PMN transendothelial migration decreases nuclear NFκB in IL-1β–activated endothelial cells: role of PECAM-1

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Introduction

A systemic inflammatory response is a frequent consequence of a severe bacterial infection or trauma and can impact on organ systems remote from the initial insult (Bone, 1995; Lush and Kvietys, 2000). The release of bacterial products and other proinflammatory mediators from the affected tissue results in an increase in plasma concentrations of cytokines (e.g., tumor necrosis factor α [TNF-α],* interleukin 1β [IL-1β], etc.). These cytokines activate both circulating neutrophils (polymorphonuclear leukocytes, PMN) and the vascular endothelium. Activated PMN increase their surface levels and/or activation state of adhesion molecules (e.g., cluster of differentiation-18, CD18) and become less deformable (Hogg and Doerschuk, 1995; Linderkamp et al., 1998; Lush and Kvietys, 2000). Endothelial cell activation also involves up-regulation of adhesion molecules (e.g., intercellular adhesion molecule 1 [ICAM-1], the ligand for CD18) and structural alterations, such as swelling and pseudopod formation (Panes et al., 1995; Goddard et al., 1998). These changes in the activation state of PMN and endothelium facilitate PMN invasion of various organ systems, where they contribute to tissue injury. If unchecked, this sequence of events can lead to multiple organ dysfunction, and ultimately, death.

The systemic inflammatory response appears to be a self-amplifying phenomenon generated by the activation of nuclear transcription factors by circulating cytokines. One transcription factor that is believed to be important in the systemic inflammatory response is nuclear factor kappa B (NFκB; Collins et al., 1995; Stancovski and Baltimore, 1997; Winyard and Blake, 1997; Mercurio and Manning,
In quiescent cells, NFκB (p50/p65 heterodimer) is localized to the cytoplasm by virtue of its association with inhibitory protein kappa B (IκB). IκB apparently masks the nuclear localization sequence on NFκB, and thereby prevents its translocation to the nucleus (Henkel et al., 1992; Lin et al., 1995; Mercurio and Manning, 1999). In cytokine-activated cells, IκB is phosphorylated, ubiquitinated, and subsequently degraded by the proteasome pathway. The loss of IκB allows NFκB to enter the nucleus and initiate the transcription of relevant proinflammatory genes, including those encoding for endothelial adhesion molecules and various cytokines. Interestingly, NFκB transcribes genes encoding for the same cytokines that mobilized it to the nucleus. This positive feedback mechanism could amplify the inflammatory state with severe consequences to the host.

Fortunately, there are negative feedback mechanisms in place that limit an excessive and prolonged inflammatory response on NFκB translocation to the nucleus. For example, NFκB transactivates the gene encoding for IκB (Brown et al., 1993; Baldwin, 1996; Bonizzi et al., 2000). The resultant generation of IκB presumably binds to cytoplasmic NFκB and prevents further translocation of this transcription factor to the nucleus. This feedback inhibition assures a transient response to the initiating signal and prevents an excessive, uncontrolled inflammatory response. Our previous preliminary works (Cepinskas et al. 1998. FASEB J. 12(5):A801. Abstract) indicated that there might be an additional negative feedback mechanism in place to control the systemic inflammatory response. In that paper, we noted that the increase in rat myocardial and lung nuclear NFκB induced by sepsis (peritonitis) was enhanced when PMN emigration was prevented by antibodies directed to CD18. This observation suggested that PMN emigration into the lungs and heart during the systemic inflammatory response could reduce tissue nuclear NFκB. Herein, we provide evidence that the IL-1β–induced increase in endothelial cell monolayer nuclear NFκB can be reduced if PMN are allowed to migrate across these monolayers. Furthermore, engagement of platelet-endothelial cell adhesion molecule 1 (PECAM-1) on endothelial cells by PMN may be the mechanism by which this negative feedback inhibition occurs.

