A CD99-related antigen on endothelial cells mediates neutrophil, but not lymphocyte extravasation in vivo

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Running title: CD99L2 participates in neutrophil diapedesis

Supported by funding through the Interdisziplinäres Klinisches Forschungszentrum (IZKF) Münster and the Max-Planck-Gesellschaft to D.V. and through the European Community (NoE MAIN 502935) to F.K.

M.G.B., B.P., A.G.K., A. K., K. W.-B., H.W., S.M. performed, designed and analyzed experiments; F.K., and D.V. initiated the study, designed and supervised the research project; M.G.B. and D.V. contributed to data analysis and interpretation and wrote the paper.

Word count: Text: 4968, abstract: 200

Scientific Heading: Immunobiology

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Abstract

CD99 is a long known leukocyte antigen that does not belong to any of the known protein families. It was recently found on endothelial cells where it mediates transendothelial migration of human monocytes and lymphocyte recruitment into inflamed skin in the mouse. Here we show that a recently cloned, widely expressed antigen of unknown function with moderate sequence homology to CD99, called CD99L2, is expressed on mouse leukocytes and endothelial cells. Using antibodies, we found that CD99L2 and CD99 are involved in transendothelial migration of neutrophils in vitro and in the recruitment of neutrophils into inflamed peritoneum. Intravital and electron microscopy of cremaster venules revealed that blocking CD99L2 inhibited leukocyte transmigration through the vessel wall (diapedesis) at the level of the perivascular basement membrane. Surprisingly and in contrast to CD99, CD99L2 was not relevant for the extravasation of lymphocytes into inflamed tissue. Although each protein promoted cell aggregation of transfected cells, endothelial CD99 and CD99L2 participated in neutrophil extravasation independent of these proteins on neutrophils. Our results establish CD99L2 as a new endothelial surface protein involved in neutrophil extravasation. In addition, this is the first evidence for a role of CD99 and CD99L2 in the process of leukocyte-diapedesis in vivo.
Introduction

Leukocytes are recruited into inflamed tissue via endothelial adhesion molecules and chemoattractants\(^1\text{-}^4\). Upon docking to the endothelial surface, which is mediated mainly via selectins and leukocyte integrins, leukocytes transmigrate through the vessel wall, a process called diapedesis\(^5\text{-}^6\). Leukocytes have been reported to traverse the endothelial cell layer either on a transcellular or a paracellular, junctional route\(^7\text{-}^8\). Almost all known endothelial membrane and adhesion molecules that participate in the diapedesis process are located at endothelial cell contacts and therefore are likely to participate in the junctional migration of leukocytes.

VE-cadherin represents a barrier on this route, since adhesion-blocking antibodies against VE-cadherin accelerate recruitment of leukocytes into inflamed tissue\(^9\). In contrast to VE-cadherin, all other endothelial cell contact proteins assist in the diapedesis process. All of these proteins, except one, are members of the immunoglobulin supergene family (Ig-SF), such as platelet-endothelial cell adhesion molecule (PECAM)-1\(^10\text{-}^11\), members of the junctional adhesion molecule (JAM) family\(^12\text{-}^14\), endothelial cell-selective adhesion molecule (ESAM)\(^15\), intercellular adhesion molecule (ICAM)-2\(^16\) and polyo virus receptor (PVR)\(^17\), a member of the nectin family. It is not yet known how they function in detail and it will be a challenging goal for the future to elucidate potential cascades and the interplay of these endothelial cell contact proteins during leukocyte diapedesis.

One of the most recently identified proteins participating in leukocyte diapedesis is CD99, a long known leukocyte surface antigen, which was found to be expressed at endothelial cell contacts and to participate in the in vitro transmigration of human monocytes through monolayers of human endothelial cells\(^18\). That CD99 is indeed relevant for leukocyte extravasation in vivo was demonstrated in the mouse. Antibodies against mouse CD99 inhibited the recruitment of lymphocytes into inflamed sites of the skin\(^19\). Whether CD99 would be relevant for the extravasation of myeloid cells in vivo has not yet been analyzed.

CD99 is a rather small membrane protein with a highly O-glycosylated extracellular part comprising only about 100 amino acids. Based on its primary structure it is not related to any known protein family. Three other genes have been described of which two are mapping in the vicinity of the CD99 gene in the human genome and displaying moderate sequence homology to CD99. One of them (MIC2R) is a pseudogene, the second, PBDX, codes for an erythrocyte cell surface antigen (Xga) of unknown function. The third related gene coding for a protein of unknown function was recently cloned in various species\(^20\). Sequencing revealed
a homology of not more than 32% amino acid identity between this gene and CD99 in the mouse. Based on in situ hybridizations the mRNA seems to be widely distributed, with prominent expression on neuronal cells, choroid plexus, sertoli cells, and granulose and theca cells of the ovary. Although no studies have been performed that would attribute any function to the corresponding protein, and expression on leukocytes has not been analyzed, it was tentatively named CD99 antigen like-2 (CD99L2).

Here we have analyzed whether CD99L2 would indeed be functionally related to CD99 and whether it would be relevant for the process of leukocyte extravasation. We generated antibodies against mouse CD99L2 with which we found that this antigen is expressed on neutrophils and lymphocytes as well as on endothelium in various tissues. Despite its strong expression on lymphocytes and in contrast to CD99, CD99L2 was not involved in the recruitment of lymphocytes into inflamed skin. However, in two different inflammation models, antibodies against CD99L2 inhibited the extravasation of neutrophils. Intravital and electron microscopy allowed to demonstrate that CD99L2 was not relevant for the contact formation between leukocytes and endothelium, but rather for the actual transmigration step. In addition, CD99 was also found to participate in neutrophil diapedesis in vivo. Our results establish CD99L2 as a new endothelial surface protein that participates in the extravasation and diapedesis of neutrophils.
Materials and Methods

Cell culture

The following cells were propagated as described: Proteinlipid protein (PLP)-specific Th1 memory/effector T cell line SJL.PLP721, Chinese hamster ovary (CHO) cells22, and bEnd.5 mouse endothelioma cells23, human umbilical vein endothelial cells24. CHO cell lines expressing the E3-E4 form or the E3A-E3-E4 form of mouse CD99L2 or the respective CD99L2-Fc fusion protein were generated by electroporation according to established procedures25. COS-7 cells were transiently transfected with mouse CD99 full length or the E3-E4 form of CD99L2 full length according to the manufacturer’s protocol (GeneJammer, Stratagene, La Jolla, CA).

