PS3, A Semisynthetic β-1,3-Glucan Sulfate, Diminishes Contact Hypersensitivity Responses Through Inhibition of L- and P-Selectin Functions

Susanne Alban[1,8], Ralf J. Ludwig[2,3,8], Gerd Bendas[4], Michael P. Schön[5,6], Gertie J. Oostingh[5,7], Heinfried H. Radeke[3], Juliane Fritzsche[4], Josef Pfeilschifter[3], Roland Kaufmann[2] and Wolf-Henning Boehncke[2]

Leukocyte extravasation is initiated by an interaction of selectin adhesion molecules and appropriate carbohydrate ligands. Targeting those interactions seems a promising approach to treat chronic inflammation. We developed a β-1, 3-glucan sulfate (PS3) with inhibitory activity toward L and P-selectins under static conditions. Here, detailed investigation showed inhibition of P- and L-selectins, but not E-selectin under flow conditions (relative reduction of interaction with appropriate ligands to 34.4 ± 16.6, 8.5 ± 3.6, or 99.5 ± 9.9%, respectively, by PS3 for P-, L- or E-selectin). Intravital microscopy revealed reduction of leukocyte rolling in skin microvasculature from 22.7 ± 5.0 to 12.6 ± 4.0% after injection of PS3. In the next experiments, mice were sensitized with 2,4,-dinitrofluorobenzene (DNFB), and lymphocytes were transferred into syngeneic recipients, which were challenged by DNFB. Inflammatory responses were reduced when immunity was generated in mice treated with PS3 or in L-selectin-deficient mice. No effect was observed when L-selectin-deficient donor mice were treated with PS3, further suggesting that PS3 acted primarily through inhibition of L-selectin. Elicitation of a contact hypersensitivity response was reduced in P-selectin-deficient and in PS3-treated mice. Again, PS3 had no effect in P-selectin-deficient mice. PS3 is a potent P- and L-selectin inhibitor that may add to the therapy of inflammatory diseases.

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INTRODUCTION

Constant migration of leukocytes through blood, lymphoid system, and organs is a basic prerequisite for immune surveillance and host defense. However, uncontrolled and/or misdirected leukocyte migration leads to immune-mediated diseases, such as inflammatory bowel diseases, multiple sclerosis, psoriasis, or rheumatoid arthritis (O'Dell, 2004; Schön and Boehncke, 2005). To move from the bloodstream into tissues, leukocytes must interact with the endothelial lining of the vasculature. This process of leukocyte extravasation (Butcher and Picker, 1996; Schön and Ludwig, 2005) is initiated by an interaction of selectin adhesion molecules with their appropriate ligands (Weninger *et al.*, 2000). Hence, selectin–ligand interactions are promising therapeutic targets in chronic inflammatory diseases (Zarbock *et al.*, 2007; Ludwig *et al.*, 2007b). In support of this hypothesis, clinical trials with pan-selectin-inhibitory compounds, for example TBC1269 (Friedrich *et al.*, 2006) or recombinant P-selectin glycoprotein ligand-1 (rPSGL-Ig; Mertens *et al.*, 2006), often showed a beneficial effect in inflammatory processes. However, specific inhibition of E-selectin alone by infusion of a function-blocking monoclonal antibody had no effect in patients suffering from psoriasis (Bhushan *et al.*, 2002).

Whereas TBC1269 and rPSGL-Ig inhibit all three selectins (Hicks *et al.*, 2003; Kranich *et al.*, 2007), heparins possess only P- and L-selectin-inhibitory activities (Borsig, 2004). Although heparins exert a wide range of biological activities (Lever and Page, 2002; Ludwig *et al.*, 2006b; Buller *et al.*, 2007), their P- and L-selectin-inhibitory activity is thought to be especially important for both their anti-inflammatory and antimetastatic activities (Ludwig *et al.*, 2006b; Buller *et al.*, 2007), may be also involved in their antithrombotic activity (Polgar *et al.*, 2005), and thus contribute to their overall therapeutic efficacy. A broad use of heparins as anti-inflammatory drugs may, however, be hindered by several

[1]*Pharmaceutical Institute, Christian-Albrechts University, Kiel, Germany;* [2]*Department of Dermatology, Clinic of the Johann Wolfgang Goethe University, Frankfurt am Main, Germany;* [3]*Pharmazentrum Frankfurt/ZAFES, Clinic of the Johann Wolfgang Goethe University, Frankfurt am Main, Germany;* [4]*Pharmaceutical Institute, University of Bonn, Bonn, Germany;* [5]*Department of Dermatology, DFG Research Center for Experimental Biomedicine, Rudolf Virchow Center, University of Würzburg, Würzburg, Germany;* [6]*Department of Dermatology and Venereology, University Medical Center Göttingen, Göttingen, Germany and* [7]*Molecular Biology, University of Salzburg, Salzburg, Austria*

[8]*These authors contributed equally to this work.*

Correspondence: Dr Ralf J. Ludwig, Department of Dermatology, University of Lübeck, Ratzeburger Allee 160, 23562 Lübeck, Germany. E-mail: ralf.ludwig@uk-sh.de

Abbreviations: DNFB, 2,4,-dinitrofluorobenzene; E-CHO, E-selectin expressing CHO; PBMC, peripheral blood mononuclear cell; PS3, β-1,3-glucan sulfate; sLe^x, sialyl Lewis X; TNF-α, tumor necrosis factor-α

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reasons (Alban, 2005a, b). Most importantly, heparins exhibit strong antithrombin-mediated anticoagulant activities, the risk of bleeding limits their therapeutic use in inflammation or cancer treatment. As natural products consisting of variable complex mixtures of glycosaminoglycans, heparins show certain batch-to-batch variation, resulting in inconsistencies with regard to selectin inhibition (Alban, 2005a; Fritzsche *et al.*, 2006). Among different heparin preparations, pronounced differences in their extent of selectin inhibition and thus pharmacological activity *in vivo* were found (Stevenson *et al.*, 2005; Ludwig *et al.*, 2006a; Simonis *et al.*, 2007). Recently, heparin-related safety issues have come into the focus of the medical community (FDA, 2008). Structural modifications, for example, reduction of uronic acid residues, have led to P-selectin-binding heparin derivatives without antithrombin-mediated anticoagulant activity (Xie *et al.*, 2000; Gao *et al.*, 2005). The other disadvantages of heparins, such as their non-human origin, shortage on resources, and their batch-to-batch variability, were not eliminated by this approach and limit the broad use as anti-inflammatory drugs.

