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

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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 IgG 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 Fcγ R 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., 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. 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 a 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. 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. 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 Her2+ 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 outcome. 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.
Figure 1 | Model of neutrophil responses to SIRPα/CD47 blockade and anti-tumor antibody.

**Genetic contributions of SIRPα and FcγR to antibody-dependent cancer cell destruction**

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.11 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 binding16. 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 developed17-19, 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 cancer20-22, 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 synapse, 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 cells.

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

<table>
<thead>
<tr>
<th>Recognition:</th>
<th>NK cell</th>
<th>Neutrophil</th>
<th>Macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signaling pathway(s):</td>
<td>FcR</td>
<td>FcR</td>
<td>FcR</td>
</tr>
<tr>
<td>Killer synapse:</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Cytotoxic mechanism:</td>
<td>perforin &amp; granzyme B</td>
<td>trogocytosis</td>
<td>phagocytosis</td>
</tr>
<tr>
<td>Cell death:</td>
<td>apoptosis</td>
<td>trogoptosis</td>
<td>intracellular degradation</td>
</tr>
<tr>
<td>Control:</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


