Targeting CD47-SIRPα interactions for potentiating therapeutic antibody-mediated tumor cell destruction by phagocytes

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Genetic variation of Fc receptors and SIRP in neutrophil ADCC towards cancer cells

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ABSTRACT

Anticancer antibodies act through different mechanisms, including antibody-dependent cellular cytotoxicity (ADCC) triggered via Fcγ receptors on phagocytes. We recently found that interference with CD47-SIRPα interactions promotes neutrophil ADCC, but the magnitude of this response varies among individuals. Both FcγR and SIRPα display genetic variation and we investigated whether this could explain the variability in ADCC. We performed an integrated genetic analysis. ADCC by activated human neutrophils towards Trastuzumab-opsonized breast cancer cells involves a combination of the different FcγRs, with FcγRIla and FcγRIIIb functioning in one activation route, whereas FcγRI primarily operates in a partially redundant parallel pathway. Neutrophils with FcγRIla-131H or FcγRIIIb-NA1 polymorphic variants displayed higher killing capacity, relative to their alternative variants FcγRIla-131R or FcγRIIIb-NA2, respectively. Furthermore, ADCC induced through the different FcγR and their genetic variants was enhanced to a similar degree (~1.5 to 2-fold) by the targeting of CD47-SIRPα interactions, and there were no significant differences between the most prevalent Caucasian SIRPα polymorphic variants. Thus, neutrophil ADCC capacity is directly related to FcγRIla or FcγRIIIb polymorphisms, and interference with CD47-SIRPα interactions enhances ADCC independently of FcγR (geno)type, suggesting that targeting of CD47-SIRPα interactions might be a generic strategy for potentiating the efficacy of antibody therapy in cancer.
INTRODUCTION

Therapeutic antibodies are widely used for the treatment of certain forms of cancer. In addition to direct growth effects on the cancer cells, monoclonal antibodies can opsonize the tumor cells, thus turning them into targets for immune-mediated destruction by either antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and/or through complement-dependent cytotoxicity (CDC). Although the exact contribution of ADCC to antibody therapy in cancer patients is not known, the reported associations between the clinical efficacy of different cancer therapeutic antibodies, including Trastuzumab, and polymorphisms in FcγRIIa expressed on myeloid cells and FcγRIIIa expressed on NK cells, suggest a role for ADCC mediated by both cell types in patients. A considerable number of genetic FcγR variants have been described within the FcγR2/3 locus. Of interest, preliminary studies indicated that some of these, such as e.g. the FcγRIIa H/R131 single nucleotide polymorphism (SNP), are linked to responsiveness to antibody therapy in cancer patients and also to the ADCC capacity of neutrophils. However, the interpretation of such findings is not straightforward at all, mainly because many of the genetic variants are in linkage disequilibrium with others, which makes a direct comparison difficult if not impossible. Thus, to obtain insight into the contribution of FcγR variation to antibody mediated cancer cell destruction, while avoiding such bias, an integrated analysis of FcγR genotype needs to be performed along with proper stratification. We have previously developed a multiplex ligation-dependent probe amplification (MPLA) assay to determine the relevant polymorphic and gene copy number (CNV) variations within the FcγR2/3 locus. In the present study we have applied this method to investigate direct associations between the relevant genetic FcγR variants and neutrophil ADCC capacity.

Another important aspect of cancer therapeutic antibodies is that their clinical efficacy is rather limited. In fact, despite their high degree of specificity, the potency of cancer therapeutic antibodies is generally too low to justify their use in the absence of additional non-specific treatment regimens, such as chemotherapy. Chemotherapeutics themselves are carcinogenic and cause many other side effects, such as leukopenia, which would be anticipated to compromise ADCC rather than to promote it. Therefore, there is a pertinent need to improve the efficacy of cancer therapeutic antibodies. We and others have previously demonstrated that targeting the interaction between CD47 expressed on cancer cells and the inhibitory immunoreceptor SIRPa expressed on myeloid cells substantially potentiates the capacity of anti-cancer antibodies, including Trastuzumab and Rituximab. Consistent with this notion the clinical response of either breast cancer patients treated with Trastuzumab or Non-Hodgkin lymphoma patients treated with Rituximab was better when CD47 expression levels in the tumor cells were lower. However, it is not exactly known how SIRPa signaling inhibits ADCC. Does it for instance do so by inhibiting all different FcγRs and their variants expressed on phagocytes, or by selectively affecting some of them? Furthermore, there are different polymorphic variants of SIRPa within the population and although the two variants most commonly found in Caucasians do not differ with respect to their CD47 binding capacity, it is not known whether they differ in other aspects of their functioning, including their capacity to signal and to modulate effector function, such as ADCC.
In the present study we have evaluated 62 healthy individuals to investigate a possible association between FcγR genetics and function in neutrophil ADCC. In addition, we have explored whether the capacity to potentiate ADCC through the manipulation of CD47-SIRPα interactions is affected by SIRPα polymorphisms. We have observed that all of the different FcγRs expressed on neutrophils participate in ADCC towards Trastuzumab-opsonized breast cancer cells. In doing so FcγRIIa and FcγRIIIb were shown to function in a common activation route, whereas FcγRI primarily operates in the parallel pathway acting in synergy. Furthermore, we have identified independent associations between either the FcγRIIa-H/R131 or the FcγRIIIb-NA1/NA2 polymorphisms and neutrophil ADCC. Finally, we show that the capacity to induce ADCC through the different FcγRs and their genetic variants can be potentiated to the same extent (~1.5 to 2-fold) by the manipulation of CD47-SIRPα interactions, and that this is not affected by the major SIRPα polymorphisms present in the Caucasian population. These findings demonstrate for the first time a direct independent link between neutrophil FcγR genetics and function in ADCC. In addition, they suggest that the interference with CD47-SIRPα interactions might be a generic method to enhance the efficacy of antibody therapy in cancer patients.

