JCB Article

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

Gediminas Cepinskas, Jurate Savickiene, Carmen V. Ionescu, and Peter R. Kvietys

Program in Vascular Biology/Inflammation, Lawson Health Research Institute, London, Ontario, Canada N6A 4G5

During the systemic inflammatory response, circulating cytokines interact with the vascular endothelium, resulting in activation and nuclear accumulation of the nuclear transcription factor, nuclear factor kappa B (NFκB). In turn, NFκB transactivates relevant proinflammatory genes, resulting in an amplification of the inflammatory response. Because this scenario is potentially detrimental to the host, mechanisms exist to limit this amplification. Using an in vitro system that mimics the vascular–interstitial interface during inflammation (cell culture inserts), we provide evidence for the existence of a novel negative feedback mechanism on NFκB activity. We show that the interleukin 1β–induced accumulation of nuclear NFκB in human umbilical vein endothelial cell monolayers is dramatically reduced when polymorphonuclear leukocytes (PMN) are allowed to migrate across these monolayers. This effect does not appear to be due to PMN-derived elastase or nitric oxide. Fixed PMN (adhere but do not migrate) did not affect nuclear NFκB. Furthermore, cross-linking of platelet-endothelial cell adhesion molecule-1 (PECAM-1), but not intercellular adhesion molecule-1, reduces human umbilical vein endothelial cell nuclear NFκB induced by interleukin 1β. Finally, interaction of PMN with PECAM-1–deficient endothelial cells does not reduce nuclear NFκB. These observations indicate that engagement of PECAM-1 by emigrating PMN is a pivotal event in this negative feedback on NFκB activity.

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,

G. Cepinskas and P.R. Kvietys contributed equally to this paper.

Address correspondence to Peter R. Kvietys, Program in Vascular Biology/Inflammation, Lawson Health Research Institute, 375 South Street, Room C210, London, Ontario, Canada N6A 4G5. Tel.: (519) 685-8300 ext. 77055. Fax: (519) 667-6629. E-mail: pkvietys@julian.uwo.ca

*Abbreviations used in this paper: CD18, cluster of differentiation-18; CLP, cecal ligation and perforation; EMSA, electrophoretic mobility shift assay; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; IκB, inhibitory protein kappa B; IL-1β, interleukin 1β; LPS, lipopolysaccharide; MPO, myeloperoxidase; NFκB, nuclear factor kappa B; NO, nitric oxide; PAF, platelet-activating factor; PECAM-1, platelet-endothelial cell adhesion molecule 1; PMN, polymorphonuclear leukocytes; TNF-α, tumor necrosis factor α.

Key words: ICAM-1; CD18; HUVEC; mice; NO

1999; Bonizzi et al., 2000). 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

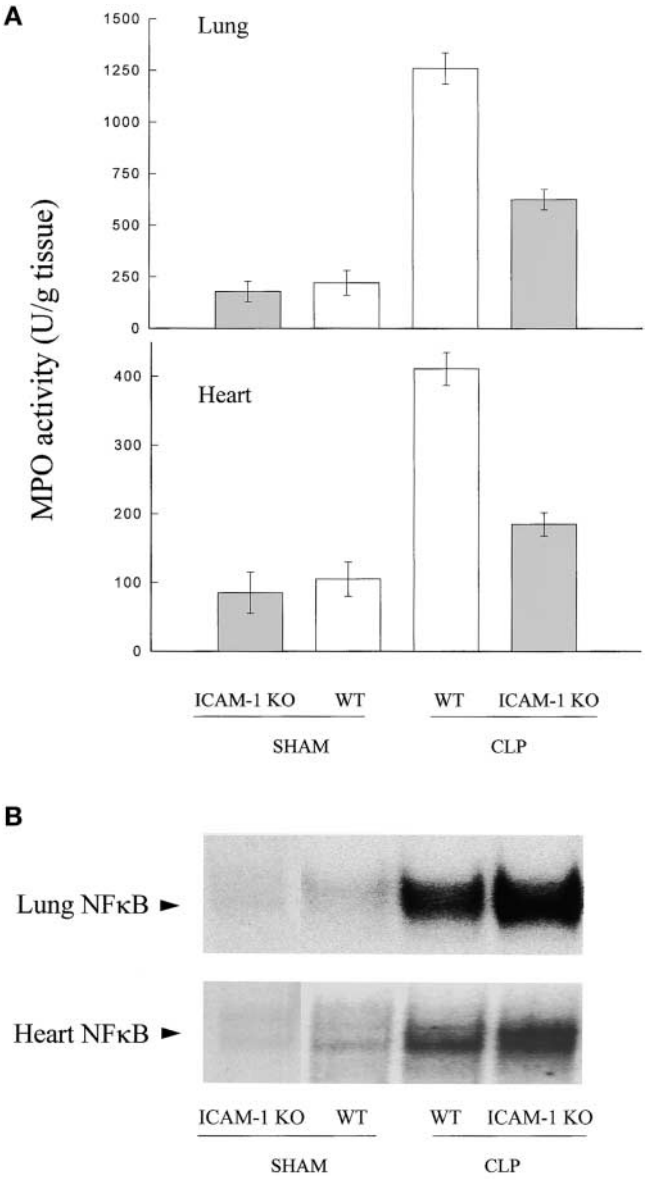

Figure 1. **PMN accumulation in the lung and heart of ICAM-1–deficient mice is decreased during peritonitis, whereas tissue nuclear NFκB is increased.** Wild-type and ICAM-1–deficient mice were subjected to peritonitis (CLP). (A) The increase in MPO activity (index of PMN accumulation) was less in the lungs and hearts of ICAM-1 knockout (ICAM-1 KO) mice as compared with their wild-type counterparts (WT). MPO activity was measured 6 h after induction of CLP. Values are means and SEM from six experiments. (B) The CLP-induced increase in NFκB in nuclear extracts of the lungs and hearts was greater in ICAM-1 knockout mice (ICAM-1 KO). The nuclear extracts were obtained 6 h after induction of CLP and EMSA performed as described in Materials and methods. Results presented are representative of six experiments.

