Cutting Edge: Endothelial-Specific Gene Ablation of CD99L2 Impairs Leukocyte Extravasation In Vivo

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Cutting Edge: Endothelial-Specific Gene Ablation of CD99L2 Impairs Leukocyte Extravasation In Vivo

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CD99-like 2 (CD99L2) is a membrane protein with moderate sequence homology to CD99, which initiates cell aggregation of transfected cells and that is strongly expressed on endothelial cells, neutrophils, and lymphocytes. We showed recently that Abs against CD99L2 inhibit neutrophil, but not T lymphocyte, recruitment into inflamed tissues. In this study, we have generated conditional gene–deficient mice for CD99L2 and show by analyzing them in various inflammation models several results. First, gene ablation of CD99L2 impairs neutrophil recruitment into inflamed cremaster and peritoneum. Second, despite the strong expression of CD99L2 on peripheral neutrophils, only gene ablation on endothelial cells but not on myeloid cells affects neutrophil extravasation. Third, in contrast to our previous Ab-based results, recruitment of activated T cells into inflamed skin was impaired in mice lacking CD99L2 on endothelial cells. We conclude that CD99L2 is an essential endothelial Ag for leukocyte extravasation, which does not require homophilic interactions with CD99L2 on leukocytes. The Journal of Immunology, 2013, 190: 892–896.

Leukocyte recruitment into sites of inflammation is mediated by a set of adhesion and signaling molecules, such as selectins, integrins, and chemokines, that determine at which site and when leukocytes interact with the blood vessel wall. Upon capture, leukocytes start to migrate through the blood vessel wall (diapedesis), a process that is mediated by various adhesion receptors, such as PECAM-1, the tight junction-associated membrane proteins JAM-A, JAM-C, ESAM, and the nectin family member PVR (1, 2).

A role for CD99 in the diapedesis process was originally demonstrated for human monocytes transmigrating through HUVEC monolayers (3). Blocking mouse CD99 in vivo with Abs revealed that CD99 was indeed involved in the recruitment of lymphocytes, neutrophils, and monocytes into various inflamed tissues (4–6).

CD99 is a membrane protein with an extracellular domain of ∼100 aa and a short C-terminal part. A related protein to CD99 (32% amino acid identity) was cloned in various species, including mice and humans, and was called CD99-like 2 (CD99L2) (7). Based on in situ hybridization, it was prominently expressed on neuronal cells, choroid plexus, Sertoli cells, and granulosa and theca cells of the ovary (7). Generating Abs to mouse CD99L2 allowed us to detect it on endothelial cells, where it is enriched at endothelial cell contacts (5), as well as on blood-derived T and B cells and neutrophils. These Abs inhibited neutrophil recruitment into thioglycollate-inflamed peritoneum and IL-1β–stimulated cremaster tissue, whereas the entry of activated T cells into inflamed skin could not be inhibited (5). These results were confirmed for neutrophils and monocytes in the peritonitis model (8).

A major question about how CD99L2 functions in leukocyte diapedesis is whether CD99L2 binds to homophilic or heterophilic ligands. Transfection of CD99L2 into either Chinese hamster ovary (CHO) cells or L cells induced cell aggregation in a divalent cation-dependent manner (5, 8). Interestingly, transfected CHO cells aggregated with mock-transfected cells equally well as with transfected cells (5), suggesting that CD99L2 may either bind to heterophilic ligands or that expressing CD99L2 in CHO cells may stimulate other adhesion mechanisms that mediate cell aggregation. In contrast, CD99L2-transfected L cells aggregated only with each other and not with control cells, arguing for a homophilic aggregation mechanism (8). These variant results highlight the importance of the question whether CD99L2 is relevant on endothelial cells and on leukocytes or only on one of the cell types during leukocyte diapedesis.

In this study, we have generated and analyzed genetically modified mice that allow cell type–specific ablation of the CD99L2 gene. We show that CD99L2 is only on endothelial cells necessary for the diapedesis process, but not on neutrophils. Our results establish, independent of potentially indirect effects of Abs, that CD99L2 is indeed required for optimal neutrophil extravasation in vivo. Additionally, we show that CD99L2 is required for the recruitment of T cells, although Abs against CD99L2 did not inhibit this process.
Materials and Methods

Generation of CD99L2 conditional knockout mice

The targeting construct was generated by PCR using the clone PAC RP21-438P10 [National Center for Biotechnology Information ID A091454] as template. The PGK-neo cassette was removed by mating with FLP-expressing mice. Animals were backcrossed to C57BL/6 at least seven times.

