iniscent of previous reports suggesting thymocyte enclosure by thymic epithelial cells (*13, 14*). This unique cellular topology might play a role in some aspect of thymic selection, perhaps serving to increase the surface area of the association.

In our system, there was a >3-fold increase in the proportion of thymocytes contacting wild-type versus $MHC^{-/-}$ stromal cells (Fig. 1C); this finding implies that most of the described interactions depended on MHC recognition. In addition, we observed multiple stable and dynamic contacts within large clusters of thymocytes (Fig. 2) (movie S3) and these structures were formed only around MHC-bearing stromal cells (Fig. 1D). Thus, although a small fraction of the described interactions may have been MHC independent, a substantial proportion of both stable of dynamic contacts observed in our system were associated with MHC recognition. Future experiments with molecular markers of TCR signaling are needed to directly identify individual MHC-driven interactions.

This study documents the cellular interactions between thymocytes and stromal cells during positive selection within a three-dimensional tissue environment. Mature T cell activation can result from a stable, long-lasting interaction with an APC (*15, 16*) but may also result from summing of multiple transient encounters with APCs (*11, 17*). Our observation that MHC recognition by thymocytes is associated with both stable and dynamic contacts with thymic stromal cells raises the possibility that both modes of TCR signaling may occur during positive selection. The basis for the observed diversity of thymocyte–thymic stromal cell interactions is unclear. One possibility is that these different patterns of interaction involve distinct types of stromal cells. This is unlikely to be the sole explanation, because both stable and dynamic contacts have been repeatedly observed at the surface of the same stromal cell (Fig. 2A) (movies S3 and S10). Alternatively, the different interaction patterns could be associated with different signals or could correspond to different stages of positive selection. Following the history of TCR signals received by an individual thymocyte during the whole process of thymocyte maturation remains challenging but ultimately should help to answer these questions. More generally, the use of TPLSM to track the fate of immune cells within lymphoid tissues should provide new insights into lymphocyte biology and development.

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Supplementary Online Material

www.sciencemag.org/cgi/content/full/296/5574/1876/DC1
Figs. S1 to S3
Movies S1 to S11

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Activation of Endothelial Cell Protease Activated Receptor 1 by the Protein C Pathway

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The coagulant and inflammatory exacerbation in sepsis is counterbalanced by the protective protein C (PC) pathway. Activated PC (APC) was shown to use the endothelial cell PC receptor (EPCR) as a coreceptor for cleavage of protease activated receptor 1 (PAR1) on endothelial cells. Gene profiling demonstrated that PAR1 signaling could account for all APC-induced protective genes, including the immunomodulatory monocyte chemoattractant protein-1 (MCP-1), which was selectively induced by activation of PAR1, but not PAR2. Thus, the prototypical thrombin receptor is the target for EPCR-dependent APC signaling, suggesting a role for this receptor cascade in protection from sepsis.

Tissue factor–initiated coagulation in sepsis triggers a lethal response (*1–3*) that may involve coagulation protease–mediated proinflammatory signaling through heterotrimeric GTP-binding protein (G-protein)–coupled protease-activated receptors (PARs) (*4–7*). The PC pathway protects animals from *Escherchia coli*–induced lethality (*8–10*) and APC reduces mortality in patients with severe sepsis (*11*). PC bound to EPCR is activated by a coagulation feedback loop in which traces of thrombin, once bound to thrombomodulin, specifically activate PC (*12*). APC is a trypsin-like coagulation protease and PARs serve as the cellular sensors for these enzymes (*4, 5*). The PC anticoagulant pathway operates on endothelial cells that express PAR1 and PAR2 along with EPCR. Proteolytic signaling by APC induces protective responses in endothelial cells (*13*), but the involvement of PARs in this process remains unclear.

PAR1-deficient murine fibroblasts are not activated by proteases unless transfected with an appropriate PAR (*6, 7*). We exploited the unresponsiveness of these cells to APC to characterize the requirement for protease signaling by APC (*14*). Stimulation with APC was performed in the presence of 100 nM hirudin, which blocks all cell-surface thrombin-mediated PAR1 signaling (*15*). PAR1-deficient fibroblasts were responsive to 20 nM APC only when EPCR was coexpressed with a PAR (Fig. 1A). Expression of EPCR or PAR2 alone or coexpression of PAR2 with an EPCR mutant (EPCR A154) deficient in APC binding (*16*) failed to support APC signaling. These results show that only EPCR-bound APC can efficiently activate PARs.