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 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

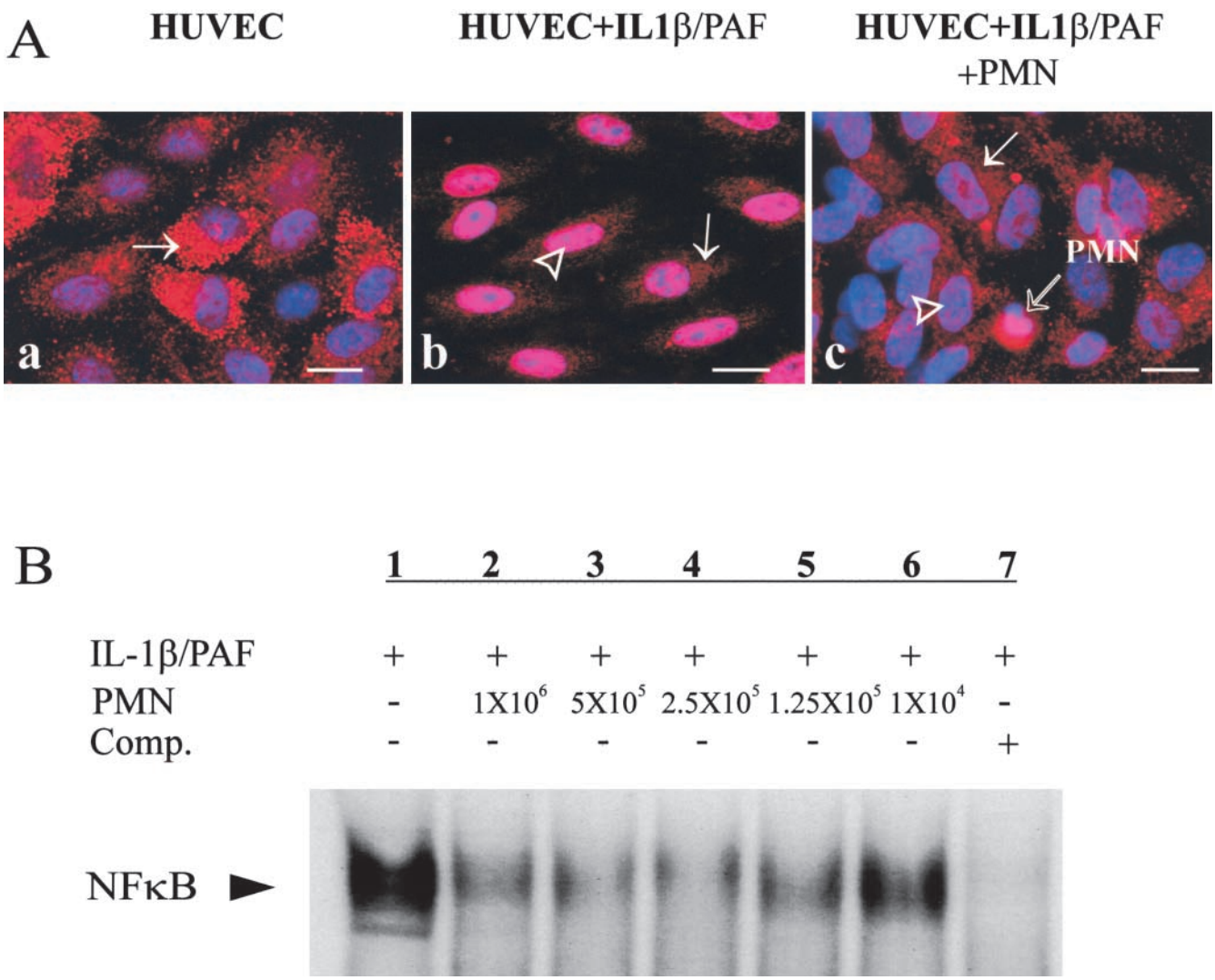

Figure 2. **PMN transendothelial migration across IL-1β–activated HUVEC monolayers reduces nuclear levels of the NFκB.** (A) In naive (unstimulated) HUVECs, the NFκB p65 subunit (red fluorescence) is confined to the cytoplasm (a, arrow), i.e., there is no detectable NFκB p65 in the nuclei. In IL-1β/PAF-stimulated HUVECs, the NFκB p65 subunit is detected in the nuclei (b, arrowhead) with very little of the NFκB p65 remaining in the cytoplasm (b, arrow). The IL-1β–induced nuclear staining for NFκB p65 is decreased after PMN migration across HUVECs (c, arrowhead), while some cytoplasmic staining is present (c, solid arrow). Although the images were obtained after the bulk of the PMN added to the HUVEC monolayers had completed their passage across the monolayers, an occasional PMN was captured still in the process of migration (c, open arrow). Bar, 20 μm. The experiment was repeated twice and similar results were obtained. Quantitation of this phenomenon was performed by counting the number of nuclei stained for NFκB in 100 random cells. Under control conditions (a), 2% of the nuclei were stained; after stimulation of HUVECs with IL-1β/PAF (b), 97% of the nuclei were stained; and after PMN transendothelial migration (c), 22% of the nuclei were stained. (B) HUVECs in the apical compartment of culture inserts were stimulated with IL-1β for 4 h, and subsequently, PMN transendothelial migration was induced by PAF introduced into the basal compartment of the system (IL-1β/PAF). After PMN transendothelial migration, nuclear extracts were obtained from the HUVEC monolayers and assessed for NFκB using EMSA. PMN transendothelial migration decreased the IL-1β–stimulated HUVEC nuclear content of NFκB. As the number of PMN allowed to interact and migrate across HUVEC monolayers was increased, the greater was the decrease in the levels of nuclear NFκB (lanes 2–6). Lane 7 shows the control competition assay (Comp.) using 50-fold excess of the unlabeled oligonucleotide. Results presented are representative of three experiments.

