Targeting CD47-SIRP interactions for potentiating therapeutic antibody-mediated tumor cell destruction by phagocytes
Zhao, Xi

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Xi Wen Zhao

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Single 411
1012 XM Amsterdam

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Paranimfen
Hanke Matlung
h.l.matlung@gmail.com
Michel van Houdt
michelynvhoudt@hotmail.com
Targeting CD47-SIRPα interactions for potentiating therapeutic antibody-mediated tumor cell destruction by phagocytes

Xi Wen Zhao
赵西雯
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Targeting CD47-SIRPα interactions for potentiating therapeutic antibody-mediated tumor cell destruction by phagocytes

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Xi Wen Zhao

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Copromotor: Dr. T.K. van den Berg

Overige leden: Prof. dr. L.A. Aarden
Dr. M. van Egmond
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Faculteit der Geneeskunde
To my Parents
致我的父亲母亲
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Introduction
INTRODUCTION

Metastatic and disseminated cancers are generally treated by chemotherapeutics and other non-specific conventional treatments. However, such therapies often cause considerable side effects that deteriorate prognosis or may even cause additional forms of cancer. The immune system, which is highly specialized for the elimination of harmful pathogens while causing minimal damage to the host, is, at least in principle, ideally suited for selective tumor cell elimination. Based on this idea different types of immune therapeutic approaches have been explored for the treatment of cancer. Some of these have already been approved for and used in clinical practice, whereas others are still under investigation. Essentially, two types of immunotherapy can be distinguished. On the one hand there are the cell-based therapies that mostly aim to raise an effective response of infused most commonly cytotoxic T (CTL) cells or NK cells against the tumor cells. This idea was originally pioneered by Rosenberg using so called lymphokine-activated killer (LAK) cells, while investigators are now exploring the potential of e.g. CTL with genetically engineered chimeric antigen receptors (CAR)\(^1\)\(^2\). On the other hand there are the therapeutic monoclonal antibodies, some of which have already been experienced in clinical practice for more than 25 years by now.

Most of the currently used anti-cancer therapeutic antibodies have been developed to induce direct or indirect elimination of tumor cells, generally by specifically targeting a tumor antigen that is more or less selectively (over)expressed on the surface of the tumor cells. Most of the time the tumor destruction of these antibodies also depends on recruitment of immune-mediated effector mechanisms, including antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC)\(^3\). ADCC involves interactions with specialized receptors for the constant regions of antibodies, known as Fc receptors (FcR), on relevant effector cells of the immune system, such as NK cells or phagocytes. Unfortunately, the overall clinical benefit of cancer therapeutic antibodies is too limited to justify their use as monotherapeutic agents, but they are still used in combination with non-specific and harmful chemotherapeutics, which by causing e.g. leukopenia further undermine the beneficial effect of antibody therapy in cancer. Therefore, there is an urgent need to improve antibody therapy in cancer. This thesis is dedicated to provide a proper understanding of the mechanism(s) by which in particular phagocytes mediate ADCC, and to explore how this can perhaps be improved by targeting immuno-regulatory pathways, such as the one mediated by CD47-SIRP\(\alpha\) interactions.

In this introduction, I will first focus on the general knowledge of antibody based therapy in cancer treatment. I will provide a description of the available antibody therapeutics for different types of cancer, their clinical effects, and the cellular and molecular mechanisms underlying these effects. In second part of the introduction, I will describe the role of interactions between the broadly expressed CD47 molecule and the inhibitory immunoreceptor SIRP\(\alpha\) in the regulation of phagocyte function. Finally, I will propose CD47-SIRP\(\alpha\) interactions as a potential molecular target for the enhancement of antibody therapy against cancer and provide an outline of the studies that are described in this thesis to explore this.
ANTIBODY THERAPY AGAINST CANCER

1. Introduction
Over the past 30 years, antibody cancer therapeutics have been developed and used clinically. This has revealed the potential of targeted immune-based therapy in cancer. Therapeutic antibodies were designed to targeted tumor antigen and induced direct and indirect tumor destruction via different mechanisms. Until now, the US Food and Drug Administration (FDA) has approved 12 monoclonal therapeutic antibodies in the field of oncology (Table 1), and given the number of agents currently in clinical trials it seems likely that this number will further increase in the near future. Among the first generation cancer therapeutic antibodies, which are obviously most extensively explored are, for example, the chimeric IgG1 antibody Rituximab (Rituxan™) against CD20 used in Non-Hodgkin lymphoma and other B cell malignancies, humanized IgG1 antibody Trastuzumab (Herceptin™) against the Her2/Neu growth factor receptor used in metastatic breast cancer, and Cetuximab (Erbitux™) which targets the epidermal growth factor receptor (EGFR) and is used for the treatment of colorectal and other types of gastro-intestinal cancer. Based on this a number of ‘biosimilar’ antibodies have subsequently been developed that target the same tumor antigens. In addition to direct tumor antigen targeting, therapeutic antibodies that modulate the tumor microenvironment have been developed, such as Bevacizumab (Avastin™) directed against vascular endothelial growth factor (VEGF), which acts by preventing new blood vessel formation within developing cancers, thereby essentially cutting off food and oxygen supply. Finally, there are a number of antibodies, partly still under development, that are aimed to decrease or limit tumor growth by enhancing the host immune response, a strategy that has collectively been termed ‘immune-checkpoint inhibition’. An example of that is Ipilimumab, directed against the co-inhibitory receptor CTLA on T lymphocytes, and different antibodies directed against the PD1-PDL1 inhibitory axis, another pathway that keeps T cell activity under control.
Table 1 | FDA/EMA approved therapeutic monoclonal antibodies in oncology. Data derived from Weiner et al[100].

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target and isotype</th>
<th>Application</th>
<th>Company</th>
<th>First approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rituxan</td>
<td>CD20; Chimeric, IgG1</td>
<td>CD20-positive B-cell non-Hodgkin lymphoma</td>
<td>Johnson &amp; Johnson</td>
<td>1986 (US)</td>
</tr>
<tr>
<td>Herceptin: Trastuzumab</td>
<td>HER2: Humanized, IgG1</td>
<td>HER2 positive metastatic breast cancer</td>
<td>Genentech/Roche</td>
<td>1998 (US)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2000 (EU)</td>
</tr>
<tr>
<td>Campath: Alemtuzumab</td>
<td>CD52: Humanized, IgG1</td>
<td>Chronic lymphocytic leukemia</td>
<td>Genzyme</td>
<td>2001 (US)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2001 (EU)</td>
</tr>
<tr>
<td>Zevalin: Ibritumomab tiuxetan</td>
<td>CD20: Murine, IgG1, radiolabeled 90Y</td>
<td>Low grade or B cell non-Hodgkin’s lymphoma</td>
<td>Biogen Idec</td>
<td>2002 (US)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2004 (EU)</td>
</tr>
<tr>
<td>Bexxar: Tositumomab</td>
<td>CD20: Murine, IgG2a, radiolabeled 131I</td>
<td>CD20-positive follicular lymphoma</td>
<td>Corixa/GSK</td>
<td>2003 (US)</td>
</tr>
<tr>
<td>Erbitux: Cetuximab</td>
<td>EGFR: Chimeric, IgG1</td>
<td>Metastatic colon cancer and head and neck cancer</td>
<td>Imclone/Lilly</td>
<td>2004 (US)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2004 (EU)</td>
</tr>
<tr>
<td>Avastin: Bevacizumab</td>
<td>VEGF: Humanized, IgG1</td>
<td>Metastatic colon cancer</td>
<td>Genentech/Roche</td>
<td>2004 (US)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2005 (EU)</td>
</tr>
<tr>
<td>Vectibix: Panitumumab</td>
<td>EGFR: Human, IgG2</td>
<td>Metastatic colon cancer</td>
<td>Amgen</td>
<td>2006 (US)</td>
</tr>
<tr>
<td>Arzerra: Ofatumumab</td>
<td>CD20: Human IgG1</td>
<td>Chronic lymphocytic leukemia</td>
<td>Genmab</td>
<td>2009 (EU)</td>
</tr>
<tr>
<td>Yervoy: Iplimumab</td>
<td>CTLA-4: Human IgG1</td>
<td>Melanoma</td>
<td>Seattle Genetics</td>
<td>2011 (US)</td>
</tr>
<tr>
<td>Perjeta: Pertuzumab</td>
<td>Her2: Humanized, IgG1</td>
<td>HER2-positive metastatic breast cancer</td>
<td>Genentech/Roche</td>
<td>2012 (US)</td>
</tr>
<tr>
<td>Kadcyla: Ado-trastuzumab emtansine</td>
<td>Her2: Humanized, IgG1, Drug-conjugate</td>
<td>HER2-positive metastatic breast cancer</td>
<td>Genentech/Roche</td>
<td>2013 (US)</td>
</tr>
</tbody>
</table>

Our own efforts have been dedicated to understanding and improving the established forms of antibody therapy, in which an antibody is targeting a tumor antigen on the surface of cancer cells. In the following parts I will highlight the important features of such anti-tumor antibodies, with a major focus on how such antibodies mobilize immune effector mechanisms for inducing tumor destruction, and how the limitations for that can potentially be relieved.
2. Antibody structure and function
Köhler and Milstein first established the hybridoma technology for the production of mouse monoclonal antibodies (mAb) in 1975, and this allowed monospecific antibodies of the appropriate specificities to be selected and produced, under controlled conditions, in essentially infinite amounts. This opened the door to produce antibodies for therapeutic purposes. Since then, many advances in antibody engineering technology have occurred that enabled the generation of chimeric mouse-human, humanized and eventually fully human mAbs, which promote immunocompatibility by minimalization of the number of mouse immunogenic determinants. IgG₁ is the most frequently used antibody isotype in oncology, and also in general. IgG antibodies are large molecules of about 150 kDa composed of two heavy (H) and (L) and two light polypeptide chains. The two heavy chains are linked to each other and to a light chain each by disulfide bonds, form a Y-shaped structure. IgG can be divided into two different parts: the antigen binding region (Fab) and constant binding region (Fc). The Fab contains of variable domains of the H and L chains, which each bear 3 complementarity-determining regions (CDRs). The CDRs contribute to antigen binding and confer the antigenic specificity (3D structure of Herceptin Fab complex with HER extracellular domain shown in Figure 1). The Fc-region consist of the flexible hinge and the constant heavy-chain domains (CH₂ and CH₃) that provides recognition sites for Fc receptors expressed on immune cells, which triggers downstream effector functions, such as ADCC. The Fc-portion is also capable of initiating complement-dependent cytotoxicity (CDC).

Figure 1 | Structure of the extracellular region of Her2/Neu alone and in complex with the Trastuzumab Fab. Green: Her2 extracellular domain; Red: Light Chain of Herceptin Fab fragment. Blue: Heavy Chain of Herceptin Fab fragment. Figure derived RCSB website http://www.rcsb.org/pdb/explore.do?structureId=1n8z (ID: In8z), data published by Cho HS et al.101
3. Mechanisms of action of cancer therapeutic antibodies

The antibodies can invoke tumor cell death by different pathways, which include targeting tumor receptors, tumor microenvironment and anticipate in immune surveillance. Here I summarize the general knowledge of therapeutic anti-tumor antibodies, with focus on such antibodies triggering tumor destructions.

3.1 Direct growth inhibitory mechanisms

Monoclonal antibodies have been generated to against antigens more or less specifically expressed on tumor cells. Some of the most commonly exploited target antigens to which therapeutic antibodies have been developed are the growth factor receptors, which are overexpressed in certain cancers. These growth factor receptors are actually partly causative to the tumorigenic potential of the cells that carry these receptors, because their overexpression amplifies the respective growth factor response. Therapeutic antibodies binding to these receptors, generally act to prevent their normal activation by their natural growth factor ligands, thereby inhibiting the growth rate of the cancer cells and/or promoting the induction of apoptosis. The exact mechanisms of action depend on the nature of the growth factor receptor, and sometimes even the targeting antibody as well.

Both EGFR and Her2/Neu are members of the EGFR receptor family of growth factor receptors that are overexpressed on certain solid tumors, including colon carcinoma and Her2/Neu-positive breast tumor, and a number of registered monoclonal antibodies are directed against these two growth factor receptors. One of the most widely used and therefore clinically best tested antibodies in oncology is Cetuximab, a chimeric IgG1 directly against epidermal growth factor receptor (EGFR). Cetuximab binds to extracellular domain of the EGFR, thereby acting as competitive antagonists of the natural ligands. As a result, Cetuximab blocks EGFR-mediated signaling, leading to the G1 cell cycle arrest that ultimately results in an inhibition of carcinoma cell proliferation. Trastuzumab, a humanized IgG1 antibody, is used for treatment of invasive breast cancer with overexpression of Her2/Neu. In contrast to EGFR, Her2/Neu has no known natural ligand, but binding of Trastuzumab inhibits Her2/Neu dimerization and thereby blocks cell cycle progression in the breast cancer cells.

Furthermore, hematopoietic malignancy associated antigen have been developed as therapeutic target and been successfully applied in varies cancer therapies, such as Rituximab, (chimeric human–murine IgG1, against CD20) and Alemtuzumab (Humanized IgG1, against CD52). Rituximab has been widely used in Non-Hodgkin’s lymphoma, which eliminated tumor cells by inducing apoptosis and complement dependent cytotoxicity (CDC). Furthermore, a response to rituximab in patients with follicular lymphoma may be correlated with the presence of an FcγRIIIA polymorphism (valine at position 158) that is associated with improved antibody binding, suggesting the importance of antibody-dependent cellular cytotoxicity (ADCC). Alemtuzumab has been reported as a single reagent for the treatment of chronic lymphocytic leukemia, and it binding to CD52 on tumor cell and induce direct killing effect by apoptosis and CDC. However, it should be noted that to some extend the antibody-mediated interference with growth promoting pathways may even help to sensitize the tumors to the cytostatic effects of chemotherapeutic drugs. In fact,
cancer therapeutic antibodies are not potent enough to cure cancer and that why these agents are generally combined in cancer therapy. On the other hand, the use of chemotherapy can also be seriously compromise the immune-mediated effects of cancer monoclonal antibodies (see also below).

Finally, as indicated above there are also indirect non-immune mechanisms by which cancer therapeutic antibodies can limit cancer cell growth. The best known example is Bevacizumab, a humanized IgG1 antibody against VEGF, which neutralizes binding of the VEGFR and thereby prevents angiogenesis in the developing tumors, which is required for further growth of these tumors14.

3.2 Immune-mediated effector mechanisms

Immune-mediated effector functions of therapeutic antibodies include two classes of mechanisms: i) cellular Fc-receptor (FcR)-dependent effector mechanism(s), including antibody dependent cellular cytotoxicity (ADCC) or antibody dependent cellular phagocytosis (ADCP), and ii) humoral complement-dependent cytotoxicity (CDC), which is mediated by complement. Both mechanisms can occur simultaneously and they are initiated by interactions between either the Fc-domain of immunoglobulin protein and FcR on effector cells, or between the antibody Fc-portion and complement components present in the plasma or extracellular compartment of tissues.

3.2.1 FcR-dependent cellular mechanisms

A number of distinct receptors for the various isotypes of the IgG class of antibodies, termed Fcγ receptors (FcγR), are present in humans, including a high-affinity FcγRI (CD64) and a number of low-affinity FcγRII (CD32) and FcγRIII (CD16)15. The FcγR are encoded by a cluster of genes located on human chromosome 1 (Fig. 2A), and they are expressed in a cell type specific fashion (Fig. 2C), have different affinities for the various IgG isotypes, and have either intracellular signaling activating potential or, in the case of FcγRIIb, signaling inhibitory capacity (Fig. 2B). Upon binding an cross-linking FcγRs, the activating FcγRs can transduce stimulatory signals through immunoreceptor tyrosine-based activation motifs (ITAMs), encoded within either the cytoplasmic domain of the receptor (in FcγRIIa) or within the associated g-chain (the others), which can trigger downstream signaling via the pivotal Src-family protein tyrosine kinases and the pivotal tyrosine kinase Syk16. Of relevance, there is genetic variation within the population with respect to FcγR, particularly within the FcγR2/3 subcluster of genes, and the different genetic variants, which include both polymorphisms and gene copy number variation (CNV) (Table 2), can differ with respect to expression, IgG affinity and/or signaling capacity15,17-19. In fact, SNPs in the FcγR2A and FcγR3A genes appear to have clinical significance as they have been reported to correlate with responses to therapeutic mAbs in cancer treatment. A coding polymorphism in the extracellular domain of FcγR2A has been described where a C>T substitution changes the amino acid from histidine to arginine at position 13120. A second important coding polymorphism occurs in extracellular domain 2 of FcγR3A. A T>G substitution changes valine to phenylalanine at position 15821. It is known, for instance, that patients with homozygous 131-histidine/
histidine alleles of FcγRIIa (FcγRIIa-131H/H) are associated with higher clinical efficacy of Trastuzumab-based therapy in Her-2/neu-positive metastatic breast cancer. Similar findings for this polymorphism have been done in the context of Rituximab treatment in Non-Hodgkin lymphoma. Other studies show that also patients with homozygous 158-valine/valine alleles of FcγRIIIa (FcγRIIIa-158V/V) display a higher response rate to rituximab treatment.

Figure 2 | The human FcγRs family. A) The genes encoding human Fcγ receptors (FcγRs) are located in a genomic cluster on chromosome 1. B) Structure of various human FcγRs and its different single nucleotide polymorphisms (SNPs). Figure is adapted from Misbah S et al. Green box represents ITAMs; and the red box represents ITIMs. C) Human FcγR expression pattern on different immune cells. Data derived from Bruhns P and van der Hijden et al. + = detectable expression; (+) = inducible expression; +/- = very low percentage or rare subsets express the receptor; − = no detectable expression. * = associated with FcγR2C-ORF donors; § = detectable and functional expression in nonconventional FcγR2C-ORF donors.
Various immune effector cells that carry Fcγ receptors (FcγR), such as natural killer (NK) cells and phagocytes, including macrophages and granulocytes, may contribute to the destruction of antibody-opsonized cancer cells. Their relative therapeutic contribution may depend on the type of tumor cells and also on the therapeutic antibody, but the genetic association studies outlined above, in which a link between amongst FcγR polymorphisms and the clinical efficacy of cancer therapeutic antibodies has been established, strongly support the idea that FcγR-dependent cellular mechanisms do play an important role. However, the actual cellular effector mechanisms by which NK cells and phagocytes in particular mediate antibody-dependent tumor cell destruction have not been thoroughly established.

