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INTERLEUKIN-15 IN THE PATHOGENESIS OF MULTIPLE SCLEROSIS AND ITS ANIMAL MODELS

par

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Résumé

L'interleukine-15 (IL-15) contribue au développement et à l'activation des lymphocytes T CD8, des cellules immunes qui ont été impliquées dans plusieurs maladies auto-immunes telle la sclérose en plaques. Des niveaux élevés de l'IL-15 ont été trouvés chez les patients atteints de cette maladie comparativement aux témoins, mais aucune étude n'a examiné les effets de tels niveaux élevés sur les lymphocytes T CD8. Les objectifs de notre étude étaient 1- de caractériser l'expression de l'IL-15 par des lymphocytes B humains et de déterminer ses effets sur les fonctions des lymphocytes T CD8, et 2- d'évaluer l'expression *in vivo* de l'IL-15 dans des modèles murins de la sclérose en plaques.

Nous avons établi que les cellules B humaines augmentaient leur expression de l'IL-15 suite à une stimulation via le CD40. De plus, les fonctions effectrices des lymphocytes T CD8 ont été significativement augmentées lors des co-cultures avec des cellules B alloréactives exprimant l'IL-15. Dans les modèles murins de la sclérose en plaques, nous avons détecté au sein du système nerveux central des cellules immunes exprimant l'IL-15 ainsi que des cellules T CD8 exprimant le récepteur pour cette cytokine à différents stades de la maladie.

Nous avons démontré que les cellules B modulent des réponses des lymphocytes T CD8 via l'IL-15, ce qui suggère un rôle pour les cellules B dans la pathogenèse de la sclérose en plaques. Nous avons aussi mis en évidence la présence de cellules exprimant l'IL-15 dans le système nerveux central dans des modèles murins de cette maladie.

Mots clés

interleukine-15

cellules B

cellules T CD8

sclérose en plaques

encéphalomyélite autoimmune expérimentale

Summary

Interleukin-15 is a cytokine involved in the homeostatic proliferation and maintenance of CD8 T cells. Activated CD8 T cells are implicated in several autoimmune diseases, including Multiple Sclerosis (MS). Elevated levels of IL-15 have been reported in serum and on peripheral leukocytes of MS patients relative to controls, yet no study has addressed the effects of elevated IL-15 levels on CD8 T cells. To study the *in vivo* effects of any molecule, the animal model for MS, EAE, is used; the expression of IL-15 during the EAE disease course has not yet been elucidated. Thus the goals of our study were to characterize surface IL-15 expression on human B lymphocytes and determine the effects on human CD8 T cell functions; and to assess the *in vivo* expression of IL-15 in MS mouse models.

We found that B cells are capable of up-regulating the expression of surface IL-15 upon CD40 stimulation, and CD8 T cell effector functions were significantly enhanced upon co-culture with alloreactive IL-15-expressing B cells. In the MS mouse models we used, we found IL-15-expressing immune cells present within the central nervous system (CNS) at various points of disease, and that CNS-infiltrating CD8 T cells were potentially responsive to IL-15.

Here, we not only demonstrate the modulation of CD8 T cell responses by IL-15 presented by B cells, implying a role for B cells in MS pathogenesis, but also show the presence of IL-15-expressing cells within the inflamed CNS of EAE.

Key Words

Interleukin-15

B cells

CD8 T cells

Multiple Sclerosis

Experimental Autoimmune Encephalomyelitis

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LIST OF ACRONYMS AND ABBREVIATIONS

A488	Alexa Fluor® 488
A700	Alexa Fluor® 700
AA	amino acid
Ab	antibody
Ag	antigen
APC	antigen presenting cells
BCR	B cell receptor
BAFF	B lymphocyte activator of the TNF family
BBB	blood-brain barrier
BFA	Brefeldin A
B6	C57BL/6
cDNA	Complementary DNA
CNS	central nervous system
CSF	cerebrospinal fluid
CFA	complete Freund's adjuvant
DC	dendritic cells
D	diversity
DLN	draining lymph nodes
DNA	Deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EAE	experimental autoimmune encephalomyelitis
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GrB	Granzyme B
HMGB1	High-mobility group protein B1
HBEC	human brain endothelial cell
Hprt1	hypoxanthine phosphoribosyltransferase 1
Ig	immunoglobulin
IBD	inflammatory bowel disease
IFN	interferon
IL-	interleukin-
ICAM	intracellular adhesion molecule
J	joining
LSP	long signal peptide
LPS	lypopolysaccharide
MHC	major histocompatibility complex

MS	Multiple Sclerosis
MBP	myelin basic protein
NCAM	neural cell adhesion molecule
PB	Pacific Blue
PBMC	Peripheral blood mononuclear cells
PFA	paraformaldehyde
PAMP	pathogen-associated molecular patterns
PRR	pattern recognition receptors
PerCP-Cy5.5	Peridinin-cholorophyll proteins Cy5.5
PE	Phycoerythrin
PECy7	Phycoerythrin-Cy7
polyI:C	polyinosinic acid: polycytidylic acid
PPMS	primary progressive MS
PLP	proteolipid protein
RAG	recombinase-activating genes 1 and 2
RRMS	relapsing-remitting MS
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
RT-PCR	Real-time polymerase chain reaction
SPLN	spleen
SPMS	secondary progressive MS
SSP	short signal peptide
STAT	signal transducer and activator of transcription
SJL	SJL/J
TCR	T cell receptor
TdT	terminal deoxynucleotide transferase
TLR	Toll-like receptors
TNF	tumor necrosis factor
UTR	untranslated region
V	variable

DEDICATION

To my parents for seeing the end from the very beginning and encouraging me to reach it

To E.T. and P.M. for the much needed push to start, helpful advice and support

To S.N.M. for believing in my capacity and keeping me company through many a sleepless night

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INTRODUCTION

1.0. THE IMMUNE SYSTEM

The immune system is divided into two main branches: the innate immune system which serves as the first line of defense, and the adaptive immune system which tailors specific responses to and develops memory against pathogens. While there are two arms of the immune system defined, they usually act together: the activation of an antigen-specific response is initiated by components of the innate system, and the adaptive response will include innate effector responses to aid in promoting pathogen clearance. Thus, the cooperative effect of the two aspects of the immune system is necessary for a complete and effective immune response.

The innate immune system has various and diverse elements: there are physical barriers preventing the entry of pathogens into the body, from the epithelial cell layers with tight cell-cell contacts, to the mucus layers covering the respiratory and gastrointestinal tracts. It also includes various proteins and compounds or molecules produced by the innate system cells, including complement and chemokines and cytokines which attract and regulate the function of other immune cells. Lastly, the cells of the innate system express a range of pattern recognition receptors (PRRs), giving it the ability to respond to a number of pathogen-associated molecular patterns (PAMPs) on pathogens. Among the PRRs are Toll-like Receptors (TLRs) which recognize PAMPs such as lipopolysaccharide (LPS) from Gram negative bacteria, viral double-stranded RNA and unmethylated DNA with CpG motifs. Among the cells comprising the innate system, are natural killer cells, macrophages and dendritic cells (DC). The latter two cell types are phagocytic and, together with B cells, are classified as professional antigen presenting cells (APC). After processing of internalized proteins, these cells can present various antigens (Ag) to other immune cells on Major Histocompatibility Complex (MHC) Class II. DC, as well as activated B cells, have the ability to cross-present antigen on MHC Class I molecules as well. The efficacy of the first line of defense, namely the innate immune response, in addition to other factors, will contribute to whether or not the adaptive response is initiated.

The adaptive immune response is largely dependent on the receptors expressed on the T or B cell surface. The varied repertoire is due to the re-arrangement of the genes encoding the receptors; this resulting receptor diversity confers the ability of the adaptive

system to specifically respond to pathogenic insult, and to generate immune memory. The response of the adaptive system can be classified under two categories: the humoral response mediated by B cells, and the cell-mediated response mediated by T cells. In the former case, upon activation via the B cell receptor (BCR), these cells will secrete antibodies which promote opsonization and phagocytosis of foreign matter. Cellular immunity involves the activation of T cells via the T cell receptor (TCR) which occurs via recognition of antigen presented by MHC in addition to a co-stimulatory signal. Activation of the immune system will result in a strong and targeted response to allow pathogenic clearance including killing of pathogen-bearing cells; thus it is important that a balance is maintained with respect to the activation status of the immune cells, and that they do not become "mis"-activated.

1.1 Adaptive immunity: Cell-mediated response

1.1.1 T cell development

It is well-established that the thymus is crucial in providing the support T cell progenitors require for development, differentiation and selection to occur (Miller, 2002; Petrie, 2002). Progenitors, upon entering the thymus, are influenced by various factors; early expansion is influenced by IL-7, an essential factor in T cell development, (von Freeden-Jeffry et al., 1995), accompanied by the induction of various transcription factors, such as Notch (Zuniga-Pflucker, 2004) which serve to commit the precursor cells to the T cell lineage and induce expression of the genes required for TCR assembly.

The genes encoding TCRs are in a non-functional state; they exist as arrays of variable (V), diversity (D; present in only the β and δ chains of the TCR) and joining (J) segments; rearrangement is required for the creation of the genes encoding for the α and β or γ and δ chains of the TCR. Rearrangements are mediated by a protein complex encoded by recombinase-activating genes 1 and 2 (RAG). RAG binds and cleaves the DNA at specific sites flanking the V, D and J segments; the DNA ends are repaired and the resulting recombination, if functional, dictates the final structure and binding specificity of the TCR (Schatz and Ji, 2011). T cells expressing the αβ TCR will become double-positive, expressing both CD4 and CD8 (co-receptors of the TCR). The subsequent selection of T cells is crucial. Cells with TCRs that do not recognize or bind to self-MHC

molecules with low avidity are eliminated (positive selection), as are those with TCRs that recognize self-peptides presented by MHC molecules with too high avidity (negative selection) (Takahama, 2006), ensuring that self-reactive T cells do not mature. Self-reactive T cells escaping such selection can result in the development of autoimmune disease.

1.1.2 The TCR complex and co-receptors

The TCR is a heterodimer of di-sulfide linked α and β or γ and δ chains, and forms a complex together with CD3. CD3 is made up of the invariant proteins of the CD3 family (γ, δ and ε) and the ζ family (Clevers et al., 1988; Fernandez-Miguel et al., 1999). The CD3 chains possess sequence motifs for tyrosine phosphorylation, known as ITAMs (immunoreceptor tyrosine-based activation motif) (Davis, 2002); it is the CD3 component of the complex which is responsible for signaling upon stimulation of the TCR (Fernandez-Miguel et al., 1999). There are two pathways, it seems, which trigger T cell activation upon TCR stimulation: the first is tyrosine phosphorylation of the ζ chain ITAMs by src kinases (Salmond et al., 2009), and the second involves a conformational change in the CD3ε chain leading to Nck (an adaptor protein) recruitment (Davis, 2002). In either case, engagement of the TCR leads to the subsequent signaling via CD3, resulting in T cell activation; however, before signaling even occurs, the TCR needs to recognize and bind to a MHC-peptide complex. Aiding this process, the TCR/CD3 complex is further associated with other molecules, which further confer a degree of specificity to the T cell.

CD4 and CD8 are co-receptors to the TCR; they are trans-membrane glycoproteins, each with immunoglobulin-like (Ig-like) extracellular domains, and a short cytoplasmic tail (Leahy, 1995; Littman, 1987) which interacts with lck (Leahy, 1995), a src-family kinase, and thus they also play a role in signaling. There still are, however, differences in their structures. CD4 is a monomer with four Ig-like domains, and CD8 exists as a dimmer of either two α chains or an α and β chain (Leahy, 1995). These co-receptors play a role in MHC recognition by the TCR complex, increasing the affinity and stabilizing the interaction; CD4 and CD8 bind to non-polymorphic regions (von Freeden-Jeffry et al., 1995) of MHC class II or MHC class I respectively.

Stimulation of the TCR, while necessary, is not sufficient to fully induce a T cell response. Co-stimulation whether positive or negative, is also required. There are various co-stimulatory molecules, some of which are constitutively expressed and others which are induced. Among the co-stimulatory molecules expressed on T cells, are CD28, ICOS, CD30, 4-1BB, which result in positive signaling, and BTLA, CTLA-4 (CD152) and PD-1 regulators, which dampen activation of T cells (Beier et al., 2007). CD28 is constitutively expressed on T cells, and interacts with CD80/CD86 on APCs. In conjunction with TCR stimulation, CD28 ligation promotes IL-4 and IL-5 production, and induces increased levels of IL-2, which enhances proliferation in an autocrine manner and induces resistance to apoptosis (Beier et al., 2007).

1.1.3 T cell effectors

The effector functions of T cells are diverse and varied: they can function as helper cells by providing various signals to enhance responses mediated by several other immune cells; they may be directly involved in eliminating pathogens by killing infected cells, and T cells can also regulate and dampen immune responses, limiting tissue damage.

1.1.3.1 CD4 T cells

Naïve CD4 T cells (Th cells) differentiate into various helper T cell subsets upon activation. After stimulation by APCs, Th cells begin to produce IL-2; as they continue to respond to activating signals, they further differentiate (Chaplin, 2010). Th1 and Th2 are the classical helper cell subtypes; a specialized subset is the follicular helper cells. Regulatory T cells (Tregs) and recently, Th17 (Steinman, 2007) and Th9 (Veldhoen et al., 2008) cells have also been described.

Th1 cells drive cell-mediated responses, via activation of mononuclear phagocytes, natural killer cells and cytolytic T cells, licensing them to kill cells infected with intracellular pathogens. They produce IFNγ and IL-2, as well as lymphotoxin (Bonilla and Oettgen, 2010; Chaplin, 2010). Naïve helper CD4 T cells differentiate into Th1 cells under the influence of IL-12, IFNγ and the T-box expressed in T cell (T-bet) transcription factor (Bonilla and Oettgen, 2010).

Th2 cells differentiate under the influence of IL-4 and the transcription factor GATA-3 (Bonilla and Oettgen, 2010). They produce IL-4, 5, 10, and 13 and are responsible for enhancing antibody production by B cells, promoting clearance of parasites and extracellular pathogens.

Follicular helper cells are memory CD4 T cells which trigger the activation of B cells and reside in the lymph nodes and spleen; they express CXCR5, which mediates their recruitment to follicles (Bonilla and Oettgen, 2010). Th17 cells are induced in response to extracellular bacteria, and serve to help recruit neutrophils (Chaplin, 2010); this cell subset is also thought to play a role in autoimmunity (Bonilla and Oettgen, 2010). Th17 cell differentiation is induced by IL-1, IL-6 and TGF-β (Chung et al., 2009), in addition to autocrine IL-21. (Korn et al., 2009) under the direction of the transcription factors RORγt and RORα (Yang et al., 2008). IL-23 is also required for the expansion and maintenance of these cells (McGeachy et al., 2009), and differentiation is prevented by IL-12 and IFNγ (Harrington et al., 2005). Th17 cells produce IL-17 (A-F), a strong proinflammatory cytokine (Harrington et al., 2005; Korn et al., 2009; Steinman, 2007), as well as IL-6, GM-CSF and TNFα. Via their cytokine profile, Th17 cells are able to suppress regulatory T cells (Pasare and Medzhitov, 2003), stimulate macrophage differentiation (Hamilton and Anderson, 2004) and induce apoptosis (Hehlgans and Pfeffer, 2005).

1.1.3.2 CD8 T cells

CD8 T cells destroy infected cells or transformed cells; they are MHC class I restricted, and thus recognize antigens derived from polypeptides present in the cytosol. Upon activation, naïve CD8 T cells differentiate into cytotoxic T lymphocytes (CTLs) and are capable of killing target cells, in a contact-dependent manner. However it must be noted that CD8 T cells have functions other than killing; they produce cytokines such as IFNg and TNF as well as chemokines which recruit and or activate macrophages, neutrophils and microbicidal effector cells (Harty and Bevan, 1999; Harty et al., 2000) and which may also have the effect of interfering with pathogen attachment or gene expression (Harty et al., 2000).

The generation of effector CTL requires multiple signals; the signaling cascades of these events have been extensively studied in mouse models of viral infections. The first of

these is the interaction between the TCR and MHC class I molecules presenting Ag. A co-stimulatory signal is required for the full activation of a CD8 T cell. The "typical" co-stimulation of CD8 T cells is the interaction between CD80 or CD86 (members of the B7 family) and CD28, which results in IL-2 production (Lenschow et al., 1996; Sharpe and Freeman, 2002). Another co-stimulatory molecule is 4-1BB, or CD137 which is expressed by activated T cells and plays a role in augmenting CTL responses during suboptimal stimulation (Williams and Bevan, 2007). The ligation of 4-1BB by T cells results in the secretion of IL-2 and the induction of cell division (Bertram et al., 2002). While the signals mediated through TCR/MHC interaction and through co-stimulatory molecules are sufficient to activate (or suppress) CD8 T cells, the development of an optimal CTL response requires a cytokine signal (Williams and Bevan, 2007). IL-12 and type 1 interferons (IFNα/β) promote the expansion and differentiation of CTLs (Mescher et al., 2006; Thompson et al., 2006). IL-12 is important in the development of CTL cytotoxicity by inducing IFNγ production (Curtsinger et al., 2003) as well as the expression of granzyme B (Curtsinger et al., 2005). The expression of various transcription factors important in CTL differentiation is also induced by, or in part by, IL-12: T-bet (Joshi et al., 2007; Takemoto et al., 2006) is crucial for differentiation of CD8 T cells into effector cells (Sullivan et al., 2003); STAT4 activation is mediated by IL-12 and IFNα/β, leading to IFNγ production (Nguyen et al., 2002); and Type 1 interferons also promote the survival of activated CD8 T cells (Marrack et al., 1999). The balance between these cytokines, IL-12 and IFNα/β, varies depending on the infection (Cousens et al., 1999; Kolumam et al., 2005). Of course, this is a rather simplified perspective, and it is more than likely that other cytokines play a role and influence the CD8 T cell-mediated response. Furthermore, much of what is known about the CD8 T cell response is due to studies in various animal viral models, and how the immune response plays out in humans in the context of pathogen clearance and autoimmune disease may not be quite the same.

After a CD8 T cell-mediated immune response, the majority of effector cells die via apoptosis, mediated through Bim (Hildeman et al., 2002; Pellegrini et al., 2003) and potentially other mechanisms. The proportion of cells that survive become memory T cells, characterized by relatively enhanced proliferation, the ability to survive for long periods of time, and increased ability to re-express effector genes (Parish and Kaech, 2009). In

addition, memory CD8 T cells express receptors for IL-7, promoting survival, and IL-15, driving antigen-independent proliferation (Williams and Bevan, 2007).

1.2 Adaptive Immunity: Humoral response

1.2.1 B cell development

B cells are derived from hematopoietic stem cells in the bone marrow. During development, they acquire antigen specificity; pro-B cells express TdT (Terminal deoxynucleotide transferase), a specialized template-independent DNA polymerase that catalyzes polymerization of deoxynucleoside triphosphates *in vitro* (Benedict et al., 2000), and RAG1 and RAG2, which are necessary for gene recombination of the B cell receptor (BCR) (Mesquita Junior et al., 2010). The BCR is an immunoglobulin, consisting of two heavy and two light chains linked by disulfide bonds. There are 5 heavy chains: α, γ, δ, ε, and μ which define the classes of immunoglobulins: IgA, IgG, IgD, IgE and IgM respectively. The light chains are kappa (κ) and lambda (λ). The heavy chain is made up of various segments: V_H, D, J_H and C_L (Bonilla and Oettgen, 2010); the segments V_H, D, and J_H are rearranged and assembled under the influence of RAG. The heavy chain complex is associated with an invariable chain and expressed on the cell surface as a pre-BCR with two peptide chains, Igα and Igβ. These peptides, which play a role in BCR trafficking and signal transduction, have an N-terminal extracellular Ig-like domain, a single trans-membrane domain and a cytoplasmic domain of 61 and 48 amino acids respectively (Defranco et al., 1995). Expression of the pre-BCR on the cell surface is followed by the rearrangement of the light chain κ; if a functional rearrangement fails, then the λ chain is rearranged. Antigen specificity is conferred by the variable regions of the heavy and light chains. The BCR of naïve B lymphocytes are IgM; co-expression of IgD marks a mature B cell (Zubler, 2001). Upon activation, the BCR undergoes class switching (Mesquita Junior et al., 2010).

Positive and negative selection follow the creation of a functional, intact BCR. Positive selection confers survival signals upon the B cells; negative selection takes place in the bone marrow. B cells that recognize self-antigen with high affinity will either undergo apoptosis or are given a second chance: RAG genes are re-activated and another light chain-VJ recombination is generated to substitute for an autoreactive combination

(Mesquita Junior et al., 2010). B cells recognizing self-antigens with weaker reactivity enter varying degrees of inactivation (i.e. anergy), but anergic cells still may be recruited in immune responses (Zubler, 2001). B cells migrating out of the bone marrow express various chemokine receptors and other molecules allowing them to migrate to specific areas within the body.

One subset of B cells, B1 in mice, migrate to the peritoneal tissues or cavities, the primary follicles of Peyer's patches, and/or to the spleen and lymph nodes. Another B cell subset, B2, migrate to the splenic marginal zone, rich in macrophages and DCs, and favoring a T-independent response (Zubler, 2001).

1.2.2 B cell activation

The BCR complex consists of the membrane immunoglobulin and two peptide chains, Igα and Igβ, which are responsible for signaling via ITAM motifs. Cross-linking of the BCR leads to clustering of the cytoplasmic domains of Igα and Igβ. Subsequent phosphorylation of the ITAM motifs mediated by src-family tyrosine kinases further recruits other tyrosine kinases (Syk or Lyn) (Defranco et al., 1995), thereby activating factors that promote the transcription of genes involved in the proliferation and differentiation of the B cell (Mesquita Junior et al., 2010). The activation of B cells can take place in one of two ways, depending on the type of antigen it interacts with. One response requires T cell help, initiated by T-dependent antigens; the other, independent of T cell help, is initiated by T-independent antigens (Bonilla and Oettgen, 2010). More recently, the role of TLRs in the activation of B cells is of increasing interest.

1.2.2.1 T-independent antigens

Some macromolecules have repeating molecular patterns and can interact with and cross-link multiple immunoglobulin receptors on B cells. The (weak) activating signals transduced by cross-linking requires additional signals from cytokines or other cell contacts, provided by DCs (Bonilla and Oettgen, 2010).

1.2.2.2 T-dependent antigens

B cells, like T cells, require more than one signal for optimal activation. B cells are activated by activated T cells which recognize the peptide presented on B cells via MHC

class II. The BCR will have recognized and captured the antigen, resulting in cross-linking of the BCR and induction of intracellular signaling, which leads to the production of additional molecules to enhance the B-T cell interaction and allows B cells to receive additional signals needed for full B cell activation (Bonilla and Oettgen, 2010). The interaction between B and T cell ultimately leads to each activating the other through the expression of various molecules and cytokines. Once activated, T cells express co-stimulatory molecules such as ICOS, which interacts with the ligand B7RP-1 (ICOS-L) (Zubler, 2001). Interactions between various other molecules expressed on B and T cells lead to further stimulation and activation. Another important interaction is that between CD40L on activated T cells and CD40 on B cells, which also leads to induction of cytokine production (Mesquita Junior et al., 2010). Overall, the T-dependent activation of B cells is complex, and is not unidirectional, but rather each cell type induces the activation of the other.

1.2.2.3 Toll-like receptor activation

TLRs are a subset of pattern recognition receptors (PRRs), usually found expressed by innate cells, but are also found in other cell types including B cells. In general, PRRs recognize PAMPs found on microbial organisms, which are foreign and absent on host cells (Janeway, 2001). TLRs are type 1 trans-membrane glycoproteins with leucine-rich repeat motifs on their extracellular domain and a Toll/interleukin-1R-interacting domain which contributes to cytoplasmic signaling via the MyD88 pathway (Booth et al., 2011), with the exception of TLR3 which mediates signaling through TRIF (Brown et al., 2011). Human B cells express moderate to high levels of TLRs 1, 6, 7, 9 and 10, and low levels of TLR 2; TLRs 3 and 4 are not found on human B cells (Booth et al., 2011). The levels of TLR found on B cells vary according to the B cell subset, thus affecting the sensitivity to TLR-mediated signaling and activation. Memory B cells, in general, express higher levels of TLR, and activation via TLR interaction with their ligands induces differentiation into Ab-producing plasma cells. On the other hand, naïve B cells express lower levels of TLR and thus respond weakly to TLR agonists (Booth et al., 2011). It has been demonstrated that activation of naïve B cells may be dependent on stimulation via TLR, and thus it has been proposed that TLR stimulation may be a required 3rd signal following BCR cross-linking and T cell help (Ruprecht and Lanzavecchia, 2006).

Following efficient activation via the TLR, B cells are able to proliferate, secrete cytokines and express co-stimulatory molecules. TLR stimulation was also shown to inhibit apoptosis (Ruprecht and Lanzavecchia, 2006). In the case of CpG, acting on TLR9, stimulation promotes BCR class-switching to IgG2a, IgG2b and IgG3, and inhibits IgG1 and IgE switching (Lin et al., 2004; Liu et al., 2003). The secretion of immunoglobulins and cytokines requires additional signals concurrent to the CpG-induced signals. BCR cross-linking, CD40 ligation and factors such as BAFF and APRIL contribute to Ig secretion, whereas secretion of IL-6, IL-12 or IL-10 requires signals from CD40L or DCs (Booth et al., 2011).

Activation of B cells can occur in various ways, stimulation leading to different signaling pathways resulting in the expression of various cytokines and molecules important to mediating an immune response. Upon activation, B cells can either respond "directly" via antibody production, or also have a role in cytokine production which can modulate the immune response mediated by other immune cells.

1.2.3 B cell responses

The primary B cell response, relatively slow to develop, involves the activation of naïve B cells and their differentiation into either short-lived plasma blasts which produce antibodies, or into memory cells. Upon activation, B cells go down one of two pathways; they either further differentiation into short-lived antibody-producing plasma blasts, or enter follicles and establish a germinal center where they undergo class-switching of the BCR (Bonilla and Oettgen, 2010). The primary response is dominated by the production of IgM Ab with relatively low affinity for Ag; other Ab classes appear later and show higher affinity for the cognate antigen (Bonilla and Oettgen, 2010). In the germinal center, the BCR changes from IgM or IgD to IgG, IgA or IgE. The gene arrangement involved in class switching is analogous to TCR and BCR rearrangement and involves genomic DNA rearrangement and alternative RNA splicing (Bonilla and Oettgen, 2010; Mesquita Junior et al., 2010). The variable regions of the immunoglobulin remain the same, so that the specificity for antigen does not change. It is the constant portion of the heavy chain which is replaced by one of the other chains, thereby diversifying the immune response since the different Ig classes each have a different function (Mesquita Junior et al., 2010). Class

switching and the resulting heavy chain is under cytokine influence. Various cytokines induce a switch to IgG (Zubler, 2001), IL-4 and IL-13 induce a switch to IgE, which is inhibited by IFNγ, and IL-10 and TGFβ have been shown to promote IgA (Bonilla and Oettgen, 2010).

In addition to producing antibodies, B cells have a role in cytokine production. For instance, B cells produce IL-10, which is primarily anti-inflammatory, but can be pro-inflammatory under some conditions (Nikolajczyk, 2010). Activated B cells produce interleukins, TNF, macrophage inflammatory protein (MIP)1α and MIP1β. These factors modulate the migration of DC, activate macrophages, regulate T cell functions and also provide feedback signals further stimulating B cells (Dalakas, 2008).

