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Françoise Gondois-Rey, Thomas Miller, Vladimir Laletin, Xavier Morelli, Yves Collette, Jacques Nunès, Daniel Olive

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# CD47-SIRP $\alpha$ Controls ADCC Killing of Primary T Cells by PMN Through a Combination of Trogocytosis and NADPH Oxidase Activation

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### Edited by:

Nicola Tumino,  
Bambino Gesù Children's Hospital  
(IRCCS),  
Italy

### Reviewed by:

Alessandro Poggi,  
San Martino Hospital (IRCCS), Italy  
Siqi Chen,  
City of Hope, United States  
Kumiko Nakada-Tsukui,  
National Institute of  
Infectious Diseases, Japan

### \*Correspondence:

Françoise Gondois-Rey,  
francoise.gondois-rey@inserm.fr  
Thomas W. Miller  
thomas.miller@inserm.fr  
Daniel Olive  
daniel.olive@inserm.fr

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Françoise Gondois-Rey<sup>1\*</sup>, Thomas Miller<sup>2\*</sup>, Vladimir Laletin<sup>1</sup>, Xavier Morelli<sup>2</sup>,  
Yves Collette<sup>2</sup>, Jacques Nunès<sup>1</sup> and Daniel Olive<sup>1\*</sup>

<sup>1</sup> Immunity and Cancer Team, Centre de Recherche en Cancérologie de Marseille (CRCM), Inserm U1068, CNRS UMR7258, Institut Paoli-Calmettes, Aix Marseille University UM105, Marseille, France, <sup>2</sup> Integrated Chemical and Structural Biology Team, Centre de Recherche en Cancérologie de Marseille (CRCM), Inserm U1068, CNRS UMR7258, Institut Paoli-Calmettes, Aix Marseille University UM105, Marseille, France

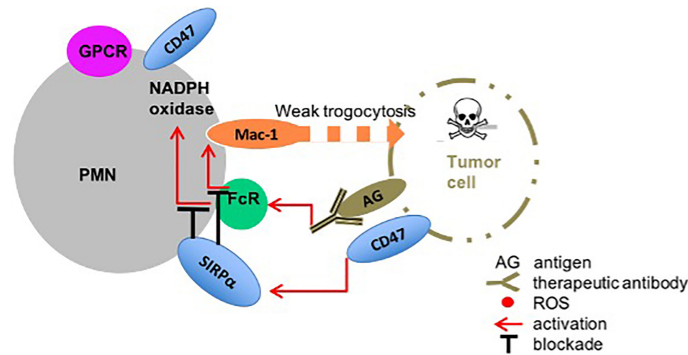
Immunotherapies targeting the “don’t eat me” myeloid checkpoint constituted by CD47 SIRP $\alpha$  interaction have promising clinical potential but are limited by toxicities associated with the destruction of non-tumor cells. These dose-limiting toxicities demonstrate the need to highlight the mechanisms of anti-CD47-SIRP $\alpha$  therapy effects on non-tumor CD47-bearing cells. Given the increased incidence of lymphopenia in patients receiving anti-CD47 antibodies and the strong ADCC (antibody-dependent cellular cytotoxicity) effector function of polymorphonuclear cells (PMNs), we investigated the behavior of primary PMNs cocultured with primary T cells in the presence of anti-CD47 mAbs. PMNs killed T cells in a CD47-mAb-dependent manner and at a remarkably potent PMN to T cell ratio of 1:1. The observed cytotoxicity was produced by a novel combination of both trogocytosis and a strong respiratory burst induced by classical ADCC and CD47-SIRP $\alpha$  checkpoint blockade. The complex effect of the CD47 blocking mAb could be recapitulated by combining its individual mechanistic elements: ADCC, SIRP $\alpha$  blockade, and ROS induction. Although previous studies had concluded that disruption of SIRP $\alpha$  signaling in PMNs was limited to trogocytosis-specific cytotoxicity, our results suggest that SIRP $\alpha$  also tightly controls activation of NADPH oxidase, a function demonstrated during differentiation of immature PMNs but not so far in mature PMNs. Together, our results highlight the need to integrate PMNs in the development of molecules targeting the CD47-SIRP $\alpha$  immune checkpoint and to design agents able to enhance myeloid cell function while limiting adverse effects on healthy cells able to participate in the anti-tumor immune response.

**Keywords:** PMN (polymorphonuclear leucocyte), SIRP $\alpha$ , NADPH oxidase, CD47 antibody, ADCC (antibody-dependent cellular cytotoxicity)

### PMN ADCC of tumor cells opsonized by therapeutic mAbs

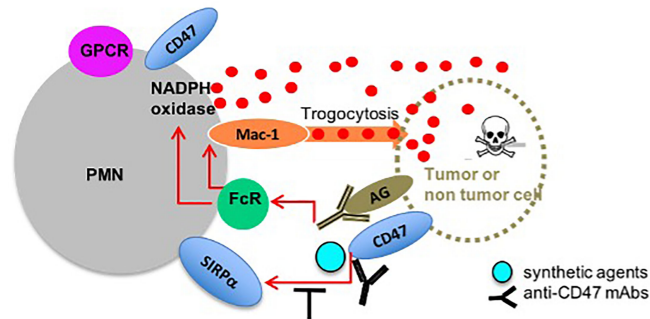
#### a) Without blockade of SIRP $\alpha$

Engagement of FcR activates Mac-1 adhesion and trogocytosis. However, the cytotoxicity is limited by the interaction of CD47 with SIRP $\alpha$ .



#### b) With blockade of SIRP $\alpha$

Blockade of SIRP $\alpha$  increases trogocytosis and liberates activation of NADPH oxidase. The resulting cytotoxicity combining trogocytosis and production of ROS is high. However collateral damages to healthy cells cannot be avoided and are devastating if anti-CD47 mAbs are used to block SIRP $\alpha$ .



GRAPHICAL ABSTRACT |

## HIGHLIGHTS

We show that potent cytotoxicity by PMN occurs through a novel combination of trogocytosis and ROS regulated by CD47-SIRP $\alpha$ . We also show that anti-CD47 antibody therapy risks collateral damage by opsonizing and directing this cytotoxicity to healthy T cells.