### Results

**In ICAM-1–deficient mice, the peritonitis-induced increase in lung and heart MPO activity is decreased, whereas the increase in tissue nuclear NFκB activity is augmented**

Previously, we have shown that cecal ligation and perforation (CLP) in mice results in peritonitis and sepsis, a systemic inflammatory response (Lush et al., 2001). As shown in Fig. 1 A, induction of peritonitis in wild-type mice resulted in an increase in myeloperoxidase (MPO) activity in the lungs and hearts of these animals. The increase in MPO activity in the lungs and heart in response to CLP was substantially diminished in ICAM-1–deficient animals. As shown in Fig. 1 B, the NFκB in nuclear extracts obtained from lungs and hearts of wild-type mice subjected to CLP was increased. The NFκB in nuclear extracts obtained from the lungs and hearts of ICAM-1–deficient mice subjected to CLP was increased to a greater extent than the levels noted in wild-type mice subjected to the same procedure (Fig. 1 B, compare lane 3 with lane 4). These observations are in agreement with our previous studies in rats (Cepinskas et al. 1998. FASEB J. 12(5):A801. Abstract). As in the present work, induction of peritonitis (CLP) resulted in an increase in MPO activity...
and nuclear NFκB in the hearts and lungs of rats. Antibodies to CD18 prevented the CLP-induced PMN accumulation in the lungs and hearts of rats, while exaggerating the increase in nuclear NFκB. Thus, collectively, these findings indicate that CD18/ICAM-1-mediated adhesive interactions between PMN and endothelial cells can modulate the systemic inflammatory response.

There are two possible explanations for these observations based on the importance of CD18–ICAM-1 adhesive interactions in PMN infiltration of tissues (Granger and Kubes, 1994; Kvietys et al., 1996; Panes and Granger, 1998; Wang and Springer, 1998). The neutralization of ICAM-1 (genetically) and CD18 (antibodies) may have prevented PMN emigration into the site of infection, the peritoneum, thereby compromising the clearance of fecal bacteria and their proinflammatory products. Ultimately, this would result in a greater systemic inflammatory response, e.g., exaggerated levels of circulating cytokines. The higher levels of circulating cytokines could, in turn, induce a greater increase in heart and lung nuclear NFκB. Alternatively, the prevention of PMN emigration into the interstitium of the target organs (heart and lung) may have directly impacted on the CLP-induced increase in nuclear NFκB in these organs. This latter possibility suggested that PMN transendothelial migration may initiate a negative feedback signal to diminish nuclear levels of NFκB. In the present work, we mimicked the interstitial–vascular interface during sepsis by activating endothelial monolayers in culture inserts with IL-1β and inducing PMN transendothelial migration from the apical to the basal aspects of the inserts by introducing platelet-activating factor (PAF) into the basal compartment. This approach allowed for a direct assessment of PMN transendothelial migration in target organs in the absence of complications induced by excessive cytokine production due to lack of clearance of bacteria from the initial site of infection. Herein, we provide evidence that PMN transendothelial migration initiates a negative feedback on endothelial cell nuclear NFκB.

**Migrating PMN decrease nuclear NFκB in IL-1β/PAF-stimulated HUVECs**

We used confocal microscopy to assess the negative impact of PMN transendothelial migration on the IL-1β–induced increase in human umbilical vein endothelial cell (HUVEC) nuclear NFκB (Fig. 2 A). Under control (unstimulated) conditions, p65 is localized to the cytoplasm of HUVECs (Fig. 2 A, a). After stimulation with IL-1β/PAF, the p65 is primarily localized to the nuclei of HUVECs (Fig. 2 A, b). When PMN were allowed to migrate across the HUVEC monolayers, there was a decrease in nuclear p65 (Fig. 2 A, c). Of interest is the observation that, although there is some staining for NFκB in the cytoplasm, the overall extent is much less than under control conditions (Fig. 2 A, compare a with c). The reason for the overall lack of cytoplasmic staining after PMN migration is not clear, but may reflect degradation of NFκB or modification of NFκB, such that it is no longer recognized by the antibody.

To further address this issue, we used electrophoretic mobility shift assay (EMSA) to monitor nuclear NFκB. As shown in Fig. 2 B (lane 1), activation of HUVECs grown to confluence in cell culture inserts with IL-1β (apical aspect) and PAF (basal aspect) resulted in nuclear accumulation of NFκB. This observation is consistent with previous reports of cytokine-induced NFκB activation and translocation to HUVEC nuclei (Stancovski and Baltimore, 1997; Lush et al., 2000). When PMN were allowed to migrate across the IL-1β/PAF-stimulated HUVEC monolayers, the nuclear level of NFκB was dramatically diminished (Fig. 2 B, lane 2). The negative effect on HUVEC nuclear NFκB induced by migrating PMN was dependent on the number of PMN interacted with HUVECs. The greater the number of PMN that were allowed to migrate across HUVEC monolayers, the greater was the decrease in nuclear NFκB (Fig. 2 B, lanes 2–6). The finding that a decrease in nuclear NFκB was noted even when the endothelial cell:PMN ratio was as low as 10:1 (Fig. 2 B, lane 6) indicates that this phenomenon is pathophysiologically relevant. Collectively, the data obtained using EMSA and confocal microscopy indicate that PMN transendothelial migration has a negative impact on HUVEC nuclear NFκB. To our knowledge, this is the first time that this phenomenon has been described.