Memory B cells can be of two sorts: the "conventional" memory B cells, or memory plasma cells (Yoshida et al., 2010). The former require restimulation with antigen, while plasma cells secrete specific antibodies for an extended time, long after the initial infection has been cleared, and persist independently of antigenic stimulation (Yoshida et al., 2010). The level of antigen required to elicit a response from memory B cells is lower than that for the response mediated by naïve B cells. In addition, the immune response mediated by memory B cells is faster, and antibody production reaches higher levels than the primary response (Mesquita Junior et al., 2010). While plasma cells depend on signals from BAFF and APRIL (see below), memory B cells require expression of a functional BCR complex, capable of mediating signaling (Yoshida et al., 2010). Interestingly, memory B cells can also be maintained via Epstein-Barr virus infection; the expression of viral receptors mimics regular activation signals, by-passing the direct need for BCR signaling (Yoshida et al., 2010).

B lymphocyte activator of the TNF family, or BAFF, is a potent stimulator of B cell maturation and survival. It is expressed by activated macrophages and DCs (Zubler, 2001) in membrane-bound or soluble form (Schneider et al., 1999; Thangarajh et al., 2004) and interacts with transmembrane activator and CamL interactor (TACI), expressed on activated B cells. TACI has been shown to have a role in B cell homeostasis in addition to being a key mediator of class switching (Castigli et al., 2005b; Seshasayee et al., 2003). TACI is expressed on mature B cells and plasma cells (Hsu et al., 2002), and mutations are associated with IgA deficiency (Castigli et al., 2005a). APRIL (a proliferation-inducing ligand) shares properties and structural homology with BAFF (Schneider et al., 1999;

Thangarajh et al., 2004), also interacts with TACI, and is expressed on a range of leukocytes (Bonilla and Oettgen, 2010; Zubler, 2001). BAFF and APRIL have been implicated in the pathogenesis of various autoimmune diseases including multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis.

2.0 INTERLEUKIN-15

Interleukin-15 (IL-15) is a pleiotropic cytokine, expressed (at the mRNA level) in many tissues including kidney, lung, heart, skeletal muscle (Grabstein et al., 1994), and by various cell types under stimulatory conditions (Blauvelt et al., 1996; Jonuleit et al., 1997; Leclercq et al., 1996; Mohamadzadeh et al., 1995; Reinecker et al., 1996). However, in spite of this, the actual protein expression is not as widespread as the mRNA would indicate, and in fact translation is tightly regulated (Fehniger and Caligiuri, 2001). IL-15 is a member of the four α-helix family of cytokines which also include IL-2, IL-3, IL-6, IL-7, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Bazan, 1990a; Bazan, 1990b).

IL-15 was independently discovered by two groups based on its ability to mimic the effects of IL-2 *in vitro* (reviewed by (Waldmann, 2006)); however, *in vivo*, the effects of these two cytokines are very different (Fehniger and Caligiuri, 2001) demonstrated by using mice deficient in IL-15 (Kennedy et al., 2000) or IL-15Rα (Lodolce et al., 1998). The IL-15 and IL-2 receptors each have a unique alpha chain (Rα) which allows for the binding of the cytokine with high affinity. IL-2Rα expression is restricted to T cells for the most part, but is also expressed by DC (Wuest et al., 2011), while IL-15Rα overlaps with IL-15 in its expression at the mRNA level, and is found in almost every cell and tissue type (Anderson et al., 1995a; Giri et al., 1995). However, unlike the cytokine, IL-15Rα protein expression is not under similar regulation, and seems to parallel the transcript expression (Anderson et al., 1995b; Giri et al., 1995; Stonier et al., 2008). Interestingly, a pool of intracellular IL-15 is present in monocytes/macrophages, which is ready to be expressed on the surface as needed and independently of new protein synthesis (Neely et al., 2001). Signaling is mediated via a shared β chain (IL-2/15Rβ or CD122) (Bamford et al., 1994; Carson et al., 1994) and the common γ chain, also shared by IL-2, IL-4, IL-7, IL-9 and IL-21 (Stonier and Schluns, 2010).

2.1 Structure of IL-15 and IL-15Rα

The IL-15 gene (Fig.1) contains 9 exons, 7 of which are coding exons (Fehniger and Caligiuri, 2001). An alternative exon has also been described, encoding for an alternative leader peptide, between exons 4 and 5 (Nishimura et al., 1998; Onu et al., 1997; Tagaya et al., 1997). The original IL-15 encodes for an IL-15 precursor protein with a long (48-amino acid (AA)) leader peptide (LSP) and a 114-AA mature protein; the alternate sequence encodes for a short signal peptide (SSP) of 21-AA (Fehniger and Caligiuri, 2001). Both isoforms of IL-15 encode identical IL-15 protein, the only difference being in the signal sequence, and the localization of the final products: the LSP is secreted from the cell and/or placed into the cell membrane (Kurys et al., 2000; Onu et al., 1997), and the SSP remains localized to the cytoplasmic and nuclear compartments (Waldmann and Tagaya, 1999). The mature IL-15 protein is encoded by exons 5 to 8 of the IL-15 gene (Anderson et al., 1995b; Krause et al., 1996) and contains two disulfide bonds, one of which is homologous to that of IL-2 (Fehniger and Caligiuri, 2001). IL-15 has been predicted to have "helical moments" at four different sites in the protein (at AA 1-15, 18-57, 65-78 and 97-114), supporting the model of a four α-helix bundle structure (Fehniger and Caligiuri, 2001).

IL-15Rα is a type-1 trans-membrane protein with eight isoforms (Waldmann and Tagaya, 1999) and has been shown to be highly homologous to the IL-2Rα subunit (Anderson et al., 1995a; Giri et al., 1995). Both contain a short cytoplasmic tail, a transmembrane domain, a hinge region and a sushi domain (most isoforms), which is required for cytokine binding (Anderson et al., 1995a; Giri et al., 1995; Waldmann, 2006). However, in contrast to IL-2Rα which has low affinity for IL-2 in the absence of IL-2Rβγ, IL-15Rα binds its cytokine with high-affinity, without the need for the rest of the receptor complex (Fehniger and Caligiuri, 2001). Furthermore, IL-15Rα is capable of reverse signaling; it was demonstrated to mediate a non-proliferative signal in the absence of IL-2/15Rβ (Stevens et al., 1997) and hyper cross-linking of surface IL-15 resulted in activation of various signaling pathways including ERK 1/2 and MAPK (Neely et al., 2004). However, for signaling to occur, the Rα chain is not absolutely necessary: IL-15 may bind to IL-2/15Rβγ with intermediate affinity, in the absence of IL-15Rα (Giri et al., 1994).

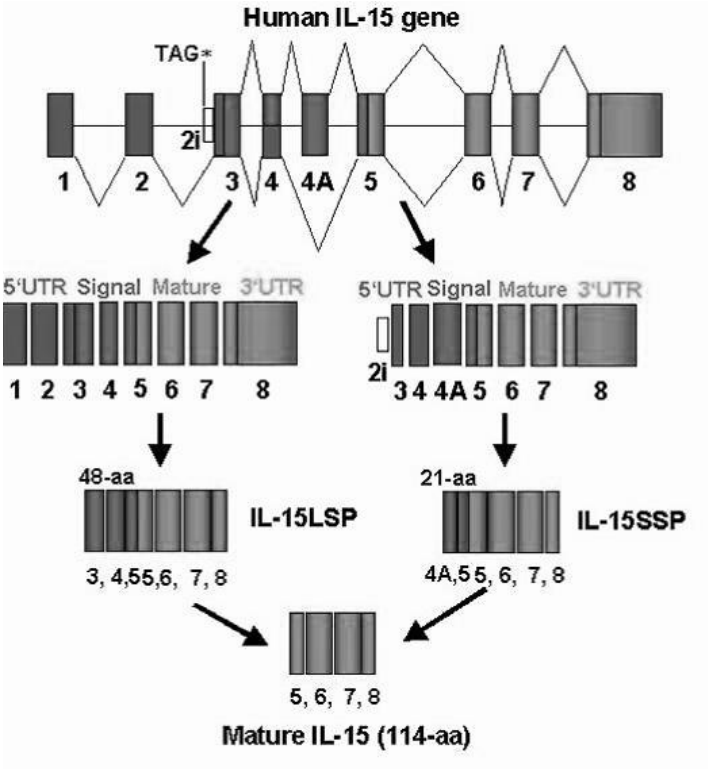


Fig.1: Diagram of IL-15 gene. IL-15 has two isoforms due to an alternate exon (4A) resulting in a short (21-AA) signal peptide (IL-15SSP) rather than the long (48-AA) signal peptide (IL-15LSP). Adapted from (Bulfone-Paus et al., 2006).

2.2 Signal transduction

The IL-15R complex signals through Jak1 and 3 as well as signal transducer and activator of transcription (STAT) 3 and 5 (Giri et al., 1995; Waldmann et al., 1998). IL-15 was shown to activate the transcription factor NF-κB in neutrophils but not AP-1, while IL-15 stimulation of whole human peripheral blood lymphocytes activated both (Fehniger and Caligiuri, 2001). Additional signaling pathways may include *src*-related tyrosine kinases and through the Ras/Raf/MAPK pathway leading to fos/jun activation (McInnes and Gracie, 2004). However, it must be noted that signaling related to IL-15 does not occur in the conventional manner as with other cytokines.

A study in which T cell proliferation mediated by IL-15 did not require IL-15Rα expression on the responding T cells but rather required IL-15Rα expression by surrounding cells (Lodolce et al., 1998) was the first indication of a unique manner by

which IL-15 signaling is mediated. As the direct effect of IL-15 in inducing T cell proliferation had already been established, it was unlikely their observations were due to an indirect effect mediated by IL-15 (Stonier and Schluns, 2010). Another group soon proposed a mechanism of IL-15 trans-presentation (depicted in Fig. 2), demonstrating a prolonged effect on T cells by IL-15 relative to IL-2, despite IL-15 withdrawal from the media (Dubois et al., 2002). This was demonstrated to be mediated by the strong association of IL-15 with IL-15Rα, allowing the receptor alpha chain to maintain IL-15 on the cell surface and enhance the persistence of IL-15 signaling (Dubois et al., 2002). Furthermore, IL-15 and IL-15Rα were shown to associate intracellularly and were followed from the endoplasmic reticulum to the cell surface (Dubois et al., 2002).

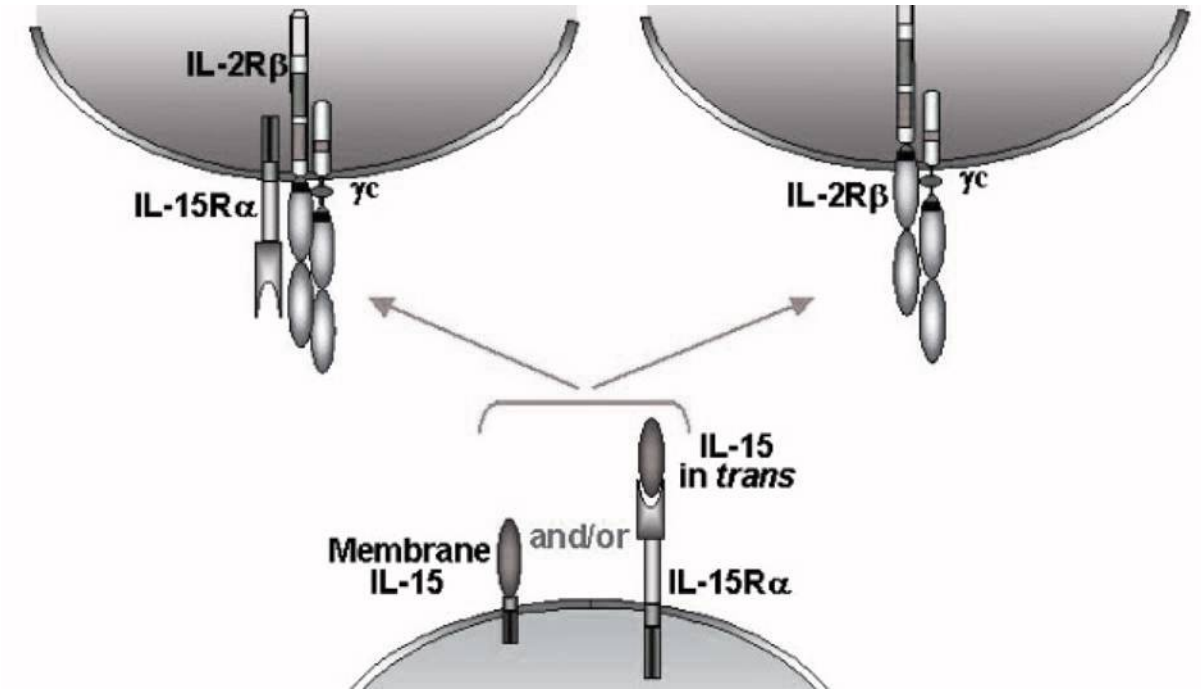


Fig.2: Trans-presentation of IL-15. IL-15/IL-15Rα-expressing cell presents IL-15 in *trans* to effector cells expressing either IL-15Rβγ or IL-15Rαβγ. Adapted from (Bulfone-Paus et al., 2006)

2.2.1 Regulation of trans-presentation

At the most basic level, trans-presentation requires IL-15 and IL-15Rα to be transcribed in the same cell. However, it is not yet known if up-regulation of transcription will be sufficient to increase the IL-15 being trans-presented (Stonier and Schluns, 2010). It is known that there are several mechanisms in place at the post-transcriptional level

(discussed later) which would limit the availability of IL-15 for trans-presentation. Similar mechanisms regulating IL-15Rα have not been identified, which perhaps explains the higher prevalence of IL-15Rα compared to IL-15. Another level of regulation after limited protein expression is the transport of IL-15 to the cell surface. It has been reported that IL-15Rα is required for IL-15 to be transported from the endoplasmic reticulum to the cell surface (Duitman et al., 2008). Furthermore, IL-15Rα bound to IL-15 can be internalized and later return to the surface (Dubois et al., 2002) which may promote the need for regulating cytokine production, or may be an additional level of control, to limit the availability of IL-15 as needed.

Another factor to consider limiting the availability of IL-15 to cells could simply be cellular localization. For trans-presentation to occur, a responsive cell must be in close proximity to the cell presenting IL-15. It is thus highly probable that rather than randomly encountering IL-15 presenting cells, responsive cells would be directed to the source (Stonier and Schluns, 2010).

2.3 Expression of IL-15: regulation

IL-15 mRNA is found in many cell types, but protein expression is limited and regulated through multiple mechanisms. IL-15 production is regulated at both the transcriptional and posttranscriptional levels. Several binding sites for transcription factors were identified in the promoter regions of human and murine IL-15 (Fehniger and Caligiuri, 2001). There are three main "checkpoints" which regulate translation of IL-15 mRNA (Fehniger and Caligiuri, 2001): first, the existence of multiple start codons in the 5' untranslated region (UTR); the LSP and SSP themselves; and negative regulation near the C terminus of the protein precursor.

The LSP IL-15 5' UTR is relatively long and contains multiple AUGs upstream of the translation start site, which impedes translational efficiency (Bamford et al., 1996; Fehniger and Caligiuri, 2001). The removal of most of the upstream AUG sequences in the 5' UTR resulted in a 5- to 10-fold increase in IL-15 protein expression (Bamford et al., 1996). It was also demonstrated that LSP IL-15 is translated with lower efficiency than SSP IL-15 (Tagaya et al., 1997); also considering the localization of each of the two isoforms, we see a tight regulation of protein expression. SSP IL-15 is more efficiently

translated, but is localized to the nucleus and cytoplasmic regions, and is not secreted. Not only is LSP IL-15 is less efficiently translated, but also is secreted from the cell and/or inserted into the cell membrane at low levels (Fehniger and Caligiuri, 2001; Kurys et al., 2000). Finally, it was suggested that a signal in the C terminus of the mature IL-15 protein is responsible for inefficient secretion (Fehniger and Caligiuri, 2001); since the activity of a LSP-IL-15-GFP construct was significantly higher than that of LSP-IL-15 (approximately 10 times more labeled IL-15 was secreted), the authors suggested the GFP tag masked a retention signal in the C terminus (Gaggero et al., 1999).

If all three of these "checkpoints" are removed – removing upstream AUG sequences, replacing IL-15 leader peptide with that of IL-2 and changing the C terminus end of the protein, synthesis of IL-15 increased by 250-fold (Bamford et al., 1998). The extent to which the expression of IL-15 is regulated indicates that overproduction would be dangerous. There is some evidence to support this: transgenic mice over-expressing IL-15 develop a fatal lymphocytic leukemia (Fehniger et al., 2001), and IL-15 is consistently upregulated in leukemic cells of childhood acute lymphoblastic leukemia patients with CNS involvement (Cario et al., 2007).

2.4 The role and effect of IL-15

IL-15 is important in the development, homeostasis and activity of NK cells and γδ T cells, and plays a role in the survival and expansion of naïve and memory CD8 T cells. It also has an effect on other immune cells, as well as on non-immune cells such as fibroblasts, epithelial and endothelial cells, and neuronal and glial cells.

IL-15 is important in the survival and expansion of naïve and memory CD8 T cells (Alves et al., 2003; Berard et al., 2003); homeostatic proliferation in response to IL-15 allows for the self-renewal and maintenance of memory CD8 T cells over long periods of time (Becker et al., 2002; Goldrath et al., 2002). Mice deficient in IL-15 or IL-15Rα have a marked decrease in the number of memory CD8 T cells and, to a lesser extent, in the number of naïve CD8 T cells, but have normal levels of CD4 and B cells (Kennedy et al., 2000; Lodolce et al., 1998); IL-15Rα deficient mice are, in addition, impaired in thymic CD8 T cell development (Lodolce et al., 1998).

Naïve T cells can proliferate in response to high concentrations of IL-2 and IL-15, as long as they also receive a signal via the TCR through MHC recognition (Cho et al., 2007; Ramsey et al., 2008). Also supporting a role for IL-15 in homeostatic proliferation, mice deficient in IL-15 have about half the number of naïve CD8 T cells relative to wild type mice (Berard et al., 2003; Ramanathan et al., 2006). Involved with the regulation of the naïve T cell response to IL-15 is SOCS-1, which is up-regulated in response to IL-15 (among other cytokines) (Yoshimura et al., 2007). Deficiency in SOCS1 increases the sensitivity of double positive thymocytes to both IL-7 and IL-15 (Chong et al., 2003; Ramanathan et al., 2006) and results in mature CD8 T cells that are hyper-sensitive to IL-15 (Davey et al., 2005; Ramanathan et al., 2006). Mice deficient in SOCS1 (restricted to T cells) have CD8 T cells of primarily a memory phenotype (Davey et al., 2005). Overall, as a regulator of the cellular response to IL-15, SOCS1 might be involved in controlling antigen-independent activation of naïve CD8 T cells under inflammatory conditions, to prevent expansion of potentially autoreactive CD8 T cells (Ramanathan et al., 2008).

Along with IL-7, IL-15 has a crucial role in the MHC-independent homeostasis of memory CD8 T cells (Surh and Sprent, 2008). While the expansion of memory CD8 T cells under the influence of IL-7 occurs only in lymphopenic hosts, IL-15 is able to support and favor the generation of memory CD8 T cells in mice with an intact T cell compartment (Surh and Sprent, 2008). One of the first indications that cytokines were involved in the maintenance of memory T cells, the injection of adjuvants resulted in an increase in the proliferation of memory CD8 T cells (Surh and Sprent, 2008). Further investigation showed that such adjuvants (e.g. LPS or PolyI:C) stimulated the production of type-1 interferons, which promoted the synthesis of IL-15 on APCs; this IL-15 was shown to act on memory CD8 T cells and enhance the rate of homeostatic proliferation (Zhang et al., 1998). Administering IL-15 to IL-15 deficient mice increased the proportion and number of CD8 memory T cells in the spleen and lymph nodes (Berard et al., 2003), and further outlining a crucial role for IL-15, memory CD8 T cells transferred into IL-15 deficient mice failed to proliferate and died rapidly (Judge et al., 2002).

2.5 IL-15 in disease

Activation of CD8 T cells, while beneficial in protective immune responses, can also contribute to chronic inflammatory disorders as well as to the progression of autoimmune diseases due to their ability to directly kill target cells. CD8 T cells have been implicated in various autoimmune diseases (reviewed by (Walter and Santamaria, 2005). IL-15, being one of the cytokines involved in the homeostatic maintenance of CD8 T cells, is an ideal candidate for potentially aberrant CD8 T cell activation. This cytokine facilitates the maintenance and triggering of autoreactive CD8 T cells (Itsumi et al., 2009; Peng et al., 2006; Ramanathan et al., 2011) and plays a crucial role in CD8 T cell migration and survival in organs (McGill et al., 2010; Verbist et al., 2011). Indeed, IL-15 has been implicated in various diseases, including those in which CD8 T cells have also been implicated: rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), type 1 diabetes, psoriasis, as well as various CNS disorders (Davey et al., 2005; Liu et al., 2000; McInnes and Gracie, 2004; McInnes and Liew, 1998; Ramanathan et al., 2011; Rentzos and Rombos, 2011; Sakai et al., 1998). Furthermore, due to its ability to activate T cells, IL-15 is also under investigation regarding its potential in anti-tumor immunotherapy (Hanson et al., 2000; Jakobisiak et al., 2011; Klebanoff et al., 2011) and as a factor to improve vaccine efficacy (Hu et al., 2009; Huntington et al., 2011; Yu et al., 2011).

2.5.1 Rheumatoid Arthritis

IL-15 protein has been detected in synovium and in RA joint lesions (Thurkow et al., 1997). Synovial fluids were demonstrated to promote the migration and activation of T cells, which was partially inhibited by the addition of antibodies antagonistic to IL-15 (McInnes et al., 1996). Furthermore, IL-15 activated T cells from RA patients were able to stimulate macrophage cell lines as well as primary monocytes and macrophages to produce TNFα *in vitro*. Phase II clinical trials using an anti-IL-15 human monoclonal antibody (AMG 714) have been completed (NCT00433875, http://www.clinicaltrials.gov), and the same antibody is also being currently tested for psoriasis in a phase II trial (NCT00443326).

2.5.2 Inflammatory Bowel Diseases

IL-15 mRNA has been detected in macrophages and epithelial cells from patients with active IBD and has been detected at the protein level in the supernatants of rectal mucosal biopsy samples (Sakai et al., 1998). Mention *et al.* showed that IL-15 was not secreted, but rather was presented at the surface of enterocytes in celiac disease (Mention et al., 2003). In addition, T cells isolated from the lamina propria of IBD patients showed a greater responsiveness to IL-15 as compared to controls, not only producing IFNγ and TNF in response to IL-15 stimulation, but also inducing the production of pro-inflammatory cytokines in monocytes *in vitro* (Liu et al., 2000). In conjunction with retinoic acid, IL-15 induced the production of IL-12p70 and IL-23 by DC (DePaolo et al., 2011). Not only does the elevated levels of IL-15 seem to induce the production of various pro-inflammatory cytokines, but also is able to stimulate CD8 T cells to be cytotoxic. Studies have shown increased induction of NKG2D in response to elevated levels of IL-15 (Meresse et al., 2004; Roberts et al., 2001), and that NKG2D serves as a co-stimulator for CTL (Roberts et al., 2001), activating CTL functions independently of TCR specificity(Meresse et al., 2004).

2.5.3 Type 1/autoimmune diabetes

Serum IL-15 has been detected in patients with type 1 diabetes and levels were significantly higher as compared to controls (Kuczynski et al., 2005). In a mouse model of autoimmune diabetes, it was shown that stimulation with IL-15 and IL-21 enabled CD8 T cells to respond to weak antigens, inducing proliferation, cytokine secretion and cytolytic activity (Ramanathan et al., 2011). Furthermore, increased sensitivity to IL-15 via SOCS1 deficiency can result in the activation of autoreactive CD8 T cells in a model of type 1 diabetes (Davey et al., 2005).

2.5.4 CNS disorders

IL-15 has been demonstrated to be elevated in various CNS disorders: patients with Alzheimer's disease or frontotemporal dementia had higher levels of IL-15 detected in their cerebrospinal fluid (CSF) as compared to patients with non-inflammatory neurological disease (Rentzos et al., 2006). An increase in circulating IL-15 was detected in levadopa-treated patients with advanced Parkinson's disease and functional impairment

(Gangemi et al., 2003). In multiple sclerosis (MS), which not only involves the CNS, but is also an autoimmune disease, IL-15 and IL-15Rα have been detected at the mRNA level in the CNS (Hanisch et al., 1997; Kurowska et al., 2002; Lee et al., 1996), and at the protein level, expressed on the surface of monocytes from MS patients (Vaknin-Dembinsky et al., 2008).

A role for IL-15 in CNS disorders is further indicated upon examination of various animal models. The neuronal localization of IL-15 and IL-15Rα has been demonstrated in mice (Wu et al., 2010), and is expressed in the cerebellum and hippocampus(Gómez-Nicola et al., 2008a). IL-15 seems to be mainly expressed by astrocytes and microglia (Gómez-Nicola et al., 2008b), but has also been described to be expressed in some projection neurons (Gómez-Nicola et al., 2008a). In a mouse model of facial nerve axotomy, fewer T cells and activated microglia were found in IL-15 or IL-15Rα knockout mice than in wild type mice (Wu et al., 2010). This cytokine seems to play a role in the development of early inflammatory events. In a model of spinal cord and sciatic nerve constriction injury, IL-15 was expressed by astroglial and microglial cells in the spinal cord and acted as a cue for macrophage and T cell activation and infiltration (Gómez-Nicola et al., 2008b). Under inflammatory conditions, as demonstrated using a model of acute inflammatory injury using LPS injection, astrocytes and microglia expressed elevated levels of IL-15 (Gómez-Nicola et al., 2008a).

The role of IL-15 in MS pathogenesis is of specific interest, when the involvement of CD8 T cells is also considered, and warrants further investigation.

3.0 MULTIPLE SCLEROSIS

Multiple Sclerosis is considered to be a T cell-mediated autoimmune disease of the central nervous system, where myelin-specific T cells enter the CNS and cause damage in genetically susceptible individuals. The disease is characterized by the infiltration of various immune cells and their presence in and around lesions (Frohman et al., 2006), demyelination, and axonal injury (Lassmann et al., 2001; Trapp et al., 1998). While under “normal” conditions the CNS retains its immune privileged status, inflammatory processes breach the integrity of the blood-brain barrier thereby allowing entry of immune cells into the CNS.

The majority of cases (80-90%) of MS begin with a relapsing-remitting disease course (RRMS). As the disease progresses, the number of relapses decreases but patients enter a secondary progressive phase (SPMS) characterized by a decrease in inflammation but an increase in disability. A fraction of patients with MS do not have relapses, but rather have a primary progressive course of disease (PPMS) (Hemmer et al., 2002). In patients with RRMS, acute CNS lesions are commonly detected (Hemmer et al., 2002) characterized by perivascular infiltration by lymphocytes, demyelination, glial cell activation, axonal damage and the presence of myelin-laden macrophages (Frohman et al., 2006). In PPMS or SPMS, inflammatory activity is not as prominent as in RRMS, but rather brain atrophy is dominant and correlates with disability (Hemmer et al., 2002).

The inflammatory processes involved in multiple sclerosis cannot be described in a linear fashion, beginning with a triggering event followed by the response from the immune system subsequently leading to damage. The initial events or the factors involved leading to the development of multiple sclerosis still remains an enigma; there are various perspectives regarding this question: whether the causes are genetic (Hillert and Olerup, 1993; International Multiple Sclerosis Genetics Consortium, 2011), environmental (Gale and Martyn, 1995), viral (Kakalacheva et al., 2010; Santon et al., 2011; Sibley et al., 1985) or due to vitamin D levels (Albert et al., 2009; Smolders et al., 2009). However, while we do not know as yet the initiating events for MS, we can indirectly observe what is going on during disease progression through the examination of factors and cells present in the blood and CSF of patients, in post-mortem tissue, or by using animal models.