## INTRODUCTION

The ability to manipulate patients' immunity with antibodies has changed the therapeutic outlook for cancer. Strategies based on the induction of antibody-dependent cell cytotoxicity (ADCC) with antibodies targeting tumor antigens like Rituximab in B-lymphomas and Trastuzumab in breast cancer have demonstrated clinical efficacy. More recently, strategies based on the blockade of the T cells inhibitory checkpoints CTLA-4 and PD-1/PD-L1 with mAbs showed spectacular efficacy but were limited to certain tumor types with high mutational burdens and T cell infiltration and associated side effects (1). To create therapies

with broader efficacy, strategies targeting the myeloid checkpoint and signal regulatory protein  $\alpha$  (SIRP $\alpha$ ) were developed to enhance myeloid ADCC induced by therapeutic antibodies (2, 3).

SIRP $\alpha$  controls phagocytosis when engaged by its ligand CD47, a molecule widely expressed in most cell types (4). This interaction constitutes the "don't eat me" regulatory axis. Inhibition results in part from the immunoreceptor tyrosine-based inhibitory motif (ITIM) phosphorylation of SIRP $\alpha$  cytoplasmic tail that prevent modifications of the membrane, leading to the formation of the phagocytic cup (5). The regulation of SIRP $\alpha$  signaling in PMN is known to be sensitive to their activation where IL-17 stimulation results in cleavage of the ITIM signaling domain of SIRP $\alpha$  (6). SIRP $\alpha$  also inhibits the activation of Mac-1, the integrin required for the spread and adhesion of myeloid cells on their target (7, 8). Mac-1 is a heterodimer of CD11b ( $\alpha$ M) and CD18 ( $\beta$ 2) integrins but only CD18 is required in ADCC-mediated adhesion (9). Whereas macrophages are capable of whole-cell phagocytosis, polymorphonuclear cells (PMNs) ingest parts of the target cell in a mechanism called trogocytosis (10, 11). In ADCC, trogocytosis is sufficient to induce necrotic cell death resulting

from lytic processes but requires high ratios of PMNs per target cell (10, 12).

PMNs are mainly known for their capacity to degranulate toxic molecules accumulated during their maturation or produce toxic reactive oxygen species (ROS) upon the “respiratory burst” within minutes of stimulation to fulfill their killing mission (13). The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a multi-subunit enzyme complex that, once assembled at the PMN membrane, generates superoxide as a precursor for other ROS ( $H_2O_2$ , HOCl), released in the milieu as an antimicrobial agent. However, ROS are also toxic to host cells and this process must be tightly controlled. NADPH oxidase is activated through membrane G protein-coupled receptors (GPCR) that sense the various molecules of the milieu, such as bacterial compounds (fMLP and LPS) (14), but little is known about its control. Recently, SIRP $\alpha$  was shown to be involved in the control of NADPH oxidase by inhibiting the expression of the gp91<sup>Phox</sup> subunit, a membrane component of NADPH oxidase complex, in immature cells (15). This inhibition required engagement of SIRP $\alpha$  by CD47 and signaling by the cytoplasmic tail of SIRP $\alpha$ . Blockade of SIRP $\alpha$  engagement resulted in enhanced production of ROS. However, NADPH oxidase and ROS are not known to be involved in ADCC (10).

CD47 is a widely expressed signaling receptor and marker of “self” involved in many biological processes through its interaction with its ligand thrombospondin-1 (*TSP-1*), an inflammatory protein that promotes migration and activation of cells (16). Different epitopes of CD47 are involved in the interaction of CD47 with TSP-1 and SIRP $\alpha$  (17). CD47 interacts also with SIRP $\gamma$ , a molecule only expressed in T cells (18). CD47 is known to signal through its lateral association with integrins and GPCR (19). For example, triggering of CD47 induces endothelial cell spreading on RGD sequences through the lateral association of CD47 with  $\beta 3$  integrins and adhesion of T cells on LDV sequences through the lateral association with  $\beta 1$  integrins (20). An interaction of CD47 with Mac-1 was recently described as one of the mechanisms involved in the fusion of macrophages (21).

The overexpression of CD47 on tumor cells suggested that blockade of the “don’t eat me” checkpoint could synergize with therapeutic mAbs (16) to enhance the elimination of tumors by myeloid cells (22). Antibodies blocking the CD47-SIRP $\alpha$  interaction increased phagocytosis of macrophages (22, 23) and cytotoxicity of PMNs (12), inhibited tumor engraftment (22), and eliminated pre-existing tumors in mice (24). Although both can block the “don’t eat me” interaction, anti-CD47 mAbs were more efficient than anti-SIRP $\alpha$  mAbs (24). This higher efficiency was thought to result from the additional ADCC resulting from the opsonization of the target with anti-CD47 mAbs (4, 12).

Despite their promising pre-clinical results, the clinical progress of anti-CD47 mAb therapies have been limited by on-target, non-tumor toxicities including anemia, neutropenia, thrombocytopenia, and lymphopenia (25). We had previously identified the CD47-SIRP $\alpha$  immune checkpoint and SIRP $\alpha$  activity as key determinants of low-density PMN-MDSCs

(myeloid-derived suppressive cells) cytotoxicity toward healthy T cells (26). Low-density PMN-MDSCs share immune-suppressive capacities while they are composed of heterogeneous populations of immature and mature cells having acquired low-density properties after activation (27). We investigated the effect of the SIRP $\alpha$ -blocking anti-CD47 mAb (clone CC2C6) on high-density mature PMN-mediated T cell cytotoxicity. By investigating their ADCC on primary T cells, we found that blockade of SIRP $\alpha$  engagement on PMNs resulted in an important cytotoxicity sustained not only by an enhancement of trogocytosis but also by induction of a strong respiratory burst, resulting in suppression of T cells.

## MATERIALS AND METHODS

### Cells

Blood samples were obtained from healthy donors (EFS, Etablissement Français du Sang, Marseille, France). High-density PMNs were separated from peripheral blood mononuclear cells (PBMC) by centrifugation on Ficoll-Hypaque gradients. Red cells were eliminated with RBC lysing buffer (eBioscience, ThermoFischer, France). PMNs were kept at 4°C in PBS supplemented with  $Ca^{2+}$  1 mM and  $Mg^{2+}$  1 mM. The purity of the PMN preparations was routinely between 70%–90%, contaminants were T cells, and monocytes were absent (**Figure S1A**). T cells were separated from frozen or fresh PBMC using CD3<sup>+</sup> magnetic beads (Miltenyi Biotech, Germany), and the purity of preparations was above 95% (not shown). A weak death of T cells (10%–20%) was observed after overnight culture (not shown). Raji B cell lines were obtained from ATCC. For the Jurkat T cell line, JA16 was initially subcloned in the lab (28), and JINB8 is a CD47deficient Jurkat cell line (29). Cells were cultivated in RPMI 1640 medium supplemented with 10% foetal calf serum and antibiotics.

