Roles of the B7-CD28 superfamily in the regulation of Experimental Autoimmune Encephalomyelitis

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To Byron H. Waksman,

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Attributions

Raymond A. Sobel performed the staining of the brains and spinal cords of mice and evaluation of lesion numbers presented in tables 3.1, 3.2, 3.3, 4.1, 4.2, 4.3, and 5.1.

Bernhard Greve performed the genomic DNA extraction, PCRs and statistical analysis for the genetic linkage presented in tables 3.4 and 3.5.

Diane van der Woude performed the mRNA analysis in RPA's presented in Figure 5.6 and 5.7.

Abbreviations

%	Percent
Ab	antibody
Ag	antigen
APC	Antigen Presenting Cell
B6	C57Bl/6
B7h	B7 homologue
BBB	Blood Brain Barrier
BC1	Backcross 1
CFA	Complete Freund's Adjuvant
CNS	Central Nervous System
Cpm	counts per minute
CTLA-4	Cytotoxic T Lymphocyte Associated protein-4
DNA	Desoxyribonucleic acid
EAE	Experimental Autoimmune Encephalomyelitis
e.g.	for example
ELISA	Enzyme-linked immunosorbent assay
F1	(B6xSJL)F1
Fig	Figure
g	gram
h	hour
ICOS	Inducible Costimulatory molecule
IDDM	Insulin Dependent Diabetes Mellitus
Ig	Immunoglobulin
IL	Interleukin
IFNγ	Interferon-gamma
i.p.	intra-peritonatally
i.v.	intra-venously
l	liter
LN	Lymph node
LNC	Lymph node cell
m	milli (10^{-3})

μ	Micro
mAb	monoclonal Antibody
max.	maximum
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
M.T.	Mycobacterium Tuberculosis
NASE	Neuraminidase
NOD	Nonobese Diabetic
OVA	Ovalbumin
p	pico
PBS	Phosphate Buffered Saline
PLP	Proteolipid Protein
P.T.	Pertussis Toxin
QTL	Quantitative Trait Loci
RNA	Ribonucleic acid
RPA	RNAse Protection assay
s/c	subcutaneously
TCR	T cell Receptor
Th	T helper
TNFα	Tumor Necrosis Factor-alpha
vs.	versus
Wt	wild-type

Abstract

Roles of the B7-CD28 superfamily in the regulation of Experimental Autoimmune Encephalomyelitis

The immune system must be activated to fulfill its role in destroying invading pathogens. For T cell activation to occur, T cells require two signals. The first signal comes from antigen recognition of pathogens by T cell receptors. The second signal is provided by a group of receptors, called costimulators, which positively and negatively regulate activation of the responding T cells. Under certain circumstances, T cells can become directed against self, and this inappropriate activation leads to autoimmune diseases. This thesis focuses on the role of members of the B7-CD28 superfamily of costimulatory molecules (B7.1, B7.2 and ICOS) in regulating the initiation and progression of an autoimmune disease called Experimental Autoimmune Encephalomyelitis (EAE).

To study the role of the B7 costimulatory pathway, we utilized B7.1 and B7.2 deficient ($B7.1^{-/-}$, $B7.2^{-/-}$ and $B7.1/B7.2^{-/-}$) mice that have been crossed onto two different EAE-susceptible mouse strains, SJL and C57Bl/6 (B6). Whereas $B7.1/B7.2^{-/-}$ mice on the B6 background did not develop EAE, $B7.1/B7.2^{-/-}$ mice on the SJL background remained susceptible to EAE induction. We were able to show that this difference between the strains was not due to the different immunogens used for the induction of EAE and could not be attributed to changes in expansion of T helper cells or in their cytokine profile. By genetic linkage analyses, we found that the differences in susceptibility in the $B7.1/B7.2^{-/-}$ mice on the two backgrounds appears to be due to the genetic differences, in particular of the MHC haplotype. We also

demonstrated that B7 costimulation has a role in both the induction and effector phases of EAE in SJL mice. B7.1 and B7.2 seem to have distinct and overlapping roles in this model.

We have examined the role of a second B7-related costimulatory pathway, the B7h-ICOS pathway, by studying ICOS deficient mice and the effect of anti-ICOS antibody treatment in EAE. Blockade of the ICOS pathway exacerbates EAE and skews effector T cells toward a Th1 phenotype. Adoptive transfer studies suggest that the manipulation of the ICOS pathway at different stages of disease can either ameliorate or exacerbate EAE. Therefore, the timing of the ICOS blockade is critical.

These studies show that B7 and ICOS costimulation are important for the pathogenesis of EAE, and that individuals of different genetic backgrounds have different requirements for costimulation. These findings have important implications for the development of therapies for autoimmune diseases that target costimulatory pathways.

Zusammenfassung

Die Funktion der B7-CD28 Superfamilie in der Regulation der Autoimmunkrankheit Experimental Autoimmune Encephalomyelitis

Das Immunsystem muss aktiviert werden, um seine Rolle erfüllen zu können, eindringende Pathogene unschädlich zu machen. T Zellen benötigen zwei Signale zu Ihrer Aktivierung. Das erste Signal ist die Antigen-Erkennung des Pathogens durch den T-Zell-Rezeptor. Das zweite Signal wird durch kostimulatorische Moleküle vermittelt. Diese können, durch positive und negative Signale, die T-Zell-Aktivierung regulieren. Unter bestimmten Umständen kann die T-Zell-Aktivierung gegen körpereigene Antigene gerichtet sein, was zu der Entwicklung einer Autoimmunkrankheit führen kann. In der vorliegenden Doktorarbeit wurde der Einfluss von Mitgliedern der kostimulatorischen B7-CD28 Superfamilie (B7.1, B7.2 und ICOS) auf die Induktion und das Fortschreiten der Autoimmunkrankheit Experimental Autoimmune Encephalomyelitis (EAE) untersucht.

Die Rolle der kostimulatorischen Moleküle der B7 Famile kostimulatorischen Pathway in der Pathogenese von EAE wurde analysiert, indem B7.1$^{-/-}$, B7.2$^{-/-}$ und B7.1/B7.2$^{-/-}$ Mäuse auf die EAE-suszeptiblen genetischen Hintergründe SJL and C57Bl/6 (B6) gekreuzt wurden. Während B7.1/B7.2$^{-/-}$ Mäuse auf dem B6 genetischen Hintergrund gegen EAE resistent waren, konnten wir in B7.1/B7.2$^{-/-}$ Mäuse auf dem SJL genetischen Hintergrund EAE induzieren. Wir konnten weiterhin zeigen, dass dies weder auf die unterschiedenen Immunogene, durch die EAE in den beiden Maus Stämmen induziert worden war, noch auf Unterschiede in der T-Helfer-Zell-Expansion oder den Zytokin-Profilen zurückzuführen war. Durch eine Linkage-

Analyse konnten wir zeigen, dass der Unterschied zwischen EAE-Empfänglichkeit und EAE-Resistenz der SJL und B6 B7.1/B7.2$^{-/-}$ Mäuse auf den jeweiligen genetischen Hintergrund zurückzuführen war, im Besonderen auf den MHC Haplotyp. Weiterhin konnten wir zeigen, dass B7 Kostimulation in der Induktions- und Effektor- Phase von EAE im SJL Modell wichtig ist. B7.1 und B7.2 scheinen spezifische, aber auch übereinstimmende Funktionen zu haben.

Wir analysierten ausserdem die Rolle eines zweiten Mitglieds der B7-CD28 Superfamilie, ICOS, in der Pathogenese von EAE. Dazu wurde EAE in ICOS$^{-/-}$ Mäusen oder in Wildtyp Mäusen, die mit einem anti-ICOS monoklonalen Antikörper behandelt wurden, induziert. Diese Studien zeigten, dass die Blockade von ICOS EAE verschlimmert und die Entwicklung von Th1-Zellen begünstigt. Adoptive Transfer Experimente legen nahe, dass die Manipulation der Signalübertragung durch ICOS während unterschiedlicher Phasen von EAE diese Autoimmunkrankheit entweder verhindern oder verschlimmern kann.

Die Studien dieser Arbeit zeigen, dass B7 und ICOS Kostimulation wichtig für die Pathogenese von EAE ist. Weiterhin scheint die individuelle genetische Ausstattung die Notwendigkeit von Kostimulation für die Krankheitsentstehung zu beeinflussen. Diese Erkenntnisse haben wichtige Implikationen für die Entwicklung von Therapien für Autoimmunkrankheiten, die auf der Manipulation von kostimulatorischen Signalen basieren.

Chapter 1.

General Introduction

Immunity and autoimmunity

The immune system has evolved to protect the individual from diseases caused by extracellular pathogens such as parasites and bacteria, as well as from intracellular pathogens such as viruses. A normal immune response involves the two key branches of the immune system.

The first is innate immunity, which is found in both lower and higher life forms. This system is the first line of defense and prevents foreign invasion via physical barriers like skin and mucous membranes, and by other defense systems including the complement system (lysing microbes), phagocytic cells (engulfing and destroying microbes), and natural killer (NK) cells (lysing virus-infected cells). Cells of the innate immune system often use receptors that recognize molecules, which are only produced by pathogens such as Lipopolysaccharide (LPS).

The second essential branch of the immune system is adaptive immunity, which is antigen-specific and is found only in higher life forms. This system can be divided into the humoral immune response, which depends on B cells, and the cell mediated immune response, which requires T cells. These systems are not clearly separated though, since there are many interactions between B and T cells. When challenged with a foreign antigen, B cells can respond via antigen specific antibodies (membrane bound or secreted), whereas T cells, cytotoxic T lymphocytes (CTLs) and T helper (Th) cells, can detect antigen and act via antigen-specific T cell receptors (TCRs). Depending on the type of pathogen, either B or T cells or both are recruited to clear the pathogen from the system. B and T cell mediated immune responses are antigen-

specific and some of the cells that survive after a pathogen invasion provide memory for future infections. It is because of this memory that the adaptive immune system can respond more rapidly and effectively upon subsequent challenges with the same pathogen. Thus, the adaptive immune system provides immunologic memory.

Because antigen specific receptors expressed on both T and B cells are generated by random recombination, the immune system must eliminate self-reactive B and T cells to become self-tolerant. B cells mature and undergo self-tolerance in the bone marrow. T cells mature in the thymus and have to pass certain checkpoints before they are released into the peripheral immune compartment. In the thymus, T cells that have affinity for self-MHC molecules are selected in a process termed positive selection. However, T cells that have high affinity for the self-antigen-MHC complex are deleted in a process termed negative selection. This process, known as central tolerance, results in the loss of self-reactive cells. However, an autoreactive T cell can escape thymic deletion if 1) the self antigen is tissue-specific and thus not expressed in the thymus; 2) the antigen is ineffectively expressed or only expressed at low levels in the thymus; 3) the TCR is of low affinity for the autoantigen and does not mediate negative selection; or 4) the MHC molecule presenting the antigen is unstable and only expressed at low density on thymic APCs, which may then select a population of T cells with increased TCR affinity to self (Ridgway et al., 1999).

According to the avidity model of thymic selection (Ridgway et al., 1999), the strength (avidity) of a signal transmitted to pre-T cells is the summation of multiple inputs (Figure 1.1). This model assumes that the avidity necessary to attain positive and negative thymocyte selection thresholds (Avidity) is constant among mouse strains. According to this model, the expression of MHC and costimulatory molecules can affect the TCR affinity. For example, an unstable MHC molecule or the loss of

costimulatory signals might result in the selection of a T cell population with increased TCR affinity to attain the avidity threshold required in thymic positive selection (Ridgway et al., 1999). The selected high-affinity self-reactive T cells can then enter the periphery.

Figure1.1. The avidity model of thymic selection [adapted from (Ridgway et al., 1999)]

[MHC-peptide][TCR avidity] + (x) = Avidity (constant)

[MHC-peptide] is composed of MHC stability, MHC density, MHC-peptide affinity and Peptide length/sequence.

[TCR avidity] is composed of TCR sequence (affinity), and TCR density.

(x) is composed of costimulatory/accessory molecules, and cytokine/chemokine signals

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Activation and regulation of self-reactive T cells in the periphery

T cells with TCRs that recognize self can be found in every individual. The autoreactive T cells that escape thymic deletion are kept in check by the mechanisms of peripheral tolerance (reviewed in (Walker and Abbas, 2002)), which include 1) clonal ignorance, in which autoreactive T cells "ignore" self-antigens in the absence of appropriate activating signals; 2) clonal anergy, which renders autoreactive T cells functionally inactive; 3) peripheral deletion of autoreactive cells; and 4) active suppression mediated by regulatory CD4+ T cells.

Regulatory CD4+ T cells. The existence of regulatory T cells as a separate lineage of cells that mediate down-regulatory functions by a variety of effector mechanisms has been a matter of great controversy over the past 40 years (for a review see (Shevach,

2000)). Many people believe that suppression is merely the result of the activity of counter-regulatory cytokines secreted by Th1 versus Th2 cells. However, in the past few years, the concept of suppressor cells that play a critical role in regulation of autoimmune disease has been resurrected. Regulatory CD4+ T cells are thought to be generated in the thymus. The target antigens for the suppressor populations and their mechanisms of action are poorly defined. Currently, regulatory T cell populations are distinguished from effector cells by the expression of certain membrane markers, including CD4, CD25 and $CD45RB^{low}$. However, identification of regulatory cells by these markers are imperfect, because all of them are also expressed on other cell types. Thus, ongoing research is being done to precisely characterize the properties of suppressor T cells, including antigen-specificity, MHC restriction, frequency of reactive cells, mechanism of action, and cellular targets of suppression. It is likely that these cells will express the same or overlapping populations of costimulatory molecules compared with effector T cells.

Thus, the mechanisms of central and peripheral tolerance act to preserve tolerance to self-antigens by preventing the generation and activation of autoreactive cells. However, when there is a breakdown of self-tolerance, self-reactive T cells become activated, expanded and differentiated into pathogenic effector cells, leading to autoimmune disease.

Two-signal hypothesis

The two-signal hypothesis was a theory that Bretscher and Cohn first proposed in 1970 to explain the phenomenon of self-tolerance of the immune system (Bretscher and Cohn, 1970). The original hypothesis was proposed to explain how B cells that generate autoreactive B cell receptors might be eliminated, and suggested a

mechanism by which cells can discriminate between self and non-self (foreign). A B cell can either be activated or enter a state of non-responsiveness in which the cell becomes inert to subsequent stimulation ("paralyzed"), depending on the signal it receives. The 2-signal-hypothesis proposes that B cells are activated when they receive specific antigenic stimulation (signal 1) along with additional signals from other B cells recognizing different determinants on the same antigen (signal 2). This theory provided an important conceptual framework. Over the years, the details of this original B cell-based model have been significantly modified into the current 2-signal hypothesis for T cell activation.

In the 1970s, it became clear that antigen recognition by T cells is MHC restricted and requires the presence of bone-marrow derived antigen-presenting cells (APCs). In 1975, Lafferty et al. proposed that T cells also may need 2 signals to be activated: signal 1, provided by the antigen and signal 2, provided by APCs (Lafferty and Cunningham, 1975).

Jenkins and Schwartz first showed in 1987 that T cell clones could not be activated by chemically fixed APCs, presumably lacking the second signal (Jenkins and Schwartz, 1987). Antigen presentation by chemically fixed APCs induced long-term functional non-responsiveness rather than activation in murine Th1 clones (Jenkins and Schwartz, 1987). Non-responsiveness was antigen-specific, but could be rescued by the addition of exogenous IL-2. The phenomenon of T cell functional non-responsiveness was termed clonal anergy.

Further studies indicated that anergy is induced by specific TCR engagement in the absence of other signals provided by the APC (Jenkins et al., 1990; Quill and Schwartz, 1987). These observations formed the basis of the "modern" 2-signal hypothesis for T cell activation proposed by Jenkins and Schwartz. T cells require 2

signals for optimal activation: Signal 1, which provides specificity for T cell activation, and is mediated by the T cell receptor-MHC peptide interaction; and signal 2, which provides the amplification of the signal, and is provided by additional antigen-non-specific “costimulatory” signals from APCs. In addition, when T cells receive only the TCR-MHC/peptide signal (signal 1) in the absence of costimulation (signal 2), T cells *in vitro* become non-responsive (anergic) (Jenkins, 1992; Schwartz, 1990).

Anergy is an active process that requires protein synthesis and Calcium (Ca^{++})-dependent signal transduction. Anergic T cells are characterized by their inability to produce IL-2 (Mueller et al., 1989). Thus, the lack of IL-2 production was postulated to be directly responsible for the induction of anergy, and signal 2 was broadly defined as any stimuli that promotes cell division (Jenkins, 1992). Studies from Jim Allison's laboratory provided direct evidence that B7 molecules expressed on APCs could block development of anergy induced by fixed APCs. Various molecules are known to provide costimulatory signals, including members of the B7 family (as discussed below), and members of the Tumor necrosis factor (TNF)/TNF receptor (TNFR) family (including Ox40-Ox40 ligand and CD40-CD40 ligand). Costimulatory stimuli may also promote T cell activation by enhancing adhesion between T cells and APCs, or by providing additional biochemical signals that complement the signals delivered through the TCR.

B7 family of costimulatory molecules

The interactions between a number of ligands expressed on APCs and their receptors expressed on T cells appear capable of providing a costimulatory signal (Clark and Ledbetter, 1994) for T cell activation and expansion. However, the critical importance

of the B7-CD28 pathway is underscored by its unique capacity to induce IL-2 production, prevent the induction of anergy, and expand and differentiate T cells (Boussiotis et al., 1993; Boussiotis et al., 1993; Boussiotis et al., 1994).

Two different B7 molecules (B7.1 and B7.2) have been identified on APCs, which bind to two different receptors expressed on T cells, CD28 and CTLA-4. Signaling through the B7.1/B7.2:CD28/CTLA-4 costimulatory pathway is complex due to dual specificity of the two B7 family members, B7.1 (CD80) (Freedman et al., 1987; Freeman et al., 1991; Freeman et al., 1989; Freeman et al., 1991; Yokochi et al., 1982) and B7.2 (CD86) (Azuma et al., 1993; Freeman et al., 1993; Freeman et al., 1993) for the two CD28 family members, CD28 (June et al., 1994) and Cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152) (Linsley et al., 1991), providing the potential for redundancy in the interactions between B7 and CD28 family members. An additional level of complexity is provided by the opposing outcomes of the CD28 and CTLA-4 mediated signaling (Gribben et al., 1994; Walunas et al., 1994). Whereas CD28 provides a positive signal for T cell activation and expansion, CTLA-4 induces a negative signal into T cells. In addition to the B7-CD28/CTLA-4 pathway, in the last two years a number of molecules with homology to B7 or CD28 have been cloned and shown to either promote or inhibit T cell activation or differentiation (see Figure 1.2). In recent years, more members of the B7-CD28 superfamily have been identified (for a review see (Sharpe and Freeman, 2002)) that make up new pathways, including the ICOS-B7h pathway, the PD-1- PD-L (ligand)1/PD-L2 pathway and B7-H3 (pathway not yet determined).

B7.1/B7.2-CD28/CTLA-4 pathway

B7.1 and B7.2 molecules show only modest (25%) amino acid conservation, and are members of the immunoglobulin superfamily. Murine B7.1 (mB7.1) is a 50- to 60-kD glycoprotein consisting of immunoglobulin (Ig) V- and Ig C-like extracellular domains, a transmembrane region, and a short cytoplasmic tail (Freeman et al., 1991). Murine B7.2 (mB7.2) is a 60- to 100-kD glycoprotein and, like B7.1, consists of Ig V- and Ig C-like extracellular domains, a transmembrane region, and a short cytoplasmic tail (Freeman et al., 1993).

Originally, B7.1 was described as a B cell-restricted activation antigen. However, B7.1 and B7.2 expression are not restricted to B cells. B7.1 expression is induced by activation on most APC populations. B7.2 is expressed constitutively at low levels on most APC populations and is rapidly upregulated (Freeman et al., 1993); Hathcock, 1994 #1019]. The constitutive or early expression of B7.2 has led to the hypothesis that B7.2 may be more important in initiating an immune response, thereby playing a pivotal role in the decision between T cell activation and anergy (Freeman et al., 1993), whereas B7.1, expressed at later timepoints, may serve to amplify or regulate the immune response. Both B7.1 and B7.2 costimulate T cell proliferation and IL-2 production (Freeman et al., 1993). In addition to APCs, B7.1 and B7.2 expression can also be found on T cells, but the function for B7 signaling on T cells is still unclear.

B7.1 and B7.2 molecules bind to both CD28 and CTLA-4. B7.1 and B7.2 have similar low affinities for CD28 and high affinities for CTLA-4 (Freeman et al., 1993). However, they have distinct kinetics of binding to CD28 and CTLA-4, with B7.2 having faster dissociation kinetics, and utilizing different binding determinants (Linsley et al., 1994), suggesting a mechanism by which B7.1 and B7.2 could generate different signals.

To evaluate the functional roles of B7.1 and B7.2 *in vivo*, mouse strains deficient in B7.1 (Freeman et al., 1993), or B7.2 (Borriello et al., 1997) or B7.1 and B7.2 (Borriello et al., 1997) were generated by gene targeting of embryonic stem cells.

The characterization of the B7.1$^{-/-}$ mice suggested that the absence of the B7.1 gene results in only a partial defect in the immune response (Freeman et al., 1993). Naïve B7.1$^{-/-}$ mice have normal numbers of B and T cells, normal concentrations of serum immunoglobulins, and respond normally to mitogens. However, analysis of the B7.1$^{-/-}$ mouse revealed a 70% reduction in costimulation of the response to alloantigen. The lack of a more dramatic phenotype was later explained by the existence of the additional CD28/CTLA-4 counter-receptor B7.2 (Borriello et al., 1997).

B7.2$^{-/-}$ mice displayed a complete absence of isotype switching and germinal center formation when immunized with antigen intravenously, suggesting an important role for B7.2 in immunoglobulin class switching and germinal center formation (Borriello et al., 1997). However, when immunized by other routes or with a strong adjuvant, B7.2$^{-/-}$ mice gave antigen-specific IgG responses comparable with wild-type mice.

B7.1/B7.2$^{-/-}$ mice fail to generate an antigen-specific IgG1 or IgG2a response, even in the presence of the potent adjuvant Complete Freund's adjuvant (CFA) (Borriello et al., 1997). IgG1 responses are thought to be T helper type 2 (Th2) dependent; IgG2a responses are thought to be Th1 dependent. Therefore, B7 costimulation seems to influence both Th1 and Th2 responses *in vivo* (Borriello et al., 1997). Thus, functional analysis of B7 single and double deficient mice suggested that B7.1 and B7.2 have both overlapping and distinct functions in immunoglobulin class switching and germinal center formation (Borriello et al., 1997; Freeman et al., 1993).

CD28 and CTLA-4

CD28 and CTLA-4 both belong to the CD28 family. CD28 and CTLA-4 are type I transmembrane glycoproteins and Ig supergene family members with a single IgV-like domain. CD28 and CTLA-4 both have a (single amino acid sequence) MYPPPY motif within the IgV-like domain that is required for binding to B7.1 and B7.2 (Peach et al., 1994).

CD28

CD28 is constitutively expressed on the surface of T cells (Gross et al., 1992). CD28 is expressed on virtually all human CD4+ T cells and 50% of human CD8+ T cells, and all murine T cells. It is also expressed on some plasma cells and natural killer (NK) cells, but its function on these cell types remains uncertain. CD28 has a cysteine residue following the IgV-like domain and this is involved in homodimerization.

CD28 provides a positive signal for activation, expansion and differentiation of T cells. When CD28 interacts with B7 molecules, it transmits a signal that synergizes with the TCR signal to promote T cell activation (Lanzavecchia et al., 1999). Costimulation by CD28 decreases the number of TCR engagements needed for effective T cell activation, and thus regulates the threshold for T cell activation (Viola and Lanzavecchia, 1996). CD28 signaling optimizes the response of previously activated T cells and promotes IL-2 production and T cell survival of naïve T cells in particular. T cell survival enables cytokines to initiate T cell clonal expansion and differentiation, thus augmenting and sustaining T cell responses (Boise et al., 1995; Lucas et al., 1995; Shahinian et al., 1993; Sperling et al., 1996; Thompson et al., 1989). CD28 costimulation also seems to upregulate CD40L expression (Sharpe et al.

unpublished and (Klaus et al., 1994)). Overall, CD28 delivers a positive signal into the T cell.

CTLA-4

CTLA-4 is the higher affinity receptor for B7.1 and B7.2 (Linsley et al., 1994; Linsley et al., 1992) with a 20-50 fold higher dissociation constant relative to CD28. The cytoplasmic domain of CTLA-4 has only ~30% homology with the CD28 cytoplasmic domain, but is 100% conserved across species, suggesting an important signaling function. In contrast to CD28, CTLA-4 (Brunet et al., 1987; Linsley et al., 1991) is an inhibitory receptor that is not constitutively expressed, but is rapidly upregulated following T cell activation, with peak expression 24-48 hours after activation (Linsley et al., 1996). Interaction of CTLA-4 with B7 molecules results in a negative signal, inhibiting TCR- and CD28-mediated signal transduction. The fatal lymphoproliferative disease that develops in CTLA-$4^{-/-}$ mice, due to uncontrolled B7.1/B7.2 costimulation, underscores the importance of CTLA-4 as a negative regulator of T cell responses (Tivol et al., 1997; Waterhouse et al., 1995) CTLA-4 inhibits IL-2 synthesis and progression through the cell cycle, and terminates T cell responses (Brunner et al., 1999; Greenwald et al., 2001; Krummel and Allison, 1995; Walunas et al., 1996; Walunas et al., 1994). In addition, CTLA-4 seems to actively induce peripheral T cell tolerance by effecting cell cycle regulation (Greenwald et al., 2001; Perez et al., 1997). Overall, CTLA-4 delivers a negative signal into the T cells.

B7h-ICOS pathway

B7h and ICOS are both relatively recently described members of the B7-CD28/CTLA-4 superfamily.

B7h

B7h (B7 homolog) is also called ICOSL (ICOS Ligand), LICOS (Ligand of ICOS), GL50, B7RP-1 (B7 Related Protein-1) and B7-H2 (B7-Homolog 2). B7h is expressed constitutively in non-lymphoid tissues such as kidney, liver, peritoneum, lung and testes (Swallow et al., 1999). B7h is expressed at low levels on resting B cells, on some macrophages and dendritic cells (Ling et al., 2000) and can be upregulated on these cell types by IFNγ cytokine (Aicher et al., 2000; Yoshinaga et al., 1999). B7h is also expressed on a small subset of CD3+ T cells (Ling et al., 2000).

The functional significance of B7h-expression in non-lymphoid cells has not yet been determined. Also, it is not yet clear which stimuli are most important for upregulating B7h expression and which cell types are the most important for delivering the B7h costimulatory signal.

ICOS

Similar to CD28 and CTLA-4, ICOS (Inducible Costimulator) is a glycosylated disulfide-linked homodimer. ICOS has a FDPPPF motif instead of the MYPPPY motif and does not bind B7.1 or B7.2 (Beier et al., 2000; Ling et al., 2000; Yoshinaga et al., 1999). In contrast to CD28, ICOS is not expressed constitutively, but is induced after T cell activation (Beier et al., 2000; Coyle et al., 2000; Hutloff et al., 1999; Mages et al., 2000; McAdam et al., 2000; Yoshinaga et al., 1999). ICOS is expressed on recently activated T cells and resting memory cells, on both Th1 and Th2 cells during differentiation, but ICOS levels remain high on Th2 cells and diminish on Th1 cells (Coyle et al., 2000; McAdam et al., 2000). ICOS has also been reported on rat B cells (Tezuka et al., 2000), but not on B cells from other species. ICOS is expressed in

human fetal and newborn thymus (Hutloff et al., 1999), and in the thymic medulla and cortico-medullary junction in mice (Mages et al., 2000), but studies in $ICOS^{-/-}$ mice do not indicate an essential role for ICOS in T cell development (Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001).

ICOS is similar to CD28 in that it mediates a positive signal to T cells and enhances cytokine production. Like CD28, ICOS has a role in providing T cell help for B cells, demonstrated *in vivo* by the profound deficits in immunoglobulin class switching accompanied by impaired germinal center formation in ICOS deficient mice (McAdam et al., 2001). Class switching was restored in $ICOS^{-/-}$ mice by CD40 stimulation, demonstrating that ICOS promotes T-B collaboration through the CD40-CD40L pathway by upregulating CD40L expression (McAdam et al., 2001).

ICOS seems to have a costimulatory role in already activated cells. ICOS upregulation is enhanced by CD28 costimulation (Aicher et al., 2000; McAdam et al., 2000), but is not entirely dependent on CD28 signals (Kopf et al., 2000). Studies of allergic airway disease and inflammatory lung disease have demonstrated that CD28 has a dominant role at the time of antigen priming, whereas ICOS regulates effector Th2 responses (Gonzalo et al., 2001; Tesciuba et al., 2001). Although ICOS can modestly stimulate T cell proliferation and the upregulation of secretion of IL-2, it is not as potent as CD28 and does not sustain proliferative responses (McAdam et al., 2001; Riley et al., 2001). Signals through ICOS seem to be more important for regulating cytokine production (IL-4, IL-5, IL-10, IFNγ, TNFα, GM-CSF) by recently activated and effector T cells (Coyle et al., 2000; Hutloff et al., 1999). Thus, in contrast to CD28, ICOS can stimulate IL-10 production, a key immunoregulatory cytokine. ICOS is upregulated on both Th1 and Th2 cells, but ICOS expression is more persistent in Th2 cells (Coyle et al., 2000; McAdam et al., 2000), suggesting

that ICOS signaling is important for Th2 development. Recent studies have shown that ICOS also regulates Th1 effector responses (Sperling, 2001; Sporici and Perrin, 2001). In addition, whereas CD28 seems to protect naïve T cells from apoptosis, ICOS signals appear to protect memory T cells (Sporici et al., 2001). Overall, ICOS seems to deliver a positive signal into the T cell.

PD-1:PD-L1/PD-L2 pathway

The newest pathway in the B7-CD28 family consists of PD-L1 (B7H1) and PD-L2 (B7-DC), both of which bind to PD-1. In contrast to other CD28 family members, PD-1 is not only expressed on T cells but also on myeloid and B cells. Its ligands, PD-L1 and PD-L2 are also not only expressed on professional APCs but also on non-lymphoid tissue. PD-L1 and PD-L2 have distinct expression patterns. PD-L1 is expressed on T cells, B cells, myeloid DCs, heart, fetal liver and placenta, and is IFNγ-induced (Dong et al., 1999; Freeman et al., 2000). PD-L2 is expressed on DCs, heart, lung, placenta, liver and pancreas, and is induced by IL-4 (reviewed in (Coyle and Gutierrez-Ramos, 2001)).

Thus, members of the B7-family of costimulatory molecules have a great impact on the immune response. Costimulatory signaling through these pathways can be important for T cell activation and differentiation, regulation of T cell expansion and cell survival, regulating B cell help, and for the regulation of tolerance.

Costimulation in Autoimmunity

To investigate whether costimulatory molecules can regulate the development of autoimmune responses, B7-CD28 costimulatory pathways have been manipulated *in vivo* to determine its effects on the development of autoimmunity.

As previously mentioned, autoimmune diseases do not occur until there is activation, expansion and differentiation of autoreactive T cells in the peripheral immune compartment. One of the best-studied models of autoimmunity is Experimental Autoimmune Encephalomyelitis (EAE), the animal model of Multiple Sclerosis (MS).

Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis (EAE)

Multiple Sclerosis (MS) is an organ specific autoimmune disease of the central nervous system (CNS). Disease syptoms vary from being mild, such as numbness in the limbs, to being severe, resulting in loss of vision or paralysis. MS can affect people of all ages, but is most commonly found in female adults, aged 20 to 50. It is still not clear what causes MS. Several factors, which influence each individual's predisposition to disease, have been identified. These include genetic predisposition, infection, stress and hormone related influences. There are different forms of MS, including acute disease, relapsing remitting disease and chronic progressive disease. The disease has a very variable course and the progress, severity and specific symptoms of MS in any one person cannot yet be predicted. Current treatments are aimed at improving symptoms and at modulation of the immune system. Current treatments are directed at modulating immune responses nonspecifically (e.g. by beta interferon).

Animal models provide a useful tool for understanding the pathogenesis of disease and for the search for treatment. EAE is a chronic inflammatory demyelinating

disease of the CNS and has been studied as an animal model for human MS. EAE is induced by autopathogenic Th1 cells that traffic to the CNS and initiate inflammation and demyelination, resulting in ascending paralysis (Gonatas and Howard, 1974; Juedes et al., 2000; Wekerle, 1991)

EAE can be induced in genetically diverse strains of mice by immunization with different myelin antigens in a strong adjuvant such as CFA, or by the transfer of myelin-specific CD4+ T helper 1 (Th1) cells (see Table 1.1, modified from (Anderson, 2002)). EAE can be induced in animals from different genetic backgrounds including SJL, PLSJLF1, NOD and C57BL/6 (B6) by immunization with different myelin-derived components such as Myelin Proteolipid protein (PLP), Myelin Basic Protein (MBP) or Myelin Oligodendrocyte Glycoprotein (MOG) peptides. Immunization of different animal strains with various myelin peptides results in different clinical disease phenotypes. While PLP 139-151 peptide induces a relapsing-remitting disease in H-2^s mice (e.g. SJL mice) (McRae et al., 1992; Tuohy et al., 1989), immunization with MOG 35-55 peptide induces a chronic or chronic-progressive disease in H-2^b mice (e.g. B6 mice) and in H-2^{g7} mice (e.g. NOD) (Kerlero de Rosbo et al., 1995; Mendel et al., 1995), and immunization with MBP1-11 induces an acute monophasic EAE in H-2^u mice (e.g. PL/J mice), providing clinical equivalents of various phenotypes of human MS.

The typical disease course is an ascending paralysis, starting with a limp tail followed by hind limb and forelimb paralysis. However, atypical forms of EAE have been reported, in which ataxia is combined with paralysis of forelimbs, but without hindlimb paralysis. For disease to occur, T cells have to be activated in the peripheral lymph nodes and then migrate across the blood brain barrier (BBB) to access the target organ, the CNS. In the target tissues, the autoreactive T cells have to be further

activated by the local APCs, to initiate inflammation and mediate tissue injury (Chang et al., 1999; Merrill et al., 1992). Autoimmune T cells, like all other T cells, also need two signals for their proper activation and differentiation.

Different phases of EAE

Currently, we distinguish between the following different phases of EAE-development and progression: 1) the development of the self-reactive endogenous repertoire, and the activation, expansion and differentiation of self-reactive cells in the periphery is considered the induction phase; 2) the trafficking of activated myelin-specific T cells to the target organ, and the reactivation of T cells within the CNS is considered the effector phase; and 3) epitope spreading, during which T cells with new epitope-specificities are recruited from the periphery.

1) The induction phase

Endogenous repertoire: a naturally occurring population of self-reactive cells in the peripheral immune system of normal individuals. In humans, a certain number of myelin-reactive T cells can be detected in the peripheral blood of healthy normal individuals (Burns et al., 1983; Ota et al., 1990; Zhang et al., 1994). It is still unclear how these self-reactive cells undergo selection (and escape thymic negative selection), how they are maintained in the periphery, and what their function is. These myelin-reactive cells might be regulatory, or might be the precursors of pathogenic cells that somehow get activated to induce MS. The existence of a substantial number of myelin-reactive cells found in a naïve repertoire (an endogenous auto-reactive repertoire) has so far been detected in one of the EAE-susceptible mouse strains, namely the SJL mouse strain (Anderson et al., 2000). The high degree of EAE-

susceptibility of the SJL mice to PLP 139-151-induced EAE was shown to be due to the presence of a very high frequency (1/20,000) of PLP 139-151-reactive T cells in the naïve repertoire. These PLP 139-151 reactive T cells are in the CD44 high population in the unimmunized SJL mice, suggesting that they belong to a memory pool of T cells (Anderson et al., 2000). However, in spite of a high number of autoreactive T cells, naïve SJL mice do not develop spontaneous EAE disease, and the question of which factors, genetic and/or environmental, are critical for the activation and expansion of the self-reactive repertoire still remains to be answered.

Several studies have suggested a role for B7.1 and B7.2 in thymic negative selection, however this is an area of controversy (Amsen and Kruisbeek, 1996; Kishimoto et al., 1996; Lucas and Germain, 2000; Punt et al., 1997). Thus, B7 costimulation may affect the generation of the endogenous repertoire (this is further discussed in Chapter 4).

Activation, expansion and differentiation of myelin-reactive T cells. Various strains of mice are susceptible to the development of EAE. To actively induce disease, myelin-reactive T cells in the periphery have to be activated, expanded and differentiated. Animals are immunized with myelin antigens in the presence of a strong adjuvant like Complete Freund's Adjuvant (CFA) that contains *Mycobacterium Tuberculosis* (M.T.) to boost the immune system. Alternatively, mice can be injected with a myelin-specific T cell line (Ben-Nun, 1982; Kuchroo et al., 1992; Zamvil and Steinman, 1990). In addition to immunization/cell-injection, the mice are also injected with *Pertussis Toxin* (P.T.). It is still not entirely clear how P.T. promotes EAE development, but it is thought to open up the blood-brain-barrier (BBB) and thus facilitate the entry of self-reactive cells into the CNS. Immunization causes myelin-specific T cells to be activated and expanded in the peripheral draining lymph nodes

(LNs). The activation of T cells in the peripheral LNs requires the interaction of self-reactive T cells with APCs that are presenting myelin antigen. In agreement with the 2-signal-hypothesis, signal 1 (TCR-MHC-peptide interaction) and signal 2 (costimulation) are required for the activation of T cells during the induction phase of EAE, thus B7 costimulation must play an important role during this process.

Cytokines in EAE: Th1 versus Th2

EAE is mainly mediated by myelin-reactive CD4+ T helper cells (Brostoff, 1984; Waldor M.K., 1985). CD4+ T cells, upon activation, differentiate into T cells producing different cytokine profiles. T helper type I (Th1) cells are characterized by secretion of Interleukin-2 (IL-2), Interferon-gamma (IFNγ), and/or Lymphotoxin-alpha (LT-α), also called Tumor Necrosis Factor-beta (TNFβ). IL-12 is critical for the induction of Th1 differentiation. Th1 cells mediate delayed-type hypersensitivity and class switching to IgG2a. T helper type II (Th2) cells are characterized by production of IL-4, IL-5 and IL-10. Th2 cells mediate recruitment of eosinophils and class switching to IgG1 and IgE. Th1 and Th2 cytokines are mutually cross-regulatory and can have profound effects on the outcome of autoimmune diseases, infectious diseases and transplantation (Mosmann and Sad, 1996). Costimulation has been shown to affect the development of Th1 and Th2 cells, but has a greater effect on Th2 cells (Rulifson et al., 1997; Salomon and Bluestone, 2001).

T helper (Th subsets) and their cytokines impact EAE development in important ways. Most evidence suggests that Th1 cells/cytokines as well as other proinflammatory cytokines such as Tumor Necrosis Factor-alpha (TNFα) are critical in the pathogenesis of EAE. Myelin-specific Th1 cells can induce EAE (Baron J.L., 1993; Kuchroo et al., 1993; Miller and Karpus, 1994), and Th1 cytokines like IFNγ

and TNFα have been found within inflammatory CNS lesions during EAE (Baker et al., 1991; Bright et al., 1998; Kennedy et al., 1992). IL-12 deficient mice are resistant to EAE (Segal et al., 1998). However, IFNγ$^{-/-}$ and TNFα$^{-/-}$ mice developed exacerbated EAE (Ferber et al., 1996; Frei et al., 1997; Korner et al., 1997; Krakowski and Owens, 1996; Liu et al., 1998), suggesting that IFNγ and TNFα are not essential for EAE disease.

Most evidence suggests that Th2 cells and their cytokines down-modulate EAE. Myelin-specific Th2 cells can protect against EAE onset and/or reverse ongoing disease (Kuchroo et al., 1995; Nicholson et al., 1995), and Th2 cytokines such as IL-4 and IL-10 are not present in inflammatory lesions, but are upregulated during disease remission (Kennedy et al., 1992; Khoury et al., 1992). Direct administration of IL-4 has been shown to dramatically reduce EAE severity (Racke et al., 1994), and transfer of Myelin-reactive T cells transfected with IL-4 or IL-10 expression vectors were able to inhibit EAE development (Mathisen et al., 1997; Shaw et al., 1997). IL-10$^{-/-}$ mice succumb to severe chronic EAE, and IL-10 transgenic mice are resistant to EAE induction (Bettelli et al., 1998; Cua et al., 1999). However, there is also evidence that Th2 cells/cytokines are not critical for EAE development. IL-4$^{-/-}$ mice developed either comparable (Bettelli et al., 1998; Liblau et al., 1997; Segal et al., 1998) or exacerbated (Falcone et al., 1998) EAE when compared to controls. Further, MBP-specific Th2 cells were able to transfer EAE into immuno-compromised mice, although EAE disease was histologically atypical (Lafaille et al., 1997).

Overall, cumulative data suggests that Th1 cells are pathogenic for EAE development, while Th2 cells are protective. However, the Th1/Th2 paradigm should not be oversimplified.

3) Effector phase

Trafficking of self-reactive cells into the target organ. Once myelin-specific T cells have been primed and activated in the peripheral lymphoid tissues, they enter the circulation and traffic to the target organ. There is evidence that activated lymphocytes can cross the BBB regardless of antigen specificity (Hicky, 1991). The expression of certain adhesion molecules seems to be critical for T cells to traffic into the CNS. T lymphocytes express chemokine receptors (seven transmembrane receptors) depending on their state of activation and differentiation. Chemokine receptors bind chemokines (for a review see (Ward et al., 1998), small basic proteins that promote chemotaxis (Baggiolini, 1998). Chemokines were initially characterized for their role in attracting cells to sites of inflammation, and have more recently been found to also direct cell movements within lymphoid tissue. A few studies have shown the relationship of chemokine expression with the development of EAE disease. These studies demonstrate that CCR1-RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) and CCR2-MCP-1 (Monocyte Chemotactic Protein-1) have important roles in the immunopathogenesis of EAE in that mice that lack the interactions of these chemokine receptors with their ligands develop less severe EAE than wild-type (Wt) mice (Izikson et al., 2000; Karpus et al., 1995; Rottman et al., 2000). This has led to the hypothesis that distinct chemokine receptors (corresponding to specific ligands) are expressed by CNS-infiltrating antigen-specific T cells as well as host-derived bystander T cells and monocytes. Indeed, recent studies have demonstrated the importance of CC Chemokine receptor expression by CD4+ encephalitogenic T cells for CNS infiltration and subsequent disease development (Fife et al., 2001). EAE-induction resulted in the expression of the chemokine receptors CCR1, CCR2, CCR3, CCR4, CCR5, CCR8, CXCR2 and

CXCR3 on T cells in the CNS (Fife et al., 2001; Fischer et al., 2000; Matejuk et al., 2000). Protection of mice from EAE by TCR vaccination or Altered Peptide Ligands (APLs) results in a selective reduction of inflammatory chemokines and chemokine receptors, and also in the selective upregulation of modulating chemokine receptors in the CNS (Fischer et al., 2000; Matejuk et al., 2000). In particular, RANTES, IP-10 (Interferon-γ inducible Protein) and MCP-1 are thought of as inflammatory chemokines. CCR5 (which binds MIP-1α = Macrophage Inflammatory Protein-1α, MIP-1β and RANTES) and CXCR3 (which binds IP-10 and MIG = Monokine Induced by interferon-γ) are both Th1 associated markers, and are thought to be inflammatory chemokine receptors. In contrast, CCR3 (which binds Eotaxin-1 and Eotaxin-2) and CCR4 (binding TARC = Thymus and Activation-Regulated Chemokine) are both Th2 associated markers, and are thought to be disease-modulating chemokine receptors (Fischer et al., 2000; Matejuk et al., 2000).

Reactivation of myelin-reactive cells within the CNS. Only antigen specific T cells seem to be retained within the CNS. In contrast, non-specific T cells exit the CNS rapidly (Hicky, 1991). Thus, T cell retention within the CNS and subsequent T cell-mediated EAE pathology seems to require antigen-specific recognition within the CNS. Several cell types may act as APCs within the CNS. The $CD45^{hi}$ perivascular macrophage is believed to be the major APC population within the CNS, but also other cells can present antigen, including $CD45^{lo}$ microglia, astrocytes and endothelial cells (Shrikant and Benveniste, 1996). The interaction of myelin-specific T cells with their antigen presented by resident CNS APCs and costimulatory signals is thought to result in the reactivation of the T cells. Subsequently, the reactivated T cells produce inflammatory cytokines such as IFNγ and TNFα, activating the endothelium and causing the BBB to become permeable to leukocytes. Activated T cells and APCs

also produce chemokines such as RANTES and MIP-1α that recruit additional lymphocytes and monocytes through the compromised BBB into the perivascular area. Nearby astrocytes, activated by increasing levels of IFNγ and TNFα, then establish an IP-10 gradient that draws the lymphocytes into the parenchyma (Karpus and Ransohoff, 1998). A positive feedback loop is established in which the inflammatory response is amplified by the accumulation of a second wave of both antigen-specific and antigen-non-specific T lymphocytes. There is evidence that tissue damage is ultimately mediated by the recruitment of activated macrophages that strip off myelin from axons via phagocytic mechanisms and release of TNFα, proteolytic enzymes, and Nitric oxide (NO). The secretion of cytokines, including IL-1 and TNFα, further perpetuates nonspecific inflammatory reactions (Oppenheim and I, 1982; Scheurich et al., 1987).

3) Epitope spreading

As explained above, the destruction of the myelin sheath leads to inflammation and tissue damage in the CNS. The resulting tissue debris is processed and presented on resident and peripheral APCs, leading to the activation and differentiation of a second wave of Th1 cells, which can re-enter the tissue and cause additional tissue destruction (reviewed in (Vanderlugt and Miller, 2002)). The tissue damage caused by the initial auto-immune response can lead to the presentation of various myelin epitopes and thus initiate the priming of self-reactive T cells that are specific to different epitopes, regardless of the specificity of the first wave of T cells, a phenomenon called epitope spreading. Epitope spreading was initially defined as the diversification of the immune response from the initial focused, dominant epitope-specific T cell response, to subdominant epitopes on that protein (Lehmann et al.,

1992; Lehmann et al., 1993). However, not only has intramolecular epitope spreading (different epitopes on the same myelin protein) been observed, but also intermolecular epitope spreading (different epitopes on different myelin proteins). Epitope spreading seems to have a pathological role in chronic disease progression (Kumar, 1998; McRae et al., 1995; Tuohy et al., 1998; Vanderlugt et al., 1998) in that it spreads and prolongs the immune response. It has been shown that functional epitope spreading is dependent on costimulatory signals. In particular, blockade of the B7-CD28 pathway (Miller et al., 1995) and the CD40-CD40L pathway (Howard et al., 1999) results in the inhibition of epitope spreading, which in turn results in the reduction of EAE relapses.

In EAE, a hierarchial order of epitope spreading has been characterized (Lehmann et al., 1998; Yu et al., 1996). In SJL mice, priming with the immunodominant PLP epitope PLP 139-151 results the induction of PLP 139-151-specific CD4+ T cell reactivity within three days of priming, and this reactivity is maintained throughout the disease course. Immediately before and continuing through the first relapse, PLP 178-191 reactivity is detected. During the second relapse, MBP 84-104 reactivity is detected. The development of these responses has been shown to correlate with the extent of myelin destruction during the acute disease phase. So far, there have been no reports on the role of epitope spreading in the model of MOG-peptide induced chronic EAE in B6 mice. It is possible that epitope spreading has a different importance for different disease models, being important for relapsing-remitting EAE, but not critical in chronic disease.

The importance of epitope spreading in human MS is still unclear and difficult to prove since MS patients are examined when disease is already established and the response to the initial epitope might have already shifted to a different epitope.

However, there is some evidence for an unfocused unstable epitope response in progressive MS (Goebels et al., 2000; Tuohy et al., 1997; Tuohy et al., 1999; Tuohy et al., 1999), indicating a role for epitope spreading in human MS.

Two signals are necessary for the activation of autoreactive T cells

Consistent with the two signal model, autoreactive T cells in the periphery do not become activated until they encounter antigen (either self or mimicry non-self peptide) in the context of relevant MHC molecules, together with second appropriate costimulatory signals (as discussed before). Activation of T cells is important at several stages of the development of an autoimmune disease, including stimulation and clonal expansion within peripheral lymphoid tissues, entry and reactivation of autoreactive T cells in the target tissue, tissue destruction within tissue parenchyma of the target tissue and activation of "new" T cells during epitope spreading (for a review, see (Hicky, 1991)). Recent studies indicate that the expression of appropriate costimulatory molecules on the APCs in the peripheral immune compartment and also at the target tissue site is essential for the induction and effector functions of the autoreactive T cells and induction of autoimmune disease (Chang et al., 1999). In addtion to the role of costimulation in the periphery, we must also consider that costimulation may play a role in thymic selection, when potential auto-reactive T cells are selected.

The role of B7 family molecules in autoimmunity

B7 family molecules seem to have a critical role in the development of autoimmune diseases. The published reports suggest that costimulation of autoreactive T cells is important not only in experimentally antigen-induced models of autoimmunity such

as EAE, but also in spontaneous models of autoimmunity such as type 1 diabetes in NOD mice [as reviewed in (Jabs et al., in press)]. This is further substantiated by genetic analysis of susceptibility/resistance to a number of experimental autoimmune diseases in animals and human autoimmune diseases, showing that susceptibility to autoimmune diseases is linked to genetic intervals that encompass B7-costimulatory receptors and ligands (Encinas et al., 1996). Interestingly, the role of costimulation in the induction or inhibition of the two autoimmune disease models (spontaneous and induced) is not always the same. This thesis focuses on the role of costimulation in EAE, an induced autoimmune disease.

Manipulation of the B7-CD28/CTLA-4 costimulatory pathway in autoimmunity.

To analyze the role of costimulation in autoimmunity, several tools have been developed to manipulate the interaction of B7 molecules and their receptors (CD28 and CTLA-4). The following describes these tools and the role of positive signals (CD28) and negative signals (CTLA-4) in autoimmune disease.

CTLA-4Ig

Studies with CTLA-4Ig, a fusion protein that binds to both B7.1 and B7.2 and blocks B7.1 and B7.2 interactions with their counter-receptors CD28 and CTLA-4 (Lenschow et al., 1992; Linsely et al., 1992), indicate an important role for the B7-CD28/CTLA-4 pathway in regulating EAE. In EAE, when CTLA-4Ig is given at the time of immunization, (Cross et al., 1995; Perrin et al., 1995; Racke et al., 1995) shortly after immunization (Perrin et al., 1996) or when MBP-specific T cells are restimulated *in vitro* in the presence of CTLA-4Ig before adoptive transfer (Perrin et al., 1995), the development of EAE can be prevented or ameliorated. However, when

activated MBP-reactive T cells are adoptively transferred and the recipient is treated with CTLA-4Ig, EAE is not inhibited (Perrin et al., 1995). In addition, treating mice with CTLA-4Ig at the onset of clinical symptoms (Perrin et al., 1996) results in the exacerbation of EAE. Therefore, blocking the B7-CD28/CTLA-4 pathway by administration of CTLA-4Ig at different stages of EAE results in different outcomes, which may be due to preferential blockade of B7-CD28 versus B7-CTLA-4 interactions. These studies suggest that the timing of B7 blockade by CTLA-4Ig administration has different effects on the clinical outcome of the disease. Whereas blocking B7-costimulation at the induction of disease is effective in inhibiting disease, perhaps by blocking the induction of autoreactive T cells, B7-blockade by CTLA-4Ig administration is not effective in inhibiting disease induced by previously activated autoreactive T cells.

Anti-B7.1 and anti-B7.2 mAbs

Anti-B7.1 and anti-B7.2 mAbs have been used to address whether differential blockade of either B7.1 or B7.2 independently might affect the development of autoimmune diseases. *In vivo* studies with anti-B7.1 and anti-B7.2 mAbs suggest that B7.1 and B7.2 can differentially regulate autoimmune responses. Anti-B7.1 mAbs administered at the time of immunization have been shown to reduce the incidence and severity of acute EAE, whereas anti-B7.2 mAb therapy either increased or had no effect on disease severity (Kuchroo et al., 1995; Miller et al., 1995; Racke et al., 1995). *In vivo* administration of both B7.1 and B7.2 antibodies in combination had no effect on EAE (Kuchroo et al., 1995). In EAE and MS, B7.1 has been shown to become a dominant costimulatory molecule, and it is possible that the blocking effects of anti-B7.1 antibody in EAE might be for this reason (Miller et al., 1995; Windhagen

et al., 1995). In searching for the mechanism by which B7.1 and B7.2 could have such contrasting effects, several studies suggested that B7.1 and B7.2 molecules differentially affect Th cell differentiation. It was clear that anti-B7 antibodies did not significantly inhibit the induction of T cells to the immunizing antigen but altered the cytokine profile of the responding T cells (Kuchroo et al., 1995). These studies suggested that administration of anti-B7.1 antibody *in vivo* at the time of immunization resulted in the generation of Th2 clones whose transfer both prevented induction of EAE and abrogated established disease. Since EAE is thought to be mediated by Th1-type cytokines (Kuchroo et al., 1993), these results suggest that anti-B7.1 mAbs block the development of Th1 T cells and consequently the progression of disease, while anti-B7.2 mAbs block the development of protective Th2 cells (Kuchroo et al., 1995), resulting in more severe disease.

