CD47-SIRPα Innate Immune Checkpoint on Neutrophils

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Immunotherapy aims to engage various immune cells in the elimination of cancer cells. Neutrophils are the most abundant leukocytes in the circulation and have unique mechanisms by which they can kill cancer cells opsonized by antibodies. However, neutrophil effector functions are limited by the inhibitory receptor SIRPα, when it interacts with CD47. The CD47 protein is expressed on all cells in the body and acts as a 'don't eat me' signal to prevent tissue damage. Cancer cells can express high levels of CD47 to circumvent tumor elimination. Thus, blocking the interaction between CD47 and SIRPα may enhance anti-tumor effects by neutrophils in the presence of tumor-targeting monoclonal antibodies. Blocking the CD47-SIRPα interaction may therefore potentiate neutrophil-mediated antibody-dependent cellular cytotoxicity (ADCC) towards cancer cells, and various inhibitors of the CD47-SIRPα axis are now in clinical studies.

tumor antibody therapy neutrophil CD47-SIRPα immune checkpoint

1. CD47-SIRPα as an Innate Immune Checkpoint in Neutrophil-Mediated Tumor Killing

The SIRP family is a multigene family consisting of five members: SIRPα, SIRPβ1, SIRPβ2, SIRPγ and SIRPδ in humans[1]. SIRPα (also known as CD172a, SHPS-1, p84, MFR, MYD-1 or PTPNS1) is an inhibitory receptor expressed on myeloid cells, including macrophages, neutrophils and myeloid dendritic cells, as well as on neuronal cells in the central nervous system[2]. The protein contains three extracellular immunoglobulin (Ig) superfamily (IgSF) domains, consisting of one V-type IgSF (IgV) domain and two C1-type IgSF (IgC) domains, one transmembrane region and an intracellular tail capable of inhibitory signaling (**Figure 1**)[3]. The intracellular tail contains four tyrosine residues, forming two typical immunoreceptor tyrosine-based inhibitory motifs (ITIM). In addition, the extracellular IgV-domain contains a ligand-binding region, allowing SIRPα to interact with its ligand, CD47[4].

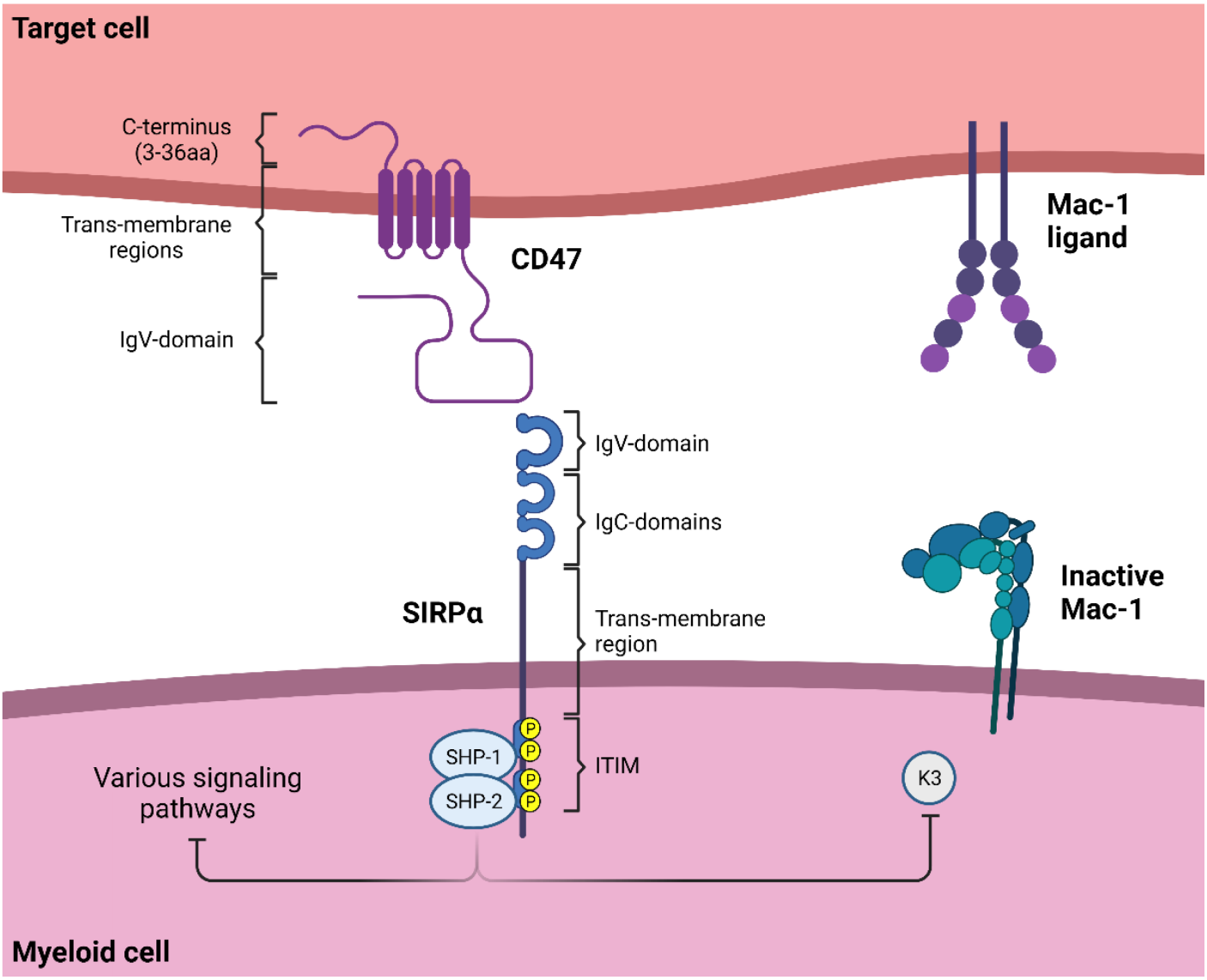

Figure 1. The CD47-SIRPα axis. Interaction between the IgV-domain of CD47 and the IgV-domain of SIRPα results in phosphorylation of the two ITIMs in the intracellular SIRPα tail. As a consequence, the phosphatases SHP-1 and SHP-2 are recruited, which are subsequently activated and able to regulate downstream cellular signaling pathways, e.g., FcR or TLR signaling, by tyrosine dephosphorylation of various mediators. In addition, neutrophil Mac-1 activation is inhibited in a Kindlin3-dependent manner. Abbreviations: K3: Kindlin3. Created with Biorender.com.

