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Anthrax toxins inhibit immune cell chemotaxis by perturbing chemokine receptor signalling

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Summary

Pathogenic strains of *Bacillus anthracis* produce two potent toxins, lethal toxin (LT), a metalloprotease that cleaves mitogen-activated protein kinase kinases, and oedema toxin (ET), a calcium/calmodulin-dependent adenylate cyclase. Emerging evidence indicates a role for both toxins in suppressing the initiation of both innate and adaptive immune responses, which are essential to keep the infection under control. Here we show that LT and ET inhibit chemotaxis of T-cells and macrophages by subverting signalling by both CXC and CC chemokine receptors. The data highlight a novel strategy of immunosuppression by *B. anthracis* based on inhibition of immune cell homing.

Introduction

Pathogenic strains of *Bacillus anthracis* produce two toxins, lethal factor (LF) and oedema factor (EF), that display their enzymatic activities in the cytosol after entry into target cells following their assembly as binary complexes with protective antigen (PA) (Collier and Young, 2003). *B. anthracis* spores enter the host through skin abrasions, ingestion or inhalation. In pulmonary anthrax spores are taken up by alveolar phagocytes which migrate to regional lymph nodes where they release vegetative bacteria that enter the bloodstream, leading to death of the host by septicaemia and toxaemia (Turnbull, 2002; Collier and Young, 2003). This general scheme holds true also for cutaneous and gastrointestinal anthrax but, at least in the more common cutaneous anthrax, bacterial dissemination is usually kept under control by the host reactions. Whichever route of entry, the host appears inhibited in its capacity to mount an effective immune response. Recent data have highlighted a potent strategy of immune evasion by *B. anthracis* based on subversion of the intracellular signalling pathways implicated in the activation of both innate and adaptive immunity by LF and EF (Pellizzari *et al*., 1999; Agrawal *et al*., 2003; Comer *et al*., 2005; Fang *et al*., 2005; 2006; Rossi Paccani *et al*., 2005; Tournier *et al*., 2005; reviewed in Baldari *et al*., 2006). Modification of signalling by these toxins results from their capacity to target crucial molecular components of these pathways. Indeed, LF is a specific metalloprotease which inactivates mitogen-activated protein kinase kinases (MAPKK), while EF is a potent adenylate cyclase (Collier and Young, 2003).

By directing their trafficking and homing to the appropriate districts, chemotaxis is crucially required for the development, activation, differentiation and effector function of all immune cells (Sallusto *et al*., 2000). For *B. anthracis* chemotaxis represents a double-edged sword, as bacteria need phagocytes to enter the body and reach the lymph node, but can be thwarted by immune cell recruitment to the site of infection. Fine-tuning of immune cell migration may therefore be of paramount importance to the outcome of infection by *B. anthracis*. Chemotaxis is orchestrated by chemokine receptors, which are serpentine transmembrane proteins coupled to heterotrimeric Gi proteins that modulate adenylate cyclase activity. Furthermore, they initiate a tyrosine kinase-dependent pathway leading to the activation of the mitogen-activated protein kinase (MAPK) cascade (Wong and Fish, 2003). Pathways initiated by chemokine receptors are therefore potential targets of the two anthrax toxins. Here we have addressed the possibility that LF and EF may inhibit chemotaxis by interfering with chemokine receptor signalling.

Results and discussion

As a paradigm to study the effect of LF or EF on T-cell chemotaxis, we used the homeostatic chemokine SDF-1α, which is the only known ligand of the widely expressed chemokine receptor CXCR4 and a potent chemoattractant for mature T-cells and monocytes

