Simultaneous Targeting of FcγRs and FcαRI Enhances Tumor Cell Killing

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Abstract

Efficacy of anticancer monoclonal antibodies (mAb) is limited by the exhaustion of effector mechanisms. IgG mAbs mediate cellular effector functions through FcγRs expressed on effector cells. IgA mAbs can also induce efficient tumor killing both in vitro and in vivo. IgA mAbs recruit FcαRII-expressing effector cells and therefore initiate different effector mechanisms in vivo compared with IgG. Here, we studied killing of tumor cells coexpressing EGFR and HER2 by the IgG mAbs cetuximab and trastuzumab and their IgA variants. In the presence of a heterogeneous population of effector cells (leukocytes), the combination of IgG and IgA mAbs to two different tumor targets (EGFR and HER2) led to enhanced cytotoxicity compared with each isotype alone. Combination of two IgGs or two IgAs or IgG and IgA against the same target did not enhance cytotoxicity. Increased cytotoxicity relied on the presence of both the peripheral blood mononuclear cell and the polymorphonuclear (PMN) fraction. Purified natural killer cells were only cytotoxic with IgG, whereas cytotoxicity induced by PMNs was strong with IgA and poor with IgG. Monocytes, which coexpress FcγRs and FcαRI, also displayed increased cytotoxicity by the combination of IgG and IgA in an overnight killing assay. Coinjection of cetuximab and IgA2-HER2 resulted in increased antitumor effects compared with either mAb alone in a xenograft model with A431-luc2-HER2 cells. Thus, the combination of IgG and IgA isotypes optimally mobilizes cellular effectors for cytotoxicity, representing a promising novel strategy to improve mAb therapy. Cancer Immunol Res; 1–9. ©2015 AACR.

Introduction

Antibody therapy with IgG monoclonal antibodies (mAb) represents part of the standard treatment in the clinic for various forms of cancer. The ErbB family members EGFR and HER2 are validated clinical targets for mAb therapy (1). Cetuximab (anti-EGFR) is approved for colon and head and neck cancers, whereas trastuzumab (anti-HER2) is used for breast and gastric cancer treatment. EGFR mAbs have a dual mechanism of action: Both Fab- and Fc-mediated antitumor effects were described (2). The direct Fab-mediated effects include occlusion of the ligand-binding site, leading to hampering of receptor dimerization, and internalization of EGFR, leading to inhibition of downstream signaling (3). The antitumor mechanisms of HER2 mAbs are less well understood, but also involve Fab-mediated direct effects, such as induction of receptor internalization and degradation, or induction of cell-cycle arrest by inhibiting downstream signaling (4, 5). Next to direct Fab-mediated effects, the mechanisms of action of both mAbs involve Fc gamma receptor (FcγR)-dependent mechanisms. The importance of Fc–FcγR interaction is supported by clinical association studies and preclinical animal studies for both EGFR (6–8) and HER2 mAb therapy (9, 10). However, IgG mAb therapy is rarely curative, and this has in part been attributed to exhaustion of cellular effector mechanisms (11).

Cetuximab and trastuzumab are, like most therapeutic mAbs, of the IgG1 subclass. IgG1 mAbs engage FcγRs on effector cells to initiate antibody-dependent cell-mediated cytotoxicity (ADCC). Each immune effector cell type has a unique expression of FcγRs. For example, in humans, natural killer (NK) cells express only FcγRIIA (and in some individuals FcγRIIC), polymorphonuclear cells (PMN) express FcγRIIA and FcγRIIB, whereas monocytes express FcγRI, FcγRIIB, and FcγRIIIA.

Coengagement of the activating FcγRs with the inhibitory FcγRIIB by IgG1 on monocytes results in inhibitory signaling and limits the cytotoxic activity of IgG1. PMNs abundantly express a GPI-linked nonsignaling FcγRIIB. The interaction of IgG1 with FcγRIIB limits IgG1-mediated PMN cytotoxicity (12, 13). Moreover, polymorphisms in several FcγRs influence IgG1 activity: Genotypes that confer to stronger IgG binding are associated with better response to mAb therapy (14).