The CD47 protein (also known as IAP, MER6 or OA3) is a transmembrane glycoprotein expressed on virtually all cells in the body, including both hematopoietic and non-hematopoietic cells[5]. It is a member of the Ig superfamily, and consists of an extracellular IgV-like domain at the N-terminus, a region with five membrane-spanning segments, and a cytoplasmic C-terminus ranging from 3–36 amino acids[6]. CD47 was identified independently on different cell types, resulting in different nomenclature. It was first described as integrin-associated protein (IAP), as it was shown to associate with integrins, e.g., $\alpha_v\beta_3$, on various cell types[7]. In addition, CD47 was identified as OA3, an antigen overexpressed on ovarian carcinoma cells[8]. As it is now clear that this molecule is expressed on

various cell types, and can interact with different proteins, including integrins, thrombospondins (TSP), VEGFR and SIRPs, the current consensus is to refer to it as CD47[6][9].

The interaction between CD47 and SIRPα was first described in 1999 in mice[10]. Using SIRPα-expressing murine brain cells, CD47 was identified as a binding partner of SIRPα. This was confirmed by anti-CD47 mAbs, which blocked the attachment of various cells to SIRPα-coated substrates[10]. Subsequently, CD47 was also recognized as a ligand for SIRPα in humans[11]. Similarly, in rats a CD47-targeting mAb was identified to prevent the adherence of SIRPα-coated beads[12]. The interaction between CD47 and SIRPα has been analyzed in detail with high-resolution X-ray crystallography and mutagenesis studies[13]. The N-terminal end of SIRPα domain 1 (IgV-domain) consists of four loops, which all contribute to binding of CD47. The N-terminal end of CD47 also forms loops, which are needed for the interaction between CD47 and SIRPα. In addition, CD47 contains a pyroglutamate at the N-terminal, that plays a significant role in the interaction[13][14].

In humans, two allelic variants of SIRPα have been identified: $SIRP\alpha_1$ and $SIRP\alpha_{BIT}$[15]. Within a healthy Caucasian population, $SIRP\alpha_{BIT}$ and $SIRP\alpha_1$ homozygotes represent 15.9 and 48.7% of the population, respectively, with 35.4% heterozygous for $SIRP\alpha_1/SIRP\alpha_{BIT}$[15]. These variants differ by as much as 13 amino acid residues in the IgV domain responsible for CD47 binding. However, no differences in CD47 binding were observed between $SIRP\alpha_1$ and $SIRP\alpha_{BIT}$[15][16][17]. This may not be surprising, as the polymorphisms occur primarily outside the CD47 binding site[15]. Whereas these polymorphisms could in principle still have an effect on downstream signaling capacities and thereby affect neutrophil effector functions, such as ADCC, no differences were observed in neutrophil-mediated ADCC of trastuzumab-coated SKBR3 cancer cells between neutrophils from donors with the three different genotypes[15]. Thus, it appears that neutrophil ADCC is not affected by the SIRPα genotype.

1.1. SIRPα Signaling

To investigate the mechanism of CD47-SIRPα signaling, the immunological synapse between target cells and neutrophils was investigated. It was already established that Mac-1 is essential for the formation of this synapse[18]. However, whether and how SIRPα signaling affects the formation or maintenance of the synapse was not yet clear. During the formation of effector-target interactions, CD47 and SIRPα are both present in the immunological synapse, as SIRPα translocates to the synapse in the presence of CD47, while it is excluded from the synapse in the absence of CD47[19][20]. Cell–cell contacts between neutrophils and CD47-expressing or CD47-deficient SKBR3 cells were analyzed and indicated that disruption of the CD47-SIRPα axis resulted in the promotion of neutrophil–tumor cell interactions in the presence of tumor-targeting antibodies[21].

After ligation by CD47, ITIM motifs in the cytoplasmic tail of SIRPα are phosphorylated, most likely by Src family kinases. This leads to recruitment of tyrosine phosphatases, in particular Src homology region 2 (SH2)-domain-containing phosphatase-1 (SHP-1) and -2 (SHP-2), which are considered to be principal mediators of SIRPα inhibitory signaling (**Figure 1**)[22][23]. After recruitment of SHP-1 and SHP-2 to SIRPα, these phosphates undergo conformational changes, allowing them to become activated[24]. The phosphatases can subsequently dephosphorylate various downstream substrates, thereby regulating pivotal intracellular signaling pathways, such

as FcR and TLR signaling[21][25][26]. Therefore, different effector functions can be regulated by inhibitory signaling through CD47-SIRPα interactions. In addition, SIRPα might associate with the inhibitory protein kinase Csk and the adaptor protein Grb-2[27], but the role of these molecules in neutrophil killing has not been explored.

