



## ADAM17 cleaves CD16b (FcγRIIb) in human neutrophils

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### ABSTRACT

CD16b (FcγRIIb) is exclusively expressed by human neutrophils and binds IgG in immune complexes. Cell surface CD16b undergoes efficient ectodomain shedding upon neutrophil activation and apoptosis. Indeed, soluble CD16b is present at high levels in the plasma of healthy individuals, which appears to be maintained by the daily turnover of apoptotic neutrophils. At this time, the principal protease responsible for CD16b shedding is not known. We show that CD16b plasma levels were significantly decreased in patients administered a selective inhibitor targeting the metalloproteases ADAM10 and ADAM17. Additional analysis with inhibitors selective for ADAM10 or ADAM17 revealed that only inhibition of ADAM17 significantly blocked the cleavage of CD16b following neutrophil activation and apoptosis. CD16b shedding by ADAM17 was further demonstrated using a unique ADAM17 function-blocking mAb and a cell-based ADAM17 reconstitution assay. Unlike human CD16, however, mouse CD16 did not undergo efficient ectodomain shedding upon neutrophil stimulation or apoptosis, indicating that this mechanism cannot be modeled in normal mice. Taken together, our findings are the first to directly demonstrate that ADAM17 cleaves CD16 in human leukocytes.

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### 1. Introduction

The human IgG Fc receptor CD16 (FcγRIII) consists of two isoforms (CD16a/FcγRIIIa and CD16b/FcγRIIIb) that are encoded by two highly homologous genes [1]. However, CD16a is a membrane-spanning protein, whereas CD16b is linked to the plasma membrane via a GPI anchor [2,3]. CD16b is expressed only by neutrophils and it primarily recognizes IgG-containing immune complexes, providing an important link between innate and adaptive immunity. Immune complexes can also promote excessive neutrophil activation that results in the release of high quantities of cytolytic and pro-inflammatory factors leading to extensive tissue injury, as is the case for neutrophils infiltrating synovial tissues during rheumatoid arthritis [4].

The surface density of CD16b is rapidly modulated by a complex interplay between mobilization from intracellular stores and proteolytic release [5]. CD16b proteolysis occurs upon neutrophil activation and apoptosis [6–8], and the maintenance of soluble CD16b in the plasma of healthy individuals indicates that its cleavage is a physiological process [6]. Serine proteases and metalloproteases have been implicated in CD16b proteolysis [8–10]; however, the primary enzyme involved in generating plasma CD16b has yet to be defined. This represents a

critical step for understanding how CD16b cleavage is regulated and for identifying potential therapeutic targets in inflammatory diseases.

Several members of the ADAM<sup>1</sup> (a disintegrin and metalloprotease) family of membrane-associated proteases facilitate ectodomain shedding of cell surface proteins [11]. The family member ADAM17 is a well described sheddase, which is expressed in resting neutrophils and its enzymatic activity is rapidly induced upon neutrophil activation and apoptosis [12–15]. Using highly selective small molecule inhibitors, a unique ADAM17 function blocking mAb, and a cell-based ADAM17 reconstitution assay, we provide the first direct evidence that ADAM17 is a sheddase of CD16b in neutrophils.

### 2. Materials and methods

#### 2.1. Human subjects and animals

The indicated patients from study INCB7839-202 (ClinicalTrials.gov Identifier: NCT00864175) were orally administered the selective ADAM10 and ADAM17 inhibitor INCB7839 (Incyte Corporation, Wilmington, DE) at 300 mg twice daily for 28 days with trastuzumab and docetaxel, and plasma samples were collected. These procedures and peripheral blood collection from normal individuals were performed in accordance with protocols approved by the Institutional Review Board at the University of Minnesota and the Incyte Corporation. Bone marrow neutrophils were isolated from wild-type C57BL/6J mice and FcγRIIb-deficient mice (C57BL/6j) (Taconic, Germantown, NY) in

Abbreviations: ADAM, a disintegrin and metalloprotease

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accordance with protocols approved by the Animal Care and Use Committee of the University of Minnesota.