Previously, we have shown that IgA antitumor mAbs can induce efficient cytotoxicity in vitro and in vivo (15, 16). IgA induces more cytotoxicity than IgG by purified PMNs and unfraccionated human leukocytes (15) and induces comparable cytotoxicity with
IgG by macrophages (16, 17). The cytotoxic activity of IgA is mediated by FcγRI, and therefore IgA induces different effector functions than IgG, for example, more efficient activation of PMNs compared with IgG (18).

EGFR and HER2 coexpression is found in a number of cancers, including pancreatic cancer, non–small cell lung cancer, and breast cancer (19–21). Moreover, EGFR and HER2 coexpression is associated with the most aggressive tumors (21). To increase IgG activity, the combination of two antitumor IgG mAbs against these two targets has been investigated before (22, 23). These studies have revealed increased antitumor effects by the combination of two IgG mAbs, which were mainly due to the dual Fab-mediated effector functions. At saturating target opsonization, this approach also increases the available Fc moieties for FcγR binding; however, tumor cell killing is still limited by the number of available FcγRs on effector cells and/or their cytotoxic capacity.

We reasoned that the combination of IgG and IgA would induce enhanced tumor cell killing through the recruitment and/or activation of both FcγR-bearing NK cells and FcγR-bearing PMNs. Coengagement of FcγRs and FcγRII on the same effector cell (monocytes/macrophages) might also result in stronger Fcγ signaling and tumor cell killing. To this end, we studied cytotoxicity induced by the combination of IgG and IgA (cetuximab or trastuzumab, as well as their IgA variants carrying the same variable region) toward tumor cells coexpressing EGFR and HER2.

Materials and Methods

Cell lines and cell culture

The human epidermoid carcinoma cell line A431 and the human breast cancer cell line SK-BR-3 (ATCC) were authenticated by STR profiling. No reauthentication assay was performed because cell lines were directly obtained from cell banks and used at early passages after receipt or resuscitation. All cell lines were tested and validated to be Mycoplasma free. Cell lines were cultured in RPMI-1640 (Gibco) supplemented with 10% FCS and penicillin and streptomycin (Gibco). A431-HER2 cells were generated by retroviral transduction of A431 cells with human HER2 (pMX-puro-HER2), and positive clones were selected using puromycin. A431-luc2 cells were generated as previously described (16) and transduced with HER2. HER2 expression on A431-luc2-HER2 cells was comparable with that in A431-HER2 cells. HER2 expression on the transduced cell lines was regularly tested by cyttofluorimetry and was found to be stable in time. The number of EGFR and HER2 molecules on A431-HER2 cells was similar to the number of A431 and A1207 cells (Supplementary Table S1).

Antibodies

The chimeric anti-human EGFR mAb cetuximab and the humanized anti-human HER2 mAb trastuzumab were purchased from the pharmacy of UMC Utrecht. Both cetuximab and trastuzumab are of IgG1 isotype. The generation and characterization of IgA2-EGFR have been previously described (15, 24). The generation and characterization of IgA2-HER2 mAb will be described in detail elsewhere (S. Meyer, submitted for publication). Briefly, the variable regions of trastuzumab were inserted into an expression vector containing the constant regions of the heavy and light chains of human IgA2 (24). IgA2 was produced by transient transfection of HEK 293F cells (Freestyle 293 Expression System; Invitrogen) and purified by affinity and size-exclusion chromatography.

Flow cytometry

Expression of HER2 and EGFR on tumor cells was determined using different concentrations of trastuzumab and cetuximab, respectively, and measured with flow cytometry using a PE-labeled anti-human IgG Ab (The Jackson Laboratory). The following mAbs were used for flow cytometry: CD3 (UCHT1; Biologend), CD56 (HCD56; Biologend), CD14 (HCD14; Biologend) and CD16 (3G8; Biologend) for isolated effector cells or CD45 (HI30; Biologend), CD3 (OKT3; Biologend), CD56 (HCD56; Biologend), CD14 (G1D3; ebioscience), and CD16 (3G8; Biologend) Abs for leukocytes. Control mouse IgG2b and mouse IgG1 mAbs did not show any background binding. BD Truccout tubes were used to determine the absolute amount of effector cells in 50 μl leukocyte suspension reconstituted to the original volume of the peripheral blood sample. During the measurement, leukocytes were identified to be CD45+. Flow-cytometry measurements were performed on FACS Cantor II (BD Biosciences). Flow-cytometry data were analyzed by FACS Diva software.