MATERIALS AND METHODS

Cells, culture and antibodies
The Her2/Neu-positive human breast cancer carcinoma cell line SKBR-3 was cultured in IMDM medium (Gibco, Paisley, UK) supplemented with 20% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (i.e. complete RPMI) at 37°C, 5% CO2. SKBR-3 cells in which CD47 had been knocked down by shRNA, referred to as SKBR-3-CD47KD cells, express 10-15% of normal CD47 surface levels, as described before 13. In experiments with SKBR-3 cells, cells expressing empty vector shRNA were used as the control. The following antibodies were used to detect expression of FcγRs: anti-human CD64 (Clone 10.1, mouse IgG1, BD Pharmingen, San Diego, CA), anti-human CD32 (Clone AT10, mouse IgG1, AbD Serotec, Oxford, U.K.), anti-human CD16 (Clone 3G8, mouse IgG1, BD Pharmingen, San Diego, CA). FcγRs antagonistic antibodies were used in ADCC at a final concentration of 10 μg/ml: anti-human CD64 (Clone 10.1, BioLegend), anti-human CD32 (Clone AT10, mouse IgG1, AbD Serotec, Oxford, U.K.), anti-human CD16 (Clone 3G8, mouse IgG1, BD Pharmingen, San Diego, CA). FcγRs antagonistic antibodies were used in ADCC at a final concentration of 10 μg/ml: anti-human CD64 (Clone 10.1, BioLegend), anti-human CD32 (Clone AT10, mouse IgG1, AbD Serotec, Oxford, U.K.), anti-human CD16 (Clone 3G8, BioLegend). Antibodies were used as either intact antibodies or F(ab’)2-fragments and pilot ADCC experiments with neutrophils from 8 different individuals demonstrated that both were equally effective (data not shown), thereby excluding the so called ‘Kurlander effect’ 17.

Isolation of human neutrophils from healthy donors
Neutrophils were isolated from n=99 healthy Caucasian volunteers 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 18. Neutrophil preparations were ≥ 95% pure, with the contaminating cells being mostly eosinophils. Cells were cultured in complete RPMI medium, in the presence of 10 ng/ml clinical grade G-CSF (Neupogen; Amgen, Breda, The Netherlands) and 50 ng/ml recombinant human Interferon-γ (Pepro Tech...
Inc, USA) at a concentration of $5 \times 10^6$ cells/ml for 16 h. Afterwards the cells were washed and prepared for analysis by ADCC assay.

**Analysis of FcγR and SIRPα polymorphisms and CNV**

Genotyping of $n=62$ out of the indicated 99 individuals for FcγRIIa, FcγRIIIb, FcγRIIc polymorphism was performed using the FcγR-specific Multiplex Ligation-dependent Probe Amplification (MLPA) assay (MRC Holland), using genomic DNA isolated from whole blood with the QIamp® kit (Qiagen, Hilden, Germany). The MLPA assay was performed essentially as described previously. For the present study, the following SNPs and CNV were determined by the MLPA assay: FcγR2A 131 H/R (rs1801274), FcγR2C 13 Q/Stop (rs10917661), FcγR2B/C promoter polymorphism -386C/G (rs3219018), FcγR3B haplotypes (NA1/NA2/SH), and FcγR2C and FcγR3B CNV. The assay also contains probes specific for the SNP determining the open reading frame in exon 3 of the FcγR2C gene, which has been described previously. A distribution of the different variants is provided in Suppl. Table 1A.

We isolated genomic DNA from PBMC of healthy Caucasian donors ($n=83$), CNV and SNPs were determined with a SIRPα specific MLPA assay. In our donor set, SIRPα haplotype was identified by sequencing the V-Ig domain encoded by the third exon (data not shown), namely SIRPα1 and SIRPα2. Probes binding to SIRPα variants which are synthetic oligonucleotides made by Invitrogen (Carsblad, CA) and were designed according to the sequencing results and the available data in http://www.ensembl.org/index.html. For an overview of the specific target sequences of the probes, see Suppl. Table 1A.

