Activated platelets in the tumor microenvironment for targeting of antibody-drug-conjugates to tumors and metastases

May Lin Yap\textsuperscript{1,2}, James D McFadyen\textsuperscript{1,3,4}, Xiaowei Wang\textsuperscript{1,3}, Melanie Ziegler\textsuperscript{1}, Yung-Chih Chen\textsuperscript{1}, Abbey Willcox\textsuperscript{1,3,4}, Cameron J Nowell\textsuperscript{5}, Andrew M Scott\textsuperscript{6,7}, Erica K Sloan\textsuperscript{5}, P Mark Hogarth\textsuperscript{2,8,9}, Geoffrey A Pietersz\textsuperscript{1,2,8,9,10#}, Karlheinz Peter\textsuperscript{1,3,9,##}

\textsuperscript{1}Baker Heart and Diabetes Institute, Melbourne, 3004, Australia
\textsuperscript{2}Department of Clinical Pathology, The University of Melbourne, Melbourne, 3010, Australia
\textsuperscript{3}Department of Medicine, Monash University, Melbourne, 3800, Australia
\textsuperscript{4}Department of Hematology, The Alfred Hospital, Melbourne, 3004, Australia
\textsuperscript{5}Drug Discovery Biology Theme, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia
\textsuperscript{6}Olivia Newton-John Cancer Research Institute, and School of Cancer Medicine, La Trobe University, Melbourne, Victoria, Australia
\textsuperscript{7}Department of Molecular Imaging and Therapy, Austin Health, and University of Melbourne, Melbourne, Victoria, Australia.
\textsuperscript{8}Burnet Institute, Melbourne, 3004, Australia
\textsuperscript{9}Department of Immunology, Monash University, Melbourne, 3800, Australia
\textsuperscript{10}College of Health and Biomedicine, Victoria University, Melbourne, 3021, Australia

#equally contributing senior authors

*To whom correspondence should be addressed:

Karlheinz.Peter@baker.edu.au
Graphical Abstract

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Abstract

Rationale

Platelets are increasingly recognized as mediators of tumor growth and metastasis. Hypothesizing that activated platelets in the tumor microenvironment provide a targeting epitope for tumor-directed chemotherapy, we developed an antibody-drug conjugate (ADC), comprised of a single-chain antibody against the platelet integrin GPIIb/IIIa (scFvGPIIb/IIIa) linked to the potent chemotherapeutic microtubule inhibitor, monomethyl auristatin E (MMAE).

Methods

We developed an antibody-drug-conjugate (ADC) comprised of three components: 1) A single-chain antibody which specifically binds to the high affinity, activated integrin GPIIb/IIIa on activated platelets. 2) A highly potent microtubule inhibitor, monomethyl auristatin E. 3) A drug activation/release mechanism using a linker cleavable by cathepsin B, which we demonstrate to be abundant in the tumor microenvironment. The ADC was conjugated with Cyanine7 for in vivo imaging.

Results

In vitro studies confirmed that this ADC specifically binds to activated GPIIb/IIIa, and cathepsin B-mediated drug release/activation results in tumor cytotoxicity. Fluorescence in vivo imaging demonstrated that the newly generated ADC localized to primary tumors and metastases in a mouse xenograft model of triple negative breast cancer, a particularly difficult to treat tumor for which a selective tumor-targeting therapy remains to be clinically established. Importantly, we demonstrated that the ADC displays marked efficacy as an anti-cancer agent, reducing tumor growth and preventing metastatic disease, without any discernible toxic effects.
Conclusion

We developed a novel ADC that targets a potent cytotoxic drug to activated platelets and specifically releases and activates the cytotoxic drug within the confines of the tumor. This unique targeting mechanism that focuses on the tumor microenvironment holds major promise as a novel therapeutic option for a broad range of primary tumors and metastatic disease, particularly those that are difficult to treat or do not express a specific molecular epitope for drug targeting.
Introduction

The mainstay of treatment for many cancers remains systemic chemotherapy. However, this approach is associated by inevitable, ‘off target’ effects of chemotherapy, resulting in systemic toxicity given these agents often have limited selectivity for tumor cells. Antibody-directed chemotherapy offers a more recent and highly promising approach to overcome these inherent limitations of systemic chemotherapy by leveraging the targeting capability of antibodies towards tumor-specific antigens or proteins for the delivery of chemotherapy. As a result, antibody-drug-conjugates (ADC) provide the means to deliver high local concentrations of chemotherapy specifically to the tumor whilst avoiding systemic toxicity. The efficacy of ADCs has been demonstrated by the success of trastuzumab emtansine for HER-2 positive breast cancer and brentuximab vedotin in anaplastic large cell lymphoma and Hodgkin lymphoma, and holds promise in malignancies with particularly limited prognosis such as glioblastoma.

The efficacy of ADCs is highly contingent upon the expression and abundancy of a target epitope on the cancer cells. However, this represents a major limitation for the use of ADCs since in several cancer types, such as triple negative breast cancer, specific antigenic molecular targets are either not identified or are yet to be proven to be clinically successful for molecular targeting. Here, we develop a unique strategy to overcome this limitation, not by choosing a molecular epitope on specific cancer cells but by targeting a specific component of the tumor microenvironment. There is growing evidence demonstrating an intimate relationship between platelets and cancer, and the abundance of activated platelets in the microenvironment of a range of tumors including ovarian cancer, colorectal cancer and oesophageal cancer. Platelets express an abundant, highly specific surface epitope, the integrin glycoprotein...
(GP)IIb/IIIa (αIIbβ3; CD41/CD61). On circulating platelets this integrin is in a low affinity conformation for ligand binding. In contrast, activated platelets present in the tumor microenvironment express the high affinity conformation of GPIIb/IIIa\textsuperscript{14}. This presents a unique opportunity to specifically target adherent platelets in the tumor microenvironment using a target epitope that is highly abundant and specific, an ideal prerequisite for molecular therapeutic targeting. Using various imaging modalities, we recently provided proof of concept that the high affinity conformation of GPIIb/IIIa can serve as a molecular target epitope of the tumor microenvironment in several types of cancers in mice and humans\textsuperscript{15}.

Here, we describe the generation of a unique ADC, scFv\textsubscript{GPIIb/IIIa}-MMAE, that targets platelets in the microenvironment of tumors as a novel approach for cancer treatment. This ADC is based on three major components: 1) The targeting single-chain antibody (scFv\textsubscript{GPIIb/IIIa}), which selectively binds to an epitope that is cryptic on the low-affinity GPIIb/IIIa on circulating platelets but becomes exposed on the high affinity GPIIb/IIIa, expressed on activated platelets\textsuperscript{16,17}. 2) A synthetic microtubule inhibitor, monomethyl auristatin E (MMAE), which is an ideal drug for targeting by utilizing its high cytotoxic potency and obviating its otherwise dominant non-specific cell toxicity. 3) A cancer-specific, localized release mechanism providing an additional layer of specificity achieved by a cathepsin B recognition sequence containing the peptide linker, Valine-citrulline (Val-Cit). This linker utilizes the presence of cathepsin B in the tumor microenvironment for cleavage and release of free MMAE, thereby locally releasing cytotoxic effects\textsuperscript{18,19}.