3.1 Animal Models of MS

Multiple sclerosis is a human disease, of unknown etiology. Furthermore, it is quite complex, involving contradictory processes: attacks and remission, demyelination and remyelination, inflammation and suppression of inflammation, all leading to progressive disability in patients. While no animal model can mimic all features of the disease, they are still useful in allowing us to learn about and understand various aspects of disease and the contributions from the immune system and CNS in neuroinflammation. Indeed, thus far they have contributed much to our understanding of some of the basic processes in MS. There are two main types of models (Pachner, 2011): autoimmune and virus-induced (although viral models also have autoimmune responses).

3.1.1 Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE), ironically, is an animal model developed based on observations in humans. In some instances of patients receiving Louis Pasteur's rabies vaccine (Pachner, 2011), patients developed an ascending paralysis from the lower limbs to the upper limbs and neck, eventually affecting swallowing and breathing. In milder cases, paralysis was mild and recovery rapid (Baxter, 2007). Later investigation using brain extracts and rhesus monkeys presented the first instances of vaccine-induced encephalomyelitis (Baxter, 2007; Pachner, 2011). Following these first experiments, other groups boosted the immune response by injecting brain emulsions mixed with paraffin oil and *Mycobacterium tuberculosis*, an adjuvant described by Freund (Baxter, 2007). The resulting EAE could now be consistently induced in animal models. Depending on the animal used, the clinical signs and disease pathology will vary (Baxter, 2007).

Rats were the animal of choice in MS research (Croxford et al., 2010); the disease is consistent and rather straightforward to induce in Lewis rats. EAE is characterized by acute onset of disease followed by spontaneous recovery, resembling the relapses and remissions in MS. However, disease is localized to the spinal cord, and no demyelination is present (Croxford et al., 2010). Over the years, mice became a more common choice for induction of EAE and MS studies. It could simply have been a matter of what tools were available; more reagents were generated for use in mice than in rats, antibodies for use in flow cytometry were generated against mouse (and human) antigens, and, finally, the existence of various inbred genetic backgrounds and knockouts in mice made it possible to study the effects and involvement of specific factors in disease (Croxford et al., 2010), while minimizing variability in disease between groups (Pachner, 2011).

3.1.1.1 Characteristics of EAE

The disease course of EAE can be acute, chronic, or relapsing-remitting, depending on the genetic background, immunogen and the use of pertussis toxin (Stromnes and Goverman, 2006a). In general, myelin-specific CD4 T cells are activated in the periphery whereupon they cross the blood-brain barrier (BBB) into the CNS. APC present in the CNS re-activate infiltrating T cells via peptide presentation on MHC class II with co-stimulatory molecules thus initiating an immune response perpetuating inflammation and

damage (Stromnes and Goverman, 2006a). Events in EAE include the secretion of chemokines which recruit macrophages to sites of T cell activation, of pro-inflammatory molecules such as TNFα and IL-1, important in inflammation and CNS tissue damage (Stromnes and Goverman, 2006a). Some other features of EAE, also seen in MS, are destruction of the myelin sheaths, the presence of CNS lesions (more in the brain stem and spinal cord of EAE models), axonal injury, the infiltration of immune cells and the presence of immunoglobulin in the CNS and CSF (Baxter, 2007).

In rodent models of EAE, disease is typically characterized by an ascending paralysis, beginning with a limp tail and progressing to the hind and forelimbs. Disease course is typically scored on a scale from 0 to 5. There is no one universal scale for scoring EAE, but typically main "points" in disease are a limp tail, ataxia, hind limb weakness and/or paralysis in one or both limbs, forelimb weakness and/or paralysis and finally, a moribund state. Variations to the classical EAE disease course do occur (Muller et al., 2000), and is termed atypical EAE. Atypical disease usually involves active induction of EAE, but it has been observed in the adoptive transfer of wild-type or IFNγ deficient T cells (Stromnes and Goverman, 2006b). While in classical EAE, symptoms are indicative of inflammation and damage localized to the spinal cord, inflammation is localized more in the brain in atypical EAE (Muller et al., 2000; Stromnes and Goverman, 2006b).

3.1.1.2 Induction: Active vs. Passive EAE

Most protocols for the induction of EAE use purified encephalitogens, including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG); recombinant myelin proteins; or synthetic peptides derived from myelin proteins (Pachner, 2011; Stromnes and Goverman, 2006a).

Active EAE is induced via immunisation of susceptible rats or mice with CNS protein in complete Freund's adjuvant, with or without simultaneous injection of pertussis toxin, depending on the strain, to promote opening of the BBB. It can take from 9-20 days, typically, for symptoms to begin to manifest (Stromnes and Goverman, 2006a).

Passive EAE involves the adoptive transfer of immune cells from immunized animals to naïve hosts. Transferred cells can be lymph node cells, T cells, or even specific subsets of cells, all of which are sufficient to induce disease (Stromnes and Goverman, 2006b). In passive EAE, cells can be labelled before transfer, so that localization and

activity can be monitored. Furthermore, the effector functions can be manipulated via pre-stimulation *in vitro* before transfer, so that the relevance of a particular cell subset may be examined in the disease pathogenesis (Stromnes and Goverman, 2006b). Most commonly, passive EAE models involve the transfer of CD4 T cells; fewer models involve transfer of CD8 T cells (Wekerle, 2008).

3.1.1.3 Transgenic mouse models

Transgenic mouse models are useful in that they can help expose and elucidate the potential role of components of the immune system in triggering disease induction. Often, these models involve constitutive expression of a molecule, or myelin-specific TCRs, resulting in spontaneous disease development.

One such model resulting in demyelination in mice, involves the constitutive expression of B7.2 (CD86), a co-stimulator of T cells, on microglia. This seems to induce the infiltration of the CNS by primarily CD8 memory-effector T cells, as well as CD4 T cells. Transfer of T cells into T cell-deficient B7.2 transgenic hosts induces disease, implying a role for the co-stimulatory signal on T cells in disease induction and development (Zehntner et al., 2003).

Another model expresses a myelin-specific T cell receptor towards MOG. While it would be expected that disease spontaneously develops in such mice, interestingly disease develops in MOG-deficient mice. Upon further examination, it was found that the MOG-specific TCR also recognizes neurofilament-M, a neuronal cytoskeletal protein (Krishnamoorthy et al., 2009). The authors suggest that cumulative responses – reactivity of specific T cells to more than one antigen – could result in spontaneous development of autoimmune disease in humans.

3.1.1.4 Limitations of EAE

Of course, being only a model for disease, there are some limitations for EAE as a model of MS. As the etiology of MS is currently unknown, EAE cannot be used to study potentially triggering events in the pathogenesis of MS especially as it is induced by immunization with myelin antigens or via adoptive transfer. Furthermore, there are no models which spontaneously develop disease except transgenic mice. Another factor to consider is that the myelin peptides used to initiate disease may be different from those actually inducing disease in multiple sclerosis and so the factors seemingly involved in

disease pathogenesis may not be relevant to the human disease. Lastly, the active induction of EAE is skewed towards a CD4 T cell response; however, increasing evidence shows that other immune cells play an important role in disease pathogenesis as well, including CD8 T cells, B cells, and various innate immune cells. However, despite limitations of the model, it has been useful thus far in understanding inflammation in the CNS not only as it relates to MS, but also to other inflammatory conditions in the CNS, and will continue to be so.

3.2 Multiple Sclerosis and the Immune System

3.2.1 The immune system and the CNS

The CNS is traditionally seen as an immune-privileged site, due to the existence of the BBB, a barrier made up of tight junctions between brain endothelial cells. The BBB serves to restrict the entry of solutes and ions (Carson et al., 2006) as well as immune cells (Hawkins and Davis, 2005; Mrass and Weninger, 2006) into the CNS. However, the selectivity of the BBB does not necessarily mean it is completely exclusive; cell migration occurs at the post-capillary venules, and is controlled by adhesion molecules, cytokines and chemokines and their corresponding receptors (Owens et al., 2008).

In general the CNS creates an immunosuppressive environment: various neuropeptides and transmitters in addition to various neuronal membrane proteins such as CX3CL1 (fractalkine), intracellular adhesion molecule (ICAM)-5, neural cell adhesion molecule (NCAM) and semaphorins all contribute to regulating inflammation (Tian et al., 2009), and the presence of factors such as transforming growth factor-1 in the microenvironment created by neurons favor the differentiation of regulatory T cells (Amor et al., 2010). Furthermore, the CNS is capable of defending itself against immune infiltration: astrocytes can suppress both Th1 and Th2 cell activation and are capable of causing T cell apoptosis (Bechmann et al., 2002), and it has also been shown that neurons, too, can induce T cell apoptosis (Tian et al., 2009). In addition, the basal expression level of MHC molecules in the CNS is quite low, thus contributing to limiting the autoimmune response against CNS cells.

The infiltration of immune cells occurs during neuroinflammation upon the release of inflammatory mediators by activated microglia which increase BBB permeability

(Popescu et al., 2009). CD4 T cells have been observed in the CNS of MS patients, and CD8 T cells have been shown to be in close contact with neurons, indicating that neuronal damage is mediated by cytotoxic CD8 T cells (Neumann et al., 2002). Neurons can also promote immune activation via the secretion of chemokines, complement factors, and damage-associated molecular pattern molecules (Amor et al., 2010). The presence of T cells in the brain also affects glial function, with Th1 and Th17 cells increasing microglial activation (McQuillan et al., 2010; Murphy et al., 2010). However, not all immune responses within the CNS are detrimental; they can also aid in repair and regeneration.

Microglia play an important role in promoting repair; for instance, they clear debris after myelin damage, and impeding this process results in delayed regeneration (Neumann et al., 2009). Interestingly, it would seem that T cells can also aid in recovery and have a role in repair and protection, although the specific mechanisms of such effects are as yet unclear (Hohlfeld, 2008; Schwartz et al., 2009). For instance, T cell responses include the production of neuroprotective factors such as brain-derived neurotrophic factor (Hohlfeld, 2008; Kerschensteiner et al., 2003). Additionally, far from being only destructive, it has been shown that autoimmune T cells can also aid in the repair of the CNS (Hofstetter et al., 2003; Nielsen et al., 2009).

3.2.2 Inflammation in MS

While MS has long been considered to be a T-cell mediated autoimmune disease, there are, of course, various other factors also at play. Not only are T cells involved, but also B cells, innate immune cells, and the cells of the CNS are all involved in modulating the immune reactions taking place.

3.2.2.1 Innate immunity

Microglia are the resident immune cells of the CNS. In their resting state, they have a ramified structure and express low levels of immunological molecules. However, while in a ‘resting’ state, they are continuously monitoring a defined area within the CNS (Davalos et al., 2005; Nimmerjahn et al., 2005). Upon injury or disruption to the BBB, microglia become activated immune-competent cells, increasing the expression of MHC Class I and II molecules (Kreutzberg, 1996) and extending their processes or migrating towards sites of injury (Cho et al., 2006; Davalos et al., 2005; Nimmerjahn et al., 2005). In

multiple sclerosis, inflamed glial cells produce pro-inflammatory cytokines such as IL-1β, tumor necrosis factor (TNF)-α, and various chemoattractant molecules such as CXCL10, CCL2 and CCL5 which attract microglia to lesion sites and which promote the invasion of peripheral immune cells (Kierdorf et al., 2010). Furthermore, with the activation of microglial cells, they serve as APCs and can potentially present brain-derived antigen to invading T cells. Facilitating the entry of immune cells into the CNS, endothelial cells produce chemokines such as CCL19 and CCL21 which serve to guide T cells to enter the injured tissue (Alt et al., 2002).

3.2.2.2 MS and inflammation: the role of T cells

The implication and role of T cells in the pathogenesis of multiple sclerosis is long-standing. Many studies have examined the frequency of myelin-specific T cells in patients with multiple sclerosis as compared to healthy controls. However, while often no significant difference in frequency was found between the two groups (Ota et al., 1990; Pette et al., 1990), it was reported that a higher frequency of myelin-specific T cells from MS patients did have a memory or activated phenotype, while the same Ag-specific T cells in healthy donors have a naïve phenotype (Bielekova et al., 2004; Lovett-Racke et al., 1998; Scholz et al., 1998). Additionally, differences in the expression of chemokine receptors and cytokines by myelin-specific T cells also suggest that these cells are more inflammatory in MS patients (Crawford et al., 2004; Kivisäkk et al., 2004).

The commonly held theory regarding the pathogenesis of MS is that it is primarily a CD4 T cell-mediated disease (Delgado and Sheremata, 2006; Kuchroo et al., 2002; Steinman, 1996). The pathology seen in EAE is similar to that in MS patients (Goverman, 2009) and supports the involvement of CD4 T cells. The induction of EAE favours the activation of CD4 T cells (Goverman, 2009), disease induction via adoptive transfer of myelin-specific CD4 T cells was shown to be possible (Baron et al., 1993), and human studies show genetic susceptibility to developing MS is associated with MHC class II genes ((Hillert and Olerup, 1993) reviewed by (Batoulis et al., 2010)). Thus, it was thought that the primary effector cells in the pathogenesis of MS were CD4 T cells.

Much early research was thus focused on the role of these cells, and more specifically on the Th1 response (Batoulis et al., 2010), but while CD4 T cells continue to be studied, further research since then has opened doors for the involvement of not only

Th1 but also Th17 cells, as well as CD8 T cells in disease pathogenesis. For instance, while relatively higher numbers of CD4 T cells are found in active MS lesions, they are not present in chronic MS lesions (Chitnis and Alireza, 2007), and in some cases greater numbers of CD8 T cells than CD4 T cells have been detected in MS lesions (Babbe, 2000; Frischer et al., 2009; Hauser et al., 1986). Generally, the accumulation of T cells in brain tissue depends on MHC restriction in addition to local antigen recognition(Flugel, 2001). Infiltrating CD4 T cells tend to be localized at the perivascular cuffs and close to the meninges, while CD8 T cells invade the parenchyma of the inflamed CNS (Kawakami et al., 2005) and are enriched at the site of actively demyelinating lesions(Neumann et al., 2002).

Research has demonstrated different roles for subsets of CD4 T cells in the pathogenesis of MS – and in other autoimmune diseases. In general, it is suggested that inflammatory responses in autoimmune disease are mediated by inflammatory Th1 cells (Liblau et al., 1995), requiring IL-12 for differentiation, and producing lymphotoxin and IFNγ, but little IL-4 (Mosmann and Coffman, 1989). Various evidence supports this idea: administration of IFNγ to MS patients exacerbated the disease (Panitch et al., 1987); myelin-reactive T cells from MS patients produce cytokines consistent with a Th1 response (Crawford et al., 2004); and increased activity in MS was related to the levels of IFNγ and IL-12 found in the CNS and CSF of patients (Gutcher and Becher, 2007). This is further supported by findings in EAE: T cells infiltrating the CNS secrete IFNγ, IL-12 is detected in inflammatory lesions (Baron et al., 1993; Segal and Shevach, 1996) and the transfer of autoreactive CD4 T cells is well established in inducting disease (Baron et al., 1993; Stromnes and Goverman, 2006b). Considering the effects of IFNγ: the ability to induce MHC Class II expression on CNS cells, activate macrophages, promote T cell homing (Navikas and Link, 1996) and even induce oligodendrocyte death (Vartanian et al., 1995), there is a strong case for the role of IFNγ-producing Th1 cells in the inflammatory response in the CNS. However, in spite of such evidence for the role of Th1 cells in MS pathogenesis, it does not quite pass muster upon further examination.

First, mice deficient in IL-12, IFNγ and TNF developed worse EAE than controls (Steinman, 2007). IL-12 is a heterodimer consisting of two subunits: p40 and p35; it was shown that depending on which subunit was lacking, the susceptibility to disease induction

in mice was varied. Mice lacking the p40 subunit did not develop EAE (Becher et al., 2002), but mice lacking the p35 subunit were more susceptible to developing disease (Gran et al., 2002). It is worth noting that IL-12 shares the p40 subunit with another interleukin, IL-23 (Gran et al., 2002). Lastly, mice deficient in IL-23 were resistant to disease, but had a normal Th1 response (Cua et al., 2003) further indicating that perhaps it is not Th1 cells which are the primary cells mediating the inflammatory response in the CNS.

IL-23 is required for the development of pathogenic Th17 cells (McGeachy et al., 2009), and the subunits of this interleukin (p40 and p19) have been detected in active lesions of patients (Li et al., 2007). In the EAE model, more severe disease was observed upon adoptive transfer of Th17 cells (Jager et al., 2009; Langrish et al., 2005), and neutralization of IL-17 ameliorated EAE (Hofstetter et al., 2005). Supporting a role for Th17 cells in multiple sclerosis, increased numbers of these cells have been detected in chronic MS lesions compared to acute lesions or to controls (no CNS pathology) (Lock et al., 2002), and IL-17 mRNA and protein in CD4 T cells were localized in active lesions and the borders of chronic lesions of MS patients (Tzartos et al., 2008).

However, once again despite supporting studies, the role of Th17 cells as the main effector cell of CNS immunity is not as clear-cut as it would seem. The effects of IL-23 and IL-17 in EAE seem to be different: while IL-23 deficient mice were resistant to EAE, mice deficient in either IL-17A or F are still susceptible to disease. In these cases, disease was occasionally milder (Haak et al., 2009; Hofstetter et al., 2005), but this was not always the case (Langrish et al., 2005; Röhn et al., 2006). Furthermore, the distinction between Th1 and Th17 cells is not always defined; a degree of plasticity is present in their phenotype. There are Th cells which express both IFNγ and IL-17, in addition to the transcription factors T-bet and RORγt (Abromson-Leeman et al., 2009), and stimulation of Th17 cells with IL-12 (in the absence of TGF-β) promotes conversion to a Th1 phenotype as well as the production of IFNγ (Lee et al., 2009). In line with such plasticity, depending on the conditions, both Th1 and Th17 cells induce EAE (Kroenke et al., 2008; Stromnes et al., 2008). The resulting effects of each cell type is different: Th1-induced disease results in increased recruitment of macrophages, whereas disease mediated by Th17 promotes infiltration of neutrophils (Kroenke et al., 2008). Furthermore, the ratio of these cell types in passive EAE influenced localization of inflammation: a high Th17 to Th1 ratio in the brain meninges resulted in infiltration of the brain and the induction of inflammation

(Stromnes et al., 2008). Overall, it is clear that in spite of the evidence supporting a role for Th1 and Th17 cells in the pathogenesis of MS, further studies are most definitely required to more fully elucidate the roles each CD4 T cell subset plays in disease pathogenesis. Further adding to the complexity, the role of CD8 T cells in multiple sclerosis must also be considered.

The traditional culprits of multiple sclerosis pathology are indeed CD4 T cells (Delgado and Sheremata, 2006; Kuchroo et al., 2002; Steinman, 1996), but more recently, a role for CD8 T cells has been considered. Animal models of demyelinating and neurodegenerative disease have been shown to be mediated by CD8 T cells (Na et al., 2008; Sanchez-Ruiz et al., 2008; Saxena et al., 2008); CD8 T cells have been detected in MS lesions in higher numbers than CD4 T cells (Babbe, 2000; Frischer et al., 2009; Hauser et al., 1986); there is clonal expansion and persistence of CD8 T cells within the CNS compartment of MS patients (Jacobsen, 2002; Junker et al., 2007; Skulina et al., 2004); and lastly, oligodendrocytes express MHC Class I, rendering them susceptible to direct attack by activated CD8 T cells (Ruijs et al., 1990). Furthermore, the fact that CD8 T cells recognize MHC class I, expressed by all cells, and mediate the direct killing of cells implies a direct role in causing the damage seen in the CNS.

CD8 T cells have three effector pathways through which they can mediate cell-killing via apoptosis: one is secretion of cytotoxic granules containing perforin and serine proteases (Kierdorf et al., 2010); the second is the interaction between CD95 on the CTLs interacting with CD95L on the target cell after Ag-MHC I recognition (Siegel et al., 2000); and lastly there is the secretion of cytokines belonging to the TNF family, where TNFα binding to its receptor activates the receptor death domain. However, to be able to mediate killing, CD8 T cells must recognize their Ag-MHC I complex. The challenge is that classical MHC class I molecules are not constitutively expressed on all neural cells, and so are not efficient at activating CD8 T cells. However MHC expression is induced on neural cells during most inflammatory and degenerative CNS diseases (Neumann et al., 2002): astrocytes, oligodendrocytes , neurons and axons in acute MS lesions upregulate the expression of MHC class I (Hoftberger et al., 2004), and thus are rendered susceptible to killing by activated CD8 T cells.

Primary cultures of microglia are inefficient at MHC class I presentation, but they are lysed when CTLs recognize their antigen-MHC combination (Bergmann et al., 1999).

CNS-invading CTLs activated in the periphery release proinflammatory cytokines (IFNγ and TNFα) which can induce expression of MHC on brain cells *in vitro* (Vass and Lassmann, 1990). MHC class I expression on astrocytes is upregulated by IFNγ, and are susceptible to lysis by CTLs via a granule mechanism, or via CD95/Fas-mediated apoptosis (Medana et al., 2001a). While oligodendrocytes express MHC I, they lack expression of co-stimulatory molecules; however, upon treatment with IFNγ, they express CD95 and are thus susceptible to apoptosis (Pouly et al., 2000). Lastly, there are the neurons. The expression of MHC I on neurons is more strictly regulated than in other cell types (Neumann, 2001), but "electrically silent" neurons do express MHC I (Neumann et al., 1995), and, as in the other neural cells, after IFNγ treatment they express MHC I (Neumann et al., 1997; Rensing-Ehl et al., 1996). CTL form stable contacts with neurites in culture suggesting they directly cause axonal damage *in vivo* (Medana et al., 2001b).

3.2.2.3 MS and inflammation: the role of B cells

Many lines of evidence exist for the involvement of B cells in the pathogenesis of multiple sclerosis. However, it has taken longer for the participation of B cells to be identified, perhaps due to the lack of their contribution in "traditional" EAE models (Batoulis et al., 2010). EAE models using proteins rather than the usual relatively short peptide sequences (Figueiredo et al., 1999; Kuerten et al., 2006; Lyons et al., 1999) have demonstrated a role for B cells in disease pathology. Further supporting a role for B cells, while the passive transfer of myelin-specific antibodies did not induce EAE, the depletion of B cells created resistance to active EAE (Batoulis et al., 2010). Finally, the transfer of MOG-primed serum into B cell deficient hosts resulted in a disease course similar to that seen in wild-type hosts (Lyons et al., 2002).

In multiple sclerosis, the role B cells play in CNS inflammation and modulating the disease course can perhaps best be indicated by the effects of treatment targeting B cells in MS. Patients treated with anti-CD20 Ab (rituximab), an antibody depleting B cells but not plasma cells and therefore not impacting antibody production, showed a reduction in gadolinium-enhancing brain lesions (Bar-Or et al., 2008; Hauser et al., 2008) as well as in the B cells found in CSF (Cross et al., 2006).

Plasma cells, myelin-specific antibodies and even B cells have been found in MS lesions (Cross and Waubant, 2011). Higher concentrations of IgG antibody displaying oligoclonal bands, is found in the brain and CSF of patients with MS as compared to controls (Hemmer et al., 2002). In addition, IgG has been observed within myelin in MS lesions and in macrophages containing myelin debris (Barnett et al., 2009); however, this was not specific to MS and seems to be associated with damage to white matter in general (Barnett et al., 2009). Antibody extracted from MS CNS tissue has been demonstrated to be reactive to myelin proteins. IgG isolated from MS plaques are specific for MBP (Warren and Catz, 1993; Wucherpfennig et al., 1997) and MOG (O'Connor et al., 2005).

Antibody-secreting B cells are present in MS lesions and CSF, and are clonally expanded (Baranzini, 1999; Colombo, 2000); furthermore, the heavy chains identified in MS plaques were not observed in the peripheral blood (Owens et al., 2001). B cell follicle-like structures have been identified in the meninges of patients with secondary progressive MS (Magliozzi et al., 2007; Serafini et al., 2004), and in some cases, their presence has been identified with a more aggressive clinical course (Magliozzi et al., 2007). This implies an environment within the CNS promoting B cell expansion and antibody production can exist (Cross and Waubant, 2011). Indeed, these follicles contain not only B cells, but also T cells, plasma cells and follicular DCs which produce chemokines such as CXCL13 (Serafini et al., 2004). CXCL13 is a B cell-attracting chemokine, and plays a crucial role in germinal center organization and the recruitment of B cells into lymphoid tissue (Reindl et al., 2010). However, other groups have not detected meningeal B cell follicles in MS (Peferoen et al., 2010; Willis et al., 2009)

3.3 Therapies

Treatments for multiple sclerosis are only partially effective. What remains a challenge is the inability of treatments to cross the BBB and enter the CNS, where activated immune cells are found; however, current treatments do have a degree of efficacy although the side effects, since they affect the immune system function, can be severe. While effective to a degree, there still is a need to develop alternative strategies that have more of an impact on ameliorating the disease course, and that are administered differently

such as fingolimod (FTY-20), which is administered orally, rather than current treatments which involve multiple injections.

IFNβ: IFNβ is an immunomodulator injected intramuscularly (weekly) or subcutaneously (three times weekly) (Cohen and Rieckmann, 2007), that decreases numerous immune responses. It inhibits adhesion of cells, as well as the synthesis and transport of matrix metalloproteinases which have a role in breaking down the integrity of the BBB (Frohman et al., 2006). IFNβ also has the ability to reduce MHC class II expression on B cells and their ability to present antigen to T cells (Boster et al., 2010).

Glatiramer Acetate: Glatiramer acetate (GA) is a random polymer of four amino acids. It is administered daily, subcutaneously (Cohen and Rieckmann, 2007) and used to treat patients with RRMS (Frohman et al., 2006). It is thought that GA can induce a shift in the activation state of T cells in the periphery from a Th1 to a Th2 phenotype (Boster et al., 2010), increasing regulatory T cell levels and suppressing inflammatory cytokines (Frohman et al., 2006). It has also been shown to modulate APC function by blocking antigen presentation (Boster et al., 2010; Frohman et al., 2006).

Natalizumab: This drug was initially developed to inhibit T cell adhesion to the inflamed endothelium in EAE; it is a monoclonal antibody that blocks α4 integrins. It is given to patients intravenously on a monthly basis (Cohen and Rieckmann, 2007). Studies have shown that natalizumab impairs CNS immune surveillance, inhibiting lymphocyte entry in the subarachnoid space. While it has been effective in treating MS patients, potential side effects can be severe, with a fraction of patients treated with natalizumab developing progressive multifocal leukoencephalopathy induced by virus infecting oligodendrocytes (Boster et al., 2010).

Mitoxantrone: Mitoxantrone is a chemotherapeutic agent, administered intravenously every three months (Cohen and Rieckmann, 2007). It is used short-term in patients with RRMS or SPMS prior to switching them to other, less severe therapies (Boster et al., 2010; Frohman et al., 2006). It reduces levels of Th1 cytokines such as lymphotoxin and TNFα and suppresses proliferation of T cells, B cells and macrophages (Boster et al., 2010).

Rituximab: Therapy with rituximab, a monoclonal antibody, results in a depletion of the CD20+ B cell population (Boster et al., 2010) without affecting the Ab-producing plasma cells. Treatment with this drug resulted in a significant reduction in gadolinium-enhancing lesions in RRMS patients (Boster et al., 2010; Hauser et al., 2008). Studies indicate that B cell depletion leading to diminished proinflammatory responses by CD4 and CD8 T cells is effective due to the removal of cytokines produced and expressed by B cells (Bar-Or et al., 2010). This drug is not yet approved, and is still in clinical trials.

