THE COAGULATION FACTORS, ACTIVATED PROTEIN C AND THROMBIN, MODULATE THE PATHOGENESIS OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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THE COAGULATION FACTORS, ACTIVATED PROTEIN C AND THROMBIN, MODULATE THE PATHOGENESIS OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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The immune and coagulation systems are closely linked. Components of the coagulation system, notably activated protein C (APC) and thrombin, have potent effects on the immune response. APC, beyond its traditional function as an anti-coagulant, has broad anti-inflammatory effects, mediated through modulation of leukocyte functions and conferring vascular barrier protection. Conversely, thrombin, the central enzyme in coagulation, induces pro-inflammatory responses and promotes vascular barrier disruption. We examined the influences of APC and thrombin on the pathogenic components of experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis (MS), an autoimmune disease characterized by infiltration of encephalotogenic $CD4^+$ T-cells in the CNS resulting in immunopathology.

We investigated the influence of APC on EAE pathogenesis by inhibiting APC in the circulation of mice induced with EAE. Interestingly, we observed that APC inhibition alleviated EAE despite increased blood brain barrier (BBB) permeability and pronounced leukocyte infiltration in the brain. We additionally observed that the encephalotogenic $CD4^+$ T-cells in the CNS is decreased, and, consistently, the CNS pathology is minimal. Moreover, inhibition of APC modulated the functional responses of peripheral leukocytes, resulting in increased and more activated $CD11b^+$ myeloid population, including the $CD11b^+$ regulatory subset referred to

as myeloid-derived suppressor cells (MDSC) and characterized to be potent T-cell suppressors. We demonstrated that the increase in MDSCs resulted in the suppression of functional responses of $CD4^+$ T-cells required for EAE progression. Thus, the net effect of APC inhibition during EAE is attenuated disease.

We additionally examined the inflammatory effects of the coagulation factor, thrombin, on an *in vitro* BBB. Overall, thrombin effectively modified the BBB, resulting in a BBB phenotype that is conducive to leukocyte recruitment, firm adhesion and extravasation. The permissiveness of the BBB to leukocyte transendothelial migration is one the major pathological components of EAE and MS. These findings, therefore, are indicative of thrombin's potential influence on the progression of these diseases.

Collectively, the results described in this dissertation strongly demonstrate the capacity of APC and thrombin in modulating the pathogenic components of EAE and further affirms that the dynamic interactions between the coagulation and immune systems in disease settings can affect disease progression.

BIOGRAPHICAL SKETCH

Leah was born in the seaside town of Agoo, La Union in the Philippines. At the age of 13, she and her family settled in the United States. She attended Kearny High School in New Jersey and earned a bachelor's degree in Biology and Computer Science at Rutgers University in New Brunswick, New Jersey. Leah entered the graduate program at Cornell University in Ithaca, New York to pursue a Ph.D. degree in the field of Immunology.

DEDICATION

To the mysteriousness of existence

“And when we meet on a cloud
I'll be laughing out loud
I'll be laughing with everyone I see
Can't believe how strange it is to be anything at all”

~ Neutral Milk Hotel

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Introduction

Significance

A complex and dynamic interaction exist between the immune and coagulation systems. Endogenous coagulation components have considerable impact on the pathogenesis of inflammatory diseases. This is demonstrated by the aberrant coagulation states observed in severe inflammatory conditions, notably in sepsis [1-3]. Understanding the interplay between the immune and coagulation systems, and how this interaction influences the pathogenesis of disease conditions will give way to novel therapeutic avenues and provide a more complete picture of the underlying biology of disease conditions. This dissertation describes the influence of the endogenous anti-coagulant, activated protein C (APC), on the inflammatory progression and pathology of experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis (MS). In addition, the effect of the coagulation factor, thrombin, on blood brain barrier (BBB) permeability, a major pathological component of neuroinflammatory diseases, including EAE and MS, is also described.

The Coagulation Process

The coagulation process has traditionally been presented as a cascade of events in which coagulation serine proteases activate the factors required for the subsequent step, culminating into the formation of a blood clot [4, 5]. This cascade model is divided into two distinct pathways, the extrinsic pathway, which is initiated outside the vasculature, and the intrinsic pathway, viewed to occur within the vasculature [6, 7]. Both pathways use a common pathway to eventually generate the hemostatic plug or blood clot [4-7]. New understanding of hemostasis, however, suggests that the cascade model is deficient in accurately depicting the coagulation process as it occurs *in vivo* [6, 7]. In recent years, a new model has been developed

that incorporates the importance of cells in hemostasis. The cell-based model of coagulation views the intrinsic and extrinsic pathways not as two separate and redundant pathways but rather as parallel pathways that occur on different cell surfaces [6, 7].

Initiation Phase

The cell-based model proposes that the initiation phase of the coagulation process *in vivo* occurs on the surface of cells that express tissue factor (TF) [8]. TF is a membrane glycoprotein expressed on cells that are normally sequestered from the vasculature, but an injury that results in the rupture of blood vessels, such as what occurs in a cut, exposes TF to coagulation factors [6-8]. The coagulation process is initiated when the active form of the coagulation factor VII (FVIIa) binds to exposed TF, and the TF-VIIa complex results in further activation of FVII, thus amplifying the number of TF-VIIa complexes [7] (Figure 1.1). The TF-VIIa complex then activates factors IX (FIX) and X (FX) [9]. The activated FX (FXa), that remains bound to the surface of TF-expressing cells, can activate Factor V (FVa), which binds to FXa and serve as its co-factor [10]. The FXa-FVa complex, also known as prothrombinase complex, remains on the surface of TF-bearing cells, where it cleaves prothrombin, generating its active form, thrombin [6, 7].

Amplification Phase

The coagulation cascade proceeds to the amplification phase if platelets leak out to the extravascular area and adhere to the TF-bearing cells [6, 7]. The small amount of thrombin generated on the surface of TF-expressing cells in the initiation phase results in the activation of platelets [11] (Figure 1.1). Activated platelets express negatively charged phospholipids on the cell membrane that will bind the glutamic acid (Gla) residues of coagulation proteins [7, 12] .

The cell membrane of activated platelets, therefore, provides a pro-coagulant surface on which coagulation proteins can localize, thus enhancing the assembly of coagulation complexes and amplifying the activation of coagulation factors [6].

In addition to platelet activation, the small amount of thrombin generated in the initiation phase will result in the activation of factors XI (FXI), FV, and factor VIII (FVIII) [6, 7, 13]. Thrombin is also responsible for the release of von Willebrand factor from FVIII, thus freeing it to mediate platelet adhesion and aggregation [14, 15].

Propagation Phase

The surfaces of activated platelets serve as the main setting for the propagation phase of the coagulation process (Figure 1.1). FIXa generated both by the TF-VIIa complex in the initiation phase and by FXIa in the amplification phase associates with FVIIIa generated during the amplification phase to form the tenase complex on the surface of activated platelets [6, 16]. The tenase complex is responsible for the rapid generation of FXa on platelets [16]. FXa then rapidly binds to FVa activated by thrombin in the amplification phase to form additional thrombinase complexes on the surface of platelets [17]. The activity of thrombinase complexes on platelets leads to a surge of thrombin generation [6, 7]. This burst of thrombin activity results in the conversion of fibrinogen to fibrin through the cleavage of fibrinopeptide A from fibrinogen [18-20]. This cleavage will reveal polymerization sites that will allow fibrin molecules to form soluble complexes with neighboring fibrin and fibrinogen molecules [19, 20]. A profuse amount of thrombin needs to be generated in order to result in a critical mass of soluble fibrin complexes that will spontaneously polymerize into fibrin strands and form an insoluble fibrin matrix, which serves as the scaffolding of a blood clot [20].

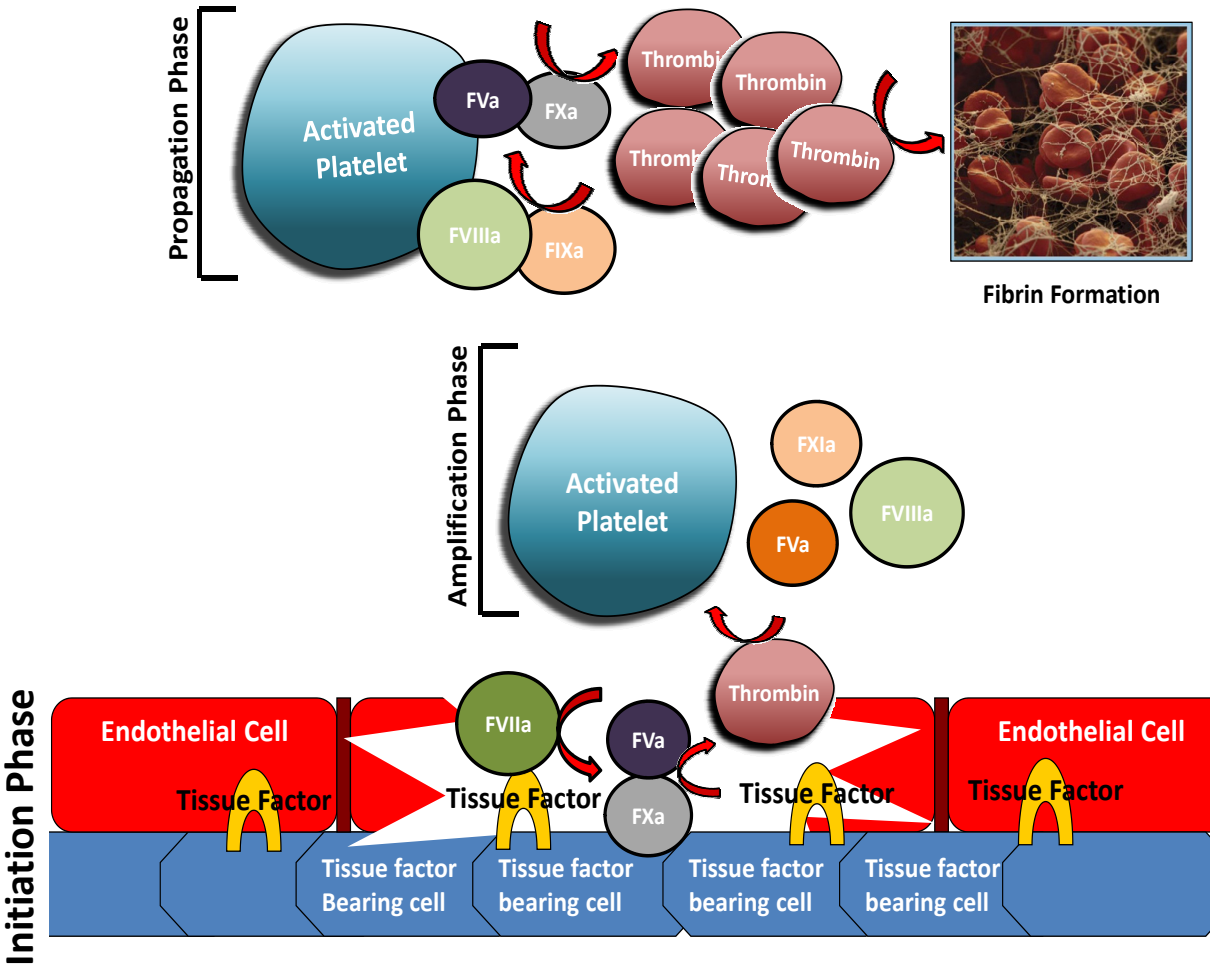

Figure 1.1 The cell-based model of the coagulation process.

The Protein C Anti-Coagulant System

The coagulation process is tightly regulated in order to localize the coagulation cascade in the area of injury and prevent systemic dissemination of activated coagulation proteins that may lead to thrombosis [21]. The protein C anti-coagulant pathway is one of several systems that regulate the coagulation process. The initiation of the protein C pathway occurs when thrombin binds to thrombomodulin, a transmembrane glycoprotein on the surface of endothelial cells [22] (Figure 1.2). This binding blocks thrombin's exosite I, a site required for thrombin's interactions with its substrates in the coagulation cascade, thus effectively abrogating thrombin's pro-coagulant activity [23]. Once thrombin is complexed to thrombomodulin, its substrate affinity is switched to the zymogen, protein C (PC), resulting in the cleavage of the activation peptide of PC to generate the serine protease, activated protein C (APC) [24]. The activation of PC is enhanced if PC is bound to the endothelial protein C receptor (EPCR), which co-localizates PC with the thrombin-thrombomodulin complex on the surface of endothelial cells [25]. APC regulates the coagulation process by proteolytically inactivating FVa and FVIIIa, thus inhibiting further generation of thrombin and consequently dampening the coagulation cascade [26, 27]. Protein S, a vitamin K-dependent glycoprotein, acts as a co-factor for APC in the inactivation of FVa and FVIIIa [28, 29].

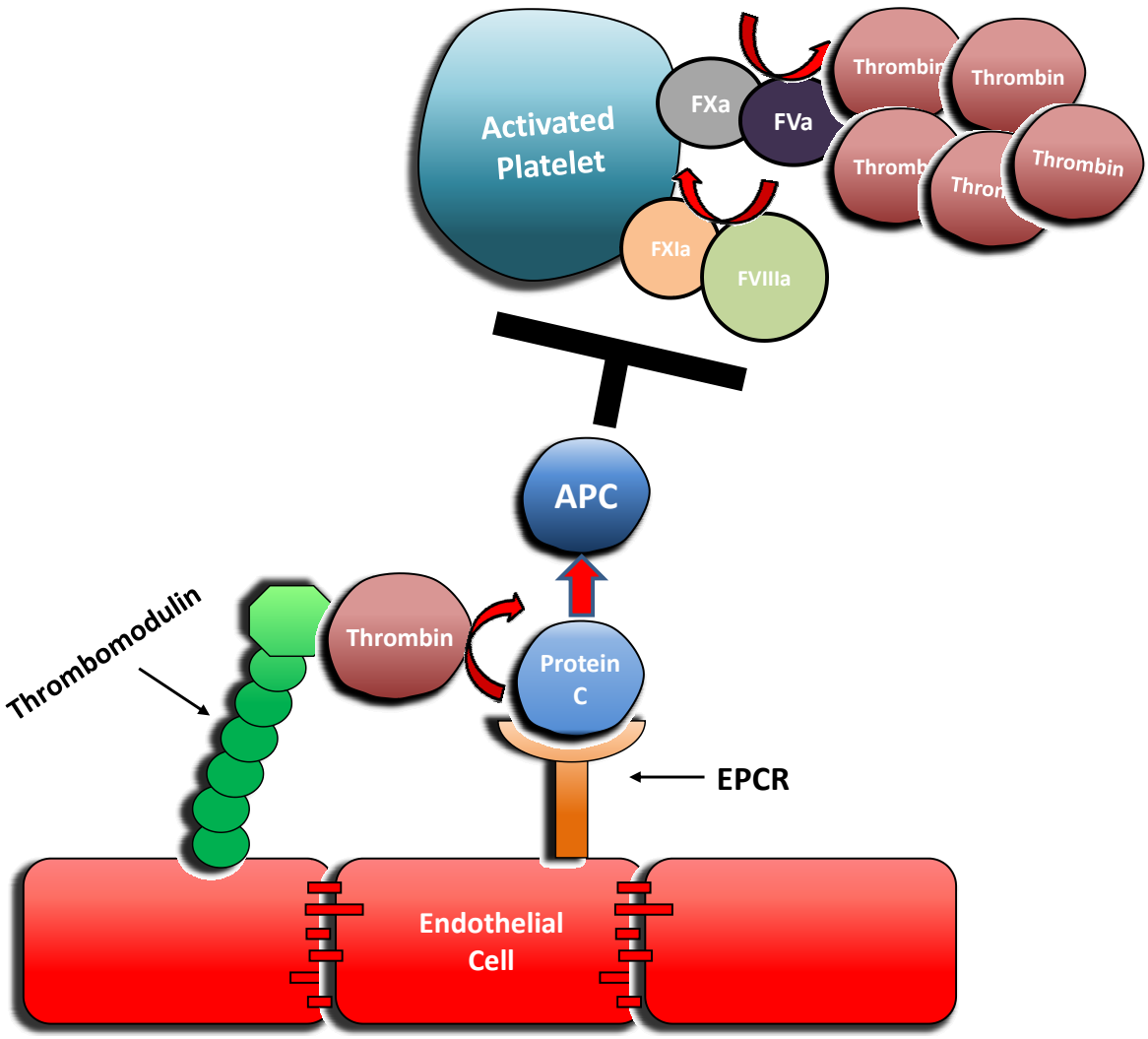

Figure 1.2 The Activated Protein C anti-coagulant pathway.

Two-way interaction between the Coagulation and Immune Systems

The existence of a link between the coagulation and immune systems is manifested in the aberrant coagulation state observed in severe inflammatory conditions [30, 31]. This is notably true in sepsis wherein coagulopathy is considered one of the pathologic hallmarks of the disease [2]. The activation of the coagulation process in sepsis is evidenced by fibrin deposition in blood vessels and various organs [1, 3]. Septic patients also exhibit low platelet counts and increased risk of bleeding, as a result of rapid consumption of platelets and coagulation factors [1]. Moreover, disseminated intravascular coagulation (DIC) correlates with sepsis severity and considered a predictor of organ failure and mortality in septic patients [1, 3]. Induction of the coagulation system has also been observed in inflammatory conditions caused by viral, fungal, protozoan, and spirochetes infection [32-35]. A number of autoimmune disorders, including inflammatory bowel disease, systemic lupus erythematosus, rheumatoid arthritis, and type-1 diabetes mellitus, have also been associated with abnormal coagulation conditions [36-39].

Initiation of the Coagulation Process and Inhibition of Anti-coagulant Pathways during Inflammatory Conditions

There is a consensus understanding that the activation of the coagulation process during severe inflammatory conditions is largely mediated by pro-inflammatory cytokines [40]. As outlined above, the coagulation process is initiated *in vivo* through exposure of coagulation components to TF, which is normally expressed on cells outside the vasculature. In inflammatory settings, the production of pro-inflammatory cytokines, particularly IL-6, results in the induced expression of TF on circulating mononuclear cells and on endothelial cells,

consequently leading to activation of the coagulation cascade [30]. The central role of IL-6 and TF in the initiation of the coagulation process in inflammatory setting have been demonstrated in a number of studies in which inhibition of either IL-6 or TF during pro-inflammatory conditions, such as in endotoxemia, abrogates the activation of the coagulation cascade and alleviates disease pathology [41, 42].

While inflammatory conditions results in the activation of the coagulation process, the anti-coagulant pathways, conversely, are actively suppressed during inflammation [43]. Levels of APC in the circulation during inflammatory settings are diminished due to decreased synthesis of PC in the liver and inhibited PC activation [43]. Pro-inflammatory cytokines, specifically TNF-α, down-regulate the expression of thrombomodulin and EPCR on the endothelium [44, 45], both of which are required for the effective activation of PC [22], thus resulting in deficient PC activation and an overall impaired protein C pathway.

How Thrombin Influences the Inflammatory Response

The cross-talk between the coagulation and immune systems is a two-way interaction, wherein both systems impact each other [46]. Accordingly, the activation of the coagulation system during inflammatory conditions results in the generation of coagulant factors that can directly affect the immune response [40]. In particular, thrombin, beyond its role as the central protease in the coagulation cascade, has considerable and well-documented impact on several aspects of the immune response [47]. The overall influence of thrombin on the immune system is to propagate inflammation, and this is mediated through thrombin's capability to activate a group of G-protein-coupled cellular receptors known as protease-activated receptors (PARs) [47-

49]. There are four identified PARs, namely PAR-1, PAR-2, PAR-3, and PAR-4; thrombin is known to activate PARs 1, 3, and 4 [48, 49]. Thrombin activation of PARs requires proteolytic cleavage of the N-terminal part of a tethered ligand on the extracellular domain of the receptor, consequently exposing a new N-terminus sequence that can associate with the extracellular loop 2 of the receptor to initiate cell signaling [48, 50]. Various cell types, including endothelial cells, fibroblasts, platelets, smooth muscle cells and several leukocyte populations express PARs, enabling thrombin to directly regulate the cellular processes and responses of these cells [30]. Thrombin propagates inflammation largely through its ability to direct cell signaling in endothelial cells and several leukocyte subsets [47].

Thrombin activation of PARs, specifically PAR-1, expressed on the endothelium induces endothelial cells to undergo phenotypic changes that include cytoskeletal rearrangements, alterations in cell shape, and disassembly of junctional proteins, leading to an overall increase in vascular permeability [47, 51, 52]. Downstream signaling of thrombin-activated PAR-1 on endothelial cells involves PAR-1 coupling with G proteins, $G\alpha_q$ and $G\alpha_{12/13}$ [53] (Figure 1.3). Thrombin-activated PAR-1 additionally results in the trans-activation of sphingosine 1-phosphate receptor 3 ($S1P_3$) [47] (Figure 1.3). $S1P_3$ is one of five G-protein-coupled receptors for the lipid derivative, sphingosine 1-phosphate (SIP) [47, 54]. Similar to PAR-1, activated $S1P_3$ also couples with $G\alpha_q$ and $G\alpha_{12/13}$ [47, 54]. Coupling of PAR-1 and $S1P_3$ to these particular G-proteins results in the stimulation of the guanosine triphosphatase (GTPase), RhoA, which mediates cytoskeletal re-organization and disassembly of the junctional proteins that connect adjacent endothelial cells, consequently increasing vascular barrier permeability [47, 54]. Thrombin has also been reported to modulate the transcription of a wide spectrum of genes in endothelial cells, notably increasing the expression of molecules that can facilitate trafficking

and leukocyte-adhesion to the endothelium [50]; these include increased expression of adhesion molecules, ICAM-1, VCAM-1 [55], and increased generation of chemokines, IL-8 and MCP-1 [56, 57]. It follows that the cumulative effect of thrombin on the endothelium during inflammatory conditions is increased vascular permeability to leukocyte transendothelial migration. In fact, it has been shown that direct inhibition of thrombin in an inflammatory setting impedes leukocyte adhesion to the endothelium and vascular leakage *in vivo* [58]. PAR-1 antagonism similarly inhibited *in vivo* leukocyte recruitment [59].

Thrombin additionally exerts its pro-inflammatory effects by directly modulating leukocyte function (Figure.1.3). The expression of PAR-1 has been observed on monocytes, macrophages, dendritic cells, and T-cells [60-63]. Thrombin can induce chemotaxis in monocytes and elicit the production of IL-6 and MCP-1 [64-66]. The proliferative responses and cytokine production in T-cells are augmented as a result of thrombin activation [67]. Thrombin-mediated signaling in dendritic cells (DC) regulates the production of cytokines and enhances the antigen presenting capacity in these cells [62]. Moreover, the PAR-1 and S1P3 signaling pathway in DCs has been implicated in the dissemination of inflammation in an animal model of sepsis [68].

Consistently, a number of studies in animal models of inflammation have demonstrated that modulation of thrombin levels and thrombin-associated signaling can reduce systemic inflammation and disease pathology. For instance, in baboons lethally challenged with Escherichia coli, treatment with recombinant human antithrombin III, an endogenous thrombin inhibitor, attenuated inflammation and improved survival [69]. Thrombin inhibition and selective PAR-1 antagonism similarly improved survival in mice challenged with lipopolysaccharide (LPS) [68].

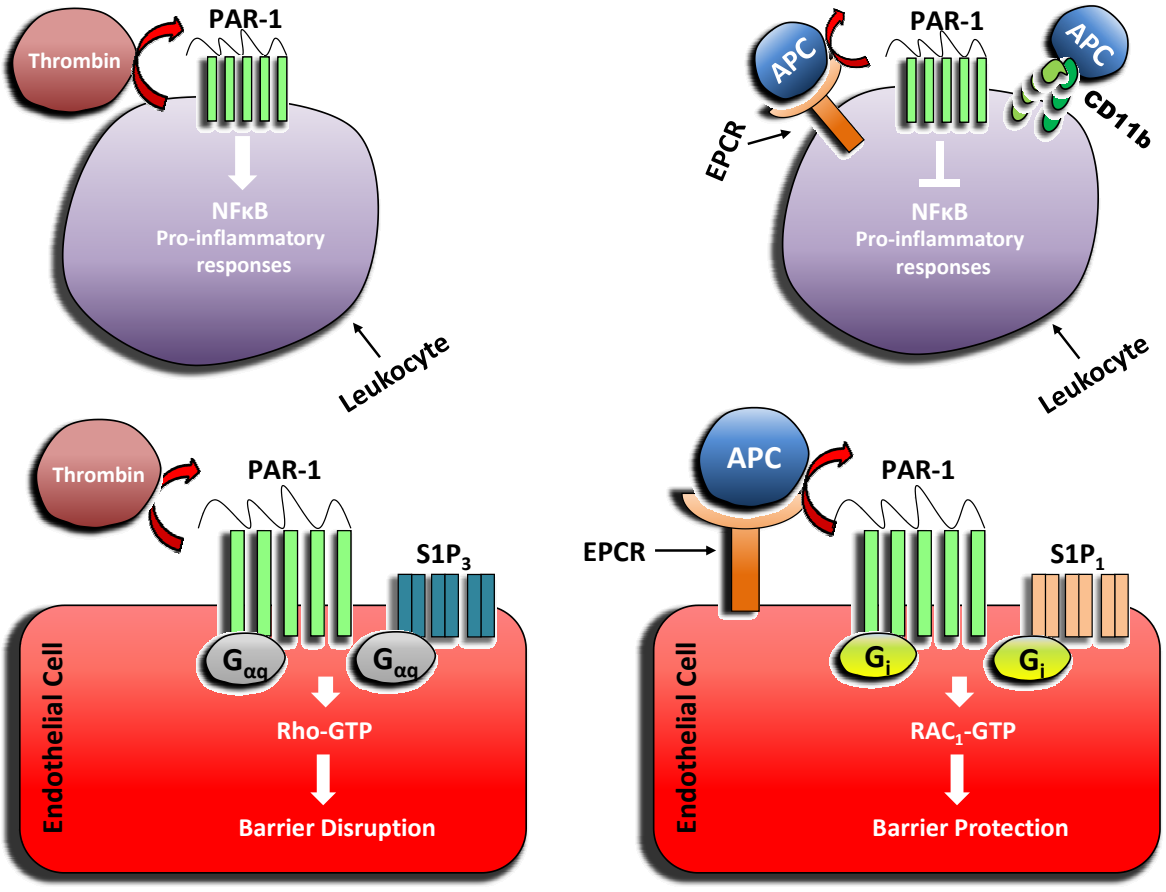

Figure1.3. Thrombin and APC have opposing influences on endothelial cells and leukocytes.

