Blockade of CD200-CD200R1 signaling axis can upregulate phagocytosis of cancer cells

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Abstract

In the field of anti-cancer immunotherapy, there is exciting progress in recent years which calls for more investigation about immunoregulation. Immune checkpoint blockade therapy is one of the most significant breakthroughs in immunotherapy. Previous research related to checkpoint blockade paid great attention to adaptive immunity, however, more and more potential therapeutic targets were discovered on innate immune cells like macrophage. Signal regulatory protein alpha (SIRPα) has been proved to be an important regulator of phagocytosis on macrophage, and the blockade of interaction between SIRPα and its ligand CD47 can trigger the phagocytosis of cancer cells. However, due to the redundancy of inhibitory signals in immunoregulation, there should be other immune checkpoints regulating phagocytosis on macrophage. CD200-CD200R1 signaling axis has been reported to be important for graft survival, hence, we hypothesize that CD200-CD200R1 signaling axis might regulate the function of macrophage. By utilizing bone-marrow-derived macrophages (BMDMs) to do in vitro phagocytosis assays, we collected supportive evidence for our hypothesis. We found out that CD200-CD200R1 signaling axis is capable of controlling the phagocytosis ability of BMDMs. Our results exhibited that CD200 expression can directly protect cancer cells from being phagocytosed by BMDMs. We further demonstrated that the blockade of interaction between CD200 and its inhibitory receptor on macrophages, CD200R1, can enhance phagocytosis. We also confirmed that knocking-out CD200R1 on BMDMs can enhance phagocytosis significantly. Moreover, we found out that the inhibitory function of CD200R1 is dependent on NPXY motif. Taken together, our results suggest that CD200-CD200R1 signaling axis is a critical regulator of the activity of macrophages, and it is a potential target for anti-

cancer immunotherapy.

Résumé

Dans le domaine de l'immunothérapie anticancéreuse, des progrès intéressants ont été réalisés ces dernières années, qui appellent de nouvelles recherches sur l'immunorégulation. La thérapie de blocage aux points de contrôle immunitaire est l'une des percées les plus importantes en immunothérapie. Des recherches antérieures sur le blocage des points de contrôle accordaient une grande attention à l'immunité adaptative. Cependant, de plus en plus de cibles thérapeutiques potentielles ont été découvertes sur des cellules immunitaires innées telles que les macrophages. La protéine régulatrice de signal alpha (SIRPα) s'est révélée être un important régulateur de la phagocytose sur les macrophages et le blocage de l'interaction entre SIRPα et son ligand CD47 peut déclencher la phagocytose des cellules cancéreuses. Cependant, en raison de la redondance des signaux inhibiteurs dans l'immunorégulation, il devrait exister d'autres points de contrôle immunitaires régulant la phagocytose sur les macrophages. L'axe de signalisation CD200-CD200R1 a été signalé comme étant important pour la survie du greffon; par conséquent, nous émettons l'hypothèse que l'axe de signalisation CD200-CD200R1 pourrait réguler la fonction du macrophage. En utilisant des macrophages dérivé de la moelle osseuse (BMDMs) pour effectuer un test de phagocytose in vitro, nous avons recueilli des preuves à l'appui de notre hypothèse. Nous avons découvert que l'axe de signalisation CD200-CD200R1 est capable de contrôler l'aptitude à la phagocytose des BMDMs. Nos résultats ont montré que l'expression de CD200 peut directement protéger les cellules cancéreuses d'être phagocytées par les BMDMs. Nous avons en outre démontré que le blocage de l'interaction entre CD200 et son récepteur sur le macrophage, CD200R1, pouvait potentialiser la phagocytose. Nous avons également confirmé que l'inactivation de CD200R1

sur les BMDMs peut considérablement améliorer la phagocytose. De plus, nous avons découvert que la fonction inhibitrice de CD200R1 dépend du motif NPXY. Dans l'ensemble, nos résultats suggèrent que l'axe de signalisation CD200-CD200R1 est un régulateur essentiel de l'activité des macrophages et constitue une cible potentielle pour l'immunothérapie anticancéreuse.

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Author contributions and statements of originality

The work described in this study was performed by the author, and this study is novel and original. This study demonstrates that CD200-CD200R signaling axis can inhibit phagocytosis by macrophage for the first time.

List of Abbreviations

Abbreviation	Definition
LPS	Lipopolysaccharide
PRR	Pattern recognition receptor
PAMP	Pathogen associated molecular pattern
TLR	Toll-like receptor
DAMP	Damage-associated molecular pattern
MHC	Major histocompatibility complex
APC	Antigen-presenting cell
IFNγ	Interferon gamma
CAR	Chimeric antigen receptor
TCR	T-cell receptor
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
ITIM	Immunoreceptor tyrosine-based inhibition motif
SIRPα	Signal regulatory protein alpha
CD200R	CD200-receptor
IgSF	Ig superfamily
SLAMF7	Signaling lymphocytic activation molecule F7
SFRKO	SLAM family receptor knock-out

1 Introduction

1.1 Background

1.1.1 Cancer

Cancer is a multi-factorial disease and the causes of cancer are quite complicated. There are many factors which can increase the risk of cancer, including viral infections, smoking and tobacco, exposure to sunlight and other forms of radiation, and sedentary lifestyle (1). Cancer is a serious health problem all over the world, which is one of the leading causes of death, constituting great burden in both economically developed and developing countries (2). It has been estimated that cancer claimed 7.6 million people's lives in 2008, 8.2 million in 2012 and 9.6 million in 2018 (3-5). The increasing deaths caused by cancer call for more advancement of medical care, more intensive research and more invention of novel therapy.

Cancer can be divided into different types based on different criteria. According to histology, cancer can be classified as carcinoma, sarcoma, myeloma, leukemia and lymphoma (6). Besides, location is another criterion for nomenclature (7), for example, "breast cancer" refers to a tumor originating from breast. Furthermore, cancer can be categorized into distinctive stages on the grounds of tumor size, depth of invasion and presence of metastasis, so as to facilitate the selection of treatment (8).

It is well-known that cancer is a tough enemy to fight against since it is capable of resisting death, sustaining proliferation and activating invasion and metastasis (9). Up to now, there are various kinds of mature treatments targeting cancer, including surgery, radiotherapy, chemotherapy, endocrine therapy and viral therapy (10). Unfortunately, these established

therapies cannot yield ideal effects as expected and they might bring about unpleasant side effects. For instance, chemotherapy cannot work anymore after cancer cells develop drug resistance; and endocrine therapy can cause bone loss and lead to fracture (11, 12). To achieve better treatment outcome for patients with cancer, a mass of research has been done with immune system in recent decades, which is a potential powerful anti-cancer weapon.

1.1.2 Immune system

Mammals live in an environment where there are numerous pathogens, toxins and allergens which can threaten their homeostasis. Under these challenges, mammals are well protected by immune system, which can defend against and eliminate vicious intruders, and can avoid exerting damages to self-tissues at the same time (13). Immune system is divided into two major groups – innate immunity and adaptive immunity, depending on the speed and specificity of response (14).

The first group, innate immunity can provide immediate host defense by utilizing hematopoietic cells (like macrophages, dendritic cells, neutrophils, mast cells and natural killer cells), non-hematopoietic cells (like epithelial cells located in the respiratory tracts) and humoral components (like compliment proteins and lipopolysaccharide (LPS) binding proteins) (15). To recognize potential pathogens and to leave self-tissue safe and sound simultaneously, innate immunity takes advantage of three strategies. First, innate immunity depends on pattern recognition receptors (PRRs), a circumscribed group of germline-encoded receptors, which are able to detect pathogen associated molecular patterns (PAMPs) - conserved and common

structures on a vast array of microbes (15, 16). PRRs are expressed on immune cells like macrophages, dendritic cells and neutrophils; and they are composed of several families including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and DNA receptors (17). As the most studied PRRs, TLRs are regarded as the major sensors of PAMPs (17). At different localizations, TLRs bind distinct ligands: TLRs on cell surfaces can detect microbial lipids and proteins, and TLRs located in intracellular vesicles can recognize microbial nucleic acids (18). Second, innate immunity is activated by the upregulation of damage-associated molecular patterns (DAMPs), which are molecules representing dangerous conditions like inflammation and infection (15). When the physiological condition is in normal state, DAMPs will be concealed and not detected by immune system; while the organism is exposed to stress or injury, stressed immune cells would secrete DAMPs actively and dying cells would release DAMPs passively (19). DAMPs are released or secreted from various origins including nucleus, cytosol, mitochondria and endoplasmic reticulum (20). Some DAMPs can recruit and activate immune cells, like High Mobility Group Box 1 (HMGB1) from nucleus and uric acid crystals from cytosol; some DAMPs are responsible for maintaining tissue homeostasis, like phosphatidylserine (PS) and B-cell CLL/lymphoma 2 (BCL2); and some DAMPs act as bystanders during the immunogenic cell death, like heat shock protein 90 kDa (HSP90) (20, 21). Third, innate immunity identifies “non-self” substances by checking molecules expressed by healthy and normal cells only (15). This strategy is well utilized by natural killer (NK) cells. NK cells express inhibitory receptors which are specific for major histocompatibility complex (MHC) class I molecules, and these receptors prevent NK cells from attacking cells with expression of MHC class I molecules (22). NK cell cytotoxicity will

only be elicited when there is engagement between activating receptor on NK cell and activating ligands on target cell, and no engagement between inhibitory receptor and MHC class I molecules (23).

In contrast to innate immunity, adaptive immunity can recognize much broader range of pathogens with high degree of precision, and it also requires much longer response time, ranging from several hours to days (24). The ability of adaptive immunity to recognize wide range of pathogens comes from a critical event - V(D)J recombination, which takes place during the early developing stages of T and B cells (25). V(D)J recombination refers to DNA rearrangements within the exons encoding antigen binding domains of immunoglobulins and T cell receptors, which involve in three kinds of gene segments – variable (V), diversity (D) and joining (J) (26). Adaptive immunity consists of two major parts – cellular immunity and humoral immunity, whose effector cells are T cells and B cells respectively (24). Progenitors of T cells migrate to thymus and generate $CD4^+CD8^+$ double-positive thymocytes; after positive selection, $CD4^+$ or $CD8^+$ single-positive thymocytes come into being (27). Naïve $CD4^+$ T cells can differentiate into T helper (Th) cells (including Th1, Th2, Th17), induced Tregs (iTregs) and T follicular helper (Tfh) cells upon different stimuli (28). Each class of $CD4^+$ T cells has distinctive functions: Th cells are cytokine-producers and they are capable of activating and recruiting immune cells; iTregs are critical for immunological tolerance and they can suppress immune response; Tfh cells are necessary for the development of germinal center and they can induce proliferation of germinal center B cells (29-31). Naïve $CD8^+$ T cells get primed by interacting with antigen-presenting cells (APCs), resulting in rapid proliferation and differentiation into cytotoxic T lymphocytes (CTLs) (32). CTLs are able to get rid of infected

cells by secreting cytokines like interferon gamma (IFNγ) and tumor necrosis factor (TNF), and secreting molecules comprising perforin, granzymes and Fas-ligand (FasL) (33). As for B cell development, it is a long process which consists of multiple stages. Common lymphoid progenitor cells develop to pro-B cells, pre-B cells, and immature B cells sequentially in bone marrow, and immature B cells migrate to spleen so as to complete maturation (34). There are three classes of mature B cells: 1) follicular B cells, which are the majority and locate in the lymphoid follicles of lymph nodes and spleen; 2) marginal zone B cells, which reside in the region between the red and white pulp in the spleen; 3) B1 cells, which have the highest chance to contact pathogens since they live in peritoneal cavity, pleural cavity and mucosal sites (35). B1 and marginal zone B cells are able to respond to T cell-independent antigens (TI antigens) and they can differentiate into short-lived plasmablasts; while follicular B cells are specialized to T cell-dependent antigens (TD antigens) and they can differentiate into memory B cells or long-lived plasma cells (35). Both plasmablasts and plasma cells are capable of producing antibodies (36). Antibodies play a significant role in immune response because of their diverse functions – antibodies can neutralize pathogens, direct cytotoxic destruction, and mediate phagocytosis (37). Apart from secreting antibodies, B cells can regulate the functions of T cells, dendritic cells and APCs by releasing cytokines; they are also in charge of the organization of lymphoid tissue; they can control wound healing and influence tumor development as well (38).

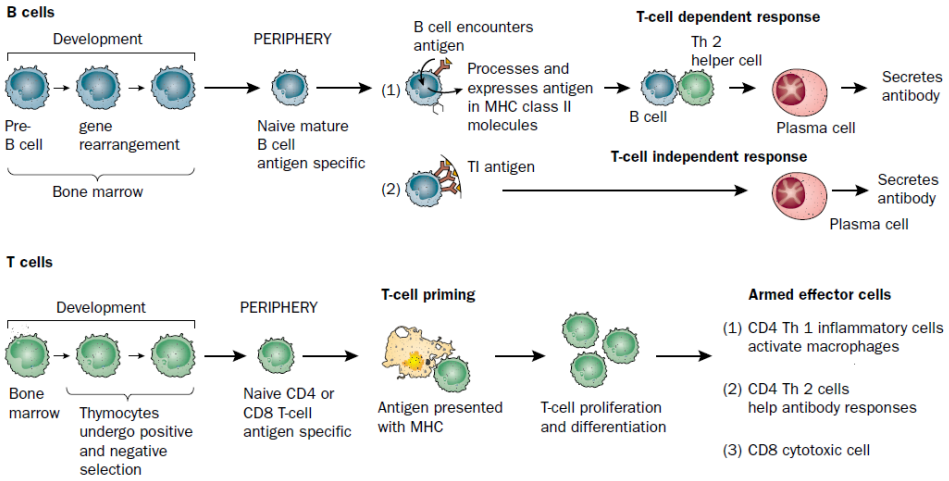

Figure 1.1. The development process and role of T and B cells (14).

