Innate immune checkpoint inhibitors for treatment of Diffuse Large B-cell Lymphoma

Bouwstra, Renée

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CHAPTER 7
Summary, discussion and perspectives
Summary

The reactivation of phagocytes, important initiators of anticancer immunity, with checkpoint inhibitors is the main focus of my thesis. Herewith I investigate this type of therapy for treatment of cancer patients, mainly patients with Diffuse Large B-cell Lymphoma (DLBCL). However, several issues remain to be addressed to optimally exploit checkpoint targeting for treatment of DLBCL and I discuss some of these issues with blockade of the prominent CD47-SIRPα innate immune checkpoint axis. In brief, binding of the receptor CD47 to signal regulatory protein alpha (SIRPα) expressed on phagocytes triggers immunoreceptor tyrosine-based inhibitory motif (ITIM)-mediated inhibitory signalling and limits phagocyte activity\(^1\).

Many different types of cancer express CD47 to misuse this mechanism and, thereby, evade immune surveillance. However, as described in chapter 2 of this thesis, although blocking CD47 in patients demonstrated prominent signs of clinical efficacy treatment also associated with significant (and sometimes lethal) toxicities, particularly toward red blood cells (RBCs).

In chapter 2, we summarize the observed toxicity in clinical trials and review strategies to circumvent RBC toxicity. Moreover, we review strategies to increase the efficacy of CD47-SIRPα blocking. For instance, to prevent toxicity triggered by CD47-SIRPα blockade, antibodies with a more tumor-restricted activity profile and tumor-directed bispecific antibodies were developed and the (pre)clinical results thereof were discussed. Further, although CD47-SIRPα blockade is sufficient to trigger phagocytosis as a single agent, the decision to phagocytose depends on the integration of a multitude of pro- and anti-phagocytic signals. Thus, to accomplish an effective tilt in the balance towards anti-tumor immunity in cancer patients, studies have been focused on identifying additional therapeutic signals to combine with CD47 checkpoint blockade. Approximately 90% of the clinical trials with CD47-SIRPα blockade investigate the combination with a variety of additional therapeutic signals. In chapter 2 we aim to summarize this data and provide directions for taking CD47-SIRPα blockade into clinical care.

In chapter 3 we reported on a new strategy to both enhance the safety and efficacy of CD47-SIRPα checkpoint inhibition. Specifically, we developed two SIRPα-based fusion proteins, SIRPα-RTX and RTX-SIRPα. The rationale behind these constructs was to generate CD47 checkpoint inhibitors that only effectively blocked CD47 after CD20-specific binding to cancer cells. We previously published an analogous concept using an anti-CD47 antibody fragment-based fusion protein that showed CD20-restricted activity\(^2\). By exploiting SIRPα as an effector domain we aim to exploit its more restricted binding profile. Moreover, the lack of an Fc domain will eliminate the thrombocytopenic side-effects reported for Fc-based SIRPα fusion proteins \(\text{in vivo}^{3}\). RTX-SIRPα or SIRPα-RTX comprise an N or C-terminal located CD20-targeting scFv antibody fragment derived from rituximab and the extracellular domain of SIRPα. The binding of RTX-SIRPα to CD20/CD47 double-positive cells effectively blocked both CD20 and CD47, whereas CD47 was not blocked on CD47 single-positive cells. In contrast
to RTX-SIRPα, SIRPα-RTX was not capable of blocking the interaction between CD20 and RTX on CD20/CD47 double-positive cells. Nevertheless, single-agent treatment with both fusion proteins triggered phagocytic removal of CD20/CD47 double-positive malignant B-cells and not of CD20-negative/CD47-positive cells. Interestingly, RTX-SIRPα triggered more phagocytosis of neutrophils than SIRPα-RTX (5-20% vs 5-10%). We hypothesized that steric hindrance hampered the functional effect of SIRPα-RTX. In conclusion, RTX-SIRPα is a CD20-directed inhibitor of CD47-SIRPα signalling that triggered phagocytosis by neutrophils and macrophages and may be a safe and tumor-selective checkpoint inhibitor.

In addition to antibody formats and therapeutic strategies, the type or subtype of cancer impacts on the effect of CD47-SIRPα signalling blockade. An example hereof was reported in chapter 4. We identified that high CD47 expression correlated with poor survival after treatment with rituximab in combination with CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) and not single CHOP therapy. More importantly, the negative impact of high CD47 expression was only evident in DLBCL patients of the non-germinal center B-cell (GCB) subtype. Correspondingly, treatment of GCB and non-GCB cell lines with rituximab and CD47 blockade augmented phagocytosis compared to single rituximab treatment only in the non-GCB subtype. Therefore, future clinical trials with CD47 blockade as treatment for DLBCL patients should be aimed at delineating whether the effect in GCB and non-GCB patients indeed is different.

