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Blocking 'don't eat me' signals CD47 and LILRB2 to enhance macrophage- and granulocyte-mediated phagocytosis of cancer cells

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Abstract

Cancer cells can evade immune surveillance by utilizing surface receptors called immune checkpoints. CD47 and LILRB2 are two of the few receptors that can provide an inhibitory signal to immune phagocytic cells, also called 'don't eat me' signals. These signals dampen the immune responses of macrophages and granulocytes by emulating the preventive signals of host cells to avoid autoimmunity. By blocking these receptors using monoclonal antibodies, the phagocytosis of cancer cells will be enhanced, thus stimulating anticancer responses by the innate immune system. As the innate immune system is the first line of defense, enhancing its activation not only helps better engulf cancer cells, but also provides long-term immunity by augmenting antigen-presentation to adaptive immune cells. Furthermore, these 'don't eat me' signals can negatively affect the efficacy of rituximab (RTX), a highly effective monoclonal antibody (mAb) targeting CD20 in B cell malignancies. Synergy between anti-CD47 mAb and RTX was achieved in clinical trial for diffused large B cell lymphoma (DLBCL) patients with rituximab-resistance or refractory. Therefore, this research aims to establish the synergy between the monoclonal antibodies targeting CD47 and LILRB2 to provide a rationale for bispecific antibody production, and to achieve synergy with RTX to further increase efficacy. The anti-CD47 and anti-LILRB2 mAbs function by blocking 'don't eat me' antiphagocytic signals, and rituximab mediate cell death by antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC). Therefore, we determine these effects by performing the granulocyte, and macrophage phagocytosis assays on B-cell lymphoma cell lines (SUDHL6, SUDHL10, U2932), and MTS cytotoxic assay on carcinoma cell lines (DLD-1, MDA-MB231, OVCAR3). Despite varying results, the combination of anti-CD47 antibody with anti-LILRB2 antibody did synergize to enhance phagocytosis of granulocyte and macrophage compared to the single agent treatment with anti-CD47 antibody of all cell lines, except for OVCAR3. Additionally, although not uniformly the combination of anti-CD47 and anti-LILRB2 antibodies with rituximab enhanced granulocyte- and macrophage-mediated phagocytosis of cell lines U2932 and SUDHL6, respectively.

Therefore, the current evidence supports the bispecific antibody production, and it is indicative that this bispecific antibody would synergy with rituximab for an enhanced treatment for B cell malignancies.

Keywords: anti-CD47, anti-LILRB2, rituximab, phagocytosis, bispecific antibody, checkpoint inhibitor

Abbreviations

ADCC: antibody-dependent cellular cytotoxicity

ADCP: antibody-dependent cellular phagocytosis

ANGPTL: angiopoietin-like protein

CD47: Cluster of Differentiation 47

DLBCL: diffuse large B cell lymphoma

FACS: Fluorescent activated cell sorting

FcR: crystallizable fragment receptor

LILRB2: leukocyte immunoglobulin-like receptor subfamily B 2

mAb: monoclonal antibody

MHC I: major histocompatibility complex class I

PMN: polymorphonuclear neutrophils

SIRPα: Signal-Regulatory Protein α

RTX: rituximab

Introduction

Cancer is a complex disease that express a variety of traits that differentiate them from normal cells called hallmarks of cancer. One of the hallmarks of cancer is immune evasion.[1] Cancer cells are often overexpress receptors, which are present on normal cells, to evade the host's immune surveillance.[2] These cell-surface receptors that interact with their ligands on immune cells to dampen the immune system, consequently inhibiting their effector functions.[3] These receptors are called immune checkpoints. In order to block the negative regulation of these ligand-checkpoint interactions, inhibitors have been produced (immune checkpoint inhibitors). The Cluster of Differentiation 47 (CD47) is an immune checkpoint that has been demonstrated as an effective cancer target.[4] This receptor is overexpressed on cancer cells and bind to the Signal-Regulatory Protein α (SIRPα) present on macrophages and granulocytes to transmit an antiphagocytic 'don't eat me' signal on cancer cells[2]. Additionally, high CD47 expression in ovarian cancer is correlated to a poor prognosis in patients, such as promoted metastasis, tumor invasion, and lower overall survival rate in diffuse large B cell lymphoma (DLBCL) patients.[5,6,7,8] Blockade of this signaling axis using anti-CD47 antibody have demonstrated to enhance macrophage phagocytosis[4] and trogoptosis[9], a process of cell membrane engulfment by granulocytes leading to cell lysis. Therefore, by using anti-CD47 antibodies, 'don't eat me' signaling is reduced.[9, 10] As such, clinical trial of single-agent therapy of anti-CD47 mAb has been documented to induce partial remission in two heavily pre-treated patients with clear cell ovarian and fallopian tube cancers.[11]

Targeting only CD47 is insufficient as cancer immunoevasion can prevail through another pathway, such as the expression of the major histocompatibility complex class I (MHC I) by cancer cells.[12] MHC I can bind to two receptors that leads to evasion of phagocytosis, the LILRB1 and LILRB2 (leukocyte immunoglobulin-like receptor subfamily B 1 and 2).[12] Barkal et al. demonstrated that by blocking LILRB1 from binding to MHC I using a monoclonal antibody enhances anti-CD47-induced phagocytosis.[12] However, the blockade of LILRB2 was not extensively studied in their paper. The LILRB2 receptor, unlike LILRB1, can bind to MHC I without β_2-microglobulin.[13] Thus, showing that LILRB2 binds to more variants of MHC I and is potentially a better therapeutic target than LILRB1. Therefore, in this study, the blockade of LILRB2 will be investigated with anti-CD47 treatment.

The LILRB2 is an inhibitory receptor that contains the immunoreceptor tyrosine-based inhibitory motifs (ITIM). [13] The binding of LILRB2 to its ligands, such as the (MHC I)[13] and angiopoietin-like (ANGPTL) protein family[15] and subsequently negatively modulate the immune cell's effector functions. Therefore, anti-phagocytic signaling occurs when LILRB2 expressed on macrophages and granulocytes bind with its ligands expressed on cancer cells.[16] LILRB2 co-expression with ANGPTL2 correlates with poor prognosis such as greater level of lymph node metastasis and shorter over survival rate.[17] Therefore, blocking LILRB2 will lead to enhanced phagocytosis by removing the anti-phagocytic signaling. By targeting

both CD47 and LILRB2, the innate immune system will be re-sensitized through the removal of these 'don't eat me' signals. Their combinations could yield a synergistic induction of phagocytosis that could potentially be produced into a bispecific antibody. Bispecific antibodies induce superior antitumor effects than monoclonal antibodies by their ability to increasing proximity of target cells and simultaneously inhibit two receptors at once.[18] Furthermore, as chemo-radiotherapy induces off-target toxicity that negatively impacts the patient,[19, 20] an alternative to this is immunotherapy targeting CD47 and LILRB2. As this treatment directs more selective killing than chemo-radiotherapy, it is safer for patients. Moreover, this bispecific antibody could induce an enhanced antitumor response when combined with rituximab, an anti-CD20 antibody currently in clinical use that is a genuine option besides chemotherapy for hematological malignancies.[21] Additionally, the combination of anti-CD47 and anti-LILRB2 antibody with rituximab could also overcome rituximab resistance.[22] As clinical trials in DLBCL patients have shown that rituximab synergize with CD47 blocking antibody[23,24], the further addition of LILRB2 blockade is expected to increase rituximab-induced phagocytosis.