Antibodies

Polyclonal rabbit antisera against mouse CD99L2 (E3-E4 form) were generated against the CD99L2-Fc fusion protein. Antibodies against the IgG1-Fc part were removed from the serum by incubation with human IgG1-coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech, Freiburg). Specific antibodies against CD99L2 were affinity-purified with CD99L2-Fc immobilized on CNBr-Sepharose. F(ab’)_2 fragments were generated with immobilized pepsin on beads (Pierce, Rockford, IL, USA) according to the manufacturer’s protocol. uncleaved IgG and Fc fragments were removed using protein A sepharose (Amersham Pharmacia Biotech, Freiburg). Purity (less than 0.3% intact IgG) and proper size of the F(ab’)_2 fragments were confirmed by SDS-PAGE. Relative binding efficiency was tested by ELISA using CD99L2-Fc as antigen. No endotoxin was detectable. Affinity purified polyclonal rabbit antibodies against mouse CD99 and mouse ESAM were generated as described previously25. Monoclonal antibodies were purified from hybridoma supernatants: anti-mouse ICAM-1 (YN1/1.7, rat IgG-2b, from ATCC)26, anti mouse L-selectin (MEL14, from ATCC); anti-mouse P-selectin (RB40.34, rat IgG1); anti mouse PECAM-1 (1G5.1, rat IgG2b; 5D2.6, rat IgG2a) (both unpublished). Purchased antibodies: anti-LFA-1 (BD Pharmingen). Cy3-conjugated anti-rabbit IgG, PE-conjugated anti-rabbit IgG, peroxidase-conjugated anti-rabbit IgG (Dianova, Hamburg, Germany); FITC-labeled and peroxidase-labeled polyclonal antibodies to rabbit IgG F(ab’)_2 (Acris, Hiddenhausen, Germany). Immunofluorescence staining and western blots were done as described25.
Glycosidase treatment
3 µg of CD99L2-Fc fusion protein as digested in 150 µl of 25 mM NaH$_2$PO$_4$, pH6.0 for 18 h at 37°C. The reaction was stopped by boiling in SDS-PAGE sample buffer and 100 ng of the reaction mixture were electrophoresed, and detected by immunoblotting with peroxidase conjugated anti-human IgG followed by ECL.

Cloning of mouse CD99L2 isoforms
For generation of a mouse CD99L2-Fc fusion protein, total cellular RNA was isolated from mouse spleen cells using the Trizol reagent (Invitrogen) according to the manufacturer’s protocol. A cDNA fragment coding for the extracellular part (amino acid 1 to 161) of the E3-E4 form of mouse CD99L2 (NM_138309, GenBank TM/EBI) was generated by RT-PCR from total spleen RNA, using a HindIII site-containing sense oligonucleotide 5’-TAGTAGAAGCTTCTGCGCCTGCTCCCAGCCAT-3’ and an EcoRI site-containing antisense oligonucleotide 5’-CTACTAGAATTCACTTACCTCCAGTTTCTGTGCGATATGCC-3’. The product was inserted into a pcDNA3 based Fc-construct vector (pcDNA3/hlgG-Fc) in frame and upstream of a fragment of human IgG1 covering bases 553-1803 (hinge, C$_\text{H2}$, C$_\text{H3}$). A full length cDNA for mouse CD99L2 was generated by RT-PCR from total RNA from mouse spleen using the sense oligonucleotide 5’-CTCCTGCGCTAACTGTGCTCCT-3’ and the antisense oligonucleotide 5’-AATCCGGGGTGCTCGGTTGATT-3’. The PCR product was inserted into pcDNA3 (Invitrogen).

For detection of the splice variants of CD99L2 in endothelial cells, the following primers were used: For mouse CD99L2: sense primer 5’-CTCCTGCGCTAACTGTGCTCCT, antisense primer 5’-GGTCACAAGCGGTGGCTTCCT; for human CD99L2: sense primer 5’-GCTCTCGCGCTAACTGTGCTC, antisense primer 5’-GTGGCACCATTGTGCATGCCTG. RT-PCR was performed with total cell RNA from mouse endothelioma cells bEnd.5 and from HUVEC.

Immunohistochemistry and flow cytometry
Animals were anaesthetized using Isoflurene anesthesia (Abbott, Wiesbaden, Germany), and were perfused with 10 ml PBS through the left ventricle of the heart. Tissue was removed, embedded in Tissue-tec, (OCT, Miles Inc., Giessen, Germany), and snap-frozen. Cryostat sections (6 µm) were stained as described$^{27}$. Flow cytometry was done as described$^{28}$. 
Aggregation assay
Our assay was modified from a previously described method and was done as described. Quantification of aggregation was estimated by the following formula: % aggregation = (N₀ – Nₜ)/N₀ x 100, where Nₜ is the total number of particles at the incubation time t, and N₀ is the total number of cells. In some experiments, a mixed population of untransfected and CD99L2-transfected CHO cells was subjected to cell aggregation assays. One cell type had been labeled (20µM/5x10⁶) with cells Cell Tracker™ Green CMFDA (5-chloromethylfluorescein diacetate) (Invitrogen) and the number of cells per cell type each cells in each aggregate was counted in a fluorescence microscope.

Adhesion assay
Adhesion assays were done as described, except for that PMNs were used instead of lymphocytes.

