

# New insights into innate immune cell-based immunotherapies in cancer

**Edited by**

Mary Poupot-Marsan, Anne Caignard, Daniela Wesch,  
Virginie Lafont and Chiara Porta

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# New insights into innate immune cell-based immunotherapies in cancer

## Topic editors

Mary Poupot-Marsan — INSERM U1037 Centre de Recherche en Cancérologie de Toulouse, France

Anne Caignard — Institut National de la Santé et de la Recherche Médicale (INSERM), France

Daniela Wesch — University of Kiel, Germany

Virginie Lafont — Institut National de la Santé et de la Recherche Médicale (INSERM), France

Chiara Porta — University of Eastern Piedmont, Italy

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# Table of contents

- 05 **Editorial: New insights into innate immune cell-based immunotherapies in cancer**  
Anne Caignard, Mary Poupot-Marsan, Virginie Lafont, Daniela Wesch and Chiara Porta
- 08 **Crosstalk between cGAS-STING pathway and autophagy in cancer immunity**  
Qijun Lu, Yukun Chen, Jianwen Li, Feng Zhu and Zhan Zheng
- 22 **Corrigendum: Crosstalk between cGAS-STING pathway and autophagy in cancer immunity**  
Qijun Lu, Yukun Chen, Jianwen Li, Feng Zhu and Zhan Zheng
- 24 **Targeting myeloid-derived suppressor cells in tumor immunotherapy: Current, future and beyond**  
Yang Zhao, Junfeng Du and Xiaofei Shen
- 41 **The application of autologous cancer immunotherapies in the age of memory-NK cells**  
Gaby D. Lizana-Vasquez, Madeline Torres-Lugo, R. Brent Dixon, John D. Powderly II and Renaud F. Warin
- 61  **$\gamma\delta$  T cells in immunotherapies for B-cell malignancies**  
Léa Rimalho, Carla Faria, Marcin Domagala, Camille Laurent, Christine Bezombes and Mary Poupot
- 73 **ChemR23 activation reprograms macrophages toward a less inflammatory phenotype and dampens carcinoma progression**  
Margot Lavy, Vanessa Gauttier, Alison Dumont, Florian Chocteau, Sophie Deshayes, Judith Fresquet, Virginie Dehame, Isabelle Girault, Charlene Trilleaud, Stéphanie Neyton, Caroline Mary, Philippe Juin, Nicolas Poirier, Sophie Barillé-Nion and Christophe Blanquart
- 90 **BAT6026, a novel anti-OX40 antibody with enhanced antibody dependent cellular cytotoxicity effect for cancer immunotherapy**  
Shizhong Liang, Dandan Zheng, Xiong Liu, Xiong Mei, Congcong Zhou, Cuizhen Xiao, Chao Qin, Haitao Yue, Jian Lin, Cuihua Liu, Shengfeng Li and Jin-Chen Yu
- 101 **NK cells in peripheral blood carry trogocytosed tumor antigens from solid cancer cells**  
Mauricio Campos-Mora, William Jacot, Genevieve Garcin, Marie-Lise Depondt, Michael Constantinides, Catherine Alexia and Martin Villalba
- 117 **The challenge of making the right choice: patient avatars in the era of cancer immunotherapies**  
Charlotte Kayser, Annika Brauer, Sebens Susanne and Anna Maxi Wandmacher

- 126 Enhanced expression of natural cytotoxicity receptors on cytokine-induced memory-like natural killer cells correlates with effector function**  
Sofia Carreira-Santos, Nelson López-Sejas, Marina González-Sánchez, Eva Sánchez-Hernández, Alejandra Pera, Fakhri Hassouneh, Esther Durán, Rafael Solana, Javier G. Casado and Raquel Tarazona
- 138 Novel NKG2D-directed bispecific antibodies enhance antibody-mediated killing of malignant B cells by NK cells and T cells**  
Sebastian Lutz, Katja Klausz, Anca-Maria Albici, Lea Ebinger, Lea Sellmer, Hannah Teipel, André Frenzel, Anna Langner, Dorothee Winterberg, Steffen Krohn, Michael Hust, Thomas Schirrmann, Stefan Dübel, Regina Scherließ, Andreas Humpe, Martin Gramatzki, Christian Kellner and Matthias Peipp
- 151 Immunotherapies inducing immunogenic cell death in cancer: insight of the innate immune system**  
Kenny Misael Calvillo-Rodríguez, Helen Yarimet Lorenzo-Anota, Cristina Rodríguez-Padilla, Ana Carolina Martínez-Torres and Daniel Scott-Algara
- 172  $\gamma\delta$  T cell-mediated cytotoxicity against patient-derived healthy and cancer cervical organoids**  
Junxue Dong, David Holthaus, Christian Peters, Stefanie Koster, Marzieh Ehsani, Alvaro Quevedo-Olmos, Hilmar Berger, Michal Zarobkiewicz, Mandy Mangler, Rajendra Kumar Gurumurthy, Nina Hedemann, Cindrilla Chumduri, Dieter Kabelitz and Thomas F. Meyer
- 185 Neutrophil extracellular traps regulating tumor immunity in hepatocellular carcinoma**  
Weixiong Zhu, Chuanlei Fan, Shi Dong, Xin Li, Haoifei Chen and Wence Zhou
- 203 Chimeric antigen-receptor (CAR) engineered natural killer cells in a chronic myeloid leukemia (CML) blast crisis model**  
Jusuf Imeri, Paul Marcoux, Matthias Huyghe, Christophe Desterke, Daianne Maciely Carvalho Fantacini, Frank Griscelli, Dimas T. Covas, Lucas Eduardo Botelho de Souza, Annelise Bennaceur Griscelli and Ali G. Turhan
- 212 Vdelta1 T cells are more resistant than Vdelta2 T cells to the immunosuppressive properties of galectin-3**  
Jan Schadeck, Hans-Heinrich Oberg, Matthias Peipp, Nina Hedemann, Wolfgang W. Schamel, Dirk Bauerschlag and Daniela Wesch
- 227 Gamma/delta T cells as cellular vehicles for anti-tumor immunity**  
Chelsia Qiuxia Wang, Pei Yu Lim and Andy Hee-Meng Tan
- 247 Seasonal influenza vaccines differentially activate and modulate toll-like receptor expression within the tumor microenvironment**  
Kajal H. Gupta, Eileena F. Giurini and Andrew Zloza



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EDITED AND REVIEWED BY  
Peter Brossart,  
University of Bonn, Germany

\*CORRESPONDENCE  
Chiara Porta  
✉ chiara.porta@uniupo.it

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# Editorial: New insights into innate immune cell-based immunotherapies in cancer

Anne Caignard<sup>1</sup>, Mary Poupot-Marsan<sup>2</sup>, Virginie Lafont<sup>3</sup>, Daniela Wesch<sup>4</sup> and Chiara Porta<sup>5,6\*</sup>

<sup>1</sup>Institut National de la Santé et de la Recherche Médicale (INSERM) U1160, Institut de Recherche Saint-Louis Hôpital Saint Louis, Paris, France, <sup>2</sup>Centre de Recherche en Cancérologie de Toulouse (CRCT), UMR1037 Institut National de la Santé et de la Recherche Médicale (Inserm)-Univ. Toulouse III Paul Sabatier-ERL5294 Centre National de la Recherche Scientifique (CNRS), 2 avenue Hubert Curien Oncopole de Toulouse, CS53717, Toulouse, France, <sup>3</sup>Institut de Recherche en Cancérologie de Montpellier (IRCM), Institut National de la Santé et de la Recherche Médicale (INSERM) U1194, Univ Montpellier, Institut du Cancer de Montpellier (ICM), Montpellier, France, <sup>4</sup>Institute of Immunology, University Medical Center Schleswig-Holstein, Christian-Albrechts University, Kiel, Germany, <sup>5</sup>Department of Pharmaceutical Sciences, University of Eastern Piedmont, Novara, Italy, <sup>6</sup>Center for Translational Research on Autoimmune & Allergic Diseases (CAAD), University of Eastern Piedmont, Novara, Italy

## KEYWORDS

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## Editorial on the Research Topic

### New insights into innate immune cell-based immunotherapies in cancer

The limited number of patients achieving an effective and durable response following T cell-centric immunotherapies indicates the urgent medical need for complementary approaches. Recent insights in innate immune cell-based cancer immunotherapies are discussed in this Research Topic, encompassing several original research manuscripts unveiling antibodies, genetic engineering and other promising strategies to enhance the anti-tumor activity of macrophages, NK and  $\gamma\delta$  T cells. Additionally, reviews within this Research Topic offer a comprehensive overview of the state-of-the-art approaches for targeting specific innate cell subtypes in cancer therapy. They also discuss emerging druggable pathways poised to advance toward clinical application in the foreseeable future, and highlight current challenges, including patient avatars as an essential tool to guide clinical decision making.

As key orchestrators of a tumor-promoting microenvironment, tumor-associated macrophages (TAM) have long been recognized as promising therapeutic targets for the development of new anti-cancer therapies. In this context, the preclinical studies of [Lavvy et al.](#) indicate the activation of ChemR23 through an agonist monoclonal Antibody (mAb) as a novel potential strategy to reprogram macrophages toward a less inflammatory and immunosuppressive phenotype and to dampen triple negative breast cancer progression. Although rare and sparse compared to macrophages, cytotoxic innate lymphoid cells infiltrate solid cancers and contribute to the elimination of tumor cells. Interestingly, the studies of [Campos-Mora et al.](#) highlight a specific CD45R0<sup>+</sup>CD107a<sup>+</sup> NK cell subset that, after having acquired antigens from breast cancer cells through trogocytosis, returned in the

blood circulation. The identification of these circulating activated NK cell subsets carrying solid tumor markers provides the rationale to argue for its use in complementing established therapies or as a theragnostic approach for solid cancer patients. For the development of effective NK-based immunotherapies, several strategies have been investigated to enhance the cytotoxic activities and prolong the half-life of NK cells. For example, [Carreira-Santos et al.](#) established a protocol to activate NK cells isolated from the blood of healthy donors with a cytokine cocktail consisting of IL-12, IL-15 and IL-18. As a result, cytokine-induced memory-like (CIML) NK cells showed increased cytotoxicity *in vitro* and a longer lifespan, valuable features for adoptive cell transfer immunotherapies. The identification of effective combination strategies to enhance anti-tumor immunity is crucial to improve the efficacy of immunotherapy. In this regard, [Lutz et al.](#) demonstrate that novel bispecific antibodies targeting the activating receptor NKG2D and the malignant B cell antigen CD20 can potentiate both antibody-dependent cell-mediated cytotoxicity (ADCC) of anti-CD38 or anti-CD19 mAbs and the effectiveness of approved bispecific mAbs directed against CD3 and CD19, suggesting a synergistic effect between NK and T cells. Another promising combination immunotherapy consists of a novel high affinity human antibody specific for the activating receptor OX-40 (BT6026) with the antibody blocking the inhibitory checkpoint PD-1. Specifically, [Liang et al.](#) show that BT6026 had an enhanced ADCC effect and a significant anti-tumor activity in an OX40-humanized mouse model of colon cancer. Moreover, when combined with an anti-PD-1 antibody, BT6026 resulted in a synergistic effect on tumor inhibition. The good safety profile observed in non-human primates further warrants additional studies of the long-term safety and efficacy of BAT6026 in clinical trials, to assess its potential as a cancer immunotherapy.

The participation of cytotoxic innate immune cells (NK,  $\gamma\delta$  T cells) in novel adoptive cell therapies using selected innate cell subtypes or chimeric antigen receptor (CAR) engineered innate cell subsets are increasing. In this perspective, [Imeri et al.](#) provides preclinical evidence of efficacy and specificity of an Interleukin-2-Receptor  $\alpha$  subunit (IL2RA/CD25) CAR-NK-based therapy for chronic myeloid leukemia (CML), thereby indicating a new potential therapeutic option for CML patients that are in blast phase and resistant to targeted therapies. [Lizana-Vasquez et al.](#) review the studies based on CIML NK cells for cell therapy in autologous settings, while [Rimailho et al.](#) and [Wang et al.](#) discuss  $\gamma\delta$  T cells in cancer immunotherapies. Specifically, [Rimailho et al.](#) focus on the current understanding of  $\gamma\delta$  T cell biology in the context of B-cell malignancies. They describe the diversity of  $\gamma\delta$  T cells in both tissues and blood, highlighting their potential functional plasticity and anti-tumor properties as exploitable features for immunotherapeutic approaches. The review also provides a comprehensive description of the strategies to harness  $\gamma\delta$  T cells, such as activation and tumor-targeting, expansion protocols and gene modification. Additionally, it summarizes ongoing clinical trials in B cell malignancies. Along the same line, [Wang et al.](#) offer a clinically focused review that points to key gaps in knowledge and proposes strategies to harness the unique properties of  $\gamma\delta$  T cells for cellular immunotherapy, drawing insights from previous clinical trials. Moreover, the review gives

an update on ongoing trials involving  $\gamma\delta$  T cells for both hematological and solid cancers and discusses strategies that have been tested or can be explored to improve anti-tumor activity and durability of  $\gamma\delta$  T cells.

The advent of immunotherapy has fostered the development of tumor models, such as organoids, organotypic tissue slice culture, organ-on-a-chip and patient-derived xenografts, in order to get insights into the cross-talk between cancer and immune cells and to test immunotherapeutic approaches. The advantages and disadvantages of each model system are discussed by [Kayser et al.](#), who highlight the need to define the rationale and requirements for their use and the importance to advance further in the development of patient avatars as a complementary tool for testing and predicting immunotherapeutic strategies for personalized tumor therapies. In addition, two original research manuscripts of this Research Topic report recent insights into the generation and utilization of these novel models to study  $\gamma\delta$  T cells in the context of cervical and ovarian cancer. In particular, [Dong et al.](#) have established patient-derived healthy and transformed cervical organoids expressing HPV16 oncogenes E6 and E7. Using bulk-RNA sequencing, they revealed differences in DNA damage and cell cycle checkpoint pathways and identified crucial molecules for  $\gamma\delta$  T cell activation. [Schadeck et al.](#) investigate the immunosuppressive effect of galectin-3 on different  $\gamma\delta$  T cell subsets using co-culture systems consisting of ovarian cancer and V $\delta$ 1 or V $\delta$ 2 T cells. Given that galectin-3 inhibits proliferation of V $\delta$ 2 T cells only, the results of this study suggest that an activation of V $\delta$ 1 T-cell proliferation, as part of a T-cell-based immunotherapy, can be advantageous due to their resistance to the immunosuppressive properties of galectin-3.

Besides boosting cytotoxic effectors, targeting myeloid cells in order to reprogram the immunosuppressive tumor microenvironment (TME) into an immunostimulatory one has emerged as a promising strategy to enhance the efficacy of cancer immunotherapy. Therefore, an in-depth understanding of the mechanisms driving myeloid-derived suppressor cell (MDSCs) generation, suppressive activities and recruitment in the TME are essential to identify effective combinatorial strategies for tumor immunotherapy. In this context, [Zhao et al.](#) summarized current understanding of functional and regulatory mechanisms of MDSCs within the TME, along with recent insights into therapeutic strategies targeting MDSCs in combination treatments for cancer patients. Additionally, [Zhu et al.](#) report on the role of neutrophils and neutrophil extracellular traps (NETs) in the initiation and progression of hepatocellular carcinoma. They also review studies conducted on NETs in other types of cancers and discuss emerging areas of interest in the field.

Finally, two reviews debate about the impact of cancer cell activities on innate and cancer immunity. [Cavillo-Rodriguez et al.](#), explore the complex interactions between immunogenic cell death (ICD) induced by cancer therapies and the innate immune system, and address the next challenges in cancer treatment. cGAS-STING pathway and autophagy have been shown to be interrelated in innate immunity. Along the same line, [Lu et al.](#) summarize the recent findings of the cGAS-STING pathway and autophagy in cancer immunity and explore their interactions in this context, theorizing that strategies targeting these processes can be exploited for novel combination cancer immunotherapies.



Lastly, we would like to express our gratitude to all the authors who have contributed to this Research Topic, as well as to the reviewers for their remarkable commitment. We hope that the insights presented in this Research Topic will serve as a source of inspiration for those already working in the field of cancer immunotherapy, and as a fruitful and engaging read for those less familiar with this area of research.

## Author contributions

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## OPEN ACCESS

## EDITED BY

Mary Poupot-Marsan,  
INSERM U1037 Centre de Recherche en  
Cancérologie de Toulouse, France

## REVIEWED BY

Mariana Pavel-Tanasa,  
Grigore T. Popa University of Medicine and  
Pharmacy, Romania  
Peter Wang,  
Bengbu Medical College, China

## \*CORRESPONDENCE

Zhan Zheng

✉ zhengzhan2696@126.com

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# Crosstalk between cGAS-STING pathway and autophagy in cancer immunity

Qijun Lu<sup>1</sup>, Yukun Chen<sup>2</sup>, Jianwen Li<sup>1</sup>, Feng Zhu<sup>3</sup>  
and Zhan Zheng<sup>1\*</sup>

<sup>1</sup>Department of Oncology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China, <sup>2</sup>Cancer Institute, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China, <sup>3</sup>Department of Laboratory Medicine, Huadong Hospital, Fudan University, Shanghai, China

The cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway is critical in cancer immunity. Autophagy is a highly conserved process that is responsible for the degradation of cytoplasmic material and is involved in both innate and adaptive immunity. Recently, cGAS-STING and autophagy have been shown to be interconnected, which may influence the progression of cancer. Although cGAS-STING and autophagy have been shown to be interrelated in innate immunity, little has been reported about cancer immunity. As cancer immunity is key to treating tumors, it is essential to summarize the relationship and interactions between the two. Based on this, we systematically sorted out the recent findings of cGAS-STING and autophagy in cancer immunity and explored the interactions between cGAS-STING and autophagy, although these interactions have not been extensively studied. Lastly, we provide an outlook on how cGAS-STING and autophagy can be combined, with the hope that our research can help people better understand their potential roles in cancer immunity and bring light to the treatment of cancer.

## KEYWORDS

antitumor, autophagy, cancer, cGAS-STING, immunity

## 1 Introduction

Cancer is one of the world's most serious threats to human health, with high morbidity and mortality rates, and according to the latest global data, 9.96 million patients will die from cancer in 2020 (1). Cancer is a genetic abnormal disease triggered by a long-term combination of multiple factors. When the human body is affected by chemical, physical, viral, and other carcinogenic substances in the environment or due to its own genetic, endocrine, gender, age, and other factors, a series of abnormal genetic changes will occur to form malignant tumors (2). Tumor cell growth is initiated by mutations that activate oncogenic drivers. This process is combined with the genetic or non-genetic activation or inactivation of genes that promote or inhibit tumor proliferation (3). In many cancers,

oncogenesis is accompanied by the accumulation of mutations, which can provide a selective advantage to cancer cell populations by increasing the degree of genetic diversity and accelerating their evolutionary adaptation (4, 5). However, this diversity comes at a cost: the more the cancer cell differs from normal cells, the more likely it is to be recognized as a foreign agent by the immune system.

Current clinical treatment of malignancies is still dominated by radiotherapy, chemotherapy and surgery, but the 5-year survival rate of patients is still very low (6). Along with the advancement of human understanding of tumor immunity, immunotherapy has become increasingly sophisticated and offers new hope for cancer treatment (7–10). Immune checkpoint inhibitors, such as therapeutic monoclonal antibodies targeting the programmed cell death protein 1/programmed cell death ligand 1 (PD-1/PD-L1) pathway, have been approved as monotherapy or combination therapy for oncology treatment (11). One of the main targets of immune checkpoint inhibitors is the release of effector T cells. The positive correlation between T-cell infiltration in the tumor stroma and prognosis, as well as the clinical success of chimeric antigen receptor (CAR) T-cell infusion in certain hematologic malignancies, suggest a critical role for T cells in tumor immunity (12). These clinical successes have led to a T-cell-centric view of tumor immunity. There is a strong link between cancer and the immune system (13). Adaptive immunity, as well as innate immunity, make up the immune system. However, the effector function of T cells is not autonomous (14). The immune system promotes or suppresses tumor growth by recognizing and killing cancer cells. The initiation and maintenance of T cell responses and the development of durable protective memory T cells are dependent on the innate immune response (15). Innate immunity involves various types of myeloid cells, including dendritic cells (DCs), monocytes, macrophages, polymorphonuclear cells, mast cells, and innate lymphocytes (ILCs), such as natural killer (NK) cells (14). Innate immunity is the host's first and fastest line of defense against invading pathogens. Different pattern recognition receptors (PRRs) are used to activate the innate immune response when host cells recognize conserved pathogen patterns known as pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (16). In eukaryotic cells, DNA is usually present in the nucleus and mitochondria. The DNA present in the cytoplasm is usually due to microbial infection or DNA damage. Thus, cytoplasmic DNA is a red flag that triggers a strong innate immune response (17). Recognition of cytoplasmic DNA is an important host defense mechanism. Cyclic GMP-AMP synthase (cGAS) is thought to be a key sensor mediating cytoplasmic DNA recognition.

The STING pathway has emerged as a promising drug target for the treatment of cancer (18). By triggering the cGAS-STING pathway, the innate immune system can be activated, promoting acquired immunity to fight cancer and thus improving survival (19). In addition, autophagy, a tightly regulated mechanism of cellular self-degradation, is essential for maintaining intracellular homeostasis under stressful conditions (20). Autophagy is extensively involved in the survival, development, and maturation

of immune cells (21). It can contribute to the initiation or inhibition of tumor growth by regulating the development of innate and adaptive immunity (22). cGAS-STING pathway can trigger autophagy in several ways, and autophagy can also regulate the cGAS-STING pathway (23). Therefore, in this review, we systematically discuss the interaction between the cGAS-STING signaling pathway and autophagy in cancer immunity, hoping to provide a direction for exploring new cancer immune mechanisms and therapeutic approaches (Figure 1).

## 2 Overview of the cGAS-STING pathway

The immune system recognizes different pathogens to protect the body and maintain homeostasis. Innate immunity functions as the first line of defense against pathogenic microorganisms and as a basis for adaptive immune responses. The host cells become aware of a pathogen invasion through pattern recognition receptors, which will initiate a series of signaling events. Many pattern recognition receptors exist, such as Toll-like receptors, Nod-like receptors, and Scavenger receptors. A recently discovered pathogen recognition receptor, cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), can activate any sequence of double-stranded DNA (dsDNA) (24) and participate in various cellular processes, including proliferation, apoptosis, differentiation, and invasion of cancer cells.

STING is a receptor protein located on the endoplasmic reticulum (ER) that is critical for the response pathway in innate immunity. It is usually observed in the resting state as a dimer. By liquid-liquid phase separation, cGAS and dsDNA interact to form micrometer-sized drops that activate cGAS. As the reactants concentrate, these lipid droplets generate cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) which can be catalyzed from ATP and GTP (25, 26). STING is activated by cGAMP in the ER and becomes a tetramer by oligomerization (27). Sulfated glycosaminoglycans induce STING to translocate into the Golgi apparatus and perinuclear endosomes from the ER (28), during which STING is palmitoylated in the Golgi apparatus and caused to activate IRF3 by recruiting TBK1 kinase, which undergoes transautophosphorylation, thus enhancing affinity for interferon (IFN) regulatory factors. When IRF3 is activated, it enters the nucleus, which works synergistically with NF- $\kappa$ B to promote the transcription of type I IFN genes and related immunomodulatory factors (29–31). STING is rapidly degraded by sorting into lysosomes after signaling (32). In addition, STING can mediate the activation of the NF- $\kappa$ B pathway downstream of DNA damage signaling independently of cGAS, and the E3 ubiquitin ligase TRAF6, P53, DNA damage kinase ataxia telangiectasia mutated, enzyme poly(ADP-ribose) polymerase 1, and interferon- $\gamma$ -inducible factor 16 combine to form a distinct STING signaling complex that induces TRAF6-dependent NF- $\kappa$ B activation (33–37). However, the exact mechanism of STING-dependent NF- $\kappa$ B pathway activation remains unknown (Figure 2).

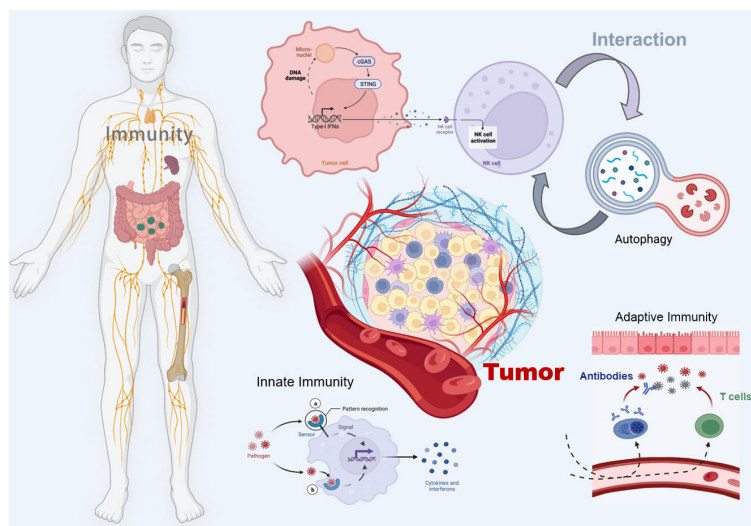


FIGURE 1

Schematic illustration of the crosstalk between the cGAS-STING pathway and autophagy in cancer immunity. The figure was created with BioRender (<https://biorender.com/>).

### 3 cGAS-STING pathway in cancer

Many tissues have been found to express STING, such as the heart, spleen, lung, ovary, and various antigen-presenting cells (APCs). However, it was less expressed in tissues such as the brain, liver, kidney, small intestine, and colon. According to The Cancer Genome Atlas dataset, cGAS and STING gene expression was detected in all types of cancer, but the expression varied according to the stage and type of cancer (38). STING expression is significantly increased in murine pancreatic cancer models and human pancreatic tumors, as well as tongue squamous cell carcinoma, while down-regulated in malignant melanoma (39, 40). In addition, patients with lung adenocarcinoma had lower cGAS expression and longer survival (41). Based on the evidence presented above, cGAS-STING is inextricably linked to cancer.

In further studies, cGAS-STING was found to have a tumor-suppressive effect. By regulating the initiation of intestinal inflammation, STING may hinder the progression of colon cancer, and it may also regulate various signaling pathways such as signal transducer and activator of transcription-3 and NF- $\kappa$ B (42). However, tumors can develop when the cGAS-STING pathway is overactivated. By activating STING, the carcinogen 7,12-dimethyl-Benz[a]anthracene can cause DNA breaks in mice, resulting in skin tumors (43). In the same way, STING activation is associated with Lewis lung cancer growth (44).

Furthermore, the cGAS-STING pathway is involved in cancer metastasis. Cancer cells can transfer cGAMP to astrocytes *via* the cancer-astrocyte gap junction channel, which activates STING in astrocytes and subsequently produces inflammatory cytokines such as IFN- $\alpha$  and TNF- $\alpha$ , which in turn activate signal transducers and activator of transcription 1 (STAT1) and NF- $\kappa$ B signaling pathways in the cancer cell, leading to brain metastasis (45). In metastatic breast cancer, cGAS-STING signaling can activate atypical NF- $\kappa$ B pathways, which can promote metastasis due to epithelial-

mesenchymal transition (EMT) (46). Meanwhile, the elimination of STING can inhibit breast cancer metastasis by reducing the expression of the EMT gene (46).

### 4 cGAS-STING pathway in cancer immunity

cGAS-STING participates in the remodeling of the tumor microenvironment (TME) (47), which induces the production of antitumor cytokines such as interleukin 10 and invariant surface glycoprotein (ISG) that inhibit tumor growth (48). Macrophages serve as powerful APCs by engulfing foreign pathogens and priming host defenses (49). The cGAS-STING pathway could significantly regulate macrophage polarization, which is considered an essential part of innate immunity and may be adopted as a target for immunotherapy-related diseases. Administration of liposome-derived cGAMP nanoparticles (cGAMP-NP) to tumor cells can activate STING in macrophages, repolarize M2-type macrophages into M1-type macrophages, improve MHC-like molecules or costimulatory molecules, and then induce differentiation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, thus producing a potent antitumor response (50).

In tumor cells, activating the cGAS-STING pass-through may inhibit the development of early tumors by upregulating type I IFN and other inflammatory genes. TME contains multiple proangiogenic factors that stimulate the formation of new blood vessels during tumor angiogenesis (51). Endothelial STING controls T-cell transendothelial migration in association with IFN-I (52). Activating STING increases the immune response to the TME and normalizes the tumor vasculature. In addition, the cGAS-STING pathway affects CD8<sup>+</sup> T cell-mediated antitumor immunity by type I IFN. Downregulation of the cGAS-STING pathway leads to a reduction in tumor-infiltrating CD3<sup>+</sup> CD8<sup>+</sup> T cells by inhibiting type I IFN downstream genes, including chemokine ligands 9 and 10 (53).

In the immune system, DCs also play an essential antitumor immunity role. The STING protein in DCs amplifies signals from cytoplasmic DNA sensors, enhancing the adaptive immune system of the tumor. After being absorbed by tumor-infiltrating DCs, exosomal DNA activates STING signaling (54). DCs respond to NARK signaling by phagocytosing dead/damaged tumor cells, transferring exosomes, and forming cGAMP gap junctions. After injecting type I IFN, DCs drain lymph nodes and trigger tumor specific CD8<sup>+</sup> T cells to migrate to the tumor. Finally, these CD8<sup>+</sup> T cells proliferate in lymph nodes, killing the tumor cells (55). During TME, phagosomes degrade mtDNA from tumor cells, causing the production of type I IFN in the DC cytoplasm; inhibiting CD47 suppresses this degradation, enhancing adaptive immunity against tumors (56). If STING is deleted in DC, the ability to present antigens is abolished, and tumor infiltrating lymphocyte abundance is decreased (57). A similar effect was observed in colon tumors with MC38 after radiation exposure by mobilizing myeloid-derived suppressor cells (MDSCs) dependent on the host STING molecule (58).

In contrast, cGAS-STING signaling may promote tumor growth and metastasis. Chronic activation may induce an immunosuppressive TME (17). STING was associated with poor prognosis in a subset of patients with colorectal cancer (38), suggesting that STING may contribute to tumor growth and immune evasion. Recent research found that STING agonists activate cell stress in T cells and trigger cell death (59). Another study found that constitutive activation of STING impaired T lymphocyte proliferation, a process dependent on NF- $\kappa$ B and triggered by STING relocation to the Golgi apparatus (60). These findings suggest that cGAS-STING, as an innate sensor, also has the potential to impair the adaptive immune system.

Immune responses to DNA in the TME are influenced by tumor antigenicity, which is underappreciated. Through the induction of indoleamine 2,3-dioxygenase (IDO), the cGAS-STING pathway promotes tumor progression with low antigenicity (44). However, it remains unclear how cGAS-STING signaling stimulates cells to express PD-L1, which is known to mediate immune evasion of cancer cells (61). Mutations in the liver kinase B1 (LKB1) cause primary resistance to immunotherapy in non-small cell lung cancer (NSCLC). When LKB1 is lost, STING is inhibited, and cytoplasmic dsDNA is not sensitive to detection. Cancers resistant to immune checkpoint blockade may benefit from reactivating the LKB1 or STING pathways (62). In tongue squamous cell carcinoma samples, STING expression increased with tumor progression, with STING protein activation seen in papillomavirus positive specimens. In contrast, STING gene silencing does not affect cell viability or apoptosis but promotes IL-10, IDO, and CCL22, thus enhancing immunosuppressive cytokines and regulatory T-cell infiltration, suggesting that STING regulates the TME and influencing tumor progression (63).

## 5 Overview of autophagy

Autophagy is a tightly regulated and stress-induced catabolic process that regulates cancer in eukaryotes (64). Macroautophagy, also known as canonical autophagy, can be divided into several stages including initiation, nucleation, or phagophore formation, elongation

of the phagophore membrane to form the autophagosome, fusion of the autophagosome with the lysosomes, and degradation of the contents of the autophagosome (65). Macroautophagy was initially thought to be a massive degradation process activated by cellular starvation. Nevertheless, new findings suggest that autophagy also functions as a quality control mechanism for specific organelles and proteins (66). Through lysosomal or endosomal invagination, cytoplasmic cargo is engulfed during microautophagy (67).

During canonical autophagy, signaling pathways such as mTOR and AMPK sense metabolic stress and thus activate the Unc-51-like kinase 1 (ULK1 and ULK2) complex (68–70). In the initiation phase, ULK1/2 activates VPS34 and the complexes of VPS34, VPS15, autophagy-related gene (ATG)14, Beclin-1, and P150 catalyze the production of phosphoinositol-3-phosphate, recruiting a further boost to the autophagic pathway (70–72). The phosphoinositide 3-kinase (PI3K) complex is responsible for the expansion and maturation of autophagic vesicles (73). Furthermore, ATG5-ATG12-ATG16 and the LC3 ubiquitin-like system contribute to the extension of autophagosome membranes (74). In particular, ATG5-ATG12 non-covalently binds to interact with ATG16 to form the ATG5-ATG12/ATG16 complex (75). ATG4, ATG7, and ATG3 cleave the precursors of LC3-like proteins, maturing and conjugating them with phosphatidylethanolamine (PE) to form LC3-II, which drives the elongation and closure of cell membranes and, ultimately, the formation of autophagosomes (76, 77). P62 via a LIR motif (LC3 interacting region) interacts with LC3. P62 has also an ubiquitin binding domain (UBD) and can bind to autophagy cargo (the ubiquitinated proteins). thus, P62 is an adaptor protein, linking LC3 to its cargo (78). At the maturity stage, LC3-II is digested and autophagosome forms that fuse with a lysosome, causing cell cargo degradation (76) (Figure 3).

Although canonical and non-canonical autophagy pathways share overlapping machinery, they differ in several important ways (79, 80). Non-canonical autophagy processes include microautophagy, chaperone-mediated autophagy (CMA), and LC3-associated phagocytosis (LAP) (81). Microautophagy occurs when lysosomes or vesicular endosomes directly engulf intracellular material for degradation (82). CMA is the process of binding intracytoplasmic proteins to molecular chaperones and transferring them to the lysosomal lumen, where lysosomal enzymes digest them (83). However, CMA is selective in removing proteins and is a soluble protein (84). During phagocytosis, pathogens engage extracellular receptors, such as Toll-like receptors, to initiate LAP, a non-canonical form of autophagy (85). Also, immune complexes and dying cells can trigger LAP (86). Furthermore, LAP is an important mediator in the response to immune tolerance, in addition to participating in the degradation of engulfed pathogens (87). With increased research on non-canonical autophagy, the concept of autophagy has been better understood and appreciated.

## 6 Autophagy in cancer

Cells need to adapt to environmental disturbances to maintain homeostasis in the body. In this process, autophagy serves as a



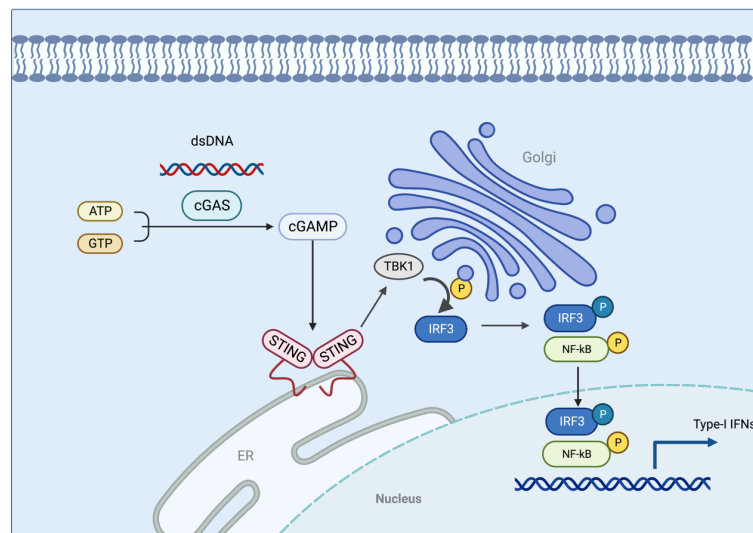


FIGURE 2

In this model, the cGAS interacts with the dsDNA via liquid-liquid phase separation, which activates the cGAS. STING is activated in the ER when cGAMP is generated in response to the concentration of the reactants. As STING is transferred to the Golgi apparatus, TBK1 is recruited to activate IRF3. When IRF3 is activated, it enters the nucleus and functions with NF- $\kappa$ B to produce type I IFN. The figure was created with BioRender (<https://biorender.com/>).

recycling pathway that participates in the turnover of cellular components (88). Also, autophagy is crucial for cancer cell survival in conditions of nutrient and oxygen deprivation by degrading protein and lipid bulks for nutrient recycling (89–91). Tumor types and tumor models affect autophagy in cancer progression (89, 90). Defects in autophagy *in vivo* have been linked to an increased risk of tumor initiation (92). However, it is still unclear how autophagy-deficient tumors sustain their growth. Hepatocellular tumors are more likely to progress in autophagy-deficient livers when the group box1 is released from autophagy-deficient hepatocytes, which increases proliferation capacity (93). A lack of autophagy inhibits the killing of triple-negative breast cancer cells both *in vitro* and *in vivo* (94). Phosphorylation of Beclin1 controls autophagy and promotes or inhibits it (95). It is reported to have increased Beclin1 expression in cancer tissues in 110 patients with prostatic carcinoma, suggesting that autophagy could promote tumorigenesis (96).

Malignant tumors are closely linked to autophagy, especially the processes of recurrence, metastasis, and drug resistance (97). Cancer progression has been characterized by metastasis. Autophagy in metastasis is quite complex as a survival pathway and quality control mechanism. During the early stages of metastasis, autophagy serves primarily as a suppressor by restricting necrosis and mediating autophagic cell death (98). On the contrary, in the advanced stages of metastasis, autophagy as a dynamic degradation and recycling system can help to cope with intracellular and environmental stresses, such as hypoxia, nutrient shortage, or cancer therapy, thus favoring tumor progression. Moreover, Autophagy is upregulated in primary human glioblastoma, melanoma, esophageal cancer, and hepatocellular carcinoma upon progression to advanced metastatic disease, and autophagy markers in these cancers are associated with poor

prognosis (99–101), indicating its importance throughout the metastatic cascade. Also, profilin 1 participates in cell proliferation and enhances autophagy-induced drug resistance by interacting with the Beclin1 complex in multiple myeloma (102).

## 7 Autophagy in cancer immunity

Autophagy influences tumorigenesis by modulating the formation of TME, and this microenvironment causes changes in autophagy signaling pathways in tumors, stroma, and innate immune cells (103). Depending on the characteristics of the tumor, autophagy can promote or suppress the immune response of the TME. Autophagy of these cells can enhance antitumor immune responses and immunotherapy. As a major innate effector component of early immunity, NKs play a crucial role. When NK cells develop, autophagy protects them by removing damaged mitochondria and reactive oxygen species (ROS) (104). As a result of its interaction with ATG7, phosphorylated Forkhead box O (FoxO) 1 induces autophagy in iNKs (104). NK cell maturation may be affected by autophagy when ATG7 and FoxO1 are disrupted in the cytosol of immature NK cells (105). CCL5 overexpression was associated with significantly improved long-term survival in patients with melanoma. Targeting autophagy in a CCL5-dependent manner improves NK cell infiltration and inhibits melanoma growth (106). Therefore, autophagy can act as an inhibitor of the expression of protumor and antitumor chemokines, thus differentially influencing tumor progression.

Autophagy is involved in the processing and presentation of major histocompatibility complex (MHC) molecular antigens and T cell-mediated immune responses, which contribute to tumorigenesis or antitumor immune responses. Pancreatic ductal adenocarcinoma

(PDAC) cells are targeted for selective degradation by the autophagy cargo receptor neighbor of BRCA1, inhibiting antigen presentation and killing T cells. On the other hand, inhibition of autophagy restored MHC I surface levels, improved antigen presentation, enhanced antitumor T cell responses, and reduced tumor growth in syngeneic host mice (107). Unlike LAP and LANDO14, canonical autophagy is required for the degradation of MHC I. This suggests that tumor cells can evade immune surveillance through autophagy-mediated degradation of MHC I. A significant component of tumor-induced immunosuppression is MDSCs, which produce DCs, macrophages, and neutrophils. Autophagy deficiency enhances the immunogenic properties of tumor-derived tumor-infiltrating autophagy-deficient monocytic MDSCs through impaired lysosomal degradation of MHC II molecules (108). Consequently, inhibition of autophagy in MDSCs may be beneficial in the treatment of cancer; however, it remains challenging to target specific myeloid subpopulations in TME. The ubiquitination of MHC II in DC affects homeostasis, phenotype, cytokine production, and Ag proteolysis by DC, affecting Ag presentation and T-cell and Ab-mediated immunity (109). By interacting with antigen-processing pathways in DCs, autophagy can effectively modulate adaptive immunity. Through autophagy, organelles and apoptotic proteins are degraded, promoting T-cell development and survival. Furthermore, autophagy in DCs was shown to process tumors intracellularly for the presentation of MHC II to CD4<sup>+</sup> T cells (110). Fusion of viral and tumor antigens into the LC3-II protein of ATG8, which is located in autophagosomal membranes, increases the presentation to CD4<sup>+</sup> T cells (111). A CD4<sup>+</sup> T helper cell activates CD8<sup>+</sup> T cells primed by DCs. An effector CD8<sup>+</sup> T cell lacking autophagy cannot establish long-term memory for effective antiviral immunity (112). Mice lacking the autophagy genes *Atg5*, *Atg14*, or *Atg16L1* suffer from synthetic tumor growth impairment (113). Also, *Atg5*<sup>-/-</sup> CD8<sup>+</sup> T cells show enhanced glucose metabolism which results in altered histone methylation and higher transcription levels (113). In contrast, limiting glucose could inhibit the *Atg5*-dependent enhancement effector, therefore directly enhancing antitumor immunity *via* autophagy (113). In addition, DC activity can be inhibited by autophagy and antigen degradation. Through autophagy induction, the immune response is activated, inhibiting T cell activation after EMT and ROS (114, 115), affecting tumor killing. Inhibition of LAP in myeloid cells induces tumor-associated macrophages (TAMs) to develop a proinflammatory phenotype and increases phagocytosis of dying tumor cells, suggesting that LAP can increase immunity (116).

Furthermore, TME galectin-1 (Gal-1) improves tumor cell adhesion, invasiveness, angiogenesis, and immune evasion and contributes to tumor progression (117, 118). Through TLR2-activated secretory autophagy and MVB/Rab11/VAMP7-mediated vesicle trafficking, Hepatocellular carcinoma (HCC) cells stimulate TAMs to actively secrete Gal-1 (119). Autophagy-secreted Gal-1 promotes the growth of HCC in mice and is associated with a poor prognosis in patients with HCC (120). HCC cells can inhibit macrophage autophagy flux *in vitro* and stimulate the expression of PD-L1 (121). Another report shows that autophagy blockade drives PDAC to up-regulate and utilize the NRF2-induced alternative macrophagocytosis nutrient procurement pathway,

which allows tumor cells to extract nutrients from extracellular sources and use them for energy production (122). As a result, combined autophagy and macropinocytosis inhibition may enhance cancer treatment.

## 8 Upstream pathway of cGAS-STING and autophagy

As part of autophagy induction, the core complex Beclin-1-PI3KC3 generates a PtdIns-3-P-rich membrane that recruits autophagy proteins and forms autophagosomes (123). Rubicon interacts with the Beclin-1-PI3KC3 core complex, negatively regulating autophagy and PI3KC3 lipid kinase activity (124). Rubicon competes with cGAS in conjunction with Beclin1. Binding of the central NTase domain of cGAS to the central CCD of Beclin 1 inhibits cGAMP synthesis and subsequent IFN production, as well as stimulates Rubicon release from the Beclin 1 complex, which induces autophagy by activating PI3KC3, clearing cytoplasmic dsDNA, inhibiting cGAS activation and sustained immune stimulation (125). In conclusion, cGAS and Beclin-1 interact to coordinate the IFN and autophagic pathways and thereby regulate the innate immune response.

cGAS contains five LC3-interacting regions (LIRs) that bind to LC3 and induce noncanonical autophagy (126). In a recent study, ATG7 and ATG14 were found to depend on the involvement of cGAS to contribute constitutively to nucleus clearance, suggesting that this pathway occurs through typical autophagy, in contrast to STING1-mediated autophagy of the non-dependent ULK1 and BECN1 pathways (127). cGAS has also been shown to bind to dsDNA to form liquid-phase condensates (25). Interestingly, liquid-like condensates can recruit autophagy-related molecules like ATG, LC3, as well as P62 to form cytosomes and participate in the mTOR-mediated autophagic pathway to facilitate cargo degradation (128, 129).

In the immune system, cGAS may be a versatile sensor. Triplet motif containing 14 (TRIM14), a mitochondrial articulator that promotes innate immune signaling, is involved in various tumorigenesis processes. Through the PRYSPRY domain and the C terminus of cGAS, TRIM14 and cGAS interact (130). Researchers demonstrated that TRIM14 inhibits autophagic degradation of cGAS by preventing its entry into the autophagosome, which promotes immune responses (130).

## 9 STING proteins and autophagy

In the drosophila model, previous research revealed that inflammation-induced STING-dependent autophagy limits Zika virus infection (131). In further experiments, it was found that STING may evolve to destroy intracellular pathogens, suggesting that cGAS/STING induces autophagy in an ancient and highly conserved way (132). Nuclear warhead protease B has been found to mediate genomic DNA damage and cell membrane DNA release, activating STING-dependent autophagy and leading to ferrotoxic death in human pancreatic cancer cells (133). This implies that

STING-mediated autophagy is potentially promising for the treatment of cancer.

When STING binds to cGAMP, it changes conformation. As the oligomerized STING migrates from the ER to the Golgi apparatus, it passes through the ER-Golgi intermediate compartment (ERGIC). In ERGIC, STING plays an essential role in the induction of autophagy. The STING translocation requires both the COP-II complex and ARF GTPases. The STING-containing ERGIC is capable of lipidating LC3 membranes and thereby triggering the formation of autophagosomes (134). In STING-induced autophagy, the transport of STING from ERGIC to Golgi is unknown. After sensing c-di-AMP, STING disrupts ER homeostasis, leading to the stress of the ER, mTOR inactivation, and ER phagocytosis to coordinate autophagy, thus rescuing dead cells. A recent study has demonstrated that activated STING can undergo intercellular transfer and stimulate RAB22A-mediated non-canonical autophagy derived from the ER, thereby propagating antitumor immunity (135).

Additionally, STING activated the unfolded protein response (UPR) (136). ER stress is induced by unfolded or misfolded proteins, which trigger the UPR to relieve it and restore ER homeostasis. The UPR signaling network activates transcription factor 6, PKR-like ER kinase (PERK), and Inositol-Requiring Protein-1 (137). UPR activation may affect autophagy (138). A lack of PERK has been implicated in converting MDSCs into antitumor CD8<sup>+</sup> T cells and myeloid immune cells, leading to STING-dependent production of type I IFN and antitumor immunity (115).

By separating ULK1 from AMP-activated proteins, cGAMP generated by cGAS promotes autophagy independent of STING. Upon activation of ULK1, STING is phosphorylated at serine 366, which is then degraded by autophagy and inhibits IRF3 activity (139). In this regard, it is essential to note that, although cGAMP stimulates STING function, it is followed by negative feedback that inhibits the expression of pro-inflammatory molecules, emphasizing the complexity of STING trafficking.

Autophagy proteins have alternative functions, such as LAP, which is involved in phagosome maturation and subsequent signaling mechanisms. Through its direct interaction with LC3, STING mediates autophagy through its classical LIRs. However, STING does not require TBK1 or IRF3 for autophagy to be induced (140). Similarly, autophagy proteins of myeloid cells in the TME are involved in the immunosuppression of T lymphocytes by affecting LAP-induced oncogene expression and triggering the STING-mediated TAM type I IFN response (116).

There is a potential connection between DNA sensing and autophagy: cytosolic DNA inhibits STING-dependent delivery of microbes to autophagosomes that destroy intracellular pathogens (141). The ATG5-dependent autophagy machinery in the ER, which is a key membrane source for autophagosome formation, may regulate innate immune signaling through STING (140). Cytosolic DNA accumulates in cells depleted of ATG5 and ATG7, induced by the expression of STING, STAT1, and ISG15. Activation of the STAT1-ISG15 axis leads to cell migration, invasion, and proliferation, suggesting that inhibition of autophagy can promote tumor-associated phenotypes by

activating STING (142). Atg9a is the only multitransmembrane protein identified as an ATG protein in mammals (143) that delivers membranes to the trans-Golgi network (TGN) to form autophagosomes between the plasma membrane and the TGN (144, 145). After dsDNA stimulation, STING colocalizes with the autophagy-associated protein Atg9a and the microtubule-associated protein LC3. When Atg9a is disrupted, the assembly of STING and TBK1 dsDNA is promoted, leading to aberrant activation of innate immunity (146, 147). Interestingly, STING can activate autophagy without Beclin1, Ulk1, or Atg9a (140). A lack of Atg9a led to enhanced STING signaling, suggesting that Atg9a is independent of autophagy in the regulation of STING signaling [118]. Furthermore, activated STING has been reported to recruit ATG16L1 to lipidated LC3 for single membrane perinuclear vesicles through its structural domain WD40, a process that bypasses the requirement for canonical upstream autophagy (148). STING-induced ERGIC or Golgi membrane damage induces the V-ATPase (vacuolar-type H<sup>+</sup>-ATPase) to lipidate LC3 on the Golgi membrane and participates in non-canonical autophagy (85, 149). These findings suggest that STING can interact with LC3 and participate in noncanonical autophagy.

## 10 Downstream of the cGAS-STING and autophagy

Activating the cGAS-STING pathway can regulate intrinsic cellular programs, such as inducing autophagy in tumor cells (150). Increasing evidence suggests that cargo receptors provide substrates for selective autophagy (151, 152). As a chaperone-like protein, ubiquitously expressed prefoldin like chaperone was vital for suppressing excessive activation of STING1-mediated type I IFN signaling through autophagic degradation of STING1 through sequestosome 1 (153). The Unc-93 homolog B1 attenuates the cGAS-STING signaling pathway by targeting STING for degradation in autophagy lysosomes (154). This provides new insight into the function of STING in innate antiviral immunity, which functions as a checker to prevent hyperactivation.

P62 has been implicated in tumor development as an autophagy selective substrate (155, 156). In cancer cells, increased expression of p62 is associated with defective autophagy, which promotes tumor growth (157). Autophagy can be induced even in the absence of p62 in the presence of ectopic expression of STING (140), indicating that p62 is not necessary for STING-dependent autophagy. The ubiquitination of STING promotes both activation and negative regulation of STING during autophagosome degradation (158). Microtubule-associated protein one LC3 promotes the recruitment of ubiquitinated carriers to the autophagosome membrane through its ubiquitin-associated structural domain. The interaction of LC3-p62 interaction and autophagic degradation is regulated by the structural domain of LIRs (78). By connecting to K63, STING is ubiquitinated and recruited into p62 positive compartments. This results in TBK1 phosphorylating p62 in a manner that depends on IRF3 but not on transcription, thus increasing the affinity of ubiquitin for it. Therefore, p62-deficient cells do not degrade STING, resulting in

elevated levels of type I IFN and ISG (159). STING and p62 interact in autophagy and immune regulation, which requires further research.

The mediator of IRF3 activation, a regulator of innate immunity, regulates autophagy flux to promote cell death in breast cancer cells (160). Autophagy may also regulate the stability of IRF3. PSMD14/POH1 deubiquitinase prevents IRF3 autophagy by cleaving its K27-linked polyubiquitin chain in lysine 313 to promote IRF3-mediated type I IFN activation (161). STING also triggers non-canonical autophagy in response to dsDNA, which is crucial for the activation of both IRF3/7 and NF- $\kappa$ B (139). Consequently, selective autophagy-mediated degradation of IRF3 causes immunosuppression by preventing excessive IFN signaling. Nevertheless, IRF3 does not appear to understand the molecular mechanisms that lead to STING degradation. The future of precise immunosuppression may involve activation of the IRF3 pathway, although autophagy may be an important contributor to IRF3-dependent type I IFN signaling (Figure 4).

## 11 Discussion and outlook

The cGAS-STING pathway has been identified as a significant immune pathway to recognize cytosolic DNA. It has now made great progress in multiple immune pathways. To support antitumor effects, the host can activate the cGAS-STING pathway, but excessive activation can also contribute to tumor progression. STING activity can be precisely modulated to affect the immune response, including terminating STING-mediated excessive immune activation, which could lead to further investigation. Autophagy exhibits similar dichotomous effects on tumor

development. With the advancement of research, autophagy is becoming a more prominent part of tumor immunity. Most of them are focused on the field of canonical autophagy, and non-canonical autophagy remains an area that needs to continue to be explored in depth, which appears to be more comprehensive for better control of mechanistic studies of autophagy in cancer immunity (162).

This review explores the interactions between the upstream and downstream regulators of cGAS-STING and autophagy-related proteins and their relevant effects on cancer immunity. Future research could focus on finding herbal medicine and ingredients that can promote immune cells with antitumor effects. Herbal medicine can be used in combination with chemotherapy or targeted drugs, or immunotherapy represented by PD-1 and PD-L1 inhibitors to have a selective synergistic effect, improving the killing effect of cancer cells, while reducing the side effects of these therapies on healthy ones. In clinical practice, this expectation is consistent with what we have observed. The combination of herbal medicine and various therapies can enhance tumor inhibition more effectively than single drugs (163). Meanwhile, we found that herbal medicine can enhance the cytotoxic effect of chemotherapy on NSCLC by inhibiting cisplatin-induced protective autophagy (164). This way, the application of synergistic treatment of tumors with herbal medicine combined with chemotherapy or targeted drugs, or immunotherapy will be appropriate. This fundamental study can better facilitate the design and development of future antitumor-targeting drugs. Based on the function of cGAS-STING, we will take this pathway as the main means to test the anticancer effect of herbal medicine.

Many interesting questions remain for future investigation and interpretation, although cGAS-STING can trigger both canonical and non-canonical autophagy through multiple pathways. First, in different types of cancer, cGAS-STING inhibits the cell growth cycle

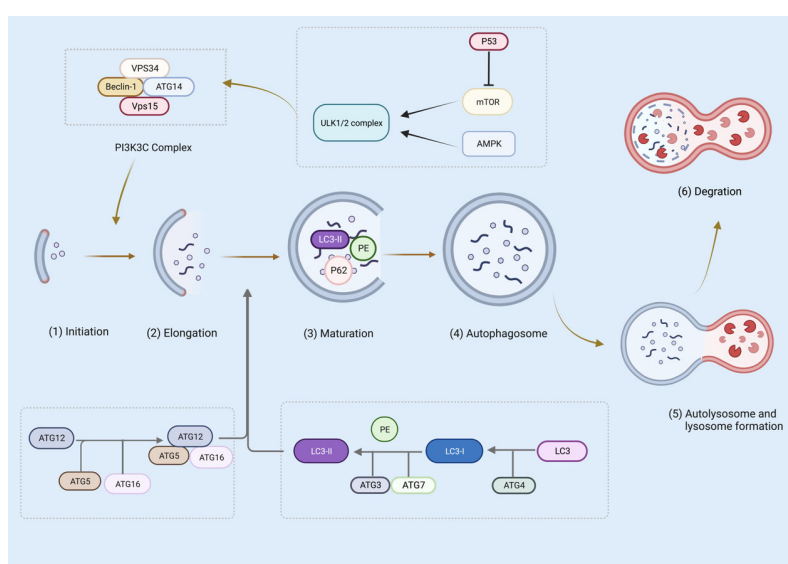


FIGURE 3

Several steps are involved in canonical autophagy: (1) initiation; (2) nucleation or phagosome extension; (3) maturation; (4) autophagosome formation; (5) autophagosome and lysosome formation; (6) degradation. The figure was created with BioRender (<https://biorender.com/>).



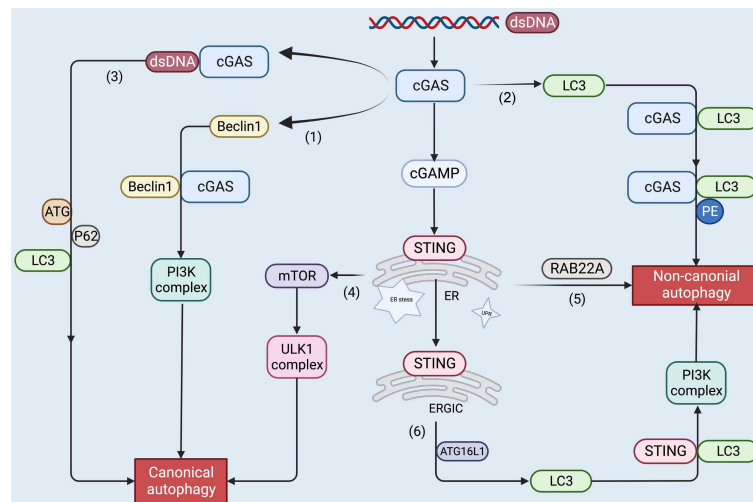


FIGURE 4

The upstream and downstream of the cGAS-STING pathway, including STING proteins, trigger autophagy by the following roughly divided mechanisms: cGAS binds to dsDNA to form liquid-phase condensates. (1) cGAS interacts with Beclin1 and triggers canonical autophagy; (2) cGAS binds to LC3 to induce non-canonical autophagy; (3) cGAS binds to dsDNA and recruits ATG, LC3, and P62 to participate in canonical autophagy; (4) STING leads to ER stress, mTOR inactivation, and coordinates autophagy; (5) STING stimulates RAB22A-mediated non-canonical autophagy derived from the ER; (6) STING recruits ATG16L1 to lipidated LC3, induces non-canonical autophagy. The figure was created with BioRender (<https://biorender.com/>).

through cellular senescence, necrosis, and apoptosis (165–168). What determines cell fate after cGAS-STING mediation? Does the presence of cGAS without transmembrane domains and its localization have an impact on this, including the onset of autophagy? Furthermore, the degree of STING activity and the intrinsic changes in the cancer cells themselves are also taken into account. Second, we need to find other pathways to connect cGAS-STING to autophagy more directly. At present, there is only a preliminary linkage between the two, but there is no more comprehensive systematic evidence to combine them and coordinate a series of downstream pathways to improve tumor immune efficiency in response to various foreign stimuli. Third, it is worthwhile to think about how to more fully elucidate the specific structures and modes of interaction between STING and some of the factors associated with autophagy along with drug trials and applications concerning each of them. Overall, combining cGAS-STING with autophagy can help to deepen the understanding of the intersection of innate and acquired immunity, which provides a new avenue for studying antitumor immunity.

