Junctional Adhesion Molecules (JAM)-B and -C Contribute to Leukocyte Extravasation to the Skin and Mediate Cutaneous Inflammation

Ralf J. Ludwig,*[1] Thomas M. Zollner,†[1] Sentot Santoso,‡ Katja Hardt,* Jens Gille,* Holger Baatz,§ Petra Schulze Johann,* Jeannette Pfeffer,* Heinfried H. Radeke,¶ Michael P. Schön,# Roland Kaufmann,* Wolf-Henning Boehncke* and Maurizio Podda*

*Department of Dermatology, Klinikum der J.W. Goethe Universität, Frankfurt am Main, Germany; †CRBA Dermatology, Schering AG, Berlin, Germany; ‡Institute for Clinical Immunology and Transfusion Medicine, Justus-Liebig-Universität, Gießen, Germany; §Klinik am Rathauspark, Recklinghausen, Germany; ¶pharmazentrum Frankfurt/ZAFES, Klinikum der J.W. Goethe Universität, Frankfurt am Main, Germany; #Rudolf Virchow Center, DFG Research Center for Experimental Biomedicine and Department of Dermatology, Würzburg, Germany

Leukocyte extravasation is a finely tuned process, in which transmigration is the final step. Transmigration depends on molecules located at borders of endothelial cells; e.g., junctional adhesion molecules (JAM-A, -B and -C). *In vivo* blockade of JAM-A lead to decreased migration of monocytes into the skin. In contrast, the role of JAM-B and -C in development of cutaneous inflammation is unknown. We therefore elicited an allergic contact dermatitis in mice using 2,4-dinitro-1-fluorobenzene. RT-PCR and immunofluorescent staining of healthy skin revealed a constitutive JAM-B (66.4% ± 6.7% of all vessels) and -C expression (88.6 ± 13.2%), which remained constant after induction of contact dermatitis. Functional studies, in which either JAM-B or -C neutralizing antibodies were injected into sensitized mice prior to allergen challenge showed a concentration-dependent reduction of the contact dermatitis. Decreased ear swelling was accompanied by reduction of leukocyte infiltration as analyzed by hematoxylin and eosin (H&E) histology and enzyme activity. Combined antibody treatment at doses of 1.25 mg per kg bodyweight lead to additive inhibition of allergic contact dermatitis, indicating that JAM-B and -C may have distinct functions. In conclusion, interactions with JAM-B and -C are essential for development of cutaneous inflammation.

Key words: adhesion molecules/cell trafficking/inflammation/skin
J Invest Dermatol 125:969–976, 2005

The extravasation of leukocytes into the skin is required for immune surveillance. Unrestrained extravasation, however, plays a key role for a number of inflammatory skin disorders, e.g., psoriasis, allergic, or contact dermatitis. The finely regulated process of leukocyte homing involves several well-defined steps (Butcher and Picker, 1996; von Andrian and Mackay, 2000; Schön *et al*, 2003): It is initiated by short-lived adhesive interactions of endothelial selectins with leukocyte carbohydrate ligands displayed on glycoprotein scaffolds. These interactions slow down leukocytes, which then roll along the endothelium. Rolling allows a chemokine-mediated interaction of leukocytes with endothelial cells, which may lead to leukocyte activation, resulting in an increased integrin avidity (Kishimoto *et al*, 1989; Lollo *et al*, 1993). This permits firm adhesion of leukocyte integrins to the endothelial ligands, mostly of the immunoglobulin superfamily (Butcher and Picker, 1996). Adhering leukocytes leave the circulation by passing between endothelial cells at their borders (Dejana *et al*, 1995). Six molecules (PECAM-1, CD99, VE-cadherin, junctional adhesion molecules (JAM)-A, -B and -C) are concentrated at the lateral borders of the endothelial cells and seem to be involved in leukocyte transmigration (Müller, 2003; Weber, 2003; Schön and Ludwig, 2005). Blockade of either PECAM-1 or CD99 in mice with antibodies leads to a 90% reduction of leukocyte transmigration *in vivo*. In contrast, treatment of mice with a VE-cadherin antibody increased vascular permeability and accelerated the entry of neutrophils into chemically inflamed mouse peritoneum, suggesting that opening of VE-cadherin mediated endothelial cell contacts may be a relevant step during neutrophil extravasation (Gothsch *et al*, 1997). *In vivo* antibodies against JAM-A reduced leukocyte extravasation in a cytokine-induced meningitis (Del Maschio *et al*, 1999) and in the air-pouch model (Martin-Padura *et al*, 1998). In contrast, the same antibody failed to inhibit leukocyte homing in viral or bacterial meningitis models (Lechner *et al*, 2000). The contribution of JAM-B and -C to leukocyte extravasation to the skin *in vivo* is not known. The expression pattern and ligand-interactions of JAM-B and -C, however, indicate a role of these JAM in leukocyte extravasation. JAM-B and -C are strongly expressed by endothelial cells of high endothelial venules—the predominant site of leukocyte extravasation (Butcher and Picker, 1996).

Abbreviations: CHS, contact hypersensitivity; DNFB, 2,4-dinitro-1-fluorobenzene; JAM, junctional adhesion molecules

[1]Both authors contributed equally to this work.

In addition, JAM-B and -C bind to the leukocyte integrins VLA-4 or Mac-1, respectively (Cunningham *et al*, 2002; Santoso *et al*, 2002). *In vitro* studies using either JAM-B neutralizing antibodies or soluble JAM-B support this hypothesis: Both substances reduced leukocyte transmigration across human umbilical vein endothelial cells (Johnson-Leger *et al*, 2002).

In an attempt to determine the contribution of JAM-B and -C in leukocyte homing to the skin, we assessed expression patterns on protein and mRNA levels in healthy and inflamed (contact dermatitis) skin, and evaluated the impact of JAM-B and -C on leukocyte extravasation to the skin using a mouse model of allergic contact dermatitis.

Results

JAM-B and -C are constitutively expressed in murine skin To quantify the mRNA expression of JAM-B and -C during development of a contact hypersensitivity (CHS) response, expression of JAM-B and -C was evaluated in vehicle treated and challenged skin using real-time PCR. At all time points, expression of JAM-B and -C mRNA remained constant in challenged ears and were similar to expression patterns of vehicle treated ears, indicating that both JAM molecules have not been up regulated during CHS (Table I).