The combination of rituximab with anti-CD47 and anti-LILRB2 antibody would enhance rituximab-induced antitumor response through the removal of antiphagocytic signals and simultaneously 'eat me' signals are enhanced from the binding of the crystallizable fragment receptor (FcR) on macrophages and granulocytes to the Fc domain of the rituximab.[24] As such, the combination with rituximab will inducing an "eat me" signal through FcR binding while the IgG4 anti-CD47 and anti-LILRB2 are also eliminating the CD47 and LILRB2 "don't eat me" signals for an enhanced innate immunity tumor clearance. Due to the presence of the Fc domain of rituximab, antibody-opsonized tumor cells would not only undergo antibody-dependent cellular phagocytosis (ADCP) but also antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity.[9, 10, 24] It is well-established that the granulocytes (also known as neutrophils) are the first line of the defense in the immune system,[25] and there are several macrophage subtypes present in the tumor microenvironment, such as the M1 (pro-inflammatory) subtype and M2 (pro-tumorigenic) subtype.[26] Therefore, to investigate the phagocytic effects of the various combinations of monoclonal antibodies anti-CD47, anti-LILRB2 and rituximab, the granulocyte phagocytosis, and M1 and M2 macrophage phagocytosis assays will be performed on DLBCL cell lines. Additionally, the MTS cytotoxic assay that measures viability of cells using colorimetric method will also be performed with granulocytes on carcinoma cell lines with anti-CD47 and anti-LILRB2.

This research will investigate whether the combination of anti-CD47 antibody with anti-LILRB2 antibody will synergize to induce enhanced phagocytosis of tumor cells by macrophages and granulocytes. This knowledge will subsequently be explored for the potential production of CD47-LILRB2 bispecific antibody for cancer immunotherapy. Additionally, the synergy of rituximab combined with monoclonal antibodies anti-CD47 and anti-LILRB2 will also be investigated to augment the efficacy of rituximab.

Materials & Methods

Cell culturing

Six human tumor cell lines were used during these experiments: three types of solid cancer (carcinoma) cell lines, three types of hematological malignancies cell lines; DLD-1 (colorectal adenocarcinoma), MDA-MB231 (invasive ductal carcinoma), OVCAR3 (high grade ovarian serous adenocarcinoma), U2932 (diffuse large B cell lymphoma), SU-DHL-6 (diffuse large B cell lymphoma), and SU-DHL-10 (diffuse large B cell lymphoma), respectively. All cell lines were obtained from the Experimental Hematology Department, University Medical Center Groningen. DLD-1, MDA-MB231, and U2932 cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS). SU-DHL-6 and SU-DHL-10 cells were grown in RPMI 1640 medium (Gibco) supplemented with 20% FBS. OVCAR3 cells were grown in DMEM medium (Lonza) + Glucose and L-glutamine supplemented with 10% FBS. All cell cultures were cultivated at 37°C in 5% CO_2 humidity and passed every two or three days. Carcinoma cells were split using trypsin/EDTA solution.

Mononuclear cell isolation from human peripheral blood

Apheresis blood was obtained from healthy donors after informed consent. Dilute blood with phosphate buffered saline (PBS) (1:3 volume ratio). Peripheral blood mononuclear cells (PBMCs) were fractionated by density gradient centrifugation over isotonic Lymphoprep™ (Axis-Shield PoC As, Norway) using the included manual. The pellets were then resuspended in RPMI 1640 medium (Gibco), counted using fluorescent activated cell sorting (FACS) analysis, diluted, and plated into a 6-well plate in the presence of macrophage colony stimulating factor (M-CSF) and granulocyte/macrophage colony stimulating factor (GM-CSF) for monocyte differentiation and proliferation into M0 macrophages. After a few days of macrophage maturation, macrophages were then polarized to M1 macrophages by adding lipopolysaccharide (LPS) at a concentration of 100 ng/ml and interferon gamma (IFNγ) at 50 ng/ml, and M2c macrophages were derived from adding interleukin 10 (IL-10) cytokines at 50 ng/ml to M0 macrophages. Macrophages were plated at 10 x 10^3 in 96-well plate for functional experiments.

Granulocyte isolation from human peripheral blood

Apheresis blood was obtained from healthy donors. Blood was diluted and underwent Lymphoprep™ (Axis-Shield PoC As, Norway) fractionation as previously described. As granulocytes and erythrocytes are denser than PBMCs, they sediment at the bottom of the tube. Therefore, to harvest granulocytes, all the supernatant was removed, leaving the pellet fraction. Erythrocyte lysis was followed by adding Red Blood Cell lysis buffer solution for 30-40 min at 4°C. The remaining granulocytes were washed twice in PBS. Granulocytes were resuspended in the X-VIVO™ 15 medium (Lonza) at a final concentration of 5 x $l0^5$ cells/mL for functional assays.

Flow cytometry-based polymorphonuclear neutrophil (PMN) phagocytosis assay

To determine the effect of anti-CD47, anti-LILRB2 antibodies and/or rituximab (RTX) on granulocyte phagocytosis of B cell lymphoma, a PMN phagocytosis assay was performed. First, tumor cells were stained with CFSE (Thermo Fisher Scientific, USA). After staining, the stain was washed away with RPMI 1640 medium (Gibco) with 10% FBS and then with PBS. Tumor cells were counted and resuspended to 5 x 10^5 cells/ml in RPMI 1640 medium (Gibco) supplemented with 10% FBS. Before tumor cells and granulocytes were co-cultured, they were pre-incubated with their according therapeutic antibodies at 37°C in 5% CO_2 humidified air. Treatment antibodies anti-human CD47 (GenScript) and RTX (Truxima) were added at a concentration of 1 µg/ml and 0.1 µg/ml, respectively and pre-incubated with tumor cells for 30 min. Anti-LILRB2 (Anti-human CD85d (ILT4) clone 42D1, eBioscience Inc., USA) was added at a concentration of 1 µg/ml into the respective tubes with granulocytes and was pre-incubated for 30 min. After the pre-incubation period, 5 x 10^4 tumor cells were co-cultured with 5 x 10^4 granulocytes for 2 h at 37°C in 5% CO_2 humidified air. After co-culture, phagocytosis was evaluated using the BD Accuri™ C6 Plus personal flow cytometer. The percentage of phagocytosis was determined by quantification of the percentage of CFSE-positive granulocytes