To demonstrate the functionality of our novel anti-cancer ADC with its unique activated platelet targeting, we first confirmed the efficacy of scFv\textsubscript{GPIIb/IIIa}-MMAE for tumor killing in
human cell lines of triple negative breast cancer, colorectal cancer, fibrosarcoma and prostate cancer. Next, we chose the particularly difficult to treat triple negative breast cancer for proof of concept in vivo studies. Treatment of mice with scFvGPllb/IIa-MMAE resulted in significant regression of primary tumors and prevented metastasis without systemic side effects. Together, these findings indicate the generation of a highly promising antibody-drug-conjugate and establishes a unique concept that holds promise as a novel, potentially broadly applicable anti-cancer therapy, which is of relevance for both difficult to treat tumors and those without specific target epitopes.
Materials and Methods

Generation of targeting antibody scFv-LPETG and coupling enzyme sortase A

The generation of the single-chain antibody directed against GPIIb/IIIa (scFv_{GPIIb/IIIa}) and a control, non-binding, single-chain antibody (scFv_{mut}) has been described previously\(^\text{20}\). Using polymerase chain reaction, an LPETG-tag (sortase A recognition sequence), a V5-tag and a His-tag were introduced to the C-terminal end of the scFv\(^\text{21}\). The entire scFv was then sub-cloned into a pSectag 2A vector (Invitrogen) for expression in human embryonic kidney (HEK) cells (Invitrogen)\(^\text{22}\). Sortase A was used to induce an enzymatic reaction used for the conjugation of the scFv, carrying an LPETG sequence to MMAE which was produced carrying a triple glycine sequence. Sortase A, a transpeptidase cloned from *Staphylococcus aureus* was produced and purified as previously described\(^\text{23}\). All proteins (scFvs and sortase A) contain a 6x His-tag, which was used for purification with nickel-based affinity chromatography (Invitrogen).

Conjugation of scFv with MMAE and Cy7

MMAE, carrying a Val-Cit linker and a triple glycine sequence (GGG-Val-Cit-PAB-MMAE) was synthesized by Levena Biopharma. The scFv_{GPIIb/IIIa} and scFv_{mut} (each constructed with a LPETG-tag) were linked to GGG-Val-Cit-PAB-MMAE using a sortase A enzyme-based protocol to produce scFv_{GPIIb/IIIa}-MMAE and scFv_{mut}-MMAE, as described previously\(^\text{21}\). Excess scFv which contains a His-tag was removed using metal affinity chromatography (Invitrogen) and excess MMAE was removed using a 10 kDa spin column. For imaging studies, Cy7 was incorporated into the conjugate by incubating scFv_{GPIIb/IIIa}-MMAE and scFv_{mut}-MMAE with 2x excess Cy7 via amine labeling (AAT Bioquest). Excess free dye was removed by dialysis in PBS. The purified scFv-Cy7-MMAE was analyzed by SDS-PAGE gel and the
protein and near-infrared signal from the band of interest was confirmed using the Odyssey Imager. Additionally, Western blot was performed with rabbit anti-MMAE antibody (Levena Biopharma), detected with an anti-rabbit HRP antibody (Cell Signaling) to confirm conjugation of MMAE to the scFv.

**Preparation of platelet rich plasma and flow cytometry**

Blood was collected from healthy volunteers in citrate and centrifuged at 180 g for 10 minutes. The platelet rich plasma was then collected, stored at 37°C and used within two hours. For flow cytometry, platelet rich plasma was diluted 1:20 in Tyrode’s buffer. To induce platelet activation, ADP was added at a final concentration of 20 μM for 5 minutes before adding the scFv. Binding was determined by anti-V5-FITC (Thermofisher Scientific) or rabbit anti-MMAE antibody (Levena Biopharma) and detected with an anti-rabbit mAb coupled to AF647 (Invitrogen). Flow cytometry was performed using a FACS Fortessa scanner (BD Biosciences). Results were analyzed using the Flowlogic software.

To determine the ability of cancer cells to activate platelets, platelet rich plasma was incubated with the cancer cell lines MDA-MB-231, HT29, HT1080 and PC3 for 6 hours at 37°C. As a positive control with the same experimental setting, ADP-activated platelet rich plasma was incubated with the cancer cells for 6 hours at 37°C. The cancer cell and platelet (resting/ADP-activated platelets) mixtures were then stained with an anti-CD41-PE monoclonal antibody and scFv<sub>GPIIb/IIIa</sub> or scFv<sub>mut</sub> binding was detected by an anti-V5-FITC monoclonal antibody. Flow cytometry and analysis was performed as described above.
Cancer cell lines

A metastatic variant of the MDA-MB-231 triple-negative breast adenocarcinoma cell line (a kind gift from Dr Zhou Ou, Fudan University Shanghai Cancer Center, China) was transduced with a lentiviral vector containing codon-optimized firefly luciferase-mCherry under the control of the ubiquitin-C promoter\(^{24,25}\) and was cultured in DMEM medium + Glutamax (GIBCO\(^\text{®}\)), supplemented with 10\% (v/v) FBS (Invitrogen), at 37°C in a 5\% CO\(_2\) humidified atmosphere. Human cancer cell lines HT29, HT1080 and PC3 were cultured in RPMI media (GIBCO\(^\text{®}\) #21870) supplemented with 10\% (v/v) FBS (Invitrogen), 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in a 5\% CO\(_2\) humidified atmosphere. Cell identity was confirmed by karyotyping.

Cytotoxicity assays

The cytotoxic activity of GGG-Val-Cit-PAB-MMAE and the scFv-MMAE conjugates were assessed in a cytotoxicity assay in the presence of cathepsin B. To induce cathepsin B cleavage, MMAE, GGG-Val-Cit-PAB-MMAE or scFv-MMAE conjugate was incubated with 0.01 units of pre-activated cathepsin B (Sigma Aldrich) for 4 hours at 37°C in 25 mM acetate buffer pH 4.8. MDA-MB-231 cells were seeded on a 96 well plate at 6000 cells/well overnight. The next day, cells were treated with MMAE, GGG-Val-Cit-PAB-MMAE or scFv-MMAE in the presence or absence of cathepsin B and incubated for 72 hours. Metabolism of the yellow tetrazolium salt (XTT) was determined according to the manufacturer’s instructions (Sigma Aldrich). Percentage killing relative to untreated control cultures was calculated using the following formula: 100 - [(test value/untreated value) x100]. Each assay was repeated in triplicate.
For platelet assays, 100 µL of platelet rich plasma (containing approximately 2x10^7 platelets) was activated with 20 µM ADP. Activated platelets were incubated with scFv-MMAE for 30 minutes. To remove unbound scFv-MMAE, the platelet-scFv-MMAE mixture was centrifuged at 2000 g for 2 minutes, the supernatant was removed, and platelets were resuspended in PBS. As a control, unwashed platelet-scFv-MMAE mixture was used. Either washed or unwashed (control) platelet-scFv-MMAE mixture was added to seeded MDA-MB-231, HT29, HT1080 and PC3 cells in the absence of exogenous cathepsin B and incubated for 72 hours before determination of XTT metabolism as above.

