

A Potential Mechanism for ADC-Induced Neutropenia: Role of Neutrophils in Their Own Demise



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Abstract

Neutropenia is a common adverse event in cancer patients treated with antibody–drug conjugates (ADC) and we aimed to elucidate the potential mechanism of this toxicity. To investigate whether ADCs affect neutrophil production from bone marrow, an *in vitro* assay was developed in which hematopoietic stem cells (HSC) were differentiated to neutrophils. Several antibodies against targets absent in HSCs and neutrophils were conjugated to MMAE via a cleavable valine-citrulline linker (vcMMAE-ADC) or MMAF via a noncleavable maleimidocaproyl linker (mcMMAF-ADC), and their cytotoxicity was tested in the neutrophil differentiation assay. Results showed that HSCs had similar sensitivity to vcMMAE-ADCs and mcMMAF-ADCs; however, vcMMAE-ADCs were more cytotoxic to differentiating neutrophils than the same antibody conjugated to mcMMAF. This inhibitory effect was not mediated by internalization of ADC either by macropinocytosis or FcγRs. Our results suggested that

extracellular proteolysis of the cleavable valine-citrulline linker is responsible for the cytotoxicity to differentiating neutrophils. Mass spectrometry analyses indicated that free MMAE was released from vcMMAE-ADCs in the extracellular compartment when they were incubated with differentiating neutrophils or neutrophil conditioned medium, but not with HSC-conditioned medium. Using different protease inhibitors, our data suggested that serine, but not cysteine proteases, were responsible for the cleavage. *In vitro* experiments demonstrated that the purified serine protease, elastase, was capable of releasing free MMAE from a vcMMAE-ADC. Here we propose that ADCs containing protease cleavable linkers can contribute to neutropenia via extracellular cleavage mediated by serine proteases secreted by differentiating neutrophils in bone marrow. *Mol Cancer Ther*; 16(9); 1866–76. ©2017 AACR.

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Introduction

Antibody–drug conjugates (ADC) are targeting antibodies conjugated to a cytotoxic agent. Currently, two ADCs, brentuximab vedotin (ADCETRIS) and T-DM1 (KADCYLA), have been approved by the FDA, whereas about 50 ADCs are in clinical development (1, 2). ADCs are designed to selectively deliver highly cytotoxic payloads to tumor cells and to minimize systemic toxicity; however, several common, off-target toxicities have been observed, one of which is neutropenia. Patients treated with ADCs conjugated to MMAE via a protease cleavable valine-citrulline linker (3, 4), that is, vcMMAE-containing ADCs, are likely to experience neutropenia independent of the target to which the ADC is directed against (5). For example, neutropenia was one of most common events leading to dose delays in brentuximab vedotin phase II trials (6). ASG-5ME is an ADC comprised of

fully human IgG2 mAb targeting SLC44A4 conjugated to MMAE via valine-citrulline linker (7, 8). In the ASG-5ME phase I study for metastatic pancreatic and gastric cancers, dose-limiting neutropenia was observed in some patients (9). Neutropenia was also observed after treatment with ADCs containing other cleavable linkers (2, 5, 10). While it has been hypothesized that free, membrane permeable payload, such as MMAE, released from the ADC is responsible for causing neutropenia, the exact mechanism is yet poorly understood.

Neutropenia is uncommon in patients treated with ADCs conjugated to monomethyl auristatin F (MMAF) via a noncleavable maleimidocaproyl linker (4, 11, 12), that is, mcMMAF-containing ADCs (2, 5, 13–17). AGS-16C3F is an ADC comprised of a fully human IgG2 mAb targeting ENPP3 conjugated to MMAF via a maleimidocaproyl linker which is currently in phase II study in metastatic renal cell carcinoma (mRCC; ref. 18). In phase I studies of AGS-16C3F and several other mcMMAF-containing ADCs, for example, SGN-75, SGN-CD19A, ABT-414, and PF-06263507, some of the common toxicities were ocular toxicity and thrombocytopenia; however, very few cases of neutropenia were observed (13–17).

Neutrophils, the most abundant type of white blood cells in humans, differentiate from hematopoietic stem cells in bone marrow (19, 20). They are an essential part of the innate immune system and use different strategies such as phagocytosis or secretion of proteases to kill invading pathogens. Upon activation,

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granules within neutrophils can undergo exocytosis and release of their contents. Azurophilic granules have high content of a family of structure-related serine proteases, neutrophil elastase, proteinase 3 and cathepsin G (21).

Mature neutrophils are short-lived and are produced at approximately 10^{11} cells per day (19, 20, 22, 23). It takes 12 days for a neutrophil to be produced and go through different stages to become mature and another 4–6 days before it is released into circulation (20, 23). The different maturation stages of neutrophils can be followed by Giemsa/Wright staining (24). Damage to bone marrow, for example, by chemotherapeutic agents, would reduce neutrophil cell counts causing neutropenia resulting in an increase in the vulnerability to infections. In this report, human hematopoietic stem cells (HSC) isolated from bone marrow were differentiated to neutrophils *in vitro*, and were used to investigate their sensitivity towards different ADCs. Our results suggest that vcMMAE-containing ADCs contribute to neutropenia via non-specific extracellular cleavage by proteases secreted by differentiating neutrophils in bone marrow. ADCs do not affect survival of mature neutrophils, and internalization by macropinocytosis or FcγR does not play a major role. Results presented here demonstrate that neutrophils can release MMAE from vcMMAE-ADC that can contribute to toxicities such as neutropenia.

Materials and Methods

Antibodies and reagents

Mouse anti-human CD66b (catalog no. 12-0666-42), anti-CD15 (catalog no. 11-0159-42), and anti-CD32 (catalog no. 11-0329-41) antibodies were from eBioscience. Halt protease inhibitor cocktail was from Thermo Fisher Scientific (catalog no. 87786). AEBSF (catalog no. A8456), aprotinin (catalog no. A1153), and E-64 (catalog no. E3132) were purchased from Sigma. Anti-MMAE/F antibody (SG15-22) was provided by Seattle Genetics, Inc. All ADCs used technology licensed from Seattle Genetics, Inc. to conjugate to MMAE/F (Supplementary Table S1) with drug-antibody ratio (DAR) of 3.5-4.7, and some were tested and reported elsewhere (7, 16, 18). Control antibodies (IgG1 and IgG2) were produced from Chinese hamster ovary (CHO) cells directed against chicken egg white lysozyme (HEWL) protein which is known not being expressed in mammalian cells and tissues. The antibodies were generated by immunizing Balb/c mice with HEWL protein. The resulting lysozyme-binding mouse hybridoma mAb variable heavy and light chain sequences were cloned into plasmids with human IgG Fc and Kappa light chain sequences to produce control IgG1 or IgG2.

