Leukocyte trafficking in a mouse model for leukocyte adhesion deficiency II/congenital disorder of glycosylation IIc

Svitalina Yakubenia,1 *David Frommhold,2 Dirk Schölch,2 Christina C. Hellbusch,3 Christian Körner,3 Björn Petri,1 Claire Jones,1 Ute Ipe,1 M. Gabriele Bixel,1 Robert Krempien,4 †Markus Sperandio,5 and †Martin K. Wild1

1Max Planck Institute for Molecular Biomedicine, Münster, Germany; 2Department of Pediatrics, Section of Neonatology, and 3Division of Inborn Metabolic Diseases, University Children’s Hospital, Heidelberg, Germany; 4Department of Radiation Oncology, University Heidelberg, Heidelberg, Germany; and 5Walter Brendel Center of Experimental Medicine, Ludwig-Maximilians-University, München, Germany

Leukocyte adhesion deficiency II (LAD II), also known as congenital disorder of glycosylation IIc (CDG-IIc), is a human disease in which a defective GDP-fucose transporter (SLC35C1) causes developmental defects and an immunodeficiency that is based on the lack of fucosylated selectin ligands. Since the study of in vivo leukocyte trafficking in patients with LAD II is experimentally limited, we analyzed this process in mice deficient for SLC35C1. We found that E-, L-, and P-selectin–dependent leukocyte rolling in cremaster muscle venules was virtually absent. This was accompanied by a strong but not complete decrease in firm leukocyte adhesion. Moreover, neutrophil migration to the inflamed peritoneum was strongly reduced by 89%. Previous reports showed surprisingly normal lymphocyte functions in LAD II, which indicated sufficient lymphocyte trafficking to secondary lymphoid organs. We now found that while lymphocyte homing to lymph nodes was reduced to 1% to 2% in Slc35c1−/− mice, trafficking to the spleen was completely normal. In accordance with this, we found a defect in the humoral response to a T-cell–dependent antigen in lymph nodes but not in the spleen. Taken together, Slc35c1−/− mice show strongly defective leukocyte trafficking but normal lymphocyte homing to the spleen, which may explain normal lymphocyte functions in LAD II. (Blood. 2008; 112:1472-1481)

Introduction

Leukocyte adhesion deficiency II (LAD II), also known as congenital disorder of glycosylation IIc (CDG-IIc), is a rare inherited human disease that is characterized by the lack of fucosylated structures, including ligands for the selectin class of adhesion molecules.1-3 Several years ago we identified the genetic defect in this disease and found that a defective nucleotide sugar transporter called solute carrier family 35 member C1 (SLC35C1), which transfers GDP-fucose into the Golgi apparatus, is responsible for the hypofucosylation in LAD II.4-5 Patients with LAD II show strong mental and growth retardation as well as leukocytosis and immunodeficiency.1,2,6,7 Whereas the cause for the developmental defects in LAD II is still unknown, the immunodeficiency can readily be explained by the absence of fucosylated selectin ligands.1,9 Selectin ligands mediate leukocyte–endothelial cell interactions by binding to the 3 selectins, E-, L-, and P-selectin. These interactions are characterized by leukocyte rolling on endothelium, which is required for subsequent leukocyte activation, arrest, and extravasation into lymph nodes (LNs) and inflamed tissues.10 Of note, migration to LNs is dependent on the presence of fucosylated selectin ligands on high endothelial venules (HEVs) in LNs, whereas trafficking to sites of acute inflammation requires selectin ligand activity on leukocytes.11

A large body of data shows that in vitro binding of leukocytes to patients with LAD II to selectins is strongly reduced.1,6,9,12 However, in vivo studies are scarce due to the limitations of studies in humans. Von Andrian et al13 analyzed rolling of neutrophils from a patient with LAD II in inflamed venules of rabbit mesenteries and found a reduction of more than 80% of the number of rolling cells. In addition, it was shown that the number of neutrophils migrating to the inflamed skin in a patient with LAD II was only 2% to 6% of normal.14 Interestingly, T-cell–dependent antibody responses to the bacteriophage ΦX174 and to keyhole limpet hemocyanin (KLH) were found to be normal in LAD II, implying that T-cell and B-cell functions are not perturbed.14,15 These findings led to the classification of LAD II in reviews and textbooks as a disease that mainly affects phagocytic cells.16-18 Importantly, normal T-cell–dependent antibody responses suggested that sufficient lymphocyte homing to secondary lymphoid organs—where such adaptive immune responses are initiated—is possible in LAD II. However, in vivo lymphocyte homing to lymph nodes cannot be studied in humans.

