IgA-Mediated Killing of Tumor Cells by Neutrophils Is Enhanced by CD47–SIRPα Checkpoint Inhibition


Abstract

Therapeutic monoclonal antibodies (mAb), directed toward either tumor antigens or inhibitory checkpoints on immune cells, are effective in cancer therapy. Increasing evidence suggests that the therapeutic efficacy of these tumor antigen–targeting mAbs is mediated—at least partially—by myeloid effector cells, which are controlled by the innate immune-checkpoint interaction between CD47 and SIRPα. We and others have previously demonstrated that inhibiting CD47–SIRPα interactions can substantially potentiate antibody-dependent cellular phagocytosis and cytotoxicity of tumor cells by IgG antibodies both in vivo and in vitro. IgA antibodies are superior in killing cancer cells by neutrophils compared with IgG antibodies with the same variable regions, but the impact of CD47–SIRPα on IgA-mediated killing has not been investigated. Here, we show that checkpoint inhibition of CD47–SIRPα interactions further enhances destruction of IgA antibody-opsonized cancer cells by human neutrophils. This was shown for multiple tumor types and IgA antibodies against different antigens, i.e., HER2/neu and EGFR. Consequently, combining IgA antibodies against HER2/neu or EGFR with SIRPα inhibition proved to be effective in eradicating cancer cells in vivo. In a syngeneic in vivo model, the eradication of cancer cells was predominantly mediated by granulocytes, which were actively recruited to the tumor site by SIRPα blockade. We conclude that IgA-mediated tumor cell destruction can be further enhanced by CD47–SIRPα checkpoint inhibition. These findings provide a basis for targeting CD47–SIRPα interactions in combination with IgA therapeutic antibodies to improve their potential clinical efficacy in tumor patients.

Introduction

Monoclonal antibodies (mAb) targeting tumor antigens are widely used and an effective treatment of various malignancies. Over the years, an increasing number of mAbs targeting different tumor antigens have been approved for use in cancer patients, including trastuzumab or cetuximab, which target HER2/neu or EGFR, respectively (1). All of the anticancer mAbs on the market are of the IgG class (2), but the potential of other isotypes, such as IgA, is also being explored. IgA is known for its antimicrobial role and is abundantly present at mucosal sites in its dimeric form (3). As a monomer, IgA is the second most prevalent antibody in serum (3). IgA is composed of two subclasses, IgA1 and IgA2, that bind with similar affinity to the myeloid IgA receptor (FcαRI, CD89; ref. 4). Many cancer therapeutic antibodies act by a combination of both direct and indirect immune-mediated effects, which include cytotoxicity induced by complement activation, antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent cellular cytotoxicity (ADCC; ref. 5). ADCC can be mediated through activation of different Fc-receptor–expressing cells, including natural killer (NK) cells, macrophages, and neutrophils (6–9). Of these Fc-receptor–expressing effector cells, macrophages and neutrophils express FcαRI and hence can kill tumor cells by ADCC or ADCP as demonstrated in vitro (9–12). Antibodies of IgA isotype are effective in inducing ADCC of various tumor targets, including HER2/neu+ and EGFR+ carcinomas and CD20+ lymphomas (9, 13, 14). When neutrophils are used as effector cells, IgA therapeutic antibodies induced significantly higher levels of ADCC compared with IgG variants of the same mAb (9, 13–17).

Checkpoint inhibition of the inhibitory receptor signal regulatory protein alpha (SIRPα) or its ligand CD47 is effective in preclinical models when combined with IgG1- and IgG2-based cancer therapies (18–23). Based on these results, CD47–SIRPα interaction blocking agents are being tested in clinical trials for hematologic and solid cancers (www.clinicaltrials.gov identifiers: NCT02216409, NCT02678338, NCT02641002, NCT02367196, NCT02890368, NCT02663518, and NCT02953509; refs. 24, 25). A published phase Ib study shows encouraging results for a blocking anti-CD47 antibody in combination with rituximab, resulting in durable complete responses in patients with non-Hodgkin lymphoma (25). SIRPα is selectively present on myeloid cells and limits ADCC by macrophages and neutrophils (18). SIRPα’s ubiquitously expressed ligand CD47 acts as a “don’t eat me” signal and was found to be often overexpressed on cancer cells, inhibiting phagocytosis and clearance by macrophages (20, 26–28). In a clinical setting, the...
expression level of CD47 on cancer cells is inversely related to patient response to anticancer antibody therapy (18, 20, 27–29). In several preclinical models, CD47–SIRPα blockade is a promising target for enhancing cancer immunotherapies when combined with IgG mAbs targeting different tumor antigens, including cetuximab, trastuzumab, and rituximab (18, 20, 21). However, for IgA antibodies directed against specific tumor antigens, this enhancing effect of additional CD47–SIRPα checkpoint inhibition has not yet been investigated. Here, we examined whether inhibiting CD47–SIRPα interactions leads to an enhancement of IgA-based cancer therapies in both in vitro systems and in vivo mouse models. We demonstrated a prominent increase in IgA-opsonized tumor cell clearance in the presence of CD47–SIRPα blockade, providing support for the idea that targeting the CD47–SIRPα checkpoint could be used to improve IgA therapeutic antibodies in cancer.

