

Enhancement of antibody-dependent cell-mediated cytotoxicity by endowing IgG with Fc α RI (CD89) binding

M Jack Borrok¹, Nadia M Luheshi², Nurten Beyaz¹, Gareth C Davies², James W Legg², Herren Wu¹, William F Dall'Acqua¹, and Ping Tsui^{1,*}

¹Antibody Discovery and Protein Engineering; Medimmune Ltd.; Gaithersburg, MD USA; ²Oncology; Medimmune Ltd.; Cambridge, UK

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Abbreviations: IgG, immunoglobulin G; IgA, immunoglobulin A; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; CDC, complement dependent cytotoxicity; TAA, tumor associated antigens; FcRn, neonatal Fc receptor; Fc γ R, Fc gamma receptor; HER2, human epithelial receptor two; SPR, surface plasmon resonance; PMN, polymorphonuclear; NK, natural killer; PBMC, peripheral blood mononuclear cell; E:T ratio, effector to target ratio; FCM, flow cytometry; LDH, lactate dehydrogenase; M Φ , macrophage; DSC, differential scanning calorimetry; PK, pharmacokinetics; AUC, area under the curve; CL, clearance rate; T $\frac{1}{2}$, half-life; V_{ss}, central compartment volume of distribution; C_{max}, maximum serum concentration

Fc effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP) are crucial to the efficacy of many antibody therapeutics. In addition to IgG, antibodies of the IgA isotype can also promote cell killing through engagement of myeloid lineage cells via interactions between the IgA-Fc and Fc α RI (CD89). Herein, we describe a unique, tandem IgG1/IgA2 antibody format in the context of a trastuzumab variable domain that exhibits enhanced ADCC and ADCP capabilities. The IgG1/IgA2 tandem Fc format retains IgG1 Fc γ R binding as well as FcRn-mediated serum persistence, yet is augmented with myeloid cell-mediated effector functions via Fc α RI/IgA Fc interactions. In this work, we demonstrate anti-human epidermal growth factor receptor-2 antibodies with the unique tandem IgG1/IgA2 Fc can better recruit and engage cytotoxic polymorphonuclear (PMN) cells than either the parental IgG1 or IgA2. Pharmacokinetics of IgG1/IgA2 in BALB/c mice are similar to the parental IgG, and far surpass the poor serum persistence of IgA2. The IgG1/IgA2 format is expressed at similar levels and with similar thermal stability to IgG1, and can be purified via standard protein A chromatography. The tandem IgG1/IgA2 format could potentially augment IgG-based immunotherapeutics with enhanced PMN-mediated cytotoxicity while avoiding many of the problems associated with developing IgAs.

Introduction

Many of the current monoclonal antibody based immunotherapeutics depend on the recruitment and activation of immune effector cells for efficacy.¹ These antibody immunotherapeutics (exclusively of the IgG isotype) utilize Fc-mediated mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP) to kill cancer cells via targeting tumor-associated antigens (TAAs)² or immunosuppressive cells via targeting cell surface receptors such as OX40.^{3,4}

Considerable effort has been put into technologies that can enhance antibody effector functions. The main technologies used to improve target cell killing focus on improving the affinity between IgG1-Fc and Fc γ RIIIa, expressed on natural killer (NK) cells. Afucosylation of the N297 Fc glycan is a well-known

method for enhancing NK-mediated ADCC.^{5,6} Concurrently, IgG variants have also been generated to enhance ADCP and complement-dependent cytotoxicity (CDC) by increasing the Fc domain's affinity to Fc γ RIIa or C1q respectively.⁷⁻⁹ These technologies are similar in that they attempt to enhance existing interactions between the IgG-Fc and immune receptors rather than introduce novel effector functions to IgG.

Serum IgA, the second most abundant immunoglobulin present in blood, can effectively engage polymorphonuclear (PMN) cells via the interaction with the Fc α RI receptor (CD89).¹⁰ Ligation of Fc α RI by IgA-containing immune complexes can trigger ADCC by neutrophils, degranulation of eosinophils, and phagocytosis by monocytes, and macrophages (M Φ).¹¹ Other myeloid-derived cells such as mast cells and basophils do not express CD89.¹²⁻¹⁴ Fc α RI engagement has long been proposed as a way to enhance the well-recognized anti-tumor ability of

*Correspondence to: Ping Tsui; Email: tsui@medimmune.com

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Table 1. Expression, antigen binding, and thermal stability of analyzed antibodies

	Expression (mg/L)	Monomer content [#] after affinity column	Her2 binding EC ₅₀ (nM)	Lowest T _m (°C)	Fab T _m (°C)
Her2-IgG1	180	97.6%	2.4	72.1	82.5
Her2-IgG1/IgA2	195	91.0%	1.5	72.4	82.0
Her2-IgA2	N.D.*	91.1%	2.1	73**	82.3

#Determined by size-exclusion chromatography.

*Her2-IgA2 expression levels were determined to be ≥ 50 mg/L by quantification from Peptide M agarose resin, but exact expression was not quantified.

