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Large Molecule Therapeutics

Molecular Cancer Therapeutics

Inhibition of Megakaryocyte Differentiation by Antibody–Drug Conjugates (ADCs) is Mediated by Macropinocytosis: Implications for ADC-induced Thrombocytopenia

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Abstract

Thrombocytopenia is a common adverse event in cancer patients treated with antibody–drug conjugates (ADC), including AGS-16C3F, an ADC targeting ENPP3 (ectonucleotide pyrophosphatase/phosphodiesterase-3) and trastuzumab emtansine (T-DM1). This study aims to elucidate the mechanism of action of ADC-induced thrombocytopenia. ENPP3 expression in platelets and megakaryocytes (MK) was investigated and shown to be negative. The direct effect of AGS-16C3F on platelets was evaluated using platelet rich plasma following the expression of platelet activation markers. Effects of AGS-16C3F, T-DM1, and control ADCs on maturing megakaryocytes were evaluated in an *in vitro* system in which human hematopoietic stem cells (HSC) were differentiated into MKs. AGS-16C3F, like T-DM1, did not affect platelets directly, but inhibited MK differentiation by the activity of Cys-mcMMAF, its active metabolite. FcγRIIA did not appear to play an important role in ADC cytotoxicity to differentiating MKs. AGS-16C3F, cytotoxic to MKs, did not bind to FcγRIIA on MKs. Blocking the interaction of T-DM1 with FcγRIIA did not prevent the inhibition of MK differentiation and IgG1-mcMMAF was not as cytotoxic to MKs despite binding to FcγRIIA. Several lines of evidence suggest that internalization of AGS-16C3F into MKs is mediated by macropinocytosis. Macropinocytosis activity of differentiating HSCs correlated with cell sensitivity to AGS-16C3F. AGS-16C3F was colocalized with a macropinocytosis marker, dextran-Texas Red in differentiating MKs. Ethyl isopropyl amiloride (EIPA), a macropinocytosis inhibitor, blocked internalization of dextran-Texas Red and AGS-16C3F. These data support the notion that inhibition of MK differentiation via macropinocytosis-mediated internalization plays a role in ADC-induced thrombocytopenia. *Mol Cancer Ther; 16(9); 1877–86.* ©*2017 AACR.*

See related article by Zhao et al., p. 1866

Introduction

Antibody–drug conjugates (ADC) are targeting antibodies conjugated to a cytotoxic agent. Two ADCs are currently marketed. Brentuximab vedotin (ADCETRIS®), approved for the treatment of Hodgkin lymphoma and anaplastic large cell lymphomas, is an anti-CD30 antibody conjugated to MMAE, a potent microtubule-disrupting agent via a protease cleavable linker (1, 2). T-DM1 (KADCYLA®), approved for treatment of HER2-positive metastatic breast cancer, is an IgG1 mAb against HER2 (trastuzumab) conjugated to maytansine derivative DM1 via a stable thioether linker (3–5). Currently, there are many ADCs in clinical development with some showing promising benefit (6–8). AGS-16C3F is an ADC currently being investigated in a phase II study in metastatic renal cell carcinoma (mRCC; refs. 9, 10). AGS-16C3F is comprised of a fully human IgG2 monoclonal ENPP3-targeting antibody conjugated to monomethyl auristatin F (MMAF) via a noncleavable maleimidocaproyl linker (Supplementary Fig. S1). ENPP3 is an ectonucleotide pyrophosphatase/phosphodiesterase protein with high expression in some tumors such as clear cell renal cell carcinoma and generally low expression in normal tissues. AGS-16C3F has potent tumor growth inhibition in animal models (9).

Antibody–drug conjugates are designed to selectively deliver highly cytotoxic payloads to tumor cells to minimize systemic toxicity. There are, however, some toxicities that are common to ADC therapies (6, 8), one of which is thrombocytopenia characterized by low platelet counts. Thrombocytopenia can cause bleeding in tissues, bruising, and slow blood clotting after injury (11). Some drugs, such as carboplatin, can cause myelosuppression that then induces thrombocytopenia (12–14). Thrombocytopenia was the dose-limiting toxicity of T-DM1 therapy during clinical development (5, 15). For the phase I clinical trial with AGS-16C3F, thrombocytopenia was observed in 11 of 34 patients. Of these 11, 6 subjects (18%) experienced grade 3 or 4 events with no obvious clinical implications (10). In general, a pattern of initial platelet count drop and establishment of lower on-treatment threshold around study day 90 was observed across all dose levels tested in this phase I study.

Platelets are produced by MKs that are derived from hematopoietic stem cells (HSC) in the bone marrow. Recently, two

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studies described potential mechanisms of thrombocytopenia induced by T-DM1 (16, 17). These studies proposed that thrombocytopenia was mediated mainly via inhibition of megakaryocyte differentiation and that T-DM1 did not have a direct effect on platelet function. The exact mechanism of T-DM1 internalization in maturing MKs is still controversial as one of the studies suggested that internalization was mediated by a FcγRIIA-dependent pathway (16), while the other proposed that it was FcγRIIA independent (17) without describing alternative mechanisms of internalization.

In this report, human hematopoietic stem cells isolated from human bone marrow were differentiated into megakaryocytes *in vitro*, and were used to investigate potential mechanisms of thrombocytopenia induced by AGS-16C3F and T-DM1. Our results suggest that AGS-16C3F, like T-DM1, can inhibit megakaryocyte differentiation, and this inhibition is mediated mainly by internalization via a macropinocytosis pathway independent of FcγRIIA.

Materials and Methods

Antibodies and reagents

Anti-human CD41 antibody R-Phycoerythrin conjugate was from Life Technologies (catalog no. MHCD4104) for FACS and naked anti-human CD41 antibody for confocal microscopy was from Abcam (catalog no. ab11024). 5-(N-ethyl-N-isopropyl) amiloride (EIPA) was from Santa Cruz Biotechnology (catalog no. sc-202458). R-Phycoerythrin streptavidin is from Jackson Immunoresearch (catalog no. 016-110-084), and anti-MMAE/F antibody (SG15-22) was provided by Seattle Genetics, Inc. T-DM1 was purchased from a pharmacy. The general structure of AGS-16C3F is shown in Supplementary Fig. S1. All ADCs conjugated to MMAE/F used technology licensed from Seattle Genetics, Inc., and they were conjugated with a similar drug–antibody ratio (DAR Supplementary Table S1). Control antibodies (IgG1 and IgG2) were produced from CHO cells directed against chicken egg white lysozyme protein that is known not to be expressed in mammalian cells and tissues. The antibodies were generated by immunizing Balb/c mice with the chicken egg white lysozyme 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 megakaryocytes

