Research Article

Genetic variation of human neutrophil Fcγ receptors and SIRPα in antibody-dependent cellular cytotoxicity towards cancer cells

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The efficacy of cancer therapeutic antibodies varies considerably among patients. Anti-cancer antibodies act through different mechanisms, including antibody-dependent cellular cytotoxicity (ADCC) triggered via Fcγ receptors (FcγR). This phagocyte ADCC can be promoted by interference with CD47-SIRPα interactions, but the magnitude of this enhancement also varies among individuals. Both FcγR and SIRPα display considerable genetic variation, and we investigated whether this explains some of the variability in ADCC. Because of linkage disequilibrium between FcγR variants the interpretation of previous reports suggesting a potential link between FcγR polymorphisms and ADCC has been troublesome. We performed an integrated genetic analysis that enables stratification. ADCC by activated human neutrophils towards Trastuzumab-coated breast cancer cells was predominantly dependent on FcγRIIa. Neutrophils from individuals with the FcγRIIa-131H polymorphic variant displayed significantly higher killing capacity relative to those with FcγRIIa-131R. Furthermore, ADCC was consistently enhanced by targeting CD47-SIRPα interactions, and there were no significant functional differences between the two most prevalent SIRPα polymorphic variants. Thus, neutrophil ADCC capacity is directly related to the FcγRIIa polymorphism, and targeting CD47-SIRPα interactions enhances ADCC independently of FcγR and SIRPα genotype, thereby further suggesting that CD47-SIRPα interference might be a generic strategy for potentiating the efficacy of antibody therapy in cancer.

Keywords: Cancer · CD47- SIRPα interactions · FcγR · Neutrophils · Trastuzumab

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 coat the tumor cells, thus turning...
them into targets for immune-mediated destruction by antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis and/or complement-dependent cytotoxicity. 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 FcγRIIA expressed on NK cells, suggest a role for ADCC mediated by both cell types in patients [1]. A considerable number of genetic Fcγ Receptor (FcγR) variants have been described within the FCGR2A/3 locus [2–4]. Preliminary studies reported 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 [5, 6] and also to the ADCC capacity of neutrophils [7]. 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 based on the analysis of individual variants 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 (MLPA) assay to determine the relevant polymorphic and gene copy number variations (CNV) within the FCGR2A/3 locus [2, 8–10]. 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 as monotherapeutics 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 unfortunately. 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 SIRPα expressed on myeloid cells substantially potentiates the capacity of anti-cancer antibodies, including Trastuzumab and Rituximab [11–13], for recent reviews see [14–16]. 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 treated with Rituximab was better when CD47 expression levels were lower [11, 13]. However, it is not exactly known how SIRPα signaling inhibits ADCC. Does it for instance do so by inhibiting all different FcγRs and their variants expressed on phagocytes, or by only affecting some of them? Furthermore, there are different polymorphic variants of SIRPα within the population [17, 18] and although the two variants most commonly found in Caucasians do not differ with respect to their CD47 binding capacity [19], it is not known whether they differ in other aspects of their functioning, including their capacity to signal and to modulate ADCC.

In the present study we have evaluated 101 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. While activated neutrophils express a range of FcγR, including FcγRI (CD64), FcγRIIA (CD32), FcγRIIB (CD16) and in some individuals also FcγRIIC we have observed that the principle FcγR on neutrophils participating in ADCC towards Trastuzumab-coated breast cancer cells is FcγRIIA. Furthermore, we have identified an independent association between the FcγRIIA/H-R131 polymorphism and neutrophil ADCC, with no significant associations for the other variants, i.e. the FcγRIIB-N1A1/N2A2 polymorphism and FcγRIIC-Stop/ORF.

Finally, we show that the capacity to induce ADCC through the different FcγRs and their genetic variants can be potentiating to a similar extent (~1.5 to 2-fold) by the manipulation of CD47-SIRPα interactions, and this is not affected either by the major SIRPα polymorphisms present in the Caucasian population. These findings demonstrate for the first time unequivocally a direct link between neutrophil FcγR genetics and function in ADCC. In addition, they support the concept that interference with CD47-SIRPα interactions constitute a generic method to enhance the efficacy of antibody therapy in cancer patients.