Cell cultures and differentiation of human neutrophils

Human hematopoietic stem cells (CD133⁺, catalog no. ABM026F) isolated from bone marrow and human mature neutrophils (catalog no. PB016) were purchased from ALL-CELLS. HSC growth media (StemSpan SFEM II, catalog no. 09600), supplements for expansion (CC100, catalog no. 02690) were from StemCell Technologies. Interleukin 3 (IL3), stem cell factors (SCF), Flt3 ligands, granulocyte-macrophage colony-stimulating factors (GM-CSF) and granulocyte colony-stimulating factors (G-CSF) were from R&D Systems. HSCs were first expanded in StemSpan SFEM II supplemented with CC100 for three days. Stem cell markers (CD133, CD34) and granulocyte marker (CD66b) were measured by FACS prior to differentiation. The method to differentiate HSCs to neutro-

phils was described previously (25). Briefly, there were three steps for neutrophil differentiation. First, expanded HSCs were incubated for 4 days in StemSpan SFEM II medium with 50 ng/mL SCF, 100 ng/mL Flt-3L, 5 ng/mL IL3, 5 ng/mL GM-CSF, and 5 ng/mL G-CSF. Cells were then incubated for 4 days in StemSpan SFEM II medium supplemented with 5 ng/mL IL3 and 30 ng/mL G-CSF. At day 8, cells were incubated in StemSpan SFEM II supplemented with 30 ng/mL G-CSF till day 14. Granulocyte markers CD66b was measured by flow cytometry analysis after 3-day expansion, at day 8 and 14. For ADC treatment, cell growth medium was changed at day 8 for last step differentiation, and ADCs were added on same day for 6 days. Effect of ADCs on neutrophil differentiation is represented by percentage of CD66b⁺ within the viable cell population. PC3 cell line was purchased from ATCC in 2012, and was banked upon receipt and passaged for fewer than 6 months before use. It was authenticated by short tandem repeat (STR) DNA profiling and tested for mycoplasma at Agensys Inc. in 2012. PC3-SLC44A4 cells were described in previous study (7), and were maintained in RPMI1640 supplemented with 10% FBS. STR DNA profiling and mycoplasma testing was done in 2013 and 2014, respectively.

Flow cytometry analysis

Cells were washed and incubated with mouse anti-human antibodies for 30 minutes in the dark in FACS stain buffer (FBS, BD Pharmingen; catalog no. 554656). Cells were then washed three times with stain buffer, and analyzed by Attune Acoustic Focusing Cytometer (Life Technologies) gated for live cells with appropriate isotype-matched controls and unstained cells as negative controls. For the macropinocytosis assay, HSCs or differentiating neutrophils were incubated with 1 mg/mL dextran-FITC (10,000 MW, Life Technologies) for 3 hours at 37°C. Flow cytometry was used to detect internalization of Dextran-FITC conjugates. Median fluorescence intensity (MFI) values from samples at 37°C were normalized against values from 4°C to determine MFIR values for macropinocytosis.

Proliferation assays

Cells (3,000 cells/well in 100 μL) were grown in 96-well plates (Corning Assay Plate, catalog no. 3903) for 6 days, CellTiter-Glo (CTG) luminescence assay kit (Promega, catalog no. G7572) was used to measure the number of viable cells. CTG values were normalized against mock-treated cells at day 6 (% max proliferation), and GraphPad Prism 6 was used to generate IC₅₀ values. Each experiment was done in triplicates and each result is from at least three experiments.

Wright Giemsa staining of differentiating neutrophils

Differentiated neutrophils (0.25×10^6 cells per well) from day 12 were plated on a Poly-D-Lysine coated 8-well culture slide (Corning, catalog no. 354632) and allowed to adhere overnight. After removal of media, slides were stained in Wright Giemsa Stain solution (Sigma, catalog no. WS16, 500 mL) for approximately 15 seconds, followed by deionized (DI) water for approximately 30 seconds, rinsing briefly to remove all traces of stain. Stained slides were then imaged using a Nikon Eclipse E400 Upright Light Microscope.

Microarray

Total RNA was extracted using TRIzol and cleaned up using RNeasy Mini kit (Qiagen). RNA quality was analyzed on

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Bioanalyzer RNA-nano chips (Agilent Technologies). Samples with RIN <7.0 were not used for expression profiling. Cyanine 3-CTP-labeled cRNA target was prepared using One-Color Low Input Quick Amp kit and One Color Spike-In kit (Agilent Technologies) using 100 ng input RNA. Labeled cRNA was purified using RNeasy Mini kit and was quantified on a NanoDrop UV Spectrophotometer. Specific activity of Cyanine 3 was calculated in pmol/ μ g with a minimum threshold of 15 pmol/ μ g. Hybridization of labeled cRNA onto Agilent Human GE 8 \times 60K v2 microarrays and subsequent washing, and processing of arrays was performed according to the manufacturer's protocols. Microarray slides were scanned in an Agilent Scanner and images extracted to data using Agilent Feature Extraction Software version 10.7 and third quantile normalization was performed on median signal values.

Elastase activity assay

To measure elastase activity, conditioned medium from neutrophil differentiation at day 14 (NCM) or hematopoietic stem cells after 3-day expansion (HSC CM) were collected and cells were removed by centrifugation. Five microliters (5 μ L) of conditioned medium was collected to measure elastase activity according to the vendor's protocol (Abcam, catalog no. ab204730). Regular growth medium or conditioned medium was incubated with reaction buffer and substrate, and fluorescence was measured in a kinetic mode, every 2–3 minutes, for 20 minutes at 37°C protected from light as suggested by vendor. Protease inhibitor cocktail (Halt, Thermo Fisher Scientific, catalog no. 87786) was used. Relative fluorescence units (RFU) from regular growth medium background were subtracted from RFU readings from conditioned medium to represent elastase activity. Elastase concentration was estimated following manufacturer's protocol. To measure cleavage of valine citrulline linker by elastase, indicated amount of human neutrophil elastase (Abcam, catalog no. 91099) was incubated with 100 nmol/L AGS5-vcE for 2 or 20 hours at 37°C prior to MMAE measurement by mass spectrometry. The buffers used for this assay were from activity kits for elastase (Abcam, catalog no. ab204730). AEBSF (1 mmol/L) and aprotinin (0.8 μ mol/L) were used for inhibition.

MMAF and MMAE quantitation in neutrophil-conditioned media by mass spectrometry

Protein precipitation was performed on neutrophil-conditioned media harvested at various timepoints prior to quantitation of MMAF (1) and MMAE (ref. 2; Supplementary Fig. S1.) by LC/MS. Samples were filtered with a 0.22- μ m filter (Millex Low Protein Binding Durapore, 13 mm), centrifuged for 5 minutes at 10,621 \times g and dried under reduced pressure (18 hours, miVac DUO). Samples were resuspended in 100 μ L of HPLC water, precipitated by 1,000 μ L of cold isopropanol (Sigma Aldrich), vortexed, and centrifuged at room temperature for 15 minutes at 20,817 \times g. Supernatants were decanted and centrifuged for 3 minutes at 10,621 \times g. Samples were dried under reduced pressure (18 hours, miVac DUO), resuspended in 10 μ L of injection buffer [10 mmol/L ammonium formate/10% methanol/2.04 nmol/L ¹³C₅-MMAE (3), Supplementary Fig. S1], vortexed, and centrifuged at 10,621 \times g for 5 minutes. Samples were transferred to HPLC vials for LC/MS analysis. Quantitation of free drugs and internal standard by LC/MS was performed on an LC/MS-8050 Shimadzu Triple Quadrupole Mass Spectrometer with sample introduction via Nexera X2 LC-30AD Shimadzu and autosampler

(Nexera X2 sil-30AC). Chromatography was performed on an Acquity UPLC BEH C18 column (1.7 μ m, 1.0 mm \times 50 mm) with an Acquity UPLC BEH C18 Vanguard Pre-column (1.7 μ m, 2.1 mm \times 5 mm) at 40°C. Chromatographic separation was achieved using a binary gradient (Supplementary Table S2). Solvent flow was 0.5 mL/minute (Solvent A: 0.1% formic acid in water and Solvent B: 0.1% formic acid in acetonitrile). Injection volume was 5 μ L (two injections per time point). LC/MS-MS data was acquired and processed using Shimadzu LabSolutions. Detection was performed in ESI positive ion mode using multiple reactions monitoring of specific transitions (Supplementary Table S3).

