

INTRODUCTION: I thank the reviewers for their insightful comments. Below I have reiterated and addressed their concerns. To conserve space, I have paraphrased some of their comments:

1. “Discussion on expected results and statistical analysis would strengthen the experimental design.” The research strategy section has been extensively revised and I have expanded the experimental designs so they include a discussion and alternative strategies. A section on statistical analysis has also been added.
2. “It is not clear whether the applicant will be encouraged to attend and present his studies at any national or international meetings.” I have been encouraged to attend and present my research studies at both national and international meetings. I have already attended three such meetings, most recently the Federation of Clinical Immunology Societies (FOCIS) in June of 2012. I anticipate attendance at the annual meeting for the American Association of Immunologists (AAI) in May 2013. Dr. Szalai has revised his mentoring plan to make his support of my attendance more clear.
3. “The personal statement in the biosketch, which should be a concise statement of research and career goals, was long with most information better suited for other sections of the application, and very little about what is to be accomplished by this application.” I have moved much of the information contained within the biosketch to other sections of the application. The resulting personal statement briefly describes how my experience and training as well as the expertise and environment provided by my mentor will facilitate completion of my doctoral dissertation and achievement of my long term career goals.
4. “There is very little preliminary data provided on the transgenic mice with no evidence provided of the extent of the human-Fc-expression and restriction to DC in the knock-ins.” The background preliminary data and methods sections have been rewritten and now include more preliminary data and descriptions of the CD11c-huFc γ RIIB mice (see Fig. 9).
5. “The research proposal is based on a single phenomena presented in Fig 1 with no statistical analysis, no explanation of what are the error bars, and it is unclear as to the relationship between rapid onset and suppression of disease specially when the severity and presumably the incidence of disease is not different between the wild type and knockout mice.” The figure in question (now figure 4) is not the basis for the proposal, but rather is intended as additional support to the premise that CRP is protective.
6. “The research training plan is presented as if the ideas are not quite clearly thought out.” As instructed by F31 guidelines and with my mentor’s encouragement, I wrote the initial proposal in its entirety. To improve my research plan, I spent considerable time with Dr. Szalai, who guided revisions that ensured improved clarity and flow of information. Although the fundamental plan remains largely the same as the original, the project as now presented is easier to comprehend.
7. “There is no description about how the applicant will be trained in making, handling, cross breeding and phenotyping of the transgenic mice.” I have completed formal trainings through UAB’s Animal Resources Program, including “Working with Mice in Research at UAB” and “Using Animals for Teaching, Testing, and Research at UAB.” Furthermore, I have contributed to the generation of the CD11c-huFc γ RIIB mouse and have been trained in transgenic genotyping and phenotyping by the UAB Transgenic Mouse Facility. I also receive training from Dr. Szalai, who has extensive experience in working with mice.
8. “Justification of mice numbers is not acceptable. Two sample T-test is not acceptable for comparing two groups of mice with EAE using a continuous scoring system. Non-parametric analysis is required. Also, it is not clear how many different experiments are needed and how many other mice for the ex vivo experiments will be used.” This serious oversight has been given careful consideration in the revised proposal. As indicated in an earlier response, I have added a ‘Statistical analysis’ section to the end of the Research Strategy. Each of these experiments will be done in triplicate to ensure reproducibility. For each EAE experiment, I will use 15 mice per genotype/treatment group, and at least 3 donating mice for each *in vitro* experiments. As the reviewer correctly points out, when the data are not continuous or normally distributed I will use parametric tests of significance.
9. The overall project is too ambitious and alternative approaches and pitfalls are not well discussed. For example, it is well established that the EAE model has many limitations and is only an approximation to the situation in MS and the applicant does not explain why she is focusing on a model of chronic EAE. It is not clear whether [the Fc γ RIIC mouse] already exists or would need to be generated” As stated in the responses to concerns 1 and 7, I have simplified the research plan to a more reasonable length and complexity while bolstering the discussion of potential pitfalls and alternative strategies. We utilize the C57BL/6 model (chronic EAE) because that is the model wherein we have the most information about CRP’s role in EAE. (Please see ‘Vertebrate Animals’ section.) We currently have available mice with B cell specific expression of Fc γ RIIC.

Human C-reactive protein is an acute phase protein whose blood concentrations rise dramatically in response to inflammatory insults. Though CRP is primarily of hepatic origin, it is also produced by neurons during times of CNS inflammation. CRP's contribution to CNS inflammation and the biological importance of neuronal CRP are unknown. Published work from our lab shows that mice containing the human CRP transgene (CRPtg) are protected from myelin oligodendrocyte glycoprotein (MOG) peptide induced experimental autoimmune encephalomyelitis (EAE), a rodent model of Multiple Sclerosis (MS). Human CRP, expressed transgenically or administered by injection, delays onset and reduces severity of EAE, and these effects rely on expression of Fc γ RIIB. We showed that transgenic or injected human CRP is unable to ameliorate EAE in Fc γ RIIB^{-/-} mice. The Fc γ RIIB-expressing cell that responds to human CRP remains unknown. The goals of this research project are (1) to determine the Fc γ RIIB expressing cell through which CRP exerts protection against EAE and (2) to determine if CNS specific expression of human CRP is sufficient to protect mice from EAE. I hypothesize that CRP mediated amelioration of EAE is manifest via CRP binding to Fc γ RIIB on dendritic cells (DCs), and that local CNS expression of CRP is sufficient to achieve this benefit. Utilizing purified human CRP, I will analyze bone marrow derived DCs from mice either sufficient or deficient for Fc γ RIIB to determine if CRP alters their activation status *in vitro* and if any CRP driven effect depends upon Fc γ RIIB. Since EAE/MS is considered to be a T cell mediated disease, I will examine CRP's effect on DCs' ability to stimulate T cell proliferation and polarization *in vitro* in DC:T cell co-cultures using wildtype versus Fc γ RIIB deficient DCs as antigen (MOG) presenting cells. I will also induce EAE in humanized mice (mice that express human CRP and express human Fc γ RIIB exclusively on DCs, but no mouse Fc γ RIIB) to investigate if the Fc γ RIIB→DC axis is sufficient for CRP mediated amelioration of EAE. To assess the requirement of DCs, I will induce EAE in CRPtg in which DCs have been selectively depleted. Finally, using mice wherein human CRP expression is limited to neurons I will determine if exclusively CNS expression of human CRP is sufficient to protect mice from EAE. I will induce EAE in nCRPtg and compare their disease to CRPtg and WT mice. Successful pursuit of the work described herein will identify the effector cell by which CRP exerts protection in EAE, confirm that a human CRP→human Fc γ RIIB axis operates *in vivo*, and elucidate the source of protective CRP. This will pave the way for development of new therapies that will benefit the ~350,000 U.S. citizens with MS.

