at the cellular and molecular level in PNO and CNO mice to be studied. CNO2 mice crossed with OT-II mice were also analysed and intended as controls. While PNO double Tg and CNO/OT-II mice did not exhibit a spontaneous neuroautoimmune disease, CNO2/OT-I mice displayed a severe form of a CNS inflammatory disease (personal communication Dr Yi Cao).

3.4.2 T cell development in primary and secondary lymphoid organs

It was primarily attempted to conduct the following phenotyping experiments with mice of the same age. This was not always possible as single Tg mice used for the following analyses were not always from the same litter as double Tg mice. Consequently it was endeavoured to investigate mice within 8-12 weeks of age. The following table depicts the relative age and sex of each of the mice used in the numbered experiments.

Expt. No.		Genotype							
		C57BL/6	PNO3	CNO2	OTI	PNO3/OTI	OTII	PNO3/OTII	CNO2/OTII
	Sex		m				f	m	
1	Age/wks		20				20	20	
	Sex		m		m	m			
2	Age/wks		9		9	9			
	Sex	m	m		m	m			
3	Age/wks	10	10		11	13			
	Sex	m	m				f	m	
4	Age/wks	10	10				9	9	
	Sex	m	m	m			m	m	m
5	Age/wks	7	8	9			9	12	9
	Sex	f	m		f	m	m	m	
6	Age/wks	9	9		10	10	9	7	
	Sex	f	m		f	m	f	m	
7	Age/wks	10	9		14	9	10	8	
	Sex	f	m		f	m	f	m	
8	Age/wks	10	9		15	6	15	13	
	Sex	f	m		f	m	f	m	

Table 5. Features of the double Tg mice analysed.

For the following FACS and proliferation experiments the relative sex and age in weeks of each mouse is listed for the PNO3/OT-I, PNO3/OT-II and CNO/OT-II mice and their respective WT counterparts as well as the control mice. m; male, f; female

Development of OT-I and OT-II thymocytes in PNO mice expressing OVA was analysed by FACS (Fig 29). Initially the cellularity of the primary and secondary lymphoid organs was compared between double Tg and their single Tg counterparts. PNO3 and PNO3/OT-II mice showed normal cellularity of thymi, spleen and LN relative to their respective C57BL/6 and OT-II controls. In contrast, total thymocyte numbers were reduced almost 3 fold in PNO/OT-I thymi when compared with control littermates, indicating that the majority of thymocytes were subjected to negative selection in the presence of intrathymic OVA expression. The cellularity of LN and spleen remained unaffected.

A Lymph nodes

B Spleen

Total cell number (10^6)

Legend:
- C57BL/6
- PNO3
- OT-I
- PNO3/OT-I
- OT-II
- PNO3/OT-II

C Thymus
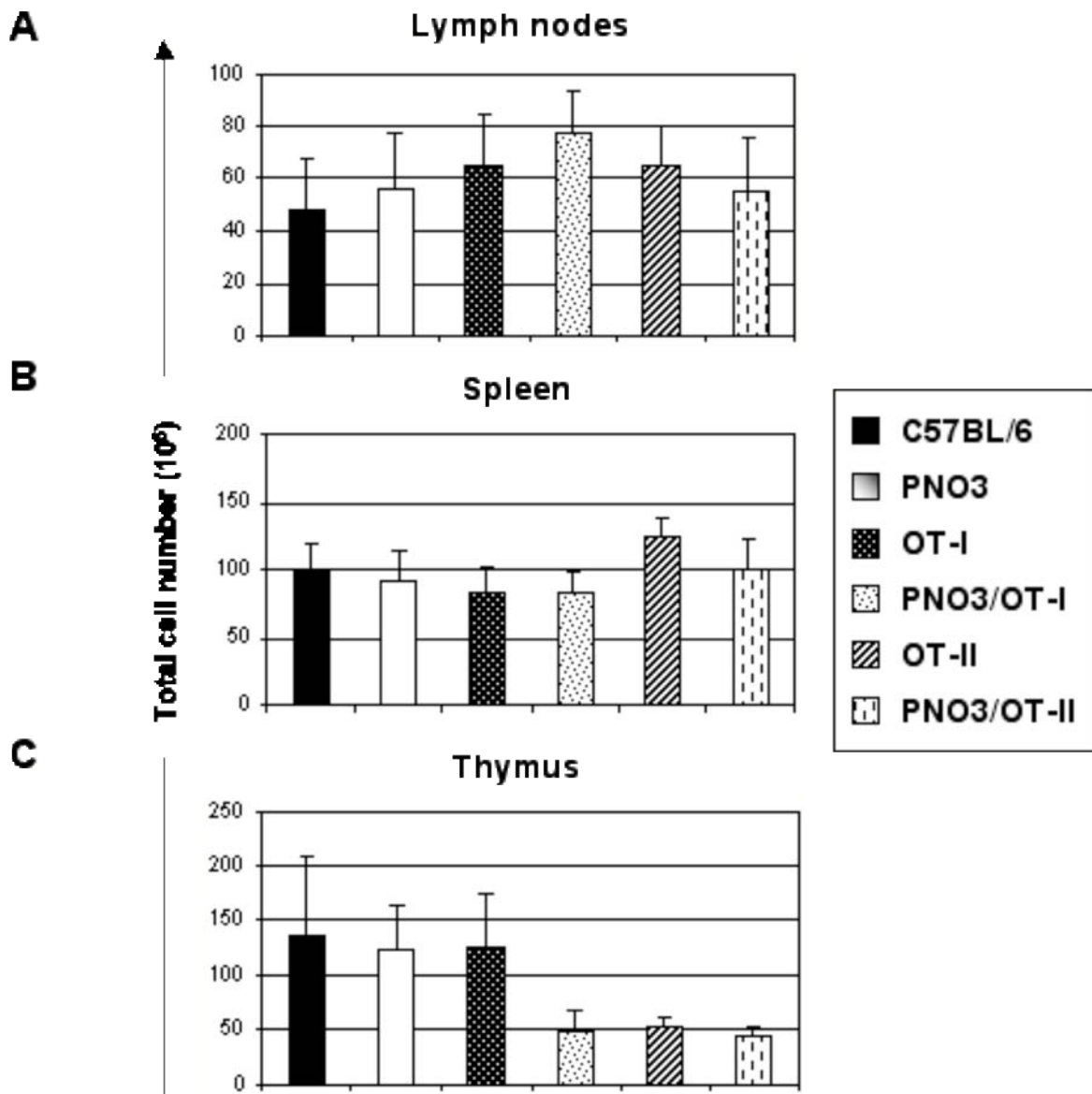
Fig. 29 Thymic cellularity is reduced in PNO3/OT-I but not in PNO/OT-II mice when compared with single Tg counterparts.

PNO3/OT-I spleen and LN cellularity appeared unaltered when compared with controls. In contrast thymic cellularity was significantly reduced. PNO/OT-II LN, spleen and thymic cellularity were comparable with control mice. Cellularity was determined by enumeration of viable cells via trypan blue exclusion of LN, spleen and thymus single cell suspensions.

Characterisation of the distribution of subpopulations within LN and thymi has formerly been shown for the OT-I (Clarke, Barnden et al. 2000) and OT-II (Barnden, Allison et al. 1998) mice. As previously described Tg cells both in the periphery and in the thymus are strongly skewed towards the CD8+ or CD4+ subset in OT-I or OT-II mice respectively. In our case the OT-I Tg thymus contains 7-11% CD8+ single positive (SP) T cells which is slightly lower compared with Clarke et al., who showed 13% of OT-I thymocytes being CD4-CD8+. Consistent with these characterisations our OT-I mice also contain a significant proportion of cells that are CD4^{int} CD8^{lo}. These cells, omitted from gating, have been shown to become mature CD8+T cells once intrathymically transferred into unirradiated C57BL/6 mice (Lundberg, Heath et al. 1995). In line with Barnden et al., our OT-II thymi carry 11% CD4+ SP T cells while the CD8+ SP T cell compartment represents 1% of the total T cell population.

Examination of the PNO3/OT-I mice revealed an 84±21% decrease in CD8+ SP cells when compared with their OT-I counterparts. This correlated with a decrease in total CD8+ SP cells from 12±6 to 2±1·10^6 cells. Although there appears to be a slight increase in the DN compartment and a slight decrease in the DP compartment this is not significant across the group of PNO3/OT-I mice investigated. This suggests that although a large proportion of PNO/OT-I thymocytes were able to differentiate into CD4+CD8+ DP thymocytes, the differentiation to the CD8+ SP stage appears to be partially blocked. In contrast, PNO3/OT-II thymi exhibit no significant differences in their thymocyte subtypes when compared with single Tg counterparts. These results indicate that intrathymic OVA expression in PNO mice led to significant clonal deletion of OT-I but not OT-II T cells.

The slight increase in CD4+ SP cells in PNO3/OT-I mice suggests that this small population underwent rearrangement of the endogenous α chain, which was then subsequently selected on the class II molecule. The mechanism of rearrangement of the endogenous α chain that competes with the Tg α chain for pairing with the Tg β chain has previously been reported. In this case a small proportion of CD8+ class II restricted heamagglutinin specific T cells were selected in Tg mice that predominantly produce CD4+ T cells (Kirberg, Baron et al. 1994).
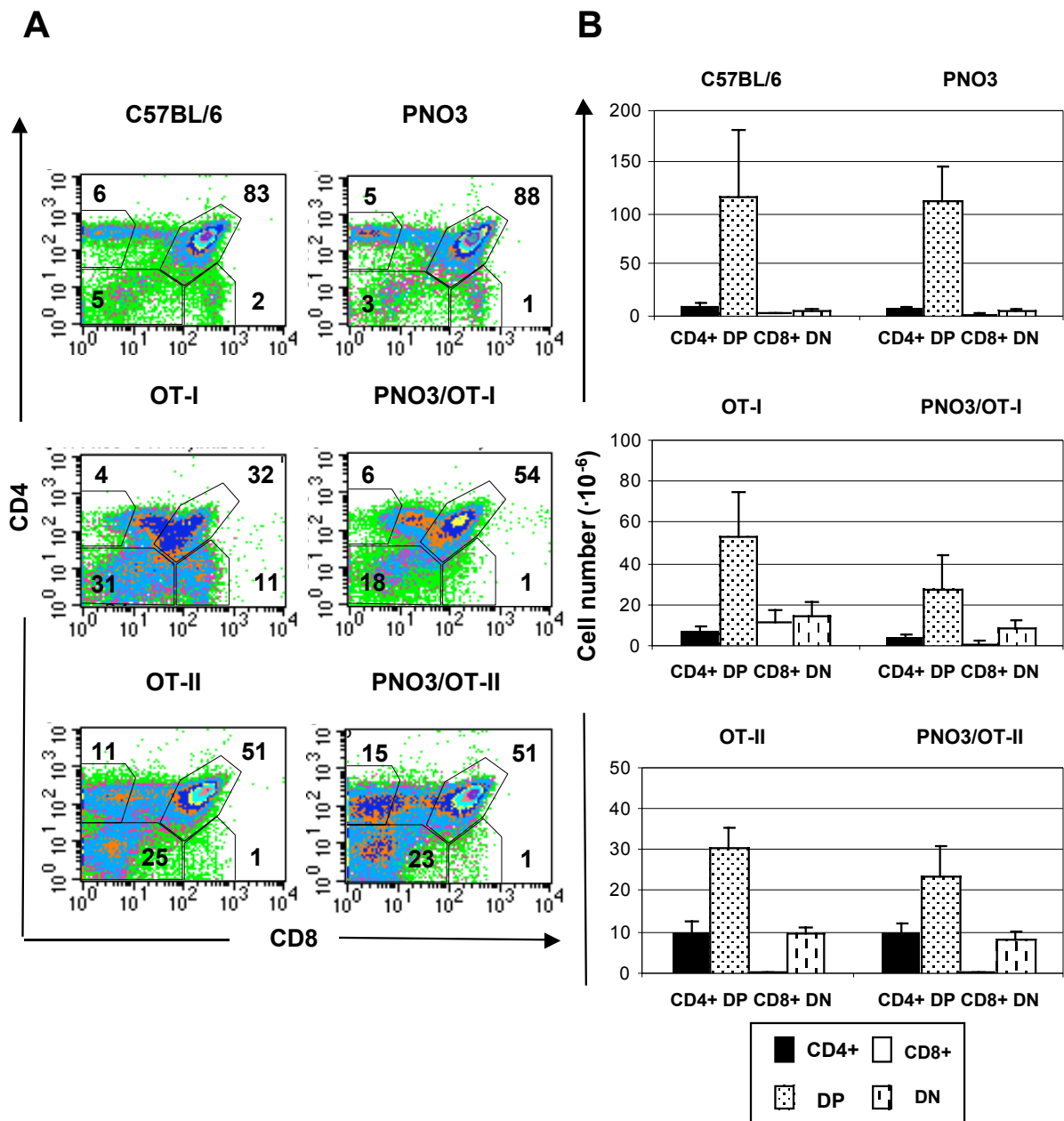
Fig. 30 CD8+CD4- thymocytes are significantly diminished in PNO3/OT-I mice.

PNO3/OT-I mice show an 84±21% decrease in the CD8+ subpopulation in comparison with their single Tg counterparts. In contrast PNO3/OT-II mice show no alterations in their subset compartments when compared with their OT-II littermates. Thymi were dissected and prepared for FACS analysis as previously described. Thymocyte diagrams were obtained from four colour stainings with anti-CD4 APC, anti-CD8 FITC, anti-Vβ5PE and anti-Vα2 bio mAb. Biotin coupled mAb was detected using the SA Cy-chrome conjugate. A) density plots depict percentages and B) column graphs total cell number within the different thymocyte subtypes. DP; double positive, DN; double negative.

As determined in previous analyses the total number of T cells in lymph nodes of OT-I and OT-II mice are comparable to their NTg counterparts; C57BL/6 or PNO3 mice. As in the thymus the peripheral Tg T cells are also strongly skewed towards the CD8+ or CD4+ subset in OT-I or OT-II mice respectively. Consistent with Barnden et al., OT-I and OT-II LN subsets show an altered CD4:CD8 ratio. While NTg mice contain a 2:1 CD4:CD8 ratio, the ratio in OT-I and OT-II mice is 1:4 and 8:1 respectively.

Similar to the thymus, LNC from PNO3/OT-I but not PNO3/OT-II mice display a decrease in the dominant subset. The CD8+ subset in PNO3/OT-I mice is decreased by 62±29%. This was paralleled by a 57±8% increase in the CD4+ compartment relative to their OT-I counterparts. This correlated with a decrease in total CD8+ T cells from 21±10 to 8±4·10^6. In contrast to the thymus, not only did the relative CD4+ T cell number from LNs increase in PNO3/OT-I mice but also the total number of CD4+ cells from 5±1 to 10±4·10^6.

In line with the thymus findings, LNC of the PNO3/OT-II mice did not exhibit any alterations in their CD4+ subsets comparative to their OT-II littermates. OT-II LNC contained 22±6·10^6 CD4+ T cells compared with 16±5·10^6 in PNO3/OT-II and 3±0,9·10^6 CD8+T cells compared with 2±0,6·10^6 in PNO3/OT-II mice.
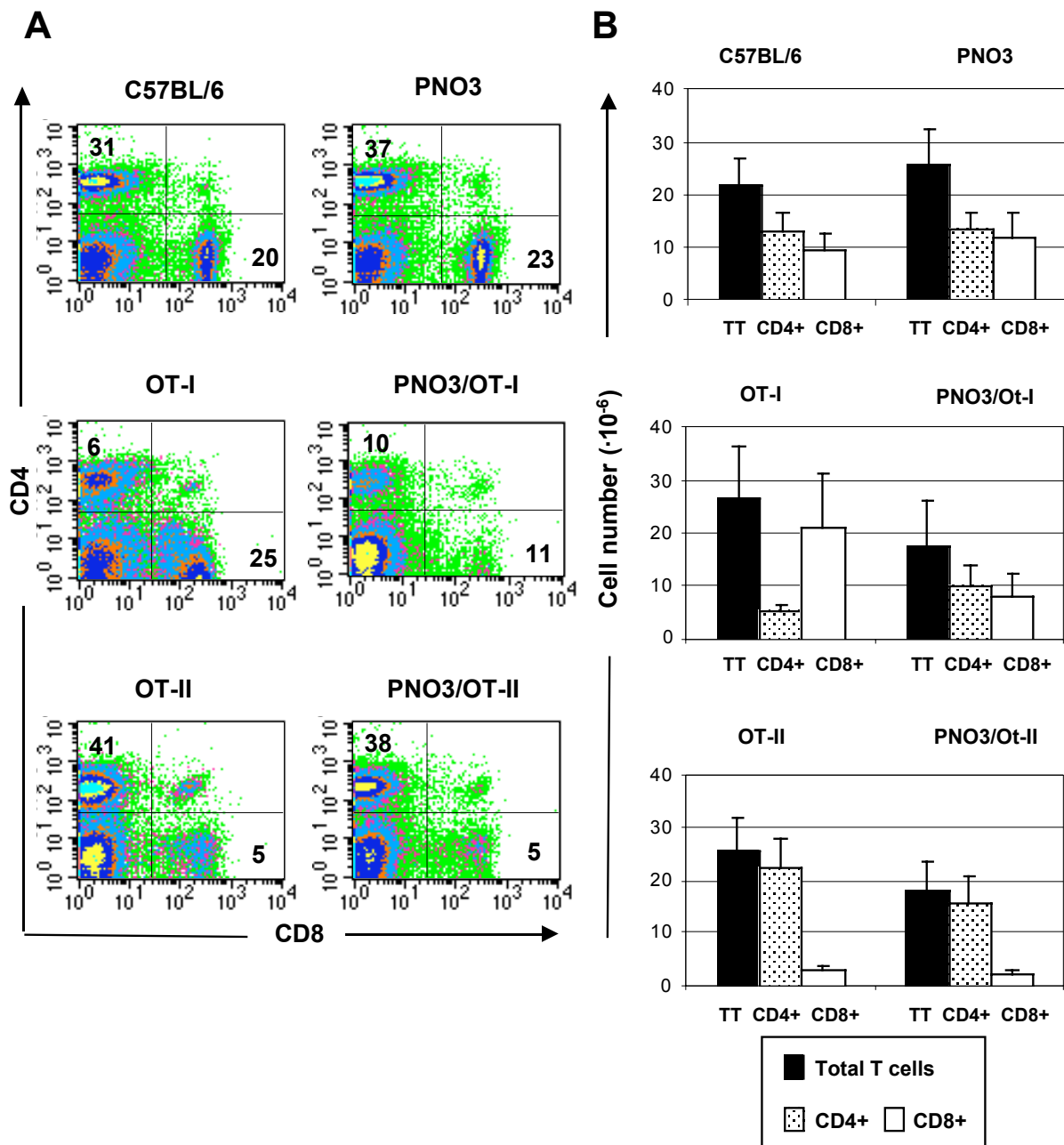
Fig. 31 TheCD8+ LNC subset is diminished in PNO3/OT-I mice.

CD8+ T cells in PNO3/OT-I mice were decreased by 62±29% and CD4+ T cells were increased by 57±8% when compared with their OT-I counterparts. PNO3/OT-II LN subsets remained unchanged relative to their OT-II littermates. LN single cell suspensions were prepared as described in methods, counted and then analysed for CD4 and CD8 expression by flow cytometry, allowing for enumeration of total CD8+, CD4+ and T cells. A) Density plots indicate the percentage of the CD4 and CD8 subset and B) total cell numbers within each T cell subset is shown

The OT-I and OT-II mice were generated from T cell clones expressing the $V\alpha2$ and $V\beta5$ variable chains of the TCR. As the initial analyses indicated that over 50% of CD8+ cells are being deleted in the thymus as well as in the periphery of PNO3/OT-I mice it was important to determine the proportion of cells that possessed the Tg $V\alpha2$ and $V\beta5$ TCR chain within the LNC population ie identification of the clonotype+ T cells. PNO3/OT-I mice contained 17±5 % $V\alpha2+V\beta5+$ T cells compared with 40±7 % in OT-I mice and therefore a decrease in the

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frequency of clonotype+ T cells by 57±17% when compared with single Tg mice. In contrast PNO3/OT-II (37±6 %) showed no decrease in Vα2+Vβ5+ T cells relative to their OT-II counterparts (33±7 %).