**Table 2** Genetic variation in human FcγR and its functional implications. Various single nucleotide polymorphisms (SNPs) have been identified in 3 of human FcγRs. Some of them are known to have functional implications related to binding affinity of IgG subtypes or/and signaling effects.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Variants</th>
<th>Functional relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγR2A</td>
<td>SNP</td>
<td>H131, R131</td>
<td>Binding affinity for hlgG1 and hlgG2a: H131 &gt; R131. R131 can bind mIgG1.</td>
</tr>
<tr>
<td>FcγR2A</td>
<td>Splice site mutation</td>
<td>c.742+871A, c.742+871G</td>
<td>c.742+871A&gt;G leads to splice variant FcγRIIA*extein871</td>
</tr>
<tr>
<td>FcγR2B</td>
<td>SNP</td>
<td>I232, T232</td>
<td>I232 inhibits FcγRI signaling more strongly than T232.</td>
</tr>
<tr>
<td>FcγR2C</td>
<td>Promoter haplotype</td>
<td>2B.1, 2B.2, 2B.3, 2B.4</td>
<td>2B.2 is linked with an ORF in FcγR2C exon 3. 2B.4 results in an increased transcriptional activity of FcγR2B</td>
</tr>
<tr>
<td>FcγR2C</td>
<td>SNP</td>
<td>Q13, Stp13</td>
<td>Q13 leads to an ORF in exon 3 and expression of FcγRIIc (when combined with c.791+1G)</td>
</tr>
<tr>
<td>FcγR2C</td>
<td>Splice site mutation</td>
<td>c.791+1G, c.791+1A</td>
<td>c.791+1A leads to misplacing and lack of expression level of FcγRIIc</td>
</tr>
<tr>
<td>FcγR2C-ORF</td>
<td>CNV</td>
<td>0, 1 or 2 copies</td>
<td>The copy number of FcγR2C-ORF relates to expression of FcγRIIc; the NK cells mediated ADCC</td>
</tr>
<tr>
<td>FcγR3A</td>
<td>SNP</td>
<td>V158, F158</td>
<td>Binding affinity for all hlgG isotypes: V158 &gt; F158</td>
</tr>
<tr>
<td>FcγR3A</td>
<td>CNV</td>
<td>1, 2 or 3 copies</td>
<td>The number of copies of FcγR3A relates to expression level of FcγRIIIa</td>
</tr>
<tr>
<td>FcγR3B</td>
<td>Polymorphic variants</td>
<td>NA1, NA2, SH</td>
<td>Binding affinity for hlgG3: NA1 and SH &gt; NA2.</td>
</tr>
<tr>
<td>FcγR3B</td>
<td>CNV</td>
<td>0, 1, 2 or 3 copies</td>
<td>The number of copies of FcγR2C-ORF relates to expression levels of FcγRIIIb; binding and uptake of immune complexes</td>
</tr>
</tbody>
</table>
3.2.1.1 NK cell-mediated ADCC
NK cells are a population of innate lymphoid cells important in the elimination of aberrant cells, including virus-infected cells and tumor cells. NK cell cytotoxicity is triggered by various conditions, including an absence of MHC class I molecules on host cells (a condition known as ‘missing self’), or by IgG-opsonized cells (i.e. ADCC), the latter occurring primarily through FcγRIIIa (CD16a). The importance of NK cell-mediated immunosurveillance in the control of tumor growth has been demonstrated in NK cell-deficient mouse models, which shown an increased susceptibility to neoplastic disease as they aged. A SCID xenograft leukemia/lymphoma mouse model also confirmed that NK-cell depletion resulted in diminished efficacy of antibody therapy.

NK cells store cytotoxic proteins in secretory lysosomes, also known as lytic or cytotoxic granules. Within these cytotoxic granules a lytic mixture perforin, a plasma membrane pore-forming protein, and a group of serine proteases called granzymes, including the apoptosis-inducing granzyme B, are engaged in this process. Upon recognition of target cells a cytotoxic ‘killer’ synapse is formed between the effector cell and target cells, directed exocytosis of secretory lysosomes is then activated, and subsequently these organelles release their cytotoxic contents into the killer synaptic interface. The granzymes, which trigger caspase-dependent apoptosis in the target cells, are then delivered by a perforin-dependent mechanism into the cytosol of the target cell and eventually induce tumor cell death.

Severely impaired NK cell cytotoxicity has been shown for cells from patients that have mutations in either perforin or in proteins that are intrinsic components of the cellular machinery required for secretory granule exocytosis. Because such defects in the killing of NK, and also cytotoxic T lymphocytes (CTL), can lead to the ineffective destruction of virus-infected cells, but have a sustained IFNγ production by such cells, this can cause a life threatening condition known as macrophage activation syndrome. One of such genetic defects known as familial hemophagocytic lymphohistiocytosis (FHL5) is caused by mutations in the gene encoding STXBP2, also known as munc18-2, which is result in impaired NK cell and CTL granule exocytosis and cellular cytotoxicity. In chapters 5 and 6, we describe studies in which NK cells and neutrophils of such patients are employed to investigate a role in the killing of both cancer cells and bacteria.

3.2.1.2 Phagocyte-mediated antibody-dependent mechanisms
Phagocytes (phagocyte is Greek for “large eater”) constitute a group of innate immune cells derived from the myeloid lineage of hematopoietic cells, including macrophages and (neutrophilic) granulocytes, which protect the body from infection by destruction and elimination of pathogens. Macrophages in particular are also responsible for the clearance of cell debris or dying (apoptotic) cells. Macrophages are very heterogeneous with respect to phenotype and function, and various subsets of specialized macrophages can be found in different tissues. Monocytes comprise a circulating pool of macrophage precursors that can also infiltrate inflammatory lesions and cancers. Macrophages are also critical mediators of inflammation during infection and other inflammatory conditions, and are able to generate a large number of inflammatory mediators, including cytokines, chemokines, lipids and others (e.g. reactive oxygen species and nitric oxide metabolites). In important part of their functions...
is to phagocyte or engulf foreign substances, infectious microbes and host cells, including cancer cells, and opsonins such as antibodies and complement can promote this significantly. Although there is still debate about the exact tumor-promoting or anti-cancer effects of tumor-associated macrophages (TAM) in the absence of cancer therapeutic antibodies, the destructive capacity of macrophages towards antibody-opsonized tumor cells is well documented, with numerous studies showing that activated macrophages are potent in killing malignant cells of various tissue origins. While a major mechanism of antibody-dependent tumor cell destruction by macrophages appears to be phagocytosis, also known as antibody-dependent cellular phagocytosis (ADCP), other mechanisms can not be excluded. For instance, macrophages secrete several substances that may be directly involved in tumor cell killing, such as e.g. tumor necrosis factor (TNF) and nitric oxide (NO).

Neutrophilic granulocytes, also known as polymorphonuclear cells (PMN), are the most abundant phagocyte subset within human blood, and they are of critical importance in the defense against invading bacteria and fungi. The two major intracellular effector mechanisms exerted by neutrophils in the context of microbial killing, include oxidative killing and granule-mediated cytotoxicity. Oxidative killing involves the phagocyte NADPH oxidase, a multi-subunit enzyme complex that is assembled at the phagosomal and plasma membrane upon activation and that mediates the production of toxic reactive oxygen species. Granule-mediated cytotoxicity involves proteases and other anti-microbial components that are preformed and stored in different classes of granules (e.g. azurophilic or specific), the contents of which are released into the phagosome or the extracellular milieu upon neutrophil activation. Apart from their clear involvement in innate immunity against infections, there is growing attention for PMN as potential effector cells in antibody-dependent destruction of cancer cells in vitro and in animal models in vivo. Other studies also confirm the importance of neutrophils in the alemtuzumab and rituximab mediated ADCC activity in vivo. However, although there were already some indications that neutrophil-mediated antibody-dependent cancer cell was NADPH oxidase independent at least, and also that neutrophils are unable to engulf entire cancer cells, the actual mechanism(s) of neutrophilic granulocyte-dependent tumor cell cytotoxicity have until now remained unclear. Regardless of the killing mechanism itself, it seems, in analogy to NK cells, that killer synapse formation between neutrophils and antibody-opsonized tumor cells is important. The work described in this thesis confirms and extends the role of cytotoxic synapse formation during neutrophil ADCC (chapter 4), and also provides preliminary evidence for a fundamentally different mechanism of cancer cell killing by human neutrophils (chapter 5).

### 3.2.2 Complement-dependent cytotoxicity

Apart from the above described FcγR-mediated mechanisms, antibodies can also bind complement through their Fc-portions and trigger downstream complement-dependent cytotoxicity (CDC). The complement system is a set of plasma and membrane proteins that upon triggering can cooperate to mediate cytotoxicity. Activation of the complement system may occur through one or more out of three proteolytic cascades known as the classical, lectin and alternative pathways. Amongst the various classes and isotypes of human antibodies, IgG₄ is the most effective isotype with respect to triggering complement
activation\textsuperscript{62}, and this may actually be one of the reasons why most of the currently available cancer therapeutic antibodies are of this isotype. Following antibody binding to C1q, an interaction for which the structural requirements have recently been clarified\textsuperscript{63}, the classical pathway of complement is activated. Cleavage of soluble C3 results in C3b formation, which covalently attaches to the target cell surface or to the antibody itself. This C3b subsequent mediates the formation of C5-C9 membrane attack complex (MAC), a pore-forming structure leading to cell lysis through osmotic shock\textsuperscript{3}. Apart from the direct killing of antibody-coated tumor cells via MAC activation, complement can also opsonize tumor cells and facilitate cellular cytotoxicity and/or phagocytosis upon engagement of bound C3 components with CR3 on effector cells\textsuperscript{64,65}.

4. Signal regulatory proteins

4.1 The SIRP family of immunoreceptors

It is of critical importance to keep an appropriate balance in the activity (or inducibility of activity) of the immune system. Excessive activation may result in autoimmunity, whereas too little activation (or too much inhibition) could cause immune-deficiency and therefore an impaired response to infection. At least in part the balance within the immune system is maintained by a variety of activating and inhibitory immunoreceptors expressed on the surface of immune cells. There are a number of such ‘paired’ receptor families within the immune system, with the term paired referring to the fact that those families harbor both activating and inhibitory family members with highly homologous extracellular domains. One such family, the Fc\textsubscript{γ}R that mediates recognition of IgG antibodies, has already been introduced in the previous part. However, there are quite a number of other paired receptor families within the human immune system and the general idea is that these act simultaneously and collectively to keep the right immune equilibrium\textsuperscript{66}.

Among the largest and probably also genetically most diverse and complex family of paired receptors is that of the killer immunoglobulin-like receptors primarily expressed on human NK cells. The inhibitory KIR can act as receptors for MHC class I molecules, such as e.g. HLA-C. Furthermore, their cytoplasmic regions encode typical immunoreceptor tyrosine-based inhibitory motifs (ITIM), which upon phosphorylation recruit the intracellular tyrosine phosphatases SHP-1 and/or SHP-2 that restrict NK mediated cytotoxicity\textsuperscript{67}. Instead, the activating KIR receptors, which only have short cytoplasmic regions without such or any other signaling motifs, associate with the transmembrane adaptor protein DAP12. DAP12 contains an immunoreceptor tyrosine-based activation motif (ITAM) and functions therefore, in a fashion analogous to that of the FcR\textgamma-chain, by generating activating signals that promote cytotoxicity towards tumor and virus-infected cells\textsuperscript{68,69}.

A family of paired immunoreceptors prominently expressed on myeloid cells is the signal-regulatory protein (SIRP) family\textsuperscript{70}. Several SIRP members have been identified in humans, including the closely homologous SIRP\textalpha SIRP\textbeta1 and SIRP\textalpha, and the more distantly related SIRP\textbeta2. SIRP\textalpha, which has also been termed Src-homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHP-S1), or BIT (brain Ig-like molecule with tyrosine-based activation motifs), macrophage fusion receptor (MFR), p84, constitutes the prototypic
and only inhibitory receptor of the family\textsuperscript{71-74}. The molecule has four ITIM motifs within its cytoplasmic domain, which upon interaction and clustering by its widely expressed cell surface ligand CD47, becomes phosphorylated. The resultant recruitment and activation of SHP1 and/or SHP2 regulates, generally in a negative fashion, the various downstream functions, such as e.g. phagocytosis (discussed in more detail below)\textsuperscript{72;75-77}. SIRP\(\beta\)1 contains a transmembrane region with a positively charged lysine residue, which mediates an association with DAP12. The consequent activating signaling results, for instance, in an enhanced phagocytosis in macrophages through Syk and the mitogen-activated protein kinase pathway\textsuperscript{78}. The natural ligand of SIRP\(\beta\)1, which at least is not CD47, is still unknown. SIRP\(\alpha\) has a short cytoplasmic domain without any (known) motifs or capacity signaling. SIRP\(\alpha\) can interact with CD47, albeit with 10-fold lower binding affinity than SIRP\(\beta\)1. Unlike other SIRP proteins, SIRP\(\alpha\) is expressed on T cells and NK cells and CD47-SIRP\(\alpha\) interactions may be involved in T-cell trans-endothelial migration\textsuperscript{79}.

4.2 The CD47-SIRP\(\alpha\) interaction
SIRP\(\alpha\) is the best characterized member of the SIRP family (reviewed in \textsuperscript{70;80;81}). This inhibitory receptor is primarily expressed myeloid cells and also on neurons\textsuperscript{82}. The extracellular region of SIRP\(\alpha\) contains 3 Ig-like super family (IgSF) domains, a transmembrane region, and a cytoplasmic region, which encodes 4 tyrosine residues that form two complete ITIM motifs. The N-terminal (V-set) domain mediates the interaction with its ligand CD47\textsuperscript{83;84}, and the structural basis for CD47 recognition by SIRP\(\alpha\) is well defined\textsuperscript{85}. Because of the characteristic ITIM motifs in the SIRP\(\alpha\) cytoplasmic tail, CD47-SIRP\(\alpha\) interactions and SIRP\(\alpha\) signaling are generally considered to participate in an inhibitory pathway, and this is indeed largely supported by functional experiments\textsuperscript{70}. Among the best documented functions of the CD47-SIRP\(\alpha\) axis is the negative regulation of host cell phagocytosis. This was originally established in the context of red blood cell clearance, where the infusion of CD47-deficient red blood cells in mice was shown to result in a very rapid clearance of these cells and the consequent identification of CD47 as a so called ‘don’t-eat-me’ signal\textsuperscript{86}. In line with a negative regulatory role for CD47-SIRP\(\alpha\) interactions in the homeostatic clearance of blood cells mice deficient in CD47 or with a defect in SIRP\(\alpha\) signaling are mildly anemic and thrombocytopenic\textsuperscript{87;88} and also the antibody-dependent clearance of these cells in models for autoimmune-mediated hemolytic anemia (AIHA)\textsuperscript{89} and idiopathic thrombocytopenic purpura (ITP)\textsuperscript{90}. Other evidence suggests a role for CD47-SIRP\(\alpha\) interactions in the control of the phagocyte NADPH oxidase\textsuperscript{91} and in osteoclast bone resorption\textsuperscript{92}, but the \textit{in vivo} phenotypes associated with this in mutant mice appear rather mild. Of interest, SIRP\(\alpha\) displays considerable polymorphic variation within the human population\textsuperscript{93}, and this is also seen amongst inbred strains of mice\textsuperscript{94}. While variation occurs specifically in the CD47-binding N-terminal Ig-like domain of SIRP\(\alpha\) polymorphic amino acid residues are mostly located outside the critical regions for CD47-SIRP\(\alpha\) interaction and do not appear to have a major impact in ligand binding\textsuperscript{85}. Nevertheless, CD47-SIRP\(\alpha\) interactions are highly species specific and a lack of ‘matching’ may contribute to graft rejection in xenotransplantation. Notably, the major reason that the NOD-SCID mouse represents a superior model for the
Engraftment of human cells and tissues seems to be that human CD47 binds with very high affinity to the mouse NOD SIRPα variant\textsuperscript{93}. Clearly, this knowledge could be exploited for improving xenotransplantation.

An area of particular interest for therapeutic interference in the CD47-SIRPα system that we have explored in the studies described in this thesis, and that also other groups have investigated in parallel, is cancer. The basic underlying idea was that CD47 expressed on cancer cells may deliver a ‘don’t-eat-me’ or ‘don’t-kill-me’ signal to phagocytes that would limit their immune-mediated elimination. In line with this hypothesis, a number of recent studies has demonstrated an elevated CD47 expression on diverse types of human tumors, including acute myeloid leukemia (AML)\textsuperscript{95,96}, non-Hodgkin’s lymphoma\textsuperscript{97}, bladder cancer\textsuperscript{98}, and several other solid tumor\textsuperscript{97,99}. Because CD47-SIRPα interactions had been demonstrated to negatively regulate the antibody- and FcγR-mediated clearance of normal (blood) cells, we were particularly interested to investigate a role for the CD47-SIRPα axis in the context of antibody therapy against cancer.

5. Scope of this thesis

The primary aim of the studies described in this thesis was to investigate the role of CD47-SIRPα interactions in therapeutic antibody-dependent tumor cell destruction by human phagocytes and also explore the killing mechanism(s) by which human phagocytes, and in particular human neutrophils, mediate therapeutic antibody-dependent cytotoxicity towards cancer cells.