How APC influences the Inflammatory Response

In much the same way that APC and thrombin are functionally opposite in their respective roles in the coagulation process, their overall influence on the immune response are similarly divergent. Whereas thrombin has primarily been associated with pro-inflammatory responses, APC, in contrast, has potent anti-inflammatory and anti-apoptotic effects [23]. Both APC and thrombin, however, mirror each other in the way they exert their influence on the immune response, which is primarily through their cell-signaling capacities that enable both molecules to directly regulate cellular functions [70]. Moreover, in somewhat of a paradoxical twist, APC and thrombin invoke opposing influences on the immune response through activation of the same cellular receptor, PAR-1 [71, 72] (Figure 1.3). APC, however, needs to bind to EPCR in order to initiate signaling through PAR-1 [72], and it has been proposed that APC's occupancy of EPCR is the mediating factor that enables APC and thrombin to initiate divergent and opposing cellular responses from one receptor [73-75] (Figure 1.3). One group has suggested that the association of APC to EPCR localizes APC in the cholesterol-enriched lipid rafts or caveolae microdomains of the cell membrane, where PAR-1 and EPCR are both co-localized [74, 75]. They propose that the binding of APC to EPCR may induce changes in the membrane localization and/or conformation of the PAR-1 exodomain that renders PAR-1 more accessible to activation [75]. Furthermore, it was demonstrated that the binding of APC to EPCR results in the migration of EPCR from the caveolar compartment, a process that appears to induce PAR-1 to switch its G-protein association to G_i, subsequently triggering cell signaling cascades that favor anti-inflammatory responses [73, 75]. This deviates from thrombin activation of PAR-1, which results in PAR-1 coupling to $G\alpha_q$ and $G\alpha_{12/13}$, thereby giving rise to pro-inflammatory processes [54].

The anti-inflammatory influences of APC are primarily mediated through its direct ability to regulate the cellular responses in endothelial cells and leukocytes [23]. The culminating effect of APC on the endothelium during inflammatory conditions is to limit the extravasation of inflammatory leukocytes into tissues [23]. This is a direct result of APC's potent ability to stabilize the integrity of the endothelial barrier. As noted above, most of the cell signaling pathways mediated by APC, including its barrier protective effects, involves the coupling of PAR-1 to the G protein, G_i. APC- mediated signaling in endothelial cells also results in the transactivation of $S1P_1$, one of the receptors for the sphingolipid, S1P, that couples with G_i [54, 76]. This is in contrast to thrombin-mediated signaling, which transactivates the $S1P_3$ receptor, resulting in mobilization of the second messenger, RhoA, known to facilitate endothelial barrier disruption. $S1P_1$ transactivation by APC, on the other hand, mobilizes the second messenger, Rac1, needed for the stabilization of cytoskeletal elements, thereby conferring vascular barrier protection [77, 78]. APC is also known to modulate the expression of transcription factors and various genes in endothelial cells. In TNF-α-treated endothelial cells, APC was shown to counter-suppress genes that were up-regulated by TNF-α [79]. These include down-regulation of the expression levels of adhesion molecules, ICAM-1, VCAM-1, and E-selectin, likely through APC's regulation of the NFκB signaling pathway [79]. APC can reduce the mRNA expression of the p52 subunit of the transcription factor, NFκB, in endothelial cells [79].

A number of studies have demonstrated that APC can directly regulate the inflammatory responses of various leukocyte populations. In monocytes stimulated with pro-inflammatory factors such as LPS and IFN-γ, APC can inhibit the production of TNF-α, IL-1β, IL-6, and IL-8 [80, 81]. APC has also been shown to decrease the generation of pro-inflammatory mediators in macrophages [81, 82]. One study has demonstrated that APC can reduce the mortality in a

mouse model of sepsis through down-regulation of inflammatory responses in DCs [83]. The ability of APC to down-regulate the generation of pro-inflammatory factors in leukocytes have been attributed to APC's capability to regulate several inflammatory signaling pathways, notably the NFκB pathway [84, 85]. One study has demonstrated that APC can similarly modulate the Wnt signaling pathway in macrophages [86]. It is, however, still unclear as to which cellular receptors are involved in the APC-mediated signaling in leukocytes. Some have implicated the EPCR/PAR-1 receptors [80, 87]; while other studies have demonstrated that the regulatory effects of APC on leukocytes are not dependent on EPCR [88, 89]. One study, in particular, has shown that APC's anti-inflammatory effects on macrophages are mediated through the association of APC to the CD11b integrin and subsequent PAR-1 activation but do not involve EPCR [90].

Beyond mediating anti-inflammatory responses, APC has also been reported to inhibit cellular apoptosis [23]. APC can down-regulate key regulators involved in the intrinsic and extrinsic apoptotic pathway. APC has been shown to decrease the expression of the tumor-suppressor protein, p53, a central protein in the intrinsic apoptotic pathway [91]. Similarly APC can modulate the extrinsic apoptotic pathway by inhibiting the activation of caspase 8 [92].

APC's potent anti-inflammatory and anti-apoptotic effects are reflected on its ability to attenuate inflammation and pathology in various disease settings. APC's efficacy as a therapeutic molecule in a human inflammatory disease setting was demonstrated in the PROWESS study, a breakthrough phase III clinical trial, wherein infusion of recombinant APC in patients with severe sepsis reduced mortality [93]. Utilizing APC in the treatment of sepsis, however, remains controversial since the efficacy of APC was not consistently demonstrated in subsequent clinical trials [94, 95]. In other disease settings, APC has also been reported to

strongly influence disease pathology. For instance, in animal models for lung injury and inflammation, APC has been shown to be protective by inhibiting leukocyte accumulation and chemotaxis [96, 97]. Similarly in murine models for cerebral ischemia and stroke, APC has been demonstrated to increase survival through its potent anti-inflammatory and neuroprotective effects [98, 99]. APC's neuroprotective effect in stroke models has been attributed to its ability to inhibit neuronal apoptosis by regulating the p53 apoptotic pathway [91]. Other disease models in which APC has been shown to confer protection include inflammatory bowel disease (IBD)[100], diabetes [101], and amyotrophic lateral sclerosis (ALS) [102].

Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Multiple Sclerosis

Multiple Sclerosis (MS) is a complex neurologic disease with diverse clinical and pathological symptoms. The hallmark pathogenesis of the remitting/relapsing phase, which constitutes the initial stage of MS, is characterized by the formation of focal demyelinated lesions in the white matter of the central nervous system (CNS) [103, 104]. As the disease enters the progressive phase, the pathogenesis in the CNS becomes more widespread, involving axonal pathology and neurodegeneration, both in the white and grey matter [103, 105]. Among the clinical symptoms manifested by MS patients include cognitive deficiencies, coordination and balance problems, and in the most severe form, paralysis [106-108]. It is believed that MS can develop because of genetic susceptibility, and some have proposed that outside environmental factors, such as a viral infection, may also trigger the disease [109-111].

It is widely accepted that the driving force behind the CNS pathology in MS is an inflammatory process, primarily mediated by autoreactive T lymphocytes (T-cells) specific for neuroantigens [109]. Demyelination in MS lesions is believed to be largely mediated by infiltrating T-cells and activated microglial cells [108-110]. Human Leukocyte Antigen (HLA) Class II-restricted $CD4^+$ T-cells have been specifically implicated in the initial formation of lesions [109]. The involvement of $CD4^+$ T-cells in MS is evidenced by the strong association of HLA class II genes with genetic susceptibility for MS [111]. Acute lesions are known to be initiated by IFNγ-producing CD4+ T helper type I (T_H1) cells [109]. The pathogenic CD4+ T helper 17 (T_H17) subset has similarly been highly implicated in MS immunopathology [108]. The mRNA expression of IL-17, the signature cytokine produced by Th17 cells, is increased in the mononuclear cells in the cerebrospinal fluid (CFS) of MS patients [112, 113]. The production of IL-17 and IFN-γ by infiltrating CD4+ T-cells induces the activation of resident cells in the CNS, notably microglial cells, which is known to generate inflammatory factors such as nitric oxide (NO) that can mediate demyelination and axonal pathology [108, 110]. While T_H1 and T_H17 cells are known to be pathogenic in MS, the $CD4^+$ T-regulatory (T-regs) subset and the T-helper 2 (T_H2), conversely, inhibit disease progression [114]. T-regs are reportedly impaired in patients with MS [115]. The importance of $CD4^+$ T-cells in MS initiation and progression is well-established; it should be noted, however, that Class-I restricted $CD8^+$ T-cells are also found in large numbers in MS lesions, suggesting their equally likely significance in MS pathology [116]. In addition to these cellular components, evidence of blood brain barrier (BBB) damage has also been observed both in MS active lesions and inactive plaques [109, 110]. The production of IL-17 has been specifically implicated in increased BBB dysfunction and the further recruitment and extravasation of peripheral leukocytes to the CNS [108].

Several pharmacological treatments were developed and approved for MS therapy. IFNβ treatment is one of the longest approved and more-widely used therapies for relapsing-remitting MS [117]. IFNβ acts on type-1 IFN transmembrane receptors to initiate signaling cascades that can directly mediate T-cell function [117]. IFNβ is known to limit T-cell activation and proliferation [118]. Moreover, IFNβ is also believed to limit T-cell extravasation into the CNS by downregulating several molecules, such as VLA-4, which is involved in T-cell adhesion to the BBB [117]. Another long-time used MS treatment is glatiramer acetate (GA), a synthetic polymer resembling myelin basic protein (MBP), a component of myelin [117]. GA interferes with the activation of myelin-specific T-cells by binding directly to MHC II on antigen presenting cells [119]. It also believed that myelin-T cells exposed to GA results in the decrease of IFN-γ production and skews the immune response to T_H2 [117].

Experimental Autoimmune Encephalomyelitis

Most of what is currently known about the immunopathology of MS and the development of disease treatments are derived from studies utilizing the animal models of the disease [120]. Experimental autoimmune encephalomyelitis (EAE), the most widely studied model for MS, closely replicates the immunologic and pathologic characteristics of MS [121]. EAE can be induced in various animals; however, in recent years, the murine EAE model has been the most commonly utilized [121]. EAE is induced through the immunization of CNS antigens or through the passive transfer of encephalitogenic T-cells [120, 121]. Immunization with neuroantigens typically requires the antigens to be emulsified in an adjuvant [120, 121]. Several EAE models co-administer pertussis toxin (PTX) to increase disease severity; PTX is known to increase BBB permeability, thus facilitating the entry of autoreactive lymphocytes into the CNS [122, 123]. The clinical course of the disease is dependent on the mouse strain and the combination of

neuroantigen and adjuvant used [121]. Immunization with the myelin peptide, myelin oligodendrocyte glycoprotein peptide$_{35-55}$ (MOG_{35-55}) in C57BL/6 mice results in a monophasic, chronic disease, with immune cell infiltration and pathology in the spinal cord and the brain [124]. Immunization with myelin proteolipid protein (PLP) in SJL mice results in chronic relapsing disease [125]. Neuroantigens are typically emulsified in Complete Freud's Adjuvant (CFA), the most commonly used adjuvant in EAE models, resulting in the induction of T_H1 and T_H17 immune response [126]. The immunopathology in EAE has been strongly attributed to both these $CD4^+$ subsets; and this is consistent with the fact that T_H1 and T_H17 cells have been detected in MS lesions and in the cerebrospinal fluid (CSF) of MS patients [114]. In EAE, it appears that a combination of both T_H1 and T_H17 is required for disease development. It has been demonstrated, for instance, that mice lacking the T_H17 subset but with intact T_H1 response fail to develop EAE [127]. Other $CD4^+$ subsets, specifically, T_H2 and T-reg cells, are non-pathogenic in EAE [114]. T-reg cells, in fact, actively confer protection in EAE by inhibiting autoreactive T-cells, likely through cell-to cell contact mechanisms and/or the production of anti-inflammatory factors such as IL-10 and TGF-β [128]. It should be noted that while $CD4^+$ T-cells are generally regarded as the main effectors cells in EAE and MS, other lymphocytes such as $CD8^+$ T-cells and B cells have been demonstrated to also cause immunopathology in the EAE disease setting [114].

Within the first five days after EAE immunization, neuroantigen-specific T-cells are activated and proliferate in secondary lymphoid organs. As early as day 5 post immunization, activated T-cells disseminate systemically, and a number of activated T-cells transmigrate across the BBB and enter the CNS [129]. It has been proposed that activated T-cells, regardless of specificity, can transmigrate through the BBB and enter the CNS; if these cells do not encounter

their antigen in the CNS, however, the cells return to the circulation [130]. In the EAE model, CNS barriers, including the BBB and the epithelial barriers within the choroid plexus, are more permeable to immune cells as a result of pertussis toxin administration and pro-inflammatory factors produced by activated lymphocytes, thus facilitating the entry of neuroantigen-specific T-cells [130]. Once neuroantigen-specific T-cells encounter their antigen in the CNS, they are stimulated to produce cytokines that not only result in the further recruitment of inflammatory leukocytes from the periphery but also results in the recruitment and activation of resident cells in the CNS, notably microglial cells [131]. Neuroantigen-specific $CD4^+$ T_H1 cells that transmigrate to the CNS produce IFN-γ resulting in the induction of microglial cells to produce inflammatory factors, such as nitric oxide (NO), oxygen radicals, and TNF-α, which are toxic to oligodendrocytes thus promoting these cells to undergo apoptosis [132]. Oligodendroctyes are glial cells that associate with neuronal axons and produce the myelin sheath that encloses the axon [132]. In EAE and MS, oligodendrocyte cell death, as a result of inflammation, greatly contribute to the demyelinating pathology [132]. Moreover, the pro-inflammatory factors produced by activated microglial cells also result in axonal pathology and neurodegeneration [114]. CD4+ T_H17 cells that are activated by neuroantigens in the CNS produce IL-17, which similarly promote microglial activation [131]. Furthermore, generation of IL-17 in the CNS has also been attributed to increased BBB disruption and enhanced recruitment of peripheral leukocytes, notably neutrophils, resulting in the sustainment of CNS inflammation and further immune pathology [108]. While $CD4^+$ T-cells are sufficient to induce disease and mediate CNS pathology, as demonstrated by adoptive transfer studies involving $CD4^+$ T-cells, other lymphocyte populations are also believed to contribute to EAE and MS pathology. $CD8^+$ T-cells, in particular, which have been observed to constitute a majority of the infiltrating cellular

components in MS lesions, have been implicated in axonal pathology and neurodegeneration in EAE models [114].

Coagulation Factors in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis

While the functions of endogenous coagulation proteins, such as APC and thrombin, in the progression and pathogenesis of EAE and MS have yet to be clearly delineated, there are accumulating studies suggesting the likely involvement of the endogenous coagulation system in EAE and MS pathology. Proteomics analysis of MS lesions revealed the presence of coagulation proteins in chronic active plaques [133]. In particular, protein C inhibitor, which is known to inhibit APC, was one of coagulation proteins detected in MS plaques [133]. Studies on the EAE model show elevated levels of thrombin inhibitors detected both in the periphery and the CNS [134, 135]. Fibrin deposition has also been observed in the brains of EAE rodents [136]. The findings of these studies strongly suggest the possible activation of coagulation components during the progression of EAE and MS. The effects of coagulation factors on specific aspects of MS and EAE pathogenesis will need to be determined. The likely influence of coagulation proteins, particularly, APC and thrombin, on the pathology of neuroinflammatory diseases is potentiated by the ability of both proteins to directly affect resident cells in the CNS. APC, for instance, has been demonstrated to be neuroprotective by downregulating apoptotic pathways in neurons during pathological conditions [91]. Moreover, glial cells express PAR-1 [137], which is indicative of receptiveness to thrombin or APC regulation. Thrombin, in fact, has been reported to regulate microglial generation of pro-inflammatory factors and astrocyte activation

[137, 138]. Thus in addition to thrombin and APC's effects on traditional cellular components of the immune system, these coagulation proteins may also influence the disease progression of EAE and MS through the capacity to mediate the cellular responses of resident cells in the CNS. Finally, it should be noted that one of the major pathological components of both EAE and MS is BBB dysfunction, which further underscores the likely relevance of APC and thrombin in disease progression, given APC's ability to confer vascular barrier protection and thrombin's promotion of vascular barrier disruption.

Synthesis

The studies presented in this dissertation describe the effects of coagulation factors, APC and thrombin on pathogenic components of EAE. In **Chapter 2**, we demonstrate the influence of APC inhibition on EAE pathogenesis and disease progression. We show that abrogation of APC activity in the systemic circulation increased BBB permeability, resulting in pronounced leukocyte infiltrates in the brains of EAE mice. However, despite the presence of considerable infiltrates, these mice exhibited minimal CNS pathology and attenuated disease. Moreover, we show that inhibition of APC activity modulated the functional responses of specific leukocyte populations, resulting in increased and more activated $CD11b^+$ myeloid cells. Among the $CD11b^+$ population that expanded as a result of APC inhibition are myeloid-derived suppressor cells (MDSC), characterized to be potent T-cell suppressors. We demonstrate that the increase in MDSCs resulted in a deficient and defective $CD4^+$ T-cell population, consequently attenuating EAE progression.

In **Chapter 3,** we demonstrate that thrombin effectively induced an inflammatory phenotype in the BBB, which plays a prominent role in EAE and MS pathology. We show that thrombin activated the BBB, resulting in increased expression of adhesion molecules and various chemokines. Furthermore, thrombin additionally elevated the paracellular permeability of the BBB *in vitro*. Overall, this study demonstrated that thrombin effectively induced the BBB to adopt a phenotype that can potentially facilitate the recruitment, firm adhesion, and diapedesis of leukocytes, suggesting thrombin's relevance in neuroinflammatory conditions such as EAE and MS.

We summarize our findings in **Chapter 4**. We additionally present our conclusions and suggest future directions.

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CHAPTER 2

Inhibition of endogenous activated protein C attenuates experimental autoimmune encephalomyelitis by inducing myeloid-derived suppressor cells

Abstract

Activated protein C (APC) is an anti-coagulant involved in the interactions between the coagulation and immune systems. APC has broad anti-inflammatory effects mediated through its capability to modulate leukocyte function and confer vascular barrier protection. In this study, we investigated the influence of APC on the pathogenesis of experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis. We modulated APC levels in the circulation during the induction of EAE through systemic administration of a monoclonal antibody against protein C/APC (anti-PC). We hypothesized that inhibition of APC may result in a heightened inflammatory environment thereby resulting in increased EAE pathogenesis. On the contrary, we observed that mice treated with anti-PC antibody (anti-PC mice) exhibited attenuated clinical signs of EAE. Most striking is that despite attenuated EAE symptoms, anti-PC mice exhibited considerable leukocyte infiltration in the brain, comparable to control mice with very severe EAE. We show that attenuated EAE in anti-PC mice is a consequence of the induction of a recently-described $CD11b^+$ population, referred to as myeloid-derived suppressor cells (MDSC) and characterized to be potent suppressors of effector T-cell function. We conclude that inhibition of APC can affect the functional responses of $CD11b^+$ cells, resulting in expansion and increased activation of MDSCs, consequently suppressing T-cell function and attenuating EAE. These findings present a novel influence of APC on the immune response and contribute to further elucidating the complex interaction between the immune and coagulation systems.

Introduction

The anti-coagulant, APC, has a prominent role in mediating the complex crosstalk between the coagulation and inflammatory responses [1-3]. APC is a serine protease derived from the zymogen protein C (PC), which is activated on the surface of endothelial cells by the coagulation factor, thrombin bound to the glycoprotein, thrombomodulin [3]. Once activated, APC in the circulation is known for regulating blood clotting through its ability to proteolytically inactivate coagulation factors Va and VIIIa, consequently dampening further generation of thrombin [4]. Independent of APC's function in the coagulation cascade, APC can affect various cellular processes through its interactions with membrane receptors. APC mediates cell signaling in endothelial cells through binding with endothelial protein C receptor (EPCR), enabling APC to activate the G-protein coupled receptor, protease-activated receptor-1 (PAR-1) [5, 6]. APC-mediated activation of PAR-1 on endothelial cells reduces endothelial permeability through stabilization of cytoskeletal components [7], consequently limiting the extravasation of inflammatory leukocytes [5]. APC additionally directs leukocyte function through alteration of signaling pathways involved in inflammatory responses [8-12]. Several studies have proposed that APC's effects on leukocytes may similarly be mediated through the EPCR/PAR-1 pathway [13, 14]. However, a more recent study has shown that APC's anti-inflammatory effects on myeloid cells are mediated through the binding of APC to the CD11b integrin [15].

The pleiotropic effects of APC, which encompasses both cell signaling and anti-coagulant properties, are indicative of its influence in various disease conditions and its potential as a promising therapeutic target. The efficacy of APC as a therapeutic molecule has, in fact, already been demonstrated for severe sepsis. In the PROWESS study, infusion of human recombinant APC improved survival among patients with severe sepsis [16]. The effectiveness

of APC in sepsis treatment however remains controversial since its efficacy was not exhibited in a subsequent trial [17], prompting the withdrawal of the drug from the market [18]. Nevertheless, APC's protective effects in other disease settings have been evidenced in various animal studies. In ischemic stroke models, APC can reduce leukocyte infiltration in the brain [19], and APC can ameliorate the animal model for amyotrophic lateral sclerosis (ALS) by conferring blood-spinal cord barrier protection [20]. APC has also been demonstrated to attenuate inflammation in mouse models for inflammatory bowel disease (IBD) [21] and lung injury model [22].

In this study, we set out to investigate the influence of endogenous APC on the pathogenesis of EAE, the animal model for multiple sclerosis (MS). EAE and MS are autoimmune disorders characterized by neuroinflammation and consequent axonal demyelination leading to clinical symptoms such as paralysis [23, 24]. The neuroinflammatory response in EAE is mainly mediated by effector $CD4^+$ T-cells that are able to infiltrate the central nervous system (CNS) as a result of permeability and dysfunction at CNS barriers [25]. Our rationale for studying APC in EAE stems from previous studies suggesting the likely involvement of endogenous coagulation components in EAE and MS pathology. In a study by Han et. al, proteomics analysis of MS lesions revealed the presence of coagulation proteins in chronic active plaques [26]. In EAE studies, fibrin deposition in the brain has been reported [27], and increased presence of thrombin inhibitors were detected in the peripheral circulation of EAE mice [28]. Moreover, APC's known anti-inflammatory effects, specifically its ability to mediate leukocyte function and confer vascular barrier protection, further underscore the relevance of studying APC in EAE, wherein the major pathological component is CNS barrier dysfunction resulting in neuroinflammation and pathology.

To investigate APC function in EAE, we inhibited endogenous APC during disease progression through systemic administration of an antibody against protein C/APC (anti-PC). We anticipated that blocking APC may lead to worsening of EAE due to CNS barrier dysfunction and/or by exacerbating systemic inflammation. Converse to our hypothesis, we observed that mice treated with anti-PC exhibited attenuated clinical symptoms despite considerable leukocyte infiltration in the brain. Moreover, we observed diminished T-cell effector function in these mice, coupled with increased expansion of MDSCs, a cell-subset characterized to be potent T-cell supressors [29]. This study shows that diminished APC activity can affect EAE pathogenesis at multiple fronts, influencing both BBB permeability and effector functions of leukocytes. The results of this study exemplify the intricate and multi-faceted interaction between the coagulation and immune systems, which is likely further complicated by each unique disease setting.

Materials and Methods

Mice: C57BL/6 (BL/6) mice and C57BL/6 2d2 TCR transgenic mice were generated from our in-house breeding colony or were purchased from Taconic (Germantown). Mice were maintained in specific pathogen-free facility at Cornell University. Procedures performed on mice were approved by the Institutional Animal Care and Use Committee of Cornell University.

EAE induction: To induce EAE, a 1:1 emulsion of myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) (Anaspec) at 1 mg/ml and complete Freund's adjuvant (CFA) (Sigma) was subcutaneously injected into mice flanks. Pertussis toxin (PTX) (List Biologicals) at 20 ng/ml was intravenously injected on the day of immunization, and a second dose was administered 48 hours later. Disease severity was assessed daily, and clinical scores were assigned as follows: 0 = no disease, 0.5 = weak tail, 1 = completely limp tail, 1.5 = impaired righting reflex, 2 = affected gait, 2.5 = partial hind limb paralysis, 3 = complete hind limb paralysis, 3.5 = hind limb and partial forelimb paralysis, 4 = moribund, 5 = death. Mice that reach a score of 4 are euthanized and scored as 5.

Antibody to protein C: Generation of mAb MPC1609 (anti-PC) was described previously [30]. Anti-PC was raised to mouse PC and cross reacts with mouse APC. Anti-PC inhibits the activation of protein C and blocks both PC and APC from binding to endothelium and phospholipid surfaces, thus effectively abrogating APC's anticoagulant and cell signaling capabilities [30]. The antibody at 1mg/kg was administered via intraperitoneal (i.p) injection.

Immunohistochemistry: CNS harvested after perfusion were snap frozen in OCT media (Tissue-Tek, CA). Frozen tissues were sectioned to 6-micron thickness using a cryostat, and sections were affixed to Superfrost Plus slides (Fisher). After fixing in acetone, slides were

washed in PBS and blocked with casein (Vector Labs) for 10 minutes. Slides were then incubated with primary antibodies for 90 minutes at 37^{o}C. Primary antibodies used are as follows: anti-CD45 (AbD Serotec) anti-Iba-1 (Wako Chemicals), anti-MOG (R&D Systems). After washing, slides were incubated with the appropriate secondary antibody for 30 minutes, washed and subsequently incubated with avidin-horseradish peroxidase (HRP) complex (Invitrogen). Slides were developed with chromogenic substrate for HRP (Zymed) and counterstained with hematoxylin. Images were captured using a Zeiss Axio Imager M1 microscope.

CNS Infiltrate and Splenocyte Isolation: CNS harvested after perfusion were homogenized using a syringe plunger. Tissues were further homogenized by repeatedly passing through a syringe attached to an 18-gauge blunt needle. CNS homogenates were fractionated on 30/70% Percoll gradient, and cells were recovered from the 30/70 interface. For splenocyte isolation, spleens were homogenized between the frosted ends of two glass slides. Homogenates were re-suspended in ACK lysis buffer to lyse erythrocytes. After washing, splenocytes were passed through a 70 μm cell strainer. Cells were then prepared for flow cytometry analysis or cutured *in vitro*.

Dextran Extravasation Assay: At day 5 post EAE induction, 2 mg of texas red-labeled dextran (10,000 molecular weight) were i.v. injected into the systemic circulation of both IgG and anti-PC treated mice. 5 hours after dextran injection, mice were perfused, and brains were harvested. Brains were homogenized in 50 mM Tris-HCl (1:1 per μl Tris-HCl to mg of brain weight). Homogenates were centrifuged and supernatants were collected. Supernatants were mixed 1:1 with methanol and centrifuged. Fluorescence in supernatants was quantified using a BioTeck (Winnoski, VT) Synergy 4 plate reader. Dextran concentration was based on a standard curve.

Flow Cytometry: Cells were re-suspended in staining buffer (PBS with 0.5% BSA, and 0.09% sodium azide). Cell suspensions were incubated with Fc block (BD Biosciences) for 10 minutes before incubation with primary antibodies for 30 minutes at 4^{o}C. Primary antibodies used are as follows: PE-conjugated anti-mouse CD4 (BD Biosciences), CD11b (eBioscience), CTLA-4 (BD Biosciences), FITC-conjugated anti-mouse CD8 (BD Biosciences), CD69, PerCP-conjugated anti-mouse CD25, NK1.1 (eBioscience), B220 (BD Biosciences), allophycocyanin-conjugated anti-mouse Ly6C, CD11c (eBioscience). Foxp3 staining was done with the Ebioscience Foxp3 staining kit in accordance with the manufacture's protocol. For iNOS intracellular staining, cells were fixed in 4% paraformaldehyde and permeabilized with .01% triton-x. Cells were incubated with polyclonal rabbit anti-mouse iNOS (BD Biosciences) for 30 minutes. Cells were washed and subsequently incubated with allophycocyanin-conjugated secondary antibody. All samples were acquired using a FACSCanto II flow cytometer (BD Biosciences). Raw data were evaluated using FlowJo Flow Cytometry Analysis Software (Treestar).