Fingolimod (FTY): FTY belongs to a new class of drugs in the treatment of MS; it is administered orally rather than via injection and has been recently approved by the FDA and in Canada. It is a sphingosine-1-phosphate (S1P) receptor agonist; S1P is a G protein-coupled receptor ligand playing a role in the migration of lymphocytes through secondary lymphoid organs. Treatment with FTY results in a dose-dependent reversible decrease in peripheral blood lymphocyte counts. Peripheral counts decrease due to the fact B and T lymphocytes are sequestered in secondary lymphoid organs (Brown et al., 2007).

However, while these various treatments can ameliorate the course and severity of disease, none of them actually cure MS, and so the development of new therapies is crucial.

HYPOTHESIS AND OBJECTIVES

RATIONALE

In our laboratory, we study various factors influencing the activation state of CD8 T cells, in the context of MS; one such factor we have selected to study is interleukin-15. This is a cytokine that plays a central role in immunity and is involved in enhancing CD8 T cell activation and proliferation. IL-15 has been shown to be up-regulated in the blood and CSF of MS patients, and our laboratory has demonstrated IL-15 to be up-regulated in MS brain lesions, as well as in the blood of MS patients as compared to healthy controls (Saikali et al., 2010; Schneider et al., in press). Furthermore, we observed that CD8 T cells from MS patients are activated to a greater degree upon the addition of recombinant IL-15 in culture relative to healthy controls and display an enhanced cytotoxic profile. We also observed elevated proportions of B cells from MS patients expressing IL-15 as compared to controls. Considering that IL-15 has been previously mainly described on macrophages/monocytes, we investigated the role of IL-15 expressed by B cells. In addition to this, to work towards understanding the role of IL-15 in disease pathogenesis, we examined the expression of IL-15 in mouse models for MS: EAE.

We hypothesize that peripheral sources of IL-15 contribute to the enhancement of CD8 T cell responses in MS patients, and that IL-15 provided by the inflamed CNS actively contributes to enhancing CD8 T cell responses.

Objectives:

1. To assess surface IL-15 and IL-15Rα expression on human B lymphocytes and to determine whether expression levels are sufficient to enhance human CD8 T cell functions; and
2. To assess and establish the *in vivo* expression of IL-15 in MS mouse models.

MATERIALS AND METHODS

1.0 ISOLATION OF HUMAN BLOOD CELLS

1.1 Isolation of peripheral mononuclear cells (PBMC) from human blood

One hundred mL of blood was collected from healthy consenting donors in 10 cc tubes (VACUTAINER®; Becton Dickinson, Oakville, ON, Canada) treated with ethylenediaminetetraacetic acid (EDTA) in accordance with the local ethical committee (HD 07.002 and BH 07.001). Around 20 mL of blood was transferred into individual 50mL conical tubes and diluted with PBS-EDTA (2mM EDTA) for a final volume of 35 mL of diluted blood. Fourteen mL of Ficoll-Paque^{TM} PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was under-laid to form a distinct layer below the diluted blood. Tubes were then centrifuged for 30 minutes at 851 *x g* at room temperature (RT) without brakes. The top layer consisting of serum was removed, and the PBMC layer collected and transferred into a fresh 50 mL tube, then washed with PBS-EDTA and centrifuged at 479 *x g* for 15 minutes. The supernatant was discarded, the pellet re-suspended and washed twice more with PBS-EDTA (10 minutes per wash at 417 *x g*). Cells from the same donor were pooled and passed through a 70μm nylon mesh (BD Biosciences, Mississauga, ON, Canada) and counted using a hemacytometer before the second wash. Finally, cells were re-suspended in cold MACS buffer (PBS containing 2mM EDTA and 0.5% (v/v) Fetal Bovine Serum (FBS), Wisent) for isolation of specific subpopulations.

1.2 Isolation of CD8 T cells and B cells

CD8 T cells and B cells were isolated using positive selection. After isolation of PBMC, cell pellet was resuspended in 90 μL of MACS buffer per 10^7 total cells and 10 μL of CD8+ or CD19+ (CD8 T or B cell isolation respectively) MicroBeads (Miltenyi Biotech Inc., Auburn, CA, USA) were added per 10^7 total cells, and incubated for 15 minutes at 4^oC. After incubation, cells were washed in 2 mL buffer per 10^7 cells and centrifuged at 417 *x g* for 10 minutes. Supernatant was removed, and up to 10^8 cells resuspended in 500 μL of buffer. Magnetic separation was carried out using LS MACS Columns (Miltenyi Biotech Inc.); column was placed in MACS Separator, and rinsed with 3 mL of MACS buffer. Cell suspension was then applied to the column; column was washed three times with 3 mL of buffer per wash; flow-through with unlabelled cell fraction was collected in 15 mL conical tubes. After the washes, column was removed from the separator and positively-labelled cells flushed out with 5 mL of buffer. Cells were counted, washed and

re-suspended in appropriate medium. Purity assessed by flow cytometry of isolated cell populations was >95%.

2.0 IN-VITRO ASSAYS

2.1 Upregulation of IL-15 on B cells

For the TLR stimulation assay, LPS from *Escherichia coli* serotype 0127:B8 (Sigma-Aldrich, Oakville, ON, Canada) (100pg/ml), or CpG oligonucleotide (CpG); Invivogen) (18μg/ml) was used as ligands for TLR4 and 9 respectively. Recombinant human high-mobility group protein B1 (HMGB1; R&D Systems, Minneapolis, MN) (2μg/ml) was also used as a ligand for TLR4. The BCR was stimulated using F(ab')2 fragment goat anti-human Ig (αhu Ig; Jackson ImmunoResearch Laboratories, West Grove, PA) (2.5μg/ml). B cells were co-cultured with one of the above stimuli for 4 days. B cells were then harvested and labeled for flow cytometry analysis or used as antigen presenting cells (APC) in co-culture with T cells.

Isolated human B cells were co-cultured with either irradiated NIH-3T3 cells expressing CD40L or with irradiated control NIH-3T3 cells, in the presence of IL-4. 3T3 cultures at 70-90% confluence were washed with PBS, trypsinated at room temperature with trypsin (0.25%) (Wisent) diluted in PBS, and washed in R10 (RPMI 1640 (Roswell Park Memorial Institute medium), 100 IU/mL Penicillin, 100 μg/mL Streptomycin, 10 % (v/v FBS, and 2mM L-glutamine; Wisent) after 2 minutes. 3T3 cells were then resuspended in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with Penicillin (100 IU/mL), Streptomycin (100 μg/mL), 10 % FBS and L-glutamine (2mM) to a concentration of $5x10^5$ cells/mL. 3T3 cells were then irradiated (1500 rad) and then plated in 6-well plates, at $5x10^5$ cells/well and incubated for at least 30 minutes to allow the 3T3s to adhere to the well bottoms. One million isolated human B cells were added to the 3T3 cells, and IL-4 was added (10 ng/mL) to promote cell-survival in a final volume of 3 mL. Kinetics to determine the optimal number of days was carried out. B cells were harvested after 2, 3, or 4 days and surface expression of IL-15 examined using flow cytometry. For subsequent experiments, B cells were incubated with 3T3 cells for 4 days prior to flow cytometry analysis.

2.2 CFSE labelling of CD8 T cells

After isolation, CD8 T cells were resuspended in RPMI (Wisent) without serum, pre-warmed to 37°C at > $5x10^6$ cells/mL. CFSE (5,6- carboxyfluorescein succinimidyl ester, Molecular Probes) was added at 1.25 μM and incubated for 10 minutes at 37°C. Dye was quenched with FBS at room temperature for 5 minutes, then washed twice with RPMI for 10 minutes at 423 *x g*. Lastly, cells were resuspended in R10 and counted.

2.3 CD8 T cell and B cell co-cultures

48-well plates were coated with α-CD3 (clone OKT3, 32.5 ng/ml) overnight. B cells activated with CD40L were harvested, washed in R10 medium and counted. Up to $7x10^6$ B cells from each donor were resuspended in 2 mL of R10 and Fc receptors blocked for 20 min with normal mouse immunoglobulin (msIgG; invitrogen); cells were subsequently divided into two parts: one half was incubated 1h with msIgG (10 μg/mL), and the other was incubated with a blocking mouse anti-human antibody (Ab) specific to IL-15 (10 μg/mL; R&D Systems) for 1h. While blocking, CD8 T cells were labelled with CFSE and then added onto α-CD3-coated plates for at least half an hour at $5x10^5$ cells/well. After blocking, R10 was added to B cells to a final concentration of $2x10^5$ cells/mL, and 500 μL of the cell suspension was added to the CFSE-labelled CD8 T cells. B cell-CD8 T cell co-cultures were incubated for 6 days at 37°C, then harvested and stained for flow cytometry.

2.4 Flow Cytometry

Stainings were performed in a V-bottom 96-well plate, using the antibodies listed in Table 1. Staining B cells for surface IL-15, cells were blocked with mouse IgG for 20 min on ice in FACS buffer, washed and then followed by surface staining. For surface stainings, cells were incubated with relevant antibodies for at least 20 min on ice, or overnight at 4°C, in FACS buffer (PBS, 1% (v/v) FBS and 0.1% (w/v) NaN_3) in a total volume of 50 μL. Unbound antibodies were removed by washing twice in the same buffer. To measure interferon-γ and granzyme B (intracellular), after 6 days in culture, CD8 T-B cell co-cultures were stimulated for 4-5 hours with PMA (1μg/mL), ionomycin (20 ng/mL) and Brefeldin A (5 μg/mL), then stained for surface markers before fixation in paraformaldehyde (PFA)-saponin buffer (Hank's Balanced Salt Solution (HBSS; Wisent), containing 4% (w/v) PFA (Sigma-Aldrich), 1mM HEPES (Wisent) and 0.1% (w/v)

saponin from Quilla JA Bark, Sigma-Aldrich) at room temperature (RT) for 10 minutes followed by two washes in saponin buffer (PBS, 0.1% (w/v) saponin, 1% (v/v) FBS and 0.1% (w/v) NaN_3). Intracellular staining was performed in saponin buffer for 30 minutes. Isotype staining was performed using the corresponding Ab at the same concentration as the staining Ab. After surface staining, cells were resuspended in 200 µL 1% formaldehyde; cells stained intracellularly were resuspended in 200 µL of FACS buffer. A FACS LSR II (BD Biosciences) was used for acquisition, and results were analyzed using FlowJo software (Treestar, San Carlos, CA).

Table 1. Antibodies used for flow cytometry analysis

Antibody	Conjugate*	Concentration	per stain	Isotype	Distributor[+]
Primary: targeting mouse					
CD3e	PerCP-Cy5.5	200 µg/mL	0.1 µg	Hamster IgG1	BD
CD4	A700	200 µg/mL	0.01 µg	Rat IgG2a	BD
CD8a	PB	200 µg/mL	0.04 µg	Rat(Lou) IgG2a	BD
CD122	PE	200 µg/mL	0.06 µg	Rat IgG2b	Cedarlane
IL-15	–	500 µg/mL	2.5 µg	Rabbit IgG	Cedarlane
IL-15Rα	(biotin)	50 µg/mL	0.25 µg	Gt IgG	Cedarlane
CD45	PECy7	200 µg/mL	0.1 µg	Rat IgG2b	BD
B220	A488	200 µg/mL	0.1 µg	Rat IgG2a	Cedarlane
CD11b	A700	200 µg/mL	0.1 µg	Rat IgG2b	Cedarlane
Primary: targeting human					
CD20	PECy7	0.2 mg/mL	0.8 µg	ms IgG1	BD
CD86	allophycocyanin	3 µg/mL	0.015 µg	ms IgG1	BD
HLA-DR	A700	unknown	5 µL	ms IgG2b	Cedarlane
IL-15	PECy7	25 µg/mL	0.25 µg	ms IgG1	Cedarlane
IL-15Rα	(biotin)	0.5 mg/mL	0.5 µg	ms IgG2b	Cedarlane
CD8	PB	0.2 mg/mL	0.1 µg	ms IgG1	BD
Granzyme B	allophycocyanin	unknown	0.2 µL	ms IgG1	Invitrogen
Interferon-γ	A700	0.2 mg/mL	0.04 µg	ms IgG1	BD
Secondary					
Streptavidin	allophycocyanin	0.2 mg/mL	0.1 µg	–	BD
Streptavidin	PerCPCy5.5	0.2 mg/mL	0.1 µg	–	Cedarlane
Gt α-Rabbit	A488	2 mg/mL	1 µg	–	Invitrogen
Gt α-Rabbit	PE	500 µg/mL	0.25 µg	–	Cedarlane
Other					
LIVE/DEAD®-Fixable Dead Cell Stain Kit	aqua fluorescent reactive dye	unknown	0.5 µL	–	Invitrogen

*Abbreviations (fluorochromes): A488, Alexa Fluor® 488; A700, Alexa Fluor® 700; PB, Pacific Blue; PerCP-Cy5.5, Peridinin-chlorophyll proteins-Cy5.5; PE, Phycoerythrin; PECy7, Phycoerythrin-Cy7. [+]BD, BD Biosciences.

2.5 Isolation of Human Brain Endothelial Cell (HBEC)* and Migration Assay

*(*procedure performed by the team of Dr. Alexandre Prat, our collaborator)*

HBECs were isolated from CNS tissue specimens of temporal lobe resections from young adults undergoing surgery for intractable epilepsy, as previously described (Prat et al., 2000b). Prior to surgery, ethical approval and informed consent were given (BH 07.001). HBECs were isolated from non-epileptic material to generate an *in vitro* model of the human BBB according to a published protocol (Prat et al., 2000b).

Migration assays were performed in a modified Boyden chamber as previously described (Prat et al., 2000b). HBECs plated on Boyden chambers (Collaborative Biomedical Products, Bedford, MA) were stimulated with IFN-γ (200U/ml) and TNF (200U/ml) for 24 hours. B cells were activated with CD40L-expressing 3T3 cells as described above, and subsequently cultured with CD8 T cells, in the presence of α-CD3. After 6 days, CD8 T cells were harvested by magnetic bead separation as previously described and added to the upper chamber and allowed to migrate for 18h across pre-activated HBECs. The ability of CD8 T cells to cross the HBEC-monolayer was assessed by counting the absolute number of cells in triplicate wells that migrated to the lower chamber after 18h.

3.0 IL-15 AND EAE

3.1 Induction of Active Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) was induced in 6-7 wk old female SJL/J (SJL) and C57BL/6 (B6) mice via subcutaneous injections of emulsions containing myelin peptide and intraperitoneal injections of pertusis toxin. Mice were shaved at the injection site (at the base of the tail) at least two days in advance. To prepare the emulsions, *Mycobacterium tuberculosis* was added to Incomplete Freund's adjuvant (DIFCO) to make Complete Freund's adjuvant (CFA), and was used at a concentration of 8mg/mL *M. tuberculosis*. Proteolipid protein 139-151 ($PLP_{139-151}$; Alpha Diagnostic) was used at a concentration of 3 mg/mL in PBS, and myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}; Alpha Diagnostic) was used at 4 mg/mL in PBS. Emulsions were made by mixing equal parts of CFA and peptide by passing solutions through a micro-emulsifying needle (Fisher Scientific), and subsequently injected subcutaneously (2 injections of 50 μL on each side) near the base of the tail of SJL or B6 mice using $PLP_{139-151}$ (150 μg/mouse)

or MOG_{35-55} (200 μg/mouse) peptide, respectively. Pertussis toxin (Cedarlane Laboratories) was injected at day 0 with the emulsions, and again at day 2: Lyophilised toxin was resuspended in HBSS to a concentration of 50 μg/mL, and 100 μL at 4 μg/mL (400 ng) was injected intraperitoneally.

3.2 Scoring

Mice were weighed and scored daily from day 7-8 post-injection. Scores were given using the criteria shown in table 2. Animals were sacrificed if reaching 20% weight loss or score of 4.0-4.5.

Table 2. Disease scores for mice with EAE

Score	Symptoms
0.5	tail does not wrap around cylindrical object
1.0	limp tail
1.5	limp tail, mild ataxia
2.0	limp tail, ataxia
2.5	ataxia and weakness in hind limbs (weak grip)
3.0	one hind limb paralyzed
3.5	one hind limb paralyzed, weakness in other
4.0	both hind limbs paralyzed
4.5	both hind limbs paralyzed, weakness in forelimbs
5.0	Moribund

3.3 Perfusion

Mice were anesthetized using isofluorane. Once they were no longer responsive to toe-pinching, they were immobilized using pins on a styrofoam board. Thoracic cage was opened and heart exposed. A small incision was made in the lower right side, and a 26G needle connected to a pump with PBS was inserted into the left-hand side. Mice were perfused with around 25 mL of saline, until PBS flowing out of heart was clear.

3.4 Processing of organs: Lymphocyte isolation

Spleen: spleen was harvested and put in 5 mL R10 on ice. Spleens were mashed through a cell strainer (70 μm, BD Biosciences) in a Petri dish to make a single cell suspension. Cell strainer and Petri dishes were rinsed with RPMI. Cells were centrifuged at 424 *x g* for 7 min. Supernatant was poured off, and cells resuspended. One mL of 0.83% (w/v) ammonium chloride was added per spleen, tubes inverted to mix, and incubated at

RT for 4 min. Medium was added to >10 mL, and centrifuged at 424 *x g* for 7 min. Cells were then resuspended, passed through a cell strainer again to remove debris, and re-centrifuged at same speed. Cells were finally resuspended and counted. Cell concentration was adjusted to 1 x 10^7 cells/mL in R10 (for culture) or FACS buffer (for staining).

Draining lymph nodes: Inguinal lymph nodes, which drained the injection sites, were collected in 5 mL R10 on ice, mashed through a cell strainer. Filter and Petri dishes rinsed with RPMI, cells collected and centrifuged at 424 *x g* for 7 min. Supernatant was removed and then cells resuspended and counted. Cell concentration was adjusted to 1 x 10^7 cells/mL in R10 (for culture) or FACS buffer (for staining).

CNS: brain and spinal cord were collected in ice-cold R10, and mashed through a cell strainer (70 μm). Cells were passed multiple times through strainer to homogenize. Strainer and Petri dish washed with RPMI to a final volume of 15 mL. Homogenates were centrifuged at 400 *x g* at 4^0C for 7 min. Supernatants carefully poured off, pellets resuspended in 4 mL 90% Percoll (VWR) solution. 3 mL of 60% Percoll was carefully added on top of 90% layer, followed by a 40% Percoll layer (4 mL) and 2-3 mL HBSS (1x) to make a total volume of 14.5 mL. Discontinuous gradients were centrifuged at 4^0C at 1000 *x g* for 15 min, without brakes. Myelin layer (0-40% interface) was discarded; liquid was collected until 60-90% interface (5-6 mL left in tube) and transferred to a fresh tube. Percoll was washed by adding HBSS to collected fraction and centrifuged for 10 min at 424 *x g* at 4^0C. Supernatant was removed, pellet resuspended and washed again in RPMI for 10 min at 424 *x g.* Supernatant was removed and pellet resuspended in 400 μL R10 or FACS buffer.

4.0 IL-15 EXPRESSION: QUANTITATIVE (REAL-TIME) PCR

4.1 RNA extraction: B cells

Cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Mississauga, On, Canada) according to manufacturer's instructions. Briefly, isolated human B cells were disrupted through the addition of RLT buffer and the lysate passed through a 1 mL RNase-free syringe (BD Bicosciences) with a 20-gauge needle. 70% Ethanol was added at an equivalent volume to the homogenized lysate, and mixed by pipetting up and down. Up to

700 µL of sample were transferred to a RNase-free spin column with a collection tube (provided in the kit), and centrifuged at 8000 *x g* for 15s. The process was repeated for samples at volumes greater than 700 µL. Flow-through was discarded, and the spin column membrane washed with 700 µL of Buffer RW1 for 15s at 8000 *x g*, followed by two washes using RPE buffer (500 µL for 15s at 8000xg, second wash for 2 min). Columns were then transferred to new collection tubes and centrifuged at full speed for 1 min. To elute the RNA, spin columns were transferred to 1.5 mL RNase-free Eppendorf tubes, 30-50 µL of RNase-free water added directly to the membrane, and centrifuged for 1 min at 8000 *x g*. RNA concentration and purity were determined using a nanodrop. RNA was then stored at -80^{o}C or immediately followed by cDNA transcription. 260/280 nm ratio was typically > 1.8.

4.2 RNA extraction: Mouse tissue

Liver, spleen, cortex, brain stem, and spinal cord were collected and put into Trizol (50-100 mg tissue/mL trizol; Invitrogen). Tissue was homogenized using a polytron and then placed at -80^{0}C. To extract RNA, tissue samples were defrosted at RT. 200 µL chloroform was added per mL trizol; sample was vortexed for 15 sec and then incubated 2-3 min at RT. Samples were then centrifuged for 15 min at 12 000 *x g* at 4^{0}C. The aqueous phase was transferred into fresh tubes, and 0.5mL isopropanol/1 mL trizol used was added, and incubated for 10min at RT. Samples were centrifuged again at the same speed and temperature, for 10 min. Supernatant was removed and pellet washed with 1 mL 75% ethanol/1 mL trizol used. Samples were vortexed and subsequently centrifuged at 7500 *x g* for 5 min at 4^{0}C. Ethanol was removed, and RNA left to dry until transparent. RNA was then resuspended in either 50 µL (spinal cord) or 100 µL (all other tissues) RNase free water. RNA concentration and purity was determined using a NanoDrop (Thermo Scientific, Wilmington, DE, USA). 260/280 nm ration was typically >1.8. RNA aliquots were adjusted to 1 mg/mL where possible, and stored at -80^{0}C.

4.3 Complementary DNA (cDNA)

The QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis, following manufacturer's instructions. Potentially contaminating genomic DNA was eliminated using gDNA Wipeout Buffer, incubated with total RNA at 42^{o}C for 2 min.

RNA was then transcribed into cDNA using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix; the reaction was incubated at 42^{o}C for 30 min, and then at 95^{o} for 3 min to inactivate the reverse transcriptase. RNase inhibitor and dNTPs were present in the kit components.

4.4 Quantitative/Real-time Polymerase Chain Reaction

B cells: qPCR was used to examine the up-reguation of human IL-15 and IL-15Rα upon activation via CD40L/CD40. Relative gene expression was determined using primers and TaqMan FAM-labeled MGB probes for human IL-15 and IL-15Rα and ribosomal 18S (VIC-labeled probe, used as an endogenous control) (Applied Biosystems, Foster City, CA) and according to the manufacturer's instructions.

Mice: The expression of IL-15 in various tissues at various points of disease was determined using qPCR. Spleen and liver were used as controls for mouse IL-15 expression. Tissue from the CNS was divided into: the cortex, brain stem, and spinal cord. Tissue was also taken from control mice which had either been injected with an emulsion of CFA and PBS (no peptide), or had not been injected. IL-15 expression was examined at onset and peak of disease (SJL mice) or at onset, peak and remission (B6 mice). Relative gene expression was determined using primers and TaqMan VIC-labeled probes for IL-15 and Hprt1 (hypoxanthine phosphoribosyltransferase 1; VIC-labeled probe, used as an endogenous control) (Applied Biosystems, Foster City, CA) and according to the manufacturer's instructions.

For all PCR reactions, RNAse-free water was used as a negative control. Each sample was run in triplicate, in a volume of 10uL in a 384-well plate. Plate was spun at high speed for several seconds to ensure air bubbles were removed. Plates were incubated in a 7900 Fast Real Time PCR system (Applied Biosystems) for 10 min at 95^{0}C, This was followed by 40 cycles of PCR: denaturation of cDNA at 95^{0}C for 15 sec, and followed by synthesis of a new DNA strand at 60^{0}C for 1 min. The threshold fluoresence (the point at which an increase in fluorescence is distinguishable from the background) and the C_T readout (the point at which the detected signal is significantly above threshold) were automatically determined by the Sequence Detection System (SDS) software.

The relative expression of each gene was compared to either 18S (human cells) or Hprt1 (murine cells) and calculated as ΔC_T, the difference in threshold cycles for target and reference genes using the following formula: $\Delta C_T = C_{T(target)} - C_{T(reference)}$. Finally, the levels of the target gene expressed were normalized to the endogenous reference, and calculated as $2^{(-\Delta C_T)}$.

5.0 STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism software (San Diego, CA). Statistical analyses include paired student's t test.

RESULTS

1.0 B CELL ACTIVATION AND IL-15 PRODUCTION

1.1 Optimizing IL-15 production by B cells

Our laboratory observed increased proportions of monocytes/macrophages and B cells carrying IL-15 in multiple sclerosis compared to controls (Schneider et al. in press 2011), with the former cell type expressing higher levels of the cytokine (Schneider et al. in press 2011). The expression of IL-15 on monocytes/macrophages in the context of MS has already been described (Vaknin-Dembinsky et al., 2008) thus we focused on the expression of IL-15 on B cells. Cytokine expression by B cells in the context of MS is also of interest due to the efficacy of the drug Rituximab, which depletes the CD20+ B cell population; its efficacy is thought to be due to the removal of cytokines produced and expressed by B cells (Bar-Or et al., 2010). IL-15 could be one such cytokine. And so, we decided to test various stimuli for their capacity to activate B cells and potentially induce an increase in surface IL-15 on B cells. We measured expression of surface IL-15 as the IL-15Rα binds its cytokine with high-affinity, and so IL-15 is essentially presented on the cell surface rather than secreted (Fehniger and Caliguri, 2001).

CD40L as a stimulus is of interest: its use as an activator of B cells has already been previously described (Arbour et al., 2006; Banchereau et al., 1991; Lapointe et al., 2003), and CD40L expression has been reported to be up-regulated in various autoimmune disorders including MS (Gerritse et al., 1996a; Peters et al., 2009a; Teleshova et al., 2000). In addition to stimulation using CD40L, there is an increasing interest in the role of Toll-like Receptors (TLRs) in the development of autoimmunity (Green and Marshak-Rothstein, 2011; Horton et al., 2010; Reynolds et al., 2010) and their role in MS or EAE are under investigation (Carty and Bowie, 2011; Marta et al., 2009; Smolianov et al., 2011). TLRs can either exacerbate or suppress CNS inflammatory demyelination, depending on the stimulus and hence which TLR is being stimulated (Brien and Gran, 2008). Stimulation of the BCR is achieved via cross-linking of the receptor, and results in down-stream signaling and the production of various factors, including cytokines.

