UNCLEAVABLE CD16A PROMOTES ANTIBODY-DEPENDENT PHAGOCYTOSIS OF INDUCED PLURIPOTENT STEM CELL-DERIVED MACROPHAGES

Bongkotchanok DANRUNGCHAROEN[1], Praewphan INGRUNGRUANGLERT[2], Netchanok LEELA-ADISORN[3] and Nipan ISRASENA[4]

1 Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; bongkotchanok.d@gmail.com
2 Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; numtip_in@hotmail.com
3 Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; yuaneye5@hotmail.com
4 Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; nipan.i@chula.ac.th

ARTICLE HISTORY
Received: 2 June 2023 **Revised:** 21 June 2023 **Published:** 3 July 2023

ABSTRACT

Macrophages have emerged as a promising cell type for cellular cancer immunotherapy due to its high penetrating capability, and ability to modulate tumor microenvironment. Although macrophages are highly resistant to genetic manipulation, overexpression of chimeric antigen receptor (CAR) has shown to improve its specificity and tumor-killing effect. Induced pluripotent stem cell is an attractive source for next-generation cellular immunotherapy due to it provide a platform for multiple genomes editing and scale-up manufacturing. CD16 is critical for antibody-dependent cell-mediated cytotoxicity of both NK- and T-cell types but the role in ADCC of macrophage remains unclear. In this study, we created human induced pluripotent stem cell (iPSC) line containing a mutant CD16 that is resistant to ADAM17 cleavage using CRISPR mediated genome editing technique. The iPSCs were then differentiated into iPSC-derived macrophages (iMacs), and CD16 expression was assessed using flow cytometry. The results demonstrated significantly higher levels of CD16 expression in non-cleavable CD16 iMacs compared to unmodified iMacs. Importantly, the engineered iMacs exhibited a significantly greater phagocytic activity (~50%) compared to normal iMacs (~36%) in co-culture experiments with a B-cell lymphoma cell line treated with anti-CD20 mAbs. These findings indicate that maintaining CD16 expression on iMacs contributes to enhancing their antibody-mediated antitumor function.

Keywords: non-cleavable CD16, induced pluripotent stem cell-derived macrophages, cancer immunotherapy

CITATION INFORMATION: Danrungcharoen, B., Ingrungruanglert, P., Leela-Adisorn, N., & Israsena, N. (2023). Uncleavable CD16a Promotes Antibody-Dependent Phagocytosis of Induced Pluripotent Stem Cell-Derived Macrophages. *Procedia of Multidisciplinary Research, 1*(7), 103.

INTRODUCTION

Macrophages, which are essential components of the innate immune system, play crucial roles in maintaining tissue homeostasis, combating infections, and coordinating immune responses. These cells exhibit a remarkable plasticity, being capable of adopting both pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes, leading to anti- and pro-tumor responses, respectively. When properly stimulated, macrophages can mount an anti-cancer response through phagocytosis and cytotoxicity, activating innate and adaptive immune system components (Mantovani et al., 2022). Additionally, their tumor-infiltrating capacity has made macrophage cell therapy an attractive approach in cancer treatment. Efforts to enhance the efficacy of macrophages in cancer therapy have involved reprogramming them into a tumor-killing phenotype and sustaining it. Gene-editing has emerged as a promising tool to achieve stable modifications and long-term effects (Poltavets et al., 2020). However, transducing primary macrophages derived from bone marrow or peripheral blood mononuclear cells (PBMCs) has proven challenging, presenting a significant obstacle in the advancement of cell therapy. Fortunately, the discovery of induced pluripotent stem cells (iPSCs) offers a homogeneous and clinically scalable approach, allowing for gene editing at the iPSC stage prior to differentiation into macrophages (Lyadova et al., 2021). Various engineered iPSC-derived macrophages (iMacs) have demonstrated promising results in inhibiting tumor growth, activating T-cells, and increasing the production of anti-cancer cytokines (Fukushima et al., 2023).