Human hematopoietic stem cells ($CD133^+$, catalog no. ABM026F) isolated from bone marrow were purchased from ALLCELLS. All HSC growth media (StemSpan SFEM II, catalog no. 09600), supplements for expansion (CC100, catalog no. 02690) and megakaryocyte differentiation (catalog no. 02696) were from Stem Cell Technologies. IL1 and IL3 were from R&D Systems. To differentiate HSCs to MKs, HSCs were first expanded in growth medium (StemSpan SFEM II) supplemented with CC100 for three days according to manufacturer's recommendation. Stem cell markers (CD133, CD34) and megakaryocyte marker (CD41) were measured by FACS prior to differentiation. Complete megakaryocyte differentiation medium is StemSpan SFEM II supplemented with Megakaryocyte Differentiation Supplement (Stem Cell Technologies), plus 10 ng/mL IL1 and 10 ng/mL IL3 (R&D Systems). After a 3-day expansion, cells were incubated in complete megakaryocyte differentiation medium for a period of time indicated by each experiment. Typically, ADCs were added to differentiation medium at the second day of differentiation and incubated for 6 days prior to flow cytometry analysis. Effect of ADCs on MK differentiation is represented by the number of CD41-positive ($CD41^+$) cells. Molm-13 cell line was purchased in 2011 from The Leibniz Institute DSMZ (Germany) and was banked upon receipt. It was authenticated by short tandem repeat (STR) DNA profiling at Agensys Inc. and tested for mycoplasma in 2012. Molm-13 cells were maintained in RPMI1640 supplemented with 10% FBS, and were used within 20 passages after thawing.

Flow cytometry analysis

After a 3-day expansion, CD133-positive ($CD133^+$) HSCs were incubated in differentiation medium for 24 hours and were then treated with ADCs at indicated concentrations for 6 days. Cells were washed and incubated with mouse anti-human CD41 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 megakaryocytes 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 by megakaryocytes.

Confocal internalization studies

Megakaryocytes were seeded on 8-well chamber slides (0.75×10^6 cells per well) and cultured for 48 hours prior to treatment and immunostaining. Cells were then incubated with 10 μg/mL AGS-16C3F with and without coincubation of 0.5 mg/mL dextran-Texas Red (Molecular Probes D18653) for 4 hours at 37°C. Inhibition of macropinocytosis was evaluated by treating megakaryocytes with 50 μmol/L 5-(N-ethyl-N-isopropyl) amiloride (EIPA) for 30 minutes prior to AGS-16C3F/dextran-Texas Red incubation. After the incubation period, unbound antibody was washed off with PBS and cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature. Cells were then permeabilized in PBS + 0.1% Triton-X-100 for 15 minutes and nonspecific labeling was blocked in PBS + 10% normal goat serum. Cell surface bound and internalized cytosolic AGS-16C3F was visualized by incubating cells with Alexa Fluor 488–labeled goat anti-human IgG (Invitrogen). Megakaryocytes were identified by staining with anti-CD41 (Abcam ab11024) and microtubules were visualized by staining with anti-α-Tubulin (clone DM1A, Sigma T9026). Indirect immunofluorescence of CD41 or α-Tubulin was visualized with a secondary antibody, Alexa Fluor 594–labeled goat anti-mouse IgG (Invitrogen). Nuclei were visualized with TOPRO-3 Iodide (Invitrogen) and coverslips were mounted using Prolong Gold anti-fade reagent (Invitrogen) for imaging. High-resolution laser confocal image sections were acquired using a Leica TCS SP5-II (63x oil immersion objective; NA = 1.4) and were scanned sequentially to minimize fluorophore cross-talk and false-positive colocalization.

Proliferation assays

HSCs (3,000 cells/well in 100 μL) were grown in 96-well plates (Corning Assay Plate, catalog no. 3903) for 6 days, CellTiter-Glo

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(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 IC_{50} values. Each experiment was done in triplicates and each result is from at least two experiments.

Antibody biotinylation

EZ-Link NHS-PEG4-biotin was from Thermo Fisher Scientific (catalog no. 21329). Biotinylation reaction was done with 12-fold molar excess of biotin to IgGs (13 mg/mL) for 2–3 hours at room temperature. Biotinylated products were dialyzed overnight at 4°C using Slide-A-Lyzer mini dialysis device (Thermo Fisher Scientific catalog no. 88402).

Results

AGS-16C3F does not bind to platelets nor does it affect platelet activation

To investigate whether AGS-16C3F has a direct effect on mature platelets, we prepared platelet rich plasma (PRP) from normal donors and first tested expression of its target, ENPP3 by FACS analysis (Supplemental Methods). ENPP3 (ectonucleotide pyrophosphatase/phosphodiesterase 3) was not detected on the surface of platelets and AGS-16C3F did not bind to platelets (Supplementary Fig. S2A). Furthermore, ENPP3 was not detected in bone marrow by IHC (Supplementary Methods; Supplementary Fig. S2B). Mature platelets can be activated by thrombin receptor-activating peptide (TRAP) or collagen, which can be measured by expression of glycoprotein IIb/IIIa (PAC-1) and P-selectin (CD62P). AGS-16C3F or control ADC IgG2-mcMMAF treatment did not cause platelet activation or affect activation induced by TRAP or collagen (Supplementary Fig. S3). These data suggest that a direct effect on platelets is not the cause of thrombocytopenia.

Effect of AGS-16C3F on megakaryocyte differentiation from HSCs

To investigate the mechanism of action of thrombocytopenia induced by AGS-16C3F, an *in vitro* model of MK maturation was developed as described by Uppal and colleagues (16). Human hematopoietic stem cells (HSCs; $CD133^+/CD34^+$ cells) were first expanded for three days after which most cells still expressed CD133 (60%) and CD34 (80%), with no expression of CD41, a MK specific marker (Fig. 1A). After differentiation for 14 days, 70%–90% viable cells expressed CD41 with undetectable CD34 (Fig. 1A), suggesting that the cells had differentiated to megakaryocytes. Megakaryocytes (defined as $CD41^+$ cells) changed in morphology and size as the process of maturation continued. Figure 1B shows a snapshot after three weeks of differentiation illustrating examples of large cells resembling mature MKs and other less differentiated cells. These large cells had rough edges and small cells resembling platelets clustering at the edge of mature megakaryocytes (Fig. 1B). To validate this *in vitro* model, T-DM1 was tested and the results compared to those of Uppal and colleagues (16). Expanded HSCs were differentiated for one day and treated with T-DM1 for six days (early differentiation assay) and the effect on MK differentiation was determined by measuring CD41 staining by FACS (Fig. 1C). As previously reported (16), T-DM1 was cytotoxic to differentiating MKs as the percentage and the total number of $CD41^+$ cells decreased significantly (55% and 97% decrease, respectively) when compared with control treated cells. These results suggest that the *in vitro* MK differentiation model is equivalent to the one described by Uppal and colleagues (16).