We assessed the ability of various stimuli to enhance the surface expression of IL-15 on B cells: HMGB1, LPS, and CpG were used to stimulate TLRs 2, 4 and 9; NIH-3T3 CD40L-expressing cells were used to stimulate CD40, and $F(ab')_2$ fragment goat anti-

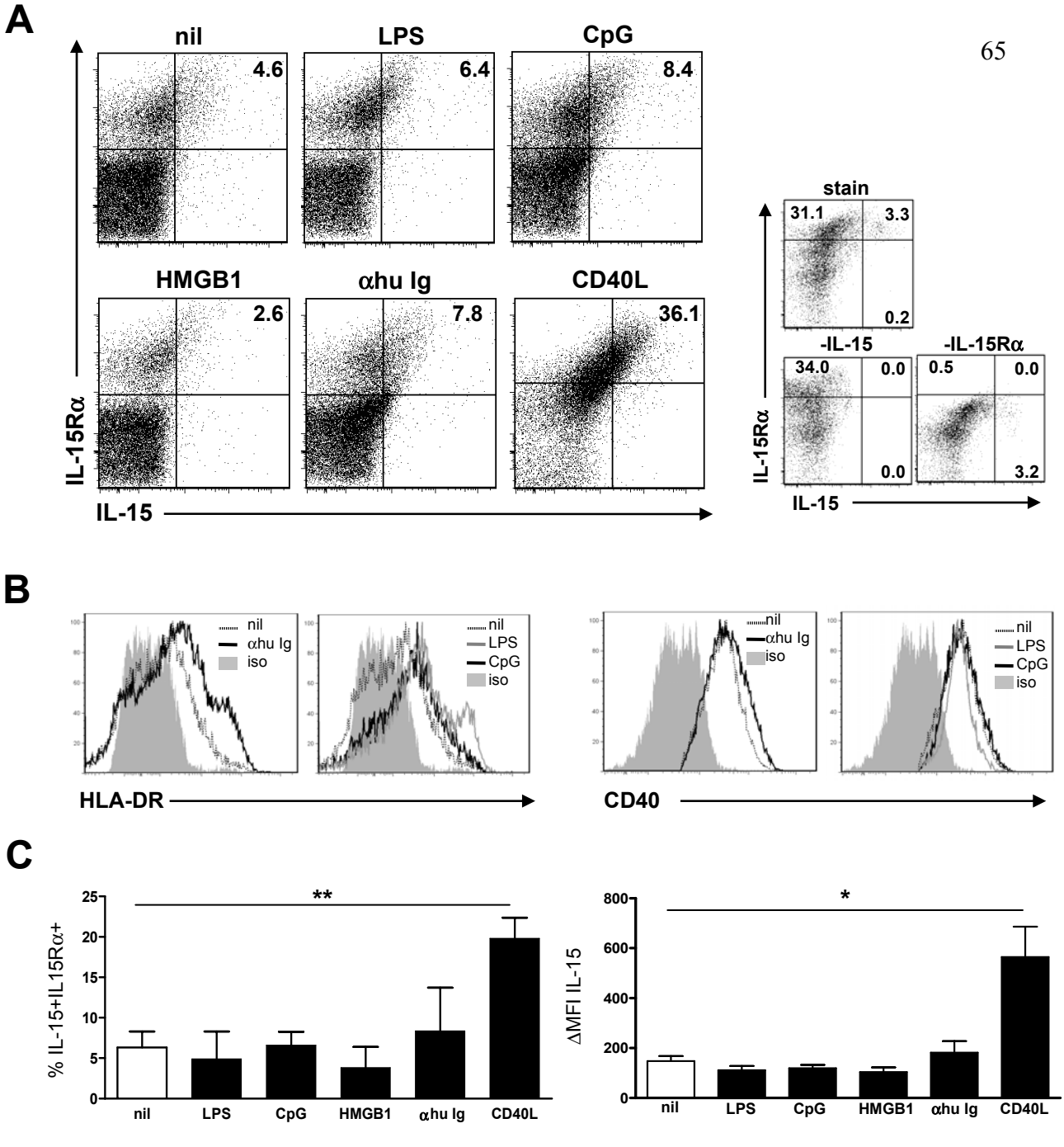


Fig.3: CD40L is a potent stimulus for the upregulation of IL-15 on B cells. B cells were cultured for 3-4 days in the presence of different stimuli, after which they were harvested, stained and assessed for IL-15 and IL-15Rα surface expression by flow cytometry. (A) Representative FACS plots of B cells cultured in the presence of various stimuli as indicated above each plot. No significant upregulation of IL-15 and its receptor was observed via TLR (LPS, CpG, HMGB1) or BCR (αhu Ig) stimulation. However, IL-15 and IL-15Rα were significantly upregulated via stimulation of CD40 on B cells. Fluoresence minus one controls for activation with CD40L shown in right panel. (B) HLA-DR and CD40 were assessed on the same cells; expression was increased upon stimulation with CpG, LPS, and αhuIg. Activation was not necessarily correlated with IL-15 expression. (C) Left panel: Pooled data showing proportion of B cells expressing IL-15 and IL-15 Rα after culture. Right panel shows difference in median fluoresence intensity (ΔMFI) of IL-15 expression on the same cells. (n=5). Cells were gated on living CD20+ B cells. No stimulus indicated by "nil". Statistics: student's t test, * $p<0.05$, ** $p<0.01$.

human Ig was used to stimulate the BCR as previously demonstrated by others (Pittner and Snow, 1998). B cells were isolated from PBMCs obtained from healthy donors using CD19+ positive selection; purity was typically over 95%. B cells were put in culture for 4 days and then analyzed by flow cytometry, gating on CD20+ living cells as determined by LIVE/DEAD staining. Untreated B cells were used as control. Representative FACS plots of IL-15 and IL-15Rα up-regulation are shown in Fig. 3A. Stimulation using LPS, CpG, HMGB1 or αhu Ig did not lead to a significant up-regulation of surface IL-15 (Fig. 3C, pooled data from n=4-8 tested donors). However, while there was no change in cytokine levels, this was not a result of B cells not being activated. BCR stimulation, as well as TLR 4 and 9 stimulation resulted in an increase of HLA-DR expression by B cells (Fig. 3B). The expression of CD40, usually responsible for co-stimulation, was also increased. In contrast, stimulation using CD40L resulted in a significant up-regulation of IL-15 (Fig. 3A, C). Not only did we observe an increased proportion of IL-15/IL-15Rα-expressing B cells, but also the ΔMFI (the difference in median fluorescence intensity between isotype and sample) of IL-15 and IL-15Rα was significantly higher on CD40L-activated B cells as compared to controls (Fig. 3C, pooled data n=4-8 donors).

1.2 CD40L as a potent stimulus of B cells

We further examined the up-regulation of Il-15 on the surface of B cells using CD40L as an activator of B cells. Co-culturing B cells with CD40L-expressing cells, we examined the kinetics of IL-15 up-regulation on the B cell surface to determine the optimal time to harvest the cells for subsequent experiments. Experiments looking at B cells after 2, 3, and 4 days in culture showed that after 4 days in culture, B cells reach a peak in their IL-15 expression on the cell surface (Fig. 4A left panel). After 4 days in culture, we also see an increased proportion of cells double positive for IL-15 and IL-15Rα (Fig. 4A right panel; from 5.7% d2 to 16.0% d4).

To confirm the specificity of the stimulus, namely that the up-regulation of IL-15 was due to interactions with CD40L expressed on the surface of the NIH-3T3 cells, we co-cultured B cells with either CD40L-expressing or non-expressing (i.e. transfected with an empty vector) NIH-3T3 cells. After four days in culture, B cells cultured with the NIH-3T3 control did not up-regulate the surface expression of IL-15 and IL-15 Rα to any great degree as compared to those cultured with NIH-3T3-CD40L cells (Fig. 4B left panel). The

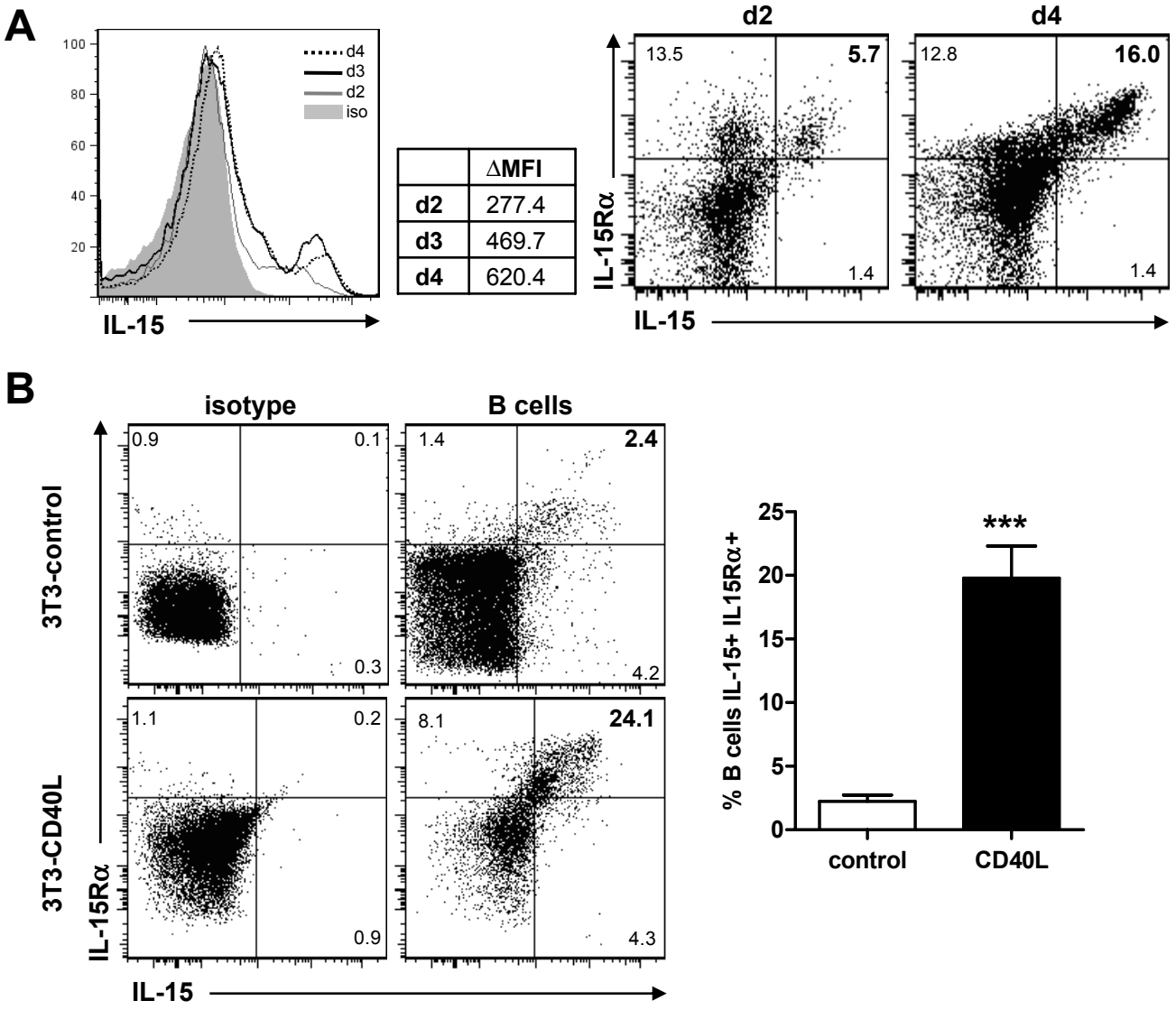


Fig.4: Upregulation of IL-15 on B cells via stimulation of CD40 using NIH-3T3 CD40L-expressing cells. B cells were co-cultured with 3T3-CD40L or control cells in the presence of IL-4, and IL-15 and IL-15Rα surface expression assessed by flow cytometry. (A) Histogram (left) depicting the kinetics of IL-15 upregulation on B cells as assessed by flow cytometry after 2, 3 and 4 days of co-culture with 3T3-CD40L cells. On average, higher levels of IL-15 expression were seen after 4 days. Dot plots illustrating the typical upregulation of IL-15 and IL-15Ra 2 and 4 days after CD40L-activation. (B) B cells cultured with CD40L-expressing 3T3 cells significantly upregulate IL-15 and IL-15Rα surface expression as compared to B cells cultured with control 3T3 cells (no CD40L). Representative FACS plots shown; gated on CD20+ B cells. Pooled data shown in right panel (n=6 donors). Statistics: student's t test, * $p<0.05$, *** $p<0.001$.

effect of CD40L stimulation resulted in a very significant increase in the double positive population of B cells expressing both IL-15 and IL-15Rα (pooled data from 6 donors, Fig. 4B right panel).

As we were adding IL-4 to our 3T3-CD40L-B cell co-cultures, to determine whether IL-4 played a role or impacted IL-15 production by B cells, we assessed its effects. While IL-4 alone did not affect IL-15 surface expression by B cells, it did have an effect on IL-15 production, increasing the impact of CD40L (Fig. 5A). Also, addition of IL-4 promoted survival of human B cells *in vitro*, as fewer cells were positive for a LIVE/DEAD staining.

To confirm that B cells indeed were producing IL-15 and presenting it on the cell surface, we carried out a series of experiments. B cells can express IL-15Rα without co-expression of IL-15. And so, using a sensitive ELISA assay with a detection limit of 4pg/mL, we examined whether the "empty" receptors would capture and bind soluble IL-15; upon addition of recombinant IL-15, no surface binding of the cytokine was detected after 5 hrs at 37^0C (data not shown). We also determined that IL-15 is not secreted from B cells into the surrounding environment: supernatant from cultures of either untreated or CD40L-activated B cells (n=4 donors) was also examined using the ELISA assay, and we did not detect any secreted IL-15 (data not shown). We also further confirmed production of IL-15 by B cells, comparing the surface and intracellular expression after culture, and upon addition of brefeldin A for the last 16h. Untreated cells had virtually no detectable IL-15 (Fig. 5B; MFI: isotype: 86; surface: 84; intracellular: 95), but when treated with CD40L and IL-4, there was an up-regulation of IL-15 in both the surface and intracellular compartments (Fig. 5B right panel; MFI: isotype 293; surface 421; intracellular: 623).

Lastly, we examined whether the up-regulation of IL-15 was due to a corresponding increase in the transcription, as opposed to a recycling of IL-15/IL-15Rα. Real-time PCR demonstrated that both IL-15 and IL-15Rα are up-regulated at the mRNA level, and that this up-regulation is specific upon stimulation with CD40L (Fig. 5C n= 3 donors). No IL-15 was detected by qPCR using RNA isolated from 3T3-CD40L cells (data not shown). Overall, our results support the notion that B cells synthesized IL-15, which was then translocated and expressed on the cell surface, a mechanism similarly seen in other cell types.

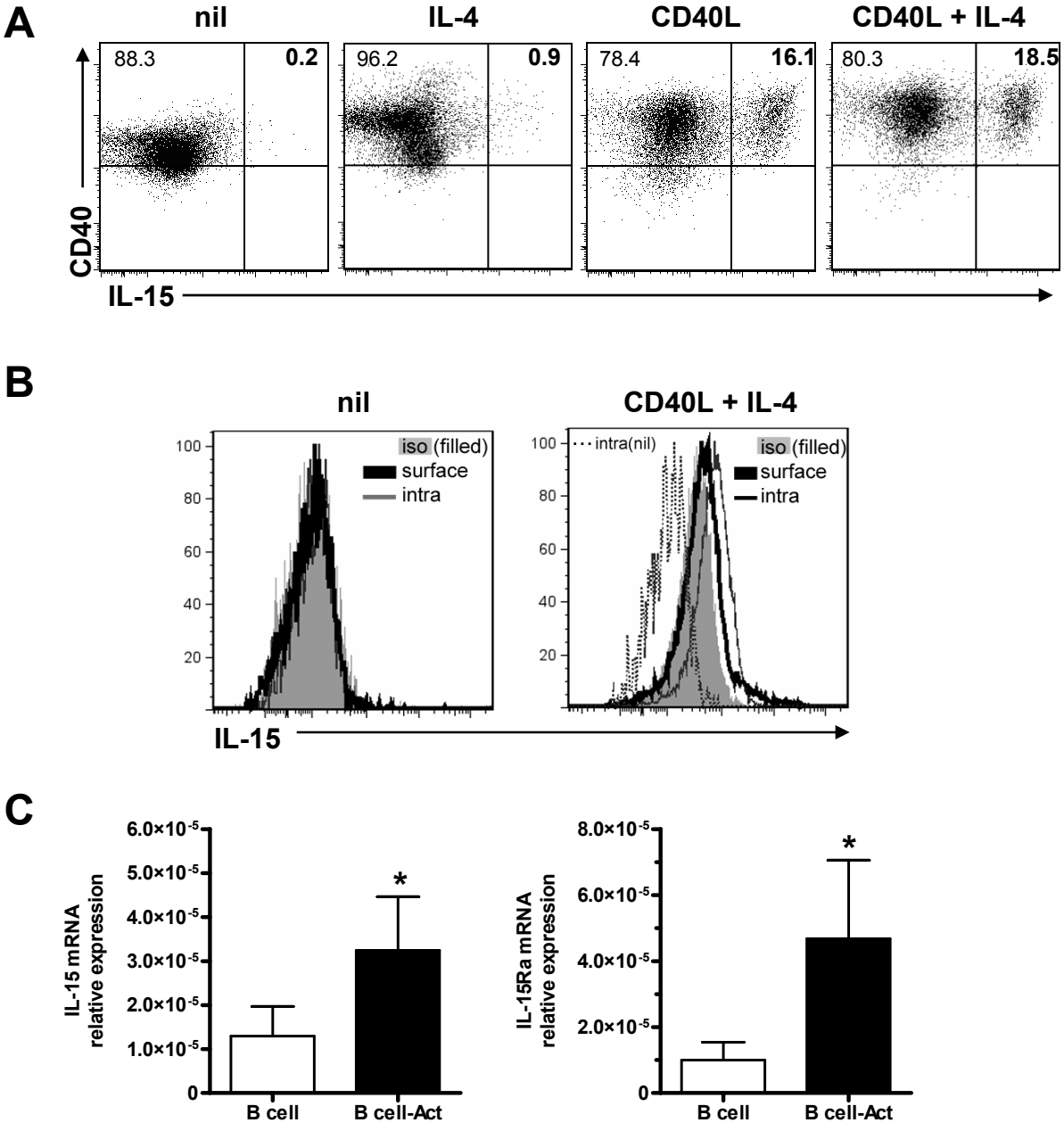


Fig 5: Specificity of the upregulation of IL-15 on B cells via stimulation of CD40 using NIH-3T3 CD40L-expressing cells. (A) B cells were co-cultured with 3T3-CD40L in the absence or presence of IL-4, or with IL-4 or alone for 4 days and then IL-15 and CD40 surface expression assessed by flow cytometry. (B) Surface and intracellular detection of IL-15 in B cells as assessed by flow cytometry after 4 days of co-culture with 3T3-CD40L cells and IL-4 or no stimulation (nil). Representative FACS plots shown; gated on CD20+ B cells. (C) IL-15 and IL-15 Rα are upregulated at the mRNA level relative to 18s rRNA expression. B cells activated with CD40L in the presence of IL-4 indicated by B cell-Act. Upregulation is specific to stimulation with CD40L-expressing 3T3 cells. Pooled data with n=3 donors. Statistics: student's t test, * $p<0.05$

Thus we conclude that IL-15 and its receptor can not only be produced by human B cells, but also that their expression can be effectively up-regulated upon CD40L stimulation.

1.3 IL-15 on B cells enhances the cytotoxic profile of CD8 T cells

To determine the functional effect of the IL-15 produced by activated B cells on CD8 T cells, B cells pre-activated via CD40L were incubated with either an IL-15 blocking Ab or with an isotype control, and subsequently co-cultured with CFSE-labelled allogeneic CD8 T cells for 6 days. After 6 days, CD8 T cells were harvested and assessed for cell proliferation in addition to granzyme B (GrB) and IFNγ production using flow cytometry. One typical example is illustrated and data obtained from 6 CD8 T cell donors with 3 donors of B cells are illustrated (Fig. 6). When IL-15 was blocked on B cells, this impacted the production of both GrB and IFNγ, resulting in decreased levels being produced by CD8 T cells (Fig. 6A and B, left panels, one typical experiment illustrated). Overall, the percentage of CD8 T cells that had proliferated and were producing granzyme B significantly decreased with the blocking of surface-expressed IL-15 on B cells, and the same held true when looking at the total production of granzyme B by CD8 T cells (Fig. 6A, right panel, n= 6). However, while the same trend was present when examining IFNγ production by proliferated CD8 T cells, there was no overall trend in the total IFNγ production by CD8 T cells (Fig. 6B, right panels). The increased proliferation of CD8 T cells as a result of exposure to surface IL-15 provided by B cells is associated with a greater proportion of CD8 T cells displaying a cytotoxic profile, expressing IFNγ and GrB.

1.4 IL-15 on B cells enhances the ability of CD8 T cells to cross an in vitro BBB

Of interest in the pathogenesis of multiple sclerosis, is the infiltration of immune cells into the CNS; once in the CNS, immune cells could cause damage to neural tissue. However, to reach the CNS, immune cells must first be able to cross the BBB; activated T cells display an enhanced capacity to cross the BBB (Engelhardt, 2006). Using a well established *in vitro* model of the BBB consisting of a confluent monolayer of human brain endothelial cells (HBECs) (Prat et al., 2000a), we investigated the ability of CD8 T cells to cross the barrier in conjunction with the influence of IL-15 expressed by B cells.

Pre-activated B cells (using CD40L) were harvested and incubated with a blocking antibody to IL-15 or the isotype control, cultured with alloreactive CD8 T cells for 5-6

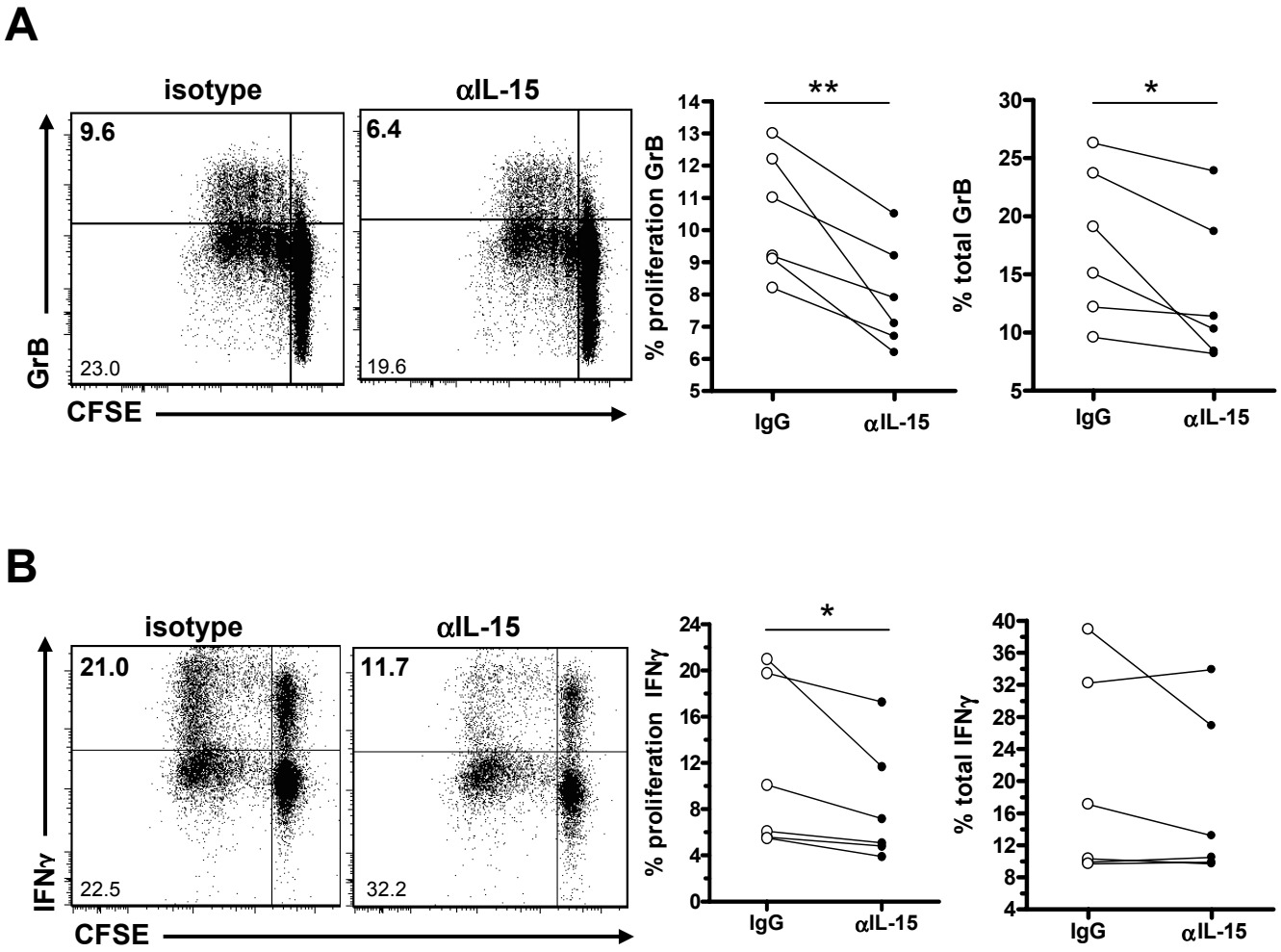


Fig.6: IL-15 presented by B cells is functional and enhances a cytotoxic profile in CD8 T cells. B cells pre-activated via CD40L were incubated with either a blocking antibody to IL-15 or with an isotype control; B cells were then co-cultured with CFSE-labelled CD8 T cells (alloreactive) and proliferation, granzyme B (A), and IFNγ (B) production by CD8 T cells assessed using flow cytometry after 6 days in culture. (A) Left panel: representative FACS plots shown. Blocking IL-15 on B cells results in decreased granzyme B production and decreased proliferation. Right: Data from multiple donors showing a significant decrease in granzyme B production upon blocking IL-15 on B cells. Each pair of dots represents an individual donor (n=6 CD8 T cells, n=3 B cells). (B) Left panel: Representative FACS plots of IFNγ production by CD8 T cells upon IL-15 exposure. Blocking IL-15 on B cells decreases IFNγ production. Summary from multiple donors on right (n=6 CD8 T, n=3 B cell donors). There is no overall trend in total IFNγ production by CD8 T cells. Statistics: student's t test, * $p<0.05$, ** $p<0.01$.