Having demonstrated the negative impact of PMN transendothelial migration on the IL-1β–stimulated increase in HUVEC nuclear NFκB using two different approaches, we assessed whether this phenomenon could be demonstrated using other cytokines to stimulate HUVECs. HUVECs were activated with 10 ng/ml TNF-α (R&D Systems) and 0.5 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich) rather than IL-1β. Similar results were noted as with IL-1β, i.e., these compounds increased HUVEC nuclear NFκB, and subsequent PMN transendothelial migration resulted in a decrease in HUVEC nuclear NFκB (unpublished data). These latter observations indicate that PMN transendothelial migration can provide a negative influence on HUVEC nuclear NFκB induced by a variety of cytokines. Because LPS, IL-1β, and TNF-α have all been implicated in the systemic inflammatory response (Lush and Kvietys, 2000), this negative feedback on HUVEC nuclear NFκB may be very relevant to this pathology. For the remainder of the experiments, we focused on IL-1β as the cytokine prototype.

**PMN-derived soluble factors do not play a role in the decrease in HUVEC nuclear NFκB induced by PMN transendothelial migration**

In these experiments, we assessed whether PMN secrete or discharge substances that are responsible for the decrease in nuclear NFκB of IL-1β–stimulated HUVECs. PMN were separated from HUVEC monolayers by placing them in the apical compartment of cell culture inserts (0.4-μm pore diameter) over the HUVEC monolayers in the basal compartment (distance between PMN and HUVECs was 0.9 mm). When PAF-activated PMN were coincubated with HUVECs in this system for 1 h, there was no detectable increase in nuclear NFκB in naive HUVECs (unpublished data). More importantly, the IL-1β–induced increase in nuclear NFκB was not affected by subsequent addition of PMN and PAF (unpublished data). These observations suggest that PAF-activated PMN do not release substances that can
traverse 0.9 mm to influence HUVEC nuclear NFκB. However, this does not preclude the possibility that contact of PMN with the endothelium is necessary for PMN-derived soluble factors to be effective. Others have shown that adhesion of PMN to biological surfaces renders them more sensitive and reactive to inflammatory stimuli (Fuortes et al., 1993; Furuno et al., 1997).

Previously, we have shown that activated PMN mobilize elastase to the cell surface, where it plays an important role in PMN transendothelial migration (Cepinskas et al., 1999a). Others have shown that neutrophil-derived elastase can also induce cell signaling in epithelial (Hashimoto et al., 1999) and endothelial (Yamaguchi et al., 1998) cells. Thus, several approaches were used to determine whether PMN-derived elastase can decrease nuclear NFκB in IL-1β/PAF-stimulated HUVECs. Neutroplasts (neutrophilic cytoplasts) prepared from 10^7 M PAF-stimulated PMN were used, rather than intact PMN. As shown in Fig. 3 A, migrating
The negative impact of PMN transendothelial migration on nuclear NFκB in IL-1β-activated HUVECs does not require PMN degranulation or PMN-derived elastase. The experimental conditions were the same as described in Fig. 2 B, and nuclear extracts were assessed for NFκB content by EMSA. (A) Neutrophils (anuclear PMN devoid of granules) decreased nuclear NFκB in HUVECs when induced to migrate across IL-1β-activated HUVEC monolayers by a PAF gradient (IL-1β/PAF). (B) An elastase inhibitor (L658 758) did not prevent the decrease in nuclear NFκB induced by PMN transendothelial migration (compare lane 2 with lane 3). Results presented are representative of three experiments.

Nitric oxide (NO) has been implicated as an inhibitor of endothelial cell NFκB (De Caterina et al., 1995; Spiecker et al., 1997, 1998; Umansky et al., 1998). In the previous works, NO donors were coincubated with the cytokines, i.e., NO was being generated during cytokine stimulation. In the present paper, we assessed the role of NO in decreasing HUVEC nuclear NFκB after its activation and translocation to the nucleus. Thus, although NO may prevent cytokine-induced activation of NFκB, it may not be capable of decreasing nuclear NFκB after its accumulation within HUVEC nuclei.