**Approximate value, determined by deconvolution of Fab T_m shoulder (see Fig. S1).

(221–229) serving as a linker between the 2 Fc domains (Fig. S1A). To prevent dimerization, the cysteine tailpiece of IgA was not included in either the Her2-IgG1/IgA2 or Her2-IgA2 construct by introducing a stop codon after K441 in the IgA2 Fc. For Her2-IgA2, the same IgA2-Fc and hinge replaces the IgG1-Fc domain and hinge region. Expression levels for Her2-IgG1/IgA2 transiently expressed in HEK 293FT cells were comparable to that of the IgG parent at ~ 200 mg/L (Table 1). Her2-IgG1/IgA2 and Her2-IgA2 retained binding to the Her2/neu antigen similarly to that of the parental IgG1 antibody (Fig. S1B, Table 1), and differential scanning calorimetry (DSC) analysis confirmed that addition or replacement with the IgA2-Fc had no negative impact on overall thermal stability (Fig. S1C, Table 1). Her2-IgG1/IgA2 monomer content (91.1%) determined by SEC after protein A purification was lower than Her2-IgG1 (97.6%) and similar to Her2-IgA2 (91.0%) (Fig. S1, Table 1).

Receptor binding to antibody variants

Surface plasmon resonance (SPR) was used to measure the affinity for various Fc γ receptors, Fc α RI, C1q and both human and murine FcRn to Her2-IgG1, Her2-IgA2, Her2-IgG1/IgA2 and Her2-IgG1-afuc (lacking fucosylation at the N297 glycan) (Table 2). Her2-IgG1 and Her2-IgG1/IgA2 binding to human and murine FcRn, C1q, Fc γ RI, Fc γ RIIa, Fc γ RIIb, and Fc γ RIIIa (158V and 158F allotypes) were similar to what has been previously reported for IgG antibodies.²⁹ Her2-IgG1-afuc showed improved Fc γ RIIIa binding as expected. Neither Her2-IgG1 nor Her2-IgG1-afuc had any detectable binding to Fc α RI, and Her2-IgA2 had no detectable binding to any of the Fc γ receptors, C1q or FcRn. Her2-IgA2 bound to soluble human Fc α RI with an affinity of 122 nM. Her2-IgG1/IgA2 bound to Fc α RI at an affinity similar to that of Her2-IgA2 while maintaining Fc γ Rs and C1q binding characteristics similarly to that of Her2-IgG1 (Table 2).

ADCC activity of Her2-IgG1/IgA2 with different effector populations

We evaluated the ability of Her2-IgG1/IgA2 to promote ADCC using different effector cell populations. To assess Fc γ RIIIa-mediated cytotoxicity, the NK-derived cell line KC1333 stably expressing Fc γ RIIIa was used as effector cells (Fig. 2A). Her2-IgG1 and Her2-IgG1/IgA2 both induced BT474 target cell killing, indicating NK-mediated ADCC activity is retained in Her2-IgG1/IgA2. Her2-IgG1-afuc displayed superior cytotoxicity in this assay as expected. Her2-IgA2 and a non-Her2 binding control IgG did not induce NK-mediated ADCC (Fig. 2A).

ADCC was then evaluated using freshly isolated PMN cells (consisting of $\sim 85\%$ neutrophils) as effector cells (Fig. 2B). At an E:T ratio of 20:1, both Her2-IgG1/IgA2 and Her2-IgA2 effectively promoted ADCC. Neither Her2-IgG1 nor Her2-IgG1-afuc elicited ADCC under these conditions. ADCC was further interrogated using combined, freshly isolated PMN and peripheral blood mononuclear cells (PBMCs), reconstituting an effector population representative of immune cell ratios found in blood. This effector cell population contains multiple cell types capable of eliciting cytotoxicity, including neutrophils and monocytes from the PMN fraction and NK cells present in the PBMC fraction. The two IgA2-containing constructs, Her2-IgA2 and Her2-IgG1/IgA2, elicited effective cytotoxicity from this effector cell population (Fig. 2C). Her2-IgG1-afuc, despite having enhanced NK-mediated ADCC, elicited much lower cytotoxicity than either 2 IgA2-Fc-containing antibodies, whereas Her2-IgG1 elicited very low levels of ADCC at this effector ratio with this combined population (Fig. 2C).

The PMN-mediated cytotoxicity by IgA2-Fc-containing antibodies was visualized using fluorescence microscopy at early (3 hr) and late (18 hr) time points (Fig. 3A). Wells incubated with either control IgG or Her2-IgG1 did not show PMN adherence to BT474 cells at either time point. Additionally, live

Table 2. Fc γ R, FcRn, C1q and Fc α RI binding of antibodies determined by surface plasmon resonance

	K _D values (nM)								
	Fc α RI	Fc γ RI	Fc γ RIIIa 158F	Fc γ RIIIa 158V	Fc γ RIIa	Fc γ RIIb	hFcRn	mFcRn	C1q
Her2-IgG1	N.B.	6.05	1,770	409	638	4,980	1,210	97.5	222
Her2-IgG1/IgA2	128	7.61	1,810	401	771	5,900	1,790	140	677
Her2-IgA2	122	N.B.	N.B.	N.B.	N.B.	N.B.	N.B.	N.B.	N.B.
Her2-IgG1-afuc	N.B.	6.27	312	132	1,220	4,240	1,250	97.9	266

N.B. indicates no detectable binding.

