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Inhibition of Endogenous Activated Protein C Attenuates Experimental Autoimmune Encephalomyelitis by Inducing Myeloid-Derived Suppressor Cells

Leah M. Alabanza,* Naomi L. Esmon,†,‡ Charles T. Esmon,†,‡,§,∥ and Margaret S. Bynoe*

Activated protein C (PC) is an anticoagulant involved in the interactions between the coagulation and immune systems. Activated PC has broad anti-inflammatory effects that are mediated through its ability to modulate leukocyte function and confer vascular barrier protection. We investigated the influence of activated PC on the pathogenesis of experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis. We modulated activated PC levels in the circulation during EAE induction through systemic administration of a mAb against PC/activated PC (anti-PC). We initially hypothesized that inhibition of activated PC may result in a heightened inflammatory environment, leading to increased EAE pathogenesis. Contrary to this hypothesis, mice treated with anti-PC Ab (anti-PC mice) exhibited attenuated EAE. Interestingly, despite reduced disease severity and minimal pathogenic conditions in the CNS, anti-PC mice exhibited considerable leukocyte infiltration in the brain, comparable to control mice with severe EAE. Furthermore, CD4+ T cells were diminished in the periphery of anti-PC mice, whereas various CD11b+ populations were increased, notably the myeloid-derived suppressor cells (MDSCs), a CD11b+ subset characterized as potent T cell suppressors. MDSCs from anti-PC mice exhibited increased expression of T cell suppressive factors and effectively inhibited T cell proliferation. Overall, our findings show that activated PC inhibition affected EAE pathogenesis at multiple fronts, specifically increasing vascular barrier permeability, as evidenced by considerable leukocyte infiltration in the brain. Additionally, inhibition of activated PC modulated the functional responses of CD11b+ cells, leading to the expansion and increased activation of MDSCs, which are suppressive to the CD4+ T cells required for EAE progression, thereby resulting in attenuated EAE. The Journal of Immunology, 2013, 191: 3764–3777.
In this study, we set out to investigate the influence of endogenously activated PC on the pathogenesis of experimental autoimmune encephalomyelitis (EAE), the animal model of 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 primarily mediated by effector CD4+ T cells that are able to infiltrate the CNS as a result of permeability and dysfunction at CNS barriers (25). Our rationale for studying activated PC 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. (26), proteomic analysis of MS lesions revealed the presence of coagulation proteins in chronic active plaques. In EAE studies, fibrin deposition in the brain was reported (27), and an increased presence of thrombin inhibitors was detected in the peripheral circulation of mice with EAE (28). Moreover, activated PC’s known anti-inflammatory effects, specifically its ability to mediate leukocyte function and confer vascular barrier protection, further underscore the relevance of studying activated PC in EAE, wherein the major pathological component is CNS barrier dysfunction resulting in neuroinflammation and pathology.

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

Materials and Methods

Mice and EAE induction

C57BL/6 (BL/6) mice and C57BL/6 2d1 TCR-transgenic mice were generated from our in-house breeding colony or were purchased from Taconic (Germantown, NY). Mice were maintained in a specific pathogen-free facility at Cornell University. Procedures performed on mice were approved by the Institutional Animal Care and Use Committee of Cornell University. To induce EAE, a 1:1 emulsion of myelin oligodendrocyte glycoprotein peptide (MOG35-55; AnaSpec; 1 mg/ml) and CFA (Sigma) was injected s.c. into mice flanks. Pertussis toxin (List Biologicals; 20 ng/ml) was injected i.p. on the day of immunization, and a second dose was administered 48 h later. 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, and 5 = death. Mice that reached a score of 4 were euthanized and scored as 5.

Ab to PC

Generation of mAb MPC1609 (anti-PC) was described previously (30). Anti-PC was raised to mouse PC, and it cross-reacts with mouse activated PC. Anti-PC inhibits the activation of PC and blocks both PC and activated PC from binding to endothelium and phospholipid surfaces, thus effectually abrogating activated PC’s anticoagulant and cell-signaling capabilities (30). The Ab (1 mg/kg) was administered via i.p. injection.

Immunohistochemistry

CNS harvested after perfusion were snap-frozen in OCT media (Tissue-Tek, Torrance, CA). Frozen tissues were sectioned to 6-μm 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 min. Slides were incubated with primary Abs for 90 min at 37°C. The following primary Abs were used: anti-CD45 (AbD Serotec), anti-Iba-1 (Wako Chemicals), and anti-MOG (R&D Systems). After washing, slides were incubated with the appropriate secondary Ab for 30 min, washed, and incubated with avidin–HRP complex (Invitrogen). Slides were developed with chromogenic substrate for HRP (Zymed) and counterstained with hematoxylin. Images were captured using a Zeiss Axios Imager M1 microscope.

CNS infiltrate and splenocyte isolation

CNS harvested after perfusion was homogenized using a syringe plunger. Tissues were further homogenized by repeatedly passing through a syringe with an 18-gauge blunt needle. CNS homogenates were fractionated on a 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 resuspended in ACK lysis buffer to lyse erythrocytes. After washing, splenocytes were passed through a 70-μm cell strainer and prepared for flow cytometry analysis or cultured in vitro.