Thus, the impact of high mRNA expression levels of CD47 on survival of GCB and non-GCB DLBCL patients upon R-CHOP treatment differs. This implies that other signals associated with high CD47 expression in non-GCB DLBCL patients likely contributed to their inferior treatment outcome. In chapter 5, we performed a large transcriptomic screen to find such factors/genes. Within this search, membrane protein CD300a was identified as associating with inferior outcome in non-GCB DLBCL patients with high CD47 expression. Interestingly, a deconvolution method that attributes gene expression to specific cell types, clarified that not the cancer cells but innate immune cells such as macrophages and neutrophils expressed CD300a. This pattern was confirmed experimentally with membrane staining and gene expression analysis on primary patient-derived tumor material. CD300a expressed on phagocytes was previously reported to interact with phosphatidylserine (PS) and phosphatidylethanolamine (PE), thereby triggering inhibitory signalling in the phagocytes that inhibit phagocytosis of cells in the process of dying. Likely, the cancer cells in non-GCB patients exploit these mechanisms, next to overexpression of CD47, to evade immune surveillance. Correspondingly, blockade of CD300a on the non-GCB DLBCL cell lines with a CD300a mAb induced phagocytosis and trogocytosis by M2c macrophages and neutrophils, respectively. In retrospective transcriptomic datasets of Uveal melanoma (UM) and mantle cell lymphoma (MCL) patients CD300a also associated with inferior survival in these cancer
types. Further, phagocytosis of MCL and UM cancer cell lines in vitro increased when treated with CD300a blockade. Taken together, the experiments conducted in this chapter identify CD300a as a novel immune checkpoint that may be of potential use as a target for checkpoint inhibition for non-GCB DLBCL, MCL and UM patients.

In chapter 6 a surface antigen, SLAMF7, that was reported to be a requisite for effective CD47 mAb treatment in hematopoietic cancer was investigated. Firstly, the effect of SLAMF7 on survival of DLBCL was investigated in a large retrospective transcriptomic dataset. Surprisingly, the mRNA expression level of SLAMF7 in DLBCL patient samples did not impact on survival of DLBCL patients, whereas CD47 expression levels did. At the protein level, SLAMF7 expression also did not impact on phagocytosis upon treatment with CD47 blockade in vitro. These findings may be of clinical importance since the prior observations suggested that SLAMF7 should be used as selection/exclusion criterion for clinical studies that evaluate the therapeutic potential of CD47-blockade or the combination with CD47 blocking therapy. The data presented in chapter 6 clearly argue against such a role for SLAMF7.
Discussion & Perspective

Restoring the cancer immune cycle with blockade of the CD47-SIRPα signalling axis

At the basis of anti-cancer immunity, innate cells such as macrophages, neutrophils and dendritic cells (DC) phagocytose cancer cell antigens. However, to restore the cancer immune cycle it is pivotal that these phagocytes also process and present antigen on their membrane and thereby activate T-cells. Most of the research conducted on CD47-SIRPα blockade focussed on the direct effect of phagocytosis of cancer cells. However, emerging evidence indicates that T-cells are important for the effect of CD47-SIRPα in vivo, as the effect of CD47 blockade is abrogated in the absence of T cells in preclinical models. Thus the presence of a functional cancer immune cycle is critical for therapeutic effect of CD47-SIRPα in vivo and this was confirmed in several publications. For example, the absence of CD8+ T cells in WT syngenic BALB/c nude mice resulted in completely abrogated therapeutic effect of anti-CD47, whereas depletion of CD4+ T cells had no effect on tumor growth. Moreover, intratumoral injection with CD47 treatment triggered good clinical responses in cutaneous T-cell lymphoma patients (CTCL), with 90% having a reduced Composite Assessment of Index Lesion Severity (CAILS) score. Interestingly, here the tumor micro-environment is composed of malignant and non-malignant T cells, including CD8+ tumor infiltrating T cells as well as DC, macrophages and mast cells and treatment resulted in amplified expansion of NK and CD8+ T-cells and increased IFNα secretion in the tumor micro-environment. Thus, emerging evidence of mouse models and clinical trials indicates that for in vivo CD47 blockade the presence of CD8+ T-cells and/or their capability to migrate into the tumor micro-environment impacts on outcome. Therefore, it will be of importance to determine whether and how T-cells are involved in the therapeutic effect of CD47 blocking in humans, e.g. by defining TCR repertoire after treatment and screening for frequency of tumor-reactivity within the T cell population. If T-cell activation proves important in humans, further combinations tailored towards synergizing innate and adaptive immunity, like the DSP107 and SL-172154 fusion proteins described in chapter 2 may yield significant advance in therapeutic outcome. In this respect it may be interesting to develop a trispecific antibody that targets a T-cell costimulatory domain as CD28 together with CD47 and CD20 that will trigger tumor directed T-cell activation and phagocytosis in CD20+ B-cell lymphomas. Of note, to activate T-cells upon CD47-SIRPα signalling blockade, for any combinatorial treatment strategy the timing and dosing regimen should be carefully calibrated. In this respect, when mice were treated with chemotherapeutic agents (cyclophosphamide and paclitaxel) after CD47 therapy the CD8+ T cell response in vivo was abrogated upon rechallenge of mice with the same tumor cell-line. In contrast, when chemotherapeutics were given one day before CD47 blocking, the blockade of CD47 did not only synergize with chemotherapy for tumor control but also preserved the host memory response against relapsing tumors.
Most likely CD47-SIRPα signalling blockade triggers T-cell activation when, after phagocytosis, tumor antigens are presented on MHC-I or MHC-II by APCs. Interestingly, although macrophages were more potent in uptake of cancer cells, the cross priming of CD8+ T cells mediated by type I interferon (IFN) largely depends on DCs. Correspondingly, the secretion of IFN type 1 was not increased upon in vivo CD47 blockade on macrophages, whereas it was increased by dendritic cells. The mechanism behind this observation might be related to the intracellular processing of tumor antigens in macrophages vs. DCs. Upon CD47 blockade, tumor mitochondrial DNA phagocytosed by macrophages was rapidly degraded, whereas phagocytosis by DCs activated the cGAS-STING-IRF3 mediated cytosolic DNA sensing that is essential for the induction of type I IFNs and correspondingly cross-priming of T cells (reviewed by). Similarly, the effect of CD47 blockade was lymphocytic choriomeningitis virus was dependent on the presence of DC and not on macrophages and required activation of CD8+ T cell responses. Moreover, the CD8+ T cell expansion was not dependent on SIRPα expression on the T-cells themselves, that implies that CD47 mAb link adaptive and innate immune responses through improved APC function. Thus future studies with new formats of CD47 blockade should also investigate their capability to enhance the APC function of DCs as emerging evidence indicates they are critical for CD47 response in vivo.

