Diminished Lymphocyte Adhesion and Alleviation of Allergic Responses by Small-Molecule- or Antibody-Mediated Inhibition of L-Selectin Functions

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Selectins are attractive targets for specific anti-inflammatory therapies. Using human lymphocytes as well as an L-selectin-transfected pre-B-cell line in dynamic flow chamber experiments, we could demonstrate that the small-molecule compound efomycine M blocks L-selectin-mediated lymphocyte rolling on sialylated Lewis^X, an action that was confirmed by plasmon resonance spectroscopy. Recruitment of naive lymphocytes to peripheral lymph nodes depends on L-selectin-mediated adhesion to high endothelial venules. We performed intravital microscopy studying lymphocyte rolling in peripheral lymph nodes and showed a 53% reduction ($P=0.0006$) of lymphocyte rolling in mice treated with efomycine M or a function-blocking antibody against L-selectin. In addition, the number of lymph node-homing T cells was reduced by >60% using either efomycine M or L-selectin-blocking antibodies. As recruitment of naive lymphocytes is a prerequisite for sensitization in T-cell-mediated immune reactions and allergic responses, mice were treated with efomycine M or an L-selectin-specific antibody during contact sensitization with DNFB. After adoptive transfer of corresponding T cells into non-sensitized recipient mice, the capacity of these cells to induce contact hypersensitivity was significantly reduced ($P=0.0002$ and $P=0.0001$, respectively). Our data demonstrate that it is possible, in principle, to diminish T-cell-mediated allergic reactions through interference with L-selectin functions during the early sensitization phase.

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INTRODUCTION

Leukocyte tethering and rolling along the vessel wall is mediated by transient adhesive interactions that are essential for and precede subsequent activation and firm adhesion of leukocytes to endothelial cells. These interactions are primarily mediated by selectins (Stoolman, 1989; von Andrian *et al.*, 1991; Smith *et al.*, 1993; Springer, 1994; Butcher and Picker, 1996), single-chain transmembrane adhesion molecules binding to carbohydrate epitopes on glycoprotein scaffolds (Stoolman, 1989; Varki, 1994; Feizi, 2001; Ley, 2001). Typically, the three members of the selectin family, E-, P-, and L-selectin, interact with glycoproteins bearing the sialylated Lewis^X (sLe^X) moiety or closely related structures, such as sialylated Lewis^a. In addition to sLe^x, P- and L-selectin require negative groups on their ligands such as tyrosine- or glycan-bound sulfate groups, that is, on GlyCAM-1, CD34, or podocalyxin (Lasky *et al.*, 1992; Baumhueter *et al.*, 1993; Mebius *et al.*, 1993). Given the pivotal role of selectins in early events of leukocyte recruitment to peripheral organs, specific suppression of selectin-mediated cellular functions may be of great utility for the treatment of inflammatory disorders such as psoriasis, rheumatoid arthritis, or inflammatory bowel diseases (Ulbrich *et al.*, 2003).

L-selectin (CD62L) is only found on hematopoietic cells and is expressed on most leukocytes, with the exception of a proportion of memory T cells and natural killer cells (Finger *et al.*, 1996; Rainer, 2002). L-selectin plays a predominant role in lymphocyte homing to lymphoid tissues (Stoolman, 1989; Picker and Butcher, 1992). Given that naive and memory T cells homing to lymph nodes express L-selectin, whereas memory T cells that home to sites of inflammation do not express this molecule, there appears to be a functional

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Abbreviations: FC, flow cytometry; PBL, peripheral blood lymphocytes; sLe^X, sialylated Lewis^X; sLe^X/TS-PAA, biotinylated polyacrylamide labeled with sialylated Lewis^x and sulfated tyrosine; SPR, surface plasmon resonance

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preference for L-selectin in lymph node homing. Lymph nodes are involved in antigen presentation to T- and B cells, as a result of which T cells mature into antigen-specific cytotoxic T cells or memory T cells and B cells into antibody-producing plasma cells. Lymphocyte homing to the lymph nodes is, therefore, of utmost importance for induction of antigen-specific immune responses (von Andrian and Mempel, 2003). It is predicted that L-selectin functions are a prerequisite for the sensitization of cells within lymph nodes (Shimada *et al.*, 2003).

We have recently described efomycine M, a small-molecule macrolide that inhibits P-selectin (CD62P)- and E-selectin (CD62E)-mediated leukocyte rolling in inflamed tissues *in vivo* and alleviates chronic inflammatory skin changes in two complementary mouse models of psoriasis (Schön *et al.*, 2002). However, the effect of efomycine M on L-selectin (CD62L)-mediated responses as well as its influence on T-cell sensitization was thus far unknown.