### Antibodies and Peptides

Purified anti-CD47 mAbs clones CC2C6 and 2D3 (BioLegend) and clone B6H12 (BD Biosciences) and purified anti-SIRP $\alpha$  mAbs (clone SE5A5) and G1 isotype mAbs were used at 10  $\mu$ g/ml (BioLegend). Anti-Mac-1 was reconstituted by mixing anti-CD11b (clone ICRF44) and CD18 (clone TS1/18) mAbs at a final concentration of 10  $\mu$ g/ml for each (BioLegend). Anti-CD3 mAbs (clone UCHT1) were prepared in the laboratory and used at 10  $\mu$ g/ml. Recombinant SIRP $\alpha$  was prepared as in (30) and was used as a monomer or multimerized with Neutravidin at a saturating concentration of 5  $\mu$ M. RGDS and LGDP were used, respectively, at 40  $\mu$ g/ml and 100  $\mu$ g/ml (Sigma Aldrich Merck). 4N1K, a CD47-binding domain adhesive peptide derived from TPS1, was used at 10  $\mu$ M (Eurogentec). CC2C6-F(ab)<sup>2</sup> was prepared using F(ab)<sup>2</sup> Preparation Kit (Pierce) as described by the manufacturer using an optimized 1-h digestion time at 37°C. Digestion was verified by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and CD47 binding activity was measured against the parent CC2C6 using AlphaScreen as described in (30).

## Cytotoxicity Assay

Targets were stained with CellTrace™ Violet (Life Technologies, France). Counting beads (BD Biosciences, France) were added to target cell suspensions. Cocultures were set using PMNs and T cells from different donors at ratios ranging from 1/1 to 5/1 PMNs to target and incubated overnight at 37°C in culture medium. An aliquot of the coculture was stained and analyzed by flow cytometry immediately after mixing to verify ratios and after overnight incubation to evaluate cytotoxicity by counting live targets. Target counts were standardized to counting beads and either compared to counts of control targets cultured overnight without PMNs or to control coculture with PMNs. T cells did not proliferate during overnight culture, Raji cells slightly proliferated, and Jurkat and JINB8 proliferated by a factor of 3–5. PMNs remained alive during overnight culture but showed a trend to display an activated phenotype characterized by a decrease of SSC and an increase of FSC and depending on the stimulation counts importantly decreased (shown in **Figure S2B**). This uncontrolled weak activation of PMNs during their preparation was previously described (31) and resulted in spontaneous cytotoxicity or protection of T cells from spontaneous death ranging between 50% and 150%. Experiments were excluded where this effect was over 20% to focus on the effects of antibodies. Inhibition of respiratory burst was performed by adding 50  $\mu$ g/ml of catalase to digest H<sub>2</sub>O<sub>2</sub> and 10  $\mu$ M of Diphenyleneiodonium chloride (DPI) to inhibit NADPH oxidase (Sigma-Aldrich, Merck) during the overnight coculture. DPI could not be used with cell lines as a target because it inhibited cell proliferation. Because cell lines

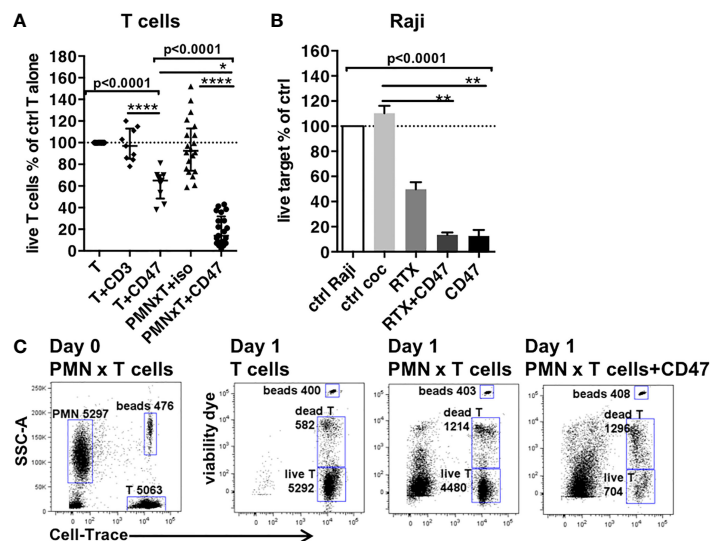
proliferated during the overnight incubation cytotoxicity could no more be quantified as it was based on the absolute counting of cells.

## Flow Cytometry

Cell suspensions were stained with Near-IR LIVE/DEAD™ (Life Technologies), CD3-PC5 or CD19-PC5, and CD11b-PE when indicated (BD Biosciences). Samples were acquired on a FORTRESSA cytometer (BD Biosciences). Data were exported and analyzed with FlowJo (RRID : SCR\_008520; version 9-2, MacOS X). Counting beads and cells were gated on forward scatter-area/side-scatter area- (FSC-A/SSC-A) (shown in **Figure 1C**). Doublets were excluded on FSC-A/FSC-H. Dead cells were excluded on the expression of the viability dye (shown in **Figure S1A**). PMNs were gated as SSC<sup>hi</sup> cells and CD11b expression. For analysis of trogocytosis of PMNs, target T cells were excluded on the expression of CD3. For cytotoxicity assays, targets (T cells, Jurkat T cell-lines, and Raji B lymphoma cell-line) were gated on SSC<sup>low</sup>FSC<sup>hi</sup>, cell-trace, and CD3 or CD19 expression.

## Trogocytosis

Target cells were stained with the membrane-dye PKH67 (Sigma-Aldrich) and cocultured with PMNs at ratios ranging from 1:1 to 3:1 for 3 h. Trogocytosis was also analyzed in PMNs recovered from cytotoxicity assays after an overnight incubation with targets stained with CellTrace. Percentages of trogocytosis were determined by the expression of the T cell dye in PMNs after setting gates on PMNs cultured alone.