Preparation of mouse bone marrow neutrophils
NMRI mice (Harlan-Winkelmann, Borchen, Germany) were sacrificed, femurs and tibias were cut out and muscles were removed. Bones were placed in Ca²⁺/Mg²⁺-free HBSS (HBSS-) to prevent dry-out. The ends of the bones were cut and the bone marrow was flushed into a 50ml tube with HBSS-/ (Gibco, Eggenstein, Germany) using a short (5/8’’) 25 G needle and a 2 ml syringe. Larger bone marrow pieces were disaggregated using a plastic pipette and a mesh (70 µm). The cell suspension was centrifuged (300 x g, 10 min) and the pellet was re-suspended in 1 ml HBSS-/-. The cell suspension was then added on top of a 10 ml tube containing 4ml Histopaque 1119 an 4 ml Histopaque 1077 (Sigma-Aldrich, Steinheim, Germany). After centrifugation for 30 min at 700 g (brake off), PMN-containing cell layer was collected and washed two times with HBSS-/-.

Transmigration assay
Transendothelial migration of SJL.PLP7 and PMNs was performed as described. 5x 10⁴ bEnd.5 cells/well of 6.5 mm Transwells (costar, Bodenheim, Germany) were grown for two days for each assay. For assays with PMNs, 5x10⁵ PMNs were added per transwell and chemoattraction was done with 40 ng/ml KC in the bottom chamber. Migrated leukocytes were collected for cell counting (CASY, Schärfe-System, Reutlingen, Germany). Confluency of the endothelial monolayer was confirmed after each assay on formalin fixed and 2.6% Giemsa stained inserts.
**Contact hypersensitivity and T cell immigration into inflamed skin**
Experiments were done as described\(^{19}\).

**Thioglycollate-induced peritonitis assay**
For each assay 5 female Balb/c mice 8-12 weeks were used. Thioglycollate-induced peritonitis assays (induced by 3% sterile thioglycollate broth) were essentially performed as described\(^{28}\), except for the peritoneal lavage was done with 20 mL PBS containing 3 mM EDTA, total cell counts were determined with a cell counter and the percentage of neutrophils was determined by flow cytometry (FACS Calibur, BD Heidelberg) using the mAb RB6-8C5 against Ly-6G and Ly6C (Gr-1).

**Intravital microscopy**
Surgical preparation of cremaster muscles and intravital microscopy were essentially done as described\(^{15,30}\). Inflammatory stimulation was achieved by intrascrotal injection of 50 ng of rmIL-1\(\beta\) (R & D Systems) diluted in 0.3 mL PBS, administered to mice 4h prior to microscopic observation (n=6 each group). At the same time 50 \(\mu\)g of affinity purified rabbit IgG (against CD99 or CD99L2), or control rabbit IgG, or 75 \(\mu\)g of F(ab’)\(2\)-fragments were injected intravenously. For each animal, 3-5 single unbranched postcapillary venules with diameters 17-35 \(\mu\)m were analyzed.

**Electron microscopy**
Four hours after inflammatory stimulation and antibody injection (as described above) the cremaster muscle was dissected and prepared for electron microscopy as described for other tissues before\(^{31}\). For each vessel, the number of leukocytes in each of the following positions was determined: A: within lumen of vessel, B: crossing the endothelium, C: between endothelium and basement membrane/pericyte; D: outside the vessel but within 50 \(\mu\)m of it. The fraction of leukocytes that were diapedesing, but were still inside the basement membrane or pericyte layer was calculated by the equation C/C+D. For each antibody type, 32-52 vessels of 2-3 mice were evaluated.
Results

**Generation and characterization of specific antibodies against CD99L2**

In the course of cloning the mouse ortholog of human CD99\(^{19}\), we found additional clones in the mouse gene database that coded for a CD99 related protein (MIC2-like1), for which later the name CD99L2 has been suggested\(^{20}\). Based on the sequence of clone NM_138309 we designed primers with which we cloned two RT-PCR products of 660 bp and 729 bp from poly A\(^+\)-RNA of mouse spleen. Sequencing the smaller product revealed full match with clone NM_138309. The larger product was identical except for an additional 69 bp insertion. During the course of our project this larger form has been described in the gene database and the inserted sequence has been designated as exon 3A\(^{20}\).

In order to study the CD99L2 protein, we generated polyclonal antibodies against a mouse CD99L2-Fc fusion protein containing the complete extracellular part of the shorter splice variant of CD99L2 (E3-E4 form) fused to the Fc part of human IgG\(_1\). Polyclonal antibodies against mouse CD99 had been raised against a CD99-Fc fusion protein and were described before\(^{19}\). Since mouse CD99L2 and CD99 show 32% amino acid identity, we tested our affinity purified antibodies against CD99L2 and CD99 for cross-reactivity. The antibody preparations had been extensively depleted for antibodies directed against the Fc-part of the fusion proteins. As shown in Fig. 1A anti CD99L2 antibodies only detected CD99L2-Fc but not CD99-Fc or ESAM-Fc in immunoblots. Likewise, anti-CD99 antibodies recognized CD99-Fc, but not CD99L2-Fc or ESAM-Fc (Fig. 1A). Furthermore, COS7 cells transfected with CD99L2 were brightly stained with anti-CD99L2 antibodies, while only background staining was observed with CD99-transfected cells. Similarly, anti-CD99 antibodies stained COS7 cells transfected with CD99, but no signal was obtained when cells were transfected with CD99L2 (Fig. 1B). Thus, our affinity-purified antibodies against CD99L2 and CD99 are strictly specific for their respective antigen.

**CD99L2 is expressed on endothelial cells**

To characterize the proteins corresponding to the two splice variants of CD99L2, CHO cells transfected with the short (CD99L2\(_S\)) and the long (CD99L2\(_L\)) splicing variant of CD99L2 were analyzed by immunoblotting. This revealed that CD99L2\(_S\) gave rise to a 40kD/37kD doublet whereas the longer form was recognized as a 56kD/40kD doublet (Fig. 2A). Specificity of the signals was confirmed by blocking studies with the CD99L2-Fc fusion protein. It is likely that the smaller molecular weight bands detected in each case in
transfected CHO cells represent either degradation products or glycosylation variants, due to ectopic expression. Since the sequence-based calculation of the molecular weight of the two splicing variants would predict sizes of 23.5 and 26.1 kDa, respectively, CD99L2 is likely to be glycosylated. The lack of N-glycosylation sites suggests O-glycosylation. Indeed, treatment of CD99L2 with neuraminidase followed by O-glycosidase caused a shift in molecular weight, demonstrating O-glycosylation (Fig. 2B).