Residual fucosylation-dependent lymphocyte homing to lymph nodes is theoretically possible in patients with LAD II. We have detected low residual fucosylation in leukocytes and fibroblasts of patients with LAD II by sensitive flow cytometry using the fucosylation-specific Aleuria aurantia lectin (AAL).6 This may also apply to HEVs, the lymphocyte entry sites within LNs, although this has not been tested. Residual fucosylation is obviously independent of SLC35C1 since it can be detected in cells from patients with nonfunctional and mislocalized SLC35C1 molecules6 as well as in Slc35c1−/− mice.19 Thus, residual fucosylation must be based on an alternative low-efficiency GDP-fucose
transport system. This transport system may be identical to an as-yet-unknown mechanism that allows efficient refucosylation in the presence of high concentrations of exogenous L-fucose, an effect that forms the basis for the successful therapy of patients with LAD II.7,12,20 Whether such an alternative transport provides sufficient residual fucosylation in the absence of exogenous fucose and would thus allow lymph node entry of lymphocytes in LAD II is not known.

An alternative site for the initiation of T cell–dependent antibody responses is the spleen. This organ is particularly adapted to mount immune responses against blood-borne antigens and pathogens.21,22 Whether fucosylation plays a role in lymphocyte homing to the spleen is not clear. On the one hand, lymphocyte homing to the spleen appears to be independent from alpha1,3-fucosyltransferases (FucT) IV and VII, the glycosyltransferases which are required for selectin ligand formation in leukocytes and HEVs.23 On the other hand, studies with the sulphated L-fucose polymer fucoidan showed that this compound inhibits lymphocyte homing to the spleen by up to 63%.24-26 raising the question whether FucTIV/VII-independent fucosylation of the spleen is required for efficient homing to the spleen and whether lymphocyte homing to the spleen may be affected in LAD II.

Recently, a mouse knock-out model for Slc35c1-deficiency was generated.19 These mice show strong hypofucosylation, severe growth retardation, changes in lung morphology that resemble those in FucTVIII−/− mice,27 and high mortality. Hypocellularity of peripheral lymph nodes and persistent leukocyte cytosis showed that the immune system is affected in Slc35c1−/− mice. Intravital microscopic observations of leukocyte rolling in HEVs of Peyer patches demonstrated a complete absence of L-selectin–mediated rolling. In addition, binding of E-selectin and P-selectin Fc-chimeric proteins to neutrophils of Slc35c1−/− mice was dramatically reduced in static in vitro assays. Finally we found that, like in patients with LAD II, exogenous L-fucose can correct the fucosylation defect in Slc35c1−/− cells, which points to the existence of an alternative Golgi GDP-fucose transport system.19 Here, we made use of Slc35c1−/− mice to study in vivo leukocyte trafficking under conditions similar to those in patients with LAD II. We show that leukocyte rolling and adhesion in cremaster muscle venules, neutrophil migration to inflamed peritoneum, and lymphocyte homing to LN are strongly reduced in the LAD II model mice. In contrast, we found that lymphocyte trafficking to the splenic white pulp is normal in Slc35c1−/− mice. Accordingly, humoral immune responses of lymph nodes but not of the spleen were defective. We therefore suggest that SLC35C1-independent lymphocyte homing to the spleen partially compensates for the lack of LN accessibility, which explains why adaptive immune responses appear to be normal in patients with LAD II.

**Methods**

**Animals**

Slc35c1−/− mice were generated as described earlier19 and kept as a heterozygous breeding colony. All mice used in these experiments (8 to 12 weeks old) were of mixed 129Sv/C57BL/6 background. Homozygous (+/+ ) littermates were used as control mice throughout. All animals were housed in a pathogen-free barrier facility, and experiments were approved by the Regierungspräsidium Karlsruhe, Germany (AZ 35-9185.81/G-69/05 and G35/06).