**FIGURE 1** | Anti-CD47 mAbs induce killing of primary T cells by PMNs. **(A)** Cytotoxicity to primary T cells induced by anti-CD47 mAb clone CC2C6 alone or in the presence of PMNs ( $n = 9$ –25 different donors; ratio of PMN to T cells = 2). Iso, isotype; CD3, anti-CD3 mAbs; CD47, anti-CD47 mAbs. **(B)** Induction of PMNs' cytotoxicity to Raji lymphoma B cells by combinations of Rituximab (RTX) plus anti-CD47 mAb clone CC2C6 (CD47) ( $n = 3$ ; ratio of PMN to target = 3). For **(A, B)**, cytotoxicity is represented by the % of live targets in indicated conditions compared to targets cultivated overnight alone. Median and IQR (interquartile range) are shown.  $P$ -values from Kruskal–Wallis test is indicated on top of groups,  $P$ -values from Dunn's multiple comparison post-test on top of pairs: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ . **(C)** Cytotoxicity assay. The left plot shows the coculture of PMN with Cell-Trace stained T cells on day 0. The following plots show cell-trace and viability staining of indicated cocultures after overnight incubation. Gates and counts of beads, live and dead Cell-Trace–stained T cells are shown.

## Intracellular Production of ROS

PMNs were stained with 25  $\mu$ M Dihydrorhodamine 123 (DHR, Sigma-Aldrich) and cultured for 1–1.5 h in the presence of catalase at 50  $\mu$ g/ml (32). Lipopolysaccharide (LPS, 100 ng/ml) plus N-Formylmethionyl-leucyl-phenylalanine (fMLP, 5  $\mu$ M) and monoclonal antibodies at 10  $\mu$ g/ml were added immediately after DHR staining. Samples were acquired on a FORTRESSA cytometer, DHR was analyzed in the 525/50 channel and the percentage was determined by setting the gate on unstimulated stained cells.

## Statistics

Statistical graphics were performed with Prism 6 (RRID : SCR\_005375) software. Mann–Whitney, Wilcoxon matched pair non-parametric test or Kruskal–Wallis test followed by multiple comparison Dunn’s post-test to compare variables between groups were used as indicated.

## Data Availability

The data generated in this study are available within the article and its supplementary data files.

## RESULTS

### Anti-CD47 mAbs Induce Killing of Primary T Cells by PMNs

To investigate the potential for CD47-dependent PMN killing of primary T cells, we treated cocultures with anti-CD47 mAb CC2C6 at a PMN to T cell ratio of 2:1. Overnight coculture with PMNs alone resulted in a variability of 50%–150% of T cells survival. The introduction of CC2C6 to the PMNs T cell coculture resulted in a much stronger cytotoxicity (mean survival of 19% of control-treated T cells; **Figures 1A, S2B**). We noted that CC2C6 had a direct weak cytotoxicity to T cells as previously shown with other antibodies to CD47 (33). As expected, percentages of dead T cells in coculture with PMNs and anti-CD47 mAbs were increased as compared to control T cells cultivated alone or with PMNs without anti-CD47 mAbs (**Figure 1D**) (67% vs. 15% and 22%, respectively). However, the most striking difference was the significant decrease of total cells suggesting that cytotoxicity manifested as necrosis rather than apoptosis, as reported for PMNs induced killing (10) but the possibility that dead T cells were removed by PMNs’ efferocytosis cannot be excluded as well.

The potent CD47 mAb-induced PMN cytotoxicity was not limited to primary T cells as we observed similar cytotoxicity with Raji tumor cells (Raji) as targets. At a ratio of PMN to a target of 3:1, RTX induced the killing of 50% of the Raji tumor cells (Raji) (**Figures 1B, S2A**). The addition of anti-CD47 mAb to RTX reduced the survival of Raji to 14% of control, but the anti-CD47 mAb alone reduced cell survival to 13%. The similarity of cytotoxicity induced by the anti-CD47 mAb whether alone or with RTX suggested that they were mainly responsible for the target cell death when used in the combination. Because of the uniquely potent nature of the

cytotoxicity observed for PMN in the presence of an anti-CD47 antibody, we sought to investigate its mechanism.

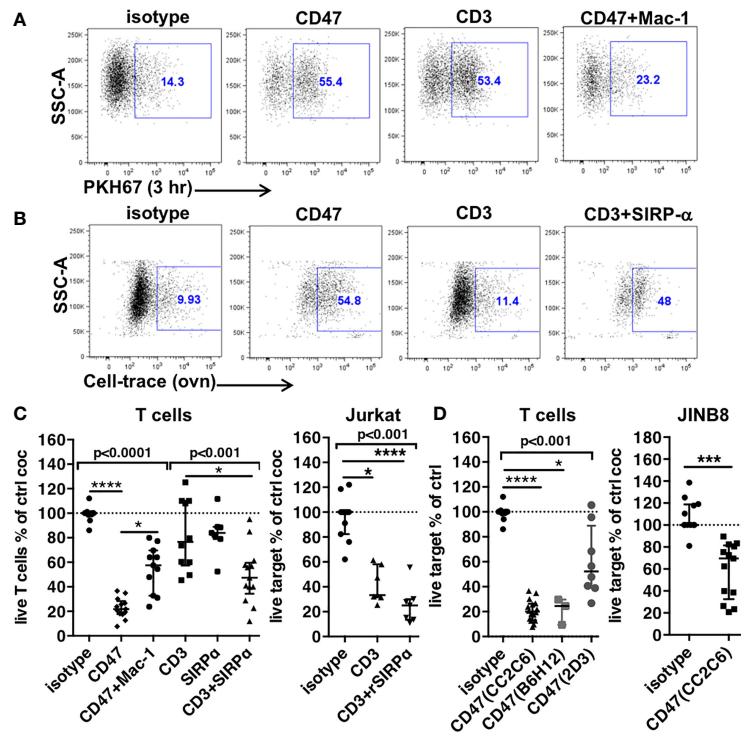
### Trogocytosis plus CD47-SIRP $\alpha$ Blockade Are Insufficient to Explain the Enhanced Cytotoxicity of PMNs in Anti-CD47 mAb-Triggered ADCC

Anti-CD47 mAbs are capable of exerting cytotoxic functions *via* a variety of mechanisms. They can simultaneously opsonize T cells to activate PMNs’ FcR providing “eat me” signals while also inhibiting “don’t eat me” signals resulting from the engagement of PMNs’ SIRP $\alpha$  by CD47 on T cells. PMN-mediated toxicity *via* ADCC was recently shown to proceed almost exclusively by trogocytosis of the target cell membrane and to be regulated by SIRP $\alpha$  (10). We reconstituted these mechanisms with anti-CD3 mAbs to opsonize T cells and anti-SIRP $\alpha$  mAbs to block “don’t eat” signals while comparing trogocytosis by PMNs and cytotoxicity to T cells.