SPECIFIC AIMS: Multiple sclerosis (MS) is a neurologic disease wherein an autoimmune and inflammatory attack leads to demyelination of white matter in the central nervous system (CNS) [1]. MS affects ~350,000 people in the United States and the fatigue, tremors, impaired coordination, and paralysis it causes can greatly reduce the quality of life of afflicted individuals [1]. The lost productivity due to MS combined with medical treatment costs our nation ~\$6.8 billion each year [2]. Current therapies slow progression of MS and lessen the severity of its relapses [1] but despite the great need, there is still no effective therapy for prevention of the disease. A cure for MS remains elusive largely because its pathogenesis is not sufficiently understood. Generally speaking, MS pathogenesis is characterized by infiltration of inflammatory cells and myelin specific CD4⁺ T cells into the CNS parenchyma, whose combined actions culminate in demyelination [1]. Both clinical and post-mortem evidence strongly support the widely-held notion that irregularities of adaptive immunity and inflammation together are responsible for the neurodegeneration seen in MS, but the causes of these irregularities remain equivocal; even less is known about the contribution of innate immunity in MS. Does innate immunity regulate autoimmunity and/or inflammation in MS? Is activation of innate immunity a cause or a consequence of demyelination? By answering these questions my research aims to address this MS knowledge gap, driving the field forward.

My long-term goal is to forge a career in neuroimmunology, to perform research that will identify and help understand interactions between the innate and adaptive immune systems that promote MS, and to use this acquired knowledge to develop novel therapies that will prevent, ameliorate, or cure the disease. The objective of the current application - which represents an important early step towards my long term goal - is to establish the means by which the innate immunity molecule C-reactive protein (CRP) protects against MS. My central hypothesis is that by binding to dendritic cells (DCs) via Fc γ RIIB, CRP diminishes proliferation of encephalitogenic T cells - thereby lessening MS severity. I formed this hypothesis based on my growing knowledge of the published MS literature, extensive and ongoing discussions with my mentor and thesis committee members, and observations I made using human CRP transgenic and CRP deficient mice. For example, though CRP levels are similar between MS patients and healthy controls CRP level is positively associated with MS severity and is increased in patients receiving interferon- β therapy [3]. The implications of these associations are unknown as the biology of CRP in MS has not been studied. However, we showed that human CRP protects mice against experimental autoimmune encephalomyelitis (EAE), an animal model of MS [4]. In the *Research Strategy* section I present evidence that this protective effect is attributable to decreased proliferation of encephalitogenic T cells and a less inflammatory cytokine profile [4]. Importantly, I also show that the beneficial effect of transgene expressed human CRP depends on mouse Fc γ RIIB, the inhibitory Fc γ receptor [5]. Indeed, injection of purified human CRP into mice is sufficient to protect them from EAE but only if they express Fc γ RIIB [5]. These data raise the exciting possibility that in MS elevation of CRP is actually a beneficial response and that CRP administration might be a useful new therapy.

The purpose of my proposed research is to identify the Fc γ RIIB expressing cell(s) via which CRP mediated protection from EAE is realized. My rationale is that by identifying these cells, we can map the complete pathway linking CRP to protection of the CNS and thus reveal a potent new therapeutic avenue for MS. I will test my central hypothesis and accomplish my objectives by pursuing the following specific aims:

Aim 1: Ascertain the influence of CRP on DC phenotype and function. The working hypothesis is that CRP binding to Fc γ RIIB on DCs alters their capacity to stimulate T cells. I will compare activation markers and cytokines expressed *in vitro* by bone marrow derived DCs (BMDCs) of wild type versus Fc γ RIIB deficient mice, and I will test T cell proliferation supported by the two genotypes of CRP stimulated BMDCs. *In vivo* I will test if BMDCs pre-conditioned by CRP are therapeutic when administered to mice with ongoing EAE.

Aim 2: Determine the contribution of DCs to CRP mediated Fc γ RIIB dependent protection from EAE. The working hypothesis is that CRP associated Fc γ RIIB-dependent protection from EAE depends on DCs. I will pursue experiments using DC-sufficient versus DC-deficient CRPtg, Fc γ RIIB sufficient versus Fc γ RIIB-deficient CRPtg, and CRPtg expressing human Fc γ RIIB exclusively on DCs.

Aim 3: Assess the protective capacity of CNS expressed CRP. The working hypothesis is that CRP produced in the CNS is sufficient to protect against EAE. Studies will rely on a novel mouse strain wherein a transgene is driven by a promoter that limits human CRP expression to neurons.

Regarding expected outcomes, successful pursuit of the work described will (i) identify the effector cell by which CRP exerts protection in EAE, (ii) confirm that a human CRP \rightarrow human Fc γ RIIB axis operates *in vivo* and (iii) elucidate the source of protective CRP. Collectively, this will pave the way for development of new therapeutics that will benefit the ~350,000 U.S. citizens with MS.