**Immunohistochemical analysis of cathepsin B in tumors**

Tumors were fixed in formalin solution (Sigma Aldrich) for 24 hours, paraffin embedded, and microtome sectioned (Leica) to 5 µm – 30 µm onto a glass slide. Sections were deparaffinized and underwent antigen retrieval with 0.01 M Citric Acid in 90°C for 20 minutes. Tumor sections were stained overnight with an anti-cathepsin B antibody (Abcam) and detected with an Alexa Fluor 488 labeled anti-mouse antibody (Life Technologies) and cell surface membrane-reactive anti-sodium/potassium ATPase antibody (Abcam), counterstained with Hoechst® (Thermo Fisher Scientific) and visualized using the Nikon A1r Plus Confocal Microscope, 20x water objective.

**Breast cancer metastasis model**

To establish metastasis, 2 x 10^5 MDA-MB-231 cells in 20 µL PBS were injected into the fourth left mammary fat pad of 5-6 weeks old anaesthetized BALB/C athymic nude mice. Primary tumor growth was measured by caliper and tumor volume was determined using the formula (length × width^2)/2. Additionally, primary tumor growth was measured via
bioluminescence using the IVIS Lumina II (Perkin Elmer) imaging system by measuring luciferase activity after a tail-vein injection of 150 mg/kg D-luciferin (Thermofisher Scientific). Metastasis development was monitored via bioluminescence using IVIS Lumina II by measuring luciferase activity in the chest region, distant from the primary tumor, for a longer time point (60 sec) as previously described\textsuperscript{25}. Metastasis development was quantified using the Living Image software v4.5.1 (Perkin Elmer) by quantifying photon/s in a region of interest around the chest bioluminescence signal.

For therapy studies, mice underwent bioluminescence imaging on day 3 and day 7 post tumor inoculation to confirm tumor growth. Mice with growing tumors were then randomly assigned to three groups and treated with either scFv\textsubscript{GPIIb/IIa}-MMAE, scFv\textsubscript{mut}-MMAE or left untreated. Treatment was initiated 7 days following tumor inoculation, via intravenous injection of 6 mg/kg body weight of either scFv\textsubscript{GPIIb/IIa}-MMAE or scFv\textsubscript{mut}-MMAE followed by three additional treatments every fourth day. Primary tumor size and metastasis development was monitored twice a week as described above.

\textit{In vivo} fluorescence imaging of scFv\textsubscript{GPIIb/IIa}-Cy7-MMAE

Animals were injected intravenously with 20 µg of scFv\textsubscript{GPIIb/IIa}-Cy7-MMAE or scFv\textsubscript{mut}-Cy7-MMAE. Fluorescence imaging of the ADC in mice was performed 24 hours later using the IVIS Lumina using the following settings (Filter Passband = Excitation 710-760 nm, Emission 810-875 nm). Following imaging, mice were sacrificed, organs perfused to remove circulating blood and reimaged.
Flow cytometry and *ex vivo* fluorescence imaging of scFvGPIIb/IIIa-GFP and GP1bβ

BALB/C nude mice with MDA-MB-231 mammary tumor, or non-tumor bearing mice were injected intravenously with 20 µg of scFvGPIIb/IIIa-GFP or scFvmut-GFP and 20 uL of DyLight 649 anti-GP1bβ. After 24 hours, animals were sacrificed, and tumor, spleen and femurs were extracted.

Tumor samples were fixed in formalin (Sigma Aldrich #HT501128) for 24 hours, paraffin embedded, and microtome sectioned (Leica) to 20 µM onto a glass slide. Sections were deparaffinized, stained with Hoechst® (Thermo Fisher Scientific #33342) and visualized using the Nikon A1r Plus Confocal Microscope using a 60x oil objective.

For flow cytometry, spleen sections were teased apart, disaggregated and filtered through a 100 µm filter into single cell suspensions. Bone marrow cells were extracted from the femurs and filtered through a 100 µm filter into single cell suspensions. Spleen and bone marrow cells were then suspended in 1 mL red cell lysis buffer (0.155 M NH₄Cl, 0.01 M KHCO₃, 0.01 mM EDTA) for 10 min and neutralized with wash buffer (PBS, 2mM EDTA, 0.1% BSA). Cells were stained with mouse CD41-PE (eBioscience) and flow cytometry was performed using a Fortessa scanner (BD Biosciences). Results were analyzed using the Flowlogic software.

**Mouse blood collection and toxicity measurements**

Mouse blood was collected via submandibular bleeds into EDTA-coated microtainer collection tubes (Becton Dickinson) and blood counts were performed using the Sysmex XS-1000i hematologic analyzer (Sysmex Corporation) to determine white blood cells (WBC) and
platelets counts. For liver and kidney function tests, 500 uL of mouse blood was collected in citrate. Plasma was collected following centrifugation of blood at 1000 g for 10 minutes and alkaline phosphatase (ALP), alanine aminotransferase (ALT) and urea levels were measured using the Beckman Coulter SYNCHRON LX20PRO System (Beckman Coulter Diagnostics) by Monash Pathology.

Statistical analyses

All data are reported as mean ± SEM of at least 3 independent assays unless otherwise noted. Statistical analyses were performed using unpaired Student’s T tests for comparison of two groups. Statistical analyses were performed using one-way ANOVA, with Dunnett’s multiple comparisons test for analysis of metastasis volume and two-way ANOVA, with Tukey’s multiple comparisons test for analysis of primary tumor growth volume. A P value of < 0.05 was considered significant.
Results