Closer examination of the gated clonotype+ T cell population in LNC from PNO/OT-I mice revealed an 80% (5 fold) decrease in CD8+ T cells relative to OT-I (Fig 32). A reduction in total T cell numbers and nearly a two fold increase in the frequency of CD4⁻CD8⁻ clonotype+ T cells (data not shown) accounted for the huge reduction in total clonotype+ CD8+ cells. As expected the total number of CD4+ T cells within the clonotype+ gated population in PNO3/OT-II LNC was similar to OT-II.
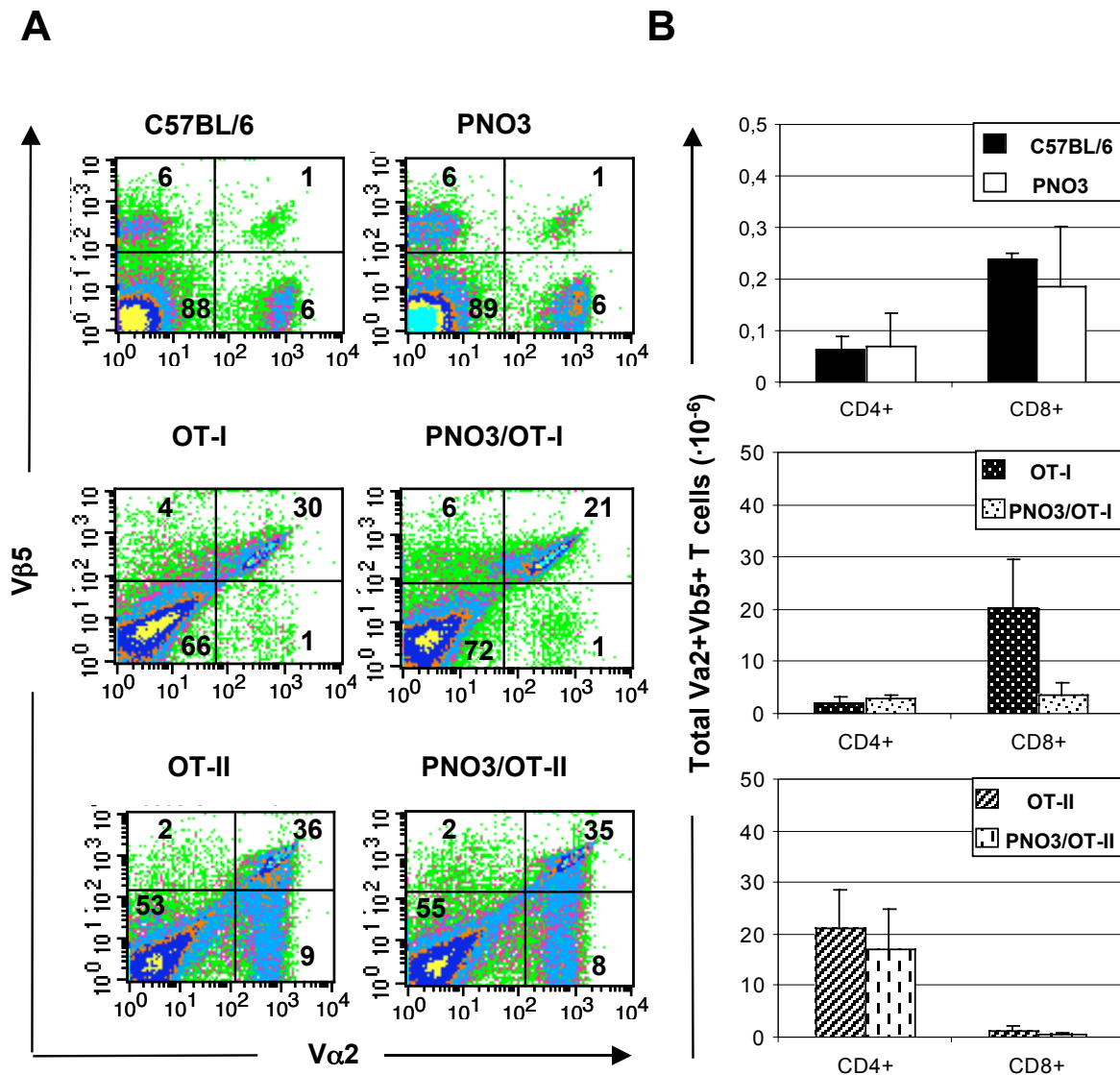
Fig. 32 The frequency of clonotype+ T cells is decreased in PNO3/OT-I LNC.

Investigation of the proportion of the CD8+ T cells within the clonotype+ gated T cell population from PNO/OT-I LNC revealed an 80% decrease relative to OT-I. PNO3/OT-II LNC did not show any significant difference in the CD4+ clonotype+ T cell population relative to OT-II. LNC suspensions were examined by flow cytometry with four colour stainings for CD4, CD8, Vα2 and Vβ5 expression. The FACS diagrams are representative of 5 OT-I/PNO3/OT-I mice and 6 OT-II/PNO3/OT-II mice. A) Density plots depict the percentage of Vα2+ Vβ5+ T cells present within LNC. B) Depicts the total number of CD4+ or CD8+ T cells present within the Vα2+Vβ5+ gated population.

Downregulation of the TCR as well as the coreceptor molecule is a common feature of central and peripheral tolerance mechanisms induced in the presence of the cognate Ag. (Schonrich, Kalinke et al. 1991). To further analyse the phenotype of the double Tg T cells it was important to investigate the expression levels of the clonotypic TCR as well as the coreceptor molecules including CD4 and CD8. Inefficient allelic exclusion of the TCR α chain enables the clonotypic TCR to not only be expressed on the dominant CD4+ or CD8+ T cell population in OT-II or OT-I mice respectively but also on the minor subpopulation. Such cells are absent in recombinase-deficient mice carrying the relevant clonotypic TCR. Therefore the clonotype+ T cell population within the CD4+ as well as the CD8+ T cell subset was analysed for each TCR Tg mouse.

Analysis of the Vα2 chain expression level on PNO3/OT-II thymic CD4+ SP cells revealed no difference when compared with OT-II mice. As expected this was similar for the DP thymocytes analysed. In contrast, all PNO3/OT-I mice thymi investigated showed a downregulation of the Vα2 chain expressed on CD8+SP but not DP cells when compared with OT-I thymocytes (Fig. 33).
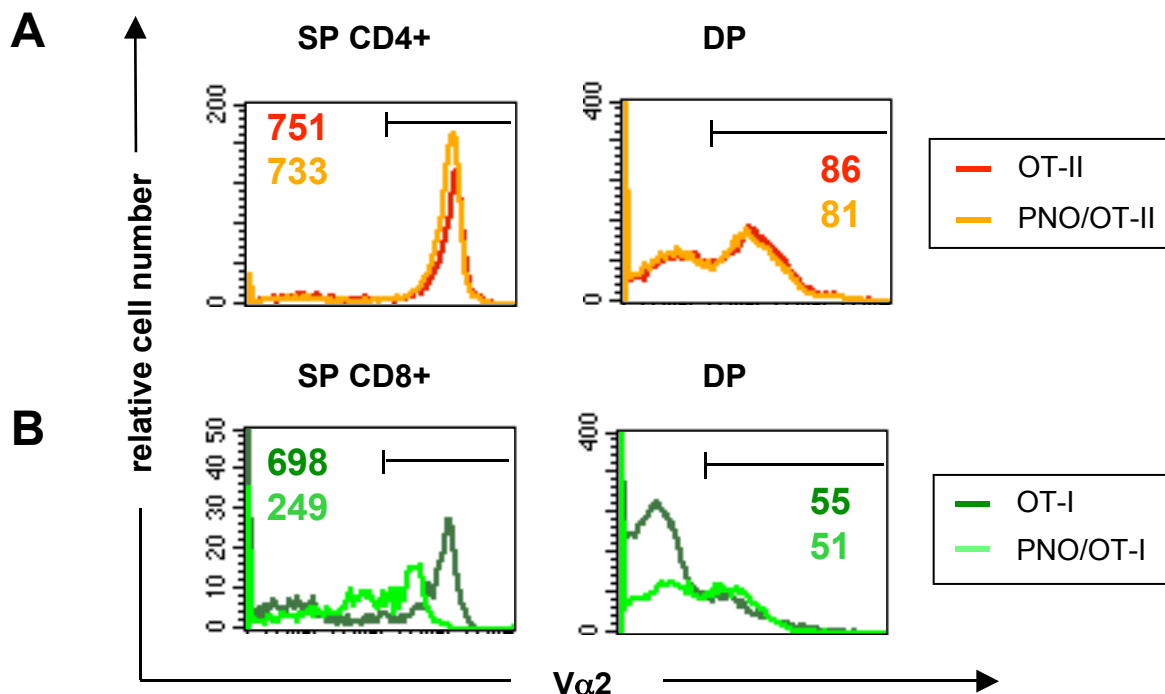
Fig. 33 Vα2 expression is downregulated on SPCD8+ PNO3/OT-I thymocytes.

Total thymocytes were stained with anti-CD4, anti-CD8 and anti- Vα2 antibodies. The histogram displays the MFI of Vα2 expression A) on gated CD4+SP and DP from OT-II (red line) and PNO3/OT-II thymocytes (orange line) in addition to B) gated CD8+SP and DP from OT-I (dark green line) and PNO3/OT-I (light green line) thymocytes.

Negative selection of thymocytes has been ascribed to various stages in thymocyte development, at the SP stage or most commonly at the DP stage. Fig. 34 shows a reduction in DPVα2+ cells in PNO3/OT-I mice when compared with OT-I mice, from $30,25\pm11,09\cdot10^6$ to $10,22\pm1,88\cdot10^6$ thymocytes. Moreover the CD8+Vα2+ SP cells are also reduced from $9,28\pm4,90\cdot10^6$ in OT-I mice to $0,84\pm0,76\cdot10^6$ cells in double Tg mice. Whether deletion in the DP thymocyte compartment resulted in the subsequent reduction of SP cells or whether SP cells were directly involved in the negative selection process can not be verified from the available data. Recently (Zhan, Purton et al. 2003) utilised an *in vivo* BrdUrd pulse – chase

technique to distinguish newly derived CD4+ SP cells from the existing pool. The authors showed that upon cognate peptide stimulation a dramatic reduction in the BrdUrd+ (newly derived) SP cells was observed after three days, indicating that it takes more than one day for the DP precursors to be eliminated while newly derived BrdUrd+ SP cells are not sensitive to Ag-induced cell death. Further analysis with Annexin V staining determined that in vivo apoptosis was increased in DP cells but that there was no change in SP cells two days after Ag administration. These published findings suggest that negative selection occurs mainly at the DP cell stage.
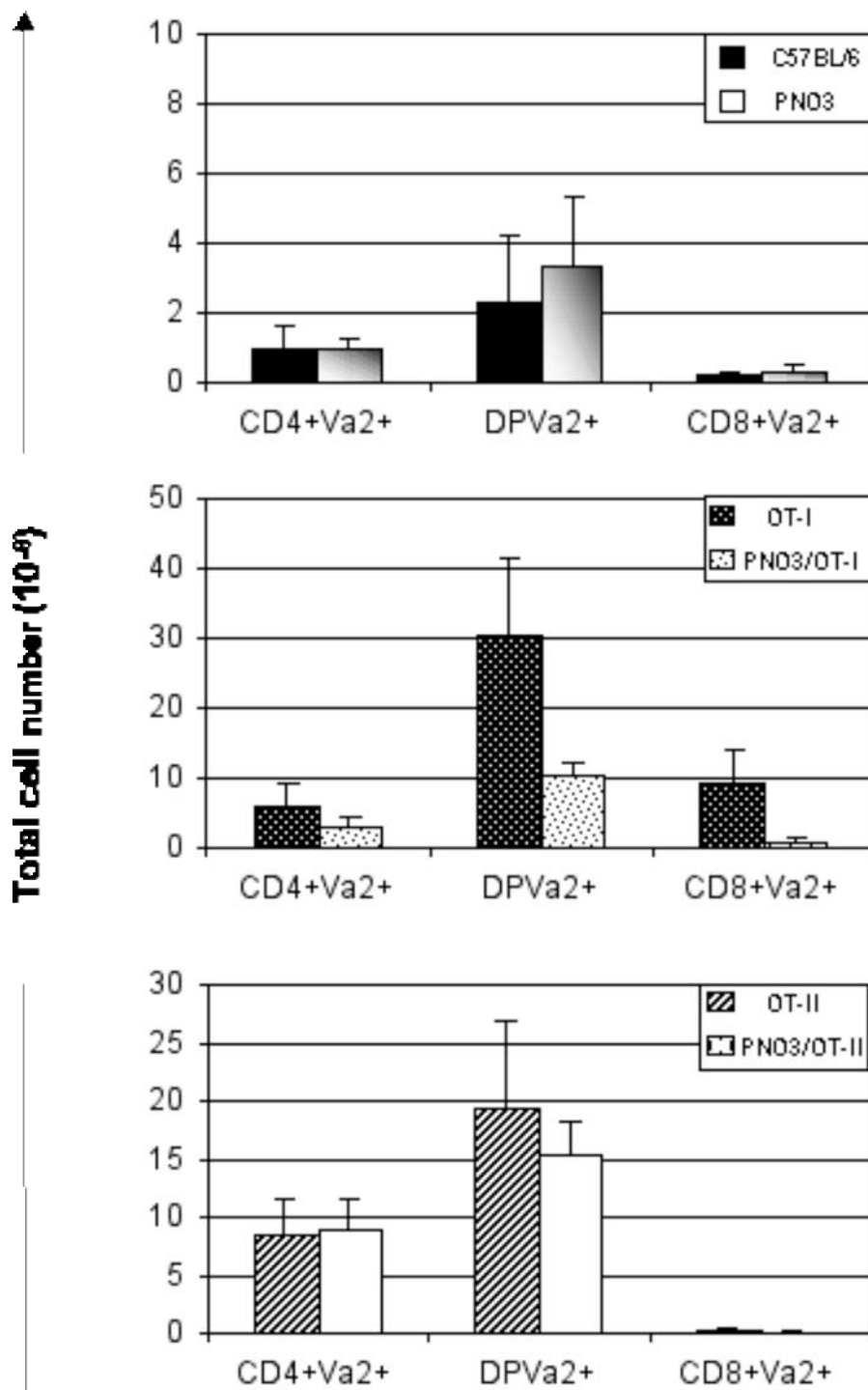
Fig. 34 PNO/OT-I thymocytes display a reduction in the total DPVα2+ compartment.

DP Vα2 + and CD8+ SP cells are dramatically reduced in PNO3/OT-I thymocytes when compared with OT-I mice. PNO3/OT-II DP and SP Vα2+ T cells remain unchanged relative to their single Tg controls. Total thymocytes were stained with anit-CD4, anti-CD8 and anti-Vα2 mAbs and viable cell numbers were enumerated by trypan blue exclusion.

Analysis of the CD4 and the CD8 molecule expression on Vα2+Vβ5+ gated PNO3/OT-II or OT-II LNC shows no downregulation in the clonotypic TCR (Fig. 35) nor in the coreceptor molecules (Fig. 36). It was expected that downregulation of the Vα2 chain in thymocytes from PNO3/OT-I mice would correlate with a downregulation of the clonotypic TCR in the LNC population. A slight downregulation of the Vα2 and Vβ5 chains on CD8+ Vα2+ gated T cells was observed in PNO/OT-I mice (Fig. 35). Further analysis of the CD8 coreceptor molecule expression revealed a downregulation in only one out of five mice analysed. This mouse also exhibited downregulated Vα2 and Vβ5 TCR chains. These results indicate that recognition of OVA by developing PNO/OT-I thymocytes results in clonal T cell deletion, while the peripheral clonotype+ T cells downmodulate their Tg TCR but not the coreceptor molecules.
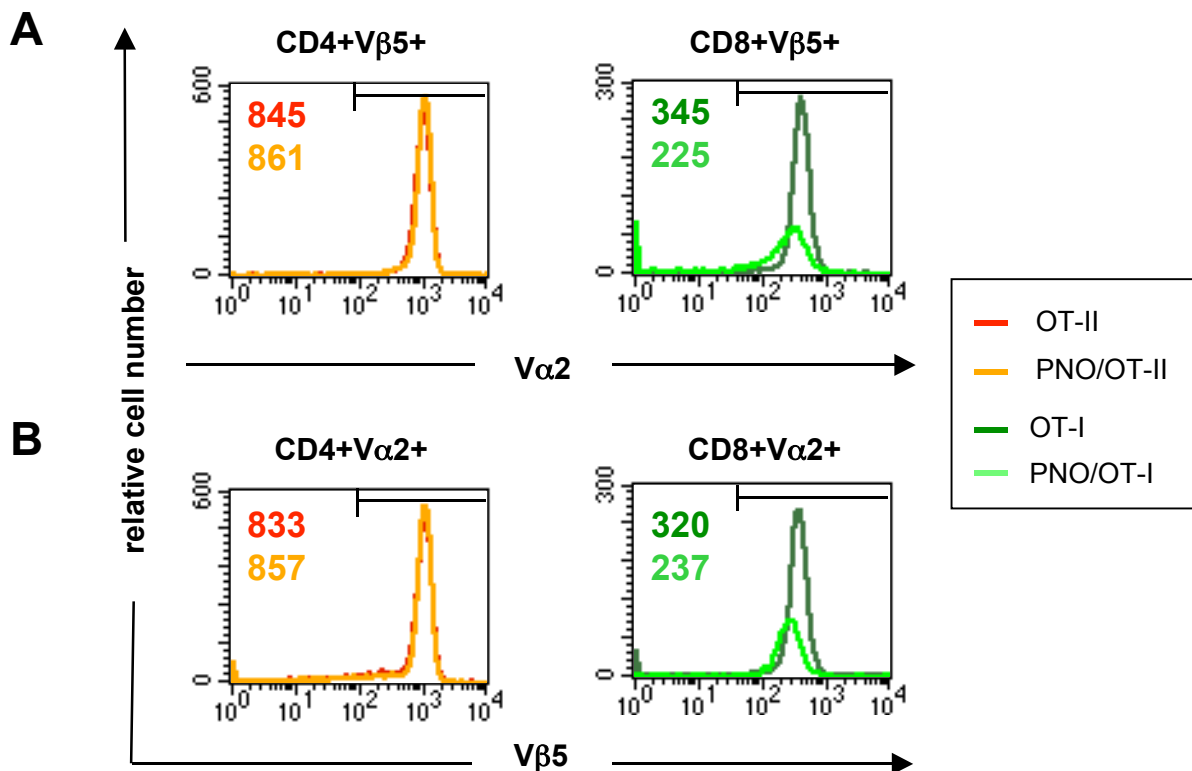
Fig. 35 The OT-I TCR is downregulated on PNO3/OT-I LNC.

The clonotypic TCR as identified by Vα2 and Vβ5 expression was downregulated in PNO/OT-I but not PNO/OT-II or control mice. Total LNC were stained with anti-CD4, anti-CD8 and anti- Vα2 mAbs. Histograms show Vα2 and Vβ5 expression on A) CD4/CD8 Vβ5+ gated or B) CD4/CD8 Vα2+ gated LNC from OT-II (red line), PNO3/OT-II (orange line), OT-I (dark green line) and PNO3/OT-I (light green line) mice. The MFI of either the Vα2 or Vβ5 TCR chain is shown.
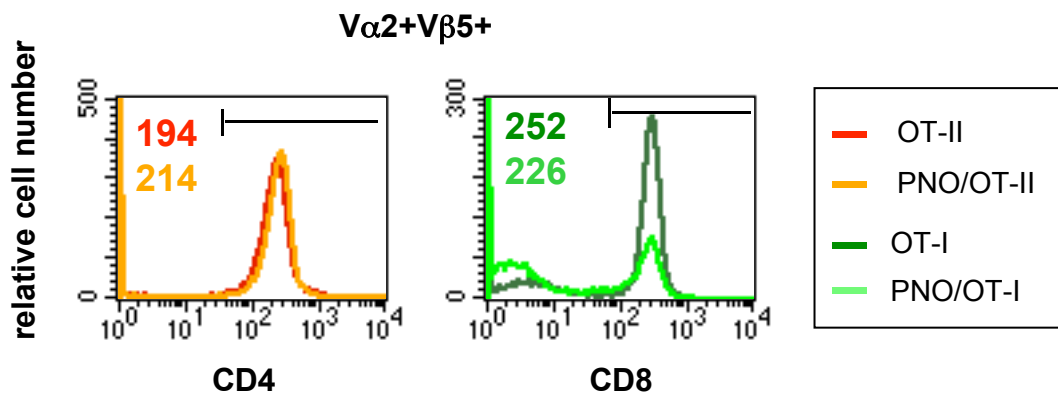
Fig. 36 The CD8 coreceptor is not downregulated on Vα2+Vβ5+ gated T cells from PNO3/OT-I LNC.

Neither the CD8 nor the CD4 coreceptor was downregulated on clonotype+ T cells from double Tg relative to single Tg LNC. Total LNC were stained with anti-CD4, anti-CD8 and anti- Vα2 mAbs. Histograms show CD4 and CD8 expression on gated Vα2+Vβ5+ LNC from OT-II (red line), PNO3/OT-II (orange line), OT-I (dark green line) and PNO3/OT-I (light green line) mice. The MFI of either the CD4 or CD8 coreceptor molecule is shown.

The expression of certain activation markers on thymocytes is also an indication of previous Ag encounter within the thymic environment. Therefore the expression pattern of a panel of activation markers was analysed on CD4+SP, CD8+SP and DP thymocytes from double Tg mice.

Activation marker analysis on CD4+SP and DP gated thymocytes from PNO3/OT-II did not reveal any alteration when compared with OT-II (Fig. 37). However, in line with clonal deletion of SP CD8+ thymocytes and downregulation of the Tg TCR in the periphery, CD8+SP thymocytes displayed an activated phenotype based on the upregulation of CD44, CD69 and CD25 in PNO/OT-I relative to OT-I mice (Fig. 38). Several reports pledging the view that negative selection occurs at the DP stage have shown that TCR ligation was found to induce CD69 upregulation on DP cells *in vitro* (Merkenschlager, Graf et al. 1997). The recent report from Zhan et al., showed upregulation of CD69 and CD44 on SP cells but not on DP cells *in vivo* after Ag stimulation. DP and CD8+ SP cells from PNO3/OT-I mice expressed upregulated levels of CD69 and CD25 with regard to OT-I mice. Cell size did not appear altered on either DP or SP gated thymocytes (data not shown).
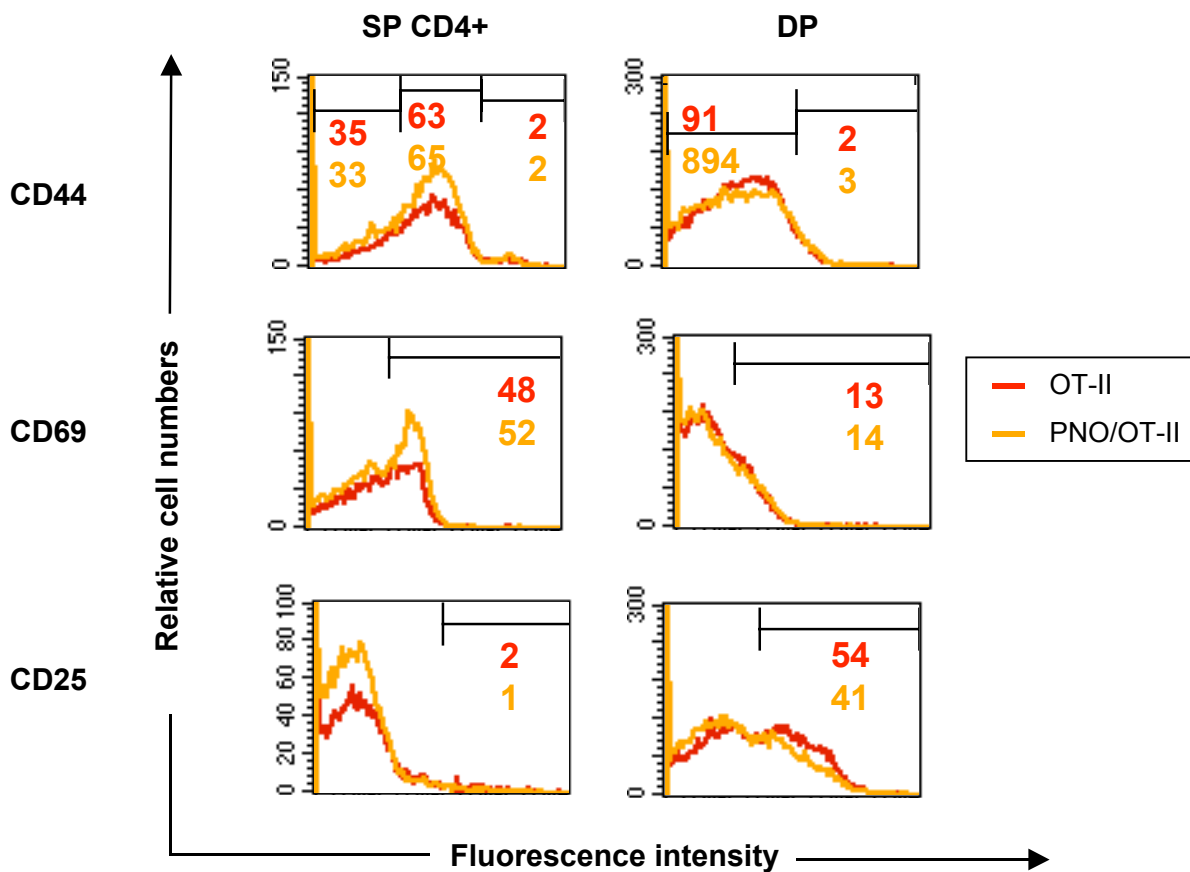
Fig. 37 The relative number of CD44^{hi}, CD69+, CD25+ cells is not increased in the PNO3/OT-II DP or CD4+ SP thymocyte compartment when compared with OT-II.