Cells

Mouse lung microvascular endothelial cells were isolated according to a described procedure (9). T cells isolated from OT-II mice were activated by coculturing with irradiated B6 splenocytes plus OVA323–339 peptide for 3 d followed by culturing 2–4 d in the presence of IL-2 before use.

Abs

Abs used included anti-mouse CD99L2 generated as described (5), anti-α-tubulin (Sigma-Aldrich), peroxidase-conjugated secondary Abs (Dianova), FITC-conjugated anti-Ly6G and Ly6C (Gr-1), anti-CD19, FITC-conjugated anti-CD45, and FITC-conjugated rat IgG2b.

Transendothelial migration assays were done as described (9). For assays with OT-II T cells, 2 × 10^6 cells were added per transwell and 100 ng/ml SDF-1 was added to the lower chamber.

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SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed as described (4, 10).

In vivo inflammation models

Thioglycollate-induced peritonitis, T cell recruitment into the skin (delayed-type hypersensitivity [DTH] model) and ear swelling, and neutrophil recruitment in the cremaster were analyzed as described (4, 5, 10). Neutrophils in inflamed peritoneum were distinguished from monocytes by FACS gating and Gr-1 expression levels.

Transplantation of bone marrow

Mice were irradiated (920 rad) and injected i.v. with at least 4 × 10^6 bone marrow cells per mouse.

Statistical analysis

Statistical significance was analyzed using the Student t test for independent samples where appropriate or otherwise by a Mann–Whitney rank-sum test. A p value < 0.05 was considered significant. Results are shown as means ± SEM.

Results and Discussion

Generation of conditional gene-deficient mice for CD99L2

To test whether CD99L2 is indeed required for leukocyte extravasation and, if so, whether it is needed on leukocytes or on endothelial cells, we designed a cell type-specific inactivation strategy of the CD99L2 gene. We inserted loxP recombination sites flanking the ATG-containing exon 1 along with a neo cassette that was flanked by FRT sites into the CD99L2 gene of mouse embryonic stem cells (Fig. 1A). A Cre-driven deleter would remove the translational start site as well as the signal peptide of CD99L2. PGK-Cre mice were used for systemic deletion and Tie-2-Cre mice for deletion in endothelial and myeloid cells. Systemic deletion of exon-1 resulted in the loss of CD99L2 protein as was documented by immunoblotting of lung lysates with polyclonal Abs against the extracellular part of CD99L2 (not shown). Similar results were found for CD99L2 Δ/Δ mice expressing the Tie-2-Cre transgene, suggesting that CD99L2 was exclusively expressed by endothelial and myeloid cells in this tissue (Fig. 1B). Investigating each of these cell types separately showed that CD99L2 was indeed lost in peripheral neutrophils of these mice (Fig. 1C) and in endothelial cells isolated from lungs of CD99L2 Δ/Δ mice. Breeding efficiency and litter size were normal (not shown). Likewise, peripheral leukocyte counts of these mice were normal (not shown). To test the relevance of CD99L2 on endothelial cells for the transmigration of leukocytes, we first isolated primary endothelial cells from the lungs of CD99L2 Δ/Δ mice (Fig. 1D) and from wild-type (wt) mice. With the help of these cells, we found that transmigration of bone marrow–derived mouse neutrophils through CD99L2− endothelial cells was reduced by 23.8% (±2%) when compared with wt endothelial cells (Fig. 2A). This is in line with our recently reported inhibitory effects of anti-CD99L2 Abs in similar assays, although the Ab effects were slightly stronger than the inhibitory effects we saw in this study with gene-deficient endothelial cells (5).

In contrast to these results with neutrophils, we had reported before that anti-CD99L2 Abs did not inhibit the transmigration of activated T cells (5). However, using our CD99L2-null endothelial cells, we now found that transmigration of Ag-stimulated T cells from OT-II mice, directed against OVA, was 28.8% (±2.3%) reduced compared with wt endothelial cells (Fig. 2B). This surprising finding was unexpected and highlights the importance to reassess results obtained with Abs by other means, such as gene deficiency. Our divergent results for T cell transmigration could suggest...
that CD99L2 may act differently in neutrophil transmigration than in lymphocyte transmigration and that our Abs may only be able to block functional sites on CD99L2, which are relevant for neutrophil diapedesis, whereas other sites are relevant for lymphocyte diapedesis, which may not be blocked by our Abs.