- Emerging evidence indicates that T-cells and dendritic cells are of critical importance for the therapeutic effect of CD47-SIRPα blockade.
- Future studies with CD47-SIRPα blockade should focus on how T cells influence the therapeutic effect.

The paradigm of GCB and non-GCB DLBCL; impact of lymphoma micro-environment

In chapter 4 and chapter 5 we observed that the expression of 2 checkpoints, CD47 and CD300a, impacted on survival of non-GCB DLBCL patients, but not on GCB DLBCL patients that were treated with R-CHOP. Importantly, the effect of CD47 and CD300a expression on survival of non-GCB was absent when these patients were treated with CHOP chemotherapy. Thus, these checkpoints impact on rituximab treatment. As non-GCB is comprised mainly of ABC and little of unclassified in these statistical analyses the results imply that the immunoregulation in GCB and ABC DLBCL patients is distinct.

The difference in response is likely related to the evolution of GCB and ABC subtypes from different stages of the germinal center reaction. In brief, GCB DLBCL cancer cells arise from the germinal center dark zone where B-cells express B-cell receptors with different affinities for the presented antigen and are densely packed with centroblasts that rapidly divide and
undergo somatic hyper mutations\textsuperscript{17,18}. Thus, GCB DLBCL arise from a part of the germinal center where there are predominantly B cells. ABC DLBCL arises from the light zone, a zone where antigen-dependent signals are delivered and B-cells compete with each other for antigen\textsuperscript{19}. Here, fit cells retrieve antigen presented on follicular dendritic cells and they also interact with T follicular helper cells (TFHs) that help to select B-cells with the highest affinity B-cell receptor\textsuperscript{20}. Thus, ABC DLBCL cancers arise from a part of the germinal center that comprises other immune cell subtypes. This differential immune environment during oncogenesis is likely important in shaping the immune evasion profile of GCB vs. ABC DLBCL. Recently it was published that the lymphoma micro-environment that associates with an inflamed phenotype associates more with ABC/non-GCB subtypes (66\% of the inflamed lymphoma micro-environment)\textsuperscript{21}. Here cancer cells develop and/or select genetic and epigenetic traits that contribute to the evasion of immune microenvironmental constrains\textsuperscript{22}, such as enrichment of immune-suppressive and pro-lymphoma cytokines (CXCL8, neutrophil attractant\textsuperscript{23}, IL-10\textsuperscript{24,25} , PD-L1 and IDO\textsuperscript{26} expression, and increased activation of the NF\textkappa B pathway\textsuperscript{27}. These observations are similar to metastatic colon rectal cancer (mCRC) patient with high CD8\+ infiltration that express a lot of T-cell checkpoint inhibitors to evade immune mediated destruction\textsuperscript{28,29}. Also for melanoma, responsiveness to antigen-specific vaccination administered in combination with system interleukin-2 is predetermined by a tumor microenvironment conducive to immune recognition\textsuperscript{10}. Moreover, the genetic alterations of malignancies interplays with the immune micro-environment of a tumor. For example, in lung cancer, the STK11 and KEAP1 mutations confer worse outcomes to PD-L1 inhibition among patients with KRAS mutant non-small cell lung cancer\textsuperscript{31}. This was also observed in large genomic analyses of the tumor immune interface of 28 different types of cancer and >8000 tumor samples\textsuperscript{32}. Here the genotype of the tumor determined the immunophenotype and tumor escape mechanism. This implies that the interaction with the immune system is predetermined by the genetic basis of the tumors. As DLBCL arises from an area with somatic hypermutation it is not surprising that the different tumor cells that arise hereof have different lymphoma micro-environments (LME). They have been subdivided into distinct subtypes based on transcriptomic analyses\textsuperscript{21} and these subtypes associate with GCB and non-GCB DLBCL subtypes. LME subtypes are composed of distinct cell types where the inflamed subtype was enriched with neutrophils, macrophages, CD8\+ T-cells, more activated NK-cells and the lowest relative number of malignant cells and 66\% of these patients were ABC DLBCL (together with unclassified non-GCB). The germinal center-like LME resembled the constitution of a normal germinal center microenvironment since it contained a relatively high proportion of follicular dendritic cells, lymphatic endothelial cells, total T-cells, several CD4\+ T-cell subpopulations including regulatory T-cells and T-helper cells. Also, the ratio of malignant to normal B-cells was lower in this subtype. Of the germinal center LME, 66\% was defined as GCB DLBCL subtype. Also, higher infiltration of phagocytes
in the inflamed LME that associates with the ABC subtype was in concordance with our observations in chapter 5 where the fraction of total phagocytes was higher in non-GCB patients. Interestingly, for both ABC and GCB DLBCL patients, the microenvironment that is depleted from immune cells had the worst overall survival upon treatment with R-CHOP suggesting that, the microenvironment may provide mechanisms to avoid lymphomagenesis.