In brief, 5µl of DNA (20 µg/µl) isolated from healthy donors was heated at 98°C for 5 minutes in 200µl tubes in a thermocycler with a heated lid (Biometra Uno II). Upon denaturation, 1µl SIRP probe mix is mixed with 1µl control probe mix, 2µl MLPA buffer (EK5 kit MRC-Holland) and 1µl water and added to the DNA at 25°C. This was incubated for 1 minute at 95 °C followed by 16 hours at 60°C. Ligation of annealed oligonucleotides was performed at 54°C by adding 32µl Ligase-65 mix and incubation at 54°C for 15 minutes. After the ligase enzyme was inactivated by heating at 98°C for 5 minutes, 10µl of ligated probes was diluted in a mixture of 4µl PCR buffer and 26µl water at 4°C. Then, 10µl of PCR buffered solution (containing labeled primers) was added at 60°C and the PCR reaction was 38 cycles of 30 second at 95°C, 30 second at 60°C and 60 second at 72°C, followed by an incubation of 20 minutes at 72°C. After that, 1µl 6-FAM-labeled forward primer amplified-probes, 8.8µl highly deionized formamide and 0.2µl LIZ marker were added to a 96 well plate and heated for 10 minutes at 90°C for further separation of the probes by capillary electrophoresis on a sequencer (ABI 3130XL, Applied Biosystems, USA). Data was analyzed using GeneMarker v1.6 sofware.
ADCC assay
ADCC was measured in a 4-hour $^{51}$Cr release assay with SKBR3 and SKBR3-CD47KD as target cells and G-CSF/IFNγ-primed human granulocyte as effector cells, as described 13. In brief, human breast carcinoma cell line were harvested by mild trypsin treatment, and washed tumor cells ($1 \times 10^6$ cells) were collected and labeled with 100 μCi $^{51}$Cr (Perkin-Elmer, USA) in 1 ml for 60 min at 37°C. The target cells ($5 \times 10^3$/well) and effector cells were co-cultured in 96-well U-bottom tissue culture plates in a ratio of E:T=50:1, in the presence or absence of 5 μg/ml trastuzumab in RPMI with 10% (v/v) FCS medium. Aliquots of supernatant were harvested and analyzed for radioactivity in a gamma counter. The percent relative cytotoxicity was determined as \[ \left( \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \right) \times 100\% \]. All conditions were tested in triplicate. In case of neutrophils of a single individual tested on multiple occasions, the average measurements were used in to avoid disturbing the population balance.

Statistical analyses
Statistical differences were determined by Students t-test, or by Grubbs outlier test, as indicated.

RESULTS
Different FcγRs contribute to neutrophil ADCC.
ADCC by both NK cells and phagocytes, including neutrophils and macrophages, contributes to the clinical responsiveness towards therapeutic antibody treatment in cancer patients 6. However, both the clinical efficacy of antibody therapy in cancer, as well effector cell ADCC capacity, varies considerably among individuals 13. We aimed to explore the mechanistic basis underlying this variability, and in particular the role of genetic and functional variation in FcγR expression. We did so by evaluating neutrophil ADCC capacity towards Trastuzumab-opsonized Her2/Neu-positive SKBR-3 breast cancer cells of 62 healthy adult Caucasian individuals. Evaluation of ADCC, performed in the presence or absence of CD47 interference, by either blocking of CD47-SIRPα interactions with antagonistic anti-CD47 F(ab’)2 antibody fragments or by the knock-down of CD47 in tumor cells, showed a substantial inter-individual variation, ranging from 15-82% in the absence of CD47 interference (Fig. 1A,B). As reported previously 13, CD47 interference gave a highly significant and consistent enhancement of ADCC, supporting the idea that CD47-SIRPα interactions and SIRPα inhibitory signaling restrict ADCC performed by phagocytes 13,22,23 (see below). The interference with CD47-SIRPα interactions resulted in a ~1.5 to 2-fold increase in cytotoxicity and this appeared independent of the method of interference used (Fig. 1C).
Figure 1 | Variability in neutrophil ADCC towards Trastuzumab-opsonized SKBR3 cells. Neutrophils from healthy Caucasian subjects (n=99) were tested for ADCC. ADCC was performed for 4 h as described in detail in the Materials and Methods section at an E:T ratio of 50:1 and with 5 mg/ml Trastuzumab. CD47-SIRPα interactions were prevented either by using F(ab’)2 fragments of the blocking anti-CD47 antibody B6H12 (10 mg/ml; black lines) as shown in A, or by using SKBR3 in which CD47 was knocked down to 10-15% of control surface levels (SKBR3-CD47KD; blue lines) as shown in B. For clarity only the values obtained in the presence of Trastuzumab are shown, with the matched values for the two conditions of each donor, i.e. control or CD47 interference by either anti-CD47 F(ab’)2 fragments (A) or by CD47 knock-down (B), connected by lines. Killing in the absence of Trastuzumab was always below 3%. Data shown are means ± SEM of triplicate measurements. Statistics were performed by Student’s t-test. C, The individual ratios of cytotoxicity of the indicated CD47 interference condition and control are shown. The logarithmically transformed data were analysed by paired t test; n.s., non-significant.
Neutrophils express different FcγRs. FcγRs are encoded by a set of distinct genes encoded on chromosome 1, of which some, in particular those of the FCGR2-FCGR3 gene cluster, are subject to considerable variation in terms of polymorphisms and/or surface expression variation due to either gene copy number variation (CNV) or promoter polymorphisms. We wanted to establish the direct contributions of these variations to neutrophil ADCC. Previous studies suggesting associations between certain FcγR variants and ADCC or the clinical response to antibody therapy in cancer patients has focused on individual variations. Since there exists strong linkage disequilibrium between the different genetic FcγR variations, this approach is prone to misinterpretation. To circumvent this we performed an integrated evaluation of the various relevant genetic variants, by means of an MLPA assay that we described before, which allows for appropriate stratification and therefore represents an unbiased way to study direct associations between FcγR genetics and neutrophil ADCC capacity.