In chapter 2 we describe studies investigating whether signaling through the SIRPα cytoplasmic tail constituted a limitation for antibody-dependent cancer cell elimination \textit{in vivo}. Here, we also examined the effect of targeting the CD47-SIRPα interaction for potentiating the \textit{in vitro} killing capacity of human phagocytes towards Trastuzumab-opsonized Her2/Neu-positive breast cancer cells. This identified the CD47-SIRPα interaction as a \textit{bona fide} therapeutic target for improving antibody-mediated cancer cell destruction. Subsequently, in chapter 3, we study in detail the role genetic variants of FcγRs and SIRPα in the ADCC of human neutrophils towards trastuzumab-opsonized SKBR-3 breast cancer cells. Chapter 4 describes studies aimed to provide an explanation for the mechanism by which CD47-SIRPα interactions modulate neutrophil-mediated ADCC, which is shown to occur at the level of inside-out affinity regulation of CD11b/CD18 integrins that are essential for cytotoxic synapse formation and killing. In chapter 5, we provide experiments aimed to understand the mechanism by which neutrophils mediate antibody-dependent cancer cell destruction. These studies essentially exclude an involvement of the classical antimicrobial killing mechanisms, including the NADPH oxidase and granule-dependent mechanisms, in antibody-mediated cancer cell destruction and provide preliminary evidence for a novel trogocytosis-based mechanism. In chapter 6, we describe the involvement of granule exocytosis in neutrophil anti-bacterial activity using cells from patients with familial hemophagocytic lymphohistiocytosis syndrome with genetically-defined mutations in the STXBP2/munc18-2 protein. The results presented in this thesis are summarized and discussed (chapter 7) in the context of recent developments in the field of targeting CD47-SIRPα interactions in cancer.
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CD47-SIRPα interactions form a barrier for antibody-mediated tumor cell destruction

Xiwen Zhao, Ellen M. van Beek, Karin Schornagel, Hans van der Maaden, Michel van Houdt, Marielle A. Otten, Pascal Finetti, Marjolein van Egmond, Takashi Matozaki, Georg Kraal, Daniel Birnbaum, Andrea van Elsaas, Taco W. Kuijpers, Francois Bertucci, Timo K. van den Berg

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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 cells1-3.

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 ADCC4-6. ADCC can be mediated by Fc-receptor expressing NK cells and phagocytes, including macrophages and granulocytes7,8 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, respectively3,9.

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 cells7. 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 SIRPα10-14. The extracellular region of SIRPα interacts with the broadly expressed surface molecule CD4715-17. 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 fashion10,11,18. It is well documented, for instance, that SIRPα acts to inhibit in the phagocytosis and the in vivo clearance of CD47-expressing host cells, including red blood cells and platelets, by macrophages19-24. CD47-SIRPα interactions also appear essential for engraftment upon hematopoietic stem cell (HSC)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 cells22,26.

Chao et al.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 \textit{in vitro} and antibody-dependent elimination of tumor cells \textit{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.

\section*{METHODS}

\subsection*{Mice and B16 melanoma model}

C57BL/6 mice with a targeted deletion of the SIRPα cytoplasmic region have been described previously\textsuperscript{24}. 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\textsuperscript{5} B16F10 tumor cells in 100 \textmu l HBSS on day 0. Mice were injected i.p. with a suboptimal dose of 10 \textmu g of TAA9 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 described\textsuperscript{28}.

\subsection*{CD47 mRNA expression in breast cancer}

We analyzed \textit{CD47} mRNA expression in 353 invasive breast carcinomas and 11 normal breast (NB) samples profiled\textsuperscript{29} using whole-genome Affymetrix oligonucleotide microarrays (GEO: GSE21653). Only two of the probe sets representing \textit{CD47}, 211075_s_at and 213857_s_at, mapped exclusively to constitutively transcribed \textit{CD47} exons according to NetAffx, RefSeq, and the UCSC Genome Browser\textsuperscript{27}. Their expression strongly correlated (Spearman correlation: 0.87). We retained that with the highest variance (211075_s_at). Before analysis, \textit{CD47} expression level for each tumor was centered by the average expression level of the NB samples. We analysed the correlation between \textit{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 described\textsuperscript{30}. 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 surgery\textsuperscript{31}. Pathological complete response was defined as the absence of invasive cancer in the breast and axillary lymph nodes at the time of surgery.

\subsection*{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-\gamma (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 ^51Cr (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. 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 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. 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 10⁵ 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 10⁵ 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\(^1\). 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\(^1\) 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](image_url)

**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\(_2\)-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\(^1\): n=22). Log\(_2\)-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 | 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\(^\text{12,16}\), 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-β-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\(_\text{BIT}\), 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 α\(_1\)/α\(_1\)-homozygous and α\(_1\)/α\(_\text{BIT}\)-heterozygous individuals. Both mAb effectively inhibited the binding of CD47-coated beads to CHO cells expressing SIRPα\(_1\) and/or SIRPα\(_\text{BIT}\) (Fig. 5A) and promoted Trastuzumab-mediated ADCC towards SKBR-3 cells by neutrophils from individuals with...
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 | Monoclonal antibodies against SIRPa that block CD47-SIRPa interactions enhance ADCC.

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 IgG$_1$ control mAb) block the binding of CD47-beads to either both SIRP$_{\alpha_1}$- and SIRP$_{\alpha_{\text{BIT}}}$-expressing CHO cells (12C4), or only to SIRP$_{\alpha_1}$-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 ($\alpha_1/\alpha_1$, $\alpha_{\text{BIT}}/\alpha_{\text{BIT}}$ 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, 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 SIRPαBIR, 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 SIRPα₁/SIRPα₁* 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 (CCGGGCACAATTACTTGGACTAGTTCTCGAGAACTAGTCCAAGTAATTGTGCTTTTT) 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 μg/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, Santa Cruz Biotechnology, Santa Cruz, CA)), anti-human SIRPβ₁ (B4B6, mouse IgG₁, clone 10.1, mouse IgG₁, BD Pharmingen), anti-human CD64 (Clone 10.1, mouse IgG₁), anti-human CD32 (Clone AT10, mouse IgG₂b, BD Pharmingen), 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, Sanquin).

The primers 5’-GAGATCGATATCCGACTACTTTATAAAACAAAATCT-3’ (forward) and 5’-GAGATCGATCTAAACCACGATATTTTGG-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 IgG1 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) equipped 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’-CACCATGGAGCCCGCCGCGCC-5’ and reverse primer 3’- GAATAGCAGTAGCAGTCACAAGAGTCGCCGCCGCT AAAC-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 technology. 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


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

Xi Wen Zhao, Joris van der Heijden, Sietse Q. Nagelkerke, Patricia Gonzalez, Martin de Boer, Hanke L. Matlung, Taco W. Kuijpers, Timo K. van den Berg

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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 FcγRIIA and FcγRIIIb functioning in one activation route, whereas FcγRI primarily operates in a partially redundant parallel pathway. Neutrophils with FcγRIIa-131H or FcγRIIIb-NA1 polymorphic variants displayed higher killing capacity, relative to their alternative variants FcγRIIa-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 FcγRIIA 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 SIRPα 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 SIRPα 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 SIRPα 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). 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 5x10^6 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 QIAamp® 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 (Carsbad, 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 (1x10⁶ 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 (5x10³/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 \[
\frac{\text{[experimental cpm-spontaneous cpm]} \times 100}{\text{[total cpm-spontaneous cpm]}}
\]
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).
Genetic variation of Fc receptors and SIRP in ADCC

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 FcγR 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 lymphoma 24, Trastuzumab treatment in Her2/Neu-positive metastatic breast cancer 6, 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 \(F_{c\gamma}R2/3\) 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 \(F_{c\gamma}R2c\) gene, with the corresponding individuals lacking Fc\(\gamma\)RIic protein expression, designated Fc\(\gamma\)RIic-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.
Figure 3 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 [23]. 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 [30]. 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.
Genetic variation of Fc receptors and SIRP in ADCC

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. 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, but DNA sequencing and MLPA analysis of our healthy Caucasian donors (n=83) identified only two polymorphic variants, SIRPα1 and SIRPα1R (Suppl. Table 1A), within this population with frequencies of 16.9% and 41.0% of SIRPα1 and SIRPα1R homozygotes, respectively, and 42.4% heterozygotes (allele frequencies: 59.3% (SIRPα1) and 83.4% (SIRPα1R)). 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 because most of these polymorphisms occur in areas not covering but just adjacent to the CD47 binding site (Suppl. Table 1B). 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 in 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. Of relevance, this variation is also associated with the clinical response to Trastuzumab and other cancer therapeutic antibodies. 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. FcγRI is constitutively expressed on monocytes and macrophages, can be induced on neutrophils in patients by treatment with cytokines, such as IFNγ and/or G-CSF, and may even be upregulated in cancer patients during chemotherapy-induced neutropenia. 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.
Genetic variation of Fc receptors and SIRP in ADCC

Polymorphisms of SIRP\(\alpha\) do not affect neutrophil ADCC towards Trastuzumab-opsonized SKBR3 cells. Results are shown for subjects with the indicated SIRP\(\alpha\) genotypes, including homozygous \(\alpha_1/\alpha_1\) (n=14), homozygous \(\alpha_{\text{BIT}}/\alpha_{\text{BIT}}\) (n=15) and heterozygous \(\alpha_1/\alpha_{\text{BIT}}\) (n=14). 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’ (for actual ADCC data and ratios represented for each of these conditions separately see suppl. Fig. 3). 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. Data shown are means ± SEM.

We have also explored a possible role for SIRP\(\alpha\) genetics in regulating neutrophil ADCC. Although it is known that there are at least 10 SIRP\(\alpha\) polymorphic variants among different ethnic groups, including African and Asian individuals \(^{14}\), 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. SIRP\(\alpha_1\) (also known as variant 2) and SIRP\(\alpha_{\text{BIT}}\) (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 SIRP\(\alpha\) \(^{34}\), and also that these particular Caucasian SIRP\(\alpha\) variants are similar with respect to their affinity for CD47 \(^{16}\), it could still be that there were functional differences in the responses downstream. However, our current results show that the both genetic SIRP\(\alpha\) 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 SIRP\(\alpha\) 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 Fc\(\gamma\)Rs 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 Fc\(\gamma\)RIIa-H/R131 and Fc\(\gamma\)RIIIb-NA1/NA2 polymorphisms. Finally, we demonstrate that CD47-SIRP\(\alpha\) interactions regulate ADCC triggered via the various Fc\(\gamma\)Rs and their genetic variants to a similar extent, and independent of the SIRP\(\alpha\) polymorphisms that are present. The latter clearly supports the idea that interference with CD47-SIRP\(\alpha\) interactions will be a broadly applicable therapeutic strategy to potentiate antibody therapy in cancer, independently of Fc\(\gamma\)R and SIRP\(\alpha\) genetics.
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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<sub>a</sub>, SIRPα<sub>1</sub> and SIRPα<sub>BIT</sub>. 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).

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FcγRIIb-allele

HNA1HNA1          | 13 (23.2%)         |
HNA1HNA2          | 24 (42.9%)         |
HNA1HNA2          | 19 (33.9%)         |

FcγRIIc exon3

Stop              | 53 (85.4%)         |
ORF               | 9 (14.5%)          |

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**B**
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 FcγRIIb 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 FcγRIIc-stop 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

CD47-SIRPα interactions restrict antibody-mediated killer synapse formation and tumor cell destruction by limiting CD11b/CD18-integrin activation

Xi Wen Zhao, Hanke L. Matlung, Michel van Houdt, Hans Janssen, Edith van de Vijver, Anton T. J. Tool, Karl Seeger, Sergio Rutella, Daria Pagliara, Taco W. Kuijpers, Timo K. van den Berg

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ABSTRACT

Antibody mediated tumor cell destruction is limited by interactions between CD47 expressed on tumor cells and SIRPα expressed on phagocytes. Therefore, targeting-of CD47-SIRPα interactions may potentiate antibody therapy in cancer-patients. However, the mechanism by which CD47-SIRPα interactions regulate cytotoxicity has remained unknown. Here we demonstrate that CD47-SIRPα interactions limit neutrophil-mediated ADCC towards Trastuzumab-opsonized breast cancer cells by controlling the inside-out-activation of CD11b/CD18-integrins that are essential for killer synapse formation. The requirement of CD11b/CD18-integrin for ADCC was demonstrated by blocking antibodies and by using neutrophils from a LAD1 patient lacking CD18-integrins. Disrupting CD47-SIRPα interactions promoted CD11b/CD18-dependent killer synapse formation during ADCC and enhanced CD11b/CD18 activation. The importance of the latter was demonstrated by the absence of killing by neutrophils from LAD3 patients, which lack kindlin-3 and have defective integrin activation. Collectively, these findings demonstrate that CD47-SIRPα interactions restrict neutrophil-mediated ADCC by controlling activation of CD11b/CD18-integrin that mediates killer synapse formation.
INTRODUCTION

Cancer therapeutic antibodies are now available for clinical use in several types of cancer. The best known examples are Trastuzumab against Her2/neu-positive breast cancer, Cetuximab against metastatic colon carcinoma and head and neck cancer, and Rituxumab against Non-Hodgkin lymphoma and other CD20-positive leukemias/lymphomas 1. Cancer therapeutic antibodies generally act via a combination of direct tumor growth inhibitory effects and immune-mediated effects, the latter including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity. ADCC can be mediated by NK cells and phagocytes, including macrophages and neutrophils, and the association between polymorphisms in the Fc-receptors FcγRIIa (CD32a) and FcγRIIIa (CD16a) and the clinical response rate of antibody therapy in cancer suggests that both phagocytes and NK cells contribute to antibody therapy in cancer 1;2. One problem with cancer therapeutic antibodies is that their clinical efficacy has remained insufficient to justify their use as single agents. Consequently, they are generally used in combination with chemotherapeutics, which by causing leukopenia, will undermine the clinical effects of therapeutic antibodies. An important question is therefore: How can the clinical efficacy of cancer therapeutic antibodies be improved?

We and others have recently demonstrated that interactions between CD47 expressed on cancer cells and the myeloid-restricted inhibitory immunoreceptor SIRPα form a restriction for antibody-dependent destruction of tumor cells in vivo and neutrophil- and monocyte-mediated ADCC in vitro 3-5. This implicates the CD47-SIRPα interaction as a potential therapeutic target for potentiating antibody therapy in cancer patients. In the present study we have further investigated the mechanism by which CD47-SIRPα interactions enhance neutrophil-mediated ADCC. Our findings show that neutrophil ADCC towards tumor cells involves CD11b/CD18-dependent killer synapse formation, and that CD47-SIRPα interactions control FcγR-induced CD11b/CD18 affinity regulation that is critical for cytotoxicity. These findings suggest that CD47-SIRPα interactions restrict ADCC, at least in part, by limiting CD11b/CD18 integrin activation.

METHODS

Cells and culture
The Her2/neu-positive human breast cancer carcinoma cell line SKBR-3 was routinely cultured in IMDM medium (Gibco, Paisley, UK) supplemented with 20% 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. SKBR-3 cells in with CD47 was knocked-down using shRNA (SKBR-3 CD47-KD cells), which express 10-15% of normal CD47 surface levels, have been described before 3. In experiments where SKBR-3 cells were used cells expressing empty vector shRNA were used as the control. The Leukocyte Adhesion Deficiency type 1 patient (LAD1) has been reported previously 6. In brief, patient carries a homozygous point mutation in CD18 gene exon 8 (c. 846G>A), resulting in an amino acid substitution (Asn282Lys) that leads to a virtual absence of CD18 and CD11b expression (Suppl. Fig. 1). Leukocyte Adhesion
Deficiency type 3 syndrome (LAD3) patient A has been described previously \textsuperscript{7,8}. In brief, this patient has a homozygous mutation in exon 12 (c.1525C>T) in the \textit{FERMT3} gene, which results in a premature stop at codon 509 (p. Arg 509X) leading to an absence of kindlin-3 protein. LAD3 patient B has been genotyped recently. We identified a new mutation (c.1173delT, p.As393Thr fsX29), in exon 10 of the \textit{FERMT3} gene. Assessment of kindlin-3 protein expression by Western Blotting (Suppl.Fig.5B) has been described before \textsuperscript{7}. Heparinized blood was collected from healthy donors and from patients after obtaining informed consent. The study was approved by the AMC Institutional Medical Ethics Committee in accordance with the 1964 Declaration of Helsinki.

\textbf{Neutrophil isolation}

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by centrifugation over isotonic Percoll (Pharmacia Uppsala, Sweden). After centrifugation of the Percoll-cell suspension the PMN and the red blood cells were recovered from the pellet. Red blood cells were lysed by two consecutive steps of isotonic ammonium chloride at 4 °C. Prior to ADCC purified PMN were cultured for either 4 or 18 h in complete RPMI1640 medium supplemented with 10% FSC, and containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 1% L-glutamin, in the presence of 10 ng/ml clinical grade recombinant G-CSF (Neupogen; Amgen, Breda, The Netherlands) and 50 ng/ml recombinant human interferon-γ (PEPROTECH, Rocky Hill, USA) at concentration of 5x10^6 cells/ml.

