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## Ectodomain shedding by ADAM17 (a disintegrin and metalloproteinase 17) in canine neutrophils

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### Abstract

ADAM17 is a transmembrane protease expressed by most cells in humans and mice that cleaves cell surface substrates primarily in a *cis* manner, a process referred to as ectodomain shedding. ADAM17 has numerous substrates and plays a broad role in various physiological processes, including as a key regulator of inflammation. At this time, little is known about ADAM17 expression and function in dogs. A well-established ADAM17 substrate is the leukocyte adhesion protein CD62L (L-selectin). We show that a selective inhibitor of ADAM17, but not an inhibitor of its most closely related family member ADAM10, blocks CD62L shedding upon canine neutrophil activation. We also tested several anti-human ADAM17 monoclonal antibodies (mAbs) for staining canine neutrophils. Although most did not recognize canine neutrophils, the mAbs MEDI3622 and D1(A12) did. They also blocked the downregulation of CD62L upon neutrophil activation. MEDI3622 is a human IgG antibody and we found that a canine chimeric version of this mAb also blocked CD62L shedding by canine leukocytes. Taken together, our findings provide the first direct evidence of ADAM17 expression and sheddase activity in dogs, establishing a potential therapeutic target for various inflammatory disorders.

### Keywords

Neutrophil; metalloprotease; inflammation; canine

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## Introduction

ADAM17 (a disintegrin and metalloproteinase 17) is a transmembrane protease that is constitutively expressed by most cells in the body (Lambrecht et al., 2018). It cleaves its substrates on the cell surface typically in a *cis* manner at a specific extracellular site, a process referred to as ectodomain shedding. Its catalytic activity is highly inducible upon cell activation by a broad range of stimuli. This process occurs very rapidly and involves ADAM17 transitioning from a low to high activity state through a conformational change and intermolecular interactions (Mishra et al., 2017). ADAM17 substrates have a key role in regulating inflammatory and immune processes and include various cytokines, their receptors, and adhesion molecules (Lambrecht et al., 2018; Mishra et al., 2017; Zunke and Rose-John, 2017). ADAM17 has been well-characterized in human and mouse neutrophils where it directly regulates various effector functions, including their activation, migration, and phagocytosis of antibody-opsonized targets (Mishra et al., 2017). Aberrant ADAM17 induction, however, has pathological consequences (Lambrecht et al., 2018; Mishra et al., 2017; Zunke and Rose-John, 2017). Using models of sepsis, we have reported that mice with ADAM17-null leukocytes have significantly reduced mortality (Long et al., 2012; Long et al., 2010; Mishra et al., 2016). This was associated with increased neutrophil recruitment, reduced bacteria levels at sites of infection, and markedly reduced systemic levels of proinflammatory cytokines, establishing a central role of ADAM17 in sepsis pathogenesis (Long et al., 2012; Long et al., 2010; Mishra et al., 2016). Moreover, in human patients, ADAM17 activity and a functional polymorphism of its gene corresponded with sepsis progression (Kermarrec et al., 2005; Shao et al., 2016).

One of the best studied substrates of mouse and human ADAM17 is the leukocyte adhesion protein CD62L (L-selectin) (Ivetic, 2018; Mishra et al., 2017). Canine CD62L has been characterized at the protein, cellular, and functional levels and is very similar to human CD62L (Abbassi et al., 1991; Crockett-Torabi and Fantone, 1997). Similar to human and murine CD62L (Jutila et al., 1990; Kishimoto et al., 1989; Kishimoto et al., 1990), canine CD62L also undergoes a rapid downregulation in expression upon neutrophil activation, suggesting it is similarly regulated by ectodomain shedding (Abbassi et al., 1991). At this time, the function of ADAM17 in canine neutrophils has not been reported. We demonstrate its expression and that blocking its activity disrupts the downregulation of CD62L expression. Given that ADAM17 has a broad immunomodulatory role in humans (Lambrecht et al., 2018; Mishra et al., 2017; Zunke and Rose-John, 2017), this sheddase may be a key therapeutic target in dogs for different inflammatory disorders.

## Materials and methods

### Animals

Peripheral blood was collected from 15 healthy pet dogs with owner consent. All animals received routine veterinary examinations and vaccines. The dog breeds represented in this study include four mixed breed dogs, three Labrador retrievers, one beagle, one Weimaraner, and one German shorthaired pointer. Breed information was not readily available for five donors due to client de-identification at time of collection. Blood collection was carried out in strict accordance with the recommendations in the Guide for the Care and Use of

Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Minnesota (Protocol Number: 21903–36913A).