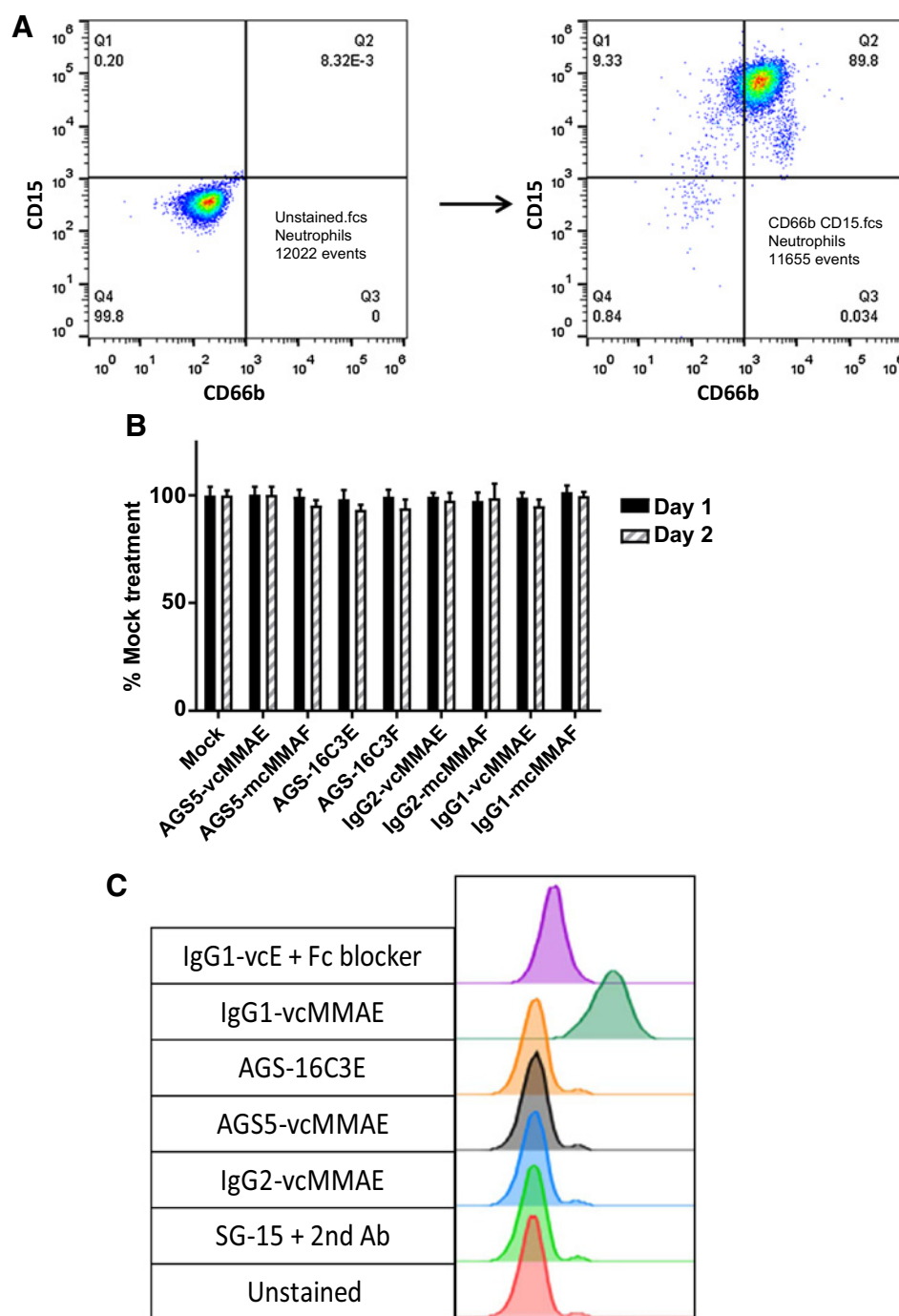
Results

ADCs did not affect viability of human mature neutrophils

Commercial mature human neutrophils were purified from blood and stained positive for CD16 (AllCells). Two other markers were used to confirm the purity of the mature neutrophils: CD66b, which is a marker specific for granulocytes and CD15, which is expressed on several cell types including neutrophils (Fig. 1A). FACS analysis showed that 90% of the mature neutrophils stained positive for CD66b and CD15 (Fig. 1A). FACS analysis with anti-CD32 antibody also showed that over 90% neutrophils expressed Fc γ RIIA (Supplementary Fig. S2). Consistent with previous publications that mature neutrophils have a short half-life (19, 23), their viability decreased by more than 90% after 1 day and by 99% after 2 days. To determine whether ADCs could have a direct effect on neutrophils, the viability of mature neutrophils treated with ADCs was investigated. The following antibodies were conjugated to either vcMMAE or mcMMAF and their effects on the viability of mature neutrophils were assessed (Fig. 1B): AGS5 (IgG2, anti-SLC44A4), AGS-16C3 (IgG2, anti-ENPP3; refs. 7, 18), and antibodies (IgG1 and IgG2) directed against hen egg white lysozyme (HEWL), a nonhuman protein (Supplementary Table S1). Compared with mock treatment, none of the ADCs tested had an obvious effect on neutrophil viability (Fig. 1B). Some ADCs were also tested for their ability to bind to neutrophils. AGS5-vcMMAE, AGS-16C3E, and IgG2-vcMMAE did not bind to neutrophils (Fig. 1C). This is consistent with microarray data showing that mature neutrophils did not express SLC44A4, ENPP3, or HEWL (Supplementary Fig. S3). IgG1-vcMMAE bound to neutrophils, which was inhibited by the presence of an Fc blocker reagent, suggesting this binding is mediated by Fc receptors (Fig. 1C). The properties of this Fc blocker reagent have been characterized in detail previously (26). Taken together, these results suggest that ADC-induced neutropenia is not caused by their effect on the viability of neutrophils in circulation.

Effect of ADCs on differentiating neutrophils

To investigate whether ADCs affect neutrophil production in the bone marrow, human hematopoietic stem cells (HSC) *in vitro* were differentiated to neutrophils. HSCs can be differentiated *in vitro* to different cell types such as megakaryocytes (27) and granulocytes (25). Following the protocol for granulocytes, HSCs were differentiated to granulocytes/neutrophils over a period of 2 weeks (Fig. 2A). Progression towards neutrophils was monitored using the neutrophil marker CD66b. Initially, most cells expressed stem cell markers, CD133 and CD34 (26), with little CD66b expression (Fig. 2A; Supplementary Table S4). On average, more than 30% cells expressed CD66b after 8-day

**Figure 1.**

ADCs did not affect viability of mature neutrophils from circulation.

A, Characterization of mature neutrophils from circulation. Human mature neutrophils isolated from circulation (AllCells) were incubated with anti-CD66b and anti-CD15 antibodies, and their binding was measured by FACS (right) compared with unstained neutrophils (left).