We then examined the expression of JAM-B and -C proteins at vessels of murine skin, staining specimen from normal and 2,4-dinitro-1-fluorobenzene (DNFB)-challenged mouse skin with JAM-B and -C antibodies. JAM expression was visualized using fluorescent-labeled secondary antibodies, and vessels were identified by their CD31 expression. We observed a constitutive high expression of both JAMs in healthy murine skin, which remained constant 24 h after induction of a CHS using DNFB (Fig 1). In normal skin, 66.4% $\pm$ 6.7% of all vessels expressed JAM-B, which was almost identical in sections from DNFB-challenged skin (68.5% $\pm$ 5.4%; p = NS; *t* test). Over 80% of all vessels in normal and inflamed skin expressed JAM-C (86.6% $\pm$ 13.2% (normal) *vs* 85.9% $\pm$ 15.2% (inflamed); p = NS; *t* test).

Table I. JAM-B and -C mRNA expression patterns remain constant during development of a CHS response

	Fold expression of HPRT				
	t = 0 h	t = 3 h	t = 6 h	t = 24 h	t = 48 h
JAM-B					
Vehicle	0.83	1.12	1.01	1.40	0.85
CHS	0.84	1.17	0.80	1.10	0.70
JAM-C					
Vehicle	0.31	0.34	0.45	0.27	0.23
CHS	0.30	0.39	0.32	0.29	0.21

Specimen from three mice were pooled and analyzed for JAM-B and -C mRNA expression in vehicle and DNFB treated ears for each time point (after DNFB challenge) indicated. Gene expression was calculated compared to HPRT expression. At all time points no significant difference in JAM-B or -C expression was detected, and expression of both JAM remained constant over the period investigated.

JAM, junctional adhesion molecule; CHS, contact hypersensitivity; DNFB, 2,4-dinitro-1-fluorobenzene; HPRT, hypoxanthine-guanine phosphoribosyl transferase.

JAM-B and -C are required for development of a CHS Next, we evaluated the impact of inhibitory JAM-B and -C antibodies on the development of cutaneous inflammation in a murine model of CHS. Mice were sensitized with DNFB and treated with different doses of JAM-B and/or -C inhibitory antibodies 60 min prior to challenge with DNFB. In the control experiments, treatment with either rat-IgG isotype control antibody, goat IgG or both control antibodies had no effect on ear swelling (ear swelling responses: 20.9 $\pm$ 11.9, 20.9 $\pm$ 9.0, 22.6 $\pm$ 15.3 and 16.7 $\pm$ 2.4 cm $\times$ 10^{-3} for untreated, rat and goat IgG isotype controls or both control antibodies). In contrast, application of either anti-JAM-B or anti-JAM-C antibody resulted in a significant and concentration dependent, but not complete inhibition of ear swelling. Although treatment with low doses (1.25 mg per kg) of anti-JAM-B or -C antibody did not lead to a statistically significant reduction in ear swelling, higher doses (2.5 mg per kg) of these antibodies had a statistically significant effect on ear swelling. Further increase of antibody concentration to 5.0 mg per kg had no additional effect for both antibodies investigated (Fig 2). Reduction of ear swelling responses in mice treated with 2.5 mg per kg antibody was accompanied by reduction of total leukocyte infiltration as analyzed by hematoxylin and eosin (H&E) histology (Fig 3).

To gain further insight into the leukocyte populations affected by either antibody treatment, sections were also stained with antibodies specific for CD3, neutrophils and macrophages/monocytes. Whereas JAM-B antibody (2.5 mg per kg) treatment significantly reduced epidermal CD3 staining and neutrophil infiltration, JAM-C blockade (2.5 mg per kg) additionally reduced dermal CD3 staining, suggesting a differential contribution of JAM-B and -C for cutaneous inflammation. In support of this hypothesis, peroxidase- and elastase-activity showed even more diverse results: Activity of both enzymes was reduced in mice treated with 2.5 mg per kg antibody. In contrast to JAM-C antibody treatment (1.25 mg per kg), injection of an identical dose of anti-JAM-B lead to a significant decrease in peroxidase and elastase activity. With respect to the nature of experiments using antibodies, this may indicate that JAM-B might be predominantly involved in neutrophil extravasation (Fig 4).

To exclude that observed changes in ear swelling and infiltration are due to alterations in leukocyte counts caused by application of the antibodies, a differential blood count was performed after treatment with antibodies. Injection of either one of the anti-JAM or isotype antibodies at 2.5 mg per kg bodyweight lead to a significant leukopenia, which was due to a reduction of the lymphocyte population. No significant difference, however, was observed between the different treatment groups. Erythrocyte and platelet count remained constant at the investigated time points (plus 4 and 24 h) after antibody injection (not shown). As no difference in differential blood count were observed between isotype and JAM antibody-treated mice, the changes in ear swelling and infiltration were not due to changes in leukocyte counts.

