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Antiinflammatory cAMP signaling and cell migration genes co-opted by the anthrax bacillus

Chun Kim*, Sarah Wilcox-Adelman†, Yasuyo Sano*, Wei-Jen Tang‡, R. John Collier§, and Jin Mo Park*¶

*Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129; †Boston Biomedical Research Institute, Watertown, MA 02472; ‡Ben-May Institute for Cancer Research, University of Chicago, Chicago, IL 60637; and §Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115

Bacillus anthracis, the etiologic agent of anthrax, avoids immune surveillance and commandeers host macrophages as a vehicle for lymphatic spreading. Here, we show that *B. anthracis* edema toxin (ET), via its adenylyl cyclase activity, dramatically increases the motility of infected macrophages and the expression of vascular endothelial growth factor. The transcription factor CREB and the syndecan-1 gene, a CREB target, play crucial roles in ET-induced macrophage migration. These molecular and cellular responses occur in macrophages engaged in antiinflammatory G protein-coupled receptor activation, thus illustrating a common signaling circuitry controlling resolution of inflammation and host cell hijacking by *B. anthracis*.

edema toxin | macrophage | *Bacillus anthracis*

Protective antigen (PA), lethal factor (LF), and edema factor (EF), the three proteins comprising anthrax toxin, are essential for the virulence of *Bacillus anthracis* in mammalian hosts (1). LF and EF translocate into the cytosol of mammalian host cells through transmembrane pores formed in the endosome by PA and modify host cell signaling across the prokaryotic–eukaryotic boundary (2). The endoprotease and adenylyl cyclase activities of LF and EF, respectively, are pivotal for this process.

Inhalation anthrax, the most lethal form of *B. anthracis* infection, begins by phagocytic engulfment of the spores by alveolar macrophages and their subsequent intracellular germination (3). Lung-resident dendritic cells also offer an intracellular niche for the establishment of the infection (4, 5). During this early phase of anthrax pathogenesis, *B. anthracis*-infected macrophages behave in a fashion reminiscent of the phenotype of macrophages during resolution of inflammation: suppression of immune responses and lymphatic migration. The major immune suppressive mechanism in anthrax pathogenesis is likely inhibition of MAPK signaling by LF (6, 7). The intracellular bacteria are carried to regional lymph nodes by migrating host cells and ultimately spread to the systemic circulation (8), causing high levels of mortality. To date, it is unclear whether *B. anthracis* actively modifies the migratory behavior of its host cells to promote efficient spreading, and if so, how.

Herein, we describe our finding that *B. anthracis* infection enhances macrophage migration through EF-mediated activation of cAMP signaling and the transcription factor CREB. Our search of the transcriptional targets activated by EF and CREB identifies the heparan sulfate transmembrane proteoglycan syndecan-1 as critically required for the pathogen-induced host cell migration. Finally, we show that certain antiinflammatory G protein-coupled receptors (GPCRs) that are highly expressed in macrophages can promote macrophage migration through a similar signaling mechanism. These results reveal a common molecular basis for macrophage trafficking in inhalation anthrax and during the resolution of inflammation.

Results

Modulation of Macrophage Signaling and Motility by Anthrax EF. It was reported that antibody-mediated neutralization of PA or loss of the plasmid encoding the anthrax toxin proteins reduces the pathogen's dissemination from the original infection site (9, 10). Therefore, in addition to playing a role in evading host immunity, anthrax toxin may contribute to the regulation of host cell migration. In this regard, we particularly noted the ability of EF to trigger cAMP signaling, as cAMP-elevating agents have been shown to facilitate motility of cells akin to macrophages in certain settings (11, 12). In our experimental approach we sought *ex vivo* models that could recapitulate the migration of *B. anthracis*-infected host cells and enable analysis of its molecular regulatory mechanism. We fed *B. anthracis* spores to mouse bone marrow-derived macrophages (BMDMs) and found that the resulting intracellular infection by the germinated bacteria caused enhanced migration of BMDMs in a transwell chamber assay (Fig. 1A). Addition of adefovir dipivoxil (AD), a specific EF inhibitor (13), significantly attenuated infection-induced migration of BMDMs, but did not affect the number of viable intracellular bacteria (Fig. 1B), arguing against a secondary, antibacterial effect of the inhibitor on the host cell behavior. EF is expected to trigger cAMP signaling in host cells through its adenylyl cyclase activity. Increased intracellular cAMP activates protein kinase A (PKA) and PKA-mediated phosphorylation of the cAMP-responsive transcription factor CREB. Correspondingly, *B. anthracis* infection induced CREB phosphorylation and transcription of the CREB target gene *Dusp1* in macrophages in an EF-dependent manner (Fig. 1 C–E). These results indicate that EF produced from intracellular *B. anthracis* can activate a transcription program within host cells and modify their cellular response to the infection.