We pursued the strategy of developing structurally defined glucan sulfates obtained by chemical modifications of neutral homoglucans produced by algae, bacteria, or fungi. The aim was to produce compounds with reduced anticoagulant activity, but stronger anti-inflammatory and/or antimetastatic activity compared to heparin (Becker *et al.*, 2003a). Studies on structure–activity relationships and optimization of the semisynthetic procedure led to the highly sulfated β-1,3-glucan sulfate (PS3; Yvin *et al.*, 2002; Becker *et al.*, 2003b). In assays under static conditions, these semisynthetic glucan sulfates structure and concentration dependently inhibited cell adhesion to P- and L-selectins, but not to E-selectin (Yvin *et al.*, 2002). Under flow conditions, they showed similar structure–activity relationships and considerably stronger P-selectin-inhibitory activity than heparin (Hopfner *et al.*, 2003; Fritzsche *et al.*, 2006). These findings suggested that P- and/or L-selectin may represent worthwhile targets for this type of sulfated polysaccharides. The aim of this study was to investigate the inhibitory activity of the PS3, which was selected because of its potent *in vitro* P- and L-selectin-inhibitory activity (Fritzsche *et al.*, 2006), on cell–cell and cell–selectin interactions and its impact on P- and L-selectin-mediated functions in animal models of cutaneous inflammation.

RESULTS

Synthesis and characterization of PS3

PS3 is a well-defined, reproducible semisynthetic β-1,3-glucan sulfate (existing as sodium salt), which is obtained by chemical modification of the uncharged, small β-1,3-glucan Phycarine (Figure 1; Yvin *et al.*, 2002; Ménard *et al.*, 2004). Phycarine is isolated from the brown algae *Laminaria digitata* and consists of a β-1,3-glucan backbone with an average degree of polymerization of 25 and one or two C6 ramifications with glucose. The chemical sulfation of Phycarine was performed with SO_3–pyridine complex according to the method described by Alban *et al.* (1992), which had been optimized to obtain regular β-1,3-glucan sulfation without any polysaccharide degradation and to control the resulting degree of sulfation. Structure analysis revealed that the primary OH group in position 6 of PS3 is fully sulfated, whereas the two secondary OH groups in positions 2 and 4 are equally sulfated to about 60% under the used conditions (Yvin *et al.*, 2002; Ménard *et al.*, 2004). The overall degree of sulfation of PS3 is 2.2 sulfate groups per glucose unit. PS3 has a molecular mass M_r of about 10,000, and like the starting polymer a low polydispersity. Similar to heparins and in contrast to Phycarine, PS3 exhibits a wide range of biological activities by interfering with processes involving endogenous glycosaminoglycans. However, it shows mostly stronger pharmacological effects than heparins in assays indicating potential anti-inflammatory and anti-metastatic activities (for example, PS3 is significantly superior to heparins in *in vitro* P- and L-selectin adhesion assays), whereas its anticoagulant activity is considerably weaker (for example, the concentration for doubling the activated partial thromboplastin time is seven times higher than that of unfractionated heparin) (Yvin *et al.*, 2002; Alban *et al.*, 2005). PS3 passed the basic toxicity tests as prerequisite for clinical studies in humans. In addition, intravenous application of PS3 in monkeys revealed that it is well tolerated and results in stronger release of tissue factor pathway inhibitor than heparin (Alban *et al.*, 1997).

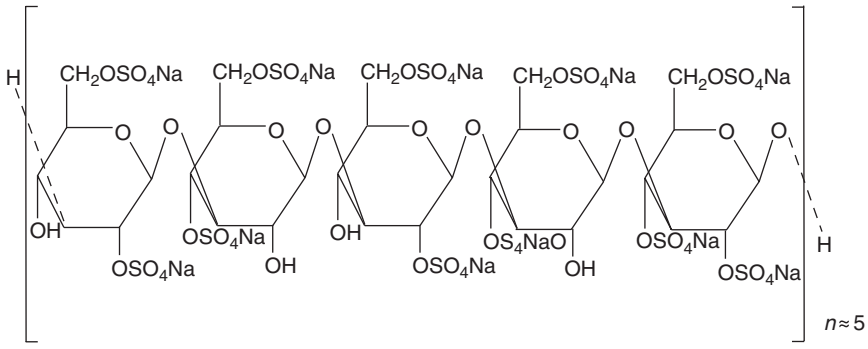

Figure 1. Structure of PS3. PS3 is the sodium salt of a β-1,3-glucan sulfate with a degree of sulfation (DS) of 2.2, a degree of polymerization (DP) of about 25 corresponding to a mean molecular mass (M_r) of 10,000.

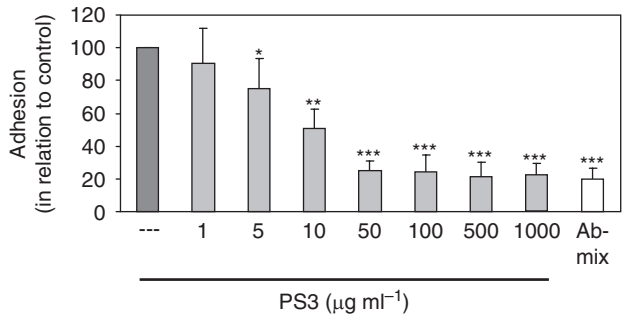

Figure 2. PS3 inhibits interactions between PBMCs and endothelial cells under conditions of shear flow. TNFα-activated endothelial cells, coated on plastic coverslips, were mounted in a flow chamber system and human PBMCs were allowed to interact with these endothelial cells under conditions of shear flow (1.12 ml per hour). Cell–cell interactions were recorded in real time after 20 minutes. The average of six independent pictures was used to analyze the number of PBMC binding to the endothelial cells and the data are presented as the relative number of adhering cells, whereby this number was set to 100% in the absence of PS3 for each PBMC donor ($n=3$) ± SEM. Concentrations as low as 5 μg ml^{-1} PS3 could significantly inhibit the adherence of PBMC to the stimulated HUVECs (*$P<0.05$, **$P<0.01$, ***$P<0.001$, one-way ANOVA with Bonferroni *t*-test for pair-wise multiple comparisons). An antibody cocktail, containing antibodies blocking all three selectins significantly inhibited the binding interactions by 80%. However, complete inhibition by either PS3 or anti-selectin-Ab cocktail was not observed. This residual binding is most likely due to an interaction of leukocyte integrins (e.g., VLA-4) with appropriate ligands on the HUVEC (e.g., VCAM-1), which have been shown to mediate rolling in the skin microvasculature (Butcher and Picker, 1996).