A similar mechanism might affect effector functions of other SIRPα-expressing cells, such as ADCP by macrophages. The interaction of CD47 with SIRPα on macrophages led to suppressed integrin activation and reduced the spreading and engulfment of mAb-opsonized beads[19]. In addition to myeloid cells, SIRPα is also expressed on B1 lymphocytes, a subtype of murine B cells, which produce natural antibodies[28]. Using transgenic mice which lack the intracellular domain of SIRPα and therefore have defective SIRPα signaling, it was observed that B1 cells produce more antibodies when SIRPα signaling is disrupted. In addition, these SIRPα-mutant B1 cells displayed enhanced Mac-1 integrin-dependent migration[28]. Taken together, these studies demonstrate that blocking SIRPα can enhance various Mac-1 integrin-dependent cellular functions, including cytotoxicity and migration, and suggest that the function of the CD47-SIRPα checkpoint may be intimately linked to that of Mac-1.

1.2. Neutrophil Effector Functions Influenced by CD47-SIRPα

Neutrophils have various effector functions essential for their role in immunity. As SIRPα signaling can regulate various signaling pathways, different effector functions may be influenced by the CD47-SIRPα interaction. In the 1990s, it was suggested that CD47 may play a role in neutrophil transmigration[29][30][31]. More recently, signaling via the CD47-SIRPα axis has been demonstrated to regulate neutrophil-mediated cytotoxicity[32].

Anti-CD47 mAbs did not disrupt neutrophil adhesion to epithelial cells, indicating that CD47 may affect the transmigration step of neutrophils across the epithelial layer[30]. This effect was mediated by tyrosine kinases, such as Src family kinases and Syk tyrosine kinases, as specific inhibition reverted the effect of anti-CD47 mAbs[33][34]. In vivo studies with CD47-deficient mice suggested a prominent defect in neutrophil extravasation leading to a lethal defect in the clearance of pathogenic bacteria. While this defect in neutrophil migration was clearly linked to β3-integrin function, it is not known whether CD47 was primarily required for pathogen recognition by neutrophils or for the actual migration process itself[31]. CD47 has a variety of well-established binding partners, including integrins, TSP-1, VEGFR and SIRPs. Therefore, it was investigated as to what extend CD47-SIRPα interactions are regulating trans-endothelial/epithelial migration. Anti-SIRP mAbs inhibited neutrophil transmigration across an epithelial monolayer and collagen-coated filters, albeit with different kinetics when compared to anti-CD47 mAbs[35]. Blocking the CD47-SIRPα interaction with a function-blocking peptide, which binds to the CD47 binding domain of SIRPα, resulted in inhibited neutrophil transepithelial migration in vitro[36]. Of note, questions with respect to the specificity of the peptide for CD47-SIRPα interactions can be raised. However, SIRPα-mutant mouse neutrophils, lacking the cytoplasmic region of SIRPα, transmigrated significantly less in vitro when compared to wild type (WT) neutrophils in response to the chemoattractant C5a[37]. In vivo, transmigration of these SIRPα-mutant neutrophils was also slightly delayed when compared to WT neutrophils[37]. This demonstrates that signaling via the intracellular tail of SIRPα may, at least to some extent, controls neutrophil transmigration. Nonetheless, it remains difficult to anticipate how much of an effect SIRPα signaling may have on the overall

accumulation of neutrophils in tissues, including tumors, even though such migration may be clearly affected by CD47-targeting agents.

In addition to an effect on neutrophil transmigration, the CD47-SIRPα axis also regulates neutrophil cytotoxicity. Pioneering studies reported by Oldenborg et al. showed that CD47 restricted the clearing of red blood cells (RBC), suggesting that the broadly expressed CD47 functions as a signal of 'self' to control the elimination of normal cells by the immune system[38]. In particular, it was found that CD47-deficient RBCs were rapidly cleared, within hours, after their infusion into healthy recipient mice due to phagocytosis by macrophages[38]. For comparison, normal CD47-expressing RBC have a lifespan of 45 days in mice. Thus, it became clear that CD47 essentially functions as a 'don't eat me' signal. It was established in subsequent studies that SIRPα was the inhibitory receptor limiting the phagocytosis of CD47-expressing erythrocytes and that CD47-SIRPα interactions were also restricting phagocytosis and clearance of IgG- or complement- opsonized erythrocytes[23][39]. It should be noted that not only macrophages but also neutrophils are able to eliminate IgG-opsonized RBCs, at least in vitro, and this process is also enhanced after blocking CD47-SIRPα[32]. This principle extends beyond red blood cells, and has now been observed for platelets and other hematopoietic cells, as well as non-hematopoietic cells[40][41][42][43][44][45]. In line with this, the lack of species compatibility between CD47-SIRPα is an important hurdle for xenotransplantation, and, inversely, an exaggerated binding of human CD47 to NOD SIRPα was found to be responsible for the superior engraftment of human tissues in immunodeficient mice in a NOD background[44][46][47]. These findings firmly established the role of the CD47-SIRPα axis in the clearance of normal cells, and also inspired the initial studies to demonstrate its role as an innate immune checkpoint in the antibody-dependent destruction of cancer cells by macrophages and neutrophils[17][48].

