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ADAM17-Mediated Shedding of FcγRIIIA on Human NK Cells: Identification of the Cleavage Site and Relationship with Activation

Laurie Lajoie,†,*,1 Nicolas Congy-Jolivet,*,∗† Armelle Bolzec,† Valérie Gouilleux-Gruart,∗† Elodie Sicard,∗ Hsueh Cheng Sung,∗ Frank Peiretti,‡ Thierry Moreau,§ Henri Vié,¶ Beatrice Clémenceau,§ and Gilles Thibault*,†

FcγRIIIA/CD16A, the low-affinity receptor for the IgG Fc portion expressed on human CD56dim NK cells and involved in Ab-dependent cell cytotoxicity, is shed upon NK cell activation. We found that recombinant a disintegrin and metalloprotease (ADAM) 17 cleaved the ectodomain of FcγRIIIA/CD16A and a peptide for which the sequence encompasses aa 191–201 of the FcγRIIIA/CD16A stalk region but not ADAM10. MALDI-TOF analysis revealed that the peptide was cleaved between Ala195 and Val196 (i.e., 1 aa upstream of the expected position). This location of the cleavage site was confirmed by the finding that ADAM17 failed to cleave a peptide in which Ala and Val were reversed. ADAM17 was found to be expressed on NK cells, and stimulation with PMA or N-ethyl-maleimide resulted in the shedding of FcγRIIIA/CD16A and CD62L, a specific substrate of ADAM17. Selective inhibition of ADAM17 prevented the shedding of both molecules. Moreover, the shedding of FcγRIIIA/CD16A was strongly correlated with degranulation when a wide range of CD56dim NK cell activating receptors were stimulated, whereas both ADAM17-dependent shedding and internalization were involved in FcγRIIIA/CD16A downmodulation when the latter was engaged. Finally, the shedding of FcγRIIIA/CD16A was restricted to activated cells, suggesting that ADAM17 acts mainly, if not exclusively, in cis. Taken together, our results demonstrated for the first time, to our knowledge, at the molecular level that ADAM17 cleaves the stalk region of FcγRIIIA/CD16A and identified its cleavage site. The shedding of FcγRIIIA/CD16A was at least partially ADAM17 dependent, and it may be considered as a marker of FcγRIIIA/CD16A-independent NK cell activation highly correlated with degranulation. The Journal of Immunology, 2014, 192: 741–751.

The low-affinity receptor for the IgG Fc portion expressed on human CD56dim NK cells, monocytes, dendritic cells and rare T cells (1) and involved in Ab-dependent cell cytotoxicity, FcγRIIIA/CD16A, is shed upon NK cell activation (2, 3). Shedding is a mechanism for irreversible removal of many transmembrane cell-surface molecules. FcγRIIIA/CD16A is shed upon FcγRIIIA/CD16A-dependent activation of CD56dim NK cells by anti-CD16 mAb (3, 4), a condition in which reversible removal of FcγRIIIA/CD16A resulting from internalization has also been described (5), or by the Fc portion of rituximab (4, 6), as well as upon FcγRIIIA/CD16A-independent activation by K562 cells (7) or by phorbol esters such as PMA (2). The identification of the main enzyme involved in the shedding of FcγRIIIA/CD16A represents a critical step toward understanding how FcγRIIIA/CD16A-dependent functions are regulated.

Several members of the a disintegrin and metalloprotease (ADAM) family of membrane-associated proteases are involved in the ectodomain shedding of cell surface proteins (8). ADAM17/TNF-α-converting enzyme, which is responsible for processing TNF-α from its membrane-bound precursor to its soluble circulating form, and ADAM10 are the most characterized members of the ADAM family, having ~50% sequence identity within the catalytic domain (9). Specific substrates exist for each protease: CD62L (ε-selectin), for example, is specifically processed by ADAM17 from leukocyte membranes (10, 11), whereas epidermal growth factor is processed exclusively by ADAM10 (12). However, several substrates can be cleaved by both enzymes, and the specific protease involved is dictated by the activating stimulus. The enzymatic activity of ADAM17 and ADAM10 is rapidly induced upon neutrophil activation (13). Several corroborating studies have shown that ADAM17 can shed FcγRIIB/CD16B on stimulated neutrophils (14–17). The role of ADAM10 is less clear: selective inhibitors of ADAM10 do not impair the shedding of FcγRIIB/CD16B (17), whereas Guo et al. (16) reported that...

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ADAM17 and ADAM10 are the main sheddases of FcγRIIIA/CD16A when cells are stimulated by PMA and calcium ionophore (CaCl₂), respectively. The shedding of FcγRIIIA/CD16A on NK cells induced by FcγRIIIA/CD16A cross linking or by stimulation of NK cells with IL-12 and IL-18 has recently been shown to be greatly attenuated in the presence of ADAM17 inhibitor (18). It is of note that FcγRIIIA/CD16B and FcγRIIIA/CD16A are encoded by two highly similar genes (19). However, FcγRIIIA/CD16A is a membrane-spanning protein (19, 20), whereas FcγRIIIA/CD16B is linked to the plasma membrane via a GPI anchor (21, 22). The sequences from Thr¹⁹¹ to Ser²⁰⁰ of FcγRIIIA/CD16A and FcγRIIIA/CD16B are identical and contain the putative cleavage site of FcγRIIIA/CD16B (23) (Fig. 1).