In immunoblots of the mouse endothelioma cell line bEnd.5, antibodies against CD99L2 recognized a major band at 56 kD and a minor band at 40 kD (Fig. 2A, lane 8). To determine whether these two bands could indeed represent the two splice variants we performed RT-PCR experiments with total RNA from bEnd.5 cells. As shown in Fig. 2C, two cDNA fragments were generated with the higher molecular weight form being the dominant one. The large splice variant was also the dominant one in HUVEC, besides two minor smaller variants (Fig. 2C). Sequencing revealed that these products originated from the known splice variants. Thus, endothelial cells express mainly the larger splice variant of CD99L2, and only very small amounts of the smaller ones.

To examine the subcellular distribution of CD99L2, unfixed bEnd.5 endothelioma cells were incubated at 4°C with anti-CD99L2 antibodies, washed, fixed and then stained with secondary antibodies. CD99L2 staining was found to be concentrated at cell-cell contacts of cultured endothelial cells, although it was also found on the entire surface of the cells (Fig. 2D). On cryosections of various tissues such as heart, kidney and liver, antibodies against mouse CD99L2 stained endothelial cells (Fig. 2E) similarly to antibodies against CD99 which were used for controls.

**CD99L2 is expressed on leukocytes**
Cell surface expression of CD99L2 on mouse leukocytes was analyzed by flow cytometry. T and B cells as well as neutrophils from peripheral blood were strongly positive for CD99L2 and the expression levels were comparable to those of CD99 (Fig. 3). Analyzing leukocytes that we routinely use in in vitro transendothelial migration assays, we found that the T cell line SJL.PLP7 expressed CD99L2 at similar levels as primary lymphocytes, whereas bone marrow derived neutrophils expressed almost undetectable levels of CD99L2 and well detectable levels of CD99. The drastic difference between the CD99L2-expression levels on peripheral and bone marrow derived neutrophils makes CD99L2 one of very few antigens induced with neutrophil maturation.
CD99L2 supports cell aggregation of transfected cells

CD99 has been described to support homotypic cell aggregation, as was demonstrated for L cells transfected with human CD99\textsuperscript{18} and for CHO cells transfected with mouse CD99\textsuperscript{19}. To test whether CD99L2 would be able to support cell aggregation we stably transfected CHO cells with either CD99L2\textsubscript{L} or CD99 L2\textsubscript{S}. As shown in Fig. 4A, each of the two splice variants of CD99L2 enabled CHO cells to aggregate and this homotypic cell aggregation required the presence of divalent cations, since it was strongly reduced in the absence of Mg\textsuperscript{2+} and Ca\textsuperscript{2+}. Surprisingly, and in clear contrast to CD99, antibodies against CD99L2 could not block CD99L2-dependent cell aggregation, whereas anti-CD99 antibodies, as we showed before, efficiently blocked aggregation of CD99 transfected cells (Fig. 4B). Thus, either our anti CD99L2 antibodies are not able to block a potential adhesive function of CD99L2, or CD99L2 is stimulating the adhesive function of other adhesion molecules.

It is unlikely that cell aggregation was dependent on homophilic CD99L2 interactions, since mixed populations of CD99L2-transfected and of mock-transfected CHO cells aggregated as well as the same number of CD99L2-transfected cells. Aggregates of mixed cell populations contained 50% transfected and mock-transfected cells, respectively (Fig. 4C). In agreement with this, we could not pull down CD99L2 from lysates of transfected cells with CD99L2-Fc (not shown).

CD99L2, in contrast to CD99, is not required for lymphocyte extravasation

We have recently found that mouse CD99 is required for the migration of T cells through the monolayer of endothelial cells, as was shown by blocking transmigration of SJL/PLP7 T cells through the monolayer of bEnd.5 endothelial cells with anti-CD99 antibodies\textsuperscript{19}. Since both cell types express CD99L2 (as described above), we tested anti-CD99L2 antibodies in this assay. As shown in Fig. 5A, no inhibitory effect of the antibodies was detected, whereas anti CD99 and anti ICAM-1 antibodies, as expected, efficiently inhibited T cell transmigration. The assays were performed for 30 min with the antibodies present during the assay and in the presence of the chemoattractant SDF-1 in the lower chamber. Comparable results were obtained when transmigration was allowed for 2 h in the absence of a chemokine (not shown).

Despite this lack of inhibition, we tested whether these antibodies would affect the recruitment of T cells into inflamed areas of the mouse skin in a typical delayed type hypersensitivity (DTH) model. In vivo activated T cells were isolated from lymph nodes of
dinitrofluorobenzene (DNFB) treated mice, labeled with $^{51}$Cr and injected i.v. into mice with a DNFB-elicited DTH reaction in the right ear. Affinity purified antibodies against CD99L2, negative control antibodies from pre-immune serum or from a serum against ESAM and positive control antibodies against E- and P-selectin were co-injected with the T cells. Mice were sacrificed 15 h later and radioactivity in the right ear was determined. As shown in Fig 5B, T cell recruitment was only inhibited with antibodies against E- and P-selectin, whereas the antibodies against CD99L2 had no effect. Thus, CD99L2 seems to differ considerably from CD99 and only antibodies against the latter interfere with lymphocyte extravasation.