Considering the advantages of modified macrophage cell therapy, combining it with antibody treatment presents a promising strategy to enhance anti-cancer capabilities. Studies utilizing in vivo macrophage depletion have highlighted the critical role of macrophages in the efficacy of anti-CD20 antibodies against both normal and malignant B cells. Conversely, mice deficient in T cells, B cells, or NK cells retained the potential of these antibodies, emphasizing the specific contribution of macrophages (Weiskopf & Weissman, 2015). One mechanism by which anti-cancer antibodies exert their action is by activating immune effector cells to mediate antibody-dependent cellular cytotoxicity (ADCC) (Zahavi et al., 2018). ADCC has been demonstrated as a crucial process for the activity of antibodies like trastuzumab and rituximab in mouse models (Clynes et al., 2000). Effector cells involved in ADCC must express Fcγ receptors (FcγRs) on their cell surface to bind with monoclonal antibodies (mAbs). Recognizing the significance of FcγRs, researchers have targeted them to enhance ADCC. Interestingly, it was found that FcγRIIIA (CD16a) on natural killer (NK) cells is rapidly cleaved after activation (Jing et al., 2015). This mechanism inspired the engineering of non-cleavable CD16 into NK cells, resulting in an increase in antibody-dependent antitumor capacity (Zhu et al., 2020). While this approach has not been explored in macrophages,

Our study suggests that maintaining CD16 on the surface of macrophages could potentially improve the anti-cancer response in antibody therapy.

RESEARCH METHODOLOGY

Cell culture

Raji cells (human B lymphoblastoid cells) were cultured with RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine and 1% Penicillin-Streptomycin (P/S). The cells were incubated with 5% CO_2 at 37ºC.

VEGF expressing C3H10T1/2 feeder and non-VEGF expressing C3H10T1/2 feeder cells were cultured in BME medium supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin-Streptomycin (P/S). The cells were incubated with 5% CO_2 at 37ºC.

Human iPSCs (hiPSCs) were cultured on matrigel-coated disk with mTeSR plus medium at 5% CO_2 and 37 ºC. The medium was changed every two days. The cells were passaged by

CTK solution (10% collagenase type IV, 0.25% trypsin, 20% knockout serum, 1mM $CaCl_2$ in PBS and ddH_2O).

Generating non-cleavable CD16 iPSCs via CRISPR/Cas9

The 176V allelic variant of CD16a was converted from the serine at position 197 to proline and became to non-cleavable CD16. Firstly, According to CRISPR-Cas9 system, gRNA and single strand oligo donor were designed to generate C12436T mutation following previous study (Jing et al., 2015). The sequence of CD16a got from NCBI (NG_009066.). The gRNA is responsible for specifying mutation sites. And CAS9 protein will generate double strand break (DSB). The oligo donor is template for gene editing. The sgRNA will be annealed and cloned into backbone vector following the protocol of Zhang lab. All components were confirmed by sequencing before they were transferred into hiPSCs via Nucleofector Transfection (Amaxa Cell Line Nucleofector Kit V). For demonstrating gene editing in iPSCs, transfected cells were sorted into single cells by flow cytometer and expanded before they were extracted DNA. The DNA will be amplified and confirmed the mutation by T7 endonuclease. After that amplified DNA are sequencing for confirming the gene engineering.

Differentiation of iPSCs into macrophages

The hiPSCs were differentiated into ES sac following previous study (Takayama et al., 2008). Briefly, the hiPSCs were dissociated with CTK into small clumps (20-30 cells/clump) and transferred into the plate covered with radiated VEGF expressing C3H10T1/2 feeder and non-VEGF expressing C3H10T1/2 feeder in ratio 1:1. After that the cells were cultured in ES-sac differentiation medium, which was refreshed every 3 days. ES-sac differentiation medium was Iscove modified Dulbecco medium supplemented with a cocktail of 10 µg/mL human insulin, 5.5 µg/mL human transferrin, 5 ng/mL sodium selenite, 2 mM L-glutamine, 0.45 mM mono-thioglycerol, 50 µg/mL ascorbic acid, and 15% FBS. Cultures were maintained in a 5% CO_2, 5% O_2 and 90% N_2 environment for the first 7 days and then transferred to a 5% CO2 air environment. ES sac media was refreshed every 3 days. On day 14, the ES sacs were ripped for harvesting cells. These cells were cultured in ES sac media with IL-3 (10 ng/ml) and M-CSF (50 ng/ml). On day 21, IL-3 was removed from the media. These cells were maintained and refreshed media every 3 days. The cells were harvested on day 28 and defined with CD14 expression via flowcytometry.