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-β/PAF-stimulated HUVECs. Neutroplasts (neutrophilic cytoplasts) prepared from 10^{-7} M PAF-stimulated PMN were used, rather that intact PMN. As shown in Fig. 3 A, migrating

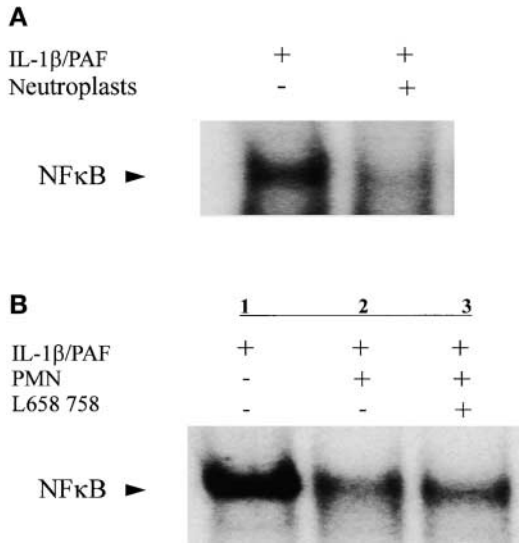

Figure 3. **The negative impact of PMN transendothelial migration on nuclear NFκB in IL-1β–activated HUVECs does not require PMN degranulation or PMN-derived elastase.** The experimental conditions were the same as described in Fig. 2 B, and nuclear extracts were assessed for NFκB content by EMSA. (A) Neutroplasts (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 (L 658 758) 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.

neutroplasts were also capable of inducing a decrease in HUVEC nuclear NFκB. This observation indicates that PMN degranulation (extracellular release of enzymes) is not required for the negative impact of PMN migration on HUVEC nuclear NFκB. However, the cell membranes of neutroplasts obtained from PAF-activated PMN are enriched in elastase (Cepinskas et al., 1999a). Thus, it was quite possible that membrane-bound elastase could be involved. However, the elastase inhibitor L658 758 (100 μM; Merck) did not affect the decrease in NFκB in HUVEC nuclei induced by migrating intact PMN (Fig. 3 B). In another series of experiments, purified human elastase (0.1–0.5 μg/ml for 15 min; DakoCytomation) was added to the IL-β/PAF-stimulated HUVECs. This maneuver also failed to affect the increase in HUVEC nuclear NFκB induced by IL-1β (unpublished data). Together, these experiments indicate that PMN-derived elastase does not play a role in the negative effect on endothelial nuclear NFκB induced by migrating PMN.

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). Thus, it is quite possible that NO released from migrating PMN could have a negative impact on nuclear NFκB in IL-1β/PAF-stimulated HUVECs. However, as shown in Fig. 4 A, inclusion of the NO inhibitor N^G-nitro-L-arginine methyl ester (100 μM; Sigma-Aldrich) did not alter the inhibitory affect of migrating PMN on HUVEC nuclear NFκB. Furthermore, addition of a NO donor (SIN at 300 μM for up to 2 h) to IL-β/PAF-stimulated HUVECs also had no impact on nuclear NFκB (unpublished data). Finally, endothelial cells isolated from the hearts of wild-type mice were stimulated with IL-β/PAF (recombinant mouse) and allowed to interact with PMN isolated from iNOS-deficient mice. These iNOS-deficient PMN were also capable of decreasing the levels of nuclear NFκB in endothelial cells (Fig. 4 B). Collectively, these observations do not support a role for NO in the negative effect of migrating PMN on endothelial cell nuclear NFκB.

Our findings that PMN-derived NO is not involved in the decrease in IL-1β/PAF-induced increase in HUVEC nuclear NFκB appear to contradict the results of previous studies indicating a role for NO in inhibiting cytokine induced activation of 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.

