

Attenuation of Leukocyte Sequestration by Selective Blockade of PECAM-1 or VCAM-1 in Murine Endotoxemia

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Key Words

Adhesion molecules · Platelet-endothelial cell adhesion molecule 1 · Vascular cell adhesion molecule 1 · *Salmonella abortus equi* endotoxin · Leukocyte-endothelial cell interaction · Microcirculation

Abstract

Background: Molecular mechanisms regulating leukocyte sequestration into the tissue during endotoxemia and/or sepsis are still poorly understood. This in vivo study investigates the biological role of murine PECAM-1 and VCAM-1 for leukocyte sequestration into the lung, liver and striated skin muscle. **Methods:** Male BALB/c mice were injected intravenously with murine PECAM-1 IgG chimera or monoclonal antibody (mAb) to VCAM-1 (3 mg/kg body weight); controls received equivalent doses of IgG2a (n = 6 per group). Fifteen minutes thereafter, 2 mg/kg body weight of *Salmonella abortus equi* endotoxin was injected intravenously. At 24 h after the endotoxin challenge, lungs, livers and striated muscle of skin were analyzed for their myeloperoxidase activity. To monitor intravital leukocyte-endothelial cell interactions, fluorescence videomicroscopy was performed in the skin fold chamber model of the BALB/c mouse at 3, 8 and 24 h after injection of endotoxin. **Results:** Myeloperoxidase activity at 24 h after the endotoxin challenge in

lungs ($12,171 \pm 2,357$ mU/g tissue), livers ($2,204 \pm 238$ mU/g) and striated muscle of the skin ($1,161 \pm 110$ mU/g) was significantly reduced in both treatment groups as compared to controls, with strongest attenuation in the PECAM-1 IgG treatment group. Arteriolar leukocyte sticking at 3 h after endotoxin (230 ± 46 cells \times mm⁻²) was significantly reduced in both treatment groups. Leukocyte sticking in postcapillary venules at 8 h after endotoxin (343 ± 69 cells/mm²) was found reduced only in the VCAM-1-mAb-treated animals (215 ± 53 cells/mm²), while it was enhanced in animals treated with PECAM-1 IgG (572 ± 126 cells/mm²). **Conclusion:** These data show that both PECAM-1 and VCAM-1 are involved in endotoxin-induced leukocyte sequestration in the lung, liver and muscle, presumably through interference with arteriolar and/or venular leukocyte sticking.

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Introduction

The characteristic findings during endotoxemia are fever, initial leukopenia which may later on lead to peripheral leukocytosis and thrombocytopenia. Cytokines, especially interleukin (IL) 1 β , tumor necrosis factor (TNF) α and γ -interferon, are the key mediators for propagation of this systemic inflammatory reaction which is

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accompanied by a systemic leukocyte activation state [1, 2]. Activation of leukocytes and endothelial cells results in aggregation of leukocytes, platelets and erythrocytes in vivo. This may favor disseminated intravascular coagulation and may result in multiple organ failure [3]. Therapeutic strategies aiming at the blockade of the involved inflammatory mediators such as TNF- α or IL-1 have shown to improve the outcome under various experimental settings [4–6], while this holds not true under clinical conditions [7].

Adhesion molecules both on leukocytes and endothelial cells can be stimulated by lipopolysaccharides or cytokines such as TNF- α or IL-1. Among those adhesion molecules, platelet-endothelial cell adhesion molecule 1 (PECAM-1, CD31) and vascular cell adhesion molecule 1 (VCAM-1, CD106) have been suggested to play a vital role for leukocyte transmigration and emigration into the perivascular tissue [8–10]. PECAM-1 is constitutively expressed on endothelial cells, platelets and several leukocyte subpopulations [11]. Therefore it can undergo multiple cellular interactions through homophilic and/or heterophilic binding. Moreover, it has been shown that PECAM-1 plays a role in cell signaling as has been described for its supportive function on the activation state of leukocyte integrins [12], the affinity state of the VCAM-1/VLA-4 adhesion system [13] and as a signaling, adaptor and adhesion molecule [14]. VCAM-1 is known to play a pathophysiological role in organ transplantation and atherosclerosis [15, 16]. The expression of VCAM-1 during endotoxemia is mediated through the release of IL-1 and TNF- α at 3 and 6 h after stimulation, respectively [8]. The biological effects of this adhesion molecule may therefore be expected at the earliest about 5–8 h after injection of endotoxin.

