Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Junctional adhesion molecule (JAM)-B supports lymphocyte rolling and adhesion through interaction with $\alpha 4\beta 1$ integrin

Ralf J. Ludwig,[1,2] Katja Hardt,[1] Max Hatting,[1] Roxana Bistrian,[3] Sandra Diehl,[1] Heinfried H. Radeke,[2] Maurizio Podda,[1] Michael P. Schön,[4] Roland Kaufmann,[1] Reinhard Henschler,[3] Josef M. Pfeilschifter,[2] Sentot Santoso[5] and Wolf-Henning Boehncke[1]

[1]*Department of Dermatology, Clinic of the J.W. Goethe University, Frankfurt am Main,* [2]*Pharmazentrum Frankfurt/ZAFES, Clinic of the J.W. Goethe University, Frankfurt am Main,* [3]*Institute for Transfusion Medicine and Immune Hematology, German Red Cross Blood Donor Service, Johann Wolfgang Goethe-University, Frankfurt am Main,* [4]*Department of Dermatology and Venereology, Georg August University, Göttingen, and* [5]*Institute for Clinical Immunology and Transfusion Medicine, Justus-Liebig-University, Gießen, Germany*

doi:10.1111/j.1365-2567.2009.03100.x
Received 25 November 2008; revised 27 February 2009; accepted 10 March 2009.
Correspondence: R. J. Ludwig, MD, Department of Dermatology, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany.
Email: ralf.ludwig@uk-sh.de
Senior author: Wolf-Henning Boehncke, Email: boehncke@em.uni-frankfurt.de

Summary

Junctional adhesion molecule-A (JAM-A), JAM-B and JAM-C have been implicated in leucocyte transmigration. As JAM-B binds to very late activation antigen (VLA)-4, a leucocyte integrin that contributes to rolling and firm adhesion of lymphocytes to endothelial cells through binding to vascular cell adhesion molecule (VCAM)-1, we hypothesized that JAM-B is also involved in leucocyte rolling and firm adhesion. To test this hypothesis, intravital microscopy of murine skin microvasculature was performed. Rolling interactions of murine leucocytes were significantly affected by blockade of JAM-B [which reduced rolling interactions from 9·1 ± 2·6% to 3·2 ± 1·2% (mean ± standard deviation)]. To identify putative ligands, T lymphocytes were perfused over JAM-B-coated slides in a dynamic flow chamber system. JAM-B-dependent rolling and sticking interactions were observed at low shear stress [0·3 dyn/cm^2: 220 ± 71 (mean ± standard deviation) versus 165 ± 88 rolling ($P < 0·001$; Mann–Whitney rank sum test) and 2·6 ± 1·3 versus 1·0 ± 0·7 sticking cells/mm^2/min ($P = 0·026$; Mann–Whitney rank sum test) on JAM-B- compared with baseline], but not at higher shear forces (1·0 dyn/cm^2). As demonstrated by antibody blocking experiments, JAM-B-mediated rolling and sticking of T lymphocytes was dependent on $\alpha 4$ and $\beta 1$ integrin, but not JAM-C expression. To investigate whether JAM-B-mediated leucocyte–endothelium interactions are involved in a disease-relevant *in vivo* model, adoptive transfer experiments in 2,4,-dinitrofluorobenzene (DNFB)-induced contact hypersensitivity reactions were performed in mice in the absence or in the presence of a function-blocking JAM-B antibody. In this model, JAM-B blockade during the sensitization phase impaired the generation of the immune response to DNFB, which was assessed as the increase in ear swelling in untreated, DNFB-challenged mice, by close to 40% [$P = 0·037$; analysis of variance (ANOVA)]. Overall, JAM-B appears to contribute to leucocyte extravasation by facilitating not only transmigration but also rolling and adhesion.

Keywords: adhesion molecules; inflammation; skin

Introduction

In order to reach inflamed tissues, leucocytes must leave the bloodstream. This process of leucocyte extravasation requires several sequential steps.[1–3] The first step, tethering and rolling, is mediated by short-lived interactions of selectins with carbohydrate ligands displayed on glycoprotein scaffolds. This allows close interactions of cytokines and chemokines with their receptors, eventually leading to activation of both leucocytes[4–6] and endothelial cells.[7–9] The next step, firm adhesion of leucocytes to the endothelium, is primarily mediated by leucocyte integrins and endothelial adhesion molecules, mainly of the immunoglobulin superfamily. Finally, adhering leucocytes leave the circulation by passing between the lateral margins of endothelial cells or, possibly, even through endothelial

cells.[10,11] Several molecules expressed at the lateral borders of endothelial cells have been implicated in this last, crucial step of extravasation. Among these molecules are the three members of the junctional adhesion molecule (JAM) family – namely JAM-A, JAM-B and JAM-C, all of which have been demonstrated to be involved in leucocyte transmigration.[12]

In addition to homophilic interactions, several heterophilic ligands have been identified for the JAMs: JAM-A has been shown to bind the leucocyte integrin lymphocyte function-associated antigen (LFA)-1;[13] and we have recently identified Mac-1 as a ligand for JAM-C.[12,14,15] Along with the detection of new ligands, additional functions of JAMs have been identified: JAM-A redistributes to the apical surface upon inflammatory stimuli,[13,16] where it becomes available for LFA-1-mediated leucocyte–endothelial cell interactions. Indeed, an interaction of LFA-1 with JAM-A appears to support firm adhesion of T lymphocytes, but not neutrophils, under flow conditions *in vitro*.[13] Furthermore, JAM-C sustains firm adhesion through an interaction with Mac-1.[17]

Consistent with these findings, recent evidence has indicated that the function of JAM-B in the extravasation cascade is not exclusively restricted to transmigration.[1,18,19] As JAM-B can directly interact with very late activation antigen (VLA)-4, an integrin supporting rolling and sticking interactions in the skin microvasculature,[3] we hypothesized that JAM-B may also mediate those two processes. This hypothesis is supported by the observation that antibody-mediated blockade of JAM-B impairs the development of cutaneous inflammation through inhibition of leucocyte extravasation.[20] However, measurements of ear swelling and infiltration are endpoint measurements, which do not allow conclusions to be drawn regarding the mechanistic basis of the reduced inflammatory response. We have, therefore, analysed the role of JAM-B in T-lymphocyte extravasation cascade *in vitro* and *in vivo*.

Materials and methods

Mice

Male C57BL/6 mice, aged 6–10 weeks, were purchased from Charles River (Sulzfeld, Germany) and housed with food and water *ad libitum*. Animal experiments have been approved by the governmental administration of Hessen (Darmstadt, Germany).