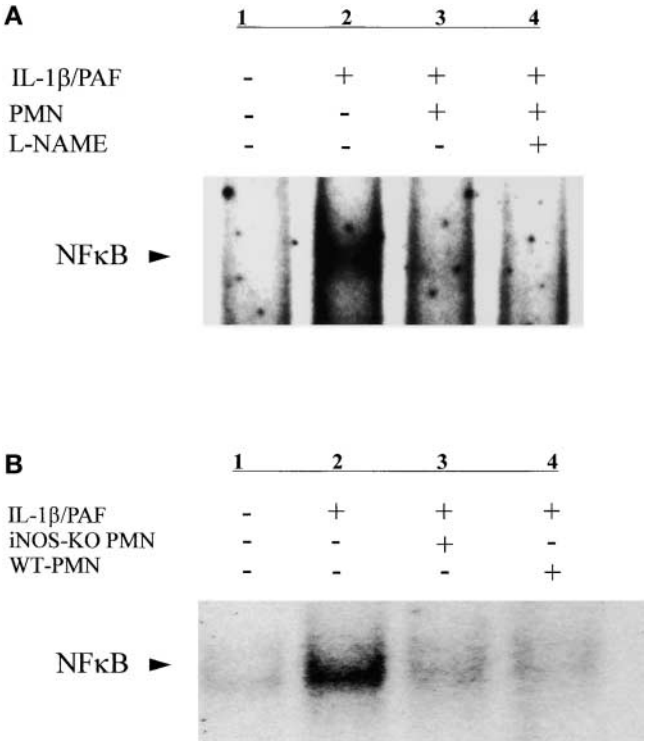

Figure 4. **PMN-derived NO does not play a role in the negative impact of PMN transendothelial migration on nuclear NFκB in IL-1β–activated HUVECs.** The experimental conditions were the same as described in Fig. 2 B, and nuclear extracts were assessed for NFκB content by EMSA. (A) An inhibitor of NO 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.

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 (IB_4; 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.

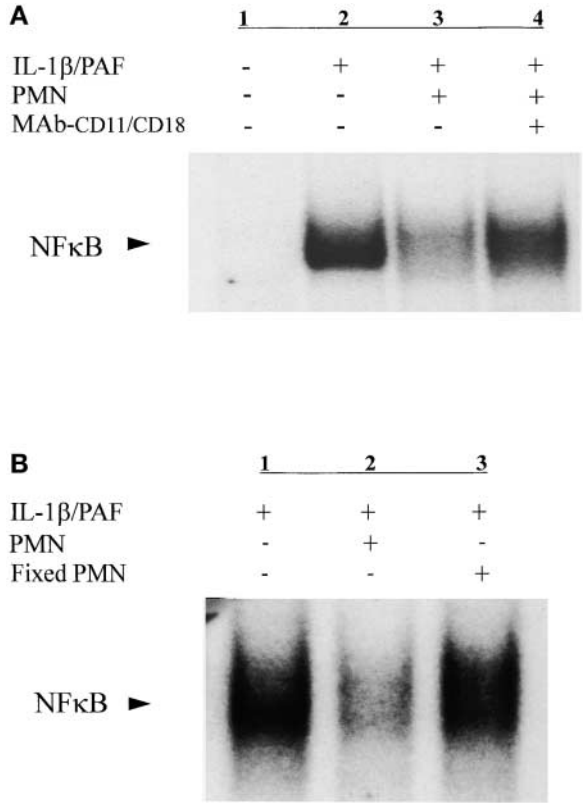

Figure 5. **PMN interactions with adhesion molecules on HUVECs play a role in the negative impact on nuclear NFκB induced by PMN transendothelial migration across IL-1β–stimulated HUVECs.** The experimental conditions were the same as described in Fig. 2 B, and nuclear extracts were assessed for NFκB content by EMSA. (A) An mAb directed to CD18 prevented the decrease in nuclear NFκB in IL-1β/PAF-stimulated HUVECs induced by migrating PMN (compare lane 3 with lane 4). (B) Interaction of fixed PMN with HUVECs did not have a negative impact on nuclear NFκB of IL-1β/PAF-stimulated HUVECs (lane 3). Results shown are representative of three experiments. 1-h transendothelial migration of PMN, PMN plus CD18 mAb, or fixed PMN were 80.3 ± 0.7, 16.4 ± 1.3, or 7.9 ± 1.4, respectively. 30-min adhesion of PMN, PMN plus CD18 mAb, or fixed PMN to HUVEC monolayers on nonporous surface were 20.1 ± 3.2, 5.7 ± 0.8, or 9.6 ± 0.9, respectively.

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 (Gurubhagavatula 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