RESEARCH STRATEGY:

(a) Significance: In the United States ~350,000 people have MS and there are no specific therapies to prevent the disease or hasten its recovery, and none that completely abrogate its life-threatening side effects [1-3]. MS carries a ~\$6.8 billion healthcare burden [3] that persists because the pathogenesis of MS is not fully understood. To address this knowledge gap and help alleviate the burden we will combine novel *in vitro* studies with unique *in vivo* experiments using new strains of mice subjected to EAE, a rodent model of MS [6]. Although adaptive immunity and inflammation are widely recognized to contribute to EAE/MS the contribution of innate immunity remains largely unknown. Also unknown is if innate immunity regulates the EAE/MS process and the mechanism by which it might do so. Answering these questions is an important step towards significantly easing the MS burden. My proposed study of the mechanism of CRP-mediated protection in EAE will address this knowledge gap and drive the MS field forward. CRP is a recognized biomarker of MS and other CNS diseases [7] but, aside from our own studies, there has been no investigation of the possibility that CRP contributes to the etiology or progression of EAE/MS. I expect the contribution of the proposed research to be the confirmation that CRP has a beneficial impact on CNS damage during EAE as well as the identification of the biological pathway by which this benefit is achieved. This contribution will be significant because the new knowledge gained will hasten development of improved algorithms for monitoring MS progression and support development of CRP-based drugs that could lessen or prevent MS symptoms.

(b) Approach: In this section I present the conceptual framework for my interest in understanding CRP's protective role in EAE. Specifically, I provide important background on CRP as it pertains to its role in the regulation of inflammation and autoimmunity and I cite previously published data and show preliminary unpublished data that have guided the formation of my current hypothesis. My specific aims are intended to localize the source of CRP that protects against EAE (CNS or soma?) and identify the cellular machinery (dendritic cells?) that manifests the beneficial effect, and how CRP engages this machinery (via Fc γ RIIB?).

(b.1) Background and Preliminary Data: Despite tremendous advances in the understanding of MS its exact etiology remains unknown. It is generally believed that abnormal activation of the immune system leads to a breach in the blood-brain-barrier (BBB), allowing entry of myelin specific T cells into the CNS (normally an area of immune privilege). Accordingly, MS therapy aims to curtail and control immune activation with the goal of diminishing axonal demyelination, the underlying cause of the clinical signs and symptoms of MS. Much of this and other existing knowledge of MS pathogenesis has been gained by study of animal models of demyelinating disease, including experimental autoimmune encephalomyelitis (EAE) in mice. Although there are established differences from MS, the EAE model has helped clarify genetic, immune, and inflammatory pathways responsible for autoimmune destruction in the CNS [8]. The EAE model I utilize is evoked by immunization of mice with myelin oligodendrocyte glycoprotein peptide (MOG₃₅₋₅₅). When MOG₃₅₋₅₅ is injected together with complete Freund's adjuvant and pertussis toxin, the highly immunogenic peptide is taken up and presented by antigen presenting cells (APCs) and thereby induces a T cell driven disease whose pathology and clinical symptoms resemble the chronic progressive form of MS [9]. MOG is vital to proper nerve myelination [10]; thus the MOG peptide guides the immune system to attack the CNS. Once MOG-specific T cells infiltrate the CNS and are stimulated they proliferate and produce cytokines that recruit additional inflammatory cells, like macrophages. The latter also infiltrate the CNS and it is thought that their destructive activity culminates in demyelination [11].

Human CRP circulates at 1-3 μ g/ml in blood during health but it increases \geq 500-fold in response to inflammation through IL-6 driven transcription and protein synthesis, the liver being the main (but not exclusive) source of blood CRP [12, 13]. Inflammation contributes to demyelination and because human CRP is an acute phase reactant [16,17], its blood concentration should rise in EAE/MS. Indeed, in humans CRP is positively associated with MS severity [14] and CRP can be produced by neurons during times of inflammation [15]. CRP interacts with phosphocholine/phosphatidylcholine (abundant in the CNS) via one of its two faces and with complement C1q and Fc receptors on the other [16, 17]. Thus, CRP can initiate complement activation, induce phagocytosis, and help clear apoptotic remnants [18, 19], functions that are likely to be relevant during EAE/MS. Indeed, of direct relevance to the current proposal is evidence from multiple independent studies suggesting that CRP can act as a tonic suppressor of immunity. For example in female NZBxNZW mice, which spontaneously develop lupus like autoimmunity, we showed that human CRP is protective i.e., onset of lupus nephritis was significantly delayed in mice carrying a human CRP transgene [20]. In fact others showed that lupus nephritis could be improved simply by administering (via s.c. injection) human CRP [21]. Conversely, we showed that mice genetically deficient in CRP (CRP^{-/-}) are more susceptible to collagen induced arthritis (CIA; an animal model of rheumatoid arthritis) [22].

Seminal work performed in the Szalai lab demonstrated that human CRP transgenic mice (CRPtg) resist MOG induced EAE [4] (**Fig. 1A**), an effect associated with (i) a CRP driven decrease in MOG dependent T cell proliferation (**Fig. 2**) and (ii) a CRP driven suppression of pro-inflammatory cytokines with concomitantly increased anti-inflammatory cytokines, e.g. IL-10 (**Fig. 3**). My more recent preliminary results suggest that CRP^{-/-} are more susceptible to EAE than wild type (**Fig. 4**). That CRP is protective against EAE and other kinds of autoimmunity in mice is now clear, but the mechanism via which CRP exerts this protection effect is still not yet understood. However, we have strong evidence that CRP's ability to protect mice from EAE relies on its interaction with Fc γ RIIB [23]. Thus human CRP, whether injected or expressed by transgene, does not protect mice from EAE if they lack Fc γ RIIB (**Figs 1B and 5**, respectively) [23]. The combined available evidence suggests CRP must interact with Fc γ RIIB in order to abrogate MOG-induced EAE. The cell type(s) on which the predicted CRP→Fc γ RIIB interaction takes place during EAE and where it occurs (CNS versus periphery) remain unknown. My project aims to determine if dendritic cells (DCs), potent antigen presenting cells that express abundant Fc γ RIIB [24] and can suppress autoimmunity [25], can mediate suppression of EAE through interaction with CRP.