**Generation of Slc35c1−/− bone marrow chimeric mice**

Bone marrow chimeric mice with a Slc35c1−/− hematopoietic system (Slc35c1pm−/−) were generated by bone marrow transplantation as described.28 Briefly, recipient littermate control mice were irradiated by the linear accelerator Mevatron KD2 (Siemens, München, Germany) in 2 doses of 3.5 Gy (350 rad) each (total dose of 7 Gy [700 rad]) approximately 3 hours apart, followed by a recovery time of 1 hour before transplantation. Approximately 10⁶ unfractionated donor Slc35c1−/− bone marrow cells in 0.5 mL media were delivered through the tail vein of each recipient mouse. More than 4 weeks after adoptive transfer, mice were used for intravital microscopic experiments. To formally test the successful adoptive transfer of Slc35c1−/− bone marrow into lethally irradiated control mice, binding of fucos-specific AAL to Gr1+ leukocytes from Slc35c1−/− mice was assessed by flow cytometric analysis. Similar to Slc35c1−/− Gr1− leukocytes, Gr1− cells from Slc35c1pm−/− mice showed dramatically reduced AAL binding when compared with Gr1+ cells from control mice, indicating that the transfer of Slc35c1−/− bone marrow cells into lethally irradiated control mice led to successful reconstitution of the hematopoietic system (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article).

**Antibodies and cytokines**

The following mAbs were used: anti–P-selectin mAb RB40.34 (rat IgG1),29,30 anti–E-selectin mAb 9A9 (rat IgG1; gift from Dr B. Wolitzky, Mitocor, Nutley, NJ), anti–L-selectin mAb MEL-14 (rat IgG2a),29,30 anti–CD99 mAb mCD99–102.2 (rat IgG2a; own production, M.G.B.), anti–MacCAM-1 mAb MECA 367 (rat IgG2a; BD Pharmingen, San Diego, CA), and granulocyte-specific anti-Ly-6G mAb Gr-1 (BD Pharmingen). Recombinant murine TNF-α (R&D Systems, Minneapolis, MN) was injected intrascrotally at a dose of 500 ng per mouse in a volume of 0.3 mL sterile saline 2 hours prior to the intravital microscopic experiment.

**Flow cytometry for lectin binding to Gr1+ leukocytes**

Flow cytometry was performed according to standard protocols12 with 10 µg/mL biotinylated AAL (Vector Laboratories, Burlingame, CA) and phycoerythrin (PE)-conjugated streptavidin (BD Pharmingen). Subsequently, cells were stained with APC-conjugated mAb Gr-1 and analyzed using the 4-decade FACS LSR II and FACSDiva and Cell Quest software package (Becton Dickinson, San Jose, CA).

**Surgical preparation and intravital microscopy**

Anesthesia and surgical preparation of the cremaster muscle for intravital microscopy experiments was performed as described previously.28 Intravital microscopy was conducted on an upright microscope (Model S12815/20; Leitz, Wetzlar, Germany) with a saline immersion objective (SW 400.75 numerical aperture). Experiments were recorded via a CCD camera system (model CF8/1; Kappa, Gleichen, Germany) on a Panasonic S-VHS recorder (Hamburg, Germany). Postcapillary venules under observation ranged from 20 to 40 µm in diameter. Systemic blood samples (10 µL) were taken before and during the experiment, stained with Tuerck solution (Merek, Darmstadt, Germany), and assessed for white blood cell count using a hemocytometer.

**Data analysis of intravital experiments**

Diameter and segment length of postcapillary venules were measured using a digital image processing system.31 Venular centerline erythrocyte velocity in the cremaster muscle was assessed using a dual photodiode and a digital online cross-correlation program (Circusoft Instrumentation, Hockessin, DE) as described.32 Wall shear rates (γw) were calculated as 4.9 (8v5d), where v5 is the mean blood flow velocity and d the vessel diameter.33 Rolling leukocyte flux fraction was defined as the percentage of rolling
leukocytes to all leukocytes passing through the same venule per unit time.30

Peritonitis assays
Experiments were performed as described previously.34,35 The percentages of neutrophils in peritoneal lavages were determined using mAb Gr-1 in flow cytometry.

Frozen section binding assays
Experiments were performed as described previously36 with the following modifications. Mesenteric lymph nodes were sectioned to 10 μm thickness. Binding was assayed with 2 × 106 lymphocytes/section. Lymphocytes were obtained from wild-type spleens by centrifugation on Lympholyte M (Cedarlane Laboratories, Burlington, ON). In some experiments lymphocytes were preincubated for 20 minutes with mAb Mel-14 (2 μg/mL) or control mAb mCD99-102.2, which binds to CD99, a protein that is expressed on lymphocytes and endothelial cells and does not participate in homing to LNs.37 Subsequently, cells were washed and allowed to bind to the sections. Following fixation sections were blocked for 1.5 hours in phosphate-buffered saline (PBS)/2% bovine serum albumin (BSA)/10% normal goat serum and incubated for 1 hour with 10 μg/mL MECA 367 mAb for HEV detection before they were stained with peroxidase-conjugated secondary antibodies. The sections were photographed under an Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany) at 100× magnification using a 10×/0.45 NA Plan-Apochromat objective (Zeiss). Lymphocyte adhesion was quantified using the ImageJ analysis program (National Institutes of Health, Bethesda, MD) and expressed as the number of bound lymphocytes per 1 mm2 of HEV area.