Given the sequence homology of FcγRIIIA/CD16A and FcγRIIIA/CD16B, we were interested to study the roles of ADAM10 and ADAM17 in the shedding of FcγRIIIA/CD16A and to identify the putative cleavage site. Our second aim was to study the relationship between the shedding of FcγRIIIA/CD16A and the functional responses of CD56dim NK cells upon FcγRIIIA/CD16A-independent stimulation.

Materials and Methods

Cells

PBMCs, NK cells, JY cells, NKL cells, and CD16γ-transduced T cells were isolated and/or cultured as previously described (1, 4, 24, 25).

Abs

The following mAbs were used: FITC- or PC7-conjugated anti-CD16 (clone 3G8), PE-conjugated anti-CD62L (clone DREG56), PC7-conjugated anti-CD3 (clone UCHT1), PE-conjugated anti-CD19 (clone J3-119), PC5-conjugated anti-CD107a (clone H4A3) and isotype control (Beckman Coulter); PC5-conjugated anti-CD56 (clone N901), and isotype control (Beckman Coulter). The following mAbs were used: FITC- or PC7-conjugated anti-CD16 (clone 1D11), NKp30/CD335 (clone 4D12), NKp46/CD335 (clone 9E2), 2B4/CD244 (clone 2-69), and DNAx accessory molecule-1 (DNAM)/CD226 (clone DX11) and isotype control from BD Biosciences; and FITC-conjugated anti-ADAM17 (clone 111633) and its isotype control from R&D Systems. Unconjugated anti-ADAM17 rabbit polyclonal Ab was from France Biochem, HRP-conjugated anti-rabbit Ab from Cell Signaling Technology, and HRP-conjugated anti-murine IgG from GE Healthcare.

Reagents and substrates

Recombinant human (rh)FcγRIIIA/CD16A tagged with a polyhistidine at the C-terminal, rhADAM17, and rhADAM10 were purchased from R&D Systems. The sp. act. of rhADAM17 and rhADAM10 was verified using fluorogenic peptide substrates III and IX, respectively (R&D Systems). PMA and Cal (A23187, Calympcin) were purchased from Sigma-Aldrich. N-ethyl-maleimide (NEM) was purchased from Calbiochem, human neutrophil elastase (HNE) from Biocentrum, and trypsin from Euromedex. Fluorogenic substrate 4-(4-dimethylaminophenyl) diazenylbenzoic acid (DABCYL)-Thr-Gln-Gly-Leu-Ala-Val-Ser-Thr-Ile-Ser-Ser-Glu 5-(2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS)-NH₂ and DABCYL-Thr-Gln-Gly-Leu-Ala-Val-Ser-Thr-Ile-Ser-Ser-Glu(NEDANS)-NH₂ were custom synthesized by Gencust Europe.

ELISA assay

Soluble rhFcγRIIIA/CD16A protein (0.4 nM) was incubated for 2 h at 37°C in the absence or presence of 0.3 μM HNE or with rhADAM10 or rhADAM17 (0.6–20 nM) in ADAM reaction buffer (25 mM Tris [pH 7.4] containing 2.5 μM ZnCl₂ and 0.005% Brij-35 [v/v]). Each sample (100 μl) was applied to the Nickel chelate plate (Fisher Scientific) for 2 h with gentle agitation at room temperature. The uncleaved rhFcγRIIIA/CD16A was detected after incubation for 1 h with anti-CD16 clone Dj130c (1 μg/ml) and then HRP-conjugated anti-murine IgG diluted to 1/5000. Absorbance at 492 and 620 nm was measured with an iEMS reader MF spectrometer (LabSystem).

Fluorogenic assay

The assay was performed with HNE (3 μM), trypsin (3 μM), rhADAM17, or rhADAM10 (10 μg/ml) in 25 μM Tris (pH 9), containing 2.5 mM ZnCl₂ and 0.005% Brij-35 (v/v). Cleavage of the fluorogenic substrate (5 μM) was measured at Ex340/Em490nm using a SPECTRAmax

![FIGURE 1. Schematic representation of FcγRIIIA/CD16B and FcγRIIIA/CD16A. FcγRIIIA/CD16B (left panel) is linked to the plasma membrane via a GPI anchor, whereas FcγRIIIA/CD16A (right panel) is a membrane-spanning protein. The residue at position 203 (Phe and Ser for FcγRIIIA/CD16A and FcγRIIIB/CD16B, respectively) in the proform of the molecules is critical for this alternative membrane form (48). The proform of FcγRIIIB/CD16B (with Ser²⁰⁰) expressed as a membrane-spanning protein in the endoplasmic reticulum before GPI anchoring is shown as a gray dotted line. The glypliation site of the mature form of FcγRIIIA/CD16B is likely to be the Ser at position 201 according to the prediction of the α-site (cleavage site) in the proprotein sequence (49–51). The sequence from Thr¹⁹¹ to Ser²⁰⁰ of the stalk regions of FcγRIIIB/CD16B and FcγRIIIA/CD16A is indicated (middle panel). The location of the cleavage site (between Val¹⁹⁶ and Ser¹⁹⁷) of FcγRIIIB/CD16B is putative (23). Black boxes represent ITAMs.](http://www.jimmunol.org/Downloaded from http://www.sajm.org/)
Results