## Author contributions

QL wrote the first version of the manuscript, and YC finalized the manuscript. JL downloaded the references and processed the figures in the manuscript. FZ collected the data. ZZ (corresponding author) conceived and coordinated the study and critically evaluated the data. All authors contributed to the article and approved the submitted version.

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## Conflict 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.

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## Glossary

PD-1/PD-L1	programmed cell death protein 1/programmed cell death ligand 1
CAR-T cell	chimeric antigen receptor T-cell
DCs	dendritic cells
ILCs	innate lymphocytes
NK	natural killer
PRRs	pattern recognition receptors
PAMPs	pathogen-associated molecular patterns
DAMPs	danger-associated molecular patterns
cGAS	cyclic guanosine monophosphate-adenosine monophosphate synthase
dsDNA	double-stranded DNA
STING	stimulator interferon gene
ER	endoplasmic reticulum
cGAMP	cyclic guanosine monophosphate-adenosine monophosphate
IFN	interferon
APCs	antigen-presenting cells
STAT1	signal transducer and activator of transcription 1
EMT	epithelial-mesenchymal transition
TME	tumor microenvironment
ISG	invariant surface glycoprotein
cGAMP-NP	cGAMP nanoparticles
MDSCs	myeloid derived suppressor cells
IDO	indolamine 2,3-dioxygenase
LKB1	liver kinase B1
NSCLC	non-small cell lung cancer
CMA	chaperone-mediated autophagy
ATG	autophagy-related gene
PI3K	phosphoinositide 3-kinase
PE	phosphatidylethanolamine
PI3KC3	class III PI3K
ROS	reactive oxygen species
FoxO	Forkhead box O
UBD	ubiquitin binding domain
MHC	major histocompatibility complex
PDAC	pancreatic ductal adenocarcinoma
LAP	LC3-associated phagocytosis
TAMs	tumor-associated macrophages
Gal-1	galectin-1

(Continued)

## Continued

HCC	Hepatocellular carcinoma
LIRs	LC3-interacting regions
TRIM14	triplet motif containing 14
ERGIC	ER–Golgi intermediate compartment
UPR	unfolded protein response
PERK	PKR-like ER kinase
TGN	trans-Golgi network



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\*CORRESPONDENCE  
Zhan Zheng  
✉ zhengzhan2696@126.com

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# Corrigendum: Crosstalk between cGAS-STING pathway and autophagy in cancer immunity

Qijun Lu<sup>1</sup>, Yukun Chen<sup>2</sup>, Jianwen Li<sup>1</sup>, Feng Zhu<sup>3</sup>  
and Zhan Zheng<sup>1\*</sup>

<sup>1</sup>Department of Oncology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China, <sup>2</sup>Cancer Institute, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China, <sup>3</sup>Department of Laboratory Medicine, Huadong Hospital, Fudan University, Shanghai, China

## KEYWORDS

antitumor, autophagy, cancer, cGAS-STING, immunity

## A Corrigendum on

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In the published article, there was an error in **Figure 1** as published. The word ‘immunity’ is not capitalized, keeping the format consistent. The corrected **Figure 1** and its caption “**Figure 1**. appear below.

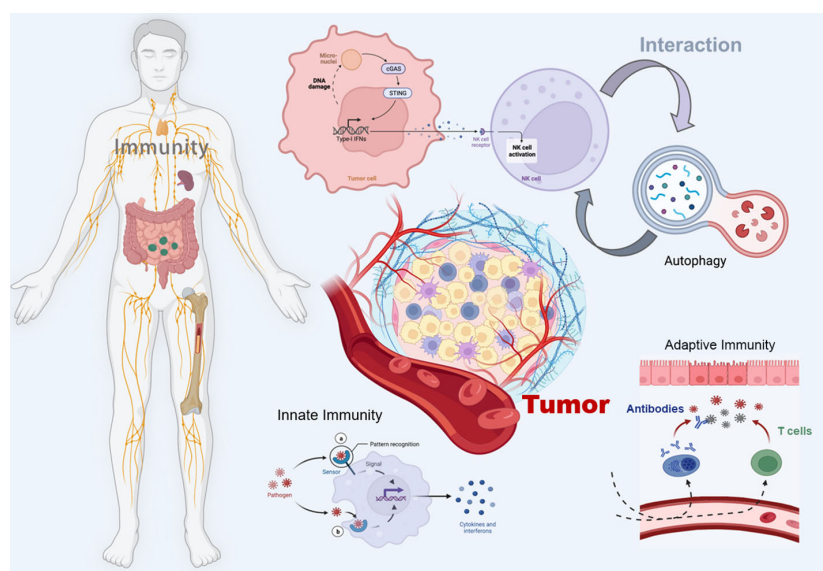


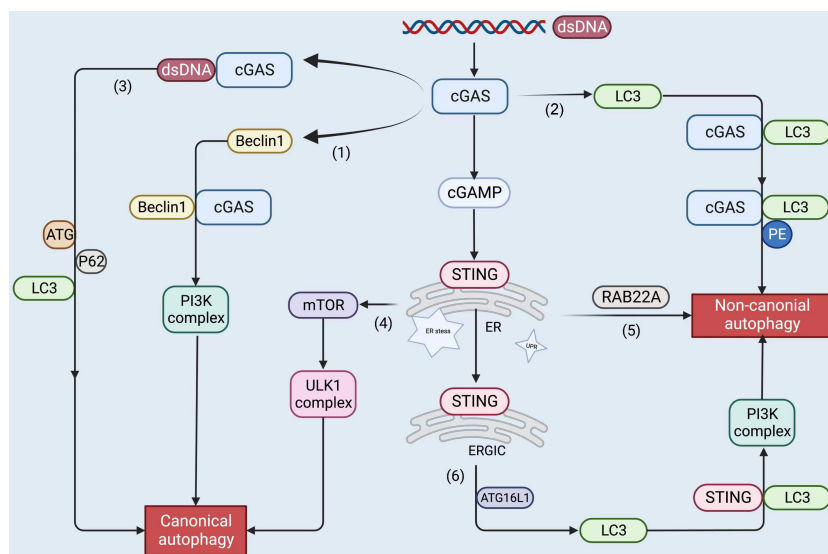
FIGURE 1

Schematic illustration of the crosstalk between the cGAS-STING pathway and autophagy in cancer immunity. The figure was created with BioRender.



In the published article, there was an error in **Figure 4** as published. The icon (2) in **Figure 4** is missing. The corrected **Figure 4** and its caption “**Figure 4** appear below.

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.



**FIGURE 4**

The upstream and downstream of the cGAS-STING pathway, including STING proteins, trigger autophagy by the following roughly divided mechanisms: cGAS binds to dsDNA to form liquid-phase condensates. (1) cGAS interacts with Beclin1 and triggers canonical autophagy; (2) cGAS binds to LC3 to induce non-canonical autophagy; (3) cGAS binds to dsDNA and recruits ATG, LC3, and P62 to participate in canonical autophagy; (4) STING leads to ER stress, mTOR inactivation, and coordinates autophagy; (5) STING stimulates RAB22A-mediated non-canonical autophagy derived from the ER; (6) STING recruits ATG16L1 to lipidated LC3, induces non-canonical autophagy. The figure was created with **BioRender**.

In the published article, there was an error in the author list, and author Yukun Chen was erroneously denoted as co-first author. The corrected author list appears below.

Qijun Lu<sup>1</sup>, Yukun Chen<sup>2</sup>, Jianwen Li<sup>1</sup>, Feng Zhu<sup>3</sup> and Zhan Zheng<sup>1\*</sup>

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## EDITED BY

Mary Poupot-Marsan,  
INSERM U1037 Centre de Recherche en  
Cancérologie de Toulouse, France

## REVIEWED BY

Eva Sahakian,  
Moffitt Cancer Center, United States  
Michela Perego,  
Wistar Institute, United States

## \*CORRESPONDENCE

Junfeng Du

✉ dujf66@126.com

Xiaofei Shen

✉ dg1535058@smail.nju.edu.cn

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# Targeting myeloid-derived suppressor cells in tumor immunotherapy: Current, future and beyond

Yang Zhao<sup>1,2</sup>, Junfeng Du<sup>3\*</sup> and Xiaofei Shen<sup>4\*</sup>

<sup>1</sup>State Key Laboratory of Membrane Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, <sup>2</sup>Beijing Institute for Stem Cell and Regenerative Medicine, Beijing, China, <sup>3</sup>Department of General Surgery, The 7th Medical Center, Chinese People's Liberation Army General Hospital, Beijing, China, <sup>4</sup>Department of General Surgery, Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, China

Myeloid-derived suppressor cells (MDSCs) are one of the major negative regulators in tumor microenvironment (TME) due to their potent immunosuppressive capacity. MDSCs are the products of myeloid progenitor abnormal differentiation in bone marrow, which inhibits the immune response mediated by T cells, natural killer cells and dendritic cells; promotes the generation of regulatory T cells and tumor-associated macrophages; drives the immune escape; and finally leads to tumor progression and metastasis. In this review, we highlight key features of MDSCs biology in TME that are being explored as potential targets for tumor immunotherapy. We discuss the therapies and approaches that aim to reprogram TME from immunosuppressive to immunostimulatory circumstance, which prevents MDSC immunosuppression activity; promotes MDSC differentiation; and impacts MDSC recruitment and abundance in tumor site. We also summarize current advances in the identification of rational combinatorial strategies to improve clinical efficacy and outcomes of cancer patients, via deeply understanding and pursuing the mechanisms and characterization of MDSCs generation and suppression in TME.

## KEYWORDS

myeloid-derived suppressor cells, tumor immunotherapy, tumor immune microenvironment, combinatorial strategies, cell therapy

## 1 Introduction

Accumulating evidence shows that the formation of the tumor immune microenvironment (TIME) is closely related to tumor malignant development and metastasis. The occurrence and progression of tumors is a complex pathophysiological process. Most researchers believe that the TIME includes: 1) secretion of immunosuppressive factors, including interleukins, chemokines, growth factors, and other cytokines, inducing inflammatory responses and forming a local milieu conducive

to tumor propagation (1, 2); and 2) many immune cell components, such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), regulatory T (Treg) cells, tumor-associated dendritic cells, mast cells, and type 2 natural killer T (NKT) cells, are involved in tumor immunosuppression (3–6). Due to their contribution to tumorigenesis and progression, MDSCs are recognized as the critical factor in TIME and their function exacerbates the disease. MDSCs may be the basis of tumor immunosuppression. First, although in tumor patients, one or several of the above-mentioned immunosuppressive cells are detected, MDSCs can be detected in most patients. Second, MDSCs suppress the innate immune response and delay the adaptive immune response. Third, MDSCs can induce expansion of immunosuppressive cells (TAMs and Treg cells).

MDSCs, have high heterogeneity, were first referred around 30 years ago and have unique characteristics and an important place in many diseases, especially cancer (7). MDSCs are a group of innate immune cells derived from myeloid lineage at different developmental stages with strong heterogeneity, which can differentiate into dendritic cells (DCs), macrophages and granulocytes under physiological conditions. However, under pathological conditions, like inflammation, trauma, tumors and autoimmune diseases, release of immunosuppressive factors block the differentiation of myeloid progenitors, promote their expansion, and further recruit them to the blood, spleen, liver, and tumor tissue (7–9). MDSCs play a pivotal and central role in governing and maintaining the TIME in solid tumors (10). MDSCs are composed of the myeloid cells with similar biological activities, but distinct phenotypes. Unlike monocytes, macrophages and DCs, which express specific molecular markers on the cell surface, MDSCs are composed of a mixture of granulocytes and monocytes, without clear and specific markers on their surface (11). In mice, MDSCs are defined as cells that co-express myeloid antigens Gr-1 and CD11b (CD11b<sup>+</sup>Gr-1<sup>+</sup>). In addition, according to the expression of Ly6C and Ly6G, CD11b<sup>+</sup>Gr-1<sup>+</sup> cells can be further divided into granulocytic MDSCs (CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>low</sup>, G-MDSC) and monocytic MDSCs (CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>high</sup>, M-MDSC) subtypes (9, 12). MDSCs induced by human solid tumors were divided into two subgroups: CD33<sup>+</sup>HLA-DR<sup>low</sup>HIF1 $\alpha$ <sup>+</sup>/STAT3<sup>+</sup> and CD11b<sup>+</sup>HLA-DR<sup>low</sup>C/EBP $\beta$ <sup>+</sup>, according to their phenotypes and molecular mechanisms impeding other immune cells (13). Human monocytic MDSCs (M-MDSCs) were defined as CD11b<sup>+</sup>CD14<sup>+</sup>HLADR<sup>low</sup>CD15<sup>+</sup>, while granulocytic MDSCs (G-MDSCs) were defined as CD11b<sup>+</sup>CD14<sup>+</sup>CD15<sup>+</sup> or CD11b<sup>+</sup>CD14<sup>+</sup>CD66b<sup>+</sup> (14, 15).

In recent years, it has been discovered that MDSCs directly participate in the promotion of tumor progression and metastasis and are closely related to the clinical treatment of malignant tumors. In this review, we describe the functional and regulatory mechanism of MDSCs within TME. Notable clinical success in tumor immunotherapies, such as immune checkpoint blockade (ICB), and adoptive cell therapy (ACT), have reinvigorated our interest in the field of immunotherapy and established it as a mode of tumor therapy along with traditional strategies, like surgery, chemotherapy and radiotherapy (16, 17). Therefore, we will discuss the emerging data associated with the therapeutic strategies that targeting

MDSCs. Further, we also highlight what the aspects of MDSCs requires an in-depth understanding to discriminate and evaluate reasonable and sensitive combinatorial strategies to increase the efficacy of tumor immunotherapy for cancer patients.

## 2 Mechanisms of MDSC-mediated immunosuppression within TME

### 2.1 Signaling pathways related to MDSCs generation and function

In the setting of cancer, MDSCs can be generated by common myeloid progenitor (CMP) in bone marrow, recruited to tumor site and expand massively by tumor-derived factors or inflammatory signals, including inflammatory cytokines, chemokines, growth factors, and other pathological mediators accelerate the expansion and recruitment of immature myeloid cells to tumor site to suppress the host antitumor response (Figure 1). Classical ideas propose that the direct immunosuppressive function of MDSCs depends on secreting inhibitory factors, including production of nitric oxide (NO), elimination of key nutritional factors by depleting L-arginine (*via* arginase1), sequestering L-cysteine, or decreasing local tryptophan levels due to the activity of indole amine 2,3 dioxygenase (IDO) (18–21) (Figure 1). The mechanisms of immunosuppression by G-MDSCs and M-MDSCs are distinct to the tumor site. Tumor-mediated G-MDSC mainly inhibited T cells *via* reactive oxygen species (ROS), whereas M-MDSC inhibited T cell mainly through arginase and inducible NO synthase (iNOS) (22–25). The deprivation of L-Arginine, catabolized by MDSC-secreted Arg-1, restrained T cells proliferation *via* disrupting the expression of CD3 $\xi$  chain (19, 26). Nitric oxide (NO) is synthesized by NOSs, which are ubiquitously expressed in MDSCs, and induce T cell apoptosis by blocking JAK/STAT/nuclear factor kappa-B (NF- $\kappa$ B) signaling pathway (27). Peroxide nitrate (PNT) is produced by MDSCs and inhibits CD8<sup>+</sup> T cells migration through reducing the integration of MHC I molecules with antigenic peptides on tumor cells and nitrate chemokines (28, 29). In response to a variety of growth factors and cytokines, the progenitor of MDSCs drive a complex transcription network, allowing for their expansion and preventing the further differentiation. The abovementioned factors trigger multiple signaling pathways in MDSCs (11, 30), and most of them converge on the activation of signal transducer and activator of transcription Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling, which upregulates immunosuppressive mediators such as iNOS, ROS, and arginase (31). STAT3 activation promoted the accumulation of MDSCs in melanoma (32), and STAT3 inhibition weakened the suppressive function of MDSCs (33). The MDSCs amplification and function are related to the downstream signals of STAT3. The calcium-binding pro-inflammatory protein factors S100A9 and S100A8, upregulated by STAT3 activation, could block the differentiation and maturation of dendritic cells (DC) and promote MDSCs accumulation (34). The exact mechanism of this process remains

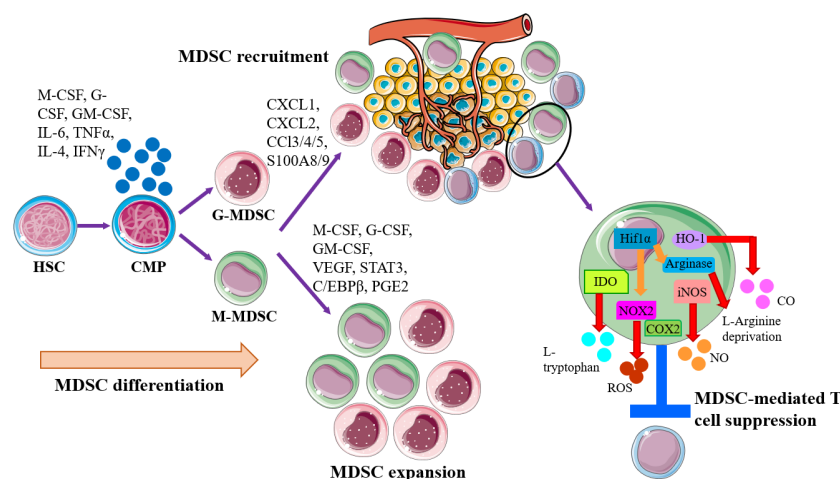


FIGURE 1

Schematic of MDSC generation, expansion, recruitment, and role in the establishment of tumor immune inhibitory microenvironment. MDSCs are generally generated by common myeloid progenitor (CMP) in bone marrow, governed by the abnormal or pathological signals, especially proinflammatory factors. Then MDSCs are recruited to tumor site by tumor-derived factors or inflammatory signals for establishing the microenvironments that promoting tumor cell escape. Within the tumor microenvironment (TME), both monocytic-MDSC (M-MDSC) and granulocytic-MDSC (G-MDSC) will expand and exert immunosuppressive functions to induce T cell suppression and anergy *via* multiple mechanisms, like arginase1, iNOS, IDO, HO-1, and NOX2.

to be explored, but some scholars have pointed out that this may be related to the S100A9 and S100A8 heterodimers participating in the formation of the nicotinamide adenine dinucleotide phosphoric acid (NADPH) oxidase complex, which increasing the production of ROS in myeloid cells. STAT1 is the main transcription factor under IFN- $\gamma$  or IL-1 $\beta$  stimulation, which is believed to have an important relationship with the activity of iNOS and arginase. Some studies point out that MDSCs lacking STAT1 are unable to suppress T cell function due to decreased secretion of iNOS and lower expression of arginase (35). STAT6 activated by IL-4 and IL-13 enhances the activity of arginase and inducing transforming growth factor  $\beta$  (TGF $\beta$ ) production by MDSCs through IL-4R $\alpha$  (36). Besides, Nuclear factor (NF- $\kappa$ B), prostaglandin E2 (PGE2)/cyclooxygenase 2 (COX2), and Ras were also of the great significance in the molecular mechanism of suppressing T cell activity mediated by specific subgroups of MDSCs (37, 38). MDSCs are affected by both novel anti-cancer immune therapies, as well as the conventional treatments such as radiotherapy. Following radiotherapy, cytoplasmic double stranded DNA stimulates the cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING) pathway, resulting in type I interferon production (39). cGAS/STING signaling becomes a key factor in inhibiting MDSC function after radiotherapy *via* multiple mechanisms. The treatment of cGAMP, the STING agonist, prevented MDSC immunosuppressive function *via* reducing NO in B16 melanoma tumor-bearing mice (40). Furthermore, STING agonist treatment combined with the STAT3 inhibitor and markedly regressed tumor growth in syngeneic mice by increasing CD8 $^{+}$  T cells and Tregs and MDSCs in TME (41). Collectively, cGAS/STING and JAK/STAT pathway are both recognized as the central signaling pathway in controlling MDSC generation, accumulations and function in tumor progression. The rationale combinatorial treatment of STAT inhibitors and STING agonists

will be potential therapeutic strategy and make advances in tumor immunotherapy.

## 2.2 Interplay between MDSCs and other immunosuppressive cells

Another major mechanism mediating immunosuppression is the induction and recruitment of other regulatory cells, like Treg cells (42). The characteristic of the TME enable crosstalk between MDSCs and Tregs that allows them to modulate each other mutually. MDSCs in TIME selectively facilitated expansion and induction of Treg cells *via* a TGF $\beta$ -dependent manner (43), or dependent on MDSC-secreted IL-10 and IFN- $\gamma$  (23). Furthermore, MDSCs can provide additional signals for Treg cell induction and development *via* upregulating ligands expressed on the surface of MDSCs for several costimulatory molecules, such as CD86, programmed death ligand (PD-L1), and leukocyte immunoglobulinlike receptor subfamily B (LILRB4). MDSCs promoted the induction and expansion of tumor-specific Treg cells *via* taking in tumor antigens and presenting them to T cells, also converted T cells in other differentiated states into Treg cells to assist tumor evasion (44, 45). The correlation of arginase-1 expression with increasing expression of immune checkpoint receptor and ligands results in more potent suppressive activity of MDSCs (46). Twofold repression caused by MDSCs and Treg cells will create strong immune tolerance and promote tumor progression and propagation.

Thus, there is a consensus that the TME can induce MDSCs with the more potent suppressive activity *via* increasing the expression of a series of immunosuppressive molecules. Exploring more suitable approaches to blockade the immunosuppressive molecules expressed by MDSCs will be hopeful to disrupt the immunosuppression mediated by MDSCs in TME.

### 3 Current approaches and strategies targeting MDSCs for tumor immunotherapy

The TME plays an important role in supporting and promoting tumor growth and metastasis, where MDSCs have an important role in immuno-suppression. More studies are trying to explore and achieve tumor therapy by changing the TME (soil) to prevent the activity of tumor cells (seeds). Targeting the TIME has become a new approach for tumor therapy in recent years. In view of the important role of MDSCs in the TIME, therapeutic strategies targeting MDSCs are being explored (Table 1): 1) promoting the differentiation and maturation of MDSCs; 2) inhibition of the

expansion and accumulation of MDSCs; 3) elimination of MDSCs in TME; 4) abolition of MDSCs immunosuppression (Figure 2).

#### 3.1 Therapies promoting differentiation of MDSCs into mature cells

MDSCs are a mixture of immature myeloid cell populations with high heterogeneity and immunosuppressive activity. All-trans retinoic acid (ARTA) could promote the differentiation of MDSCs into granulocytes, macrophages and DC, improve the host anti-tumor immune response *via* neutralizing the production of ROS (79–81). For example, the administration of formic acid receptor

TABLE 1 The therapeutic strategies of targeting MDSCs in preclinical cancer trials.

Strategy	Drug	Combinatorial partner	Tumor model	Mechanism	References
Promoting differentiation of MDSCs	ATRA	DC101 (antibody targeting murine VEGFR2)	The syngeneic models of breast cancer, 4T1 and TS/A	Blockade of the antiangiogenic therapy-induced expansion of MDSC secreting high levels of vessel-destabilizing S100A8	(47)
	Ibrutinib (BTK inhibitor)	None	The orthotopic mouse breast cancer model	To promote MDSCs develop into mature DCs	(48)
	Dihydroorotate dehydrogenase inhibitors (DHODH)	PD-1 inhibitor	The metastatic TNBC models, 4T1 and E0771.ML-1	To facilitate MDSCs maturation and differentiation	(49)
	JSI-124 (STAT3 inhibitor)	Sialidase	In mice bearing two transplantable tumors (EL4, CT26) and two transgenic tumors (Ret melanoma and TRAMP prostate carcinoma)	To control the differentiation of MDSC into macrophage <i>via</i> decreasing STAT3 activity	(50)
	VSSP	Anti-TLR4/anti-TLR2 mAb	Mice bearing MCA203 or the tumor-bearing mouse model using the G-CSF-producing 4T1 cell line	To induce MDSC differentiation to DC or macrophage	(51, 52)
Inhibiting MDSCs generation, recruitment and trafficking	Calcitriol (1 $\alpha$ ,25-dihydroxyvitamin D3)	None	The ectopic mouse tumor implantation model, CE81T and TE2	Inhibiting IL-6 signaling	(53)
	Entinostat	5-azacytidine	The NSG mice were transplanted subcutaneously of LLC tissue (Patient) and HNM007 tissue (Patient)	Downregulation of CCR2 and CXCR2, and promoting MDSC differentiation into a macrophage-like phenotype	(54)

(Continued)



TABLE 1 Continued

Strategy	Drug	Combinatorial partner	Tumor model	Mechanism	References
	SX-682 (CXCR1/2 inhibitor)	CAR-NK	The mice bearing murine oral cancer 2 or cells from HNSCC patients <i>in vitro</i> or the MOC1 oral carcinoma and LLC mouse tumor models	To abrogate MDSC accumulation and trafficking, and enhance adoptive transferred NK tumor infiltration	(55, 56)
	Olaparib	EGFRvIII-targeted CAR-T	4T1EGFRvIII tumor-bearing mice	To inhibit MDSC migration <i>via</i> the SDF1 $\alpha$ /CXCR4 axis	(57)
	JBSNF-000088 (NNMT inhibitor)	None	The xenografted tumor models overexpressing NNMT GBC cells	To inhibit MDSCs generation by decreasing IL-6 and GM-CSF expression on an epigenetic modified manner	(58)
	Icariside II	$\alpha$ -PD-1 mAb	LLC tumor-bearing mice	To suppress the chemotactic migration of MDSCs by downregulating the expression of CXCL2 and CXCL3	(59)
	SB225002 (CXCR2 inhibitor)	JNJ-40346527 (CSF1R inhibitor)	LLC, CT26, EL4, or 4T1 tumor-bearing mice	To block G-MDSCs infiltration and decrease TAMs	(60)
	PLX647 (CSF1R inhibitor)	Indoximod/D-1MT (IDO inhibitor)	B16-IDO tumor-bearing mouse model	To block tumor infiltrating MDSCs	(61)
	Maraviroc (CCR5 inhibitor)	anti-PD1 mAb	4T1 and PyMT breast tumor model or from patients with gastric cancer <i>in vitro</i>	To result in strong reductions of MDSCs <i>via</i> targeting autocrine CCL5-CCR5 axis	(62, 63)
	Trametinib (MEK1/2 inhibitor)	$\alpha$ PD-1-supplementation	4NQO-L- and B16-bearing mice	To reduce the abundance of CSF-1R <sup>+</sup> CD11c <sup>+</sup> MDSC populations	(64)
Preventing suppressive activity of MDSCs	Vitamin D	None	CLL cells from patients <i>in vitro</i>	Downregulating MDSC function as negative regulator of miR155	(65)
	Entinostat	anti-PD1 mAb	The murine models of lung and renal cell carcinoma	Inhibition of immunosuppressive function of G- and M-MDSC populations by reducing arginase-1, iNOS and COX-2 levels	(66, 67)
	UNC4241 (pan-TAM inhibitor)	$\alpha$ -PD-1 mAb	Melanoma tumor-bearing mice	To diminish MDSC suppression and differentiation in part through regulation of STAT3 serine phosphorylation and nuclear localization	(68)
	Difluoromethylornithine	None	B16 tumor-bearing mice	Inhibition of ODC by DFMO is to impair MDSCs suppressive activity <i>via</i> reducing arginase expression and inhibiting the CD39/CD73-mediated pathway	(69)
	Ibrutinib (BTK inhibitor)	Anti-PDL1 checkpoint inhibitor	Neuroblastoma tumors-bearing mice	To alter NO production, and decrease expression of IDO, Arginase, TGF $\beta$	(70)
Elimination of MDSCs	Gemtuzumab ozogamicin	CAR-T	NSCLC; PA; B16; CA; PDA cell lines <i>in vitro</i>	To deplete MDSCs for reactivating CAR-T cell responses against multiple cancers	(71)

(Continued)

TABLE 1 Continued

Strategy	Drug	Combinatorial partner	Tumor model	Mechanism	References
	5-Fluorouracil or capecitabine (5-FU pro-drug)	Gemcitabine	EL4-bearing mice or pancreatic cancer patients	To eliminate MDSCs <i>via</i> selectively induce MDSCs apoptosis	(72, 73)
	MD5-1 (anti-DR5 antibody)	Anti-PD-L1 antibody		To deplete MDSCs and induce enrichment of CD8 <sup>+</sup> T cells	(74)
	Cabozantinib	Anti-HER2 mAb	4T1-HER2 murine breast cancer model	To delete MDSCs and improve the efficacy of anti-HER2	(75)
<b>Decreasing immune checkpoint receptors expression on MDSCs</b>	Anti-CD200 mAb	Anti-PD-1 antibody	MT-5 tumor-bearing mice and genetically engineered PDAC mouse model	To limit CD200R <sup>+</sup> MDSCs expansion	(76)
	Anti-gp49B (LILRB4) antibody	Anti-PD-1 antibody	LLC-tumor bearing mice	To decrease M-MDSCs infiltration	(77)
	HMBD-002 (anti-VISTA antibody)		CT26, HCT15, A549, and 4T1 tumor-bearing	To decrease the infiltration of MDSCs and increase T cell activity	(78)

ATRA, all-trans retinoic acid; BTK, bruton's tyrosine kinase; TNBC, triple-negative breast cancer; VSSP, very small size proteoliposomes; RCA, renal cell carcinoma; DCs, dendritic cells; LLC, lewis lung carcinoma; ESCC, esophageal squamous cell carcinoma; MOC2, murine oral cancer 2; CLL, chronic lymphocytic leukemia; PDAC, pancreatic ductal adenocarcinoma; TMA RTK, transmembrane receptor tyrosine kinases; ODC, ornithine decarboxylase; DFMO, difluoromethylornithine; mAb, monoclonal antibody; NSCLC, non-small cell lung carcinoma; PA, prostate adenocarcinoma; BIDC, breast invasive ductal carcinoma; CA, colon adenocarcinoma; PDA, pancreas duct adenocarcinoma; NNMT, nicotinamide N-methyltransferase; GBC, gallbladder carcinoma; CXCL2, CXC chemokine ligands 2; CXCL3, CXC chemokine ligands 3; ICB, immune checkpoint blockade; HNC, head and neck cancer; IDO, indoleamine 2,3-dioxygenase; APC, advanced pancreatic cancer; 5-FU, 5-Fluorouracil; LILRB4, leukocyte immunoglobulin-like receptor subfamily B member 4; VISTA, V-domain Ig suppressor of T cell activation.

(RAR) antagonist (which does not affect retinol X receptor (RXR)) in mice can cause the accumulation of granulocytes in various hematopoietic organs, including bone marrow, suggesting that the RAR pathway blocks the differentiation and maturation of granulocyte precursors. 1,25-dihydroxy vitamin D3 (1,25(OH)

2D3), which is the active metabolite of vitamin D3, has been identified as a potent natural modulator of innate and adaptive immunity. Vitamin D3 combined with various cytokines induced the differentiation of CD34<sup>+</sup> progenitors isolated from patients with head and neck squamous cell carcinoma (HNSCC), resulting in

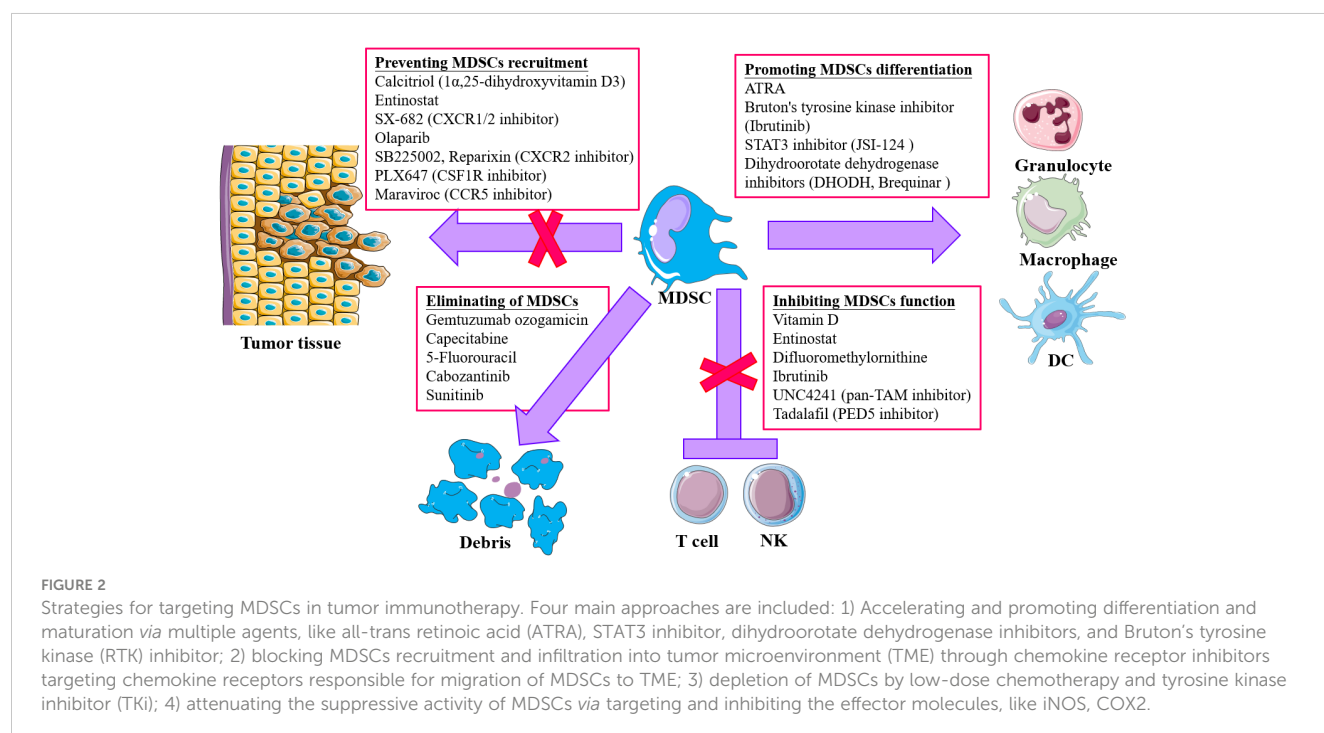


FIGURE 2

Strategies for targeting MDSCs in tumor immunotherapy. Four main approaches are included: 1) Accelerating and promoting differentiation and maturation *via* multiple agents, like all-trans retinoic acid (ATRA), STAT3 inhibitor, dihydropyrimidine dehydrogenase inhibitors, and Bruton's tyrosine kinase (RTK) inhibitor; 2) blocking MDSCs recruitment and infiltration into tumor microenvironment (TME) through chemokine receptor inhibitors targeting chemokine receptors responsible for migration of MDSCs to TME; 3) depletion of MDSCs by low-dose chemotherapy and tyrosine kinase inhibitor (TKi); 4) attenuating the suppressive activity of MDSCs *via* targeting and inhibiting the effector molecules, like iNOS, COX2.

increased numbers of cells phenotypically similar to mature DCs (82). Recent studies have provided important insights that primitive myeloid leukemic cell lines can be driven to differentiate into monocyte-like cells by 1,25(OH)<sub>2</sub>D<sub>3</sub>, which may be useful in differentiation therapy of myeloid leukemia and myelodysplastic syndromes (MDS) (83, 84). However, the role of vitamin D<sub>3</sub> in myeloid cell differentiation remains controversial. A recent study showed that DCs treated with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (calcitriol) did not differentiate or mature, locking the cells in a tolerogenic/immature state (85). Vitamin D<sub>3</sub>-induced tolerogenic DCs are thought to develop their regulatory properties through a semimature profile, inhibition or reduction of T-cell responses, and switching the immune response to a Th2 profile (86–89). Vitamin-D<sub>3</sub>-induced tolerogenic DCs with the semimature phenotype, anti-inflammatory profile, and low capacity to induce T-cell proliferation, can be used clinically for inducing immunotolerance (90). Calcitriol attenuated the recruitment of MDSCs and increased infiltration of cytotoxic T cells following radiotherapy in hepatocellular carcinoma and prostate cancer (82, 91). Thus, the role of vitamin D<sub>3</sub> in tumor therapy is complex and the application of vitamin D<sub>3</sub> for clinical use by targeting MDSCs still needs more study. By promoting the development of MDSCs into normal monocytes and granulocytes, not only reduces MDSCs, but also increases the mature myeloid cells in TIME, thereby inhibiting tumor growth.

### 3.2 Strategies that inhibiting the expansion and recruitment of MDSCs

As mentioned above, the expansion of MDSCs is regulated by tumor-derived suppressive factors secreted by tumor cells and released by the TME. It mainly includes IL-6, GM-CSF, G-CSF, VEGF, COX-2 and other cytokines, which can trigger a variety of different signal transduction and signal activation pathways in MDSC. The STAT3 signaling pathway is an important regulator for MDSC amplification mediated by these factors and could be the ideal target. STAT protein has an N-terminal DNA-binding domain and C-terminal protein-binding domain. Tumor-derived suppressive factors binding to corresponding receptors leads to continuous activation of STAT3, which then upregulates expression of STAT3-related genes and produces proteins (survivin and cyclin) and matrix metalloproteinase-9 (MMP-9) that promote MDSCs expansion. Targeting the STAT3 signaling pathway has become a research hotspot in the inhibition of MDSC expansion (92). MMP-9 is an important target for tumor therapy *via* inhibiting amplification of MDSCs and facilitating formation of the TME. MMP-9 inhibitors promote the normalization of hematopoietic function, thus reducing the production of MDSCs in tumor-bearing BALB-neuT mice expressing an activated rat c-erbB-2/neu transgene model (93). VEGF is currently recognized as the most powerful angiogenic factor, which can specifically act on vascular endothelial cells and promote vascular endothelial hyperplasia. Studies have confirmed that VEGF in tumor tissues can promote the generation of neovascularization, inhibit the development of DCs and induce the generation of MDSCs (94, 95). Therefore,

blockade of VEGF could be another approach for tumor therapy, by removing immunosuppression. In a tumor-bearing mouse model, tumor growth was significantly inhibited after administration of anti-VEGF antibody, and there was a reduced number of MDSCs in tumor tissue and peripheral blood. However, the mechanism by which anti-VEGF monoclonal antibody (mAb) inhibits the expansion of MDSCs remains to be elucidated (96, 97). Bevacizumab was the first FDA-approved monoclonal antibody against VEGF, which brings hope to patients with advanced tumors by anti-tumor microangiogenesis and inhibiting the progression of metastatic lesions. Although, the application of anti-VEGF mAb has been verified and evaluated in a multitude of clinical trials for tumor therapy, because of the multiple effects mediated by blocking VEGF, the efficacy could not be only attributed to MDSC reduction. Sunitinib, as the anti-angiogenic drug, is a receptor tyrosine-kinase inhibitor and immunomodulator, that potently prevents MDSC accumulation and restores normal T-cell function in tumor-bearing mice, independent of its capacity to inhibit tumor progression, as well as reverses MDSC accumulation and T-cell inhibition even in the blood of non-responder renal cell carcinoma (RCC) patients (98).

Chemokines as key mediators of MDSCs recruitment have been extensively studied in many tumor models and cancer patients (Table 1). The recruitment of MDSCs from bone marrow and spleen to tumor tissues is mainly through multiple signaling pathways. A pivotal role of CCL2-CCR2 signaling in MDSC recruitment and tumor progression has been demonstrated in melanoma and hepatocellular carcinoma (HCC) mouse models (99, 100). Thus, blocking CCL2 with soluble CCR2 fragment or inhibition of CCR2 with blocking antibody could decrease tumor accumulation of MDSCs in the tumor bone metastases model by injecting prostate cancer cells directly into murine tibiae, making it a potential target for anti-tumor therapy (101). mCCR5-Ig fusion protein, anti-CCR5 antibody, or even CCL5-neutralizing mAb were all found that could reduce the number and suppressive capacity of tumor infiltration MDSCs, prevent the tumor metastasis, promote the survival of B16 tumor-bearing mouse and even improve the efficacy of anti-PD-1 tumor therapy (102, 103). Chemokine (C-X-C motif) ligand 8, also known as IL-8, highly expressed in various tumors, including colon, ovarian, breast, pancreatic, prostate, and hematological malignancies (104–106), has been demonstrated that could recruit MDSCs to tumor sites *via* CXCR1/CXCR2 (107). The treatment of Reparixin, the pharmacological inhibitor of CXCR1 and CXCR2, caused the significant reduction of G-MDSCs numbers, in colon adenocarcinoma HT29 xenograft tumor and colon carcinoma CT26-GM-derived subcutaneous tumor models (108–110). Furthermore, inhibition of MDSC trafficking by SX-682, a CXCR1/2 inhibitor, enhanced NK-Cell immunotherapy in head and neck cancer models (55). HuMax-IL8, an anti-IL-8 mAb, reduced the number of G-MDSCs in MDA-MB-231 breast cancer xenografts (111). CXCL8 levels determine the efficacy of sunitinib treatment, which was demonstrated to effectively target MDSCs, suggesting that CXCL8 acts as a potential target in anti-tumor therapy (112). Blocking MDSC recruitment to tumor tissues may be an effective approach for disrupting the formation of TIME and improving the efficacy of anti-tumor therapy.

### 3.3 Strategies abolishing MDSC immunosuppression

MDSCs mainly express reactive ROS, arginase1, NOS and peroxynitrite to exert their immunosuppressive function (28, 113). Therefore, appropriate inhibition of those factors, serving as important potential therapeutic targets, can eliminate the immunosuppression of MDSCs. ROS, as part of the major mechanism by which MDSCs suppress T-cell responses, activate anti-oxidative pathways and induce transcriptional programs that regulate the fate and function of MDSCs. Nuclear factor erythroid 2-related factor 2 (Nrf2) activation in regulating the constitutive activation and availability of antioxidant enzymes, including NADPH, NADPH quinone oxidoreductase 1 (NQO1), hem oxygenase (HO), might be a central mechanism enabling cells to increase mitochondrial ATP production by simultaneously counteracting subsequent high ROS levels (114). Selective activation of Nrf2 can decrease the intracellular ROS production, inhibit the immunosuppression of MDSCs, prevent tumor metastasis, and induce tumor regression. Furthermore, the synthetic triterpenoid C-28 methyl ester of 2-cyano-3,12-dioxooleana-1,9,-dien-28-oic acid (CDDO-Me) completely abolished the immunosuppressive activity of MDSCs by reducing ROS production in mouse tumor models (115). Moreover, the treatment of pancreatic cancer patients with CDDO-Me did not affect the number of MDSCs in peripheral blood of patients but significantly improved the immune response (115). Agents that target MDSCs, such as sanguinarine (SNG), are now being considered for treatment of lung cancer. SNG was found to inhibit the immunosuppressive activity of MDSCs *via* decreasing the expression of Arg-1, iNOS, and ROS, as well as inducing the differentiation of MDSCs into macrophages and DCs through the NF- $\kappa$ B pathway *in vitro* from Lewis lung cancer mouse model (116). The type I interferons pathway is well known to promote anti-tumor immunity by diverse mechanisms. Emerging evidence shows that the downregulation of the IFNAR1 chain is found in MDSC from cancer patients and mouse tumor models. The decrease in IFNAR1 depends on the activation of the p38 protein kinase and is required for activation of the immunosuppressive phenotype (117). Stabilizing IFNAR1 using p38 inhibitor combined with IFN induction therapy elicits a robust anti-tumor effect *via* undermining suppressive activity of MDSCs in tumor bearing mice (117). The JAK/STAT signaling pathway is one of the well-known pathways induces immune escape of tumors *via* cytokines and growth factors to control MDSC generation and differentiation (118, 119). Blockade of STAT3, STAT5 or even NF- $\kappa$ B by the selective inhibitors can inhibit the immunosuppression of MDSCs (120). AMP-activated protein kinase  $\alpha$  (AMPK $\alpha$ ) signaling was increased in tumor-MDSCs from tumor-bearing mice and patients with ovarian cancer, which was induced by tumor-derived GM-CSF and occurred in a STAT5-dependent manner (121). In addition, genetic deletion of *ampk $\alpha$ 1*-coding gene, *prkaa1* antagonized M-MDSC differentiation to macrophages and re-routed M-MDSC, but not G-MDSC, into cells that elicited direct antitumor cytotoxic effects through NOS2-mediated actions, suggesting the therapeutic

use of AMPK inhibitors to overcome MDSC-induced T-cell dysfunction and AMPK inhibition as a potential therapeutic strategy to restore protective myelopoiesis in cancer. G-MDSCs in the TME spontaneously die by ferroptosis, inducing the release of oxygenated lipids and limiting the activity of human and mouse T cells, although decreasing the presence of G-MDSCs (122). Thus, genetic and pharmacological inhibition of ferroptosis by liproxtatin-1, abolishes suppressive activity of G-MDSCs, reduces tumor progression and synergizes with immune checkpoint blockade (ICB) to suppress tumor growth in immunocompetent mice (122). However, induction of ferroptosis in immunocompetent mice promotes tumor growth. Therefore, ferroptosis is a unique and targetable immunosuppressive mechanism of G-MDSCs in TME that can be pharmacologically modulated to limit tumor progression. In human hepatocellular carcinoma (HCC), the tumor-surrounding fibrotic livers were markedly enriched with M-MDSCs, along with the poor survival rates. Mechanistically, activated hematopoietic stem cell (HSC) induced monocyte-intrinsic p38 mitogen-activated protein kinase (MAPK) signaling to trigger enhancer reprogramming for M-MDSC development and immunosuppression (123). Treatment with p38 inhibitor inhibited HSC-M-MDSC crosstalk to prevent HCC growth (123). Concomitant with patient-derived M-MDSC suppression by i-BET762, combined treatment with anti-PD-L1 synergistically enhanced tumor-infiltrating lymphocytes, resulting in tumor eradication and prolonging survival in the fibrotic-HCC mouse model (123). It has been reported that mouse and human G-MDSCs exclusively upregulate fatty acid transport protein 2 (FATP2), which was controlled by GM-CSF, through activation of the STAT5 (124). The selective pharmacological inhibition of FATP2 abrogated the suppressive activity of G-MDSCs and substantially delayed tumor progression (124). In combination with immune checkpoint inhibitors (ICIs), FATP2 inhibition blocked tumor progression in mice and has the potential to improve the efficacy of cancer therapy.

### 3.4 Therapies eliminating MDSC within TME

An initial attempt was made to clear the MDSCs with antibodies against Gr-1, but since Gr-1 is not specifically expressed by MDSCs, which is also expressed by mature granulocytes. Therefore, the elimination of MDSCs may also lead to a decline in normal immune cells. In addition, once the plasma concentration of the antibody in plasma decreases or the immune system responds to the antibody, the number of MDSCs increases rapidly, which enhances the immunosuppressive function of MDSCs in the TME. Low dose of chemotherapy, such as 5-fluorouracil (5FU), paclitaxel, cisplatin and gemcitabine, has been shown to effectively eliminate MDSC in tumor-bearing mice, and enhanced anti-tumor immunity (72, 73, 125–127). The number of MDSCs in the spleen was significantly reduced, although DCs, T cells, NK cells, macrophages and B cells were not significantly affected. The mechanism may be that the chemotherapy drugs belong to base analogs, which can prolong and

block the DNA synthesis in the cell cycle and induce cell death. However, the mechanism of selective killing of MDSCs needs to be further clarified. Additionally, subclinical doses of platinum-based drugs, such as cisplatin, prevented the generation and suppressive activity of M-MDSCs by inhibiting STAT3-COX2 signaling pathway, along with decreasing COX2 and arginase1 expression in M-MDSCs of melanoma and head and neck squamous cell carcinoma (HNSCC) patients (128). Therefore, some chemotherapy drugs can play an active role in anti-tumor immunotherapy by targeting MDSCs in a certain dosage and course of treatment.

The remodeling of metabolic states also contributes to the shape of the TIME and plays an important role in regulating MDSCs in the TME. The radiotherapy-augmented Warburg effect helps myeloid cells to acquire an immunosuppressive phenotype, resulting in limited treatment efficacy for pancreatic ductal adenocarcinoma (PDAC) (129). Sustained increase in lactate secretion, resulting from the radiation augmented Warburg effect, was responsible for the enhanced immunosuppressive phenotype of MDSCs after radiotherapy (129). Thus, targeting lactate derived from tumor cells and the hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) signaling in MDSCs could reinstate antitumor T-cell responses and inhibit tumor progression after radiotherapy in pancreatic cancer, indicating distinct promise for clinical therapies to alleviate radio resistance in PDAC. Glutamine metabolism is a crucial element of cancer cell metabolism. Glutamine is important for nucleotide synthesis, amino acid production, redox balance, glycosylation, extracellular matrix production, autophagy, and epigenetics (130, 131). Emerging evidence shows that targeting tumor glutamine metabolism leads to a decrease in G-CSF and hence recruitment and generation of MDSCs as well as immunogenic cell death, leading to an increase in inflammatory TAMs (132). Alternatively, inhibiting glutamine metabolism of the MDSCs themselves not only led to activation-induced cell death and conversion of MDSCs to inflammatory macrophages, also impaired suppressive function of MDSCs *via* inhibiting IDO expression in the tumor and MDSCs, that resulted in a marked decrease in kynurenine levels, and rendered checkpoint blockade-resistant tumors susceptible to immunotherapy in tumor-bearing mice (132). Therefore, the application of glutamine antagonism in synergistic targeting inhibition of tumor and MDSCs may hold promise for clinical therapy to inhibit tumor growth and metastasis. Therapeutic liver-X nuclear receptor (LXR) agonism was also found to reduce MDSC abundance in murine models and in patients treated in a first-in-human dose escalation phase 1 trial, accompanied with the activation of cytotoxic T lymphocyte (CTL) responses in mice and patients (133).

## 4 significance of tumor immunotherapy combined with MDSC-targeted therapies

### 4.1 Combination of MDSC-targeted therapies with adoptive cell therapy

Tumor immunotherapy, such as ICB and adoptive cell therapy, has attracted much attention in recent years due to its remarkable

efficacy. However, preliminary and limited success is achieved in hematological malignancies and in certain solid tumors, owing to the limitations in curative effect, or in technology. Combined treatment strategy is suggested which may improve the efficacy of mono-immunotherapy and compensate for the deficiency of monotherapy. MDSCs mediate tumor metastasis and are implicated in immune evasion through shaping the TME, and are referred as the “queen bee” of the TME (134). Strategies to reverse the suppressive TME should also attract and activate immune effectors with antitumor activity (Table 2). Cytokine-induced killer (CIK) cell-based immunotherapy is effective as adjuvant therapy in HCC with early stage but lacks efficacy in advanced HCC. MDSCs are increased in response to CIK cell therapy and subsequently may be targeted to provide an additional therapeutic benefit. A study on immunosuppressive mechanisms focusing on CIKs found that combination treatment with a PDE5 inhibitor reversed the MDSC suppressor function *via* arginase-1 and iNOS blockade and systemic treatment with a PDE5 inhibitor prevented MDSC accumulation in the TME of the tumor bearing mice (135). Similarly, treatment with a PDE5 inhibitor suppressed CD14<sup>+</sup>HLA-DR<sup>-/low</sup> MDSCs immunosuppressive activity and enhanced CIK activity against human HCC cell lines *in vitro*, suggesting targeting MDSCs is an efficient strategy to enhance the antitumor efficacy of CIKs for the treatment of patients with HCC. The possible combination of olaparib with EGFRvIII-targeting CAR (806-28Z CAR) T cells has been explored (57). The hostile TME is also one of the major obstacles to the efficacy of chimeric antigen receptor modified T (CAR-T) cells, and the recruitment of MDSCs within the TME may contribute to the unsatisfactory performance of CAR-T cells in solid tumors. Olaparib might suppress the recruitment of MDSCs to improve the TIME, which contributes to the infiltration and survival of CAR-T cells on breast cancer in mice (57). The additional mechanistic rationale for combining the third-generation PARPi (olaparib) with CAR-T therapy for the treatment of breast cancer was supported. GPC3-CAR T cell treatment together with C1632, the inhibition of Lin28, which targets IDO1 and PDL1, led to enhanced anti-tumor activity in a HCC xenograft mouse model (136). Combination of targeting IDO1 and PDL1 with CAR-T cells serves as a dual targeting agent against tumor cells and MDSCs in TME and enhances immunotherapeutic potential of CAR-T cells against tumor.

### 4.2 Combination of MDSC-targeted therapies with immune checkpoint therapy

Although ICB therapy has made remarkable achievements in tumor immunotherapy, there is still a large proportion of patients that do not respond to ICIs or develop resistance (137, 138). Furthermore, ICB therapy is disappointing with a response rate < 10% in cancers with a poorly immunogenic or “cold” TIME, requiring further strategies for effective immunotherapy (139, 140). Immunotherapy non-responders often harbor high levels of circulating MDSCs which can predict the response to cancer immunotherapies, which is an important factor in developing resistance to ICB therapy and mediates immunosuppression in TME, hindering the efficacy of such therapy (141). In particular, the



TABLE 2 Summary of clinical trials targeting MDSCs in cancer.

Drug	Target	Combinatorial partner	Cancer	Outcome	ClinicalTrials.gov identifier
ATRA	Retinoic acid receptor	Ipilimumab	Melanoma	None of the patients in the Ipilimumab plus ARTA group had signs of disease progression	NCT02403778
ATRA	Retinoic acid receptor	Pembrolizumab	Advanced melanoma	Ongoing	NCT03200847
HF1K16	ATRA	None	RST	Recruiting and ongoing	NCT05388487
Entinostat	Class I HDAC	Azacitidine	NSCLC	Combined epigenetic therapy decreases relapses after curative surgery	NCT01207726
Entinostat	Class I HDAC	Clofarabine	ND ALL/ABL; R/R ALL/ABL	Entinostat plus clofarabine appears to be tolerable and active in older adults with ND ALL/ABL, but less active in R/R patients	NCT01132573
Entinostat	Class I HDAC	Exemestane	ER <sup>+</sup> breast cancer	8.3-mo improvement in OS among patients who received entinostat	NCT02115282
SX-682	CXCR1/2	Pembrolizumab	Melanoma	Recruiting and ongoing	NCT03161431
Capecitabine	DNA/RNA synthesis	Bevacizumab	GBM	Circulating MDSCs were lower and the increased cytotoxic immune infiltration was observed after low-dose capecitabine treatment	NCT02669173
Tadalafil	PDE5	None	HNSCC	Significantly reducing both MDSCs and Treg and increasing CD8 <sup>+</sup> T cells reactive to autologous tumor antigens	NCT00843635
Gemcitabine	DNA/RNA synthesis	Nivolumab	NSCLC	Decreasing MDSCs to enhance anti-PD1 therapy	NCT03302247
Omaveloxolone (RTA 408)	Nrf2	Ipilimumab or nivolumab	Melanoma	The best overall in omaveloxolone (5 mg) & ipilimumab group is up to 100%	NCT02259231
Dasatinib	Tyrosine kinase	DC vaccines	Metastatic melanoma	Combined treatment was safe and resulted in coordinating immunologic and/or objective clinical responses in 6/13 (46%) evaluable patients	NCT01876212
MTL-CEBPA	C/EBP $\alpha$	Pembrolizumab	AST	Causing inactivation of MDSCs with potent antitumor responses across different tumor models and in cancer patients	NCT04105335
Reparixin	CXCR2	Paclitaxel	TNBC	Weekly combinatorial treatment in MBC appeared to be safe and tolerable, with demonstrated responses in the enrolled population	NCT02370238
RGX-104	LXR	Nivolumab, ipilimumab, docetaxel	EC, NSCLC	Recruiting and ongoing	NCT02922764
Tasquinimod	S100A9	None	mCRPC	Tasquinimod significantly improved rPFS compared with placebo	NCT01234311
Sunitinib	VEGF and c-KIT	None	RCC	The therapy is feasible, safe and an effective method to manage toxicity in metastatic renal cell carcinoma	NCT01499121
Aspirin	COX2	Ipilimumab, Pembrolizumab	Melanoma	To inhibit the function of tumor MDSCs	NCT03396952
Maraviroc	CCR5	None	CRC	Mitigation of tumor-promoting inflammation within the tumor tissue and objective tumor responses in CRC were observed.	NCT01736813

ATRA, all-trans retinoic acid; RST, refractory solid tumors; ND ALL/ABL, newly diagnosed acute lymphoblastic leukemia/acute biphenotypic leukemia; R/R ALL/ABL, relapsed/refractory acute lymphoblastic leukemia/acute biphenotypic leukemia; NSCLC, non-Small Cell Lung Carcinoma; HDAC, histone deacetylase; ER, estrogen receptor; OS, overall survival; GBM, glioblastoma brain tumors; PED5, phosphodiesterase-5; HNSCC, neck squamous cell carcinoma; Nrf2, erythroid 2-related factor 2; DC, dendritic cell; AST, advanced solid tumor; TNBC, triple-negative breast cancer; MBC, metastatic breast cancer; EC, endometrial cancer; mCRPC, metastatic castration-resistant prostate cancer; rPFS, radiographic progression-free survival; RCC, renal cell carcinoma; CRC, colorectal cancer; COX2, cyclooxygenase-2; CXCR2, CXC chemokine receptor 2; CCR5, C-C motif chemokine receptor type 5.

levels of MDSCs indicate whether the patients will respond to ICIs, which the close association between MDSCs level in patients with the efficacy of anti-PD1 or anti-CTLA4 therapy has been observed (142–144). PD-L1 is usually expressed in the majority of cancers, and PD-L1 expression by host myeloid cells is more effective than

that on cancer cells in suppressing CTL function (145–149). MDSCs may also suppress CTL activity by PD-L1-dependent and -independent mechanisms (29). Therefore, combining TAM and G-MDSC inhibitors reduced both populations in the tumor site, and dramatically enhanced the effect of ICB with anti-PD-1 in our

preclinical model of cholangiocarcinoma (CCA) (150). Combining ICI treatment with MDSC depletion has been successful and has been investigated in some pre-clinical studies. Combined treatment using entinostat and 5-azacytidine, epigenetic modulatory drugs, with ICB antibodies (anti-PD1 and anti-CTLA4), led to complete tumor regression and metastatic progression in the aggressive triple-negative breast cancer (TNBC) model 4T1, with >80% survival rate 100 days after tumor implantation (54, 151). IL-6 plays a major role in the accumulation and activation of MDSCs during tumor development. Importantly, increased IL-6 levels positively correlate with disease progression and MDSC enrichment in cancer patients (152). Preclinical studies of IL-6/IL-6R blockade to target MDSCs in cancer have been conducted. IL-6 blockade or anti-IL-6R monoclonal antibody reversed the effect of ICIs in HCC, colorectal cancer, melanoma, triple negative breast cancer, and squamous cell carcinoma, along with marked reduction of MDSCs, decreased suppressive activity of MDSCs, or an increase in tumor infiltrating CD8<sup>+</sup> effector T cells (153–157). Only combination therapy in targeting MDSCs and immune checkpoints was more effective for anti-tumor therapy, while only using the epigenetic modulatory drugs did not mediate the anti-tumor immunity (66). Mechanistic insight into the reversibility of epigenetic modification through small-molecule inhibitors has unlocked the possibility of targeting specific epigenetic pathways to reprogramme the MDSC population into an immunostimulatory phenotype. The histone deacetylases inhibitor, entinostat, was shown to block the formation of the premetastatic niche *via* promoting MDSCs differentiation into pro-inflammatory macrophages and the therapeutic use of entinostat has been observed limited efficacy in some clinical trials (NCT01207726, NCT01132573, NCT02115282) (54). However, entinostat-driven inhibition of MDSC activity combined with ICI resulted in the tumor regression and longer tumor-free survival by improving the infiltration and function of granzymeB<sup>+</sup>CD8<sup>+</sup> T cells in mouse models of HER2 transgenic breast cancer and the Panc02 metastatic pancreatic cancer mouse models (3, 67). The m6A demethylase Alkbh5 has effects on m6A density and splicing events in tumors during ICB therapy and modulates MDSCs accumulation in TME by regulating Mct4/Slc16a3 expression and lactate content of the TME in the employed melanoma and colon syngeneic mouse models (158). Thus, a small-molecule Alkbh5 inhibitor enhanced the efficacy of ICB cancer immunotherapy. ATRA can have positive effects on anti-tumor therapy by reprogramming MDSCs state within the TME. Some clinical trials have also demonstrated the potential significance of ATRA alone or in combination with ICB or target-orientated anticancer drug in anti-tumor therapy (NCT02403778, NCT03200847). It has been reported that addition of ATRA, which reduces expression of immunosuppressive genes including *PD-L1*, *IL-10*, and *IDO* by MDSCs, to standard of care ipilimumab appeared safe (159). Finally, ATRA significantly decreased the frequency of circulating MDSCs compared to ipilimumab alone in advanced-stage melanoma (159). Additionally, ATRA has been demonstrated to increase the efficacy of anti-VEGFR2 antibodies alone and in combination with chemotherapy in preclinical breast cancer models, reverse the anti-VEGFR2-induced accumulation of

intratumoral MDSCs, alleviate hypoxia, and counteract the disorganization of tumor microvessels (47). Although, the clinical efficacy of ATRA has been evaluated, the more effective combination treatment needs to be further explored. We highlight the current clinical trials ongoing and testing the combination of targeting-MDSC with ICB, chemotherapy in Table 2.