First, we investigated the involvement of the various FcγRs in our neutrophil ADCC model by using blocking antibodies against the different FcγRs. Neutrophils cultured in the presence of G-CSF and IFNγ, as used herein and also in a previous study express FcγRI, FcγRIIa, FcγRIIIb, and in a minority of about 15-18% of Caucasian individuals also FcγRIIc. As shown in Fig. 2, blocking experiments with inhibitory antibodies against the different types of FcγRs revealed that Trastuzumab-mediated killing of SKBR-3 cells by activated neutrophils involves a combination of the different FcγRs. In particular, blocking all FcγRs as well as blocking the combination of only the FcγRs with activating signaling capacity, i.e. FcγRI (CD64) and FcγRIIa/c (CD32a/c) essentially abrogated the cytotoxicity. Inhibition of either of the latter two receptors resulted in only minor inhibition, consistent with the anticipated redundancy among these FcγRs. The blocking of FcγRIIIb (CD16b), which lacks intrinsic signaling capacity but is highly expressed on neutrophils (see also supplementary figure 1) and can act as a tethering receptor, also resulted in ~50% inhibition. Interestingly, it appeared that anti-FcγRIIIb antibody could further inhibit ADCC in the presence of anti-FcγRI antibodies, but was hardly able to provide a further reduction in killing through FcγRIIa/c, suggesting that FcγRIIIb primarily functions to support FcγRII, and less so to facilitate binding and signaling by the high affinity FcγRI. It should be noted that when considering human neutrophils as effector cells in ADCC towards tumor cells some studies have also indicated an involvement of both FcγRII and FcγRIIIb, while others show a more exclusive involvement of FcγRII apparently. These differences are likely to be related, at least in part, to differences in target cells and opsonizing antibodies employed, and in the activation state of the neutrophils. Collectively, these results suggest that, at least in our experimental system, all of the available FcγRs contribute to ADCC.
Figure 2 | Involvement of FcγRs in neutrophil-mediated ADCC towards Trastuzumab-opsonized SKBR3.
A) Blocking FcγRs during ADCC by antagonistic antibodies (10 µg/ml) against FcγRI (mAb 10.1), FcγRII (mAb AT10) and/or FcγRIII (mAb 3G8). Experiments were performed with either SKBR3 cells ('control') or with SKBR3-CD47KD cells ('CD47KD'). B) Ratio of cytotoxicity towards CD47 and control SKBR3 cells. Data shown are means ± SEM with neutrophils from n=18 healthy controls tested in 6 independent experiments. Statistics were performed by Students t-test and the p-values are indicated.

**FcγRIIa and FcγRIIIb polymorphisms are associated with neutrophil ADCC capacity**

Previous studies have demonstrated that some of the relevant polymorphisms of FcγRs on neutrophils are associated with the clinical efficacy of therapeutic antibodies. In particular, the higher affinity (at least with respect to human IgG2 binding) allele of FcγRIIa, FcγRII-H131, is associated with a better clinical responsiveness as compared to the alternative FcγRII-R131 allele, and this has been observed for Rituximab treatment in Non-Hodgkin lymphoma²⁴, Trastuzumab treatment in Her2/Neu-positive metastatic breast cancer⁶, and
Cetuximab treatment in colorectal cancer \(^{29}\). However, as indicated above, additional Fc\(\gamma\)R polymorphisms and gene copy number variation (CNV), which are actually known to be in linkage disequilibrium with each other, also exist at the \(Fc\gamma R2/3\) locus \(^{30}\). Thus, to make the appropriate comparisons, these variations need to be evaluated in an integrated fashion. Therefore, all our neutrophil donors were genotyped by MLPA for all Fc\(\gamma\)R variants, and the relationship between Fc\(\gamma\)R genotype and target cell killing in ADCC was explored after appropriate stratification.