More recent studies suggest that B7.1 on T cells can regulate IL-4 production from the T cells since the loss of B7.1 from the T cells (in the B7.1 deficient mice) leads to a preferential development of IL-4 producing Th2 cells (Schweitzer and Sharpe, 1999). Activated T cells express B7.1 on their surface (Prabhu Das et al., 1995; Sansom and Hall, 1993) and the blocking effect of anti-B7.1 antibody might be due to its effects on blocking B7.1 on the surface of T cells rather than APCs.

Mice deficient in B7 or CD28 expression

CD28 has been shown to induce a positive signal in T cells. $CD28^{-/-}$ mice on the NOD background are resistant to EAE when actively immunized with PLP 56-70 which induces disease in Wt littermate controls (Girvin et al., 2000). $B7.1/B7.2^{-/-}$ and $CD28^{-/-}$ mice on the B6 background are resistant to EAE when actively immunized with one dose of MOG 35-55 which induces disease in Wt littermate controls. Histological

analyses suggest that the inflammatory cells are generated, but are retained in the meninges of the CNS (Chang et al., 1999; Girvin et al., 2000; Perrin et al., 1999). However, induction of EAE in $CD28^{-/-}$ mice on the B6 background has been demonstrated. When MOG 35-55-primed T cells from B6 Wt donor mice were transferred into $CD28^{-/-}$ recipients and the recipients were afterwards immunized with MOG 35-55, they developed EAE (Chitnis et al., 2001). Also, when B6 $CD28^{-/-}$ mice were actively immunized twice with MOG 35-55 they developed EAE that was comparable in severity but was delayed, as compared to B6 Wt mice that were immunized once with MOG 35-55 (Chitnis et al., 2001). These observations demonstrate that the lack of CD28 can be overcome by increasing the dose of immunizing encephalitogenic antigen.

Anti-CTLA-4 Ab and CTLA-$4^{-/-}$ mice.

CTLA-4 is known to induce a negative signal in T cells. CTLA-4 deficient mice develop a severe lymphoproliferative disorder and die at a young age due to multi-organ autoimmune disease (Tivol et al., 1995; Waterhouse et al., 1995). Inhibition of CTLA-4 by antibody blockade exacerbates EAE. Several laboratories have reported that *in vivo* administration of anti-CTLA-4 antibody exacerbated EAE induced either by active immunization or by adoptive transfer of encephalitogenic T cells (Hurwitz et al., 1997; Karandikar et al., 1996; Karandikar et al., 1998; Perrin et al., 1996). Administration of anti-CTLA-4 at the onset of clinical EAE increased mortality and enhanced subsequent relapses by increasing epitope spreading, clearly supporting a role of the negative signal from CTLA-4 in downregulating autoreactive T cells and inhibiting autoimmune reactions. Besides its role in inhibiting T cell activation, CTLA-4 has also been shown to play a role in inducing T cell anergy *in vivo*

(Greenwald et al., 2001). Furthermore, studies suggest that preferential ligation of CTLA-4 by B7s may induce T cell non-responsiveness (Perez et al., 1997). Studies with CTLA-$4^{-/-}$ T cells have shown that CTLA-4 has an essential role in determining the outcome of T cell encounter with a tolerogenic stimulus (Greenwald et al., 2001). Following exposure to a tolerogenic stimulus, CTLA-$4^{-/-}$ T cells but not Wt T cells were still able to proliferate and produce IL-2. In contrast to wild-type T cells, which became tolerant and were blocked in the late G1 to S restriction point of the cell cycle, CTLA-$4^{-/-}$ T cells entered the S phase of the cell cycle, proliferated and produced effector cytokines. Thus, tolerance resistance in the CTLA-$4^{-/-}$ T cells was shown to be related to a role for CTLA-4 in regulating cell cycle progression (Greenwald et al., 2001).

Recent studies suggest that the B7-CD28/CTLA-4 pathway might also be critical for the induction and/or function of CD4+CD25+ regulatory T cells. NOD B7.1/B7.2 deficient mice have an increased incidence and severity of autoimmune diabetes, which can be corrected by the adoptive transfer of CD4+CD25+ cells (Salomon et al., 2000). This observation further supports the notion that B7-CD28 may play a crucial role in the generation or regulatory function of CD4+CD25+ T cells. In addition, CD4+CD25+ regulatory cells express CTLA-4 on their surface (Read et al., 2000; Takahashi et al., 2000). Therefore, CTLA-4 may exert its downregulatory effects on autoimmunity by multiple mechanisms, including inhibition of T cell activation, induction of anergy, regulation of CD4+CD25+ regulatory T cells and/or production of immuno-suppressive cytokines that may inhibit the induction or progression of an autoimmune disease.

Manipulation of the ICOS-B7h costimulatory pathway in autoimmunity.

Several studies suggest a crucial role for the ICOS-B7h pathway in autoimmunity. ICOS deficient mice on the 129xB6 mixed background show exacerbated MOG 35-55-induced EAE as compared to Wt littermate controls (Dong et al., 2001). Exacerbation of EAE can be demonstrated by higher severity of clinical disease, more inflammatory foci in the brain, and more CD4+ T cells in the CNS producing IFNγ. The increased severity of EAE in the $ICOS^{-/-}$ mice was attributed to a decrease in IL-13 and increase in IFNγ production by the responding T cells. *In vivo* blockade of ICOS-B7h pathway by administration of anti-ICOS antibody (Rottman et al., 2001) or ICOS-Ig (Sporici et al., 2001) further supported these studies.

PD-1:PD-L1/PD-L2 pathway and autoimmunity.

A role for PD-1 in regulating tolerance is indicated by the phenotype of PD-1 deficient mice. Aged $PD\text{-}1^{-/-}$ mice develop a spontaneous lupus-like autoimmune disease on the B6 background (Nishimura et al., 1999) and autoimmune dilated cardiomyopathy resulting in heart failure and sudden death on the Balb/c background (Nishimura et al., 2001). $PD\text{-}1^{-/-}$ mice show significantly augmented serum levels of IgG2b, IgG3 and IgGA and a reduction of CD5 on B-1 cells, a molecule known to negatively regulate B cell responses, suggesting that the PD-1/PD-L pathway may not only affect T cell activation but may also regulate certain aspects of B cell activation and differentiation. PD-1 deficient mice develop autoimmune disease of peripheral organs. Since PD-L1 and PD-L2 are expressed on the parenchymal cells of various organs, this suggests that the PD-1/PD-L pathway might provide a negative signal to T cells, and suggests a mechanism by which tissue tolerance may be maintained. This provides a unique mechanism for regulating T cell tolerance in peripheral tissues and

preventing organ-specific autoimmune diseases. Since PD-1 is expressed on B cells and T cells, PD-1 may regulate both B and T cell tolerance. It is not clear whether the B cell defects are secondary to T cell defects or whether both B and T cells are primarily affected because of loss of PD-1. No studies have yet been reported on the consequence of manipulating this pathway in various models of autoimmunity.

Conclusions

Since the first description of the B7 family members and the identification of their role in T cell costimulation, the field has become complex with the identification of new members of the family and potential for both positive and negative signaling within and outside the immune system (see Figure 1.2). Thus the members of the B7-family are not only important in enhancement and inhibition of T and B cell responses, but the expression of their ligands in the parenchyma of the peripheral tissues provides a way of regulating autoimmune diseases at sites of inflammatory damage and inducing and/or maintaining tissue tolerance. The newly identified pathways may provide avenues for the inhibition of undesired activation or effector functions of autoreactive T cells and provide new drug targets for the regulation and treatment of autoimmune diseases.

The studies in this thesis investigate the role of the B7-CD28/CTLA-4 and B7h-ICOS pathways in regulating EAE.

We have examined the roles of B7.1 and B7.2 in regulating PLP 139-151-induced EAE by studying B7.1, B7.2 and B7.1/B7.2 deficient mice on the SJL background. Our studies of the SJL B7-deficient mice in the PLP-peptide EAE model differ significantly from our studies using B6 B7-deficient mice in the MOG-peptide EAE

model. We found that the EAE susceptibility of SJL B7.1/B7.2$^{-/-}$ mice versus the EAE resistance of B6 B7.1/B7.2$^{-/-}$ mice is not dependent on the encephalitogenic peptide, but is strongly dependent on the MHC, and is also influenced by other genes (Chapter 3). Since B7.1 and B7.2 have overlapping but also distinct functions, studying EAE in the B7.1$^{-/-}$ and B7.2$^{-/-}$ mice gave us a tool to investigate the roles of the individual B7 molecules. Our studies demonstrate that B7 costimulation is important both in the induction and effector phase of PLP 139-151-induced EAE in SJL mice (Chapter 4).

Finally, we examined the role of a relatively recently discovered pathway, the ICOS-B7h pathway. Studying ICOS$^{-/-}$ mice and using anti-ICOS monoclonal antibodies (mAbs) revealed that inhibiting ICOS signaling during the induction phase of EAE results in exacerbation of EAE. In contrast, inhibiting ICOS during the effector phase of EAE results in amelioration of EAE. By studying the upregulation of ICOS in B7.1/B7.2$^{-/-}$ mice on the SJL and B6 background, we found that the expression of ICOS depends on B7 costimulation (Chapter 5).

Thus, this thesis emphasizes important roles for B7-CD28/CTLA-4 and ICOS-B7h costimulation in the initiation and progression of EAE. It is especially interesting that different genetic backgrounds seem to have different requirements for costimulation. The results of this thesis are important in the context of therapeutic manipulation of B7-CD28/CTLA-4 and ICOS-B7h costimulation as a treatment-option for MS patients. MS patients have diverse genetic backgrounds, which may alter the outcome of such treatments.

Chapter 2

Materials and Methods

Mice

SJL and B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). These mice or heterozygous or homozygous Wt littermates of the B7 deficient mice were used as wild-type (Wt) controls. 129/S4SvJae wild-type mice were obtained from Arlene Sharpe's breeding colony.

B7.1$^{-/-}$, B7.2$^{-/-}$ and B7.1/B7.2$^{-/-}$ mice were generated in Arlene Sharpe's laboratory by gene targeting as described previously (see references (Borriello et al., 1997; Freeman et al., 1993)). The 3 strains (originally on the 129/S4SvJae background) were backcrossed for 10 generations onto the C57Bl/6 (B6) background. B7.1$^{-/-}$ and B7.2$^{-/-}$ mice were backcrossed for 7-8 generations onto the SJL background, and B7.1/B7.2$^{-/-}$ mice were backcrossed for 5-6 generations onto the SJL background.

(B6xSJL)F1 mice. (B6xSJL)F1 Wt mice were either purchased from The Jackson Laboratory (Bar Harbor, ME) or generated by crossing female B6 Wt with male SJL Wt mice or by crossing female SJL Wt with male B6 Wt mice. (B6xSJL)F1/B7.1/B7.2$^{-/-}$ mice were generated by crossing female B6 B7.1/B7.2$^{-/-}$ mice with male SJL B7.1/B7.2$^{-/-}$ mice.

BC1 mice. Backcross 1 (BC1) mice were generated by crossing (B6xSJL)F1 B7.1/B7.2$^{-/-}$ mice with SJL B7.1/B7.2$^{-/-}$ mice.

ICOS$^{-/-}$ mice. The ICOS$^{-/-}$ mice were generated by gene targeting as previously described (McAdam et al., 2001). Mice used in experiments were on the 129/S4SvJae background or were backcrossed from the 129 background to the B6 background for 1 generation, thereby generating a mixed 129xB6 background.

Antigens

PLP 139-151 (HSLGKWLGHPDKF) and PLP 178-191 (NTWTTCQSIAFPSK) were synthesized by Quality Controlled Biochemicals, Inc (Hopkinton, MA). The control Neuraminidase 101-120 peptide (NASE 101-120; EALVRQGLAKVAYVYKPNNT) was synthesized by Dr. R. Laursen (Boston University, Boston, MA) on a Milligen model 9050 synthesizer using F-moc chemistry. MOG 35-55 (MEVGWYRSPFSRVVHLYRNGK), MOG 92-106 (DEGGYTCFFRDHSYQ) and ovalbumin (OVA) 323-336 peptide (ISQAVHAAHAEINE) were synthesized by Dr. David Teplow at the Biopolymer Facility (Center for Neurological Diseases, Brigham and Women's Hospital) on an Applied Biosystems 430A peptide synthesizer (Foster City, CA) using F-moc chemistry. All peptides were HPLC purified, and peptide identity was confirmed by mass spectroscopy. The peptides were over 90% pure, as determined by HPLC.

Induction and assessment of EAE

By Active Immunization

Groups of 6-12 week old female mice (three to six per group) were immunized with 25-100 μg of PLP 139-151 or PLP 178-191 or MOG 35-55 in PBS emulsified 1:1 in Complete Freund's Adjuvant (CFA) supplemented with 400 μg/ml of *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI) in the two hindflanks subcutaneously. PLP-immunized mice were injected with 100 ng of pertussis toxin (P.T.), and MOG immunized mice were injected with 150 ng of P.T. (List Biological Laboratories, Campbell, CA) intravenously (i.v.) on the day of immunization and two days later.

By Adoptive Transfer

Female mice were immunized with 100 μg of PLP 139-151 or MOG 35-55 in PBS emulsified 1:1 in Complete Freund's Adjuvant (CFA) in the two hind-flanks and the two hind-footpads subcutaneously. 10 days later, draining lymph nodes were harvested, and LNCs were resuspended at a concentration of 5-10 x 10^6 cells per ml in culture medium in the presence of 20 μg/ml PLP 139-151 or 30 μg MOG 35-55 and 20 ng/ml of murine rIL-12 (Genetics Institute) (MOG cultures only).

Short-term-cultures. After four days of *in vitro* culture, cells were harvested, purified over a Ficoll/Hypaque gradient (PLP 139-151-reactive cells only), washed and resuspended in PBS at 50-60 x 10^6 cells per ml. Recipient mice received 10-12 x 10^6 cells per mouse (0.2 ml), as well as 100 ng (PLP 139-151-reactive cells) or 150 ng (MOG 35-55-reactive cells) of P.T. (List Biological Laboratories, Campbell, CA) i.v. immediately after cell transfer.

Long-term-cultures (PLP 139-151-reactive cells only). Cells were fed every 3 days with culture medium containing 0.8% recombinant IL-2. In addition, cells were restimulated every 7 days with antigen (20 μg/ml PLP 139-151) plus freshly isolated and irradiated (3000 rad) splenocytes from SJL Wt mice as a source of antigen presenting cells (APCs). After four weeks of *in vitro* culture, cells were harvested, purified over a Ficoll/Hypaque gradient, washed and resuspended in PBS at 50 x 10^6 cells per ml. Recipient mice received 10 x 10^6 cells per mouse (0.2 ml), as well as 100 ng of P.T. (List Biological Laboratories, Campbell, CA) i.v. immediately after cell transfer.

Assessment of EAE

Mice were observed daily for clinical signs of EAE and scored on a scale of 0-5: 0 - no disease; 1 - limp tail; 2 - hindlimb weakness; 3 - hindlimb paralysis; 4 - hind and forelimb paralysis; 5 - moribund state or death. In our experience, this scale correlates with weight loss and number of CNS inflammatory foci. Mean clinical score was calculated by averaging the scores of all mice in each group, including animals that did not develop EAE.

Histology

Brains and spinal cords were removed at the peak of disease or at the end of the experiment and fixed in 10% formalin. The staining of the brains and spinal cords of mice and evaluation of tissue sections was done in collaboration with Dr. Raymond Sobel. Paraffin-embedded sections were stained with Luxol fast blue-hematoxylin and eosin for light microscopy. Inflammatory foci, identified as perivascular clusters containing at least 20 mononuclear cells, were counted in leptomeninges, gray matter, and choroid plexus. Areas of loss of Luxol fast blue parenchymal staining indicative of demyelination were measured using a Bioquant System image analyzer (R & M Biometrics, Nashville, TN) attached to an IBM personal computer. Microscopic fields with white matter lesions were selected from coded slides. The images were projected from fields magnified to 1,117 x on a monitor and areas of white matter staining loss were outlined on a digitizer pad. Mean area per field and total lesion areas were determined for each mouse. Sections were evaluated in a blinded fashion (Sobel et al., 1990).

T cell response to PLP or MOG antigens

T cell proliferation. Mice were immunized subcutaneously with 100 μg of PLP 139-151, PLP 178-191 or MOG 35-55 in PBS emulsified 1:1 in CFA (Difco Laboratories). Draining lymph nodes were harvested ten days later and single cell suspensions prepared. Whole LNCs were cultured in 96-well plates at $5x10^5$ cells/well with medium alone or a range of concentrations of PLP 139-151, PLP 178-191, NASE 101-120 (for the SJL or F1 mice) or MOG 35-55, MOG 92-106 or OVA 323-336 (for B6 or F1 mice) in HL-1 medium (Bio Whittaker, Walkersville, MD). Plates were pulsed with ^{3}H-thymidine (New England Nuclear, Boston, Massachusetts) at 1 μCi/well on day two of culture for the final 18 hours. Mean incorporation of thymidine in DNA was measured in quadruplicate wells by liquid scintillation counting (model LS 5000; Beckman Instruments, Fullerton, CA). Background levels from medium controls were subtracted and data are presented as ΔCPM.

Cytokine ELISA. Lymph node cells were prepared and cultured as above. Supernatants were harvested at 42 hours of culture. Cytokines (IL-2, IL-4, IL-10, TNFα and IFNγ) were determined by sandwich Enzyme-linked immunosorbent assay (ELISA), as described previously (Nicholson et al., 1995). Monoclonal antibody pairs and recombinant cytokine standards were purchased from PharMingen (San Diego, CA). The following mAb pairs were used: IL-2 (JES6-1A12, capture; JES6-5H4 detection); IL-4 (BVD4-1D11 capture; BVD6-24G2 detection); IL-10 (JES5-2A5 capture; SXC-1 detection); TNFα (MP6-XT22, capture; MP6-XT3, detection); IFNγ (R4-6A2 capture; XMG1.2 detection). The lower limit of detection for IL-2 and IL-10 was 50 pg/ml, for IL-4 was 25 pg/ml, for TNFα was 200 pg/ml, and for IFNγ was 100 pg/ml.

RNAse Protection assay (RPA)

Mice were immunized subcutaneously with 100 μg of MOG 35-55 in PBS emulsified 1:1 in CFA (Difco Laboratories). Draining lymph nodes were harvested ten days later and single cell suspensions prepared. Whole LNCs were cultured in 24-well plates at $3x10^6$ cells/well with MOG 35-55 at a concentration of 100 μg/ml in HL-1 medium (Bio Whittaker, Walkersville, MD). After 2 or 3 days of culture, cells were transferred into TRIzol® reagent (Life technologies) at $5x10^6$ cells/ml. The analyzation of mRNA was done in collaboration with Diane van der Woude. mRNA was isolated from $5x10^6$ cells using TRIzol® reagent. mRNA was analyzed for cytokine and chemokine receptor expression by RNAse protection assay using RiboQuant multiprobe template set mCK1 and a custom-made template set, according to the manufacturer's instructions (Pharmingen).

Linkage analysis

A total of 95 BC1 B7.1/B7.2$^{-/-}$ mice, generated by crossing (B6xSJL)F1 B7.1/B7.2$^{-/-}$ mice with B7.1/B7.2$^{-/-}$ SJL mice, plus control mice were immunized to induce EAE with the PLP peptide 139-151. Clinical disease was monitored for 40 days, and livers were obtained from 88 BC1 B7.1/B7.2$^{-/-}$ mice (the livers from 7 mice could not be obtained) plus control mice at the end of the experiment and frozen immediately at –70^{o}C. The genominc DNA preparation, the performance of Polymerase Chain Reactions (PCRs) and statistical analyzation was done in collaboration with Bernhard Greve. Genomic DNA was prepared by digesting liver samples with proteinase K and subsequent phenol/chloroform extraction. MHC class II genotype of the BC1 progeny was analyzed by a PCR strategy designed to differentiate SJL class II (IA^{s}) homozygotes from the B6 class II (IA^{b}) SJL class II heterozygotes. The following

primers were used to distinguish between b and s MHC haplotype: the forward primer (5'-GGCATTTCGTGTACCAGTTC-3') with the B6 allele-specific reverse primer (5'-TCCAGGATCTCCGGCTGG-3') amplified a 195 base pair product from the I-A^b B6 allele. The SJL allele-specific reverse primer (5'-CATCTACATAGACAGGTGTCTA-3') with the same forward primer resulted in amplification of an 85 base pair product from the IA^s SJL allele. All primers were custom synthesized (Sigma Genosys, The Woodlands, Tx, USA). PCR amplification was performed using a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) in 20 μl volumes using approximately 100 ng genomic DNA, 0.5 μM of each Primer, 0.2 mM of each dNTP, 2.5 Units Taq DNA Polymerase in PCR buffer containing 2mM $MgCl_2$ (all Qiagen Inc., Valencia, CA, USA). The following cycling conditions were used: initial denaturation for 3' at 94^0C followed by 35 cycles of denaturation at 94^0C (30"), annealing at 58^0C (30"), extension at 72^0C (50") and final extension at 72^0C for 10'. PCR products were electrophoresed on a 3% Agarose gel and stained with ethidium bromide. The MHC genotypes (s/s and b/s) were then correlated with disease susceptibility and severity.

Fluorescence activated cell sorter (FACS) analysis of ICOS expression

Lymph node cells or splenocytes were either analyzed *ex vivo* (0 hours) or cultured in medium alone or with 0.5 μg/ml anti-CD3 Ab (clone 145-2C11) (Pharmingen San Diego, CA) for 48 hours. Cells were stained with directly conjugated antibodies specific for the cell surface molecules CD4 (clone RM4-5), CD8 (clone 53-6.7) (Pharmingen San Diego, CA) and ICOS (McAdam et al., 2000) (Pharmingen San Diego, CA) or the appropriate matched isotype control IgG2b (clone A95-1) (Pharmingen San Diego, CA). Samples were analyzed on a FACSort (Becton

Dickenson, San Jose CA), calibrated with appropriately stained control cells, using CellQuest software (Becton Dickenson, San Jose CA). Data was analyzed using Flow Jo software (TreeStar, Inc.).

Statistics

The P values for the Incidence of clinical EAE and Mortality were calculated using the Fishers exact test. The Mann-Whitney U test was used to calculate the P values for day of onset and mean maximal score of clinical EAE and the Histology data. P values are mentioned only when statistically significant ($P<0.05$).

To assess linkage between the MHC haplotype and disease susceptibility both a $\chi 2$ test of independence and the likelihood ratio statistics using Mapmaker QTX (http://mcbio.med.buffalo.edu/mmQTX.html) were used to analyze the data. A P-value of 0.0001 (Lod 3.3) was chosen as a cutoff value for significant linkage.

Chapter 3

The MHC and background genes determine the requirement for B7 costimulation in EAE induction

Abstract

To study the role of B7 costimulatory molecules in a relapsing-remitting model of EAE, we backcrossed B7.1/ B7.2$^{-/-}$ mice onto the EAE susceptible SJL background. In spite of loss of B7.1 and B7.2 costimulatory molecules, SJL B7.1/B7.2$^{-/-}$ mice were susceptible to EAE by active immunization with PLP 139-151 and also with PLP 178-191. This is in contrast to previous observations showing that B7.1/B7.2$^{-/-}$ mice on the B6 background are resistant to MOG 35-55-induced EAE by active immunization. These data suggest that B7 costimulation is important in the induction phase of EAE on the B6 background, but not on the SJL background. This difference in EAE-susceptibility in the B7.1/B7.2$^{-/-}$ mice on the two backgrounds could be due to the genetic contribution of the backgrounds or the encephalitogenic peptides used for disease induction. Therefore, we explored the influence of the disease-inducing peptides and the genetic backgrounds on EAE-susceptibility. We generated (B6xSJL)F1 mice by crossing B6 and SJL mice. F1 wild-type (Wt) mice were susceptible to MOG- and PLP-induced EAE. In contrast, F1 B7.1/B7.2$^{-/-}$ mice were resistant to EAE induced with either MOG- or PLP- peptide, suggesting that the EAE-inducing peptide does not determine susceptibility versus resistance, but that the genetic background is the important determinant. To determine the genetic elements that influence disease susceptibility on the two genetic backgrounds, we generated Backcross 1 (BC1) B7.1/B7.2$^{-/-}$ mice by breeding SJL B7.1/B7.2$^{-/-}$ mice with F1 B7.1/B7.2$^{-/-}$ mice and then induced EAE by immunization with PLP 139-151. Genetic linkage analysis of the immunized BC1 B7.1/B7.2$^{-/-}$ mice revealed that EAE-

susceptibility is strongly linked to the MHC, though other genes also seem to determine the requirement for B7 costimulation in inducing EAE. Thus, the role for B7.1/B7.2 costimulation in regulating EAE varies with genetic background. Genetic modifiers of B7.1/B7.2 include MHC, but other genes outside the MHC are also important and remain to be identified. These data have important implications for developing B7 based immunotherapies for human diseases.

Introduction

The B7 costimulatory pathway has been shown to play a crucial role in the induction of autoimmunity (reviewed in (Anderson et al., 1999, and Jabs et al., in press), tumor immunity (Melero et al., 1997; Schlom and Hodge, 1999) and transplant rejection (Dai and Lakkis, 1999). Studies using B7 antagonists (such as CTLA-4Ig) have demonstrated that blockade of the B7-CD28/CTLA-4 pathway can ameliorate multiple autoimmune diseases in experimental animals and inhibit rejection of organ transplants and graft versus host disease. Similarly, over-expression of B7 molecules in various tumors leads to their rejection. Based upon these promising results in animal models, B7-based therapies are currently being investigated in humans for treating autoimmune diseases like MS and transplant rejection. Since most B7-based therapeutic approaches have been tested on inbred strains of mice that are genetically homogenous, it remains to be determined whether B7-blockade will be equally efficacious in humans that are genetically heterogenous. The importance of genetic heterogeneity is shown by several studies, which have demonstrated an important role for the genetic background in susceptibility versus resistance to EAE.

The role of genetic background in inducing EAE

The fact that only certain strains of mice are susceptible to EAE first suggested a role for genetic influences on disease susceptibility or resistance. Genetic studies of EAE and other autoimmune diseases conducted since the 1970's have identified many genetic loci that are linked to EAE susceptibility. MHC class II has been identified as a major susceptibility locus in many of these studies. The encephalitogenicity of specific MBP and PLP peptides has been shown to be MHC-restricted. However, even different mouse strains with the same MHC haplotype are not necessarily

equally susceptible to disease induced with the same peptide. For example SJL mice (H-2^s) are highly susceptible, whereas B10.s mice (also H-2^s) are resistant to PLP 139-151-induced EAE (Encinas et al., 1996). Thus, while MHC genes are clearly involved, genes outside the MHC are also important determinants of disease susceptibility.

Initial genetic studies of EAE took a candidate gene (or candidate locus) approach and attempted to correlate the inheritance of specific genetic polymorphisms with susceptibility to disease. Polymorphisms are allelic differences in genes that can be located in the intron, exon and/or untranslated (promoter/enhancer) regions of a gene. Depending on the location of the polymorphism, it can e.g. lead to differences in the level of protein expression (polymorphisms in untranslated regions) or to structural changes in the expressed protein (polymorphisms in exons). Polymorphisms can be responsible for significant changes in protein function, which can affect the induction of autoimmune diseases. Candidate gene approach studies have implicated a role for the MHC (specifically the allele at the I-A locus of the H-2 haplotytpe) in determining the epitope preference of the SJL and PL/J strains (Fritz et al., 1985). More recently, the technology of genome-wide scanning has made it possible to identify regions that associate with disease. This approach uses microsatellite markers which encompass the entire mouse genome at high density (Copeland et al., 1993; Dietrich et al., 1992; Dietrich et al., 1994). Microsatellite markers, also known as simple sequence length polymorphisms, consist of di-nucleotide (or tri- or tetra-nucleotide) repeats flanked by unique sequences, which are present at high density throughout the whole genome, and are highly polymorphic. With an appropriate set of markers one can compare different genetic backgrounds at a density of one marker per 10-20 cM. This relatively unbiased technique has been used successfully in the identification of

several disease-associated loci, and has begun to allow the systematic genetic analysis of complex traits, which have a polygenic pattern of inheritance.

Using this technique, several loci have been identified that show linkage with EAE disease incidence, severity, duration, onset and weight loss (Baker et al., 1995; Butterfield et al., 1999; Butterfield et al., 1998; Croxford et al., 1997; Encinas et al., 1996; Encinas et al., 2001; Sundvall et al., 1995). For example, EAE-related weight loss has been linked to a chromosome 2 locus, the development of paralysis has been linked to a chromosome 3 locus, and both development of paralysis and incidence of CNS inflammation have been linked to a locus on chromosome 8 (Encinas et al., 2001).

Several candidate genes have been identified that are linked to EAE susceptibility. The IL-2 locus has been closely linked to disease susceptibility, as have other loci, which include Th2 cytokine genes. One study has linked polymorphisms of the CTLA-4 gene to genetic susceptibility to human MS, suggesting that a dysregulation of CTLA-4 driven downregulation of T cell activation could be involved in the pathogenesis of MS (Ligers et al., 1999). The role for CTLA-4 in EAE susceptibility is yet to be determined.

In a number of autoimmune diseases, genome wide scans have shown that there is a genetic linkage to the CD28/CTLA-4/ICOS locus (on mouse chromosome 1). However, which of the costimulatory molecules within this locus affect disease susceptibility and the cellular mechanism by which the locus affects susceptibility/resistance to autoimmune diseases has not been clarified.

Differences between SJL and B6 EAE model

Is the B7-CD28/CTLA-4 pathway equally important in different models of EAE or do different genetic backgrounds and EAE models have different needs for B7 costimulation? To address this question, we compared the induction of EAE in B7.1/B7.2$^{-/-}$ mice on the SJL versus the B6 genetic background. Several differences distinguish these two models.

SJL mice share a number of autoimmune susceptibility loci with NOD mice (Encinas et al., 1999). SJL mice are of the IA^{s} haplotype and, when immunized with PLP 139-151, develop a relapsing-remitting type of EAE. Animals experience a moderate to severe acute episode of disease followed by remission and one or more relapses. PLP comprises 50% of CNS myelin (Folch, 1951) and is an abundant transmembrane protein on the surface of oligodendrocytes.

B6 mice are of the IA^{b} haplotype and, when immunized with MOG 35-55, they develop a chronic form of EAE. Animals get progressively worse after the initiation of disease and do not recover. MOG comprises only 0.01-0.05% of CNS myelin and is found on the outermost lamellae of the myelin sheath (Amiguet et al., 1992; Brunner et al., 1989; Mendel et al., 1995).

Thus, these two models differ in 1) genetic background, including MHC haplotype; 2) distinct encephalitogenic peptides used for EAE induction (PLP vs. MOG); 3) development of different forms of EAE (relapsing-remitting vs. chronic); 4) an exceedingly high precursor frequency of PLP 139-151 specific T cells in naïve SJL mice (Anderson et al., 2000).

To analyze whether these two models differ in the requirement for B7 costimulation for the induction of autoimmune disease, we induced EAE in B7.1/B7.2$^{-/-}$ mice on the

SJL and B6 backgrounds by immunization with PLP 139-151 or MOG 35-55, respectively.

In this chapter we demonstrate that 1) SJL B7.1/B7.2$^{-/-}$ mice were susceptible to EAE by active immunization with PLP 139-151 and also PLP 178-191. This is in contrast to B7.1/B7.2$^{-/-}$ mice on the B6 background, which were resistant to EAE by active immunization with MOG 35-55; 2) the differences between EAE-susceptibility versus resistance could not be attributed to changes in Th cell expansion or the cytokine profile; 3) the difference in EAE-susceptibility could not be attributed to differences in the encephalitogenic peptides used for the induction of disease on the two genetic backgrounds (PLP vs. MOG peptide); and 4) the difference in EAE-susceptibility seems to be due to the difference in the genetic background, in particular MHC class II.

Results

B7.1/B7.2$^{-/-}$ mice on the SJL background are susceptible to EAE.

Our observations regarding the role of B7.1/B7.2 costimulation in EAE induced by MOG 35-55 on the B6 genetic background have been published previously and are referred to as reference (Chang et al., 1999). In these studies, we showed that B7.1/B7.2 deficient mice on the B6 background do not develop EAE when immunized with the encephalitogenic peptide MOG 35-55. For a reprint of this paper, see Appendix A.

To examine the role of B7.1 and B7.2 in induction of relapsing-remitting EAE, we studied the development of EAE in SJL mice lacking both B7.1 and B7.2. The B7.1/B7.2$^{-/-}$ mice used in this study were originally generated on the 129 background (Borriello et al., 1997) and have been crossed onto the SJL genetic background for 5 to 6 generations.

Strikingly, SJL B7.1/B7.2$^{-/-}$ mice remained susceptible to EAE when immunized with either PLP 139-151 or another PLP-peptide, PLP 178-191 (Figure 3.1). PLP 178-191 is a second encephalitogenic antigen that induces EAE in the SJL strain. It induces EAE in SJL wild-type mice as well as PLP 139-151-peptide does, but differs from this antigen in that SJL mice do not have a high frequency of endogenous repertoire to PLP 178-191 as they have for PLP 139-151. EAE incidence and mortality were comparable in Wt and B7.1/B7.2$^{-/-}$ SJL mice in which EAE was induced with either PLP 139-151 or PLP 178-191 (Table 3.1). However, the EAE severity was lower and the day of onset was delayed in B7.1/B7.2$^{-/-}$ SJL mice immunized with either PLP 139-151 or PLP 178-191, as compared to Wt SJL mice (Table 3.1). Histological analyses revealed lesions both in the meninges and the parenchyma of the brains and

spinal cords of SJL B7.1/B7.2$^{-/-}$ mice, with modestly reduced lesion-numbers compared to the Wt mice (Table 3.1). These data are in contrast to the results obtained with the B6 B7.1/B7.2$^{-/-}$ mice on the B6 background, which are resistant to clinical EAE and have only very few lesions in the CNS (Chang et al., 1999). Taken together, these results demonstrate that, unlike B6 B7.1/B7.2$^{-/-}$ mice, SJL B7.1/B7.2$^{-/-}$ mice remain susceptible to EAE. The lack of both B7.1 and B7.2 molecules in SJL B7.1/B7.2$^{-/-}$ mice leads to only slightly milder clinical EAE course, with modestly reduced CNS inflammatory foci as compared to Wt controls.

Markedly impaired proliferative responses, but abundant IFNγ production by the LNCs of SJL B7.1/B7.2$^{-/-}$ mice.

To determine whether the proliferative responses of T cells were altered in SJL mice lacking both B7.1 and B7.2, we immunized B7.1/B7.2$^{-/-}$ mice and Wt SJL mice with PLP 139-151 and isolated the draining LNCs ten days following immunization. Lymph node cells were restimulated *in vitro* with PLP 139-151 and/or a control peptide, and proliferative responses and cytokine production were determined (Figure 3.2). Proliferative responses of B7.1/B7.2$^{-/-}$ LNCs were markedly impaired compared to the Wt (Figure 3.2.A). Even at the highest peptide concentrations examined, proliferation of B7.1/B7.2$^{-/-}$ LNCs was only 10% of Wt levels. Similarly reduced proliferative responses were observed in SJL B7.1/B7.2$^{-/-}$ mice immunized with PLP 178-191 (data not shown). However, even though B7.1/B7.2$^{-/-}$ cells proliferated very little, they still produced levels of IFNγ comparable to Wt cells (Figure 3.2.B). No IL-2 production could be detected in LNCs from B7.1/B7.2$^{-/-}$ mice, whereas IL-2 production could be detected in the culture supernatant of Wt mice (Figure 3.2.C). IL-

4 and IL-10 were below detection limits in both the Wt and B7.1/B7.2$^{-/-}$ mice (data not shown).

These data are consistent with data on B7.1/B7.2$^{-/-}$ mice on the B6 background. LNCs from B6 B7.1/B7.2$^{-/-}$ mice proliferate poorly when restimulated with MOG 35-55, but produce abundant IFNγ, comparable to Wt cells (Chang et al., 1999).

Breeding scheme for generating F1 progeny

The difference in susceptibility of SJL and B6 B7.1/B7.2$^{-/-}$ mice could be due to differences in the EAE inducing peptides (PLP versus MOG) or the genetic background (SJL versus B6). To address this issue we generated F1 mice. SJL and B6 mice were crossed to generate F1 progeny (for the breeding scheme, see Figure 3.3). We crossed Wt female SJL with male B6 (F1 SJL) and also Wt female B6 with male SJL (F1 B6). SJL mice are of white coat color, B6 mice are of black coat color. The cross of these mice yielded F1 progeny of brown coat color. SJL mice are of MHC genotype H-2^{s} (s/s), B6 mice are H-2^{b} (b/b), therefore the F1 progeny was of the genotype s/b.

F1 wild-type mice are susceptible to EAE induced with either PLP 139-151 or MOG 35-55 peptide

F1 Wt (B7.1/B7.2$^{+/+}$) mice were immunized with PLP 139-151 or MOG 35-55 to test for susceptibility to EAE disease. Following immunization with PLP 139-151, F1 Wt mice from both crosses, F1 SJL and F1 B6, developed EAE with very similar severity, onset of disease and maximum disease score (Figure 3.4.A). However, EAE disease was less severe than in SJL Wt control mice. B6 Wt mice did not, as expected, develop EAE with the PLP-peptide. Following immunization with MOG 35-55, the

F1 mice developed EAE very similarly, especially until day 20 post-immunization (Figure 3.4.B). After day 20 post-immunization, F1 B6 (derived from male SJL mice crossed with female B6) disease was slightly more severe. Disease development was very similar to MOG-peptide-induced EAE in B6 Wt mice. SJL mice did not, as expected, develop EAE with MOG peptide.

Our results demonstrate that F1 Wt mice are susceptible to EAE when immunized with either PLP 139-151 or MOG 35-55, although the disease is slightly less severe than in SJL or B6 Wt mice, respectively (Figure 3.4). Since the disease course of F1 SJL mice and F1 B6 mice with either encephalitogenic peptide was very similar, we excluded the influence of significant X-linked-effects. All further matings were thus conducted breeding female B6 mice with male SJL mice, because of breeding difficulties with SJL mothers.

Histologically, F1 progeny that are immunized with PLP 139-151 develop slightly fewer lesions in the CNS than SJL/Wt mice (Table 3.2.A). In contrast, F1 progeny immunized with MOG 35-55 have more lesions in the CNS than B6/Wt mice (Table 3.2.B).

F1 B7.1/B7.2$^{-/-}$ mice are resistant to EAE induced with either PLP 139-151 or MOG 35-55 peptide

Since F1 Wt mice were susceptible to EAE with either the PLP or MOG peptide, we could determine whether the difference in susceptibility to disease in the B7.1/B7.2$^{-/-}$ mice on the SJL or B6 background was due to the encephalitogenic peptide or the genetic background. We crossed male SJL B7.1/B7.2$^{-/-}$ mice with female B6 B7.1/B7.2$^{-/-}$ mice to generate F1 B7.1/B7.2$^{-/-}$ mice. These mice were immunized with either PLP 139-151 or MOG 35-55 and observed for the development of EAE.

Strikingly, the majority of the F1 B7.1/B7.2$^{-/-}$ mice were resistant to EAE when immunized with either peptide (Figure 3.5 and Table 3.2). The mice that did develop disease experienced very mild clinical EAE with delayed onset of disease and reduced mean maximal score (Table 3.2 A and B). By histological analysis, 100% of F1 B7.1/B7.2$^{-/-}$ mice immunized with PLP 139-151 and 87% of F1 B7.1/B7.2$^{-/-}$ mice immunized with MOG 35-55 did have lesions in the brain and spinal cord, but the numbers of foci in meninges and parenchyma were significantly lower compared to the F1Wt mice (Table 3.2 A and B).

Lymph node cells from F1 B7.1/B7.2$^{-/-}$ mice proliferate poorly, but produce IFNγ after stimulation with either PLP 139-151 or MOG 35-55.

To examine T cell proliferation and cytokine production in F1 B7.1/B7.2$^{-/-}$ mice, we immunized F1 B7.1/B7.2$^{-/-}$ and Wt mice with PLP 139-151 or MOG 35-55, harvested draining LNCs ten days after immunization, and restimulated the draining LNCs with PLP 139-151 or MOG 35-55 or control peptides (IA^{s}-binding NASE 101-120 or IA^{b}-binding OVA 323-336) *in vitro*. The proliferation of F1 Wt LNCs to PLP 139-151 (Figure 3.6.A) and MOG 35-55 (Figure 3.6.B) was not significantly different when compared to SJL or B6 Wt LNCs, respectively. LNCs from B6 Wt mice did not proliferate to the PLP peptide and SJL Wt derived LNCs did not proliferate to the MOG peptide. However, the proliferative responses of F1 B7.1/B7.2$^{-/-}$ LNCs to either the PLP or the MOG peptide were markedly reduced (Figure 3.6.A, B) compared to F1 Wt LNCs.

LNCs from F1 B7.1/B7.2$^{-/-}$ mice produced levels of IFNγ comparable to SJL and B6 Wt cells when restimulated with PLP 139-151 or MOG 35-55, respectively (Figure 3.7.A, B). SJL Wt LNCs that were restimulated with MOG 35-55 and B6 Wt LNCs

that were restimulated with PLP 139-151 did not produce any detectable IFNγ. Therefore, although F1 B7.1/B7.2$^{-/-}$ mice are resistant to induction of EAE with either encephalitogenic PLP or MOG peptide, resistance to EAE induction does not correlate with lack of IFNγ production.

After restimulation with PLP 139-151, LNCs from SJL Wt produced IL-2 (max. 2870 pg/ml), but no IL-4 or IL-10. LNCs from B6 Wt mice did not produce any IL-2, IL-4 or IL-10. LNCs from F1 Wt mice produced IL-2 (max. 770 pg/ml) and IL-10 (max. 160 pg/ml), but no IL-4. Similarly to LNCs from B6 Wt mice, LNCs from F1 B7.1/B7.2$^{-/-}$ mice did not produce significant amounts of IL-2, IL-4 or IL-10 after restimulation with PLP 139-151 (data not shown).

After restimulation with MOG 35-55, LNCs from SJL Wt, B6 Wt or F1 B7.1/B7.2$^{-/-}$ mice did not produce IL-2, IL-4 or IL-10. Only LNCs from F1 Wt mice produced IL-2 (max. 120 pg/ml) and IL-10 (max. 130 pg/ml), but no IL-4.

Breeding scheme for generating BC1 B7.1/B7.2$^{-/-}$ mice

As a first step in identifying genetic elements that may be responsible for the difference in EAE susceptibility in the B7.1/B7.2$^{-/-}$ SJL mice, we backcrossed female F1/B7.1/B7.2$^{-/-}$ with male SJL B7.1/B7.2$^{-/-}$ mice to generate Backcross 1 (BC1) B7.1/B7.2$^{-/-}$ progeny for genetic analysis (for the breeding scheme see Figure 3.3). F1/B7.1/B7.2$^{-/-}$ mice are of brown coat color, SJL B7.1/B7.2$^{-/-}$ mice are of white coat color. Of the 95 BC1 B7.1/B7.2$^{-/-}$ mice, 52 mice were of white, 38 were of brown, 4 were of agouti and 1 was of black coat color. On average, the BC1 mice have a random 50% of the genome homozygous and identical to the parental strain (SJL B7.1/B7.2$^{-/-}$), and the other 50% will be heterozygous and a hybrid of the two parental

strains (SJLxB6 B7.1/B7.2$^{-/-}$). In the case of the MHC, 50% of all BC1 B7.1/B7.2$^{-/-}$ mice will be s/s genotype and 50% will be s/b genotype.

EAE in BC1 B7.1/B7.2$^{-/-}$ mice

A total of 95 BC1 B7.1/B7.2$^{-/-}$ mice were obtained and immunized to induce EAE, using PLP 139-151. Animals were scored for clinical EAE and, as a second measure of EAE severity, mice were also weighed daily. Weight loss occurs prior to paralysis and is thought to be a consequence of the release of inflammatory mediators (particularly TNFα) into the circulation, causing loss of appetite and dehydration. After paralysis begins, inability to reach food and water also contributes to weight loss. At the end of the experiments, brains and spinal cords were isolated for histological analysis and liver DNA was obtained from 88 (the livers from 7 mice could not be obtained) of the BC1 B7.1/B7.2$^{-/-}$ mice for genetic analysis (see below).

Cumulative results of EAE in control and BC1 B7.1/B7.2$^{-/-}$ mice are shown in Figure 3.8.A and Table 3.3. The BC1 progeny showed about 66% (63 out of 95 BC1 B7.1/B7.2$^{-/-}$ mice tested) disease incidence. The average day of onset of disease in BC1 B7.1/B7.2$^{-/-}$ mice was later, compared to SJL Wt mice, but similar to the later onset of disease of SJL B7.1/B7.2$^{-/-}$ mice (Table 3.3). Mortality and mean maximal score was lower than SJL Wt and B7.1/B7.2$^{-/-}$ controls (Table 3.3). However, analyzing the disease development in individual mice, striking differences could be observed. Some BC1 B7.1/B7.2$^{-/-}$ mice got very severe disease (similar to SJL wild-type mice) and some only developed very mild or no clinical disease (similar to B6 and F1 B7.1/B7.2$^{-/-}$ mice). BC1 B7.1/B7.2$^{-/-}$ mice were thus categorized into three groups, according to disease severity. Group 1 including mice of EAE score 0, Group 2 including mice of EAE scores 0.5-2 (mild/medium disease) and group 3 including

mice of EAE scores 2.5-5 (severe disease). Categorizing the mice into these groups was important for our linkage analysis (see below).

In general, weight loss correlated with EAE disease development. BC1 B7.1/B7.2$^{-/-}$ mice lost less weight than SJL Wt and B7.1/B7.2$^{-/-}$ mice (Figure 3.8.B), correlating with less severe disease in these mice. The greatest weight loss could be observed at the peak of disease (around day 18 post-immunization), followed by weight gain during disease remission. Surprisingly, B6 B7.1/B7.2$^{-/-}$ mice lost weight between days 7 and 8 post-immunization, but gained weight from day 9 post-immunization on, without having developed clinical signs of EAE.

Cumulative histological data show that PLP-peptide immunized BC1 B7.1/B7.2$^{-/-}$ mice have significantly more lesions in the CNS than F1 B7.1/B7.2$^{-/-}$ mice, and a comparable number of lesions in the CNS to SJL/Wt and SJL B7.1/B7.2$^{-/-}$ mice (Table 3.3). Interestingly, individual histological analysis of the brains and spinal cords from SJL B7.1/B7.2$^{-/-}$ mice and especially BC1 B7.1/B7.2$^{-/-}$ mice revealed a high incidence of edema associated with the lesions (data not shown). In SJL Wt mice which develop severe clinical signs of EAE, no such edema were detected. In addition, analysis of the CNS from BC1 B7.1/B7.2$^{-/-}$ mice revealed vacuolation associated with demyelination. These differences in histological EAE might indicate different mechanisms of disease pathology in Wt and B7.1/B7.2$^{-/-}$ mice.

Identification of genetic elements that contribute to disease susceptibility in the B7.1/B7.2$^{-/-}$ SJL mice.

Liver DNA was obtained from 88 out of 95 BC1 mice (and also from control mice) at the end of the experiment. The genomic DNA was subjected to genetic analysis by utilizing PCR primers that distinguish between SJL (IA^{s}) and B6 (IA^{b}) MHC

haplotypes. Since the MHC is known to positively select an autoreactive PLP 139-151-T cell repertoire (Anderson et al., 2000) which contributes to EAE susceptibility, we first analyzed the role of the SJL versus B6 MHC genotype in susceptibility to EAE in the B7.1/B7.2$^{-/-}$ mice. The MHC genotype of the BC1 mice was compared to the disease susceptibility in regard to incidence (Table 3.4) and severity (Table 3.5).

Linking EAE incidence with MHC haplotype. Using Chi square analysis, the data showed very significant linkage ($P< 5x10^{-8}$) of the disease incidence (susceptibility) in the BC1 B7.1/B7.2$^{-/-}$ mice with SJL derived MHC (Table 3.4). When the disease incidence in the s/s and s/b MHC haplotypes in the BC1 was compared with the parent s/s (SJL) and s/b (F1) B7.1/B7.2$^{-/-}$ mice, the incidence in the BC1 B7.1/B7.2$^{-/-}$, s/s genotype was similar to the parent SJL B7.1/B7.2$^{-/-}$ mice (88% vs. 91%). However, when the incidence of BC1 B7.1/B7.2$^{-/-}$ s/b genotype was compared to the parent F1 (B6 x SJL) B7.1/B7.2$^{-/-}$, disease incidence was higher (32% vs. 17%). These data suggest that the SJL derived (s/s) MHC haplotype might contribute to disease susceptibility in the B7.1/B7.2$^{-/-}$ mice.

Linking EAE severity to MHC haplotype. To further support the linkage data between the MHC genotype and disease in the B7.1/B7.2$^{-/-}$ BC1 mice, we analyzed the MHC genotype data with the severity of disease using disease severity (score) as a quantitative trait. As shown in Tables 3.4 and 3.5, mice with a homozygous s/s genotype in the B7.1/B7.2$^{-/-}$ BC1 mice, not only had higher disease incidence but also had more severe EAE than the heterozygous b/s, suggesting a significant linkage of SJL MHC (s/s) haplotype to the severity of EAE in B7.1/B7.2$^{-/-}$ mice.

Segregating BC1 s/s versus s/b. We further compared the disease course in the SJL (s/s) B7.1/B7.2$^{-/-}$, B6 (b/b) B7.1/B7.2$^{-/-}$, and F1 (s/b) B7.1/B7.2$^{-/-}$ to the BC1 (s/s and s/b) B7.1/B7.2$^{-/-}$ mice. The mean disease score profile plotted over time of all BC1

mice was intermediate between the F1 B7.1/B7.2$^{-/-}$ and SJL B7.1/B7.2$^{-/-}$ (Figure 3.9). When the disease score profile of the BC1 mice was segregated based on s/s or s/b genotype, it became clear, that the BC1 B7.1/B7.2$^{-/-}$ mice bearing the b/s-genotype had a lower group disease score and BC1 B7.1/B7.2$^{-/-}$ mice bearing the s/s-genotype had a higher mean group disease score profile when compared to the whole BC1 group (Figure 3.9). This further confirms the data presented in Table 3.4 and 3.5, that the MHC class II genotype contributes to disease incidence and severity in the B7.1/B7.2$^{-/-}$ mice. However, the disease profiles presented in Figure 3.9 show that s/s genotype of BC1 mice do not get as severe disease as the parent SJL (s/s) B7.1/B7.2$^{-/-}$ mice, and that s/b BC1 B7.1/B7.2$^{-/-}$ get a slightly more severe disease than the parent F1 B7.1/B7.2$^{-/-}$ mice, suggesting that besides IA^{s}, additional genes must contribute to the disease susceptibility in the SJL (s/s) B7.1/B7.2$^{-/-}$ mice. These linkage data show that in the absence of B7-costimulation, MHC haplotype of the individual might play a major role in overcoming a need for B7-costimulation to induce autoimmunity.

Discussion

B7.1/B7.2$^{-/-}$ mice on the SJL background remain susceptible to EAE, in contrast to B7.1/B7.2$^{-/-}$ mice on the B6 background which are relatively resistant to the development of clinical EAE. Thus, there are differences in the requirement for B7 costimulation in EAE development on two different backgrounds, SJL versus B6 mice. Our model of susceptible SJL B7.1/B7.2$^{-/-}$ mice and resistant B6 B7.1/B7.2$^{-/-}$ mice gives us a unique tool to investigate, which genes compensate for the loss of B7 costimulation in the induction of EAE.

SJL susceptibility versus B6 resistance

The EAE susceptibility of SJL B7.1/B7.2$^{-/-}$ mice was unexpected and contrasts with the EAE resistance of B6 B7.1/B7.2$^{-/-}$ mice. However, EAE-susceptibility of SJL B7.1/B7.2$^{-/-}$ mice agrees with previous antibody studies, demonstrating that EAE in SJL mice cannot be inhibited by blocking both B7 molecules by co-administration of anti-B7.1 and anti-B7.2 monoclonal antibodies (mAbs) (Kuchroo et al., 1995; Perrin et al., 1996).