We were interested in studying the effect of L-selectin inhibition on lymphocyte rolling and homing as well as on the pathogenesis of T-cell-dependent allergic reactions. Toward this end, we have analyzed L-selectin-mediated adherence of lymphocytes to sLe^X combined with sulfated tyrosine (sLe^x/TS-PAA) under shear stress in a dynamic flow chamber system. L-selectin interactions with sLe^x/TS-PAA were also assessed on the molecular level using surface plasmon resonance (SPR) spectroscopy (VanCott *et al.*, 1992). In addition, we performed intravital microscopy experiments in mice, looking at the influence of efomycine M or L-selectin-specific antibodies on lymphocyte rolling on high endothelial venules and homing to peripheral lymph nodes. Moreover, a murine adoptive transfer model was used to study the effect of L-selectin-mediated lymphocyte adhesion on the pathogenesis of a T-cell-dependent allergic response. We show that L-selectin-mediated responses could be significantly inhibited by efomycine M *in vitro* and *in vivo* and that interference with L-selectin-mediated lymphocyte adhesion could significantly reduce T-cell-mediated allergic responses *in vivo*. These results indicate that it is possible to therapeutically influence the pathogenesis and clinical outcome of T-cell-mediated allergic responses at a very early stage through interference with L-selectin functions by antibodies or small-molecule compounds.

RESULTS

L-selectin-dependent cell adhesion to sLe^X under shear stress can be inhibited by efomycine M

Approximately 65% of peripheral blood lymphocytes (PBL) freshly isolated by density-gradient centrifugation expressed high levels of L-selectin (mean fluorescence intensity >100), but neither E- nor P-selectin (Figure 1a, left panel). The observation that a function-blocking antibody (Mel-14) directed against L-selectin completely abrogated rolling and binding of PBL ($n=5$ different donors) on a matrix of sLe^X and sulfated tyrosine (sLe^X/TS-PAA) under shear flow (150 seconds^{-1}) suggested that leukocyte rolling and adhesion in this system was dependent on L-selectin functions (Figure 1b). Efomycine M, a novel small-molecule macrolide compound (Schön *et al.*, 2002), also had a profound and significant dose-dependent inhibitory effect on leukocyte rolling in this system, similar to the effect observed with the L-selectin-blocking antibody (Mel-14). Significant inhibition of PBL adhesion was observed at concentrations as low as 1 μM ($P<0.001$ as compared to untreated controls; Figure 1b). These findings indicated that efomycine M inhibits L-selectin functions.

To prove formally that the rolling and binding seen in the cellular assay was strictly owing to L-selectin, a pre-B-cell line (NALM-6; Figure 1a, middle panel), which did not express L-selectin and did not roll or adhere to sLe^X/TS-PAA, was employed. When NALM-6 cells were transfected with human L-selectin (Figure 1a, right panel) and used in the flow chamber system, they had acquired a profound capacity to roll and adhere on the sLe^X/TS-PAA matrix (21 ± 5 cells/field), as compared to their mock-transfected counterparts, which did not adhere (0 cells/field; Figure 1c and d, and Videos S1 and S2). Again, the L-selectin-dependent binding was significantly inhibited in a dose-dependent manner either by the L-selectin-blocking antibody or by efomycine M ($P<0.05$ at concentrations $\geqslant 1$ μM; Figure 1c and d, and Videos S3 and S4).

Complementary experiments showed no increase in the number of apoptotic or dead cells as determined by flow cytometry (FC, annexin V binding to phosphatidylserine and propidium iodide staining of dead cells) after 30 minutes of incubation with 300 μM efomycine M (the limit of solubility in this system) at 37°C (data not shown). Thus, the inhibition of L-selectin-mediated adhesion by efomycine M on the cellular level was not owing to cytotoxic effects or the induction of apoptosis in lymphocytes (data not shown). As reported previously (Schön *et al.*, 2002), longer exposure of endothelial cells to efomycine M (up to 10 μM) for 72 hours also did not result in significantly decreased viability or reduced cell proliferation as compared to untreated control cells. In addition, as detected by two-color FC, incubation with efomycine M did not alter the expression of several activation markers, including CLA, CD25, CD69, CD54, and HLA-DR on $CD3^+$ T cells ($n=6$ donors in each case, data not shown), suggesting that efomycine M did not exert detectable effects on lymphocyte activation. Moreover, the expression of L-selectin was not altered after incubation with efomycine M, indicating that efomycine M did not induce L-selectin shedding (Figures S1 and S2).

