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

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CD47-SIRPα interactions form a barrier for antibody-mediated tumor cell destruction

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ABSTRACT

Monoclonal antibodies are among the most promising therapeutic agents for treating cancer. Therapeutic cancer antibodies bind to tumor cells, turning them into targets for immune-mediated destruction. We show here that this antibody-mediated killing of tumor cells is limited by a mechanism involving the interaction between tumor cell expressed CD47 and the inhibitory receptor SIRPα on myeloid cells. Mice that lack the SIRPα cytoplasmic tail, and hence its inhibitory signaling, display increased antibody-mediated elimination of melanoma cells in vivo. Moreover, interference with CD47-SIRPα interactions by CD47 knock-down or by antagonistic antibodies against CD47 or SIRPα significantly enhances the in vitro killing of Trastuzumab-opsonized Her2/Neu-positive breast cancer cells by phagocytes. Finally, the response to Trastuzumab therapy in breast cancer patients appears correlated to cancer cell CD47 expression. These findings demonstrate that CD47-SIRPα interactions participate in a homeostatic mechanism that restricts antibody-mediated killing of tumor cells. This provides a rational basis for targeting CD47-SIRPα interactions, using for instance the novel antagonistic antibodies against human SIRPα described herein, in order to potentiate the clinical effects of cancer therapeutic antibodies.
INTRODUCTION

Therapeutic monoclonal antibodies (mAb) directed against tumor cells have become a valuable alternative or addition to conventional cancer treatment modalities. However, in spite of the beneficial effects documented for various therapeutic antibodies against different types of cancer, antibodies alone are not curative and methods to improve their efficacy are warranted. Therapeutic cancer antibodies may act by one or more of several mechanisms, including immune mediated effects, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) mechanisms, as well as by direct growth-inhibitory effects on the tumor cells\textsuperscript{1-3}.

Currently the most widely used examples of therapeutic antibodies are Rituximab and Trastuzumab. Trastuzumab (Herceptin) is a humanized IgG1 monoclonal antibody approved for the treatment of Her2/Neu-positive breast cancer. While Trastuzumab binds to the extracellular domain of Her2/Neu the mechanism(s) of action in patients is not exactly clear. In vitro and in vivo studies in mice suggest that Trastuzumab acts by inducing direct G1 growth arrest in breast cancer cells as well as by mediating ADCC\textsuperscript{4-6}. ADCC can be mediated by Fc-receptor expressing NK cells and phagocytes, including macrophages and granulocytes\textsuperscript{7,8} and a link between FcγRIIa (CD32a)- and FcγRIIIa (CD16)- polymorphisms and clinical Trastuzumab responsiveness in patients with breast cancer suggests an involvement of both types of Fc-receptors expressed on phagocytes and NK cells, respectively\textsuperscript{3,9}.

NK cell-mediated ADCC is controlled by interactions between ‘self’ MHC class I molecules on (malignant) host cells and inhibitory killer immune receptors (KIR) expressed on NK cells. Upon ligand binding inhibitory KIR recruit and activate the cytosolic tyrosine phosphatases SHP-1 and/or SHP-2 that limit Fc-receptor signaling and, consequently, ADCC towards host cells\textsuperscript{7}. An inhibitory receptor on myeloid cells, including macrophages and granulocytes, that may potentially act in a similar fashion to restrict antibody-mediated tumor cell elimination is SIRP\textsubscript{α}10-14. The extracellular region of SIRPα interacts with the broadly expressed surface molecule CD47\textsuperscript{15-17}. CD47 binding to SIRPα triggers the recruitment and activation of SHP-1 and SHP-2 to immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within the SIRPα cytoplasmic region, and this regulates intracellular signaling pathways and associated downstream functions, usually in a negative fashion\textsuperscript{10,11,18}. It is well documented, for instance, that SIRPα acts to inhibit in the phagocytosis and the \textit{in vivo} clearance of CD47-expressing host cells, including red blood cells and platelets, by macrophages\textsuperscript{19-24}. CD47-SIRPα interactions also appear essential for engraftment upon hematopoietic stem cell (HSC)\textsuperscript{25}. Based on this, it has been proposed that the broadly expressed CD47 functions, in analogy to MHC class I molecules, acts as a ‘self’ signal to control immune effector functions of myeloid cells\textsuperscript{22,26}.

Chao et al.\textsuperscript{27} have recently reported that antibodies against CD47 synergize with the therapeutic cancer antibody Rituximab in the phagocytosis of Non-Hodgkin lymphoma by macrophages in immunodeficient mice. However, this study does not provide conclusive evidence for the role of CD47-SIRPα interactions in the context of antibody therapy against cancer. In the present study we demonstrate that CD47-SIRPα interactions and SIRPα-
signaling negatively regulate Trastuzumab-mediated ADCC in vitro and antibody-dependent elimination of tumor cells in vivo. These findings support the idea that CD47-SIRPα interactions create a barrier for antibody-mediated tumor cell elimination and provide a rational basis for targeting CD47-SIRPα interactions in order to potentiate the clinical effects of cancer therapeutic antibodies.