Antibody dependent cellular phagocytosis (ADCP) assay

Raji cells were stained with 2 µg/ml pHrodo Red, succinimidyl ester (Invitrogen) for 30 min at room temperature in the dark. The stained Raji cells with 2.5 $x10^6$ cell/ml were seeded into ultra-low attachment (ULA) 96-well plate (100 µl/well) and incubated with various concentrations of rituximab for 30 min in 5% CO_2 at 37 ^OC. After that 5 x 10^4 iMacs were added and incubated in 5% CO_2 at 37 ^OC for 4 hours. The cells were harvested and labeled with anti-CD14 antibodies to define iMac in the dark for 15 min at room temperature for flow cytometric assay.%ADCP was calculated as the percentage of pHrodo red^+ cells within the $CD14^+$ population. Statistical analysis will be performed by using SPSS software. This experiment will be used mean ± standard deviations (SD) for analyzed triplicate experiments. Unpaired two tailed Student's t-tests will be used for comparisons between groups.

RESEARCH RESULTS

ADCP activity of iMacs

The process of generating macrophages from human induced pluripotent stem cells (hiPSCs) involved several steps. Initially, C3H10T1/2 feeder cells were seeded and allowed to incubate overnight. Subsequently, hiPSCs were seeded onto these feeder cells to form embryoid bodies (EBs). Over the course of 14 days, the cells within the culture were treated with IL-3 and M-CSF to facilitate macrophage differentiation (fig. 1A). As the culture progressed, round-shaped floating cells became apparent, while the number of plate-attached cells increased. The

resulting differentiated macrophages, known as iMacs, exhibited distinctive features such as a larger size, abundant pseudopodia, and vacuoles (fig. 1B). Furthermore, the iMacs were evaluated for the expression of macrophage markers, including CD14 and CD16 (fig 1C). According to identifying iMac by CD14 expression, efficacy of differentiation is around 50-70% of the total differentiated cells. These analyses confirmed that iMacs possessed the typical morphology and phenotype of macrophages.

In addition, flow cytometric analysis revealed that iMacs expressed markers associated with both M1 (CD86) and M2 (CD163) activated macrophages, although the expression level of M1 markers was relatively low (fig. 1D). This indicated that the differentiated macrophages did not conform strictly to the conventional "M1/M2" paradigm.

The expression of CD16 in macrophages is known to be relevant to antibody-mediated phagocytotic activity (Vermi et al., 2018). To assess the phagocytic function of iMacs, they were incubated with a Burkitt's lymphoma cell line in the presence of varying concentrations of rituximab, a therapeutic antibody. The phagocytic activity of iMacs was subsequently evaluated using flow cytometry (fig. 1E). The results demonstrated that the presence of therapeutic antibodies significantly enhanced the phagocytosis of Raji cells compared to the absence of antibodies. This experiment provided evidence of the antibody-dependent cellular phagocytosis (ADCP) function in iMacs.

Transfection and generation of non-cleavable CD16 iPSC

According to Jing et al. (2015), ADAM17 cleavage site of CD16 was defined and disrupted resulting in non-cleavable CD16 NK cells. The disruption was generated by introducing 12436T>C mutation which change TCA (Serine) into CCA (Proline). In this study, we use CRISPR/cas9 system for providing DSB and gene-editing via 12436T>C donor (fig. 2A). When gRNA was cloned into px458 vector, its subclones from transformation were validated with BpiI restriction enzyme. To confirm the cloning results, the subclones were analyzed with DNA sequencing. The subclone and the donor were transfected into iPSCs through SF primary cell 4D-Nucleofector® X kit (Lonza). After transfection 48 h, iPSCs were sorted into single cell for homogeneous clone and expanded for DNA extraction. To investigate the mutation, their DNA was determined with T7 endonuclease. The modified DNA exhibited mismatch and was cleaved with T7 endonuclease (fig. 2B). The transfected iPSCs was DNA sequence analysis (fig. 2C). The sequencing results showed both edited and non-edited patterns in the same clone. Thus, this iPSC clone was gene-edited into a heterogeneous gene.