## 2.2. Cell isolation and treatment

Human peripheral blood neutrophils and mouse bone marrow neutrophils were isolated as previously described [15,16]. NK cells were purified using a human NK cell isolation kit (Miltenyi Biotec, Auburn, CA), as per the manufacturer's instructions, resulting in >90% enrichment of CD56<sup>+</sup>CD3<sup>-</sup> lymphocytes. Cell activation with PMA (10 ng/ml; Sigma, St. Louis, MO), formyl peptide receptor-like 1 agonist (1 µg/ml; Sigma), human TNFα (5 µg/ml; PeproTech Inc, Rocky Hill, NJ) or induction of apoptosis with anti-human Fas mAb CH-11 (500 ng/ml) was performed as previously described [15,17]. Mouse neutrophil activation was induced by formyl peptide receptor-like 1 agonist or LPS from *E. coli* O111:B4 (100 µg/ml; Sigma). Mouse neutrophil apoptosis was induced by mouse TNFα (20 ng/ml; PeproTech) and cycloheximide (35 µM), which reproducibly induces apoptosis [18–21]. Mouse TNFα was initially titrated down to a concentration that caused nominal neutrophil activation during the timeframe of the assay, as we have previously reported [17]. Some cells were pre-incubated for 30 min with the broad-spectrum metalloprotease inhibitor TAPI-I (Peptides International, Louisville, KY) at 50 µM, the selective ADAM17 specific inhibitors SP26 [22] (MERCK, Whitehouse Station, NJ) at 5 µM and BMS566394 referred to as inhibitor 32 in Ref. [23] (Bristol-Myers Squibb Company, Princeton, NJ) at 5 µM, the selective ADAM10 inhibitor GI254023X (kindly provided by Dr. Andreas Ludwig, Rhein-Westphalian Technical University, Aachen, Germany) at 0.5 µM, which is 10-fold selective for ADAM10 over ADAM17 in cellular assays [24], the anti-human ADAM17 function blocking mAb D1(A12) at 50 nM (kindly provided by Dr. Gillian Murphy, University of Cambridge, Cambridge, United Kingdom), or isotype-matched negative control antibody.

The EC2 fibroblast cell line derived from ADAM17-deficient mouse embryos has been previously described [14,25,26]. The two allelic forms of CD16b (NA1 and NA2) were amplified from human neutrophil cDNA, cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA), and expressed in a stable manner in EC2 cells using described procedures [14,26]. The EC2 cells were then reconstituted with wild-type mouse ADAM17 using a bicistronic retroviral vector co-expressing eGFP, as previously described [14,26]. Apoptosis was induced by UV irradiation using a UV-C light source at a dosage of 60 mJ/cm<sup>2</sup>, followed by incubation at 37 °C in 5% CO<sub>2</sub> for 2 h.

## 2.3. Flow cytometry

Flow cytometric analyses were performed on a FACSCanto instrument (BD Biosciences), as described [15,16]. Human CD16 was detected by the mAb 3G8 (Biolegend). The mAb 196001 (R&D Systems, Minneapolis, MN) detects mouse CD16 but not FcγRIV, and the mAb 2.4G2 (Santa Cruz Biotech, Santa Cruz, CA) detects mouse FcγRIIB, CD16, and FcγRIV [27]. Mouse L-selectin was detected with Mel-14 (eBioscience, San Diego, CA). Externalized phosphatidylinositol on apoptotic cells was detected by fluorochrome-conjugated annexin-V, as per the manufacturer's instructions (BD Biosciences, San Jose, CA).

## 2.4. SDS-PAGE and immunoblotting

Western blotting was performed as previously described [14,15]. Human CD16 was detected by the mAb DJ130c (Santa Cruz Biotech, Santa Cruz, CA), mouse and human caspase-3 was detected by antibody #9662 (Cell Signaling, Beverly, MA), and mouse GAPDH was detected by antibody G9545 (Sigma).

## 2.5. Cytometric bead assay

A well established, commercially available human CD16 ELISA is not currently available. We developed a quantitative immunosorbent assay using cytometric functional beads A8 and A5 (BD Biosciences) conjugated with the anti-CD16 mAb 3G8 and an IgG1 isotype-matched negative control antibody, respectively, as per the manufacturer's instructions. A multiplexed quantitative cytometric bead assay was performed by flow cytometry, as previously described with some modifications [15]. Briefly, a suspension of A8 and A5 beads was incubated with supernatants from treated neutrophils or with human plasma diluted by 2-fold serial dilutions, followed by PE-conjugated anti-human CD16 mAb DJ130c (10 µg/ml). DJ130c detects an epitope distinct from 3G8 [28]. Soluble CD16 concentrations were determined from a standard curve obtained from serial dilutions of recombinant human CD16b containing BSA (R&D Systems).