Flow cytometry

Cells were resuspended in staining buffer (PBS with 0.5% BSA and 0.09% sodium azide). Cell suspensions were incubated with Fc block (BD Biosciences) for 10 min before incubation with primary Abs for 30 min at 4°C. The following primary Abs were used: 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), allyophycocyanin-conjugated anti-mouse Ly6C, and CD11c (eBioscience). Foxp3 staining was done with the eBioscience Foxp3 staining kit, in accordance with the manufacturer’s protocol. For inducible NO synthase (iNOS) intracellular staining, cells were fixed in 4% paraformaldehyde and permeabilized with 0.01% Triton X-100. Cells were incubated with polyclonal rabbit anti-mouse iNOS (BD Biosciences) for 30 min. Cells were washed and subsequently incubated with allyophucyanin-conjugated secondary Ab. For annexin V staining, we used the BD Pharmingen Apoptosis Detection Kit and followed the manufacturer’s protocol. All samples were acquired using a FACSCanto II flow cytometer (BD Biosciences). Raw data were evaluated using FlowJo Flow Cytometry Analysis Software (TreeStar).

Dextran-extravasation assay

At day 5 post-EAE induction, 2 mg Texas Red–labeled dextran (10,000 m.w.) was injected i.v. into the systemic circulation. Five hours after dextran injection, mice were perfused, and brains were harvested. Brains were homogenized in 50 mM Tris-HCl (1:1 ratio of Tris-HCl [μl]/brain [mg]). Homogenates were centrifuged, and supernatants were collected. Supernatants were mixed 1:1 with methanol and centrifuged. Fluorescence in supernatants was quantified using a Synergy 4 plate reader (BioTek, Winooski, VT). Dextran concentration was based on a standard curve.

Cell culture and ELISA

Cells were cultured in complete RPMI 1640 (10% FBS, penicillin/streptomycin, 1-glutamine, sodium pyruvate, 50 mM 2-ME, 25 mM HEPES buffer). Samples were cultured untreated or were stimulated with MOG35-55 (10 μg/ml) for 48 h. After culture, supernatants were collected for ELISA. ELISA for IFN-γ and IL-10 (eBioscience) was performed according to the manufacturer’s protocol.

Proliferation assay

Splenocytes were labeled with CFSE (Molecular Probes, Carlsbad, CA), according to the manufacturer’s protocol. T cells in whole-splenocyte culture were stimulated with plate-bound anti-CD3 (1 μg/ml; eBioscience) and soluble anti-CD28 (1 μg/ml; BioLegend) for 96 h. After culture, splenocytes were labeled with PE-conjugated mouse anti-CD4 and acquired by flow cytometry to assess CFSE dilution. For CD11b+/CD4+ cell coculture, splenic CD11b+ cells were isolated using an EasySep Positive Selection Kit, and splenic MOG-specific CD4+ T cells from naïve BL/6 2D2 transgenic mice were isolated using an EasySep CD4+ Selection Kit (both from STEMCELL Technologies). Cell isolation was done according to the manufacturer’s protocol. CD4+ T cells were labeled with CFSE, stimulated with...
Inhibition of endogenous activated PC attenuates EAE

To determine whether activated PC can influence EAE pathogenesis, we inhibited endogenous activated PC in the circulation through administration of a mAb to PC/activated PC (Materials and Methods). Anti-PC or IgG isotype (10 mg/kg) was administered at days 0, 2, 4, and 6 post-EAE induction. Administration of anti-PC within this timeframe inhibits activated PC during the initiation phase of the immune response, as well as at the outset of the effector phase of EAE, which is notably characterized by leukocyte infiltration into the CNS. We observed that the onset of clinical signs in anti-PC mice was delayed significantly (Fig. 1A, 1B). The mean day of onset for anti-PC mice was 16.7 d post-EAE induction, whereas the controls exhibited clinical symptoms as early as day 7, with mean onset at 10.5 d postimmunization. Additionally, the incidence of disease was higher in the control group; 80% of controls developed EAE by day 16, and 100% exhibited paralysis by day 22 (Fig. 1C). In contrast, only 44% of anti-PC mice exhibited clinical signs on day 16, and 66% exhibited clinical signs by day 22. These data show that inhibition of endogenous activated PC can alter the disease course of EAE, albeit in a rather unexpected way, given that inhibition of activated PC, an anti-inflammatory molecule, attenuated EAE rather than increasing disease severity.