HSCs at different differentiation stages were tested for their sensitivity to AGS-16C3F. First, proliferating HSCs were kept in stem cell growth medium after 3-day expansion. HSCs were then treated with AGS-16C3F for 6 days and the effect on HSC growth was assessed. HSC proliferation was sensitive to AGS-16C3F treatment with an IC_{50} of 16.9 $\pm$ 2.2 nmol/L (Fig. 2A; Table 1). Next, megakaryocytes ($CD41^+$ cells) at different maturation stages were compared for their sensitivity to AGS-16C3F treatment (Fig. 2A; Table 1). AGS-16C3F was tested in the early differentiation assay (i.e., day 1–7 treatment) or in the later differentiation assay (i.e., day 8–14 treatment) for its effect on $CD41^+$ cells. MKs during the first week of differentiation were more sensitive (IC_{50} = 41 $\pm$ 8 nmol/L) than those during the second week of differentiation (IC_{50} = 98 $\pm$ 2.4 nmol/L). Similarly, a previous study also showed that the T-DM1 cytotoxic effect is more pronounced in early differentiating MKs than in maturing MKs (16).

We next investigated the effect of AGS-16C3F (at 100 nmol/L based on IC_{50} values from Fig. 2A) on the number of mature megakaryocytes defined by the presence of large cells as shown in Fig. 1B. Expanded HSCs were differentiated for either 7 days or 14 days and then treated with AGS-16C3F for two weeks, and mature megakaryocytes were counted at the end of treatment (Fig. 2B). AGS-16C3F treatment decreased the number of mature megakaryocytes by at least 50% in both cases compared with mock treatment. These results indicate that AGS-16C3F-induced inhibition of MK differentiation leads to a decrease in the number of mature megakaryocytes.

The effect of different ADCs on differentiating megakaryocytes was investigated next. Naked antibody (AGS-16C3), AGS-16C3F or ENPP3 non-binding control ADCs (IgG1-mcMMAF, IgG2-mcMMAF; 100 nmol/L) were tested in the early differentiation assay (Fig. 2C). The number of $CD41^+$ cells was greatly decreased by AGS-16C3F treatment (77%). The anti-ENPP3 naked antibody, AGS-16C3 did not have effect on $CD41^+$ cell number, and the presence of 100-fold excess of this antibody (10 μmol/L) did not block AGS-16C3F cytotoxicity to MKs. This suggests that AGS-16C3F cytotoxicity is not mediated by its target, ENPP3. Furthermore, neither of the two control ADCs, IgG1-mcMMAF or IgG2-mcMMAF induced significant inhibition at the same dose, suggesting that unknown biophysical properties of the antibody might be determinants of this inhibition. The lack of activity of the naked antibody points to the active metabolite (Cys-mcMMAF) as the cause of the inhibition, similar to what has been shown for T-DM1 (16). Maleimide-based ADCs are prone to drug-linker loss in the presence of free thiol groups via a retro-Michael type reaction (18, 19). Interestingly, the lack of cytotoxic activity of the two controls, IgG1 and IgG2 ADCs conjugated to mcMMAF suggest that inhibitory activity is not mediated by mcMMAF adducts that may be formed by retro-Michael reaction during the 6 days of incubation.

Mature megakaryocytes or platelets do not express ENPP3 (Supplementary Fig. S2A–S2D), and anti-ENPP3 naked antibody cannot block AGS-16C3F cytotoxicity to differentiating MK cells (Fig. 2C). These data suggest that AGS-16C3F inhibition of differentiating MKs is not dependent upon its target,

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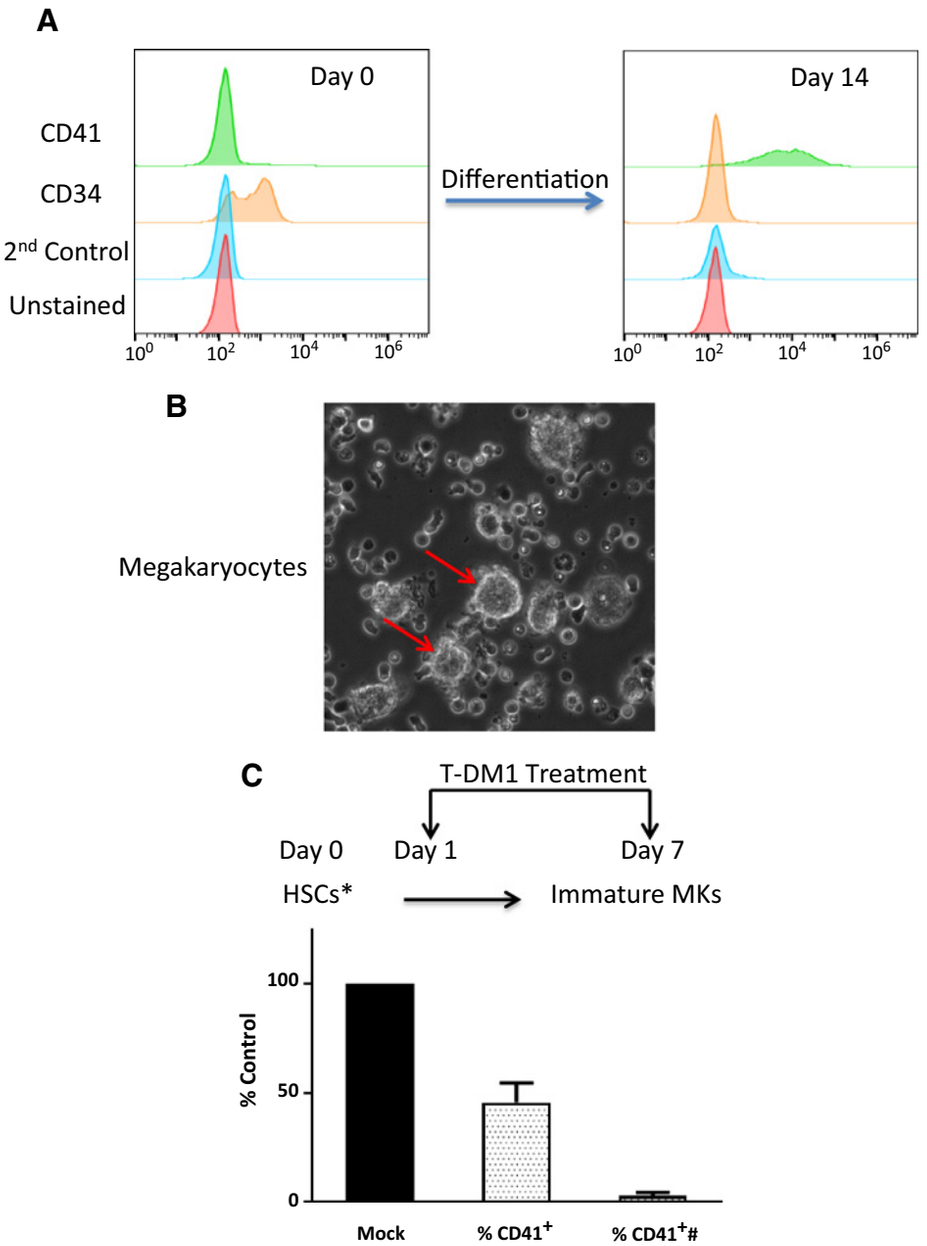