Materials and Methods

Cells and culture

Cell lines were from ATCC (A431, SKBR3, BT-474, and RAW264.7) or DSMZ (Kyse-30). Ba/F3 cells were a kind gift from the group of Dr. Leo Koendeman and were reauthenticated by PCR analysis by IDEXX in 2017. All other cells were kept in culture according the suppliers’ recommendations and were obtained between 2011 and 2018. Cells were tested for Mycoplasma and kept in culture and used for experiments for a maximum of three months.

Generation of genetically modified cells

A431-CD47KO cell lines were generated by lentiviral transduction of pLentiCrispR-v2—CD47KO (pLentiCrispR-v2 was a gift from Feng Zhang, Addgene plasmid #52961); A431 cells expressing HER2/neu (A431HER2/neo) were generated by retroviral transduction, followed by positive selection based on puromycin resistance, as previously described (10). SKBR3-CD47KD cells were generated by lentiviral transduction of pLKO.1-puro—CD47KD. Transduced and afterward puromycin selected cells showed a CD47 expression of 10% to 15% of normal. As control cell line, a scrambled shRNA was used. Knockdown and knockout of antigens on cell lines was routinely verified by flow cytometry. Ba/F3 cells expressing EGFR were transfected with WT EGFR (Upstate) and EGFR-expressing clones were selected using neomycin. Ba/F3 cells expressing HER2/neu were generated by retroviral transduction, followed by positive selection using puromycin resistance and limiting dilution.

Antibodies and reagents

SIRPα or CD47 were blocked using human SIRPα mAb 12C4 or F(ab′)2 fragments of human CD47 antibody B6H12 (both at 10 μg/mL), respectively (18). IgG trastuzumab (Roche), IgG cetuximab (Merck KGaA), anti-HER2-IgA2 and anti-EGFR-IgA2 were generated as described (15) and used at a final concentration of 5 μg/mL, unless stated otherwise. To block mouse SIRPα, we used the rat IgG2 antibody MY-1, generously gifted to us by the group of Prof. Dr. Takashi Matozaki (University of Kobe, Kobe, Japan), at a final concentration of 10 μg/mL in our in vitro studies (30).

Neutrophil isolation

Human neutrophils were isolated either by density centrifugation of heparinized blood over isotonic Percoll (Pharmacia Uppsala, Sweden) followed by red cell lysis with hypotonic ammonium chloride solution at 4°C (31) or by using Polymorph Prep (catalog number 1114683, ProGen). After isolation, neutrophils were cultured in HEPES+ medium (containing 132 mmol/L NaCl, 6.0 mmol/L KCl, 1.0 mmol/L CaCl2, 1.0 mmol/L MgSO4, 1.2 mmol/L [NH4]2HPO4, 20 mmol/L HEPES, 5.5 mmol/L glucose and 0.5% HSA) and immediately used at a concentration of 5 × 10⁶ cells/mL, unless stated otherwise, or stimulated for 30 minutes at 37°C with 50 U/mL GM-CSF (CellGenix) before use. All blood was obtained from healthy individuals after written informed consent. Studies on human blood samples were conducted after written informed consent and according to the Declaration of Helsinki 1964. Blood from healthy volunteers was collected after approval by the Sanquin Research institutional medical ethical committee, in accordance with the standards laid down in the 1964 Declaration of Helsinki.

Two FHL5 patients were used in this study; for patient characteristics, see patients B and C described in ref. 32. Four individual chronic granulomatous disease (CGD) patients were used in this study: 2 male patients with mutations in the gp91 gene CYBB and 2 female patients with mutations in the p47phox gene NCF1.

Mouse neutrophils were isolated from mouse bone marrow as follows: Femurs were crushed in a mortar, washed with MACS buffer (containing phosphate-buffered saline (PBS) and filtered through a 40-μm filter. Rat anti-mouse-CD16/CD32 (BD Pharmingen, clone 2.4G2) was incubated in 1 mL MACS buffer, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mmol/L EDTA at a concentration of 25 μg/mL for 20 minutes on ice. After incubation, anti-Ly6G-APC (clone 1A8, BD Biosciences) was directly added at a concentration of 1 μg/mL for 30 to 45 minutes on ice. After washing the cells in MACS buffer, finally, 20 μL anti-APC MicroBeads (Miltenyi Biotec) were added per 10⁶ cells for 60 minutes on ice. After isolation, mouse neutrophils were cultured overnight in RPMI supplemented with 10% (v/v) FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at a concentration of 5 × 10⁶ cells/mL, in the presence of 50 ng/mL mouse IFNγ (PeproTech) and 10 ng/mL clinical-grade G-CSF (Neupogen), after which the cells were used in assays the following day.