ADCC

ADCC with 51Cr-labeled target cells was described previously (25). Briefly, 1 × 10⁶ target cells were labeled with 100 μCi (3.7 MBq) 51Cr for 2 hours. After extensive washing, cells were adjusted to 10⁷/mL. Blood was obtained from healthy donors at the UMC Utrecht. In leukocyte ADCC, erythrocytes were lysed by incubation in water for 30 seconds, and then total leukocytes were resuspended in medium. The number of leukocytes used per well corresponded to the number of leukocytes present in 50 μL of blood before lysis. Effector cells, sensitizing mAbs at various concentrations, medium, and tumor cells were added to round-bottom microtiter plates (Corning Incorporated). After 4 or 20 hours (overnight) of incubation at 37°C, ⁵¹Cr release was measured in counts per minute (cpm). The percentage of specific lysis was calculated using the following formula: [(experimental cpm – basal cpm)/(maximal cpm – basal cpm)] × 100, with maximal lysis determined in the presence of 3% triton and basal lysis in the absence of Abs and effector cells. We included only those donors in our analysis that induced a minimum of 5% and maximum of 80% lysis with both single mAbs (Supplementary Table S2).

PMN and peripheral blood mononuclear cell (PBMC) fractions were isolated from blood by Ficoll/Histopaque separation (GE Healthcare; Sigma-Aldrich). Monocytes were isolated from the PBMC fraction using CD14 magnetic beads (Miltenyi Bioscience). NK cells were isolated from the CD14-negative fraction by negative selection using the EasySep Human NK Cell Enrichment Kit (Stemcell Technologies). Purity of effector cells was analyzed by flow cytometry and was approximately 85% for NK cells, 70% for monocytes, and close to 100% for PMNs. The effector-to-target (E:T) ratios for purified fractions were 70:1 (PBMC), 40:1 (PMN), 15-25:1 (monocytes), or 10:1 (NK cells). The E:T ratio in the leukocyte ADCC was 1:1 for both NK cells and monocytes and 12:1 (with a range of 6–18:1) for PMNs. IgG and IgA mAbs were combined at a 1:1 ratio.

In certain experiments, A431-HER2 or SK-BR-3 cells were incubated for 6 hours with both PMNs (E:T = 40:1) and PBMCs.
(E:T = 70:1), and specific lysis was compared with conditions using either PMNs (E:T = 80:1) or PBMCs (E:T = 140:1) alone. For the experiments using sequential incubation with effector cells, tumor cells were first incubated with PMNs (E:T = 40:1) for 3 hours, then PBMCs (E:T = 70:1) were added, and incubated with the target cells for another 3 hours. As controls, tumor cells were incubated either with PMNs (E:T = 80:1) or with PBMCs (140:1) twice for 3 hours. Cetuximab and IgA2-HER2 were added at 1:1 ratio at the indicated concentrations.

A431-luc2-HER2 xenograft tumor model

The in vivo xenograft experiment was performed as described previously (16). FcεRI Tg or nontransgenic littermate SCID mice were injected i.p. with $5 \times 10^5$ A431-luc2-HER2 cells in 100 μL PBS. Tumor outgrowth was monitored by serial bioluminescent imaging (BLI; PhotonImager; Biospace Lab). BLI images were processed using M3Vision software (Biospace Lab) and edited in Adobe Photoshop. Mice with tumor outgrowth were randomized in different treatment groups on day 13. Cetuximab was injected once on day 14 (10 μg), whereas 10 μg IgA2-HER2 was injected daily between days 14 and 23 (in total 10 times) to compensate for its shorter half-life. The combination group was injected with both cetuximab and IgA2-HER2. Tumor growth is expressed as percentage signal compared with the beginning of the treatment. Circulating serum mAb levels were monitored in serial blood samples taken from alternating mice (2 mice/time point). Serum levels of human IgG and IgA were measured by ELISA as previously described (16). The experiment was approved by the local Animal Ethical Committee.