Figure 1.
Differentiation of human hematopoietic stem cells to megakaryocytes *in vitro*. **A,** Human hematopoietic stem cells (HSC) were grown in differentiation medium for 14 days, and $CD41^+$ (megakaryocyte marker) and $CD34^+$ (stem cell marker) were measured by flow cytometry. **B,** HSCs were differentiated for 21 days, and pictures were taken to see appearance of mature megakaryocytes indicated by arrows. **C,** Effect of T-DM1 on MK differentiation. After HSCs were grown in stem cell growth medium (HSC^+), PBS (Mock), or 100 nmol/L T-DM1 was added to HSCs in differentiation medium on day 1 for 6 days. Effect of T-DM1 was shown as % $CD41^+$ which represents percentage of CD41-positive cells within viable cells gated in FACS analysis, or $CD41^+$# which is total cell number with $CD41^+$ normalized against mock-treated samples. Values represent average of four experiments from 4 different HSC donors.

ENPP3. To further confirm this, we investigated ENPP3 expression in differentiating MKs (Supplementary Fig. S4). FACS analysis of differentiating MKs with anti-ENPP3 antibody showed an MFIR of 1.4 that is consistent with minimal expression of ENPP3 on differentiating MKs (Supplementary Fig. S4).

Internalization of AGS-16C3F in megakaryocytes

Generation of the active metabolite of AGS-16C3F, Cys-mcMMAF, a nonmembrane permeable microtubule-disrupting agent generated after complete digestion of the ADC in lysosomes, requires internalization of the ADC. To confirm internalization of AGS-16C3F, MKs differentiated for 1 week were incubated with AGS-16C3F or control ADC IgG2-mcMMAF and their localization was investigated by confocal microscopy. No internalization was observed for control ADC IgG2-mcMMAF (Fig. 3A–C), while significantly more AGS-16C3F was internalized (Fig. 3D). Furthermore, intracellular staining of AGS-16C3F was observed in cells which also stained positive for plasma membrane CD41 (Fig. 3E and F) suggesting that differentiating, $CD41^+$ MKs, which are ENPP3-negative as shown above, can internalize AGS-16C3F. Cells were also costained with anti-α-tubulin antibody to test the effect of AGS-16C3F on microtubule structure. Cells that stained positive for AGS-16C3F showed an aberrant microtubule structure when compared with nontreated cells (Fig. 3G–L), implying that the active metabolite, Cys-mcMMAF, was generated after internalization into $CD41^+$ MKs and caused disruption of the tubulin network.

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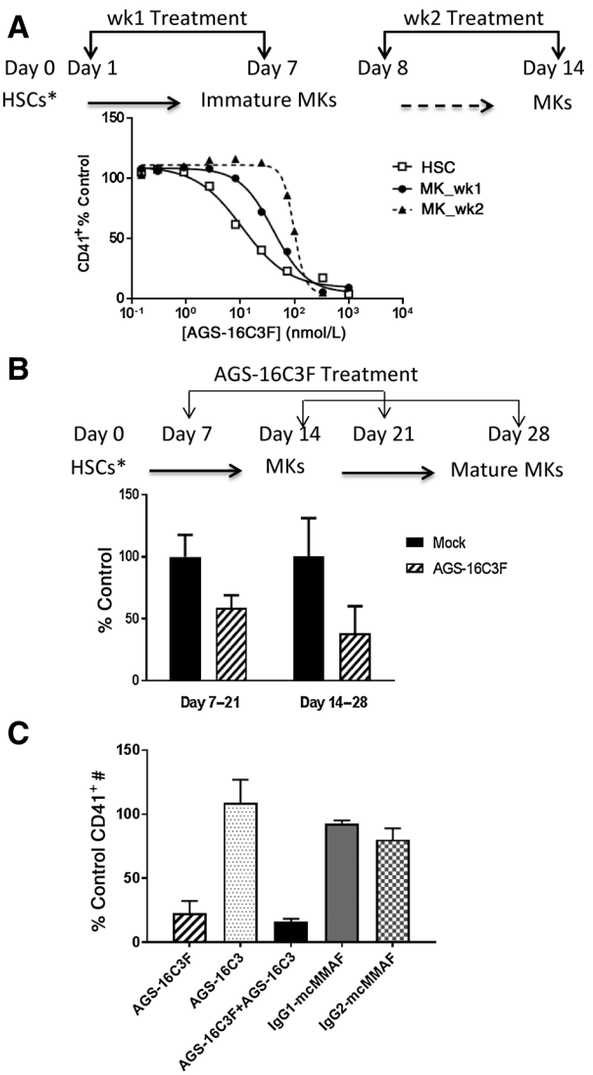

Figure 2.