PMN-derived NO does not play a role in the negative impact of PMN transendothelial migration on nuclear NFκB in IL-1β-activated HUVECs. The experimental conditions were the same as described in Fig. 2 B, and nuclear extracts were assessed for NFκB content by EMSA. (A) An inhibitor of NO synthase (N^G-nitro-l-arginine methyl ester) did not prevent the decrease in nuclear NFκB in IL-1β-activated HUVECs induced by migrating PMN (compare lane 3 with lane 4). (B) PMN isolated from iNOS-deficient mice (iNOS-KO PMN) reduced endothelial cell nuclear NFκB to the same extent as PMN isolated from their wild-type counterparts (WT-PMN) when induced to migrate across IL-1β-activated mouse cardiac endothelial cells by a PAF gradient (IL-1β/PAF).

Results are representative of three experiments.

![Figure 3](image-url)

![Figure 4](image-url)
Adhesive interactions with HUVECs play an important role in the decrease in HUVEC nuclear NFκB during PMN transendothelial migration

PMN adhesive interactions with endothelial cells mediated by CD18–ICAM-1 are a prerequisite for PMN transendothelial migration (Granger and Kubes, 1994; Kvietys et al., 1996; Panes and Granger, 1998; Wang and Springer, 1998). Thus, we assessed whether these adhesive interactions play a role in the decrease in HUVEC nuclear NFκB during PMN migration across HUVEC monolayers. To this end, we assessed the effects of an mAb directed to CD18 (IB4; 40 μg/ml) on the PMN-mediated decrease in nuclear NFκB in IL-β/PAF-stimulated HUVECs. As shown in Fig. 5 A, inclusion of the mAb prevented the decrease in HUVEC nuclear NFκB; the NFκB level was the same as in the absence of PMN–endothelial cell interactions. Based on previous works (Yoshida et al., 1992) and the present paper (legend to Fig. 5), the mAb directed to CD18 decreases adhesion of PMN to HUVECs and prevents the subsequent transendothelial migration (legend to Fig. 5). Although this approach provided useful information, it did not address the issue of whether PMN adhesion to HUVECs or PMN transendothelial migration, per se, was the critical event involved in the decrease in HUVEC nuclear NFκB. Thus, we next assessed the effects of fixed PMN in this system. Fixed PMN adhere to HUVECs, but do not migrate across the monolayers (Cepinskas et al. 1999. *FASEB J.* 13(4):A178, Abstract and legend to Fig. 5). Fixed PMN failed to induce a decrease in nuclear levels of NFκB in IL-β/PAF-stimulated HUVECs (Fig. 5 B). Together, these findings suggest that the process of PMN transendothelial migration may be more important than the initial PMN adhesive interactions with HUVECs.

PMN adhesion to HUVECs and transendothelial migration is dependent on the interaction of adhesion molecules on both PMN and HUVECs. The firm adhesion of PMN to HUVECs is mediated by CD18–ICAM-1 (Granger and Kubes, 1994; Kvietys et al., 1996; Panes and Granger, 1998; Wang and Springer, 1998), and transendothelial migration is dependent on homotypic adhesive interactions between PECAM-1 on PMN and HUVECs ( Muller, 1995; Liao et al., 1997; Thompson et al., 2001). Both ICAM-1 and PECAM-1 are constitutively expressed on HUVECs; with ICAM-1 expression being punctate on the entire surface, whereas PECAM-1 expression being more concentrated at the interendothelial junctions (Muller et al., 1989; Kishimoto et al., 1999). Furthermore, both endothelial ICAM-1 and PECAM-1 can induce intracellular signaling (Guruhagavatula et al., 1998; Etienne-Manneville et al., 2000; O’Brien et al., 2001). Thus, we assessed whether cross-linking of ICAM-1 or PECAM-1 (in the absence of PMN) would influence the nuclear levels of NFκB in IL-β/PAF-stimulated HUVECs. As shown in Fig. 6 A, cross-linking of ICAM-1 did not affect the nuclear levels of NFκB in IL-β/PAF-stimulated HUVECs (Fig. 6 A, compare lane 3 with lane 1). By contrast, cross-linking of PECAM-1 resulted in a decrease in HUVEC nuclear levels of NFκB (Fig. 6 A, compare lane 1 with lane 2). To further probe for a role for PECAM-1, endothelial cells derived from the hearts of PECAM-1–deficient mice were activated with mouse recombinant IL-1β/PAF and allowed to interact with mouse PMN in the migration assay. As shown in Fig. 6 B, PMN did not decrease the levels of nuclear NFκB in PECAM-1–deficient endothelial cells. In control experiments, we noted that PMN migrating across endothelial cells from wild-type mice (expressing PECAM-1) did decrease nuclear NFκB (Fig. 6 C). Collectively, these observations suggest that engagement of PECAM-1 (but not ICAM-1) on endothelial cells by migrating PMN plays an important role in decreasing the IL-1β/PAF-induced accumulation of NFκB in endothelial cell nuclei.