Inhibition of PBMC–endothelial cell interactions by PS3 under flow conditions

To investigate whether PS3 inhibited selectin-dependent leukocyte–endothelial cell interactions on the cellular level, a dynamic flow chamber system with a matrix of human umbilical vein endothelial cells and suspended peripheral blood mononuclear cells (PBMCs) was used. PS3 significantly inhibited the binding interactions between PBMC and tumor necrosis factor-α (TNF-α)-activated human umbilical vein endothelial cells at concentrations as low as 5 μg ml^{-1} ($P=0.02$ as compared to control conditions; Figure 2). This inhibition dose dependently increased up to 75% at a concentration of 50 μg ml^{-1} PS3 ($P<0.001$ compared to control conditions). Higher PS3 concentrations up to 1,000 μg ml^{-1} did not further improve the inhibitory effect. A cocktail of antibodies directed against E-, P- and L-selectins had a similar inhibiting effect (80% inhibition, $P<0.001$ compared to control conditions). On the basis of leukocyte and endothelial expression patterns of adhesion molecules, and the effects on selectins under static conditions (Yvin *et al.*, 2002; Becker *et al.*, 2003b), the effect by PS3 is most likely to be mediated by an interaction of leukocyte PSGL-1 with endothelial P-selectin. To further investigate which pathways are impaired by PS3, we performed experiments with defined selectin ligands.

PS3 impairs P- and L-, but not E-selectin-mediated interactions under flow conditions

To gain further insight into the molecular basis of PS3's ability to inhibit binding interactions between PBMC and activated endothelial cells, we used defined conditions that allowed to investigate the effects of PS3 on each selectin separately. First, the inhibitory effect of PS3 on interactions of the PSGL-1 expressing human monocytic cell line U937 with immobilized P-selectin was investigated under conditions of shear flow. Presence of PS3 in the flow chamber led to a significant reduction of U937 interactions with immobilized P-selectin by 65% ($P=0.016$; Figure 3). To further validate a specific P-selectin inhibition by PS3 we tested for dose–response relationship. Whereas PS3 at a concentration of 5 μg ml^{-1} had no effect on U937 and P-selectin interaction, doses of 50, 100, and 500 μg ml^{-1} had a profound effect. Maximal inhibition was observed with 100 μg ml^{-1}, and an increase of PS3 concentration did not lead to a further reduction (Figure 3b). The inhibitory effect of PS3 on the interaction between human PBMC and immobilized sialyl Lewis X (sLe^x)/TS-PAA, a synthetic ligand for all three selectins with a strong affinity for E-, and L-selectins, was tested under conditions of shear flow. As L-selectin is the only selectin expressed on PBMC, it is reasonable to assume that any inhibitory effects in this system were because of the interference with L-selectin functions. Similar to the U937/P-selectin assay, PS3 turned out to be a potent inhibitor of the interactions between PBMC and sLe^x/TS-PAA at concentrations as low as 5 μg ml^{-1} ($P<0.001$). This inhibitory effect was dose dependent, and at concentrations >500 μg ml^{-1}, almost complete inhibition (that is, >90%) could be observed (Figure 3). L-selectin-specific antibodies at a final concentration of 10 μg ml^{-1} also significantly inhibited this interaction ($P<0.001$), albeit to a somewhat lesser extent as compared to higher concentrations of PS3 (not shown). The effect of PS3 on the interaction of E-selectin expressing CHO (E-CHO) cells with the E-selectin ligand sLe^{x}Lac1 was evaluated. In contrast to the effects observed with P and L-selectins, adherence of E-CHO cells under shear remained unchanged in the presence of PS3 (Figure 3). This is in accordance with previous results under static conditions, where PS3 concentration dependently reduced the cell adhesion to immobilized P- and L-selectins, but not to E-selectin (Yvin *et al.*, 2002).

PS3 impairs P-selectin function *in vivo*

In the next series of experiments, we assessed whether the *in vitro* observed effects of PS3 on P- and L-selectin function were also relevant *in vivo*. For this purpose, superantigen-activated PBMCs were fluorescently labeled and injected into C57Bl/6 mice or P-selectin-deficient mice. Rolling interactions of fluorescent cells within the same vessels before and after PS3 treatment were then observed by intravital microscopy in the skin microvasculature. As expected (Weninger *et al.*, 2000), rolling interactions of leukocytes were reduced in P-selectin-deficient mice (rolling fraction: 3.6% ± 1.8%) as compared to wild-type C57Bl/6 mice (rolling fraction: 22.7% ± 5.0%; $P=0.006$). In wild-type mice, a single injection of 25 mg kg^{-1} PS3 reduced the rolling interactions to 12.6 ± 4.0% (decrease by 45%; $P=0.014$, paired *t*-test). In contrast, no further effect of PS3 was seen in P-selectin-deficient mice, suggesting that the observed reduction in