METHODS

Mice and B16 melanoma model
C57BL/6 mice with a targeted deletion of the SIRPα cytoplasmic region have been described previously24. These mice, originally generated onto the 129/Sv background and backcrossed onto C57BL/6 mice for ten generations, were bred and maintained under SPF conditions, together with wild type C57BL/6 mice from the same genetic background, and used between 8 and 12 weeks of age. Age-matched wild type and SIRPα-mutant mice were injected i.v. with 1.5x10^5 B16F10 tumor cells in 100 μl HBSS on day 0. Mice were injected i.p. with a suboptimal dose of 10 μg of TA99 antibody (or PBS as control) on days 0, 2, and 4. At day 21 the mice were sacrificed. Their lungs were excised and scored for number of metastases and tumor load as described28.

CD47 mRNA expression in breast cancer
We analyzed CD47 mRNA expression in 353 invasive breast carcinomas and 11 normal breast (NB) samples profiled29 using whole-genome Affymetrix oligonucleotide microarrays (GEO: GSE21653). Only two of the probe sets representing CD47, 211075_s_at and 213857_s_at, mapped exclusively to constitutively transcribed CD47 exons according to NetAffx, RefSeq, and the UCSC Genome Browser27. Their expression strongly correlated (Spearman correlation: 0.87). We retained that with the highest variance (211075_s_at). Before analysis, CD47 expression level for each tumor was centered by the average expression level of the NB samples. We analysed the correlation between CD47 expression and patients’ age (≤/>50y), pathological tumor size (≤/>2cm), axillary lymph node status (negative/positive), and grading (I/II/III), IHC oestrogen and progesterone receptors (ER and PR) status (negative/positive; positivity threshold 10% of tumor cells), and molecular subtypes (luminal A/luminal B/basal/Her2/Neu+/normal-like), defined as described30. We also analyzed a public (http://caarraydb.nci.nih.gov/caarray/) expression dataset of Her2/Neu+ breast cancers treated with primary Trastuzumab plus vinorelbine weekly for 12 weeks followed by surgery31. Pathological complete response was defined as the absence of invasive cancer in the breast and axillary lymph nodes at the time of surgery.

ADCC assay
Neutrophils were isolated by density centrifugation from heparinized blood obtained from healthy volunteers using isotonic Percoll (Pharmacia Uppsala, Sweden) followed by red cell lysis with hypotonic ammonium chloride solution. 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 5x10^6 cells/ml for 16-20h. Monocytes were isolated from the PBMC fraction by magnetic cell sorting by using anti-CD14 coated beads according to the manufacturer’s instructions (Miltenyi Biotec B.V., Utrecht, The Netherlands) or by counterflow elutriation. Washed tumor cells (5-8x10^6 cells) were collected and labeled with 100μCi^{51}Cr (Perkin-Elmer, USA) in 1 ml for 90 min at 37°C. The cells were preincubated with anti-CD47 and/or the therapeutic antibodies, as indicated, and washed again. The target cells (5x10^3/well) and effector cells were co-cultured in 96-well U-bottom tissue culture plates in complete medium in a ratio of E:T=50:1, unless indicated otherwise, for 4 hours at 37°C in 5% CO₂ in RPMI with 10% FCS medium. Aliquots of supernatant were harvested and analyzed for radioactivity in a gamma counter. The percent relative cytotoxicity was determined as [(experimental cpm- spontaneous cpm)/(Total cpm- spontaneous cpm)] x 100%. All conditions were measured in triplicate.

Statistical analyses
Statistical differences were determined using ANOVA or students t-test as indicated.

Antibodies, cell lines and culture conditions, procedures for the production of monoclonal antibodies, CD47-beads binding assay, and flow cytometry are described in supplementary information (SI methods).