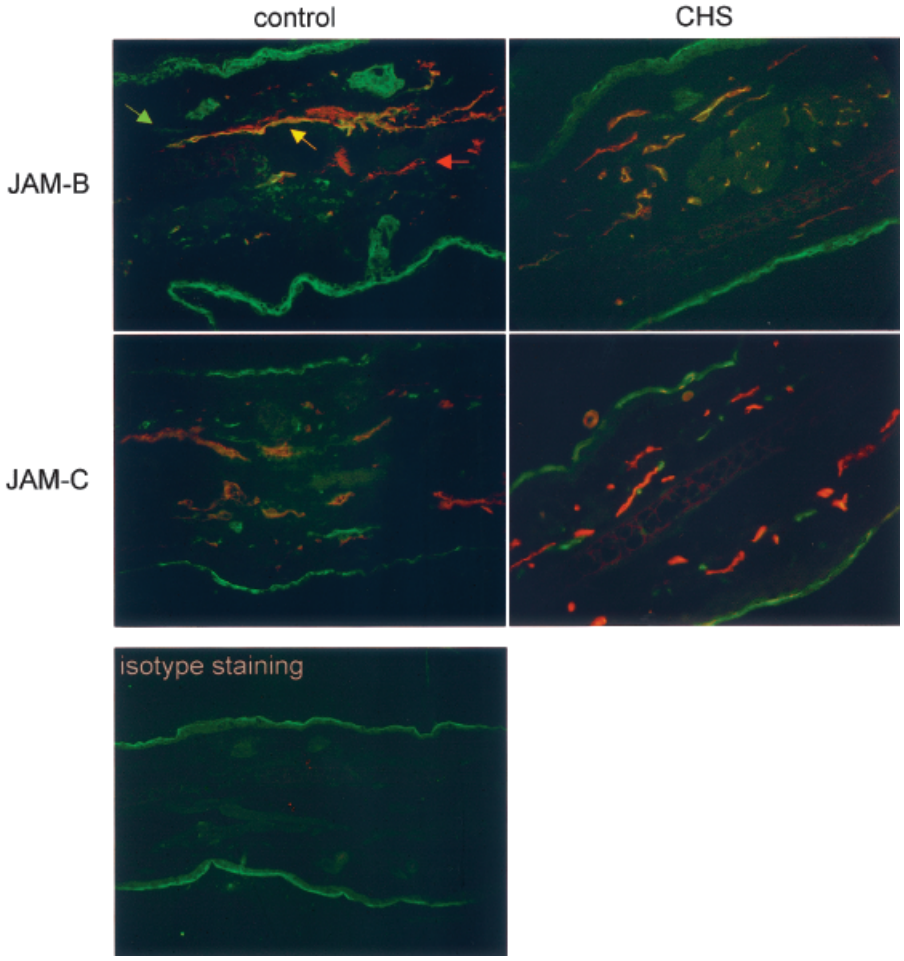


Figure 1
Junctional adhesion molecule (JAM)-B and -C expression in murine skin. Normal and inflamed (CHS) mouse skin were stained with JAM-B or -C antibodies (green) as outlined in Materials and Methods. Vessels were identified by their expression of CD31 (red). In normal skin 66.4% ± 6.7% and 86.6% ± 13.2% all vessels expressed JAM-B or -C respectively. Development of a CHS had no effect on the expression of both JAM-B and -C (68.5% ± 5.4% or 85.9% ± 15.2%, *t* test, n = 3 mice/group). As staining with isotype control, antibody showed fluorescence in the epidermal layer, epidermal staining has to be considered unspecific. Exemplary, arrows indicate vessels staining for CD31 or JAM only (*red or green, respectively*) and double positive staining vessels (*orange*).

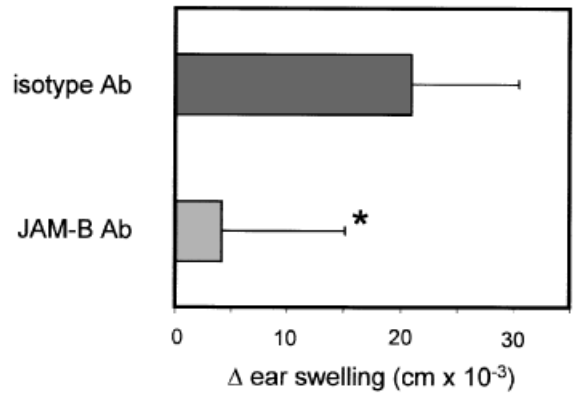
Combined treatment with JAM-B and -C antibodies has additive anti-inflammatory effects As JAM-B and -C bind to different ligands they may play distinct roles in leukocyte extravasation. Consequently, combined inhibition of these JAM may lead to additive or synergistic effects. We therefore evaluated the impact of simultaneous JAM-B and -C antibody-treatment prior to challenge with DNFB. Injection of JAM-B and -C antibodies at concentrations of 2.5 mg per kg bodyweight had no additional effect compared to JAM-B or -C blockade alone (Table II and Fig 5). Combined treatment with concentrations which if applied separately, however, failed to show any effects on CHS responses (1.25 mg per kg) lead to a marked decrease in ear swelling (Table II and Fig 5). The reduction in ear swelling in mice treated with JAM-B or -C (1.25 mg per kg) was 34.1% and 28.3% (p = NS *vs* appropriate isotype controls, ANOVA on ranks). Hence, a combined blockade of JAM-B and -C has additive effects (Fig 6).

Discussion

Leukocyte extravasation is mediated by interactions of leukocytes with endothelial cells in a stepwise process, which is initiated by leukocyte rolling and completed with transmigration across the endothelial barrier (von Andrian and Mackay, 2000; Schön *et al*, 2003; Weber, 2003). The adhering leukocytes leave the circulation by passing between endothelial cells at their borders (Dejana *et al*, 1995). Yet, the molecular mechanisms of this process are far from being completely understood. Recently, JAM-A has been shown to be involved in leukocyte transmigration in a number of *in vivo* models (Martin-Padura *et al*, 1998; Del Maschio *et al*, 1999). In contrast, the contribution of JAM-B and -C to leukocyte extravasation to the skin *in vivo* is not known. Our results provide the first evidence that JAM-B and -C play a role in leukocyte extravasation to the skin.

The recently described differential expression of the JAMs also support the idea, that JAM-B and -C are involved in leukocyte extravasation to sites of inflammation. In contrast to JAM-A, JAM-B and -C are highly expressed at HEV (Aurrand-Lions *et al*, 2001), the predominant site of leukocyte extravasation (Butcher and Picker, 1996). In addition, JAM-B and -C bind to the leukocyte integrins VLA-4 or Mac-1, respectively (Cunningham *et al*, 2002; Santoso *et al*, 2002). *In vitro* studies using either JAM-B antibodies or soluble JAM-B support this hypothesis. Both substances reduced leukocyte transmigration across human umbilical vein endothelial cells (Johnson-Leger *et al*, 2002). Similar results were obtained in recent studies investigating the role of JAM-C in leukocyte transmigration *in vitro* and *in vivo* (Chavakis *et al*, 2004; Zen *et al*, 2004). Furthermore, despite the constant expression during a cutaneous inflamma-