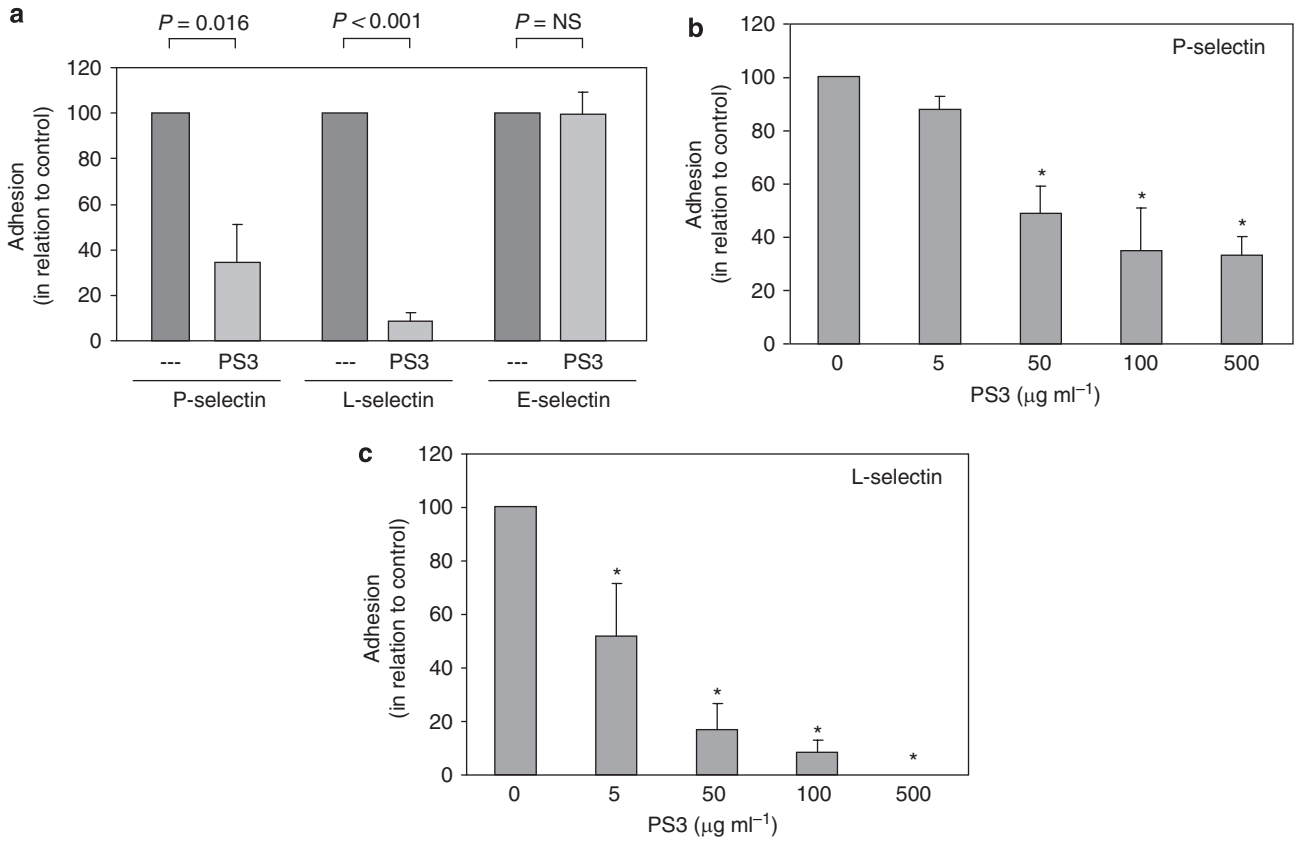

Figure 3. PS3 inhibits P- and L-, but not E-selectin function under conditions of shear flow. Flow chamber experiments to assess the influence of PS3 on P-, L-, or E-selectin function were performed as described in Materials and Methods. (**a**) Although a profound inhibition P- and L-selectin functions is observed after PS3 treatment, E-selectin functions remain unaffected (data showed are from experiments using 100 μg ml^{-1} for all selectins). (**b**) Inhibition of U937 cells with P-selectin-coated surfaces by PS3 was dose depended. A significant inhibition was observed at a concentration of 50 μg ml^{-1}, but not at lower concentrations. Maximum inhibition was detected at a dose of 100 μg ml^{-1}, and an increase to 500 μg ml^{-1} did not yield additional effects. (**c**) Likewise, the effect of PS3 on L-selectin function also proved to be dose dependent. Compared to the inhibition of P-selectin function, the effect on L-selectin was even more pronounced, with no detectable interactions at PS3 concentrations of 500 μg ml^{-1} or more. Data have been obtained from at least four independently performed experiments for each selectin. Statistical evaluation performed with either paired *t*-test or signed-rank test, as appropriate (*$P<0.05$).

rolling interactions was primarily due to inhibition of P-selectin (Figure 4).

Adoptive transfer of immunity toward a contact allergen is reduced after treatment with PS3

To evaluate if above *in vitro* and *in vivo* findings were relevant in an inflammatory disease model, we sought to investigate the effects of PS3 in a murine cutaneous hypersensitivity response model. Given that L-selectin functions are relevant to generate immunity by facilitating homing of naive T lymphocytes to peripheral lymph nodes (Catalina *et al.*, 1996; Oostingh *et al.*, 2007a), we expected that PS3 treatment during the sensitization against a topically applied contact allergen would impair generation of immunity. For this purpose, wild-type or L-selectin-deficient mice were sensitized with 2,4,-dinitrofluorobenzene (DNFB) and simultaneously treated with PS3. Once immunity was established (5 days after sensitization), isolated lymph node and spleen cells from these mice were adoptively transferred into syngeneic wild-type recipient mice, which were then exposed to DNFB. As reported earlier (Catalina *et al.*, 1996), L-selectin deficiency led to a decreased immune response in adoptive transfer experiments (Δ ear swelling $35.6 \pm 4.0\,\text{cm} \times 10^{-3}$ in wild-type animals compared to $21.0 \pm 1.5\,\text{cm} \times 10^{-3}$ in mice lacking L-selectin expression). Likewise, PS3 treatment of wild-type donor mice leads to a diminished immune response in recipient mice (Δ ear swelling $13.2 \pm 3.1\,\text{cm} \times 10^{-3}$). Injection of PS3 in L-selectin-deficient mice had no additional effects in recipient wild-type animals (Δ ear swelling $12.3 \pm 3.1\,\text{cm} \times 10^{-3}$; Figure 5). Therefore, the immunosuppressive effect of PS3 can, at least in part, be attributed to inhibition of L-selectin during the sensitization phase.

Contact hypersensitivity reactions respond to treatment with PS3

In addition to hindrance of the generation of immunity by PS3, the compound could also affect the P-selectin-dependent extravasation of leukocytes to the skin (Subramaniam *et al.*, 1995) following exposure to DNFB (elicitation phase). To test this hypothesis, wild-type or P-selectin-deficient mice were sensitized with DNFB. Concomitantly with the second exposure to DNFB, both groups of mice were injected with PS3 or solvent alone. In this model of DNFB-induced contact

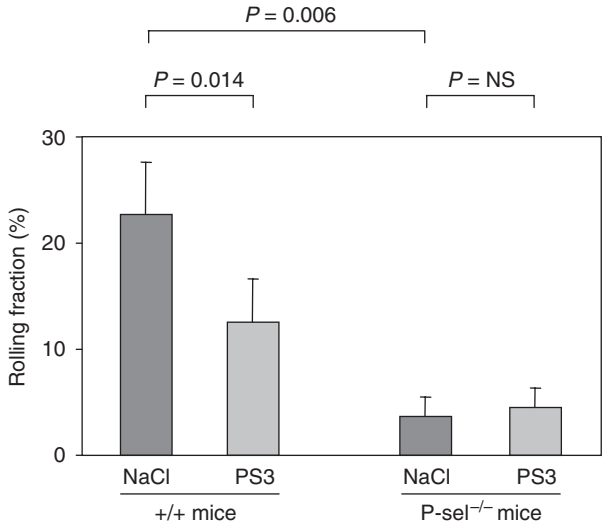

Figure 4. PS3 impairs P-selectin function *in vivo*. Superantigen-stimulated PBMCs were injected into C57Bl/6 (+/+) or P-selectin-deficient (P-sel$^{-/-}$) mice. Rolling interactions of cells in the skin microvasculature before and after i.v. treatment with 25 mg kg^{-1} bodyweight PS3 were observed using intravital microscopy. As expected, rolling interactions are impaired in P-selectin-deficient mice (Weninger *et al.*, 2000). In addition, PS3 reduces rolling interactions in the skin microvasculature through inhibition of P-selectin. Data are presented as mean ± standard error. Results are from nine vessels from three C57Bl/6 and seven vessels of two P-selectin-deficient mice. Comparisons within one group were performed using the paired *t*-test, whereas the *t*-test was used for comparison of rolling in C57Bl/6 and P-selectin-deficient mice.