## Antibodies

D1(A12) (MilliporeSigma, St. Louis, MO) is an anti-human ADAM17 mAb and has been shown to block its proteolytic activity (Tape et al., 2011). MEDI3622 is also an anti-human ADAM17 mAb that has been shown to block its proteolytic activity (Peng et al., 2016; Rios-Doria et al., 2015). For this study, MEDI3622 was generated as human IgG (Syd Labs, Natick, MA) and canine IgG (Creative Biolabs, Shirley, NY) using its variable heavy and variable light chain sequences. The anti-human ADAM17 mAbs 111633 and 111623 were purchased from R&D Systems (Minneapolis, MN). Anti-CD4 FITC (clone YKIX302.9) was purchased from eBioscience (San Diego, CA). Anti-CD5 PE (clone YKIX322.3), anti-CD3 PE (clone CA17.2A12), anti-CD8 APC (clone YCATE55.9), and anti-CD45 APC (clone YKIX716.13) were purchased from Bio-Rad Laboratories (Hercules, CA). The mAb CL2/6, which recognizes canine CD62L (Abbassi et al., 1991), and anti-CD22 FITC (clone RFB-4) were purchased from (ThermoFisher Scientific, Waltham, MA). The mouse anti-human ADAM17 mAb M220 (generously provided by Dr. R. Black, Immunex, Seattle, WA) has been previously described (Doedens and Black, 2000). The mAb R15.7 (generously provided by Dr. R. Rothlein, Boehringer Ingelheim, Ridgefield, CT) has been previously shown to recognize canine CD18 (Entman et al., 1990). Isotype-matched negative control mouse and human antibodies were purchased from R&D Systems and MilliporeSigma, respectively. Allophycocyanin-conjugated F(ab')<sub>2</sub> goat anti-mouse or donkey anti-human IgG (H+L) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

## Leukocyte isolation and treatment

Canine peripheral blood was collected from healthy donors in K<sub>2</sub>-EDTA blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). Whole blood was diluted ten-fold with red blood cell lysis buffer (ammonium chloride, 155 mM; potassium bicarbonate, 10 mM; and disodium EDTA, 0.1 mM) and incubated for 10 minutes at room temperature. Leukocytes were washed in PBS buffer (without Ca<sup>+2</sup> and Mg<sup>+2</sup>) (Lonza, Walkersville, MD) and cell viabilities were assessed using trypan blue exclusion, which was > 95%. Leukocytes (2.5×10<sup>6</sup>/ml in PBS) were stimulated with 1–15ng/mL phorbol-12-myristate-13-acetate (PMA) (MilliporeSigma), 1μg/mL formyl peptide receptor-like 1 agonist (MilliporeSigma), or 10μg/mL *Pseudomonas* lipopolysaccharide (LPS) (MilliporeSigma), as we have previously described (Mishra et al., 2015). Cells were stimulated for 30 minutes at 37°C, which was stopped by washing three times with ice cold PBS. Some cells were pre-incubated for 30 minutes on ice with ADAM10 and ADAM17 hydroxamate-based small molecule inhibitors or function blocking antibodies prior to activation. The ADAM17 inhibitor BMS566394 (MedKoo Biosciences, Morrisville, NC), referred to previously as inhibitor 32 (Ott et al., 2008), selectively blocks ADAM17 at 5 μM (Wang et al., 2013); the ADAM10 inhibitor GI254023X (R&D Systems) is 10-fold more selective for ADAM10 than ADAM17 in cellular assays and preferentially blocks ADAM10 in cells at a concentration ranging from 0.2–1 μM (Hundhausen et al., 2003; Wang et al., 2010). GI254023X was used

at 0.5  $\mu\text{M}$ , as we have previously described (Wang et al., 2013). INCB3619 (MedKoo Biosciences) blocks the activity of ADAM10 and ADAM17 (Zhou et al., 2006). INCB3619 was used at 10 $\mu\text{M}$ , as previously described (Wang et al., 2013). All inhibitors were resuspended in DMSO and used at the indicated concentrations. The anti-ADAM17 monoclonal antibodies MEDI3622 and D1(A12) were used at 10  $\mu\text{g/ml}$ .

