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The Physiological and Therapeutic Role of CD47 in Macrophage Function and Cancer

Shelby N. Bess, Matthew J. Igoe, and Timothy J. Muldoon

Department of Biomedical Engineering, University of Arkansas, Fayetteville, Arkansas, USA

ABSTRACT

Background: Immunotherapy is an emerging strategy in cancer therapeutics aimed at modulating the immune system to inhibit pro-tumor pathways and increase a tumor's sensitivity to chemotherapy. Several clinically approved immunotherapy treatments, such as monoclonal antibody treatments, have been successful in solid tumors such as breast, colorectal, and pancreatic. However, an outstanding challenge of these strategies is tumor cell resistance. One target of interest for immune cell modulation is targeting macrophages that enter the tumor microenvironment. More specifically, an immune checkpoint of interest is CD47. CD47 is a transmembrane protein that inhibits phagocytic activity by acting as a "don't eat me" signal. In both mice and humans, healthy cells can express CD47, while solid malignancies like colorectal and breast cancer express it most strongly.

Methods: Analysis of literature data on the physiological and functional roles of tissue-resident macrophages, along with the structure and mechanisms of action of the CD47 pathway was explored. We also explored how CD47 can influence different aspects of the tumor microenvironment (i.e. cellular metabolism and hypoxia) in addition to current clinical strategies and challenges associated with targeting CD47.

Results: Overall, it was discovered that CD47 is overexpressed in a variety of cancer types in addition to normal tissue, making it a promising treatment regimen to enhance the capability of macrophages to phagocytose tumor cells. However, treatment efficacy is varied in pre-clinical and clinical models due to various challenges such as off-target effects.

Conclusion: This review emphasizes the diverse functionality of macrophages in normal and cancerous tissue, while also emphasizing the importance of macrophage targeting and their clinical significance.

KEYWORDS

CD47; immunotherapy; macrophages; phagocytosis; tumor

Introduction

Cancer is characterized by several hallmark characteristics, including prolonged proliferation, inhibition of growth suppressors, resistance to cell death, the ability to reproduce immortally, induction of angiogenesis, activation of invasion, and resistance to immune destruction (Chao et al., 2012; Hanahan & Weinberg, 2011). Since the earliest discussion of tumor immune surveillance more than a century ago, numerous immune system components have been involved in the detection and removal of cancer cells by the immune system (Swann & Smyth, 2007). The innate immune system, notably the macrophage, has only

CONTACT Timothy J. Muldoon tmuldoon@uark.edu Department of Biomedical Engineering, University of Arkansas, Fayetteville, AR 72701

recently been proven to have a significant role in regulating tumor pathogenesis, whereas the adaptive immune response is well known to play a major role in anti-tumor immunity (Jaiswal et al., 2010). As part of a process known as "cancer immunoediting," the immune system integrates its dual host-protective and tumor-promoting functions (Dunn et al., 2002; Hao et al., 2023; Schreiber et al., 2011). By identifying tumor-associated antigens, the immune system keeps an eye out for and destroys cancerous cells. However, immune resistance and evasion are frequently brought on by malignancies' hijacking of immune-inhibitory pathways (Dunn et al., 2002; Hao et al., 2023; Schreiber et al., 2011).

In tumor immunotherapy, immune checkpoint (surface proteins that regulate immune system responses from attacking healthy cells) treatments that improve T cell activity have made significant strides (X. Jiang et al., 2019; Y. Wang et al., 2022). However, the rate of treatment response is typically constrained by the immunosuppressive milieu created by solid tumors (Binnewies et al., 2018; Y. Wang et al., 2022). The most prevalent immune cells in the tumor microenvironment (TME) are macrophages that can make up to 50% of the solid tumor mass, unlike T cells, which need to infiltrate the TME. These macrophages can support tumor growth when controlled by tumor cells, reducing T-cell activation and encouraging immune escape (Cassetta & Pollard, 2018). There is a growing understanding of the significance of innate immune checkpoints in cancer cell monitoring and elimination even though FDA-approved cancer immunotherapies targeting adaptive immunological checkpoints have significantly improved patient outcomes. Antigen-presenting cells (APCs), like monocytes, macrophages, and dendritic cells, capture antigens through phagocytosis and then present them to naive T and B cells, which causes them to become activated. This process is known as cross-priming of the adaptive immune system (Iwasaki & Medzhitov, 2010; Jutras & Desjardins, 2005; van Duijn et al., 2022). Tumor cells either disguise themselves as self-cells or block signals that the innate immune system can detect to avoid identification and phagocytosis. As a result, innate immune checkpoints are crucial for immunological escape caused by tumors. They could therefore serve as immunotherapy targets for cancer.

To escape being ingested, tumor cells can also overexpress "Don't eat me" molecules, particularly cluster of differentiation 47 (CD47), which interacts with signal regulatory protein (SIRP), on macrophages (Jaiswal et al., 2009; Y. Wang et al., 2022). Importantly, many tumor cells express CD47 extensively (Willingham et al., 2012). The ability of macrophages to phagocytose tumors may therefore be restored through proper therapies that block the transmission of "Don't eat me" signals, which would have a significant impact on tumor immunotherapy (Majeti et al., 2009). However, due to the ubiquitous expression of CD47 on normal cells such as red blood cells (RBCs), the therapeutic ability of CD47 blockade is compromised due to toxicity and fast clearance. In this review, we will discuss tissue resident macrophage origins and functional roles, structure and mechanisms of CD47 and its influence on the tumor microenvironment, and current clinical strategies and challenges associated with targeting CD47.

Macrophage origins

Ilya Metchnikoff first described macrophages in detail in terms of their ability to engulf and destroy cellular components from living and dead microbial and host cells (Munro & Hughes, 2017). In 1924, a new term, "reticuloendothelial system," was used to describe

the system of phagocytic cells (Munro & Hughes, 2017). In 1969, it was decided by a group of immunologists that the term "reticuloendothelial" was no longer adequate to describe this system of phagocytic cells (Munro & Hughes, 2017). Instead, the system was renamed "the mononuclear phagocyte system" to include the functions and morphologies of dendritic cells, monocytes, and macrophages and how those cells were derived from the bone marrow (Munro & Hughes, 2017). Macrophages differentiate from circulating peripheral blood mononuclear cells (PBMCs) that migrate into the tissue either in response to inflammation or in a steady state (Mosser & Edwards, 2008). The PBMCs develop from common myeloid progenitor cells in the bone marrow that can also be precursors for many different cell types, such as eosinophils, basophils, dendritic cells, neutrophils, mast cells, and macrophages (Figure 1) (Mosser & Edwards, 2008). During monocyte development, myeloid progenitor cells give rise to monoblasts, pro-monocytes, and monocytes, which are then released into the bloodstream from the bone marrow. These monocytes then migrate into the tissue through a specialized process called diapedesis (squeezing through a blood vessel wall) to replenish long-lived tissue-specific macrophages of the central nervous system (microglial cells) (Ginhoux et al., 2010), gastrointestinal tract, bone (osteoclasts),

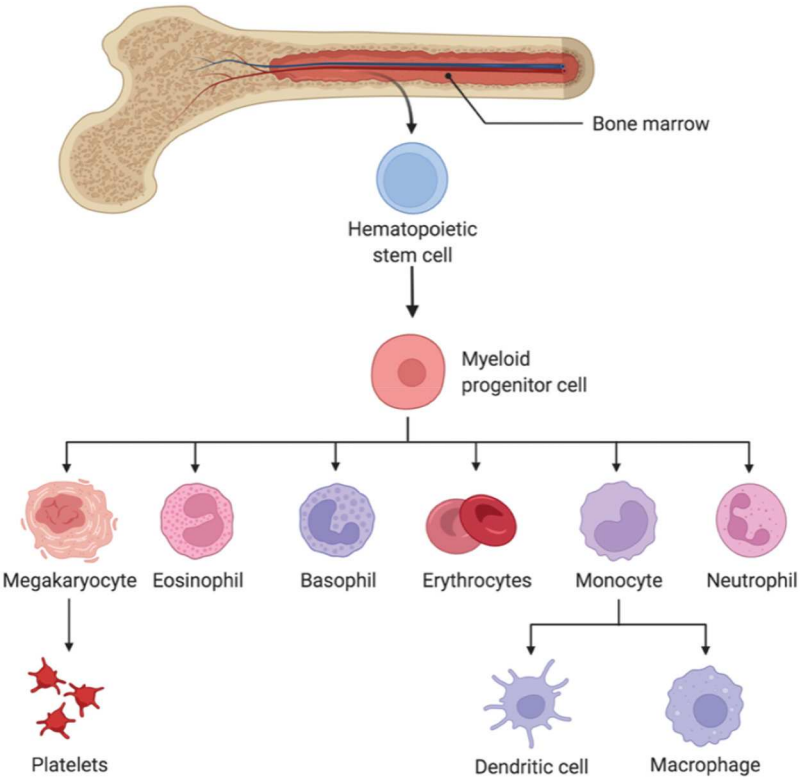

Figure 1. Graphical representation of a simplified view of the myeloid lineage derived from the bone marrow and hematopoietic stem cells. Myeloid progenitor cells can differentiate into megakaryocytes (which can further differentiate into platelets), eosinophils, basophils, erythrocytes (RBCs), neutrophils and monocytes. Monocytes can further differentiate into dendritic cells (DCs) and several macrophage phenotypes. Figure was created with BioRender.

alveoli, liver (Kupffer cells), peritoneum, and spleen (S. Gordon & Plüddemann, 2017; Mosser & Edwards, 2008). There is some debate about whether specific monocyte populations can give rise to specific tissue macrophages in the blood since it is known that monocytes are not a homogeneous population of cells (Mosser & Edwards, 2008). One theory suggests that monocytes continue to develop in the blood and are then recruited into the tissues (Mosser & Edwards, 2008). Depending on the location and endogenous and exogenous stimuli, the point at which the monocytes leave the blood can define their overall function as tissue-resident macrophages (S. Gordon & Plüddemann, 2017).