The decrease in nuclear NFκB induced by PMN transendothelial migration has functional consequences

Next, we assessed whether any functional consequences relevant to inflammation were realized by this ability of migrating PMN to decrease nuclear NFκB in IL-1β/PAF-activated
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HUVECs. Our approach was to stimulate HUVEC monolayers with IL-1\(\beta\)/PAF as in the previous experiments. Subsequently, these monolayers were either interacted with PMN or not. 1 or 12 h later, the HUVEC monolayers were restimulated with IL-1\(\beta\)/PAF, and three endpoints relevant to inflammation were assessed: (1) nuclear mobilization of NF\(\kappa\)B; (2) HUVEC surface levels of ICAM-1; and (3) PMN transendothelial migration. Because the results obtained with a 1-h hiatus between cytokine challenges were qualitatively and quantitatively similar, only the data obtained with a 12-h hiatus are presented and discussed.

The effects of PAF-induced PMN transendothelial migration across IL-1\(\beta\)-stimulated HUVECs on the nuclear levels of NF\(\kappa\)B after a subsequent stimulation of HUVECs with IL-1\(\beta\) are shown in Fig. 7 A. Lanes 1–3 of Fig. 7 A show that IL-1\(\beta\) can induce an increase in HUVEC nuclear NF\(\kappa\)B that is dramatically decreased if PMN are allowed to migrate across the HUVEC monolayers. If PMN were not interacted with the HUVEC monolayers, a second challenge with IL-1\(\beta\) resulted in a greater increase in nuclear accumulation of NF\(\kappa\)B than that noted with the first challenge (Fig. 7 A, lane 4). If PMN were allowed to migrate across IL-1\(\beta\)-stimulated HUVEC monolayers, the second challenge with IL-1\(\beta\) did not result in an increase in HUVEC nuclear NF\(\kappa\)B. These findings indicate that allowing PMN to migrate across HUVEC monolayers initially challenged with IL-1\(\beta\) renders them refractory to a second challenge in terms of NF\(\kappa\)B mobilization to the HUVEC nucleus.

The effects of PAF-induced PMN transendothelial migration across IL-1\(\beta\)-stimulated HUVECs on ICAM-1 expression on the surface of HUVECs after a subsequent challenge of HUVECs with IL-1\(\beta\) are shown in Fig. 7 B. The initial stimulation increased ICAM-1 surface expression on HUVECs. The surface levels of ICAM-1 were not affected by subsequent induction of PMN transendothelial migration. If PMN were not interacted with the HUVEC monolayers after the initial challenge, a second challenge with IL-1\(\beta\) resulted in a greater increase in surface levels of ICAM-1 than that observed after the first challenge. If PMN were allowed to migrate across IL-1\(\beta\)-stimulated HUVECs, the second challenge with IL-1\(\beta\) resulted in a diminished increase in ICAM-1 surface levels on HUVECs as compared with the initial challenge with IL-1\(\beta\). In general, cytokine-induced expression of ICAM-1 on HUVECs reaches maximal levels within 6–8 h, and this enhanced expression is maintained for up to 72 h (Kvietys and Granger, 1993). In the present work, ICAM-1 expression on HUVECs was less after the second cytokine challenge than the first, if PMN were allowed to migrate across the monolayers (Fig. 7 B; compare bar 4 with bar 5). The exact explanation for this observation is not entirely clear. One explanation may be that during PMN transendothelial migration, PMN-derived elastase becomes associated with HUVECs (Cepinskas et al., 1999a) and proteolytically cleaves ICAM-1 (Champagne et al., 1998). Irrespective of the explanation, our findings suggest that allowing PMN to migrate across HUVEC monolayers renders them refractory to a second challenge with respect to induction of an increase in HUVEC surface levels of ICAM-1.