### Cell staining and flow cytometric analysis

For cell staining, Fc receptor and nonspecific antibody binding sites were blocked for 30 minutes using 25% canine serum and 25% FBS in PBS prior to their staining with antibodies. All cell staining was analyzed on FACSCanto or FACSCelesta instruments (BD Biosciences, San Jose, CA). For controls, fluorescence minus one was used as well as appropriate isotype-matched antibodies since the cells of interest expressed Fc receptors. An FSC-A/SSC-A plot was used to set an electronic gate on leukocyte populations and an FSC-A/FSC-H plot was used to set an electronic gate on single cells. Fixable viability dyes eFluor 506 (eBioscience) or Zombie Violet (BioLegend, San Diego, CA) were used to assess live vs. dead cells, as per the manufacturers' instructions. Canine neutrophils were identified based on their forward and side light-scattering characteristics (Weiss et al., 2000; Weiss et al., 2004). Canine neutrophils express high levels of CD4 (Moore et al., 1992) and their identification was confirmed based on CD4 staining and lack of CD22, CD5, and CD8 staining (data not shown).

### Pairwise Sequence Alignment

To identify the MEDI3622 binding site for ADAM17 across several species, amino acid sequences of human, mouse, rat, canine (predicted), feline (predicted), and rabbit (predicted) ADAM17 were retrieved from UniProtKB. Amino acid sequences were aligned using Clustal Omega (Dublin, Ireland).

### Statistical analyses

All experiments were performed using three biological replicates. Flow cytometry data was analyzed using FlowJo. Statistical significance was determined between two groups by using an unpaired Student's t test.

## Results and Discussion

### Effects of selective ADAM17 inhibitors on CD62L downregulation by activated canine neutrophils.

The mAb CL2/6 recognizes canine CD62L and it has been reported that canine neutrophils rapidly downregulate CD62L upon their activation (Abbassi et al., 1991). We observed that neutrophil staining with this mAb rapidly and efficiently decreased upon their treatment with various stimuli, as determined by flow cytometry (Fig 1A,B). To investigate the involvement of ADAM17 in the downregulation of canine CD62L, we utilized several small molecule hydroxamate-based metalloproteinase inhibitors. These compounds were used at concentrations previously established to have selective inhibitory activity, as described in the Materials and Methods. INCB3619 is a selective inhibitor of ADAM10 and ADAM17 (Zhou et al., 2006). ADAM10 is the most similar ADAM family member to ADAM17 in terms of

amino acid sequence and structure (Takeda, 2016). INCB3619 was found to block CD62L downregulation following canine neutrophil activation with PMA (Fig 1A). GI254023X is a well described selective ADAM10 inhibitor (Hundhausen et al., 2003), but it was not found to significantly block the downregulation of CD62L following neutrophil activation (Fig 1A). BMS566394 has a potency orders of magnitude higher for ADAM17 than ADAM10 (Ott et al., 2008), and this compound did effectively block CD62L downregulation upon neutrophil activation (Fig 1A). BMS566394 also blocked the downregulation CD62L following canine neutrophil activation with physiological stimuli, such as the bacterial components formyl peptide and LPS (Fig 1B). Our findings based on the use of selective small molecule inhibitors suggests a role by ADAM17 but not ADAM10 in shedding canine CD62L. In humans, ADAM17 cleaves CD62L at a specific extracellular site proximal to the cell membrane (Kahn et al., 1994). The cleavage region of human CD62L is 87.5% identical and 100% homologous at the amino acid level to the membrane proximal region of canine CD62L (Fig 1C), which suggests that CD62L in canine leukocytes may be cut at the same location by ADAM17. However, we were not able to successfully immunoblot membrane or soluble CD62L from canine neutrophils, and thus were unable to confirm the presence and molecular mass of soluble CD62L or its membrane-associated cleavage fragment.

CD18 integrins are additional adhesion molecules expressed by neutrophils that are also important for their attachment and transmigration through the vascular endothelium (Ley et al., 2007). CD62L and Mac-1 (CD11b/CD18) surface levels are inversely regulated upon neutrophil activation (Kishimoto et al., 1989). Mac-1 resides in intracellular granules that are very rapidly translocated to the cell surface after neutrophil stimulation, providing a sensitive and quantifiable measure of cell activation (Berger et al., 1984). We found that neither INCB3619 nor BMS566394 treatment of canine neutrophils affected the upregulation of CD18 expression upon their stimulation (Fig 2). These findings thus indicate that the inhibitors at the concentrations used did not overtly impair neutrophil activation and prevent CD62L downregulation in that manner.