Results

The principal FcγR mediating neutrophil ADCC is FcγRIIA

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 in individual ADCC potential, and in particular the role of genetic and functional variation in FcγR expression. We did so by evaluating neutrophil ADCC capacity towards Trastuzumab-coated Her2/Neu-positive SKBR-3 breast cancer cells of 101 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 20–82% in the absence of CD47 interference (Fig. 1A and B). Killing in the absence of Trastuzumab was always below 3%. As reported previously [13], CD47 interference gave a highly significant and also consistent enhancement of neutrophil ADCC, supporting the idea that CD47-SIRPα interactions and SIRPα inhibitory signaling restrict ADCC performed by phagocytes [13, 20, 21] (Fig. 1A and B). The interference with CD47-SIRPα interactions resulted in an average ~1.5 to 2-fold increase in cytotoxicity independent of the method of interference used (Fig. 1C).
Neutrophils express different FcγRs, which could all contribute to neutrophil ADCC. To investigate the involvement of the various FcγRs in our neutrophil ADCC model, we used blocking antibodies and other antagonists against the different FcγRs. Neutrophils cultured in the presence of G-CSF and IFNγ, as used in this study and also in a previous study [13], express FcγRI, FcγRIIa, FcγRIIb, and in a minority of about 15–20% of Caucasian individuals also FcγRIIc [2]. As shown in Fig. 2, blocking experiments with antagonists, i.e. monovalent Fc-fragments for blocking FcγRI (Supporting Information Fig. 1) or F(ab′)2-fragments of blocking antibodies against FcγRII or FcγRIII, revealed that Trastuzumab-mediated killing of SKBR-3 cells by activated neutrophils predominantly involves FcγRIIa. Of interest, there was no effect of the blocking of FcγRI, which represents the high affinity FcγR, but is expressed at relatively low levels and may potentially already be saturated with monovalent IgG from human plasma and/or the culture [22]. The blocking of FcγRIIb (CD16b), which lacks intrinsic signaling capacity but is highly expressed on neutrophils and can potentially act as a tethering receptor [23–25] did not result in inhibition. Instead, if anything there might be a negligible enhancement.

The FcγRIIa polymorphism is associated with neutrophil ADCC capacity

The FcγRs are encoded on chromosome 1, of which some, in particular those of the FCGR2/3 cluster, are subject to considerable variation in terms of polymorphisms and/or surface expression variation due to either gene CNV or promoter polymorphisms [2, 3]. We wanted to know whether the variation in neutrophil ADCC between neutrophil donors was related to the known genetic variations in neutrophil FcγR expression and function. Previous studies have already 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’ allele of FcγRIIa, FcγRII-H131, is associated with a better clinical responsiveness as compared to the alternative FcγRII-R131 allele, as has been observed for Rituximab treatment in Non-Hodgkin lymphoma [26], Trastuzumab treatment in Her2/Neu-positive metastatic breast cancer [5], and Cetuximab treatment in colorectal cancer [27]. However, as indicated above, additional FcγR polymorphisms and gene CNV, which are actually known to be in linkage disequilibrium with the FcγRIIa-H/R131 polymorphism and with each other, also exist at the FCGRs locus [28].

Thus, to make the most appropriate comparisons, these variations need to be evaluated in an integrated fashion. Therefore, all our neutrophil donors were genotyped by MLPA for all FcγR variants, and the relationship between FcγR genotype and target cell killing in ADCC was explored after appropriate stratification. An overview of the FCGRs SNP and CNV in our cohort can be found in Table 1. When considering the FcγRIIa H/R131 polymorphism, the results demonstrated that there is a trend that homozygous H131 donors induce higher cytotoxicity toward target cells than homozygous R131 donors when considering the entire study population (Fig. 3A). After stratifying for the three most common
Table 1. Polymorphisms and gene copy number variation in FCGR2/FCGR3 and SIPRA genes