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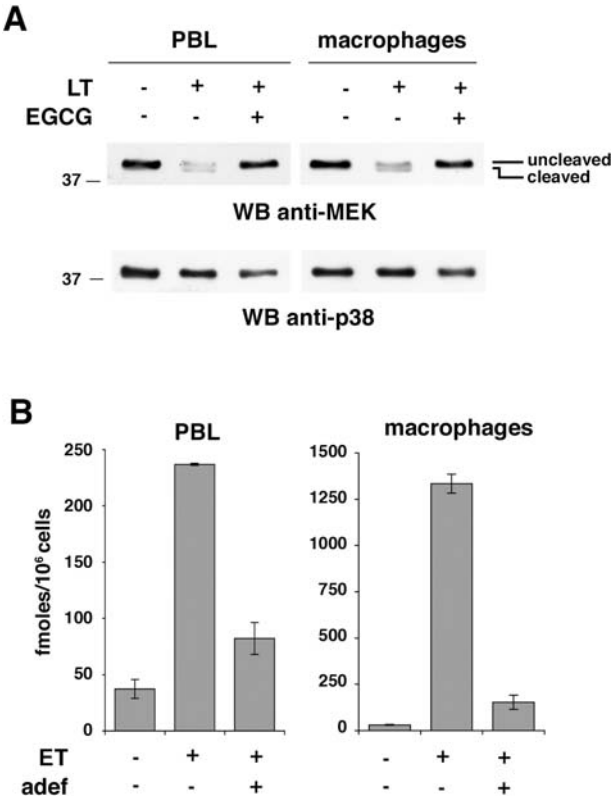

Fig. 1. MEK1 cleavage by LT and cAMP production by ET in PBL and macrophages.
A. Immunoblot analysis of post-nuclear supernatants from PBL (left) or macrophages (right) in the presence or absence of 8 μg ml^{-1} PA and 5 μg ml^{-1} LF, in combination with 10 μM EGCG ($n \geq 3$).
B. Quantification of cAMP in lysates of PBL treated with 8 μg ml^{-1} PA and 5 μg ml^{-1} EF, in combination with 10 μM adefovir dipivoxil. The levels of cAMP are expressed as fmoles/10^6 cells. The results of duplicate samples from a representative experiment are shown ($n \geq 3$). Error bars, SD; *n*, number of experiments.

(Sallusto *et al*., 2000). As expected, LF + PA [(lethal toxin (LT)] cleaved the prototype MAPKK, MEK1, in peripheral blood lymphocytes (PBL), as shown by the electrophoretic mobility shift which results from the loss of seven amino acid residues from the N-terminus (Fig. 1A). EF + PA [oedema toxin (ET)] promoted cAMP production in these cells (Fig. 1B). These effects were prevented by the respective selective inhibitors, epigallocatechin-3-gallate (EGCG; Dell'Aica *et al*., 2004) and adefovir dipivoxil (Shen *et al*., 2004) (Fig. 1). PBL treatment with LT or ET resulted in dose-dependent inhibition of SDF-1α-induced chemotaxis. Inhibition was reversed by EGCG and adefovir dipivoxil respectively (Fig. 2A).

MAPK activation is required for the migration of a wide variety of cell types in response to binding of extracellular matrix proteins, growth factors and other stimuli (Huang *et al*., 2004). PBL treatment with the MEK inhibitor, PD098059, resulted indeed in inhibition of SDF-1α-mediated chemotaxis (Fig. 2A), supporting a causal role of MEK/Erk in this process. We have reported that TCR-dependent activation of Erk1/2, the MAPKs downstream of MEK, is suppressed by both LT and ET (Rossi Paccani *et al*., 2005). This convergence of the activities of LT and ET on Erk1/2 is achieved at different steps of the MAPK cascade, as LT cleaves and inactivates the upstream kinase MEK1, while ET may inhibit the MAPK pathway a step upstream by promoting the inhibitory phosphorylation of Raf by protein kinase A (PKA; Dhillon *et al*., 2002). We therefore assessed the effect of either toxin on MAPK activation. Treatment with LT or ET resulted in inhibition of Erk1/2 activation in response to CXCR4 engagement by SDF-1α in PBL (Fig. 2B), suggesting that the inhibitory activity of the two toxins on T-cell chemotaxis results, at least in part, from their capacity to block MAPK activation. Of note, at variance from the synergistic activity of LT and ET on T-cell activation (Rossi Paccani *et al*., 2005), no further inhibition of chemotaxis was observed when PBL were treated with a combination of LT and ET (data not shown), suggesting that additional, MAPK-independent pathways which are not affected by the toxins are implicated in this process.