We next tested whether purified *B. anthracis* toxin proteins enhance macrophage migration and CREB activation without other infection-associated events. Edema toxin (ET), a mixture of PA and EF, but not either protein alone, dramatically increased macrophage migration and CREB phosphorylation (Fig. 1 F and G). LF, alone or in conjunction with PA, was without effect. The enhanced migration after ET exposure correlated with changes in cell shape and cytoskeletal structure. The initial well spread morphology of the cells was dramatically reduced as early as 6 h after ET treatment [Fig. 1H and supporting information (SI) Fig. S1]. The ET-treated cells became more rounded and formed filopodial protrusions. Concomitantly, actin was redistributed to the cell margin and filopodia, likely rendering ET-exposed macrophages poised for rapid movement.
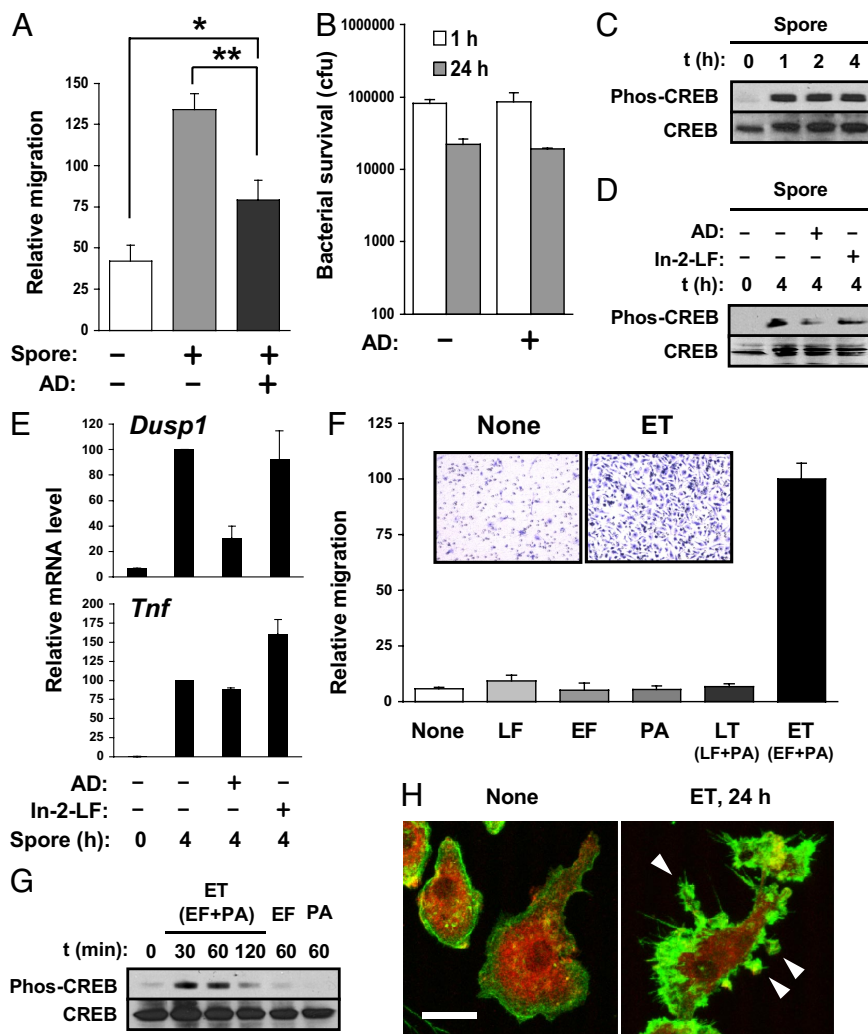
Fig. 1. Enhanced macrophage migration and CREB activation after *B. anthracis* infection mediated by EF action. (*A*) Migration of BMDMs infected with *B. anthracis* spores at moi of 10 was analyzed by a transwell assay without or with the EF inhibitor AD (20 μM) (**, $P < 0.01$; *, $P < 0.05$). (*B*) *B. anthracis* survival (cfu) in BMDMs was determined at the time points indicated. (*C–E*) BMDMs were infected with spores without or with AD and the LF inhibitor In-2-LF (20 μM). (*C* and *D*) At the indicated time points, cell lysates were prepared and analyzed by immunoblotting with antibodies against Ser-133-phosphorylated (Phos) and total CREB. (*E*) Cellular RNA was isolated, and relative expression of the indicated genes was determined by real-time qPCR. (*F*) Migration of BMDMs incubated with purified recombinant toxin proteins (1 μg/ml each) was examined by a transwell assay. (*Inset*) Cells that had migrated to the lower surface of the transwell chamber were visualized by crystal violet staining. (*G*) CREB phosphorylation in BMDMs incubated with toxin proteins was analyzed as in *C*. (*H*) BMDMs before and after incubation with ET (24 h) were stained for vinculin (red) and F-actin (green). The micrographs show merged images. White arrowheads indicate actin-rich filopodial protrusions. (Scale bar: 25 μm.)