SJL mice are a highly autoimmune prone disease strain, and SJL Wt mice develop very severe EAE when immunized with PLP 139-151 (Tuohy et al., 1989). As mentioned before, SJL mice have a very high frequency of PLP 139-151 reactive T cells in the naïve repertoire, which seem to belong to a memory pool of T cells (Anderson et al., 2000). T cells of a memory phenotype have been shown to be less dependent on costimulation than naïve T cells (Kuiper et al., 1994; London et al., 2000). Thus, PLP 139-151 reactive cells may be more readily activated even in the absence of B7.1/B7.2 costimulation and induce EAE in the SJL B7.1/B7.2$^{-/-}$ mice. To

determine whether EAE susceptibility of SJL B7.1/B7.2$^{-/-}$ mice depended upon a high frequency of the PLP 139-151 reactive T cells, we immunized SJL mice with a second encephalitogenic epitope of PLP, PLP 178-191, which binds to IA^{s} with similar affinity, but does not have an expanded repertoire *in vivo* in SJL Wt mice. The SJL B7.1/B7.2$^{-/-}$ mice, when immunized with this peptide, were also susceptible to EAE. These data suggest that either 1) the endogenous repertoire of PLP 139-151-reactive cells is not essential to the susceptibility of SJL B7.1/B7.2$^{-/-}$ mice to EAE or 2) due to the loss of B7.1 and B7.2, SJL B7.1/B7.2$^{-/-}$ mice have generated a high frequency of PLP 178-191 reactive cells, thus making them susceptible to disease induced with this peptide, even in the absence of B7 costimulation. To test this directly, naïve SJL B7.1/B7.2$^{-/-}$ mice should be analyzed for the frequency of PLP 139-151- and PLP 178-191-reactive T cells in the naïve repertoire (the way in which this may be approached is further discussed in Chapter 4).

The encephalitogenic peptide does not determine susceptibility versus resistance

The EAE susceptibility of SJL B7.1/B7.2$^{-/-}$ mice and EAE resistance of B6 B7.1/B7.2$^{-/-}$ mice raised the question of whether this difference is due to the genetic background or to the encephalitogenic peptides used for the induction of EAE. Different peptides have different characteristics, because they can vary in length, sequence and charge. Therefore, each MHC molecule binds a unique set of peptides.

B6 Wt mice do not develop EAE when immunized with PLP 139-151. Draining LNCs from these mice don't proliferate when restimulated *in vitro* with PLP-peptide, and conversely draining LNCs from MOG 35-55-immunized SJL Wt mice do not proliferate when restimulated with MOG 35-55 *in vitro*. This suggests that the IA^{s}

MHC molecule cannot successfully present the MOG-peptide, nor can the IA^b MHC molecule present the PLP-peptide.

To investigate the role of the encephalitogenic peptide in EAE, we generated F1 progeny by crossing SJL (IA^s) and B6 (IA^b) mice. MHC class II molecules are heterodimers consisting of one α and one β chain, and thus heterologous molecules containing one maternal-derived and one paternal-derived chain are produced. Each F1 ($IA^{s/b}$) mouse is expected to express on average about 25% of each of the four different MHC class II haplotypes s/s, b/b, s/b (symbolizing: α chain from SJL, and β chain from B6 genetic background) and b/s (symbolizing: α chain from B6, and β chain from SJL genetic background). The background (non-MHC) genes in F1 mice are all s/b. We assumed that these haplotypes should be able to bind both PLP and MOG peptide. The F1 Wt and B7.1/B7.$2^{-/-}$ mice were then tested for EAE susceptiblility by immunizing them with PLP 139-151 and MOG 35-55.

We found that EAE can be induced in F1 Wt mice with either PLP- or MOG-peptide. In addition, draining LNCs from these mice proliferated when restimulated *in vitro* with either of the MHC binding peptides. Each F1 ($IA^{s/b}$) mouse expresses the MHC class II haplotypes s/s (25%), b/b (25%), s/b (25%) and b/s (25%). Thus, each F1 mouse expresses homozygous IA^s and IA^b haplotypes. Is it likely that in PLP-peptide-immunized F1 mice only IA^s MHC molecules present the PLP-peptide, and similarly, only IA^b MHC molecules present the MOG-peptide in MOG-peptide-immunized mice. Our EAE-disease data shows, that F1 Wt mice develop EAE induced with PLP- or MOG-peptide with a lower severity than SJL or B6 Wt mice, respectively (Figure 3.4 and 3.5). However, the frequency of disease is comparable between the F1 and Wt mice, so although homozygous haplotypes may only be expressed at a low percentage (25%) they can be sufficient for EAE induction.

In contrast to F1 Wt mice, the F1 B7.1/B7.2$^{-/-}$ mice were resistant to the development of EAE with either PLP- or MOG-peptide. This suggests that the striking difference in susceptibility of SJL B7.1/B7.2$^{-/-}$ mice versus resistance in B6 B7.1/B7.2$^{-/-}$ mice was not due to different immunogens, but to other genetic differences.

This hypothesis is further substantiated by testing B7.1/B7.2-deficient mice on the NOD background for the development of acute EAE induced by PLP 56-70. The blockade of the B7-CD28 pathway on the NOD background either by using CD28$^{-/-}$ mice or by treating Wt mice with anti-B7.1/B7.2 mAbs results in significantly reduced EAE severity and delayed disease induction (Girvin et al., 2000). The myelin peptide-specific T cells were shown to be effectively primed, however the peptide-specific delayed-type hypersensitivity responses were significantly decreased, suggesting a critical role for B7-CD28 costimulation in *in vivo* trafficking and systemic immunity (Girvin et al., 2000). Thus differences in the susceptibility of B7.1/B7.2 deficient mice to EAE on the three different genetic backgrounds suggests that the requirement for B7 costimulation to induce autoimmunity will vary depending upon the genetic background of the individual.

Previous observations suggest that susceptibility to EAE in SJL mice is a polygenic disease and multiple loci contribute to disease susceptibility (Encinas et al., 1996). Of interest is that loci on Chromosome 1 and Chromosome 16 which include CD28/CTLA-4/ICOS and B7.1/B7.2, respectively, show linkage to disease susceptibility (Encinas et al., 1996) (and unpublished data). It is not yet clear whether any polymorphisms exist in the B7 costimulatory ligands and/or receptors on SJL versus B6 backgrounds and whether such polymorphisms could contribute to the functional differences observed here.

The influence of MHC on susceptibility versus resistance

Our finding that the encephalitogenic peptide does not determine susceptibility versus resistance gave impetus to investigate the genetic basis for susceptibility to EAE in SJL mice in the absence of B7.1/B7.2 costimulation.

In our study, we are combining the classical genetic approach with the approach of a genome-wide screen for susceptibility loci. The work presented in this chapter reflects the first step, the classical approach to link genetics with susceptibility. This approach is used to study the association between a known polymorphic gene (in our case: MHC) and a trait (in our case: susceptibility to EAE).

We have set up a test cross between susceptible (SJL B7.1/B7.2$^{-/-}$) and resistant (B6 B7.1/B7.2$^{-/-}$) strains, yielding F1 progeny. The F1 mice were then crossed with one of the parental strains (the susceptible SJL strain B7.1/B7.2$^{-/-}$), to yield backcross 1 progeny (BC1 B7.1/B7.2$^{-/-}$). Due to the random assortment of chromosomes and the occurrence of crossovers during meiosis, the BC1 progeny will have some of their genes from the susceptible parent and the rest from the resistant parent. Thus every BC1 mouse will have an average of a random 50% of the genome homozygous and identical to the parental strain (SJL B7.1/B7.2$^{-/-}$), and the complimentary 50% will be heterozygous and a hybrid of the two parental strains (SJLxB6 F1 B7.1/B7.2$^{-/-}$). In the case of the MHC, 50% of all BC1 B7.1/B7.2$^{-/-}$ mice will have the s/s genotype and 50% will have the s/b genotype. If there is a significant correlation between phenotype and genotype, the gene studied can be said to be linked with the gene controlling the trait.

The BC1 progeny was immunized with PLP 139-151 and the susceptible versus resistant mice were tested for MHC (s/s or b/s) genotype. Thus we could study the linkage of the candidate gene (MHC) to EAE susceptibility. We have found a

significant linkage between the incidence and severity of EAE (phenotype) and MHC (genotype). Therefore, the MHC is strongly linked to susceptibility in mice deficient in B7.1/B7.2 expression. Our data would suggest that SJL derived MHC ($H\text{-}2^s$) is crucial in overcoming the need for B7.1/B7.2-costimulation for the induction of EAE in the $B7.1/B7.2^{-/-}$ mice or, alternatively, that the presence of the $H\text{-}2^b$ allele is protective.

Thus, the genetic approach has enabled us to identify one of the genes that is responsible for the difference in susceptibility versus resistance in SJL $B7.1/B7.2^{-/-}$ mice versus B6 $B7.1/B7.2^{-/-}$ mice. How can the MHC haplotype influence the requirement for B7 costimulation in the induction of EAE? In order to answer this question we need to consider 1) the strength of MHC signaling; and 2) the influence of MHC on the generation of the endogenous repertoire.

Strength of MHC signaling. Previous *in vitro* observations have shown that the intensity and magnitude of signal 1 (MHC-peptide-TCR) can overcome the need for signal II (costimulation). It is possible that certain MHC haplotypes (e.g. IA^s) can mediate a stronger signal to the TCR than other MHC haplotypes (e.g. IA^b). Previous data have demonstrated successful induction of EAE in $CD28^{-/-}$ mice on the B6 or B10.PL background by increasing the dose and number of immunizations with the encephalitogenic peptides (Chitnis et al., 2001; Oliveira-dos-Santos et al., 1999). Thus, a stronger signal might be mediated by the IA^s haplotype. This hypothesis is supported by previous data, where EAE could be successfully induced in $CD28^{-/-}$ mice on the B6 or B10.PL background by amplifying signal 1 through increasing the dose and number of immunizations with the encephalitogenic peptides (Chitnis et al., 2001; Oliveira-dos-Santos et al., 1999).

Endogenous repertoire. SJL mice have been associated with the development of multiple autoimmune diseases and have been implicated in positively selecting a self reactive T cell repertoire. According to the Avidity model of thymic selection (Ridgway et al., 1999), the characteristics of the MHC haplotype, including MHC stability, MHC density and MHC-peptide affinity can influence the positive selection of thymocytes. For example, NOD mice express IA^{g7}, which was found to be a poor peptide binder and to have structural instability (Carrasco-Marin et al., 1996). These features seem to be responsible for the defective thymic selection mediated by the IA^{g7} haplotype (Ridgway and Fathman, 1998; Ridgway et al., 1998), which seems to cause the selection of a T cell population with an increased mean affinity for self peptide-MHC and thus leads to increased T cell autoreactivity in NOD mice (Kanagawa et al., 1998). It is possible that the characteristics of the IA^{s} haplotype, similarly to IA^{g7}, cause the generation of a greater population of self-reactive T cells with a high-affinity TCR.

Thus, a combination of a larger self reactive repertoire in the naïve SJL mouse, together with increasing the TCR affinity of self-reactice T cells might contribute to overcoming the requirement for B7.1/B7.2-costimulation in the induction of EAE in the SJL B7.1/B7.$2^{-/-}$ mice.

The segregation of all BC1 B7.1/B7.$2^{-/-}$ mice into BC1 B7.1/B7.$2^{-/-}$ (s/s) mice and BC1 B7.1/B7.$2^{-/-}$ (s/b) mice allowed us to look at these populations separately.

BC1 B7.1/B7.$2^{-/-}$ (s/s) mice. BC1 B7.1/B7.$2^{-/-}$ (s/s) mice express homozygous s/s MHC haplotype, but their background (non-MHC) genes are 50% s/s and 50% s/b. As demonstrated in Figure 3.9, BC1 B7.1/B7.$2^{-/-}$ (s/s) mice do not develop as severe EAE as SJL B7.1/B7.$2^{-/-}$ (s/s) mice. The difference between BC1 (s/s) and SJL (s/s) is in

their non-MHC genes, where BC1 have a lower percentage of SJL-derived genes (50% s/s and 50% s/b) than SJL mice (100% s/s). Thus, the influence of genes derived from the B6 background might render the BC1 B7.1/B7.2$^{-/-}$ mice less susceptible to PLP-peptide induced EAE than SJL B7.1/B7.2$^{-/-}$mice. Thus, in addition to H-2^{b}, other genes coming from B6 mice might play a role in disease resistance.

BC1 B7.1/B7.2$^{-/-}$ (s/b) mice. BC1 B7.1/B7.2$^{-/-}$ (s/b) mice express homozygous s/s and b/b haplotypes and also heterozygous s/b and b/s haplotypes. The background (non-MHC) genes are 50% s/s and 50% s/b. As demonstrated in Figure 3.9, BC1 B7.1/B7.2$^{-/-}$ (s/b) mice develop slightly more severe EAE than F1 B7.1/B7.2$^{-/-}$ (s/b) mice. The difference between BC1 and F1 mice is in their non-MHC genes, where BC1 mice have a higher percentage of SJL background (50% s/s and 50% s/b) than F1 mice (100% s/b). Thus, it is plausible that additional (background) genes derived from the SJL background may increase susceptibility to EAE in the BC1 B7.1/B7.2$^{-/-}$ mice.

Thus, our analysis suggests that background genes outside the MHC also influence EAE susceptibility, since MHC is not linked 100% to EAE-susceptibility. This hypothesis is further supported by our B7 blocking studies using CTLA-4Ig in two SJL mouse strains derived from two different breeding colonies. Harlan SJL mice were protected from EAE after treatment with CTLA-4Ig, whereas Jackson SJL mice were still susceptible to EAE (data not shown). Jackson SJL mice were also used as breeders for B7 deficient mice, which confirms that these mice can develop EAE independently from B7 expression. Interestingly, Harlan and SJL mice seem to differ in their requirement for B7 costimulation, even though they express the same MHC and also express mostly the same background genes. Through breeding the same mouse strain in different facilities though, these mice could have experienced a

"genetic drift", causing slight changes in the genetic background. These slight differences may affect susceptibility versus resistance to EAE in a B7 deficient environment.

These data suggest that, although the MHC has an important role in overcoming the need for B7.1/B7.2 costimulation in the induction of EAE, additional genes outside the MHC must also influence EAE susceptibility. These genes outside MHC might work additively or by epistatic interaction with the MHC to determine a susceptible or resistant phenotype in the B7.1/B7.2$^{-/-}$ SJL vs. B6 mice.

In the future we plan to extend our studies using the approach of a genome-wide screen for susceptibility loci. We are planning to test the DNA of BC1 B7.1/B7.2$^{-/-}$ mice using microsatellites to identify additional genes that might be responsible for the difference in EAE susceptibility.

The studies in this chapter demonstrate that 1) SJL B7.1/B7.2$^{-/-}$ mice are susceptible to EAE induced by PLP 139-151, which is in contrast to B6 B7.1/B7.2$^{-/-}$ mice that are resistant to EAE induced by MOG 35-55; 2) F1 B7.1/B7.2$^{-/-}$ mice are resistant to the development of clinical EAE with either PLP 139-151 or MOG 35-55 encephalitogenic peptides, whereas F1 Wt mice are susceptible to EAE induced by either of these peptides, suggesting that the disease-inducing peptide does not determine EAE-susceptibility and that the resistance to EAE seen in the B6 B7.1/B7.2$^{-/-}$ mice is dominant; 3) Analysis of the BC1 mice generated by crossing F1 B7.1/B7.2$^{-/-}$ with SJL B7.1/B7.2$^{-/-}$ mice demonstrated a significant linkage between inheritance of MHC from the SJL mice with susceptibility to EAE in the B7.1/B7.2 double deficient mice; and 4) linkage of EAE susceptibility to MHC haplotype is not 100%, supporting evidence from other studies that non-MHC genes are involved in

determining EAE susceptibility, and suggesting these effects can also be detected in B7.1/B7.2$^{-/-}$ mice.

These results suggest that the genetic background modulates the requirement for B7.1/B7.2 costimulation and inheritance of appropriate MHC might overcome the requirement of B7-costimulation for the induction of EAE.

Considering that humans are outbred, these results raise the possibility that different individuals may have distinct requirements for costimulatory molecules in the selection of an autoreactive repertoire and in the induction of autoimmune disease. These genetic differences in the requirements for costimulatory molecules may have profound effects on the efficacy of B7-based immunotherapies currently being investigated for the treatment of human autoimmune diseases and other immune-mediated disorders. It may be that certain MHC alleles and/or other genes will modify efficacy of B7.1/B7.2 blockade in the treatment of MS. Thus, the identification of certain MHC and/or other genes may be useful diagnostically in determinig which patients will benefit from B7.1/B7.2 blockade. Since resistance to EAE is dominant in B7.1/B7.2$^{-/-}$ F1 mice, it is possible that B7 blockade will be beneficial in many populations.

Chapter 4

Distinct roles for B7.1 and B7.2 during the induction and effector phases of EAE in SJL mice

Abstract

To compare the roles of B7.1 and B7.2 in a relapsing-remitting model of EAE, we have backcrossed B7.1$^{-/-}$ and B7.2$^{-/-}$ mice onto the EAE susceptible SJL background, and examined the effect of B7.1 or B7.2 deficiency on the induction and effector phases of PLP 139-151-induced EAE.

When immunized with PLP 139-151, SJL B7.1$^{-/-}$ mice developed exacerbated PLP 139-151-induced disease in comparison to wild-type (Wt) controls. Exacerbation of EAE might be partly due to an increased production of the Th1 cytokine IFNγ by draining LNCs. SJL B7.2$^{-/-}$ mice developed only modestly exacerbated EAE and produced comparable amounts of cytokines to Wt mice. These data suggest that B7.1 and B7.2 have slightly different roles during the induction of EAE in SJL mice. The adoptive transfer of PLP 139-151-primed T cells from SJL B7.1$^{-/-}$ or B7.2$^{-/-}$ donor mice into Wt littermates did not induce EAE in these recipients, suggesting that B7.1$^{-/-}$ and B7.2$^{-/-}$ T cells either die from overstimulation in the CNS of Wt mice, or that B7.1 and B7.2 have a critical role on T cells in the effector phase of EAE. In SJL B7.1/B7.2$^{-/-}$ mice, adoptive transfer of PLP 139-151-primed cell lines from Wt donors did not induce EAE. Thus, in contrast to active induction of EAE in B7.1/B7.2$^{-/-}$ mice, adoptive transfer studies show that B7 costimulation during the effector phase of EAE is critical for normal SJL T cells, suggesting an important role for B7 expression on APCs in the CNS during the effector phase of EAE in SJL mice. These studies suggest different roles for B7 molecules during the induction and effector phases of EAE development.

Introduction

Our studies in chapter 3 suggested that B7.1/B7.2 costimulation is not essential for the induction of EAE by immunization with PLP 139-151 in SJL mice. This is consistent with published data in that administration of anti-B7.1 and anti-B7.2 mAbs together did not block the deveopment of EAE in SJL mice (Kuchroo et al., 1995; Perrin et al., 1996). However, these studies showed that when anti-B7.1 or anti-B7.2 mAbs were administered separately, anti-B7.1 mAbs blocked EAE in the SJL mice, whereas anti-B7.2 mAbs either enhanced or had no effect on the induction of EAE. These data gave us an impetus to test the development of EAE in B7.1$^{-/-}$ or B7.2$^{-/-}$ mice.

There are inherent problems in interpreting data describing administration of antibodies *in vivo*. Antibodies and fusion proteins can have different effects due to 1) induction of stimulatory signals rather than inhibitoy/blocking signals; 2) Fc-receptor cross-linking; 3) variable penetration of the antibodies/fusion proteins during *in vivo* treatment; 4) differing affinities of the antibodies/fusion proteins to their respective ligands; and/or 5) induction of anti-Ig antibodies that might affect antibody treatment studies. We have therefore addressed the role of B7 costimulation in EAE by using B7.1 and B7.2 single deficient and B7.1/B7.2 double deficient mice.

B7.1$^{-/-}$, B7.2$^{-/-}$ and B7.1/B7.2$^{-/-}$ mice

The advantage of using B7 deficient mice as a tool to analyze the role of B7 costimulation is that the expression of B7 molecules in these mice is disrupted, thus eliminating uncertainties about the effectiveness of antibodies (see above). On the other hand, B7 deficient mice lack B7 function throughout development, which can result in intrinsic defects. B7.2 and B7.1/B7.2 deficient mice have defects in immunoglobulin class switching and germinal center formation. In addition, the lack

of B7 molecules can result in compensatory up- or down-regulation of other molecules. It has been observed that B7.1 is over-expressed in B7.2$^{-/-}$ mice on the NOD background (Salomon et al., 2001). B7.1/B7.2$^{-/-}$ mice on the BALB/c background have been shown to over-express CD28 in thymus and peripheral T cells (Yu et al., 2000). Further, B7 molecules are expressed in the thymus and may have a role in thymic negative selection (Punt et al., 1997; Samoilova et al., 1997). Loss of B7 molecules might therefore affect the formation of the T cell repertoire. Thus, the loss of B7 molecules in B7 deficient mice can effect the overall immune response, which is an important consideration when analyzing EAE in these mice.

The initial characterization of B7 single and double deficient mice suggested that B7 costimulation affects Th1/Th2 development and that B7.1 and B7.2 have overlapping, but also distinct functions in immunoglobulin class switching and germinal center formation (Borriello et al., 1997; Freeman et al., 1993).

EAE in B7.1$^{-/-}$, B7.2$^{-/-}$ and B7.1/B7.2$^{-/-}$ mice

To determine the distinct roles of B7.1 or B7.2 in the induction phase of relapsing-remitting EAE, we induced EAE by immunizing B7.1$^{-/-}$ and B7.2 $^{-/-}$ mice on the SJL background with PLP 139-151. Our results suggest that B7.1 and B7.2 have overlapping, but also distinct roles in EAE pathogenesis, as shown by EAE clinical disease and cytokine profiles after immunization of SJL B7.1$^{-/-}$ and B7.2 $^{-/-}$ mice with PLP 139-151. Further, we addressed the role of B7.1 and B7.2 in the effector phase of EAE. Adoptive transfer of PLP 139-151-primed T cells from SJL B7.1$^{-/-}$ and B7.2 $^{-/-}$ mice into Wt donor mice did not induce EAE. In addition, adoptive transfer of PLP 139-151-primed T cells from Wt donors into SJL B7.1/B7.2 $^{-/-}$ recipient mice did not

induce EAE, demonstrating that B7 costimulation is important during the effector phase of this model.

Results

B7.1 deficiency exacerbates EAE in SJL mice.

B7.1$^{-/-}$and B7.2$^{-/-}$ mice were generated on the 129 background and have been crossed onto the SJL background for 7 generations. To compare the roles of B7.1 and B7.2 during the initiation of PLP-induced EAE, we immunized Wt SJL mice and SJL mice lacking either B7.1 or B7.2 with PLP 139-151 and observed the mice for clinical signs of EAE. Following this immunization, mice lacking B7.1 developed more severe clinical EAE than B7.2$^{-/-}$ or Wt mice (Figure 4.1). The incidence of EAE in B7.1$^{-/-}$ and B7.2$^{-/-}$ mice was slightly higher than the Wt controls but this difference was not significant (Table 4.1). However, the severity of EAE was different in the groups: the mean maximal disease score and the mortality rate were both significantly higher in the B7 single deficient mice compared to the Wt controls. The day of onset was not significantly different between the three groups. Histological analysis revealed more lesions in the meninges and the parenchyma of the brains and spinal cords of both the B7.1$^{-/-}$ and B7.2$^{-/-}$ mice (Table 4.1).

Thus, EAE induced in SJL B7.1$^{-/-}$ and B7.2$^{-/-}$ mice is more severe as compared to Wt mice. However, the extent of the exacerbation is different, in that B7.1$^{-/-}$ mice develop more severe EAE than B7.2$^{-/-}$ mice. These observations might indicate that B7.1 and B7.2 have overlapping, but also distinct roles in the induction of EAE on the SJL genetic background.

SJL B7.1$^{-/-}$ lymph node cells are primed to PLP 139-151 and secrete abundant IFNγ.

To study the mechanism for the difference in disease incidence and severity in the SJL B7.1$^{-/-}$ and B7.2$^{-/-}$ mice, we analyzed draining lymph node cells (LNCs) for proliferative responses and cytokine production from the B7.1$^{-/-}$ and B7.2$^{-/-}$ mice. The B7 deficient and Wt mice immunized with PLP 139-151 10 days previously were restimulated with PLP 139-151 *in vitro* to determine their ability to proliferate and produce cytokines (Figure 4.2). The proliferation of LNCs from B7.1$^{-/-}$ and B7.2$^{-/-}$ was comparable to the cells from Wt mice (Figure 4.2.A). The IFNγ production was increased in cell cultures from B7.1$^{-/-}$ mice (Figure 4.2.B). IFNγ production of cells from B7.2$^{-/-}$ mice, however, was comparable to the IFNγ production in Wt mice. IL-2 was produced in comparable amounts in all the three groups (Figure 4.2.C). A small amount of IL-10 production could be detected only in B7.2$^{-/-}$ mice. No IL-4 or TNFα was detected in any of the culture supernatants tested (data not shown). Thus, increased production of IFNγ from B7.1$^{-/-}$ cells might increase their encephalitogenic potential and contribute to the exacerbation of EAE disease in the SJL B7.1$^{-/-}$ mice.

SJL wild-type mice are resistant to EAE by adoptive transfer of PLP 139-151 primed cells from SJL B7.1$^{-/-}$ or B7.2$^{-/-}$ donor mice.

When SJL mice that lack B7.1 or B7.2 molecules are actively immunized for EAE, they develop more severe EAE than Wt controls (see above). This might mean that more effector cells of a pathogenic phenotype are produced in the B7.1$^{-/-}$ and B7.2$^{-/-}$ mice. We wanted to determine if T cells primed to the PLP 139-151 peptide in a B7.1 or B7.2 deficient environment are potent in inducing EAE in Wt littermate recipient mice. Due to breeding difficulties with the SJL mouse strain, SJL B7.1$^{-/-}$ and B7.2$^{-/-}$

mice are only backcrossed to generation F7. Thus, they still express about 0.8% of genes from the original 129 genetic background. To reduce the probability of deletion of T cells originating from B7.1$^{-/-}$ and B7.2$^{-/-}$ mice on transfer to an environment that might delete cells that express foreign (129) genes, we transferred the T cells into F7 wildtype littermate recipients (B7.1$^{+/+}$ and B7.2$^{+/+}$ mice). We immunized SJL B7.1$^{-/-}$ and B7.2$^{-/-}$ or Wt donor mice with PLP 139-151. After ten days, draining LNs were harvested and whole LNCs were restimulated with PLP 139-151. After four days of *in vitro* culture, the cultures were purified for T cells by Ficoll gradient and transferred into SJL wildtype littermates. The Wt recipients receiving primed Wt T cells developed clinical EAE with 100% disease incidence (Figure 4.3 and Table 4.2). Histological analysis of the brains and spinal cord confirmed evidence of lesions in the meninges and parenchyma (Table 4.2). In contrast, SJL Wt mice receiving T cells from B7.1$^{-/-}$ and B7.2$^{-/-}$ donors were quite resistant to EAE induction (Figure 4.3 and Table 4.2). T cells from B7.1$^{-/-}$ donor mice induced clinical EAE in only 1 out of 21 (5%) Wt recipients and T cells from B7.2$^{-/-}$ donors induced clinical EAE in only 2 out of 18 (11%) Wt recipients. When T cells originated from B7.1$^{-/-}$ or B7.2$^{-/-}$ donors, in addition to the low disease incidence, mortality was reduced to 0% instead of 36% in Wt, the day of onset was delayed to day 22 post-immunization instead of day 11 in Wt, and the mean maximum score was reduced to 2 instead of 4 in Wt-donor T cells (Table 4.2). Histological analysis revealed that the number of lesions in brains and spinal cords was reduced to a total of 0-4 instead of 127 in recipients that received T cells from Wt donors (Table 4.2).

Overall, T cells that are deficient in B7.1 or B7.2 and that are primed in an environment that is deficient in either B7.1 or B7.2 are much less potent to induce clinical and histological EAE in SJL Wt mice than T cells primed in a Wt

environment. Thus, B7.1 and B7.2 expressed on T cells may be important for regulating T cells, since B7.1$^{-/-}$ and B7.2$^{-/-}$ T cells are not able to induce EAE in Wt SJL mice. This seems contradictory with our previous data (Figure 4.1), where we show that active immunization induces severe EAE in B7.1$^{-/-}$ and B7.2$^{-/-}$ mice. However, in active immunization, PLP-peptide is given in a strong adjuvant (CFA), whereas no such adjuvant affects the recipients in an adoptive transfer. This may at least in part account for the differences seen between active and passive induction of EAE.

SJL B7.2$^{-/-}$ mice are highly susceptible to EAE by adoptive transfer of PLP 139-151 primed cells from SJL B7.2$^{-/-}$ donor mice.

B7.1$^{-/-}$ and B7.2$^{-/-}$ T cells that are primed to PLP 139-151 peptide in B7.1$^{-/-}$ and B7.2$^{-/-}$ mice, respectively, do not induce EAE in SJL Wt mice as well as Wt T cells primed in a Wt environment (see above). We wanted to examine whether T cells, which originate and are primed in a B7.1 or B7.2 deficient environment are pathogenic or defect in their encephalitogenic potential. Therefore, we transferred B7.1$^{-/-}$ or B7.2$^{-/-}$ T cells back into B7.1$^{-/-}$ or B7.2$^{-/-}$ mice.

T cells from B7.1$^{-/-}$ donors did not induce disease in B7.1$^{-/-}$ recipients (Figure 4.4). In contrast, the T cell line originating from B7.2$^{-/-}$ mice could induce EAE in B7.2$^{-/-}$ recipients (Figure 4.4). The same T cell line could not induce EAE in Wt recipients, which was consistent with our previous observations (see Figure 4.3 and 4.4). B7.2$^{-/-}$ mice that had received PLP 139-151-primed B7.2$^{-/-}$ T cells developed very severe EAE disease and all 3 recipients had succumbed to EAE by day 15 post-immunization. Wt and B7.1$^{-/-}$ recipients were followed until day 23 post-immunization, but neither group developed EAE disease.

PLP 139-151-primed cells from Wt donor mice are unable to induce EAE in SJL B7.1/B7.2$^{-/-}$ mice.

We wanted to examine the importance of B7 molecule expression during the effector phase of relapsing-remitting EAE. Therefore we adoptively transferred peptide-primed wild-type/costimulation competent cell lines into B7 deficient recipients and thus examined the importance of B7 costimulation for T cell entry and reactivation in the CNS.

We immunized SJL wild-type mice with the PLP 139-151 peptide and harvested the draining lymph nodes after ten days. The lymph node cells were cultured with PLP 139-151 for four days and after the purification for T cells by Ficoll, the PLP-peptide-primed cells were adoptively transferred into either SJL Wt or B7.1/B7.2$^{-/-}$ recipient mice. Interestingly, SJL B7.1/B7.2$^{-/-}$ mice were resistant to EAE induction by adoptive transfer in contrast to Wt controls, which developed clinical and histological EAE (Figure 4.5 and Table 4.3). Histological analysis of the brains and spinal cords from SJL B7.1/B7.2$^{-/-}$ mice showed only a very low number of lesions in meninges and parenchyma (Table 4.3), demonstrating that the Wt T cell lines that were shown to be encephalitogenic in Wt controls, were not able to induce tissue damage in SJL mice deficient in B7.1 and B7.2 expression. These observations suggest that B7 costimulation is important for T cells to enter the CNS and/or is important for the reactivation of T cells in the CNS by the local APCs.

Similarly, the adoptive transfer of MOG 35-55-primed T cells from B6 Wt donors did not induce EAE in B7.1/B7.2$^{-/-}$ recipient mice on the B6 background [for details see (Chang et al., 1999)].

These results suggest that the expression of B7.1 and B7.2 molecules during the effector phase of EAE is important in the progression of EAE in the SJL and B6 background, since the adoptive transfer of encephalitogenic T cells did not induce EAE. In contrast, the induction of EAE by active immunization, using the very strong adjuvant CFA, seems to be able to overcome the requirement for B7 costimulation in the SJL background, but not the B6 background.

Discussion

In this chapter, we have analyzed the distinct roles of B7.1 and B7.2 costimulatory molecules in relapsing-remitting EAE by studying the B7 deficiency on the SJL background. Our results obtained with B7.1$^{-/-}$, B7.2$^{-/-}$ and B7.1/B7.2$^{-/-}$ mice demonstrate that B7.1 and B7.2 have distinct and overlapping roles during induction and effector phases of EAE in the SJL genetic background.

It should be noted that due to breeding constraints with the SJL mouse strain, we had to be selective with the experiments that were conducted. Future experiments that are planned in order to further elucidate the role of B7 costimulation in the SJL-EAE-model, are discussed below.

The role of B7.1 and B7.2 in the induction phase of EAE in SJL mice

Previous studies have shown that B7.1 may be a dominant costimulatory molecule involved in the induction of PLP- or MBP- induced EAE on the SJL background (Kuchroo et al., 1995; Miller et al., 1995; Perrin et al., 1996; Racke et al., 1995). Overall, these studies show that administration of anti-B7.1 antibody resulted in partial or complete inhibition of EAE. In contrast, anti-B7.2 antibody administration either exacerbated or had no effects on disease outcome. These results are in sharp contrast to the knockout data presented here in that the B7.1$^{-/-}$ mice on the SJL background developed more severe disease. When trying to explain these apparent differences, we first have to consider that we are comparing antibody studies with studies using genetically engineered mice. Antibody treatment may not entirely block the B7-CD28/CTLA-4 pathway due to the antibody-effects already discussed earlier. In contrast, B7 signaling is entirely inhibited in B7 deficient mice, which might

promote the development of alternative costimulatory pathways. Thus, we might be comparing two different scenarios: a semi-blocked or even activated B7 pathway (antibodies) with a true blockade of the B7 pathway (B7 deficient mice).

An expanded endogenous repertoire may exacerbate EAE in B7 deficient mice. The complete loss of B7.1 or B7.2 during development in B7.1$^{-/-}$ and B7.2$^{-/-}$ mice may affect and shape the peripheral (autoreactive) T cell repertoire by affecting thymic selection (central tolerance). B7.1 is expressed on thymic epithelial and dendritic cells in the medulla of the thymus, but is virtually absent from the cortex and seems to be co-expressed with MHC class II molecules (Degermann et al., 1994; Reiser and Schneeberger, 1994). It has been suggested that the combined expression of B7.1 and ICAM-1 may define the medulla as the principal site of negative selection (Kishimoto et al., 1996; Lucas and Germain, 2000). On the other hand, B7 mediated costimulatory signals do not seem to be involved in the process of positive selection, which occurs in the cortex and seems to involve ICAM-1 (Jenkinson et al., 1994; Lucas and Germain, 2000). The expression of B7.1 seems to be up-regulated during CD4+ thymocyte negative selection (Degermann et al., 1994), and B7.1 signaling seems to result in the deletion of significant numbers of CD4+ thymocytes (Burns et al., 1999; Yu et al., 2000). Overexpression of B7.1 or B7.2 (in transgenic mice) results in the decrease of CD4/CD8 ratio in peripheral T cells and also downregulates CD28 expression in the thymus and periphery (Yu et al., 2000). Deficiency in B7.1 and B7.2 expression (in B7.1/B7.2$^{-/-}$ mice) results in the increase of CD4/CD8 ratio in peripheral T cells and also upregulates CD28 expression in the thymus and periphery (Yu et al., 2000). Thus, B7 expression can effect CD4+ and CD8+ T cell homeostasis and CD28 expression.

Both B7.1 and B7.2 have been implicated in promoting thymic negative selection and inducing activation-induced cell death of MHC class II-restricted thymocytes (Amsen and Kruisbeek, 1996; Samoilova et al., 1997). Thus, MHC/peptide induced signals through the TCR and B7-induced signals through CD28 can induce clonal deletion in the thymus. However, B7-CD28 does not seem to be the only pathway able to provide costimulation for TCRs during negative selection, and other B7-independent costimulatory (so far undefined) signals seem able to fulfill this function as well (Amsen and Kruisbeek, 1996). In addition, studies with $CD28^{-/-}$ mice have demonstrated that CD28 signaling is not essential for positive and negative selection, but may increase the efficiency of (negative) selection and/or expansion of peripheral T cell populations (Walunas et al., 1996). However, the use of signals from different costimulatory receptors might synergize differently with signals from the TCR and represent two distinct clonal deletion strategies, with different TCRs being selected by CD28-dependent and CD28-independent pathways (Punt et al., 1997). Signals through CTLA-4 on the other hand do not seem to be involved in thymic selection since thymocytes develop normally in CTLA-$4^{-/-}$ mice (Chambers et al., 1997). Thus, the loss of B7.1 and/or B7.2 may increase the threshold for thymic deletion of autoreactive T cells and may change the frequency with which they escape to the periphery.

Our preliminary studies of the endogenous repertoire in naïve B7.$1^{-/-}$ and B7.$2^{-/-}$ mice support this hypothesis (data not shown). We have determined the proliferation from LNCs from naïve SJL B7.$1^{-/-}$ and B7.$2^{-/-}$ mice that were activated with PLP 139-151 *in vitro*. These studies suggest that both SJL B7.$1^{-/-}$ and B7.$2^{-/-}$ mice have an increase in PLP 139-151-reactive T cells in the periphery as compared to SJL Wt mice. It is plausible that the increased autoreactive repertoire in B7 deficient mice is at least

partly responsible for the development of exacerbated EAE in B7.1$^{-/-}$ and B7.2$^{-/-}$ mice. If a greater number of autoreactive cells is already present in the naïve mice, this repertoire can expand more quickly when activated and therefore cause more damage.

As well as repeating the endogenous repertoire experiments as more mice become available, we plan to complement these results with studies of the immune response to PLP 178-191. In addition, it will be interesting to examine the cytokine profile of PLP-peptide specific T cells from naïve mice, which may suggest if the self-reactive T cells fulfill a potentially pathogenic or protective function.

A limitation of using a proliferation assay to detect self-reactive T cells is that we can only indirectly estimate the size of the endogenous repertoire. A method to directly quantify the endogenous repertoire of PLP 139-151- and PLP 178-191- reactive cells in B7.1$^{-/-}$ and B7.2$^{-/-}$ mice and compare it to SJL wild-type mice is to use MHC tetramers. MHC tetramers are the artificial combination of four (or more) MHC-peptide complexes that are linked to a molecule that can be detected by flow cytometry. The tetramer can be used to visualize T cells whose TCRs are specific for the MHC-peptide complex and thus bind the tetramer. The advantage of a tetramer is that it can detect very small populations of T cells with a particular antigen-specificity. Recently, MHC class I and MHC class II tetramers have been constructed and used to monitor antigen-specific CD4+ and CD8+ T cell responses (Nepom et al., 2002; Pittet et al., 2001). Our laboratory is currently constructing MHC II IA^{s} tetramers that are specific for PLP 139-151 or PLP 178-191. These tetramers can be used in future experiments to detect PLP 139-151 and PLP 178-191 reactive T cells in naïve mice. Thus we will be able to compare the endogenous repertoire in naïve SJL wildtype and B7 deficient mice by directly staining the self-reactive T cells.

Does B7 costimulation affect the TCR affinity of T cells? In addition, costimulation might also effect the TCR affinity of T cells. Based on the avidity model of thymic selection ((Ridgway et al., 1999) for details see General Introduction), the loss of costimulatory signals might result in the generation of T cells with increased TCR affinity, in order to attain the avidity threshold. Thus, in a B7 deficient environment, we would not only generate an increased pool of self-reactive T cells, but these T cells might also have a high affinity TCR, with increased disease-producing potential.

Does the loss of B7 costimulation result in the decrease of regulatory cells? The role of regulatory cells in the maintenance of peripheral tolerance is still unclear. However, it is plausible that regulatory cells exist that keep self-reactive T cells in the periphery in check and prevent autoimmunity. In fact, Salomon et al have demonstrated, that B7-CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes (Salomon et al., 2000). This paper shows that the CD4+CD25+ T cell population is decreased in B7.1/B7.2$^{-/-}$ and CD28$^{-/-}$ mice on the NOD background, and that reconstitution of this population can control the development of diabetes in NOD CD28$^{-/-}$ mice. Similarly, a population of B7-costimulation-dependent immunoregulatory cells might exist that controls the self-reactive cells that induce EAE in SJL mice. Thus, if B7 costimulation is inhibited or constrained in B7 deficient mice, a lower number of these regulatory cells might be generated, which may result in the exacerbation of EAE disease. This hypothesis could be tested by flow cytometric analyses of the populations of CD4+CD25+ regulatory cells in naïve and immunized SJL B7 deficient mice as compared to SJL Wt mice, possibly combined with MHC class II tetramer staining.

Does the loss of B7 molecules result in the compensatory up- or down- regulation of other molecules? It has been observed that B7.1 is over-expressed in B7.2$^{-/-}$ mice on

the NOD background (Salomon et al., 2001). Studies of CD28 expression in B7.1/B7.2$^{-/-}$ mice on the BALB/c background have demonstrated, that the loss of B7 molecules led to the over-expression of CD28 in thymic and peripheral T cells (Yu et al., 2000). Thus, it is plausible that in B7 deficient mice on the SJL background, the expression of other molecules is changed as compared to Wt mice. In SJL B7.1$^{-/-}$ mice for example, B7.2 might be upregulated on PLP 139-151-reactive T cells. Previous studies have suggested that B7.2 is the dominant B7-costimulator during the induction phase of EAE (Miller et al., 1995). Thus, an up-regulation of B7.2 in B7.1$^{-/-}$ mice might favor the activation of self-reactive cells in the LNs. In addition, an upregulation of CD28 on encephalitogenic T cells might favor their reactivation by APCs in the CNS. Thus, changes in the expression-patterns of other molecules might affect the severity of EAE in B7 deficient mice.

B7 deficient mice on the SJL versus B6 background

Our studies with B7 deficient mice on the SJL background are in contrast with studies with the same B7 deficient mice crossed onto the B6 background.

B7.1$^{-/-}$ and B7.2$^{-/-}$ mice on the B6 background develop MOG 35-55-induced EAE comparable to B6 Wt mice (Chang et al., 1999) (instead of developing more severe EAE as in SJL B7 single deficient mice). T cells from B7.1$^{-/-}$ and B7.2$^{-/-}$ mice that are primed to MOG 35-55 proliferate and produce cytokines (IL-2 and TNFα) comparably to Wt, although IFNγ levels seem to be slightly increased in B7.1 and B7.2 deficient mice, especially the B6 B7.2$^{-/-}$ mice. Thus, the phenotype of the responding autoreactive cells in B7 single deficient mice on the SJL and B6 background seems to be similar, but the disease outcome is very different. The different outcomes of EAE in these different EAE models might be due to the same

factors as we have previously discussed in chapter 3, including genetic background (in particular MHC haplotype), and the fact that B6 mice do not seem to have an expanded endogenous repertoire of myelin-reactive T cells.

The role of B7.1 and B7.2 in the effector phase of EAE in SJL mice

To analyze the role of B7.1 and B7.2 during the effector phase in EAE, we conducted adoptive transfer studies.

Does the expression of B7.1 and B7.2 on T cells have a role in EAE-progression? To address this question, PLP-peptide-primed B7.1$^{-/-}$ or B7.2$^{-/-}$ T cells from B7.1$^{-/-}$ or B7.2$^{-/-}$ donor mice were transferred into Wt recipients. These T cells induced clinical EAE in only a very low percentage of Wt donor mice, and EAE in the few mice that did develop disease was very mild, with a late onset of disease and a very short duration of clinical disease. These studies suggest that either 1) B7.1 and B7.2 expression is important on T cells during the effector phase of EAE; 2) the peptide-primed B7.1$^{-/-}$ or B7.2$^{-/-}$ T cells were deleted in Wt recipients; or 3) B7.1$^{-/-}$ or B7.2$^{-/-}$ mice upregulate an alternative costimulatory pathway which isn't active in Wt mice.

The first possibility is that the expression of B7.1 and B7.2 on T cells within the CNS increases the potential for autoreactivity. Earlier reports have demonstrated that a high percentage of T cells that are infiltrating the CNS during clinical EAE express B7.1 and B7.2 (Cross et al., 1999). In Wt SJL mice, B7 expression on T cells might function to provide encephalitogenic T cells with the ability to stimulate other T cells that they encounter in the CNS. T cell-T cell-interactions might be a mechanism by which the immune response in the CNS is amplified. Without the expression of B7 molecules (in B7.1$^{-/-}$ and B7.2$^{-/-}$ T cells), T cells are not able to activate "bystander" T cells and thus might not be able to successfully induce EAE.

The second possibility is, that B7.1$^{-/-}$ and B7.2$^{-/-}$ T cells simply get deleted by the Wt recipient due to overstimulation by the Wt APCs. T cells that are generated in B7.1$^{-/-}$ and B7.2$^{-/-}$ mice have been primed by the interaction with B7-comprised APCs in thymus and periphery. When they are transferred into a Wt mouse that is fully costimulation-competent, expressing both B7.1 and B7.2 molecules, the signal through CD28 on the T cells might be too strong and thus induce apoptosis. This hypothesis is supported by our data showing that a B7.2$^{-/-}$ T cell line is able to induce EAE in B7.2$^{-/-}$ mice (and thus confirming that the B7.2$^{-/-}$ T cell line is properly primed and pathogenic), while the same B7.2$^{-/-}$ T cell line did not induce EAE in Wt recipients. Interestingly, B7.1$^{-/-}$ T cells were not able to induce EAE in B7.1$^{-/-}$ recipient mice. This observation might be due to distinct roles of B7.1 and B7.2 during the initiation of EAE disease. To test the hypothesis that B7.1$^{-/-}$ and B7.2$^{-/-}$ T cells are deleted in the CNS by Wt APC-overstimulation, we propose to test recipient brain tissue for apoptosis, by TUNEL staining. Alternatively, PLP 139-151-primed B7.1$^{-/-}$ and B7.2$^{-/-}$ T cells could be activated *in vitro* by PLP-peptide-presenting Wt APCs, and T cells could be tested for apoptosis.

It has been suggested that the expression and function of B7.1 and B7.2 during EAE is tissue specific (Karandikar et al., 1998). In PLP 139-151-induced EAE on the SJL background, B7.2 seems to be functionally predominant in the lymph nodes (early/induction phase), whereas B7.1 seems to be functionally predominant in the spleen and CNS (later/effector phases) as shown by selective upregulation of B7.1 in nervous tissue of mice with EAE (Karandikar et al., 1998). Also, B7.1-mediated costimulation seems to be the predominant mechansim of epitope spreading and relapse induction (Miller et al., 1995).

It has also been shown that the loss of B7.1 on T cells in B7.1$^{-/-}$ mice leads to a preferential development of IL-4 producing Th2 cells (Schweitzer and Sharpe, 1999). B7.1 signaling on T cells seems to downregulate IL-4 production, and IL-4 seems to down-regulate B7.1 expression, resulting in a modulatory feedback loop (Schweitzer and Sharpe, 1999).

Interestingly, B7.2$^{-/-}$ mice on the NOD background develop spontaneous autoimmune peripheral polyneuropathy (SAPP), where mice have severe demyelination in the peripheral nerves, but not in the CNS (Salomon et al., 2001). Analysis of these mice suggests a striking over-expression of B7.1, especially effecting nervous tissue. Thus, NOD B7.2$^{-/-}$ mice seem to compensate for the loss of B7.2 with the over-expression of B7.1. In contrast, B7.2$^{-/-}$ mice on the B6 background do not develop SAPP and do not overexpress B7.1. This study emphasizes that the loss of B7.2 on certain backgrounds can lead to the development of autoimmune disease and can also lead to over-expression of B7.1 (Salomon et al., 2001).

Taking together these and our studies, we suggest the following scenario. When B7.1$^{-/-}$ mice are immunized with PLP-peptide in CFA, B7.1$^{-/-}$ T cells are properly primed and expanded since they express B7.2, the costimulatory molecule functionally predominant in the initial/priming phase. After the B7.1$^{-/-}$ T cells are adoptively transferred into B7.1$^{-/-}$ recipient mice however, they are not able to induce EAE since they lack B7.1 expression, important for the effector phase. In addition, the loss of B7.1 promotes the production of IL-4, a cytokine known to rather downregulate EAE. In contrast, when B7.2$^{-/-}$ mice are immunized with PLP-peptide in CFA, B7.2$^{-/-}$ T cells are also properly primed even though they lack B7.2 expression, probably because 1) a strong adjuvant like CFA reduces the need for costimulation; and 2) B7.2$^{-/-}$ mice may have an expanded repertoire of PLP 139-151 reactive cells,

probably also of a memory phenotype as has been shown in SJL Wt mice (Anderson et al., 2000) which are less dependent on costimulation for their activation (further discussed below). After the B7.2$^{-/-}$ T cells are adoptively transferred into B7.2$^{-/-}$ recipient mice, they are able to reach the CNS, because 1) they express B7.1 which seems to be functionally predominant in the spleen and CNS; and 2) the loss of B7.2 may promote the production of Th1-type cytokines, which are implicated with EAE pathogenesis. and 3) the B7.2$^{-/-}$ recipient mouse might over-express B7.1 in the CNS.

Future studies should analyze the expression-patterns of B7.1, B7.2 and CD28 in the LNs and CNS of naïve and immunized SJL Wt and B7-deficient mice to determine compensatory up- or down-regulation of these molecules. Also, the activation of purified T cells from SJL B7.1$^{-/-}$ and B7.2$^{-/-}$ mice with irradiated APCs from Wt or B7-deficient mice could clarify if B7-deficient T cells promote the production of Th1 or Th2 cytokines in the SJL genetic background.

These studies raise the possibility that B7.1 and B7.2 on T cells have a role in the induction of EAE. Furthermore, these studies support the hypothesis, that B7.1 and B7.2 have distinct roles during EAE pathogenesis.

Does the expression of B7.1 and B7.2 on APCs have a role in EAE-progression? To determine if B7 costimulation on APCs is important during the effector phase of EAE, we have adoptively transferred PLP 139-151-primed T cells from SJL Wt donor mice into SJL B7.1/B7.2$^{-/-}$ recipients. SJL B7.1/B7.2$^{-/-}$ mice did not develop EAE, suggesting that B7 costimulation plays a key role in the effector phase of EAE in SJL mice. The lack of clinical and histological EAE in SJL B7.1/B7.2$^{-/-}$ recipient mice suggests that PLP 139-151-primed, encephalitogenic Wt T cells were unable to enter the CNS and/or were unable to be reactivated in the CNS, because of lacking B7

costimulation that is required for T cells to infiltrate the CNS and mediate tissue damage.

The inability of Wt T cells to induce EAE in SJL B7.1/B7.2$^{-/-}$ mice is in contrast to the EAE-suceptibility of SJL B7.1/B7.2$^{-/-}$ mice by active immunization. However, in active immunization, B7-deficient T cells interact with B7-deficient APCs. We have previously discussed the possibility of intrinsic modifications (e.g. up- or down-regulation of other costimulators to compensate for loss of B7 molecules), which seem to be crucial for B7.1/B7.2$^{-/-}$ mice to develop EAE disease (Chapter 3). In an adoptive transfer, Wt T cells without these intrinsic modifications seem to be unable to be activated by B7-deficient APCs, and therefore do not mediate an autoimmune response.

Our observations that B7 costimulation is important in the effector phase of EAE in SJL mice is consistent with our previous results, demonstrating that B7 costimulation is crucial in the effector phase of EAE in B6 mice (Chang et al., 1999).

B6 B7.1/B7.2$^{-/-}$ mice were relatively resistant to EAE by adoptive transfer of MOG 35-55-primed Wt cells. Adoptively transferred encephalitogenic T cells from B6 Wt donor mice accumulated in the meninges of B6 B7.1/B7.2$^{-/-}$ recipient mice. Recent evidence suggests that the T cells die in the CNS from B6 B7.1/B7.2$^{-/-}$ recipient mice due to lack of costimulation (Chang et al., submitted). Overall, these studies suggest that B7 expression on APCs in the effector phase of EAE is important in both, the SJL and B6, mouse strain.

In summary, the studies of this chapter demonstrate that 1) Induction of EAE by active immunization with PLP 139-151 induces exacerbated EAE disease and increased IFNγ production in SJL B7.1$^{-/-}$ mice as compared to SJL Wt mice; induction

of EAE in SJL B7.2$^{-/-}$ mice induces moderately exacerbated EAE and a similar cytokine profile as compared to SJL Wt mice, suggesting that B7.1 and B7.2 have distinct roles during the induction phase of PLP 139-151-induced EAE in SJL mice; 2) the adoptive transfer of PLP 139-151-primed T cells from SJL B7.1$^{-/-}$ or B7.2$^{-/-}$ mice does not induce EAE in SJL Wt recipients, and PLP 139-151-primed T cells from B7.1$^{-/-}$ mice do not induce EAE in B7.1$^{-/-}$ recipients,but PLP 139-151-primed T cells from B7.2$^{-/-}$ mice induce EAE in B7.2$^{-/-}$ recipients, suggesting that the expression of B7.1 and B7.2 on T cells may have distinct roles in the effector phase of PLP 139-151-induced EAE in SJL mice; and 3) the adoptive transfer of PLP 139-151-primed T cells from SJL Wt donors does not induce EAE in SJL B7.1/B7.2$^{-/-}$ mice, and the adoptive transfer of MOG 35-55-primed T cells from B6 Wt donors does not induce EAE in B6 B7.1/B7.2$^{-/-}$ mice, suggesting that B7 expression on APCs is important during the effector phase of EAE in both, the SJL and B6 genetic background.

Chapter 5

The role of ICOS costimulation in the induction and effector phase of Experimental Autoimmune Encephalomyelitis

Abstract

In this chapter, we investigated the role of Inducible Costimulator (ICOS) molecule in the induction and effector phases of EAE by studying the disease in $ICOS^{-/-}$ mice or in wild-type (Wt) mice following administration of anti-ICOS monoclonal antibodies (mAbs) *in vivo*.