The aim of this study was to investigate the role of PECAM-1 and VCAM-1 during severe endotoxemia on (i) leukocyte sequestration into the lung, liver and striated muscle of skin and (ii) microvascular leukocyte-endothelial cell interaction in arterioles and postcapillary venules of striated muscle of skin.

Material and Methods

Antibodies

The following antibodies against murine antigens were used: PECAM-1 IgG was prepared as described by Liao et al. [12]. This chimeric PECAM-1 construct has been shown to competitively block PECAM-1 both in vitro and in vivo with a long-lasting in vivo half-life of more than 24 h and without activating the complement cascade or interfering with the peripheral leukocyte count in vivo [17].

Rat IgG2a served as control antibody (Dianova, Hamburg, Germany). Aliquots of the injectable solutions were tested for endotoxin with the limulus amoebocyte test (Boehringer, Mannheim, Germany). The maximal endotoxin dose an animal received by injection of the solution was <0.75 ng.

Monoclonal antibodies (mAbs) to VCAM-1 were obtained from Prof. Dr. D. Vestweber (Institute for Immunobiology, University of Münster, Germany). Anti-VCAM-1 mAb was a IgG2a rat antibody, clone AK2-C5. This antibody was purified and concentrated by column chromatography (Prof. Dr. G. Enders, Institute for Surgical Research, University of Munich, Germany). The specificity of this antibody has been tested earlier [18].

Endotoxin

Endotoxin of *Salmonella abortus equi* was obtained from Prof. C. Galanos (Max Planck Institute for Immunobiology, Freiburg, Germany) and administered intravenously at a dose of 2 mg/kg body weight. Endotoxin was regularly aliquoted before the experiment and assessed with the limulus amoebocyte test before injection.

Myeloperoxidase

Myeloperoxidase (MPO) activity was assessed in the tissues of the animals at the end of the observation period of 24 h. For this purpose, lungs, livers and the striated skin muscle tissues were excised under ketamine hydrochloride (125 mg/kg body weight, Ketavet[®], Parke Davis, Freiburg, Germany) and xylazine (15 mg/kg, Rompun[®], Bayer, Leverkusen, Germany) anesthesia. The measurement of enzyme activity was performed according to the technique by Kuebler et al. [19].

Animal Model

Intravital fluorescence microscopy was used for investigation of leukocyte-endothelial cell interactions in the dorsal skin fold chamber model of the striated muscle of skin of BALB/c mice. The surgical technique has been described previously [20]. All experiments have been approved by the Local Ethical Committee of the Government of Bavaria (AZ: 211-2531-4693).

Intravital Fluorescence Microscopy

A 25-fold water immersion objective (total magnification: 560-fold; E. Leitz Inc., Wetzlar, Germany) was used to select 4–6 sites of interest per chamber, each containing one or several postcapillary venules (between 10–40 μ m and 40–80 μ m in diameter) and arterioles (10–60 μ m in diameter). By means of a computer-controlled stepping-motor-driven platform [21], the identical vessel segments were investigated with respect to leukocyte/endothelium interaction, vessel diameter and red blood cell velocity before administration of the antibody solutions and at 3, 8 and 24 h after injection of *S. abortus equi* endotoxin (2 mg/kg body weight). For visualization of leukocytes, the in vivo fluorescent marker rhodamine 6G (Sigma Chemical Co., Deisenhofen, Germany) was infused intravenously as a bolus of 0.15 mg/kg body weight 10 min before the intravital microscopic recordings. Epi-illumination was achieved with an HBO mercury lamp attached to a Ploemopak illuminator with an N₂ filter block (Leitz). For visualization of the plasma, the I2/3 filter block (Leitz) was used after bolus infusion of the in vivo fluorescent plasma marker fluorescein isothiocyanate (FITC)-dextran (Pharmacia, Uppsala, Sweden; molecular weight 150 kDa; 15 mg/kg body weight i.v.). This double fluorescence technique allowed for sequential investigation of vessel diameter and red blood cell velocity on the one hand, and