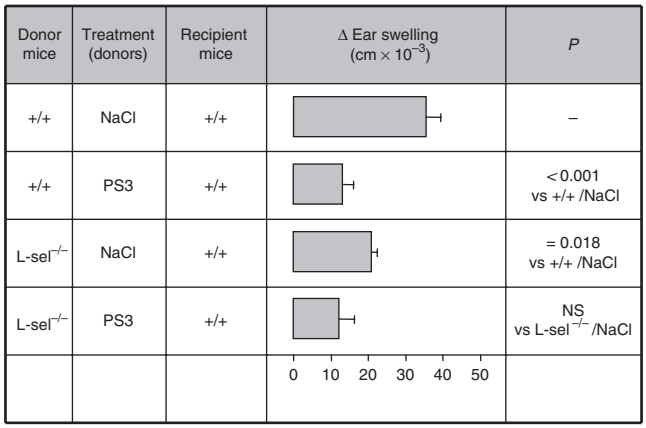

Figure 5. PS3 treatment hinders generation of immunity through inhibition of L-selectin. C57Bl/6 (+/+) or L-selectin-deficient (L-sel$^{-/-}$) mice were sensitized using DNFB. Before topical application of the chemical, mice were either i.v. treated with 25 mg kg^{-1} PS3 or solvent alone. Donor mice were killed and leukocytes were isolated from peripheral lymph nodes and spleen, which were transferred into recipient C57Bl/6 mice. Recipients were simultaneously challenged with DNFB. After 24 hours, ear swelling response was evaluated. In accordance with previous reports (Catalina *et al.*, 1996), we confirm a major, but not sole, role of L-selectin for generation of immunity in peripheral lymph nodes. We furthermore provide evidence that PS3 hinders generation of immunity through inhibition of L-selectin. Data are presented as mean ± standard error. Results are from 11 to 18 mice per experimental condition. One-way ANOVA was used for statistical comparisons; pair-wise multiple comparisons were performed using the Bonferroni *t*-test.

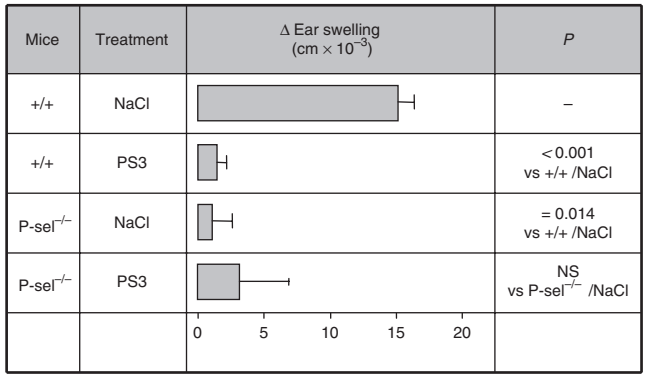

Figure 6. PS3 treatment diminishes the effector phase of a contact hypersensitivity response. C57Bl/6 (+/+) or P-selectin-deficient (P-sel$^{-/-}$) mice were sensitized and challenged with DNFB. Before challenge with the chemical, mice were either treated with 25 mg kg^{-1} PS3 or solvent alone. After 24 hours, ear swelling response was evaluated. Here we show that treatment of wild-type mice with PS3 leads to a profound inhibition of the contact hypersensitivity response. As no effect of PS3 is observed in P-selectin-deficient mice, this effect of PS3 seems predominantly mediated through an inhibition of P-selectin. Data are presented as mean ± standard error. Results are from 6 to 18 mice per experimental condition. One-way ANOVA was used for statistical comparisons; pair-wise multiple comparisons were performed using the Bonferroni *t*-test.

hypersensitivity response, P-selectin-deficient mice showed significantly diminished ear swelling ($1.1 \pm 1.5\,\text{cm} \times 10^{-3}$) as compared to wild-type mice ($15.1 \pm 1.3\,\text{cm} \times 10^{-3}$; $P = 0.014$; Figure 6). In addition, a significant decrease of the inflammatory response was observed when wild-type mice were treated systemically with PS3 (Δ ear swelling $1.5 \pm 0.7\,\text{cm} \times 10^{-3}$; $P < 0.001$ compared to vehicle-treated mice; Figure 5). In mice lacking P-selectin expression, PS3 has no further inhibitory effect (Figure 5). Thus, the apparent therapeutic effect of PS3 treatment was presumably due to inhibition of P-selectin function in the wild-type mice.

DISCUSSION

Comparing several glucan sulfates (phycarin, curdlan, or pullulan sulfates) for their P-selectin-inhibitory capacity *in vivo* to identify structural parameters attributable to P-selectin inhibition, we identified a phycarin sulfate with a profound inhibitory activity comparable to a function-blocking P-selectin-directed antibody (Fritzsche *et al.*, 2006). Using this highly active P-selectin-inhibitory glucan sulfate (termed PS3), we have shown an additional L-selectin but not E-selectin-inhibitory activity of PS3 under *in vitro* flow conditions. In addition, we could show inhibition of lymphocyte rolling by PS3 *in vivo*. As both P- and L-selectins contribute to the pathogenesis of chronic inflammatory disease (Uchimura and Rosen, 2006; Ludwig *et al.*, 2007b), we evaluated the use of PS3 as an anti-inflammatory agent in a murine model of contact hypersensitivity. Our results show a clear reduction of the inflammatory response by PS3 in two complementary models, the adoptive transfer of immunity (sensitization phase), and the challenge with contact allergen (elicitation phase). We confirmed in the adoptive transfer model that L-selectin deficiency of donor mice diminishes the

inflammatory response in wild-type donor mice (Catalina *et al.*, 1996; Diacovo *et al.*, 1998). In these donor mice, PS3 treatment had no additional effect on ear swelling of the recipients. Thus, the immunosuppressive effects of PS3 are most likely mediated, at least in part, through L-selectin inhibition in the phase of sensitization in the lymph nodes. PS3 treatment of P-selectin-deficient mice during challenge with DNFB had no effect, indicating that PS3's anti-inflammatory activity is mainly because of inhibition of P-selectin function.