### 4.3 Engineering CAR-T cells to deliver the targeting agents against MDSC

Genomic and epigenomic editing provides more opportunities for immunotherapies to create and alter properties. Furthermore, gene editing for immune cell therapies saves the cost and labor participating in the manufacture of the cell products. Gene-modified T cell therapy has been developed as a way to deliver T cells targeting different targets of tumor. However, the immunosuppressive tumor microenvironment is still one of multiple barriers existing in solid tumors that continue to hinder the efficacy of CAR-T cells. Thus, gene modification may enable CAR-T cells acting as a dual targeting agent against tumor cells and MDSCs. One study has developed a modified CAR T cells with IL15, targeting the receptor IL15 receptor alpha (IL15R $\alpha$ ) expressed on MDSC in human and murine glioblastomas (GBMs) (160). The fusion of IL15 to the antibody part of CAR T cells generates a dual targeting system that diminishes the frequency MDSC and tumor cells and improved the survival of mice in two GBM models (160). Another study engineered CAR-T cells to deliver RN7SL1, an endogenous RNA that activates RIG-I/MDA5 signaling (161). RN7SL1 promotes expansion and effector-memory differentiation of CAR-T cells, and transferred RN7SL1 restricts MDSC development, decreases TGF $\beta$  in myeloid cells, and fosters DC subsets with costimulatory features, which enables CAR-T cells to enhance autonomous and endogenous immune function (161). To reverse the suppressive tumor microenvironment, some study developed gene modified T-cells bearing a chimeric receptor in which activating receptor NKG2D fused to intracellular domains of 4-1BB and CD3 $\zeta$  (NKG2D CAR) (162). The NKG2D CAR-T cells target MDSCs, which overexpress Rae1 (NKG2D ligands) within the TME. NKG2D CAR-T cells eliminated MDSCs and improved antitumor activity of subsequently infused CAR-T cells in a novel orthotopic implantation of syngeneic pancreatic ductal adenocarcinoma (PDAC) tissue slices mice model (162). Similar results were observed in the study that used gene-modified NK cells bearing a chimeric receptor in which the activating receptor NKG2D was fused to the cytotoxic  $\zeta$ -chain of the T-cell receptor (NKG2D. $\zeta$ ), and targeted MDSCs that overexpressed NKG2D ligands within the TME (163, 164). Confirmed in the clinical trial, NKG2D. $\zeta$ -NK cells generated from patients with neuroblastoma killed autologous intra-tumoral MDSCs capable of suppressing CAR-T function (NCT03373097) (164). Combination therapy with NKG2D. $\zeta$ -NK cells and CAR-T cells for solid tumors may provide superior efficacy compared to CAR-T cells therapy alone. PD-L1 axis is a key immunosuppressive signal provided by tumor cells and MDSCs in TME, which limits CAR-T cell function. Some studies designed the CAR-T cells secreting anti-PD-L1 single-chain variable fragment (scFv) or generated a novel PD-L1-targeting

chimeric switch receptor (PD-L1.BB CSR) (165–167). The former CAR-T cells secreting anti-PD-L1 scFv which could bind to PD-L1 on PD-L1<sup>high</sup> tumor cells and MDSCs competitively and block their binding with anti-PD-L1 monoclonal antibodies, leading to increased efficacy (165). The latter CAR-T cells can bind to PD-L1, switching the inhibitory signal into an additional 4-1BB signal, displayed superior fitness and enhanced functions in culture medium, causing rapid and durable eradication of pleural and peritoneal metastatic tumors in xenograft models (166). Furthermore, a phase I clinical trial related to this study, was initiated in patients with pleural or peritoneal metastasis (NCT04684459). Thus, those studies open the opportunity for investigating other targeting moieties on the surface of MDSCs, specifically those enriched in cells of TME, and applying these modifications to CAR T cells for their direct dual functions against glioma cells and immunosuppressive MDSC.

#### 4.4 Combination of MDSC-targeted therapies with tumor vaccine

Recent report showed that a vaccine based on heat-killed pathogens induced spleen M-MDSCs that can be activated to kill dendritic cells (DCs), an additional mechanism that may help to explain the difficulties found to develop a very successful anti-pathogen vaccine (168). Tumor vaccines harness the tumor as the source of antigens and implement sequential immunomodulation to generate systemic and lasting antitumor immunity. Mechanism accounting for these is based on isolated patient-derived DCs, through pulsing them with tumor-associated antigens (TAAs) or neoantigens and maturation signals, followed by their reinfusion, or directly inject the antigens subcutaneously by activating DCs *in vivo* in patients (169). A major challenge facing the future of tumor vaccines for cancer treatments is to persist the cytotoxic T cell responses and overcome inhibitory signals from MDSCs in TME, understand the mechanisms of resistance to vaccines and to develop combination therapies that enhance antitumor immunity and durable responses. In a syngeneic B16F0 melanoma model and using tyrosinase related protein 1 (TRP1) as a vaccine antigen, it has been found that simultaneous delivery of IL-12 and a PD-L1-silencing shRNA was the only combination that exhibited therapeutically relevant anti-melanoma activities (170). Interestingly, the lentivector co-expressing IL-12 and the PD-L1 silencing shRNA was the only one that counteracted MDSC suppressive activities, potentially underlying the observed anti-melanoma therapeutic benefit (170). Prospective evaluation of candidate cancer treatment using *ex vivo* differentiated MDSCs highlights therapies with significant therapeutic potential and the therapy of the IL12-encoding combined with PD-L1 silencing lentivector vaccines demonstrated promising anti-melanoma activities. A prophylactic vaccine by employing exosomes derived from murine ESCs engineered to produce GM-CSF (ES-exo/GM-CSF vaccine) successfully protects mice from the outgrowth of an implantable form of murine lung cancer and provides protection against metastasized pulmonary tumors, by decreasing the frequencies of tumor infiltrating immunosuppressive immune cells, including Treg cells and MDSCs (171). Similar to this idea,

treatment with sunitinib, which inhibits G-MDSCs prior to the vaccine composed of peptides of the tumor antigen survivin (SVX vaccine), could magnify the vaccine-mediated immune responses in a colorectal carcinoma mouse model (172). IFN $\alpha$  and 5-Aza-2'-deoxycytidine combined with a DC targeting DNA vaccine (a MIP3a fused vaccine targeting two common melanoma antigens, gp100 and trp2) exhibited greater tumor infiltration of DCs, and NK cells, as well as reduced levels of MDSCs in vaccinated groups in the B16F10 melanoma model (173). The combination therapy alters the tumor immune cell infiltration and elicits protective immune responses, but the underlying mechanisms needs to be explored. The advent of vaccines in multiple solid tumors has prompted the development of new therapeutic combinations that target MDSCs, modulating the TIME and the systemic antitumor response. Moreover, to explore new strategies to optimize the efficacy of standard immunotherapies, it is essential to find approaches that target MDSCs in antitumor immunization.

## 5 Concluding remarks

Tumor immunotherapy has undergone remarkable advances in recent years and has shown great potential for cancer patients. For most cancer patients, a favorable initial response to immunotherapy, is followed by limited responses and cancer relapse and recurrence, due to the multiple mechanisms inducing tumor immunosuppression (174). It also becomes more urgent and possible to reinforce the immune responses against tumors by ICB, adoptive cell transfer (ACT) therapy or tumor vaccines, and abolish TIME. Many studies found nonresponses to those therapies, mostly due to ignorance of the shape of the TIME after treatment. Thus, exploration of combination immunotherapeutic strategies coupled with other immunotherapy with reprogramming the TIME will be the top priority. It should be noted that MDSCs are known to suppress the anti-tumor immune response to induce host tolerance, support cancer stem cells and increase tumor angiogenesis and vascular maturation (175–178), suggesting that MDSC-targeted therapeutic approaches have broad implications in a wide range of cancer therapies in addition to immunotherapy. A growing number of studies have demonstrated that circulating MDSCs in cancer patients are a negative prognostic biomarker in predicting disease course, tumor stage, or metastatic spread (179). Thus, MDSCs have been recognized as a promising therapeutic target and prognostic biomarker for cancer patients, while the diversity, complexity, and heterogeneity of human MDSCs make it difficult to define their phenotypes accurately and uniformly in cancer. Therefore, it is necessary to investigate the phenotypes and characterizations of MDSCs in different types of tumors to establish the precise means to eliminate MDSCs. Although, the phenotypes and suppressive mechanisms seem to be shared among tumor MDSC subsets, it is necessary to identify and distinguish the differences in detail, in order to make more use of accurate individualized treatment regimens. We have reported here that numerous preclinical trails in mouse tumor models, have exhibited favorable efficacy by targeting MDSCs (61, 180).

The field of MDSC research still has more questions than answers. Better characterization of human MDSCs and a clearer understanding of whether MDSC-targeted cues will be of clinical significance are main priorities in this field. We do not yet know the outcomes of the ongoing clinical trials, including inhibition of MDSC immunosuppressive activity, blockade of MDSC recruitment and expansion, and promotion of MDSC differentiation into mature non-suppressive cells. However, reprogramming MDSCs in tumors, combined with newly developing immunotherapy, like ICB or ACT, seems to be a potential new approach to improve antitumor immunity, although adverse events of the treatment strategies should be taken into consideration. Removing the negative or suppressive immune response and improving the positive immune response is a theoretically ideal scheme to mediate anti-tumor immunity. However, the systemic depletion of suppressive cells, like Treg cells, also causes serious immune-related adverse events (181, 182). Hence, how to realize the immunoregulation in or around the tumor sites to augment antitumor immunity will be challenging. Therefore, developing therapeutic strategies targeting MDSC subpopulation is of paramount importance to improve the effectiveness of tumor therapy. We are at an interesting point in the translation of cancer immunotherapies where an improved knowledge of targeting MDSCs will be critical.

## Author contributions

All authors contributed to the article and approved the submitted version. XS and JD was responsible for the overall conceptualization, supervision and writing of the review. YZ was responsible for the draft of the original article, figures and tables.

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## Conflict 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.

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## EDITED BY

Daniela Wesch,  
University of Kiel, Germany

## REVIEWED BY

Xiaofei Shen,  
Nanjing Drum Tower Hospital, China  
Eleonora Ponterio,  
National Institute of Health (ISS), Italy  
Christian Kellner,  
LMU Munich University Hospital, Germany

## \*CORRESPONDENCE

Renaud F. Warin  
✉ rwarin@carolinabiooncology.org

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# The application of autologous cancer immunotherapies in the age of memory-NK cells

Gaby D. Lizana-Vasquez<sup>1,2</sup>, Madeline Torres-Lugo<sup>1</sup>,  
R. Brent Dixon<sup>2,3</sup>, John D. Powderly II<sup>2,3</sup> and Renaud F. Warin<sup>2,3\*</sup>

<sup>1</sup>Department of Chemical Engineering, University of Puerto Rico-Mayagüez, Mayagüez, Puerto Rico,

<sup>2</sup>Cancer Research Clinic, Carolina BioOncology Institute (CBOI), Huntersville, NC, United States,

<sup>3</sup>Human Applications Lab (HAL) - BioCytics, Huntersville, NC, United States

Cellular immunotherapy has revolutionized the oncology field, yielding improved results against hematological and solid malignancies. NK cells have become an attractive alternative due to their capacity to activate upon recognition of “stress” or “danger” signals independently of Major Histocompatibility Complex (MHC) engagement, thus making tumor cells a perfect target for NK cell-mediated cancer immunotherapy even as an allogeneic solution. While this allogeneic use is currently favored, the existence of a characterized memory function for NK cells (“memory-like” NK cells) advocates for an autologous approach, that would benefit from the allogeneic setting discoveries, but with added persistence and specificity. Still, both approaches struggle to exert a sustained and high anticancer effect *in-vivo* due to the immunosuppressive tumor micro-environment and the logistical challenges of cGMP production or clinical deployment. Novel approaches focused on the quality enhancement and the consistent large-scale production of highly activated therapeutic memory-like NK cells have yielded encouraging but still inconclusive results. This review provides an overview of NK biology as it relates to cancer immunotherapy and the challenge presented by solid tumors for therapeutic NKs. After contrasting the autologous and allogeneic NK approaches for solid cancer immunotherapy, this work will present the current scientific focus for the production of highly persistent and cytotoxic memory-like NK cells as well as the current issues with production methods as they apply to stress-sensitive immune cells. In conclusion, autologous NK cells for cancer immunotherapy appears to be a prime alternative for front line therapeutics but to be successful, it will be critical to establish comprehensive infrastructures allowing the production of extremely potent NK cells while constraining costs of production.

## KEYWORDS

autologous immunotherapy, solid tumor, memory-like natural killer cells, cellular stress, upscale production, point-of-care manufacturing

# 1 Introduction

Immunotherapy is a therapeutic approach that harnesses the immune system against cancer. It has revolutionized the oncology field in terms of effectiveness and offers personalized, targeted treatment options that ultimately yield improved results when compared to surgery, chemotherapy, and/or radiation treatments. The key attributes of cancer immunotherapy are detecting, surveilling, and destroying neoplasm cells using immune cells. To date, six cellular therapy products, all autologous CAR-T cells against hematological malignancies, have been approved by the U.S. Food and Drug Administration (FDA) (<https://www.cancer.gov>). These treatments require a tailored product for each individual patient. As a consequence of the successful results obtained with CAR-T cells, the use and engineering potentiation to fight cancer of different immune cell types (1), such as dendritic cells, macrophages, and natural killer (NK) cells, has seen increased interest (2).

NK cells, monocytes, macrophages, and to some degree, dendritic cells constitute the innate lymphoid cell family, which is the first line of defense against invasive pathogens and transformed cells in the human body (3, 4). The discovery and characterization of NK cells dates back to 1975 when Herberman et al. and Kiessling et al. found a natural cytotoxic activity of a subpopulation of lymphoid cells against cancer cells in mouse studies (5, 6). The use of innate lymphocytes NK cells is attractive because of their unique ability to recognize cancer cells and exert antitumor cell cytotoxicity (7). Innate NK cells offer a very attractive approach to cancer therapy. Since NK cells base their initial recognition on particular “stress” or “danger” signals, tumor cells become a perfect target for NK cells.

NK cell cytotoxicity is exerted through a delicate balance mechanism of activating and inhibiting surface receptors, without an antibody or MHC-I strict dependence, as detailed in Figure 1 (1, 8, 11). The lack of expression of CD3 characterizes the human NK cell population, but it expresses the CD56 and CD16 cell surface markers and is classified into two main subsets (3). CD56<sup>bright</sup>CD16<sup>lo/-</sup> NK cells are poorly cytotoxic and mainly produce pro-inflammatory cytokines after cytokine stimulation. CD56<sup>dim</sup>CD16<sup>+</sup> NK cells, which represent about 90% of peripheral blood NK cells, are highly cytotoxic, but expand poorly and do not show a significant *de-novo* cytokine production (3, 12). Unsurprisingly CD16, also known as FcγRIIIA, was characterized early as critical to the proper activation of the ADCC (Antibody-Dependent Cell Cytotoxicity) response (13). These NK cell subsets also possess metabolic differences; for example, under cytokine stimulation, the CD56<sup>bright</sup> subset is more susceptible to upregulating expression of nutrients receptors, amino acid transporters, and transferring receptors than the CD56<sup>dim</sup> subset (12, 14, 15). However, several studies agree that both subsets, CD56<sup>bright</sup> and CD56<sup>dim</sup>, can be cytotoxic or produce cytokines after suitable *in vitro* stimulation (3).

Target cell recognition and NK cell activation occur through different mechanisms (see Figure 1). For example, target cells can be recognized through the overexpression of ligands for the NK cell activating receptors such as natural killer group 2D (NKG2D), costimulatory receptors (DNAX accessory molecule-1 DNAM-1),

and natural cytotoxicity receptors (NKP46). The ligands for these receptors are MHC class I chain-related MIC or UL16-Binding Proteins ULBPs (16), Nectin-2 or Poliovirus Receptor PVR (17), and B7H6 (18), respectively. Another target cell recognition mechanism is through a low-level expression of ligands for NK cell inhibitory receptors (e.g., KIRs family) such as MHC-I or the non-classical HLA-E for NKG2A (8, 19, 20). NK cells will kill cells that express low levels of MHC-I or HLA-E. Therefore, cancer cells that downregulate MHC-I molecules to evade T cell cytotoxicity can be targets for NK cells (21). Once target cells are recognized, NK cells exert their cytotoxicity by secreting cytokines and chemokines. They release perforin and granzyme cytolytic granules upon direct contact with the target malignant cell to cause cell apoptosis. NK cells also generate interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), which exert antitumor effects and upregulate other immune responses (11, 19, 22).

All these features make NK cells particularly useful for autologous and allogeneic cancer immunotherapy, since they were recognized early on for their potential use in the allogeneic setting as an “off-the-shelf” product (23). Still, some challenges are standing before a successful anticancer treatment can be implemented, including short-term persistence, sensitivity, and clinical-grade *ex vivo* expansion (24). Despite the encouraging results of NK cells against hematological malignancies and solid tumors, the tumor microenvironment presents a steep challenge to the success of cancer immunotherapies. In the past decade, it was found that NK cells can exhibit memory-like functions under certain circumstances. This memory function can provide superior host protection compared to naive NK cells, due to their faster recognition and higher cytotoxic responses against tumor cells (25, 26). It could be a powerful property if successfully leveraged against solid tumors.

In this review, we will provide the traditional overview of the use of NK cells in cancer immunotherapy along with their autologous and allogeneic approaches. Allogeneic NK cell therapy dominates the adoptive NK cell-mediated cancer immunotherapy. Despite this fact, ongoing research and some clinical trials with autologous settings suggest its use as an alternative when the allogeneic setting is not applicable or unavailable (27). Then, the characteristics of NK cells memory function and the memory-like NK cell production for NK cell-mediated cancer immunotherapy will be described. Finally, the enhancement of non-genetically modified autologous NK cells will be shown along with their cancer therapeutic advantages when compared to genetically engineered or allogeneic cells.

## 2 NK cells in cancer immunotherapy

Since the identification of the NK cell population in the 1970s (5, 6), this immune cell lineage has been used as an anticancer treatment because of its capability to identify and kill malignant cells without priming, in an MHC-I non-restricted manner (28–30). In contrast to T cells, NK cells do not need prior antigen priming by MHC-I molecules to mediate their killing capability (28, 30), instead relying on a state of equilibrium that can be influenced by



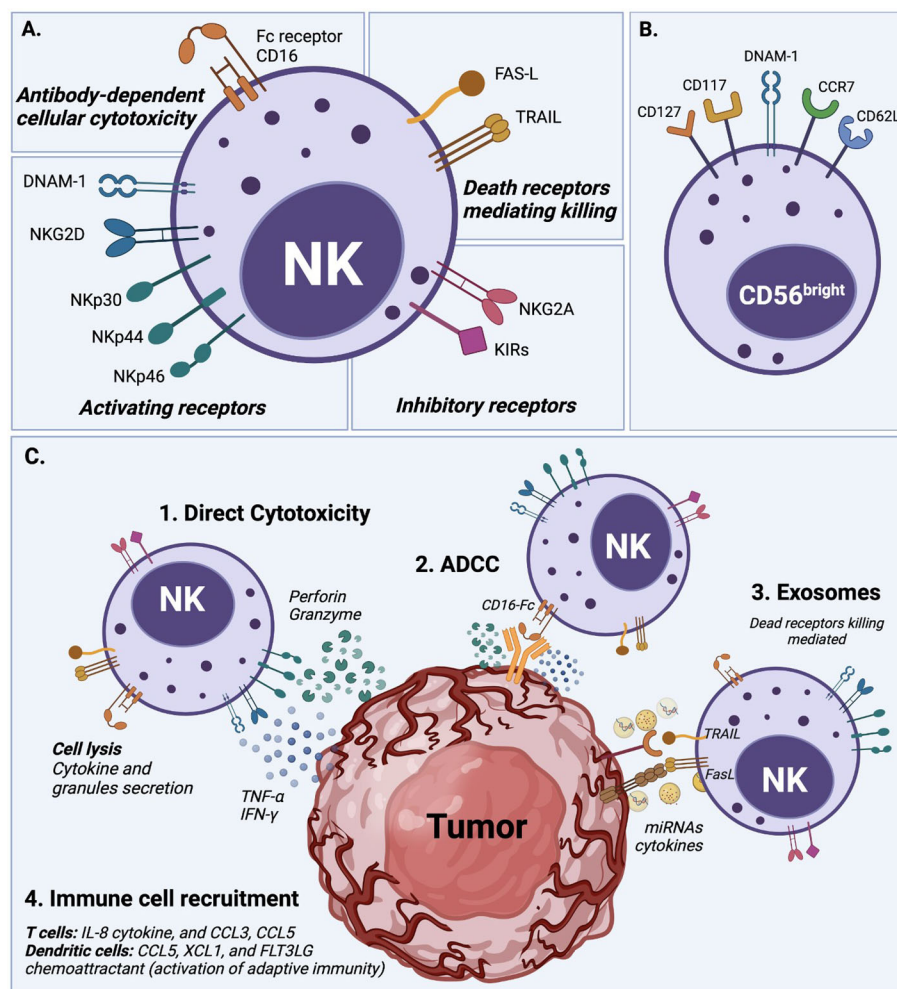


FIGURE 1

**NK cell activation and natural cytotoxicity.** (A) NK cells have a natural ability to recognize abnormal cells and exert cell cytotoxicity through different mechanisms. (1) NK cells exert direct cytotoxicity against compromised cells mainly by the secretion of IFN- $\gamma$  and TNF- $\alpha$  cytokines, and the release of perforin and granzyme cytolytic granules. (2) Antibody-dependent cellular cytotoxicity (ADCC) is another mechanism of NK cell cytotoxicity, which is mediated by the CD16 (FcR111) receptor after recognizing and bound target cells. (3) NK cells display dead receptors, FAS-L and TRAIL, and exert their killing capability against compromised cells through the release of exosome that contains small non-coding RNAs (miRNAs) and cytokines (8–10). (4) The immune cell recruitment of T cells and dendritic cells also plays an essential role in NK cell cytotoxicity to enhance their action through soluble cytokine IL-2 and chemokine (CCL3, CCL5, XCL1, and FLT3LG) secretion. (B) Overview of NK cell receptors. All described cytotoxicity mechanisms are in response to an orchestrated balance of activating receptors (e.g., DNAM-1, NKG2D, Nkp30, Nkp44, Nkp46) and inhibitory receptors (e.g., NKG2A, KIRs family) that determine the NK cell activation state. (C) Phenotype of circulating CD56<sup>bright</sup> NK cells which typically express DNAM-1, CD62L (L-selectin), and CCR7, but also in some cases CD117 (c-kit), and CD127 (IL-7R $\alpha$ ).

recognition of multiple markers of “missing self” (missing classical of non-classical MHC markers) or “induced self-recognition” (induced markers of abnormal-self, for example generated upon virus infection). The use of NK cells to fight hematological malignancies, including leukemia, lymphoma, and multiple myeloma, yielded encouraging results in pre-clinical and clinical studies (31). These favorable outcomes stem from different mechanisms that NK cells exert against tumor cells. In normal conditions, healthy cells of the human body have either absent or low expression of ligands for activating receptors. When compromised, stressed, or modified, cells upregulate these ligands, becoming more sensitive to NK cells (32). This mechanism, along with the tolerance of a KIR-HLA mismatch between recipient and donors, makes NK cells suitable for an “off-the-shelf” allogeneic

cancer immunotherapy product. This allogeneic approach is based on the NK cells’ ability to recognize, direct, and rapidly lyse cancerous, stressed, and viral-infected cells without MHC-dependent priming (33–35). It avoids the rejection of self-MHC recognition and has a low risk of cytokine release syndrome (“cytokine storm”), graft-versus-host disease (GvHD), and other immune-associated off-target events observed with CAR-T cells (2, 24). Additionally, it was corroborated that autologous and allogeneic NK cells are a fantastic alternative therapy when cancer cells become resistant to chemotherapy, T cells, or checkpoint immunotherapy (29, 30, 36).

Allogeneic NK cell immunotherapy has been investigated in several clinical trials. It has been used not only against hematologic malignancies, but also against solid tumors, yielding encouraging

results even with the immunocompromising tumor microenvironment. Melanoma, neuroblastoma, breast cancer, hepatocellular cancer, ovarian cancer, renal cell carcinoma, and colorectal cancer are examples of treated solid tumors (1, 4, 37). To date, more than 150 clinical trials of NK cells against cancer have been developed. Table 1 illustrates 15 out of 150 trials of NK cells against cancer. These 15 trials explicitly focused on fighting solid tumors using allogeneic cells; most of them were completed, but two were terminated due to toxicity (NCT00582816) or the death of 2 patients (NCT01337544).

### 3 Autologous NK therapy against cancer

The principle of allogeneic transplant has been explored since the early 1950s (39), and allogeneic therapies are defined by the transplant of biological material from a donor to a host in order to cure a disease. This approach has been intensively sought by researcher as a tool against cancer since it would allow for an “off-the-shelf”, universal solution (40). NK cells are a privileged tool for allogeneic immunotherapies mainly because, unlike cytotoxic T-Cells, their MHC independence does not trigger Graft vs. Host Disease (GvHD) (41). Because of this obvious advantage, the consideration to use the own patient NK cells (autologous setting) has received little attention. Ongoing research with autologous settings suggests it is a promising strategy when allogeneic cells are limited or unavailable (27). Autologous products are simpler to obtain and avoid some challenges of human leukocyte antigen (HLA) mismatched cellular therapy, such as the failure to persist for more than a few weeks in an immune incompatible environment (42–44). Another example is the off-target activation of allogeneic cell products due to the abundance of targets in the non-immune compatible recipient that can result in NK cell misdirection, exhaustion, or anergy (44). Additionally, the use of chemotherapy is needed before allogeneic therapy injection to avoid immunological rejection of the recipient (42, 45). Table 2 summarizes the advantages and limitations offered by both types of NK cell therapy, autologous and allogeneic settings.

The initial clinical autologous NK cell therapy was conducted in 2003 and used IL-2 *ex vivo* cytokine-activated NK cells. The results of this trial did not produce significant improvements in patient disease outcomes of lymphoma and breast cancer (46). More recently, Nahi et al. performed multiple infusion doses of *ex vivo* activated and expanded autologous NK cells in multiple myeloma (MM) patients. This treatment resulted in a reduction or minimal residual disease with increased NK cell circulation and granzyme B levels, persisting for several weeks after the last infusion (27). Another clinical trial used autologous NK cells combined with Bortezomib against different solid tumors such as Chronic Myeloid Leukemia (CML), MM, carcinoma, pancreatic, colon/rectal, and non-small-cell lung cancer (NCT00720785). Also, 12 young patients with recurrent or refractory brain tumors were the target in a phase I clinical trial (NCT02271711) that evaluated autologous *ex vivo* expanded NK cells. These two trials yielded no conclusive data.

In another study, patients with metastatic digestive cancer, whose standard therapy failed, were enrolled in a phase I clinical trial to be treated with *ex vivo* expanded NK cells (UMIN000007527). Expanded and  $\gamma$ -irradiated PBMCs were used as feeder cells to stimulate NK-cell expansion. Injected NK cells were demonstrated to have high *in vitro* lytic action and strong expression of NKG2D and CD16. Even when no clinical responses were observed, the therapy did not show severe adverse events, and the cytotoxicity of peripheral NK cells was elevated up to 4 weeks following the last transfusion (47). Bae et al. performed a phase I clinical trial of autologous expanded and activated NK cell therapy. Cells were administered for 5 consecutive days in a dose-escalating manner combined with chemotherapy in 11 hepatocellular carcinoma patients. They observed a disease control rate of 81.8% with no decompensation or adverse events (48). Similarly, pediatric medulloblastoma and ependymoma were treated with intraventricular infusions of *ex vivo* expanded autologous NK cells. NK cells were expanded from PBMCs by co-culturing with irradiated K562 feeder cells genetically modified to express costimulatory molecules. In this case, 8 of the 9 patients still showed progressing disease. Nevertheless, NK cells increased in the cerebrospinal fluid when higher dose levels with repetitive infusions were used. The authors suggested that these findings support additional studies of NK cell infusions against brain malignancies in children (49).

Although many strategies efficiently generate large quantities of expanded NK cells *ex vivo*, it is still unclear whether the expanded NK cells can persist/proliferate *in vivo* without exogenous human cytokines. Vahedi et al., in a pre-clinical study with humanized mice, demonstrated that autologous human immune cells support the *in vivo* survival of *ex vivo* expanded human NK cells. They administered *ex vivo* expanded human cord blood (HCB) derived NK cells into humanized mice reconstituted with autologous HCB immune cells. In contrast to the control mice lacking human immune cells, NK cells could survive and possibly proliferate *in vivo* without exogenous cytokine administration (50). This study underlines the benefits of the autologous setting to maintain the correct immune homeostasis and feedback mechanisms.

Despite these efforts, the success of autologous NK cell therapies has been affected by the aggressiveness of malignant cells, tumor escape, and manufacturing letdowns due to the low number and compromised function of patient-derived NK cells. For that reason, several approaches emerged to enhance their antibody-dependent cellular cytotoxicity (ADCC). Some examples include the addition of checkpoint receptor blockers, antitumor monoclonal antibodies, bi-/tri-specific killer engagers (BiKEs and TriKEs), and the use of memory-type NK cells, which showed beneficial effects (51). Additionally, therapeutic applications recently explored the targeting of NK cells with memory functions, acquired during a previous activation (52, 53).

### 4 Memory-type NK cells

Under certain conditions, NK cells display adaptive and memory-like features against different pathogens, such as viruses, including HCMV (54), HIV (55), chikungunya (56), and influenza

TABLE 1 Allogeneic NK cells clinical trials against solid tumors.

Identifier	Condition or disease	Additional treatment	Study phase	Recruitment Status	Participants	Initial date	Goals and Outcome
NCT00640796	Ewing sarcoma family of tumors (ESFT) and rhabdomyosarcoma (RMS)	Chemotherapy	I	Completed	22	September 2008	Determine the maximum tolerable dose of expanded NK cells in patients. No results have been posted.
NCT00582816	Solid Tumors	Chemotherapy	I/II	Terminated (toxicity)	9	August 2008	Engraftment Failure was reported in 3 patients within 28 of therapy. Many patients developed grade III or IV GVHD.
NCT00823524	Brain and Central Nervous System Tumors	HLA-haploidentical hematopoietic cell transplantation	I/II	Completed	47	January 2009	Evaluation of safety and side effects of donor NK cell infusion. No results have been posted.
NCT00855452	Metastatic Breast Cancer Malignant Melanoma Renal Cell Cancer Gastrointestinal Cancer	NA	II	Completed	20	January 2009	Evaluation of cancer progression on days 7, 17, and 28 post-cell therapy. No results have been posted.
NCT01212341	Solid Tumors	NA	I	Completed	18	September 2010	Determine the MTD of allogeneic NK cells within 4 to 5 weeks. No results have been posted.
NCT01287104	Solid Tumors	Stem Cell Infusion		Completed	34	January 2011	Five of 9 transplant recipients experienced acute GVHD following therapy (grade 4 was observed in 3 patients) (38).
NCT01337544	Childhood Solid Tumor	Haploidentical Stem Cell Transplantation	I	Terminated	6	January 2011	Measure safety and tolerability of the therapy. No results have been posted and the study was halted due to a claim against the hospital by parents of two patients who died.
NCT01875601	Solid Tumors Brain Tumors Sarcoma Neuroblastoma	RhIL-15	I	Completed	16	June 2013	Assess the toxicity of infusing escalating doses of autologous NK cells and the feasibility of harvesting and expanding activated NK cells to meet the proposed escalating dose. No results have been posted.
NCT02100891	Ewing Sarcoma Neuroblastoma Rhabdomyosarcoma Osteosarcoma CNS Tumors	NA	II	Active, not recruiting		March 2014	Disease control rate and overall survival after 6 months and 1 year cell therapy respectively. No results have been posted.
NCT02130869	Neuroblastoma High-risk Tumor	CD133+ autologous stem cell infusion Several drugs	I	Completed	8	October 2014	Determine the positive ANC engraftment in patients. No results have been posted.
NCT03213964	Epithelial Ovarian Cancer Fallopian Tube Cancer Primary Peritoneal Cancer	IL-2	I	Completed	10	October 2017	Determine the maximum tolerated dosage of NK cell therapy in patients. No results have been posted.
NCT03319459	Gastric Cancer Colorectal Cancer Melanoma	Cetuximab Trastuzumab	I	Completed	44	January 2018	Determine the incidence of DLT within each dose, 28 days after administration. No results have been posted.

(Continued)

TABLE 1 Continued

Identifier	Condition or disease	Additional treatment	Study phase	Recruitment Status	Participants	Initial date	Goals and Outcome
	Pancreatic Cancer Breast Cancer						
NCT02857920	Solid Tumors	Bevacizumab	I/II	Completed	45	August 2016	Determine the PFS and OS in patients over 1 and 3 years respectively. No results have been posted.
NCT02853903	Malignant Solid Tumor	NA	II	Completed	20	August 2016	Determine the relief degree of tumors within 3 months. No results have been posted.
NCT03420963	Relapsed or Refractory solid tumors	Cyclophosphamide Etoposide	I	Recruiting	38	August 2021	Determine the incidence of adverse events and maximum tolerated dose of expanded allogeneic NK cells. No results have been posted.

PFS, progress-free survival; OS, overall survival; DLT, dose-limiting toxicities; ANC, absolute neutrophil count; MTD, maximum tolerable dose. Source: <https://clinicaltrials.gov>.

TABLE 2 NK cell therapies comparison.

Autologous	Allogeneic
HLA compatibility	KIR-HLA mismatch with donor may trigger alloreactivity of NK cells
Low risk of getting graft versus host disease	Potential risk of getting graft versus host disease
Faces a known environment	Non-immune incompatible environment
Designed to be used in the same patient	Can be used with any recipient ("off-the-shelf" product)
Compatible environment facilitates the NK cell expansion and persistence	More probability of reaching cell exhaustion or anergy
No need to use immunosuppressive regimen	To avoid immunological rejection, chemotherapy is needed before the therapy
The cell source is the patient, who usually possesses cells with reduced effector function ("exhausted")	Wide range of NK cells sources

(57). These findings primed the interest of researchers to explore the NK cell memory potential for cancer treatment of hematologic malignancies and solid tumors. The ability of the immune system to remember and rapidly respond against prior-faced pathogens is defined as immunological memory, which is mainly considered a characteristic of adaptive immune cells. Recent studies indicate that innate immune cells can also exert memory function against a previously encountered pathogen (52, 53). Memory-like (ML) NK cells can ignore some inhibitory receptors, such as KIR, and exhibit higher expression of the activating receptors, such as NKG2D, NKp46, and DNAM-1, suggesting their potential effectiveness in recognizing additional tumor types (26). ML NK cells show various features, such as longer persistence, higher proliferation, and extended effector function (58), that provide superior host protection, higher expansion, and higher IFN- $\gamma$  production than conventional NK cells (59). In humans, the increased output of IFN- $\gamma$  by ML NKs was correlated to the expression of NKp46, CD94, NKG2A, and CD69 receptors, as well as the lack of CD57 and KIR receptors (56). Additionally, it was found that the inhibitory receptor NKG2A is a dominant checkpoint for the ML NK-cell phenotype, and that CD8 $\alpha^+$  donor NK cells correlate to

treatment failure against AML acute myeloid leukemia (AML) (60). We summarized the specific features of ML NK cells in comparison to classical NK features in Table 3.

The use of ML NK cells has been considered reliable and sufficient to stimulate remissions in patients with hematological malignancies such as AML and multiple myeloma (MM) patients (60, 70). Activated NK cells effectively targeted and killed compromised cells in pediatric and adult patients with acute lymphoblastic leukemia (ALL). Boieri et al. demonstrated that Roser leukemia was highly resistant to autologous NK cells. That resistance could be overcome using autologous NK cells pre-activated with IL-12, IL-15, and IL-18 cytokines *in vitro* and *in vivo* (71). Romee et al. demonstrated that ML NK cells induced with IL-12, IL-15, and IL-18 cytokines had highly functional responses, enhanced INF- $\gamma$  production, and higher cytotoxicity when stimulated *in vitro* with primary human AML blasts and against several myeloid leukemia cell lines. Also, good clinical responses were observed in five of nine patients, including four complete remissions (72). The *in vitro* activation of ML NK cells was also shown to have a greater and longer capacity to contain the *in vivo* growth of multiple myeloma cell lines compared to IL-2 activated NK cells (73).

TABLE 3 Types of memory-like NK cells.

NK cell type	Memory-Like	Phenotype	Transcription factors	Specific features
Traditional	No	CD3 <sup>+</sup> CD56 <sup>+</sup> lymphocytes with different expressions of CD16 and CD56	• GATA2, EOMES, and T-bet commitment to transitional NK cells promote the transition from immature and mature NK cell stages (61).	• Natural cytotoxic activity. • Mediation of anti-viral and anti-tumor responses.
Hapten-induced	Yes	Expression of CXCR6 chemokine receptor (62).	• Aryl hydrocarbon receptor (AhR) is required for NK hapten-memory function (63).	• Long-lived immune memory cells. • Increased cytotoxicity.
Virus-induced	Yes	NKG2C <sup>+</sup> NK cell subset found after viral exposure (64, 65).	• IRFB8 and BTB-ZF (Zbtb32) transcription factors and BNIP3- and BNIP3L genes are essential for adaptive NK cell antiviral immunity (25, 66, 67).	• Increased cytotoxicity. • Long-lived memory NK cell population.
Cytokine-induced	Yes	CD117 (c-Kit), CD127 (IL-7R $\alpha$ ), and CD122 (IL-2R $\beta$ ) cytokine receptors outline the stages of NK cells (61).	• STAT4 signaling is required to express several genes when <i>in vitro</i> cytokine-stimulated NK cells are generated. • STAT4 binding directly influences the expression and upregulation of IFN, STAT1, Zbtb32, Tbx21, Runx1, and Runx3 (68, 69).	• Increased cell sensitivity. • Reduced risks of irradiation and contamination during large-scale NK cell expansion.

ML NK cells also exhibited promising *in vitro* results for treating solid tumors, encouraging their use in the early phases of clinical studies (see Table 1). For example, human ML NK cells were injected in NSG (NOD scid gamma) mouse models, demonstrating higher control of melanoma xenografts compared to conventional NK cells maintained with IL-15 (26). Uppendahl et al. studied the interaction of ML NK cells from healthy donors and melanoma patients against melanoma targets and established the superior cytokine production (IFN- $\gamma$ ) and cytotoxicity of ML NKs. They correlated that these ML NK-cell responses against autologous targets were related to the activatory receptors NKG2D and NKP46. ML NK cells also showed enhanced *in vitro* production of TNF- $\alpha$  and IFN- $\gamma$  against different ovarian cancer cell lines, including SKOV-3, MA148, OVCAR5, and A1847, but also controlled the MA148 tumor growth of an *in vivo* xenogeneic mouse model (74). Moreover, ML NK cells isolated from hepatocellular carcinoma (HCC) patients and healthy donors were identified as potential effectors against HCC by trafficking to the liver to exert a cytolytic activity responsible for lower levels of spontaneous HCC formation (75). Tanzi et al. studied ML NK cells against solid tumors and demonstrated that these cells were able to lyse autologous tumors, including lung, liver, and peritoneum tumors. Of note, in this study, the antitumor activity of IL-12, IL-15, and IL-18 cytokine-activated NK cells was superior to that obtained with IL-2 stimulated NK cells (76). Another study demonstrated that ML NK cells cultured in the presence of IL-2 and IL-21 showed enhanced expansion and cytotoxicity against K562 cells. This enhanced bioactivity was correlated to the upregulation of the markers CD69 and CD25, along with phosphorylation of signal transducer and activator of transcription (STAT) 1 and 3 (68).

In summary, the use of memory-like NK cells has shown to be advantageous and a promising alternative to improve the current results obtained with NK cell immunotherapy. However, to translate these advantages into a clinical setting, it is imperative that efficient methods can be deployed for the large, stable production of these cells.

## 5 Targeting the immunosuppressive tumor microenvironment

One of the significant challenges in using NK cell-mediated immunotherapy is the cells' low activity when deployed against solid tumors, due to the several immunosuppressive mechanisms that the tumor microenvironment (TME) uses, as illustrated by Figure 2. The TME establishes immune evasion mechanisms that include acidification (low pH), hypoxia, decreased expression of tumor-associated antigens, and low nutrients. Also, the TME upregulates the expression of inhibitory ligands and downregulates transcriptional ligands complementary to NK cell-activating receptors (4, 77, 78).

For example, the activating natural killer group 2D (NKG2D) receptor is highly involved in NK cell-mediated tumor surveillance. Specific tumor cells developed a mechanism to evade NKG2D recognition by shedding and saturating the NKG2D receptor with NKG2D soluble ligand (79). This action, in addition to allowing cancer cells to hide from the effect of NK cells, also leads to the desensitization of NKG2D-mediated NK cell activation since high levels of NKG2D ligand shedding cause the downregulation of NKG2D signaling (80). Another immunosuppressive mechanism example is the increase of associated factors, including tumor-secreted factors (TSF) and tumor-secreted exosomes (TSE) (2). Some examples of TSE or tumor cytokine expression are the transforming growth factor (TGF)- $\beta$ , tumor necrosis factor (TNF), vascular endothelial growth factor (VEGF), and IL-10 that induce the upregulation of key molecules for tumor cell proliferation and suppress the adaptive antitumor immune response (2, 4, 81). It was demonstrated that TGF- $\beta$  leads to NK cell differentiation and reduces the expression of NK cell activating receptors NKG2D, NKP30, and DNAM-1, which is related to their lack of efficient activation (81, 82). Another factor that represents a challenge for NK cells, when facing cancer cells, is their ability to maintain normal MHC-I expression. This results in the inhibition of NK cell activation by binding to NKG2A and KIR (Killer cell



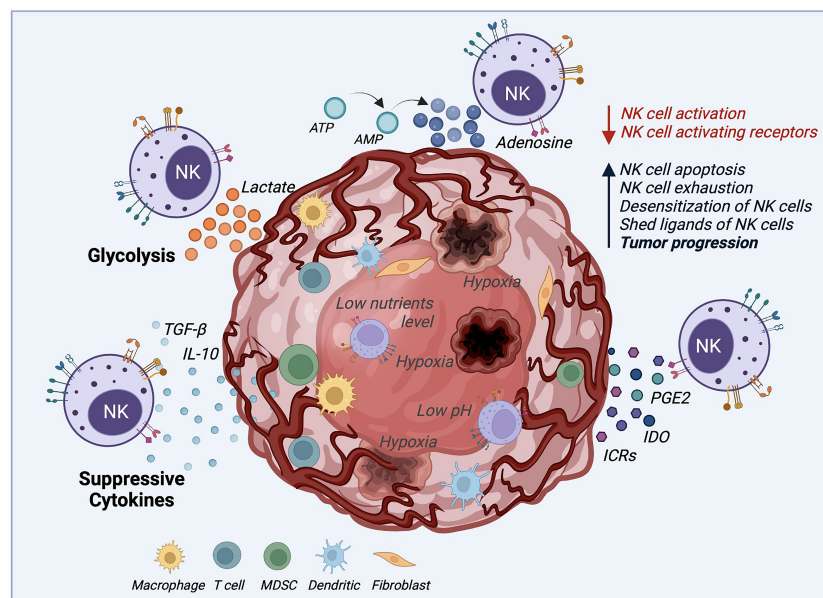


FIGURE 2

**Tumor microenvironment (TME) mechanisms inhibiting NK cell cytotoxicity.** The TME deploys several mechanisms to survive and increase the tumor progression rate by desensitizing NK cells and decreasing NK cell activation. These mechanisms negatively impact NK cell cytotoxicity by boosting NK cell apoptosis, exhaustion, and proliferation. Suppressive cytokines such as TGF- $\beta$  and IL-10, immune-checkpoint receptors (ICRs), indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2) are secreted by the TME to desensitize NK cells. Also, the production of lactate and adenosine, along with hypoxia, and low levels of nutrients and pH, inhibits the NK cell function against solid tumors.

Immunoglobulin-like Receptors). These receptors act as immune checkpoints, preventing NK-mediated cancer cell destruction (83).

Moreover, tumor metabolic activity, mitophagy, and oxygen availability also affect the NK cell responses (24). Tumor glycolysis can drive lactic acid accumulation in the TME. NK cells *ex vivo* culture with 15 mM lactic acid results in a totally blocked IFN- $\gamma$  production, inhibiting NK cell proliferation and tumor surveillance in melanomas (84). Similarly, the mitochondrial fragmentation caused by hypoxia may be an essential factor to consider for NK cell therapy success (24). Zheng et al. found that mitochondrial fragmentation induced a loss of NK cell cytotoxicity, helping tumor evasion from NK cell-mediated surveillance (85). Hypoxia, along with nutrient shortage, acidic environment, abnormal vasculature, and high pressure, also plays an essential role in TME. Hypoxia not only stimulates the TSE release and attracts bone marrow-derived cells (BMDCs), but it may also increase neoplastic growth, slow down the tumor cell death rate, and promote cancer cells genomic variability leading to increased tumorigenesis (2). Similar to the TGF- $\beta$  mechanism, hypoxia affects NK cell cytotoxicity by decreasing the expression of activating receptors Nkp46 and CD16. Hypoxia can also induce overexpression of the adenosine nucleotidase CD73, upregulate checkpoint molecules such as PD-L1, and down-regulate the activating ligand MICA on tumor cells (51).

All of these survival TME mechanisms are challenges that autologous and allogeneic NK cells must overcome to be successful as an immunotherapy option. Lately, one alternative explored for therapeutic purposes is to target and boost NK cells that were previously activated to awaken their memory functions (52, 53).

## 6 Memory-like NK cell production

The consensus is that innate memory forms in three stages (1). The cell expansion phase upon an activation event (encounter of the antigen/activator by naïve cells) (2); the contraction phase, when cells that return to baseline function undergo apoptosis; and survival cells enter (3) the memory phase, which produces enhanced response of remembering a prior-faced stimulation. However, NK cell memory differs from a classic adaptive memory, varying accordingly to the employed activation stimulus with unique functional and molecular characteristics (25, 86). Current strategies to produce a large number of NK cells are in development, with the hope of allowing the potential treatment of many patients from a single source, as observed in Figure 3 (24). Several studies demonstrated that the generation of ML NK and memory-like responses could be achieved under specific circumstances such as hapten-specific exposure, viral infection, feeder cell exposure, and cytokine activation (25, 53).

ML NK cells obtained by antigen-specific exposure, were first described by O'Leary et al. when studying hapten-induced contact hypersensitivity (CHS) response in mice lacking T cells and B cells (87). Hapten-induced CHS is a typical example of adaptive immunity, where the epithelial surface is exposed to molecules that chemically modify proteins. Those "haptened molecules" becomes foreign antigens triggering the formation of hapten-specific and long-lived immune memory cells. In this study, it was found that the Ly49C-I+ NK subpopulation localized in the donor's liver exhibited responses specifically to DNFB (2,4-dinitro-1-fluorobenzene) and OXA (oxazolone) haptens that persisted for at least 4 weeks (87). After

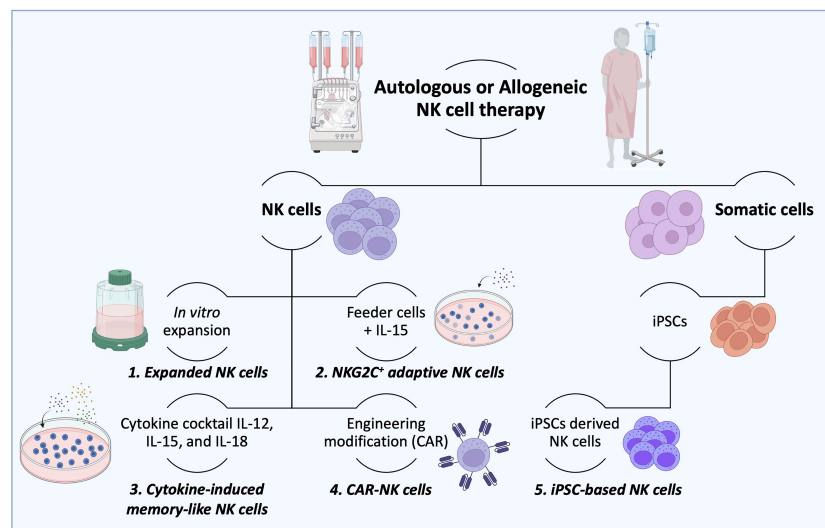


FIGURE 3

**Production of therapeutic NK cells.** For autologous or allogeneic clinical purposes, a considerable number of therapeutic NK cells are needed to destroy cancer cells effectively. To this end, several ways to obtain the desired number of therapeutic NK cells have been established. (1) After isolation, NK cells could be mass expanded *in vitro* in culture flasks, reactors, and bags until the needed cell number is achieved. (2) Irradiated feeder cells (to suppress their expansion) in the presence of IL-15 are employed to stimulate the NK cell activation and expansion. (3) The combination of cytokines such as IL-12, IL-15, and IL-18 is used to expand NK cells and generate memory functions (memory-like NK cells). (4) NK cells can also be potentiated by engineering modifications with CAR constructs to overcome their current limitations. (5) More recently, induced pluripotent stem cells (iPSCs) have been considered an advantageous source of NK and CAR-NK cells over peripheral and cord blood due to their usage in both autologous and allogeneic settings. (6) The IL-2-dependent immortalized NK-92 cell line is another type of NK cell source that possesses features of immune cell types and has been used in clinical trials.

that, it was demonstrated that the CXCR6 (C-X-C chemokine receptor type 6) chemokine receptor plays a critical role in NK memory response to haptens and viruses. CXCR6 was present on hepatic NK cells and was responsible for their persistence and homeostasis, but not for antigen recognition. Although the CXCR6 receptor expression is not limited to the liver, only hepatic NK cells mediated the memory response (62). Another study of classical CHS protocols with immunocompromised mice that lacked T and B cells, but not NK cells, demonstrated that liver NK cells induced specific memory responses to haptens. This behavior was confirmed upon transfer in CD3-deficient mice, confirming the existence of “memory-like” NK cells (88). Consequently, it was established that DNFB-induced liver NK cells were potent effector cells that appeared within 1 hour after immunization. Authors suggested that hapten-specific effector NK cells are pre-existing in naive hosts, ready to exert inflammatory responses immediately after antigen contact (89). Antigen-specific NK cell memory was also found in primate species (rhesus macaques) after SIVmac251- and SHIV162P3 infections and AD26 vaccination, suggesting a potential use in vaccines against HIV-1 and pathogens (55). Likewise, it was found that specific subpopulations infected with human cytomegalovirus (HCMV) possess an NKG2C<sup>+</sup> NK cell subset that exhibits clonal-like expansion and partially mimics an anti-viral adaptive response (64). Another study showed that NK cells exposed to therapeutic antibodies such as AFM13 (CD30/CD16A) produced more IFN- $\gamma$  and showed increased cytotoxicity upon restimulation with lymphoma tumor cells, indicating memory-like functionality (65).

The use of feeder cells such as PBMCs, Jurkat T cells, K562 leukemia cells, and Wilms tumor HFWT have also been explored to

produce ML NK cells. This method shows promising survival and growth results even for large-scale NK cell expansion. Feeder cells can promote cell growth by triggering the extracellular secretions of growth factors, detoxification of the culture media, synthesis of extracellular matrix proteins, or improved cell attachment (90). For example, NK cells expanded in a co-culture setting with irradiated (40 Gy) 721.221-AEH cells showed a high killing capability against acute lymphoblastic leukemia cells, that could be easily scaled up into a GMP-compliant process. Interestingly, even when the cytotoxicity response was optimal, less than 30% of the cell population showed CD107a marker upon degranulation, which was explained by the different killing mechanisms that natural killer cells could perform in response to the target (91). Jurkat T cells sub-line KL-1 induced a 100-fold NK cell expansion with almost 90% purity of NK cells from PBMCs, with potent antitumor capability. The *in vitro* and *in vivo* tumor-lytic capacity was associated with the expression of natural cytotoxicity receptors (NKG2D, DNAM-1), and adhesion molecules (CD11a, ICAM-1) (92). Authors also suggested that the positive results using feeder cells could be enhanced using genetically modified cells. Indeed K562-mb15-4-1BBL cells (K562 cells modified to express membrane-bound IL-15 and 4-1BBL) are already used in several clinical trials of allogeneic NK cells (93). Membrane-bound cytokines IL-2, IL-15, or IL-21 in K562 feeder cells, lacking HLA, but expressing costimulatory molecules, can be used for effective NK cell *ex vivo* expansion. Concurrently, Min et al. combined the use of irradiated autologous PBMCs and the monoclonal antibody OKT3 in the presence of IL-2 to large-scale NK cell expansion for 3 weeks and evaluated the effect of a freeze/

thaw cycle. NK cells reached approximately 15,000-fold expansion, and while their viability was slightly reduced after thawing, their cytotoxicity and cytokine secretion against hepatocellular carcinoma cells were not affected (94). Rezaeifard et al., as part of an autologous phase I clinical trial, executed a pre-clinical NK cell enrichment employing anti-CD56 and anti-CD3 in PBMCs with the addition of IL-2. After two weeks, these expanded NK cells (CD3-CD16+/-CD56+) presented highly lytic properties against K562 cells, reaching a 510-fold average with a strong expression of NKG2D and CD16 (95).

Another method to support the activation, expansion, and survival of NK cells is the addition of cytokines to the culture medium, traditionally using IL-2 and, most recently, IL-15 (72, 96). The rationale behind using cytokines to stimulate NK cells is to increase cell sensitivity by targeting different costimulatory molecules and activating receptors (97). Various combinations and permutations of the cytokines IL-12, IL-15, IL-18, and IL-21 were shown to produce ML NK cells for pre-clinical and clinical studies. Terren et al. studied different combinations of IL-12, IL-15, and IL-18 cytokines to generate human cytokine-induced memory-like (CIML) NK cells. They found that IL-15 significantly contributes to NK cell cytotoxicity, but combining the three cytokines induced a synergistic effect that granted the cells the best polyfunctional profile. That profile includes proper degranulation and production of multiple cytokines and chemokines such as INF- $\gamma$ , TNF- $\alpha$ , and CCL3 (C-C motif chemokine ligand 3) (98). Romee et al. developed the first-in-human phase I clinical trial of CIML NK cells with low dose IL-2, demonstrating high functional responses enhancing INF- $\gamma$  production and cytotoxicity. This study showed promising results, with good clinical response in five of nine treated myeloid leukemia patients (72). Shapiro et al. performed a phase I clinical trial infusing allogeneic CIML NK cells in acute AML patients after lymphodepletion and subcutaneous IL-2 dosage. NK cells stimulated for 12–16 hours with the cytokine cocktail (IL-12, IL-15, and IL-18) and infused at a range of 5 to 10 million cells/kg demonstrated a rapid *in vivo* expansion and long-term persistence after infusion (59). Other research focused on the large-scale production of ML NK cells through cytokine stimulation. Liu et al. established a strategy to develop a high-efficient large-scale generation of NK cells from peripheral blood. In that study, NK cells stimulated with IL-2, IL-15, and IL-18 showed prolonged persistence, high cytotoxicity against K562 cells, and high levels of activating molecules such as CD16 and NKG2D (99). While feeder cells have previously been employed for large-scale expansion (100), the use of cytokine-induced stimulation could avoid the problems that come along with the use of feeder cells. Some examples of those problems include irradiation for inactivation, product contamination by feeder cells, and overall safety risk that can limit the large-scale production of NK cells for clinical purposes (99).