Quantitative real-time PCR

Mice were perfused and CNS were harvested then homogenized. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Extracted RNA was treated with Baseline-Zero DNAse (Epicentre) to remove contaminating genomic DNA. 1.5 µg of total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems CA, USA). Real Time PCR was performed using SYBR Green technology (SYBR FAST Master Mix KAPAbiosystems) on a CFX96 Real Time System Thermal Cycler (Bio-RAD). The cycling conditions were as follows: Enzyme activation 95° C for 3 minutes followed by 40 cycles of denaturation 95°C for 3 secs, annealing 60° C for 30 seconds, elongation 72° C for 5 seconds. BioRad CFX Manager software was used to determine cycle

threshold (C_t) values, and gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The expression levels of all genes of interest were normalized to the internal control gene, GAPDH.

***In vitro* cell culture and ELISA:** Cells were cultured in complete RPMI (10% FBS, Penicillin/Streptomycin, L-glutamine, Sodium Pyruvate, 50mM 2-Mercaptoethanol, 25mM HEPES buffer). Samples were cultured untreated or stimulated with MOG_{35-55} (10 μg/ml) for 48 hours. After culture, supernatants were collected for ELISA. ELISA kits for IFN-γ and IL-10 were from eBioscience, and assays were performed according to the manufacturer's protocol.

Proliferation Assay: Splenocytes were labeled with CFSE (Molecular Probes, NY) according to the manufacturer's protocol. T-cells in whole-splenocyte culture were stimulated with plate-bound anti-CD3 (1μg/ml) (Ebioscience) and soluble anti-CD28 (1μg/ml) (Biolegend) for 96 hours. After culture, splenocytes were labeled with PE-conjugated mouse anti-CD4 and acquired by flow cytometry to assess CFSE dilution. For CD11b+/CD4+-cell co-culture, splenic CD11b+ cells were isolated using Easy Sep positive selection kit (Stem Cell Technologies), and splenic MOG-specific CD4+ T-cells from naive BL/6 2D2 transgenic mice were isolated using Easy Sep CD4+ selection kit (Stem Cell Technologies). Cell isolation was done according the manufacturer's protocol. CD4+ T-cells were labeled with CFSE, stimulated with MOG_{35-55} (10 μg/ml) and co-cultured with CD11b+ cells for 96 hours at a ratio of 1:4. Proliferation was assessed by flow cytometry based on CFSE dilution.

CD11b+ cells and CD4+ T-cells co-culture for T-reg expansion: CD11b+ and CD4+ cells were isolated as described above. CD4+ T-cells were stimulated with anti-CD3/anti-CD28, as described above, and co-cultured with CD11b+ cells for 96 hours at a ratio of 1:2. After culture,

CD4+ T-cells were stained with PerCP-conjugated anti-CD25 and allophycocyanin-conjugated anti-Foxp3. Samples were acquired by flow cytometry to assess T-reg frequency.

Arginase Activity: Splenocytes were lysed with lysis buffer (0.1% triton-X, pepstatin 5µg, aprotinin 5µg, and antipain 5µg). Arginase activity was measured as described previously [31]. Sample absorbance was read with a Biotek EL x 50 plate reader at 550 nm.

Nitrate/Nitrite Assay: Splenocytes were cultured and supernatants were collected after 48 hours. Nitrate/Nitrite assay was done as described previously [32]. Absorbance was measured in a Biotek EL x 50 plate reader at 550 nm.

Reactive Oxygen Species: Splenocytes were labeled with anti-Ly6C and anti-CD11b. Cells were washed and incubated with 10 uM of 2′,7′-Dichlorofluorescein diacetate (Sigma) for 30 minutes at 37^{o}C. Cells were washed and incubated in 37^{o}C for 30 mins. Cells were acquired by flow cytometry to assess fluorescence corresponding with ROS generation.

Immunofluorescence: Splenocytes were cultured on coverslips in 24-well plates. After culture, cells were washed and stained with FITC-conjugated anti-mouse CD11b (eBioscience) and allophycocyanin-conjugated anti-Ly6C (Ebioscience). Cells were fixed with 4% PFA and permeabilized with 0.1% triton-X. Cells were stained with polyclonal anti-arginase I (Genetex) and subsequently incubated with the appropriate secondary antibody. After washing, coverslips were treated with Prolong Gold with Dapi (Molecular Probes) and mounted on slides. Images were captured using a Zeiss Axio Imager M1 microscope.

Activated Protein C interaction with MDSC: APC was labeled with FITC as previously described [30]. Splenic cells were labeled with anti-Ly6C and subsequently incubated with APC-FITC in HBSS buffer containing 0.5% BSA, 3mM $CaCl_2$ and 0.6mM MgCl2 in the absence or presence of anti-PC antibody. Cells were incubated on ice for 15 minutes, then acquired by flow cytometry to assess APC binding to Ly6C+ cells.

Statistical Analyses: Statistical analyses were performed using GraphPad Prism 5 software. Statistical significance was assessed by unpaired Student *t* test or by two-way ANOVA/Bonferroni post-test as indicated in figure legends.

Results

Inhibition of endogenous APC results in attenuated EAE.

To determine whether APC can influence EAE pathogenesis, we inhibited endogenous APC in the circulation through administration of a monoclonal antibody to PC/APC (See Materials and Methods). Anti-PC or IgG isotype at 10 mg/kg was administered at days 0, 2, 4, and 6 post EAE induction. Administration of anti-PC within this timeframe inhibits APC during the initiation phase of the immune response, as well as at the outset of the effector phase of EAE, which is characterized by leukocyte infiltration into the CNS. We observed that the onset of clinical symptoms in mice treated with anti-PC (anti-PC mice) was significantly delayed (Figure 2.1 and Table 2.1). The mean day of onset for anti-PC mice was 16.7 days post EAE induction while controls exhibited clinical symptoms as early as day 7 with mean onset at 10.5 days post immunization. Additionally, incidence of disease was higher in the control group; 80% of controls developed EAE by day 16 and 100% exhibited symptoms by day 22 (Table 2.1). In contrast, only 44% of anti-PC mice exhibited clinical symptoms on day 16 and 66% by day 22. These data show that inhibition of endogenous APC can alter the disease course of EAE, albeit in a rather unexpected way, given that inhibition of APC, an anti-inflammatory molecule, resulted in attenuation of EAE rather than increasing disease severity.

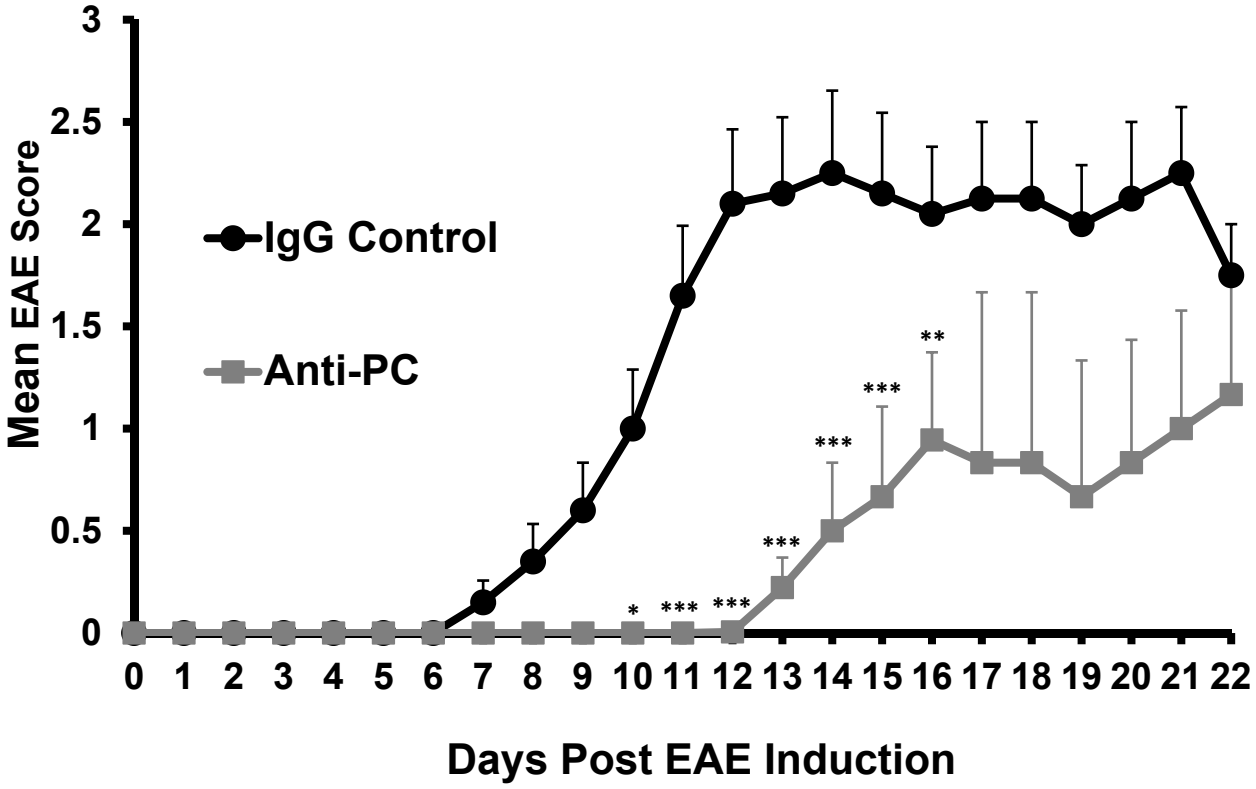

Figure 2.1 Mice treated with anti-PC antibody exhibit attenuated EAE. To induce EAE, BL/6 mice were immunized with MOG_{35-55} in CFA; pertussis toxin was i.v. injected on the day of immunization, and a second dose was injected 48 hours later. Anti-PC or IgG control was i.p. injected on days 0, 2, 4, and 6 post EAE induction. Mice were monitored daily to assess clinical symptoms, and scores were assigned based on the scoring system outlined in materials and methods. Scores were plotted as the mean $\pm$ S.E.M (anti-PC n = 9, IgG control n = 10. On day 16, mice from both groups were randomly selected for tissue collection.

* $p < 0.01$, *** $p < 0.001$ by two-way ANOVA/Bonferroni post-test; Statistical analysis up to day 16 included all mice. Statistical analysis up to day 22 did not include mice taken for tissue collection on day 16). The result shown is representative of four independent experiments.

Table 2.I Anti-PC mice exhibit attenuated EAE

	IgG Control	Anti-PC
Mean Day of Onset [a]	10.5 (± 3.4)	16.7 (± 3.3)*
Mean Maximum Clinical Score[b] by Day 16	2.2	0.94
Incidence by Day 16	80%	44%
Mean Maximum Clinical Score[b] by Day 22[#]	3.0	1.6
Incidence by Day 22[#]	100%	66%

[a] Day of Onset denotes the first day each mouse exhibited clinical symptoms after EAE induction. For mice that were not showing clinical symptoms at the time of tissue collection, the day following tissue collection was assigned as the day of onset. ± S.E.M are indicated inside the parenthesis; * $p < 0.05$.

[b]Maximum clinical score denotes the highest score each mouse reached during the course of EAE progression.

[#]Day 22 calculation for mean maximum clinical score and incidence does not include the mice that were taken for tissue collection on day 16.

Anti-PC mice have considerable leukocyte infiltration in the brain despite exhibiting attenuated clinical symptoms.

The major pathological component in EAE is leukocyte infiltration into the CNS, which typically correlates with the severity of clinical signs [24, 25]. To investigate why anti-PC mice had attenuated disease, we assessed the severity of CNS infiltration at the peak of disease (days 14-17). We observed that the extent of leukocyte ($CD45^+$ cells) infiltration in the brains of control mice positively correlates with disease severity (Figure 2.2A-C). Interestingly, 33% (Appendix-A Table I) of anti-PC mice with no clinical symptoms had considerable CD45+ infiltrates in the brain, comparable to controls with severe EAE (Figure 2.2A-C). We noted that cellular infiltrates were located in similar areas of the brain in both groups, notably in the hippocampal area (Figure 2.2 A), the cerebellar parenchyma (Figure 2.2B), and at the meninges (Figure 2.2C). We additionally observed that the extent of infiltration in the spinal cords (SC) of controls corresponds with the severity of symptoms (Figure 2.2D). Interestingly, the degree of cellular infiltration in the SC of anti-PC mice was minimal despite considerable infiltration in the brain (Figure 2.2D). Perivascular cuffing, characterized by cellular infiltrates encircling blood vessels in the CNS, is a hallmark of EAE pathology and is indicative of increased BBB permeability [33]. We observed rampant perivascular cuffing in the brain parenchyma of anti-PC mice (Figure 2.2E), suggesting increased BBB permeability in these mice. To confirm whether inhibition of APC resulted in increased BBB permeability, fluorescent dextran molecules were injected into the systemic circulation of both control and anti-PC mice, and we assessed the degree of dextran extravasation into the brain. We consistently observed increased dextran extravasation in the brains of anti-PC mice compared to controls (Fig 2.2F), further exhibiting increased BBB permeability in anti-PC mice. Overall, these data demonstrate that

despite exhibiting attenuated clinical symptoms, inhibition of APC during EAE resulted in increased BBB permeability as evidenced by pronounced leukocyte infiltration and increased dextran extravasation in the brains of anti-PC mice.

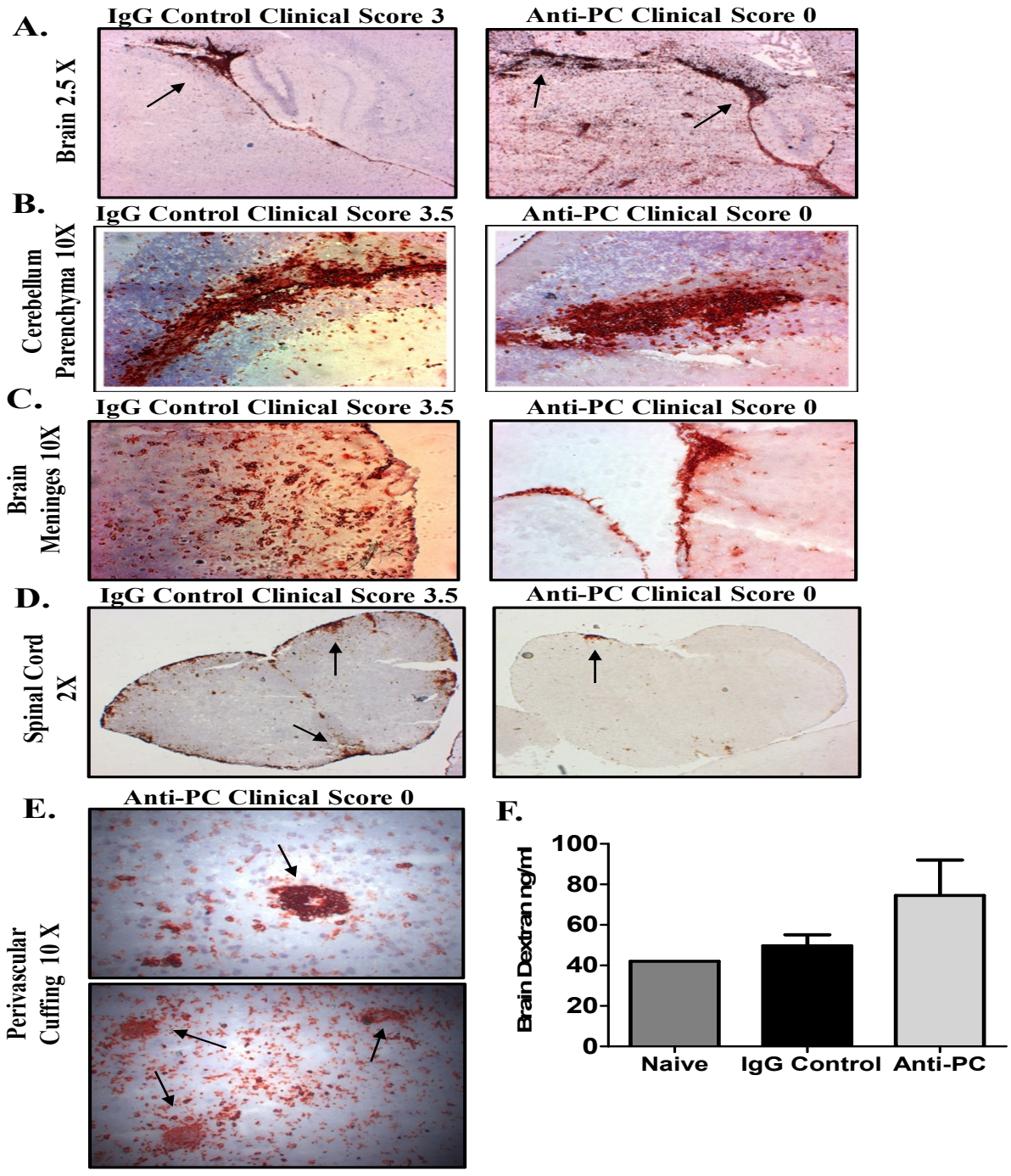
A.
IgG Control Clinical Score 3
Anti-PC Clinical Score 0
Brain 2.5 X
B.
IgG Control Clinical Score 3.5
Anti-PC Clinical Score 0
Cerebellum Parenchyma 10X
C.
IgG Control Clinical Score 3.5
Anti-PC Clinical Score 0
Brain Meninges 10X
D.
IgG Control Clinical Score 3.5
Anti-PC Clinical Score 0
Spinal Cord 2X
E.
Anti-PC Clinical Score 0
Perivascular Cuffing 10 X
F.
Brain Dextran ng/ml
100
80
60
40
20
0
Naive
IgG Control
Anti-PC

Figure 2.2 Anti-PC mice show considerable infiltration in the brain despite exhibiting attenuated clinical symptoms. CNS were harvested at the peak of disease (days 14-17) following EAE induction. CNS sections were stained with anti-CD45 (red) and various areas of the CNS, specifically (**A**) the hippocampal area, (**B**) cerebellar parenchyma, (**C**) brain meninges, and (**D**) spinal cord were assessed for the presence of cellular infiltrates. Arrows indicate areas of the CNS with pronounced $CD45^+$ staining. (**E**) Histological representation showing $CD45^+$ cellular infiltrates surrounding blood vessels in the brain parenchyma of anti-PC mice. (**F**) At day 5 post EAE induction, fluorescent dextran molecules (2 mg) were i.v. injected into the systemic circulation of anti-pc and control mice. Brains were harvested 5 hours later to assess the degree of dextran extravasation. The concentration of extravasated dextran in brain homogenates was determined based on a standard curve.

Anti-PC mice exhibit reduced $CD4^+$ pathogenic subsets in the brain while the $CD4^+$ T-regulatory subset is increased.

To further investigate the disparity in disease course between anti-PC and control mice, we next examined the frequency of specific leukocyte infiltrates in the CNS. We did not observe significant differences in $CD4^+$ T-cell frequency in the brains (Figure 2.3A) and in the SC (Figure 2.3C) between the two groups. Various $CD4^+$ T-cell subsets have differing effects on EAE severity; notably, the T-regulatory (T-regs) subset ($CD4^+CD25^+Foxp3^+$) confers protection in EAE, while T-helper 1 (T_H1) and T-helper 17 (T_H17) subsets are pathogenic [25]. We observed significantly increased T-reg frequency in the brains of anti-PC mice compared to controls (Figure 2.3B). T-reg frequency is similarly increased in the SC of anti-PC group, although the difference did not reach statistical significance (Figure 2.3C). The protective effect of T-regs is partly mediated through the production of the anti-inflammatory cytokine, IL-10 [34]. Consistent with increased T-reg frequency, we detected increased IL-10 production from cellular infiltrates in the brains and SC of anti-PC mice (Figure 2.3D). In contrast, the production of the T_H1 cytokine, IFN-γ, is reduced in brain infiltrates (but not from SC infiltrates) from anti-PC mice (Figure 2.3D). We similarly detected decreased IL-17 mRNA expression in the brains and SC of anti-PC compared to controls (Figure 2.3 E), suggesting that the presence of the pathogenic T_H17 subset in the CNS of anti-PC mice is reduced. Collectively, these data show that despite considerable infiltration in the brains of anti-PC mice, the pathogenic $CD4^+$ subsets and their respective signature cytokines required for disease progression are reduced in the brains of anti-PC mice while the suppressive T-reg population is increased. We additionally examined the frequency of non-CD4 leukocytes in the brain but found no significant differences (Appendix-B).

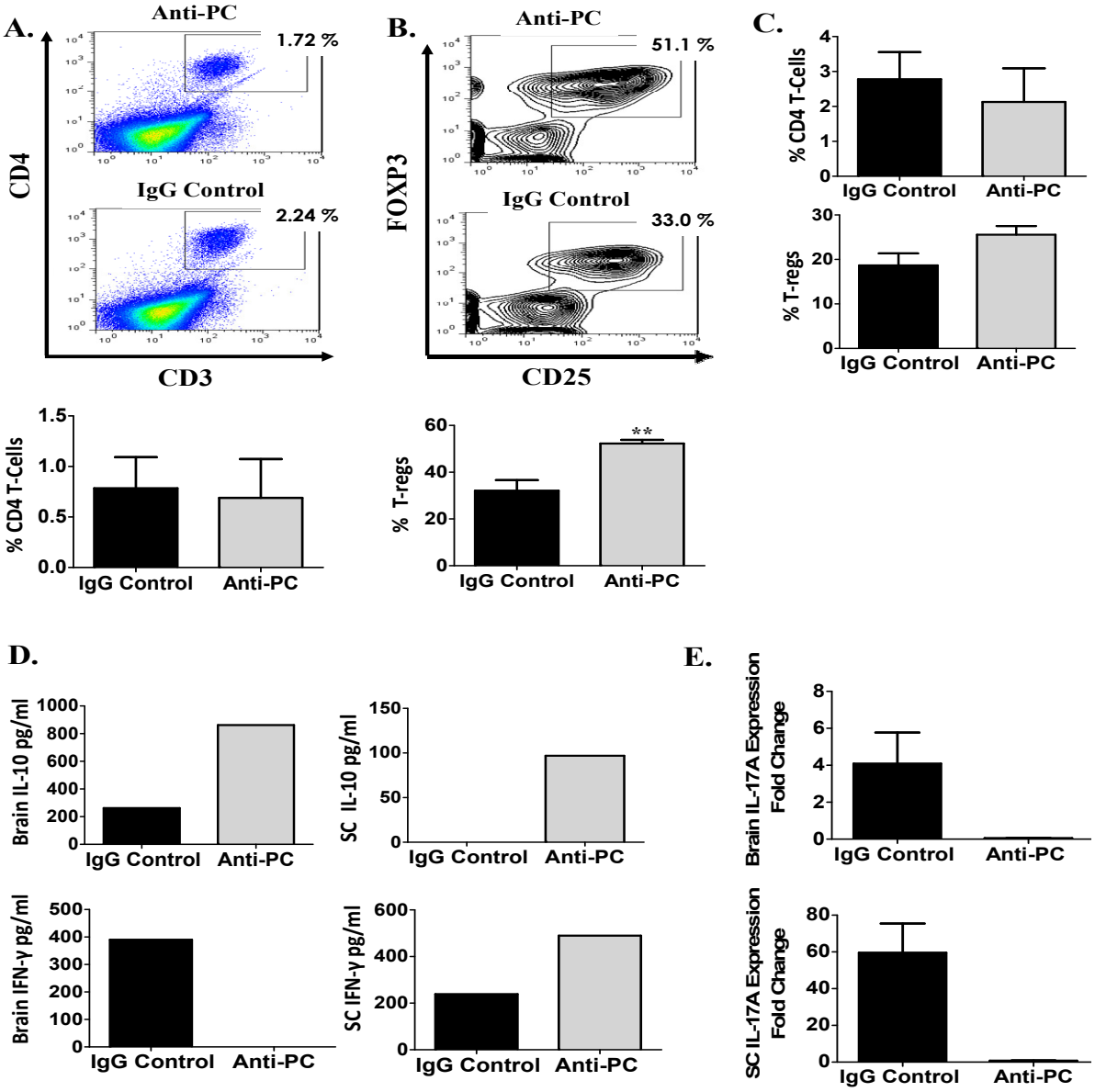
A.
Anti-PC
1.72 %
IgG Control
2.24 %
CD4
CD3
B.
Anti-PC
51.1 %
IgG Control
33.0 %
FOXP3
CD25
C.
% CD4 T-Cells
IgG Control
Anti-PC
% T-regs
IgG Control
Anti-PC
% CD4 T-Cells
IgG Control
Anti-PC
% T-regs
**
IgG Control
Anti-PC
D.
Brain IL-10 pg/ml
IgG Control
Anti-PC
SC IL-10 pg/ml
IgG Control
Anti-PC
Brain IFN-γ pg/ml
IgG Control
Anti-PC
SC IFN-γ pg/ml
IgG Control
Anti-PC
E.
Brain IL-17A Expression Fold Change
IgG Control
Anti-PC
SC IL-17A Expression Fold Change
IgG Control
Anti-PC

Figure 2.3 Anti-PC mice show increased T-regulatory cells and IL-10 production in the CNS. (**A**) At day 17 following EAE induction, cellular infiltrates from brains of anti-PC and control mice were isolated by percoll gradient, and cells were subjected to flow cytometry analysis to assess the frequency of $CD4^+$ T-cells. Flow cytometric dot plot is shown on the upper panel, and data are represented as means $\pm$ SEM at the bottom panel (Control n = 6, anti-PC n = 4). (**B**) The frequency of T-regs (CD25+, Foxp3+) among $CD4^+$ T-cell infiltrates in the brain was assessed by flow cytometry. Data are shown as flow cytometric contour plot (gated on $CD3^+CD4^+$ cells) on the upper panel and as means $\pm$ SEM on the bottom panel. (Control n = 6, anti-PC n = 4, ** $p < 0.01$ by Student *t* test) (**C**) Leukocyte infiltrates from the spinal cords of anti-PC mice and controls were isolated by percoll gradient and assessed for the frequency of $CD4^+$ T-cells (upper panel) and T-regs among $CD4^+$ T-cells (bottom panel) at day 16 following EAE induction. Data are represented as means $\pm$ SEM (n = 5-6). (**D**) CNS infiltrates from both anti-PC and control mice were isolated as described above. Cells from individual mouse ($n \leq 3$) were pooled for each group and re-stimulated with MOG_{35-55} *in vitro*. After 48 hours, supernatants were collected and the production of IL-10 (upper panel) and IFN-γ (bottom panel) were measured by ELISA. Cytokine concentrations were determined based on standard curves. (**E**) Brains and SC were homogenized, and whole-cell mRNA was isolated using the Trizol-based extraction process. IL-17A mRNA was quantified using real-time quantitative PCR, and expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The expression levels of IL-17A were normalized to the internal control gene, GAPDH. IL-17A expression is represented as fold change in expression relative to naïve mice (non-EAE induced).