MTS Cytotoxic Assay

To determine the effect of granulocytes incubated with anti-CD47 and/or anti-LILRB2 antibodies on carcinoma cell lines, cell viability (MTS assay) assay was performed. First, DLD-1, MDA-MB231, and OVCAR3 were plated at density 1 x 10^4 cells/well in a 96-well plate. Treatment antibody anti-human CD47 (GenScript) was added at a concentration of 1 µg/ml and pre-incubated with adherent cells for 30 min. On the other hand, granulocytes (5 x 10^4 cells/well) were pre-incubated with anti-LILRB2 antibody (Anti-human CD85d (ILT4) clone 42D1, eBioscience Inc., USA) at concentration 1 µg/ml for 30 min. After pre-incubation, tumor cells and granulocytes were co-cultured overnight at 37°C in 5% CO_2 humidified air. After co-culture, granulocytes were washed away twice with 200 µl of RPMI 1640 (Gibco) with 10% FBS. After washing, plates were checked under the light microscope to ensure granulocytes were well-washed away. The MTS tetrazolium compound was diluted with RPMI 1640 medium in a 1:5 volume ratio and 50 µl of this solution was added to the wells. The 96-well plate was incubated for 1 to 3 h. Reduction of MTS tetrazolium compound was quantified at optical density (OD)= 490 nm wavelength using microplate reader (iMark).

Fluorescent microscopy-based macrophage phagocytosis assay

To determine the effect of anti-CD47, anti-LILRB2 antibodies and/or rituximab on macrophage phagocytosis of B cell lymphoma, a macrophage phagocytosis assay was performed, visualized using fluorescent microscopy, and quantified by manual counting. Staining of tumor cells with CSFE occurred in a similar fashion as described in previous sections. Tumor cells (5 x 10^4 cells/sample) were pre-incubated with therapeutic antibodies occurred in a similar fashion as the PMN phagocytosis. Macrophages were plated in a 96-well plate (1 x 10^4 macrophages/well) and pre-incubated with anti-LILRB2

antibody (Anti-human CD85d (ILT4) clone 42D1, eBioscience Inc., USA) at concentration of 1 μg/ml for 30 min at 37°C in 5% CO_2 humidified air. After pre-incubation, tumor cells and macrophages were co-cultured for 2 h at 37°C in 5% CO_2 humidified air. After co-culture, macrophages were stained for CD11b by Alexa Fluor 594 conjugated mouse anti-human CD11b antibody (BioLegend, USA) that was diluted 1:200 with RPMI 1640 medium (Gibco) with 10% FBS. The plate was incubated for 20 min at room temperature. CD11b stain was washed away twice with RPMI 1640 (Gibco) supplemented with 10% FBS. PBS was added back into the samples. Thereafter, phagocytosis was evaluated using the IncuCyte® S3 live-cell analysis system and macrophage phagocytosis was counted. Phagocytosis was quantified by counting the macrophages exhibiting phagocytosis and the total macrophage number to find the percentage of engulfing macrophages.

Results

The single-agent αCD47 treatment induced PMN phagocytosis of all 3 B-cell lymphoma cell lines, while single-agent treatment of αLILRB2 increased phagocytosis of U2932 and SUDHL6