Coexpression of EPCR and PAR1 also resulted in responsiveness to APC (Fig. 1B). Like thrombin signaling, APC signaling was inhibited by PAR1 cleavage–blocking antibodies, whereas antibodies to PAR1 did not prevent signaling by the

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direct PAR1 agonist, excluding nonspecific PAR1 desensitization. These results indicate that APC signals through a proteolytic mechanism and not through a protease-independent receptor crosstalk between EPCR and PARs. There was no difference in the APC dose response of PAR1 versus PAR2 activation; thus, presentation of APC by binding to EPCR, rather than specific features in the scissile bond of the PARs, determined the efficiency of PAR cleavage. The finding that the prototypical thrombin receptor PAR1 could be activated by other proteases agrees with recent data indicating that coagulation factor Xa also cleaves PAR1 (*6*, *7*, *15*). EPCR has a potential cytoplasmic palmitoylation site that may influence the cosignaling properties of the receptor. Mutation of this single cytoplasmic Cys to Ser did not influence signaling of EPCR-bound APC through PAR1 or PAR2 (Fig. 1, A and B). Thus, palmitoylation of EPCR is not a strict requirement for APC-dependent cleavage of PARs.

Although the heterologous overexpression experiments demonstrated that EPCR-bound APC could activate PAR1 and PAR2, PAR cleavage specificity of EPCR-bound APC in primary endothelial cells remained to be established. In endothelial cells, APC induced mitogen-activated protein kinase (MAPK) phosphorylation, a common response of PAR1 and PAR2 signaling (Fig. 1, C and D). Active site–blocked APC failed to induce MAPK phosphorylation, demonstrating a proteolytic mechanism. Moreover, APC, but not thrombin, responses were inhibited by a 10-fold molar excess of active site–blocked APC that competed for EPCR binding, confirming receptor dependence of APC signaling in endothelial cells. Cleavage-blocking antibodies to PAR1 inhibited APC signaling without affecting the response to the PAR2 agonist peptide; therefore, MAPK phosphorylation by the PC pathway on endothelial cells was dependent on PAR1.

To determine whether PAR activation accounts for APC-dependent gene induction in endothelial cells, large scale mRNA expression profiles were determined for endothelial cells stimulated with APC or with selective agonist peptides for PAR1 or PAR2 (*14*). Stimulation for 90 min was chosen to capture both early transcription-related and delayed effector gene induction events. In three independent experiments, 1% of the ~7000 represented genes were reproducibly up-regulated by stimulation with APC or a PAR agonist (Fig. 2). PAR1 and PAR2 agonists induced most genes to a similar extent (Fig. 2A), with the prominent exception of the transcript of MCP-1, which was induced only by the PAR1 agonist.

Overall, gene induction by APC correlated with direct agonist stimulation of PAR1 (Fig. 2B) as well as PAR2 (Fig. 2C). Some genes were induced by PAR agonist peptides but not by APC stimulation. Most important, APC induced the PAR1-specific MCP-1 transcript, but none of the transcripts were induced by APC or by the PAR1 agonist. An additional 1% of the genes represented on the microarray had higher interexperimental variability in agonist induction. None of the PAR2-selective transcripts in this gene set was induced by APC, but APC up-regulated other PAR1-induced genes (table S1). Hierarchical clustering of the larger gene set further confirmed the similarity between APC signaling and the PAR1 response. All transcriptional responses to APC signaling of endothelial cells were accounted for by PAR1 signaling.

In time-course experiments in primary endothelial cells (*14*), MCP-1 was similarly induced by APC and the PAR1 agonist but not by direct activation of PAR2, whereas all three agonists induced the nuclear hormone receptor TR3 with similar kinetics (Fig. 3A). The transcript for Down syndrome critical