Effects of engineering non-cleavable CD16 in iMacs

To determine the effects of the S197P mutation on CD16, genetically modified iPSCs were differentiated into macrophages. On day 28, iMacs were dissociated with TrypLE enzyme (Gibco) and stained with anti-CD14 and anti-CD16 for flow cytometric analysis. The iMacs were gated with CD14 to identify macrophages before demonstrated CD16 expression. Non-cleavable CD16 iMacs exhibited higher MFI of CD16 than unmodified iMacs (Fig.3A). This finding indicated that interrupting cleavage site of CD16 in macrophages relates to CD16 expression.

According to role of FcγRs in ADCP, non-cleavable CD16 iMacs were investigated phagocytic activity. B cell lymphoma cell lines were stained with pHrodo Red SE and incubated with rituximab. Next, these cells were co-cultured with iMacs in presence of therapeutic mAbs for 4 h. The cells were harvested and stained with FITC-anti-CD14 antibody before flow cytometry. The engineered iMacs showed a higher percentage of engulfment compared to normal iMacs (Fig. 3B). Thus, the increase of CD16 expression in iMacs contributes to stronger ADCP capacity.

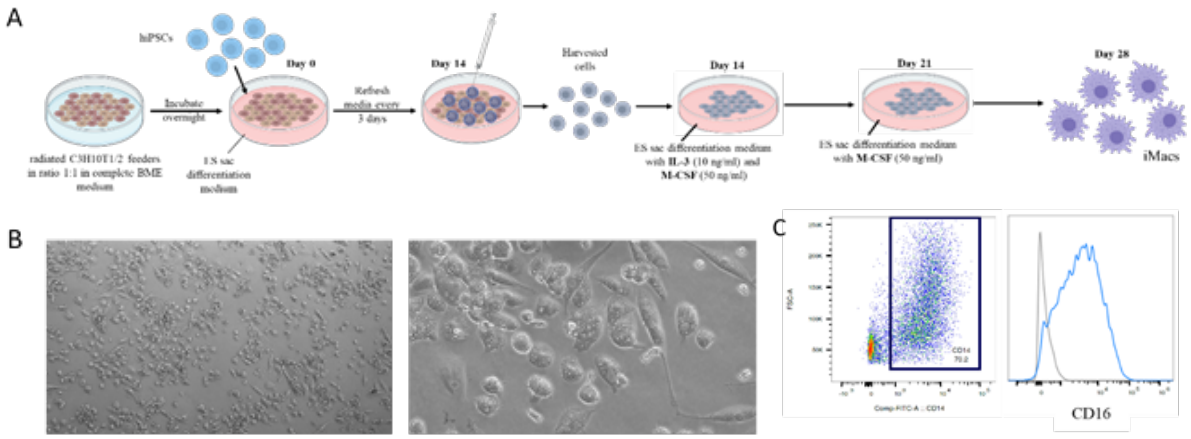
DISCUSSION & CONCLUSION

This study demonstrates enhanced antibody-dependent cellular phagocytosis (ADCP) of genetically modifying non-cleavable CD16 iMac. This data suggests the presence of CD16 shedding in iMacs, similar towhat observed in monocytes derived from peripheral blood mononuclear cells (PBMCs) stimulated with various stimuli (Waller et al., 2019).

Our finding showed that disrupting CD16 shedding lead to increase CD16 expression resulting in upregulation of antibody-mediated anti-cancer capacity in iMacs. However, it is important to note that macrophages are highly plastic and can polarize into M1 or M2 subtypes in response to external factors (Larionova et al., 2019). M1 polarization, characterized by classical activation stimulated by lipopolysaccharide (LPS) or Th1 cytokines such as IFN-γ and GM-CSF, promotes a pro-inflammatory state and tumor resistance (Shapouri-Moghaddam et al., 2018). Therefore, generating M1 macrophages and preserving its phenotype represent a feasible approach to develop "anti-tumor" macrophages for cell-based therapy. Multiplexing genome editing of iPSCs to enhance macrophage M1 polarization is one strategy that could further enhance its antitumor properties.

