CD99 is essential for leukocyte diapedesis *in vivo*[*]

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Abstract

Recruitment of leukocytes into inflamed tissue requires migration of leukocytes from the blood stream across the endothelial lining and the basement membrane of the local blood vessels. CD99 in humans is a 32 kD highly O-glycosylated cell surface protein expressed on most leukocytes. We recently found CD99 to be expressed in leukocytes and at human endothelial cell contacts. Human CD99 is involved in homophilic interaction between the two cell types and participates in the transendothelial migration of monocytes and PMN *in vitro.*

To test the role of CD99 *in vivo*, we cloned murine CD99 (muCD99), expressed it *in vitro*, and generated a blocking monoclonal antibody against it. We first showed that muCD99 is expressed on mouse leukocytes as well as enriched at the endothelial cell borders. Transfection of cells with muCD99 imparts on them the ability to aggregate in a CD99-dependent homophilic manner. Cells expressing muCD99 did not bind to cells expressing murine or human PECAM or human CD99. In the thioglycollate peritonitis model of inflammation, anti-CD99 monoclonal antibody blocked the recruitment of neutrophils and monocytes by over 40 % and 80%, respectively, at 18 hours. Microscopy showed that this blocking occurred at the luminal surface of venules. We conclude that CD99 plays a major role in the emigration of leukocytes *in vivo*.

Keywords

CD99; transendothelial migration; endothelial cells; adhesion molecules; inflammation

INTRODUCTION

Extravasation of leukocytes from blood into inflamed tissue is a critical step in the innate immune response. Endothelial cells at the site of inflammation recruit leukocytes locally.

DISCLOSURES
The authors have no financial or competing interests to disclose.

Leukocytes are captured out of the flowing bloodstream and roll along the luminal wall predominantly using selectin molecules and their counter-receptors. G protein coupled receptors on the leukocytes sense their cognate chemokines presented on the endothelial surface. This triggers inside-out activation of leukocyte integrins to stop the rolling and induce firm adhesion. The adherent leukocytes crawl along the endothelial cell surface to the junction where they squeeze themselves between two or three cells at the borders (diapedesis) and transmigrate to the basal lamina and the tissue beneath.

While the first steps in leukocytes emigration are reversible, diapedesis is a point of no return. Therefore, it represents a unique and crucial step in inflammation and a potential target to prevent unwanted inflammation. Compared to the earlier steps of rolling and adhesion relatively little is known about the diapedesis process. However, several molecules expressed at the endothelial borders including ICAM-1 and -2, JAM-A, the Polio Virus Receptor (PVR), platelet/endothelial cell adhesion molecule (PECAM) and CD99 (reviewed in (Muller, 2002; Muller, 2003; Vestweber, 2007) and CD99L2 (Bixel et al., 2007; Schenkel et al., 2007) have been implicated in diapedesis. This large array of molecules may underscore the complexity of diapedesis. On the other hand, specific molecules may be relatively more important in the response to a specific stimulus or the recruitment of selected leukocyte subsets.

Among these molecules, PECAM and CD99 play a major role in humans. Both have been reported to form homophilic interactions *in vitro* between endothelial cells and leukocytes. PECAM is involved in the initiation of transmigration (Muller et al., 1993) whereas CD99 is involved in the completion of the diapedesis for both monocytes (Schenkel et al., 2002) and neutrophils (Lou et al., 2007). The role for PECAM in diapedesis of neutrophils, monocytes, (Bogen et al., 1994; Liao et al., 1997; Liao et al., 1999; Schenkel et al., 2004; Vaporciyan et al., 1993) and activated murine T cells (Qing et al., 2001; Reinke et al., 2007) has been clearly confirmed *in vivo*. However, only one report, using a polyclonal antibody, has investigated a role for CD99 (Bixel et al., 2004) *in vivo*. Furthermore, these studies were restricted primarily to T cell emigration.

In order to analyze the relevance of CD99 for diapedesis of myeloid cells *in vivo*, we used a blocking monoclonal antibody against the homologue mouse CD99. Our results demonstrate in a peritonitis model, that CD99 is necessary for extravasation of both monocytes and neutrophils.

MATERIALS AND METHODS

Mouse Strains

FVB/n wild-type mice were raised and housed at Weill Cornell Medical College. All procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College.