AGS-16C3F inhibits megakaryocyte differentiation. **A,** Sensitivity of differentiating HSCs to AGS-16C3F. After 3-day expansion, differentiating HSCs (HSCs*) continued to grow in stem cell growth medium (CC100) for 1 day, and were then treated with different concentrations of AGS-16C3F for 6 days. CellTiter-Glo kit was used to measure cell proliferation after 6 days (open square). CTG values were normalized against mock-treated samples and presented as % max proliferation. Differentiating HSCs (HSCs*) were grown in differentiation medium for 1 day (wk1 treatment, solid circle) or 8 days (wk2 treatment, solid triangle), and were treated with different concentrations of AGS-16C3F for 6 days. Total $CD41^+$ cells were measured by FACS and normalized against mock-treated sample, and data from three different donors were analyzed by GraphPad Prism 6 for IC_{50} values. **B,** HSCs were differentiated for 7 or 14 days and were then treated with 100 nmol/L AGS-16C3F or vehicle for another 14 days. The number of MKs, characterized by their larger size was counted with a hemocytometer. MK number was normalized against mock-treated samples. **C,** After 3-day expansion, differentiating HSCs (HSCs*) were grown in MK differentiation medium for 1 day and were treated for 6 days with 100 nmol/L AGS-16C3F, its naked antibody AGS-16C3 only, 100 nmol/L AGS-16C3F in the presence of 10 μmol/L AGS-16C3, or two control ADCs, IgG1-mcMMAF and IgG2-mcMMAF. $CD41^+$ cells were measured with FACS, and the number of $CD41^+$ cells was normalized against mock-treated samples. Values were derived from at least two donors.

FcγRIIA is not required for receptor-independent ADC effect on maturing megakaryocytes

The FcγRIIA is the only Fc receptor expressed in MK and platelets (20, 21), and was recently proposed to mediate binding and internalization of T-DM1 as the mechanism for inhibition of MK differentiation (16). However, AGS-16C3 is an IgG2 antibody with lower binding affinity to FcγRIIA than an IgG1 antibody (22), such as T-DM1 (Supplementary Table S2), which led us to investigate the role of FcγRIIA in the internalization and cytotoxic effects of AGS-16C3F on differentiating MKs. The levels of FcγRIIA in HSCs and differentiating MKs were investigated by FACS with an anti-CD32 antibody. Differentiating HSCs expressed little FcγRIIA on the cell membrane which increased significantly during MK differentiation (Fig. 4A; Table 1), confirming the presence of this receptor. The Fc region of an IgG1 antibody containing S239D/I332E mutations that have been shown to enhance the affinity for Fc gamma receptors (23, 24), "Fc blocker", was used to inhibit IgG/FcγR interactions. We confirmed that the Fc blocker had a higher affinity for FcγRI, FcγRIIA, and FcγRIIIA than an IgG1 antibody (Supplementary Methods; Supplementary Table S2). A significant number of MKs were bound by ADCs of IgG1 isotype, control IgG1-mcMMAF (38%), and T-DM1-biotin (63%; Fig. 4B). Addition of Fc blocker reduced the binding to MKs of the IgG1-mcMMAF and of T-DM1 to 2.4% and 20%, respectively (Fig. 4B) suggesting that binding was through the FcγRIIA. On the other hand, MKs did not bind to the control antibody, IgG2-biotin, and the majority of MKs did not bind to AGS-16C3-biotin. The AGS–16C3F interaction was not inhibited by Fc blocker suggesting that the FcγRIIA was not involved. Biotin-T-DM1 was bound to approximately 20% of differentiating MKs in the presence of Fc blocker despite lack of Her-2 expression (16). Likewise, around 18% of ENPP3-negative MKs bound AGS-16C3F. The cause and significance of this minor binding are not currently known.

To continue investigating the potential contribution of FcγRIIA, maturing MKs were treated with AGS-16C3F in the presence of Fc blocker (100-fold excess) and the number of $CD41^+$ cells was measured by flow cytometry in the early differentiation assay (Fig. 4C). The presence of Fc blocker had no effect on AGS-16C3F–induced cytotoxicity to differentiating MKs. Surprisingly, the Fc blocker had no effect on the T-DM1–induced cytotoxicity to MK differentiation as well. Two control experiments were carried out to confirm that the Fc blocker was able to compete for binding to Fc receptors. First, MK-cell medium taken after 6-day incubation with Fc blocker inhibited IgG1-mcMMAF binding to MK cells (Fig. 4D). Second, Molm-13, an acute monocytic leukemia cell line that expresses a moderate level of FcγRIIA (25), was used to test the effects of the Fc blocker (Supplementary Fig. S5). Control IgG1-mcMMAF showed moderate cytotoxic effects after 6-day incubation despite lack of a specific antigen, presumably through binding and internalization by FcγRIIA. A 100-fold excess of Fc blocker almost completely inhibited IgG1-mcMMAF cytotoxicity to Molm-13 cells, indicating that the Fc blocker can inhibit the FcγRIIA-mediated cytotoxic activity of an IgG1 ADC. Taken together, these results suggest that FcγRIIA does not play a critical role in the inhibition of differentiating MKs by AGS-16C3F or T-DM1.

Macropinocytosis mediates receptor-independent internalization of AGS-16C3F

Seeking alternative mechanisms for ADCs to account for the internalization by differentiating HSCs, macropinocytosis was

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Table 1. Macropinocytosis, FcγRIIA and sensitivity to AGS-16C3F

		Megakaryocytes	
	HSCs	**Wk1**	**Wk2**
AGS-16C3F IC_{50} (nmol/L)	16.9 ± 2.2	41 ± 8	98 ± 2.4
FcγRIIA expression (MFIR)	1.4 ± 0.2	5.8 ± 2.8	6.4 ± 2.1
Macropinocytosis (MFIR)	14.4 ± 4.7	5.3 ± 0.4	

investigated as it is used by cells to internalize large molecules in a receptor-independent way (26–28). The uptake of dextran-fluorophores by FACS, a well-accepted method to measure macropinocytosis (29), was measured in HSCs and maturing MKs (Fig. 5). Results showed that both cell populations have measurable uptake of the dextran-fluorophore suggesting active macropinocytosis. Interestingly, the degree of macropinocytosis of the HSCs (MFIR 14.4) was higher than that in the population of cells in the maturing MKs (MFIR 5.3) which correlated with their higher sensitivity to AGS-16C3F (Table 1) and T-DM1 (15).