Taken together, it is not be surprising that expression of two checkpoints; CD47 and CD300a, impact on survival after R-CHOP treatment of non-GCB (ABC and unclassified subgroup together) as they need immune-suppressive signals to evade of immune microenvironmental constrains whereas GCB does not. Moreover, the distinct LME of GCB and non-GCB may be related to their genetic differences. The tumor micro-environment of DLBCL may be of particular importance for innate checkpoint inhibitors as the phagocytes that respond to these therapies must be present in the tumor tissue in order to engulf them. This was observed in melanoma metastases where responsiveness to antigen-specific vaccination administered in combination with system interleukin-2 is predetermined by a tumor microenvironment conducive to immune recognition30. Thus it is of pivotal importance that innate checkpoint inhibitors are evaluated only in DLBCL patients with an inflamed tumor microenvironment. This is different from CAR-T cell therapy that can travel easily throughout the whole body33 and in the case of second or third generation CARs do not require additional costimulatory domains of other immune cells to kill the tumor cell34. This was confirmed in with tisagenlecleucel, the second CD19 directed CAR-T therapy (with a 4-1BB costimulatory domain) that gave no difference in objective response rate across GCB and ABC DLBCL patients35.

Regarding the selection of DLBCL patients with an inflamed environment the ABC accounts for approximately 66% leaving another 33% for GCB DLBCL patients that also have an inflamed micro-environment. Thus the ABC/GCB COO is not an optimal method to select patients for checkpoint inhibition but does work as a good model system to demonstrate the importance of the interaction between tumors and their immune environment for therapeutic outcome of innate checkpoint inhibitors. To determine patient populations in the clinic that might benefit from CD47-SIRPa blockade and other checkpoint inhibitors it may be better to select those patients that express checkpoint inhibitors such as PD-1/PD-L1, CD300a, LILRB1 and or loss of MHC I or II and have infiltration of macrophages and CD8+ T-cells. This could be analysed with immunohistochemistry or with single cell flow cytometry analyses.

- The immune environment during oncogenesis is of critical importance for the development of checkpoint on the tumor cells.
- The tumor micro-environment may be of particular importance for innate checkpoint inhibitors, as the phagocytes must be in contact with the tumor cells in order to engulf them.
Checkpoints that associated with GCB or non-GCB and their response to CD300a and CD47 blockade \textit{in vitro}

In this thesis we observed that with the use of different non-GCB and GCB DLBCL cell-lines in similar conditions \textit{in vitro} the effect on phagocytosis was different. In brief, we observed in our \textit{in vitro} analyses with allogenic phagocytoses that the effect of CD300a blockade on phagocytosis by neutrophils and macrophages was present with non-GCB cell-lines (U2932, SUDHL2, OCI-LY3) and absent in GCB cell-lines (SUDHL4, SUDHL6 and SUDHL10) (\textit{chapter 5}). Moreover, single treatment with CD47-IgG4 mAb and CD47F(ab)'2 triggered similar response in GCB and non-GCB (\textit{chapter 6}) whereas combination with rituximab only improved phagocytosis of non-GCB cell-lines (\textit{chapter 4}). Thus, the immunoregulatory make-up on GCB and non-GCB DLBCL cell-lines is distinct. The observations correspond with the pattern we observe in transcriptomic data analyses. Together these results have implications for the design of new and interpretation of ongoing clinical trials. In order to design optimal treatment for GCB and non-GCB separately, it seems warranted to further elucidate the background of the differential response to CD47 and CD300a blockade as well as identify other factors that impact on phagocytosis of DLBCL cells.