When considering the Fc\(\gamma\)RIIa H/R131 polymorphism, the results demonstrated that homozygous H131 donors induce significantly higher cytotoxicity towards target cells than homozygous R131 donors (Fig. 3). This was observed both in the entire group (Fig. 3A) and also when stratified for the most common genotypes having: i) the p.Q13X modification in exon3 of the \(Fc\gamma R2C\) gene, with the corresponding individuals lacking Fc\(\gamma\)RIlc protein expression, designated Fc\(\gamma\)RIlc-STOP, and ii) having 2 gene copies of Fc\(\gamma\)RIIIb, termed Fc\(\gamma\)RIIIb-2x (Fig. 3B). It should be noted that this observation is perhaps somewhat surprising. Trastuzumab is a human IgG1 antibody and although Fc\(\gamma\)RIIa-H131 has a substantially higher affinity for human IgG2 in comparison to Fc\(\gamma\)RIIa-R131, only minor differences in terms of human IgG1 binding have been observed, at least when binding to these Fc\(\gamma\)RIIa variants was evaluated in an ectopic system \(^{31}\). Therefore, these two Fc\(\gamma\)RIIa variants may also differ in other functional properties relevant for cytotoxicity, such as e.g. membrane mobility, complex formation, signaling capacity and/or other relevant parameters. Alternatively, they are associated with different Fc\(\gamma\)Rs expression levels or other relevant, but as yet unknown, phenotypic differences. Firstly, no relationship is observed between Fc\(\gamma\)R expression level and ADCC capacity (Suppl.Fig1 A), suggesting that Fc\(\gamma\)R expression levels are not an important determinant in this context at least. Secondly, as can be seen in Suppl. Fig 1B there were at least no differences in Fc\(\gamma\)RIIa/c expression between H131 and R131 individuals, essentially excluding this possibility too. Of note, there did seem to be a significant difference in Fc\(\gamma\)RI expression levels, with H131 expressing individuals expressing relatively low levels of Fc\(\gamma\)RI (Suppl. Fig.1B), but this could clearly not explain the higher capacity for killing observed in this subgroup, since the H/H131 genotype showed a higher killing capacity in spite of these lower Fc\(\gamma\)RI surface expression levels. The measured difference in ADCC capacity may therefore rather have been an underestimation of the real difference in functional capacity between the Fc\(\gamma\)RIIa H131 and R131 variants. Testing for the Fc\(\gamma\)RIIa-exon6 polymorphism \(^{3}\) (not shown) and the Fc\(\gamma\)RIIc-Stop/ORF variation (not shown) did not reveal a significant relationship with neutrophil killing capacity, although there was a trend that neutrophils of individuals with a Fc\(\gamma\)RIIc-ORF allele, which is known to encode an activating Fc\(\gamma\)R \(^{19}\), had higher levels of cytotoxicity. The lack of significance could well have been caused by the low number (n=8) of individuals with an Fc\(\gamma\)RIIc-ORF allele in our study.
The FcγRIIa R/H131 polymorphism affects neutrophil ADCC towards Trastuzumab-opsonized SKBR3 cells. A) Results for all, including homozygous H131 (n=17), heterozygous H/R131 (n=28) and homozygous R131 (n=17) subjects, or (B) stratified for subjects carrying the FcγRIIC-Stop allele and 2 copies of FcγRIIIb: homozygous H131 (n=12), heterozygous H/R131 (n=21) and homozygous R131 donors (n=12). Experiments were performed with either SKBR3 cells ('control') or with SKBR3-CD47KD cells ('CD47KD'). The ratio of cytotoxicity towards CD47 and control SKBR3 cells is shown in the panels on the right. Data shown are means ± SEM. Statistics were performed by students t-test; *, p<0.05. For analysis ratio t test in Figure 3, data were transformed as logarithm Y=Log (Y), then the transformed data were analysed by paired t test.