Host Target Genes Activated by Anthrax EF. Although EF and cAMP may possibly activate a PKA-independent signaling module via the guanine nucleotide exchange factor EPAC (14, 15), migration of BMDMs was greatly promoted by incubation with a PKA-specific, but not EPAC-specific, cAMP analog (Fig. S2). This finding suggests that EF- and cAMP-induced macrophage migration, like CREB activation, is linked to the PKA signaling module. PKA is known to regulate several other transcriptional targets besides CREB. We therefore hypothesized that *B. anthracis* mobilizes host macrophages by activating host gene expression through EF- and PKA-regulated transcription factors. To substantiate this idea, we analyzed whole-genome expression profiles of control and ET-treated BMDMs. Of the 41,534 DNA microarray spots scanned for RNA levels after 2 and 4 h of ET treatment, 59 and 81 spots, respectively, were identified as ET-inducible (Fig. S3 *A* and *B*). Only a few genes showed repressed expression in ET-treated cells. Validation of ET-induced gene expression by real-time quantitative PCR (qPCR) analysis and an RNase protection assay (Fig. 2 *A* and *B*)

focused our attention on host genes that may regulate the migration of *B. anthracis*-infected macrophages. Among these genes are those that encode VEGF and other secreted signaling molecules. In our independent analysis of macrophage culture supernatants, VEGF was identified as the single most prominent cytokine released from ET-exposed BMDMs (Fig. 2*C*). VEGF, a potent inducer of edema and lymphangiogenesis (16), may function to create an environment amenable to the efflux of *B. anthracis*-infected macrophages via the lymphatics. Other notable ET-inducible genes whose function has