Four colour stainings with anti- CD4, CD8, Vα2 mAbs and the mAb for the relevant activation marker were performed on thymocytes. Histograms show the activation marker-positive population present within gated CD4+ SP or DP thymocytes. Percentages for each activation marker-positive thymocyte population are indicated for OT-II (red) and PNO3/OT-II (orange) mice.
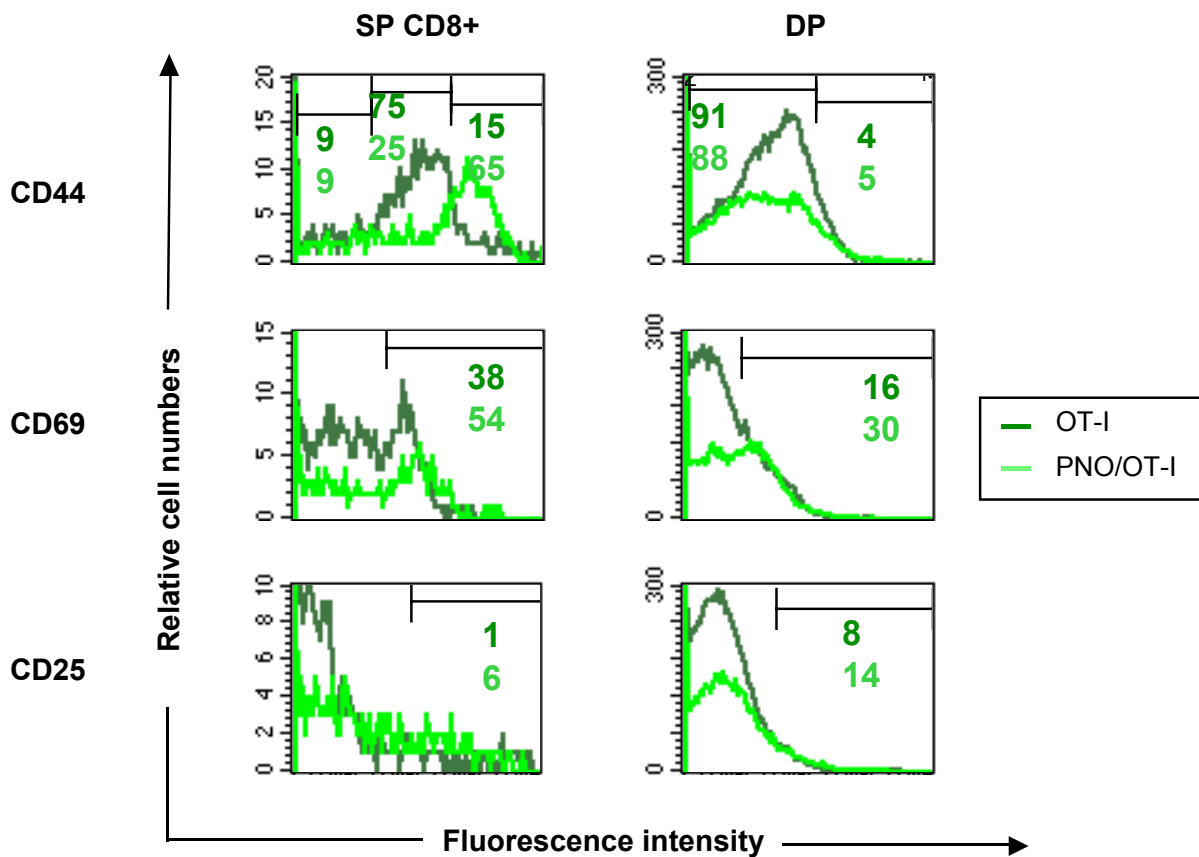
Fig. 38 the relative number of CD44^hi, CD69+ and CD25+ cells is increased in PNO3/OT-I CD8+SP but not DP thymocytes when compared with OT-I.

Four colour stainings with anti- CD4, CD8, Vα2 mAbs and the mAb for the relevant activation marker were performed on thymocytes. Histograms show the activation marker-positive population present within gated CD8+ SP or DP thymocytes. Percentages for each activation marker-positive thymocyte population are indicated for OT-I (dark green) and PNO/OT-I (light green) mice.

In contrast to OT-I controls, the activated phenotype exhibited by PNO3/OT-I thymocytes was mirrored in the peripheral LNC population in which CD8+Vα2+ gated T cells showed an increase in the CD44^hi, CD69+, CD25+, a decrease in the CD62L^lo T cell (Fig. 39). As expected CD4+Vα2+ gated T cells from PNO3/OT-II mice did not show any differences in activation marker expression levels when compared with OT-II mice (Fig. 39).
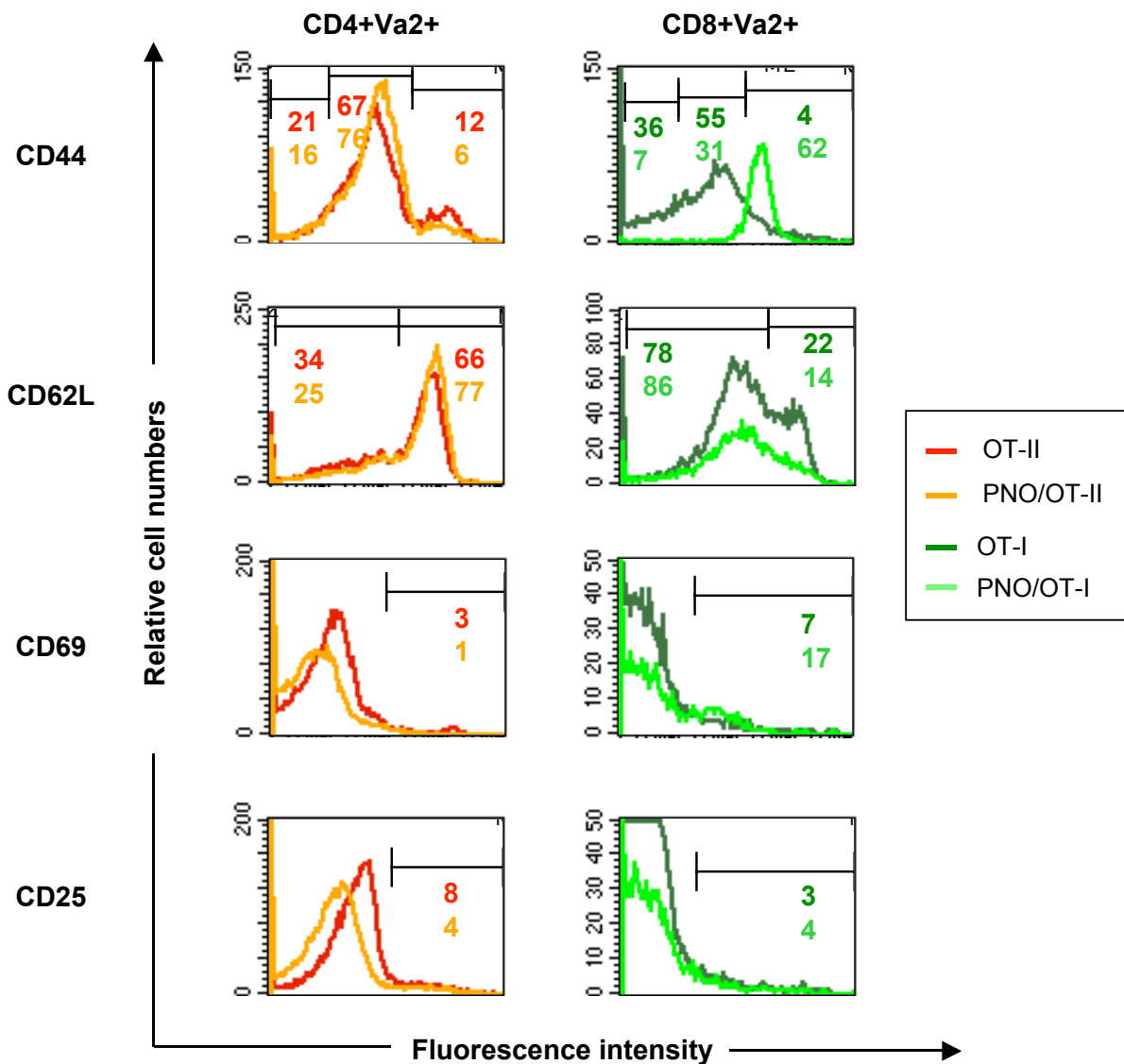
Fig. 39 The relative number of CD44^{hi}, CD62L^{lo}, CD69+, CD25+ PNO3/OT-I CD8+Vα2+ T cells is increased when compared with OT-I.

PNO3/OT-I CD8+Vα2+ T cells presented an activated phenotype in contrast to PNO/OT-II LNC. Four colour stainings were performed on LNC with anti- CD4, CD8, Vα2 mAbs and the mAb for the relevant activation marker. Percentages for the activation marker+ cells present within the gated CD4+/CD8+ Vα2+ T cells are indicated for OT-II (red), PNO3/OT-II (orange), OT-I (dark green) and PNO3/OT-I (light green) mice.

3.4.3 In vitro proliferative response of double Tg T cells to cognate Ag

Based on the above altered phenotype findings of thymocytes and LNC from OT-I double Tg mice relative to their single Tg counterparts it was important to investigate the affected effector function with regard to their proliferative response to cognate Ag *in vitro*. To this end, proliferation assays containing mixed LNC and either the antigenic peptide, OVA protein or anti-CD3 mAb were conducted. The proliferative response of clonotype+ T cells was calculated by dividing the (Brockes, Fields et al. 1979)-Thymidine incorporation counts by the percentage of clonotype+ T cells as determined from FACS analysis multiplied by 100. This

ensured that the proliferative response analysed was based on the fraction of clonotype+ T cells present within each well.

OT-II clonotype+ T cells responded slightly more to OVAp$_{(323-339)}$ peptide stimulation than PNO/OT-II. Saturation point of stimulation for both double Tg and single Tg cells was achieved with 0.1μg/mL peptide. The overall proliferative response was greater with peptide than when stimulated with OVA protein. Again OT-II clonotype+ T cells responded 1.5-3 fold better to whole OVA stimulation than PNO3/OT-II over a concentration range of 4-100μg/mL. While OT-II cells were optimally stimulated at 500μg/mL PNO3/OT-II cells needed 1000μg/mL (ie 2 fold more). PNO3/OT-II clonotype+ T cells responded on average 2.4 fold less well to anti-CD3 mAb than OT-II cells.
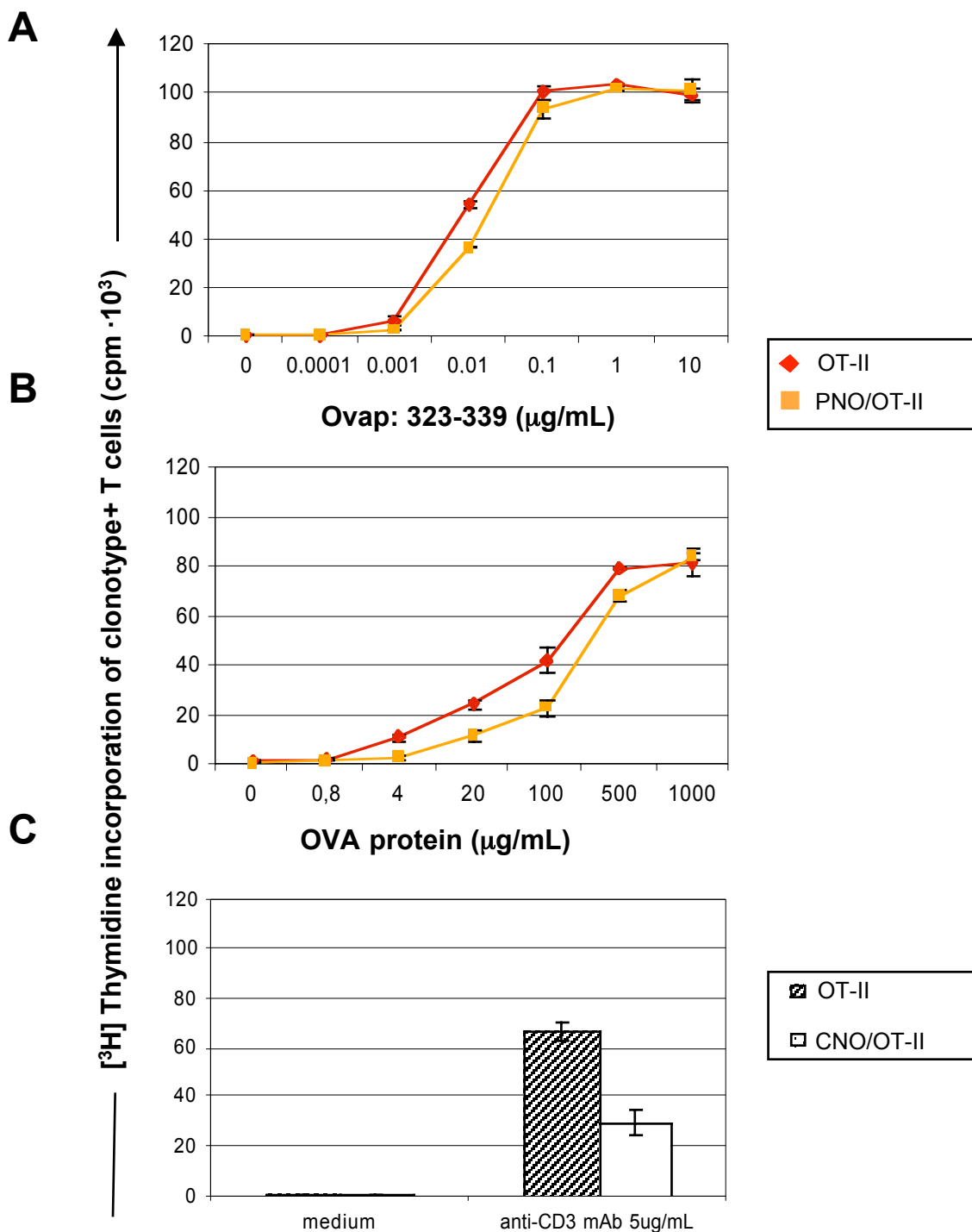
Fig. 40 the proliferative response of PNO/OT-II clonotype+ T cells to antigenic peptide was reduced when compared with OT-II.

PNO3/OT-II and OT-II clonotype+ T cell proliferative response to either A) OVAp$_{(323-339)}$ or B) OVA protein and C) anti-CD3 mAb was studied in a 72h in vitro assay during which the last 16h of culture [^{3}H]-Thymidine incorporation of proliferating LNC was measured. The proliferative response of clonotype+ T cells is given as mean cpm of triplicate wells / % of clonotype+ T cells x 100. The experiment was repeated 4 times with similar results.

The proliferative response of 5 month old PNO/OT-II mice was further diminished when compared with 3 month old counterparts, (Fig. 41). The difference in the proliferative response to whole OVA between PNO/OT-II and OT-II LNC was increased from 1.5-2.5 fold over a concentration range of 20-500μg/mL. The reduced proliferative response can not be based on the small age differences in immune systems. Rather the continued exposure of self-reactive T cells to the self-Ag over time is likely to have influenced the observed reduced proliferation. Furthermore the stronger signal provided by anti-CD3 in contrast to peptide/MHC stimulation is likely to have enabled anergy to be overcome and thereby accounted for the differences in the anti-CD3 proliferative recall response between three and five month old double Tg LNCs.
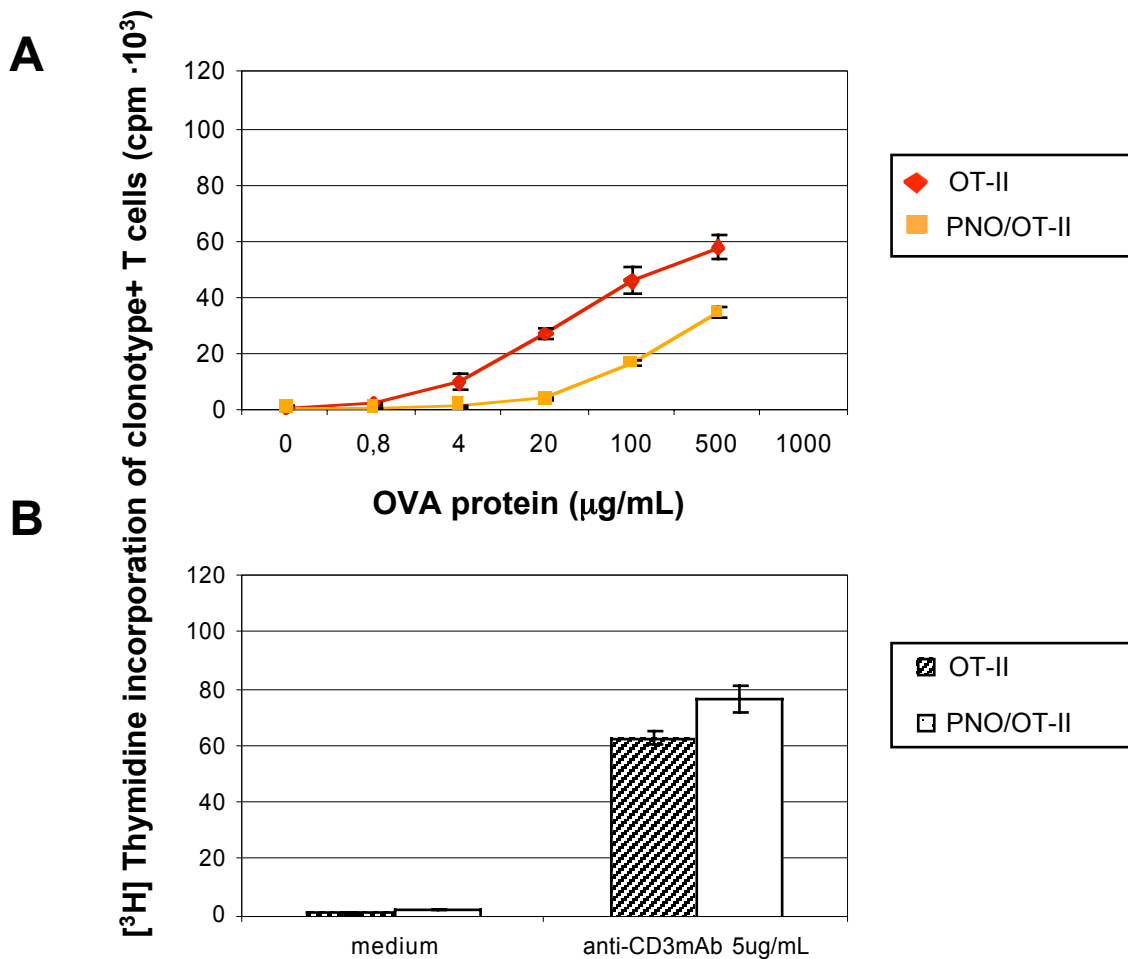
Fig. 41 Five month old PNO/OT-II clonotype+ T cells displayed a further reduction in their proliferative response to whole OVA stimulation when compared with three month old LNCs.

PNO3/OT-II and OT-II clonotype+ T cell response to either A) OVAp$_{(323-339)}$ or B) OVA protein and C) anti-CD3 mAb was investigated in a 72h in vitro assay during which the last 16h of culture [^{3}H]-Thymidine incorporation of proliferating LNC was measured. The proliferative response of clonotype+ T cells is given as mean cpm of triplicate wells / % of clonotype+ T cells x 100.