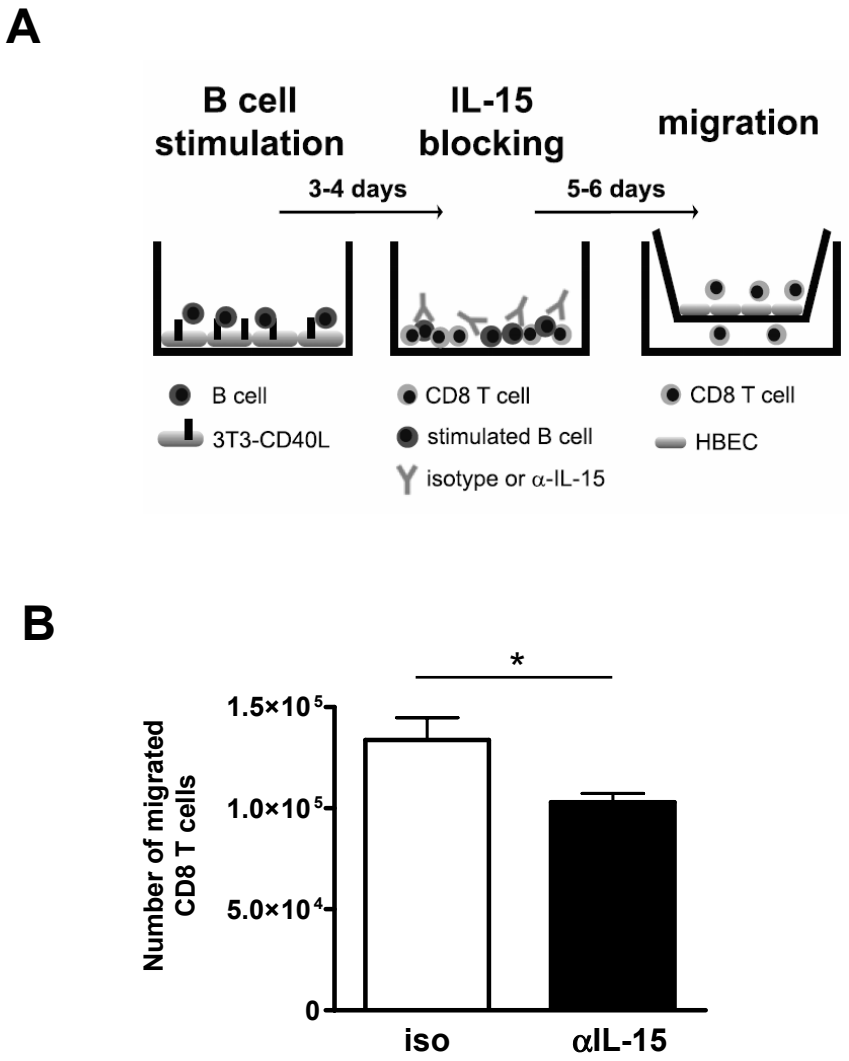


Fig.7: IL-15 presented by B cells enhances the ability of CD8 T cells to cross an *in vitro* blood-brain-barrier. (A) B cells were cultured and pre-activated with CD40L, then harvested after 3-4 days. B cells were then incubated with either a blocking antibody to IL-15 or an isotype control, and then cultured with alloreactive CD8 T cells for 5-6 days, after which CD8 T cells were harvested and placed on a confluent monolayer of inflamed HBECs (an *in vitro* model of the blood-brain-barrier), and allowed to migrate for 18h. Cells in the upper and lower chambers were then counted. (B) Blocking IL-15 on B cells before co-culture significantly (* $p<0.05$, student's t test) decreases the ability of CD8 T cells to cross the inflamed HBEC layer. n=4 CD8 T cell donors

days. Activated CD8 T cells were harvested and placed in an upper chamber on top of a monolayer of inflamed HBECs (Fig. 7A), and allowed to migrate for 18h. Cells from the upper and lower chambers were then counted. Blocking IL-15 on B cells before co-culture with CD8 T cells significantly decreased the ability of CD8 T cells to subsequently cross the *in vitro* BBB (Fig. 7B, pooled data n=4 donors). B cells are able to upregulate surface expression of IL-15 upon stimulation via CD40-CD40L interaction. Furthermore, not only do B cells produce and present IL-15 on the cell surface, these enhanced levels are sufficient to enhance the cytotoxic profile of CD8 T cells, inducing them to upregulate the production of Granzyme B and IFNγ. Lastly, IL-15 presented by B cells to CD8 T cells enhances their ability to migrate across an inflamed *in vitro* model of the BBB.

2.0 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS AND IL-15

2.1 Inducing Experimental Autoimmune Encephalomyelitis: the disease course

The EAE model, the most commonly used animal model of MS, was established, with mice consistently becoming sick, and with similar patterns of disease progression between mice of the same background. EAE was induced in SJL/J (SJL) or C57BL/6 (B6) mice using either $PLP_{139-151}$ or MOG_{35-55} peptides respectively. Disease progression is characterized by an ascending paralysis beginning with a limp tail and progressing to hind limb then fore-limb paralysis (clinical scoring criteria shown in Fig. 8C). Symptoms typically start to show about 7-10 days post-injection. Mice are weighed and scored daily according to symptoms; upon 20% weight loss, mice were sacrificed regardless of score.

In our hands, B6 mice either had acute EAE progressing towards a peak in the disease (Fig. 8A left panel), or entered a chronic phase and then went into remission (Fig. 8A right panel). SJL mice never went into remission, but rather the disease continued to progress (Fig. 8B). Furthermore, SJL mice in general had more severe EAE, reaching a peak score (≤ 3.0) sooner than B6 mice.

2.2 IL-15 expression in EAE: mRNA

To determine whether IL-15 expression is up-regulated during EAE, mRNA levels in different organs was first assessed. All mice in these and in subsequent experiments were perfused with PBS to avoid any contamination from blood cells.

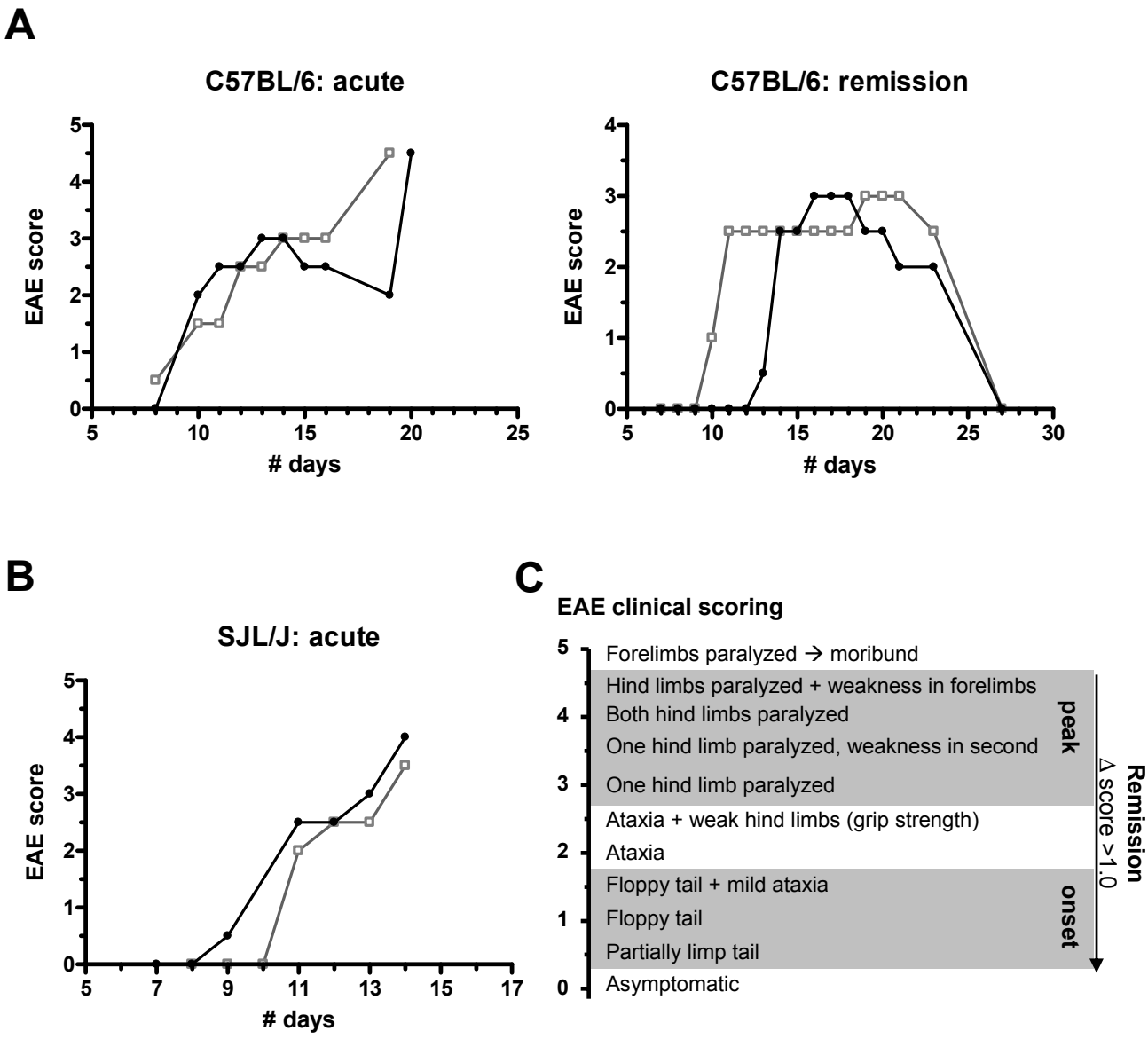


Fig.8: Typical clinical scores and disease progression of active EAE induced in C57BL/6 and SJL/J mice. Active EAE was induced in both C57BL/6 and SJL mice using MOG_{35-55} or $PLP_{139-151}$ peptides respectively. (A) C57BL/6 mice progressed to either a peak in disease (score 3.5 – 4.5) or plateau followed by remission to a score of 0. (B) SJL/J mice progress to a peak in disease (score 3.5 – 5.0). Clinical scoring criteria shown in (C). Mice were sacrificed upon reaching a score of 4.5 or at a an end-point defined by 20% weight loss. Representative disease course in two individual mice out of n=8-10 for acute disease and n=3 for remission of the corresponding genetic background are shown for each case.

2.2.1 IL-15 in spleen and liver

IL-15 mRNA was detectable in the spleen and liver of both B6 and SJL mice using qPCR (Fig. 9), but levels were significantly greater in B6 mice compared to SJL (scale Fig. 9). Mice were sacrificed at the onset (score 0.5-1.5) or peak (score $\leq$ 3.0) of disease, and in the case of B6 mice, when in remission (Δscore $\leq$ 1.0). Control mice were either injected with an emulsion containing CFA and PBS or were not injected. B6 mice at onset, peak and/or in remission did not show large differences in IL-15 expression relative to expression levels seen in control mice in either the spleen or liver (Fig. 9A). In SJL mice, at the onset of disease there was slightly less IL-15 at the mRNA level; however at the peak of disease, the expression of IL-15 in the spleen and liver was almost 5x lower than those seen in control mice (Fig. 9B). Spleen obtained from very sick SJL animals were notably smaller in size compared to controls or mice at onset.

2.2.2 IL-15 in the CNS

Distinct areas of the CNS were analyzed by qPCR for IL-15 mRNA levels. CNS lesions are more abundant in the spinal cord than in the cortex or brainstem, in the conventional EAE induced in SJL mice or B6 (Sobel, 2000). However, lesions in the brainstem have been reported under certain circumstances (Lees et al., 2008; Muller et al., 2000). Also, with time post-disease induction, demyelination progresses from the (lower) spinal cord, towards the brainstem and cerebellum (Cross et al., 1993; Sobel, 2000). IL-15 was expressed in the CNS in both B6 and SJL mice at the mRNA level (Fig. 10). At the onset of disease (score 1.5, n=3), there were higher levels of IL-15 mRNA found in the brainstem and spinal cord in B6 mice (Fig. 10A) relative to the controls. In the cortex, however, at disease onset there was slightly less IL-15 to be found. At the peak of disease (score 3.0, n=2), IL-15 mRNA was present and at its highest in the brainstem. Mice in remission (score 0, n=3) have almost no IL-15 in the brainstem, but it is detectable in the spinal cord, as well as in the cortex. While the highest levels of IL-15 were to be found in the brainstem at the onset and peak of disease, and the lowest levels found at remission again in the brainstem, the greatest change in IL-15 expression with disease progression occurred in the spinal cord at onset and the peak of disease (Fig. 10A, fold increase of 3.4 and 3.7 at onset and peak respectively in the spinal cord, compared to 1.8 and 2.1 in the brainstem).

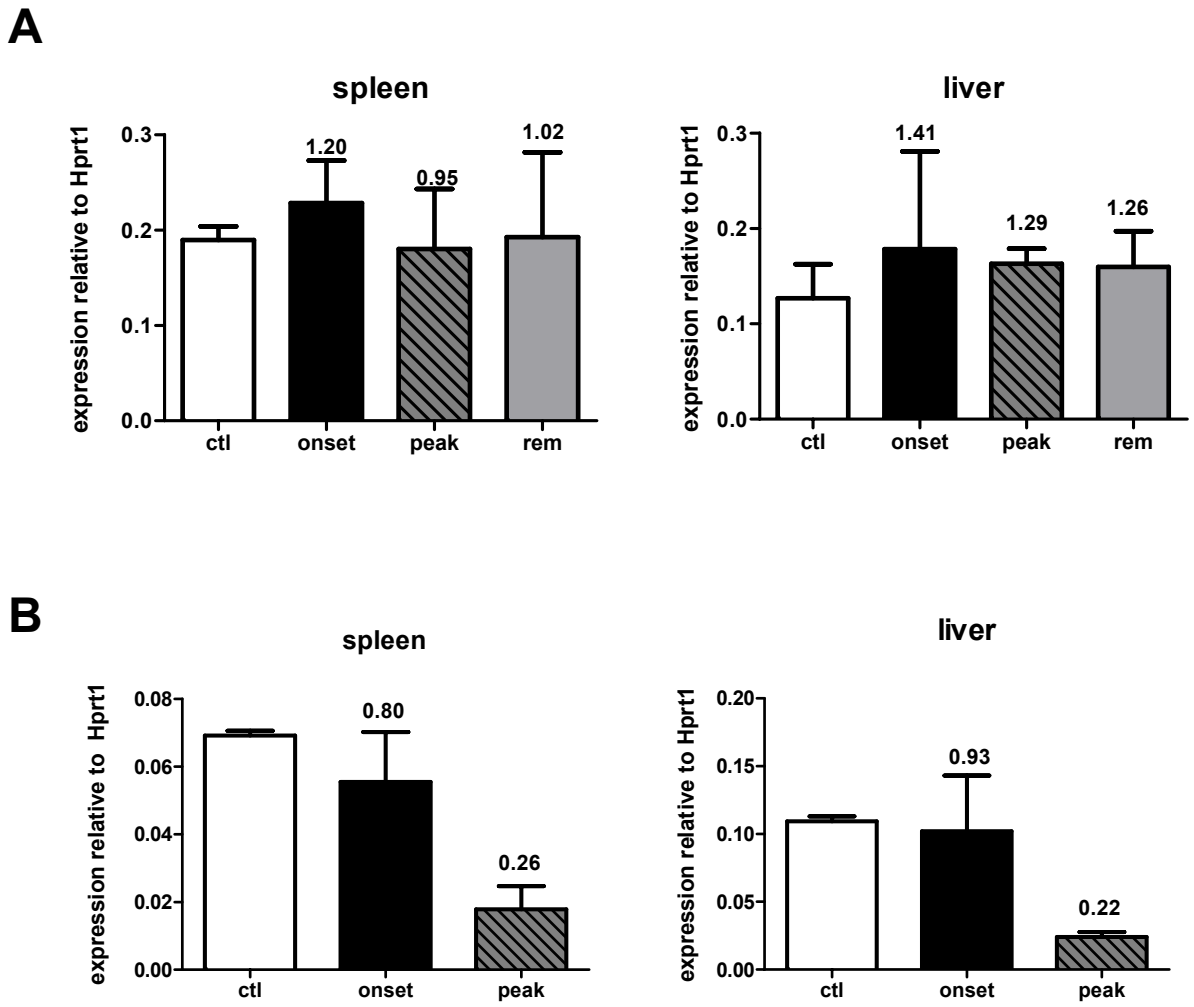
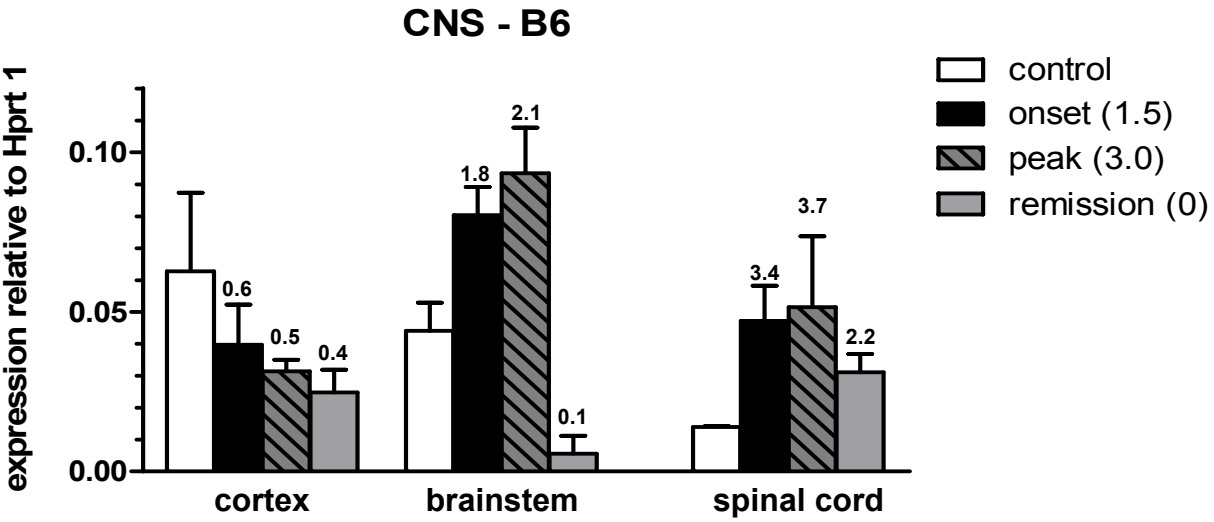


Fig.9: Quantification of IL-15 mRNA levels in spleen and liver of B6 and SJL mice using qPCR. Spleen and liver were harvested from PBS-perfused mice, RNA extracted, transcribed into cDNA and qPCR performed as described in methods. Results are presented as relative expression compared to the endogenous Hprt1 gene. Number above column indicates expression level compared to control group. (A) Organs harvested from B6 mice. Small differences or changes in expression level of IL-15 as compared to controls or at different points of disease (onset, peak or remission). (B) Organs from SJL mice. Expression level of IL-15 is decreased during disease relative to controls (n= 2-3 for each group) ctl, control; onset, score 0.5-1.5; peak, score 3.0-4.5; rem, remission Δ score >1.0.

A



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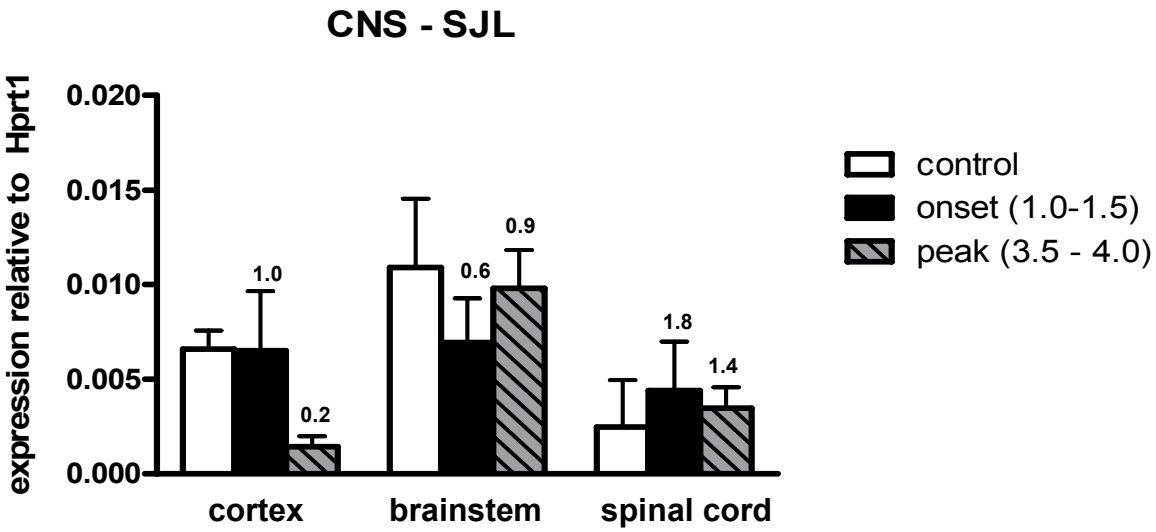

Fig.10: Expression of IL-15 in various parts of the CNS in B6 and SJL mice. Cortex, brainstem and spinal cord were harvested from PBS-perfused mice, RNA extracted, transcribed into cDNA and qPCR performed as described in methods. Results are presented as relative expression compared to the endogenous Hprt1 gene. Number above column indicates expression level compared to control group. (A) Organs harvested from B6 mice (B) Organs harvested from SJL/J mice. n=2-3 mice for each stage of disease.

Overall and in general, there were relatively higher levels of IL-15 detected in B6 mice as compared to SJL mice. In the cortex, there was no change in IL-15 expression at the onset of disease; however at the peak of disease, IL-15 mRNA levels decreased in SJL/J mice. There was no substantial change in IL-15 expression in the brainstem during the course of disease. In the spinal cord, while changes were not as large as those seen in the B6 mice during disease, there was an increase in the IL-15 mRNA levels at both onset and peak of disease (Fig. 10B).

Thus, IL-15 mRNA levels were up-regulated in the spinal cord, the main site of CNS lesions, of both strains at onset and peak of disease.

2.3 IL-15 in EAE: infiltrating immune cells express IL-15 and IL-15Rα

As the surface-bound form of IL-15 is mainly responsible for cellular signaling to neighboring cells (Budagian et al., 2006), surface expression of IL-15 and IL-15Rα on *ex vivo* immune cells was assessed by flow cytometry. In order to be certain that what we were seeing were cells positive or negative for IL-15 and/or the receptor alpha chain, we chose fluorochromes for each of these two molecules so as to not have potential influence due to compensation problems. And so, for the following experiments, IL-15 was labelled with an anti-IL-15 PE-labelled antibody, and IL-15Rα was first labelled with a biotinylated antibody followed by streptavidin conjugated to APC. Leukocytes were isolated from the spleens, draining lymph nodes (DLN) (to the emulsion injection site) and the CNS of both B6 and SJL mice at onset and peak of disease.

As the qPCR results indicated that IL-15 was expressed during EAE disease progression in the CNS, we examined the profile of immune cells present in each of the harvested organs using flow cytometry, with regards to IL-15 expression. Gating on CD3 negative cells, preliminary results showed the presence of IL-15 and IL-15Rα-expressing immune cells in the CNS of B6 mice and SJL mice at the onset (Figs. 11 and 12 respectively, panel A; representative of 2-3 mice) and peak of disease (Figs. 11 and 12, panel B, representative of 4 mice). This expression of IL-15 and IL-15Rα on non-T cells seemed to be specifically localized to immune cells present in the CNS, although slightly more cells expressing IL-15Rα were present in the DLN and the spleen in B6 mice at the onset of disease compared to mice at the peak of disease (Fig. 11A dot plots for DLN and SPLN vs. Fig. 11B DLN and SPLN). Also at the onset of disease, IL-15+/IL-15Rα- cells

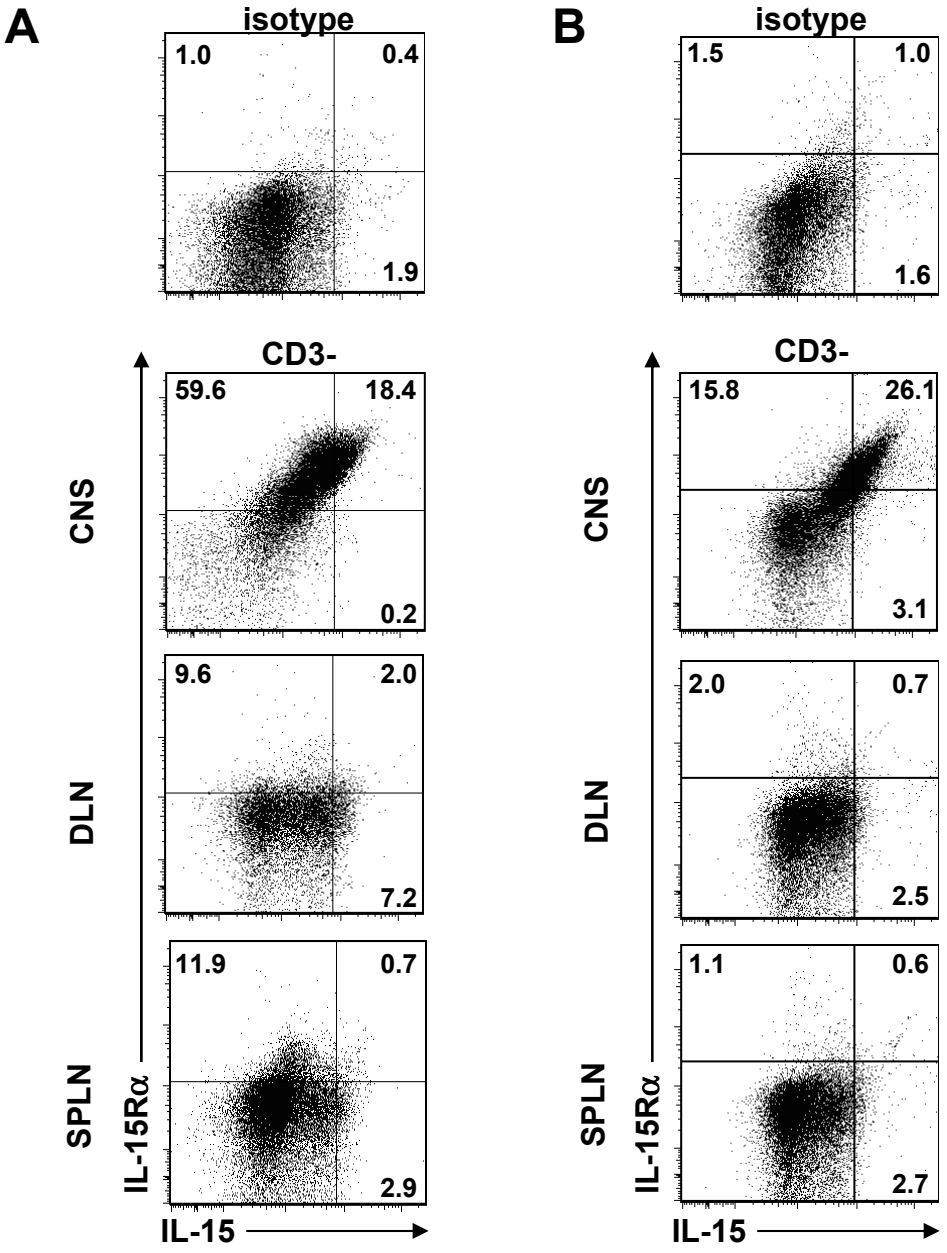

Fig.11: Immune cells found in the CNS of B6 mice express both IL-15 and its receptor alpha-chain. B6 mice were sacrificed (perfused with PBS) at either the onset or peak of disease. Leukocytes were isolated from various organs (spleen (SPLN); draining lymph nodes (DLN); or CNS) and subsequently stained and analyzed by flow cytometry. Plots are gated on CD3 negative cells (CD3-). IL-15 and IL-15Rα-expressing cells were specifically found in the CNS, and were not present in the DLN or SPLN of the same animal. Representative FACS plots for mice at onset (n=3) or peak (n=4) of disease. B6 mouse at (A) onset (score 1.5) and (B) peak of disease (score 4.5).