| FCGRs SNP and CNV         | Number (frequency) |
|---------------------------|--------------------|
| FcγRIla                   |                    |
| 131HH                     | 33 (32.7%)         |
| 131HR                     | 42 (41.6%)         |
| 131RR                     | 26 (25.7%)         |
| FcγRIIC exon3 Stop        | 81 (80.2%)         |
| ORF                       | 20 (19.8%)         |
| FcγRIIC-allele            |                    |
| NA1NA1                    | 17 (16.8%)         |
| NA1NA2                    | 38 (37.6%)         |
| NA2NA2                    | 29 (28.7%)         |
| Other                     | 17 (16.8%)         |
| FcγRIIC-CNVI              |                    |
| NA1                       | 1 (0.99%)          |
| NA2                       | 6 (5.94%)          |
| NA1NA1                    | 17 (16.8%)         |
| NA1NA2                    | 38 (37.6%)         |
| NA1NA2 SH                 | 1 (0.99%)          |
| NA2NA2                    | 29 (28.7%)         |
| NA2NA2 SH                 | 1 (0.99%)          |
| NA1NA1NA2                 | 2 (1.98%)          |
| NA1NA2NA2                 | 4 (3.96%)          |
| NA1NA2NA2NA2 SH           | 2 (1.98%)          |
| SIPRA SNP                 | Number (frequency) |
| SIPRA1                    | 13 (15.9%)         |
| SIPRA2                    | 40 (48.7%)         |
| SIPRA2/SIPRA1             | 29 (35.4%)         |

observed no relationship between the FcγR expression level and ADCC capacity (data not shown), suggesting at least that FcγR expression levels per se are not an important determinant in this context. Secondly, as can be seen in Supporting Information Fig. 2, there were at least no differences in FcγRIIa/c expression between H131 and R131 individuals, essentially excluding this possibility too. We also determined that there is no significant difference in our ADCC assays between individuals who have FcγRIIc-ORF or FcγRIIc-STOP (Supporting Information Fig. 3).

There are various levels of potentially relevant genetic variation in FcγRIIb, which is the only FcγRII isoform expressed by neutrophils [23]. First, there is FcγRIIb gene CNV, with individuals expressing 0–5 copies of the gene. The number of encoded FcγRIIb copies is strongly associated with surface FcγRIIb expression and function [30, 31]. Because in the group that we tested the vast majority (n = 86) had 2 gene copies of FcγRIIb gene copies (FcγRIIb-2x) we did not have the power to test for a possible relationship between FcγRIIb CNV and killing capacity. The most common polymorphic variants of FcγRIIb are designated FcγRIIb-NA1 and -NA2. The FcγRIIb-NA1 and -NA2 nucleotide sequences differ at five positions, with four 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 FcγRIIb-NA1NA1 individuals are known to bind and phagocytize IgG-opsonized bacteria and red blood cells more efficiently than those from -NA1NA2 and -NA2NA2 individuals [32, 33]. We therefore hypothesized that the FcγRIIb NA1/NA2 polymorphism may also play a role in ADCC. We stratified our analysis to the neutrophil donors with 2 copies of FcγRIIb (FcγRIIb-2x). As shown in Supporting Information Fig. 4A, the various genotypes induced no significantly higher cytotoxicity to tumor cells. Further stratification for the most common FcγRIIc-Stop allele and having no SH variant gave essentially the same results (Supporting Information Fig. 4B). Again, there were no significant differences in the surface expression of FcyRI, FcγRII or FcγRIIb among the NA1/NA2 variants (Supporting Information Fig. 5). Finally, we investigated potential functional interactions between the relevant genotypes.
Figure 3. The FcγRIa R/H131 polymorphism affects neutrophil ADCC towards Trastuzumab-coated SKBR3 cells. ADCC assay was performed as described in the materials and methods. ADCC capacity for donors genotyped for the FcγRIa R/H131 polymorphism were compared. Experiments were performed with either SKBR3 cells (‘control’) or when CD47-SIRPα interactions were inhibited with blocking antibody or using SKBR3-CD47KD cells (‘CD47 interference’). (A) Results for all, including homozygous H131 (n = 33), heterozygous H/R131 (n = 42) and homozygous R131 (n = 26) subjects, or (B) stratified for subjects carrying the FcγRIIc-Stop allele and 2 copies of FcγRIIIb and no SH variant: homozygous H131 (n = 16), heterozygous H/R131 (n = 32) and homozygous R131 donors (n = 17). The ratio of cytotoxicity toward CD47 and control SKBR3 cells is shown in the panels on the right. Data shown are means ± SEM and are pooled from over 60 experiments with 2–3 donor samples per experiment. Data shown are all individual donors and represented by a single dot in the graph. Statistics between the polymorphisms was performed by one way ANOVA with Tukey’s post-test. Statistical significance between control and CD47 interference was performed by paired Student’s t-test resulting in ****p < 0.0001 for H/H, H/R and R/R (not shown in graph). ns = non-significant, *p < 0.05.

