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

Zhao, X.W.

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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)\textsuperscript{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)\textsuperscript{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\textsubscript{a} 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\textsubscript{a} in the regulation of phagocyte function. Finally, I will propose CD47-SIRP\textsubscript{a} 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\textsuperscript{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:</td>
<td>HER2: Humanized, IgG1</td>
<td>HER2 positive metastatic breast cancer</td>
<td>Genentech/ Roche</td>
<td>1998 (US) 2000 (EU)</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campath:</td>
<td>CD52: Humanized, IgG1</td>
<td>Chronic lymphocytic leukemia</td>
<td>Genzyme</td>
<td>2001 (US) 2001 (EU)</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zevalin:</td>
<td>CD20: Murine, IgG1, radiolabeled,\textsuperscript{90}Y</td>
<td>Low grade or B cell non-Hodgkin’s lymphoma</td>
<td>Biogen Idec</td>
<td>2002 (US) 2004 (EU)</td>
</tr>
<tr>
<td>Ibritumomab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiuxetan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bexxar:</td>
<td>CD20: Murine, IgG2a, radiolabeled \textsuperscript{111}I</td>
<td>CD20-positive follicular lymphoma</td>
<td>Corixa/GSK</td>
<td>2003 (US)</td>
</tr>
<tr>
<td>Tositumomab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erbitux:</td>
<td>EGFR: Chimeric, IgG1</td>
<td>Metastatic colon cancer and head and neck cancer</td>
<td>Imclone/ Lilly</td>
<td>2004 (US) 2004 (EU)</td>
</tr>
<tr>
<td>Cetuximab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avastin:</td>
<td>VEGF: Humanized, IgG1</td>
<td>Metastatic colon cancer</td>
<td>Genentech/ Roche</td>
<td>2004 (US) 2005 (EU)</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vectibix:</td>
<td>EGFR: Human, IgG2</td>
<td>Metastatic colon cancer</td>
<td>Amgen</td>
<td>2006 (US)</td>
</tr>
<tr>
<td>Panitumumab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arzerra:</td>
<td>CD20: Human IgG1</td>
<td>Chronic lymphocytic leukemia</td>
<td>Genmab</td>
<td>2009 (EU)</td>
</tr>
<tr>
<td>Ofatumumab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yervoy:</td>
<td>CTLA-4: Human IgG1</td>
<td>Melanoma</td>
<td>Seattle Genetics</td>
<td>2011 (US)</td>
</tr>
<tr>
<td>Ipilimumab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perjeta:</td>
<td>Her2: Humanized, IgG1</td>
<td>HER2-positive metastatic breast cancer</td>
<td>Genentech/ Roche</td>
<td>2012 (US)</td>
</tr>
<tr>
<td>Pertuzumab</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kadcyla:</td>
<td>Her2: Humanized, IgG1, Drug-conjugate</td>
<td>HER2-positive metastatic breast cancer</td>
<td>Genentech/ Roche</td>
<td>2013 (US)</td>
</tr>
<tr>
<td>Ado-trastuzumab emtansine</td>
<td></td>
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</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 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](http://www.rcsb.org/pdb/explore.do?structureId=1n8z) (ID: In8z), data published by Cho HS et al.
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 extent the antibody-mediated interference with growth promoting pathways may even help to sensitize the tumors to the cytostatic effects of chemotherapeutic drugs.
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 tumors.

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 Syk. Of relevance, there is genetic variation within the population with respect to FcγR, particularly within the FcγRII/III 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 FcγRIIA and FcγRIIA 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 FcγRIIA 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 FcγRIIA. 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 antibody25,26, 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 hIgG1 and hIgG2a: H131 &gt; R131. R131 can bind mIgG1.</td>
</tr>
<tr>
<td>FcγR2A</td>
<td>Splice site mutation</td>
<td>c.742+871A</td>
<td>c.742+871A&gt;G leads to splice variant FcγRIIA&lt;sup&gt;extremo&lt;/sup&gt;</td>
</tr>
<tr>
<td>FcγR2A</td>
<td>Splice site mutation</td>
<td>c.742+871G</td>
<td></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>FcγR2B</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</td>
<td>ORF 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\(^3\). 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 Fc\(\gamma\)RIIIa (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\(^27\). A SCID xenograft leukemia/lymphoma mouse model also confirmed that NK-cell depletion resulted in diminished efficacy of antibody therapy\(^28\).