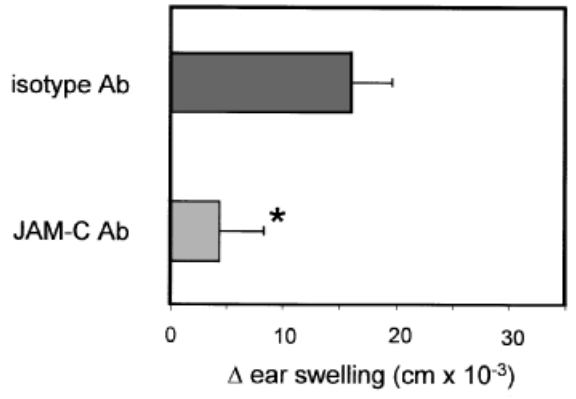
A



B

Treatment	Dose	Δ ear swelling (cm x 10^{-3})
NaCl	-	22.0 ± 13.2
Isotype	5.00 mg/kg bw	18.3 ± 9.1
Isotype	2.50 mg/kg bw	20.9 ± 9.5
JAM-B antibody	5.00 mg/kg bw	6.4 ± 4.9*
JAM-B antibody	2.50 mg/kg bw	4.1 ± 10.9*
JAM-B antibody	1.25 mg/kg bw	12.0 ± 10.1

C



D

Treatment	Dose	Δ ear swelling (cm x 10^{-3})
NaCl	-	19.4 ± 9.9
Isotype	5.00 mg/kg bw	16.1 ± 3.5
Isotype	2.50 mg/kg bw	22.6 ± 15.3
JAM-C antibody	5.00 mg/kg bw	6.5 ± 2.9*
JAM-C antibody	2.50 mg/kg bw	4.3 ± 4.0*
JAM-C antibody	1.25 mg/kg bw	10.0 ± 4.6

E

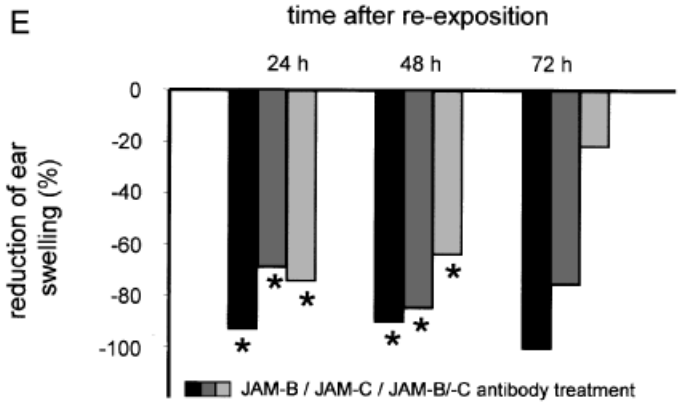


Figure 2

Ear swelling in 2,4-dinitro-1-fluorobenzene (DNFB)-induced contact hypersensitivity (CHS) is reduced in mice treated with either junctional adhesion molecule (JAM)-B or -C antibodies. Mice were sensitized with DNFB and treated with indicated doses of JAM-B and/or -C function blocking antibodies one hour before challenge. Data shown corresponds to the difference in ear swelling 24 h after challenge with DNFB or solvent. (*A*) Ear swelling response in mice treated with either 2.5 mg per kg JAM-B antibody or an appropriate control antibody at the same dose. (*B*) Inhibition of ear swelling with anti-JAM-B antibody is dose dependent. Although 1.25 mg per kg bodyweight have no significant effect on ear swelling, 2.5 mg per kg profoundly inhibit the inflammatory response after DNFB challenge. Further increase of antibody dose has no additional effect. *C* and *D* show the results for experiments using a JAM-C antibody and are otherwise similar to *A* and *B*. Six to eight mice per group were investigated. *$p < 0.05$ (*t* test) compared to isotype control antibodies. (*E*) Persistant effects of JAM-B-, JAM-C- or combined JAM-B/-C-blockade: Mice were treated with either isotype (1.25 mg per kg or 2.5 mg per kg), JAM-B (2.5 mg per kg), JAM-C (2.5 mg per kg) or JAM-B/-C (1.25 mg per kg) antibodies. Ear swelling responses were evaluated 24, 48, 72 and 96 h after reexposition with DNFB. Significant ear swelling responses persisted until 72 h past reexposition, whereas at 96 h no significant differences were observed (not shown). Treatment of C57Bl/6 mice with 2.5 mg per kg JAM-B or -C antibody, as well as using a combination of JAM-B and -C (each at 1.25 mg per kg) lead to an inhibition of ear swelling lasting 48 h after reexposition. At 72 h, no significant effect of either antibody treatment is observed, which is most likely due to the relatively small, jet significant, difference in ear swelling in the isotype antibody treated mice. Data from five mice per experimental condition. *$p < 0.05$ (one-way ANOVA, Bonferroni procedure) against appropriate isotype control antibodies treated animals.