Administration of anti-ICOS mAb in SJL Wt mice during the induction of EAE with PLP 139-151 resulted in exacerbated EAE when mice treated with anti-ICOS mAb were compared with control (rat IgG) Ab treated animals. Similarly, $ICOS^{-/-}$ mice (on 129 or 129xB6 mixed background) showed increased severity and duration of MOG 35-55-induced EAE compared with Wt controls. The proliferation of lymph node cells (LNCs) from $ICOS^{-/-}$ mice was comparable to that of LNCs from Wt mice following immunization and reactivation *in vitro* with MOG 35-55, but $ICOS^{-/-}$ LNCs produced increased amounts of the Th1 cytokine IFNγ and decreased amounts of the Th2 cytokines IL-4 and IL-10. We conclude that ICOS plays an important regulatory role during the induction of EAE induced with either PLP- or MOG-peptide. Further, we transferred PLP-peptide primed T cell lines into SJL Wt mice and treated these recipients with either anti-ICOS or control Ab. Treatment of recipients of a differentiated T cell line with anti-ICOS mAb induced amelioration of EAE in comparison with control Ab treated mice. Thus, the effects of ICOS during the induction and effector phases of EAE are distinct. Finally, expression studies showed a difference in ICOS expression on different genetic backgrounds. CD4+ T cells from SJL mice expressed higher levels of ICOS after activation *in vitro* than B6 Wt mice.

The loss of B7 costimulation led to decreased levels of ICOS in both the SJL and B6 genetic backgrounds. Therefore, ICOS expression is dependent on B7 costimulation and may vary between genetic backgrounds.

Introduction

In chapter 3 and 4 we have addressed the role of B7 costimulation in EAE. This chapter focuses on the role of the ICOS-B7h pathway in EAE. Both, ICOS and B7h, are members of the B7-CD28 superfamily and have been described relatively recently.

The ICOS-B7h pathway was of particular interest because of its proposed role in the development of Th2 cells. Although more recent observations suggest that ICOS can regulate the production of both Th1 and Th2 cytokines, its main function seems to be in the promotion of Th2 cell responses. Since EAE is a Th1 cell-mediated disease, ICOS may be important in the down-regulatory aspects of a pathogenic autoreactive immune response.

ICOS was also of interest since it seems to be involved in regulating the later phases of an immune response by regulating the effector responses of T cells. It may play a different role in the development of EAE than CD28, which seems to be rather important in inducing the T cell response.

We addressed the role of the ICOS-B7h pathway in EAE, using $ICOS^{-/-}$ mice and anti-ICOS monoclonal antibodies (mAbs). We have induced EAE by active immunization and also by adoptive transfer to address the role of the ICOS-B7h pathway in the induction versus effector phase. We have also begun to explore the role of ICOS in different genetic backgrounds, as we want to determine if different genetic backgrounds have a different requirement for ICOS costimulation.

Our data show that ICOS has an important role during both the induction and effector phase of EAE. However, the role of ICOS signaling during the different stages of EAE seems to change, since blockade of ICOS during the induction phase exacerbates EAE, whereas blockade of ICOS during the effector phase ameliorates EAE.

The previous chapters have investigated how the requirement for B7 costimulation in the induction of EAE can vary under different experimental conditions. Our results comparing the susceptible B7 deficient mice on the SJL background, with resistant B7 deficient mice on the B6 background, have suggested roles for the endogenous repertoire, the MHC haplotype, and other yet undefined "background genes". Another method to investigate the factors that compensate for a lack of B7 costimulation in the induction of EAE, is to analyze what other molecules are affected by the lack of B7 molecule expression. Does the loss of B7.1 and B7.2 cause other molecules to be upregulated or downregulated?

The loss of B7 molecules in B7.1/B7.2$^{-/-}$ mice could result in the upregulation of a molecule, because 1) B7 signaling might directly or indirectly inhibit or downregulate the expression of other molecules; 2) there may be compensation for the loss of B7 signalling by upregulation of other receptors; an example for such compensation is the upregulation of CD28 in B7.1/B7.2$^{-/-}$ mice (Yu et al., 2000).

In contrast, the loss of B7 molecules in B7.1/B7.2$^{-/-}$ mice could result in the downregulation of a molecule, because the upregulation of a molecule is directly or indirectly dependent on signals derived from the B7-CD28/CTLA-4 pathway.

We decided to study the effect of B7 deficiency on the expression of the ICOS-B7h pathway. Previous studies have shown that CD28 signaling enhances ICOS expression, which provides more evidence for ICOS as a down-stream molecule. Our study suggests that B6 and SJL wildtype mice express ICOS at different levels following the primary activation of CD4+ T cells. The loss of B7 molecules in SJL and B6 B7.1/B7.2$^{-/-}$ mice results in lower expression levels of ICOS on the CD4+ T cells on both backgrounds.

Results

Treatment with anti-ICOS mAb exacerbates PLP 139-151-induced EAE in SJL mice.

To analyze the role of ICOS in the induction phase of EAE on the SJL genetic background, we immunized SJL Wt mice with PLP 139-151 and treated these mice with anti-ICOS mAb or control (rat IgG) Ab. Mice were immunized with 75 μg PLP 139-151 and treated with 50 μg anti-ICOS mAb or control Ab from the day of immunization on (day 0) every alternate day until day 18 (Figure 5.1). Each mouse therefore received a total of 10 injections and a total of 500 μg antibody. Mice that received the anti-ICOS mAb demonstrated more severe clinical EAE than control Ab treated mice in the initiation phase of EAE and also had higher disease scores in the later stages of EAE (Figure 5.1). Thus, treatment of the ICOS pathway in SJL mice resulted in exacerbation of EAE.

As we discussed earlier, it is difficult to assess the function of an antibody *in vivo*. The anti-ICOS mAb used in this study has been shown to block ICOS-B7h interactions *in vitro*. However, even if anti-ICOS mAb does block ICOS-B7h interactions, it might still induce ICOS signaling *in vivo*. To clarify this matter, we induced EAE in $ICOS^{-/-}$ mice (see below).

ICOS deficient mice develop more severe MOG 35-55-induced EAE than wild-type controls.

To examine the role of ICOS costimulation in the development of MOG peptide-induced EAE, we immunized wild-type and $ICOS^{-/-}$ mice with MOG 35-55. $ICOS^{-/-}$ mice were generated on the 129 background and are currently being crossed onto the

B6 background. Thus, the mice used in these experiments were either on a 129 or on a 129xB6 mixed genetic background. Wt mice on a 129 genetic background are not as susceptible to MOG 35-55 induced EAE as B6 Wt mice, so we expected a milder form of EAE in the wild-type mice of the 129 and also on the 129xB6 mixed backgrounds. Following the immunization the mice were observed for clinical signs of EAE. Mice deficient in ICOS developed more severe EAE than control mice (Figure 5.2). The most striking difference was in the duration of clinical disease. Wild-type mice developed a comparable mean maximal score to $ICOS^{-/-}$ mice, but recovered quickly and did not relapse. In contrast, $ICOS^{-/-}$ mice showed clinical disease for a longer period of time, remitted and then relapsed around day 40 post-immunization (Figure 5.2 and Table 5.1). Histological analysis revealed a comparable number of lesions in the meninges, but more lesions in the parenchyma of the brains and spinal cords of the $ICOS^{-/-}$ mice (Table 5.1). These results suggest that ICOS plays an important role in the induction of MOG 35-55-induced EAE, since mice lacking this molecule develop more severe disease than the Wt controls.

In addition, the results with the $ICOS^{-/-}$ mice suggest that the anti-ICOS mAb used in SJL mice (Figure 5.1) is blocking ICOS signaling *in vivo* (since it causes exacerbation of EAE), but is not very effective (with only modest effect, compared to $ICOS^{-/-}$ mice). We speculate that treatment with a more efficient anti-ICOS Ab or with a higher dose of the Ab that we used in our experiments, would be more effective in exacerbating EAE in SJL and B6 mice.

$ICOS^{-/-}$ T cells are primed to MOG 35-55 and have a strong Th1 phenotype.

To study the mechanism for the exacerbation of disease in the ICOS deficient mice, we analyzed draining LNCs from the $ICOS^{-/-}$ mice for proliferative responses and

cytokine production. The KO and Wt mice were immunized with MOG 35-55 and ten days later, the draining lymph nodes were harvested and restimulated *in vitro* with MOG peptide, to determine their ability to proliferate (Figure 5.3) and produce cytokines (Figures 5.4-5.6). Cytokine production was analyzed both by ELISA from the supernatants of cell cultures (Figure 5.4 and 5.5), and also by RNAse Protection assays (RPAs) from cells (Figure 5.6) on days 2 and 3 of *in vitro* culture. The proliferation of LNCs from $ICOS^{-/-}$ was comparable to the cells from Wt mice on Day 2 (Figure 5.3.A) and Day 3 of culture (Figure 5.3.B). In contrast, the cytokine profile of Wt and $ICOS^{-/-}$ cells analyzed by ELISA showed striking differences on both day 2 (Figure 5.4) and day 3 (Figure5.5) of culture. The IFNγ production was increased in cell cultures from $ICOS^{-/-}$ mice (Figure 5.4.A and 5.5.A). In contrast, the production of Il-4 (Figure 5.4.B and 5.5.B) and IL-10 (Figure 5.4.C and 5.5.C) was decreased in cell cultures from $ICOS^{-/-}$ mice. IL-2 was not detected in any of the groups (data not shown). Therefore, cells from $ICOS^{-/-}$ mice showed an increase in production of Th1 type cytokines (IFNγ) and a decrease in production of Th2 type cytokines (IL-4 and IL-10).

For the RPAs, cells were harvested and RNA expression of certain cytokines was determined (Figure 5.6). The RNA expression levels of cytokines were quantified and related to the expression of the house-keeping gene GAPDH (Glyceraldehyde 3-Phosphate Dehydrogenase). Cytokine RNA levels are relative to GAPDH levels, and thus are shown in percent. The data from RPAs further supported the finding of a skewing towards a Th1 phenotype of cells from $ICOS^{-/-}$ mice. Cells from $ICOS^{-/-}$ mice produced significantly higher levels of IFNγ RNA than wild-type controls (Figure 5.6). In addition, there was a decrease in the RNA of the Th2 type cytokines IL-4, IL-5 and IL-10 in cells from $ICOS^{-/-}$ mice, although the RNA levels of these cytokines

were low (Figure 5.6). We also detected a moderate increase in the RNA of the Th2 type cytokine IL-13 in cells from ICOS$^{-/-}$ mice. This seems to be in contrast to a recent publication where no IL-13 protein production could be detected in ICOS$^{-/-}$ mice of the same genetic background (Dong et al., 2001). This may be because although we detect IL-13 on an RNA level in ICOS$^{-/-}$ mice, the protein is not able to leave the cell, or the assay for IL-13 protein is not as sensitive as the RPA.

Taken together, ELISA and RPA show that cells from ICOS$^{-/-}$ mice are primed for activation by the MOG 35-55 peptide, and that these cells are biased towards the Th1 phenotype, suggesting a mechanism by which ICOS$^{-/-}$ mice can develop a more severe form of the Th1 cell mediated disease EAE.

Moderate differences in the RNA levels of chemokine receptors on lymph node cells from ICOS$^{-/-}$ and Wt cells.

The expression of chemokine receptors on T cells can influence their ability to traffic and migrate into the CNS. We therefore analyzed draining LNCs for upregulation of chemokine receptors from Wt and ICOS$^{-/-}$ mice that were immunized with MOG35-55. The ICOS$^{-/-}$ and Wt mice were immunized with MOG 35-55 and ten days later, the draining LNs were harvested and restimulated *in vitro* with MOG peptide. After 2 and 3 days of culture, cells were harvested and RNA expression of certain chemokine receptors was determined by RPA (Figure 5.7). The expression levels of chemokine RNA were quantified and related to the expression of the house-keeping gene GAPDH. Thus, chemokine receptor RNA levels are shown in percent, relative to GAPDH levels.

Our data shows, that the chemokine receptors CCR1, CCR2, CCR3, CCR4 and CCR5 are expressed on draining LNCs from ICOS$^{-/-}$ and Wt mice that were immunized with

MOG 35-55. This is consistent with previous observations, showing that these chemokine receptors are expressed on T cells in the CNS of SJL mice with PLP 139-151-induced EAE disease (Fischer et al., 2000). In our study, RNA levels of the Th2 associated chemokine receptor CCR4 were modestly decreased in cells from $ICOS^{-/-}$ mice as compared to Wt on days 2 (3% in $ICOS^{-/-}$ vs. 5% in Wt) and 3 (4% in $ICOS^{-/-}$ vs. 6% in Wt). Cells from $ICOS^{-/-}$ and Wt mice expressed comparable RNA levels of CCR1 and CCR3. CCR5 RNA was upregulated at moderately higher levels in $ICOS^{-/-}$ cells on day 2 compared to Wt cells (8% in $ICOS^{-/-}$ vs. 5% in Wt on day 2), but comparable on day 3. CCR2 RNA was upregulated at moderately higher levels in $ICOS^{-/-}$ cells on day 2 compared to Wt cells (3% in $ICOS^{-/-}$ vs. 2% in Wt), but did not reach RNA-levels of Wt CCR2 expression on day 3 (3% in $ICOS^{-/-}$ vs. 5% in Wt). However, the differences between chemokine receptor expression in $ICOS^{-/-}$ vs. Wt cells were small, and it is not clear if the slight differences in chemokine receptor expression on T cells may impact their ability to traffic.

Treatment with anti-ICOS mAb exacerbates or ameliorates adoptively transferred EAE in SJL mice, depending on how established the T cell lines are.

We have attempted to address the role of ICOS in the effector phase of EAE, by adoptive transfer studies transferring MOG 35-55-primed T cells from $ICOS^{-/-}$ donor mice into Wt recipient mice, and also by transferring MOG 35-55-primed T cells from Wt donor mice into $ICOS^{-/-}$ recipient mice (data not shown). However, the adoptive transfers were unsuccessful, since in the control experiment, MOG 35-55-primed T cells from Wt donors into Wt recipients only induced very mild EAE. The unsuccessful outcome of these adoptive transfers is probably due to the genetic background, since both donors and recipients were of 129 genetic background. Once

the ICOS$^{-/-}$ mice are further crossed onto the B6 background, the adoptive transfer studies will be repeated.

In the meanwhile, we used anti-ICOS mAb as a tool to address the role of ICOS in the effector phase of PLP 139-151-induced EAE on the SJL background, by conducting adoptive transfers of PLP 139-151-primed cells and treatment of the recipients with anti-ICOS mAb.

We immunized SJL Wt mice with PLP 139-151 and harvested the draining lymph nodes after ten days. The lymph node cells were restimulated with PLP peptide to establish PLP 139-151-reactive cell lines. We generated two types of PLP 139-151-reactive cell lines: A) a short-term cell line which was kept in culture for only four days and were therefore still at an early stage of Th differentiation and B) a long-term cell line which was kept in culture for four weeks and was restimulated with PLP peptide a total of three times. Cells from the long-term cell line are thus in a more differentiated state. We then adoptively transferred either the short-term line (Figure 5.8.A) or the long-term lines (Figure 5.8.B) into SJL wild-type recipient mice. Recipients were treated with anti-ICOS mAb or a control rat IgG Ab.

When short-term lines were transferred, anti-ICOS mAb treated recipients showed modest exacerbation of clinical EAE in contrast to rat IgG treated mice (Figure 5.8.A). The effect of anti-ICOS mAb was thus similar to treatment of mice with actively induced EAE. In contrast, when long-term, more established cell lines were transferred, anti-ICOS treated mice showed amelioration of clinical EAE in contrast to control Ab treated mice (Figure 5.8.B). As mentioned before, a more efficient ICOS blockade may increase the differences seen between anti-ICOS and control Ab treated groups.

These results support the hypothesis that blockade of the ICOS pathway can have very different outcomes on disease, depending on the stage of EAE at which the antibody is given. Treatment with anti-ICOS mAb seems to have an exacerbating effect on disease when cells are in a state of differentiation. Later, when cells are more primed/differentiated, anti-ICOS mAb treatment has a rather ameliorating effect.

Differences in ICOS expression between SJL and B6 mice.

Previously, we have shown that mice with different genetic backgrounds (SJL versus B6 backgrounds) have different requirements for B7 costimulation (see Chapters 3 and 4). Thus, we were interested to investigate if these genetic backgrounds also have different requirements for ICOS costimulation. We compared the expression-levels of ICOS between T cells from SJL and B6 Wt mice after activation. In addition, we compared ICOS upregulation in SJL and B6 wild-type mice to ICOS upregulation in SJL and B6 B7.1/B7.2$^{-/-}$ mice, to further analyze the mechanism that is responsible for the difference in EAE susceptibility of the B7.1/B7.2$^{-/-}$ mice derived from B6 and SJL backgrounds.

To study the upregulation of ICOS during the initiation of an immune response, we analyzed the expression of ICOS on CD4+ cells before and 48 hours after activation with the anti-CD3 Ab *in vitro* (Figure 5.9). Without activation, CD4+ T cells from either the SJL or B6 strains did not show a significant expression on the cell surface, both *ex vivo* (white columns) or after 48 hours of culture in medium (gray striped columns). This is consistent with the characteristics of ICOS as an induced molecule. The slight upregulation of ICOS after 48 hours in medium (as compared to 0 hours in medium) can be explained by the ability of tissue culture to activate cells. After 48

hours of anti-CD3 stimulation, ICOS was upregulated on CD4+ T cells from SJL and B6 mice. However, the SJL and B6 strains exhibited significant differences in ICOS expression following activation. Anti-CD3 activated splenocytes and LNCs (gray shaded columns) from the Wt SJL mice expressed significantly higher levels of ICOS on CD4+ T cells (Figure 5.9) when compared to the B6 Wt cells, suggesting a significant difference in the expression and upregulation of ICOS between the two strains.

The SJL B7.1/B7.2$^{-/-}$ T cells showed a lower (50-60%) expression of ICOS following activation as compared to the Wt mice suggesting that ICOS expression in the SJL mice is highly dependent on B7.1/B7.2 costimulation on CD4+ cells (Figure 5.9). CD4+ cells from B6 B7.1/B7.2$^{-/-}$ showed a more modest decrease (30-50%) in the ICOS expression as compared to the activated Wt B6 T cells. These findings suggest that the expression of ICOS on CD4+ cells in SJL and B6 mice is dependent on B7 costimulation. This data complements previous observations, showing that the expression of ICOS on CD4+ cells is B7-CD28 costimulation dependent in mice on the 129 background (McAdam et al., 2000), for a reprint see Appendix B).

In addition, expression of ICOS in SJL mice might be more dependent on B7 costimulation than in B6 mice, since the loss of B7 molecules in SJL B7.1/B7.2$^{-/-}$ mice causes a greater drop in ICOS levels. However, overall CD4+ cells from SJL B7.1/B7.2$^{-/-}$ mice express greater ICOS levels than cells from B6 B7.1/B7.2$^{-/-}$ mice.

We then wanted to study the upregulation of ICOS later in an immune response. We therefore immunized SJL and B6 Wt and B7.1/B7.2$^{-/-}$ mice with the encephalitogenic peptides, PLP 139-151 or MOG 35-55, respectively. After ten days, the draining lymph nodes were harvested and lymph node cell cultures were restimulated with the PLP or MOG peptide. ICOS upregulation was determined after 48 or 72 hours of

culture. In contrast to ICOS upregulation on cells from naïve mice (primary activation of T cells) (see Figure 5.9), we could detect only a very moderate upregulation of ICOS on cells from immunized mice that were subjected to peptide-restimulation (reactivation of T cells) (Figure 5.10). However, peptide-restimulation activates only PLP- or MOG- specific T cells in SJL and B6 mice, respectively. Thus, ICOS expression is induced in a smaller number of T cells, in contrast to our previous study (Figure 5.9) where T cells were stimulated with anti-CD3 Ab which affects all T cells regardless of their antigen-specificity.

CD4+ cells that were restimulated with peptide (gray shaded columns) upregulated ICOS rather comparably to cells that were cultured in medium (white columns) (Figure 5.10). Also, in contrast to ICOS upregulation in primary activated cells (Figure 5.9), ICOS expression levels were comparable between CD4+ cells from SJL and B6 Wt mice. In spite of low levels of ICOS upregulation, we could still detect the tendency for lower ICOS upregulation on CD4+ cells in B6 B7.1/B7.2$^{-/-}$ mice (Figure 5.10).

Our results suggest that the expression of ICOS between the SJL and B6 mice show two essential differences: 1) ICOS is upregulated in SJL and B6 Wt mice, but the basic expression levels seem to be different, since CD4+ T cells from B6 Wt mice express much less ICOS than CD4+ T cells from SJL Wt mice after primary activation; 2) ICOS expression is partly dependent on B7-CD28 signaling since the expression levels of ICOS is reduced in B7.1/B7.2$^{-/-}$ mice as compared to Wt mice (in both SJL and B6 backgrounds).

These differences in the upregulation of ICOS, and their dependence on the B7-CD28 costimulation, may partly account for the difference in susceptibility of the B7.1/B7.2$^{-/-}$ mice.

Discussion

Our studies in this chapter demonstrate a critical role for ICOS in the induction and effector phase of EAE. Further, we demonstrate that the primary upregulation of ICOS is different in SJL and B6 genetic backgrounds, but that ICOS expression is partly dependent on B7 costimulation in both the SJL and B6 background.

ICOS in the induction phase of EAE

Our previous studies (Chapter 3 and 4) have shown that different genetic backgrounds have different requirements for B7 costimulation. This led us to examine if there is a different requirement for ICOS costimulation in 129/B6 versus SJL backgrounds. Since $ICOS^{-/-}$ mice are not yet available on the SJL background, we used anti-ICOS Ab to manipulate the ICOS pathway in the SJL EAE-model. Treatment of PLP-immunized SJL mice with anti-ICOS mAb resulted in exacerbation of EAE, demonstrating a role for ICOS in the induction of EAE on the SJL background. Our study complements a previous study, also showing that *in vivo* blockade of the ICOS-B7h pathway by administration of anti-ICOS antibody (Rottman et al., 2001) enhances EAE in SJL mice. However, this study uses a different antibody, a higher dose of antibody and a different treatment schedule, which results in a very high mortality rate in the anti-ICOS Ab treated group. This report demonstrated that treatment with anti-ICOS antibody during the induction phase of EAE resulted in exacerbated IFNγ production (Rottman et al., 2001).

Mice that are deficient in ICOS expression develop an exacerbated EAE disease when EAE is induced by active immunization with MOG 35-55, despite being on a 129xB6 mixed genetic background. We would expect EAE in Wt mice to be more severe on a

pure B6 background and the effects of ICOS deficiency may be even greater in these mice. The most striking clinical finding was a longer duration of clinical EAE in $ICOS^{-/-}$ mice. This might relate to a failure of normal regulatory mechanisms following the initial induction of disease. ICOS deficiency does not seem to affect the proliferation of MOG-peptide-specific T cells, but affects their cytokine profile. Comparing MOG-peptide primed cells from wild-type and $ICOS^{-/-}$ mice, we found that cells from $ICOS^{-/-}$ mice produced enhanced levels of the Th1 cytokine IFNγ, and produced lower levels of the Th2 type cytokines IL-4 and IL-10. Since EAE is a Th1 disease, the skewing towards a Th1 cytokine profile is consistent with a reduction in the number of Th2 cytokine secreting MOG 35-55-specific cells.

These results complement recent findings, also showing exacerbated disease in $ICOS^{-/-}$ mice (Dong et al., 2001). This study, using a very high dose of antigen and Pertussis Toxin to induce disease, demonstrated exacerbation of EAE by a very high mortality rate of $ICOS^{-/-}$ mice. By histology, more inflammatory foci were observed in the brain. The increased severity of EAE in the $ICOS^{-/-}$ mice was attributed to a decrease in IL-13 and increase in IFNγ production by the responding T cells. Even though our studies could not confirm a lack in IL-13 on the RNA level, it is possible that IL-13 protein is not able to be produced by $ICOS^{-/-}$ mice.

To examine if ICOS signaling affects T cell trafficking, we examined the effect of ICOS deficiency on various chemokine receptors. However, the expression of chemokine receptors CCR1, CCR2, CCR3, CCR4 and CCR5 were low in LNCs from $ICOS^{-/-}$ and Wt mice. In addition, the differences between chemokine receptor RNA levels of $ICOS^{-/-}$ and Wt LNCs were very small and might not influence the ablility of T cells to traffick. Preliminary experiments have addressed the question, whether ICOS has a role in inducing tolerance (data not shown), since a defect in tolerance

could contribute to the exacerbation of EAE disease in ICOS$^{-/-}$ mice. We chose to tolerize ICOS$^{-/-}$ and Wt mice, by injecting soluble MOG- or control (OVA) peptide intraperitonatally (i.p.). The injection of soluble peptide intraperitonatally has been shown to induce tolerance against the particular peptide. Mice were then actively immunized with MOG 35-55 for EAE disease. Preliminary results suggest that ICOS is not required for tolerance induced by the i.p. injection of peptide, since ICOS$^{-/-}$ mice showed comparable tolerance to Wt mice. However, the mechanism for tolerance induction by i.p. injections may be independent of the IL-10 pathway. Data by Akbari et al. has shown that the induction of tolerance by nasal exposure to antigen is mediated by IL-10 (Akbari et al., 2001). Therefore a future direction should address the induction of nasal tolerance in ICOS$^{-/-}$ mice.

In summary, the blockade of B7h-ICOS pathway at the induction of EAE affects T cell differentiation and promotes the generation of pathogenic, strongly Th1 biased cells.

ICOS in the effector phase of EAE

In contrast to the exacerbation of EAE observed after ICOS manipulation during the induction phase, blockade of ICOS during the effector phase seems to ameliorate disease.

We have generated two different PLP-peptide-specific T cells lines. A short-term T cell line where LNCs from immunized mice are restimulated with PLP 139-151 *in vitro* for only four days before being adoptively transferred. This T cell line is primed towards PLP 139-151, but includes cells that are still developing towards a Th1 or Th2 phenotype. In addition, we generated a long-term T cell line where LNCs from immunized mice are restimulated with PLP 139-151 *in vitro* over a period of four

weeks, once a week, before they are adoptively transferred. This culture contains cells that are thought to be fully differentiated. By treating the donors of each cell line with anti-ICOS mAb, we were able to study the effect of ICOS-manipulation on differentiating versus differentiated cells. Treatment with anti-ICOS-Ab exacerbated adoptively transferred EAE in SJL mice when short-term lines were transferred. In contrast, treating recipients of long-term lines with anti-ICOS mAb resulted in amelioration of EAE disease. Thus, the manipulation of ICOS can result in exacerbation of EAE, when cells are in the priming/induction phase, and can also ameliorate EAE, when cells are in the differentiated/efferent phase of EAE.

Our studies complement previous studies examining the role of ICOS during the effector phase, using anti-ICOS Ab (Rottman et al., 2001) or ICOS-Ig (Sporici et al., 2001) in SJL or MBP-specific TCR transgenic B10.PL mice, respectively. Treating PLP-immunized SJL mice with anti-ICOS Ab during a late stage of EAE induction (days 9-20 post-immunization) resulted in ameriolation of clinical disease as compared to control mice, possibly due to decreased IFNγ production of primed splenocytes of mice treated with anti-ICOS Ab (Rottman et al., 2001).

Blocking the ICOS-B7h pathway with ICOS-Ig (Sporici et al., 2001) during the *in vitro* activation of MBP-reactive transgenic CD4+ T cells, inhibited the ability of these T cells to transfer EAE, even though they entered the brains of recipient mice. Similarly, ICOS-Ig treatment of mice after the onset of EAE ameliorated clinical disease. ICOS-Ig was found to increase apoptosis in the transgenic T cells, particularly affecting the memory population. ICOS-Ig did not prevent IL-2 production, but suppressed IFNγ and IL-10 production. Therefore signaling through the ICOS-B7h pathway seems to promote activation and viability of previously

activated encephalitogenic T cells and blocking this pathway therefore inhibits EAE (Rottman et al., 2001).

Taken together, these studies suggest that administration of anti-ICOS antibody in the effector phase of the disease might delete or inhibit effector functions of the previously activated autopathogenic T cells, suggesting a mechanism by which ICOS signaling on effector T cells could affect autoimmune disease. Manipulation of this pathway might have very different outcomes (exacerbation or amelioration), depending on the timing of ICOS-B7h blockade.

Taken together the data presented above raises the following questions. What is the role of ICOS in the development of EAE in normal mice? How does ICOS mediate two very different disease outcomes? How does the loss of B7 affect the ICOS pathway in an EAE setting? Do different genetic backgrounds have different requirements for ICOS signaling in the development of EAE?

What is the role of ICOS in the development of EAE in normal mice?

ICOS can have a direct and/or indirect affect on encephalitogenic cells.

Direct effect of ICOS on encephalitogenic cells. When self-reactive T cells are initially activated in the LNs, ICOS is induced and costimulates the T cell by upregulating CD40L, enhancing Th2 cytokine production and possibly enhancing Th2 chemokine receptor expression. One could imagine a mechanism by which ICOS provides Th2-inducing signals to balance other Th1-inducing signals. However, (in the case of the development of a pathogenic T cell), the T cell develops a Th1 phenotype despite of Th2 inducing signals from ICOS, and ICOS is downregulated. In later phases, when encephalitogenic T cells have acquired a memory phenotype, ICOS may play a role in their survival by inhibiting apoptosis. In this scenario, ICOS

signals are over-ruled by other Th1-inducing signals. Inducing EAE in ICOS transgenic mice could test this hypothesis. The overexpression of ICOS might lead to exacerbated Th2 signaling and may prevent the induction of pathogenic Th1 cells.

Indirect effect of ICOS on encephalitogenic cells. ICOS might be expressed on a sub-population of self-reactive T cells with a regulatory (rather than a pathogenic) function. If signaling via ICOS is important in the development of a Th2 phenotype, the regulatory cells might not act as efficiently in its absence.

In this scenario, ICOS is expressed on a functionally distinct population. The development of exacerbated EAE would suggest that disease caused by pathogenic cells is more severe in the absence of ICOS expressing (regulating) T cells. Analyzing the expression of ICOS on the regulatory CD4+CD25+ cells may give supporting correlative data. If a CD4+CD25+ICOS+ T cell population exists, the transfer of these cells into EAE-primed mice might prevent the induction of autoimmune disease.

How does inhibition of ICOS signaling exacerbate EAE?

When ICOS signaling is inhibited (in $ICOS^{-/-}$ mice or by treatment with anti-ICOS Ab) self-reactive T cells can still be effectively primed (due to other costimulatory signals). In addition, since ICOS signaling is defective, its inhibitory effects on Th1 priming are lost, and self-reactive T cells can acquire a phenotype strongly biased to Th1. Thus, the priming of T cells in an environment that lacks the regulatory influences of ICOS might result in the generation of encephalitogenic T cells that are more pathogenic in that they produce exacerbated Th1 cytokines and upregulate Th1 chemokine receptors (direct effect). Cells in this scenario are not of a memory phenotype. Thus, they would not rely on ICOS for anti-apoptotic signals.

In part, this hypothesis is confirmed by the observations that EAE induction in $ICOS^{-/-}$ mice leads to exacerbated disease and the generation of T cells with a strongly biased Th1 phenotype (Figure 5.2 and (Dong et al., 2001)). Similarly, mice that are actively immunized for EAE induction and treated with anti-ICOS Ab develop exacerbated EAE (Figure 5.1 and (Rottman et al., 2001)).

Treating mice with anti-ICOS Ab after the transfer of encephalitogenic T cells resulted only in a moderate exacerbation of EAE. This could be due to the down-regulation of ICOS expression after the primary T cell activation (see Figure 5.9 and 5.10). Thus, the lack of ICOS expression on the cell surface would render the treatment with anti-ICOS mAb ineffective.

In addition to the hypothesis that ICOS-blockade affects encephalitogenic T cells directly, there might be a reduction in the generation of ICOS-expressing regulatory cells that would normally keep the pathogenic T cells in check (indirect effect).

Thus, a higher number of pathogenic T cells with a stronger pathogenic phenotype and/or a lower number of regulatory cells might induce an exacerbated EAE disease in mice where ICOS signaling is blocked during the differentiating stages of enephalogenic T cells.

How does inhibition of ICOS signaling ameliorate EAE?

The blockade of ICOS in later stages of EAE ameliorates this autoimmune disease. How does the blockade of ICOS, which has previously shown to exacerbate EAE, ameliorate EAE in a different scenario? Based on the observations by Sporici et al (Sporici et al., 2001), we propose that fully differentiated encephalitogenic T cells (of a memory phenotype) are deleted before they are able to induce EAE, due to the lack of survival signals normally provided by ICOS signaling. This hypothesis could be

tested by tracking encephalitogenic T cells in order to determine if they are able to reach the CNS, for example by adoptively transferring CFSE (5/6-Carboxy-Fluoresceindiacetate, Succinimidyl-Ester, for reference see (Lyons and Parish, 1994)) labeled cells or transgenic cells (with cell specific marker) that can be visualized by flow cytometry assays.

Overall, ICOS signaling seems to have different functions during different stages in T cell development and thus during different stages of EAE induction and maintenance. First, ICOS provides "differentiating signals" (affecting cytokine and chemokine profiles) in differentiating T cells. Later, ICOS provides survival signals to differentiated T cells of a memory phenotype. Thus, manipulation of ICOS during different developmental stages of the T cell can affect either the differentiation of T cells or their viability. Also, T cells in different developmental stages seem to express variable levels of ICOS, thus rendering anti-ICOS Ab treatment more or less efficient.

Loss of B7 signaling influences ICOS upregulation

As discussed and shown in the results (Figure 5.9), the loss of B7 molecules can result in the downregulation or upregulation of other molecules. The EAE susceptibility of B7.1/B7.2$^{-/-}$ SJL mice raises the question of whether genetic deletion of B7.1/B7.2 has a distinct outcome on B6 and SJL strains due to differences in the expression of other costimulatory molecules that control EAE-induction and regulation. We studied the affect of B7 deficiency on ICOS upregulation.

Overall, ICOS was more strongly upregulated when T cells were activated by anti-CD3, in contrast to restimuled cells from immunized mice. This may indicate that 1) anti-CD3 is a more potent T cell activator and restimulation with peptide was only sub-optimal; 2) ICOS is highly upregulated during the initial immune response and is

not as highly expressed in the later response; or 3) in draining LNs, there is only a small number of Ag specific cells, whereas all T cells (Ag-specific and Ag-unspecific) are activated with anti-CD3 Ab.

Our data indicate that the SJL and B6 strains have a significant difference in the level of ICOS expression following primary activation. In addition there seems to be a difference in the B7.1 and B7.2 dependence of ICOS between SJL and B6 strains. Following activation, the WT SJL T cells displayed a significantly higher expression of ICOS than did Wt B6 mice. The loss of B7 resulted in a dramatic impairment in ICOS upregulation in both B6 and SJL B7.1/B7.2$^{-/-}$ mice. If ICOS is involved in regulating Th2 development and can function to limit the expansion of the encephalitogenic T cells, the minimal upregulation of ICOS in the B7.1/B7.2$^{-/-}$ mice may not be sufficient to limit the expansion of encephalitogenic T cells and regulate EAE. Thus the loss of B7 signaling results in a loss of ICOS expression in B7.1/B7.2$^{-/-}$ mice of both backgrounds, skewing activated T cells towards a Th1 phenotype and enhancing the development of encephalitogenic cells.

The expression-studies of ICOS in Wt and B7.1/B7.2$^{-/-}$ mice on the SJL and B6 genetic backgrounds demonstrated that ICOS upregulation is dependent on B7 signaling in both genetic backgrounds. Further studies are needed to determine whether the different expression of ICOS may in part account for the functional difference in EAE-susceptibility in B7.1/B7.2$^{-/-}$ mice on the SJL and B6 genetic background.

How does B7-CD28 affect ICOS signaling after ICOS upregulation?

As shown, ICOS is dependent on the B7-CD28 pathway (Figures 5.9 and 5.10 and (McAdam et al., 2000), in that CD28 signaling is important for ICOS upregulation.

However, it is not clear if CD28 signaling is also important for the functioning of ICOS, once it is upregulated.

In B7.1/B7.2$^{-/-}$ mice, ICOS have not been observed to be able to reach normal expression levels as seen in Wt mice. The lower levels of ICOS expression in B7 deficient mice may affect the induction and progression of EAE in these mice. Since ICOS seems important for the differentiation and survival of T cells, lower expression-levels of this molecule might have consequences during early and late phases of EAE development.

The role of ICOS in B7.1/B7.2$^{-/-}$ mice during early stages of EAE. The lower levels of ICOS expression due to lack of B7-CD28 signaling, might have an effect on the T cells when they differentiate into pathogenic T cells. Similarly to anti-ICOS Ab treatment during the induction phase of EAE, the reduced ICOS signal may allow the amplification of the Th1 phenotype. The analyses of draining LNs from B7.1/B7.2$^{-/-}$ mice have shown that MOG 35-55-reactive cells from B7.1/B7.2$^{-/-}$ mice on the B6 background produce abundant Th1 cytokine (IFNγ) (Chang et al., 1999), similarly to PLP 139-151-reactive cells from B7.1/B7.2$^{-/-}$ mice on the SJL background that also produce abundant IFNγ (Fig 3.2). The exacerbated production of this Th1 cytokine in the B7.1/B7.2$^{-/-}$ mice might be the effect of abnormally low expression of ICOS in these mice.

In addition, lower levels of ICOS expression in B7.1/B7.2$^{-/-}$ mice might reduce the number of regulatory cells and thereby indirectly effect EAE disease

The role of ICOS in B7.1/B7.2$^{-/-}$ mice during later stages of EAE. Due to the reduction in ICOS-mediated survival signals, peptide-primed cells (with a memory phenotype) may be deleted. However, clinical EAE can be induced and maintained in SJL B7.1/B7.2$^{-/-}$ mice suggesting that 1) the loss of B7 signaling upregulates a different

molecule that can mediate survival signals to effector and memory cells, which sustain disease or 2) if SJL B7.1/B7.2$^{-/-}$ mice have an increased endogenous repertoire, EAE disease might be sustained by continuous activation of "new" autoreactive cells instead of reactivating memory T cells to sustain the immune response.

Overall, the lower levels of ICOS during the early stages of EAE induction might promote the induction of a Th1 phenotype and thus have a role in the development of EAE in SJL B7.1/B7.2$^{-/-}$ mice. What the precise effects of ICOS-dysregulation in B7.1/B7.2$^{-/-}$ mice are remains to be determined, but the data is consistent with the hypothesis that this may be one of the factors determining susceptibility versus resistance.

B7.1/B7.2$^{-/-}$ mice on the B6 background are resistant to EAE, thus the effects of abnormally low ICOS expression do not overcome the need for B7 costimulation in this genetic background.

Do different genetic backgrounds have a different requirement for ICOS costimulation in the induction of EAE?

Our results have demonstrated that the blockade of ICOS seems to exacerbate EAE in Wt mice on 129, 129xB6 and SJL backgrounds, suggesting that ICOS has a similar role in these backgrounds. However, we have compared results from studies using either ICOS deficient mice (on 129 and 129xB6 backgrounds) or anti-ICOS antibodies (SJL background) to manipulate the ICOS-B7h pathway. We have previously discussed the problems of both tools. Thus we would find a comparison between genetic backgrounds more satisfactory, if the same tools were used for both backgrounds. Once ICOS$^{-/-}$ mice on the SJL background are available or a more

efficient anti-ICOS Ab treatment is established, we would plan to reinvestigate the role of ICOS on different genetic backgrounds.

In summary, the studies in this chapter demonstrate that 1) the treatment of PLP 139-151 immunized SJL Wt mice with anti-ICOS mAb induces an exacerbated EAE as compared to control Ab treated mice; and MOG 35-55-immunized ICOS$^{-/-}$ mice on the 129 or 129xB6 mixed backgrounds develop exacerbated EAE disease as compared to Wt mice, suggesting that ICOS signaling normally functions to regulate EAE disease in SJL, B6 and 129 genetic backgrounds; 2) draining LNCs from MOG 35-55-immunized ICOS $^{-/-}$ mice produce higher amounts of the Th1 cytokine IFNγ and lower amounts of the Th2 cytokines IL-4 and IL-10 and also express lower RNA levels of the Th2 associated chemokine receptor CCR4 as compared to LNCs from Wt mice, suggesting that ICOS $^{-/-}$ mice are very Th1 biased in regard to cytokines and chemokine receptors; 3) the treatment of SJL Wt recipients of differentiated PLP 139-151-primed T cell lines with anti-ICOS mAb induces ameliorated EAE as compared to control Ab treated recipients, suggesting that ICOS signaling in the effector phase functions to maintain EAE disease in SJL mice.

The blockade of the ICOS-B7h pathway for the treatment of autoimmune disease has already been suggested (Sporici and Perrin, 2001). ICOS seems to be an attractive target since it is an induced molecule and seems to be important for previously activated and memory T cells. Thus it could be an option for therapy in individuals that already have an established autoimmune disease like MS. Compared with treatment directed at the B7-CD28 pathway, anti-ICOS treatment may have the advantage that it targets the previously activated and memory population.

Chapter 6

General Discussion

In this thesis, we have examined the role of B7 and ICOS costimulation in EAE. Through these studies, we have gained a better understanding of how these costimulatory pathways influence susceptibility to CNS autoimmunity on different genetic backgrounds. We have shown that B7.1 and B7.2 play different roles in the pathogenesis of EAE in gene targeted mutants, but that the genetic background has a profound influence on the phenotype of these effects. We have shown linkage of these effects to the MHC and found evidence that other, yet to be defined non-MHC genes, influence the requirement for costimulation. These results have important implications for the development of therapies that target costimulation pathways in human autoimmune diseases like MS.

Re-addressing the 2-signal hypothesis

Does Signal 2 provide more than just amplification of signal 1? According to the 2-signal hypothesis, T cells require two signals for activation. Signal 1 is generated by the TCR-MHC-peptide interaction and provides specificity. Signal 2 amplifies the effects of signal 1, leading to effective clonal expansion (as discussed in chapter 1). However, our and other data suggest that signal 2 can have other functions beyond amplifying signal 1. It serves to provide differentiation (e.g. B7.1 and B7.2 in Th1/Th2 differentiation) and survival signals (e.g. ICOS in memory T cells) to the activated T cell and therefore plays an important part in determining the fate of the T cell and thus the immune response.

As more of the molecules involved in costimulation are identified, it becomes clear that different members of these pathways have various functions, some of which overlap and some of which are unique. In addition, some members of costimulatory family of molecules are constitutively expressed, while others are upregulated by activation signals, which may themselves depend on costimulatory signals or on the products of those signals (e.g. via the production of cytokine/cytokine receptor interactions).

Costimulatory pathways are interdependent (as illustrated in Figure 6.1.A):

The CD40-CD40L pathway has been described as being critical for the differentiation of a naïve T cell into a pathogenic Th1 effector cell (Chang et al., 1999). The upregulation of CD40L seems to be dependent on TCR signals, and signals from CD40L seem to upregulate B7.1 and especially B7.2 expression (Roy et al., 1995).

Thus, maximum activation of the B7-CD28/CTLA-4 pathway seems dependent on the CD40-CD40L pathway. However, B7.2 is constitutively expressed, while CD40L requires induction, therefore the two molecules may work in parallel to reinforce each others effects (Figure 6.1.A). The B7-CD28/CTLA-4 pathway has been ascribed many functions. B7-CD28 costimulation has been shown to promote T cell activation (Lanzavecchia et al., 1999); to regulate the threshold for T cell activation (Viola and Lanzavecchia, 1996); to promote T cell proliferation and IL-2 production (Freeman et al., 1993); and to promote T cell survival, of naïve T cells in particular, and thereby to augment and sustain T cell responses (Boise et al., 1995; Lucas et al., 1995; Shahinian et al., 1993; Sperling et al., 1996; Thompson et al., 1989). B7-CTLA-4 costimulation has been shown to inhibit IL-2 synthesis and progression through the cell cycle and to terminate T cell responses (Brunner et al., 1999; Greenwald et al., 2001; Krummel and Allison, 1995; Walunas et al., 1996; Walunas et al., 1994), and to actively induce

peripheral T cell tolerance (Greenwald et al., 2001; Perez et al., 1997). Our data complements previous data in showing that the B7-CD28/CTLA-4 pathway also induces, at least in part, the upregulation of other costimulatory pathways (Figure 6.1.A). For example, B7 costimulation is important for ICOS upregulation (Figure 5.9, 5.10 and (Aicher et al., 2000; McAdam et al., 2000) and for Ox40 upregulation (our data, not shown).

Thus, ICOS expression is in part dependent on the B7-CD28/CTLA-4 pathway, but can also be induced by non-CD28 signals (Kopf et al., 2000). Several functions have been ascribed to the B7h-ICOS pathway, including modest stimulation of T cell proliferation and IL-2-secretion (although not as effective as CD28 signaling) (McAdam et al., 2001; Riley et al., 2001), regulation of cytokine production (IL-4, IL-5, IL-10, IFNγ, TNFα, GM-CSF) by recently activated and effector T cells (Coyle et al., 2000; Hutloff et al., 1999), regulation of Th1 and Th2 responses (Figure 5.4-5.6 and (Coyle et al., 2000; Gonzalo et al., 2001; McAdam et al., 2000; Sperling, 2001; Sporici and Perrin, 2001; Tesciuba et al., 2001)), signaling to memory T cells (Sporici et al., 2001), and the provision of T cell help to B cells, promoting Ig class switching and germinal center formation (McAdam et al., 2001). Finally, the B7h-ICOS pathway can itself upregulate CD40L expression (McAdam et al., 2001), thus giving memory cells the potential for rapid upregulation of activation via a positive feedback loop.

This example emphasizes several key features of costimulatory pathways. 1) Different pathways have different functions to fulfill in the costimulation-apparatus. 2) The upregulation of costimulatory molecules is not only dependent on the signals from the TCR, but also on signals from other costimulatory pathways (e.g. signals from CD28 enhances ICOS expression) and these signals may amplify the expression of an

already activated molecule in a feedback-loop (e.g. ICOS enhances CD40L). Thus, costimulatory pathways are extremely interdependent. 3) As more costimulatory pathways are described, it becomes clear that some functions that have so far been ascribed to one costimulatory pathway may actually just reflect the functions of another pathway (e.g. the functions ascribed to CD28 may in part be the result of ICOS signaling). Overall, this example serves to demonstrate that the interactions of costimulatory molecules are highly complex.

The role of the kinetics of expression in costimulation. Costimulatory molecules have different kinetics. Some molecules are constitutively expressed (e.g. CD28), while other molecules are upregulated on activation (e.g. ICOS). Some molecules are expressed for a longer time on the cell surface (e.g. ICOS), some only for a very short time (e.g. CTLA-4). Some molecules are expressed shortly after T cell activation (e.g. B7.2), while some are expressed after a few days of T cell activation (e.g. ICOS), possibly reflecting the fact that these molecules are upregulated by other costimulators and are therefore further downstream.

Therefore it is possible that different costimulatory pathways may subserve the same functions at different times, while on the other hand some functions may only occur once a molecule is upregulated. Finally, some functions may depend on a molecule being expressed at a specific time. Therefore, in a CD28 deficient environment, although other signals may also upregulate ICOS expression, changes in the kinetics of expression may alter the function of ICOS.

Redundancy in costimulation. Studies of costimulatory pathways suggest that different molecules can have both distinct and overlapping functions. CD28 and ICOS both induce T cell proliferation, although ICOS signaling is not as potent (McAdam et al., 2001; Riley et al., 2001). In autoimmune disease, B7.1$^{-/-}$ or B7.2$^{-/-}$ mice on the B6

background develop EAE that is comparable to Wt mice, but B6 B7.1/B7.2$^{-/-}$ mice are resistant to disease induction (Chang et al., 1999). These observations indicate that in this model, B7.1 can fulfill the role of B7.2 in B7.2$^{-/-}$ mice, and that B7.2 can fulfill the role of B7.1 in B7.1$^{-/-}$ mice, but when both molecules are inhibited in B7.1/B7.2$^{-/-}$ mice, their function cannot be substituted by another costimulatory molecule. However, as shown in chapter 4, the phenotype of the B7.1 and B7.2 single deficient mice can change in a different background.

In addition, it is possible that lack of costimulatory signals can be substituted by a very strong TCR-signal and/or a persistent TCR signal (Nicholson et al., 1998). For example, CD28$^{-/-}$ mice on the B6 background have been shown to be resistant to EAE induction in contrast to B6 Wt mice (Chang et al., 1999; Chitnis et al., 2001) (Figure 6.1.B). However, when the signal through the TCR was enhanced by actively immunizing CD28$^{-/-}$ mice twice instead of once, or by combining adoptive transfer of MOG-peptide-primed Wt T cells into CD28$^{-/-}$ mice with active immunization, the CD28$^{-/-}$ mice developed EAE comparable to Wt mice (Chitnis et al., 2001). These studies support the hypothesis that a stronger signal through the TCR can overcome CD28 signaling. In addition, not only were the T cells activated, but they were also able to induce EAE, indicating that the strong TCR signal may have induced the upregulation of costimulatory pathways that in other circumstances require CD28 engagement (Figure 6.1.C).

We have described in chapter 3, that SJL B7.1/B7.2$^{-/-}$ mice develop EAE that is comparable to Wt mice, and we have shown that EAE- susceptibility and severity are strongly linked to the MHC (Tables 3.4 and 3.5). According to the avidity model of thymic selection, the MHC can influence TCR affinity. Thus, one possibility is that in SJL mice, self-reactive T cells with a high TCR affinity for autoantigens are selected,

resulting in strong signals through the TCR when T cells encounter MHC-peptide presented by APCs. In this scenario, the unusually strong signals through the TCR might render the T cells from SJL mice less dependent on B7-CD28 costimulation than B6 mice.

Overall, costimulation seems to not only amplify signal 1, but also functions to promote the differentiation and maintenance of a T cell response. In addition, the concept of costimulation involves a growing number of molecules that make up a very interactive and dynamic system. The system appears to be promiscuous, in that the functions of different molecules seem to overlap, and redundant in that signaling through different pathways may induce the expression of the same costimulatory molecule. Possibly, this is a means by which the immune system retains the ability to activate T cells, even if one part of the costimulatory apparatus fails.

Readdressing the roles of costimulatory pathways at different stages of EAE pathogenesis.

As we discussed in chapter 1, there are different stages of pathogenesis in EAE. Most publications recognize at least two broad stages: priming/induction and effector phases (Anderson, 2002). In light of our observations and the costimulation-EAE literature, we think it is important to define additional phases and also to separate induction and effector phases into different components of the response. Thus, in respect to costimulation, the different stages of EAE should be separated into 1) thymic selection: generation of self-reactive T cells; 2) maintenance of myelin-reactive T cells in the periphery; 3) early induction phase (activation of T cells); 4) late induction phase (differentiation of activated T cells); 5) trafficking through the lymphatic system; 6) crossing the BBB; 7) early effector phase (reactivation of T

cells); 8) late effector phase (possible interaction of effector T cells with other T cells; maintenance of effector T cells); and 9) epitope spreading. We and others have shown that B7.1/B7.2-CD28/CTLA-4 and ICOS-B7h costimulation pathways are involved during several of these stages (as illustrated in Figure 6.2).

1) *Thymic selection.* Both B7.1 and B7.2 have been implicated in promoting thymic negative selection and inducing activation-induced cell death (AICD) of MHC class II-restricted thymocytes (Amsen and Kruisbeek, 1996; Samoilova et al., 1997). Thus, B7 costimulation may be important in deleting self-reactive cells and may therefore serve to inhibit the generation of an endogenous repertoire of myelin-reactive T cells. Our preliminary studies of the endogenous repertoire in naïve B7.1$^{-/-}$ and B7.2$^{-/-}$ mice on the SJL background support this hypothesis, as these mice seem to have an expanded repertoire of PLP 139-151-reactive cells. ICOS expression has also been detected on a subpopulation of CD4^{-}8^{-} and CD4^{+}CD8^{-} thymocytes in Balb/c mice, which raises the possibility that ICOS may also be involved in thymic selection (McAdam et al., 2000).

2) *Maintenance of myelin-reactive T cells in the periphery.* So far, there have not been any reports that directly connect B7 or ICOS costimulation with the maintenance of myelin-reactive cells in the periphery. However, B7 has been implicated in the maintenance of naïve cells and ICOS has been implicated in the maintenance of memory cells.

3) *Early induction phase.* During the early induction phase, myelin-reactive T cells are activated for the first time. Experimentally, we may address this stage by

determining the proliferation profiles of cells from draining LNs. Our data supports an important role for B7.1/B7.2 costimulation at this stage, since draining LNCs from PLP 139-151- or MOG 35-55- immunized B7.1/B7.2$^{-/-}$ mice on the SJL and B6 backgrounds, respectively, show impaired proliferative responses, compared to LNCs from Wt mice (Figure 3.2 and (Chang et al., 1999)). However, an analysis of draining LNCs from PLP 56-70-immunized B7.1/B7.2$^{-/-}$ mice on the NOD background, showed only a very moderate reduction in proliferation compared to Wt controls (Girvin et al., 2000), indicating that B7 costimulation is not critical in this model. Similarly, CD28$^{-/-}$/CD4+ T cells from CD28$^{-/-}$anti-MBP transgenic mice on the PL/J background, showed only a moderate reduction in proliferation when activated with MBP 1-17 and compared to cells from CD28$^{+/-}$ anti-MBP transgenic mice (Oliveira-dos-Santos et al., 1999).