Many signals have been described to impact on phagocytosis of cancer cells, such as LILRB1\textsuperscript{36}, PD-1/PD-L1\textsuperscript{113} that are anti-phagocytic signals and pro-phagocytic signals such as, PS, PE and calreticulin\textsuperscript{115} (Fig. 1). In particular PS and PE might impact on the above-described observation for CD300a. In \textit{chapter 5} we found that CD300a, expressed on phagocytes only impacts on phagocytosis of non-GCB. However, although the ligands of CD300a, PS and PE were not significantly different expressed on the extracellular domain of GCB and non-GCB, emerging evidence indicates that not all externalized PS is functionally equivalent. For example, two scramblases; TMEM16F and Xkr8 externalize PS by distinct regulatory mechanisms. TMEM16F externalizes PS by Ca\textsuperscript{2+} and Xkr8 is activated by a caspase 3/7-dependent pathway. Interestingly, when a mutant TMEM16F was introduced into a mouse lymphoma cell to achieve constitutive PS exposure, PS-positive tumor cells (assessed as annexin V positive) were not engulfed by professional DCs and only became phagocytosed after activation of caspase 3 and Xkr8 with Fas antibody\textsuperscript{41}. Moreover, a loss-of-function mutation of TMEM30A, a transmembrane protein that maintains asymmetric distribution of PS, did not result in increased PS expression. However, loss-of-function resulted in increased phagocytosis of lymphoma cells in a mouse model, with higher infiltration of M1 macrophages and increased efficacy of CD47 blockade\textsuperscript{42}. Interestingly, this mutation of TMEM30A occurs more often in ABC DLBCL patients\textsuperscript{21}. This suggest that there may be different topologies of PS arranged on the surface of non-GCB and GCB DLBCL cells that engage receptors in distinct ways.
Another interesting signalling axis for DLBCL is the leukocyte immunoglobulin like receptor B (LILRB) 1, a negative regulator of myeloid activation (figure 1). LILRB1 is the most widely distributed member of the LILRB family, with expression on monocytes/macrophages, eosinophils, basophils, dendritic cells, certain NK cells, subsets of T cells, B cells, progenitor mast cells, and osteoclasts. LILRB1 interacts with the β2 microglobulin (β2M) subunit of the MHC I complex. This interaction triggers inhibitory signalling that reduces phagocytic clearance. Correspondingly, deletion of MHC I or LILRB1 potentiated phagocytosis and increased expression of β2M associated with worse survival of DLBCL upon R-CHOP treatment. However, MHC-I and MHC-II are required for T cell recognition by presentation of antigens to T-cells. Downregulation of MHC I has been commonly observed to render tumor cells resistant to T cell elimination. Thus an yet unexplored question is how cancer cells emerge by immunoselection given the pro- and anti-immunogenic role of MHC-I?

Interestingly, loss of HLA is more common in the non-GCB DLBCL patient population. Thus, loss of MHC in non-GCB patients might help the tumor cells to avoid cytotoxic T cells in an inflamed microenvironment and associates with inferior survival upon R-CHOP treatment. Consequently, the LILRB1-MHC I axis might not have a strong impact on phagocytosis on non-GCB patients and may be of more importance for GCB patients. To investigate this the transcriptome data of the DLBCL population could be used to investigate whether there is an effect of LILRB1 and/or MHC expression on GCB CD47 high or non-GCB CD47 high. Simultaneously, the effect of blockade of LILRB1 on phagocytosis of GCB and non-GCB cell lines in vitro can be investigated.

MYC is a oncogenic driver that codes for a transcription factor and associates with BCL-2 expression in DLBCL. The co-expression of these two genes associates with non-GCB DLBCL patients (35.9% GCB, 64.1% non-GCB). In pre-clinical mouse models the suppression of MYC in both human and mouse tumor cells reduced the expression levels of CD47 and PD-L1. MYC regulates the expression through binding of the promotor region of the genes encoding CD47/PD-L1. However, other regulators of CD47 expression include the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, that is more often activated in ABC DLBCL. As the analyses in chapter 4 and chapter 5 were performed with GCB and non-GCB patients with similar CD47 expression levels, it may not be the expression but the regulation of the transcription that impact on the survival and therapeutic effect of CD47 blockade.
Another interesting signalling protein is CD24, a heavily glycosylated GPI-anchored surface protein, known to interact with siglec-10 on innate immune cells. This interaction elicits an inhibitory signalling cascade in macrophages. CD24 is overexpressed by cancer cells as a mechanism to evade immnosurveillance. Further, siglec-10 is upregulated by cytokines as IL-10 and TGFβ present in the TME. Blockade of CD24 can increase phagocytosis of cancer cells by siglec 10+ macrophages. Thus, CD24 acts as a checkpoint for tumor cells. Interestingly, DLBCL patients seem to either express CD24 or CD47 with a minority expressing both (10/48 patients). Moreover, CD24+ lymphoma B-cells were well phagocytosed by CD24.
Chapter 7

blockade\textsuperscript{61}. This opens up the possibility that some tumors of patients might respond better to CD47 blockade, whereas others might respond better to CD24 blockade\textsuperscript{61}. Therefore, the determination of the collective pro- and anti-phagocytic signals expressed on DLBCL and associated macrophages could help to predict optimal treatment strategy. Perhaps the effect of CD47 blockade on GCB patients is hampered by expression of CD24.