### **ADAM17 expression and function on canine neutrophils.**

To date, antibodies that detect canine ADAM17 have not been described. The predicted amino acid sequence of canine ADAM17 based on cDNA sequence reveals a 96% identity to human ADAM17 (Sarasa et al., 2010). Therefore, we examined the reactivity of several anti-human ADAM17 mAbs with canine neutrophils. We have previously demonstrated that the anti-ADAM17 mAbs 111633, 111623, and m220 all stain ADAM17 on human cells by flow cytometry (Buchanan et al., 2017; Jing et al., 2015; Walcheck et al., 2006; Wang et al., 2010). In Figure 3A, these mAbs are shown to stain human peripheral blood neutrophils, but not canine peripheral blood neutrophils above that of an isotype-matched negative control antibody (Fig 3B).

MEDI3622 is a human IgG mAb that specifically recognizes ADAM17 and is well characterized at blocking its function (Mishra et al., 2020; Mishra et al., 2018; Rios-Doria et al., 2015). MEDI3622 also blocks the downregulation of CD62L on human leukocytes upon their activation (Mishra et al., 2018). Its epitope has been mapped to a surface loop that is located within the catalytic domain of ADAM17, between amino acids P<sup>366</sup>-N<sup>381</sup>, which

does not occur in other ADAM family members (Peng et al., 2016). Alignment of this region with the predicted amino acid sequence for canine ADAM17 shows it to be nearly identical to human ADAM17. Additionally, this region is also conserved in other animal species, including mouse, rat, feline, and rabbit (Fig 4A). Indeed, MEDI3622 has been shown to recognize mouse ADAM17 (Mishra et al., 2018; Rios-Doria et al., 2015). We found that it also stained canine neutrophils and blocked the downregulation of CD62L upon their activation (Fig 4B,C).

MEDI3622 was generated by screening human scFv phage libraries with human ADAM17 (Peng et al., 2016; Rios-Doria et al., 2015). Using corresponding nucleotide sequences of the amino acids comprising the variable heavy and light regions of MEDI3622, we generated a chimeric antibody consisting of the constant heavy and light regions of canine IgG (D isotype), referred to as cIgG-B07, which also blocked the downregulation of surface CD62L expression upon canine neutrophil activation (Fig 4D).

D1(A12) is another anti-human ADAM17 mAb that blocks its function (Tape et al., 2011). We found this mAb also stained canine neutrophils and blocked the downregulation of CD62L upon their activation (Fig 4E,F), but not as effectively as MEDI3622 (Fig 4C). However, though MEDI3622 and D1(A12) were used at saturating concentrations, a comprehensive comparison of the inhibitory activity of these mAbs was not performed. D1(A12) is a human mAb engineered from phage display and its variable heavy and light chains were designed to recognize distinct epitopes in different regions of ADAM17 (Tape et al., 2011). To our knowledge, the specific locations of these epitopes have not been determined. D1(A12) has been reported to not recognize mouse ADAM17 (Tape et al., 2011), indicating less cross-reactivity than MEDI3622. The apparent decreased efficiency of blocking canine CD62L shedding by D1(A12) compared to MEDI3622 may be the result of either its heavy or light chain not effectively recognizing its respective epitope on canine ADAM17.

In summary, we demonstrate for the first time the function of ADAM17 in canine neutrophils and its role in the downregulation of CD62L upon their activation. We show that selective small molecule inhibitors of ADAM17, but not ADAM10, as well as the function-blocking ADAM17 mAbs MEDI3622 and D1(A12) block canine CD62L shedding from the neutrophil surface. ADAM17 has an important role in regulating the release of assorted soluble factors and the cell surface density of receptors and adhesion molecules; however, its excessive or prolonged induction has pathological consequences (Lambrecht et al., 2018; Mishra et al., 2017; Zunke and Rose-John, 2017). Indeed, systemic CD62L shedding by circulating neutrophils impairs their critical recruitment to sites of infection in animal models of sepsis and in septic patients (Kermarrec et al., 2005; Long et al., 2012; Long et al., 2010; Mishra et al., 2016). Neutrophil dysfunction is also a characteristic of the early stages of sepsis in dogs (Webb et al., 2007). ADAM17 therefore may serve as an important therapeutic target to reduce hyperinflammation and maintain proper leukocyte function during sepsis or other inflammatory disorders (Mishra et al., 2017).