been related to cell migration include *Sdc1* (17, 18), which encodes the transmembrane proteoglycan syndecan-1, *Ccrl2* (19), an orphan chemokine receptor gene, and *Gja1*, the connexin-43 gene (20, 21). Expression of these genes was also responsive to the adenylyl cyclase activator forskolin and to *B. anthracis* spore infection in a fashion inhibitable by AD (Fig. S3 *C* and *D*). Collectively, these data demonstrate that cAMP signaling is a major pathway activating the expression of *Vegfa*, *Sdc1*, and other ET-inducible genes in host macrophages during *B. anthracis* infection.
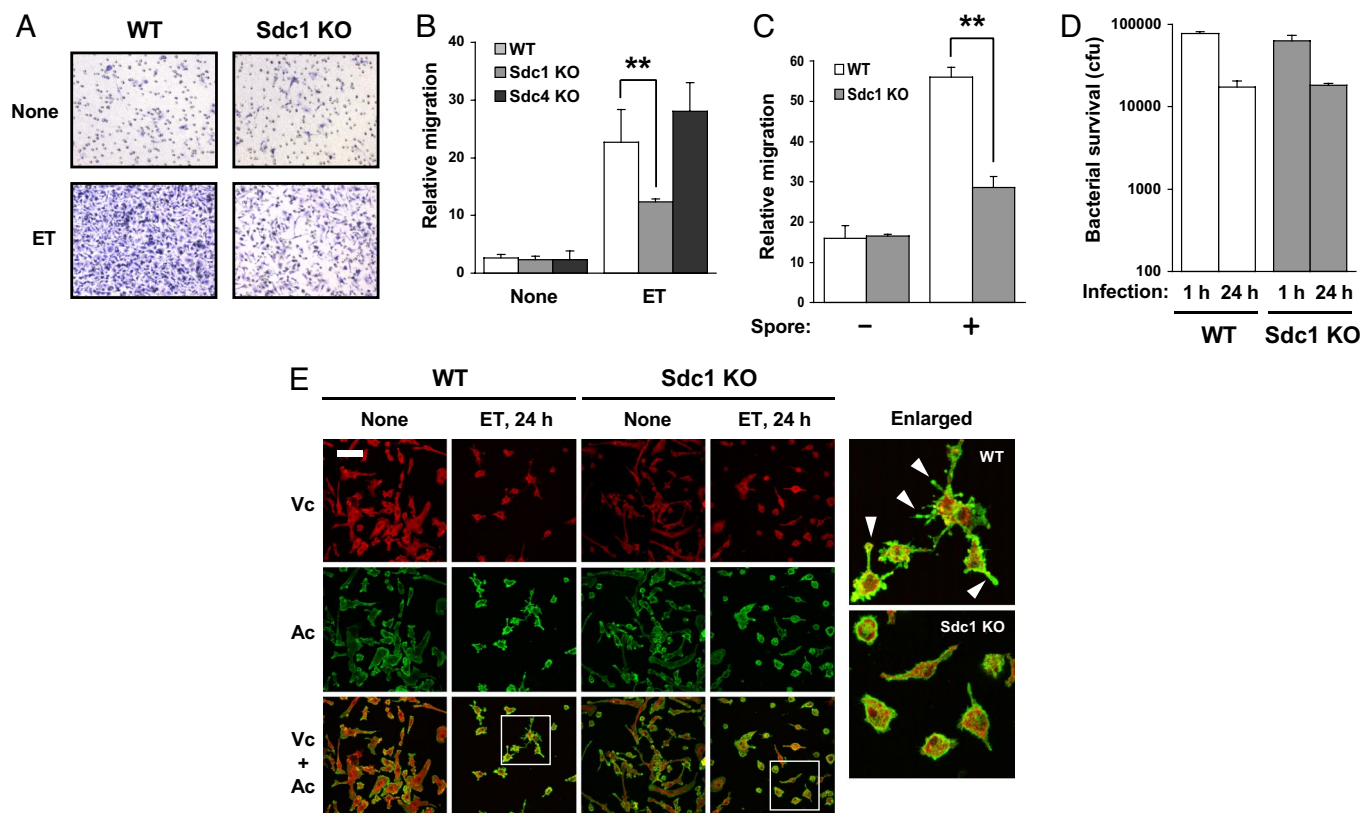
Fig. 2. A role for CREB-dependent ET-inducible genes in macrophage migration. (*A*) The expression levels of genes induced in ET-exposed BMDMs were determined by DNA microarray analysis and validated by real-time qPCR. Shown is the fold induction of genes in cells treated with ET (1 μg/ml PA + 1 μg/ml EF) for the indicated durations versus those left untreated. (*B*) Expression of selected ET-inducible genes in *A* was analyzed by RNase protection assay. (*C*) The concentration of various cytokines was determined from culture supernatants of BMDMs left untreated or treated with ET or LPS for 24 h. (*D*) BMDMs were infected with lentiviruses expressing GFP or the dominant-negative CREB inhibitor A-CREB and treated with ET. After 4 h, cellular RNA was isolated, and the expression levels of the genes were determined by real-time qPCR. Individual bars denote the percent of ET induction of each gene in A-CREB-expressing cells compared with that in GFP-expressing cells. (*E*) Migration of BMDMs infected with lentiviruses expressing GFP or A-CREB was measured in the absence or presence of ET as in Fig. 1*F*.