Data processing and statistical analyses

Statistical analyses were performed in R (3.1.0) or in Graph Pad 6.0 software. Data are represented as mean ± SEM, unless indicated otherwise. Dose–response curves for the ADCC experiments were calculated via nonlinear regression [log(agonist) vs. response] using Graph Pad 6.0 software.

Maximal specific lysis was analyzed by using the triplets of a concentration level on which maximal average specific lysis was attained in an individual ADCC experiment. Treatment effects were analyzed by using two-way full factorial linear mixed-effects modeling. The model consisted of two indicator variables for drugs applied (yes or no) as fixed-effects terms, and subject and triplets nested within subject as random-effect terms. The least-square mean differences in the maximal lysis among the full factorial interaction groups were estimated with the Tukey adjustment. Inferential statistical analysis regarding the effect of treatments on the time course of tumor growth in vivo was carried out by using linear mixed-effects modeling. The log-transformed tumor size was described by a model consisted of fixed-effect terms, such as time since xenograft (days) as a continuous covariate, and its second- and third-order interactions with the cetuximab and IgA2-HER2 treatments as factors, and two random-effect terms allowing individual differences in tumor size at day 1 and in tumor growth time course.

Results

IgG and IgA mAbs to both EGFR and HER2 induce cytotoxicity of A431-HER2 cells

To test cytotoxicity induced by the combinations of two different isotypes, we used IgG1 and IgA specific for EGFR and HER2.

lgA variants of the chimeric IgG1-EGFR mAb cetuximab were previously generated (15, 24). Following a similar approach, we generated IgA variants of the human HER2 mAb trastuzumab (S. Meyer, submitted for publication). The IgA2 subclass was used in our experiments because it induces stronger cytotoxicity than the IgA1 subclass (15, 16).

IgA2-EGFR and IgA2-HER2 induced significantly higher cytotoxicity than cetuximab and trastuzumab, respectively, by leukocytes in a 4-hour killing assay with tumor cell lines expressing high levels of endogenous targets (Supplementary Fig. S1A and S1B). To study the effects of different mAb combinations against EGFR and HER2, we established a target cell line with high expression of both EGFR and HER2 (A431-HER2 cells), because HER2 expression on A431 cells was too low for efficient cytotoxicity by trastuzumab (Supplementary Fig. S2; Supplementary Table S1 and unpublished results).

Both IgG- and IgA-mediated targeting of either EGFR or HER2 induced specific lysis of A431-HER2 cells using leukocytes as effector cells and, as expected, maximal lysis by IgA was higher than by IgG for both targets (Fig. 1A). No specific lysis was observed in the absence of mAbs or effector cells, nor was the lysis induced by tumor-specific mAbs, influenced by the presence of nonspecific antibodies. At low mAb concentrations, cetuximab was more effective than trastuzumab, most likely due to the higher expression of EGFR than HER2 by A431-HER2 cells.

Cytotoxicity by IgG and IgA is mediated by distinct populations of effector cells within the leukocytes

Leukocytes contain a heterogeneous population of effector cells [NK cells, monocytes, and PMNs] that are all capable of inducing cytotoxicity. We therefore purified the different effector cells to determine their individual cytotoxic capacity. PMNs and monocytes expressed both FcγRs and FcεRI, whereas NK cells only expressed FcγRIIIa (Supplementary Fig. S3). In accordance with their FcR expression, NK cells only induced cytotoxicity with IgG (Fig. 1B). PMNs were more efficient with IgA2-EGFR than with cetuximab, and they induced tumor cell killing with IgA2-HER2 but not with trastuzumab (Fig. 1C). Monocytes did not induce cytotoxicity after 4 hours with any of the mAbs. However, monocytes induced potent cytotoxicity in an overnight killing assay with all four mAbs, which is in line with previous findings showing that monocytes require a longer incubation time for efficient cytotoxicity (Fig. 1D; refs. 16, 17).

In conclusion, IgG-mediated cytotoxicity is mediated mainly by NK cells, whereas IgA-mediated cytotoxicity is mediated predominantly via PMNs. Monocytes are unlikely to directly contribute to tumor cell killing in a 4-hour assay, but mediate cytotoxicity with both IgG and IgA in an overnight assay.