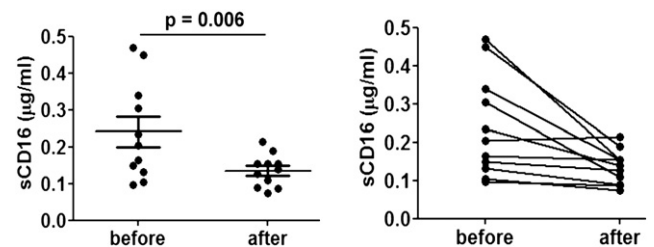
## 3. Results and discussion

### 3.1. Effect of an ADAM inhibitor on plasma CD16 levels

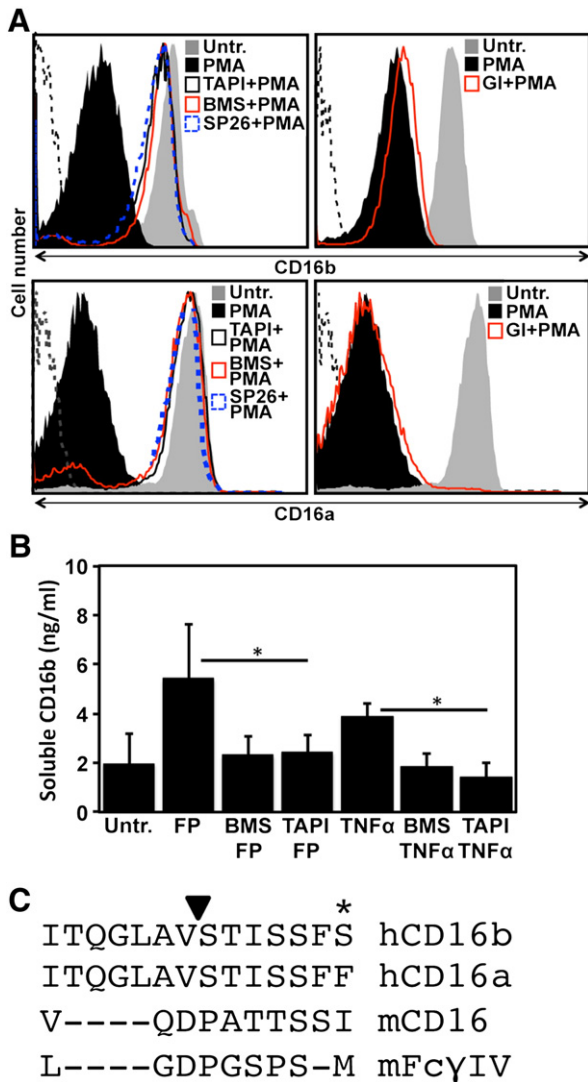
INCB3619 is a potent and selective inhibitor that targets both ADAM10 and ADAM17 when compared with a panel of matrix metalloproteases and ADAM family members [29,30]. The second-generation inhibitor INCB7839, which has a specificity profile identical to INCB3619 [31], has been examined in clinical trials in HER2-positive metastatic breast cancer patients, and found to cause a marked reduction in plasma levels of the ADAM product, soluble HER2 [32]. Using clinical samples from those studies, we assessed the plasma levels of soluble CD16 pre- and 28 days post-treatment with INCB7839. As shown in Fig. 1, CD16 plasma levels were significantly reduced in patients following INCB7839 treatment, with the highest level of reduction being 67%. These data suggest that human plasma CD16 levels are regulated by ADAM10 and/or ADAM17 activity.

### 3.2. Effects of selective ADAM10 and ADAM17 inhibitors on CD16 shedding

We next examined the effects of other hydroxamate-based metalloproteinase inhibitors on CD16 shedding, which differ in their selectivity for ADAM10 and ADAM17. GI254023X is an ADAM10 inhibitor that blocks ADAM10, but not ADAM17, in cells at a concentration ranging from 0.2 to 1 µM [33,34]. Using GI254023X within this concentration range, we found the inhibitor had little to no effect on the down-regulation of CD16b surface expression upon neutrophil treatment with PMA, a cell activator that induces robust CD16b shedding [8,9,35,36]. SP26 is a highly potent inhibitor as well, but with a selectivity more than 10,000-fold greater for ADAM17 than ADAM10 [22]. SP26 selectivity has been assessed in cellular assays and *in vivo* [33]. SP26 markedly attenuated CD16b down-regulation from PMA-activated neutrophils (Fig. 2A). BMS566394 is another highly selective ADAM17 inhibitor, again with a potency orders of