As shown here and elsewhere (Arbones *et al.*, 1994; Catalina *et al.*, 1996; Maly *et al.*, 1996; Collins *et al.*, 2001), interactions of L-selectin with appropriate ligands, for example, peripheral lymph node adressin, significantly contribute to the generation of immunity. Interactions of L-selectin with its ligands allow rolling of naive T lymphocytes along class I–III vessels in peripheral lymph nodes (von Andrian, 1996), which initiates extravasation (Butcher and Picker, 1996) to and antigen presentation in the lymph node. In addition to L-selectin, P-selectin may contribute to the generation of immunity (at least in L-selectin-deficient mice), as infusion of P-selectin-expressing, activated human platelets into L-selectin-deficient mice during sensitization with DNFB led to reconstitution of immunity in those mice, by forming platelet–leukocyte aggregates, which allow leukocyte rolling in lymph nodes (Diacovo *et al.*, 1998). This contribution of P-selectin to the generation of immunity might explain why treatment of wild-type donor mice with PS3 led to a more pronounced reduction of the CHS response as compared to L-selectin donor mice. Extravasation of leukocytes into sites of inflammation also relies on the expression of L- and P-selectins: several sites of inflammation express L-selectin ligands (Michie *et al.*, 1993; Arvilommi *et al.*, 1996), which may allow L-selectin-dependent leukocyte extravasation to those inflamed tissue sites (Arbones *et al.*, 1994; Tedder *et al.*, 1995; Collins *et al.*, 2001). Deficient P-selectin expression in mice (Mayadas *et al.*, 1993) directly and indirectly leads to reduced inflammatory responses in several model systems and can presumably be attributed to the lack of both platelet and endothelial P-selectin (Subramaniam *et al.*, 1995; Robinson *et al.*, 1999; Schober *et al.*, 2002; Huo *et al.*, 2003; Manka *et al.*, 2004). This profound contribution of L- and P-selectins to the pathogenesis of several inflammatory conditions may explain the significant effects observed by PS3 treatment in both of our models of cutaneous inflammation.

Although PS3 turned out to antagonize L- and P-selectins, it did not influence E-selectin. Also, heparin fractions and heparin sulfate were found to be inactive against E-selectin (Skinner *et al.*, 1991; Albelda *et al.*, 1994). All three selectins bind to sLe^x, but in contrast to the natural P/L-selectin ligands, the E-selectin ligands contain no negatively charged substituents (McEver, 2001). The E-selectin binding to its ligand seems to occur independently of ionic interactions. Therefore, polyanions such as PS3 may be unable to displace the ligands from E-selectin. As P- and L-selectins were found to be more important for the initial leukocyte rolling than E-selectin (Labow *et al.*, 1994), inhibition of P/L-selectin might be sufficient to efficiently inhibit extravasation.

Current treatment strategies for patients with chronic inflammatory diseases are mainly based on immunosuppression (Weinblatt *et al.*, 1999; Schön and Boehncke, 2005). Despite its pronounced anti-inflammatory activities, PS3 differs in several aspects from classical immunosuppressant drug substances. First, the binding of glycans to their targets is typically reversible and not as tight as those of antibodies to their antigens or tailored xenobiotic chemical entities to their target molecules (Mulloy, 2005). These distinct binding characteristics become obvious regarding the selectin–glycan interactions mediating the rolling of leukocytes and the interactions between proteins responsible for firm cell adhesion. Second, PS3 is structurally comparable with endogenous glycosaminoglycans. Among them, especially the heparan sulfates are known to exhibit important (patho-) physiological functions by mediating cell–cell and cell–extracelullar matrix interactions and binding to a large number of biomolecules such as enzymes, enzyme inhibitors, growth factors, chemokines, or cell receptors (Lindahl *et al.*, 1998). Heparan sulfates and also other glycans are thus strongly involved in the (patho-) physiological network of inflammation, hemostasis, and tumor progression and metastasis. Sulfated glycans may interfere with processes where heparan sulfates or other glycan structures are involved (Alban, 2008a). Their three-dimensional structure and patterns of charge distribution are responsible for differential affinities for biomolecules (Stringer *et al.*, 2002). A third characteristic of sulfated glycans such as heparin and PS3 is that they do not only highly specifically interact with one single target structure, but rather act like multivalent biomodulators and exhibit a wide range of activities contributing to its overall therapeutic benefit (Alban, 2008b). Similar to combination therapies, the synergism of various relatively moderate actions finally results in efficacy with fewer side effects. But their individual activity profiles are dependent on their individual structure and may significantly differ. As an example, 30–50% of the molecules of an unfractionated heparin preparation bind to antithrombin and thus inhibit factor Xa and thrombin, whereas PS3 does not at all exhibit antithrombin-mediated anticoagulant activity (Yvin *et al.*, 2002). However, PS3 is superior to heparin in many of its other biological activities (Becker *et al.*, 2003a). Besides the L- and P-selectin antagonizing effect, it was shown, to inhibit the chemotaxis of monocytes, the complement activation, enzymes such as elastase, hyaluronidase, and heparanase, the release of cytokines, or to mobilize tissue factor pathway inhibitor.

Compared to heparin doses used to observe a significant reduction in the inflammatory response in various mouse models of inflammation (Ludwig *et al.*, 2007b) and also compared to the anti-inflammatory effect of PS3 in the croton oil-induced mouse ear edema model (Yvin *et al.*, 2002), the dose of PS3 ($25\,mg\,kg^{-1}$ bodyweight) used here to achieve a significant immunosuppressive (Figure 5) and anti-inflammatory activity (Figure 6) in mice is higher. However, as we did not examine the dose–response relationship in this model,

activity of PS3 at lower doses cannot be excluded. Further, PS3 used at 25 mg kg^{-1} bodyweight has only marginal effects on coagulation, and was about 10 times more effective than heparin in experiments directly comparing the effect of PS3 and heparin in models of metastasis (Ludwig and Alban, unpublished), a process also depending on P- and L-selectin expression (Borsig *et al.*, 2002).

Supporting the mechanistic linkage between hemostasis and inflammation, recent clinical research has clearly documented an increased incidence of cardiovascular disease in patients suffering from chronic inflammatory diseases (Maradit-Kremers *et al.*, 2005; Gelfand *et al.*, 2006; Ludwig *et al.*, 2007a). As numerous immunological factors identified as relevant in the pathogenesis of atherosclerosis are also known to have key functions in other chronic systemic inflammatory diseases such as rheumatoid arthritis or psoriasis, a higher than expected rate of cardiovascular disease could be explained based on shared pathogenic pathways (Arad *et al.*, 2005; Hansson, 2005; Boehncke *et al.*, 2007; Ludwig *et al.*, 2007b). P-selectin is a likely candidate molecule to mediate the close interaction of chronic inflammation and cardiovascular disease. This notion is best supported by the defects in leukocyte extravasation and coagulation in P-selectin-deficient mice (Mayadas *et al.*, 1993; Subramaniam *et al.*, 1996). Extending these findings, we have recently provided experimental evidence that P-selectin-dependent functions of platelets are important in myocardial infarction and that targeting these functions by using the small-molecule P-selectin inhibitor efomycine M (Schön *et al.*, 2002) has a significant therapeutic potential in relevant animal models (Oostingh *et al.*, 2007b). In addition to P-selectin inhibition, PS3 possesses a similar *in vivo* antithrombotic activity as unfractionated heparin, whereas its anticoagulant properties are reduced to 20% of unfractionated heparin (Alban *et al.*, 1995).