T cells were stained by PKH67 a lipophilic dye that accumulates in the plasma membrane and whose transfer to non-labeled cells indicates trogocytosis (34). Transfer of the membrane dye to PMNs was analyzed by flow cytometry after a 3-h incubation. Similar percentages of PKH67<sup>+</sup>PMNs were found with anti-CD47 and anti-CD3 mAb treatment (59% and 60%, respectively, **Figure 2A**) suggesting an equivalent interaction of PMNs with T cells whether opsonized with anti-CD3 or anti-CD47 mAbs. To control for non-specific cellular adhesion or ADCC mediated adhesion, we included an anti-Mac-1 mAb that would block specific ADCC mediated adhesion *via* integrins  $\alpha_M\beta_2$ . Anti-Mac-1 mAbs inhibited PMNs trogocytosis induced by anti-CD47 mAbs. Given the lateral interactions of CD47 with integrins (17), we verified whether  $\beta_1$  and  $\beta_3$  (as control) integrins were involved, but only antibodies to the  $\beta_2$  integrin CD18 inhibited anti-CD47 mAbs-induced trogocytosis, as reported for PMNs’ ADCC (**Figure S3B**).

These results suggested that this acute trogocytosis was not controlled by CD47-SIRP $\alpha$  signaling but *via* FcR activation. We further investigated trogocytosis in different experimental conditions using CellTrace Violet-labeled T cells incubated overnight with PMNs [as in (10)] (**Figures 2B, S3A**). The uptake of CellTrace in PMNs triggered by anti-CD3 mAbs was not different from control (11% vs. 9.9%, respectively). Blockade of CD47-SIRP $\alpha$  interaction with anti-SIRP $\alpha$  targets only the PMNs as T cells do not express SIRP $\alpha$ . Anti-SIRP $\alpha$  antibodies increased trogocytosis to a level similar to anti-CD47 mAbs (48% and 54.8%, respectively), demonstrating CD47-SIRP $\alpha$  regulation of PMNs’ trogocytosis and again showing an equivalent interaction of PMNs with T cells.

Next, we compared the cytotoxicity induced in overnight cocultures. At ratios ranging from 1 to 3:1, anti-CD47 mAbs-ADCC induced by clone CC2C6 or clone B6H12 decreased T cells viability of a factor of 5 (22% survival) (**Figures 2C, D, S2B**). Moreover, B6H12 is not directly cytotoxic (35) showing that the equivalent effect of the anti-CD47 antibodies is due to ADCC. Anti-Mac-1 mAbs, while fully inhibiting trogocytosis, only partially restored T cells survival (54% vs. 22%). This suggested that adhesion-mediated ADCC was involved in cytotoxicity but



**FIGURE 2** | Trophocytosis and cytotoxicity in PMNs' ADCC. **(A)** Expression of the PKH67 T cells membrane dye in PMNs after a 3-h coculture in the presence of indicated mAbs. **(B)** Uptake of Cell-Trace-stained T cells components in PMNs after overnight coculture in the presence of mAbs. For **(A, B)**, PMNs are gated after exclusion of doublets and dead cells. **(C, D)** Cytotoxicity of PMNs to targets indicated on top of graphs, evaluated by the percentages of live targets compared to control after overnight coculture.  $N = 6-12$  different donors, ratios of PMNs to target = 1–3. CD3, anti-CD3 mAbs; CD47, anti-CD47 mAbs clone CC2C6, B6H12, or 2D3 as indicated; Mac-1, anti-CD11b+anti-CD18 mAbs. SIRP $\alpha$ , anti-SIRP $\alpha$  mAbs. rSIRP $\alpha$ , recombinant SIRP $\alpha$  protein. Median and IQR are shown.  $P$ -values from Kruskal–Wallis test indicated on top of groups,  $P$ -values from Dunn's multiple comparison post-test on top of pairs: \* $P < 0.05$ ; \*\*\*\* $P < 0.001$ . For **(D)**,  $P$ -values from Wilcoxon matched pair test is shown.

does not account for the complete effect. Consistent with a trophocytosis mechanism, PMNs' cytotoxicity induced by anti-CD3 mAbs was weak but became significant when anti-SIRP $\alpha$  mAbs were added (82% vs. 50% survival, respectively). Similarly, blockade of SIRP $\alpha$  with a recombinant SIRP $\alpha$  protein (rSIRP $\alpha$ ) [as in (36)] increased cytotoxicity of PMNs to Jurkat T cells opsonized with anti-CD3 mAbs by a factor of 1.6-fold (41% vs. 25%, respectively) (Figure 2C). In the two systems, however, blockade of SIRP $\alpha$  did not result in the same level of cytotoxicity induced by anti-CD47 mAbs. Although the direct effect of anti-CD47 mAbs on T cells must slightly contribute to the whole effect in the presence of PMNs (see Figure 1B), the combination of anti-CD3 plus anti-SIRP $\alpha$  mAbs seemed unable to induce similar level of PMN-mediated cytotoxicity despite inducing similar trophocytosis.