Due to the limited group size we were forced to restrict our analysis to the most common FcγRIa-H/R131 and the FcγRIIIb-NA1/NA2 variations. As can be seen in Supporting Information Figs. 6 and 7 the contributions of these two variants described above were independent and were not linked to each other.

Potentiation of neutrophil ADCC by interference with CD47-SIRPα interactions

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 one or more FcγR 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 either CD47 knock-down (CD47KD) was performed or when an inhibiting F(ab’)2 antibody for CD47 was used. As can be seen in Fig. 2, CD47 interference consistently enhanced ADCC towards the tumor cell targets irrespective of the FcγRs that were functional. The same was found when the effect of CD47 interference in combination with the different FcγRIa-H/R131 and FcγRIIIb-NA1/NA2 polymorphisms were evaluated (Fig. 3 and Supporting Information Fig. 4). These data are consistent with a common pathway of regulation in ADCC by CD47-SIRPα that is independent of FcγR genotype.

SIRPα polymorphisms do not affect neutrophil ADCC capacity

Finally, we investigated the potential contribution of SIRPα polymorphisms. It has been reported that a considerable number of SIRPα polymorphisms exist in different ethnic groups [17],
Polymorphisms of SIRPα do not affect neutrophil ADCC toward Trastuzumab-coated SKBR3 cells. ADCC assay was performed as described in the materials and methods. ADCC capacity for donors genotyped for the SIRPα polymorphisms were compared. (A) Results for all, including subjects with the indicated SIRPα genotypes, including homozygous α1/α1 (n = 13), homozygous αBIT/αBIT (n = 40) and heterozygous α1/αBIT (n = 29), or (B) stratified for subjects carrying the FcγRIIc-Stop allele, 2 copies of FcγRIIIb and no SH variant: homozygous α1/α1 (n = 9), homozygous αBIT/αBIT donors (n = 26) and heterozygous α1/αBIT (n = 18). Experiments were performed with either SKBR3 cells ('control') or with SKBR3 cells in which CD47 was manipulated 'CD47 interference' either by 'anti-CD47 F(ab)′2'-blocking or 'CD47KD'. The individual ratios of cytotoxicity of the indicated CD47 interference condition and control are shown in the panels on the right. Statistical significance between control and CD47 interference was performed by paired Student’s t-test resulting in ****p < 0.0001 for α1/α1, α1/αBIT and αBIT/αBIT (not shown in graph). Data shown are means ± SEM and are pooled from over 50 experiments with 2–3 donor samples per experiment. Data shown are all individual donors and represented by a single dot in the graph. Statistics were performed by one way ANOVA with Tukey’s test for multiple comparisons. ns = non-significant.

Figure 4. Polymorphisms of SIRPα do not affect neutrophil ADCC toward Trastuzumab-coated SKBR3 cells. ADCC assay was performed as described in the materials and methods. ADCC capacity for donors genotyped for the SIRPα polymorphisms were compared. (A) Results for all, including subjects with the indicated SIRPα genotypes, including homozygous α1/α1 (n = 13), homozygous αBIT/αBIT (n = 40) and heterozygous α1/αBIT (n = 29), or (B) stratified for subjects carrying the FcγRIIc-Stop allele, 2 copies of FcγRIIIb and no SH variant: homozygous α1/α1 (n = 9), homozygous αBIT/αBIT donors (n = 26) and heterozygous α1/αBIT (n = 18). Experiments were performed with either SKBR3 cells ('control') or with SKBR3 cells in which CD47 was manipulated 'CD47 interference' either by 'anti-CD47 F(ab)′2'-blocking or 'CD47KD'. The individual ratios of cytotoxicity of the indicated CD47 interference condition and control are shown in the panels on the right. Statistical significance between control and CD47 interference was performed by paired Student’s t-test resulting in ****p < 0.0001 for α1/α1, α1/αBIT and αBIT/αBIT (not shown in graph). Data shown are means ± SEM and are pooled from over 50 experiments with 2–3 donor samples per experiment. Data shown are all individual donors and represented by a single dot in the graph. Statistics were performed by one way ANOVA with Tukey’s test for multiple comparisons. ns = non-significant.
Discussion