\textbf{Antibodies, reagents and flow cytometry}

The primary monoclonal antibodies (mAb) were used: anti-CD11b (clone 44A, ATCC, Rockville, MD, USA); anti-CD18 (clone IB4, ATCC) in the first step of the staining. Alexa 633-labeled anti-mouse IgG antibody (Invitrogen, Molecular Probes, USA) was used as the conjugate. The samples were analyzed on an LSRII flow cytometer equipped with a plate reader (BD Biosciences, San Jose, CA). For blocking experiments, the anti-CD11b/CD18 mAbs 44a and IB4 were pre-incubated with PMN at 10 μg/ml for 20 minutes and then used in ADCC or cell-cell conjugate formation experiments. For blocking FcγRs, a combination of mAb 10.1 (AbD Serotec, Oxford, U.K.) anti-FcγRI, mAb AT10 (azide-free AbD Serotec, Oxford, U.K.) anti-FcγRII, and mAb 3G8 (low endotoxin azide-free; BioLegend) anti-FcγRIII pre-incubated with PMN for 20 minutes at 10 μg/ml. Other antagonistic antibodies: CLA-LFA1/2 (Sanquin, Amsterdam, The Netherlands) anti-CD11a \textsuperscript{9} and CBR-p150/4G1 (AbD Serotec) anti-CD11c \textsuperscript{10} were used at 10 μg/ml in a similar manner. Detection of activation of CD11b/CD18 has been described previously \textsuperscript{11}. In brief, APC-conjugated mAb24 (10 μg/ml) was incubated with cells for 20 minutes at room temperature, washed twice with ice cold PBS containing 0.1% BSA, and analyzed within 15 minutes as described above. Total CD11b/CD18 expression on neutrophil upon ADCC were analyzed by flowcytometry by using mAbs 44a and IB4. Syk kinase-specific inhibitor BAY 61-3606 was used in cell-cell conjugate formation assay at a concentration of 10μM (Sigma, USA) \textsuperscript{12}. 
**ADCC assay**

The ADCC assay was performed as described before 3. Briefly, target cells were labelled with 100 μCi ⁵¹Cr (Perkin Elmer Life and Analytical Sciences, USA) for 90 minutes. After four washes the cells were harvested and suspend in RPMI medium with 10% FCS, target cells seeded in 96-well U-bottom tissue culture plate as 5000 cells/well. Target cells were opsonized with Trastuzumab (human IgG₁, Roche) at a final concentration of 5 μg/ml. The viability of neutrophils was determined by Annexin V staining and it always exceeded 80%. Viable neutrophils were co-incubated at ratio of E:T=50:1 with target cells for 4 hours at 37°C with 5% CO₂. After the incubation, aliquots of supernatant were harvested and analyzed for radioactivity in a gamma counter (Wallac). The percent relative cytotoxicity was determined as [(experimental cpm- spontaneous cpm)/(Total cpm- spontaneous cpm)] x 100%. All incubations were performed in triplicate.

**Electron microscopy**

Killing synapse formation between human neutrophils and tumor cells in ADCC was visualized by electron microscopy. For EM, cells were fixed in Karnovsky’s fixative. Postfixation was done with 1% Osmiumtetroxide in 0.1 M cacodylatebuuffer, after washing the pellets were stained en bloc with Ultrastain 1 (Leica, Vienna, Austria), followed by ethanol dehydration series. Finally the cells were embedded in a mixture of DDSA/NMA/ Embed-812 (EMS, Hatfield, U.S.A), sectioned and stained with Ultrastain 2 (Leica, Vienna, Austria) and analyzed with a CM10 electron microscope (FEI, Eindhoven, the Netherlands).

**Conjugate formation assay**

A total of 1x10⁶ G-CSF/IFNγ primed human neutrophil effector cells labelled with calcein-AM fluorescent dye (Invitrogen, Grand Island, USA) were incubated with 2x10⁵ SKBR3 cells stained with CMTPX/cell-tracker red fluorescent dye (Invitrogen) (E:T ratio=5:1) in the absence or presence of 5 μg /ml Trastuzumab for 30 minutes at 37°C in 5% CO₂. After incubation, the cells were fixed with 1.2% paraformaldehyde in PBS. Samples were run in an ImageStreamX imagine flow cytometer (Amnis Corporation, Seattle, WA) and images were acquired for 10,000 evens/sample. Cell images of double-positive events CMTPX+/Calcein+ were analyzed to visualize and quantify effector-target cell conjugate formation using the Amnis’ IDEAS® data analysis package.

**Data analysis and statistics**

Flow cytometry data were analyzed by Flowjo software (Tree Star, Inc, USA). Statistical differences were determined using students t-test by GraphPad Prism (GraphPad Software, San Diego, USA). For analysis ratio t test in Figure 4C, data were transformed as logarithm Y=Log (Y), then the logarithmed data were analyzed by paired t test. P value of ≤ 0.05 was considered significant.
RESULTS AND DISCUSSION

CD11b /CD18 integrin is required for neutrophil-mediated ADCC

We have previously shown that the interaction between CD47 on human breast carcinoma cell line SKBR3 and SIRPα expressed on neutrophils restricts ADCC towards the breast cancer cells in the presence of the therapeutic antibody Trastuzumab. However, the mechanism(s) by which CD47-SIRPα interactions regulate neutrophil-mediated ADCC have not been clarified. Previous studies have established an important role for the neutrophil CD11b/CD18 integrin in ADCC against various tumor cell targets, including Trastuzumab-opsonized breast cancer cells, by monoclonal antibody (mAb) blocking experiments and by using cells from CD11b-deficient mice. These studies also suggested that CD11b/CD18 mediates direct cell-cell interactions between neutrophils and antibody-opsonized target cells and is important for killer synapse formation. We confirmed and extended the role of CD11b/CD18 in our model. First, we tested the effects of blocking mAbs against the CD11b/CD18 integrin during ADCC. The blocking mAb 44a against the αMac1-chain (CD11b) and mAb IB4 against the β2-chain (CD18), or combination of mAb44 and IB4, but not isotype control antibodies, completely abrogated neutrophil-mediated ADCC (Fig. 1A), and this was observed for both SKBR3 and SKBR3 CD47 knock-down cells, the latter of which are more prone to killing because CD47-SIRPα interactions are prevented as demonstrated by us before. To further assess the requirement for the CD11b/CD18 integrin in ADCC neutrophils from a Leukocyte Adhesion Deficiency type 1 syndrome (LAD1) patient were analyzed. This LAD1 patient had a complete lack of expression of CD11/CD18 integrin on resting neutrophils (Suppl. Fig. 1A) due to a homozygous mutation c.846C>A in the CD18 gene. Consistent with the antibody blocking experiments the LAD1 patient neutrophils displayed completely defective ADCC, against both opsonized SKBR3 and SKBR3 CD47 knock-down cells (Fig. 1B).
Figure 1 | The requirement for CD11b/CD18 for neutrophil-mediated ADCC. A, Blocking mAb 44a anti-CD11b and/or mAb IB4 anti-CD18 (10 mg/ml), but not isotype control antibodies (‘control’), prevent neutrophil-mediated ADCC against Trastuzumab-opsonized SKBR3 cells and SKBR3 cells in which CD47 was knocked-down (SKBR3-CD47KD). The ADCC assay was performed as described in detail in “Materials and Methods”. Data are from a representative experiment out of three in which all incubations are performed in triplicate. B, Defective ADCC using neutrophils from a LAD1 patient, control is from a healthy donor. Results are presented as mean±SEM of one experiment which all incubations are performed in triplicate.
Although the above suggested an absolute requirement for the CD11b/CD18 integrin both in the presence or absence of CD47-SIRPα interactions, we also wanted to investigate the potential complementary involvement of other integrins, including other β2-integrins, and β1-integrins. As can be seen in Suppl. Fig. 2A, B, antagonistic antibodies CLA-LFA1/2 and CBR-p150/4G1 against other β2-integrin α-subunits CD11a and CD11c respectively, did not inhibit cytotoxicity. Furthermore, upon blocking β1-integrins using mAb LIA1/2.15 (Suppl. Fig. C) no significant effects on ADCC were observed. These data implicate that ADCC is strictly dependent on the CD11b/CD18 β2-integrin, but not on other hematopoietic integrins expressed by neutrophils.

**CD47-SIRPα interactions control CD11b/CD18-dependent killer synapse formation**

As ADCC appeared completely CD11b/CD18-integrin dependent, both in the presence or absence of CD47-SIRPα interactions, and because CD11b/CD18 integrin is apparently involved in killer synapse formation between mouse neutrophils and antibody-opsonized cancer cells14,16, we investigated whether CD47-SIRPα interactions could regulate killer synapse formation between human neutrophils and antibody-opsonized tumor cells during ADCC. For this purpose a quantitative flow cytometric assay for the analysis of neutrophil-SKBR3 cell conjugate formation was developed. Neutrophils and SKBR3 breast cancer cells were labeled with different fluorescent dyes, incubated together, fixed, and the proportion of conjugates was determined using Amnis Imagesteam-X technology. As can be seen in Fig. 2 interactions between effector and target cells were induced by antibody opsonization and were completely dependent on CD11b/CD18 integrin. Both killer synapse formation (Fig. 2B) as well as ADCC (Suppl. Fig. 3) were strictly dependent on both Fc-receptor binding and signaling, as it could be prevented by a cocktail of blocking antibodies against FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), as well as by BAY 61-3606, an enzymatic inhibitor of the tyrosine kinase Syk that plays a pivotal role in FcγR signaling17. Importantly, neutrophil-SKBR-3 killer synapse formation could be significantly enhanced by CD47 KD (Fig. 2B) or by prevention of CD47-SIRPα interactions using antagonistic monoclonal antibodies against CD47 or SIRPα (not shown) that we have previously reported to potentiate cytotoxicity3. Electron microscopy showed extensive neutrophil spreading onto the tumor target cells in the presence of Trastuzumab, consistent with killer synapse formation14, and the appearance of these interactions did not change notably in the absence of CD47 on the target cells (Suppl. Fig. 4). Collectively, this suggested that disruption of CD47-SIRPα interactions promotes CD11b/CD18 integrin-dependent killer synapse formation during ADCC.
CD47-SIRPa interactions limit conjugate formation between neutrophils and Trastuzumab-opsonized SKBR3 breast cancer cells. Calcein-labelled neutrophils were incubated, in the presence or absence of Trastuzumab, with cell-tracker red (CTR) labeled SKBR3 cells at a 5:1 ratio for 30 minutes and fixed. The cell conjugates were analyzed by ImageStream-X flow cytometry. A total of 10,000 events for each sample was evaluated. A, Dot plot image (left panel) showing the double positive events representing neutrophil-SKBR3 conjugates (yellow window) and the associated brightfield and single and overlaid fluorescence images of a selected conjugate (right panel). B, Percentage of conjugates observed under various conditions, including CD11b/CD18 blocking (using mAb 44a and IB4), FcγR blocking (using the cocktail of antibodies described in Materials and Methods) and in the presence of Syk kinase inhibitor BAY 61-3606. Each bar represents that mean +/- SEM from 5-10 different donors evaluated in several independent experiments, with each incubation performed in triplicate. P-values of statistically significant differences, as determined by students t-test, are indicated.

**CD47-SIRPa interactions limit CD11b/CD18-integrin activation that is essential for ADCC**

In principle, there could be different ways in which CD47-SIRPa interactions might control CD11b/CD18-integrin-dependent killer synapse formation and cytotoxicity, involving regulation of either receptor expression or function, or both. Expression of CD11b/CD18 integrin might be modulated, for instance, by mobilization from specific granules that are known to contain substantial amounts of this integrin in neutrophils. We tested this by...
performing flow cytometry for total expression of CD11b/CD18 during ADCC (Fig. 3A). Although the recognition of antibody-opsonized tumor cells led to a substantial upregulation of surface CD11b/CD18 integrin expression on the neutrophils, this was not further enhanced by CD47 KD. Alternatively, CD11b/CD18 integrin function might be modulated by CD47-SIRPα interactions. Indeed, the function of integrins in general depends on so called inside-out activation, thereby establishing a high-affinity ligand binding conformation, and this is also the case for CD11b/CD18 on neutrophils 19. The CD11b/CD18 active conformation can preferentially by recognized by the mAb24 11 and we used this to evaluate CD11b/CD18 integrin affinity modulation by CD47-SIRPα signaling. As can be seen in Fig. 3B and C, elevated integrin activation was observed during Fc-receptor triggering during ADCC in neutrophils, and this was further enhanced by interference with CD47-SIRPα interactions, suggesting that CD11b/CD18 is subject to affinity regulation by CD47-SIRPα interaction and signaling downstream of SIRPα.

Finally, to directly address the role of integrin affinity regulation in neutrophil-mediated ADCC, we evaluated the ADCC capacity of cells from two unrelated patients with LAD3 syndrome (also known as LAD1/variant syndrome), which have mutations in the FERMT3 gene, encoding the intracellular protein kindlin-3 that is required for the activation of various integrins, including neutrophil CD11b/CD18 7. Characteristic for the LAD3 syndrome, the expression of CD11b/CD18 on neutrophils was not affected (Suppl. Fig. 5A), but the CD11b/CD18-dependent zymosan-induced respiratory burst was essentially absent (Suppl. Fig. 5B), as reported previously 8. Clearly, neutrophil ADCC was completely defective in the two patients evaluated, demonstrating the absolute requirement for CD11b/CD18 integrin activation during ADCC (Fig. 3D).
Figure 3 | CD47-SIRPα interactions restrict CD11b/CD18 integrin activation which is essential for ADCC. Analysis of total (A) and active (B) surface CD11b expression during ADCC of neutrophils towards Trastuzumab-opsonized SKBR3 cells at a 5:1 ratio analyzed after 30' determined by flow cytometry. Active β2-integrin was analyzed by using APC conjugated mAb24 (grey histogram). Open histogram, isotype control. C, The ratio of active integrin and total integrin expression was calculated from the respective MFIs of mAb24 and the total CD11b staining. Data shown as mean±SEM from 4 healthy donors. D, ADCC of neutrophils towards Trastuzumab-opsonized SKBR3 and SKBR3-CD47KD cells mediated by neutrophil from healthy controls (n=3) and neutrophil from LAD3 patient A (n=1) and patient B (n=2) in the absence (empty bar) and presence (black bar) of Trastuzumab (5μg/ml). Cytotoxicity was analyzed after 4 hours at a E:T ratio of 50:1. Results are shown as mean±SEM with all incubations performed in triplicate, **p<0.05, ***p<0.01.
CONCLUDING REMARKS

Taken together, our results confirm and extend the evidence for CD11b/CD18-dependent killer synapase formation and cytotoxicity during neutrophil-mediated ADCC. Furthermore, they demonstrate for the first time that CD47-SIRPα interactions restrict antibody-dependent tumor cell destruction, at least in part, by limiting the CD11b/CD18 integrin activation that occurs upon FcγR triggering in neutrophils and that is apparently critical for cytotoxicity. These findings are of clinical relevance because CD47-SIRPα interactions have been implicated, by us and by others, as a promising and likewise generic therapeutic target for potentiating antibody therapy in cancer. SIRPα is a typical myeloid-restricted inhibitory immunoreceptor that upon CD47 triggering recruits and activates the cytosolic tyrosine phosphatases SHP-1 and SHP-2. SHP-1 in particular seems a good candidate to mediate the effects on integrin activation. Previous findings have already provided evidence that the Src kinase Lyn plays a critical role in neutrophil SIRPα phosphorylation and subsequent SHP-1 recruitment, and this finding together with the hyperadhesive phenotype of Lyn-deficient neutrophils suggested that plasma membrane-associated SHP-1 negatively regulates CD11b/CD18 integrin-dependent adhesion. Clearly, one remaining question is at what level(s) do the FcγR- and SIRPα- signaling pathways converge. Based on the selectivity of integrin affinity regulation by CD47-SIRPα interactions, which in contrary did not detectably regulate FcγR-dependent CD11b/CD18 mobilization, it would seem that the most upstream and common FcγR signaling events are at least not the only and/or primary ones to be controlled by CD47-SIRPα interactions. However, further efforts are clearly necessary to shed light more on this, as well as to identify additional possible levels of regulation by CD47-SIRPα interactions in ADCC. This information will further help to establish the CD47-SIRPα axis as a bona fide therapeutic target for improving antibody therapy against cancer.

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CD47-SIRPa interactions restrict integrin activation

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SUPPLEMENTARY FIGURE LEGENDS:

Supplementary Figure 1 | Defective CD11b/CD18 expression on LAD1 neutrophils. Expression of CD11b and CD18 on resting neutrophil was analyzed by flow cytometry by using mAb 44a and IB4, respectively. Note the virtual absence of CD11b/CD18 expression on the LAD1 neutrophils.

Supplementary Figure 2 | Characterization of LAD3 patient B neutrophils. A, expression of CD11b and CD18 on resting neutrophils were analyzed by flow cytometry by using mAb 44a and IB4, respectively. Note the normal surface levels of CD11b/CD18. B, Lack of kindlin-3 protein in neutrophils of LAD3 patient B. C, Hydrogen peroxide production (nmol/min/10^6 cells) by control and LAD3 patient neutrophil in response to indicated stimuli. Note that the zymosan-induced respiratory burst is strongly impaired in LAD3 neutrophils, whereas the response towards PMA is normal. This represents a typical feature of LAD3 neutrophils (Kuijpers et al. (2009) Blood 113:4740-4746).
Supplementary Figure 3 | The role of β1- and β2-integrins in ADCC. ADCC was performed in the presence of: A, blocking mAb against CD11a (using mAb CLA-LFA1/2). B, blocking mAb against CD11c (using mAb CBR-p150/4G1). C, blocking mAb against β1 integrin (using mAb LIA1/2.1). Neutrophils were pre-incubated the antibodies at 10 μg/ml for 20 minutes. Data represent the mean±/SEM from 3 independent experiments using 3 different donors with each incubation being performed in triplicate.

Supplementary Figure 4 | Dependency on FcγRs signaling in neutrophil ADCC. A, Effect of the combination of blocking antibodies against FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) each used at 10 μg/ml in the ADCC. B, Effect of the Syk kinase inhibitor BAY 61-3606 used at 10 μM in the ADCC. Data represent the means±/SEM from 3 independent experiments using 3 different donors with each incubation being performed in triplicate.
Supplementary Figure 5 I Neutrophil killer synapse formation visualized by electron microscopy. After co-incubation of neutrophils and Trasuzumab-opsonized SKBR3 or SKBR3-CD47-KD cells for 30 minutes (E:T ration=5:1) cells were fixed in Karnovsky’s fixative. Post-fixation was done with 1% Osmiumtetroxide in 0.1 M cacodylatebuffer, after washing the pellets were stained en bloc with Ultrastain 1 (Leica, Vienna, Austria), followed by ethanol dehydration series. Finally the cells were embedded in a mixture of DDSA/NMA/Embed-812 (EMS, Hatfield, U.S.A), sectioned, stained with Ultrastain 2 (Leica, Vienna, Austria) and analyzed with a CM10 electron microscope (FEI, Eindhoven, the Netherlands). Note that neutrophils form killer synapse structures.
Neutrophil antibody-dependent cellular cytotoxicity mediated by trogocytosis


* These authors contributed equally

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Both natural killer (NK) cells and phagocytes, including neutrophils and macrophages, can mediate antibody-dependent cellular cytotoxicity (ADCC) and this contributes to the clinical efficacy of cancer therapeutic antibodies. However, whereas the effector mechanisms underlying NK cell- and macrophage-mediated ADCC are relatively well defined, the way in which neutrophils kill antibody-opsonized cancer cells has remained an enigma. We investigated the mechanism of human neutrophil ADCC towards cancer cells. Our findings establish that the killing of antibody-opsonized tumor cells does not require the classical antimicrobial mechanisms of neutrophils, including the NADPH oxidase and/or the secretion of granule-derived mediators. Instead, we provide preliminary evidence for ‘trogocytosis’ as a mechanism of ADCC by neutrophils. Trogocytosis (‘trogo’ is Greek for gnaw) is a process in which neutrophils mechanically tear off fragments from the plasma membrane of antibody-coated cancer target cells, thereby causing a type of necrotic cell death that we propose to term trogoptosis. Triggering of neutrophil trogoptosis requires intracellular signaling involving phosphoinositol-3 kinase (PI3K), and it can be promoted by interference with CD47-SIRPα interactions that were previously shown to restrict neutrophil ADCC towards cancer cells in vitro and in vivo. Collectively, our findings provide insight into the mechanism of neutrophil ADCC against tumor cells, and suggest that this can occur through trogocytosis. Future studies should be directed to establish the in vivo relevance of neutrophil trogocytosis and the underlying mechanism, and the latter may also identify molecular targets for enhancing the clinical efficacy of antibody therapy in cancer.