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 EAE, we assessed the severity of CNS infiltration at the peak of disease (days 14–17). The extent of leukocyte (CD45+ cells) infiltration in the brains of control mice correlated positively with disease severity (Fig. 1E). Interestingly, 33% of anti-PC mice (Supplemental Table I) with no clinical signs had considerable CD45+ infiltrates in the brain, comparable to controls with severe EAE (Fig. 1E, 1F). We noted that cellular infiltrates were located in similar areas of the brain in both groups, notably in the brain parenchyma adjacent to the lateral ventricles (Fig. 1E, upper panels), in the cerebellar parenchyma (Fig. 1E, lower panels), and at the meninges (data not shown). We additionally observed that the extent of infiltration in the SC of controls corresponded with the severity of paralysis (Fig. 1G). Interestingly, the degree of cellular infiltration in the SC of anti-PC mice was minimal, despite considerable infiltration in the brain (Fig. 1G, 1H). Perivascular cuffing, characterized by cellular infiltrates encircling blood vessels in the CNS, is a hallmark of EAE pathology and is indicative of BBB permeability (33). We observed perivascular cuffing in the brain parenchyma of anti-PC mice (Fig. 1I). To assess any alterations in the degree of BBB permeability between the two groups, fluorescent dextran molecules were injected into the systemic circulation of IgG and anti-PC mice, and we examined the degree of dextran extravasation into the brain. We consistently observed increased dextran extravasation in the brains of anti-PC mice compared with controls and naive mice (Fig. 1J), indicating increased BBB permeability in anti-PC mice. Overall, these data show that, despite showing attenuated clinical signs, anti-PC mice exhibit increased BBB permeability as evidenced by pronounced leukocyte and dextran extravasation in the brain.

Anti-PC mice exhibit reduced pathogenic and inflammatory conditions in the CNS

To further investigate the disparity in disease course between anti-PC and control mice, we next examined the composition of cellular infiltrates in the CNS. We did not observe significant differences in CD4+ T cell frequency and absolute numbers in the brains (Fig. 2A, 2B) and SC (Fig. 2E) between the two groups. Various CD4+ T cell subsets have differing effects on EAE severity; specifically, the regulatory T cell (Treg) subset (CD4+CD25+Foxp3+) confers protection in EAE, whereas Th1 and Th17 subsets are pathogenic (25). We observed significantly increased Treg frequency in the brains of anti-PC mice compared with controls (Fig. 2C, 2D). Treg frequency was similarly increased in the SCs of the anti-PC group, although the difference did not reach statistical significance (Fig. 2F). The protective effect of Tregs is mediated, in part, through the production of the anti-inflammatory cytokine IL-10 (34). Consistent with increased Treg frequency, we detected increased IL-10 production from cellular infiltrates in the brains and SCs of anti-PC mice (Fig. 2G). In contrast, the production of the Th1 cytokine, IFN-γ, is reduced from brain infiltrates (but not from SC infiltrates) in anti-PC mice (Fig. 2H). We similarly detected decreased IL-17 mRNA expression in the brains and SCs of anti-PC mice compared with controls (Fig. 2I), suggesting a reduced pathogenic Th17 subset in the CNS of anti-PC mice. 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, whereas...
the suppressive Treg population is increased. We also examined the frequency and absolute numbers of other leukocytes populations in the brain but found no considerable differences (Supplemental Fig. 1).

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). Control mice with severe EAE showed markedly pronounced staining for the microglial marker Iba-1 (Fig. 2J), suggesting increased microglial activation (35, 36) in these mice. In contrast, the brains of anti-PC mice showed reduced Iba-1 staining (Fig. 2J). We also assessed the extent of demyelination in the CNS of both groups and detected extensive demyelination in the brains and SCs of control mice, correlating with the severity of clinical signs (Fig. 2K). Conversely, the CNS of anti-PC mice showed minimal demyelination.
elination (Fig. 2K). Taken together, the reduced microglial activation and demyelination further demonstrate the decreased inflammatory and pathogenic conditions in the CNS of anti-PC mice, despite the presence of cellular infiltrates.

Peripheral CD4+ T cells in anti-PC mice are significantly reduced

In EAE, leukocyte infiltration in the CNS is preceded by initial activation of the immune response in the periphery (37). Because activated PC is known to directly modulate various leukocyte functions (9, 13), we hypothesized that abrogating activated PC in the circulation at the initiation of EAE likely can affect the activation and functional responses of peripheral leukocytes. Therefore, we 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 with controls (Fig. 3A, 3B). Consistent with what we observed in the CNS, the frequency of Tregs was also significantly increased in the periphery of anti-PC mice (Fig. 3C, 3D). We evaluated the expression levels of various cell surface markers on splenic T cells.
Interestingly, we observed that the ratio of CD3^{hi}/CD3^{lo} T cells was consistently reduced in anti-PC mice compared with controls (Fig. 3E). However, the expression levels of other CD4^{+} T cell surface markers were not altered considerably (Fig. 3F). We next assessed the proliferative capacity of splenic CD4^{+} T cells from both experimental groups. CD4^{+} T cells in whole-splenocyte cultures were restimulated in vitro. We observed that CD4^{+} T cells from control mice exhibited robust proliferation, whereas the proliferative capacity of CD4^{+} T cells from anti-PC mice was significantly diminished (Fig. 3G). Taken together, these data are indicative of a decreased and deficient CD4^{+} T cell effector function in anti-PC mice.