Anti-PC mice exhibit reduced microglial activation and minimal demyelination in the CNS.

We next examined the extent of CNS pathology in control and anti-PC mice. Activated microglial cells greatly contribute to CNS pathology in EAE, including demyelination, axonal pathology and neurodegeneration, leading to the clinical signs of the disease [35, 36]. To assess the degree of microglial activation, CNS sections were stained for the microglial marker Iba-1, which is upregulated on activated microglial cells [35, 36]. Control mice with severe EAE showed markedly pronounced staining for Iba-1 (Figure 2.4A), suggesting increased microglial activation in the brains of these mice. In contrast, Iba-1 staining in the brains of anti-PC mice was less prominent (Figure 2.4A), indicative of reduced microglial activation in these mice. The hallmark pathology in EAE is demyelination. We assessed the extent of demyelination in the CNS of both groups. We detected extensive demyelination in the brains and SC of control mice (Figure 2.4B), corresponding to the severity of clinical signs and the degree of leukocyte infiltrates in the CNS. Conversely, the CNS of anti-PC mice exhibit minimal demyelination (Figure 2.4B). Taken together, these data further demonstrate the decreased inflammatory and pathogenic conditions in the CNS of anti-PC mice despite the presence of cellular infiltrates.

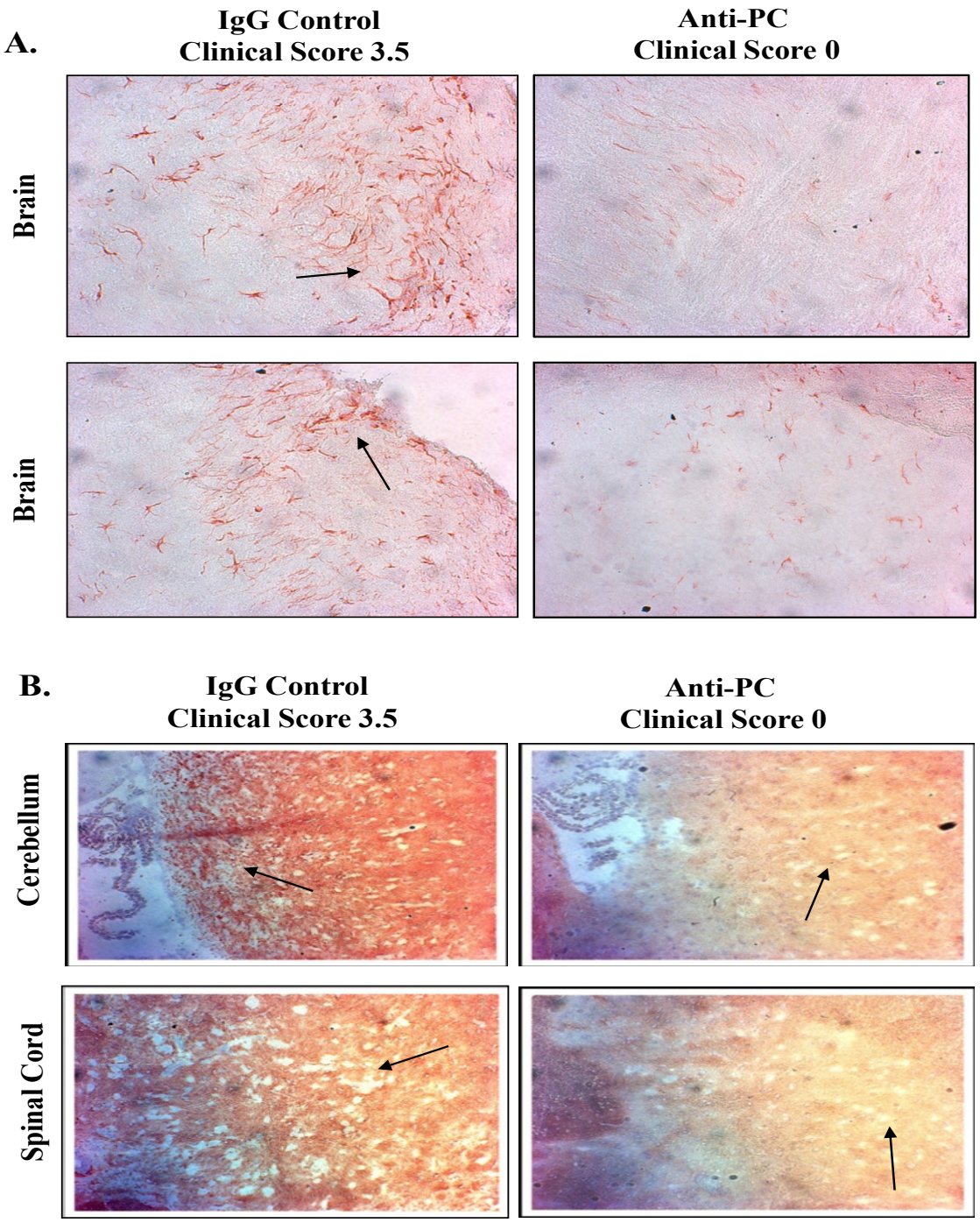
A.
IgG Control
Clinical Score 3.5
Anti-PC
Clinical Score 0
Brain
Brain
B.
IgG Control
Clinical Score 3.5
Anti-PC
Clinical Score 0
Cerebellum
Spinal Cord

Figure 2.4 Anti-PC mice exhibit minimal microglial activation and demyelination in the CNS. CNS were harvested at the peak of disease (days 14-17) following EAE induction. (**A**) Brain sections from control and anti-PC mice were stained with anti-Iba-1 (red) to assess microglial activation. Arrows indicate pronounced Iba-1 staining. (**B**) CNS sections were examined for severity of demyelination by staining with anti-MOG antibody. Areas in the CNS that do not positively stain for MOG denote demyelination as indicated by arrows.

Peripheral $CD4^+$ T-cells in anti-PC mice are significantly decreased and functionally deficient.

In EAE, leukocyte infiltration in the CNS is preceded by initial activation of immune populations in the periphery [37]. Since APC can directly modulate various leukocyte functions [9, 13], we hypothesize that abrogating APC activity in the circulation at the initiation of EAE can likely affect the activation and functional responses of peripheral leukocytes. We, therefore, examined the frequency and functional characteristics of various leukocyte populations in the periphery. We observed significantly decreased splenic $CD4^+$ T-cells in anti-PC mice compared to controls (Figure 2.5A). Moreover, consistent with what we observed in the CNS, the frequency of T-regs is significantly increased in the periphery of anti-PC mice (Figure 2.5B). We evaluated the expression levels of various cell surface markers on splenic T-cells. Interestingly, we observed that the ratio of $CD3^{high}$ to $CD3^{low}$ T-cells is consistently reduced in anti-PC mice compared to controls (Figure 2.5C). Reduced CD3 expression has been attributed to lowered T-cell activation [38]. The expression levels of other $CD4^+$ T-cell surface markers, however, were not altered (Appendix-C). We next assessed the proliferative capacity of splenic $CD4^+$ T-cells from both experimental groups. $CD4^+$ T-cells in whole-splenocyte culture were re-stimulated *in vitro*, and we observed that $CD4^+$ T-cell from control mice exhibited robust proliferation, while the proliferative capacity of $CD4^+$ T-cells from anti-PC mice is significantly diminished (Figure 2.5D). Taken together, these data are indicative of a decreased and deficient $CD4^+$ T-cell effector function in anti-PC mice. Furthermore, the suppressive T-reg population is significantly increased in the periphery of anti-PC mice.

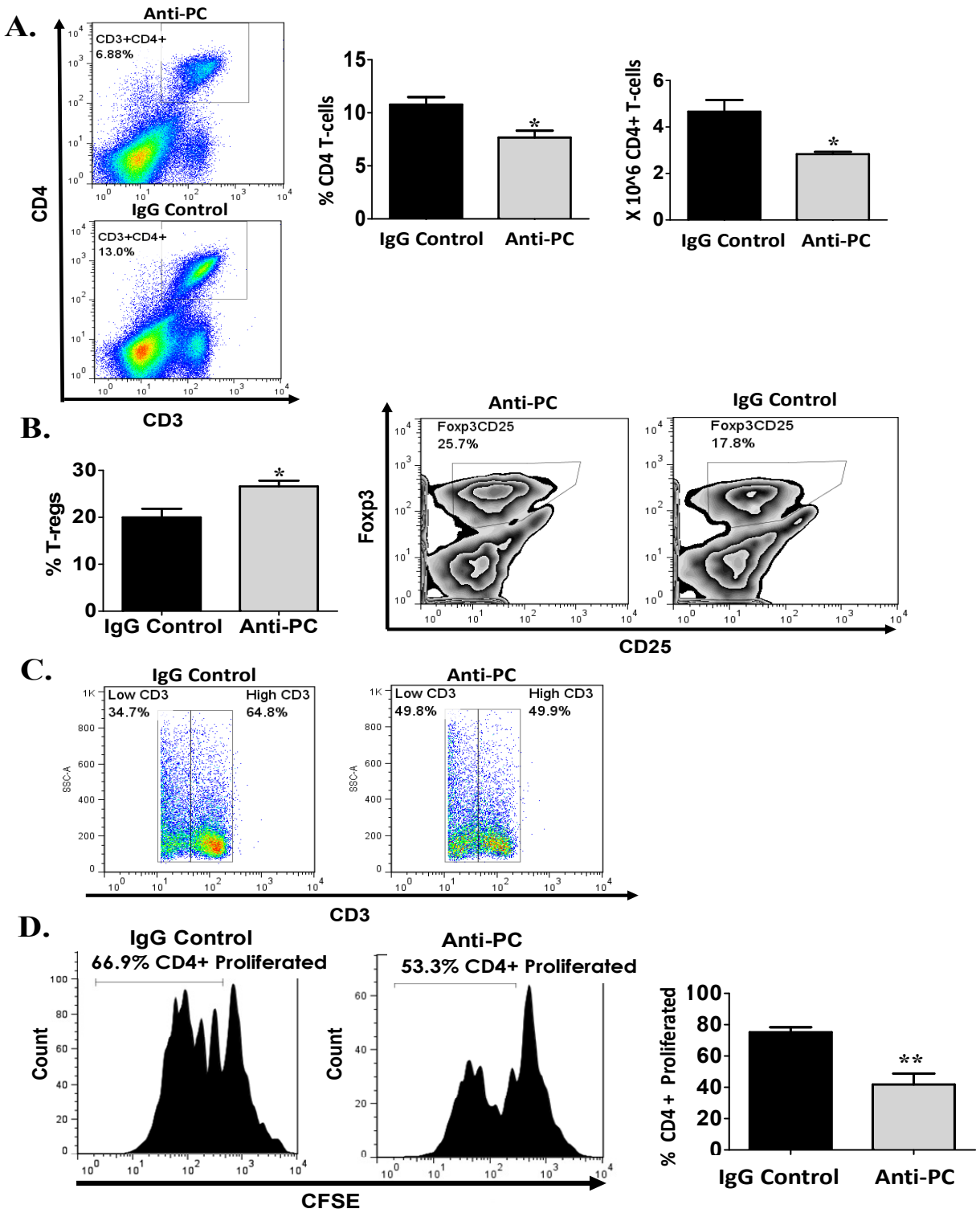
A.
Anti-PC
CD3+CD4+
6.88%
IgG Control
CD3+CD4+
13.0%
CD4
CD3
% CD4 T-cells
IgG Control
Anti-PC
X 10^6 CD4+ T-cells
IgG Control
Anti-PC
B.
% T-regs
IgG Control
Anti-PC
Anti-PC
Foxp3CD25
25.7%
IgG Control
Foxp3CD25
17.8%
Foxp3
CD25
C.
IgG Control
Low CD3
34.7%
High CD3
64.8%
Anti-PC
Low CD3
49.8%
High CD3
49.9%
SSC-A
CD3
D.
IgG Control
66.9% CD4+ Proliferated
Anti-PC
53.3% CD4+ Proliferated
Count
CFSE
% CD4 + Proliferated
IgG Control
Anti-PC

Figure 2.5 Peripheral CD4+ T-cells in anti-PC mice are significantly decreased and functionally deficient. (**A**) The frequency of splenic $CD4^+$ T-cells from anti-PC and control mice was examined by flow cytometry at day 17 following EAE induction. Data are represented as flow cytometric dot plots on the left panel. Middle panel and right panel are the graphical representation of the mean percentage of splenic $CD4^+$ T-cells and the mean absolute cell counts of splenic $CD4^+$ T-cells, respectively. Data are represented as means $\pm$ SEM (control n = 6; anti-PC n = 4; * $p < 0.05$ by Student *t* test). (**B**) The frequency of splenic T-regs ($CD4^+$, $CD25^+$, $Foxp3^+$) among $CD4^+$ T-cells was assessed by flow cytometry. Left panel shows the graphical representation of the mean percentage of T-regs among $CD4^+$ T-cells. Data are shown as means $\pm$ SEM (control n = 6; anti-PC n = 4; * $p < 0.05$ by Student *t* test). Middle and right panels show data as flow cytometric contour plots (gated on $CD3^+CD4^+$). (**C**) Flow cytometric dot plot representing the percentage of $CD3^{high}$ and $CD3^{low}$ splenic cells from both experimental groups (gated on $CD3^+$ cells). (**D**) Splenocytes from control and anti-PC mice were labeled with CFSE and cultured *in vitro*. T-cells in whole splenocyte culture were stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 96 hours. The proliferative capacity of $CD4^+$ T-cells was assessed by flow cytometry based on CFSE dilution. Left panels show the histogram representation of CFSE labeled $CD4^+$ T-cells from both experimental groups (gated on $CD4^+$ cells). Data on right panel are the graphical representation of the mean percentage of $CD4^+$ T-cells that have proliferated based on CFSE dilution. Data represented as means $\pm$ SEM (n=4; ** $p < 0.01$ by Student *t* test).

$CD11b^+$ cells are significantly increased in the periphery of anti-PC mice.

We next examined the frequencies of other splenic populations from both control and anti-PC mice. Similar to $CD4^+$ T-cells, the frequency of $CD8^+$ T-cells is significantly decreased in anti-PC mice (Figure 2.6A). Moreover, while we did not observe significant changes in the frequencies of various non-T-cell populations between the two experimental groups (Figure 2.6A), we did, however, observe increased splenic $CD11b^+$ cells in anti-PC mice compared to controls, and this was consistent through different time points in EAE (Figure 2.6B). Since various leukocyte subsets are known to express CD11b, we next determined the frequency of specific CD11b-expressing leukocyte subsets. We observed significantly increased macrophages ($CD11b^+F480^+$) and dendritic cells (DC) ($CD11b^+CD11c^+$) in anti-PC mice (Figure 2.6C). A population of blood monocytes known as myeloid-derived suppressor cells (MDSC), and characterized to be potent T-cell suppressors, has also been shown to express CD11b [39]. Various studies have used the co-expression of CD11b and Ly6C as identifying markers for MDSC in mice [29, 40]. Consistent with other $CD11b^+$ populations, we observed increased frequency of MDSCs in anti-PC mice, and this was observed through various stages in EAE (Figure 2.6D).

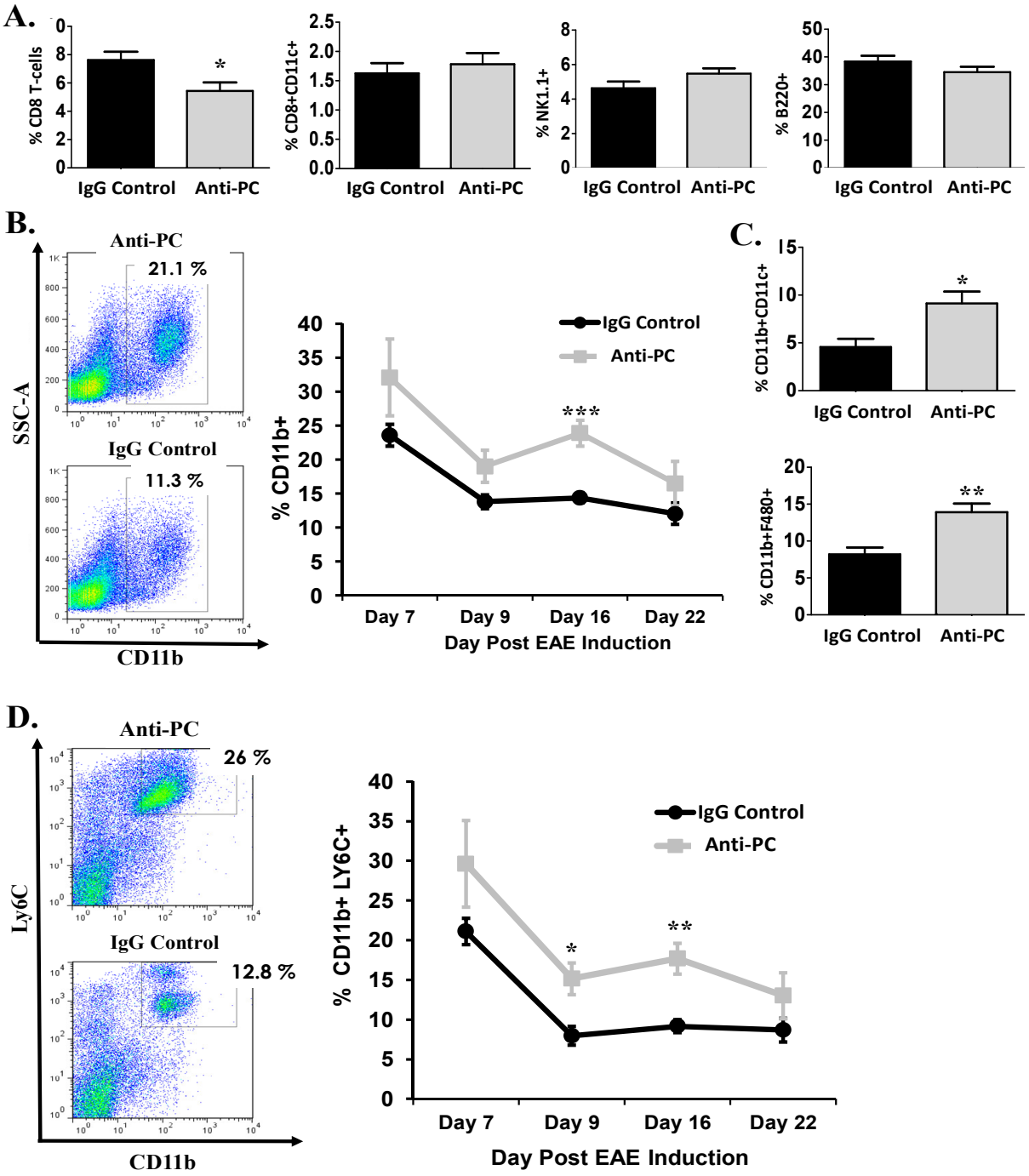
A.
% CD8 T-cells
IgG Control
Anti-PC
% CD8+CD11c+
% NK1.1+
% B220+
B.
Anti-PC
21.1 %
IgG Control
11.3 %
SSC-A
CD11b
% CD11b+
Day 7
Day 9
Day 16
Day 22
Day Post EAE Induction
C.
% CD11b+CD11c+
% CD11b+F480+
D.
26 %
12.8 %
Ly6C
% CD11b+ LY6C+

Figure 2.6. $CD11b^+$ cells in the periphery of anti-PC mice are significantly increased.

(**A-D**) Splenocytes were harvested from the anti-PC and IgG control mice at day 17 following EAE induction, and the frequency of several leukocyte populations was assessed by flow cytometry. (**B**) Left panel shows the flow cytometric dot plot representing the splenic CD11b frequency of both experimental groups. The right panel shows the frequency of $CD11b^+$ cells in the spleen at various stages of EAE progression as assessed by flow cytometry. (**C**) Splenocyte frequency of specific Cd11b expressing populations, namely macrophages ($CD11b^+$ $F480^+$) (upper panel) and DC's ($CD11b^+$ $CD11c^+$) (bottom panel), was examined by flow cytometry. (**D**) The frequency of MDSCs was examined in the spleen by staining for cell surface markers, CD11b and Ly6C. Frequencies were analyzed by flow cytometry. Left panel represents flow cytometric dot plot of $CD11b^+LyC^+$ cells from both anti-PC and control mice. Right panel exhibits the percentage of MDSC's populations in the spleen from both experimental groups at various stages of EAE progression. Data are represented as means $\pm$ SEM (n = 3-6; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Student *t* test).

$CD11b^+$ cells from anti-PC mice suppress the proliferation of $CD4^+$ T-cells and increase the proliferation of T-regulatory cells

We have observed that the frequency and effector functions of $CD4^+$ T-cells in anti-PC mice are significantly diminished (Figure 2.5). Studies have shown that certain populations of $CD11b^+$ cells, specifically MDSCs [29], are effective regulators of T-cell function. We, therefore, hypothesize that the deficit in $CD4^+$ T-cell numbers and diminished $CD4^+$ effector functions observed in anti-PC mice is a consequence of increased expansion of suppressive $CD11b^+$ cells. We tested this hypothesis by isolating $CD11b^+$ cells from both groups and examined their ability to suppress the proliferation of MOG_{35-55}-specific $CD4^+$ T-cells. We observed that $CD11b^+$ cells from anti-PC mice were more effective in suppressing MOG_{35-55}-induced proliferation of $CD4^+$ T-cells compared to $CD11b^+$ cells from controls (Figure 2.7A). No difference in proliferation, however, was observed if $CD4^+$ T-cells were co-cultured with non-$CD11b^+$ cells from either anti-PC or control mice (Figure 2.7B). Moreover, we have previously observed that the cell-surface expression of CD3 is reduced on splenic T-cells in anti-PC mice (Figure 2.5C). Consistently, we observed that $CD4^+$ T-cells co-cultured with $CD11b^+$ cells from anti-PC exhibit significantly lowered CD3 expression compared to $CD4^+$ T-cells co-cultured with $CD11b^+$ cells from control mice (Figure 2.7C). A number of studies have shown that MDSCs can directly mediate T-reg expansion [29, 40, 41]. We, therefore, hypothesize, that the increased frequency of T-regs, which was observed both in the CNS (Figure 2.2B) and periphery (Figure 2.5B) of anti-PC mice, is similarly mediated by the increased $CD11b^+$ suppressor cells in these mice. We tested this hypothesis by co-culturing *in vitro*- stimulated $CD4^+$ T-cells with $CD11b^+$ cells from either anti-PC or control mice. We observed significantly

higher frequency of T-regs following co-culture with $CD11b^+$ cells from anti-PC mice (Figure 2.7E). Altogether, these data exhibit the increased suppressive capability of $CD11b^+$ cells in anti-PC mice, mediated through direct suppression of T-cell function and the induced expansion of T-regs.

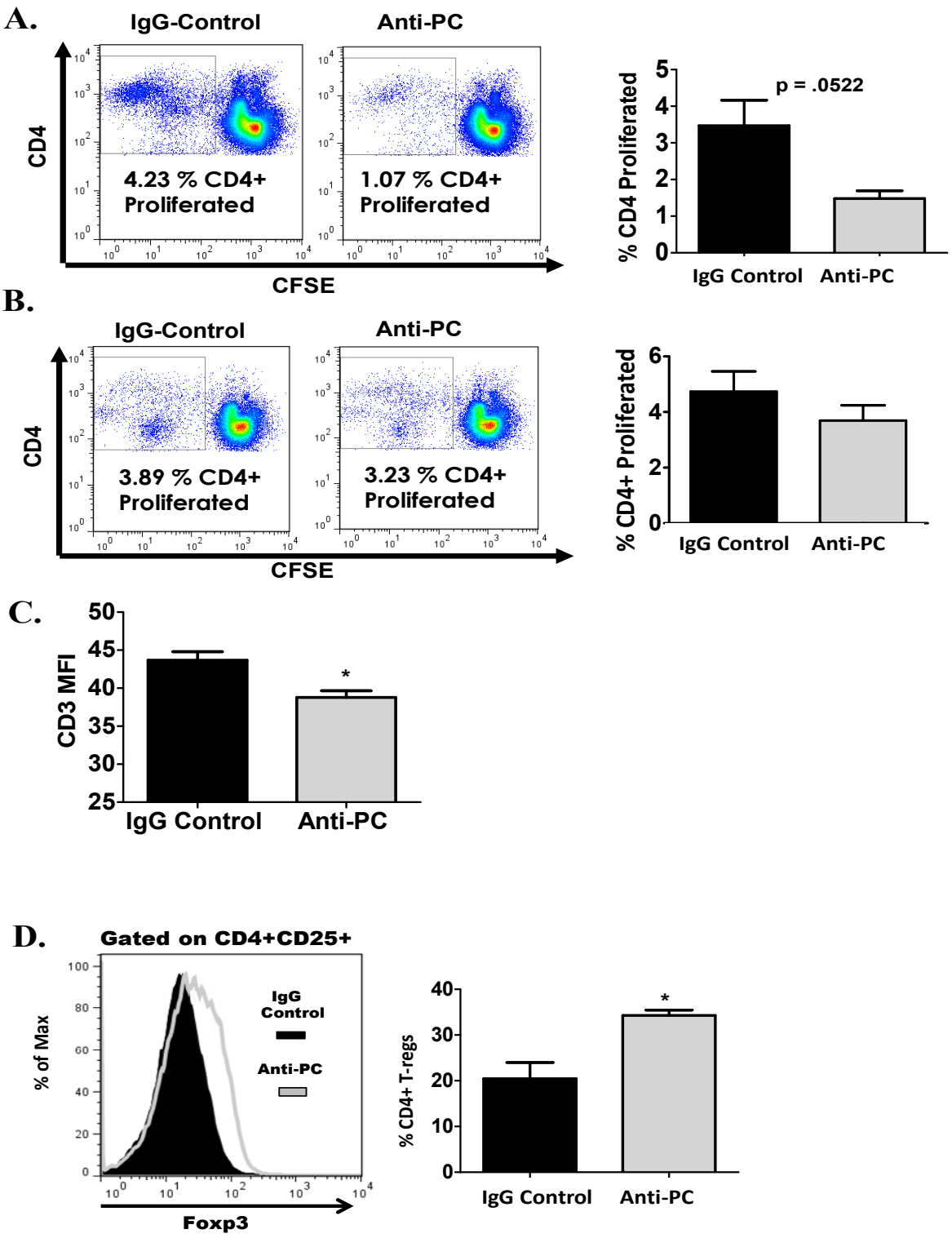
A.
IgG-Control
Anti-PC
CD4
CFSE
4.23 % CD4+ Proliferated
1.07 % CD4+ Proliferated
% CD4 Proliferated
p = .0522
IgG Control
Anti-PC
B.
IgG-Control
Anti-PC
CD4
CFSE
3.89 % CD4+ Proliferated
3.23 % CD4+ Proliferated
% CD4+ Proliferated
IgG Control
Anti-PC
C.
CD3 MFI
IgG Control
Anti-PC
D.
Gated on CD4+CD25+
% of Max
IgG Control
Anti-PC
Foxp3
% CD4+ T-regs
IgG Control
Anti-PC

Figure 2.7. CD11b$^+$ cells from anti-PC mice suppress CD4$^+$ T-cell proliferation and increase CD4+ T-regulatory cells. (**A**) Following EAE induction, splenic CD11b$^+$ isolated from anti-PC or control mice were co-cultured for 96 hours with MOG_{35-55}-specific CD4$^+$ T-cells isolated from BL/6 2D2 mice. To assess proliferation, CD4$^+$ T-cells were labeled with CFSE and stimulated with MOG_{35-55}. CFSE dilution was assessed by flow cytometry. Left panel represents flow cytometric dot plot of CFSE labeled CD4$^+$ T-cells co-cultured with CD11b$^+$ cells from either anti-PC or control mice. Right panel shows percentage of CD4$^+$ T-cells that have proliferated based on CFSE dilution. Data are represented as means $\pm$ SEM (n = 3; p calculated by Student *t* test). (**B**) Non-CD11b$^+$ splenocytes from anti-PC or control mice were co-cultured for 96 hours with CD4$^+$ T-cells from BL/6 2D2 mice. CD4$^+$ T-cells were labeled with CFSE and stimulated with MOG_{35-55} for 96 hrs. Proliferation of CD4$^+$ T-cells was analyzed by flow cytometry based on CFSE dilution. Left panel represents flow cytometric dot plot of CFSE labeled CD4$^+$ T-cells co-cultured with non-CD11b$^+$ cells from anti-PC or control mice. Right panel shows percentage of CD4$^+$ T-cells that have proliferated based on CFSE dilution. Data are represented as means $\pm$ SEM (n = 3). (**C**) The expression of cell-surface CD3 on CD4$^+$ T-cells that were co-cultured with CD11b$^+$ cells from either anti-PC or control mice was assessed by flow cytometry, and the difference in CD3 expression levels was determined by mean fluorescence intensity (MFI). Data shown as mean MFI $\pm$ SEM (n=4; * $p < 0.05$ by Student *t* test). (**D**) CD11b$^+$ cells were isolated from anti-PC or IgG control mice following EAE induction, and co-cultured for 96 hours with CD4$^+$ T-cells that were stimulated with plate-bound anti-CD3 and soluble anti-CD28. After co-culture, the frequency of T-regs among CD4$^+$ T-cells was assessed by flow cytometry. Left panel is the histogram representation of foxp3 expression in CD4$^+$CD25$^+$ cells from the two co-culture conditions. Right panel shows the mean percentage

of foxp3+ cells among $CD4^+$ $CD25^+$ T-cells. Data are represented as means $\pm$ SEM (n = 3; * $p < 0.05$ by Student *t* test).