ABSTRACT

Both natural killer (NK) cells and phagocytes, including neutrophils and macrophages, can mediate antibody-dependent cellular cytotoxicity (ADCC) and this contributes to the clinical efficacy of cancer therapeutic antibodies. However, whereas the effector mechanisms underlying NK cell- and macrophage-mediated ADCC are relatively well defined, the way in which neutrophils kill antibody-opsonized cancer cells has remained an enigma. We investigated the mechanism of human neutrophil ADCC towards cancer cells. Our findings establish that the killing of antibody-opsonized tumor cells does not require the classical antimicrobial mechanisms of neutrophils, including the NADPH oxidase and/or the secretion of granule-derived mediators. Instead, we provide preliminary evidence for ‘trogocytosis’ as a mechanism of ADCC by neutrophils. Trogocytosis (‘trogo’ is Greek for gnaw) is a process in which neutrophils mechanically tear off fragments from the plasma membrane of antibody-coated cancer target cells, thereby causing a type of necrotic cell death that we propose to term trogoptosis. Triggering of neutrophil trogoptosis requires intracellular signaling involving phosphoinositol-3 kinase (PI3K), and it can be promoted by interference with CD47-SIRPα interactions that were previously shown to restrict neutrophil ADCC towards cancer cells in vitro and in vivo. Collectively, our findings provide insight into the mechanism of neutrophil ADCC against tumor cells, and suggest that this can occur through trogocytosis. Future studies should be directed to establish the in vivo relevance of neutrophil trogocytosis and the underlying mechanism, and the latter may also identify molecular targets for enhancing the clinical efficacy of antibody therapy in cancer.
INTRODUCTION

Antibody therapy is used for the treatment of certain forms of cancer, and immune-mediated mechanisms, including complement-mediated cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), are known to account for at least part of the cancer cell destruction. However, the clinical efficacy of cancer therapeutic antibodies remains much too limited, and as a result such these presumed selective antibody therapeutics still have to be used generally in combination with non-specific and harmful (e.g. mutagenic) chemotherapy. Unfortunately, chemotherapy also undermines the clinical benefit of antibody therapy as it causes leukopenia, thereby strongly reducing the availability of relevant effector cells. ADCC is triggered via FcγR expressed on NK-cells and phagocytes in both experimental animal models and also during therapeutic antibody treatment of cancer patients. The latter is supported by strong indirect evidence from genetic studies in which a link between the efficacy of treatment and polymorphisms in the patient FcγR have been shown. The fact that significant independent associations were observed for either FcγRIIIa and FcγRIIa furthermore suggested a contribution of both NK cells and phagocytes to the cellular destruction of cancer cells. The effector mechanism of NK cell ADCC towards cancer cells is well established. It involves tumor cell apoptosis caused by the regulated release of granzyme-B and perforin from preformed and stored cytotoxic granules. NK cell ADCC is known to involve signaling downstream of Fcγ-receptors and the formation of cytotoxic (or ‘killer’) synapses, which ensure a localized and directed release of granule contents at the surface of the target cell. In contrary, macrophages have been reported to eliminate antibody-opsonized cancer cells by phagocytosis, also known as antibody-dependent (cellular) phagocytosis (ADCP). However, the exact mechanism by which neutrophils kill cancer cells has remained unknown. In contrary to macrophages neutrophils appear incapable of phagocytosing intact opsonized cancer cells and there is debate as to whether they might express perforin and granzymes. Nevertheless, evidence presented in chapter 4 and by others has clearly demonstrated that neutrophil ADCC also requires direct cell-cell interactions with the tumor cells and the formation of killer synapses, and that these critically depend on the CD11b/CD18 integrin.

In the present study we provide preliminary evidence with respect to the effector mechanism by which neutrophils mediate ADCC towards cancer cells. First, we demonstrate that neutrophil ADCC surprisingly does not depend on either of the two ‘classical’ anti-microbial effector functions of neutrophils, i.e. the production of toxic reactive oxygen species (ROS) by the phagocyte NADPH oxidase, or the mobilization of cytotoxic granule components, including the neutrophil serine proteases. Instead, our results provide evidence that neutrophil ADCC occurs by a trogocytosis-related process in which neutrophils actively tear-off fragments of target cell membrane in an antibody-dependent fashion, which results in type of necrotic cell death in the target cell, which we propose to term ‘trogoptosis’. Finally, we demonstrate that interference with CD47-SIRPa interactions between respectively tumor cells and neutrophils, which was previously demonstrated to selectively enhance antibody-mediated tumor cell destruction, strongly potentiates neutrophil trogocytosis. Collectively, these findings identify trogocytosis as an effector mechanism by which neutrophils perform antibody-dependent killing of cancer cells.
METHODS

Cells and culture

The Her2/Neu-positive human breast cancer carcinoma cell line SKBR3 was routinely cultured in IMDM medium (Gibco, Paisley, UK) supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO₂. CD47 was knocked-down in SKBR3 cells using shRNA (SKBR3-CD47KD cells), resulting in 10-15% expression of normal CD47 surface levels as shown previously[13]. Knock-down was routinely verified by flow cytometry and was found to be very stable.

Effector cell isolation

Neutrophils (PMNs) were isolated as previously described[14]. After isolation, neutrophils were cultured for either 4 h or overnight in RPMI culture medium, supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1% L-glutamin, in the presence of 10 ng/ml clinical grade recombinant G-CSF (Neupogen) and 50 ng/ml recombinant human interferon-γ (PEPROTECH) at a concentration of 5x10⁶ cells/ml. Neutrophils from healthy donors or from chronic granulomatous disease (CGD) patients and FHL5 patients were also used (Suppl. Table1). NK cells were isolated from the PBMC fraction of blood after Percoll fractionation using anti-CD56 coated MACS beads (Miltenyi Biotech), according to procedures provided by the manufacturer.

ADCC assays

SKBR3 cells and SKBR3-CD47KD cells were used as target cells and labeled with 100 μCi ⁵¹Cr (Perkin-Elmer) for 90 minutes at 37°C. After 3 washes with PBS, 5x10³ cells were incubated in RPMI culture medium supplemented with 10% FCS for 4 hours at 37°C and 5% CO₂ in a 96-wells U-bottom plate together with PMNs in an E:T ratio of 50:1 or with NK cells at an E:T ratios of 6:1 and 12:1. After the incubation supernatant was harvested and analyzed for radioactivity using a gamma counter (Wallac). The percentage of cytotoxicity was calculated as [(experimental cpm - spontaneous cpm)/(total cpm - spontaneous cpm)] x 100%. All conditions were measured in triplicate.

Respiratory burst and myeloperoxidase (MPO) production during ADCC

NADPH oxidase activity was evaluated during ADCC by measuring extracellular hydrogen peroxide (H₂O₂) release using Amplex Red assay kit (Molecular Probes) as previously described[15]. Samples were measured in the presence of Amplex Red (0.5 μM) and horseradish peroxidase (1 U/ml). Fluorescence was measured at 30-second intervals for 30 minutes with the HTS7000+ plate reader (Perkin Elmer, Norwalk, CT). The maximal slope of H₂O₂ release was assessed over a 2-minute interval as a measure of the activity of the NADPH oxidase. Myeloperoxidase (MPO) release from PMNs during ADCC of cancer cells was measured using an ELISA kit (HyCult Biotechnology), according to manufacturers’ instructions. Abolished ROS production with treatment of DPI (Diphenylene iodonium), a NADPH oxidase inhibitor, was reported previously[16]. A final concentration of 20 μM (1:200; Sigma-Aldrich) DPI was added during 4 hours ADCC assay to inhibit NADPH-dependent ROS production.
**Live cell imaging**
Target cells were cultured on glass coverslips and labeled with the membrane dye DiO (5μM, Invitrogen) for 30 minutes at 37°C. Target cells were incubated with PMNs in E:T ratio 5:1 at 37°C in 5% CO₂ in IMDM culture medium supplemented with 20% FCS and imaging was performed at various indicated times and intervals using a LSM 510 META laser scanning microscope (Carl Zeiss).

**Trogocytosis assay**
During trogocytosis the transfer of membrane between tumor cell and neutrophil was quantified by FACS. SKBR3 cells and SKBR3-CD47KD cells were labeled with DiO as indicated above. After washing with PBS, cells were incubated together with neutrophils in a U-bottom 96-wells plate at a E:T ratio of 5:1 in the absence or presence of Trastuzumab (Roche). Subsequently, the samples were directly fixed with 0.5% PFA at several timepoints between 0 and 120 minutes and measured on FACS flow cytometer (LSRII/CantoII, BD Biosciences) and data analyzed by FACSDiva software (BD Biosciences). After gating for individual neutrophils by forward and side scatter profiles, the mean fluorescent intensity (MFI) and the percentage of cells positive for DiO were determined.

**RESULTS**

**Neutrophil ADCC is independent of NADPH oxidase and granule secretion**
In order to identify the mechanism of killing by human neutrophils during ADCC we first aimed to investigate the contribution of the two classical anti-microbial effector mechanisms, i.e. the NADPH oxidase and cytotoxic granules. During ADCC using the Her2/Neu-positive breast cancer cell line SKBR-3 and Trasuzumab we measured the extracellular production of ROS and the azurophilic granule constituent MPO. The generation of both components was increased during ADCC (Figure 1A,B). Furthermore, upon interference with CD47-SIRPα interactions by knock-down of CD47 in the cancer cells, which had previously been shown reduced CD47 surface levels to 10-15% of control levels and to potentiate ADCC, a further enhancement of both components was seen. This suggested that at least NADPH oxidase activation and granule exocytosis were triggered during neutrophil ADCC. To investigate the relevance of NADPH-mediated ROS production during ADCC, we used neutrophils from patients with chronic granulomatous disease (CGD), which have defective ROS production. As shown in Figure 1C CGD neutrophils induce similar Trastuzumab-dependent cytotoxicity towards cancer cells compared to control neutrophils, demonstrating that NADPH-mediated ROS production is not required for ADCC.

**Figure1 A,B,C (first option)**

Because NK cell ADCC towards tumor cells requires granule secretion, we investigated ADCC by neutrophils from FHL5 patients which carry mutations in the STXBP2 gene, encoding the STXBP2/munc18-2 protein, and therefore have a virtually absolute defect in granule exocytosis, which in turn compromises bacterial killing (chapter 6, Zhao et al.)
Clearly, FHL5 patient neutrophils were comparable to control neutrophils with respect to their ADCC capacity (Figure 1D), showing that killing did not require neutrophil granule secretion. In contrary and as expected, NK cells from FHL-5 patients displayed strongly defective ADCC against Trastuzumab-opsonized SKBR-3 cells (Figure 1E). Finally, to rule out possible redundancy among the two effector pathways we used DPI (Diphenylene iodonium), an effective flavoprotein inhibitor that completely abrogates ROS production in combination with neutrophils from FHL5 patients. Incubation of the FHL5 patient PMNs with DPI (20μM) also did not significantly affect the capacity of cytotoxicity towards cancer cells (Figure 1F), confirming indeed that neither NADPH oxidase-derived ROS nor granule release from PMNs are essential for neutrophil ADCC towards cancer cells.

Figure 1 | A, B, C
Figure 1 | continued

**A:** Extracellular H$_2$O$_2$ production, quantified by Amplex Red assay, during neutrophil ADCC of Trastuzumab opsonized SKBR3 cells. Note that incubation with Trastuzumab increases the amount of hydrogen peroxide produced substantially. Knockdown of CD47 in SKBR3 cells further enhances the production of ROS. **B:** MPO production measured by ELISA in supernatant of ADCC assay. Knockdown of CD47 in SKBR3 cells is enhancing the exocytosis of MPO. **C:** Antibody-dependent tumor cell cytotoxicity by PMNs in the absence and presence of Trastuzumab and knockdown of CD47. PMNs from 5 individual CGD patients do not influence the extent of cytotoxicity towards cancer cells. **D:** Neutrophils of FHL5 patients, which are unable to exocytose their granules have normal ADCC capacity towards Trastuzumab-opsonized SKBR3 cells. **E:** FHL-5 patient NK cell ADCC towards Trastuzumab-opsonized SKBR3 cells is virtually absent compared to control cells (n=3). **F:** SKBR3 cells were opsonized by Trastuzumab and incubated with neutrophils of two FHL5 patient in an effector:target ratio of 50:1. To simultaneously inhibit ROS production by the NADPH oxidase cells were incubated with the ROS inhibitor DPI. Defective protease release combined with inhibition of ROS did not influence the amount of cytotoxicity towards cancer cells compared to controls. * = P≤0.05, n.s. = not significant.
Cancer cell killing by NK cells and cytotoxic T cells depends on the release of perforin and granzyme B, leading to the activation of apoptotic caspases. To investigate the contribution of apoptosis to cancer cell killing by PMNs, ADCC was performed in the presence of 20 μM of the pan-caspase inhibitor ZVAD-FMK. This concentration was shown to potently inhibit neutrophil apoptosis in parallel experiments as also reported by us before. However, the inhibition of caspase did not decrease ADCC of PMNs towards cancer cells compared to controls (Figure 2) indicating that, unlike NK cells and CTL, PMNs induce cancer cell death by a caspase-independent pathway.

Neutrophil ADCC occurs by a trogocytosis-related process

We and others have previously shown that neutrophil ADCC involves the formation of cytotoxic synapses. In order to visualize the interaction between tumor cells and neutrophils and to explore the mechanism of killing we performed life cell imaging using confocal microscopy. As can be seen in the stills provided in Figure 3B, neutrophils form transient but intimate interactions with their cancer cell targets, which were labeled using the membrane dye DiO. Typically, neutrophils were observed to migrate towards the cancer cell, after which they adhered to and spread onto their targets forming cytotoxic synapses (see also chapter 4). Intriguingly, during rounding up and detaching from the tumor cells the neutrophils were observed to take up parts of the target cell membrane (Figure 3B) and this could also be confirmed by electron microscopy (Figure 3A). This process, which was reminiscent of trogocytosis previously described during e.g. antigen presentation, could further be quantified using flow cytometry (Figure 3C). As can be seen trogocytosis could be observed as early as 10-20’ after initiation of the experiment, and this clearly preceded the detection of cell lysis in ADCC (Figure 3D). After 60’ the vast majority of neutrophils had
acquired cancer cell membrane. Importantly, the process absolutely required Trastuzumab, suggesting that it was induced by active FcγR cross-linking and signaling in the neutrophil, and it was strongly enhanced by interference with CD47-SIRPα interactions, either by using CD47KD target cells (Fig. 3 C and D), or by using antibodies that block CD47-SIRPα interactions (data not shown).

**Figure 3** | Neutrophil ADCC is associated with trogocytosis. A: EM pictures of a neutrophil forming a synapse with an SKBR3 cell (left image); after 90 minutes of incubation large invaginations of the tumor cell into the neutrophil can be seen (right image). B: Confocal imaging (63x objective) of trogocytosis during neutrophil ADCC. Trastuzumab opsonized SKBR3 cells, labelled with DiO (green fluorescence), were incubated in a E:T ratio of 5:1 with neutrophils. Example of a neutrophil that can be seen to: i) migrate towards an SKBR3 cell, ii) spread upon the surface of its target cell to form a cytotoxic synapse, and iii) tear away a fragment of the SKBR3 membrane, before iv) detaching and moving away with this fragment of tumor cell membrane. C: Flow cytometric quantification of trogocytosis. SKBR3 cells were labeled green with DiO and incubated in the absence or presence of Trastuzumab with either control SKBR3 or SKBR3-CD47KD cells. Samples were fixed at the indicated timepoints and the transferred target cell membrane was quantified by determining the % of individual neutrophils that had trogocytosed or by expressing the mean fluorescent intensity (MFI) within the entire neutrophil population. Trastuzumab-dependent trogocytosis is observed as early as 10-20’ after the initiation of the experiment and it is strongly enhanced by interference with CD47-SIRPα interactions. D: Tumor cell lysis (ADCC) was measured at the indicated timepoints (n=1), methods is described in Figure 1 C, color bar indicated the tumor cell in the presence of Trastuzumab.
Antibody-dependent neutrophil trogocytosis is PI3K-dependent

We wanted to investigate whether signaling in neutrophils was important for antibody-induced trogocytosis. As previously reported in the context of T-cell trogocytosis, the PI3K inhibitor Wortmannin is inhibiting membrane transfer between antigen presenting cells and T-cells. Moreover PI3K can act downstream of FcγR signaling in neutrophils\(^\text{23}\). Therefore, we tested the inhibition of the PI3K pathway using Wortmannin (100 nM concentration) in neutrophils ADCC against cancer cells and trogocytosis. Wortmannin significantly inhibited Trastuzumab-induced cancer cells lysis as compared to that of control cells (Figure 4A). Furthermore, PMN-mediated trogocytosis induced by opsonization of cancer cells by Trastuzumab was essentially abolished when treating the cells with Wortmannin (Figure 4B). This suggests that PI3K is a critical intermediary in neutrophil FcγR signaling-induced trogocytosis and killing.