Roles for CREB Target Genes in ET-Induced Macrophage Migration. We examined ET-inducible gene promoters and found that many possess cAMP-responsive elements (CREs), CREB-binding sites (Fig. S4). To directly assess the requirement for CREB in ET-induced gene expression and macrophage migration, a dominant-negative CREB inhibitor, A-CREB, was lentivirally expressed in BMDMs. CREB inhibition prevented transcriptional induction of many, but not all, of the ET-inducible genes (Fig. 2*D*), revealing both CREB-dependent and -independent transcription programs activated in response to ET action. A-CREB-expressing macrophages displayed a significantly reduced migration after ET exposure without incurring diminished survival (Fig. 2*E*). Hence, it appears that CREB and its transcriptional targets are partially responsible for EF-regulated changes in host gene expression and the macrophage migration response. Following this reasoning we explored the contribution of *Sdc1* because of the clear CREB dependency of its induction in macrophages and its potential role in leukocyte migration. Intriguingly, the migration response of

Sdc1-null BMDMs to purified ET and *B. anthracis* infection was significantly reduced compared with wild-type cells, whereas the mutant cells did not manifest any defect in basal migration (Fig. 3 *A–C*). BMDMs lacking syndecan-4, another syndecan family member highly expressed in macrophages and implicated in cell migration, did not exhibit similar migration phenotypes (Fig. 3*B*). Bacterial survival in infected *Sdc1*-deficient macrophages was comparable to that in wild-type macrophages (Fig. 3*D*), indicating that the different outcomes of host cell migration did not reflect nonidentical viabilities of the pathogen within the host cells. Consistent with the migration phenotype, *Sdc1*-null macrophages did not evoke actin redistribution or form actin-rich filopodial protrusions in response to ET (Fig. 3*E*). *Sdc1* deficiency, however, did not preclude the shift of the cells from a well spread to a rounded shape. These findings show that EF, through its ability to activate cAMP signaling and CREB-dependent transcription, induces *Sdc1* expression and thereby increases the motility of *B. anthracis*-infected macrophages.

Fig. 3. Requirement of syndecan-1 in the macrophage migration response to ET and *B. anthracis* infection. (*A* and *B*) Migration of BMDMs isolated from WT, Sdc1, and Sdc4 mutant mice was examined in the absence or presence of ET. (*A*) WT and Sdc1 KO macrophages that migrated to the lower surface of the transwell chamber were visualized by crystal violet staining. (*B*) Migration of WT, Sdc1 KO, and Sdc4 KO macrophages was quantified (∗∗, *P* < 0.01). (*C* and *D*) Macrophage migration (*C*) and bacterial survival in infected macrophages (*D*) were examined after *B. anthracis* spore infection as in Fig. 1 *A* and *B* by using WT and Sdc1 KO macrophages. (*E*) (*Left*) WT and Sdc1 KO BMDMs were left untreated or treated with ET and stained for vinculin (Vc; red) and F-actin (Ac; green). Merged images (Vc + Ac) are also shown. (*Right*) Images enlarged from the areas enclosed in white lines from *Left*. White arrowheads indicate actin-rich filopodial protrusions. (Scale bar: 50 μm.)