Lymphocyte homing assay
Lymphocytes from peripheral LNs (PLNs) and mesenteric LNs (MLNs) or from spleens for the spleen homing assays (Figures 6B and 7) of wild-type mice were obtained from wild-type spleens by centrifugation on Lympholyte M. A total of 6 × 107 lymphocytes in Hanks balanced salt solution (PAA Laboratories, Pasching, Austria) were injected into tail veins of 10- to 12-week-old mice and L-selectin–dependent rolling more than 4 hours after TNF-α treatment in 14 venules of 3 mice as compared with control mice. L-selectin–dependent rolling more than 4 hours after TNF-α application.40 We first investigated leukocyte rolling 2 to 4 hours after TNF-α injection, where rolling is mediated by P-, E-, and P-selectin blocking mAb RB40.34 reduced rolling dramatically, whereas blockade of E-selectin using mAb 9A9 led to an increase in rolling flux fraction, which is due to significantly higher rolling velocities and an impairment of leukocyte extravasation as described before.41 In line with published results, P-selectin blocking mAb RB40.34 reduced rolling dramatically, whereas blockade of E-selectin using mAb 9A9 led to an increase in rolling flux fraction, which is due to significantly higher rolling velocities and an impairment of leukocyte extravasation as described before.41

Results
Leukocyte rolling in TNF-α–induced inflammation
In order to analyze in vivo leukocyte migration and trafficking in Slc35c1-deficient mice we first studied leukocyte rolling under inflammatory conditions by means of intravital microscopy of cremaster muscle venules. Since breeding resulted in very few Slc35c1−/− mice, we also analyzed chimeric mice (Slc35c1mwm−/−), which were obtained by transplanting bone marrow from Slc35c1−/− mice into lethally irradiated recipient animals. Slc35c1mwm−/− mice showed full chimerism as confirmed by strong hypofucosylation in all blood leukocytes (Figure S1). Microvascular parameters for the groups are presented in Table 1 and show similar vessel diameters, centerline velocities, and wall shear rates, but significant leukocytosis in Slc35c1−/− and Slc35c1mwm−/− mice as compared with control mice.

Intracrotal injection of TNF-α leads to E- and P-selectin–dependent rolling 2 to 4 hours after TNF-α injection and to E-, P-, and L-selectin–dependent rolling more than 4 hours after TNF-α application.40 We first investigated leukocyte rolling 2 to 4 hours after TNF-α treatment in 14 venules of 3 Slc35c1−/− mice and 7 venules of 2 Slc35c1mwm−/− mice and compared the results with rolling in 20 venules of 6 control animals.

We found that the leukocyte rolling flux fraction in cremaster muscle venules of control mice was 15% plus or minus 2%, which is similar to previous reports (Figure 1A).31 In line with published results, P-selectin blocking mAb RB40.34 reduced rolling dramatically, whereas blockade of E-selectin using mAb 9A9 led to an increase in rolling flux fraction, which is due to significantly higher rolling velocities and an impairment of leukocyte extravasation as described before.42 In contrast, leukocyte rolling was completely absent in Slc35c1−/− and Slc35c1mwm−/− mice (Figure 1A), suggesting that both P- and E-selectin–mediated leukocyte rolling is impaired in the absence of Slc35c1.

Next, we investigated leukocyte rolling more than 4 hours after TNF-α injection, where rolling is mediated by P-, E-, and
L-selectin. To isolate for L-selectin–mediated rolling, we injected blocking mAbs against P-selectin and E-selectin, leading to a decrease in rolling flux fraction to 4% plus or minus 2% in littermate control mice, which could be completely abolished by additional injection of L-selectin–blocking mAb Mel-14 (Figure 1B). In Slc35c1+/− and Slc35c1bm−/− mice we did not observe any rolling more than 4 hours after TNF-α injection and after application of anti-P-selectin mAb RB40.34 and anti-E-selectin mAb 9A9, suggesting that L-selectin ligand function is dependent on Slc35c1 in this setting. These results do not only indicate the importance of posttranslational fucosylation for P-, E-, and L-selectin ligand activity during inflammation in vivo, the experiments with the Slc35c1bm−/− mice also confirm that all of the relevant selectin ligand activity in the model of TNF-α–induced inflammation of the cremaster muscle is found on leukocytes.