Figure 4 | Inhibition of PI3K reduces trogocytosis and ADCC. A: Inhibition of PI3K activity by Wortmannin (100 nM) reduces antibody dependent cytotoxicity of neutrophils towards SKBR3 cells (n=7). B: Neutrophil trogocytosis is completely inhibited upon incubation with Wortmannin at timepoint of 90 mins (n=3).
DISCUSSION

In this study we have investigated the nature of the antibody-dependent mechanism(s) of cancer cell killing by neutrophils. There has been some controversy about whether neutrophil granules contain perforin and granzyme B, and might use these compounds to induce caspase-dependent apoptosis of cancer cells, like NK cells\textsuperscript{24}. However, this seems rather unlikely because of several reasons. Firstly, we have not detected any granzyme B and perforin in neutrophils (Zhao, unpublished). Secondly, during neutrophil ADCC the tumor target cells do not show the typical morphological features of apoptosis (Fig. 3B) as seen in e.g. NK cell-mediated cytotoxicity\textsuperscript{19}, and also because the pan-caspase inhibitor ZVAD apparently did not affect the killing process (Fig. 2). Third, and most importantly, by using neutrophils from FHL5 patients, which have a profound defect in granule exocytosis (see chapter 6)\textsuperscript{19}, we have found that neutrophil ADCC does not depend on release of serine proteases nor any other granule-derived components to induce antibody-dependent killing of cancer cells. Furthermore, the other major anti-microbial mechanism of neutrophils, i.e. the production of ROS by the phagocyte NADPH oxidase, was also not required for ADCC of cancer cells, as shown by the normal killing capacity of CDG patient neutrophils. The latter is also in agreement with data from a report Horner et al., who previously also reported effective killing of Trastuzumab opsonized Her2/Neu transfected Raji cells by neutrophils from a CGD patient.

It has been shown by us (chapter 4) and others\textsuperscript{10} that neutrophil-mediated ADCC of cancer cells depends on the CD11b/CD18-dependent cytotoxic synapse formation between effector cell and target cell. Here we show that upon synapse formation between neutrophils and cancer cells, the effector cells actively acquire pieces of membrane from the cancer cell, as demonstrated by both live cell imaging, ultrastructural analysis, and a newly developed flow cytometric assay. This transfer of membrane from target cell to effector cell is remarkably similar to what has been described as ‘trogocytosis’ between antigen presenting cells and T-cells\textsuperscript{12}. This process is induced in T-cells upon activation of the TCR through its associated cytoplasmic ITAM motifs. Interestingly, the ITAM motifs associated with Fc\textgamma{}R-signaling on the various effector cells, including neutrophils and NK cells, provide a very similar signaling platform in the context of trogocytosis and ADCC. Furthermore, the completely antibody-dependent trogocytosis by neutrophils was shown to be strongly enhanced after disruption of CD47-SIRPa signaling, correlating with an enhanced antibody-dependent cytotoxicity\textsuperscript{13}, and supporting the idea that ADCC and trogocytosis are causally related processes. Clearly, at this point more evidence is required to substantiate a causative role of trogocytosis in neutrophil-mediated ADCC.

The regulation of this trogocytosis-related process involves signaling through PI3K, as shown by inhibition by Wortmannin. This finding further strengthens the hypothesis that trogocytosis and ADCC of cancer cells are related processes. Similarly, data from Bonnema et al. showed that in the case of ADCC mediated by NK-cells, killing of cancer cells is depending on Fc\textgamma{}R-ligation and downstream activation of PI3K\textsuperscript{25}. Nevertheless, trogocytosis and killing may not actually be exactly synonymous in terms of regulation. For instance it appears that e.g.
the final transfer of target cell membrane material to neutrophils is completely inhibited by Wortmannin (Fig. 4B), whereas there appears to be a partial inhibition of ADCC (Fig. 4A). This could suggest either an additional parallel PI3K-independent mechanism of killing and/or an additional requirement for PI3K in the final uptake of target cell fragments. The interpretation of this is further complicated because our preliminary findings have shown that PI3K inhibition is also influencing, to some extent, the killer synapse formation between effector and target cells during neutrophil-mediated ADCC (Zhao et al., data not shown), which will obviously also impact the downstream events that includes the effector killing mechanism(s). Therefore, one of the main challenges will be to identify the molecular mechanism(s) that act downstream of synapse formation and are instrumental in the process of trogocytosis itself. This could perhaps not only help to identify components suitable for interference that may help to reveal the contribution of trogocytosis to tumor elimination in vivo, but perhaps also targets for improving antibody-dependent destruction of cancer cells by neutrophils.

Clearly, the exact mechanism(s) by which the exchange of membrane from cancer cell to neutrophil leads to lysis of the tumor cells remains to be further investigated. As outlined above the available evidence argues strongly against an apoptotic form of cell death, as seen in NK-cell mediated ADCC and CTL-mediated killing of tumor cells\(^\text{18}\). Besides apoptosis, autophagy has been suggested to induce antibody dependent cancer cell killing by neutrophils in the presence of tumor-cell specific FcγRI bispecific or IgA Abs, but direct evidence by interfering with components of the autophagic machinery was not provided\(^\text{26}\). Using EM imaging and confocal imaging for LC3, a marker of autophagosomes, we could not detect autophagy-related processes during IgG/Trastuzumab-dependent SKBR3 cell killing by neutrophils (unpublished results).

Taken together, we have shown that antibody mediated lysis of cancer cells by neutrophils is not depending on any of the two classical anti-microbial effector mechanisms of neutrophils, but rather seems to be regulated by trogocytosis in which neutrophils are actively tearing membrane-parts of cancer cells in an antibody and FcγR fashion. This preliminary evidence furthermore indicates that this leads to a form of immune cell-mediated necrotic target cell death that we propose to term ‘trogoptosis’. Future efforts should be dedicated to studying the underlying mechanism(s) of neutrophil-mediated trogocytosis and its contribution to the elimination of cancer cells in vivo.
REFERENCE LIST


## Supplementary Table 1 | Patients information.

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Defects in neutrophil granule mobilization and bactericidal activity in Familial Hemophagocytic Lymphohistiocytosis type 5 (FHL-5) syndrome caused by STXBP2/Munc18-2 mutations


* These authors contributed equally to this work

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ABSTRACT

Familial hemophagocytic lymphohistiocytosis (FHL) is caused by genetic defects in cytotoxic granule components or their fusion machinery, leading to impaired Natural Killer (NK) cell and/or T lymphocyte (CTL) degranulation and/or cytotoxicity. This may accumulate into a life-threatening condition known as macrophage activation syndrome. STXBP2, also known as MUNC18-2, has recently been identified as the disease-causing gene in FHL5. A role for STXBP2 in neutrophils, and for neutrophils in FHL in general, has not been documented thus far. Here, we report that FHL5 neutrophils have a profound defect in granule mobilization, resulting in inadequate bacterial killing, in particular of Gram-negative *Escherichia coli*, but not of *Staphylococcus aureus*, which rather depends on intact NADPH oxidase activity. This impairment of bacterial killing may contribute to the apparent susceptibility to gastrointestinal inflammation in FHL5 patients.
INTRODUCTION

Rare mutations (incidence: ~1-4 cases/million depending on ethnicity) in four different genes, including PRF1 (encoding perforin-1) in FHL2, UNC13D (encoding Munc13-4) in FHL3, STX11 (encoding syntaxin 11) in FHL4, and, more recently, also STXBP2 (also known as MUNC18-2), encoding the granule-associated docking protein syntaxin binding protein 2 (STXBP2), in FHL5, have been identified as disease-causing in FHL patients. FHL-5 accounts for an estimated 10% of all FHL cases. STXBP2 is important for regulating intracellular granule trafficking and docking at the plasma membrane. Previous reports have documented the role of STXBP2 in NK, CTL and platelet degranulation in FHL type 5 patients (FHL-5). Some of the clinical features of FHL5, such as the increased susceptibility to gastrointestinal bacterial infection, have remained unexplained and it therefore seems possible that STXBP2 plays a role in other leukocytes, such as neutrophils. Indeed, STXBP2 has been reported to localize to the different types of granules in human neutrophils. Neutrophil granules contain an array of anti-microbial constituents and proteases that will be secreted and/or released into the phagosome upon mobilization and this process contributes to microbial killing. Although there is some evidence for an involvement of STXBP2 in neutrophil granule function, compelling evidence for a direct role has not been documented. Here, we demonstrate that STXBP2 is involved in neutrophil granule mobilization and bacterial killing using neutrophils from three genetically-defined FHL5 patients.

METHODS

FHL5 patients, control subjects and neutrophil isolation

Heparinized blood was collected, after informed consent and according to the declaration of Helsinki 1964, from three unrelated FHL-5 patients (supplementary table 1) and healthy controls, which included umbilical cord blood and blood from healthy adults. Granulocytes were isolated by density gradient centrifugation with isotonic Percoll (Pharmacia, Uppsala, Sweden) and erythrocyte lysis, as described before. Granulocytes were washed and resuspended in Hepes-buffered saline solution (HBSS containing 132 mM NaCl, 6.0 mM KCl, 1.0 mM CaCl$_2$, 1.0 mM MgSO$_4$, 1.2 mM potassium phosphate, 20 mM Hepes, 5.5 mM glucose and 0.5% (w/v) human serum albumin, pH 7.4). The purity of granulocytes always exceeded 95%

Degranulation assays

Neutrophil degranulation was examined as described before. Briefly, neutrophils (2×10$^6$/ml) were incubated in Hepes-buffered saline solution (Hepes-buffer) at 37°C in a shaking water bath before adding the (priming) agents PAF (1 μM, Sigma, 5 minutes) or cytochalasin B (5 μg/ml, Sigma, 5 minutes) and were subsequently stimulated with fMLP (1 μM, Sigma, 15 minutes). After stimulation, cells were put on ice, washed with Hepes-buffer once, and subsequently stained with antibodies against neutrophil-granule markers: CD63-PE (IgG1, 435); CD66b-FITC (IgG1, CLB-B13.9). Data are expressed as mean fluorescence intensities (MFI). The cells were analyzed on an LSRII flow cytometer equipped with FACSDiva software (BD). The release of elastase and lactoferrin was evaluated using ELISA kits (HyCult Biotechnology) according to the manufacturer’s instructions.
Bacterial killing
Granulocyte bactericidal activity was determined using, *Escherichia coli*, strain ML-35, and *Staphylococcus aureus*, strain 502A. Bacterial survival was measured by assaying bacterial colony formation as previously described\(^{10}\).

Statistics
Statistical significance was determined where possible using Students t-test, or otherwise by Grubb's outlier test; \(p < 0.05\) was considered to be significant.

RESULTS AND DISCUSSION

In order to characterize a role for STXBP2 in neutrophil function and a possible contribution of neutrophils to FHL5, we analyzed three unrelated patients with defined mutations in STXBP-2 (Suppl. Table 1). The absence of STXBP2 protein in neutrophils of patients A and C was confirmed by Western blot analysis, whereas patient B carrying a homozygous exon 15 splice site mutation, which is known to be associated with residual NK function and a milder clinical phenotype\(^{11}\), did express substantial levels of a STXBP2 protein (Suppl. Fig.1A). As reported before\(^{12}\), this splice site mutation yields a variant STXBP2 protein (Suppl. Fig.1B), most likely with a partially impaired function. In line with this, and also with previous findings\(^{11}\), a complete (patients A and C) or partial (patient B) absence of NK cell-mediated degranulation and cytotoxicity towards target cells was observed (Suppl. Fig.2). The exocytosis of the different types of neutrophil granules\(^6\), induced by either cytochalasin-B/fMLP or PAF/fMLP, was evaluated by monitoring secretion of the granule components elastase (azurophilic granules) or lactoferrin (LAF) (specific granules) and the exposure of cell surface CD63 (azurophilic granules) or CD66b (secretory, tertiary and specific granules) (Fig.1). This revealed a pronounced defect in the release of both types of vesicles in FHL5 neutrophils of all three patients. Consistently, the cytoB/fMLP-stimulated release of extracellular proteolytic activity, analyzed by DQ-BSA proteolysis assay, which primarily measures neutrophil-derived serine proteases, appeared completely absent in FHL5 neutrophils, although the total cellular content of the proteases was not different (Suppl. Fig.3). Immuno-EM analysis of the tertiary, specific and azurophilic granules, using respectively gelatinase, lactoferrin and MPO as markers, showed a normal granule appearances and frequencies (Suppl. Fig.4), thereby excluding defects in granule biosynthesis. Measurement of intra-phagosomal serine protease activity suggested that mobilization of neutrophil granules to phagosomes was also substantially impaired in FHL-5 neutrophils (Suppl. Fig.5), although it was certainly not completely absent as also shown by the presence of both specific and azurophilic granule components in *E.coli*-containing phagosomes (Suppl. Fig.6). Collectively, these findings suggested, for the first time, that STXBP2 is required for neutrophil granule exocytosis, and likewise also, at least in part, for granule mobilization to the phagosome.
Impaired neutrophil granule mobilization and bactericidal activity in FHL-5

Figure 1 | Defective granule mobilization by FHL5 neutrophils.
Degranulation of neutrophils was examined in one day old healthy adult (n=11) and fresh cord blood (n=6) controls, and the FHL-5 patient A (fresh), B and C (both one day old) by monitoring the release of (A) lactoferrin (LAF, specific granules) and surface exposure of (B) CD66b (secretory, tertiary and specific granules) upon stimulation with PAF/fMLF, and the release of (C) elastase (azurophilic granules) and surface exposure of (D) CD63 (azurophilic granules) upon stimulation with cytoB/fMLF, respectively. Concentrations (ng/ml) of released factors, or the mean fluorescence intensities (MFI) of surface markers for stimulated (white, blue, green, red) and unstimulated (black parts of the bars) cells are shown. The values shown are averages ± SEM, from at least two independent experiments, each performed in triplo, with the exception of patient A, which was evaluated once; *, p < 0.05; n.s., non-significant; Grubb’s outlier test. Note that granule release is essentially absent in all three FHL-5 patients.
Neutrophils are essential for controlling bacterial and fungal infections. There are two mechanisms that primarily contribute to neutrophil-mediated killing of microbes: (a) the NADPH oxidase, which upon assembly and activation in the phagosomal- and/or plasma-membrane produces toxic reactive oxygen species, and (b) proteolytic killing, which is assumed to require the fusion of the protease-loaded azurophilic granules with phagosomal- and/or plasma-membrane. Although gene targeting experiments in the mouse have demonstrated that both mechanisms may act independently or in concert to achieve full destructive power, the relative contribution of each activity depends on the nature of the microbe as well.13-15

However, in spite of abundant information on neutrophil microbial killing in a human context with respect to the role of the NADPH oxidase, an activity that is defective in chronic granulomatous disease (CGD), much less is know about the contribution of granule mobilization in human neutrophils. Clearly, the availability of FHL5 neutrophils with a granule mobilization defect allowed us to explore this directly. Neutrophil-mediated killing of \textit{S. aureus} and \textit{E. coli} was evaluated. Figure 2 shows that the \textit{S. aureus} killing was virtually unaffected in FHL5 neutrophils, but the killing of \textit{E. coli} was substantially impaired in cells from all patients investigated. Of relevance, the phagocytosis of the bacteria was apparently not significantly affected (Suppl.Fig.7). In line with a complementary role for the NADPH oxidase in this context, \textit{S. aureus} killing was more or less abolished in cells treated with the NADPH oxidase inhibitor diphenylene iodonium (DPI), while the killing of \textit{E. coli} was only partially impaired (Suppl.Fig.8). Similar abnormalities in killing have been observed with neutrophils from CGD patients16 (and data not shown). It should be noted that the production of reactive oxygen species by the NADPH oxidase in response to particulate stimuli, such as unopsonized or opsonized zymosan (Suppl.Fig.9A), or soluble stimuli, such as PAF/fMLP (not shown), was not impaired in FHL5 neutrophils. However, the intra-phagosomal oxidation of DHR-coated \textit{E.coli} appeared slightly delayed in FHL-5 neutrophils (Suppl. Fig.9B). Because DHR oxidation requires MPO activity17 this is consistent with the impaired mobilization of granules to phagosomes noted above. Collectively, these findings suggest that, while \textit{S. aureus} killing depends primarily on the NADPH oxidase, both the oxidase as well as granule mobilization-dependent mechanisms, either to the plasma membrane and/or to the phagosomal membrane, play a role in \textit{E. coli} killing. Finally, chemotaxis of FHL5 neutrophils was apparently normal (Suppl. Fig.10).
Figure 2 | Impaired bacterial killing by FHL5 neutrophils.

Killing of *S. aureus* (A) and *E. coli* (B) was assessed in cord blood (control A; n=6) and normal (control B/C; n=10) controls and the FHL-5 patients as described in the Materials and Methods section (see also the legend of Fig. 1). Remaining viable bacteria were quantified as colony forming units (CFU) and expressed as % of CFU at t=0. For the control groups the values shown are averages ± SEM, and for the patients the average of two measurements from two (patient A/B) or three (patient C) independent experiments are shown; *, p < 0.05; Grubbs’ outlier test (patient A/B) or Student’s-t-test (patient C). Note that the killing of *S. aureus* is virtually normal, while *E. coli* killing is significantly impaired for all three FHL-5 patients.

Taken together, to our knowledge these findings provide the first direct demonstration of a requirement for STXBP2 in particular, and granule mobilization in general, in the killing of bacteria by human neutrophils. This defect in bacterial killing may potentially also explain some of the unexplained symptoms, such as the increased susceptibility to gastrointestinal inflammation, which has previously been reported in FHL5 patients.