On the other hand, there several ways to enhance the efficacy of therapeutic antibodies via modified macrophages. While most FcγRs are associated with inducing an antitumor response, FcγRIIB acts as an inhibitory receptor that counteracts the function of activating FcγRs, resulting in negative regulation of ADCP (Bournazos et al., 2009). Therefore, the use of anti-FcγRIIB monoclonal antibodies along with therapeutic antibodies holds promise (Gül & van Egmond, 2015). In ex vivo experiment, bone marrow-derived macrophages were CRISPR-mediated knockdown CSF1R (Cao et al., 2022). The results indicate that modified macrophages exhibited stronger effect on promoting phagocytosis of Raji and DLD1 cells with presence of rituximab and cetuximab, respectively, similarly with knocking down SIRPα. The data indicated that both CSF1R and SIRPα play an inhibitory role in ADCP. Eventually enhancement of cancer immunotherapy is performed in term of promoting and preserving activating molecules and suppressing inhibitory molecules.

In summary, this study emphasizes the importance of CD16 expression in iMacs for antibody-mediated phagocytosis. The successful generation of non-cleavable CD16 iMacs using gene editing techniques provides a valuable tool for studying and enhancing the anti-cancer capabilities of macrophages in immunotherapy. Further investigations can explore the specific signaling pathways and mechanisms underlying the enhanced anti-cancer activity in iMacs, potentially leading to the development of more effective cancer treatment strategies.



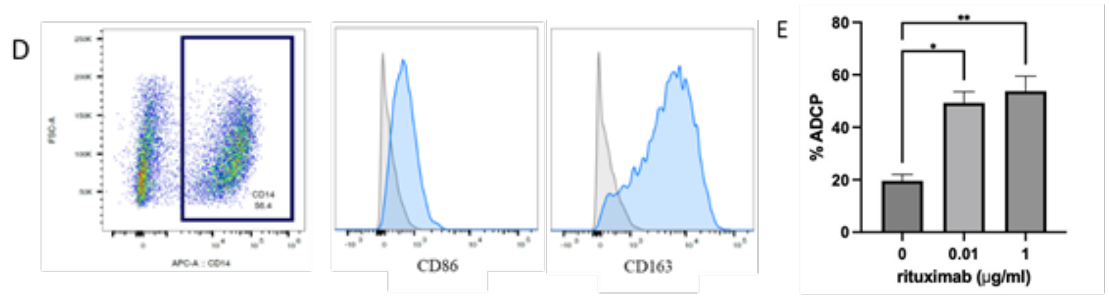
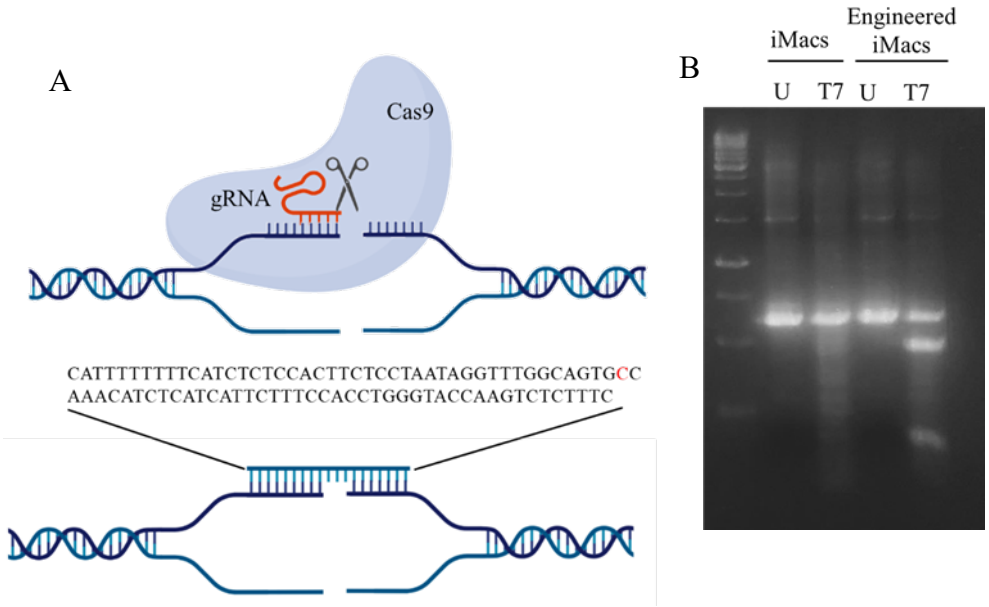