Arginase I activity, iNOS expression, and reactive oxygen species production are increased in CD11b$^+$ cells from anti-PC mice

Several studies have demonstrated that the suppressive capacity of MDSC on T-cells is largely mediated through one or a combination of arginase I (Arg I) activity, nitric oxide (NO) production, and generation of reactive oxygen species [29, 39]. Arg I activity converts L-Arginine (L-Arg), into urea and L-ornithine [39]. Increased Arg I activity in MDSCs depletes L-Arg from the microenvironment, resulting in suppression of various T-cell responses, including inhibition of T-cell proliferation, reduced CD3ζ chain expression, and in some studies increased Arg I activity has been implicated in MDSC-mediated T-reg expansion [38, 39, 42]. Consistent with the increase of MDSCs, we measured higher arginase activity in splenocytes from anti-PC mice (Figure 2.7A), and we additionally show by immunofluorescence that Arg I is specifically expressed by CD11b$^+$ and CD11b$^+$ Ly6C$^+$ cells (Figure 2.7B). MDSCs are also known to express elevated levels of iNOS, which utilizes L-Arg as a substrate to produce nitric oxide (NO), contributing to L-Arg depletion [29, 39]. Moreover, NO production is known to directly inhibit T-cell functions, including the induction of T-cell apoptosis [29, 39]. We observed significantly increased iNOS expression in CD11b$^+$ and CD11b$^+$Ly6C$^+$ populations from anti-PC mice (Figure 2.7C), coupled with elevated NO production (Figure 2.7D). Another mechanism for MDSC-mediated T-cell suppression has been attributed to the generation of ROS, which can inhibit antigen-specific T-cell proliferation and downregulate CD3ζ chain expression [29, 39]. We observed significantly higher generation of ROS in CD11b$^+$ cells (Figure 2.7E) and Ly6C$^+$ cells (Figure 2.7F) from anti-PC compared to controls. Collectively, these data demonstrate that CD11b$^+$ cells from anti-PC mice express and generate elevated levels of specific factors that have been implicated in the suppression of T-cell functions.

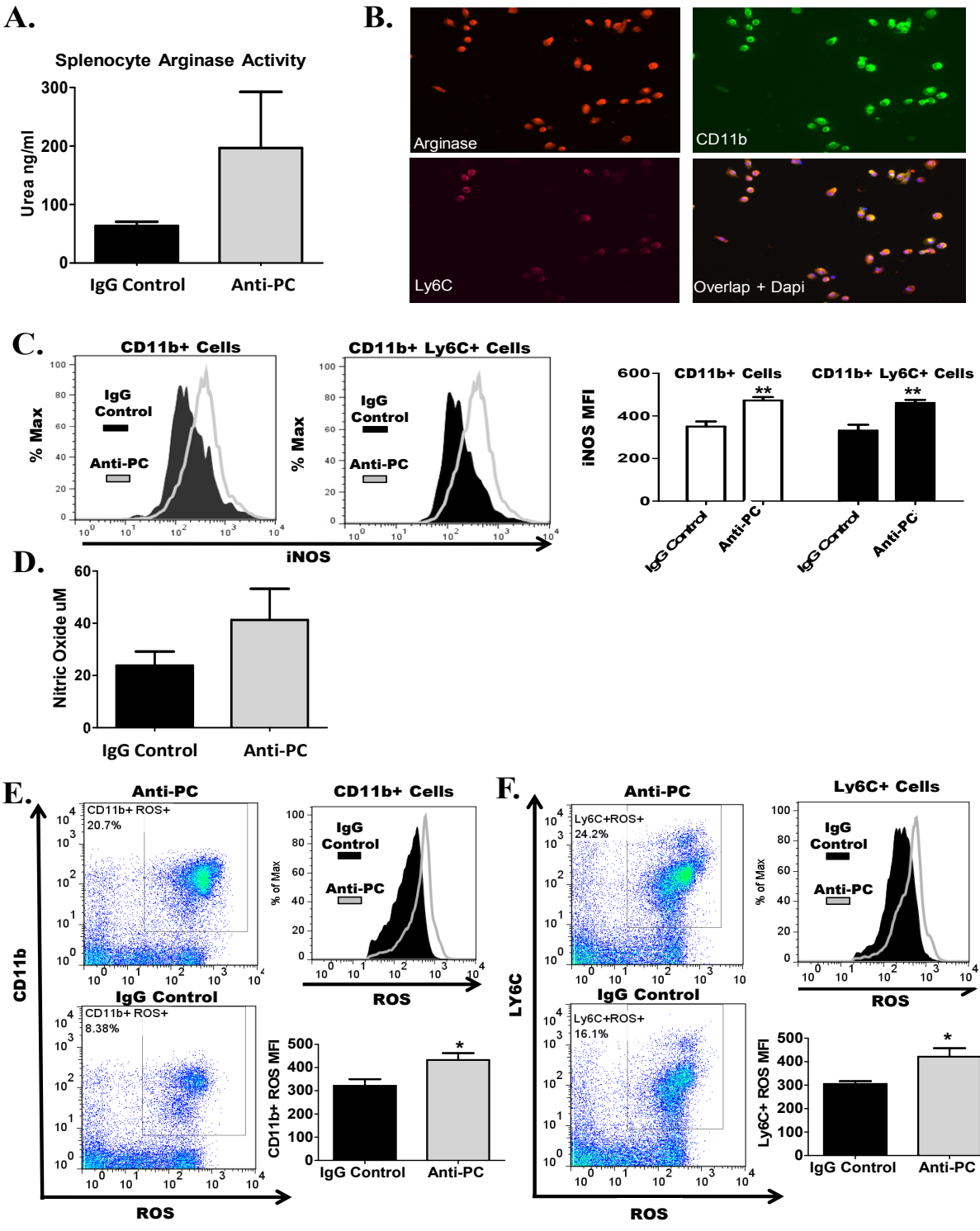
A.
Splenocyte Arginase Activity
Urea ng/ml
300
200
100
0
IgG Control
Anti-PC
B.
Arginase
CD11b
Ly6C
Overlap + Dapi
C.
CD11b+ Cells
CD11b+ Ly6C+ Cells
% Max
IgG Control
Anti-PC
iNOS
iNOS MFI
600
400
200
0
**
D.
Nitric Oxide uM
60
40
20
0
IgG Control
Anti-PC
E.
Anti-PC
CD11b+ ROS+
20.7%
IgG Control
CD11b+ ROS+
8.38%
CD11b
ROS
% of Max
CD11b+ ROS MFI
500
400
300
200
100
0
*
F.
Anti-PC
Ly6C+ Cells
Ly6C+ROS+
24.2%
Ly6C+ROS+
16.1%
LY6C
Ly6C+ ROS MFI

Figure 2.8 Arginase I activity, iNOS expression, and reactive oxygen species production are increased in CD11b$^+$ cells from anti-PC mice. (**A**) Splenocytes were collected following EAE induction, and arginase activity in splenocytes was measured based on urea generation. Data are represented as means $\pm$ SEM (n = 4). (**B**) Splenocytes were cultured on cover slips overnight, and cells were stained with fluorochrome-conjugated antibodies to CD11b, Ly6C, and arginase. Coverslips were mounted on slides, and images were captured using a Zeiss Axio Imager M1 microscope. (**C**) The expression of iNOS in CD11b$^+$ and CD11b$^+$ Ly6C$^+$ cells was assessed by flow cytometry. Left and middle panel show the flow cytometric histogram of iNOS expression in CD11b$^+$ and CD11b$^+$ Ly6C$^+$ cells, respectively. Right panel represents the mean fluorescence intensity (MFI) of iNOS expression in CD11b$^+$ and CD11b$^+$Ly6C$^+$ cells. Data are represented as means $\pm$ SEM (n = 3; ** $p < 0.01$ by Student *t* test). (**D**) Splenocytes were cultured for 48 hours, and supernatants were collected to assess NO production using Griess assay. Data are represented as means $\pm$ SEM (n = 3). (**E – F**) ROS generation is assessed by incubating splenocytes with 2',7' –dichlorofluorescein diacetate, a compound which diffuses into cells and fluoresces upon oxidation by ROS. Degree of cell fluorescence, which increases with elevated ROS generation, was assessed by flow cytomerty. (**E**) Left panel shows the flow cytometric dot plot of ROS$^+$ CD11b$^+$ cells from both experimental groups. Right upper panel is the histogram representation of the degree of ROS production in CD11b$^+$ cells, and bottom right panel shows the average MFI correlating to ROS generation in CD11b$^+$ cells. Data are represented as means $\pm$ SEM (n = 4; * $p < 0.05$). (**F**) Left panel shows the flow cytometric dot plot of ROS$^+$ Ly6C$^+$ cells. Right upper panel is the histogram representation of the level of ROS production in Ly6C$^+$ cells, and bottom right panel shows the average MFI of ROS generation in Ly6C$^+$ cells. Data are represented as means $\pm$ SEM (n = 4; * $p < 0.05$).

Signal transducer and activator of transcription 3 (Stat3) is significantly increased in CD11b+ cells from anti-PC mice

The transcription factor, Stat3, has been shown to have a critical role in the expansion and suppressive capacity of MDSCs [29, 39]. Several studies have demonstrated that inhibition of Stat3 abrogated the expansion and suppressive activities of MDSCs [43, 44]. We therefore specifically examined the expression of Stat3 in $CD11b^{+}Ly6C^{+}$ cells from both anti-PC and control mice. We observed significantly increased Stat3 expression in splenic $CD11b^{+}Ly6C^{+}$ cells from anti-PC mice (Figure 2.9A). We additionally observed increased phosphorylated Stat3 (pStat3) in $Ly6C^{+}$ cells from anti-PC mice (Figure 2.9B), suggesting elevated Stat3 activation in these cells. These data further confirm that the $CD11b^{+}Ly6C^{+}$ population in anti-PC mice demonstrate the suppressive MDSC phenotype.

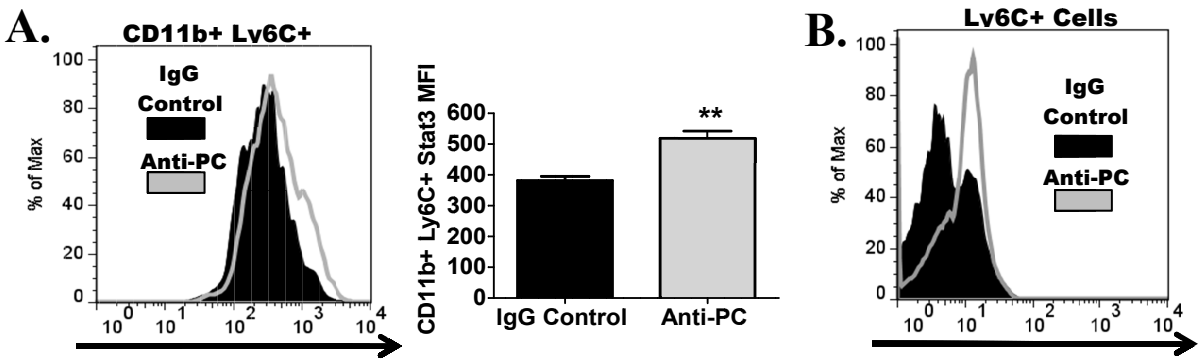

Figure 2.9 Stat3 expression and activation is increased in CD11b+Ly6C cells from anti-PC mice. (**A**) Stat3 expression in splenic $CD11b^{+}Ly6C^{+}$ cells was assessed by flow cytometry. Left panel shows the histogram representation of Stat3 expression in $CD11b^{+}Ly6C^{+}$ cells from anti-PC and control mice. Right panel is the graphical representation of the average MFI corresponding to Stat3 expression in $CD11b^{+}Ly6C^{+}$ cells. Data are represented as means $\pm$ SEM ($n = 4$; * $p < 0.05$). (**B**) The phosphorylation status of Stat3 in $Ly6C^{+}$ cells was assessed by flow cytometry. Levels of phosphorylated Stat3 (pStat3) in $Ly6C^{+}$ cells from anti-PC and control mice are represented as flow cytometric histograms.

APC directly interacts with MDSCs

APC has traditionally been known to regulate various cell types, including leukocytes, through its direct interaction with the cellular receptor, EPCR, subsequently enabling APC to activate PAR-1 [3]. A number of studies, however, have demonstrated that APC can regulate leukocyte functions through its interactions with receptors other than EPCR [45, 46]. One particular study has shown that APC can directly regulate the inflammatory responses of myeloid cells by binding to the CD11b integrin expressed on the cell surface [15]. In this study, Cao et. al demonstrated that APC can bind CD11b on macrophages, subsequently enabling APC to activate PAR-1 and resulting in the inhibition of pro-inflammatory responses, including downregulation of iNOS and Stat3 [15]. Based on this particular study, which demonstrates that APC is a potent negative regulator of $CD11b^+$ cells though direct interaction with the CD11b integrin, we hypothesize that inhibition of APC during EAE resulted in the increased expansion and activation of various $CD11b^+$ populations, as was observed in anti-PC mice. Moreover, we propose that among the $CD11b^+$ populations that increased and become more activated as a result of APC inhibition is the suppressive MDSC population, which ultimately contributed to attenuating EAE. We confirmed that MDSCs express PAR-1 (Figure 2.10A), suggesting that these cells express the necessary receptor to be responsive to APC regulation. We next determined whether APC can directly interact with MDSCs. Fluorochrome-conjugated APC's were incubated with $Ly6C^+$ cells, and we show by flow cytometry that APC directly binds $Ly6C^+$ cells (Figure 2.10B). Moreover, we confirm that the monoclonal antibody to PC used to inhibit APC in EAE mice can abrogate the direct binding of APC to $Ly6C^+$ cells (Figure 2.10C). Collectively these data demonstrate that APC directly interacts with $Ly6C^+$ cells, indicative of

the potential regulatory capability of APC on the MDSC population, which can be abrogated with anti-PC treatment.

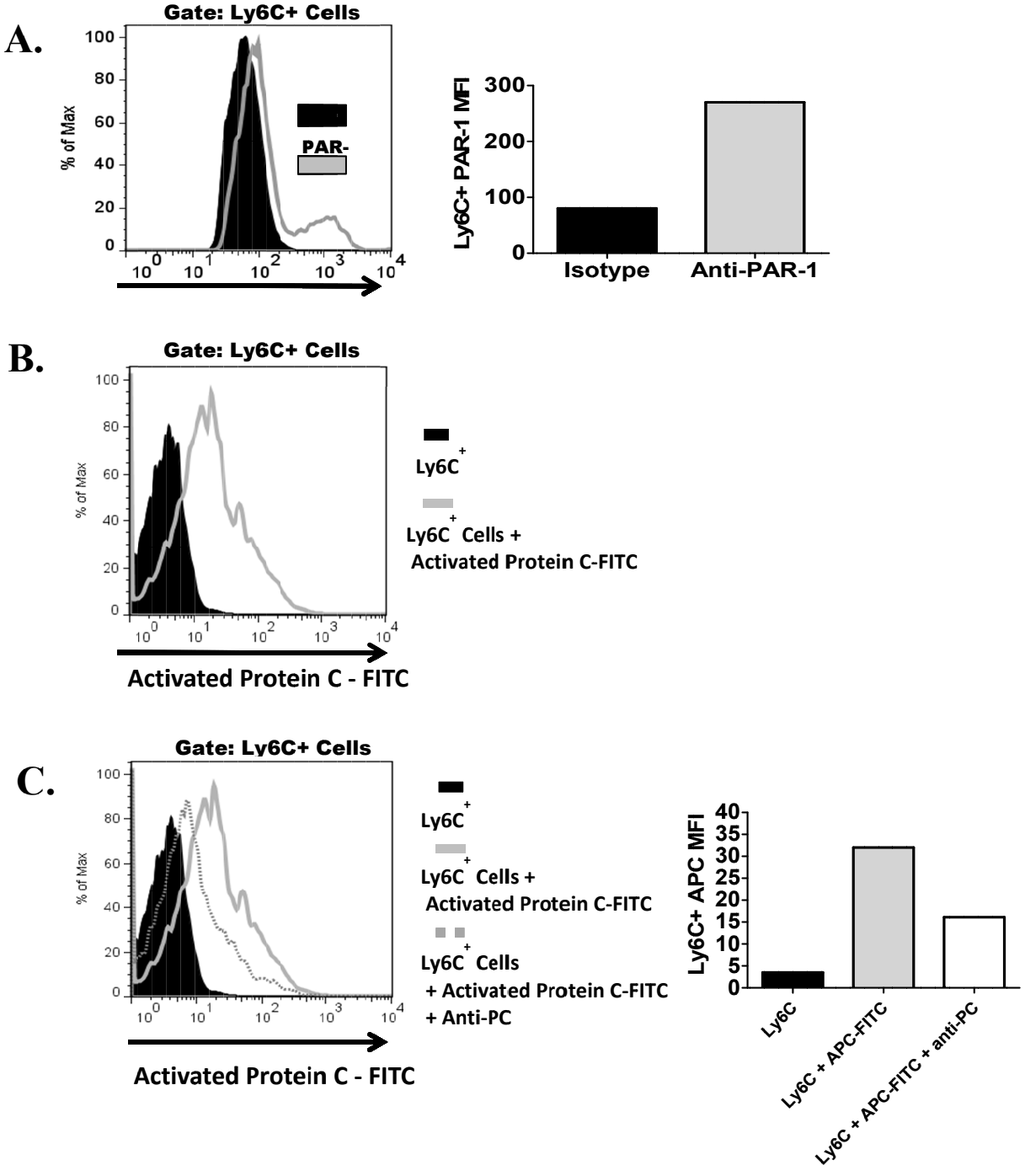

Figure 2.10 APC directly interacts with Ly6C$^+$ cells. (**A**) Splenic cells were stained with anti-PAR-1 to determine the expression of PAR-1 on Ly6C$^+$ cells. Left panel shows the histogram representation of PAR-1 expression on Ly6C$^+$ cells stained with either isotype control or anti-PAR-1. Right panel shows the graphical representation of MFI corresponding to PAR-1 expression on Ly6C$^+$ cells. (**B**) To determine whether APC can directly interact with Ly6C$^+$ cells, fluorochrome-conjugated APC were incubated with splenic cells, and the binding of APC to Ly6C$^+$ cells was assessed by flow cytometry. (**C**) To examine whether anti-PC can decrease the interaction of APC to Ly6C$^+$ cells, fluorochrome-conjugated APC were incubated with splenic cells with or without anti-PC, and the binding of APC to Ly6C$^+$ cells was assessed by flow cytometry. Left panel exhibits the histogram representation of the extent of APC binding to Ly6C$^+$ cells, and right panel is the graphical representation of MFI corresponding to APC binding to Ly6C$^+$ cells.

Discussion

In addition to its traditional function as an anti-coagulant, APC can influence various aspects of the pathological setting by directing cellular processes involved in inflammatory responses, vascular integrity, and cell survival [1-3]. Our study further exemplifies the broad-ranging effects of APC in pathological conditions. We observed that depletion of APC during EAE affected disease pathogenesis at multiple fronts. What is interesting and unexpected, however, is that the various effects of APC-depletion on the inflammatory response in EAE have opposing and incongruent consequences on the progression and severity of the disease. We observed that inhibition of APC increased BBB permeability as evidenced by considerable leukocyte infiltration and increased dextran extravasation in the brains of anti-PC mice. However, APC depletion also had considerable effects on the inflammatory responses of various leukocyte populations. Inhibition of APC effectively resulted in increased and more activated $CD11b^+$ myeloid population, including the expansion of the MDSC subset that is capable of negatively regulating the effector T-cells required for disease progression. Consequently, the net effect of APC inhibition in EAE is attenuated disease.

The ability of APC to influence inflammatory conditions has been largely attributed to its cell signaling capabilities [1-3]. APC can regulate the functions of various cell types including several leukocyte populations. The cellular receptors that are involved in APC- mediated signaling in leukocytes, however, is still unclear. In endothelial cells, APC controls cellular processes by interacting with EPCR, a receptor which localizes APC on the cell surface and within the lipid rafts of the cell membrane, allowing APC to activate PAR-1 and initiate various cell signaling cascades [5, 7]. Several studies, however, have shown that APC's effects on

myeloid cells are not dependent on EPCR and may involve other cellular receptors [46, 47]. A recent study by Cao *et. al*. has identified the CD11b integrin as the facilitator of APC's anti-inflammatory effects on macrophages [15]. In this study, the group demonstrated that APC can effectively inhibit the pro-inflammatory responses in macrophages, including down-regulation of iNOS, STAT3, and NF-κB expression, through APC's interaction with CD11b on the cell surface [15]. They propose that CD11b expressed on leukocytes can serve a similar function as EPCR on endothelial cells, in that CD11b can bind APC and co-localize it with PAR-1 within the lipid rafts of the cell membrane, thereby facilitating PAR-1 activation, and inhibiting inflammatory signaling cascades [15].

In accordance with the study by Cao *et. al,* we observed that inhibition of APC during EAE resulted in the significant increase in various $CD11b^+$ populations. Since APC has been shown to be a negative regulator of $CD11b^+$ cells, we hypothesize that inhibition of APC during EAE likely resulted in the increased and more activated $CD11b^+$ subsets. Further, we propose that certain $CD11b^+$ populations, notably MDSCs, which similarly expanded as a result of APC inhibition, can have suppressive effects on the progression of EAE, thus resulting in the attenuated disease observed in anti-PC mice.

MDSCs are a heterogenous population of immature myeloid cells characterized to be potent T-cell suppressors [29, 39, 40]. In mice, MDSCs are identified by cell surface co-expression of CD11b and Gr-1 [29]. Antibodies to Gr-1 bind two epitopes, Ly6C and Ly6G. MDSCs are categorized into two subsets based on the cell surface expression of these two molecules [29, 39, 40]. The $CD11b^+Ly6C^{hi}Ly6G^+$ subset has a monocytic morphology while the $CD11b^+Ly6C^{low}Ly6G^-$ subset was described to have a granulocytic morphology [29, 39, 40]. Each subset employs distinct mechanisms for suppressing T-cell function. Since both MDSC

subsets express Ly6C, we utilized the co-expression of CD11b and Ly6C as identifying markers for the MDSC population; thus, the MDSC population identified in our study incorporates both MDSC subsets. The immunoregulatory effects of MDSCs on T-cells has initially been described in tumor micro-environments, but recent studies have also reported the suppressive capabilities of MDSCs in various pathological settings, including parasitic infections and autoimmunity [40]. The suppressive capacities of these cells have been specifically observed in EAE. $CD11b^{+}LyC^{high}$ cells were shown to increase in the spleen following EAE induction, and these cells are capable of suppressing the proliferation of $CD4^{+}$ and $CD8^{+}$ T-cells [48]. In another study, MDSCs were observed in the spinal cord (SC) during EAE where they can promote T-cell apoptosis, thus limiting inflammation [49]. In agreement with these studies, we observed that anti-PC mice with attenuated EAE have increased splenic $CD11b^{+}Ly6C^{+}$ cells. Moreover, we observed that the frequency and the proliferative capacity of $CD4^{+}$ T-cells are significantly reduced in these mice, consistent with the known suppressive effects of MDSCs on T-cell proliferation. We also observed that the proliferation of MOG_{35-55}-specific T-cells is inhibited when co-cultured with $CD11b^{+}$ cells isolated from anti-PC mice, confirming the direct suppressive capability of the $CD11b^{+}$ population from the anti-PC mice on antigen-induced T-cell proliferation. The mechanisms utilized by monocytic MDSCs to suppress T-cell proliferation involve Arg I activity and /or the production of NO [29]. Increased Arg I activity depletes L-arginine from the microenvironment, consequently inhibiting T-cell cycle progression [50], as well as downregulating CD3 expression [38]. Similarly, increased production of NO can limit T-cell proliferation through a mechanism involving inhibition of the IL-2 receptor downstream pathway [51]. The suppressive capability of granulocytic MDSCs has been attributed to the generation of ROS, which is also known to suppress antigen-induced T-cell

proliferation [29, 39, 40]. Consistent with these characterized features of MDSCs, we observed increased Arg I activity in splenocytes and significantly higher expression of iNOS and ROS generation in $CD11b^+$ and $CD11b^+Ly6C^+$ cells in anti-APC mice, confirming the increased expansion and activation of the suppressive MDSC population in these mice.