RESULTS

Antibody-mediated cancer elimination in vivo is restricted by SIRPα signaling
We postulated that interactions between CD47, expressed broadly on normal and tumor cells, and the myeloid inhibitory immunoreceptor SIRPα, would negatively regulate phagocyte-mediated ADCC induced by cancer therapeutic antibodies, and that targeting of CD47-SIRPα interactions would comprise a generic strategy to improve antibody therapy against cancer. In line with this, Chao et al.^{27} have recently shown that antibodies against human CD47 synergize with Rituximab in the elimination of Non-Hodgkin lymphoma cells in immunodeficient mice and in in vitro phagocytosis experiments. Instead, we used mutant mice lacking the SIRPα cytoplasmic tail^{24} to investigate whether inhibitory signaling via SIRPα could regulate the antibody-mediated elimination of syngeneic tumor cells in immunocompetent mice. In particular, we employed the well established mouse metastatic B16 melanoma model, in which the therapeutic antibody TA99, directed against the melanoma gp75 tumor antigen, has shown prominent beneficial effects in tumor cell clearance^{28}. First, B16F10 cells, that expressed surface CD47 (Fig. 1A), were injected i.v., in the absence of therapeutic TA99 antibody, into wild-type and SIRPα-mutant mice, and this resulted in a similar tumor formation in both strains of mice (Fig. 1B), indicating that SIRPα signaling did not affect tumor cell metastasis and outgrowth per se. Next, these experiments were performed in mice that were treated with suboptimal concentrations of TA99 antibody. TA99 antibody treatment resulted only in a minimal reduction in tumor cell outgrowth in wild type mice, but tumor formation was essentially abrogated in SIRPα-mutant animals under these conditions (Fig. 1C). This demonstrated directly that SIRPα-derived signals can form a limitation for antibody-dependent tumor cell elimination in vivo.
Figure 1 | SIRPα signaling limits antibody-mediated destruction of melanoma cells in vivo. (A) CD47 expression on B16F10 mouse melanoma cells as demonstrated by flow cytometry using anti-mouse CD47 antibody (Miap301) and PE-labeled anti-mouse IgG (closed histogram). The open histogram represents the isotype control. (B) Comparable outgrowth of B16 melanoma in wild type and SIRPα-mutant mice in the absence of therapeutic antibody. Wild type and SIRPα-mutant mice were injected i.v. with 1.5 x 10^5 B16F10 tumor cells. After 21 days, mice were sacrificed, lungs were excised, photographed (representative examples are shown), and tumor loads were determined and expressed as the sum of the following scores: metastases less than 1 mm were scored as 1; metastases between 1 and 2 mm were scored as 3; and metastases larger than 2 mm were scored as 10. Measurements from individual mice are shown, with means indicated by bars, and statistical differences between groups (n=10) were determined by ANOVA. Note that comparable tumor loads occur in wildtype (34.7 ± 9.5 (mean ± SEM)) and SIRPα-mutant mice (35.9 ± 5.2). Data are from one representative experiment out of three. (C) Enhanced antibody-mediated clearance of B16 melanoma cells in SIRPα-mutant mice. Wild type and SIRPα-mutant mice were challenged i.v. with 1.5 x 10^5 B16F10 tumor cells, and, where indicated, with a suboptimal dose of 10 mg of TA99 antibody (or PBS as control) on day 0, 2, and 4. After 21 days, mice were sacrificed and analyzed as in panel (B). Measurements from individual mice are shown, with means indicated by bars, and statistical differences between groups (n=8) were determined by ANOVA. Note the black nodules of melanoma lung metastases in (B) and (C). Note in the graph in panel (C) that TA99 antibody treatment resulted only in a minimal non-significant reduction in tumor cell outgrowth in wild type animals (47.9 ± 9.4 (mean ± SEM) in PBS-treated mice compared to 29.0 ± 7.8 in TA99 treated mice), but tumor formation was essentially absent in SIRPα-mutant animals treated with TA99 antibody (4.5 ± 1.0). Data are from one representative experiment out of three.
Expression of CD47 in breast cancer correlates with adverse features and resistance to trastuzumab

In line with the above we hypothesized that CD47-SIRPα interactions were restricting the clinical efficacy of Trastuzumab in the treatment of patients with Her2/Neu-positive breast cancer. To test this hypothesis we explored a possible relationship between CD47 expression and breast cancer pathological features and clinical Trastuzumab responsiveness. In order to do so, we analysed breast cancer tissue CD47 mRNA expression in our 353 breast cancer patient cohort as well as in a public data set\textsuperscript{11}. CD47 mRNA was overexpressed in many tumors, and expression correlated with poor-prognosis molecular subtypes (i.e. basal, Her2/Neu+) (Fig. 2A) and with adverse pathological features (i.e. high grade, ER-, PR-). Furthermore, analysis of a relatively small public data set\textsuperscript{31} of Her2/Neu-positive breast cancer patients treated with Trastuzumab plus vinorelbine revealed an inverse correlation between CD47 expression level and pathological response to the therapy (Fig. 2B), with significantly lower CD47 expression in complete responders. Although the latter finding clearly requires confirmation in a larger and independent patient cohort, it is consistent with an adverse role of CD47 in the Trastuzumab-mediated elimination of breast cancer cells.