## 7 Enhancing NK cells potency against cancer

Current NK cells-mediated cancer immunotherapy produced encouraging results against hematological malignancies and solid

tumors (1, 4). Still, these results can likely be potentiated in combination with other cell lines (101), nanoparticles (102–104), as well as chemotherapy and radiotherapy (105).

The combination of NK cell therapy with cytotoxic T cells was demonstrated to kill more cells than the sum of individual treatments. Notably, these 2 cell types are the most widely used lymphocytes in cancer immunotherapy, which aims to target cancer cell populations through opposites mechanisms, suggesting the existence of cytotoxic synergies (37, 101). Additionally, INF- $\gamma$  production of NK cells in the TME upregulates MHC-I expression by tumor cells, increasing activatory targets for cytotoxic T cells (106, 107). Therefore, combining NK cells with T cells is a potential mechanism to achieve tumor regression. For example, Zhang et al. studied this combined human lymphocyte treatment against primary tumor tissue of lung cancer *in vitro* and *in vivo*. They found that allogeneic NK cells were more cytolytic against cancer cells than autologous NK cells; activated T cells primarily impacted MHC class I-positive cancer cells, while NK cells focused on MHC class I-negative cancer cells (101). This result could be correlated to the fact that CD8<sup>+</sup> T cell response and expansion increased with the presence of NK cells in the B16F10 melanoma (2). Friedmann et al. used melanoma cells as a cancer model to study the combinatorial cytotoxicity of NK cells and cytotoxic T lymphocytes (CTL). They found that after 24 hours of co-culture using high effector-to-target (E:T) ratios, melanoma cells were fully lysed, while at lower E:T ratios (simulating human cancer conditions), a significant number of melanoma cells survived and became more resistant to further NK cell exposure. Interestingly, this detrimental effect could be reversed if the initial NK exposure is followed by CTL exposure, which the authors suggest could be due to the release of exogenous antigens (108). The engineering modification of NK cells in combination with CAR-T cells has also been explored to enhance the potency of cell therapy treatments, showing that NK cells support the anticancer CAR-T cell response (109). In combination with CD19 CAR-T cells, cord blood-derived NK cells induced an enhanced antitumor efficacy of CAR-T cells against multiple myeloma, by facilitating their earlier activation and improving their migration to the tumor cells (109). It was also observed that in malignant mesothelioma patients, the physiological levels of CD56<sup>Bright</sup> and CD56<sup>Dim</sup> NK cell subset populations were abnormal. However, the addition of an anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA4) checkpoint restored those NK-subset levels, activity, and killing capability (110).

NK cells have also been associated with other immune cells than T cell lymphocytes to enhance the cytotoxic response against cancer. In human melanoma, stimulatory dendritic cell abundance was found to be related to the intra-tumoral expression of the gene encoding the cytokine FLT3LG, which is principally produced by NK cells. Also, Böttcher et al. found that the interplay between NK cells and conventional type 1 dendritic cells (cDC1) has numerous therapeutic implications within the TME (111). For instance, intra-tumoral transcripts CCL5, XCL1, and XCL2 closely correlate with NK cells and cDC1 gene signatures that were also associated with patient survival (111). This cell interaction suggested an excellent tool for the success of cell

immunotherapy since NK cells are required to promote the optimal cytotoxicity response of T cells through the recruitment of dendritic cells in the tumor site (21, 107, 111, 112).

Nanoparticles have also been explored to enhance NK cell therapy in multiple research groups (103). Magnetic (113), chitosan (114), lipid nanoparticles (115), liposomal polymeric gel (116), and PLGA microspheres (117) are examples of nanoparticle substrates employed in previous studies to augment NK cell immunotherapy against several types of cancers. Namely, Adjei et al. used manganese dioxide nanoparticles as a vehicle to deliver siRNA (small interfering RNA) for the NK cell TGF- $\beta$  receptor 2, which resulted in the restoration of NK cell activity against lung cancer cells (102). Most recently, Biber et al. developed a non-viral lipid nanoparticle to encapsulate and deliver small interfering RNAs (siRNAs) to overcome current obstacles in NK cell-based immunotherapies. They showed that their nanoparticles targeted NK cells *in vivo*, silencing their intrinsic inhibitory checkpoints and triggering NK cell activity to kill tumors (118).

Currently, the combination of chemotherapy with NK cell therapy is tested in multiple cancer clinical trials (see Table 1). This combination can be a synergistic strategy to improve outcomes in order to facilitate effector cell trafficking, cell infiltration, NK cell-mediated cytotoxicity, lysis, and cancer antigen release (105, 119). Several chemo-drugs also promote the expression of NK cell activating receptors and their ligands expression on tumor cells, and enhance the secretion of selected chemokines, making cancer cells more vulnerable to NK cell cytotoxicity (37, 105). A vast list of available drugs can be considered to potentiate the NK cell therapy results. Antitumor antibiotics, antimetabolic and alkylating agents, plant alkaloids, proteasome, histone deacetylase, and tyrosine kinase inhibitors are types of drugs commonly used in chemotherapy (105). That potentiation has been demonstrated with different types of cancer. For example, Bae et al. demonstrated the safety of a high-dose autologous NK cell therapy combined with chemotherapy in a phase I clinical trial. They employed 4 cycles of hepatic arterial infusion chemotherapy (HAIC) of 5- fluorouracil (750 mg/m<sup>2</sup>) and cisplatin (25 mg/m<sup>2</sup>) (48). Additionally, it was confirmed that combining NK cells with alkylating agents sensitizes melanoma cells, allowing the control of tumor growth. Using dacarbazine in melanoma patients showed an increased expression of the NKP46 receptor (120), and a higher NK cell activation and cytolytic activity (121). Another study revealed that using lymphodepletion chemotherapy before injecting NK cells in patients with AML combined with a low dose of IL-2, increased IL-15 production and promoted NK cell persistence and expansion (42). The chemotherapy regimen included cyclophosphamide and methylprednisolone, and fludarabine (42). Other types of cancer, including ovarian cancer (122) and advanced colon carcinoma (123), have also been treated with NK cells and chemotherapy combinations. Moreover, NK cells combined with chemoradiotherapy induced long-term tumor control against metastatic nasopharyngeal carcinoma (124). An intriguing avenue to enhance the cytotoxicity of NK cells in the TME is to use artificial engagers that both support NK function and lower the TME capacity for immune inhibition while lowering the potentials for side-effects. For example, a fusion complex combining an IL-15 agonist coupled with a TGF- $\beta$  molecular sink showed increased infiltration of both NK

and CD8+ cytotoxic cells and lower tumor burden in a syngeneic mouse B16F10 melanoma model (125).

Other combinatorial studies have also demonstrated additional avenues for potentiating the anti-cancer effect of NK-based therapies. For instance, it was reported that the combined treatment of NK cells with oncolytic viruses (OV, therapeutically beneficial viruses that have the ability to replicate and kill cancer cells) enhanced the cytotoxicity of OV and induced NK cell infiltration (107, 126–128). Another study found that using a checkpoint antibody blocking TIGIT, an inhibitory cell surface receptor, reversed the NK cell exhaustion and enhanced NK cell cytotoxicity, inhibiting *in vivo* colon, breast, and melanoma tumor growth (129).

In addition to the potentiated NK cell therapy combinations already mentioned, engineered modifications of NK cells is a fast-growing field. Further details are mentioned in the next section.

## 8 Engineered modification of NK cells

The genetic modification of NK cells to express CAR (Chimeric Antigen Receptor) constructs has recently gained significant attention, due to the success met using this technology with T cells. CAR were first described in 1987 (31) and are bioengineered cell receptors coded by “chimeric genes composed of immunoglobulin (Ig)-derived variable (V) regions and T-cell receptor (TCR)-derived constant (C) regions”, allowing for the specific of cancer cells while activating the engaged immune cell. Since NK possess several activation receptors, this concept was ideal to be used for the design of CAR-NK cells (130). These modifications intend to overcome the limitations of non-modified NK cells when facing tumor cells with genetic or epigenetic variations that bypass the NK immunological surveillance (51, 58). CAR-NK cells can be produced from multiple sources (such as peripheral blood, cord blood, iPSCs, and NK-92 cell line), adopting the basic methods borrowed from CAR-T cell generation (131). The CAR molecule, a synthetic hybrid antigen receptor (132), can redirect the NK cell-killing capability to a specific antigen target. The main characteristics of CAR-NK cells versus traditional NK cells have been summarized in Table 4.

To date, NK cells have been engineered and modified with CARs in order to overcome challenges such as the limited persistence after infusion without cytokine support, slow trafficking to tumor beds, and overcoming the immunosuppressive TME (131). Similar to CAR-T cells, CAR-NK cell functionality was initially tested against hematological malignancies. In a pre-clinical study, the combination of CIML NK cells expressing an anti-CD19 specific CAR was tested to enhance the response rate in NK-resistant cancers. Results demonstrated an increased functional response (degranulation, IFN- $\gamma$  production, and specific killing) against B cell lymphomas by CD19-CIML NK cells compared with CD19-NK, CD33-NK, and ML NK cells (133). These results underline the great potential that CAR-NK cell therapy could offer compared with non-modified NK cells. In addition to CD19, there are almost 50 other surface target antigens, including CD5 (134), EGFR or EGFRvIII (135–138), HER-2 (139–142), NKG2D (143–146), and GD2 (147, 148) that have been considered to enhance CAR-NK cell responses (149).



TABLE 4 Comparison of NK and CAR-NK cell products.

Feature	NK cells	CAR-NK cells
Specificity	Do not have preferential targeting (targets any compromised cells).	Specific to target antigen expresses on the cancer cell surface. But many healthy cells also express cancer-associated antigens.
Killing mechanism	NK cell receptors mediate the cell-killing capability.	NK cell receptors mediate the cell-killing capability.
Gene editing/ CAR	N/A	After modification, cells maintain their natural ability to detect stress-evoked ligands. Low genetic transfection levels <40% were obtained compared with CAR-T cells.
Dosage*	A single dose of $1 \times 10^7$ to $1 \times 10^8$ cells. NCT03383978. One to three cycles of $3\text{--}5 \times 10^7$ cells/kg. NCT00855452. Daily scalable dosage $2.5 \times 10^8$ , $5 \times 10^8$ , $10 \times 10^8$ NK cells. KCT0003973	Weekly scalable dosage ( $3 \times 10^6$ , $1 \times 10^7$ , $3 \times 10^7$ , $1 \times 10^8$ cells). NCT02439788. Weekly infusion of $2 \times 10^9$ cells. NCT04847466.
Manufacturing	Therapeutic NK cell expansion could take 2 to 5 weeks (up to the expansion method).	Additional time and cost to engineering expanded NK cells. After engineering, a lower survival cell rate is observed, and it takes additional recovery time before using cells.

\*Dosage based on the available information on glioblastoma, neuroblastoma, and gastric or head/neck treatments. Source: <https://clinicaltrials.gov>.

Additionally, it is known that NK cells are characterized by a short-lived persistence in the body after infusion in the absence of cytokine support, approximately 14 days, which limits the efficacy of NK cell immunotherapy. That limitation was intended to be overcome by the genetic modification of NK cells with transgenes encoding cytokines IL-2 or IL-15 either for secretion or membrane expression. This strategy yielded the suppression of inhibitory signals of the TME as well as enhancing the NK cell killing capability in several studies (131, 150, 151). Also, to modulate the CAR-NK cell functionality, the expression of cytotoxic ligands and chemokine receptors such as CXCR4, EGFRvIII, and the combined expression of FR $\alpha$  and DR4/5 were included in the engineering modification design (137, 152, 153). The NK cell metabolism, cell homing, and tumor infiltration were also enhanced with engineering modification (154). For example, to improve the metabolism within the TME, maintaining the levels of cMyc signaling with kinase glycogen synthase kinase-3 (GSK3) inhibitors (155, 156), as well as the deletion of hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) (157) were a target of modifications. Cell homing is another critical factor considered in the NK cell therapy success, which was suggested to be targeted with different mechanisms (154). For example, lymphodepletion complemented with cytokine support (158), expression of the CXCR4 gene (152), pharmacologic combination (139, 159), and others were employed, having encouraging results in cell homing that also impacted the cell trafficking and tumor infiltration.

The first clinical trial of CAR-NK cell treatment focused on CD19 as a target (see Table 5), which showed a 73% positive response rate without evidence of GvHD, development of cytokine release syndrome, or neurotoxicity. Infused CD19-CAR-NK cells also demonstrated expansion and persistence for at least 12 months at low levels (160). After that trial, other phase I and II clinical studies were performed in USA and China. Some of these studies focused on hematological malignancies such as lymphomas (13 trials) and multiple myeloma (NCT05182073, NCT03940833). Others focused on solid tumors (131), including colorectal (NCT05213195, NCT05248048), glioblastoma and neuroblastoma (NCT03383978, NCT02439788), prostate (NCT03692663), ovarian

(NCT03692637), metastatic gastric or head and neck cancer (NCT04847466), and pancreatic (NCT03941457) cancer. Some of these studies were withdrawn because of funding issues (NCT03579927) and methodology improvement (NCT02439788), while others have unknown statuses (NCT03940833, NCT03941457, NCT03692637, NCT03415100, NCT03940820).

Nevertheless, before translating the use of CAR-NK cells in clinical trials, some challenges must be addressed. Their limited persistence/expansion and their difficulty in being genetically modified must be addressed (152, 154, 161, 162). Some examples of successful genetic modification include the work of Tang et al., which reached over 90% transduction efficiency of CD33-CAR construct in the NK-92 cell line (163). But, NK cells are generally heterogeneous and have proven to be more challenging to expand and engineer *in vitro* by transduction, transfection, or nucleofection than T cells. During and after the engineering process, they show high sensitivity to apoptosis and low gene expression levels, which implies instability of the product and that the cost of obtaining therapeutic CAR-NK cells could be significantly higher than other alternatives (164).

The CAR transduction techniques employed for NK are primarily based on viral transduction and non-viral mediated transfection. The latter is the preferred method as it will induce fewer toxicities (149). The most successful current techniques yield a rapid, high, but transient expression (electroporation) or a sustained but low gene expression (viral vectors) (165). Despite the limitations, the most successful non-viral gene delivery technique in NK cells is the rapid transient expression by electroporation (166). Electroporation yields highly efficient T-cell transfection, but that result was not observed at a similar level with NK cells (154). For example, in terms of engineered modification of blood-derived NK cells, lipid-based and electroporation methods typically result in less than 5% transfection; retrovirus vector-based yield a 27–50% efficiency, and lentivirus-based transfection only yields a 20–40% efficiency (167). Nevertheless, even when viral transduction produces better results, they are associated with decreased cell viability, NK cell apoptosis, and reduced efficacy (154). To date, some *in vitro* and *in vivo* attempts at genome editing



TABLE 5 Current CAR-NK cells clinical trials.

Identifier	Cancer type	Target	Additional treatment	Recruitment Status	Phase	Goals and Outcome
NCT05020678	B cell cancers	CD19	NA	Recruiting	Phase I	Determine the incidence of treatment-emergent adverse events and the proportion of subjects experiencing dose-limiting toxicities of NKX019 therapy. No results have been posted.
NCT04623944	Hematological Malignancies or Dysplasias	NKG2D ligands	Cyclophosphamide, Fludarabine, Cytarabine (ara-C)	Recruiting	Phase I	Determine the incidence of treatment-emergent adverse events and the proportion of subjects experiencing dose-limiting toxicities of NKX101 therapy. No results have been posted.
NCT05215015	Acute Myeloid Leukemia	Anti-CD33/CLL1	NA	Recruiting	Early phase I	Determine the incidence of DLT within each dose, 28 days after administration. No results have been posted.
NCT03056339	B Lymphoid Malignancies	CD19-CD28-zeta-2A-iCasp9-IL15	Fludarabine, Cyclophosphamide, Mesna, AP1903	Active, not recruiting	Phase I and II	Determine the optimal NK Cell dose level, toxicity, and efficacy of cell product within 45, 14, and 30 days after infusion, respectively. No results have been posted.
NCT05379647	B-Cell Malignancies	CD19	QN-019a, Rituximab, Cyclophosphamide, Fludarabine, VP-16	Recruiting	Phase II	Determine the incidence of subjects with DLT within each dose level cohort and treatment-emergent adverse events (28 days). No results have been posted.
NCT05092451	Relapse/Refractory Hematological Malignances	CAR.70/IL15	Cyclophosphamide, Fludarabine phosphate	Not yet recruiting	Phase I and II	Determine the number of participants with treatment-related adverse events, complete or partial response, and who are alive and in remission. No results have been posted.
NCT05487651	B-Cell Malignancies	CD19	NA	Not yet recruiting	Phase I	Determine the incidence rate and the grade (severity) of DLTs based on adverse events (AEs). No results have been posted.
NCT04288726	Lymphomas	CD30	NA	Recruiting	Phase I	Determine the DLT rate. No results have been posted.
NCT04952584	Lymphomas	CD30	NA	Not yet recruiting	Phase I	Determine the DLT rate. No results have been posted.
NCT03579927	B-cell Lymphoma	CD19-CD28-zeta-2A-iCasp9-IL15	Carmustine, Cytarabine, Etoposide, Filgrastim, Melphalan, Rituximab,	Withdrawn (Lack of Funding)	Phase I and II	Determine the incidence of adverse events, and CR or PR. No results have been posted.
NCT05182073	Multiple Myeloma	BCMA	Cyclophosphamide, Fludarabine, Daratumumab	Recruiting	Phase I	Determine the incidence and nature of adverse events and DLTs, along with the RP2D. No results have been posted.
NCT03940833	Multiple Myeloma	BCMA	NA	Unknown	Phase I and II	Determine the occurrence of treatment related adverse events. No results have been posted.
NCT05213195	Refractory Metastatic Colorectal Cancer	NKG2D	NA	Recruiting	Phase I	Determine the DLT (safety) and MTD (tolerability evaluation). No results have been posted.
NCT05248048	Metastatic Colorectal Cancer	NKG2D	NA	Recruiting	Early phase I	Determine the DLT (safety) and MTD (tolerability evaluation). No results have been posted.
NCT04847466	Metastatic Gastric or Head and Neck Cancer	PD-L1	Pembrolizumab, PD-L1 t-haNK	Recruiting	Phase II	Determine the response rate with irradiated PD-L1 CAR-NK cells in combination with N-803 plus pembrolizumab in enrolled patients. No results have been posted.
NCT03383978	Glioblastoma	HER2	Ezabenlimab	Recruiting	Phase I	Determine the number of participants with treatment-related adverse events, MTD or MFD, and period of detectability of

(Continued)

TABLE 5 Continued

Identifier	Cancer type	Target	Additional treatment	Recruitment Status	Phase	Goals and Outcome
						NK-92/5.28.z cells in blood and cerebrospinal fluid. No results have been posted.
NCT02439788	Neuroblastoma	GD2	Cyclophosphamide, Fludarabine	Withdrawn	Phase I	Determine the number of patients with DLT within 4 weeks. No results have been posted.
NCT03692663	Prostate Cancer	PSMA	Cyclophosphamide, Fludarabine	Recruiting	Early phase I	Determine the occurrence of treatment related adverse events. No results have been posted.
NCT03941457	Pancreatic Cancer	ROBO1	NA	Unknown	Phase I and II	Determine the occurrence of treatment related adverse events. No results have been posted.
NCT03692637	Epithelial Ovarian Cancer	Mesothelin	NA	Unknown	Early phase I	Determine the occurrence of treatment related adverse events. No results have been posted.
NCT03415100	Metastatic Solid Tumors	NKG2D	NA	Unknown	Phase I	Determine the number of treatment adverse events and anti-tumor response due to CAR-NK cell infusions. No results have been posted.
NCT05194709	Advanced Solid Tumors	5T4	NA	Recruiting	Early phase I	Evaluate the safety and tolerability of anti-5T4 CAR-NK cells. No results have been posted.
NCT03940820	Solid Tumors	ROBO1	NA	Unknown	Phase I and II	Determine the occurrence of treatment related adverse events. No results have been posted.

RP2D, highest dose with acceptable toxicity; MFD, maximum feasible dose; CR, complete response; PR, partial response. Source: <https://clinicaltrials.gov>.

techniques aim to design suitable CAR-NK cell products (168). For example, the NK cells modification with a chimeric receptor consisting of activating receptor NKG2D (143), DNAX-activation protein 12 (DAP12) with CD3 $\zeta$  (169), and NKG2D- $\zeta$  (170) were used to improve NK cell cytotoxic activity. But even when CAR-NK cells are designed to enhance further the results obtained with normal NK cells, the use of engineering NK cells does not always appear to produce optimal results. For example, Bachiller et al. studied the therapeutic effect of CAR-T cells with CAR-NK cells and NK cells combinations against non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM) using cord blood as an NK cell source. The effectiveness of CAR-NK cells was observed only when using higher doses of cells, with a rapid loss of activity over time compared with CAR-T cells, while the control NK cells produced a synergistic anticancer CAR-T cell response (109).

## 9 Challenges and future perspectives

The principal feature of NK cells is their natural ability to protect the host by recognizing, directing, and executing the rapid lysis of cancerous, stressed, and viral-infected cells without dependent priming (33–35). However, several challenges overshadow the effective NK cell-mediated cancer immunotherapy. Those challenges, including short-term persistence, sensitivity, immunosuppression of the TME, and clinical-grade *ex vivo* expansion, should be addressed for a successful cell therapy treatment (24). The potential for NK therapies, demonstrated in pre-clinical studies, has clearly supported sustained research but outside of the field of hematological cancers, very little to no progress

have been reported in the clinic for any type of NK therapies against solid tumors with mostly due to a lack of results or early discontinuation of the trial. These discontinuations are in majority due to either off-target toxicities or a lack of response. While it seems that NK-based immunotherapies are of very little benefit to the patient, it is worth looking at the potential for optimizations that might be responsible for some of these early lackluster results.

NK cells have a highly sensitive response to external stresses, such as cryopreservation. The wellness, stability, and proper therapeutic responses of NK cells throughout the entire manufacturing process are highly dependent on maintaining stable, stress-free physiological settings (171). NK cells are very susceptible to stress stimuli, particularly cryopreservation, which is reflected in their loss of viability and cytotoxic function after thawing (131, 167): the average rate of cryopreserved NK cell survival is less than 50%. Even when the cytotoxicity of viable cells can be recovered overnight in the presence of IL-2, recovering the pre-freezing cell numbers could take several days of *in vitro* expansion. Similarly, exposure to low temperatures, such as overnight refrigeration, affects cytokine-activated NK cells, as observed by a reduction of their cytotoxicity, even when their viability is not affected (167). In a recent study, cryopreserved NK cells demonstrated a 5.6-fold reduced killing activity against K562 tumor cells embedded in a 3D collagen matrix compared with fresh NK cells. This behavior may be explained by two mechanisms affected by cryopreservation: (1) loss of NK cell activation through cleavage of CD16, and (2) reduction of NK cell motility, preventing contact with target cells (172). Other factors affected by cryo-injury in NK cells are cell metabolic activity, cytokine activation, cell culture density, and persistence (131). Since this particular issue negatively impacts

(through the use of cryopreservation, long-term storage, and shipping process) general immunotherapies before infusion, it is essential to intensify efforts and optimize procedures to reduce the NK cell dysfunction after thawing. Preserving the viability and cytotoxic function post-thaw is critical in a clinical setting to ensure a high consistency and quality of the products. Due to these issues observed after thawing during the *in vitro* culture, the direct infusion of freshly expanded NK cells “warm chain” may be advantageous. Another advantage to such “warm chain” would be the capability to add other immune fractions that could benefit from minimal handling and that can work in synergy with NK cells.  $\gamma\delta$ T cells or CD8<sup>+</sup> cytotoxic cells would be prime candidates for such combinations and could be obtained for the same leukapheresis product, for example. However, the realization of such personalized, combinatorial immune cell therapy would require the creation of a structure allowing the harvesting, isolation, expansion, re-invigoration and formulation of the immune cells at the point of care.

Regarding engineering modification, challenges such as the difficulty of transferring genetic material into NK cells in pre-clinical studies, selecting the most suitable and effective technique to accomplish the GMP grades should be a priority before scaling up the CAR-NK cell production. One of the primary issues with CAR-NK stem from either their lack of efficacy, or too much off-target cytotoxicity when applied in the clinic setting. These issues have been intensively scrutinized and researchers have been considering many variables to optimize therapies (149, 173, 174): (i) different cell sources such as immortalized cell lines (NK-92 or YT cell lines, that require irradiation before injection), peripheral NKs, stem-cell, umbilical or placenta-derived NKs, and ML-NKs, (ii) CAR design and use of the correct promoter, (iii) transduction methods such as retroviruses or electroporation. While these approaches have yielded encouraging pre-clinical results, it is still unclear as to how this can translate into the clinical setting. As underlined in Table 5, the lack of positive progress report or the discontinuation for current clinical trial are a testament of the difficulties to port these therapies to the clinic. Additionally, engineering of NK cell carries an additional production cost compared with non-engineered NK cells, and in some cases, the therapeutic response does not have the best cost-effectiveness ratio. Nevertheless, using NK cells over T cells as a vehicle to carry CAR expression has several advantages, such as the different NK cell sources and the donor-patient compatibility, which could reduce the average therapy manufacturing cost. Using iPSCs to obtain NK cells is a promising alternative to producing a significant number of therapeutic NK cells and then genetically modifying them. They are considered an advantageous source of NK and CAR-NK cells over peripheral and cord blood due to their versatility, homogeneity, high proliferation capacity, and usage in both autologous and allogeneic settings (4). This alternative is currently addressed in several research groups. For example, in 2019, Fate Therapeutics Company published clinical data about an iPSC-derived CAR-NK cell therapy against B Cell malignancies. In combination with rituximab, this therapy demonstrated prolonged *in vivo* cell survival and enhanced tumor-killing capacity compared with

rituximab therapy alone (4, 175). Moreover, against solid tumors (e.g., ovarian cancer), iPSC-derived CAR-NK also showed enhanced anti-tumor activity, inhibition of tumor growth, and prolonged survival compared with iPSC-NK cells, T-CAR-iPSC-NK cells, or PB-NK cells (176). These results underline the great potential that iPSCs-derived CAR-NK cells could offer for cancer immunotherapy. One intriguing possibility would be to combine the high cytotoxic and survival of ML-NK cells with the increased specificity and targeting conferred by CAR modifications. However, while this combination might yield significant clinical effect, it is unclear how the downside of CAR modifications would ultimately negatively affect the outcome.

While significant technical progress has been made to enhance the properties of NK cells, most of the progress has sought to correct issues that stem from the allogeneic and bioengineered nature of current NK cell therapies: short persistence of the product and off-target cytotoxicity are instances described in this review. Additionally, we reviewed evidence here that at least some cell anergies could be linked to a decentralized process, introducing detrimental inefficiencies such as product lead time, shipping, and freezing/thawing. Typically, a cell therapy designed by a biopharmaceutical entity would have to be produced and shipped to a clinical site specialized in infusion, where the processes specific to the product needs to be executed. These processes are extremely specific, from shipping/receiving to handling and dosing, and in return require extreme expertise from the staff involved. Meanwhile, the possible complications linked to novel NK cell therapies will always require the availability of specialized post-care monitoring and care by a hospital system or a primary oncologist. However, both the medical and regulatory fields have been slow to recognize NK cells and their unique abilities (and cell therapy as a whole) as a new class of therapies, so it follows that only highly trained and specialized staff should be monitoring and managing patients for these early therapies and their specific needs. The fragmentation of the process, and lack of specialization of the involved structures (especially the hospital system), are clear hurdle to the obtention of stable and interpretable results. The slow progress of NK cell immunotherapy, and the *de-facto* focus on allogeneic use of the cells that can only exacerbate possible cytotoxicities, may be linked to the inefficiencies listed above. These problems could be addressed with a new clinical paradigm, where the patient's cells could be directly harvested, expanded, and administered at the point of care, removing inefficiencies and lowering the cost of therapy (177), which remains one of the highest obstacles to the universal adoption of cellular immunotherapies. Patient's safety and deviations would consequently be minimized and improve trials success and patient care. Even if this model could counteract to some of the downsides of CAR-NK production, it would still not answer the lackluster clinical observations from CAR-NK therapies in the solid tumor space. Autologous therapies and patients would be the main beneficiaries of this model, since the need for an “off-the-shelf” product would become obsolete as safe and longer-lasting therapies can be created on-site directly from the patient and become a prime solution for cancer immune cell therapies.

## Author contributions

RW contributed to the conception, design, revision and approval of this manuscript. GL-V contributed to the writing of the sections and the design of figures in the manuscript. MT-L, RD and JP contributed to the manuscript revisions and approved the submitted version. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

JP is the founder/owner of CBOI and BioCytics. RW and RD are employees of CBOI and BioCytics.

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The remaining 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.

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## EDITED BY

Trent Spencer,  
Emory University, United States

## REVIEWED BY

Christopher Doering,  
Emory University, United States  
Gabriela Denning,  
Expression Therapeutics (United States),  
United States

## \*CORRESPONDENCE

Mary Poupot  
✉ mary.poupot@inserm.fr  
Christine Bezombes  
✉ christine.bezombes@inserm.fr

<sup>†</sup>These authors have contributed  
equally to this work

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# $\gamma\delta$ T cells in immunotherapies for B-cell malignancies

Léa Rimailho<sup>1†</sup>, Carla Faria<sup>1†</sup>, Marcin Domagala<sup>1†</sup>,  
Camille Laurent<sup>1,2</sup>, Christine Bezombes<sup>1\*</sup> and Mary Poupot<sup>1\*</sup>

<sup>1</sup>Cancer Research Center of Toulouse (CRCT), UMR1037 Inserm-Univ. Toulouse III Paul Sabatier-ERL5294 CNRS, Toulouse, France, <sup>2</sup>Department of Pathology, Institut Universitaire du Cancer de Toulouse - Oncopôle, Toulouse, France

Despite the advancements in therapy for B cell malignancies and the increase in long-term survival of patients, almost half of them lead to relapse. Combinations of chemotherapy and monoclonal antibodies such as anti-CD20 leads to mixed outcomes. Recent developments in immune cell-based therapies are showing many encouraging results.  $\gamma\delta$  T cells, with their potential of functional plasticity and their anti-tumoral properties, emerged as good candidates for cancer immunotherapies. The representation and the diversity of  $\gamma\delta$  T cells in tissues and in the blood, in physiological conditions or in B-cell malignancies such as B cell lymphoma, chronic lymphoblastic leukemia or multiple myeloma, provides the possibility to manipulate them with immunotherapeutic approaches for these patients. In this review, we summarized several strategies based on the activation and tumor-targeting of  $\gamma\delta$  T cells, optimization of expansion protocols, and development of gene-modified  $\gamma\delta$  T cells, using combinations of antibodies and therapeutic drugs and adoptive cell therapy with autologous or allogenic  $\gamma\delta$  T cells following potential genetic modifications.

## KEYWORDS

immunotherapy,  $\gamma\delta$  T cells, lymphoma, leukemia, myeloma

## 1 Introduction

T lymphocytes play a critical role in anti-tumor immunity. Besides broadly discussed conventional  $\alpha\beta$  T lymphocytes,  $\gamma\delta$  T cells are also now recognized in the context of cancer inhibition. In the blood, among peripheral mononuclear cells (PBMC),  $\gamma\delta$  T cells generally account for 1 to 5% whereas they are predominant in tissues such as skin and intestine (1). Both residents, as well as circulating  $\gamma\delta$  T cells upon migration to the tumor site, can display an anti-tumor effect. With a structural difference between the  $\gamma$  and  $\delta$  chains,  $\gamma\delta$  T cells can be divided into three main groups, V $\delta$ 1, V $\delta$ 2 and V $\delta$ 3 T cells, all of which recognize antigens independently of the major histocompatibility complex (MHC) molecules.

In B-cell malignancies, such as B-cell lymphomas, chronic lymphocytic leukemia (CLL) or multiple myeloma (MM), tumor cells can be found both in peripheral blood (PB) and in lymphoid organs, such as bone marrow (BM) or lymph nodes (LN). Therefore, these malignant cells can interact with other cell types constituting a specific microenvironment,



in which infiltrating  $\gamma\delta$  T cells can play an important role. V $\delta$ 1 and V $\delta$ 2 T cells have been described to participate in the anti-cancer responses in B-cell malignancies with sometimes different proportions and different modes of action.

In this review, we first described  $\gamma\delta$  T cell diversity in B-cell lymphomas, CLL and MM. We then focused on  $\gamma\delta$  T cell activation and finally we presented attractive candidates for immunotherapies (IT) in B-cell malignancies.

## 2 $\gamma\delta$ T cell diversity in B-cell lymphomas, CLL and multiple myeloma

Following T cell receptor (TCR) rearrangement,  $\gamma\delta$  T cells can be categorized into three main groups: the variable V $\gamma$ 9 chain paired with V $\delta$ 2 (V $\gamma$ 9V $\delta$ 2 T cells, also known as V $\delta$ 2 cells) (2), the variable V $\delta$ 1 chain with different V $\gamma$  chains (3) and V $\delta$ 3 T cells. Lymphocytes expressing heterodimers of V $\delta$ 2 and V $\gamma$ 9 chains are predominant in the blood where they account for most (50–95%) of the  $\gamma\delta$  T cells, whereas V $\delta$ 1 T cells (paired with various V $\gamma$  chains) are more abundant in tissues, including healthy epithelia or solid tumors (4). V $\delta$ 3 like V $\delta$ 1 T cells were shown as dominant in the intestinal mucosa, skin, and liver (3), and to actively participate in cancer immunobiology.

These lymphocytes can differentiate into different T helper-like cells (Th1-, Th2-, Th9-, and Th17-like cells), producing a wide range of cytokines to fulfill their physiological role (5–7). More precisely,  $\gamma\delta$  T cells can harbor different phenotypes, such as: naive, central memory (CM), effector memory (EM) or RA<sup>+</sup> effector memory (TEMRA) (8, 9). Moreover,  $\gamma\delta$  T cells co-express other functional receptors, including activating natural killer receptors (NKR: NKG2D, NKp30 and NKp44) (10, 11) and various Toll-like receptors (TLRs) (12). However, they can also express inhibitory NKR such as CD94/NKG2A or immune checkpoints (ICP), such as: PD-1, TIM3, LAG3 or CD39. Interestingly, NKG2A<sup>+</sup> V $\delta$ 2 T cells were shown to exert higher anti-tumor potential (13).

Patients with Hodgkin's Lymphoma (HL) were characterized with a marginally higher level of circulating  $\gamma\delta$  T cells, compared to healthy donors (14). The tumor escape from immune surveillance by the  $\gamma\delta$  T cells in these patients could therefore be due to the immunosuppressive profile of these cells plus an increase of soluble MICA derived from its shedding at the surface of lymphoma cells. Interestingly, HIV-infected individuals developing HL were also shown to display a significant expansion of the V $\delta$ 1 T cell subset compared to those without HL. To go further, the authors showed a high expression of CD16 and the inhibitory receptor CD158b by these V $\delta$ 1 T cells, concomitantly with a low expression of CCR5, CXCR4 and CXCR3, thus decreasing their homing to the tumor site (15). This discrepancy could point to a causal role in the pathogenesis of HL.

On the other hand, in B-cell non-Hodgkin's lymphomas (B-NHL), the major subtypes of circulating  $\gamma\delta$  T cells were shown to be V $\gamma$ 1, V $\delta$ 1 and V $\delta$ 2 (16). Compared to healthy donors, patients exhibit an absence of V $\gamma$ 2 TCR subfamily in PB, BM, and LN. This

implies a widespread restriction of the V $\gamma$  gene expression repertoire that may be a feature in patients with B-NHL. Moreover, the distribution of V $\gamma$  and V $\delta$  subfamilies varied between PB, BM, or LN, and this may be due to the distribution or expansion of  $\gamma\delta$  T cells in different immune organs and to local immune responses (16).

In the case of diffuse large B-cell lymphoma (DLBCL), an aggressive form of B-NHL,  $\gamma\delta$  T cells represent a substantial population among infiltrating T lymphocytes. Amongst this population, V $\delta$ 1 T cells were shown as the major  $\gamma\delta$  T cell subset in both tumor and PBMC, whilst V $\delta$ 2 T cells were the most common subset in PBMC of healthy donors (17). In this study, the V $\delta$ 1 T displaying a naive phenotype (whether in blood or in LN) were shown as functional, however the authors did not observe any correlation between the rate of V $\delta$ 1 T and well-established prognostic factors, clinical responses or progression-free survival (PFS). Interestingly, the germinal center (GC) subtype of DLBCL was associated with an increase in V $\delta$ 1 cells in the tumors, whereas the non-GC subtype was associated with a lower frequency of  $\gamma\delta$  T cells (17). Activation or reactivation of V $\delta$ 1 T cells in DLBCL patients either by using *ex-vivo* expanded cells or by promoting their expansion *in vivo*, could represent a therapeutic outcome.

In the case of follicular lymphoma (FL), Braza and collaborators showed that  $\gamma\delta$  T cells as well as CD8<sup>+</sup> T lymphocytes were located in the perifollicular zone of the LN of FL patients and not inside follicles. The majority of FL-LN  $\gamma\delta$  T cells are V $\delta$ 2 CCR7<sup>+</sup> unlike circulating ones, whereas expression of the chemoattractant CCL19 chemokine is lower in FL-LN than in inflamed LN, explaining the low  $\gamma\delta$  T cell count in FL-LN (18). However,  $\gamma\delta$  T cells from FL patients displaying good cytolytic properties against lymphoma cells, *ex-vivo* expansion or promotion of *in vivo* expansion could be a therapeutic option if expanded  $\gamma\delta$  T cells can home in to the tumor site. Nevertheless, in this study, the authors considered only the V $\delta$ 2 subtype without taking into account the V $\delta$ 1 cells, which can counterbalance the decrease of V $\delta$ 2 T cells. In another study, the authors compared reactive LN from lymphoma-free individuals with FL-LN. Unlike Braza's work, they showed no significant difference in the percentage of the cytolytic  $\gamma\delta$  T cell population between reactive LN and FL-LN (19). The immune microenvironment of LN can therefore have an important impact on the phenotypical and functional characteristics of infiltrated T cells.

Increase in V $\delta$ 1 T cells was also observed in CLL and MM patients. The analysis of PB of patients with CLL revealed a general prevalence of the V $\delta$ 1 T cell subtype, with an increased cell count in more severe stages of the disease (20). This increasing percentage of V $\delta$ 1 cells was also observed as belonging to the CD27<sup>+</sup> compartment from controls to advanced stages of CLL patients, in particular in Binet B and C CLL groups, exhibiting a cytotoxic phenotype with the expression of granzyme B (11). Another study showed an increase of V $\delta$ 1 cells in the blood of CLL patients with stable disease, which were able to proliferate and produce TNF- $\alpha$  and IFN- $\gamma$  in response to autologous CLL cells suggesting the potential of V $\delta$ 1 cells based therapies in this disease (21). These contradictions could be explained by the expression of some exhaustion markers, not determined in this study which can thwart the cytotoxic efficacy. An increase of exhaustion markers



such as PD-1, TIGIT, TIM3 and CD39, expressed by V $\delta$ 1 cells was also shown in BM of MM patients. Whilst these patients displayed no difference regarding an overall level of  $\gamma\delta$  T cells in comparison to healthy donors, they showed a higher proportion of V $\delta$ 1 over V $\delta$ 2 T cells (22). Elevated percentage of  $\gamma\delta$  T cells with an exhausted phenotype (PD-1<sup>+</sup>), associated with a decreased expression of genes involved in effector functions was also found in patients with relapsed/refractory MM (23). However, exhaustion problems could be overcome by using ICP inhibitors such as anti-PD-1 antibodies. Additionally, inhibition of the anti-tumor immune response was associated with elevated levels of  $\gamma\delta$  regulatory T cells in the PB of MM patients with a bad prognosis (24) as well as in CLL patients (25). Besides, the increase of circulating cytotoxic  $\gamma\delta$  T in PB of MM patients after autologous hematopoietic stem cell transplantation was associated with improved PFS and overall survival (OS) (26).

Finally,  $\gamma\delta$  T cells were detected in all B-cell malignancies with an exhausted phenotype. Their reactivation or manipulation with immunotherapeutic approaches could represent a promising therapeutic option for patients with these diseases as developed in the section 4.

### 3 $\gamma\delta$ T cell activation in B-cell lymphomas, CLL and multiple myeloma

Depending on their TCR variant,  $\gamma\delta$  T cells, can respond to a variety of antigens. V $\delta$ 1 and V $\delta$ 3 cells can recognize, *via* their bound TCR glycolipids, MHC-related class Ib molecules CD1c for V $\delta$ 1 (27) and CD1d for V $\delta$ 3 (28). On the other hand, V $\delta$ 2 cells recognize non-peptidic antigens in the form of small pyrophosphate molecules called phosphoantigens (PAg), that can be found endogenously, such as hydroxymethyl-butyl-pyrophosphate (HMBPP/HDMAPP) or metabolites of the mevalonate pathway, or synthetic molecules, such as BrHPP (bromohydrine pyrophosphate) (29, 30). Zoledronate, a third generation aminobisphosphonate, a farnesyl pyrophosphate synthase inhibitor, widely used for osteolysis, has been shown to enhance antitumor V $\delta$ 2 cell responses, through the overexpression of endogenous PAg by tumor cells (31, 32). MHC-class I molecules are not involved in PAg recognition by V $\delta$ 2 T cells, but other molecules including butyrophilins (BTN3A1/BTN2A1), the ABCA1 transporter, the intracellular RHOB or periplakin molecules were shown to be involved in their activation (33–37). Besides TCR involvement,  $\gamma\delta$  T cells expressing NKR and TLRs, are also able to respond to stress-induced NKR ligands such as the ribonucleic acid export 1 (RAE1), MHC class I-related molecule A or B (MICA/MICB), UL16-binding proteins (ULBPs) (11, 38) amongst other DAMPs or PAMPs (12). Interaction of  $\gamma\delta$  T cells with cancer cells expressing these molecules, leads to the formation of an immunological synapse (39, 40) resulting in  $\gamma\delta$  T cell proliferation, cytokine release and tumor cell lysis (41). However, other molecules such as CD226 (DNAX accessory molecule-1), adhesion molecules (ICAM-1), CD3 or CD2, can also be involved in

$\gamma\delta$  T cell activation and favor their immune responses. These different activation modes are summarized in Figure 1.

Considering the maturation stages,  $\gamma\delta$  TEM cells expressing high levels of chemokine receptors, produce large amounts of IFN- $\gamma$  and TNF- $\alpha$  in response to TCR stimulation and  $\gamma\delta$  TEMRA cells, which express several NKR but low levels of chemokine receptors, are highly active against tumoral target cells and efficient to ADCC thanks to CD16 expression. These  $\gamma\delta$  T effector cells express their cytotoxicity through the production of high amounts of perforin and granzyme. Naive and CM cells do not display effector functions but are able to proliferate.

$\gamma\delta$  T cells from patients with B-cell malignancies and particularly V $\delta$ 2 T cells were evaluated for their functionality, by the *in vitro* sensitization of cancer cells or cells of the tumor microenvironment with zoledronate (31, 32). In NHL, LN mesenchymal stromal cells were shown to interfere with V $\delta$ 2 lymphocyte cytolytic function and differentiation into Th-1 or EM cells but pre-treatment of these immunosuppressive cells with zoledronate can rescue lymphoma cell killing *via* the TCR and NKG2D (32). V $\delta$ 2 T cells from patients with B-cell lymphoma and MM, expanded *in vitro* by culture with zoledronate and IL-2, displayed enhanced cytotoxic effects towards MM/B-cell lymphoma cell lines and autologous tumor cells, without cytotoxicity against normal cells in these patients (31). However, approximately 50% of untreated MM patients showed V $\delta$ 2 T cells that were unable to proliferate upon stimulation with zoledronate and IL-2, but had strong effector properties exhibiting TEM or TEMRA phenotypes (42, 43). Similar results have been described for untreated CLL patients, who were classified as responders and displayed proliferation of zoledronate-stimulated V $\delta$ 2 T cells (25). Interestingly, the low-responders showed significantly greater baseline peripheral V $\delta$ 2 T cell counts than the responders, ruling out a quantitative defect. Indeed, the low-responder patients showed an accumulation of TEM and TEMRA V $\delta$ 2 cells with high effector functions and low capacity to proliferate, whereas naive and CM were preferentially found in responding patients (25, 44). In addition, Coscia and collaborators showed that a low proliferation capacity of V $\delta$ 2 cells was correlated with subsets of CLL patients with unmutated immunoglobulin heavy variable (IGHV) genes (U-CLL). This is in agreement with the upregulation of NKG2D on  $\gamma\delta$  T cells of CLL patients responding to zoledronate (25). V $\delta$ 2 T cells isolated from PBMC of MM patients were also shown to upregulate NKG2D upon *in vitro* expansion with zoledronate. Additionally, a low-dose treatment with bortezomib (proteasome inhibitor typically used in MM) sensitized MM cells to *in vitro* lysis by V $\delta$ 2 cells through NKG2D (45). V $\delta$ 1 cells may also be involved in the anti-cancer response towards CLL cells through NKG2D activation. Indeed, V $\delta$ 1 cells, which are enriched in PB of CLL patients, can kill neoplastic CLL cell lines transfected with MICA, and blocking anti-NKG2D antibody largely decreases autologous leukemic cell lysis (21). In addition, purified V $\delta$ 1 cells isolated from the PB of MM patients can kill MM cell lines, and produce cytokines involving their TCR and NKG2D, DNAM-1 and adhesion molecules (46). Unfortunately, cancer cells are able to express some enzymes such as ADAM 10 and 17 and are able to shed the stress molecules MIC-A and -B and

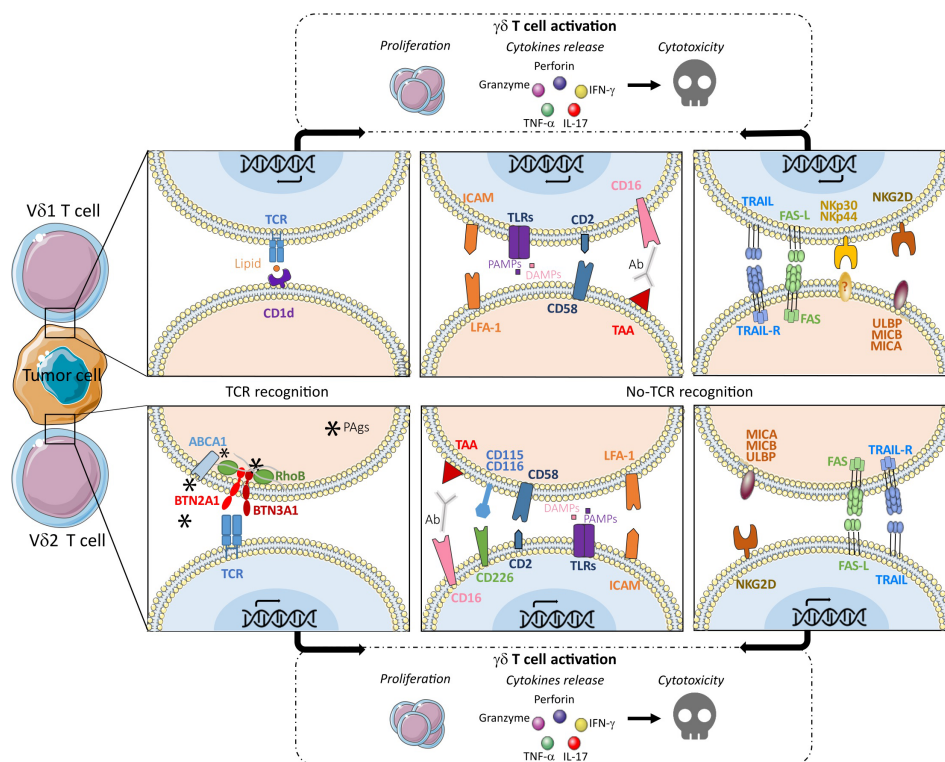


FIGURE 1

$\gamma\delta$  T cell activation through different TCR-dependent and TCR-independent pathways leading to proliferation and/or cytokine release and/or cytotoxic signals.

ULBPs from their surface, therefore decreasing the  $\gamma\delta$  T response through NKG2D (47).

As  $\gamma\delta$  T cells, whether V $\delta$ 1 or V $\delta$ 2, can be activated by B-cell lymphoma, CLL or MM cells, these cells represent essential actors in anti-tumor responses against B-cell malignancies and can definitely be good targets for IT in these diseases.

## 4 $\gamma\delta$ T cell-based immunotherapies in B-cell malignancies

For a long time neglected,  $\gamma\delta$  T cells have emerged as a key immune cell type in cancer biology, representing very attractive and promising candidates for cancer IT. Their therapeutic potential in solid and hematological cancers have been extensively reviewed elsewhere (48–53) and here we focused on their exploitation in B-cell malignancies.

$\gamma\delta$  T cells can be used in several strategies, based on the *in vivo* activation to potentiate the tumor-targeting or the optimization of *in vivo* or *ex vivo* expansion protocols. These approaches consist of: i) combination with therapeutic drugs or antibodies, ii) adoptive cell transfer (ACT) using of autologous or allogenic  $\gamma\delta$  T cells expanded *ex vivo* and iii) ACT using genetically modified  $\gamma\delta$  T cells. The timeline of  $\gamma\delta$  T cell-based IT is presented in the Figure 2 and the various cell-based IT, ongoing clinical trials, and associated sponsors are summarized in the Table 1.

### 4.1 Combination of $\gamma\delta$ T cells with therapeutic drugs or antibodies

$\gamma\delta$  T cells can be directly activated by drugs or antibodies modifying their effector properties and/or potentializing their *in vivo* expansion, but also indirectly by increasing the sensitization of cancer cells.

Concerning sensitization of tumor cells, Poggi and collaborators showed that trans-retinoic acid, an active metabolite of vitamin A, was able to induce MICA expression at the surface of CLL cells from patients, leading to an increase of their lysis by autologous V $\delta$ 1 T cells (21). Other drugs such as bortezomib, were also involved in the up-regulation of NKG2D and DNAM-1 ligand expression by MM cells, leading to the enhancement of the V $\delta$ 2 T cell cytotoxic effect (45). Moreover, the use of ADAM 10 and 17 inhibitors on HL cell lines revealed an increase of their sensitivity to NKG2D-dependent cell killing mediated by NK and  $\gamma\delta$  T cells (47). Another class of molecule, HDAC inhibitors, were also able to increase expression of NKG2D ligands by pancreatic or prostate cancer cells (54) and could be interesting in B-cell malignancy therapies. However, these molecules also suppress the  $\gamma\delta$  T cell anti-tumor functions inducing a non-functional truncated form of NKG2D and increasing ICP expression (55, 56). Another way to increase the NKG2D mediated anti-tumor effect of  $\gamma\delta$  T cells, is to use recombinant immunoligands consisting of a CD20 single-chain fragment variable (scFv) linked to MICA or ULBP2. Indeed, killing by both

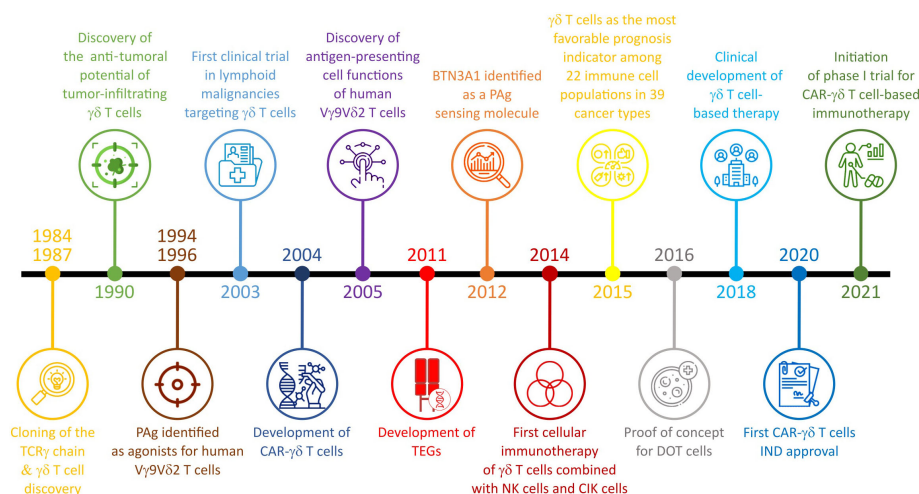


FIGURE 2

Discovery timeline of the  $\gamma\delta$  T cell role in cancer and  $\gamma\delta$  T cell-based IT (adapted from *Silvia-Santos et al., Nature Review 2019* and *Bhat et al., Frontiers in Immunology 2022*).

TABLE 1 Summary of  $\gamma\delta$  T cell-based IT, ongoing clinical trials and associated sponsors.

Cell-Based Immunotherapy approaches in B-cell malignancies				
Type of therapy	Disease	Agents in development	Sponsors	Reference/Clinical trial number
Combination with therapeutic drugs or antibodies				
Zoledronate/IL-2	MM	NA	NA	(60)
Pamidronate/IL-2	Relapsed/refractory low-grade NHL and MM	NA	NA	(61)
BrHPP/IL-2	FL	NA	NA	(63)
Zoledronate	ALL and AML	NA	NA	(65, 66)
Zoledronate/IL-2	Hematological malignancies	NA	Nantes University Hospital	NCT03862833
Anti-BTN3A1+anti-PD-1	R/R DLBCL and FL	ICT01	ImCheck Therapeutics	NCT04243499
$\gamma\gamma$ TCRxCD1d bAb	R/R CLL, AML and MM	LAVA-051	Lava Therapeutics	NCT04887259 (74),
$\gamma\gamma$ TCRxCD40 bAb	CLL, MM	LAVA-1278	Lava Therapeutics	(75)
CD19xCD16bAb	ALL	NA	NA	(73)
Adoptive cell transfer using of autologous				
Zoledronate-activated V $\delta$ 2 T cells	CLL, AML, ALL		University of Kansas Medical Center & In8bio Inc.	NCT03533816
Adoptive cell transfer using allogeneic $\gamma\delta$ T cells expanded ex vivo				
Zoledronate/IL-2 activated allogeneic $\gamma\delta$ T cells from healthy donors	Advanced refractory MM			(83)
2 cycles of 2 dosage escalated manner infusions at 14 days intervals	R/R NHL		Institute of Hematology & Blood Diseases Hospital	NCT04696705

(Continued)

TABLE 1 Continued

Cell-Based Immunotherapy approaches in B-cell malignancies				
Type of therapy	Disease	Agents in development	Sponsors	Reference/ Clinical trial number
			& Beijing GD Initiative Cell Therapy Technology Co	
Dose escalation between 3 cohorts (negative MRD or SD, positive MRD but not HR, HR)	AML, ALL, Lymphoma		Chinese PLA General Hospital & Beijing	NCT04764513
Allogenic vd1 and T cell therapy	AML, CLL	GDX012	GammaDelta Therapeutics Limited	NCT05001451, (84, 97)
Adoptive cell transfer using $\gamma\delta$ T cells genetically modified				
CD20 directed CAR- $\delta$ 1 T cells	FL, MCL, MZL, DLBCL, NHL	ADI-001	Adicet Bio, Inc	NCT04735471 (89, 90),
$\alpha\beta$ T cell product retrovirally transduced with $\gamma\delta$ 2 TCRs	AML R/R MM	TEG001 TEG002	Gadeta B.V.	NCT04688853, (93, 94)
CD19-CAR (Ab)TCR	R/R CD19 <sup>+</sup> NHL	ET190L1 ARTEMIS <sup>TM</sup>	Duke University & Duke Clinical Research Institute Peking University & Eureka(Beijing) Biotechnology	NCT03379493, NCT03415399 (95),
CD19-directed CAR $\gamma\delta$ 2 T cells	ALL	NA		(86)
Anti-CD19 CAR- $\gamma\delta$ T cells	R/R CD19 <sup>+</sup> B-cell leukemia and lymphoma	ET019003-T Cells	Wuhan Union Hospital, China &Eureka(Beijing) Biotechnology	NCT04014894

NA, not applicable.

V $\delta$ 2 and V $\delta$ 1 T cells, of CLL cells from patients and lymphoma cell lines sensitized by these two immunoligands was significantly increased (57).

The direct activation of  $\gamma\delta$  T cells can be achieved by the TCR dependent pathway through exogenous or endogenous PAg. As mentioned previously, aminobisphosphonates induce the production of endogenous PAg in tumor cells. Treatment of BM mononuclear cells from MM patients with aminobisphosphonates induced an *in vitro* stimulation of  $\gamma\delta$  T cell-mediated anti-plasma cell activity, as well as a tumor regression in myeloma xenografted mouse models (58, 59). However, a phase II clinical trial of zoledronate/IL-2 treated MM patients after BM transplantation led to only 18% of complete remission (CR) due to a progressive reduction of  $\gamma\delta$  T cells *in vivo* expansion despite several cycles of zoledronate/IL-2 injections (60).