In this thesis, we used mRNA data of bulk tumor biopsies to detect expression levels of CD47 and CD300a. Using deconvolution methods such as CIBERSORT\textsuperscript{x} and Quantiseq immune infiltrates within the tumor were detected and quantified. However, these methods can only estimate, though with good accuracy, the immune infiltration and expression levels of checkpoint inhibitors\textsuperscript{62,63}. To improve accuracy, it would be of interest to perform single cell RNA sequencing and determine checkpoint inhibitors on a single cell level. For example droplet barcoding is an interesting technique to detect whole transcriptome scRNA-seq that requires small amounts of tumor samples that can be used in routine setting\textsuperscript{64}. However, both these methods measure mostly mRNA that is not fully representative for protein levels as mRNA undergoes translation and post translational modifications. The integrative analyses of transcriptome and proteome data in single cells would be the ideal analysis of DLBCL tumor tissue. An interesting technique to perform this type of analyses is cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq)\textsuperscript{65}. Here antibodies that are DNA-barcoded are mixed with tumor bulk tissue and single cell in combination with one bead are encapsulated in one droplet. The amplified antibody derived tags and cDNA molecules can be separated by size that allows the evaluation of the membrane proteins and mRNA separately\textsuperscript{65}. Using CITE-seq analyses of a large number of tumor tissues may be the most optimal method to find markers that define the optimal treatment for each patient.

- As the effect of similar expression levels of CD300a and CD47 is different in GCB and non-GCB there may be other immunoregulatory proteins involved that impact on this observation.
- To analyse the expression patterns of these immunoregulatory proteins it might be interesting to use CITE-seq analyses for DLBCL tumor biopsies.

Dual targeting strategies that block CD47-SIRP\textsubscript{\textalpha} immunotherapy

Next to patient selection for innate checkpoint inhibition, therapeutic effect can also be improved by design of therapeutic formats and combinatory strategies as discussed in chapter 2. Particularly, the CD47-SIRP\textsubscript{\textalpha} signalling axis is a prominent checkpoint for innate immune cells and hijacked by many types of cancer to circumvent immune surveillance. However, the applicability of CD47 checkpoint inhibitors is clearly hampered by side-effects such as
anaemia and thrombocytopenia. The use of bispecific tumor-directed antibodies is perhaps one of the most promising approaches to not only increase tumor-selective activity, but also to reduce possible toxicity. An important feature of bsAbs is higher avidity to dual antigen-expressing cells relative to single antigen-expressing cells\(^{66}\). Thus, to create this higher avidity of bsAbs it is important that both arms bind to the tumor cell. This was described in chapter 3 where the effect of SIRP\(\alpha\)-RTX on neutrophil mediated phagocytosis was less as compared to RTX-SIRP\(\alpha\) bsAbs. This was likely due to the weaker interaction with CD20 as SIRP\(\alpha\)-RTX could not block the interaction between CD20 and rituximab. In actual fact, for an ideal tumor-restricted pattern it would be of interest to lower the affinity of SIRP\(\alpha\) to steer the dependence of binding to CD20. In this respect, Weiskopf et al. developed different SIRP\(\alpha\) sequences with mutations that lead to increased affinity of SIRP\(\alpha\)\(^{67}\), thereby demonstrating that steering the affinity is possible. Notably, the effect size of both RTX-SIRP\(\alpha\) and SIRP\(\alpha\)-RTX already correlated with CD20 expression, with higher CD20 expression triggering more phagocytosis.

A important remark to such target antigen dependency is the fact that the expression of CD20 in DLBCL patients is variable, with some patients having “dim” CD20 expression\(^{68}\). Further, expression of CD20 is at least reduced in approximately 11% of DLBCL patients upon relapse\(^{69}\). These patients have inferior survival upon R-CHOP treatment\(^{70}\) and might either be refractory to CD47-blocking therapy by RTX-SIRP\(\alpha\) or reversely, the additional CD47-blocking by RTX-SIRP\(\alpha\) might tip the balance towards removal. Thus, to determine which patients will benefit from treatment with RTX-SIRP\(\alpha\) it will be important to delineate the expression level cut-off needed \textit{in vitro} and possibly in murine models for optimal treatment effect. This information could be used to stratify patients for CD20 directed and combination treatment. To circumvent inhibitory effects of low CD20 expression, development of an analogous anti-CD19-SIRP\(\alpha\) fusion protein is of interest. CD19 is another well-known B cell antigen and has been used in the clinic to direct CAR T cells\(^{71}\) and has been reported as target in a CD19xCD47 bispecific antibody\(^{72}\). A CD19-targeted SIRP\(\alpha\) fusion protein would be expected to also synergize RTX-mediated phagocytosis without the possibility of competitive inhibition and would be of particular interest for patients with dim CD20 expression that relapsed or are refractory DLBCL patients.