The direct antiadhesive activity of efomycine M toward L-selectin on the molecular level was confirmed by dynamic real-time biomolecular interaction analysis, using SPR technology. Real-time biomolecular interaction analysis monitors the formation and dissociation of biomolecular complexes on a sensor surface as the interaction occurs. By covalently attaching sLe^X/TS-PAA to the surface, the interaction of L-selectin present in free solution with the ligand was followed. Measurements were made under conditions of continuous flow. It was found that efomycine M had a highly significant, albeit biphasic, inhibitory effect on L-selectin adhesion to immobilized sLe^X/TS-PAA with inhibition by approximately 50% at 20 μM ($P=0.0003$ compared to

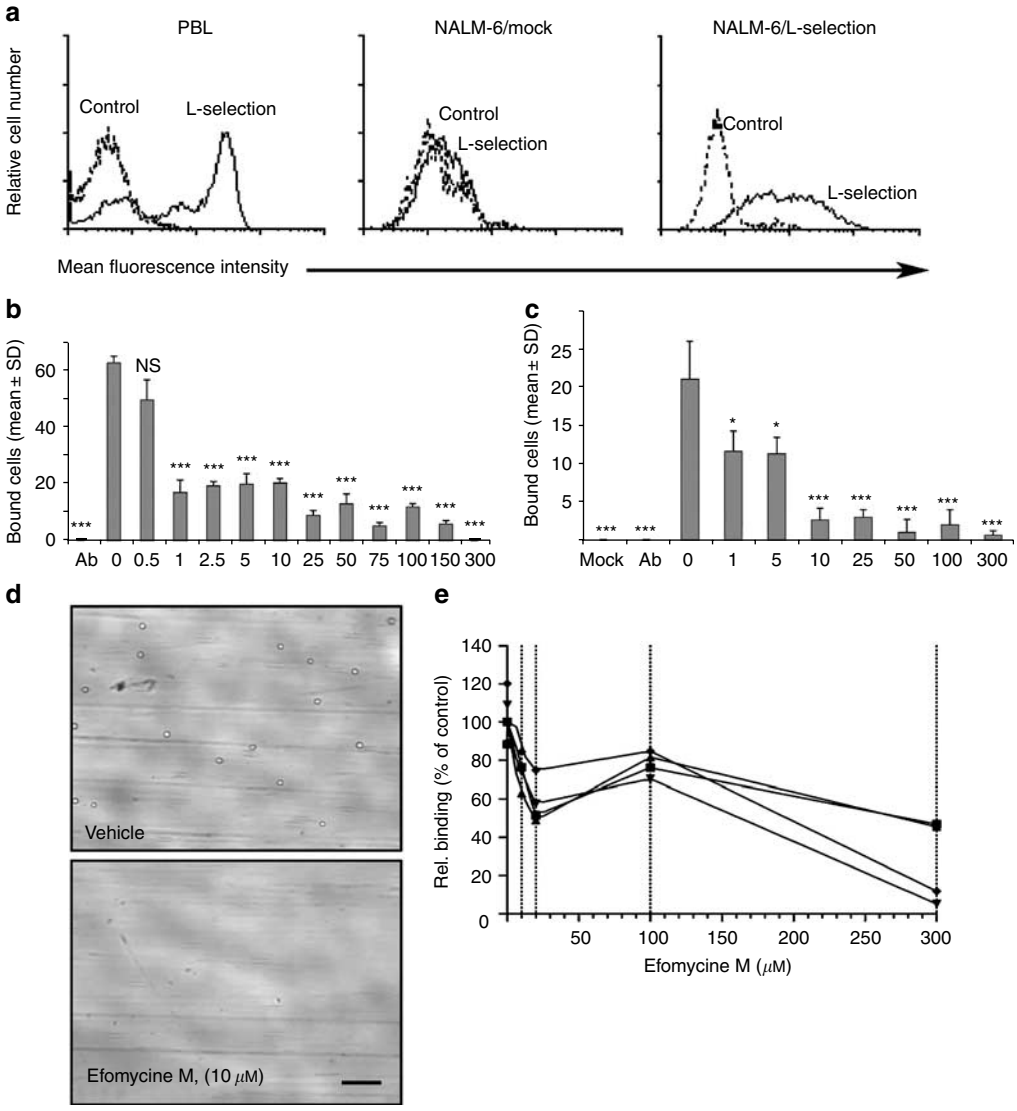

Figure 1. L-selectin-mediated adhesion *in vitro* can be inhibited by L-selectin-specific antibody or efomycine M (corresponds to Videos S1–S4). (**a**) FACS analysis demonstrating expression of L-selectin in PBL (left panel), mock-transfected NALM-6 cells (middle panel), and NALM-6 cells transfected with human L-selectin. (**b**) Rolling of PBL in a dynamic flow chamber system in the presence of a function-blocking mAb directed against L-selectin (mAb Mel-14; column 1) or various concentrations of efomycine M (in μM) as indicated (***$P<0.001$ as compared to untreated controls; NS, not significant). This experiment was repeated three times with similar results in each experiment. (**c**) Rolling of mock-transfected NALM-6 cells (column 1) and NALM-6 cells transfected with L-selectin (other columns) in a dynamic flow chamber system in the presence of a function-blocking mAb against L-selectin (column 2) or various concentrations of efomycine M (in μM) as indicated (*$P<0.05$, and ***$P<0.001$ as compared to untreated controls). The experiment was repeated twice. (**d**) Microscopic image of NALM-6 cells transfected with L-selectin in a dynamic flow chamber system in the presence of vehicle (DMSO 0.1%; upper panel) or efomycine M (10 μM, bottom panel). Cell adhesion to a matrix of sLe^X/PAA was analyzed at 1.04 dyn/second^2. Bar = 50 μm. (**e**) SPR analysis of the binding of a multimerized L-selectin-IgG chimera to immobilized sLe^X/TS-PAA in the presence of the indicated concentrations of efomycine M. SPR was used for real-time biomolecular interaction analysis under conditions of continuous flow (20 μl/minute). Each data point represents a binding signal relative to the signal obtained by control measurements (buffer only). Each of the indicated concentrations of efomycine M were measured in four independent experiments.

vehicle control, calculated from data of four independent experiments; Figure 1e).