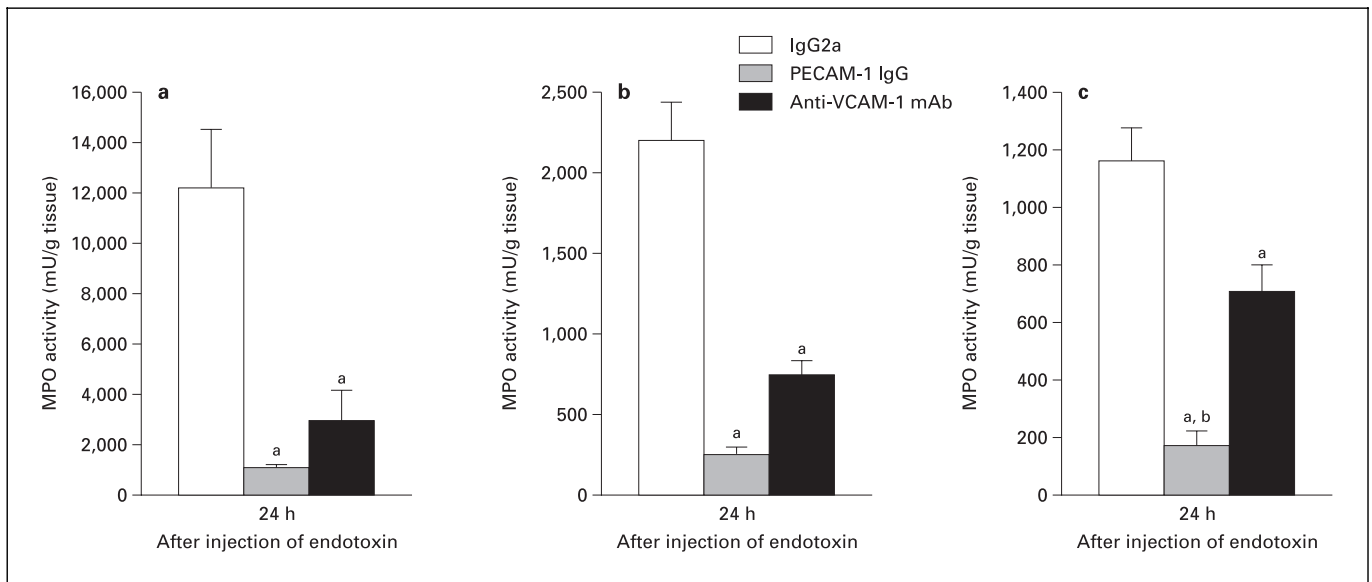


Fig. 1. MPO activity in lung (a), liver (b) and striated muscle of skin (c) of BALB/c mice taken at 24 h after injection of 2 mg/kg body weight *S. abortus equi* endotoxin. Control animals received 3 mg/kg body weight of an isotype-matched antibody (rat IgG2a, n = 7). Animals of the treatment groups received equivalent doses of PECAM-1 IgG or anti-VCAM-1 mAb (each n = 6). Note the different scaling of

the vertical axis: MPO activity in the lung was approximately 10-fold that of the striated muscle of skin tissue, whereas MPO activity in the liver was approximately 2-fold that of the striated skin muscle tissue. Mean \pm SEM, ^a p < 0.05 versus IgG2a, ^b p < 0.05 versus anti-VCAM-1 mAb, Kruskal-Wallis test.

leukocyte/endothelium interaction on the other hand in the identical vessel segment [20, 22]. The microscopic images (Camera COHU 4400; Prospective Measurements, San Diego, Calif., USA) were recorded on videotape for 30 s using both filter blocks consecutively, providing enough time for analysis of the video images by playback employing a computer-assisted microcirculation analysis system [21]. Red blood cell velocity was assessed offline by a cross-correlation technique using the Capiflow system (Capiflow version 3.1; Lawrence Co., Sulzbach, Germany).