1.3. The Innate Immune Checkpoint CD47-SIRPα in Cancer

In the clinic, high CD47 expression has been correlated with a worse prognosis of patients with non-small cell lung cancer (NSCLC)[49]. Interactions between CD47 on tumor cells and SIRPα on neutrophils inhibit neutrophil effector functions, allowing the tumor to escape immune surveillance (**Figure 2**A)[48][50]. Therefore, targeting the innate immune checkpoint CD47-SIRPα could be a potential way to improve current antibody therapies, as it can stimulate neutrophil-mediated tumor killing (**Figure 2**B). Blocking CD47-SIRPα can be established by various methods: i.e., anti-CD47 mAbs, anti-SIRPα mAbs, or alternative ways, e.g., by downregulating CD47 or by affecting the SIRPα binding site. In vitro, macrophages can eliminate various opsonized solid[51][52][53][54][55][56][57] and hematological cancer[48][58][59][60] cell types via ADCP, which can be further promoted by treatment with anti-CD47 mAbs. Similarly, anti-CD47 mAbs enhance neutrophil-mediated ADCC of solid cancers in vitro, such as neuroblastoma[61]. However, it appears that neutrophils are less capable of killing hematologic cancer cells, and blockade of the CD47-SIRPα axis with anti-CD47 mAbs is not enough to promote tumor elimination. For example, neutrophils were unable to eliminate rituximab-opsonized B cell lymphoma cells[58]. Even when the CD47-SIRPα axis was disrupted using anti-CD47 Fab fragments, neutrophil-mediated ADCC was not improved, although tumor cell elimination was significantly increased when combined with sodium stibogluconate (SSG; an alleged inhibitor of SHP-1)[58]. It is important to note that some anti-CD47 antibodies can by themselves opsonize tumor cells, depending on their ability to still bind Fc-receptors, and hence act as a two-edged sword, i.e., by opsonizing tumor

cells for phagocytosis and simultaneously inhibiting CD47-SIRPα interactions. In some cases, it therefore appears that anti-CD47 antibodies were sufficient for killing by myeloid cells, without the need of additional anti-TAA mAbs[53][59][62][63]. Since CD47 is broadly expressed on normal cells, it is highly undesirable to therapeutically use an anti-CD47 antibody with a functional Fc tail, as this would also trigger effector responses against the patient's healthy cells. As an alternative to anti-CD47 antibodies, anti-SIRPα antibodies have also been studied for their ability to promote tumor elimination. By targeting SIRPα, neutrophil-mediated ADCC of various opsonized cancer cells, such as breast cancer[21], neuroblastoma[61], and colorectal adenocarcinoma[64][65] was promoted.

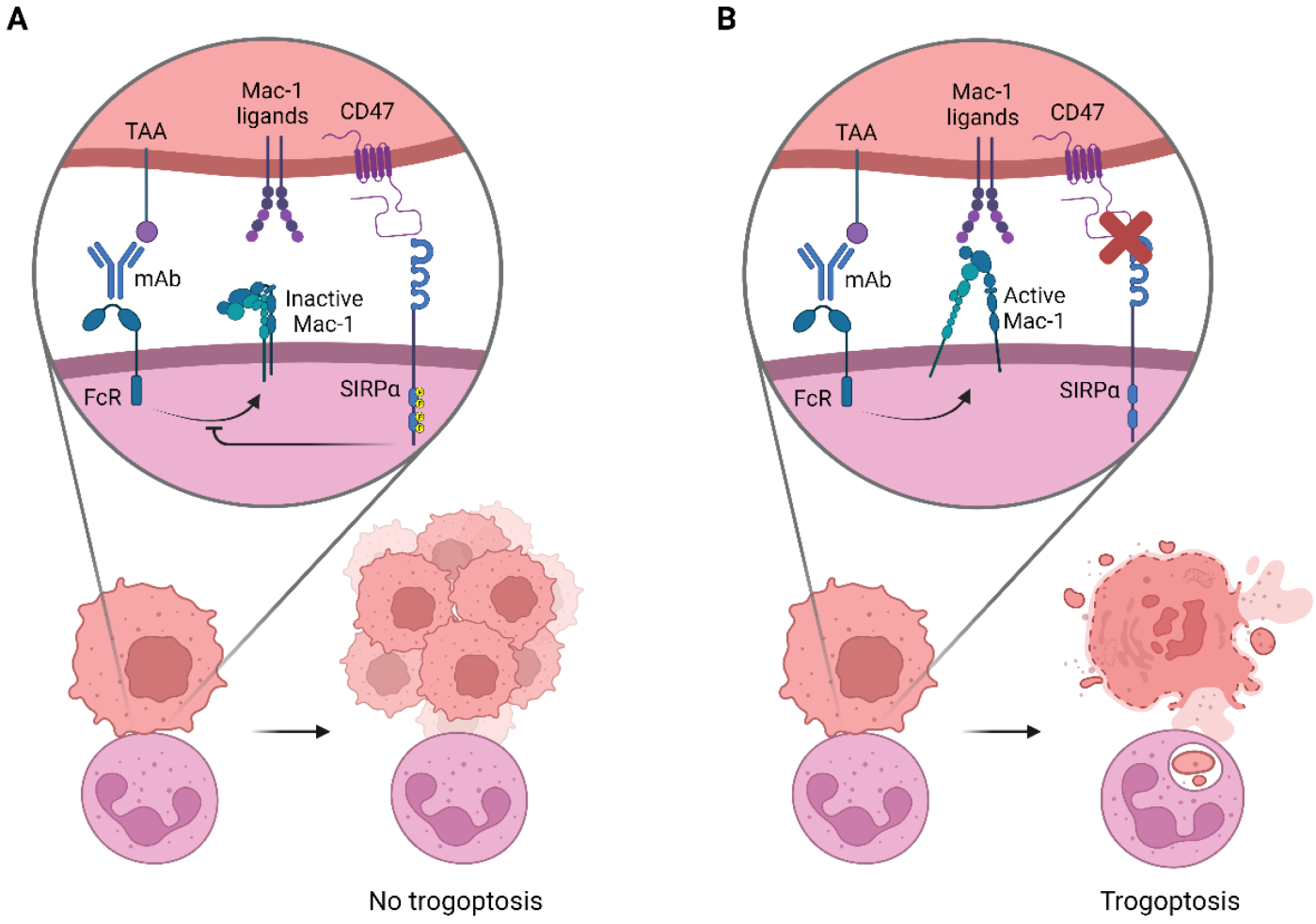

Figure 2. CD47-SIRPα signaling prevents neutrophil-mediated tumor cell killing. (**A**) Ligation of CD47 and SIRPα controls integrin (Mac-1) activation on neutrophils. This subsequently results in less cell–cell contacts between neutrophils and tumor cells, limiting trogoptosis of tumor cells. (**B**) Disruption of the CD47-SIRPα interaction allows Mac-1 activation, resulting in enhanced synapse formation, trogocytosis and eventually trogoptosis of antibody-opsonized cancer cells.