Figure 2 | CD47 mRNA expression in breast cancer. (A) Correlation with molecular subtypes: basal, Her2/Neu-positive, luminal A, luminal B, and normal-like (Institut Paoli-Calmettes series: n=353). Log\textsubscript{2}-transformed expression levels in tumors are reported as box plots relatively to expression in normal breast (NB, horizontal solid line). Overexpression (ratio T/NB ≥2; horizontal dashed line) of CD47 was found in 63% of tumors. Note that the poor-prognosis subtypes (i.e. basal and Her2/Neu+) have the highest CD47 expression levels. Differences in expression levels between the five subtypes were tested for significance using one-way ANOVA, and between two subtypes using Student t-test. (B) Correlation with pathological response to Trastuzumab plus vinorelbine treatment (public dataset\textsuperscript{31}; n=22). Log\textsubscript{2}-transformed expression levels in tumors are reported as box plots relatively to median expression in all samples (median, horizontal solid line). Note that patients with a pathological complete response (pCR; n=3) have significantly lower CD47 expression than patients with an incomplete response (no pCR; n=19).
Targeting CD47-SIRPα interactions potentiates Trastuzumab-mediated ADCC against breast cancer cells

In order to directly investigate whether CD47-SIRPα interactions play a role in the Trastuzumab-dependent destruction of breast cancer cells by phagocytes, we established an in vitro ADCC assay employing Trastuzumab-opsonized human SKBR-3 breast cancer cells expressing surface Her2/Neu and CD47 (Fig. 3A) as targets and human neutrophils as effector cells. Trastuzumab-mediated ADCC by neutrophils was potently and synergistically enhanced by F(ab)’2-fragments of the B6H12 mAb that blocks CD47 binding to SIRPα (Fig. 3B-E). The enhancing effect of blocking anti-CD47 F(ab)’2 was observed at different E:T ratios (Fig. 3C) and appeared to act by both decreasing the threshold as well as by increasing the magnitude of killing (Fig. 3D). Importantly, in the absence of Trastuzumab no detectable tumor killing effect of anti-CD47 F(ab)’2 was observed, suggesting that CD47-SIRPα interactions do not control antibody-independent mechanisms of killing. This observation is in apparent contrast with the results of Chao et al.27,32 who also reported significant effects on lymphoma phagocytosis with the anti-CD47 mAb B6H12 alone. The latter may possibly relate, at least in part, to their use of intact B6H12 mAb that according to our own results can indeed cause direct ADCC in SKBR3 cells (Figure S1).

In the numerous independent experiments (n>50) that were performed with neutrophils as effector cells for killing of Trastuzumab-opsonized SKBR-3 cells a consistent enhancing effect of the anti-CD47 F(ab)’2 was observed, although the degree of killing (with Trastuzumab alone) varied considerably for different effector cell donors (Fig. 3B). The latter appeared to be related to factor(s) intrinsic to the effector cells, including individual differences in the expression of FcγRI and FcγRIIIb receptors, that are pivotal for the induction of ADCC (Fig. S2).

![Figure 3](image-url)
CD47-SIRPa interactions restrict ADCC

Interference with CD47-SIRPα interactions using blocking anti-CD47 antibody B6H12 potentiates Trastuzumab-mediated ADCC of neutrophils towards Her2/Neu-positive SKBR-3 breast cancer cells. A) Flow cytometric analysis of Her2/Neu and CD47 surface expression on SKBR-3 breast cancer cells (filled histograms), using respectively Trastuzumab and B6H12 mAb against CD47. Isotype controls are shown in the open histograms. B) ADCC of neutrophils against Trastuzumab-opsonized SKBR-3 cells (E:T ratio=50:1) in the absence or presence of B6H12 anti-CD47 F(ab’)2. Shown is a representative example. Results are expressed as means ± SD of triplicate measurements and statistical differences were shown by students t-test. Note that anti-CD47 F(ab’)2 do not affect cytotoxicity alone, but do synergize with Trastuzumab. C,D) Blocking CD47-SIRPα interactions using anti-CD47 F(ab’)2 enhances the ADCC of neutrophils against Trastuzumab-opsonized SKBR-3 cells at different E:T ratios (C) and Trastuzumab concentrations (D). Shown is a representative experiment out of three. E) The effects of anti-CD47 F(ab’)2 on ADCC towards Trastuzumab-opsonized SKBR-3 cells using neutrophils from different donors in multiple independent experiments (n=53). For clarity only the values in the presence of Trastuzumab ± anti-CD47 F(ab’)2 are shown, with the matched values of the two conditions for each donor connected by lines. Killing in absence of Trastuzumab ± anti-CD47 F(ab’)2 was always below 5%. P-values of statistically significant differences, as determined by students t-test, are indicated.