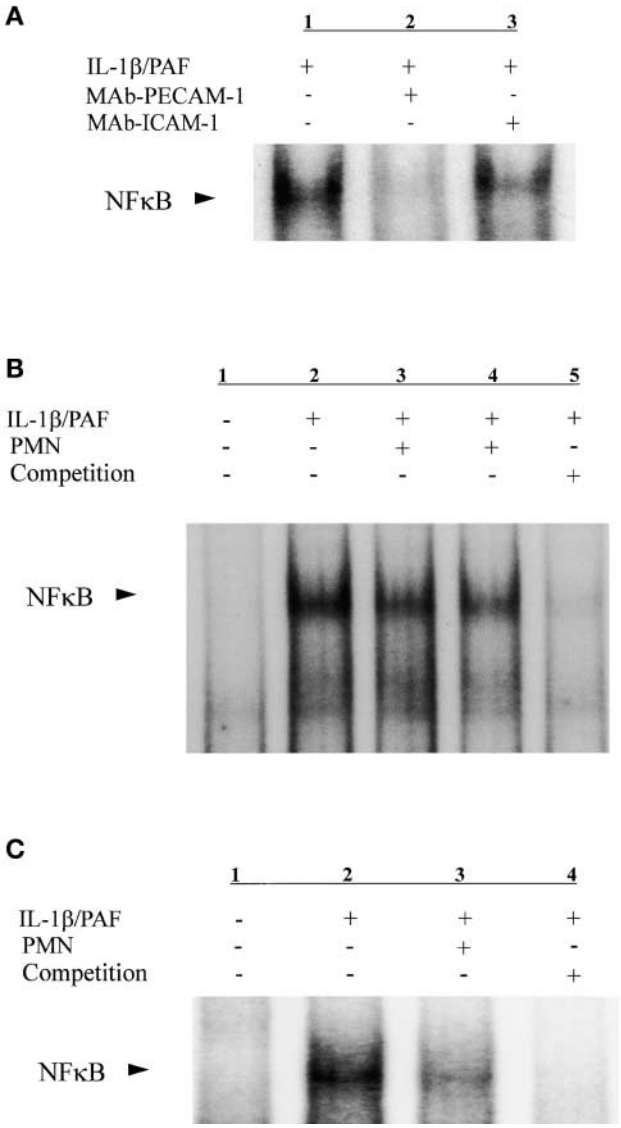

Figure 6. **Engagement of PECAM-1 on endothelial cells plays a role in the decrease in nuclear NFκB in IL-1β–activated endothelial cells.** (A) HUVEC monolayers were stimulated with IL-1β/PAF and ICAM-1 or PECAM-1 were cross-linked as described in Materials and methods. Cross-linking of PECAM-1 (mAb-PECAM-1), but not ICAM-1 (mAb-ICAM-1), resulted in a decrease in nuclear NFκB in IL-1β/PAF-stimulated HUVECs in the absence of PMN. (B) Cardiac endothelial cells were isolated from wild-type mice (lanes 1, 2, and 5) or PECAM-1–deficient mice (lane 3, The Jackson Laboratory; lane 4, obtained from Dr. W.A. Muller). Mouse PMN adhesive interactions with PECAM-1–deficient endothelial cells did not affect the increase in nuclear NFκB induced by IL-1β/PAF. In these experiments, 58% (lane 3) and 54% (lane 4) of the PMN migrated across PECAM-1–deficient endothelial cells after 1 h of coincubation. (C) Mouse PMN adhesive interactions with endothelial cells from wild-type mice (expressing PECAM-1) reduced endothelial cell nuclear NFκB induced by IL-1β/PAF. Results shown are representative of three experiments.

HUVECs. Our approach was to stimulate HUVEC monolayers with IL-1β/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-1β/PAF, and three endpoints relevant to inflammation were assessed: (1) nuclear mobilization of NFκB; (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-1β–stimulated HUVECs on the nuclear levels of NFκB after a subsequent stimulation of HUVECs with IL-1β are shown in Fig. 7 A. Lanes 1–3 of Fig. 7 A show that IL-1β can induce an increase in HUVEC nuclear NFκB 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-1β resulted in a greater increase in nuclear accumulation of NFκB than that noted with the first challenge (Fig. 7 A, lane 4). If PMN were allowed to migrate across IL-1β–stimulated HUVEC monolayers, the second challenge with IL-1β did not result in an increase in HUVEC nuclear NFκB. These findings indicate that allowing PMN to migrate across HUVEC monolayers initially challenged with IL-1β renders them refractory to a second challenge in terms of NFκB mobilization to the HUVEC nucleus.

The effects of PAF-induced PMN transendothelial migration across IL-1β–stimulated HUVECs on ICAM-1 expression on the surface of HUVECs after a subsequent challenge of HUVECs with IL-1β 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-1β 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-1β–stimulated HUVECs, the second challenge with IL-1β resulted in a diminished increase in ICAM-1 surface levels on HUVECs as compared with the initial challenge with IL-1β. 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-1β–stimulated HUVECs on the PMN transendothelial migration after a subsequent challenge of HUVECs with IL-1β are shown in Fig. 7 C. The initial stimulation with IL-1β 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