In the present study we have investigated whether differences in FcγR and SIRPα genetics may provide an explanation for the inter-individual variation in neutrophil ADCC capacity, and the potential of the latter to be enhanced by manipulation of CD47-SIRPα interactions, respectively. This 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 in an integrated fashion, which is important given the known linkage disequilibrium within the FcγR locus. This analysis allowed us, upon stratification for the two most frequent variations in the other neutrophil FcγR, namely FcyRIIc-Stop and FcyRIIib-2x, to define a significant association between the FcγRIIa H/R131 polymorphism and neutrophil ADCC towards Trastuzumab-coated breast cancer cells. It should be mentioned that there is as yet no definitive mechanistic explanation available for the observed association of FcγRIIa, because the affinity of FcγRIIa-H131 (Kd 5.2 × 10^{-6}) for human IgG1 appears only slightly higher than that for FcγRIIa-R131 (Kd 3.5 × 10^{-6}), as measured by surface plasmon resonance [34]. It appears unlikely that this slight difference is the sole cause of the difference in ADCC. By comparison, the difference between the two FcγRIIa alleles for e.g. IgG2 binding is much more prominent and this also translates into differences in neutrophil ADCC capacity as observed for the IgG2 anti-EGFR antibody Panitumumab [35]. There was also no difference in expression levels between the different alleles that could explain the results, thus, receptor properties other than IgG1 binding capacity, such as membrane mobility or interactions/cooperation with other molecules that could potentially affect intracellular signaling and consequently ADCC could perhaps contribute for the differences observed. Whatever the mechanism, there is good reason to believe that the observed association is relevant in vivo as it is also linked to the clinical response to Trastuzumab [5] and other cancer therapeutic antibodies of the IgG1 subclass [6, 36].

Since the Kurlander-effect could not be excluded when using intact antibodies for FcγR blocking, which may interfere with the activity of other FcγR as well by a phenomenon that has become known as the 'Kurlander-effect' [45].

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 especially when combined with SIRPα-CD47 interference therapies [46]. FcγRI is constitutively expressed on monocytes and macrophages [47], can be induced on neutrophils in patients by treatment with cytokines, such as IFNγ and/or G-CSF [26, 48, 49], and may even be upregulated in cancer patients during chemotherapy-induced neutropenia [50]. Since the Kurlander-effect could not be excluded when using intact monoclonal antibody 10.1, a known blocker of FcγRI, and the F(ab')2 fragments of this antibody are known not to be able to fully block the receptor [22] we used monovalent Fc-fragments to inhibit FcγRI. Due to the receptors high affinity to binding IgG-Fc fragments they are able to inhibit FcγRI (Supporting Information Fig. 1), and potentially also partially other receptors. However, as shown in Fig. 2, monovalent Fc fragments are unable to block FcγRIIa/c and FcγRIIib in our system. Our findings demonstrate ADCC with IFNγ and G-CSF treated neutrophils involves mainly FcγRIIa/c, also when this is further enhanced by CD47-SIRPα checkpoint blockade, with no significant role found for FcγRI. It should be noted that when using a different effector cell, like macrophages or CD16+ monocytes, that the FcγRs required for ADCC and phagocytosis could be different then when looking at neutrophils, since they express a different combination of FcγRs.