There are various levels of potentially relevant genetic variation in FcγRIIIb, which is the only FcγRIII isoform expressed by neutrophils. First, there is FcγRIIIb gene CNV, with individuals expressing 0-3 copies of the gene. The number of encoded FcγRIIIb copies is strongly associated with surface FcγRIIIb expression and function. However, because in the group that we tested the vast majority (n=52) had 2 gene copies of FcγRIIIb gene copies (FcγRIIIb-2x) we could not reliably test for a possible relationship between FcγRIIIb CNV and killing capacity. The most common polymorphic variants of FcγRIIIb are designated FcγRIIIb-NA1 and -NA2. The FcγRIIIb-NA1 and -NA2 nucleotide sequences differ at five positions, with four predicted amino acid differences. As a consequence, the NA2 variant has two additional N-linked glycosylation sites as compared to NA1, and this might have functional consequences. For instance, neutrophils from FcγRIIIb-NA1NA1 individuals are known to bind and phagocytize IgG-opsonized bacteria and red blood cells more efficiently.
than those from -NA1NA2 and -NA2NA2 individuals. We therefore hypothesized that the FcγRIIIb NA1/NA2 polymorphism may also play a role in ADCC. We stratified our analysis to the neutrophil donors with 2 copies of FcγRIIIb (FcγRIIIb-2x). As shown in Figure 4A, individuals with the FcγRIIIb-NA1NA1 genotype induced significantly higher cytotoxicity to tumor cells than individuals with the other genotypes. Further stratification for the most common FcγRIIC-Stop allele gave essentially the same results (Fig. 4B). Again, there were no significant differences in the surface expression of FcγRI, FcγRIIa/c or FcγRIIIb among the NA1/NA2 variants, although there was a tendency that FcγRIIIb-NA1NA1 individuals expressed somewhat higher levels of FcγRIIIb (suppl. Fig. 1C and 1D). Finally, we investigated potential functional interactions between the relevant genotypes. Due to the limited group size we were forced to restrict our analysis to the most common FcγRIIa-H/R131 and the FcγRIIIb-NA1/NA2 variations. As can be seen in Suppl. Fig. 2 the contributions of these two variants described above were not linked to each other and thus independent.

**Figure 4** The FcγRIIIb NA1/NA2 polymorphism affects neutrophil ADCC towards Trastuzumab-opsonized SKBR3 cells. A) Results for subjects stratified for 2 copies of FcγRIIIb, including homozygous NA1 (n=11), heterozygous NA1/NA2 (n=24) and homozygous NA2 (n=17) subjects, or (B) stratified for subjects carrying the FcγRIIC-Stop allele and 2 copies of FcγRIIIb: homozygous NA1 (n=10), heterozygous NA1/NA2 (n=20) and homozygous NA2 donors (n=14). Experiments were performed with either SKBR3 cells ('control') or with SKBR3-CD47KD cells ('CD47KD'). The ratio of cytotoxicity towards CD47 and control SKBR3 cells is shown in the panels on the right. Data shown are means ± SEM. Statistics were performed by students t-test; *, p<0.05. For analysis ratio t test in Figure 4, data were transformed as logarithm Y=Log (Y), then the transformed data were analysed by paired t test.
**Potentiation of neutrophil ADCC by interference with CD47-SIRPα interactions and the contribution of SIRPα polymorphisms**

We have previously demonstrated that CD47-SIRPα interactions restrict neutrophil-mediated ADCC, suggesting that interference with such interactions could be a promising strategy for enhancing therapeutic antibody-dependent tumor cell destruction \(^{13}\). However, it is not known whether CD47-SIRPα interactions have a generalized effect on FcγR signaling, or whether the effects are restricted to specific FcγRs or their variants. To test the contribution of CD47-SIRPα interactions and inhibitory signaling to killing through the various FcγRs and their genetic variants, parallel testing in ADCC was performed with SKBR3 target cells in which CD47 knock-down (CD47KD) was performed. As can be seen in Fig. 2 CD47KD consistently enhanced ADCC towards the tumor cell targets irrespective of the FcγRs involved. Furthermore, it appeared that ADCC via the different available FcγRs, or their combinations, was modulated to the same extent by CD47KD (Fig. 2B). The same was found when the effect of CD47 interference in combination with the different FcγRIIa-H/R131 and FcγRIIIb-NA1/NA2 polymorphisms were evaluated (Figs. 3 and 4, right panels). These data are consistent with a common pathway of regulation in ADCC by CD47-SIRPα that applies to all neutrophil FcγR that can contribute to cytotoxicity.

Finally, we investigated the potential contribution of SIRPα polymorphisms. There exist a considerable number of SIRPα polymorphisms in different ethnic groups \(^{14}\), but DNA sequencing and MLPA analysis of our healthy Caucasian donors (n=83) identified only two polymorphic variants, SIRPα\(_1\) and SIRPα\(_{HRT}\) (Suppl. Table 1A), within this population with frequencies of 16.9% and 41.0% of SIRPα\(_1\) and SIRPα\(_{HRT}\) homozygotes, respectively, and 42.4% heterozygotes (allele frequencies: 59.3% (SIRPα\(_1\)) and 83.4% (SIRPα\(_{HRT}\))). SIRPα gene copy number variation was not observed in our MLPA analysis (not shown). Although these two variants differ in 13 out of 118 amino acid residues in the N-terminal immunoglobulin-like domain responsible for CD47 binding (Suppl. Table 1B), there appear to be no detectable differences in affinity for CD47 \(^{16}\) because most of these polymorphisms occur in areas not covering but just adjacent to the CD47 binding site (Suppl. Table 1B)\(^{34}\). Nevertheless, it seemed possible that there are other differences between these variants, such as differences in expression levels or signaling capacity that could have an impact on downstream signaling and neutrophil ADCC capacity. To our knowledge functional differences among SIRPα polymorphic variants have not been investigated before in primary cells. However, as can be seen in Suppl. Fig. 3 there were no measurable differences in the ADCC capacity between the different SIRPα genotypes. Furthermore, the relative modulatory effect of interference with CD47-SIRPα interactions, which again was ~1.5 to 2-fold, did not differ among the SIRPα genotypes tested, and it was also independent of the method of interference used. It seems therefore that the enhancement of ADCC by manipulation of CD47-SIRPα interactions is independent of FcγR usage and genotype, and independent of SIRPα genotype, supporting the idea that this may provide a generic method for potentiating the efficacy of cancer therapeutic antibodies.
DISCUSSION