DCs express both MHC I and MHC II molecules and their ability to cross-present antigen allows them to mount an effective adaptive immune response. According to the conventional paradigm, when immature DCs are exposed to antigen they phagocytize it, migrate to the lymph node, and therein present the processed antigen to T cells to drive their expansion. DCs also play an important role in shaping the T cell response i.e., DC activation can drive polarization of different T cell subsets, thus ultimately guiding the effector response towards damage or protection. This ability is important because the nature of T cell polarization can significantly alter the course of EAE. For example, EAE was once considered to be primarily Th1 mediated but it is now recognized to have an important Th17 component [6, 26, 27]. DCs can also promote proliferation of regulatory T cells (Tregs) which can diminish the autoimmune response [28]. The importance of DCs to EAE is demonstrated by a decrease in EAE severity in mice that have been genetically depleted of DCs [29].

CRP is known to interact with Fc receptors [30] and thus connect the cell-mediated and humoral arms of the adaptive and innate immune systems. Activating Fc receptors (Fc γ RI, Fc γ RIIA, Fc γ RIII, and Fc γ RIV) utilize a common γ -chain that encodes an intracellular immunoreceptor tyrosine based activation motif (ITAM). It is generally accepted that activating Fc γ Rs promote autoimmune disease [31]. Conversely, Fc γ RIIB is the sole Fc γ receptor to contain an intracellular immunoreceptor tyrosine based inhibition motif (ITIM). Thus when engaged by CRP, Fc γ RIIB negatively regulates the activating Fc γ Rs [35] and this action could potentially promote peripheral tolerance. CRP is known to bind Fc γ Rs on DCs [32] and my preliminary data shows that human CRP affects the expression of surface

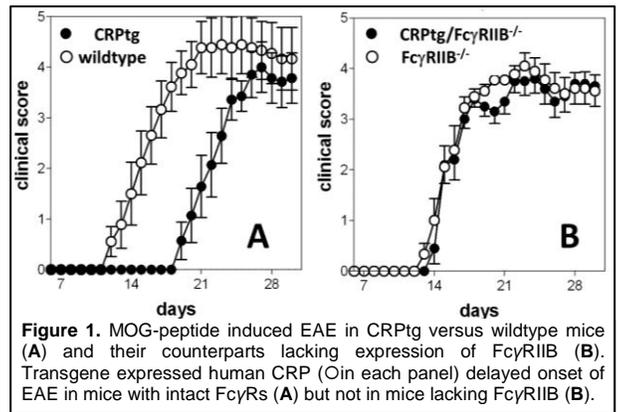


Figure 1. MOG-peptide induced EAE in CRPtg versus wildtype mice (A) and their counterparts lacking expression of Fc γ RIIB (B). Transgene expressed human CRP (O in each panel) delayed onset of EAE in mice with intact Fc γ Rs (A) but not in mice lacking Fc γ RIIB (B).

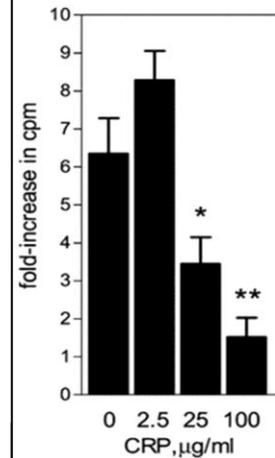


Figure 2. T cells enriched from spleens of mice with EAE were co-cultured with irradiated syngeneic splenic APCs plus MOG peptide (5 μ g/ml) and 0 to 100 μ g/ml human CRP. Cells were pulsed with [³H]-thymidine and ³H incorporation (cpm) measured 6 h later. Significantly less proliferation of T cells occurred in the presence of human CRP compared to cells with no human CRP added (*, p < 0.05; **, p < 0.005; Fisher's protected least squares difference tests).

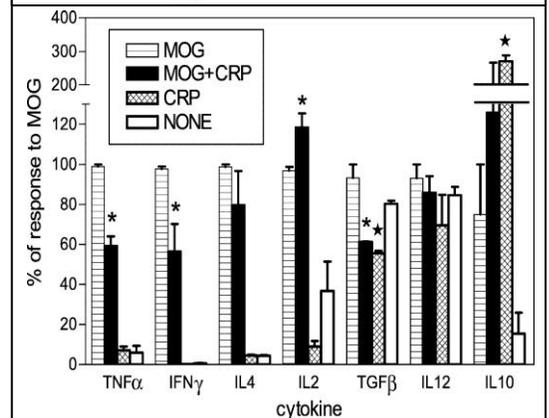


Figure 3. Encephalitogenic T cells from spleens of mice with EAE were co-cultured with irradiated APCs from spleens of naive donors and stimulated with MOG (5 μ g/well), MOG plus human CRP (100 μ g/ml), human CRP, or left untreated. Cytokines were measured at 48 h by ELISA and their production amount is shown relative to the amount measured after stimulation with MOG peptide. * = significantly less/more cytokine compared to cultures stimulated with MOG only (p < 0.05; t tests). ★ = significantly less/more cytokine compared to unstimulated cultures (p < 0.05; t tests).