Microcirculatory Parameters

According to their interaction with the microvascular endothelium, sticking leukocytes were defined as cells that do not move or detach from the endothelial lining within an observation time of 30 s and are expressed as cells per square millimeter of endothelial surface, calculated from diameter and length (200 μ m) of the vessel segment under investigation. The microhemodynamic parameters vessel diameter and red blood cell velocity were assessed in each vessel segment.

Experimental Protocol

For quantification of the effects of the antibodies on leukocyte sticking during endotoxemia, PECAM-1 IgG and mAb against VCAM-1 were administered 15 min prior to infusion of endotoxin as intravenous bolus injection of 3 mg/kg body weight, respectively. Intravital microscopic measurements were made before antibody administration and at 3, 8 and 24 h after injection of endotoxin. Animals were randomly assigned to control and treatment groups. Control animals received equivalent doses of an isotype-matched control

antibody (rat IgG2a, n = 7). Treatment animals received 3 mg/kg body weight of either PECAM-1 IgG (n = 6) or anti-VCAM-1 mAb (n = 6).

Statistical Analysis

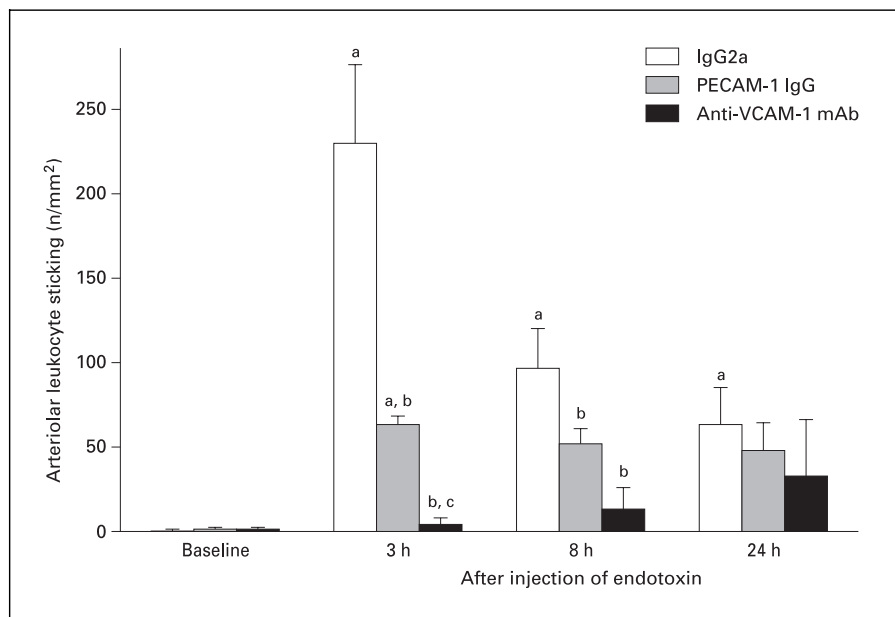
Paired data of more than two samples were analyzed using Friedman's nonparametric test for two-way analysis of variance and comparison on ranks for multiple samples. For unpaired data of multiple samples the Kruskal-Wallis test was used with Bonferroni correction of p (Unistat[®] Statistical Package Version 1.2, 1984–1993, Unistat Ltd., UK). p values < 0.05 were considered significant. Despite non-parametric data distribution, data are given in text and figures as arithmetic means \pm SEM to facilitate interpretation of data published in the literature.

