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Activated Protein C Inhibits Pancreatic Islet Inflammation, Stimulates T Regulatory Cells, and Prevents Diabetes in Non-obese Diabetic (NOD) Mice*[S]

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Background: aPC is a natural anticoagulant with strong anti-inflammatory properties.
Results: aPC reduced diabetes by 90% in NOD mice and induced Tregs. These cells suppressed T cell proliferation and prevented adoptive transfer of diabetes.
Conclusion: aPC inhibits inflammation, induces Tregs, and prevents diabetes in NOD mice.
Significance: This work indicates a potential role of aPC in the prevention of type 1 diabetes in at-risk individuals.

Activated protein C (aPC) is a natural anticoagulant with strong cyto-protective and anti-inflammatory properties. aPC inhibits pancreatic inflammation and preserves functional islets after intraportal transplantation in mice. Whether aPC prevents the onset or development of type 1 diabetes (T1D) is unknown. In this study, when human recombinant aPC was delivered intraperitoneally, twice weekly for 10 weeks (from week 6 to 15) to non-obese diabetic (NOD) mice, a model for T1D, the incidence of diabetes was reduced from 70% (saline control) to 7.6% by 26 weeks of age. Islets of aPC-treated mice exhibited markedly increased expression of insulin, aPC/protein C, endothelial protein C receptor, and matrix metalloproteinase (MMP)-2 when examined by immunostaining. The insulitis score in aPC-treated mice was 50% less than that in control mice. T regulatory cells (Tregs) in the spleen, pancreatic islets, and pancreatic lymph nodes were increased 37, 53, and 59%, respectively, in NOD mice following aPC treatment. These Tregs had potent suppressor function and, after adoptive transfer, delayed diabetes onset in NOD.severe combined immunodeficiency mice. The culture of NOD mouse spleen cells with aPC reduced the secretion of inflammatory cytokines interleukin (IL)-1β and interferon-γ but increased IL-2 and transforming growth factor-β1, two cytokines required for Treg differentiation. In summary, our results indicate that aPC prevents T1D in the NOD mouse. The aPC mechanism of action is complex, involving induction of Treg differentiation, inhibition of inflammation, and possibly direct cyto-protective effects on β cells.

Type 1 diabetes mellitus (T1D)[2] is an autoimmune disease that results in the destruction of insulin-producing β cells in the pancreatic islets of Langerhans (1). T1D has two distinct phases as follows: subclinical, where mononuclear cells infiltrate the islets (insulitis) selectively destroying β cells, and clinical, where most β cells have been destroyed leading to inadequate insulin production and hyperglycemia (1). Apoptosis is the dominant form of β cell death in T1D (2). Studies in the non-obese diabetic (NOD) mouse model of T1D demonstrate that β cell destruction is mediated by auto-reactive T cells (1, 3). $CD4^+$ and $CD8^+$ T cells are required to transfer diabetes from recently diabetic mice to young irradiated NOD mice or NOD. severe combined immunodeficiency (SCID) mice (1, 3). Pathogenic T cells are normally held in check by a variety of T regulatory cells (Tregs). Prototypic $CD4^+CD25^+$ Tregs are programmed by the forkhead box transcription factor (FoxP3) (4). The critical role of FoxP3 is illustrated by its natural mutation in scurfy mice and in humans with immune dysregulation polyendocrinopathy enteropathy, X-linked syndrome, which leads to multiorgan-specific autoimmune disease in infancy (5). Immune dysregulation polyendocrinopathy enteropathy, X-linked, is the genetic equivalent of the scurfy mouse, and they have overlapping phenotypes (6). $CD4^+CD25^+$ Tregs suppress differentiation of islet-reactive $CD8^+$ T cells to cytotoxic T cells (7) and protect against diabetes development in the NOD mouse (8).

Activated protein C (aPC) is a natural anticoagulant derived from an endogenous vitamin K-dependent precursor protein, protein C (PC). aPC also possesses strong anti-inflammatory, anti-apoptotic, and endothelial barrier stabilizing properties (9–12). Many of the anti-inflammatory properties of aPC are mediated through its specific receptor, endothelial protein C receptor (EPCR) (13). aPC is reported to have anti-inflammatory protective effects in the pancreas. In rats with experimental acute necrotizing pancreatitis, aPC inhibits pancreatic inflammation and reduces tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels in sera (14). aPC preserves functional islets after intraportal transplantation in a mouse model (15) and

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[2] The abbreviations used are: T1D, type 1 diabetes; aPC, activated protein C; EPCR, endothelial protein C receptor; NOD, non-obese diabetic; SCID, severe combined immunodeficiency; Treg, T regulatory cell; NF-κB, nuclear factor-κB; PC, protein C.

protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis (16). Interestingly, plasma levels of aPC are reduced in humans with T1D (17, 18), whereas the levels of soluble EPCR, which retains its affinity for but inhibits the activity of aPC, are elevated (19). This study was designed to investigate whether aPC is protective in T1D and to understand the mechanisms of its protection.

EXPERIMENTAL PROCEDURES

Mouse Model—Female NOD/Lt mice were used as a T1D model and BALB/c mice as normal controls in this study. Initially, 6-week-old NOD mice were treated intraperitoneally with human recombinant aPC (Xigris, Eli Lilly) at 0.5, 1, 2, and 4 mg/kg or the same volume of phosphate-buffered saline (PBS) for 5 weeks to optimize the aPC dose. aPC at 2 mg/kg showed the best protective effect with no observed toxicity. NOD mice were then treated with 2 mg/kg aPC or PBS intraperitoneally twice weekly for 5 or 10 weeks. The onset of diabetes was monitored by measuring tail vein blood glucose twice weekly until 26 weeks of age. Diabetes was defined as blood glucose >16.7 mM, confirmed on a subsequent measurement.

To investigate whether aPC can reverse established diabetes, female NOD mice were left untreated until they developed diabetes and then were treated with aPC (2 mg/kg intraperitoneally) or PBS every 2nd day. Diabetic mice also received 1 unit/day long acting insulin. Blood glucose was measured daily for 5 weeks.

To test whether aPC-induced reduction in diabetes is a general immunogenic effect, the precursor of aPC, PC, was used to treat mice at the same concentration of aPC, and diabetic incidence was monitored until week 26.

Mice were housed in a specific pathogen-free room and handled as approved by Royal North Shore Hospital Animal Care and Ethics Committee.

Intraperitoneal Glucose Tolerance Test—After overnight fasting, mice were injected intraperitoneally with 2 g/kg glucose as a 200 mg/ml solution. Glucose was measured in tail vein blood at base line and 30, 60, 90, 120, and 180 min after injection.

Histology and Grading of Islet Infiltrates—Pancreata from mice were fixed in 10% PBS-buffered formalin and embedded in paraffin. Insulitis was graded in at least 10 islets per pancreas as described previously (20). The mean insulitis score of each pancreas was calculated by dividing the sum of graded islets by the total number of islets analyzed.

Islet Isolation—Mouse pancreatic islets were isolated by collagenase digestion as described by Li *et al.* (21). The purity of isolated islets was examined under an inverted microscope and were visually identified as >98% free of exocrine tissue. Islet yield ranged from 25 to 81/mouse at 18 weeks of age. Islets from six mice were pooled together to obtain sufficient and relatively similar number of islets for each group.