Several studies have demonstrated that in addition to suppressing T-cell function, MDSCs have the capacity to induce the generation of T-regs [52, 53]. The mechanism through which MDSCs induce T-reg expansion has yet to be fully elucidated; interestingly a study has implicated Arg I activity in MDSCs as a possible contributing factor in T-reg generation [42]. Various studies have independently linked MDSCs to T-regs expansion. For instance, the accumulation of T-regs within the tumor microenvironment has been associated to MDSCs, and in vitro studies have confirmed that MDSCs co-cultured with T-cells can result in T-reg expansion [52, 53]. In our study, we similarly observed that the expansion of $CD11b^+Ly6C^+$ population in anti-PC mice was accompanied by an increase in T-reg subset, both in the CNS and in the periphery. We further showed that co-culture of $CD4^+$ T-cells with $CD11b^+$ cells from anti-APC mice increased T-reg frequency. These findings exhibit the increased suppressive capability of $CD11b^+$ cells from anti-PC mice, not only via direct suppression of T-cell function but also through the induction of T-reg expansion.

The accumulation of MDSCs is known to be critically mediated by the transcription factor, Stat3 [54, 55]. Inhibition of Stat3 signaling *in vivo* abrogated the expansion of MDSCs in tumor-bearing mice. Moreover, the suppressive activities of MDSCs, specifically the generation of ROS, are also reportedly regulated by Stat3 [29]. Consistent with these studies, we observed increased Stat3 expression and Stat3 activation in the $CD11b^+Ly6C^+$ population in anti-PC mice, further confirming the MDSC phenotype of these cells.

The influence of APC on inflammatory settings can be mediated at two different fronts, namely APC's direct effects on leukocytes and APC's effects on vascular barrier permeability [3]. Consistently, we observed that inhibition of APC during EAE not only affected leukocyte function but additionally increased BBB permeability as evidenced by considerable leukocyte infiltration in the brains of anti-PC mice and the extensive perivascular cuffing observed in the brain parenchyma of these mice. Despite exhibiting attenuated clinical symptoms, anti-PC mice had heavy cellular infiltrates in the brain, and the degree of infiltration is comparable to control mice with severe clinical symptoms. Interestingly, cellular infiltration in the SC of anti-PC mice was less pronounced. This deviates from the known pattern in classical EAE models, which is characterized by predominant infiltration and pathology in the SC [56, 57]. The reason for the disparity in infiltration between the two sites is unclear. It should be noted, however, that there are reported molecular differences governing leukocyte trafficking events in the brain and in the SC [58]. Thus, it is likely that modulation of APC in the circulation can have separate and varying effects on leukocyte extravasation in the brain and SC, accounting for the observed disparity in cellular infiltration between the two sites.

Despite considerable leukocyte infiltration in the brain, the pathological condition in the CNS of anti-APC mice, specifically demyelination and microglial activation, is minimal compared to control mice, and it follows that these mice exhibited attenuated clinical signs. This is an indication that the infiltrating population in the CNS of anti-PC mice is less inflammatory. In fact, we observed increased T-reg frequency in the CNS of these mice, as well as increased production of anti-inflammatory cytokine, IL-10, from cellular infiltrates. Further, we detected decreased pathogenic cytokines in the brains of anti-PC mice, specifically IFN-γ and IL-17, the signature cytokines produced by encephalitogenic $CD4^+$ T-cell subsets in EAE. We postulate

that the decreased encephalitogenic $CD4^+$ T-cells in the CNS of anti-PC mice is a consequence of increased expansion of MDSCs in the periphery, which we have demonstrated can effectively suppress the effector responses of $CD4^+$ T-cells, notably MOG_{35-55}-induced proliferation of $CD4^+$ T-cells, while concurrently inducing T-reg expansion.

It is widely recognized that APC's influences on the immune response, which is mediated through its direct regulation of leukocyte responses and its capability to confer vascular barrier protection, are pre-dominantly anti-inflammatory [3]. Therefore, it was largely unexpected that inhibition of APC during EAE resulted in the attenuation of disease. We propose that depletion of APC, through its pleiotropic nature, affected EAE pathogenesis at multiple fronts, resulting in pathogenic conditions with opposing overall influences on the progression of EAE. Specifically, APC inhibition resulted in increased BBB permeability as evidenced by increased dextran extravasation in the brain and leading to the considerable leukocyte infiltration in the brains of anti-PC mice. This is in line with the well-documented effects of APC on vascular barrier function during pathogenic conditions [3]. Additionally, depletion of APC during EAE also affected the inflammatory responses of specific leukocyte populations. APC's ability to downregulate the pro-inflammatory responses of leukocytes has been notably observed in myeloid cells, with a recent study identifying the CD11b integrin as the facilitator of APC's anti-inflammatory effects on CD11b expressing myeloid cells [15]. Consistently, we observed that APC inhibition resulted in the increased frequency of the $CD11b^+$ myeloid population. We speculate that the absence of APC as a negative regulator resulted in the increased expansion and activation of various $CD11b^+$ cells, including the suppressive MDSC population, during the EAE inflammatory setting. It should be noted that MDSCs are known to expand in various inflammatory settings, including in EAE [39, 40]. It is likely that inhibition of APC contributed

to the further accumulation and activation of these cells as EAE progressed. One likely mechanism through which APC can regulate MDSCs is through Stat3 expression. Stat3 has been established to be critical in the expansion and suppressive activities of MDSCs [29]. In the study by Cao et. al, APC has been demonstrated to suppress the expression of Stat3 and in myeloid cells through engagement with the CD11b integrin [15]. Moreover APC can similarly downregulate the expression of iNOS, which is utilized in T-cell suppression by MDSCs [15]. It is likely, therefore, that APC inhibition can lead to increased expansion and activation of MDSC as observed in anti-PC mice, and consequently resulting in suppressed T-cell responses. This, in effect, explains why inhibition of APC, generally known as an anti-inflammatory molecule, unexpectedly resulted in alleviated EAE severity. APC inhibition increased BBB permeability and resulted in a more activated CD11b+ myeloid population, including a myeloid subset known to be a potent T-cell suppressor. Since EAE is a pre-dominantly T-cell mediated disease, the net effect of APC depletion during EAE is alleviated disease severity (See proposed model Figure 2.11).

APC's influence on the MDSC population is presented here for the first time. The ability of APC to affect the functional responses of MDSCs opens a novel therapeutic avenue through which APC can influence disease conditions. MDSCs, for instance, are widely-reported to be a major hindrance in tumor immunity [59]. The findings in this study, which suggests that APC can negatively regulate MDSCs, bring to the fore, the possible therapeutic relevance of APC in cancer immunotherapies.

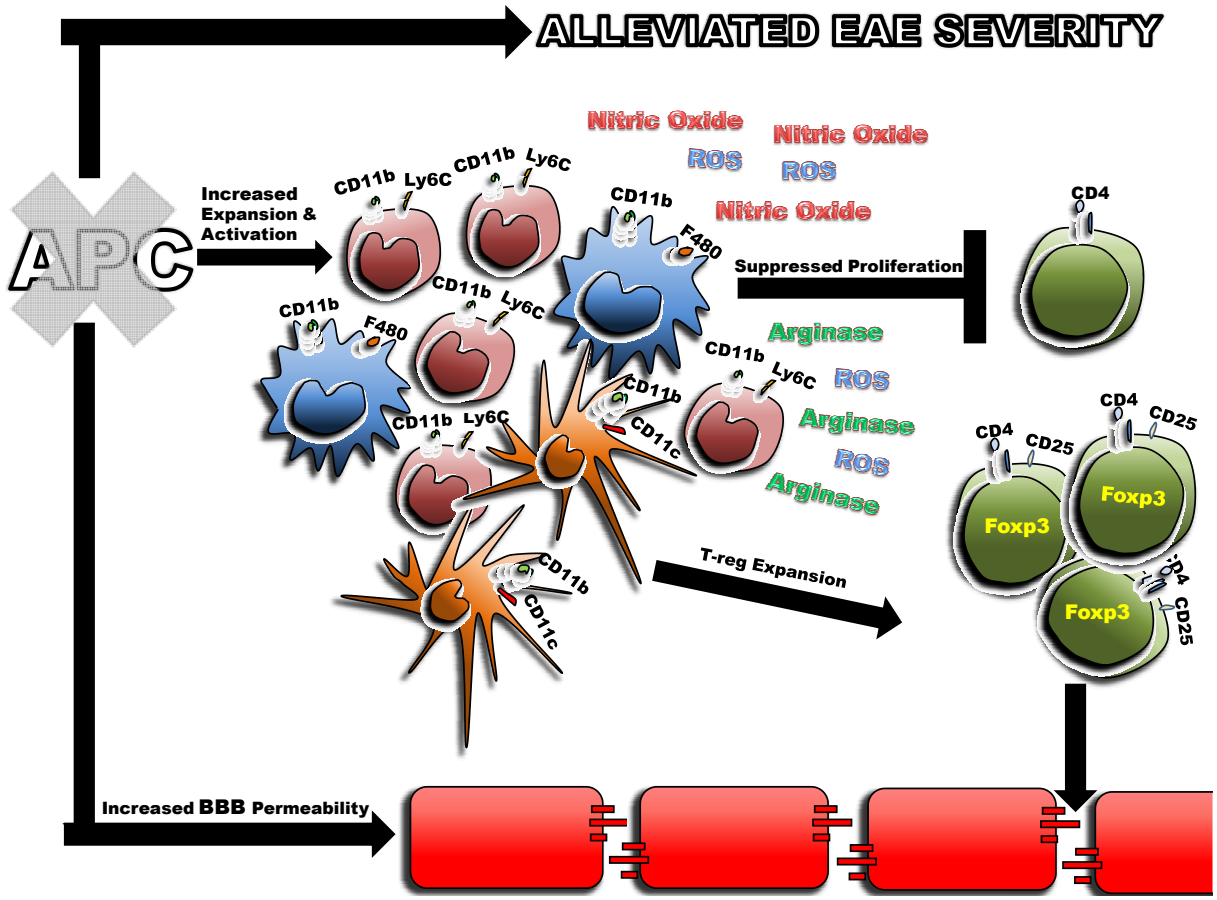

Figure 2.11 Proposed model. Inhibition of APC results in increased expansion of $CD11b^{+}$ cells, including the suppressive $CD11b^{+}$ subset known as MDSCs. The increased MDSCs mediate T-reg expansion and suppress T-cell proliferation through expression of Arg I, NO and ROS. Inhibition of APC also resulted in increased BBB, however, since the effector T-cell population is diminished and the suppressive T-reg subset is increased, pathology and inflammation in the CNS is minimal.

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CHAPTER 3

Thrombin induces an inflammatory phenotype in a human brain endothelial cell line[1]

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Abstract

In this study, we utilized the human brain endothelial cell line, hCMEC/D3, to determine the effects of the coagulation factor, thrombin, on the human blood-brain barrier (BBB). We show that thrombin increased the mRNA and cell surface levels of ICAM-1 and VCAM-1 in hCMEC/D3 cells. Thrombin similarly upregulated several chemokines implicated in human neurological conditions. Additionally, the paracellular permeability of the human BBB *in vitro* was increased following thrombin treatment. Overall, this study demonstrates that thrombin can effectively induce an inflamed phenotype in an *in vitro* human BBB, suggesting the potential relevance of thrombin in neuroinflammatory diseases such as multiple sclerosis, wherein leukocyte extravasation into the CNS as a consequence of increased BBB permeability is a primary pathological component.

Introduction

The blood-brain barrier (BBB) is structured to limit the influx of potentially harmful blood-borne molecules into the CNS. The BBB endothelium, however, is responsive to various stimuli resulting in a BBB phenotype that can dynamically change based on the conditions in the microenvironment [1]. In neuroinflammatory settings, for instance, an inflamed BBB becomes more permeable to leukocytes in the circulation as a consequence of phenotypic changes that include the disassembly of tight junction molecules, increased expression of cell adhesion molecules (CAMs), and synthesis of chemotactic molecules [1, 2].

In recent years, there has been accumulating evidence of a complex, two-way interaction between the immune and coagulation systems [3, 4]. Specific components of the coagulation system can directly influence the immune response. The coagulation factor, thrombin, in particular, is widely-regarded to have a major influence on inflammation [5-7]. Thrombin is a serine protease that is responsible for the formation of fibrin, the primary component of blood clot [8]. Beyond its central role in the coagulation process, thrombin can directly affect the cellular processes of various cell types, including leukocytes and endothelial cells, largely through the activation of G protein-coupled receptors known as protease-activated receptors (PARs) [9, 10].

While the inflammatory influences of thrombin have been predominantly observed in the periphery, there are now growing reports that thrombin may also be involved in neuroinflammatory and neurodegenerative conditions [11, 12]. Thrombin in the circulation can enter the CNS in conditions where the BBB is compromised [13]. Additionally several coagulation factors including prothrombin and its activator, factor X, are locally synthesized in

the brain [14, 15]. Increased thrombin activity and/or expression in the CNS have been observed in a number of neurological diseases. It has been reported that there is increased thrombin inhibitors in the CNS and in the periphery during experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis (MS). In Parkinson's disease and human immunodeficiency virus encephalitis, thrombin and prothrombin expression have been shown to be upregulated in astrocytes and neurons [16, 17]. Ischemic brains are characterized by high thrombin levels as a result of thrombin extravasation from the circulation and local prothrombin synthesis [18, 19]. Elevated thrombin levels have also been observed in brain microvessels in Alzheimer's disease [20]. While the specific role of thrombin in the progression of these diseases is still not well-delineated, it is known that resident cells in the CNS can be responsive to thrombin activity. Thrombin can induce microglia and astrocytes to release proinflammatory cytokines including IL-1β, IL-6, and TNF-α [17, 21]. High thrombin concentrations can also result in neurodegeneration [22].

Similarly, *in vivo* studies in animal models have shown that the BBB can be responsive to high levels of thrombin in the CNS. For instance, intrathecal injection of thrombin into rat brains can result in brain edema as a result of BBB permeability [23, 24]. The collective effects of thrombin on the phenotype of the specialized endothelial cells that comprise the human BBB however have not been thoroughly investigated. While there are a number of studies that have examined thrombin's effects on endothelial cells, these studies have exclusively utilized endothelial cells from the periphery. The specific responses of peripheral endothelial cells to thrombin, however, may not necessarily reflect the responses of the distinct endothelial cells that comprise the BBB. It is, therefore, warranted to particularly investigate the effects of thrombin activity on brain endothelial cells. Furthermore, given that thrombin's presence is heightened in

various human neuroinflammatory and neurological disorders it is pertinent to study thrombin's effects specifically on human brain endothelial cells.

The purpose of this study is to utilize a recently developed *in vitro* model of the human BBB to examine the responses of human brain endothelial cells to thrombin activity. We utilized the hCMEC/D3 cell line, an immortalized primary human brain endothelial cell line that stably retain the morphological characteristics of the BBB without the need for supporting glial cells [25], as an *in vitro* model for studying the responses of human brain endothelial cells to thrombin. In this study, we show that thrombin induces specific responses in brain endothelial cells *in vitro*, effectively inducing an inflamed BBB phenotype that is conducive to the potential recruitment, capture, and diapedesis of leukocytes. The results of this study may provide insight into the possible contribution of thrombin in the pathogenesis of neuroinflammatory and neurodegenerative conditions.

Materials and methods

Cell Culture

The hCMEC/D3 cell line was generated by Weksler *et.al* [25] and kindly provided to us by Dr. Babette Weksler. The cells were cultured in EBM-2 (Lonza, Wokingham UK) or MCDB 131 (Invitrogen, CA U.S.A) with 2.5% fetal bovine serum (FCS) and supplemented with hydrocortisone, ascorbate, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-1), epidermal growth factor (EGF), and gentamycin sulfate. Supplements were purchased from Lonza (Wokingham, UK), and concentrations used were according to the manufacturer's protocol. The cells were maintained at 37^{o} C in a humidified atmosphere with 5% CO_2. All culture wares were coated with 0.1 mg/ml rat-tail collagen type I (BD Pharmingen, CA U.S.A.). In all the experiments, the cells were grown to confluency for 7-10 days in fully supplemented MCDB-131 or EBM-2 media, which was changed every 2-3 days. Eighteen to twenty hours before each assay, the media was switched to reduced FBS and growth-factor depleted medium (MCDB 131 or EBM-2 supplemented with 0.25% FBS, 1 ng/ml bFGF, 10 mM Hepes, and .55 μM hydrocortisone). This media was used throughout the entire duration of all experiments. For thrombin treatments, human thrombin (Sigma-Aldrich MO, U.S.A.) was solubilized in molecular grade H_2O and further diluted in media.

Quantitative real-time PCR

Cells were seeded on collagen-coated 12-well plates (BD Falcon, CA U.S.A). After thrombin (0.5 – 60 U/ml) or vehicle treatments, total RNA was extracted using Trizol (Invitrogen CA, U.S.A) according to the manufacturer's protocol. Extracted RNA was treated with Baseline-Zero DNAse (Epicentre, WI U.S.A.) to remove contaminating genomic DNA. 1.5 µg of total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems CA, USA). Real Time PCR was performed using SYBR Green technology (SYBR FAST Master Mix KAPAbiosystems, MA U.S.A) on a CFX96 Real Time System Thermal Cycler (Bio-RAD, CA U.S.A). The cycling conditions were as follows: Enzyme activation 95° C for 3 minutes followed by 40 cycles of denaturation 95°C for 3 secs, annealing 60° C for 30 seconds, elongation 72° C for 5 seconds. BioRad CFX Manager software was used to determine cycle threshold (C_t) values, and gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The expression levels of all genes of interest were normalized to the internal control gene, GAPDH.

Flow Cytometry Analysis

Cells were seeded on 24-well plates (BD Falcon). After thrombin or vehicle treatments, cells were gently detached from culture plates using polyethylene cell scrapers (BD Falcon). The cells were washed and re-suspended in staining buffer (PBS with 0.5% BSA, and 0.09% sodium azide). Cell suspensions were incubated with antibody against the protein of interest for 30 minutes at 4^{o}C. Antibodies used are as follows: biotin anti-human ICAM-1, (BD Pharmingen, CA USA), APC-conjugated anti-human VCAM-1 (Biolegend, CA U.S.A), anti-

human PAR-1 (Enzo Life Sciences, NY U.S.A). The cells were subsequently washed twice in staining buffer and incubated with the appropriate secondary antibody for 30 minutes at 4°C. The cells were subjected to two final rounds of washing and resuspended in a final volume of 300 µl of staining buffer. The cells were acquired using a FACSCanto II flow cytometer (BD Biosciences, CA U.S.A). The raw data was evaluated using FlowJo Flow Cytometry Analysis Software (Treestar, OR U.S.A).

Western Blot Analysis

Cells were seeded on collagen-coated 12-well plates (BD Falcon). After thrombin or vehicle treatments, the supernatants and whole-cell lysates were collected. Cells were lysed on ice using lysis buffer (10 mM Tris-HCl, pH 7.4, 150mM NaCl, 1 mM EDTA, 10 mM Na_3VO_4, 10 mM Sodium Fluoride, 10 mM Sodium Pyrophosphate, 1% NP-40, 1mM PMSF, 1µM Pepstatin A). Laemmli buffer at 5x (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) was added to the supernatant and whole-cell lysate samples. Samples then were heated to 99°C for 5 minutes. For detection of CCL2 or CXCL8, the supernatant samples were loaded in 18% SDS-PAGE gel (1.5 M Tris-HCl pH 8.8, 20% SDS, Acrylamide/Bis-acrylamide, 10% ammonium persulfate, TEMED). For CX3CL1 detection, the whole-cell lysate samples were loaded in 7% SDS-PAGE gel. The gels were ran in running buffer (25mM Tris, 192 mM glycine, 0.1% SDS pH 8.5) at 100 V. Proteins were transferred to nitrocellulose membrarane (Whatman, NJ USA) by standard wet transfer protocol. After transfer, membranes were washed with TBS-Tween (100mM Tri-HCl pH 7.5, 0.9% NaCl, 0.1% Tween-20) for 5 minutes. Membranes were blocked with 5% BSA in TBS-Tween for 1 hour at

4°C. After blocking, membranes were incubated with primary antibody in 1% BSA in TBS-Tween overnight at 4°C. Primary antibodies used are as follows: rabbit anti-human IL-8 (Peprotech NJ, USA), rabbit anti-human MCP-1 (Hycult Biotech, USA), rat anti-mouse CX3CL1 (R&D Systems). Membranes were washed 3 times with TBS-Tween and incubated with the appropriate HRP-conjugated secondary antibody for 1 hour at room temperature. Membranes were washed 3 times with TBS-Tween and incubated with chemiluminescent substrate for HRP (Thermo Scientific Super Signal). X-ray films were exposed to membranes to visualize proteins.

Transendothelial Electrical Resistance Measurement

Cells were seeded on collagen-coated transwell polyester membrane inserts (for 24-well plate) with 8 μm pore size (BD Falcon). Transendothelial Electrical Resistance (TEER) was measured using volt ohm meter, EVOMX, equipped with STX100 electrodes (World Precision Instruments, FL USA). TEER readings were recorded 10 minutes before thrombin (5, 20, 60 U/ml) or vehicle treatment and every ten minutes for 1.5 hours after treatments. Cells were maintained on a 37°C heat block for the duration of the TEER measurements. Changes in TEER at the given timepoints were expressed as fold change relative to TEER readings that were recorded 10 minutes before treatment.

FITC-Dextran Permeability Assay

hCMEC/D3 cells were grown to confluence on collagen-coated transwell inserts (for 24-well plate) with 3 μm pore size (BD Falcon). Thrombin at 5, 20, 60 U/ml, or vehicle and 1 mg/ml of FITC-dextran in assay media were added to the apical chamber. The media from the basolateral lower chamber was sampled after 15, 30, 60, 90, and 180 minutes of treatment to assess the degree of FITC-dextran flux across the endothelial barrier. Fluorescence intensity was measured at excitation wavelength 488 nm and at emission wavelength 519 nm using Synergy 4 microplate reader (Biotek, VT U.S.A). The degree of diffused FITC-dextran at each given timepoint was expressed as fold change in fluorescence intensity relative to media from vehicle controls.

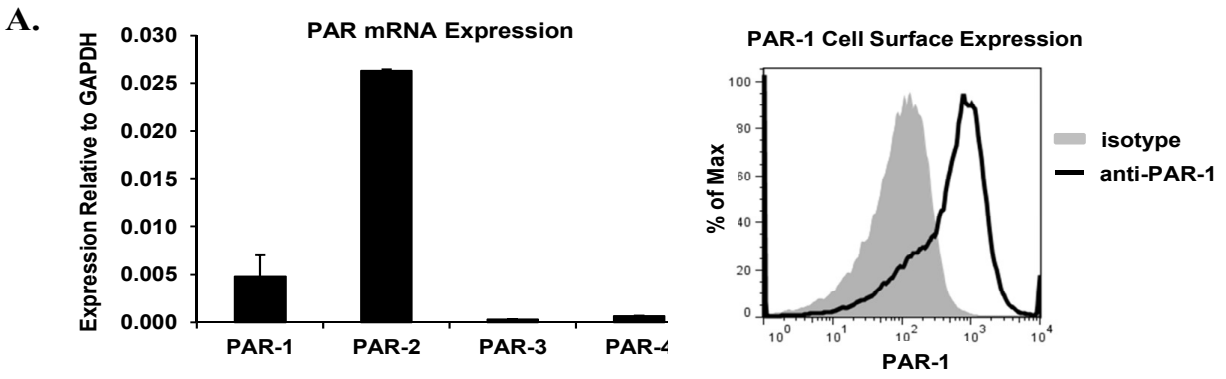
Statistical Analysis

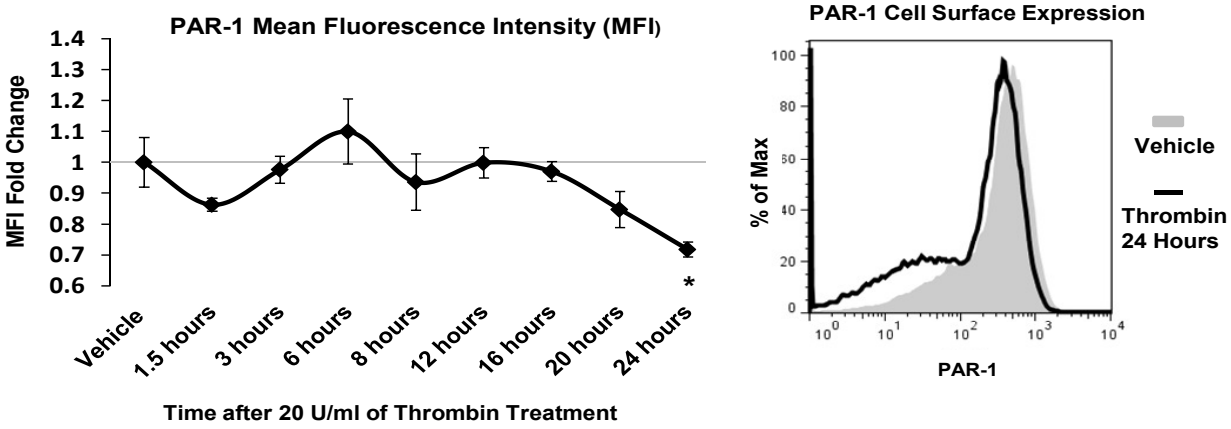
Results were represented as means $\pm$ S.E. All experiments were done in triplicates. Two-tailed student's T-test was used to analyze the differences between two groups using GraphPad Software. A difference was considered statistically significant if the p value is < 0.05.

Results

The hCMEC/D3 cell line expresses the necessary receptors to be responsive to thrombin

To determine whether hCMEC/D3 cells can be responsive to thrombin activity, we examined whether these cells constitutively express the cellular receptors for thrombin. We show by real-time quantitative PCR that hCMEC/D3 cells express all four PARs, three of which (PAR-1, PAR-3, and PAR-4) can be activated by thrombin (Figure 3.1A). PAR-1, the predominant thrombin receptor on endothelial cells [26], is the most highly expressed among all three thrombin receptors in hCMEC/D3 cells. Additionally, we confirmed by flow cytometry analysis that hCMEC/D3 cells express PAR-1 at the cell surface (Figure 3.1A). We also examined whether cell surface expression of PAR-1 is altered in response to thrombin activity. We observed a slight decrease in PAR-1 expression after 1.5 hours of thrombin treatment (Figure 3.1B). At 3 hours, PAR-1 levels returned to baseline and remained close to these levels until 20 - 24 hours of thrombin treatment. After 24 hours of thrombin treatment, we observed a significant decrease in cell surface expression of PAR-1 (Figure 3.1B). These data show that thrombin can affect the expression of PAR-1 on hCMEC/D3 cells and is consistent with previous studies on other endothelial cell systems [27]. We also show that the hCMEC/D3 cell line express thrombomodulin and endothelial protein C receptor (EPCR) (Figure 3.1C). Both receptors are involved in regulating thrombin activity; the former acts as a thrombin regulator through direct interactions with thrombin, while the latter is involved in the activation of protein C, a serine protease known to inhibit thrombin activation [28]. Overall, these data show that the hCMEC/D3 cells express the necessary receptors to respond to thrombin activity.