Results

Effects of PECAM-1 IgG and Anti-VCAM-1 mAb on Endotoxin-Induced Leukocyte Sequestration into Lung, Liver and Striated Muscle of Skin in BALB/c Mice

MPO activity was assessed in lungs, livers and striated skin muscle tissues of BALB/c mice both under baseline conditions and 24 h after endotoxin administration. Under baseline conditions, there was a trend for enhanced MPO activity to be observed in the lung (479 \pm

Fig. 2. Leukocyte sticking in arterioles of the striated muscle of skin in BALB/c mice before and at the given time points after injection of 2 mg/kg body weight *S. abortus equi* endotoxin. Control animals received equivalent doses of an isotype-matched control antibody (rat IgG2a, n = 7). Animals of the treatment groups received equivalent doses of 3 mg/kg body weight PECAM-1 IgG or anti-VCAM-1 mAb (n = 6). Mean \pm SEM, ^a p < 0.05 versus baseline, Friedman test; ^b p < 0.05 versus IgG2a, ^c p < 0.05 versus PECAM-1 IgG, Kruskal-Wallis test.



250 mU/g tissue) and liver (281 ± 72 mU/g tissue) when compared to the striated skin muscle (193 ± 98 mU/g tissue). At 24 h of endotoxemia, MPO activity was dramatically increased in all tissues of IgG2a-treated control animals (n = 7) compared to animals treated with PECAM-1 IgG or anti-VCAM-1 mAb (fig. 1a–c). There was a tendency for PECAM-1 IgG to be more effective than anti-VCAM-1 mAb treatment in the prevention of leukocyte sequestration into all tissues, which reached significance only in striated skin muscle.

Effects of PECAM-1 IgG and Anti-VCAM-1 mAb on Endotoxin-Induced Leukocyte Sticking in Arterioles and Postcapillary Venules of Murine Striated Muscle of Skin

Leukocyte sticking was observed both in arterioles and postcapillary venules after injection of endotoxin. In arterioles, a significant increase in leukocyte sticking was found with a maximum at 3 h after endotoxin (fig. 2). This increase was effectively reduced by PECAM-1 IgG as compared to IgG2a-treated control animals and was further attenuated by mAb to VCAM-1. In animals treated with anti-VCAM-1 mAb, the reduction of arteriolar sticking was significantly more effective at 3 h after endotoxin as compared with PECAM-1-treated animals. At 24 h after endotoxin there were no more significant differences of leukocyte sticking to be detected.

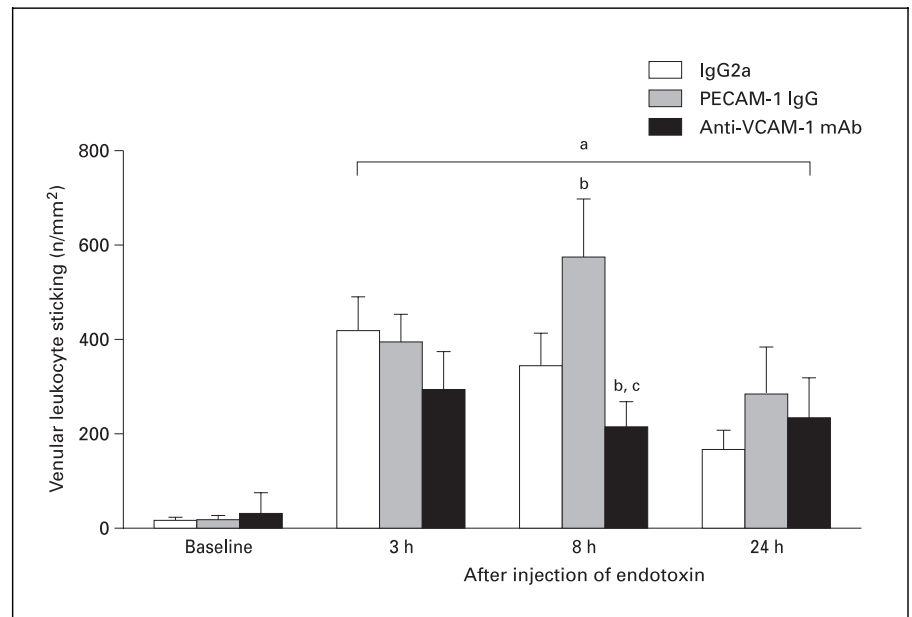
In postcapillary venules of the striated muscle of skin, leukocyte sticking under baseline conditions ranged be-

tween 20 and 40 cells/mm² endothelial surface (fig. 3). A significant increase in venular leukocyte sticking was observed in IgG2a-treated animals after the endotoxin challenge. The response in postcapillary venules of animals treated with PECAM-1 IgG or VCAM-1 mAb was different from that observed in arterioles. There was no attenuation, but in contrast an enhancement of leukocyte sticking in animals receiving the PECAM-1 IgG chimera at 8 h after endotoxin, while a reduction of leukocyte sticking by about 40% was seen at 8 h after endotoxin in animals treated with mAb to VCAM-1.