Real Time RT-PCR—Total RNA was extracted from isolated mouse islets using TRI Reagent. Single-stranded cDNA was synthesized from total RNA using avian myeloblastosis virus-reverse transcriptase and oligo$(dT)_{15}$ as a primer (Promega Corp., Madison, WI). The levels of mRNA were semi-quantified using real time PCR on a Rotor-gene 3000A (Corbett Research, Sydney, Australia). Samples were normalized to the housekeeping gene GAPDH, and results were reported for each sample relative to the control. Primers used were as follows: PC (238 bp), 5′-GGTGCTCATCCACACTTCCT and 5′-GCAGATGGGCACTATGGTTT; EPCR (167 bp), 5′-ACAGAGAGTGGCCTGCAGAT and 5′-TCGAAGAAGACATGGGGTTC; GAPDH (171 bp), 5′-ACCCAGAAGACTGTGGATGG and 5′-CACATTGGGGGTAGGAACAC.

Immunohistochemistry/Immunofluorescent Staining—Deparaffinized pancreatic tissue sections were incubated with antibodies against mouse PC, EPCR, MMP-2, MMP-9 (R&D Systems, Minneapolis, MN), FoxP3 (eBioscence, San Diego), and insulin and then stained by LSAB + Systems stain kit (DAKO Corp., Glostrup, Denmark). For immunofluorescent staining, after incubation with primary antibodies to EPCR and insulin, tissue sections were incubated with fluorescence-conjugated secondary antibodies (Invitrogen), counterstained with DAPI. and observed under a fluorescent microscope.

Cell Culture—Mouse spleen cells were isolated and maintained in RPMI 1640 medium with 10% fetal calf serum (FCS) (Invitrogen). Before treatment, cells were switched to serum-free medium overnight and then to fresh serum-free medium.

Chemotaxis Assay—Mouse peritoneal macrophages obtained by lavaging the peritoneal cavity were cultured in 10% FCS/RPMI 1640 medium for 2 h. Nonadherent cells were then removed by washing with serum-free medium. The adherent cells (macrophages) and $CD4^+$ T cells isolated from spleens of NOD mice were used for the chemotaxis assay, as described previously (22).

aPC Activity Assay—The activity of aPC in plasma was measured by the chromogenic substrate Spectrozyme PCa (American Diagnostica Inc., Stamford, CT).

Gelatin Zymography—MMP-2 and MMP-9 were measured using gelatin zymography under nonreducing conditions, as described previously (23).

Adoptive Transfer of Diabetes—Single cell suspension was prepared from pooled spleens of NOD mice. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated using antibody-coated magnetic microbeads (Invitrogen). Cells were resuspended in PBS and injected into the tail vein of 6-week-old female NOD.SCID mice. Blood glucose levels of recipient NOD.SCID mice were monitored twice weekly until the mice were 20 weeks old.

Enzyme-linked Immunosorbent Assay (ELISA)—IL-1β, IL-2, IL-6, IL-10, interferon (IFN)-γ, and transforming growth factor (TGF)-β1 in culture supernatants of spleen cells were assayed by ELISA kits (R&D Systems).

Treg Detection—$CD4^+CD25^+$ $FoxP3^+$ cells were detected by flow cytometry using the mouse Treg flow cytometry kit (BioLegend, San Diego).

T Cell Proliferation—$CD4^+CD25^-$ T cells isolated from spleen cells of NOD mice were labeled with 1 μM carboxyfluorescein succinimidyl ester (Invitrogen) and cultured in 96-well plates coated with anti-CD3 and anti-CD28 antibodies (eBioscience). Cells were treated with aPC or co-cultured with $CD4^+CD25^+$ T cells. After 3 days, cells were harvested and directly analyzed by flow cytometry. Data were analyzed using FlowJo software.

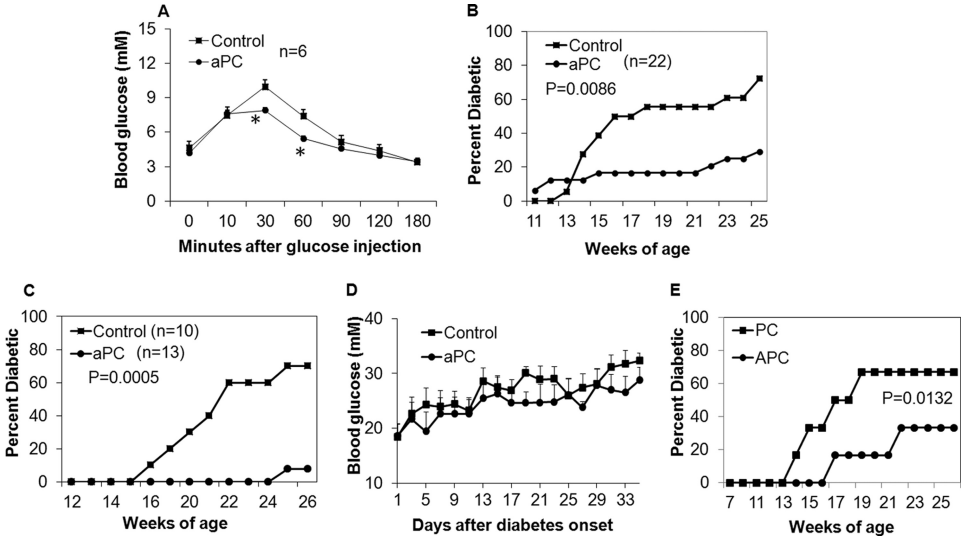

FIGURE 1. **aPC treatment delays the onset and decreases the incidence of diabetes in NOD mice.** *A,* glucose tolerance of nondiabetic NOD mice at 11 weeks of age ($n = 6$) after treatment with aPC (2 mg/kg) or PBS for 5 weeks from 6 to 10 weeks of age. Glucose was measured in blood from the tail vein at base line (0) and 30, 60, 90, 120, and 180 min after glucose administration. *B,* diabetes incidence in NOD mice treated with aPC (2 mg/kg) or PBS for 5 weeks from 6 to 10 weeks of age ($n = 22$). *C,* diabetes incidence in NOD mice treated with aPC (2 mg/kg) for 10 weeks from 6 to 15 weeks of age (aPC group: $n = 13$; PBS group, $n = 10$). *D,* blood glucose levels of mice treated with aPC or PBS after diabetes had developed ($n = 8$). Mice were treated with aPC twice a week for 5 weeks and received basal insulin daily. *E,* diabetic incidence in NOD mice treated with aPC or PC (2 mg/kg, six mice/group) for 5 weeks from 6 to 10 weeks of age. All results are expressed as mean ± S.E. For *B, C,* and *E*, *p* value represents statistical difference between the curves using Survival plots (Kaplan-Meier) and log rank analysis. *, $p < 0.05$.

Apoptosis Detection—T cell apoptosis was detected using annexin V surface staining and propidium iodide DNA staining by flow cytometry.

Statistical Analysis—Analysis of variance and Student's *t* test were used to compare means, followed by appropriate post-comparison tests. Survival plots (Kaplan-Meier) and log rank analysis were used to compare diabetes incidence in NOD and NOD.SCID mice.

