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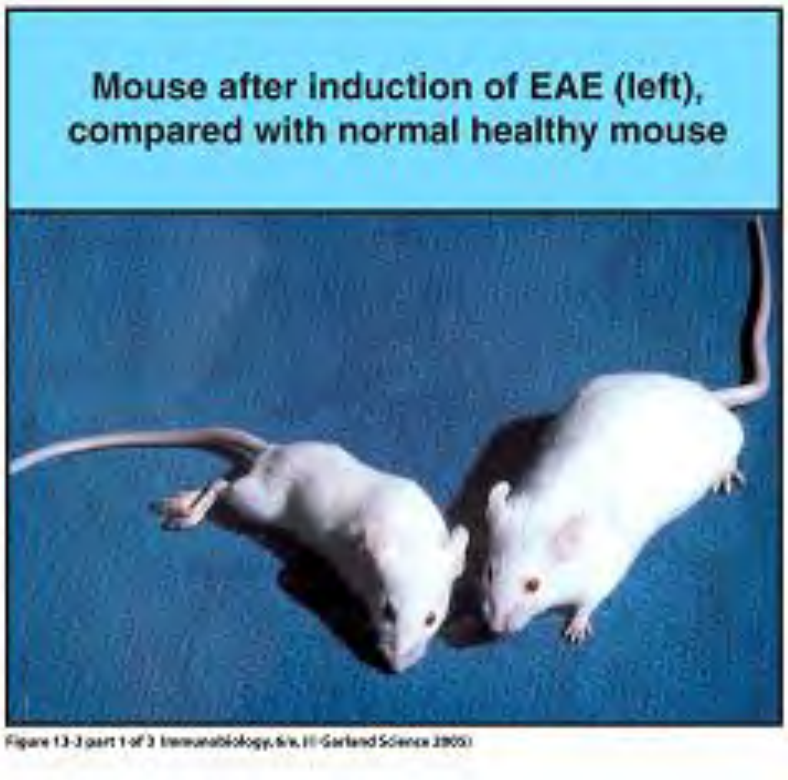
Experimental Autoimmune Encephalomyelitis

Identification of the Antigen Presenting Cell Responsible for Priming Th17 Lymphocytes in EAE



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Abstract
The study of experimental autoimmune encephalomyelitis (EAE) as an animal model disease for human Multiple Sclerosis (MS) brings more options that are otherwise unavailable. Thanks to this model, many issues have been deciphered paving the way to take a step closer toward understanding not only MS, but rather autoimmunity including many other autoimmune diseases. In our experiment, we aim to investigate which Antigen Presenting Cell (APC) presents Myelin Oligodendrocyte Glycoprotein (MOG) to T helper 17 (T_h17) lymphocytes in EAE and induce its initiation. This is done by the introduction of the Cre LoxP system into the mouse genome, a system that allows researchers to manipulate the immune system in ways that enable studying certain functions of its different components. The expression of the Cre recombinase in cells with a gene flanked with directly oriented repeats of LoxP sites allows knocking the gene out forming a circular DNA fragment that is unable of replication and transcription initiation. In our model, LoxP sites flanked Exon I in the A complex of the beta chain of Major Histocompatibility Complex (MHC) class II. The introduction of the gene encoding Cre recombinase under cell-type specific promoters ensures the expression of the enzyme conditionally in the respective cell type. The three C57BL/6 mouse strains we used in our model were trans-genetically engineered to clone the Cre gene into the DNA downstream of three promoters which are specifically expressed in one of the three APCs. The three strains: CD11c-Cre, CD19-Cre and Lyz2-Cre- have the Cre recombinase encoded under the DC-specific promoter, CD11c; the B-cell-specific promoter, CD19; and the macrophage-specific promoter, Lyz2; respectively. We assume that by crossing these three strains with the IA-b fl/fl strain, we will be able to identify the MOG-presenting APC to T_h17 lymphocytes.

1. Introduction

1.1 The Immune System

1.1.1 Definition

The Immune system has evolved to protect the body from infections and cancers. It is divided into innate and adaptive immunity that work in concert to preserve the integrity of the internal milieu. The immune system is composed of organs, tissues, cells and molecules that work together. Any disturbance in any component of the immune system might lead to immunodeficiency or autoimmunity.

1.1.2 Lymphocytes

Lymphocytes are produced in the bone marrow from which B lymphocytes exit as fully mature while T lymphocytes exit as immature and migrate to the thymus, the site of their maturation. Auto-reactive B lymphocytes are deleted by apoptosis in the bone marrow, while auto-reactive T lymphocytes are deleted in the thymus. Additionally, apoptosis is induced in T lymphocytes that cannot bind to MHC molecules in animals, or human leukocyte antigen (HLA) molecules in humans. Cytotoxic T lymphocytes (CTL) bind to class I MHC molecules that are expressed on almost all nucleated cells, whereas T_h lymphocytes bind to class II MHC molecules that are expressed only on APCs. Mature naïve CTL and T_h lymphocytes exit the primary lymphoid organs and reside in the peripheral organs waiting for activator stimuli.

1.1.3 Lymphocyte Activation

The rapid somatic mutation of B lymphocytes increases the possibility of the generation of auto-reactive clones posing a major threat on health. However, B cell and CTL cell activation is controlled by T_h lymphocytes that need at least 3 signals to be activated, a mechanism that normally ensures the prevention of autoimmunity. The first signal needed for T_h cell activation is comprised by binding of their T cell receptors (TCRs) to antigen-MHC class II complexes. This signal might only be provided by the three MHC II-expressing cells that are: DCs[39], the professional antigen presenting cells, macrophages[14] the professional phagocytes, and B cells. This binding, accompanied by the Cluster of Differentiation 4 (CD4) to the MHC II-antigen complex, induces a conformational change in the TCR or the MHC, or even both, resulting in a congregation of the complexes into microclusters that allow the CD3 and TCR molecules to come in close proximity, forming TCR-CD3 complexes. The complexes then allow further activation of the T cell[13] and mediate signalling pathways that direct the T_h differentiation pathway[30]. The second signal is the linkage of trans-membrane co-stimulatory receptors to their cellular ligands expressed by the same APC. This binding can result in the promotion or inhibition of T cell survival and expansion. The absence of the second signal induces cellular anergy in T_h lymphocytes, which is a state of dormancy. The binding of CD28 to its ligands represents the major second signal. It binds to CD80 (B7.1) or CD86 (B7.2) expressed also on the same APC inducing the expression of the T-cell growth factor interleukin-2 (IL-2) and its high affinity receptor on T lymphocytes that act in a positive feedback loop resulting in proliferation and differentiation. The third signal is the secretion of cytokines such as IL-6, IL-12 and TGF-β by the APC and CD40-CD40L interactions. The innate immune system cues manifested by such cytokines induce several intracellular biochemical events leading to the full maturation and differentiation of naïve CD4+ T lymphocytes into effector T_h1, T_h2 or T_h17 lymphocytes. T_h17 lymphocytes are responsible for the secretion of the six members of the IL-17 family that are: IL-17A, IL-17B, IL-17C, IL-17D, IL-17F and IL-17E (IL-25) that seem to have many roles in inflammation[4, 5, 15, 25, 43].

When a pathogen enters the body, Toll-like receptors (TLRs), expressed on many cell types, bind to their pathogen associated molecular patterns (PAMPs) and induce the secretion of various cytokines. They also lead to engulfment of the TLR-bound antigen by phagocytes. Pathogenic antigens are then presented on MHC class II molecules on the surface of APCs to T_h lymphocytes constituting the first signal needed for their activation. Secreted cytokines and membrane bound co-stimulatory molecules constitutes the second and third signals and induce differentiation of naïve precursor T_h lymphocytes into differentiated T_h lymphocytes.

1.1.4 Molecular Mimicry and Autoimmune Diseases

Despite this control on lymphocytes, autoimmune diseases continue to be diagnosed in a relatively large number of humans some leading to death and others causing chronic illnesses. The reasons behind the initiation and development of autoimmune diseases are not known. However, despite that the role of viral infections has never been conclusively proven, it is speculated that certain infections might be linked to autoimmune diseases[1, 7, 38]. This is supported by the exacerbation of EAE in mice lacking TLR4 or TLR9; the increased susceptibility of EAE in TLR6-deficient mice[28], the resistant state of MyD88$^{-/-}$ mice to EAE[28], and the ability to induce type-1 diabetes by using a virus[7]. In such cases, the pathogen would carry a gene encoding a protein

that is sequentially close and structurally similar to a self protein. This mimicry might disrupt the immune system's ability to discriminate between a foreign pathogen and a self component[38]. When this loss of tolerance occurs, the immune system reacts by mounting an immune response against both, the pathogen associated antigen and its structurally mimicked self-antigen destroying all tissues, organs, or any site where those antigens exist leading to diseases such as CNS autoimmune demyelinating disease[6].