The expression levels of CD20 are also important when RTX-SIRP\(\alpha\) treatment is combined with rituximab as these therapeutics target the same epitope. This may create competitive inhibition for CD20 particularly in case of saturating doses of rituximab in patients. This was in concordance with our observations \textit{in vitro} where treatment of SUDHL6 (cell line with lower CD20 expression than U2932) with rituximab + RTX-SIRP\(\alpha\) did not synergize to increase phagocytosis whereas similar treatment in U2932 did synergize. In contrast combination with CD19 minibody did enhance phagocytosis of SUDHL6 \textit{in vitro}. Thus it would be of interest to combine RTX-SIRP\(\alpha\) with CD19 directed antibodies or with CD20 directed antibody ofatumumab or obinutuzumab that target a different epitope of CD20 than rituximab\(^{73,74}\).
In the research described in chapter 6, we observed that CD47 F(ab')2 triggered an increase in macrophage-mediated phagocytosis of lymphoma cell lines. Thus, the effect of blockade of CD47-SIRPα signalling on phagocytosis is not due to FcR-opsonization. In literature, the effect of CD47 based therapeutics in the absence of an functional Fc domain have been described extensively, with several IgG4 based antibodies\(^{75,76}\) and CD47 F(ab')2\(^{77,78}\) preparations yielding phagocytosis. For SIRPα-based fusion proteins, TTI-622 (SIRPα fused to an IgG4) demonstrated that Fc-opsonization is not required to trigger phagocytosis. In line with this, Dual signalling protein (DSP)-107 (composed of the extracellular domains of 4-1BB ligand and SIRPα) was able to block the interaction between CD47 and phagocyte-expressed SIRPα and, thereby, increased phagocytosis of cancer cells as single agent\(^79\). Interestingly, treatment of breast cancer cell line with CD47F(ab')2 required the addition of Fc-R opsonization to trigger phagocytosis of cancer cells\(^80\). Moreover, Fab fragments of high affinity SIRPα variants did trigger phagocytosis of Raji (B-cells) and did not trigger phagocytosis of DLD_1 (colon cell line)\(^67\). Taken together, the above described articles demonstrate that B-cell lymphoma cell-lines are phagocytosed upon CD47 blockade in the absence of FcR opsonization whereas for other types of cancer e.g. colon cancer and breast cancer single blockade of CD47 did not trigger phagocytosis.

However, whether the effect of blockade of CD47-SIRPα signalling on phagocytosis will be enough to cure patients remains to be seen. Based on clinical data of Hu5F9-G4 or TTI-622 that are CD47-IgG4 and SIRPα-IgG4 respectively, toxicity was manageable\(^81,82\). However single treatment with Hu5F9-G4 triggered partial response in 2 out of 62 treated solid cancer\(^81\), with no data available on single agent treatment of lymphoma patients. TTI-622 triggered 20% objective response in relapsed/refractory DLBCL\(^82\). Thus taken together, additional “eat me” signalling may be required in vivo to trigger sufficient activation of phagocytes to kill tumor cells and cure patients. These “eat me” signals can be obtained with combinatory strategies as discussed in chapter 2. Most successful are those strategies that increase tumor specific FcR crosslinking or upregulation of calreticulin, PS and many more “eat me” signals on the membrane of cancer cells.

As neutrophils are the most abundant leukocytes, representing 60% of the whole leukocyte population they are an interesting phagocyte to activate with immunotherapy. However, most of the tumor-directed CD47-SIRPα blockade was evaluated in vitro on their therapeutic ability to trigger macrophage-mediated phagocytosis. In this thesis, we demonstrated that CD47-SIRPα blockade can also potentiate neutrophil activity in vitro, which corresponds with our findings on the RTX-CD47 fusion protein\(^2\). Additional, Zhao et al. demonstrate that the FcR-opsonization of neutrophils by trastuzumab was enhanced by anti-CD47 F(ab')2\(^80\). Moreover, as they are the most abundant leukocytes, they might have a major part in the therapeutic effect of CD47-SIRPα in vivo. Thus, it would be of interest to improve the anti-cancer response.
of neutrophils to CD47-SIRPα blockade. Neutrophil activity can entail both neutrophil-mediated phagocytosis, i.e. a whole tumor cell is eaten, and trogocytosis, i.e. “bites” are taken out of a cancer cell membrane resulting in loss of membrane integrity and cell death\(^83\). In the tumor micro-environment neutrophils have both anti- and pro- tumoral activity. They can mediate tumor progression via the promotion of proliferation, angiogenesis, invasion and metastasis and limit activity of T-cells. Anti-tumoral properties include the direct tumor killing trough release of \(\text{H}_2\text{O}_2\), Fas interaction and comple ment dependent cytotoxicity (CDC), antibody directed cellular cytotoxicity (ADCC) and antibody directed cellular phagocytosis (ADCP) (reviewed by\(^84,85\)). Moreover, it was reported that neutrophil mediated trogocytosis decreased the level of antibodies bound to receptors and more importantly, did not trigger upregulation of 7-AAD, a late apoptosis marker, suggesting that B-cell lymphoma cell lines are not killed by trogocytosis\(^86\). Recently a pre-clinical study with B cell lymphoma antibody-dependent neutrophil trogocytosis of B-cell lymphoma cells can be potentiated through combination of SSG (sodium stibogluconate) that was reported to inhibit the tyrosine phosphatase SHP-1\(^87\). Interestingly, SSG potenti ates trogocytosis of neutrophils through interaction with the FcγRI and not FcγRIIa\(^87\). Similar observation of increased potentiation through FcγRI interaction on neutrophils were done with Fc-domains of therapeut ic mAbs.