Reduction of CD47 in breast cancer cells promotes Trastuzumab-mediated ADCC

To further study a regulatory role of CD47-SIRPα interactions in ADCC, siRNA-mediated knock-down of CD47 expression was performed in SKBR-3 target cells. This yielded cells with 80-90% reduced surface CD47 expression (Fig. 4A). These cells were significantly more sensitive towards neutrophil-mediated ADCC, consistent with a role for CD47-SIRPα interactions in restricting tumor cell killing (Fig. 4B). The increase was comparable to levels seen with wild-type SKBR-3 cells in the presence of blocking anti-CD47 F(ab’)2.
Unique mAb against SIRPα potentiates Trastuzumab-mediated ADCC against breast cancer cells

While the above strongly supported the idea that CD47-SIRPα interactions regulate ADCC in vitro and tumor elimination in vivo, it was important to confirm these findings with blocking antibodies against SIRPα. In fact, because of its much more restricted expression\(^{12,16}\), we anticipate that SIRPα, rather than the ubiquitous CD47, constitutes the preferred target for potential future therapeutic intervention. Since the previously reported antibodies against human SIRPα available to us either lacked the proper specificity or the ability to block interactions with CD47, we generated novel blocking mAb against SIRPα. One antibody, designated 1.23A, was generated by the method described by Steenbakkers et al.\(^{(37)}\) following negative selection on CHO cells expressing the myeloid-specific SIRP-family member SIRPβ\(_1\), whereas the other, designated 12C4, was generated by conventional hybridoma technology. Both of the two SIRPα polymorphic variants predominating in the Caucasian population, SIRPα\(_1\) and SIRPα\(_{bim}\), as well as the highly homologous myeloid SIRPβ\(_1\) and non-myeloid SIRPγ family members were recognized by 12C4, but the 1.23A mAb exclusively recognized the SIRPα\(_1\) variant (Fig. S3A,B). Staining of leukocytes from SIRPα genotyped individuals was consistent with this specificity (Fig. S3C), with the mAb 1.23A selectively recognizing monocytes and neutrophils from both \(\alpha_{\gamma/\gamma}\)-homozygous and \(\alpha_{\gamma/\text{bim}}\)-heterozygous individuals. Both mAb effectively inhibited the binding of CD47-coated beads to CHO cells expressing SIRPα\(_1\) and/or SIRPα\(_{bim}\) (Fig. 5A) and promoted Trastuzumab-mediated ADCC towards SKBR-3 cells by neutrophils from individuals with

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**Figure 4** Knock-down of CD47 in SKBR-3 breast cancer target cells enhances Trastuzumab-dependent neutrophil-mediated ADCC. A) Flow cytometric analysis for Her2/Neu and CD47 surface expression in SKBR-3 cells transfected with empty vector (‘control’) or CD47 shRNA (‘CD47-KD’). Note that CD47 expression is strongly decreased in the CD47-KD cells (MFI=358 in CD47-KD cells vs MFI=4.187 in control), but Her2/Neu levels are unaltered (MFI=18.638 in CD47-KD cells and MFI=18.993 in control). B) Neutrophil-mediated ADCC using control and CD47-KD SKBR-3 cells opsonized with Trastuzumab in three independent experiments with three different effector cell donors. Note that a similar level of enhancement occurs with anti-CD47 F(ab)’\(_2\)-mediated blocking and CD47 knock-down. P-values of statistically significant differences, as determined by students t-test, are indicated.
different genotypes (Fig. 5B,C). For the 1.23A mAb, enhanced killing was only observed when neutrophils from $\alpha_1/\alpha_1$ homozygous individuals were employed. When $\alpha_{\text{BIT}}/\alpha_{\text{BIT}}$ homozygous or $\alpha_1/\alpha_{\text{BIT}}$ heterozygotic donor cells were used, 1.23A did not enhance SKBR-3 killing by Trastuzumab, suggesting that presence of a single functional allele of SIRPa is sufficient to restrict ADCC, and that both alleles have to be inhibited simultaneously in order to achieve a beneficial effect, accordingly.

**Figure 5**

**A)** CD47-coated fluorescent bead binding to CHO cells expressing empty vector (i.e. ‘CHO’), SIRPa$_1$ or SIRPa$_{\text{BIT}}$. The 12C4 and 1.23A mAb (but not isotype IgG1 control mAb) block the binding of CD47-beads to either both SIRPa$_1$- and SIRPa$_{\text{BIT}}$-expressing CHO cells (12C4), or only to SIRPa$_{\text{BIT}}$-expressing CHO cells. The proportion (in %) of cells binding CD47-beads is indicated in the upper right part of each panel. Shown is one representative experiment out of three. **B)** Enhancing effect of 12C4 mAb on ADCC towards Trastuzumab-opsonized SKBR-3 cells using neutrophils from (n=12) individuals in 4 independent experiments. **C)** Enhancing effect of 1.23A mAb on ADCC towards Trastuzumab-opsonized SKBR-3 cells using neutrophils from (n=9) individuals with different SIRPa genotypes ($\alpha_1/\alpha_{\text{BIT}}$ or $\alpha_{\text{BIT}}/\alpha_{\text{BIT}}$ homozygotes or $\alpha_1/\alpha_{\text{BIT}}$ heterozygotes) in 3 independent experiments. P-values of statistically significant differences, as determined by students t-test, are indicated; n.s.=non-significant.
DISCUSSION

In the present study we have investigated the role of CD47-SIRPα interactions in the context of antibody therapy against cancer. In general our results provide evidence that CD47-SIRPα interactions, and the resultant intracellular signals generated via SIRPα in myeloid cells, suppress antibody-mediated destruction of tumor cells.