**Fig. 1.** Role of ADAM proteases in the homeostatic maintenance of CD16 plasma levels. Plasma levels of CD16 from 11 individuals before and after treatment with INCB7839 were quantified by ELISA. Shown in the left plot is the mean ± SD. In the right plot, patient CD16 plasma levels were compared before and after treatment.



**Fig. 2.** ADAM17 inhibitors block CD16 shedding upon leukocyte activation. **A.** Human peripheral blood leukocytes were activated with PMA in the presence or absence of TAPI, SP26, BMS566394 (BMS), or GI254023X (GI) for 30 min at 37 °C. Relative CD16b and CD16a expression levels on neutrophils (top panels) and NK cells (bottom panels), respectively, are shown. Negative control antibody staining of untreated cells is shown as a dashed line. The x-axis = Log 10 fluorescence. Data are representative of at least 3 independent experiments using leukocytes isolated from separate donors. **B.** Peripheral blood neutrophils were activated with formyl peptide (FP) or TNF $\alpha$  in the presence or absence of BMS566394 (BMS) or TAPI for 30 min at 37 °C. Cell supernatant levels of soluble CD16b were quantified by ELISA. Shown is the mean ( $\pm$ SD) of 3–5 independent experiments. \*,  $p < 0.05$  versus formyl peptide or TNF $\alpha$  treatment alone. **C.** Alignment of the extracellular juxtamembrane regions of human and mouse CD16 and mouse Fc $\gamma$ RIV. Dashes indicate gaps introduced to maximize homology. The arrowhead indicates the putative CD16b cleavage site [36] and the asterisk indicates the CD16b GPI-linkage site [50,51]. The alignment of human and mouse CD16 is based on the amino acid sequence comparison described by Mechetina et al. [52].

magnitude higher for ADAM17 [23]. BMS566394 also blocked the down-regulation of CD16b surface expression upon neutrophil activation with PMA (Fig. 2A). Moreover, BMS566394 blocked CD16b shedding by neutrophils activated with the physiological activators formyl peptide and TNF $\alpha$  (Fig. 2B). The broad-spectrum metalloprotease inhibitor TAPI did not further block CD16b shedding (Fig. 2A,B), indicating a predominant role by ADAM17 in cleaving CD16b.

CD16 expression occurs at high levels in human NK cells as well, but these cells express the transmembrane form (CD16a) and not the GPI-linked form (CD16b) [1]. CD16a also undergoes efficient ectodomain shedding by an unknown metalloprotease upon cell

activation [37]. Similar to CD16b in neutrophils, inhibition of ADAM17, but not ADAM10, blocked the down-regulation of CD16a upon NK cell activation (Fig. 2A). Hence, both the GPI-linked and transmembrane forms of CD16 appear to undergo ectodomain shedding by ADAM17 upon cell activation. A putative cleavage site in the extracellular juxtamembrane region of CD16b has been identified between amino acids Val-196 and Ser-197 proximal to the GPI-linkage site [10,36]. CD16b differs from CD16a by six amino acids in the extracellular domain [38]. However, none of these amino acid alterations occur at the putative CD16b cleavage site (Fig. 2C), indicating that the two CD16 isoforms may be cleaved by ADAM17 at the same location. Serine proteases have also been implicated in CD16b proteolysis [8–10]. In contrast to the plasma membrane-associated ADAMs, serine proteases appear to be released upon azurophil degranulation and their digestion of CD16b has been reported to produce nonfunctional proteolytic fragments [10]. Serine proteases are not involved in CD16b proteolysis following neutrophil apoptosis [8], consistent with their being little degranulation during this process.