**CD99L2 as well as CD99 are involved in the transendothelial migration of neutrophils in vitro**

Since human CD99 had been reported to be involved in the transmigration of human monocytes, we tested, whether mouse CD99 or CD99L2 are relevant for the transendothelial migration of mouse myeloid cells. Due to the low yields of mouse neutrophils that can be obtained from blood, we used neutrophils isolated from bone marrow, although they expressed only low amounts of CD99 and almost undetectable levels of CD99L2. Remarkably and in contrast to the situation with lymphocytes, antibodies against each antigen, CD99 as well as CD99L2, clearly inhibited transmigration of neutrophils (Fig. 6A). A similar result was found for F(ab’)2 fragments of both antibody preparations, excluding potential problems with myeloid Fc-receptors (Fig. 6A). This establishes for the first time CD99 and CD99L2 as molecules involved in the transendothelial migration of mouse neutrophils. In addition, it demonstrates that our anti CD99L2 antibodies are indeed able to block a function of this antigen.

The low expression of CD99 and the hardly detectable expression of CD99L2 on bone marrow derived neutrophils already suggested that the antibodies affected transmigration via binding to the endothelial antigens. To test this more directly, we pre-incubated bEnd.5 cells with either anti CD99 or anti CD99L2 antibodies and washed them away prior to the assay. We found that this treatment reduced neutrophil transmigration almost as efficiently as when antibodies were still present during the assay (Fig. 6B), demonstrating that indeed each of the two antigens act on the endothelial side. Pre-incubating only neutrophils with antibodies prior to the assay did not yield conclusive results, since the test antibodies as well as negative control antibodies from the pre-immune sera inhibited transmigration, possibly due to unspecificly activating neutrophils via Fc-receptors. In order to avoid this problem, we pre-incubated neutrophils with F(ab’)2-fragments of anti CD99, anti CD99L2 and pre-immune
antibodies prior to the assay. As shown in Fig. 6C, neither anti CD99 nor anti CD99L2 F(ab’)_2-fragments inhibited neutrophil transmigration. This had been expected for CD99L2, since this antigen had not been detected in significant amounts on neutrophils. However, it is surprising that CD99 on neutrophils was not involved in neutrophil transmigration, since the transmigration of lymphocytes requires CD99 on the lymphocyte side as well as on the endothelial surface.

To determine whether CD99 and CD99L2 act in independent pathways, we tested whether F(ab’)_2-fragments of antibodies against each antigen would have additive inhibitory effects. As shown in Fig. 6D, both types of F(ab’)_2-fragments together had no stronger inhibitory effect than F(ab’)_2-fragments against each antigen alone. We conclude that the CD99-independent extravasation of neutrophils is also CD99L2-independent.

In order to distinguish whether any of the two antigens would participate in neutrophil transmigration via mediating adhesion of neutrophils to endothelial cells or via participating in the transendothelial migration step, we tested whether the antibodies would inhibit adhesion of neutrophils to bEnd.5 cells. No such inhibitory effect was found (Fig. 6E). We conclude that CD99 as well as CD99L2 on endothelial cells are involved in the migration of neutrophils through endothelial cell layers.

**CD99 and CD99L2 participate in the migration of neutrophils into inflamed peritoneum.**

Chemically induced peritonitis is a well-characterized model to study neutrophil recruitment into sites of acute inflammation in vivo. We tested whether i.v. injection of either anti CD99 or anti CD99L2 antibodies immediately before i.p. injection of thioglycollate would affect the recruitment of neutrophils into the peritoneum within a 4 h period after stimulation. We found that antibodies against CD99 reduced neutrophil accumulation by 59% (±12%), whereas anti CD99L2 antibodies inhibited by 69% (±5%) when compared with negative control antibodies from the pre-immune sera (Fig. 7A). Combined treatment with anti L- and anti P-selectin antibodies blocked neutrophil recruitment by more than 85%. Injection of PBS without antibodies gave similar results as negative control antibodies (not shown). To exclude the possibility that interactions with Fc-receptors or complement would interfere with our results, we also tested F(ab’)_2-fragments. As shown in Fig. 7A, F(ab’)_2-fragments of anti CD99 as well as of CD99L2 antibodies reduced neutrophil recruitment into inflamed peritoneum significantly, although the inhibitory effect was less pronounced compared to the decrease observed with intact antibodies.
Antibodies against CD99 or CD99L2 inhibit neutrophil extravasation in vivo at the level of transmigration

To clarify in vivo at which step during the process of leukocyte extravasation CD99L2 and CD99 are involved, we visualized the process in the cremaster muscle using intravital near-infrared reflected light oblique transillumination microscopy. Under the experimental conditions used, the vast majority (more than 80%) of extravasating leukocytes are neutrophils. Mice were intravenously injected with antibodies and stimulated intrascrotally with IL-1β for 4 h prior to microscopic analysis. As can be seen in (Fig. 7B), neither anti CD99 nor anti CD99L2 antibodies affected rolling or adhesion of leukocytes in the observed cremaster venules. Instead, antibodies against both antigens significantly reduced the number of extravasated neutrophils accumulating in the observation area. Similar results were obtained with F(ab')2-fragments (Fig. 7B). Thus, CD99 as well as CD99L2 are involved in vivo in the diapedesis of neutrophils.

To determine at which step of the diapedesis process leukocytes are arrested by the antibodies against CD99L2, the cremaster tissue was analyzed by electron microscopy. Animals treated with antibodies against CD99L2 or against PECAM-1 had significantly larger numbers of leukocytes trapped between the endothelium and the basement membrane/pericyte layer than animals treated with control antibodies (Fig. 7C and D). Independent of the type of antibody used, the number of leukocytes trapped between endothelial cells (category B) was very small (3–5% of all extravasating cells: B/B+C+D) with no significant changes between the different groups.
Discussion

CD99L2 was recently cloned as a CD99-related gene coding for a protein of unknown function, which is widely distributed in various tissues. Here we have identified CD99L2 as a novel cell surface protein on endothelial cells and leukocytes that participates in vivo in the recruitment of neutrophils into inflamed tissue.