**Leukocyte adhesion upon TNF-α stimulation**

Next, we investigated the number of adherent leukocytes in cremaster muscle venules of Slc35c1+/− and Slc35c1bm−/− mice 2 to 4 hours after TNF-α stimulation. We found a significant reduction, but not a complete loss, of adherent leukocytes in Slc35c1+/− (245 ± 45 cells/mm²) and Slc35c1bm−/− mice (234 ± 81 cells/mm²) when compared with littermate control mice (697 ± 102 cells/mm²; Figure 2A), suggesting that selectin-independent mechanisms exist that allow some leukocyte adhesion during inflammation. To further assess the observed residual adhesion, we compared leukocyte adhesion efficiency between the groups (Figure 2B). Leukocyte adhesion efficiency is defined as the number of adherent cells per vessel surface area divided by systemic leukocyte count and takes into account differences in systemic leukocyte count between the groups. We found a dramatic (13- to 18-fold) reduction in leukocyte adhesion efficiency in Slc35c1+/− (0.015 ± 0.002) and Slc35c1bm−/− mice (0.021 ± 0.008) when compared with littermate control mice (0.27 ± 0.04; Figure 2B), demonstrating that Slc35c1+/− leukocytes are severely impaired in their ability to firmly adhere to the inflamed endothelium.

Taken together, theses results suggest that the lack of GDP-fucose transporter Slc35c1 causes absence of leukocyte rolling, which translates into a very poor endothelial adherence of each individual leukocyte. However, the strong leukocytosis that prevails in the LAD II model mice still allows a surprisingly high residual number of leukocytes to adhere to inflamed vessel endothelium.

**Recruitment of granulocytes to the inflamed peritoneum**

Next we were interested in how leukocytes in Slc35c1+/− mice migrate to sites of inflammation. To test this we made use of a thioglycollate-induced peritonitis model. At 4 hours after injection of thioglycollate into the peritoneum of littermate control and Slc35c1+/− mice, the number of peritoneal granulocytes was determined. Figure 3 shows that the migration of granulocytes to the inflamed peritoneum was dramatically reduced by 89% plus or minus 4% in Slc35c1+/− mice compared with control animals. The residual migration was not far above the background level which was determined by PBS treatment of control mice. These results show that, despite of the leukocytosis in Slc35c1+/− mice, the
absence of the GDP-fucose transporter strongly compromises leukocyte migration to sites of inflammation.

**Lymphocyte homing to LNs**

T cell–dependent antibody responses were shown to be normal in LAD II,14,15 which suggested that sufficient lymphocyte entry into LNs might be possible in this disease. This prompted us to analyze lymphocyte homing to LNs in Slc35c1−/− mice. In vivo homing was assessed with fluorescently labeled wild-type lymphocytes, which were injected intravenously into wild-type and Slc35c1−/− mice. After 3 hours, the percentages of labeled cells in PLNs and MLNs were determined by flow cytometry. Figure 4A shows that the percentages of homed lymphocytes in PLNs and MLNs of Slc35c1−/− mice were strongly decreased by 87% plus or minus 6% and 72% plus or minus 12%, respectively, when compared with control values. In addition, LNs of Slc35c1−/− mice were strongly hypocellular (Figure 4B). Consequently, the calculated absolute numbers of lymphocytes that accumulated in Slc35c1−/− PLNs and MLNs during the chosen time window were drastically reduced by 99% plus or minus 1% and 98% plus or minus 1%, respectively, as compared with control lymph nodes (Figure 4C).

To directly analyze the ability of lymphocytes to interact with Slc35c1-deficient HEVs, we prepared frozen sections of MLNs and allowed lymphocytes to bind. Lymphocytes bound to MLN sections from wild-type mice in a HEV-specific manner with little background binding to the LN parenchyma (Figure 5A). The figure also shows that considerably fewer lymphocytes bound to HEVs of Slc35c1-deficient MLNs. Cell binding was quantified and expressed as the number of HEV-bound cells per square millimeter of HEV area on the sections. In comparison with control HEVs, the Slc35c1-deficient HEVs bound 82% plus or minus 11% less lymphocytes per square millimeter of HEV area (Figure 5B). Interestingly, we found that the residual binding to Slc35c1-deficient HEVs could be blocked by 91% with an L-selectin–specific antibody (Figure 5B; inset).