**Various CD11b^{+} populations, including MDSCs, are increased significantly in the periphery of anti-PC mice**

Although we did not observe significant changes in the frequencies of various non-T cell populations between the two experimental groups (data not shown), we did observe increased frequencies (Fig. 4A, 4B) and absolute cell counts (Fig. 4C) of splenic CD11b^{+} cells in anti-PC mice compared with controls, and this was consistent through different time points in EAE (Fig. 4B). Because various leukocyte subsets are known to express CD11b, we next determined the frequency of specific CD11b^{+}-expressing leukocyte subsets. We observed significantly increased dendritic cells (CD11b^{+}CD11c^{+}) (Fig. 4D* upper panel*) and macrophages (CD11b^{+}F4/80^{+}) (Fig. 4D* lower panel*) in anti-PC mice. MDSCs, which are a subset of cells notably characterized to be potent T cell suppressors, also express CD11b (29, 38, 39). Various studies used the coexpression of CD11b and Ly6C as identifying markers for MDSC in mice (29, 40). Consistent with other CD11b^{+} populations, we observed increased frequency (Fig. 4E, 4F) and absolute cell numbers (Fig. 4G) for MDSCs in anti-PC mice, and this was observed through various stages of EAE (Fig. 4F). MDSCs are divided into monocyteic (CD11b^{+}Ly6C^{lo}/Ly6G^{+}) and granulocytic (CD11b^{+}Ly6C^{hi}/Ly6G^{hi}) subsets (29). We observed significantly increased numbers of both subsets in anti-PC mice (Fig. 4H). MDSCs from anti-PC mice also exhibit significantly increased IL-4Rα (Fig. 4I), a characteristic cell surface marker on some MDSC subpopulations that has been implicated in their suppressive functions (39). We also examined the expression of other surface markers on MDSCs, specifically PD-L1, CD40, MHCII, and B7.1, but found no significant differences between control and anti-PC cells (Fig 4I).

**CD11b^{+} cells from anti-PC mice exhibit high levels of arginase I activity, iNOS expression, and increased ROS production**

Several studies demonstrated that the suppressive effects of MDSCs on T cells are largely mediated through arginase I (Arg I) activity, NO production, and generation of ROS (29, 39). Increased Arg I activity in MDSCs depletes L-arginine from the microenvironment, resulting in suppression of various T cell responses, including inhibition of T cell proliferation and reduced CD3ζ-chain expression (39–41). We assessed higher arginase activity in splenocytes from anti-PC mice (Fig. 5A), and we also showed, by immunofluorescence staining, that Arg I is expressed by CD11b^{+} and CD11b^{+}Ly6C^{hi} cells (Fig. 5B). MDSCs are also known to express elevated levels of iNOS, which similarly use L-arginine as a substrate to produce NO, thus contributing to l-arginine depletion (29, 39). We observed significantly increased iNOS expression in CD11b^{+} and CD11b^{+}Ly6C^{hi} populations from anti-PC mice (Fig. 5C), coupled with elevated NO production (Fig. 5D). Another mechanism for MDSC-mediated T cell suppression has been attributed to the generation of ROS, known to inhibit Ag-specific T cell proliferation and downregulate CD3ζ-chain expression (29, 39). There were significantly increased ROS^{*} CD11b^{+} (Fig. 5E) and Ly6C^{+} (Fig. 5G) cells from anti-PC mice, as well as elevated generation of ROS in these cells (Fig. 5F, 5H). Collectively, these data demonstrate that CD11b^{+} cells from anti-PC mice express and generate elevated levels of T cell–suppressive factors.

**CD11b^{+} cells from anti-PC mice suppress CD4^{+} T cell proliferation and mediate Treg expansion**

We hypothesized that the diminished CD4^{+} T cell numbers (Fig. 3A, 3B) is a consequence of increased expansion of suppressive CD11b^{+} cells, notably MDSCs. 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 than were CD11b^{+} cells from controls (Fig. 6A). However, no difference in proliferation was observed if CD4^{+} T cells were cocultured with non-CD11b^{+} cells from either anti-PC or control mice (Fig. 6B). Moreover, we observed that CD4^{+} T cells cocultured with CD11b^{+} cells from anti-PC mice resulted in a significantly decreased percentage of CD3^{hi} T cells (Fig. 6C). A number of studies showed that MDSCs can directly mediate Treg expansion. We postulated that the increased frequency of Tregs, which was observed both in the CNS (Fig. 2C, 2D) and periphery (Fig. 3C, 3D) of anti-PC mice, is similarly mediated by MDSCs in these mice. We tested this hypothesis by coculturing in vitro–stimulated CD4^{+} T cells with CD11b^{+} cells from either anti-PC or control mice. We noted a significantly higher frequency of Tregs (Fig. 6D) and increased CD25 expression on Tregs (Fig. 6E) following coculture with CD11b^{+} cells from anti-PC mice. We next assessed the capacity of CD11b^{+} cells from anti-PC mice and control mice to directly suppress EAE progression by adoptively transferring CD11b^{+} cells from either group to EAE recipient mice. We observed that recipient mice that received CD11b^{+} cells from anti-PC mice developed attenuated EAE (Fig. 6F). Altogether, these data demonstrated the capacity of CD11b^{+} cells from anti-PC mice to suppress T cell function and EAE progression.