ADCC

Cancer cell lines were labeled with 100 μCi 51Cr (PerkinElmer) for 90 to 120 minutes at 37°C. After 3 washes with PBS, 5 × 10⁶ cancer cells were cocultivated with neutrophils [see figure legends for effectortarget (ET) ratios] in 96-well U-bottom plates. The coculture was in IMDM cell culture medium (SKBR3 and BT-474) or RPMI culture medium (A431, Kyse-30, and Ba/F3) supplemented with 20% or 10% (v/v) FCS, respectively, and 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin for 3 to 4 hours at 37°C and 5% CO2 and contained therapeutic antibodies against HER2/neu or EGFR (see figure legends for concentrations used). After incubation, the supernatant was harvested and analyzed for radioactivity using a gamma counter (Wallac). The percentage of cytotoxicity was calculated as [(experimental cpm – spontaneous cpm)/total cpm – spontaneous cpm] × 100%. All conditions were measured in triplicate.

Trogocytosis assay

To determine tumor membrane uptake by neutrophils, a FACS-based assay was used. Cancer cells were labeled with a lipophilic membrane dye (DiO/DiD, 5 μmol/L, Invitrogen) for 30 minutes at 37°C. After washing the target cells with PBS, they were incubated with neutrophils in a U-bottom 96-well plate at a 5:1 ET ratio in the absence or presence of 5 μg/mL therapeutic antibodies against HER2/neu or EGFR. Samples were fixed with stopbuffer containing 0.5% PFA, 1% BSA, and 20 mmol/L NaF and measured by flow cytometry as described below. After gating for the neutrophil population, the mean fluorescent intensity of cells positive for DiO/DiD were determined.
Live-cell imaging

Target cells were cultured on glass coverslips (25 mm or 30 mm diameter), and their membranes were labeled with DiD according to the manufacturer’s instructions (5 μmol/L, catalog number V22887; Invitrogen). Target cells were incubated with neutrophils in a 5:1 E:T ratio for time periods up to 4 hours at 37°C and 5% CO2 in IMDM culture medium (Gibco) supplemented with 20% FCS. Imaging was started within 5 minutes after initiation of the experiment and was performed at the indicated times and intervals using a Leica TCM sp8 confocal microscope (Leica).

Mouse models

Experiments for the Ba/F3 peritoneal model (discussed below) were performed with 12- to 38-week-old male and female human FcαR transgenic (Tg) mice, which were generated at UMC Utrecht (33) and backcrossed on a Balb/cByJRj background (Janvier) and maintained in hemizygous breeding. Transgene-negative (NTg) littermates were used as control mice. Experiments for the A431 in vivo model (discussed below) were performed with 17- to 70-week-old female human FcαR transgenic (Tg) mice and backcrossed on an SCID background (CB17/lcr-Prkdcscid/crl, Charles River) and maintained in hemizygous breeding. Mice on SCID background were used to allow engraftment of A431 cells and has the advantage to not exaggerate the role of CD47–SIRPα interactions, as is the case in, i.e., NOD SCID mice, in which NOD SIRPα exhibits a ~50 times higher affinity for human CD47 (34). All mice were bred in the specific pathogen-free facility of the Central Animal Laboratory of Utrecht University; all animal experiments were conducted in accordance with, and with the approval of, the Animal Ethical Committee of the UMC Utrecht (license A.V.D115002016410).

The Ba/F3 peritoneal model was described previously (9). Briefly, Ba/F3-HER2 and Ba/F3 cells were labeled with, respectively, 2 μmol/L or 10 μmol/L CT violet (Invitrogen, Thermo Fisher) for 15 minutes at room temperature and mixed thereafter at a 1:1 ratio. In total, 1 × 10⁷ cells were injected per mouse intraperitoneally (i.p.) in 200 μL PBS.

My-1 (200 μg) was used to block mouse SIRPα in vivo, which was injected 2 days before tumor cell injection and mixed with the treatment consisting of anti-HER2-IgG1 or anti-HER2-IgA2 (100 μg) injected intraperitoneally directly after the injection of tumor cells. Antibodies were diluted in PBS and injected in a volume of 200 μL. Sixteen hours later, these mice were euthanized, the peritoneum washed with PBS containing 5 mmol/L EDTA, the absolute number of Ba/F3-HER2 and Ba/F3 was determined by flow cytometry using TruCount tubes (BD Biosciences), and the ratio of Ba/F3-HER2 and Ba/F3 was calculated. Effector cells in the peritoneum were determined using specific antibodies (described below), and their relative amount was normalized to a constant amount of beads (Invitrogen). Gating strategies for defining the different subpopulations are depicted in Supplementary Fig. S1.

For the A431 in vivo model, mice were injected with 5 × 10⁵ A431-CD47KO cells on the right flank, and 5 × 10⁵ A431 scrambled

![Figure 1. Downregulation of CD47 expression promoted IgA-mediated ADCC by neutrophils.](image)
(A431-SCR) control cells were injected in the same mouse on the left flank. On day 6, all the mice had palpable A431-CD47KO and A431-SCR control tumors; thus, intravenous treatment via the tail vein was started with a single injection of 50 μg IgG cetuximab or 250 μg anti-EGFR-IgA2. Antibodies were diluted in PBS and injected in a volume of 100 μL. Anti-EGFR-IgA2 has a shorter half-life compared with cetuximab; therefore, anti-EGFR-IgA2 treatment was continued by intraperitoneal injections on days 8, 10, 13, 15, and 17 (250 μg). Tumor outgrowth was measured twice a week with calipers, and volume was calculated as length × width × height.