Comparing the function of CD99L2 with that of CD99 in more detail revealed important similarities and striking differences. There are four important characteristics that both proteins share. First, both proteins are structurally related with 32% amino acid identity between mouse CD99 and CD99L2. They are both type 1 transmembrane proteins with extracellular domains only slightly larger than 100 amino acids, rich in O-linked carbohydrates, with no resemblance to any known protein family. Second, both proteins are expressed on endothelium as well as on lymphocytes and neutrophil granulocytes. Third, both proteins support cell aggregation upon ectopic expression in CHO cells. Fourth and most importantly, both proteins participate in the diapedesis process and not in the docking of leukocytes to the vessel wall. While this was already anticipated for CD99 from in vitro transmigration assays, our results demonstrate this for the first time in vivo, for CD99 as well as for CD99L2. Thus, CD99 and CD99L2 represent a new class of membrane proteins clearly distinct from the Ig-SF, that participate in leukocyte diapedesis.

Importantly, we could demonstrate by electron microscopy that anti-CD99L2 antibodies trap neutrophils between the basal side of the endothelium and the basement membrane/ pericyte layer similarly as anti PECAM-1 antibodies. This confirms other studies for PECAM-1 and establishes CD99L2 as the second endothelial cell contact protein for which it has been determined in vivo at which step during diapedesis it is required.

Despite the many similarities, CD99 and CD99L2 differ in important aspects. Most importantly, they participate in the extravasation of different repertoires of leukocytes. Whereas both proteins mediate the extravasation of neutrophils, only CD99 is relevant for the extravasation of lymphocytes, despite strong expression of CD99L2 on lymphocytes. The possibility that CD99L2 might even participate in lymphocyte extravasation and that our antibodies are merely unable to block this process is unlikely, although cannot be ruled out.

It has been suggested that CD99 may participate in lymphocyte diapedesis via homophilic interactions. This is based on the findings that transmigration required CD99 on lymphocytes and on endothelial cells and that CD99 supports homotypic cell aggregation of transfected cells. Similar results have been demonstrated for human CD99 and its role in...
transmigration of monocytes. Obviously CD99L2 on lymphocytes is not able to function in the same way, since it is not involved in lymphocyte diapedesis although it is present on the surface of both cell types.

Interestingly, neutrophils seem to extravasate in a different way than lymphocytes with respect to the participation of CD99 and CD99L2. Both proteins on endothelial cells are involved in the transendothelial migration of neutrophils, since antibodies against each of the two proteins inhibited transmigration, when incubated with endothelial cells only. It is unlikely that endothelial CD99 or CD99L2 participated in diapedesis by homophilic binding to their respective counterpart on neutrophils since, first, CD99L2 was almost undetectable on bone marrow derived neutrophils and CD99 was only weakly expressed. Second and more importantly, even if the weak expression levels of CD99 and CD99L2 on bone marrow derived neutrophils would have been sufficient to serve as binding partners for endothelial CD99 or CD99L2, it would have been expected that incubating neutrophils with antibodies against CD99 or CD99L2 would have reduced transmigration. Since this was not the case, we conclude that endothelial CD99 and CD99L2 probably can mediate neutrophil transmigration independent of their respective counterparts on the neutrophil surface. However, we cannot exclude that, in addition, CD99 or CD99L2 on peripheral neutrophils could be involved in diapedesis, since both proteins are expressed at much higher levels on these cells than on bone marrow-derived neutrophils.

A role of CD99L2 in supporting cell interactions independent of homophilic adhesive interactions is also suggested by our cell aggregation assays with CD99L2-transfected CHO cells. Although CD99L2 promoted cell aggregation, this could not be blocked with antibodies, despite their ability to block the function of CD99L2 in diapedesis. Thus, it is uncertain whether the cell aggregation-promoting adhesion function of CD99L2 is involved in diapedesis. CD99L2 could trigger homotypic cell aggregation by stimulating other adhesion molecules or effects on the membrane underlying cytoskeleton. While this is possible, we can exclude that anti CD99 or anti CD99L2 antibodies inhibit diapedesis by simply affecting adhesive mechanisms that mediate the binding of leukocytes to endothelial cells, since the antibodies had no effects in adhesion assays with lymphocytes (not shown) or neutrophils (Fig. 6) on endothelial cell monolayers.

It is remarkable that the anti CD99 and anti CD99L2 antibodies had no additive inhibitory effects on neutrophil transmigration. Thus, they do not act independently. Potential heterophilic binding of CD99 to CD99L2 in pull down experiments, however, could not be demonstrated (not shown).
Further investigations are necessary to reveal the mechanisms that enable CD99 and CD99L2 to control and mediate leukocyte diapedesis. Whatever mechanism will be elucidated for CD99 and CD99L2, our present study establishes CD99L2 as a cell surface protein on endothelium that represents a novel target to block infiltration of neutrophils into inflamed tissue.

Acknowledgements

We thank Birgit Kempe, Yeliz Donat, Ria Knittel, and Gabi Frommer-Kästle for excellent technical assistance.
References

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Figure Legends

**Fig. 1: Anti-CD99L2 and anti-CD99 antibodies specifically react only with their corresponding antigen**

(A) Immunoblots of CD99-Fc, CD99L2S-Fc and ESAM-Fc (as indicated above) with affinity purified antibodies against CD99 and CD99L2 (as indicated below). (B) Immunofluorescence staining of COS7 cells transiently transfected with full length CD99L2_s (upper panel) or full length CD99 (lower panel) using affinity purified antibodies against CD99 or CD99L2 (as indicated on the left). Bar = 25 µm.

**Fig. 2 Expression of CD99L2 on endothelial cells.**

(A) Immunoblots of cell lysates of CHO cells stably transfected with the short E3-E4 splice variant (CD99L2_s; lanes 1-2 and 4-5), with the long E3A-E3-E4 splice variant of mouse CD99L2 (CD99L2_l; lane 6), or mock-transfected (lane 3), and of lysates of bEnd.5 endothelioma cells (lanes 7-8) were analyzed with pre-immune serum IgG (co-IgG: lanes 1 and 7) or affinity purified antibodies against CD99L2 (anti-CD99L2: lanes 2-6, 8). To control the specificity anti-CD99L2 antibodies were pre-incubated either with recombinant CD99L2-Fc (lane 4) or with ESAM-Fc (lane 5). (B) Purified CD99L2-Fc was digested without enzymes or with neuraminidase (NA) or with neuraminidase and O-glycosidase (O-Gly), followed by immunoblotting with peroxidase-conjugated anti human IgG antibodies. (C) Detection of CD99L2 splice variants in bEnd.5 endothelioma cells and HUVEC by RT-PCR. (D) Immunofluorescence staining of bEnd.5 endothelioma cells with affinity purified antibodies against CD99L2 and negative control IgG from the respective pre-immune serum (as indicated). First antibodies were detected with Cy3-conjugated secondary antibodies and visualized by fluorescence microscopy. Bar = 20 µm. (E) Immunoperoxidase staining of cryostat sections of mouse heart, kidney and liver with affinity-purified antibodies against mouse CD99L2, mouse CD99 and control rabbit IgG from pre-immune serum (as indicated). Tissue sections were incubated with first antibodies, followed by washing and incubation with secondary and tertiary reagent. Bar = 70 µm.