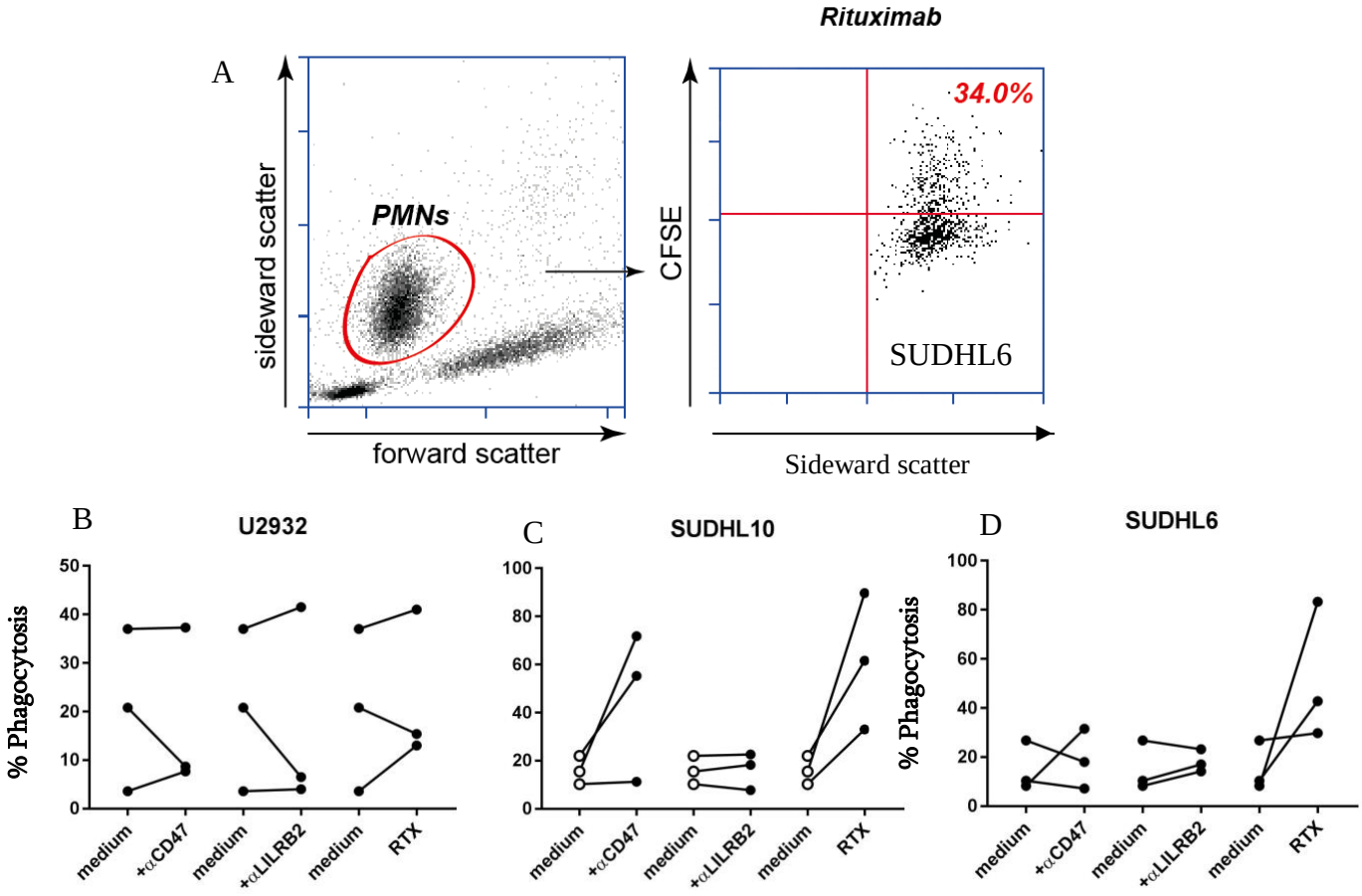

Figure 1. Percentage phagocytosis of tumor cells *in vitro* by PMN upon incubation with anti-CD47 antibody, anti-LILRB2 antibody, or rituximab. a. Representative graphs of gating strategy for flow cytometry-based analysis for phagocytosis of tumor cells by PMNs with rituximab in SUDHL6. The PMNs were gated based on the forward scatter/side scatter plot, a gate was thereafter drawn to determine the amount of CSFE positive PMNs. Flow cytometry–based measurement of the phagocytosis of three cell lines of diffused large B cell lymphoma **b.** U2932 **c.** SUDHL10 and **d.** SUDHL6 by donor-derived human granulocytes in the presence of anti-CD47, anti-LILRB2 or rituximab (RTX). Each point represents an individual donor, n = 3 donors.

In order to elucidate the effects of the combination of the mAbs, we first sought to investigate the effect of individual mAbs on granulocytic phagocytosis of different DLBCL cell lines. After 2 h of incubation, the samples were quantified using a flow cytometer. Monoclonal antibodies targeting CD47 and LILRB2 induced varying effects, while rituximab, significantly enhanced phagocytosis in most of the samples by 5-80%, depending on the cell line (Fig.1a,c,e). Generally, anti-CD47 antibody modulated increased phagocytosis, but the efficacy varied per experiment and per cell line. It seems that anti-

CD47 antibody induced an increase of 40-60% phagocytosis in SUDHL10 comparing to the control sample, while other cell lines either had a 5-10% increase or even less phagocytosis than the control (Fig.1a,c,e). Anti-LILRB2 antibody also did not induce a large increase in PMN phagocytosis comparing to the control (5-10% increase at maximum) (Fig.1a,c,e).

The co-treatment of αCD47 and αLILRB2 antibodies synergized to enhance PMN phagocytosis of SUDHL10, but with the addition of rituximab, only U2932 exhibited enhanced PMN phagocytosis

After analyzing single agent activity, the combination activity of CD47 and LILRB2 mAbs were evaluated for synergism. Additionally, the synergy of the various combinations of mAbs anti-CD47, anti-LILRB2 and rituximab were analyzed through the phagocytic potential of granulocytes on different B cell lines.

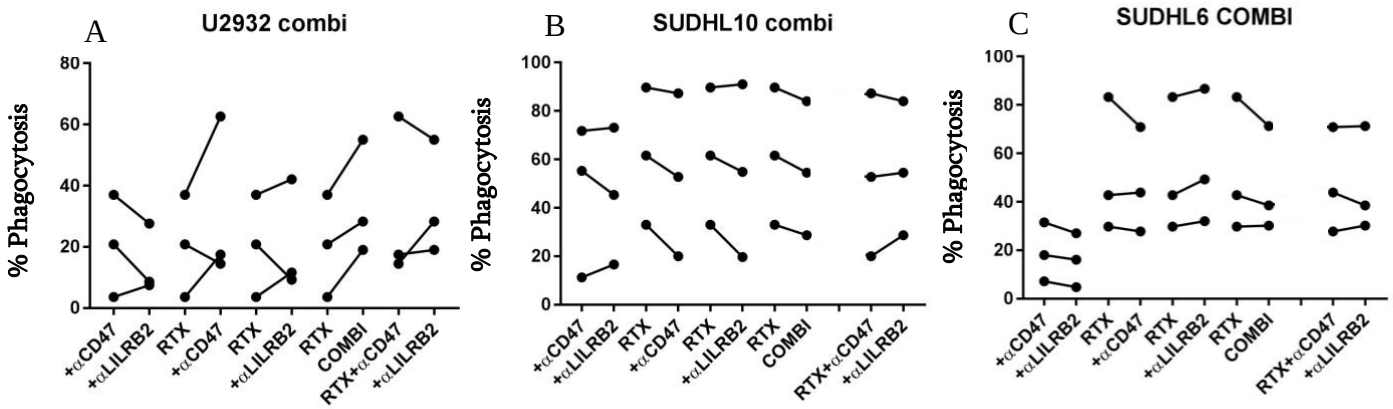

Figure 2. Percentage of phagocytosis of tumor cells *in vitro* by PMN upon the combination treatment between monoclonal antibodies anti-CD47, anti-LILRB2 and/or rituximab. Flow cytometry-based measurement of the phagocytosis of diffused large B cell lymphoma cell lines **a**. U2932 **b.** SUDHL10 and **c.** SUDHL6 by donor-derived human granulocytes in the presence of the various antibody combinations of anti-CD47, anti-LILRB2, and/or rituximab (RTX). The phagocytosis of each combinations is compared. Y axis from left to right: αCD47 vs. αCD47+αLILRB2, RTX vs. RTX+αCD47, RTX vs. RTX+αLILRB2, RTX vs. COMBI (RTX+αCD47+αLILRB2), RTX+αCD47 vs. RTX+αCD47+αLILRB2. Each point represents an individual donor, n = 3 donors.

The co-treatment with anti-LILRB2 antibody had an added effect of 5% on the anti-CD47 antibody in 1 out of 3 triplicates of the cell line U2932 (Fig. 2a). RTX-induced phagocytosis of cell line U2932 was also enhanced by 10-20% with the addition of both the anti-CD47 and anti-LILRB2 mAbs. Of which, the addition of anti-LILRB2 mAb increased phagocytosis by 5-15% in addition to the synergistic effects of the co-treatment of rituximab and anti-CD47 mAb (Fig. 2a).

A 5% increase in granulocyte phagocytosis was induced upon stimulation with both anti-CD47 and anti-LILRB2 mAbs on SUDHL10 cell line (Fig. 2b). In both SUDHL6 and SUDHL10, the co-treatment of either anti-CD47 or anti-LILRB2 with rituximab did not significantly increase efficacy of rituximab (Fig. 2b and c). As such, the combination of rituximab with both anti-CD47 and anti-LILRB2 mAb did not induce an added effect on top of the effect by rituximab for SUDHL10 and SUDHL6 cell lines (Fig. 2b and c).

αCD47 and αLILRB2 synergize to induced decreased cell viability of cell lines DLD-1 and MDA-MB231 but not in OVCAR3

Phagocytosis of cancer cells by granulocytes can cause them to lose membrane integrity and die. Therefore, the cell viability assay by MTS was performed to quantify the tumor cell viability with single agent treatment of anti-CD47 or anti-LILRB2 mAb, or the co-treatment of both antibodies.