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats using density gradient sedimentation. Negative isolation of highly purified T lymphocytes was performed using a T-cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for magnetic cell separation as described previously.[21] Expression of JAM-C and VLA-4 on isolated cells was determined by incubation with the respective fluorescently labelled antibody [fluorescein isothiocyanate (FITC)-anti-human CD49d, clone 44H6, from Acris Antibodies, Hiddenhausen, Germany, or allophycocyanin (APC)-anti-human JAM-C antibody, clone 208212, from R&D Systems, Minneapolis, MN]. Appropriate isotype antibodies were used as controls (FITC-IgG1 from BD Biosciences, Heidelberg, Germany, and APC-mouse IgG2b from R&D Systems). After washing, expression was determined using a FACSCalibur (BD Biosciences) and CELLQUEST software (BD Biosciences). Binding of the JAM-B-E-selectin and P-selectin-IgG fusion proteins (all from BD Biosciences) to the T lymphocytes was determined by incubating the cells with the appropriate fusion protein for 30 min at a concentration of 1 μg/1 × 10^5 cells. Binding was visualized using phycoerythrin (PE)-anti-human IgG (Dianova, Hamburg, Germany), and evaluated by flow cytometry. Incubation with the secondary antibody without prior incubation with the fusion protein served as a negative control.

Immunofluorescent staining of JAM-B

Human umbilical vein endothelial cells (HUVEC; 20 000 cells/well) were plated onto eight-well chamber slides (Permanox, Nunc, Denmark), which were pre-coated with 0·2% gelatin, and were allowed to adhere overnight at 37°. Thereafter, HUVEC were washed and treated with or without 20 ng/ml tumour necrosis factor (TNF)-α in 2% fetal calf serum (FCS) containing medium for 12 hr at 37°. After extensive washing, the slides were fixed with ice-cold methanol and blocked with 3% [weight/volume (w/v)] bovine serum albumin (BSA). The slides were then incubated with polyclonal goat anti-human JAM-B (5 μg/ml; R&D Systems) for 2 hr at 22°. After washing, secondary FITC-coupled anti-goat IgG (1 : 100; Dianova) was added for 1 hr at 22°. The cells were counterstained with 4′,6-diamidin-2′-phenylindoldihydrochloride after extensive washings and mounted in Vectashield (Vector Laboratories, Burligame, CA). Double-stained images were merged to analyse co-localization.

Intravital microscopy

Intravital microscopy of the cutaneous microvasculature was performed as described previously.[18,22,23] In brief, lymphocytes were obtained by gently homogenizing peripheral lymph nodes from C57Bl/6 mice through a nylon mesh (70 μm; BD Biosciences), and stained using carboxyfluoresceindiacetate-acetoxymethyl ester (CFDA-AM). After washing, stained lymphocytes were injected downstream into the right carotid artery of an

anaesthetized C57Bl/6 mouse. The left ear of the mouse was gently placed on a microscope slide, and vascular architecture and labelled cells were visualized during their passage through vessels under fluorescent epi-illumination using a multiband-filter system (XF 53; Omega Optical, Brattleboro, VT). Continuous digital recordings of the microcirculation were obtained using a 1/3″ DSP 3-CCD camera (DXC-390; Sony, Köln, Germany) mounted on a modified[24] Zeiss microscope (Axiotech Vario 100 HD; Zeiss, Oberkochen, Germany) equipped with a 10× saltwater immersion objective (Nikon, Düsseldorf, Germany). Images were recorded digitally using Ulead MediaStudio Pro 7·0 (Ulead, Kaarst, Germany). For each experiment, lymphocytes were injected along with the isotype control antibody (rat IgG2a, clone 54447; R&D Systems), followed by a second injection of cells with the function-blocking anti-mouse JAM-B antibody (clone 150005; R&D Systems) at the same concentration. Cell behaviour in individual vessel segments was determined by playback of video files. Cells were considered non-interacting when they moved at the velocity of the mean blood flow, whereas cells with detectably lower velocities were defined as rolling.

Experiments investigating interactions of endogenous leucocytes with the vasculature of lymph nodes were carried out with slight modifications: injections of rhodamine 6G and antibodies (the above-mentioned function-blocking anti-JAM-B and the appropriate isotype control antibody, both at 4 mg/kg bodyweight) were performed using a catheter placed downstream in the right carotid artery. The superficial inguinal lymph node was microsurgically dissected and leucocyte interactions with post-capillary venules were analysed after staining with rhodamine 6G using the above-described set-up for intravital microscopy. Again, cells were considered non-interacting when they moved at the velocity of the mean blood flow, whereas cells with detectably lower velocities were defined as rolling.

Flow chamber experiments on immobilized JAM-B

For flow chamber assays, glass slides were coated with the JAM-B-IgG fusion protein (R&D Systems) as described previously.[25–27] In addition, the presence of JAM-B was confirmed using immunofluorescent staining. The flow chamber system and the experimental conditions have been described previously.[28] In brief, JAM-B-IgG fusion protein-coated slides were mounted on a parallel plate flow chamber system (Circular Parallel Plate Flow Chamber Kit; GlycoTech, Gaithersburg, MD). For negative controls, slides were coated with buffer containing BSA, or Fc fragments. No difference was observed between those two controls (not shown). A syringe pump (Perfusor secura FT; B. Braun, Heidelberg, Germany) was connected to the inlet port of the flow chamber system, simulating a uniform laminar flow field at a shear stress of 0·3 or 1·0 dyn/cm^2 by adequately adjusting the pump rate. Interactions of isolated T lymphocytes at a final concentration of 10^6 cells/ml with JAM-B-IgG fusion protein-coated slides were evaluated in the absence or presence of antibodies (mouse IgG1 isotype control from Acris Antibodies; anti-human α4-integrin antibody, clone 2B4, from R&D Systems; anti-β1 integrin antibody, clone Lial/2, from Acris Antibodies; goat IgG from Dianova, Hamburg, Germany; and polyclonal anti-human JAM-C from R&D Systems) at concentrations indicated in the figure legends. Continuous real-time digital video recordings of the chamber were obtained using a CCD camera (Sony, Cologne, Germany), mounted on an inverted-stage microscope (Axiovert 135 from Zeiss) equipped with a 10× objective (Zeiss). By off-line analysis of digital recordings from 30-second time frames of a window sized to correspond to an area of 15 mm^2, cells were considered non-interacting when they moved at the velocity of the flow, whereas cells moving at lower velocities were defined as rolling. If a cell remained stationary for over 10 seconds it was considered as firmly adherent (sticking). The number of rolling and sticking cells was counted per field of view (related to mm^2/second).[29] Rolling velocities were determined from at least 60 cells per experimental condition.