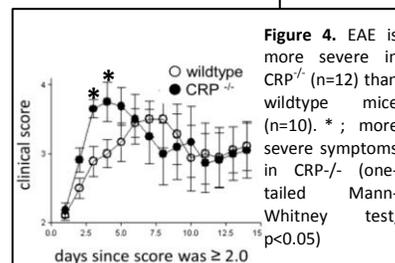


Figure 4. EAE is more severe in CRP^{-/-} (n=12) than wildtype mice (n=10). * ; more severe symptoms in CRP^{-/-} (one-tailed Mann-Whitney test, p < 0.05)

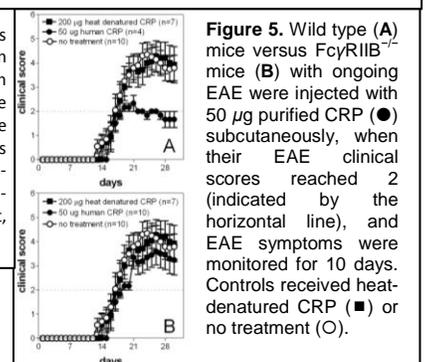


Figure 5. Wild type (A) mice versus Fc γ RIIB^{-/-} mice (B) with ongoing EAE were injected with 50 μ g purified CRP (●) subcutaneously, when their EAE clinical scores reached 2 (indicated by the horizontal line), and EAE symptoms were monitored for 10 days. Controls received heat-denatured CRP (■) or no treatment (O).

markers on bone marrow derived dendritic cells (BMDCs), upregulating MHCII (Fig. 6) and CD40, CD80, and CD86 (data not shown). The effect of CRP on these DC surface markers is reminiscent of the effect Flt-3L has on DCs [33], and like we predict for CRP stimulated DCs, BMDCs stimulated with Flt-3L inhibit autoimmunity when injected into mice [33]. On the other hand CRP stimulated BMDCs express less CD1d [34] and this decrease is FcγRIIB dependent (Fig. 7). The influence of CRP on BMDC activation markers and cytokines and ability to stimulate T cell proliferation and polarization, and the requirement of FcγRIIB for CRP mediated changes in these parameters, will be fully investigated under Aims 1 and 2. The demonstration that DCs are present in the brain during inflammation [35, 36] means it is possible that small amounts of CRP in the CNS are sufficient to modify DC function during EAE/MS. Alternatively microglia, a CNS resident APC [37], might replace the function of DCs in the brain and spinal cord. This possibility will be fully explored under Aim 3.

(b.2) Experimental Design:

The goal of my proposed project is to better understand the cellular mechanism(s) by which CRP protects mice from EAE. To achieve this goal I will utilize unique and informative animal models to dissect the requirement of DCs and FcγRIIB for CRP-mediated resistance. For example unlike human CRP, mouse CRP maintains a low level even during inflammation (i.e. it is not an acute phase reactant) [38]. We will take advantage of this species difference and by using wild type versus CRP^{-/-} mice [22], isolate the contribution of baseline CRP to protection from EAE. Importantly, because we have both CRP^{tg} and CRP^{-/-} we can by selective breeding produce humanized mice that express only human CRP and as an acute phase protein. To address the contribution of CNS versus hepatic expression of CRP, we will utilize mice that express human CRP exclusively in the CNS (see *General Methods* section). Finally, we have produced mice that are deficient in murine FcγRIIB but selectively express human FcγRIIB only on their DCs. In conjunction with commercially available DC-deficient mice, the latter will be used to demonstrate the importance of the DC for the CRP mediated shift in onset and progression of EAE. The remaining pages of this proposal are dedicated to describing the experimental design I will use to test each working hypothesis. Each aim's design will be discussed separately. Pertinent experimental procedures are described in section **b.2d General Methods**.

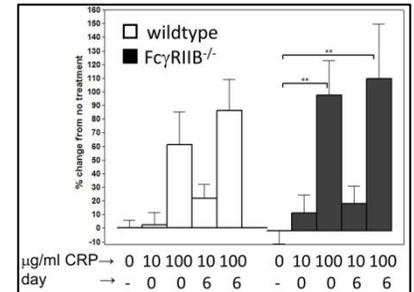


Figure 6. BMDC's from wildtype and FcγRIIB^{-/-} received recombinant human CRP on day 0 or day 6 of culture, then on day 7 cells were analyzed by flow cytometry for expression of **MHCII**. For each genotype, values are normalized to untreated cells.

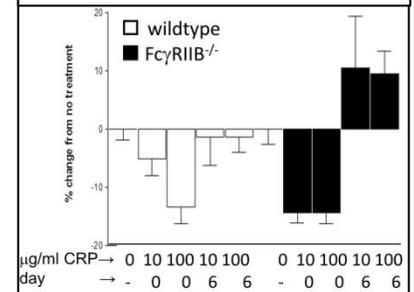


Figure 7. BMDC's treated with CRP as described for Figure 6 and analyzed for expression of **CD1d**. For each genotype, values are normalized to untreated cells.

(b.2a) Experimental Design for Aim 1:

Ascertain the influence of CRP on DC phenotype and function

The working hypothesis is that CRP binding to FcγRIIB on DCs alters their capacity to stimulate T cells. Activation markers and cytokines expressed *in vitro* by DCs derived from bone marrow (BMDCs) of wild type versus FcγRIIB deficient mice will be compared, as will T cell proliferation. *In vivo* we will test if CRP pre-conditioned BMDCs are therapeutic when administered to mice with ongoing EAE.