**ScFvGPIIb/IIIa-Cy7-MMAE binds to activated platelets**

To allow the unique targeting of a potent anti-mitotic agent to activated platelets, we developed a novel antibody-drug-conjugate combining several advanced biotechnological tools and methods. We utilized a unique scFvGPIIb/IIIa, which specifically targets activated platelets, combined with a sortase A based site-directed biological conjugation method to produce a novel ADC, scFvGPIIb/IIIa-MMAE incorporating the highly potent anti-mitotic agent (MMAE). In order to facilitate its conjugation to the scFv and enzymatic release, a MMAE linker was designed with a triglycine (GGG) sequence, a cathepsin B cleavable peptide (Val-Cit) and a para-aminobenzylalcohol (PABA) self-immolative spacer (Figure 1). Each scFv has a sortase A recognition sequence, LPETG, at the C-terminus used for a site-directed, enzymatic coupling to the glycine sequence of GGG-Val-Cit-PAB-MMAE. To enable *in vivo* detection, the scFv-MMAE was labeled with a Cy7 dye by NHS labeling to yield, scFvGPIIb/IIIa-Cy7-MMAE (Figure 1). Analysis of this final construct using SDS page electrophoresis and visualized using the Odyssey reader confirmed successful Cy7 labeling of the scFv which has a molecular weight of 34 kDa (Figure 2A). The scFvGPIIb/IIIa carries a hexahistidine tag downstream of the LPETG sequence, which is cleaved following sortase A reaction, resulting in a final scFvGPIIb/IIIa-Cy7-MMAE product with reduced molecular weight compared to the uncleaved scFvGPIIb/IIIa. MMAE conjugation to the scFv was analyzed via Western blotting using an anti-MMAE antibody and confirmed by the presence of a band at ~33 kDa (Figure 2B). Additionally, flow cytometry experiments demonstrated that specific binding of the scFvGPIIb/IIIa-Cy7-MMAE construct to activated platelets was maintained post conjugation (Figure 2C). The scFvGPIIb/IIIa used in this study binds to only the activated form of platelet-specific GPIIb/IIIa, importantly both in human, as well as in mouse platelets. As a negative
control, we used an scFv<sub>mut</sub>, which we have previously developed with a point mutation, such that it acts as a non-binding control (Figure 2D)<sup>16</sup>.

**Cathepsin B cleaves scFv<sub>GPIIb/IIa</sub>-MMAE and scFv<sub>mut</sub>-MMAE to its potent and cytotoxic form**

Next, we assessed the cytotoxic activity of MMAE on tumor cells in culture after the drug was released from the conjugate by exogenous cathepsin B and confirmed equal dose-dependent killing of the triple-negative breast adenocarcinoma cell line MDA-MB-231 by scFv<sub>GPIIb/IIa</sub>-MMAE and scFv<sub>mut</sub>-MMAE. On MDA-MB-231 cells, the IC<sub>50</sub> of scFv<sub>GPIIb/IIa</sub>–MMAE was 1.98x10<sup>-10</sup> M while the IC<sub>50</sub> of the scFv<sub>mut</sub>–MMAE was 1.78x10<sup>-10</sup> M (Figure 3A). These studies confirmed that our approach of utilizing a sortase A conjugation of MMAE to the scFv allowed for a controlled and equal coupling of MMAE to the scFv and the equivalent cathepsin B-mediated release of cytotoxic MMAE by scFv<sub>GPIIb/IIa</sub> and scFv<sub>mut</sub>.

The incorporation of a cathepsin B cleavable linker enables the activation of an inactive prodrug to an active cytotoxic form by utilizing the abundance of cathepsin B in the tumor microenvironment<sup>26</sup>. Prior to animal studies, *in vitro* studies were performed to demonstrate that the conjugated MMAE is released and maintains cytotoxicity upon exposure to cathepsin B. MDA-MB-231 cells were incubated with either MMAE, GGG-Val-Cit-PAB-MMAE or scFv<sub>GPIIb/IIa</sub>-MMAE in the absence and presence of cathepsin B. The IC<sub>50</sub> killing efficacy of GGG-Val-Cit-PAB-MMAE on MDA-MB-231 tumor cells was 1.86x10<sup>-8</sup> M and the addition of cathepsin B increased the cellular killing efficacy by approximately 40-fold to 4.70x10<sup>-10</sup> M (Figure 3B), which is similar to the IC<sub>50</sub> of the unmodified MMAE. The IC<sub>50</sub> killing efficacy of scFv<sub>GPIIb/IIa</sub>-MMAE was 2.21x10<sup>-8</sup> M and the addition of cathepsin B enhanced the killing
efficacy to $1.96 \times 10^{-10}$ M (Figure 3B). We confirmed an increased cell killing by the GGG-Val-Cit-PAB-MMAE and scFv-MMAE in the presence of cathepsin B, indicating release of MMAE. We also observed a lower level ($\sim 10^8$ M) of non-specific killing of cancer cells with scFv-MMAE and GGG-Val-Cit-PAB-MMAE in the absence of exogenous Cathepsin B. We expect this to be due to the secretion of small amounts of cathepsin B from the cancer cells, or release of other cathepsins and proteases that can also cleave the Val-Cit linker, as previously reported\(^{27-29}\). These findings confirm the cytotoxic potency and the efficacy of the cathepsin B dependent release and activation of the potent MMAE from its inactive form in the newly created scFv\(_{\text{GPIIb/IIIa}}\)-MMAE-conjugate.

To investigate if the therapeutic efficacy of scFv\(_{\text{GPIIb/IIIa}}\)-MMAE is based on its specificity for activated platelets, the scFv\(_{\text{GPIIb/IIIa}}\)-MMAE or scFv\(_{\text{mut}}\)-MMAE was incubated with ADP-stimulated platelets \textit{in vitro}. Platelets were subsequently washed to remove unbound scFv\(_{\text{GPIIb/IIIa}}\)-MMAE or scFv\(_{\text{mut}}\)-MMAE and then added to MDA-MB-231 cells (Figure 3C). Consistent with the specific binding of the targeted ADC to activated GPIIb/IIIa, incubation of scFv\(_{\text{GPIIb/IIIa}}\)-MMAE with activated platelets resulted in increased MDA-MB-231 cell killing, with an IC\(_{50}\) of $5.9 \times 10^{-9}$ M. In contrast, activated platelets incubated with scFv\(_{\text{mut}}\)-MMAE demonstrated no cellular killing. As a control to confirm the equal coupling of MMAE to the scFv\(_{\text{GPIIb/IIIa}}\) and scFv\(_{\text{mut}}\), a mixture of activated platelets and either scFv\(_{\text{GPIIb/IIIa}}\)-MMAE or scFv\(_{\text{mut}}\)-MMAE, which did not undergo washing, were added to MDA-MB-231 tumor cells (Figure 3C). No significant differences in killing were observed between the scFv\(_{\text{GPIIb/IIIa}}\)-MMAE and scFv\(_{\text{mut}}\)-MMAE. The IC\(_{50}\) killing efficacy of scFv\(_{\text{GPIIb/IIIa}}\)-MMAE on MDA-MB-231 was $1.27 \times 10^8$ M and the IC\(_{50}\) killing efficacy of scFv\(_{\text{mut}}\)-MMAE on MDA-MB-231 was $1.38 \times 10^8$ M, thereby confirming equal coupling efficacy of MMAE to both, scFv\(_{\text{GPIIb/IIIa}}\) and scFv\(_{\text{mut}}\).
To examine whether our novel ADC displays efficacy against different tumor types, these experiments were repeated using a human colorectal cell line (HT29), a human fibrosarcoma cell line (HT1080) and a human prostate cancer cell line (PC3). We first demonstrated that MDA-MB-231, HT29, HT1080 and PC3 tumor cells cultured in GGG-Val-Cit PAB-MMAE and scFvGPIIb/IIIa-MMAE, in the presence of cathepsin B were able to induce cellular killing, albeit at slightly varying concentrations (Supp Fig 1A). Next, HT29, HT1080 and PC3 cell lines were cultured in ADP-activated platelets mixed with scFvGPIIb/IIIa-MMAE and scFvmut-MMAE (Supp Fig 1B). These studies confirmed that the scFvGPIIb/IIIa-MMAE bound to activated platelets and induced cell killing of the HT29, HT1080 and PC3 cell lines, thus supporting the notion that this platelet-targeted ADC is active across a broad range of tumor cell types.