In the present study we have investigated whether differences in FcγR and SIRPα genetics may form the basis for variation in neutrophil ADCC capacity, and its modulation by manipulation of CD47-SIRPα interactions, respectively. This to our knowledge represents the largest study thus far conducted with respect to either of these issues. Furthermore, this is the first study that investigates FcγR genetic variation in antibody-mediated destruction of cancer cells an integrated fashion, which is important given the linkage disequilibrium within the FcγR locus. Nevertheless, our findings confirm earlier preliminary observations with a very limited number (i.e. n=3) of individuals on the link between FcγRIIa 131H/R polymorphism in neutrophil ADCC 8. Of relevance, this variation is also associated with the clinical response to Trastuzumab 6 and other cancer therapeutic antibodies 7,35. Our results also demonstrate a hereto unreported link between the FcγRIIIb NA1/NA2 allele and neutrophil ADCC capacity. Moreover, we show that the associations between neutrophil ADCC and FcγRIIa or FcγRIIIb variations were clearly independent. As discussed above there is currently no clear mechanistic explanation available for the observed differences in the context of responses through IgG1 antibodies, such as Trastuzumab.

Although resting neutrophils do not express significant levels of FcγRI, it represents the only high affinity receptor for IgG and may therefore be important for successful therapy with cancer therapeutic antibodies 36. FcγRI is constitutively expressed on monocytes and macrophages 37, can be induced on neutrophils in patients by treatment with cytokines, such as IFNγ and/or G-CSF 28,38,39, and may even be upregulated in cancer patients during chemotherapy-induced neutropenia 40. Our findings demonstrate that ADCC with IFNγ and G-CSF treated neutrophils involves all of the available FcγR, including FcγRI, FcγRIIa/c, and FcγRIIIb. More precisely, our findings suggest, for the first time, that these receptors function on two partially redundant parallel pathways, one involving FcγRI and the other involving both FcγRIIa/c and FcγRIIIb. This is perhaps not surprising given the fact that FcγRIIa/c is a low-affinity receptor and would be expected to benefit strongly from the presence of FcγRIIIb which is very highly expressed on neutrophils. FcγRIIIb does not possess intrinsic signaling capacity and can thus be expected to act as a tethering receptor. FcγRI, in contrary, acts as a high affinity IgG receptor and may thus be less dependent on FcγRIIIb.

We have also studied in detail whether the beneficial effect of targeting CD47-SIRPα interactions, with either antagonists or CD47 knock-down in the tumor cells, is associated with the available FcγR (geno)type. Our findings essentially show that the potentiating effect of CD47-SIRPα targeting occurs independent of the FcγR type available (Fig. 2) and FcγRIIa or FcγRIIIb polymorphic variant(s) encoded (Figs. 3 and 4), and that even the magnitude of the enhancing effect is very similar on average.
We have also explored a possible role for SIRPα genetics in regulating neutrophil ADCC. Although it is known that there are at least 10 SIRPα polymorphic variants among different ethnic groups, including African and Asian individuals, the actual diversity within the Caucasian population had not been determined. We show that within our 83 healthy Caucasian donors of neutrophils evaluated there are only two variants present, i.e. SIRPα₁ (also known as variant 2) and SIRPα₆ (also known as variant 1), with allele frequencies of 60 and 84%, respectively. Whereas it was known that amino acid variation primarily occurs in the region flanking the CD47 binding site within the N-terminal Ig-like domain of SIRPα, and also that these particular Caucasian SIRPα variants are similar with respect to their affinity for CD47, is could still be that there were functional differences in the responses downstream. However, our current results show that the both genetic SIRPα variants have very similar ADCC restricting capacity, which is represented by the typical 1.5- to 2-fold potentiation observed upon interference (Fig. 5). This demonstrates, for the first time, that the two SIRPα polymorphisms within the Caucasian population do not show differences in their overall function as determined here by ADCC using primary neutrophils.