Bead binding assay

The SIRPα-expressing murine macrophage cell line RAW 264.7 was used to show the blocking capabilities of the mouse anti-SIRPα antibody MY-1, using a bead-binding assay. In short, 10^6 RAW 264.7 cells were seeded in a V-shaped 96-well plate and washed with PBS containing 0.1% BSA. Cells were then incubated in PBS containing 0.1% BSA and 10 μg/mL anti-SIRPα blocking antibody (MY-1) and kept on ice for 30 minutes. Another vial containing 1 μg/mL mouse CD47-Fc together with 20 μg/mL goat anti-human Alexa647 and 1% normal rat serum was incubated on ice for 30 minutes. Cells were washed with PBS containing 0.1% BSA, after which 50 μL mouse CD47 goat anti-human Alexa647-NRS complex was added to cells and kept on ice for 30 minutes. Finally, cells were washed in HEPES buffer, and binding was determined using flow cytometry as described below.

Flow cytometry

Effector cells in the peritoneum were determined after incubation with PBS containing 5% normal mouse serum (Equitech-bio) for 45 minutes at 4°C to 7°C. Subsequently, the following fluorescently labeled antibodies were used for 45 to 60 minutes at 4°C to 7°C to stain for different effector cell types: B220 (RA3-6B2), I-A/I-E (M5/114.15.2), CD8 (1H10), CD45 (30-F11), CD4 (RM4-5), F4/80 (BM8), all from Biolegend, and CD11b (m1/70, BD Biosciences).

After excluding Ba/F3 cells from the analysis based on their CT violet staining, granulocytes were identified as Ly-6G^+/CD11b^+ and F4/80^-; macrophages were identified as F4/80^-/CD11b^+; and Ly-6G lymphocytes were analyzed by first excluding Ba/F3 cells, F4/80^-/CD45^- macrophages and dead cells (7AAD^+) followed by CD45 selection where B cells were identified as B220^-/I-A/I-E^+ and T cells as being CD4^+ or CD8a^-.

Saturation of SIRPα in vivo was determined by comparing staining for the injected MY-1 with anti-rat Ig (BD Biosciences) with ex vivo-added MY-1 or isotype control and anti-rat Ig both followed by staining for macrophages and granulocytes. Measurements were performed on a FACSCanto II (BD Biosciences); data were analyzed using FACSDiva software (BD Biosciences).

For the CD47-bead binding assay, goat anti-human Alexa647 IgG (H + L; Invitrogen) was used at a concentration of 20 μg/mL.

Cell lines were analyzed for expression of HER2 using trastuzumab, EGFR using cetuximab, human CD47 using B6H12 (18), murine CD47 using miap301 (eBioscience), and murine SIRPα using MY-1 (30).

Data analysis and statistics

Statistical differences between two groups were tested using (paired) t test; multiple comparisons were tested using two-way ANOVA with Tukey correction for multiple tests or one-way ANOVA test followed by Sidak or Dunett post hoc test for correction of multiple comparison by GraphPad Prism (GraphPad Software, versions 7 and 8). A P value less than 0.05 was considered statistically significant.
Results

IgA-mediated ADCC was restricted by CD47–SIRPα interactions

IgA-mediated ADCC by neutrophils is superior to IgG-mediated ADCC (9–11). However, it is still unknown whether this is also controlled by CD47–SIRPα checkpoint inhibition, as is seen with IgG antibodies (10–12). This is particularly relevant as IgA-mediated killing is induced by a unique FcR, i.e., FcαRI (CD89), not by FcγR. Inhibition of CD47–SIRPα interactions significantly increases IgG-mediated phagocytosis of cancer cells by macrophages and killing by stimulated neutrophils, while neutrophils are

Figure 3.