Immune system holds a strong bond with human diseases, and either hyperactivity or hypoactivity of immune system can lead to complications. When immune system has trouble in distinguishing pathogens from self-tissues, incorrect immune response will come up and exert damage on healthy tissues, leading to autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (39). Defects of immune system development or function can render primary immunodeficiency disorder (PID), which consists of more than 250 disorders and makes individuals more susceptible to infection (40). Besides, uncontrolled overgrowth of immune cells can cause hematologic malignancies directly, like lymphoma, myeloma and leukemia (41). Lymphoma origins from B, T and NK cells; myeloma comes from plasma cells; and leukemia rises from leukocytes in blood and bone marrow (42-44).

1.1.3 Anti-cancer immunotherapy

More than 100 years ago, people started trying to harness immune system to combat cancer. In 1868, a German physician named Busch was the first one to inoculate cancer patients with

erysipelas on purpose, and such treatment could shrink tumor (45). In 1882, Fehleisen, another German physician, repeated such treatment and he figured out that *Streptococcus pyogenes* were the causative agent of erysipelas (45). 9 years later, William B. Coley, an American surgeon, injected streptococcal organisms into a patient bearing sarcoma so as to cause erysipelas and boost the activity of immune system (46). As a result, tumor in the patient vanished due to the attack from stimulated immune system (46). Coley was honored as the "Father of Immunotherapy" because of his astounding contribution and lifelong dedication to the development of immunotherapy (46). However, since Coley could not provide reasonable interpretation for how such treatment worked, there were a lot of controversies and criticism about his practice (46). Unfortunately, even though Coley's discovery implied the tremendous power of immunotherapy, the pace of development of immunotherapy was nearly static until 1957, in which year interferon was discovered (47). In the following decades, immunotherapy regained attention and got back on track: dendritic cells and NK cells were discovered in 1973 and 1975 respectively; in 1986, interferon-α (IFNα) was approved for treatment of hairy cell leukemia; in 1990, a kind of germ called Bacillus Calmette-Guerin was approved for treatment of bladder cancer; and in 1992, interleukin-2 (IL-2) was approved for treatment of renal cell carcinoma (47-49). After entering the 21st century, the development of novel immunotherapy got increasingly rapid. Up to now, immunotherapy can be classified into different types: non-specific immunotherapy, cancer vaccine, oncolytic virus therapy, adoptive cell transfer therapy and checkpoint blockade therapy.

1.1.3.1 Non-specific immunotherapy

The principle of non-specific immunotherapy is relatively simple – to stimulate and boost immune system by cytokines so as to increase the possibility of eliminating cancer cells. Many cytokines have been studied in preclinical murine cancer models, and some of them demonstrated anti-cancer capacity, such as IL-2, IL-12, IL-15, IL-21, and IFNα (49). IL-2 and IFNα have already been approved by FDA for cancer treatment in last century.

IL-2 is majorly produced by antigen-stimulated $CD4^+$ T cells, and it is critical for both the differentiation of $CD4^+$ T cells into different subsets and the maintenance of Treg cells (50). However, the application of IL-2 is highly restricted due to several drawbacks of IL-2. First, IL-2 is able to exert effects on both effector T cells and Treg cells, leading to dual functions; second, high dose of IL-2 is required to obtain the optimal effects, which brings about toxicities like vascular leak syndrome and hypotension (50). As for IFNα, it is predominantly produced by plasmacytoid dendritic cells; it can enhance the antigen-presenting ability of dendritic cells, activate NK and $CD8^+$ T cells, and down-regulate the proliferation of Treg cells (51). Similarly, IFNα treatment can also cause strong side effects like flu-like symptoms, elevated transaminases, nausea and fatigue, which might outweigh therapeutic benefits (52). To minimize side effects and achieve better clinical results, cytokines have been combined with other forms of immunotherapies. For instance, combinational therapy using both IL-15 and anti-CD40 antibody can significantly prolong the survival of mice bearing colon cancer, compared with monotherapy using either IL-15 or anti-CD40 (53).

1.1.3.2 Cancer vaccine

Cancer vaccine is divided into two categories: preventive vaccines and therapeutic vaccines. Currently, there are two successful preventive vaccines: human papillomavirus (HPV) vaccine and hepatitis B vaccines, which can block the infection of HPV and HBV, so as to avoid cervical cancer and liver cancer respectively (54).

Cancer cells can be distinguished from normal cells by immune system because of their specific antigens which are not expressed or significantly less expressed by normal cells (55, 56). These antigens on tumor cells have been taken advantaged by therapeutic cancer vaccine, which loads tumor antigens or manipulated immune cells into patients by injection. At injection site, tumor antigens would encounter and be taken up by APCs like dendritic cells; next, APCs would transfer to lymph nodes and present tumor-derived peptides to $CD4^+$ and $CD8^+$ T cells, so as to activate T cells and promote tumor-specific T cell responses; finally, activated T cells would move to tumor sites and kill tumor cells that express cognate antigens (57). In 2010, Sipuleucel-T was approved by FDA for the treatment of prostate cancer, and it is the first FDA-approved therapeutic cancer vaccine (58). Sipuleucel-T is composed of 3 steps: 1) Isolate autologous dendritic cells from patients' blood by leukapheresis; 2) culture dendritic cells with a fusion protein named PA2024, which is composed of the antigen prostatic acid phosphatase (PAP) and granulocyte-macrophage colony stimulating factor (GM-CSF) (PAP is expressed by prostatic cells only; GM-CSF helps dendritic cells to mature and enhances immune responses); 3) activated dendritic cells expressing PAP are reinfused into patients so as to stimulate T cells to attack prostate cancer cells (59, 60). One study showed that treatment with Sipuleucel-T could prolong survival for 4.1 months among men with metastatic castration-resistant prostate

cancer, proving that it is worthwhile to investigate more about cancer vaccine (61).

1.1.3.3 Oncolytic virus therapy

Virus is like a double-edged sword – not only can it cause a wide array of diseases, it can also stimulate immune system and treat diseases like cancer. Upon infection with virus, normal cells and cancer cells react differently: normal cells would down-regulate protein translation and initiate apoptosis so as to block viral expansion; on the contrary, cancer cells strongly resist death and favor high level of translation, providing cellular resources for assembly of new viruses (62). This is the reason why many viruses prefer cancer cells as host, and such preference is the ground of oncolytic virus therapy - oncolytic viruses can selectively attack cancer cells and nearly do no harm to normal cells, which makes them potential for cancer treatment (63). There are three major mechanisms utilized by oncolytic virus to debulk tumor: 1) directly lyse tumor cells after infection; 2) destruct tumor blood vessels and 3) boost antitumor immunity by releasing tumor antigens (64). Oncolytic viruses can be further genetically engineered for various purposes, like to eliminate viral pathogenicity, to improve replication ability, and to circumvent viral neutralization (65).

In 2015, FDA approved the first oncolytic virus therapy - Talimogene laherparepvec (T-VEC) for the treatment of skin and lymph node melanoma (66). T-VEC is a genetically engineered herpes simplex virus, type 1 (HSV-1), which can cause fever blister disease (67). The neurovirulence genes which lead to fever blister and a viral gene which impedes antigen presentation have been deleted from T-VEC (66). T-VEC preferentially replicates in cancer

cells because of their aberrant protein kinase R (PKR) and type I IFN pathways (66). In normal cells, viral infection would activate PKR, which mediates the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF-2α), leading to the block of translation of proteins (68). However, cancer cells prefer continuous protein synthesis and cell growth, so they choose to disrupt PKR-eIF-2α pathway, providing virus with opportunity to replicate (66). As for type I IFN, it is a critical mediator for the apoptosis of cells infected by virus; it can also cause cancer regression by inducing apoptosis, cell cycle arrest and MHC-I expression (69, 70). To circumvent the anticancer effects of type I IFN, cancer cells tend to downregulate the expression level of the receptor for type I IFN, so as to create a better microenvironment (71). Because of disrupted type I IFN pathway, cancer cells would not undergo apoptosis after viral infection, allowing virus to expand rapidly. Finally, mature virions would induce cell lysis and infect more cancer cells. Apart from new generation of virus, many pro-immunogenic factors would also be released, including viral based PAMPs, DAMPs and cytokines (66). These factors would recruit immune cells and help them to get mature, inducing host immune responses (66).

1.1.3.4 Adoptive cell therapy

Adoptive cell therapy is a personalized cancer therapy in which anticancer immune cells are administrated into patients (72). Currently there are two types of adoptive cell therapy: tumor-infiltrating lymphocyte (TIL) therapy and chimeric antigen receptor T-cell (CAR-T) therapy.

Back to 1980s, in vitro experiments demonstrated that TIL was capable of recognizing autologous tumors and leading to the regression of tumors in patients with metastatic melanoma (72). Following clinical trials also proved that TIL therapy is effective for the treatment of metastatic melanoma, leading to durable responses (73). The process of TIL therapy is not complicated. First, a tumor specimen is resected from patient and divided into fragments which are cultured with IL-2; next, after obtaining overgrown lymphocytes from those fragments, desired lymphocytes that can react to tumor are selected through coculture assay; last, ideal lymphocytes undergo quick expansion and around 10^{11} lymphocytes can be generated for the infusion into patients (72).

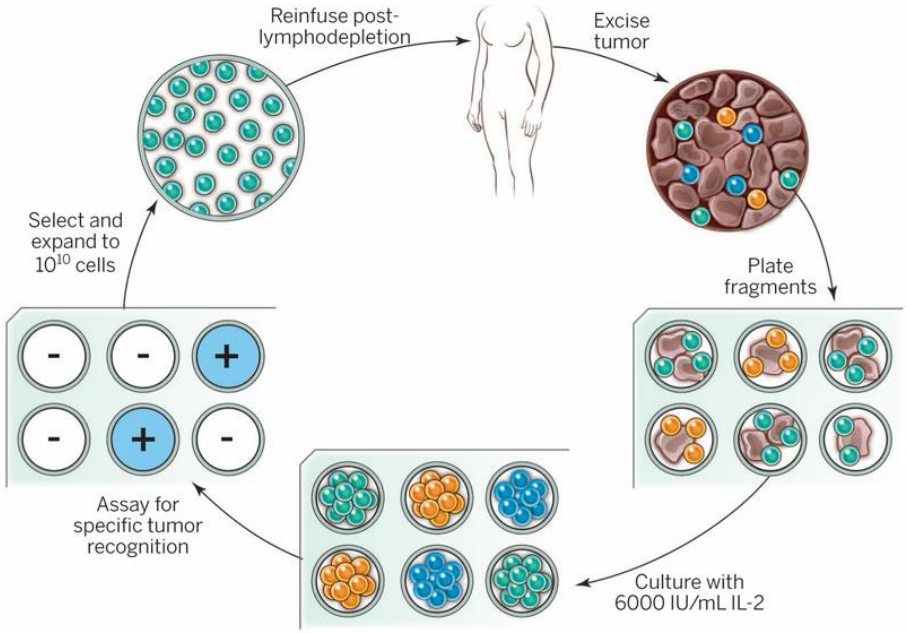

Figure 1.2. Schema of TIL therapy (72).

As for CAR-T therapy, it starts with the extraction of T cells from patients' blood, followed by adding chimeric antigen receptors (CARs) to the surface of T cells. CARs are artificially synthetic receptors which recognize tumor cells with high specificity (74). CARs are composed of an ectodomain which can identify tumor antigens with high affinity, a flexible hinge, a

transmembrane segment, and an intracellular signaling domain which is derived from the CD3z domain (74). Unlike classical T-cell receptors (TCRs), CARs do not require neither dendritic cell activation nor MHC expression for the recognition of tumor antigens (74). After being genetically engineered, T cells are infused back into patients by intravenous injection so as to attack tumor cells. In 2017, FDA approved the first CAR-T therapy for the treatment of patients with B-cell precursor acute lymphoblastic leukemia (74).

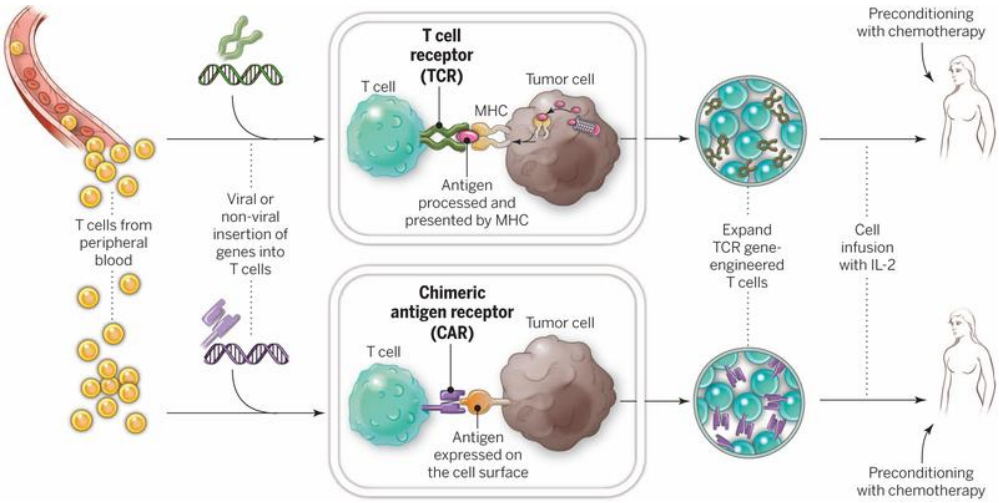

Figure 1.3. Schema of CAR-T therapy (72). Top panel: classical TCR is inserted into T cells, and MHC is necessary for the recognition of tumor antigen. Bottom panel: CAR is inserted into T cells, and the recognition of tumor antigen is independent on MHC expression.

1.1.3.5 Checkpoint blockade therapy

Immune system functions under sophisticated regulation. For instance, faced with acute infection by pathogens, immune functions should be accelerated to get rid of threats; after clearing all dangerous substances, immune responses should be immediately terminated so as to avoid the destruction to normal tissues. To achieve such regulation, there are both "gas pedal" and "brake" on immune cells, for acceleration and termination respectively. On immune cells, stimulatory receptors work as "gas pedal", and inhibitory checkpoint molecules serve as

“brake”. Inhibitory checkpoint molecules are extremely important for maintaining homeostasis and tolerance. Unfortunately, inhibitory checkpoint molecules have been utilized by cancer cells to escape from immunosurveillance. By upregulating the expression of ligands for inhibitory checkpoint molecules, cancer cells can get away from being attacked and suppress immune responses. Using monoclonal antibody to block the interaction between inhibitory checkpoint molecules and their ligands is an efficient strategy for cancer treatment. Up to now, FDA has approved immune checkpoint therapy for a wide range of cancers, like melanoma, non-small cell lung cancer and Hodgkin lymphoma (75).

Programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are two important inhibitory checkpoints on T cells, and they are ideal targets of immune checkpoint blockade therapy. When PD-1 was discovered, it was thought to be responsible for cell death, but later research demonstrated that PD-1 is capable of down-regulating T cell functions through interacting with programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2) (76). Naïve T cells have no expression of PD-1 until they get stimulated by cytokines or through TCR engagement (77). On the intracellular tail of PD-1, there is an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (77). When PD-1 is engaged with its ligands, the tyrosine residue on PD-1 would be phosphorylated, and phosphatases would be recruited (77, 78). Thus, the positive signals delivered by TCR and co-stimulatory receptor CD28 are countered by these phosphatases, resulting in the inhibition of T cell functions (77). In various types of cancers like lung cancer, ovarian cancer, gastric cancer, cervical cancer and colorectal cancer, the increasing expression of PD-L1 has been observed (79). FDA has approved two PD-1 blocking

antibodies (Pembrolizumab and Nivolumab) and three PD-L1 blocking antibodies (Atezolizumab, Durvalumab and Avelumab) for cancer treatment (80).

CTLA-4 and costimulatory receptor CD28 are homologous receptors that share same ligands – B7 molecules (CD80/CD86) (81). CTLA-4 is continuously expressed on Treg cells, and it is induced by T cell activation (81). Compared with CD28, CTLA-4 has higher affinity to B7 molecules, which means that CTLA-4 competes with CD28 for ligand binding and CTLA-4 can obstruct the co-stimulation mediated by CD28 (81). Consequently, blocking CTLA-4 can remove the inhibition of T cell function and mediate tumor regression. FDA has approved a CTLA-4–blocking antibody, ipilimumab, for the treatment of melanoma in 2011 (82).

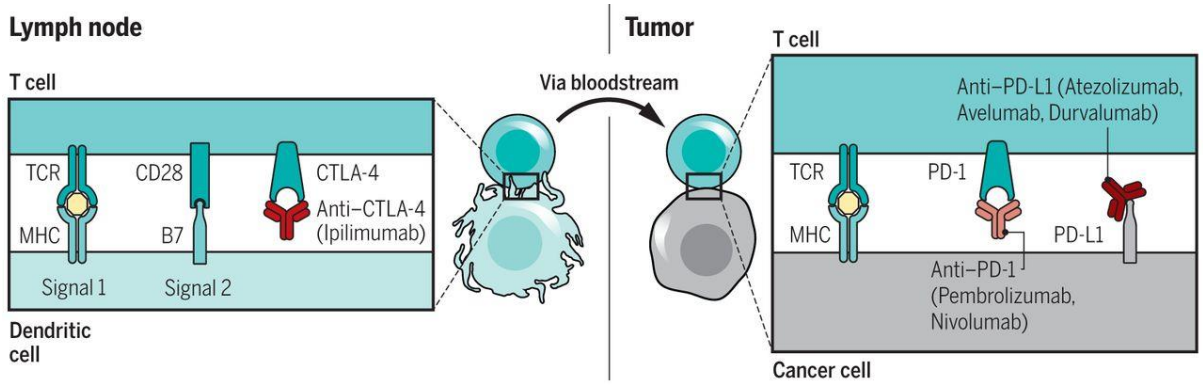

Figure 1.4. Antitumor responses can be induced by blockade of CTLA-4, PD-1 and PD-L1 (82).

1.1.4 Macrophage

Macrophage is a critical component of innate immune system, which takes part in multiple biological activities including development, homeostasis, tissue repair and immune surveillance (83). Literally, “macrophage” means “big eater” in Greek, and macrophage is indeed a fan of eating: it can phagocytose and digest dead cells, cell debris, foreign substances

and tumor cells. It is worthwhile to study macrophage and convert it into a weapon of immunotherapy.

In mouse model, the first appearance of macrophage is observed in yolk sac during early gestation (embryonic day 6.5 [E6.5]–E8.5); around E9.5, primitive macrophages circulate throughout the whole embryonic tissue; around E10.5, definitive hematopoietic stem cells (HSCs) come into being from the aorta-gonad-mesonephros (a region of embryonic mesoderm) and they colonize the fetal liver (84, 85). Such definitive hematopoiesis in fetal liver produces all kinds of major hematopoietic lineages (85). Monocytes generated from fetal liver enter peripheral tissues (exclude central nervous system) and differentiate into tissue-resident macrophages (85). In different tissues, the microenvironment is totally different. The identity and functions of tissue-resident macrophage are decided by cytokines and metabolites in the tissue, which can cause the expression of specific transcription factor (86). For example, in the lungs, fetal monocytes differentiate into alveolar macrophages because of the exposure to colony-stimulating factor 2 (CSF2) (86). Macrophages derived from yolk sac and fetal liver are long-lived and can self-renew (85). After birth, hematopoietic progenitors colonize spleen and bone marrow, where HSCs are hosted (85). In adulthood, blood monocytes derived from bone marrow can replenish tissue-resident macrophage but the monocyte-derived macrophage has limited lifespan (85).

Macrophage exerts its function through phagocytosis, which is an elegant and complicated process. The first step of phagocytosis is to detect targets by three kinds of receptors on macrophage: PRRs, opsonic receptors and apoptotic body receptors (87). Macrophage expresses various kinds of PRRs like CD36 (recognizes lipoprotein components of bacterial

cell walls, β-glucans on fungi and plasmodium falciparum-infected erythrocytes), scavenger receptor A (recognizes LPS), and mannose receptor (recognizes terminal mannose residues) (87, 88). Opsonic receptors are able to recognize opsonins including antibody and complement components (87). Opsonins are capable of tagging to pathogens and attracting phagocytes. Fc receptors and Mac-1 are typical examples of opsonic receptors, which can bind to the Fc portion of antibody and complement component iC3b, respectively (87). Apoptotic body receptors like T cell immunoglobulin mucin-1 (TIM-1) and TIM-4 can identify phosphatidylserine on apoptotic cells (89). When a target interacts with these receptors on macrophage, a series of signaling activities would take place, triggering actin polymerization and membrane remodeling (87). The membrane of macrophage would invaginate and generate a phagocytic cup to surround the target (87). After the membrane closes at the distal end, a new phagosome would be formed successfully (87). To get mature, phagosome would reconstruct its membrane components (90). Phagosome would fuse with early endosome to form early phagosome, fuse with late endosome to form late phagosome, and eventually fuse with lysosomes to form phagolysosome (90). During the maturation of phagosome, it gets increasingly acidic, providing digestive enzymes with optimal conditions to degrade contents inside phagosome (90).

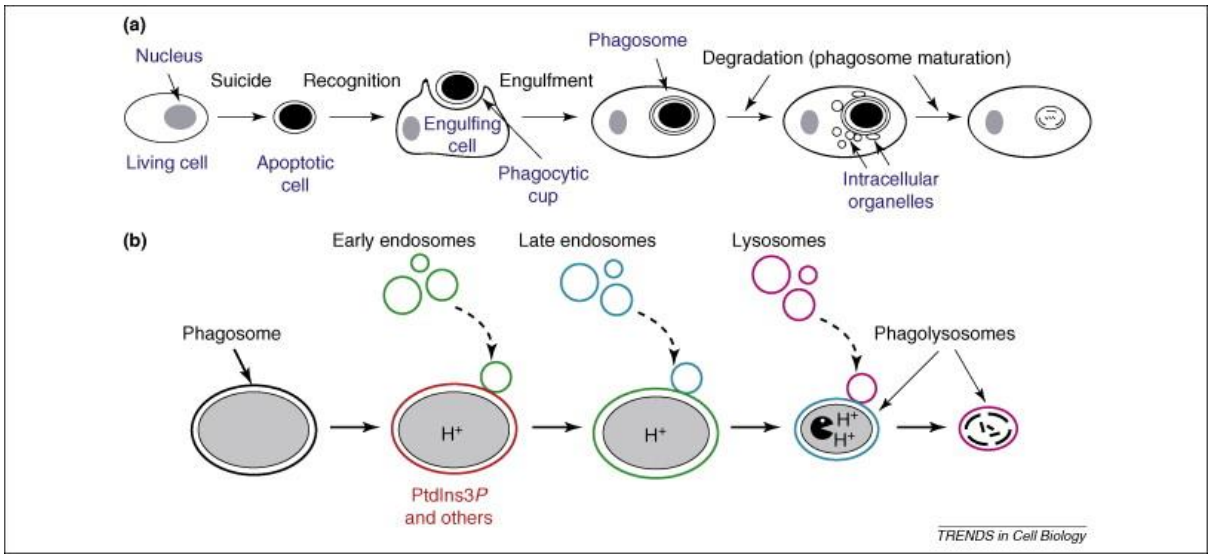

Figure 1.5. The process of phagocytosis and phagosome maturation (91).

To avoid destroying normal cells, checkpoints on macrophage block phagocytosis when they are engaged with corresponding "don't eat me" signal. Signal regulatory protein alpha (SIRPα) is an inhibitory immunoreceptor which is expressed on myeloid cells restrictively; while the ligand of SIRPα is CD47, which is expressed on nearly all kinds of cells and overexpressed on tumor cells (92). Upon engagement with CD47, the tyrosine residues in ITIM of SIRPα get phosphorylated, recruiting Src homology region 2 (SH2)-domain-containing phosphatase-1 (SHP-1) and SHP-2, both of which can prevent cell activation by suppressing the function of non-muscle myosin IIA (93). Up to now, a wide variety of CD47-SIRPα blocking agents like human anti-CD47 antibodies and anti-SIRPα antibodies have demonstrated antitumor efficacy based on in vitro assay and preclinical studies (93). Another checkpoint on macrophage is leukocyte immunoglobulin like receptor B1 (LILRB1), whose ligand is β_2-microglobulin (β2M), a MHC class I component (94). The expression of MHC class I on tumor cells can protect them from phagocytosis directly and the blockade of MHC class I–LILRB1 signaling can enhance the anti-CD47-mediated phagocytosis (94). Taken

together, CD47-SIRPα and MHC class I–LILRB1 interactions are promising therapeutic targets for human cancer.

According to activation profile, macrophage can be classified into two polarities: M1 and M2. M1 refers to classically activated macrophages while M2 represents alternatively activated macrophages (95). M1 macrophages are induced by IFN-γ, TNF and LPS, while M2 macrophages are stimulated by IL-4 and IL-13 (96). M1 macrophages are characterized by anti-microbial and anti-cancer abilities, which can secrete pro-inflammatory cytokines and nitric oxide (96). They can also eliminate pathogens by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system and producing reactive oxygen species (ROS) (96). M1 macrophages are able to cause damage to the tissue of host so their activation should be properly regulated (95). On the contrary, M2 macrophages favor secreting anti-inflammatory cytokines and they can promote tissue remodeling, wound healing, angiogenesis, tumor formation and progression (96). However, such linear classification is not suitable to describe highly plastic macrophages. A new perspective to classify macrophages is based on their fundamental functions including host defense, wound healing and immune regulation (95). This idea facilitates the understanding and illustration of macrophages which possess the characteristics of both M1 and M2. Under specific conditions, macrophages would change from one class to another. For instance, within tumor microenvironment, classical activated macrophages would switch to regulatory macrophages, which produce a large number of IL-10 and suppress the function of other nearby macrophages (95).

1.1.5 CD200 and CD200R1

CD200 is a membrane glycoprotein expressed on various cell types including thymocytes, activated T cells, B cells, dendritic cells, endothelial cells and neurons (97). Moreover, CD200 is overexpressed in various kinds of malignancies like renal carcinoma, colon carcinoma, multiple myeloma, B cell neoplasms, chronic lymphocytic leukemia and so on, manifesting that CD200 might be a prognostic factor for cancer and a therapeutic target as well (98-100). CD200 has two Ig superfamily (IgSF) domains (which are responsible for the cell-cell interaction) in its extracellular domain, a single transmembrane domain, and only 19 amino acids in cytoplasmic domain which contains no signaling motif (97).

Compared with CD200, the distribution profile of its receptor, CD200-receptor 1 (CD200R1) is significantly narrower – its expression is mainly restricted on dendritic cells and monocytes/macrophages (97). Similar to CD200, CD200R1 also contains two IgSF domains but its cytoplasmic domain is longer and able to signal (97). Different from human, there are 4 members in mouse CD200R family: one inhibitory receptor designated as CD200R1, and three activating receptors designated as CD200R2, CD200R3 and CD200R4 (101). However, CD200 only bind to the inhibitory CD200R in both human and mouse (102). Murine CD200R1 signaling is reported to be dependent on a NPXY motif in the cytoplasmic region, which can recruit docking protein 2 (Dok2) and RAS GTPase-activating protein (RasGAP) to mediate inhibitory signals (103).

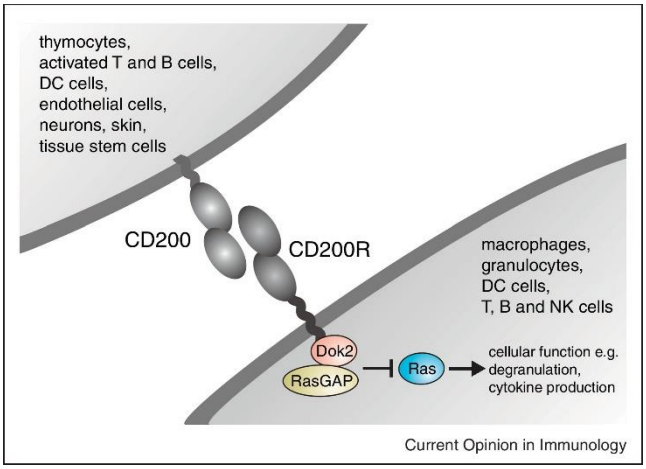

Figure 1.6. CD200R can recruit Dok2 and RasGAP to inhibit immune responses when it is engaged with CD200 (102).