The effect of LT and ET was also assessed on macrophages, where they were found to cleave MEK1 and increase cAMP respectively (Fig. 2). Macrophage treatment with LT or ET resulted in inhibition of SDF-1α-induced chemotaxis (Fig. 3A) which, as in PBL, correlated with an impairment of Erk1/2 activation (Fig. 3B). To test whether this activity is limited to CXCR4 signalling, or whether *B. anthracis* may display a general perturbing effect on signalling by chemokine receptors, we assessed the effect of the two anthrax toxins on macrophage chemotaxis and MAPK activation induced by the inflammatory CC-chemokine MIP-1α, which interacts with CCR5 and promotes monocyte extravasation and migration to inflamed tissues (Sallusto *et al*., 2000). Treatment with LT or ET resulted in impaired MIP-1α-dependent chemotaxis (Fig. 3A), as well as in suppression of Erk1/2 activation (Fig. 3C). Both these effects were largely reversed by EGCG and adefovir dipivoxil respectively. Inhibition of macrophage migration by LT and ET was also observed when the formylated bacterial tripeptide, fMLP, which interacts with a Gi-coupled receptor, was used as chemoattractant (data not shown). Both SDF-1α- and MIP-1α-dependent macrophage chemotaxis was inhibited by PD098059 (Fig. 3D), supporting the notion that the suppressive effects of LT and ET on this process are, at least in part, the result of their inhibitory activity on Erk1/2 activation.

At present, the only known molecular targets of LT are MAPKKs (Collier and Young, 2003). The well-established role of MAPKs in cell migration, resulting from phosphorylation of structural and regulatory components of the