1.2 Molecular events of MS and EAE

1.2.1 Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Human MS is an autoimmune disease affecting young adults with prevalence in women, being twice as high as for men. Neither the reason leading to the development of the disease is known and characterized, nor are treatments or vaccines identified. Therefore, researchers are not sparing any effort to reveal the causes leading to the development of MS and find a way to prevent or even cure it as well as other autoimmune diseases.

Genome wide studies have implicated cell mediated immune mechanisms in MS, revealed the presence of several disease-promoting HLA-DR risk alleles, and also the association of a protective HLA-A class I allele with MS has been confirmed and identified[36]. MS develops when the immune system reacts toward certain proteins in the central nervous system (CNS). These candidate auto-antigens include myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). MOG is a major component of the myelin sheath that encapsulates the axons of certain nerve cells. The immune response against myelin leads to the destruction of the myelin sheath impairing the normal conduction of electrical impulses through the axons. Axons with damaged myelin sheaths lose their optimal working ability leading to paralysis, sensory disturbances, in-coordination and visual impairment[38]. Multiple sclerosis progresses in an exacerbating relapsing-remitting mode of events where the relapses are often triggered by infections with common viruses such as influenza and EBV, which encode proteins mimicking myelin components[38].

In the CNS of MS patients, pro-inflammatory cytokines such as TNF-α are released at the site of inflammation and lead to the expression of adhesion molecules on endothelial cells so that activated lymphocytes can bind to them and penetrate the blood barrier into the CNS by diapedesis[38]. TNF-α secreted by activated T lymphocytes[38] and macrophages[12] induces the expression of the inducible nitric oxide synthase (iNOS) in macrophages and the subsequent release of the free radical nitric oxide (NO). These events lead to the recruitment of lymphocytes and myeloid cells into the CNS and result in the demyelination process that occurs in MS pathophysiology.

1.2.2 Interleukin-12

In an earlier study, EAE was prevented by antibodies against the cytokine IL-12[27]. This notion of IL-12 as the central factor of neuro-inflammation is a misinterpretation that was refuted upon the identification of the two proteins, p35 and p40, that join together to form IL-12; and the identification of a novel p19 protein that engages the IL-12p40 to form the cytokine IL-23[31]. Hence, IL-23 rather than IL-12 was then

characterized as the cytokine critical for autoimmune inflammation of the brain[12]. This was shown when $p19^{-/-}$, $p35^{-/-}$ and $p40^{-/-}$ mice were generated and tested for their susceptibility for EAE[12]. Only $p35^{-/-}$ mice, that specifically lacked IL-12, were highly susceptible to EAE whereas the other two were resistant to it[12].

1.2.3 Interleukin-23

IL-23 is one of the essential factors required for the expansion of a pathogenic CD4+ T cell population characterized by the production of IL-17[26]. The T_h17 subset is implemented in the establishment of organ-specific autoimmune diseases such as collagen-induced arthritis and autoimmune inflammation associated with the CNS such as EAE[12, 26]. However IL-23 is not required for the de novo differentiation of T_h17 from naïve CD4+ T lymphocytes[26], but rather important for their survival and expansion because neutralization of IL-23 by using anti-p40 antibodies does not suppress the generation of IL-17 producing T lymphocytes[42]; which suggests the involvement of other cytokines in the process. Nevertheless, IL-23R is only present on memory T lymphocytes[2].

1.2.4 Interleukin-6

Studying which cytokines are crucial for T_h17 differentiation led to the identification of IL-6 as a key factor. IL-6 and IL-23p40 are closely related members of the subfamily of helical cytokines that encompasses a set of factors with shared signal-transducing receptors and thus overlapping biological functions[31]. To test this, the addition of anti-IL-6 neutralizing antibodies abolished the differentiation of naïve CD4+ T lymphocytes into T_h17 lymphocytes[42]. IL-6 is produced by activated DCs stimulated by the ligation of different TLRs to their respective ligands. However, the addition of DC-derived IL-6 to naïve CD4+ T lymphocytes causes differentiation of IFN-ɣ-producing T_h1 lymphocytes[42] that control infections with intracellular pathogens including viruses and bacteria[7]. Thus, IL-6 alone is not sufficient for the de novo differentiation of T_h17. The presence of naïve CD4+ T lymphocytes, Treg lymphocytes, and LPS-activated DCs induced the differentiation of T_h17 lymphocytes. Furthermore, neutralizing the transforming growth factor (TGF-β1) by adding neutralizing antibodies to the medium suppresses the process and skews it back towards the differentiation of T_h1 lymphocytes[42]. This indicates that TGF-β1 together with IL-6 induce the de novo differentiation of T_h17 lymphocytes.

1.2.5 *TGF-β1*

The role of TGF-β1 is more of a paradox[19]. It was initially identified as a proinflammatory cytokine with an important role in chemotaxis; then it was considered as a potent immune suppressor. Interestingly, T_h1 lymphocytes express Smad7, the negative regulator of TGF-β, but neither T_h2 nor T_h17 express it[42]. This might explain the ability of TGF-β to affect T_h17 and to induce its differentiation[42].

1.2.6 ROR-ɣt

In comparison with wild type mice, intestinal CD4+ T lymphocytes from IL-6 deficient mice, did not express the orphan nuclear receptor ROR-ɣt, IL-17F and the IL-23-specific chain of the IL-23R[23]. Additionally, IL-17+ lymphocytes were reduced by about 10-fold in IL-6 deficient mice, an observation similar to that seen with ROR-ɣt-deficient mice[23]. This similarity in the observations suggested the involvement of ROR-ɣt as a downstream regulator that is affected by IL-6 and that directs the differentiation program of pro-inflammatory IL-17+ T_h lymphocytes[23].

1.2.7 GM-CSF

As mentioned earlier, IL-23 plays an important role in the effector phase of EAE by maintaining and supporting the expansion of pro-inflammatory T_h17 lymphocytes. To analyse the role of IL-23, IL-12Rβ1-deficient mice, unresponsive to IL-12 only, and IL-12Rβ2-deficient mice, unresponsive to IL-12 and IL-23, were compared with wild type mice for their pro-inflammatory cytokine secretion after immunization with MOG[9]. The levels of GM-CSF differed greatly; it was higher in IL-12-unresponsive mice compared to wt mice, while it was almost zero in the combined IL-12-unresponsive IL-23-unresponsive mice[9]. The expression of GM-CSF, that is upregulated by IL-1β, is driven by ROR-ɣt and mice lacking ROR-ɣt fail to secrete GM-CSF even under conditions that favour its secretion[9]. The dependence of the increased GM-CSF secretion levels on ROR-ɣt and IL-23, and its inhibition by IL-27 and the T_h1 cytokines, IFN-ɣ and IL-12, suggest an involvement of GM-CSF in CNS inflammation during EAE[9, 16].

GM-CSF-secreting T_h lymphocytes appear to be highly encephalitogenic and GM-CSF secretion seems to be crucial for the functioning of these lymphocytes and sufficient to render them pathogenic[9]. The mechanism delineating the ability of GM-CSF to induce CNS neuro-inflammation was analyzed in mice that lacked the csf2rb, the receptor of GM-CSF[9]. In the early phase of EAE, the invasion of leukocytes and myeloid cells into the CNS was similar in the wt and in the csf2-deficient mice; whereas their frequencies dropped down in the csf2-deficient mice and they did not accumulate during the effector phase of the disease, after which the clinical scores of EAE dropped down back to zero and inflammation deceased[9].

1.3 Transgenic mouse strains

1.3.1 MOG-Presentation

All the events that lead to the development of MS in humans and EAE in the animal models are initiated when MOG is presented by APCs to T_h lymphocytes; where MHC class II plays a very important and fundamental role. This limitation of the entire process to MHC II-expressing cells makes it tempting to identify the specific APC that presents MOG to T_h lymphocytes.

1.3.2 MHC II-Negative Mice

MHC class I molecules are constitutively expressed on almost all nucleated cells, whereas MHC class II is expressed on all APCs constitutively, or in an inducible fashion controlled by the class II trans-activator (CIITA)[37]. The generation of cells lacking MHC II molecules on their surface might be done by inactivating the beta chain of the A complex of MHC II (H2-IAb), a method that was used to successfully generate MHC class II-negative mice[10]. This was achieved by cloning an expressible neomycin resistance gene into the second Exon of the A_β chain of MHC II gene[10]. The drastically mutated DNA was then electroporated into cells from which, neomycin resistant clones were injected into C57BL/6 (B6) blastocysts and the embryos implanted into foster mothers[10]. Furthermore, iab^{neo} heterozygous offspring were crossed to produce $iab^{neo/neo}$ homozygous genotypes[10].