Tissue resident macrophages

Tissue-resident macrophages have a wide range of homeostatic functions, such as inflammation resolution, cellular debris clearance, and tissue immune surveillance (Davies et al., 2013). Macrophages can respond to endogenous or exogenous stimuli produced by innate immune cells or antigen-specific cells, which can affect their overall physiology (Mosser & Edwards, 2008). Macrophages can also produce several cytokines and chemokines that can influence their physiology and function (S. Gordon, 2007). The biological complexity of how the tumor microenvironment can change macrophages with an anticancer phenotype into macrophages with a pro-tumor phenotype, lowering patient survival across a wide range of cancer types, has been investigated through more thorough research. Macrophages can be divided into phenotypes M1 and M2. The M1 phenotype can be induced by bacterial products like lipopolysaccharides and pro-inflammatory cytokines like interferons (Mackaness, 1977; O'Shea & Murray, 2008). This phenotype can produce IL-12 and CXCL10, which are angiostatic molecules linked to antitumor immunity. On the other end of the spectrum, immunoregulatory cytokines (IL-4 and IL-10) can generate the M2 phenotype (Kreider et al., 2007). This phenotype oversees tissue remodeling factors linked to pro-tumor function and pro-angiogenic (MMPs and VEGF) secretion. Recent research has revealed that macrophages rarely exhibit a single distinct phenotype, preventing a binary classification of the complexity of these immune cells (Mosser & Edwards, 2008). To account for macrophage subtypes that can co-express M1 and M2 gene signatures, researchers are aiming to divide macrophage populations into other categories such as M2a, M2b, and M2c (Mosser & Edwards, 2008). For this review, we will describe the functions of tissue-resident M1 macrophages.

Phagocytosis: bacterial/microbial clearance

A major function of macrophages in host defense is the ingestion of microbes and non-self-antigens. Macrophages engulf, digest, and destroy these microbes and cells through a process called phagocytosis. Phagocytosis is an energy-dependent process that engulfs large particles (>5 μm in diameter) into vesicles (Abbas et al., 2018; Hirayama et al., 2017). To start the process, when macrophages arrive at a site of infection or injury, they will express microbe-specific receptors to engage the binding of the foreign microbes. These receptors can include pattern recognition receptors such as C-type lectins and scavenger receptors such as the mannose receptor. Phagocytes also have high-affinity receptors for opsonins, including complement proteins, plasma lectins, and antibody molecules, which is a critical step for

phagocytosis for microbes that are coated with opsonins. After a microbe or foreign particle binds to its respective receptor, the macrophage's plasma membrane begins to extend out into a cup-shaped projection around the microbe. Once the cup extends beyond the diameter of the microbe, the top portion of the phagocytic cup closes and pinches off to form an intracellular vesicle called a phagosome. The phagosome then breaks away from the plasma membrane. Signals from pattern recognition receptors also work to activate macrophages to kill ingested microbes. Phagosomes then fuse with lysosomes to create a phagolysosome, where most of the microbicidal mechanisms for killing the microbe occur (Figure 2) (Abbas et al., 2018; Hirayama et al., 2017).

Two classes of microbicidal molecules include reactive oxygen species and nitric oxide. Activated macrophages convert molecular oxygen into reactive oxygen species (ROS), with the primary system generated being the phagocyte oxidase system (Abbas et al., 2018; Roos, 1991). Phagocyte oxidase is a multi-subunit enzyme assembled during active phagocytosis in the phagolysosomal membrane. Phagocyte oxidase is activated through stimuli such as toll-like receptors (TLRs) and interferon-y (IFN-y) and functions to reduce molecular oxygen into superoxide radicals, with the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) acting as a cofactor. The superoxide radicals that are generated are modified into hydrogen peroxide and used to convert unreactive halide ions into reactive hypohalous acids that are toxic to bacteria.

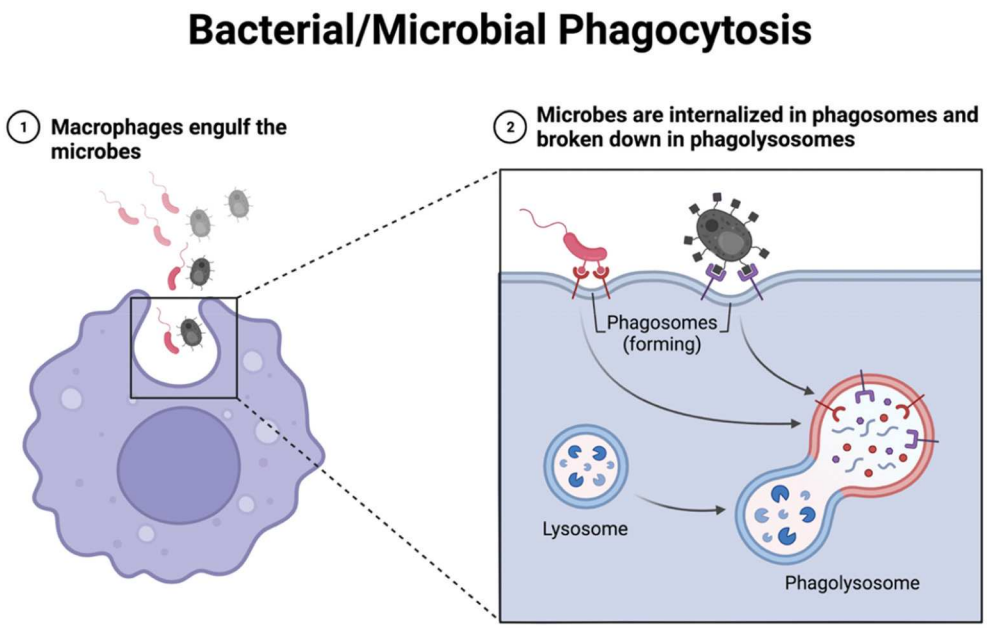

Figure 2. Bacterial and microbial phagocytosis process. 1) macrophages engulf bacteria and microbes after binding with pattern recognition receptors. The plasma membrane of the macrophage binds to extend out and form a cup-shaped projection around the foreign body. 2) Once the phagocytic cup extends beyond the microbe, the cup pinches off and forms a phagosome. The phagosome then breaks away and fuses with a lysosome to form a phagolysosome where the process of digestion the microbe begins. Figure was created with BioRender.

This process is called the respiratory burst (Abbas et al., 2018; Roos, 1991). Macrophages produce nitric oxide (NO) with the help of an enzyme called inducible nitric oxide synthase (iNOS). iNOS is a cytosolic enzyme that is normally dormant in resting macrophages but can be activated in response to TLRs, especially in combination with IFN-y. iNOS catalyzes arginine and converts it to citrulline, which releases freely diffusible nitric oxide (Abbas et al., 2018).

Antigen presentation and T-Cell activation

After a microbe has been broken down into smaller fragments or antigens, the fragments are then loaded onto an MHC class I (viral) or class II (bacteria or other extracellular antigens) complex and transported to the surface of a macrophage for antigen presentation. The antigen that is presented on the MHCs is recognized by the T cell receptor (TCR) on the surface of T cells (Abbas et al., 2018; Hirayama et al., 2017). Each T cell has a wide range of TCRs, each uniquely specific to an antigen. Not only are the TCRs specific to a particular antigen, but they also have specificity for a specific MHC molecule (CD4 or CD8). CD4 is present on T helper (Th) cells and only binds to the MHC class II complexes, while CD8 is present on cytotoxic T cells (CTLs). Th1 cells are known for their effects on antigen presentation and T-cell and macrophage activation (Abbas et al., 2018; Hirayama et al., 2017).

When a Th cell recognizes an antigen on the macrophage surface, the TCR-CD3 complex binds to the peptide-MHC complex, while CD4 binds to another section of the MHC complex (Abbas et al., 2018; Hirayama et al., 2017). Once this occurs, CD4 recruits a kinase called Lck, which phosphorylates immunotyrosine activation motifs (ITAMs) present in CD3's gamma, delta, epsilon, and zeta chains. The protein ZAP-70 binds to the phosphorylated ITAMs through its SH2 domain, which makes it phosphorylated and orchestrates the downstream signaling required for T-cell activation (Abbas et al., 2018). Once the Th1 cells have received the initial TCR/CD3 signal, the naive T cell must activate a second independent chemical pathway to ensure that a T cell is correctly responding to foreign antigens. This second pathway involves an interaction between CD28 on the CD4 T cell and the protein CD80 or CD86 (also called co-stimulatory molecules) on the macrophage. Once both pathways are activated, the biochemical changes from the initial TCR/CD3 signal are altered, and the T cell is now activated, as shown in Figure 3 (Abbas et al., 2018; Hirayama et al., 2017).