Discussion

In this study, we demonstrate that the sequestration of leukocytes into the lung, liver and striated skin muscle of BALB/c mice is effectively attenuated by blockade of either PECAM-1 or VCAM-1. Leukocyte adhesive interactions were monitored at 3, 8 and 24 h after injection of endotoxin. We found that both adhesion molecules, PECAM-1 and VCAM-1, are involved in arteriolar leukocyte sticking in the striated skin muscle, while in postcapillary venules no inhibitory effects were observed after blockade of PECAM-1 and only temporary effects were seen after blockade of VCAM-1 at 8 h after the endotoxin challenge. In control animals, there was a massive (25-fold) increase in MPO activity in the lungs, followed by an 8-fold increase in the liver and a 6-fold one in striated skin

Fig. 3. Leukocyte sticking in postcapillary venules of the striated muscle of skin in BALB/c mice before and at the given time points after injection of 2 mg/kg body weight *S. abortus equi* endotoxin. Control animals received 3 mg/kg body weight of an isotype-matched control antibody (rat IgG2a, n = 7). Animals of the treatment groups received equivalent doses of 3 mg/kg body weight PECAM-1 IgG or anti-VCAM-1 mAb (n = 6). Mean \pm SEM, ^a p < 0.05 versus baseline, Friedman test; ^b p < 0.05 versus IgG2a, ^c p < 0.05 versus PECAM-1 IgG, Kruskal-Wallis test.



muscle tissue (fig. 1a–c) as compared to baseline values assessed in normal BALB/c mice. Both blockade of VCAM-1 and PECAM-1 resulted in a significant reduction of leukocyte accumulation in these organs whereby blockade of PECAM-1 seemed to be more effective.

Our findings using mAb to VCAM-1 to reduce *pulmonary* injury (fig. 1a) are supported by other studies demonstrating in remote organ injury models of obstructive cholangitis or mesenteric ischemia-reperfusion an upregulation of this adhesion molecule associated with pulmonary sequestration [23, 24]. Upregulation of VCAM-1 in response to TNF- α has been reported in the lung, liver and kidneys [25, 26]. Callicutt et al. [27] have shown in a pancreatitis-induced lung injury model in mice that therapeutic blockade of VCAM-1 reduces pulmonary leukocyte sequestration and, in consequence, lung injury.

However, there are only few data on the role of PECAM-1 in endotoxin-induced pulmonary injury. Vaporciyan et al. [9] have shown in three different (nonendotoxin) in vivo models of inflammation that PECAM-1 is involved in neutrophil transmigration into the alveolar compartment of the lung. There is both in vitro and in vivo evidence for expression of PECAM-1 in the pulmonary vascular bed as assessed in oxidative stress models [28]. Moreover, it has been reported that catalase conjugated to PECAM-1 mAb rapidly accumulates in the lungs after intravenous injection into mice and protects from pulmonary injury in a murine lung model of oxidative stress [29]. Hence, our findings provide first in vivo evi-

dence for the functional role of PECAM-1 in pulmonary leukocyte sequestration during endotoxemia in mice (fig. 1a).

Both adhesion molecules have been shown to play significant roles in leukocyte-mediated *hepatocellular* tissue damage. This finding is supported by our MPO data demonstrating a significant reduction of leukocyte accumulation in the liver in response to endotoxemia (fig. 1b). Expression of VCAM-1 in rat liver cells is strongly enhanced upon injection of endotoxin [30]. Essani et al. [31] have demonstrated that neutrophils use the VLA-4/VCAM-1 adhesion system for transmigration into the liver parenchyma in vivo. The selective blockade by anti-VCAM-1 mAb protected from hepatocellular injury in endotoxic shock [31].