EXPT 1: To establish the time-frame of FcγRIIB expression by BMDCs, and thus refine the conditions for maximized CRP→FcγRIIB interaction, I will perform RT-PCR analysis on mRNA collected from DCs derived from wild type mouse bone marrow (n=3 marrow donors per BMDC culture) daily from initiation of the BMDC culture to its end on day 7. I will also add purified human CRP to these cultures to determine if CRP alters FcγRIIB expression levels. **EXPT 2:** To verify that human CRP→ mouse FcγRIIB interaction occurs *in vitro* on BMDCs, cells expressing maximal levels of FcγRIIB will be pulsed with purified human CRP (1, 10, 100 μg/ml) in the presence or absence of saturating amounts of the FcγRIIB blocking antibody 2.4G2 (BD Biosciences), then subjected to FACS analysis to quantitate CRP binding. Since antibody 2.4G2 has known cross-reactivity with mouse FcγRIII and CRP also binds that receptor, to confirm that 2.4G2 mediated inhibition of CRP binding is via blockade of FcγRIIB we will repeat the antibody inhibition experiments using BMDCs derived from our FcγRIIB^{-/-} mice. **EXPT 3:** To ascertain the influence of the CRP→FcγRIIB axis on BMDC phenotype I will culture wild type versus FcγRIIB^{-/-} BMDCs with increasing amounts of purified human CRP and (24 h later) measure expression of surface markers of DC activation and T cell co-stimulating capacity (e.g. MHC I and II, CD80, CD86, CD1d, CD11b). **EXPT 4:** To ascertain the influence of the CRP→FcγRIIB axis on BMDC

supported T cell proliferation and T cell cytokine production, I will use mixed cultures of CRP treated wild type versus Fc γ RIIB^{-/-} BMDCs and CFSE-loaded CD4⁺ T cells. For these experiments T cells will be harvested from mice expressing a transgenic MOG peptide-specific T cell receptor (2D2; available for purchase from Jackson Laboratories, Bar Harbor, ME) and BMDCs will be pulsed with MOG peptide. I will then use FACS to determine T cell proliferation, T cell expression of the CD80 and CD86 counter-receptors CD28 and CTLA-4, and T cell expression of; IFN γ (for a Th1 response), IL-4 (for a Th2 response), IL-17 (for a Th17 response), and IL-10 and TGF- β (for a Treg response) and ELISA to determine cytokines secreted by both DCs and T cells.

Expected Outcomes, Potential Pitfalls, and Alternative Strategies:

① Based on published reports [39] we expect maximal Fc γ RIIB expression by day seven of culture. ② We expect CRP binding to be dose-dependent and inhibited by 2.4G2 if wild type BMDCs are used but not Fc γ RIIB^{-/-} BMDCs. To ensure that exposure to CRP does not reduce expression of Fc γ RIIB we will perform real-time PCR on mRNA isolated from parallel BMDC cultures treated with CRP. ③ Based on our preliminary data we expect that CRP will alter BMDC expression of cell surface markers and that certain of these CRP driven changes will depend on Fc γ RIIB. By using BMDCs expressing maximal amounts of Fc γ RIIB in these experiments we can avoid prolonged cultures and thus ensure that any observed CRP mediated effects on BMDC phenotype are not due to CRP-mediated changes in viability or maturation of BMDCs. Preliminary experiments I have already performed (data not shown) indicate that CRP neither promotes BMDC death nor delays their development. ④ Decreased proliferation of damaging T cells or increased proliferation/polarization towards beneficial T cells would both support our position that CRP provides a tonic T cell suppressive effect in EAE. Likewise decreased T cell expression of CD28 or increased expression of CTLA-4 would be in alignment with our expectations. To test if CRP causes increased proliferation/polarization of regulatory T cells [21] I will monitor *in vitro* expression of CD25 and Foxp3 by FACS. Extrapolating from our previous data I anticipate that CRP will reduce T cell secretion of IFN γ and IL-17 while increasing IL-4, IL-10, and TGF- β . The dependence of these T cell outcomes on BMDC expressed Fc γ RIIB are difficult to predict.

(b.2b) Experimental Design for Aim 2:

Determine the contribution of DCs to CRP mediated Fc γ RIIB dependent protection from EAE

Success under Aim 1 will establish that a CRP→Fc γ RIIB axis operates *in vitro* and influences BMDC function. Under this aim we will pursue studies to show that this system operates *in vivo* and influences EAE outcome. The working hypothesis is that CRP associated Fc γ RIIB-dependent protection from EAE depends on DCs. Experiments will be pursued using DC sufficient versus DC-deficient CRPtg, Fc γ RIIB sufficient versus Fc γ RIIB-deficient CRPtg, and CRPtg expressing human Fc γ RIIB exclusively on DCs.

EXPT 5: BMDCs expressing high amounts of MHC class II and co-stimulatory molecules have been shown to alter the course of autoimmune disease when administered to mice [33]. I will harness this approach to determine the effect of CRP pre-conditioning on BMDCs' ability to influence ongoing EAE. Wild type and Fc γ RIIB^{-/-} BMDCs will be pulsed with human CRP *in vitro* as outlined under Aim 1, then harvested, washed, and administered (10⁶ cells per mouse via i.v. injection [40]) to mice with ongoing EAE. **EXPT 6:** Direct proof that human CRP mediated protection against EAE relies on Fc γ RIIB expressed by DCs, and evidence that a CRP→Fc γ RIIB axis should operate in humans with MS, will be obtained using Fc γ RIIB^{-/-} mice that express human Fc γ RIIB driven by a mouse CD11c promoter (CD11c-huFc γ RIIB mice; see **Fig. 9**). EAE will be induced in wild type, Fc γ RIIB^{-/-}, and CD11c-huFc γ RIIB mice and the protective capacity of i.p. administered purified human CRP assessed as described (**Fig. 5**). **EXPT 7:** As a corollary to experiment 6 I am breeding CRPtg with CD11c-DTR mice (see part **b3.d General Methods**) and will thus generate CRPtg mice whose CD11c⁺ DCs can be selectively depleted via injection of diphtheria toxin. Together with experiment 6, this experiment will establish the strength of the requirement for dendritic cells for CRP-mediated protection from EAE.