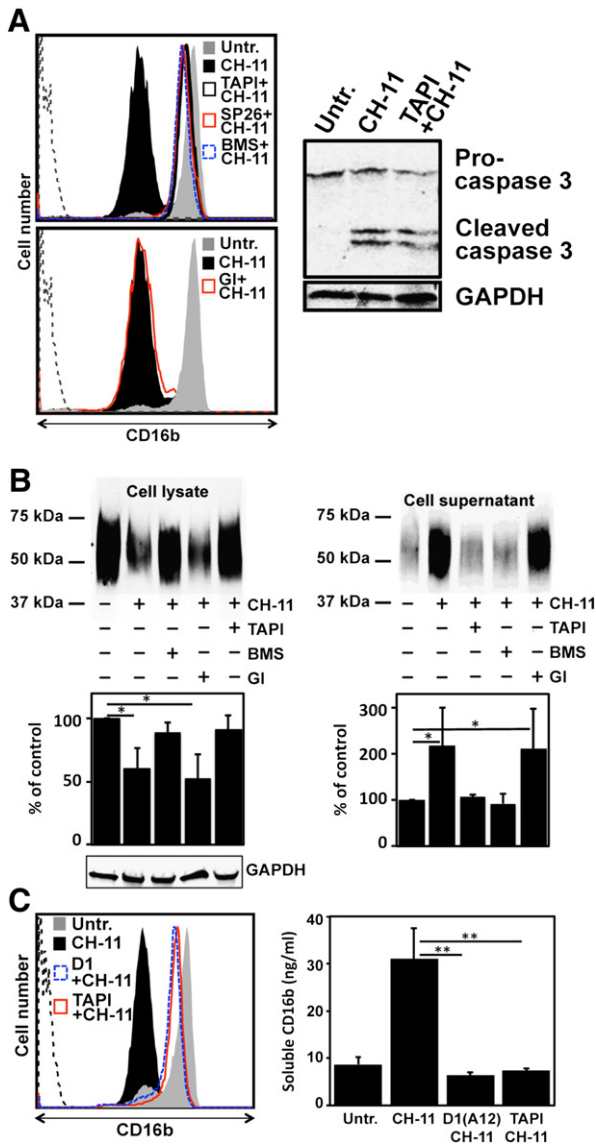
### 3.3. Role of ADAM17 in CD16b cleavage during neutrophil apoptosis

Neutrophils are the most abundant leukocyte in humans. These cells are short-lived and their turnover is governed by apoptosis, which occurs on the order of  $10^{11}$  cells per day in the average adult [39]. CD16b undergoes shedding upon the induction of neutrophil apoptosis [6–8], and considering that the vast majority of plasma CD16 is derived from neutrophils [6,40,41], this process may account for the homeostatic maintenance of plasma CD16 [8,42]. Fas is a key death receptor expressed by neutrophils and induction of this physiological signaling pathway resulted in substantial CD16b shedding (Fig. 3A and B). Indeed, by immunoblotting we observed a significant decrease in cell-associated CD16b and a corresponding increase in the levels of soluble CD16b in the cell supernatant (Fig. 3B). Similar to neutrophil activation, selective inhibitors of ADAM17, but not ADAM10, greatly impaired CD16b shedding by apoptotic neutrophils, and additional inhibition was not observed with a broad-spectrum metalloprotease inhibitor (Fig. 3A and B). In light of limitations in specificity by small molecule inhibitors, we also utilized a specific ADAM17 inhibitory mAb generated by phage display that contains individual antibody variable domains to two distinct ADAM17-specific epitopes [43]. In Fig. 3C, we show that the ADAM17 inhibitory mAb also blocked CD16b shedding by apoptotic neutrophils.

As an additional approach to investigate CD16b shedding by ADAM17, we used immortalized fibroblasts derived from ADAM17-deficient mice. CD16b occurs in two allelic forms termed NA1 and NA2, which are 95% homologous [1,44], and we expressed both forms in a stable manner in the ADAM17-null cells. The cells were then transduced with a bicistronic retroviral vector containing wild-type ADAM17 and GFP, which are expressed in a proportional manner [14,26]. In order to directly compare cells expressing and lacking ADAM17, GFP $^{+}$  cells (expressing ADAM17) and GFP $^{-}$  cells (lacking ADAM17) within the same population were assessed for their expression levels of CD16b-NA1 and CD16b-NA2 before and after the induction of apoptosis. The GFP $^{+}$  cells demonstrated a significant down-regulation in surface expression of CD16b-NA1 and CD16b-NA2 upon the induction of apoptosis, whereas the GFP $^{-}$  cells lacking ADAM17 maintained high levels of both forms of CD16b (Fig. 4). We confirmed the induction of cell apoptosis, in part, by assessing the activation of caspase-3 (Fig. 4).