1.3.3 Cre-LoxP System

An elegant genetic engineering system, the Cre-*Lox*P, offers a way to induce conditional knockout of MHC II from specific cells. The system is based on two components that can be used to mediate DNA excision, inversion or even insertion[35, 40]. The first component is the Cre recombinase, which is an enzyme that, when expressed, binds to specific DNA sites and efficiently mediates the recombination of the DNA portion in between them. Those DNA sites are the second component of the system. Each one is a 34-bp *Lox*P site that consists of two 13-bp inverted repeats- the binding sites of Cre- and an 8-bp asymmetric region at which recombination occurs. The orientation of the *Lox*P sites dictates whether DNA would be excised or inverted. An opposite orientation induces DNA inversion while a direct orientation induces excision of the *Lox*P-flanked DNA fragment into a circular DNA loop that will eventually be lost due to its inability to replicate. To generate cells carrying Cre recombinase or floxed genes, embryonic stem cells (ES) are transfected by electroporation with a linearized DNA targeting construct after which the homologous recombinants are identified by Southern blot hybridization[18], injected in blastocysts and implanted in foster mothers[10].

1.3.4 IA-b fl/fl

The two methods described above:- the inactivation of the IA-b chain to abrogate MHC II expression, and the Cre-*Lox*P system- were combined to generate a conditional null allele of the major histocompatibility complex IA-b chain[20]. The strategy targeted the IA-b locus, located on chromosome 17 at 18.6 cM, and via homologous recombination, introduced a neomycin resistance gene along with *Lox*P sites cloned around Exon I of the IA-b chain. To test this, Cre recombinase was coupled to a HIV-derived TAT protein to create a cell-permeable TATCre[24] that was used to mediate the DNA recombination in a phenomenon referred to as DNA-recombination-after-transduction, or DRAT activity. These cells, harbouring the neomycin resistance gene with Exon I of the IA-b chain of MHC II flanked by *Lox*P sites, were used to generate IA-b fl/fl transgenic mice, just as described earlier.

With this IA-b fl/fl mouse strain in hand; it was obviously possible to generate new strains in which MHC II is knocked out specifically from one type of the three APC types, but not the other two. This might be done by crossing IA-b fl/fl mice with a cell type-specific Cre deletion strain, that is, a mouse strain carrying the gene coding for the Cre recombinase in their genome, under the control of a cell-type specific promoter. Accordingly, the next task was to identify specific promoters for each type of the APCs.

1.3.5 Dendritic Cells and CD11c-Cre

DCs have a pivotal role in activating immune responses upon their maturation[39]. Therefore, they were targeted to induce antigen-specific immune responses by limiting vaccine antigen expression to DCs, in an attempt to improve the safety of vaccination in clinical applications[29]. For this purpose, several transcription factor binding sites (TFBS) were analyzed in a bioinformatics analysis that revealed the presence of four genes that seemed to be DC-associated and thus to be good candidates for DC-transcriptional targeting[29]. The expression levels of CD11c, a gene among the four DC-specific genes, was tested for its expression in non-DC and DC cell lines[29]. No expression was detected in non-DC cell lines except for the macrophages that express it while differentiating towards a DC-like phenotype in

response to GM-CSF and IL-4[29]. Considering CD11c as a relatively specific DC marker, it was used to create a system for gene targeting in the DC lineage in a murine animal model. A DC-specific deletion transgenic mouse strain was generated using an artificial bacterial chromosome within which a Cre recombinase was cloned into the CD11c gene[3]. This CD11c-Cre strain was crossed to a Rosa26-StopFlox-enhanced yellow fluorescent protein (R26-EYFP) reporter mice, a cross that resulted in the expression of EYFP in >95% of splenic DCs, <10% in lymphocytes and <1% in myeloid cells[3].

1.3.6 B Cells and CD19-Cre

The B-cell specific marker is the CD19 molecule. CD19 is a cell-surface protein with two extracellular domains and a cytoplasmic tail that mediates signal transduction[44]. Its expression was revealed to be present in pre-B and B cell lines but not in two myeloma and four T cell lines tested for CD19 mRNA expression levels[44]. CD19 mRNA was also absent in the liver, brain, lung, kidney, muscles and thymus[44]. For this reason, CD19 was considered as a suitable molecule within which a Cre recombinase sequence can be integrated and thus mediate B-cell specific recombination. Therefore, embryonic stem cells were generated, by homologous recombination, to include a Cre expression cassette within the second Exon of CD19[33]. The efficiency and specificity of the system was tested by crossing CD19-Cre mice to mice having the DNA polymerase beta (*pol*β) flanked by *Lox*P sites[34]. *Pol*β was deleted in 90-95% of splenic B lymphocytes whereas its germ-line configuration was maintained in T lymphocytes and non-lymphoid organs[34].

1.3.7 Macrophages and Lyz2-Cre

Finally, it was shown that lysozyme M (LyzM) is weakly expressed in myeloblasts, not so much in immature macrophages but highly expressed in mature macrophages and thus is considered to be macrophage-specific in mice[11]. Therefore, LyzM-Cre mice, that harbour Cre recombinase within their LyzM locus, were generated. The deletion efficiency achieved by using this strain appears to be 83-98% in mature macrophages, 100% in granulocytes and 16% in CD11c+ splenic DCs when tested by crossing LyzM-Cre[8] with mice having their *pol*β flanked by *Lox*P sites[18]. The efficiency of deletion was also tested by crossing LyzM-Cre mice with $RFX5^{flox}$ mice; this cross resulted in knocking out RFX5 in 83.2% of macrophages and their subsequent loss of MHC II expression, in 22.5% of peritoneal cells and in 2.5% of splenocytes[8].

1.4 Aim of the Experiments

All these studies have made it possible to investigate which APC mediates the presentation of MOG to T_h17 lymphocytes thus inducing EAE in animal models. Despite all of the uncertainties and the dissimilarities between animal models of human diseases[32] our observations and attempts to identify the primary APC in EAE might be correlated to human MS. These three strains: CD11c-Cre, CD19-Cre and LyzM-Cre were successively crossed with MHC II IAb-fl/fl strain to obtain, after several generations, offspring with the following genotypes: CD11c-Cre; IAb-fl/fl, CD19-Cre; IAb-fl/fl and LyzM-Cre; IAb-fl/fl, respectively. The aim of the project is then to immunize the three strains mentioned above by injecting them with 200-250 µg of MOG emulsified in CFA mixed with heat-killed Mycobacteria per mouse. The clinical symptoms of the disease will be scored as mentioned in earlier[22]. The deletion of

MHC II molecules in one type of the three APC types might render its specific strain resistant to EAE. If this is seen after MOG immunization, then it would be concluded that the respective APC is the responsible cell that presents the MOG antigen to T_h17 lymphocytes, priming them and leading to the development of EAE.

If the results of the study on the animal model disease EAE are representative of the human disease MS, it might give a deeper insight on MS and takes us one step ahead closer to understanding MS and maybe other autoimmune diseases. This understanding of autoimmunity might help developing new treatments for such disease or even ways to completely prevent it.

2. Abbreviations

APC:	Antigen Presenting Cell
BSA:	Bovine Serum Albumin
CD:	Cluster of Differentiation
CFA:	Complete Freund's Adjuvant
CNS:	Central Nervous System
csf2rb:	Colony Stimulating Factor 2 Receptor beta
CTL:	Cytotoxic T Lymphocyte
DMEM:	Dulbecco's Modified Eagle Medium
DRAT:	DNA-Recombination After Transduction
EAE:	Experimental Autoimmune Encephalomyelitis
EBV:	Epstein-Barr Virus
EDTA:	Ethylene Di-amine Tetra-Acetic acid
FBS:	Fetal Bovine Serum
GM-CSF:	Granulocyte-Macrophage Colony Stimulating Factor
HLA:	Human Leukocyte Antigen
IFN:	Interferon
IL:	Interleukin
iNOS:	inducible Nitric Oxide Synthase
LyzM:	Lysozyme M
MBP:	Myelin Basic Protein
MHC II:	Major Histocompatibility Complex Class II
MOG:	Myelin Oligodendrocyte Glycoprotein
MS:	Multiple Sclerosis
NO:	Nitric Oxide
PAMP:	Pathogen Associated Molecular Pattern
PBS:	Phosphate Buffered Saline
PCR:	Polymerase Chain Reaction
PLP:	Proteo-Lipid Protein
R26-EYFP:	Rosa26-Enhanced Yellow Florescent Protein
RT-PCR:	Real-Time Polymerase Chain Reaction
SVA:	Statens Veterinärmediciniska Ansualt
TCR:	T-Cell Receptor
TFBS:	Transcription Factor Binding Site
TGF:	Transforming Growth Factor
T_h:	T Helper Lymphocyte
TLR:	Toll-Like Receptor
TNF-α:	Tumor Necrosis Factor-Alpha

3. Results

3.1 Breeding and genotyping

3.1.1 Breeding of Cre-Expressing Mouse Strains

Male mice heterozygous for CD11c-Cre (CD11c Cre/wt) were bred with female mice homozygous for the *Lox*P flanked IA-b (IA-b fl/fl). This Breeding patterns occurs in a Mendelian fashion and results in generation one (Gen 1) offspring heterozygous for the *Lox*P-flanked IA-b (IA-b fl/wt) and around 50% having CD11c-Cre (CD11c-Cre/wt or CD11c-wt/wt). Note that IA-b fl/wt has one wt allele and one transgenic allele, flanked with two *Lox*P sites. Consecutive breeding between the offspring and an IA-b fl/fl mother (backcross) were done to achieve a final generation in which the offspring are heterozygous for Cre and homozygous for IA-b fl/fl (CD11c-Cre/wt; IA-b fl/fl) (Figure 1).