**Fig. 3 Expression of CD99L2 on leukocytes and comparison with CD99.**

Detection of CD99L2 (left panels) and CD99 (right panels) primary T cells, B cells and neutrophils isolated from blood and on neutrophils derived from bone marrow and on the T
cell line SJL.PLP7 was analyzed by flow cytometry. Binding of affinity purified anti-CD99L2 or anti-CD99 antibodies (black lines) and control IgG (grey lines).

**Fig. 4 CD99L2 supports aggregation of CD99L2-transfected CHO cells.**
(A) Mock-transfected CHO cells (CHO) or CHO cells stably transfected with CD99L2s (CHO-CD99L2s) or CD99L2L (CHO-CD99L2L) were allowed to aggregate in the presence (+) or in the absence (-) of Ca²⁺/Mg²⁺ as described under Materials and Methods. ***p<0.001 CHO-CD99L2s with Mg²⁺/Ca²⁺ vs. CHO-CD99L2s without Mg²⁺/Ca²⁺, ***p<0.001 CHO-CD99L2L with Mg²⁺/Ca²⁺ vs. CHO-CD99L2L without Mg²⁺/Ca²⁺. (B) Mock-transfected CHO cells (CHO) or CHO cells transfected with CD99L2L (CHO-CD99L2L) or CD99 (CHO-CD99) were allowed to aggregate in the presence of 30 µg/ml of the following antibodies: control antibodies (co-IgG), affinity-purified antibodies against CD99L2 (anti-CD99L2) or CD99 (anti-CD99) as indicated. ***p<0.001 CHO-CD99 + anti-CD99 vs. CHO-CD99 + co-IgG. (C) Mock-transfected CHO cells (CHO) or CHO cells stably transfected with CD99L2s (CHO-CD99L2s; labelled with Cell Tracker™ Green) or CD99L2L (CHO-CD99L2L; labelled with Cell Tracker™ Green) or mixed populations of mock-transfected or transfected cells (as indicated) were allowed to aggregate. Cell aggregates were analyzed by immunofluorescence microscopy and the number of mock-transfected cells (grey part of bar) or transfected cells (black part of bar) per aggregate were counted. Statistical analysis was done by Student t test. Each panel is representative of at least 3 experiments; each measurement was done in triplicate.

**Fig. 5 Antibodies against CD99L2 do not affect lymphocyte transmigration in vitro and lymphocyte extravasation in vivo**
(A) Transendothelial migration of SJL.PLP7 T lymphocytes is not affected by anti-CD99L2 antibodies. SJL.PLP7 cells were allowed to transmigrate through a monolayer of bEnd.5 cells grown on transwell filters for 30 min in the presence of 100 ng/ml SDF-1 in the lower chamber. Endothelial cells were incubated for 30 min before the assay either without antibodies (w/o Ab) or with pre-immune control IgG (co-IgG), affinity purified anti-CD99 IgG (anti-CD99), affinity purified anti-CD99L2 IgG (anti-CD99L2), or a monoclonal antibody against ICAM-1 (anti-ICAM-1). Antibodies (each 30 µg/ml) remained present during the assay. Each panel is representative of at least 3 experiments; each measurement was done in triplicate. ***p<0.001 anti-CD99 vs. co-IgG. (B) Migration of lymphocytes into inflamed skin is not affected by anti-CD99L2. Radiolabeled in vivo-activated T cells were
injected intravenously into hapten challenged mice together with (50 µg each) control IgG from pre-immune serum (co-IgG), or monoclonal antibodies against P-selectin and E-selectin (anti-P/E-selectin), or affinity purified antibodies against ESAM (anti-ESAM) or affinity purified antibodies against CD99L2 (anti-CD99L2). Radioactivity accumulating in the non-inflamed ear is represented by the bar on the left. Statistical analysis was done by Student t test. Results are representative for three similar experiments, for each determination four mice were analyzed. Numbers on the left refer to the percentage of injected cells that were found in the analyzed ear.

Fig. 6 Antibodies against CD99L2 and CD99 block transendothelial migration of neutrophils in vitro.