IgA-mediated trogocytosis of cancer cells by neutrophils. A–F, Representative live-cell imaging stills at different time points of a neutrophil binding to an anti-HER2-IgA2-opsonized cancer cell and trogocytosing pieces of fluorescent cancer cell membrane. Scale bar, 10 μm. G, Trogocytosis of SKBR3 cells opsonized with increasing concentrations of trastuzumab or anti-HER2-IgA2. Tmab, trastuzumab. ADCC (H) and trogocytosis (I) of anti-EGFR-IgA2-opsonized A431 cancer cells by freshly isolated neutrophils isolated from healthy controls or FHL5 patients (for patient characteristics, see patients B and C described in ref. 32) combined without (white background) or with CD47–SIRPα inhibition by knockout of CD47 in the target cells (gray background). ADCC (J) and trogocytosis (K) of anti-HER2-IgA2-opsonized SKBR3 cells by freshly isolated neutrophils isolated from healthy controls or CGD patients (for patient characteristics, see Materials and Methods), combined without (white background) or with CD47–SIRPα inhibition by knockdown of CD47 in the target cells (gray background). Data shown are means ± SEM pooled from two separate experiments with two replicates of 2 individual FHL5 patient samples and 11 total healthy controls (H and I), three separate experiments with 1 to 2 donor samples per experiment with n = 4 (G) individual donors. Statistics shown in G are for the highest antibody concentrations using two-way ANOVA with Tukey correction for multiple tests. No statistics could be performed on FHL5 patient data in H and I due to low patient count (n = 2). Statistics shown in J and K using one-way ANOVA with Sidak correction for multiple tests (ns, nonsignificant; *, P < 0.05; **, P < 0.01; ***, P < 0.001). MFI, mean fluorescence intensity.
normally quite inefficient in performing IgG-mediated ADCC of cancer cells (18, 20, 21). To investigate the role of SIRPα as a checkpoint in IgA-mediated ADCC by freshly isolated neutrophils, we used the HER2/neu-positive breast cancer cell line SKBR3 and the EGFR-positive epidermoid carcinoma cell line A431 (Fig. 1A). Furthermore, we verified CD47 expression across the two different cell lines (Fig. 1A). Disruption of CD47–SIRPα interactions was achieved by either genetically decreasing CD47 expression (CD47KD) on SKBR3 or by genetically deleting CD47 expression (CD47KO) on A431 cells (Fig. 1A), without interfering with HER2/neu or EGFR (Fig. 1A). In 3Cr-release assays, the different IgA2 antibodies targeting HER2/neu or EGFR significantly increased neutrophil ADCC toward the opsonized target cells compared with their respective IgG antibodies (Fig. 1B and C). Downregulation of CD47 by either CD47KD or by CD47KO led to significant increases in IgA-mediated ADCC by freshly isolated neutrophils compared with unmodified target cells (Fig. 1B and C). Even at lower E:T ratios (below 25:1), this enhancement in neutrophil-mediated killing of antibody-opsonized A431 cells was observed for both IgG and IgA antibodies when deleting CD47–SIRPα interactions (Supplementary Fig. S2A). Stimulation of neutrophils by GM-CSF dose dependently increased their potency to perform ADCC with either IgG or IgA therapeutic antibodies in the absence or presence of CD47 on the tumor cells (Supplementary Fig. S2B). Experiments shown in Fig. 1 demonstrated the importance of CD47–SIRPα interactions on IgA-mediated neutrophil ADCC by genetically interfering with CD47 expression on tumor cells.

Next, we wanted to investigate the effect of directly blocking CD47–SIRPα interactions. We compared IgA-mediated killing by neutrophils against wild-type and CD47KD SKBR3 or CD47KO A431 cells (Fig. 2A and B) and with the addition of either CD47 (Fig. 2B) or SIRPα (Fig. 2C) blocking antibodies. Both the blockade of CD47 or SIRPα by genetic disruption or antibodies resulted in significantly enhanced neutrophil-mediated ADCC by IgA antibodies (Fig. 2A–C). Thus, enhancement of neutrophil ADCC was independent of the method targeting the CD47–SIRPα axis. CD47–SIRPα checkpoint inhibition by CD47 or SIRPα antibodies significantly increased neutrophil-mediated ADCC of additional target cells such as EGFR+ Kyse-30 and HER2/neu+ BT-474 cells by IgA2 variants of cetuximab and trastuzumab, respectively (Supplementary Fig. S2C and S2D). Together, these results showed that the ADCC capacity of IgA antibodies is enhanced by CD47–SIRPα checkpoint blockade, similar to ADCC mediated by IgG therapeutic antibodies.

**Trogocytosis by neutrophils of IgA-opsonized cancer cells**

The cytotoxic mechanism by which neutrophils perform ADCC against cancer cells has not been well characterized. Recently, we described that IgG-mediated neutrophil ADCC involves a unique cytotoxic process, termed trogocytosis, and that trogocytosis (the acquisition of target cell plasma membrane fragments by neutrophils) is instrumental in this trogocytic killing mechanism (32). To investigate whether IgA opsonization of cancer cells also induced trogocytosis, as described for IgG (32), we used live-cell imaging. IgA antibody–opsonized cancer cells were labeled with a fluorescent membrane dye, and neutrophils were added as effector cells. We detected neutrophils taking up pieces of IgA-opsonized cancer cell membrane (Fig. 3A–F), similar to IgG-opsonized cancer cells. This was further quantified using a FACS-based trogocytosis assay, which showed enhanced trogocytosis of anti-HER2-IgA2 opsonized breast cancer cells compared with the trastuzumab control (Fig. 3G). CD47–SIRPα checkpoint inhibition led to a significant increase in neutrophil-mediated trogocytosis compared with anti-HER2-IgA2 alone (Fig. 3G; Supplementary Fig. S2E).