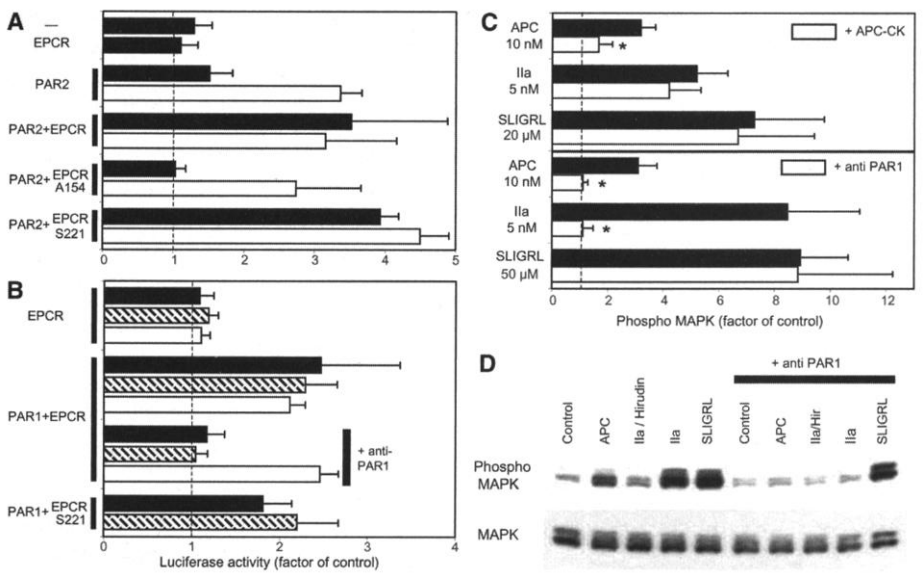

Fig. 1. Cell activation by APC is dependent on EPCR and PAR expression. (**A** and **B**) Induction of Egr-1 promoter activity in PAR1-deficient fibroblasts transfected with human EPCR, EPCR variants with $Tyr^{154}\rightarrow Ala^{154}$ (EPCR A154) or $Cys^{221}\rightarrow Ser^{221}$ (EPCR S221) substitutions, or human PAR2 or PAR1. Fold induction of luciferase activity upon stimulation with 20 nM APC (solid bars), PAR agonist peptide [open bars; 100 μM SLIGRL (*28*) in (A) and 10 μM TFLLRNPNDK in (B)], or 5 nM thrombin (crosshatched bars) is shown (n = 3 repeat experiments, mean ± SD). (**C** and **D**) APC signaling in human umbilical vein endothelial cells (HUVECs) measured as Erk1/2 phosphorylation. Stimulation with the indicated agonists in the absence (solid bars) or presence of 100 nM chloromethylketone-modified APC (APC-CK) (open bars, n = 3 repeat experiments, mean ± SD) or cleavage blocking antibodies to-PAR1 (open bars, n = 5 repeat experiments, mean ± SD), * $P <$ 0.05. A representative Western blot is shown in (D).

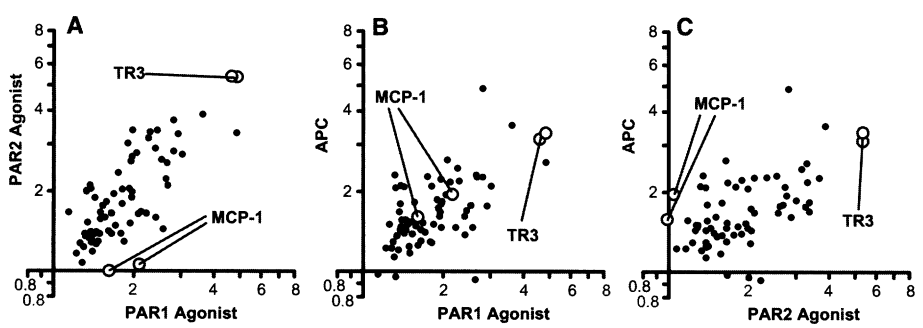

Fig. 2. APC- and PAR1-specific agonist induced similar genes in human endothelial cells. (**A**) A plot of fold induction (average of three experiments) by PAR1 versus PAR2 agonist peptides demonstrated selective up-regulation of MCP-1 by the PAR1 agonist. Both MCP-1 and the nuclear receptor TR3 were represented by two independent probe sets. Comparison of genes induced by APC versus PAR1 (**B**) and PAR2 (**C**) agonist peptides showed up-regulation of MCP-1 by APC stimulation.

region gene 1 (DSCR1), a negative regulator of calcineurin, was not induced by APC stimulation but rather showed selective up-regulation upon PAR2 stimulation (Fig. 3A). These results show that APC cannot induce a response that is typical for PAR2 agonist stimulation. As expected, antibodies to PAR1 blocked the induction of the PAR1-specific MCP-1 gene by APC, but antibodies to PAR1 also inhibited the APC-dependent induction of genes responsive to either PAR1 or PAR2 stimulation (Fig. 3B). These experiments demonstrate that PAR2 cannot substitute for PAR1 in APC signaling of endothelial cells, because PAR1-selective and PAR2-permissive APC responses were blocked by antibodies to PAR1. Simultaneous expression of PAR1 and PAR2 in knockout fibroblasts did not produce a similar PAR1 specificity of APC signaling, excluding the possibility that PAR1 coexpression restricts PAR2 cleavage by EPCR-bound APC. The selective APC–mediated activation of PAR1 in endothelial cells may result from a cell type–specific colocalization of PAR1 and EPCR in a particular microenvironment or from potential posttranslational modifications of PAR2 (*17*) that may restrict cleavage by APC.