CD200-CD200R1 signaling is crucial for the maintenance of homeostasis after immune responses. CD200R1 is highly expressed on alveolar macrophages, controlling the magnitude of inflammation to protect respiratory tract (104). It was found out that CD200-knockout (KO) mice are more susceptible to develop autoimmune diseases like encephalomyelitis and collagen induced arthritis than wildtype mice (102). Due to the absence of CD200, the activity of myeloid cells cannot be properly limited, leading to immune pathology. Moreover, the expression of CD200 and CD200R1 in tissue grafts is important for graft survival (105). However, the underlying mechanism of how CD200 and CD200R1 influence graft survival is not fully understood. Besides, cancer cells take advantage of CD200-CD200R1 signaling to suppress immune responses. By expressing CD200, cancer cells can inhibit the cytotoxic activity of NK cells, T cells and myeloid cells (102). Therefore, CD200-CD200R1 signaling is a potential therapeutic target of cancer.

1.2 Rationale

There is increasing enthusiasm in the research related to immunotherapy which demonstrates excellent anticancer potential. Checkpoint blockade therapy is a successful instance of immunotherapy, but its major advancement lies in adaptive immunity rather than innate immunity. It is necessary and worthwhile to investigate more about checkpoints on immune cells which are in charge of innate immunity like macrophage, in order to develop novel therapeutic strategy utilizing macrophage to attack tumor cells. One important checkpoint which regulates phagocytosis on macrophage is SIRPα. Blocking SIRPα and its ligand by various kinds of agents can promote macrophage to engulf tumor cells. However, due to the redundancy of immune inhibitory receptors, there should be additional checkpoints that suppress phagocytosis. Previous studies have proved CD200R1, whose ligand is CD200, to be an inhibitory receptor for inflammation, but no studies have shown its ability to regulate phagocytosis on macrophage. Herein, to have a better understanding of the function of CD200-CD200R1 signaling axis on macrophage, we take advantage of in vitro phagocytosis assay to figure out whether CD200R1 is a new regulator of phagocytosis, and to evaluate whether CD200-CD200R1 axis holds potential to be a therapeutic target.

1.3 Hypothesis

CD200 can protect tumor cells from being phagocytosed by macrophage and CD200R1 is an immune checkpoint of phagocytosis on macrophage.

1.4 Aim

The objective of this study is to demonstrate that CD200-CD200R1 axis can inhibit phagocytosis and might be potential therapeutic target on macrophage.

1.5 Significance

Macrophage can be utilized as powerful weapon to fight against cancer. This study firstly indicates that CD200R1 is a checkpoint of macrophage which regulates phagocytosis. In addition, this study provides basic evidence that CD200-CD200R1 interaction might be a therapeutic target for cancer treatment.

2 Materials and Methods

2.1 Mice

SFRKO mice were described in paper previously published by our lab (106). CD200R1KO mice were a gift from Dr. Amy Saunders. Wildtype C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). All mice were housed in SPF+/SPF facility of Institut de recherches cliniques de Montréal (IRCM). All experiments were performed in accordance with the guidelines of IRCM.

2.2 Cells

To produce mouse bone-marrow-derived macrophages (BMDMs), femora and tibiae were flushed with sterile PBS with 2% FBS and cultured in bacterial Petri dishes in DMEM medium supplemented with 30% (v/v) L929 cell-conditioned medium for 7 days. A20 (TIB-208), J558 (TIB-6), WEHI-231 (CRL-1702) cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA). Culture method recommended by ATCC is used for all cells obtained from ATCC. A20-CD200KO cells were generated by CRISPR-Cas-mediated genome editing, using the guide RNA sequences TCTCCACCTACAGCCTGATT (KO1), CTGCTGCCATGCCCCAAATC (KO2), AGGCTGGATTACAACCCCAT (KO3). J558-CD200KO cells were generated by CRISPR-Cas-mediated genome editing, using the guide RNA sequences TCTCCACCTACAGCCTGATT (KO1), AGGCTGGATTACAACCCCAT (KO2). Fresh splenic $CD4^+$ T cells are purified from total splenocytes by using Easysep mouse $CD4^+$ T cell isolation kit (Stemcell Technologies,

Vancouver, British Columbia, Canada). Activated $CD4^+$ T cells were obtained by culturing purified splenic $CD4^+$ T cells with concanavalin A (4 μg/ml; Sigma-Aldrich, St. Louis, Missouri, United States) for 48 h, followed by IL-2 (50 U/ml) for 24 h. Unstimulated normal B cells were acquired by isolating total splenocytes from T-cell deficient mice.

2.3 Antibodies

For flow cytometry, the following monoclonal antibodies were used. Anti-CD11b (M1/70) was obtained from eBioscience (San Diego, California, USA). Anti-F4/80 (BM8), anti-CD18 (M18/2), anti-CD16/32 (93), anti-CD200R1 (OX-110), anti-SLAMF7 (4G2), anti-CD200 (OX-90), anti-SIRPα (P84), anti-CD47 (Miap301), anti-CD64 (X54-5/7.1), anti-CD19 (6D5), and anti-B220 (RA3-6B2) were obtained from Biolegend (San Diego, California, USA). Polyclonal antibody goat anti-rat IgG (Poly4054) was obtained from Biolegend (San Diego, California, USA).

For blocking, the following monoclonal antibodies were used. Anti-CD47 (Miap301) and its corresponding isotype control were purchased from eBioscience (San Diego, California, USA). Anti-CD200 and its corresponding isotype control were purchased from Biolegend (San Diego, California, USA).

2.4 Flow cytometry

Cells were harvested and re-suspended in FACS buffer (2% FBS in PBS (PH=7.2)). To

stain for surface markers, cells were blocked with 2.4G2/7G7 on ice for 30 minutes, then incubated with corresponding antibodies on ice for 30 minutes in dark condition. Cells were washed with FACS buffer and were analyzed on CyAn ADP flow cytometer (Beckman Coulter, Brea, California, United States). As for antibody without fluorescent labels, secondary antibody is necessary. After 30-minute staining with primary antibody, cells were washed once and then were stained with secondary antibody for another 30 minutes. After being washed again, cells were analyzed on cytometer.

2.5 Conjugate assay

2×10^5 macrophages were stained with 2.5 μM CellTrace Violet (CTV; Life Technologies) plated in a cover slide overnight. The next day, target cells were washed and labelled with 2.5 μM of carboxyfluorescein succinimidyl ester (CFSE), using a CFSE Cell Proliferation Kit (C34554; Life Technologies, Burlington, Ontario, Canada). 1×10^6 CFSE-labelled target cells were added to the macrophages, in the presence of anti-CD47 antibodies or control IgG (10 μg/ml). After incubation for 30 min at 37 °C, cover slides were extensively washed and fixed on a glass slide by Dako fluorescent mounting media and nail oil. Slides were imaged with a confocal microscope. The conjugate formation rate was calculated as the number of macrophages contacting $CFSE^+$ target cells per 100 macrophages.

2.6 Phagocytosis assay

Microscopy-based phagocytosis assay: 5×10^4 macrophages were plated in a 24-well tissue

culture plate overnight, with/out 18 h pre-treatment of 100 ng/ml LPS, 100 ng/ml IFNγ or together. The next day, target cells were washed and labelled with 2.5 μM of carboxyfluorescein succinimidyl ester (CFSE), using a CFSE Cell Proliferation Kit (C34554; Life Technologies, Burlington, Ontario, Canada). After incubating macrophages in serum-free medium for 2 h, 2.5 × 10^5 CFSE-labelled target cells were added to the macrophages, in the presence of anti-CD47, anti-CD200 antibodies or control IgG (10 μg/ml). Target cells and macrophages were co-incubated for 2 h at 37 °C. After incubation, macrophages were extensively washed and imaged with an inverted microscope (Carl Zeiss Axiovert S100 TV). The phagocytosis rate was calculated as the number of macrophages containing $CFSE^+$ target cells per 100 macrophages.

Flow-cytometry-based phagocytosis assay: 5×10^4 macrophages were plated in a 24-well tissue culture plate overnight, with/out 18 h pre-treatment of 100 ng/ml IFNγ. The next day, target cells were washed and labelled with 100 ng/ml of pHrodo Green AM Intracellular pH Indicator (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After incubating macrophages in serum-free medium for 2 h, 2.5×10^5 pHrodo-labelled target cells were added to the macrophages, in the presence of anti-CD47 antibodies, anti-CD200 or control IgG (10 μ g/ml). After 2 h incubation, supernatant medium was collected, and macrophages were detached by Accutase. Harvested cells were stained with anti-F4/80-APC for 30 min on ice in dark to distinguish macrophages, then were analyzed by flow cytometry using CyAn ADP flow cytometer. Phagocytosis efficiency was determined as the percentage of $F4/80^+$ cells containing pHrodo-derived green fluorescence (detected in FL1 channel).

2.7 Plasmid construction

The cDNA of CD200R1 with tag (MR204778) was obtained from OriGene (Rockville, Maryland, United States). The pure cDNA of CD200R1 without tag was obtained by PCR using specially designed primers. Then the cDNA was inserted into pFB-GFP plasmid through double digestion and overnight ligation. Ligated products were transformed into competent cells by heat shock. Competent cells were then applied onto agar plate with ampicillin and cultured in incubator at 37°C overnight. The positive clones were picked up and cultured in LB medium with ampicillin separately in shaker at 37°C overnight. The plasmids produced by competent cells were collected by mini-prep kit (Qiagen, Hilden, Germany) and these collected plasmids were sequenced to make sure there is no mutation. After confirming that the plasmids have correct sequence, large amount of competent cell culture was used to obtain large quantity of plasmids by using maxi-prep kit (Invitrogen, Carlsbad, California, United States).

To produce plasmids with ideal mutation, special primers containing altered nucleotides were designed. Overlap PCR was done using these primers and final products were inserted into pFB-GFP plasmids, followed by identical procedures as mentioned above. 5 pFB plasmids were used in experiments: original pFB-GFP as empty vector, pFB-GFP vector with wildtype CD200R1 cDNA (pFB-WT CD200R1), pFB-GFP vector with mutant CD200R1 cDNA (pFB-Y-F CD200R1, pFB-N-Q CD200R1, and pFB-P-A CD200R1).

2.8 Transfection and infection

Phoenix cells were transfected with pFB plasmids by lipo2000 (Thermo Fisher Scientific,

Waltham, Massachusetts, United States). 48 h after transfection, the supernatants of phoenix cells were collected, which contain retroviruses. BMDMs were infected with these supernatants and 8 μg/ml polybrene for 48 h. After infection, BMDMs were detached from culture plates, and GFP^+ BMDMs were sorted and cultured for additional 48 h in growth medium before used for experiments.

2.9 F(ab')$_2$ antibody production

To generate F(ab')$_2$ fragments, anti-CD47 monoclonal antibody and its corresponding isotype control antibody were digested by pepsin (Sigma-Aldrich, St. Louis, Missouri, United States) in 37°C water bath for 48 h. After digestion, antibody underwent dialysis in PBS solution, followed by concentration measurement. 2 μg antibody was used to run SDS-PAGE and protein gel was stained with Coomassie Blue in order to confirm that Fc portion of antibody has gone.

2.10 Statistical analysis

One-way ANOVA was performed using Prism 7 (GraphPad Software) to test for statistical significance between data groups.

3 Results

3.1 CD200 expression can protect cells from being phagocytosed

To study the relationship between CD200 and phagocytosis, target cells with CD200 expression are required. Besides, since signaling lymphocytic activation molecule F7 (SLAMF7) was reported by our laboratory to be critical for anti-CD47 mediated phagocytosis (107), we added SLAMF7 expression as a criterion for target cell selection. By flow cytometry, we found three cells lines expressing both CD200 and SLAMF7: A20 (B lymphocyte; reticulum cell sarcoma), J558 (B lymphocyte, plasmacytoma; myeloma) and WEHI-231 (B lymphocyte, immature; B cell lymphoma) (Figure 3.1A). We also found out that CD4 T cells activated by concanavalin A have both expression of CD200 and SLAMF7 (Figure 3.1B). Non-activated normal B cells (resting B cells) have expression of CD200 (Figure 3.1C), and they have been reported to express SLAMF7 (107). In this study, these 5 kinds of cells were used as target cells for experiments.

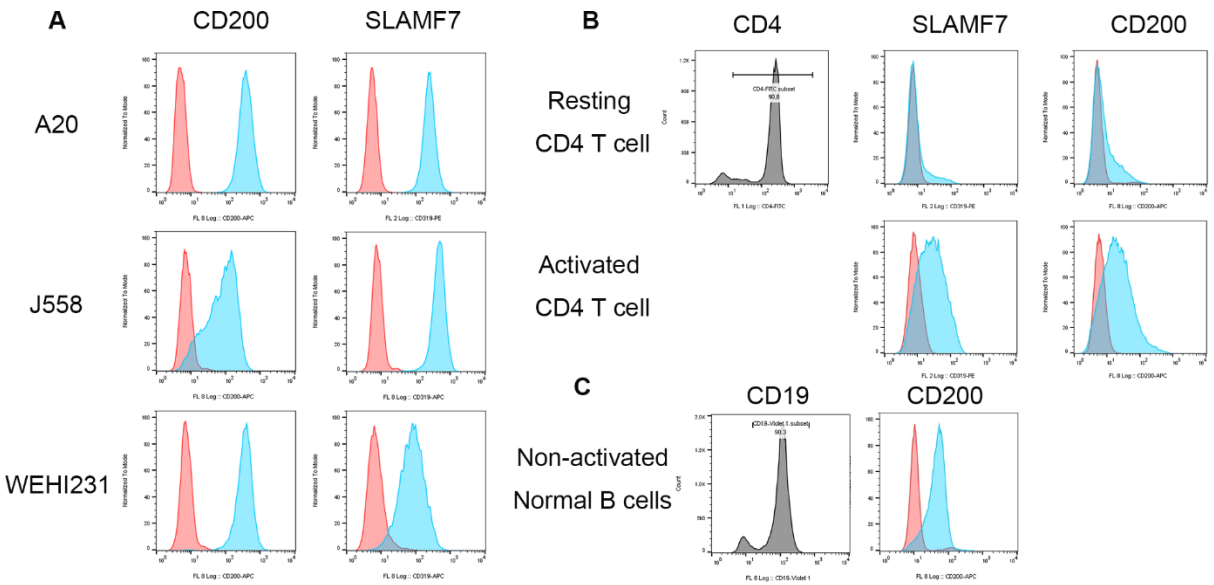

Figure 3.1. CD200 and SLAMF7 expression on certain tumor cells and immune cells. A, flow cytometry analyzing the expression of CD200 and SLAMF7 on A20, J558 and WEHI-231 cell lines. B, flow cytometry analyzing the expression of CD200 and SLAMF7 on resting and activated CD4 T cells. C, flow cytometry analyzing the

expression of CD200 on resting B cells. CD4 and CD19 staining demonstrates the high purity of CD4 T cell and B cell, respectively. Red curve, isotype control; blue curve, antibody staining.