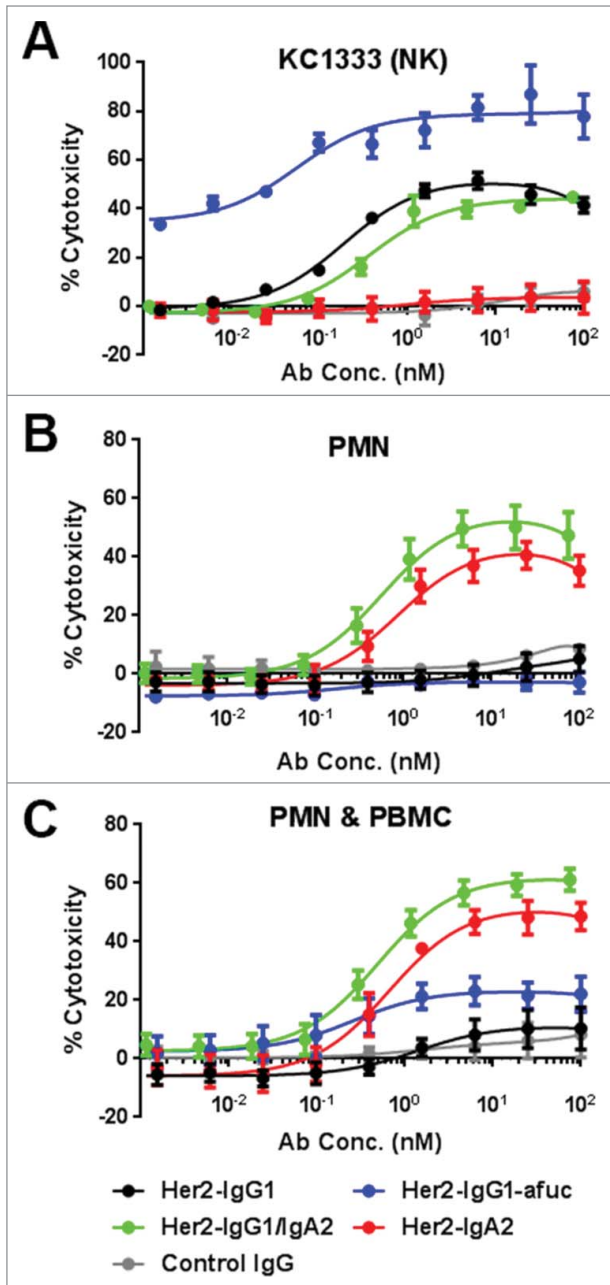


Figure 2. ADCC with different effector cell types. ADCC results are shown using BT474 cells as targets and either NK cell line KC1333 (A), the freshly isolated PMN cells (B) or both the PMN and PBMC cell fractions combined (C). For PMN and PMN plus PBMC experiments, data are the average of 5 different experiments with 5 separate donors. KC1333 data is combined from 3 separate experiments. E:T ratios were 2.5:1 when KC1333 cells were used as effector cells, and 20:1 with PMN and PMN plus PBMC effector cells.