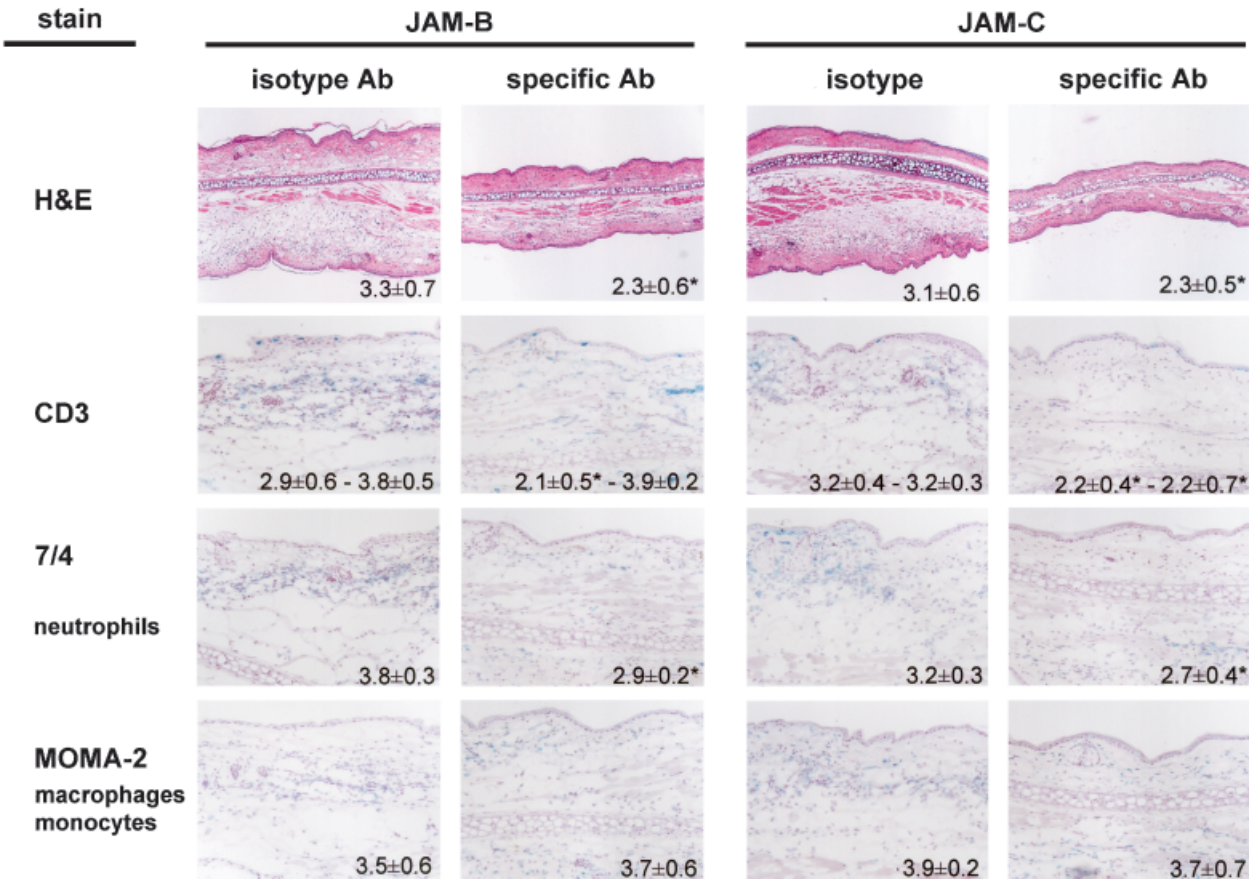
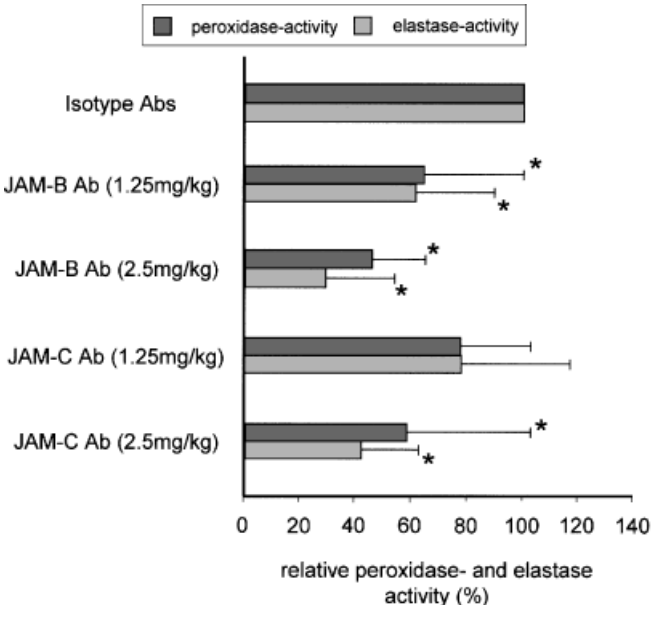


Figure 3
Leukocyte infiltration in 2,4-dinitro-1-fluorobenzene (DNFB)-induced contact hypersensitivity (CHS) is reduced in mice treated with either junction adhesion molecule (JAM)-B or -C antibodies. Mice were sensitized with DNFB and treated with of JAM-B and/or -C function blocking antibodies (2.5 mg per kg) just before challenge. 24-h after reexposition specimen from ears were stained with hematoxylin and eosin (H&E) and immunohistochemisty was performed (CD3, clone 7/4 (neutrophils) and MOMA-2 (macrophages/monocytes). To evaluate infiltration/staining intensity slides were analyzed by two pathologists unaware of the applied treatment protocol and assigned a score ranging from one to four, corresponding to no, mild, moderate or severe infiltration/staining, respectively. Numbers in lower right indicates the average score (mean ± standard deviation) of all animals per group ($n = 6$). $^*p < 0.05$ (*t* test) compared to the corresponding isotype antibody treated group.



tion (Fig 1), JAM-B and -C may contribute to constitutive leukocyte extravasation, required for maintenance of immunosurveillance (Dailey, 1998) and may also show activation-depended changes in phosphorylation or localization, as described for JAM-A (Ozaki *et al*, 1999, 2000).

Hence, we treated sensitized mice with JAM-B or -C antibodies before challenge with DNFB. Both antibodies inhibited the CHS response in a dose depended manner. Side by side titration of antibody effects showed, that both antibodies had a similar influence on ear swelling. Indicating, that antibody effects can be compared despite their

Figure 4
Reduction of contact hypersensitivity (CHS) in mice treated with junctional adhesion molecule (JAM)-B or -C antibodies is accompanied with reduced neutrophil infiltration. Mice were sensitized with 2,4-dinitro-1-fluorobenzene and treated with different doses of JAM-B and/or -C function blocking antibodies just before challenge ($n = 6$/group). Peroxidase and elastase activity, corresponding to total and neutrophil granulocyte infiltration, respectively, were analyzed in normal and challenged ears. The difference of peroxidase and elastase activity (normal *vs* inflamed ear) is expressed in relation to the difference in mice treated with the appropriate control antibodies (Ab) (normal *vs* inflamed ear). No difference was detected in untreated and isotype Ab-treated specimen (not shown). $^*p < 0.05$ (ANOVA on ranks, Dunn's method).

Table II. JAM-B and -C synergistically mediate ear swelling in a CHS response

	Ear swelling
Isotype (JAM-B and -C, both at 2.5 mg per kg)	
Anti-JAM-B + anti JAM-C (1.25 mg per kg)	*
Anti-JAM-B + anti JAM-C (2.5 mg per kg)	*
Δ (cm $\times$ 10^{-3})	0 5 10 15 20 25 30

Mice were sensitized with DNFB and treated with indicted doses of JAM-B and -C inhibitory antibodies 60 min before re-exposition ($n = 6$ per group). Twenty-four hours after re-exposition, ear swelling was evaluated. *$p < 0.05$ ANOVA on ranks (Dunn's method). No difference was observed between untreated and isotype treated mice, as well as animals treated with 1.25 mg per kg and 2.5 mg per kg (ANOVA on ranks).

JAM, junctional adhesion molecule; CHS, contact hypersensitivity; DNFB, 2,4-dinitro-1-fluorobenzene.