Taken together, LN homing and binding data show that HEVs of Slc35c1−/− mice have a strong defect in supporting lymphocyte binding that translates into an almost absent accumulation of lymphocytes in PLNs and MLNs from the circulation. In addition, although L-selectin mediates the low residual lymphocyte binding to Slc35c1-deficient HEVs of frozen sections, this adhesion molecule can maximally account for the 1% to 2% residual lymphocyte homing in Slc35c1−/− mice in vivo.

**Lymphocyte homing to the spleen**

To address the role of the GDP-fucose transporter in lymphocyte homing to the spleen, we investigated spleen size and cellularity as well as lymphocyte homing to this organ in Slc35c1−/− mice. We found that spleens of Slc35c1−/− mice were devoid of detectable fucosylation (Figure 6A) but showed normal size and cellularity (Slc35c1−/−: 3.93 ± 0.60 × 10^7 vs wild-type: 4.04 ± 0.48 × 10^7 leukocytes/spleen; data from 5 spleens in each group). Importantly, spleen homing of fluorescently labeled lymphocytes obtained from lymph nodes or spleens of wild-type mice was absolutely normal in Slc35c1−/− mice (Figure 6B). Perhaps not surprisingly, homing of
hypofucosylated *Slc35c1*-deficient lymphocytes to the spleens of wild-type mice was not reduced either (data not shown).

It has been shown that fucoidan specifically blocks lymphocyte entry into the white pulp, the compartment in which T cell/B cell interactions can take place.25,26 This prompted us to analyze splenic white and red pulp in *Slc35c1*/H11002/H11002 mice. We first visualized the marginal zone that surrounds the white pulp in spleen sections and found that white pulp/red pulp architecture and distribution are unperturbed in LAD II model mice (Figure 7A). Specific white pulp homing in the deficient mice was assessed using fluorescently labeled wild-type splenic lymphocytes. At 3 hours after intravenous injection of these cells, spleens were harvested, sectioned, and stained with a marginal zone–specific antibody (Figure 7B).

Quantification of fluorescent cells in the white pulp of *Slc35c1*/H11002/H11002 mice disclosed that the number of homed lymphocytes in this compartment was rather slightly (but insignificantly) increased than decreased in comparison with control mice (Figure 7C). These data show that lymphocyte trafficking to the spleen is independent from *Slc35c1*, and that the spleen has the potential to serve as a substitute for the inaccessible lymph nodes in LAD II.

**Humoral response to a T cell–dependent antigen**

Similar to *Slc35c1*/H11002/H11002 mice, L-selectin–deficient mice show strongly reduced lymphocyte trafficking to LNs.39 Steeber et al39 subcutaneously immunized L-selectin/H11002/H11002 mice. This route of immunization primarily stimulates regional LNs. In accordance with the cellular trafficking defect, the same authors found that within the first week after subcutaneous immunization with KLH, a T cell–dependent antigen, KLH-specific antibody production was impaired. Following the immunization protocol of Steeber et al,39 we subcutaneously immunized *Slc35c1*/H11002/H11002 and littermate control mice with KLH. Table 2 shows that 7 days after immunization, the generation of KLH-specific IgM and IgG is reduced by 45% and 88%, respectively, in *Slc35c1*/H11002/H11002 mice. This resembles the data obtained in L-selectin/H11002/H11002 mice.39 In contrast to the subcutaneous route of immunization, intravenous injection of antigen directly induces immune responses in the spleen. As would be predicted from the normal lymphocyte homing to the spleen in *Slc35c1*/H11002/H11002 mice, we find that
generation of specific IgM and IgG antibodies upon intravenous immunization with KLH is as strong as in wild-type mice (Table 2). These data strongly support the view that impaired lymphocyte homing to LNs of \textit{Sle35c1}^{--} mice causes reduced or delayed immune responses in these organs, whereas unperturbed lymphocyte homing to the spleen allows normal spleen responses in the LAD II model mice.