Blockade of the CD47-SIRPα axis generally only enhances tumor killing in the presence of tumor-targeting antibodies[15][58][17][61]. Consequently, not only enhanced CD47 expression on the tumor, but also decreased expression of TAAs on tumor cells can reduce or preclude neutrophil-mediated killing, as observed, e.g., in neuroblastoma cells of the mesenchymal phenotype that have lost GD2 expression[61].

Tumor-targeting mAbs stimulate neutrophil activation and tumor killing via FcR binding and signaling. As neutrophils express a variety of FcRs, different antibody isotypes can be used to stimulate neutrophils. Currently, most therapeutic mAbs are of the IgG1 isotype, which bind most FcγRs on neutrophils, including the highly expressed FcγRIIIb, which acts as a decoy receptor[66][67]. Treatment with IgG1 mAbs alone can enhance tumor killing by neutrophils. However, combination with CD47-SIRPα blockade significantly enhanced the cytotoxic capabilities of neutrophils[17]. In addition, some IgG2 mAbs are used in the clinic. IgG2 is able to effectively trigger myeloid cells, like neutrophils, at least as effective as IgG1, since it has a high affinity for FcγRIIa, which is the main FcγR involved in ADCC, and lower affinity for the decoy receptor FcγRIIIb[15][67][68].

Within bispecific antibodies (BsAb), opsonization and CD47-SIRPα blocking activity can be combined in one antibody, as Fab regions can target different antigens[69]. By combining a TAA-targeting mAb and anti-CD47 or anti-SIRPα mAb, immune cells can be recruited to the tumor and become fully activated by one antibody. For example, the GPC3xCD47 BsAb targets the TAA GPC3, expressed on hepatocellular carcinoma (HCC) cells, and CD47, as well as FcγRs via a functional IgG1 Fc tail[70]. Both in vitro and in vivo, GPC3xCD47 BsAb promoted neutrophil- and macrophage-mediated tumor killing of GPC3-expressing Raji cells. Similarly, the CD47xEGFR-IgG1 BsAb enhanced neutrophil ADCC of EGFR-expressing cancer cells[71]. The CD70/KWAR23 BsAb targets the TAA CD70 and SIRPα[65]. This BsAb significantly enhanced phagocytosis of CD70-expressing cancer cell lines in vitro. Furthermore, CD70/KWAR23 BsAbs limited the growth of Burkitt's lymphoma cells in vivo[65]. These preclinical studies have demonstrated that blocking the CD47-SIRPα interaction, by either mAbs or BsAbs, may promote tumor cell killing by myeloid cells such as neutrophils and macrophages.

2. Targeting CD47-SIRPα to Potentiate Antibody Therapy

The preclinical evidence that inhibition of the CD47-SIRPα checkpoint may promote the efficacy of tumor-directed therapeutic antibodies has prompted the clinical development of a variety of compounds targeting CD47-SIRPα. Currently, different agents, such as antibodies against either CD47 or SIRPα, or other therapeutic biologics directed against CD47, are being investigated for their ability to block the CD47-SIRPα axis to promote tumor reduction. Whereas CD47-SIRPα targeting is often referred to as a method to improve macrophage mediated-phagocytosis, it is clear that neutrophils may also play a critical role as effector cells towards cancer cells during tumor-targeting antibody therapy in general[65][72][73][74][75]. Moreover, neutrophils may also prominently contribute to the enhanced tumor elimination after CD47-SIRPα disruption[65]. In addition, there is accumulating evidence that also adaptive T cell-mediated anti-cancer immunity can be promoted by CD47-SIRPα blockade[76][77]. Clearly, this also sets the stage for a combination of CD47-SIRPα antagonists with PD1–PDL1 inhibitors[78][79][80][81]. Along these lines, there is even initial evidence that CAR-T cell activity may be promoted by CD47-SIRPα inhibitors[82].

2.1. CD47-Targeting Agents

Many different CD47-targeting agents have been developed, of which various agents have entered the clinical stage. Magrolimab (also known as GS-4721 or Hu5F9-G4) is a humanized anti-CD47 blocking antibody with a IgG4 tail modified to prevent Fab arm exchange[83]. As the IgG tail is still functional, at least to some extent with

respect to FcγRI binding[84], the anti-CD47 antibody may simultaneously function as an opsonizing antibody. In pre-clinical studies, combined treatment with Magrolimab and trastuzumab resulted in enhanced anti-tumor effects in NSG and C57BL/6 mice that had been xenografted with human SKBR3 cancer cells[85]. Treatment with Magrolimab or trastuzumab alone did not decrease tumor size in vivo. Due to these promising pre-clinical results, Magrolimab was the first in class anti-CD47 mAb that entered clinical trials, and is currently also the most clinically advanced CD47-SIRPα-targeting agent. Treatment with various doses of Magrolimab induced mainly grade I and II adverse events, including but not limited to transient anemia (57% of patients), lymphopenia (34%) and hyperbilirubinemia (34%). Moreover, partial remissions were observed in two patients with ovarian/fallopian tube cancers[86]. Currently, a large variety of clinical trials are ongoing to investigate the effect of Magrolimab for the treatment of various cancers.