**Activated PC binds directly to MDSCs**

Although activated PC has traditionally been known to regulate leukocyte function through its interaction with the receptor, EPCR, several studies also demonstrated that activated PC can regulate the cellular processes of leukocytes by binding with other receptors (42, 43). One particular study by Cao et al. (15) showed that activated PC directly binds the CD11b integrin on CD11b^{+} cells, enabling activated PC to colocalize and activate PAR-1 on the cell surface, subsequently resulting in the downregulation of proinflammatory responses in CD11b^{+} cells. Based on this study, we hypothesized that activated PC can negatively regulate the CD11b^{+} population, including MDSCs, during the progression of EAE. We confirmed that MDSCs express PAR-1 (Fig. 7A), suggesting that these cells express the necessary receptor to be responsive to activated PC regulation. We next determined whether activated PC can directly interact with MDSCs. Fluorochrome-conjugated activated PC was incubated with splenocytes, and we showed by flow cytometry that activated PC directly binds the Ly6C^{+} population (Fig. 7B). Moreover, we confirmed that the mAb used to inhibit activated PC in mice with EAE can abrogate the direct binding of activated PC to Ly6C^{+} cells (Fig. 7C). We also showed that blocking the CD11b integrin similarly decreased the binding of activated PC to Ly6C^{+} cells (Fig. 7D), confirming that activated PC binds the CD11b^{+} integrin on these cells. MDSCs are known to expand under various inflammatory conditions, including during EAE. We postulated that because activated PC was shown to be a negative regulator of CD11b^{+} cells (15), inhibiting activated PC in the circulation...
during inflammatory conditions in EAE likely contributed to the increased expansion and activation of CD11b+ cells, notably the MDSCs. We observed that inhibiting activated PC in the circulation of naive mice did not affect the frequency of CD11b+ cells and the MDSC population (Supplemental Fig. 2), indicating that activated PC’s effect on CD11b+ and MDSC expansion is mediated under

FIGURE 3. Peripheral CD4+ T cells in anti-PC mice are significantly decreased and functionally deficient. (A) The frequency of splenic CD4+ T cells was examined by flow cytometry at day 17 following EAE induction. Data are represented as flow cytometric dot plots. (B) Graphical representation of the mean percentage of splenic CD4+ T cells (upper panel). Absolute cell counts of splenic CD4+ T cells (lower panel). Data are mean ± SEM (control, n = 6; anti-PC, n = 4). (C) The frequency of splenic Tregs (CD4+, CD25+, Foxp3+) among CD4+ T cells was assessed by flow cytometry. Data are shown as cytometric contour plots (gated on CD3+CD4+). (D) The percentages of Tregs among CD4+ T cells in the periphery. Data are mean ± SEM (control, n = 6; anti-PC, n = 4). (E) CD3 expression on splenic CD4+ T cells was assessed by flow cytometry. Data are shown as flow cytometric contour plots (gated on CD3+ cells; left and middle panels). Percentage of CD3high splenocytes from both groups (right panel). Data are mean ± SEM (n ≥ 3). (F) The cell surface expression of CD44, CD69, VLA-4, and CTLA-4 on splenic CD4+ was assessed by flow cytometry. 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 h. The proliferative capacity of CD4+ T cells was assessed by flow cytometry based on CFSE dilution. Counts of CFSE-labeled CD4+ T cells from both experimental groups (gated on CD4+ cells) (left and middle panels). Graphical representation of the percentage of CD4+ T cells that proliferated based on CFSE dilution (right panel). Data are mean ± SEM (n = 4). *p < 0.05, **p < 0.01, Student t test.
FIGURE 4. CD11b+ cells in the periphery of anti-PC mice are significantly increased. (A) Splenocytes were harvested at day 17 following EAE induction, and the frequency of CD11b+ cells was assessed by flow cytometry. Data are presented as flow cytometric dot plots. (B) The frequency of splenic CD11b+ cells in anti-PC or IgG mice at various stages of EAE progression was assessed by flow cytometry. Data are mean ± SEM (n = 3). ***p < 0.001, Student t test. (C) The absolute cell counts of splenic CD11b+ cells from both groups. Data are mean ± SEM (n = 3). **p < 0.01. (D) Frequency of specific CD11b+ subsets, namely macrophages (CD11b+F480+) (upper panel) and dendritic cells (CD11b+CD11c+) (lower panel), was examined by flow cytometry. Data are mean ± SEM (n = 3). p * < 0.05, **p < 0.001, Student t test. (E) The frequency of splenic MDSCs was examined by staining for cell surface markers CD11b and Ly6C and analyzed by flow cytometry. Data are represented as flow cytometric dot plots. (F) The frequency of splenic MDSCs in anti-PC or IgG mice at various stages of EAE progression was assessed by flow cytometry. Data are mean ± SEM (n = 3). *p < 0.05, **p < 0.01, Student t test. The absolute cell counts of splenic MDSCs (G) and the two MDSC subsets (H). Data are mean ± SEM (n = 3). **p < 0.01. (I) The expression of cell surface markers on splenic MDSCs were assessed by flow cytometry. Expression levels are presented as histograms (upper panels) and as mean MFI ± SEM (lower panels) (n ≥ 3). *p < 0.05. n.s., Not significant.
inflammatory conditions. The expansion and increased survival of MDSCs during pathogenic conditions have been attributed to Stat3 signaling (39, 44). Interestingly, it was demonstrated that activated PC can decrease the expression of Stat3 in CD11b+ cells through activated PC's interaction with the CD11b integrin (15). Consistently, we observed increased Stat3 expression (Fig. 7E) and activation (Fig. 7F) in CD11b+ cells from anti-PC mice. Stat3 expression in MDSCs is known to drive cell survival by upregulating antiapoptotic genes. Accordingly, we observed decreased apoptosis (Fig. 7G) in MDSCs from anti-PC mice, likely contributing to the increase in MDSC numbers in these mice. Taken together, these data demonstrate that activated PC directly interacts with Ly6C+ cells, which is indicative of the potential regulatory capability of activated PC on MDSCs, possibly by modulating Stat3 expression and, consequently, affecting expansion and cell survival of this leukocyte subset.