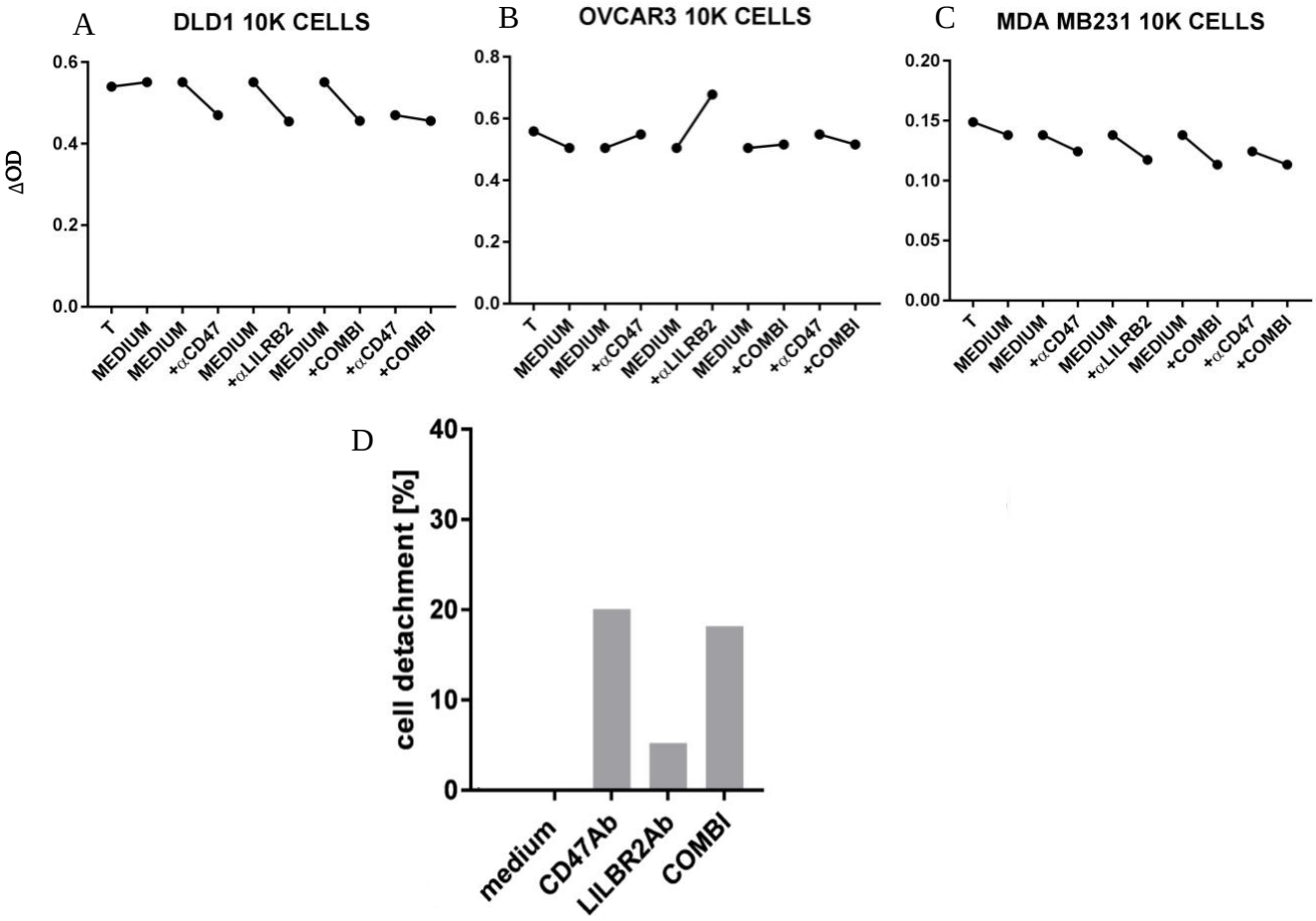

Figure 3. The viability of tumor cells after incubation with granulocytes in the presence of anti-CD47 antibody, anti-LILRB2 antibody, or a combination of both. MTS reagent-based measurement of cell viability of adherent tumor cell lines *in vitro* **a.** DLD-1 **b**. OVCAR3 and **c.** MDA-MB231 by donor-derived human granulocytes in the presence of anti-CD47 antibody, anti-LILRB2 antibody, and combination of both antibodies. The cell viability of each combinations is compared. Y axis from left to right: T (tumor only control) vs. medium control (tumor cells and granulocytes without mAb), medium vs. αCD47, medium vs. +αLILRB2, medium vs. COMBI (αCD47+αLILBR2), αCD47 vs. COMBI. Each point represents the mean of 2 replicates (donors).**d.** Incucyte-based measurement of confluency was used to calculate cell detachment of OVCAR3 induced by donor-derived human granulocytes in the presence of anti-CD47 antibody, anti-LILRB2 antibody, and the combination of both antibodies. N=1 donor.

In cell lines DLD-1 and MDA-MB231 a decrease in cell viability was induced by the antibody treatments, with anti-LILRB2 mAb inducing lower cell viability than anti-CD47

(Fig. 3a and c). The combination treatment yielded the lowest cell viability with a decrease of OD by 21% and 21,5% compared to the medium control for DLD-1 and MDA-MB231, respectively (Fig. 3a and c). Of which, the anti-LILRB2 mAb was responsible for the 3% and 9.7% decreased in cell viability of DLD-1 and MDA-MB231, respectively (Fig. 3a and c).

In the OVCAR3 cell line, the single agent treatment of the antibodies and the combination treatment did not decrease cell viability. Tumor cell viability even increased, particularly of the anti-LILRB2 mAb treatment where cell viability increased by 40% (Fig. 3b). We suspected that the cytotoxicity assay using MTS may be yielding incorrect results due to the residual granulocytes present on the plate causing a high background, as seen in OVCAR3 (Fig. 3b). This was confirmed when analyzing for confluency. In OVCAR3, cell detachment induced by anti-CD47 mAb was enhanced by 20% compared to the medium control (Fig. 3d). The single agent treatment of anti-LILRB2 mAb increased cell detachment of OVCAR3 by 4%. However, the co-treatment of anti-CD47 and anti-LILRB2 mAb only increase cell detachment by 18%, which is less than that of the single agent treatment of anti-CD47 mAb for OVCAR3 (Fig. 3d). This data shows that the addition of anti-LILRB2 mAb does not enhance the effects of anti-CD47 mAb to increase granulocyte phagocytosis of OVCAR3.