In this study, we introduce a novel approach of utilizing Val-Cit-PAB-MMAE, a cathepsin B dependent prodrug, conjugated to an antibody that targets activated platelets as a component of the tumor microenvironment. Previous studies have indicated the abundance of secreted or cell surface-associated cathepsin B within tumor cells\textsuperscript{30,31}. To confirm the suitability of the platelet-targeting approach for therapeutic application, we investigated the presence and localization of cathepsin B within the tumor via immunofluorescence imaging of tumor xenograft sections stained with a cathepsin B antibody. Immunostaining confirmed the presence of cathepsin B within MDA-MB-231 tumor cells and the tumor microenvironment (Figure 3D). To ascertain the cellular localization of cathepsin B in tumor cells, a magnified image was acquired (Figure 3E). This confirms the intracellular and pericellular location (indicated by white arrows) of cathepsin B within tumor cells.
Cancer cells induce platelet activation and can be imaged using scFvGPIIb/IIIa-Cy7-MMAE

The ability of cancer cells to activate platelets and the functionality of the scFvGPIIb/IIIa-MMAE to target activated platelets in the tumor microenvironment was first analyzed in vitro via fluorescence imaging. MDA-MB-231, HT29, HT1080 and PC3 cells were incubated with washed human platelets and stained with anti-CD41 antibody as a platelet marker and scFvGPIIb/IIIa as a platelet activation marker. Strikingly, flow cytometry analysis of the platelet population (as defined by CD41 expression) revealed that incubation of platelets with MDA-MB-231, HT29, HT1080 or PC3 tumor cells resulted in activation of the entire platelet population, to a similar extent as ADP-activated platelets, thus confirming the ability of scFvGPIIb/IIIa to detect tumor cell-induced platelet activation (Figure 4A and 4B, red line). In contrast, scFvmut, as negative control, showed no binding to platelets incubated with cancer cells (Fig 4B, blue line). Furthermore, gating on the cancer cell population confirmed that platelets bind directly to cancer cells, to varying degrees, depending on the cell line (Figure 4A and 4C). Indeed, the percentage of MDA-MB-231, HT29, HT1080 and PC3 cells with adherent platelets were 10%, 15%, 44% and 11%, respectively (Figure 4C). To further illustrate the platelet-cancer cell interactions, we performed immunofluorescence imaging, demonstrating that platelets can bind directly to cancer cells and that cancer cells induce platelet activation, which can be detected by binding of the activation-specific scFvGPIIb/IIIa (Figure 4D).

scFvGPIIb/IIIa-Cy7-MMAE localizes to activated platelets in the tumor microenvironment and sites of metastasis

For in vivo characterization, scFvGPIIb/IIIa-MMAE and scFvmut-MMAE were conjugated to Cy7 and either ADC injected intravenously to mice with MDA-MB-231 primary tumor that had already metastasized. Fluorescence imaging of mice performed 24 hours post injection of scFv-
Cy7-MMAE demonstrated enrichment of scFvGPIIb/IIIa-Cy7-MMAE at the primary tumor region, with no tumor localization of the scFvmut-Cy7-MMAE (Figure 5A). As the MDA-MB-231 cells had been previously transduced with a lentiviral vector to express luciferase, bioluminescence imaging was used to detect areas of primary tumor as shown in Figure 5B. Concordant with in vivo imaging, ex vivo analyses showed significant enrichment of scFvGPIIb/IIIa-Cy7-MMAE but not scFvmut-Cy7-MMAE in the primary tumors (Figure 5C and 5D). Bioluminescence imaging of lung and lymph node metastases was performed by covering the primary tumor site and imaging with a longer exposure time (60 sec) (Figure 5E and F). We further demonstrated that the scFvGPIIb/IIIa-Cy7-MMAE localized in the lungs of MDA-MB-231 tumor-bearing mice with lung metastases (Figure 5E), but not in mice without lung metastases (Figure 5F), confirming that scFvGPIIb/IIIa-Cy7-MMAE localization was specific to activated platelets within tumors. Again, no tumor localization of the scFvmut-Cy7-MMAE was observed in the lungs in the presence or absence of metastases (Figure 5E and F).

**scFvGPIIb/IIIa localizes to activated platelets in the tumor microenvironment but not to resting platelets in the spleen and bone marrow**

To further confirm the selectivity of the targeted ADC for platelets in the tumor microenvironment in vivo, MDA-MB-231 tumor-bearing mice were injected with scFvGPIIb/IIIa-GFP or scFvmut-GFP and a Dylight 649 anti-GP1bβ antibody. This in vivo approach for immunofluorescence was used as the activated form of GPIIb/IIIa undergoes a conformational change and antigen masking upon tissue fixation and is not recognized by the scFvGPIIb/IIIa. After 24 hours, tumors were extracted, sectioned and imaged using fluorescence microscopy to demonstrate the localization of platelets, detected by GP1bβ staining co-localizing with activated platelets, as indicated by scFvGPIIb/IIIa-GFP in the tumor microenvironment (Figure
In contrast, we saw no GFP signal in mice injected with scFv\textsubscript{mut}-GFP (Figure 6B), indicating the specificity of the scFv\textsubscript{GPIIb/IIIa} targeting to activated platelets.

To demonstrate the specificity of the scFv\textsubscript{GPIIb/IIIa} to activated platelets in the tumor microenvironment, we next investigated whether the scFv localized to the spleen and bone marrow, which are two abundant sources of non-activated platelets. BALB/C nude mice were injected with scFv\textsubscript{GPIIb/IIIa}-GFP or scFv\textsubscript{mut}-GFP and the Dylight 649 anti-GP1b\(\beta\) antibody and the femurs and spleen were extracted after 24 hours and stained for CD41. Single cell analysis of the spleen and bone marrow via flow cytometry, gated on CD41-positive cells displayed binding of the GP1b\(\beta\) antibody to the spleen and bone marrow but no scFv\textsubscript{GPIIb/IIIa} binding to these regions (Figure 6C and 6D), thus confirming the specificity of scFv\textsubscript{GPIIb/IIIa} for activated platelets \textit{in vivo}.