Another anti-CD47 antibody is CC-90002, which has a humanized IgG4-PE (S228P and L235E mutation) tail, preventing FcγR interactions[87]. In pre-clinical studies, CC-90002 induced anti-tumor activity in vitro and in vivo against various hematological and solid cancers[88]. In a phase I trial (NCT02641002), patients with relapsed and/or refractory (r/r) AML and MDS were treated with CC-90002. Serious treatment-related adverse events were observed in 82% of patients and included febrile neutropenia (10/23) and bacteremia (4/23). In addition, no objective responses were observed in the treated patients. Due to the lack of a clinically sufficiently encouraging profile, as well as frequent anti-drug antibodies (ADA) development, this program was discontinued. CC-90002 treatment was also investigated as therapy for NHL patients in combination with rituximab (NCT02367196)[89], but this trial also showed low efficacy and was discontinued.

Letaplimab (also referred to as IBI188) is another anti-CD47 IgG4 antibody. Similar to the other anti-CD47 mAbs, Letaplimab was able to promote macrophage ADCP in vitro. In addition, it stimulated anti-tumor effects in NHL and AML/MDS xenograft mouse models in combination with rituximab or azacitidine[90]. In an initial phase Ia clinical trial (NCT03763149), the tolerability and safety of Letaplimab were assessed in patients with advanced or refractory solid tumors or lymphoma[91]. In general, treatment was well tolerated, with mainly grade I or II adverse events. Three out of twenty patients experienced adverse events of grade III or higher, i.e., hyperbilirubinemia, thrombocytopenia or anemia, each in one patient. Currently, five other clinical trials are ongoing with Leraplimab as a monotherapy, or in combination with rituximab, anti-PD-1, or chemotherapy in patients with various cancers, including solid tumors, lymphomas, MDS or AML.

Lemzoparlimab (also referred to as TJ011133 or TJC4) is also a fully human anti-CD47 IgG4 antibody. As most anti-CD47 antibodies cause anemia due to phagocytosis of RBCs, Lemzoparlimab was generated to specifically target CD47 on malignant cells while not recognizing CD47 on RBCs, due to unique CD47 binding properties[92]. In a phase I study (NCT03934814), patients with solid tumors were treated with monotherapy of Lemzoparlimab[92]. During this trial, only grade I or II adverse events were observed, including anemia in 30% of patients. In addition, one out of three patients treated with 30 mg/kg Lemzoparlimab had a partial response, and three out of sixteen patients in the trial achieved stable disease[92]. In the same trial, patients with r/r NHL were treated with a combination of Lemzoparlimab and rituximab[93]. Most adverse events were grade I and II, and anemia and thrombocytopenia were observed as one isolated episode. In addition, three out of seven patients had a CR, one

had a partial response, and three achieved stable disease. In an ongoing phase I/II clinical trial (NCT04202003), r/r AML and MDS patients were treated with monotherapy of Lemzoparlimab[94]. Most adverse events were grade I or II, but one patient experienced grade III thrombocytopenia. As recruitment is still ongoing, no results are yet available on response rates in this trial.

Another method to target CD47 is with a fusion protein consisting of the N-terminal IgV-domain of SIRPα and a functional Fc region, also known as SIRPα-Fc. These proteins basically function as a decoy receptor and prevent CD47 binding to SIRPα. In addition, the functional Fc tail can interact with FcγRs, to further enhance anti-tumor activity through, e.g., ADCP or ADCC. An example of a SIRPα-Fc in clinical trials is TTI-621, a fully human SIRPα-Fc with a functional IgG1 Fc region[95]. In vitro, TTI-621 was able to strongly bind various tumor cell lines and primary patient tumors[95]. In addition, TTI-621 also bound to cells in peripheral blood, as CD47 is widely expressed on normal cells. In co-cultures, the addition of TTI-621 significantly enhanced macrophage phagocytosis of various hematologic and solid tumors[95][96]. The *in vivo* treatment of AML xenografted mice with TTI-621 resulted in significantly reduced tumor burden[95]. Similar results were observed with B cell lymphoma xenograft models. Treatment tolerance and adverse events were therefore assessed in phase I clinical trials (NCT02663518, NCT02890368), in which patients with relapsed or refractory hematologic or solid malignancies were treated with TTI-621 alone or in combination with rituximab or nivolumab (anti-PD-1, a checkpoint molecule on T cells)[97][98]. These initial trials demonstrated that treatment with TTI-621 does not cause severe toxicities (at the maximally tolerated dose) and has some anti-tumor effects in various cancer types.

TTI-622 also is a fully human SIRPα-Fc, consisting of the CD47-binding domain of SIRPα and an IgG4 Fc tail. It was suggested that TTI-622 does not bind to RBCs, unlike many anti-CD47 agents, thereby limiting adverse events such as anemia. In an ongoing phase I trial (NCT03530683), preliminary results were published of 25 patients with r/r lymphoma, who were treated with various doses of TTI-622 monotherapy[99]. In 48% of patients, treatment-related adverse events were reported, mostly being grade I or II. Grade III adverse events observed included neutropenia (9%), thrombocytopenia (5%) and anemia (2%). Objective responses were observed in nine patients, and included two CRs and seven PRs[100]. In this ongoing trial, combinations of TTI-622 with azacitidine or other chemotherapeutic agents are also being investigated in hematologic cancers. Moreover, a clinical trial (NCT05139225) has started in which the toxicity and efficacy of TTI-621 and TTI-622 are being compared in combination with the anti-CD38 antibody daratumumab in relapsing multiple myeloma patients.