A.
PAR mRNA Expression
Expression Relative to GAPDH
0.030
0.025
0.020
0.015
0.010
0.005
0.000
PAR-1
PAR-2
PAR-3
PAR-4
PAR-1 Cell Surface Expression
% of Max
PAR-1
isotype
anti-PAR-1


B.
PAR-1 Mean Fluorescence Intensity (MFI)
MFI Fold Change
Vehicle
1.5 hours
3 hours
6 hours
8 hours
12 hours
16 hours
20 hours
24 hours
*
Time after 20 U/ml of Thrombin Treatment
PAR-1 Cell Surface Expression
% of Max
PAR-1
Vehicle
Thrombin
24 Hours

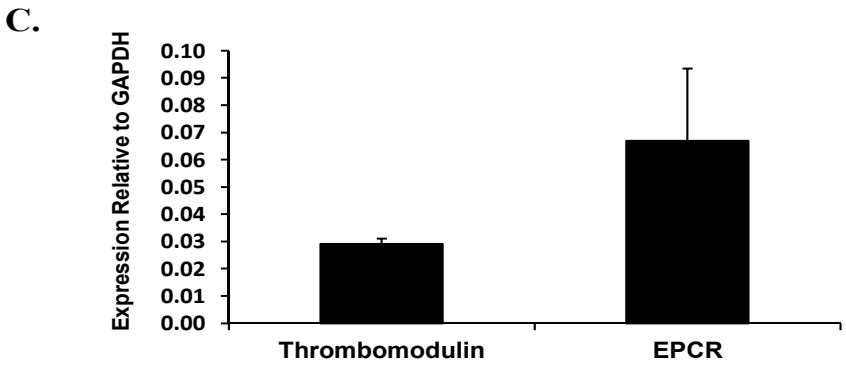

C.
Expression Relative to GAPDH
Thrombomodulin
EPCR

Figure 3.1 hCMEC/D3 cells express the necessary receptors to respond and regulate thrombin activity. A) Real-time quantitative PCR was performed to assess the expression of PARs in hCMEC/D3 cells. The mRNA levels are expressed relative to the internal control gene, GAPDH. Data are represented as means $\pm$ S.E. (n=3). Fluorescence histogram on the right represents hCMEC/D3 cells that were stained with either fluorescently-labeled antibody to PAR-1 or isotype control. Cell fluorescence was assessed by flow cytometry. B) hCMEC/D3 cells were stained with fluorescently-labeled antibody to PAR-1 after treatment with 20 U/ml of thrombin for the timepoints given. Cell fluorescence was examined by flow cytometry, and levels of cell surface PAR-1 at each timepoint was shown as fold change in mean fluorescence intensity relative to vehicle controls. Data represented as means $\pm$ S.E. (n = 3). * $p < 0.05$ versus vehicle control (two-tailed Student's t-test). Fluorescence histogram on the right represents cell surface expression of PAR-1 at 24 hours after vehicle or thrombin treatment. C) The mRNA expression of thrombomodulin and EPCR was examined by real-time quantitative PCR. The mRNA levels are expressed relative to the internal control gene, GAPDH. Data are represented as means $\pm$ S.E. (n=3).

Thrombin induces brain endothelial cells to increase the mRNA and cell surface expression of ICAM-1 and VCAM-1

The BBB can increase the expression of cell adhesion molecules (CAMs) in response to stimulating factors in the microenvironment. The resting BBB typically expresses low levels of CAMs, however in certain conditions such as in neuroinflammatory settings, the BBB upregulates various CAMs to capture inflammatory immune cells and facilitate the entry of leukocytes into the CNS [29]. ICAM-1 and VCAM-1 are believed to have a predominant involvement in leukocyte infiltration into the CNS [29]. We examined whether thrombin is capable of altering the expression of both ICAM-1 and VCAM-1 in human brain endothelial cells. Our data show that thrombin can induce a dose-dependent increase in mRNA expression of both CAMs in hCMEC/D3 cells (Figure 3.2A). The increase in mRNA levels reached statistical significance after treatment with thrombin at 0.5 U/ml, the lowest concentration used, and reached maximal upregulation after treatment with 60 U/ml of thrombin. The increase in adhesion molecule expression was observed after one hour of thrombin treatment and further increase was observed at three hours (Figure 3.2B). The mRNA levels return close to basal levels after six hours of thrombin treatment. We observed a second increase in mRNA levels after 18 hours of treatment, subsequently declining to basal levels at 24 hours (Figure3.2B).

We next examined whether the cell surface levels of both CAMs are similarly increased after thrombin treatment. Flow cytometry analysis shows detectable increase in VCAM-1 cell surface expression after 24 hours of thrombin treatment (Fig 3.2C, left). The up-regulation in cell surface VCAM-1, as demonstrated by increase in mean fluorescence intensity (MFI), is observed after treatment with thrombin concentrations in the range of 0.5 – 10 U/ml (Figure 3.2C, right). Higher concentrations of thrombin (20 – 60 U/ml), however, decreased cell surface

VCAM-1 expression at 24 hours (Figure 3.2C, right). Conversely, we observed maximal increase of ICAM-1 cell surface levels at 24 hours with higher concentrations of thrombin (20-40 U/ml) (Figure 3.2D). We did not observe significant changes in cell surface ICAM-1 after treatment with lower concentrations of thrombin (data not shown). Overall these data demonstrate that thrombin can effectively induce brain endothelial cells to express VCAM-1 and ICAM-1, both at the mRNA and cell surface levels.

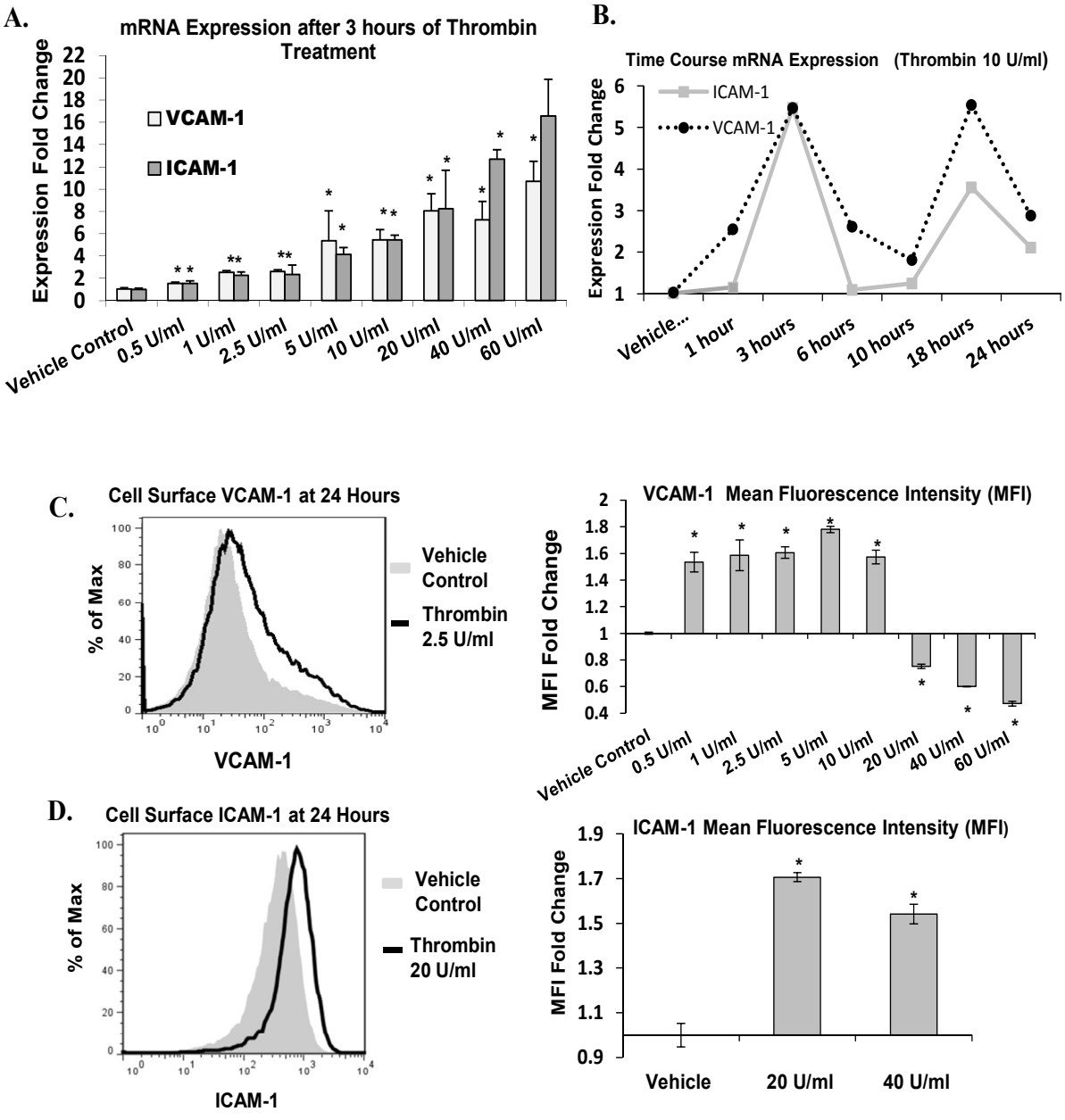

A.
mRNA Expression after 3 hours of Thrombin Treatment
Expression Fold Change
VCAM-1
ICAM-1
Vehicle Control
0.5 U/ml
1 U/ml
2.5 U/ml
5 U/ml
10 U/ml
20 U/ml
40 U/ml
60 U/ml
B.
Time Course mRNA Expression (Thrombin 10 U/ml)
Expression Fold Change
ICAM-1
VCAM-1
Vehicle...
1 hour
3 hours
6 hours
10 hours
18 hours
24 hours
C.
Cell Surface VCAM-1 at 24 Hours
% of Max
Vehicle Control
Thrombin 2.5 U/ml
VCAM-1
VCAM-1 Mean Fluorescence Intensity (MFI)
MFI Fold Change
Vehicle Control
0.5 U/ml
1 U/ml
2.5 U/ml
5 U/ml
10 U/ml
20 U/ml
40 U/ml
60 U/ml
D.
Cell Surface ICAM-1 at 24 Hours
% of Max
Vehicle Control
Thrombin 20 U/ml
ICAM-1
ICAM-1 Mean Fluorescence Intensity (MFI)
MFI Fold Change
Vehicle
20 U/ml
40 U/ml

Figure 3.2 Thrombin upregulates the mRNA and cell surface expression of VCAM-1 and ICAM-1 in human brain endothelial cells. A) hCMEC/D3 cells were activated for 3 hours with thrombin at the given concentrations, and the mRNA expression of VCAM-1 and ICAM-1 were assessed by real-time quantitative PCR. Expression levels are shown as fold change relative to vehicle-treated controls. Data represented as means $\pm$ S.E. (n = 3). * $P < 0.05$, versus vehicle control (two-tailed Student's t-test). B) hCMEC/D3 cells were treated with 10 U/ml of thrombin for the given timepoints. The mRNA expression levels of adhesion molecules at each timepoint were evaluated by real-time quantitative PCR. Mean expression levels are shown as fold change relative to vehicle treated controls for each given time point (n=3). C) and D) hCMEC/D3 cells were stained with fluorescently-labeled antibodies to VCAM-1 and ICAM-1 after treatment with the given concentrations of thrombin for 24 hours. Cells were analyzed by flow cytometry, and changes in cell surface expression of VCAM-1 and ICAM-1 were assessed by comparing mean fluorescence intensity (MFI) of thrombin-treated samples to vehicle-treated controls. Fluorescence histograms for VCAM-1 and ICAM-1 are shown on the left. Bar graphs on the right show fold change in MFI relative to vehicle treated controls. Data represented as means $\pm$ S.E. (VCAM-1 n = 3, ICAM-1 n = 3). * $P < 0.05$ versus vehicle control (two-tailed Student's t-test).

Thrombin induces brain endothelial cells to increase the expression of chemokines

Under pathological conditions, the endothelial cells that form the BBB can synthesize chemokines to recruit leukocytes to the site of CNS inflammation [30]. In addition to their chemotactic functions, chemokines secreted by endothelial cells can enhance leukocyte firm adhesion to CAMs on the endothelium, thus directly facilitating the subsequent diapedesis of leukocytes to the CNS parenchyma [29]. We examined whether thrombin can induce CNS endothelial cells to express chemokines that are known to be involved in various neuroinflammatory conditions. We examined the expression of CXC chemokines, a family of chemokines known to be primarily chemotactic for neutrophils. In particular, we looked at the mRNA expression of CXCL1 (GRO-alpha), CXCL2 (GRO-beta), CXCL8 (IL-8), and CXCL10 (IP-10); all have been reportedly involved in various neuropathologies [31, 32]. Thrombin activity significantly resulted in increased mRNA expression of all four CXC chemokines (Figure 3.3A). CXCL8 a strong neutrophil chemoattractant, with likely involvement in neurological conditions like MS, ischemic brain injury and traumatic brain injury [33], was the most highly upregulated with maximal mRNA upregulation reaching 40 fold. Western blot analysis confirms a time-dependent increase in soluble CXCL8 in the supernatant of thrombin treated hCMEC/D3 cells (Figure 3.3B).

We also examined the mRNA expression of selected members of the CC chemokine family which have been recognized to play roles in neuroinflamatory diseases; these include CCL2 (MCP-1), CCL3 (MIP-α), and CCL5 (RANTES) [30]. Thrombin can induce a robust dose-dependent increase in mRNA levels of CCL2 (Figure 3.3C), a potent chemoattractant for monocytes and activated T cells [33]. CCL2 mRNA expression was increased by 15.2 fold after treatment with 60 U/ml of thrombin. Additionally, soluble CCL2 was also increased over time

in the supernatant of thrombin treated hCMEC/D3 cells (Figure 3.3D). Thrombin treatment, however, did not significantly affect the mRNA expression of either CCL3 or CCL5 (Figure 3.3C).

We next examined the CX3C chemokine group, which is comprised of CX3CL1 (fractalkine). CX3CL1 is a unique chemokine in that it is synthesized as a membrane-bound molecule allowing it to directly capture circulating leukocytes. It can also be cleaved from the cell membrane by metalloprotenaises to generate a soluble form that can function as a traditional chemoattractant [34]. CX3CL1 has similarly been linked to the pathogenesis of several neuroinflammatory diseases, including Parkinson's disease and experimental autoimmune encephalomyelitis (EAE) [35]. Our data show that thrombin activity can dose-dependently induce brain endothelial cells to significantly upregulate the mRNA expression of CX3CL1 (Figure 3.3E). The highest upregulation was observed at 9.3 fold relative to vehicle control. We also show that CX3CL1 protein expression was increased by thrombin in whole-cell lysates of hCMEC/D3 cells (Figure 3.3F).

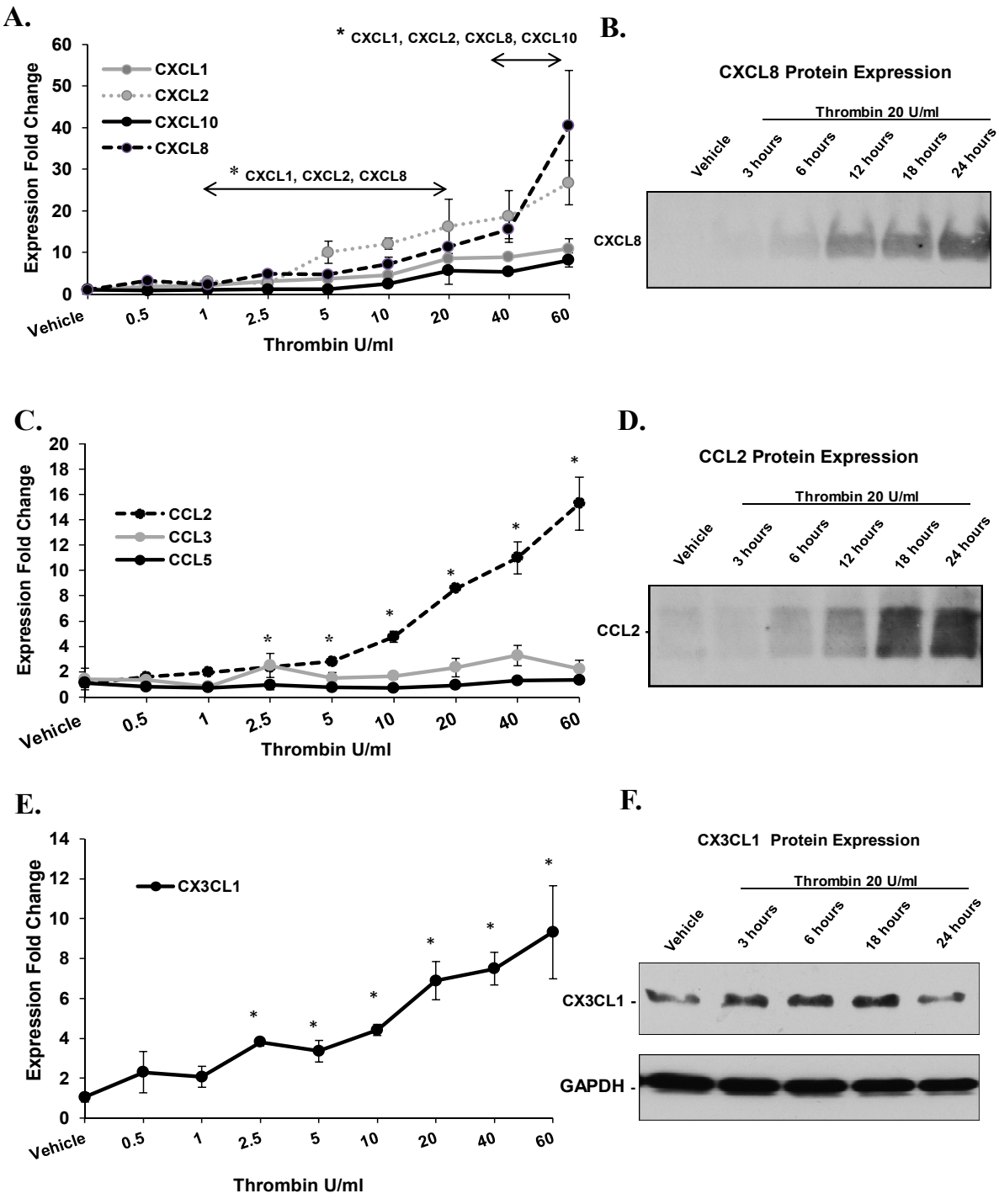

A.
B.
C.
D.
E.
F.
Expression Fold Change
Thrombin U/ml
Vehicle
0.5
1
2.5
5
10
20
40
60
CXCL1
CXCL2
CXCL10
CXCL8
* CXCL1, CXCL2, CXCL8, CXCL10
* CXCL1, CXCL2, CXCL8
CXCL8 Protein Expression
Thrombin 20 U/ml
3 hours
6 hours
12 hours
18 hours
24 hours
CCL2
CCL3
CCL5
CCL2 Protein Expression
CX3CL1
CX3CL1 Protein Expression
GAPDH

Figure 3.3 Thrombin induces the expression of neuroinflammatory-implicated chemokines in human brain endothelial cells. hCMEC/D3 cells were activated with the given concentrations of thrombin for three hours, and the mRNA levels of selected chemokines from A) CCL, C) CXC, and E) CX3C chemokine families were assessed by real time quantitative PCR. The mRNA levels are expressed as fold change relative to vehicle treated controls. Data represented as means $\pm$ S.E. (n = 3). *P < 0.05 versus vehicle controls (two-tailed Student's t-test). Arrows span the concentrations in which the chemokines indicated have been significantly upregulated. B) and D) The supernatant of thrombin-treated hCMEC/D3 cells at the given timepoints were examined by western blot for soluble CXCL8 or soluble CCL2 . F) The protein expression of CX3CL1 in whole-cell lysates of thrombin-treated hCMEC/D3 cells was assessed by western blot.

Thrombin increases the paracellular permeability of brain endothelial cells.

One of the more drastic changes in BBB functionality in response to factors in the microenvironment is the increased permeability to cells and compounds in the circulation [1]. Increase in BBB permeability is a consequence of various processes in endothelial cells which include cytoskeletal rearrangements and disassembly of junctional proteins, ultimately resulting in the formation of spaces in between endothelial cells to allow for paracellular diapedesis of leukocytes and paracellular flux of blood-borne molecules [29]. We also examined if thrombin activity can directly affect the permeability of the human BBB *in vitro*. We assessed permeability changes by measuring the transendothelial electrical resistance (TEER) of hCMEC/D3 cells after thrombin treatment. The cells were seeded in transwell inserts and treated with vehicle or thrombin at 5, 20, or 60 U/ml. TEER was measured over time before and after treatment; a decrease in TEER correlates with increase in barrier permability. Thrombin at all three concentrations resulted in detectable decrease in TEER of hCMECD/3 cells within 30 minutes after treatment (Figure 3.4A). Maximal and statistically significant TEER decrease was observed after 1 hour of treatment at all three concentrations. We also observed that the decrease in TEER was not sustained; as TEER started to increase 70 minutes after treatment (Fig 3.4A).

The paracellular permeability of thrombin-treated hCMEC/D3 cells was further investigated by assessing the paracellular flux of 10 kDa FITC-Dextran at various timepoints after treatment with thrombin. We observed statistically significant increases in dextran permeability 15 minutes after thrombin treatment at 5, 20, 60 U/ml (Figure 3.4B). Maximal permeability was observed after 60-90 minutes of treatment. We did not observe continued increase in dextran permeability at 3 hours (180 minutes) (Figure 3.4B), suggesting that similar

to what was observed with TEER measurement, the increased paracellular permeability to dextran molecules was not sustained for prolonged periods.

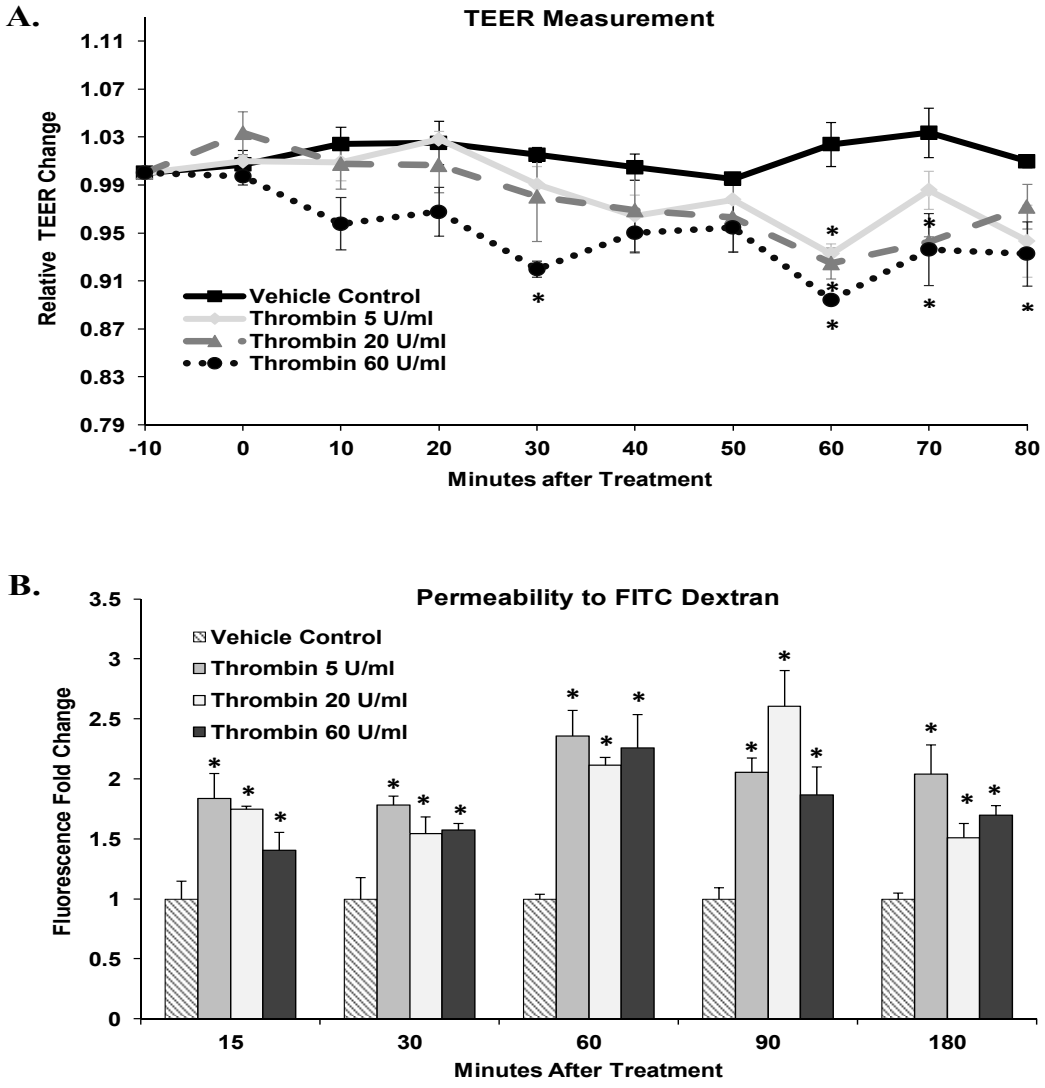


Figure 3.4. Thrombin increases the paracellular permeability of human brain endothelial cells. A) hCMEC/D3 cells were seeded on transwell inserts and treated with vehicle or thrombin at 5, 20, or 60 U/ml. Permeability was assessed by measuring TEER changes every 10 minutes after treatment. TEER changes at each given timepoint are shown as fold change relative to TEER readings recorded 10 minutes prior to treatment. Data represented as means $\pm$ S.E. (n = 3). *P < 0.05 versus vehicle control at each timepoint (two-tailed Student's t test). B) hCMEC/D3 cells were seeded in transwell inserts; 10 KDa FITC-dextran and thrombin at the concentrations shown were added to the apical chamber. After treatment, media from the lower chamber were sampled at the timepoints given to assess the degree of FITC-dextran paracellular flux. Fluorescence intensity in the basolateral media is expressed as fold change relative to vehicle control for each respective timepoint. Data represented as means $\pm$ S.E. (n = 3) * P < 0.05 versus vehicle control at each timepoint (two-tailed Student's t test).