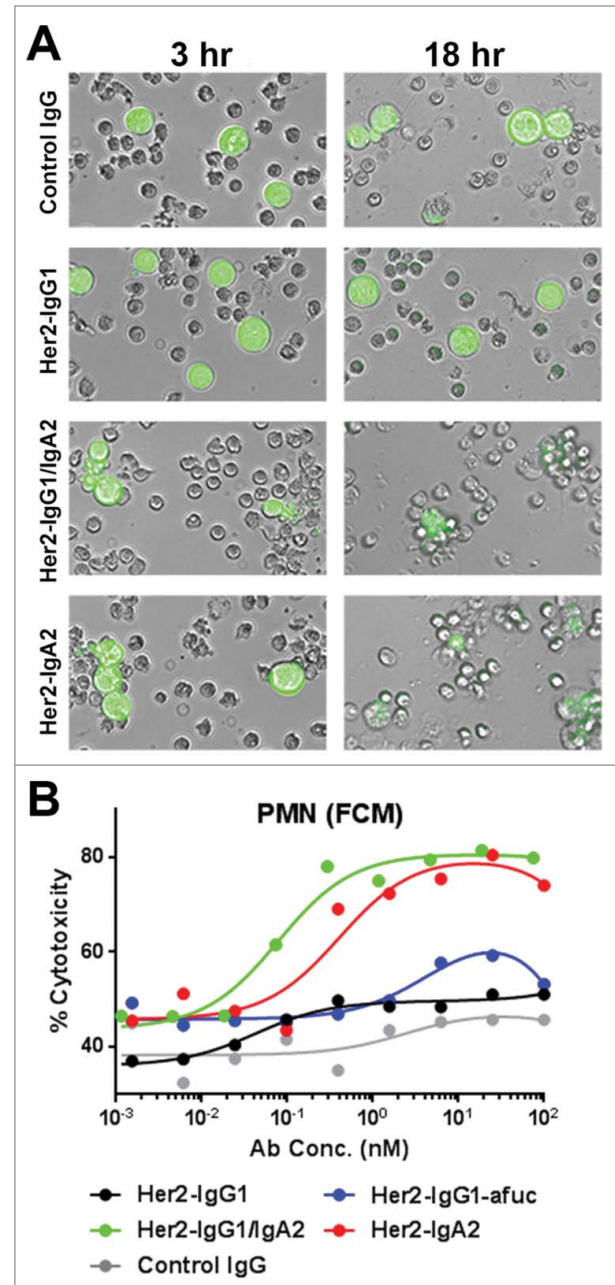


Figure 3. Visualizing PMN-mediated ADCC of mAb variants via microscopy and FCM. (A) Freshly isolated PMN cells (unlabeled) were incubated with Her2⁺⁺⁺ BT474 cells (green) at an E:T ratio of 10:1 with either IgG isotype control, Her2-IgG1, Her2-IgG1/IgA2 or Her2-IgA2 at a concentration of 6 nM and visualized via fluorescence microscopy. PMN mediated ADCC (B) was assessed using an FCM based 3 color assay to measure target cell killing. An E:T ratio of 10:1 PMN:BT474 cells was used. Data are representative from one donor of 3 tested.

BT474 cells could be observed at 18 hours. In contrast, at 3 hours PMN cells could be seen clumping near BT474 cells in both Her2-IgG1/IgA2 and Her2-IgA2-containing wells (Fig. 3A). Few intact BT474 cells could be visualized in these fields containing Her2-IgA2 or Her2-IgG1/IgA2 at the 18 hour time point. Those that were discernable were usually surrounded

by multiple neutrophils and staining appeared weak, suggesting on-going cell lysis.

To further evaluate and confirm the PMN-mediated cytotoxicity of Her2-IgG1/IgA2, a 3-color flow cytometry (FCM) assay was employed. PMN cells (labeled with Calcein AM Violet) and BT474 cells (labeled with Calcein AM) were incubated with

antibodies at an E:T ratio of 10:1 for 18 hrs. Afterwards, cells were stained with a near-IR live/dead stain to assess membrane integrity. Populations of PMN and BT474 cells were differentiated by their fluorescent label and the percent cytotoxicity of the target cell population was determined (Fig. 3B). The results mirrored those of the PMN-mediated ADCC assays using LDH release as the read out. Again, only the IgA2-Fc-containing formats, Her2-IgA2 and Her2-IgG1/IgA2, had improved cytotoxicity compared to Her2-IgG1 or Her2-IgG1-afuc (Fig. 3B).

Macrophage mediated ADCP of antibody variants

Macrophage are an additional myeloid-derived cell population expressing both FcγRs and FcαRI capable of tumor cell killing, mainly by ADCP.³⁰ We evaluated the ability of our antibodies to facilitate ADCP using monocyte derived MΦ by FCM. MΦ were labeled with celltrace violet and target cells were labeled with CFSE. In the presence of antibodies to the target

cells, phagocytosis could be observed as an increase in the number of double positive cells (Fig. 4A). Phagocytosis of both a Her2+++ (SK-BR-3) and a lower expressing Her2++ cell line (MDA-MB-453) were assessed with the panel of anti-Her2 antibodies (Fig. 4B). SK-BR-3 and MDA-MB-453 target cells were both phagocytosed in the presence of all anti-Her2 antibodies tested. Phagocytosis of the Her2-IgG1/IgA2 was slightly improved compared to Her2-IgG1 or Her2-IgG1-afuc in both cell lines, and was further improved compared to Her2-IgA2.

Pharmacokinetics of antibody variants in Balb/C mice

The serum persistence of Her2-IgG1/IgA2 was determined in mice. As Her2-IgG1/IgA2 contains Fcs from 2 different isotypes, we sought to confirm that the antibody could still be recycled efficiently by FcRn, resulting in IgG1-like serum persistence. Serum concentration-time profiles of Her2-IgG1, Her2-IgA2, and Her2-IgG1/IgA2 in Balb/C mice are shown in Figure 5, and