ALX148 (also known as Evorpacept) is another SIRPα-Fc fusion protein. More specifically, ALX148 consists of an inactive human IgG1 Fc region that is fused to a modified N-terminal IgV-domain of SIRPα, which enhances CD47 binding[101][102]. As ALX148 has ~50,000× higher binding affinity to CD47 compared to wild-type SIRPα, it prevents SIRPα ligation by acting as a potent decoy receptor. The Fc region is able to interact with neonatal Fc receptors, allowing for extended pharmacokinetics. Contrarily, the Fc tail is unable to bind human FcγRs, preventing targeting of immune cells to normal cells[102]. In pre-clinical studies, ALX148 improved the phagocytosis of OE19, DLD-1, MM1.R, and Daudi tumor cells opsonized with trastuzumab, cetuximab, daratumumab (anti-CD38), and obinutuzumab (anti-CD20), respectively[102]. Mice engrafted with human B cell mantle cell lymphoma were treated with ALX148 or obinutuzumab alone or as combination therapy[102]. Combination treatment significantly inhibited

tumor growth when compared to monotherapies. Similar results were observed in mice engrafted with OE19 gastroesophageal tumors, treated with ALX148 and trastuzumab, and mice harboring Raji B cell lymphoma tumors, treated with ALX148 and rituximab[102]. In a phase I clinical trial (NCT03013218; ASPEN-1), 110 patients with advanced or metastatic solid tumors were treated with various doses of ALX148 alone or in combination with pembrolizumab (anti-PD-1) or trastuzumab[103]. All treatments were well tolerated, with four serious adverse events in patients treated with ALX148 alone, five in patients treated with ALX148 and pembrolizumab, and one serious adverse event related to ALX148 plus trastuzumab treatment. The most common serious adverse events were thrombocytopenia and neutropenia. In addition to toxicity, the preliminary therapeutic effects of ALX148 were assessed. Of patients treated with monotherapy with ALX148 18% had stable disease. Currently, other clinical trials are ongoing in which ALX148 is being given in combination with various mAbs and chemotherapeutic agents to treat patients with hematologic or solid cancers.

Targeting CD47 on tumor cells allows for simultaneous tumor cell opsonization when the compounds contains a functional Fc tail. However, as indicated above, CD47 is widely expressed on virtually all cells in the body, and, particularly, hematologic adverse events are often observed in patients treated with anti-CD47 mAbs, e.g. anemia, thrombocytopenia, lymphopenia, and neutropenia. In addition, anti-CD47 antibodies may not only disrupt interactions with SIRPα, but also with other CD47 ligands, e.g., thrombosponin-1 or integrins, which could cause other adverse events[104]. Therefore, anti-SIRPα antibodies may in principle provide a better alternative.

2.2. Anti-SIRPα mAbs

Since SIRPα expression is much more restricted, with its expression largely confined to myeloid immune cells, it may be easier to saturate. Therefore, lower antibody concentrations may be needed to obtain beneficial clinical responses[104]. Nonetheless, an important aspect to consider is the large homology between SIRP family members. For example, SIRPγ also binds CD47, but is expressed on T cells, and has been suggested to play a role in T cell activation and transmigration in vitro[89]. Thus, the specificity of anti-SIRPα antibodies is key, and if such antibodies cross-react with other SIRP family members, their potential associated effects on safety and efficacy should be considered.

The first anti-SIRPα mAb entering clinical trials was CC-95251, a fully human IgG1 anti-SIRPα antibody with a K322A mutation, rendering the Fc tail inactive in terms of complement activation, but maintaining FcγR binding capacity[105]. In pre-clinical studies, CC-95251 demonstrated a synergistic effect with rituximab to promote phagocytosis of tumor cells by macrophages. In addition, intravenous administration appeared to be safe in cynomolgus monkeys, as no significant depletion of blood cell counts was observed[105]. Following these results, a phase 1 clinical trial (NCT03783403) was initiated, in which 230 patients with advanced solid or hematologic malignancies are intended to be treated with CC-95251 monotherapy or in combination with cetuximab or rituximab. Recently, the first interim results of 17 NHL patients treated with CC-95251 and rituximab were published[106]. In these patients, grade 3 or higher adverse events included neutropenia (53%), infections (24%) and thrombocytopenia (6%). The ORR was 56% and 25% of patients achieved a CR[106]. This trial is still ongoing,

and recently another trial (NCT05168202) has been announced investigating the effect of CC-95251 in combination with azacitidine on r/r AML and MDS.

BI765063 (also referred to as OSE-172) is a humanized IgG4 anti-SIRPα antibody with S229P and L445P mutations, which only binds to one of the major SIRPα polymorphic variants (V1, also known as $SIRP\alpha_{BIT}$) present in the population. BI765063 is reported to be unable to bind SIRPγ, and thus should preserve T cell activation and migration[81]. In vivo, a murine variant of BI765063 promoted ADCC and ADCP of triple-negative breast cancer cells. In addition, anti-tumor effects were enhanced even further in combination with other checkpoint blockades, e.g., anti-PD-L1 antibodies. Analysis of the TME demonstrated that T lymphocytes accumulated in the tumor in mouse models[81]. BI765063 has entered an initial phase I clinical trial (NCT03990233), in which it is used to treat patients with advanced solid tumors as a monotherapy, or in combination with an anti-PD-1 antibody (BI754091). Preliminary results have been presented at ASCO and ESMO meetings. Fifty patients with solid cancer have received monotherapy BI765063[107]. No dose-limiting toxicities were observed and mostly grade I and II adverse events were reported. Only one patient experienced a grade III infusion-related reaction and none of the patients had anemia or thrombocytopenia as a result of the treatment. One patient showed durable PR, and had increased CD8 T-cell infiltration into the TME upon BI765063 treatment. After two weeks, an increased expression of PD-L1 was measured on the tumor[107]. Thus, combination with anti-PD-1 or anti-PD-L1 antibodies may further enhance clinical benefit.