B7.1 and B7.2 seem to have more or less overlapping functions at this stage, since LNCs from B7.1$^{-/-}$ and B7.2$^{-/-}$ mice on the SJL background proliferate at a level that is comparable to Wt controls (Figure4.2). On the B6 background, LNCs from B7.1$^{-/-}$ mice proliferate at a level that is comparable to Wt controls, and LNCs from B7.2$^{-/-}$ mice show a modest reduction when compared with Wt controls (Chang et al., 1999). On the NOD background, proliferative responses in B7.1$^{-/-}$ mice were significantly greater than those seen in Wt controls, while proliferative responses in B7.2$^{-/-}$ mice were significantly lower than controls (Girvin et al., 2000).

These observations indicate that B7 costimulation promotes T cell activation during this stage in some backgrounds (SJL and B6), while it is not critical in other backgrounds (NOD and PL/J). In addition, the roles of B7.1 and B7.2 seem to be overlapping in some backgrounds (SJL and B6), while costimulation by B7.1 versus B7.2 has very distinct effects on the NOD background (Girvin et al., 2000).

Our data suggests that ICOS costimulation does not seem to be involved in early activation since LNCs from ICOS$^{-/-}$ mice proliferate at levels that are comparable to Wt controls on 129 and 129xB6 backgrounds (Figure5.3). However, in SJL mice immunized with PLP 139-151 and treated with anti-ICOS Ab on days 1-10 post-immunization, draining LNCs proliferated more than LNCs from control Ab-treated mice (Rottman et al., 2001). This data would suggest that ICOS normally suppresses T cell activation during the early induction phase of EAE in SJL mice.

4) *Late induction phase*. During the late induction phase, activated T cells differentiate into effector cells. Experimentally, we may address this stage by determining the cytokine profiles of cells from draining LNs, which gives an indication of the pathogenicity of the differentiated cell. Studies with B7 deficient mice indicate that B7 costimulation inhibits the development of a pathogenic phenotype in the B6 background, since mice deficient in B7.1 and B7.2 produce higher amounts of the pro-inflammatory cytokines IFNγ and TNFα production (Chang et al., 1999). This effect might be primarily due to B7.2 costimulation, since LNCs from B7.2$^{-/-}$ mice produce abundant IFNγ and TNFα, while LNCs from B7.1$^{-/-}$ mice show only a moderate increase in IFNγ production (Chang et al., 1999). On the SJL background, IFNγ production is moderately increased in LNCs from B7.1/B7.2$^{-/-}$ mice compared to Wt controls (Figure 3.2). In contrast to the B6 background, this effect seems to be primarily due to B7.1 costimulation, since IFNγ production is increased only in LNCs from B7.1$^{-/-}$ mice, whereas B7.2$^{-/-}$ mice produce IFNγ levels comparable to Wt controls (Figure 4.2).

Studies with anti-B7 Abs suggest distinct roles for B7.1 and B7.2 for T cell differentiation. When spleen cells from a naïve MBP-specific TCR transgenic mouse

on the B10.PL background were activated with MBP 1-11 and anti-B7.1 Ab, the splenocytes produced less IFNγ, and produced increased amounts of the Th2-associated cytokine IL-4 (Kuchroo et al., 1995). In contrast, the activation of these cells with anti-B7.2 led to the increased production of IFNγ, suggesting that B7.1 and B7.2 differentially activate the Th1/Th2 developmental pathways (Kuchroo et al., 1995).

Overall, these studies suggest that the production of IFNγ (and thus the promotion of a Th1 phenotype) may be inhibited primarily by B7.1 (SJL background) or B7.2 (B6 and B10.PL backgrounds), while the production of IL-4 (and thus the promotion of a Th2 phenotype) is induced by B7.2 (B10.PL background). Thus, B7.1 and B7.2 seem to have distinct roles during this phase in different models of EAE.

Our studies with $ICOS^{-/-}$ mice on the 129 and 129xB6 backgrounds demonstrate that ICOS is very critical in this differentiation phase. It seems to promote the production of Th2-type cytokines (IL-4 and IL-10) and suppresses the production of IFNγ (Figure 5.4-5.6). Similarly, Dong et al show that the production of the Th2-associated cytokine IL-13 is reduced in splenocytes from MOG 35-55-immunized $ICOS^{-/-}$ mice on the 129xB6 background, and that CD4+ T cells from CNS tissues produce increased amounts of IFNγ,possibly reflecting the effect of ICOS during T cell differentiation (Dong et al., 2001). These data are complimented by anti-ICOS Ab treatment studies on the SJL background, where the treatment with anti-ICOS Ab on days 1-10 post-immunization enhanced IFNγproduction from draining LNCs (Rottman et al., 2001). ICOS promotes the differentiation of Th2 cells, and may therefore function to inhibit the induction of EAE.

5) *Trafficking through the lymphatic system.* There have not been any reports that directly connect B7 costimulation with T cell trafficking through the lymphatic system. Data presented in chapter 5 shows that ICOS costimulation may have a role at this stage by influencing the upregulation of chemokine receptors in T cells. However, the differences in chemokine receptor mRNA levels between $ICOS^{-/-}$ and Wt LNCs were very moderate and may thus not suffice to influence T cell trafficking.

6) *Crossing of the BBB.* The roles of B7 and ICOS costimulation may be similar at the BBB to their roles during the late induction phase and T cell trafficking. This phase is correlated with the expression of chemokine receptors and the production of Th1 cytokines. A study by Rottman et al demonstrated a role for ICOS costimulation in the expression of chemokine receptors and chemokines in the brain. SJL mice immunized with PLP 139-151 and treated with anti-ICOS Ab on days 1-10 post-immunization, showed higher RNA expression levels of the chemokine receptor CCR1 and higher RNA levels of the chemokines RANTES, MIP-2 and MCP-1 on day 14 post-immunization when compared with untreated mice (Rottman et al., 2001). CCR1 interacts with RANTES, and this interaction has been shown to promote EAE development (Rottman et al., 2000). Similarly, the interaction of MCP-1 with its receptor CCR2 has been shown to be crucial for EAE development (Izikson et al., 2000; Karpus et al., 1995).

It is possible that different chemokine receptor-chemokine-interactions may be important during different stages of EAE development. Th1-associated chemokine receptors (e.g. CCR4) may be important for the homing of effector T cells to the target organ during the trafficking stage, whereas other chemokine receptors (including CCR1 and CCR2) are important for effector T cells to cross the BBB and

reach their target site in the CNS (Izikson et al., 2000; Karpus et al., 1995; Rottman et al., 2000).

7) *Early effector phase.* During the early effector phase, T cells are reactivated in the target organ. Experimentally, we may address the role of specific molecules during the effector phase by adoptively transferring encephalitogenic Wt cells into mice deficient in that molecule. Alternatively, we can induce EAE in a Wt mouse, and then treat the mouse with a reagent that blocks the expression of this molecule. The treatment needs to be given several days after the immunization, so that T cells have already started to cross the BBB, but have not yet been reactivated (since disease onset varies in different experiments, this phase is challenging to address by Ab treatment).

Adoptive transfer studies suggest that B7 costimulation is critical during the early effector phase. In this thesis, we show that B7.1/B7.2$^{-/-}$ recipient mice are resistant to EAE after they receive PLP 139-151-primed encephalitogenic Wt T cells, in contrast to SJL Wt mice that developed clinical and histological EAE after they receive the same T cell line (Figure 4.5 and Table 4.3). Histological analysis of the SJL B7.1/B7.2$^{-/-}$ recipient mice revealed very few lesions, which suggests that the encephalitogenic T cells cannot be activated by APCs that lack B7.1 and B7.2 and thus are unable to induce tissue damage.

Similarly, we have shown that B7.1/B7.2$^{-/-}$ recipient mice on the B6 background developed less severe EAE upon adoptive transfer of MOG 35-55-primed T cells from Wt donors, in contrast to B6 Wt recipients that developed clinical and histological EAE (Chang et al., 1999). The histological picture in B7.1/B7.2$^{-/-}$ recipient mice on the B6 background is similar to B7.1/B7.2$^{-/-}$ recipient mice on the

SJL background, in that B7.1/B7.2$^{-/-}$ recipients of both backgrounds had fewer CNS inflammatory infiltrates compared to Wt recipient mice. However, it is also different in that lesions were detected in the meninges of the CNS from B7.1/B7.2$^{-/-}$ recipients of the B6 background, indicating that T cells accumulate in the meninges without being able to enter the CNS parenchyma. Recent studies have demonstrated that T cells die in the meninges, probably due to the lack of restimulation (Chang et al, submitted).

These studies complement previous observations, showing that treatment with CTLA-4Ig (blocking both B7.1 and B7.2) injected directly into the CNS on day 13 post-immunization, inhibited EAE induced with spinal cord homogenate in Biozzi ABH Wt mice, in contrast to control treated mice that developed EAE (Croxford et al., 1998).

However, when MBP-specific T cell lines were transferred into SJL or (PLxSJL)F1 mice and the recipients were treated with CTLA-4Ig intraperitonatally (i.p.) after transfer, they developed clinical EAE with a severity that was comparable to control Ab treated mice (Perrin et al., 1995), suggesting that B7 costimulation is not critical in this model.

Overall, B7 costimulation seems to be important during the early effector phase of EAE in that it promotes the reactivation of effector T cells in the CNS.

So far, it is unclear whether ICOS costimulation is important during the reactivation of T cells during the early effector phase. Expression studies of ICOS and B7h have shown that these molecules are upregulated in the brain tissue of PLP 139-151-immunized SJL mice on days 10-20 post-immunization (Rottman et al., 2001). Considering the kinetics of expression of these molecules, the ICOS-B7h pathway is more likely to be important during the late effector phase.

8) *Late effector phase.* During the late effector phase, the myelin sheath is damaged, which disrupts normal nerve function and results in clinical disease. Damage of the myelin sheath also creates debris, which contributes to epitope spreading (discussed below). Effector T cells may possibly cause tissue damage by the production of inflammatory cytokines (including lymphotoxin and IFNγ), but they may also induce mononuclear cells in the CNS to cause tissue destruction (bystander tissue destruction). It is possible that effector T cells also interact with other T cells through B7-CD28/CTLA-4 interactions, and thereby amplify the T cell response. Finally, the T cell response is terminated. T cells that do not undergo programmed cell death become resting memory cells.

During this phase, B7.1 has been shown to be upregulated in the CNS of PLP 139-151-immunized SJL Wt mice with ongoing disease and during remissions (Karandikar et al., 1998; Miller et al., 1995). Its expression is higher than B7.2, which suggests that B7.1 is the dominant molecule during the late effector phase.

During the late effector phase, B7-CD28 costimulation seems to have a role in maintaining the immune response. Treatment with anti-CD28 after the onset of MBP-induced EAE in (PLxSJL)F1 mice resulted in the amelioration of the subsequent disease course, and no significant relapses (Perrin et al., 1999). Thus, anti-CD28 was able to block established disease, suggesting that CD28 signaling is crucial to maintaining the T cell response. Another study showed that when SJL Wt mice were immunized with CNS myelin and were treated with CTLA-4Fc (blocking B7.1 and B7.2) after the onset of clinical signs, full clinical remission occurred twice as often in the CTLA-4Fc group as in those mice receiving placebo (Cross et al., 1999). However, CTLA-4Fc treatment had no effect on the relapse rate.

In contrast to B7-CD28 costimulation, B7-CTLA-4 costimulation seems important for terminating the T cell response. Karandikar et al have shown that the adoptive transfer of PLP 139-151-primed Wt T cells into SJL Wt mice resulted in exacerbated EAE, when recipients received anti-CTLA-4 Ab at the time of adoptive transfer in comparison with control Ab-treated recipients (Karandikar et al., 1996). Also, when recipients received anti-CTLA-4 Ab at the time of disease remission, the incidence of clinical relapses and EAE severity were increased (Karandikar et al., 1996). Similarly, (PLxSJL)F1 mice that were immunized with MBP and treated with anti-CTLA-4 Ab at disease onset developed more severe EAE than control Ab-treated mice (Perrin et al., 1996). These observations suggest a role for B7-CTLA-4 costimulation during the effector phase in terminating the immune response. Thus, B7 costimulation can have either EAE promoting or inhibiting effects during the late effector phase, depending on which molecule, CD28 or CTLA-4, the B7 molecules engage.

ICOS seems to be important for the survival of effector and memory T cells at this stage. Our data show that the adoptive transfer of long-term PLP 139-151-primed T cell lines induces ameliorated EAE when recipients are treated with anti-ICOS mAb, compared to severe EAE in control Ab-treated recipients (Figure 5.8.B). Similarly, Rottman et al. show that treatment of PLP 139-151-immunized SJL Wt mice with anti-ICOS Ab during days 9-20 post-immunization results in less severe disease as compared to immunized mice that received no Ab-treatment (Rottman et al., 2001). In addition, Sporici et al. found that the induction of EAE by adoptive transfer of MBP-reactive transgenic T cells in B10.PL Wt mice resulted in amelioration of EAE when recipients were treated with ICOSIg (which blocks the ICOS ligand B7h) at the onset of disease (Sporici et al., 2001). B7h was found to provide an anti-apoptotic signal through ICOS to the encephalitogenic T cell. Thus, ICOS signaling during the late

effector phase seems to be critical in providing survival signals to effector and memory T cells, which could serve to maintain the autoimmune response.

9) *Epitope spreading*. During epitope spreading, the specificity of the immune response spreads to include self-epitopes other than those which initiated the inflammatory process (reviewed in (Vanderlugt and Miller, 2002)). For example, EAE may have been induced by immunization with PLP 139-151 in an SJL mouse. When tissue destruction occurs in the CNS, tissue debris is taken up by resident APCs, which can either stay in the CNS or can re-enter the lymphatic system where they can prime unactivated myelin-reactive T cells. Since the APCs now present different epitopes (e.g. PLP 178-191 or MBP 84-104), they will activate self-reactive T cells with a different epitope-specificity than PLP 139-151. In this way, a second wave of Th1 cells is induced that causes more tissue destruction.

B7-CD28 costimulation seems to promote epitope spreading. Studies by Miller et al. suggest that B7.1-CD28 costimulation is mainly responsible for this. SJL Wt mice were immunized with PLP 139-151 and, after recovery from the acute paralytic episode (after day 25 post-immunization), these mice were treated with B7.1 or B7.2 F(ab) fragments, blocking B7.1 or B7.2, respectively (Miller et al., 1995). Mice treated with anti-B7.2 F(ab) fragments developed clinical relapses comparable to control mice. In contrast, anti-B7.1 F(ab) fragment therapy resulted in blockade of clinical relapses, amelioration of CNS pathology and blockade of epitope spreading. Inhibition of epitope spreading was shown by the reduced Delayed Type Hypersensitivity (DTH) response to PLP 178-191 in anti-B7.1 F(ab) fragment treated mice, as compared to control treated mice (Miller et al., 1995). When anti-B7.1 F(ab) fragments were administered before the first relapse, this treatment resulted in PLP

178-191-specific tolerance and protection from disease relapse, even though peripheral Th1 responses to the initiating PLP 139-151 epitope were unaffected. Therefore, CD28 blockade seemed to lead to specific tolerance of T cells to the relapse-associated PLP 178-191 epitope. As of yet, there are no studies implying a role for ICOS in epitope spreading.

In summary, B7 and ICOS costimulation have many important roles during particular stages of EAE pathogenesis. Overall, B7 costimulation seems to promote the autoimmune response in that it costimulates the activation and differentiation of pathogenic T cells. In contrast, ICOS costimulation seems to counteract the development of pathogenic cells by downmodulating the differentiation of pathogenic cells. During later stages, however, ICOS costimulation seems to provide critical survival signals to effector T cells, and these are important for sustaining the autoimmune response.

These conclusions are very general and represent an overview of the different roles of costimulation in EAE. The mechanisms involved are complex and this is reflected by the many contradictory studies in the literature. Using the techniques described in this thesis has allowed us to separate, to some extent, the roles of different molecules at different times during EAE pathogenesis. Future progress depends on being able to refine these distinctions further.

Genetics and susceptibility to EAE

Predisposition to autoimmune disease is heritable and therefore it is of great importance to define the different genes, which are involved. In recent years, genetic analyses have been conducted in order to determine the genetic components that

determine susceptibility to EAE (for a review see (Anderson, 2002)). Genetic analyses have either used the classical approach or genome wide screening to identify loci that associate with a susceptible phenotype (discussed in more detail in chapter 3). In EAE, the suceptible phenotype has been defined primarily by paralysis, but intermediate disease phenotypes including CNS inflammation and demyelination, weight loss and drop in body temperature have been valuable in genetic studies since they depend on a smaller number of genes than the complete disease phenotype, and are therefore easier to analyze.

Genome wide screening has successfully identified several loci that influence susceptibility to EAE. However, many of the loci-intervals are relatively large and thus may contain more than one susceptibility or resistance allele. One of the best characterized susceptibility-loci is the locus Idd3 (located on murine chromosome 3), which was originally identified as a susceptibility locus for diabetes in the NOD mouse (Wicker et al., 1994), and later for EAE in mice (Encinas et al., 1999) and rats (Dahlman et al., 1999), and for experimental allergic orchitis (Teuscher et al., 1996). Idd3 contains *Fgf2* and *Il2*. *Il2* is the obvious candidate gene for an autoimmunity gene and polymorphisms in exon 1 are shared by the susceptible NOD and SJL mouse strains (Encinas et al., 1999). However, the mechanism by which IL-2 polymorphisms affect disease susceptibility is not known. Another important locus is on chromosome 11 and contains a Th2 cytokine gene cluster (Butterfield et al., 1998). With a combination of genome mapping and candidate gene analysis, it is likely that many autoimmunity-associated genes will be defined in the next decade.

The insights obtained with genome wide screening have led to two important observations. First, susceptibility loci identified in several autoimmune diseases overlap, raising the possibility that autoimmune gene(s) common to multiple

autoimmune diseases exist (Becker et al., 1998; Encinas et al., 1999; Teuscher et al., 1996; Vyse and Todd, 1996). Secondly, not all alleles from resistant mouse strains necessarily confer resistance since the threshold for disease depends on the sum of effects of genes of both susceptible and resistant phenotypes.

Overall, predisposition to EAE appears to be polygenic, meaning that disease susceptibility seems to be the result of multiple genetic factors acting together. The current concensus is that disease predisposition follows a threshold liability model, where effects of alleles at several loci incrementally contribute to susceptibility and define a threshold for disease induction. In this model, any different combination of alleles that contributes to susceptibility will produce a susceptible phenotype, as long as their cumulative effect surpasses the threshold. Therefore, different strains may be susceptible to EAE due to the inheritance of different sets of disease-associated alleles.

Human autoimmune diseases are clearly associated with genes at the HLA locus, prompting the study of MHC genes in mice and rats. In murine EAE models, the effect of H-2 haplotype on disease susceptibility has been studied using inbred and congenic lines. However, the association between MHC haplotype and EAE susceptibility is still unclear. In 1974, Levine and Sowinski showed that the replacement of H-2 haplotypes that were generally found in resistant strains (a or k) with those that were found in susceptible strains (s, b, or q) did not enhance susceptibility (Levine and Sowinski, 1974). In addition, an extensive study of congenic strains conducted by Montgomery and Rauch in 1982, showed that susceptibility correlated better with background genotype than with H-2 haplotype (Montgomery and Rauch, 1982). The only haplotype that appeared to have any effect

was H-2^b. They concluded that in mice the primary control of EAE susceptibility is outside the H-2 complex.

Our studies support the hypothesis that non-MHC genes are involved in determining EAE susceptibility in addition to the MHC. We have shown that a low number of BC1 B7.1/B7.$2^{-/-}$ mice homozygous for IA^s were resistant to EAE. In addition, a low number of s/b BC1 B7.1/B7.$2^{-/-}$ mice developed severe EAE (Table 3.5). These observations are consistant with a model in which additional genes outside the MHC can contribute to susceptibility.

We have demonstrated a clear linkage between the MHC haplotype and the EAE susceptibility phenotype. Our study has analyzed Backcross 1 (BC1) progeny generated by crossing EAE-resistant (SJLxB6)F1 B7.1/B7.$2^{-/-}$ (s/b) mice with EAE-susceptible SJL B7.1/B7.$2^{-/-}$ (s/s) mice. Immunization with PLP 139-151 resulted in the development of moderate to severe EAE in some BC1 mice, while other BC1 mice were resistant. Our genetic linkage analysis demonstrated that susceptibility and severity of disease were strongly linked to the MHC haplotype (Tables 3.4 and 3.5).

The mechanism of the association of MHC class II molecules with autoimmune disease is not well understood. The currently favored hypothesis proposes that disease-associated MHC molecules do not bind self-antigens well and are therefore unable to mediate effective negative selection in the thymus. This hypothesis is supported by studies that show the association of diabetes in the NOD mouse with the class II MHC-IA^{g7} molecules (Carrasco-Marin et al., 1996; Ridgway et al., 1999). IA^{g7} was found to be a poor peptide binder and to have structural instability (Carrasco-Marin et al., 1996). It was proposed that the unstable IA^{g7} would produce an effectively decreased density of MHC-peptide complexes on thymic APCs, which would select a population of T cells with increased TCR affinity (according to the

avidity model of thymic selection, see chapter 1 and (Ridgway et al., 1999)). Thus, IA^{g7} would select high-affinity self-reactive T cells which would enter the periphery and, in collaboration with other disease-related genes, mediate autoimmunity once an inflammatory event broke self-tolerance (Ridgway et al., 1999). Several studies showing that IA^{g7} allows the thymic selection of autoreactive T cells support this hypothesis, and this leads to an increase in autoreactive cells in the periphery (Kanagawa et al., 1998; Ridgway and Fathman, 1998; Ridgway et al., 1998). These studies propose a mechanism where IA^{g7} acts in the thymus of NOD mice.

In the context of our studies in chapter 3, where we show that the IA^{s} molecule is associated with EAE-susceptibility in BC1 B7.1/B7.2$^{-/-}$ mice, this raised the possibility that the IA^{s} molecule selects high-affinity self-reactive T cell populations in the thymus, similar to the IA^{g7} molecule. A study addressing the biochemical characteristsics of the IA^{s} molecule may thus reveal that it is also an unstable molecule, similarly to the IA^{g7} molecule. On the other hand, previous studies from our laboratory have shown that SJL mice (IA^{s}) have an endogenous repertoire of PLP 139-151-reactive T cells (Anderson et al., 2000), and this may indicate a more specific mechanism for the selection of a pathogenic repertoire.

Another study of diabetes proposes a mechanism whereby certain MHC class II molecules can mediate positive selection of T cells with additional specificities which act as regulatory T cells (Luhder et al., 1998). This study used a mouse model that carried the rearranged TCR transgene from a diabetogenic T cell clone (BDC2.5) derived from a NOD mouse. The BDC2.5 TCR transgenic mice on a B6 background but expressing homozygous IA^{g7} molecules exhibited strong insulitis at about 3 weeks of age and most mice developed diabetes a few weeks later. When one of the IA^{g7} alleles was replaced by the $H\text{-}2^{b}$ allele, to generate a $IA^{g7/b}$ mouse, insulitis was still

severe, but diabetes was markedly inhibited. The protective effect was shown to be mediated by the $A\beta^b$ gene and by T cells which do not use the transgenic BDC2.5 TCR. This study suggests that the $H\text{-}2^b$ allele present in the $IA^{g7/b}$ mice positively selects regulatory T cells that protect against diabetes (Luhder et al., 1998).

Therefore, based on the above studies, there are at least two mechanisms by which MHC molecules can confer susceptibility or resistance to autoimmune disease. One mechanism by which MHC molecules positively select a quantitatively increased repertoire of high-affinity autoreactive T cells (conferring susceptibility); the other by positively selecting regulatory T cells (conferring resistance). Whether these mechanisms are similar to the $B7.1/B7.2^{-/-}$ EAE model needs to be determined.

Implications for human MS

EAE and MS share many clinical and histological features. MS is an inflammatory disease of the CNS white matter characterized by demyelination and focal T cell and macrophage infiltrates. Like EAE, the loss of neurological functions can be either chronic or relapsing-remitting. Mice and humans seem to use similar mechanisms to generate their endogenous repertoires of autoreactive encephalitogenic cells, and the frequency of myelin-specific CD4+ cells is increased in the peripheral blood and cerebrospinal fluid in humans with MS (Allegretta et al., 1990; Bieganowska et al., 1997; Zhang et al., 1994). These similarities have led to the hypothesis that MS is caused by autoreactive T cells reacting against myelin antigens in much the same way as in actively induced EAE.

However, there are also distinct differences between murine EAE and human MS. Mice and humans have different MHC haplotypes and differ in various non-MHC background genes. In addition, there might be a difference in the critical

encephalitogen(s). The inciting myelin antigen for MS has not yet been identified, but it is likely that a variety of myelin proteins and epitopes play a role.

EAE is induced by the immunization of animals with myelin peptides in strong adjuvants or by adoptively transferring cells lines that are primed to a specific myelin epitope. The reasons why humans develop MS are still only poorly understood. Disease occurs because of a combination of environmental factors like viruses or stress, combined with a genetic predisposition. Patients are diagnosed with MS when disease is already fully established, so that pathogenic autoreactive T cells have already been induced and have reached an activated or memory-stage when therapy begins.

It has been demonstrated that MBP-specific T cells from the peripheral blood of MS patients have been previously activated and can expand independently of B7 costimulation *in vitro* (Scholz et al., 1998; Windhagen et al., 1995; Zhang et al., 1994). This is not too surprising since previously activated/memory cells have been found to be rather costimulation-independent also in the murine system. However, as we have demonstrated, B7 signaling is not only critical for the priming phase (at least in the B6 genetic background), but also in the effector phase, for autoreactive cells to enter the target organ (CNS). Thus, anti-B7 treatment might still be beneficial even in a later stage of autoimmune disease, even though most cells will have been activated by then. In addition if epitope spreading plays a similar role in human MS to its function in murine EAE, B7-blocking treatment might prevent the induction of autoreactive cells with additional specificities.

This thesis emphasizes that two factors are very important when analyzing the role of costimulation in EAE, timing and genetic background. Treatments for MS that target costimulatory molecules may need to be modified based on the stage of disease in the

individual. Our and other data clearly demonstrate that costimulatory molecules have different roles during various stages of disease and their blockade or activation at different timepoints may result in very different outcomes.

Individuals of different MHC haplotypes and different background genes may respond differently to the loss or manipulation (blockade versus activation) of costimulatory molecules. When designing treatments to ameliorate EAE or MS by manipulating costimulatory pathways, the influence of genetic background on the therapeutic target needs to be better understood. In human populations, the vast genetic heterogeneity among MS patients, may make it challenging to predict which patients will respond to anti-costimulation therapy. Therefore it will be valuable to determine genetic modifiers of B7 costimulation as these will have a predictive value for therapy.

Overall, targeting of costimulatory molecules provides a very attractive option for therapy in diseases like MS. Manipulating costimulatory molecules has the potential to be more specific than treating patients with immuno-suppressive or cytotoxic drugs.

Conclusions

The studies in this thesis were undertaken to examine the role of B7 and ICOS costimulation in EAE. SJL mice were susceptible to EAE, despite loss of the important B7-CD28 pathway. These results were in contrast to B6 mice that are resistant to EAE when the B7-CD28 pathway is disrupted. Studies using SJL B7.1 and B7.2 single and double deficient mice demonstrate that B7.1 and B7.2 have both distinct and overlapping roles in the induction and effector phases of EAE. The B7-CD28/CTLA-4 pathway also affects the expression of the costimulatory molecule

ICOS. We have shown that ICOS costimulation has a role during the induction and effector phases of EAE. Thus, the manipulation of one pathway may result in changing the profile of other pathways, contributing to different outcomes of EAE disease.

Our studies further demonstrate that the timing of costimulation-manipulation is critical and may determine whether the intervention exacerbates, ameliorates or has no effect on EAE. In addition, the genetic background, in particular the MHC haplotype, determines if the blockade of a costimulatory pathway results in susceptibility or resistance to EAE.

The elucidation of the role of the B7 and ICOS pathways in EAE is particularly challenging because 1) B7 costimulation through receptors can mediate both positive (e.g. CD28) or negative (e.g. CTLA-4) signals into the T cells, 2) of the presence of many other costimulatory pathways, many of them only partly understood or not yet identified; 3) of different roles of these molecules at different stages of disease; and 4) of their different roles on different genetic backgrounds.

The findings of this thesis emphasize the different requirements for costimulation on different genetic backgrounds and during different stages of EAE disease. They will become particularly relevant when therapies for human autoimmune diseases are developed that are based on the manipulation of B7 and ICOS costimulatory pathways.

Bibliography

Aicher, A., Hayden-Ledbetter, M., Brady, W. A., Pezzutto, A., Richter, G., Magaletti, D., Buckwalter, S., Ledbetter, J. A., and Clark, E. A. (2000). Characterization of human inducible costimulator ligand expression and function. J Immunol *164*, 4689-96.

Akbari, O., DeKruyff, R. H., and Umetsu, D. T. (2001). Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat Immunol *2*, 725-31.

Allegretta, M., Nicklas, J. A., Sriram, S., and Albertini, R. J. (1990). T cells responsive to myelin basic protein in patients with multiple sclerosis. Science *247*, 718-721.

Amiguet, P., Gardinier, M. V., Zanetta, J. P., and Matthieu, J. M. (1992). Purification and partial structural and functional characterization of mouse myelin/oligodendrocyte glycoprotein. J Neurochem *58*, 1676-82.

Amor, S., Baker, D., Groome, N., and Turk, J. L. (1993). Identification of a major encephalitogenic epitope of proteolipid protein (residues 56-70) for the induction of experimental allergic encephalomyelitis in Biozzi AB/H and nonobese diabetic mice. J Immunol *150*, 5666-72.

Amor, S., Groome, N., Linington, C., Morris, M. M., Dornmair, K., Gardinier, M. V., Matthieu, J. M., and Baker, D. (1994). Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. J Immunol *153*, 4349-56.

Amsen, D., and Kruisbeek, A. M. (1996). CD28-B7 interactions function to co-stimulate clonal deletion of double- positive thymocytes. Int Immunol *8*, 1927-36.

Anderson, A. C., L.B. Nicholson, and V.K. Kuchroo. (2002). Experimental Autoimmune Encephalomyelitis. In The Molecular Pathology of Autoimmune Diseases, A. N. T. a. C. Bona, ed. (New York: Taylor and Francis), pp. 734-751.

Anderson, A. C., Nicholson, L. B., Legge, K. L., Turchin, V., Zaghouani, H., and Kuchroo, V. K. (2000). High frequency of autoreactive myelin proteolipid protein-specific T cells in the periphery of naive mice: mechanisms of selection of the self-reactive repertoire. J Exp Med *191*, 761-70.

Anderson, D. E., Sharpe, A. H., and Hafler, D. A. (1999). The B7-CD28/CTLA-4 costimulatory pathways in autoimmune disease of the central nervous system. Curr Opin Immunol *11*, 677-83.

Azuma, M., Ito, D., Yagita, H., Okumura, K., Phillips, J. H., Lanier, L. L., and Somoza, C. (1993). B70 antigen is a second ligand for CTLA-4 and CD28. Nature. *366*, 76-79.

Baggiolini, M. (1998). Chemokines and leukocyte traffic. Nature *392*, 565-8.

Baker, D., O'Neill, J. K., and Turk, J. L. (1991). Cytokines in the central nervous system of mice during chronic relapsing experimental allergic encephalomyelitis. Cell Immunol *134*, 505-10.

Baker, D., Rosenwasser, O. A., O'Neill, J. K., and Turk, J. L. (1995). Genetic analysis of experimental allergic encephalomyelitis in mice. Journal of Immunology *155*, 4046-51.

Baron J.L., M., J.A., Ruddle, N.H., Hashim, G., and Janeway Jr., C.A. (1993). Surface expression of α4 intergin by CD4 T cells is required for their entery into brain parenchyma. J. Exp. Med. *177*, 57-68.

Becker, K. G., Simon, R. M., Bailey-Wilson, J. E., Freidlin, B., Biddison, W. E., McFarland, H. F., and Trent, J. M. (1998). Clustering of non-major histocompatibility complex susceptibility candidate loci in human autoimmune diseases. Proc Natl Acad Sci U S A *95*, 9979-84.

Beier, K. C., Hutloff, A., Dittrich, A. M., Heuck, C., Rauch, A., Buchner, K., Ludewig, B., Ochs, H. D., Mages, H. W., and Kroczek, R. A. (2000). Induction, binding specificity and function of human ICOS. Eur J Immunol *30*, 3707-17.

Ben-Nun, A., and I.R. Cohen. (1982). Experimental autoimmune encephalomyelitis (EAE) mediated by T cell lines: Process of selection of lines and characterization of cells. J. Immunol. *129*, 303-308.

Bettelli, E., Prabhu Das, M., Howard, E. D., Weiner, H. L., Sobel, R. A., and Kuchroo, V. K. (1998). IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. Journal of Immunology *161*, 3299-3306.

Bieganowska, K. D., Ausubel, L. J., Modabber, Y., Slovik, E., Messersmith, W., and Hafler, D. A. (1997). Direct ex vivo analysis of activated, Fas-sensitive autoreactive T cells in human autoimmune disease. J Exp Med *185*, 1585-94.

Boise, L., Minn, A., Noel, P., and Thompson, C. (1995). CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-xl. Immunity *3*, 87-98.

Borriello, F., Sethna, M. P., Boyd, S. D., Schweitzer, A. N., Tivol, E. A., Jacoby, D., Strom, T. B., Simpson, E. M., Freeman, G. J., and Sharpe, A. H. (1997). B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. Immunity *6*, 303-13.

Boussiotis, V. A., Freeman, G. J., Gray, G., Gribben, J., and Nadler, L. M. (1993). B7 but not intercellular adhesion molecule-1 costimulation prevents the induction of human alloantigen-specific tolerance. J Exp Med *178*, 1753-63.

Boussiotis, V. A., Freeman, G. J., Gribben, J. G., Daley, J., Gray, G., and Nadler, L. M. (1993). Activated human B lymphocytes express three CTLA-4 counterreceptors that costimulate T-cell activation. Proc Natl Acad Sci U S A *90*, 11059-63.

Boussiotis, V. A., Gribben, J. G., Freeman, G. J., and Nadler, L. M. (1994). Blockade of the CD28 co-stimulatory pathway: a means to induce tolerance. Curr Opin Immunol *6*, 797-807.

Bretscher, P., and Cohn, M. (1970). A theory of self-nonself discrimination. Science *169*, 1042-9.

Bright, J. J., Musuro, B. F., Du, C., and Sriram, S. (1998). Expression of IL-12 in CNS and lymphoid organs of mice with experimental allergic encephalitis. J Neuroimmunol *82*, 22-30.

Brostoff, S. W., and Mason, D.W. (1984). Experimental allergic encephalomyelitis: successful treatment in vivo with a monoclonal antibody that recognizes T helper cells. J. Immunol. *133*, 1938-1942.

Brunet, J. F., Denizot, F., Luciani, M. F., Roux-Dosseto, M., Suzan, M., Mattei, M. G., and Golstein, P. (1987). A new member of the immunoglobulin superfamily - CTLA-4. Nature *328*, 267-270.

Brunner, C., Lassmann, H., Waehneldt, T. V., Matthieu, J. M., and Linington, C. (1989). Differential ultrastructural localization of myelin basic protein,

myelin/oligodendroglial glycoprotein, and 2',3'-cyclic nucleotide 3'-phosphodiesterase in the CNS of adult rats. J Neurochem *52*, 296-304.

Brunner, M. C., Chambers, C. A., Chan, F. K., Hanke, J., Winoto, A., and Allison, J. P. (1999). CTLA-4-Mediated inhibition of early events of T cell proliferation. J Immunol *162*, 5813-20.

Burns, J., Rosenzweig, A., Zweiman, B., and Lisak, R. P. (1983). Isolation of myelin basic protein-reactive T-cell lines from normal human blood. Cell Immunol *81*, 435-40.

Burns, R. P., Jr., Nasir, A., Haake, A. R., Barth, R. K., and Gaspari, A. A. (1999). B7-1 overexpression by thymic epithelial cells results in transient and long-lasting effects on thymocytes and peripheral T helper cells but does not result in immunodeficiency. Cell Immunol *194*, 162-77.

Butterfield, R. J., Blankenhorn, E. P., Roper, R. J., Zachary, J. F., Doerge, R. W., Sudweeks, J., Rose, J., and Teuscher, C. (1999). Genetic analysis of disease subtypes and sexual dimorphisms in mouse experimental allergic encephalomyelitis (EAE): relapsing/remitting and monophasic remitting/nonrelapsing EAE are immunogenetically distinct. J Immunol *162*, 3096-102.

Butterfield, R. J., Sudweeks, J. D., Blankenhorn, E. P., Korngold, R., Marini, J. C., Todd, J. A., Roper, R. J., and Teuscher, C. (1998). New genetic loci that control

susceptibility and symptoms of experimental allergic encephalomyelitis in inbred mice. J Immunol *161*, 1860-7.

Carrasco-Marin, E., Shimizu, J., Kanagawa, O., and Unanue, E. R. (1996). The class II MHC I-Ag7 molecules from non-obese diabetic mice are poor peptide binders. J Immunol *156*, 450-8.

Chambers, C. A., Cado, D., Truong, T., and Allison, J. P. (1997). Thymocyte development is normal in CTLA-4-deficient mice. Proc Natl Acad Sci U S A *94*, 9296-301.

Chang, J. T., Shevach, E. M., and Segal, B. M. (1999). Regulation of interleukin (IL)-12 receptor beta2 subunit expression by endogenous IL-12: a critical step in the differentiation of pathogenic autoreactive T cells. J Exp Med *189*, 969-78.

Chang, T. T., Jabs, C., Sobel, R. A., Kuchroo, V. K., and Sharpe, A. H. (1999). Studies in B7-deficient mice reveal a critical role for B7 costimulation in both induction and effector phases of experimental autoimmune encephalomyelitis. J Exp Med *190*, 733-40.

Chitnis, T., Najafian, N., Abdallah, K. A., Dong, V., Yagita, H., Sayegh, M. H., and Khoury, S. J. (2001). CD28-independent induction of experimental autoimmune encephalomyelitis. J Clin Invest *107*, 575-83.

Clark, E. A., and Ledbetter, J. A. (1994). How B and T cells talk to each other. Nature *367*, 425-428.

Copeland, N. G., Jenkins, N. A., Gilbert, D. J., Eppig, J. T., Maltais, L. J., Miller, J. C., Dietrich, W. F., Weaver, A., Lincoln, S. E., Steen, R. G., and et al. (1993). A genetic linkage map of the mouse: current applications and future prospects [see comments]. Science *262*, 57-66.

Coyle, A. J., and Gutierrez-Ramos, J. C. (2001). The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. Nat Immunol *2*, 203-9.

Coyle, A. J., Lehar, S., Lloyd, C., Tian, J., Delaney, T., Manning, S., Nguyen, T., Burwell, T., Schneider, H., Gonzalo, J. A., Gosselin, M., Owen, L. R., Rudd, C. E., and Gutierrez-Ramos, J. C. (2000). The CD28-related molecule ICOS is required for effective T cell- dependent immune responses. Immunity *13*, 95-105.

Cross, A. H., Girard, T. J., Giacoletto, K. S., Evans, R. J., Keeling, R. M., Lin, R. F., Trotter, J. L., and Karr, R. W. (1995). Long-term inhibition of murine experimental autoimmune encephalomyelitis using CTLA4-Fc supports a key role for CD28 costimulation. Journal of Clinical Investigation *95*, 2783-2789.

Cross, A. H., Lyons, J. A., San, M., Keeling, R. M., Ku, G., and Racke, M. K. (1999). T cells are the main cell type expressing B7-1 and B7-2 in the central nervous system

during acute, relapsing and chronic experimental autoimmune encephalomyelitis. Eur J Immunol *29*, 3140-7.

Cross, A. H., San, M., Keeling, R. M., and Karr, R. W. (1999). CTLA-4-Fc treatment of ongoing EAE improves recovery, but has no effect upon relapse rate. Implications for the mechanisms involved in disease perpetuation. J Neuroimmunol *96*, 144-7.

Croxford, J. L., O'Neill, J. K., Ali, R. R., Browne, K., Byrnes, A. P., Dallman, M. J., Wood, M. J., Fedlmann, M., and Baker, D. (1998). Local gene therapy with CTLA4-immunoglobulin fusion protein in experimental allergic encephalomyelitis. Eur J Immunol *28*, 3904-16.

Croxford, J. L., O'Neill, J. K., and Baker, D. (1997). Polygenic control of experimental allergic encephalomyelitis in Biozzi ABH and BALB/c mice. J Neuroimmunol *74*, 205-11.

Cua, D. J., Groux, H., Hinton, D. R., Stohlman, S. A., and Coffman, R. L. (1999). Transgenic interleukin 10 prevents induction of experimental autoimmune encephalomyelitis. J Exp Med *189*, 1005-10.

Dahlman, I., Jacobsson, L., Glaser, A., Lorentzen, J. C., Andersson, M., Luthman, H., and Olsson, T. (1999). Genome-wide linkage analysis of chronic relapsing experimental autoimmune encephalomyelitis in the rat identifies a major susceptibility locus on chromosome 9. J Immunol *162*, 2581-8.

Dai, Z., and Lakkis, F. G. (1999). The role of cytokines, CTLA-4 and costimulation in transplant tolerance and rejection. Curr Opin Immunol *11*, 504-8.

Degermann, S., Surh, C. D., Glimcher, L. H., Sprent, J., and Lo, D. (1994). B7 expression on thymic medullary epithelium correlates with epithelium- mediated deletion of V beta 5+ thymocytes. J Immunol *152*, 3254-63.

Dietrich, W., Katz, H., Lincoln, S. E., Shin, H. S., Friedman, J., Dracopoli, N. C., and Lander, E. S. (1992). A genetic map of the mouse suitable for typing intraspecific crosses. Genetics *131*, 423-47.

Dietrich, W. F., Miller, J. C., Steen, R. G., Merchant, M., Damron, D., Nahf, R., Gross, A., Joyce, D. C., Wessel, M., Dredge, R. D., and et al. (1994). A genetic map of the mouse with 4,006 simple sequence length polymorphisms. Nat Genet *7*, 220-45.

Dong, C., Juedes, A. E., Temann, U. A., Shresta, S., Allison, J. P., Ruddle, N. H., and Flavell, R. A. (2001). ICOS co-stimulatory receptor is essential for T-cell activation and function. Nature *409*, 97-101.

Dong, H., Zhu, G., Tamada, K., and Chen, L. (1999). B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med *5*, 1365-9.

Encinas, J. A., Lees, M. B., Sobel, R. A., Symonowicz, C., Greer, J. M., Shovlin, C. L., Weiner, H. L., Seidman, C. E., Seidman, J. G., and Kuchroo, V. K. (1996). Genetic analysis of susceptibility to experimental autoimmune encephalomyelitis in a cross between SJL/J and B10.S mice. J Immunol *157*, 2186-2192.

Encinas, J. A., Lees, M. B., Sobel, R. A., Symonowicz, C., Weiner, H. L., Seidman, C. E., Seidman, J. G., and Kuchroo, V. K. (2001). Identification of genetic loci associated with paralysis, inflammation and weight loss in mouse experimental autoimmune encephalomyelitis. Int Immunol *13*, 257-64.

Encinas, J. A., Wicker, L. S., Peterson, L. B., Mukasa, A., Teuscher, C., Sobel, R., Weiner, H. L., Seidman, C. E., Seidman, J. G., and Kuchroo, V. K. (1999). QTL influencing autoimmune diabetes and encephalomyelitis map to a 0.15-cM region containing Il2 [letter]. Nat Genet *21*, 158-60.

Endoh, M., Kunishita, T., Nihei, J., Nishizawa, M., and Tabira, T. (1990). Susceptibility to proteolipid apoprotein and its encephalitogenic determinants in mice. Int Arch Allergy Appl Immunol *92*, 433-8.

Falcone, M., Rajan, A. J., Bloom, B. R., and Brosnan, C. F. (1998). A critical role for IL-4 in regulating disease severity in experimental allergic encephalomyelitis as demonstrated in IL-4-deficient C57BL/6 mice and BALB/c mice. J Immunol *160*, 4822-30.

Ferber, I. A., Brocke, S., Taylor-Edwards, C., Ridgway, W., Dinisco, C., Steinman, L., Dalton, D., and Fathman, C. G. (1996). Mice with disrupted IFN-g gene are susceptible to the indcution of experimental autoimmune encephalomyelitis (EAE). J. Immunology *156*.

Fife, B. T., Paniagua, M. C., Lukacs, N. W., Kunkel, S. L., and Karpus, W. J. (2001). Selective CC chemokine receptor expression by central nervous system- infiltrating encephalitogenic T cells during experimental autoimmune encephalomyelitis. J Neurosci Res *66*, 705-14.

Fischer, F. R., Santambrogio, L., Luo, Y., Berman, M. A., Hancock, W. W., and Dorf, M. E. (2000). Modulation of experimental autoimmune encephalomyelitis: effect of altered peptide ligand on chemokine and chemokine receptor expression. J Neuroimmunol *110*, 195-208.

Folch, J., and M. Lees (1951). Proteolipides, a new type of tissue lipoproteins. Their isolation from the brain. J. Biol. Chem. *191*, 807-817.

Freedman, A. S., Freeman, G. J., Horowitz, J. C., Daley, J., and Nadler, L. M. (1987). B7, a B cell restricted antigen which identifies pre-activated B cells. J. Immunol. *137*, 3260-3267.

Freeman, G., Gray, G., Gimmi, C., and et.al. (1991). Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. Journal of Experimental Medicine *174*, 625-631.

Freeman, G. J., Boriello, F., Hodes, R. J., Reiser, H., Gribben, J. J., Ng, J. W., Kim, J., Goldberg, J. M., Hathcock, K., G., L., Lombard, L. A., Wang, S., Gray, G., Nadler, L. M., and Sharpe, A. H. (1993). Murine B7-2, an alternative CTLA4 counter-receptor that costimulates T cell prolifeartion and interleukin-2 production. J. Exp. Med. *178*, 2185-2192.

Freeman, G. J., Borriello, F., Hodes, R. J., Reiser, H., Hathcock, K. S., Laszlo, G., McKnight, A. J., Kim, J., L., D., Lombard, D. B., Gray, G. S., Nadler, L. M., and Sharpe, A. H. (1993). Uncovering of functional alternative CTLA4 counter-receptor in B7-deficient mice. Science *262*, 907-909.

Freeman, G. J., Freedman, A. S., Segil, J. M., Lee, G., Whitman, J. F., and Nadler, L. M. (1989). B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. J. Immunol. *143*, 2714-2722.

Freeman, G. J., Gribben, J. G., Boussiotis, V. A., Ng, J. W., Restivo, V., Lombard, L., Gray, G. S., and Nadler, L. M. (1993). Cloning of B7-2: a CTLA4 counter-receptor that costimulates human T cell proliferation. Science. *262*, 909-911.

Freeman, G. J., Gribben, J. G., Gimmi, C. D., Koyasu, S., Moebius, U., Reinherz, E. L., and Nadler, L. M. (1991). B7 enhances MHC class II restricted antigen specific cytolysis by a cytolytic T cell clone. Blood *78, Suppl. 1*, 264a.

Freeman, G. J., Long, A. J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L. J., Malenkovich, N., Okazaki, T., Byrne, M. C., Horton, H. F., Fouser, L., Carter, L., Ling, V., Bowman, M. R., Carreno, B. M., Collins, M., Wood, C. R., and Honjo, T. (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med *192*, 1027-34.

Frei, K., Eugster, H. P., Bopst, M., Constantinescu, C. S., Lavi, E., and Fontana, A. (1997). Tumor necrosis factor alpha and lymphotoxin alpha are not required for induction of acute experimental autoimmune encephalomyelitis. J Exp Med *185*, 2177-82.

Fritz, R. B., Skeen, M. J., Chou, C. H., Garcia, M., and Egorov, I. K. (1985). Major histocompatibility complex-linked control of the murine immune response to myelin basic protein. Journal of Immunology *134*, 2328-32.

Girvin, A. M., Dal Canto, M. C., Rhee, L., Salomon, B., Sharpe, A., Bluestone, J. A., and Miller, S. D. (2000). A critical role for B7/CD28 costimulation in experimental autoimmune encephalomyelitis: a comparative study using costimulatory molecule-deficient mice and monoclonal antibody blockade. J Immunol *164*, 136-43.

Goebels, N., Hofstetter, H., Schmidt, S., Brunner, C., Wekerle, H., and Hohlfeld, R. (2000). Repertoire dynamics of autoreactive T cells in multiple sclerosis patients and healthy subjects: epitope spreading versus clonal persistence. Brain *123 Pt 3*, 508-18.

Gonatas, N. K., and Howard, J. C. (1974). Inhibition of experimental allergic encephalomyelitis in rats severely depleted of T cells. Science *186*, 839-41.

Gonzalo, J. A., Tian, J., Delaney, T., Corcoran, J., Rottman, J. B., Lora, J., Al-garawi, A., Kroczek, R., Gutierrez-Ramos, J. C., and Coyle, A. J. (2001). ICOS is critical for T helper cell-mediated lung mucosal inflammatory responses. Nat Immunol *2*, 597-604.

Greenwald, R. J., Boussiotis, V. A., Lorsbach, R. B., Abbas, A. K., and Sharpe, A. H. (2001). CTLA-4 regulates induction of anergy in vivo. Immunity *14*, 145-55.

Greer, J. M., Kuchroo, V.K., Sobel R.A., and Lees, M.B. (1992). Identification and characterization of a second encephalitogenic determinant of myelin proteolipid protein (residues 178-191) for SJL mice. J. Immunol. *149*, 783-788.

Gribben, J. G., Freeman, G. J., Boussiotis, V. A., Rennert, P., Jellis, C. L., Greenfield, E., Barber, M., Gray, G. S., and Nadler, L. M. (1994). CTLA4 mediated costimulation induces apoptosis of activated human T lymphocytes. Proceedings of the National Academy of Science USA *in press*.

Gross, J., Callas, E., and Allison, J. (1992). Identification and distribution of the costimulatory receptor CD28 in the mouse. Journal of Immunology *149*, 380-388.

Hashim, G. A. (1978). Myelin basic protein: structure, function and antigenic determinants. Immunol Rev *39*, 60-107.

Hicky, W. (1991). T lymphocyte entry and antigen recognition in the central nervous system. Academic Press (New York) *Ader R, Felton D, Cohen N., ed. Psycoimmunoloty*, 149-176.

Howard, L. M., Miga, A. J., Vanderlugt, C. L., Dal Canto, M. C., Laman, J. D., Noelle, R. J., and Miller, S. D. (1999). Mechanisms of immunotherapeutic intervention by anti-CD40L (CD154) antibody in an animal model of multiple sclerosis. J Clin Invest *103*, 281-90.

Hurwitz, A. A., Sullivan, T. J., Krummel, M. F., Sobel, R. A., and Allison, J. P. (1997). Specific blockade of CTLA-4/B7 interactions results in exacerbated clinical and histologic disease in an actively-induced model of experimental allergic encephalomyelitis. J Neuroimmunol *73*, 57-62.

Hutloff, A., Dittrich, A. M., Beier, K. C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., and Kroczek, R. A. (1999). ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature *397*, 263-6.

Izikson, L., Klein, R. S., Charo, I. F., Weiner, H. L., and Luster, A. D. (2000). Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2. J Exp Med *192*, 1075-80.

Jabs, C., A.H. Sharpe, and V.K. Kuchroo. (in press). B7 family molecules in autoimmunity. In The B7 family and the immune responses: R.G. Landes Publisher.

Jenkins, M., Chen, C., Jung, G., Mueller, D., and Schwartz, R. (1990). Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immorbilized anti-CD3 monoclonal antibody. J. Immunol. *144*, 16-24.

Jenkins, M. K. (1992). The role of cell division in the induction of clonal anergy. Immunology Today *13*, 69-73.

Jenkins, M. K., and Schwartz, R. H. (1987). Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. Journal of Experimental Medicine *165*, 302-319.

Jenkinson, E. J., Anderson, G., Moore, N. C., Smith, C. A., and Owen, J. J. (1994). Positive selection by purified MHC class II+ thymic epithelial cells in vitro: costimulatory signals mediated by B7 are not involved. Dev Immunol *3*, 265-71.

Juedes, A. E., Hjelmstrom, P., Bergman, C. M., Neild, A. L., and Ruddle, N. H. (2000). Kinetics and cellular origin of cytokines in the central nervous system: insight into mechanisms of myelin oligodendrocyte glycoprotein- induced experimental autoimmune encephalomyelitis. J Immunol *164*, 419-26.

June, C. H., Bluestone, J. A., Nadler, L. M., and Thompson, C. B. (1994). The B7 and CD28 receptor families. Immunol. Today. *15*, 321-31.

Kanagawa, O., Martin, S. M., Vaupel, B. A., Carrasco-Marin, E., and Unanue, E. R. (1998). Autoreactivity of T cells from nonobese diabetic mice: an I-Ag7- dependent reaction. Proc Natl Acad Sci U S A *95*, 1721-4.

Karandikar, N., Vanderlugt, C., Walnus, T., Miller, S., and Bluestone, J. (1996). CTLA-4-a negative regulator of autoimmune disease. Journal of Experimental Medicine *184*, 783-788.