Production of Stable Transfectants Expressing CD99-EGFP

A full length murine CD99 cDNA (kindly provided by Drs. Cheryl Guyre and Johanne Kaplan, Genzyme, Inc., Framingham, MA) was inserted in the pEGFP-N1 plasmid. L-cell

fibroblasts were transfected with the CD99-EGFP cDNA as described (Muller et al., 1992). Briefly, 20 μg of a full-length CD99-EGFP cDNA construct in the pEGFP-N1 vector was mixed with 10^7 L-cells in 0.5 ml of RPMI medium and subjected to electroporation at 250 mV, 960 μF. After 2 days of culture in nonrestrictive medium (RPMI 1640 + 10% fetal bovine serum (FBS), Gibco-BRL, Grand Island, NY), transfected cells were selected by the addition of 0.5 mg/ml of geneticin (G418, Gibco). Colonies representing single clones were selected, tested for EGFP expression and subcloned by limiting dilution.

Western Blot

Clones expressing mCD99 were harvested either by trypsin or using 10 mM EDTA in Hanks' Balanced Salt Solution (HBSS). Cells were washed twice in PBS and lysated with 0.4% NP40 saline buffer on ice, centrifuged at 14,000 rpm for 5 min at 4 °C and the clarified lysates were collected. Lysates were first fractionated on 4–16% SDS-PAGE, then proteins immobilized on a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and incubated with an anti-EGFP antibody (Santa Cruz sc-8334) diluted in PBS + 5% BSA. The membrane was then washed and incubated in horseradish peroxidase–conjugated goat anti–mouse IgG and detected by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ).

Flow Cytometry

Adherent clones expressing mCD99 were resuspended for flow cytometry by incubation in 10 mM EDTA in HBSS. Cells were washed in PBS + 2.5 % FBS and analysed for EGFP expression. Alternatively, cells were incubated with a rat anti-mCD99 primary antibody, clone EJ2, (Park et al., 2005) or isotype control, diluted in PBS + 2.5 % FBS, incubated for 30 min on ice, washed by repeated centrifugation in PBS + 2.5 % FBS, then incubated for 30 min on ice in PE-labeled F(ab′)$_2$ fragments of goat anti–rat IgG (Beckman Coulter PN IM1623). After extensive washing, cells were analyzed on a FACScalibur (Becton Dickinson, Mountainview, CA) flow cytometer using Cell Quest Software.

Flow Cytometry of Mouse Leukocytes

Blood was collected from mice by cardiac puncture into heparinized syringes. After pre-blocking with 2.5% FBS, anti-muCD99 antibody was incubated with 10^5 leukocytes on ice, washed twice in cold PBS, incubated with FITC-labeled F(ab′)2 fragments of goat anti–rat IgG (Beckman Coulter PN IM1623). PE-labeled antibodies against T cells, B cells and myeloid cells (anti-CD3, anti-CD19, and anti-Ly6C/G, respectively, BD Biosciences) were added to asses the different expression for each of the subsets.

Murine Endothelial Cell Isolation and CD99 Staining

Murine endothelial cells were isolated based on published procedures (Mason et al., 2007). Six mice were euthanazied and the hearts removed. Cardiac tissue was submitted to two enzymatic digestion rounds with collagenase II and trypsin. Endothelial cells were purified from cells in suspension using an anti-murine endoglin (CD105) monoclonal antibody (MJ7/18, BD Biosciences Pharmingen, San Diego, CA) on a magnetic column (miniMACS, Miltenyi Biotech, Inc., Auburn, CA). Then endothelial cells were cultured for 3-4 days in

DMEM + 10% FCS. For anti-CD99 staining, endothelial cells were fixed with 2% paraformaldehyde and labeled with the EJ2 anti-mCD99 for 1h followed by fluorescent secondary antibody.

Aggregation Assay

Aggregation of L cell transfectants was performed and quantified as described (Muller et al., 1992; Schenkel et al., 2002). Briefly, L-cells transfected with muCD99, huCD99, huPECAM-1, muPECAM-1 or vector alone were nonenzymatically resuspended to 10^6 cells/ml in HBSS with or without divalent cations, transferred in 1 ml aliquots to 24-well tissue culture plates, and incubated at 37 °C on a gyrotory shaker at 90 rpm. In some experiments, the transfectants were preincubated with antibody (20 μg/ml) then washed free of unbound antibody before incubation. The aggregation reaction was stopped by adding 5% glutaraldehyde to a final concentration of 2%. In experiments to determine whether aggregation was heterophilic or homophilic, distinct populations of cells were prelabeled before mixing with 5-(and 6-) Carboxyfluorescein (CFSE) (Molecular Probes, Eugene, OR). For these experiments, the cells were examined live at the end of the aggregation period by fluorescence microscopy (Muller et al., 1992; Schenkel et al., 2002).