Chosay et al. [32] have shown in a previous study that endotoxin alone or in combination with galactosamine elicits neutrophil margination in liver sinusoids and post-sinusoidal venules while emigration occurred only after administration of the combination of endotoxin with galactosamine at the site of the sinusoids. Our finding of an enhanced leukocyte accumulation upon injection of endotoxin alone may be attributed to the significantly higher dose of endotoxin used in this study. At the dose of 2 mg/kg body weight *S. abortus equi* used in our study, as compared to 100 μ g/kg body weight used by Chosay et al. [32], additional trigger mechanisms for leukocyte transmigration may be activated, perhaps in a dose-dependent fashion. Moreover, the authors demonstrated in an im-

munohistological investigation that PECAM-1 is expressed in postsinusoidal veins of the liver, but not in sinusoidal venules [33], making them conclude that PECAM-1 does not play a functional role for leukocyte sequestration in the liver. This finding is contrary to our results showing that blockade of PECAM-1 was associated with a significant reduction of MPO activity (fig. 1b). In a recent study by Neubauer et al. [34] however, constitutional expression of PECAM-1 has been reported both in rat and human livers as well as isolated rat sinusoidal endothelial cells, hepatic stellate cells and hepatocytes. The discrepancy between these studies may in part be due either to species- or endotoxin-dependent (*Escherichia coli* vs. *S. abortus equi*) differences. It may furthermore be speculated that in the absence of PECAM-1 expression in sinusoidal endothelial cells as observed by Chosay et al. [33], platelet deposition may taper the endothelial lining of the sinusoids, thus allowing platelet-derived PECAM-1 to support leukocyte transmigration through the sinusoidal endothelium. Nevertheless, our study clearly supports the functional role of PECAM-1 for leukocyte sequestration into the livers of BALB/c mice during severe endotoxemia.

In the present study, we moreover demonstrate a role for both VCAM-1 and PECAM-1 in endotoxin-induced leukocyte sticking and sequestration into the striated muscle of the skin as assessed by the MPO activity at 24 h after endotoxin (fig. 1c). These findings have to be interpreted in view of the intravital videomicroscopic observations: a significant increase in arteriolar leukocyte sticking was observed with a maximum at 3 h after injection of endotoxin (fig. 2) as has previously been described by our group [35]. Other investigators have also reported on arteriolar leukocyte rolling and sticking upon injection of *E. coli* endotoxin into hamsters [36]. Since injection of TNF- α can also elicit arteriolar rolling in vivo [37], the release of this cytokine in response to endotoxin may account for this observation. We herein for the first time demonstrate that blockade of either PECAM-1 or

VCAM-1 effectively reduces arteriolar leukocyte sticking, indicating that both molecules are involved in this scenario. This finding is not surprising in view of the role of VCAM-1 which has been shown to play a crucial role in atherogenesis [16].

At the venular side of the microcirculatory bed, leukocyte sticking increased significantly after endotoxin, but was attenuated by mAb to VCAM-1 at 8 h after endotoxin (fig. 3). This finding coincides with the upregulation of this adhesion molecule in response to endotoxin [30]. Interestingly, there was no reduction of endotoxin-induced leukocyte sticking visible in animals treated with PECAM-1 IgG. On the contrary, a significant increase in leukocyte sticking at this time point of investigation in animals treated with PECAM-1 IgG was observed. Similar findings have been reported by other investigators suggesting that PECAM-1 may facilitate endothelial tightening by the intravascularly trapped leukocytes prevented from transmigration [10]. Indeed, in a previous study from our laboratory we have demonstrated macromolecular leakage of FITC-dextran 150 kDa from postcapillary venules to be significantly attenuated in animals treated with PECAM-1 IgG [38]. Therefore, increased leukocyte sticking in postcapillary venules may not necessarily be equated with increased endothelial cell injury. In conclusion, both PECAM-1 and VCAM-1 are involved in leukocyte adhesion and transmigration in postcapillary venules of the striated skin muscle, however with different emphasis on the step involved in the adhesion cascade.

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