Karandikar, N. J., Vanderlugt, C. L., Bluestone, J. A., and Miller, S. D. (1998). Targeting the B7/CD28:CTLA-4 costimulatory system in CNS autoimmune disease. J Neuroimmunol *89*, 10-8.

Karandikar, N. J., Vanderlugt, C. L., Eagar, T., Tan, L., Bluestone, J. A., and Miller, S. D. (1998). Tissue-specific up-regulation of B7-1 expression and function during the course of murine relapsing experimental autoimmune encephalomyelitis. J Immunol *161*, 192-9.

Karpus, W. J., Lukacs, N. W., McRae, B. L., Strieter, R. M., Kunkel, S. L., and Miller, S. D. (1995). An important role for the chemokine macrophage inflammatory protein-1 alpha in the pathogenesis of the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis. J Immunol *155*, 5003-10.

Karpus, W. J., and Ransohoff, R. M. (1998). Chemokine regulation of experimental autoimmune encephalomyelitis: temporal and spatial expression patterns govern disease pathogenesis. J Immunol *161*, 2667-71.

Kennedy, M. K., Torrance, D. S., Picha, K. S., and Mohler, K. M. (1992). Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. Journal of Immunology *149*, 2496-505.

Kerlero de Rosbo, N., Mendel, I., and Ben-Nun, A. (1995). Chronic relapsing experimental autoimmune encephalomyelitis with a delayed onset and an atypical clinical course, induced in PL/J mice by myelin oligodendrocyte glycoprotein (MOG)-derived peptide: preliminary analysis of MOG T cell epitopes. Eur J Immunol *25*, 985-93.

Khoury, S. J., Hancock, W. W., and Weiner, H. L. (1992). Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis as associated with downregulation of inflammatory cytokines and differential upregulation of tranforming growth factor β, interleukin 4, and prostaglandin E expression in the brain. J. Exp. Med. *176*, 1355.

Kishimoto, H., Cai, Z., Brunmark, A., Jackson, M. R., Peterson, P. A., and Sprent, J. (1996). Differing roles for B7 and intercellular adhesion molecule-1 in negative selection of thymocytes. J Exp Med *184*, 531-7.

Klaus, S. J., Pinchuk, L. M., Ochs, H. D., Law, C. L., Fanslow, W. C., Armitage, R. J., and Clark, E. A. (1994). Costimulation through CD28 enhances T cell-dependent B cell activation via CD40-CD40L interaction. J. Immunol. *152*, 5643-52.

Kopf, M., Coyle, A. J., Schmitz, N., Barner, M., Oxenius, A., Gallimore, A., Gutierrez-Ramos, J. C., and Bachmann, M. F. (2000). Inducible costimulator protein (ICOS) controls T helper cell subset polarization after virus and parasite infection. J Exp Med *192*, 53-61.

Korner, H., Riminton, D. S., Strickland, D. H., Lemckert, F. A., Pollard, J. D., and Sedgwick, J. D. (1997). Critical points of tumor necrosis factor action in central nervous system autoimmune inflammation defined by gene targeting. J Exp Med *186*, 1585-90.

Krakowski, M., and Owens, T. (1996). Interferon-gamma confers resistance to experimental allergic encephalomyelitis. Eur J Immunol *26*, 1641-6.

Krummel, M., and Allison, J. (1995). CD28 and CTLA4 have opposing effects on the response of T cells to stimulation. J Exp. Med. *182*, 459-466.

Kuchroo, V., Prabhu Das, M., Brown, J. A., Ranger, A. M., Zamvil, S. S., Sobel, R. A., Weiner, H. L., Nabavi, N., and Glimcher, L. H. (1995). B7-1 and B7-2 costimulatory molecules differentially activate the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell *80*, 707-716.

Kuchroo, V. K., Martin, C. A., Greer, J. M., Ju, S. T., Sobel, R. A., and Dorf, M. E. (1993). Cytokines and adhesion molecules contribute to the ability of myelin

proteolipid protein-specific T cell clones to mediate experimental allergic encephalomyelitis. Journal of Immunology *151*, 4371-82.

Kuchroo, V. K., Sobel, R. A., Laning, J. C., Martin, C. A., Greenfield, E., Dorf, M. E., and Lees, M. B. (1992). Experimental allergic encephalomyelitis mediated by cloned T cells specific for a synthetic peptide of myelin proteolipid protein. Fine specificity and T cell receptor V beta usage. Journal of Immunology *148*, 3776-82.

Kuiper, H., Brouwer, M., de Boer, M., Parren, P., and van Lier, R. A. (1994). Differences in responsiveness to CD3 stimulation between naive and memory CD4+ T cells cannot be overcome by CD28 costimulation. Eur J Immunol *24*, 1956-60.

Kumar, V. (1998). Determinant spreading during experimental autoimmune encephalomyelitis: is it potentiating, protecting or participating in the disease? Immunol Rev *164*, 73-80.

Lafaille, J. J., Keere, F. V., Hsu, A. L., Baron, J. L., Haas, W., Raine, C. S., and Tonegawa, S. (1997). Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. J Exp Med *186*, 307-12.

Lafferty, K. J., and Cunningham, A. J. (1975). A new analysis of allogeneic interactions. Aust J Exp Biol Med Sci *53*, 27-42.

Lanzavecchia, A., Lezzi, G., and Viola, A. (1999). From TCR engagement to T cell activation: a kinetic view of T cell behavior. Cell *96*, 1-4.

Lehmann, P., Forsthuber, T., Miller, A., and Sercarz, E. (1992). Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature *358*, 155.

Lehmann, P. V., Sercarz, E. E., Forsthuber, T., Dayan, C. M., and Gammon, G. (1993). Determinant spreading and the dynamics of the autoimmune T-cell repertoire. Immunol Today *14*, 203-8.

Lehmann, P. V., Targoni, O. S., and Forsthuber, T. G. (1998). Shifting T-cell activation thresholds in autoimmunity and determinant spreading. Immunol Rev *164*, 53-61.

Lenschow, D. J., Zeng, Y., Thistlethwaite, J. R., Montag, A., Brady, W., Gibson, M. G., Linsley, P. S., and Bluestone, J. A. (1992). Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. Science *257*, 789-92.

Levine, S., and Sowinski, R. (1974). Experimental allergic encephalomyelitis in congenic strains of mice. Immunogenetics *1*, 352-356.

Liblau, R., Steinman, L., and Brocke, S. (1997). Experimental autoimmune encephalomyelitis in IL-4-deficient mice. Int Immunol *9*, 799-803.

Ligers, A., Xu, C., Saarinen, S., Hillert, J., and Olerup, O. (1999). The CTLA-4 gene is associated with multiple sclerosis. J Neuroimmunol *97*, 182-90.

Ling, V., Wu, P. W., Finnerty, H. F., Bean, K. M., Spaulding, V., Fouser, L. A., Leonard, J. P., Hunter, S. E., Zollner, R., Thomas, J. L., Miyashiro, J. S., Jacobs, K. A., and Collins, M. (2000). Cutting edge: identification of GL50, a novel B7-like protein that functionally binds to ICOS receptor. J Immunol *164*, 1653-7.

Linsely, P., Wallace, P., Johnson, J., Gibson, M., Greene, J., Ledbetter, J., Singh, C., and tepper, M. (1992). Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. Science *257*.

Linsley, P., Bradshaw, J., Greene, J., Peach, R., bennett, K., and Mittler, R. (1996). Intracellular trafficking of CTLA4 and focal localization towards sites of TcR engagement. Immunity *4*, 535-543.

Linsley, P., Brady, W., Urnes, M., Grosmaire, L., Damle, N., and Ledbetter, J. (1991). CTLA-4 is a second receptor for the B cell activation antigen B7. Journal of Experimental Medicine *174*, 561-569.

Linsley, P. S., Greene, J. L., Brady, W., Bayorath, J., Ledbetter, J. A., and Peach, R. (1994). Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA4 receptors. Immunity *1*, 793-801.

Linsley, P. S., Greene, J. L., Tan, P., Bradshaw, J., Ledbetter, J. A., Anasetti, C., and Damle, N. K. (1992). Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. J . Exp. Med. *176*, 1595-1604.

Liu, J., Marino, M. W., Wong, G., Grail, D., Dunn, A., Bettadapura, J., Slavin, A. J., Old, L., and Bernard, C. C. (1998). TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. Nat Med *4*, 78-83.

London, C. A., Lodge, M. P., and Abbas, A. K. (2000). Functional responses and costimulator dependence of memory CD4+ T cells. J Immunol *164*, 265-72.

Lucas, B., and Germain, R. N. (2000). Opening a window on thymic positive selection: developmental changes in the influence of cosignaling by integrins and CD28 on selection events induced by TCR engagement. J Immunol *165*, 1889-95.

Lucas, P. J., Negishi, I., Nakayama, K., Fields, L. E., and Loh, D. Y. (1995). Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific Immune response. Journal of Immunology *154*, 5757-5768.

Luhder, F., Katz, J., Benoist, C., and Mathis, D. (1998). Major histocompatibility complex class II molecules can protect from diabetes by positively selecting T cells with additional specificities. J Exp Med *187*, 379-87.

Lyons, A. B., and Parish, C. R. (1994). Determination of lymphocyte division by flow cytometry. J Immunol Methods *171*, 131-7.

Mages, H. W., Hutloff, A., Heuck, C., Buchner, K., Himmelbauer, H., Oliveri, F., and Kroczek, R. A. (2000). Molecular cloning and characterization of murine ICOS and identification of B7h as ICOS ligand. Eur J Immunol *30*, 1040-7.

Matejuk, A., Vandenbark, A. A., Burrows, G. G., Bebo, B. F., Jr., and Offner, H. (2000). Reduced chemokine and chemokine receptor expression in spinal cords of TCR BV8S2 transgenic mice protected against experimental autoimmune encephalomyelitis with BV8S2 protein. J Immunol *164*, 3924-31.

Mathisen, P. M., Yu, M., Johnson, J. M., Drazba, J. A., and Tuohy, V. K. (1997). Treatment of experimental autoimmune encephalomyelitis with genetically modified memory T cells. J Exp Med *186*, 159-64.

McAdam, A. J., Chang, T. T., Lumelsky, A. E., Greenfield, E. A., Boussiotis, V. A., Duke-Cohan, J. S., Chernova, T., Malenkovich, N., Jabs, C., Kuchroo, V. K., Ling, V., Collins, M., Sharpe, A. H., and Freeman, G. J. (2000). Mouse Inducible Costimulatory Molecule (ICOS) Expression Is Enhanced by CD28 Costimulation and Regulates Differentiation of CD4(+) T Cells. J Immunol *165*, 5035-5040.

McAdam, A. J., Greenwald, R. J., Levin, M. A., Chernova, T., Malenkovich, N., Ling, V., Freeman, G. J., and Sharpe, A. H. (2001). ICOS is critical for CD40-mediated antibody class switching. Nature *409*, 102-5.

McRae, B. L., Kennedy, M. K., Tan, L. J., Dal Canto, M. C., Picha, K. S., and Miller, S. D. (1992). Induction of active and adoptive relapsing experimental autoimmune encephalomyelitis (EAE) using an encephalitogenic epitope of proteolipid protein. J Neuroimmunol *38*, 229-240.

McRae, B. L., Vanderlugt, C. L., Dal Canto, M. C., and Miller, S. D. (1995). Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. J Exp Med *182*, 75-85.

Melero, I., Bach, N., and Chen, L. (1997). Costimulation, tolerance and ignorance of cytolytic T lymphocytes in immune responses to tumor antigens. Life Sci *60*, 2035-41.

Mendel, I., Kerlero de Rosbo, N., and Ben-Nun, A. (1995). A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells. Eur J Immunol *25*, 1951-9.

Merrill, J. E., Kono, D. H., Clayton, J., Ando, D. G., Hinton, D. R., and Hofman, F. M. (1992). Inflammatory leukocytes and cytokines in the peptide-induced disease of experimental allergic encephalomyelitis in SJL and B10.PL mice [published erratum appears in Proc Natl Acad Sci U S A 1992 Nov 1;89(21):10562]. Proc Natl Acad Sci U S A *89*, 574-8.

Miller, S., and Karpus, W. (1994). The immunopathogenesis and regualtion of T-cell mediated demyelinating diseases. Immunology Today *15*, 356-361.

Miller, S., Vanderlugt, C., Lenschow, D., Pope, J., Karandikar, N., Canto, M. D., and Bluestone, J. (1995). Blockade of CD28-B7-1 interaction prevents epitope spreading and clinical relapses of murine EAE. Immunity *3*, 739-745.

Montgomery, I. N., and Rauch, H. C. (1982). Experimental allergic encephalomyelitis (EAE) in mice: primary control of EAE susceptibility is outside the H-2 complex. Journal of Immunology *128*, 421-5.

Mosmann, T. R., and Sad, S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more [see comments]. Immunol Today *17*, 138-46.

Mueller, D. L., Jenkins, M. K., and Schwartz, R. H. (1989). An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. J. Immunol. *142*, 2617-2628.

Nepom, G. T., Buckner, J. H., Novak, E. J., Reichstetter, S., Reijonen, H., Gebe, J., Wang, R., Swanson, E., and Kwok, W. W. (2002). HLA class II tetramers: tools for direct analysis of antigen-specific CD4+ T cells. Arthritis Rheum *46*, 5-12.

Nicholson, L. B., Greer, J. M., Sobel, R. A., Lees, M. B., and Kuchroo, V. K. (1995). An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. Immunity *3*, 397-405.

Nicholson, L. B., Waldner, H., Carrizosa, A. M., Sette, A., Collins, M., and Kuchroo, V. K. (1998). Heteroclitic proliferative responses and changes in cytokine profile induced by altered peptides: implications for autoimmunity. Proc Natl Acad Sci U S A *95*, 264-9.

Nishimura, H., Nose, M., Hiai, H., Minato, N., and Honjo, T. (1999). Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity *11*, 141-51.

Nishimura, H., Okazaki, T., Tanaka, Y., Nakatani, K., Hara, M., Matsumori, A., Sasayama, S., Mizoguchi, A., Hiai, H., Minato, N., and Honjo, T. (2001). Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science *291*, 319-22.

Oliveira-dos-Santos, A. J., Ho, A., Tada, Y., Lafaille, J. J., Tonegawa, S., Mak, T. W., and Penninger, J. M. (1999). CD28 costimulation is crucial for the development of spontaneous autoimmune encephalomyelitis. J Immunol *162*, 4490-5.

Oppenheim, J., and I, G. (1982). Interleukin 1 is more than an interleukin. Immunology Today *3*, 113.

Ota, K., Matsui, M., Milford, E. L., Mackin, G. A., Weiner, H. L., and Hafler, D. A. (1990). T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. Nature *346*, 183-187.

Peach, R. J., Bajorath, J., Brady, W., Leytze, G., Greene, J., Naemura, J., and Linsley, P. S. (1994). Complementarity determining region 1 (CDR1)- and CDR3-analogous regions in CTLA-4 and CD28 determine the binding to B7-1. J Exp Med *180*, 2049-58.

Perez, V., Vanparijis, L., Biuckians, A., Zheng, X., Strom, T., and Abbas, A. (1997). Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. Immunity *6*, 411-417.

Perrin, P. J., June, C. H., Maldonado, J. H., Ratts, R. B., and Racke, M. K. (1999). Blockade of CD28 during In vitro activation of encephalitogenic T cells or after disease onset ameliorates experimental autoimmune encephalomyelitis [In Process Citation]. J Immunol *163*, 1704-10.

Perrin, P. J., Lavi, E., Rumbley, C. A., Zekavat, S. A., and Phillips, S. M. (1999). Experimental autoimmune meningitis: a novel neurological disease in CD28-deficient mice. Clin Immunol *91*, 41-9.

Perrin, P. J., Maldonado, J. H., Davis, T. A., June, C. H., and Racke, M. K. (1996). CTLA-4 blockade enhances clinical disease and cytokine production during experimental allergic encephalomyelitis. J Immunol *157*, 1333-6.

Perrin, P. J., Scott, D., Davis, T. A., Gray, G. S., Doggett, M. J., Abe, R., June, C. H., and Racke, M. K. (1996). Opposing effects of CTLA4-Ig and anti-CD80 (B7-1) plus anti-CD86 (B7-2) on experimental allergic encephalomyelitis. J Neuroimmunol *65*, 31-9.

Perrin, P. J., Scott, D., Quigley, L., Albert, P. S., Feder, O., Gray, G. S., Abe, R., June, C. H., and Racke, M. K. (1995). Role of B7:CD28/CTLA-4 in the induction of chronic relapsing experimental allergic encephalomyelitis. J Immunol *154*, 1481-90.

Pittet, M. J., Speiser, D. E., Valmori, D., Rimoldi, D., Lienard, D., Lejeune, F., Cerottini, J. C., and Romero, P. (2001). Ex vivo analysis of tumor antigen specific CD8+ T cell responses using MHC/peptide tetramers in cancer patients. Int Immunopharmacol *1*, 1235-47.

Prabhu Das, M. R., Zamvil, S. S., Borriello, F., Weiner, H. L., Sharpe, A. H., and Kuchroo, V. K. (1995). Reciprocal expression of co-stimulatory molecules, B7-1 and B7-2, on murine T cells following activation. European Journal of Immunology *25*, 207-11.

Punt, J. A., Havran, W., Abe, R., Sarin, A., and Singer, A. (1997). T cell receptor (TCR)-induced death of immature CD4+CD8+ thymocytes by two distinct mechanisms differing in their requirement for CD28 costimulation: implications for negative selection in the thymus. J Exp Med *186*, 1911-22.

Quill, H., and Schwartz, R. H. (1987). Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. J Immunol *138*, 3704-12.

Racke, M., Bonomo, A., Scott, D., Cannella, B., Levine, A., Raine, C., Shevach, E., and Rocken, M. (1994). Cytokine induced immune deviation as a therapy for inflammatory autoimmune disease. Journal of Experimental Medicine *180*, 1961-1966.

Racke, M., Scott, D., Quigley, L., Gray, G., Abe, R., June, C., and Perrin, P. (1995). Distinct roles of B7-1 (CD-80) and B7-2 (CD-86) in the initiation of Experimental Allergic Encephalomyelitis. J. Clinical Investigation *96*, 2195-2203.

Read, S., Malmstrom, V., and Powrie, F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med *192*, 295-302.

Reiser, H., and Schneeberger, E. E. (1994). The costimulatory molecule B7 is expressed in the medullary region of the murine thymus. Immunology *81*, 532-7.

Ridgway, W. M., Fasso, M., and Fathman, C. G. (1999). A new look at MHC and autoimmune disease. Science *284*, 749, 751.

Ridgway, W. M., and Fathman, C. G. (1998). The association of MHC with autoimmune diseases: understanding the pathogenesis of autoimmune diabetes. Clin Immunol Immunopathol *86*, 3-10.

Ridgway, W. M., Ito, H., Fasso, M., Yu, C., and Fathman, C. G. (1998). Analysis of the role of variation of major histocompatibility complex class II expression on nonobese diabetic (NOD) peripheral T cell response. J Exp Med *188*, 2267-75.

Riley, J. L., Blair, P. J., Musser, J. T., Abe, R., Tezuka, K., Tsuji, T., and June, C. H. (2001). ICOS costimulation requires IL-2 and can be prevented by CTLA-4 engagement. J Immunol *166*, 4943-8.

Rottman, J. B., Slavin, A. J., Silva, R., Weiner, H. L., Gerard, C. G., and Hancock, W. W. (2000). Leukocyte recruitment during onset of experimental allergic encephalomyelitis is CCR1 dependent. Eur J Immunol *30*, 2372-7.

Rottman, J. B., Smith, T., Tonra, J. R., Ganley, K., Bloom, T., Silva, R., Pierce, B., Gutierrez-Ramos, J. C., Ozkaynak, E., and Coyle, A. J. (2001). The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. Nat Immunol *2*, 605-11.

Roy, M., Aruffo, A., Ledbetter, J., Linsley, P., Kehry, M., and Noelle, R. (1995). Studies on the interdependence of gp39 and B7 expression and function during antigen-specific immune responses. Eur J Immunol *25*, 596-603.

Rulifson, I. C., Sperling, A. I., Fields, P. E., Fitch, F. W., and Bluestone, J. A. (1997). CD28 costimulation promotes the production of Th2 cytokines. J Immunol *158*, 658-65.

Sakai, K., Zamvil, S. S., Mitchell, D. J., Lim, M., Rothbard, J. B., and Steinman, L. (1988). Characterization of a major encephalitogenic T cell epitope in SJL/J mice with synthetic oligopeptides of myelin basic protein. Journal of Neuroimmunology *19*, 21-32.

Salomon, B., and Bluestone, J. A. (2001). Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu Rev Immunol *19*, 225-52.

Salomon, B., Lenschow, D. J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., and Bluestone, J. A. (2000). B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity *12*, 431-40.

Salomon, B., Rhee, L., Bour-Jordan, H., Hsin, H., Montag, A., Soliven, B., Arcella, J., Girvin, A. M., Padilla, J., Miller, S. D., and Bluestone, J. A. (2001). Development of spontaneous autoimmune peripheral polyneuropathy in B7-2- deficient NOD mice. J Exp Med *194*, 677-84.

Samoilova, E. B., Horton, J. L., Bassiri, H., Zhang, H., Linsley, P. S., Carding, S. R., and Chen, Y. (1997). B7 blockade prevents activation-induced cell death of thymocytes. Int Immunol *9*, 1663-8.

Sansom, D. M., and Hall, N. D. (1993). B7/BB1, the ligand for CD28, is expressed on repeatedly activated human T cells in vitro. Eur. J. Immunol *23*, 295-298.

Scheurich, P., Thoma , B., and Ucer, U. (1987). Immunoregualtory activity of recombinant human tumor necrosis factor (TNF)-a: induction of TNF receptors on human cells and TNF-a mediated enhancement of T cell responses. J. Immunol. *138*, 1786.

Schlom, J., and Hodge, J. W. (1999). The diversity of T-cell co-stimulation in the induction of antitumor immunity. Immunol Rev *170*, 73-84.

Scholz, C., Patton, K. T., Anderson, D. E., Freeman, G. J., and Hafler, D. A. (1998). Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation. J Immunol *160*, 1532-8.

Schwartz, R. H. (1990). A cell culture model for T lymphocyte clonal anergy. Science *248*, 1349-1356.

Schweitzer, A. N., and Sharpe, A. H. (1999). Mutual regulation between B7-1 (CD80) expressed on T cells and IL-4. Journal of Immunology *in press*.

Segal, B. M., Dwyer, B. K., and Shevach, E. M. (1998). An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. J Exp Med *187*, 537-46.

Shahinian, A., Pfeffer, K., Lee, K. P., Kundig, T. M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P. S., Thompson, C. B., and Mak, T. W. (1993). Differential T cell costimulatory requirements in CD28-deficient mice. Science *261*, 609-12.

Sharpe, A. H., and Freeman, G. J. (2002). The B7-CD28 superfamily. Nature Rev Immunol *2*, 116-26.

Shaw, M. K., Lorens, J. B., Dhawan, A., DalCanto, R., Tse, H. Y., Tran, A. B., Bonpane, C., Eswaran, S. L., Brocke, S., Sarvetnick, N., Steinman, L., Nolan, G. P., and Fathman, C. G. (1997). Local delivery of interleukin 4 by retrovirus-transduced T lymphocytes ameliorates experimental autoimmune encephalomyelitis. J Exp Med *185*, 1711-4.

Shevach, E. M. (2000). Regulatory T cells in autoimmmunity*. Annu Rev Immunol *18*, 423-49.

Shrikant, P., and Benveniste, E. N. (1996). The central nervous system as an immunocompetent organ: role of glial cells in antigen presentation. J Immunol *157*, 1819-22.

Slavin, A., Ewing, C., Liu, J., Ichikawa, M., Slavin, J., and Bernard, C. C. (1998). Induction of a multiple sclerosis-like disease in mice with an immunodominant epitope of myelin oligodendrocyte glycoprotein. Autoimmunity *28*, 109-20.

Sobel, R. A., Tuohy, V. K., Lu, Z. J., Laursen, R. A., and Lees, M. B. (1990). Acute experimental allergic encephalomyelitis in SJL/J mice induced by a synthetic peptide of myelin proteolipid protein. Journal of Neuropathology & Experimental Neurology *49*, 468-79.

Sperling, A. I. (2001). ICOS costimulation: is it the key to selective immunotherapy? Clin Immunol *100*, 261-2.

Sperling, A. I., Auger, J. A., Ehst, B. D., Rulifson, I. C., Thompson, C. B., and Bluestone, J. A. (1996). CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. J Immunol *157*, 3909-17.

Sporici, R. A., Beswick, R. L., von Allmen, C., Rumbley, C. A., Hayden-Ledbetter, M., Ledbetter, J. A., and Perrin, P. J. (2001). ICOS ligand costimulation is required for T-cell encephalitogenicity. Clin Immunol *100*, 277-88.

Sporici, R. A., and Perrin, P. J. (2001). Costimulation of memory T-cells by ICOS: a potential therapeutic target for autoimmunity? Clin Immunol *100*, 263-9.

Sundvall, M., Jirholt, J., Yang, H.-T., Jansson, L., Engström, Å., Pettersson, U., and Holmdahl, R. (1995). Identification of murine loci associated with susceptibility to chronic experimental autoimmune encephalomyelitis. Nature Genetics *10*, 313-7.

Swallow, M. M., Wallin, J. J., and Sha, W. C. (1999). B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. Immunity *11*, 423-32.

Tafuri, A., Shahinian, A., Bladt, F., Yoshinaga, S. K., Jordana, M., Wakeham, A., Boucher, L. M., Bouchard, D., Chan, V. S., Duncan, G., Odermatt, B., Ho, A., Itie, A., Horan, T., Whoriskey, J. S., Pawson, T., Penninger, J. M., Ohashi, P. S., and Mak, T. W. (2001). ICOS is essential for effective T-helper-cell responses. Nature *409*, 105-9.

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W., and Sakaguchi, S. (2000). Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med *192*, 303-10.

Tesciuba, A. G., Subudhi, S., Rother, R. P., Faas, S. J., Frantz, A. M., Elliot, D., Weinstock, J., Matis, L. A., Bluestone, J. A., and Sperling, A. I. (2001). Inducible costimulator regulates Th2-mediated inflammation, but not Th2 differentiation, in a model of allergic airway disease. J Immunol *167*, 1996-2003.

Teuscher, C., Wardell, B. B., Lunceford, J. K., Michael, S. D., and Tung, K. S. (1996). Aod2, the locus controlling development of atrophy in neonatal thymectomy-

induced autoimmune ovarian dysgenesis, co-localizes with Il2, Fgfb, and Idd3. J Exp Med *183*, 631-7.

Tezuka, K., Tsuji, T., Hirano, D., Tamatani, T., Sakamaki, K., Kobayashi, Y., and Kamada, M. (2000). Identification and characterization of rat AILIM/ICOS, a novel T-cell costimulatory molecule, related to the CD28/CTLA4 family. Biochem Biophys Res Commun *276*, 335-45.

Thompson, C. B., Lindsten, T., Ledbetter, J. A., Kunkel, S. L., Young, H. A., Emerson, S. G., Leiden, J. M., and June, C. H. (1989). CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. Proc. Natl. Acad. Sci. USA *86*, 1333-1337.

Tivol, E. A., Borriello, F., Schweitzer, A. N., Lynch, W. P., Bluestone, J. A., and Sharpe, A. H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity *3*, 541-7.

Tivol, E. A., Boyd, S. D., McKeon, S., Borriello, F., Nickerson, P., Strom, T. B., and Sharpe, A. H. (1997). CTLA4Ig prevents lymphoproliferation and fatal multiorgan tissue destruction in CTLA-4-deficient mice. J Immunol *158*, 5091-4.

Tuohy, V. K., Lu, Z., Sobel, R. A., Laursen, R. A., and Lees, M. B. (1989). Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. Journal of Immunology *142*, 1523-7.

Tuohy, V. K., Lu, Z., Sobel, R. A., Laursen, R. A., and Lees, M. B. (1988). A synthetic peptide from myelin proteolipid protein induces experimental allergic encephalomyelitis. Journal of Immunology *141*, 1126-30.

Tuohy, V. K., and Thomas, D. M. (1995). Sequence 104-117 of myelin proteolipid protein is a cryptic encephalitogenic T cell determinant for SJL/J mice. J Neuroimmunol *56*, 161-70.

Tuohy, V. K., Yu, M., Weinstock-Guttman, B., and Kinkel, R. P. (1997). Diversity and plasticity of self recognition during the development of multiple sclerosis. J Clin Invest *99*, 1682-90.

Tuohy, V. K., Yu, M., Yin, L., Kawczak, J. A., Johnson, J. M., Mathisen, P. M., Weinstock-Guttman, B., and Kinkel, R. P. (1998). The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis. Immunol Rev *164*, 93-100.

Tuohy, V. K., Yu, M., Yin, L., Kawczak, J. A., and Kinkel, P. R. (1999). Regression and spreading of self-recognition during the development of autoimmune demyelinating disease. J Autoimmun *13*, 11-20.

Tuohy, V. K., Yu, M., Yin, L., Kawczak, J. A., and Kinkel, R. P. (1999). Spontaneous regression of primary autoreactivity during chronic progression of experimental autoimmune encephalomyelitis and multiple sclerosis. J Exp Med *189*, 1033-42.

Vanderlugt, C. L., Begolka, W. S., Neville, K. L., Katz-Levy, Y., Howard, L. M., Eagar, T. N., Bluestone, J. A., and Miller, S. D. (1998). The functional significance of epitope spreading and its regulation by co-stimulatory molecules. Immunol Rev *164*, 63-72.

Vanderlugt, C. L., and Miller, S. D. (2002). Epitope spreading in immune-mediated diseases: implications for immunotherapy. Nature Rev Immunol *2*, 85-95.

Viola, A., and Lanzavecchia, A. (1996). T cell activation determined by T cell receptor number and tunable thresholds [see comments]. Science *273*, 104-6.

Vyse, T. J., and Todd, J. A. (1996). Genetic analysis of autoimmune disease. Cell *85*, 311-8.

Waldor M.K., S., S., Hardy, R., Herzenberg, L. A., Herzenberg, L. A., Lanier, L., Lim, M.,and Steinman , L. (1985). Reversal of experimental allergic encephalomyelitis with a monoclonal antibody to a T cell subset marker (L3T4). Science *227*, 415-417.

Walker, L. S., and Abbas, A. K. (2002). The enemy within: keeping self-reactive T cells at bay in the periphery. Nature Rev Immunol *2*, 11-9.

Walunas, T. L., Bakker, C. Y., and Bluestone, J. A. (1996). CTLA-4 ligation blocks CD28-dependent T cell activation. J Exp Med *183*, 2541-50.

Walunas, T. L., Lenschow, D. J., Bakker, C. Y., Linsley, P. S., Freeman, G. J., Green, J. M., Thompson, C. B., and Bluestone, J. A. (1994). CTLA-4 can function as a negative regulator of T cell activation. Immunity. *1*, 405-413.

Walunas, T. L., Sperling, A. I., Khattri, R., Thompson, C. B., and Bluestone, J. A. (1996). CD28 expression is not essential for positive and negative selection of thymocytes or peripheral T cell tolerance. J Immunol *156*, 1006-13.

Ward, S. G., Bacon, K., and Westwick, J. (1998). Chemokines and T lymphocytes: more than an attraction. Immunity *9*, 1-11.

Waterhouse, P., Penninger, J., Timms, E., Wakeham, A., Shahinian, A., Lee, K., Thompson, C., Griesser, H., and Mak, T. (1995). Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. Science *270*, 985-988.

Wekerle, H. (1991). Immunopathogenesis of multiple sclerosis. Acta Neurol (Napoli) *13*, 197-204.

Whitham, R. H., Bourdette, D. N., Hashim, G. A., Herndon, R. M., Ilg, R. C., Vandenbark, A. A., and Offner, H. (1991). Lymphocytes from SJL/J mice immunized with spinal cord respond selectively to a peptide of proteolipid protein and transfer relapsing demyelinating experimental autoimmune encephalomyelitis. Journal of Immunology *146*, 101-7.

Wicker, L. S., Todd, J. A., Prins, J. B., Podolin, P. L., Renjilian, R. J., and Peterson, L. B. (1994). Resistance alleles at two non-major histocompatibility complex-linked insulin-dependent diabetes loci on chromosome 3, Idd3 and Idd10, protect nonobese diabetic mice from diabetes. J Exp Med *180*, 1705-13.

Windhagen, A., Newcombe, J., Dangond, F., Strand, C., Woodroofe, M. N., Cuzner, M. L., and Hafler, D. A. (1995). Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. J Exp Med *182*, 1985-96.

Yokochi, T., Holly, R. D., and Clark, E. A. (1982). Lymphoblastoid antigen (BB-1) expressed on Epstein-Barr virus-activated B cell blasts, B lymphoblastoid lines, and Burkitt's lymphomas. J. Immunol. *128*, 823-827.

Yoshinaga, S. K., Whoriskey, J. S., Khare, S. D., Sarmiento, U., Guo, J., Horan, T., Shih, G., Zhang, M., Coccia, M. A., Kohno, T., Tafuri-Bladt, A., Brankow, D., Campbell, P., Chang, D., Chiu, L., Dai, T., Duncan, G., Elliott, G. S., Hui, A., McCabe, S. M., Scully, S., Shahinian, A., Shaklee, C. L., Van, G., Mak, T. W., and et al. (1999). T-cell co-stimulation through B7RP-1 and ICOS. Nature *402*, 827-32.

Yu, M., Johnson, J. M., and Tuohy, V. K. (1996). A predictable sequential determinant spreading cascade invariably accompanies progression of experimental autoimmune encephalomyelitis: a basis for peptide-specific therapy after onset of clinical disease. J Exp Med *183*, 1777-88.

Yu, X., Fournier, S., Allison, J. P., Sharpe, A. H., and Hodes, R. J. (2000). The role of B7 costimulation in CD4/CD8 T cell homeostasis. J Immunol *164*, 3543-53.

Zamvil, S. S., Mitchell, D. J., Moore, A. C., Kitamura, K., Steinman, L., and Rothbard, J. B. (1986). T-cell epitope of the autoantigen myelin basic protein that induces encephalomyelitis. Nature *324*, 258-260.

Zamvil, S. S., and Steinman, L. (1990). The T lymphocyte in experimental allergic encephalomyelitis. Annual Review of Immunology *8*, 579-621.

Zhang, J., Markovic-Plese, S., Lacet, B., Raus, J., Weiner, H. L., and Hafler, D. A. (1994). Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. J Exp Med *179*, 973-84.

Zhao, W., Wegmann, K. W., Trotter, J. L., Ueno, K., and Hickey, W. F. (1994). Identification of an N-terminally acetylated encephalitogenic epitope in myelin proteolipid apoprotein for the Lewis rat. J Immunol *153*, 901-9.

Appendix A

Reprint of:

Chang T.T., C. Jabs, R.A. Sobel, V.K. Kuchroo, and A.H. Sharpe. 1999. Studies in B7-deficient mice reveal a critical role for B7 costimulation in both induction and effector phases of Experimental Autoimmune Encephalomyelitis. *J Exp Med.* 190:733-740.

Appendix B

Reprint of:

McAdam A.J., T.T. Chang, A.E. Lumelsky, E.A. Greenfield, V.A. Boussiotis, J.S. Duke-Cohan, T. Chernova, N. Malenkovich, C. Jabs, V.K. Kuchroo, V. Ling, M. Collins, A.H. Sharpe, and G.J. Freeman. 2000. Mouse Inducible Costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. *J Immunol.* 165:5035-5040

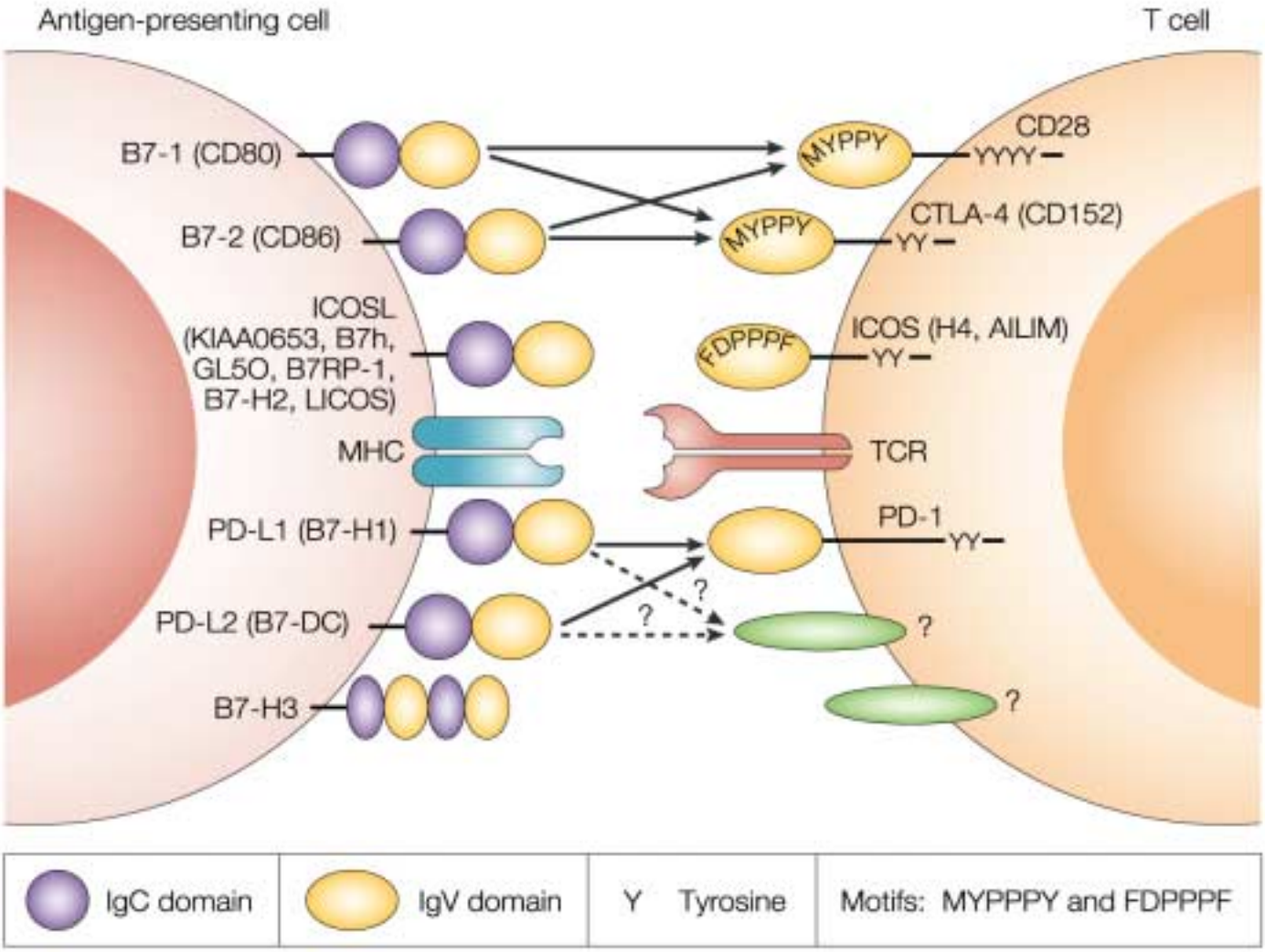

Figure 1.2. Members of the B7-CD28 Superfamily.
(Figure adapted from Sharpe and Freeman, 2002).
CD28 family members are immunoglobulin superfamily members with a single immunoglobulin V-like domain. CD28 and CTLA-4 have a MYPPPY motif that is essential for binding B7.1 and B7.2, whereas ICOS has a FDPPPF motif and binds B7h (ICOSL) but not B7.1 and B7.2. PD-1 is a receptor for both PD-L1 and PD-L2, which might also bind to other, as yet unidentified, receptors on T cells (indicated by the dotted arrows and the question mark).
B7 family members are immunoglobulin superfamily members with immunoglobulin-V-like and immunoglobulin-C-like domains. Although one form of human B7-H3 has one V-like and one C-like domain, the most common form is a four-immunoglobulin extracellular domain consisting of IgV-IgC-IgV-IgC, and this is the version shown.
Abbreviations: CTLA-4, cytotoxic T-lymphocyte antigen 4; ICOS, inducible T-cell costimulator; ICOSL, ICOS ligand; MHC, major histocompatibility complex; PD-1, programmed death 1; PD-L1, PD-L2, PD-1 ligands 1 and 2; TCR, T-cell receptor.

Table 1.1. Encephalitogenic myelin epitopes in different rodent strains.

(Table modified from (Anderson, 2002)

Species	Strain	MHC haplotype	Myelin epitope	Reference
Mouse	129/SvS4	H-2^{b}	MOG 35-55	as described in this thesis
	Biozzi AB/H	H-2^{dg1}	MOG 1-22	(Amor et al., 1994)
			MOG 43-57	(Amor et al., 1994)
			MOG 134-148	(Amor et al., 1994)
			PLP 56-70	(Amor et al., 1993)
	C3H	H-2^{k}	PLP 215-232	(Endoh et al., 1990)
	C57Bl/6	H-2^{b}	MOG 35-55	(Mendel et al., 1995)
	NOD	H-2^{g7}	PLP 56-70	(Amor et al., 1993)
			MOG 35-55	(Slavin et al., 1998)
	PL/J	H-2^{u}	MBP 1-11	(Zamvil et al., 1986)
			PLP 43-64	(Whitham et al., 1991)
	SJL	H-2^{s}	MBP 84-104	(Sakai et al., 1988)
			PLP 104-117	(Tuohy et al., 1995)
			PLP 139-151	(Tuohy et al., 1989)
			PLP 178-191	(Greer et al., 1992)
			MOG 92-106	(Amor et al., 1994)
	SWR	H-2^{q}	PLP 103-116	(Tuohy et al., 1988)
Rat	Lewis	$RT1^{1}$	MBP 68-84	(Hashim et al., 1978)
			PLP 217-240	(Zhao et al., 1994)

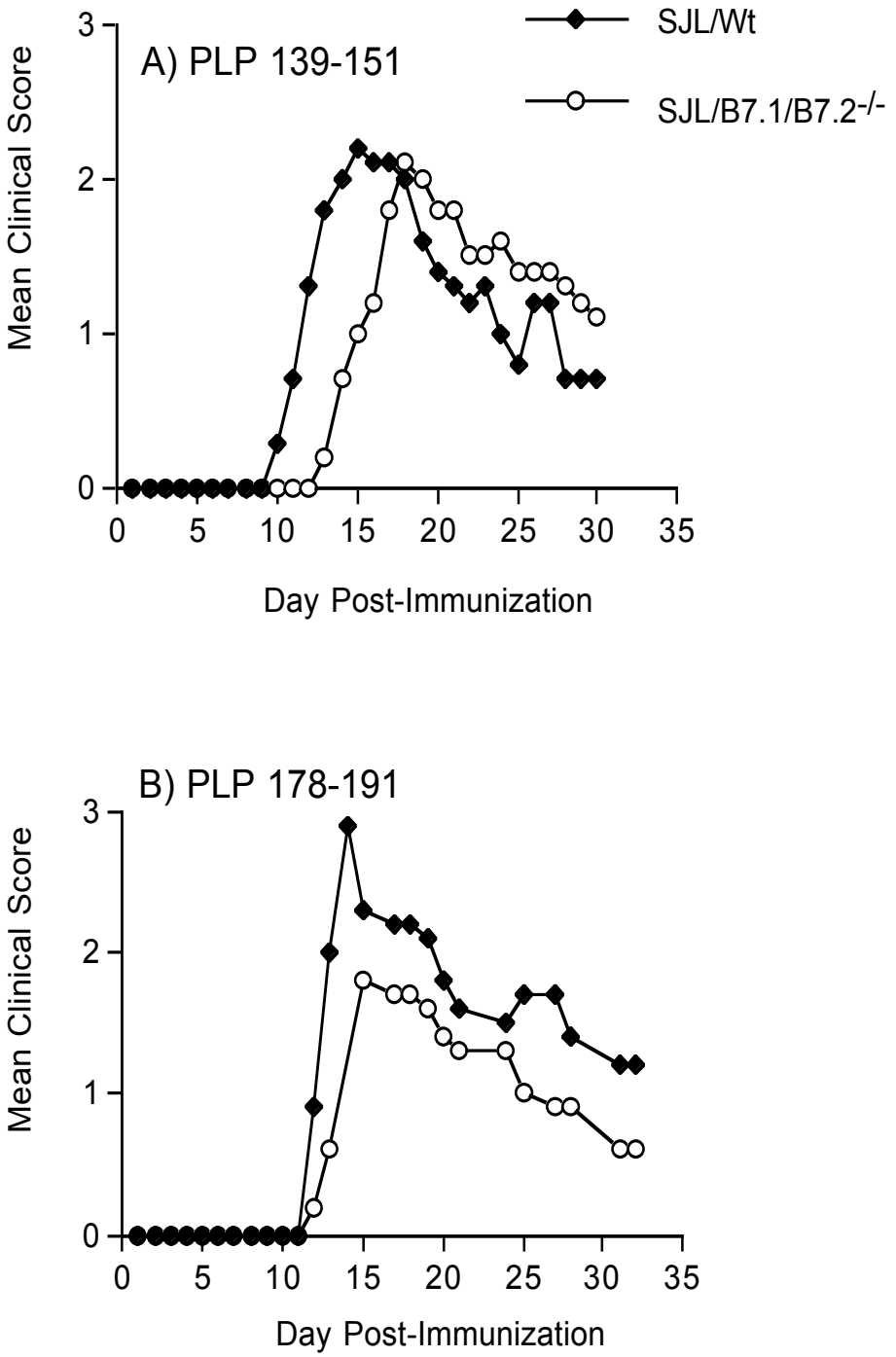

Figure 3.1. SJL B7.1/B7.2$^{-/-}$ mice are susceptible to EAE induced with PLP 139-151 and PLP 178-191.
EAE disease course in SJL Wt (filled diamonds, n=18 in A; n=7 in B) and B7.1/B7.2$^{-/-}$ mice (open circles; n=18 in A; n=12 in B). Mice were immunized with 25-100 mg of PLP 139-151 (A) depending on the experiment or with 50 mg PLP 178-191 (B) subcutaneously and injected twice with 100 ng Pertussis Toxin intravenously. Mice were observed daily and scored as described in Materials and Methods. Data represent mean clinical score of each group plotted against time.

Table 3.1. *EAE in B7.1/B7.2 $^{-/-}$ SJL mice induced by active immunization.*

		Clinical [A]EAE			
		Incidence	Mortality	Day of onset	Mean maximal score
				mean ± SE	*mean ± SE*
SJL/Wt	PLP 139-151	16/20 (80%)	2/20 (10%)	12.9 ± 0.5	3.1 ± 0.2
SJL/B7.1/B7.2$^{-/-}$	PLP 139-151	17/18 (94%)	0/18 (0%)	15.6 ± 0.5[C]	2.4 ± 0.2
SJL/Wt	PLP 178-191	7/7 (100%)	1/7 (14.3%)	12.7 ± 0.3	3.5 ± 0.4
SJL/B7.1/B7.2$^{-/-}$	PLP 178-191	11/12 (91.6%)	0/12 (0%)	14.5 ± 0.7[B]	2.4 ± 0.2[B]
		Histological EAE			
		Incidence	Meningeal foci	Parenchymal foci	Total foci
			mean ± SE	*mean ± SE*	*mean ± SE*
SJL/Wt	PLP 139-151	10/10 (100%)	58.5 ± 14.1	42.5 ± 10.8	101 ± 23.7
SJL/B7.1/B7.2$^{-/-}$	PLP 139-151	12/13 (92%)	49.2 ± 10.2	36.5 ± 10.9	85.8 ± 20.4
SJL/Wt	PLP 178-191	5/5 (100%)	65.2 ± 27	59.6 ± 26.6	124.8 ± 53.3
SJL/B7.1/B7.2$^{-/-}$	PLP 178-191	11/12 (92%)	49.7 ± 10.9	36.6 ± 7.7	86.4 ± 18

[A]EAE was induced by immunization with PLP 139-151 (25-100 μg) or PLP 178-191 (50 μg) in CFA subcutaneously and two injections of 100 μg Pertussis Toxin. Animals were scored for disease as described in Materials and Methods.
All P values are compared with Wt control and are indicated only when statistically significant ($P < 0.05$). [B]$P < 0.05$; [C]$P < 0.01$.

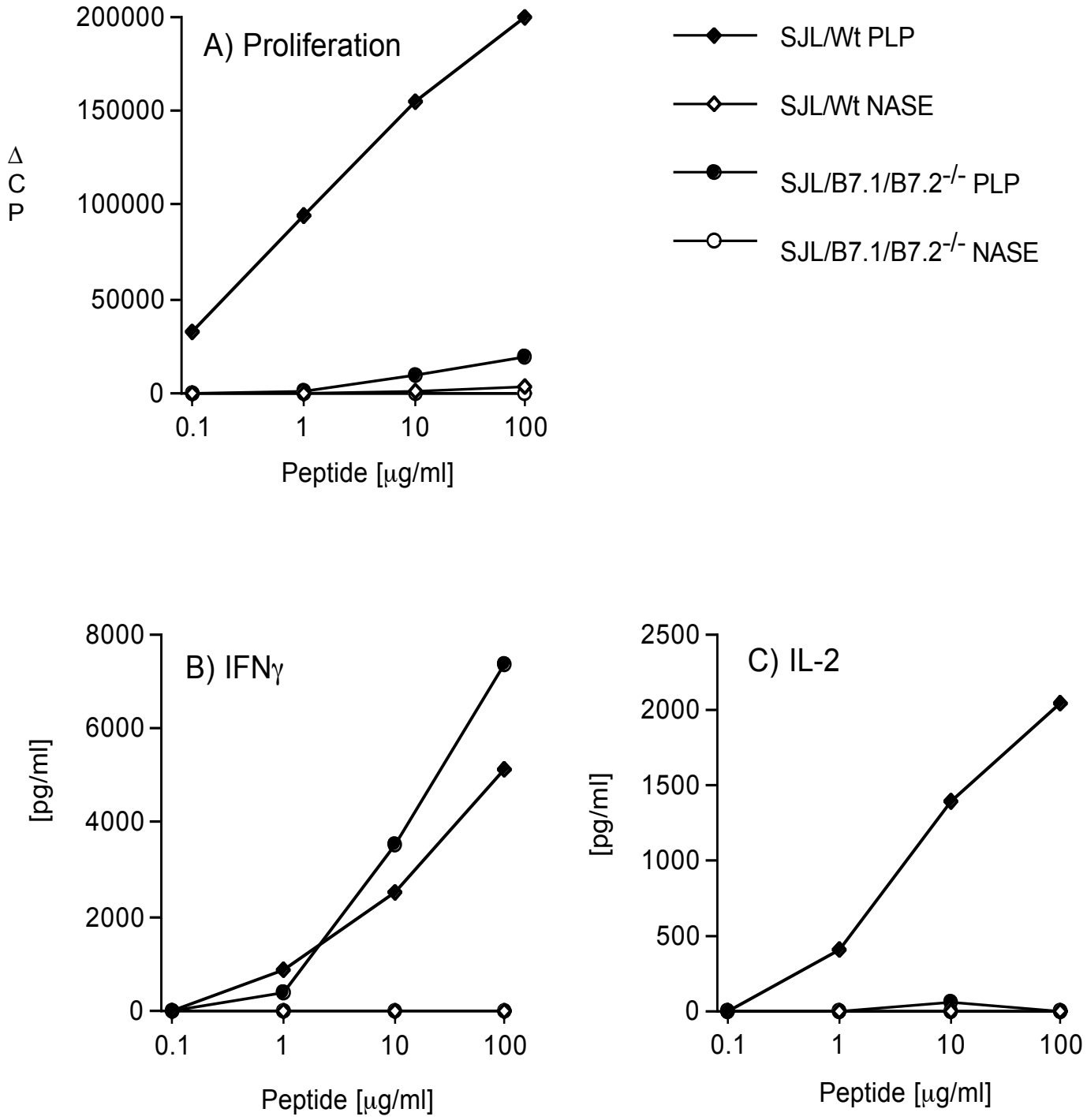

Figure 3.2. LNCs from SJL B7.1/B7.2$^{-/-}$ mice proliferate little, but produce abundant IFNg. Proliferation (A) and cytokine production (B,C) of LNCs from SJL Wt and B7.1/B7.2$^{-/-}$ mice challenged with PLP 139-151. Mice were immunized with 100 mg of PLP 139-151 in CFA. Ten days later, draining LNCs from Wt (diamonds) or B7.1/B7.2$^{-/-}$ (circles) SJL mice were restimulated *in vitro* with a titration of PLP 139-151 (filled symbols) or NASE 101-120 (open symbols) or medium alone. Background levels from medium controls (ranging from 3400 to 6000) were subtracted in proliferation assays and presented as ΔCPM. Culture supernatants were collected at 42 h and assayed for cytokines as described in Material and Methods. Data are representative of five independent experiments.