Thioglycolate-induced Aseptic Peritonitis

We used the sterile peritonitis model of acute inflammation to define the role of CD99 in vivo (Bogen et al., 1994; Schenkel et al., 2004). Mice were injected i.v. with 100 μg of a rat anti-muCD99 Ab (clone EJ2) or a rat IgG as a control 1 h before i.p. injection of 1 ml of 4% thioglycollate broth (Difco Laboratories, Sparks, MD). We also simultaneously injected age- and sex-matched controls with PBS as control for trauma. Experiments were conducted as described previously (Bogen et al., 1994; Schenkel et al., 2004). Briefly, at 18 h after thioglycolate injection, mice were sacrificed. The peritoneal cavity was washed with 5 ml of 10 mM EDTA in HBSS. Cytospin preparation and cell counts were done immediately. Blood was drawn by cardiac puncture into heparinized syringes for white blood cell and differential counts. All internal organs were harvested and fixed in 10% buffered formalin for later examination.

Microscopy

Evaluation of the positions of leukocytes relative to the venular wall were performed as previously described (Bogen et al., 1994; Liao et al., 1997). Sections of mesentery were embedded in paraffin and coded H&E sections were examined by an experienced pathologist blinded to the identity of the specimens. Ten representative profiles of postcapillary and small venules were examined for each slide. The number of leukocytes apparently adherent to the vessel wall and the total number of leukocytes in the profile were recorded.

Statistics

All data were analyzed by pairwise comparison using a two-tailed t test assuming unequal variances.

RESULTS

Subcloning mCD99 and Transfection

The muCD99 cDNA sequence we identified was identical to the NM.025584.2 Genbank sequence (not shown). We reamplified the muCD99 sequence and subcloned it into the pEGFP-N1 vector. The cDNA encoding the fusion protein was 1086 base pairs long. The muCD99-EGFP sequence was then transfected into L-cell fibroblasts. Expression of the fusion protein was assessed by microscopy, western blot and flow cytometry. Microscopy showed the fusion protein at the plasma membrane as well as in the cytoplasm (Fig. 1A). The EGFP-fusion protein ran at 50 kD on SDS-PAGE, as predicted (Fig. 1B). Comparing trypsinized cells to non-enzymatically harvested cells, we confirmed the presence of about 1/3 of the fusion protein on the plasma membrane surface (Fig. 1B). Flow cytometry allowed us to quantify muCD99-EGFP expression on the FITC channel and show that that clones had uniform expression (Fig. 1C). Flow cytometry performed on intact cells using an anti muCD99 antibody confirmed the presence of muCD99 at the plasma membrane surface (Fig. 1D).

MuCD99 is Expressed on Leukocytes and Endothelial Cells

The monoclonal antibody EJ2 was previously shown to bind to CD99 on thymocytes and endothelial cells *in situ* (Park et al., 2005). To determine if its expression were potentially relevant to inflammation, we investigated its expression on peripheral blood leukocytes and localization on endothelial cells. We found that mouse peripheral blood leukocytes stain strongly for CD99. B cells expressed a lower and broader expression (Fig. 2A) whereas T cells (Fig. 2B) and the myeloid cells (Fig. 2C) have a high and relatively homogenous expression. In cultured mouse heart endothelial cells CD99 expression was found to be enriched at the cell borders (Fig. 2D), as expected based on results with human cells (Schenkel et al., 2002).

MuCD99 Supports Homophilic Cell Aggregation in Dose-dependent Manner

CD99 is mediates homophilic cell aggregation as demonstrated with human CD99 in L-cells (Schenkel et al., 2002). We tested the ability of muCD99 to support cell adhesion by generating L-cells that stably express the muCD99-EGFP fusion protein. We performed the aggregation assay using two different clones expressing different levels of muCD99-EGFP expression. L-cells expressing PECAM were used as positive control for homophilic aggregation whereas L-cells transfected with EGFP or an inverted PECAM sequence were used as negative control.

L-cells expressing muCD99-EGFP aggregated in suspension in a calcium dependent manner (Fig. 3A). Aggregation increased with time over 60 min as previously seen for PECAM and huCD99 (Muller et al., 1992; Schenkel et al., 2002) and muCD99L2 (Bixel et al., 2007; Schenkel et al., 2007). Moreover, the monoclonal antibody against mCD99 blocked aggregation (Fig. 3B), consistent with the idea that aggregation depended on surface expression of CD99. Furthermore, L-cells aggregated in a muCD99 dose dependent manner since the low level expressing cells consistently aggregated slower (Fig. 3C,D).