Adoptive transfer of immunity

Adoptive transfer experiments were performed according to previously published protocols.[30] Mice were sensitized by painting 75 μl of 2,4,-dinitrofluorobenzene (DNFB; Sigma, St Louis, MO) solution (0·5% in acetone:olive oil, 4 : 1) on the back of each mouse on day 0 as previously described.[20] Mice were either left untreated, or received an intravenous (i.v.) injection of an isotype control antibody (rat IgG2a, clone 54447; R&D Systems) or a function-blocking anti-mouse JAM-B antibody (clone 150005; R&D Systems) at doses indicated in the figure legends immediately after application of DNFB. Alternatively, JAM-B-deficient mice[31] could have been used as donors. However, at the time of the experiments, those were not available to us. 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 nylon mesh (70 μm; BD Biosciences). Recipient mice were injected with 4×10^7 cells in 200 μl, followed by immediate application of 20 μl of DNFB at 0·3% to the right ears of the mice.

Statistics

All data are expressed as mean ± standard deviation. Evaluation of statistical significance was performed as

indicated in the text. A P-value < 0·05 was considered to be statistically significant.

Results

Human T lymphocytes express VLA-4 and JAM-C, and bind to JAM-B under static conditions

To test our hypothesis that JAM-B mediates rolling and sticking of T lymphocytes, we assessed the expression of JAM-C and VLA-4, which are known leucocyte ligands for JAM-B,[14,19] using flow cytometry. As expected, a high proportion of lymphocytes expressed VLA-4 ($\alpha4\beta1$ integrin). Only a few T lymphocytes stained positive for JAM-C (Fig. 1a). In addition, we repeatedly observed binding of the JAM-B protein to lymphocytes under static conditions, confirming previously published work.[15,19] The observed frequency of JAM-B binding to lymphocytes of 10·0 ± 1·6% was similar to that of binding to the E-selectin fusion protein and lower than that of P-selectin fusion protein binding (Fig. 1b).

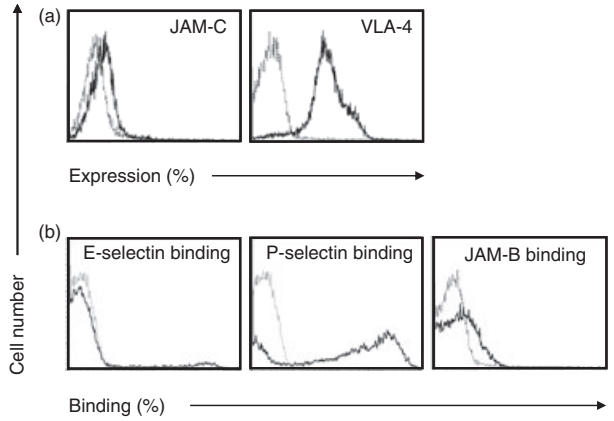


Figure 1. Human CD3$^+$ lymphocytes constitutively express very late activation antigen (VLA)-4 and junctional adhesion molecule (JAM)-C – and bind to soluble JAM-B-Ig fusion protein. Freshly isolated human T lymphocytes were analysed for their expression of VLA-4 and JAM-C, as well as for their capacity to bind to soluble JAM-B fusion protein using flow cytometry. (a) Representative overlays from at least five experiments (expression of VLA-4 and JAM-C) performed per group are shown. The thin line corresponds to staining with isotype control antibodies. Overall, 89·0 ± 2·3% of CD3$^+$ cells expressed VLA-4. JAM-C expression was detected in 2·1 ± 0·7% of CD3$^+$ cells. (b) Representative overlays from at least five fusion protein binding experiments performed per group are shown. The thin line corresponds to staining with omission of recombinant protein. Overall, 10·0 ± 1·6% of cells bound to the JAM-B fusion protein, which is very similar to the frequency of binding to the E-selectin fusion protein (12·4 ± 3.8%), but considerably lower than the frequency of P-selectin fusion protein binding (66·4 ± 11·6%). (c) Co-incubation with JAM-C function-blocking antibody has no influence on JAM-B binding, whereas $\alpha4$ or $\beta1$ function-blocking antibodies, or a combination of $\alpha4$ and $\beta1$ antibodies, significantly reduced JAM-B binding to human T lymphocytes. For each condition, one representative of at least three independent experiments is shown.

Expression of JAM-B on HUVEC

We next investigated the expression of JAM-B on endothelial cells, to determine if JAM-B is at all 'accessible' to lymphocyte VLA-4. Using immunofluorescent staining, we detected little to no JAM-B expression on quiescent HUVEC (Fig. 2). However, given that JAM-B expression was detected regularly around the edges, as well as on the cellular surfaces of TNF-α-stimulated cells (Fig. 2), it is conceivable that endothelial JAM-B is capable of interacting with ligands expressed by flowing/rolling leucocytes – in particular those expressed on the tips of leucocyte microvilli, such as VLA-4.

Rolling interactions *in vivo* are sensitive to treatment with a function-blocking antibody directed against JAM-B

To evaluate whether JAM-B mediated interactions of T lymphocytes with endothelial cells *in vivo*, interactions of murine T lymphocytes in the skin microvasculature were investigated. This particular vascular bed was chosen, because we had previously shown that JAM-B (and JAM-C) are constitutively expressed at high levels in murine skin.[20] When lymphocytes isolated from peripheral lymph nodes of C57Bl/6 mice were fluorescently labelled and injected into healthy syngeneic donor mice, interactions of labelled cells with skin post-capillary venules can easily be observed by epifluorescence microscopy. In the presence of the isotype antibody, 9·1 ± 2·6% of the lymphocytes adopted a rolling motion, which was sensitive to treatment with a function-blocking anti-JAM-B antibody (rolling fraction 3·2 ± 1·2%; $P = 0·046$; paired t-test; Fig. 3; Video Clips S1 and S2). Thus, JAM-B supports rolling interactions *in vivo*.