2.3. Alternative Ways to Disrupt CD47-SIRPα Interactions

Besides CD47- or SIRPα-targeting antibodies, the CD47-SIRPα axis can be disrupted in alternative ways, for example by downregulating CD47. Galectin-9 (Gal-9) is a β-galactoside-binding galectin, and has been described for its role in cancer, as loss of Gal-9 is associated with tumor progression and metastasis[108]. However, recently it has been identified that Gal-9 also affects CD47 expression[109]. Associated with this finding, in co-cultures, treatment with Gal-9 significantly enhanced trogocytosis of FaDu cells by neutrophils, but not phagocytosis by macrophages. In addition to downregulation of CD47 on tumor cells, it was shown that the treatment of neutrophils with Gal-9 induced neutrophil activation, such as induced calcium flux, and degranulation, measured by upregulation of CD11b, CD18, CD11c, CD15, CD66b and CD63 on the cell's surface. In co-cultures with FaDu or Caco2 cancer cell lines, neutrophils were able to kill significantly more tumor cells after Gal-9 treatment[109].

Recently, a small molecule, RRx-001, was identified as a tumor targeting agent, as it also downregulates CD47 on tumor cells[110]. RRx-001 activates the peroxisome proliferator-activated receptor gamma (PPAR-γ), which is a nuclear receptor transcription factor that inhibits Myc by heterodimerizing with retinoid X receptor. Inhibition of the transcription factor Myc subsequently results in downregulation of CD47[111]. RRx-001 treatment decreased both the expression of CD47 on A549 lung cancer cells, and SIRPα expression on monocytes and macrophages in vitro[112]. Consequently, enhanced phagocytosis of A549 lung cancer, or AU-565, MCF-7, and MDA-MDB-231 breast cancer cells was observed. Treatment of A549-bearing nude mice with RRx-001 resulted in a significant reduction of tumor growth[112]. A phase I trial (NCT01359982) with 25 patients with advanced soluble cancers showed that treatment with RRx-001 was well tolerated with no clinically significant toxicity[113]. In addition, 67% of

patients had stable disease and 5% had a partial response. A phase II clinical trial (NCT02489903) showed that RRx-001 is also able to downregulate PD-L1 on small cell lung cancer cells[114]. Moreover, RRx-001 can have a direct anti-tumor effect through epigenetic modulation in multiple myeloma cells[115]. Currently, RRx-001 is tested in various clinical trials[116].

CD47-SIRPα interactions can also be disrupted by modulating enzymatic modifications of the SIRPα-binding domain in CD47. In a FACS-based haploid genetic screen, the gene encoding glutaminyl-peptide cyclotransferase-like (QPCTL, isoQC) was identified to significantly reduce the binding capabilities of SIRPα to CD47[111][117]. QPCTL is an enzymatic modifier, which adds pyroglutamate modifications to proteins. It has previously been demonstrated that CD47 contains an N-terminal pyroglutamate, which is involved in SIRPα binding[110]. Knockout of QPCTL decreased SIRPα binding in various human cell lines (HAP1, A375, A431, A549, DLD1, and RKO), while the overall expression of CD47 remained unaffected[111]. Similarly, treatment with small molecule inhibitors targeting QPCTL, i.e., SEN177 and PQ912, significantly reduced binding to SIRPα. In a co-culture of human macrophages and anti-CD20 treated Raji cells, the addition of SEN177 significantly increased phagocytosis[111]. Neutrophil-mediated ADCC of cetuximab-treated A431 cells or trastuzumab-treated Ba/F3 cells was significantly enhanced by treatment with SEN177 or knockout of QPCTL as well. These results were confirmed by other studies, showing that SEN177 treatment significantly enhanced ADCP by macrophages and neutrophil-mediated ADCC[118][119][120]. Another QPCTL inhibitor, luteolin, also abrogated the interaction between CD47 and SIRPα[121][122]. In addition, in co-cultures of H929 or DLD1 cancer cells with mouse bone-marrow-derived macrophages, phagocytosis was significantly improved after treatment with luteolin. To determine the effect in vivo, human FcαRI transgenic BALB/c mice were injected with 1:1 WT and QPCTL knockout Ba/F3 cells[111]. Mice were subsequently treated with anti-Her-2/neu IgA antibodies or PBS. Only in the IgA anti-HER-2/neu treated mice was profound killing of QPCTL knockout cells observed. In addition, an influx of neutrophils into the tumor was observed as a result of anti-HER-2/neu IgA treatment in combination with QPCTL knockout. The specific depletion of neutrophils with anti-Ly6G antibodies abrogated the treatment effect, demonstrating that neutrophils were the main effector cells eliminating QPCTL-deficient tumor cells[111]. These studies demonstrate that alternative ways of targeting the CD47-SIRPα axis may perhaps also have potential to promote tumor elimination. However, more pre-clinical and clinical studies are needed to demonstrate whether these compounds are well tolerated and effective in patients.

Despite the promising preliminary results observed in the various clinical trials targeting the CD47-SIRPα axis, it is important to consider the ways by which tumor cells may adopt resistance against these therapies. Since neutrophils, but also macrophages, require tumor opsonization with anti-TAA antibodies, loss of TAA expression will prevent tumor opsonization and thereby reduce killing by these immune cells. This has already been observed for neutroblastoma cells, where the TAA expression of GD2 can decrease during anti-GD2 mAb therapy[61]. Moreover, tumor cells may upregulate other (perhaps less well defined) checkpoint molecules to limit immune activation and tumor killing. Lastly, tumor cells could escape elimination by preventing immune cell infiltration, by creating an immunosuppressive microenvironment. Thus, although CD47-SIRPα appears to enhance tumor killing, the therapy is dependent on the opsonization of the tumor cells and possibly also the immunosuppressive state of the TME.

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