PNO/OT-I clonotype+ T cells responded slightly better to OVAp$_{(254-267)}$ peptide stimulation than OT-I. Both double Tg and single Tg T cells were optimally stimulated with 0.109μg/mL peptide. PNO/OT-I clonotype+ T cells also responded 1.3-2.5 fold better to OVA protein

stimulation than OT-I over a concentration range of 100-1000μg/mL. PNO3/OT-I clonotype+ T cells responded on average 1.3 fold less well to anti-CD3mAb than OT-I.
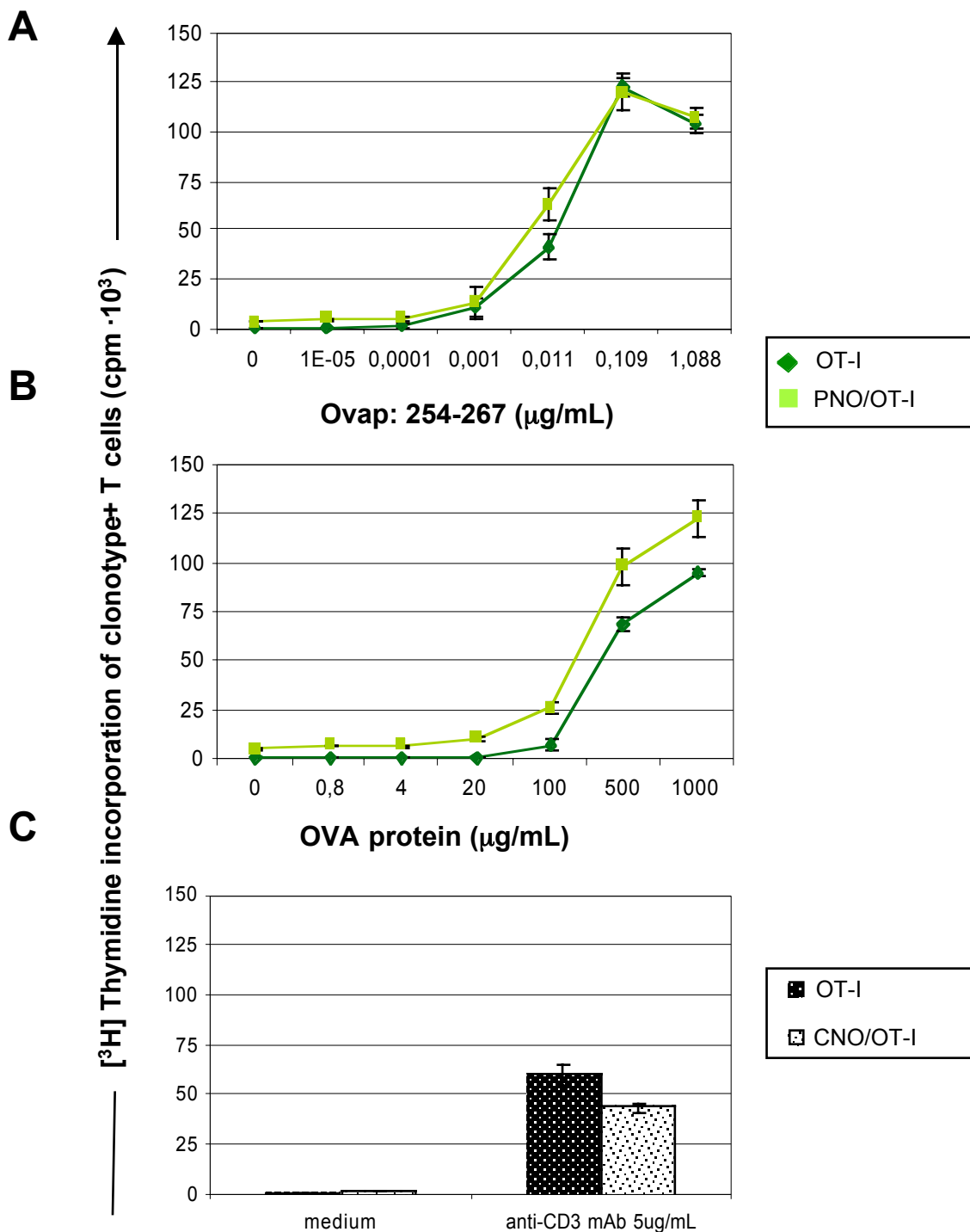
Fig. 42 PNO3/OT-I proliferative response to antigenic peptide is comparable with OT-I while the response to OVA protein is nearly 2 fold greater.

PNO3/OT-I and OT-I clonotype+ T cell response to either A) OVAp$_{(254-267)}$ or B) OVA protein and C) anti-CD3 mAb was determined in a 72h in vitro assay during which the last 16h of culture [^{3}H]-Thymidine incorporation of proliferating LNC was measured. The proliferative response of clonotype+ T cells is given as mean cpm of triplicate wells / % of clonotype+ T cells x 100. The experiment was repeated 3 times with SIINFEKL and 4 times with whole OVA with similar results.

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OT-I and OT-II clonotype+ T cells were more efficiently stimulated in the presence of small concentrations of cognate peptide than OVA protein. This was to be expected, as not all epitopes displayed after processing of whole OVA would be antigenic in nature. It was only at higher concentrations of whole OVA ie 100μg/mL that OT-I and PNO3/OT-I clonotype+ T cells began to be stimulated in contrast to OT-II and PNO/OT-II which could be stimulated with 20μg/mL of OVA protein. Interestingly the overall proliferative response to antigenic peptide and OVA protein, as measured by thymidine uptake, was greater for OT-I and PNO/OT-I than for OT-II and their double Tg clonotype+ T cells. It is interesing that OT-I and PNO/OT-I LNC respond to OVA protein stimulation in the first place. Calculation of the molarity of the OVA peptide giving half the maximal stimulation as compared to that of OVA protein results in almost a 1000 fold difference in the proliferative response. The fact that the OT-I and PNO/OT-I response to OVA protein stimulation was so successful indicated either the presence of small breakdown products (ie peptides) in the OVA preparation which could be directly loaded onto the MHC class I molecules or successful processing and presentation of OVA protein in the class I pathway of APCs in culture. The above results indicate that the slight hyperresponsiveness displayed by PNO/OT-I clonotype+ T cells is in line with their activated phenotype.

Could the proliferative response of both OT-I and OT-II clonotype+ T cells be improved in the presence of exogenous IL-2? This question was answered by adding soluble rIL-2 to cell cultures. Addition of 100U/mL soluble IL-2 to PNO3/OT-II clonotype+ T cells only slightly improved the proliferative response to either cognate peptide or OVA protein (Fig. 43). This implied that PNO/OT-II T cells showed mild signs of anergy, which could be reversed in the presence of IL-2. Addition of exogenous IL-2 to PNO/OT-I LNC cultures doubled and tripled the proliferative response to peptide and OVA protein stimulation respectively. These results suggested that exogenous IL-2 could augment the mild hyperresponsiveness exhibited by PNO/OT-I clonotype+ T cells.

A

Ovap: 254-267 (μg/mL)

Ovap: 323-339 (μg/mL)

B

OVA protein (μg/mL)

[³H]-Thymidine incorporation of clonotype+ T cells (cpm ·10³)

Legend:
- OT-I
- OT-I + IL-2
- PNO3/OT-I
- PNO3/OT-I + IL-2
- OT-II
- OT-II + IL-2
- PNO3/OT-II
- PNO3/OT-II + IL-2
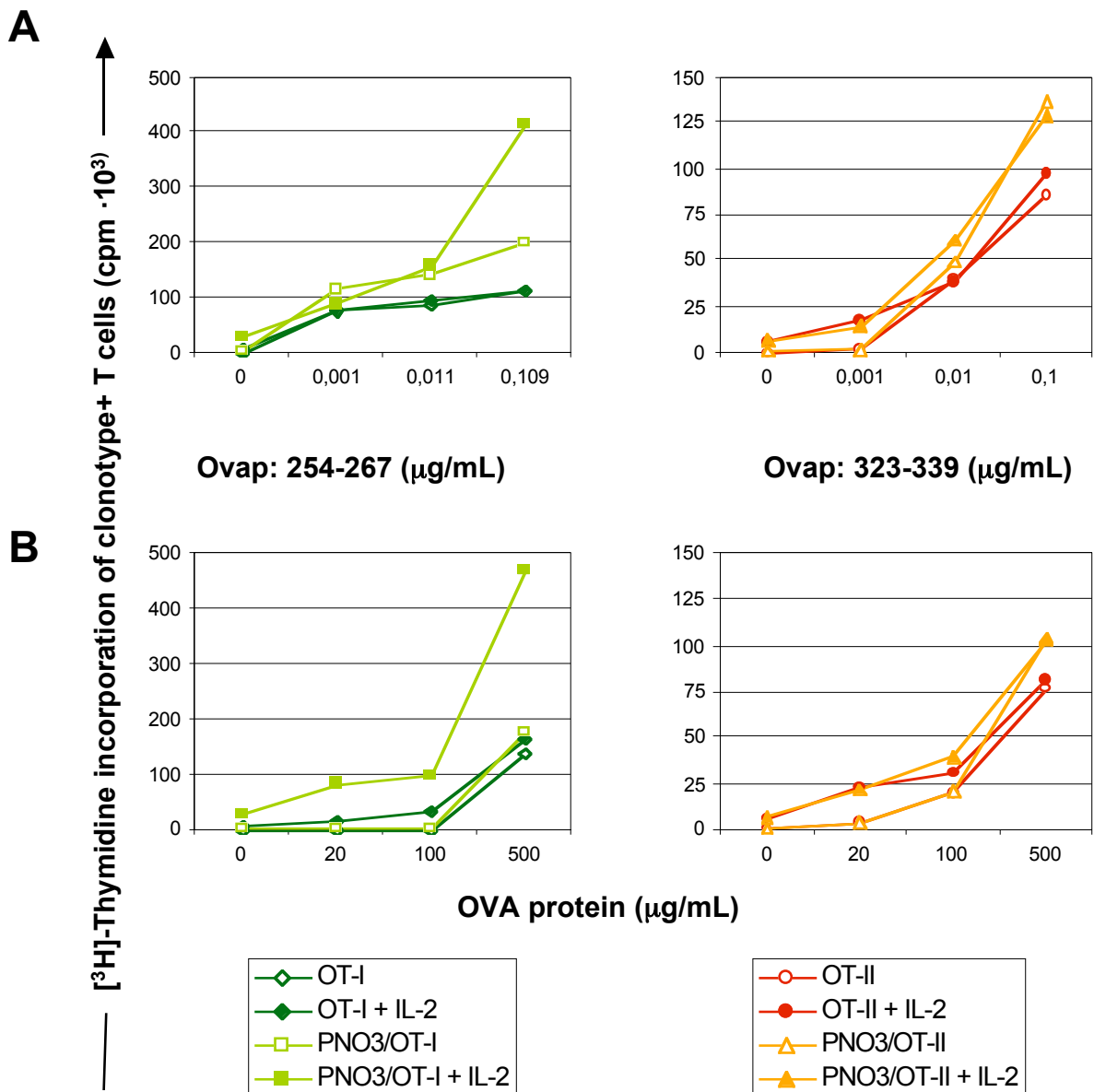
Fig. 43 the proliferative response of PNO/OT-I and PNO/OT-II clonotype+ T is not significantly increased in the presence of exogenous IL-2.

Proliferative response to 100U/mL soluble IL-2 added to LNC cultures stimulated with either A) cognate peptide or B) OVA protein was determined in a 72h in vitro assay during which the last 16h of culture [³H]-Thymidine incorporation of proliferating LNC was measured. The proliferative response of clonotype+ T cells is given as mean cpm of triplicate wells / % of clonotype+ T cells x100.

The PNO double Tg mice served to confirm intrathymic expression of OVA and assisted in explaining the resulting tolerogenic state observed in PNO mice. Clonal deletion of CD8+ T cells is more significant in the thymus and periphery of PNO/OT-I than the deletion of CD4+ T cells in PNO/OT-II mice. However the clonotype+ T cells present in PNO/OT-I mice are more reactive to OVA stimulation than those found in PNO/OT-II mice.

3.4.4 Characterisation of CD4+ OVA specific T cells generated in CNO mice

CNO mice were also crossed with OT-I and OT-II mice generating double Tg mice in whom the development of clonotype+ CD4 or CD8 T cells could also be studied. Furthermore as for the PNO double Tg mice, the presentation efficiency of minuscule amounts of intrathymic OVA could also be assessed. As conducted for PNO/ OT-II or OT-I mice, cellularity, expression of the Tg TCR and of activation markers was compared between double and single Tg CNO mice. (Dr Cao investigated the CNO/OT-I mice). LN and thymus cellularity remained unchanged in CNO/OT-II when compared with OT-II mice. Furthermore CNO/OT-II mice displayed the marked skewing towards the peripheral CD4+ T cell subset and CD4 SP thymocytes including a slight reduction in CD4 CD8 double positive thymocytes which is characteristic of OT-II mice.
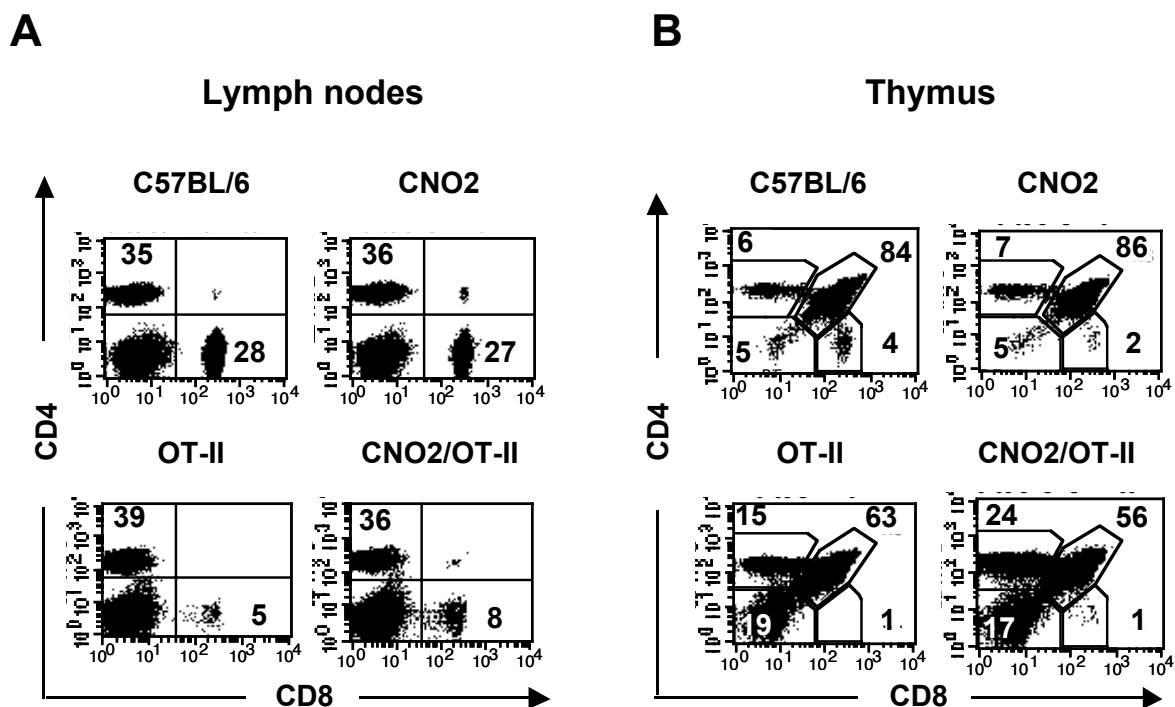
Fig. 44 Lymph node and thymus development remained unaltered in CNO/OT-II when compared with OT-II mice.

The biased skewing towards the CD4+ T cell subset including a slight decrease in the double positive CD4+CD8+ thymocyte compartment was preserved in CNO/OT-II mice. LN single cell suspensions and thymi were dissected and prepared for FACS analysis as previously described. Dot plots were obtained from stainings of A) LNC and B) thymocytes with anti-CD4 APC and anti-CD8 FITC mAb. Percentages of the relevant T cell compartments are shown.

Furthermore Tg TCR levels of expression (Fig. 45A) as well as the CD4 or CD8 coreceptor (Fig. 45B) on cells expressing the OT-II receptor were indistinguishable between single and double Tg mice. This illustrated that neither immature thymocytes nor mature CD4 T cells were recognising their cognate Ag. Similar levels of activation markers including CD44, CD62L, CD69 and CD25 on clonotype+ OT-II and CNO/OT-II LNC extended this assumption (data not shown).

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A

B
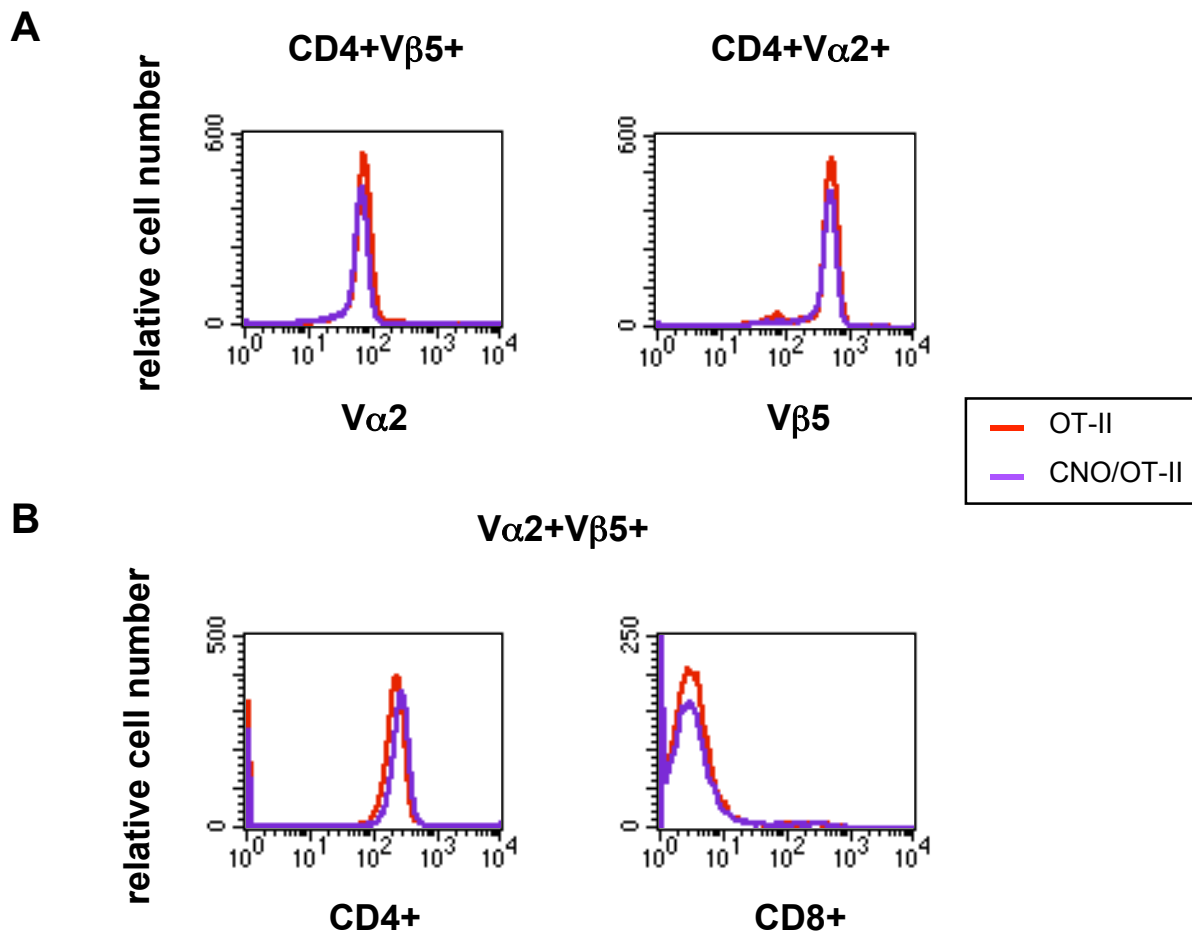
 Fig. 45 Neither Vα2 or Vβ5 TCR chain nor coreceptor expression was altered on CNO/OT-II when compared with OT-II LNC.

Total LNC were subjected to four colour staining with anti-CD4, anti-CD8, anti- Vα2 and anti-Vβ5 mAb. The MFI is shown for A) Vα2 and Vβ5 expression on gated CD4+Vb5+ and CD4+Vα2+ T cells respectively from OT-II (red line) and CNO/OT-II (purple line) mice. B) Also depicts the MFI for CD4 and CD8 expression on clontoype+ gated T cells.

Double Tg CD4+ T cells were tested for functional anergy by in vitro stimulation with OVA$_{(323-339)}$ peptide or OVA protein. As expected CNO/OT-II LNC responded equally well to stimulation with cognate Ag when compared with OT-II.
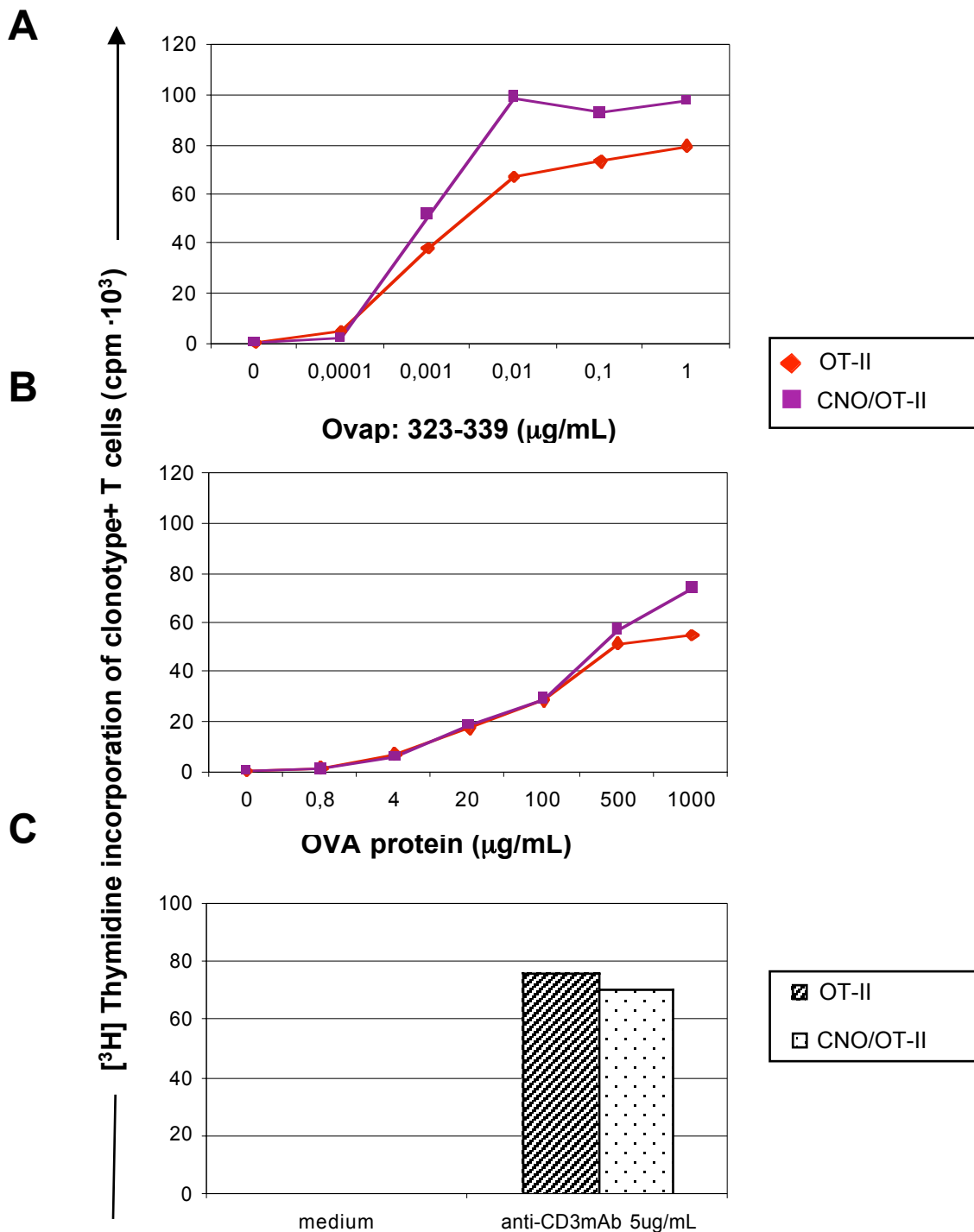
Fig. 46 CNO/OT-II responded equally well to stimulation with cognate Ag as did OT-II LNC.