IgA Fc-domains interact through FcγRI and are thereby likely superior compared to IgG Fc-domains for the exploitation of neutrophil anticancer activity. Whereas IgA trigger activation of four immunoreceptor Trosine-based Activating motifs (ITAMs) via FcαRI and FcγRI, IgG Fc-domains trigger single ITAM activation upon FcγRIIa\(^88\). This was confirmed with several anti-cancer mAbs as neutrophils are superior in eliminating IgA-opsonized tumor cells compared to IgG using EGFR, CD20, Her2, HLA II as targets\(^89–92\). Thus, in order to use the full potential of neutrophil-mediated anti-cancer immunity it seems warranted to activate the FcγRI on neutrophils. The above-described paper demonstrates that the combination with SSG can accomplish that in vitro. However, as both SSG and CD47-SIRPα are not tumor-selective, there might be severe toxicity in humans or in murine models that can mimic the CD47 sink in humans. Therefore, it would be interesting to develop tumor directed blockade of CD47-SIRPα with simul atious activation of the FcγRI receptor on neutrophils through an IgA Fc-domain. One of the disadvantages of IgA Fc domains is their relatively low half life compared to IgG due to lack of binding to neonatal Fc receptor (FcRn)\(^93\). Several modification can increase the half life such as development of aglycosylated IgA molecules\(^94\), inducing binding to the FcRn via protein engineering\(^95\) or IgG-IgA fusion proteins\(^96\). To circumvent toxicity by the strong neutrophil opsonization and off-target activity towards CD47 expressing cells, the use of a tumor-selective CD47 or a tumor-directed bispecific protein directed towards an antigen such as CD19 on malignant B-cells. Thus, although RTX-SIRPα in the absence of an Fc-domain can trigger phagocytosis by neutrophils, the effect on phagocytosis may be improved by developing an IgA based bispecific protein targeting CD20 and CD47.
In order to develop a tumor-directed bispecific fusion protein or antibody it is important that the target antigen is truly tumor specific. However, for many types of cancer a truly tumor-specific antigen does not exist, meaning such bispecific antibodies are also directed towards healthy target antigen-expressing cell types with high avidity, such as healthy epithelial cells upon EGFR targeting or cardiomyocytes upon Her2 targeting. Moreover, epithelial cell adhesion molecules (Epcam) directed bispecific T-cell engager precluded dose escalation due to dose limiting toxicities97. This might unmask additional toxicity issues not previously encountered with CD47-based therapeutics. For B cell lymphoma, neurotoxicity was present upon CD19 based CAR T-cell98 and BITE99 therapy. However, the neurotoxicity associated with appearance of the cytokine release syndrome that may change the blood brain barrier that allows CAR T-cell to infiltrate10. Thus the toxicity of CAR T-cells may not be directly related to CD19 expression in the brain101. Therefore, it is expected that such toxicity issues as well as depletion of healthy B cells are of less concern than for EGFR or Epcam. Furthermore, targeting of CD47 to a particular tumor-associated antigen carries an inherent risk in often heterogenous cancers. Indeed, many models used to validate bispecific antibody formats employ xenografted tumor cell-lines that do not represent the heterogenous make-up of tumors in patients. For example, in AML several distinct clones can co-exist within one patient, with distinct membrane receptor-expression profiles102. Furthermore, therapy can change the clonality and drive resistance to targeted therapy103. Indeed, in DLBCL CD20 expression is an independent risk factor for worse prognosis of DLBCL patients104. The phenotypic transformation of decreased CD20 expression upon rituximab treatment was observed in patients and in B-lymphoma cell lines and may be related to down-regulation of the CD20 gene, epigenetic mechanisms and TrkB signalling105–108. Overcoming such heterogeneity in cancer using a CD47-based bispecific antibody might be achieved by use of an anti-tag approach, such as targeting of biotin or the fluorescent label FITC, as previously demonstrated by us for targeted activation of TNFR superfamily signalling109.

- To further improve bispecific fusion proteins or antibodies the impact of differences in expression levels of both targets should be evaluated.
- Changing the Fc domain of current immunotherapeutics my improve neutrophil mediated anti-tumor effects
- therapy can change the clonality and drive resistance to targeted therapy
Conclusions

Taken together the data presented in the chapters of this thesis the effect of checkpoint inhibitors, such as therapeutics that block the CD47-SIRPα signalling, require a shift in balance toward phagocytosis by additional stimuli. Additionally, checkpoint inhibitors require the selection of DLBCL patient that have a tumor micro-environment that will benefit most from these therapies. Although the GCB/non-GCB model mirrors the difference between a suitable immune micro-environment and one that is not. This is most likely a suboptimal patient selection method. Therefore it is pivotal to develop techniques that enable the distinction of these patients.
REFERENCES


Summary, discussion and perspectives


CHAPTER 7