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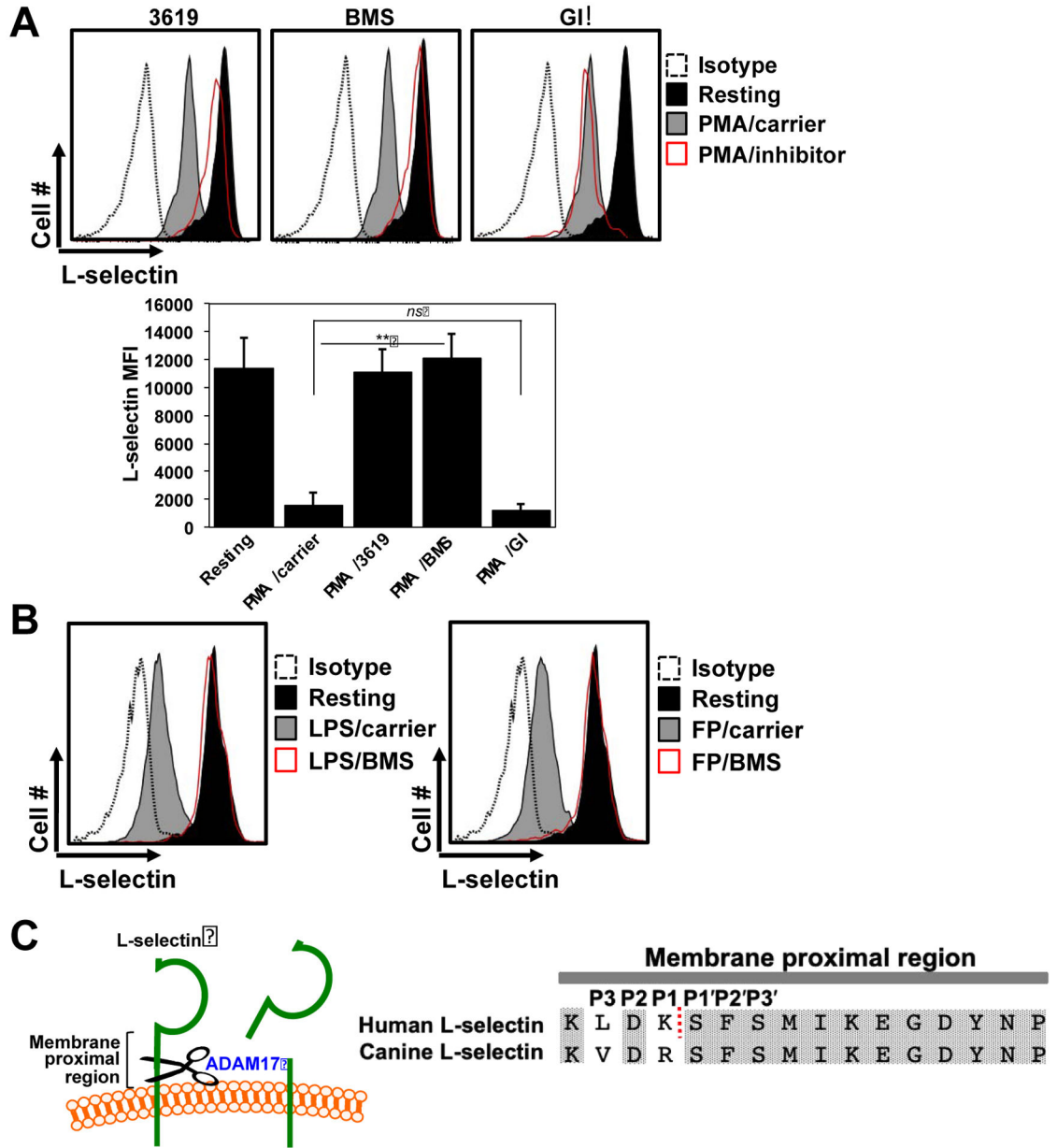
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**Fig 1. ADAM17 activity in canine neutrophils.**

**A.** Canine peripheral blood leukocytes were treated with or without PMA in the presence or absence of the ADAM10 and ADAM17 inhibitor INCB3619 (3619), the ADAM17 inhibitor BMS566394 (BMS), the ADAM10 inhibitor GI254023X (GI), or DMSO carrier for 30 minutes at 37°C, as indicated. The leukocytes were then stained with an anti-CD62L mAb or an appropriate isotype negative control antibody and assessed by flow cytometry. Neutrophils were gated based on their characteristic forward and side light scatters. All histograms show representative data and the bar graphs show mean ± SD with at least 3 animals per treatment. The x-axis of all histograms = Log 10 fluorescence. Statistical significance is indicated as \*\*p < 0.01 PMA/carrier vs. PMA/3619 and PMA/BMS. PMA/carrier vs. PMA/GI was not significant (*ns*). MFI = Mean fluorescence intensity. **B.**