To study the role of SIRPα in vivo we used mutant mice lacking the SIRPα cytoplasmic tail to investigate whether inhibitory signaling via SIRPα could regulate the antibody-mediated elimination of syngeneic B16F10 melanoma cells in immunocompetent mice. Our results demonstrate that SIRPα signaling does indeed limit the capacity of cancer therapeutic antibodies to eliminate tumor cells in vivo. The effects could not be attributed to direct effects of SIRPα on tumor homing or outgrowth as identical tumor development was shown in absence of therapeutic antibody. To our knowledge this provides the first evidence for a role of SIRPα in antibody mediated tumor cell destruction in vivo.

The role of CD47-SIRPα interactions in a human context was investigated with an in vitro ADCC method using Trastuzumab-opsonized Her2/Neu-positive SKBR-3 breast cancer cells as target cells and neutrophils as the effector cells. In this assay the addition of F(ab’2)-fragments of the antibody B6H12, which is known to block CD47-SIRPα interactions,33, substantially enhanced Trastuzumab-mediated cancer cell killing, supporting the idea that CD47-SIRPα interactions negatively control ADCC. Of note, the interference with CD47-SIRPα interactions in the absence of Trastuzumab did not enhance ADCC. The latter is in apparent contrast with the results of Chao et al. who did show significant effects of anti-CD47 antibody alone on tumor cell phagocytosis in vitro and in vivo. However, Chao et al. used intact B6H12 anti-CD47 antibody in the vast majority of their experiments, including all of their in vivo experiments. We now demonstrate that this intact anti-CD47 antibody causes direct ADCC in neutrophils (Fig. S1) and similar observations have also been done for monocytes/macrophages, thereby indicating, in retrospect, that the results of Chao et al. did not really justify the conclusion that the effects were due to the interference with CD47-SIRPα interactions. In contrary, our findings, which are based on both antibody blocking experiments performed with anti-CD47 F(ab’2)-fragments as well as CD47 knock-downs in breast cancer cells, do indeed exclude alternative explanations and thereby provide direct evidence for a regulatory role of CD47-SIRPα interactions in antibody-dependent cancer cell destruction.

Although the above clearly supported a role for CD47-SIRPα interactions in antibody-dependent tumor cell elimination it was considered important to confirm these results with antagonistic antibodies against SIRPα. Moreover, because of its much more limited tissue distribution as compared to CD47, SIRPα appears to be the preferred target for potential future therapeutic intervention. Since antagonistic antibodies of the appropriate specificity were unavailable we attempted to generate new reagents. Two antagonistic antibodies were identified and characterized that reacted with one or both of the two major (and apparently equally functional34) polymorphic SIRPα variants, SIRPα1 and SIRPα2, found in the Caucasian population and both were shown to be able to enhance Trastuzumab-
mediated ADCC in breast cancer cells. Notably, the inability of the SIRPα1-specific antibody to enhance antibody-dependent tumor cell elimination when effector cells from heterozygote SIRPαα/SIRPαβ individuals were used suggests that inhibitory signals from both alleles are required to provide substantial control over antibody-mediated cytotoxicity. It will be of interest to test the in vivo efficacy of our antibodies in appropriately humanized mouse xenograft tumor models.

Clearly, an interesting and clinically highly relevant question is whether CD47-SIRPα interactions play a regulatory role in the context of antibody therapy in human cancer patients, and whether antagonists targeting the CD47-SIRPα interaction, such as the antibodies against SIRPα described herein, can be used to enhance the clinical efficacy of Trastuzumab. While the present study does not provide direct evidence for this, our findings do suggest a preliminary link between CD47 expression on breast cancer cells and clinical Trastuzumab responsiveness in breast cancer. In particular, pathologically complete responders were found to have significantly lower CD47 mRNA levels compared to Trastuzumab-treated patients lacking a pathologically complete response.

It should be emphasized that CD47-SIRPα interactions may not form the only mechanism by which tumor cells can evade phagocyte-mediated immune destruction. In fact, recent studies have shown that the interaction between the ‘self’ CD200 molecule, expressed on tumor cells and many other cell types, and the non-conventional (i.e. ITIM-lacking) inhibitory CD200 receptor (CD200R) on myeloid cells may also limit the immune-mediated elimination of leukemic cells, such as B-CLL. However, this can apparently occur in absence of therapeutic antibodies, and may also be mediated by a different effector mechanism involving cytotoxic T cells. The observation that different non-redundant mechanisms may actually underlie the regulatory effects of the CD47-SIRPα and the CD200-CD200R interaction may actually generate opportunities for simultaneous targeting of these pathways to increase therapeutic benefit.