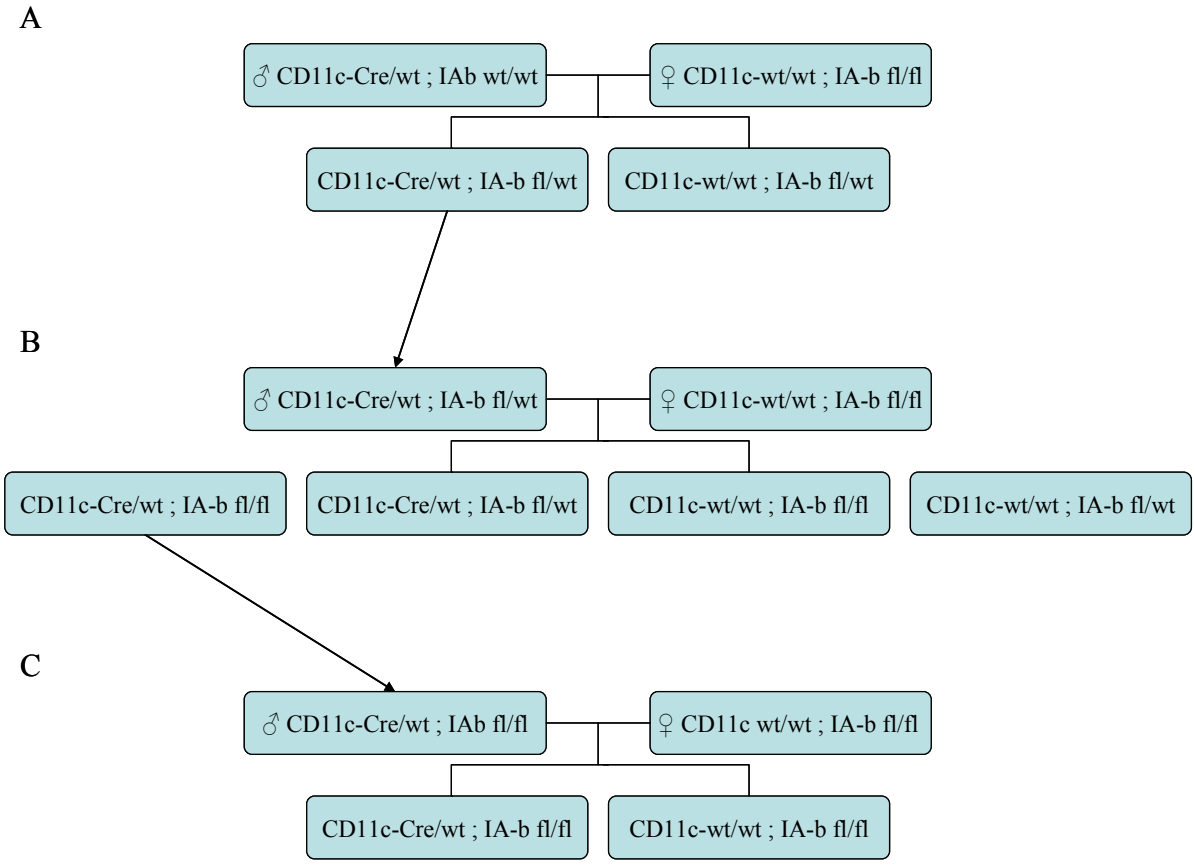


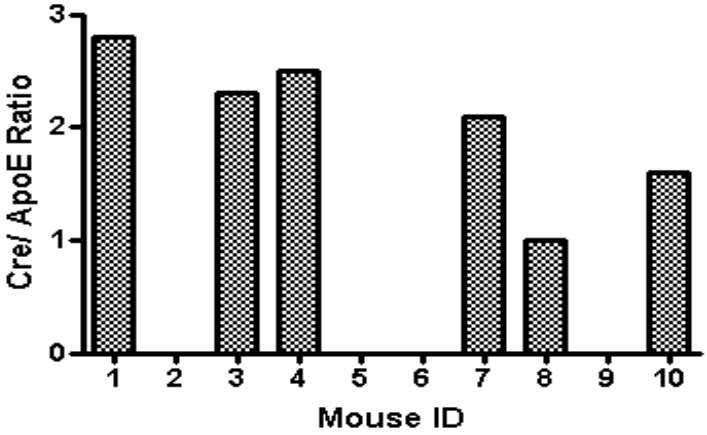
Figure 1: *The breeding scheme to achieve CD11c-Cre/wt; IAb- fl/fl genotype A. Male CD11c-Cre/wt; IA-b wt/wt mice crossed with female CD11c-wt/wt; IA-b fl/fl mice. B. Male mice from generation 1 with genotypes CD11c-Cre/wt; IA-b fl/wt are crossed with female CD11c-wt/wt; IA-b fl/fl mice. C. Male mice from generation 2 with genotypes CD11c-Cre/wt; IA-b fl/fl are crossed with female CD11c-wt/wt; IA-b fl/fl mice. Around 50% of generation offspring will have the correct genotype- CD11c-Cre/wt; IA-b fl/fl.*

Male mice from the CD19-Cre and the Lyz2-Cre mouse strains were also crossed with female IA-b fl/fl in a similar breeding pattern to that of the CD11c-Cre strain to achieve CD19-Cre/wt; IA-b fl/fl and Lyz2-Cre/wt; IA-b fl/fl genotypes, respectively (Supplementary Figures 1 and 2). However, due to experimental issues, the correct generations of CD19 and Lyz2 strains were achieved at the fourth generation instead of the third.

3.1.2 *Genotyping of Cre Transgene*

DNA from mouse ear tags and tail tips was extracted and used for genotyping of mice by PCR to identify what genes each mouse has. CD19-cre and Lyz2-cre strains were analyzed for carrying the CD19-Cre and Lyz2-Cre transgenes by melt-curve analysis (Supplementary Figures 3 and 4). This method allows for identification of the carriers of the Cre transgene by comparison with homozygous, heterozygous and wt mice. CD11c-Cre mice were tested for the presence of Cre by a RT-PCR method in which the DNA starting quantity (SQ) of CD11c-Cre was divided on the DNA SQ of ApoE, a house keeping gene, to obtain a ratio that is clearly correlated to the presence or absence of the transgene (Figure 2). All protocols were developed by the vendor of the mice: (Jackson Laboratories, USA).

Figure 2: *Genotyping of Cre Transgene. Bar graph showing the ratio of the DNA SQ of Cre/ApoE. Mice carrying Cre are clearly discriminated from those lacking it.*



3.1.3 *Genotyping of IA-b fl/fl*

Starting from the Cre-expressing strains that have the wt IA-b (IA-b wt/wt) and lack transgenic IA-b (IA-b fl/fl), the offspring of each of the consecutive generations were genotyped by the melt-curve method that allows direct identification of IA-b fl/fl carriers when compared to homozygous IA-b fl/fl, heterozygous IA-b fl/wt and wt mice (Data not shown).

3.1.4 *Germ-Line Deletion*

Meiosis is the process of cell division that occurs during gametogenesis and results in the production of four gametes. During gametogenesis, identical homologous chromosomes aline in front of each other forming tetrads. The chromatids of the tetrads cross over each other during prophase 1 forming chiasma, the site at which homologous recombination occurs and results in the production of four gametes carrying different sets of genetic material. The frequency of the occurrence of chiasmata is so high in female gametogenesis (oogenesis) compared to male gametogenesis (spermatogenesis), where chiasmata formation is relatively low, or even absent. This spontaneous homologous recombination during oogenesis imposes choosing male mice to be the parent carrying the Cre gene so that Cre expression, induced by its gene translocation, would be avoided and accordingly, germ-line deletion of IA-b fl/fl would also be avoided.

Therefore, during breeding of the mice, and more importantly, after achieving the Cre-expressing generations that are assumed to be 100% IA-b fl/fl, testing germ-line deletion was required so that mice with deletion of IA-b in all their cells are neither

chosen to be parents and used for breeding of the next generation, nor to be immunized with MOG and induce EAE. This is done by RT-PCR where the value of the DNA SQ of Exon I of IA-b is divided by the value of the DNA SQ of ApoE to obtain a ratio that is clearly correlated to germ-line deletion in the mouse samples when compared to the ratios of wt mice, heterozygous IA-b fl/fl, homozygous IA-b fl/fl and wt mice (Figure 3). However, no incident of germ-line deletion was detected. Thus no germ-line deletion of IA-b was observed.