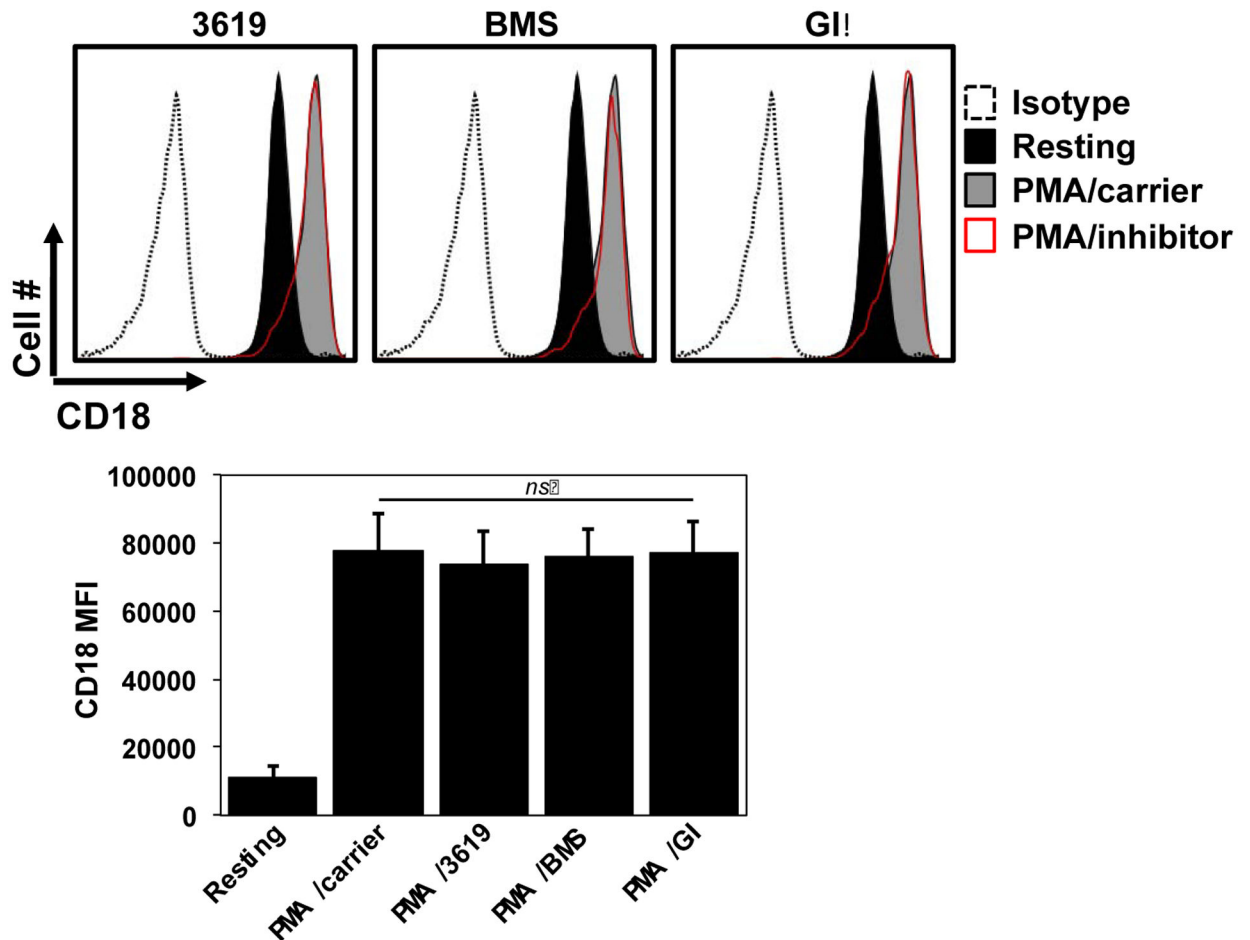
Leukocytes were treated with the ADAM17 inhibitor BMS as described above and LPS or formyl peptide (FP) were used as stimuli. C. CD62L undergoes ectodomain shedding by ADAM17 within a membrane proximal region, as indicated. Alignment of the extracellular membrane proximal regions of human and canine CD62L. The dashed line indicates the site of cleavage in human CD62L (Kahn et al., 1994). The amino acid sequences of human and canine CD62L are from the NCBI reference sequences NM\_000655.4 and XM\_537201.4, respectively.