NK cells store cytotoxic proteins in secretory lysosomes, also known as lytic or cytotoxic granules\(^29\). 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\(^30;31\), 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\(^32;33\).

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\(^34\). 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 IFN\(\gamma\) 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\(^35-37\). In chapters 5 and 6\(^38\), 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 FcR-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, IgG is the most effective isotype with respect to triggering complement
activation\(^4\), 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\(^6\), 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\(^3\). 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\(^6^4,6^5\).

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\(\gamma\)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\(^6^6\).

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\(^6^7\). 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 \(\gamma\)-chain, by generating activating signals that promote cytotoxicity towards tumor and virus-infected cells\(^6^8,6^9\).

A family of paired immunoreceptors prominently expressed on myeloid cells is the signal-regulatory protein (SIRP) family\(^7^0\). Several SIRP members have been identified in humans, including the closely homologous SIRP\(\alpha\) SIRP\(\beta\)1 and SIRP\(\alpha\), and the more distantly related SIRP\(\beta\)2. SIRP\(\alpha\), which has also been termed Src-homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1), 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. 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). SIRPβ1 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. The natural ligand of SIRPβ1, which at least is not CD47, is still unknown. SIRPα has a short cytoplasmic domain without any (known) motifs or capacity signaling. SIRPα can interact with CD47, albeit with 10-fold lower binding affinity than SIRPβ1. Unlike other SIRP proteins, SIRPα is expressed on T cells and NK cells and CD47-SIRPα interactions may be involved in T-cell trans-endothelial migration.

4.2 The CD47-SIRPα interaction

SIRPα is the best characterized member of the SIRP family (reviewed in ). This inhibitory receptor is primarily expressed myeloid cells and also on neurons. The extracellular region of SIRPα 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, and the structural basis for CD47 recognition by SIRPα is well defined. Because of the characteristic ITIM motifs in the SIRPα cytoplasmic tail, CD47-SIRPα interactions and SIRPα signaling are generally considered to participate in an inhibitory pathway, and this is indeed largely supported by functional experiments. Among the best documented functions of the CD47-SIRPα 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. In line with a negative regulatory role for CD47-SIRPα interactions in the homeostatic clearance of blood cells mice deficient in CD47 or with a defect in SIRPα signaling are mildly anemic and thrombocytopenic and also the antibody-dependent clearance of these cells in models for autoimmune-mediated hemolytic anemia (AIHA) and idiopathic thrombocytopenic purpura (ITP). Other evidence suggests a role for CD47-SIRPα interactions in the control of the phagocyte NADPH oxidase and in osteoclast bone resorption, but the in vivo phenotypes associated with this in mutant mice appear rather mild. Of interest, SIRPα displays considerable polymorphic variation within the human population, and this is also seen amongst inbred strains of mice. While variation occurs specifically in the CD47-binding N-terminal Ig-like domain of SIRPα polymorphic amino acid residues are mostly located outside the critical regions for CD47-SIRPα interaction and do not appear to have a major impact in ligand binding. Nevertheless, CD47-SIRPα 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. 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), non-Hodgkin’s lymphoma, bladder cancer, and several other solid tumor. 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 in vivo. Here, we also examined the effect of targeting the CD47-SIRPα interaction for potentiating the in vitro killing capacity of human phagocytes towards Trastuzumab-opsonized Her2/Neu-positive breast cancer cells. This identified the CD47-SIRPα interaction as a 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.
REFERENCE LIST