Increased cytotoxicity by the combination of IgG- and IgA-targeting EGFR and HER2

We first investigated the effect of combining two IgGs (cetuximab and trastuzumab) or two IgAs (IgA2-EGFR and IgA2-HER2) on the specific lysis of A431-HER2 cells by leukocytes. These combinations did not decrease EC50 values or increase maximal cytotoxicity compared with the best mAb alone (Fig. 2A–D), likely because the Fc parts of both mAbs competed for the same set of FcRs.
Given that IgG and IgA recruit predominantly different cellular effectors (NK cells and PMNs), we hypothesized that the combination of IgG and IgA would result in increased cytotoxicity by leukocytes containing both NK cells and PMNs. The combination of IgG and IgA directed against the same target (cetuximab and IgA2-EGFR or trastuzumab and IgA2-HER2) did not increase maximal tumor cell killing compared with the best mAb alone (Fig. 2E–H). This is most likely due to competition for the target by both IgG and IgA. IgG might decrease IgA-mediated recruitment and activation of PMNs from the leukocytes. Indeed, IgA2-EGFR-induced cytotoxicity by purified PMNs was inhibited by the addition of cetuximab (Supplementary Fig. S4).

Next, we investigated the combination of IgG and IgA directed against two different targets. Cetuximab and IgA2-HER2 were mixed at varying ratios while keeping the total mAb concentration constant. The combination of cetuximab and IgA2-HER2 resulted in increased lysis of A431-HER2 cells compared with cetuximab or IgA2-HER2 alone (Fig. 3A). Using broad mAb concentrations with multiple donors, the maximal lysis induced by the combination cetuximab of and IgA2-HER2 was significantly increased compared with the maximal lysis by the best single mAb (Fig. 3B and C).

The combination of trastuzumab and IgA2-EGFR did not increase maximal lysis compared with IgA2-EGFR alone (Fig. 3D). This might be due to the relatively high cytotoxicity induced by IgA2-EGFR alone as a result of the high EGFR expression by A431-HER2 cells.

These data show that the combination of IgG and IgA against two different tumor targets can result in increased cytotoxicity by leukocytes. Increased cytotoxicity by the combination of IgG and IgA requires multiple effector cell types

Whereas NK cells express only FcγRIIa, human PMNs may express both FcγRs (FcγRIa, FcγRIIa, and FcγRIIb) and FcγRI, and are capable of tumor cell killing with both IgG and IgA (Supplementary Fig. S3). Because trastuzumab alone did not induce cytotoxicity of A431-HER2 cells with PMNs (Fig. 1C), we studied only the combination of cetuximab and IgA2-HER2 on PMN-induced cytotoxicity. Cetuximab alone induced 5% to 20% maximal lysis depending on the donor. The combination of cetuximab and IgA2-HER2 did not increase cytotoxicity by purified PMNs (Supplementary Fig. S5A and S5B).

The combination of cetuximab and IgA2-HER2 resulted in increased maximal cytotoxicity of A431-HER2 cells when PBMCs and PMNs were combined compared with either fraction individually (Fig. 4A). Similar effects were observed using SK-BR-3 cells expressing both EGFR and HER2 endogenously (Fig. 4B). In addition, the sequential incubation of A431-HER2 or SK-BR-3 cells with PBMCs and PMNs in the presence of cetuximab and IgA2-HER2 resulted in increased cytotoxicity compared with incubation with either PBMCs or PMNs alone (Fig. 4C and D).

Monocytes express high levels of both FcγRs and FcγRI (Supplementary Fig. S3) and induced comparable cytotoxicity by both IgG and IgA (Fig. 1D). Both combinations of IgG and IgA against different targets increased maximal cytotoxicity induced by monocytes in an overnight killing assay (Fig. S5A–D).

These results show that increased cytotoxicity induced by the combination of IgG and IgA by leukocytes in a 4-hour assay...
requires the presence of at least two effector cell types, most likely NK cells and PMNs. Furthermore, the combination of IgG and IgA can also increase cytotoxicity induced by monocytes alone.