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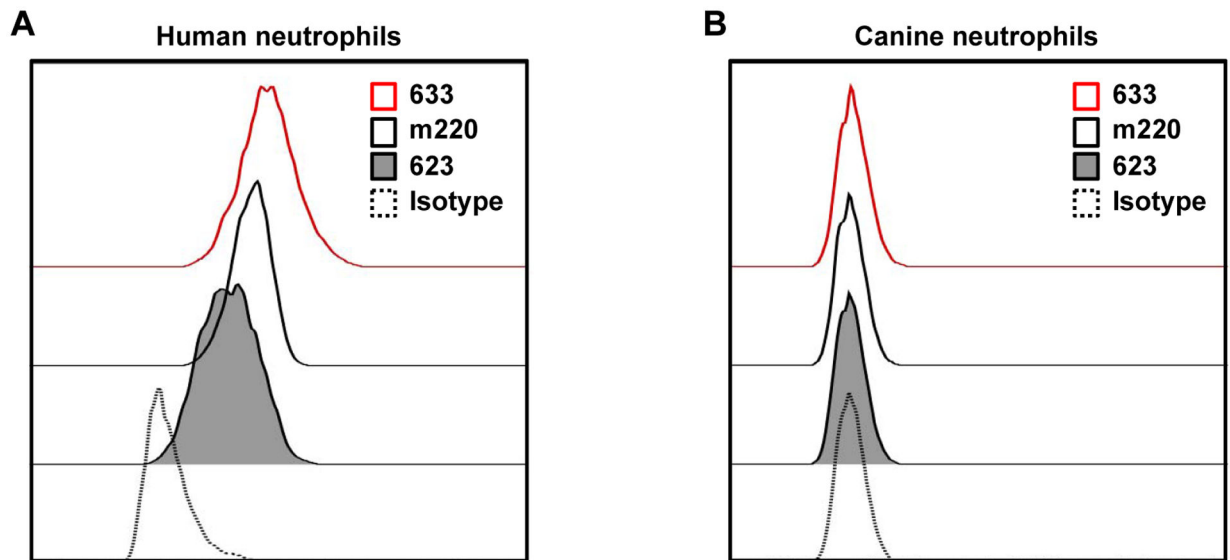
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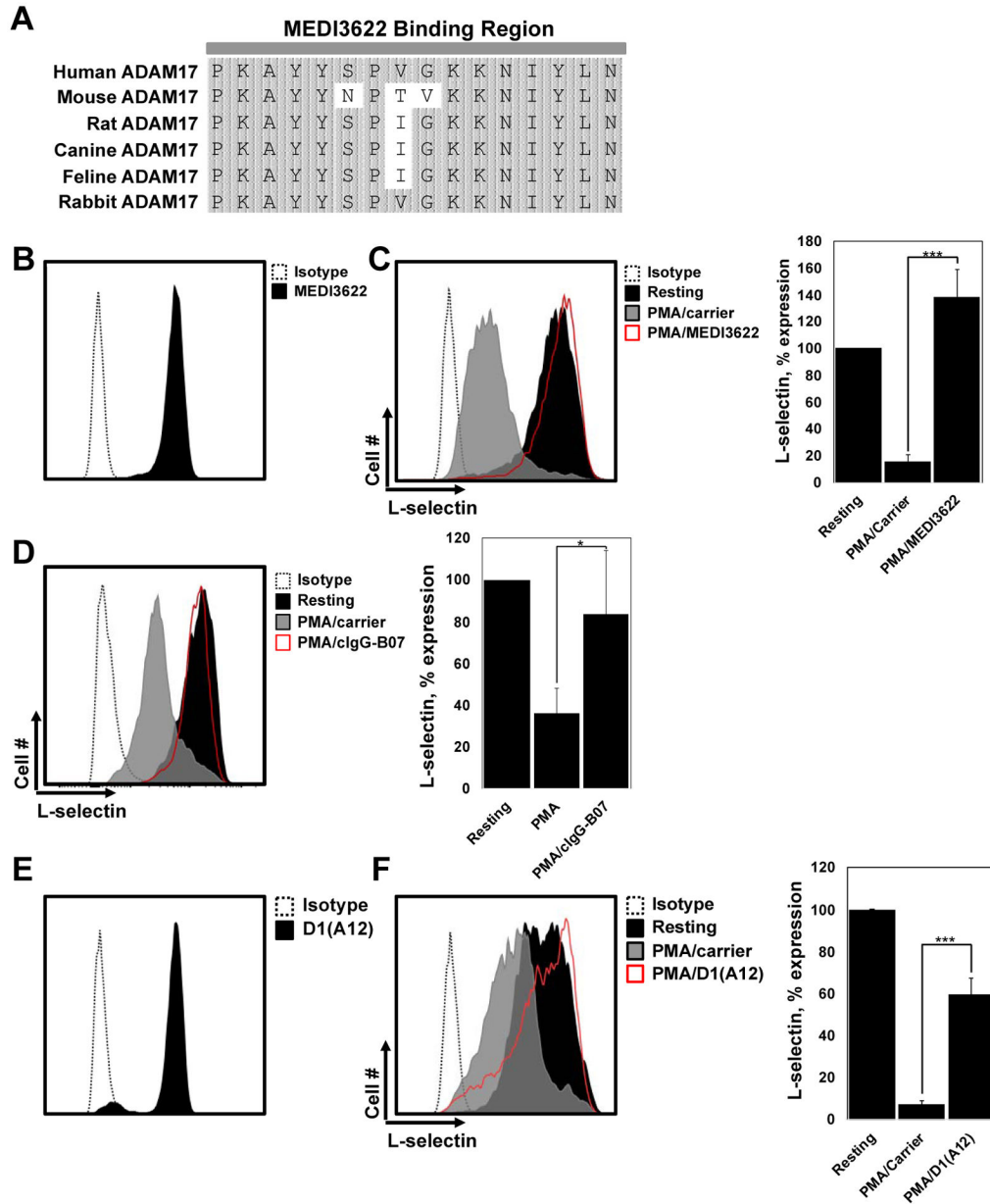
**Fig 2. ADAM10 and ADAM17 inhibitors do not affect Mac-1 upregulation.**