**Discussion**

In this report we have analyzed in vivo leukocyte trafficking in mice deficient for the GDP-fucose transporter \textit{Sle35c1} and show strongly defective leukocyte rolling in inflamed cremaster muscle venules, impaired neutrophil trafficking to the inflamed peritoneum, virtually absent lymphocyte homing to LNs, but normal homing of lymphocytes to the spleen.

Similar to results reported in an earlier in vivo study in which leukocytes from patients with LAD II were injected into inflamed vessels of the rat mesentery,\textsuperscript{13} we found an almost complete absence of leukocyte rolling in TNF-\alpha (2-4 hours)--stimulated cremaster muscle venules of \textit{Sle35c1}--deficient mice. Leukocyte rolling in this setting is mostly dependent on interactions of P- and E-selectin with fucosylated core-2--decorated O-glycans on selectin ligands.\textsuperscript{41} A total of 2 fucosyltransferases, FucT-VII and FucT-IV, have been implicated in the posttranslational fucosylation of inflammatory selectin ligands in vivo. In mice double-deficient for these enzymes, leukocyte rolling in inflamed venules of the ear and the cremaster muscle was almost entirely lost.\textsuperscript{23,44} Together with our results, this implies that the GDP-fucose transporter is essential for maintaining sufficient transport of GDP-fucose into the Golgi apparatus, where this nucleotide sugar is used for posttranslational fucosylation of selectin ligands by FucT-VII and -IV.

In addition to the profound defect in leukocyte rolling, we also found a dramatic reduction in firm leukocyte arrest, although the observed reduction was less pronounced than anticipated. Furthermore, neutrophil recruitment to the inflamed peritoneum was also reduced by 89% in \textit{Sle35c1}^{--} mice. This shows that both leukocyte adhesion and transmigration into tissue are largely dependent on \textit{Sle35c1} expression. The residual adhesion and recruitment seen in \textit{Sle35c1}^{--} mice may in part be due to the 5-fold increase of neutrophils in the circulation of these mice. Taking this neutrophilia into account, a very low adhesion and recruitment efficiency can be calculated for \textit{Sle35c1}^{--} mice. Whether residual leukocyte arrest was preceded by occasional rolling events which escaped our detection or whether arrest was completely independent of rolling is unknown. Residual adhesion has been observed in P-, E-, and L-selectin triple-deficient mice, suggesting that selectin-independent mechanisms exist for the successful transition of free-flowing leukocytes via leukocyte rolling to firm leukocyte arrest.\textsuperscript{45,46} We and others have reported that \alpha_4-integrin can mediate selectin-independent leukocyte rolling and adhesion during inflammation.\textsuperscript{47,48} This mechanism may be responsible for the occurrence of residual adhesion in the absence of \textit{Sle35c1}.

The reduction of neutrophil recruitment to the inflamed peritoneum in \textit{Sle35c1}^{--} mice is very similar to that induced by blocking P- and L-selectins (89%--90%)\textsuperscript{49} and to that seen in FucT-IV/VII double-deficient mice (95%).\textsuperscript{23} Thus, the peritonitis data confirm that these 4 proteins appear to be largely dependent on \textit{Sle35c1} with little or no contribution of additional GDP-fucose transport systems. Price et al\textsuperscript{14} measured neutrophil emigration into the inflamed skin of a patient with LAD II. A 94% to 98% reduction of neutrophil recruitment was found. These results are consistent with our data from the murine peritonitis model.
Besides the perturbated neutrophil function in Slc35c1−/− mice, we show for the first time that the absence of a functional GDP-fucose transporter also affects a critical function of lymphocytes, the homing to LNs. We found that absolute numbers of lymphocytes that homed to PLNs and MLNs in Slc35c1−/− mice were reduced by 99% and 98%, respectively. This homing defect is at least as strong as that in FucT-IV-VII double-deficient mice (reduction of 99% and 96%, respectively). This implies that the residual binding of L-selectin is largely dependent on L-selectin. The basis for the homing defect in Slc35c1−/− mice is identical to the reduction in lymphocyte homing in the presence of L-selectin blocking mAb Mel-14 and in L-selectin–deficient mice. We now show that lymphocyte homing to the splenic white pulp is independent of L-selectin. Moreover, lymphocyte homing to the spleen appears to be independent from L-selectin, P-selectin, P-selectin glycoprotein ligand-1, MadCAM-1, and the modifying enzymes FucT-IV and FucT-VII.