ADAM17 cleaved the stalk region of FcγRIIIA/CD16A between Ala195 and Val196 residues

We first investigated the ability of ADAM10 and ADAM17 to cleave FcγRIIIA/CD16A (Fig. 1). Soluble rhFcγRIIIA/CD16A was incubated in the presence of increasing concentrations of rhADAM17 or rhADAM10 or in the presence of HNE used as positive control. Each sample was then deposited on a nickel plate to capture polyhistidine-tagged rhFcγRIIIA/CD16A. Uncleaved rhFcγRIIIA/CD16A protein was detected by ELISA using the anti-CD16 mAb D130c. Detection of rhFcγRIIIA/CD16A was reduced by 67.5% after incubation with HNE, indicating effective cleavage of rhFcγRIIIA/CD16A (Fig. 2A). rhADAM17 was also effective and cleaved rhFcγRIIIA/CD16A in a dose-dependent manner, whereas no cleavage was detected after incubation with a high concentration of rhADAM10.

We then used a fluorogenic peptide, DABCYL-Thr-Gln-Gly-Leu-Ala-Val-Ser-Thr-Ile-Ser-Ser-Glu(EDANS)-NH2 (designated with a fluorochrome and a quencher at the N- and C-terminal ends of the peptide, respectively), for which the sequence encompasses aa 191–201 of FcγRIIIA/CD16A and FcγRIIB/CD16B. Fluorescence was measured after incubation of the peptide in the presence of HNE, trypsin (used as a negative control), rhADAM17, or rhADAM10. HNE effectively cleaved the peptide, whereas trypsin had no significant detectable effect (Fig. 2B). rhADAM17 induced substantial cleavage of the peptide after 10 min of exposure, reaching a plateau similar to that of HNE after 45 min. By contrast, no cleavage was observed in the presence of rhADAM10. These results are therefore in accordance with those obtained with the complete ectodomain of FcγRIIIA/CD16A (Fig. 2A) and demonstrate the ability of rhADAM17 to cleave the stalk regions of FcγRIIIA/CD16A and FcγRIIB/CD16B.

Analyses of the cleavage products by MALDI-TOF–mass spectrometry are shown in Fig. 2C. Two peaks, one corresponding to the intact peptide DABCYL-Thr-Gln-Gly-Leu-Ala-Val-Ser-Thr-Ile-Ser-Ser-Glu(EDANS)-NH2 (m/z 1688.763) and one corresponding to a peptide containing degraded DABCYL (m/z 1556.696), were detected in the absence of the enzyme (Fig. 2C, top panel). A single peak was observed in the sample containing only rhADAM17 (Fig. 2C, middle panel). Two additional peaks were identified in the cleavage products (Fig. 2C, bottom panel) (i.e., DABCYL-Thr-Gln-Gly-Leu-Ala [m/z 738.274] and Val-Ser-Thr-Ile-Ser-Ser-Glu (EDANS)-NH2 [m/z 967.435]), indicating that ADAM17 cleaves the peptide substrate between Ala195 and Val196 residues.

An Ala residue at P1 position immediately upstream of the cleavage site favors the catalytic action of ADAM17, whereas a Val residue at this position is detrimental (29). We therefore designed a second fluorogenic peptide in which Ala and Val were reversed. As shown in Fig. 2D, the fluorescence observed after incubation of rhADAM17 with the peptide containing the reverse sequence was dramatically decreased compared with that observed with the wild-type (WT) sequence, confirming the specificity of the cleavage site in the peptide corresponding to the stalk regions of FcγRIIIA/CD16A and FcγRIIB/CD16B. We concluded that rhADAM17 cleaves FcγRIIIA/CD16A and probably FcγRIIB/CD16B between the Ala195 and Val196 amino acids.

ADAM17 was present on the surface of NK cells and mediated the shedding of FcγRIIIA/CD16A