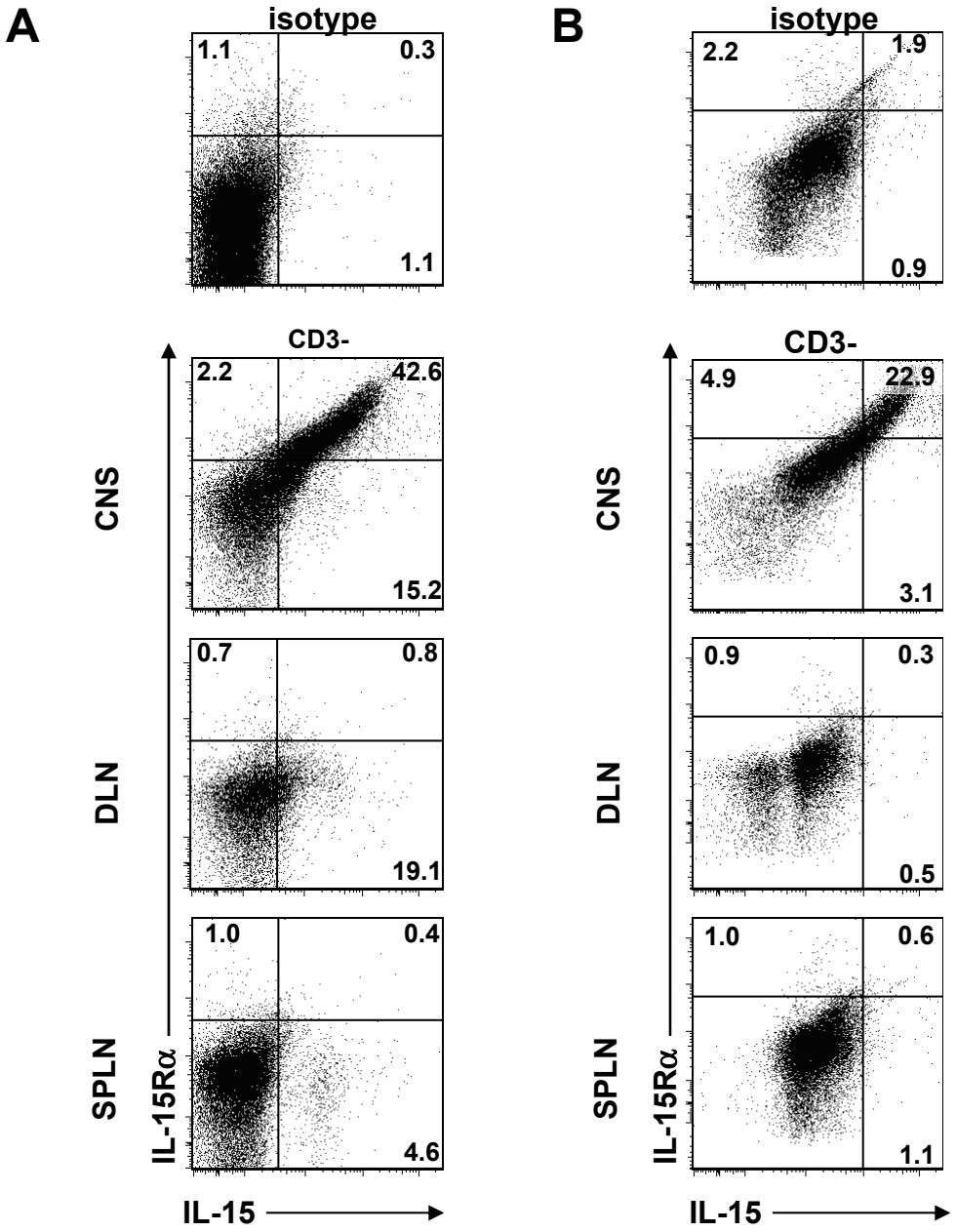

Fig.12. IL-15 and IL-15Rα-expressing cells are specifically found in the CNS of SJL mice, and are not present in the DLN or SPLN. SJL mice were sacrificed (perfused with PBS) at either the onset or peak of disease, leukocytes isolated from various organs (spleen (SPLN); draining lymph nodes (DLN); or CNS) and subsequently stained and analyzed by flow cytometry. Plots are gated on CD3 negative cells (CD3-). Representative FACS plots for mice at onset (n=2) or peak (n=4) of disease. SJL mouse at (A) onset (score 1.0) and (B) peak of disease (score 4.5).

were detected in lymphoid organs of both strains, but not at the peak of disease. This was not seen in the SJL mice (Fig. 12A and B). It was not possible to analyze CNS cells from control animals as very few cells could be recovered from these mice.

2.4 CNS-infiltrating immune cells expressing IL-15

We further characterized the immune cells present in the CNS of SJL and B6 mice, expressing IL-15 and IL-15Rα using flow cytometry. As a preliminary screening, we chose the markers CD11b (indicative of a macrophage/microglial population) and B220, a marker found predominantly on B cells. Again to avoid problems with compensations, Ab-fluorochrome combinations were carefully chosen: CD11b was labeled with Alexa 700 and B220 with Alexa 488. Upon examination of the CNS-infiltrating cells in B6 mice, the proportion of CD3- cells expressing IL-15 also expressed IL-15Rα (20.5%; Fig. 13A); a proportion of these cells were CD11b+ (13.2%; Fig. 13A ii), but the majority were double positive for CD11b and B220 (86.8%). A proportion of cells expressing only the IL-15Rα (67.2% CD3- cells; Fig. 13A), when further characterized, also showed a CD11b+ and CD11b+/B220+ profile (Fig. 13A i). When compared to the peak of disease, the proportion of CD3- cells IL-15+/IL-15Rα+ found in the CNS of B6 mice were CD11b+ and B220- (Fig. 13B ii). There was also a small subset of immune cells expressing IL-15, but negative for the receptor; these too were CD11b+ (Fig. 13B iii).

In contrast to the results seen in B6 mice, the profile of the immune cells found in the CNS of SJL mice at the same points of disease is different. At the onset of disease, cells positive for IL-15Rα are CD11b+ (Fig. 14A i). The proportion of double positive cells for IL-15 and the Rα, unlike those in the CNS of B6 mice, are positive for only CD11b (Fig. 14A ii). Cells positive for only IL-15 are either CD11b+ or B220+, with the majority being CD11b+ (54.7% compared to 26.2%; Fig. 14A iii). A similar profile exists for the CNS immune cells at the peak of disease (Fig. 14B i, ii, iii). The B220+ CD11b- population expressing IL-15 was not observed in B6 mice.

This characterization of immune cells present in the CNS of mice with active EAE at either onset or peak of disease indicated that further analysis of IL-15-expressing cell populations using additional markers will more precisely identify the immune cells expressing IL-15 in the CNS. For instance, immune cells double positive for CD11b and B220 have been previously described (Davoust et al., 2006) in the context of chronic EAE;

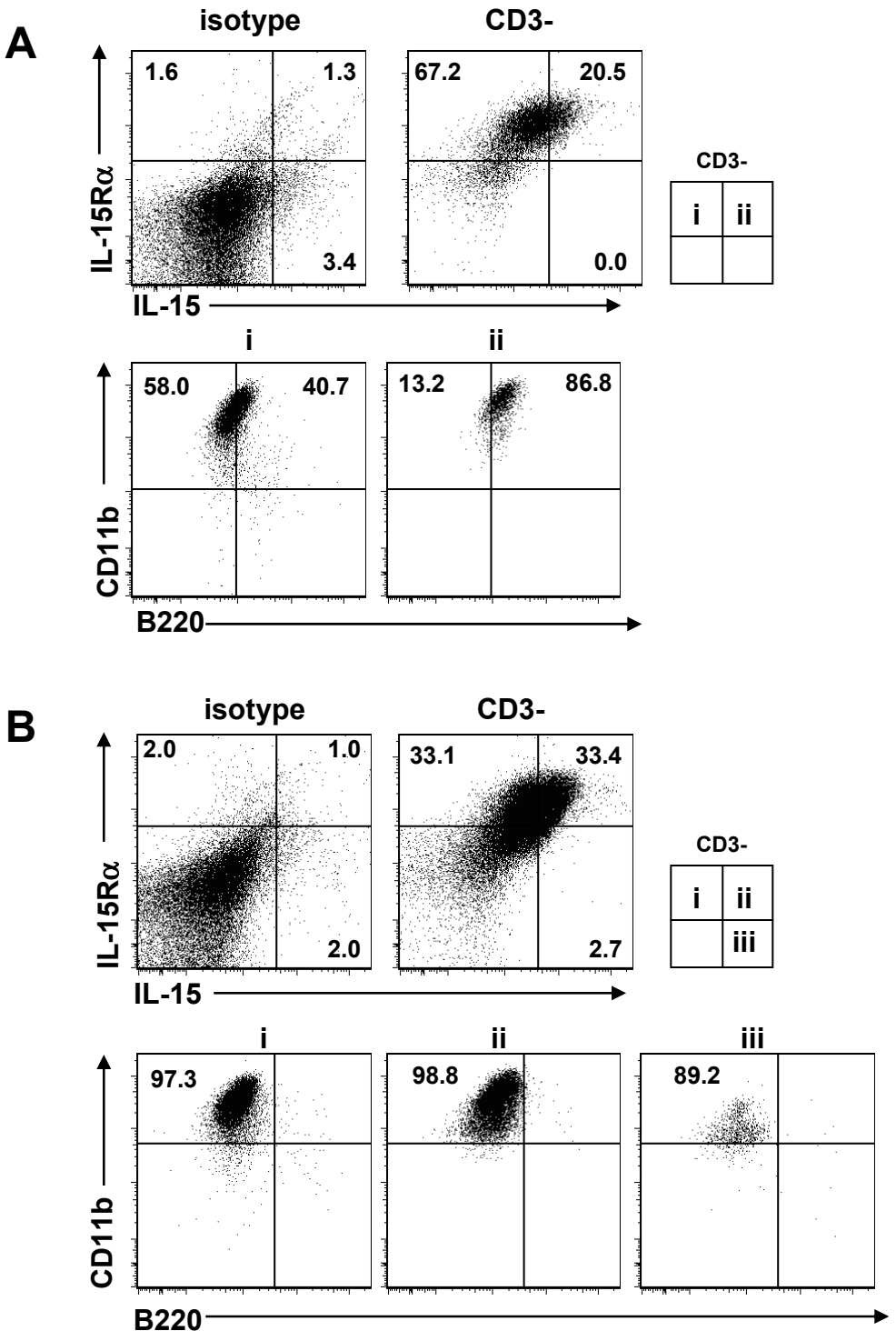

Fig.13. Characterization of IL-15-expressing lymphocytes isolated from CNS of B6 mice. Leukocytes were isolated from the CNS of PBS-perfused B6 mice with EAE, using a Percoll gradient. Cells were then stained and analyzed by flow cytometry (A) B6 mice at onset of disease (score 1.5). Proportion of cells co-expressing IL-15 and IL-15Rα are CD11b+ and B220-. (B) B6 mice at peak of disease (score 4.0). Cells positive for IL-15 are also CD11b+. Top panels of (A) and (B) gated on CD3 negative (CD3-) cells. Representative FACS plots of n=3-4 mice for each stage of disease.

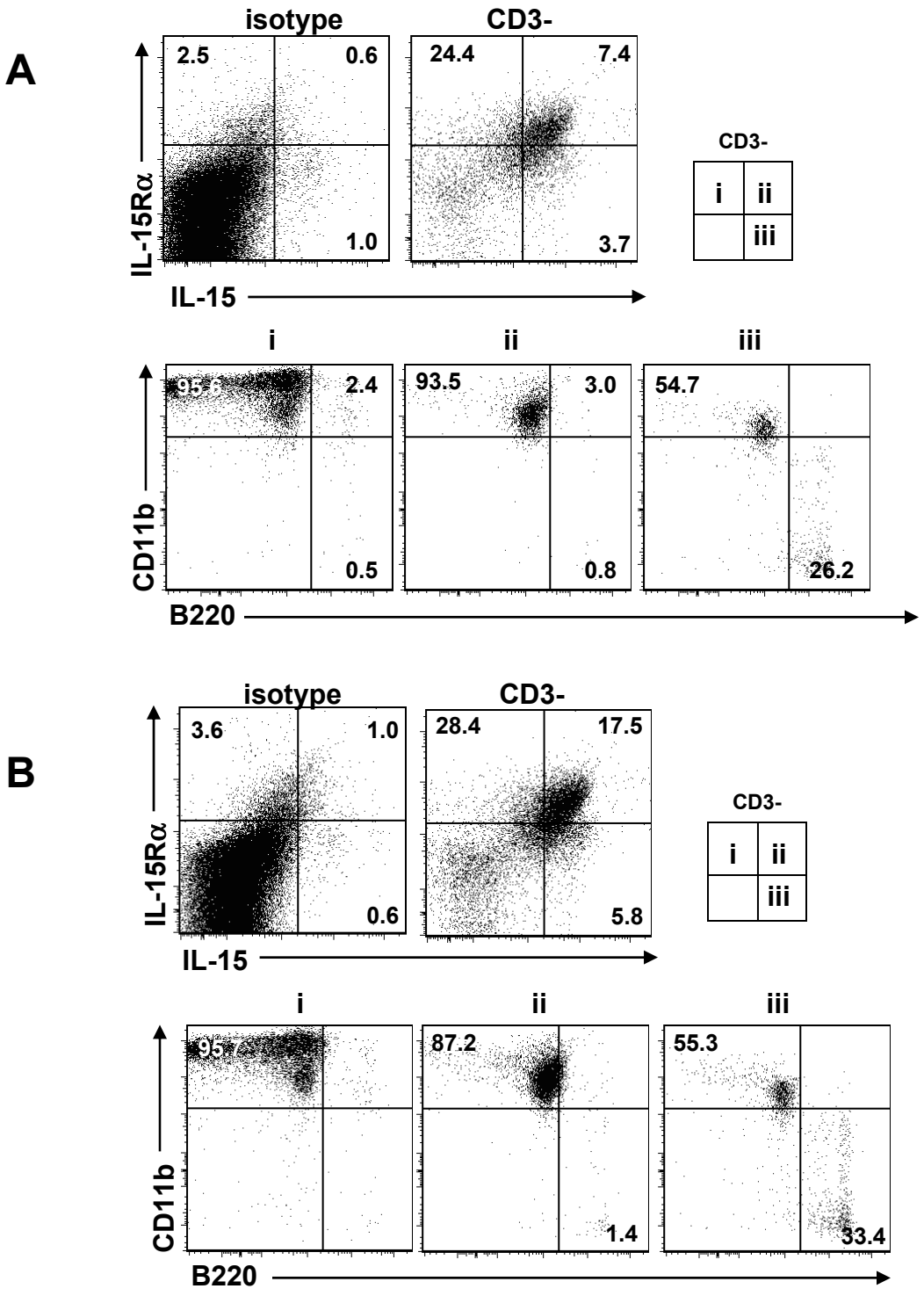

Fig.14. Characterization of IL-15-expressing lymphocytes isolated from the CNS of SJL mice. Leukocytes were isolated from the CNS of PBS-perfused SJL mice using Percoll gradient. Cells were then stained and analyzed by flow cytometry (A) SJL mice at onset of disease (score 1.0). IL-15+IL-15Rα+ cells are CD11b+B220-. IL-15+ cells are either CD11b+B220- or CD11b-B220+. (B) SJL mice at peak of disease (score 4.0). Double positive cells are also CD11b+. Cells positive for only IL-15 are CD11b-B220+ or CD11b+B220-. Top panels of (A) and (B) gated on CD3 negative cells. Representative FACS plots of n=2-4 mice for each disease stage.

their data suggest that these CD11b+/B220+ cells (also CD34+ in their study) develop into a subpopulation of microglial cells under neuroinflammatory conditions. However, we can still conclude that although further characterization of these immune cells is required, immune cells were present in the CNS of B6 and SJL mice expressing IL-15. Furthermore, IL-15 expression was specific to the immune cells found in the CNS and was not seen in those found in the spleen or in the draining lymph nodes. Results are representative of 2-4 mice; percentages varied between mice, but the overall trend remained the same.

2.5 T cells found in the CNS of mice with active EAE are responsive to IL-15

Signaling occurs through the IL-15Rβ (CD122) chain, shared with IL-2, and common γ chain, shared with other interleukins. IL-15Rα is required to bind strongly to IL-15 and to present the cytokine to the other receptor components. To determine whether T cells present in the target organ of EAE could respond to these increased levels of IL-15, leukocytes isolated from spleen, draining lymph nodes and the CNS of B6 and SJL mice at the onset and peak of disease were assessed for IL-15 receptor expression using flow cytometry (Figs. 15 and 16; representative of 2-4 mice). Overall, a greater proportion of T cells found in the CNS expressed both the alpha and beta chains (IL-15Rα and CD122 respectively) of the IL-15 receptor than did T cells found in the draining lymph nodes and spleen of the same animals. However, an important proportion of the T cells in the DLN and spleen did express the beta chain, which is shared with IL-2.

At the onset and peak of disease, a higher proportion of CD8 T cells found in the CNS of B6 mice expressed the alpha chain as compared to CD4 T cells (Fig. 15A and B, CNS panels). At onset, in the DLN and spleen, a proportion of T cells were double positive for both IL-15Rα and CD122; again greater in the CD8 T cell population than in the CD4 population (Fig. 15A). However, this diminished at the peak of disease, where we no longer saw the IL-15Rα positive cells present in either the DLN or spleen (Fig. 15B). In the CNS of SJL mice, we saw the same trend in the CNS, with a greater proportion of CD8 T cells expressing IL-15Rα and CD122 than CD4 T cells (Fig. 16A, B). However, the difference between onset and peak of disease with regards to the expression of IL-15Rα was not as pronounced in the DLN and spleen of SJL mice (Fig. 16A, B, DLN and SPLN panels). Overall, this indicates T cells found in the CNS were potentially responsive to IL-15.

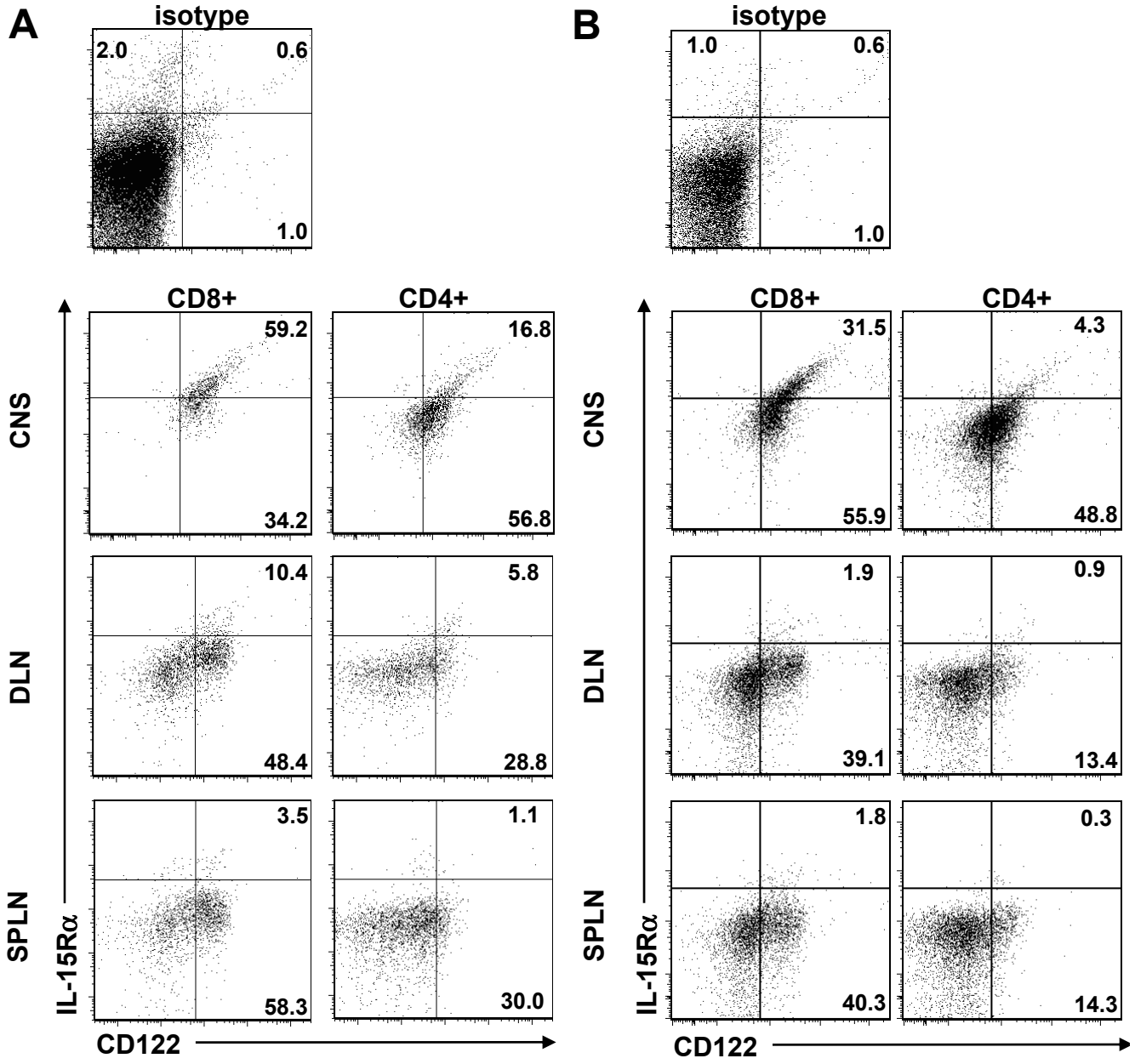

Fig.15. T cells found in the CNS of B6 mice are potentially responsive to IL-15. Leukocytes were isolated from organs obtained from PBS-perfused mice. Cells were then stained and analyzed by flow cytometry A greater proportion of CD8 T cells express CD122 in the CNS compared to T cells found in the draining lymph nodes (DLN) or in the spleen (SPLN). Representative FACS plots for mice at onset (n=3) or peak (n=4) of disease. B6 mouse at (A) onset, score 1.5 or (B) peak of disease, score 4.5.

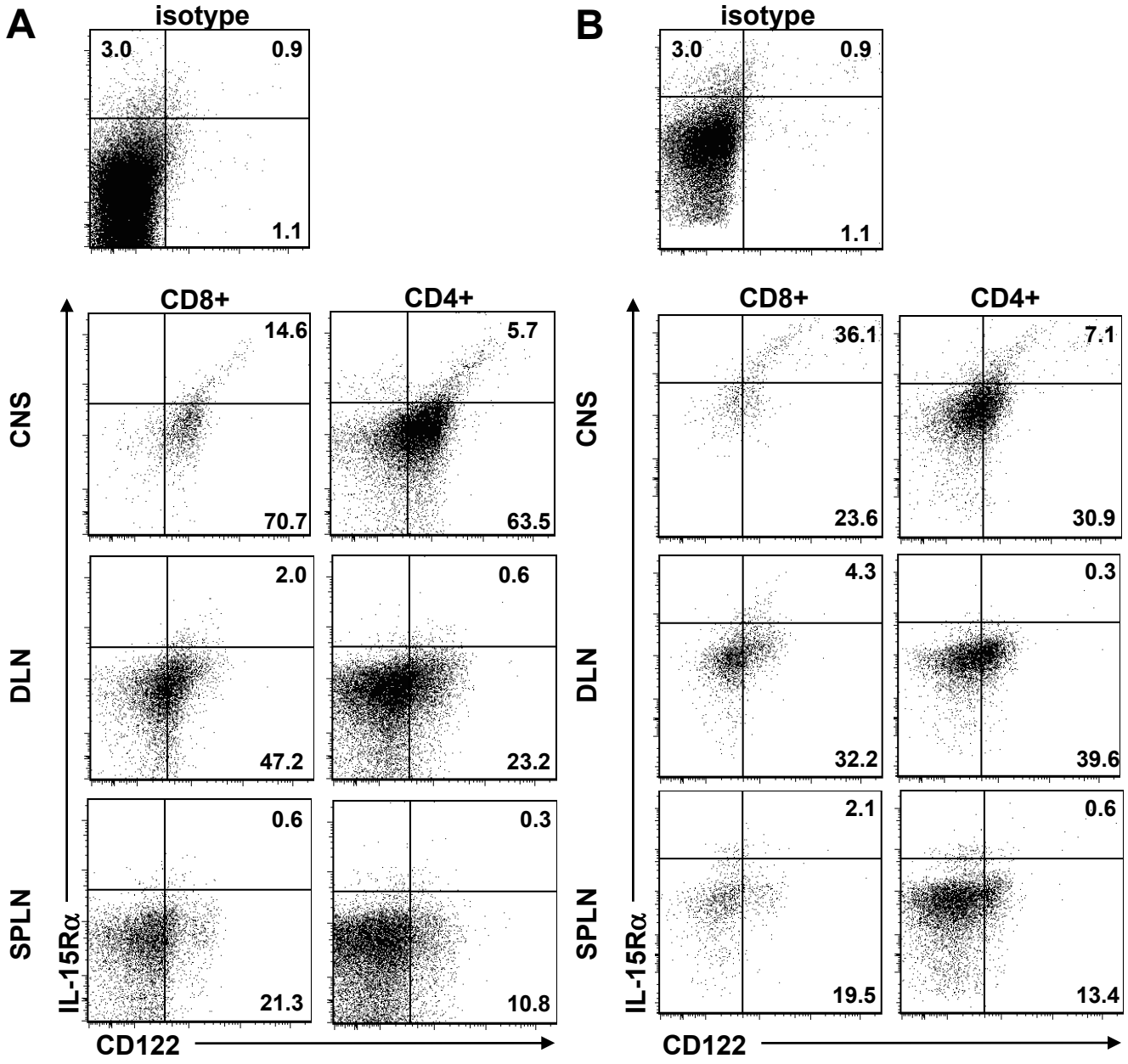

Fig.16: T cells found in the CNS of SJL mice are potentially responsive to IL-15. Leukocytes were isolated from organs obtained from PBS-perfused mice. Cells were then stained and analyzed by flow cytometry A greater proportion of CD8 T cells express CD122 in the CNS compared to T cells found in the draining lymph nodes (DLN) or in the spleen (SPLN). Representative FACS plots for mice at onset (n=2) or peak (n=4) of disease. SJL mouse at (A) onset, score 1.5 or (B) peak of disease, score 4.5.

DISCUSSION

IL-15 is a pleiotropic cytokine expressed in various tissues and cells at the mRNA level. However, at the protein level, IL-15 is not as extensively expressed; translation is under several regulatory checkpoints (Fehniger and Caligiuri, 2001). IL-15 is important for the development, homeostasis and activity of NK cells, as well for the survival and expansion of CD8 T cells. In conjunction with IL-7, IL-15 plays a crucial role in the MHC-independent homeostasis of memory CD8 T cells (Surh and Sprent, 2008). It is the role this cytokine plays in the maintenance of CD8 T cells that makes it a candidate for aberrant activation of T cells. CD8 T cells are already implicated in various diseases, due to their ability to directly target and kill cells. Under normal conditions, such an immune response is useful and necessary in the clearance of intracellular pathogens, but when disregulated, can lead to autoimmune disorders. IL-15 is a cytokine implicated in the maintenance and triggering of autoreactive CD8 T cells (Ramanathan et al., 2011) and also plays a crucial role in CD8 T cell migration and survival in organs (McGill et al., 2010; Verbist et al., 2011). Furthermore, it has already been implicated in various autoimmune diseases. In the context of MS, elevated levels of IL-15 and IL-15Rα, required for trans-presentation of this cytokine to effector cells, are found in the CNS at the mRNA level (Kurowska et al., 2002), surface-expression of IL-15 on monocytes has been reported in MS patients (Vaknin-Dembinsky et al., 2008), and our lab has shown elevated levels of IL-15 expressed by astrocytes and microglia/macrophages in MS lesions (Saikali et al., 2010).

1.0 EXPRESSION OF IL-15 BY HUMAN B CELLS

We have shown that with specific stimulation, B cells up-regulate the expression of both IL-15 and IL-15Rα, and present it on the cell surface. Furthermore, the IL-15 levels presented by activated B cells are sufficient to induce activation of CD8 T cells, enhancing the production of both granzyme B and IFNγ. Not only does IL-15 presented on B cells promote a cytotoxic profile of CD8 T cells, but also the ability of CD8 T cells to migrate across an *in vitro* BBB is significantly enhanced upon exposure to IL-15-expressing B cells.

1.1 Up-regulation of IL-15 expression

From among the various stimuli including HMGB1, CpG, LPS and αhu Ig, we tested, only one, CD40L, was sufficient to significantly enhance expression of IL-15 by B cells (Fig. 3A and C; Fig. 5C). The stability of surface IL-15 on B cells is unknown; however IL-15 has been described to be recycled through endosomal vesicles of monocytes and DCs, allowing for the persistence of membrane-bound IL-15 for many days (reviewed in (Waldmann, 2006)). The expression of IL-15 on B cells may perhaps be maintained via the same mechanism; furthermore it would be of interest to investigate whether the stimulation of B cells triggers a transport of intracellular pools of IL-15 to the cell surface in response.