T lymphocytes roll on and firmly adhere to immobilized JAM-B under flow conditions

To gain insights into the molecular basis of JAM-B-mediated lymphocyte rolling, glass slides were coated with JAM-B protein. Successful and homogeneous coating of the slides was confirmed by immunofluorescence (Fig. 4a). Interactions of lymphocytes with immobilized JAM-B protein were then evaluated by applying different shear forces. At a calculated shear stress of 1·0 dyn/cm^2 no rolling or sticking motions were observed (not shown). In contrast, when a lower shear (0·3 dyn/cm^2) was applied, rolling and sticking interactions of human T lymphocytes were readily observed in JAM-B-coated slides: 220 ± 71 cells/mm^2/second on JAM-B-coated slides compared with 165 ± 88 cells/mm^2/

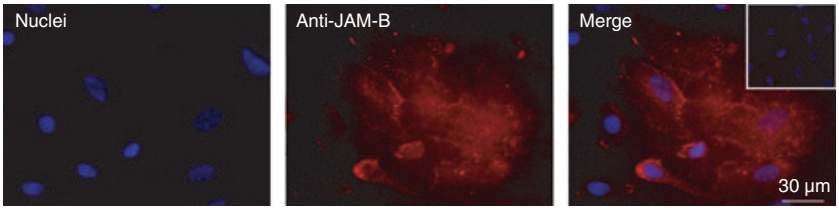


Figure 2. Expression of junctional adhesion molecule (JAM)-B on endothelial cells. Human umbilical vein endothelial cells (HUVEC) cultured until confluence were analysed for their expression of JAM-B using immunofluorescent staining. While quiescent cells did not express JAM-B (insert on the right), cells stimulated with tumour necrosis factor (TNF)-α stained positive for JAM-B protein expression (red). JAM-B expression was observed on the entire surface, and was not restricted to the borders of the endothelial cells. This indicates that JAM-B is also located on the luminal side of endothelial cells, and is thus able to contact ligands displayed by flowing leucocytes. Nuclei were stained using 4′,6-diamidin-2′-phenylindoldihydrochloride (blue). Representative staining from three independent experiments is shown.

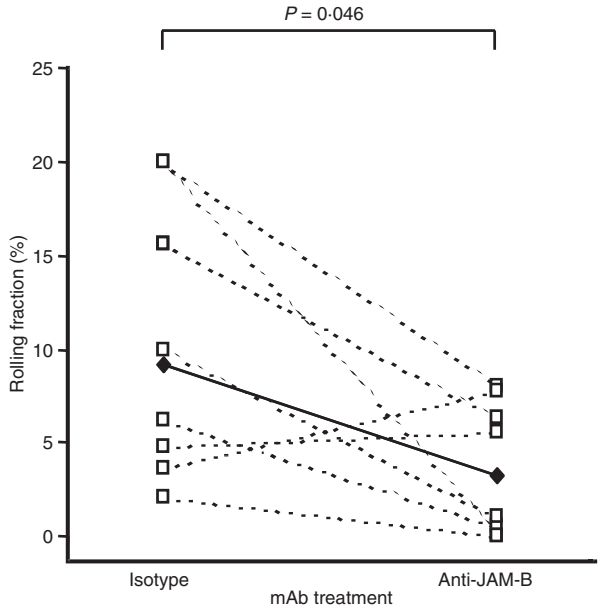


Figure 3. Murine junctional adhesion molecule (JAM)-B mediates T-lymphocyte rolling *in vivo*. Freshly isolated murine T lymphocytes were fluorescently labelled using carboxyfluoresceindiacetate-acetoxymethyl ester (CFDA-AM) and visualized during their passage in murine skin post-capillary venules. Rolling interactions of T lymphocytes with endothelial cells in the presence of either isotype monoclonal antibody (mAb) or function-blocking anti-JAM-B antibody are shown. Square data points connected by dotted lines represent the rolling fraction in the same vessel after injection of isotype mAb (left) or anti-JAM-B mAb (right). Diamond-shaped data points connected by the bold line correspond to the mean rolling observed in all experiments obtained from investigation of nine vessels from three mice. Statistical significance was calculated using the paired *t*-test.

second on control slides ($P < 0{\cdot}001$; Mann–Whitney rank sum test; Fig. 4b and Video Clips S3 and S4). The increase in sticking was even more pronounced: $2{\cdot}6 \pm 1{\cdot}3$ sticking cells/mm^2/second on JAM-B versus $1{\cdot}0 \pm 0{\cdot}7$ cells/mm^2/second on BSA ($P = 0{\cdot}026$; Mann–Whitney rank sum test; Fig. 4c and Video Clips S3 and S4). As the observed increase in sticking was greater than the increase in rolling interactions, JAM-B-mediated sticking was not merely attributable to the higher number of rolling cells. Data were derived from at least seven independently performed experiments.

Rolling and adhesion of T lymphocytes to JAM-B depend on VLA-4, but not JAM-C

To assess which T-lymphocyte adhesion molecules were involved in JAM-B-mediated rolling and sticking interactions, flow chamber experiments were performed in the presence of function-blocking antibodies directed against the α4 and/or β1 integrins, or JAM-C. While anti-JAM-C antibodies had no effect on JAM-B-mediated rolling and sticking interactions, blockade of α4 and/or β1 integrin significantly reduced lymphocyte rolling and sticking on JAM-B protein to baseline. In comparison to sticking, JAM-B-supported rolling was more sensitive to α4-/β1 blockade: antibody concentrations of 1 μg/ml significantly impaired rolling, and combination of the two antibodies did not lead to a further decrease in rolling (Fig. 4b; Video Clips S3–S5). When the rolling velocity of JAM-B-mediated T-lymphocyte rolling was analysed, an average rolling velocity of 292 ± 79 μm/second was detected. This value was significantly increased by inhibition of either α4 or β1 integrin, or a combination of the two antibodies [$369 \pm 47{\cdot}8$, 526 ± 87 or 484 ± 76 μm/second, respectively; $P < 0{\cdot}05$ for all antibody-treated groups versus control; analysis of variance (ANOVA)]. In contrast to rolling, both antibodies (at 1 μg/ml) failed to exert a significant effect on JAM-B-mediated sticking interactions; only the combination of the two antibodies yielded a significant inhibitory effect (Fig. 4c; Video Clips S3–S5), indicating that sticking on JAM-B also depends on α4β1 integrin.