After the two-signal activation is complete, the Th cell allows itself to proliferate through the release of interleukin-2 (IL-2) (Abbas et al., 2018; Janeway et al., 2001). When a Th cell receives both signals of activation and proliferation, it will now become a T helper 0 (Th0) cell, which can secrete IL-2, IL-4, and IFN-y and differentiate into either a Th1 or Th2 cell depending on the cytokine environment. More specifically, the Th1 subset of Th lymphocytes is driven by IL-12 and IFN-y, which activate the transcription factors T-bet, STAT1, and STAT4 (Abbas et al., 2018; Janeway et al., 2001). T-bet is induced in naive CD4+ T cells in response to antigens and IFN-y. STAT1 activation induced by IFN-y stimulates T-bet expression, which then promotes IFN-y production through a combination of inducing chromatin remodeling of the IFN-y promoter region and direct transcriptional activation of the IFNG gene. This creates a positive amplification loop that drives the differentiation of T cells towards the Th1 phenotype. IL-12 binds to receptors on antigen-stimulated CD4+ T

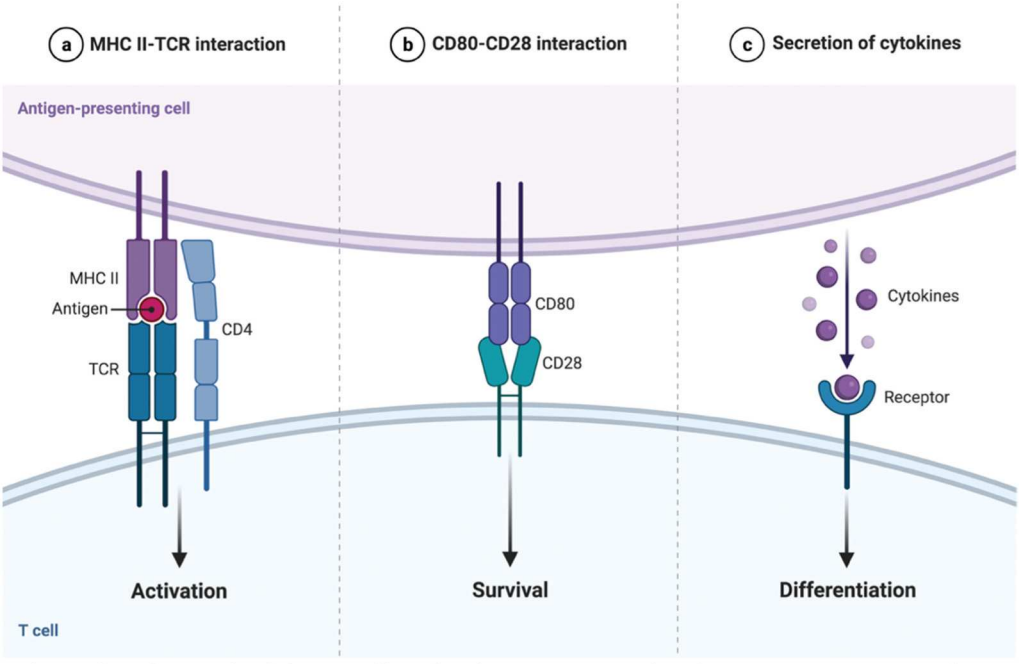

Figure 3. Three signals required for T cell activation. 1) interaction between MHC-II on the macrophage and TCR/CD4 complex on the T-cell to induce T-cell activation. 2) interaction between CD80 on the macrophage and CD28 on the T-cell to promote T-cell survival. 3) secretion of cytokines from the antigen-presenting cell to kickstart T-cell differentiation. Figure was created with BioRender.

cells and activates the transcription factor STAT4, which further enhances IFN-y production (Figure 4) (Abbas et al., 2018; Janeway et al., 2001).

Th1 cells help activate macrophages through contact-mediated signals through CD40L-CD40 binding and IFN-y. After a Th1 cell is stimulated by an antigen, it expresses CD40L on its surface. This, along with IFN-y secretion, is a potent stimulus for macrophage activation. CD40 signals on the macrophage activate the nuclear factor kB (NF-kB) and activation protein 1 (AP-1), while IFN-y activates STAT1 (Abbas et al., 2018; Janeway et al., 2001). Together, they stimulate the expression of several enzymes in the phagolysosomes of macrophages, including iNOS and NO. The interactions between CD40 on macrophages and CD40L on T cells ensure that the macrophages that are presenting the antigens are also the macrophages that will be in contact with the T cells to ensure the positive feedback loop of macrophage activation through T-cell activation (Figure 5) (Abbas et al., 2018; Janeway et al., 2001).

Clearance of apoptotic cells

Along with the phagocytosis of pathogens by phagocytes during infection, host cells that undergo apoptosis are cleared by macrophages using a specific type of phagocytosis called efferocytosis (Kourtzelis et al., 2020). The phagocytic clearance of apoptotic cells consists of

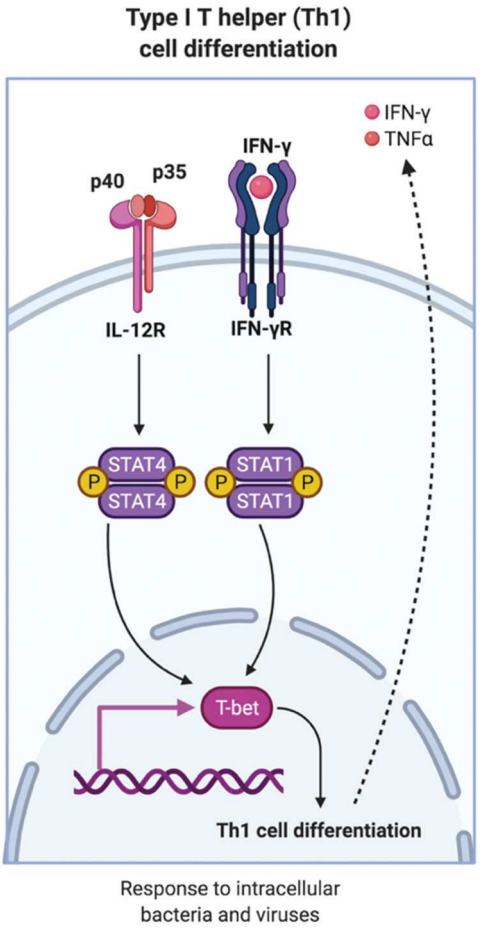

Figure 4. Th1 cell differentiation. After a T-cell receives the activation and survival signals, ifn-y, IL-12, and other cytokines will be released. Once ifn-y and IL-12 binds to their respective receptors, the STAT1 and STAT4 pathways will be activated which stimulates T-bet expression. This induces the chromatin remodeling of the ifn-y promoter region increasing production of ifn-y and tnf-α. Figure was created with BioRender.

four distinct steps: 1) recruitment of phagocytes; 2) apoptotic cell recognition through receptors; 3) engulfment; and 4) processing of the engulfed cell (Erwig & Henson, 2007).

At sites of inflammation, where a large number of apoptotic cells are generated, an accumulation of phagocytes can be seen. Before the apoptotic cells can be removed, they undergo secondary necrosis, which necessitates phagocyte migration. This has led to the assumption that apoptotic cells release extracellular signals to direct phagocytes to their location (Erwig & Henson, 2007). The role of "find me" signals is to establish a chemotactic gradient to simulate the migration of phagocytes (Hochreiter-Hufford & Ravichandran, 2013). To date, there are several proposed "find me" signals released by apoptotic cells (Table 1).

Once the phagocytes are in proximity to the apoptotic cells, the macrophages must be able to distinguish live cells from dead cells. Apoptotic cells will display "eat me" signals on

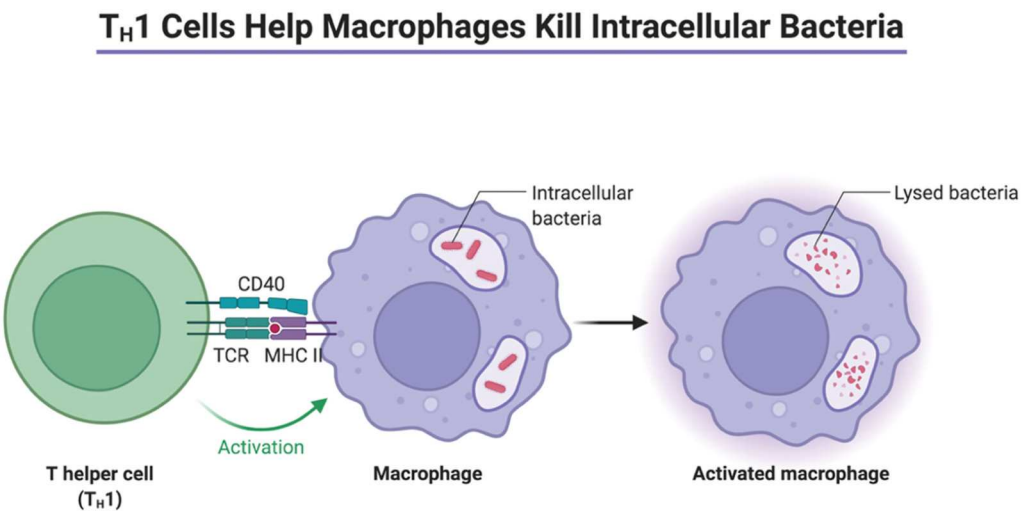

Figure 5. Th1 T-cells and macrophages interact through the binding of CD40/CD40L and TCR/MHCII in addition with the release of ifn-y. This stimulates the nf-kB pathway along with STAT1, which produces iNOS and NO, which is needed to lyse bacteria within the phagolysosome. Figure was created with BioRender.

Table 1. Common "find me," "eat me," and "don't eat me" signals involved in phagocytosis of apoptotic cells.