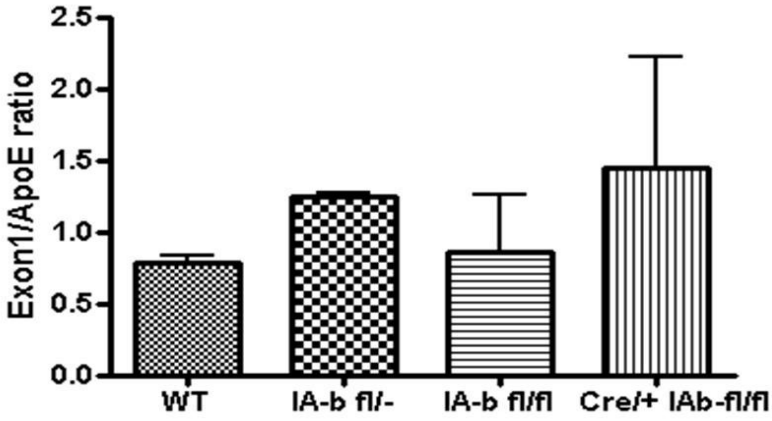


Figure 3: *Germ-line deletion testing in CD19-Cre strain. The ratio of the DNA starting quantity of Exon 1 of the IA-b gene to DNA starting quantity of ApoE.*

3.2 Flow Cytometry

3.2.1 MHC II Expression on Dendritic Cells in the CD11c-Cre Strain

Identification of CD11c-Cre mice within the CD11c-Cre/wt; IA-b fl/fl generation is done by genotyping as described. These Cre-expressing mice are supposed to express the Cre recombinase conditionally in CD11c+ cells, which are DCs, and not anywhere else. After testing for germ-line deletion by RT-PCR, mice are euthanized and their spleens were ablated to run flow cytometry and analyze the expression pattern of MHC II molecules on DCs. The experiment was done using rat-anti-mouse antibodies that are anti-CD11b, anti-CD11c and anti-MHC II antibodies. This combination of antibodies allows for making a gate around a population of DCs characterized by their high expression levels of CD11c ($CD11c^{hi}$) and their expression of CD11b ($CD11b^{+}$). DCs were analyzed for their MHC II expression were the deletion frequency was almost 90% (Table 1 and Figure 4).

Table 1: *Deletion efficiency percentage of MHC II on APCs on the three strains*

Strain	Genotype	Macrophages	B cells	Dendritic cells
CD11c-Cre	CD11c-Cre; IA-b fl/fl	93.5%	70.8%	89.7%
CD19-Cre	CD19-Cre; IA-b fl/fl	75,0%	92.9%	2.1%
Lyz2-Cre	Lyz2-Cre; IA-b fl/fl	79.6%	52.7%	49.6%

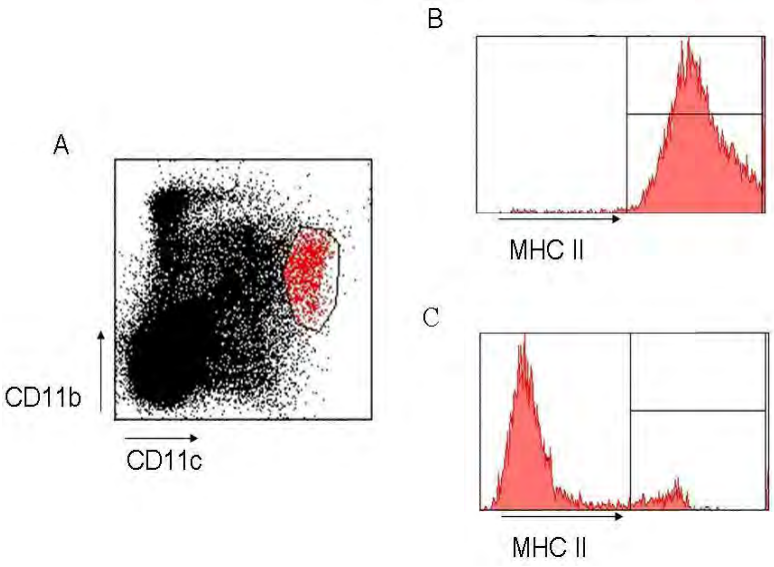


Figure 4: *Flow cytometry analysis of MHC II expression levels on splenic dendritic cells in the CD11c-Cre mouse strain. A. Gating of $CD11c^{hi}$, $CD11b^{+}$ DCs from the parent population. B. MHC II expression level on DCs from a control group. C. MHC II expression level on DCs from a mouse with a CD11c-Cre; IA-b fl/fl genotype.*

3.2.2 MHC II Expression on B cells in the CD19-Cre Strain

The CD19-Cre strain sample was genotyped and tested for germ-line deletion by RT-PCR before splenectomy and flow cytometry analysis. B cells are characterized by their unique expression of CD19. Therefore, a combination of rat-anti-mouse antibodies, that are anti-CD19 and anti-MHC II antibodies, was used to gate B cells and analyse their expression levels of MHC II. Figure 5 indicates a clear knockout of

MHC II molecules from B cells as compared to the control group. The deletion efficiency of MHC II from B cells reached 93% (Table 1).

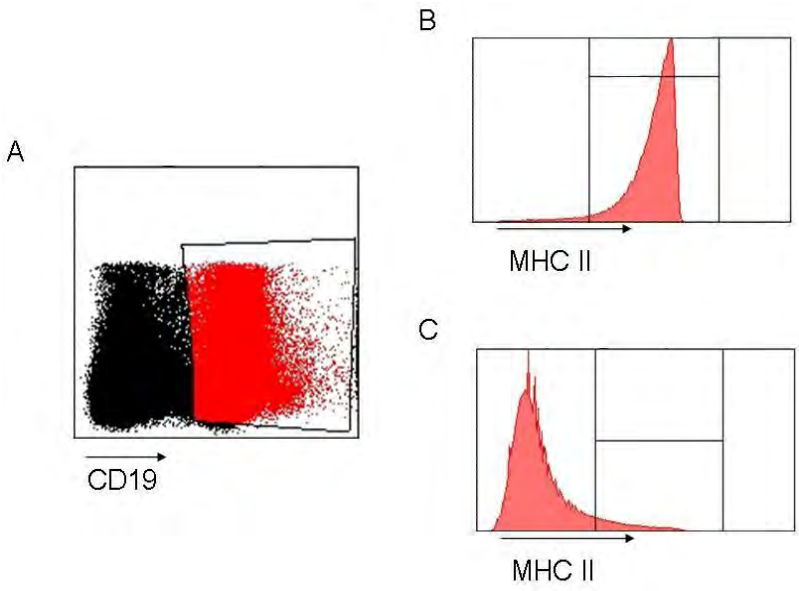


Figure 5: *Flow cytometry analysis of MHC II expression levels on splenic B cells in the CD19-Cre mouse strain. A. Gating of CD19^{+} B cells from the parent population. B. MHC II expression level on B cells from a control group. C. MHC II expression level on B cells from a mouse with a CD19-Cre; IA-b fl/fl genotype.*

3.2.3 MHC II Expression on Macrophages in the Lyz2-Cre Strain

Similar to the CD19- Cre strain, the Lyz2-cre strain was genotyped by the standard-curve method, tested for germ-line deletion by RT-PCR, before continuing with splenectomy and analysis of MHC II expression by flow cytometry. Macrophages were gated by using a mixture of rat-anti-mouse antibodies that were anti-CD11b, anti-F4/80 and anti-MHC II despite the fact that F4/80 is expressed on many cell types[17]. However, it is still possible to gate macrophages by using anti-F4/80 antibodies in a mixture with anti-CD11b antibodies were they are positively stained for both CD11b and F4/80[21]. Figure 6 depicts a clear total inhibition of the expression of MHC II on macrophages in the Lyz2-Cre; IA-b fl/fl mouse strain and Table 1 indicates a deletion efficiency of 80%.