CNO/OT-II (purple line) and OT-II (red line) clonotype+ T cell response to either A) OVAp$_{(323-339)}$ or B) whole OVA and C) anti-CD3 mAb was determined in a 72h in vitro assay during which the last 16h of culture [^{3}H]-Thymidine incorporation of proliferating LNC was measured. The proliferative response of clonotype+ T cells is given as mean cpm of triplicate wells / % of clonotype+ T cells.

The significant clonal deletion, activated phenotype and mild hyperresponsiveness to cognate Ag exhibited by OVA specific T cells in PNO/OT-I mice inferred the efficient

presentation of K^b/OVA complexes in the thymus and peripheral organs. In contrast the stimulatory activity of IA^b/OVA peptide complexes was inefficient in significantly deleting or activating OT-II T cells in OVA double Tg mice. In conclusion OVA derived peptide presented by K^b but not IA^b are recognised by developing and mature T cells in PNO or CNO mice. These results also serve to illustrate the efficiency with which class I Ags can be presented on a range of different cell types compared with the need for class II Ags to be transferred to MHC II positive cells prior to CD4+ T cell activation.

4. DISCUSSION

4.1 Generation of OVA transgenic mice

The present project attempted to generate a murine model in which to further elucidate the cellular and molecular interactions that regulate the immune response in MS or GBS. While the animal models EAN and EAE have already identified the importance of a T cell dominated immune response this project sought to extend existing autoimmune models with a novel murine transgenic system. In this system the well characterised model Ag, OVA, to be expressed exclusively as a neo-self-Ag, either in the CNS or PNS would enable investigation into the mechanisms involved in the initiation, propagation and termination of an autoimmune disease of the nervous system. The availability of CD4+ and CD8+ OVA specific TCR Tg cells would allow the dynamic role for each T cell subset in the immune response against a CNS or PNS autoAg to be further analysed.

4.1.1 OVA transgenic constructs

The creation of the pSCova and pMBPova constructs used to generate the PNO and CNO mice respectively, was based on the reported ability of different lengths of 5' flanking sequences of the MBP gene to target reporter gene expression exclusively either to oligodendrocytes or Schwann cells (Forghani, Garofalo et al. 2001; Farhadi, Lepage et al. 2003). The 5.5kb core MBP promoter located at –6.5kb within the 5' MBP flanking sequence was shown to specifically direct expression of the lacZ reporter gene in oligodendrocytes. In contrast the 0.6kb SCE1 sequence located at –8.9kb within the 5' MBP flanking sequence ligated to the heterologous hsp68 promoter was found to direct reporter gene expression exclusively to Schwann cells. Therefore PNO and CNO mice were generated by microinjection of linearised plasmids, containing either the isolated SCE-hsp68 or –6.5kb 5' MBP promoter fused upstream of the complete OVA cDNA, into C57BL/6 oocytes.

The development of gene targeting technology including the Cre/loxP system enables specific genes to be inactivated in vivo. To provide an additional control lever on the expression of the OVA transgene, two directly repeated loxP sites were inserted. It was considered that future undesired ectopic OVA expression could be removed by pairing OVA Tg mice with a tissue specific Cre Tg mouse. For example potential leaky CNS expression in PNO mice could be deleted by mating PNO mice with MBPCre mice which express oligodendrocyte specific Cre recombinase (Niwa-Kawakita, Abramowski et al. 2000). Likewise, shut down of OVA expression within the CNS or PNS tissues could provide an additional facet to the model in which to study persistence of encephalitogenic T cells, their interaction with glial cells and the impact of effector molecules on the CNS or PNS tissue in the absence of the neuroAg.

In vitro transfection assays can offer insights into the functionality of the transgene. This has been demonstrated for many different reporter gene assays including one in which the human MBP promoter was able to regulate expression of the chloramphenicol acetyl transferase (CAT) reporter gene in primary oligodendrocyte cultures but not in unrelated cell lines (Wrabetz, Taveggia et al. 1998). In our case, the pSCova plasmid was transiently active in L929 and 293T cells. This implied that the SCE-hsp68 promoter was intrinsically able to direct expression of OVA. Although the SCE-hsp68 promoter was not active within the Schwann cell line this did not exclude its ability to regulate Schwann cell specific OVA expression in vivo. Indeed early studies of the MBP promoter were shown to direct reporter

gene expression in Schwann cells in vitro but not in vivo (Asipu and Blair 1994). This indicates that regulatory constraints of neighbouring genes and chromatins can ultimately influence promoter activity. Although in vitro transfection experiments can indicate the activity of a certain promoter, caution needs to be taken in placing too great an emphasis on negative results.

4.1.2 Analysis of *in situ* OVA expression

Stable chromosome integration of the OVA transgene was determined via PCR or Southern blot in 23-25% of PNO and CNO founder mice. This lies within the integration frequency range of 10-40% as described by other laboratories (Palmiter and Brinster 1986) (Gordon and Ruddle 1981) (Wagner, Hoppe et al. 1981).

The absence of detectable PNS specific OVA protein expression in PNO mice may be due to a number of different factors. Detection of OVA by Western blot or immunohistochemistry was unsuccessful perhaps due to very low protein expression. The main distinguishing problems that are associated with the generation of Tg mice are positioning effects and epigenetic factors. The PNO mice were generated by microinjection of the linearised pSCova DNA directly into the pronuclei of C57BL/6 fertilised mouse eggs. This standard method of generating Tg mice results in the random integration of the transgene into the host chromosome. The random insertion event determines that each founder will contain the transgene at a different site in the genome. Positioning effects may not only have influenced the expression of the transgene but also the ensuing phenotype. Endogenous transcription regulatory elements present at or near the site of insertion may have conveyed new instructions onto the transgene resulting in aberrant expression. In addition promoter occlusion effects could have been responsible for variegated expression, as neighbouring promoters can influence each other. Epigenetic factors can regulate gene expression through DNA methylation, modification of the chromatin structure and chromosome positioning in the nucleus. These factors may have negatively influenced OVA transgene expression if the site of integration was within a closed chromatin region. Other epigenetic factors including allelic variations and interactions between different alleles may also have influenced OVA expression in PNO mice.

Frequently foreign DNA integrates as a tandem linear array consisting of a few to a hundred copies of the transgene. High copy number can have a deleterious, silencing rather than an additive effect on the transgene expression. The copy number of the OVA transgene was not investigated by dot blot assays in either the CNO or PNO mice. However, the relative band intensity of the PNO mice investigated via Southern blot indicated that the three positive lines contained differing amounts of the transgene. Based on band intensity, PNO9 contained the highest; PNO3 contained the lowest while PNO8 contained a medium amount of the transgene. It is not apparent from these data if the 'higher copy number' in PNO9 mice led to gene silencing. Generally the integration event occurs at a single chromosomal locus. However, if integration had occurred in two chromosomal loci then breeding to the next generation ensured segregation of the transgene sites. Nevertheless, transgene expression frequently does not correlate with copy number. Therefore it was important to examine all three PNO mouse lines to acquire consensus of the protein expression pattern.

Some of the above mentioned problems could perhaps have been circumvented by targeted transgenesis (Bronson, Plaehn et al. 1996). Introduction of the transgene into a defined genomic locus via homologous recombination in ES cells can control both the site of expression and copy number of the transgene. This method requires knowledge of the expression pattern of the desired gene locus but does not require analysis of the regulatory gene elements. Gene targeting has been used to drive heterologous gene expression from endogenous promoters. In the case of the PNO mice an alternative method for their generation could be considered. This might include targeting the protein zero (PO) gene, the

major protein of the peripheral myelin sheath. As PO-/- and PO+/- mice develop a peripheral neuropathy two alternative methods of targeting the OVA to the PO gene would have to be employed. Insertion of the OVA gene into the PO gene without disrupting it could be accomplished by the creation of a bicistronic locus. In this case an internal ribosomal entry site could be incorporated upstream of the transgene allowing for the generation of a dicistronic fusion mRNA (Mountford and Smith 1995) (Mohrs, Shinkai et al. 2001). In the case of the PNO mouse this might be useful with regard to coupling the expression of the OVA gene to that of the intact PO genomic locus. Alternatively it might be feasible to generate a PO-OVA fusion protein. Recently Ekici et al., investigating the adhesion abilities of mutated forms of PO protein generated a PO-GFP fusion protein, whereby GFP was expressed at the carboxyl terminal end in frame with PO without hampering the adhesion function of the wild type PO protein (Ekici, Oezbey et al. 2002). Whether the expression of the PO-OVA protein would be expressed at high enough levels to induce an immune response would need to be determined.

Leaky OVA mRNA expression within kidney, lung and thymus of PNO8 and PNO3 mice appears to correlate with reports from Forghani et al., from which we obtained the SCE-hsp68 promoter. In their early characterisation studies only 2 out of 9 founder mice expressed the lacZ reporter gene exclusively in PNS tissue (Forghani, Garofalo et al. 2001). Four mice showed CNS postnatal lacZ gene expression, which they attributed to enhancer trapping. Which non-neuronal cells expressed lacZ was not mentioned. The remaining 3 mice expressed lacZ neither in the CNS nor in the PNS. These results suggest that SCE-hsp68 directed transgene expression is generally leaky and not only Schwann cell specific.

In our case only 3 positive PNO founder lines carrying the OVA transgene were identified within the group of 13 founder mice. Further investigation of OVA mRNA and protein expression failed to detect OVA within the PNS tissues, suggesting that OVA may be present at levels below assay detection limit. However identification of OVA mRNA in the kidney, lung and thymus implied aberrant tissue expression. Backcrossing PNO mice onto anti-OVA TCR Tg mice further substantiated evidence for intrathymic OVA expression within PNO3 mice (see section 4.3).

Histological examination of paraffin embedded thymus sections, stained with anti-OVA serum, identified some thymic epithelial cells in the medulla to be positively stained. Three populations of self-Ag presenting cells residing in the thymus include the medullary epithelial and to a lesser extent the cortical epithelial and haematogenous dendritic cells (Derbinski, Schulte et al. 2001). Which cell type might be expressing OVA could be further immunohistochemically investigated (Surh, Gao et al. 1992). This might include selective staining of the medullary or cortical epithelium and anti-OVA Ab counterstaining. Previous studies have shown that low-level expression of a self-Ag in the cortical epithelium can lead to an increase in the selection of CD4+CD25+ regulatory T cells (Jordan, Boesteanu et al. 2001), supporting the view that the cortical region is devoid of cells capable of deletion (Laufer, Glimcher et al. 1999) (Capone, Romagnoli et al. 2001). Although there is considerable discrepancy between reports on efficient clonal deletion mediated by either cortical (Mayerova and Hogquist 2004) or medullary (Smith, Olson et al. 1997) epithelial cells, Zhang et al., observed in their model that presentation of soluble Ag led to deletion of thymocytes in the cortex whereas when the same Ag was membrane bound thymocytes were deleted in the medulla (Zhang, Vacchio et al. 2003). The expression of a soluble self-Ag makes it difficult to determine which APC is responsible for the thymic selection process as OVA could diffuse throughout the thymus and be taken up and presented by either medullary or cortical epithelial cells in addition to BM derived APCs. Although medullary epithelial cells have been considered to be poor Ag presenting cells bone marrow chimera studies revealed a dynamic cooperation between medullary epithelial cells and bone marrow derived DCs in capturing and presenting Ag to delete autoreactive T cells (Gallegos and Bevan 2004). Therefore cross-presentation of Ag by BM DCs may augment the negative selection process induced by direct Ag presentation on medullary epithelial cells. Gathering

information on the location and type of APC in PNO mice will assist in understanding the central tolerance mechanisms that may support deletion as well as simultaneous generation of regulatory T cells.

Although Forghani's 9 founder mice displayed a similar integration frequency for the SCE-hsp68 promoter when compared with our PNO founders, successful exclusive and robust Schwann cell targeted expression could not be correlated with our own. The probability of identifying a PNO positive founder mouse with exclusive PNS tissue expression may have been greater in a larger population of positive founder mice.

As alluded to in the introduction, an additional interesting facet concerning the SCE exists. It appears that the SCE1 is primarily active in myelinating SC. This was demonstrated by positive β-galactosidase staining in juvenile mice containing the SCE-hspLacZ promoter but no staining at all when the same mice reached sexual maturity (Forghani, Garofalo et al. 2001). Therefore additional analysis of pre-weaned PNO mice would most likely have detected OVA positive SCs in sciatic nerve. Recently Peterson et al., have successfully circumvented this problem of low expression in post weaned mice by integration of the construct as a single copy at the HRPT site. Importantly from this location the SCE1 driven lacZ construct delivers low but significant and robust expression of the reporter gene in mature mice (personal communication A. Peterson).

Although the frequency of 3 CNO mouse lines obtained from within a group of 34 PCR positively identified founders was not very high, 2 lines expressed OVA within CNS tissues in the absence of aberrant expression (Dr Yi Cao personal communication). CNS specific OVA expression in CNO mice reflects the previous successful oligodendrocyte targeting capability of the −6.5kb 5'MBP promoter (Gow, Friedrich et al. 1992) (Foran and Peterson 1992) (Goujet-Zalc, Babinet et al. 1993) (Stankoff, Demerens et al. 1996).

In summary, use of the −6.5kb 5'MBP promoter was successful in directing Tg OVA expression specifically to oligodendrocytes. In contrast use of the SCE-hsp68 promoter failed to direct OVA expression exclusively to Schwann cells. Other than the study by Forghani et al., there are no successful reports of Schwann cell targeted transgene expression when employing the SCE-hsp68 promoter.

4.2 Active disease induction

The development of an immunological response in PNO mice was investigated by immunisation with PT and whole OVA in CFA. By assuming that PNO mice were expressing undetectable low levels of OVA in PNS tissue, immunised mice were assessed for a PNS based autoimmune disorder. Immunised PNO animals did not present a form of a PNS tissue disorder based on the graded clinical score and weight assessment. Subclinical signs in the form of perivascular mononuclear infiltrates into the PNS or CNS tissues were also absent in histological analyses. In contrast OVA immunised CNO mice displayed EAE symptoms including animals with a maximum score of becoming moribund. Additionally, perivascular mononuclear infiltrates were observed in brain sections of diseased mice (Dr Yi Cao personal communication).

4.2.1 Tolerance versus immunity in immunised OVA transgenic mice

In contrast to CNO mice, both cellular and humoral immune responses to whole OVA in PNO mice were diminished. An impaired OVA specific T cell proliferative response and a reduction in anti-OVA serum Ab titres demonstrated this.

The immunogenicity of the OVA protein was evaluated at the T cell level by conducting a proliferation assay in which OVA primed T lymphocytes were restimulated in vitro with whole OVA. Although PNO mice were profoundly resistant to disease induction in vivo, LNC still showed appreciable proliferative responses in vitro. Consistent with the humoral response, T lymphocytes from immunised PNO mice failed to proliferate to the extent that control C57BL/6 T lymphocytes did. The ability of LNC from all groups of primed mice to respond equally well to the polyclonal activator, ConA, indicated that PNO T lymphocytes did not have an intrinsic defect in their proliferating capability. The epitope generated by processing exogenous OVA protein might have varied from that generated by processing of endogenous OVA, providing additional antigenicity. The presentation of this 'foreign antigenicity' would have been irrelevant to disease induction but evident by in vitro lymphocyte restimulation. Alternatively OVA reactive cells generated within the thymus might have been incompletely deleted or anergised. In this case, high affinity clones were preferentially affected while low affinity clones were unable to cause disease. However, after escaping into the periphery their reduced responsiveness to restimulating Ag was measured in vitro.

The time point for harvesting the autoreactive T cells may also have influenced the potential proliferative response to cognate Ag in vitro. Abdul-Majid et al., found in their MOG_{1-125} induced EAE model, that LNC harvested prior to clinical EAE (one week post immunisation) were unresponsive to in vitro restimulation in contrast to LNC removed at the peak of clinical disease (Abdul-Majid, Jirholt et al. 2000). The authors argued that non-specific immunosuppressive events were acting on the autoreactive T cells prior to clinical disease while specific suppressive mechanisms were enforced during the initiation phase. Of course it is also possible that by virtue of their high numbers during the peak clinical phase, some autoreactive T cells escaped any suppressive mechanisms which may have restricted their response during the early EAE phase. Clearly it is important to pinpoint the correct time point for characterisation of an in vitro recall response for a particular autoimmune model. It is unlikely in the absence of a marked autoimmune response in PNO mice, that high numbers of autoreactive LNC were present at the harvesting time point of day 11-16. This time point was chosen as it correlates with the advent of the clinical phase active PO-EAN in C57BL/6 mice. Of course the clinical course of a potential EAN like disease in PNO mice might not necessarily correlate with that found in the classical EAN. Although further kinetic studies might identify the peak clinical phase of a potential PNS autoimmune disease in PNO mice this is highly unlikely in the absence of detectable target Ag expression.

In contrast to CNO mice, sera from OVA immunised PNO mice at day 11-16 post immunisation contained almost 3-fold fewer anti-OVA IgM and IgG antibodies relative to WT controls. The reduced humoral immune response in PNO mice suggested partial tolerisation of T helper cells and/ or B cells.

The increased levels of anti-OVA IgG1 in C57BL/6 OVA immunised mice but not in PNO mice suggested a successful isotype switch from IgM to IgG during the T dependent immune response. T helper cells play a pivotal role in antibody production against T-dependent antigens. Upon recognition of the MHCII : peptide on B cells, naïve T helper cells expressing antigen specific TCRs are induced to undergo activation, clonal expansion and differentiation. Engagement of this complex by the TCR induces expression of CD40L that

binds to CD40 on the B cell leading to several B cell functions including further stimulation of T cells (Grewal and Flavell 1998). Subsequently activated T helper cells differentiate into either the Th1 or Th2 subsets. IL-4 induces T helper cells to become committed Th2 cells secreting IL-4, IL-5 and IL-6. These Th2 type cytokines in turn induce the proliferation of antigen-specific B cells, isotype switching and differentiation into plasma cells (Mosmann and Sad 1996). OVA challenged PNO mice showed decreased levels of IgG1 suggesting a partial inhibition in the Th2 type response. Future measurements of IgG2a isotype autoAbs could exclude or include tolerance mechanisms affecting Th1 type responses.

The reduced immunogenic response in PNO mice led us to consider a role for the generation of regulatory T cells and their role in mediating partial tolerisation of T helper cells. Recent studies have assigned the thymus a role in directing the selection of regulatory CD4+CD25+ T cells. In PNO mice the generation of regulatory T cells may have led to thymic derived tolerance. Mice doubly Tg for the influenza hemagglutinin protein and TCR demonstrated the increased generation of CD4+CD25+ T cells indicating a role for the agonist ligand in thymic development of regulatory T cells (Jordan, Boesteanu et al. 2001). Additionally the development of self-peptide-specific CD4+CD25+ regulatory T cells was demonstrated in mice expressing varying amounts of the peptide-Ag and Tg anti-peptide specific TCR T cells (Lerman, Larkin et al. 2004).

The CD4+CD25+ regulatory T cell population has been shown to effectively suppress the functions of transferred Th1 and Th2 cell lines in vitro and in vivo. In the colitis model the suppression of both Th1 and Th2 cells resulted in disease resolution (Xu, Liu et al. 2003). Furthermore CD4+CD25+ cells have been shown to modulate the T helper response towards a Th2 type by inhibiting Th1 differentiation in an allergic airway model (Suto, Nakajima et al. 2001). Importantly these studies provide evidence for suppression of Th1 and Th2 T cell subtypes by the CD4+CD25+ regulatory T cell population in vivo.

To gain an insight into the role of regulatory T cells maintaining immunologic self-tolerance in PNO mice the CD4+CD25+ T cell population could firstly be phenotypically characterised followed by analysis of CD4+CD25+ mediated suppression. The frequency of CD4+CD25+ T cells was comparable between WT and PNO mice comprising between 5-10% of the total CD4+ T cell population (data not shown). Further investigation could assess the suppressive capacity of the CD4+CD25+ population by isolation and adoptive transfer into CNO mice undergoing EAE symptoms. Conversely depletion of CD4+CD25+ T cells prior to immunising PNO mice with whole OVA might indicate the extent to which suppressors are influencing the proliferative recall response. As previously shown, CD4+CD25+ T cells can be efficiently deleted from blood in C57BL/6 mice for about 30 days post ip injection of anti-CD25 mAb (Oldenhove, de Heusch et al. 2003).

Further investigation into the cytokines expressed by T cells in immunised PNO mice would provide a greater insight into the peripheral tolerance mechanisms operating in PNO mice. Either RT-PCR or intracellular FACS analysis could determine the levels of certain regulatory cytokines including IL-10 and TGF-β. This would provide a greater insight into the tolerogenic response operating in OVA immunised PNO mice.

4.2.2 Optimisation of the disease induction protocol employed for PNO mice

The data on EAN in C57BL/6 mice is scarce, as the EAN model has primarily been studied in the rat. Immunisation with common neuroAgs such as the P2 protein or BPM has been unsuccessful in inducing EAN in the C57BL/6 strain (Taylor and Hughes 1985) (Zhu, Nennesmo et al. 1999). However, unsuccessful EAN induction in the C57BL/6 mouse strain was solved by immunising with a more abundant Schwann cell specific neuroAg, the PO

glycoprotein (Zou, Ljunggren et al. 2000). Immunisation of mice with PO in CFA was carried out sc on days 0 and 6. Additionally mice received PT iv on days –1 (400ng) 0, 1, 3 (200ng). Conversely in the EAE mouse model MOG35-55 has strong encephalitogenic properties in C57BL/6 mice (Kerlero de Rosbo, Mendel et al. 1995). The author's active immunisation protocol included one sc injection of 200-400ug MOG35-55 in CFA (containing 400ug H37RA) with PT (300ng) being injected iv on days 0 and 2 pi (Johns, Kerlero de Rosbo et al. 1995). Various laboratories have modified induction protocols by challenging with alternative neuroAgs and adjuvants at different time points. By considering these parameters a variation of the above mentioned protocols (materials and methods) was carried out in an attempt to induce a PNS autoimmune disease. While the immunisation schedule was unsuccessful in inducing a PNS type autoimmune disease in PNO mice it was successful in inducing EAE like symptoms in the CNO mice (Dr Cao personal communication).