The potential role of macropinocytosis as the main mechanism of action for AGS-16C3F's effect on differentiating HSCs was further investigated by determining whether AGS-16C3F would colocalize with dextran-Texas Red by confocal microscopy (Fig. 5). The results showed that AGS-16C3F and dextran-Texas Red were colocalized to the same intracellular compartments (Fig. 5B–D). Internalization by macropinocytosis can be blocked by ethyl isopropyl amiloride (EIPA; ref. 28), an inhibitor of Na^+/H^+ exchange, and EIPA was used to test whether macropinocytosis is required for AGS-16C3F internalization into MK cells. Early differentiating MK cells were treated with AGS-16C3F or dextran-Texas red in the presence of EIPA. Internalization and colocalization of AGS-16C3F and Dextran-Texas Red were monitored by confocal microscopy (Fig. 5E–H). Interestingly, EIPA treatment significantly inhibited dextran accumulation in MK cells as expected (Fig. 5F and H), but it also blocked the internalization of AGS-16C3F (Fig. 5E and G) suggesting that early differentiating

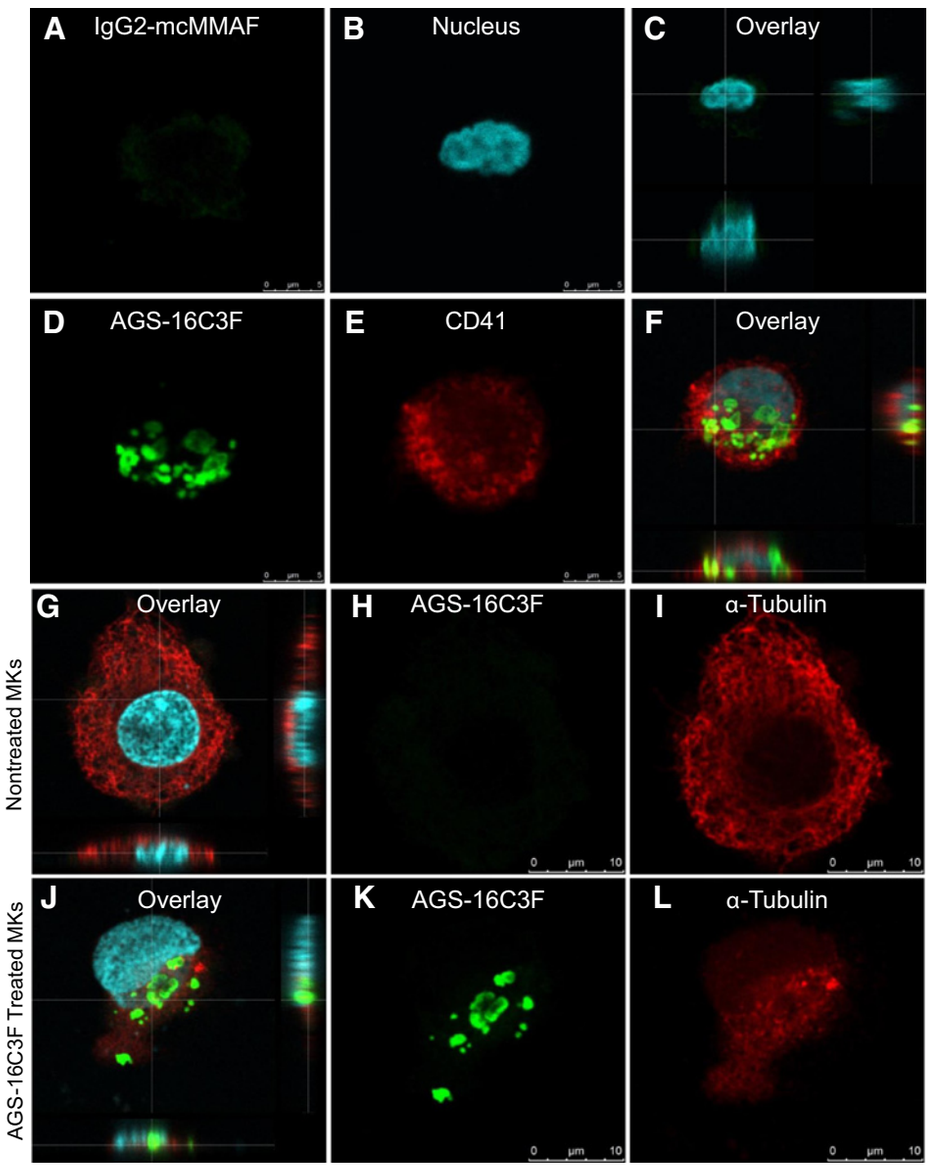

Figure 3.
Internalization of AGS-16C3F disrupts microtubule structure in megakaryocytes. HSCs were differentiated for 7 days before treatment for confocal microscopy. **A–F,** $CD41^+$ MKs can internalize AGS-16C3F. MKs at day 7 were treated with 10 μg/mL IgG2-mcMMAF (**A–C**) or AGS-16C3F (**D–F**) for 4 hours for confocal microscopy. Cytosolic IgG2-mcMMAF (**A**) and AGS-16C3F (**D**) was visualized by incubating cells with Alexa Fluor 488–labeled goat anti-human IgG, megakaryocytes were identified by staining with anti-CD41 antibody (**E**), and nuclei were visualized with TOPRO-3 Iodide (blue color, **B**, **C**, and **F**). **G–L,** Effect of AGS-16C3F on microtubule structure. MKs (day 7) were treated with vehicle (Top) or 10 μg/mL AGS-16C3F (Bottom) overnight, and α-tubulin structure (**I** and **L**) and AGS-16C3F localization (**H** and **K**) were analyzed in MKs individually or together with nuclei (**G** and **J**).

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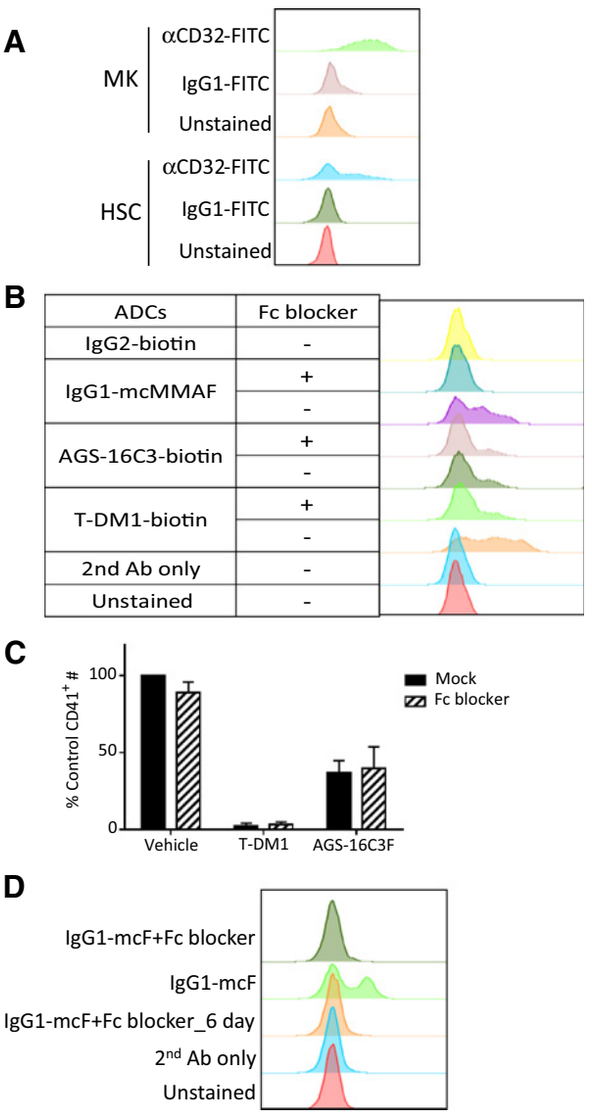

Figure 4.