	Description	Signal Name	Reference
Find Me	Mediators that aid in macrophage chemotaxis to apoptotic cells	Fractalkine (CX3CL1)	Hochreiter-Hufford and Ravichandran (2013), Peter et al. (2010)
		Lysophosphatidylcholine (LPC)	Lauber et al. (2004), Peter et al. (2008), Truman et al. (2008)
		Sphingosine-1-phosphate (S1P)	Nagata et al. (2010), Gude et al. (2008)
		ATP and UTP	Elliott et al. (2009), Rosen and Goetzl (2005)
Eat Me	Surface molecules that is exposed to induce phagocytosis	Thrombospondin (TSP1)	Moodley et al. (2003)
		ICAM3	Kristóf et al. (2013), Torr et al. (2012)
		Gas6	Dransfield et al. (2015), Grommes et al. (2008), Qingxian et al. (2010)
		C1q	Fraser et al. (2009), Galvan et al. (2012)
Don't Eat Me	Surface mediators on cells used to evade detection by innate immune cells	CD24	Bradley (2019)
		PD-1	Feng et al. (2019)
		LILRB1	Feng et al. (2019)

the cell surface, which macrophages can detect through receptors on their cell surface (Hochreiter-Hufford & Ravichandran, 2013). The detection of apoptotic cells is not always direct. Some receptors can directly engage with the ligands on the dying cells, but other receptors use soluble bridging molecules as intermediates to recognize the "eat me" signals on the dying cells (Hochreiter-Hufford & Ravichandran, 2013). Some cells also have mechanisms to prevent their unwanted removal. For example, cancer cells can overexpress both pro- and anti-phagocytic signals that can either inhibit or induce phagocytosis

(Cendrowicz et al., 2021). The balance between these signals in the TME results in the level of cancer cells engulfed by macrophages. Pro-phagocytic signals (i.e., phosphatidylserine (PtdSer)) can originate from cancer cells or external factors like opsonizing antibodies, while anti-phagocytic signals (i.e., CD47 and PD-L1) are proteins that are upregulated on cancer cells to help avoid the immune response (Cendrowicz et al., 2021).

The most widely studied and universally detected "eat me" signal is the exposure of the phospholipid phosphatidylserine (PtdSer) (Fadok et al., 2001). In living cells, PtdSer is kept in the inner leaflet of the lipid bilayer with the help of ATP-dependent phospholipid flippases (Bosurgi & Rothlin, 2021). During apoptosis, there is a loss of flippase activity and scramblase activation, which leads to PtdSer shifting to the outer leaflet of the cell membrane. This lets PtdSer serve as an "eat me" signal for neighboring macrophages (Barth et al., 2017). Exposure to PtdSer affects the organization of the plasma membrane through the recruitment of proteins to PtdSer-enriched regions via electrostatic interactions (Moodley et al., 2003). Macrophages and other phagocytes express transmembrane receptors that bind directly to PtdSer (i.e., brain-specific angiogenesis inhibitor-1 (BAI-1) and stabilin-2). A critical threshold of PtdSer exposure may be critical to trigger efferocytosis since it has been identified that phagocytes fail to engulf viable cells that expose low levels of PtdSer during activation or when the phospholipid scramblase transmembrane protein 16F (TMEM16F) is overexpressed, leading to PtdSer exposure.

CD47 structure and mechanisms of action

Structure

Cluster of differentiation 47 (CD47) is a 50 kDa transmembrane integral protein belonging to the immunoglobulin superfamily that is located on the surface of a variety of different cell types (Lindberg et al., 1994; Reinhold et al., 1995; Soto-Pantoja et al., 2015). CD47 has an N-terminal extracellular IgV domain from the immunoglobulin superfamily, followed by a presenilin domain with five segments that span the membrane and a brief, variable-spliced cytoplasmic region (Brown & Frazier, 2001; Mushegian, 2002). The N-terminal IgV domain is composed of a complex of integrins, G proteins, and cholesterol (Lindberg et al., 1994; Subramanian et al., 2007). The C-terminal intracellular tail can exist in four alternatively spliced isoforms, which differ only in the length of their cytoplasmic tail (Soto-Pantoja et al., 2015). Some of the signaling capabilities of CD47 depend on a long-range disulfide bond that connects Cys^{33} in the IgV domain to Cys^{263} in the last extracellular loop. An O-linked glycosaminoglycan is added to and N-glycosylated onto the IgV domain (Kaur et al., 2011; Mushegian, 2002). SIRP-α binding is not dependent on the five N-glycosylation sites, but thrombospondin-1 (TSP1) signaling through CD47 is dependent on the glycosaminoglycan modification (Barclay & Van den Berg, 2014; Kaur et al., 2011).

Mechanisms of action

The IgV domain of CD47 interacts with two extracellular ligands: signal regulatory protein alpha (SIRP-α) and thrombospondin-1 (TSP-1). For this review, we will focus on SIRP-α (Figure 6). SIRP-α is a transmembrane protein located on the surface of myeloid cells such as macrophages, granulocytes, monocytes, and myeloid dendritic cells (Lindberg et al.,

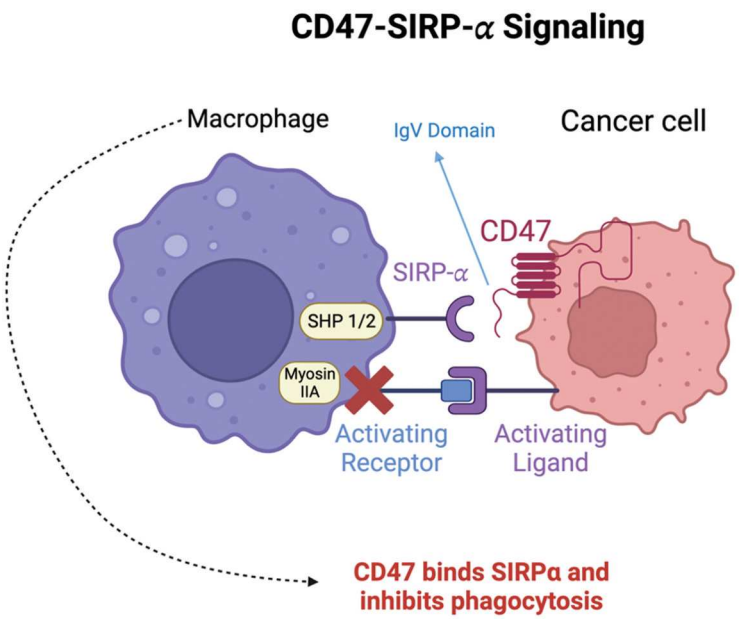

Figure 6. CD47 structure and binding with sirp-α. The inhibition of phagocytosis through the CD47/SIRP-α axis starts when the macrophage sirp-α interacts with the CD47 ligand on cancer cells. When this occurs, sirp-α undergoes tyrosine phosphorylation to recruit SHP-1 and SHP-2 tyrosine phosphatases. SHP-1/SHP-2 inhibits the prophagocytic receptors and their ability to trigger phagocytosis. Figure was created with BioRender.

1994). It is associated with the Src homology region 2 (SH2) domain-containing phosphatases: SHP-1 and SHP-2 (Lindberg et al., 1994; Takada et al., 1998). SIRP-α has an extracellular region that contains three immunoglobulin superfamily-like regions: an NH2-terminal V-like structural domain that can directly bind to CD47 and two C1-like IgSF domains (Kristóf et al., 2013). SIRP has four tyrosine residues in its cytoplasmic domain, which combine to form two standard immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Lorenz, 2009; Takada et al., 1998). They can recruit and activate the cytosolic tyrosine phosphatases SHP-1 or SHP-2 upon phosphorylation (but not the inositol phosphatases SHIP-1 or SHIP-2), which can then dephosphorylate a variety of substrates and control downstream signaling pathways, most frequently in an inhibitory manner. For SHP-2 binding, two Tyr residues (449 and 473) were found to be crucial. SHP-1 and SHP-2 are mainly inactive when found in the cytosol due to an autoinhibitory process involving the SH2 domains. However, the phosphatase activity is revealed when the SH2 domains are recruited to the phosphorylated ITIMs of SIRP or other inhibitory receptors (van Beek et al., 2012). SHP-2 has a widespread expression pattern and may therefore be important for SIRP signaling in either myeloid or neuronal cells, in contrast to SHP-1, which is mostly expressed in hematopoietic and epithelial cells. Therefore, SHP-1 can only function when myeloid cells are present. Even though it seems logical to infer that SHP-1 and/or SHP-2 mediate many of the inhibitory effects of SIRP, direct proof for this, such as through mutagenesis of the ITIM tyrosines, is not always available (Alenghat et al., 2012). Consequently, some of the other signaling molecules that can bind to SIRP may facilitate

some of the signaling capabilities of SIRP, as shown by biochemical investigations (Carter-Su et al., 2000; Timms et al., 1999; Veillette et al., 1998). It is probable that the two proline-rich regions in the cytoplasmic tail of SIRP interact with SH3 domains in specific signaling proteins. These regions are situated between the tandem tyrosines of the ITIMs. The fact that tyrosines in the SIRP cytoplasmic domain must first be phosphorylated to attract phosphatases is a crucial aspect of SIRP signaling. It is possible that the phosphorylation state is normally modest, at least in cultured resting phagocytes (Johansen & Brown, 2007), but it is also possible that the integrin-mediated adhesion can increase it (Tsai & Discher, 2008). It is yet unknown how much SIRP is phosphorylated in the different cell populations that express the receptor *in vivo*. However, activation of SIRP by CD47 leads to the phosphorylation of SIRP ITIM, most likely due to the activity of Src family kinases, which increases phosphatase recruitment and activity in the phagocytic synapse and limits phagocytosis. The motor protein myosin IIA, which is dephosphorylated upon SIRP activation in macrophages, is at least one probable crucial step in this situation (Oldenborg, 2013). However, CD47 interaction can also result in signaling. SIRP signaling can be used to analyze several functional facts. Even though we do not go into great depth about CD47 signaling, it is possible that it involves both heterotrimeric Gi protein-dependent and protein-independent pathways. Additionally, signaling may potentially take place through the cytoplasmic tail of an integrin (Johansen & Brown, 2007; Matozaki et al., 2009).