Successful active induction of EAN in C57BL/6 mice might have been influenced by the amount of *M. tuberculosis* (H37RA) present in the CFA. The amount of M. tuberculosis generally administered varies between studies from 0.2-4mg although Sun et al., argue that if less than 0.6mg of *M. tuberculosis*, H37RA, is used mice undergo a shorter duration of disease varying from 20-60 days (Sun, Whitaker et al. 2001). Perhaps the use of only 0.1mg in the immunisation protocol for PNO mice needs to be increased to provide adequate benefits of the adjuvant stimulating properties.

In addition to altering the concentrations of various adjuvant constituents, past reports have shown that resistance to a CNS or PNS autoimmune disorder could be overcome by combining adoptive transfer of encephalitogenic T cells with antigenic challenge (Shaw, Kim et al. 1996), or even treating mice with anti-IFNγ mAbs (Duong, Finkelman et al. 1994). Moreover the development of aggressive, demyelinating EAE in rats has been related to the presence of anti-MOG Abs in the presence of autoreactive T cells (Steffel A et al., 1999). Additionally in rats and in marmoset monkeys a second injection of soluble MOG into MOG:CFA preimmunised animals caused a Th2 shift that eventually resulted in a delayed and more severe form of EAE (Genain, Abel et al. 1996). Whether this disease inducing protocol would apply to the PNO mouse would need to be determined in further experiments.

The inclusion of PT in an induction regime increases the incidence and severity of EAE or EAN. Early on it was recognised that PT is able to bypass several genetic checkpoints associated with EAE by converting some resistant mouse strains to susceptible ones. Bernard et al., established an immunisation schedule that included whole spinal cord homogenate emulsified in CFA containing 2 types of mycobacteria, followed by two doses of PT iv (Bernard and Carnegie 1975). It has also been recognised that early administration of PT and not several weeks after immunisation can contribute to successful disease induction (Hofstetter, Shive et al. 2002). Numerous experiments in C57BL/6 mice have led to the standard EAE protocol, which includes the ip or iv injection of 400ng PT at days 0 and day 2 post immunisation.

Interestingly previous reports have shown that CFA with PT in the absence of neuroAg is also able to induce EAE in the B10.RIII mouse strain (Jansson, Olsson et al. 1991). Furthermore the incidence and severity of EAE induced by immunisation with the neuroAg in CFA was shown to be enhanced by the coinjection of PT (Linthicum, Munoz et al. 1982) (Yong, Meininger et al. 1993). Treatment of MBP TCR Tg mice with only PT was also reported to induce EAE (Goverman, Woods et al. 1993).

The mechanism by which PT facilitates the induction of EAE or EAN remains controversial in light of its enhancing versus inhibitory properties. Its pleiotropic effects on the immune system include T cell mitogenesis, an increase in cytokine and Ab production and the promotion of DTH responses (Sewell, Munoz et al. 1983). One popular model proposes that PT facilitates immune cell entry to the CNS by increasing permeability across the BBB. Conversely it can facilitate cellular penetration through the BNB. This may be achieved by

histamine induced vascular leakage of the respective endothelial cell layer (Yong, Meininger et al. 1993). However, it was recently shown via intravital microscopy of the cerebromicrovasculature that recruitment of leukocytes and activated T cells is the primary action of PT when administrated alone, while permeability is the consequence (Kerfoot, Long et al. 2004). This would support evidence presented by Hofstetter et al., that showed PT to promote Th1 differentiation and clonal expansion of encephalitogenic T cells via its activation of APCs either in the lymphoid tissues (Hofstetter, Shive et al. 2002) (Shive, Hofstetter et al. 2000) or CNS. Furthermore Kerfoot et al., were able to determine that PT-induced leukocyte recruitment was TLR4 receptor dependent (Kerfoot, Long et al. 2004). This provided an important link to the innate immune system as signalling through a receptor, which is linked to the innate immune response, may partly promote CNS disease inducing mechanisms.

Although adjuvants might be considered as an effective experimental substitute for environmental factors that support the induction of EAE or EAN, their usage can skew the immune response. Clearly it would be an advantage to remove or reduce the amount of adjuvants from the immunisation protocol, as they are unlikely to play a physiological role in the corresponding human disease. Recently Hofstetter et al., demonstrated that injection of PT in conjunction with the neuroAg:IFA emulsion prevented tolerance induction as conferred by injection of Ag:IFA and therefore provided an alternative to the standard CFA-based induction of EAE in the PLP:SJL/J mouse model (Hofstetter, Shive et al. 2002). Alternatively DBA/1 mice were found to be susceptible to EAE upon a single injection of MOG_{1-125} or MOG_{79-96} peptide in the absence of PT (Abdul-Majid, Jirholt et al. 2000). This is consistent with findings in other groups which left out PT but required a second immunisation with the MOG_{35-55} peptide in either PL/J or C57BL/6 mice (Kerlero de Rosbo, Mendel et al. 1995). However, removal or alterations to adjuvant dosage, administration or even type, can only be considered if the more common protocols prove successful. This is important, as certain adjuvants are needed in overriding specific tolerance mechanisms that might impede the development of a PNS autoimmune disease. Further critical appraisal of the above mentioned parameters might result in the successful induction of a PNS autoimmune disease in PNO mice.

In summary, OVA immunised PNO mice did not display clinical or subclinical signs of a PNS type disease. Furthermore they demonstrated partial tolerisation of their T helper cell compartment by presenting a reduced in vitro proliferative capacity and Th2 humoral response when compared with their NTg counterparts. These results further support the notion that expression of OVA in the target tissue (PNS) was absent.

4.3 Passive disease induction

In order to follow an immune response to low levels of OVA expressed in either oligodendrocytes or Schwann cells, we wanted to bypass intrathymic induced tolerance mechanisms acting on anti-OVA specific T cells by utilising a modification of the TCR Tg T cell adoptive transfer system (Pape, Kearney et al. 1997). Previous adoptive transfer models have also utilised OVA Tg mice to study organ specific immunity. In these disease models anti-OVA specific T cells were adoptively transferred into mice expressing OVA in pancreatic islets and kidney (Kurts, Heath et al. 1996) (Kurts, Kosaka et al. 1997) or the small intestine (Vezys, Olson et al. 2000). In our model activated OT-I and OT-II cells (which have the capacity to enter tissues) were transferred into naïve irradiated or nonirradiated PNO or CNO recipients in an attempt to passively transfer a PNS or CNS type disease. This system of adoptive cell transfer allowed for a closer examination of the fate of clonotype+ T cells in PNO and CNO mice in vivo.

Moreover, previous reports suggested that passive induction of a CNS autoimmune disease could result in a more severe clinical and pathological form of the disease than that induced

by active immunisation. For instance, Sun et al., were able to demonstrate a more progressive form of EAE in immunocompetent C57BL/6 mice following adoptive transfer of pMOG$_{35-55}$ specific CD8+ T cells when compared with their active immunisation procedure (Sun, Whitaker et al. 2001).

4.3.1 Inclusion of IL-12 in the in vitro activation protocol

IL-12 is produced mainly by pathogen stimulated macrophages and dendritic cells and provides an important link between the innate and adaptive immune responses. Signalling through a heterodimeric receptor it induces IFN-γ production by T and NK cells and is therefore the pivotal cytokine in promoting development of a Th1 type immune response. IL-12 has been shown to enhance the encephalitogenicity of neuroAg reactive T cells upon stimulation in vitro (Leonard, Waldburger et al. 1995) (Leonard, Waldburger et al. 1996; Waldburger, Hastings et al. 1996). Neutralising IL-12 mAbs prevented active as well as passive induction of EAE, indicating a crucial role for the cytokine in the development of EAE (Leonard, Waldburger et al. 1995). Leonard et al., were able to demonstrate in an SJL/J mouse AT-EAE model that *in vitro* stimulation of encephalitogenic T cells resulted in a rapid onset of clinical symptoms as well as a prolonged and exacerbated form of EAE (Leonard, Waldburger et al. 1995).

IL-12 has been shown to enhance CTL responses (Kieper, Prlic et al. 2001). Adoptive transfer of CD8+ T cells primed in vitro with IL-12 led to an increase in the primary and memory T cell population (Chang, Cho et al. 2004). In a recent novel murine model of autoimmune myocarditis, Grabie et al., were able to demonstrate the importance of IL-12 in the *in vitro* generation of pathogenic effector CD8+ T cells (Grabie, Delfs et al. 2003). In their model, CMy-mOVA Tg mice did not show humoral or cellular immune responses upon different routes of immunisation with OVA. However, adoptive transfer of OT-I T cells stimulated in vitro with SIINFEKL in the presence of IL-12 were not only able to home to the draining lymph nodes of the heart but were also able to cause myocarditis in contrast to IL-12 untreated cells. Further investigation determined IL-12 stimulation to upregulate the expression of CCR5, which increased the homing ability of OT-I CD8+ effector cells to myocarditic hearts expressing cognate chemokines (Grabie, Delfs et al. 2003).

These published studies provided evidence for the importance of IL-12 in rendering in vitro stimulated T cells more encephalitogenic either by affecting the T cell effector function or their number. Initially in our case intracellular cytokine analysis confirmed successful *in vitro* stimulation of OT-I and OT-II T cells and polarisation to the Tc1 or Th1 subtype respectively.

4.3.2 Transferred CD8+ OT-I and CD4+ OT-II T cells remain silent in PNO and CNO mice

Preliminary adoptive transfers in immuno-competent and –compromised PNO mice with a total of 10·10^6 cognate peptide stimulated OT-I and OT-II LNC did not result in any form of a PNS type autoimmune disease as based on weight analysis and the graded clinical score. Furthermore the number of clonotype+ T cells recovered from peripheral lymph nodes day 10 and onwards was below background level demonstrating either non-specific homing to tissues other than the PNS post restimulation, in situ apoptosis or foremost differentiation of autoreactive T cells *in vivo* into a non-pathogenic, potentially protective phenotype.

Further adoptive transfer experiments using immunocompetent CNO mice showed similar tolerogenic results as for PNO mice. At all time points OT-II CD4+ T cells were recovered from the peripheral lymphoid organs in similar numbers from immunocompromised CNO and NTg controls. In contrast, recovered OT-I CD8+ T cell numbers were significantly reduced in

CNO mice when compared to NTg mice day on 3 post transfer. Unfortunately, in preliminary immunohistological stainings of CNS tissues, CD3+ T cells went undetected in CNS tissues suggesting that either transferred T cells were dying in situ or relocating to extralymphoid sites other than the CNS.

Potential suppressor cells that might influence the ability of transferred OVA specific TCR T cells to proliferate upon secondary exposure to the native autoAg were transiently removed by sublethal irradiation. Sublethal irradiation has in the past been used to remove the endogenous cell population to facilitate the exclusive study of transferred cells *in vivo*. In particular Romagnoli et al., showed that the kinetic repopulation of CD4+CD25+ regulatory thymocytes in sublethally irradiated adult C57BL/6 mice was similar to that of the mature CD4+ thymocyte compartment (Romagnoli, Hudrisier et al. 2002) Fig. 3A). Both populations re-accumulated over a 1-week period from day 9-15 post irradiation. This time frame was utilised in our experiments to investigate the fate of transferred CD4+ and CD8+ T cells in PNO and CNO mice. A more stringent strategy to remove thymic tolerogenic influences might involve thymectomy followed by grafting with a NTg C57BL/6 thymus. This would remove intrathymic OVA expression. Subsequent lethal irradiation and bone marrow reconstitution with either OT-I or OT-II BM would enable autoimmunity or tolerance to tissue expressed OVA to be investigated independently of thymic influences.

CD4+ as well as CD8+ T cells are known to be involved in the induction and down-regulation phase of a neuroautoimmune disease. According to past experimental data CD4+ Th1 cells are generally considered as effectors while Th2 cells take the role of regulators during tissue damage in organ-specific autoimmunity. For instance Th1 polarised and not Th2 Ac 1-16 MBP peptide specific CD4+ T cell clones were successful inducers of disease (Baron, Madri et al. 1993). Additionally Vizler C et al., were able to demonstrate that the Tc1 pathogenic ability to induce IDMM was greater than when transferring Tc2 polarised CD8+ Ag-specific T cells (Vizler, Bercovici et al. 2000). Considering this hypothesis, OT-I and OT-II T cells were stimulated in vitro with the respective peptide and skewed towards the Th1/Tc1 phenotype in the presence of IL-2 and IL-12 before being tested for their encephalitogenic potential in either non-irradiated or irradiated PNO or CNO mice.

One disadvantage of adoptively transferring *in vitro* activated cells is that preactivated cells will travel to all tissues of the body (Wack, Corbella et al. 1997) and therefore not exclusively to desired target organs. Furthermore the polarisation to a Th1 or Tc1 subtype inferred their secretion of cytokines, which might have caused initial cell damage in organs other than the target organ. For the initiation of a cellular response to be more effectively observed our adoptive transfer model could be modified so as to prime purified naive transferred cells *in vivo*.

In published attempts at inducing a CNS disease model, Shaw et al., demonstrated successful induction of AT-EAE by challenging MBP specific T cell clone recipient mice on day 16 with MBP-CFA (Shaw, Kim et al. 1996). Conversely another study in the Lewis rat was able to exacerbate AT-EAE by intrathecal injection of the neuroAg 4-5 days post transfer of weakly pathogenic T cells (Kawakami, Lassmann et al. 2004). However, local injection of a soluble Ag has been shown to enhance AICD mechanisms within the CNS (Critchfield, Racke et al. 1994). Future experiments would need to determine the efficacy of challenging AT-PNO recipient mice with soluble OVA.

The requirement of CD4 T cell help for CD8 T cells remains controversial. Whereas CTL responses against certain viruses can be carried out in the absence of CD4+ T cell help, in the RIP-OVA model CTL cross priming depends on OVA-specific CD4+ T cell help. The latter was demonstrated by Kurts et al., where cross-priming was successful when CD4+ T cells activated the DC that was cross-presenting the MHCI/peptide complex (Kurts, Carbone et al. 1997). CD4+ T cell help therefore appears to be necessary for the induction of some CD8+ T cell responses either directly or indirectly (Husmann and Bevan 1988) (Guerder and

Matzinger 1992). In particular, when male specific Tg TCR CD8+ T cells were confronted with cognate Ag a transient response was observed in the absence of CD4+ T cells after which all Ag specific T cells died (Kirberg, Bruno et al. 1993). Additionally in the RIP-mOVATg mouse model it was observed that coinjection of OT-II CD4+ T cells prolonged the lifespan of OT-I CD8+ T cells and led to diabetes in 68% of mice. AI-EAE models have indicated CD4+ T cells to be primarily involved in the initiation of the CNS autoimmune disease, while AT-EAE assigns activated myelin specific CD8+ T cells an important role in the development and progression of a demyelinating disease either directly via cytotoxic activity or indirectly via bystander mechanisms. This suggests that while CTL precursors initially need the help of CD4+ T cells either directly or indirectly via DC they are able to mediate pathogenesis in their absence. Based on these reports our adoptive transfers were conducted with the rational being to employ activated OT-II CD4+ T cells for their support in initial OT-I CTL effector responses. Therefore activated OT-I and OT-II T cells were injected consecutively or simultaneously into PNO or CNO mice. Transfer of either combination proved unsuccessful in inducing a nervous system disorder in either PNO or CNO mice.

Successive transfer experiments were conducted with an increase in the total number of anti-OVA specific T cells when considering that normal tolerogenic mechanisms would be unable to cope with a large number of autoreactive T cells. A previous report by Kurts et al., determined that transfer of $5 \cdot 10^6$ OT-I T cells into RIPmOVATg mice resulted in diabetes in contrast to when only $0.25 \cdot 10^6$ T cells were transferred (Kurts, Kosaka et al. 1997). The success rate of our adoptive transfers inducing either a CNS or PNS autoimmune disease may have been related to the number of transferred encephalitogenic T cells. This was described early on when Bernard et al., showed that disease induction was reproducible in SJL/J mice with the transfer of $6 \cdot 10^7$ LNC but not $1.5 \cdot 10^7$ LNC (Bernard and Carnegie 1975). The number of PNS specific T cells transferred was also determined to be critical in determining the degree, type of pathology and severity of AT-EAN in Lewis rats (Heininger, Stoll et al. 1986). However increasing the number of transferred OT-I and OT-II cells (6 fold) from $2 \cdot 5 - 15 \cdot 10^6$ cells each did not induce a CNS or PNS immunogenic response as determined by weight analysis and immunohistological examination of the relevant organs. While transfer of high numbers of autoreactive T cells caused tissue damage in other models but not in ours this might again reflect distinct anatomical mechanisms of tolerance induction or limited pathogenic potential of transferred T cells. More likely in the case of PNO mice the absence of the target Ag was responsible for the failure of disease induction upon transfer to immuno -competent or –compromised hosts

Inappropriate retrieval time points and impurity of transferred OVA specific T cells are further factors which may have hampered the optimisation of our desired disease models. The time points studied to investigate the fate of potentially encephalitogenic T cells were generally based on published EAE and EAN adoptive transfer models. It is plausible that in our specific model the time points would vary greatly depending on the exact site, quantity and quality of OVA presentation. In addition the individual avidity of OT-I and OT-II TCR and their ability to migrate to the site of Ag presentation is another factor to consider. As has been shown a particular avidity can influence for example the helper dependence of CD8+ T cells (Heath, Kjer-Nielsen et al. 1993). Although mixed LNC and not RAG-/- or column purified T cells were used in the transfers, it is fair to argue that after 6 days in culture with the cognate peptide FACS analysis determined ≤10% of T cells that remained were clonotype-. This indicated a minimum loss in cognate Ag recognition potential.

During the initiation of an encephalitogenic attack the mechanisms of BBB/ BNB activation remain to be elucidated. Kawakami et al. suggested that following adoptive transfer of GFP transduced encephalitogenic T cells, only those that were highly activated could cross the BBB. These T cells could potentially provide a conducive environment for subsequent autoimmune T cells by supporting Ag processing and presentation of Ag by glial cells through cytokine secretion. The failure of transferred T cells to accumulate within the CNS of CNO mice may have been due to inefficient local presentation of OVA as well as the differing

TCR avidity between OT-I and OT-II T cells. As contact with the target autoAg did not provide a strong enough TCR activation stimulus or was completely excluded, a conducive environment, which might initiate and propagate a full inflammatory response, may have been hindered. Perhaps overall low-level restimulation in CNO and PNO mice will remain undetected. In a healthy CNS tissue, the production of MHC determinants, costimulatory molecules and proinflammatory mediators are normally suppressed. Local APCs including astrocytes which may only partially have activated T cells by inefficient costimulation may have led to T cell anergy or deviation of activated effector cells to a Th2 type phenotype (Aloisi, Ria et al. 2000). It is conceivable that low amounts of OVA being presented in either CNO or PNO mice may have rendered weakly pathogenic transferred T cells anergic. This could be confirmed in future experiments by re-stimulation of retrieved cells with cognate peptide either in situ or after isolation ex vivo.

An additional mechanism that may have rendered transferred T cells non-pathogenic is the action of IL-12 *in vitro*. Recently it was determined that *in vitro* stimulation with cognate Ag and IL-12 rendered T cells hyporesponsive upon re-exposure to Ag *in vitro*. Hyporesponsiveness was secondary to FAS/FAS-L mediated AICD. Interestingly the degree of AICD induced was directly correlated with the concentration of IL-12 used in the priming cultures and reports have indicated that Th$_1$ cells are more susceptible to AICD than Th2 cells (Mendel, Natarajan et al. 2004). Whether the IL-12 signal sensitised OT-I and OT-II cells to AICD, which would influence their *in vivo* potential, would have to be evaluated by *in vitro* challenge with cognate peptide.

As already referred to, a second endogenous TCR is expressed on a small population of TCR Tg T cells (Heath, Kjer-Nielsen et al. 1993) (Hardardottir, Baron et al. 1995) (Balomenos, Balderas et al. 1995). In published transgenic models, an increasing number of T lymphocytes carrying an endogenous α-chain were recovered from recipient mice. Due to the preferential expansion of T cells with an endogenous alpha chain, those carrying the Tg α-chain were progressively diluted from the total population and therefore could no longer be detected in a significant proportion from recipient organs. Examination of the clonotype-population did not provide evidence for a preferential expansion of the endogenous α chain carrying T cell population in either CNO or PNO recipient mice. Therefore the significant decrease in OT-I cells in CNO irradiated recipients could not be accounted for by an increase in clonotype- T cells, ie a dilution effect in Tg TCR CD8+ T cells.

It is generally considered that activated T cells can enter the CNS irrespective of their Ag specificity (Owens, Renno et al. 1994). As shown by Flugel et al., not only encephalitogenic T cells but also those specific for a non CNS Ag such as OVA migrate into the CNS (Flugel, Berkowicz et al. 2001). In fact, the different brain-specific T cells invaded the CNS at day 3-4 post injection, with similar intensity. Considering these points, it is unlikely that the activated OT-I or OT-II clonotype- and clonotype+ T cells could not gain access to the CNS or PNS. Activated OT-II and/or OT-I T cells may have entered the CNS/PNS but did not persist due to lack of restimulation with the cognate Ag in situ. In the case of the PNO mice it is more likely that in the absence of the target Ag activated OT-I and OT-II T cells were unable to be restimulated.

4.3.3 Considering the fate of transferred OVA specific T cells in immunocompromised CNO mice

In preliminary experiments CD3+ T cells were unable to be detected via immunohistochemistry within the brain or spinal cords of CNO recipient mice. Recently it was shown in the Lewis rat EAE model, that the majority of neuroAg specific T cells migrated deeply into the CNS parenchyma (Kawakami, Lassmann et al. 2004) in contrast to the mouse EAE model in which encephalitogenic T cells were located mainly within the

perivascular space (Cross, Cannella et al. 1990). Therefore the in situ localisation of transferred T cells may not be as easily determined when looking at the CNS as a whole but rather be restricted to certain areas of the CNS. Considering localisation of a cellular response would require further histological analysis of the CNS tissues.