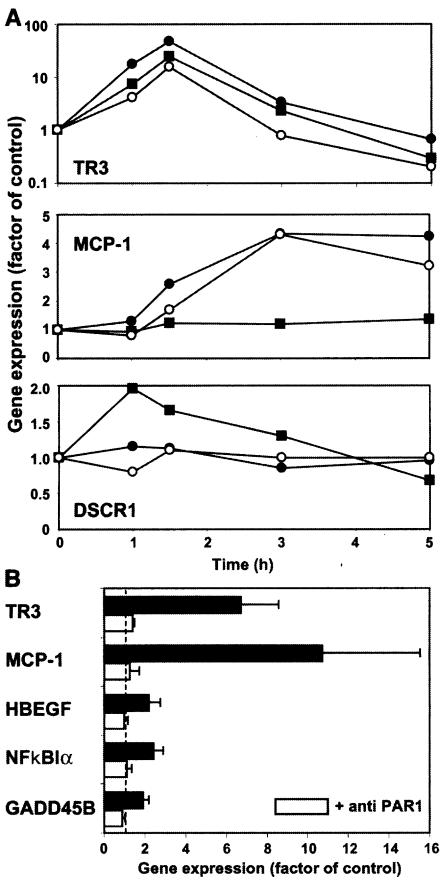

Fig. 3. (**A**) The time course of TR3, MCP-1, and DSCR1 induction in HUVECs by PAR1 agonist (solid circles), PAR2 agonist (solid squares), and APC (open circles) was analyzed by quantitative PCR. Fold inductions, normalized to glyceraldehyde phosphate dehydrogenase levels, are shown for a typical experiment. (**B**) Fold inductions of the indicated genes after stimulation with 10 nM APC/100 nM hirudin in the absence and presence of antibody to PAR1 were determined by quantitative PCR (n = 3 repeat experiments, mean ± SD). HBEGF, heparin-binding EGF-like growth factor; NF κBIα, NFκB Inhibitor α; GADD45B, growth arrest and DNA damage-inducible gene beta.

Pretreatment of cells with ~10-fold higher concentrations of APC inhibits inflammatory signaling and induces cell survival in endothelial cells and monocytes (*13*, *18*, *19*). Prolonged stimulation with APC also up-regulates genes in endothelial cells (*13*); and two of the previously identified genes—the anti-apoptotic BCL2-related protein A1 and inhibitor of apoptosis protein 1—were induced by APC and PAR agonists at the early time point of our experiments (table S2). APC or PAR stimulations induced additional counterregulatory mechanisms of proinflammatory signaling pathways (table S2). These included negative regulators of the G protein-coupled receptor (AKAP12), the MAPK/Egr (DUSP1 and -5, NAB1), or the nuclear factor kappa B (NF-κBIα) pathway. Other APC- and PAR-induced genes have crucial functions in terminating tumor necrosis factor (TNF) signaling in vivo [TNFAIP3 (*20*) and ZFP36 (*21*)] or are anti-apoptotic (TNFAIP3, IER3, or GADD45B). The concordant up-regulation of protective genes by PAR1 agonist and APC suggests that all anti-inflammatory and anti-apoptotic effects of APC signaling are PAR-mediated in endothelial cells.

How can activation of the prototypical thrombin receptor PAR1 by APC be relevant if the generation of APC is thrombin-dependent? Infusion of low concentrations of thrombin into primates does not elicit platelet activation, a highly sensitive PAR-dependent response. Rather, thrombin activates the PC pathway (*22*) because of thrombin's binding to endothelial cell thrombomodulin, which inhibits thrombin-dependent PAR signaling (*23*). Given that thrombin-thrombomodulin activates EPCR-bound PC (*24*) and that EPCR-bound APC activates PAR1, the physiological activation of the PC pathway emerges as a highly relevant activator of protective PAR1 signaling on endothelial cells. EPCR's protective roles in endotoxin-challenged animals (*8*) emphasize the importance of this receptor cascade for sepsis.

The production of inflammatory cytokines in sepsis disables the physiological anticoagulant pathway by down-regulating thrombomodulin, but EPCR remains detectable on thrombomodulin-depleted endothelial cells (*25*). Therapeutically administered APC may use residual EPCR as a coreceptor in signaling to achieve protection from severe sepsis (*11*). In the escalation of sepsis syndrome, thrombin is unlikely to reproduce endothelium-restricted, protective PAR1 signaling of APC, because thrombin's targets include PARs on a number of cell types that are activated in the context of microthrombotic organ dysfunction. Unexpectedly, MCP-1 was identified as the product of a gene that is selectively upregulated by APC-dependent PAR1 signaling in endothelial cells. In systemically and locally induced sepsis models, MCP-1 is protective (*26*, *27*). In addition to the direct endothelial protective functions of APC, local MCP-1 induction by APC may promote indirect anti-inflammatory effects through an immunomodulatory chemokine network that controls the host defense in sepsis.

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28. Single-letter abbreviations for the amino acid residues are as follows: D, Asp; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; R, Arg; S, Ser; and T, Thr.

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Supporting Online Material

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Materials and Methods
Tables S1 and S2

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