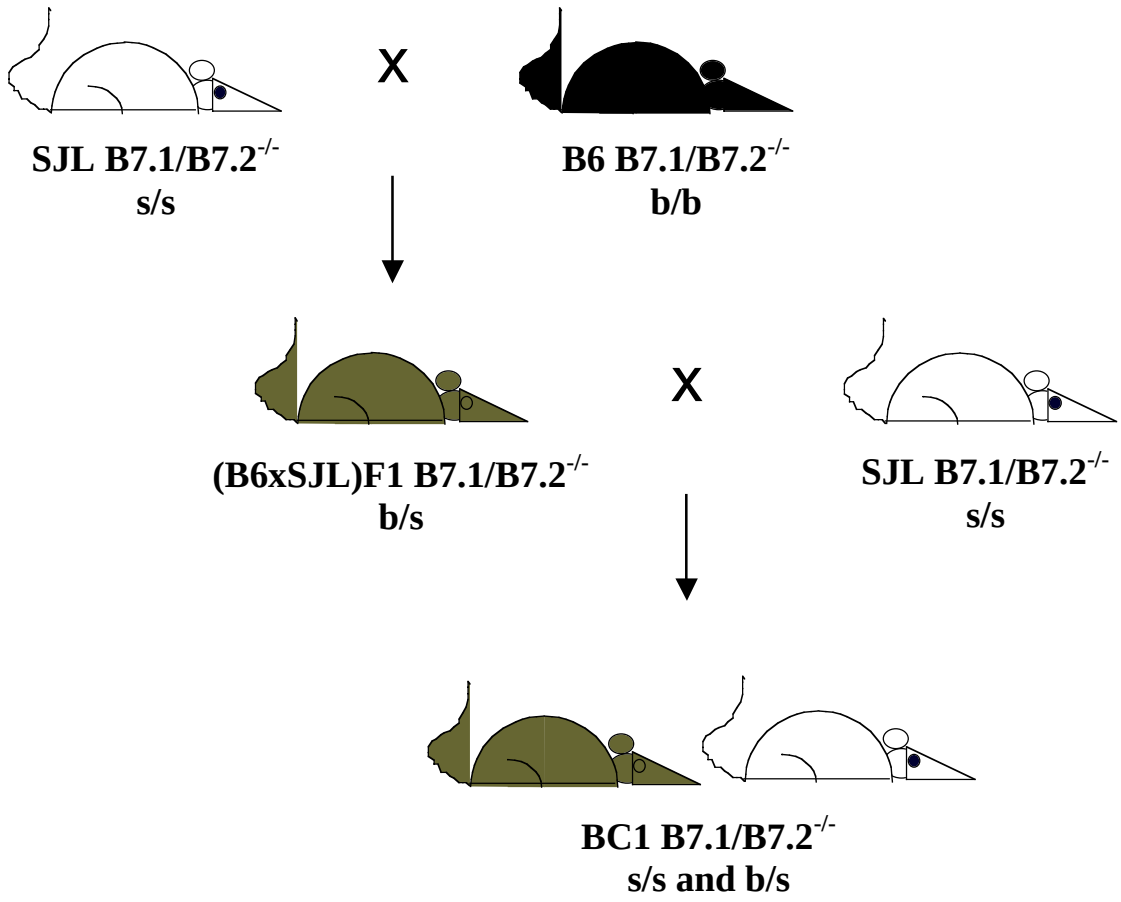

Figure 3.3. Breeding scheme for generation of F1 and BC1 B7.1/B7.2$^{-/-}$ mice.

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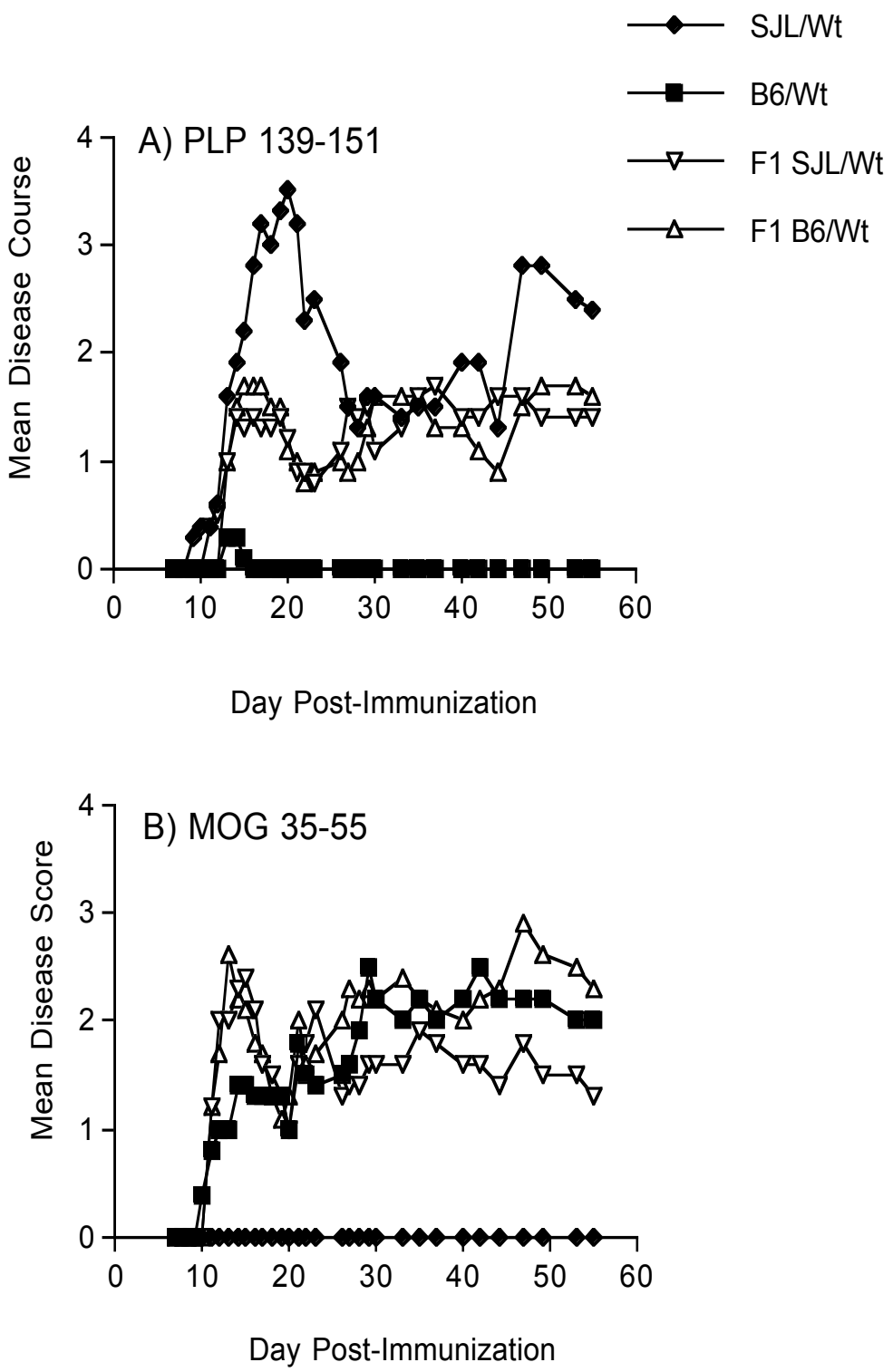


Figure 3.4. F1 B7.1/B7.2$^{+/+}$ mice are susceptible to EAE induced with PLP 139-151 or MOG 35-55.
EAE disease course in SJL Wt (filled diamonds; n=5 in A; n=5 in B), B6 Wt (filled squares; n=5 in A; n=4 in B), (B6xSJL)F1 SJL (open up-side-down triangles; n=5 in A; n=5 in B) and (B6xSJL)F1 B6 (open triangles; n=6 in A; n=5 in B). Mice were immunized with 100 mg PLP 139-151 (A) or with 100 mg MOG 35-55 (B) subcutaneously and injected twice with 100 ng (A) or 150 ng (B) Pertussis Toxin intravenously. Mice were observed daily and scored as described in Materials and Methods. Data represent mean clinical score of each group plotted against time.

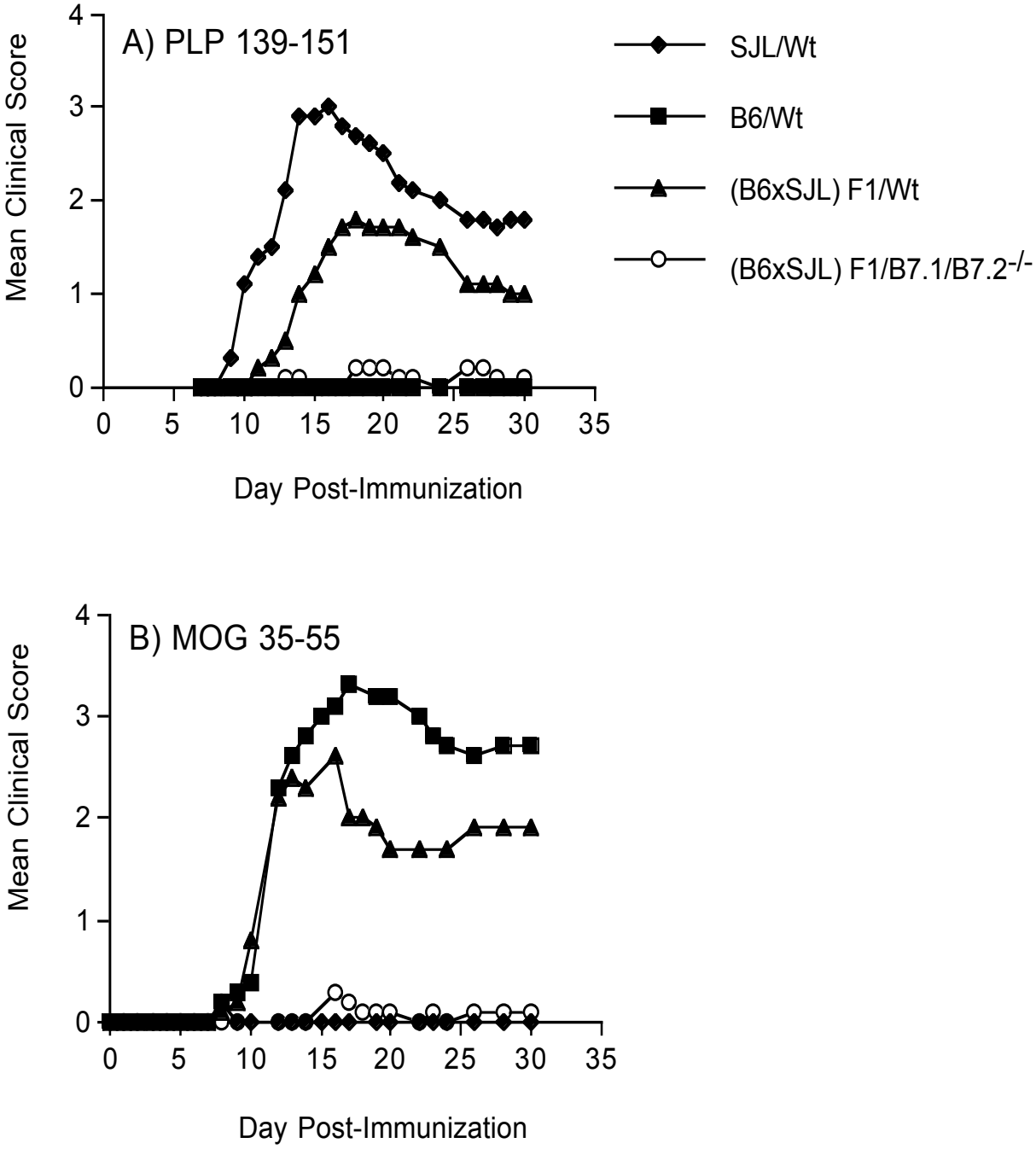


Figure 3.5. F1 B7.1/B7.2$^{-/-}$ mice are resistant to EAE induced with PLP 139-151 or MOG 35-55.

EAE disease course in SJL Wt (filled diamonds; n=12 in A; n=7 in B), B6 Wt (filled squares; n=7 in A; n=8 in B), F1 Wt (filled triangles; n=12 in A; n=18 in B) and F1 B7.1/B7.2$^{-/-}$ mice (open circles; n=12 in A; n=15 in B). Mice were immunized with 100 mg PLP 139-151 (A) or with 100 mg MOG 35-55 (B) subcutaneously and injected twice with 100 ng (A) or 150 ng (B) Pertussis Toxin intravenously. Mice were observed daily and scored as described in Materials and Methods. Data represent mean clinical score of each group plotted against time.

Table 3.2. *EAE in (B6xSJL)F1 B7.1/B7.2 $^{-/-}$ mice induced by active immunization.*

A) PLP 139-151	Clinical [A]EAE			
	Incidence	Mortality	Day of onset	Mean maximal score
			mean ± SE	*mean ± SE*
SJL/Wt	12/12 (100%)	3/12 (25%)	11.4 ± 0.6	3.6 ± 0.3
B6/Wt	0/7 (0%)	-	-	-
(B6xSJL)F1/Wt	10/12 (83.3%)	0/10 (0%)	13.9 ± 0.5	2.4 ± 0.2
(B6xSJL)F1/B7.1/B7.2$^{-/-}$	4/12[B] (33.3%)	0/4 (0%)	21 ± 3.4	1.3 ± 0.2
	Histological EAE			
	Incidence	Meningeal foci	Parenchymal foci	Total foci
		mean ± SE	*mean ± SE*	*mean ± SE*
SJL/Wt	9/9 (100%)	66.8 ± 6.8	50 + 6	116.8 ± 9.5
B6/Wt	0/7 0%	0	0	0
(B6xSJL)F1/Wt	12/12 (100%)	47.8 ± 6	59.5 ± 9.4	107.3 ± 14.8
(B6xSJL)F1/B7.1/B7.2$^{-/-}$	12/12 (100%)	13.3 + 2.3[E]	9.8 + 2.3[E]	23.1 + 0.2

[A]EAE was induced by immunization with 100 μg PLP 139-151in CFA subcutaneously and two injections of 100 ng Pertussis Toxin. Animals were scored for disease as described in Materials and Methods.

All P values are compared with (B6xSJL)F1/Wt control and are indicated only when statistically significant; ($P < 0.05$). '[B]$P < 0.05$; [C]$P < 0.01$; [D]$P < 0.001$; [E]$P < 0.0001$.

Table 3.2. *EAE in (B6xSJL)F1 B7.1/B7.2 $^{-/-}$ mice induced by active immunization.*

B) MOG 35-55	Clinical [A]EAE			
	Incidence	Mortality	Day of onset	Mean maximal score
			mean ± SE	*mean ± SE*
SJL/Wt	1/7 (14.3%)	0/7 (0%)	9	1
B6/Wt	8/8 (100%)	2/8 (25%)	11.3 ± 0.6	3.9 ± 0.4
(B6xSJL)F1/Wt	18/18 (100%)	1/18 (5.6%)	11.7 ± 0.4	3.3 ± 0.2
(B6xSJL)F1/B7.1/B7.2$^{-/-}$	2/15[E] (13.3%)	0/2 (0%)	16[E]	1.8 ± 0.8
		Histological EAE		
	Incidence	Meningeal foci	Parenchymal foci	Total foci
		mean ± SE	*mean ± SE*	*mean ± SE*
SJL/Wt	3/7 (42.9%)	1.9 ± 0.9	0.6 ± 0.4	2.4 ± 1.2
B6/Wt	6/6 (100%)	35 ± 4.9	36.3 ± 13.6	71.3 ± 17.5
(B6xSJL)F1/Wt	17/17 (100%)	48 ± 6.7	47.1 ± 7.6	95.1 ± 13.8
(B6xSJL)F1/B7.1/B7.2$^{-/-}$	13/15 (86.7%)	7.5 ± 1.3[E]	1.7 ± 0.6[E]	9.2 ± 1.5[E]

[A]EAE was induced by immunization with 100 μg MOG 35-55 in CFA subcutaneously and two injections of 150 ng Pertussis Toxin. Animals were scored for disease as described in Materials and Methods.

All P values are compared with (B6xSJL)F1/Wt control and are indicated only when statistically significant; ($P < 0.05$). '[B]$P < 0.05$; [C]$P < 0.01$; [D]$P < 0.001$; [E]$P < 0.0001$.

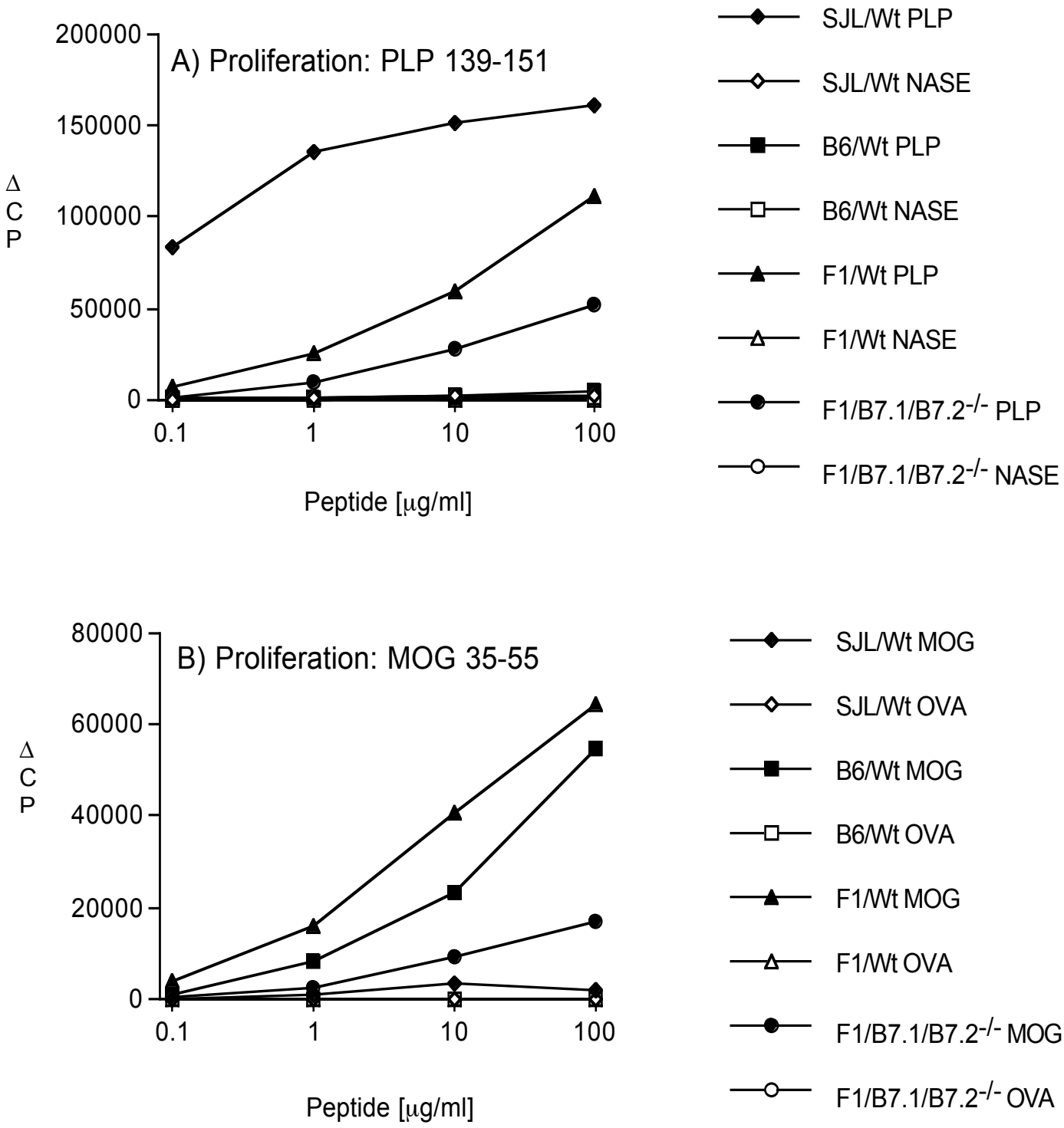


Figure 3.6. LNCs from F1 B7.1/B7.2$^{-/-}$ mice show impaired proliferation. Proliferation of LNCs from wild type B6 and SJL, B7.1/B7.2$^{-/-}$ SJL and B6 and (B6xSJL)F1 B7.1/B7.2$^{-/-}$ mice immunized with PLP 139-151 (A) or MOG 35-55 (B). Mice were immunized with 100 mg of PLP 139-151 or MOG 35-55 in CFA. Ten days post-immunization, draining LNCs from SJL Wt (diamonds), B6 Wt (squares), (B6xSJL)F1 Wt (triangles) or (B6xSJL)F1 B7.1/B7.2$^{-/-}$ (circles) mice were restimulated *in vitro* with a titration of PLP 139-151 or MOG 35-55 (filled symbols) or a control peptide (NASE 101-120 or OVA 323-336). Data are representative of two independent experiments.

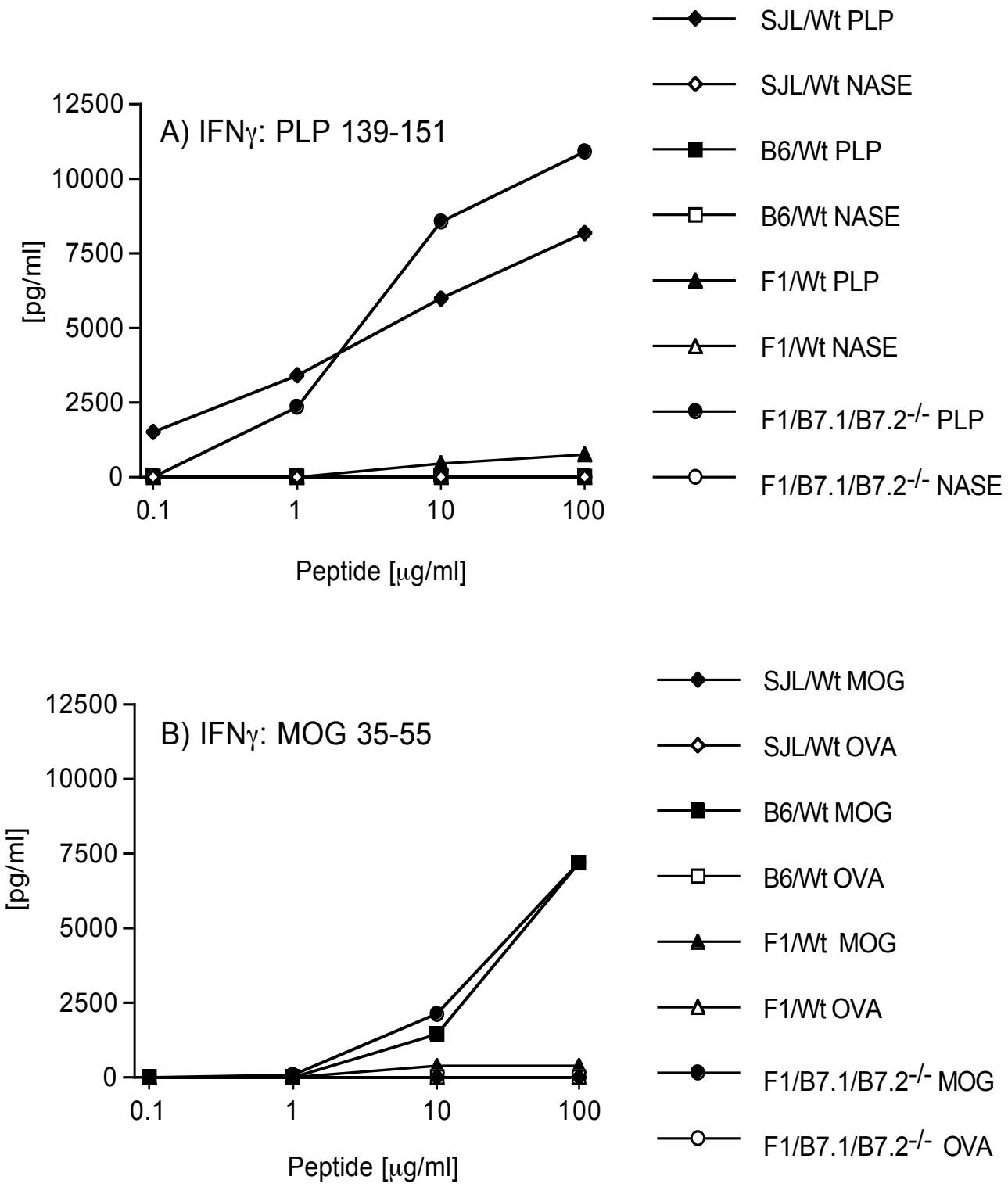


Figure 3.7. LNCs from F1 B7.1/B7.2$^{-/-}$ produce IFNg.
Cytokine production of LNCs from control and (B6xSJL)F1 B7.1/B7.2$^{-/-}$ mice immunized with PLP 139-151 (A) or MOG 35-55 (B). Supernatants from cell-cultures (see Figure 3.6 legend) were collected after 42 h and assayed for cytokines by sandwich ELISA. Data are representative of two independent experiments.

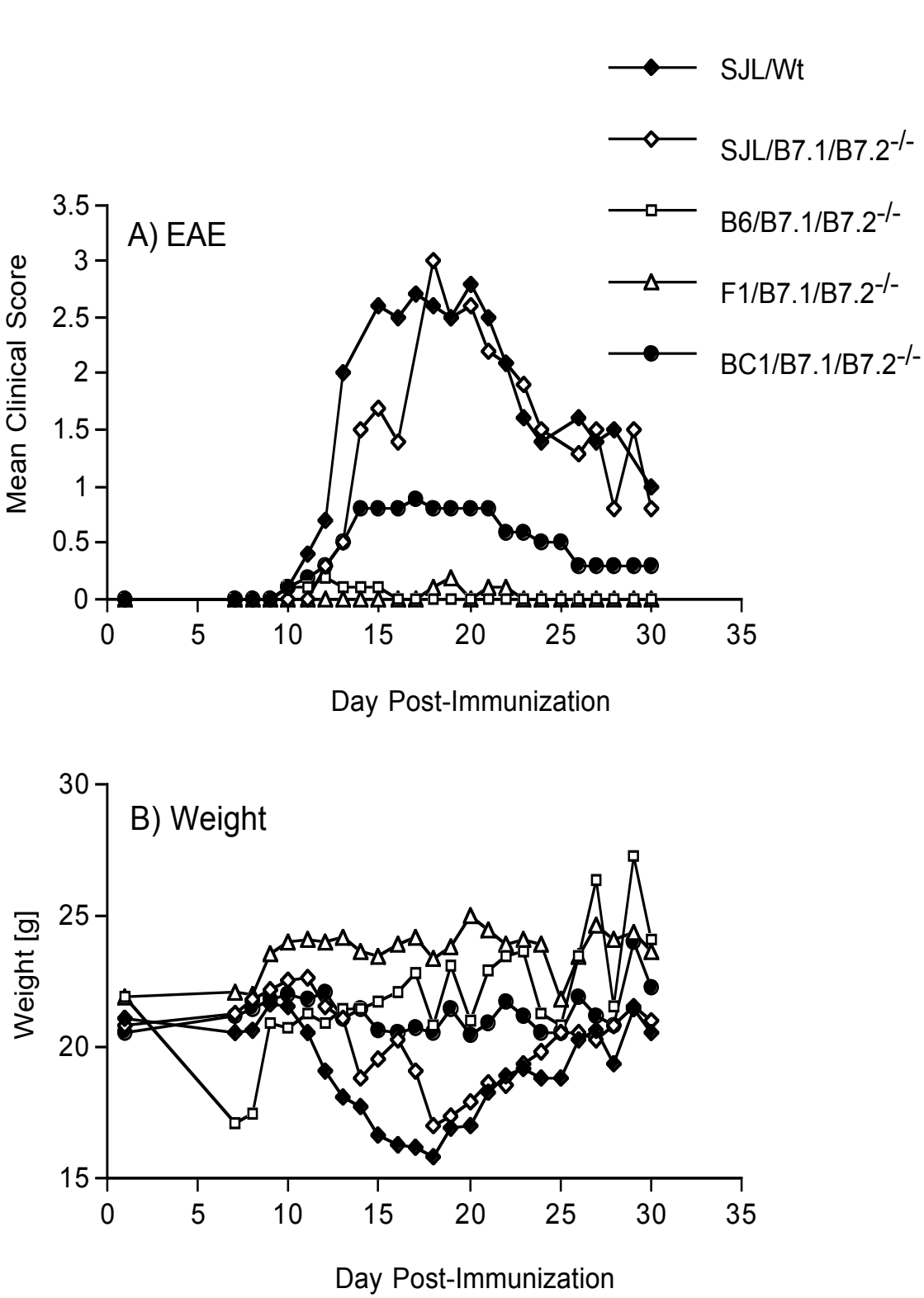


Figure 3.8 EAE and Weight loss in Backcross 1 (BC1) B7.1/B7.2$^{-/-}$ mice. EAE was induced by immunization with the PLP 139-151 peptide in SJL/Wt (filled diamonds; n=14), SJL B7.1/B7.2$^{-/-}$ (open diamonds; n=11), B6 B7.1/B7.2$^{-/-}$ (open squares; n=8), F1 B7.1/B7.2$^{-/-}$ (open triangles; n=12) and BC1 B7.1/B7.2$^{-/-}$ (closed circles; n=95) mice. Mice were observed daily and scored as described in the materials and methods. Clinical disease (A) and weight loss (B) was determined. Data represent mean clinical score of each group over time and represent three pooled experiments.

Table 3.3. *Clinical analysis of Backcross 1 B7.1/B7.2 $^{-/-}$ mice.*

		Clinical EAE[A]			
	Haplotype	Incidence	Mortality	Day of onset *mean ± SE*	Mean max. score *mean ± SE*
SJL/Wt	s/s	14/14 100%	3/14 (21.4%)	13.6 ± 0.2	3.6 ± 0.2
SJL/B7.1/B7.2$^{-/-}$	s/s	10/11 (90.9%)	4/10 (40%)	15.3 ± 0.8	3.9 ± 0.3
B6/B7.1/B7.2$^{-/-}$	b/b	1/8 (12.5%)	0/1 (0%)	10 ± 0	1 ± 0
F1/B7.1/B7.2$^{-/-}$	s/b	2/12 (16.7%)	0/2 (0%)	18.5 ± 0.5	1 ± 0
BC1/B7.1/B7.2$^{-/-}$	s/s and s/b	63/95 (66.3%)	1/63 (1.6%)	15.2 ± 0.6	2.1 ± 0.1

		Histological EAE			
	Haplotype	Incidence	Meningeal foci *mean ± SE*	Parenchymal foci *mean ± SE*	Total foci *mean ± SE*
SJL/Wt	s/s	12/12 100%	60.4 ± 8.6	59.7 ± 11.5	124.3 ± 21.7
SJL/B7.1/B7.2$^{-/-}$	s/s	8/8 100%	53.3 ± 4.8	57 ± 9.9	110.3 ± 12.3
B6/B7.1/B7.2$^{-/-}$	b/b	4/7 (57.1%)	9.7 ± 5	0.3 ± 0.3	10 ± 5.2
F1/B7.1/B7.2$^{-/-}$	s/b	8/11 (72.7%)	7.1 ± 2.5	3.5 ± 1.5	10.6 ± 3.6
BC1/B7.1/B7.2$^{-/-}$	s/s and s/b	91/94 (96.8%)	58.9 ± 4.5	47.1 ± 4.1	105.9 ± 8.1

[A]EAE was induced by immunization with 75 μg PLP 139-151 and two injections of 100 ng Pertussis Toxin. Animals were scored daily for disease as described in Materials and Methods.

Table 3.4. *Linkage analysis of EAE incidence in BC1 B7.1/B7.2 $^{-/-}$ mice with MHC genotype*

	MHC (I-A)		
EA	b/	s/s	tota
no	2	6	3
diseas	1	4	5
tota	3	5	8

$\chi 2$ $p = 5.0 \times 10^{-8}$

Table 3.5. *Linkage analysis of EAE severity in BC1 B7.1/B7.2$^{-/-}$ mice with MHC genotype*

	MHC (I-A)		
EAE	b/	s/s	tota
0	2	6	3
0.5-	9	1	2
2.5-	3	2	2
tota	3	5	8

$\chi 2$ $p = 7.8 \times 10^{-8}$

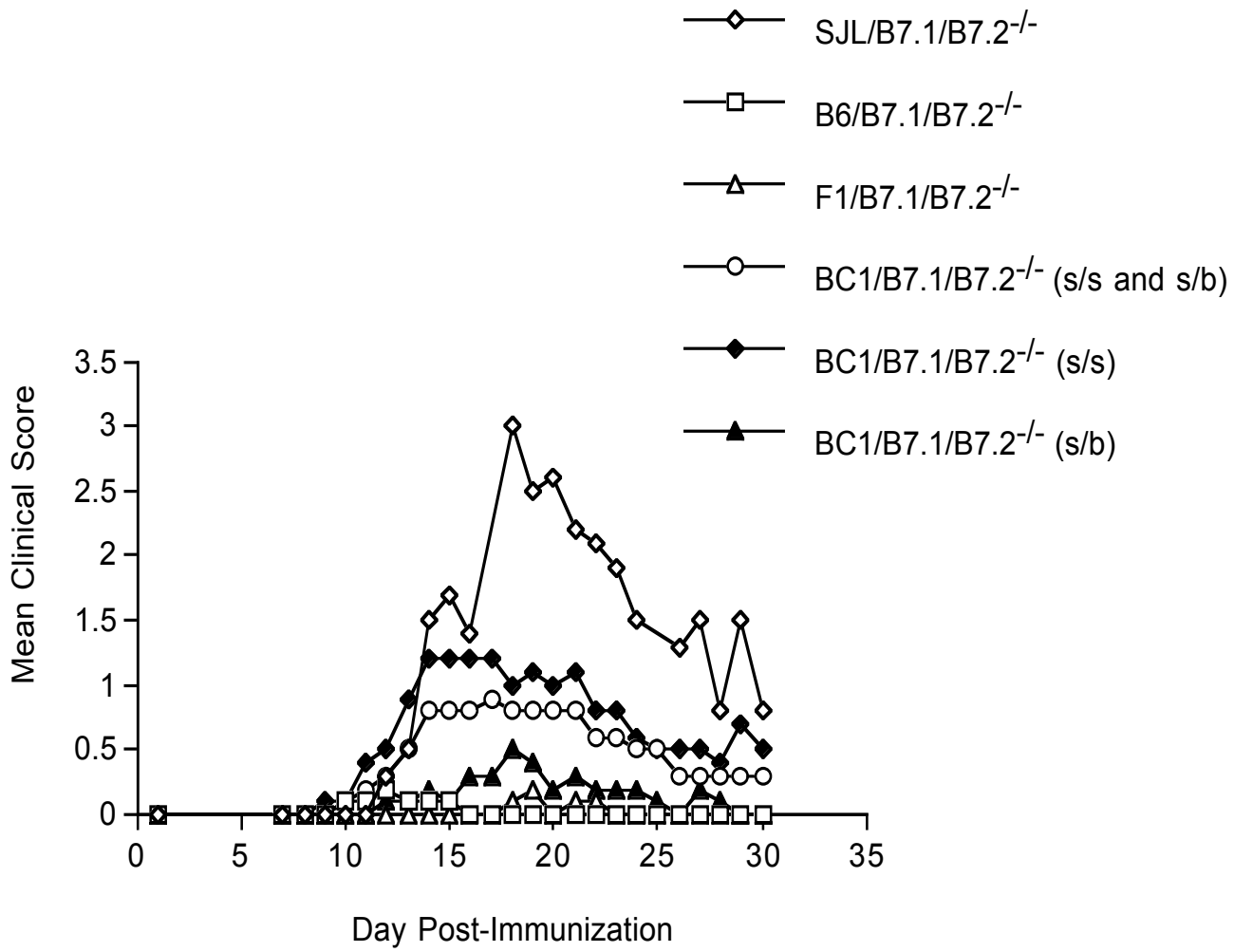


Figure 3.9 EAE in Backcross 1 (BC1) B7.1/B7.2$^{-/-}$ mice: s/s versus s/b haplotype

The disease profile of the BC1 B7.1/B7.2$^{-/-}$ mice (from Fig. 3.8) was further segregated based on the s/s and s/b MHC IA genotype and plotted over time. For clarity, SJL/Wt scores were omitted for clarity. Mice were observed daily and scored as described in the materials and methods. Data represent mean clinical score of each group over time and represent three pooled experiments.

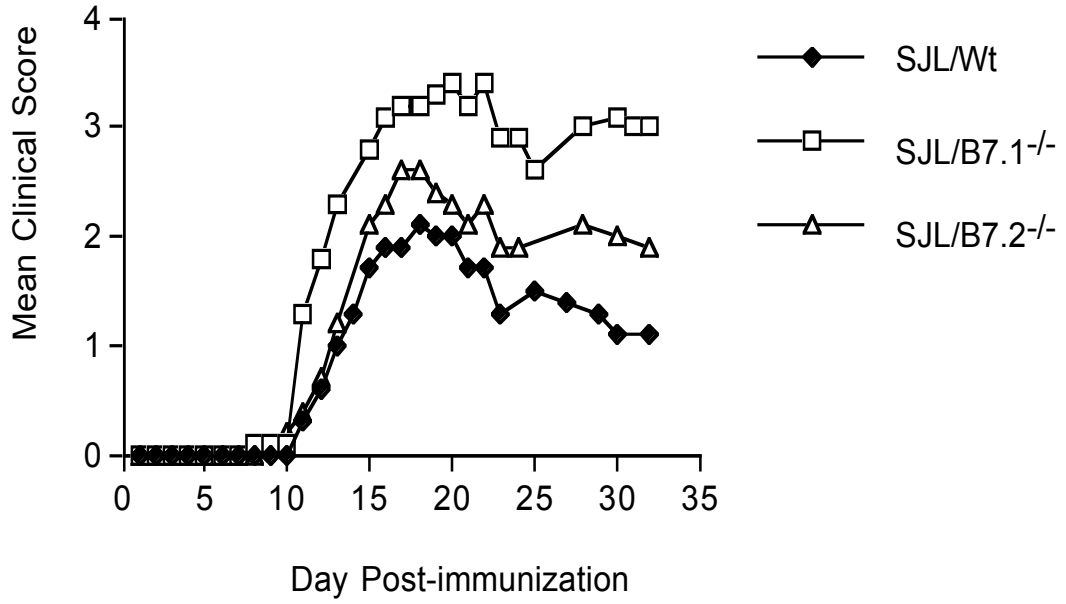


Figure 4.1. SJL B7.1$^{-/-}$ mice develop more severe EAE than SJL Wt mice.
EAE disease course in SJL Wt (filled diamonds, n=42), B7.1$^{-/-}$ (open squares, n=29) and B7.2$^{-/-}$ mice (open triangles, n=33). Mice were immunized with 25-100 μg of PLP 139-151 subcutaneously and injected twice with 100 ng Pertussis Toxin intravenously. Mice were observed daily and scored as described in Materials and Methods. Data are pooled from four experiments and represent mean clinical score of each group plotted against time.

Table 4.1. *EAE in B7.1 $^{-/-}$ and B7.2 $^{-/-}$ mice induced by active immunization.*

	Clinical EAE[A]			
	Incidence	Mortality	Day of onset *mean ± SE*	Mean maximal score *mean ± SE*
SJL/Wt	34/42 (81%)	4/34 (11.8%)	13.8 ± 0.5	3.0 ± 0.2
SJL/B7.1-/-	28/29 (96.6%)	12/28[C] (42.9%)	13.4 ± 0.7	4.0 ± 0.2[C]
SJL/B7.2-/-	29/33 (87.9%)	10/29[B] (34.5%)	14.2 ± 0.5	3.6 ± 0.2[B]
	Histological EAE			
	Incidence	Meningeal foci *mean ± SE*	Parenchymal foci *mean ± SE*	Total foci *mean ± SE*
SJL/Wt	27/29 (93.1%)	41.0 ± 4.9	36.4 ± 5.2	77.3 ± 9.6
SJL/B7.1-/-	13/13 (100%)	49.2 ± 5.6	49.1 ± 7.3	98.3 ± 12.4
SJL/B7.2-/-	17/17 (100%)	49.1 ± 7.3	56.9 ± 8.0[B]	109.9 ± 13.8[B]

[A]EAE was induced by immunization with PLP 139-151 (25-100 μg) in CFA subcutaneously and two injections of 100 ng Pertussis Toxin. Animals were scored for disease as described in Materials and Methods.
All P values are compared with Wt control and are indicated only when statistically significant ($P < 0.05$). [B]$P < 0.05$; [C]$P < 0.01$.

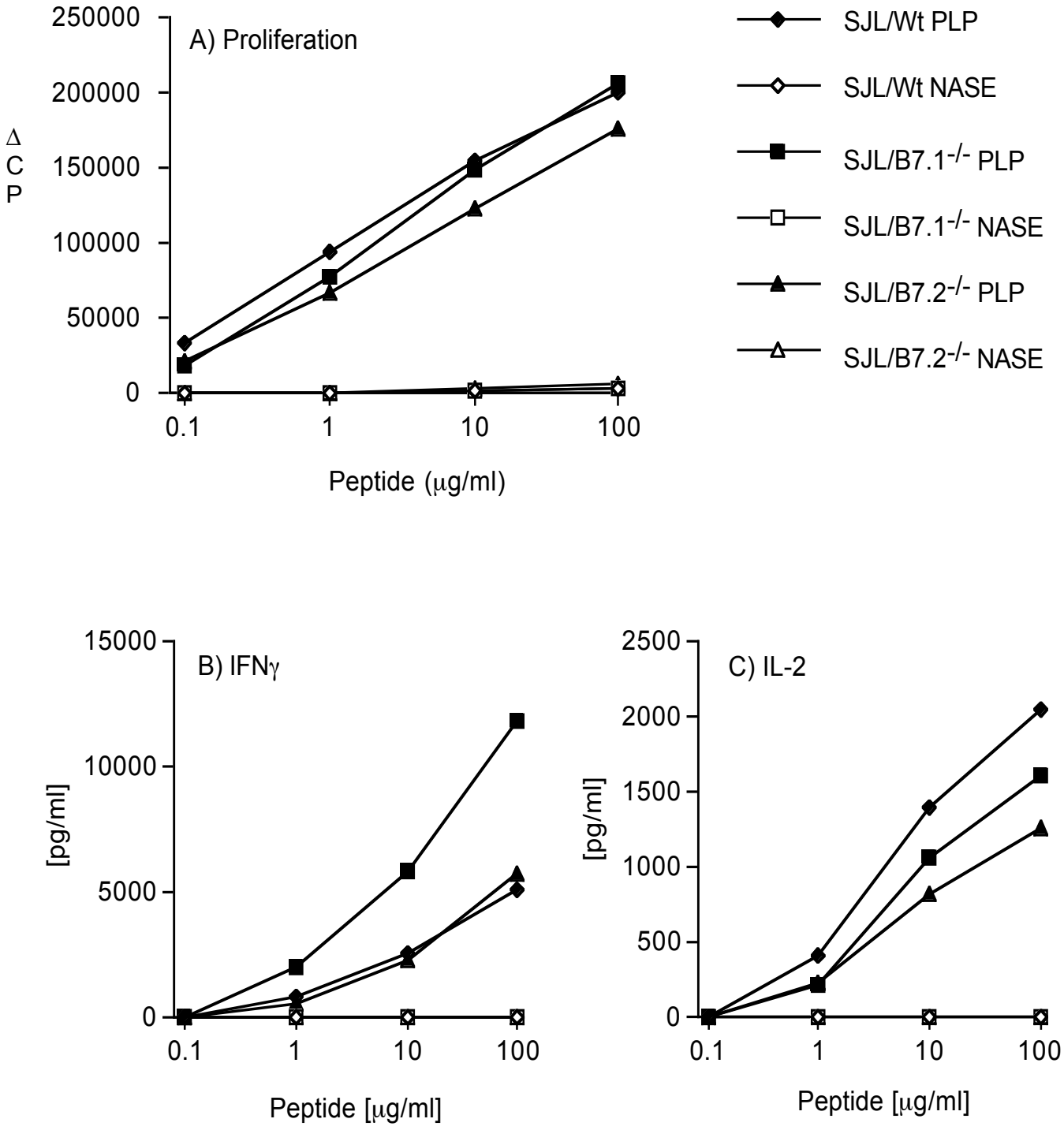


Figure 4.2. T cells from SJL B7.1$^{-/-}$ mice are primed to PLP 139-151 and secrete abundant IFNγ. Proliferation and cytokine production of lymph node cells from SJL Wt, B7.1$^{-/-}$ and B7.2$^{-/-}$ mice challenged with PLP 139-151. Mice were immunized with 100 μg of PLP 139-151 in CFA. 10 days later, draining lymph node cells from Wt (diamonds), B7.1$^{-/-}$ (squares), or B7.2$^{-/-}$ (triangles) mice were restimulated *in vitro* with a titration of PLP 139-151 (filled symbols) or NASE 101-120 (open symbols) or media alone. Culture supernatants were collected after 42 h. Cytokines were measured by sandwich ELISA. Background levels from media controls were subtracted in proliferation and cytokine assays. Data are representative of four independent experiments.

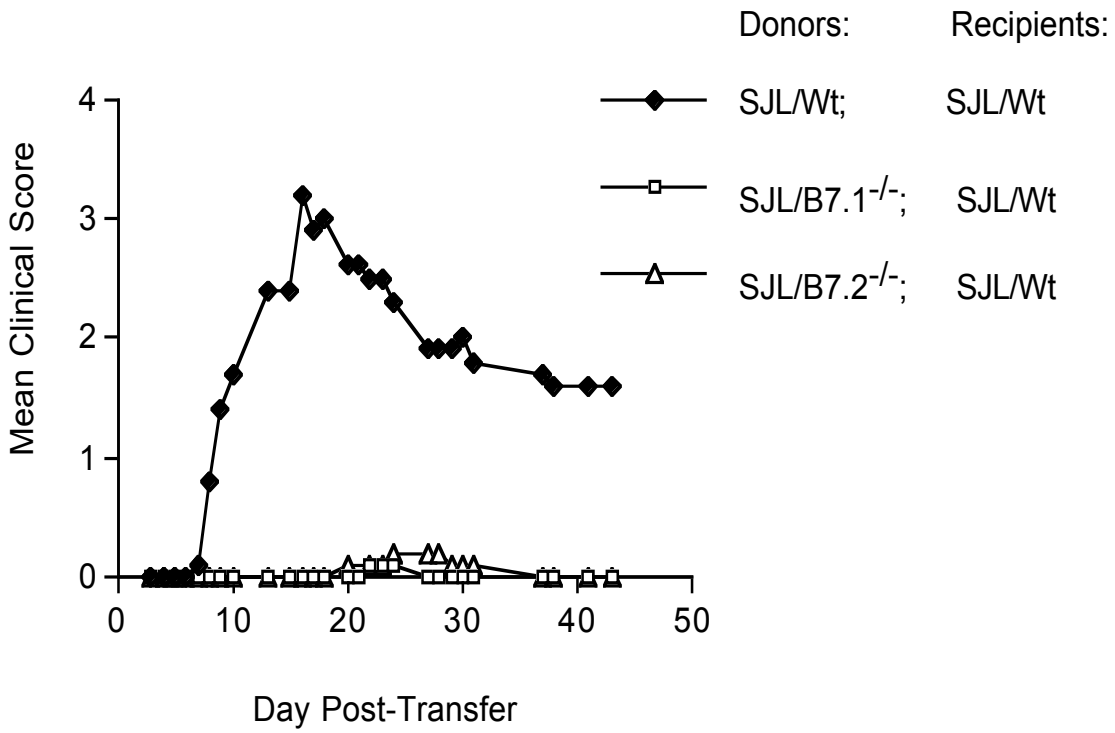


Figure 4.3. PLP 139-151 primed T cells from SJL B7.1$^{-/-}$ or B7.2$^{-/-}$ Donor mice are unable to induce EAE in wildtype recipients.
SJL/Wt, B7.1$^{-/-}$ or B7.2$^{-/-}$ mice were immunized with 100 μg PLP 139-151. After ten days, draining lymph node cells were restimulated *in vitro* with 20 μg/ml PLP 139-151 and kept in culture for four days. 10 x 10^6 of these PLP 139-151 primed cells were then adoptively transferred into SJL/Wt Recipients. SJL/Wt mice received cells from either SJL/Wt Donors (filled diamonds; n=11) or from B7.1$^{-/-}$ Donors (open squares; n=21) or from B7.2$^{-/-}$ Donors (open triangles; n=18). Recipients also received 100 ng Pertussis Toxin. Mice were observed daily and scored as described in Materials and Methods. Data represent mean clinical score of group plotted against time and represent two pooled experiments.

Table 4.2. *EAE in SJL/Wt induced by adoptive transfer of SJL/Wt, B7.1 $^{-/-}$ or B7.2 $^{-/-}$* cells.

		Clinical EAE[A]			
Donor	Recipient	Incidence	Mortality	Day of onset *mean ± SE*	Mean maximal score *mean ± SE*
SJL/Wt	SJL/Wt	11/11 (100%)	4/11 (36.4%)	10.9 ± 1	4.1 ± 0.3
SJL/B7.1$^{-/-}$	SJL/Wt	1/21 (4.8%)	0/1 (0%)	22	2
SJL/B7.2$^{-/-}$	SJL/Wt	2/18 (11.1%)	0/2 (0%)	22 ± 2	2.3 ± 0.3
		Histological EAE			
		Incidence	Meningeal foci *mean ± SE*	Parenchymal foci *mean ± SE*	Total foci *mean ± SE*
SJL/Wt	SJL/Wt	6/6 (100%)	69 ± 10.3	57.5 ± 9.9	126.5 ± 19.5
SJL/B7.1$^{-/-}$	SJL/Wt	0/21 (0%)	0	0	0
SJL/B7.2$^{-/-}$	SJL/Wt	6/18 (33.3%)	3.2 ± 1.5	1.1 ± 0.6	4.3 ± 1.9

[A]EAE was induced by adoptively transferring 10 x 10^6 cells (from SJL/Wt, B7.1$^{-/-}$ or B7.2$^{-/-}$ Donors) i.v. and one injection of 100 ng Pertussis Toxin i.v. Animals were scored daily for disease as described in Materials and Methods.

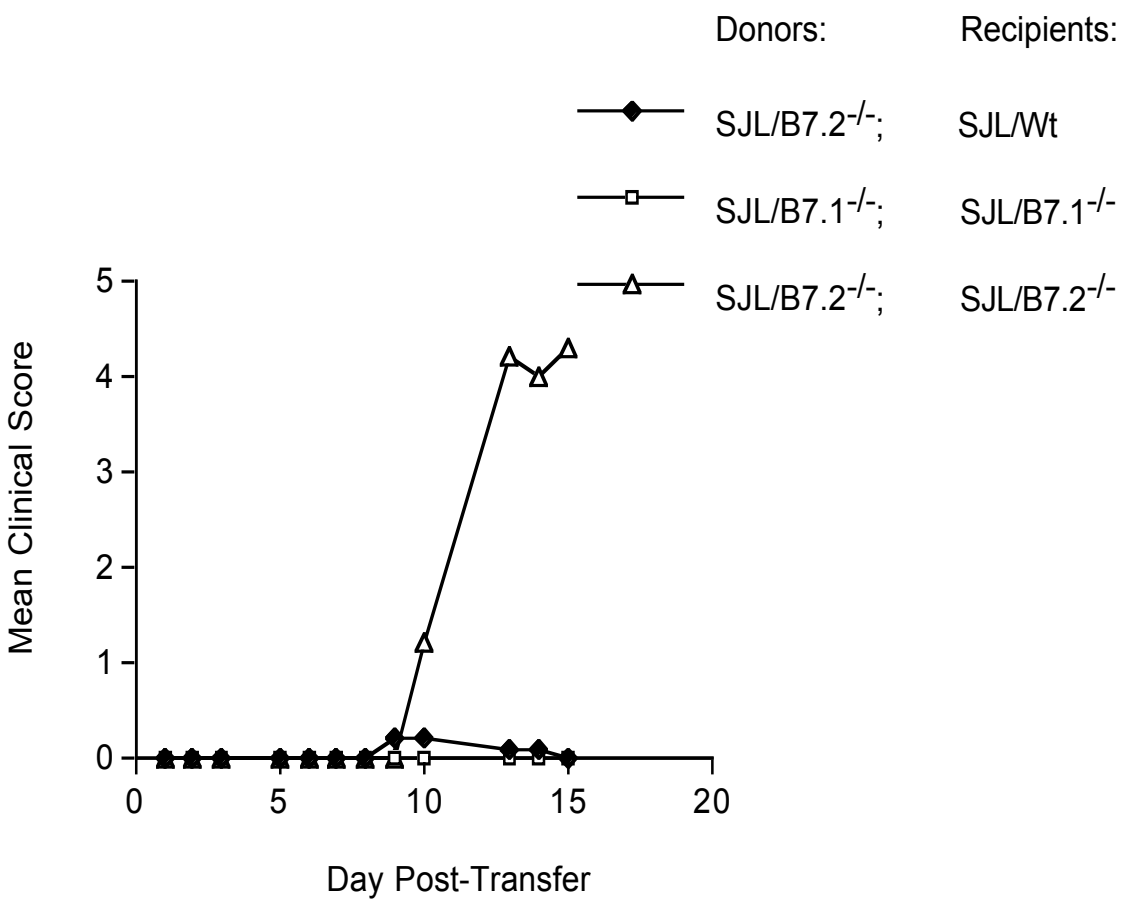


Figure 4.4. PLP 139-151 primed T cells from B7.2$^{-/-}$ Donor mice induce severe EAE in B7.2-/- Recipient mice.
B7.1$^{-/-}$ or B7.2$^{-/-}$ mice were immunized with 100 μg PLP 139-151. After ten days, draining lymph node cells were restimulated *in vitro* with 20 μg/ml PLP 139-151 and kept in culture for four days. 10 x 10^6 of the PLP 139-151-primed cells originating from B7.2$^{-/-}$ Donors were then adoptively transferred into either SJL/Wt (closed diamonds; n=8) or B7.2$^{-/-}$ (open triangles; n=3) Recipients. 10 x 10^6 of the PLP 139-151 primed cells originating from B7.1$^{-/-}$ Donors were adoptively transferred into B7.1$^{-/-}$ mice(open squares; n=7). Recipients also received 100 ng Pertussis Toxin. Mice were observed daily and scored as described in Materials and Methods. Data represent mean clinical score of group plotted against time and represent two pooled experiments.