There is a study showing that CD200 is important for graft survival (105), consequently, we hypothesize that CD200 expression can protect cells from being phagocytosed. Herein, we produced A20-CD200KO cells by using CRISPR-Cas9 based genome editing. We inserted three guide RNA into PX458 plasmids and used them to transfect A20 cells to generate three strains of A20-CD200KO cells. In parallel, original PX458 was used as empty vector (EV) to transfect A20 cells so as to generate A20-EV cells. To confirm the knock out efficiency of CD200 and to check whether there is any off-target effect, we did flow cytometry to determine the expression of some surface proteins on A20 cells. These selected surface proteins are common B cell markers. In A20-CD200KO cells, there is no detectable CD200 expression, while in A20-EV cells, CD200 expression is obvious. As for other surface proteins, A20-EV and A20-CD200KO cells show nearly the same expression pattern – they express CD47, SLAMF7, CD19, B220 but not CD16/32 (Figure 3.2).

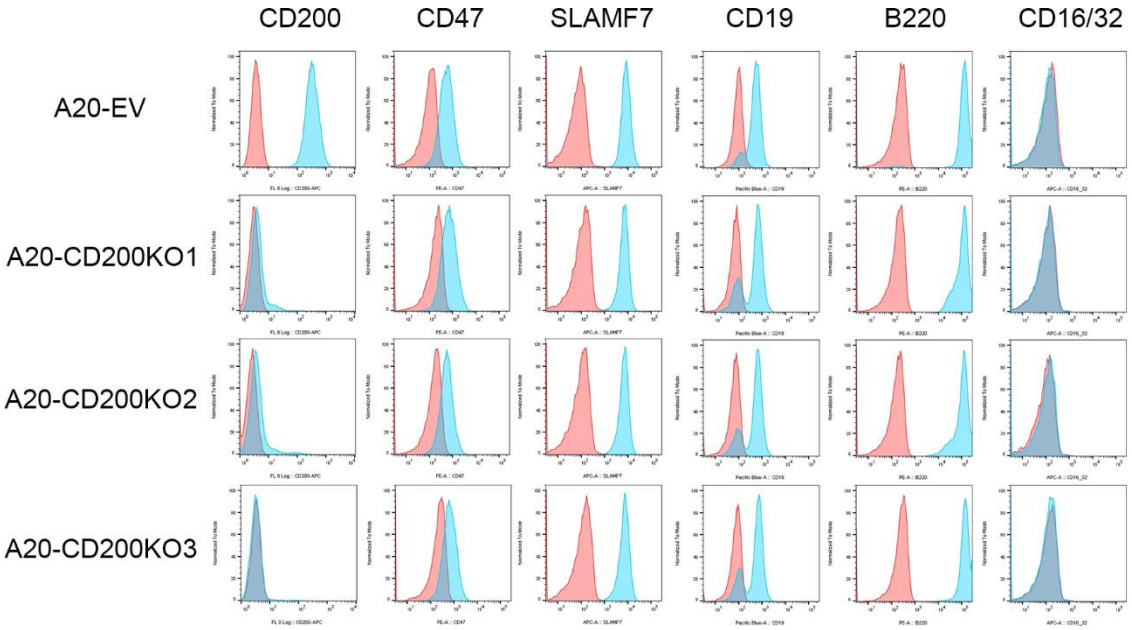

Figure 3.2. Surface protein expression on A20-EV and A20-CD200KO cells. Red curve, isotype control; blue curve, antibody staining.

To test whether CD200 has inhibitory effects for phagocytosis, phagocytosis assay has been done. Target cells have been stained with CFSE, which shows green fluorescence, and incubated with BMDMs with IgG or blocking antibody for 2 hours (Figure 3.3A). Pictures have been taken under fluorescence microscope and the phagocytosis percentage has been determined by counting the number of BMDMs that have phagocytosed target cells (Figure 3.3B). Our results show A20-EV cells are very reluctant to be phagocytosed by wildtype (WT) BMDMs – less than 2% of macrophage can phagocytose A20-EV cells with or without blockade of CD47 (Figure 3.3C). As for A20-CD200KO cells, when CD47 is not blocked, similarly, phagocytosis percentage is around 2%; however, when CD47 is blocked by antibody, phagocytosis percentage reaches approximate 5%, suggesting CD200 has protective functions against phagocytosis (Figure 3.3C). Since the results of three strains of A20-CD200KO cells show high consistency, in the following experiments they have been pooled together.

IFNγ and LPS are capable of inducing classical activation of macrophages (M1) which are significant for tumor suppression (108), so we pre-treated BMDMs with IFNγ and LPS before phagocytosis assay, wondering whether IFNγ and LPS can further enhance phagocytosis of A20-CD200KO cells. Our results show that LPS and IFNγ indeed help to elevate phagocytosis percentage. When WT BMDMs have been pre-treated with LPS, phagocytosis percentage of A20-EV cells is more or less 5% with or without CD47 blockade; phagocytosis percentage of A20-CD200KO cells is 7% when there is no CD47 blockade, while with blockade, percentage increases to 11% (Figure 3.3D). With IFNγ pre-treatment, results are similar to LPS pre-treated group. When we combine LPS and IFNγ together to pre-treat BMDMs, phagocytosis percentage has been improved further: in IgG treated group, the phagocytosis percentage of

A20-EV and A20-CD200KO cells is around 5% and 7% respectively; with anti-CD47 treatment, percentage increases to 6% and 15% respectively.

Previously, Chen et al., reported that SLAMF7 has a critical function for phagocytosis of hematopoietic tumor cells (107), so we wonder whether SLAMF7 is also required for phagocytosis of A20 cells. SLAM family receptor knock-out (SFRKO) mice have been used for experiments. Our results demonstrate that SFRKO BMDMs' ability to phagocytose A20 cells is not compromised (Figure 3.3E), so SFR is not required for the phagocytosis of A20 cells.

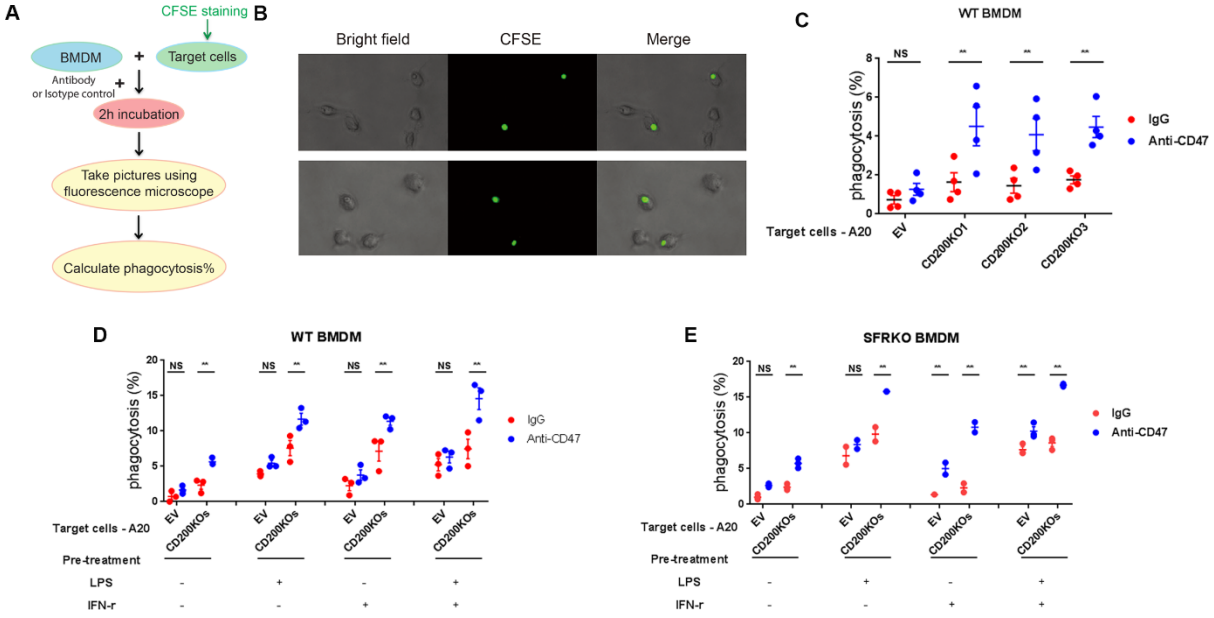

Figure 3.3. CD200 protects A20 cells from being phagocytosed. A, experimental scheme of phagocytosis assay. B, pictures taken during phagocytosis assay. Target cells have been stained with CFSE, showing green fluorescence. Green cells within macrophage are identified as phagocytosed cells. C, microscopy-based measurement of phagocytosis of A20-EV and A20-CD200KO cells by WT BMDMs (n=4). D, microscopy-based measurement of phagocytosis of A20-EV and A20-CD200KO cells by pre-treated WT BMDMs (n=3). E, microscopy-based measurement of phagocytosis of A20-EV and A20-CD200KO cells by pre-treated SFRKO BMDMs (n=2-3). Error bars stand for standard error of the mean (SEM).

Before engulfment of targets, targets should adhere or tether to the surface of macrophage, forming conjugates. We hypothesize that CD200 might play a role in conjugate formation,

which influences the occurrence of phagocytosis, so we have done conjugate formation assay to check the ability of WT BMDMs to form conjugates with A20-EV and A20-CD200KO cells. We stained WT BMDMs with CellTrace Violet (shows blue fluorescence) before plating, and stained target cells with CFSE. Target cells have been incubated with BMDMs for half an hour and confocal microscope has been used for picture taking (Figure 3.4A, B). Less than 5% of BMDMs can form conjugates with A20-EV cells with or without CD47 blockade; while 11% of BMDMs can form conjugates with A20-CD200KO cells when CD47 is not blocked, 14% when CD47 is blocked (Figure 3.4C). Our results prove that CD200 can prevent conjugate formation between A20 cells and WT BMDMs, which partially explains why A20 cells are reluctant to be phagocytosed.

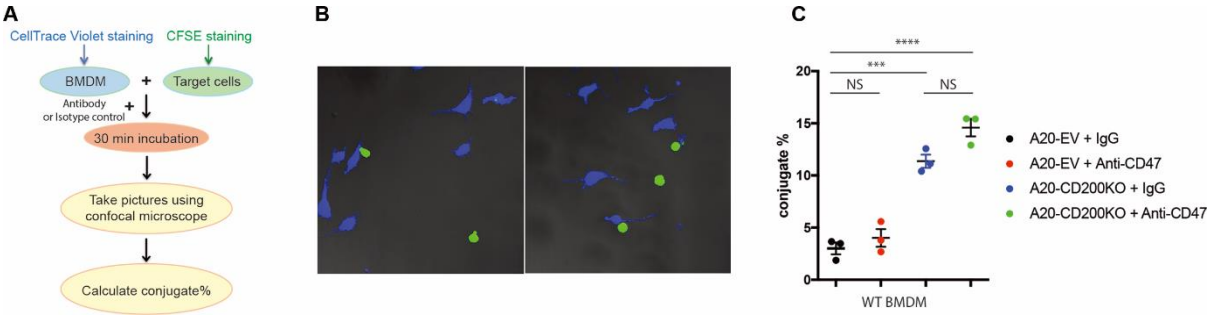

Figure 3.4. CD200 prevents A20 cells to form conjugates with BMDMs. A, experimental scheme of conjugate assay. B, pictures taken during conjugate assay. Blue cells are macrophages, and green cells are target cells. C, microscopy-based measurement of conjugate formation between WT BMDMs and A20-EV/CD200KO cells (n=3). Error bars stand for SEM.

We also produced J558-EV and J558-CD200KO cells. The surface protein expression has been detected by flow cytometry as well. We confirmed that parental J558 and J558-EV cells have expression of CD200 while J558-CD200KO cells have not. As for other surface markers, parental J558, J558-EV and J558-CD200KO cells show the same expression pattern: all of them have expression of CD47 and SLAMF7, but have no expression of CD19, B220 and

CD16/32 (Figure 3.5A). Phagocytosis assay has been done using J558-EV and J558-CD200KO cells. Our results demonstrate that anti-CD47 can increase phagocytosis of J558-CD200KO cells significantly, but cannot improve phagocytosis of J558-EV cells (Figure 3.5B). Anti-CD47 increases the phagocytosis percentage of J558-CD200KO cells from 5% to 10% when WT BMDMs have not been pre-treated. In addition, IFNγ pre-treatment can enhance phagocytosis of both J558-EV and J558-CD200KO cells. With IFNγ pre-treatment, anti-CD47 can improve the phagocytosis percentage of J558-CD200KO cells from 10% to 15%.