(A) Antibodies against CD99 or CD99L2 inhibit transendothelial migration of PMNs through a bEnd.5 monolayer. Bone-marrow derived PMNs were allowed to transmigrate for 30 min through a monolayer of bEnd.5 cells grown on transwell filters in the presence of 100 ng/ml KC in the lower chamber. Endothelial cells were incubated for 30 min with 30 µg/ml of antibody prior to the start of the assay as indicated. Left panel: with pre-immune control IgG (co-IgG) or affinity purified anti-CD99 IgG (anti-CD99) or F(ab′)2 fragments of control IgG (co-IgG F(ab′)2) or anti-CD99 IgG (anti-CD99 F(ab′)2) or a monoclonal antibody against ICAM-1 (anti-ICAM-1). **p<0.01 anti-CD99 vs. co-IgG, *p<0.05 anti-CD99 F(ab′)2 vs. co-IgG F(ab′)2. Right panel: with pre-immune control IgG (co-IgG) or affinity purified anti-CD99L2 IgG (anti-CD99L2) or F(ab′)2 fragments of control IgG (co-IgG F(ab′)2) or anti-CD99L2 IgG (anti-CD99L2 F(ab′)2) or a monoclonal antibody against ICAM-1 (anti-ICAM-1). **p<0.01 anti-CD99 vs. co-IgG, *p<0.05 anti-CD99 F(ab′)2 vs. co-IgG F(ab′)2. Antibodies remained present during the assay. (B) After pre-incubation of the endothelial cells with the indicated antibodies, the antibodies either remained present during the assay (first four bars) or were washed away before adding the PMNs (indicated by “pre-inc.” underneath the last four bars). ***p<0.001 anti-CD99 vs. co-IgG, **p<0.01 anti-CD99L2 vs. co-IgG, ***p<0.001 anti-CD99 pre-inc. vs. co-IgG pre-inc., *p<0.05 anti-CD99L2 pre-inc. vs. co-IgG pre-inc. (C) PMNs were preincubated with medium without antibody (w/o Ab) or with F(ab′)2 fragments of control IgG (co-IgG F(ab′)2), or anti-CD99 IgG (anti-CD99 F(ab′)2) or anti-CD99L2 IgG (anti-CD99L2 F(ab′)2). Antibodies were washed away prior to adding the PMNs into the assay. Each panel is representative of at least 3 experiments; each measurement was done in triplicate. (D) No additive effect of anti CD99 and anti CD99L2 F(ab′)2-fragments: Experiments were done as in (A), endothelial cells were preincubated with
F(ab')₂ fragments of control IgG (co-IgG F(ab')₂) or anti-CD99L2 F(ab')₂ (anti-CD99L2 F(ab')₂) or anti-CD99 F(ab')₂ (anti-CD99 F(ab')₂) or a mixture of anti CD99L2 and CD99 F(ab')₂ (anti CD99L2/CD99 F(ab')₂) or with monoclonal antibody against ICAM-1 (anti-ICAM-1). (E) Binding of PMNs to endothelial cells is not affected by antibodies against CD99 or CD99L2: Bone-marrow derived PMNs were allowed to adhere to a monolayer of bEnd.5 cells in the absence of antibody (w/o Ab), or in the presence of control antibodies (co-IgG) or antibodies against LFA-1 (anti-LFA-1), affinity-purified antibodies against CD99 (anti-CD99) or CD99L2 (anti-CD99L2). ***p<0.001 anti-LFA-1 vs. co-IgG. Statistical analysis was done by Student t test. Each panel is representative of at least 3 experiments; each measurement was done in triplicate.

**Fig. 7 Antibodies against CD99L2 and CD99 inhibit neutrophil extravasation in vivo by blocking diapedesis**

(A) Peritonitis assay: Mice were intravenously injected with 50 µg antibody: pre-immune control IgG (co-IgG) for negative controls, or with monoclonal antibodies against P-selectin and L-selectin (anti-P-/L-selectin) for positive controls, or with affinity purified anti-CD99L2 IgG (anti-CD99L2) or F(ab')₂ fragments of anti-CD99L2 IgG (anti-CD99L2 F(ab')₂) (upper panel) or with affinity purified anti-CD99 IgG (anti-CD99) or F(ab')₂ fragments of anti-CD99 IgG (anti-CD99 F(ab')₂) (bottom panel) in PBS immediately followed by i.p. administration of thioglycollate. Peritoneal leukocytes were removed at 4 hours after stimulation, and neutrophil counts were determined. Results represent 3 independent experiments, for each determination of each experiment 5 mice were analyzed. Left panel: ***p<0.001 anti-P-/L-selectin vs. co-IgG, ***p<0.001 anti-CD99L2 vs. co-IgG, **p<0.01 anti-CD99L2 F(ab')₂ vs. co-IgG. Right panel: ***p<0.001 anti-P-/L-selectin vs. co-IgG, ***p<0.001 anti-CD99L2 vs. co-IgG, *p<0.05 anti-CD99L2 F(ab')₂ vs. co-IgG. Statistical analysis was done by Student t test. (B) Intravital microscopy of cremaster muscle venules: Mice were intrascrotally stimulated with IL-1β and intravenously injected with 50 µg antibody of pre-immune control IgG (co-IgG), or affinity purified anti-CD99L2 IgG (anti-CD99L2) or affinity purified anti-CD99 IgG (anti-CD99) (left panels) or with 75 µg of control-IgG-F(ab')₂-fragments (co-IgG-F(ab')₂), or anti-CD99L2- F(ab')₂ or anti-CD99- F(ab')₂ (as indicated) (right panels). Four hours later, the cremaster muscle was surgically prepared, and the number of rolling
leukocytes per second per mm of vessel diameter, the number of adherent leukocytes per $10^4 \mu m^2$ of venule surface area, and the number of extravasated leukocytes from cremasteric venules per $10^4 \mu m^2$ tissue area were determined by intravital near-infrared reflected light oblique transillumination microscopy. *p<0.05 vs. co-IgG, Statistical analysis was done by ANOVA. (C) Proportion of neutrophils trapped between endothelial cells and the basement membrane/pericyte layer in IL-1\(\beta\) treated cremaster muscle. Analysis was done by transmission electron microscopy. Animals were treated with pre-immune control antibodies (co-IgG), or anti CD99L2 antibodies (anti-CD99L2) or with a mixture of two mAb against mouse PECAM-1 (anti-PECAM-1). The graph shows the number of neutrophils found between the endothelium and the underlying layer of basement membranes and pericytes, given as percentage of leukocytes that had passed the endothelial cell contacts. For each determination 30-52 randomly selected vessel segments were analyzed. (D) Electron micrographs of vessel segments of IL-1\(\beta\) activated cremaster muscle of mice treated with control antibody (ctrl: a), anti-CD99L2 antibodies (anti-CD99L2: b) or anti-PECAM-1 mAbs (anti-PECAM-1: c). E indicates endothelium, L leukocyte, P pericyte. The lumen of the vessel is indicated. Bar: 2 \(\mu m\).
A CD99-related antigen on endothelial cells mediates neutrophil, but not lymphocyte extravasation in vivo

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