RESULTS

aPC Treatment Delays the Onset and Decreases the Incidence of Diabetes in NOD Mice—At 11 weeks of age, when subjected to a glucose tolerance test, NOD mice treated with aPC from 6 to 10 weeks had significantly lower levels of blood glucose at 30 and 60 min (Fig. 1*A*), indicating better glucose tolerance than untreated mice. Blood glucose levels, in mice treated similarly for 5 weeks with either aPC or PBS (22 mice/group), were monitored weekly until mice were 26 weeks of age. aPC treatment delayed the onset and decreased the incidence of diabetes (Fig. 1*B*). By 26 weeks of age, 16 of 22 (~73%) PBS-treated mice had developed diabetes, compared with 8 of 22 (~36%) aPC-treated mice ($p = 0.009$) (Fig. 1*B*). aPC treatment was then extended for a further 5 weeks, *i.e.* from 6 to 15 weeks of age. Under this regimen, by 26 weeks of age only 1 of 13 (7.7%) aPC-treated mice had developed diabetes, whereas 7 of 10 (70%) control mice were diabetic ($p = 0.008$) (Fig. 1*C*). We then examined whether aPC could reverse diabetes by treating NOD mice after the disease was fully developed. aPC treatment for 5 weeks did not induce remission of diabetes, and all mice became progressively more hyperglycemic (Fig. 1*D*).

PC, the nonactivated form of APC, was also used to treat NOD mice at the same concentration of aPC. At week 26, the diabetes incidence was significantly higher in PC-treated mice than that in aPC-treated mice (Fig. 1*E*), indicating that aPC-induced reduction in diabetes is specific for aPC and not a general immunogenic effect.

aPC Treatment Reduces Inflammation and Increases aPC/EPCR Expression in Islet Cells—Pancreata from 26-week-old NOD mice were processed for histological examination to grade insulitis. As expected, pancreata from PBS-treated diabetic mice exhibited almost complete islet loss, associated with massive infiltration by leukocytes. The insulitis score was two times higher in nondiabetic control (PBS-treated) than that in aPC-treated nondiabetic mice (Fig. 2*D*). In the nondiabetic control NOD mice, inflammatory cells typically composed of >50% of islet volume and insulin expression was weak and restricted to the central regions of islets (Fig. 2*A*). In aPC-treated NOD mice, however, inflammatory cells comprised <10% islet volume on average and islets stained strongly for insulin (Fig. 2*A*).

Islet cells from BALB/c mice expressed both EPCR and PC/aPC, although islets from control NOD mice expressed some PC/aPC but minimal EPCR (Fig. 2*A*). However, aPC treatment stimulated PC/aPC and EPCR expression in NOD mouse islet cells, resembling that of islet cells in BALB/c mice (Fig. 2*A*). Dual staining of insulin and EPCR revealed their co-localization, confirming that EPCR is expressed by β cells (Fig. 2*B*). RT-real time PCR confirmed that islet cells from both BALB/c and NOD mice expressed PC and EPCR at the mRNA level (Fig. 2*C*). Cells from BALB/c mice expressed approximately two times more EPCR mRNA than those from NOD mice. aPC treatment stimulated EPCR but not PC mRNA expression in NOD mice (Fig. 2*C*). Interestingly, there was no significant difference in PC mRNA expression among the three groups, even though immunohistochemistry results showed variations in protein levels, possibly indicating that PC protein remains on the cell surface bound to the high levels of EPCR in BALB/C and aPC-treated NOD mice.

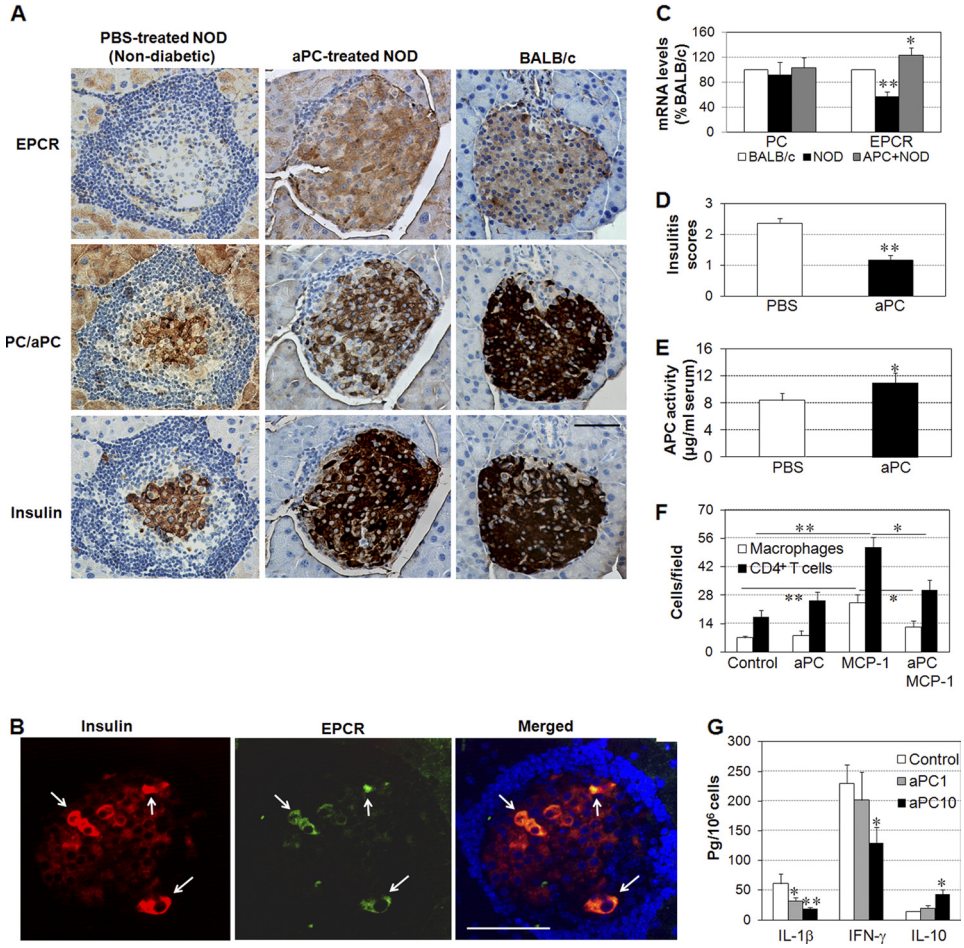

FIGURE 2. **aPC treatment reduces inflammation and increases EPCR/PC/aPC in islet cells.** NOD mice were treated with aPC (2 mg/kg) or PBS for 10 weeks from 6 to 15 weeks of age. Pancreata or sera were collected from NOD mice at 26 weeks of age. *A,* immunostaining for EPCR, PC/aPC, or insulin in pancreatic islets from nondiabetic mice. BALB/c mice (no treatment) at the same age served as positive controls. *B,* dual staining of EPCR (*green*) and insulin (*red*) in islets, and cell nuclei (*blue*) were stained with DAPI. Images were merged with Image J. *C,* PC and EPCR mRNA levels in isolated mouse islets detected by RT-real time PCR. Data are expressed as % of BALB/c mouse ± S.E. ($n = 4$). *D,* insulitis scores of aPC- or PBS-treated mice (six mice/group, all were nondiabetic). *E,* aPC activity in plasma, measured by Spectrozyme assay. Data are expressed as mean ± S.E. ($n = 6$, each plasma sample was pooled from two mice, and the glucose levels and diabetic incidence for control *versus* APC were 8.2 mM *versus* 23.4 mM and 75% *versus* 9.1%, respectively). *F,* chemotaxis of peritoneal macrophages and $CD4^+$ T cells from NOD mice in response to aPC (10 μg/ml), MCP-1 (10 ng/ml), or aPC plus MCP-1. Data are expressed as mean ± S.E. ($n = 3$). *G,* IL-1β, IL-10, and INF-γ in media of spleen cells treated with aPC (0, 1, and 10 μg/ml, *e.g.* control, aPC1, and aPC10) *in vitro* for 3 days and measured by ELISA. Data are expressed as mean ± S.E. ($n = 3$). *Scale bar,* 100 μm. *, $p < 0.05$; **, $p < 0.01$.