The effects of PAF-induced PMN transendothelial migration across IL-1\(\beta\)-stimulated HUVECs on the PMN transendothelial migration after a subsequent challenge of HUVECs with IL-1\(\beta\) are shown in Fig. 7 C. The initial stimulation with IL-1\(\beta\) resulted in an increase in PMN transendothelial migration in response to a chemotactic gradient induced by PAF. If PMN were not allowed to interact with the HUVEC monolayers after the initial challenge with
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IL-1β/PAF (I) PMN IL-1β/PAF (II)
- - + + - -

PMN IL-1β/PAF (II)
- - + + - -

Figure 7. The decrease in nuclear NFκB induced by PMN migrating across IL-1β/PAF-stimulated HUVECs has functional consequences with respect to the inflammatory process. HUVEC monolayers were stimulated with IL-1β/PAF (I) for 4 h, and subsequently were interacted with PMN or not interacted with PMN. 12 h later, the HUVEC monolayers were rechallenged with IL-1β/PAF (II) for 4 h, and various endpoints relevant to inflammation were assessed. (A) If PMN were not reacted with HUVECs after the first challenge, the second challenge resulted in a greater level of nuclear NFκB as detected by cell ELISA (lane 4). If PMN transendothelial migration was induced after the first challenge, the second challenge did not result in nuclear accumulation of NFκB (lane 5). (B) Lane 6 shows data obtained when IL-1β/PAF stimulation was maintained over the entire experimental period of 16 h (control). Results shown are representative of three experiments. (B) If PMN were not interacted with HUVECs after the first challenge, the second challenge resulted in greater HUVEC surface levels of ICAM-1 as detected by cell ELISA (bar 4). If PMN transendothelial migration was induced after the first challenge, the second challenge resulted in less ICAM-1 induction when compared with the surface of ICAM-1 (compare bar 4 with bar 5). n = 3. (C) If PMN were not interacted with HUVECs after the first challenge, the second challenge resulted in the same degree of PMN transendothelial migration as after the first challenge (compare bar 2 with bar 3). If PMN transendothelial migration was induced after the first challenge, the second challenge resulted in less PMN transendothelial migration (bar 4). n = 3. Dark bars in B and C show data obtained when IL-1β/PAF stimulation was maintained over the entire experimental period of 16 h (control).

Discussion

During the systemic inflammatory response, circulating cytokines interact with the vascular endothelium, resulting in the activation and nuclear translocation of NFκB (Collins et al., 1995; Winyard and Blake, 1997). In turn, NFκB transactivates genes encoding for various cytokines. This results in a positive feedback loop that could amplify the inflammatory response, a situation that could be potentially detrimental to the host. Fortunately, there are negative feedback mechanisms in place that limit an excessive and prolonged inflammatory response on NFκB translocation to the nucleus. For example, in addition to transactivating various proinflammatory genes, NFκB also transactivates the gene encoding for its inhibitory protein, IκB (Brown et al., 1993; Baldwin, 1996). IκB binds to NFκB and prevents further translocation of this nuclear transcription factor, thereby preventing an excessive, uncontrolled inflammatory response. In the present paper, we provide evidence for the existence of another negative feedback mechanism impacting on NFκB. We show that the IL-1β-induced increase in HUVEC nuclear NFκB can be dramatically reduced by PMN transendothelial migration (Figs. 2–5). In addition, we provide evidence to implicate PECAM-1 in this negative feedback mechanism (Fig. 6). To our knowledge, this is first demonstration of the existence of this novel negative feedback on NFκB.

During the course of our experiments, we were aware of one potential caveat to the interpretation of the data. Previous works have shown that, during preparation of samples of endothelial cell monolayers for assay, any PMN associated with the monolayers could lead to artificial proteolysis of proteins under investigation (Moll et al., 1998). In our experiments, we minimized the potential for this artifact to impact on our results in two ways. For EMSA, we allowed the PMN to completely leave the apical aspect of the HUVEC monolayers, and we maximized the antiproteolytic activity of the buffers used to extract nuclear proteins (McDonald et al., 1997). In addition, we used another approach to assess the negative impact of PMN transendothelial migration on the IL-1β-induced increase in HUVEC nuclear NFκB, i.e., confocal microscopy (Fig. 2).
volved the fixation of all cells under study, and thus prevented PMN degranulation during sample preparation. Finally, addition of the PMN protease (elastase) directly to IL-1β–stimulated HUVECs had no impact on nuclear NFκB. Together, these observations indicate that artificial proteolysis was not an issue in our experiments.