**Treatment with scFv\textsubscript{GPIIb/IIIa}-Cy7-MMAE reduces tumor growth and metastasis**

To investigate the \textit{in vivo} effect of the scFv\textsubscript{GPIIb/IIIa}-MMAE therapeutic, MDA-MB-231 metastatic tumor-bearing mice were imaged for bioluminescence signal at day 3 post MDA-MB-231 inoculation, and again at day 7 to confirm the presence of growing tumors (Fig 7A). Mice with established tumors were then divided into three groups and treated with scFv\textsubscript{GPIIb/IIIa}-MMAE or scFv\textsubscript{mut}-MMAE, or left untreated, and the effect of therapy on tumor growth and metastasis formation was assessed. Strikingly, treatment of tumor-bearing mice with scFv\textsubscript{GPIIb/IIIa}-MMAE at a dose of 6 mg/kg (administered every 4 days) resulted in a marked reduction in the primary tumor size by over 70% as compared to mice treated with scFv\textsubscript{mut}-MMAE or mice in the untreated group (Figure 7B). Accordingly, a significant reduction in the development of lung and lymph node metastases on day 23, as measured by total
bioluminescence, was seen in scFvGPIIb/IIIa-MMAE treated mice compared to non-targeted and vehicle controls (Figure 7C). Significantly, treatment with scFvGPIIb/IIIa-MMAE prevented the development of metastasis in 40% of mice (3 out of 7 mice), as indicated by bioluminescence imaging, while all mice in the untreated group or treated with scFvmut-MMAE developed metastases. Importantly, no therapeutic effects were observed with scFvmut-MMAE, thus highlighting the impressive ability of our targeting approach to concentrate therapeutic doses of MMAE specifically within the tumor and to prevent metastases. Consistent with our data demonstrating the high degree of specificity of scFvGPIIb/IIIa-MMAE for activated platelets, we did not observe any off-target side effects in treated mice. Mice were healthy during the course of the treatment, did not lose weight (Supp Fig 2A), and hematological parameters, liver function and renal function were within normal ranges (Supp Fig 2B). Taken together, these findings demonstrate the potential of therapeutic targeting activated platelets as a strategy for the delivery of chemotherapy to maximize potency whilst sparing systemic side effects.
Discussion

In this study we introduce the targeting of activated platelets within the tumor microenvironment as a novel strategy for the treatment of primary tumors and metastatic disease. This approach is based on the development of a unique ADC, which targets tumor-associated platelets in the tumor microenvironment using the activated GPIIb/IIIa as an epitope for localized delivery of MMAE, a highly potent synthetic anti-mitotic agent. Importantly, this novel approach allows the specific targeting and controlled-release of MMAE, via tumor-derived cathepsin B, whilst sparing untoward systemic side effects.

The efficacy of ADC is highly contingent upon the presence and abundance of the antibody target within the tumor, representing a major limitation for the use of ADCs in several cancer types, which do not express a clinically validated specific antigenic molecular target. In this regard, a particular advantage of our approach is the possible ubiquitous nature of activated platelets in a broad range of human tumors, including breast\textsuperscript{32}, colorectal\textsuperscript{33}, lung\textsuperscript{34}, ovarian\textsuperscript{11} and esophageal\textsuperscript{13} tumors, indicating that our platelet-targeting strategy holds promise for a large range of tumors.

Additionally, we show the ability to target the tumor stroma as a novel approach to deliver a pro-drug for the killing of tumor cells. Valine-citrulline (Val-Cit), a cathepsin B cleavable linker requires the presence of cathepsin B for cleavage and release of free MMAE, the active anti-cancer agent. The protease cathepsin B is tightly regulated in healthy tissues. However it is well established that cathepsin B is overexpressed in tumor cells\textsuperscript{30,31}. Moreover, tumor cells as well as cells in the tumor microenvironment, such as stromal fibroblasts and macrophages, can secrete cathepsin B, resulting in the accumulation of cathepsin B within the tumor.
There are opposing views on the role of cathepsin B either to be a promoting factor in cancer, by facilitating tumor progression and metastasis, or to be protective by promoting tumor apoptosis. Nevertheless, there is a general consensus affirming the high abundance of cathepsin B in the tumor microenvironment. Therefore, incorporating a cathepsin B cleavable linker, which allows for the specific release of the potent cytotoxic MMAE at the tumor site, represents a major advantage of our ADC design.

Therapeutic approaches using ADCs, such as MMAE are commonly used to target antigens on the cancer cell surface followed by internalization. However, studies by Dal Corso et al, have demonstrated the functionality of ADCs relying on a cathepsin B-cleavable linker coupled to an antibody with a specificity to non-internalizing targets in the tumor microenvironment. In this study, we further illustrate the tumor-specific cytotoxic potency of scFvGPIIb/IIIa-MMAE, conjugated via a Val-Cit linker targeting activated platelets within the tumor microenvironment. Thus, specific targeting of the tumor microenvironment via a single-chain antibody directed against activated platelets combined with a cathepsin B release mechanism, localizes MMAE-mediated cytotoxicity to tumor cells and potentially other cells types in the tumor stroma. A similar elegant and successful targeting approach for MMAE to the tumor microenvironment was recently reported. The Fc-FcγR interaction was used to deliver an MMAE-conjugated ADC to tumor-associated macrophages resulting in the release of MMAE in the tumor environment. Our approach demonstrates an alternative mechanism for targeting and release. Nevertheless, the outcome of both selective deliveries of MMAE to the tumor and the resulting effective anti-cancer therapy is highly promising using both therapeutic approaches.
Whilst many ADCs have utilized whole antibodies, we chose to use a scFv as an antibody format, as it offers several advantages. The small size allows quick and efficient penetration of the tumor environment. The recombinant nature of design offers flexibility with respect to conjugation with drugs to generate a homogenous ADC, as we have described using a sortase A conjugation method. This production method also allows for easy modification of the scFv and the incorporation of additional tags for the development of theranostic agents. Furthermore, the scFvGPIIb/IIIa possesses two additional advantages for future work aiming for clinical translation: First, the scFvGPIIb/IIIa was developed from a human scFv library, reducing the risk of antigenicity. Moreover, scFvGPIIb/IIIa is cross reactive between mice and humans, and binds with similar affinity to activated platelets from mice and humans.

Our data suggests that this newly designed ADC has the capability to target and efficiently kill a broad range of tumor cells in vitro. Therefore, future studies will be aimed at investigating the efficacy of scFvGPIIb/IIIa-MMAE across different tumor types in vivo. In addition, whilst tumors can display or acquire resistance mechanisms to MMAE, a major advantage of our scFv platform is the ability to conjugate our scFv with different cytotoxic drugs, thus providing a major scope in both the range of tumors which can be targeted, and the cytotoxic payload to be delivered. Whilst we observed no off target toxicity associated with our scFvGPIIb/IIIa-MMAE, it is important to note that mice are approximately 3 to 6 times less sensitive to the toxic effects of MMAE compared to humans. Thus, studies to examine for signs of systemic toxicity in other species, which share a similar MTD to humans, such as rats and cynomolgus monkeys, will be important for clinical translation.
Cancer associated thrombosis in general, and in the context of paraneoplastic thrombocytosis, is one of the major causes of mortality in cancer patients and it is therefore common for cancer patients to be treated with anti-thrombotics\textsuperscript{47}. As such, another important aspect for clinical translation relates to the question of whether the concurrent use of anti-thrombotic drugs impacts on the therapeutic efficacy of our ADC. Indeed, the use of GPIIb/IIIa inhibitors during anti-cancer therapy might reduce the effective binding of the GPIIb/IIIa targeting ADC. However, GPIIb/IIIa inhibitor therapy is only used, albeit infrequently, in the acute setting of percutaneous coronary intervention\textsuperscript{14}. In addition, GPIIb/IIIa inhibitors are associated with a high bleeding risk and would be contraindicated in patients with active cancer\textsuperscript{48}. Nevertheless, cancer patients might receive treatment with aspirin or P\textsubscript{2}Y\textsubscript{12} inhibitors. Although platelet function is partially inhibited with these two drug classes, they do not fully prevent the conformational change of the GPIIb/IIIa receptor\textsuperscript{49}, which exposes the targeting epitope of our ADC. Furthermore, given the multiple mechanisms by which tumors can activate platelets, and the high abundance of the GPIIb/IIIa receptor on platelets, the ADC targeting epitope should still be available in high abundance following aspirin or P\textsubscript{2}Y\textsubscript{12} therapy. Interestingly, anticoagulants such as heparin, have been demonstrated to increase cathepsin B activity\textsuperscript{50}, which could potentiate the effect of our ADC therapy. Overall, we expect that the described activated platelet targeting approach will work in the presence of anti-thrombotic drugs. However, during the clinical development of scFv\textsubscript{GPIIb/IIIa}-MMAE, the issue of concomitant anti-thrombotic therapies clearly requires further investigation.