aPC treatment significantly raised plasma aPC activity at week 26, 10 weeks after the final aPC administration (Fig. 2*E*). A chemotaxis assay was performed to determine whether aPC inhibits leukocyte infiltration, a critical step leading to diabetes. In basal conditions, aPC had no significant effect on the migration of macrophages and T cells. However, in response to monocyte chemotactic protein (MCP)-1, aPC suppressed macrophage and $CD4^+$ T cell migration by 50 and 40%, respectively (Fig. 2*F*). In addition, the inflammatory/anti-inflammatory cytokines in the culture supernatants of spleen cells treated with aPC were measured by ELISA. aPC at 10 μg/ml decreased IL-1β and IFN-γ secretion by 80 and 44%, respectively, whereas it increased IL-10 by ~3 times (Fig. 2*G*).

aPC Differentially Regulates MMP-2 and MMP-9 Expression by Islets and Spleen Cells—We have previously shown that aPC activates and stimulates MMP-2 although it inhibits MMP-9 (24). Immunohistochemical staining of islets from BALB/c mice showed intensive expression of MMP-2 and faint or absent expression of MMP-9 (Fig. 3*A*). Nondiabetic NOD mice had a similar paucity of islet staining for MMP-9 regardless of whether they were PBS- or aPC-treated. In contrast, islets from aPC-treated NOD mice expressed very high levels of MMP-2, similar to that in BALB/c mice, yet MMP-2 was barely detectable in PBS-treated NOD mice (Fig. 3*A*). Spleen cells isolated from NOD mice produced substantial amounts of MMP-9 after 24 h in culture, whereas MMP-9 was barely detectable in spleen cells from BALB/c mice (Fig. 3*B*). aPC treatment of spleen cells from NOD mice caused a dose-dependent increase of MMP-2 and a decrease of MMP-9 (Fig. 3*B*).

aPC Promotes Tregs Both in Vitro and in Vivo—Tregs suppress the differentiation of islet-reactive $CD8^+$ T cells to cytotoxic T cells (7) and protect against diabetes development in the NOD mouse (8). To investigate the effects of aPC on Tregs, NOD mice were treated with aPC or PBS for 10 weeks and Tregs investigated by immunostaining and flow cytometry. Immunostaining of pancreata indicated that there was a substantial increase in FoxP3 expression in aPC-treated NOD mice compared with control mice, and a further increase in BALB/c

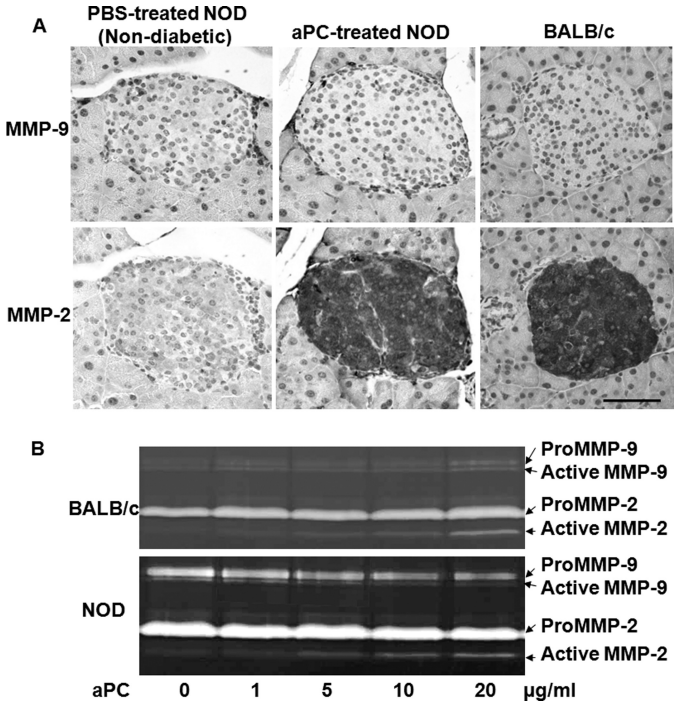

FIGURE 3. **aPC increases/activates MMP-2 and decreases MMP-9 by islets or spleen cells of NOD mice.** *A,* MMP-2 and -9 expression in islets from 26-week-old aPC- or PBS-treated nondiabetic NOD mice or BALB/c mice. *Scale bar,* 100 μm. *B,* MMP-2 and -9 in media of spleen cells from 10-week-old BALB/c or nondiabetic NOD mice in response to aPC treatment for 24 h, as detected by zymography. Images are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

pancreatic islets (Fig. 4*A*). To confirm these results, the frequency of $CD4^+FoxP3^+$ T cells was detected. aPC increased Tregs in spleens, pancreatic islets, and draining pancreatic lymph nodes by 37, 53, and 59%, respectively (Fig. 4*B* and see supplemental Fig. 1 for $CD4^+CD25^+$ plots). When spleen cells from NOD mice were incubated with aPC *in vitro* for 3 days, the proportion of $CD4^+$FoxP3-positive cells increased by ~30% (Fig. 4*C*), as did the expression of FoxP3 protein (Fig. 4*D*). To explore how aPC might promote differentiation of Tregs, we examined its effect on the expression of cytokines involved in Treg differentiation. aPC dose-dependently increased the production of TGF-β1 and IL-2, two cytokines that are required for the development, maintenance, and function of Tregs, but had no effect on IL-6, a cytokine that favors Th17 development (Fig. 4*E*).

Tregs from aPC-treated Mice Suppress T Cell Proliferation in Vitro and Prevent Adoptive Transfer of Diabetes in Vivo—To investigate the suppressive capacity of $CD4^+CD25^+$ T cells following aPC treatment, we first measured their effect on proliferation of $CD4^+CD25^-$ T cells from diabetic NOD mice *in vitro*. $CD4^+CD25^+$ T cells from aPC-treated mice showed a greater suppressive effect on the proliferation of $CD4^+CD25^-$ T cells than $CD4^+CD25^+$ T cells from control mice ($p = 0.021$) (Fig. 5*A*). Incubation of $CD4^+CD25^+$ T cells with aPC *in vitro* for 3 days did not increase their capacity to suppress proliferation (data not shown), suggesting that aPC does not act directly on Tregs.

We next examined whether aPC affected Treg cell survival/apoptosis. *In vitro*, aPC had minimal direct effect on purified Treg survival/apoptosis as determined by flow cytometry using annexin V and propidium iodide staining (Fig. 5*B*). This was further confirmed by trypan blue exclusion, which showed that in response to aPC treatment (0, 0.1, 1 and 10 μg/ml) the proportion of live cells was 53, 59, 61, and 63%, respectively. However, when added to $CD4^+CD25^-$ T cells, stimulated with anti-CD3 and anti-CD28 antibody-coated plates for 3 days, aPC dose-dependently inhibited proliferation (Fig. 5*C*). At 10 μg/ml, aPC almost completely abolished proliferation of $CD4^+CD25^-$ T cells.