Figure 1 Characteristics of iMacs (A) Schematic showing the protocol for differentiating iMacs from iPSCs. (B) Representative light microscopy of iMacs. Original magnification, 50x (left) and 200x (right). (C) CD16 expression in iMacs determined by flow cytometry. (D) iMacs were co-cultured with rituximab opsonized Raji cells (E:T ratio 5:1) for 2.5 h and evaluated phagocytic capacity with flow cytometry. Data are representative of two independent experiments. Bar graphs show mean ± SD. Statistical significance is indicated as * (P<0.05).



C

Non-cleavable CD16 #1	CTAATAGGTTTGGCAGTGTCAAACATCTCATCATTCTTTC
Non-cleavable CD16 #2	CTAATAGGTTTGGCAGTGTCAAACATCTCATCATTCTTTC
Non-cleavable CD16 #3	CTAATAGGTTTGGCAGTGCCAAACATCTCATCATTCTTTC

Figure 2 Engineering S197P mutation in iPSCs. (A) FCGR3A gene was generated DSB by specific gRNA with Cas9 and repaired with donor template. (B) T7 endonuclease I mismatch detection of 12436T>C mutation in iPSCs. (C) Representative sequence patterns of mutant clones.

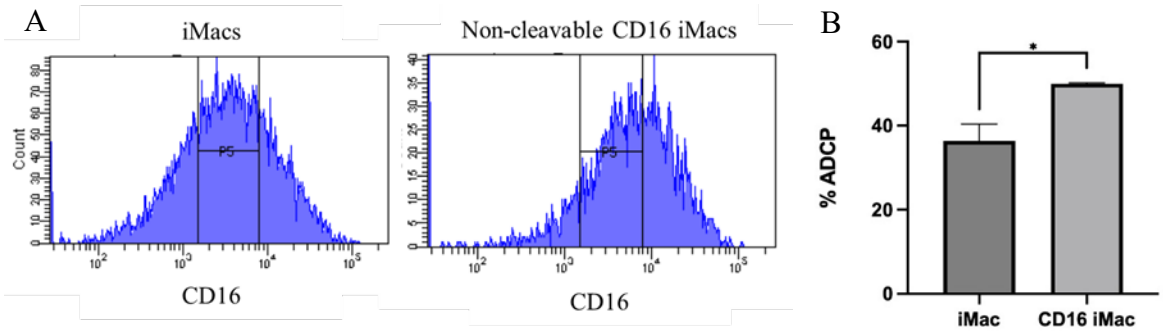


Figure 3 Effects of the engineered S197P mutation in iMacs. (A) CD16 expression in iMacs and non-cleavable CD16 iMacs determined by flow cytometry. (B) iMacs and non-cleavable CD16 iMacs were co-cultured with 10 μg/ml rituximab-opsonized raji cells (E:T ratio 1:1) for 4 h and evaluated phagocytic capacity with flow cytometry. Data are representative of at least two independent experiments. Bar graphs show mean ± SD. Statistical significance is indicated as * ($P<0.05$).

REFERENCES

Bournazos, S., Woof, J., Hart, S., & Dransfield, I. (2009). Functional and clinical consequences of Fc receptor polymorphic and copy number variants. *Clinical & Experimental Immunology, 157*(2), 244-254.