The co-treatment of αCD47 and αLILRB2 synergized to enhance M1 macrophage phagocytosis potential and also enhance rituximab efficacy

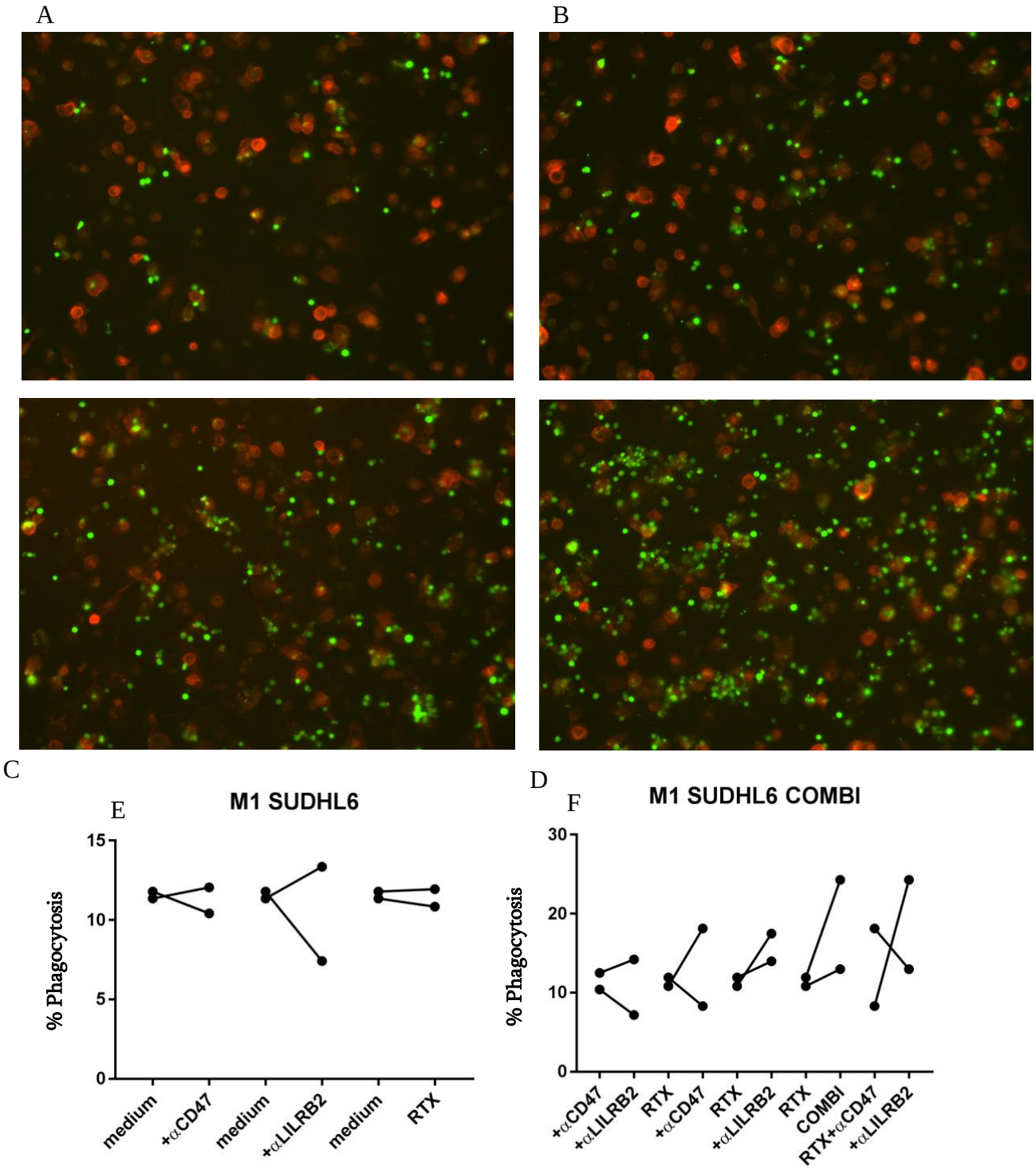

Figure 5. Phagocytosis of tumor cells *in vitro* by M1 macrophages upon incubation with anti-CD47 antibody, anti-LILRB2 antibody, rituximab, or their different combinations. Fluorescent microscopy-based visualization of macrophage phagocytosis of DLBCL cell line SUDHL6 by donor-derived human M1-polarized macrophages in the presence of anti-CD47 antibody, anti-LILRB2, rituximab (RTX) or their various combinations. The representation of fluorescent microscopy visualization used to quantify phagocytosis of **a.** medium control (untreated sample), **b.** anti-CD47 mAb treated, **c.** RTX treated, **d.** combination of anti-CD47, anti-LILRB2 mAbs and RTX. Engulfment of tumor cells were determined

by the clear internalization of tumor cells (green) by macrophages (red). Tumor cell attachment to macrophages were not quantified as phagocytosis. The phagocytosis potential of M1 macrophages are represented as **e**. mono-treatments, and **f.** combined treatments where the phagocytosis of each combinations is compared. Y axis from left to right: αCD47 vs. αCD47+αLILRB2, RTX vs. RTX+αCD47, RTX vs. RTX+αLILRB2, RTX vs. COMBI (RTX+αCD47+αLILRB2), RTX+αCD47 vs. RTX+αCD47+αLILRB2. Each point represents an individual donor, n = 2 donors.

To investigate the phagocytosis potential of M1 macrophages when 'don't eat me' signals CD47, LILRB2 and/or CD20 are inhibited, CSFE-stained tumor cells were mixed with M1-polarized macrophages.

The visualized effects were significantly different for each antibody treatment. The combination treatment of anti-CD47 and anti-LILRB2 mAb with rituximab induced significant tumor cell clustering and adherence to macrophages (Fig. 5d). Not only did the quantity of engulfing macrophages were enhanced, but the magnitude of engulfing also increase as some macrophages engulfed multiple tumor cells (Fig. 5d). Rituximab treatment also augmented ample recruitment of macrophages to tumor cells, as tumor cell attachment to macrophages are observed (Fig. 5c). However, phagocytosis does not seem to increase with rituximab compared to the anti-CD47 mAb treatment (Fig. 5c and b, respectively). There was no significant different between the untreated sample or the anti-CD47 treatment, except for the enhanced macrophage recruitment to tumor cells (Fig. 5a,b). As such, we did not observe a clear increase in phagocytosis upon stimulation with anti-CD47 mAb compared to the medium control (Fig. 5e). Similarly, rituximab did not enhance phagocytosis, even though ample tumor cell attachment was visualized (Fig. 5 c,e). The combination therapy samples, on the other hand, induced a more promising response. The additional blockade of LILRB2 induced an increase in anti-CD47 mAb-induced phagocytosis of tumor cells by 3% (Fig. 5f). The addition of anti-CD47 or anti-LILRB2 antibody increased macrophage phagocytosis by 9% or 5%, respectively, compared to rituximab alone. As such, the addition of both these antibodies with rituximab significantly enhanced phagocytosis compared to rituximab alone by 3-15% (Fig. 5f). Of which, anti-LILRB2 mAb is responsible for around 10% of the increase, compared to the phagocytosis of rituximab and anti-CD47 combination (Fig. 5f). Thus, in SUDHL6 the combination of anti-CD47 mAb, anti-LILRB2 mAb and rituximab synergized to maximize macrophage phagocytosis.