In vivo blockade of JAM-B impairs generation of the immune response to DNFB

An important role for JAM-B in contact hypersensitivity reactions has been shown by us previously[20] – in the

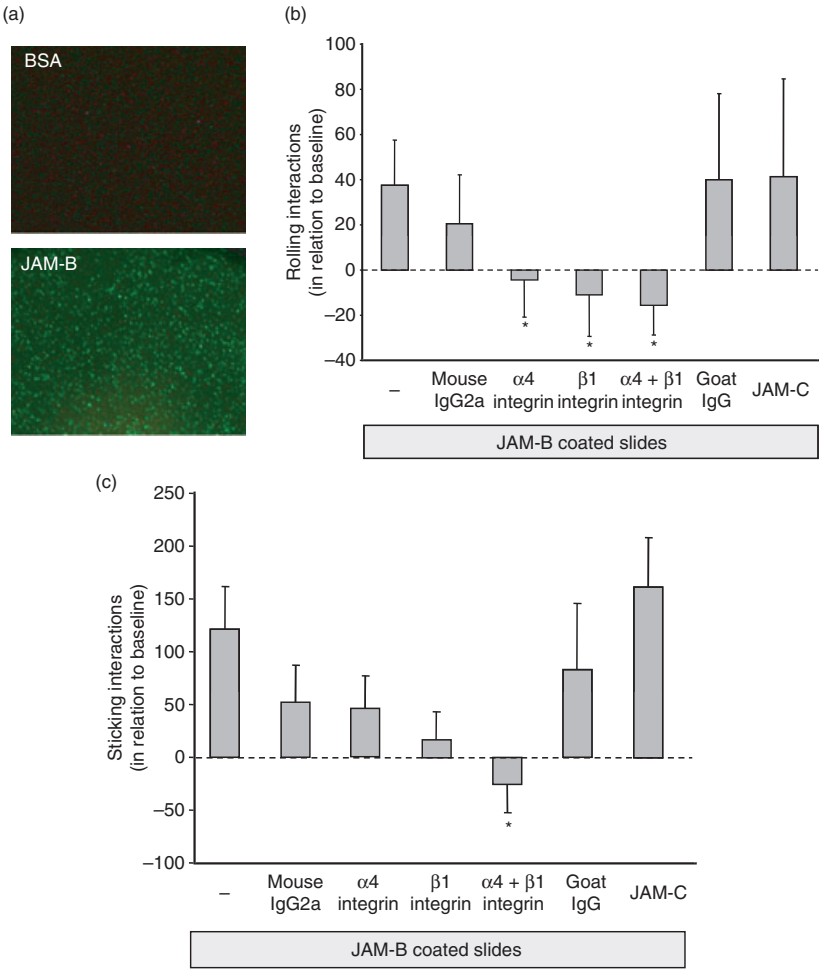
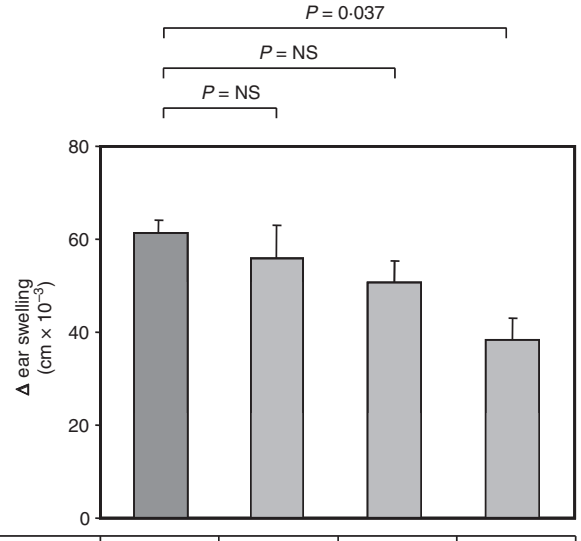


Figure 4. Human junctional adhesion molecule (JAM)-B supports T-lymphocyte rolling and sticking under flow. (a) Representative immunofluorescent detection of JAM-B coating on glass slides incubated with JAM-B-IgG fusion protein. (b) Rolling interactions in comparison to baseline [bovine serum albumin (BSA)-coated slides] in the absence or presence of indicated monoclonal antibodies at 0·3 dyn/cm^2 [all antibodies with the exception of goat IgG and anti-human JAM-C (2 μg/ml) at 1 μg/ml]. Blockade with either α4 or β1 antibodies, or a combination of the two, led to a significant inhibition of rolling along JAM-B-coated slides. This effect was not observed when function-blocking anti-JAM-C antibodies were present. Data are from at least seven independent experiments. Differences in the treatment groups were assessed using one-way analysis of variance (ANOVA) (Bonferroni procedure for multiple comparisons). (c) Using the same experimental set-up described in the legend to Fig. 4, sticking of T cells was sensitive to blockade of α4β1 integrin. However, the doses required to achieve this effect were higher than those required for inhibition of rolling. All antibodies were used at 1 μg/ml with the exception of mouse IgG2a, goat IgG and anti-human JAM-C antibody, which were used at 2 μg/ml. Data are from at least seven independent experiments. Differences in the treatment groups were assessed using one-way ANOVA (Bonferroni procedure for multiple comparisons).

current work, a contribution of JAM-B to T-lymphocyte extravasation has been shown (Figs 1, 3 and 4.) T-lymphocyte extravasation is known to be required for the effector phase of the contact hypersensitivity reaction – now it shall be addressed whether JAM-B also co-operates critically in the sensitization phase of the contact hypersensitivity reaction inflammation model. For this purpose, C57Bl/6 mice were sensitized using the hapten DNFB. Mice were simultaneously treated with solvent, isotype-matched control antibodies or anti-JAM-B antibodies at different doses. After completion of sensitization, lymphocytes from peripheral lymph nodes were adoptively transferred into syngeneic recipient mice, which were simultaneously challenged with DNFB. In recipient mice, we observed a moderate, but significant, reduction of ear swelling responses in mice treated with the JAM-B antibodies. The changes in ear swelling values expressed as cm $\times 10^{-3}$ were: 61·4 ± 2·7 (untreated), 55·7 ± 7·2 (isotype at 5·0 mg/kg; not significant compared with control), 47·3 ± 5·0 (anti-JAM-B antibody at 2·5 mg/kg; not significant compared with control) and 38·3 ± 4·7 (anti-JAM-B antibody at 5·0 mg/kg; $P = 0{\cdot}037$ compared with control; one-way ANOVA – Bonferroni procedure) (Fig. 5). On the basis of these data, the possibility of an influence of



NaCl	+	–	–	–
Isotype Ab	–	5·0 mg/kg bw	–	–
JAM-B Ab	–	–	2·5 mg/kg bw	5·0 mg/kg bw

Figure 5. Murine junctional adhesion molecule (JAM)-B is involved in the generation of an immune response to the hapten 2,4,-dinitrofluorobenzene (DNFB) in mice. Donor mice were treated with indicated doses of antibodies and simultaneously sensitized using DNFB. Five days later, mice were killed and leucocytes isolated from spleens and peripheral lymph nodes. Leucocytes were transferred into syngenic recipient mice, which were concurrently challenged with DNFB. Twenty-four hours later the inflammatory response was assessed by analysing ear swelling. As shown, only high doses of function-blocking anti-JAM-B monoclonal antibody (mAb) led to a significant reduction in the inflammatory response after adoptive transfer of immunity in recipient mice. Data are from at least five mice per group. Differences in the treatment groups were assessed using one-way analysis of variance (ANOVA) (Bonferroni procedure for multiple comparisons). bw, body weight; NS, not significant.