Common Physiological Outcomes of Antiinflammatory GPCR Signaling and Anthrax EF Action. Lymphangiogenesis and lymphatic macrophage efflux are hallmarks of tissues undergoing inflammatory resolution (22). The ET-induced cellular responses that we have identified, namely, VEGF production and enhanced macrophage motility, are well suited to supporting those vascular and leukocytic activities. In fact, several endogenous antiinflammatory mediators are known to activate, via GPCRs, cAMP signaling within target cells and are thus expected to act like EF in regulating macrophage physiology. To investigate whether such GPCRs use signaling pathways and bring about macrophage responses in common with anthrax EF, we first analyzed GPCRs with reported antiinflammatory functions that are highly expressed in macrophages. GPCRs for prostaglandin (PG) E$_2$ (EP2 and EP4), adenosine (A$_{2A}$), and endocannabinoids (CB2) met such criteria (Fig. 4*A*). PGE$_2$ is a lipid inflammatory mediator produced by the cyclooxygenase (COX) pathway. Besides its well understood proinflammatory functions, COX-2, the inducible isoform of the enzyme, was recently found to perform key antiinflammatory functions through PGE$_2$ and possibly other PG products in tissues undergoing inflammatory resolution (23, 24). We used PGE$_2$ in macrophage stimulation and observed that it can actively induce CREB phosphorylation (Fig. 4*B*). We also confirmed by gene expression profiling that PGE$_2$ activates the expression of almost all ET-inducible genes (Fig. S5). These PGE$_2$-responsive signaling and transcriptional cascades culminated in VEGF production and enhanced macrophage migration (Fig. 4 *C* and *D*). We extended these tests to include another antiinflammatory GPCR ligand, adenosine (25).

Treatment of macrophages with adenosine resulted in CREB phosphorylation, albeit with a duration and magnitude different from those of the PGE$_2$-induced response (Fig. 4*B*). Although its ability to induce VEGF production was modest in our experimental condition (Fig. 4*C*), adenosine was also capable of promoting macrophage migration (Fig. 4*D*). As in ET-induced migration, syndecan-1 was critically required for the macrophage migration response to both GPCR ligands tested (Fig. 4*D*). Therefore, we conclude that *B. anthracis* and certain antiinflammatory GPCRs promote macrophage migration and VEGF production, most likely through common regulatory mechanisms (Fig. 5).