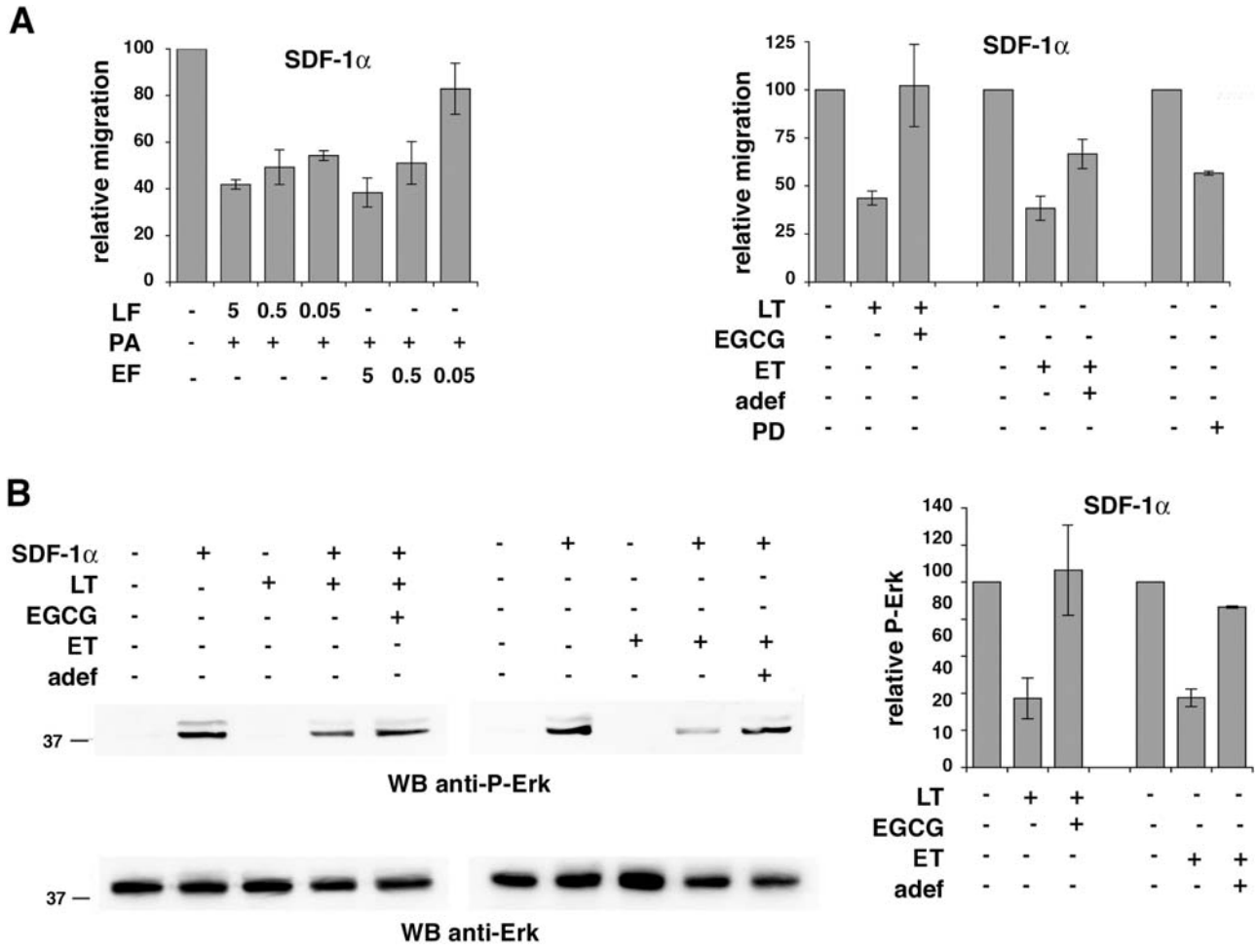

Fig. 2. LT and ET inhibit SDF-1α-dependent chemotaxis and Erk phosphorylation in PBL.
A. *Left*. PBL chemotaxis induced by SDF-1α (10 ng ml^{-1}) in the presence of 8 μg ml^{-1} PA and the indicated concentrations of LF and EF (μg ml^{-1}). The data are presented as relative migration, with the migration index of untreated cells taken as 100% ($n \geq 2$). *Right*. PBL chemotaxis induced by SDF-1α (10 ng ml^{-1}) in the presence of 8 μg ml^{-1} PA and 5 μg ml^{-1} LF (LT) or 5 μg ml^{-1} EF (ET), in the presence or absence of 10 μM EGCG or 10 μM adefovir dipivoxil. Alternatively, PBL were treated with 15 μM PD098059 ($n \geq 3$).
B. *Left*. Immunoblot analysis, using a phospho-specific antibody, of SDF-1α-dependent Erk1/2 phosphorylation (5 min at 37°C) in post-nuclear supernatants from PBL treated with 8 μg ml^{-1} PA and 5 μg ml^{-1} LF (LT) or EF (ET) ($n \geq 3$). Where indicated, PBL were pretreated with EGCG or adefovir dipivoxil as above. Representative experiments are shown. *Right*. Quantification by laser densitometry of the relative levels of Erk1/2 phosphorylation in PBL treated as above with LT or ET, in the absence or presence of the respective inhibitors, and subsequently stimulated with SDF-1α (Erk1/2 phosphorylation in control cells taken as 100%) ($n \geq 3$). No effect on either chemotaxis or Erk phosphorylation was observed when cells were treated with PA, LF, EF or either inhibitor alone, nor did the toxins affect cell viability at the concentrations used in the experimental time frame (data not shown). Error bars, SD; *n*, number of experiments.