In addition, an *in vivo* amplification of autologous  $\gamma\delta$  T cells has been shown following injection of aminobisphosphonates and IL-2. The first study was conducted by Wilhelm and collaborators, where a low-dose of IL-2 in combination with pamidronate was tested, according to two different schedules, in 19 patients with relapsed/refractory low-grade NHL (FL, CLL, mantle cell lymphoma-MCL) and MM (61). The first treatment schedule consisted of administration of pamidronate on day 1 followed by increasing dose levels of IL-2 from day 3 to day 8. Unfortunately, only 1 out of 10 treated patients achieved a stable disease. The other treatment

schedule consisted of pamidronate infusion, followed directly by IL-2 administration from day 1 to day 6. In that case, a significant *in vivo* activation/proliferation of  $\gamma\delta$  T cells was observed in 5 out of 9 patients and objective responses were achieved in 3 patients (33%). Interestingly, *in vivo* proliferation of  $\gamma\delta$  T cells was associated with tumor regression confirming a  $\gamma\delta$  T cell-mediated anti-lymphoma effect. This correlation was also observed in a B-cell depletion assay from cynomolgus monkeys injected with the regimen combining an anti-CD20 mAb (rituximab) with BrHPP and IL2 (62). Thanks to promising results from pre-clinical studies (18), it was possible to enter clinical phase I/II studies where the effect of BrHPP (IPH1101) combined with low doses of IL2 was evaluated in 45 FL patients. The treatment induced a strong and specific amplification of  $\gamma\delta$  T cells with a 45% overall response rate (63). Unfortunately, the final outcomes were never published. Besides, Zoledronate exhibited promising results in hematological malignancies, such as acute myeloid leukemia (AML) (64) where 25% of partial response was reached. In pediatric acute lymphocytic leukemia (ALL) and AML, after B- and  $\alpha\beta$  T-cell-depleted and HLA-haploidentical hematopoietic stem cell transplantation (HSCT), infusion of zoledronate lowered transplantation-related mortality, increased the number of circulating  $\gamma\delta$  T cells and improved disease-free survival (65, 66). Currently, a phase I clinical trial is open to determine the maximum tolerated dose of early administration of increasing doses of IL-2 in combination



with a fixed dose of zoledronate, in order to expand V $\delta$ 2 cells in patients with a hematological disease eligible for a haplo-stem cell transplantation (NCT03862833).

Some antibodies have also been shown as activating  $\gamma\delta$  T cell anti-tumor functions. A first-in-class humanized anti-BTN3A1 antibody was designed to harness and enhance V $\delta$ 2 cell-driven anti-tumor activity against multiple tumor cell lines and primary tumor cells (67), opening promising perspectives. A phase I/IIa trial in patients with advanced-stage relapsed and/or refractory cancers including DLBCL and FL (NCT04243499) are currently opened to assess the safety, tolerability and efficacy alone or in combination with the anti-PD-1 mAb pembrolizumab.

ICP blocking antibodies can also favor the anti-tumor cytotoxic potential of  $\gamma\delta$  T cells, against the Burkitt lymphoma cell line Raji for instance (55), arguing that in some cases, ICP can be the only barrier to the cytotoxic functionality of these effector cells. These antibodies in MM treatment were shown to improve PAg-activation of V $\delta$ 2 T cells as, in the BM microenvironment, these cells largely express PD-1 hampering their PAg-reactivity (68, 69). This anergy was also detected in CLL with a reduction of cytotoxicity related to reduced granzyme secretion (44), opening up the possibility of using anti-ICP antibodies in CLL treatment.

Antibodies targeting Fc receptors, such as CD16 expressed by  $\gamma\delta$  T cells, can also artificially enhance their cytolytic function *via* ADCC. Efficacy of ADCC in B-cell malignancies has been shown using anti-CD20 (19, 62, 70–72), anti-CD52 (62) or anti-CD38 (72) mAbs, all these antibodies target different molecules at the surface of cancer cells. Moreover, potentiation of ADCC using anti-CD20 was observed with the BrHPP/IL-2 stimulation of V $\delta$ 2 T cells from PBMC of CLL patients in autologous co-cultures (62).

Finally, another category of antibodies consists of bispecific Ab (bsAb) that simultaneously bind  $\gamma\delta$  T cells and a tumor antigen. These bsAb strongly enhanced lysis mediated by  $\gamma\delta$  T cells, as shown for the SPM-1 Ab, a single chain trispecific Ab (triplebody or tribody) directed against CD19-CD19-CD16 that efficiently redirected lysis of CD19-bearing target cells (73). De Weert and collaborators, showed a robust activation and degranulation of V $\delta$ 2 T cells in co-culture with autologous CLL cells expressing CD1d treated with  $\gamma\gamma$ TCRxCD1d bsAb (LAVA-051) (74). As CD40 is also overexpressed in CLL and MM, the bsAb CD40-V $\gamma$ 9V $\delta$ 2 T cell engager (LAVA-1278) was shown as promoting a potent V $\delta$ 2 T cell degranulation and cytotoxicity against CLL and MM cells *in vitro* and *in vivo* (75). Recently, a novel bispecific molecule was developed by linking the extracellular domains of tumor-reactive V $\gamma$ 9V $\delta$ 2 TCR to a CD3-binding moiety, creating  $\gamma\delta$ TCR-anti-CD3 bispecific molecules (GABs). The high affinity of V $\delta$ 2 for PAg enriched in tumor cells favored the recruitment of other CD3<sup>+</sup> T cells in the TME enhancing the *in vivo* targeting of MM cells and leading to tumor regression (76). Altogether, these bsAbs represent promising candidates for the development of novel treatments for B-cell malignancies and for now, only one phase I/II clinical trial is opened (NCT04887259) to assess the efficacy of LAVA-051 ( $\gamma\gamma$ TCRxCD1d bAb) in patients with relapsed/refractory CLL and MM in whom it appears to be well tolerated (77).

## 4.2 Adoptive cell transfer with autologous $\gamma\delta$ T cells

One of the biggest advantages of using autologous  $\gamma\delta$  T cells in adoptive cell transfer for IT is the lack of graft versus host disease (GVHD). The ACT of autologous  $\gamma\delta$  T cells requires *ex vivo* expansion of  $\gamma\delta$  T cells thanks to the activation of purified PBMC from the blood of the patient by IL2 and either natural (isopentenyl pyrophosphate-IPP and HMBPP) or synthetic (BrHPP) PAg. The prerequisite being the capacity of patient  $\gamma\delta$  T cells to be expandable in *in vitro* culture. In the case of activation with exogenous PAg added to IL-2, PBMC of CLL patients showed a significant *ex vivo* expansion of V $\delta$ 2 T cells with the ability to secrete lytic granules leading to the efficient killing of autologous CLL cells (62). *Ex vivo* expansion of  $\gamma\delta$  T cells offers an opportunity to characterize their phenotype and sort the cells with the highest anti-tumoral potency, prior to their reinfusion into patients. So far, this approach has only been described for solid tumors (78). In MM patients, high-dose administration of *ex vivo* zoledronate-activated V $\delta$ 2 T cells resulted in a measurable increase of V $\delta$ 2 cell number in PB and BM, which was correlated with an anti-tumoral effect in 4 of 6 patients (79). A phase I clinical trial is currently opened in CLL (NCT03533816) in order to extract, concentrate, and activate  $\gamma\delta$  T cells from the PB to provide an innate anti-tumor effect.

## 4.3 Adoptive cell transfer with allogeneic $\gamma\delta$ T cells

In almost all autologous studies, treatments showed the reduction of tumor burden some patients, but the effects were inconsistent. It becomes clear, that the failure of these strategies can be due to the poor T-cell fitness of patients heavily pre-treated with chemotherapy. Thus, to overcome this issue, a huge effort has been developed to propose “off-the-shelf” therapies using allogeneic  $\gamma\delta$  T cells isolated from healthy donors. Due to their unique property to recognize antigen in a MHC independent manner and that they do not require HLA-matching of donors and recipients,  $\gamma\delta$  T cells are ideal candidates to develop ACT strategies. ACT with allogeneic V $\delta$ 2 cells is more often tested in patients harboring solid cancers (80–82). In hematological malignancies, only one study reported the infusion of allogeneic  $\gamma\delta$  T cells from healthy donors, in patients harboring, amongst others, advanced refractory MM who were not eligible for allogeneic transplantation (83). Proliferation of  $\gamma\delta$  T cells peaked after 8 days and donor cells persisted up to 28 days. Although refractory to all prior therapies, 3 out of 4 patients achieved a CR, which lasted for 8 months in a patient with plasma cell leukemia. Thus, this pilot study indicated that the use of allogeneic  $\gamma\delta$  T cells, from selected donors who were half-matched (HLA-haploidentical) family members, is feasible and safe, and that zoledronate/IL-2 infusions can activate and expand allogeneic  $\gamma\delta$  T cells *in vivo* to achieve promising therapeutic responses.



#### 4.4 Adoptive cell transfer with allogenic genetically modified $\gamma\delta$ T cells

The V $\delta$ 1 subset of  $\gamma\delta$  T cells is a promising candidate for cancer IT but suffers from the lack of a suitable expansion/differentiation method. Thus, without any genetic modification, Sebestyen's group was the first to develop a robust and reproducible clinical-grade method for generating cytotoxic V $\delta$ 1 T cells called Delta One T (DOT) cells that have been expanded and differentiated (84). Based on studies of CLL models, DOT exhibited cytotoxic features and specifically targeted leukemic *in vitro* and in preclinical *in vivo* models (cell line- or patient derived-xenograft), controlling the burden and dissemination of cancer cells (84).

Concerning CAR-T cells,  $\alpha\beta$  T cells were the first T cells developed for ACT. However, even though CAR- $\alpha\beta$  T cells are still developed in cancer IT, potential GVHD apart from cytokine toxicity and antigen escape pose limitations to this approach. CAR- $\gamma\delta$  T cells rapidly become a highly interesting alternative due to their HLA-independent antitumor immunity. Thanks to the progress made in the field of engineering and expansion protocols consistent with current good manufacturing practices, CAR- $\delta$ 1 and CAR- $\delta$ 2 T cells were developed during the recent years. Therefore, CAR- $\gamma\delta$  T cells appeared to have their niche in situations where conventional CAR therapy is less suitable. In 2004, Rischer and collaborators were pioneers to demonstrate that zoledronate-activated CD19-CAR- $\gamma\delta$  T cells exhibited a potent and specific anti-tumor activity against B cell malignancies *in vitro* (85). Ten years later, the group of L.J.N. Cooper developed a CD19-directed CAR- $\gamma\delta$  T cell that displayed enhanced killing of CD19<sup>+</sup> tumor cells *in vitro* and in leukemia xenograft models (86). These observations were also obtained by other groups (87).

Due to their long persistence *in vivo*, V $\delta$ 1 T cells represent attractive candidates for ACT. Based on the "DOT protocol" (84), a clinically translatable protocol for V $\delta$ 1 T cell expansion allowed the development of CAR- $\delta$ 1 T cells that exhibited highly consistent innate cytotoxicity against different leukemic cell lines (88). Of note, CD20 directed CAR- $\delta$ 1 T cells (under the name ADI-001) exhibited a potent anti-cancer activity both *in vitro* and *in vivo*, in B-cell lymphoma xenografts in NSG mice bringing strong evidence to propose the assessment of its efficacy in phase I clinical trial (NCT04735471) in patients with B-cell malignancies (89). The first results showed that ADI-001 maintained a favorable safety profile, and preliminary efficacy showed very encouraging CR rate (4/5) and sustained durability in patients, including those previously exposed to conventional CAR-T therapy (90). Based on the same strategy, a 4-1BB-based CAR-DOT directed against CD123 was generated and preclinically validated in AML, with a potent cytotoxicity against cell lines and primary samples both *in vitro* and *in vivo*, even following a tumor rechallenge (91).

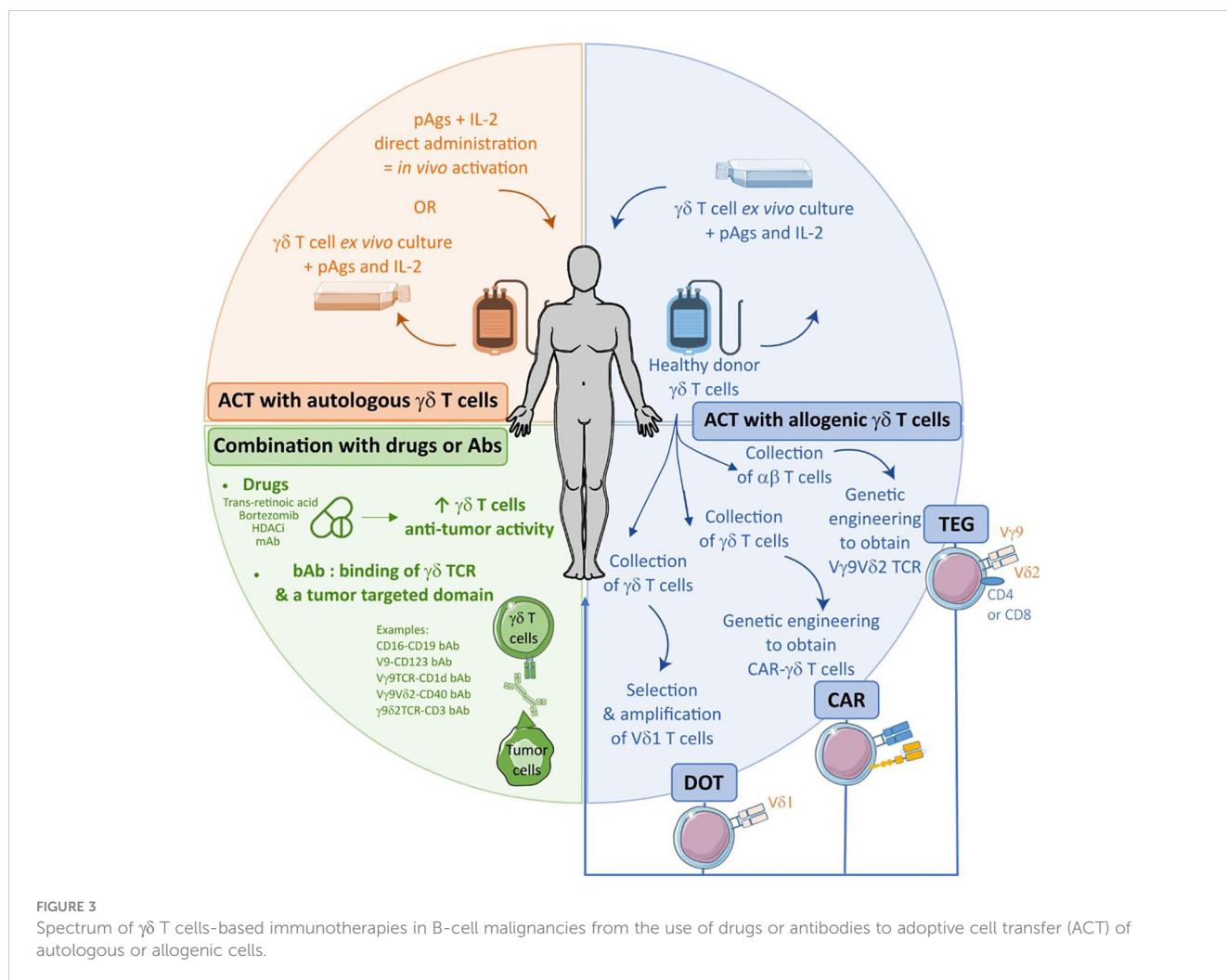
Although promising results were obtained with CAR- $\gamma\delta$  T cells, limited proliferative capacity of V $\delta$ 2 cells and their underestimated diversity, led to the development of  $\alpha\beta$  T cells engineered to express a defined  $\gamma\delta$  TCR, the so-called TEGs (92), that can target a broad range of hematological tumors (93, 94). Interestingly, these cells not only exhibited strong anti-tumor reactivity and potent proliferative

capacity of  $\alpha\beta$  T cells, leading to tumor eradication in leukemic PDX models, they also retained both CD4<sup>+</sup> and CD8<sup>+</sup> effector cell functions (94). Currently, a phase I clinical trial (NCT04688853) is testing the TEG002, an autologous T cell transduced with a specific  $\gamma\delta$  TCR, in relapsed/refractory MM patients. Another strategy is based on the combination of the Fab domain of an antibody with the  $\gamma$  and  $\delta$  chains of the TCR (AbTCR) as the effector domain (95). This CD19-CAR (Ab)TCR (ET190L1 ARTEMIS<sup>TM</sup>) triggered Ag-specific cytokine production, degranulation and killing of CD19<sup>+</sup> cancer cells *in vitro* and in a xenograft mouse model. Whether these pre-clinical findings for AbTCR translate into clinical settings has been assessed in two clinical trials in relapsed and refractory CD19<sup>+</sup> NHL (NCT03379493, NCT03415399). Very recently, a novel anti-CD19 CAR-T cell system was obtained by fusing the anti-CD19 antibody Fab domain with the transmembrane and intracellular domains from the  $\gamma\delta$  TCR with addition of an ET190L1-scFv/CD28 co-stimulatory molecule (ET019003 T cells) (96). ET019003 T cells were tested in preclinical studies followed by a phase I clinical trial in relapsed/refractory CD19<sup>+</sup> B-cell leukemia and lymphoma (NCT04014894). So far, it was shown that these CAR-T cells presented a good safety profile and could induce rapid responses and durable CR in patients with relapsed or refractory DLBCL. Although, these results are preliminary and are limited to a small sample size, they offer new promising therapeutic strategies for patients with high-risk profiles. The spectrum of  $\gamma\delta$  T cells based IT in B-cell malignancies is summarized in the Figure 3.

## 5 Discussion and conclusion

Human  $\gamma\delta$  T cells present several specific characteristics that make them very attractive for their use in anti-cancer therapy in general and anti-lymphomatous in particular. Firstly, in contrast to  $\alpha\beta$  T cells, their anti-tumoral activity does not depend on mutational burden, thus rendering them efficient against tumors harboring few somatic mutations. Secondly, as they do not act dependently of MHC I-mediated Ag presentation, unlike CD8<sup>+</sup>  $\alpha\beta$  T cells, they exhibit an anti-tumoral efficacy against tumors harboring a downregulation of surface MHC class I molecules. This characteristic is particularly well suited for the "off-the-shelf" allogenic cell therapy. Thirdly, they exhibit increased anti-tumoral activity due to their particular activation mechanisms present on both adaptive cells through the TCR signaling and innate cells through NK signaling (NKG2D, DNAM-1, NKp46, NKp44, NKp30). This is amplified by their low expression of killer inhibitor receptor.

Although V $\delta$ 2 T cell-based IT exhibited safety and good tolerance in patients, they also demonstrated limited success due to several reasons among which a highly variable capacity to recognize tumoral cells, functional instability, dysfunction or exhaustion of chronically activated V $\delta$ 2 T cells. Thus, innovative strategies were developed to improve tumoral cell recognition, promote durable persistence and circumvent exhaustion mechanisms involving V $\delta$ 2 but also V $\delta$ 1 T cells. In this context, engineered cells such as DOT and CAR-T offer very encouraging perspectives as well as combination of V $\delta$ 2 T cells with antibodies targeting ICP or neutralizing inhibitory cytokines to



counteract immune suppression TME and exhaustion processes or transduction of selected high affinity V $\gamma$ 9V $\delta$ 2 TCR into  $\alpha\beta$  T cells in order to induce a durable and memory-based response.

Several challenges remain, among them the difference of efficacy between cell engagers and ACT involving  $\gamma\delta$  T cells considering their logistical requirements and costs. Another important point to be considered is the justification for the selection of patients to be treated by such IT based on the identification of tumor Ag recognized by  $\gamma\delta$  T cells. All these issues will help to better understand, use and develop next-generation  $\gamma\delta$  T cell-based IT.

## Author contributions

LR, CF and MD contributed equally to the work of this review. CL participated in the writing. MP and CB wrote the review and supervised the work. All authors contributed to the article and approved the submitted version.

## Conflict 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.

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## EDITED BY

Mary Poupot-Marsan,  
INSERM U1037 Centre de Recherche en  
Cancérologie de Toulouse, France

## REVIEWED BY

Sushil Kumar,  
Oregon Health and Science University,  
United States  
Alexei Gratchev,  
Russian Cancer Research Center NN  
Blokhin, Russia

## \*CORRESPONDENCE

Christophe Blanquart  
✉ christophe.blanquart@inserm.fr

<sup>†</sup>These authors have contributed  
equally to this work and share  
first authorship

<sup>†</sup>These authors have contributed  
equally to this work and share  
last authorship

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# ChemR23 activation reprograms macrophages toward a less inflammatory phenotype and dampens carcinoma progression

Margot Lavy<sup>1†</sup>, Vanessa Gauttier<sup>1†</sup>, Alison Dumont<sup>2</sup>,  
Florian Chocteau<sup>2</sup>, Sophie Deshayes<sup>2</sup>, Judith Fresquet<sup>2</sup>,  
Virginie Dehame<sup>2,3</sup>, Isabelle Girault<sup>1</sup>, Charlene Trilleaud<sup>1</sup>,  
Stéphanie Neyton<sup>1</sup>, Caroline Mary<sup>1</sup>, Philippe Juin<sup>2,4</sup>,  
Nicolas Poirier<sup>1†</sup>, Sophie Barillé-Nion<sup>2†</sup>  
and Christophe Blanquart<sup>2\*\*</sup>

<sup>1</sup>OSE Immunotherapeutics, Nantes, France, <sup>2</sup>Nantes Université, Inserm UMR 1307, CNRS UMR 6075, Université d'Angers, CRCI2NA, Nantes, France, <sup>3</sup>Nantes Université, CHU Nantes, service de pneumologie, l'institut du thorax, Nantes, France, <sup>4</sup>ICO René Gauducheau, Saint Herblain, France

**Introduction:** Tumor Associated Macrophages (TAM) are a major component of the tumor environment and their accumulation often correlates with poor prognosis by contributing to local inflammation, inhibition of anti-tumor immune response and resistance to anticancer treatments. In this study, we thus investigated the anti-cancer therapeutic interest to target ChemR23, a receptor of the resolution of inflammation expressed by macrophages, using an agonist monoclonal antibody,  $\alpha$ ChemR23.

**Methods:** Human GM-CSF, M-CSF and Tumor Associated Macrophage (TAM)-like macrophages were obtained by incubation of monocytes from healthy donors with GM-CSF, M-CSF or tumor cell supernatants (Breast cancer (BC) or malignant pleural mesothelioma (MPM) cells). The effects of  $\alpha$ ChemR23 on macrophages were studied at the transcriptomic, protein and functional level. Datasets from The Cancer Genome Atlas (TCGA) were used to study *CMKLR1* expression, coding for ChemR23, in BC and MPM tumors. *In vivo*,  $\alpha$ ChemR23 was evaluated on overall survival, metastasis development and transcriptomic modification of the metastatic niche using a model of resected triple negative breast cancer.

**Results:** We show that ChemR23 is expressed at higher levels in M-CSF and tumor cell supernatant differentiated macrophages (TAM-like) than in GM-CSF-differentiated macrophages. ChemR23 activation triggered by  $\alpha$ ChemR23 deeply modulates M-CSF and TAM-like macrophages including profile of cell surface markers, cytokine secretion, gene mRNA expression and immune functions. The expression of ChemR23 coding gene (*CMKLR1*) strongly correlates to TAM markers in human BC tumors and MPM and its histological detection in these tumors mainly corresponds to TAM expression. *In vivo*, treatment with  $\alpha$ ChemR23 agonist increased mouse survival and decreased metastasis

occurrence in a model of triple-negative BC in correlation with modulation of TAM phenotype in the metastatic niche.

**Conclusion:** These results open an attractive opportunity to target TAM and the resolution of inflammation pathways through ChemR23 to circumvent TAM pro-tumoral effects.

#### KEYWORDS

macrophage, cancer, ChemR23 receptor, agonist, antibody

## Introduction

Inflammation is a hallmark of cancer and cancer cells can hijack inflammatory mechanisms to promote their own growth and survival (1). Activation of pro-tumorigenic factors and secretion of pro-inflammatory cytokines by tumor cells and/or immune cells present in the tumor microenvironment (TME), including macrophages, contribute to tumor promotion (2). This inflammatory TME favors all stages of tumor progression such as angiogenesis and metastases and often modulates responses to cancer treatment (3). Inflammation is a natural and physiological process triggered after injury or infection that aims to restore tissue homeostasis and normally resolved spontaneously in a few days. However, when improperly terminated, inflammation evolves towards a chronic form contributing to tissue damages. Chronic inflammation is mainly associated with abnormal non-phlogistic clearance of apoptotic cells (efferocytosis) by macrophages and a defect of the resolution of inflammation (4). Anti-inflammatory drugs were shown to limit tumor inflammation however, in parallel they dampen both innate and adaptive immune responses, so their use in oncology was overall disappointing. The resolution of inflammation is an active immunological process mediated by specialized pro-resolving mediators (SPM) which target specific resolutive G-protein coupled receptors (GPCR) expressed by different immune cells and participates in the return to tissue homeostasis after injury without being immunosuppressive (5). Defects in this process fuels chronic inflammation and contributes to carcinogenesis and exacerbates tumor growth. In contrast, administration of SPM controls tumor growth in several preclinical models (4).

The GPCR ChemR23, encoded by the gene *CMKLR1*, contributes to both initiation and resolution of inflammation, depending on the ligand that binds to it, in various acute or chronic inflammation models (6, 7). Two ligands were described for ChemR23, the pro-resolutive lipidic resolvin E1 (RvE1) and the chemoattractant protein Chemerin, encoding by the gene *RARRES2*. ChemR23 is mainly expressed on the innate immune cells including monocytes, macrophages, dendritic cells and some Natural Killer (NK) cells as well as on adipocytes and endothelial cells (8–11). Its activation by RvE1 has been reported to increase phagocytic activity of macrophages and to induce a proresolutive

phenotype (12). In addition to RvE1, Chemerin, widely recognized as an adipokine particularly abundant in inflammatory fluids (9), binds ChemR23 at the site of inflammation (8), where it promotes recruitment of monocytes/macrophages and their adhesion to extracellular matrix proteins (13). Chemerin-derived peptides processed on the site of inflammation have been reported to have anti-inflammatory effects that contribute to inflammation resolution in ChemR23 dependent manner (14, 15). The biology of ChemR23 is complex as its activation can trigger distinct inflammatory or resolving pathways, which determine the outcome of inflammation.

Macrophages critically orchestrate chronic inflammation and related diseases. They display high intrinsic plasticity and adaptability based on epigenetic regulation relying on various signals emanating from the microenvironment. Beyond the canonical M1/M2 macrophage phenotype dichotomy, single-cell analysis has recently emphasized macrophage diversity during differentiation and activation processes especially during cancers (16, 17). Even though macrophage phenotypes in cancers appeared to be part of a continuum contributing to either pro- (mainly immunosuppressive) or anti-tumor activities, they mainly exhibit a protumoral M2-like phenotype favored by their reeducation by cancer cells, that often reduces cancer therapy efficacy (18). Reprogramming tumor-associated macrophages (TAM) has thus been viewed as therapeutic opportunities to treat cancers (7).

Although ChemR23-mediated activation in macrophages during inflammation has been recently documented (19), its role in cancer progression has not been yet fully explored. The objective of this study was to evaluate the effect of an agonist antibody directed against ChemR23 ( $\alpha$ ChemR23) (19) in the pathological context of cancers. We thus first evaluated ChemR23 expression in our models of GM-CSF and M-CSF differentiated macrophages.  $\alpha$ ChemR23 effect *in vitro* on M-CSF macrophages has been explored at transcriptomic, protein and functional levels. We then extended our study to cancer contexts, using TAM-like models (differentiation of monocytes in tumor cell supernatants), focusing on BC and MPM, in which TAM exert a decisive impact on tumor progression (20).

Finally, *in vivo* activation of ChemR23 with  $\alpha$ ChemR23 was evaluated in immunocompetent orthotopic murine model of triple-negative BC on tumor growth, metastasis and survival after tumor

resection. Altogether, our data indicate that ChemR23 regulates macrophage phenotype and cancer-related inflammation in tumors and that targeting ChemR23 may contribute to control tumor progression and metastasis.

## Materials and methods

### ChemR23 agonist

The pro-resolutive agonist  $\alpha$ ChemR23 mAb was produced and purified by OSE Immunotherapeutics as previously reported and characterized (19). The hIgG1 control mAb (clone MOTA-hIgG1) was produced in parallel by Evitria (Switzerland).

### Cells

Human monocytes were freshly isolated by magnetic sorting from PBMC of healthy volunteers following the manufacturer's protocol (classical monocyte isolation kit, Miltenyi Biotec). The MPM cell line Meso13 was established from the pleural fluid of a MPM patient (21), characterized for its karyotype (GSE 134349) and for their mutational status using targeting sequencing (*CDKN2A*del, *CDKN2B*del). Cal51 cell line, derived from metastatic site of a triple negative BC (pleural effusion), and the murine triple-negative BC 4T1-luc2 cell line were purchased from DSZM (Braunschweig, Germany) and ATCC, respectively. These cell lines were cultured in complete RPMI-1640 (Gibco) or DMEM 4.5g/l Glucose media (Gibco) supplemented with 2mM L-glutamine, 100IU/mL penicillin, 0.1mg/mL streptomycin (Gibco) and 10% heat-inactivated fetal calf serum FCS (Gibco) at 37°C and 5% CO<sub>2</sub> atmosphere.

### Analysis of tumor gene expression profiling

All RNAseqv2 samples from the The Cancer Genome Atlas (TCGA)-MESO dataset (n=87 patients) and BRCA dataset (n=1997) are available on the Broad's Genome Data Analysis Centre (<http://gdac.broad-institute.org/>). Gene expressions as RNA-seq by expectation maximization values (RSEM values) were analyzed. Clinical data for these samples were downloaded from FireBrowse (<http://firebrowse.org>; version 2018\_02\_26 for MESO and BRCA). The Breast Cancer Gene-Expression Miner v4.8 (<http://bcgenex.ico.unicancer.fr/BC-GEM/GEM-Accueil.php?js=1>) was used to analyze *CMKLR1* expression in BC with macrophage markers.

### Macrophage polarization

Monocytes were seeded, in 12-wells plates, at  $0.5 \times 10^6$  cells/ml in 2.5ml of complete medium supplemented with GM-CSF at 20ng/ml (Cellgenix, 001412-050) or with M-CSF at 50ng/ml (Isokine, 01-

A0220). To obtain TAM-like macrophages, monocytes were differentiated with undiluted supernatant from MPM (Meso13) or BC (Cal51) cell lines as previously described (22). Macrophages were kept in culture for 3 days at 37°C and 5% CO<sub>2</sub> atmosphere and analyzed after treatment as described below at days 4-5.

### ChemR23 signaling

M-CSF differentiated macrophages were starved for 4h in medium then stimulated with hIgG1 or  $\alpha$ ChemR23 (10 $\mu$ g/ml) for different times. Cells were lysed with 200 $\mu$ l of RIPA (Sigma-Aldrich) buffer 1X supplemented with protease inhibitor cocktail (Fast protease inhibitor, Sigma-Aldrich) and phosphatase inhibitors (Phos STOP, Roche). Cell lysates were centrifuged for 25min at 800g at 4°C to remove debris and the supernatants were stored at -80°C. Proteins were quantified using Bradford assay (Interchim). 7 $\mu$ g of proteins were loaded in 4-20% Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup> Precast Protein Gel (#4561093, Biorad) and then transferred onto a nitrocellulose membrane. After 2h of saturation in 5% milk/TBS-Tween 0,05%, the membranes were incubated with primary antibodies anti-phospho(Thr202/Tyr204) - ERK1/2 (p44/42 MAPK) (#4370, Cell Signaling) (1:1000), anti- ERK1/2 (p44/42 MAPK) (#9102S, Cell Signaling) or anti-phospho(Ser473) -AKT (#4051S, Cell Signaling) (1:1000), anti-AKT (#4691S, Cell Signaling) or anti-Actin M(AB1501 Millipore) for 1h at room temperature. Proteins were incubated with Goat anti-Rabbit (#111-001-003) or Goat anti-Mouse (115-035-008) secondary antibodies (Jackson ImmunoResearch) for 1h at room temperature and then revealed with the Immoblie Western Chemiluminescent HRP Substrate (WBKLS0500, EMD Millipore). The data were analyzed with the Fusion FX device (Vilber).

### Immunophenotyping by flow cytometry

Macrophages were detached using phosphate-buffered saline (PBS)-EDTA at 4°C for 30min and labeled with live/dead marker FVS510 (BD, 564406) for 30min at 4°C in PBS. After washing, the cells were labeled with anti-CD14 APC-Vio770 (clone REA599, Miltenyi Biotec), anti-HLA-DR FITC (clone L243, BD), anti-CD16 BV421 (clone 3G8, Biolegend), anti-CD163 AF647 (clone GHI/61, BD) and anti-ChemR23 PE (clone 84939, R&D system) antibodies for 30 min at 4°C in PBS-0,01% bovine serum albumin (BSA) (Sigma Aldrich). IgG1k-AF647 (clone MOPC-21, BD), REA control-APCviolet770 (clone REA293, Miltenyi Biotec), IgG3-PE (clone 133316, R&D system), IgG1,k-FITC (MOPC-21, BD) and IgG1,k-BV421 (clone MOPC-21, Biolegend) isotypes were used as controls. Analysis was performed by flow cytometry using FlowJo v10 software. The results are expressed as the ratio of the median fluorescence obtained with the specific antibody to the median fluorescence obtained with the corresponding isotype (RFMI=Ratio of Median fluorescent intensity).

For tSNE data projection, M-CSF differentiated macrophages were incubated with 10 $\mu$ g/ml  $\alpha$ ChemR23 or hIgG1 antibodies for



24h and processed for flow cytometry staining as mentioned above with the following antibodies: anti-CD14 APC (clone MSE2, Biolegend), anti-CD163 (clone GHI/61.1, Miltenyi), anti-CD192 BV605 (clone K036C2, Biolegend), anti-CD206 BB515 (clone 19.2, BD), anti-CD274 PE (clone MIH1, BD), anti CD45 APC R700 (clone HI30, BD), anti-CD209 BV421 (clone DCN46, BD), anti-CD80 BUV395 (clone L307.4, BD), anti-CD16 BUV737 (clone 3G8, BD), anti HLA-DR BV711 (clone L243, Biolegend).

Analysis of the data was performed using FlowJo v10 software.

## Chemokine secretion assays

After 3 days in culture for differentiation, macrophages were incubated with 10µg/ml αChemR23 or hIgG1 antibodies for 24h. Then, cells were stimulated with 200ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich) and the supernatant were collected after 24h for TNF-α, IL-1β, IL-1RA, IL-6, IL-10, IL-12p70, IL-12p40, CCL17, IL-23 and IP-10 quantification using the LEGENDplex Human M1/M2 macrophages panel (BioLegend) according to the manufacturer's recommendations.

## Transcriptomic analysis

After 3 days in culture, macrophages were incubated with 10µg/ml of αChemR23 or hIgG1 antibodies for 24h. Then, cells were stimulated with 200ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich) for 6h and cells were lysed using the RLT buffer (Qiagen) supplemented by 1% β-mercaptoethanol (Sigma-Aldrich). Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen).

For RT-qPCR experiments, 0.5µg of total RNA was reverse transcribed using MMLV reverse transcriptase (Invitrogen). PCR reactions were performed using QuantiTect Primer Assays (Qiagen) and RT<sup>2</sup> Real-time SYBR-Green/ROX PCR mastermix (Qiagen) and carried out using QuantStudio<sup>TM</sup> Real-Time PCR system 3 (ThermoFisher). *RPLP0*, *ACTB* and *GAPDH* gene expression were used as internal standards.

For Nanostring analysis, gene expression was quantified with the NanoString nCounter platform using 50ng of total RNA in the nCounter Human (594 genes) or Mouse (561 genes) Immunology Panel (NanoString Technologies). The code set was hybridized with the RNA overnight at 65°C. RNA transcripts were immobilized and counted using the NanoString nCounter Sprint. Normalized expression data were analyzed with the nSolver software. Standardized not log2-transformed counts were used for differential gene expression analysis with the R package DESeq2 (23). Genes were ranked in order of differential expression and *P* value score. Gene set enrichment analysis was performed with the GSEA software with 1000 permutations. Score signatures of transcriptomic analysis (single sample GSEA score) were obtained calculating a gene set enrichment score per sample as the normalized difference in empirical cumulative distribution functions of gene expression ranks inside and outside the gene set with the R package GSVA.

## ChemR23 immunolabelling of human breast and mesothelial tumors

Human tumor specimens were collected from 7 treatment-naïve patients with invasive BC after surgical resection at the Institut de Cancérologie de l'Ouest, Nantes/Angers France, and from 3 patients with MPM. Informed consent was obtained from enrolled patients and protocol was approved by Ministère de la Recherche (agreement n°: DC-2012-1598) and by local ethic committee (agreement n°: CB 2012/06) or through the biological resource center (CHU Nantes, Hôtel Dieu, Tumorothèque, Nantes, France BRIF: BB-0033-00040, transfer number 122C366) for patients with BC tumors or MPM, respectively. Three-micrometers-thick tissue sections of formalin-fixed, paraffin-embedded (FFPE) breast cancers were treated with protease for 4min (Protease 1, Ventana, 760-2018) to achieve epitope retrieval. Samples were then incubated during 32min at 37°C with the anti-ChemR23 antibody at a dilution of 1:200 (clone 1A7, mouse monoclonal, Origene) and with the anti-Iba1 antibody at a dilution of 1:1000 (clone EPR16588, rabbit monoclonal, Abcam). Protein expression was detected with an OptiView DAB IHC Detection Kit (Roche Diagnostics, 760-700), optimized for automated immunohistochemistry (Benchmark GX stainer, Ventana Medical Systems, Roche Diagnostics). ChemR23 and Iba1-immunolabelled macrophages were quantified on 10 High Power Fields (HPFs, Fields of 1590 µm<sup>2</sup> at 400X magnification) in tumor and peritumor (comprising a thickness of 200µm around the periphery of the tumor) areas.

## Phagocytosis assay

For induction of tumor cell apoptosis, the BC cell line Cal51 was treated with 70nM paclitaxel (Sigma-Aldrich) plus 100nM of the BCLxL antagonist A1331852 (SelleckChem) for 24h, as previously described in (24). Meso13 cells were treated with 1.6 µg/ml cisplatin (Merck) and 80 µM pemetrexed (Sigma-Aldrich) for 72h, as previously described in (16). Apoptotic and control untreated cells were then harvested, washed and labelled with 1µg/ml of the pH sensitive dye pHrodo<sup>®</sup> (Incucyte pHrodo Red cell labelling Kit for phagocytosis, Sartorius, 4649) following the manufacturer's protocol. In parallel, differentiated macrophages were seeded at 10<sup>4</sup> cells in 96-well plates in 200µl of complete medium and treated with 10µg/ml αChemR23 or hIgG1 antibodies for 24h at 37°C. Then, labelled apoptotic or untreated cancer cells were added to macrophages at a ratio of 1:5 for 24h and phagocytosis was measured by live cell imaging using Incucyte<sup>®</sup> technology (Sartorius). Phagocytosis was quantified using fluorescence intensity ("Total integrated intensity") in integrated software.

## Polyclonal T cell proliferation assay

Differentiated macrophages treated with 10 µg/ml αChemR23 or hIgG1 antibodies for 24h were stimulated with 200 ng/ml LPS for

additional 24h, supernatants (conditioned media) were collected and centrifuged to remove cell debris, aliquoted and stored at  $-80^{\circ}\text{C}$ .

Human T lymphocytes were freshly isolated by negative magnetic sorting (EasySep Human T cell isolation kit, Stemcell technologies) from PBMC of healthy volunteers following the manufacturer's protocol. Isolated T lymphocytes were labelled with  $0.5\ \mu\text{M}$  of cell proliferation dye (CPD) following the manufacturer's protocol (eBioscience<sup>TM</sup> Cell proliferation dye efluor 670, ThermoFischer).  $0.1 \times 10^6$  labelled T lymphocytes were activated with CD3/CD28 beads at a ratio 1:40 or 1:100 for 5 days (Dynabeads Human T-Activator CD3/CD28, ThermoFischer) in 96-well U-bottom plates and treated with conditioned media from macrophages, IL-2 at 75 U/ml or IL-10 at 50 ng/ml (R&D system). After 5 days in culture, T lymphocytes were washed twice with PBS and labeled with live/dead dye APC-H7, anti-CD3 FITC (clone REA163), anti-CD4 PE (clone RPA-T4) and anti-CD8 BV421 (clone RPA-T8) antibodies. Analysis was performed by flow cytometry using DIVA software.

## Preclinical tumor model

Animal housing and surgical procedures were conducted according to the guidelines of the French Agriculture Ministry (APAFIS 8629-2017011915305978) and were approved by the local animal ethics committee (CEEA-PdL n°6 (pour Comité d'éthique en expérimentation animale\_Pays de la Loire n°6)). BALB/c mice were purchased from Janvier Laboratories and kept in the UTE Nantes SFR Bonamy animal facility. 4T1-luc2 cells were harvested and resuspended in PBS for animal inoculation. Metastasis spreading was monitored using bioluminescence (Biospace Imager) by injecting  $100\ \mu\text{l}$  of D-luciferin (Interchim) at  $33.33\text{mg/ml}$  through intraperitoneal route. 4T1-luc2 cells ( $0.25 \times 10^6/\text{mouse}$  in PBS) were injected into the fat pad of mammary gland of 8-week-old BALB/c female mice (day 0). Mice were treated with  $\alpha\text{ChemR23}$  or hIgG1 mAbs intraperitoneally at  $1\text{mg/kg}$  3 times a week for 3 weeks from day 7 to day 28. At day 13, primary tumors were surgically removed from mammary glands. Tumor spreading was analyzed by overall survival and by *in vivo* bioluminescence on lungs every week using a bioimager. Mice were euthanized when critical endpoints were reached according to criteria defined by ethical committees and lungs were harvested for further experiments including transcriptomic analysis (Nanostring technology and RT-qPCR) and flow cytometry, as previously described.

## Statistical analysis

The two data groups were compared with the nonparametric test Mann-Whitney. To assess the significance of the effect of  $\alpha\text{ChemR23}$  compared with hIgG on macrophages, the Wilcoxon paired t-test was used. The log-rank test was used to compare survival times between the two groups. Errors bars represent standard errors of mean (SEM). The symbols correspond to a *P*-value inferior to \*0.05, \*\*0.01, \*\*\*0.001, \*\*\*\*0.0001. All statistical

analyses were performed using GraphPad Prism software (version 8.0).

## Results

### ChemR23 expression and activation in human M-CSF macrophages

We first evaluated the expression of ChemR23 in M-CSF or GM-CSF differentiated human macrophages. M-CSF is a cytokine usually secreted by tumor cells and found in malignant tissues (25). M-CSF macrophages present phenotypic and functional characteristics different from M2a-c macrophages (26–28), but close to those of TAM as described in ovarian cancer (28) thus representing an interesting model of macrophages with immunomodulatory properties in the context of a malignancy. In contrast, using GM-CSF usually found elevated in the context of inflammation or immune response (29) leads to macrophages with proinflammatory properties however different from macrophages obtained using IFN- $\gamma$  +/- LPS corresponding to a particular context of host defense (30). As previously observed, M-CSF macrophages expressed a significantly higher level of CD14 and CD163 (27), and a significantly lower level of CD80 and HLA-DR than GM-CSF macrophages (Figure S1) (28). GM-CSF expressed a higher level of CD206 and CD192 than M-CSF macrophages whereas the latter expressed a significantly higher level of CD16 than GM-CSF macrophages (Figure S1). No evident differences in CD209, CD45 and CD274 expression were observed on both model of macrophages (Figure S1). As expected, GM-CSF macrophages secreted significant higher level of IL-12p70 and TNF $\alpha$ , and significant lower level of IL-10, IL-1RA and IP-10 than M-CSF macrophages (Figure S2A) (30, 31). No differences in the secretion of IL-6 and IL-1 $\beta$  was observed between M-CSF and GM-CSF macrophages (Figure S2A). M-CSF macrophages expressed significantly more ChemR23 than GM-CSF macrophages (Figure 1A). To evaluate the biological activity of ChemR23 agonist antibody, previously reported to activate the pro-resolutive signaling of ChemR23 (19), on macrophages, we measured cytokine secretion. Treatment with  $\alpha\text{ChemR23}$  mAb significantly decreased IL-10, IP-10 and IL-6 secretion, and significantly increased TNF $\alpha$  and IL-1RA secretion while not changing IL-12p70 and IL-1 $\beta$  secretion of M-CSF macrophages (Figures 1B, S3A). On GM-CSF macrophages,  $\alpha\text{ChemR23}$  treatment led to a significant decrease of IP-10 secretion only (Figure S3B). These results suggest that  $\alpha\text{ChemR23}$  mAb mainly impact M-CSF macrophages functions thus, we focused our study on this macrophage subtype.

To confirm that  $\alpha\text{ChemR23}$  triggered ChemR23 activation in M-CSF macrophages, we studied intracellular signaling pathways. Treatment by  $\alpha\text{ChemR23}$  mAb induced phosphorylation of ERK1/2 within 30min or 1h (Figure 1C) without triggering AKT pathway (Figure 1D). These results demonstrate the capacity of the agonist mAb to induce an intracellular signaling pathway in M-CSF macrophages.

In order to better characterize the impact of ChemR23 activation on M-CSF macrophages, we performed a

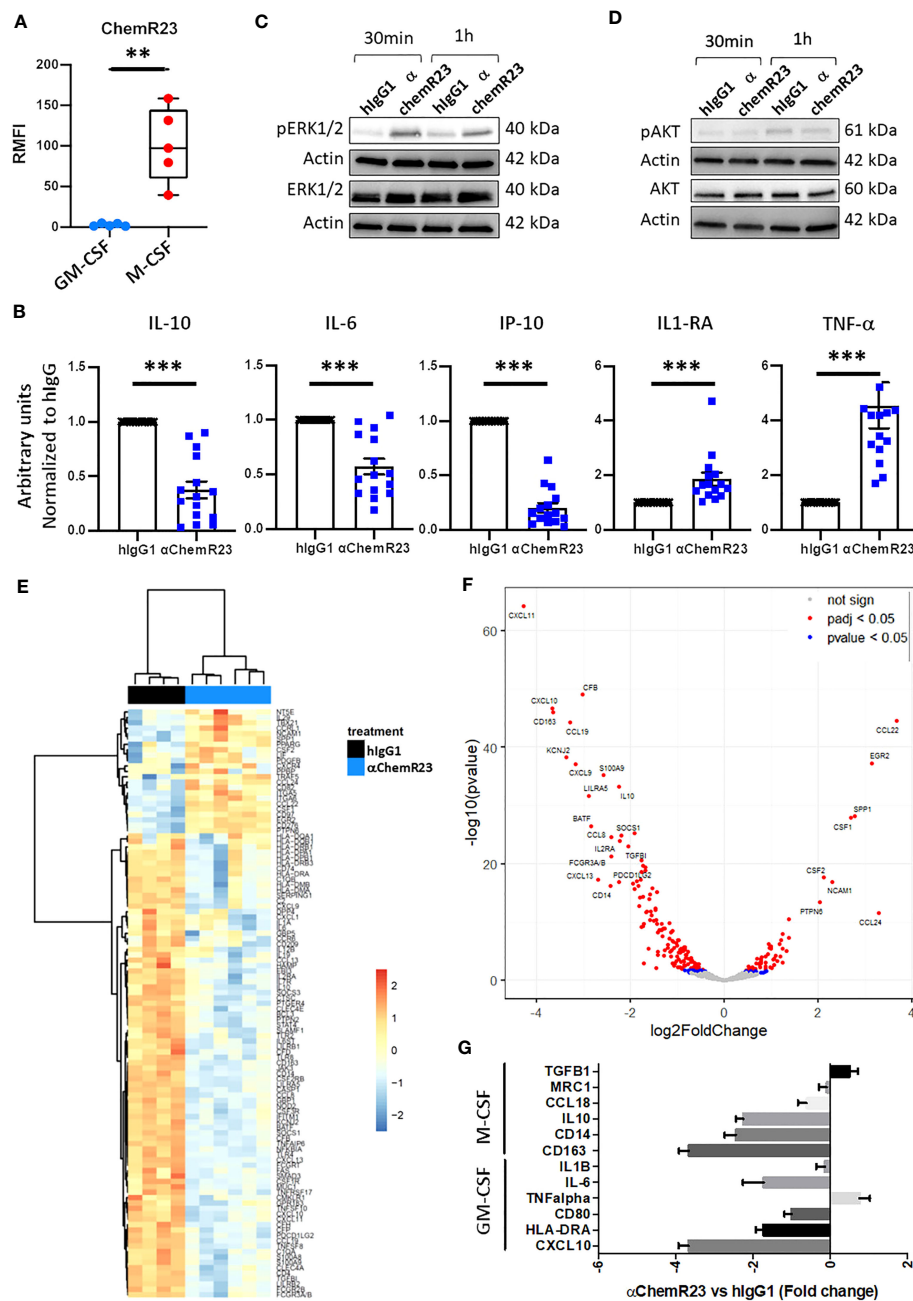


FIGURE 1

ChemR23 expression and activation in M-CSF macrophages. (A) Human monocytes from 5 healthy donors were differentiated *in vitro* using GM-CSF (20ng/ml) or M-CSF (50ng/ml) for 3 days, then ChemR23 expression was analyzed by flow cytometry. RFI=Ratio of Median fluorescent intensity \*\*,  $p < 0.01$ . (B) M-CSF macrophages were treated with αChemR23 or control hlgG1 for 24h. Then, cells were stimulated with LPS (200 ng/ml) for 24h and supernatants were collected. The indicated cytokines were quantified using multiplex ELISA in the corresponding supernatants.  $n = 15$ . \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . pERK1/2(Thr202/Tyr204) (C) and pAKT(Ser473)/AKT (D) ChemR23 signaling assessed by immunoblot in M-CSF macrophages treated with either 10μg/ml of αChemR23 agonist antibody or with control hlgG1 for 30min or 1h. (E–G) M-CSF differentiated macrophages were treated with αChemR23 agonist or control hlgG1 for 24h followed by LPS stimulation (200ng/ml) for 6h. mRNA were extracted and analyzed using NanoString Technology (Human immunology panel). (E) Heatmap of genes differentially expressed between αChemR23 agonist versus control hlgG1 treated macrophages from 4 and 6 donors respectively with a  $p$  value adjusted  $\leq 0.05$  and absolute log2 fold change  $\geq 1$ . (F) Volcano plot showing differentially expressed genes following αChemR23 agonist treatment, and genes with absolute value of the log2 fold change  $\geq 2$  and  $p$  value adjusted  $\leq 0.05$  are highlighted with their gene code name. (G) Graphic showing variation of expression of a panel of genes specific of GM-CSF or M-CSF macrophages.  $n = 4$ –6.

transcriptomic analysis using NanoString Technology with the Human immunology panel. Unsupervised hierarchical clustering representation shows that ChemR23 triggering by the agonist antibody induced strong gene expression modifications compared

to hlgG1 control (Figure 1E). Volcano plot representation (Figure 1F) indicates in red points significant ( $\text{padj} < 0.05$ ) gene expression modifications with 53 induced and 137 repressed genes on a total of 594 analyzed genes, and genes with an absolute log2

fold change  $\geq 2$  are highlighted by their gene code name (8 induced and 19 repressed). Using discriminating genes between M1-like and M2-like signatures (32), we evidenced a decrease of both M2-associated markers *CCL18*, *IL10*, *CD14*, *CD163*, *IL6* expression as well as M1-associated ones *CD80*, *HLA-DRA* and *CXCL10*, encoding IP-10, and a slight increase of *TNFA* and *TGFB1* (M1 associated) (Figure 1G). In contrast *MRC1* and *IL1B* were not affected. Interestingly, we found that *IDO1* expression, coding for Indoleamine 2,3-dioxygenase-1 a potent T-cell immunosuppressive enzyme, was also reduced by anti-ChemR23 treatment on M-CSF macrophages (Figure S4A). These results argue for the induction of an intermediate phenotype different from the GM-CSF or M-CSF-induced macrophages polarization following ChemR23 activation.

## ChemR23 activation modulates the expression of macrophage surface markers

In order to study the impact of  $\alpha$ ChemR23 mAb on macrophage phenotype, we measured the expression of 10 cell surface markers, CD14, CD163, CD192, CD206, CD274, CD45, CD209, CD80, CD16, HLA-DR, using flow cytometry. According to our transcriptomic study, the expression of CD14, CD16, CD163 and HLA-DR was decreased whereas CD206 was not modified (Figure S5). tSNE representation (Figure 2A) shows that the decrease of marker expression was not homogenous and led to a redistribution of macrophage subpopulations. Indeed, as examples, we observed an increase of CD14 high, CD163 med, CD16 low and HLA-DR low cells (16.8% to 57.3%) (Figure 2B, pop 3), CD14 high, CD163 -, CD16 -, and HLA-DR low cells (2.06% to 14.1%) (Figure 2B, pop 5) and CD14 low, CD163 -, CD16 -, and HLA-DR low cells (0.95% to 3.82%) (Figure 2B, pop 4), and a decrease of CD14 high, CD163 high, CD16 high and HLA-DR med (62.7% to 11.6%) (Figure 2B, pop 1) macrophage subpopulations. CD14 high, CD163 med, CD16 med and HLA-DR med macrophage subpopulation was weakly impacted by  $\alpha$ ChemR23 mAb (16.3% to 12.6%) (Figure 2B, pop 2).

## ChemR23 activation modulates macrophage activities

Since M-CSF macrophages were deeply impacted in response to ChemR23 activation, we further extended our study on two macrophage main functions, phagocytosis and T-cell proliferation modulation. Phagocytic activity of macrophages during inflammation is fundamental for its resolution, we thus evaluated macrophage phagocytosis capacity, i.e. their ability to phagocytose apoptotic tumor cells, here BC and MPM apoptotic cells, following ChemR23 activation. We found that phagocytosis capacity was, as expected, higher in M-CSF (3.5 fold) compared to GM-CSF macrophages and only occurred towards apoptotic cancer cells (Figures S6A, B). Treatment of M-CSF macrophages with  $\alpha$ ChemR23 induced a significant reduction of their capacity to internalize apoptotic cancer cells (Figures 3A, B). No effect of the agonist mAb was observed on GM-CSF macrophages phagocytosis

activity (Figures S6C, D). These results illustrate that ChemR23 activation by the antibody deeply affects human M-CSF macrophages including modification of their capacity to phagocytose apoptotic tumor cells.

Furthermore, macrophages can also exert their immunomodulatory function through regulation of T-cell proliferation, hence we incubated T lymphocytes from PBMC from 3 healthy donors with macrophages supernatants previously incubated with ChemR23 agonist antibody or with the isotype control antibody. T-cell activation was induced by stimulation with CD3-CD28 beads for 5 days, then cell-proliferation dye dilution was measured by flow cytometry in total CD3+ T cells (Figure 3C) or CD3+ CD8+ T lymphocytes (Figure 3D) and CD3+ CD4+ T lymphocytes (Figure S7). As positive control, IL-2 potently increased T cell proliferation while IL-10 decreased T-cell proliferation at the concentration used in this assay. Of interest, we found that the supernatants from M-CSF macrophages also reduced the proliferation of human T-cell but this immunosuppressive effect was reversed when macrophages were priorly incubated with the agonist anti-ChemR23 mAb (Figures 3C, D). Of note, this effect was not significant on CD4 T-cells (Figure S7).

These results demonstrate that ChemR23 triggering by an agonist mAb strongly modifies human M-CSF macrophages polarization as illustrated by the profound transcriptomic, phenotypic, cytokines secretion and immune functions modifications.