To determine whether aPC prevents diabetes by modifying T cell function *in vivo*, adoptive transfer experiments were performed. Spleen cells were isolated from PBS- or aPC-treated NOD mice at 26 weeks of age and injected into 6-week-old NOD.SCID mice, and diabetes in recipients was monitored for up to 20 weeks. Spleen cells from PBS-treated mice transferred diabetes to all recipients by week 10. In contrast, diabetes onset was delayed for 2 weeks and not until week 15 did all recipients develop diabetes after transfer of spleen cells from aPC-treated NOD mice (Fig. 5*D*). In further experiments, $CD4^+$ ($CD25^-$ or $CD25^+$) cells were isolated from spleens of 16-week-old nondiabetic NOD mice treated with PBS or aPC for 10 weeks. Cells were co-transferred with spleen cells from recent onset diabetic mice in a ratio of 2×10^6:10×10^6 ($CD4^+$/spleen cells) into NOD.SCID recipients. Although $CD4^+CD25^-$ cells had no effect, $CD4^+CD25^+$ cells from both PBS- and aPC-treated mice delayed the onset of diabetes in NOD.SCID recipients ($p < 0.01$); however, cells from aPC-treated mice had a significantly greater protective effect ($p = 0.015$) (Fig. 5*E*). All NOD.SCID recipients developed diabetes at week 17 after adoptive transfer of $CD4^+CD25^+$ cells from PBS-treated mice. In contrast, after adoptive transfer of $CD4^+CD25^+$ cells from aPC-treated mice, there remained 30% of recipients that had not developed diabetes at week 20.

DISCUSSION

aPC exerts cytoprotective effects in a number of disease models, including spinal cord injury, chronic wounds, asthma, kidney injury, lung injury, and sepsis (25). A human recombinant form of aPC is Food and Drug Administration-approved for use in patients with severe sepsis, and evidence from human pilot studies shows that aPC may be an effective treatment for chronic wounds (26) and lung injury (27). This is the first report to show that aPC can prevent T1D in an animal model. When administered over 10 weeks, starting at 6 weeks of age, aPC almost completely prevented spontaneous diabetes in NOD mice. The protective effect of aPC was long lasting, being sustained for at least 10 weeks after the last treatment. aPC appears to act by inhibition of inflammation and promotion of Treg differentiation with suppression of T cell proliferation.

aPC significantly decreased the severity of insulitis in NOD mice. Histologically, pancreatic sections exhibited a marked reduction in the immune/inflammatory infiltrates into islets. Additionally, aPC inhibited MCP-1-induced chemotaxis of macrophages and T cells from NOD mice and reduced inflammatory cytokines IL-1β and IFN-γ. During the development of autoimmune diabetes, islets are infiltrated by macrophages followed by $CD4^+$ and $CD8^+$ T cells. These invading immune

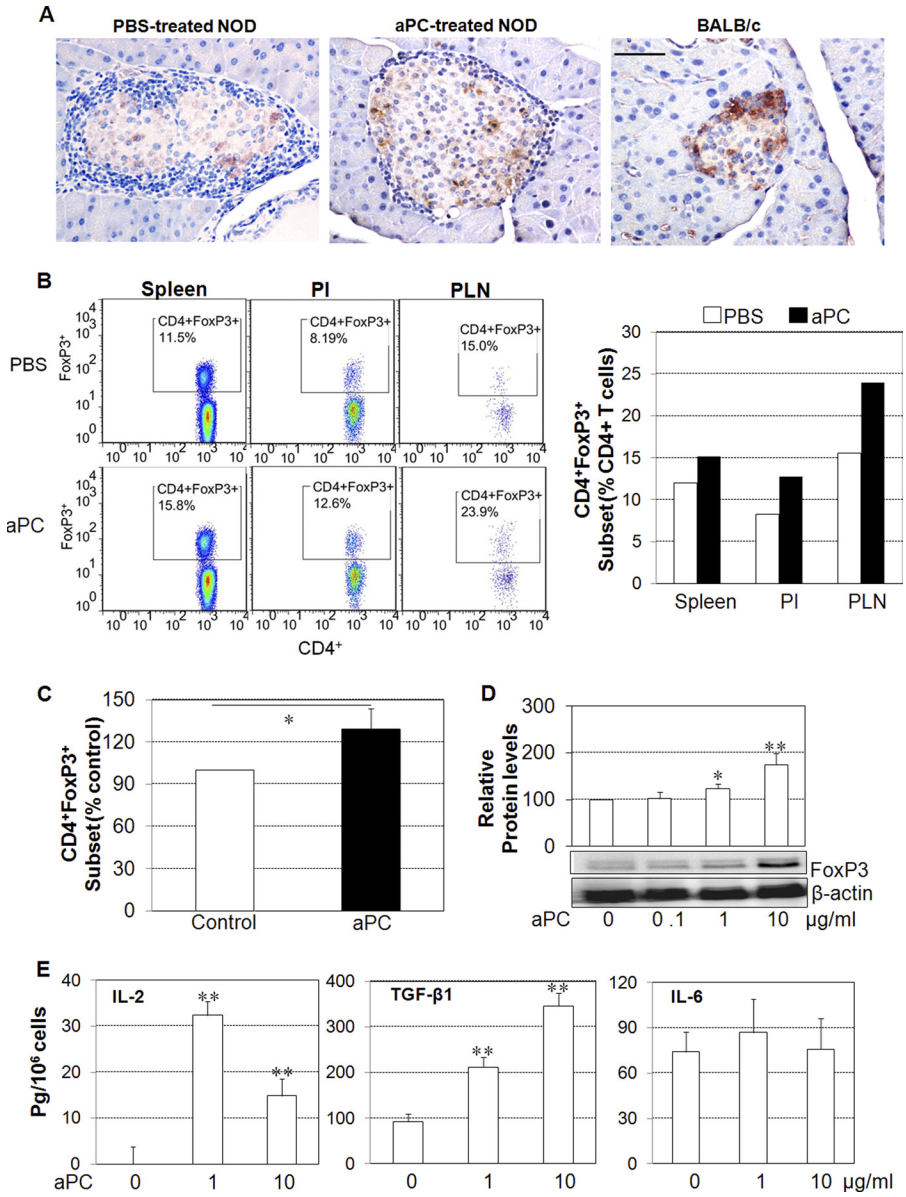

FIGURE 4. **aPC induces Tregs *in vivo* and *in vitro*.** *A,* immunostaining for FoxP3 (*brown*) in pancreatic islets of NOD and BALB/c nondiabetic mice at 18 weeks of age. NOD mice were treated with aPC (2 mg/kg) or PBS for 10 weeks. BALB/c mice at the same age served a positive control. *Scale bar,* 100 μm. *B, left,* flow cytometry showing the frequency of $CD4^+FoxP3^+$ cells in total $CD4^+$ T cells in spleens, pancreatic islets (*PI*), and pancreatic lymph nodes (*PLN*) isolated from either PBS or aPC-treated 18-week-old NOD mice, detected by a Treg cell kit. *Right,* flow cytometry data are shown as % $CD4^+$ $FoxP3^+$ cells of total $CD4^+$ T cells, from six mice for each group (PBS group: mean blood glucose 12.6 mM, one mouse was diabetic; APC group: mean blood glucose 7.9 mM, all nondiabetic). *C,* percentage of $CD4^+FoxP3^+$cells in spleen cells from 10-week-old nondiabetic NOD mice treated with aPC (10 μg/ml) for 3 days *in vitro*, compared with control, detected by flow cytometry using a Treg detection kit. Data are shown as mean ± S.E. ($n = 3$. *D,* FoxP3 in spleen cells treated with aPC for 3 days and detected by Western blotting, semi-quantified by image analysis, and expressed as relative protein levels (percent of control, mean ± S.E., $n = 3$). *E,* IL-2, IL-6, and TGF-β1 in media of spleen cells treated with aPC *in vitro* for 3 days and measured by ELISA. Data are expressed as mean ± S.E. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$.