Discussion

In addition to its traditional function as an anticoagulant, activated PC affects 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 activated PC in pathological conditions. We observed that depletion of activated PC during EAE affected disease pathogenesis at multiple fronts. Interestingly, these effects have opposing and incongruent consequences on the progression and severity of the disease. We observed that inhibition of activated PC increased BBB permeability, as evidenced by considerable leukocyte infiltration in the brains of anti-PC mice. However, activated PC depletion also had profound effects on the inflammatory responses of various leukocyte populations, resulting in an increased and more activated CD11b+ myeloid population, including the MDSC subset, which is suppressive to the effector T cells required for disease progression. Consequently, the net effect of activated PC inhibition in EAE is attenuated disease.

The ability of activated PC to influence inflammatory conditions has been largely attributed to its cell-signaling capabilities (1–3). Activated PC is known to regulate the functions of various cell types, including several leukocyte populations. However, the cellular receptors that are involved in activated PC–mediated signaling in leukocytes are unclear. In endothelial cells, activated PC controls cellular processes by interacting with EPCR, a receptor that localizes activated PC on the cell surface and within the lipid rafts of the cell membrane, allowing activated PC to activate PAR-1 and initiate

FIGURE 5. CD11b+ cells from anti-PC mice express T cell suppressive factors. (A) Splenocytes were collected following EAE induction, and arginase activity in splenocytes was measured based on urea generation. Data are mean ± SEM (n = 4). (B) Splenocytes were cultured on coverslips overnight, and cells were stained with fluorochrome-conjugated Abs to CD11b, Ly6C, and arginase. Coverslips were mounted on slides, and images were captured using a Zeiss Axio Imager M1 microscope at 10× magnification. (C) The expression of iNOS in CD11b+ and CD11b+ Ly6C+ cells was assessed by flow cytometry. Flow cytometric data of iNOS expression in CD11b+ Ly6C+ cells (left panel). MFI of iNOS expression in CD11b+Ly6C+ and CD11b+ cells (right panel). Data are mean ± SEM (n = 3). **p < 0.01, Student t test. (D) Splenocytes were cultured for 48 h, and supernatants were collected to assess NO production by Griess assay. Data are mean ± SEM (n = 3). (E–H) ROS generation was assessed by incubating splenocytes with 2′,7′-dichlorofluorescein diacetate. The degree of cell fluorescence, which increases with elevated ROS generation, was assessed by flow cytometry. The frequencies of ROS+CD11b+ (E) and ROS+Ly6C+ (G) cells are shown as flow cytometric dot plots. Levels of ROS generation in CD11b+ cells (F, left panel) and Ly6C+ cells (H, left panel). MFI correlating to ROS generation in CD11b+ cells (F, right panel) and Ly6C+ cells (H, right panel). Data are mean ± SEM (n = 4). *p < 0.05.
various cell-signaling cascades (5, 7). However, several studies showed that activated PC’s effects on myeloid cells are not dependent on EPCR and may involve other cellular receptors (42, 43, 45). A recent study by Cao et al. (15) identified the CD11b integrin as the facilitator of activated PC’s anti-inflammatory effects on macrophages. The group demonstrated that activated PC can effectively inhibit the proinflammatory responses of macrophages, including the downregulation of iNOS, STAT3, and NF-kB expression, through activated PC’s interaction with CD11b on the cell surface (15). They proposed that CD11b expressed on leukocytes serves a similar function to EPCR on endothelial cells; specifically, CD11b binds activated PC and colocalizes it with PAR-1 within the lipid rafts of the cell membrane, thereby facilitating PAR-1 activation and resulting in the downregulation of inflammatory-signaling cascades (15).