Lymphocyte rolling on lymph node vessels and lymph node homing *in vivo* is inhibited by small-molecule- or antibody-mediated inhibition of L-selectin functions

To confirm the inhibitory activity on L-selectin-mediated adhesion *in vivo*, the recruitment of lymphocytes to peripheral lymph nodes was studied, a process that is critically dependent on L-selectin functions (von Andrian and Mempel, 2003).

As has been shown previously, efomycine M reaches maximum serum levels 30 minutes after intraperitoneal injection, and the elimination half-life of efomycine M is more than 2 hours (Schön *et al.*, 2002). These kinetics result in serum concentrations sufficient for *in vitro* inhibition of

selectin functions for up to 8 hours following a single injection. In the first series of experiments, intravital microscopy of peripheral lymph nodes of C57BL6 mice showed that the addition of efomycine M significantly reduced the number of fluorescently labeled leukocytes that rolled along the wall of high endothelial venules of the subiliac lymph node (Figure 2a and Videos S5 and S6). The rolling fraction in vehicle-treated control mice ($n=6$) was 56.3% (SEM = 5.55) as compared to 26.7% (SEM = 3.22) in efomycine M-treated mice ($n=6$; $P=0.0006$ as compared to vehicle control) and 31.7% (SEM = 3.31) in mice treated with a function-blocking antibody against L-selectin ($n=6$; $P=0.02$ compared to vehicle control).

To confirm that decreased rolling of lymphocytes along the lymph node vessel walls was functionally relevant and resulted in decreased homing, freshly isolated lymph node cells were fluorescently labeled with the intravital dye PKH26 and injected intravenously into syngeneic recipient mice (10^7 cells/mouse). As detected by FC of single-cell suspensions of lymph nodes after 2 hours, labeled cells could be retrieved from the recipients' peripheral lymph nodes (0.11%, SEM = 0.01 in mice treated with vehicle, and 0.11%, SEM = 0.01 in mice treated with vehicle and an additional isotype-matched antibody; Figure 2b). Of note, the number of retrieved cells was dramatically reduced by 72.7% in mice pretreated with efomycine M (0.03%, SEM = 0.01; $P=0.0016$ as compared to isotype-treated controls) and by 68.2% in mice treated with an L-selectin-blocking antibody (0.035%, SEM = 0.005; $P=0.0012$ as compared to isotype-treated controls).

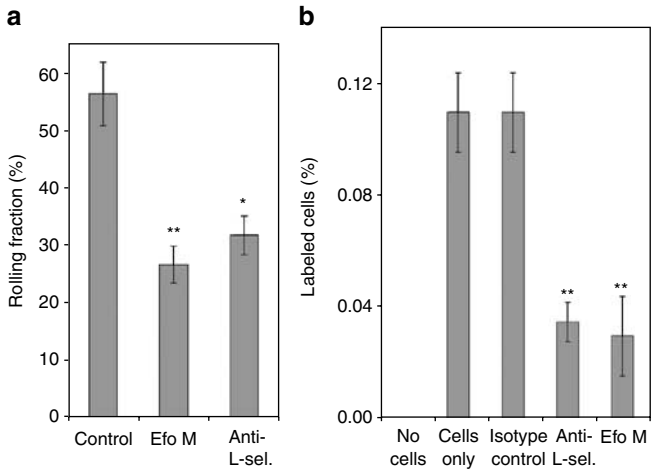

Figure 2. Inhibition of L-selectin-dependent lymphocyte homing *in vivo* (corresponds to Videos S5 and S6). (**a**) Intravital microscopy assessing the rolling fraction of fluorescently labeled lymphocytes in a lymph node *in vivo*. Rolling of fluorescently labeled lymphocytes along the venules of the subiliac lymph node was visualized by intravital microscopy in untreated mice (column 1) and after injection of efomycine M (4 mg/kg, column 2) or a L-selectin-blocking antibody (4 mg/kg, column 3) of the same lymph node (*$P<0.05$, **$P<0.01$, two-tailed *t*-test as compared to untreated control). Three vessels of two mice ($n=6$) were included in this study. The data shown are representative of three experiments. (**b**) Homing of fluorescently labeled lymphocytes to peripheral lymph nodes in the absence (column 2) or presence of isotype control antibody (column 3), L-selectin-specific antibody (column 4) or efomycine M (5 mg/kg mouse; column 5), $n=2$ for each group. The analysis was performed in triplicate. **$P<0.01$ as compared to the controls.