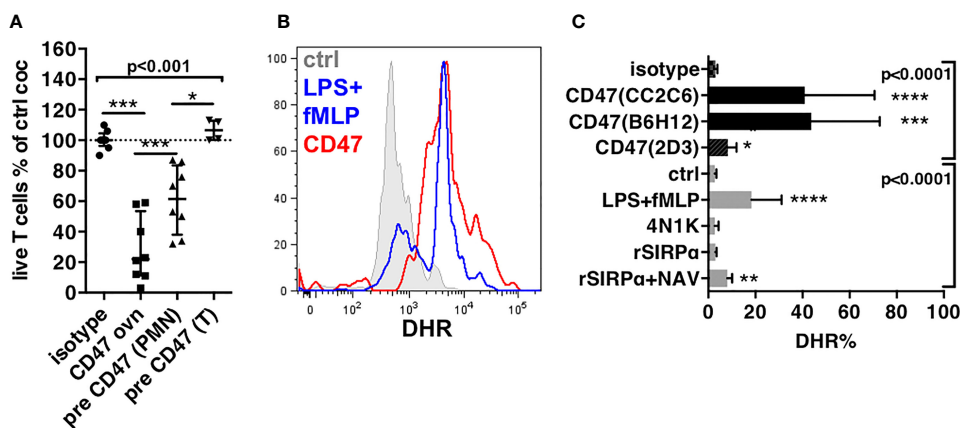
Thus, trophocytosis is not sufficient for cytotoxicity. Our results suggested a harmless propensity of PMNs to engulf parts of the membrane of opsonized cells not followed by death of targets. Overnight trophocytosis of internal cell components was better correlated to cytotoxicity but not quantitatively. These results suggested that another mechanism than trophocytosis plus blockade of the engagement of SIRP $\alpha$  was involved in the cytotoxicity induced by anti-CD47 mAbs. To

demonstrate more directly this hypothesis, we used an anti-CD47 mAb targeting an epitope outside the interaction site with SIRP $\alpha$  (2D3) to induce ADCC against T cells and a CD47-deficient Jurkat T cell line as target for PMNs in the presence of anti-CD47 mAb CC2C6 (Figures 2D, S1B, and S2C). Although a modest effect was expected, PMNs displayed cytotoxicity in the two systems demonstrating the contribution of additional mechanisms likely involving CD47 on PMNs in the strong cytotoxicity.

## Anti-CD47 mAbs Induced Strong ROS Production in PMN

To address the role of PMN CD47 engagement vs. T cell CD47 engagement in cytotoxicity, we pre-treated PMNs or T cells for 30 min with the anti-CD47 mAb CC2C6 then washed the antibody before coculture with T cells. Only pre-treatment of PMNs resulted in a significant cytotoxicity to T cells, however weaker than that obtained when the antibody was present throughout the coculture (57% and 18%, respectively, Figure 3A).

Because PMN are potent producers of ROS we investigated whether PMNs stimulated by anti-CD47 mAbs produced ROS using DHR staining. LPS plus fMLP were used as a positive control for ROS induction, and isotype as a negative control.



**FIGURE 3** | Induction of production of ROS in PMNs. **(A)** Effect of pre-treatment of cells by the anti-CD47 mAb clone CC2C6 on cytotoxicity of PMNs to T cells. Cytotoxicity is represented by the percentages of live T cells compared to control coculture (ctrl coc). Pre-CD47, pre-treatment 30 min at 4°C. CD47 ovn, anti-CD47 mAb during overnight culture,  $n = 8$ , median and IQR. **(B)** Histograms of DHR staining in PMNs after exclusion of dead cells and doublets after 1-h stimulation. **(C)** Percentages of expression of DHR in PMNs after 1-h stimulation.  $N = 5-15$  for antibodies,  $n = 5$  for recombinants proteins or peptides. Gates are set on unstimulated stained cells.  $P$ -values from Kruskal–Wallis test indicated on top of groups,  $P$ -values from Dunn's multiple comparison post-test on top of pairs: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .

Anti-CD47 mAb CC2C6 induced a rapid increase of DHR fluorescence in PMNs similar to LPS *plus* fMLP (**Figures 3B** and **S4A**). CC2C6 was described to induce hemagglutination through clustering of CD47 (35, 37). To evaluate whether this mechanism accounted for activation of NADPH oxidase, we used B6H12 an anti-CD47 mAb that similarly targeted the epitope responsible for interaction with SIRP $\alpha$  but did not induce clustering of CD47. B6H12 also induced a strong production of ROS, suggesting that activation of NADPH oxidase was rather resulting from triggering of CD47 than its clustering. This hypothesis was consistent with the known lateral association of CD47 with GPCR involved in NADPH oxidase activation (19). Consistently, the 2D3 mAb also induced ROS, however, to a lower level (**Figures 3C** and **S4A**). Likewise, weaker binding recombinant ligands of CD47 like the 4N1K peptide (38) and recombinant SIRP $\alpha$  (30) failed to induce ROS except when rSIRP $\alpha$  was multimerized with neutravidin suggesting nevertheless that avidity or membrane clustering might be important factors of direct ROS induction through CD47 (**Figure 3C**). Alternatively, the weak levels reached rather suggested that other mechanisms than direct engagement of CD47 on PMNs were involved.

### Production of ROS During PMN ADCC is Activated by FcR Stimulation and Tightly Controlled by SIRP $\alpha$

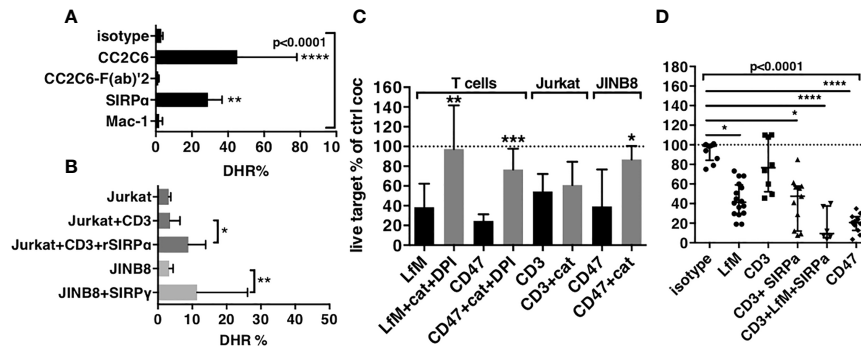
Anti-CD47 mAbs CC2C6, B6H12, and 2D3 have an intact Fc domain; thus, the contribution of FcR in the induction of ROS could not be excluded. This scenario might result from reciprocal FcR activation in PMNs, or activation through trimolecular complex between known as the scorpion effect (39) previously observed with anti-SIRP $\alpha$  antibodies on the macrophage. We investigated the contribution of FcR using an F(ab')<sub>2</sub> fragment of

CC2C6 or whole antibodies targeting other molecules on PMNs. CC2C6-F(ab')<sub>2</sub> and anti-Mac-1 mAbs failed to induce ROS, whereas anti-SIRP $\alpha$  mAbs induced significant levels (**Figures 4A** and **S4A**). Thus, FcR stimulation induced ROS in PMNs only in the context of the simultaneous blockade of SIRP $\alpha$ . Consequently, FcR activation of NADPH oxidase was simultaneously and strictly controlled by the engagement of SIRP $\alpha$ , a feature not yet described in PMN.