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 (Fig. 3 and Supporting Information Fig. 4), and that also 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 has been reported that there are at least 10 SIRPα polymorphic variants found [41]. Nevertheless, these data do not necessarily exclude a role for FcγRIIib in neutrophil ADCC and antibody therapy. In fact, we found a small but significant increase in neutrophil ADCC in our study, which may be indicative for an ‘inhibitory’ role of FcγRIIib in certain cases. We believe that the role of FcγRIIib warrants further evaluation, especially because we have used in vitro activated neutrophils in our study in which FcγRIIib expression is relatively low.

It should be noted that when considering human neutrophils as effector cells in ADCC towards tumor cells some studies have also indicated a requirement for both FcγRII and FcγRIIib [42, 43], while others show a more exclusive involvement of Fcγ-RII [44]. 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. One potential pitfall that could also have affected the results, at least in some of the reported studies, is the use of intact antibodies for FcγR blocking, which may interfere with the activity of other FcγR as well by a phenomenon that has become known as the ‘Kurlander-effect’ [45].
among different ethnic groups, including African and Asian individuals [17], the actual diversity within the Caucasian population has not been determined. We show that within our 82 healthy Caucasian neutrophil donors evaluated there are only two variants present, i.e. SIRPα (also known as variant 2) and SIRPα<sub>BAT</sub> (also known as variant 1), with allele frequencies of 34 and 66% respectively. Whereas it was known that amino acid variation primarily occurs in the regions 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 [19], there could still be functional differences in the responses downstream. However, our current results show that the both genetic SIRPα variants have very similar ADCC suppressing capacity, which is represented by the typical ~1.5- to 2-fold potentiation observed upon interference (Fig. 4). 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, at least when using neutrophils.

Collectively, our findings show that FcyRIIa expressed by neutrophils can effectively trigger ADCC against antibody-coated cancer cells. They also demonstrate that the magnitude of the response is affected by the FcyRIIa-H/R131 polymorphism.

Finally, we demonstrate that CD47-SIRPα interactions regulate ADCC triggered via FcyRIIa and its 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 FcyR and SIRPα genetics.

Isolation of human neutrophils from healthy donors

Neutrophils were isolated from n = 101 healthy Caucasian volunteers by density centrifugation of heparinized blood over isotypic Percoll (Pharmacia Uppsala, Sweden) followed by red cell lysis with hypotonic ammonium chloride solution at 4°C [51]. Cells were cultured in RPMI (Gibco) medium, supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine and 100 μg/ml streptomycin (i.e. complete RPMI) or HEPES medium (containing 132 mM NaCl, 6.0 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgSO₄, 1.2 mM K₂HPO₄, 20 mM Hepes, 5.5 mM glucose and 0.5% HSA), 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 × 10⁶ cells/ml for 16 h. Afterward cell viability was determined by the amount of FITC-Annexin V (BD Pharmingen, San Diego, CA) positive cells on FACS, after which the cell concentration was corrected to have 5 × 10⁶ viable cells/ml. Cells were consequently washed and prepared for analysis by ADCC assay.

Materials and methods

Cells, culture and antibodies

The Her2/Neu-positive human breast carcinoma cell line SKBR3 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 IMDM) at 37°C, 5% CO₂. SKBR3 cells in which CD47 had been knocked down by shRNA, referred to as SKBR3-CD47KD cells, express 10–15% of normal CD47 surface levels, as described before [13]. In experiments with SKBR3 cells, cells expressing an oligo containing scrambled shRNA not targeting any transcript before [13]. In experiments with SKBR3 cells, cells expressing an oligo containing scrambled shRNA not targeting any transcript 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 5 μg/ml: monovalent human Fc fragments (Bethyl, USA) for blocking FcγRI, anti-human CD32 F(ab’)2 (Clone 7.3, Ancell) to block FcγRIa/c, anti-human CD16 F(ab’)2 (Clone 3G8, Ancell) to block FcγRIIb.

Analysis of FcγR and SIRPα polymorphisms and CNV

Genotyping of 101 individuals for FcγRIa, FcγRIIb, and FcγRIc polymorphisms was performed using the FCGR-specific Multiplex Ligation-dependent Probe Amplification (MLPA) assay (MRG Holland), using genomic DNA isolated from whole blood with the QIAamp® kit (Qiagen, Hilden, Germany). The MLPA assay was performed essentially as described previously [2, 8, 52].