**CD99L2 on endothelial cells but not on leukocytes is required for leukocyte extravasation in the cremaster**

We have tried before to settle the question whether CD99L2 on endothelial cells or on leukocytes is relevant for leukocyte transmigration. With Abs this question could not be studied in vivo. In vitro transmigration experiments had shown that Abs against CD99L2 could block transmigration when they were exclusively incubated with endothelial cells, but not when they were incubated exclusively with neutrophil granulocytes (5). However, owing to the low yield of neutrophils isolated from blood, these experiments had been performed with bone marrow–derived neutrophils. Because they have much lower expression levels of CD99L2 than do peripheral blood neutrophils, our previous results did not allow determination of whether CD99L2 on peripheral neutrophils is indeed relevant for the process of neutrophil extravasation.

Therefore, in this study we investigated this question using a combination of cell type–specific gene ablation and bone marrow transplantation. To obtain mice that lost CD99L2 exclusively on leukocytes, we transplanted irradiated C57BL/6 wt mice with bone marrow of CD99L2-deficient mice (Δ/Δ). Endothelial cells were activated with TNF-α 15 h prior to the assay and leukocytes were allowed to transmigrate for either 45 min toward the chemokine KC (neutrophils) or for 90 min toward the chemokine SDF-1 (T cells). Data shown are representative of three independent experiments. **p < 0.01. PMN, Polymorphonuclear neutrophil.

**FIGURE 2.** CD99L2 deficiency on endothelial cells inhibits transmigration of leukocytes in vitro. (A) Bone marrow–derived neutrophils from wt mice or (B) cultured, activated T cells isolated from the lymph nodes of OT-II mice were allowed to transmigrate through a monolayer of primary lung endothelial cells grown on transwell filters and isolated from either wt mice (+/+ or CD99L2-deficient mice (Δ/Δ). Endothelial cells were activated with TNF-α 15 h prior to the assay and leukocytes were allowed to transmigrate for either 45 min toward the chemokine KC (neutrophils) or for 90 min toward the chemokine SDF-1 (T cells). Data shown are representative of three independent experiments. **p < 0.01. PMN, Polymorphonuclear neutrophil.

To determine the requirement of CD99L2 on endothelial cells for the recruitment of neutrophils into inflamed cremaster tissue, we transplanted wt bone marrow into irradiated CD99L2Δ/Δ/Tie-2-Cre mice. For controls, we used wt mice transplanted with wt bone marrow. Hemodynamic parameters were similar in both types of mice (Supplemental Table I). Intravital microscopy of the cremaster of these mice 4 h after intrascrotal injection of IL-1β revealed that extravasation of neutrophils was reduced by 36.3% (±2.9%) in mice lacking CD99L2 on endothelial cells (Fig. 3B). Leukocyte dynamic parameters were similar in +/+ and Δ/Δ mice (Supplemental Table I). Thus, despite the high expression level of CD99L2 on peripheral neutrophils, it is either redundant or not relevant for the process of neutrophil extravasation. This excludes the possibility that a homophilic interaction of CD99L2 is essential for the extravasation of neutrophils in vivo.

**FIGURE 3.** CD99L2 is required on endothelial cells but not on neutrophils for extravasation from cremaster venules. (A) Wild-type mice transplanted with wt bone marrow (black bars) and wt mice transplanted with CD99L2-deficient bone marrow (gray bars) or (B) wt mice transplanted with wt bone marrow (black bars) and CD99L2Δ/Δ/Tie-2-Cre mice transplanted with wt bone marrow (gray bars) were analyzed by intravital microscopy of cremaster tissue after 4 h IL-1β stimulation. As indicated on the left, numbers of rolling flux fraction, adherent leukocytes inside vessels, and extravasated leukocytes were determined. Results are displayed as means ± SEM of four to five animals per group with five vessel segments per animal. ***p < 0.001. n.s., Not significant, PMN, polymorphonuclear neutrophil.
rolling and adhesion within venules were unaffected compared with control mice. Collectively, these results show that despite its strong expression on peripheral neutrophils (5), CD99L2 is exclusively required on endothelial cells for optimal neutrophil recruitment into inflamed tissue. Thus, CD99L2 on endothelial cells most likely has a heterophilic ligand on neutrophils. Alternatively, CD99L2 may not even act as an adhesion receptor and may rather function as a membrane protein that interacts in cis with other endothelial membrane proteins involved in the diapedesis process.

Because CD99L2 is enriched at endothelial cell contacts (5), we tested in the Miles assay whether CD99L2 is relevant for the control of vascular permeability. However, we found that neither baseline permeability nor vascular endothelial growth factor–induced permeability in the skin differed between CD99L2fl/fl/Tie-2-Cre and wt mice (data not shown).