Expected Outcomes, Potential Pitfalls, and Alternative Strategies

① I can routinely generate up to 10⁷ BMDCs from n=3 donor mice so generating sufficient numbers of BMDCs for these experiments is not anticipated to be problematic. Depending on the strength and duration of the CRP pre-conditioning effect (to be determined), wild type BMDCs (but not Fc γ RIIB^{-/-} ones) pre-conditioned with human CRP should be able to slow, stop, or even reduce progression of clinical signs of EAE. ② Replacement of endogenous mouse Fc γ RIIB with human Fc γ RIIB expressed exclusively on CD11c⁺ DCs is expected to fully restore the ability of injected human CRP to protect against EAE, thus confirming that the human CRP→human Fc γ RIIB axis is functional and therefore likely to operate in humans with MS. If this outcome is obtained then CRPtg/Fc γ RIIB^{-/-} mice will be mated to our CD11c-huFc γ RIIB to generate CRPtg and

non-tg CD11c-huF γ RIIB. These can be used to see if CRP \rightarrow F γ RIIB is protective in a humanized system. ③ DCs will be depleted in CD11c⁺-DTR/CRPtg mice injected with DTx, and I anticipate this will render human CRP unable to confer protection from EAE. Accordingly, the disease course in CRP transgenic CD11c-DTR mice should be lessened compared to their CRP non-transgenic counterparts, but not if they are DTx treated (i.e. not if they are DC depleted). ④ If DC depletion does not ablate CRP protection then we will consider the possibility that other potent F γ RIIB expressing APCs play a role, e.g. B cells and macrophages [41, 42]. For example we could test if CRP alters macrophage polarization during EAE [perhaps away from classical activation and towards alternative activation?] [43] and we could test if CRP drives B cell production of IL-10, for example. To study further if CRP's beneficial effect is manifest through B cells, I could induce EAE in CRPtg that express human F γ RIIC specifically on their B cells. These mice are also already available in the lab. F γ RIIC is known to negatively regulate the inhibitory effects of F γ RIIB. Thus if CRP's protective effect requires F γ RIIB on B cells, CRPtg expressing B cell specific human F γ RIIC should not be protected.

(b.2c) Experimental Design for Aim 3:

Assess the protective capacity of CNS expressed CRP

The outcomes of the experiments described under Aims 1 and 2 will reveal the influence of CRP on DC function and the extent to which this cell type (or other APCs) is required for CRP mediated protection from EAE. The experiments under this aim are designed to reveal the site of operation of the CRP \rightarrow F γ RIIB \rightarrow APC pathway. The working hypothesis is that CRP produced in the CNS is sufficient to protect against EAE. Studies will rely on a novel mouse strain wherein a human CRP transgene is driven by a promoter that limits its expression to neurons.

Although DCs have been identified in the CNS [44], microglial cells are the resident APCs of the central nervous system. There they exist in three states: (1) ramified microglia that rest within the parenchyma; (2) activated, non-phagocytic microglia found in regions of inflammation; (3) reactive phagocytic microglia found in areas of infection or neurodegeneration. Like DCs, microglia are believed to originate from monocytes within the bone marrow and migrate to the CNS during embryonic development. During EAE, when the BBB is breached, DCs and monocytes/macrophages are able to freely pass from the periphery into the CNS. Since there is no reliable marker to distinguish between microglia and these cells from the periphery it is difficult to determine if the CRP driven protective cells in EAE are CNS resident ones or infiltrating ones. Our preliminary evidence indicates expression of CD68, a marker of microglial activation, is significantly attenuated in CRPtg compared to wild type mice (data not shown). This suggests that in addition to an effect on DCs, human CRP can also reduce activation of CNS resident microglia. Importantly microglia also express F γ RIIB [45]. For these reasons under this aim I will examine the contribution of CNS expressed human CRP and the contribution of CNS resident microglia to CRP-mediated suppression of EAE. **EXPT 8:** Our existing CRP^{-/-} mice will be crossed with our new mutants that express human CRP exclusively from their neurons (nCRPtg; see the *General Methods* section) to generate humanized mice with CNS restricted expression of human CRP. EAE will be induced in nCRPtg/CRP^{-/-} versus CRP^{-/-}. Since our preliminary data show that CRP^{-/-} are susceptible to EAE (**Fig. 4**) we will be able to determine if CNS restricted production of human CRP is sufficient to alter the onset and course of EAE. **EXPT 9:** Using the same approach as in experiment 8 nCRPtg/CRP^{-/-} versus CRP^{-/-} will be subjected to EAE and at 2 weeks after appearance of EAE symptoms, I will isolate CD11b⁺ cells from the cerebral cortex and assess differences in levels of MHCII and CD68, a microglia activation marker. **EXPT 10:** Wild type versus F γ RIIB^{-/-} microglia (from n=9 postnatal day 0-2 mice per genotype) will be treated with human CRP (see Aim 1) to assess differences in microglial activation.

Expected Outcomes, Potential Pitfalls, and Alternative Strategies

① I anticipate that CRP^{-/-} will be more susceptible to EAE than wild type and that nCRPtg/CRP^{-/-} will be protected from EAE compared to CRP^{-/-}. These outcomes would indicate that mouse CRP is able to limit EAE development in wild type mice and that human CRP expressed locally in the CNS is sufficient to protect against EAE in CRP^{-/-}. The protective action of CNS-expressed human CRP should be evidenced by decreased expression of activation markers by CD11b⁺ cells *ex vivo*, and this should be recapitulated using microglia cultured *in vitro*. ② If, as we expect, neuronally-produced CRP is sufficient to protect mice against EAE, by inducing EAE in nCRPtg mice that are deficient in F γ RIIB we can determine if neuronal CRP mediated protection from EAE is dependent upon F γ RIIB in the brain.