In short, CNV of FCGR2C and FCGR3B was detected by genespecific MLPA probes designed on multiple sites in these genes. Probes were also included to detect the FCGRA2 [p.131H/R] (rs1801274) SNP and haplotypes in FCGR2C and FCGR3B. Specific probes were included for the FCGR3B haplotypes NA1/NA2/SH, which respectively encode for the HNA1a/HNA1b/HNA1c alloantigens of human neutrophil antigen 1 [53]. To construct FCGR2C haplotypes, the assay contained a probe specific for the stop codon in exon3 of the FCGR2C gene [p. 57X] and a non-specific FCGR2B/C probe to detect the ORF in exon3 [p. 57Q], which together can determine the rs759550223 SNP. Probes were also included for the splice site mutation at the border of exon7-intron 7 in FCGR2C (rs76277413 c.798 +1 A>G), to distinguish the non-expressed ‘nonclassical’ FCGR2C–ORF variant from the ‘classical’ FCGR2C–ORF that is typically expressed on natural killer (NK) cells, monocytes and neutrophils [2]. Because the nonclassical FCGR2C–ORF variant is not expressed [2], it was grouped with FCGR2C-Stop in all analyses. An overview of the probes used is shown in Supporting Information Table 1. The frequency distribution of the different variants within our study population is provided in Table 1.

With genomic DNA from PBMC of healthy Caucasian donors (n = 82), SIRPα CNV and SNPs were determined with a SIRPα specific MLPA assay and confirmed with regular sequencing. In our donor set, SIRPα haplotype was identified by sequencing the
V-Ig domain encoded by the third exon (data not shown), namely SIRPa and SIRPaArr. MLPA probes, binding to SIRPa variants, are synthetic oligonucleotides made by Invitrogen (Carslbad, CA) and were designed according to the sequencing results and the available data in http://www.ensemble.org/index.html. The regular sequencing primers were located in the introns surrounding exon 3, thus sequencing the whole exon. For an overview of the specific target sequences of the probes and the forward and reverse primer used for the regular sequencing, see Supporting Information Table 1. The MLPA assay was performed essentially as described previously [2, 8, 52].

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 previously [13]. In brief, human breast carcinoma cell lines were harvested by mild trypsin treatment, and washed tumor cells (1 × 10$^6$) were collected and labeled with 100 μCi $^{51}$Cr (Perkin-Elmer, USA) in 500 μL for 90 min at 37°C. The target cells (5 × 10$^4$/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 or IMDM supplemented with 10% (v/v) FCS medium. Aliquots of supernatant were harvested and analyzed for radioactivity in a gamma counter. The percentage relative cytotoxicity was determined as [(experimental cpm−spontaneous cpm)/ (total cpm−spontaneous cpm)] × 100%. All conditions were tested in triplicate. In case of neutrophils of a single individual tested on multiple occasions, the average measurements were used to avoid disturbing the population balance.

Rosetting assay with 293T cells and RBCs

As described earlier [22] 293T cells (ATCC) (which have no endogenous FcγR expression) were transfected with a FcGr1A1-GFP fusion construct, or GFP alone as a control. RhD positive erythrocytes, uncoated or coated with human anti-RhD IgG (RheDQuin, Sanquin, The Netherlands), stained with DiD (5 μM, Invitrogen), were combined in a ratio of 1:10 (293T:RBC) in combination with known FcγRI blocking antibody 10.1 and monovalent Fc-fragments for blocking FcγRI with various concentrations for 15 minutes at 37°C after which samples were fixed with PFA. Samples were run in an ImageStreamX flow cytometer (Amnis Corporation, Seattle, WA) to determine their blocking capacity.

Statistical analyses

Statistical differences were determined by either paired or ordinary one way ANOVA, with Sidak or Tukey's post-test, or by paired Student’s t-test, as indicated in the figure legend.

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

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Abbreviations: ADCC: Antibody-dependent cellular cytotoxicity  · CNV: copy number variation  · FeγR: Fcγ receptor  · MLPA: multiplex ligation-dependent probe amplification  · SNP: single nucleotide polymorphism

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