### Table 2. Humoral responses to a T-cell–dependent antigen

<table>
<thead>
<tr>
<th>Route of Immunization</th>
<th>IgM, µg/mL</th>
<th>IgG, µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td><strong>Subcutaneous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Day 3</td>
<td>2.22 ± 0.25</td>
<td>0.93 ± 0.21</td>
</tr>
<tr>
<td>Day 5</td>
<td>3.01 ± 0.35</td>
<td>1.47 ± 0.32</td>
</tr>
<tr>
<td>Day 7</td>
<td>3.23 ± 0.35</td>
<td>1.78 ± 0.18</td>
</tr>
<tr>
<td><strong>Intravenous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Day 5</td>
<td>5.0 ± 0.25</td>
<td>5.5 ± 0.21</td>
</tr>
<tr>
<td>Day 9</td>
<td>5.0 ± 0.18</td>
<td>6.0 ± 0.22</td>
</tr>
<tr>
<td>Day 14</td>
<td>8.0 ± 1.27</td>
<td>8.5 ± 0.35</td>
</tr>
</tbody>
</table>

*Slc35c1−/− and wild-type littermate mice were immunized subcutaneously or intravenously with 100 µg KLH at day 0. At the indicated times the concentrations of KLH-specific antibodies in the sera were determined by ELISA. Values (± SD) are from 6 mice in each group.

It should be noted that the strong LN homing defect in L-selectin−/− mice does not preclude the LNs from participating in later phases of an immune response, probably because a low influx of cells is sufficient once activation signals have impaired the egress of lymphocytes from the LNs. Such an effect may also apply to Slc35c1−/− mice and patients with LAD II.

It is interesting to compare lymphocyte homing in Slc35c1−/− mice with homing in the presence of L-selectin blocking mAb Mel-14 and in L-selectin–deficient mice. Although all these data suggest that spleen homing is independent from the conventional role of fucosylation (ie, allowing interactions between selectins and their fucosylated ligands), several studies have shown that the sulphated L-fucose polymer fucoidan efficiently inhibits lymphocyte homing to the spleen. Moreover, fucoidan alters the distribution of the homed cells within the spleen, allowing fewer cells to enter the white pulp. These data suggested that fucoidan inhibits cellular recognition of fucosylated and/or sulphated structures that would normally promote entry to the spleen.

We now show that lymphocyte homing to the splenic white pulp is not perturbed in the strongly hypofucosylated Slc35c1−/− mice. This finding argues against a role of fucosylation in spleen homing. Thus, the data obtained with fucoidan may rather be interpreted as an effect of the multiple sulfate groups that are present in this compound.
limited, adaptive immunity in patients with LAD II. Spleen-mediated immunity may explain why the immunodeficiency in LAD II is milder than one would expect from the complete lack of selectin interactions that is seen in this disease.

Acknowledgments
We thank Dr Barry Wolitzky (MitoKor, San Diego, CA) for providing the E-selectin blocking antibody 9A9, Viktor Wixler (University of Münster) and Birgit Kempe for help with immunizations, and Dietmar Vestweber (Max Planck Institute for Molecular Biomedicine, Münster, Germany) and Kerstin Lühn (University of Oxford, United Kingdom) for very helpful discussions.

This work was supported by the SFB 293 of the Deutsche Forschungsgemeinschaft (DFG) (M.K.W.), by the Max Planck Society (M.K.W.), by DFG grant SP621/3-1 (M.S.), by a fellowship of the International Graduate Research School “Molecular Basis of Dynamic Cell Processes,” GRK 1050 (S.Y.), by Ludwig-Maximilians-University Innovative BioImaging, and by a PhD fellowship of the Ev. Studienwerk e.V. Villigist (C.C.H.).

Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Martin K. Wild, Max Planck Institute for Molecular Biomedicine, Rönntgenstr. 20, 48149 Münster, Germany; e-mail: mwild@mpi-muenster.mpg.de; or Markus Sperandio, Walter Brendel Center of Experimental Medicine, Ludwig-Maximilians-Universität, Marchioninistr. 15, 81377 München, Germany; e-mail: markus.sperandio@med.uni-muenchen.de.

References


Leukocyte trafficking in a mouse model for leukocyte adhesion deficiency II/congenital disorder of glycosylation IIc

Sviatlana Yakubenia, David Frommhold, Dirk Schölich, Christina C. Hellbusch, Christian Körner, Björn Petri, Claire Jones, Ute Ipe, M. Gabriele Bixel, Robert Krempien, Markus Sperandio and Martin K. Wild