The combination of αLILRB2 and αCD47 enhanced the efficacy of rituximab-induced phagocytosis of SUDHL6, but not of SUDHL10 in M2c macrophages

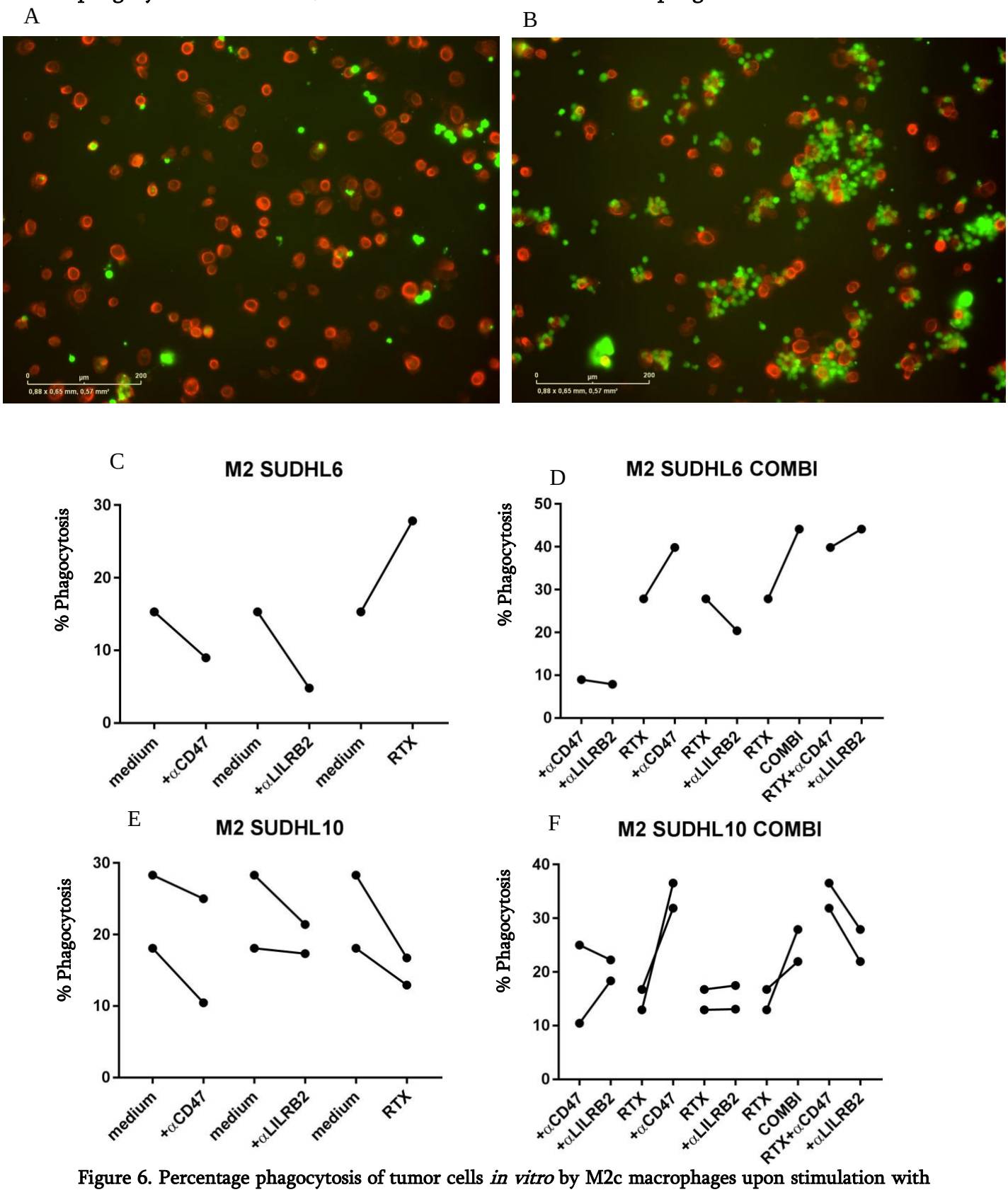

Figure 6. Percentage phagocytosis of tumor cells *in vitro* by M2c macrophages upon stimulation with anti-CD47 antibody, anti-LILRB2 antibody, rituximab, or their different combinations. Fluorescent microscopy-based visualization of donor-derived human M2c-polarized macrophage phagocytosis of **a.**

Anti-CD47 mAb treated SUDHL6 and **b**. anti-CD47 mAb treated SUDHL10. After visualization and counting of engulfing M2c macrophages, phagocytosis is quantified for **c, d.** SUDHL6 and **e,f.** SUDHL10 in the presence of anti-CD47 antibody, anti-LILRB2, rituximab (RTX) or their different combinations. **f.** Y axis from left to right: αCD47 vs. αCD47+αLILRB2, RTX vs. RTX+αCD47, RTX vs. RTX+αLILRB2, RTX vs. COMBI (RTX+αCD47+αLILRB2), RTX+αCD47 vs. RTX+αCD47+αLILRB2. Each point represents an individual donor, n = 2 donors.

The cell lines SUDHL6 and SUDHL10 react differently to the same antibody treatment, in this case anti-CD47 mAb (Fig. 6a,b). Substantial tumor cell adhesion to macrophages is visualized in SUDHL10 upon stimulation with antibodies, while almost no cell adhesion is observed in SUDHL6. Despite increased cell adhesion, enhance phagocytosis was not induced in SUDHL10 (Fig. 6e). Rituximab also did not enhance phagocytosis of M2c macrophages of SUDHL10 cell lines (Fig. 6e). However, 13% increase phagocytosis was induced by rituximab in SUDHL6 (Fig. 6,c). The combination of anti-CD47 with anti-LILRB2 mAbs did not synergize to enhance macrophage phagocytosis of SUDHL6, but a 9% increased phagocytosis was induced by the addition of anti-LILRB2 mAb to anti-CD47 mAb in SUDHL10 (Fig. 6d and f, respectively). In SUDHL6, the synergistic effect from the combination of anti-CD47 and anti-LILRB2 mAbs resulted in an additional 17% phagocytosis, in addition to the effects of rituximab (Fig. 6d). Additionally, it is indicative that the anti-LILRB2 mAb is effective as its co-treatment induced an additional 5% increase to the effects of the combination of Rituximab and anti-CD47 mAb (Fig. 6d). The synergistic effect of blocking both CD47 and LILRB2 was not observed when co-treated with rituximab in SUDHL10. The combination treatment did increase the efficacy of rituximab by almost 20%, however, this increase was not induced by the anti-LILRB2 mAb as the combination treatment of three antibodies show a 10% lower phagocytosis than rituximab with anti-CD47 (Fig. 6f).