Since aggregation occurred only in presence of muCD99, we tested whether these cell-cell interactions were due to heterophilic or homophilic muCD99 adhesion. We performed mixed aggregation assays as previously described (Muller et al., 1992; Schenkel et al., 2002). When muCD99 transfectants were mixed with equal number of CFSE labeled control cells, they aggregated only with other cells that were displaying muCD99 (Fig. 4A,C). We also demonstrated that L-cells expressing muCD99 do not aggregate with cells expressing human PECAM (Fig. 4B,E). In the mixed aggregation studies, PECAM-bearing cells adhered to PECAM-bearing cells only, whereas muCD99 bound only to other cells expressing muCD99. Furthermore, cells expressing muCD99 aggregated preferentially with themselves when mixed with cells expressing huCD99 (Fig. 4D) or murine PECAM (Fig. 4F).

Blocking Diapedesis *in vivo*

We have previously shown that CD99 is involved in the diapedesis process *in vitro* on human cells, where homophilic interaction between CD99 on endothelial cells and CD99 on neutrophils or monocytes is required (Lou et al., 2007; Schenkel et al., 2002). In order to determine if CD99 is really involved *in vivo*, we tested the ability of our monoclonal antibody to block extravasation in a peritonitis model in FVB mice. As shown in Fig. 5 peritoneal thioglycollate injection stimulated a robust inflammatory response. After 18h abundant neutrophils were still present in the peritoneal cavity and the number of monocytes/macrophages had increased two-fold compared to control.

However, when mice were pretreated with an anti-muCD99 antibody, the neutrophil influx was reduced by 40% (Fig. 5A) and the influx of monocytes was blocked almost down to baseline levels (Fig. 5B). The total number of circulating leukocytes was not affected by this treatment (data not shown). Therefore, the failure of leukocytes to accumulate in the peritoneal cavity in response to thioglycollate was not due to leukocyte sequestration or elimination.

To determine the site of leukocyte blockade, we examined small venules in the mesenteries of the mice following the experiments. These were fixed in formalin immediately after harvesting of the peritoneal cells. We noticed that in the mice that had received the anti-CD99 mAb, a significant number of leukocytes appeared to be arrested on the surface of venules at sites of inflammation (Fig. 6A,B). This is similar to the appearance of leukocytes in vessels in which PECAM activity had blocked, selectively blocking diapedesis (Bogen et al., 1994; Liao et al., 1997). In those studies, the percentage of leukocytes adherent to the vascular wall and the number of vessels with more than one leukocyte adherent correlated well with blockade of leukocyte emigration and demonstrated that PECAM arrested leukocytes on the apical surface of the endothelium in this model of inflammation. Using the same method of quantification, we found that within all vessels examined, 15% of the leukocytes appeared in contact with the wall in control mice whereas 50% appeared adherent when the mice had been treated with anti-CD99 (Fig. 6C). In addition, while 15% of vessel profiles in control mice had more than one leukocyte apparently adherent, 70% of vessel profiles in mice that received anti-CD99 mAb had more than one leukocyte adherent

(Fig. 6D). Thus, blockade of CD99 appears to block leukocyte transmigration *in vivo* as it does *in vitro* (Schenkel et al., 2002).

DISCUSSION

Diapedesis is a critical step in inflammation, and CD99 as been shown to be involved *in vitro* in this process. CD99 is an integral membrane glycoprotein with no homology with any other protein family, and has been reported involved also to be involved in apoptosis (Bernard, 1997; Pettersen et al., 2001), differentiation of thymocytes (Alberti et al., 2002), T-cell activation (Waclavicek et al., 1998; Wingett et al., 1999) and in migration in tumor cells (Kreppel et al., 2006; Manara et al., 2006). This receptor is broadly expressed on cells of the hematopoietic system and at the junction of endothelial cells (Schenkel et al., 2002). Several studies showed that two splice variants of CD99 are found in humans (Hahn et al., 1997). A long form of 32 kD is the most abundant while a short form of 28 kD is only expressed in immature thymocytes. The short form seems to have the opposite effect of the long one on adhesion and migration (Bernard et al., 1997; Scotlandi et al., 2007). Interestingly, in mouse, only one DNA sequence has been found and shows a highest homology to the short huCD99. Therefore, it was not clear whether the muCD99 would behave the same way as huCD99.