**ACKNOWLEDGEMENTS**

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### SUPPLEMENTARY FIGURE LEGENDS AND METHODS

**Supplementary Table 1 | STXBP2/MUNC18-2 gene mutations and clinical features of FHL-5 patients A, B and C.**

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</table>
| 4–6w | allele 1: c.1621G>A (p.Gly541Ser)  
allele 2: c.1621G>A (p.Gly541Ser) | not detectable | none (sibling died of MAS in HLH); successful HSCT at 6-7w |
| 13y | allele 1: 1247-1G>C (exon 15 splice site)  
allele 2: 1247-1G>C (exon 15 splice site) | yes; variant | history of mild HLH |
| 7y  | allele 1: c.1621G>A (p.Gly541Ser)  
allele 2: 1247-1G>C (splice variant) | not detectable | mild HLH |

1As determined by Western blotting; see also Suppl. Fig. 1
Supplementary Figure 1 | STXBP2/Munc18-2 protein expression in FHL-5 patient neutrophils. A) Total lysates from PMN of control and FHL-5 patient neutrophils were resolved on SDS-PAGE and subjected to immunoblotting with rabbit polyclonal antibodies specific for human STXBP2/Munc18-2 (GeneTex, USA). Staining for β-actin of α-tubulin was done as a loading control. B) STXBP2/Munc18-2 protein variant expressed in patient B. As a result of the 1247-1G>C exon 15 splice site mutation a 19 amino acid sequence of intron 14 is instead of the normal 17 amino acid sequence encoded by exon 15 (blocked area). This was verified by mass spectrometry, which identified the peptide indicated in red in blood cell lysates of the patient.
Supplementary Figure 2 | Defective degranulation and cytotoxicity by FHL-5 patient NK cells. A) Degranulation was assayed as cell surface appearance of CD107a on NK cells (CD3-CD16+CD56+) (Alter G et al. (2004) J Immunol Meth. 294:15-22). Briefly, PBMC were incubated for 4 hrs with K562 cells at a 1:1 ratio in the presence of FITC-labeled CD107a. Cells were harvested and CD107a expression was measured on the CD3-CD16++CD56 dim population by flow cytometry and analyzed with CellQuest Pro software. Values shown were corrected for the % of surface expression at t=0, which was <1% in both controls and patients A and B. Note that NK cell degranulation is essentially absent for patient A, while there is some rest activity in patient B. B) Spontaneous and antibody (Trastuzumab)-dependent NK cell cytotoxicity against SKBR-3 breast cancer cells was measured in a ⁵¹Cr release assay (Kiesling R et al.(1975) Eur J Immunol. 5:112-117) using NK cells isolated with anti-CD56 MACS beads as effector cells at the indicated target:effector (T:E) ratios. Note that NK cell cytotoxicity in patients A and C is virtually absent, while there is considerable rest activity for patient B. Similar findings were obtained using patient NK cells with K562 cells as targets (data not shown).
Supplementary Figure 3 | Extracellular release of neutrophil granule proteases triggered by cytoB/fMLP as measured by DQ-Green BSA assay, in which proteases cause an increase in fluorescence, expressed in relative fluorescence units (RFU), upon the loss of auto-quenching of the substrate. In particular, the release of neutrophil granule-derived protease activity was determined by incubating neutrophils (2.5×10⁶/ml in Hepes-buffer) with DQ-Green BSA (10 μg/ml, Molecular Probes). Upon stimulation with cytoB/fMLP the fluorescence was monitored at 120-second intervals for 1 hour by SPECTRAFluor Plus plate reader (Exitation 485 nm; Emission 535 nm). Neutrophils of FHL-5 patients A, B and C are compared to their respective controls. Values between brackets are the relative fluorescence units (RFU) signals obtained after 1 hour incubation with 1% Triton X-100, which demonstrates that there are no significant differences in cellular protease content between patients and controls.
Impaired neutrophil granule mobilization and bactericidal activity in FHL-5

Supplementary Figure 4 | Immuno-electron microscopic identification of A) gelatinase-positive tertiary granules, B) lactoferrin-positive specific granules, C) myeloperoxidase (MPO)-positive azurophilic granules in neutrophils from FHL-5 patient A and corresponding control neutrophils. Note that the FHL-5 patient has apparently normal granules with immunoreactive material (arrow-heads). Bar = 1 μm. Sample preparation and staining as described previously by Herrero-Turrión MJ et al. (2008) J Immunol. 181:3793-803. D) FHL-5 patient neutrophils have normal quantities of gelatinase-, lactoferrin- and MPO- positive granules. The average numbers of gelatinase-, lactoferrin- and MPO-positive granules/cell was determined in neutrophils from the control and the FHL-5 patient by counting positively stained vesicles (≥ 4 gold particles) in 20-25 cells from the preparations shown in panels A-C.

Supplementary Figure 4 - continued
Supplementary Figure 5 | Intra-phagosomal protease release in FHL-5 (patient B) and control neutrophils measured during uptake of human IgG-opsonized Alexa633 beads coated with A) DQ-BSA or B) DQ-cathepsin G-substrate peptide. Proteolytic activity was determined as described before by Yates RM et al. (2009) Methods Mol Biol. 531:157-71 using a BD Canto flow cytometer simultaneously measuring phagocytosis (exitation at 633 nm/emission at 650 nm) and proteolytic activity (exitation at 488 nm/emission at 520 nm for the DQ-BSA, or exitation at 405 nm/emission at 460 nm for the DQ-cathepsin G) for 5000 cells/sample. The ratios of the proteolytic MFI/phagocytic MFI were used as a measure for proteolytic activity. Incubations performed in the presence of the phagocytosis inhibitor cytochalasin B (5 μg/ml) were performed to control for extracellular proteolytic activity (RFU_{cytoB}) and these values were subtracted from the values measured in absence of cytoB, such that the intra-phagosomal proteolytic activity is represented by RFU-RFU_{cytoB} = ΔRFU, which is shown in the graphs. Data shown are from one representative experiment out of three performed for patients B and C. Note that intra-phagosomal protease activity in FHL-5 neutrophils is considerably reduced but not absent.
Supplementary Figure 6 | Localization of lactoferrin (LAF) and myeloperoxidase (MPO) in *E. coli*-containing phago-lysosomes in neutrophils of FHL-5 patient B and control individual. Neutrophils of patient and corresponding controls were allowed to phagocytose serum-opsonized *E. coli* bacteria for 30 minutes and processed for immuno-EM staining for LAF and MPO as in Suppl. Fig. 4. Note that the phagosomes in FHL-5 neutrophils, which were identified by the presence of distinguishable *E. coli* bacteria (phagosomal membranes indicated with arrow-heads), contain a substantial amount of LAF and MPO, indicative of residual specific and azurophilic granule fusion to *E. coli* phago-lysosomes.
Supplementary Figure 7 | Phagocytosis of *E. coli* and *S. aureus* by FHL-5 patient B and C and corresponding control neutrophils. Neutrophils of patient and corresponding controls were allowed to phagocytose FITC-labelled serum-opsonized *E. coli* and *S. aureus* bacteria for the indicated times as described previously (Brouwer N et al. (2008) J Immunol. 180:4124-32) and the % of neutrophils that had phagocytosed bacteria was quantified by flow cytometry. Note that there is no meaningful difference in bacterial uptake between FHL-5 and control neutrophils.

Supplementary Figure 8 | Effects of the flavoprotein/NADPH oxidase inhibitor DPI on bacterial killing by neutrophils. Killing of *S. aureus* is completely inhibited by DPI, whereas *E. coli* killing is only partially reduced. Remaining viable bacteria were quantified as colony forming units (CFU) and expressed as % of CFU at t=0. Other experimental details as in Figure 2. A representative experiment out of three is shown. Control experiments (not shown) demonstrated that DPI had no effect on bacteria itself.
Supplementary Figure 9 | Normal respiratory burst in FHL-5 patient neutrophils, but delayed intraphagosomal reduction of DHR-\textit{E. coli}. Respiratory burst in response to various particulate stimuli, including zymosan (1 mg/ml) and serum-opsonized zymosan (STZ; 1 mg/ml), was measured with the Amplex Red assay as described previously\(^9\). Mean ± SEM of triplicate measurements is shown. Control neutrophils include cord blood controls (i.e. controls A; n=5) and adult controls (i.e. controls B/C; n=82). Note that the respiratory burst in FHL-5 neutrophils may be slightly higher, but certainly not lower, than in controls.

Supplementary Figure 10 | Chemotaxis of FHL-5 patient A and C neutrophils. Chemotaxis in response to C5a, IL-8 and PAF was measured with calcein-labelled neutrophils in a Transwell chemotactic assay as described (Van Bruggen et al. (2010) J.Innate.Immun. 2:280-287), with the results represented in relative fluorescent units (RFU). Controls represent n=82 independent measurements of individual healthy donors (mean +/- SEM).
Summary and general discussion
SUMMARY AND GENERAL DISCUSSION

**CD47-SIRPα interactions as a target for potentiating antibody therapy in cancer**

The primary aim of the work presented in this thesis was to address the role of CD47-SIRPα interactions in the antibody-dependent destruction of cancer cells. This is of particular relevance in the context of the treatment of metastatic or disseminated cancer patients with cancer therapeutic antibodies, such as e.g. Trastuzumab, Rituximab and Cetuximab, because the clinical efficacy of such biologicals is as yet too limited to justify their use in absence of non-specific and harmful chemotherapeutics. In chapter 2 we first provide the general proof-of-concept that signaling via the inhibitory CD47-SIRPα axis constitutes a limiting factor for antibody therapy *in vivo* using a syngeneic immunocompetent mouse model. In particular, we used mice which express a mutant variant SIRPα receptor that lacks the cytoplasmic tail, and therefore its capacity for inhibitory signaling. When these mice are subjected to the well-characterized B16 mouse metastatic melanoma model, in the presence of suboptimal treatment with a ‘therapeutic’ anti-melanoma antibody, we observed a much more effective cancer cell destruction than in mice expressing normal SIRPα receptor. This demonstrates that SIRPα signaling forms a barrier for antibody-dependent cancer cell destruction. These findings were the first indications for a role of the CD47-SIRPα pathway using a syngeneic cancer model in an immunocompetent context. Prior, Chao et al. reported that anti-human CD47 antibodies could potentiate Rituximab-mediated elimination of human Non-Hodgkin lymphoma (NHL) cells in immunodeficient NOD mice. Importantly, the above study, as well as a considerable number of other studies from the same group, also demonstrated tumor cell elimination by anti-CD47 in the absence of Rituximab or other cancer cell-targeting therapeutic antibodies. This was in sharp contrast to our findings, in which the CD47-SIRPα axis was only affecting the antibody-dependent tumor cell destruction, as we have argued in several commentaries. We feel that the data from these studies in xenogeneic models and their interpretation are misleading for several reasons. First, the use of intact anti-human CD47 antibodies is inappropriate for addressing the role of the CD47-SIRPα interaction in these cancer models, because these mouse IgG1 antibodies do not only interfere with CD47-SIRPα interaction, but may also trigger direct antibody effector function, such as ADCC, as we have demonstrated. Instead, when we performed *in vitro* experiments using F(ab’)_2-fragments of the same anti-CD47 antibody, which lacks the Fc-portion and therefore also the ability of the intact antibody to mediate interaction with FcγR and downstream effector functions, cancer cell destruction by either human neutrophils or macrophages is not observed in the absence of anti-tumor antibodies (chapter 2 and Zhao et al). Importantly, in these experiments it was shown that the selective blocking of CD47-SIRPα interactions with these anti-CD47 F(ab’)_2-fragments was able to potentiate ADCC in the presence of anti-cancer antibodies. The question arises as to whether CD47-SIRPα targeting is strictly applicable in combination with cancer therapeutic antibodies, or may also act in absence of those, is very important when considering the development of this concept as a therapeutic strategy, also when considering potential side effects of CD47-SIRPα targeting. It should be mentioned that a recent report by Weiskopf et al appears to more or less resolve the debate in the literature as to whether CD47-SIRPα targeting can be applied alone or only in combination.
with cancer therapeutic antibodies. The investigators involved developed a recombinant affinity-enhanced antagonist SIRPα protein and demonstrated, in a variety of models, that this CD47 blocking protein was only facilitating cancer cell destruction in the presence of cancer-therapeutic antibodies such as Trastuzumab, Rituximab and Cetuximab. Another problem of the experiments with the xenogeneic models, which actually also includes the studies by Weiskopf et al.⁹, is that other homeostatic interactions between the human cancer cells and the mouse immune system, which could potentially act in a redundant fashion in conjunction with the CD47-SIRPα interaction, may not ‘match’, thereby creating a situation in which the contribution of CD47-SIRPα interactions may be exaggerated. The fact that most of these experiments were done in immune-deficient mice of the NOD background, may already potentially lead to an over-interpretation of the contribution of the CD47-SIRPα pathway, because the mouse NOD SIRPα allelic variant has been shown to demonstrate an unusually high affinity for human CD47\(^{10,11}\). Furthermore, although it could be argued that the immunodeficiency of the mice used in these studies may more or less resemble the condition in which therapeutic antibodies are applied together with chemotherapy in patients. The ultimate goal is to perform antibody therapy in absence of such therapy, and it is therefore important to demonstrate the enhancing effect of interfering with the CD47-SIRPα interaction in an immune competent context, as we did in chapter 2 for the first time. Finally, the therapeutic efficacy of the anti-CD47 of Chao et al.⁵, and the other studies⁴, along the same lines by this group of which might be largely exaggerated. This is because a human-specific anti-CD47 antibody was used in these experiments, and as such it might be much easy to saturate the CD47 molecules on the cancer cells in a xenogeneic context, in contrast to a syngeneic therapeutic situation where an enormous ‘sink’ of CD47 would be anticipated, due to the abundance of CD47 on other cells. Therefore, we believe that the targeting of SIRPα offers a better opportunity, and this is the reason that we have generated antibodies against human SIRPα that are able to block interactions with CD47 and that were shown to potentiate neutrophil-mediated ADCC towards antibody-opsonized cancer cells (chapter 2). Clearly, it will be of interest to develop these antibodies against SIRPα, for clinical application. In principle such anti-SIRPα antibodies could be combined with different cancer therapeutic antibodies, thereby providing a generic method to potentiate the clinical efficacy of cancer therapeutic antibodies. Finally, it has become clear in recent years that many cancers have an overexpression of CD47\(^{12-15}\). This could clearly protect them from the cellular effector functions of myeloid cells during antibody therapy. In line with this hypothesis we show in chapter 2, based on a data from a small and therefore somewhat preliminary study, that the clinical efficacy of Trastuzumab treatment in Her2⁹ Neu-positive breast cancer is linked to the expression levels of CD47 on the cancer cells, with the cancers having the lowest CD47 levels responding best to the treatment. Moreover, also in NHL patients treated with Rituximab, CD47 expression was demonstrated to have an association with the clinical outcome². These findings together provide indirect evidence for the importance of CD47-SIRPα interactions during antibody therapy in cancer, and are clearly also in line with the general concept that CD47-SIRPα interactions form a barrier against antibody-mediated tumor cell destruction by phagocytes.
In chapter 3 we have addressed the potential role of genetic variation in SIRPα as well as FcγR, in neutrophil-mediated cancer cell killing. Takenaka et al. found a high number of polymorphic variants of SIRPα in the general human population (i.e. 10 variants identified in 39 individuals), which includes Caucasian, Asian and African populations. Notably, the polymorphic residues are exclusively present within the N-terminal Ig-like domain of SIRPα, adjacent to the areas important for CD47 recognition and binding. It has been proposed that the polymorphic residues in SIRPα generally do not seem to affect CD47 binding. Nevertheless, with respect to understanding the general applicability of targeting CD47-SIRPα interactions in cancer patients, it was important to investigate the functional contribution of the different SIRPα variants in more detail. In Caucasian individuals we identified only two polymorphic variants, SIRPα1 and SIRPαBIT, and experiments described in chapter 3 show that the level to which an enhancement of ADCC can be achieved by the interference with CD47-SIRPα interactions, does not differ between the relevant genotypes. All combined findings supports the idea that the targeting of CD47-SIRPα interactions may be a generic approach for potentiating antibody therapy in cancer. Our parallel analysis of FcγR polymorphisms and gene copy number variation (CNV), a number of which are known able to affect FcγR expression and/or function (see chapter 1 table 2), did however reveal significant associations with the intrinsic capacity of neutrophils to kill cancer cells by ADCC. Our integrated genetic analysis using the multiplex ligation-dependent probe amplification (MLPA) that we previously developed, allowed for an unbiased approach circumventing the linkage disequilibrium that is known to exist among the various FcγR variants. Our results demonstrated that neutrophils use a combination of the different FcγR present, including FcγRI, FcγRIIa (and in some individuals also FcγRIIc), and FcγRIIIb. We also observed associations between the capacity of neutrophil to kill Trastuzumab-opsonized breast cancer cells and the FcγRIIa-131H/R and FcγRIIIb NA1/NA2 polymorphisms. Although these associations are significant and can, at least in part, provide an explanation for individual differences in neutrophil ADCC capacity (chapter 3) and also in the clinical efficacy of antibody therapy in cancer, the level by which these polymorphisms
affect cancer cell destruction is not sufficient to exclude patients from antibody therapy. In addition, no selectivity for individual FcγR genotypes was observed, with respect to the level by which manipulation of the CD47-SIRPα pathway could promote ADCC. Adding support to the idea that targeting of CD47-SIRPα interactions may comprise a generic strategy for potentiating antibody-mediated tumor cell destruction by the immune system.

The mechanism by which CD47-SIRPα interactions restrict neutrophil ADCC

When considering targeting of CD47-SIRPα interactions for improving antibody therapy in cancer it is important to understand the underlying mechanism(s). The studies described in chapter 4 address this issue. Our results demonstrate that neutrophil ADCC involves the critical formation of a so called cytotoxic synapses, which ensures an intimate, albeit transient (chapter 4), association between effector and antibody-coated target cells. Based on antibody blocking experiments in conjunction with the analysis of neutrophils deficient of CD18 integrin, from very rare patients with leukocyte adhesion deficiency syndrome type 1 (LADI), it is shown that both neutrophil killer synapse formation as well as the resultant cytotoxicity are dependent on the CD11/CD18 integrin that is prominently expressed on human neutrophils. Moreover, we show that CD47-SIRPα interactions promote cytotoxic synapse formation by controlling the inside-out regulation of integrin affinity that occurs during ADCC. Finally, we employed neutrophils from patients with LADIII syndrome, which lack the integrin affinity regulator protein kindlin3, to show that kindlin3, and therefore also integrin affinity regulation, plays an essential role in the antibody-dependent destruction of cancer cells by neutrophils. These findings establish that at least one level at which CD47-SIRPα interactions act to limit neutrophil ADCC is through restricting neutrophil CD11b/CD18 integrin affinity during this process.