Moreover, the ability of the scFv\textsubscript{GPIIb/IIIa}-MMAE to act as an imaging agent to detect activated platelets within tumors is an advantage in the context of concurrent treatment modalities that could affect platelet activation, and more generally to identify various tumor types with abundant platelets as a predictor of treatment response. Therefore, as part of developing a
personalized medicine approach, patients could first be imaged with a specific imaging version of scFvGPIIb/IIIa, e.g. that is coupled to a PET/MRI contrast agent, allowing highly sensitive detection of tumors and metastases. Upon confirming the presence of platelet-associated tumors, patients could then be given therapy with scFvGPIIb/IIIa conjugated to the cytotoxic drug. This personalized theranostic approach is a particularly attractive capability of our activated-platelet targeting strategy.

In conclusion, we introduce a novel approach for the safe and effective delivery of anti-cancer therapy within the tumor microenvironment, based on the targeting of activated platelets. We designed and successfully tested a novel antibody-drug-conjugate incorporating a single-chain antibody targeting activated GPIIb/IIIa and an additional selective drug activation/release by cathepsin B, for delivery of the cytotoxic agent, monomethyl auristatin E specifically to the tumor site. Addressing a major clinical need, we provide proof of concept of the therapeutic efficacy of our novel ADC in treating both primary tumor and preventing metastasis using a murine model of the difficult to treat triple negative breast cancer. Moreover, we have previously established the ability of the activated platelet-specific scFv for imaging a broad range of tumors which presents the possibility to integrate this approach for cancer diagnosis and therapy- by utilizing molecular imaging as a means to detect tumor-associated platelets and therefore predict those likely to respond to platelet-targeting therapy. Together, these findings demonstrate the broad potential of our unique activated platelet-targeting approach as a novel theranostic anti-cancer strategy, especially for cancers that do not have specific molecular epitopes suitable for therapeutic targeting.
References


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Author contributions: MLY, JDM, XW, MZ and AW designed and performed the experiments. MLY, JDM, XW, MZ, YCC, AW, CJN, EKS and GAP analyzed the data. MLY, JDM, XW, MZ, PMH, EKS, GAP and KP wrote the manuscript. AMS, EKS, PMH, GAP and KP supervised the project. GAP and KP developed the ADC and conceived the project.

Disclosure of Conflict of Interest: KP is an inventor on patents describing activated platelet-targeting recombinant antibodies. All other authors have declared that no conflict of interest exists.

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**List of abbreviations.**

scFv: single-chain antibodies; ADC: antibody-drug-conjugate; Manna: monomethyl auristatin E; ADP: adenosine diphosphate; GPIIb/IIIa: Glycoprotein IIb/IIIa; IVIS: In vivo imaging system; Mut: Mutant; Cy7: Cyanine 7; AF: Alexa Fluor; NHS: N-hydroxysuccinimide; Val-Cit: Valine-citrulline; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; HRP: horseradish peroxidase; XTT: tetrazolium salt; WBC: white blood cells; ALT: alanine aminotransferase and ALP: alkaline phosphatase
Figure Legends

**Figure 1.** Structure of GGG-Val-Cit-PAB-MMAE and labeling strategy for the generation of scFvGPIIb/IIIa-Cy7-MMAE and cathepsin B drug release. scFvGPIIb/IIIa was conjugated to GGG-Val-Cit-PAB-MMAE using an enzymatic sortase A reaction (I), followed by Cy7 conjugation, via NHS labelling to produce scFvGPIIb/IIIa-Cy7-MMAE (II). In vitro, cathepsin B cleaves the scFvGPIIb/IIIa-Cy7-MMAE at the MMAE Val-Cit linker, releasing the potent MMAE for tumor killing (III).

**Figure 2.** scFvGPIIb/IIIa-Cy7-MMAE characterization and specific binding to activated platelets. (A) Coomassie (red) and near infrared (green) imaging of scFvGPIIb/IIIa-Cy7-MMAE (I), unmodified scFvGPIIb/IIIa (II), scFv_mut-Cy7-MMAE (III) and unmodified scFv_mut (IV). Yellow color indicates the overlap between Coomassie in red and near infrared in green. (B) Western blot of scFvGPIIb/IIIa-Cy7-MMAE (I), unmodified scFvGPIIb/IIIa (II), scFv_mut-Cy7-MMAE (III) and unmodified scFv_mut (IV). (C) Flow cytometry profile showing comparable binding of scFvGPIIb/IIIa (red) and scFvGPIIb/IIIa-Cy7-MMAE (blue) to activated platelets. The scFvGPIIb/IIIa contains a V5 tag, which allows using an anti-V5-FITC secondary antibody for detection. The anti-V5-FITC antibody alone was used as a negative control (grey) (all n=3). (D) Flow cytometry profile of scFvGPIIb/IIIa-Cy7-MMAE and scFv_mut-Cy7-MMAE binding to ADP-activated platelets (red), resting platelets (black), and secondary anti-V5 antibody (grey filled) only.
Figure 3. Conjugation of MMAE to scFvGPIIb/IIIa and scFvmut produces an active and highly potent MMAE in the presence of cathepsin B present in tumor cells and the tumor microenvironment. (A) Cytotoxicity assay of triple negative breast adenocarcinoma MDA-MB-231 cells, cultured in MMAE, GGG-Val-Cit PAB-MMAE and scFvGPIIb/IIIa-MMAE, with (+C) or without (-C) cathepsin B. (B) Cytotoxicity assay of MDA-MB-231 cells that were cultured in scFvGPIIb/IIIa-MMAE versus scFvmut-MMAE in the presence of cathepsin B. (C) Cytotoxicity assay of MDA-MB-231 cells, which were cultured in activated platelets that had been pre-incubated with scFvGPIIb/IIIa-MMAE (■) or scFvmut-MMAE (■), and undergone washing to remove unbound scFv (wash) in the absence of exogenous cathepsin B. As a control, MDA-MB-231 cells were cultured in activated platelets that had been pre-incubated with scFvGPIIb/IIIa-MMAE (▼) or scFvmut-MMAE (▼), which did not undergo a washing step to remove unbound scFv. (D) MDA-MB-231 tumor sections were stained with an anti-cathepsin B antibody (green) and anti-sodium/potassium ATPase antibody (red) and counterstained with Hoechst® nucleic acid stain (blue). Imaging at 20x, demonstrating the abundance of cathepsin B (green) within the tumor microenvironment (left panel). Secondary antibody staining of the sections showed no fluorescence signal (right panel). (E) Further 4x magnification of the anti-cathepsin B antibody stained MDA-MB-231 tumor sections demonstrating the abundance of cathepsin B (green) within the tumor. Cathepsin B is localized pericellular (indicated by white arrows) and intracellular in perinuclear granules.