In our study, we observed that inhibition of activated PC during EAE resulted in the significant increase in various CD11b+ populations. Because activated PC was shown to be a direct negative regulator of CD11b+ cells, we hypothesize that inhibition of activated PC during EAE likely resulted in the increased and more activated CD11b+ subsets. Further, we propose that certain suppressive CD11b+ populations, notably MDSCs, expanded as a result of activated PC inhibition, culminating in the attenuated disease observed in anti-PC mice. MDSCs are a heterogeneous population of immature myeloid cells characterized as potent T cell suppressors (29, 39, 40). In mice, MDSCs are identified by cell surface coexpression of CD11b and Gr-1 (29). Abs to Gr-1 bind two epitopes: Ly6C and Ly6G. MDSCs are categorized into two subsets based on the cell surface expression

**FIGURE 6.** CD11b+ cells from anti-PC mice suppress CD4+ T cell proliferation and increase CD4+ Tregs. (A) Following EAE induction, splenic CD11b+ cells were cocultured for 96 h with MOG35–55-specific CD4+ T cells. To assess proliferation, CD4+ T cells were labeled with CFSE and stimulated with MOG35–55. CFSE dilution was assessed by flow cytometry. Flow cytometric dot plots of CFSE-labeled CD4+ T cells cocultured with CD11b+ cells from either anti-PC or control mice were assessed by flow cytometry. Expression levels of CD3 as flow cytometric contour plots (gated on CD3 cells) (left and middle panels; n = 4). MFI (mean ± SEM) corresponding to CD3 levels (right panel; n = 4), *p < 0.05, Student t test. (B) Non-CD11b+ splenocytes from anti-PC or control mice were cocultured for 96 h with MOG35–55-specific CD4+ T cells. CD4+ T cells were labeled with CFSE and stimulated with MOG35–55. Flow cytometric dot plots of CFSE-labeled CD4+ T cells cocultured with CD11b+ cells from anti-PC or control mice (left and middle panels). Percentage of CD4+ T cells that have proliferated based on CFSE dilution (right panel). Data are mean ± SEM (n = 3). (C) The expression of cell surface CD3 on CD4+ T cells that were cocultured with CD11b+ cells from either anti-PC or control mice was assessed by flow cytometry. Expression levels of CD3 as flow cytometric contour plots (gated on CD3 cells) (left and middle panels; n = 4). MFI (mean ± SEM) corresponding to CD3 levels (right panel; n = 4), *p < 0.05, Student t test. (D and E) CD11b+ cells were isolated from anti-PC or IgG mice following EAE induction and cocultured for 96 h with CD4+ T cells that were stimulated with plate-bound anti-CD3 and soluble anti-CD28. After coculture, the frequency of Tregs among CD4+ T cells was assessed by flow cytometry. Data are mean ± SEM (n = 3), **p < 0.01, Student t test. (E) The expression levels of CD25 on Tregs were assessed by flow cytometry. (F) CD11b+ cells isolated from IgG or anti-PC mice were adoptively transferred to EAE recipient mice on days 3 and 7 post-EAE induction. The progression of EAE was monitored daily by assessing clinical signs. Scores are plotted as mean ± SEM (n = 4).
of these two molecules (29, 39, 40). The CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup> subset has a monocytic morphology, whereas the CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>−</sup> subset is granulocytic (29, 39, 40). Because both MDSC subsets express Ly6C, we used the coexpression of CD11b and Ly6C as identifying markers for MDSCs. Thus, the MDSC population identified in our study incorporates both subsets. The immunoregulatory effects of MDSCs on T cells were initially described in tumor microenvironments, but recent studies also reported the presence 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<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>−</sup> cells increased in the spleen following EAE induction, and these cells are capable of suppressing the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (46). In another study, MDSCs were observed in the SC during EAE, where they promote T cell apoptosis (47). Consistent with these studies, we observed that anti-PC mice with attenuated EAE have increased splenic CD11b<sup>+</sup>Ly6C<sup>+</sup> cells. Moreover, we observed that the frequency and the proliferative capacity of CD4<sup>+</sup> 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<sub>35–55</sub>-specific T cells is inhibited when they are cocultured with CD11b<sup>+</sup> cells from anti-PC mice, confirming the direct suppressive effects of the CD11b<sup>+</sup> population on Ag-induced T cell proliferation. The mechanisms used by monocytes 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 both T cell cycle progression (48) and CD3 expression (49). Similarly, increased production of NO limits T cell proliferation by inhibiting the IL-2R–downstream pathway (50). The suppressive capability of granulocytic MDSCs has been attributed to the generation of ROS, which suppresses Ag-induced T cell proliferation (29, 39). Accordingly, we observed increased Arg I activity in splenocytes and significantly higher expression of iNOS and ROS generation in CD11b<sup>+</sup>Ly6C<sup>+</sup> cells in anti-activated PC mice, confirming the increased activation of the suppressive MDSC population in these mice.

Several studies demonstrated that MDSCs exhibit the capacity to induce the generation of Tregs (51, 52). The accumulation of Tregs within the tumor microenvironment has been associated with MDSCs (52), and in vitro studies confirmed that coculture of MDSCs and T cells results in Treg expansion (51). The mechanism through which MDSCs induce Treg expansion has yet to be elucidated; interestingly, Arg I activity in MDSCs has been implicated in Treg induction (29, 39). Accordingly, we observed increased Arg I activity in splenocytes and significantly higher expression of iNOS and ROS generation in CD11b<sup>+</sup>Ly6C<sup>+</sup> cells in anti-activated PC mice, confirming the increased activation of the suppressive MDSC population in these mice.
PC mice increased Treg frequency. These findings further demonstrate the suppressive capability of CD11b+ cells from anti-PC mice via direct suppression of T cell function, as well as through inhibition of Tregs.