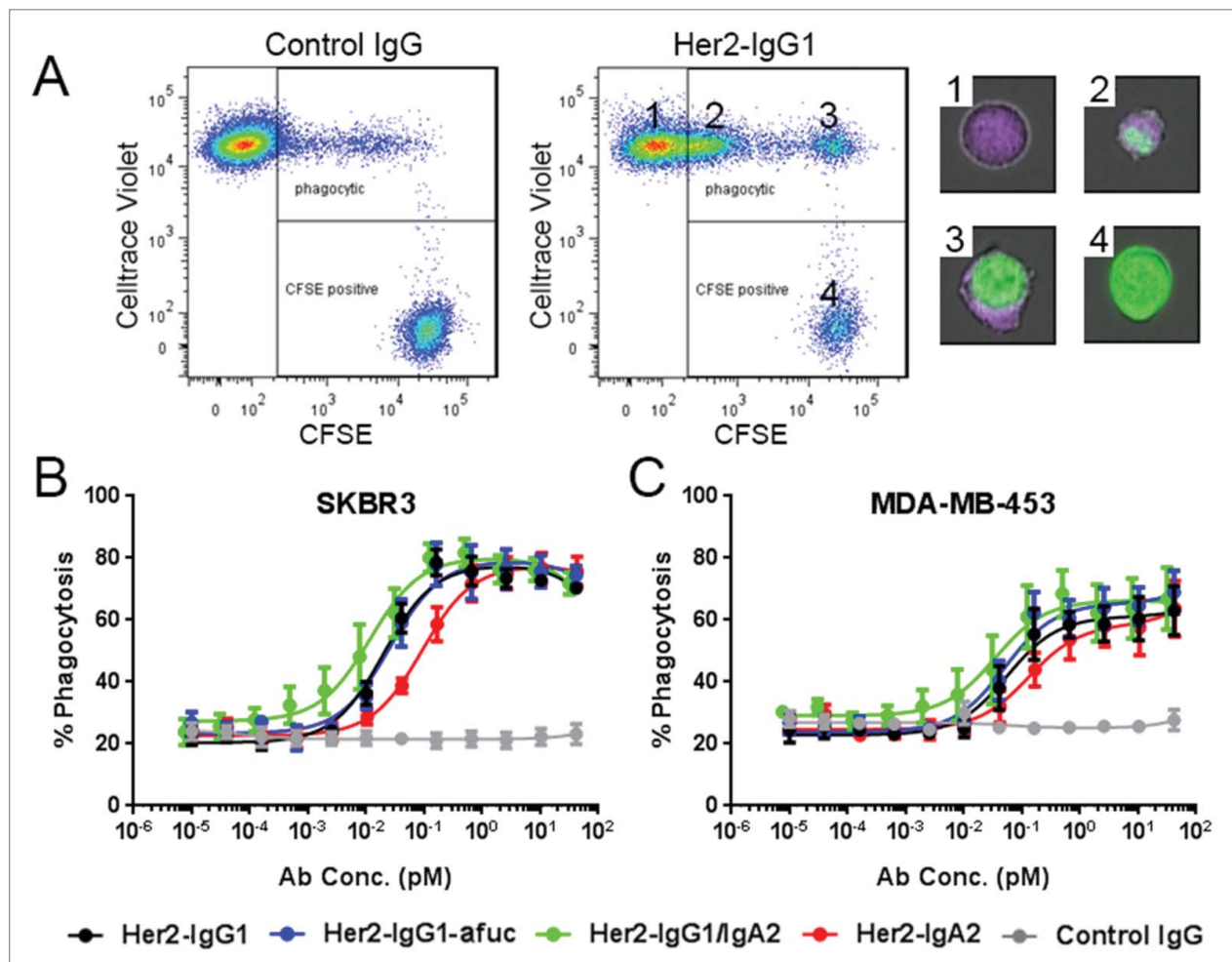


Figure 4. ADCP of SK-BR-3 and MDA-MB-453 cells with mAb variants. Human monocyte-derived MΦ were labeled with celltrace violet and co-cultured with CFSE-labeled Her2 expressing cells at an 8:1 effector: target ratio for 2 h in the presence of the indicated antibodies. Cells were collected and stained with live dead far red, and analyzed by FCM. Dot plots (A) are of live cells in SK-BR-3 cell / MΦ co-cultures in the presence of 650pM IgG isotype control or Her2-IgG1. Images of MΦ (purple), SK-BR-3 (green) and double positive cells from the indicated regions of the dot plot were captured using an imaging cytometer. Phagocytosis of SK-BR-3 cells (B) and MDA-MB-453 cells (C) was quantified as the percent double positive cells of CFSE positive cells. Data are mean ± SEM. of experiments performed with MΦ from $n \geq 4$ independent donors.

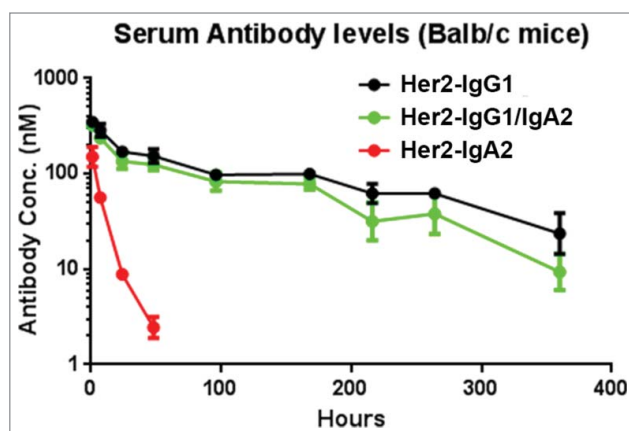


Figure 5. Pharmacokinetic profiles of mAb variants in BALB/c mice. Serum levels for Her2-IgG1 (black), Her2-IgG1/IgA2 (green), and Her2-IgA2 (red) in BALB/c mice were quantified by ELISA detecting human Fab. Mice were injected with a single IV bolus dose of 2.5 mg/kg. Each data point represents the average serum concentration of the mAb in 4 mice for up to 14 days. Error bars represent standard deviation. The data was fit to a 2 compartment model for the calculation of pharmacokinetic parameters (Table 3).

the relevant pharmacokinetic (PK) parameters were summarized in Table 3. Her2-IgG1 has a half-life of 5.2 days whereas Her2-IgA2 exhibits rapid clearance in comparison (half-life of 9 hrs). Her2-IgG1/IgA2 exhibits clearance at a rate similar to that of the parental IgG1 molecule, suggesting that FcRn recycling is unimpaired in the tandem IgG1/IgA2-Fc format.