## Expression of *CMKLR1* in BC and MPM correlates to TAM markers

Regarding the involvement of M2-like macrophages in cancers (33), we extended our study to two malignancies in which the presence of these cells was described as associated with the severity of the disease, breast cancer (34) and malignant pleural mesothelioma (35). We first studied the expression of *CMKLR1* in BC and MPM, using the dataset of The Cancer Genome Atlas (TCGA), where these pathologies appear to express high level of *CMKLR1* among 37 cancers analyzed (Figure S8A). We observed that *CMKLR1* was expressed in all subtypes of BC or MPM with a significant lower expression in luminal breast tumors (Figure S8B) and epithelioid mesothelioma tumors (Figure S8C). Importantly correlation analyses showed significant co-expression between *CMKLR1* and genes specific of tumor associated macrophages (TAM) such as *CD14*, *CD163*, *MRC1* and *HLA-DRA* in BC (Figure 4A) and MPM (Figure 4B). Of note, IHC analysis performed in breast and mesothelioma tumors from patients indicated that ChemR23 expression could be detected in a part of macrophages evidenced by Iba1 staining in tumor or in peritumoral areas (Figures 4C, D). Using a public database of single cell RNA sequencing performed on 8 breast tumors ((36), <http://panmyeloid.cancer-pku.cn/>), we observed that *CMKLR1* is mainly expressed in a macrophage subpopulation with a high expression of *CD14*, *CD163* and *HLA-DRA*, and with a low expression of *CD80* and *CD86*. Additionally, expression of *CMKLR1* was not observed in macrophage subpopulations expressing high levels of *HLA-DRA*, *CD80* and *CD86* (Figure S9A). The high expression of *CMKLR1* in macrophages expressing high level of *CD14* and *CD163* was confirmed using two additional public databases (Figures S9B, C)



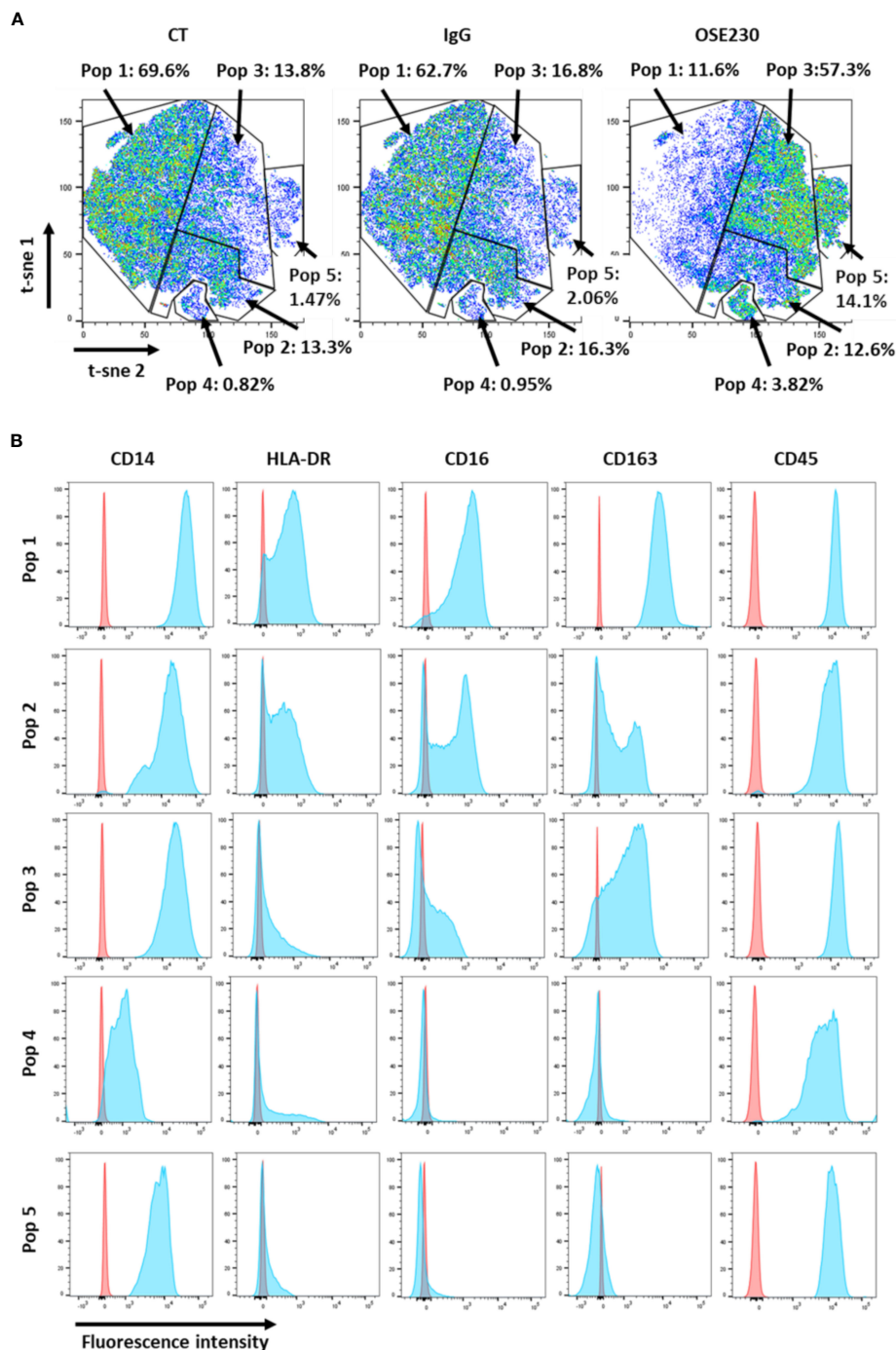


FIGURE 2

$\alpha$ ChemR23 agonist modifies M-CSF macrophages cell surface markers expression. M(M-CSF) macrophages were treated for 24h with  $\alpha$ ChemR23 or control hlgG1 and cells were collected and labelled with a panel of 10 antibodies including: CD209, CD163, CD14, CD16, CD206, HLA-DR and CD45 and analyzed using flow cytometry. **(A)** Macrophage subpopulations represented using t-SNE. **(B)** Phenotype of the different macrophage subpopulations impacted by treatment with  $\alpha$ ChemR23. Experiments were performed using monocytes from 3 different healthy donors and results include data from the 3 donors.

(37, 38). Based on these observations, we evaluated the expression of ChemR23 on macrophages obtained by incubation of monocytes from healthy donors with BC or MPM cancer cell culture supernatants, as model of TAM (TAM-like), and compared to the one of M-CSF and GM-CSF macrophages. Figure S2B shows TAM-like exhibited a phenotype close to the M-CSF one with high expression

of CD14 and CD163, low/med expression of HLA-DR, high/med secretion of IL-10 and IP-10, and low secretion of TNF $\alpha$  and IL12p70 (Figure S2A). Importantly, TAM-like macrophages expressed significantly higher levels of ChemR23 compared to GM-CSF macrophages and similar levels of ChemR23 compared to M-CSF macrophages (Figure S2B).

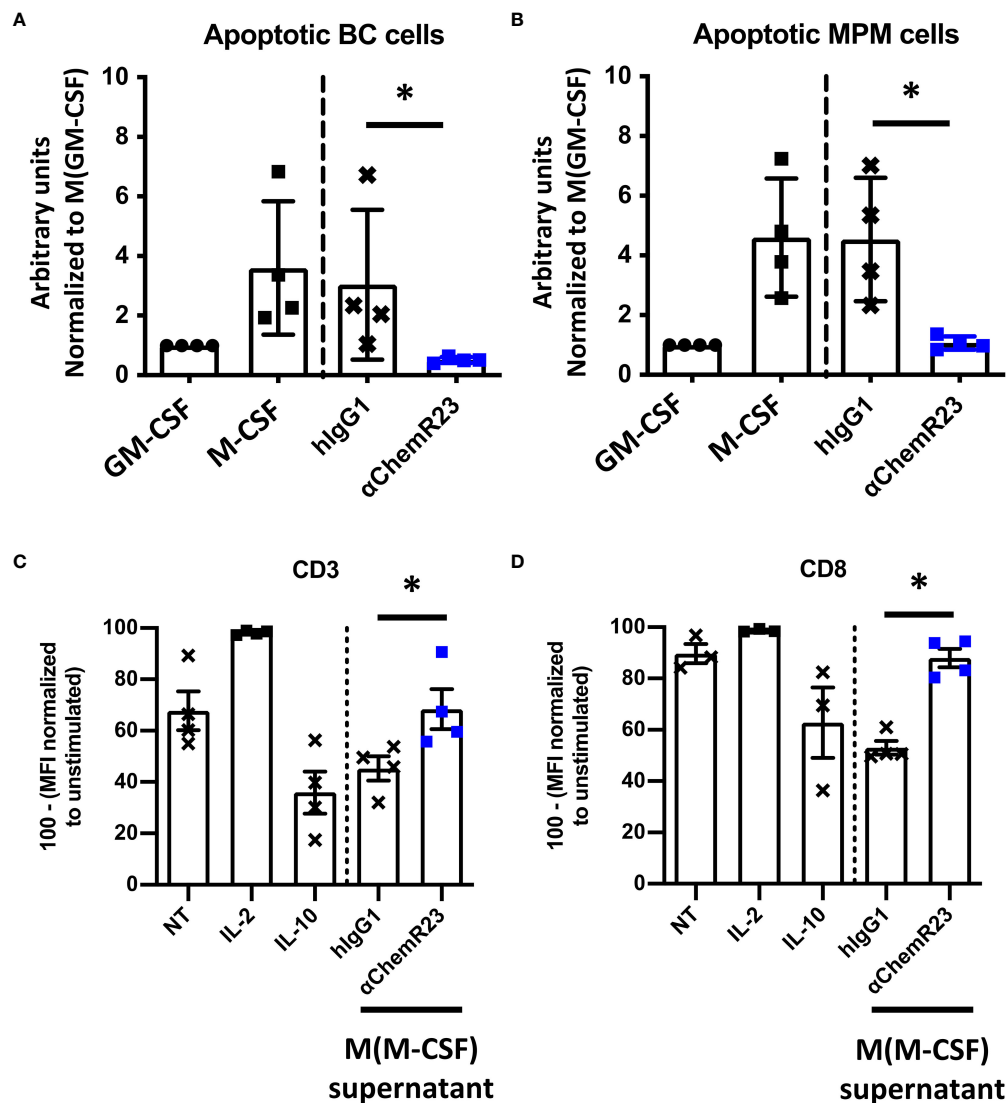


FIGURE 3

αChemR23 modulates functional properties of M-CSF macrophages. For phagocytosis assay, (A) breast cancer or (B) mesothelioma (MPM) apoptotic cells were incubated with untreated GM-CSF macrophages or M-CSF macrophages (left panels) and M-CSF macrophages (M(GM-CSF)) pretreated or not with human hlgG1 control or αChemR23 mAb for 24h (right panels). Efferocytic activity was quantified by live-cell imaging (Incucyte®). Results represent the maximum fluorescence intensity normalized to M(GM-CSF) used as reference. n=4. \*, p < 0.05. (C, D) For T cell proliferation assay, CPD stained CD3+ T-cells were incubated for 5 days with CD3/CD28 beads and either IL-2 (75 U/ml), or IL-10 (50 ng/ml), or supernatants of M(M-CSF) macrophages (MΦ) treated with αChemR23 agonist (10μg/ml) or hlgG1 control (10μg/ml) and T-cells were assessed by flow cytometry. Frequency of non-fluorescent proliferating cells was evaluated after gating on CD3+ cells (C) and after gating on CD8+ cells (D) and normalized to the unstimulated condition. n=4. \*, p < 0.05.

Altogether, these data illustrate that ChemR23 is expressed by TAM in human tumors. Moreover, our *in vitro* models of TAM seem to reproduce, at least partially, pathological situation.

### Activation of ChemR23 in TAM-like macrophages also modulates their phenotype

In order to appreciate the potential of ChemR23 triggering on human macrophages, we used our models of BC and MPM TAM-

like macrophages. As observed for M-CSF macrophages, ChemR23 activation by the agonist antibody significantly reduced IL-10, IP-10 and IL-6 secretion, and significantly induced TNFα and IL-1RA secretion while not affecting IL-12p70 in both BC (Figures 5A, S10A) and MPM TAM-like (Figure 5B, S10B). Using RT-PCR, we also observed a significant decrease of *CD163* and *CXCL10* genes, a tendency for *IL-10* expression to decrease, and a significant increase of *CCL22* and a tendency for *TGFB1* genes to increase in both BC (Figure 5C) and MPM (Figure 5D) TAM-like macrophages. As in M-CSF macrophages, we observed that *IDO1* expression tended to be reduced by anti-ChemR23 treatment on MPM TAM-like

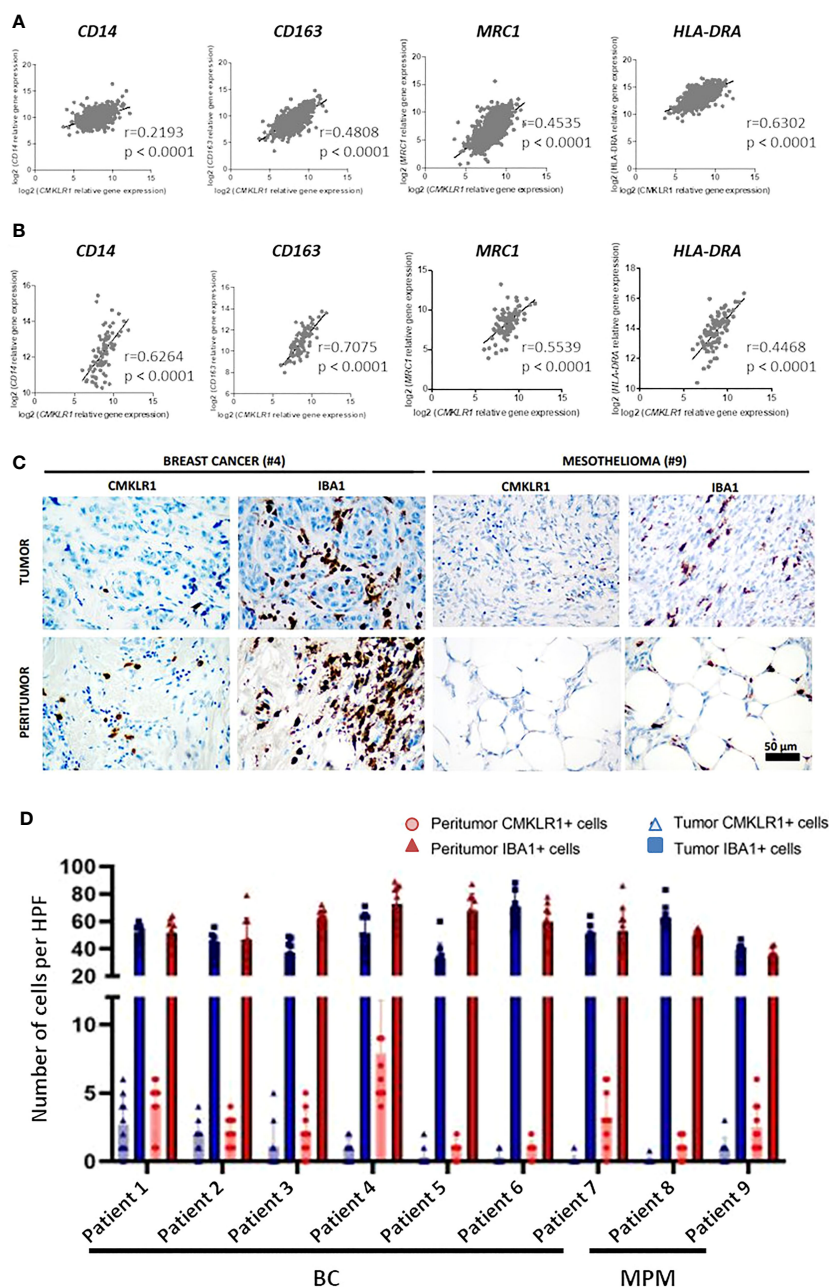


FIGURE 4

*CMKLR1* expression in breast cancer and mesothelioma tumors correlates to TAM markers and ChemR23 is expressed in *in vitro* TAM-like models. Correlation of *CMKLR1* expression with macrophage markers *CD14*, *CD163*, *MRC1* and *HLA-DRA* in breast cancers (n=1034) (A) and mesothelioma (n=87) (B) tumors using transcriptomic data from the TCGA database. (C) IHC staining of ChemR23 and Iba1 in breast tumors and MPM from 9 patients in tumor and peritumoral areas. (D) Quantification of ChemR23 IHC staining by HPF (high power field, 1590  $\mu$ m<sup>2</sup>) for each patient.

macrophages, but not significantly due to the limited number of donors (decreased expression of *IDO1* in 4 out of 5 donors,  $p=0.0625$ ), (Figure S4B). This tendency was not obtained in BC TAM-like where *IDO1* expression was poorly detectable compared to MPM (Figure S4C). These data indicate that ChemR23 receptor strongly controls macrophage phenotype and that its activation promotes a different macrophage polarization with a phenotype potentially less inflammatory and less immunosuppressive. Moreover, the tumor microenvironment could drive angiogenesis

by modulating macrophage activity, thus to address the possibility of the  $\alpha$ ChemR23 antibody modulation on this function in macrophages, we studied the expression of two well-known genes involved in angiogenesis and vessel remodeling, *VEGFA* and *MMP9* (Figure S11A, B). First, we observed a difference of expression of *VEGFA* and *MMP9* by TAM-like depending on the cell culture supernatant was from BC or MPM. Indeed, BC TAM-like highly expressed *VEGFA* compared to MPM TAM-like (Figure S11A right panel) and MPM TAM-like expressed more *MMP9* than BC TAM-

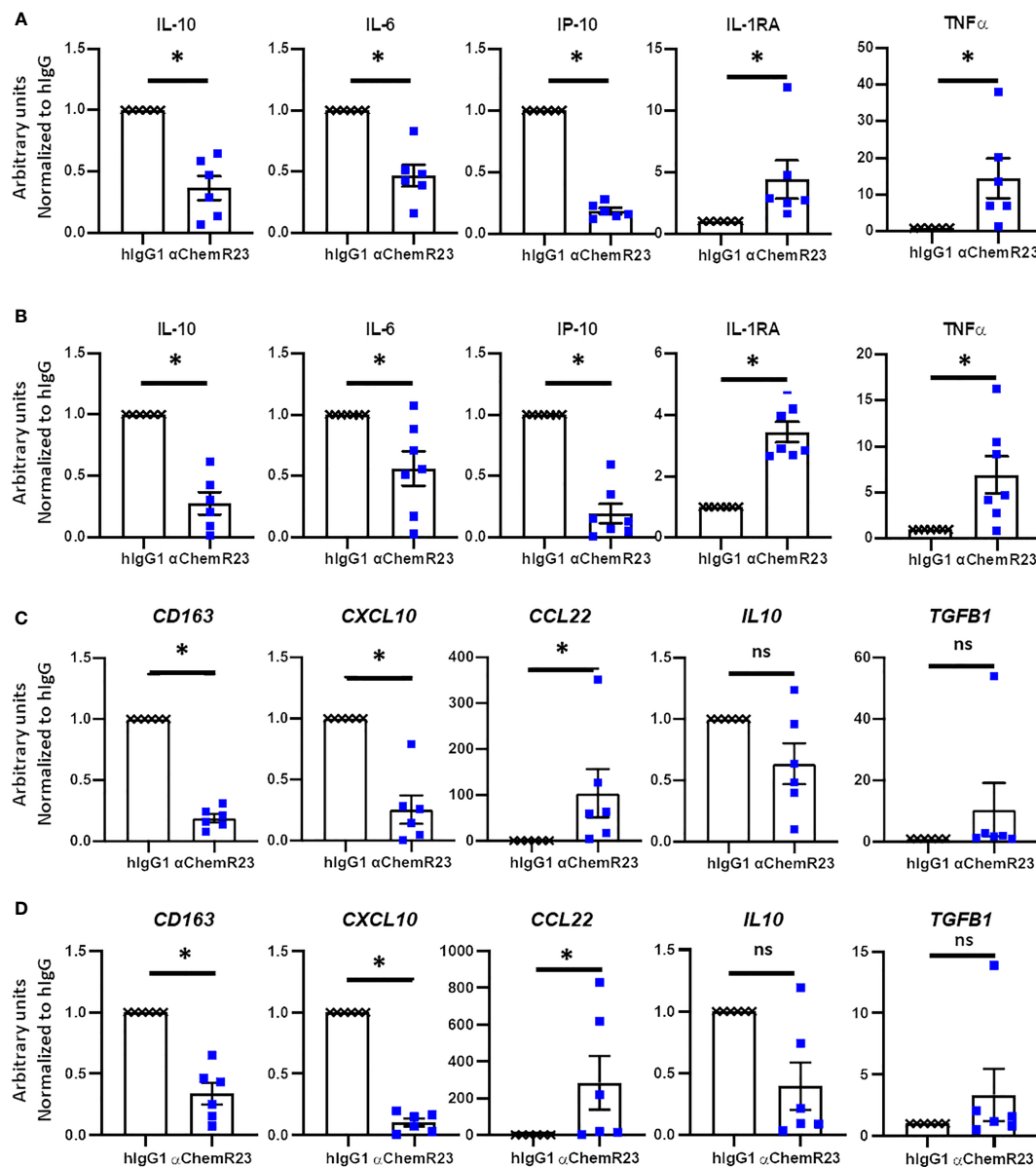


FIGURE 5

$\alpha$ ChemR23 agonist modulates TAM-like macrophages cytokine secretion and mRNA expression profiles. Monocytes from healthy donors were incubated with conditioned media of BC cells (A, C) or MPM cells (B, D) for 3 days were treated with 10  $\mu$ g/ml of  $\alpha$ ChemR23 agonist or control hlgG1 for 24h. For cytokine quantification (A, B), supernatants were collected after 24h stimulation with LPS (200ng/ml) then the indicated cytokines were quantified using multiplex ELISA in the supernatants.  $n = 6$ . \*,  $p < 0.05$ . For mRNA analyses (C, D), cells were lysed after 6h stimulation with LPS (200ng/ml) then mRNA were extracted and gene expressions were measured using RT-PCR.  $n = 5$ . \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

like (Figure S11B right panel). ChemR23 activation with the agonistic antibody decreased the *VEGFA* expression by BC TAM-like (Figure S11A), the *MMP9* expression by MPM TAM-like and tended to decrease *MMP9* expression by BC TAM-like (Figure S11B).

We further assessed the effect of  $\alpha$ ChemR23 mAb on TAM-like phagocytosis activity. BC and MPM TAM-like macrophages displayed a phagocytosis activity similar to the one of M-CSF macrophages (Figures S7A, B). As previously observed for M-CSF macrophages, a reduction of phagocytosis activity in BC and MPM TAM-like by 4 and 3 folds, respectively, was observed following treatment with  $\alpha$ ChemR23 (Figures 6A, B).

## Anti-ChemR23 mAb treatment alters metastasis development and immune microenvironment in murine breast cancers

$\alpha$ ChemR23 antibody being able to activate human as well as mouse ChemR23 receptor (Figure S12), we further investigate the interest to target ChemR23 in cancers, we evaluated agonist anti-ChemR23 mAb treatment *in vivo* on BC progression using the orthotopic syngeneic triple-negative 4T1-luc2 model. Mice treated with ChemR23 agonist antibody in monotherapy initiated 7-8 days after tumor implantation, displayed no significant tumor growth



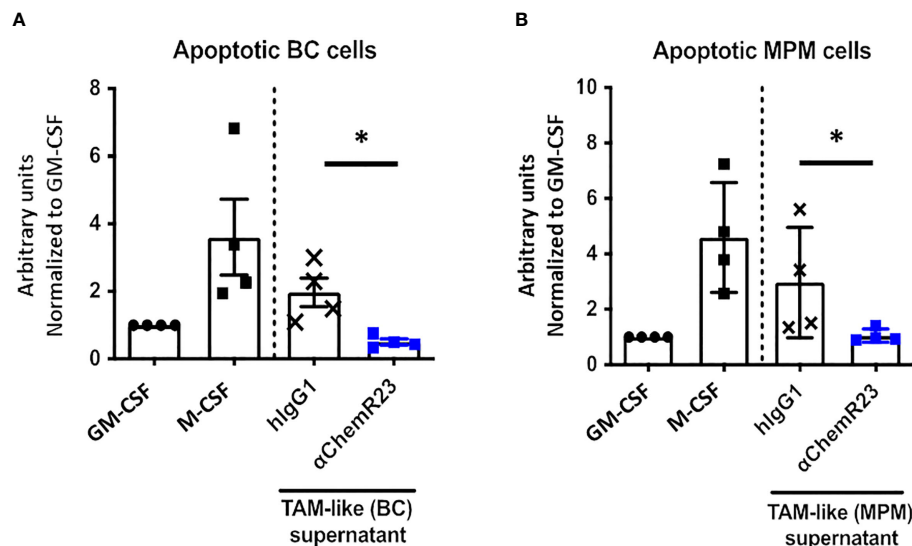


FIGURE 6

$\alpha$ ChemR23 decreased phagocytic properties of TAM-like macrophages. Phagocytosis of apoptotic (A) breast cancer (BC) or (B) mesothelioma (MPM) cell lines by untreated GM-CSF and M-CSF macrophages or TAM-like macrophages pretreated with  $\alpha$ ChemR23 or hlgG1 control (10  $\mu$ g/ml) for 24h and quantified by live-cell imaging (Incucyte®). Results represent the maximum fluorescence intensity normalized to GM-CSF macrophages condition. n=4. \*, p < 0.05.

modification compared to hlgG1 control at the dose tested (Figure S13). To closer mimic clinic situation for most patients with BC, tumor resection 13 days after tumor cell injection was performed in mice treated with anti-ChemR23 mAb 7-8 days after tumor implantation and 5-6 days before tumor resection (corresponding to the peak of metastatic risk), in order to reprogram ChemR23 expressing cells before the tumor resection and to evaluate the subsequent effect on long term metastatic development. Our results indicate that mice survival was significantly increased in the treated group resulting in 7/15 mice still alive (complete response) 100 days after tumor injection while only 2/14 in the control group survive at long-term (Figure 7A). In addition, 4 mice in the treated group had a partial response that improved their survival. Importantly, this observation correlated with significant decreased lung metastasis detection in treated mice using *in vivo* bioluminescence (Figure 7B) compared to the control group. No lung metastasis 30 days after tumor cell injection could be detected in 55% of treated mice compared to 18% in the control group. Transcriptomic analysis of lung metastasis (using the murine immunology panel from Nanostring technology) indicated that  $\alpha$ ChemR23 treatment induced significant gene expression modifications compared to hlgG1 control as shown in unsupervised hierarchical clustering representation in Figure 7C and showed, in particular, an increase of the resolution score in  $\alpha$ ChemR23 mAb treated group, although macrophages or M1/M2 scores were not modified (Figure 7D). Importantly, based on the metastasis associated gene expression *SPP1*, coding for Osteopontin, as a marker of the 4T1-luc2 metastatic cells, we defined 2 mice groups corresponding to responders, low *SPP1* expression, and non-responders, high *SPP1* expression (Figure 7E). Using Nanostring data, we observed that M1 score signature was strongly increased in the responding mice compared to the non-responding ones (Figure 7F). This was

confirmed by the increase of CCR7 positive macrophages detected in metastasis microenvironment by flow cytometry (Figure S14A). Moreover, neutrophil chemotaxis score significantly decreased in responders (Figure 7G, S14B), as previously observed in inflammatory disease model (19). Finally, using the IFN $\gamma$  score that predicts the response to T-cell immunotherapy (39) and is correlated to the NK and T cell activity, we found a significant increase of the IFN $\gamma$  score in responder animals compared to non-responders even at this very early stage of metastasis development (Figure 7H).

Altogether, these results illustrate that an anti-ChemR23 agonist mAb can modify *in vivo* ChemR23 positive cells and limits distant metastasis development in monotherapy.

## Discussion

Dysregulation of macrophage biology during carcinogenesis fuels cancer progression and resistance to treatments. In this study, we explored the opportunity to reprogram TAM by targeting the receptor ChemR23 using an agonist pro-resolutive antibody (19). Our data indicate that ChemR23 was preferentially expressed on M-CSF and TAM-like macrophages compared to GM-CSF macrophages. We report that the expression of ChemR23 coding gene, *CMKLR1*, in breast and mesothelioma tumors positively correlates to the expression of TAM markers such as *CD14*, *CD163*, *MRC1* and *HLA-DRA*, in agreement with ChemR23 detection restricted to macrophages in tumors from patients. We show that triggering ChemR23 signaling with the  $\alpha$ ChemR23 mAb deeply modulated these macrophages at the transcriptomic, phenotypic, cytokine secretion and functional levels with a resulting less inflammatory and less immunosuppressive profile

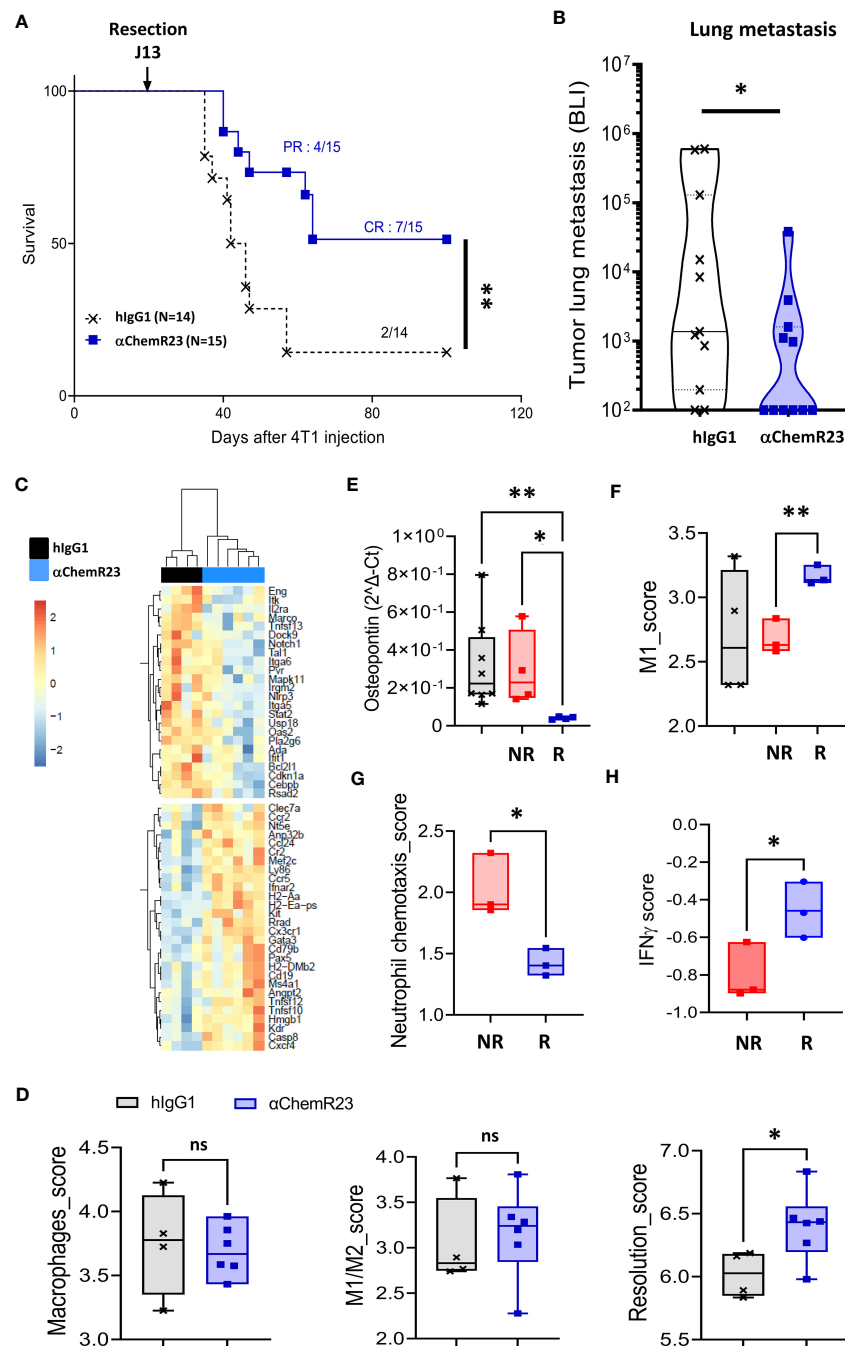


FIGURE 7

αChemR23 monotherapy increases survival and reduces metastasis in a murine orthotopic breast tumor model. Survival (A) and lung metastasis count by bioluminescence (BLI) (B) of 4T1-luc2 bearing mice treated i.p. with hlgG1 control or αChemR23 mAb (1mg/kg, 3 times a week from d7 to d28). Representative heatmap of clustered differential gene expression (C) and scores of transcriptomic analysis (D) of lungs in the αChemR23 (blue; n=7) or isotype control (black; n=4) treated 4T1-luc2-bearing mice that have been resected at d13 using the Nanostring mouse PCIP panel. (E) Osteopontin mRNA expression, measured using RT-PCR, in lung tumors of mice treated with hlgG1 control (black, n=8) or αChemR23 non-responders (red, n=4) or αChemR23 responders (blue, n=4) mAb. (F) M1 score signature of transcriptomic analysis of lungs from αChemR23 and isotype hlgG1 (black; n=4 or 8) mAb-treated mice. (G) Neutrophil chemotaxis scores and (H) IFNγ scores of transcriptomic analysis of lungs in the αChemR23 (red for non-responders n=3-4; blue for responders n=3) mAb-treated mice. ns, non-significant; \*, p < 0.05; \*\*, p < 0.01. NR, non-responders n=3-4; R, responders n=3-4.

different from the M1 and M2 dichotomy. Interestingly, *in vivo* treatment in monotherapy with this mAb, in an aggressive triple-negative breast cancer model, significantly reduced tumor dissemination and increased overall survival in correlation to TAM modulation in metastatic niche.

In this work, we show the higher expression of ChemR23 in M-CSF macrophages compared to GM-CSF macrophages. Previously, few studies described the expression of ChemR23 in human macrophages. In 2015, Herová et al. described an expression of ChemR23 in IFNγ or LPS stimulated macrophages higher than in

IL-4 or IL-13 stimulated macrophages (40). In 2016, Peyrassol et al. showed the expression of this receptor at the surface of M-CSF macrophages as in our study however without comparing to a model of pro-inflammatory macrophages (41). Regarding the experimental condition used in these two studies, the comparison is difficult and could only suggest that depending on the protocol, ChemR23 expression should be different. However, using scRNASeq data from breast tumors, we mainly observed an expression of *CMKLR1* in a subset of TAM C1QC<sup>+</sup> that expressed usual M2 signature, including high expression of CD14 and CD163, whereas *CMKLR1* expression was low in a subset of TAM ISG15<sup>+</sup> that expressed classical M1 signature (36). These results confirm our observations and support that our models of macrophages reproduce, at least in part, TAM from tumors.

We report here that agonist  $\alpha$ ChemR23 potentially decreased LPS stimulated-IL-10, IP-10 and IL-6 secretion and expression of cell surface markers such as CD163, CD14 and HLA-DR by either M-CSF or TAM like macrophages in *in vitro* experiments, as well as angiogenic modulators (*VEGFA*, *MMP9*). In the opposite, TNF $\alpha$  and IL-1RA, a natural inhibitor of IL-1 $\beta$ , were significantly increased, suggesting that ChemR23 activation led to a hybrid macrophage phenotype, as further confirmed by deeper transcriptomic analysis using Nanostring technology and flow cytometry characterization. Importantly, functional analysis of these macrophages indicated that their immunosuppressive properties towards T cell proliferation were also reduced. Of specific interest, IL-10 that is considered as a potent immunosuppressive cytokine in tumors thus promoting their immune escape, was strongly down-regulated after ChemR23 activation, as much as *IDO1* expression and to a lesser extent *ARG1* expression (data not shown) depending on the macrophage differentiation, and then could contribute to the effect observed. Other cytokines such as IP-10 that was also significantly repressed upon ChemR23 activation, may also contribute to alleviate tumor progression since it also modulates angiogenesis (42). Altogether, these data argue for a profound reprogramming of human M-CSF or TAM-like macrophages, upon exposure to agonist anti-ChemR23 mAb, to a phenotype different from the M1-M2 dichotomy with less inflammatory, less immunosuppressive functions and less phagocytosis activity.

The macrophage phenotype obtained after  $\alpha$ ChemR23 treatment seems also different from the one of resolutive macrophages previously reported since characterized by an increase of secretion of IL-10 and TGF- $\beta$ , and of efferocytosis activity (43–45). The differences observed in our study can be attributed to the pathological context and the type of macrophages studied. Indeed, in the majority of cases, inflammatory macrophages were used (M1, M2a, M2b and M2c) whereas in our study, we used M-CSF macrophages, named M2d, with a specific and different transcriptomic profile close to TAM *in vivo* (26, 46). Indeed, M-CSF was described to be produced by cancer cells, including MPM cells (22), and cells from the TME and therefore highly present in malignant tissues (25). Moreover, cytokine secretion was measured here after LPS stimulation to activate macrophages (31), a situation completely different from the one used in previous study where macrophages were not stimulated and studied in a context of inflammatory pathology (19). Finally, resolutive macrophages have a high efferocytosis activity, however in this study we have studied phagocytosis of apoptotic tumor cells but not of apoptotic neutrophils

(efferocytosis). The description of human resolutive macrophages remains also poorly documented and requires further studies to clearly define this population (47). A recent single cell RNA Sequencing study in mice has identified several populations of phagocytic or non-phagocytic macrophages coexisting within the same tissue (48). Moreover, several subpopulations of phagocytic macrophages exist, one of which is described as “satiated” after pro-resolving lipid mediators treatment such as resolvin E1 (44). The particularities of these macrophages are a low expression of CD11b and a low efferocytosis and phagocytosis activity. Macrophages resulting from  $\alpha$ ChemR23 treatment could be closer to one of them, and might be characterized as “satiated” or “post-resolving” (44, 49).

Of major importance, our results revealed that ChemR23 activation by the agonist antibody led to decreased occurrence of lung metastasis and increased survival in a preclinical model of aggressive triple-negative breast tumors, when primary tumors were resected. Interestingly, stimulating resolution of inflammation using AINS or resolvins before tumor surgery decreased micrometastases in multiple tumor resection preclinical models through induction of T cell response (50) which is in accordance with the increase of IFN $\gamma$  score observed in  $\alpha$ ChemR23 responding mice. Our results strongly suggest that *in vivo* ChemR23 activation modulated tumor cell metastasis onset that may rely on decrease of dissemination and/or modification of the metastatic niche. Complementary experiments would be necessary to further dissect which cellular and molecular events are in play during the formation of this process. Importantly, Sulciner and colleagues previously reported that using proresolving lipids such as RvE1 in preclinical models of tumor growth, that relied on chemotherapy-induced cell debris, counteracted tumor progression through stimulation of macrophage phagocytic activity and decrease of their proinflammatory cytokine (TNF $\alpha$ , IL-6) release that occurred in a ChemR23-dependent process (4). The other ligand of ChemR23, chemerin, was previously shown to suppress breast cancer growth through recruitment of mainly NK dependent immune effectors in the tumor microenvironment (51). However, it may also exert a protumoral activity directly on tumor cells or through tumor associated mesenchymal or endothelial cells (52). In addition, chemerin is released by cancer-associated myofibroblasts in mammary tumors where it contributes to cancer cell invasion (53). Previously reported characterization of the agonist pro-resolutive anti-ChemR23 mAb have however excluded a chemerin-like activity of the antibody based on different signaling induction and opposite effect in chemoattraction assay (19).

In conclusion, in line with previous reports demonstrating the therapeutic interest to promote inflammation resolution during cancer treatments, our results also argue that targeting ChemR23 using an agonist antibody may improve cancer evolution in limiting metastasis occurrence. Using human M-CSF and TAM-like macrophages, we showed that targeting ChemR23 deeply affected their phenotype and functions leading to a less inflammatory and immunosuppressive profile. These changes could contribute to improve anti-tumor immune response and then, disease outcome. Our results fuel the proof of concept that modulating TAM phenotype to harness their antitumor potential, would improve cancer therapy efficacy.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repository and accession number(s) can be found below: NCBI via accession ID GSE218330.

## Ethics statement

Informed consent was obtained from enrolled patients and protocol was approved by Ministère de la Recherche (agreement n°: DC-2012-1598 and DC-2011-1399) and by local ethic committee (agreement n°: CB 2012/06) or through the biological resource center (CHU Nantes, Hôtel Dieu, Tumorothèque, Nantes, France BRIF: BB-0033-00040, transfer number 122C366) for patients with breast tumors or mesothelioma, respectively. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Comité d'éthique en expérimentation animale\_Pays de la Loire n°6.

## Author contributions

NP, SB-N, and CB conceived the study and the experimental setup, analyzed the experiments, wrote the manuscript with input from all authors and are responsible for the overall content of this manuscript; ML and VG performed the experiments and the data analysis and wrote the manuscript; CM produced  $\alpha$ ChemR23 antibody; IG and VG performed the analysis of nanostring experiments; SD performed RT-PCR and analysis; ML, JF, and VD performed cytometry experiments on human cells and analysis; AD performed western-blot; FC carried out histology experiments and analysis; ML and VG performed the mouse experiments; CM and SN carried out the follow-up of the *in vivo* experiments and biological studies; and PJ gave critical input to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

CT, VG, CM, and NP are inventors on a patent application (no. WO 2019/193029, filed 3 April 2019, published 8 October 2019) and product (WO2021/069709, filed 09 October 2019, published 15 April 2021) related to this work filed by OSE Immunotherapeutics and employees of OSE Immunotherapeutics with IG and SN. The remaining 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. This study was partially supported by OSE Immunotherapeutics funding.

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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.2023.1196731/full#supplementary-material>

### SUPPLEMENTARY FIGURE 1

Characterization of GM-CSF and M-CSF macrophages. Monocytes were incubated with GM-CSF (20 ng/ml) or M-CSF (50ng/ml) for 72h and analyzed by flow cytometry using a panel of 10 antibodies. n = 3. \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

### SUPPLEMENTARY FIGURE 2

Characterization of MPM and BC TAM-like macrophages. Monocytes were incubated with GM-CSF (20 ng/ml), M-CSF (50ng/ml) or conditioned media from breast (TAM-like (BC)) or mesothelioma (TAM-like (MPM)) cell lines for 3 days and stimulated with LPS (200ng/ml) for 24h. (A) Supernatants were collected and the indicated cytokines were analyzed by ELISA. n = 5. \*, p < 0.05; \*\*, p < 0.01. (B) Cells were analyzed by flow cytometry for the expression of the indicated markers. n = 5. \*, p < 0.05; \*\*, p < 0.01.

### SUPPLEMENTARY FIGURE 3

$\alpha$ ChemR23 agonist modulates macrophage's secretion profile (raw data related to). The indicated cytokines were quantified by ELISA in the supernatants of (A) M-CSF (n=15) or (B) GM-CSF (n=4) differentiated macrophages treated with hlgG1 or  $\alpha$ ChemR23 (10 $\mu$ g/ml) for 24h followed by LPS stimulation for 24h. \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001, \*\*\*\*, p < 0.0001.

### SUPPLEMENTARY FIGURE 4

Effect of  $\alpha$ ChemR23 antibody on *IDO1* expression in M-CSF and TAM-like macrophages. M-CSF and MPM cell culture supernatant differentiated



macrophages were treated with  $\alpha$ ChemR23 agonist or control hlgG1 for 24h followed by LPS stimulation (200ng/ml) for 6h. mRNA were extracted and expression of *IDO1* was analyzed using (A) Nanostring Immunology Panel (M-CSF macrophages, n=6) or (B) RT-PCR (MPM TAM-like macrophages, n=5). (C) Differential expression of *IDO1* in BC and MPM TAM-like. \*, p < 0.05; \*\*, p < 0.01, \*\*\*\*, p < 0.001.

#### SUPPLEMENTARY FIGURE 5

$\alpha$ ChemR23 agonist modifies M-CSF macrophage cell surface markers expression. M-CSF macrophages were treated for 24h with  $\alpha$ ChemR23 or control hlgG1 and cells were collected and labelled with a panel of 10 antibodies including: CD209, CD163, CD14, CD16, CD206, HLA-DR and CD45 and analyzed using flow cytometry Graphics presented are representatives of experiments performed using monocytes from 3 different healthy donors.

#### SUPPLEMENTARY FIGURE 6

Macrophage phagocytosis. GM-CSF, M-CSF and TAM-like macrophages were respectively incubated with untreated or apoptotic (A) BC or (B) MPM cell lines and phagocytosis was measured every hour by live cell imaging (Incucyte®), n=3. Phagocytosis of (C) BC or (D) MPM apoptotic cells by GM-CSF macrophages pretreated with  $\alpha$ ChemR23 or control hlgG1 for 24h and quantified by live-cell imaging (Incucyte®). Results represent the maximum fluorescence intensity normalized to GM-CSF macrophages condition, n=4.

#### SUPPLEMENTARY FIGURE 7

CD4 T-cell proliferation regulation by supernatants of M-CSF macrophages treated with  $\alpha$ ChemR23 agonist. For T cell proliferation assay, CPD stained CD3+ T-cells were incubated for 5 days with CD3/CD28 beads and either IL-2 (75 U/ml), or IL-10 (50 ng/ml), or supernatants of M(M-CSF) macrophages (MΦ) treated with  $\alpha$ ChemR23 agonist (10μg/ml) or hlgG1 control (10μg/ml) and T-cells were assessed by flow cytometry. Frequency of non-fluorescent proliferating cells was evaluated after gating on CD3+/CD4+ cells and normalized to the unstimulated condition. n=4. \*, p < 0.05.

#### SUPPLEMENTARY FIGURE 8

*CMKLR1* expression in breast cancer and mesothelioma tumors. (A) Expression of *CMKLR1* using transcriptomic data of breast cancer (n=1034) (B) and MPM tumor samples (n=87) (C) from the TCGA database. *CMKLR1* expression in (B) breast tumor subtypes (BRCA) and in (C) mesothelioma (MPM) tumor subtypes. EM: Epithelioid mesothelioma, BM: Biphasic mesothelioma, SM: Sarcomatoid mesothelioma. \*, p < 0.05; \*\*, p < 0.01; \*\*\*\*, p < 0.0001. (D)

#### SUPPLEMENTARY FIGURE 9

*CMKLR1* expression in breast cancer tumors using single cell RNA sequencing database. (A) Expression of *CMKLR1*, *CD14*, *CD163*, *HLA-DRA* and *CD80* in breast tumors (n=8) monocyte/macrophage subpopulations using single cell RNA sequencing database from <http://panmyeloid.cancer-pku.cn/>. Left panel, boxplot of gene signature expression level per cell calculated with the geometric mean of all gene expression levels. Right panel, heatmap representation of the median gene expression levels in monocyte/macrophage subgroups. (B) tSNE representation of the expression of *CMKLR1*, *CD14* and *CD163* in five triple negative breast cancers

using single cell RNA sequencing database from [https://singlecell.broadinstitute.org/single\\_cell/study/SCP1106/stromal-cell-diversity-associated-with-immune-evasion-in-human-triple-negative-breast-cancer](https://singlecell.broadinstitute.org/single_cell/study/SCP1106/stromal-cell-diversity-associated-with-immune-evasion-in-human-triple-negative-breast-cancer). (C) tSNE representation of the expression of *CMKLR1*, *CD14* and *CD163* in 26 breast cancers (11 ER+, 5 HER2+ and 10 TNBCs) using single cell RNA sequencing database from [https://singlecell.broadinstitute.org/single\\_cell/study/SCP1039/a-single-cell-and-spatially-resolved-atlas-of-human-breast-cancers](https://singlecell.broadinstitute.org/single_cell/study/SCP1039/a-single-cell-and-spatially-resolved-atlas-of-human-breast-cancers).

#### SUPPLEMENTARY FIGURE 10

$\alpha$ ChemR23 agonist effect on TAM-like macrophages' cytokine secretion profile (raw data related to ). Human monocytes were differentiated using breast (A) mesothelioma (B) cell culture supernatants for 3 days. TAM-like macrophages were treated with 10μg/ml of  $\alpha$ ChemR23 agonist or human control IgG1 for 24h and stimulated with LPS (200 ng/ml) for 24h. Supernatants were collected and the indicated cytokines were quantified using multiplex ELISA in the supernatants. n= 6-7. \*, p < 0.05; \*\*, p < 0.01.

#### SUPPLEMENTARY FIGURE 11

$\alpha$ ChemR23 agonist affects expression of genes involved in immune response modulation and angiogenesis. BC or MPM cell culture supernatant differentiated macrophages were treated with  $\alpha$ ChemR23 agonist or control hlgG1 for 24h followed by LPS stimulation (200ng/ml) for 6h. mRNA were extracted and expression of *VEGFA* (A) and *MMP9* (B) was analyzed using RT-PCR. n=5. \*, p < 0.05; \*\*, p < 0.01.

#### SUPPLEMENTARY FIGURE 12

$\alpha$ ChemR23 agonist activity on human, mouse and cynomolgus ChemR23. Recombinant human, cynomolgus and mouse ChemR23 was produced and purified by G-CLIPS Biotech company (Labège, France) after HEK transfection with a plasmid encoding the ChemR23 sequence with a fluorescent conformational dye on the cytoplasmic end of the TM6 and using an appropriate formulation buffer. Receptor activation can be assessed by monitoring the shift delta of between the TM6 opening or closing. After production, ChemR23 recombinant was encapsulated in appropriated lipid micelles to better recapitulate the conformation of the receptor on a cell-surface membrane. (A) Dose response of human, mouse and cynomolgus ChemR23 activation curves, (B) table of kinetics parameters of activation (Bmax and Kd) with  $\alpha$ ChemR23 antibody.

#### SUPPLEMENTARY FIGURE 13

$\alpha$ ChemR23 monotherapy effect on primary tumor growth in murine breast tumor model. (A) Primary tumor volume (mm<sup>3</sup>) measured in the 4T1-luc2 bearing mice treated i.p. with a control mAb or  $\alpha$ ChemR23 mAb (1mg/kg, 3 times a week from d7 to d28).

#### SUPPLEMENTARY FIGURE 14

$\alpha$ ChemR23 monotherapy strongly modifies the metastasis microenvironment by controlling the macrophage function. (A) Percent of CCR7+ M1 macrophages into lungs in the  $\alpha$ ChemR23 (isotype hlgG1 (black; n=4 or 8); red for non-responders n=3-4; blue for responders n=3-4) mAb-treated mice. (B) Representative heatmap of clustered differential gene expression of lungs in the  $\alpha$ ChemR23 (red for non-responders n=3-4; blue for responders n=3) mAb-treated mice.

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## EDITED BY

Mary Poupot-Marsan,  
INSERM U1037 Centre de Recherche en  
Cancérologie de Toulouse, France

## REVIEWED BY

Yoshinobu Koguchi,  
Providence Portland Medical Center,  
United States  
Pooya Farhangnia,  
Iran University of Medical Sciences, Iran

## \*CORRESPONDENCE

Jin-Chen Yu  
✉ jcyu@bio-thera.com

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# BAT6026, a novel anti-OX40 antibody with enhanced antibody dependent cellular cytotoxicity effect for cancer immunotherapy

Shizhong Liang<sup>1</sup>, Dandan Zheng<sup>1</sup>, Xiong Liu<sup>1</sup>, Xiong Mei<sup>2</sup>,  
Congcong Zhou<sup>2</sup>, Cuizhen Xiao<sup>1</sup>, Chao Qin<sup>1</sup>, Haitao Yue<sup>1</sup>,  
Jian Lin<sup>1</sup>, Cuihua Liu<sup>2</sup>, Shengfeng Li<sup>1</sup> and Jin-Chen Yu<sup>1\*</sup>

<sup>1</sup>Department of Discovery Research, Bio-Thera Solutions, Ltd., Guangzhou, Guangdong, China,

<sup>2</sup>Department of Technology Development, Bio-Thera Solutions, Ltd., Guangzhou, Guangdong, China

OX40 (CD134), a member of the TNF receptor superfamily, is a widely studied costimulatory immune checkpoint. Several OX40 agonistic antibodies are in the clinical stage for cancer treatment, among which PF-04518600 is the leader and currently in phase II trial. It has been recognized that one potential mode of action for anti-OX40 antibodies is the deletion of intratumoral Tregs. Thus, a novel human anti-OX40 antibody, BAT6026, was generated with enhanced antibody dependent cellular cytotoxicity (ADCC) *via* fucose deletion to strengthen its Treg depletion activity. This characteristic of BAT6026 differentiates it from other previously reported anti-OX40 antibodies in the field of tumor therapy. The affinity of BAT6026 to OX40 was 0.28nM, approximately 8 times stronger than that of PF-04518600. BAT6026 effectively competed for the binding of ligand OX40L to OX40, whereas PF-04518600 only partially competed. Moreover, compared to PF-04518600, BAT6026 activated T cells more effectively when clustered by FcγRs engagement and stimulated SEB-pretreated PBMCs to secrete IL-2 cytokines *in vitro*. In addition, BAT6026 demonstrated stronger anti-tumor activity than PF-04518600 in an OX40-humanized mouse MC38 tumor model. BAT6026 also showed a significantly synergistic effect on tumor inhibition when combined treatment with PD-1 antibody. Analysis of tumor-infiltrating T cells revealed that BAT6026 treatment significantly reduced Treg cells and increased CD8+ T cells in tumor. Preclinical safety assessment in non-human primates demonstrated a good safety profile for BAT6026. Together these data warrant further development of BAT6026 into clinical trials for patients with cancer.

## KEYWORDS

OX40, antibody, ADCC-enhanced, immune checkpoint, cancer immunotherapy, BAT6026

## Introduction

In recent years, the landscape of cancer treatment has been altered by the advent of immunotherapy, which has offered improved survival in several solid cancers and established itself as a new therapeutic modality. Among these immunotherapies, Ipilimumab, an antibody targeting CTLA-4, was the first approved checkpoint inhibitor. PD-1 and PDL-1 antibodies, blocking PD-1 pathway and thus activating T cells, are the second wave receiving regulatory approval (1). Recently, Relatlimab, an anti-LAG3 antibody, was approved for the treatment of unresectable or metastatic melanoma by FDA in combination with anti-PD-1 Nivolumab (2). Although these immunotherapies provide a new direction for cancer treatment, their overall response rates (ORR) as single treatment were generally only ~20% (3). Hence, the development of other novel immunotherapies to enhance clinical effectiveness is required (4). Since the approved anti-checkpoint antibodies all belong to inhibitory immune checkpoints, now targeting on stimulatory immune checkpoints may have a better chance of success as they also share responsibility in regulating immune cells (5, 6).

Many immunostimulatory checkpoints belong to the tumor necrosis factor receptor superfamily (TNFRSF), such as OX40, GITR, 4-1BB, CD40, and CD27 (7). OX40 (CD134) is a type I transmembrane glycoprotein composed of 275 amino acids and mainly expressed on activated immune cells, primarily CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and intratumoral Tregs (8). Its only known ligand, OX40L (CD252), is a type II transmembrane glycoprotein and expressed mainly on activated antigen-presenting cells induced by CD40, toll-like receptors, and inflammatory cytokines (9, 10). When binding to one OX40L trimer, three OX40 molecules are clustered, which directly activate NF- $\kappa$ B signal pathway as well as augment PI3K/PKB and NFAT pathways of the T cell receptor (TCR) (11, 12). These signal pathways account for the functional consequences of the division, survival and cytokine secretion of T cells. Although the cellular mechanism of OX40 antibody underlying anti-tumor immunity is not completely clear, its action has been widely recognized to contain three potential modes: (1) directly stimulating CD4<sup>+</sup> and CD8<sup>+</sup> T cells by enhancing their proliferation and survival; (2) inhibiting Tregs by reducing their suppressive function; (3) directly deleting intratumoral Tregs by engaging Fc $\gamma$  receptors on effector cells (13, 14).

In mouse tumor models, previous agonistic OX40 antibodies have shown remarkable anti-tumor efficacy as a single treatment or in combination with other immunotherapies (5). The antitumor efficacy was further reported to be mainly caused by the depletion of intratumoral regulatory T cells *via* ADCC effect (15, 16). In addition, OX40 was found to be expressed at high level on tumor Treg cells but at low level on tumor Teff cells in many types of human tumor (17–19). These data suggest that strengthening Treg depletion function of OX40 antibody may largely improve its antitumor activity in cancer patients. Besides Treg depletion mechanism, the agonistic anti-OX40 can also inhibit tumor by augmenting activation and proliferation of CD4<sup>+</sup> T cells that results into activation of CD8<sup>+</sup> T cells (20, 21).

To date, there are several anti-OX40 antibodies in clinical stage for cancer indication, including PF-04518600 (Pfizer), BMS-986178 and GSK3174998. The former two are in phase II and the latter two are in phase I trials, yet none of them is ADCC-enhanced (22). Considering the important role of Treg depletion in the anti-tumor effect of OX40 antibodies, we hypothesized that ADCC-enhanced OX40 antibodies may have stronger clinical efficacy than non-ADCC-enhanced OX40 antibodies. Herein we developed a novel ADCC-enhanced anti-OX40 antibody aiming to strengthen its Treg depletion activity, BAT6026. BAT6026 demonstrated favorable *in vitro* characteristics, mouse tumor model efficacy, and a good safety profile in monkey toxicity study. It is currently being tested in phase I trial.

## Materials and methods

### Cell lines and reagents

Raji cells were purchased from the National Collection of Authenticated Cell Culture. Jurkat cells stably expressing human OX40 were prepared in house. Jurkat cells stably expressing human OX40 in pCMV vector (SinoBiological) and NF- $\kappa$ B-luciferase construct in pGL4.32 vector (Promega) were prepared in house. Jurkat cells stably expressing human Fc $\gamma$ RIIIa (158V) in pCMV vector (SinoBiological) and NFAT-luciferase construct in pGL4.32 vector (Promega) were prepared in house. Recombinant extracellular domains (ECD) of OX40 from different species and OX40L-mFc (human OX40 ligand fused with mouse Fc domain) were purchased from ACRO. Recombinant human CD27, CTLA-4, GITR, CD40 and PD-1 were also purchased from ACRO. Goat anti-human kappa light chains-HRP secondary antibody was purchased from Sigma-Aldrich. ONE-Glo<sup>TM</sup> Luciferase Assay System and CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay kit were purchased from Promega. Human peripheral blood mononuclear cells (PBMCs) were purchased from Leide Bioscience. Staphylococcus enterotoxin B (SEB) was purchased from Invitrogen. The IL-2 detection kit was purchased from Mabtech.

### Antibody screening, optimization and generation

Using the method described in the literature of Michael et al, a yeast display library of completely human antibodies was constructed (23). First, the DNA fragments of the heavy chain variable region (VH) and the light chain variable region (VL) of human IgM and IgG gene were obtained by PCR technique. These VH and VL fragments were then assembled into scfvs *via* overlapping PCR reactions. The scfvs were inserted into the yeast display plasmid PYD1. Finally, a large number of these PYD1 plasmids were transduced into *Saccharomyces cerevisiae* by electroporation to obtain a yeast display library with an approximate size of  $5 \times 10^9$ .

To obtain candidate antibodies specifically targeting OX40, the yeast antibody library was screened and enriched using OX40-



coupled magnetic beads and fluorescence activated cell sorting (FACS). After initial screening and cell-based binding analysis, the positive candidates were subjected to further examination in cell-based function assays and animal efficacy studies to select the clinical candidates. The affinity of the clinical candidate was further improved by applying “DNA walking” technique (24).

BAT6026 was expressed in a FUT8 (alpha-(1,6)-fucosyltransferase)-knockout CHO cell line established in house. BAT6026-wt is a regularly fucosylated form of BAT6026, and was expressed in wild type CHO cell. PF-04518600 was prepared in house using heavy and light chain sequences from a patent (US 9,028,824 B2). BAT6026-mIgG2a is a fusion antibody with Fab domain of BAT6026 and Fc domain of mouse IgG2a lacking fucose modification, while BAT6026-mIgG2a-wt has the Fc domain of mouse IgG2a regularly fucosylated. PF-04518600 and BAT6026-mIgG2a-wt were transiently expressed in HEK293 cells, and BAT6026-mIgG2a was transiently expressed in a FUT8-knockout CHO cell line.