Discussion

We have found that *B. anthracis* exploits a CREB-dependent transcription program to facilitate the migration of host macrophages. cAMP signaling triggered by EF played a crucial role in this pathogen–host interaction. Besides *B. anthracis*, many other bacterial pathogens produce exotoxins that act to increase intracellular cAMP levels in the host cell through their own adenylyl cyclase activity (e.g., *Bordetella pertussis* CyaA, *Pseudomonas aeruginosa* ExoY, and *Yersinia pestis* Cya). Others stimulate host adenylyl cyclases via modulating G protein activity (e.g., *Vibrio cholerae* cholera toxin). These toxins may conceivably act similarly to EF to modulate host cell signaling and behavior. Further characterization of host genes whose expression and function are regulated by EF will offer a deeper understanding of the pathogenic mechanism of anthrax and other infectious diseases caused by pathogens producing cAMP-elevating toxins.
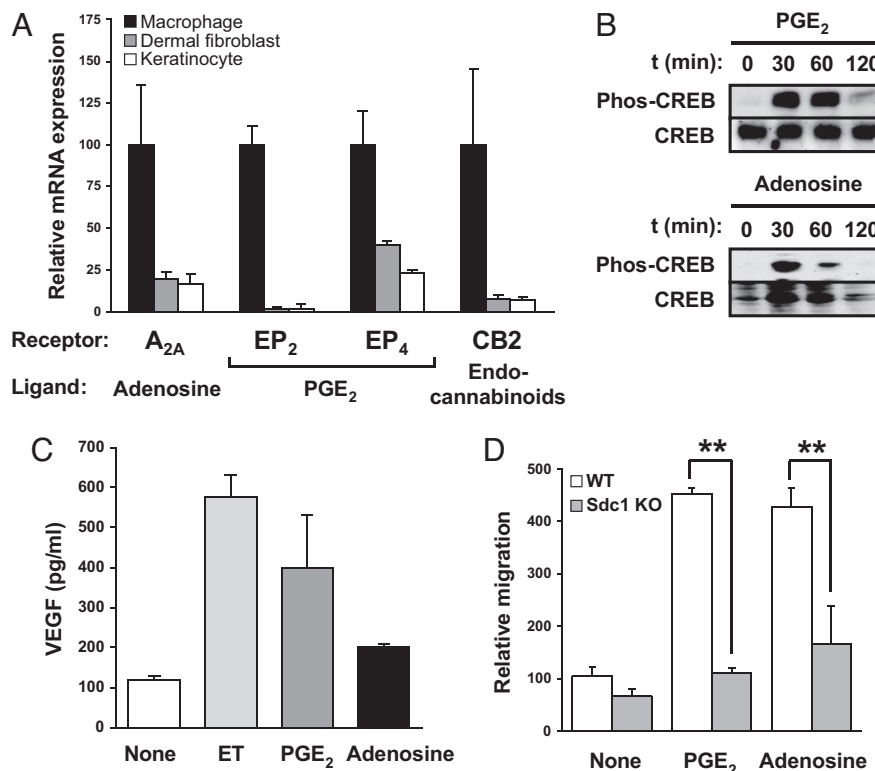
Fig. 4. CREB activation, VEGF production, and syndecan-1-dependent macrophage migration in response to antiinflammatory GPCR ligands. (*A*) Relative expression of GPCR genes in distinct types of primary mouse cells was analyzed by real-time qPCR. The cognate GPCR ligands are indicated at the bottom. (*B*) CREB phosphorylation in BMDMs incubated with PGE$_2$ (1 μM) and adenosine(100 μg/ml) was analyzed as in Fig. 1C. (*C*) The concentration of VEGF was determined from culture supernatants of BMDMs left untreated or treated with ET (1 μg/ml PA + 1 μg/ml EF), PGE$_2$ (1 μM), and adenosine(100 μg/ml) for 24 h. (*D*) Migration of WT and Sdc1 KO BMDMs in the absence or presence of the GPCR ligands indicated was quantified (∗∗, $P < 0.01$).

Of note, the EF- and cAMP-inducible genes identified in this study include those encoding COX-2 (*Ptgs2*) and histidine decarboxylase (*Hdc*). These two enzymes are responsible for the production of PGE$_2$ and histamine, respectively. As described above, PGE$_2$ itself can induce cAMP-mediated responses in macrophages. Histamine is also known to bind to and activate a cAMP-elevating GPCR (H2) in blood leukocytes (26). These findings raise the possibility that ET may promote sustained cAMP signaling in macrophages by establishing a feed-forward cascade driven by newly induced GPCR ligands.

Unlike neutrophils whose inflammatory function is temporally limited by their short lifespan, macrophages, which are long-lived inflammatory cells, are cleared from inflamed tissues through their

efflux to draining lymph nodes (27–29). Our findings of EF function and its regulatory targets in host macrophages may elucidate as-yet-unidentified mechanisms regulating the resolution of inflammation and GPCR-regulated immune cell trafficking. In addition, newly identified EF-inducible genes will serve as targets for drugs for preventing dissemination of *B. anthracis* within the host and possibly other forms of pathologic cell migration as well.

Materials and Methods

Mice, Macrophages, and Bacteria. BMDMs and fetal liver-derived macrophages were prepared from C57BL/6J mice as described (6). *Sdc1* and *Sdc4* knockout mice have been described (30, 31). For infection experiments, spores were prepared with the *B. anthracis* Sterne strain 7702 and added to macrophage cultures as described (32). In brief, bacteria were grown to stationary phase in 2× SG medium with shaking at 37°C, then diluted 5-fold with water, and further grown for an additional 40 h. The spores formed were harvested by centrifugation, washed with and resuspended in PBS, and heat-inactivated for 40 min at 70°C. The spore preparation contained no vegetative bacteria.

Recombinant Proteins and Chemical Reagents. Recombinant PA (PA$_{63}$), EF, and LF were expressed in and purified from *Escherichia coli* strain BL21 (DE3) bearing the appropriate plasmid constructs as described (33). The EF inhibitor AD (Bis-POM-PMEA) has been described (13). In-2-LF was from Calbiochem, PGE$_2$ was from Cayman Chemical, forskolin and adenosine were from Sigma-Aldrich, and cAMP analogs were from BIOLOG.