CD47 expression

Normal cells

Macrophages can discriminate cells between "self" and "non-self" based on the expression of CD47 on normal cells (Lindberg et al., 1994; Wu et al., 2018). The SIRP transmembrane protein that is present on myeloid cells, particularly macrophages, binds to CD47 to create the CD47-SIRP signaling complex (Lindberg et al., 1994; McCracken et al., 2015). CD47 can be expressed on the surface of both non-malignant and malignant cells. The extracellular IgV domain of SIRPα will bind to CD47, leading to tyrosine phosphorylation on the intracellular ITIM motif; SIRPα can also bind to the SH2 domain-containing tyrosine phosphatases, to inhibit the accumulation of myosin IIA to facilitate the release of "don't eat me" signals that inhibit macrophage-mediated phagocytosis (McCracken et al., 2015; Per-Arne, 2012). The CD47-SIRP signaling pathway is impaired when the expression of CD47 is diminished (Doyen et al., 2003; Pan et al., 2014). When this occurs, macrophages can approach and phagocytose these cells (Burger et al., 2012; Ishikawa-Sekigami et al., 2006; Klimp et al., 2002). In addition to controlling the immune response, CD47 and its ligands also serve a regulatory role in immunological tolerance, T-cell activation, and mediating pathological processes such as neutrophil chemotaxis and nervous system development (Ratnikova et al., 2017).

Cancer

Cancer cells exploit the "don't eat me" function of CD47 by expressing higher levels of CD47 on their surface compared with non-malignant cells; numerous studies have shown that CD47 is overexpressed in different types of tumors (Kim et al., 2012), including

myeloma (Edris et al., 2012), leiomyosarcoma (Chao, Alizadeh, et al., 2011), acute lymphocytic leukemia (Chao, Tang, et al., 2011), non-Hodgkin's lymphoma (Mohanty et al., 2019), breast cancer (Willingham et al., 2012), osteosarcoma (Russ et al., 2018), and head and neck squamous cell carcinoma (McCracken et al., 2015). For instance, research by Russ et al. showed that higher levels of CD47 expression were linked to a worsened therapeutic response and prognosis in acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) cells when compared to normal myeloid cells from healthy mice or humans (Yang et al., 2019). Normal hematopoietic stem cells' expression of CD47 is elevated during inflammatory conditions and cytokine mobilization (Jaiswal et al., 2010), and leukemia progenitor cells exploit this strategy to avoid macrophage-mediated phagocytosis (Jaiswal et al., 2009). Majeti et al. (2009) and Yang et al. (2019) (Galli et al., 2015) showed that leukemia stem cells from AML patients had higher amounts of CD47 mRNA and protein than normal healthy stem cells and that these increases were linked to a poor prognosis. According to Galli et al. (2015), the expression of CD47 is significantly expressed in 25% of the initial AML samples and is inversely correlated with treatment response and a good prognosis. In contrast to samples of healthy bone, Mohanty et al. discovered that human osteosarcoma samples had greater levels of CD47 expression (Russ et al., 2018). Wang et al. (2015) used immunohistochemistry to detect CD47 in ovarian clear cell carcinoma tissues and demonstrated that patients with low CD47 levels have a higher survival rate than patients with high levels and that CD47 level is a good indicator of a patient's disease stage, resistance to chemotherapy, and prognosis. Brightwell et al. (Nagahara et al., 2010) tested the relationship between CD47 expression and clinical outcome in epithelial ovarian cancer patients using the cancer genome atlas. Immunohistochemical analysis of 265 patient specimens revealed that CD47 expression was found in 210 of 265 (79%) patients, and that of these patients, high levels of CD47 protein expression were found in 129 of 265 (49%) patients. The survival percentage of patients with high CD47 expression in their tumors was significantly lower than that of patients with low CD47 expression levels, according to research by Nagahara et al. (2010), who examined CD47 and SIRP mRNA levels in the bone marrow and peripheral blood of 738 breast cancer patients. On the surfaces of lung cancer cells, SCLC tumors, and non-small cell lung cancer (NSCLC) tumors, CD47 protein, and mRNA levels are also elevated (Stone, 2015). More than 80% of bladder cancer cells have been shown to express CD47, and both muscular invasive bladder cancer (MIBC) and non-muscle invasive bladder cancer (NMIBC) exhibit CD47 expression that is much higher than that of healthy urothelial cells (Yoshida et al., 2015). Yoshida et al. (Sudo et al., 2017) demonstrated that gastric cancer cells that express high amounts of CD47 exhibit greater proliferative potential and spheroid colony formation than do those that do not. In contrast, another study found no difference in CD47 mRNA expression between primary gastric cancer and healthy stomach tissue (Li et al., 2018). Additionally, increased CD47 mRNA expression is not a poor prognostic indicator in primary gastric tumors, suggesting that post-transcriptional variations may result in increased CD47 protein expression and that the dependence of disease progression on CD47 expression may differ between various cancer types and patient populations. Future research should keep attempting to clarify the specific role(s) of CD47 in other cancer types, such as gastric and pancreatic cancer (Li et al., 2018). Table 2 displays an overview of RNA expression (FPKM) in a variety of cancer types in humans.

Table 2. RNA expression of CD47 in various human cancer types based on the human protein atlas (Human Protein Atlas, n.d.; Li et al., 2018).

Cancer Type	RNA Expression (Fragments per Kilobase of transcript per Million mapped reads, FPKM)
Glioma	16.4
Thyroid	24.8
Lung	22.5
Colorectal	15.7
Head and Neck	21.1
Stomach	15.2
Liver	5.3
Pancreatic	15.9
Prostate	19.3
Breast	14.9
Cervical	22.1
Ovarian	40.5
Melanoma	11.6

Impacts of CD47/SIRP-α binding on solid tumors

Hypoxia

Hypoxia-inducible factor-1 (HIF-1) has been shown to activate highly expressed CD47 in various cancer types (Bai et al., 2022; Jia et al., 2021; Martins et al., 2020; H. Zhang et al., 2015). Recent research from the Semenza group demonstrated that HIF-1 activated CD47 transcription in mesenchymal triple-negative primary breast cancer cells (SUM159) and promoted the breast cancer stem cell phenotype, further protecting cancer cells from phagocytosis by bone marrow-derived macrophages (Bai et al., 2022; Viola et al., 2019). According to research by H. Zhang et al. (2015), hypoxia-inducible factor 1 (HIF-1) increases the expression of CD47 in breast cancer cells to aid in phagocytosis avoidance. The inflammatory cytokine-induced elevation of CD47 expression in other malignancies or cancer cells cultivated under normoxic conditions, however, was not explained by that study. However, through the SIRP-CD47 axis, hypoxia may have positive impacts on cancer therapy. Due to increased levels of macrophage infiltration and HIF-1 expression in colon cancer compared to other cancer types, the prognosis is better (Viola et al., 2019). Hypoxia can lower the expression of SIRP in macrophages while raising the expression of CD47 in colon cancer cells. By lowering the level of SIRP expression, the heightened signal of "don't eat me" is offset, enhancing macrophages' ability to phagocytose (Viola et al., 2019). As a result, and depending on the type of malignancy, HIF-1 does have the capacity to accelerate macrophage phagocytosis (Brightwell et al., 2016).

Cellular metabolism

Recent research has shown that macrophages have distinct metabolic traits that are related to their state of function. Inducible nitric oxide synthase (iNOS) is normally expressed by M1 macrophages to create nitric oxide (NO), which is then used to boost glycolytic metabolism while impairing the tricarboxylic acid (TCA) cycle and decreasing oxidative phosphorylation (OXPHOS). Glycolysis is a critical event for M1 macrophages, and the inhibition of glycolysis affects several of the M1 functions, such as phagocytosis, ROS production, and the secretion of pro-inflammatory cytokines.

Glycolytic metabolic reprogramming relies on the activation of many transcription factors, such as hypoxia-inducible factor 1-alpha (HIF-1), which plays a vital role in the commitment to glycolysis under normoxic circumstances (Davies et al., 2019; Viola et al., 2019). Additionally, pyruvate is prevented from entering the TCA cycle by HIF-1α, which promotes pyruvate's conversion to lactate. This conversion is necessary to restore NAD+ and maintain flux through the glycolytic pathway because M1 macrophages have limited OXPHOS. The accumulation of succinate from the TCA cycle's succinate dehydrogenase (SDH) break can also stabilize HIF-1α. This results in the release of ROS, which in turn activates HIF-1α. Succinate also works as a signaling molecule. Prolyl hydroxylases (PHDs), a group of alpha-ketoglutarate (αKG)-dependent dioxygenases that control HIF-1 stability in an oxygen-dependent manner, are inhibited by succinate, which prevents HIF-1 degradation in the presence of oxygen (Davies et al., 2019).

Phagocytic cells use fuels to power cellular metabolism and adapt to microenvironments. Additionally, because every cell is sensitive to changes in its milieu, it is obvious that metabolic changes in immune cells are at the root of a significant portion of disease. In cancer, the competitive advantage of tumor cells in consuming nutrients such as glucose has the profound potential to limit the availability of fuels necessary for immune cell function. Although the metabolism of tumor cells has been extensively studied, it is remarkable that little is known about how tumor cells affect macrophages and other immune cells' immunometabolism (Roberts & Isenberg, 2021). Several mechanisms have been proposed to try to understand the changes in metabolism during active phagocytosis.