Targeting of L-selectin functions impairs the pathogenesis of contact hypersensitivity reactions in mice

Several lines of evidence, including the observation that L-selectin-deficient mice are unable to mount a delayed-type hypersensitivity response after conventional immunization (Xu *et al.*, 1996), have indicated that L-selectin-mediated homing to peripheral lymph nodes is critical for sensitization of T cells, an essential requirement in the pathogenesis of allergic responses to contact allergens. Intrigued by our findings that efomycine M diminished the recruitment of lymphocytes to peripheral lymph nodes, we have tested the hypothesis that efomycine M is beneficial for the prevention of a T-cell-mediated allergic response. Toward this end, immunocompetent C57BL6 mice were injected intravenously with a single dose of either vehicle, efomycine M (4 mg/kg) or an L-selectin-blocking antibody (Mel-14, 4 mg/kg). One hour thereafter, the mice were subjected to epicutaneous sensitization with the contact allergen DNFB. In order to avoid a direct effect of therapeutic compounds on the elicitation phase of contact hypersensitivity, peripheral lymph node cells of the sensitized animals were isolated and adoptively transferred into untreated syngeneic recipients 5 days after sensitization (5×10^7 cells/mouse, intravenous transfer). The recipients' ears were challenged with DNFB immediately after adoptive transfer of cells.

Average ear swelling responses (expressed as the difference between thicknesses of vehicle- *versus* DNFB-challenged ears) 24 hours after challenge were 0.385 mm in recipients of lymphocytes from untreated donors ($n=10$ mice; SEM = 0.0299). In contrast, this response was significantly diminished by 56.2% when recipients of cells from efomycine-treated mice were analyzed ($n=10$; average swelling 0.168 mm; SEM = 0.0258; $P=0.0002$ as compared to the vehicle-treated controls; Figure 3a). When recipients of cells obtained from mice treated with both efomycine M and the L-selectin-blocking antibody were assessed, no significant additional effect of antibody-mediated L-selectin inhibition was observed ($n=10$; average swelling 0.147 mm; SEM = 0.0204; $P=0.0001$ as compared to controls; Figure 3a). The results were confirmed histopathologically by a dermatopathologist in a blinded manner and clearly showed a diminished density of infiltrating immune cells in recipients of cells from pretreated donors (Figure 3b). In addition, the dermal edema was markedly weaker in recipients of cells from efomycine M- or antibody-pretreated donors. Treatment with efomycine M did not result in significant loss of L-selectin expression in lymph node lymphocytes under the conditions used for the adoptive transfer experiments (Figure S2).

DISCUSSION

Selectins are involved in the recruitment of lymphocytes from blood vessels to sites of inflammation. However, L-selectin (CD62L) has another, specific, function in the homing of

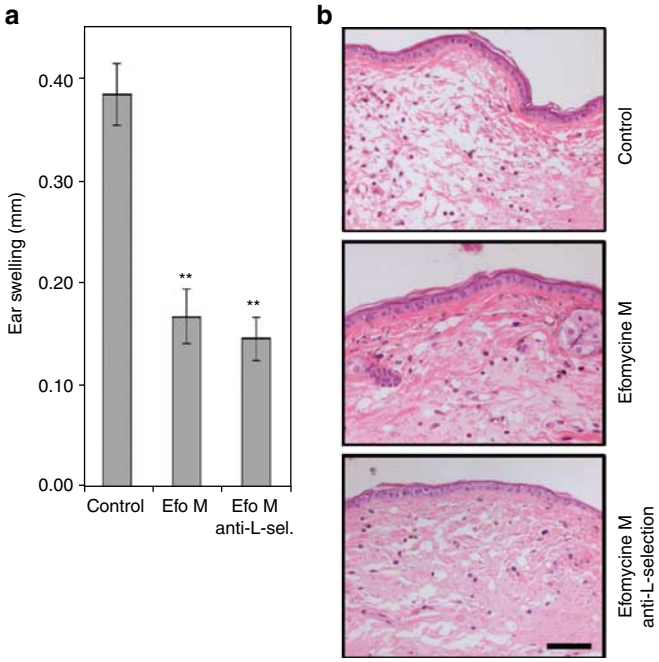

Figure 3. Blocking of L-selectin functions interferes with the pathogenesis of an allergic response in a mouse model of contact hypersensitivity. (**a**) Ear swelling responses (expressed as difference between vehicle- and DNFB-challenged ears) in a contact hypersensitivity/adoptive transfer model comparing recipients of lymphocytes from untreated donors ($n=14$) and donors treated with efomycine M ($n=15$) or a combination of efomycine M and anti-L-selectin ($n=10$) during the sensitization phase (**$P<0.01$, two-tailed *t*-test). Three separate experiments were performed and the data of all experiments was combined. (**b**) Histopathology showing inflammation and edema in ear skin of recipients of lymphocytes from untreated donors (top panel) and donors treated with efomycine M (middle panel) or a combination of efomycine M and anti-L-selectin (bottom panel) during the sensitization phase as outlined in the text. Please note reduced density of infiltrating immunocytes and reduced severity of dermal edema in recipients of lymphocytes from donors treated with efomycine M or an L-selectin-directed antibody. Bar = 50 μm.