cytoskeleton (reviewed in Huang *et al*., 2004) strongly supports the notion that disruption of MAPK cascades through MAPKK cleavage underlies the inhibitory activity of LT on immune cell chemotaxis. Interestingly, LT has been reported to inhibit actin-based motility in polymorphonuclear cells through a MEK-independent mechanism (During *et al*., 2005), suggesting that, at variance with macrophages, LT might impair PMN migration by directly affecting the dynamics of the actin cytoskeleton through as as-yet unidentified mechanism. On the other hand, by catalysing the massive production of cAMP, ET can potentially impair a number of pathways, other than the MAPK cascades, implicated in cytoskeletal dynamics. PKA has indeed been shown to phosphorylate at negative regulatory sites both key structural components of the cytoskeleton, such as actin and vasodilator-stimulated phosphoprotein, and proteins implicated in the control of cell motility, such as Rho GTPases and PAK1. Furthermore, phosphorylation of paxillin, a focal adhesion adaptor essential for cell migration, is negatively regulated by PKA-dependent activation of the tyrosine phosphatase, SHP2 (reviewed in Howe, 2004). Nevertheless, the significant levels of inhibition of cell migration achieved by the MEK inhibitor, PD098059, particularly on

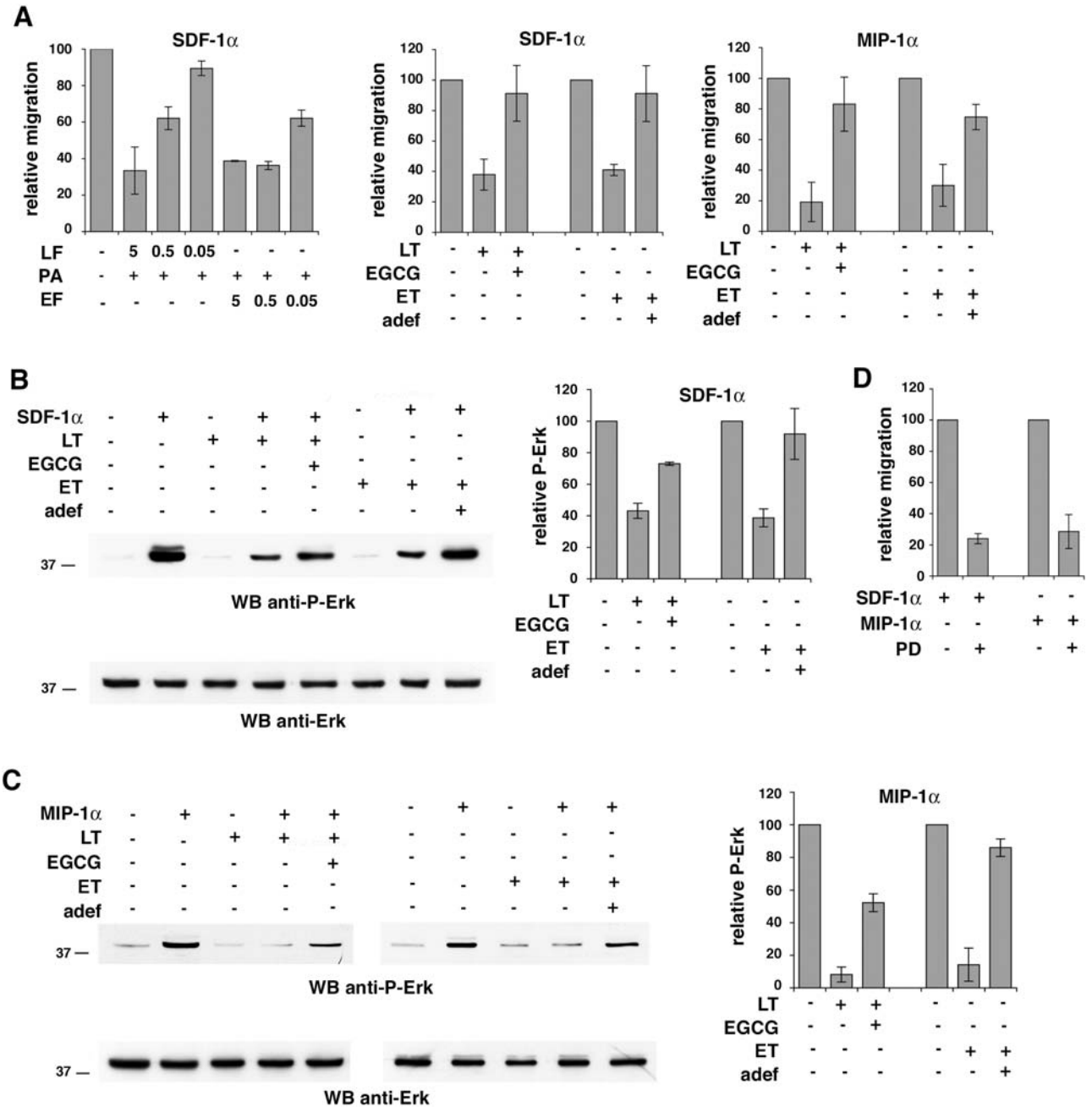

Fig. 3. LT and ET inhibit SDF-1α- and MIP-1α-dependent chemotaxis and Erk phosphorylation in macrophages.
A. *Left*. Macrophage chemotaxis induced by SDF-1α (10 ng ml^{-1}) in the presence of 8 μg ml^{-1} PA and the indicated concentrations of LF and EF (μg ml^{-1}). The data are presented as relative migration, with the migration index of untreated cells taken as 100% ($n = 2$). *Middle* and *Right*. Macrophage chemotaxis induced by SDF-1α (10 ng ml^{-1}) (*middle*) or MIP-1α (25 ng ml^{-1}) (*right*) after treatment with 8 μg ml^{-1} PA and 5 μg ml^{-1} LF (LT) or EF (ET), in the presence or absence of 10 μM EGCG or 10 μM adefovir dipivoxil ($n \geq 3$).