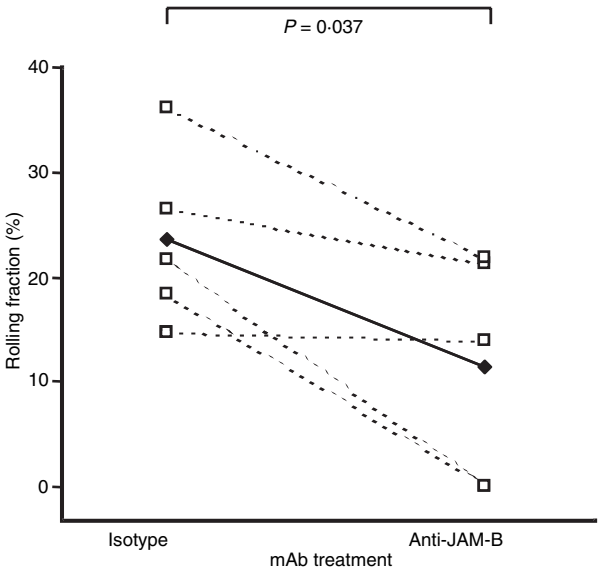


Figure 6. Murine junctional adhesion molecule (JAM)-B is involved in leucocyte rolling in peripheral lymph nodes. Interactions of fluorescently labelled endogenous leucocytes were observed in the microvasculature of inguinal lymph nodes. In comparison to basal rolling interactions, inhibition of JAM-B by a function-blocking antibody led to reduced rolling. Square data points connected by dotted lines represent the rolling fraction in the same vessel after injection of isotype monoclonal antibody (mAb) (left) or anti-JAM-B mAb (right). Diamond-shaped data points connected by the bold line correspond to the mean rolling observed in all experiments obtained from investigation of five vessels from two mice. Statistical significance was calculated using the paired *t*-test.

JAM-B on antigen-presenting cell trafficking and/or antigen presentation cannot be excluded. Yet, the expression pattern of JAM-B strongly suggests that hindrance of adoptive transfer of the immune response to DNFB is probably attributable to inhibition of extravasation of naïve T lymphocytes into lymph nodes. This assumption is further supported by results from intravital microscopy experiments on the inguinal lymph node: observation of interactions of endogenous leucocytes in this vascular bed (Fig. 6 and Video Clips S6 and S7) revealed a reduction of leucocyte rolling from 23·5 ± 8·3% (isotype antibody) to 11·9 ± 10·9% (function-blocking anti-JAM-B antibody; $P = 0{\cdot}037$; paired *t*-test; $n = 5$ vessels from two mice).

Discussion

JAM-B and JAM-C adhesion molecules have been implicated in regulating leucocyte extravasation by affecting different steps in this process through their interaction with endothelial cells.[12] Our data provide experimental evidence that JAM-B is involved in multiple steps in the process of lymphocyte extravasation. They indicate broader functions of JAM-B than could be discerned from previous publications. These findings differentiate the (patho)physiological role of JAM-B from that of most other known adhesion molecules: with the exceptions of $\alpha4\beta1$ integrin/VCAM-1, which are known to mediate both slow rolling and firm adhesion of lymphocytes in the skin,[3] JAM-A/LFA-1, which sustain firm adhesion and transmigration,[13] and Mac-1/JAM-C, which are involved in firm adhesion and transmigration,[14,32] all other adhesion molecules appear to contribute to a distinct step of the extravasation cascade,[2] thus allowing an organ- and leucocyte subset-specific extravasation process. In contrast to these 'specialized' adhesion molecules, JAM-B seems to exert somewhat boarder functions, as it is involved in several steps of the extravasation cascade.

In addition, our observations further support the concept of heterogeneity of endothelial junctions as described by Aurrand-Lions *et al.*[33] These authors demonstrated lack of JAM-B expression on brain endothelial cells, while

high endothelial venules in lymph nodes and Peyer's patches showed high expression of JAM-B, both predominant sites of leucocyte extravasation.[3] In contrast, JAM-A was highly expressed on brain endothelial cells, while absent or only weakly expressed at sites of leucocyte extravasation, such as high endothelial venules in lymph nodes and Peyer's patches.[33] This heterogeneity of endothelial junctions is also reflected in functional studies: treatment with a function-blocking anti-JAM-A antibody blocked monocyte infiltration upon chemokine administration in subcutaneous air pouches.[34] Rather unexpectedly, mice lacking expression of JAM-A showed an increased inflammatory response in a model of delayed type hypersensitivity. This finding was related to increased trafficking of JAM-A-deficient dendritic cells to peripheral lymph nodes.[35] In contrast, inhibition of JAM-B function led to alleviation of a cutaneous response,[20] and displayed a weak effect on the generation of an immune response to DNFB, as shown here. Thus, differential expression patterns (and functions) of members of the JAM family appear to contribute to the spatial compartmentalization and organ specificity of immune responses. The members of the JAM family not only contribute to the heterogeneity of endothelial junctions, but also display a heterogeneity with regard to their capacity to bind haematopoietic cells. This is, on the one hand, reflected in the different ligands of the JAM family members. On the other hand, *in vivo* data suggest that JAM-A binds to neutrophils, not T lymphocytes, in a model of ischemia reperfusion *in vivo*,[36] while JAM-B interacts with T lymphocytes under static and dynamic flow conditions, as demonstrated here.