Overall, PS3 or related substances might target both inflammation and cardiovascular comorbidity in patients suffering from chronic inflammatory diseases. However, this appealing hypothesis remains to be tested.

MATERIALS AND METHODS

Mice

Male C57Bl/6 mice, aged 4–8 weeks, were purchased from Charles River, Bar Harbor, Maine and kept under specific pathogen-free conditions with water and food *ad libitum*. P-selectin-deficient mice on the C57Bl/6 background were obtained from Jackson Laboratories (www.jax.org). L-selectin-deficient mice on the same genetic background (Borsig *et al.*, 2002) were kindly provided by R Hynes (Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA). All animal experiments were approved by the governmental administration of Hessen (Darmstadt, Germany).

Cells and culture conditions

Human PBMCs were prepared by Ficoll–Hypaque density gradient sedimentation (Pharmacia, Uppsala, Sweden) from buffy coats. U937 cells, a human monocytic cell line expressing the P-selectin ligand PSGL-1, were cultured at 37 °C and 5% CO_2 in RPMI 1640 medium containing 10% fetal calf serum and 1% penicillin–streptomycin solution (all supplements from Sigma, Deisenhofen, Germany). PSGL-1 expression was confirmed by flow cytometry (data not shown). Cells were used for the rolling experiments within 4 hours after separation, centrifugation, and resuspension in serum-free medium. E-CHO were grown in MEM-α (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum, 2 mM L-glutamine, and 1% penicillin–streptomycin solution. The cells were cultured for 3–4 days to near confluency. After splitting using 0.25% trypsin–EDTA solution (Sigma), centrifugation, and resuspension in serum-free medium, the cell suspension was transferred to slowly rotating plastic tubes. The cells remained in suspension for up to 4 hours. The rolling experiments were performed within this time period.

Synthesis and characterization of PS3

The synthesis and chemical characterization of PS3 has been described in detail previously (Yvin *et al.*, 2002; Ménard *et al.*, 2004). The starting polymer Phycarine was obtained from Goemar Laboratoires (Saint-Malo, France). PS3 was produced under Good Manufacturing Practice conditions. Chemical characterization and purity tests were performed by means of elemental analysis, conductivity-detected ion chromatography of the sulfate ions released by trifluoroacetic acid hydrolysis, conductimetric NaOH titration of PS3 transformed into the acid form by ion exchange, size exclusion chromatography, a modified ''methylation analysis'' (that is, gas-chromatography-mass spectrometry after derivatization) (Alban and Franz, 1994), high-pressure anion exchange chromatography with pulsed amperometric detection analysis, C^{13}NMR, and UV spectroscopy.

Flow chamber experiments on endothelial cells

Interactions of PBMC with endothelial cells under conditions of shear flow mimic the situation in blood vessels. This system was used to determine the effect of selectin inhibition by PS3 or a cocktail of E-, P-, and L-selectin-directed antibodies on the interaction between PBMCs and endothelial cells. Human endothelial cells were gently detached from the tissue culture flask by trypsin/EDTA (PAA Laboratories, Pasching, Germany), followed by immediate addition of serum-containing medium to inhibit the action of trypsin. Rectangular plastic tissue culture-treated Thermanox coverslips (22 × 60 mm Plano, Wetzlar, Germany) were coated with 2×10^4 endothelial cells and left at 37 °C/5% CO_2 overnight to allow the cells to attach, form a normal monolayer, and reexpress trypsin-sensitive surface antigens. Four hours before the start of the experiment, recombinant human (rh-) TNF-α was added to the endothelial cell-coated coverslips at a final concentration of 25 ng ml^{-1}. TNF-α is known to induce surface expression of E-selectin and other membrane glycoproteins involved in interactions with flowing lymphocytes. Isolated human PBMCs were washed and resuspended at 10^6 cells per milliliter in Hank's balanced salt solution containing 2 mM $CaCl_2$. An aliquot of 100 µl PS3 at concentrations of 10 mg ml^{-1} to 10 µg ml^{-1} dissolved in 0.9% NaCl, or of a mix of E-, P-, and L-selectin-specific antibodies (all at a final concentration of 10 µg per sample) was added to 900 µl PBMC suspension and incubated for at least 20 minutes. Transparent flow chambers with a slit depth of 50 µm and a slit width of 5 mm, equipped with the endothelial cell-coated coverslips, were briefly rinsed with Hank's balanced salt solution + 2 mM $CaCl_2$, and

connected to a syringe containing the preincubated cell suspension. Perfusion was performed at 37 °C using a pulse-free pump under low shear stress conditions (flow rate of 1.12 ml per hour, equivalent to a wall shear rate of 150 per second). Microscopic phase-contrast images were recorded in real time. At least six different microscopic fields were recorded for each condition. The number of adhering cells in all six fields was counted for analysis. Data presented are from three independently performed experiments.

Flow chamber experiments on immobilized P-selectin

Microscope glass slides (diameter of 18 mm, thickness of 0.2 mm) were used as transparent supports. To achieve a highly homogeneous surface, the slides were first treated with a concentrated H_2SO_4/H_2O_2 mixture (7/3 v/v; Fluka, Neu-Ulm, Germany) for 30 minutes at 80 °C under ultrasonic conditions and rinsed with ultrapure water for 30 minutes at room temperature. A cleaning procedure with $NH_3/H_2O_2/H_2O$ (1/1/5 v/v/v; Fluka) was performed, followed by a final rinse with ultrapure water and drying of the slides. For covalent immobilization of selectins, cyanuric chloride (Sigma) was used as cross-linker: it was dissolved in chloroform (Riedel-de Haën, Seelze, Germany) and added to the glass under ultrasonication. After drying, slides were incubated with a mixture of 3.75 μg ml^{-1} P-sel-Fc-fusion protein (R&D Systems, Wiesbaden, Germany) and 0.5% (m/V) BSA (Sigma) in borate buffer (pH 8.8) for 2 hours at room temperature. Immediately before the flow chamber experiments, slides were thoroughly rinsed with ultrapure water and incorporated into the flow chamber. Binding of selectin-fusion protein was confirmed by immunofluorescence (not shown).