B and C. *Left*. Immunoblot analysis, using a phospho-specific antibody, of SDF-1α-dependent (B) or MIP-1α-dependent (C) Erk1/2 phosphorylation (5 min at 37°C) in post-nuclear supernatants from macrophages treated with 8 μg ml^{-1} PA and 5 μg ml^{-1} LF (LT) or EF (ET) ($n \geq 3$). Where indicated, PBL were pretreated with EGCG or adefovir dipivoxil as above. Representative experiments are shown. *Right*. Quantification by laser densitometry of the relative levels of Erk1/2 phosphorylation in PBL treated as above with LT or ET, in the absence or the presence of the respective inhibitors, and subsequently stimulated with SDF-1α (B) or MIP-1α (C) (Erk1/2 phosphorylation in control cells taken as 100%) ($n \geq 3$). No effect on either chemotaxis or Erk phosphorylation was observed when cells were treated with PA, LF, EF or either inhibitor alone, nor did the toxins affect cell viability at the concentrations used in the experimental time frame (data not shown).
D. Macrophage chemotaxis induced by SDF-1α (10 ng ml^{-1}) (*middle*) or MIP-1α (25 ng ml^{-1}) (*right*) after treatment with 15 μM PD098059. The data are presented as relative migration, with the migration index of untreated cells taken as 100% ($n \geq 2$). Error bars, SD; *n*, number of experiments.

macrophages, indicates that disruption of MAPK signalling underlies to a significant extent the suppressive effects of ET on immune cell chemotaxis.

Collectively, the results presented in this report show that the two anthrax toxins inhibit chemotaxis of cellular components of both innate and adaptive immunity and provide a novel twist to the multifaceted immune evasion strategy evolved by *B. anthracis*. This finding has profound implications for anthrax pathogenesis. First, once vegetative bacteria start producing the toxins in the lymph node, LT and ET will co-ordinately antagonize correct homing of APCs and T-cells to these sites. Together with the inhibitory effects of the toxins on dendritic cell and T-cell activation (Agrawal *et al*., 2003; Comer *et al*., 2005; Fang *et al*., 2005; Rossi Paccani *et al*., 2005; Tournier *et al*., 2005), this activity is expected to effectively prevent initiation of adaptive immune responses. Second, resolution of inflammation and wound healing in cutaneous anthrax is slow and correlates with deficient phagocyte infiltration (Cromartie *et al*., 1947). Inhibition of inflammatory cell extravasation to the site of infection by the toxins secreted by the vegetative bacteria is likely to underlie the delay in bacterial clearance and wound healing.