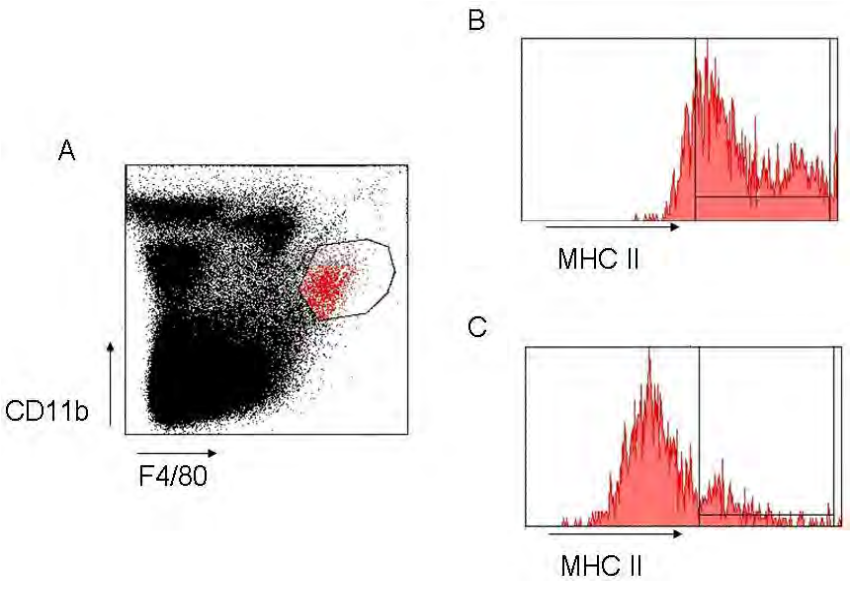


Figure 6: *Flow cytometry analysis of MHC II expression levels on splenic macrophages in the Lyz2-Cre mouse strain. A. Gating of F4/80^{hi}, CD11b^{lo} macrophages from the parent population. B. MHC II expression level on macrophages from a control group. C. MHC II expression level on macrophages from a mouse with a Lyz2-Cre; IA-b fl/fl genotype.*

3.2.4 MHC II Expression on B cells and Macrophages in CD11c-Cre Strain

After assuring a successful deletion of MHC II from DCs in the CD11c-Cre strain, it was necessary to elucidate its expression on B cells and macrophages in the same strain. This was done by using the respective antibody mixtures that are anti-CD19 for gating B cells, and anti-CD11b and anti-F4/80 for gating macrophages. Anti-MHC II antibody was also used. Figure 7 illustrates the effect exerted by CD11c-Cre on B cells and macrophages were it is obvious that B cell expression of MHC II is decreased by 70.8%, while its expression on macrophages is decreased by 93.5% (Table 1)

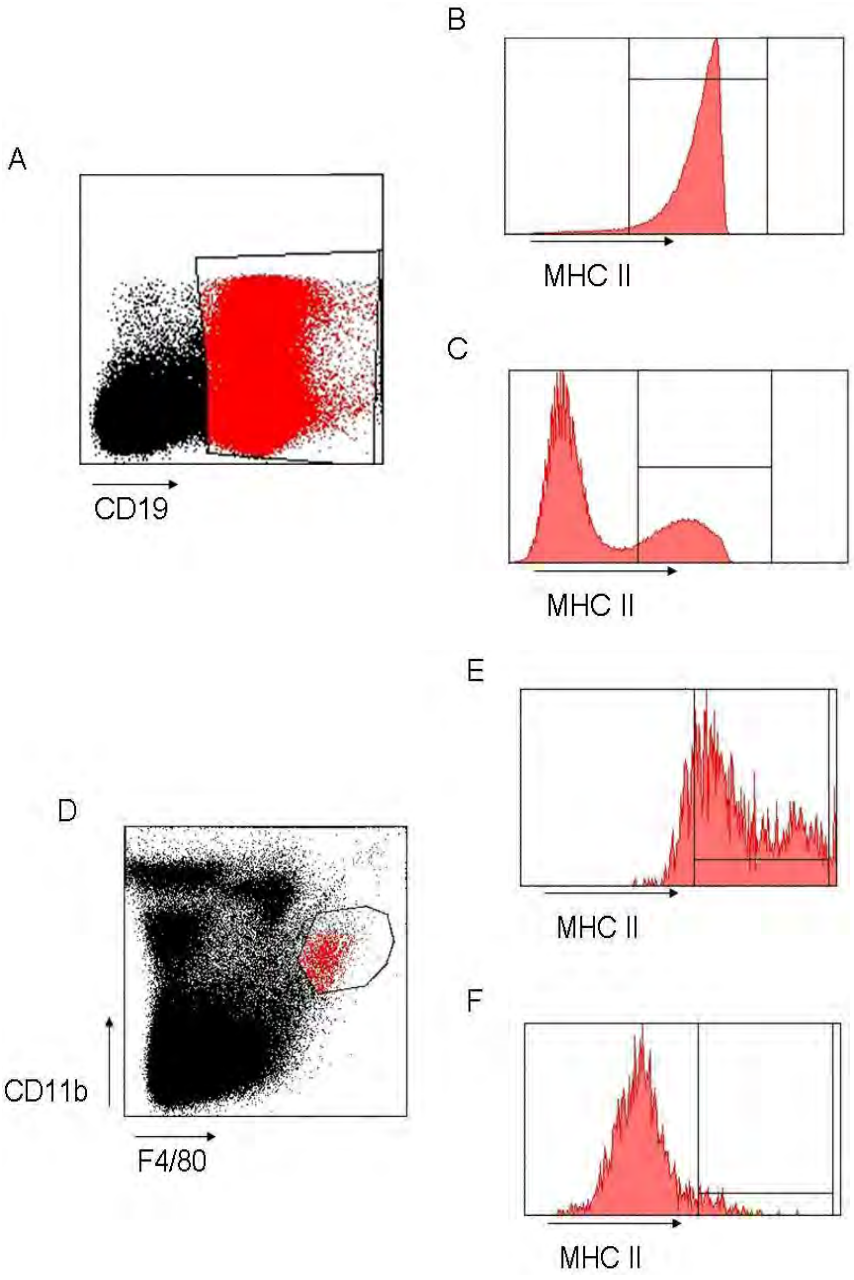


Figure 7: *Flow cytometry analysis of MHC II expression levels on splenic B cells and macrophages in the CD11c-Cre mouse strain. A. Gating of $CD19^{+}$B cells from the parent population. B. MHC II expression level on B cells from a control group. C. MHC II expression level on B cells from a mouse with a CD11c-Cre; IA-b fl/fl genotype. D. Gating of $F4/80^{hi}$, $CD11b^{lo}$ macrophages from the parent population. E. MHC II expression level on macrophages from a control group. F. MHC II expression level on macrophages from a mouse with a CD11c-Cre; IA-b fl/fl genotype.*

3.2.5 MHC II Expression on DCs and Macrophages in CD19-Cre Strain

Furthermore, MHC II expression on DCs and macrophages was analyzed in the CD19-Cre strain. CD19-Cre did not affect MHC II expression by DCs that seem to be unaffected where the deletion efficiency is 2.1%. On the other hand, macrophages show a deletion frequency of 75% (Figure 8 and Table 1).