lymphocytes to peripheral lymph nodes (Xu *et al.*, 1996; Ley, 2001; Ley and Kansas, 2004). The selectin ligand, 6′-sulfo sialylated Le^X is highly expressed in the high endothelial venules of peripheral lymph nodes and L-selectin preferentially binds to this specific ligand, which contributes to homing of L-selectin-expressing lymphocytes to lymph nodes (Hiraoka *et al.*, 1999). This function of L-selectin is of great clinical importance inasmuch as it is a prerequisite for T-cell sensitization in the pathogenesis of allergies. The relentless increase in the prevalence of T-cell-dependent allergic disorders, such as occupational contact allergies (Andersen, 2003) or atopic dermatitis (Williams, 2005), represents a major medical problem and highlights the need for effective preventive strategies. In this light, interference with L-selectin functions at a very early stage in the pathogenic cascade in individuals at risk may be beneficial in terms of supporting strategies of primary prevention (Kutting *et al.*, 2004; Arruda *et al.*, 2005; Arshad, 2005).

In order to provide a proof-of-principle for the hypothesis that blocking of L-selectin functions would result in decreased lymphocyte homing, thereby exerting a preventive effect toward T-cell-mediated hypersensitivity reactions, efomycine M, a small-molecule macrolide compound, as well as L-selectin-specific antibodies were used to inhibit L-selectin-mediated responses *in vitro* and *in vivo*. Using a novel docking engine, it has recently been predicted that efomycine M binds stronger to the ligand-binding domain as compared to the natural ligand sLe^X, at least with regard to E-selectin (Wienrich *et al.*, 2006). Our *in vitro* results showed that efomycine M as well as specific antibodies could significantly inhibit L-selectin-dependent lymphocyte rolling on a selectin ligand. Based on our intravital microscopy of peripheral lymph nodes and homing experiments in mice, both efomycine M and L-selectin-directed antibodies had an immediate and profound effect on lymphocyte homing to peripheral lymph nodes. Several explanations for the reduced numbers of labeled lymphocytes detected in the peripheral lymph nodes can be delineated: first and most likely, it may reflect diminished trafficking of lymphocytes through high endothelial venules; second, it may be owing to increased emigration of lymphocytes from the lymph nodes, or third, retention of labeled cells could have occurred in other organs. However, given that significantly decreased rolling of lymphocytes in peripheral lymph nodes after treatment with efomycine M or L-selectin-specific antibodies was observed, the reduced number of lymphocytes detected within the lymph nodes after treatment with efomycine M was most likely caused by reduced trafficking to peripheral lymph nodes following L-selectin inhibition. Although antibodies against L-selectin have been used previously to interfere with peripheral tolerance induction (Bai *et al.*, 2002), and diminished mast cell recruitment alleviated type-I allergic reactions in L-selectin-deficient mice (Shimada *et al.*, 2003), our results for the first time allowed to test the hypothesis that interference with L-selectin functions by a small-molecule compound can be employed to interfere with the pathogenesis of a T-cell-mediated allergic disorder and to alleviate its clinical outcome.

Indeed, the results from our adoptive transfer experiments, in which a potential beneficial effect of functional blocking of L-selectin for the prevention of a T-cell-mediated allergic response was tested, indicated that inhibition by an antibody or a small-molecule compound during the sensitization phase, that is, a very early event in the generation of allergic responses, can diminish a T-cell-dependent immune response in an experimental model relevant for the pathogenesis of allergic disorders. Although expression of selectin ligands on Langerhans cells has been reported (Ross *et al.*, 1994), it is not clear if and how these ligands contribute to T-cell sensitization *in vivo* since (I) E- and P-selectin are not expressed in normal HEV and (II) previous studies have shown that mice deficient for α-1,3-fucosyltransferase VII (which are characterized by deficient E-, P-, and L-selectin ligand activity) showed normal Langerhans cell migration to local lymph nodes following contact sensitization (Erdmann *et al.*, 2002). In any case, the clinical response to challenge with the contact allergen in our experiments was significantly weakened, but not completely abrogated. These observations

are consistent with the known functions of L-selectin, which is particularly important for lymph node recruitment of naive lymphocytes, but plays a lesser role for the recruitment of immune cells to peripheral sites during the elicitation phase of inflammatory reactions. This notion is supported by a recent clinical trial in which antibody-mediated blockade of L-selectin did not alleviate psoriasis, a common inflammatory skin disorder (Hardtke *et al.*, 2005). In contrast, based on our experimental evidence, it is conceivable that early interference with L-selectin functions during the T-cell-dependent sensitization phase by a small-molecule compound can alleviate the clinical outcome of an allergic response. Overall, inhibition of L-selectin-mediated responses using the small-molecule efomycine M or L-selectin-specific antibodies can diminish lymphocyte recruitment *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell preparation, generation of transfectants, and analysis of surface antigen expression