We describe that IgG-mediated ADCC by neutrophils is not dependent on their classic antimicrobial effector mechanisms, such as granule exocytosis and NADPH oxidase activity (32, 35, 36). To rule out the contribution of these antimicrobial effector functions in IgA-mediated tumor cell destruction, as seen with IgG (32), we made use of neutrophils from rare familial hemophagocytic lymphohistiocytosis (FHL)-5 patients, which have defective granule release due to a mutation in the STXBP2 gene encoding the munc18-2 protein (37). Neutrophils from CGD patients are unable to produce reactive oxygen species, which is caused by mutations in components of the NADPH oxidase (38, 39). IgA-mediated trogocytosis and ADCC by neutrophils of these patients were not abrogated, confirming that neutrophil NADPH oxidase and granules and their components do not indeed form an absolute requirement for ADCC of IgA-opsonized cancer cells (Fig. 3H–K).

To further evaluate the direct contribution of neutrophil-induced trogocytosis to FcεR-mediated ADCC, we used a number of selected inhibitors of intracellular targets (targeting the tyrosine kinase Syk, PI3K, myosin light chain kinase, intracellular calcium) that we previously identified operating downstream of FcεR during IgG-mediated neutrophil trogocytosis of cancer cells (32). Our results demonstrated that these inhibitors, as well as CD11b/CD18-...
blocking antibodies, prevented IgA-mediated trogocytosis and IgA-mediated ADCC in all of the tested cases (Fig. 4A and B). This was true with or without CD47–SIRPα inhibition, indicating that inhibition of CD47–SIRPα directly promoted the neutrophil intracellular signaling that drives these pathways of trogocytosis and/or killing, rather than inducing alternative signaling downstream of the FcγR (Fig. 4A and B).

**IgA induced cytotoxicity of tumor cells during inhibition of CD47–SIRPα interactions in mouse models**

To further investigate the effect of blocking CD47–SIRPα interactions in combination with IgA therapeutic antibody in an *in vivo* setting, we used mice expressing human FcγRI (9) on a SCID background. With these mice, we established a long-term mouse xenograft model using the human epidermoid cell line A431. We compared tumor growth of CD47-expressing A431 cells (A431 ctrl) to A431-CD47KO. These two cell lines were injected in the right or left flank of the same mouse (Fig. 5A). Groups of mice were systemically treated with either PBS, cetuximab, or anti-EGFR-IgA2 when palpable tumors formed (day 6; Fig. 5A). After 17 days, tumor volume was significantly reduced only in the CD47KO A431 tumors after anti-EGFR-IgA2 treatment compared with cetuximab, whereas control tumors displayed no volume alteration (Fig. 5B and C).

To further examine the role of neutrophils as effector cells *in vivo*, we made use of a syngeneic mouse model expressing human FcγRI (9) as previously described (9), where we compared the efficacy of anti-HER2/neu or EGFR antibodies against HER2/neu or EGFR in combination with MY-1, blocking mouse SIRPα. No antibody-dependent neutrophil-mediated killing of the target cell lines was observed in the presence of an intact CD47–SIRPα signaling axis (Fig. 6A and C). However, the IgA2 variant of the anti-HER2/neu therapeutic antibody significantly enhanced ADCC by neutrophils expressing FcγRI (Fig. 6B). This effect could be further increased by SIRPα checkpoint blockade for both HER2/neu and EGFR-expressing Ba/F3 cells (Fig. 6B and C). These data indicated that the use of IgA therapeutic antibodies caused significantly higher ADCC compared with IgG therapeutic antibodies, specifically after blocking SIRPα with MY-1, which enhanced killing of HER2/neu and EGFR-expressing tumor cells by murine neutrophils.

MY-1 was used to block SIRPα in a syngeneic *in vivo* mouse model as previously described (9), where we compared the efficacy of anti-HER2/neu or EGFR antibodies against HER2/neu or EGFR in combination with MY-1, blocking mouse SIRPα. No antibody-dependent neutrophil-mediated killing of the target cell lines was observed in the presence of an intact CD47–SIRPα signaling axis (Fig. 6A and C). However, the IgA2 variant of the anti-HER2/neu therapeutic antibody significantly enhanced ADCC by neutrophils expressing FcγRI (Fig. 6B). This effect could be further increased by SIRPα checkpoint blockade for both HER2/neu and EGFR-expressing Ba/F3 cells (Fig. 6B and C). These data indicated that the use of IgA therapeutic antibodies caused significantly higher ADCC compared with IgG therapeutic antibodies, specifically after blocking SIRPα with MY-1, which enhanced killing of HER2/neu and EGFR-expressing tumor cells by murine neutrophils.