Discussion

The potential clinical benefits of utilizing the IgA isotype as a cancer immunotherapeutic to engage PMN cells have been recognized for some time.¹⁰ Nevertheless, multiple obstacles have slowed validation of this approach. Foremost, the dissimilarity between IgA systems in human and rodents and the lack of Fc α RI in mice have slowed the *in vivo* investigation of therapeutic IgAs in oncology until recently.²³ Additionally, IgA immunotherapies face challenges related to the relatively complex structure of IgA and the shorter serum half-life compared to IgG.

We developed the tandem IgG1/IgA2-Fc format to augment IgG with an improved ability to engage abundant cytotoxic PMN cells in addition to NK-cells. By linking IgG and IgA Fcs in tandem, binding to IgG Fc γ receptors, C1q, FcRn, and

Protein A is retained while Fc α RI binding is endowed to the antibody. We demonstrated, using the Her2-IgG1/IgA2 as a test case, that this antibody format has IgG1-like expression levels and comparable thermal stability (Table 1, Fig. S1). Her2-IgG1/IgA2 soluble aggregate levels were higher than Her2-IgG1 (Table 1, Fig. S1), however, we expect that monomeric content could be improved upon optimization of elution and neutralization conditions.

A critical advantage of the tandem IgG1/IgA2 format is its improved developability compared to other IgA isotypes. IgA2-Fc was utilized rather than IgA1 because IgA1 has a long, protease-susceptible, heavily O-glycosylated hinge region³¹ that would likely pose problems for downstream development. Additionally, IgA2 has been shown to have a more potent ADCC response than IgA1.^{19,32} Notably, by fusing the Fc of IgA2(m1) to the C-terminus of IgG1-Fc, we eliminate the reported instability of heavy and light chain pairing observed for the IgA2 allotype.²² The IgA tailpiece was deleted in IgG1/IgA2 to avoid undesirable intermolecular interactions or protein aggregation. Another advantage of the IgG1/IgA2 tandem Fc format is its modularity. The preserved binding to Fc γ receptors in the tandem format (Table 2) suggests that IgG1/IgA2-Fc is likely compatible with IgG-Fc modifications³³ that have been shown to improve NK-mediated ADCC in the IgG-Fc.

Our work shows that IgG1/IgA2-Fc antibody can have superior ADCC or ADCP activities over either parental (IgG1 or IgA2) formats (Figs. 2–4). This format compares favorably to other engineered hybrid Fc approaches that do not retain the full effector receptor binding repertoire of both IgG1 and IgA or lack of FcRn mediated long serum persistence.^{26–28} The IgG1/IgA2 tandem Fc also retains C1q binding (Table 2), and exhibits CDC activity with an anti-CD20 IgG1/IgA2 antibody against CD20 expressing cell lines (data not shown). Future work using relevant *in vivo* models (such as human CD89 transgenic mice) will ultimately be necessary to validate IgG1/IgA2 (and other IgA based approaches) as viable cancer immunotherapies. As different types of malignancies will vary in both susceptibility to CD89 mediated killing by myeloid cells and levels of PMN infiltrate within tumor tissues, it will be important to match immunotherapies targeting CD89 with tumors sensitive to such therapy.

IgG1/IgA2-Fc not only has the ability to effectively engage multiple cell types, it can likely engage both Fc γ and Fc α receptors on individual cells co-expressing those receptors. One concern when engaging both receptor classes on an individual PMN cell is that the levels of intracellular FcR γ chain (used for signaling by both Fc γ RI and Fc α RI receptors) could be a limiting

Table 3. Pharmacokinetic parameters for antibody variants in Balb/c mice

	CL (mL/day/kg)	T _{1/2} (days)	V _{ss} (ml/kg)	AUC _{inf} (day*ug/ml)	C _{max} (ug/ml)
Her2-IgG1	10.7 ± 1.7	5.2 ± 1.6	76 ± 15	239 ± 34	52.4 ± 5.2
Her2-IgG1/IgA2	11.9 ± 2.5	4.0 ± 1.3	67 ± 8.7	219 ± 56	60.4 ± 8.7
Her2-IgA2	308 ± 52	0.38 ± 0.027	134 ± 30	8.29 ± 1.3	22.7 ± 6.0

Parameters are estimated from mean data calculated from 4 mice at each time point per group. AUC stands for area under curve; CL, clearance rate; T_{1/2}, half-life; V_{ss}, central compartment volume of distribution.

factor. A previously study by van Egmond et al.³⁴ has shown that with dual engagement of these receptors, the common signaling FcR γ chain used by the Fc γ RI, Fc γ RII and Fc α RI does not limit the activities of the respective receptors on PMN cells. Indeed, simultaneous engagement of these receptors on PMN cells was shown to lead to enhanced ADCC beyond that seen with individual engagement.³⁴ This may explain the small but consistently observed increases in PMN-mediated ADCC with Her2-IgG1/IgA2 compared with Her2-IgA2 (Fig. 2). Such improved ADCC activity is particularly significant when compared with Her2-IgG1, which had only limited activity when both PBMC and PMN are used as effector cells, despite Her2-IgG1 being able to engage Fc γ RIIIa on neutrophils. The low level of ADCC induced by Her2-IgG1 in this case is likely due to the small number of NK cells present, and would probably rise if effector cell concentrations were increased.