PBL were isolated from human blood under aseptic conditions by density-gradient centrifugation (Ficoll-Paque™ Plus; Amersham Biosciences, Uppsala, Sweden). PBL were washed twice ($340 \times g$ for 5 minutes) in phosphate-buffered saline and resuspended at 2×10^6 cells/ml in Hank's balanced salt solution containing 2 mM $CaCl_2$. Human pre-B cells (NALM-6) were transfected with plasmid pCR 3.1/L-sel containing the coding sequence of full-length human L-selectin or, as control, with plasmid pCR 3.1 only (mock-transfected cells). The cells were cultured in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid-buffered Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum, penicillin, streptavidin, and geneticin (used as selection antibiotic).

Cell adhesion under flow conditions

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 previously been 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 cover slips were air-dried and blocked for at least 1 hour with 0.5% BSA in phosphate-buffered saline. Cell suspensions in Hank's balanced salt solution with 2 mM $CaCl_2$ were incubated for 30 minutes at 37°C in the presence or absence of efomycine M (99.9% pure; kindly provided by T Krahn, BayerHealthCare, Wuppertal, Germany) at final concentrations ranging from 0.1 to 300 μM or an L-selectin-blocking antibody (Mel-14, 10 μg/ml). Transparent flow chambers with a slit depth of 50 μm and a slit width of 5 mm, equipped with coated or uncoated cover slips, 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/hour, equivalent to a wall shear rate of 150/seconds). Microscopic phase-contrast images were recorded in real time. At least three different microscopic fields were recorded for each condition. Image analysis was performed off-line using MetaView Imaging software (Universal Imaging Corporation, Downington, PA). The number of adhering cells from all three fields was counted and the mean value (±SD) was used for analysis.

Flow cytometric analysis

Cells were incubated at 4°C for 45 minutes with 10 μg/ml of the primary mAb, washed three times in FC buffer (phosphate-buffered saline containing 1% BSA and 0.002% NaN_3), and incubated with FITC-conjugated goat anti-mouse IgG for 45 minutes at 4°C. The cells were washed again three times in FC buffer and analyzed using a fluorescence-activated cell scanner (FACScan, Becton Dickinson, Heidelberg, Germany) and the Cell Quest software (Becton Dickinson).

Commercially available murine mAbs against the following surface antigens were used: CD3, CD25, CD62L, CD54, CD69, CLA (cutaneous lymphocyte-associated antigen), and HLA-DR. Isotype-matched murine IgG was used as control (Dako, Hamburg, Germany). In order to assess whether efomycine M affected cell surface expression of L-selectin (CD62L), lymphocytes (PBL) were incubated for 60 minutes with efomycine M at the indicated concentrations. Thereafter, surface expression of L-selectin was assessed by FC.

Apoptosis assay

Apoptosis induced by incubating PBL or NALM-6 cells for 30 minutes at 37°C with 75 or 300 μM efomycine M was determined using the commercially available annexin V-FITC apoptosis detection kit (Sigma, Deisenhofen, Germany) following the manufacturer's instructions.

SPR spectroscopy

SPR experiments were performed on a BIACORE X instrument (Biacore AB, Uppsala, Sweden) at 25°C. Biotinylated sLe^X/TS-PAA (Lectinity) was immobilized on a sensor chip SA (Biacore), whose surface consists of a carboxymethylated dextran matrix preimmobilized with streptavidin. Biotinylated *N*-acetyllactosamine-PAA (LacNAc-PAA) was immobilized as reference on a second lane of the same chip. The loading of the chip with both conjugates was carried out up to a baseline shift of 900 resonance units (RU), respectively. Recombinant L-selectin-IgG chimera was produced in the human myeloid cell line K562 (Harms *et al.*, 2001), purified by affinity chromatography and used in a multimeric form (2 nM per sample) by immobilization on Protein A-labeled gold beads (∅ 20 nm, Sigma). Before passing the sensor surface, multimerized L-selectin was incubated with efomycine M for 18 minutes at room temperature at final concentrations of 0, 0.1, 10, 20, 100, or 300 μM. Binding was recorded in running buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$) at a flow rate of 20 μl/min. Each cycle consisted of a 1-minute waiting period to monitor the baseline stability, an association phase of 105 seconds, and a dissociation phase of 180 seconds. Regeneration of the surface was performed by injection of 4 M $MgCl_2$ at 100 μl/minute use for 60 seconds. Two injections of running buffer were carried out between each sample to check carry-over effects. Data obtained in the reference lane were subtracted from that obtained in the sLe^X/TS-PAA lane. Responses of the sample injections were extracted between report points set at injection start (0 second) and the end of the dissociation phase (285 seconds). Each data point was measured in quadruplicate and the mean value (±SD) was used for analysis.