## Generation of the Fut8-knockout CHO cell line

The Fut8-knockout CHO cell line was established using TALEN gene editing technology, described in patent ZL201810910890. Firstly, the genome sequence of Fut8 (Gene ID: 100751648) was obtained by analyzing the complete genome sequence of Chinese hamster ovary cells, CHO-K1 (NW-003613860). Because the activity center of FUT8 enzyme resides in the region encoded by exon 1, the left and right flank sequences of exon 1 were chosen as the targeting sequences of TALEN technology. The Fut8-targeting TALEN plasmids were constructed and transfected into CHO-K1 cells using Lipofectamine 2000 reagent. Subsequently, the positive candidate clones with inactivated FUT8 enzyme were obtained by flow cytometry screening of cell surface characteristics. Genome sequencing was used to confirm the homozygous knockout of the Fut8 gene in the selected CHO cell line.

## OX40 specific binding assay

Microtiter plates were coated with the ECD of human OX40, cynomolgus OX40, mouse OX40, rat OX40, human CD27, CTLA-4, GITR, CD40 or PD-1 at 4°C overnight. After washing, the coated antigens were incubated with serial dilutions of BAT6026 for 1 h at 37°C. Goat anti-human kappa light chains-HRP secondary antibody was added to wells after washing and incubated for 30 min at 37°C. Color appeared after HRP substrate TMB was added to the wells, and the plates were read on the SpectraMax (Molecular Devices) at 450 nm.

## Binding to target-expressed cells

Jurkat cells stably expressing human OX40 were incubated with serial dilutions of BAT6026 or PF-04518600 antibody for 60 min in PBS with 1% BSA at 4°C, followed by washing and subsequent

incubation with PE-labeled goat anti-human Fc secondary antibody for 25 min at 4°C. Then cells were washed and resuspended in PBS with 1% BSA at 4°C, followed by flow cytometry analysis and calculation of mean fluorescence intensity (MFI) performed on an Accuri C6 system (BD Biosciences).

## Affinity measurement

Surface plasmon resonance (SPR) analysis was carried out using Protein A sensor chips (GE Healthcare) for measuring affinity kinetics between antibodies and OX40 antigen. Antibodies were diluted with HBS EP+ running buffer to 5 µg/ml and were first immobilized onto the sample flow cell of Protein A sensor chip with a flow rate of 10 µl/min at 25°C, and the reference flow cell was left blank. OX40 antigens were serially diluted with HBS EP+ running buffer, then injected over the two flow cells at a range of eight concentrations using a single-cycle kinetics program. HBS EP+ running buffer was also injected using the same program for background subtraction. All data were fitted to a 1:1 binding model using Biacore T200 Evaluation Software 3.1.

## OX40L blocking assay

Jurkat cells stably expressing human OX40 were incubated with OX40L-mFc and serial dilutions of BAT6026 or PF-04518600 antibody for 1 h in PBS containing 1% BSA at 4 °C. After cells were washed, the bound OX40L-mFc was detected by incubation with PE-labeled goat anti-mouse Fc secondary antibody. Then cells were washed and resuspended in PBS with 1% BSA at 4 °C, followed by flow cytometry analysis and calculation of MFI performed on an Accuri C6 system.

## T cell activation measured by a luciferase reporter assay

Jurkat cells stably expressing human OX40 and NF-κB-luciferase construct and equal number of Raji cells were mixed and co-incubated with serial dilutions of BAT6026, PF-04518600 antibody or an control IgG1 for 5 h at 37°C. Then the luciferase activity of samples were determined by a SpectraMax reader following the user guide of the ONE-Glo™ Luciferase Assay System (Promega).

## PBMCs activation assay

$2 \times 10^5$  PBMCs were added into each well of 96-well microtiter plates and pre-incubated with 90 ng/mL SEB for 24 h at 37°C. Then serial dilutions of BAT6026, PF-04518600 antibody or an isotype-matched negative control human IgG1 were added. After incubation at 37°C for 4 days, IL-2 secreted from activated PBMCs was determined by using an IL-2 detection kit (Mabtech).

## ADCC assay with luciferase reporter

Jurkat cells stably expressing human FcγRIIIa (158V) and NFAT-luciferase construct were used as effector cells, and Jurkat cells stably expressing human OX40 were used as target cells. The effector-to-target cell ratio in the assay was 2.5:1. Serial dilutions of BAT6026 or BAT6026-wt antibody were added to the cells and incubated for 4 hrs at 37°C. Then the luciferase of samples were determined by a SpectraMax reader following the user guide of the ONE-Glo<sup>TM</sup> Luciferase Assay System (Promega).

## ADCC assay with PBMCs

SEB-activated human PBMCs from healthy donors were used as effector cells and Jurkat cells stably expressing human OX40 were used as target cells. The effector-to-target cell ratio in the assay was 25:1. The cell ratios in this and above ADCC assays were optimized using various effector-to-target cell ratios, and selected based on good assay reproducibility and large signal-to-noise window. Serial dilutions of BAT6026 or BAT6026-wt antibodies were added to the cells, and incubated for 4 hrs at 37°C in RPMI 1640 with 10%FBS. Released lactate dehydrogenase (LDH) in culture supernatants was measured using a SpectraMax reader and CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay kit (Promega), according to the manufacturer's instructions.

## MC38 tumor model in OX40-humanized and PD-1/OX40-dual-humanized mice

1×10<sup>6</sup> MC38 cells were implanted subcutaneously into the right flank of OX40-humanized or PD-1/OX40-humanized female mice, whose ECD of OX40 or PD-1/OX40 was replaced by human counterpart (Biocytogen). When the tumor volume reached approximately 100 mm<sup>3</sup>, mice were randomly allocated into each study group and intraperitoneally injected with test antibodies once every 3 days for a total of 6 times. Tumor volume and body weight were measured twice a week, and mice were euthanized when the tumor volume reached 3000 mm<sup>3</sup>, or the percentage of body weight loss exceeded 20%.

## Analysis of tumor infiltrating lymphocytes and splenocytes with flow cytometry

OX40-humanized mice were implanted with MC38 tumor cells and treated with OX40 antibodies on days 9 and 14 post implantation. On day 17, tumors were collected and dissociated into single cell suspensions by using a digestive solution (1640 medium + 2%FBS + Collagenase IV (Sigma, C5138) + DNase I (Sigma, D5025)), and spleens were ground with sterilized glass slides and filtered through a steel mesh. Red blood cells were lysed using red cell lysing buffer (TIANGEN, RT122). Single cell suspensions were first incubated with the LIVE/DEAD<sup>TM</sup> Fixable

Green Dead Cell Stain Kit (Invitrogen, L34970), then labeled with the following antibodies in flow cytometric analyses: Brilliant Violet 421<sup>TM</sup> anti-mouse CD3 (BioLegend, 100228), PE anti-mouse Ki-67 (BioLegend, 652404), PerCP anti-mouse CD8a (BioLegend, 100732), APC/Cyanine7 anti-mouse CD45 (BioLegend, 103116), PE/Cyanine7 anti-mouse IFN-γ (BioLegend, 505826), eFluor<sup>TM</sup> 450 anti-FoxP3 (Invitrogen, 48-5773-82) and eFluor<sup>TM</sup> 506 anti-CD4 (Invitrogen, 69-0042-82). Intracellular FoxP3, IFN-γ and Ki67 were labeled following the product manual. Flow cytometry analysis was performed using Cytex<sup>®</sup> Aurora (Cytex Biosciences).

## GLP toxicity study of BAT6026 in cynomolgus monkeys

In the repeated-dose toxicology study, cynomolgus monkeys were injected intravenously with BAT6026 at doses of 1, 5 and 30 mg/kg (5 male and 5 female monkeys in each group) once per week (QW), for a total of 5 doses, followed by a 4-week recovery period. After the 5th dose, 3 monkeys per gender/group were euthanized and autopsied, and the remaining 2 monkeys in each gender/group were observed for an additional 28 days prior to being euthanized. The following parameters were examined during the study: clinical observations, ophthalmology, food consumption, body weight, body temperature, clinical pathology, lymphocyte subpopulation, immunoglobulins, toxicokinetics, immunogenicity, local skin reactions at the injection site, safety pharmacology, organ weight and ratios, gross pathology and histopathology.

## Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 8). Statistical significance between groups was determined using t-test or one-way ANOVA. A p value of ≤ 0.05 was considered statistically significant (\*p ≤ 0.05, \*\*p ≤ 0.01, \*\*\*p ≤ 0.001, and \*\*\*\*p ≤ 0.0001).

## Result

### Generation and affinity determination of BAT6026

A yeast display library of full human antibodies was constructed by cloning the DNA fragments of the VH and VL from human IgM and IgG gene in the form of scFv structure (23). Using human OX40-coupled magnetic beads and fluorescence activated cell sorting to screen the human antibody library, several candidate antibodies specifically targeting OX40 were obtained (25). These candidates were further selected using purified antigen and cell-based binding assays, cell-based function assays and animal efficacy studies, as well as through process of affinity maturation using "DNA walking" technique (24), to achieve the clinical candidate BAT6026.

Many characterization assays of BAT6026 were performed, including PF-04518600 as a comparator since it is the currently leading antibody in the anti-OX40 field (26). The binding affinity of BAT6026 to purified human OX40 antigen was evaluated utilizing SPR technology (BIAcore 2000). As shown in Figure 1A, the equilibrium dissociation constant (KD value) of BAT6026 to OX40 was 0.282nM, approximately 8-fold stronger than that of PF-04518600. The ability of BAT6026 to bind to OX40 on cells was measured using Jurkat cells expressing human OX40. BAT6026 bound OX40 expressed on Jurkat cells with an EC50 value of 1.91nM, similar to that of PF-04518600. However, the maximal binding signal value of BAT6026 was about twice higher than that of PF-04518600 (Figure 1B).

## Binding selectivity and ligand blocking of BAT6026 to OX40

To study binding specificity, the affinity of BAT6026 binding to human, cynomolgus, mouse and rat OX40 were examined by ELISA. These OX40 antigens were complete ECD of OX40s of each species. As shown in Figure 1C, BAT6026 had a high and similar affinity to human and monkey OX40 with an EC50 value of 0.034ug/ml and 0.035ug/ml, respectively. In contrast, BAT6026 did not bind to ECD of mouse or rat OX40 (Figure 1C). The binding specificity of BAT6026 against other immune checkpoint proteins was further examined. Results demonstrated that BAT6026 did not

bind to ECD of CD27, CTLA-4, GITR, CD40 and PD-1 at all (data not shown).

Next, we tested whether BAT6026 could block the binding of OX40L to OX40, using Jurkat cells overexpressing OX40. As shown in Figure 1D, BAT6026 significantly blocked the binding of OX40L to OX40 on the cell surface with an IC50 of 1.124nM, while PF-04518600 only moderately blocked. Since the binding affinities of BAT6026 and PF-04518600 to OX40 on the cell surface are similar, this difference in OX40L blocking is likely due to different binding regions of OX40 they recognize.

## Biological effect of BAT6026 on T cell activation *in vitro*

Clustered OX40 can stimulate T cells and strengthen the survival of T cells through pathways including NF- $\kappa$ B signal (13). Therefore, an NF- $\kappa$ B luciferase reporter assay was developed to determine the effect of BAT6026 on T cell activation. This reporter assay included Jurkat cells overexpressing human OX40 and NF $\kappa$ B-driven luciferase, and Raji cells expressing endogenous Fc $\gamma$ Rs (Figure 2A). As shown in Figure 2B, following binding to OX40 on T cell and subsequent crosslink offered by Fc $\gamma$ Rs on the Raji cells surface, BAT6026 demonstrated a dose-dependent fashion on T cell activation, with an EC50 of 0.0722 ug/ml. In this study, PF-04518600 exhibited a comparable EC50 in the study, however the maximal luciferase activity it provoked was much lower than that induced by

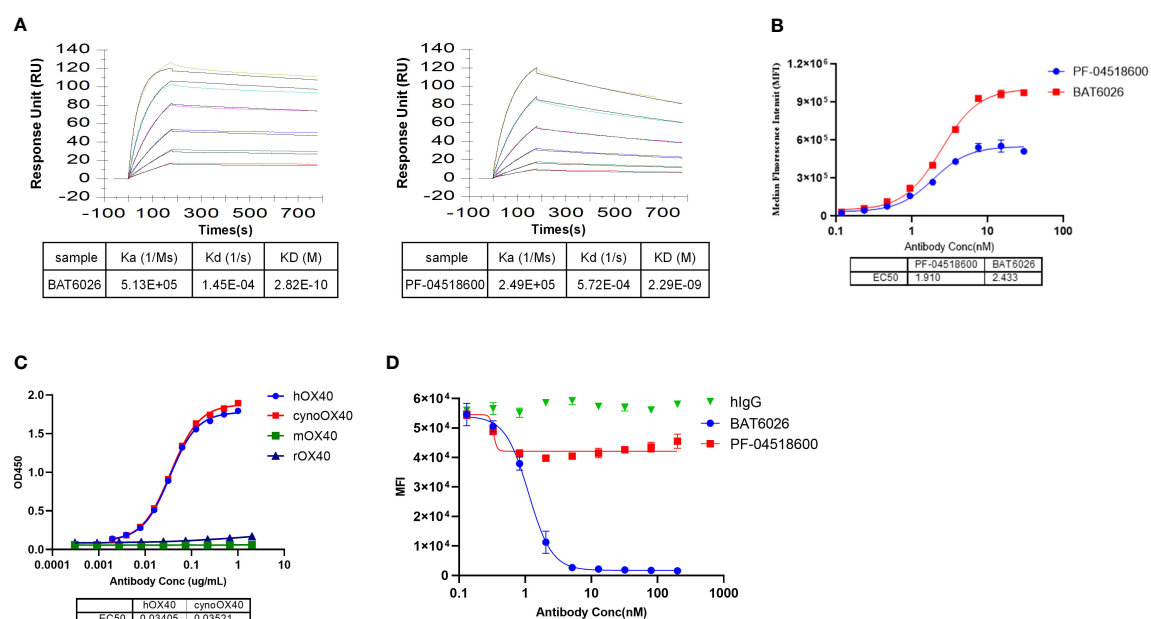


FIGURE 1

Affinity determination, binding selectivity and ligand blocking of BAT6026 to OX40. (A) Dynamic Curves of BAT6026 and PF-04518600 binding to human OX40 (His Tag) was detected using BIAcore T200 in 1:1 binding model. BAT6026 or PF-04518600 was set as ligands immobilized on CM5 chips. Human OX40 in different concentrations were set as analytes. (B) Applying cell-based binding assay and FACS to profile the affinity of BAT6026 or PF-04518600 to human OX40 expressed on Jurkat cells. (C) Using ELISA to compare the affinity of BAT6026 to extracellular domains of human OX40 (hOX40), monkey OX40 (cynoOX40), mouse OX40 (mOX40) or rat OX40 (rOX40). (D) Cell-based ligand competition assay. Increasing amounts of BAT6026 or PF-04518600 were incubated with Jurkat cells overexpressing human OX40 in the presence of 50ug/ml OX40L. The cell-bound OX40L was subsequently detected using Streptavidin R-Phycoerythrin Conjugate.

BAT6026. This is consistent with the lower maximal binding signal value of PF-04518600 than that of BAT6026 (Figure 1B).

To further assess the *in vitro* functional activity of BAT6026 on T cells, we measured its ability to provoke IL-2 cytokines release from SEB-pretreated PBMCs. In this assay, all concentrations (0.32–200 µg/ml) of BAT6026 induced approximately 4-fold IL-2 secretion over the background level of IgG control (Figure 2C). Although PF-04518600 appeared to show a minor dose-dependent increase of IL-2 release at these concentrations, it induced significantly lower IL-2 release than BAT6026, even at the highest concentration.

The mechanism of action of BAT6026 is to strengthen the immune function by activating T cells. Such type of agonistic antibody may over-activate the immune system and cause severe cytokine storms (27, 28). To examine whether BAT6026 may cause this harm, its ability to provoke cytokines release from unactivated PBMCs was measured. As shown in Figure S1, BAT6026 did not stimulate unactivated PBMCs to produce IL-2, IFN-γ, IL-6, and TNF-α even at concentration up to 200 µg/ml, compared to the positive control anti-CD28 antibody which caused thousands-fold more cytokines release at 200 µg/ml. The data implicated a high safety margin of BAT6026 in clinical trials.

## Enhanced ADCC of BAT6026 *in vitro*

Considering that one potential mode of action for OX40 antibodies is directly deleting intratumoral OX40-expressed Tregs (17–19), we hypothesized that it might be beneficial in clinical trials to enhance ADCC effect on OX40 antibody. Therefore, BAT6026 was expressed in a Fut8- knockout CHO cell line. Antibody expressed in this cell line was completely devoid of fucose modification (Supplementary Table 1), thus harboring a strengthened ADCC activity (29, 30). BAT6026 expression plasmid was also transfected into a wt CHO cells, and the product with normal level of fucose modification and ADCC activity was designated as BAT6026-wt for comparison. As the gamma Fc receptor IIIa (Fcγ-RIIIa) is the major Fc receptor

mediating ADCC effect (31), we first used SPR technology to evaluate the affinity of BAT6026 and BAT6026-wt to Fcγ-RIIIa. Table 1 shows that afucosylated BAT6026 exhibited approximately 10-fold higher binding ability than BAT6026-wt to Fcγ-RIIIa 158V and 158F both variants.

A cell-based functional assay, ADCC Reporter Bioassay system, was developed to measure the ADCC activity of BAT6026 (Figure 3A). In this assay, the target cell was Jurkat cell stably overexpressing OX40, and the effector cell was Jurkat cell stably overexpressing Fcγ-RIIIa and a luciferase reporter driven by an NFAT response element (32). Following OX40 antibody binding to OX40 on the target cell and crosslinked by Fcγ-RIIIa on the effector cell surface, the signal pathway downstream of Fcγ-RIIIa was activated and culminated in activation of the luciferase reporter gene (Figure 3A) (33). As shown in Figure 3B, BAT6026 exhibited an EC<sub>50</sub> value of 4.73 ng/ml, while BAT6026-wt exhibited an EC<sub>50</sub> value of 19.11 ng/ml, indicating the ADCC activity of BAT6026 was enhanced approximately 4-fold. Human IgG (hIgG) was nonspecific IgG, which did not bind to OX40-expressing cells and was used as a negative control.

Another cell-based assay was applied to detect the *in vitro* ADCC activity of BAT6026. PBMCs from healthy human as the effector cell were incubated with anti-OX40 antibody and the target cell, Jurkat cell expressing OX40. The effector cells would directly kill the target cells upon activated by antibody-mediated FcγRs clustering (33). BAT6026 showed obviously stronger ADCC activity than BAT6026-wt in this assay (Figure 3C), similar to the results of another assay (Figure 3B).

## Anti-tumor effect of BAT6026 in humanized syngeneic mice tumor model

BAT6026 did not bind to murine OX40, thus OX40-humanized mice were used to study the effect on tumor growth. OX40-humanized mice were generated by replacing the ECD of mouse OX40 with that of human OX40 in mouse blastocyst. After establishing the homozygous humanized OX40 mice, the

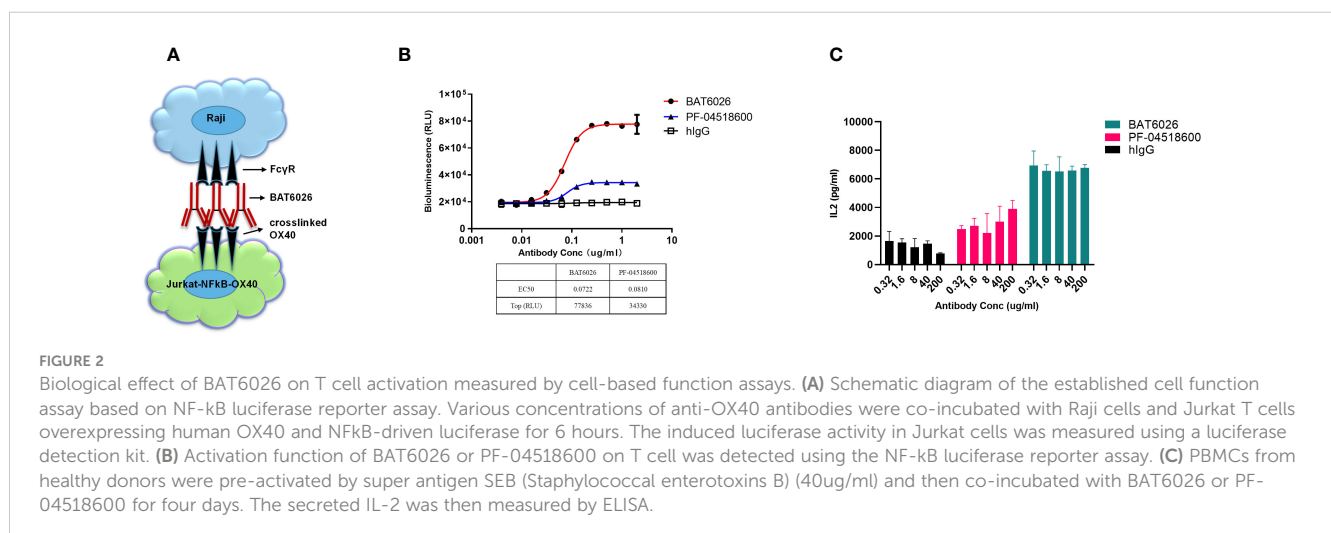




TABLE 1 Kinetic parameters of BAT6026 and BAT6026-wt binding to FcγR1IIa 158V or FcγR1IIa 158F.

Sample	FcγR1IIa 158F			FcγR1IIa 158V		
	$k_{on}(1/Ms)$	$k_{dis}(1/s)$	$K_D^a (M)$	$k_{on}(1/Ms)$	$k_{dis}(1/s)$	$K_D^a (M)$
BAT6026	3.37E+05	6.32E-02	1.88E-07	3.30E+05	1.87E-02	5.67E-08
BAT6026-wt <sup>b</sup>	4.52E+04	1.13E-01	2.51E-06	2.17E+05	1.05E-01	4.81E-07

FcγR, gamma Fc receptor;  $k_{on}$ , association rate;  $k_{dis}$ , dissociation rate;  $K_D$ , equilibrium dissociation constant.  
<sup>a</sup> $K_D$  values are calculated from the ratio of the kinetic constants as  $K_D = k_{dis}/k_{on}$ .  
<sup>b</sup>BAT6026-wt, the BAT6026 Fc variant with wilde type IgG1 Fc.

expression of humanized OX40 in T cells upon activation has been confirmed (data not shown). As shown in Figure 4A, BAT6026 demonstrated a dose-dependent efficacy in inhibiting syngeneic MC38 mouse tumor growth. The mean tumor volume of BAT6026 in the 1 and 0.2 mg/kg groups at 31 days post-tumor inoculation showed a statistically significant difference compared with that of the isotype control group. The relative tumor growth inhibition (TGI) of 1 and 0.2 mg/kg BAT6026 groups was 62.8% and 37.7%, respectively. In contrast, the relative TGI of the same dose groups of PF-04518600 were merely 6.6% and 16.1%, respectively. These data demonstrated that the efficacy of BAT6026 on tumor growth inhibition was significantly stronger than that of PF-04518600.

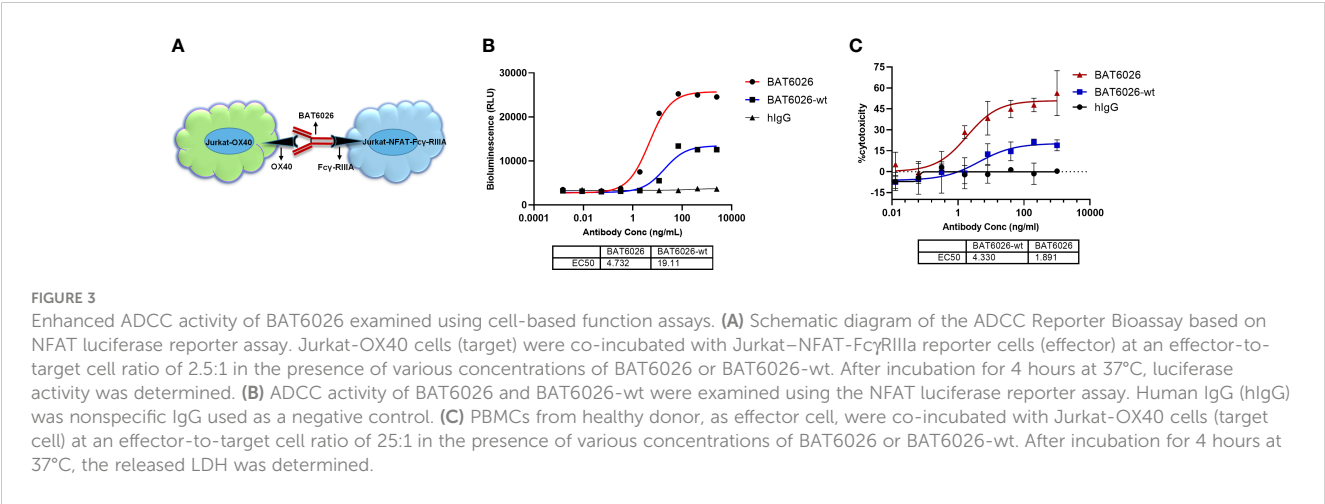
To enhance T cells activation, combination treatment with PD-1, PD-L1, or CTLA-4 antibodies is a feasible approach for anti-OX40 immunotherapy. Therefore we examined the effect of combination treatment of BAT6026 with an anti-PD1 antibody, BAT1308, in a syngeneic MC38 mouse tumor model. Since BAT1308 did not bind to murine antigen either, PD-1/OX40-dual-humanized mice were used for the study. As shown in Figure 4C, combination treatment of 0.2 mg/kg BAT6026 plus 0.3 mg/kg BAT1308 resulted in a statistically significant difference in the mean tumor volume compared to that caused by each monotherapy ( $p=0.0025$  for BAT6026 and  $p=0.0027$  for BAT1308) at 35 days post-tumor inoculation (Supplementary Table 2). The relative TGI of 0.2 mg/kg BAT6026 group and 0.3 mg/kg BAT1308 group were 35.2% and 34.4%, respectively, while that of the combination treatment group was 72.7%. A similar trend of efficacy enhancement was also observed in the combination treatment of 1 mg/kg BAT6026 plus 0.3 mg/kg BAT1308 compared to BAT6026 monotherapy. However the enhancement was not as apparent as

with 0.2 mg/kg BAT6026 dosing, likely due to the already high TGI (65.7%) of 1 mg/kg BAT6026 monotherapy (Figure 4C and Supplementary Table 2). Together, these results showed that combination treatment of BAT6026 and BAT1308 was significantly more effective than monotherapy on tumor growth inhibition.

Safety evaluation in the mouse tumor studies was based on animal death and body weight changes (Figures 4B, D). All treatment groups showed no deaths or signs of serious toxicity and the drug was well tolerated throughout the treatment (data not shown).

### BAT6026 reduced Tregs proportion and increases CD8+ T cells proportion in tumors

To further explore the pharmacodynamic effects of BAT6026 and its mechanistic difference from BAT6026-wt in tumor, we analyzed the proportion and activation status of tumor-infiltrated T cells in mouse tumors treated with these two OX40 antibodies. Because human IgG1 is equivalent to mouse IgG2a in terms of interaction between FC domain and FC receptors (34, 35), we replaced the FC domain of BAT6026s with the mouse IgG2a FC domain to form two hybrid antibodies, BAT6026-mIgG2a-wt (normal ADCC) and BAT6026-mIgG2a (enhanced ADCC), which was completely devoid of fucose modification (Supplementary Table 3). OX40-humanized mice inoculated with MC38 tumors were treated with BAT6026-mIgG2a, BAT6026-mIgG2a-wt or vehicle at days 9 and 14 post-tumor inoculation. Tumors and spleens were collected and analyzed using flow cytometry on day 15.



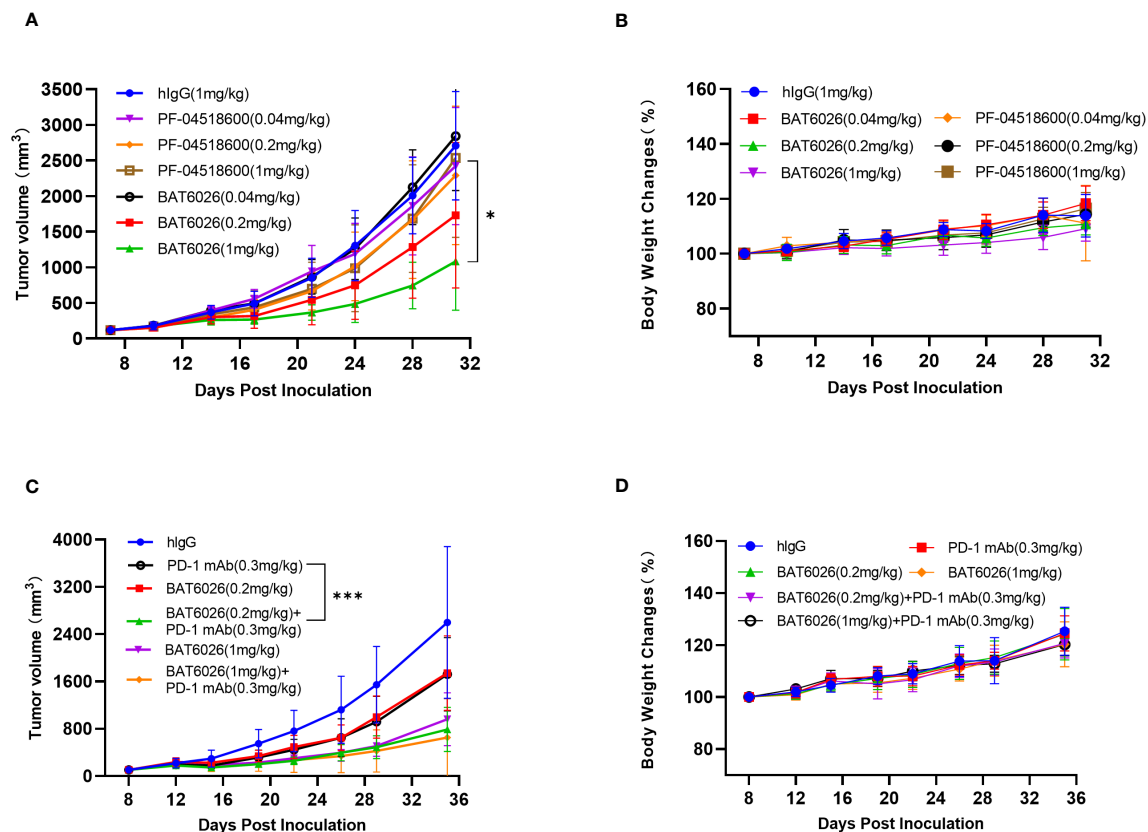


FIGURE 4

BAT6026 showed significant efficacy alone and in combination with anti-PD1 in mouse MC38 tumor model. (A) MC38 murine colon carcinoma cells were subcutaneously inoculated in OX40-humanized mice. When tumor reaches a mean volume of 119 mm<sup>3</sup> at day 8 post tumor inoculation, animals were grouped (n=8) and dosed with hlgG control (1mg/kg), BAT6026 (1, 0.2, and 0.04mg/kg) or PF-04518600 (1, 0.2, and 0.04mg/kg) by intraperitoneal injection once every three days for a total of 6 times. (B) The body weight changes of the mice were measured twice a week and recorded after dosing. (C) MC38 cells were subcutaneously inoculated in OX40/PD1-dual-humanized mice. When tumor reaches about 100 mm<sup>3</sup> at day 8 post tumor inoculation, hlgG control (1mg/kg), BAT6026 (0.2mg/kg), PD-1 antibody (BAT1308) (0.3mg/kg) or the combination dose was administered to the mice (n=8) by intraperitoneal injection once every three days for a total of 6 times. (D) The body weight changes of the mice were measured twice a week and recorded after dosing. All data was shown as mean  $\pm$  SEM. \*p < 0.05, \*\*\*p < 0.001.

As shown in Figure 5A, the proportions of CD4<sup>+</sup> T cells and Treg cells in tumors of the BAT6026-mIgG2a-wt group and BAT6026-mIgG2a group were significantly reduced than those of the vehicle control group. This effect was more pronounced in the BAT6026-mIgG2a group, likely because of the stronger ADCC effect. Meanwhile, the proportion of intra-tumoral CD8<sup>+</sup> T cells was significantly increased in BAT6026-mIgG2a-wt and BAT6026-mIgG2a groups, with the latter group being even more apparent (Figure 5A). These data suggest that ADCC-enhanced BAT6026-mIgG2a may deplete more intra-tumoral Treg cells than BAT6026-mIgG2a-wt, thus resulting in more CD8<sup>+</sup> T cells infiltration inside the tumor. In spleen, the same trend of effect on CD4<sup>+</sup> and CD8<sup>+</sup> T cells caused by treatment with these two OX40 antibodies was detected. However, unlike in tumors, the proportion of Tregs in spleen did not change significantly after OX40 antibodies treatment (Figures 5B).

The activation status of T cells in mice treated with these OX40 antibodies were also examined. Compared with the control group, the percentage of IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> or IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells was significantly increased in tumor after treatment with either OX40 antibody, and the level of increase was similar between these two antibodies (Figures 5C). These data suggest that treatment with the two OX40

antibodies may cause different levels of decrease on CD4<sup>+</sup> T cells and increase on CD8<sup>+</sup> T cells in tumor (Figures 5A), yet they cause a similar level of IFN- $\gamma$  expression, implicating T cell activation, on CD4<sup>+</sup> and CD8<sup>+</sup> cells, in tumor. Roughly similar phenomena were observed in the spleen following treatment with these OX40 antibodies (Figures 5B, D). Furthermore, the proportion of proliferating tumor-infiltrated CD8<sup>+</sup> T cells (Ki67<sup>+</sup>), but not CD4<sup>+</sup> T cells, was significantly increased in tumor after administration of either OX40 antibodies (Figures 5C).

## Safety profile of BAT6026 in cynomolgus monkeys

Prior to clinical trial in human, a GLP toxicology study of BAT6026 was performed in cynomolgus monkeys. In a repeat-dose toxicology study, 40 cynomolgus monkeys (20 females and 20 males) were administered BAT6026 (1, 5 or 30 mg/kg) *via* intravenous infusion once a week for a total of 5 doses, then the animals were allowed for a 4-week recovery phase. The results showed that repeated infusions of BAT6026 to monkeys were well tolerated at these doses,

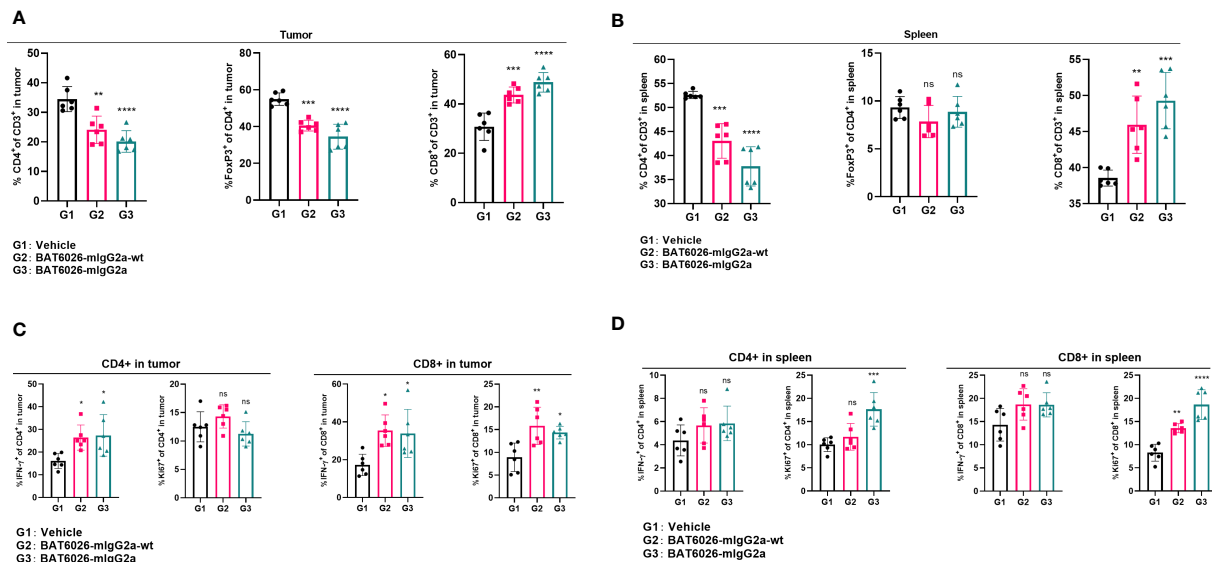


FIGURE 5

BAT6026 treatment decreased Tregs proportion, increased CD8<sup>+</sup> T cell proportion and activated both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in tumor. OX40-humanized mice bearing MC38 tumor were dosed with 5 mg/kg BAT6026-mIgG2a or BAT6026-mIgG2a-wt on days 9 and 14 post tumor injection. Single cell suspensions from tumors and spleens were collected and analyzed by flow cytometry 48 hrs after the last dose. (A) The percentage of CD4<sup>+</sup> T cells, Treg and CD8<sup>+</sup> T cells in tumor. (B) The percentage of CD4<sup>+</sup> T cells, Treg and CD8<sup>+</sup> T cells in spleen. (C) The percentage of IFN- $\gamma$ <sup>+</sup> or Ki67<sup>+</sup> CD4<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> or Ki67<sup>+</sup> CD8<sup>+</sup> T in tumor. (D) The percentage of IFN- $\gamma$ <sup>+</sup> or Ki67<sup>+</sup> CD4<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> or Ki67<sup>+</sup> CD8<sup>+</sup> T cells in spleen. Data shown as mean  $\pm$  SEM, N=6. Ordinary one-way ANOVA was used (\* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001. ns, no significant difference).

with main changes observed of decreased neutrophils, transient increased IL-6 and slight histological changes in spleen/liver (Supplementary Table 4). Therefore, the highest non-severely toxic dose (HNSTD) in the study was determined to be 30 mg/kg.

## Discussion

As a novel and fully human IgG1 monoclonal antibody, BAT6026 was obtained through screening a yeast display library and affinity maturation process. BAT6026 demonstrated a high and specific affinity to purified antigen and cell-surface human OX40. To strengthen one of its modes of action on Treg depletion, BAT6026 was expressed as an ADCC-enhanced antibody, which differentiates it from other anti-OX40 antibodies for cancer indication. Compared with current anti-OX40 field leader, PF-04518600, BAT6026 demonstrated superior activities on binding to OX40, blocking binding of OX40L to OX40, activation of T cells and SEB-pretreated PBMCs, as well as tumor inhibition in MC38 tumor model of OX40-humanized mice. BAT6026 also showed a significantly synergistic effect on tumor inhibition when combined with an anti-PD-1 antibody. We further investigated the effect of ADCC enhancement of BAT6026 on the proportions of CD4<sup>+</sup> T cells and Tregs in mouse tumor, and the results showed that these cells were significantly reduced in mice treated with BAT6026 compared to its cognate antibody with regular ADCC, BAT6026-wt.

It has been reported that the expression level of OX40 on Tregs is much higher than that on CD8<sup>+</sup> T cells in many types of tumors (17–19), suggesting the tumor inhibition mechanism of OX40 antibody may be predominantly mediated *via* Treg suppression. Indeed, Bulliard et al. demonstrated that anti-OX40 antibody

treatment caused mice Colon26 tumor regression and concomitant elimination of intratumoral Treg cells *via* Fc $\gamma$ R-mediated ADCC effect (15). We used a pair of OX40 antibodies with approximately four-fold difference in their ADCC activity, and found that treatment with ADCC-enhanced BAT6026 resulted in significantly fewer CD4<sup>+</sup> T cells and Treg cells in mice tumor than with BAT6026-wt. The *in vivo* efficacy of these two antibodies was also compared in an MC38 syngeneic mouse tumor model. Although not with statistically significant difference, BAT6026 showed a trend of stronger anti-tumor effect in the OX40-humanized mice model than BAT6026-wt (TGI 38.3% versus 21.1%) (data not shown). The reason that difference in TGI was not large could be because both antibodies were human IgG1 and thus in mouse model the ADCC activity difference was reduced (34). Afterall, our data were generally consistent with the report of Bulliard et al. and support the notion that the predominant anti-tumor mechanism of OX40 antibody is through Treg suppression.

In Figure 5A, we observed that Treg in mouse tumor was reduced when treated with BAT6026, but not completely depleted. This may be due to the fact that some Treg cells express little or no OX40 inside the tumor. Moreover, the number of effector cells, such as NK cells, inside the tumor is limited, which may lead to limited depletion of Treg *via* ADCC effect. Treg depletion/reduction was not observed in spleen (Figure 5B). Since Treg cells in spleen may be inactive, the expression level of OX40 on these cells is none or very low, which may prevent Treg depletion/reduction.

BAT6026 demonstrated a dose-dependent and powerful efficacy in inhibiting MC38 tumor growth in syngeneic mice, with a stronger efficacy than PF-04518600 and BAT6026-wt. Furthermore, in an OX40/PD1 dual humanized mouse tumor

model, combination treatment of BAT6026 and anti-PD1 BAT1308 was significantly more effective than single treatment. A manuscript by Messenheimer et al. reported that concurrent administration of PD-1 and OX40 antibodies could suppress the therapeutic effects of OX40 antibody in an orthotopically transplanted MMTV-PyMT mammary tumor model. However, sequential combination treatment of OX40 antibody followed by PD-1 antibody (but not the reverse order) resulted in significant increases in therapeutic efficacy (36). Another research found that addition of PD-1 antibody exhibited a detrimental effect on the antitumor response of OX40 antibody when they were concurrently administered in TC-1 tumor model (37). So far these are the only two manuscripts describing such attenuation effect on tumor inhibition by combination treatment. Many other preclinical studies in the field have reported that simultaneous administration of OX40 and PD-1 antibodies resulted in a significant synergistic antitumor effect (5, 38), which is consistent with our report here. These different results could be due to the different mouse tumor models used. To clarify the consequences of different administration orders for combination therapy of OX40 and PD-1 antibodies, more in-depth researches are required.

BAT6026 was expressed as an antibody completely devoid of fucose modification on N297 of Fc domain. Nonfucosylated modification can increase the affinity of Fc domain to FcγRIIIa 5–10 folds or even more (29, 39). Besides enhancing ADCC effect through stronger binding to NK cell, the increased Fc-FcγRIIIa interaction has recently been reported to promote the communication between APC and T cells bound with immune checkpoint antibodies, which further activated T cells and strengthened their tumoricidal activity (40). Thus, nonfucosylated BAT6026 can enhance the activation of CD4+ and CD8+ T cells, as well as depletion of Tregs. Compared to PF-04518600, another advantage BAT6026 holds is its ability to induce cross-linking provided by FcγRs. OX40 is a member of TNFRSF and its activation requires aggregation induced by binding to cell surface trivalent ligand OX40L, or to antibodies and subsequently cross-linked by FcγRs (13). BAT6026 is an ADCC-enhanced IgG1 antibody, while PF-04518600 is a regular IgG2 antibody, which has lower affinity to FcγRs (41), and thus weaker ability to induce cross-linking. In the T cell activation experiment, PF-04518600 exhibited a notably lower level of maximal activation signal than BAT6026 (Figure 2B). This difference could be due to its weaker ability to induce cross-linking provided by FcγRs. Recently, two articles reported that the reduced affinity of costimulatory receptors (CD40/4-1BB/OX40) antibodies let them achieve superior agonism by Fc receptors-independent clustering (42, 43). In our hands, we have also observed a similar phenomenon on OX40 antibodies. OX40 antibodies with relatively low affinity (10–20nM), not high affinity (<1nM), can activate OX40 in the absence of Fc receptors. However, when Fc receptors are present, OX40 antibodies with high affinity can activate OX40 at similar level as low-affinity antibodies (data not shown). After all, leaning toward more effectively depleting OX40+ Treg in tumors, BAT6026 with high affinity was chosen as the clinical candidate.

BAT6026 did not show significant side effects in preclinical toxicology study. Also, most OX40 antibodies did not show significant side effects in clinical trials. This may be due to the fact that OX40 is inducible and mainly expressed within 24–72 hours after activation of CD4+ and CD8+ T cells, but not on resting

T cells. BAT6026 is currently in phase I clinical trial administered alone (NCT05105971) and in combination with PD-1 antibody (NCT05109650). With these superior characteristics and a favorable safety profile in preclinical GLP toxicity research, we look forward to exposing the clinical superiority of BAT6026.

## 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 author.

## Ethics statement

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee of Biocytogen Pharmaceuticals (Beijing) Co., Ltd.

## Author contributions

ShL, DZ, XL, XM, CZ, CX, HY carried out the experiments. ShL, CQ, JL, CL, Sfl, J-CY conceived of the study and participated in its design and coordination. ShL and J-CY drafted the manuscript. All authors read and approved the final manuscript.

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## Conflict of interest

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2023.1211759/full#supplementary-material>



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## OPEN ACCESS

## EDITED BY

Chiara Porta,  
University of Eastern Piedmont, Italy

## REVIEWED BY

Karine Crozat,  
INSERM UMR1236 Microenvironnement,  
Différenciation Cellulaire, Immunologie et  
Cancer, France  
Scott Wetzell,  
University of Montana, United States

## \*CORRESPONDENCE

Martin Villalba  
✉ martin.villalba@inserm.fr

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# NK cells in peripheral blood carry trogocytosed tumor antigens from solid cancer cells

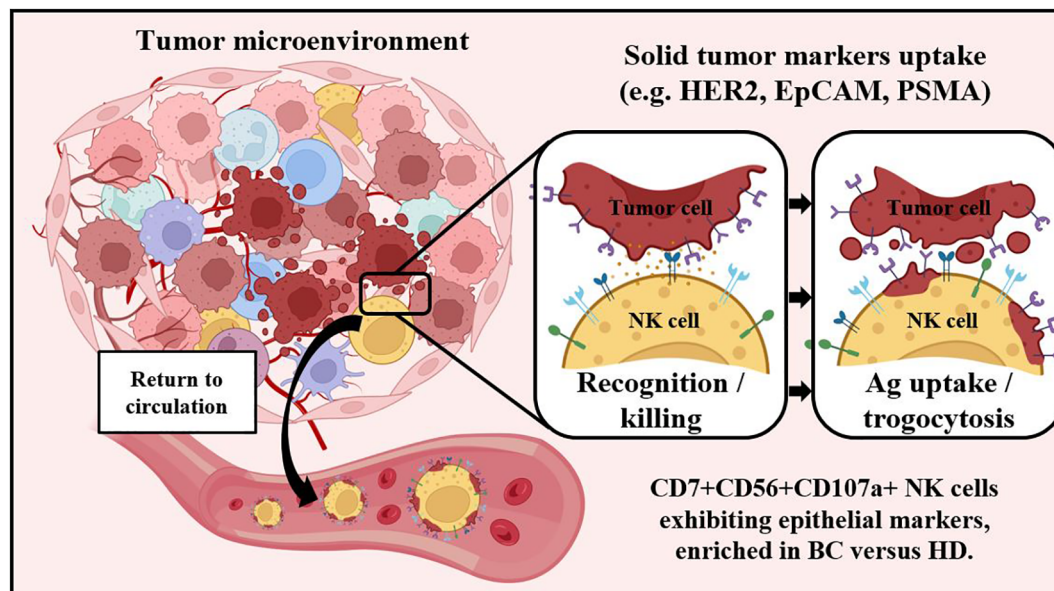
Mauricio Campos-Mora<sup>1</sup>, William Jacot<sup>2</sup>, Genevieve Garcin<sup>1</sup>,  
Marie-Lise Depondt<sup>1</sup>, Michael Constantinides<sup>1</sup>,  
Catherine Alexia<sup>1</sup> and Martin Villalba<sup>1,3,4\*</sup>

<sup>1</sup>IRMB, Univ Montpellier, INSERM, Montpellier, France, <sup>2</sup>Institut du Cancer de Montpellier (ICM) Val d'Aurelle, Montpellier University, INSERM U1194, Montpellier, France, <sup>3</sup>IRMB, University of Montpellier, INSERM, CNRS, Montpellier, France, <sup>4</sup>Institut du Cancer Avignon-Provence Sainte Catherine, Avignon, France

The innate immune lymphocyte lineage natural killer (NK) cell infiltrates tumor environment where it can recognize and eliminate tumor cells. NK cell tumor infiltration is linked to patient prognosis. However, it is unknown if some of these antitumor NK cells leave the tumor environment. In blood-borne cancers, NK cells that have interacted with leukemic cells are recognized by the co-expression of two CD45 isoforms (CD45RARO cells) and/or the plasma membrane presence of tumor antigens (Ag), which NK cells acquire by trogocytosis. We evaluated solid tumor Ag uptake by trogocytosis on NK cells by performing co-cultures *in vitro*. We analyzed NK population subsets by unsupervised dimensional reduction techniques in blood samples from breast tumor (BC) patients and healthy donors (HD). We confirmed that NK cells perform trogocytosis from solid cancer cells *in vitro*. The extent of trogocytosis depends on the target cell and the antigen, but not on the amount of Ag expressed by the target cell or the sensitivity to NK cell killing. We identified by FlowSOM (Self-Organizing Maps) several NK cell clusters differentially abundant between BC patients and HD, including anti-tumor NK subsets with phenotype CD45RARO+CD107a+. These analyses showed that *bona-fide* NK cells that have degranulated were increased in patients and, additionally, these NK cells exhibit trogocytosis of solid tumor Ag on their surface. However, the frequency of NK cells that have trogocytosed is very low and much lower than that found in hematological cancer patients, suggesting that the number of NK cells that exit the tumor environment is scarce. To our knowledge, this is the first report describing the presence of solid tumor markers on circulating NK subsets from breast tumor patients. This NK cell immune profiling could lead to generate novel strategies to complement established therapies for BC patients or to the use of peripheral blood NK cells in the theranostic of solid cancer patients after treatment.

## KEYWORDS

NK cells, t-SNE, trogocytosis, breast cancer, CD45



## GRAPHICAL ABSTRACT

Human NK cells that encounter cancerous cells uptake epithelial antigens (Ag) and can be identified on blood of breast tumor patients comprising specific NK subsets.

## Introduction

Natural killer (NK) cells are a subset of lymphoid cells and part of the innate immune compartment. As blood-circulating cells with cytotoxic activity, NK cells screen for damaged or stressed cells, and they are readily able to kill virus-infected or transformed tumor cells, contributing to immune surveillance (1). These cells are mainly classified as CD56<sup>+</sup>CD3<sup>-</sup> innate lymphoid cells, but they constitute a heterogeneous population comprising NK cell subsets with different cytotoxic potential. Based on CD56 and CD16 surface expression, they subdivide into CD56<sup>+</sup>CD16<sup>high</sup> blood-circulating NK cells, with stronger cytotoxic activity after target cell recognition, and CD56<sup>high</sup>CD16<sup>low</sup> cells cytokine-producing NK cells with poor cytolytic activity, mostly present in secondary lymphoid tissues (2). However, recent studies have challenged this classical view, and high dimensionality, single-cell proteomic analysis have revealed a striking NK cell phenotype diversity, which might be influenced by both genetic differences between individual humans and environmental conditions (3). Considering that NK

cell function is tightly modulated by the expression of several inhibitory and activating receptors, the determination of NK cell subsets repertoire and their contribution to physiological processes could be of paramount importance for generation of NK cell-based therapies against malignant diseases (4, 5).

The anti-tumor properties of NK cells have been previously discussed (6). NK cells play an essential role in tumor clearance by recognizing and killing abnormal tumor cells without the need of prior activation. After recognition of target cells, different target cell-derived proteins can be acquired in NK cell membrane surface in a cell-to-cell contact-dependent manner, a process called trogocytosis (7–11). Trogocytosis involves an intercellular transfer of membrane patches, and it has been shown to occur in different immune cell types, albeit the physiological relevance of this process is not fully understood (9, 11–13). This transference of functional proteins to cell surface could modulate NK function *in vitro* and *in vivo* (14–20). Trogocytosis is receiving high interest from the clinic for this possibility to modulate NK cell (18, 20) or CAR T cell function (21, 22).

We have reported the identification of NK cell populations with anti-tumor activity in hematological cancer patients (23–26). The highly activated CD56<sup>+</sup>CD16<sup>high</sup> NK cells found in these patients exhibit the expression of activation markers, such as NKP46 and NKG2D, and low expression levels of inhibitory markers, such as NKG2A and CD94. Interestingly, these NK cells also present non-NK, tumor cell-derived antigens on their surface, which can be an indicative of trogocytosis during cell killing (23–26). We found that this subset of anti-tumor NK cells is also characterized by degranulation and co-expression of both CD45RO and CD45RA (CD45RARO cells) (23–26). Further high-dimensionality, multiparametric flow cytometry and unsupervised analyses in multiple hematological tumor patients showed that NK subsets

**Abbreviations:** Ag, antigens; BC, breast cancer; CITRUS, cluster identification, characterization and regression; CytD, cytochalasin D; EBV, Epstein-Barr Virus; eNK, expanded human NK cell; EpCAM, epithelial cell adhesion molecule; FBS, fetal bovine serum; FDR, false discovery rate; HD, healthy donor; HER2, human epidermal growth factor receptor 2; MUC1, mucin-1; NK, natural killer cell; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffer saline; PDL, poly-D-lysine; PD1, programmed cell death protein 1; PSMA, prostate-specific membrane antigen; SAM, significance analysis of microarrays; SOM, self-organizing maps; TNBC, triple-negative breast cancer; t-SNE, t-stochastic neighbor embedding; UCB, umbilical cord blood; UCBMC, UCB mononuclear cells; viSNE, Barnes-Hut implementation of t-SNE.

presenting CD45RARO phenotype and evident tumor-antigen derived trogocytosis responds directly to the oncologic status of patients, which suggest that the frequency of the function of these NK subsets depend of the presence of targets (23, 24).

NK cells infiltrate solid cancers, as well as tumor-infiltrated lymph nodes and metastases (27–29). NK cell infiltration of most solid tumor is rather sparse and depends on tumor localization and the nature of the cancer (30). To our knowledge, detection of anti-tumor NK cell subsets expressing trogocytosed tumor-derived markers in peripheral blood of solid tumor patients have not been reported. Here we investigated whether NK cells could acquire solid tumor antigens by trogocytosis *in vitro* and *in vivo*. Additionally, we studied the presence of anti-tumor, blood-circulating, NK cells subsets exhibiting these solid tumor antigens in breast cancer patients by multiparametric flow cytometry and high-dimensionality unsupervised analyses.

## Patients, materials and methods

### Ethical statement

The use of human specimens for scientific purposes was approved by the French National Ethics Committee. All methods were carried out in accordance with the approved guidelines and regulations of this committee. Written informed consent was obtained from each patient or donor prior to collection.

### Breast tumors patients

Data and samples from patients were collected at the Institute for Cancerology of Montpellier (ICM), France, after patient's

written consent and following French regulations. Patients were enrolled in the ICM-BDD 2017/37 (ID-RCB: 2017-A01940-53) clinical program approved by the “Comités de Protection des Personnes Sud-Ouest et Outre-Mer III” with the reference 2017/45. Blood samples were collected at diagnosis. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll® gradient and stored frozen in liquid nitrogen until use. Patients' status is described in Table 1.

### Healthy donor

HD samples were obtained from written informed donors, collected by clinicians of the CHU Montpellier and collected and processed as the patient's samples.

### Cell lines

Breast cancer cell lines BT-20, SKBR3 and MDA-MB-468, pancreatic adenocarcinoma cell line LNCaP, colorectal adenocarcinoma cell line HCT116, and the Epstein-Barr Virus (EBV)-transformed lymphoblastoid B cell line PLH were grown in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum (FBS). Cells were used for experiments at confluency of ~80%. Cell line identity was confirmed by flow cytometry when possible, and cells were regularly tested for mycoplasma.

### UCBMC purification

Umbilical cord blood (UCB) units obtained from healthy donors from CHU Montpellier. UCB mononuclear cells

TABLE 1 Description of patients.

Patient Number	Pathology	Estrogen Receptor (%)	Progesterone Receptor (%)	HER2 Overexpression	Patients ER/PR Receptor status	Clinical Status
1	Breast cancer	0	0	3+	–	Alive
2	Breast cancer	100	70	2+/FISH-	+	Alive
3	Breast cancer	100	100	1+	+	Alive
4	Breast cancer	100	70	0	+	Alive
5	Breast cancer	90	90	1+	+	Alive
6	Breast cancer	60	30	1+	–	Alive
7	<i>In situ</i> breast cancer carcinoma	ND	ND	ND	–	Alive
8	Breast cancer	95	5	2+/FISH-	+	Alive
9	Atypical ductal hyperplasia	ND	ND	ND	–	Alive
10	Breast cancer	100	5	2+/FISH-	+	Alive

Blood samples were collected from patients and PBMCs were frozen until use. The clinical status of the patients is depicted regarding expression of estrogen and progesterone receptors and HER2 status (0, 1+, 2+/FISH- are, altogether “HER2-negative”, and 2+/FISH+ and 3+ are “HER2-positive”). Patients 7 and 9 were included in this study, but were not considered per se cancers. Patient 7 developed an *in situ* carcinoma and consequently ER, PR and HER2 were not tested. Patient 9 showed atypical ductal hyperplasia at recruitment, indicative of an increased risk of cancer, and, indeed, the patient developed invasive cancer the following year. ER, PR and HER2 are typically not tested in this population. The column “Patient Receptor status” describes the patients who are considered in the group of receptor-negative patients (–) or receptor-positive patients (+).

FISH, fluorescence *in situ* hybridization; ER, estrogen receptor; HER2, human epidermal growth receptor 2; ND, not determined; PR, progesterone receptor.



(UCBMC) were collected from UCB units using Ficoll® Paque Plus (Sigma) by density gradient centrifugation. Briefly, one volume of Ficoll® Paque Plus were added to conical tubes, and two volumes of blood (previously diluted 1:1 with RPMI media) were slowly deposited at the top. Tubes were centrifuged at 425 x g for 30 min at room temperature, without brake. Mononuclear cells were collected from buffy coat layer, washed in RPMI and resuspended in RPMI media supplemented with 10% FBS.