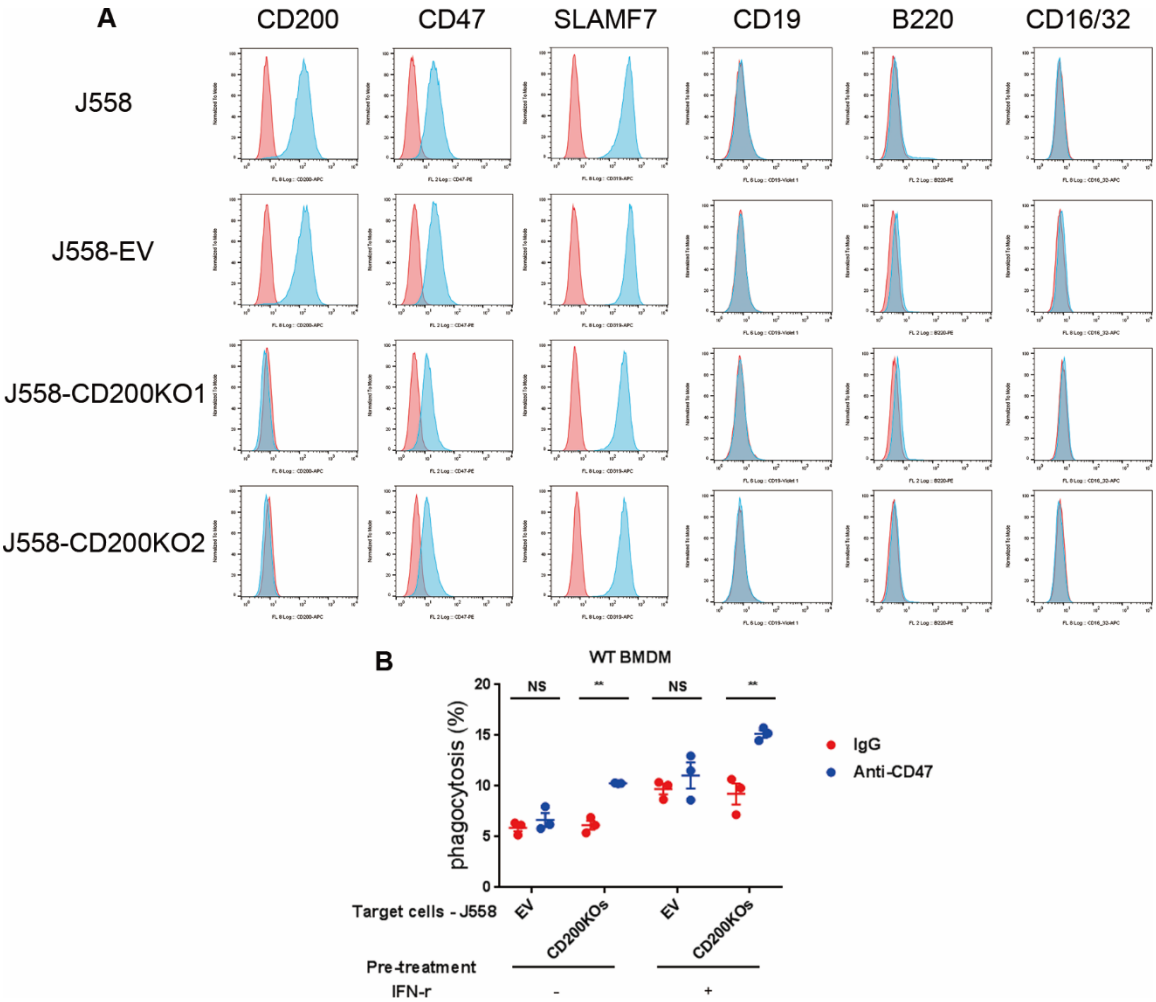

Figure 3.5. CD200 protects J558 cells from being phagocytosed. A, surface protein expression on J558-EV and J558-CD200KO cells. Red curve, isotype control; blue curve, antibody staining. B, microscopy-based measurement of phagocytosis of J558-EV and J558-CD200KO cells by WT BMDMs, with or without IFNγ pre-treatment (n=3). Error bars stand for SEM.

3.2 Blockade of CD200-CD200R1 interaction by antibody can promote phagocytosis

Blockade of checkpoint is a successful therapeutic strategy which works well in cancer treatment. To find out whether blockade of CD200-CD200R1 interaction can facilitate phagocytosis, we did phagocytosis assay using blocking antibodies against CD47 and CD200. We found out that single blockade of CD47 or CD200 can only promote phagocytosis of parental A20 cells by WT BMDMs in a very limited way, but combined blockade of CD47 and CD200 can improve phagocytosis significantly (Figure 3.6A). In non-pre-treated WT BMDMs, phagocytosis percentage increases from 2% in IgG+IgG group, to 5% in anti-CD47+anti-CD200 group; while in IFNγ-pre-treated WT BMDMs, phagocytosis percentage increases from 3% in IgG+IgG group, to 10% in anti-CD47+anti-CD200 group. To further confirm this phenomenon, we did flow-cytometry-based phagocytosis assay. A20 cells were stained with a special dye called pHrodo, an ideal dye for phagocytosis assay, which shows green fluorescence in acidic environment only, so it can exclude conjugates and distinguish cells that entered macrophages from cells outside macrophages. Results gained from flow-cytometry-based phagocytosis assay is accordant with previous results generated from microscopy-based phagocytosis assay: phagocytosis percentage increases from 4.75% in IgG+IgG group to 10.9% in anti-CD47+anti-CD200 group (Figure 3.6B).

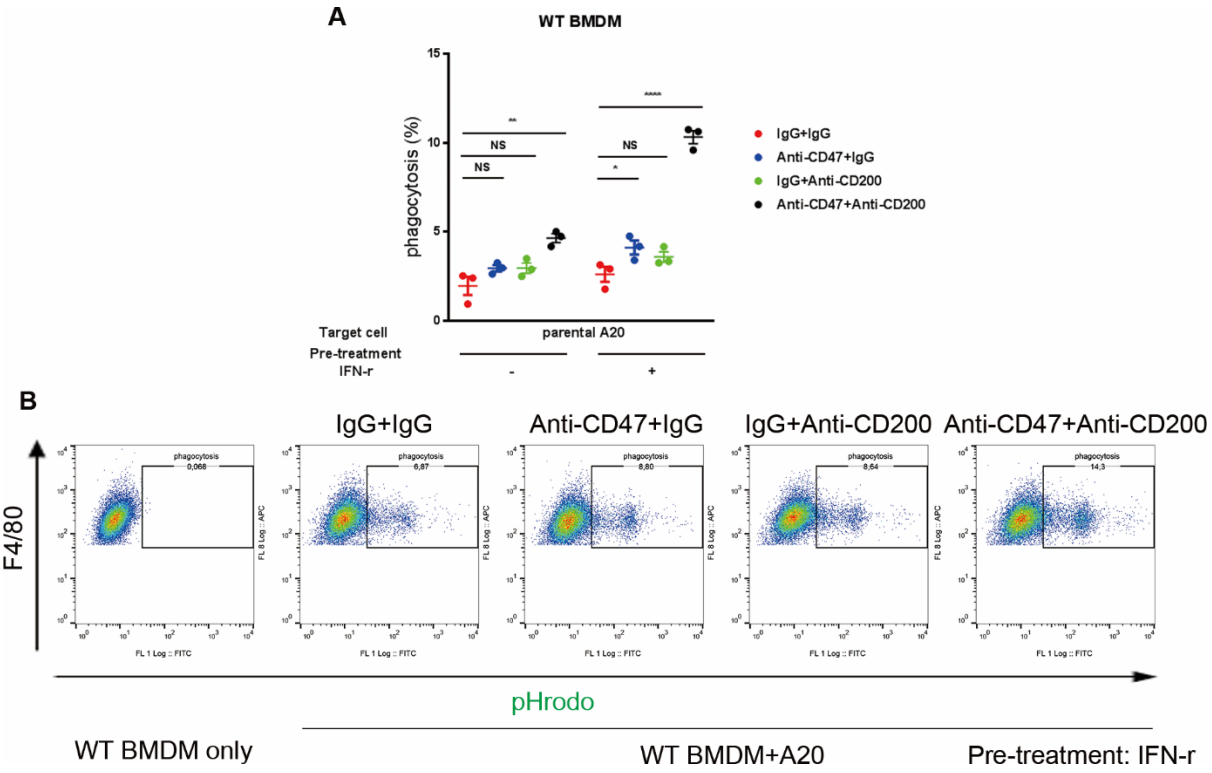

Figure 3.6. Blockade of CD200-CD200R leads to more phagocytosis of A20 cells by WT BMDMs. A, microscopy-based measurement of phagocytosis of A20 cells by WT BMDMs with/out IFNγ pre-treatment (n=3). B, flow cytometry-based measurement of phagocytosis of A20 cells by WT BMDMs pre-treated with IFNγ (n=1). Error bars stand for SEM.

WEHI-231 cells were used as target cells for both microscopy-based and flow-cytometry-based phagocytosis assay. Results generated by microscopy-based phagocytosis assay show that in non-pre-treated WT BMDMs, blockade of both CD47 and CD200 increased phagocytosis percentage from 6% to 12%; in IFNγ-pre-treated group, from 6% to 15% (Figure 3.7A). Using SFRKO BMDMs, we obtained highly similar results (Figure 3.7B). By flow-cytometry-based phagocytosis assay, we found out that in non-pre-treated WT BMDMs, blockade of both CD47 and CD200 increased phagocytosis percentage from 6% to 13% (Figure 3.7C, D).

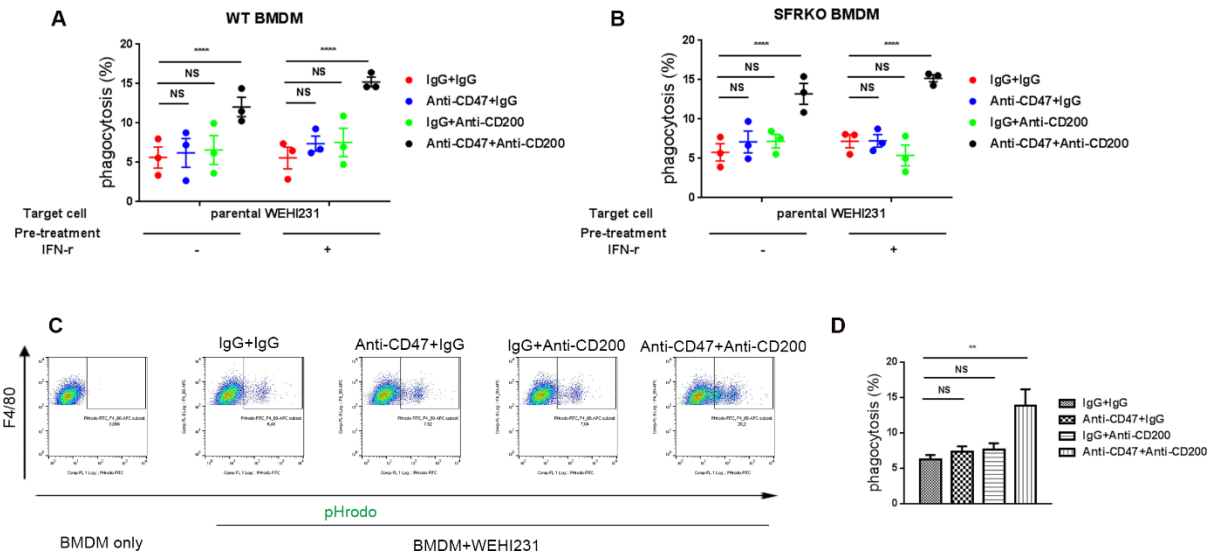

Figure 3.7. Blockade of CD200-CD200R1 leads to more phagocytosis of WEHI-231 cells by BMDMs. A, microscopy-based measurement of phagocytosis of WEHI-231 cells by WT BMDMs with/out IFNγ pre-treatment (n=3). B, microscopy-based measurement of phagocytosis of WEHI-231 cells by SFRKO BMDMs with/out IFNγ pre-treatment (n=3). C, flow-cytometry-based measurement of phagocytosis of WEHI-231 cells by WT BMDMs without pre-treatment. D, quantification of C (n=4). Error bars stand for SEM.

We wonder whether blockade of CD200 can also affect the phagocytosis of immune cells, so we did microscopy-based phagocytosis assay using ConA-activated CD4 T cells and non-activated normal B cells. Around 10% of non-pre-treated WT BMBM phagocytosed activated CD4 T cells in IgG-treated group, 15% in anti-CD47-treated group, 12% in anti-CD200-treated group, and 20% in anti-CD47+anti-CD200-treated group (Figure 3.8A), indicating that combined blockade of CD47 and CD200 can promote the phagocytosis of activated CD4 T cells significantly. As for normal B cells, they are extremely difficult to be phagocytosed. The phagocytosis percentage of normal B cells by non-pre-treated and IFNγ-pre-treated WT BMDMs is always around 2%, no matter what kinds of antibodies were used (Figure 3.8B).

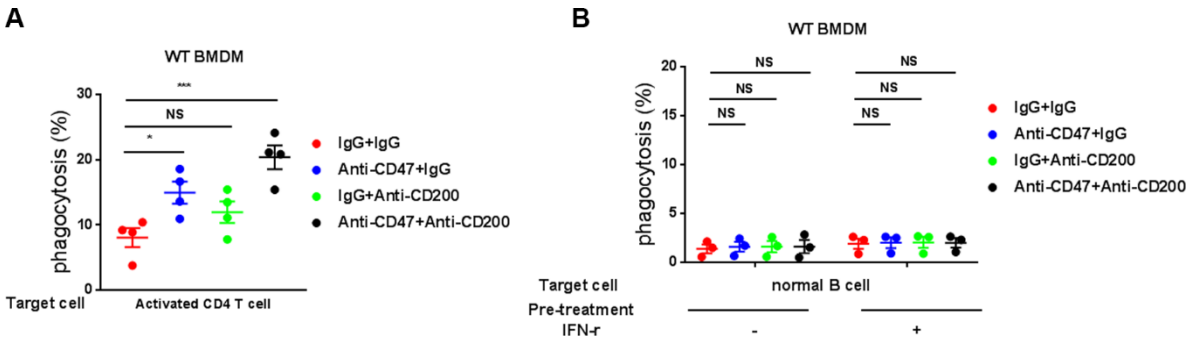

Figure 3.8. Blockade of CD200-CD200R1 leads to more phagocytosis of activated CD4 T cells by WT BMDMs, but non-activated normal B cells are not affected. A, microscopy-based measurement of phagocytosis of activated CD4 T cells by non-pre-treated WT BMDMs (n=4). B, microscopy-based measurement of phagocytosis of normal B cells by WT BMDMs with/out IFNγ pre-treatment (n=3). Error bars stand for SEM.