Experimental procedures

Cells, reagents and antibodies

Peripheral blood lymphocytes were purified from healthy donors by density gradient centrifugation and subsequent depletion from monocytes by adherence. Adherent monocytes differentiated to macrophages after 48–72 h (Fig. S1 in *Supplementary material*). Both PBL and macrophages expressed surface CXCR4, and macrophages expressed CCR5 (Fig. S1). Phospho-specific antibodies recognizing the active form of Erk1/2 were purchased from Cell Signalling Technology (Beverly, MA), anti-Erk2 and an anti-MEK1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorochrome-labelled anti-CD86 mAb was purchased from BD Biosciences (Leiden, the Netherlands), fluorochrome-labelled secondary antibodies from Dako Denmark A/S (Glostrup, Denmark), peroxidase-labelled antibodies from Amersham Biosciences Europe GmbH (Milan, Italy). Anti-CXCR4 and CCR5 antibodies were kindly provided by J. Hoxie, Leukosite and the MRC AIDS Reagent Project. PA, LF and EF were expressed in *Escherichia coli* and purified as described (Tonello *et al*., 2004; Rossi Paccani *et al*., 2005). EGCG, PD098059, SDF-1α, MIP-1α and fMLP were purchased from Sigma-Aldrich srl, adefovir dipivoxil was provided by Gilead Sciences. Cells were plated in 48 well plates at 5×10^6 cells ml^{-1} in RPMI 1640 supplemented with 7.5% FCS, added with LT or ET, and incubated at 37°C for 2 h. EGCG was pre-incubated with LT for 15 min before addition to the cells. Cells were pre-incubated with adefovir dipivoxil at 37°C for 2 h before addition of ET to allow conversion of the drug to its active cellular metabolite, adefovir diphosphate (Shen *et al*., 2004). Cell viability after toxin treatment was assessed by Trypan Blue exclusion. Chemokine treatment was carried out at 37°C.

Chemotaxis assays

Chemotaxis assays were carried out using 48 well Transwell chambers with 5 μm pore size polycarbonate membranes (Corning Life Sciences B.V., Schiphol-Rijk, the Netherlands) essentially as described (Zhang *et al*., 2001). Filters were soaked overnight in 0.2% BSA in HBSS without Ca^{2+}/Mg^{2+}. The chemotaxis medium (500 μl RPMI containing 1% BSA) with or without the selected chemokine, was placed in the lower chamber, and 100 μl of the cell suspension (5×10^5 cells/sample) in chemotaxis medium was placed in the upper chamber. After a 2 h incubation at 37°C in humidified air with 5% CO_2, the upper chamber was emptied, filters were removed and the contents of the lower chamber recovered. After two washes in PBS, cells in the upper and lower chamber were counted by flow cytometry. The migration index was calculated by determining the ratio of migrated cells in chemokine-treated versus untreated cells.

Immunoblots, flow cytometry, cAMP measurements

Cell lysis and immunoblots were performed as described (Boncristiano *et al*., 2003). Immunoblots were quantified by laser densitometry (Kodak Digital Science Electrophoresis Documentation and Analysis System 120). Flow cytometry was carried out using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). cAMP was quantified by enzyme-linked immunoassay (Biotrak EIA; Amersham Biosciences).

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Supplemental material

The following supplementary material is available for this article online:

Fig. S1. Expression of CXCR4 and CCR4 on PBL and macrophages. Flow cytometric analysis of PBL (A) and monocytes (B) after indirect labelling with anti-CXCR4 or anti-CCR5 antibodies, followed by FITC-labelled secondary antibody. Experiments were carried out on PBL and monocytes cultured for 48 h. At this time point expression of CXCR4 on PBL and of CXCR4 and CCR5 on monocytes was maximal. Furthermore, monocytes had adhered and differentiated to macrophages, as determined both by microscopic inspection (data not shown) and by expression of CD86 (C).

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