In addition to their potential in cancer immunotherapy, IgA antibodies are an important isotype for fighting infectious diseases. Serum IgA has been shown to play a key role in clearance of pathogens via Fc α RI expressing Kupffer cells in the liver or PMN cells in lung.^{35,36} It has recently been demonstrated that passive transfer of human IgA mAbs against a *Mycobacterium tuberculosis* antigen protects human CD89 transgenic mice (but not CD89-negative littermates) from infection.³⁷ Additionally, IgA can clear bacteria by neutrophil NETosis, i.e., the formation of an extracellular fibril/chromatin matrix by neutrophils.³⁸ In light of these studies, we foresee that the IgG1/IgA2 format described in this work could improve the efficacy of antibody-based immunotherapy against infectious disease in addition to cancer immunotherapy.

Methods

Antibody expression and purification

All constructs (except Her2-IgG1-afuc) were transiently expressed in HEK 293FT cells using 293fectinTM and FreestyleTM medium (Life Technologies). The culture medium was collected 10 days after transfection. IgG- and IgG1/IgA2-Fc antibodies were purified by standard protein A affinity chromatography. Her2-IgA2 was purified via Peptide M agarose resin (Invivogen). Monomer content after purification was determined by analytical size-exclusion chromatography. Preparative size-exclusion chromatography was used to remove soluble aggregate from Her2-IgG1/IgA2 and Her2-IgA2 and to >98% monomeric protein. The afucosylated antibody Her2-IgG1-afuc was generated by expressing Her2-IgG1 in a fucosyltransferase-deficient producer Chinese hamster ovary cell line (BioWa Potelligent Technology) and purified via standard Protein A chromatography.

Her2 ELISA

Human HER2/ErbB2 extracellular domain (Sino Biological) was coated on 96-well maxisorp plates (Nunc) overnight at 4°C (2 μ g/ml in phosphate-buffered saline (PBS)). Plates were then blocked with 2% BSA in PBS for 2 hours at room temperature. After washing, titrated antibodies were added and allowed to

incubate for 1 hour. After three washes in PBS, peroxidase-conjugated anti-Human Fd (Southern Biotech) was used for detection. The wells were washed 3 times, and TMB substrate solution was added. The reaction was stopped with 2 N H₂SO₄ and the absorbance at 450 nm was measured.

Differential scanning calorimetry

A Microcal VP-DSC scanning microcalorimeter (Microcal) was used for all DSC experiments. All solutions and samples used for DSC were filtered using a 0.22 μ m filter prior to loading into the calorimeter. Samples were analyzed in PBS with PBS serving as the reference buffer for the DSC experiments. Prior to sample measurement, baseline measurements (buffer-versus-buffer) were obtained to be subtracted from the sample measurement. Dialyzed samples (at a concentration of 1 mg/mL) were added to the sample well and DSC measurements were performed at a 1°C/min scan rate. Data analysis was carried out using the OriginTM DSC software provided by Microcal.

Surface plasmon resonance measurements

The binding of Fc α RI (Sino biological), Fc γ Rs, C1q (Qidel), and both murine (mFcRn) and human FcRn (hFcRn) to immobilized antibody variants was monitored by SPR with a ProteOnTM XPR36 (Bio-Rad). Fc γ RIIIa (158V and 158F), Fc γ RIIIa, Fc γ RIIb, Fc γ RIa, hFcRn and mFcRn were all generated at Medimmune. Antibodies were first coupled to a GLC sensor chip according to manufacturer's instructions. Excess reactive groups were blocked with a 6-min injection of 1 M ethanolamine. Antibodies were immobilized at a surface density of ~5000 RUs for equilibrium measurements and <200 RUs for kinetic binding experiments. FcRn was used at concentrations ranging from 0.45 nM to 3 μ M at a flow rate of 25 μ L/min for steady-state measurements and 75 μ L/min for kinetic measurements. One channel was always left unmodified to provide a blank reference surface. Dilutions and binding experiments were carried out at 25°C in PBS pH 6.0 or pH 7.4, containing 0.05% Tween 20. Steady-state binding data were collected for 10 min. Antibody surfaces were regenerated with a 15-second injection of 5 mM HCl. Binding affinities were determined by using the ProteOnTM Manager software. Dissociation constants (K_{D} s) were determined by fitting the corresponding binding isotherms for steady-state data or by fitting the kinetics for association and dissociation employing a 1:1 Langmuir or a 1:1 Langmuir Mass Transfer model.

Isolation of human effector cells and description of cell lines

Blood samples were obtained from healthy donors after obtaining informed consent. PMN and PBMCs were isolated from freshly drawn peripheral blood using a PolymorphprepTM (Axis-Shield) gradient following manufacturer's directions. PMN cells isolated using this method were found to have ~85% neutrophil content as determined by Giemsa staining. The PMN & PBMC effector cell population is a mixture of the PMN and PBMC fractions isolated from the PolymorphprepTM gradient. Residual RBCs in both effector cell populations were lysed with ACK lysing buffer. The KC1333 NK cell line (BioWa)

expressing human CD16 was maintained in RPMI 1640 medium, supplemented with 10% FBS. BT474 (Her2+++), SK-BR-3 (Her2+++), and MDA-MB-453 (Her2++) human breast cancer cells were obtained from ATCC (Rockville, MD) and maintained in DMEM supplemented with 10% FBS. Her2 expression (and assignment of “+” scoring)³⁹ was confirmed with HercepTestTM (Dako A/S, Glostrup, Denmark).