Nearly a 2-fold reduction in CD8+Vα2+Vβ5+ transferred T cells, was observed between days 7 and 12 in CNO and not NTg immuno-compromised recipients. One possibility is the migration of activated OVA specific T cells into the brain where the target Ag is expressed. However, preliminary immunohistochemical examination of the brain revealed no perivascular infiltration of CD3+ cells (data not shown). The diminished Tg T cell number may also be explained by a decreased ability in homing to LN; an increase in the number of cells undergoing apoptosis or a decrease in Tg T cell division.

Interestingly the CD4+Vα2+Vβ5+ T cells did not appear to have left the peripheral lymphoid organs when compared with the OT-I T cells. Their behaviour with respect to numbers being recovered in peripheral LN was mirrored in the NTg counterparts. OVA being presented in the CNS of CNO mice may have been invisible to OT-II T cells with a lowered TCR avidity and therefore allowed their recirculation through the CNS and back into draining lymph nodes, thereby excluding in situ activation (Hickey, Hsu et al. 1991).

A pathogenic role for CD8+ T cells has been proposed by Huseby et al., amongst other laboratories (Huseby, Liggitt et al. 2001). Via adoptive transfer Huseby et al., were able to show that transfer of $2 \cdot 10^7$ MBP CD8+ CTLs into sublethally irradiated C3H recipient mice induced a severe form of CNS autoimmune disease (Huseby, Liggitt et al. 2001). Pathological and clinical signs were distinct from the prototypic CD4+ T cell mediated EAE disease with the major difference seen in the predominance of lesions in the brain versus the spinal cord. Using a retroviral gene transfer system, Flugel et al., were able to follow the fate of GFP+ encephalitogenic T cells before and after active induction of EAE in rats (Flugel, Berkowicz et al. 2001). In their model $5\text{-}10 \cdot 10^6$ activated GFP+ retroviral transduced MBP specific T cells could be easily traced in immunocompetent Lewis rats. The authors determined that at 36h pi MBP specific T cells appeared in the draining LN of the peritoneal cavity. At 48h the MBP specific T cells entered the blood and spleen before migrating into the CNS at 72-108 h. The onset of EAE correlated with the migration of the encephalitogenic T cells and their myelin specific reactivation into effector cells (Flugel, Berkowicz et al. 2001). Notably, the early CNS infiltrates consisted mainly of CD4+ T cells, which declined rapidly by day 7 pi. While CD8+ T cells were determined to be present in the meninges, their further migratory activity was not investigated. In the recent GFAP-HA mouse model in which the neo-self Ag is primarily expressed in astrocytes of the CNS, adoptive transfer of activated CTLs led to lesion formation within the grey matter of the spinal cord and brain stem in the absence of clinical symptoms and macrophage recruitment (Cabarrocas, Bauer et al. 2003). These models demonstrate that CD8+ Tc$_1$ cells can play a major role in the induction and propagation of brain inflammation by selectively destroying cells expressing the target Ag. In contrast Sun et al., described an enhanced pathology of the CNS following adoptive transfer of CD8+ MBP specific T cells which was attributed to non-specific cytolytic activity (Sun, Whitaker et al. 2001). However, a consensus on the exact CD8+ T cell actions during the initiation and propagation of nervous system disease has not been reached.

In the above mentioned models the onset of inflammation began at days 7-8 post transfer (Sun, Whitaker et al. 2001) (Cabarrocas, Bauer et al. 2003). The time of peak inflammation at day 7 in the CNS of the GFAP-HA mouse model could correlate with the absence of OT-I T cells from LNs of CNO mice ie the neuroAg specific cells left the peripheral lymph node system and relocated to the CNS. The absence of clinical symptoms or macrophage recruitment into the CNS was observed in both the GFAP-HA and CNO model. Unlike in the GFAP-HA model at early time points CD3+ T cells could not be detected in close proximity to cells (oligodendrocytes) expressing the target Ag. It is of course a prerequisite that further

modifications to the transfer studies will be carried out in the CNO mouse model to identify the exact roles each T cell subset has in inducing a CNS autoimmune response.

Before we can determine whether low levels of OVA expression in the periphery induced CD8+ T cell deletion, we would need to ensure that the transferred OT-I T cells were successfully restimulated in vivo. To this end the proliferation status of the transferred clonotype+ T cells could be investigated by using the CFSE dilution technique. Deletion has been reported as a likely mechanism of extrathymic tolerance for self-Ag in other transgenic models (Fields and Loh 1992; Bertolino, Heath et al. 1995). Initially the fate of transferred CD8+ T cells was considered to end in the liver (Huang, Soldevila et al. 1994). However further TUNEL staining studies revealed that upon in vivo activation, OT-I CD8+ T cells underwent apoptosis in spleen and LN, 7 days post activation with SIINFEKL (Koniaras, Bennett et al. 1997) (Wack, Corbella et al. 1997). This time frame is compatible with the findings in the OT-I AT-CNO model. The inability to detect CD3+ T cells within the CNS may be due to the fact that day 7 post transfer CD8+ T cells underwent apoptosis within the peripheral lymphoid system. Furthermore OT-I CD8+ but not OT-II CD4+ T cells retrieved from LN and spleen were found to display an activated phenotype based on the relative increase in the number of clonotype+ T cells expressing CD25 and CD69. In some models of peripheral CD8+ T cell tolerance, recognition of self-Ag leads to proliferation and partial activation without gain of effector function finally culminating in apoptosis. Similarly the fate of autoreactive CD8+ T cells in the RIP-mOVA model is deletion within lymph nodes of OVA expressing tissues (Kurts, Heath et al. 1996; Kurts, Kosaka et al. 1997). This mechanism of peripheral tolerance was also observed in the InsHA mice (Morgan, Kreuwel et al. 1999; Morgan, Kurts et al. 1999). In both systems activation and proliferation in the draining LN precedes deletion. In the RIP-mOVA model this appears to involve CD95 signalling which is consistent with an activation-induced cell death model (Kurts, Heath et al. 1998) while in the InsHA model Fas plays no role suggesting autoreactive T cells undergo passive cell death (Hernandez, Aung et al. 2001). Which exact mechanisms of tolerance induction are responsible for the reduction in the anti-OVA Tg TCR CD8+ and the unresponsiveness observed in the CD4+ T cell subset remains to be investigated.

4.4 Characterisation of OVA/ anti-OVA TCR double Tg mice

To further examine the tolerogenic conditions operating in PNO mice, they were crossed with either OT-I or OT-II mice to generate double Tg mice. Clonal thymic deletion as well as peripheral T cell activation can determine the presence of very low amounts of self-Ag. The anti-OVA Tg TCR provided a sensitive means of detecting minute amounts of OVA being expressed either in the thymus or periphery. Furthermore the ability to track these clonotype+ T cells would enable the consequences of ectopic OVA expression to be determined by examining the generation, fate and effector function of self-reactive CD4+ or CD8+ T cells. To study the central and peripheral tolerance mechanisms acting in double Tg mice, the number of cells expressing the clonotypic TCR in the thymus, LN and spleen, including the expression pattern of a panel of activation markers, as well as the lymphocyte responsiveness to OVA was measured.

It should be noted that the PNO line 3 containing the lowest level of the transgene according to the Southern blot data was used for the following experiments. Although OVA mRNA expression was inconsistent in the thymi of PNO3 mice, suggesting mRNA expression levels to be near the threshold detection limit of the RT-PCR assay, anti-OVA Ab staining of PNO3 thymi revealed above background low levels of OVA expression. Ultimately the following PNO double Tg mice characterisations confirmed intrathymic OVA expression.

4.4.1 Clonal deletion of OVA specific CD8+ T cells leads to tolerance induction in PNO/OT-I mice

The induction of an autoimmune disease is a common feature of double Tg mice, which express the neoself Ag in the presence of T cells carrying a TCR with specificity for it. It was possible that even in the presence of intrathymic OVA expression, double Tg mice (PNO/OT-II or PNO/OT-I) might have exhibited an autoimmune disease such as the diabetes seen in RIP-HA double Tg mice (Morgan, Liblau et al. 1996) or ocular inflammation in HEL double Tg mice (Zhang, Vacchio et al. 2003) or the severe skin autoimmune disease in OT-I/K14-OVAp double Tg mice (McGargill, Mayerova et al. 2002). PNO double Tg mice did not experience a lethal autoimmune disease as their lifespan was normal and outward signs of autoimmunity such as hair loss, skin lesions, and a hunched appearance were not observed. Although PNO/OT-I or PNO/OT-II mice did not exhibit autoimmune disease it is possible that organs containing OVA mRNA transcripts such as the lungs and kidneys contained small numbers of autoreactive T cell infiltrates resulting in low-level organ injury. It is also possible that low grade tissue injury was occurring in single Tg mice but went undetected due to the low number of autoreactive T cells enabling tissue repair mechanisms to keep pace with the level of target organ injury.

Interestingly an early CNS inflammatory disease has been observed in CNO/OT-I mice (Dr Cao personal communication) but not in CNO/OT-II mice (own observation). In particular CNO/OT-I mice displayed hind limb paralysis shortly after birth in addition to perivascular infiltrates of the brain suggesting CNO/OT-I mice to have either more efficient presentation of OVA or a greater TCR avidity. These results again illustrate the efficiency with which OVA is likely to have been presented by the MHC class I pathway. Class I Ag presentation can occur on a wide range of different cells in contrast to class II Ags which require successful transfer to MHC II positive cells prior to CD4+ T cell recognition.

Primarily the tolerant state in PNO/OT-I mice appeared to be mediated by the clonal deletion of OVA specific CD8+ T cells. While developing thymocytes are subjected to clonal deletion in the thymus based on TCR specificity, deletion is also seen to operate in the peripheral T cell compartment (Rocha and von Boehmer 1991). The average 84% reduction in CD8+SP thymocytes suggested intrathymic clonal deletion of high avidity OVA specific T cells, and may have accounted for the 60% decrease in peripheral clonotype+ CD8+ T cells in PNO/OT-I when compared with OT-I mice. This was observed by FACS analysis which showed a significant reduction in the $V\alpha2+^{hi}$ CD8+SP thymocyte population in PNO/OT-I mice. The clonal deletion of OVA specific CD8+ thymocytes might have either been due to their high TCR avidity and/or efficient MHCI/OVA presentation. It is generally known that maturing thymocytes with high affinity TCRs undergo apoptosis when encountering their specific target (Sprent and Kishimoto 1998). PNO/OT-I clonotype+ thymocytes with high avidity presumably underwent apoptosis after encountering low levels of OVA presented by thymic epithelial cells (Klein and Kyewski 2000). Other experiments in the RIP-OVA mouse have also shown that those thymocytes expressing the highest density of the autoAg specific Tg TCR will be deleted by aberrant thymic presentation of autoAg (Heath, Karamalis et al. 1995). Medullary epithelial cells have been found to express numerous peripheral tissue-specific Ags, including insulin or myelin proteolipid protein thereby preventing autoimmune diseases against these tissues. More importantly the recent report by Gallegos et al., points to medullary epithelial cells as inducers of tolerance by deletion of T cells with high TCR-self-peptide/ MHC avidity (Gallegos and Bevan 2004).

Previous double Tg mouse models expressing a neo-Ag and T cells carrying TCRs specific for it, have shown a similar induction of peripheral tolerance. The RIP-K^b x Des-TCR Tg mice revealed that while intrathymic deletion of K^b specific CD8+ T cells did occur, those Kb-specific CD8+ T cells that escaped into the periphery were inefficient at rejecting Kb-bearing skin grafts supporting the idea that low avidity T cells remained in the periphery. Furthermore

they ignored Kb expressing islet cells even after priming. Only in the presence of local IL-2 were they able to cause autoimmune diabetes (Heath, Allison et al. 1992) showing that exogenous help maybe required for these autoreactive T cells with low avidity to return to a functional immunogenic state. Normally avidity is affected by the affinity of individual TCR molecules. However, in the Tg TCR model all T cells express a clonotypic TCR. Therefore, variations in TCR density would result in variations in avidity but not affinity and therefore one could assume that negative selection of thymocytes with Tg TCR would be analogous to deletion of thymocytes with high affinity in WT animals. Intrathymic deletion of CD8+ OVA specific TCR Tg thymocytes therefore indirectly provided evidence for minuscule amounts of OVA being expressed within the thymus.

Although double Tg mice in this study did not develop outward signs of an autoimmune disease tissue localised sites of inflammation can not be ruled out. FACS analysis of peripheral clonotype+ T cells showed foremost a significant reduction in their number. Further analysis revealed them to be activated as based on the upregulation of CD44, CD69 and downregulation of the L-selectin lymph node homing receptor. This suggested that PNO/OT-I clonotype+ T cells were being primed in the periphery, which would correlate with their ability to proliferate equally well as single Tg cells in response to low OVA concentrations in vitro. Further FACS analysis indicated only a slight reduction in the $V\alpha 2 + V\beta 5 +^{hi}$ CD8+ T cell population suggesting that most cells with a high avidity TCR bypassed negative selection. The question remained as to how PNO/OT-I mice maintained peripheral T cell tolerance.

Once self-reactive OT-I CD8+ T cells escaped into the periphery there are several mechanisms in which peripheral T cell unresponsiveness may have been maintained. Other double Tg mice displaying tolerance have revealed that low level self-Ag expression in the thymus is sufficient to induce tolerance via a non-deletional mechanism (Husbands, Schonrich et al. 1992). These include non-deletional means of modulating the level of surface molecules such as the TCR or coreceptor in addition to secondary TCR gene rearrangement occurring during allelic inclusion and receptor editing.

Previous double Tg mice, containing T cells carrying a TCR specific for an expressed neoself Ag, were found to downregulate either their TCR or CD8 coreceptor upon *in vivo* Ag encounter (Schonrich, Kalinke et al. 1991) (Rocha and von Boehmer 1991) (Husbands, Schonrich et al. 1992). Schonrich et al., showed that mice carrying anti H-2K^b TCR Tg T cells were tolerant to the H-2K^b Ag expressed in cells of neuroectodermal origin (Schonrich, Kalinke et al. 1991). CD8+CD4- thymocytes bearing H-2K^b reactive receptors were not deleted intrathymically; instead downregulation of the TCR and CD8 on peripheral T cells mediated tolerance. T lymphocytes with a decreased expression of TCR or CD8 cannot respond properly to Ag and therefore appear anergic. However, this peripheral tolerance mechanism does not appear to have been used by PNO/OT-I CD8+ T cells, as FACS analysis of the TCR and CD8 coreceptor did not show any downregulation when compared with NTg littermates. Moreover the *in vitro* proliferative response to cognate Ag did not depict induction of anergy.

In double Tg mice the generation of T cells with dual receptor reactivity has been shown to rescue self-reactive thymocytes from negative selection by reducing their avidity and therefore enabling their exit into the periphery. The dualling α-chain model proposes this to be a result of allelic exclusion of the TCRβ chain not being fail proof and less stringent for the TCRα chain resulting in the expression of two TCR on a single T cell. FACS analysis of thymocytes revealed the level of the Vβ5 chain to remain unaltered when compared with single Tg counterparts. In contrast a reduction in the Vα2 chain expression not only on clonotype+ thymocytes but also on LNC and splenocytes pointed towards the usage of an endogenous α chain. It is interesting to note that PNO/OT-I mice showed a slight increase in the CD4+ LNC population relative to OT-I mice suggesting that endogenous α chains had contributed to the formation of class II restricted TCR. Previous studies have shown that T

cells carrying two functional TCRs have a reduced avidity for their cognate Ag (Heath, Carbone et al. 1995). Dual TCR reactivity mediated by receptor editing may have facilitated the escape of PNO/OT-I thymocytes into the periphery and thereby ensured their survival by reducing their avidity. The slight increase in the Vα2-CD8+ thymocyte population in PNO/OT-I compared with single Tg mice does suggest that clonotype+ thymocytes expressed an endogenous TCRα chain. The minor increase in the Vα2-CD8+ T cell population is mirrored in the peripheral clonotype+ CD8+ T cell population. Crossing PNO/OT-I to RAG-/- mice could further test for the potential role of dual reactive T cells in contributing to peripheral tolerance. In the absence of the RAG protein, rearrangement of endogenous TCR chains cannot occur, thereby eliminating dual reactive T cells and supporting the generation of autoreactive T cells with higher avidity for the target Ag.

Double Tg CD8+ T cells could be sufficiently restimulated in vitro, in the absence of additional stimuli including IL-2 but appeared to be encumbered at becoming efficient autoreactive effectors in vivo. Although PNO/OT-I LNC do not appear to exhibit T cell anergy, accounted for by a similar proliferative response to cognate Ag as that of OT-I LNC, addition of saturating amounts of IL-2 to PNO/OT-I LNC resulted in a greater proliferative response than that seen for OT-I. Whether the presence of high concentrations of endogenous cytokines such as IL-2 would reverse an inability to become an autoreactive effector T cell remains to be determined.

The importance of CD4+ T cell help has been demonstrated in providing optimal clonal expansion of CD8+ T cells in vivo. For instance Heath et al., Janssen et al., and Sun et al., demonstrated that CD8+ T cells with low avidity were dependent on CD4+ helper T cells to cause disease (Heath, Kjer-Nielsen et al. 1993) (Janssen, Lemmens et al. 2003) (Sun and Bevan 2003). Therefore in the absence of sufficient clonotype+ CD4+ T cells in PNO/OT-I mice the autoaggressive activity of CD8+ T cells may have been impeded.

In summary PNO/OT-I mice present a model which is consistent with the idea that avidity mediated selection for self-reactivity in the thymus leads to central and peripheral in vivo tolerance induction. Although peripheral clontotype+ CD8+ T cells appear to be activated in the periphery an autoimmune disease is bypassed. Whether the reduction in clonotype+ T cells or their low TCR avidity contributed to the peripheral tolerance remains to be answered. Before the tolerance mechanisms acting on CD8+ T cells can be investigated in more detail the extent of tolerance affecting their effector function must be further demonstrated. Further CD8+ functional analysis would predict these clonotype+ T cells to reject skin grafts at an early stage and to efficiently generate an anti-OVA CTL response. In future experiments the in vitro OVA specific lytic activity could be measured by assessing the ability to lyse the H-2b EL-4 cell line and its (E.G7) OVA transfectant. Additionally it is possible that a viral infection might reverse the in vivo tolerogenic state whereby self-reactive CD8+ T cells can be induced to become autoaggressive by cross-reactive pathogen associated Ags.

Previous studies have demonstrated that anergic T cells exhibit reduced phosphorylation of signalling molecules such as Erk or JNK (Li, Whaley et al. 1996) (Tham and Mescher 2001). Furthermore a reduction in phosphorylation of these molecules could be correlated with a reduced susceptibility to deletion (Yadav, Judkowski et al. 2004). In PNO/OT-I mice an increase in the expression of phospho-Erk might correlate with an increase in clonal deletion. Therefore further analysis of the inter/ intramolecular signalling transduction pathways operating in CD8+ double Tg T cells might provide a platform on which to develop therapeutic drugs with the aim of effectively deleting potentially autoreactive T cells. The PNO/OT-I mouse might provide a greater insight into how tolerance is induced in vivo when mediated by clonal deletion and ignorance rather than by anergy.

4.4.2 Tolerance induction in PNO/OT-II mice by the generation of unresponsive CD4+ T cells

Similar to PNO/OT-I mice, PNO/OT-II displayed signs of functional tolerance in vivo. In contrast to the PNO/OT-I mice, the clonotype+ T cell population in PNO/OT-II mice was only slightly depleted in the thymus and in the periphery. Moreover PNO/OT-II peripheral T cells did not display an activated phenotype. The tolerant state did not appear to be characterised by downregulation of the TCR or coreceptor molecule either in the thymus or in the periphery. Similar to the PNO/OT-I mice, PNO/OT-II mice did not show any outwardly signs of an autoimmune disease although local inflammatory infiltrates could not be ruled out. Furthermore in vitro proliferative response assays determined PNO/OT-II LNCs at 5 months to be less responsive to cognate Ag than at 3 months which could most likely be accounted for by the continued exposure to self-Ag.

At lower concentrations of whole OVA, PNO/OT-II LNC and splenocytes showed a diminished response when compared with OT-II controls. The reduced proliferative response could not be clarified by FACS analysis of LNC. Indeed four colour stainings determined $V\alpha2+V\beta5+^{hi}$ CD4 T cells to be equal in both PNO/OT-II and control mice. Therefore a diminished proliferative response could not be accounted for by a reduction in the number of T cells with high avidity TCRs.

The exact site and OVA expression level in the periphery may have induced PNO/OT-II CD4+ T cells to become anergic. Clonal anergy is defined as a state of long-lasting, partial or total unresponsiveness induced by partial activation and characterised by the inability of the T cell to proliferate in response to cognate Ag or produce the autocrine growth factor IL-2. As the overall proliferative response of PNO/OT-II CD4+ T cells was diminished the potential role of anergy was further examined by the addition of exogenous IL-2 to OVA stimulated cultures. Stimulation of anergic cells with exogenous IL-2 has previously been shown to sometimes reverse the anergic state (Beverly, Kang et al. 1992) as also observed in the moth cytochrome c specific double Tg model (Girgis, Davis et al. 1999). In particular it can abrogate the suppressive function of CD4+CD25+ T cells (Thornton and Shevach 1998) (Takahashi, Kuniyasu et al. 1998). Other reports have also demonstrated the proliferative response to be slightly improved upon the addition of exogenous IL-2 (Mueller and Jenkins 1995). However, our results are similar with those from the RIP-OVAxDO.11.10 model in which the hyporesponsiveness of the CD4+ clonotype+ cells could not substantially be reversed by exogenous IL-2 (Kohyama, Sugahara et al. 2004). This is similar to other reports that have shown in vivo rendered anergy not to be reversed by the addition of IL-2 (Lanoue, Bona et al. 1997). These published findings suggest that depending on the signals received to induce anergy as well as the starting state of the responsive T cell population, different levels of anergy exist. Furthermore it is likely that in vivo induced anergy is different to that induced in vitro. Our limited results suggest that the reduced responsiveness of PNO/OT-II CD4+ T cells to OVA may be partially attributable to the induction of anergy.