cells, especially the macrophages, secrete inflammatory mediators such as IL-1β, TNF-α, and iNOS that activate the NF-κB pathway, signaling gene regulatory networks that contribute to β cell apoptosis (2, 28). aPC directly inhibits the expression and activation of NF-κB and blocks expression of NF-κB-regulated genes such as cell adhesion molecule expression thus preventing transmigration of leukocytes (29). aPC has recently been shown to inhibit the inflammatory response of conventional dendritic cells and suppress INF-γ production by natural killer-like dendritic cells (30). Mice with genetically determined low endogenous PC develop life-threatening inflammatory responses to endotoxin challenge (31). In the rat pancreatitis (12) and mouse islet transplant (15) models, the protective effect of aPC is associated with significant reduction in proinflammatory cytokine expression. These data, together with our results, indicate that the protective role of aPC in NOD mice occurs, at least partly, via inhibition of inflammation.

Many of the anti-inflammatory and anti-apoptotic effects of aPC are mediated through EPCR, which itself is anti-inflammatory (9, 10, 13). This is the first report of EPCR expression by islet β cells, and aPC treatment stimulated EPCR expression in NOD mice. EPCR is a 46-kDa type I transmembrane glycopro-

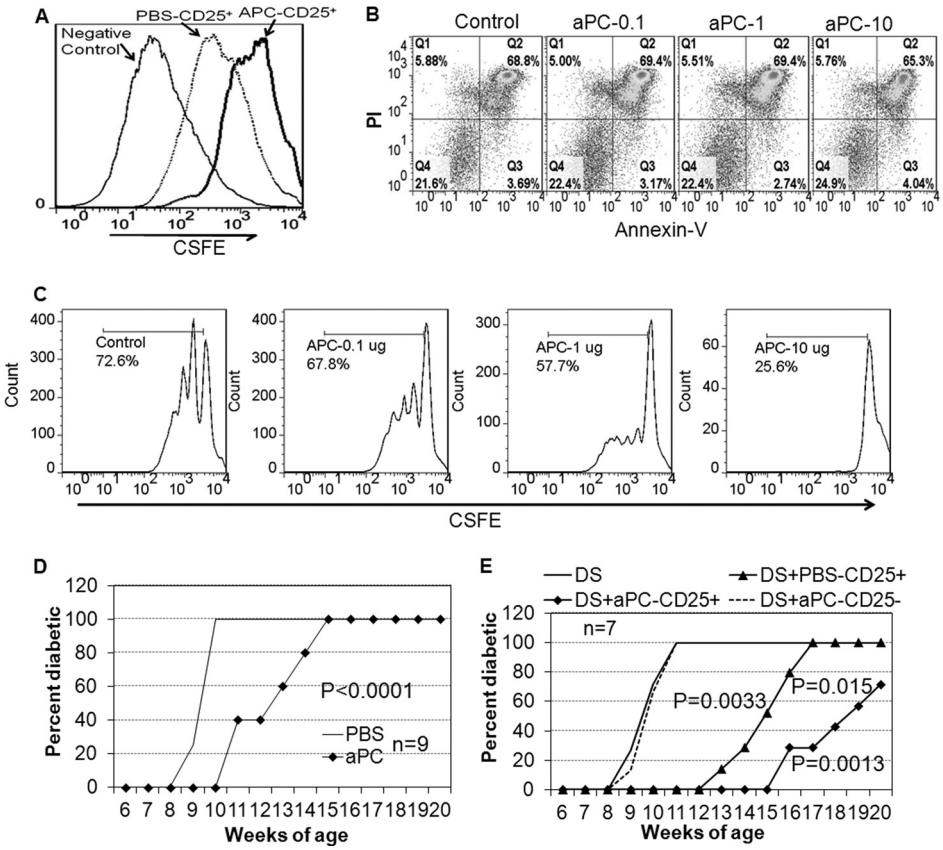

FIGURE 5. **aPC-stimulated Tregs inhibit T cells proliferation and prevent adoptive transferred diabetes in NOD.SCID mice.** *A,* $CD4^+CD25^-$ T cells (1×10^6 cells/ml) from spleens of NOD mice were labeled with carboxyfluorescein succinimidyl ester and incubated for 72 h in anti-CD3 and anti-CD28 antibody-coated plates either alone (negative control) or with $CD4^+CD25^+$ T cells (1:1) from aPC- or PBS-treated mice. Cell proliferation was analyzed by flow cytometry. *B,* survival/apoptosis of Tregs from mouse spleens in response to aPC (0, 0.1, 1, and 10 μg/ml) for 24 h, detected by annexin V with propidium iodide (*PI*) staining using flow cytometry. *C,* proliferation of $CD4^+CD25^-$ cells (4×10^6 cells/ml) from diabetic NOD mice in response to aPC (0. 0.1, 1, and 10 μg/ml) for 72 h. Cells were stimulated with anti-CD3 and anti-CD28 antibodies, and proliferation was detected by flow cytometry. Data were analyzed with FlowJo software, and representative histogram plots are from triplicate wells of two independent experiments. *D,* diabetes incidence in NOD.SCID mice after adoptive transfer of spleen cells (1×10^7 cells/mouse) from 26-week-old PBS- or aPC-treated NOD mice. Data are expressed as mean ± S.E. ($n = 9$). *E,* diabetes incidence in NOD.SCID mice after adoptive co-transfer of total spleen cells from recently diabetic mice (*DS*) with splenic $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from 10-week aPC-treated NOD mice or $CD4^+CD25^+$ from control mice. DS cells were injected i.v. alone (1×10^7) or mixed (5:1) with $CD4^+CD25^+$ cells or $CD4^+CD25^-$ cells (*DS + aPC-CD25*$^-$). Data are expressed as mean ± S.E. ($n = 7$).

tein homologous to the major histocompatibility complex class I/CD1 family proteins, expressed by a range of cell types, including leukocytes (32, 33). When it binds to EPCR, aPC cleaves protease-activated receptor-1 to exert anti-inflammatory and anti-apoptotic effects (9, 10). The presence of EPCR (and aPC) on β cells found in this study suggests that aPC exerts similar direct cytoprotective effects on these cells. The down-regulation of aPC/EPCR in PBS-treated NOD mice would likely accelerate β cell apoptosis. The reason why PC/EPCR expression is lower in NOD diabetic mice compared with its background strain BALB/c mice is not clear; however, a similar situation is seen in human circulation, as plasma levels of aPC are reduced in humans with type 1 diabetes (17, 18).