To sum up, we found out that the combined blockade of CD47 and CD200 can promote phagocytosis of A20, WEHI-231 and activated CD4 T cells significantly, but the phagocytosis of normal B cells is not affected by such blockade.

3.3 Knocking out CD200R1 on macrophage can upregulate phagocytosis

Based on previous results, we realized the importance of CD200 expression in phgocytosis regulation. We hypothesize that the receptor of CD200, CD200R1 should be an immune checkpoint on macrophage which suppresses phagocytosis ability. Therefore, we used CD200R1KO BMDMs to do experiments so as to find out whether there is any difference from WT BMDMs.

To begin with, we checked the expression of surface proteins on both WT and CD200R1KO BMDMs by flow cytometry. Our data confirm that CD200R1KO BMDMs have

no expression of CD200R1 but they have the same expression profile of other surface proteins as WT BMDMs (Figure 3.9). Both WT and CD200R1KO BMDMs have expression of SLAMF7, CD47, SIRPα, F4/80, CD11b, CD18, CD64 and CD16/32.

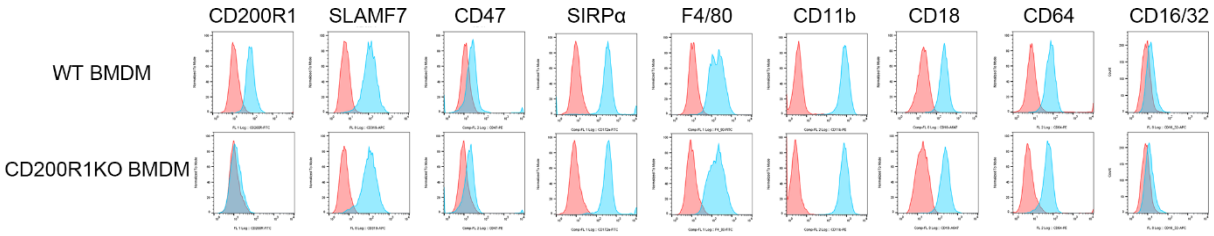

Figure 3.9. The expression of surface proteins on WT and CD200R1KO BMDMs. Red curve, isotype control; blue curve, antibody staining.

Next, we used WT and CD200R1KO BMDMs to do phagocytosis assay. The phagocytosis percentage of A20 cells by WT BMDMs could not be elevated by anti-CD47 treatment, no matter what kinds of pre-treatment were given to WT BMDMs; on the contrary, in CD200R1KO BMDMs pre-treated with LPS, IFNγ or LPS+IFNγ, the phagocytosis percentage of A20 was enhanced significantly by CD47 blockade: with LPS pre-treatment, from 7% to 11%; with IFNγ pre-treatment, from 4% to 8%; with LPS+IFNγ pre-treatment, from 9% to 13% (Figure 3.10A). When using J558 as target cells, similarly, CD47 blockade cannot improve phagocytosis of J558 by WT BMDMs, but can enhance phagocytosis by CD200R1KO BMDMs: without pre-treatment, from 7% to 13%; with IFNγ pre-treatment, from 8% to 18% (Figure 3.10B). When using WEHI-231 as target cells, CD47 blockade can only promote phagocytosis of WEHI-231 by CD200R1KO BMDMs: without pre-treatment, from 8% to 15%; with IFNγ pre-treatment, from 9% to 19% (Figure 3.10C). We also did flow-cytometry-based phagocytosis assay using non-pre-treated WT BMDMs and WEHI-231 cells. We obtained consistent results as above – anti-CD47 can increase the phagocytosis of WEHI-231 by

CD200R1KO BMDMs significantly but cannot enhance the phagocytosis by WT BMDMs (Figure 3.10D, E). Blockade of CD47 increased the phagocytosis percentage of WEHI-231 by CD200R1KO BMDMs from 7% to 13%. Taken together, our data suggest that CD200R1 has inhibitory effects for phagocytosis.

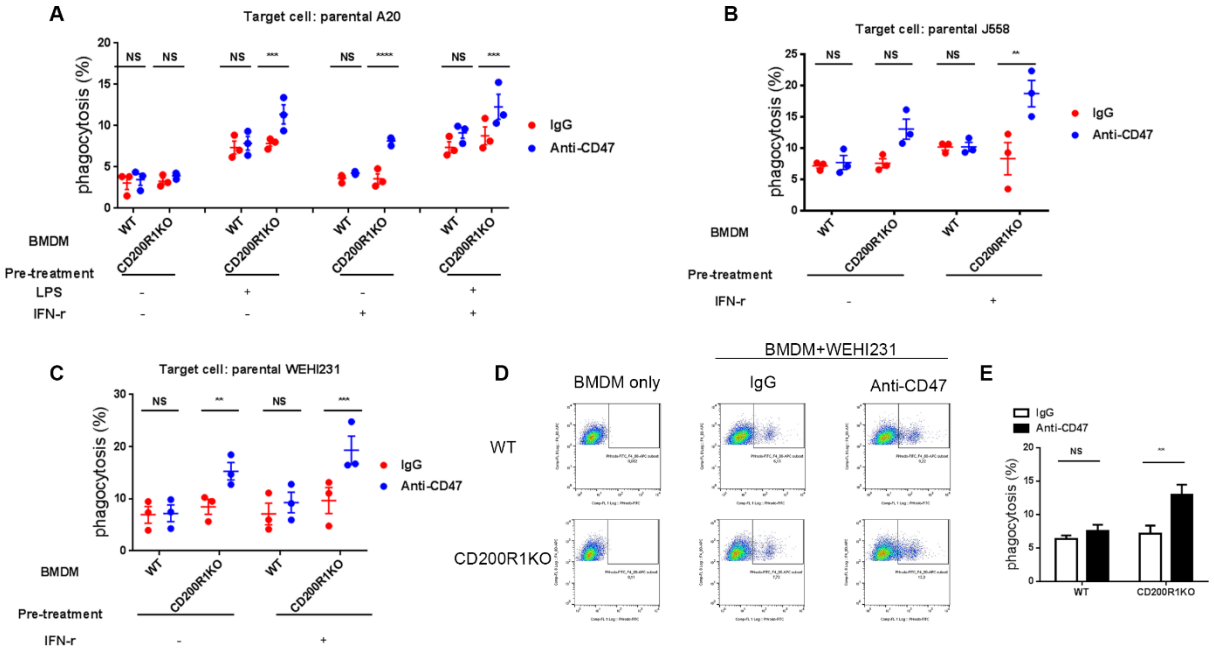

Figure 3.10. CD200R1KO BMDMs have stronger ability to phagocytose tumor cells than WT BMDMs. A, microscopy-based measurement of phagocytosis of A20 cells by WT and CD200R1KO BMDMs, with/out LPS and IFNγ pre-treatment (n=3). B, microscopy-based measurement of phagocytosis of J558 cells by WT and CD200R1KO BMDMs with/out IFNγ pre-treatment (n=3). C, microscopy-based measurement of phagocytosis of WEHI-231 cells by WT and CD200R1KO BMDMs with/out IFNγ pre-treatment (n=3). D, flow-cytometry-based measurement of phagocytosis of WEHI-231 cells by WT and CD200R1KO BMDMs without pre-treatment. E, quantification of D (n=3). Error bars stand for SEM.

3.4 NPXY motif is critical for the inhibitory function of CD200R1

It has been reported that in mast cells, CD200R1 signaling relies on a NPXY motif which binds to proteins with phosphotyrosine-binding (PTB) domain (109). Upon engagement with CD200, the NPXY motif in the cytoplasmic domain of CD200R1 gets phosphorylated,

recruiting Dok1 and Dok2 to mediate inhibition of mast cell activation (109). Therefore, we wonder whether the inhibitory function of CD200R1 for phagocytosis also relies on NPXY motif. We generated 4 kinds of pFB plasmids – one contains the original cDNA of CD200R1, while the other three contain mutated cDNA of CD200R1. By DNA sequencing, we confirmed that we generated correct mutations (Figure 3.11). We mutated tyrosine (Y) to phenylalanine (F), asparagine (N) to glutamine (Q), and proline (P) to alanine (A) respectively.

Figure 3.11. DNA sequencing results of plasmids containing mutant cDNA of CD200R1. A, amino acid Y is mutated to F by changing codon TAT to TTT; B, N is mutated to Q by changing AAT to CAA; C, P is mutated to A by changing CCA to GCA. Upper line, the original cDNA sequence of CD200R1; lower line, the sequencing results of plasmids.

By retro-virus infection, we expressed mutant CD200R1 in CD200R1KO BMDMs. We confirmed the expression of CD200R1 by flow cytometry (Figure 3.12A). Next, we did phagocytosis assay using these infected BMDMs. We found out that after restoring the expression of WT CD200R1 in CD200R1KO BMDMs, the phagocytosis percentage of WEHI-231 cells was significantly lower than BMDMs without CD200R1 expression; however, after restoring the expression of mutant CD200R1 in CD200R1KO BMDMs, the phagocytosis of WEHI-231 cells was as strong as BMDMs without CD200R1 expression (Figure 3.12B). Our data suggest that NPXY motif is important for the inhibitory function of CD200R1 in BMDMs,

and a single mutation of NPXY motif can abolish the inhibitory function of CD200R1.

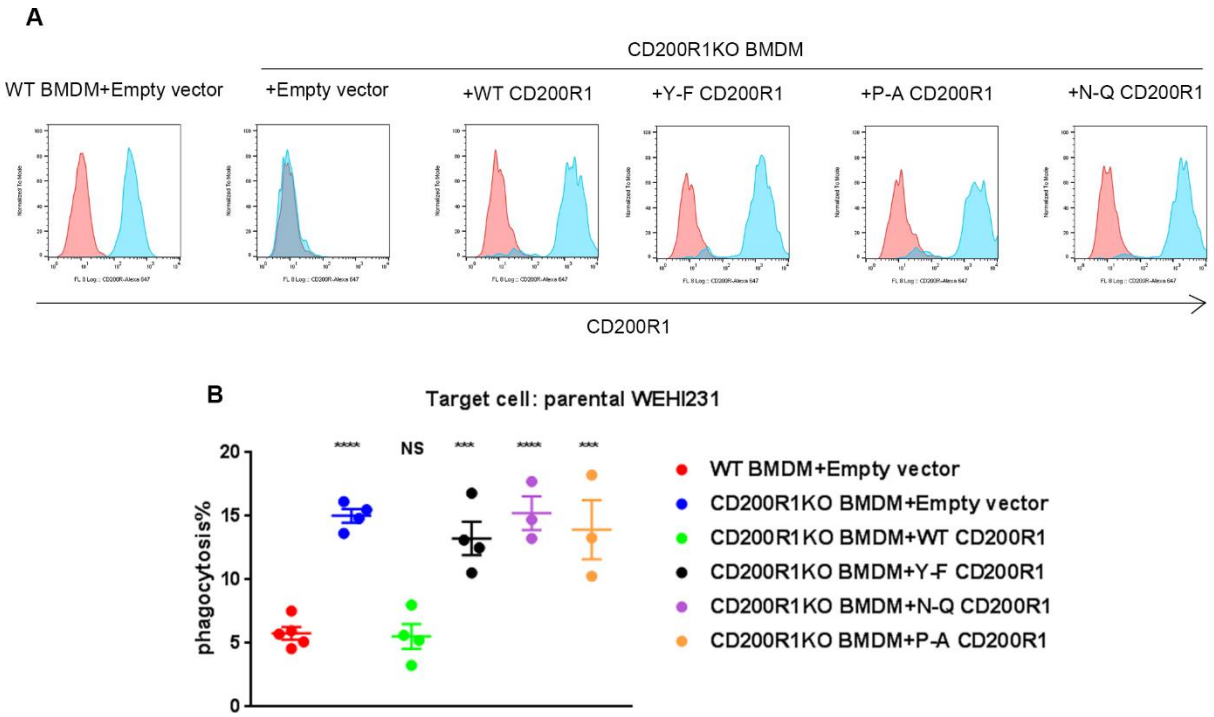

Figure 3.12. NPXY motif is critical for CD200R1 to exert its inhibitory function. A, expression of CD200R1 on infected WT and CD200R1KO BMDMs. B, microscopy-based measurement of phagocytosis of WEHI-231 cells by infected WT and CD200R1KO BMDMs (n=3-5). Error bars stand for SEM. WT BMDMs + Empty vector group was set as control group for statistical analysis.

3.5 Fc receptor is involved in phagocytosis

Fc receptor is able to induce phagocytosis and degranulation due to their ability to bind to Fc portions of immunoglobulins (110). We wonder if Fc receptor plays a role in phagocytosis of A20 and WEHI-231 cells, so we made $F(ab')_2$ antibodies of IgG and anti-CD47, which are devoid of Fc portions (Figure 3.13A). Our results show that anti-CD47 $F(ab')_2$ antibody cannot increase phagocytosis of A20-CD200KO cells by WT or SFRKO BMDMs, with or without LPS+IFNγ pre-treatment, indicating the increase of phagocytosis of A20-CD200KO cells is dependent on Fc receptor. The phagocytosis percentage is 1% for both A20-EV and A20-

CD200KO cells when WT BMDMs have not been pre-treated, 5% when pre-treated with LPS+IFNγ, no matter what antibodies were used (Figure 3.13B). Similar results have been obtained using SFRKO BMDMs (Figure 3.13C). We also observed that in non-pre-treated WT BMDMs, the phagocytosis percentage of WEHI-231 cells is always around 3% no matter what antibodies were used; in non-pre-treated CD200R1KO BMDMs, only intact anti-CD47 can enhance phagocytosis percentage significantly, while anti-CD47 $F(ab')_2$ did not work (Figure 3.13D), suggesting that Fc receptor is involved in the phagocytosis of WEHI-231 cells by CD200R1KO BMDMs.