Enhanced antitumor effect by the combination of IgG and IgA in an in vivo xenograft tumor model

Using an in vivo xenograft tumor model in human FcRRI transgenic SCID mice, we have earlier shown that administration of cetuximab and IgA2-HER2 was able to decrease intraperitoneal outgrowth of A431-luc2-HER2 cells (16). PMNs and macrophages expressing mouse FcγRs as well as human FcRI were both recruited to the peritoneal cavity and could participate in tumor killing (16). We now show that treatment of established tumors with either cetuximab or IgA2-HER2 alone reduced tumor growth compared with the control group. Mice that received both cetuximab and IgA2-HER2 displayed a reduced tumor volume compared with those treated with single mAbs (Fig. 6A and B).

The concentration of circulating cetuximab (1 μg/mL) in serum during the first 10 days after treatment was higher than that of IgA2-HER2 (~1 μg/mL; Fig. 6C). At these mAb concentrations, maximal
lysis was achieved in vitro with both mouse PMNs and macrophages with both mAbs (unpublished results; ref. 16).

These data demonstrate that the combination of IgG and IgA enhances antitumor effects of mAbs in vivo.

Discussion

Intense efforts are being undertaken to increase efficacy of antitumor IgG mAbs. However, in vivo activity of IgG is limited by the number of available FcγRs, their biologic distribution, and the cytotoxic capacity of the recruited effector cells. Here, we present evidence that the combination of IgG and IgA isotypes can increase mAb-induced tumor cell killing by more optimally harnessing the cytotoxic potential of a heterogeneous population of immune effector cells.

Increased maximal killing in the leukocyte ADCC assay results from the recruitment of both NK cells and PMNs that—as purified effector cells—only, or preferentially, mediate cytotoxicity by either IgG or IgA. The different antitumor effector mechanisms used by NK cells and PMNs could also contribute to the increased killing. NK cells induce apoptosis in tumor cells through the release of granzymes and perforin. Cytotoxicity mediated by PMNs is less well understood, but it was reported that PMNs can mediate frustrated phagocytosis or induce autophagy in tumor cells by IgA (26).

Purified PMNs preferentially induced cytotoxicity by IgA and only poorly with IgG due to the high expression of the non-signaling FcγRIIB, which scavenges IgG away from FcγRIa, and therefore decreases IgG activity (12, 13, 27). The combination of IgG and IgA did not increase cytotoxicity using purified PMNs. In contrast, purified monocytes induced comparable cytotoxicity by both IgG and IgA and mediated increased cytotoxicity by the combination of IgG and IgA. As both FcγRs and FcεRI signal through the same Fcγ-chain ITAM, the combination of IgG and IgA could result in stronger cellular signaling in monocytes, resulting in increased cytotoxicity.

IgG-opsonized tumor cells in leukocyte ADCC recruit NK cells, eliciting efficient cytotoxicity, but also recruit PMNs and monocytes, leading to less efficient cytotoxicity in a 4-hour assay. Although monocytes alone did not induce cytotoxicity within 4 hours, they could have indirectly contributed to tumor cell killing, for example, through stimulation of NK cells (28). Triggering of FcγRs on monocytes could result in the release of cytokines or other mediators, which may enhance FcεRI- or FcγRI-mediated killing by PMNs (or by monocytes) through activation via inside-out signaling. Cross-talk between effector cells is suggested by the fact that when purified effector cells were used at E:T ratios present in leukocyte ADCC, they were only poorly cytotoxic.

The combination of IgG and IgA led to increased cytotoxicity only when directed against two different tumor targets. The combination of IgG and IgA against the same target did not increase tumor cell killing possibly due to competition for tumor target binding by IgG and IgA. The combination of two IgGs or two IgAs against two different targets also did not increase tumor cell killing, which could be due to saturation of the corresponding receptors.

In our in vitro leukocyte ADCC assays, we have found that IgA-EGFR alone already induced relatively high maximal tumor cell lysis, and this was not further enhanced by adding trastuzumab.
The combination of trastuzumab and IgA-EGFR, however, enhanced cytotoxicity by monocytes, a major cellular effector population of both IgG and IgA, suggesting that this combination can also have a beneficial effect in vivo.