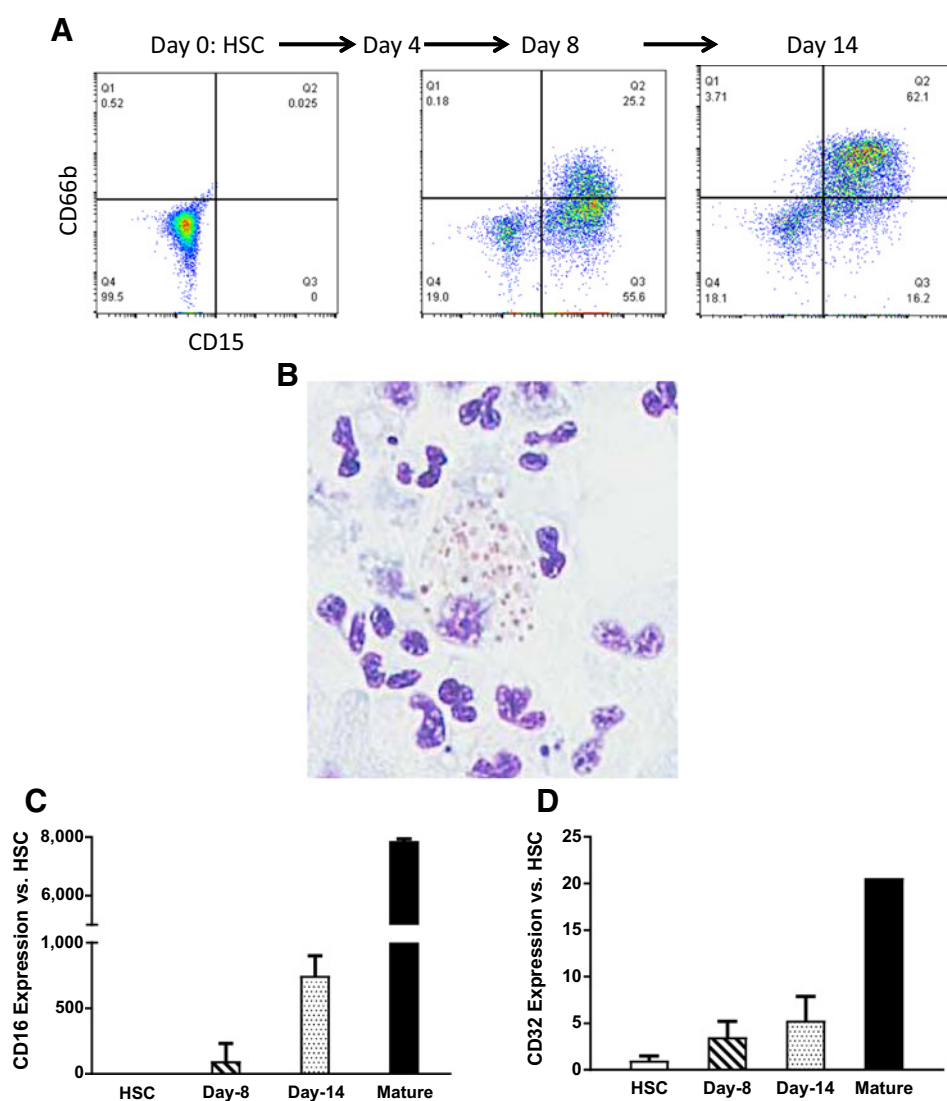
B, Cytotoxicity of ADCs to mature neutrophils. Mature neutrophils (1.5×10^5 cells) from circulation were treated with indicated ADCs (100 nmol/L) for 1 or 2 days. CellTiter Glo (CTG) values were measured and normalized to CTG values of mock treatment. **C**, Binding of ADCs to mature neutrophils. Mature neutrophils isolated from circulation were incubated with 100 nmol/L IgG2-vcMMAE, AGS5-vcMMAE, AGS-16C3E, or IgG1-vcMMAE in the absence or presence of 1 μ mol/L Fc blocker, and their binding was detected by biotinylated anti-MMAE antibody (SG15.22) and analyzed by FACS.

differentiation which increased to 60%–76% of cells at the end of 2-week differentiation (Fig. 2A; Supplementary Table S4). Several studies were performed to assess the differentiation status of these neutrophils at day 14. Wright Giemsa stain is commonly used in histology to differentiate blood cell types. First, after 14-day differentiation, cells were stained with Wright Giemsa which showed some mature but mostly immature neutrophils at different stages of differentiation (Fig. 2B). Second, microarray data showed low levels of CD16 and CD32 mRNA in HSCs which gradually increased during neutrophil differentiation (Fig. 2C and

D). Fully mature neutrophils from circulation expressed even higher level of CD16 and CD32 than neutrophils at day 14 of differentiation, suggesting that day 14 neutrophils were not yet fully mature (Fig. 2C and D). As neutrophils are the most abundant type of granulocytes, CD66b positivity was then used as a surrogate for neutrophils for the following experiments.

Next, the effect of MMAE on differentiating neutrophils was tested. Different concentrations of MMAE were added at day 8 of differentiation and flow cytometry was used to analyze CD66b positivity after a 6-day treatment. The ratio of viable cells positive

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**Figure 2.**

Characterization of immature neutrophils differentiated from HSCs *in vitro*. **A**, The scheme of differentiation of HSCs to neutrophils. After 3-day expansion (day 0), HSCs were treated with different growth factors at day 0, 4 and 8 to be differentiated into CD66b⁺ granulocytes. Differentiating neutrophils were identified by FACS with anti-CD66b antibody, these cells were also positive for CD15. **B**, Wright staining of differentiating neutrophils. Differentiating neutrophils after 14-day differentiation from HSCs were incubated with Wright stain solution, and their differentiation status was visualized by microscope. **C** and **D**, Increase of CD16 (**C**) or CD32 (**D**) expression during neutrophil differentiation. Microarray data were generated from HSCs (Open bar) or differentiating neutrophils at day 8 (slashed bar) or 14 (dotted bar), or mature neutrophils from circulation (black bar). CD16 or CD32 expression level was normalized against that of HSCs.