The results of this assay indicate that the anti-CD47 and anti-LILRB2 antibodies together did synergize to enhance the immunogenic effects of M2-like macrophages in SUDHL10 cell lines but not SUDHL6. However, the addition of anti-LILRB2 mAb and anti-CD47 mAb further promote phagocytosis induced by rituximab of SUDHL6 cancer cells by M2-like macrophage.

Discussion

From previous research, anti-CD47 mAb had resulted in partial remission of ovarian cancer and fallopian tube patients.[11] Additionally, this mAb had shown to synergize with rituximab that resulted in partial or complete response in patients with non-Hodgkin lymphoma.[23] In a recent study, the MHC I had been presented as a 'don't eat me' signal that works cooperatively with CD47 to prevent cancer cell phagocytosis.[12] In this study, by preventing interaction of MHC I to its receptor LILRB1, enhanced phagocytosis was induced with anti-CD47 mAb treatment.[12] LILRB2, another receptor of the LILRB family, is also a receptor of MHC I on phagocytes that can negatively modulate immune responses.[13, 15, 16] Therefore, the research question that is being investigated in our study is whether blockade of CD47 and LILRB2 using monoclonal antibodies will synergize to enhance innate immune responses to implement this rationale for the production of a CD47-LILRB2 bispecific antibody. Additionally, it is also investigated whether with blocking LILRB2 in addition to anti-CD47 mAb will enhance rituximab-induced phagocytosis. Donor-to-donor variability and susceptibility of granulocytes to spontaneously apoptosis create a challenge to correct execution and getting reproducible data. Nevertheless, the data presented here have indicated that the simultaneous inhibition of CD47 and LILRB2 'don't eat me' signals synergized as improved phagocytosis of two DLBCL cell lines (SUDHL6 and SUDHL10) and two carcinoma cell lines (DLD-1 and MDA-MB231) were observed. On average, the anti-LILRB2 mAb induced an increase in phagocytosis of 5-15%. Furthermore, rituximab's efficacy enhanced from the blockade of two 'don't eat me' signals to promoted improved phagocytosis by 10-20% in DLBCL cell lines U2932 and SUDHL6. Both granulocytes, M1 and M2c macrophages exhibited increase phagocytosis upon antibody stimulation. Therefore, our data provides the first indication that co-treatment of two innate immune checkpoint inhibitors may synergize, and additionally augment high rituximab-induced antitumor responses by removing the phagocytic constraints. Due to the synergy observed, it is thus worthwhile for the production of CD47-LILRB2 bispecific antibody.

For effective phagocytosis, there should be more pro-phagocytic signals than anti-phagocytic signals.[10, 27] This can explain the observation that the blockade of both CD47 and LILRB2 failed to induce enhanced granulocyte-mediated phagocytosis of U2932 and M1 and M2c macrophage-mediated phagocytosis of SUDHL6. However, with the addition of rituximab containing the pro-phagocytic Fc domain, the balance is tipped, and phagocytosis is enhanced.[10,27] Thus, a pro-phagocytic signal is required for the CD47-LILRB2 bispecific antibody to induce phagocytosis.

As demonstrated by Advani et al., rituximab synergized with an antibody blocking CD47 to induce substantial antitumor responses in patients with rituximab-refractory DLBCL.[24] With our findings, a more potent antitumor response was induced by further removing the anti-phagocytic signal LILRB2. By blocking both antiphagocytic signals and induction of 'eat me' signal by FcR-mediated ADCP by macrophages and granulocytes, this combination

therapy can resensitize the immune suppressive responses of rituximab-refractory patients. Furthermore, as complete response was not met for all patients that received rituximab with anti-CD47 antibody,[24] another underlying pathway could possibly be at play for immune evasion. Therefore, this pathway could be LILRB2 and its blockade could stimulate a stronger response in patients.

The presence of more 'don't eat me' signals could also explain why phagocytosis did not increase in cell line SUDHL10 even in the presence with all three antibodies. Additionally, it explains the resistance to anti-CD47 mAb treatment even with anti-LILRB2 mAb in OVCAR3. It is possible that in addition to LILRB2 there are more 'don't eat me' signals available to hinder phagocytosis, such as the aforementioned LILRB1 receptor that also binds to MHC I.[12] Therefore, the presence of this receptor can prevent rituximab-induced phagocytosis. Additionally, Barkal et al. also demonstrated that LILRB2 blocking did not increase phagocytosis, as opposed to LILRB1 blocking, as its expression is minimal compared to LILRB1.[12] Thus, in addition to anti-CD47 and anti-LILRB2 mAbs, LILRB1 blockade could be tested to enhance phagocytosis of ovarian cancer cell line OVCAR3, and to enhance efficacy of rituximab-induced granulocyte- and macrophage-mediated phagocytosis in cell line SUDHL10. Additionally, this would be imperative in advancing our knowledge on specific profiles of 'don't eat me' signals on tumor types and/or subtypes for the development of appropriate treatment strategies for patients.

In addition to LILRB1 and LILRB2, another immune inhibitory LILRB receptor is the LILRB4. LILRB4 is expressed on immune cells, such as macrophages and monocytes.[28] It is particularly highly expressed on the M5 subtype of human acute myeloid leukemia cells[29], and 50% of B cell chronic lymphocytic leukemia cells.[30] Silencing of this receptor in human acute myeloid leukemia cell lines have shown to inhibit cell growth *in vitro*.[31] LILRB4 could potentially be a safe therapeutic target as it is not expressed in normal B cells.[32] Additionally, increased LILRB4 expression is also associated with tumor progression spontaneous ovarian cancer in an animal model.[33] Therefore, blockade of this receptor would be most effective in these specific types of cancers types and subtypes. Thus, blockade of LILRB4 together with anti-CD47 mAb and/or rituximab could enhance phagocytosis of hematological and solid cancers by removing more anti-phagocytic signals.

Our study also provides an insight into an alternative to chemotherapy by more specific targeting of cancer cells. Chemotherapy is a cancer treatment that non-specifically targets actively proliferating cells, which includes cancer cells, hair cells and hematological cells, hence hair loss and reduced hematological cells being the common adverse effects in chemotherapy-treated patients.[34,35] These toxicity profiles, thus, delay life-prolonging treatment as dose reduction is often required.[35] As our study targets specific biomarkers such as CD47, LILRB2, and CD20, less off-target killing will occur. CD47 is present on both normal blood cells and malignant cells. However, delivery of anti-CD47 mAb does not induce phagocytosis of healthy blood cells as they lack calreticulin, a pro-phagocytic

signal only on malignant cells and aged blood cells.[36] The LILRB receptors are also specifically present on cancer cells and is not involved in normal development and hematopoiesis, making them appropriate therapeutic targets.[32] Lastly, CD20 is a B cell specific target, making it an ideal target for B cell malignancies.[37] Therefore, the use of therapeutic mAbs as an immunotherapy is safer due to its enhanced specificity.

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