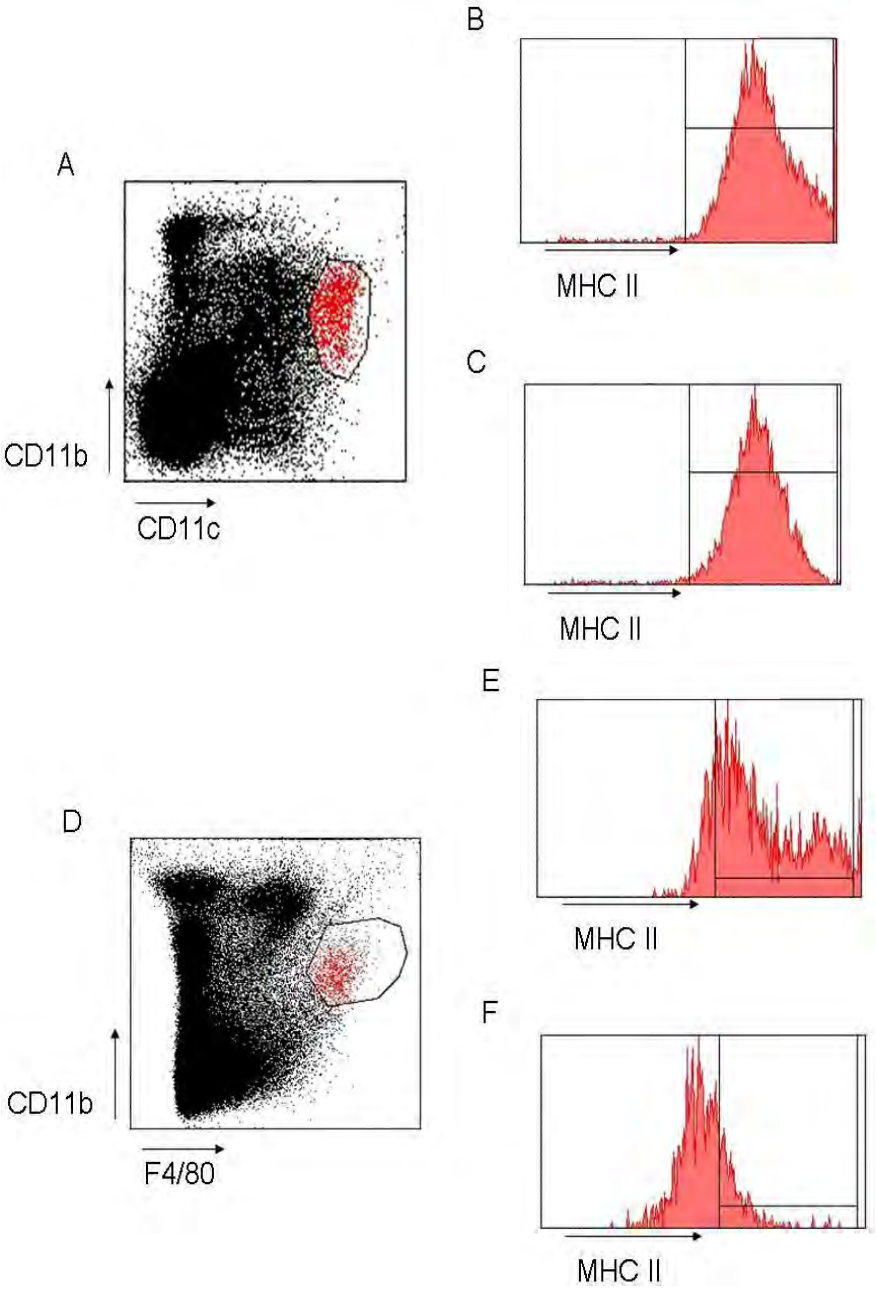


Figure 8: *Flow cytometry analysis of MHC II expression levels on splenic DCs and macrophages in the CD19-Cre mouse strain. A. Gating of $CD11c^{hi}$, $CD11b^{+}$ DCs from the parent population. B. MHC II expression level on DCs from a control group. C. MHC II expression level on DCs from a mouse with a CD19-Cre; IA-b fl/fl genotype. D. Gating of $F4/80^{hi}$, $CD11b^{lo}$ macrophages from the parent population. E. MHC II expression level on macrophages from a control group. F. MHC II expression level on macrophages from a mouse with a CD19-Cre; IA-b fl/fl genotype.*

3.2.6 MHC II Expression on B cells and DCs in Lyz2-Cre Strain

Finally, the expression level of MHC II molecules on the surface of B cells and DCs is analyzed in the Lyz2-Cre strain. As observed in Figure 9, the histograms of the patterns of MHC II expression levels indicate a reduction of MHC II by 52.7% and 49.6% on B cells and DCs, respectively.

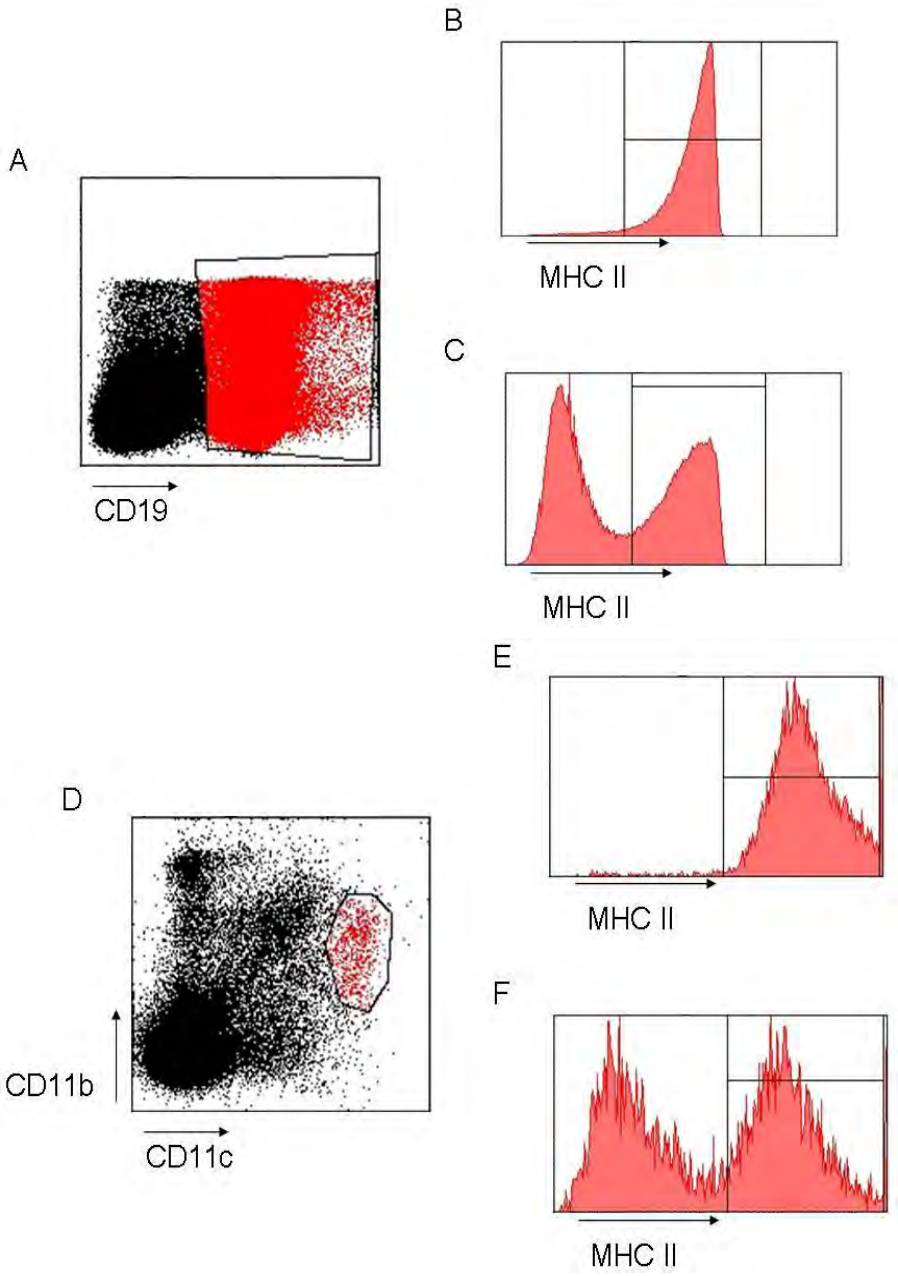


Figure 9: *Flow cytometry analysis of MHC II expression levels on splenic B cells and DCs in the Lyz2-Cre mouse strain. A. Gating of CD19^{+} B cells from the parent population. B. MHC II expression level on B cells from a control group. C. MHC II expression level on B cells from a mouse with a lyz2-Cre; IA-b fl/fl genotype. D. Gating of CD11c^{hi}, CD11b^{+} DCs from the parent population. E. MHC II expression level on DCs from a control group. C. MHC II expression level on DCs from a mouse with a Lyz2-Cre; IA-b fl/fl genotype.*

4. Discussion

Male mice of the three Cre-expressing mouse strains were crossed with female mice from a fourth strain that has the Exon I of the beta chain of the A complex of MHC II molecule, IA-b, flanked with repeats of *Lox*P sites. Successive back-breeding of the three strains with the IA-b fl/fl strain (Figure1 and supplementary Figures 1 and 2) were done to achieve generations with CD11c-Cre; IA-b fl/fl, CD19-Cre; IA-b fl/fl and Lyz2-Cre; IA-b fl/fl genotypes. However, the spontaneous homologous recombination occurring during gametogenesis alerts for the risk of expressing Cre in all of the cells of the body. This phenomenon required monitoring germ-line deletion of IA-b in the IA-b fl/fl generations that express Cre.

The deletion efficiency of MHC II on the three APCs within the three strains is puzzling and indicates an overlapping effect in a direct or an indirect manner. Within the CD11c-Cre strain, MHC II is deleted up to 90% from macrophages and DCs, and up to 70% from B cells. This suggests that CD11c is expressed in all of the three APC types. Within the CD19-Cre strain, the MHC II deletion efficiency from B cells exceeds 90%, up to 75% from macrophages, but most importantly, less than 3% on DCs. The Lyz2-Cre strain shows a deletion efficiency of MHC II from macrophages to be about 80%, while it is about 50% on both, B cells and DCs (Table 1).

A thorough comparison of the perplexing results and a careful correlation with the clinical scores upon MOG immunization, might make is possible to identify the primary APC of EAE. If CD19-Cre strain shows a normal disease progression while the other two strains show normal disease or mitigated clinical scores, it indicates that DCs are the MOG-presenting APCs. Conversely, if CD19-Cre strain is rendered resistant to EAE, while the other strains show a normal or a milder disease progression, B cells are implicated in MOG presentation. At last, macrophages are considered likely to present MOG if the three strains are resistant to EAE.

5. Material and Methods

5.1 Mice Genotyping and Breeding:

5.1.1 Transgenic Mouse Strains

C57BL/6 transgenic mice were genetically engineered to encode Cre recombinase under cell-type-specific promoters. CD11c-Cre, CD19-Cre and Lyz2-Cre transgenic, along with IA-b fl/fl mouse strains were obtained from Jackson Laboratories, USA. All mice were kept at the laboratory animal house at sterile pathogen-free conditions at Rudbeck Laboratories, Uppsala University.