We then investigated the presence of ADAM17 on NK cells. First, cell extracts from PBMC (used as a positive control), from purified human NK cells, from the JY lymphoblastoid cell line and the NKL cell line were analyzed by Western blotting. We used a rabbit polyclonal Ab against human ADAM17, allowing simultaneous detection of several bands corresponding to the catalytic form (80 kDa), the precursor (proform 110 kDa), and glycosylated ADAM17 (130 kDa). The results showed the presence of an 80-kDa band (Fig. 3A, bottom, arrow) and a 110-kDa band (Fig. 3A top, arrow) in all cell extracts. However, the mature
form of ADAM17 was detected at higher intensity in NK cell extracts than in PBMCs, the JY cell line, or the NKL cell line. The activity of rhADAM10 and rhADAM17 was checked using commercial fluorogenic peptide (L. Lajoie, unpublished observations). This experiment was repeated twice, and the results from one representative experiment are shown (mean ± SD of triplicate). (B) Hydrolysis of the fluorogenic peptide DABCYL–Thr-Gln-Gly-Leu-Ala-Val-Ser-Thr-Ile-Ser-Ser-Glu(EDANS)-NH₂, corresponding to the stalk regions of FcγRIIIB/CD16B and FcγRIIIA/CD16A, was analyzed by fluorescence after incubation in the absence or presence of HNE, trypsin used as a negative control, rhADAM10, or rhADAM17. This experiment was repeated four times, and one representative experiment is shown. (C) MALDI-TOF analysis of the fluorogenic peptide DABCYL–Thr-Gln-Gly-Leu-Ala-Val-Ser-Thr-Ile-Ser-Ser-Glu(EDANS)-NH₂ (top panel) (each amino acid residue is indicated by single letter code), rhADAM17 (middle panel), and the cleavage products obtained after incubation with rhADAM17 (bottom panel). (D) Hydrolysis of the fluorogenic WT peptide DABCYL–Thr-Gln-Leu-Ala-Val-Ser-Thr-Ile-Ser-Ser-Glu(EDANS)-NH₂ (black bars) and of the peptide DABCYL–Thr-Gln-Leu-Ala-Ser-Thr-Ile-Ser-Ser-Glu(EDANS)-NH₂ (white bars) was analyzed by fluorescence after incubation in the absence or presence of HNE, rhADAM17, or rhADAM10. This experiment was repeated twice, and one representative experiment is shown.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** The stalk region of FcγRIIIA/CD16A cleaved by ADAM17 between Ala¹⁹⁵ and Val¹⁹⁶. (A) Uncleaved recombinant human FcγRIIIA/CD16A was detected by ELISA using anti-CD16 mAb Dj130c, which recognizes an epitope located in the EC1 domain of FcγRIIIA/CD16A (4) after incubation in the absence (black bar) or in the presence of HNE used as positive control (white bar), or in the presence of rhADAM10 (gray hatched bar) or rhADAM17 (gray bars). The activity of rhADAM10 and rhADAM17 was checked using commercial fluorogenic peptide (L. Lajoie, unpublished observations). This experiment was repeated twice, and the results from one representative experiment are shown (mean ± SD of triplicate). (B) Hydrolysis of the fluorogenic peptide DABCYL–Thr-Gln-Gly-Leu-Ala-Val-Ser-Thr-Ile-Ser-Ser-Glu(EDANS)-NH₂, corresponding to the stalk regions of FcγRIIIB/CD16B and FcγRIIIA/CD16A, was analyzed by fluorescence after incubation in the absence or presence of HNE, trypsin used as a negative control, rhADAM10, or rhADAM17. This experiment was repeated four times, and one representative experiment is shown. (C) MALDI-TOF analysis of the fluorogenic peptide DABCYL–Thr-Gln-Gly-Leu-Ala-Val-Ser-Thr-Ile-Ser-Ser-Glu(EDANS)-NH₂ (top panel) (each amino acid residue is indicated by single letter code), rhADAM17 (middle panel), and the cleavage products obtained after incubation with rhADAM17 (bottom panel). (D) Hydrolysis of the fluorogenic WT peptide DABCYL–Thr-Gln-Leu-Ala-Val-Ser-Thr-Ile-Ser-Ser-Glu(EDANS)-NH₂ (black bars) and of the peptide DABCYL–Thr-Gln-Leu-Ala-Ser-Thr-Ile-Ser-Ser-Glu(EDANS)-NH₂ (white bars) was analyzed by fluorescence after incubation in the absence or presence of HNE, rhADAM17, or rhADAM10. This experiment was repeated twice, and one representative experiment is shown.

The shedding of FcγRIIIB/CD16B induced by CaI and PMA on neutrophils is mainly ADAM10 and ADAM17 dependent, respectively (16). We therefore evaluated the shedding of FcγRIIIA/CD16A on NK cells under stimulation with CaI and/or PMA. As shown in Fig. 4A, stimulation with PMA induced dose-dependent downmodulation of CD16. Moreover, this effect was not modified in the presence of CaI, whereas CaI used alone was ineffective.

CD62L (L-selectin), a known specific substrate of ADAM17 (10, 11), was expressed on a fraction of NK cells (30). We therefore studied the comodulation of CD16 and CD62L after stimulation of NK cells with NEM, a direct activator of ADAM (31), and after stimulation with PMA. First, stimulation with NEM resulted in dose- and time-dependent downmodulation of CD62L and CD16 (Fig. 4B). Downmodulation of both CD16 and CD62L was detected after 5 to 10 min and was complete after 30 to 45 min, suggesting that the ADAM(s) responsible for the cleavage is (are) expressed on the membrane of NK cells. We did not observe CD62L expression on cells that have downmodulated CD16 (CD62L⁺CD16low) after NEM stimulation, showing that downmodulation of CD16 and CD62L occurred simultaneously and suggesting that the same ADAM was responsible for the shedding of both molecules. This was confirmed when NK cells were stimulated with PMA (Fig. 4C): CD16 and CD62L were simultaneously and dramatically downregulated. Preincubation of NK cells prior to PMA stimulation with increasing concentrations of TIMP-3 or TIMP-2 [physiologic and selective pharmacologic...
induce degranulation (Fig. 5A). The coengagement of at least two or more ARs was required to five mAbs targeting NKG2D, NKp30, NKp46, 2B4, and DNAM. Cells were incubated in plates sensitized by combinations of one to downmodulation in response to stimulation of NK cell ARs. NK and DNAM (34). We therefore studied degranulation and CD16
tions requires a combination of activator signals mediated by ac-
Fc
Fc
Fc
RIIIA/CD16A engagement, the triggering of NK cell func-
RIIIA/CD16A on stimulated NK cells.