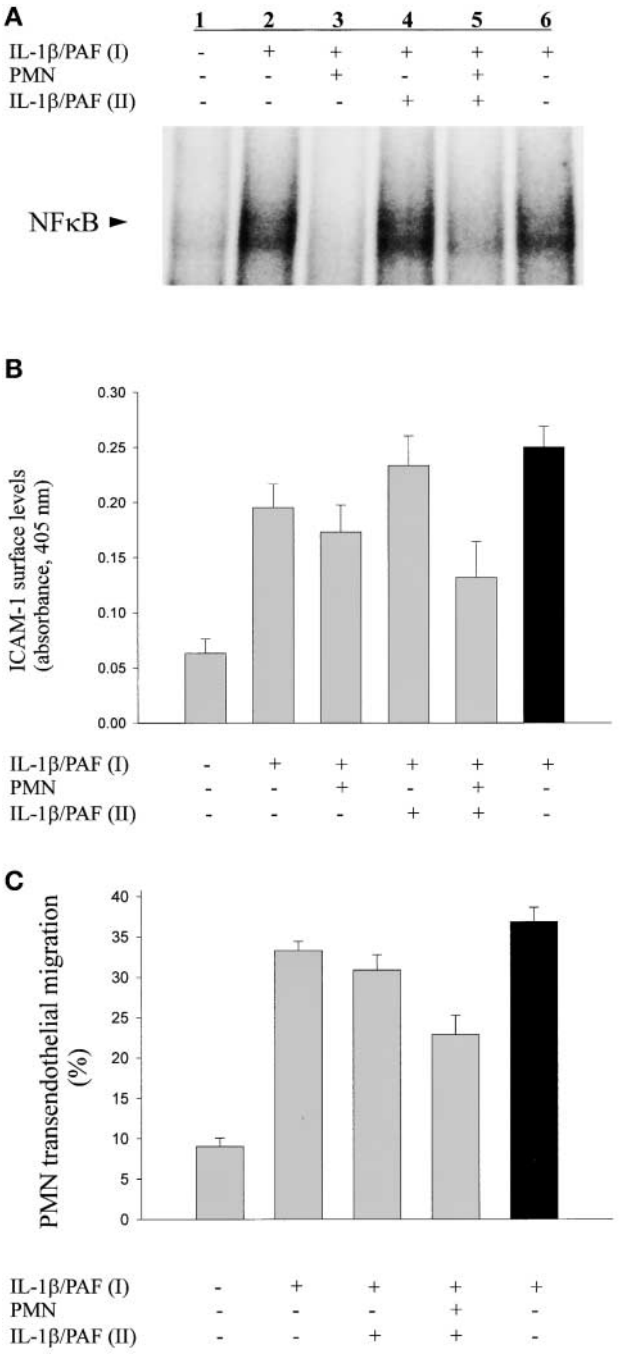

Figure 7. **The decrease in nuclear NFκB induced by PMN migrating across IL-1β/PAF-stimulated HUVECs has functional consequences with respect to the inflammatory process.** HUVEC monolayers were stimulated with IL-1β/PAF (I) for 4 h, and subsequently were interacted with PMN or not interacted with PMN. 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 EMSA (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). 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 on 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).

IL-1β, PAF-induced PMN migration after the second challenge with IL-1β was similar to that noted after the first IL-1β challenge. If the PMN were allowed to migrate across the HUVEC monolayers after the initial challenge with IL-1β, the PAF-induced PMN transendothelial migration after the second IL-1β challenge was reduced by ~45% (Fig. 7 C, compare bar 2 with bar 4).

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). This approach in-

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 artifactual 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; Kvietys 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-I 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 maybe 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 (Fraker et al., 1988; Laegreid 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-1–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 HISTOPAQUE®-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

81,000 *g*. Neutroplasts were harvested from the 12.5%/16% interface. This procedure yields a population of anucleated PMN devoid of granules. For some experiments, PMN were fixed for 5 min using 3% PFA in PBS. When necessary, PMN were isolated from whole blood of mice (C57BL/6 wild type or iNOS-deficient on C57BL/6 background; The Jackson Laboratory) by centrifugation on a NIM-2 gradient (Cardinal Associates, Inc.).

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^{-10} 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 ^{51}Cr, 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 γ[^{32}P]ATP end-labeled oligonucleotides containing consensus binding sequences for NF-κB (sense strand 5′-AGGGACTTTC-CGCTGGGGACTTTCC-3′) in a binding buffer (10 mM Hepes, pH 7.9, 80 mM NaCl, 3 mM $MgCl_2$, 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.

Laser scanning confocal microscopy for NFκB

HUVECs were prepared for immunofluorescence analysis as described previously (Cepinskas et al., 1999a). HUVECs were treated 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 NFκB p65 subunits was analyzed by confocal microscopy.

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 primary mAb directed against ICAM-1 (RR1/1, IgG_1; Biosource International). 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, IgG_1; 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, IgG_{2a}; a gift from Dr. W.A. Muller, Cornell University School of Medical Sciences, New York, NY).

The authors wish to thank Dr. W.A. Muller for his gifts of the PECAM-1 antibody and PECAM-1–deficient mice.

This work was supported by the Canadian Institutes of Health Research grants MOP 44010 (to G. Cepinskas), and MOP 37758 and 13668 (to P.R. Kvietys).

Submitted: 5 December 2002
Revised: 21 March 2003
Accepted: 25 March 2003

References

Astiz, M.E., D.C. Saha, C.M. Carpati, and E.C. Rackow. 1994. Induction of endotoxin tolerance with monophosphoryl lipid A in peritonitis: importance of localized therapy. *J. Lab. Clin. Med.* 123:89–93.

Baldwin, A.S., Jr. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14:649–683.

Berman, M.E., and W.A. Muller. 1995. Ligation of platelet/endothelial cell adhesion molecule 1 (PECAM-1/CD31) on monocytes and neutrophils increases binding capacity of leukocyte CR3 (CD11b/CD18). *J. Immunol.* 154:299–307.

Bielinska, A., R.A. Shivdasani, L. Zhang, and G.J. Nabel. 1990. Regulation of gene expression with double-stranded phosphorothioate oligonucleotides. *Science.* 250:997–1000.

Bone, R.C. 1995. Sepsis, sepsis syndrome, and the systemic inflammatory response syndrome (SIRS). Gulliver in Laputa. *JAMA.* 273:155–156.

Bonizzi, G., J. Piette, M.P. Merville, and V. Bours. 2000. Cell type-specific role for reactive oxygen species in nuclear factor-kappaB activation by interleukin-1. *Biochem. Pharmacol.* 59:7–11.

Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist. 1993. Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. *Proc. Natl. Acad. Sci. USA.* 90:2532–2536.

Cepinskas, G., C.W. Lush, and P.R. Kvietys. 1999b. Anoxia/reoxygenation-induced tolerance with respect to polymorphonuclear leukocyte adhesion to cultured endothelial cells - A nuclear factor-kappa B-mediated phenomenon. *Circ. Res.* 84:103–112.

Cepinskas, G., R. Noseworthy, and P.R. Kvietys. 1997. Transendothelial neutrophil migration. Role of neutrophil-derived proteases and relationship to transendothelial protein movement. *Circ. Res.* 81:618–626.

Cepinskas, G., M. Sandig, and P.R. Kvietys. 1999a. PAF-induced elastase-dependent neutrophil transendothelial migration is associated with the mobilization of elastase to the neutrophil surface and localization to the migrating front. *J. Cell Sci.* 112:1937–1945.

Champagne, B., P. Tremblay, A. Cantin, and Y. Stpierre. 1998. Proteolytic cleavage of ICAM-1 by human neutrophil elastase. *J. Immunol.* 161:6398–6405.