PMN adhesion to HUVECs and transendothelial migration is dependent on the interaction of adhesion molecules on both PMN and HUVECs. The firm adhesion of PMN to HUVECs is mediated by CD18–ICAM-1 (Granger and Kubes, 1994; Kvetys et al., 1996; Panes and Granger, 1998; Wang and Springer, 1998), whereas transendothelial migration is dependent on homotypic adhesive interactions between PECAM-1 on PMN and HUVECs (Muller, 1995; Liao et al., 1997; Thompson et al., 2001). Furthermore, both endothelial ICAM-1 and PECAM-1 can induce intracellular signaling. Cross-linking of ICAM-1 on rat brain endothelial cells promotes intracellular calcium signaling that results in cytoskeletal rearrangement and facilitates lymphocyte transendothelial migration (Etienne-Manneville et al., 2000). Interestingly, recent works indicate that ICAM-1 transfected into CHO cells can support PMN migration across these transfected CHO monolayers; an effect requiring the presence of the cytoplasmic domain of ICAM-1 (Sans et al., 2001). This latter observation indicates that ICAM-1 can contribute to PMN transendothelial migration. Finally, engagement of PECAM-1 on HUVECs with antibodies results in intracellular signaling (increased intracellular calcium); an effect that requires the presence of the intracellular domain (Gurubhagavatula et al., 1998; O’Brien et al., 2001). Thus, we assessed whether cross-linking of either ICAM-1 or PECAM-1 (in the absence of PMN) would have an impact on the levels of nuclear NFκB in IL-1β–stimulated HUVECs. Of these two endothelial cell adhesion molecules implicated in PMN transendothelial migration, only engagement and cross-linking of PECAM-1 was capable of mimicking the effects of PMN transendothelial migration on nuclear NFκB in cytokine-stimulated HUVECs (Fig. 6 A). Furthermore, the IL-1β–induced increase in nuclear NFκB was unaffected by PMN adhesive interactions with endothelial cells derived from PECAM-1–deficient mice (Fig. 6 B). A role for PECAM-1 (but not ICAM-1) in intracellular signaling has also been shown in another system (Ferrero et al., 2003). The specific intracellular signals that are initiated by engagement of endothelial cell PECAM-1 by PMN to decrease nuclear NFκB are, at present, unclear and warrant further attention.

Our findings also indicate that the decrease in nuclear NFκB in IL-1β–stimulated HUVECs by migrating PMN has functional consequences relevant to the inflammatory process. When PMN transendothelial migration was allowed to occur across IL-1β–stimulated HUVECs, a subsequent challenge with IL-1β resulted in less (1) nuclear NFκB accumulation; (2) ICAM-1 surface levels; and (3) PMN transendothelial migration (Fig. 7). It is worth noting that although there is a striking loss of NFκB under these circumstances, there are only modest decreases in surface levels of ICAM-1 (48%) and PMN transendothelial migration (45%). These observations indicate that other factors besides NFκB may be involved in the residual proinflammatory response observed after the second challenge with the cytokine. There may be nuclear transcription factors that are not negatively impacted by PMN migration which may contribute to the inflammatory response. Alternatively, the residual response may be independent of nuclear transcription factors, i.e., other as yet unidentified proinflammatory mediators may be involved. Irrespective of the explanation, these observations indicate that allowing PMN to migrate across IL-1β–stimulated HUVECs results in a decreased proinflammatory response to a second IL-1β challenge.

The phenomenon described herein is very reminiscent of the development of tolerance to cytokine or LPS stimulation in a variety of cells (Framer et al., 1988; Leduc et al., 1995; Lush et al., 2000). However, there is a major difference in the development of this refractoriness to cytokine stimulation between classical tolerance and the tolerance described in the present paper. In classical tolerance, an initial challenge with a cytokine or oxidant stress results in a decreased responsiveness to a subsequent challenge with the same stimulus (Cepinskas et al., 1999b; Lush et al., 2000); an effect independent of PMN interactions with the cells. In the present paper, we have provided evidence that allowing for PMN transendothelial migration between the two challenges can also result in the development of tolerance. To our knowledge, this is the first direct demonstration of such a phenomenon. Further studies are warranted to unravel the mechanisms involved in the development of this form of tolerance.

Materials and methods

Induction of sepsis in mice

Sepsis was induced in wild-type (C57BL/6) and ICAM–deficient mice (C57BL/6 background; Jackson ImmunoResearch Laboratories) by CLP as described previously (Astiz et al., 1994; Kato et al., 1995; Lush et al., 2001). The mice were anesthetized with a subcutaneous injection of 150 mg/kg ketamine and 7.5 mg/kg xylazine. After a midline incision, the cecum was exposed, ligated at the level of the ileo-cecal valve, and its contents expelled by an incision at the antimesenteric border. The laparotomy was closed and a 1-ml subcutaneous injection of saline was given for fluid resuscitation. Sham-operated mice underwent laparotomy, but no cecal ligation or perforation. 6 h after induction of CLP, the hearts and lungs of the mice were harvested and tissue processed for assessment of (1) tissue accumulation of PMN; and (2) the presence of NFκB in nuclear extracts. PMN accumulation was assessed by measuring tissue MPO activity as described previously (Lush et al., 2001). NFκB accumulation in nuclear extracts was assessed by the EMSA as described in the EMSA for NFκB section (Neviere et al., 1999).