Collectively, our findings show that the different FcγRs expressed by neutrophils can effectively collaborate and synergize to trigger ADCC against antibody-opsonized cancer cells. They also demonstrate that the magnitude of the response is independently affected by the FcγRIIa-H/R131 and FcγRIIib-NA1/NA2 polymorphisms. Finally, we demonstrate that CD47-SIRPα interactions regulate ADCC triggered via the various FcγRs and their genetic variants to a similar extent, and independent of the SIRPα polymorphisms that are present. The latter clearly supports the idea that interference with CD47-SIRPα interactions will be a broadly applicable therapeutic strategy to potentiate antibody therapy in cancer, independently of FcγR and SIRPα genetics.
REFERENCE LIST


32. Bredius RG, Fijen CA, de HM et al. Role of neutrophil Fc gamma RIIa (CD32) and Fc gamma RIIB (CD16) polymorphic forms in phagocytosis of human IgG1- and IgG3-opsonized bacteria and erythrocytes. Immunology 1994;83:624-630.


40. McCarthy DA, Macey MG, Streetly M, Schey SA, Brown KA. The neutropenia induced by the thalidomide analogue CC-4047 in patients with multiple myeloma is associated with an increased percentage of neutrophils bearing CD64. Int.Immunopharmacol. 2006;6:1194-1203.
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Table 1 | A. Polymorphisms and gene copy number variation in FCGR2-FCGR3 and SIRPα genes. Primer sets used in the SIRPα MLPA assay. Each set consists of a left and a right probe. B. Alignment of the N-terminal V-set Ig-like domain amino acid sequences of the two Caucasian polymorphic variants of SIRPα, SIRPα₁ and SIRPα₂. Note that the 13 residues in which polymorphic variation occurs (grey shading) are located outside the regions that comprise the CD47 binding site (red shading) as determined in (1).

A

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Supplementary Figure 1 | A. Correlation between neutrophil ADCC capacity (cytotoxicity %) and neutrophil surface FcγR expression levels. Each point represents neutrophil ADCC towards Trastuzumab-opsonized SKBR3 cells and expression of FcγRI (CD64); FcγRIIa/c (CD32a/c) and FcγRIII(CD16) of a single subject. Surface expression levels were analyzed by flow cytometry as described in the materials and methods section, and mean fluorescent intensity (MFI) values are shown. The coefficient of multiple correlation ($R^2$) was determined with GraphPad Prism 5 software (GraphPad Software, Inc. La Jolla, CA). B. Comparison of FcγR expression among all individuals with different FcγRIIa variants, FcγRIIa-131HH (n=17), FcγRIIa-1131HR (n=28) and FcγRIIa-1131RR (n=17). C. Comparison of FcγR expression among individuals carrying 2 copies of FcγRIIIb variants, including FcγRIIIb-NA1NA1 (n=11), FcγRIIIb-NA1NA2 (n=24) and FcγRIIIb-NA2NA2 (n=17). D. Comparison of FcγR expression among individuals carrying the FcγIIc-Stop allele as well as 2 copies of FcγRIIIb, including the variants FcγRIIIb-NA1NA1 (n=10), FcγRIIIb-NA1NA2 (n=20) and FcγRIIIb-NA2NA2 (n=13). Data shown are means ± SEM of mean fluorescence intensities (MFI). Statistics were performed by Student's t-test; n.s. = non-significant.
Supplementary Figure 2. A. Association between FcγRIIIb NA1/NA2 alleles and neutrophil ADCC capacity towards Trasuzumab-opsonized control SKBR3 cells in individuals carrying 2 copies of FcγRIIIb and either of the FcγRIIa variations: homozygous H131 (n=16), heterozygous H/R131 (n=23) and homozygous R131 (n=13). Data shown are means ± SEM as described in detail in the materials and methods section. Statistics were performed by Student’s t-test; n.s. = non-significant. In case of the single subject with the FcγRIIa-RR and FcγRIIIb-NA1NA1 variations statistics was performed with the Grubb’s outlier test (Indicated as §). B. Same as shown in A, but performed with Trasuzumab-opsonized SKBR3-CD47KD cells. C. Association between FcγRIIIb NA1/NA2 alleles and neutrophil ADCC capacity towards Trasuzumab-opsonized control SKBR3 cells in individuals carrying 2 copies of FcγRIIIb and the FcγRIIc-stop allele and either of the FcγRIIa variations: homozygous H131 (n=16), heterozygous H/R131 (n=23) and homozygous R131 (n=13). D. Same as shown in C, but performed with Trasuzumab-opsonized SKBR3-CD47KD cells.
Supplementary Figure 3 | Polymorphisms of SIRPα do not affect neutrophil ADCC towards Trastuzumab-opsonized SKBR3 cells. Actual ADCC data and ratios of the data shown in Fig.5 represented here separately for the different CD47 interference conditions i.e. ‘anti-CD47 F(ab’)2’-blocking or ‘CD47KD’.

REFERENCE LIST