First, it has been observed that extracellular citrate, which is present in the blood in minute amounts, can be absorbed into tumor cells at physiological levels and is amplified in hypoxic, glucose-starved environments like those observed in tumors (Roberts & Isenberg, 2021). It would be intriguing to postulate that macrophage activation in tumors can provide extracellular citrate for tumor growth since activated macrophages collect citrate. Furthermore, tumors with high glycolysis rates produce lactic acid, which has the potential to polarize tumor-associated macrophages into a pro-tumor phenotype. There are probably a great number of unidentified fuels and signals linked to tumors that are involved in metabolic changes and macrophage polarization. The caspase-dependent cleavage of peroxisome PPAR, which prevents fatty acid oxidation and causes an accumulation of lipid droplets that promote tumor-associated macrophage (TAM) growth, highlights the possibility of other pathways (Roberts & Isenberg, 2021).

It is well-established that TSP1 promotes cell death via CD47 and that mitochondrial dysfunction is related to this. Genes involved in glycolysis and gluconeogenesis had higher mRNA levels when CD47 was overexpressed. It is still unknown how CD47 controls basal metabolism. Nevertheless, several studies point to a function for the key transcription factor, the avian myelocytomatosis viral oncogene homolog (cMyc). TSP1-CD47 signaling, through regulation of phosphorylation, destabilized cMyc. Additionally, Thbs1- and Cd47-null cells displayed higher basal levels of cMyc mRNA and protein as well as increased proliferation with asymmetric cell division that protects stem cells. Since cMyc is essential for controlling glycolysis, fatty acid balance, and metabolism, it has been hypothesized that TSP1-CD47 negatively affects cellular metabolism by reducing cMyc expression (Maute et al., 2022).

In cancer cells where the MYC promoter has been disrupted, Myc is not suppressed by TSP1-CD47 signaling, in contrast to non-cancerous cells. Epidermal growth factor (EGF) receptor signaling was reduced in triple-negative breast cancer cells by a CD47 antibody that prevents TSP1 binding; EGF itself controls glycolysis. While oxygen is necessary for mitochondria to perform aerobic glucose metabolism, oxygen also regulates the expression of TSP1 and CD47 through its effects on the master transcription factor hypoxia-inducible factor (HIF). It is necessary to confirm TSP1-CD47's capacity to regulate metabolism in hypoxic environments. Even after reoxygenation, certain effects of hypoxia-mediated activation of TSP1-CD47 signaling on gene expression in primary human cells persisted (Maute et al., 2022).

Cancer therapy applications

The increase in CD47 in some cancer types limits their phagocytic clearance by macrophages. Based on the general hypothesis that the interaction between CD47/SIRP-α is a major innate immune checkpoint in cancer, several CD47-targeted antibodies and other CD47 antagonists are entering the early phases of clinical trials. In this review, we will focus on using CD47 antibodies as a monotherapy and in combination with conventional cancer therapeutics (i.e., chemotherapy and immunotherapy, etc.) in preclinical and clinical settings (J. Liu et al., 2015).

Monotherapy

Monoclonal antibodies targeting CD47 or SIRP-α have been developed to block the interaction between SIRP-α and CD47, providing several ways to kill tumor cells depending on the mechanism of action and the agent being used. For example, the direct blocking of CD47 or SIRP-α decreases the "don't eat me" signal, allowing macrophages to perform phagocytosis (J. Liu et al., 2015). Some antibodies that can activate Fc-dependent mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC). In addition to those antibodies, there are also classes of antibodies that can induce apoptosis directly and induce the presentation of tumor antigens by phagocytes to induce CD8+ T cell activation (J. Liu et al., 2015).

The blockade of the CD47/SIRP-α axis with the use of anti-CD47 antibodies has the potential to induce anti-tumor effects in preclinical models (J. Liu et al., 2015). For example, anti-CD47 antibodies have anticancer effects in preclinical animals when the link between CD47 and SIRP is blocked. A humanized anti-CD47 antibody called magrolimab (formerly known as Hu5F9-G4) is based on an Ig G4 scaffold and binds CD47 with a low nanomolar affinity to reduce Fc-mediated effector toxicity for non-tumor cells expressing CD47 (Gholamin et al., 2017). However, it does not directly activate ADCC or CDC or cause apoptosis. Instead, magrolimab binding is sufficient to cause macrophages to phagocytose cancer cells *in vitro* (Maute et al., 2022). Magrolimab exhibits potent monotherapy activity against human hematological malignancies in immunodeficient [NOD/SCID/IL-2Rgnull (NSG)] models (Kathawala et al., 2016), ovarian cancer cell lines, and patient-derived xenograft models, including taxane-resistant tumors, as well as orthotopic xenografts of aggressive pediatric brain

tumor types, without causing harm to other central nervous system cells (Kathawala et al., 2016, Narla et al., 2017). Hematologic and solid tumor cells were phagocytosed by CC-90002 *in vitro*, and multiple myeloma (MM) cell-line xenograft models in vivo demonstrated strong dose-dependent anticancer effects. Significant tumor regression was also seen in xenograft models of acute myeloid leukemia (AML) and triple-negative breast cancer (TNBC) obtained from cell lines and patients (Meng et al., 2019). In the xenograft model, lemzoparlimab (TJC4) treatment fully eliminated Raji cell tumors and prolonged survival in AML xenografted mice (Peluso et al., 2020). Significant antitumor activity with sustained tumor regression was observed across several xenograft models of hematologic malignancies. This anti-tumor activity was accompanied by sustained tumor regression. SRF231-induced phagocytosis of human hematopoietic tumor cell lines by human macrophages (Wilson et al., 2020). AO-176 increased dose-dependent phagocytosis of various hematologic and solid tumor cell types and preferentially induced death in tumor cells while sparing normal cells, including activated T cells. Inhibition of tumor growth was seen in xenograft models of gastric carcinoma, ovarian cancer, TNBC, and lymphoma. In one MM xenograft model, AO-176 caused total remission in every treated mouse that lasted up to 120 days (Sikic et al., 2019).

Phase I clinical trials of anti-CD47 monotherapy have often produced less evidence of efficacy than combination approaches. In 62 extensively pretreated patients with solid tumors or lymphoma, magrolimab monotherapy was assessed. Two ovarian cancer patients experienced objective partial responses (PRs), while one patient with diffuse large B-cell lymphoma (DLBCL) experienced a mixed response (Zeidan et al., 2019). Due to an inadequately encouraging clinical profile, CC-90002 monotherapy in AML and high-risk MDS was discontinued in stage 86; the best response was stable disease (SD) in two patients, and anti-drug antibodies were seen in the majority of patients at all doses (Horwitz et al., 2020; A study of CC-90002). Twenty percent of the 71 individuals who received TTI-621 with dose escalation for R/R NHL ($n = 214$) had objective responses, according to preliminary data (Querfeld et al., 2021). The Composite Assessment of Index Lesion Severity Response (reduction of 50%) was seen in 34% of 29 patients as a result of intra-lesional delivery of TTI-621 in cutaneous T-cell lymphoma, according to preliminary studies (Patnaik et al., 2020). Despite the extended SD being reported, interim SRF231 outcomes in 37 patients with R/R solid tumors showed no complete response (CR) or partial response (PR) (Burris et al., 2021). In an interim review of 27 patients with advanced solid tumors expressing high levels of CD47, AO-176 monotherapy resulted in 1 PR and 7 SD responses (Champiat et al., 2021). Finally, after dose escalation, monotherapy with the SIRP-binding drug BI 765,063 produced clinical benefit in 45% of patients with advanced solid tumors, including one PR (Ye et al., 2023). Table 3 lists more pre-clinical and clinical studies that target CD47 using a monotherapy regimen. Overall, these studies show how preclinical cancer models cannot accurately predict the effectiveness of treatments for actual malignancies.

Combination therapy

The cornerstones of CD47-based agents are SIRP fusion proteins and anti-CD47 antibodies, which have experienced the quickest development and have accumulated a large body of clinical data. After a review of these drugs' effectiveness in treating cancer as

Table 3. Monotherapy regimens targeting CD47 entering pre-clinical or clinical trials.

Drug Name	Drug Classification	Cancer Target	Clinical Trial Phase	Results	NCT Identifier
CC-90002	anti-CD47 mAb	Non-Hodgkin Lymphoma/ Acute Myeloid Leukemia	Phase I	All patients were discontinued (Death/ Progressive Disease/ Lack of Monotherapy Activity)	NCT02641002
AK117	anti-CD47 mAb	Advanced Solid Tumors	Phase I	Completed No results as of the writing of this article	NCT04728334
AO-176	anti-CD47 mAb	Multiple Myeloma	Phase I/II	Completed No results as of the writing of this article	NCT04445701
IBI188	anti-CD47 mAb	Advanced Solid Tumors	Phase I	Completed No results as of the writing of this article	NCT03717103

monotherapies, however, it is uncommon to find positive clinical results. Only 2 of 62 patients with advanced solid cancer showed decreased tumor lesions after receiving magrolimab monotherapy (NCT02216409), the first-in-class anti-CD47 antibody with an IgG4 component (N. Lakhani, 2018). Another anti-CD47 antibody called CC-90002's clinical study (NCT02641002) was halted due to its subpar effectiveness in treating patients with hematological malignancies. Additionally, the SIRP-Fc fusion protein ALX148 (NCT03013218) demonstrated only little anti-cancer activity when used alone (Daver et al., 2022). Contrarily, combination therapies based on CD47 showed more promising therapeutic results than monotherapy. In this review, we will focus on CD47-targeted therapies combined with chemotherapy and immunotherapy.