Figure 4. Tumor cells induce platelet activation. (A) Washed platelet rich plasma was added to MDA-MB-231, HT29, HT1080 and PC3 tumor cells and stained with a PE-conjugated anti-CD41 antibody and scFvGPIIb/IIIa or scFvmut, detected with an anti-V5-FITC antibody. Flow cytometry dot plots represent the gating strategy differentiating regions of cancer cells and platelets. The cancer cell region was further gated to select for CD41-positive cancer cells.
(CD41+ve). (B) Analysis of the CD41-positive region demonstrated that 100% of platelets were activated upon incubation with cancer cells for 6 hours, determined by positive scFvGPIIb/IIIa binding (red), which was equivalent to scFvGPIIb/IIIa binding to ADP-activated platelets (green). As a negative control, platelets incubated with cancer cells were also stained with scFvmut (blue) and displayed negative binding. (C) Analysis of the region of cancer cells positive for CD41 (red), confirming that platelets can bind directly to cancer cells. (D) Fluorescence imaging (20x) demonstrated that MDA-MB-231 cells bind directly to platelets (red) and induces platelet activation as shown by positive scFvGPIIb/IIIa staining (green) and negative scFvmut staining.

Figure 5. Activated platelets are present in the tumor microenvironment and can be detected and imaged using the scFvGPIIb/IIIa. (A) In vivo fluorescence imaging of MDA-MB-231 tumor-bearing mice, injected with scFvGPIIb/IIIa-Cy7-MMAE and scFvmut-Cy7-MMAE. (B) In vivo bioluminescence imaging of MDA-MB-231 tumor-bearing mice confirming primary tumor localization in the mammary gland. (C) Ex vivo biodistribution of the primary tumor of MDA-MB-231 tumor-bearing mice injected with scFvGPIIb/IIIa-Cy7-MMAE and scFvmut-Cy7-MMAE. (D) Quantification of fluorescence signal of MDA-MB-231 tumor-bearing mice, injected with scFvGPIIb/IIIa-Cy7-MMAE and scFvmut-Cy7-MMAE (all n=3). (E) Representative bioluminescence in vivo lung imaging of MDA-MB-231 tumor-bearing mice demonstrated the presence of lung metastases (left panel) and representative ex vivo fluorescence imaging of the lung (right panel) demonstrating positive scFvGPIIb/IIIa-Cy7-MMAE signal but negative scFvmut-Cy7-MMAE. (F) Representative in vivo lung imaging of MDA-MB-231 tumor-bearing mice with no lung metastasis (left panel) and representative ex vivo fluorescence imaging, demonstrating negative scFvGPIIb/IIIa-Cy7-MMAE and scFvmut-Cy7-MMAE signal (right panel).
Figure 6. Activated platelets are present in the tumor microenvironment but absent in the spleen and bone marrow. (A, B) Immunofluorescence imaging of tumor sections of MDA-MB-231 tumor-bearing mice injected with DyLight 649 anti-GP1bβ (red) and scFvGPIIb/IIa-GFP or scFvmut-GFP (green), counterstained with Hoechst® (blue). (A) Immunofluorescence imaging (20x) demonstrated the abundance of platelets within the tumor microenvironment (red), and the specificity of the platelet binding of scFvGPIIb/IIa to tumor-associated platelets in vivo (green). (B) In contrast, the scFvmut does not bind platelets in vivo. (C, D) Flow cytometry of the spleen and bone marrow of BALB/C nude mice injected with PBS (control) or DyLight 649 anti-GP1bβ and scFvGPIIb/IIa-GFP or scFvmut-GFP. (C) Flow cytometry of spleen cells, which were co-stained for CD41 and gated on the CD41-positive region, demonstrated the presence of platelets in the spleen via DyLight 649 anti-GP1bβ staining, but the absence of activated platelets, demonstrated by the absence of scFvGPIIb/IIa-GFP staining. (D) Flow cytometry of bone marrow cells from the femur, co-stained for CD41 and gated on the CD41-positive region, demonstrates the presence of platelets in the bone marrow via DyLight 649 anti-GP1bβ staining, but the absence of activated platelets, demonstrated by the absence of scFvGPIIb/IIa-GFP staining.

Figure 7. scFvGPIIb/IIa-MMAE treatment inhibits tumor growth and metastasis development in a murine model of triple negative breast cancer. Bioluminescence imaging and signal quantification of MDA-MB-231 tumor-bearing mice on day 3 and day 7 post tumor inoculation, which were then randomly assigned to scFvGPIIb/IIa-MMAE, scFvmut-MMAE or untreated groups (B) Primary tumor volume of MDA-MB-231 tumor-bearing mice treated with 6 mg/kg of scFvGPIIb/IIa-MMAE ( ■ ) (n=7), scFvmut-MMAE ( ● ) (n=6) or untreated ( ▲ ) (n=6). (C) Metastasis burden of MDA-MB-231 tumor-bearing mice treated with 6 mg/kg of scFvGPIIb/IIa-MMAE ( ■ ) (n=7), scFvmut-MMAE ( ● ) (n=6) or untreated ( ▲ ) (n=6), measured
bioluminescence imaging, 60 sec exposure, and representative images of lung and lymph node metastasis from each group. ****P<0.0001 between untreated and scFvGPIIb/IIIa-MMAE and ###P<0.0001 between scFvmut-MMAE and scFvGPIIb/IIIa-MMAE analyzed by two-way ANOVA with Tukey’s multiple comparison’s test. *P<0.05 analyzed by one-way ANOVA with Dunnetts’s multiple comparison’s test.
Figure 1.
Figure 2.
Figure 3.
Figure 4.

A. Cancer Cells

B. Platelets - CD41+ve

C. Cancer cells

D. CD41

- scFv_{premix} (Platelets + Cancer cells)
- scFv_{mix} (Platelets + Cancer cells)
- scFv_{active} (Activated Platelets + Cancer cells)
Figure 5.
Figure 7.