(b.2d) General Methods: A variety of specialized mouse strains will be used. Expression of the human CRP transgene in CRPtg mice mimics the human acute phase response, i.e. blood human CRP levels are low

at baseline (1 to 30 mg CRP/ml serum) and high during an inflammatory response (100 to 500 mg/ml). Thus, CRPtg provide an excellent *in vivo* model for studying the role of CRP within EAE/MS. CRPtg contain a 31kb segment of human DNA that contains the CRP gene, the CRP promoter and all other cis-regulatory elements [46]. Our lab has recently engineered CRP deficient mice by crossing floxed Crp with protamine Cre-recombinase transgenic mice. The mice are fully described elsewhere [22]. We also generated mice expressing the human CRP transgene exclusively in neurons. In these nCRPtg mice human CRP expression is driven by the CAMKII α promoter to ensure neuronal expression (**Fig. 8**). Mice that are deficient in murine Fc γ RIIB but selectively express human Fc γ RIIB on their DCs (driven by the CD11c promoter; **Fig. 9**) have also been made. To complement these we will use CD11c-DTR mice (Jackson Labs, Bar Harbor, ME) which allow for the selective ablation of DCs via injection of diphtheria toxin [47]. Finally, mice lacking Fc γ RIIB already are available in the Szalai laboratory.

To induce EAE, on day 0 mice will receive 150 μ g MOG₃₅₋₅₅ peptide in complete Freund's adjuvant containing 400 μ g/ml *Mycobacterium tuberculosis*. On days 0 and 2 mice also receive an i.p. injection of 200ng pertussis toxin. EAE development is monitored daily between 7-10 am using the following clinical scale: 0, no symptoms; 1, loss of tail tone; 2, tail flaccidity; 3, incomplete hind limb paralysis; 4, total hind limb paralysis; 5, hind and forelimb paralysis (animals are euthanized); 6, death. Mice will be observed for at least 30 days. Mice with a score of ≥ 2 for ≥ 2 consecutive days will be considered to have EAE. For comparison scores will be average for each genotype. **To deplete DCs** prior to induction of EAE, CD11c-DTR mice will be injected with 100ng DTx in 100 μ L of PBS (controls will receive PBS).

Bone marrow cells will be used to derive DCs and macrophages. Briefly, marrow cells from femurs and tibiae are grown in RPMI with 10% FBS and Pen/Strep. The cells are supplemented with IL-4 (50 μ g/ml) and GM-CSF (10 μ g/ml) to generate CD11c⁺ DCs. F4/80⁺ macrophages are derived from bone marrow but require no IL-4 or GM-CSF stimulation. Instead, I will supplement them with media from L929 cells (a source of M-CSF).

CD4⁺ T cells are obtained from spleens and lymph nodes via negative selection using a kit from Stem Cell Technologies. CD4⁺ **To culture microglia**, cerebral cortices are taken from pups at post natal day 0-2, trypsinized, and then passed through 70 μ L nylon filters. Cells are then suspended in DMEM-F12, 10%FBS, and penicillin/streptomycin and incubated for 14-16 days at 37°C at 5% CO₂. At day 14-16, cells are then separated using a CD11b positive selection kit (STEMCELL technologies).

To assess T cell proliferation T cells are loaded with CFSE (Life technologies) before being co-cultured with BMDCs for 72 hours at 37°C at 5% CO₂. Supernatants are aspirated and stored at -20°C. Cells are stained for cell surface markers and then cell surface expression and proliferation are measured by flow cytometry.

Statistical analysis: All *in vivo* experiments comparing the course of EAE will be performed with a minimum of 15 mice per genotype or treatment group with replication, and all *in vitro* experiments will be performed at least three times to ensure reproducibility of the findings. These experimental group sizes are based on power calculations and feasibility. Statistical analyses will be done in coordination with biostatistics resources and experts available at our institution. Pooled data will be expressed as mean \pm SEM for *in vivo* and mean \pm SD for *in vitro* experiments, and longitudinal measures assessed by repeated-measures ANOVA. When significant differences are observed ($p < 0.05$) post-hoc pair-wise least-squared difference tests will be performed with Bonferroni corrections. When data are not normally distributed analysis will be performed after transformation.

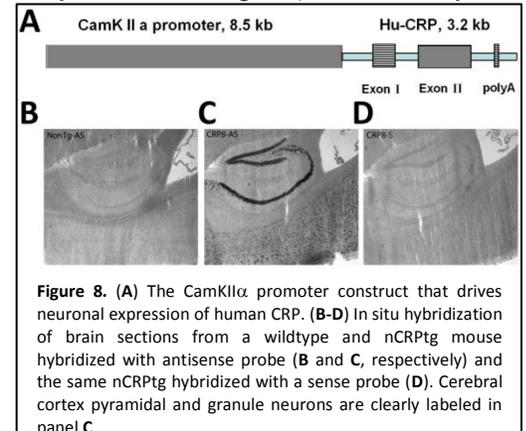


Figure 8. (A) The CamKII α promoter construct that drives neuronal expression of human CRP. (B-D) In situ hybridization of brain sections from a wildtype and nCRPtg mouse hybridized with antisense probe (B and C, respectively) and the same nCRPtg hybridized with a sense probe (D). Cerebral cortex pyramidal and granule neurons are clearly labeled in panel C.

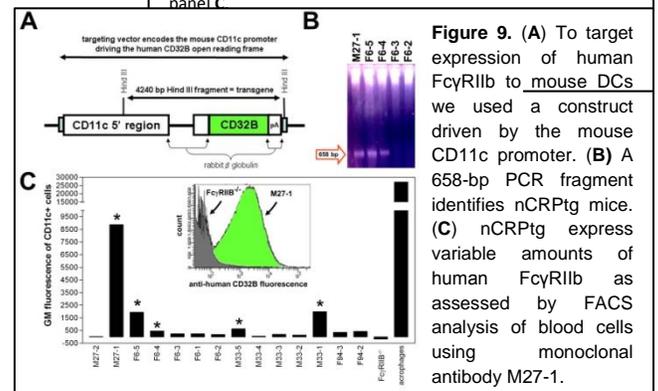


Figure 9. (A) To target expression of human Fc γ RIIB to mouse DCs we used a construct driven by the mouse CD11c promoter. (B) A 658-bp PCR fragment identifies nCRPtg mice. (C) nCRPtg express variable amounts of human Fc γ RIIB as assessed by FACS analysis of blood cells using monoclonal antibody M27-1.