Antibody-dependent cell-mediated cytotoxicity assays

ADCC assays were performed at an effector to target (E:T) ratio of 20:1 PMN or PMN & PBMC and 2.5:1 using KC1333 cells. Cell killing was measured using the Cytotox 96[®] Non-Radioactive Cytotoxicity Assay (Promega). Briefly, cells (10,000 target cells/well in a U bottom 96-well plate) were incubated at 37°C for 20 hrs in RPMI supplemented with 2.5 mM glutamine, 10% FBS, and 1% Pen-Strep prior to removing supernatant for LDH release measurements. The percentage of cellular cytotoxicity was calculated using the formula: % cytotoxicity = (experimental value - spontaneous effector - spontaneous target) / (maximal target - spontaneous target) × 100. Data were graphed and analyzed using GraphPad Prism 6.0. Curves were fitted using a nonlinear regression model with a sigmoidal dose response.

ADCC FCM analysis and microscopy

ADCC experiments for flow cytometric analysis were performed as above; however PMN and BT474 cells (at a ratio of 10:1 PMN:BT474 cells) were labeled with cell-permeable Calcein-AM violet and Calcein-AM, respectively, prior to the experiment. After 20 hours at 37°C, cells were then labeled with LIVE/DEAD[®] Fixable Near-IR Dead Cell Stain (Life Technologies) according to manufacturer's directions. FCM data were collected via LSR II (BD bioscience) and analyzed in FlowJo v7.6.1 (Tree Star Inc.). No compensation was necessary for the 3-color experiment. Percent cytotoxicity was calculated as follows: % cytotoxicity = 100 × (count Calcein-AM⁺, LIVE/DEAD[®] Fixable Near-IR⁺ cells)/(total count Calcein-AM⁺ cells). For microscopy experiments BT474 cells were labeled with Calcein-AM, and PMN cells were unlabeled. An E:T ratio of 10:1 was used with antibodies added at a concentration of 6 nM. Fluorescence microscopy was performed using a FLoId Cell Imaging Station (Life Technologies).

ADCP FCM analysis

Preparation of human monocyte-derived MΦ and quantification of target cell phagocytosis was performed as described previously.⁴⁰ Briefly, PBMC were purified from healthy donor blood, monocytes were enriched by adherence and differentiated over 6 days in RPMI with 100ng/mL human M-CSF (R & D Systems), 10% FBS and penicillin-streptomycin solution (Life Technologies). MΦ were detached with accutase (PAA laboratories) followed by scraping, labeled with celltrace violet (3 μM, Life Technologies) and seeded at 8 × 10⁴ cells/well in flat-bottomed 96-well plates. After resting overnight, 1 × 10⁴ CFSE-labeled SK-BR-3 or MDA-MB-453 (10 μM, Life Technologies) were

added per well along with the indicated concentrations of antibodies. After 2 h, adherent cells were detached with accutase, combined with non-adherent cells, stained with live dead far red (Invitrogen), and fixed in 1% PFA. Phagocytosis was quantified by FCM (FACS Canto II with 405 nm, 488 nm and 633 nm lasers, BD Biosciences), with data analysis in FlowJo v7.6.1. Data were gated on single, live cells, and % phagocytosis was calculated as follows: % phagocytosis = 100 × (count CFSE⁺, celltrace violet⁺ cells)/(total count CFSE⁺ cells)

Data were graphed and analyzed using GraphPad Prism 6.0. Curves were fitted using a nonlinear regression model with a sigmoidal dose response. Images of different cell populations in MΦ/tumor cell co-cultures were captured with Imagemaster^X imaging cytometer and analyzed with IDEAS software (Amnis Corporation).

In vivo pharmacokinetics in BALB/C Mice

Female (8 weeks old) BALB/c mice were given a bolus intravenous (IV) dose of 2.5 mg/kg antibody on day 0. Eight mice were used per antibody tested, with 2 groups of 4 (A group or B group) bled at alternate time points. Blood samples were obtained from the retro-orbital plexus using capillary pipettes at different time points throughout the 25 day study. A quantitative ELISA was used to monitor the serum concentrations of the tested antibodies. Briefly, 96-well plates were coated with 2 μg/ml of AffiPure Goat anti-human F(ab')₂ fragment-specific antibody (Jackson ImmunoResearch). Plates were blocked with 3% BSA in PBS for an hour, and then incubated with appropriately diluted serum samples (1:300 for earlier time points and 1:50 or 1:100 for later time points). Goat anti-human Fd-specific HRP-conjugated antibody (Southern Biotechnology Associates) was used to detect the human antibody (dilution 1:10000). Absorbance at 450 nm was measured after development with TMB substrate (KPL) according to manufacturer's directions. Standard curves were generated for each antibody variant diluted into 1:100 pre-bleed mouse serum (taken at day -3). The linear portions of standard curves generated in GraphPad Prism 6.0 were then used to quantify human IgG in the serum samples.

Disclosure of Potential Conflicts of Interest

The authors have no conflict of interest and financial interest to disclose.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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