CD99L2 is required for the recruitment of neutrophils into the inflamed peritoneum

We verified the involvement of CD99L2 in neutrophil extravasation for the peritonitis model. To this end, we injected CD99L2fl/fl/Tie-2-Cre mice or wt mice with thioglycollate and determined the number of neutrophils accumulated in the peritoneum 4 h later. Neutrophil recruitment was reduced by 43.0% (±5%) in CD99L2-deficient mice when compared with wt mice (Fig. 4). This is in agreement with previously reported inhibitory effects of anti-CD99L2 Abs in peritonitis experiments (11). We conclude that CD99L2 is indeed essential for optimal extravasation of neutrophils in various tissues.

Recruitment of activated T cells into inflamed skin is reduced in CD99L2-deficient mice

Because T cell transmigration through monolayers of CD99L2-deficient primary isolated endothelial cells was reduced (Fig. 2B), whereas our previous results demonstrated that anti-CD99L2 Abs did not inhibit T cell migration through endothelial cells in vitro or the recruitment of activated T cells into inflamed skin (5), we analyzed the effect of CD99L2 gene deficiency on T cell recruitment in vivo in a typical DTH reaction. For this, activated T cells were isolated from lymph nodes of 2,4-dinitro-1-fluorobenzene (DNFB)–treated wt mice, labeled with 51Cr, and injected i.v. into CD99L2fl/fl/Tie-2-Cre mice or wt mice with a DNFB-elicited DTH reaction in the right ear. Mice were killed 5 h later and radioactivity was determined in the right ear and as negative control in the left ear. T cell recruitment and ear swelling were reduced by 25% (±8.9%) and 21.5 (±5.2%), respectively, in CD99L2fl/fl/Tie-2-Cre mice compared with wt mice (Fig. 5A, 5C). This inhibitory effect is similar to what we observed in the in vitro transmigration assays (Fig. 2B). To test whether CD99L2 on T cells is involved in the recruitment of these cells into inflamed skin, we performed similar DTH experiments, except for using DNFB-treated CD99L2 Δ/Δ mice as T cell donor mice and DNFB-treated wt mice as recipients. As controls, DNFB-treated wt mice were used as T cell donors. We found that CD99L2-deficient T cells entered inflamed skin as efficiently as did wt T cells (Fig. 5B).

Thus, similar to neutrophils, T cells do not require CD99L2 on their cell surface, but they depend on CD99L2 on endothelial cells for optimal leukocyte extravasation. Importantly, this function of CD99L2 could not be blocked with Abs (5). Because the same Abs could efficiently inhibit neutrophil extravasation, it is possible that CD99L2 may act via different binding partners during the recruitment of the two different types of leukocytes.

It is important that our Ab blocking effects on neutrophil extravasation could be verified by analyzing CD99L2-deficient mice. This shows that the Abs did not act via any side effect or via other stimulating functions that might not be relevant during the physiological leukocyte recruitment process. Our results clearly establish that CD99L2 needs to be present on endothelial cells for optimal extravasation of leukocytes. For PECAM-1, it has been shown that blocking Abs and gene

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** CD99L2 deficiency impairs neutrophil recruitment into chemically inflamed peritoneum. Wild-type mice (+/+) or CD99L2fl/fl/Tie-2-Cre mice (Δ/Δ) were stimulated i.p. with thioglycollate and 4 h later, accumulation of recruited neutrophils was determined from the peritoneal lavage and given as percentage of cells recruited in wt mice. The assay shown is mean of three independent experiments with four in each assay for wt (total = 12) and four in two assays and three in one assay for Δ/Δ mice (total = 11). #p < 0.05 determined by Mann–Whitney rank-sum test. PMN, Polymorphonuclear neutrophil.
ablation can yield different results. In the peritonitis model, Abs against PECAM-1 could efficiently impair neutrophil extravasation, whereas no such inhibitory effect was initially found in PECAM-1–deficient mice (12). Interestingly, this lack of effect was only seen on the C57BL/6 background but not in FVB/n mice (13). Because CD99L2 gene deficiency and Ab effects are of similar efficiency in C57BL/6 mice, we assume that CD99L2 mediates different steps in the diapedesis process than PECAM-1. This is in agreement with our findings that CD99L2 acts at a similar site but independently of PECAM-1 during the diapedesis process (11).

The fact that CD99L2 is strongly expressed on peripheral neutrophils and T cells but irrelevant for leukocyte extravasation is surprising, especially in the light of the homotypic cell aggregation that was observed for CD99L2-transfected L cells (8). Thus, although homophilic interactions between leukocyte and endothelial CD99L2 cannot be completely ruled out, our results exclude that such interactions, if existing, are required for the leukocyte diapedesis process.

Disclosures
The authors have no financial conflicts of interest.

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