It is worth speculating that our PNO/OT-II CD4+ in vivo Ag stimulated so called anergic T cells may have assumed the regulatory T cell phenotype. Recent Tg mouse models have demonstrated the generation of CD4+CD25+ regulatory T cells in response to intrathymic self-Ag expression (Kawahata, Misaki et al. 2002) (Hori, Haury et al. 2002) (Jordan, Boesteanu et al. 2001) (Apostolou, Sarukhan et al. 2002). In particular Jordan et al., were able to demonstrate that hemagglutinin (HA)-reactive T cells were positively selected as CD4+CD25+ regulatory T cells and not deleted in double Tg mice expressing the influenza HA protein (Jordan, Boesteanu et al. 2001). In another model, CD4+ HA specific T cells removed from TCR-HAxIgHA double Tg mice were anergic in terms of proliferation when restimulated in vitro and unable to cause diabetes when transferred into INS-HA Tg mice. Further analysis revealed the in vitro release of IL-10 at the mRNA and protein level (Buer, Lanoue et al. 1998). DOxOVA^{high} CD4+ T cells were also found to exhibit regulatory T cell

properties based on their IL-10 dependent in vivo and in vitro mediated suppressive activities.

Pilot studies in which in vitro co-culture assays were conducted with varying ratios of double Tg LNC to single TCR Tg LNC (data not shown) were unsuccessful at determining a suppressive ability of either OT-I or OT-II double Tg LNC. Generation of a regulatory T cell repertoire is dependent on the level of self-Ag expression as demonstrated in the TS1-HA model (Lerman, Larkin et al. 2004). Therefore analysis of the remaining PNO mouse lines may reveal that differing levels of intrathymically expressed OVA would not generate similar T cell phenotypes as seen using the PNO3 mouse line and either lead to more efficient deletion or selection of Tg regulatory T cells.

In the analysis of the PNO double Tg CD4+ T cells another question arrises as to whether the induction of clonal anergy in vivo resulted in the complete functional inactivation of the T cells. Furthermore whether the autoreactive CD4+ T cells that bypassed negative selection were altered in their ability to differentiate into Th1 versus Th2 cells remains to be answered. These questions are important as the type of effector cells they differentiate into following activation influences the capacity for autoreactive CD4+ T cells to mediate autoimmune pathology. Numerous factors have been identified in influencing Th cell differentiation including the genetic background, the dose of the stimulating Ag, duration of TCR stimulation, the avidity with which the TCR and/or co-receptors interact with the stimulating peptide, activation of specific co-stimulatory pathways and the degree of cell division. Cytokine production analysis in a study of the HAxTS1 system showed that CD4+ T cells that evaded negative selection to a self-peptide displayed an intrinsic ability to differentiate into Th_1 type effectors (Riley, Shih et al. 2001). It is possible that in the context of the distinctive level of OVA expressed in PNO3 mice in combination with the certain TCR avidity of OT-II T cells the in vivo processes that lead to differentiation of a particular CD4+ effector subtype could be further analysed.

Greater sensitivity to OVA by OT-I in contrast to OT-II cells might reflect a higher TCR avidity and/or more efficient Ag presentation. Although the OT-I TCR affinity for its ligand has been measured (Alam, Travers et al. 1996) no information exists on the OT-II avidity. When examining our previous peptide titration assays (data not shown) OT-I cells were more responsive than OT-II cells to peptide stimulation suggesting the TCR affinity for its ligand to be greater on OT-I cells. The differing Tg TCR avidity may have led to the different modes of tolerance induction when OT-I and OT-II cells encountered OVA presented in the PNO3 mouse thymus and periphery. PNO mice carry the OVA transgene, which ecodes for a cytoplasmic form of whole OVA. The biased deletion and activation of OT-I over OT-II T cells suggested easier access to OVA via the constitutive MHC class I restricted presentation pathway. This is also confirmed phenotypically by the T cell response in the CNO/OT-I mice mice (Dr Cao personal communication), which display early signs of a CNS autoimmune disease in contrast to CNO/OT-II (results section). In contrast cross-presentation by specialised APCs is necessary for recognition by CD4+ OT-II T cells. Moreover, if the stability of the peptide/MHC complexes were different between MHC class I and MHC class II, then this would also effect the availability of one of the peptide/MHC complexes and thereby influence access to a particular presentation pathway.

Foremost the PNO double Tg mice served to confirm intrathymic expression of minuscule amounts of OVA and assisted in explaining the resulting tolerant state observed in PNO mice. The studies presented here add to existing models of CD8+ and CD4+ self-tolerance, in which tolerance is induced in response to extremely low levels of intrathymic protein Ag by two different mechanisms. Selective clonal deletion of CD8+ T cells in the thymus and periphery accounted for the peripheral tolerance observed in PNO/OT-I mice. In the PNO/OT-II mouse anergy rather than clonal deletion appeared to play the major role in tolerance induction. The PNO mouse will ideally allow for further investigation into deletional and non-deletional tolerance mechanisms acting separately on the CD8+ and CD4+ T cell

subsets in vivo. Further investigation into the mechanisms of T cell tolerance induction may be beneficial in elucidation of those factors that are important in restoring self-tolerance to tissue specific Ag in patients with autoimmunity. The adoptive T cell transfer system could be further developed to transfer either CD4+ or CD8+ PNO double Tg T cells into CNO mice to explore the mechanisms by which the tolerised subsets might regulate the different EAE phases. Finally the PNO double Tg mice will be invaluable for a more detailed examination of the phenotypic and molecular pathways involved in T cell tolerance induction.

5. SUMMARY

In this project two novel murine autoimmune models were to be established in an attempt to further investigate the nervous system disorders of Multiple Sclerosis and Guillain Barré Syndrome. Previous experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune neuritis (EAN) models have demonstrated that T cells play a major role in these diseases. Which roles CD4 and CD8 T cells specifically have in the initiation, propagation and termination of an autoimmune nervous system disorder remains controversial. To this end two transgenic mice specifically expressing the neo-antigen (Ag) ovalbumin (OVA) in either the central nervous system (CNS) or peripheral nervous system (PNS) were to be generated. The myelin basic protein (MBP) is a major component of the myelin sheath both within the CNS and the PNS. Therefore the MBP promoter was employed for its distinct regulatory elements to facilitate exclusive CNS or PNS OVA expression. The adoptive transfer of OVA specific MHCI restricted (OT-I) and MHCII restricted (OT-II) TCR Tg T cells extended the OVA Tg mouse model by allowing potentially encephalitogenic T cells to be tracked *in vivo*. Specificity for the target Ag should enable the dynamic role of antigen specific T cells in neuroinflammatory diseases to be revealed in more detail.

1. The main goal of this project was the establishment of two OVA Tg mouse lines. In the first line the Schwann Cell Enhancer (SCE1) was employed to target OVA protein to Schwann cells of the PNS. In the second line the –6.5kb MBP promoter targeted OVA protein exclusively to oligodendrocytes in the CNS. Cloning of both constructs was successfully completed. After microinjection of fully characterised vectors into C57BL/6 oocytes, 3 peripheral nerve OVA (PNO) and 2 central nerve OVA (CNO) Tg mice were obtained all of which transmitted the transgene through the germline. Southern blot analysis of PNO Tg genomic DNA implied integration of different copy numbers of the OVA transgene. However, expression of OVA in sciatic nerve could not be detected at the mRNA or protein level. RT-PCR analysis determined abberant expression of OVA within lungs, kidney and thymus. In contrast CNO mice expressed OVA specifically in oligodendrocytes (manuscript in preparation).

2. In order to investigate a PNS autoimmune disease its inducibility in PNO mice needed to be established. Active disease was attempted by sc injection of OVA emulsified in CFA and PT in PNO mice. OVA immunised mice did not display signs of an autoimmune disease based on weight analysis and a graded clinical score. Further histological analysis of the PNS and CNS organs did not indicate a subclinical form of disease. The *in vitro* proliferative recall response of lymph node cells and splenocytes was significantly reduced in comparison to non-transgenic mice. Furthermore IgM and IgG levels were reduced approximately 2 fold. These results suggest a diminished primary immune response in PNO mice due to tolerance mechanisms acting upon the T helper cell compartment.

3. Transfer of Th1/Tc1 polarised OT-I and/or OT-II effector T cells into immuno-competent and -compromised PNO mice did not induce detectable disease. Similarly transfer of increased numbers of primed OT-I and OT-II lymphnode cells into irradiated CNO recipient mice did not result in a CNS autoimmune disease based on the graded clinical score and outward physical signs. However, individual mice did display slight weight reductions in contrast to their non-Tg counterparts. Retrieved clonotype+ CD4 T cell numbers remained unchanged when comparing CNO and WT recipients. In contrast clonotype+ CD8 T cell numbers recovered from CNO LN and spleen at days 7-12 post transfer were decreased almost two-fold and displayed signs of activation. Histological analysis of the CNS tissues failed to detect significant numbers of infiltrating CD3+ T cells. This implied that the transferred CD8+ in contrast to CD4+ T cells had easier access to the neo-self Ag but alone could not induce a CNS autoimmune disease.

4. By backcrossing PNO mice to OT-I or OT-II mice the resulting double Tg mice provided a means of investigating the cellular mechanisms of central and peripheral tolerance operating in PNO mice. Moreover, double Tg mice served to confirm intrathymic expression of minuscule amounts of OVA and thereby assisted in clarifying the resulting tolerant state observed. Both PNO/OT-I and PNO/OT-II mice did not develop spontaneous autoimmune disease based on outward signs. Over half of the clonotype+ CD8+ cells in PNO/OT-I mice were deleted intrathymically. Those that escaped into the periphery showed signs of activation and were more reactive to further stimulation with cognate Ag ex vivo. In contrast clonotype+ CD4+ T cells in PNO/OT-II mice did not show signs of intrathymic deletion. Furthermore those clonotype+ cells that escaped into the periphery did not exhibit an activated phenotype and were less responsive to cognate Ag stimulation when compared with their single Tg counterparts. Exogenous IL-2 improved the proliferative response of PNO/OT-I but not PNO/OT-II LNC cells. The results illustrated that extremely low levels of self-Ag in the thymus can lead to tolerance induction via deletional or non-deletional mechanisms.

The PNO mouse model will be important in the further elucidation of specific tolerance mechanisms acting upon the CD4+ or CD8+ T cell compartment when the autoAg is expressed both in the thymus and in peripheral organs. Elucidation of certain cellular and molecular pathways that lead to tolerance induction could be used to block mechanisms of autoimmune disease such as those seen in EAE and EAN.

In contrast the establishment of the novel EAE disease in the CNO mice will enable tracking of the location and nature of the cellular and molecular initiation, propagation and termination of a CNS autoimmune disease. With the use of characterised reagents in the OVA system including peptide agonists and antagonists as well as monoclonal antibodies and tetramers, this mouse model may provide a platform on which to develop therapies that will prevent or modulate a CNS autoimmune response at the level of Ag recognition.

6. ZUSAMMENFASSUNG

Im Rahmen der vorliegenden Arbeit wurden zwei neue Mausmodelle für Autoimmunerkrankungen etabliert, um weitere Fortschritte bei der Aufklärung der zellulären und molekularen Interaktionen bei den Erkrankungen des Nervensystems Multiple Sklerose und Guillain Barré Syndrom zu erzielen. In früheren Experimenten mit EAE (experimentelle autoimmune Enzephalomyelitis) und EAN (experimentelle autoimmune Neuritis) konnte bereits gezeigt werden, dass T-Zellen eine Hauptrolle bei diesen Erkrankungen spielen, wobei jedoch die Bedeutung von CD4 bzw. CD8 T-Zellen im Einzelnen noch nicht aufgeklärt ist. Zu diesem Zwecke sollten zwei transgene (Tg) Mauslinien generiert werden, die speziell entweder im peripheren (PNS) oder im zentralen (ZNS) Nervensystem das Zielantigen OVA exprimieren. MBP ist eine Hauptkomponente der Myelinscheide sowohl im ZNS als auch im PNS. Daher kam der Myelin Basic Protein (MBP) Promoter zum Einsatz, dessen unterschiedliche regulatorischen Elemente eine Expression von intaktem OVA ausschließlich im ZNS bzw. ausschließlich im PNS steuern können. Eine Erweiterung dieser OVA tg Mausmodelle stellte der adoptive Transfer von OVA spezifischen MHCI-restringierten OTI und MHCII-restringierten OTII T-Zellen dar, da es so möglich wurde, potentiell enzephalitogene T-Zellen *in vivo* zu verfolgen. Dadurch sollte ebenfalls eine detailliertere Darstellung der dynamischen Rolle von antigenspezifischen T-Zellen bei neuroinflammatorischen Erkrankungen ermöglicht werden.

1. Das Hauptziel dieser Arbeit bestand in der Etablierung von zwei OVA Tg Mauslinien. Zum einen kam der Schwann Cell Enhancer (SCE1) zu Einsatz, um eine Expression von OVA-Protein in Schwannzellen des PNS zu erreichen. Die zweite Mauslinie erhielt den –6.5'kb MBP Promotor, mit der eine Expression des OVA-Proteins ausschließlich in den Oligodendrozyten des ZNS erzielt werden sollte. Die Klonierung beider Vektoren wurde erfolgreich abgeschlossen. Nach einer Mikroinjektion von charakterisierten Vektoren in C57BL/6 Oozyten wurden drei periphere Nerven-OVA- (PNO) und zwei zentrale Nerven-OVA (CNO)–transgene Mäuse identifiziert, die alle das Transgen durch die Keimbahn weitergaben. Southern Blot Analysen genomischer DNA aus PNO Mäusen zeigten die Integration einer verschiedenen Anzahl von Kopien des OVA-Transgens. RT-PCR-Analysen ergaben eine Expression von OVA innerhalb der Lungen, der Nieren und des Thymus. Eine Expression von OVA im Ischiasnerv des PNS wurde jedoch anhand von Protein- bzw. mRNA-Bestimmungen nicht beobachtet. Im Gegensatz dazu exprimieren CNO-Mäuse ein Oligodendrozyten-spezifisches OVA (Manuskript in Arbeit).

2. Für die Untersuchung des neuen PNS-Autoimmunmodells musste zuerst die Induzierbarkeit der Autoimmunkrankheit in PNO-Mäusen geprüft werden. Zu diesem Zwecke wurden PNO Mäusen eine OVA:CFA-Emulsion und Pertussis Toxin (PT) subkutan injiziert. Beobachtet wurde in diesem Falle eine verminderte primäre Immunantwort im Vergleich mit nicht transgenen Tieren. PNO Mäuse, welche mit OVA immunisiert wurden, wiesen keine Anzeichen einer autoimmunen Erkrankung auf, bestimmt anhand von Gewichtsmessungen und einem klinischen Parameter für Autoimmunerkrankungen des PNS. Des weiteren ergaben auch histologische Analysen des peripheren bzw. zentralen NS keine Hinweise auf eine subklinische Form der Erkrankung. Die proliferative Recall-Antwort von Lymphknoten- und Milzzellen auf das OVA Peptid waren deutlich reduziert im Vergleich mit den Werten von nicht transgenen Mäusen. Weiterhin war der IgG- sowie auch das IgM-Spiegel ca. um die Hälfte erniedrigt im Vergleich mit Wildtyptieren, ein Hinweis darauf, dass hier Toleranzmechanismen auf das T-Zell-Kompartiment wirken.

3. Der Transfer von Th1/Th2 polarisierten OTI und/oder OTII Effektor-T-Zellen in immunkompetente und immunsupprimierte PNO-Mäuse führte ebenfalls zu keiner Erkrankung. Erhielten bestrahlte CNO-Mäuse eine erhöhte Anzahl von OTI- und OTII-Lymphknotenzellen, so führte dies zwar nicht zu einer Autoimmunerkrankung im ZNS,

gemessen anhand eines klinischen Parameters sowie der Beobachtung äußerer physischer Anzeichen einer Erkrankung. Vereinzelte Tiere zeigten jedoch einen geringen Gewichtsverlust im Vergleich zu den Wildtyptieren. Während es keine Unterschiede zwischen CNO- und Wildtyp-Empfängern im Bezug auf die Anzahl der wiedergewonnenen klonotyp-positiven CD4 T-Zellen gab, wurde eine um die Hälfte reduzierte Zellzahl der klontyp-positiven CD8 T-Zellen an den Tagen 7 bis 12 nach Transfer in den Lymphknoten und der Milz von CNO-Mäusen beobachtet. Weiterführende histologische Untersuchungen von Gewebe aus dem ZNS zeigten jedoch keine erhöhte Anzahl infiltrierender CD8 T-Zellen. Dies wies darauf hin, dass die übertragenen CD8 T-Zellen im Gegensatz zu CD4 T-Zellen zwar einen leichteren Zugang zum künstlichen Auto-Antigen hatten, aber allein nicht im Stande waren, eine inflammatorische Erkrankung des ZNS zu induzieren.

4. Um die Toleranzmechanismen im zentralen und peripheren Nervensystem näher untersuchen zu können, wurden PNO-Mäuse mit OTI- oder OTII-Mäusen gekreuzt. Die hieraus entstandenen doppelt transgenen Tiere konnten zum einen eine OVA-Expression bestätigen zum anderen den Toleranzstatus in PNO-Mäusen erklären. Sowohl bei den PNO-OTI- wie auch bei den PNO-OTII-Mäusen wurde eine spontane Autoimmunerkrankung auf Grund fehlender äußerlicher Symptome ausgeschlossen. In PNO-OTI-Mäusen kam es zu einer Deletion von mehr als der Hälfte aller klonotypischen CD8 T-Zellen. Die in die Peripherie gelangten Zellen wiesen Anzeichen einer Aktivierung (CD44hi, CD25+, CD69+, CD69Llo) auf und waren in einer *in vitro* Stimulation mit kognatem Antigen leicht hyperaktiv. Im Gegensatz hierzu war die Deletion von klonotyp-positiven CD4 T Zellen in PNO-OTII-Mäusen nicht so stark ausgeprägt. Außerdem zeigten klonotyp-positiven T-Zellen in der Peripherie keine Anzeichen eines aktivierten Zustandes und konnten auch weniger leicht stimuliert werden als T-Zellen aus einfach-transgenen OT-II-Mäusen. Anhand dieser Ergebnisse ist zu erkennen, dass sehr geringe Mengen an Selbst-Antigen im Thymus ausreichen, um über Deletions- bzw. Nicht-Deletionsmechanismen Toleranz zu induzieren.

Das vorgestellte PNO-Maus-Modell könnte in der Zukunft eine wichtige Rolle spielen bei der Erkennung von spezifischen Toleranzmechanismen, die sich auf CD4 bzw. CD8 T-Zellen auswirken, wenn das Ziel-Antigen im Thymus und in den sekundären Organen (Lunge und Niere) exprimiert wird. Die Aufklärung von bestimmten zellulären und molekularen Wegen, die zu Toleranz führen, könnte zur Inhibierung von Autoimmunerkrankungen wie EAE und EAN genutzt werden.

Im Gegensatz dazu könnte die erfolgreiche Etablierung der EAE-Erkrankung in CNO-Mäusen dazu dienen, die Initiierung, Propagierung und Termination einer Autoimmunerkrankung des ZNS zu verfolgen. Mit der Bereitstellung von charakterisierten OVA-Reagenzien, wie zum Beispiel Peptid- Agonisten und Antagonisten, monoklonalen Antikörpern und Tetrameren, könnte dieses Mausmodell eine Plattform für die Entwicklung neuer Therapien darstellen, die eine Autoimmunerkrankung des ZNS auf der Basis von Antigenerkennung modulieren oder sogar verhindern könnte.

7. LITERATURE

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7.2 Publications

7.2.1 Publications from this PhD

Induction of experimental autoimmune encephalomyelitis in transgenic mice expressing ovalbumin in oligodendrocytes, *(manuscript submitted)*
Cao, Y., * **Toben CG.,** * Na, S-Y., Stark, K., Nitschke, L., Peterson, A., Gold, R., Schimpl A., Hünig, T.

*: both authors contributed equally to this work

Toben CG., Cao Y., Stark, K., Nitschke, L., Gold, R., Schimpl A., Hünig, T. Development of two novel murine EAN and EAE models *Immunobiology* 208 (2003) 1-3 *(Meeting abstract)*

7.2.2 Presentation on Congress

Poster presentation at the „34th" Annual meeting of the German society of immunology 2003 in Berlin, Germany. Development of two novel murine EAN and EAE models.

8. APPENDIX

8.1 Abbreviations

Abbreviation	Meaning
Ab	antibody
Ag	antigen
AICD	activation induced cell death
APC	antigen presenting cell
BCR	B cell receptor
bio	biotin
bp	base pairs
BSA	bovine serum albumin
CD	cluster of differentiation
CFA	complete Freund's adjuvant
Ci	Curie
CIP	calf intestinal phosphatase
CNO Tg	Central nerve OVA Tg (mouse)
CNS	central nervous system
Cy	cychrome
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfide
dNTP	deoxyribonucleotide triphosphate
EAE	experimental autoimmune encephalomyelitis
EAN	experimental autoimmune neuritis
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	feotal calf serum
FITC	fluoresceinisothiocyanate
GBS	Guillain Barre syndrome
h	hour/s
H&E	hematoxylin and eosin
HRP	horse raddish peroxidase
Ig	immunglobulin
IL	interleukin
ip	intraperitoneal
iv	intraveneous
KF	Klenow-Fragment
LPS	lipopolysaccharide
MBP	myelin basic protein
MHC	major histococompatibility complex
MFI	mean fluorescence intensity
mg	miligram
mL	milliliter

MS	multiple sclerosis
NTg	non transgenic
OT-I	OVA specific class I restricted TCR Tg (mouse)
OT-II	OVA specific class II restricted TCR Tg (mouse)
OVA	ovalbumin
pA	poly A tail
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PNO Tg	Peripheral nerve OVA Tg (mouse)
PNS	peripheral nervous system
PT	pertusis toxin
rpm	revolutions per minute
RPMI	Roswell-Park Memorial Institute (Medium)
RT	room temperature
TCR	T cell receptor
sc	subcutaneous
SCE1	Schwann cell enhancer I
SDS	sodium dodecyl sufate
TBE	tris borate EDTA buffer
TCR	T cell receptor
TE	tris EDTA buffer
Th1	T helper 1 (cell)
Th2	T helper 2 (cell)
Tg	transgenic
Tris	tris-hydroxymethylaminomethane
TSR	template suppressive reagent
U	units
ug	microgram
uL	microlitre
v/v	volume/ volume
w/v	weight/ volume
WT	wild type

9. CURRICULUM VITAE

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