The role of FcγRIIA in ADC-induced cytotoxicity to megakaryocytes. **A,** FcγRIIA expression on megakaryocyte membrane. HSCs or MKs differentiated for 10 days were stained with control IgG1-FITC or anti-CD32-FITC for 30 minutes in the dark on ice, and subjected to FACS. **B,** Binding of ADCs to MKs. MKs differentiated for 7 days were incubated with 10 μg/mL control ADCs, IgG1-mcMMAF, or biotinylated T-DM1, IgG2 (IgG2-biotin), or AGS-16C3 (AGS-16C3-biotin) for 1 hour on ice, and binding of ADCS to MKs were detected with either anti-MMAF antibody for IgG1-mcMMAF or streptavidin-PE for the latter three. 10× Fc blocker (100 μg/mL) was added to inhibit binding to FcγRIIA (+). **C,** FcγRIIA is not required for ADC-induced cytotoxicity to megakaryocytes. HSCs were grown in differentiation medium for 1 day and were incubated with 100 nmol/L T-DM1 or AGS-16C3F for 6 days in the absence (mock, black bar) or presence of 10 μmol/L Fc blocker (slashed bar). $CD41^+$ cell number was identified by FACS analysis and normalized against mock-treated samples (*y*-axis). Results were generated from 4 donors. **D,** Fc blocker activity in **C** after 6-day incubation was analyzed. MKs from **C** were incubated with 10 μmol/L Fc blocker for 6 days, and medium was obtained and tested for activity inhibiting FcγRIIA. Binding of IgG1-mcMMAF (100 nmol/L) in presence of this medium was analyzed by FACS (IgG1-mcF+Fc blocker_6 day). As control, fresh Fc blocker was added to inhibit IgG1-mcMMAF binding (IgG1-mcF + Fc blocker).

MKs use macropinocytosis to internalize AGS-16C3F. EIPA was shown to be toxic to HSCs or differentiating MKs at longer incubation times, precluding testing the effect of EIPA in the 6-day MK-differentiating assay.

Discussion

Thrombocytopenia has been reported in cancer patients treated with antibody–drug conjugates such as AGS-16C3F and T-DM1. AGS-16C3F was well tolerated without evidence of generalized myelosuppression in the phase I study where the ADC was given every 3 weeks in doses up to 4.8 mg/kg (10). Thrombocytopenia was observed but was not dose limiting in all but one subject at the 3.6 mg/kg dose level in the AGS-16C3F study. Thrombocytopenia had a general pattern of early platelet count drop in cycle 1 shortly after the first dose followed by a spontaneous recovery to a level adequate to receive the next dose on day 22. A slow reduction in platelet count was also observed in long-term exposure that spontaneously recovered to baseline levels after treatment cessation.

It was previously proposed that FcγRIIA-mediated internalization of T-DM1 in the bone marrow plays an important role in inhibition of megakaryocyte differentiation (16). The data presented here support that FcγRIIA may not be primarily responsible for the internalization of ADCs and subsequent toxic effects on megakaryocytes. These conclusions are supported by the following findings: (i) Some ADCs with IgG1 isotype that bind to FcγRIIA on MKs were not cytotoxic in the MK differentiation assay, while IgG2 ADCs that bind with lower affinity were. (ii) Inhibition of ADC-binding to FcγRIIA did not prevent T-DM1–induced cytotoxicity to megakaryocytes. (iii) During HSC differentiation into megakaryocytes, FcγRIIA expression increased with a concomitant decrease in cell sensitivity to both T-DM1 and AGS-16C3F. These data suggest that the IgG subclass of an ADC is not a major determinant on cytotoxicity to differentiating HSCs; however, some questions remain to be answered. It is not clear whether ADCs of IgG1 isotype are internalized upon binding of the FcγRIIA on differentiating MKs. If they are internalized, the ADCs do not appear to be digested to release the corresponding active metabolites. A modified T-DM1 containing impaired FcγRIIA-binding mutations decreased binding to differentiating MKs, but its cytotoxic activity against differentiating MKs was not tested (16); it will be interesting to investigate whether that mutant T-DM1 and/or other similarly mutated ADCs are also less cytotoxic to differentiating MKs.

Several lines of evidence presented here suggest that thrombocytopenia induced by AGS-16C3F and possibly other ADCs can be mediated through internalization by macropinocytosis. First, the macropinocytosis activity of differentiating HSCs correlated with cell sensitivity to ADCs. Second, AGS-16C3F colocalized with a macropinocytosis marker, Dextran-Texas Red, in differentiating megakaryocytes. Third, EIPA, a macropinocytosis blocker, inhibited internalization of both Dextran-Texas Red and AGS-16C3F. A drawback of this study was that macropinocytosis inhibitors could not be used for the time needed because of their cytotoxicity to investigate whether they can block the cytotoxic effects of AGS-16C3F and other ADCs on differentiating HSCs. In addition, we have only confirmed macropinocytosis as a possible mechanism of thrombocytopenia for AGS-16C3F. Our group is currently extending this investigation to other ADCs.