Collins, T., M.A. Read, A.S. Neish, M.Z. Whitley, D. Thanos, and T. Maniatis. 1995. Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. *FASEB J.* 9:25–35.

De Caterina, R., P. Libby, H.B. Peng, V.J. Thannickal, T.B. Rajavashisth, M.A.J. Gimbrone, W.S. Shin, and J.K. Liao. 1995. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J. Clin. Invest.* 96:60–68.

Etienne-Manneville, S., J.B. Manneville, P. Adamson, B. Wilbourn, J. Greenwood, and P.O. Couraud. 2000. ICAM-1 coupled cytoskeletal rearrangements and transendothelial lymphocyte migration involve intracellular calcium signalling in brain endothelial cell lines. *J. Immunol.* 165:3375–3383.

Ferrero E., D. Belloni, P. Contini, C. Foglieni, M.E. Ferrero, M. Fabbri, A. Poggi, and M.R. Zocchi. 2003. Transendothelial migration leads to protection from starvation-induced apoptosis in $CD34^+$ $CD14^+$ circulating precursors: evidence for PECAM-1 involvement through Akt/PKB activation. *Blood.* 101:186–193; first published on August 15, 2002; 10.1182/blood-2002-03-0768.

Fraker, D.L., M.C. Stovroff, M.J. Merino, and J.A. Norton. 1988. Tolerance to tumor necrosis factor in rats and the relationship to endotoxin tolerance and toxicity. *J. Exp. Med.* 168:95–105.

Fuortes, M., W.W. Jin, and C. Nathan. 1993. Adhesion-dependent protein tyrosine phosphorylation in neutrophils treated with tumor necrosis factor. *J. Cell Biol.* 120:777–784.

Furuno, T., T. Mitsuyama, K. Hidaka, T. Tanaka, and N. Hara. 1997. The role of neutrophil elastase in human pulmonary artery endothelial cell injury. *Int. Arch. Allergy Immunol.* 112:262–269.

Goddard, C.M., B.Y. Poon, M.E. Klut, B.R. Wiggs, S.F. vanEeden, J.C. Hogg, and K.R. Walley. 1998. Leukocyte activation does not mediate myocardial leukocyte retention during endotoxemia in rabbits. *Am. J. Physiol.* 275: H1548–H1557.

Granger, D.N., and P. Kubes. 1994. The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion. *J. Leukoc. Biol.* 55:662–675.

Gurubhagavatula, I., Y. Amrani, D. Pratico, F.L. Ruberg, S.M. Albelda, and R.A. Panettieri, Jr. 1998. Engagement of human PECAM-1 (CD31) on human endothelial cells increases intracellular calcium ion concentration and stimulates prostacyclin release. *J. Clin. Invest.* 101:212–222.

Hashimoto, S., S. Maruoka, Y. Gon, K. Matsumoto, I. Takeshita, and T. Horie. 1999. Mitogen-activated protein kinase involves neutrophil elastase-induced

morphological changes in human bronchial epithelial cells. *Life Sci.* 64: 1465–1471.

Henkel, T., U. Zabel, K. van Zee, J.M. Muller, E. Fanning, and P.A. Baeuerle. 1992. Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-kappa B subunit. *Cell* 68: 1121–1133.

Hogg, J.C., and C.M. Doerschuk. 1995. Leukocyte traffic in the lung. *Annu. Rev. Physiol.* 57:97–114.

Kato, T., A. Murata, H. Ishida, H. Toda, N. Tanaka, H. Hayashida, M. Monden, and N. Matsuura. 1995. Interleukin 10 reduces mortality from severe peritonitis in mice. *Antimicrob. Agents Chemother.* 39:1336–1340.

Kishimoto, T.K., E.T. Baldvin, and D.C. Anderson. 1999. The role of β2-integrins in inflammation. *In* Inflammation: Basic Principles and Clinical Correlates. 3rd ed. D.T. Fearon, B.F. Hynes, and C. Nathan, editors. Lippincott Williams & Wilkins, Philadelphia. 537–569.

Kvietys, P.R., and D.N. Granger. 1993. The vascular endothelium in GI inflammation. *In* Immunopharmacology of the Gastrointestinal System. J. Wallace, editor. Academic Press Ltd., London. 69–94.

Kvietys, P.R., G. Cepinskas, and D.N. Granger. 1996. Neutrophil-endothelial cell interactions during ischemia/reperfusion. *In* Tissue Perfusion and Organ Function: Ischemia/Reperfusion Injury. T. Kamada, T. Shiga, and R.S. McCuskey, editors. Elsevier, Amsterdam. 179–191.

Laegreid, A., L. Thommesen, T.G. Jahr, A. Sundan, and T. Espevik. 1995. Tumor necrosis factor induces lipopolysaccharide tolerance in a human adenocarcinoma cell line mainly through the TNF p55 receptor. *J. Biol. Chem.* 270: 25418–25425.

Liao, F., J. Ali, T. Greene, and W.A. Muller. 1997. Soluble domain 1 of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration in vitro and in vivo. *J. Exp. Med.* 185:1349–1357.

Lin, Y.-Z., S.Y. Yao, R.A. Veach, T.R. Torgerson, and J. Hawiger. 1995. Inhibition of nuclear translocation of transcription factor NFκB by synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J. Biol. Chem.* 270:14255–14258.

Linderkamp, O., P. Ruef, B. Brenner, E. Gulbins, and F. Lang. 1998. Passive deformability of mature, immature, and active neutrophils in healthy and septicemic neonates. *Pediatr. Res.* 44:946–950.