Cao, X., Chen, J., Li, B., Dang, J., Zhang, W., Zhong, X., Wang, C., Raoof, M., Sun, Z., & Yu, J. (2022). Promoting antibody-dependent cellular phagocytosis for effective macrophage-based cancer immunotherapy. *Science Advances, 8*(11), eabl9171.

Clynes, R. A., Towers, T. L., Presta, L. G., & Ravetch, J. V. (2000). Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. *Nature medicine, 6*(4), 443-446.

Fukushima, S., Miyashita, A., Kuriyama, H., Kimura, T., Mizuhashi, S., Kubo, Y., Nakahara, S., Kanemaru, H., Tsuchiya, N., & Mashima, H. (2023). Future prospects for cancer immunotherapy using induced pluripotent stem cell-derived dendritic cells or macrophages. *Experimental Dermatology, 32*(3), 290-296.

Gül, N., & van Egmond, M. (2015). Antibody-dependent phagocytosis of tumor cells by macrophages: a potent effector mechanism of monoclonal antibody therapy of cancer. *Cancer research, 75*(23), 5008-5013.

Jing, Y., Ni, Z., Wu, J., Higgins, L., Markowski, T. W., Kaufman, D. S., & Walcheck, B. (2015). Identification of an ADAM17 cleavage region in human CD16 (FcγRIII) and the engineering of a non-cleavable version of the receptor in NK cells. *PloS one, 10*(3), e0121788.

Larionova, I., Cherdyntseva, N., Liu, T., Patysheva, M., Rakina, M., & Kzhyshkowska, J. (2019). Interaction of tumor-associated macrophages and cancer chemotherapy. *Oncoimmunology, 8*(7), e1596004.

Lyadova, I., Gerasimova, T., & Nenasheva, T. (2021). Macrophages derived from human induced pluripotent stem cells: the diversity of protocols, future prospects, and outstanding questions. *Frontiers in Cell and Developmental Biology, 9*, 640703.

Mantovani, A., Allavena, P., Marchesi, F., & Garlanda, C. (2022). *Macrophages as tools and targets in cancer therapy*. Nature Reviews Drug Discovery, 1-22.

Shapouri-Moghaddam, A., Mohammadian, S., Vazini, H., Taghadosi, M., Esmaeili, S. A., Mardani, F., Seifi, B., Mohammadi, A., Afshari, J. T., & Sahebkar, A. (2018). Macrophage plasticity, polarization, and function in health and disease. *Journal of cellular physiology, 233*(9), 6425-6440.

Takayama, N., Nishikii, H., Usui, J., Tsukui, H., Sawaguchi, A., Hiroyama, T., Eto, K., & Nakauchi, H. (2008). Generation of functional platelets from human embryonic stem

cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. *Blood, The Journal of the American Society of Hematology, 111*(11), 5298-5306.

Vermi, W., Micheletti, A., Finotti, G., Tecchio, C., Calzetti, F., Costa, S., Bugatti, M., Calza, S., Agostinelli, C., & Pileri, S. (2018). slan+ monocytes and macrophages mediate CD20-dependent B-cell lymphoma elimination via ADCC and ADCP. *Cancer research, 78*(13), 3544-3559.

Waller, K., James, C., De Jong, A., Blackmore, L., Ma, Y., Stagg, A., Kelsell, D., O'Dwyer, M., Hutchins, R., & Alazawi, W. (2019). ADAM17-mediated reduction in CD14++ CD16+ monocytes ex vivo and reduction in intermediate monocytes with immune paresis in acute pancreatitis and acute alcoholic hepatitis. *Frontiers in immunology, 10*, 1902.

Zahavi, D., AlDeghaither, D., O'Connell, A., & Weiner, L. M. (2018). Enhancing antibody-dependent cell-mediated cytotoxicity: a strategy for improving antibody-based immunotherapy. *Antibody Therapeutics, 1*(1), 7-12.

Zhu, H., Blum, R. H., Bjordahl, R., Gaidarova, S., Rogers, P., Lee, T. T., Abujarour, R., Bonello, G. B., Wu, J., & Tsai, P.-F. (2020). Pluripotent stem cell–derived NK cells with high-affinity noncleavable CD16a mediate improved antitumor activity. *Blood, 135*(6), 399-410.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.