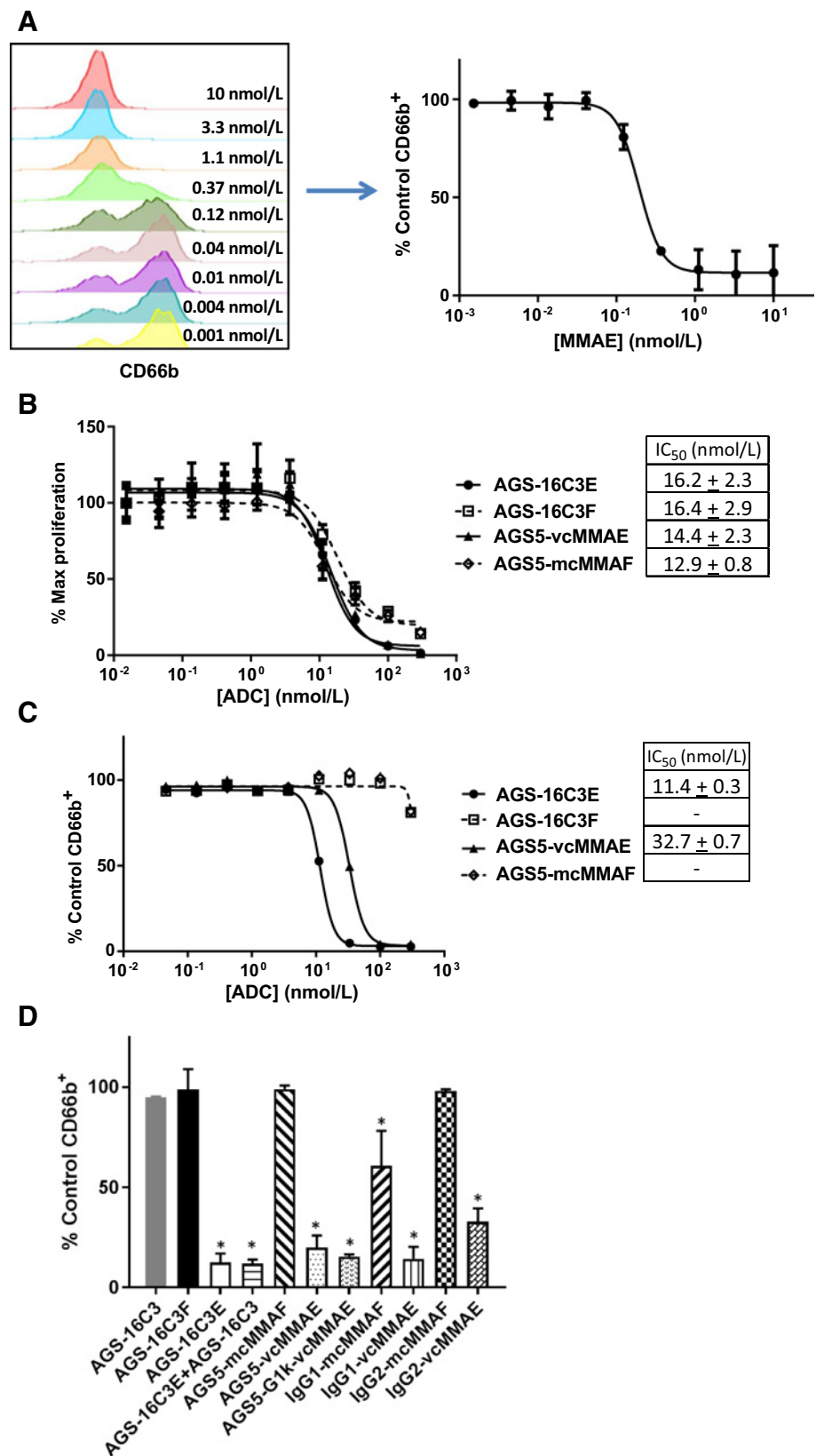
for CD66b staining (% CD66b⁺) to mock-treated samples (represented by % control CD66b⁺) was used to measure cytotoxicity (Fig. 3A). The data showed that differentiating neutrophils were very sensitive to MMAE treatment with an apparent IC₅₀ of 0.19 ± 0.05 nmol/L. Several antibodies conjugated to vcMMAE or mcMMAF were tested to compare the effect of different linkers and payloads on ADC cytotoxicity to HSCs and differentiating neutrophils. AGS5-vcMMAE, AGS5-mcMMAF, AGS-16C3F, and AGS-16C3E showed similar potency inhibiting HSC proliferation (IC₅₀ = 13–16 nmol/L; Fig. 3B). In contrast, differentiating neutrophils were resistant to both AGS5-mcMMAF and AGS-16C3F, while AGS-16C3E and AGS5-vcMMAE inhibited neutrophil differentiation with an apparent IC₅₀ of 11 nmol/L and 33 nmol/L, respectively (Fig. 3C). These data suggested that cellular changes during the differentiation from HSC to neutrophil provoked differential sensitivity to vcMMAE or mcMMAF-containing ADCs. These data are consistent with clinical observations that AGS5-vcMMAE induced neutropenia and that no significant neutropenia was reported in AGS-16C3F clinical trials (9, 16). To further investigate the effect of linker/payload, we tested four

pairs of ADCs that were conjugated to both vcMMAE and mcMMAF. At 100 nmol/L, AGS-16C3E, AGS5-vcMMAE, nonhuman targeting IgG1-vcMMAE and IgG2-vcMMAE showed much higher cytotoxicity to differentiating neutrophils than AGS-16C3F, AGS5-mcMMAF, nonhuman targeting IgG1-mcMMAF and IgG2-mcMMAF, respectively (Fig. 3D). These data suggest that antibodies conjugated to MMAE via a valine-citrulline linker were significantly more cytotoxic to differentiating neutrophils than the same antibodies conjugated to MMAF via a noncleavable maleimidocaproyl linker. To better understand the role of IgG subclass in this process, AGS5-G1k-vcMMAE, an IgG1 version of AGS5-vcMMAE (IgG2) was produced and tested. Both IgG1 and IgG2 versions of these anti-SLC44A4 ADCs were equally cytotoxic to differentiating neutrophil (Fig. 3D) suggesting that IgG isotype was not critical for the cytotoxic activity.

Inhibition of neutrophil differentiation induced by vcMMAE-containing ADCs was not due to target expression. In addition to the fact that IgG1-vcMMAE and IgG2-vcMMAE are ADCs targeting a nonhuman protein, the corresponding AGS16C3E naked antibody, AGS-16C3, could not block AGS-16C3E cytotoxic effect on

Figure 3.

Sensitivity of differentiating neutrophils and HSCs to ADCs. **A**, Differentiating neutrophils were sensitive to MMAE treatment. At day 8 of differentiation, cells were treated with indicated concentrations of MMAE for 6 days. CD66b⁺ cells were then identified by FACS (left), percentage of CD66b⁺ was normalized against mock-treated samples (% control CD66b⁺) which was used to generate IC₅₀ values. IC₅₀ values were derived from 3 different donors and the result from one donor is shown here. **B**, HSCs were equally sensitive to vcMMAE and mcMMAF-containing ADCs. After 3-day expansion, HSCs were treated with indicated ADCs at different concentrations, and CellTiter Glo (CTG) values were then measured after 6 days and normalized against mock-treated samples to generate IC₅₀ values. **C**, Dose response of differentiating neutrophils to ADCs. Differentiating neutrophils at day 8 were treated with ADCs at indicated concentration for 6 days. Percentage of CD66b⁺ generated from FACS was normalized against mock-treated samples (% control CD66b⁺) which was used to generate IC₅₀ values. IC₅₀ values were derived from three different donors. **D**, Effect of vcMMAE and mcMMAF-containing ADCs on differentiating neutrophils. Day 8 differentiating neutrophils were treated with indicated ADCs or the naked Ab, AGS-16C3 at 100 nmol/L for 6 days, and % control CD66b⁺ was generated to compare their effect. Ten micromolar (10 μmol/L) AGS-16C3E was used to compete with AGS-16C3F in the same assay. The data were generated from a minimum of two experiments. Statistical analyses were performed with *t* tests using a pooled SD, and significant difference (*P* ≤ 0.05) between treatment and mock was indicated by asterisks.



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differentiating neutrophils (Fig. 3D). Furthermore, microarray data showed that HSCs, differentiating neutrophils at day 8 and day 14 or mature neutrophils from circulation had negligible SLC44A4 or ENPP3 gene expression compared with tumor cells (Supplementary Fig. S3).

Roles of macropinocytosis and FcγRs

We set to elucidate the mechanism of ADC inhibition of differentiating neutrophils. Internalization mediated by macropinocytosis or FcγRs have been proposed to mediate nonspecific ADC internalization and cytotoxicity to megakaryocytes (26, 27). However, the drastic difference between sensitivity to vcMMAE- and mcMMAF-containing ADCs with the same antibody suggested that internalization by macropinocytosis or FcγRs was not a major determinant of cytotoxicity for differentiating neutrophils. Macropinocytosis is commonly measured by determining internalization of a dextran-fluorophore by flow cytometry (26, 28). Hematopoietic stem cells, which had shown similar sensitivity to antibodies conjugated with vcMMAE or mcMMAF (Fig. 3B), had appreciable macropinocytosis activity with MFIR of 16 ± 4 (Fig. 4A; Supplementary Table S5). These suggest that ADC internalization by macropinocytosis could explain the HSC sensitivity to ADCs. After a 14-day differentiation, the macropinocytosis activity of neutrophils decreased by 75% to MFIR of 4.1 ± 0.1 , while the sensitivity of differentiating HSCs and differentiating neutrophils to AGS-16C3E was similar. This suggests that this pathway does not mediate the sensitivity of differentiating neutrophils to vcMMAE ADCs, different from the effect on megakaryocytes (26). However, this decrease in macropinocytosis may explain the significant reduction in sensitivity of differentiating neutrophils to mcMMAF-containing ADCs.