Collectively, our results provide direct evidence for a homeostatic regulatory role of CD47-SIRPα interactions in the context of antibody-mediated destruction of tumor cells by myeloid cells. Together with the findings of Chao et al., this provides a strong rational basis for combining therapeutic antibodies against cancer cells with antagonists of the CD47-SIRPα interaction, such as the mAb against SIRPα described here. This is anticipated to enhance the clinical efficacy of cancer-targeting therapeutic antibodies and/or reduce the need for chemotherapy or other non-specific treatment regimens.

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REFERENCE LIST


SUPPLEMENTARY METHODS AND FIGURE LEGENDS

Cell lines and culture
All cell lines were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (i.e. complete RPMI) at 37°C 5% CO₂. B16F10 and SKBR-3 cells were passaged and harvested by enzymatic treatment with trypsin (0.05%)-EDTA (0.02%)-PBS solution. Knock-down of CD47 in SKBR-3 cells was performed using CD47 shRNA (CCGAGCACAATTACTGGACTATCTCTCAGAATGTTGCTTTTT) that was introduced into the cells by lentiviral transduction according to instructions provided by the manufacturer (Sigma, USA). CHO cells were transfected with full length human SIRPa₁, SIRPa₂, SIRPβ₁ and SIRPα constructs, cloned by PCR from appropriate donors into pcDNA3.1 (Invitrogen), using FuGene transfection reagent (Roche) as described by the manufacturer and cells were selected with 50 mg/ml G418 (Gibco). Constructs were verified by Sanger sequencing. SIRPa₁ and SIRPa₂ expression was verified by Western blotting using rabbit anti-human SIRPa directed against the invariable C-terminal cytoplasmic region (Abcam8120, Abcam, Cambridge, UK) and mouse anti-human β-actin (clone AC-15, Sigma, USA) as a loading control. IRDye® 800CW conjugated goat anti-rabbit IgG and IRDye® 680 conjugated donkey anti-mouse IgG (both LI-COR Corporate, Nebraska USA) were used as secondary antibodies and signals were visualized in an Odyssey Infrared Imaging System (LI-COR Bioscience, Nebraska USA). SIRPβ₁ and SIRPα expression was verified by flow cytometry using B4B6 and MRC OX119 mAb, respectively.

Antibodies, CD47-beads binding assay, and flow cytometry
The following primary antibodies were used: anti-mouse CD47 (Miap301, rat IgG₂a), anti-human CD47 (B6H12, mouse IgG₁), either as unconjugated intact antibody, F(ab)’₂-fragments, or as PE-labeled intact antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-human SIRPβ₁ (B4B6, mouse IgG₁) and anti-human SIRPα (MRC OX119, mouse IgG₁), anti-human CD64 (Clone 10.1, mouse IgG₁, BD Pharmingen), anti-human CD32 (Clone AT10, mouse IgG₁, AbDserotec), anti-human CD16 (Clone 3G8, mouse IgG₂a, BD Pharmingen), anti-mouse melanoma antigen gp75 (TA99, mouse IgG₂a), anti-human Her2/Neu (Trastuzumab, human IgG₁, Roche). Where applicable antibodies were produced in protein-free hybridoma medium containing 2% low IgG FCS and purified by protein G- or protein A-Sepharose affinity chromatography. B6H12 F(ab)’₂-fragments were generated by pepsin digestion. For flow cytometry cells were collected as indicated, washed, and incubated in FACS buffer (PBS containing 0.1% BSA) with saturating concentrations of primary antibody for 30’ on ice. After washing the cells were incubated with either Alexa 633-labeled anti-rat IgG antibody (Molecular Probes, Eugen, OR, USA) Alexa 633-labeled anti-mouse IgG antibody (Invitrogen, Molecular Probes, USA), or FITC-labelled anti-human IgG (M1325, Mouse IgG₂a, Sanquín).

The primers 5’-GAGATCGATATGCCAGCTACTATTTTAATATATGAAATTTTAT-3’ (forward) and 5’-GAGATCGATACATGAAAACATCATGCAATTTTAG-3’ (reverse) were
used to amplify cDNA encoding the extracellular Ig-domain of human CD47 and to clone it, employing the EcoRV and BglII restriction sites, into the pFUSE IgG Fc vector. The construct was verified by sequencing. The resulting fusion protein was produced by transfecting freestyle HEK293T cells (Invitrogen) according to instructions provided by the manufacturer. The CD47-Fc protein was purified by protein G-sepharose affinity chromatography from the culture supernatant and tested for reactivity in Western blotting with anti-human CD47 and anti-human IgG antibodies. Fluorescent beads (carboxylate-modified TransFluoSpheres 488/645, Invitrogen) were covalently coated with streptavidin and were subsequently used to capture biotinylated goat anti human Fc (Jackson Immunoresearch, USA), followed by CD47-Fc. The beads were stored in PBS 0,5% BSA 0,02% NaN3 (stock 1% NaN3 in H2O) and used for experiments within 3 months. Binding to cells was done by incubating 50x10^3 cells with 20ul CD47-beads diluted 1:10 in PBS/0,1%BSA for 45 min. at 37°C followed by a single washing step and flow cytometric analysis on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equiped with FlowJo Software (Tree Star, Inc, USA).