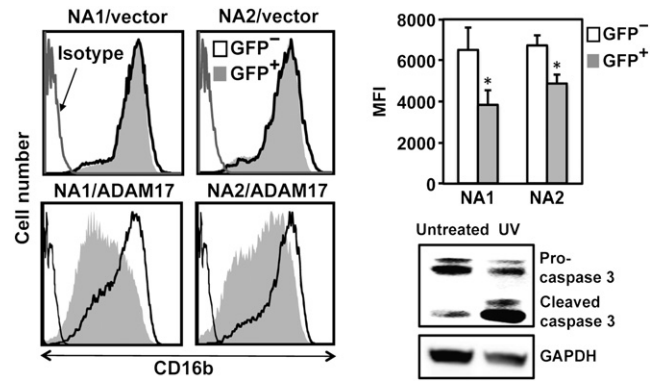
### 3.4. Ectodomain shedding of CD16 in mouse neutrophils

Unlike humans, only one gene encodes for CD16 in the mouse, and this is expressed as a transmembrane protein with an extracellular region highly homologous to mouse Fc $\gamma$ RIIB [38]. A literature review failed to reveal whether mouse CD16 shedding has been directly

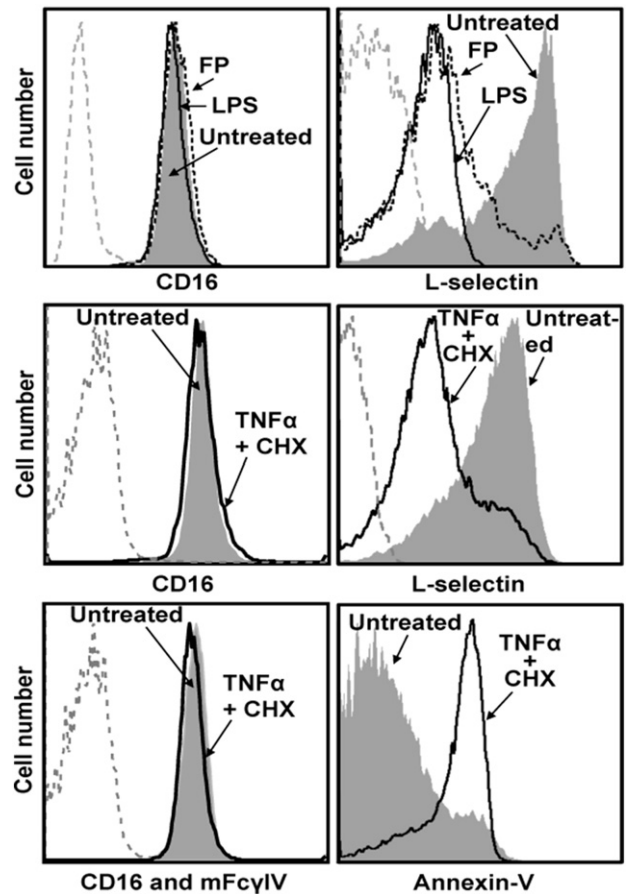


**Fig. 3.** ADAM17 inhibitors block CD16b down-regulation by apoptotic neutrophils. **A.** Neutrophils were treated with the anti-Fas antibody CH-11 in the presence or absence of TAPI, SP26, BMS566394 (BMS), or GI254023X (GI) for 6 h at 37 °C. Cell apoptosis was assessed, in part, by caspase-3 activation. Detergent lysates from CH-11-treated and untreated neutrophils cells were subjected to Western blotting with antibodies to caspase-3 or GAPDH (loading control). The anti-caspase-3 antibody detects full-length caspase-3 (pro-caspase-3) and activated caspase-3 (cleaved caspase-3). **B.** Neutrophils were treated as described above and detergent lysates (left figure) as well as cell media supernatants (right figure) from equivalent cell numbers were subjected to Western blotting with antibodies to CD16 or GAPDH (loading control). The histogram plots and immunoblots are representative of 3 independent experiments using neutrophils isolated from separate donors. Densitometric data are from 3 separate experiments (expressed as mean ± SD) and shown as percent of control. \*, *p* < 0.05 versus control. **C.** Neutrophils were treated with the anti-Fas antibody CH-11 in the presence or absence of D1(A12) (indicated as D1) for 6 h at 37 °C. Data are representative of at least three independent experiments using neutrophils isolated from separate donors. Cell supernatant levels of soluble CD16b were also quantified by ELISA. Shown is the mean (± SD) of three independent experiments. \*\*, *p* < 0.01 versus CH-11 treatment alone.