**Figure 5.**

Interference with CD47–SIRPα interactions enhanced tumor eradication in a xenograft long-term *in vivo* model. A, Schematic overview of the *in vivo* xenograft model and the injection scheme. Long-term *in vivo* tumor growth comparing the maternal A431-SCR cell line (B) with one with no expression of CD47 (A431-CD47KO; C). Treatment was started when mice had palpable tumors (day 6) with either PBS (light gray line), a single intravenous injection of 50 μg cetuximab (black line), or an intravenous injection of 250 μg anti-EGFR-IgA2 followed by four intraperitoneal injections of 250 μg (dark gray line) to compensate for the shorter half-life of IgA compared with IgG. Tumor outgrowth was measured with calipers, and volume was calculated as length × width × height. Data shown are means ± SEM from one experiment with 8 mice per group in B and C. Statistics performed are for day 17 using two-way ANOVA with Tukey correction for multiple tests (ns, nonsignificant; *, P < 0.05). Cmab, cetuximab.
Figure 6. Inhibition of SIRPα effectively triggered syngeneic tumor cell killing by neutrophils. A, Expression of CD47 and HER2 or EGFR in Ba/F3 cells transfected with human HER2 or EGFR, respectively. B, ADCC of trastuzumab or anti-HER2-IgA2 (either at 5 μg/mL)–opsonized Ba/F3-HER2 cells by mouse neutrophils isolated from wild-type (NTg) or FcγR-transgenic (FcγR-Tg) mice combined without (white background) or with inhibition of SIRPα (MY-1; gray background). C, Same as B but with cetuximab or anti-EGFR-IgA2–opsonized Ba/F3-EGFR cells. D, In vivo inhibition of SIRPα (MY-1) was combined with trastuzumab or anti-HER2-IgA2 treatment in a short intraperitoneal model. The ratio of Ba/F3-HER2 and Ba/F3 cells in wild-type mice (PBS, isotype + Tmab, anti-SIRPα (MY-1) + Tmab) or FcγR-transgenic mice (isotype + anti-HER2-IgA2, anti-SIRPα (MY-1) + anti-HER2-IgA2) in the peritoneal washes was determined by flow cytometry. E, Number of granulocytes (Ly6G+/CD11b+) present in the peritoneal cavity at the end of the experiment in D, F, Overview of all cells present in the peritoneal cavity during the experiment shown in D. G, Inhibition of SIRPα (MY-1) is combined with anti-HER2-IgA2 and neutrophil depletion (Ly6G) in an in vivo mouse experiment, showing the ratio of Ba/F3-HER2 and Ba/F3 cells in wild-type mice (isotype, anti-SIRPα (MY-1), anti-SIRPα (MY-1) + anti-HER2-IgA2 (NTg)) or FcγR-transgenic mice (isotype + anti-HER2-IgA2, anti-SIRPα (MY-1) + anti-HER2-IgA2, anti-SIRPα (MY-1) + anti-HER2-IgA2 + Ly6G). Data shown for B and C are means ± SEM pooled from two experiments with 2 mice per experiment with a total of n = 4 individual mice. Data shown for D–F are means ± SEM from one experiment with 6 mice per group with A representative of n = 2, B n = 3–6, C n = 6, and D n = 6 individual mice. Statistics shown for B and C are calculated by one-way ANOVA with Dunnett correction for multiple tests. Statistics shown for D and G are calculated by paired one-way ANOVA with Sidak correction for multiple tests. Statistics performed for E are calculated by one-way ANOVA with Sidak correction for multiple tests (ns, nonsignificant; *, P < 0.05; **, P < 0.01; ***, P < 0.001). Cmab, cetuximab; Tmab, trastuzumab.
HER2 IgG or IgA subclasses in the presence or absence of SIRPα blockade. Fluorescent Ba/F3 and Ba/F3-HER2 cells were injected in the peritoneal cavity and combined with a therapeutic antibody against HER2/neu in the presence or absence of SIRPα inhibition. Saturation of the SIRPα receptor with MY-1 on both macrophages and neutrophils was confirmed using flow cytometry (Supplemental Fig. S3C and S3D). Comparable to the in vitro setting, blocking SIRPα in vivo by MY-1 led to a significantly and substantially increased reduction in tumor load compared with the use of either IgG or IgA therapeutic antibodies alone (Fig. 6D). As previously reported (40), the antibody-mediated reduction in tumor load was accompanied by a significant influx of granulocytes, particularly in the condition where MY-1 was combined with therapeutic antibody against HER2/neu. This neutrophil influx was more pronounced when using anti-HER2-IgA2 compared with trastuzumab (Fig. 6E and F; ref. 41). Only a small decrease in macrophages, no alterations in the influx of other leukocyte populations, was detected under this condition (Fig. 6F; Supplemental Fig. S3E–S3H). Together, our data showed that checkpoint blockade of CD47–SIRPα increased the therapeutic potential of anti-HER2-IgA2 in a syngeneic mouse model, which was accompanied by an increase in granulocyte recruitment to the tumor site.

To confirm that neutrophils are the effector cell population responsible for the killing of the Ba/F3–HER2 cells in our syngeneic mouse model, we depleted neutrophils by the use of anti-Ly6G antibody (Supplemental Fig. S4A). This depletion did not result in significant changes in other leukocyte populations (Supplemental Fig. S4B–S4E). When neutrophils were depleted from the mice, only limited ADCC occurred when using both anti-EGFR-IgA2 and MY-1 (Fig. 6G). These results show that in this model neutrophils are the dominant effector cells required for therapeutic clearance with anti-HER2-IgA2 in the presence of CD47–SIRPα checkpoint inhibition.