Cell Migration Assay. BMDMs were resuspended at 1 × 10^6/ml in DMEM containing macrophage colony-stimulating factor (M-CSF; 10 ng/ml; Peprotech) and 0.5% FBS and loaded onto the upper chamber of a transwell plate with a pore size of 8.0 μm (Corning). DMEM containing M-CSF and 10% FBS was placed in the lower well, and macrophages were incubated at 37°C in a 5% CO$_2$-supplied incubator for 24 h. Anthrax toxin proteins were included in the upper chamber. To analyze the migration of *B. anthracis*-infected macrophages, BMDMs growing in a plastic culture dish were infected with spores at a multiplicity of infection
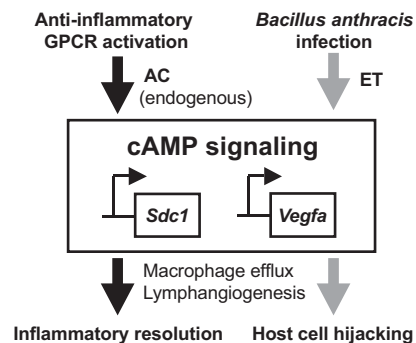

Fig. 5. Induction of cAMP signaling, target gene expression, and physiological outcomes in response to antiinflammatory GPCR activation and *B. anthracis* infection.

(moi) of 10. One hour after spore addition, cells were harvested, resuspended in DMEM containing M-CSF, 0.5% FBS, and gentamycin (5 μg/ml), and transferred into the transwell chamber. AD was added to macrophages 2 h before infection and also included in the migration chamber. Macrophages that had migrated to the lower surface of the membrane were stained with 0.09% crystal violet and counted under bright-field microscopy. In each experiment, the numbers of stained macrophages in three independent image fields of the same size were averaged and indicated as relative migration.

Protein and RNA Analysis. Whole-cell extracts for immunoblot analysis were prepared and analyzed as described (34). Antibodies against phospho-CREB (R&D Systems) and CREB (Cell Signaling Technology) were used in immunoblotting. Total RNA was isolated by using TRIzol (Invitrogen). Microarray analysis was performed with GeneChip Mouse 430 2.0 Array (Affymetrix) at the Massachusetts General Hospital Cancer Center DNA Microarray Core. RNA analysis using real-time PCR was performed as described (34). Individual primer sequences are available on request.

Immunofluorescence Microscopy. The cells fixed with 4% formaldehyde and permeabilized with cold 0.5% Triton X-100 were incubated with a monoclonal vinculin antibody (clone hVIN-1; Sigma) and an anti-mouse IgG conjugated to Cy5 (Jackson ImmunoResearch Laboratories) plus Alexa-488-conjugated phalloidin (Molecular Probes–Invitrogen). After treatment with the antifading agent Flu-oromount-G (Southern Biotech), the signal was visualized by using a Leica TCS5 confocal imaging system with a 40× oil-immersion lens (Leica). Double-labeling experiments exhibited no evidence of bleed-through between channels. Cell images were processed with Adobe Photoshop software.

Lentivirus Vectors. To construct the vector for A-CREB expression, an A-CREB DNA fragment was amplified by PCR using oligonucleotide primers with the sequences 5'-caagcagatctatggactacaaggacgacgatgacaag-3' and 5'-ctctaggaattcgaattaatct-gacttgtggcagtaaag-3' and pcDNA3-A-CREB (35) as a template. The PCR product was cloned into the BglII and EcoRI sites of pEGFP-c1, retrieved by digestion with BglII and SalI, and finally cloned into the BamHI and XhoI sites of FURY, an IRES-GFP-containing lentiviral vector based on FUGW (36). GFP- and ACREB-expressing lentiviruses were produced as described (37). For lentiviral infection, BMDMs were incubated with viruses for 48 h before ET treatment.

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