**Production of monoclonal antibodies**

The extracellular domains (ECD) of human SIRPα1 corresponding to amino acids 1-364 and 1-365 were cloned using forward TOPO primer 3’-CACCATGGAGCCCGCCGCCC-5’ and reverse primer 3’- GAATGCAATAGCAGTAGTACAAGAGTCGCCGCCCATAAAC-5’ into pCDNA 3.1D/V5-HIS-TOPO vector (Invitrogen). The C-terminal HIS-tagged SIRPα1-ECD proteins were produced in freestyle HEK293T cells as indicated above and purified on Ni-columns using Ni-NTA Fast Start kit (Qiagen). The HIS-tags were removed by enterokinase cleavage, using enterokinase cleavage capture kit (Novagen), and the remaining SIRPα1-ECD protein was dialyzed against PBS. Removal of the HIS-tag was confirmed by Western blotting. Female Balb/c mice were immunized 5x with 20 µg of SIRPα1-ECD protein or 5x with SIRPα1-expressing CHO cells diluted in 50µl of PBS and 50µl of montanide adjuvant. The hybridomas 12C4 and 1.23A were generated, respectively, by standard hybridoma technology, or by ii) negative and positive panning, respectively, on SIRPβ1- and SIRPα1-expressing CHO cells followed by electrofusion technology6. This was followed by repeated screening by ELISA and flow cytometry for reactive clones and subcloning by limiting dilution. Antibodies were produced by culture in serum-free/IgG-low medium and purified by protein-G-sepharose affinity chromatography and dialysed against PBS. Both antibodies were of the IgG1 isotype.
Supplementary Figure 1 | Antibody-dependent cellular cytotoxicity of human neutrophils toward SKBR-3 breast cancer cells after preincubation with trastuzumab (10 μg/mL) and/or B6H12 (10 μg/mL) anti-CD47 F(ab')2 or intact IgG. Values shown are means ± SD (n = 3) from a representative experiment out of three. Note that intact anti-CD47 IgG alone but not anti-CD47 F(ab')2 induces neutrophil-mediated cytotoxicity. P values of statistically significant differences, as determined by Student’s t test, are indicated. ns, nonsignificant.

Supplementary Figure 2 | Relationship between trastuzumab-induced neutrophil-mediated cytotoxicity against SKBR-3 cells and FcR expression. Experiments (n = 25) with individual neutrophil donors were divided into two groups based on high (above 50%) or low (below 50%) trastuzumab-dependent killing capacity. Surface expression levels (MFI) measured by flow cytometry of FcγRI (A) (using mAb 10.1, mouse IgG1; BD Pharmingen), FcγRII (B) (using mAb AT10, mouse IgG1; AbD Serotec), and FcγRIII (C) (clone 3G8, mouse IgG2b; BD Pharmingen) combined with Alexa 633-labeled anti-mouse IgG antibody (Invitrogen, Molecular Probes) are shown. Note that statistically higher killing is associated with higher FcγRI and FcγRIII levels. P values of statistically significant differences, as determined by Student’s t test, are indicated.
Supplementary Figure 3 | Characterization of mAb against SIRPα. (A) Expression of the two SIRPα variants was verified by Western blotting using a polyclonal antibody against the invariant C terminus. (B) The 1.23A and 12C4 mAbs were generated as described in SI Methods and tested for binding to CHO cells transfected with the fulllength SIRP family members SIRPα (including the two polymorphic variants SIRPα1 and SIRPαBIT), SIRPβ1, and SIRPα (filled histograms). The open histogram represents the isotype control. Note that the 1.23A mAb selectively recognizes SIRPα1, whereas 12C4 recognizes all of the indicated family members. (C) Flowcytometric surface staining of phagocytes, namely freshly isolated neutrophils (PMN) and monocytes, from individuals with α1/α1 or αBIT/αBIT homozygotes or α1/αBIT heterozygotes using mAbs 1.23A and 12C4 (filled histograms). The open histogram represents the isotype control. Note that whereas mAb 12C4 recognizes both SIRPα alleles (and perhaps also SIRPβ1), mAb 1.23A specifically recognizes the α1 allele, with higher levels in the homozygote than in the heterozygote individual. Stainings shown are a representative example of 12 experiments.
REFERENCE LIST