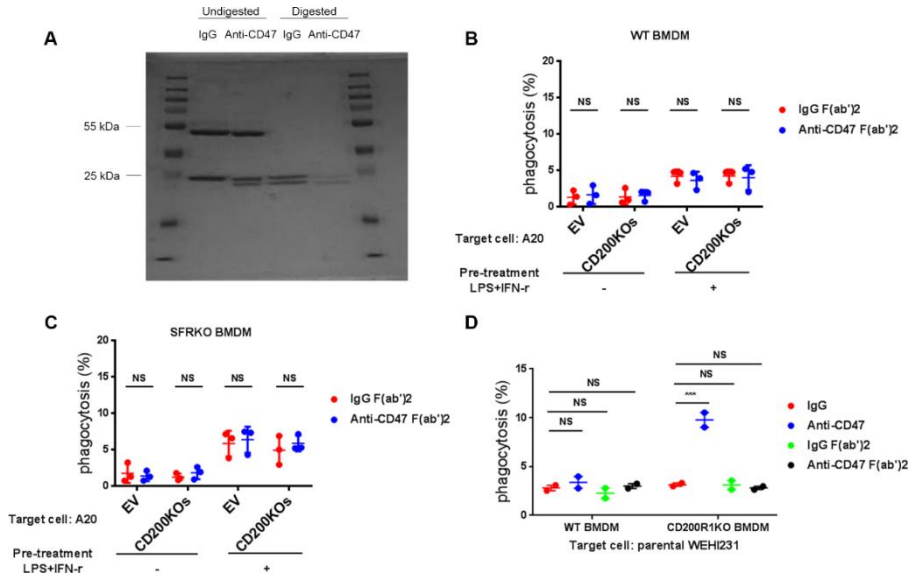

Figure 3.13. Fc receptor is involved in the phagocytosis of A20 and WEHI-231 cells. A, Coomassie-blue-stained protein gel. B, microscopy-based measurement of phagocytosis of A20 cells by WT BMDMs with/out LPS+IFNγ pre-treatment (n=3). C, microscopy-based measurement of phagocytosis of A20 cells by SFRKO BMDMs with/out LPS+IFNγ pre-treatment (n=3). D, microscopy-based measurement of phagocytosis of WEHI-231 cells by WT and CD200R1KO BMDMs without pre-treatment (n=2). Error bars stand for SEM.

4 Discussion

Our study expands the understanding of CD200-CD200R signaling, which has been proved to be an anti-inflammatory regulator. The data presented above demonstrate that murine CD200-CD200R signaling axis can down-regulate the phagocytosis ability of BMDMs. We found out that after knocking out CD200 expression, target cells became more vulnerable to phagocytosis, and they formed more conjugates with BMDMs. We also observed that using blocking antibody against CD47 and CD200 together could enhance the phagocytosis of target cells which express CD200. Compared with WT BMDMs, we found out that CD200R1KO BMDMs have stronger phagocytosis ability, indicating that CD200R can inhibit phagocytosis. In addition, we figured out that CD200R-mediated inhibition of phagocytosis is dependent on NPXY motif, which binds to PTB domain. Besides, we identified the involvement of Fc receptor in the phagocytosis of A20 and WEHI-231 cells. At last but not least, by immunoprecipitation and mass spectrometry, we discovered several proteins which interact with CD200R1 in BMDMs.

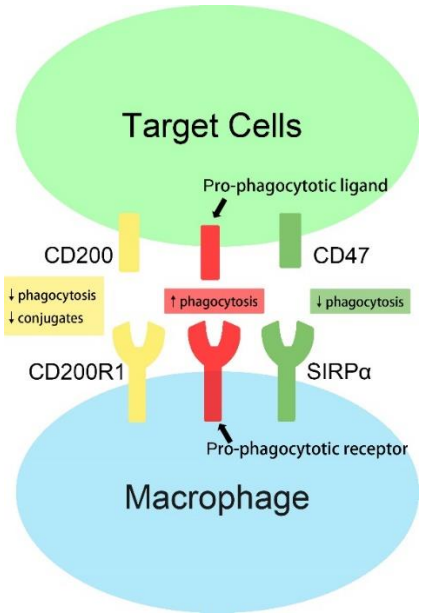

Figure 4.1. Graphic summary. Macrophage activity is regulated by different surface receptors. CD200-CD200R1 interaction can downregulate phagocytosis and conjugates formation; SIRPα-CD47 inhibits phagocytosis as well; pro-phagocytotic receptors like Fc receptor can activate macrophage to phagocytose targets. All in all, regulation of macrophage is complicated and cooperative.

To escape from being eliminated by immunosurveillance, tumor cells choose to overexpress some negative signals, like PD-L1 and CD47, so as to suppress the activation of immune cells. In our experiments, we identified high expression level of CD200 in three tumor cell lines, implying that CD200 might be another negative signal utilized by tumor cells, which protects tumor cells from phagocytosis. Interestingly, we found out that CD200 can avoid A20 cells to form conjugates with WT BMDMs. This phenomenon partially explains why the basal level of phagocytosis percentage of A20 cells is very low: less conjugate formation leads to less opportunity for macrophage to initiate phagocytosis. Considering the broad distribution profile of CD200 among various cell types, one possibility is that CD200 serves as a "safe signal". When macrophage recognizes CD200 expression on target cell, it determines this target is safe and leaves to check next target; when macrophage cannot find CD200 expression, it requires more time to search for other "safe signal", resulting in longer duration of conjugate formation. However, this theory needs to be validated by more experiments.

In our experiments, we found out that LPS and IFNγ pre-treatment is capable of enhancing the phagocytosis ability of BMDMs. LPS and IFNγ can polarize macrophage to M1, which has pro-inflammatory and anti-cancer effects. However, LPS is a kind of bacterial endotoxin so it is not suitable for therapeutic use. As for IFNγ, it has been approved by FDA in 1999 for the treatment of osteopetrosis, so it is safe as a therapeutic agent. IFNγ stimulates macrophage to become M1 through the Janus kinase/signal transducers and activators of transcription

(JAK/STAT) pathway (111). Seemingly, our results exhibited that combining IFNγ and checkpoint blockade therapy might be a good strategy for anti-cancer treatment. However, IFNγ can also display pro-tumor activities, depending on the signal intensity and microenvironment (111).

One special finding in our study is that normal B cells are extremely reluctant to be phagocytosed by WT BMDMs, even though we pre-treated BMDMs with IFNγ and used antibodies to block CD47 and CD200. Based on this finding, we speculate that on B cells, there are other redundant unidentified "don't eat me" signals except CD47 and CD200. Such redundancy is important for immune system to minimize incorrect responses towards normal cells. Another explanation for this finding is that B cells contain certain characteristics which resist conjugate formation with macrophages or subsequent phagocytosis. There are various factors that can influence phagocytosis. For instance, the size and shape of particles can decide the degree of difficulty of phagocytosis. Particles whose diameter is between 2–3 μm are most easily phagocytosed (112); besides, ellipsoidal particles are phagocytosed more slowly than spherical particles (113). Consequently, the small size and shape of B cells might account for why they are difficult to be phagocytosed. Furthermore, phagocytosis can be influenced by antigen height. It was reported that the phagocytosis of antibody-opsonized particles is dependent on the height of antigen: shorter antigen leads to the separation of Fc receptor from the inhibitory phosphatase CD45, activating the phosphorylation of Fc receptor and enhancing phagocytosis (114). When we try to explain the reason why a target is phagocytosed or not phagocytosed, it is necessary to take into account all relevant factors.

Interestingly, we found that while A20 and WEHI-231 expressed SLAMF7, the

enhancement of phagocytosis by anti-CD47 (alone or in combination with CD200R1 blockade) was independent of SFRs. Rather, it was dependent on the Fc portion of the anti-CD47 antibodies, implying that it was mediated by the concomitant ability of the anti-CD47 antibodies to engage the pro-phagocytic Fc receptors on macrophages. This finding is distinct from that previously reported by the Veillette laboratory for other immune target cells, in which anti-CD47-mediated phagocytosis was dependent on SFRs (107). In addition to implying that expression of SLAMF7 alone on targets can be insufficient to mediate phagocytosis, as already reported for normal B cells (107), these findings indicate that, for some target cells such as A20 and WEHI-231, blockade of inhibitory receptors influences the ability of Fc receptors to signal. It will be interesting to see if the increase in phagocytosis of J558 cells or activated $CD4^+$ T cells during combined CD47-CD200 blockade reported herein is mediated by SLAMF7, Fc receptors or both. Along these lines, it was previously reported that phagocytosis of activated $CD4^+$ T cells during pure CD47 blockade was dependent on SLAMF7 (107), suggesting that, at least for this cell type, a SLAMF7-dependent mechanism will be involved. Clearly, the mechanisms underlying the differential utilization of various pro-phagocytic pathways under different conditions of immune checkpoint blockade is an interesting focus for future investigations.

As a corollary, a recent study of human macrophages suggested that expression of SLAMF7 on targets may not always be needed for phagocytosis during CD47 blockade (115). Of note, however, in this study, macrophages were pre-treated with the cytokine GM-CSF, which induces the generation of inflammatory macrophages. Recent work in the Veillette laboratory showed that stimulation of mouse macrophages with inflammatory stimuli like IFNγ

or LPS can also bypass the need for SLAMF7 in phagocytosis (D. Davidson and J. Chen, unpublished results). Hence, the different nature of the macrophages used in this other study likely explained the differential involvement of SLAMF7 in these various settings. It is also possible that the different results stem from different cell lines that were used to do experiments. As discussed above, SLAMF7 may be required for certain cell lines to be phagocytosed, but not for all target cells. Another possibility is that some target cells may secrete some cytokines like IFNγ that can stimulate macrophages; as a result, SLAMF7 may not be necessary to promote phagocytosis anymore, as reported here for A20 and WEHI-231. Future studies will be needed to clarify these various issues.

The involvement of Fc receptor in phagocytosis is critical. In our study, we confirmed that the phagocytosis of A20 and WEHI-231 cells requires the engagement of Fc receptor as a pro-phagocytotic receptor. Therefore, it is reasonable to propose that using CD47-SIRPα and CD200-CD200R blocking agents which contain Fc portion might be therapeutically advantageous since they can prevent inhibition by immune checkpoints and activate Fc receptor. However, such idea is sort of risky, because CD47 and CD200 are expressed by normal cells. As a result, these Fc-portion-bearing blocking agents might target normal cells as well, inducing macrophage to eliminate normal cells. Such phenomenon might account for several side effects of CD47-SIRPα blockade like anemia, thrombocytopenia, and leukopenia. It is necessary to utilize other strategy so as to avoid the potential risk of blockade therapy. For instance, we can use some blocking agents devoid of Fc portion to prevent inhibition by immune checkpoints, at the same time use intact antibody which selectively targets tumor cells in order to activate Fc receptor-mediated phagocytosis. In this way, we can limit the bystander

effects on normal cells to a relatively low level.

The major functional assay we did in this study is phagocytosis assay, which is divided into microscopy-based and flow-cytometry-based. Microscopy-based phagocytosis assay provides us with visible results, and we can distinguish real phagocytosis from conjugates by our own eyes, offering excellent reliability. However, since we need to count the number of macrophages which phagocytosed cells manually, the results we obtained are kind of subjective. As for flow-cytometry-based phagocytosis assay, this method is highly objective, however, macrophages have to be detached from plates to run flow cytometry. Due to their strong adherence to plates, not all macrophages can be detached, so the final sample we got might be a specific population of macrophages, which can be detached more easily. Consequently, it is necessary to combine these two experiments together so as to obtain more convincing results. In our data, results obtained from microscopy-based assay and flow-cytometry-based assay are similar, suggesting that they are reliable.

One drawback of our study is that all experiments are in vitro tests. Besides, we only used BMDMs to study phagocytosis, however, considering that BMDMs have been cultured in vitro for 7 days, BMDMs might behave differently from in vivo macrophages.

5 Future Directions

In our study, we only used BMDMs to do phagocytosis assay. In order to further demonstrate that CD200-CD200R1 signaling axis can inhibit the phagocytosis ability of macrophage, it is necessary to carry out phagocytosis assay using other types of macrophages like peritoneal macrophages. We should also design some in vivo experiments in mouse model to find out whether CD200-CD200R1 signaling axis is important for phagocytosis in internal environment. For instance, we can inject tumor cells subcutaneously into mice and figure out whether blocking CD200-CD200R1 interaction can restrain the growth of tumor cells. Besides, to evaluate the function of CD200-CD200R1 signaling axis in human, we are supposed to obtain human macrophage samples to do phagocytosis assay. Moreover, since the downstream signaling of CD200R1 was majorly studied in mast cells, it is worthwhile to confirm whether the downstream signaling of CD200R1 in macrophage is also dependent on Dok2 and RasGAP. We can design specific peptides containing phosphorylated NPXY motif to do pull-down assay with the lysates of BMDMs so as to identify potential mediators for signaling. Furthermore, to figure out the relationship between CD200R1 and SIRPα, we should generate SIRPαKO mice and CD200R1-SIRPα-double-KO mice so as to compare the functional difference of macrophage from different mice. At last but not least, we can try to combine the blockade of CD200-CD200R1 interaction with other therapeutic monoclonal antibodies in order to achieve better elimination of tumor cells.

6 Conclusion

In this study, we demonstrated that CD200 expression can protect cells from being phagocytosed by BMDMs. We also showed that the blockade of CD200-CD200R1 and CD47-SIRPα can enhance phagocytosis of cells that express CD200 significantly, except for normal B cells. We exhibited that knocking out CD200R1 on BMDMs can promote phagocytosis remarkably. We confirmed that the inhibitory function of CD200R1 relies on NPXY motif which locates in the intracellular domain of CD200R1. We also showed that the involvement of Fc receptor is important for phagocytosis. Furthermore, we identified several receptors which interact with CD200R1 on BMDMs, however, at present we cannot explain why such association exists. In conclusion, our study indicates that CD200-CD200R1 signaling axis might be a target of immune checkpoint blockade therapy.

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