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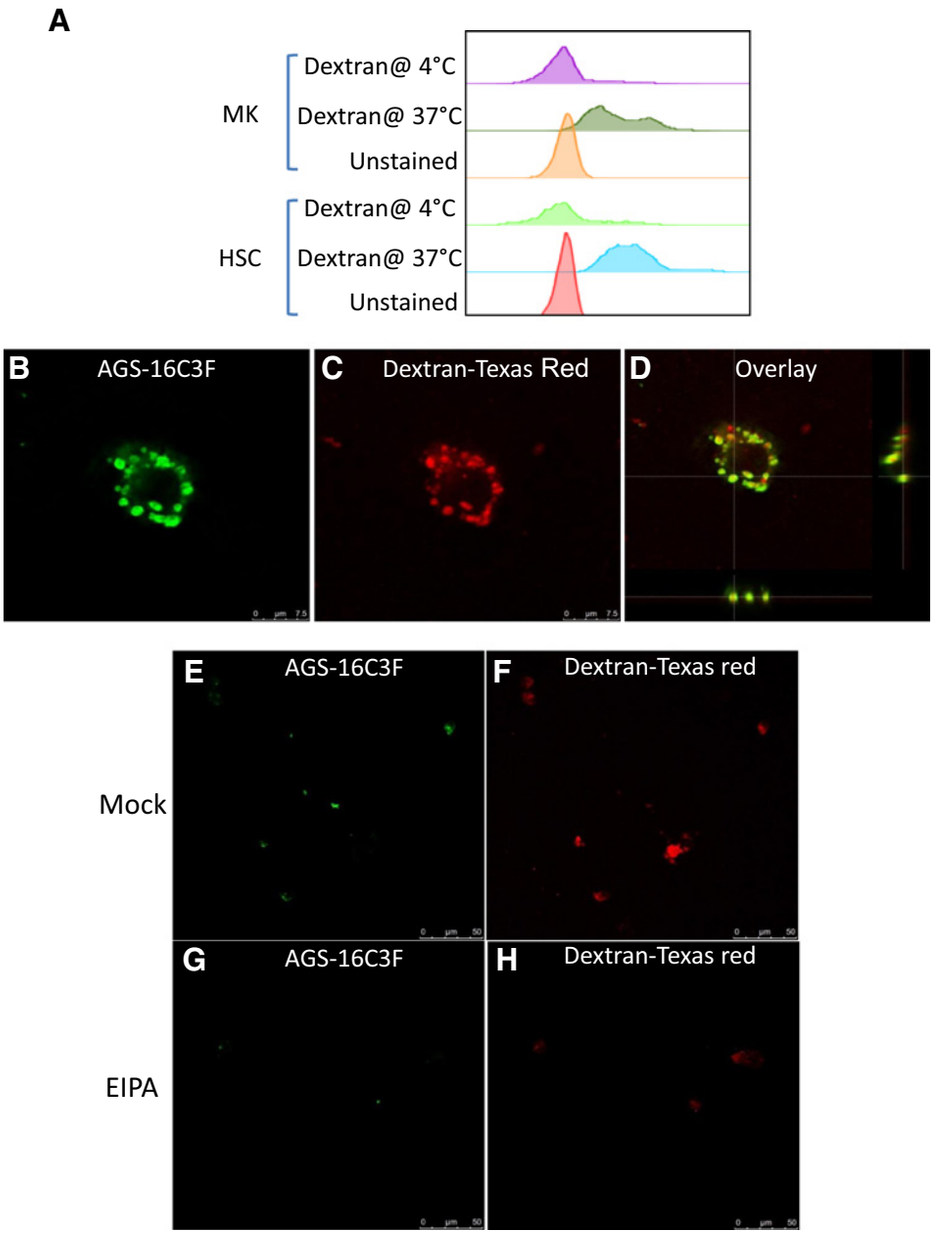

Figure 5.
Macropinocytosis mediates internalization of AGS-16C3F into MKs. **A,** Macropinocytosis activity in HSCs and MKs. HSCs or MKs differentiated for 7–9 days were incubated with dextran-FITC for 3 hours at 37°C and internalization of dextran conjugates were measured by FACS. MFI (median fluorescence intensity) was normalized against 4°C control to get MFIR values in Table 1. Result from one donor is shown here, and average MFIR of three donors are shown in Table 1. **B–D,** AGS-16C3F co-localized with macropinocytosis marker in MKs. MKs after 1 week differentiation were incubated with AGS-16C3F together with dextran-Texas Red for 4 hours. Their intracellular localization was analyzed by confocal microscopy. **E–H,** Macropinocytosis is important for AGS-16C3F internalization into MKs. MK cells were preincubated with 50 μmol/L EIPA for 30 minutes prior to AGS-16C3F and dextran-Texas Red incubation of 4 hours, were then analyzed by confocal microscopy.

Macropinocytosis has long been known as a primary method for cellular uptake of fluid-phase and membrane-bound bulk cargo which requires extensive membrane ruffling within the plasma membrane; however, it remains unclear how membrane ruffling is regulated (30). It has been observed that different proteins may not be pinocytosed at the same rate, different cells may have different levels of macropinocytosis activity, and macropinocytosis can be activated by certain mechanisms (30). Consistent with this, it appears that the biophysical properties of the antibody may play a significant role as exemplified by the different cytotoxicity of ADCs conjugated to the same drug linker (mcMMAF). Both AGS-16C3F and T-DM1 showed minor binding to differentiating MKs even in the presence of Fc blocker and despite their corresponding targets not being expressed. While the significance and cause of this binding to their MK cytotoxicity is not clear and needs further investigation, it is possible that these two ADCs can interact with unknown receptors or certain regions of ruffled membranes of MK cells that could influence MK macropinocytosis. Better understanding and proper manipulation of determinants of macropinocytosis in ADCs may result in a better toxicity profile and improved therapeutic index.

Thrombocytopenia can be caused by a decrease in platelet production or an increase in platelet destruction. The kinetics of thrombocytopenia for T-DM1 and AGS-16C3F are similar with nadirs observed by approximately day 8 or 7 and recovery by day 15 and 21, respectively, which are consistent with impaired MK formation in the bone marrow as a mechanism of action. However, other mechanisms may also play a role. The lack of cytotoxic activity of the control ADCs conjugated to mcMMAF (IgG1 and IgG2) supports that mcMMAF adducts that may have been formed

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by retro-Michael reaction during incubation were not responsible for the cytotoxic activity against differentiating HSCs. However, it cannot be ruled out that the toxicity of T-DM1 had contribution from the possible transfer and internalization of the active metabolite from the antibody during the assay. Furthermore, it was proposed recently that for some ADCs, target-independent damage to liver sinusoidal endothelial cells might cause acute thrombocytopenia (31).

In summary, the data presented here increased our understanding of the mechanism of ADC-induced thrombocytopenia, which suggests an important role of macropinocytosis-mediated internalization independent of FcγRIIA in the production of the corresponding active metabolites. It also offers the ability to alter the way ADCs are screened to avoid unwanted toxicity. Further detailed studies on the parameters affecting internalization via this mechanism are warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Study supervision: H. Zhao, K. Morrison, D.R. Stover, F. Doñate

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Inhibition of Megakaryocyte Differentiation by Antibody–Drug Conjugates (ADCs) is Mediated by Macropinocytosis: Implications for ADC-induced Thrombocytopenia

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