Altogether, our results demonstrated that IgA-mediated tumor therapy was restricted by CD47–SIRPα interactions in vitro and in vivo—using both syngeneic and xenogenic mouse models. This restriction could be therapeutically overcome by CD47– or SIRPα-blocking antibodies.

**Discussion**

In this article, we demonstrated that neutrophil-mediated cytotoxicity of cancer cells by IgA antibodies against HER2/neu or EGFR was limited by CD47–SIRPα checkpoint inhibition, both in vitro and in vivo. This new finding implies that CD47–SIRPα axis may be a promising strategy for enhancing IgA-mediated antitumor effects with neutrophils as effector cells. For IgG, the combination of therapeutic antibodies, including rituximab, cetuximab, and trastuzumab, and inhibition of CD47–SIRPα interactions show promising results in enhancing antibody therapy in several preclinical mouse models (18, 20, 21, 42). Thus, CD47 antibodies and SIRPα-Fc proteins are currently being tested in phase I/II clinical trials in combination with IgG therapeutic antibodies (24, 25). Our study implies that the combination of innate checkpoint inhibition and IgA antibodies against tumor antigens may be similarly promising.

We provide evidence that neutrophils are the dominant effector cells against IgA antibody–opsonized cancer cells in our syngeneic in vivo model. This is in contrast to our previous work where macrophages were the dominant effector cells eliminating IgA-opsonized tumor cells in Ba/F3 tumors overexpressing EGFR (9). In this particular model, the CD47–SIRPα axis was still intact, despite the use of different systems and therapeutic antibodies. This could contribute to a different effector cell population being activated and recruited against the antibody-opsonized cancer cells. Collectively, our data demonstrate that, depending on the therapeutic antibody–antigen combination, both neutrophils and macrophages can mediate cytotoxicity by IgA antitumor antibodies. These data suggest that upon the additional blockade of the CD47–SIRPα axis, neutrophils are recruited and contribute to the killing process. This neutrophil recruitment could potentially be the result of an IgA-induced feed-forward loop that depends on paracrine effects of neutrophil chemottractants such as LTB4 (40).

Most research on the development of cancer immunotherapies has focused on either stimulating antibody-mediated effector functions of the innate immune system or enhancing an adaptive immune response. In the case of targeting CD47–SIRPα interactions, the initial focus of research has been on activation of the innate immune system leading to increased ADCC by neutrophils and enhanced ADCP by macrophages (18, 20, 21, 42). Checkpoint inhibition of the CD47–SIRPα axis increases effective T-cell responses against tumors by activating CD8+ T cells and suppressing CD4+ T cells (43). The mechanism by which CD47–SIRPα checkpoint inhibition stimulates a cytotoxic T-cell response against tumors could possibly involve enhanced macrophage–myeloid dendritic cell–mediated antigen presentation in response to an increased uptake of tumor material (43, 44), or perhaps even an increase and contribution of neutrophil antigen presentation (45, 46). Although further research is needed to confirm this possibility, combining IgA-based antibody therapy against cancer with inhibition of CD47–SIRPα interactions could lead to an efficient long-lasting adaptive immune response.

An important drawback of using IgA anticancer antibodies clinically is the relatively short half-life of IgA antibodies in vivo (9), even though this drawback in half-life is more apparent in mice. In mice, the neonatal Fc receptor (FcRn) has a stronger binding capacity for human IgG, which thereby exaggerates the half-life of human IgG compared with human IgA in mice (47). There may be various ways to solve the relatively short half-life of IgA antibodies in humans. We and others have described ways to extend the serum half-life of IgA antibodies via glyco-engineering strategies (48, 49) or by inducing their binding to the neonatal Fc receptor (50, 51). Another potential solution for increasing both the half-life and engaging an optimal population of effector cells was suggested by Georgiou and colleagues, who generated an antibody with an Fc region that could simultaneously bind both Fcγ receptors and FcRα, a so-called IgGA antibody (52). This antibody engages NK cells, macrophages, monocytes, and neutrophils and activates the complement system (52). Future study is required to determine whether combining engineered IgA antibodies with inhibitors of CD47–SIRPα interactions is effective in triggering anticancer neutrophils.

Collectively, our findings show that IgA cancer immunotherapy is restricted by CD47–SIRPα checkpoint inhibition, providing support for the idea that targeting the CD47–SIRPα checkpoint could be used to potentiate the antitumor efficacy of IgA therapeutic antibodies in cancer.

**Disclosure of Potential Conflicts of Interest**

K. Franke has a research collaboration regarding CD47/SIRPα targeting in oncology. T.K. van den Berg reports receiving a commercial research grant from Synthon Biopharmaceuticals and is an inventor on patent application WO2009/131453 A1, owned by Sanquin, licensed to Synthon Biopharmaceuticals, related to the targeting of CD47–SIRPα in cancer. J.H.W. Leunis has ownership interest (including
patents) in and is a consultant/advisory board member for TigaTx. H.L. Matlung has a research collaboration regarding CD47/SIRPα targeting in oncology. No potential conflicts of interest were disclosed by the other authors.

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