ADAM17-dependent shedding of FcγRIIIA/CD16A induced by FcγRIIIA-independent NK cell activation was highly correlated with degranulation

The FcγRIIIA/CD16A engagement by anti-CD16 or rituximab led to simultaneous CD16 downmodulation and degranulation (4). Moreover, CD16 downmodulation has been observed following interaction between NK cells and K562 target cells, which is independent of FcγRIIIA/CD16A engagement (7). In addition to FcγRIIIA/CD16A engagement, the triggering of NK cell functions requires a combination of activator signals mediated by ac-
tivating receptors (ARs) such as NKGD2, NKp30, NKp46, 2B4, and DNAM (34). We therefore studied degranulation and CD16 downmodulation in response to stimulation of NK cell ARs. NK cells were incubated in plates sensitized by combinations of one to five mAbs targeting NKGD2, NKp30, NKp46, 2B4, and DNAM. The coengagement of at least two or more ARs was required to induce degranulation (Fig. 5A, top panel), CD16 downmodulation (Fig. 5A, bottom panel), or IFN-γ production (Supplemental Fig. 1A) in a significant proportion of NK cells. The percentage of degranulating cells depended on the combination used to stimulate the cells, tending to increase with the number of ARs simultaneously engaged (Fig. 5A, top panel). Using the 31 possible combinations of 1–5 mAbs, we obtained a wide range of responses. Importantly, we observed that the profiles of CD16 downmodulation and CD107a expression according to the stimulation combina-
tion used were very similar (compare Fig. 5A, top and bottom panels), although the percentages of cells downmodulating CD16 were always substantially higher than the percentages of CD107a+ cells. The calculated coefficient of correlation was therefore very high (R² = 0.97), whereas a weaker correlation was also observed between CD16 downmodulation and IFN-γ production (Supplemental Fig. 1B). It is unlikely that this weaker correlation resulted from the fact that CD56bright cells, which do not express CD16, might represent a significant proportion of IFN-γ–producing cells. Indeed, IFN-γ+ NK cells observed after stimulation by the effective combination of mAbs targeting NKGD2, NKp30, NKp46, and 2B4 were almost totally confined to CD56dim NK cells (Supplemental Fig. 1C). Finally, we studied the downmodulation of CD16 and CD62L induced by the combination of mAbs target-
ing NKGD2, NKp30, NKp46, and 2B4. This stimulation combination led to downmodulation of both molecules (Fig. 5B). Preincubation of NK cells with TIMP-3 or TMI-2 led to dose-
dependent inhibition of downmodulation of both molecules. We concluded that the ADAM17-dependent shedding of FcγRIIIA/ CD16A occurred after FcγRIIIA/CD16A-independent stimulation of NK cells and was strongly correlated with degranulation. It is of note that the FcγRIIIA/CD16A engagement by plate-bound anti-CD16 3G8 led upregulation of CD107a on 17.7 ± 3.4% of NK cells and to downregulation of FcγRIIIA/CD16A on 95.4 ± 3.9% (n = 6) (i.e., a percentage similar to and substantially higher than that obtained when NK cells were stimulated by the combination of mAbs targeting NKGD2, NKp30, NKp46, and 2B4) (Fig. 5A). Thus, CD16 downmodulation was weakly correlated with upregulation of CD107a when cells were incubated with 3G8 compared with experiments in which other stimuli were used to activate NK cells. We cannot exclude the possibility that the detection of CD16 by fluorescent mAb might be hampered by potential epitope interference (due to cross linking or conformational change) (18) or by a putative masking effect due to detachment of the unconjugated stimulating anti-CD16 from the microplate (4). However, this hypothesis is unlikely because: 1) we observed simultaneous and dramatic downmodulation of CD62L (see below) when NK cells were stimulated by plate-bound 3G8; and 2) we found that ADAM17 inhibitors substantially inhibited the downmod-
ulation of FcγRIIIA induced in this condition (Supplemental Fig. 2A). Another explanation might be that FcγRIIIA/CD16A may be internalized when it is engaged. An internalization method (35) therefore tested in which the cells were incubated with sol-
uble Alexa 488–labeled 3G8, in the presence of an anti–Alexa 488
Ab, which quenches fluorescence on the cell surface but not from internalized molecules (Supplemental Fig. 2B). Using this approach, we found that FcγRIIIA/CD16A downmodulation induced by soluble 3G8 was partially due to internalization. However, dramatic downmodulation of CD62L on CD56dim NK cells stimulated by soluble 3G8 was observed at the same time, showing that ADAM17-dependent shedding was induced in this condition (Supplemental Fig. 2C, right panel). We concluded that ADAM17-dependent shedding and internalization may both be involved in the loss of CD16 when the latter is engaged and that the relative contribution of each mechanism might depend on the conditions of engagement (for instance, immobilized versus soluble anti-CD16). By contrast, ADAM17-dependent shedding was the main, if not the exclusive, mechanism after FcγRIIIA/CD16A-independent stimulation of NK cells and strongly correlated with degranulation in this situation.