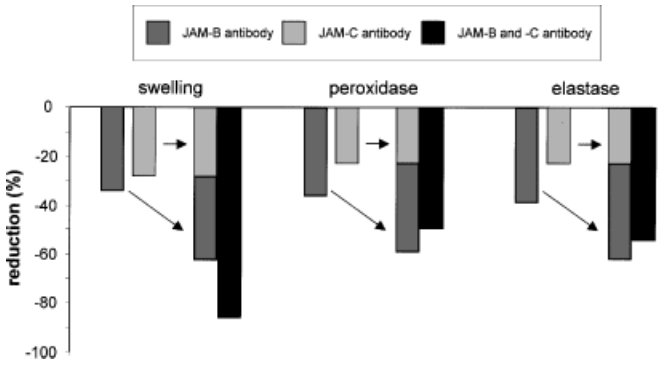


Figure 6
Combined treatment with junctional adhesion molecule (JAM)-B and -C antibodies leads to a additive inhibition of ear swelling and additive inhibition of neutrophil infiltration in a contact hypersensitivity (CHS) response. Treatment of mice with either JAM-B or -C antibodies at 1.25 mg per kg 60 min prior to allergen challenge had no significant effect on ear swelling. If both antibodies were, however, injected simultaneously, a significant reduction in ear swelling was observed. This inhibitory effect was additive. Analysis was performed in six to eight mice per group.

different nature (monoclonal vs. polyclonal). This in mind, JAM-B- and -C-antibodies seem to have differential effects on the CHS: Although 1.25 mg per kg b.w. anti-JAM-B or anti-JAM-C antibody failed to significantly reduce ear swelling, the same dose of anti JAM-B antibody lead to a significant decrease in neutrophil infiltration (peroxidase and elastase activity), which was not the case for anti-JAM-C treatment (Fig 4). Thus, JAM-B may predominately govern neutrophil transmigration, whereas JAM-C may govern edema formation. The idea, that JAM-B and -C have overlapping, but distinct functions in regulating leukocyte transmigration is supported by the results of combined

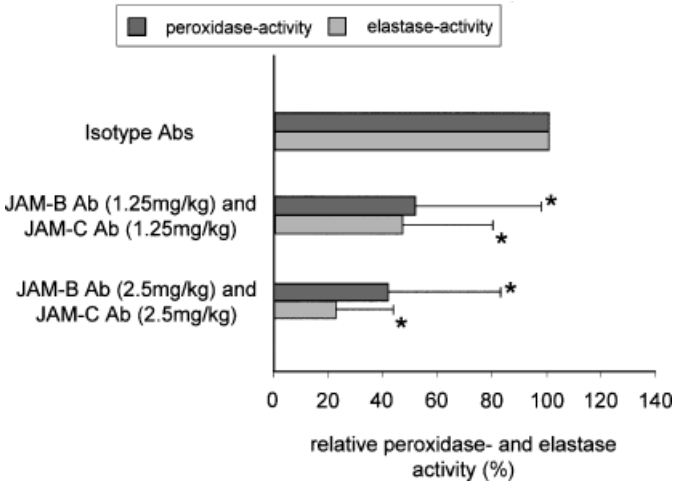


Figure 5
Reduction of contact hypersensitivity (CHS) in mice treated with junctional adhesion molecule (JAM)-B and -C antibodies is accompanied with reduced leukocyte infiltration. Mice were sensitized with 2,4-dinitro-1-fluorobenzene and treated with different doses of JAM-B and -C function blocking antibodies just before challenge ($n = 6$/group). Peroxidase and elastase activity, corresponding to total and neutrophil granulocyte infiltration, respectively, were analyzed in normal and challenged ears. The difference of peroxidase and elastase activity (normal *vs* inflamed ear) is expressed in relation to the difference in mice treated with the appropriate control antibodies (normal *vs* inflamed ear). *$p < 0.05$ (ANOVA on Ranks, Dunn's method).

treatment with JAM-B- and -C antibodies: Injection of 1.25 mg per kg b.w. anti JAM-B and -C prior to challenge with DNFB led to an additive inhibition of the CHS response.

In our study, ear swelling and leukocyte infiltration were used as endpoints. Hence, no conclusions can be drawn on where and how JAM-B and -C are involved in leukocyte extravasation. JAM-B and -C may have an impact on leukocyte rolling, firm adhesion and transmigration, as JAM-B interacts with the $\alpha 4\beta 1$ integrin (Cunningham *et al*, 2002), which is involved in leukocyte rolling and recruitment to the skin (Butcher and Picker, 1996). Indeed, we have gathered preliminary data, showing, that JAM-B mediates rolling and firm adhesion of leukocytes on endothelial cells in a flow chamber assay (Ludwig *et al*, in preparation).[1] In addition to its role in transmigration JAM-C also binds the leukocyte integrin Mac-1 (Santoso *et al*, 2002), which is involved in mediating firm adhesion of leukocytes in the skin compartment (Schön *et al*, 2003). Recent work (Aurrand-Lions *et al*, 2005) has elegantly demonstrated, that JAM-C besides mediating transmigration is also involved in firm adhesion of leucocytes).

Still, further studies are required to investigate the molecular mechanisms of JAM-B and -C mediated leukocyte homing to the skin. In summary, our data illustrates the importance of JAM-B and -C in the development of cutaneous inflammation. This makes them interesting targets for the development of novel anti-inflammatory agents.

Materials and Methods

Induction of a murine allergic contact dermatitis reaction Male C57BL/6 mice, aged 6–10 wk were purchased from Charles River (Sulzfeld, Germany) and housed with food and water *ad lib*. Animal experiments have been approved by the governmental administration of Hessen (Darmstadt, Germany). An allergic contact dermatitis (CHS) was elicited using DNFB according to standard

[1]Ludwig RJ, Hardt K, Hatling M, *et al*; Lymphocyte rolling and firm adhesion mediated by an interaction of JAM-B and VLA-A. In preparation.

protocols (Schwarz *et al*, 2000). In brief, mice were sensitized by applying 75 μL of 0.5% DNFB (Sigma, Seelze, Germany) solution (4 vol acetone/1 vol olive oil) on the back on day 0. On day 5, 20 μL of 0.25% DNFB or vehicle was applied on the left and right ear, respectively. 24 h after challenge, CHS response was 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 sensitized animals and were expressed as centimeters $\times 10^{-3}$ (mean $\pm$ SD).