To demonstrate this hypothesis, we investigated intracellular induction of ROS in short cocultures of PMNs with targets (**Figures 4B** and **S4B**). Coculture with Jurkat opsonized by anti-CD3 mAbs did not increase DHR in PMNs (5% and 4%, respectively), whereas the presence of rSIRP $\alpha$  (to block interaction with SIRP $\alpha$ ) significantly increase DHR to 10%. Similarly, coculture of PMNs with CD47-deficient target cells (JINB8) opsonized by anti-SIRP $\gamma$  mAbs significantly increased DHR in PMNs to 20%. These results suggested that stimulation of PMNs through FcR induced ROS but only when PMN-SIRP $\alpha$  was not engaged by target cell CD47, either by blockade of CD47 with rSIRP $\alpha$  or by the absence of CD47 on target.

Next, we evaluated the contribution of ROS in cytotoxicity induced by PMNs' ADCC on T cells using catalase, an H<sub>2</sub>O<sub>2</sub> scavenger *plus* diphenyleneiodonium chloride (DPI), an NADPH oxidase inhibitor, to counteract ROS effects during coculture. LPS *plus* fMLP used to induce ROS by a pathway independent of ADCC resulted in a significant cytotoxicity fully blocked by catalase *plus* DPI, whereas the cytotoxicity of anti-CD47 mAbs was significantly but partially reduced (56% vs. 26% survival, respectively, **Figure 4C**). Only catalase could be used in assays with Jurkat and JINB8 because DPI is toxic to these cell lines. Catalase alone showed no inhibition of the cytotoxicity induced by anti-CD3 mAbs on Jurkat T cells, suggesting no contribution of ROS but alternatively no inhibition of NADPH





**FIGURE 4 |** Production of ROS is stimulated in ADCC and contributes to cytotoxicity. **(A)** Stimulation of ROS in PMNs by mAbs to PMNs. **(B)** Stimulation of ROS in PMNs by cocultures with targets and mAbs. For **(A, B)**, median and IQR of percentages of expression of DHR in PMNs after 1-h stimulation. For **(A)**,  $n = 6-15$  different donors.  $P$ -values from Kruskal–Wallis test indicated on top,  $P$ -values from Dunn’s multiple comparison post-test on top of pairs:  $**P < 0.01$ ;  $****P < 0.0001$ . For **(B)**,  $n = 6-10$ .  $P$ -values of Wilcoxon matched pair test:  $*P < 0.05$ ;  $**P < 0.01$ . **(C)** Inhibition of cytotoxicity by catalase (cat), in the presence of DPI when indicated,  $n = 6-25$ . Median and IQR,  $P$ -values of Wilcoxon matched pair test:  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ . **(D)** Reconstitution of the cytotoxicity induced by anti-CD47 mAb CC2C6 (CD47) using anti-CD3 (CD3) + anti-SIRP $\alpha$  (SIRP $\alpha$ ) + LPS + fMLP (LfM),  $n = 6-14$ . Median and IQR,  $P$ -values from Kruskal–Wallis test indicated on top,  $P$ -values from Dunn’s multiple comparison post-test on top of pairs:  $*P < 0.05$ ;  $**P < 0.01$ ;  $****P < 0.0001$ .

oxidase. Conversely, the almost full inhibition of the cytotoxicity of anti-CD47 mAbs on CD47-deficient target cells (JINB8) by catalase showed both the primary use of ROS in this cytotoxicity and that catalase was sufficient for its inhibition (**Figure 4C**).

These results suggested that ROS produced during ADCC in the context of blockade of SIRP $\alpha$  contributed to the cytotoxicity of PMNs. Finally, we verified our hypothesis by using together anti-CD3 mAbs to induce ADCC, anti-SIRP $\alpha$  mAbs to block the inhibitory checkpoint, and LPS *plus* fMLP to induce ROS. This combination reached the levels of cytotoxicity induced by anti-CD47 mAbs, suggesting the important contribution of ROS in the process (**Figures 4D** and **S3B**).

## DISCUSSION

In the context of the toxicities generated by blockade of the SIRP $\alpha$ -CD47 checkpoint in cancer therapies, we sought to address the role of PMN in the lymphopenia observed with anti-CD47 antibody treatment (25). We found that the strong PMN-mediated ADCC induced by the anti-CD47 mAbs CC2C6 was sustained not only by trogocytosis but also by a strong respiratory burst, both controlled by SIRP $\alpha$ . The cooperation of both results in a uniquely efficient killing of T cells in a low effector cell to target cell ratio.

The anti-CD47 mAb clone CC2C6 can induce a weak T cells death through direct triggering (33). In coculture with PMNs, this antibody can stimulate FcR on PMNs and simultaneously blocks CD47 interaction with SIRP $\alpha$ , resulting in potent ADCC (4, 40). In this regard, we found that PMNs killed leukemic B cells in the presence of anti-CD47 antibodies regardless of the presence of the well-known ADCC antibody Rituximab. This result suggested that anti-CD47 mAbs alone were sufficient for a cytotoxic response and explained why PMNs also killed T cells. However, the reconstitution of this scenario using a T cell

opsonizing antibody (anti-CD3) that induces ADCC *plus* anti-SIRP $\alpha$  mAbs or recombinant SIRP $\alpha$  protein to block the “don’t eat me” checkpoint failed to induce such strong killing suggesting that another mechanism must contribute to the cytotoxicity induced by anti-CD47 mAbs.

The most likely hypothesis was that this additional mechanism was triggered by the interaction of the antibody with CD47 on PMNs. This was addressed by measuring the anti-CD47–induced cytotoxicity of PMN cells in coculture with CD47-deficient T cells (Jurkat T cell clone JINB8). Although less toxicity was observed when compared to CD47<sup>+</sup> T cells, we still observed a significant increase in killing as compared to the control treatment. These observations suggest that targeting CD47 on PMN is sufficient to activate cytotoxic mechanisms.