aPC-treated mice expressed higher levels of MMP-2 in pancreatic islets compared with control NOD mice. Furthermore, aPC activated MMP-2 in mouse spleen cells, which agrees with previous reports on endothelial cells, fibroblasts, and keratinocytes (34, 35). Although the best known function for matrix metalloproteinases is the degradation of extracellular matrix components, these enzymes can also regulate cytokine and chemokine activity by proteolytic processing. For example, MMP-2 efficiently cleaves and inactivates MCP-3, a CC chemokine that promotes leukocyte chemotaxis (36). This action of MMP-2 not only blocks the initiation of an inflammatory response *in vivo* but also completely abrogates pre-existing inflammation (36, 37). The specificity of aPC to activate MMP-2 is evidenced by its opposing effect on MMP-9 (24), which has similar matrix substrate specificity but amplifies inflammation by activating IL-8 (38). Although we found that treatment of NOD mice with aPC strongly increased MMP-2 expression by β cells, it did not induce MMP-9 and in fact reduced MMP-9 secretion by NOD mice spleen cells. These disparate actions of aPC on these two gelatinases may partly contribute to its inhibitory role in inflammation and in β cell death in the NOD mouse.

Considering that aPC is thought to have a biological half-life of ~20 min (39), it is noteworthy that its protective effect persisted for at least 10 weeks after the last treatment. This suggests that aPC treatment between 6 and 16 weeks of age permanently ameliorated the autoimmune process leading to β cell destruction. This long term effect could be explained by the ability of aPC to promote the number and function of Tregs and

directly suppress T cell activation and proliferation. The frequency of $CD4^+CD25^+$ T cells in the spleen, pancreatic islets, and lymph nodes was considerably higher in aPC-treated mice than control mice. Splenic $CD4^+CD25^+$ T cells from aPC-treated mice had greater suppressive capacity in adoptive transfer of diabetes into NOD.SCID recipients than cells isolated from control mice. $CD4^+CD25^+$ T cells from aPC-treated mice also displayed a stronger inhibitory effect *in vitro* on proliferation of $CD4^+CD25^-$ cells. In spleen cells, aPC significantly increased secretion of TGF-β1 and IL-2, two critical cytokines that promote differentiation of naive $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ $FoxP3^+$ Tregs (7, 40, 41) and have the potential therapeutic effects in T1D (40–42). aPC also increased expression of FoxP3, a key transcriptional regulator required not only for differentiation but also maintenance of the differentiated suppressor function of Tregs (5). These effects of aPC on Tregs are likely to occur indirectly via an action on other cells such as antigen-presenting cells. The exact mechanisms require further investigation. In addition to increasing Treg number and function, aPC appears to directly suppress $CD4^+CD25^-$ T cell activation/proliferation and therefore may also reduce the generation and migration of pathogenic T cells that mediate β cell destruction.

Although aPC can prevent diabetes in NOD mice, our results indicate that it is unable to reverse overt diabetes, when glucose levels were >16.7 mM before aPC treatment began. This may be because the dose of aPC used, which was the same as for the preventative study, was inadequate. Alternatively, most islet β cells could have been irreversibly destroyed at this stage. It is possible, however, that aPC could be therapeutically synergistic in combination with another agent that reduces the burden of pathogenic effector T cells.

In summary, we describe a novel effect of the anticoagulant aPC to prevent development of autoimmune diabetes. aPC appears to act by dampening inflammation and suppressing the autoimmune response by increasing the frequency and function of Tregs. These findings, with Food and Drug Administration approval of aPC for the treatment of sepsis, lend support for a trial of aPC to prevent T1D in at-risk individuals.

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REFERENCES

1. Bluestone, J. A., Herold, K., and Eisenbarth, G. (2010) Genetics, pathogenesis, and clinical interventions in type 1 diabetes. *Nature* **464,** 1293–1300
2. Cnop, M., Welsh, N., Jonas, J. C., Jörns, A., Lenzen, S., and Eizirik, D. L. (2005) Mechanisms of pancreatic beta cell death in type 1 and type 2 diabetes. Many differences, few similarities. *Diabetes* **54,** S97–S107
3. Adorini, L., Gregori, S., and Harrison, L. C. (2002) Understanding autoimmune diabetes. Insights from mouse models. *Trends Mol. Med.* **8,** 31–38
4. Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008) Regulatory T cells and immune tolerance. *Cell* **133,** 775–787
5. Ochs, H. D., Ziegler, S. F., and Torgerson, T. R. (2005) FOXP3 acts as a rheostat of the immune response. *Immunol. Rev.* **203,** 156–164
6. Wildin, R. S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J. L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., Bricarelli, F. D., Byrne, G., McEuen, M., Proll, S., Appleby, M., and Brunkow, M. E. (2001) X-linked neonatal diabetes mellitus, enteropathy, and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* **27,** 18–20
7. Green, E. A., Gorelik, L., McGregor, C. M., Tran, E. H., and Flavell, R. A. (2003) $CD4^+CD25^+$ T regulatory cells control anti-islet $CD8^+$ T cells through TGF-β-TGF-β receptor interactions in type 1 diabetes. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 10878–10883
8. Salomon, B., Lenschow, D. J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., and Bluestone, J. A. (2000) B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity* **12,** 431–440
9. Griffin, J. H., Fernández, J. A., Mosnier, L. O., Liu, D., Cheng, T., Guo, H., and Zlokovic, B. V. (2006) The promise of protein C. *Blood Cells Mol. Dis.* **36,** 211–216
10. Jackson, C. J., and Xue, M. (2008) Activated protein C. An anticoagulant that does more than stop clots. *Int. J. Biochem. Cell Biol.* **40,** 2692–2697
11. Grey, S. T., Tsuchida, A., Hau, H., Orthner, C. L., Salem, H. H., and Hancock, W. W. (1994) Selective inhibitory effects of the anticoagulant activated protein C on the responses of human mononuclear phagocytes to LPS, IFN-γ, or phorbol ester. *J. Immunol.* **153,** 3664–3672
12. Taylor, F. B., Jr., Chang, A., Esmon, C. T., D'Angelo, A., Vigano-D'Angelo, S., and Blick, K. E. (1987) Protein C prevents the coagulopathic and lethal effects of *Escherichia coli* infusion in the baboon. *J. Clin. Invest.* **79,** 918–925
13. Esmon, C. T. (2004) Structure and functions of the endothelial cell protein C receptor. *Crit. Care Med.* **32,** S298-S301
14. Yamanel, L., Yamenel, L., Mas, M. R., Comert, B., Isik, A. T., Aydin, S., Mas, N., Deveci, S., Ozyurt, M., Tasci, I., and Unal, T. (2005) The effect of activated protein C on experimental acute necrotizing pancreatitis. *Crit. Care* **9,** R184–R190
15. Contreras, J. L., Eckstein, C., Smyth, C. A., Bilbao, G., Vilatoba, M., Ringland, S. E., Young, C., Thompson, J. A., Fernández, J. A., Griffin, J. H., and Eckhoff, D. E. (2004) Activated protein C preserves functional islet mass after intraportal transplantation. A novel link between endothelial cell activation, thrombosis, inflammation, and islet cell death. *Diabetes* **53,** 2804–2814
16. Isermann, B., Vinnikov, I. A., Madhusudhan, T., Herzog, S., Kashif, M., Blautzik, J., Corat, M. A., Zeier, M., Blessing, E., Oh, J., Gerlitz, B., Berg, D. T., Grinnell, B. W., Chavakis, T., Esmon, C. T., Weiler, H., Bierhaus, A., and Nawroth, P. P. (2007) Activated protein C protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis. *Nat. Med.* **13,** 1349–1358
17. Gruden, G., Olivetti, C., Cavallo-Perin, P., Bazzan, M., Stella, S., Tamponi, G., and Pagano, G. (1997) Activated protein C resistance in type I diabetes. *Diabetes Care* **20,** 424–425
18. Vukovich, T. C., and Schernthaner, G. (1986) Decreased protein C levels in patients with insulin-dependent type I diabetes mellitus. *Diabetes* **35,** 617–619
19. Wu, J., Zhou, Z., Ye, S., Dai, H., Ma, L., Xu, X., and Li, X. (2000) Detection of soluble endothelial protein C receptor (sEPCR) in patients with CHD, DM, and SLE. *Zhonghua Xue Ye Xue Za Zhi* **21,** 472–474
20. Takei, I., Asaba, Y., Kasatani, T., Maruyama, T., Watanabe, K., Yanagawa, T., Saruta, T., and Ishii, T. (1992) Suppression of development of diabetes in NOD mice by lactate dehydrogenase virus infection. *J. Autoimmun.* **5,** 665–673
21. Li, D. S., Yuan, Y. H., Tu, H. J., Liang, Q. L., and Dai, L. J. (2009) A protocol for islet isolation from mouse pancreas. *Nat. Protoc.* **4,** 1649–1652
22. Thakur, A., and Willcox, M. D. (1998) Cytokine and lipid inflammatory mediator profile of human tears during contact lens associated inflammatory diseases. *Exp. Eye Res.* **67,** 9–19
23. Herron, G. S., Banda, M. J., Clark, E. J., Gavrilovic, J., and Werb, Z. (1986) Secretion of metalloproteinases by stimulated capillary endothelial cells. II. Expression of collagenase and stromelysin activities is regulated by endogenous inhibitors. *J. Biol. Chem.* **261,** 2814–2818
24. Xue, M., March, L., Sambrook, P. N., and Jackson, C. J. (2007) Differential regulation of matrix metalloproteinase 2 and matrix metalloproteinase 9 by activated protein C. Relevance to inflammation in rheumatoid arthritis. *Arthritis Rheum.* **56,** 2864–2874