Quantification of JAM-B and -C mRNA Total RNA from skin samples was prepared using Strata Prep Total mini RNA Kit (Stratagene, La Jolla, California) according to the manufacturer's instruction. RNA concentration and integrity was controlled using Agilent 2100 Caliper LabChip BioAnalyzer (Agilent Technologies, Palo Alto, California). Synthesis of cDNA from total RNA samples was performed with First Strand cDNA synthesis kit (Amersham Pharmacia, Freiburg, Germany) according to the recommended protocol. Primers and probes used were purchased from Applied Biosystems, Darmstadt, Germany. Murine JAM-B (NM_023844) and -C (NM_023277) are available as "Assays on demand", assay numbers Mm00470197_mL and Mm00499214_mL, respectively. Sequences are not disclosed by the provider. An ABI prism 5700 detector sequence was programmed for the initial sequence of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15s at 95°C and 1 min at 60°C. Each measurement was set up in triplicates. For each time point indicated (Table I), specimen from three mice were pooled. Gene expression was calculated compared to hypoxanthine-guanine phosphoribosyl transferase (HPRT) expression. Specific gene regulation was calculated by comparing allergen-challenged skin with vehicle-treated skin at the respective time points.

Evaluation of JAM-B and -C expression To measure JAM-B and -C expression on protein level, cryostat sections from inflamed (CHS) and normal mouse skin were assessed for their JAM-B and -C expression ($n = 3$ for each condition). Specimens were stained with JAM-B or -C antibodies (goat anti-mouse VE-JAM polyclonal Ab, goat anti-mouse JAM-3 polyclonal Ab; R&D Systems, Wiesbaden, Germany). Alexa Fluor 488 labeled rabbit anti-goat IgG antibodies were used to visualize the JAM (Molecular Probes, Karlsruhe, Germany). In order to quantify JAM expression, sections were simultaneously stained for CD31 using a PE-labeled rat anti-mouse CD31 (clone 390, Acris, Hiddenhausen, Germany). Specimen stained with appropriate isotype control antibodies served as negative controls. The percentage of JAM-B and -C expression on vessels sections was evaluated independently by two histologists.

Treatment of mice with JAM-B and-C antibodies Mice sensitized with DNFB were i.v. treated with the following antibodies 60 min prior to challenge: Monoclonal rat anti-mouse JAM-2 (VE-JAM, JAM-B) antibody (clone 150015), polyclonal goat anti-mouse JAM-3 (JAM-C), rat IgG_1 isotype control (clone 43414.11) and purified goat IgG. All antibodies were purchased from R&D systems with the exception of goat IgG (Sigma). Antibodies were diluted in phosphate-buffered saline and mice received either 5.0, 2.5 or 1.25 mg per kg bodyweight of antibody in a volume of 100 μL. 24 h after challenge, CHS response was determined in a blinded fashion as ear swelling responses of DNFB-challenged ears compared to the response of the vehicle-treated ear in sensitized animals and was expressed as centimeters $\times 10^{-3}$ (mean $\pm$ SD). To evaluate the long-term impact of antibody treatment, groups of mice were treated with isotype, JAM-B, -C or both antibodies and ear swelling responses were monitored at 24, 48, 72 and 96 h after re-exposition. Ear swelling responses were evaluated in at least six mice per group.

Differential blood counts To investigate if antibody treatment lead to changes in the differential blood count, mice were treated with either one of the antibodies used in this study at 2.5 mg per kg bodyweight. 24 h before, 4 and 24 h past i.v. injection of antibodies blood was drawn from the retrobulbar plexus and analyzed on a CellDyn 3500 (Abbott, Wiesbaden, Germany). For each subgroup analysis blood from two mice was pooled, and repeated three times.

Evaluation of leukocyte infiltration Specimen from normal and inflamed mouse skin were H&E stained and infiltration was scored independently by two histologists and assigned a score ranging from one to four, corresponding to no, mild, moderate or sever infiltration, respectively. Sections were also stained for infiltration by CD3, neutrophils and monocytes/macrophages. Six μm thick cryostat sections were incubated with an antibody specific for either CD3 (polyclonal rabbit, DakoCytomation, Denmark), neutrophils (clone: 7/4, Serotec, Oxford, UK), monocytes/macrophages (clone: MOMA-2, Serotec) or isotype control (clone: LO-DNP-11, Serotec). Staining was visualized by addition of appropriate second step antibodies ((a) peroxidase-conjugated goat anti-rat IgG, Dianova, Hamburg, Germany; (b) peroxidase-conjugated goat anti-rabbit, Dianova), using HistoGreen Chromogen (Linaris, Wertheim-Bettingen, Germany). In analogy to the scoring system used for H&E-stained sections, staining was assigned a score ranging from one to four, corresponding to no, weak, moderate or strong staining, respectively. CD3-stained sections were evaluated for epidermal and dermal expression. In addition, peroxidase activity, corresponding to total granulocyte infiltration, was assessed in homogenized ear-specimen according to standard protocols (Schottelius *et al*, 2002; Schäcke *et al*, 2004). Briefly, tetramethylbenzidine (TMB) dihydrochloride was used as a sensitive chromogen substrate for peroxidase. To convert TMB into TMB dihydrochloride, 34 μL of 3.7% hydrochloric acid (equimolar) was added to 5 mg of TMB. Then, 1 mL of dimethyl sulfoxide (DMSO) was added. This stock solution was slowly added to sodium acetate–citric acid buffer (0.1 mol per L, pH 6.0) in a ratio of 1:100. Two hundred microliters of this TMB solution, 40 μL of the homogenized sample, and 25 μL of 1 mM H_2O_2 were added to a microtiter plate to start the reaction. The reaction was stopped after 30 min with 45 μL of 1 N H_2SO_4. Changes in OD were monitored at 450 nm at 25°C against the mixture of all solutions without the added sample homogenate. Absolute extinction numbers were used to express peroxidase activity. Elastase activity, corresponding to neutrophil infiltration (Schottelius *et al*, 2002), was measured by fluorescence of 7-amino-4-methyl-coumarin (AMC) that is released from the substrate MeO–Succ–Ala–Ala–Pro–Val–AMC (Bachem, Torrance, California). Homogenized samples in HTAB were diluted 1/10 in cetrimide buffer (0.3% cetrimide, 0.1 M Tris, and 1 M NaCl, pH 8.5). The substrate MeO–Succ–Ala–Ala–Pro–Val–AMC (300 mM in DMSO) was diluted 1/100 in cetrimide buffer to a working concentration of 3 mM. In cetrimide buffer, diluted samples were pipetted in multiwell plates, and the reaction was started by addition of the AMC substrate at 37°C. The reaction was stopped after 1 h with ice-cold 100 mM Na_2CO_3, and samples were measured in a Spectra Max Gemine (Molecular Devices, Menlo Park, California) at 380 nm and compared against a standard curve with the AMC standard 7-amino-4-methylcoumarin (5 mM in ethanol). For each parameter investigated, at least six animals per group were investigated.