The data presented here, and elsewhere,[12] suggest that JAM-B impacts T-lymphocyte extravasation at several steps of the leucocyte extravasation cascade: homing of leucocytes is initiated by slowing down the cells in the blood stream, a process termed 'rolling'. Leucocyte rolling strongly depends on an interaction of selectins with their appropriate carbohydrate ligands. A chemokine-mediated interaction of rolling leucocytes and endothelial cells then leads to avidity changes in integrin adhesion molecules expressed by the leucocytes, which then mediate firm adhesion to the endothelial cells.[1–3] The function-blocking JAM-B antibody impaired rolling and sticking of T lymphocytes – and sticking to a greater extent than rolling (Fig. 4). Compared with the appropriate isotype control antibody, rolling was reduced after either $\alpha 4$ or $\beta 1$ antibody inhibition in the flow chamber experiments, whereas adhesion was only affected by combined inhibition of the $\alpha 4$ and $\beta 1$ chains. However, given the high variation and the more pronounced impact of antibody treatment on adhesion, interpretation of the relative contributions of the $\alpha 4$ and $\beta 1$ chains to adhesion and sticking is speculative (Fig. 4). Hence, JAM-B has to be considered an additional molecule allowing T lymphocytes to roll along the vasculature. In addition, as shown elsewhere,[12] JAM-B is also involved in leucocyte transmigration. Hence, the second step of the leucocyte extravasation cascade is the only event that is independent of JAM-B expression.

Our data do not allow us to draw conclusions regarding the relative importance of JAM-B for the extravasation of T lymphocytes in comparison to other adhesion molecules interacting with VLA-4, for example VCAM-1. A dose of 2·5 mg of anti-JAM-B antibody treatment during the elicitation phase reduces the contact hypersensitivity response by over 80%,[20] while twice the amount of antibody was required to observe a much smaller reduction in the contact hypersensitivity response in the adoptive transfer experiments (Fig. 5). Hence, it is tempting to speculate that JAM-B preferentially binds to adhesion molecules expressed by activated rather than naïve T lymphocytes. Whether this phenomenon is a result of differential expression or changes in avidity remains to be investigated. Furthermore, it remains unknown whether JAM-B has an impact on T-lymphocyte priming/activation.[37]

In summary, despite being rather unselective with regard to the process of extravasation itself, JAM-B appears to contribute differentially to organ-specific extravasation of lymphocytes: while antibody-mediated blockade of JAM-B inhibited cutaneous inflammatory responses by over 80%,[20] the same antibody only marginally affected the generation of the immune response to DNFB (Fig. 5). This diverse contribution of JAM-B to extravasation to the skin compared with generation of the immune response to DNFB indicates that interference with JAM-B functions may be a promising target for the development of therapies for the treatment of so far difficult-to-treat and severe chronic inflammatory skin diseases, such as psoriasis.[38] The advantage of such an approach would be rather limited immunosuppressive effects, whereas a potent effect on cutaneous inflammation could be expected.

Acknowledgements

This work was supported by a research grant from the Clinic of the J.W. Goethe University (Patenschaftsmodell 2005) to RJL and HHR, DFG grant Lu877/3-1 to RJL and a grant from the Deutsche Forschungsgemeinschaft to MPS.

Disclosures

No conflict of interest for all the authors.

References

1 Schön M, Ludwig RJ. Leukocyte trafficking to inflamed skin – molecular mechanisms and implications for therapeutic target molecules. *Expert Opin Ther Targets* 2005; **9**:225–43.

2 von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. *N Engl J Med* 2000; **5**:343.

3 Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science* 1996; **272**:60–6.

4 Kinashi T, Aker M, Sokolovsky-Eisenberg M *et al.* LAD-III, a leukocyte adhesion deficiency syndrome associated with defective Rap1 activation and impaired stabilization of integrin bonds. *Blood* 2004; **103**:1033–6.

5 Jakubowski A, Rosa MD, Bixler S, Lobb R, Burkly LC. Vascular cell adhesion molecule (VCAM)-Ig fusion protein defines distinct affinity states of the very late antigen-4 (VLA-4) receptor. *Cell Adhes Commun* 1995; **3**:131–42.

6 Masumoto A, Hemler ME. Mutation of putative divalent cation sites in the alpha 4 subunit of the integrin VLA-4: distinct effects on adhesion to CS1/fibronectin, VCAM-1, and invasin. *J Cell Biol* 1993; **123**:245–53.

7 Furie B, Furie BC, Flaumenhaft R. A journey with platelet P-selectin: the molecular basis of granule secretion, signalling and cell adhesion. *Thromb Haemost* 2001; **86**:214–21.

8 Hidari KI, Weyrich AS, Zimmerman GA, McEver RP. Engagement of P-selectin glycoprotein ligand-1 enhances tyrosine phosphorylation and activates mitogen-activated protein kinases in human neutrophils. *J Biol Chem* 1997; **272**: 28750–6.

9 Weyrich AS, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA. Monocyte tethering by P-selectin regulates monocyte chemotactic protein- 1 and tumor necrosis factor-alpha secretion. Signal integration and NF- kappa B translocation. *J Clin Invest* 1995; **95**:2297–303.

10 Dejana E, Corada M, Lampugnani MG. Endothelial cell-to-cell junctions. *FASEB J* 1995; **9**:910–8.

11 Carman CV, Springer TA. A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J Cell Biol* 2004; **167**:377–88.

12 Muller WA. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol* 2003; **24**:327–34.

13 Ostermann G, Weber KS, Zernecke A, Schroder A, Weber C. JAM-1 is a ligand of the beta (2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat Immunol* 2002; **3**: 151–8.

14 Santoso S, Sachs UJ, Kroll H, Linder M, Ruf A, Preissner KT, Chavakis T. The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. *J Exp Med* 2002; **196**:679–91.

15 Chavakis T, Preissner KT, Santoso S. Leukocyte trans-endothelial migration: JAMs add new pieces to the puzzle. *Thromb Haemost* 2003; **89**:13–7.

16 Ozaki H, Ishii K, Horiuchi H, Arai H, Kawamoto T, Okawa K, Iwamatsu A, Kita K. Cutting edge: combined treatment of TNF-alpha and IFN-gamma causes redistribution of junctional adhesion molecule in human endothelial cells. *J Immunol* 1999; **163**:553–7.

17 Lamagna C, Meda P, Mandicourt G *et al.* Dual interaction of JAM-C with JAM-B and alpha(M)beta2 integrin: function in junctional complexes and leukocyte adhesion. *Mol Biol Cell* 2005; **16**:4992–5003.

18 Radeke HH, Ludwig RJ, Boehncke WH. Experimental approaches to lymphocyte migration in dermatology in vitro and in vivo. *Exp Dermatol* 2005; **14**:641–66.