**Shedding of FcγRIIIA/CD16A was restricted to activated cells**

Given that the percentages of cells downmodulating CD16 were substantially higher than the percentages of degranulating cells, we...
wondered whether ADAM17 expressed on an activated cell could mediate the shedding of FcγRIIIA/CD16A on the cell surface of a neighboring cell (i.e., in trans). Expression of CD62L and CD16 was therefore evaluated on CD16/γ-transduced T cells (36) and NK cells. As shown in Fig. 6 (top panels), stimulation of NK cells with anti-CD3 mAb did not modify CD16 or CD62L expression, whereas stimulation with mAbs targeting NKG2D, NKp30, NKp44, and 2B4 induced the shedding of both molecules. In contrast, stimulation of CD16/γ-transduced T cells led to the opposite result: CD3 engagement induced substantial shedding of FcγRIIIA/CD16A, whereas coengagement of NKG2D, NKp30, NKp44, and 2B4 was ineffective (Fig. 6, middle panel). When NK cells and CD16/γ-transduced T cells were mixed and stimulated in these conditions, FcγRIIIA/CD16A shedding was restricted to T cells after CD3 stimulation (Fig. 6, bottom middle panel) and to NK cells after NKG2D, NKp30, NKp44, and 2B4 stimulation (Fig. 6, bottom right panel). These results demonstrated that the FcγRIIIA/CD16A shedding was restricted to appropriately stimulated cells and suggest that this is likely to occur mainly, if not exclusively, in cis.

Discussion
This study was undertaken to investigate the roles of ADAM10 and ADAM17 in the shedding of FcγRIIIA/CD16A. We found that only the latter was able to cleave FcγRIIIA/CD16A, and we determined the cleavage site, which was located in the stalk region between Ala195 and Val196 (i.e., 1 aa upstream of the expected position). We also demonstrated that the shedding of FcγRIIIA/CD16A on
NK cells was at least partially mediated by ADAM17. Moreover, we showed that the ADAM17-dependent shedding of FcγRIIIA/CD16A on CD56<sup>dim</sup> NK cells was strongly correlated with degranulation when a wide range of NK cell ARs were stimulated. ADAM17-dependent shedding of FcγRIIIA/CD16A was the main, if not the exclusive, mechanism of FcγRIIIA modulation after FcγRIIIA/CD16A-independent stimulation of NK cell, whereas both ADAM17-dependent shedding and internalization may be involved after FcγRIIIA/CD16A-dependent stimulation. Finally, we provided evidence that the ADAM17-dependent shedding of FcγRIIIA/CD16A was restricted to activated cells, suggesting that ADAM17 acts mainly, if not exclusively, in cis. The shedding of FcγRIIIA/CD16A may therefore be considered as a marker of FcγRIIIA-independent NK cell activation correlated to functional responses.

Using specific inhibitors (14, 15, 17) or overexpressing transfected cells (16, 17), previous studies have reported that FcγRIIB/CD16B may be cleaved by ADAM17 (14–17) and/or ADAM10 (16), depending on the stimulation conditions. The sequences from Thr<sup>191</sup> to Ser<sup>197</sup> of the stalk regions of FcγRIIIA/CD16A and FcγRIIB/CD16B are identical and contain the putative cleavage site of the latter, which was reported to be located between Val<sup>196</sup> and Ser<sup>197</sup> (23). We therefore evaluated the ability of recombinant ADAM10 and ADAM17 to cleave the complete ectodomain of FcγRIIIA/CD16A or a fluorogenic peptide encompassing the stalk regions of FcγRIIIA/CD16A and FcγRIIB/CD16B. We demonstrated that ADAM17 was able to cleave both compounds, whereas ADAM10 was ineffective. To our knowledge, this is the first demonstration at the molecular level that ADAM17 cleaves FcγRIIIA/CD16A. Furthermore, MALDI-TOF analysis of the cleavage products revealed that the cleavage site was located between Ala and Val in the WT peptide but not if it is downstream from the cleavage site, with a Val being the most favored amino acid, whereas ADAM10 can accommodate aromatic amino acids at that position. Moreover, they demonstrated selectivity for small residues such as Ala at P1 (14–17) immediately upstream of the cleavage site. They showed that ADAM17 is selective for smaller α-helical hydrophobic residues at the P1<sup>9</sup> position (immediately upstream from the cleavage site), with a Val being the most favored amino acid, whereas ADAM10 can accommodate aromatic amino acids at that position. Moreover, they demonstrated selectivity for small residues such as Ala at P1 (immediately upstream of the cleavage site). They compared their peptide library to sequences that have been identified as being cleaved by ADAMs and confirmed that an Ala at P1 and a Val at P1’ were frequent at ADAM17 cleavage sites (found for instance in the cleavage site of TNF, TGF-α, and Notch). By contrast, a Val at P1 was detrimental for both enzymes. Our MALDI-TOF results were in line with these findings. Moreover, it was expected from the results of Caescu et al. (29) that reversing the Ala and Val in our peptide (resulting in a Val-Ala-Ser sequence rather than an Ala-Val-Ser sequence) would reduce the cleavage if the cleavage site is located between Ala and Val in the WT peptide but not if it is between Val and Ser. Our finding that the Ala-Val permutation completely abolished cleavage by ADAM17 substantiated the former assumption and confirmed the findings obtained by MALDI-TOF. We concluded that ADAM17 (but not ADAM10) cleaves the stalk region of FcγRIIIA/CD16A and probably FcγRIIB/CD16B between Ala<sup>196</sup> and Val<sup>196</sup>. It is of note that the activation of mouse neutrophils, which resulted in substantial mouse l-selectin downregulation and indicated ADAM17 induction, did not lead to a significant downregulation of mouse CD16 or FcγRI (17). The fact that mouse CD16 and FcγRI are not regulated by ectodomain shedding may be related to the weak amino acid sequence similarity between the stalk region of human CD16 and the corresponding region of mouse CD16 or FcγRI (17).