Neutrophils express high levels of FcγRIIA and FcγRIIIA (29, 30) that could mediate binding and internalization of ADCs. Gene expression analysis showed increasing levels of FcγRIIIA mRNA (Fig. 2C) and to a lesser extent of FcγRIIA mRNA (Fig. 2D) during differentiation from HSCs to neutrophils. Similar to previous results with mature neutrophils (Fig. 1C), flow cytometry data indicated that IgG2 ADCs did not bind, whereas IgG1-vcMMAE and IgG1-mcMMAF showed significant binding to differentiating neutrophils (Fig. 4B). Binding of ADCs of IgG1 isotype to neutrophils was completely inhibited by the presence of an Fc blocker reagent confirming that the binding was mediated via FcγRs. Next, we tested whether binding to FcγRs was required for cytotoxicity to differentiating neutrophils. Differentiating neutrophils were treated with IgG1-vcMMAE, IgG1-mcMMAF or AGS-16C3E (IgG2) in the presence of 100-fold excess of this Fc blocker reagent and the cytotoxicity was assessed by FACS (Fig. 4C). As expected for being an IgG2 isotype, the activity of AGS-16C3E was not affected by the presence of the Fc blocker. IgG1-mcMMAF had modest cytotoxicity activity against neutrophils (40% inhibition) which was completely inhibited by the Fc blocker reagent indicating that it was due to binding and possible internalization via FcγRs. This result and previous data (26) suggested that the Fc blocker reagent was still functional after 6-day incubation. However, the presence of the Fc blocker reagent had little effect on the cytotoxicity induced by IgG1-vcMMAE suggesting that binding and possible internalization by FcγRs did not play a major role in the cytotoxicity of vcMMAE-containing ADCs to differentiating neutrophils.

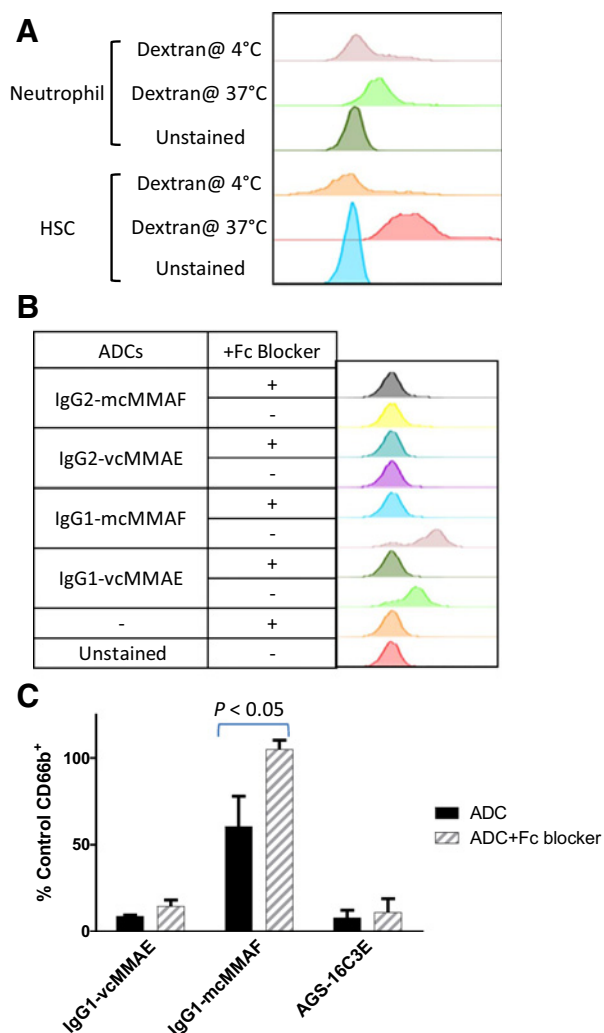
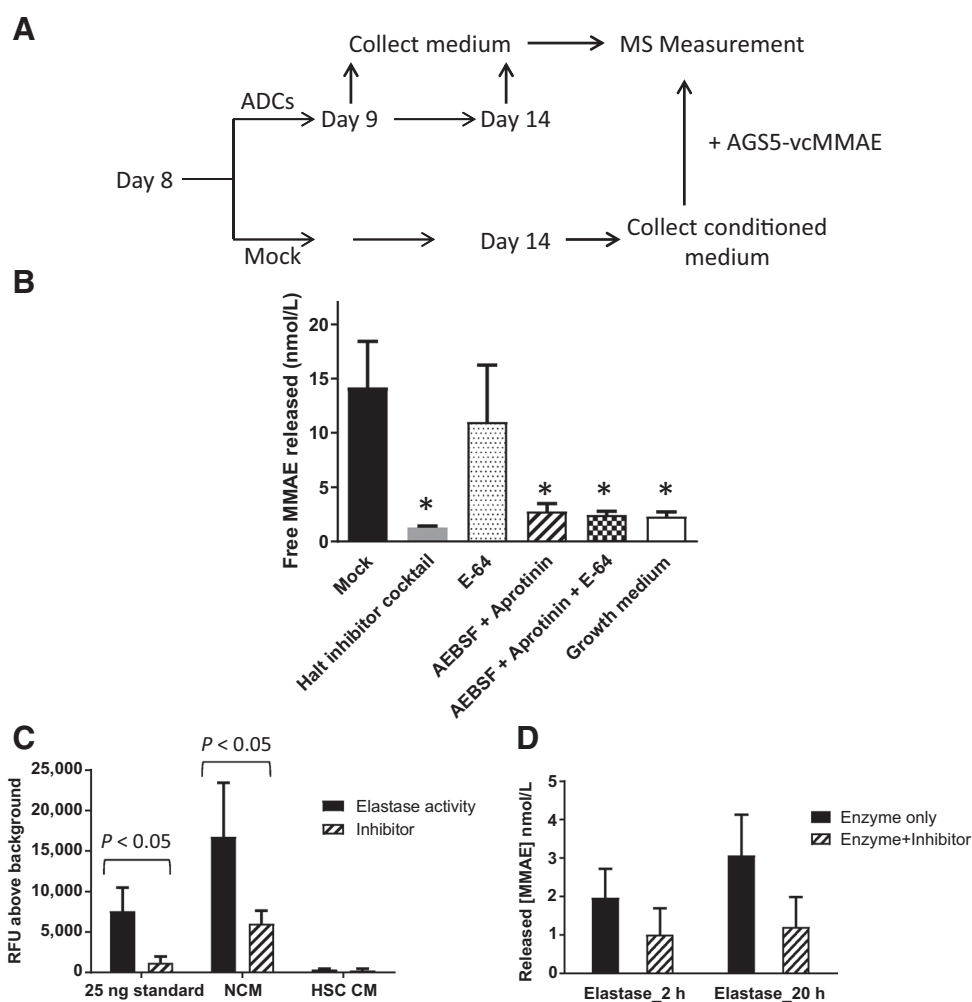


Figure 4.