The flow apparatus was mounted onto an inverted fluorescence microscope Axiovert 200 (Carl Zeiss, Germany) as described previously (Fritzsche *et al.*, 2006). In brief, phosphate-buffered saline (pH 7.4) containing 1 mM $CaCl_2$ and $MgCl_2$ was used as flow medium at a shear rate of 200 per second, driven by either hydrostatic pressure or a pump system, respectively. For the flow experiments using the pump system, 2×10^6 U937 cells were injected, and a video sequence was captured after 5 minutes of cell rolling. Then, PS3 (100 μg ml^{-1}) was added to the streaming medium, and after 5 minutes a second video sequence was recorded. To quantify a reduction in cell adhesion, the cell number after PS3 injection was compared to that observed in the control experiment. The video sequences were recorded capturing 25 frames per second with a CCD camera (CSC 795) using a long-distance objective of ×20. The images were morphometrically analyzed by the Imagoquant software (Imagoquant Multitrack-AVI-2; Mediquant, Halle, Germany) resulting in detailed and automated analysis of the experimental data. All cells (about 100) per image were included in the calculations.

Flow chamber experiments on immobilized sLe^{x}Lac1

For experiments using E-CHO cells, cleaned glass slides (described above) were incubated with monochloro-dimethyl-octadecyl-silane (Fluka) at 70 °C for 10 minutes, which binds covalently to the glass slides resulting in a hydrophobic monolayer. The binding reaction was stopped by rinsing with chloroform, washing with water and drying under air stream conditions. In the next step, lipid monolayers of 1,2-distearoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids Inc., Alabaster, AL) as matrix lipid containing 0.01 mol% of the ligand sLe^{x}Lac1 were preformed on the air–water interphase of the Langmuir trough. The glycolipid ligand sLe^{x}Lac1 was synthesized as previously published (Gege *et al.*, 2000). After condensing the lipid layer at a lateral pressure of 38 mN m^{-1}, the glass slides were vertically driven through the layer resulting in a complete bilayer. The transfer ratios were between 0.95 and 1.0. The freshly prepared bilayers were immediately used for the rolling experiments with E-CHO cells by incorporating the slides into the flow chamber under exclusion of air contact to warrant the integrity of the bilayers. Experiments under flow and data analysis were performed as described above.

Flow chamber experiments on immobilized sLe^x

The effect of PS3 or an L-selectin-specific antibody on the adherence and rolling of PBMCs to multimeric sLe^x was tested under conditions of shear stress. Rectangular coverslips (24 × 60 mm) were coated with 2.5 or 5.0 μg biotinylated polyacrylamide labeled with 20% (molar ratio) sLe^x and 5% (molar ratio) sulfated tyrosine (sLe^x/TS-PAA, 30–40 kDa; Lectinity Holdings Inc., Moscow, Russia). This ligand has been previously used to analyze L-selectin-mediated interactions and was shown to have several characteristics in common with natural L-selectin ligands (Galustian *et al.*, 2002). The coverslips were air-dried and blocked for at least 1 hour with 0.5% BSA in phosphate-buffered saline. An aliquot of 100 μl PS3 at concentrations of 100 μg ml^{-1} dissolved in 0.9% NaCl, or of L-selectin-specific antibody (at a final concentration of 10 μg per sample) was added to 900 μl peripheral blood lymphocyte suspension and incubated for at least 20 minutes. Transparent flow chambers with a slit depth of 50 μm and a slit width of 5 mm, equipped with coated or uncoated coverslips, were briefly rinsed with Hank's balanced salt solution + 2 mM $CaCl_2$, and connected to a syringe containing the preincubated cell suspension. Perfusion was performed at 37 °C using a pulse-free pump under low shear stress conditions (flow rate of 1.12 ml per hour, equivalent to a wall shear rate of 150 per second). Microscopic phase-contrast images were recorded in real time. At least six different microscopic fields were recorded for each condition. Image analysis was performed offline using MetaView Imaging software (Universal Imaging Corporation, Downingtown, PA).

Intravital microscopy of skin microvasculature

Rolling of leukocytes in the skin microvasculature depends on an interaction of P- and E-selectins with their appropriate leukocyte ligands (Weninger *et al.*, 2000). To evaluate the influence of P-selectin inhibition by PS3 *in vivo*, intravital microscopy of the skin microvasculature in mice was performed (Ludwig *et al.*, 2004; Rubant *et al.*, 2008) using human PBMC, whose selectins and ligands efficiently interact across this species barrier (Robert *et al.*, 1999). To increase expression of selectin ligand expression (Zollner *et al.*, 2002), cells were cultured in the presence of the superantigen TSST-1 for 5 days. Thereafter, cells were labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein and injected retrograde into the aortic arch of anesthetized mice. Labeled cells in cutaneous microvessels of the left ear were visualized by fluorescence epi-illumination. Rolling fractions were determined as percentages in relation to the total flux. In total, nine vessels from three C57Bl/6 and seven vessels of two P-selectin-deficient mice were investigated.

Adoptive transfer of immunity

For adoptive transfer experiments mice were sensitized by painting 75 µl of DNFB (Sigma) solution (0.5% in acetone/olive oil, 4:1) on the back of the mice on day 0. Mice were either left untreated, or received treatments as indicated in the figure legends immediately after application of DNFB. On day 5, spleens and regional lymph nodes were removed from DNFB-sensitized mice, and single cell suspensions were prepared by homogenizing lymph nodes and spleens from donor mice through a nylon mesh (70 µm pore width; BD Biosciences, Heidelberg, Germany). Recipient mice were injected i.v. with 4×10^7 cells in 200 µl total volume, followed by immediate application of 20 µl DNFB at 0.3% to the right ears of the mice. At 24 hours after challenge, CHS responses were determined using a spring-loaded micrometer (Mitutoyo, Neuss, Germany) in a blinded fashion. Ear swelling responses of DNFB-challenged ears were compared to the response of the vehicle-treated ear in and were expressed as $\text{cm} \times 10^{-3}$. Data presented are based on results obtained from 5 to 10 mice per group.

Cutaneous contact hypersensitivity reaction

Mice were sensitized as described above. On day 5, 20 µl of 0.3% DNFB or vehicle was applied on the left and right ear, respectively. At 24 hours after challenge, CHS responses were determined using a spring-loaded micrometer (Mitutoyo) in a blinded fashion. Ear swelling responses of DNFB-challenged ears were compared to the response of the vehicle-treated ear in sensitized animals and were expressed as $\text{cm} \times 10^{-3}$. Mice were either i.v. treated with PS3 at 25 mg kg^{-1} bodyweight, or solvent (0.9% NaCl) immediately before challenge with the allergen.

Statistical analysis

Data are presented as mean ± standard error if not indicated otherwise. Statistical analysis was performed using SigmaStat 3.5 (Systat Software Inc., www.systat.com). Applied tests are indicated at the figure legends. *P*-values <0.05 were considered statistically significant. All statistical tests were two sided.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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