We generated cells expressing murine CD99. This allowed us to show that muCD99 is involved in cell-cell contact and *in vitro* cell aggregation as previously found for human CD99 (Schenkel et al., 2002) and in mouse (Bixel et al., 2004). Moreover, experiments with CFSE clearly demonstrated that muCD99 is involved in homophilic adhesion similar to huCD99. MuCD99 expressing cells did not adhere to muPECAM expressing cells, suggesting that the extracellular domains of the two proteins do not interact with each other. This is consistent with the idea that they act at two distinct steps in the diapedesis process as suggested by microscopy and blocking assays (Schenkel et al., 2002). Interestingly, in this experiment, cells expressing muCD99 did not aggregate with cells expressing huCD99.

Aggregation in L-cells or COS cells may not reflect the behavior of endothelial cells or leukocytes, since protein partners downstream of the receptor may differ. However, aggregation occurred only with cells expressing the same receptor, CD99 or PECAM, strongly suggesting that it is relevant at least for homophilic aggregation. We cannot rule out that muCD99 binding by itself is insufficient to promote adhesion, but instead homophilic interaction of CD99 activates an adhesion signaling cascade and/or recruits other molecules into the zones of cell-cell interaction to promote adhesion.

To study the role of CD99 *in vivo*, we used a specific monoclonal antibody against muCD99 that worked for immunocytochemistry, western blot and flow cytometry. This antibody inhibited homophilic CD99-mediated adhesion in our aggregation assay (Fig. 3). We then used it *in vivo* in a peritonitis model, since studies using a monoclonal antibody against murine CD99 had not been reported. After 18h, neutrophil transmigration was reduced by 40% (Fig.5a). Bixel *et al.*, showed using an affinity purified polyclonal antibody that transmigration of neutrophils was blocked in vivo by 60% after 4h, a time corresponding to the peak of neutrophil extravasation (Bixel et al., 2007). Our data show that long after their

recruitment peak, 40% of the neutrophils were still blocked by anti-muCD99. This demonstrates the importance of muCD99 for continued neutrophil extravasation. Moreover, we demonstrate in this report that blocking muCD99 also blocked monocyte transmigration to baseline levels. This was the same dramatic response of monocytes to anti-CD99 mAb that observed *in vitro* (Schenkel et al., 2002).

Microscopy showed that many leukocytes in the mice treated with anti-CD99 mAb appeared to be stuck at the luminal surface of venule, similar to the appearance of cells blocked by anti-PECAM (Bogen et al., 1994), whereas in absence of any blocking antibody the cells entered the site of inflammation and were not seen on the vessel wall. At the level of resolution of the light microscope, we cannot determine whether the leukocytes are, in fact, trapped part way through the endothelial border, as found *in vitro* (Lou et al., 2007; Schenkel et al., 2002). However, the leukocyte nuclei appeared on the luminal surface of the endothelial cells as they did in the previous *in vitro* studies (Lou et al., 2007; Schenkel et al., 2002).

Bixel *et al.* (Bixel et al., 2004) reported that a polyclonal antibody against mCD99 partially inhibited migration of T-cells as well as swelling into inflamed skin. More recently they showed that polyclonal antibodies against mCD99 inhibit neutrophil extravasation *in vivo* at an early time (4 hours) following inflammatory challenge (Bixel et al., 2007). We demonstrate here that a monoclonal antibody blocked neutrophils as well as monocytes over a longer time span. It is interesting to note that blocking CD99 blocks monocyte transmigration more efficiently than neutrophil transmigration *in vivo* (Fig. 5) as well as *in vitro* (Lou et al., 2007). Since monocytes are important for chronic inflammation, CD99 may be a good therapeutic target for blocking chronic as well as acute inflammation. Antibodies against the related molecule CD99L2 have recently been shown to block inflammation *in vivo* including in the thioglycollate peritonitis model (Bixel et al., 2007; Schenkel et al., 2007). It will be interesting to see whether these molecules work in series or in parallel to promote transmigration. The mAb used in these studies does not cross-react with murine CD99L2 (data not shown.) Development of mice deficient in these molecules will be useful in this regard.

Further investigation will be necessary to fully understand the exact biological roles of CD99. In addition to a role in leukocyte emigration, this molecule has been reported to be involved in thymocyte development, cell motility, adhesion, and apoptosis. Since blocking CD99 blocks the completion of diapedesis, it may also be involved in cell "detachment". We showed here that despite the fact that muCD99 corresponds to the short form of huCD99, muCD99 can mediate homophilic cell adhesion *in vitro* and is required for efficient transendothelial migration *in vivo*.