FcγRs and macropinocytosis did not play an important role in sensitivity of differentiating neutrophils to ADCs. **A**, Macropinocytosis of HSCs and differentiating neutrophils. HSCs after 3-day expansion or differentiating neutrophils at day 12 were incubated with Dextran-FITC for 3 hours at 37°C or 4°C (control) followed by FACS analysis. **B**, Binding of ADCs to differentiating neutrophils. Differentiating neutrophils at Day 14 were incubated with 100 nmol/L indicated ADCs in the absence or presence of 1 μmol/L Fc blocker (10 ×) for 1 hour, and binding of ADCs to neutrophils were analyzed by FACS using anti-MMAE/F antibody (SG15.22). **C**, FcγRs were not critical for cytotoxicity of vcMMAE-containing ADCs to differentiating neutrophils. Differentiating neutrophils at day 8 were incubated with 100 nmol/L of indicated ADCs in the absence or presence of 10 μmol/L Fc blocker (100 ×) for 6 days, and % control CD66b⁺ was generated from FACS analysis of at least two donors. Statistical significance of differences between ADC treatment with and without Fc blockers was analyzed by *t* tests.

Extracellular cleavage by serine proteases mediates cytotoxicity to differentiating neutrophils

The data presented here showed that antibodies conjugated to MMAE via a cleavable linker caused higher cytotoxicity than those with a noncleavable linker. Neutrophils express high levels of proteases which can be secreted during degranulation as part of cellular immune or inflammatory response (21) and could

**Figure 5.**

Extracellular proteases mediated nonspecific cleavage of vcMMAE-containing ADCs. **A**, Experimental design to investigate extracellular cleavage of AGS5-vcMMAF. To measure cleavage of AGS5-vcMMAF by differentiating neutrophils (top half), neutrophils at day 8 were incubated with 100 nmol/L AGS5-vcMMAF or AGS5-mcMMAF, and medium was collected after 1 day or 6 days, and free MMAE was measured by mass spectrometry (MS). To measure ADC cleavage by neutrophil conditioned medium (bottom half), neutrophils at day 8 continued to differentiate without ADC, neutrophil conditioned medium (NCM) was collected at day 14. After any residual cells were removed by centrifugation, NCM was incubated with 100 nmol/L AGS5-vcMMAE or IgG1-vcMMAE for 1 day, and free MMAE was measured by MS. The values of free MMAE or MMAE from MS measurement were reported in Table 1. **B**, Serine proteases were important for extracellular cleavage of vcMMAE-containing ADCs. Neutrophil conditioned medium collected at Day 14 was incubated with 100 nmol/L AGS5-vcMMAE in the presence of indicated protease inhibitors or cocktails for 1 day, and free MMAE was measured by mass spectrometry. Individual protease inhibitors were used at the same concentration as those in Halt inhibitor cocktail. The average results from two donors were presented here. Statistical analyses were performed with *t* tests using a pooled SD, and significant difference ($P \leq 0.05$) between treatment and mock is indicated by asterisks. **C**, Elastase activities in neutrophil conditioned medium. Neutrophil conditioned medium (5 μ L) collected at day 14 (NCM) or HSC conditioned medium (HSC CM) was incubated with elastase substrate provided in the kit for 20 minutes in the absence or presence of serine protease inhibitors, and fluorescence was read. NCM data were from 6 different donors, and HSC CM data were from 4 donors. Statistical significance of differences between elastase activities with and without inhibitors was analyzed by *t* tests. **D**, Elastase can cleave valine-citrulline linker. Purified elastase (10 μ g/mL) was incubated with 100 nmol/L AGS5-vcMMAE for 2 or 20 hours at 37°C, and free MMAE from cleavage was measured by mass spectrometry. AEBSF (1 mmol/L) and aprotinin (0.8 μ mol/L) were used as inhibitors. The background free MMAE present in the buffer was subtracted from the concentrations of released MMAE after enzyme digestion. Data from two experiments each with duplicate samples was presented.

potentially cleave the valine-citrulline linker liberating MMAE. Thus, we next investigated whether vcMMAE-containing ADCs were cleaved by proteases in the extracellular compartment of neutrophil cultures. Free MMAE or MMAF from this cleavage can be detected by mass spectrometry (11). As MMAE is membrane permeable, free MMAE from intracellular lysosomal protease cleavage could potentially leak out to the extracellular compart-

ment. Thus, it is technically difficult to determine the levels of MMAE produced extracellularly as it may be contaminated by MMAE that leaks from inside the cell. In contrast, MMAF has poor membrane permeability (11). For this reason, AGS5, an anti-SLC44A4 antibody (7), was conjugated to MMAF via a valine-citrulline cleavable linker to minimize potential contamination from intracellular cleavage. We first tested if vcMMAF conjugation

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to AGS5 was cytotoxic to PC3 tumor cells overexpressing SLC44A4 (7). While AGS5-vcMMAF was not cytotoxic to differentiating neutrophils (5% inhibition at 100 nmol/L), AGS5-vcMMAF, AGS5-vcMMAE, and AGS5-mcMMAF showed similar activity inhibiting PC3-SLC44A4 cell growth, suggesting that vcMMAF and vcMMAE are processed similarly in the lysosomal compartment (Supplementary Fig. S4). Next, differentiating neutrophils from two different donors at day 8 were incubated with 100 nmol/L AGS5-vcMMAF or AGS5-mcMMAF, growth medium was collected after 1-day or 6-day incubation and free MMAF was measured by mass spectrometry (Fig. 5A; Table 1). Free MMAF in samples treated with AGS5-mcMMAF was below detection limit. In contrast, for differentiating neutrophils treated with AGS5-vcMMAF, an average ($n = 2$) of 1.8 nmol/L free MMAF was detected after 1-day incubation increasing to 9.3 nmol/L after 6-days (Table 1). These suggest that differentiating neutrophils secrete proteases that can cleave the valine-citrulline linker. To test whether proteases are secreted into the extracellular compartment and/or were bound to neutrophils' plasma membrane, neutrophil conditioned medium at day 14 was collected, and protease activity measured against AGS5-vcMMAE (Fig. 5A; Table 1). Incubation of day-14 neutrophil conditioned medium with 100 nmol/L AGS5-vcMMAE for 1 day produced more than 10 nmol/L free MMAE (Table 1) while no cleavage of AGS5-mcMMAF was observed. IgG1-vcMMAE was also tested using the same experimental approach resulting in the detection of 7 nmol/L free MMAE. We next investigated whether HSCs can secrete proteases than can cleave ADCs. When AGS5-vcMMAE was incubated with HSC conditioned medium (HSC CM), no free MMAE was detected as compared with growth medium incubation (Table 1). These indicate that differentiating neutrophils, but not HSCs, secrete proteases to the extracellular compartment which can cleave the valine-citrulline linker to release the active payload.

We next aimed to identify the class of proteases that were active in the neutrophil-mediated extracellular cleavage. Neutrophil-conditioned medium collected at day 14 was incubated with 100 nmol/L AGS5-vcMMAE for 1 day in the presence of different protease inhibitors and free MMAE was measured by mass spectrometry (Fig. 5B). As shown previously, neutrophil conditioned medium (day-14) cleaved AGS5-vcMMAE *in vitro* (Mock), while addition of a protease inhibitor cocktail (Halt) completely inhibited cleavage of AGS5-vcMMAE. To narrow down the type of proteases that mediate cleavage of AGS5-vcMMAE, the individual components of the cocktail were tested. Combination of two serine protease inhibitors, AEBSF and Aprotinin present in the Halt cocktail showed potent activity inhibiting the cleavage, whereas the cysteine protease inhibitor E-64 did not have a significant effect on the MMAE released. Interestingly, this is different from what has been shown for the cleavage of valine-

citrulline linkers in lysosomes, which is mediated by cysteine proteases (31). All together, these data suggest that secreted serine proteases, but not cysteine proteases, mediated the extracellular cleavage of the valine-citrulline linker by differentiating neutrophils.