Neutrophil ADCC occurs by trogocytosis, and not by NADPH oxidase activity or cytotoxic granule-dependent mechanisms

In order to further understand how CD47-SIRPα interactions regulate neutrophil-mediated ADCC towards cancer cells, we needed to understand more about the actual effector mechanism of neutrophil cytotoxicity. Apart from a requirement for cytotoxic synapse formation not much was known about this, and this prompted us to study this in more detail. The findings of these studies are described in chapter 5. Given the critical role of neutrophils in the host defense against in particular bacterial and fungal infections, we first explored the role of the two anti-microbial killing mechanisms exerted by neutrophils, i.e. the NADPH oxidase and the neutrophil cytotoxic granules. For these studies we used neutrophils from chronic granulomatous disease (CGD) or familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) patients deficient in the NADPH oxidase or the STXBP2/munc18-2 protein that is essential for the exocytosis of the neutrophil granules, respectively (chapter 6). The completely normal ADCC observed with neutrophils from these patients provided compelling evidence that neither of these anti-microbial mechanisms played a significant role in the antibody-dependent killing of cancer cells. Next, we attempted to create insight into the actual effector mechanism of neutrophil cytotoxicity. Our preliminary experiments
provide evidence for trogocytosis as a completely novel mechanism of neutrophil ADCC. During trogocytosis neutrophils mechanically tear off fragments from the cancer target cell and ingest these. We speculate that this leads to rupture of the target plasma membrane, and thus a type of necrotic cell death that we suggest to term trogoptosis. Preliminary life cell imaging experiments (Matlung et al., unpublished) already support this.

It therefore seems that there are both similarities and differences with respect to the mechanisms by which the various effector cells, including NK cells, neutrophils and macrophages, mediate ADCC (Table 1). What all of these immune effector cells share with respect to their killing of target cells is that the formation of a killer synapse is absolutely essential, and that in all cases integrins play a pivotal role in the formation of this. However, whereas NK cells kill target cells by a well-defined granule-mediated mechanism, in which the directed release of granzyme B and perforin triggers target cell apoptosis, and whereas macrophages are able to ingest intact antibody-opsonized cancer cells by phagocytosis, neutrophils may use trogocytosis as an important mechanism of antibody-dependent tumor cells destruction. In addition, in all cases there appear to exist homeostatic inhibitory mechanisms that control the process, probably for the purpose of preventing damage by excessive immune cell activation to the host. These inhibitory feedback pathways involve CD47-SIRPα interactions in the case of myeloid cells as described herein, and analogous interactions between MHC class I and inhibitory killer immunoglobulin-like receptors (KIR) for NK cells24-25.

Table 1 | Cellular mechanisms of antibody-mediated tumor cell destruction.

<table>
<thead>
<tr>
<th></th>
<th>NK cell</th>
<th>Neutrophil</th>
<th>Macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recognition:</strong></td>
<td>FcR</td>
<td>FcR</td>
<td>FcR</td>
</tr>
<tr>
<td><strong>Signaling pathway(s):</strong></td>
<td>similar (?)</td>
<td>similar (?)</td>
<td>similar (?)</td>
</tr>
<tr>
<td><strong>Killer synapse:</strong></td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Cytotoxic mechanism:</strong></td>
<td>perforin &amp; granzyme B</td>
<td>trogocytosis</td>
<td>phagocytosis</td>
</tr>
<tr>
<td><strong>Cell death:</strong></td>
<td>apoptosis</td>
<td>trogoptosis</td>
<td>intracellular degradation</td>
</tr>
<tr>
<td><strong>Control:</strong></td>
<td>MHCI-KIR</td>
<td>CD47-SIRPα</td>
<td>CD47-SIRPα</td>
</tr>
</tbody>
</table>
Finally, the availability of FHL-5 patients, with neutrophils deficient in exocytosis of granules, created the opportunity to investigate for the first time the overall contribution of neutrophil granules in microbial killing. The studies described in chapter 6 were dedicated to do so for gram-positive and gram-negative bacteria. Our findings show that neutrophils from FHL-5 patients has a profound deficiency in the release of the different subsets of granules (e.g. azurophilic and specific granules) and that this is associated with selective defects in the killing of bacteria. Thus, the killing of gram-negative *Escherichia coli* bacteria was partly affected by STXBP2/munc18-2 mutation, whereas a role for granule release in the killing of *Staphylococcus aureus* was negligible. In line with this, defects in the killing of fungi have been detected (Gazendam et al., submitted). This demonstrates that, granule-dependent cytotoxicity, it is important in the context of microbial killing although it is clearly dispensable for cancer cell destruction. Whether these defects in the anti-microbial response of neutrophils may also be relevant in the context of the clinical symptoms of FHL-5 patients or patients with comparable hemophagocytic lymphohistiocytosis (HLH) syndromes remains to be established.

**In conclusion**

The results of the studies described in this thesis, in conjunction with those of other laboratories, make a strong case for further development of strategies for targeting the CD47-SIRPα pathway for the purpose of potentiating antibody therapy in cancer. Currently, a lot of attention has been allotted to therapeutic intervention of these so called ‘immune check-points’ from both academia as well as the pharmaceutical industry. It is therefore not surprising perhaps that Science magazine recently declared immunotherapy in cancer the breakthrough of the year 2013. It appears that the targeting of CD47-SIRPα interactions in cancer, caught to attention of a number of biotech companies. Nevertheless, the two most important points that remain are, first, whether such intervention with CD47-SIRPα interactions, either alone or in combination with other immunotherapeutic approaches, can actually provide the level of efficacy required to drastically reduce the need for chemotherapy during cancer therapeutic antibody treatment in cancer. If so this would not only directly reduce chemotherapy-related morbidity, but also create a much more optimal immunological environment for performing immunotherapy in the patient at all and thereby tilt the balance onto the direction of a better outcome. The second point is whether targeting the CD47-SIRPα interaction can be done without causing substantial ‘collateral damage’ to the host. CD47-SIRPα interaction is considered a relevant homeostatic interaction that is regulating a variety of phagocyte functions. However disruption of the CD47-SIRPα interaction, by genetic ablation of CD47 or the signaling capacity of SIRPα, or the injection of CD47-SIRPα antagonists into mice, rats (Van der Goes et al., unpublished), and cynomolgus macaques has revealed, apart from a very mild anemia, remarkably few side effects. This, together with the resistance, rather than susceptibility, of SIRPα mutant mice towards autoimmunity, provides further support for the CD47-SIRPα interactions as a promising target for therapeutic intervention. When the appropriate agents for targeting the CD47-SIRPα pathway in a human context have become available the time will be right to study their benefit in clinical studies.
REFERENCE LIST


Appendices
MONOKLONALE ANTILICHAMEN ZIJN EEN VAN DE OPKOMENDE GENEESMIDDELEN IN DE BEHANDELING TEGEN KANKER. DEZE THERAPEUTISCHE ANTILICHAMEN TEGEN KANKER, BINDEN AAN DE TUMOR CELLEN, WAARDOOR DE TUMOR CELLEN GEMARKEERD WORDEN VOOR VERNIETIGING DOOR HET IMMUNSYSTEEM. VERSCHILLENDE ONDERLiggende mechanISMES ZIJN BEKEN, ZOALS DE ANTILICHAAM AFDANKELijke CELLulaire cytotoxiciteit (ADCC). ER ZIJN OP DISt MOMEIt VERSCHILLENDE THERAPEUTISCHE ANTILICHAMEN TEGEN Verschillende soorten kanker op de markt. Ondanks de gunstige gedocumenteerde effecten, zijn behandelingen met alleen antilichamen nog niet geneZend en zijn er manieren nodig om de efficiency van de behandeling te verhogen. WE hebben een mogelijk mechanisme ontdekt, dat de antilichaam afhankelijke doding van tumor cellen negatief beïnvloed, namelijk het intrinsieke mechanisme rondom de interactie tussen CD47 (wat aanwezig is op tumor cellen) en SIRPα (wat aanwezig is op macrofagen en granulocyten).

Het voornaamste doel dit proefschrift is het bestuderen van de rol van de CD47-SIRPα interactie in het proces van antilichaam gemedieerde vernietiging van kankercellen, zoals dit het geval is voor de behandeling van metastaseerende kanker met therapeutische antilichamen met bijvoorbeeld Trastuzumab, Rituximab en Cetuximab. DESE antilichamen zijn gericht op receptoren, die in hoge mate aanwezig zijn op de tumor cellen. Dit is vooral van belang, omdat bewijs voor de klinische efficiency van deze biologische geneesmiddelen nog te beperkt is om deze middelen in afwezigheid van schadelijke niet-specifieke chemokuren te gebruiken. Verder wordt er in dit proefschrift in gegaan op het mechanisme waarmee humane fagocyten, in het bijzonder neutrofelen, de antilichaam afhankelijke doding van kankercellen bewerkstelligd.

In hoofdstuk 2, beschrijven wij hoe de signalering afkomstig van de cytoplasmatische deel van SIRPα, een beperking vormt voor de antilichaam afhankelijke doding van kankercellen in vivo. In de B16 melanoom metastases model in muizen, zien we dat muizen zonder het cytoplasmatische deel van SIRPα, gevoeliger zijn voor de suboptimale behandeling met therapeutische antilichamen dan muizen met een normaal SIRPα. DESE bevindingen suggerer voor het eerst een rol voor de CD47-SIRPα interactie, in een immuun competent systeem. Daarnaast laten we in hoofdstuk 2 ook zien dat het gericht verstoren van de CD47-SIRPα interactie, met behulp van agonisten tegen zowel CD47 als SIRPα, de in vitro vernietigings capaciteit van humane fagocyten verhoogt in Her2/Neu positieve borst kanker cellen, dat met behulp van het therapeutische antilichaam Trastuzumab naar de kanker cellen worden geleid. Alles bij elkaar gezien wijst dit op het belang van de CD47-SIRPα interacties in behandelingen met antilichamen. Daarnaast onderbouwt de data de algemene hypothese, dat de CD47-SIRPα een negatief effect hebben en als een soort barrière functioneert op het antilichaam gemedieerde vernietiging van kanker cellen door fagocyten.

Vervolgens, in hoofdstuk 3, wordt het effect van de genetische variatie van de FcγRs en SIRPα op de efficiency van de antilichaam afhankelijke cellulaire cytotoxiciteit van menselijke neutrofelen op SKBR-3 borst kanker cellen in de aanwezigheid van Trastuzumab. Van de geteste neutrofelen, afkomstig van verschillende individuen, zien we een scala aan
verschillende sterktes in de cytotoxiciteit. Er is van zowel in de FcγRs en SIRPα, van de geteste neutrofielen, polymorfisme/genetische varianten te vinden. Wij onderzochten of deze genetische effecten de verschillende uitslagen van de antilichaam afhankelijke cellulaire cytotoxiciteit testen konden verklaren. We hebben van ongeveer 100 individuen, mede de genetische variant, als de antilichaam afhankelijke cellulaire cytotoxiciteit bepaalt. Hieruit is gebleken dat neutrofielen met een FcγRIIa-131H of een FcγRIIIb-NA1 genetische variant een hogere vernietigings capaciteit hadden vergeleken met de varianten FcγRIIa-131R of FcγRIIIb-NA2. Daarnaast konden we de antilichaam afhankelijke cellulaire cytotoxiciteit met 1,5 tot 2 voud verhogen door de CD47-SIRPα interactie te blokkeren, ongeacht de genetische identiteit van de FcγR en waren en geen kenbare verschillen tussen de meest voorkomende varianten/polymorfisme van SIRPα in de caucasische populatie. Daaruit kunnen we concluderen dat de antilichaam afhankelijke cellulaire cytotoxiciteit capaciteit direct gerelateerd is aan FcγRIIa or FcγRIIIb polymorfisme en dat het interferen van de CD47-SIRPα interactie de cytotoxiciteit kan verhogen en mogelijkerwijs een algemene strategie kan vormen om de effectiviteit van het gebruik van antilichamen tegen kanker kan verhogen.

Hoofdstuk 4 is er op gericht om inzicht te krijgen in het mechanisme waarmee de CD47-SIRPα interacties de antilichaam afhankelijke cellulaire cytotoxiciteit van neutrofielen moduleren. Dit gebeurt op het niveau van de regulatie van de binnenste buiten affiniteit van CD11b/CD18 integrin, die essentieel zijn voor de cytotoxicse synaps vorming and cel doding. De vereisten om CD11b/CD18 integrin/activatie voor antilichaam afhankelijke cellulaire cytotoxiciteit was aangetoond met ofwel neutrofielen afkomstig van een LAD1 patiënten die geen CD18 integrin hebben, ofwel neutrofielen van LAD3 patiënten, die geen kindlin-3 hebben en daardoor een defect hebben in integrin activatie. Bij elkaar, laten deze bevindingen zien dat CD47-SIRPα interacties de antilichaam afhankelijke cellulaire cytotoxiciteit beperken, door de activatie te beheersen van CD11b/CD18-integrin.

In hoofdstuk 5, tonen we een serie experimenten die inzicht geven in het mechanisme waarmee neutrofielen het antilichaam gemedieerde kankercellen vernietigen. Deze studies sluiten een rol uit voor de klassieke anti-microbiologische mechanismes in het antilichaam gemedieerde kanker cel vernietiging, inclusief de NADPH oxidase en de granulen afhankelijke mechanismes. Dit hebben we gedaan door neutrofielen te gebruiken van patiënten die lijden aan een chronische granulomatous ziekte, deze hebben een defect in ROS productie. Daarnaast hebben we ook neutrofielen gebruikt van patiënten die lijden aan familiaire hemophagocytic lymphohistiocytosis (FHL-5), (beschreven in hoofdstuk 6), deze neutrofielen zijn zwaar beperkt in de proteolytische granule exocytose. Belangrijkerwijs leveren we bewijs voor ‘trogocytosis’ as een mechanisme voor de antilichaam afhankelijke cellulaire cytotoxiciteit door neutrofielen. Trogocytosis (‘trogo’ is Grieks voor knagen) is een proces waarin neutrofielen het plasma membraan antilichaam bedekte kanker cellen mechanisch fragmenteren en daardoor een type van een necrotische cel dood is, voor dit mechanisme stellen wij de term trogoptosis voor.

In hoofdstuk 6 beschrijven we de betrokkenheid van de exocytose van granulen in de anti-microbiologische activiteit van neutrofielen, door te neutrofielen te bestuderen van patiënten...
met familiaire hemofagocytaire lymfohistiocytosis syndroom (FHL). Deze patiënten hebben een gedefinieerde genetische mutatie in de STXBP2/munc18-2 gen. Voorals nog is er geen rol beschreven voor STXBP2 in neutrofielen en voor neutrofielen in FHL in het algemeen. Hier beschrijven we dat FHL5 neutrofielen zwaar beperkt zijn in hun granulen mobilisatie, wat resulteert in inefficiënte doding van bacteriën, in met name Gram-negatieve Escherichia coli, maar niet Staphylococcus aureus. Dit is afhankelijk van een intacte NADPH oxidase activiteit. Deze vermindering in bacterie doding, draagt mogelijk bij aan de vatbaarheid van FHL5 patiënten voor gastro-intestinale onstekingen in FHL5 patiënten.

Concluderend, deze studies beschreven in de dit proefschrift leveren indirect bewijs voor het belang van CD47-SIRPα interacties tijdens de antilichaam behandelingen tegen kanker en zijn in lijn met de algemene concept dat CD47-SIRPα interacties, een barrière vormen tegen antibody gemedieerde tumor cel vernietiging/doding door fagocyten.
CURRICULUM VITAE

Xi Wen Zhao (赵西雯) was born on 22nd of June 1983 at Nanchang, Jiangxiang Province, China. In 2001 she graduated from Nanchang Number 3 High School. In the same year, she enrolled in the Department of Microbiology, College of Agronomy at Southeast Tropical Agriculture University. During bachelor study, she attended the project of genetic polymorphism of Mangeanthracnose (Colletotrichum gloesporioides) and fungicide resistance under supervision of Prof. Gui Xiu Huang. In 2005 she came to Amsterdam to start her master study at Department of Life Science, University of Amsterdam. In 2006 she finished 8 month internship study at the Molecular Biology Department of Nederland Cancer Institute. Later she joined the group of Cees van Kooten at the Department of Nephrology, Leiden University Medical Center for 9 months as the second internship. In May 2008 she started to work on her PhD project under the supervision of Dr. Timo K. van den Berg and Prof. Taco. W. Kuijpers at the Department of Blood cell Research, Sanquin Institute. Recently she receives a post-doc fellowship from the prestigious ERC grant, which she will continue her carrier at the Neurochirurgie Department of VU University Medical Center Amsterdam.

AWARDS

- Chinese Government Award for Outstanding PhD Self-financed Students Abroad, China Scholarship Council, 2013
- Award of the best poster of Sanquin Science Day, 2010
- DELTA Scholarship, Faculty of Science, University of Amsterdam, 2005
LIST OF PUBLICATIONS


- J van der Heijden, S Nagelkerke, XW Zhao, J Geissler, T Rispens, TK van den Berg, TW Kuijpers.


- XW Zhao, HL. Matlung, M van Houdt, TW Kuijpers, TK van den Berg. CD47-SIRPα interactions restrict neutrophil-mediated ADCC and killer synapse formation by limiting CD11b/CD18 integrin activation. *Submitted*.

- XW Zhao, J van der Heijden, S Nagelkerke, J Geissler, M de Boer, TW Kuijpers, TK van den Berg. Genetic variation of Fcγ receptors and SIRPα in neutrophil ADCC towards cancer cells. *Submitted*

- HL. Matlung, XW Zhao, M van Houdt, TW Kuijpers, TK van den Berg. The mechanism of neutrophil-mediated ADCC. *Manuscript in preparation*

*equal contribution
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