Acknowledgements

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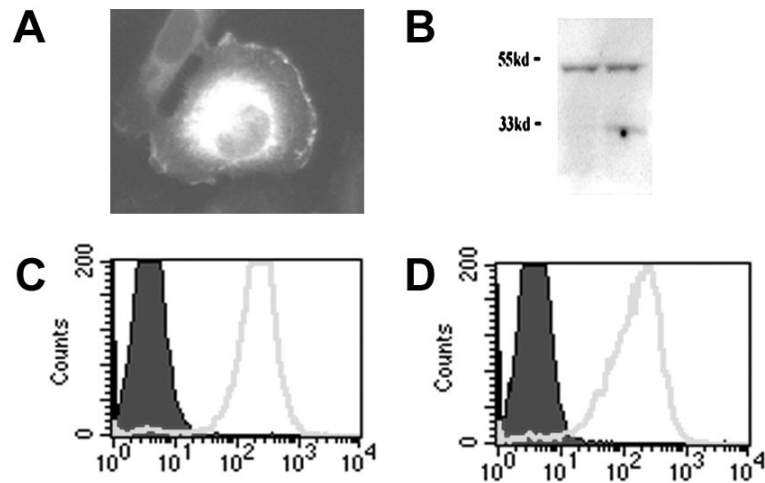
Figure 1. Murine CD99-EGFP is expressed at the cell surface
A) muCD99 is expressed on the plasma membrane of the cell as well as perinuclearly. B) Western blot against EGFP showing native muCD99-EGFP (left lane) and trypsin digested muCD99-EGFP (right lane). A shorter band around 33 kDa represents ~ 1/3 of the muCD99-EGFP that is present on the cell surface and susceptible to extracellular proteolysis. C and D) Flow cytometric analysis of a clone expressing muCD99-EGFP (light line) shows a uniform population expressing EGFP (C), while staining of the same clone with a monoclonal antibody against muCD99 (light line; detected with a PE-labeled secondary antibody) shows uniformity of muCD99 expression (D). In C and D filled histograms represent nontransfected cells and isotype control, respectively.
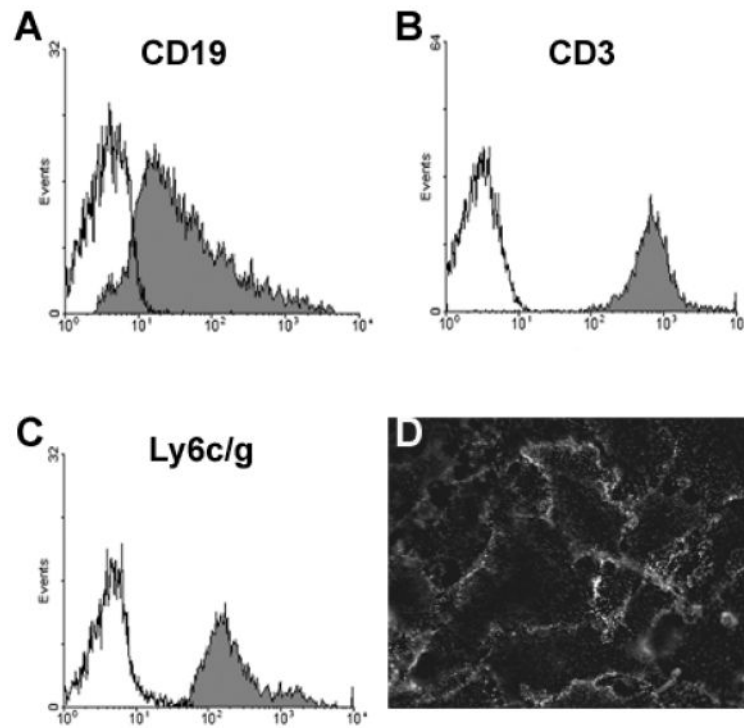
Figure 2. Murine CD99 is expressed on leukocytes and at endothelial cell borders
A-C) Mouse blood was stained with anti-muCD99 and gated by other fluorescent antibodies into leukocyte subpopulations. Expression of muCD99 on leukocytes gated for subpopulations: A) B-cells (CD19 positive). B) T-cells (CD3 positive). C) Myeloid cells (Ly6 C/G positive cells). D) Cultured murine endothelial cell monolayers were fixed and stained with anti-muCD99 mAb, which was detected with fluorescent secondary antibody. MuCD99 is restricted to cell borders.
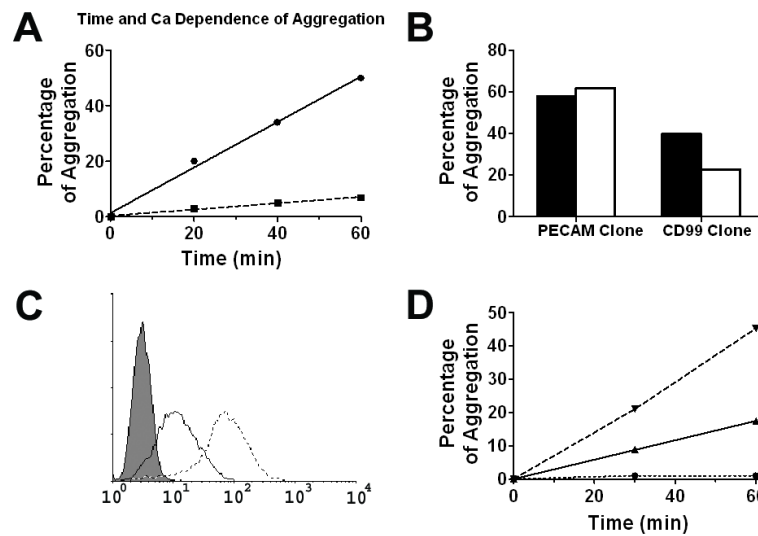
Figure 3. Expression of muCD99 imparts on cells the ability to aggregate