We clearly detected ADAM17 within cells by Western blotting and also at the cell surface by FCM. It is of note that the membrane expression was relatively weak. Interestingly, ADAM17 was also present on the cell surface of both B cells and T cells, as previously reported (37, 38). In accordance with the membrane expression of ADAM17 on NK cells, the shedding of FcγRIIIA/CD16A was very rapidly induced by stimulation with NEM, which interferes with the bond formed between the cysteine proform and the catalytic zinc ion, thus bypassing the necessity to cleave the proform (39, 40). In addition, our results showed that the shedding of FcγRIIIA/CD16A was induced by stimulation with PMA, a known activator of ADAM17 (31, 41), whereas CaI [which activates
ADAM10 (42) was ineffective. Moreover, CD62L, a well-known specific substrate of ADAM17 (10, 11), was comodulated with FcyRIIIA/CD16A in a time- and concentration-dependent manner after NEM or PMA stimulation of NK cells. Finally, the shedding of both molecules was inhibited when PMA-stimulated NK cells were preincubated with similar concentrations of ADAM17 inhibitors such as TIMP-3 or the more selective pharmacological inhibitor TMI-2 (33). Our results agree with those recently reported by Romee et al. (18) showing that human NK cells express ADAM17 and that NK cell activation by PMA, cytokine exposure, cross linking of activating receptors, or exposure to K562 target cells resulted in decreased expression of FcyRIIIA/CD16A and CD62L, which was blocked by another selective ADAM17 inhibitor (BMS566394). Taken together, these results strongly support the conclusion that ADAM17 is at least partially involved in the shedding of NK cell FcyRIIIA/CD16A.

We and others have previously shown that NK cell FcyRIIIA is downmodulated after incubation with the anti-CD16 mAb 3G8 (3, 4) or the Fc portion of rituximab (4, 6). Such modulation may result from shedding and/or internalization. Several lines of evidence suggest that the former is the main mechanism when cells are stimulated by immobilized anti-CD16. First, immunochromatography and immunoblot analysis revealed little cell-associated CD16 after stimulation of NK cells by 3G8 cross linked with goat antimouse–coupled beads or by plate-bound 3G8 (3, 18). Second, incubation of NK cells in the presence of anti-CD16 mAb immobilized on microplates induced a simultaneous dramatic downmodulation of CD62L, a known substrate of ADAM17 (18) (Supplemental Fig. 2C, left panel). Third, FcyRIIIA downmodulation was strongly inhibited in the presence of 1,10-phenanthroline, an inhibitor of Zn2+-dependent metalloprotease (3), and in the presence of different ADAM17 inhibitors (18) (Supplemental Fig. 2A).

Finally, the fact that the frequency of NK cells expressing IFN-γ or TNF-α was greater in the presence of the ADAM17 inhibitor BMS566394 after cross linking with CD16 is consistent with the maintenance of CD16 on the cell surface and not with internalization (18). In contrast, when the NK cell responses following engagement of FcyRIIIA and other ARs were compared, we found that FcyRIIIA downmodulation induced by the former was substantially higher but not degranulation. It may therefore be assumed that internalization is involved in the FcyRIIIA/CD16A downmodulation induced by its engagement. Indeed, Cecchetti et al. (5) clearly demonstrated internalization of CD16 on NK cells using confocal microscopy and fluorescent soluble 3G8. CD16 is internalized through a very rapid process (10 min), and newly synthesized CD16 is rapidly re-expressed on the membrane (10 min). In our hands, FcyRIIIA downmodulation was not detectable before 1 h of stimulation by plate-bound anti-CD16 mAb or by other stimuli. However, when NK cells were incubated with Alexa 488–labeled 3G8 in the presence of an anti-Alexa 488 Ab, which quenches fluorescence on the cell surface (Supplemental Fig. 2B), we confirmed that internalization actually occurred when FcyRIIIA was engaged by soluble 3G8. However, the total fluorescence (membrane and intracellular) was decreased after incubation at 37°C for 3 h, showing that the Alexa 488–labeled 3G8 was partially degraded after internalization and/or that shedding was also effective in this condition (Supplemental Fig. 2B, right bottom panel). The fact that CD62L was strongly downregulated in this condition demonstrated that the latter was involved and that it was ADAM17 dependent. These results suggest that both ADAM17-dependent shedding and internalization may be involved in FcyRIIIA downmodulation when the latter is engaged by anti-CD16 mAb and that the relative contribution of each mechanism might depend on the conditions of the engagement (for instance, immobilized versus soluble mAb). Finally, activation of NK cells by rituximab-opsonized cells leads to downmodulation of CD16 (18, 43), which correlates with ICAM1 up-regulation (43). Romee et al. (18) have shown that when the human CD20-positive Burkitt’s lymphoma cell line Raji precoated with rituximab was incubated with purified NK cells in the presence of the ADAM17 inhibitor BMS566394, modulation of both CD16 and CD62L from NK cells was significantly attenuated, showing that ADAM17-dependent shedding of FcyRIIIA is at least partially involved when FcyRIIIA is engaged by rituximab-opsonized CD20 positive target cells. Finally, other mechanisms may also be involved when an NK cell interacts directly with a target cell opsonized by an Ab. For instance, it has been shown that both rituximab and CD20 are triglycotyzed by NK cells stimulated by rituximab-opsonized cells (44). It is likely that internalization of CD16 is required in this process. The evaluation of the relative involvement of these different mechanisms in the downmodulation observed after FcyRIIIA engagement requires further investigations.