We investigated whether respiratory burst, the most common mechanism used by PMNs to efficiently kill their targets in a short time, was involved. Not surprisingly, ROS contributed to the strong killing of T cells by PMNs induced by anti-CD47 mAbs. This was demonstrated by i) direct induction of ROS in PMNs by stimulation with anti-CD47 mAbs, ii) partial blockade of cytotoxicity by catalase and DPI, and iii) reconstitution of equivalent cytotoxicity by induction of ADCC with anti-CD3 mAbs *plus* blockade of SIRP $\alpha$  with antibodies *plus* stimulation of ROS with LPS and fMLP.

The next question was to determine how anti-CD47 mAbs stimulated NADPH oxidase in PMNs. The known lateral association of CD47 with GPCR, themselves involved in NADPH oxidase activation (14, 19), suggested at first that binding of CD47 by antibodies was involved. This hypothesis was confirmed by the induction of ROS by all anti-CD47 mAbs tested, including 2D3 that targets an epitope outside the interaction site with SIRP $\alpha$  (35, 40). Monomeric ligands failed to induce ROS, but a higher affinity multimer of the recombinant SIRP $\alpha$  protein was obtained with neutravidin [KD = 16 nM (36)], suggesting that affinity or CD47 clustering might be

critical. Indeed, CC2C6 reportedly induced cell aggregation through CD47 clustering (37), but B6H12, although not described to possess such property, was efficiently activating NADPH oxidase as well suggesting that clustering of CD47 may not be a determinant. The weak levels reached with SIRP $\alpha$  rather than antibodies showed that activation of ROS through the direct triggering of CD47 was limited and prompted us to consider an alternative mechanism based on stimulation of FcR either by reciprocal interactions between PMNs or by the formation of trimolecular complexes with FcR on each cell (39). This hypothesis was addressed by the use of the F(ab)'2 fragment of CC2C6 to induce ROS. We found that induction of ROS occurred only with antibodies that simultaneously engage FcR and blocked SIRP $\alpha$  (CC2C6, B6H12, and anti-SIRP $\alpha$  mAbs) but not by non-SIRP $\alpha$ -CD47-interacting antibodies (like anti-Mac-1) or non-Fc-containing SIRP $\alpha$ -CD47 blockers [F(ab)'2 fragment of CC2C6].

Together, our results suggested that if NADPH oxidase activation was engaged through FcR stimulation, then it was simultaneously controlled by the blockade of SIRP $\alpha$ . Considering the potential cytotoxicity of ROS, this tight control is not surprising. Support for this hypothesis was demonstrated by the induction of ROS in PMNs cocultivated with opsonized targets either deficient for CD47 or where engagement of SIRP $\alpha$  by CD47 was blocked by recombinant SIRP $\alpha$  protein. Such control of SIRP $\alpha$  on NADPH oxidase activation was previously shown during myeloid cell differentiation by the restriction of the expression of the gp91<sup>Phox</sup> subunit of NADPH oxidase (15). The mechanism may be different in mature PMNs considering the short time of response to FcR stimulation and the basal expression of gp91<sup>Phox</sup> in mature cells and will deserve further studies.

Although our results confirmed the activation of NADPH oxidase in FcR stimulation previously proposed by van Spruiel *et al.* (7), recent work based on PMNs from NADPH oxidase-deficient patients claimed that trogoptosis, a mechanism resulting from lytic processes induced by trogocytosis, was solely responsible for ADCC of PMNs (10). Our results do not contradict this statement. The authors reported the use of high ratios of effector to target, suggesting that ADCC without blockade of SIRP $\alpha$  is efficient provided that many PMNs focused on one target. Ratios of 50:1 are used to kill 30% of SKBR3 breast cancer cells opsonized with Trastuzumab (10, 12), and killing increases only two-fold on a CD47-deficient target. On the contrary, trogocytosis at low ratios of PMN to CLL-B cells opsonized with anti-CD20 mAbs does not induce significant death (11) unless SIRP $\alpha$  is blocked allowing activation of NADPH oxidase and strong killing, as we show here for ratios between 3 and 0.5 PMN to 1 target cell.

Hence, cytotoxicity can either result from trogocytosis alone or ROS alone but when the two mechanisms cooperate a single PMN becomes a potent killer. It is tempting to speculate that the spill of ROS into the intracellular milieu of targets through membrane holes created by trogocytosis is the mechanism of optimal function of PMNs. This relationship between the number of PMNs focusing on a target and underlying

mechanisms of killing opens new perspectives on the fundamental biology of PMNs where SIRP $\alpha$ -CD47 appears as a key regulator of PMN capacity to differentiate between physiological trogocytosis and cytotoxicity by unleashing trogocytosis and ROS.

The new information brought by our work describing the role of NADPH oxidase activation during ADCC by PMN and its regulation by SIRP $\alpha$  might help to design new agents to enhance myeloid cell function in the treatment of cancer while limiting adverse effects on healthy cells. Although ROS are expected to be released into tumor cells carrying opsonized by tumor-specific therapeutic antibodies triggering trogocytosis, collateral damages resulting from local production of ROS could also suppress T cells infiltrated in the tumor. This could be further exacerbated by the use of non-tumor-specific anti-CD47 antibodies triggering the potent combination of PMN-mediated cytotoxicity described herein. Targeting CD47 with non-FcR activating agents or directly targeting SIRP $\alpha$  could avoid an important part of the killing of non-tumor cells bearing CD47 while preserving the tumor cytotoxic capacity of PMN.

This work was undertaken following the finding that PMN-MDSCs devoid of SIRP $\alpha$  in patients with metastatic melanoma demonstrated a strong capacity to kill T cells using trogocytosis and production of ROS, without *in vitro* activation (26). Activated mature high-density PMNs reproduce such behavior once SIRP $\alpha$  is neutralized. This builds on previous studies pointing to the central role of SIRP $\alpha$  in controlling many aspects of PMNs biology and warrants further studies to elucidate the underlying mechanisms to further refine immunotherapy activity in the immune microenvironment.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

FG-R, TM, JN, and DO designed research; FG-R, VL, and TM performed research; TM, XM, and YC contributed new reagents; FG-R and TM analyzed data; and FG-R, TM, JN, and DO wrote the paper. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.899068/full#supplementary-material>

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