Statistical analysis Was performed using SigmaStat (SPSS, Chicago, Illinois). The methods used for evaluation of statistical significance are indicated at figure and table legends. p-Values of < 0.05 were considered as statistically significant.

This work was in part supported by a grant from the Deutsche Forschungsgemeinschaft (Lu877/1-1) to R. J. L.

DOI: 10.1111/j.0022-202X.2005.23912.x

Manuscript received January 7, 2005; revised June 10, 2005; accepted for publication June 24, 2005

Address correspondence to: Ralf J. Ludwig, Department of Dermatology, J.W. Goethe-University, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany. Email: r.ludwig@em.uni-frankfurt.de

References

Aurrand-Lions M, Johnson-Leger C, Wong C, Du PL, Imhof BA: Heterogeneity of endothelial junctions is reflected by differential expression and specific subcellular localization of the three JAM family members. Blood 98: 3699–3707, 2001

Aurrand-Lions M, Lamagna C, Dangerfield JP, Wang S, Herrera P, Nourshargh S, Imhof BA: Junctional adhesion molecule-C regulates the early influx of leukocytes into tissues during inflammation. J Immunol 174:6406–6415, 2005

Butcher EC, Picker LJ: Lymphocyte homing and homeostasis. Science 272: 60–66, 1996

Chavakis T, Keiper T, Matz-Westphal R, *et al*: The junctional adhesion molecule-C promotes neutrophil transendothelial migration *in vitro* and *in vivo*. J Biol Chem 279:55602–55608, 2004

Cunningham SA, Rodriguez JM, Arrate MP, Tran TM, Brock TA: JAM2 interacts with alpha4beta1. Facilitation by JAM3. J Biol Chem 277:27589–27592, 2002

Dailey MO: Expression of T lymphocyte adhesion molecules: Regulation during antigen-induced T cell activation and differentiation. Crit Rev Immunol 18:153–184, 1998

Dejana E, Corada M, Lampugnani MG: Endothelial cell-to-cell junctions. FASEB J 9:910–918, 1995

Del Maschio A, De Luigi A, Martin-Padura I, *et al*: Leukocyte recruitment in the cerebrospinal fluid of mice with experimental meningitis is inhibited by an antibody to junctional adhesion molecule (JAM). J Exp Med 190: 1351–1356, 1999

Gothsch U, Borges E, Bosse R, *et al*: VE-cadherin antibody accelerates neutrophil recruitment *in vivo*. J Cell Sci 110 (Pt 5):583–588, 1997

Johnson-Leger CA, Aurrand-Lions M, Beltraminelli N, Fasel N, Imhof BA: Junctional adhesion molecule-2 (JAM-2) promotes lymphocyte transendothelial migration. Blood 100:2479–2486, 2002

Kishimoto TK, Larson RS, Corbi AL, Dustin ML, Staunton DE, Springer TA: The leukocyte integrins. Adv Immunol 46:149–182, 1989

Lechner F, Sahrbacher U, Suter T, *et al*: Antibodies to the junctional adhesion molecule cause disruption of endothelial cells and do not prevent leukocyte influx into the meninges after viral or bacterial infection. J Infect Dis 182:978–982, 2000

Lollo BA, Chan KWH, Hanson EM, Moy VT, Brian AA.: Direct evidence for two affinity states for LFA-1 on activated T-cells. J Biol Chem 268: 21693–21700, 1993

Martin-Padura I, Lostaglio S, Schneemann M, *et al*: Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 142:117–127, 1998

Müller WA: Leukocyte–endothelial–cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol 24:327–334, 2003

Ozaki H, Ishii K, Arai H, *et al*: Junctional adhesion molecule (JAM) is phosphorylated by protein kinase C upon platelet activation. Biochem Biophys Res Commun 276:873–878, 2000

Ozaki H, Ishii K, Horiuchi H, *et al*: Cutting edge: Combined treatment of TNF-alpha and IFN-gamma causes redistribution of junctional adhesion molecule in human endothelial cells. J Immunol 163:553–557, 1999

Santoso S, Sachs UJ, Kroll H, *et al*: The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. J Exp Med 196:679–691, 2002

Schäcke H, Schottelius A, Docke WD, *et al*: Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. Proc Natl Acad Sci USA 101:227–232, 2004

Schön MP, Ludwig RJ: Lymphocyte trafficing into inflamed skin–molecular mechanisms and implications for therapeutic target molecules. Expert Opin Ther Targets 9:225–243, 2005

Schön MP, Zollner TM, Boehncke WH: The molecular basis of lymphocyte recruitment to the skin: Clues for pathogenesis and selective therapies of inflammatory disorders. J Invest Dermatol 121:951–962, 2003

Schottelius AJ, Giesen C, Asadullah K, *et al*: An aspirin-triggered lipoxin A4 stable analog displays a unique topical anti-inflammatory profile. J Immunol 169:7063–7070, 2002

Schwarz A, Beissert S, Grosse-Heitmeyer K, *et al*: Evidence for functional relevance of CTLA-4 in ultraviolet-radiation-induced tolerance. J Immunol 165:1824–1831, 2000

von Andrian UH, Mackay CR: T-cell function and migration. Two sides of the same coin. N Engl J Med 343:1020–1034, 2000

Weber C: Novel mechanistic concepts for the control of leukocyte transmigration: Specialization of integrins, chemokines, and junctional molecules. J Mol Med 81:4–19, 2003

Zen K, Babbin BA, Liu Y, Whelan JB, Nusrat A, Parkos CA: JAM-C is a Component of Desmosomes and a Ligand for CD11b/CD18—mediated Neutrophil Transepithelial Migration. Mol Biol Cell 15:3926–3937, 2004