5.1.2 DNA Extraction

DNA from mouse ear tags and tail tips was extracted according to the protocol of the DNeasy Blood and Tissue Kit (Qiagen). Tissues were lysed overnight in Lysis Buffer followed by protocol procedures for DNA purification and extraction. DNA was finally eluted in 100 µl RNase-free water and stored at -20^{o}C.

5.1.3 PCR and Quantitative Real-Time RT-PCR

Polymerase chain reaction (PCR) and real-time RT-PCR were performed using MyiQ single color Real-Time PCR Detection System, MyiQ Cycler Software and CFX Manager (All from Bio-Rad) with SYBR Green (Qiagen). Amplification efficiencies were validated and normalized against ApoE.

5.1.4 Breeding

Female IA-b fl/fl mice were crossed with male mice from the Cre-expressing strains. Cre-expressing offspring were consecutively backcrossed with female IA-b fl/fl to achieve Cre-expressing; IA-b fl/fl generations apt for EAE induction.

5.2 EAE and Flow Cytometry

5.2.1 MOG

Rat MOG_{1-125} derived from recombinant Escherichia *coli* was produced as described earlier[41] as part from my research training period.

5.2.2 EAE

Female mice with CD11c-Cre IA-b fl/fl, CD19-Cre IA-b fl/fl and Lyz2-Cre IA-b fl/fl genotypes were immunized with 200 µg of MOG emulsified in CFA at the age of 8-16 weeks. Intraperitonial (i.p.) injections of 200 ng of Pertussis toxin (Sigma-Aldrich) in calcium (Ca) free and Magnesium (Mg) free 200 µl Phosphate Buffered Saline (PBS - Statens Veterinärmediciniska Ansualt - SVA, Uppsala, Sweden) were given at the day of MOG immunization and after 2 days. EAE scoring according to the symptoms developed daily by immunized mice are: Score 1, tail paralysis or weakness; score 2, paraparesis of hind legs; score 3, partial paralysis of hind legs; score 4, complete paralysis of hind legs; 5, tertraplesia or animal deceasing induced by EAE[22].
All studies have been reviewed and approved by the local ethical committee.

5.2.3 Dissection of spleen

Spleens were ablated from mice in sterile conditions and resuspended in Dulbecco's Modified Eagle Medium (DMEM - SVA, Uppsala, Sweden) and filtered through 40 µm wide cell strainers (Falcon BD). Cell concentrations were determined using a

nucleocounter.10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/mL Penicillin, 100 µg/mL Streptomycin and 292 µg/mL $_L$-glutamine (Invitrogen, USA) were added to the 2x10^6 splenocytes cultured in DMEM.

5.2.4 Macrophage Activation and MHC II Expression Induction

Recombinant mouse rmIFN-ɣ was prepared with 0.1% Bovine Serum Albumin (BSA - Sigma-Aldrich) in PBS. Increasing concentrations of 0, 0.5, 1.0, 1.5 and 2.0 ng/mL of rmIFN- ɣ were added to 2x10^6 cell cultured in 12-well plates (Nunc) for 6 hours at 37^{o}C in 5% CO_2.

5.2.5 Cell Fixation

Cells are suspended, for 30 minutes on ice, in 500 µL, 1% Paraformaldehyde in PBS (PH 7.4) at a concentration of 2x10^6 cells/mL. Cells were washed and resuspended in staining buffer: 2 mM Ethylene Di-amine Tetra-Acetic acid (EDTA) (Sigma-Aldrich).

5.2.6 Cell Staining and Flow Cytometry

Splenocytes were incubated with Fc-Block antibodies (Table 2) for 15 minutes at 4^{o}C to inhibit unspecific binding of flourochrome-conjugated antibodies to Fc-ɣ III/II receptors. The following rat or hamster-derived anti-mouse antibodies were used for cell surface staining: MHC II-FITC (anti-IA/IE), B220-PerCP (anti-CD45R/B220), F4/80-Alexa 647 (Anti-F4/80), CD4-APC (anti-CD4), CD8b-PE (anti-CD8b), CD11b-PerCP-Cy5.5 (anti-CD11b), CD11c-PE (anti-CD11c), CD19-Alexa 647 (anti-CD19) and PDCA1-PE (anti-CD317/PDCA1) (Table 2). Flow cytometry experiments ware performed on BD FACSCanto II and analyzed on BD FACSDiva v6.0 software.

Table 2: *Flourochrome conjugated anti-mouse antibodies used in flow cytometry experiments*

Antibody	Isotype	Flourocrome	Company
Anti-CD16/32 Fc-Block	Rat IgG2b, κ	-	BD Pharmingen™
Anti-CD45R/B220	Rat IgG2a, κ	PerCP	BD Pharmingen™
Anti-IA/IE (MHC II)	Rat IgG2a, κ	FITC	BD Pharmingen™
Anti-CD4	Rat IgG2a, κ	APC	BD Pharmingen™
Anti-CD8b	Rat IgG2b, κ	PE	BD Pharmingen™
Anti-CD11b	Rat (DA) IgG2b, κ	PerCP-Cy5.5	BD Pharmingen™
Anti-CD11c	Armenian Hamster IgG1, λ2	PE	BD Pharmingen™
Anti-CD19	Rat IgG2a, κ	Alexa Flour® 647	Biolegend
Anti-F4/80	Rat IgG2a, κ	Alexa Flour® 647	Biolegend
Anti-CD317 (PDCA1)	Rat IgG2b	PE	eBioscience

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أسألُ اللهَ العزيزَ الحكيمَ أنْ يتقبلَ مني عملي هذا خالصاً لجلالِ وجهِهِ وعظيمِ سلطانِهِ، وأدعوهُ بقولِهِ تعالى:
بسمِ اللهِ الرحمنِ الرحيمِ {وَقُلْ رَبِّ زِدْنِي عِلْمًا} صدقَ اللهُ العظيم.

أمّا بعد فأوجّهُ خالصَ التقديرِ والامتنانِ إلى كل منْ ساهمَ في نجاحِ عملي هذا، وساعدَني على الوصولِ إلى ما أنا عليهِ. فأشكرُ ربَّ الأسرةِ أبي الذي ربّانا وتعبَ في سبيلِ تعليمِنا وعلى إيمانِهِ بنا. فهو ضحّى عطفاً وصبراً لهُ كلُّ الامتنانِ ولقد كنتَ دائماً بجواري. كما أشكرُ أمّي الحبيبةَ على تعبِها وتربيتِها لي ولإخوتي، وعلى دعمِها لي منذُ ولادتي. أعطيتِني حناناً بلا انقطاعٍ ومحبةً لا نظيرَ لها. أشكركما على جميعِ ما قدّمتُماهُ لنا من تضحيةٍ وتكريسِ حياتكما لصالحِ أولادكما. أسألُ اللهَ تعالى أنْ يرحمَكُما كما ربّيتُماني صغيراً، وأنْ يجزيَكُما عني خيراً فأنتما عنواني وقدوتي ومهما فعلتُ لن أجزيَكُما حقَّكُما.

كما أقدّمُ أيضاً جزيلَ الشكرِ لأخواتي وأخي الذينَ كانوا دائماً عوناً لي في كل محنةٍ ألقاها ويتواجدونَ بدون ملل لمشاركتي في جميعِ أفراحي. أنتم بسمتي وسندي الدائمُ لي وجزءٌ لا يتجزأُ من حياتي فلا غنى لي عنكم.

ولكلُّ الشكرِ والامتنانِ لمنْ هي قدوتي في حياتي والمنارةُ التي تنيرُ دربي، إلى جدّتي وأمّي، لكنتِ دائماً حاضرةً لتؤمّني لي كل أنواعَ الدعمِ ونصيحتُكِ حجرُ أساسٍ في بناءِ شخصيتي منذُ طفولتي. تربيتُ وترعرعتُ في أحضانِكِ بينَ أولادِكِ فغدوتُ بينَ أولادِكِ أخاً لهم. أشكرُهم جميعاً على محبتِهم الغيرِ محدودةٍ وعلى مساهمتِهم في تكوينِ شخصيتي. أنتم عزوتي وأهلي.

أوجهُ شكري أيضاً إلى أهلي من وقفَ بجانبي وعلى وجهِ الخصوصِ جدّتي وعمّتي على دعائِهن لي عندَ الصلاةِ وعندَ قيامِ الليلِ وحينَ التوجُهِ إلى الكعبةِ.

أشكرُ أيضاً أصدقائي الذينَ كانوا عوناً لي خلالَ سفري هذا، أذكر على رأسهم فوزي الشيّار وأخي أحمد العامودي.

وأخيراً وليسَ آخراً أقدّم جزيلَ الشكرِ لمن لم أذكرهُ ولكنه كان عوناً لي.

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