Under certain conditions, neutrophils release the contents from azurophilic granules which contain serine proteases (3). Among those, elastase is one of the most abundant serine proteases and reagents are readily available to measure its activity. To investigate whether differentiating neutrophils secrete elastase, its activity was measured in conditioned media. Results indicated that conditioned media from differentiating neutrophils at day 14 (NCM), but not from hematopoietic stem cells (HSC CM), cleaved a commercially available elastase substrate (Fig. 5C). This is consistent with data that HSC CM cannot cleave vcMMAE-containing ADCs, but neutrophil conditioned medium can (Table 1; Supplementary Table S5). To test whether neutrophil's elastase was capable of releasing MMAE from vcMMAE-ADCs, purified elastase was incubated with 100 nmol/L AGS5-vcMMAE, and free MMAE measured by mass spectrometry. Data showed that elastase released significant amount of free MMAE (3 nmol/L) after 20 hours' incubation, which was sensitive to serine protease inhibitors (Fig. 5D). The MMAE concentration measured in this experiment was lower than that observed with condition media from differentiating neutrophils (Table 1). This may be due to the assay conditions and/or contributions from other serine proteases secreted by differentiating neutrophils. Altogether, these data suggest that differentiating neutrophils secrete elastase and probably other serine proteases that could cleave the valine citrulline linker and liberate MMAE.

Discussion

Neutropenia is a common off-target toxicity for ADCs conjugated to MMAE via cleavable linkers. However, little is known about the potential mechanism. Our data showed no direct effect of ADCs on mature neutrophils, probably due to their short half-life and well-differentiated state, suggesting that neutropenia is caused by an effect on neutrophil production from bone marrow stem cells. Here, hematopoietic stem cells (HSC) isolated from human bone marrow were differentiated into neutrophils and were used to investigate the potential mechanisms of neutropenia. In this *in vitro* system, all ADCs containing vcMMAE tested were more cytotoxic to neutrophil differentiation than the same antibodies conjugated to mcMMAF. This is in agreement with the clinical experience in which mcMMAF ADCs have not induced significant neutropenia, whereas those containing vcMMAE have (5, 10). Furthermore, cytotoxicity of vcMMAE-containing ADCs to neutrophils was caused by extracellular

Table 1. Detection of cleaved MMAF/E by mass spectrometry

| Donors | Neutrophils | | Conditioned medium | |
|--------|-------------|-------------|--------------------|------|
| | 1 day | 6 days | Neutrophils | HSCs |
| BM3339 | 1.2 nmol/L | 11.5 nmol/L | 16.8 nmol/L | – |
| BM3477 | 2.4 nmol/L | 7.2 nmol/L | 11.9 nmol/L | – |

NOTE: Extracellular cleavage by proteases secreted by differentiating neutrophils. HSCs from two donors (BM3339, BM3477) were differentiated to neutrophils. Differentiating neutrophils at day 8 were incubated with 100 nmol/L AGS5-vcMMAF, and medium was collected after 1 day or 6 days, and free MMAF was measured by mass spectrometry (left side). Neutrophil conditioned medium was collected at day 14. Conditioned medium was also collected from HSCs. Conditioned media from day 14 neutrophils or HSCs were incubated with 100 nmol/L AGS5-vcMMAE for 1 day, and free MMAE was measured by mass spectrometry (right side). "–" indicates below detection limit (0.2 nmol/L).

cleavage by proteases secreted from differentiating neutrophils. Serine proteases, including neutrophil elastase and possibly other neutrophil serine proteases, seem to play an important role in this extracellular proteolysis of the valine citrulline linker. Both cleavability of the linkers and membrane permeability of the payloads appear to contribute to the cytotoxicity of ADCs to differentiating neutrophils.

HSCs are similarly sensitive to vcMMAE and mcMMAF-containing ADCs which could result in cytotoxicity to multiple hematopoietic cell types; however, pancytopenia is not commonly seen with ADCs containing either vcMMAE or mcMMAF suggesting that HSCs are not the target for these ADCs. One of the reasons for this lack of sensitivity may be the low proliferation rate of human HSCs which replicate on average once every 40 weeks (32). The significant decrease in macropinocytosis activity observed during differentiation from HSCs to neutrophils correlated well with the decrease in sensitivity observed for AGS-16C3F and AGS5-mcMMAF. This suggested that internalization of mcMMAF-containing ADCs into HSCs was mediated by macropinocytosis as it has been shown for differentiating megakaryocytes (Supplementary Table S5; ref. 26). In contrast, differentiating neutrophils remained sensitive to vcMMAE-containing ADCs due to an increase in expression and secretion of proteases, cleavability of valine-citrulline linker and membrane permeability of the released toxin (Supplementary Table S5).

Similar to differentiating neutrophils in bone marrow, activated neutrophils present in areas of inflammation and/or infection secrete proteases that, based on data presented here, have the capability of releasing MMAE from ADCs. This may cause local increases in the levels of MMAE which may lead to toxicities. An unresolved issue from this *in vitro* work is the true contribution to neutropenia or other toxicities from local activation and release of MMAE from ADCs, whether in bone marrow or in inflamed/infected tissues, compared with total, circulating free MMAE. The latter is generated by mechanisms not yet fully understood, but which may include activated neutrophils or other sources of proteases. Studies are ongoing in our laboratory to better understand this issue.

FcγRIIA and FcγRIIIA are highly expressed in differentiating neutrophils which ADCs with IgG1 isotype can bind. IgG1-mcMMAF had modest cytotoxicity against differentiating neutrophils, suggesting that FcγRs-binding and likely internalization can moderately contribute to neutrophil cytotoxicity. Some vcMMAE-containing ADCs with IgG2 isotype are slightly more cytotoxic than those with IgG1 isotype. FcγRs, by bringing ADCs proximal to the neutrophil cell membrane, may facilitate cleavage since a fraction of secreted serine proteases could remain bound to neutrophil membrane (21). Factors other than the IgG subtype are also relevant for the activity of ADC against differentiating neutrophils. For instance, AGS-16C3E, an IgG2, was more cytotoxic to differentiating neutrophils than the nontargeting ADC,

IgG2-vcMMAE. This suggested that biophysical properties of the antibody contribute to the sensitivity of ADCs to proteases secreted by neutrophils.

The data presented here suggest that neutrophils can contribute to toxicities such as neutropenia, but also suggest a potential contribution to antitumor activity of vcMMAE containing ADCs. Tumor-associated neutrophils (TAN) can be abundant and play significant roles in tumorigenesis and metastasis (33, 34). TANs release proteases (34), which based on the results presented here have the potential to contribute to the antitumor activity of vcMMAE-containing ADCs.

In conclusion, this work increases our understanding of the mechanism of action for ADC-induced neutropenia. Our data suggest that vcMMAE-ADCs can be cleaved by serine proteases secreted by differentiating neutrophils releasing MMAE, which may cause cytotoxic effects. The enzymes responsible for these toxic effects are different from those mediating the target-dependent intracellular cleavage in lysosomes, which are mainly cysteine proteases such as cathepsin B. This difference could be exploited to possibly increase the therapeutic index of cleavable ADCs by designing linkers with higher specificity for cysteine proteases than for serine proteases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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A Potential Mechanism for ADC-Induced Neutropenia: Role of Neutrophils in Their Own Demise

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