Lush, C.W., and P.R. Kvietys. 2000. Microvascular dysfunction in sepsis. *Microcirculation.* 7:83–101.

Lush, C.W., G. Cepinskas, and P.R. Kvietys. 2000. LPS tolerance in human endothelial cells: reduced PMN adhesion, E-selectin expression, and NF-kappaB mobilization. *Am. J. Physiol.* 278:H853–H861.

Lush, C.W., G. Cepinskas, W.J. Sibbalad, and P.R. Kvietys. 2001. Endothelial E- and P-selectin expression in iNOS-deficient mice exposed to polymicrobial sepsis. *Am. J. Physiol.* 280:G291–G297.

McDonald, P.P., A. Bald, and M.A. Cassatella. 1997. Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. *Blood.* 89:3421–3433.

Mercurio, F., and A.M. Manning. 1999. Multiple signals converging on NF-kappaB. *Curr. Opin. Cell Biol.* 11:226–232.

Moll, T., E. Dejana, and D. Vestweber. 1998. In vitro degradation of endothelial catenins by a neutrophil protease. *J. Cell Biol.* 140:403–407.

Muller, W.A. 1995. The role of PECAM-1 (CD31) in leukocyte emigration: studies in vitro and in vivo. *J. Leukoc. Biol.* 57:523–528.

Muller, W.A., C.M. Ratti, S.L. McDonnel, and Z. Cohn. 1989. A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. *J. Exp. Med.* 170:399–414.

Neviere, R.R., G. Cepinskas, W.S. Madorin, N. Hoque, M. Karmazyn, and W.J. Sibbalad. 1999. LPS pretreatment ameliorates peritonitis-induced myocardial inflammation and dysfunction: role of myocytes. *Am. J. Physiol.* 277: H15–H25.

O'Brien, C., G. Ji, Y. Wang, J. Sun, V. Krymskaja, F. Ruberg, M. Kotlikoff, and S.M. Albelda. 2001. PECAM-1 (CD-31) engagement activates a phosphoinositide-independent, nonspecific cation channel in endothelial cells. *FASEB J.* 15:1257–1260.

Panes, J., and D.N. Granger. 1998. Leukocyte-endothelial cell interactions: molecular mechanisms and implications in gastrointestinal disease. *Gastroenterology.* 114:1066–1090.

Panes, J., M.A. Perry, D.C. Anderson, A. Manning, B. Leone, G. Cepinskas, C.L. Rosenbloom, M. Miyasaka, P.R. Kvietys, and D.N. Granger. 1995. Regional differences in constitutive and induced ICAM-1 expression in vivo. *Am. J. Physiol.* 269:H1955–H1964.

Rui, T., G. Cepinskas, Q. Feng, Y.S. HO, and P.R. Kvietys. 2001. Cardiac myocytes exposed to anoxia-reoxygenation promote neutrophil transendothelial migration. *Am. J. Physiol. Heart Circ. Physiol.* 281:H440–H447.

Sans, E., E. Delachanal, and A. Duperray. 2001. Analysis of the roles of ICAM-1 in neutrophil transmigration using a reconstituted mammalian cell expression model: implication of ICAM-1 cytoplasmic domain and Rho-dependent signaling pathway. *J. Immunol.* 166:544–551.

Spiecker, M., H.B. Peng, and J.K. Liao. 1997. Inhibition of endothelial vascular cell adhesion molecule-1 expression by nitric oxide involves the induction and nuclear translocation of IkappaBalpha. *J. Biol. Chem.* 272:30969–30974.

Spiecker, M., H. Darius, K. Kaboth, F. Hubner, and J.K. Liao. 1998. Differential regulation of endothelial cell adhesion molecule expression by nitric oxide donors and antioxidants. *J. Leukoc. Biol.* 63:732–739.

Stancovski, I., and D. Baltimore. 1997. NF-kappaB activation: the I kappa B kinase revealed? *Cell.* 91:299–302.

Thompson, R.D., K.E. Noble, K.Y. Larbi, A. Dewar, G.S. Duncan, T.W. Mak, and S. Nourshargh. 2001. Platelet-endothelial cell adhesion molecule-1 (PECAM-1)-deficient mice demonstrate a transient and cytokine-specific role for PECAM-1 in leukocyte migration through the perivascular basement membrane. *Blood.* 97:1854–1860.

Umansky, V., S.P. Hehner, A. Dumont, T.G. Hofmann, V. Schirrmacher, W. Droge, and M.L. Schmitz. 1998. Co-stimulatory effect of nitric oxide on endothelial NF-kappaB implies a physiological self-amplifying mechanism. *Eur. J. Immunol.* 28:2276–2282.

Wang, J., and T.A. Springer. 1998. Structural specializations of immunoglobulin superfamily members for adhesion to integrins and viruses. *Immunol. Rev.* 163:197–215.

Winyard, P.G., and D.R. Blake. 1997. Antioxidants, redox-regulated transcription factors, and inflammation. *Adv. Pharmacol.* 38:403–421.

Yamaguchi, Y., F. Matsumura, F.S. Wang, E. Akizuki, J. Liang, T. Matsuda, K. Okabe, H. Ohshiro, T. Horiuchi, S. Yamada, et al. 1998. Neutrophil elastase enhances intercellular adhesion molecule-1 expression. *Transplantation.* 65:1622–1628.

Yoshida, N., D.N. Granger, D.C. Anderson, R. Rothlein, C. Lane, and P.R. Kvietys. 1992. Anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. *Am. J. Physiol.* 262:H1891–H1898.