Previously, the combination of EGFR and HER2 IgG1 mAbs has been described to lead to a synergistic increase of antitumor effects, which was mainly attributed to increased direct Fab-mediated antitumor effects (22, 23). Although Fab-mediated antitumor effects are readily induced by both IgG and IgA, in this study, we focused on Fc-mediated effector functions because we do not expect differences mediated by the Fab portion of the mAbs within the time frame of our in vitro experiments. However, we expect that combination therapy with IgG and IgA against EGFR and HER2 in patients might also result in enhanced Fab-mediated antitumor effects. Despite the fact that maximal specific killing by the combination of IgG and IgA in a 4-hour in vitro assay does not reach 100%, the measured increase (~20%) might still have profound consequences in vivo and may lead to therapeutic synergy.

At the site of the tumor, the magnitude of tumor lysis will be determined by the number of available Fc moieties (influenced by the target expression, orientation of the Fc part, clustering of target in the cell membrane), the available FcRs (influenced by the numbers and types of effector cells present), and the killing capacity of the effector cells. Studies using engineered mAbs that have a higher affinity for activating FcRs suggest that increasing cellular signaling above a certain threshold does not further increase cytotoxicity (29). Cytotoxicity can thus be limited by both saturation of the target and the saturation of the FcRs. This could explain why cytotoxicity by leukocytes was enhanced only by the combination of IgG and IgA against two targets and not by the combination of two IgGs, or two IgAs against the same target or by the combination of IgG and IgA against the same target.

Monocytes express high levels of FcεRI and have a favorable expression pattern of FcγRs (high expression of FcγRII and no expression of FcγRIII). This may explain why cytotoxicity was also enhanced by the combination of IgG and IgA against two targets when monocytes alone were used as effector cells. Individual donors showed variability in the extent by which the combination of IgG and IgA increased cytotoxicity, which could be a result of polymorphisms in FcγRs. Analysis of a number of SNP and CNV polymorphisms as tested by multiplex ligation–dependent probe amplification (30) in various FcγR genes did not

Figure 5. Improved Cytotoxicity by the Combination of IgG and IgA. Dose–response curves from a representative experiment in an overnight cytotoxicity assay using A431-HER2 cells and purified monocytes (E:T = 25:1) with cetuximab and IgA2-HER2 (A) and trastuzumab and IgA2-EGFR (C). IgG and IgA mAbs were combined at a 1:1 ratio. B and D, maximal lysis was determined and plotted from 5 independent experiments, with a total of 7 donors performed with E:T = 15:1 and 25:1 (median, box extends from the 25th to 75th percentiles, whiskers to minimum and maximum; ANOVA, Tukey multiple comparison test, **, P < 0.01; *** P < 0.001). Ab, antibody.
reveal a clear correlation with increased cytotoxicity, although the numbers analyzed were small (data not shown).

Intimate contact between effector and target cells is required for efficient cytotoxicity (31). Our preliminary data suggest that the combination of trastuzumab and IgA2-EGFR, but not cetuximab and IgA2-EGFR or cetuximab and trastuzumab, increased the formation of conjugates between A431-HER2 cells and monocytes or PMNs. Thus, it appears that for PMNs increased binding does not directly translate to more efficient killing, most likely reflecting a contribution of the nonsignaling FcγRIIB to the formation of conjugates.

In the xenograft tumor model, both PMNs and macrophages were recruited to the tumor site (16). In FcRRI Tg mice, both PMNs and macrophages express FcγR and FcγRI. In preclinical studies, macrophages as well as PMNs were found to mediate effects of IgG in vivo (25, 32, 33), whereas in a different model, we previously identified macrophages as primary effectors of IgA (16). However, because PMNs as well as macrophages are effective in vitro, it is reasonable to assume that both effector cell types contribute to cytotoxicity in vivo. In our mouse model, the increased tumor killing by the combination of IgG and IgA likely results from the recruitment of PMNs and macrophages and increased FcγR triggering on both cell types.

Compared with IgG mAbs, the biology of IgA is less well understood, and the production and purification processes are not well established. However, replacing IgA with a bispecific mAb targeting both a tumor target and FcγRI might be an alternative strategy to overcome this.

In conclusion, we have presented evidence that the simultaneous targeting of both FcγRs and FcγRI appears to more optimally mobilize the cytotoxic resources of effector cells and therefore represents a conceptually new approach to improve the efficacy of mAb therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Brandsma, T. ten Broeke, L.A.P.M. Meulenbroek, S. Meyer, J.H.M. Jansen, M.A. Beltrán Buitrago, J.H.W. Leusen
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