MDSCs are known to expand in various pathogenic and inflammatory conditions, including during EAE (39, 40). We postulate that inhibition of activated PC contributed to the further accumulation and activation of these cells as EAE progressed. It is widely proposed that the expansion and cell survival of MDSCs in pathogenic conditions are regulated by the transcription factor, Stat3 (44, 54, 55). Inhibition of Stat3 signaling in vivo abrogated the expansion of MDSCs in tumor-bearing mice (55). Stat3 expression in MDSCs has been known to drive cell survival by up-regulating antiapoptotic genes (39). Accordingly, we observed increased Stat3 expression and Stat3 activation in CD11b+Ly6C+ cells in anti-PC mice, coupled with decreased apoptosis in these cells. Moreover, the suppressive activities of MDSCs, specifically the generation of ROS, also are reportedly regulated by Stat3 (29). One likely mechanism through which activated PC inhibition affected the increase in MDSC numbers and the suppressive activities of these cells is through activated PC’s capability to suppress Stat3 expression. In the study by Cao et al. (15), activated PC specifically suppressed the expression of Stat3 in myeloid cells through engagement with the CD11b integrin. Furthermore, activated PC is known to regulate the expression and nuclear translocation of NF-κB, which is also central in regulating the immunosuppressive capabilities of MDSCs (29). Based on these studies, we postulate that activated PC is a negative regulator of MDSCs, likely by binding the CD11b integrin and downregulating Stat3 and/or NF-κB signaling. Therefore, it follows that inhibition of activated PC results in the increased activation of Stat3 and/or NF-κB in MDSCs, culminating in the increased expansion and activation of this leukocyte subset.

The influence of activated PC on inflammatory settings can be mediated at two fronts: activated PC’s direct effects on leukocytes and on vascular barrier permeability (3). Consistently, we observed that inhibition of activated PC during EAE affected leukocyte function and also 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 SCs 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. However, it should be noted that there are reported molecular differences governing leukocyte trafficking events in the brain and SC (58). Thus, it is likely that modulation of activated PC 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–activated PC mice, specifically demyelination and microglial activation, is minimal compared with controls, 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 Treg frequency in the CNS of these mice, as well as increased production of the 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 (25). We postulate that the decreased encephalitogenic CD4+ T cells in the CNS of anti-PC mice are a consequence of increased expansion of MDSCs in the periphery, which we showed can effectively suppress the effector responses of CD4+ T cells, notably MOG35-55-induced proliferation of CD4+ T cells, while concurrently inducing Treg expansion.

Our findings demonstrate the complex and multifaceted effects of activated PC inhibition in the EAE inflammatory setting, where it affects both vascular permeability and leukocyte function, ultimately resulting in pathogenic conditions that have opposing overall influences on the progression of EAE. In addition, the effect of activated PC inhibition on the final outcome of EAE progression is likely dependent on the specific stage of the disease during which activated PC is inhibited. We inhibited activated PC during the induction stage of EAE. Therefore, the effects of activated PC inhibition specifically influenced the initial activation and functional responses of leukocyte populations in the periphery, culminating in an increased and more activated myeloid subset, whereas the effector CD4+ effector T cells are diminished and suppressed. However, inhibiting activated PC after disease onset did not attenuate disease; it actually resulted in a slightly increased disease severity (Supplemental Fig. 3A). We postulate that, at this point, the effector T cells have proliferated sufficiently and have already infiltrated the CNS; therefore, inhibition of activated PC in the periphery, at this stage, would not profoundly affect the T cell response. We attribute the slight increase in disease severity to the effects of activated PC inhibition on increased BBB permeability, further increasing the infiltration of pathogenic leukocytes (Supplemental Fig. 3B) into the CNS. These findings demonstrate that the overall outcome of activated PC modulation in disease settings can be governed by a number of factors, including the specific stage of the disease.

In summary, activated PC inhibition during induction of EAE affected its pathogenesis at multiple fronts, specifically increasing BBB permeability and inducing a more activated CD11b+ myeloid population, including the MDSC subset known to be potent T cell suppressors. Because EAE is a predominantly T cell–mediated disease, the net effect of activated PC depletion during EAE is alleviated disease severity. To our knowledge, activated PC’s influence on the MDSC population is presented in this article for the first time. The ability of activated PC to affect the functional responses of MDSCs opens a novel therapeutic avenue through which activated PC can influence disease conditions. MDSCs, for instance, are widely reported to be a major hindrance in tumor immunity. The findings in this study, which suggest that activated PC can negatively regulate MDSCs, bring to the fore the possible therapeutic relevance of activated PC in cancer immunotherapies. Overall, these findings present a novel influence of activated PC on the immune response and contribute to elucidating the complex interaction between the immune and coagulation systems.

Disclosures
The authors have no financial conflicts of interest.

References