Clinical trials are being conducted using chemotherapy drugs such as azacitidine (AZA), paclitaxel, doxorubicin, and docetaxel in combination with various CD47 inhibitors. Magrolimab plus AZA produced the most promising phase Ib clinical trial findings of all chemotherapy-related combination methods (NCT03248479). AZA is used to treat individuals with acute myeloid leukemia (AML) and high-risk myelodysplastic syndrome (MDS). According to the phase Ib data, after receiving a six-month combination therapy regimen, patients with MDS had an objective response rate (ORR) of 91% (30/33) and a complete remission rate (CR) of 56% (14/33) of their disease. The ORR and CR/CRi (complete remission/complete remission with partial count recovery) of the combination group were, respectively, 64% (16/25) and 56% in all patients with AML. Thus, the combination therapy demonstrated a higher therapeutic efficacy than AZA alone (S. H. Chen et al., 2021).

Innate and adaptive immune cells are directly stimulated by combination methods between drugs that target PD-L1 (or PD-1) and CD47, increasing their anti-cancer efficacy. After receiving either a CD47/PD-L1 bispecific antibody treatment (Sockolosky et al., 2016) or a CD47 antagonist in combination with an anti-PD-L1 antibody (S. R. Gordon et al., 2017), increased macrophage phagocytosis was seen. The discovery that the PD1-PD-L1 axis is yet another anti-phagocytosis checkpoint (Roohullah et al., 2021) may help to explain this result. The primary function of T cells in the combination scenario, aside from macrophages, is the eradication of cancer cells. The effectiveness of PD-1-PD-L1 and CD47-SIRP inhibition was required for a robust CD8+ T cell response, and their absence eliminated the impact.

In the clinical stage, the combination strategies for targeting PD-L1 (or PD-1) and CD47 might be classified into bispecific antibodies and co-treatment with anti-PD-L1 (or anti-PD-1) antibodies and drugs targeting CD47. Bispecific antibodies have been studied in clinical trials up to this point, including PF-07257875 (CD47/PD-L1 bispecific antibody), HX009 (PD-1/CD47 bispecific antibody), and IBI322 (CD47/PD-L1 bispecific antibody). The phase I outcome of HX009, which was employed for the treatment of advanced solid tumors, showed that doses up to 7.5 mg/kg of HX009 were well-tolerated without any dose-limiting toxicities (J. Wang et al., 2022). Furthermore, 1 and 5 mg/kg cohorts showed anticancer efficacy with objective responses in a variety of tumor types (NCT04097769). IBI322 preliminary phase I data in patients with advanced solid tumors were published. IBI322 demonstrated a good safety profile and was well tolerated. After receiving IBI322 at active doses (N. J. Lakhani et al., 2020), 4 of the evaluable 20 patients showed partial response (PR), and 7 showed complete response (SD). Treatments using anti-PD-L1 and anti-CD47 antibodies also merited clinical investigation. In recruited platinum-resistant or refractory ovarian cancer patients, a phase Ib clinical study about magrolimab paired with anti-PD-L1 antibody avelumab observed a 56% SD rate (De Silva et al., 2021).

The immunosuppressive properties of CTLA-4 are caused by its expression on Tregs. Anti-CTLA-4 use can reduce Treg numbers, partially through ADCP (Ingram et al., 2017). In a coculture system between Treg and macrophages, an inhibitor targeting CD47 along with an anti-CTLA-4 antibody demonstrated an accelerated phagocytosis of Tregs (A. Zhang et al., 2021). However, occasionally immune-related adverse effects (irAEs) are caused by the unselective depletion of Tregs in the periphery caused by anti-CTLA-4 antibodies. An anti-CTLA-4-SIRP (hIgG1 with effector function) heterodimer was created to lessen the binding of drugs targeting CD47 to RBCs. In many immunocompetent mouse models, anti-CTLA-4-SIRP treatment showed superior tumor suppression compared to the co-treatment with anti-CTLA-4 and SIRP-Fc. Mechanism research showed that the heterodimer's synergistic action was dependent on the precise ADCP of macrophages to tumor Tregs (ICOS-high immunosuppressive Tregs) (Olaoba et al., 2023). Table 4 lists more pre-clinical and clinical studies that target CD47 in a combination regimen.

Table 4. Combination therapy regimens targeting CD47 entering pre-clinical or clinical trials.

Drug Name	Drug Classification and Co-Treatment	Cancer Target	Clinical Trial Phase	Results	NCT/CRT Identifier
CC-90002	anti-CD47 mAb + Rituximab	Hematological Cancer	Phase I	Overall Response Rate: 13% Disease Control Rate: 25% One patient achieved complete response with two partial responses	NCT02367196
IBI188	anti-CD47 mAb + Azacitidine	Acute Myeloid Leukemia	Phase I/II	Completed No results as of the writing of this article	CTR20200938
TG-1801	CD47-CD19 + Ublituximab	B-cell Lymphoma	Phase I	Combination therapy was well tolerated without dose limiting toxicity 6 partial responses with one complete response	NCT03804996
IBI-322	CD47 + PD-L1 Bispecific Antibody	Advanced Solid Cancer	Phase I	11 achieved disease control rate with no complete responses and 4 partial responses	NCT04912466

Challenges

With the promising potential of CD47 antibodies entering the preclinical immunotherapy field, there is an ongoing interest in expanding CD47 antibodies into clinical trials. Phase I studies have shown that treating tumors with CD47 antibodies can result in a significant reduction in tumor burden. However, the target dose for future Phase II clinical trials for CD47-targeting antibodies has not been determined. Therefore, several challenges must be examined to determine the risks associated with biosafety and the efficient development of these antibodies to help understand potential clinical outcomes.

Off target effects

The therapeutic efficacy of CD47-SIRP-blocking mAbs is severely constrained due to significant RBC toxicity and quick target-mediated clearance because CD47 is widely expressed on normal cells. According to Buatois et al.'s findings, Hu5F9-G4 by itself or in combination with other antibodies may unintentionally destroy normal RBCs, perhaps leading to anemia (Advani et al., 2018; Buatois et al., 2018). To lessen this on-target cytotoxicity, many techniques are being used in preclinical research for CD47-targeting bsAbs. Using a priming strategy, RBC depletion toxicity can be avoided. Before inducing compensatory hematopoiesis and eradicating aging RBCs, patients get low-dose therapy.

To overcome these drawbacks and enhance therapy effectiveness, new generation CD47 antibodies were discovered. These antibodies effectively target tumor cells while having little effect on RBCs, preventing severe anemia. In addition, a few carefully crafted bsAbs have been created that selectively bind to CD47 on tumor cells with the aid of more potent bispecific molecules targeting an additional tumor target (Z. Jiang et al., 2021). IMM2902 for CD47-Her2, IMM0306 for CD47-D20, and IBI322 for CD47-PD-L1 are a few of these (Weiskopf et al., 2013). They have minimal impact on RBCs but dramatically improve in vitro tumor cell phagocytosis and efficiently suppress the CD47-SIRP signal. Hu5F9-G4 and TTI-621, two CD47-targeting medications, have been proven to cause severe anemia and thrombocytopenia in humans (Sikic et al., 2019). Anti-CD47 antibody toxicity in platelets and erythrocytes appears to be Fc-dependent. In contrast, high-affinity SIRP monomers, anti-CD47 antibodies, and SIRP-Fc fusion proteins are hazardous (J. Liu et al., 2020). These findings suggest that to create novel therapies with minimal side effects, future research should concentrate on optimizing the structure of anti-CD47 bsAbs. Liu et al. successfully treated anemia using the creation of 5F9, which is currently undergoing multiple clinical trials and did not see any major recurrent side effects of thrombocytopenia, thrombosis, or hypertension (Weiskopf, 2017).

Antigen sink

The presence of CD47 on the surface of normal tissues may have an "antigen sink" impact during the production of anti-CD47 therapeutic antibodies, preventing them from attaching to the intended tumor cells *in vivo* (Lindberg et al., 1994). Due to CD47's widespread expression, high beginning dosages and/or frequent administrations may be necessary for a medication to effectively block CD47 for therapeutic purposes. For instance, an "initiation dose" was followed by a "therapeutic dose" in a phase I trial of Hu5F9-G4 (Z. Jiang et al.,

2021). In contrast, SIRP has a more constrained histological distribution compared to CD47, which may result in less toxicity and more blockage when therapeutically targeted (Q. Chen et al., 2019). However, SIRP is highly expressed on myeloid cells as well as cells of the central and peripheral nervous systems; therefore, when employing therapies that target SIRP, the risk of neurological adverse effects should be considered. Additionally, because of their sequence closeness, additional SIRP family members (SIRP-β and SIRP-α) may react with one another, and human SIRP has at least 10 known polymorphisms. It is still unclear what will happen if these various receptor isoforms are targeted. Future research should investigate ways to specifically target CD47 and its ligands on tumor cells; these could be new drug delivery systems like modified biomimetic nanoparticles or quorum-sensing bacteria. A recent study, for instance, demonstrated that a bio-responsive fibrin gel solution containing anti-CD47-conjugated nanoparticles modulates the immune response and induces the phagocytosis of tumor cells by blocking CD47; in addition, this treatment led to an enhanced T cell-mediated immune response and the activation of tumor-associated macrophages (Chowdhury et al., 2019).