Discussion

There are growing reports that thrombin, the central enzyme in the coagulation process, is increased in the CNS during various neuroinflammatory and neurodegenerative disorders [11, 12]. In this study, we investigated whether increased thrombin presence in the CNS can potentially affect the phenotype and functionality of the BBB. We utilized the hCMEC/D3 cell line, a recently-developed model of the human BBB [25], to assess the collective effects of thrombin on brain endothelial cells. We demonstrate that thrombin can induce the expression of chemokines and CAMs in hCMEC/D3 cells. These results corroborate previous studies that have been conducted on peripheral endothelial cells [36, 37]. There is, however, an apparent difference in the degree of thrombin responsiveness in human brain endothelial cells compared to what has been reported on peripheral endothelial cells. A study conducted on human umbilical vein endothelial cells (HUVECS) reported seeing robust mRNA upregulation of ICAM-1 (16.0 fold), and VCAM-1 (12.2 fold) after treatment with a relatively low dose of thrombin (2 U/ml for four hours) [37]. In comparison, our study showed that hCMEC/D3 cells reached a comparable level of mRNA upregulation after treatment with 60 U/ml of thrombin. The upregulation in ICAM-1 and VCAM-1 cell surface levels were similarly reported to be more robust in thrombin-treated HUVECS [36] compared to what we observed in hCMEC/D3 cells. It has been proposed that cells detect and respond to differences in thrombin concentrations based on the rates of receptor activation [38]. The observation that HUVECS are more responsive that hCMEC/D3 cells to thrombin at low concentrations suggests that thrombin receptors on HUVECS are likely being activated at a faster rate than on hCMEC/D3 cells. Any differences in the expression levels of thrombin receptors on the two cell systems may likely affect the rate of receptor activation. Moreover, thrombomodulin, a cell surface glycoprotein expressed on

endothelial cells, including hCMEC/D3 cells (Figure1C) and HUVECS [39], is known to neutralize the pro-coagulant and pro-inflammatory activities of thrombin. Differences in thrombomodulin cell surface levels on hCMEC/D3 cells and HUVECS may also affect the activation rate of thrombin receptors on both cell types. It should be noted, however, that while hCMEC/D3 cells may be less responsive than HUVECS to thrombin activity, the thrombin-induced cell surface levels of VCAM-1 (1.8 fold) and ICAM-1 (1.7 fold) on hCMEC/D3 are levels that have been shown to be biologically functional. Recent studies by Roh et al. [40] and Rains et al. [41] show that a 1.25 to 2 fold increase in ICAM-1 expression on endothelial cells resulted in increased monocyte adhesion. Another group [42] showed that a 2 fold increase in VCAM-1 cell surface levels is likewise sufficient to increase monocyte adhesion.

While HUVECS and hCMEC/D3 may differ in the degree of thrombin responsiveness, our data showed that the two cell systems likely have similar mechanisms for clearance of activated thrombin receptors and re-expression of new intact receptors. In HUVECS, activation of thrombin receptors results in rapid internalization of receptors, resulting in 60% clearance of cleaved receptors within 10 minutes [27]. Moreover, HUVECS have intracellular pools of new thrombin receptors, allowing for immediate re-expression of new receptors on the cell surface [27]. HUVECS can restore nearly a full set of new receptors within a few hours after the initial thrombin receptor activation and clearance [27]. In hCMEC/D3 cells, PAR-1 surface expression decreased after treatment with thrombin for 1.5 hours, suggesting that similar to HUVECS, hCMEC/D3 likely internalize thrombin receptors immediately after activation. We also observed that PAR-1 cell surface expression returned to basal levels within three hours of thrombin activation, suggesting that hCMEC/D3 cells like HUVECS have an existing intracellular pool of new receptors that can immediately replace activated and internalized

receptors. Treatment of hCMEC/D3 cells for various timepoints longer than 3 hours did not significantly alter cell surface levels of PAR-1, likely due to the continued process of receptor internalization and receptor re-expression. We did eventually observe a significant decrease in PAR-1 expression after 24 hours of continued thrombin treatment, which may suggest that, at this point, the intracellular reserve of intact receptors have been depleted.

We investigated whether thrombin can affect the paracellular permeability of brain endothelial cells. For the endothelium to be permissive to paracellular diapedesis, endothelial cells must undergo various processes which include cytoskeletal rearrangements and disassembly of junctional proteins, ultimately resulting in the formation of spaces in between endothelial cells to allow for paracellular transmigration [43]. We assessed if thrombin can affect the paracellular permeability of brain endothelial cells by measuring TEER changes and permeability to dextran molecules after thrombin treatment. We observed that thrombin can rapidly but transiently increase the paracellular permeability of brain endothelial cells. These observations are consistent with previous studies on peripheral endothelial cells [44]. Increases in endothelial permeability in response to thrombin activity have been attributed to the disassembly and disappearance of junctional proteins at cell to cell contacts [43]. The thrombin-induced retraction of junctional proteins, however, is not accompanied by protein degradation, and thus can be rapidly reversible [44]. This is the likely mechanism behind the transient permeability observed in thrombin-treated hCMEC/D3 cells.

Thrombin's effects on endothelial permeability and CAM expression are known to be mediated through different signaling pathways and therefore subject to different regulatory mechanisms [26, 43]. This may explain why thrombin at certain concentrations can have opposing effects on CAM expression and paracellular permeability. For instance, we observed

that thrombin at 60 U/ml rapidly resulted in increased permeability in hCMEC/D3 cells, but this same concentration decreased the expression of cell surface VCAM-1 at 24 hours (Figure 2C right). Thrombin induction of VCAM-1 expression is mediated through the NF-κB signaling pathway, specifically the p65 subunit of NF-κB [26]. However, it has also been observed that thrombin can induce the p50 subunit of NF-κB, which has been suggested to be a negative transcriptional regulator of the VCAM-1 gene [26]. Thus, it has been proposed that there is a possible negative-feedback mechanism in the thrombin-VCAM-1 signaling pathway [26]. The observed downregulation of VCAM-1 at higher concentrations of thrombin is likely due to this negative feedback mechanism.

The overall effect of thrombin on brain endothelial cells *in vitro* results in an endothelium phenotype that is conducive to the multiple stages of leukocyte extravasation. The question remains whether thrombin can have the same effects on the BBB *in vivo*. Previous studies have shown that intracerebroventricular injection of thrombin into rat brains resulted in increased brain edema [45], suggesting that thrombin is capable of affecting the functionality of the BBB *in vivo.* It remains to be seen, however, if *in vivo* thrombin activity can result in increased leukocyte extravasation into the CNS. Furthermore, the source of endogenous thrombin that can affect the BBB during neurological diseases also needs to be considered. While thrombin is predominantly found in the circulation, thrombin can be locally synthesized in the CNS [14]. Moreover, thrombin activity and/or expression have been shown to be increased in the CNS in a number of neurological diseases [12]. The fact that intracerebroventricularly-injected thrombin in rat brains can affect BBB permeability [24], suggests that thrombin from within the CNS is capable of affecting BBB function. Thus, it is possible for thrombin synthesized within the CNS to induce the phenotypic changes in brain endothelial cells that have been shown in this current

study. Additionally, it also needs to be taken into account that thrombin can induce glial cells to produce pro-inflammatory cytokines such as IL-1β and TNF-α [13], which are cytokines that have been traditionally implicated in BBB dysfunction [46]. While we have shown in this study that thrombin can independently affect the functionality of the BBB, it is very likely that thrombin can act in concert with various pro-inflammatory factors during neurological conditions to affect BBB functionality. In fact, it has been observed that thrombin and TNF-α can synergistically affect endothelial barrier function *in vitro* [47].

In summary, this study shows that thrombin can induce brain endothelial cells to express chemokines and CAMs, which can potentially augment the recruitment and capture of leukocytes from the circulation. In addition, thrombin can increase the paracellular permeability of brain endothelial cells, resulting in a BBB that is more permeable to leukocyte diapedesis. Therefore, thrombin can modify the functionality of brain endothelial cells *in vitro*, resulting in a BBB phenotype that can likely facilitate the successive stages of leukocyte extravasation. Overall, this current study lends better understanding to the implications of increased thrombin presence in the CNS on the pathology of neurological conditions.

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CHAPTER 4

Summary, Conclusions, and Future Directions

Previous studies have demonstrated that coagulation components are effective regulators of the immune response [1-3]. Ongoing interest lies in determining the influence of coagulation factors on the pathogenesis of specific disease conditions, which may potentially lead to the formulation of novel therapeutic strategies. There is already a well-known involvement of coagulation components, specifically the anticoagulant, APC, on the systemic inflammatory condition, sepsis [4], leading to the development of a novel, albeit controversial, sepsis treatment [5, 6]. Moreover, there are convincing studies linking coagulation factors to other disease conditions [4, 7]. Strong indications suggest the activation of the coagulation system during disease progression in several neuroinflammatory conditions, including MS and EAE [7-9]. The specific mechanisms on how coagulation components affect MS and EAE progression, however, are still unclear. In this dissertation, we described the influence of endogenous APC on disease development and pathogenesis of EAE, the animal model for MS. We demonstrated that modulating APC levels in the circulation affected disease pathogenesis at multiple fronts, increasing BBB permeability but also concurrently altering the functional responses of leukocyte populations. Particularly, the effector functions of $CD4^+$ T-cells are diminished, consequently resulting in attenuated disease progression. We additionally described the direct inflammatory effects of the coagulation factor, thrombin, on an *in vitro* model of the human BBB. Our study exhibited that thrombin effectively modified the BBB, resulting in a phenotype that is conducive to leukocyte recruitment and extravasation. BBB permissiveness to leukocyte extravasation is one of the major pathological components of MS and EAE [10-12]; this particular study, therefore, pinpoints thrombin's potential influence on the progression of these neuroinflammatory diseases. Overall, the findings described in this dissertation unequivocally demonstrate the capacity of coagulation factors in effectively influencing pathogenic components

of EAE and MS, and further affirms that the dynamic interactions between the coagulation and immune systems can affect disease pathogenesis and progression.

We inhibited APC activity in the circulation during EAE through administration of an antibody that blocks the activation of PC and prevents the engagement of APC with its cellular receptors [13]. Abrogation of APC activity attenuated EAE severity, and we attributed the attenuation of disease to the increased expansion and activation of $CD11b^+$ myeloid cells, particularly the suppressive MDSC subset, which was capable of suppressing the responses of effector T-cells required for disease progression. Inhibition of APC during EAE, additionally, resulted in increased BBB permeability, as evidenced by pronounced leukocyte infiltration in the brains of anti-PC mice. However, despite the considerable presence of cellular infiltrates in the brains of anti-PC mice, we observed decreased inflammatory and pathogenic conditions in the CNS of these mice. We notably observed that the pathogenic cytokines produced by encephalitogenic $CD4^+$ T-cell subsets are decreased in the CNS; whereas the frequency of the anti-inflammatory T-reg subset is increased, both in the CNS and in the periphery of anti-PC mice. We hypothesize that the decrease in encephalitogenic CD4+ T-cells and the increase of the T-reg subset is a result of the expansion of $CD11b^+$ MDSCs in anti-PC mice. This was directly confirmed by demonstrating that the co-culture of isolated $CD11b^+$ cells from anti-PC mice resulted in the suppression of MOG_{35-55}-induced $CD4^+$ T-cell proliferation and additionally resulted in the increase of T-reg frequency. Overall, we conclude that inhibition of APC during EAE affected disease pathogenesis at multiple fronts, specifically increasing BBB permeability and concurrently modulating leukocyte frequency and functional responses, resulting in an increased and more activated $CD11b^+$ myeloid population, including the suppressive MDSC

subset, which subsequently suppressed effector T-cells required for disease progression. Therefore, the net effect of APC inhibition during EAE is attenuated disease progression.

We speculate that the increase of CD11b+ myeloid cells in anti-PC during EAE progression is a direct consequence of APC inhibition. This hypothesis is based on a recent study demonstrating that APC is an effective suppressor of the pro-inflammatory responses of $CD11b^+$ myeloid cells through direct engagement with the CD11b integrin [14]. Thus, it is likely that abrogated APC activity in the EAE inflammatory setting can result in an increased expansion and activation of the $CD11b^+$ myeloid population, including the suppressive, $CD11b^+$ MDSC subset. MDSCs are observed to expand in various inflammatory and pathogenic settings [15-17], and the expansion and suppressive capabilities of these cells have been demonstrated to be mediated by Stat3 [18, 19]. We hypothesize that APC acts as a direct negative regulator of MDSCs, thus decreased APC activity can result in the greater expansion and activation of MDSC in inflammatory settings. This hypothesis is supported in a study by Cao et. al, wherein it was demonstrated that APC can specifically downregulate the expression of Stat3 in $CD11b^+$ myeloid cells through interactions with the CD11b integrin and subsequent PAR-1 activation [14]. Moreover, we have shown that APC directly binds MDSCs, and we have confirmed that MDSCs express both CD11b and PAR-1, suggesting that these cells are potentially responsive to APC regulation. However, we have yet to demonstrate that APC can directly regulate the cellular processes of MDSCs, and we, therefore, want to confirm this in a future study. We specifically want to determine if APC can inhibit the expression and/or activation of Stat3 in MDSCs; this will directly implicate APC in the regulation of MDSC expansion. The suppressive capability of MDSCs on T-cells is mediated through one or a combination of Arg I activity, iNOS expression, and ROS generation. We observed elevated levels of all three factors in

MDSCs as a result of APC inhibition. In a future study, we, therefore, also want to confirm whether APC can directly inhibit Arg I activity and the generation of NO and ROS in MDSCs. One specific experiment we want to carry out is to isolate CD11b+ cells from anti-PC mice, and assess whether treatment with exogenous APC results in the decrease of Stat3 expression and/or Stat3 activation, as well as inhibition of Arg I activity, iNOS expression, and ROS generation.

We have attributed the attenuation of EAE severity in anti-PC mice to a defective and deficient effector $CD4^+$ T-cell population as a result of increased expansion of the suppressive MDSC subset. We observed significantly decreased $CD4^+$ T-cells in the periphery of anti-PC mice compared to controls, and we additionally observed lowered cell surface CD3 expression, which may potentially have resulted in an overall lowered T-cell activation. Consistently, we observed that there is decreased pathogenic cytokines associated with encephalotogenic CD4+ T-cell subsets in the CNS of anti-PC mice. Furthermore, we detected significantly increased frequency of T-regs both in the CNS and in the periphery in anti-PC mice, likely contributing to attenuated EAE pathogenesis. We postulated that the deficient and defective $CD4^+$ T-cell subset is a result of the increased and more activated MDSCs, which are potent T-cell suppressors. The production of Arg I, NO, and/or ROS by MDSCs, all of which are increased in MDSCs in anti-PC mice, suppresses antigen-induced proliferation of T-cells [17, 20-23]. Arg I and ROS are additionally known to downregulate the expression of CD3ζ, resulting in the downregulation of the T-cell receptor complex (TCR) and lowered T-cell activation [21]. Moreover, MDSCs have also been directly implicated in the expansion of T-regs [24, 25]. Since EAE progression and disease pathogenesis are primarily mediated by $CD4^+$ T-cells [26, 27], we hypothesize that the decreased and defective effector $CD4^+$ T-cell population, coupled with the shift towards an increase in the T-reg subset is the primary cause for the attenuated EAE severity observed in

anti-PC mice. To confirm whether the $CD4^+$ T-cell population from anti-PC mice are incapable of mediating EAE progression and are thus responsible for the decreased EAE severity in anti-PC mice, a future experiment that we want to carry out is to isolate $CD4^+$ T-cells from anti-PC and control mice and adoptively transfer these cells into recipient mice that lack endogenous $CD4^+$ T-cells. We can then monitor and assess EAE progression in the recipient mice that received $CD4^+$ T-cells from either donor groups. If the $CD4^+$ T-cells from the donor anti-PC mice are inherently defective in inducing EAE, we expect that the recipient mice that received these cells will develop less severe EAE relative to recipient mice that received $CD4^+$ T-cells from control mice.

We strategically administered anti-PC antibody on days 0, 2, 4, and 6 post EAE induction to inhibit APC during the induction phase of EAE, which encompasses the peripheral activation of leukocytes after MOG_{35-55} immunization. Administration of anti-PC antibody within this timeframe also abrogates APC activity at the outset of the effector phase of EAE, which is characterized by leukocyte infiltration into the CNS, typically starting at around day 5 post EAE induction [28]. Thus, we inhibited APC during two phases of EAE progression, and consistently the effects of APC inhibition was observed in both stages of EAE. Specifically, we hypothesize that APC inhibition during the induction stage of EAE led to the increase in MDSCs and the consequent suppression of T-cell responses. We also postulate that inhibition of APC resulted in increased BBB permeability, leading to the pronounced leukocyte infiltration in the brain as EAE proceeded to the effector stage of the disease. We believe that these multiple effects of APC inhibition on various components of EAE pathogenesis have conflicting influences on the overall EAE progression, but, ultimately, the net effect is attenuated EAE. In a future study, we want to alter the timeframe of APC inhibition to determine if abrogation of APC activity during different

stages of EAE progression can have varying effects on EAE pathogenesis. An alternate anti-PC treatment, for instance, is to administer the antibody during the peak of the effector phase of EAE (days 12 – 18 post EAE induction). Treatment with anti-PC within this timeframe will inhibit APC activity specifically during the effector phase, while the influence of APC inhibition on the induction stage of EAE is minimized. Therefore, the complexity of abrogating APC during multiple stages of EAE, which resulted in the conflicting influences on disease progression is eschewed. We anticipate that limiting APC activity within the effector stage of EAE would predominantly increase the infiltration of leukocytes in the brain, as a consequence of increased BBB permeability and dysfunction, thus leading to increased EAE severity.

The increased BBB permeability observed in anti-PC mice as a result of APC inhibition is consistent with the well-established cytoprotective effects of APC on endothelial cells [29, 30]. APC is known to confer vascular barrier protection resulting in decreased leukocyte extravasation into tissues during pathological settings [31, 32]. APC mediates cell signaling in endothelial cells through interactions with EPCR and PAR-1 expressed on endothelial cells, resulting in an overall enhancement of vascular barrier protection through increased expression of endothelial junctional proteins that mediate the connectivity of endothelial cells [4, 29, 33, 34]. Moreover, APC signaling is also known to induce endothelial cells to downregulate cellular adhesion molecules, which facilitate the rolling, firm adhesion and the subsequent transendothelial migration of leukocytes [35]. It should also be noted that APC's protective effects on vascular barriers have been observed in several disease conditions and pathological settings [4, 35]. BBB dysfunction is one of the hallmark pathogenesis of EAE and MS, which is induced in EAE by pertussis toxin (PTX) administration combined with increased production of pro-inflammatory factors both in the CNS and systemically [10, 11, 27, 36, 37]. We postulate

that decreased APC in the circulation of anti-PC mice further increases the susceptibility of the BBB to the effects of PTX and inflammatory factors, thus resulting in increased permissiveness to leukocyte extravasation, potentially even to non-encephatologenic and naive leukocyte populations, which would explain the minimized pathogenic conditions in the CNS of anti-PC mice despite the pronounced presence of leukocyte infiltrates. In a future study, we want to assess the phenotype of the BBB in anti-PC mice. We specifically want to determine if APC inhibition during EAE can alter the BBB's expression of adhesion molecules and endothelial junctional proteins.

The primary function of APC as an anti-coagulant is to inhibit the further production of thrombin, which serves as the central enzyme of the coagulation cascade [38]. Therefore, it is probable that inhibition of APC during EAE progression not only modulated the immune response but also potentially affected the coagulation response. Several studies have detected the elevation of thrombin activation in the course of EAE progression [8, 9]. With APC inhibition, it is likely that thrombin is further elevated during EAE. We want to confirm in a future study whether there is increased thrombin in the circulation of anti-PC mice compared to controls during EAE.

If thrombin is increased in anti-PC mice as a result of APC inhibition, it is likely that the increase in thrombin activity can be a contributing factor in the pathogenesis that we have observed in anti-PC mice, notably the increase in BBB permeability. APC and thrombin are functionally opposite, both in their respective roles in the coagulation process and their influence on the immune response [38]. Their effects on vascular barrier permeability are polar opposites; thrombin activity results in increased vascular barrier disruption while APC enhances vascular barrier stability [29, 39]. We hypothesize that elevation of thrombin in EAE can contribute to

BBB dysfunction in EAE, and potentially in MS. In fact, we have demonstrated that thrombin effectively induces an inflammatory phenotype in an *in vitro* human BBB. Thrombin treatment of human brain endothelial cells resulted in significantly increased expression of cell adhesion molecules, ICAM-1 and VCAM-1. Both adhesion molecules have been implicated in leukocyte transmigration across the BBB in EAE and MS [40, 41]. We observed significantly increased mRNA expression of both adhesion molecules in brain endothelial cells even at low thrombin treatment (0.5 – 5 U/ml), suggesting that the BBB is highly responsive to thrombin activation. Thrombin additionally induced brain endothelial cells to secrete various chemokines from different chemokine families. A number of these chemokines have been implicated in EAE and MS pathology; notably the neutrophil chemoattractant, CXCL8, which has been detected in the CSF of MS patients, as well as CCL2, a potent chemoattractant for monocytes and have been suggested to be involved in EAE progression [42]. Furthermore, we observed that thrombin effectively increased the paracellular permeability of the BBB, evidenced by lowered transendothelial electrical resistance (TEER), and increased permeability to dextran molecules. Overall, the collective effects of thrombin on the BBB resulted in the induction of an inflammatory phenotype that can potentially facilitate the recruitment, firm adhesion, and diapedesis of leukocytes. In neuroinflammatory settings, such as in EAE or MS, we postulate that elevated thrombin levels in the systemic circulation can be a contributing factor to leukocyte extravasation into the CNS. As previously mentioned, several studies have detected that thrombin is elevated in the course of EAE progression, and our study has provided a potential mechanism through which thrombin can influence EAE pathogenesis.

Overall, we have shown that APC and thrombin, the major factors of the coagulation system, are effective modulators of EAE pathogenesis. We have demonstrated that thrombin

can directly regulate the BBB phenotype, which plays a prominent role in EAE and MS pathology. Additionally we have shown that alteration of APC levels in the circulation during EAE progression affects BBB permeability as well as the functional responses of leukocytes, demonstrating that APC can modulate EAE pathogenesis at multiple fronts. It should be noted that inhibition of APC, generally known as anti-inflammatory molecule, unexpectedly resulted in alleviated EAE, further highlighting the complexity of APC's multi-leveled influence on inflammatory conditions. Therefore, it has to be taken into account that while the broad-ranging influence of APC and thrombin on pathological conditions makes them attractive targets in therapeutic avenues, our study shows that it is the pleiotropic nature of these coagulation components that also makes it difficult to accurately extrapolate their global effects in each unique disease setting. In assessing the potential of thrombin and APC as therapeutic targets, their multi-faceted influence on various areas of the pathogenic setting need to be carefully considered; keeping in mind that each of thrombin's and APC's effects on individual components of the disease pathogenesis may have separate and, at times, opposing influence on the overall disease progression.

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APPENDIX

APPENDIX-A

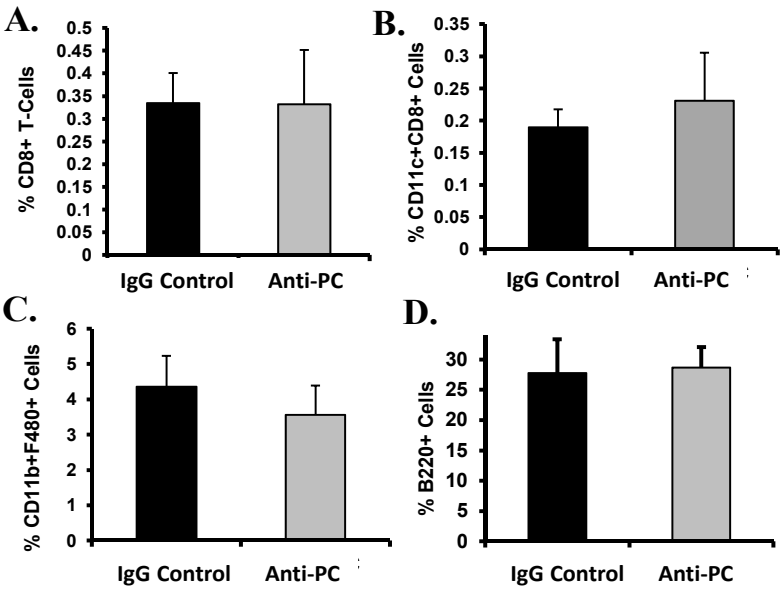
Percentage of anti-PC mice with considerable infiltrates in brain despite attenuated EAE

Appendix-A. Anti-PC mice exhibit considerable infiltration in the brain despite attenuated symptoms.

	IgG Control	Anti-PC
Severe CNS infiltration with clinical symptoms (Day 14-17)	62.5 %	16.6 %
Severe CNS infiltration with no clinical symptoms (Day 14-17)	0 %	33.3 %
No CNS infiltration with no clinical symptoms (Day 14 – 17)	37.5 %	50 %

APPENDIX-B

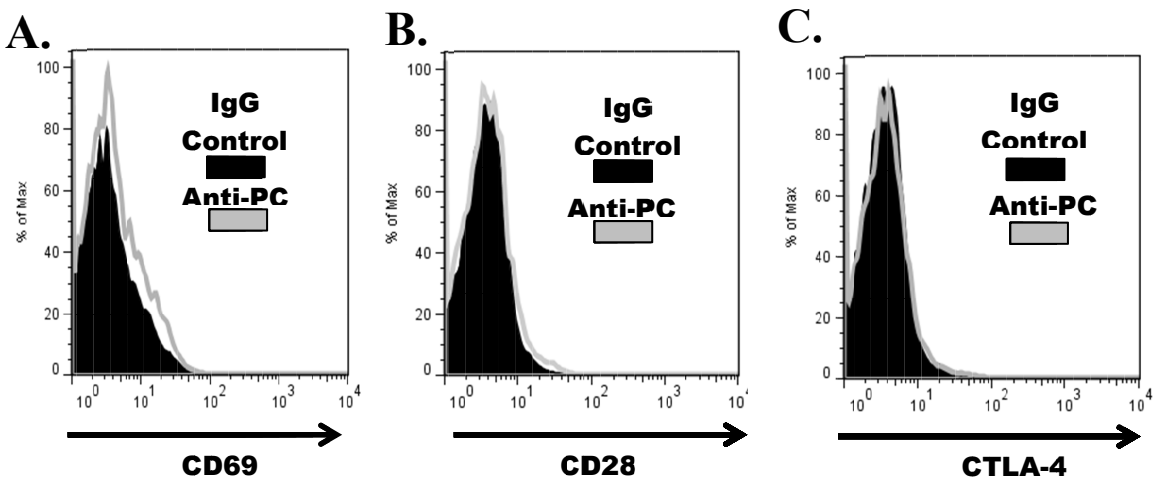
Frequency of cellular infiltrates in the brains of anti-PC and control mice



Appendix-B. Anti-PC and IgG Control mice exhibit comparable frequency in various infiltrating leukocyte populations in the CNS. Cellular infiltrates from the brains of anti-PC and control mice were isolated by percoll gradient, and cells were subjected to flow cytometry to assess the frequency of A) CD48+ T-cells, B) CD8+ CD11c+ cells, C) CD11b+F480+ cells, and D) B220+ cells. Data are represented as means $\pm$ SEM.

APPENDIX-C

Expression of various cell surface markers on splenic $CD4^+$ T-cells from control and anti-PC mice



Appendix-C. No difference in the expression of various cell surface markers on splenic CD4+ T-cells in anti-PC and control mice. CD4+ T-cells in the periphery were examined for the expression of A) CD69, B) CD28, and C) CTLA-4 by flow cytometry. Expression levels are represented as histograms.