A) L-cells expressing muCD99 aggregate with themselves in a time and calcium dependent manner, whereas L-cells transfected with a vector only do not aggregate (see part D). Solid line, with 1 mM Ca $^{+2}$, dashed line, no calcium. B) A monoclonal antibody against muCD99 blocks aggregation by 50% (white bar) compared to control IgG (black bar). The same antibody does not affect aggregation of clones expressing PECAM. C & D) Two clones expressing muCD99-EGFP at different levels (solid line for lower expression, dashed line for higher expression) are shown by FACS (C). The L-cell aggregation is muCD99 concentration dependent (D), since the clone with the higher expression aggregates two times faster in the presence of calcium. High expressor (▼), low expressor (▲), transfected with EGFP vector only (■). The data shown are experiments representative of 3 – 6 independent experiments. Experimental errors were always less than 10% of the absolute value and are omitted for clarity.
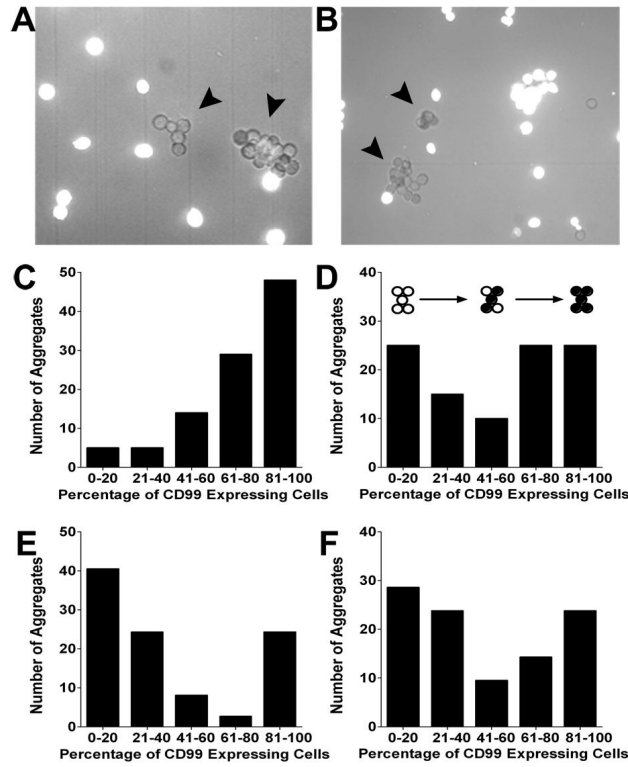
Figure 4. L-cell aggregation induced by muCD99 is due to homophilic interactions
A) muCD99 transfectants mixed with an equal number of CFSE-labeled control cells aggregated only with other cells that expressed muCD99. Note only unlabeled cells in aggregates (arrowheads). B) Unlabeled muCD99 expressing cells (arrowheads) were mixed with CFSE-labeled L-cells expressing huPECAM. Both cell types aggregated, but only in a homophilic manner. (C-F) The Y-axis indicates the number of aggregates counted containing the percentage of muCD99 expressing cells (indicated on the X-axis) when muCD99 transfectants are mixed with an equal number of cells expressing empty vector (C), or transfectants expressing huPECAM (D), huCD99 (E), or muPECAM (F). The parabola shaped pattern on D-F is expected if each cell-type aggregates homophilically. Thus, most aggregates have either a very low percentage of muCD99+ cells in them or a very high percentage of muCD99+ cells. See diagram above part D, where filled circles represent muCD99-expressing cells. Data shown are representative of at least three independent experiments.
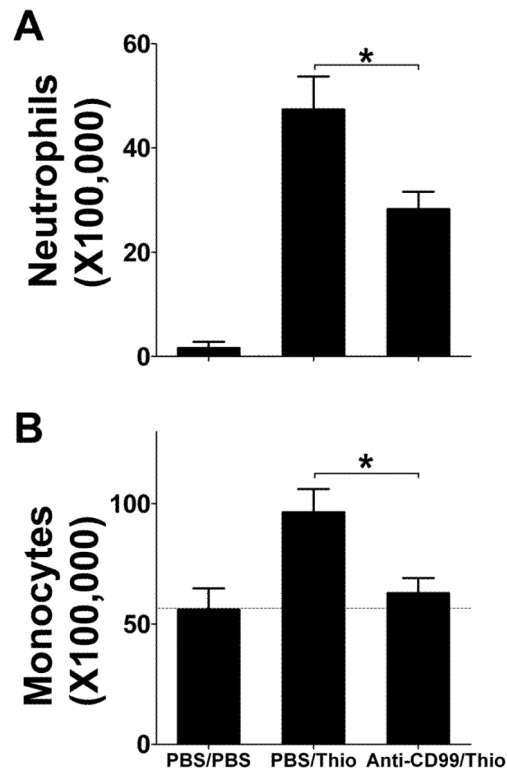
Figure 5. Anti-muCD99 blocks diapedesis *in vivo*