Another recent study by Chowdhury and colleagues showed that using quorum-sensing bacteria to deliver a single-chain antibody fragment that targets CD47 results in systemic anti-tumor immunity and slows the growth of tumors (Trabulo et al., 2017). Iron oxide magnetic nanoparticles with several functions have been created as delivery systems for gemcitabine and anti-CD47 antibodies, as well as for the targeted therapy of pancreatic cancer (X. J. Liu et al., 2017). Nanoparticle-mediated delivery of the anti-CD47 antibody to tumor cells generated more PDX models of PDAC apoptosis compared with free antibodies in the treatment of pancreatic cancer. The medicines supplied by nanoparticles did not show extra cytotoxicity. Nanoparticles with mitomycin A can also be employed to reduce CD47 expression and improve the therapeutic effectiveness of BxPC-3 tumor xenografts in athymic mice (Davis et al., 2018). In addition, Davis et al. loaded SERS nanoparticles with anti-CD47 antibodies and demonstrated using Raman spectroscopy that the antibody-laden nanoparticles specifically targeted ovarian cancer cells (Tseng et al., 2013). Future research could improve nanoparticle delivery techniques, for instance, to stop the reticuloendothelial system from clearing the circulating nanoparticles. Drug-loaded nanoparticles that target CD4 may eventually be created for the treatment of cancers that overexpress CD47 if nanomedical technologies continue to develop at the current rate. It is also likely that intra-tumor drug administrations will result in higher drug delivery and efficacy and lessened toxicity as compared to intra-peritoneal or subcutaneous delivery routes. This is in addition to innovative delivery modalities.

Efficacy in combination therapy regimens

The effectiveness of anti-CD47 mAb therapy may also be diminished using T-cell checkpoint inhibitors in conjunction with tumor cell-specific antibodies. Because CD47 is typically blocked when cancer cells are phagocytosed by macrophages or dendritic cells, these phagocytes may transmit tumor antigens to T cells, inducing anti-tumor T-cell responses (Obeid et al., 2007). The timing of multiple treatment plans may potentially have an impact on the anti-CD47 antibody activity in combination therapy. For instance, the off-target effect may be accentuated if anti-CD47 antibody therapy is paired with radiation or chemotherapy. Calreticulin expression can be increased in normal cells in

response to stressors like radiation or chemotherapy that uses anthracyclines. By boosting calreticulin overexpression, which may limit the expression of surface proteins and accelerate apoptosis, radiation or chemotherapy combined with an anti-CD47 treatment regimen may have unintended side effects (X. Liu et al., 2015). Similar to this, when administered right before anti-CD47 antibodies in syngeneic cancer models, paclitaxel and cyclophosphamide can work together to enhance the development of memory responses and promote the development of anti-tumor immunity. However, they decreased immune-mediated cancer treatment when these chemotherapeutics were administered after anti-CD47 antibodies (Chao et al., 2010).

Anti-CD47 mAb must be paired with an IgG1 due to its capacity to connect firmly with FcRs to activate complement and elicit the 'eat me signal' because it is a human IgG4 antibody with modest FcR interaction activity and cannot elicit the 'eat me signal' alone. IgG4-specific antibodies, however, might be an alternative due to the hematological toxicity brought on by IgG1. Combining CD47 mAb with another human monoclonal IgG4-specific antibody, such as anti-PD-1, may be a viable cancer treatment. According to a recent study, using anti-CD47 with human IgG1 can prevent the hematological toxicity of IgG1. Use of IMM01, which may activate the "Eat me signal" and "Don't eat me signal" (Weiskopf et al., 2013).

In a mouse model of non-Hodgkin lymphoma, the combination of an anti-CD20 antibody (Rituximab) and an anti-CD47 antibody (BRIC126 or B6H12) led to the ablation of human cancer cells (Buatois, Johnson, Salgado-Pires, Papaioannou, Hatterer, Chauchet, Richard, Barba, Daubeuf, Cons, Broyer, D'Asaro, et al., 2018). Such strategies can be applied to boost tumor selectivity and reduce on-target toxicity in non-malignant cells that express CD47. Another bispecific antibody (NI-1701) that targets CD47 and CD19 was created for B-cell lymphoma and refractory leukemia using a similar strategy (Weiskopf et al., 2016). A fusion protein that targets CD47 and PD-L1 has also been found to have anti-tumor efficacy by inducing an adaptive immune response. High-affinity SIRP proteins that block CD47 also have stronger phagocytotic and anti-tumor effects when paired with tumor-opsonizing antibodies like rituximab, trastuzumab, or cetuximab, or the anti-CD56 antibody, Lorvotuzumab (Hoos et al., 2010). Anti-SIRP-α antagonists have also been used in combination with tumor opsonizing antibodies (i.e., Rituximab) to show anti-tumor efficacy *in vitro* and xenograft lymphoma mouse models and syngeneic colon cancer mouse models. Future advancements in cancer screening and precision medicine may make it possible to identify and stratify tumor types and/or stages of cancer that would be most amenable to treatment with a certain type or types of anti-CD47 treatment. Clinical trials have already started to evaluate CD47-blocking agents in combination with rituximab, cetuximab, and trastuzumab.

Treatment resistance

The emergence of resistant cancer cells and tumor heterogeneity are common causes of therapeutic failures in cancer treatment when utilizing an immunotherapy approach. The adaptability and plasticity of cancer signaling networks have been known to contribute to the development of treatment resistance. Intrinsic mechanisms of tumor immune resistance include modifications to tumor cell signaling pathways, adjustments to anti-tumor

immune response pathways, and other modifications to tumor cells to support an immunosuppressive microenvironment. There are also external factors that can contribute (i.e., an increase in tumor cell proliferation and immune checkpoint inhibitor resistance) to supporting an immunosuppressive environment. Changes in the tumor microenvironment, drug inactivation, reduced drug absorption or greater drug release from the tumor cells, activation of tumor cell survival pathways, and epigenetic modifications are other mechanisms that lead to treatment resistance. The overall response of the anti-CD47 therapy in pre-clinical research may be impacted by the variance in the type of tumor model utilized to examine the therapeutic efficacy of medicine (Buatois et al., 2018).

Clinical study design

It is crucial to consider the roles and phenotypes of immune cells, such as tumor-specific cytotoxic T lymphocytes while determining the endpoints of a clinical trial (Buatois, Johnson, Salgado-Pires, Papaioannou, Hatterer, Chauchet, Richard, Barba, Daubeuf, Cons, Broyer, D'Asaro, et al., 2018). Instead of attacking and eliminating tumor cells, cancer immunotherapy includes stimulating the immune system. As a result, their actions may be postponed during the clinical trial. It is crucial to check if the endpoints employed in anti-CD47 clinical studies are sufficient for assessing the effectiveness of cancer immunotherapy (Hoos & Britten, 2012). Endpoints should be considered when assessing the effectiveness of cancer immunotherapy to gauge long-term disease-free survival since they occur with different cancer immunotherapies, which cause the establishment of immunological memory. For instance, anti-CTLA-4 ICB tremelimumab clinical research was abruptly terminated due to a lack of progress. As a result, it became clear that evaluating cancer immunotherapies would require its own set of clinical trial approaches. After a follow-up evaluation at 24 months into a clinical trial, a split in the survival curves for the study population was noted. To allow for the establishment of an improvement in OS, the endpoints of two additional phase 3 trials of ipilimumab were extended. This provided enough time for the FDA to approve this drug for the treatment of melanoma (Sosale et al., 2015). Minimizing or preventing injury to normal cells while exerting anticancer effects is one of the challenges to consider while creating anti-CD47 medicines.

Other challenges

There is a chance that some tumor cells lack the physiological ability to be phagocytized. The research by Discher and colleagues showed that the size, shape, and rigidity of antibody-coated red blood cells affected their capacity to be absorbed. Small, rigid, spherical red blood cells were phagocytized more efficiently than huge, squishy, or irregularly shaped red blood cells. It is reasonable to infer that after anti-CD47 therapy, excessively large, severely distorted, or irregularly shaped tumor cells may not be successfully absorbed by macrophages (Sosale et al., 2015).

Autoimmunity may be one of the most significant side effects of CD47-SIRP immune checkpoint immunotherapy, comparable to other treatments that use immune checkpoint inhibitors to treat cancer. Due to increased myelin absorption and presentation by APC, the disruption of CD47–SIRP interactions in an experimental model of autoimmune

encephalomyelitis worsened the disease's symptoms. It has been established that both SIRP and CD47 are essential for controlling leukocyte migration. For the elimination of cellular waste, restoration of tissue homeostasis, and resolution of inflammation, leukocyte recruitment to tissues during inflammation is crucial (Bian et al., 2016; Y. Liu et al., 2001).

Anti-CD47 monoclonal antibodies have been demonstrated to inhibit neutrophil migration. Due to the body's inability to utilize stored iron to produce enough healthy red blood cells, this could result in the development of anemia or inflammation. Based on this hypothesis, Bian and colleagues demonstrated in their study that LPS- or cytokine-driven inflammation, colitis, or persistently generated peritonitis all resulted in severe anemia in CD47-/- and SIRP-/- animals. This is a result of the splenic macrophages' quick phagocytosis of their red blood cells. The shortened erythrocyte lifespan brought on by increased erythrophagocytosis by cytokine-activated macrophages cannot be compensated for by the reduced erythropoiesis brought on by anemia from inflammation. When used in conjunction, anti-CD47 antibody delivery in individuals with chronic inflammatory disease may result in undesirable side effects.

Conclusions

CD47 is overexpressed in a variety of cancer types, and the blockade of the CD47-SIRP-α axis has been known to enhance the capability of macrophages to phagocytose tumor cells, making it a promising treatment regimen. However, the efficacy of anti-CD47 treatment in tumors has varied in pre-clinical and clinical models due to various challenges such as antigen sinks and off-target effects. This makes evaluating the true clinical efficacy of these therapies difficult. Therefore, it is necessary to consider these challenges when investigating the biology behind the CD47-SIRP-α axis concerning macrophage function and expression in cancer.

Disclosure statement

No potential conflict of interest was reported by the authors.

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