In the context of disease, CD40L as a stimulus is highly relevant: MS patients have a higher frequency of CD40L-expressing T cells than controls (Teleshova et al., 2000), and activated CD4 T cells expressing CD40L are found in MS patient brains (Gerritse et al., 1996b). In addition, CD40-expressing cells are also found in MS patient brain tissue, most of which are either CD11b+ macrophages/microglia or B cells (Gerritse et al., 1996b). Astrocytes have also been found to express CD40 (Kim et al., 2011). Other stimuli tested (HMGB1, CpG, LPS, αhu Ig) did not result in up-regulation of IL-15, although HLA-DR expression was enhanced (Fig 1B), indicating the cells were activated. While activation via TLR stimulation did not induce a significant up-regulation of IL-15 (Fig. 3A and C), using such stimuli still warrants further investigation. Levels of TLR expressed by B cells vary according to the subset under consideration (Booth et al., 2011), and thus the varied or lack of response to TLR agonists may differ according to the proportions of cell subsets and to TLR levels.

Although we did not look at specific B cell subsets, it would be interesting to examine the response of these subsets to TLR stimulation. For instance, while TLR4 and TLR1 mRNA is detected in all B cell subpopulations, TLR7 and TLR9 levels vary depending on the B cell subset (Bekeredjian-Ding and Jego, 2009); TLR9 while not on naïve B cells, is expressed by memory B cells (Gururajan et al., 2007). TLR3 mRNA is detected only in marginal zone B cells, but TLR2 is most prominent in B-1 B cells (Bekeredjian-Ding and Jego, 2009). Thus the response to TLR stimulation would most likely vary, depending on the activation state and the subset of B cell present in culture. Further investigation into the response to TLR stimulation by B cells is warranted, using

such markers as IgD and CD27 to differentiate between naïve and memory B cells (IgD is down-regulated in B cells acquiring the memory marker CD27) (Bleesing and Fleisher, 2001); CD23 and CD21 can be used to identify marginal zone B cells ($CD23^{-}CD21^{+}$) from follicular B cells ($CD23^{+}CD21^{-}$) (Gururajan et al., 2007).

Several lines of evidence indicate a role for TLRs in the pathogenesis of MS, and in EAE, and considering the effects of TLR stimulation on B cells including up-regulation of cytokine secretion, there might very well be a role for TLR stimulation in the production of IL-15 under specific conditions. For instance, TLR9 is constitutively expressed by B cells and DC (Marshak-Rothstein and Rifkin, 2007). Stimulation of TLR9 results in exacerbation of disease in an animal model of MS (Brien and Gran, 2008) and conversely, the absence of TLR9 (i.e. TLR9 -/- mice) results in a delayed EAE induction and decreased disease severity (Prinz et al., 2006); furthermore, a decrease in the expression of TLR4 and/or 9 has been associated with amelioration of EAE (Chearwae and Bright, 2008). While normally TLR9 recognizes viral and bacterial DNA, it has also been shown to recognize host genetic material (Marshak-Rothstein and Rifkin, 2007), which may very well be found in active MS lesions with ongoing cell damage. However stimulation through TLR9 alone may be insufficient to induce up-regulation of cytokines, and additional stimuli through other TLRs or other means may be required.

TLRs can regulate the expression of other TLRs, as in the case with TLR 2 and 9 regulating TLR4 expression on B cells (Jagannathan et al., 2009). In the case of TLR4, B cells typically express very low levels, but upon activation or under inflammatory conditions, they are capable of up-regulating expression of TLR4 (Jagannathan et al., 2009; Mita et al., 2002). Here, we used LPS from *E. coli* as a stimulus; however considering the normally low expression levels of TLR4, it may indicate pre-activation or simultaneous stimulation of B cells is required to induce a response in spite of the typically potent effects of LPS in the immune response. Alternatively, perhaps stimulation using such agonists as would be found in MS lesions would be more relevant, as they may have additional (inflammatory) effects via pathways other than TLR stimulation. While typically TLR ligands have been described to be PAMPs, non-pathogenic molecules have also been described to trigger inflammation: host genetic material via TLR9 (Marshak-Rothstein and Rifkin, 2007), and agonists of TLR2 and TLR4 include heat shock proteins (Hsp), proteolytic cleavage products and extracellular matrix components (Kigerl et al.,

2007). One such possible stimulus could be Hsp70, which has been found in MS lesions and associated with MBP and PLP, the two most abundant proteins of the CNS myelin sheath (Aquino et al., 1997; Cwiklinska et al., 2003). Over-expression of Hsp70 *in vitro* enhances the activation of APCs, and induces immune cells to produce cytokines and chemokines (Turturici et al., 2011); an enhanced production of IL-15 could be possible using this stimulation. Of course, it is also quite possible that the stimulation of TLRs on B cells has no direct effect of the production of IL-15, but rather simply serves to enhance or support the activation state of B cells, and prolong cell survival.

Thus, while stimulation of various TLRs did not induce an up-regulation of surface IL-15, this could be due to B cells expressing low basal levels of the TLR, as in the case of TLR4, the need to use a more disease-relevant stimulus, or the requirement for additional stimulus, either through another TLR or other means such as BCR cross-linking. Multiple stimuli, or combinations of stimuli might also be required to have an effect; for instance, in inducing an up-regulation of IL-15 on astrocytes, a combination of cytokines was required rather than any one stimulus alone (Saikali et al., 2010). CD40L, in contrast to TLR stimulation, proved to be a sufficient stimulus for B cells in significantly up-regulating the expression of IL-15, and IL-15Rα, by B cells at the mRNA and protein levels. Contributing to the different effects of the various B cell stimuli, CD40 signals through a different pathway, stimulation recruiting various TNFR associated factors (Peters et al., 2009b) as opposed to activation of the MyD88 pathway, providing an explanation as to the differing effects of CD40 vs. TLR stimulation of B cells in promoting IL-15 expression.

1.2 Effect of IL-15 on CD8 T cells

We examined the functional effects of IL-15-expressing B cells on CD8 T cells, and found that the cytotoxic profile was enhanced. Exposure to surface IL-15 expressed by B cells increased proliferation and the expression of granzyme B (Fig. 6A) and IFNγ (Fig. 6B). However, there was no overall trend in the effect of IL-15 on total IFNγ production whereas there was a significant production of granzyme B by proliferated and total CD8 T cells, in response to IL-15. A previous study has shown IL-15 to be more important in the up-regulation of granzyme B than for that of IFNγ by CD8 T cells (Marshall et al., 2010). Furthermore, the ability of CD8 T cells to migrate across an *in vitro* BBB was affected by the presence or absence of IL-15. Blocking IL-15 on B cells led to a significant decrease in

the number of CD8 T cells which migrated across the BBB (Fig. 7B). We confirmed that the increased migration of CD8 T cells in the presence of IL-15 was not due to a breach in the integrity of the *in vitro* BBB (data not shown). Interestingly, a study done by Suidan et al. demonstrates a non-apoptotic perforin dependent mechanism for CD8 T cells to disrupt the tight junctions of the BBB and migrate into the CNS, in a viral model of MS (Suidan et al., 2008). Since it seems CD8 T cells activated by IL-15 migrated across our model of the BBB without damaging the cells, it would be interesting to examine the role of perforin in our model of BBB *trans*-migration, and the role IL-15 plays, if any, in influencing its expression levels by CD8 T cells. It would also be interesting to look at the possible up-/down-regulation of various other molecules found on activated CD8 T cells, such as PD-1 and Fas.

The effect IL-15 presented by B cells has on the cytotoxicity and migration ability of CD8 T cells is of interest, considering the effects of B cell depletion in multiple sclerosis. B cell depletion with rituximab, a CD20 monoclonal Ab, has been effective in that the occurrence of relapses and new gadolinium-enhancing lesions in MS patients are reduced (Hauser et al., 2008). The effects of this treatment are not due to depletion of Ab-producing plasma cells and only a modest reduction of anti-myelin antibodies has been reported (Cross et al., 2006); the long-term effect of this treatment on antibody levels are as yet unknown. As it seems B cell depletion in MS patients does not have an effect via Ab depletion, there must be an alternative mechanism. It is likely that removing B cells from the peripheral blood interferes with the activation of T cells mediated by pro-inflammatory cytokines and co-stimulatory molecules such as CD86 provided by B cells (Bar-Or et al., 2010). Interestingly, we also observed elevated levels of CD86 expressed on B cells after stimulation with CD40L; thus IL-15 could be one such cytokine influencing the activation state of T cells and their cytotoxic response in MS.

Considering the involvement of IL-15 in MS and other inflammatory diseases, the question must be asked whether there are any polymorphisms in either the cytokine or receptor genes which are associated with disease. Polymorphisms in IL-15 have been shown in specific populations of patients with psoriasis (Weger et al., 2008; Zhang et al., 2007), and have been indicated in an increased risk for coronary heart disease (Gokkusu et al., 2010). However, in rheumatoid arthritis, a screen of IL-15 polymorphisms did not demonstrate a role for IL-15 gene polymorphisms in genetic predisposition to developing

rheumatoid arthritis. Overall, there does not seem to be a strong relationship between variants of IL-15 or IL-15Rα and any one disease; further studies perhaps need to be carried out.

In conclusion, IL-15 is up-regulated on B cells under inflammatory-like conditions, and is capable of promoting proliferation in addition to enhancing the cytotoxic profile and *trans*-migration capacity of CD8 T cells. Elevated IL-15 levels have been implicated in various inflammatory diseases, and interestingly CD8 T cells have also been implicated in many of them as well (Liu et al., 2000; Mention et al., 2003; Ramanathan et al., 2011; Sakai et al., 1998). Clinical trials targeting IL-15 in various inflammatory diseases have been completed or are ongoing. These potential therapies block IL-15 using soluble anti-IL-15 antibodies. Therapeutic strategies specifically targeting this cytokine could equally be beneficial in ameliorating the course and severity of MS, by decreasing CD8 T cell cytotoxicity as well as by limiting access to the inflamed CNS thus diminishing CD8 T cell-mediated damage. On the other hand, boosting the levels and effects of IL-15 on CD8 T cells would be beneficial, in such cases as cancer, where a cytotoxic response is wanted.

2.0 CHARACTERIZATION OF IL-15 IN CLASSICAL EAE

EAE is a much used animal model for Multiple Sclerosis. While it is not able to fully mimic the disease as it is found in humans, various aspects of the disease can be studied and the influences and roles of various factors examined so as to extrapolate potential culprits of disease pathogenesis back to the human disease. There are studies examining the role of IL-15 in EAE (Gomez-Nicola et al., 2010; Wu et al., 2010) as well as in other neuroinflammatory models (Gómez-Nicola et al., 2008b). However the expression pattern of IL-15 during the course of disease has not been characterized. Characterizing the expression of IL-15: when it is expressed, where it is expressed, to what degree is expression up- or down-regulated, is relevant to understanding the effects and role of IL-15 in disease exacerbation, or amelioration.

We compared the course of active EAE induced in two different strains of mice: SJL/J and C57BL/6, and examined the IL-15 expression in each at various stages of disease. All mice chosen for this study had similar disease courses. However, it would be interesting in future studies to expand the study to include mice with atypical EAE, as well

as mice which had a more rapid disease progression to be able to examine the potential variation that exists not only between genetic backgrounds, but also if there are differences in IL-15 expression related to the severity and course of disease. We were able to detect IL-15 at both the mRNA and protein level in the CNS of these mice during the course of disease. The presence of IL-15 was specific to the CNS, and was not found in either the spleen or draining lymph nodes.

2.1 Detection of IL-15

In general, it is difficult to detect IL-15 in mice, thus we elected to first look for IL-15 expression using qPCR, and to see if there were any differences in the expression levels in various components of the CNS of this cytokine during the course of disease in mice with EAE.

We detected IL-15 at the mRNA level in both SJL and B6 mice, at the onset and peak (and remission) of disease. In B6 mice, there were no significant differences in IL-15 expression levels detected in the spleen and liver of these animals during disease as compared to control mice (Fig. 9A). However, upon examining component parts of the CNS, we saw differences in IL-15 expression during the course of disease, with IL-15 levels in the cortex lower than that of controls during the course of disease; in the brainstem, at the onset and peak there was increased IL-15 expression but in remission almost no IL-15 was detected; and in the spinal cord, IL-15 was more expressed during the course of disease (Fig. 10A). In SJL mice, we did not detect differences in IL-15 expression in the CNS to the extent observed in B6 mice (compare Fig 10B with 10A), with the exception of the decrease in IL-15 expression at the peak of disease in the cortex. However, it seems that IL-15 may be down-regulated during the course of disease in general, but not in the CNS: in the spleen and liver of SJL mice at the peak of disease, there was decreased IL-15 expression as compared to controls (Fig. 9B). It must be noted that EAE induced in SJL mice is primarily a CD4 T cell-mediated disease. While IL-15 has been demonstrated to have an effect on CD4 T cells (Vaknin-Dembinsky et al., 2008), but its effects have primarily been described on CD8 T cells. Thus perhaps the levels of IL-15 in SJL mice are reflective of the extent to which CD8 T cells play a role in disease pathogenesis in this particular model.

The detection of IL-15 mRNA and the differences in expression level found in various components of the CNS indicated there might be differences in the expression of IL-15 at the protein level during the course of disease, even though the presence of mRNA does not always necessarily correspond to translated protein and thus protein levels. However, we were able to detect IL-15 expressed on the surface of immune cells isolated from the CNS (brain and spinal cord) of PBS-perfused animals.

The expression of IL-15 protein was limited specifically to the CNS, and was not detected in the spleen or in the draining lymph nodes to the emulsion site. Furthermore, we also examined the expression profile of the IL-15Rα chain, and found that it was also expressed specifically in the CD3 negative cells found in the CNS. However, a small proportion (10-12%; Fig. 11A) of CD3 negative cells in the spleen and draining lymph node at the onset of disease expressed IL-15Rα. This was specific to B6 mice only. In SJL mice at the onset of disease, a proportion of CD3 negative cells in the draining lymph node and, to a lesser extent, in the spleen, expressed IL-15, without co-expression of the Rα.

Interestingly, using CD11b and B220 as markers for macrophages/microglia and B cells respectively, we found differences in the cell profiles of IL-15-expressing immune cells in the CNS of B6 and SJL mice. In B6 mice, we observed a population of $CD11b^{+}B220^{+}$ cells present in the CNS at the onset of disease, which was not seen in the SJL mice (Fig. 13A). Normally CD11b is a marker for macrophages/microglia and B220 for B cells. However, a previous study has described a population of $CD11b^{+}B220^{+}$ (and $CD34^{+}$) cells found in the murine brain under neuroinflammatory conditions (Davoust et al., 2006). Their study suggests that a pool of $CD34^{+}CD11b^{+}B220^{+}$ cells migrating from the periphery into the CNS may proliferate and give rise to activated microglia, double positive for CD11b and B220, and thus promote ongoing CNS inflammation. Interestingly, they also define a population of cells which are $CD34^{+}B220^{+}$ but CD11b-, suggesting that they may be microglial progenitors, based on a study identifying CD11b- myeloid progenitors in the CNS (Alliot et al., 1991; Davoust et al., 2006). In our study, we did not see a population of $CD11b^{-}B220^{+}$ cells in the CNS of B6 mice. However, such a population was identified in the CNS of SJL mice. Whether these cells have the capacity to proliferate and differentiate into microglial cells would be interesting to determine. Further characterization of the immune cells found in the CNS at both peak and onset of disease is necessary. In addition, it would be interesting to compare the cell profile of immune cells

found in the CNS of mice in remission during the course of EAE, as well as those of IL-15^{+}IL-$15R\alpha^{+}$ vs. IL-15^{+}IL-$15R\alpha^{-}$ cells. B220, while commonly used as a pan-marker for B cells, is also expressed on activated NK and T cells, as well as on NK progenitors (Lai et al., 1998), and so other markers may be used to identify more precisely the IL-15^{+}IL-$15R\alpha^{-}$ expressing cells in the SJL CNS. These could include CD19, a marker more restricted to the B cell lineage, CD80 and CD86 in conjunction with CD19 would identify activated B cells, CD23, CD5 and CD11b expression can be used to distinguish between "conventional" and "non-conventional" B cells (Lai et al., 1998).

In the B6 mice, the profile of cells expressing IL-15 and/or IL-15Rα had similar profiles. At onset, there was a population of cells double positive for CD11b and B220, as well as a population expressing only CD11b. At the peak of disease, cells were found to be mainly CD11b positive. However, cells expressing only IL-15 without detection of the IL-15Rα, while expressing CD11b, seemed to express it at lower levels as compared to cells expressing IL-15 and/or IL15Rα. In SJL mice, the profiles of IL-15-expressing immune cells were considerably different from those seen in B6 mice. First, the cell population positive for only IL-15Rα were mostly positive for CD11b. Interestingly, there seems to be two populations: one that expresses high levels of CD11b, and another still expressing CD11b but lower levels. The IL-15/IL-15Rα double positive population of CD3 negative cells was CD11b+. Lastly, the cells expressing IL-15 but not IL-15Rα were either CD11b+ (approximately 55%) or B220+ (26-33%). The differences in the profile of cells found in the CNS of B6 and SJL mice indicate that there are differences in IL-15 expression due to genetic background and course of disease. SJL mice often showed symptoms sooner than B6 mice and followed a more severe course of disease, reaching our defined end-points sooner than their B6 counterparts. The presence of immune cells presenting IL-15 in the CNS as well as the differences noted, seem to indicate that perhaps IL-15 does have a role in the course of EAE, either in promoting inflammation or influencing the course and severity of disease. Further work needs to be done, to quantify the number of cells expressing IL-15.

There are various reports of the role of IL-15 in neuroinflammation. On the one hand, IL-15 has been reported to have beneficial effects in EAE (Wu et al., 2010), and that IL-15 knockout mice have more severe EAE (Gomez-Nicola et al., 2010). However, mice

deficient in IL-15 have an impaired immune system to begin with, with low numbers of NK and memory CD8 T cells (Kennedy et al., 2000), and the average clinical score of the wild-type mice in these studies (Gomez-Nicola et al., 2010; Wu et al., 2010) are quite low (average score of 1.0-1.5), as compared to other reports and to what we obtained in our study. This would indicate that conclusions drawn from these studies using IL-15 knockout mice must be made with caution. Indeed, on the other hand there are reports of IL-15 playing a role in aggravating neuroinflammation and that inhibiting its effects would be beneficial. One study found that the expression of IL-15 served as a signal for macrophage and T cell activation and infiltration into the CNS (Gómez-Nicola et al., 2008b). Blocking the activity of IL-15 in the CNS under inflammatory conditions prevented glial activation and reduced the production of pro-inflammatory molecules such as IL-1β. Thus the potential role of IL-15 in neuroinflammation, more specifically in EAE, is still uncertain, and requires further investigation.

Further adding to the complexity, $CD122^+$ CD8 T cells have been reported to have a regulatory role in EAE (Lee et al., 2008). Depletion of these cells resulted in persistent disease and increased infiltration and cytokine production of T cells in the CNS. Transfer of $CD122^+$ CD8 T cells at the peak of EAE resulted in diminished EAE symptoms, especially in the recovery phase of disease (Lee et al., 2008). Studies have shown these cells to be involved in the regulation of $CD122^-$ CD8 T cells independent of Ag (Rifa'i et al., 2004) and suppress proliferation of $CD122^-$ cells by secreting IL-10 (Endharti et al., 2005). These regulatory CD8 T cells are potentially responsive to IL-15; however they are also responsive to IL-2. The relative contribution of each of these cytokines may be one of the determining factors for the development of a regulatory response by CD8 T cells. Examination of the role of IL-15 in promoting a regulatory response by CD8 T cells would contribute to understanding the role of this cytokine in the progression of EAE, whether it acts to ameliorate or exacerbate disease.

We found that CNS-infiltrating T cells are potentially responsive to IL-15, expressing CD122 (IL-2/15Rβ) in addition to IL-15Rα. While signaling is mediated through CD122 and the common gamma chain, there seems to be a role for IL-15Rα expressed on T cells as well. The unique presence of IL-15Rα-expressing T cells in the CNS and not in the draining lymph nodes and spleen (Figs. 15&16) is indicative of their

activation state and responsiveness to IL-15. One study detected IL-15Rα expression on dividing T cells, and demonstrated that IL-15 was necessary for stimulating cell division *in vivo* (Li et al., 2001). Furthermore, in a transgenic mouse model (for leukemia) constitutively expressing IL-15, it was found that aggressive uncontrolled expansion of CD8 T cells was dependent on IL-15Rα expression; non-activated T cells were not found to express IL-15Rα (Sato et al., 2011). Thus the presence of IL-15 in the CNS would be responsible for the activation and proliferation of CNS-infiltrating CD8 and, to a lesser extent, CD4 T cells expressing IL-15Rα. This would also serve to explain the different profiles of T cells found in the CNS, and those in the spleen or draining lymph nodes. IL-15Rα-expressing T cells were only found in the CNS. Not only can IL-15 promote activation and infiltration of macrophages and T cells (Gómez-Nicola et al., 2008b), but also, once within the CNS, the activation and proliferation of T cells promoted by IL-15 in EAE may be restricted to those cells expressing the IL-15Rα as already indicated in other models (Li et al., 2001; Sato et al., 2011). Further studies will need to be carried out, examining more mice at the onset and peak of disease, to determine the role of IL-15 and possibly the role of IL-15Rα during the course of disease.

The implicated importance of both IL-15 and IL-15Rα indicates that further characterization of the role of IL-15Rα expressed by CNS-infiltrating CD8 T cells would be of great interest in addition to determining the effect of IL-15 on these cells and in modulating disease course. As IL-15 has a great influence on memory CD8 T cells, it would be interesting to characterize specific cell subsets of CD8 T cells, to differentiate between CD8 effector and central memory cells. Future studies could include identifying subsets of T cells expressing IL-15Rα, whether it is limited to one subset or if it is ubiquitously expressed among the T cell population; this would involve examining cells infiltrating the CNS, compared to cells found in the spleen and draining lymph node. In addition, the role of IL-15 in non-specific (i.e. antigen independent) activation and proliferation of T cells isolated from organs could be determined, and compared to specific re-activation of immune cells by corresponding peptides (i.e. immune cells isolated from B6 mice would be reactivated by MOG_{35-55}). The proportions of CD8 and CD4 T cells as well as the proportions of each expressing IL-15Rα determined and compared.

We have preliminary data indicating that cells from the spleen and draining lymph nodes are potentially responsive to reactivation with peptide, in that they produce IFNγ. It would be of great interest to determine whether the presence or absence of IL-15 can influence the response of T cells to peptide restimulation, and whether there is a relationship with the proportion of responsive T cells expressing IL-15Rα.

To be able to determine the role of IL-15 in influencing the course of EAE, it is useful to use animals deficient in the molecule of interest. However, as previously noted, the immune system and response of IL-15 knockout mice are skewed (the numbers of NK and CD8 T cells are substantially decreased (Kennedy et al., 2000)) and thus inducing active EAE will not give reliable results regarding the role of IL-15. Studies using IL-15 knockout mice have been done, but it must be noted that in these studies, the severity of disease was not very great. In one study, the highest score observed in wild type mice was around 1.6; in the knockout, scores averaged at 2.4 (Gomez-Nicola et al., 2010). In another, wild type mice with active EAE hovered at around 1.0, while the knockout reached scores of 2.5; administering IL-15 reduced the severity of EAE, with mice showing scores of around 2.0 (Wu et al., 2010). While it seems IL-15 has a beneficial effect on the course of EAE, there already is a difference in the induction of disease due to immune system differences between wild type and IL-15 knockout mice, thus creating a bias to the results observed. This potential bias may be overcome by adoptive transfer of immune cells into knockout mice, and inducing passive EAE.

In addition to the role of IL-15, the effect of IL-15 on specific immune cell subsets can also be examined. For example, CD8 or CD4 T cells reactivated *in vitro* may be transferred, and the effect of IL-15 on disease mediated by each of these cell types determined. The role of IL-15 on immune cells found in the CNS, spleen and draining lymph nodes should perhaps be determined prior to adoptive transfer. Considering the previously described role for IL-15Rα in T cell responsiveness to IL-15 and if the addition of IL-15 promotes the re-activation of a specific subset of immune cells, then it may be these cells which need to be transferred to determine the effects of IL-15 in the course of EAE. Results thus far would indicate that CNS-infiltrating T cells are more likely to respond to the addition of IL-15, and thus might be preferable for transfer. However, the numbers of immune cells isolated from the CNS of sick animals is variable, and perhaps too little to efficiently induce passive EAE. To examine the effects of IL-15 provided by

cells of the CNS vs. peripheral cells, mice deficient in IL-15 in either the CNS or in the peripheral cells may be used.

Lastly, further experiments can be extended to immunohistochemistry. We have frozen sections of CNS tissue, in addition to spleen and liver, from mice at various stages of disease. IL-15 has proven thus far to be difficult to detect in murine brain sections, but this is currently being optimized in our lab. We are planning to examine the expression and presence of T cells, B cells, macrophages/microglia, and astrocytes in relation to IL-15. We will also try to correlate the spatial relationship of cells with each other, to see what cell types are interacting with each other, as well as examine the pathogenesis of disease, including demyelination and axonal loss.

The presence of IL-15 expressed on immune cells found in the CNS of mice with active EAE, as well as the specific expression of IL-15Rα on T cells also found in the CNS in addition to CD122 all indicate that IL-15 likely has a role in the course of disease. However, further experiments are required to further and more specifically characterize the CNS-infiltrating immune cells expressing IL-15, and to determine the role and influence of IL-15 in modulating the disease course. For instance, using a blocking antibody, IL-15 can be blocked in the periphery, which would demonstrate the effects and role of peripheral IL-15 as opposed to the IL-15 to be found within the CNS – whether peripheral IL-15 enhances immune cell infiltration into the CNS, whether the extent to which it activates cells in the periphery influences the ultimate effects observed within the CNS, or whether, indeed, it has a role in promoting the development of regulatory CD8 T cells. The presence of CD122+ CD8 T cells, potentially responsive to IL-15, has already been shown to have a regulatory role in EAE (Lee et al., 2008), supporting a positive effect of IL-15 on disease pathogenesis through promotion of regulatory T cell development.

CONCLUSION

IL-15 is a cytokine that has already been implicated in various autoimmune diseases (Davey et al., 2005; DePaolo et al., 2011; McInnes and Gracie, 2004; Thurkow et al., 1997). Its role in such cases are most likely mediated through CD8 T cells, as it is these cells that IL-15 affects the most. Furthermore, as CD8 T cells are capable of directly causing damage to cells, non-specific activation (i.e. Ag-independent) of these cells could lead to grave consequences, such as the activation of self-reactive cells (Ramanathan et al., 2011). Self-reactive cells that are aberrantly activated have the potential to recognize self-Ag and cause damage, resulting in disease.

While IL-15 is detected in MS patients (Saikali et al., 2010; Schneider et al., in press; Vaknin-Dembinsky et al., 2008), its contribution to disease pathogenesis remains unknown; whether it plays a role in the initiation of disease, in relapses, or in recovery needs to be identified. Using animal models to mimic aspects of MS have proved to be useful thus far, and will continue to be so. Characterizing the expression profile of IL-15 during disease course of EAE in the mouse model allows us to take the first steps towards understanding the contribution and role of IL-15 in disease pathogenesis in EAE, which can then be extrapolated back to MS. Of course, it is hoped that developing an understanding of the role of this, or any, molecule in disease pathogenesis will lead to the identification of new therapeutic targets. There is great potential for IL-15 as a target in MS; it has already been demonstrated to be an effective target in the treatment of rheumatoid arthritis and psoriasis. Understanding when IL-15 plays a significant role in disease pathogenesis will hopefully allow the development of additional treatments for MS.

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