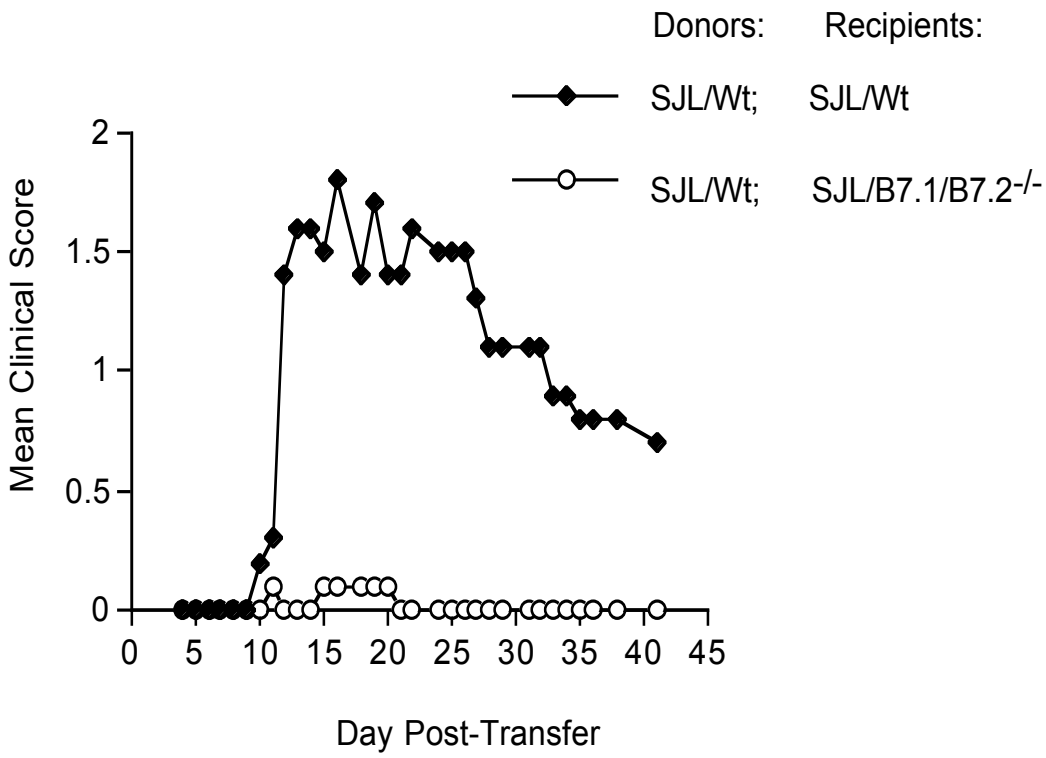


Figure 4.5. PLP 139-151 primed T cells from SJL wildtype donor mice are unable to induce EAE in SJL/B7.1/B7.2$^{-/-}$ mice. SJL/Wt mice were immunized with 100 μg PLP 139-151. After ten days, draining lymph node were restimulated *in vitro* with 20 μg/ml and kept in cuture for four days. 10x10^{6} of these PLP 139-151 primed cells were then adoptively transferred into SJL/Wt (closed diamonds; n=8) or SJL/B7.1/B7.2$^{-/-}$ (open circles; n=8) Recipients. Recipients also received 100 ng Pertussis Toxin. Mice were observed daily and scored as described in Materials and Methods. Data represent mean clinical score of group plotted against time.

Table 4.3. *EAE in SJL/B7.1/B7.2 $^{-/-}$ mice by adoptive transfer.*

	Clinical EAE*			
	Incidence	Mortality	Day of onset *mean ± SE*	Mean maximal score *mean ± SE*
SJL/Wt	7/8 (87.5%)	1/8 (12.5%)	14.4 ± 1.7	3.3 ± 0.4
SJL/B7.1/B7.2$^{-/-}$	0/8 (0%)			
	Histological EAE			
	Incidence	Meningeal foci *mean ± SE*	Parenchymal foci *mean ± SE*	Total foci *mean ± SE*
SJL/Wt	7/7 (100%)	33 ± 8.2	17 ± 2	50 ± 9.6
SJL/B7.1/B7.2$^{-/-}$	4/8 (50 %)	1.1 ± 0.7	2.3 ± 1	3.4 ± 1.4

* EAE was induced by adoptive transfer of $10x10^6$ cells (SJL/Wt-LNCs primed to PLP 139-151) i.v. and 1 x 100 ng Pertussis Toxin i.v. per mouse. Mice were observed daily and scored as described in Materials and Methods.

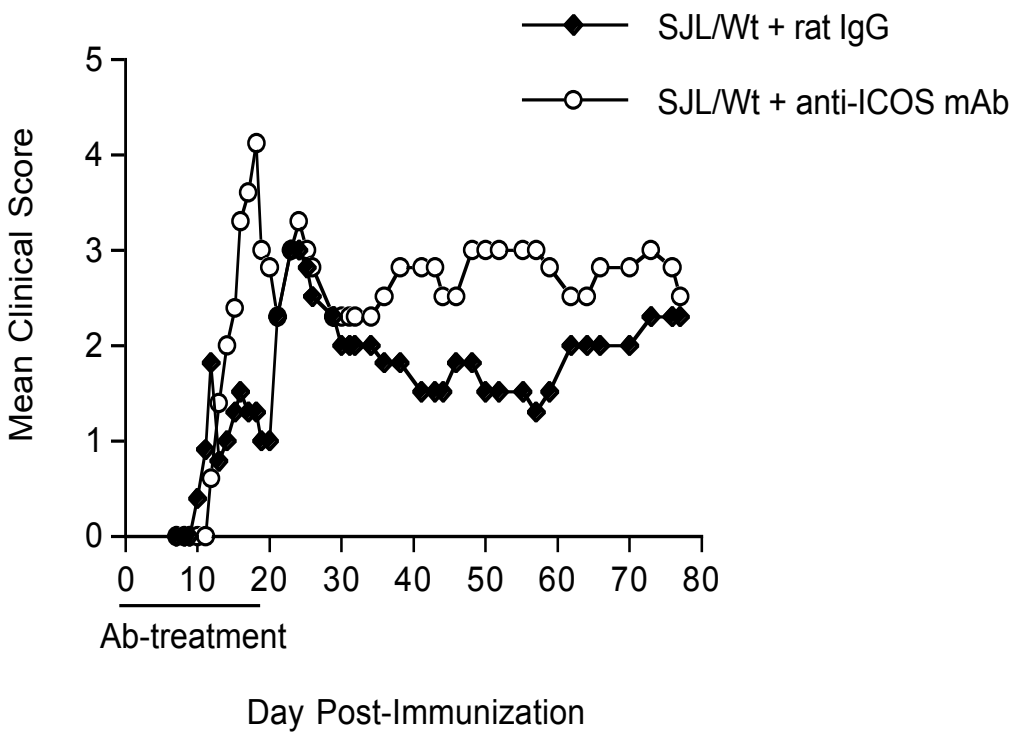


Figure 5.1. Treatment with anti-ICOS mAb exacerbates EAE in SJL mice immunized with PLP 139-151.
Mice were immunized with 75 μg PLP 139-151 subcutaneously and injected twice with 100 ng Pertussis toxin intravenously and then treated with 50 μg of anti-ICOS mAb (17G9; open circles; n=5) or control rat IgG (closed diamonds; n=5) intraperitonally on day 0, 2, 4, 6, 8, 10, 12, 14, 16, 18. Mice were observed daily and scored as described in Materials and Methods.

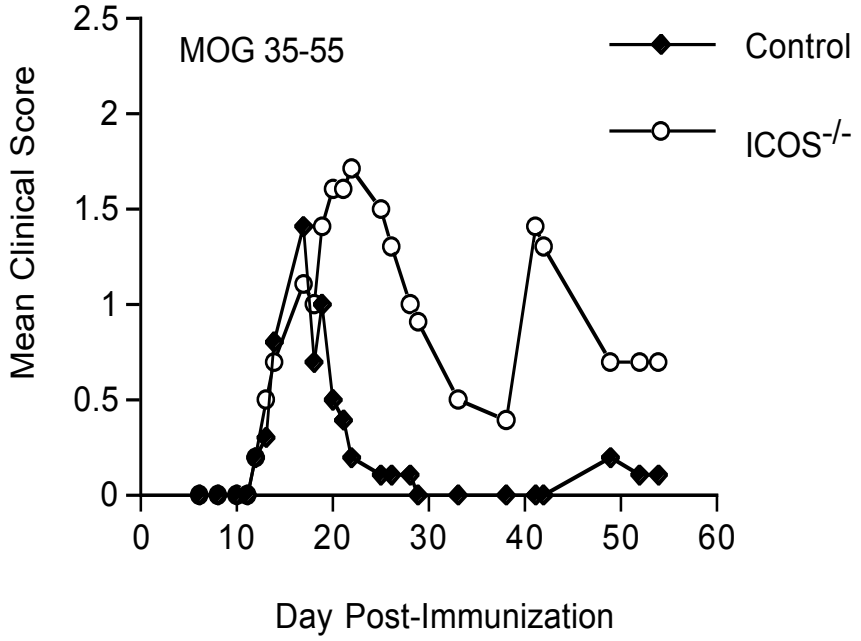


Figure 5.2. ICOS$^{-/-}$ mice develop more severe EAE than wildtype controls. ICOS$^{-/-}$ (open circles; n=7) or wildtype control (closed diamonds; n=8) mice on 129 or 129xB6 background were immunized with 100 μg MOG 35-55 subcutaneously and injected twice with 150 ng Pertussis Toxin intravenously. Mice were observed daily and scored as described in Materials and Methods. Data are pooled from two experiments and represent mean clinical score of both groups plotted against time.

Table 5.1. *EAE in ICOS $^{-/-}$ mice induced by active immunization*

	Clinical[A] EAE				
	Incidence	Mortality	Day of onset *mean ± SE*	Mean max. score *mean ± SE*	Duration of clinical EAE (in days) *mean ± SE*
Wt	7/8 (87.5%)	1/8 (12.5%)	18.6 ± 4.1	2.3 ± 0.5	5.4 ± 1.6
ICOS$^{-/-}$	7/7 (100%)	0/7 (0%)	16.6 ± 3	2.6 ± 0.4	15.4 ± 2.7
	Histological EAE				
	Incidence	Menigeal foci *mean ± SE*	Parenchymal foci *mean ± SE*	Total foci *mean ± SE*	
Wt	5/7 (71.4%)	11.3 ± 4.2	6.6 ± 4.2	17.9 ± 7.7	
ICOS$^{-/-}$	5/7 (71.4%)	14.3 ± 8	16.3 ± 7.8	30.6 ± 15.4	

[A]EAE was induced in mice on 129 or 129xB6 background by active immunization with 100 μg MOG 35-55 in CFA subcutaneously and 2x 150 ng Pertussis Toxin intravenously. Animals were scored for disease as described in Materials and Methods.

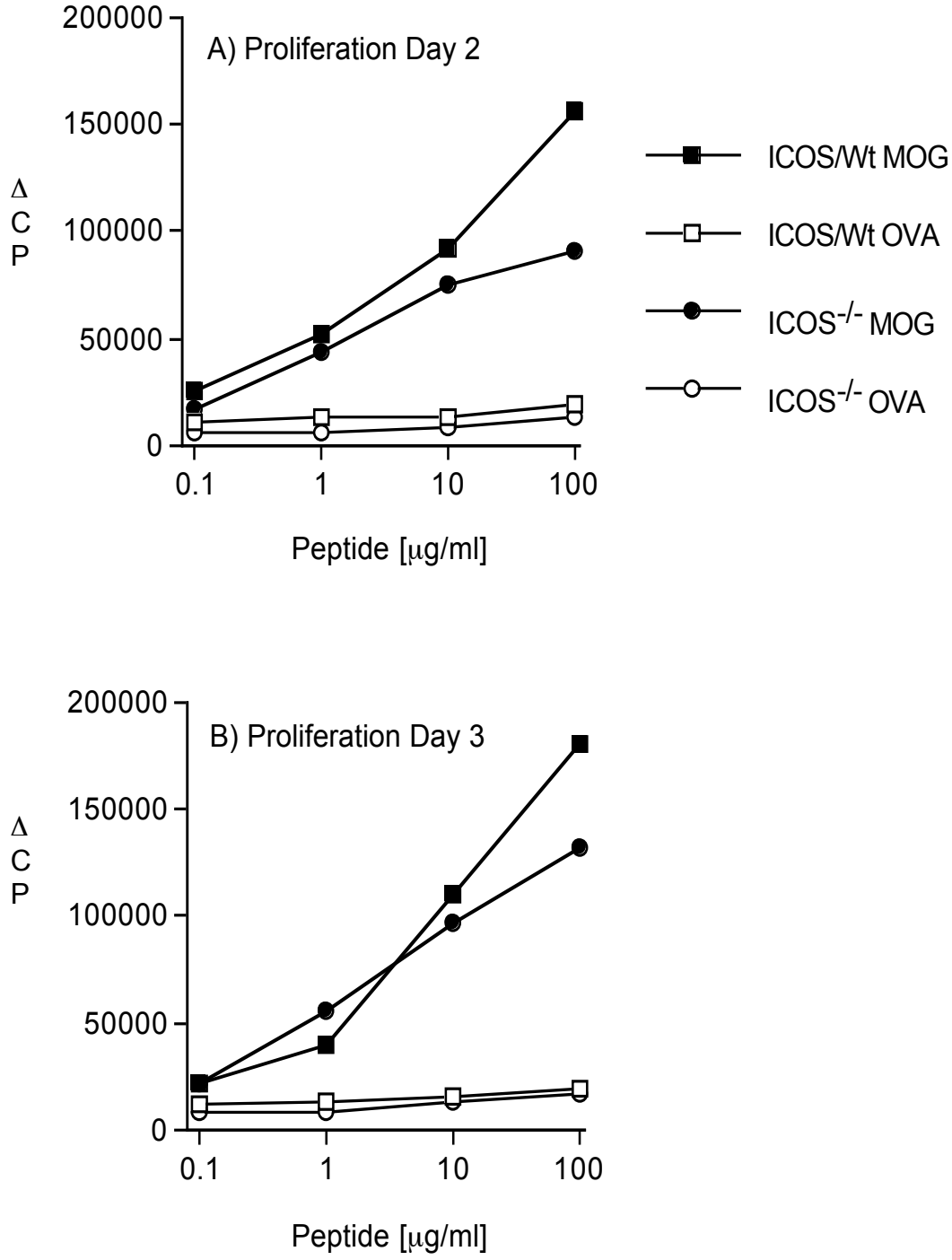


Figure 5.3. T cells primed to MOG 35-55 from ICOS$^{-/-}$ mice (129xB6 mixed background) proliferate comparably to wildtype control cells.
Wildtype (squares) and ICOS$^{-/-}$ (circles) mice on the 129xB6 background were immunized with MOG 35-55 in CFA and draining lymph nodes were harvested 10 days later. Whole draining lymph node cultures were restimulated *in vitro* with a titration of MOG 35-55 (filled symbols) or OVA 323-336 (open symbols) and kept in culture for 2 days (A) or 3 days (B). Data are representative of 3 independent experiments. Proliferation is presented as counts per minute (cpm) above media background for each condition (ΔCPM).

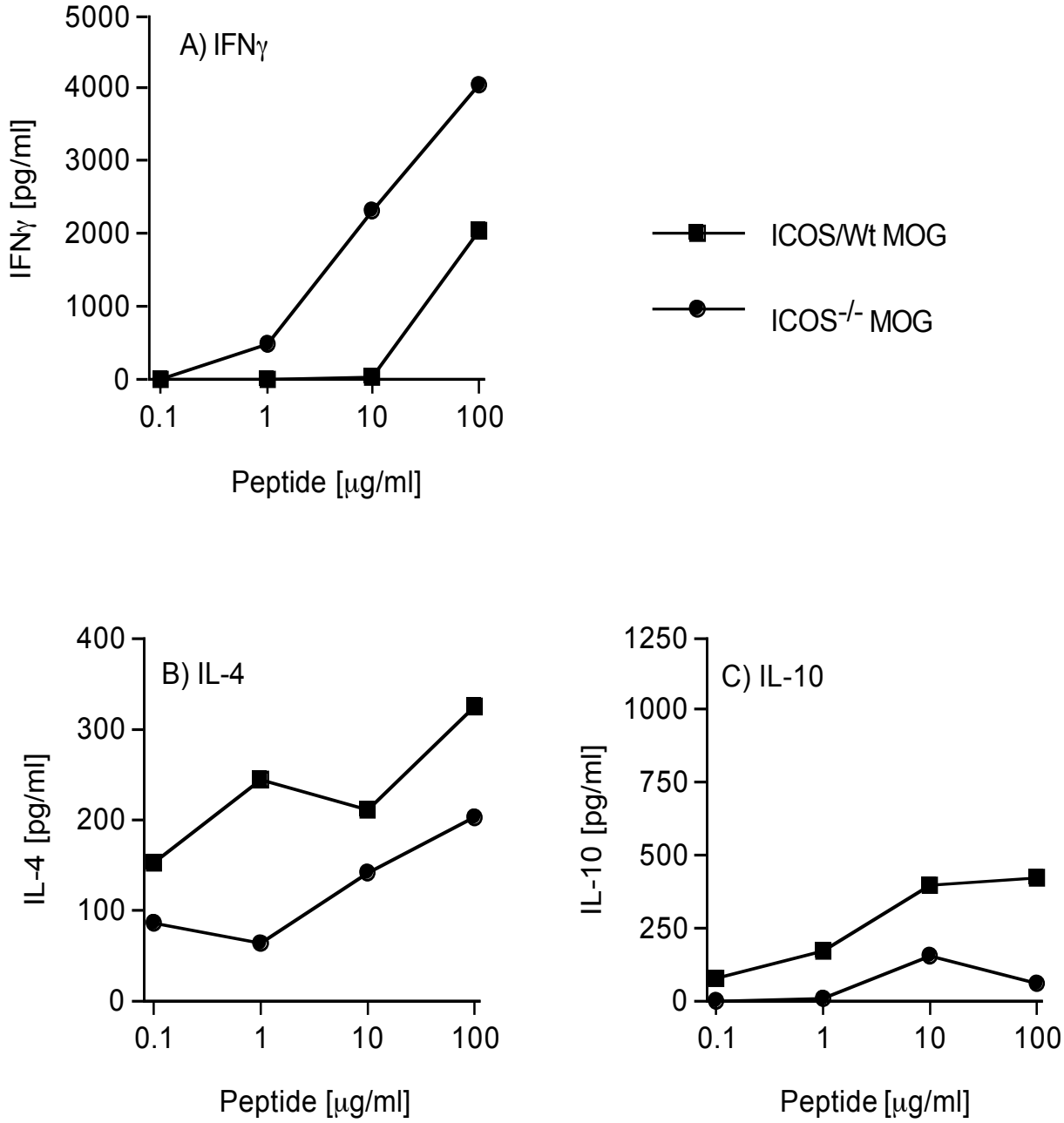


Figure 5.4. MOG 35-55 primed cells from ICOS$^{-/-}$ mice produce more IFNγ and less IL-4 and IL-10 than cells from Wildtype controls (B6x129 mixed background) on Day 2 post-restimulation (by ELISA).
Wildtype and ICOS$^{-/-}$ mice on the 129xB6 background were immunized with MOG 35-55 in CFA and draining lymph nodes were harvested 10 days later. Whole draining lymph node cultures were restimulated *in vitro* with a titration of MOG 35-55. Supernatants were taken from Wildtype (filled squares) and ICOS$^{-/-}$ (filled circles) cultures after 2 days of restimulation. IL-2, IL-4, IL-10 and IFNγ cytokine levels were measured by ELISA. IL-2 was not detected. Data are representative of 3 independent experiments.

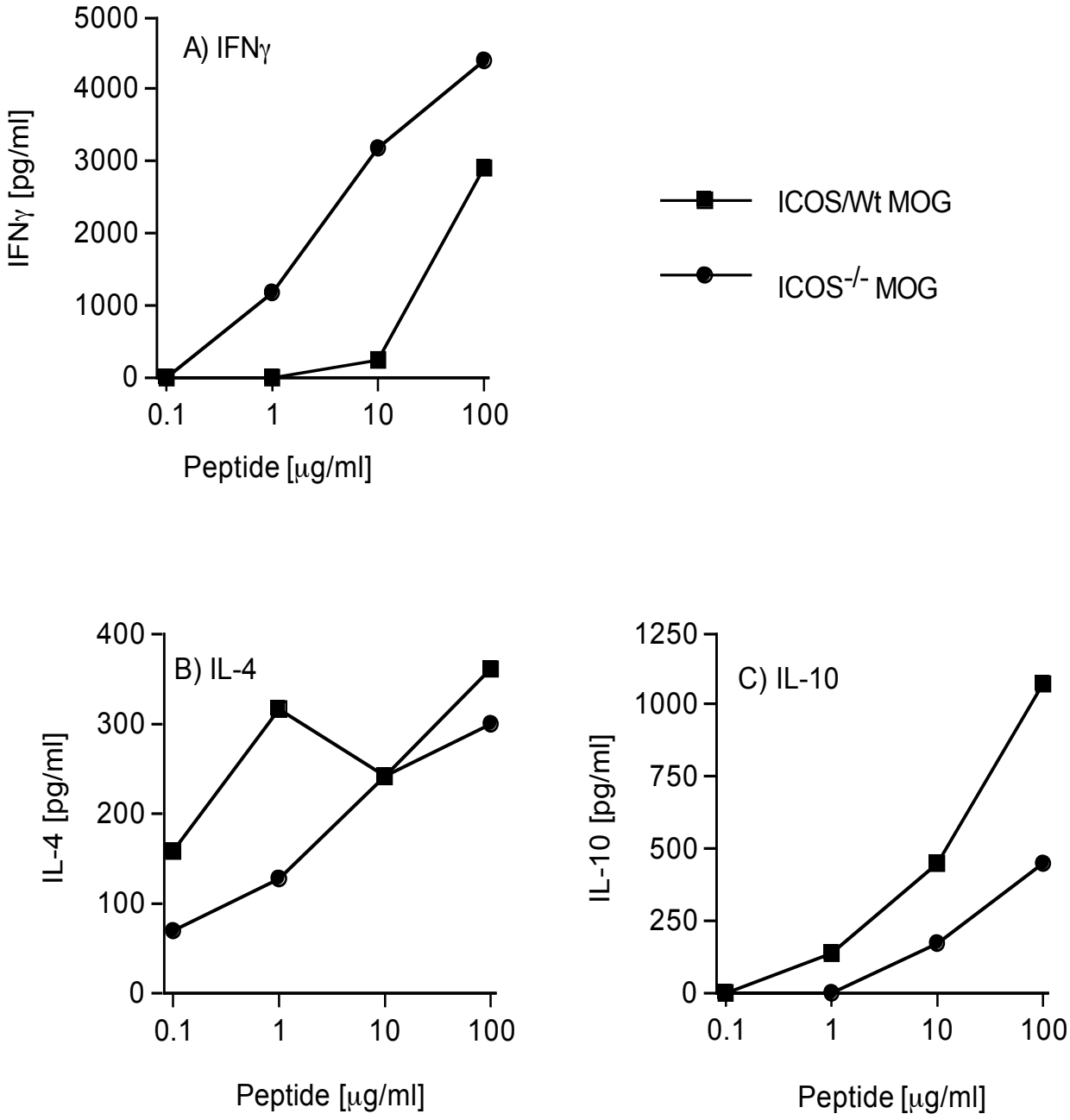
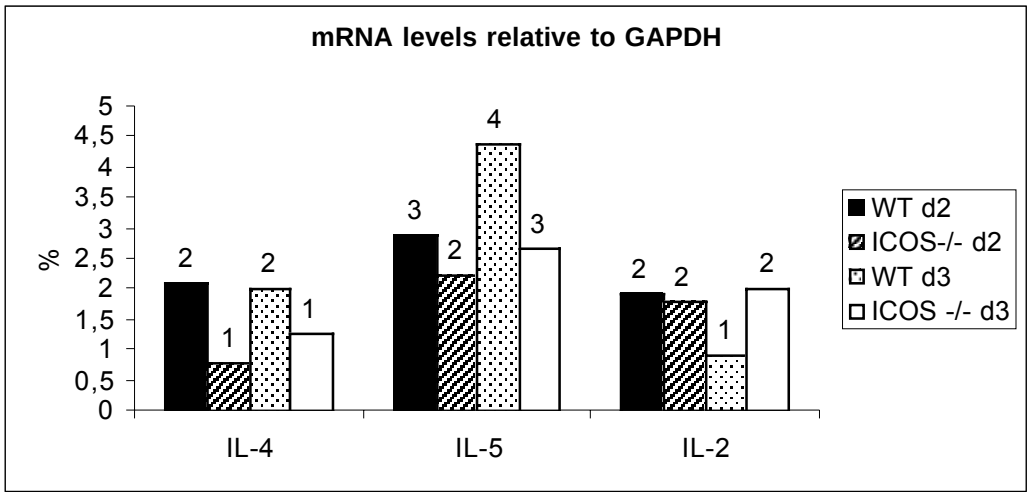


Figure 5.5. MOG 35-55 primed cells from ICOS$^{-/-}$ mice produce more IFNγ and less IL-4 and IL-10 than cells from Wildtype controls (B6x129 mixed background) on Day 3 post-restimulation (by ELISA).

Wildtype and ICOS$^{-/-}$ mice on the 129xB6 background were immunized with MOG 35-55 in CFA and draining lymph nodes were harvested 10 days later. Whole draining lymph node cultures were restimulated *in vitro* with a titration of MOG 35-55. Supernatants were taken from Wildtype (filled squares)and ICOS$^{-/-}$ (filled circles) cultures after 3 days of restimulation. IL-2, IL-4, IL-10 and IFNγ cytokine levels were measured by ELISA. IL-2 was not detected. Data are representative of 3 independent experiments.



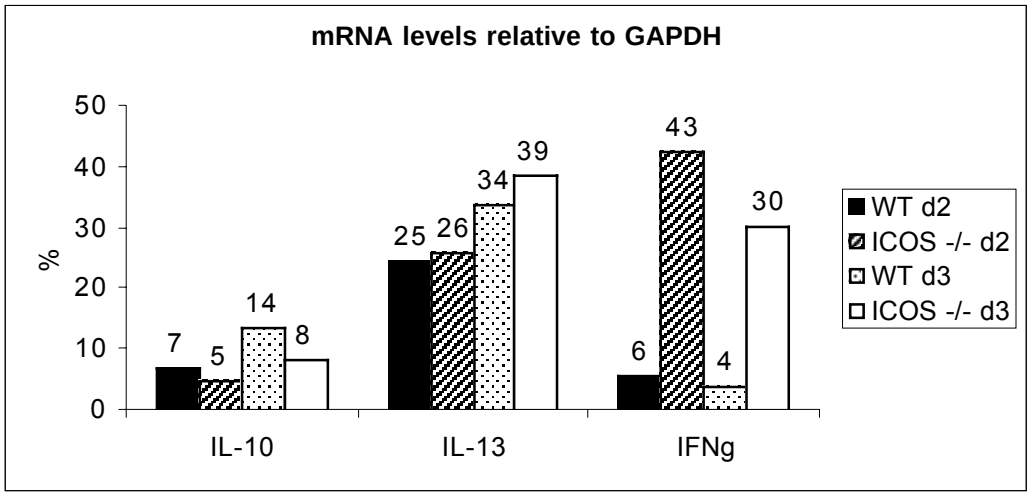


Figure 5.6. MOG 35-55 primed cells from ICOS$^{-/-}$ mice produce more IFNγ and less IL-4 and IL-10 than cells from Wildtype controls on Day 2 and 3 post-restimulation (by RPA).
Wildtype and ICOS$^{-/-}$ mice on the 129xB6 background were immunized with MOG 35-55 in CFA and draining lymph nodes were harvested 10 days later. Whole draining lymph node cultures were restimulated *in vitro* with 100 μg/ml MOG 35-55 for 2 (d2) or 3 (d3) days. mRNA was isolated from 5x10^6 cells using TRIzol® reagent and analyzed for cytokine expression by RNase protection assay. Data represent 3-6 mice per group.

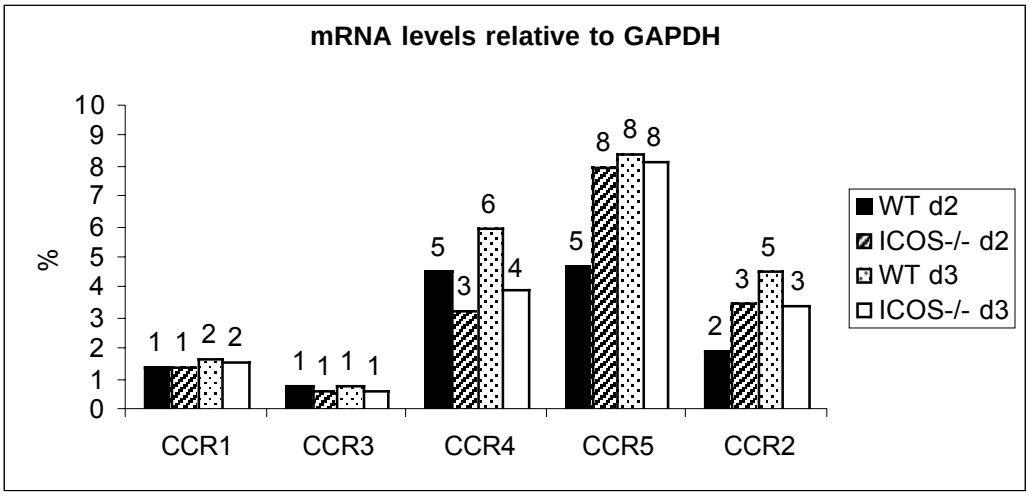


Figure 5.7. Chemokine-Receptor Expression in MOG 35-55 primed cells from ICOS$^{-/-}$ mice on Day 2 and 3 post-restimulation (by RPA).
Wildtype and ICOS$^{-/-}$ mice on the 129xB6 background were immunized with MOG 35-55 in CFA and draining lymph nodes were harvested 10 days later. Whole draining lymph node cultures were restimulated *in vitro* with 100 μg/ml MOG 35-55 for 2 (d2) or 3 (d3) days. mRNA was isolated from 5x10^6 cells using TRIzol® reagent and analyzed for chemokine receptor expression by RNase protection assay. Data represent 3-6 mice per group.

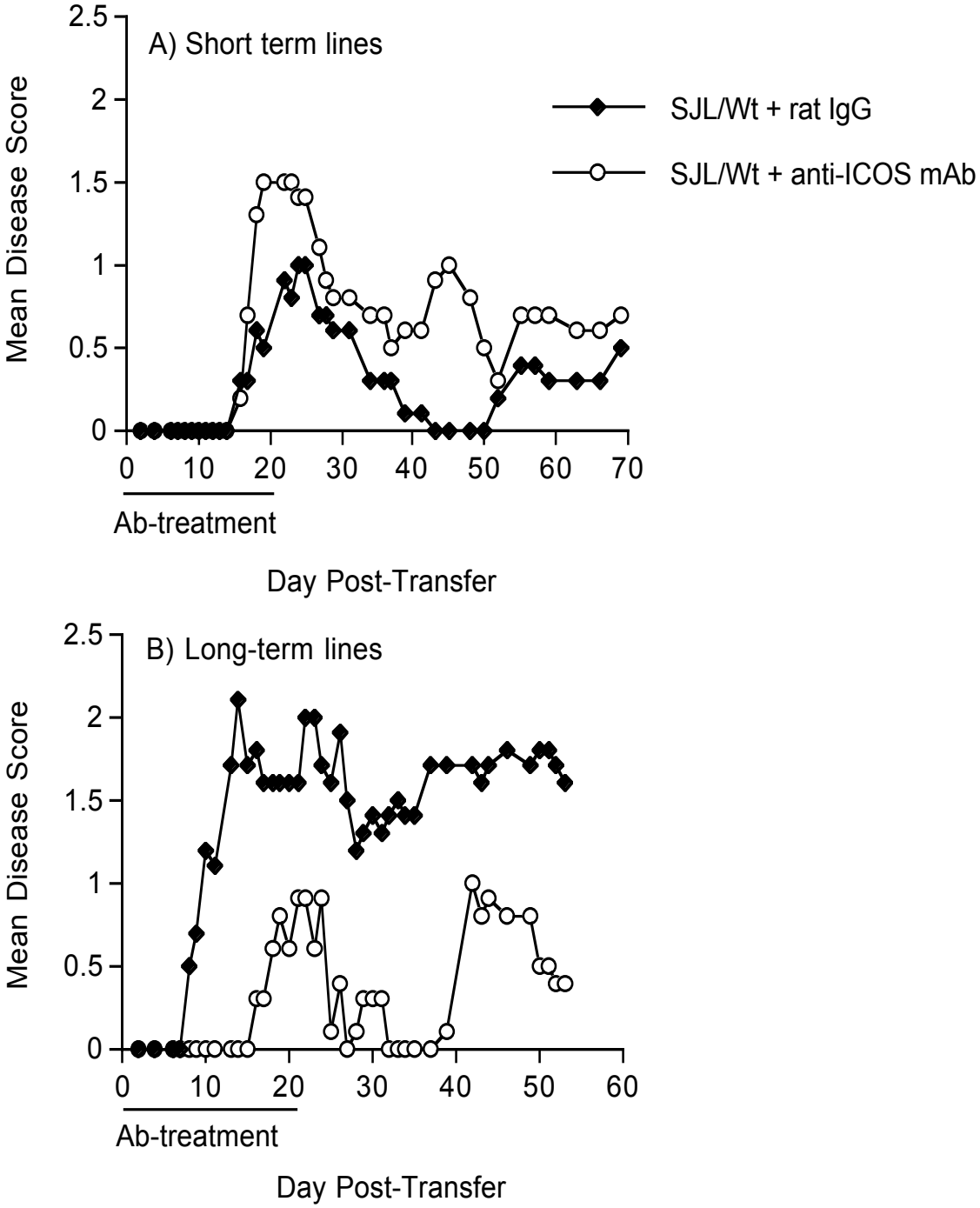


Figure 5.8. Treatment with anti-ICOS mAb exacerbates or ameliorates adoptively transferred EAE in SJL mice, depending on the timing of Antibody-treatment.

SJL/Wt mice were immunized with 100 μg PLP 139-151 subcutaneously. After ten days, draining lymph node cells were restimulated *in vitro* with 20 μg/ml PLP 139-151 and kept in culture for four days (A) or kept in culture for 4 weeks and restimulated 3 times (B) . 10 x 10^6 of the PLP 139-151 primed cells were then adoptively transferred into SJL/Wt Recipients and injected with 100 ng Pertussis toxin intravenously. Recipients were then treated with 50 μg of anti-ICOS mAb (17G9; open circles; n=6 in A; n=4 in B) or control rat IgG (closed diamonds; n=6 in A; n=8 in B) intraperitonally on day 0, 2, 4, 6, 8, 10, 12, 14, 16, 18. Mice were observed daily and scored as described in Materials and Methods.

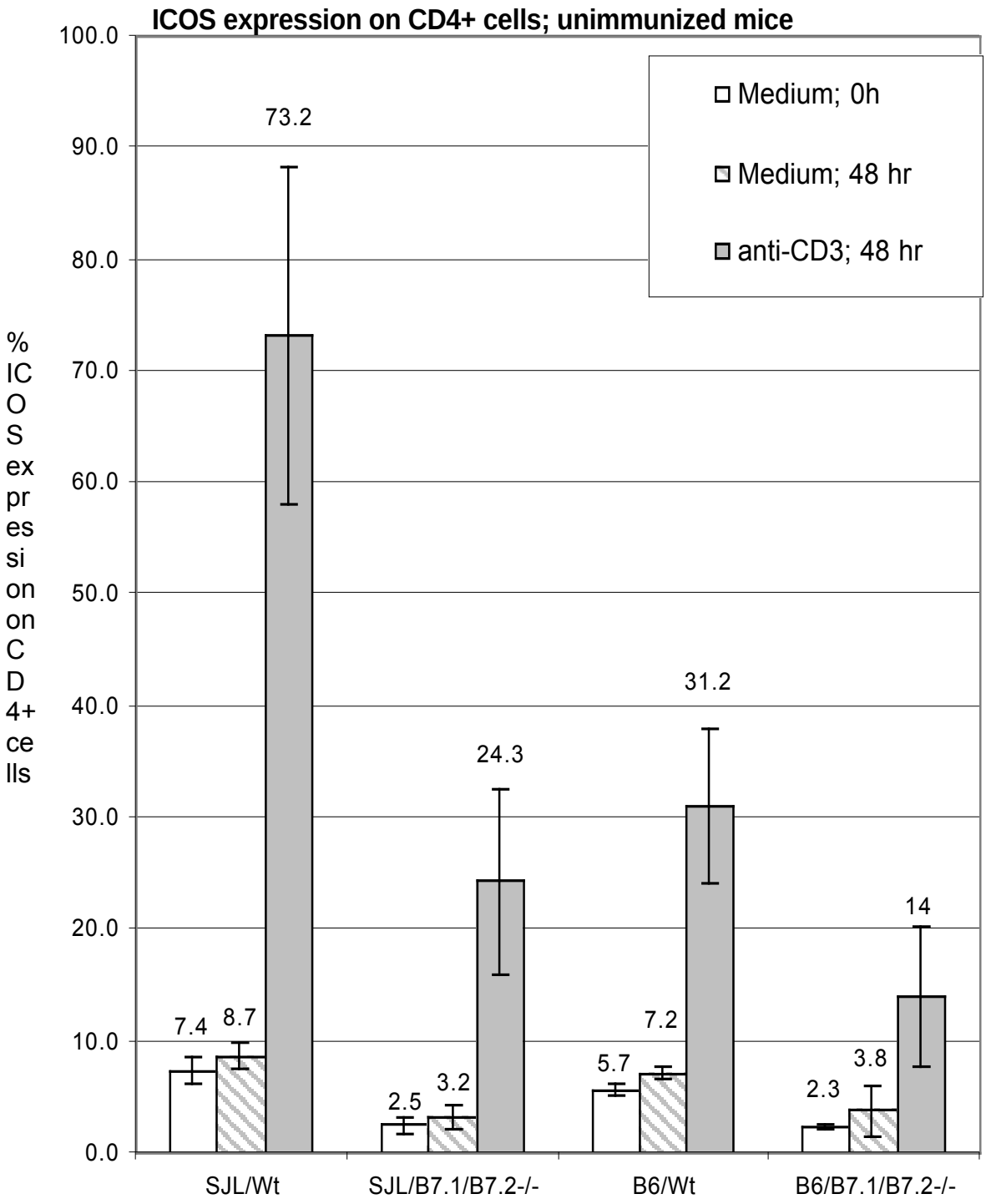


Figure 5.9. ICOS expression on CD4+ cells from unimmunized SJL/Wt, SJL/B7.1/B7.2$^{-/-}$, B6/Wt and B6/B7.1/B7.2$^{-/-}$ mice.
Lymph node cells from unimmunized mice were either analyzed *ex vivo* (Medium; 0 h) or cultured for 48 hours in medium (Medium; 48 hr) or with anti-CD3 Ab (aCD3; 48hr). Cells were stained with directly conjugated antibodies specific for the cell surface molecules CD4 and ICOS or the appropriate matched isotype control IgG2b. Samples were analysed on a FACSort. Background levels from isotype control staining was subtracted. Data are pooled from 3 independent experiments. Values are represented as X $\pm$ SEM.

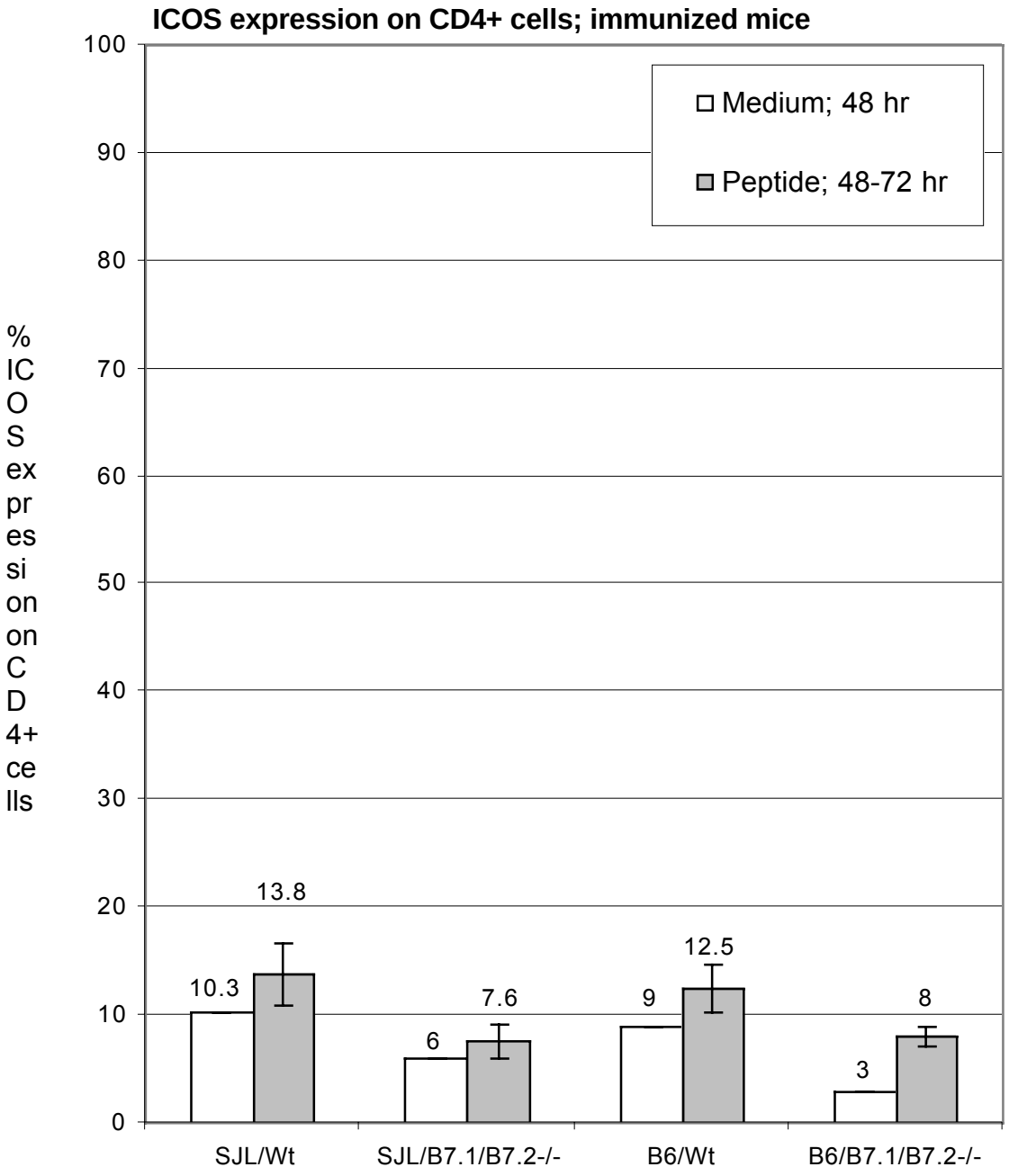


Figure 5.10. ICOS expression on CD4+ cells from immunized SJL/Wt, SJL/B7.1/B7.2$^{-/-}$, B6/Wt and B6/B7.1/B7.2$^{-/-}$ mice.
SJL/Wt and SJL/B7.1/B7.2$^{-/-}$ mice were immunized with 100 μg PLP 139-151, B6/Wt and B6/B7.1/B7.2$^{-/-}$ mice were immunized with 100 μg MOG 35-55. After 10 days, draining lymph nodes were either restimulated with the appropriate peptide (PLP or MOG peptide) or were cultured in medium for 2 to 3 days. Cells were then stained with directly conjugated antibodies specific for the cell surface molecules CD4 and ICOS or the appropriate matched isotype control IgG2b. Samples were analysed on a FACSort. Background levels from isotype control staining was subtracted. Data are pooled from two independent experiments.

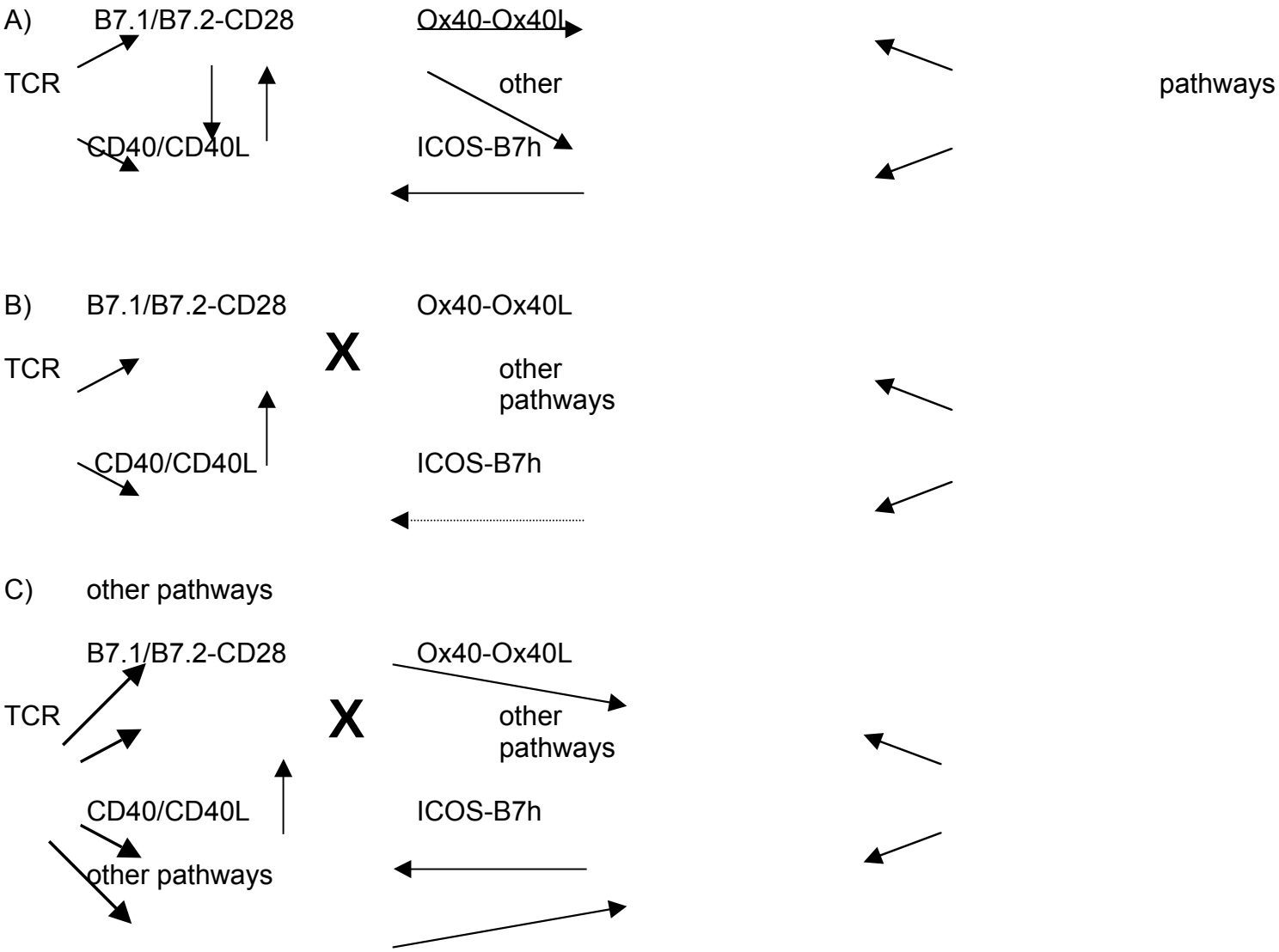


Figure 6.1 The interdependence of costimulatory pathways.
When a T cell is activated through its TCR, this signal contributes to the upregulation of costimulatory molecules. The upregulation of certain costimulatory pathways has been shown to result in the upregulation of other costimulatory pathways (see text for references) as indicated by the arrors. These signals and also the kinetics of costimulatory molecules determine the order in which a certain costimulatory molecule is upregulated (A).
However, this order can be disrupted if the expression of a certain costimulatory molecule (e.g. CD28, as indicated by the cross) is inhibited. The loss of CD28 may result in the inability of the T cell to upregulate maximum expression levels of other costimulators that are normally dependent on CD28-signaling, as e.g. ICOS and Ox40. The suboptimal upregulation of ICOS may then result in an impaired ICOS-dependent upregulation of CD40L (as indicated by the dotted arrow) (B).
Alternatively, the loss of CD28 signaling may be substituted by a very strong signal through the TCR (C).

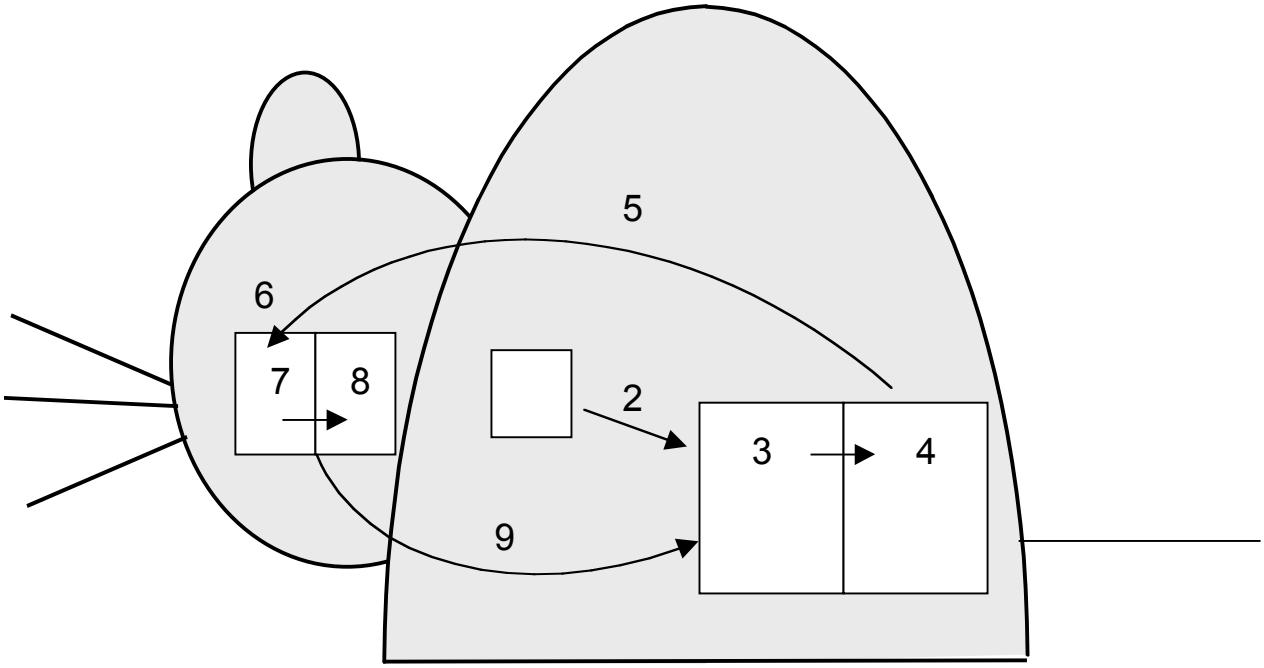


Figure 6.2 The different stages of EAE pathogenesis during which costimulation might be critical.

1) Thymic selection: The generation of myelin-reactive T cells in the thymus;
2) Maintenance of myelin-reactive T cells in the periphery;
3) Early induction phase: initial activation of myelin-reactive T cells in the lymph nodes;
4) Late induction phase: differentiation of myelin-reactive T cells in the lymph nodes;
5) Trafficking of the differentiated myelin-reactive T cells from the lymph nodes to the target organ, the central nervous system (CNS);
6) T cells crossing the Blood brain barrier;
7) Early effector phase: myelin-reactive T cells are reactivated in the CNS;
8) Late effector phase: effector T cells induce tissue damage (by production of pro-inflammatory cytokines and by recruiting other cells, including macrophages, from the periphery); possible interaction of effector T cells with other T cells (T-T signaling); termination of T cell response; maintenance of self-reactive memory T cells;
9) Epitope spreading: the specificity of the immune response spreads to include self-epitopes other than that which initiated the inflammation process.