FVB mice received anti-muCD99 or control IgG i.v. one hour before they were injected i.p. with PBS or thioglycolate (Thio) and were sacrificed 18h later for peritoneal lavage. Cell count for neutrophils (A) or monocytes/macrophages (B) showed that anti-muCD99 blocked leukocyte emigration. Data are mean ± S.E.M. for three independent experiments and are representative of a total of six independent experiments. * $p < 0.01$.
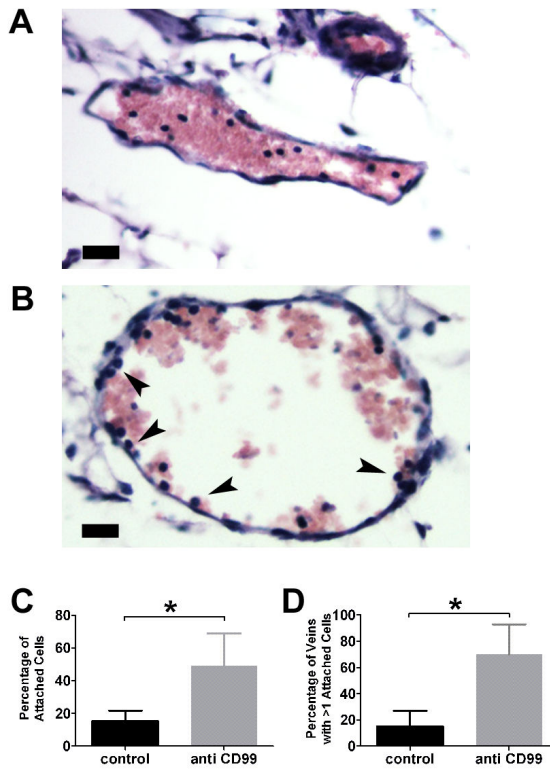
Figure 6. The block in diapedesis is at the level of the endothelial wall

Mice were pretreated with control rat IgG (A) or anti-muCD99 (B) and injected with thioglycollate as in Figure 5. Venules from mice pretreated with control rat IgG showed few leukocytes attached the venular wall (A) whereas venules from mice treated with an anti-muCD99 (B) displayed numerous leukocytes apparently interacting with the endothelial cells (arrowheads). C) Percentage of attached leukocytes in each group. D) Percentage of venules with more than one leukocyte attached. Scale bar = 20 μm. In parts C and D control is significantly different from anti-CD99 treated $p < 0.02$