Modulation of FcyRIIIA/CD16A has also been observed after incubation of NK cells with K562 cells (i.e., independently of FcyRIIIA/CD16A engagement) (7). We therefore simultaneously evaluated the degranulation and the shedding of FcyRIIIA/CD16A after coengagement of different NK cell ARs. Using 31 combinations of plate-bound mAbs targeting NKG2D, NKp30, NKp46, 2B4, and DNAM (34), we obtained a wide range of responses. We observed a correlation between IFN-γ production and shedding of FcyRIIIA/CD16A (Supplemental Fig. 1B), as previously reported (18). However, in our hands, the shedding of FcyRIIIA/CD16A was more highly correlated with degranulation according to the combination used. It has been suggested that the major effector function of CD56dimCD16+ NK cells is cytotoxicity, whereas CD56brightCD16dim/− NK cells act mainly by secretion of cytokines. However, we observed that IFN-γ-producing NK cells in response to plate-bound mAbs were CD56dim (Supplemental Fig. 1C). This is in agreement with several studies showing that NK cells producing IFN-γ upon stimulation by K562 (7), Ab-coated target (45), *Drosophila* cells expressing ligands for the NK cell ARs (46) and plate-bound mAbs against ARs (47) belong to the CD56dim subset. In contrast, NK cells that readily respond to monokines such as IL-12 or IL-15 belong to the CD56bright NK cell subset (45, 47). It appears therefore more appropriate to define CD56dim and CD56bright NK cells as target cell responsive and cytokine responsive, respectively (45). It is of note that all degranulating cells downmodulated their FcyRIIIA/CD16A, whereas the converse was not true. The shedding of FcyRIIIA/CD16A and CD62L induced by coengagement of NKG2D, NKp30, NKp46, and 2B4 was inhibited in the presence of TIMP-3 and TMI-2, showing that the ADAM17-dependent shedding of FcyRIIIA/CD16A was not restricted to stimulation conditions in which FcyRIIIA/CD16A was engaged, but extended to a wide range of FcyRIIIA/CD16A-independent stimuli. We found that the percentages of cells downmodulating CD16 were substantially higher than the percentages of degranulating cells. This may be explained by the heterogeneous response of NK cells to a given stimulus, as demonstrated by Fauriat et al. (46). Indeed, these authors showed that within stimulated NK cells, CD107a− cells were confined to a subset expressing MIP-1β, whereas the reverse was not true: the expression of MIP-1β was the sole functional response observed in a very large proportion of cells. In addition, some cells produced two or more responses in this stimulation condition, but CD107a expression did not necessarily correlate with the production of TNF-α or IFN-γ. It is thus likely that the NK cells downmodulating CD16 (CD16−) without degranulating (CD107−)
that we observed in our stimulation conditions had other functional responses such as chemokine or cytokine production. Another possible explanation for this finding was that ADAM17 expressed on an activated cell may shed the FcγRIIIA/CD16A on the cell surface of a neighboring cell (i.e., in trans). However, when CD16γ-transduced T cells and NK cells were mixed, the shedding of FcγRIIIA/CD16A was restricted to T cells after CD3 engagement and to NK cells after NKGD2, NKp30, NKp46, and 2B4 coengagement. The shedding of FcγRIIIA/CD16A was limited to appropriately stimulated cells and is therefore likely to occur mainly, if not exclusively, in cis.

We conclude that FcγRIIIA/CD16A shedding may be considered as a marker of FcγRIIIA-dependent activation of human CD56dim NK cells strongly correlated with degradation. On the basis of our results obtained with CD3-stimulated T cells, it is tempting to speculate that ADAM17-dependent shedding of membrane proteins is a general mechanism taking place during the process of lymphocyte activation. The identification of the cleavage site may help in the designing of inhibitors that could enhance FcγRIIIA-dependent functions of NK cells such as Ab-dependent cell cytotoxicity.

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References