Intravital microscopy of peripheral lymph nodes

All animal experiments were performed according to the institutional guidelines and were approved by the respective governmental authorities (Regierungspräsidium Darmstadt). Intravital microscopy of peripheral lymph nodes was performed as described (von Andrian, 1996) with minor modifications. Briefly, C57BL6 mice were anesthetized by intraperitoneal injection of Ketamin (Schwabe-Curamed, Karlsruhe, Germany) and Rompun (Bayer, Leverkusen, Germany), and placed on a homeothermic blanket. The right carotid artery was prepared microsurgically and a catheter was inserted downstream into this vessel for injection of rhodamine 6G, efomycine M (4 mg/kg bodyweight), or Mel-14, a L-selectin-specific antibody (4 mg/kg). The subiliac (superficial inguinal) LN was microsurgically dissected and leukocyte interactions with post-capillary venules were analyzed after staining with rhodamine 6G using a previously described setup for intravital microscopy (Ludwig *et al.*, 2004). Rolling fractions were determined as percentages of rolling cells in relation to the total flux of fluorescent T cells that passed through a defined venule.

Homing of lymph node cells to peripheral lymph nodes

Peripheral lymph nodes of mice were gently dissociated into single-cell suspensions in DMEM. The tissue remnants were removed and the cells were counted. Cells were than stained using the PKH26 red fluorescent cell linker kit (Sigma) according to the manufacturer's instructions. Syngeneic recipient mice received an intraperitoneal injection of either vehicle (1% DMSO, 5% ethanol, 5% Solutol-HS15 (BASF), and 89% H_2O) or 5 mg/kg efomycine M dissolved in vehicle. After a 2-hour incubation, 10^7 stained donor cells were injected intravenously. The following groups were included in this analysis: (I) mice treated with vehicle, but without cells (control I), (II) mice treated with vehicle and cells (control II), (III) mice treated with vehicle and with cells incubated after staining for 15 minutes with rat IgG2a isotype control (10 μg antibody/10^7 cells; control III), (IV) mice treated with vehicle and with cells incubated after staining for 15-minutes with L-selectin-specific antibody (10 μg antibody/10^7 cells), and (V) mice treated with efomycine M and cells. The PKH26 red-stained cells were left for 2 hours in the mice before these mice were killed and the lymph nodes from each individual mouse were collected, the cells were dissociated and the PKH26 signal was measured on a FACScan (Becton Dickinson, Heidelberg, Germany).

Induction of contact hypersensitivity and adoptive transfer of lymph node cells

Donor C57BL6 mice were injected intravenously with either NaCl (0.9%), efomycine M (4 mg/kg), or the L-selectin-blocking antibody Mel-14 (4 mg/kg). One hour later, mice were sensitized by painting 75 μl DNFB (Sigma) solution (0.5% in acetone/olive oil, 4/1) on the back. Peripheral lymph nodes from sensitized mice were removed on day 5, and single-cell suspensions of donor lymph node cells (5×10^7 cells) were injected intravenously into non-sensitized syngeneic C57BL6 mice. The recipients were challenged with DNFB (0.3% in acetone/olive oil, 4/1) immediately thereafter. Twenty-four hours later, ear-swelling responses were measured in a blinded manner using a spring-loaded caliper (Mitutoyo, Neuss, Germany). Ear swelling responses were expressed as difference in ear thickness in micrometer between DNFB- and solvent-treated ears. In addition, ears were analyzed histologically in hematoxylin- and eosin-stained paraffin-embedded tissues by a dermatopathologist in a blinded manner. Moreover, the contact hypersensitivity model was used to analyze the effect of treatment with efomycine M on L-selectin expression *in vivo*. One group of animals was treated with efomycine M and one group with vehicle only ($n = 3$ in each group) and sensitization occurred for 5 days using DNFB. Thereafter, lymphocytes were isolated from lymph nodes and tested for L-selectin expression by flow cytometric analysis as described above.

Statistical analysis

All statistical analyses were performed using the Excel software (Microsoft GmbH, Munich, Germany). Data are displayed as mean ($\pm$SD or SEM as indicated); *P*-values were determined using the two-tailed *t*-test, and *P*-values <0.05 (confidence interval of 95%) were considered statistically significant. All statistical tests were two-sided.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Videos S1–S4. Abrogation of L-selectin-mediated rolling and adhesion under shear flow by efomycine M or L-selectin-directed antibodies.

Videos S5 and S6. Lymphocyte rolling on lymph node vessels *in vivo* is diminished by efomycine M.

Figure S1. Efomycine M does not induce L-selectin shedding *in vitro*.

Figure S2. Efomycine M does not induce significant L-selectin shedding *in vivo*.

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