Canine peripheral blood leukocytes were treated with or without PMA in the presence or absence of ADAM10 and/or ADAM17 inhibitors, as described in Figure 1. Relative cell-staining levels of CD18 were determined by flow cytometry. Neutrophils were gated based on their characteristic forward and side light scatters. The x-axis of all histograms = Log 10 fluorescence. All histograms show representative data and the bar graphs show mean  $\pm$  SD with at least 3 animals per treatment.



**Fig 3. Anti-ADAM17 mAbs stain human but not canine neutrophils.**

Human (A) and canine (B) peripheral blood leukocytes were stained by different anti-ADAM17 mAbs and examined by flow cytometry, as indicated. Neutrophils were gated based on their characteristic forward and side light scatters. The y-axis of all histograms = cell number and the x-axis = Log 10 fluorescence. Data is representative of at least 3 independent experiments using leukocytes from separate human and canine donors.



**Fig 4. ADAM17 mAbs block the function of canine ADAM17.**

**A.** Alignment of amino acid sequences for ADAM17 across several species shows a high level of conservation in the MEDI3622 binding site, which occurs between amino acids P<sup>366</sup>-N<sup>381</sup>. Primary accession number (UniProt) for amino acid sequences are as follows: human (P78536), mouse (Q9Z0F8), rat (Q9Z1K9), canine predicted (M1V4E8), feline predicted (M3W8I1), and rabbit predicted (G1SZC8). **B.** Canine peripheral blood neutrophils were stained with MEDI3622 or an IgG1 isotype negative control antibody and assessed by flow cytometry. **C and D.** Canine peripheral blood neutrophils were treated with or without PMA in the presence of MEDI3622 (C) or clgG-B07 (D). **E.** Canine peripheral blood neutrophils were stained with D1(A12) or an IgG1 isotype negative control antibody and assessed by flow cytometry. **F.** Canine peripheral blood neutrophils were treated with or

without PMA in the presence of D1(A12). CD62L shedding was determined via flow cytometry. The y-axis of all histograms = cell number and the x-axis = Log 10 fluorescence. Data is representative of at least 3 independent experiments using leukocytes from separate animals. The bar graph shows mean  $\pm$  SD with at least 3 animals per treatment. Statistical significance is indicated as \* $p < 0.001$  PMA/carrier vs. PMA/cIgG-B07, \*\*\* $p < 0.001$  PMA/carrier vs. PMA/MEDI3622 or PMA/carrier vs PMA/D1(A12).

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