19 Cunningham SA, Rodriguez JM, Arrate MP, Tran TM, Brock TA. JAM2 interacts with alpha4beta1. Facilitation by JAM3. *J Biol Chem* 2002; **277**:27589–92.

20 Ludwig RJ, Zollner TM, Santoso S *et al.* Junctional Adhesion Molecules (JAM)-B and -C contribute to leukocyte extravasation to the skin and mediate cutaneous inflammation. *J Invest Dermatol* 2005; **125**:969–76.

21 Zollner TM, Podda M, Pien C, Elliott PJ, Kaufmann R, Boehncke WH. Proteasome inhibition reduces superantigen-mediated T cell activation and the severity of psoriasis in a SCID-hu model. *J Clin Invest* 2002; **109**:671–9.

22 Schön MP, Krahn T, Schon M *et al.* Efomycine M, a new specific inhibitor of selectin, impairs leukocyte adhesion and alleviates cutaneous inflammation. *Nat Med* 2002; **8**:366–72.

23 Ludwig RJ, Schultz JE, Boehncke WH *et al.* Activated, but not resting platelets increase leukocyte rolling in murine skin utilizing a distinct set of adhesion molecules. *J Invest Dermatol* 2004; **122**:830–6.

24 Baatz H, Steinbauer M, Harris AG, Krombach F. Kinetics of white blood cell staining by intravascular administration of rhodamine 6G. *Int J Microcirc Clin Exp* 1995; **15**:85–91.

25 Hopfner M, Alban S, Schumacher G, Rothe U, Bendas G. Selectin-blocking semisynthetic sulfated polysaccharides as promising anti-inflammatory agents. *J Pharm Pharmacol* 2003; **55**:697–706.

26 Ludwig RJ, Alban S, Bislian R, Kaufmann R, Diehl S, Henschler R, Gille J. The ability of different forms of heparins to suppress P-selectin function in vitro correlates to their inhibitory capacity on blood-borne metastasis in vivo. *Thromb Haemost* 2006; **95**:535–40.

27 Rüster B, Götting S, Ludwig RJ, Bistrian R, Müller S, Seifried E, Jille J, Henschler R. Mesenchymal stem cells (MSCs) display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 2006; **108**:3938–44.

28 Richter R, Bistrian R, Escher S *et al.* Quantum proteolytic activation of chemokine CCL15 by neutrophil granulocytes modulates mononuclear cell adhesiveness. *J Immunol* 2005; **175**:1599–608.

29 Gill V, Doig C, Knight D, Love E, Kubes P. Targeting adhesion molecules as a potential mechanism of action for intravenous immunoglobulin. *Circulation* 2005; **112**:2031–9.

30 Schwarz A, Beissert S, Grosse-Heitmeyer K, Gunzer M, Bluestone JA, Grabbe S, Schwarz T. Evidence for functional relevance of CTLA-4 in ultraviolet-radiation-induced tolerance. *J Immunol* 2000; **165**:1824–31.

31 Sakaguchi T, Nishimoto M, Miyagi S *et al.* Putative 'stemness' gene jam-B is not required for maintenance of stem cell state in embryonic, neural, or hematopoietic stem cells. *Mol Cell Biol* 2006; **26**:6557–70.

32 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* 2005; **174**:6406–15.

33 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* 2001; **98**:3699–707.

34 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* 1998; **142**:117–27.

35 Cera MR, Del Prete A, Vecchi A *et al.* Increased DC trafficking to lymph nodes and contact hypersensitivity in junctional adhesion molecule-A-deficient mice. *J Clin Invest* 2004; **114**:729–38.

36 Khandoga A, Kessler JS, Meissner H *et al.* Junctional adhesion molecule-A deficiency increases hepatic ischemia-reperfusion injury despite reduction of neutrophil transendothelial migration. *Blood* 2005; **106**:725–33.

37 Grabbe S, Varga G, Beissert S *et al.* Beta2 integrins are required for skin homing of primed T cells but not for priming naive T cells. *J Clin Invest* 2002; **109**:183–92.

38 Schön M, Boehncke WH. Psoriasis – immunopathogenesis, genetics and therapeutic perspectives. *N Engl J Med* 2005; **352**:1899–912.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Video Clip S1. IVM isotype mAb.mpg. Images from an intravital microscopy experiment, carried out as described in detail in Materials and methods. Within the 15 seconds shown, two cells display rolling, whereas 11 cells in the venule architecture can be observed in total. Images were captured with isotype antibody present in the circulation.

Video Clip S2. IVM JAM-B mAb.mpg. Images from the same intravital microscopy experiment as in Video Clip S1. However, after injection of function-blocking anti- junctional adhesion molecule (JAM)-B antibody, 15 cells are visible in the venular tree, none of which shows a rolling motion.

Video Clip S3. Flow chamber – BSA.mpg. Interactions in the described flow chamber system at 0·3 dyn/cm^2 of freshly isolated human CD3$^+$ lymphocytes on glass slides coated with bovine serum albumin (BSA) are shown. Only a few of the cells can be observed to undergo into a rolling or sticking motion.

Video Clip S4. Flow chamber – JAM.mpg. Interactions in the described flow chamber system at 0·3 dyn/cm^2 of freshly isolated human CD3$^+$ lymphocytes on glass slides coated with junctional adhesion molecule (JAM)-B-Ig fusion protein. In contrast to the interactions observed on bovine serum albumin (BSA)-coated slides, rolling and sticking can now be frequently observed.

Video Clip S5. Flow chamber – JAM plus a4 b1 AB.mpg. Interactions in the described flow chamber system at 0·3 dyn/cm^2 of freshly isolated human CD3$^+$ lymphocytes on glass slides coated with junctional adhesion molecule (JAM)-B-Ig fusion protein in the presence of function-blocking α4 and β1 integrin antibodies. Interactions of the CD3$^+$ lymphocytes are significantly reduced in the presence of the indicated antibodies.

Video Clip S6. IVM LN – isotype AB.mpg. Interaction of rhodamine 6G-labelled endogenous leucocytes in the microvasculature of the inguinal lymph node. Within the 10-second clip of an experiment with isotype antibody, most of the cells transit into a rolling motion – especially in the vessel on the right, flowing from bottom to top.

Video Clip S7. IVM LN – anti-JAM-B AB.mpg. Images were obtained from the same vascular bed as detailed in 'IVM LN – isotype AB.mpg', but after injection of a function-blocking anti- junctional adhesion molecule (JAM)-B antibody. Fewer slowly rolling cells are visible, and the total number of cells passing the vasculature is increased.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than about missing material) should be directed to the corresponding author for the article.