25. Jackson, C., Whitmont, K., Tritton, S., March, L., Sambrook, P., and Xue, M. (2008) New therapeutic applications for the anticoagulant, activated protein C. *Expert Opin. Biol. Ther.* **8,** 1109–1122
26. Whitmont, K., Reid, I., Tritton, S., March, L., Xue, M., Lee, M., Fulcher, G., Sambrook, P., Slobedman, E., Cooper, A., and Jackson, C. (2008) Treatment of chronic leg ulcers with topical activated protein C. *Arch. Dermatol.* **144,** 1479–1483
27. Husari, A. W., Khayat, A., Awdeh, H., Hatoum, H., Nasser, M., Mroueh, S. M., Zaatari, G., El-Sabban, M., and Dbaibo, G. S. (2010) Activated protein C attenuates acute lung injury and apoptosis in a hyperoxic animal model. *Shock* **33,** 467–472
28. Eldor, R., Yeffet, A., Baum, K., Doviner, V., Amar, D., Ben-Neriah, Y., Christofori, G., Peled, A., Carel, J. C., Boitard, C., Klein, T., Serup, P., Eizirik, D. L., and Melloul, D. (2006) Conditional and specific NF-κB blockade protects pancreatic beta cells from diabetogenic agents. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 5072–5077
29. Joyce, D. E., Gelbert, L., Ciaccia, A., DeHoff, B., and Grinnell, B. W. (2001) Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. *J. Biol. Chem.* **276,** 11199–11203
30. Kerschen, E., Hernandez, I., Zogg, M., Jia, S., Hessner, M. J., Fernandez, J. A., Griffin, J. H., Huettner, C. S., Castellino, F. J., and Weiler, H. (2010) Activated protein C targets $CD8^+$ dendritic cells to reduce the mortality of endotoxemia in mice. *J. Clin. Invest.* **120,** 3167–3178
31. Lay, A. J., Donahue, D., Tsai, M. J., and Castellino, F. J. (2007) Acute inflammation is exacerbated in mice genetically predisposed to a severe protein C deficiency. *Blood* **109,** 1984–1991
32. Feistritzer, C., Mosheimer, B. A., Sturn, D. H., Riewald, M., Patsch, J. R., and Wiedermann, C. J. (2006) Endothelial protein C receptor-dependent inhibition of migration of human lymphocytes by protein C involves epidermal growth factor receptor. *J. Immunol.* **176,** 1019–1025
33. Galligan, L., Livingstone, W., Volkov, Y., Hokamp, K., Murphy, C., Lawler, M., Fukudome, K., and Smith, O. (2001) Characterization of protein C receptor expression in monocytes. *Br. J. Haematol.* **115,** 408–414
34. Nguyen, M., Arkell, J., and Jackson, C. J. (2000) Activated protein C directly activates human endothelial gelatinase A. *J. Biol. Chem.* **275,** 9095–9098
35. Xue, M., Thompson, P., Kelso, I., and Jackson, C. (2004) Activated protein C stimulates proliferation, migration, and wound closure, inhibits apoptosis, and up-regulates MMP-2 activity in cultured human keratinocytes. *Exp. Cell Res.* **299,** 119–127
36. McQuibban, G. A., Gong, J. H., Wong, J. P., Wallace, J. L., Clark-Lewis, I., and Overall, C. M. (2002) Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties *in vivo*. *Blood* **100,** 1160–1167
37. Itoh, T., Matsuda, H., Tanioka, M., Kuwabara, K., Itohara, S., and Suzuki, R. (2002) The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis. *J. Immunol.* **169,** 2643–2647
38. Van den Steen, P. E., Proost, P., Wuyts, A., Van Damme, J., and Opdenakker, G. (2000) Neutrophil gelatinase B potentiates interleukin-8 10-fold by amino-terminal processing, whereas it degrades CTAP-III, PF-4, and GRO-α and leaves RANTES and MCP-2 intact. *Blood* **96,** 2673–2681
39. Knoebl, P. N. (2008) Severe congenital protein C deficiency. The use of protein C concentrates (human) as replacement therapy for life-threatening blood-clotting complications. *Biologics* **2,** 285–296
40. Tang, Q., Adams, J. Y., Penaranda, C., Melli, K., Piaggio, E., Sgouroudis, E., Piccirillo, C. A., Salomon, B. L., and Bluestone, J. A. (2008) Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* **28,** 687–697
41. Zheng, S. G., Wang, J., Wang, P., Gray, J. D., and Horwitz, D. A. (2007) IL-2 is essential for TGF-β to convert naive $CD4^+CD25^-$ cells to $CD25^+Foxp3^+$ regulatory T cells and for expansion of these cells. *J. Immunol.* **178,** 2018–2027
42. Park, L., Lee, E., Lee, S., Lim, M., Hong, H., Shin, G., and Park, Y. (2008) TGFβ plasmid construction and delivery for the prevention of type 1 diabetes. *Ann. N.Y. Acad. Sci.* **1150,** 177–182