Endothelial cells

HUVECs were harvested from umbilical cords by collagenase treatment and cultured as described previously (Yoshida et al., 1992; Cepinskas et al., 1999a). Primary through second passage HUVECs were used in experiments. When necessary, mouse (C57BL/6 wild type or PECAM-1 deficient on C57BL/6 background; The Jackson Laboratory and gift from Dr. W. Muller) myocardial endothelial cells were harvested as described previously (Rui et al., 2001).

Leukocytes

Human neutrophilic PMN were isolated from venous blood of healthy adults using standard dextran sedimentation and gradient separation on HISTOPAQ®-1077 (Sigma-Aldrich; Cepinskas et al., 1999b). This procedure yields a PMN population that is 95–98% viable (trypan blue exclusion) and 98% pure (acetic acid-crystal violet staining). When necessary, PMN cytoplasts (neutroplasts) were prepared as described previously (Cepinskas et al., 1999a). In brief, PMN in 12.5% Ficoll solution were layered over a discontinuous gradient of 16 and 25% Ficoll and centrifuged at
PMN transendothelial migration
PMN transendothelial migration was assessed as described previously (Cepinskas et al., 1997, 1999a). Endothelial cells were grown to confluence on 25 μg/ml fibronectin-coated Falcon cell culture inserts (3-μm-diam pores) and stimulated with 1 ng/ml IL-1β (R&D Systems) for 4 h. Neutrophils were added to the endothelial cell monolayers and allowed to migrate across them for 1 h in the presence of PAF (10−6 M; Sigma-Aldrich) in the basal compartment. This period of time was sufficient to ensure that the bulk of the PMN had traversed the monolayer. To quantitate changes in the rate of PMN transendothelial migration, PMN were labeled with 51Cr and PMN migration was assessed 30 min after coincubation with HUVEC monolayers, i.e., before PMN transendothelial migration was complete.

EMSA for NFκB
Nuclear protein from whole tissue or endothelial cells was extracted as described previously (McDonald et al., 1997; Cepinskas et al., 1999b). For EMSA, 3 μg of total nuclear proteins was incubated with 1.0 pmol of double-stranded (32)P-end-labeled oligonucleotides containing consensus binding sequences for NFκB (sense strand 5’-AGGACTTTCC-GGTCTGGGACTTCC-3’) in a binding buffer (10 mM Hepes, pH 7.9, 80 mM NaCl, 3 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10% glycerol) as described previously (Bielinska et al., 1990; Cepinskas et al., 1999b). After electrophoresis under nondenaturing conditions (0.5% TBE buffer), the gels were dried and the radioactive bands were visualized on X-ray films.

Cell ELISA for ICAM-1
Cell surface levels of ICAM-1 were measured as described previously (Lush et al., 2000). In brief, PFA-fixed (3%) HUVECs were incubated with 2 μg/ml rabbit pAb NFκB p65 (A: Santa Cruz Biotechnology, Inc.) and Texas red-conjugated secondary goat anti-rabbit IgG (Molecular Probes, Inc.). The nuclei were stained with Hoechst 33342. The distribution of HUVEC conjugated secondary goat anti–rabbit IgG (Molecular Probes, Inc.). The antibody binding intensity was evaluated using a Mouse Extravidin Peroxidase Staining Kit (Sigma-Aldrich).

Adhesion molecule cross-linking
Cross-linking of ICAM-1 was induced as described previously (Etienne-Manneville et al., 2000) with some modifications. In brief, HUVECs were grown in 35-mm Petri dishes and stimulated with 1 ng/ml IL-1β for 4 h. HUVEC ICAM-1 was engaged by treatment of HUVECs with a primary mAb (20 μg/ml) directed against ICAM-1 (RR1/1, IgG1; Biosource International) for 30 min, and cross-linking was induced by subsequent addition of 5 μg/ml rabbit anti-mouse antibodies (DakoCytomation) for an additional 30 min. Cross-linking of PECAm-1 was done in a similar manner (Berman and Muller, 1995; Gurubhagavatula et al., 1998) using an mAb (20 μg/ml) against PECAm-1 (hec7, IgG2a, a gift from Dr. W.A. Muller, Cornell University School of Medical Sciences, New York, NY).

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