examined. Of interest is that following the induction of neutrophil activation or apoptosis, we did not observe a significant down-regulation in CD16 surface expression (Fig. 5). L-selectin in humans and mice is a well established ADAM17 substrate [15,45,46], and in contrast to mouse CD16, mouse L-selectin underwent an appreciable down-regulation in surface expression upon neutrophil activation and apoptosis, indicating ADAM17 induction (Fig. 5). It is unlikely that potential anti-CD16 antibody reactivity with FcγRIIB confounded



**Fig. 4.** ADAM17 reconstitution restores CD16b shedding during apoptosis. ADAM17-deficient EC2 cells expressing NA1-CD16b or NA2-CD16b were transduced with a bicistronic retroviral vector for co-expression of ADAM17 and GFP. EC2 cells expressing NA1- or NA2-CD16b were also transduced with an empty retroviral vector (vector). Apoptosis was induced by UV light exposure. GFP-expressing cells (GFP<sup>+</sup>) and non-GFP-expressing cells (GFP<sup>-</sup>) were each electronically gated and the relative expression levels of NA1-CD16b and NA2-CD16b were determined. Mean fluorescence intensity (MFI) of NA1-CD16b and NA2-CD16b staining from three experiments are expressed as mean ± SD. \*, *p* < 0.05 versus GFP<sup>-</sup> EC2 cells. Detergent lysates from UV-treated and untreated EC2 cells were subjected to Western blotting with antibodies to caspase-3 or GAPDH (loading control).



**Fig. 5.** Mouse neutrophils do not efficiently shed CD16. Bone marrow neutrophils were either untreated or treated with formyl peptide (FP) or LPS for 30 min at 37 °C to induce activation, or TNFα and cycloheximide (CHX) for 180 min at 37 °C to induce apoptosis. Cell staining by antibodies was determined using flow cytometry. Mouse CD16 was detected by the mAb 196001 and mouse CD16 and FcγRIV were detected by the mAb 2.4G2. Cell apoptosis was assessed by annexin-V reactivity. Negative control antibody staining of untreated cells is indicated (dashed line). The x-axis = Log<sub>10</sub> fluorescence. Data are representative of at least 3 independent experiments using leukocytes isolated from separate mice.

our findings considering that mouse neutrophils express little to none of this receptor [47]. In addition, neutrophils obtained from FcγRIIB-deficient mice failed to down-regulate their CD16 upon activation or apoptosis (data not shown). Mouse neutrophils also express high levels of FcγRIV, which has been proposed to be an orthologue of human CD16a [38]. We are not aware of a commercially available antibody specific to FcγRIV, but staining by the mAb 2.4G2, which detects CD16 and FcγRIV on mouse neutrophils [27], also did not decrease following the induction of neutrophil apoptosis (Fig. 5). Taken together, these findings reveal that mouse CD16, which functions differently than human CD16, is not regulated by ectodomain shedding. Indeed, there is little amino acid sequence similarity between the cleavage region of human CD16 and the corresponding juxtamembrane region of mouse CD16 or FcγRIV (Fig. 2C).

### 3.5. Conclusions

Our findings are the first to directly demonstrate that ADAM17 is a sheddase of CD16b in human neutrophils. Ectodomain shedding of CD16b upon neutrophil activation may be an important tuning mechanism to rapidly control the receptor's surface density and neutrophil stimulation by immune complexes. CD16b shedding during neutrophil apoptosis, however, may have an anti-stimulatory role by reducing neutrophil activation by the inflammatory milieu. Indeed, CD16b joins an increasing list of activating receptors on the surface of neutrophils cleaved by ADAM17 upon neutrophil apoptosis, which includes TNF-RI, TNF-RII, IL-6R, and L-selectin [15,48,49]. ADAM17 induction during apoptosis by senescent neutrophils may also be a key mechanism underlying the homeostatic maintenance of plasma CD16b in healthy individuals (see Graphical abstract). It is tempting to speculate that plasma CD16b may serve as an inflammation buffer that reduces the potential of activating circulating neutrophils by physiological immune complexes.

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