## Enrichment, activation and expansion of human NK cells

Expanded NK (eNK) cells were obtained as previously described (31–34). Briefly, UCBMC were depleted of T cells by using EasySep™ CD3 Positive Selection Kit II (STEMCELL Technologies). Cells were cultured in the presence of  $\gamma$ -irradiated PLH cells at ratio NK-to-accessory cell of 1:1 in RPMI 10% FBS media supplemented with human IL-2 (100UI/mL, Peprotech) and human IL-15 (5 ng/mL, Miltenyi Biotec) for 14-to-21 days. Once every 3 days, cells were counted and fresh culture media with FBS, IL-2 and IL-15 was added to the culture, along with additional  $\gamma$ -irradiated PLH cells. Purity of human CD3-CD56+ eNK cells at the end of the culture was always  $\geq 90\%$ .

## Trogocytosis *in vitro* and staining

Adherent tumor cells were seeded in 48-well flat-bottom plates (200,000 cells/mL in culture medium) and incubated overnight at 37°C. Trogocytosis *in vitro* of tumor markers was attained by co-culturing eNK cells (acceptor cells) with BT20, LNCaP, SKBR3, MDA-MB-468 or HCT116 (donor cells) in several acceptor-to-donor ratios. For experiments under inhibition of actin recruitment, eNK cells were previously treated with 2  $\mu$ g/mL cytochalasin D (CytD, Sigma-Aldrich) for 30 min at 37°C. In experiments where Src kinase was inhibited, NK cells were pre-treated with 10  $\mu$ M PP2 (Sigma) for 10 min and then maintained during the co-culture. NK cells were also co-cultured with tumor cells at 4°C as a control. For membrane dye transfer experiments, SKBR3 or MDA-MB-468 cells were stained with Vybrant™ DiD Cell-Labeling Solution (Invitrogen) for 20 min, and then co-cultured with NK cells. Plates were centrifuged at 150 x g for 30 seconds to favor cell contact, and cells were incubated at 37°C for 2 h or overnight (16 h). After trogocytosis, eNK cells were recovered and washed several times in FACS buffer (PBS, 2% FBS). For experiments to measure tumor marker trogocytosis, cells were stained for surface markers with the following fluorochrome-coupled antibodies: CD56-V450; CD19-ECD; HER2-PE (all from BD); CD16-KO (Beckman); CD3-APC; CD335-PE-Vio770; CD45RO-FITC; CD45RA-APC-Vio770; and PSMA-PE or EpCAM-PE (all from Miltenyi Biotec). For experiments to determine measuring membrane dye transfer, cells were for surface markers with the antibodies CD56-V450 and CD3-FITC (all from BD). Cell viability was determined using 7-AAD exclusion (Miltenyi Biotec). Staining was performed in FACS buffer at 4°C for 25–30 min, and then cells were washed three times before

FACS acquisition and analysis in Gallios flow cytometer instrument (Beckman Coulter). After exclusion of doublets and dead cells, tumor marker detection on CD3-CD56+CD335+ NK cells was evaluated by analyzing FCS files using FlowJo software v10.6.1 (Tree Star Inc.).

## Trogocytosis *ex vivo*

In brief, healthy donors and patient's total peripheral blood mononuclear cells (PBMC) were stained for surface markers with the following fluorochrome conjugated antibodies: CD4-BUV737, CD56-BUV395, CD3-BV786, CD16-BV711, CD7-BV421, HER2-BV650, CD326-BV605, PD1-FITC, MUC1-PerCP, CD107a-PE-CF594, CD14-AF700, CD19-AF700, PSMA-PE, CD45RO-VioGreen, NKp46-PE-Vio770, CD45RA-APC-Vio770 and CD138-APC (all from Miltenyi Biotec). Cell viability was determined using DAPI exclusion (BD Biosciences). Cells were stained with antibodies cocktail in FACS buffer at 4°C for 25–30 min and washed twice with the same FACS buffer and acquired on BD LSR-Fortessa instrument (Blue-Yellow/Green-Red-Violet-Ultraviolet) (BD Bioscience). FCS files were analyzed using FlowJo software v10.6.1 (Tree Star Inc.).

## Fluorescence microscopy

Breast cancer cell line SKBR3 cells were seeded in 24-well flat-bottom plates (200,000 cells/mL in culture medium) on coverslips previously coated with poly-D-lysine (Sigma), and incubated overnight at 37°C. Trogocytosis *in vitro* of tumor markers was attained by co-culturing eNK cells (acceptor cells) with SKBR3 (donor cells) in effector-to-target ratio of 1:1, leaving some coverslips with eNK alone. Cells were incubated overnight at 37°C and after media removal and wash with PBS, attached cells were fixed using 4% paraformaldehyde diluted in PBS and blocked with 5% FBS diluted in PBS. Cells were then incubated with CD56-AlexaFluor™488 and CD326-PE antibodies (all from BD Biosciences) diluted in blocking solution for 2 h at room temperature, protected from light. Some coverslips with eNK cells were stained with CD45-PE antibody (Beckman) diluted in blocking solution. Hoechst 33342 was used for nucleus staining. After several washing steps, coverslips were mounted on microscope glass slides using Prolong Gold mounting media (ThermoFisher). Cell samples were visualized using a Leica SP5 fluorescence microscope (Carl Zeiss, Germany) and images analyzed using the Las X Life Science software (Carl Zeiss).

## High dimensional reduction analysis

To generate tSNE or UMAP embedding, a pre-gated NK cell population from each sample with the same number of cells per patient and timepoint was selected using FlowJo Downsample plugin (v3.1.0) and merged before uploading in the Cytobank cloud-based platform (Cytobank, Inc.). High-dimensional single-cell data dimensionality reduction was performed by viSNE, which is based

upon the t-Distributed Stochastic Neighbor Embedding (t-SNE) implementation of Barnes-Hut (35). viSNE was used to visualize FACS data as 2D t-SNE maps, using the following parameters: Desired Total Events (Equal sub-sampling): 50,000; Channels: selected all 16 surface markers; Compensation: “File-Internal Compensation”; Iterations: 1000; Seed: “Random”; Theta: 0.5. FlowSOM was used with default settings unless otherwise noted. FlowSOM uses Self-Organizing Maps (SOMs), based on marker expression phenotype, to assign all individual cells into clusters and metaclusters (that is, group of clusters) (36). FlowSOM was performed with the following parameters: Event Sampling Method: “Equal”; Desired events per file: “2.736”; Total events actually sampled: “27.160”; SOM Creation: “Create a new SOM”; Clustering Method: “Hierarchical Consensus”; Number of metaclusters: “12”; Number clusters: “256”; Iterations: “100”; Seed: “4567”. CITRUS (cluster identification, characterization, and regression) is an algorithm designed for the discovery of statistically significant stratifying biological signatures within single cell datasets containing numerous samples across associated conditions or correlated with clinical phenotype of interest (e.g. responders versus non-responders) (37). The output is a network topology of cell subpopulations divided in sub-clusters that represents a hierarchical stratification of the original sample. Median expression levels of functional markers measured across each population can drive the differentiation between phenotypes. CITRUS was performed using the Significance Analysis of Microarrays (SAM) correlative association model (Benjamin-Hochberg-corrected  $P$  value, false discovery rate (FDR) < 0.01), with the following parameters: Clustering channels: “selected all surface markers except CD107a and tumor markers”; Compensation: “File-Internal Compensation”; Statistic channels: “CD107a and tumor markers”; Association Models: “Significance Analysis of Microarrays (SAM) – Correlative”; Cluster Characterization: “Medians”; Event sampling: “Equal”; Event sampled per file: “2000”; Minimum cluster size (%): “1”; Cross Validation Folds: “5”; False Discovery Rate (%): “1”. Identification of NK cell subsets between the group “Healthy Donors” and “Breast Cancer patients” was performed by comparing the relative expression of CD107a and tumor markers of the specified FlowSOM metacluster.

## Statistical analysis

Experimental figures and statistical analysis were performed using GraphPad Prism (v8.0). All statistical values are presented as \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$ . Mean values are expressed as mean plus or minus the standard error of the mean (SEM).

## Results

### NK cells perform trogocytosis *in vitro* on solid tumor cells

The breast cancer cell lines BT-20 and SKBR3, the human prostate adenocarcinoma cell line LNCaP and the human colon

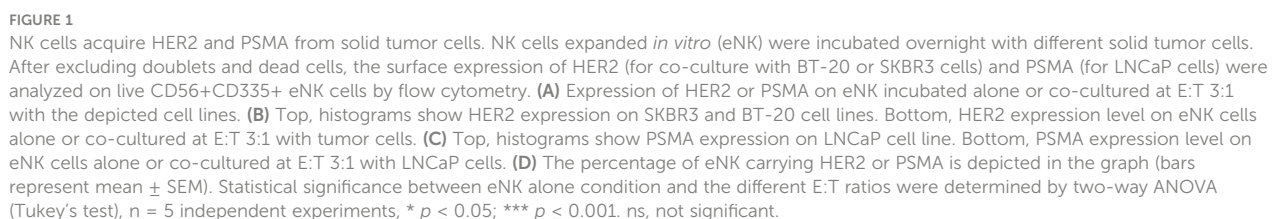
cancer cell line HCT116 lack expression of CD3 (T cell marker) and CD56 (NK cell marker) and show very low expression of CD335 (NK cell marker), also known as Nkp46/NCR1 (Figure S1). In contrast, they do express human epidermal growth factor receptor 2 (HER2) and Epithelial cell adhesion molecule (EpCAM or CD326).

We expanded NK cells (eNK) *in vitro* as previously described (31–34) and incubated them with BT-20, SKBR3 and LNCaP cell lines. All these cell lines were sensitive to eNK cell killing (Figures S2A, B).

As expected, eNK do not express HER2 or prostate-specific membrane antigen (PSMA) (Figure 1A). When eNK were incubated with BT-20 and SKBR3 cells, they gained HER2 expression (Figure 1A), although the levels were 10 times lower than those found in target cells (Figures S1, 1B). eNK also gained PSMA when incubated with LNCaP cells that largely express this Ag (Figures 1A, C). Again, the expression of PSMA was 10 times lower for eNK than for LNCaP (Figure 1C). Optimal trogocytosis on all cell lines was observed at 3:1 E:T ratio (Figures 1D, S3). Of note, eNK encountering BT-20 cells acquire less HER2 expression than those encountering SKBR3 (Figure 1A, S3), although the tumor cell sensitivity to NK cytotoxicity was similar (Figure S2).

After confirming that NK cells can capture tumor antigens from solid cancer cells, we wanted to elucidate whether this gaining of expression was caused by trogocytosis. For this objective, as trogocytosis is a very rapid process, we restricted our co-cultures down to 2 h of incubation (38, 39). There is a lack of specific inhibitors for trogocytosis, but it has been described its interference by disruption of actin polymerization, inhibition of kinases (such as Src-kinase and Syk-kinase) and low temperature (4°C) (40, 41). Before performing the co-culture with SKBR3 tumor cells, we pre-treated eNK cells with cytochalasin-D (CytD) for inhibition of actin recruitment, or incubated the cells in the presence of PP2 for inhibition of Src-tyrosine kinase, which resulted in a decrease of HER2 acquisition by NK cells (Figure S4A). Moreover, we carried out these co-cultures at 4°C which completely reduced tumor marker acquisition (Figure S4B). We complemented these observations by using MDA-MB-468 as breast cancer donor tumor cells, which are negative for HER2 in comparison to SKBR3 (Figure S4C). Consistent with the idea that HER2 is acquired from donor cells via trogocytosis, NK cells co-cultured with MDA-MB-468 cells at different ratios did not increase HER2 expression after incubation (Figure S4D). However, this result would not be due to the absence of trogocytosis from this cell line, as we confirmed by membrane dye transfer experiments, in which NK cells were co-cultured with SKBR3 or MDA-MB-468 breast tumor cells previously labeled with the lipid intercalant dye DiD (Figure S4E). Even only after 2 h of co-culture, NK cells became strongly positive for this dye, and pre-treatments with CytD and PP2 along with 4°C incubation blocked the acquisition of donor membrane lipids from NK cells (Figure S4E). Considering that transfer of proteins via trogocytosis goes together with transfer of membrane lipids, altogether these results suggest that HER2 and other solid tumor markers are acquired by NK cells via trogocytosis.

To further confirm that eNK cells captured tumor cell-expressed receptors by trogocytosis, we analyzed the interaction between solid tumor cells (donor cells) and eNK cells during co-



We next investigated if different Ags were differently uploaded from the same targets. eNK efficiently gained EpCAM, but much less HER2, from HCT116 or LNCaP cells (**Figure 3**), whereas these

We used a cohort of patients operated for a breast tumor (8 invasive breast cancers, one ductal *in situ* carcinoma, one atypical ductal hyperplasia) to analyze several phenotypic markers on

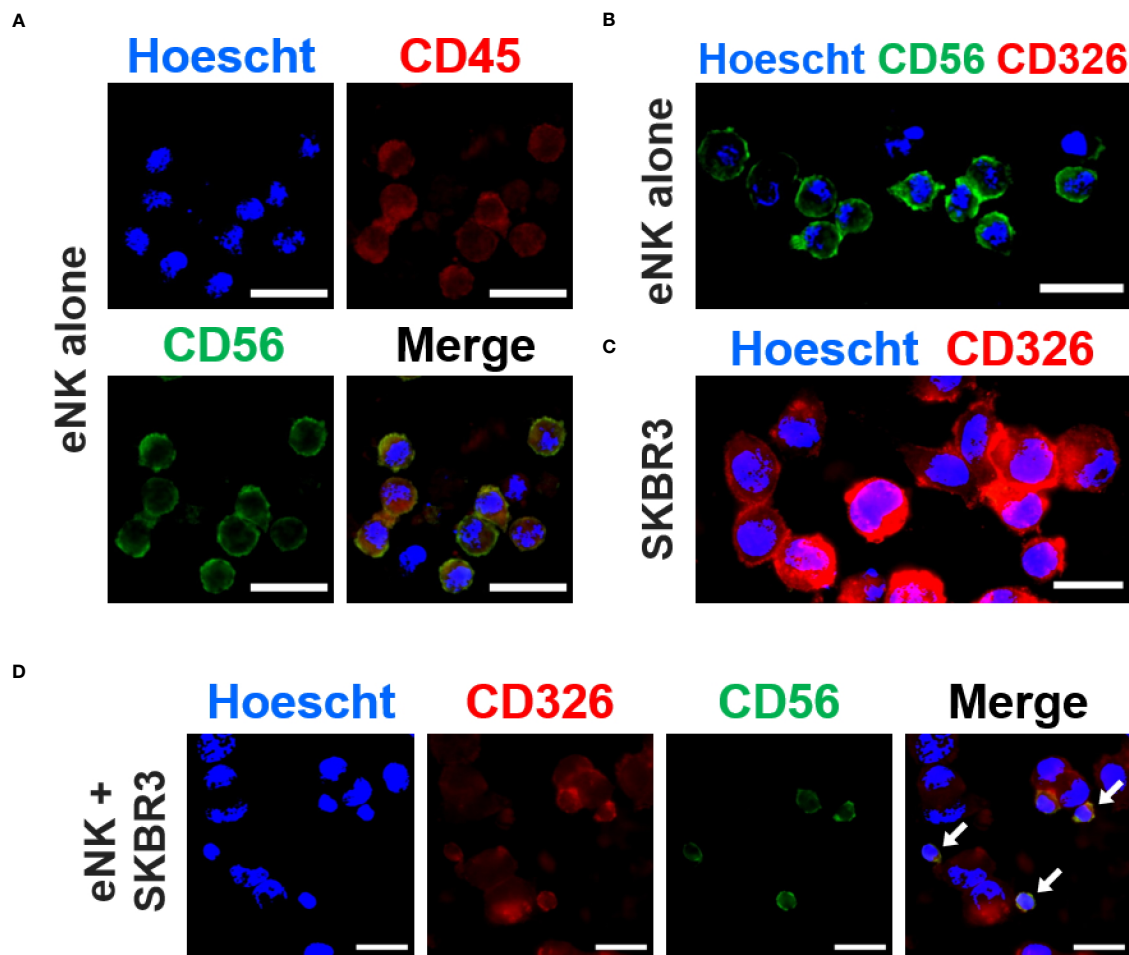


FIGURE 2

eNK cells capture solid tumor cell antigens *in vitro*. *In vitro* expanded NK cells (eNK) were incubated overnight alone or with SKBR3 solid tumor cells and, after antibody staining, the expression of several markers was analyzed by fluorescence microscopy. (A) eNK cells were stained with PE-conjugated anti-CD45 (red) and AlexaFluor@488-conjugated anti-CD56 (green) antibodies. The nucleus was stained with Hoescht (blue). (B) eNK cells were stained with PE-conjugated anti-CD326 (red) and AlexaFluor@488-conjugated anti-CD56 (green) antibodies. The nucleus was stained with Hoescht (blue). (C) SKBR3 cells were stained with PE-conjugated anti-CD326 (EpCAM, red) antibodies and Hoescht. (D) eNK cells co-cultured with SKBR3 cells at E:T ratio of 1:1 were stained for CD326 and CD56 as previously described. The representative micrograph panel show acquisition of CD326 expression by eNKs (yellow) via trogocytosis (white arrows). Scale bars (white): 20  $\mu$ m.

peripheral NK cells. We collected and analyzed blood samples at diagnosis and compared them to NK cells of a cohort of 5 healthy donors (HD) by multiparametric flow cytometry (Figure S5). Cancer patients expressed different values of estrogen and progesterone receptors and few of them overexpressed HER2. For future analysis in this manuscript, we could independently analyze patients with high expression of estrogen and/or progesterone receptors (patients 2, 3, 4, 5, 8, 10; receptor-positive or Receptor+ or R+) and those with low or unknown expression (patients 1, 6, 7, 9; receptor-negative or Receptor- or R-). Of note, estrogen and progesterone receptors are nuclear proteins and hence, NK cells should not perform trogocytosis on them.

The percentage of CD7<sup>+</sup> cells, which mainly include T and NK cells tended to decrease in patients; and in fact, the percentage of CD56<sup>+</sup> cells decreased (Figures 4A, B). However, the CD56<sup>+</sup>/CD16<sup>+</sup> cells, which represents the mature NK cells, remained stable (Figure 4C). The CD56<sup>+</sup> cell subset that decreased were CD3<sup>+</sup>

and should represent NK T cells (Figure 4D). In contrast, the CD56<sup>+</sup>/CD3<sup>-</sup> population, which represents NK cells, remained stable.

In hematological cancers, the main antitumor NK cell population is recognized by the expression of CD45RO (CD45RO cells), generally together with CD45RA (CD45RARO cells (23–26)). We observed a small, but significant, increase of this CD45RARO population in peripheral blood NK cells of patients (Figure 4E). This was associated with a decrease in cells expressing low CD45RA levels (CD45RA<sup>dim</sup>) and CD45RO (CD45RA<sup>dim</sup>RO cells). On the other side, the CD45RO<sup>+</sup> NK subset frequency was found similar between BC patients and HD (Figure 4E).

We next evaluated degranulation, i.e. CD107a<sup>+</sup> cells, in the lymphoid, i.e. CD7<sup>+</sup>, compartment and observed an increase in patients (Figure S6A). Exclusion of CD14<sup>+</sup>/CD19<sup>+</sup> cells did not change our observation (Figure S6B). The differences were not statistically different when we focused on receptor-negative patients



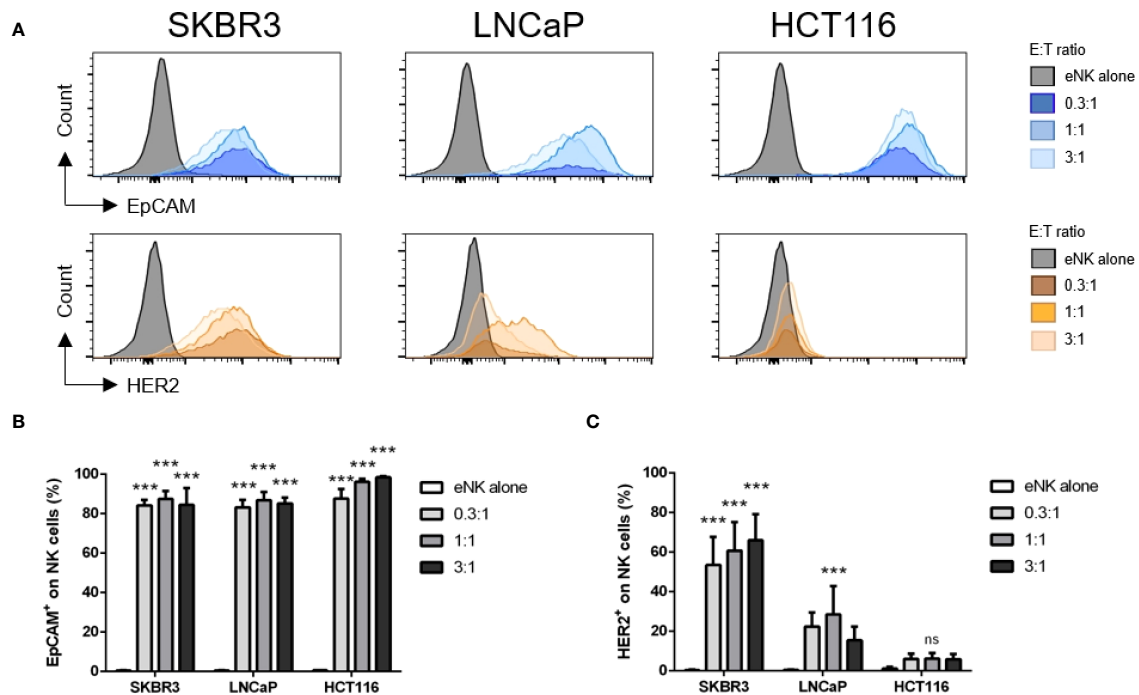


FIGURE 3

Different Ags are differently trophocytosed from different cell lines. *In vitro* expanded NK cells (eNK) were incubated alone or at different effector:target (E:T) ratios with solid tumor cells, and after exclusion of doublets and dead cells, the surface expression of HER2 and EpCAM was analyzed on live CD56+CD335+ NK cell population. (A) Representative histograms depict EpCAM (CD326) and HER2 expression on eNK cells. (B, C) Bars represent mean  $\pm$  SEM of  $n = 4$  independent experiments. Statistical significance between untreated cells and different E:T ratios were determined by two-way ANOVA (Tukey's test); \*\*\*  $p \leq 0.001$ . ns, not significant.

(Figure S6C) and hence, the increase mainly relied in receptor-positive patients (Figures S6D, E). Analysis of *bona fide* CD7<sup>+</sup>CD56<sup>+</sup>CD3<sup>-</sup> NK cells (42) confirmed the higher degranulation of NK cells in BC patients (Figure 4F).

We analyzed trophocytosis of 4 endothelial/tumor markers, which are not expressed by NK cells. These were: HER2 (human epidermal growth factor receptor 2, which is overexpressed in certain patients), CD326 (EpCAM), Mucin 1 cell surface associated (MUC1) and PSMA. The membrane localization of all these proteins makes them candidates to be trophocytosed and exposed in NK cell plasma membrane. Patients showed higher trophocytosis of all markers except EpCAM (Figure 5A), suggesting that this Ag is not well uptaken by NK cells *in vivo*. However, differences were not statistically significant in the total NK cell population (Figure 5B), nor in the cells that had degranulated (Figure 5C). Notably, when we focused the analysis on receptor-positive patients, the frequency of total CD7+CD56+ NK cells exhibiting the tumor markers MUC1 and HER2 appeared to be increased (Figure 5D). The percentage of cells that have performed trophocytosis on the epithelial markers was significantly higher in CD107a<sup>+</sup> NK cells than in the bulk of NK cells, principally in patient's samples (Figure S6F). We also observed this increase in CD45RARO cells regarding the bulk of NK cells (Figure S6G). However, we did not observe significant differences between HD NK CD45RARO+ and BC NK CD45RARO+ populations, nor between CD45RARO+CD107a<sup>+</sup> NK cells from both groups (Figure S6H). This indicates that CD45RARO and CD107a<sup>+</sup> cells

are the NK cell populations that are interacting with target cells and recovering antigens by trophocytosis. Although there were not significant differences between receptor-negative BC patients and HDs, some of the tumor markers analyzed were found upregulated on CD7+CD56+ NK cells from receptor-positive BC patients when compared with HD (Figure 5D). The fact that HDs show a similar pattern suggests that NK cells can interact and probably kill endothelial cells, which could be stress or damaged cells.

### Differential trophocytosed-receptor expression pattern in NK cell subsets between BC patients and healthy donors by viSNE

In order to further characterize the expression of these tumor markers present on NK cell surface due to trophocytosis, we used the high-dimensional reduction algorithm viSNE (for visualization of t-Distributed Stochastic Neighbor Embedding or t-SNE) (35, 43). By viSNE, we generated unsupervised 2D t-SNE maps showing the expression of 12 surface markers on NK cells from BC patients and healthy donors (Figure S7). The resulting t-SNE maps exhibit several spatial regions with differences in marker expression, suggesting that NK cell subsets with different abundance and expression of those markers were present.

To evaluate the differences in NK cell subset distribution between BC patients and healthy donors, we applied the

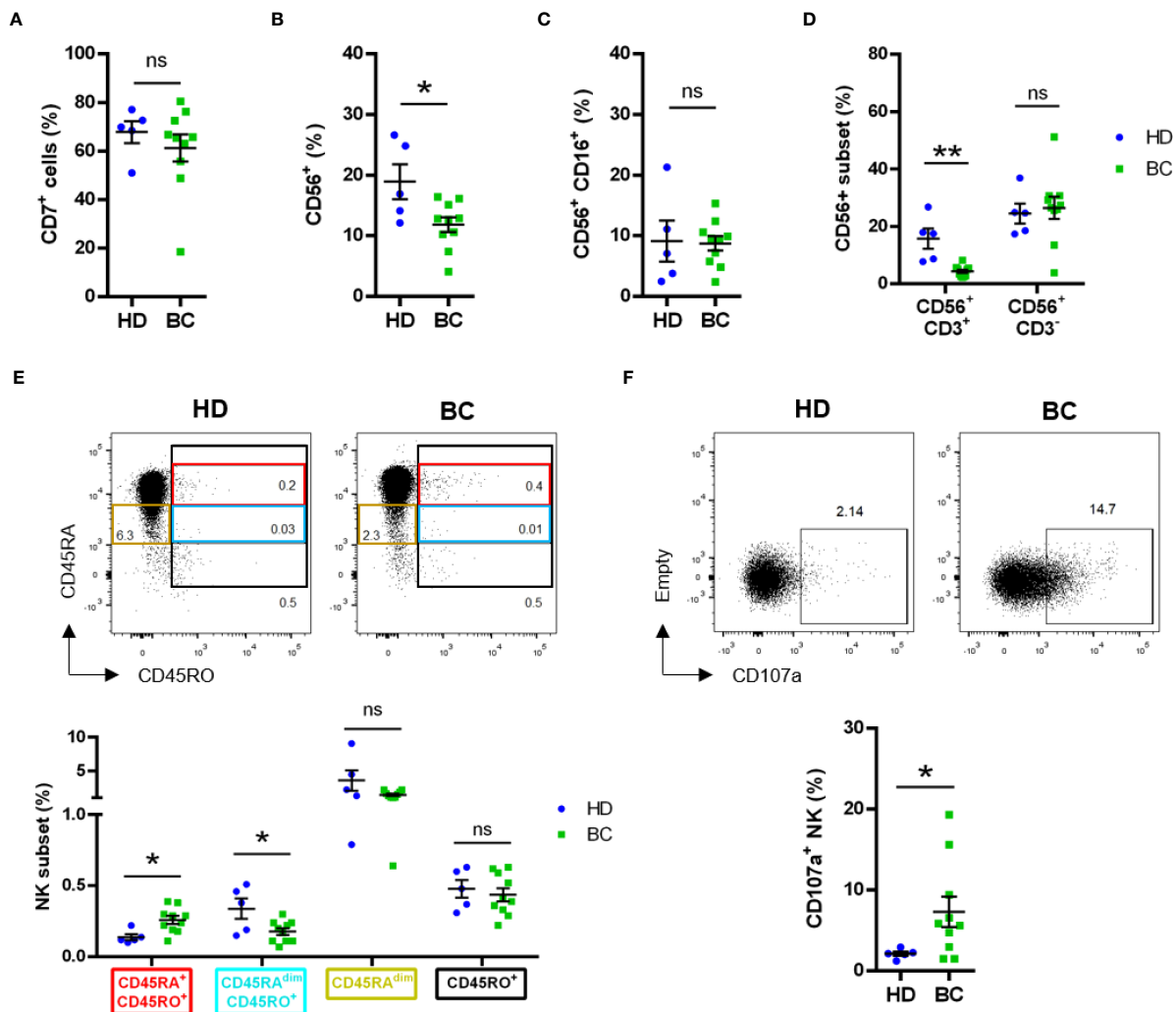


FIGURE 4

Phenotype analysis of peripheral NK cells analyzed by manual gating. Blood samples from healthy donors (HD) and breast tumor (BC) patients were analyzed by FACS for expression of lymphoid and NK cell subset markers. Graphs represent compiled data of (A) frequency of live CD7<sup>+</sup> cells; (B) frequency of live total CD56<sup>+</sup> cells and (C) frequency of live total CD56<sup>+</sup>CD16<sup>+</sup> cells. (D) Frequency of live CD56<sup>+</sup>CD3<sup>-</sup> or CD56<sup>+</sup>CD3<sup>+</sup> between HD and BC patients. (E) Frequency of live CD3<sup>-</sup>CD4<sup>-</sup>CD7<sup>+</sup>CD56<sup>+</sup> NK subsets based on CD45RA and CD45RO expression between HD and BC patients. (F) Expression of CD107a on live CD3<sup>-</sup>CD4<sup>-</sup>CD56<sup>+</sup>CD7<sup>+</sup> NK cells. Graphs represent mean  $\pm$  SEM; statistical significance between HD (n = 5) and BC (n = 10) was determined by Student t-Test (A–C, F) or two-way ANOVA (D, E); \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ . ns, not significant.

FlowSOM method which uses Self-Organizing Maps (SOMs) to cluster together cell events based on clustering channels (markers) and assign them to metaclusters, grouping them into distinct populations by an unsupervised approach (36). By this method we identified twelve metaclusters, of which two, i.e. 8 and 12, were found to be differentially abundant between BC patients and healthy donors (Figures 6A, B, Table S1). Both mainly consisted in NK cells with phenotype CD56<sup>+</sup>CD16<sup>high</sup>CD45RA<sup>+</sup> CD107a<sup>+</sup> and tumor markers expression (Table S1), Metacluster-8 was found enriched in BC patients in comparison with healthy controls and contained CD45RO<sup>+</sup> cells (10.7% versus 6.9% of control,  $p < 0.05$ ) (Figure 6B). This difference was even more evident when we compared receptor-positive patients with healthy controls (12.4% versus 6.9% of control,  $p < 0.01$ ) (Figure S8A, Table S1). On the other side, metacluster-12 was found to be decreased in BC patients

compared to healthy donors (15.5% versus 19.5% of control,  $p < 0.05$ ). This difference was higher when controls were compared with receptor-negative patients (13.2% versus 19.5% of control). In contrast to metacluster-8, this metacluster-12 mostly comprised CD45RO<sup>-</sup> NK cells (Table S1).

When we analyzed the degranulation on NK cells, we found that CD107a expression levels on Metacluster-7, Metacluster-8 and Metacluster-12 were significantly higher in BC patients compared to controls (Figure 6C). CD107a<sup>+</sup> was also found increased in NK cells from Metacluster-10 when comparing receptor-positive BC patients and healthy donors (Figure S8B). Thus, considering that NK cells having a CD45RA<sup>low</sup>/CD107a<sup>+</sup> phenotype previously exhibited higher degree of presumed trogocytosed proteins, we analyzed the expression levels of these solid tumor markers on these metaclusters. Interestingly, among all FlowSOM-identified

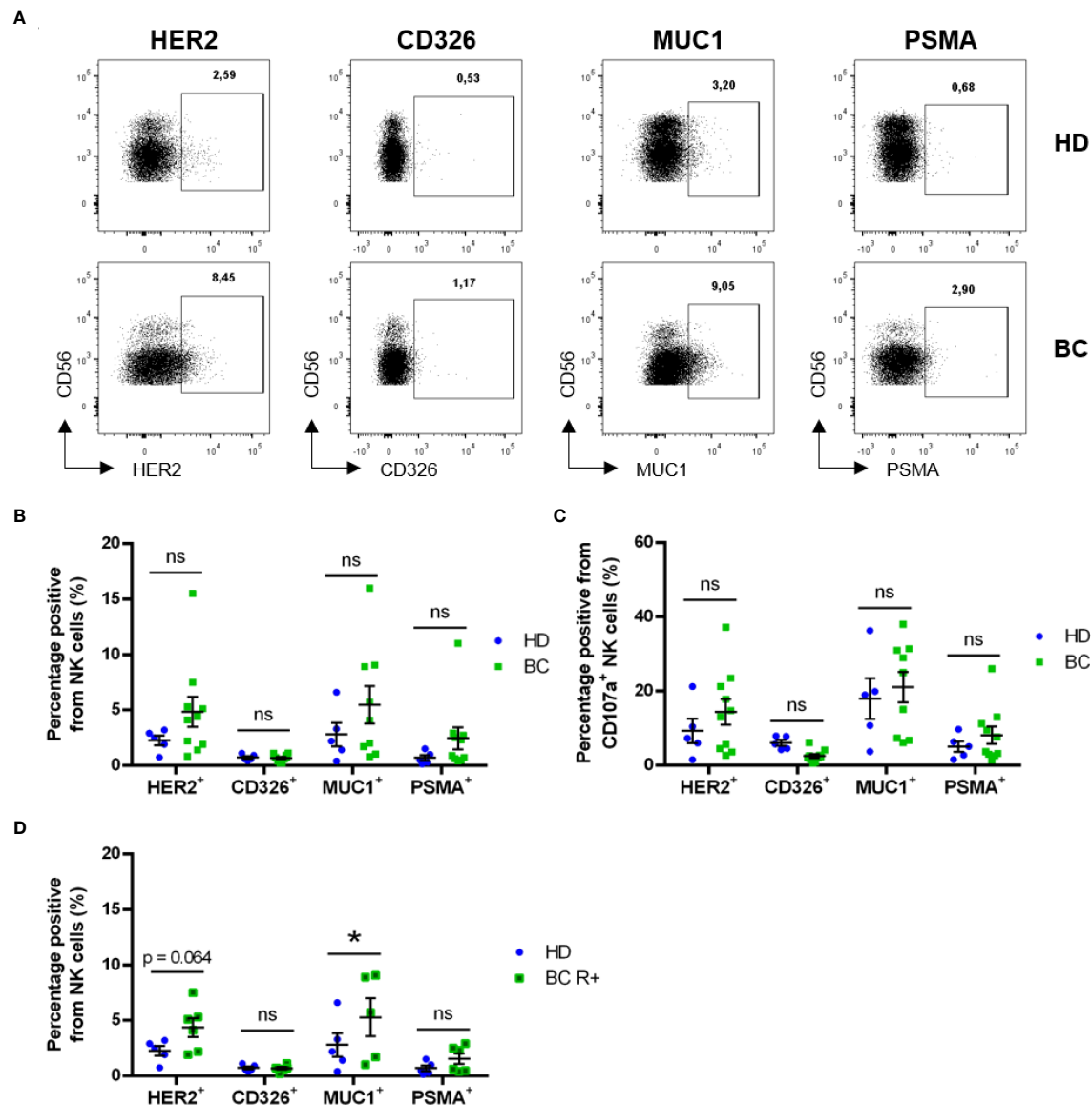


FIGURE 5

Circulating NK cells exhibit trogocytosis of solid tumor-expressed markers. Blood samples from healthy donors (HD) and breast cancer (BC) patients were analyzed by FACS for expression of tumor cell markers on CD7+CD56+ NK cell subsets. **(A)** Representative dot plots showing expression of tumor markers in circulating NK cells. **(B)** Frequency of NK cells presenting surface expression of HER2, CD326 (EpCAM), MUC1 or PSMA between HD and BC patients. **(C)** Frequency of trogocytosed markers on CD107a<sup>+</sup> NK cells. **(D)** Frequency of NK cells presenting surface expression of tumor markers in Receptor-positive patients (BC R+) versus HD. Graphs represent mean  $\pm$  SEM; statistical significance between HD (n = 5) and BC (n = 10), BC-R+ (n = 6) and BC-R- (n = 4) was determined by two-way ANOVA (B–D); \*  $p \leq 0.05$ . ns, not significant.

metacusters, we found that NK cells contained in Metacuster-8 showed the higher levels of tumor markers and, compared to healthy controls, BC patients exhibited significantly higher levels for the tumor markers CD326 and PSMA (Figure 6C). Moreover, HER2 was found particularly increased on NK cells from Metacuster-8 of receptor-positive BC patients in comparison to healthy donors (Figure S8B). Lastly, the expression of the immune checkpoint receptor programmed cell death-1 protein (PD1) was also analyzed on these NK cells, and when we compare its expression between healthy control and BC samples (total or receptor-positive patients), there was not significative difference between both groups (Figures 6C, S8B). Altogether, these results

suggest that *bona-fide* NK cells with an activated phenotype (CD107 + CD45RARO) are found increased on BC patients and, additionally, these NK cells exhibit high degree of potentially trogocytosed tumor markers on their surface.

To complement these observations with FlowSOM analyses, we further examine the cell activation and trogocytosis profile of BC patients and controls, focusing on NK cells contained in Metacuster-8 and using CITRUS (37). This algorithm allows the identification of stratifying sub-populations in multidimensional flow cytometry datasets, and can be used to distinguish single-cell signatures that might be associated with clinical outcomes (44). Among NK cells in Metacuster-8, CITRUS identified a total of 153

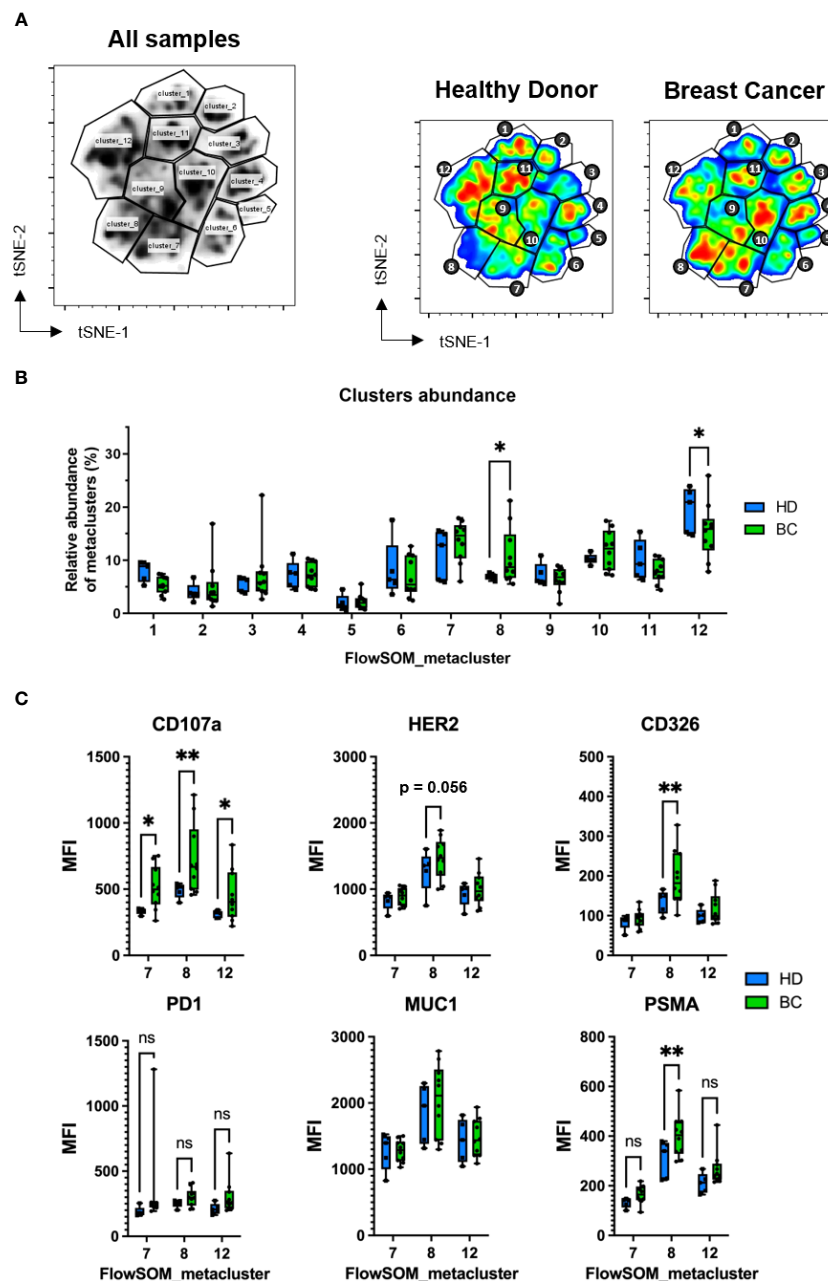


FIGURE 6

Identification of NK cell clusters with acquired tumor markers by FlowSOM. FACS samples from 5 healthy donors and 10 BC patients were concatenated and randomly subsampled into 100,000 total events which were analyzed by t-SNE and displayed using viSNE. **(A)** FlowSOM-identified twelve metaclusters visualized in the concatenated file. To the right, metacluster-derived gating applied to concatenated HD and BC patient samples. Differences in color on the viSNE map correspond to cell abundance density. **(B)** Comparison of relative frequency of each FlowSOM metacluster between HD and BC patients. **(C)** Median fluorescence intensity (MFI) of CD107a, PD1 and trogocytosed tumor markers expressed on CD7+CD56+ NK cells present on selected metaclusters (7, 8 and 12). Graph represent box and whiskers (Min to Max), and statistical significance between HD ( $n = 5$ ), BC ( $n = 10$ ) was determined by two-way ANOVA; \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ . ns, not significant.

clusters, of which four were found to be statistically associated to the BC patient's group, in accord to their CD107a and tumor marker expression (Figures 7A-D). By associating this CITRUS map with maps showing surface phenotype markers intensities (Figure 7E), we could identify these clusters specifically as subsets of CD56+ NK cells expressing high levels of CD16, CD45RA, NKp46 and intermediate levels of CD45RO, with high expression of CD326, PSMA, HER2 and MUC1 (Figure 7).

## Discussion

Individual humans have their own NK cell repertoire, which changes during development and is different in diverse tissues. In addition, multifactorial environmental events affect NK cells and generate new subsets (45) and make challenging to identify NK populations associated to a specific disease and shared by multiple patients. Conventional NK cells, which should correlate with the



population that we have studied, have a short half-life (5, 46). Hence, the amount of NK cells that has infiltrated the tumor and interacted with targets, then performed trogocytosis and came back to the blood stream should be scarce. In this current work, we have found a very low number of NK cells carrying tumor Ags, showing that their numbers are very low or, alternatively, to find and identify them is extremely difficult. Moreover, we observed that NK cells

from HD also carry some Ags typical of endothelial cells that we have used as trogocytosis markers. Probably, these NK cells have interacted with “stressed” endothelial cells. In case of breast cancer patients, the amount of “stressed” endothelial cells should be larger, because tumor cells are supposed to be stressed and recognized by NK cells, increasing the portion of NK cells that carry endothelial markers. However, most of the NK cells that have interacted with

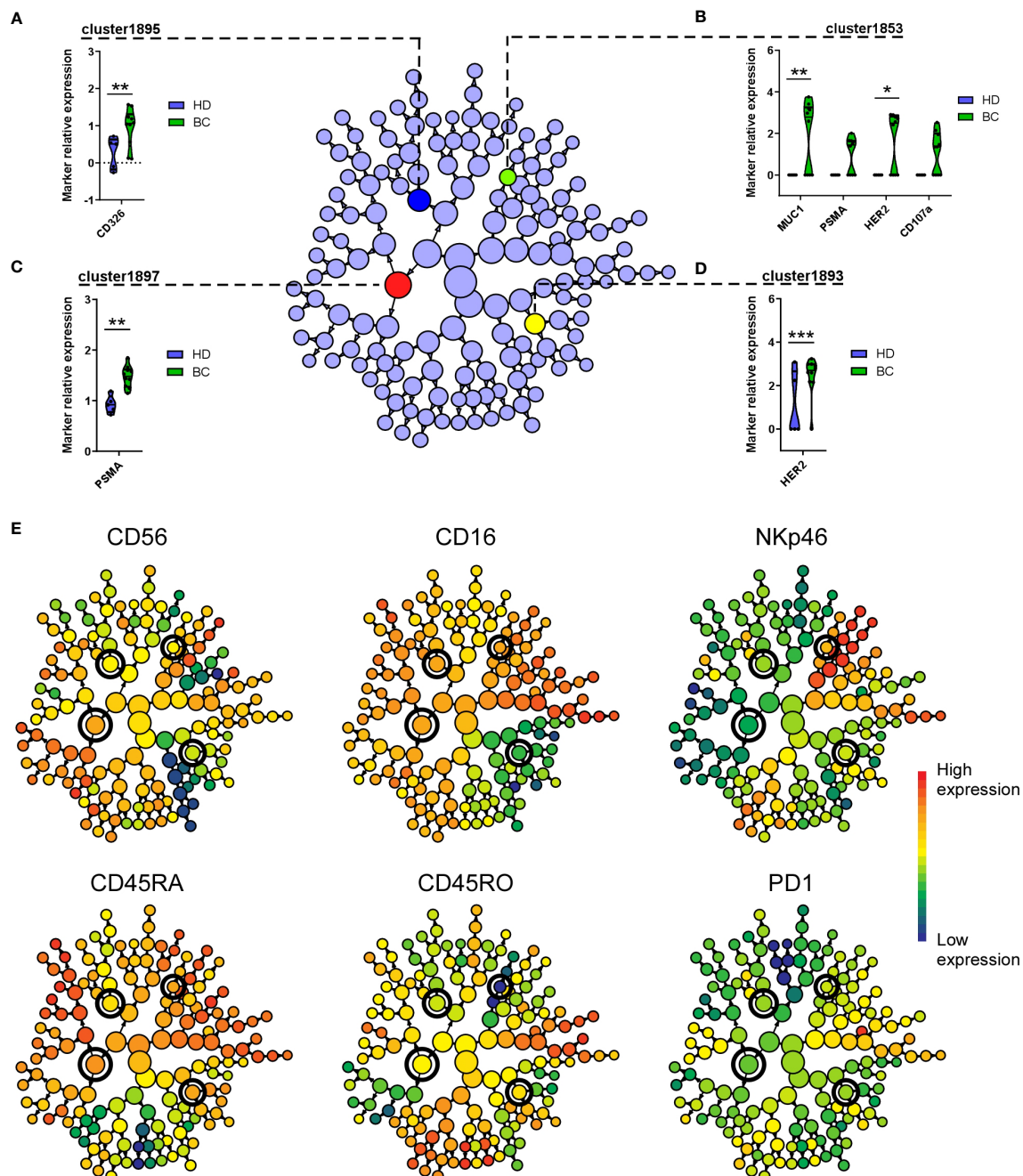


FIGURE 7

CITRUS identifies NK cell clusters with differential expression of tumor markers on BC patients. Cluster identification, characterization, and regression (CITRUS) algorithm identifies cell subsets (clusters) with significantly differential marker expression between BC patients and HD. Graphs displayed as CITRUS maps show the normalized expression (arcsinh) of CD107a and trogocytosed tumor markers on Metacluster-8 NK cells contained in (A) cluster-1895, (B) cluster-1853, (C) cluster-1897 and (D) cluster-1893, displayed as violin plots. (E) CITRUS maps overlaid with marker-intensities show relative expression of mentioned phenotype markers. Highlighted nodes in CITRUS maps correspond to cell subsets with differential tumor marker expression obtained using a significance analysis of microarray (SAM) correlative association model (Benjamini-Hochberg, adjusted  $p$ -value < 0.01).

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

tumor cells should be in the tumor microenvironment as shown previously (47). Here we show that effectively their frequency in periphery is very low, and to find differences between HD and patients we needed to unveil new populations (Figures 6, 7). Of note, NK cells eliminate senescent cells, which are considered “stressed”, favoring the clearing of this population and keeping an adequate cell population (48). In support of this hypothesis, NK cells carrying endothelial markers have predominantly degranulated, i.e. they are CD107<sup>+</sup>. This observation is consistent with previous reports describing higher degranulation and expression of activation markers on NK cells that acquired tumor cell markers by trogocytosis, proposing the surface expression of trogocytosed tumor markers as indicative of contact between these NK cells and marker-positive cancer cells (47, 49).

Our observations from *in vitro* experiments strongly imply that transference of HER2, EpCAM and other solid tumor cell markers on NK cells occurs via trogocytosis, proposing this process as the mechanism of capture of epithelial and tumor markers by NK cells under physiological and pathological conditions, as reported in tumor biopsies from BC patients (47). However, it is not possible to definitively conclude that HER2 and the rest of tumor markers found on circulating NK cells from BC patients would have been transferred via trogocytosis. These membrane proteins are normally absent and not expressed by NK cells, and our unsupervised multiparametric analyses found specific subsets of NK cells with high surface levels of these epithelial and solid tumor markers present in blood samples of BC patients; therefore, this correlation should not be completely ruled out, this point remaining pending of further elucidation.

In our study we focused on *bona fide* NK cells (CD7<sup>+</sup> CD56<sup>+</sup> CD3<sup>-</sup>) from patient's peripheral blood. It has been reported that tumor-infiltrating monocytes and NK cells that can be found in breast tumors could show high HER2-trogocytosis, mainly when patients are treated with mAbs systemic treatment, which facilitate NK cell recruitment and activation (47). These observations make us suggest that the amount of Ag capture by trogocytosis on tumor-infiltrating NK cells could be higher and more measurable than the surface levels detected on circulating NKs. However, analysis of infiltrated, tissue-specific human NK populations could prove more challenging and complex (45). For this reason, we did not investigate tissue-specific NK cells that would require biopsies. Hence, we have not probably identified all disease-associated NK populations, but only those that could exit the tumor environment. In addition, we have used a defined panel of NK-associated markers, whereas NK cells can express hundreds of them (50). In summary, other anti-tumor NK populations probably exist and perform degranulation and trogocytosis.

The identification of subsets of circulating NK cells, consisting of activated cells with evident degranulation carrying epithelial and tumor cell markers, raises the issue of the relevance that holds this tumor-trogocytosed marker harboring on NKs cellular and immune functions. Several reports in mice observed that MHC-I acquisition from target cells decreases acceptor NK cell immune recognition and cytotoxic functions (51–54). Correspondingly, it was reported

that human NK cells that capture HLA-G (a non-classical MHC-I immunosuppressive molecule) from melanoma solid tumor cells not only massively reduce their proliferation and their cytotoxicity, but also are able to suppress the cytotoxic function of other bystander NK cells expressing the HLA-G ligand and inhibitory receptor ILT2 (14). Similarly, trogocytosis of CD9 molecules from ovarian carcinoma cells to NK cells renders them less cytotoxic and poorer producers of anti-tumor cytokines, consistent with the identification of a CD9-positive NK cell subset in tubo-ovarian carcinoma samples which presence correlates with tumor progression (55). In line with this, a recent report showed that murine NK cells can acquire by trogocytosis the immune checkpoint inhibitor programmed cell death protein 1 (PD1) from leukemia cells both *in vitro* and *in vivo*, suppressing anti-tumor NK cell immunity (20).

However, this might not be the case for tumor receptors like HER2, because similarly to our present results, it has been observed that NK cells co-cultured with trastuzumab-opsonized HER2<sup>+</sup> breast cancer cells can acquire HER2 receptor via trogocytosis and exhibit higher expression of CD107a than non-HER2-trogocytosed NK cells (47). In addition, NK cells are capable to obtain the tyrosine kinase receptor TYRO3 from leukemia cells *in vitro* and *in vivo*, displaying higher levels of activation markers, enhanced cytotoxicity and interferon- $\gamma$  secretion (49), thus providing opposing evidence that tumor receptor trogocytosis on NK cells could also translate into gain of anti-tumor activity and effector function (56). This study was focused on the identification of NK cell subsets harboring trogocytosed markers from breast cancer cells, and even if experiments to elucidate the functional outcome of the acquisition of these markers by trogocytosis are pending to perform, we observed high levels of CD107a and low levels of PD1 expression on these NK cells from BC patients, which considering the mentioned literature is consistent with high anti-tumor function of these NK cells.

We have found also that degranulation and trogocytosis is higher in patients that expressed estrogen and/or progesterone receptors. This could be related to the main historical view that triple-negative breast cancer (TNBC) is a cold tumor (57). Hence, it is expected that NK cell infiltration and interaction with tumor target cells is poor in these patients. This should explain the lower level of NK cells that have degranulated and performed trogocytosis in peripheral blood from these patients in our study. However, a larger cohort of patients including both estrogen/progesterone receptor-positive and -negative would be necessary to conclude with greater certain this point.

We expanded our observations by performing dimensionality reduction analysis with viSNE, and we complemented it by using FlowSOM and CITRUS algorithms to further determine NK cell subsets carrying tumor cell antigens. Our unsupervised analyses identified clusters over-represented in BC patients containing tumor markers-expressing NK cells, i.e. metacluster 8. This cluster consisted mostly of CD45RARO<sup>+</sup> cells, with high level of degranulation that acquired surface expression of tumor markers, most probably by trogocytosis. These cells also exhibited relative

expression levels of PD1, although we found no significant difference for this marker between BC and HD group ( $p = 0.61$ ). Complementing this information, we have observed a very low PD1 levels (from 1% to 5% positive tumor cells) in our cell lines. This could suggest that PD1 is probably from NK cell origin in our settings, in accord with previous reports (58). Conversely, we cannot definitively rule out the likelihood that PD1 expression detected on NK cells derives from cancer cells, considering that PD1 expression on tumors derived from BC patients included in this study was not determined, and it has been already described that in different experimental settings NK cells can gain also PD1 by trogocytosis (20). Therefore, one possibility is that PD1 expression could increase on NK cells once they have acquired cancer cell markers. This could be due to NK cell activation after recognition of target cells, which have been reported on both circulating and tumor-infiltrating NK cells from several types of solid tumors (59–62). We favor this hypothesis, but we cannot exclude that PD1+ NK cells are more prone to acquire cancer markers. The physiological relevance could be important, because PD1+ NK cells with cancer antigens may participate in maintaining an immunosuppressive state and affect some immunotherapy treatments. Thus, trogocytosis of these types of receptors can show strong immunomodulatory capacities on NK cell immune function and capabilities.

Finally, our *in vitro* approach shows that different Ags are differently trogocytosed from different target cells and this is independent of the sensitivity to NK cells. NK cells extract for example HER2 and EpCAM from SK-BR3 or LNCaP cells; but almost exclusively EpCAM from HCT116 or BT20 cells. Meanwhile, the sensitivity of all these cell lines to NK cells was comparable. Hence, which molecules are trogocytosed depend on the donor cell and the nature of the molecule. The understanding of the precise mechanism of trogocytosis will be essential to unveil the reasons of the disparity between Ags and donor cells.

## 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 author.

## Ethics statement

The studies involving human participants were reviewed and approved by ICM-BDD 2017/37 (ID-RCB: 2017-A01940-53) clinical program approved by the “Comités de Protection des Personnes Sud-Ouest et Outre-Mer III”. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

WJ provided essential material and follow patient's status. GG, M-LD and MC performed the experiments. MC-M and MV performed study design and wrote the article. 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.2023.1199594/full#supplementary-material>



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## EDITED BY

Virginie Lafont,  
Institut National de la Santé et de la  
Recherche Médicale (INSERM), France

## REVIEWED BY

Narendra Sankpal,  
Saint Joseph Hospital Medical Center  
Phoenix, United States  
Dietmar Herndler-Brandstetter,  
Medical University of Vienna, Austria

## \*CORRESPONDENCE

Sebens Susanne

✉ susanne.sebens@email.uni-kiel.de

<sup>†</sup>These authors have contributed  
equally to this work and share  
first authorship

<sup>‡</sup>These authors have contributed  
equally to this work and share  
last authorship

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# The challenge of making the right choice: patient avatars in the era of cancer immunotherapies

Charlotte Kayser<sup>1†</sup>, Annika Brauer<sup>1†</sup>, Sebens Susanne<sup>1\*‡</sup>  
and Anna Maxi Wandmacher<sup>1,2‡</sup>

<sup>1</sup>Group of Inflammatory Carcinogenesis, Institute for Experimental Cancer Research, University Hospital Schleswig-Holstein (UKSH), Kiel University, Kiel, Germany, <sup>2</sup>Department of Internal Medicine II, University Hospital Center Schleswig-Holstein, Kiel, Germany

Immunotherapies are a key therapeutic strategy to fight cancer. Diverse approaches are used to activate tumor-directed immunity and to overcome tumor immune escape. The dynamic interplay between tumor cells and their tumor (immune) microenvironment (T(I)ME) poses a major challenge to create appropriate model systems. However, those model systems are needed to gain novel insights into tumor (immune) biology and a prerequisite to accurately develop and test immunotherapeutic approaches which can be successfully translated into clinical application. Several model systems have been established and advanced into so-called patient avatars to mimic the patient's tumor biology. All models have their advantages but also disadvantages underscoring the necessity to pay attention in defining the rationale and requirements for which the patient avatar will be used. Here, we briefly outline the current state of tumor model systems used for tumor (immune) biological analysis as well as evaluation of immunotherapeutic agents. Finally, we provide a recommendation for further development to make patient avatars a complementary tool for testing and predicting immunotherapeutic strategies for personalization of tumor therapies.

## KEYWORDS

organoids, organotypic tissue slice culture, organ-on-a-chip, patient-derived xenografts, tumor (immune) microenvironment, precision oncology, translational oncology

**Abbreviations:** BRGS, BALB/cRag<sup>2-/-</sup>IL2rg<sup>-/-</sup>Sirpa<sup>NOD</sup> mice; CAF, Carcinoma associated fibroblasts; CAR-NK cells, Chimeric antigen receptor-natural killer cells; CAR-T cells, Chimeric antigen receptor-T cells; ECM, Extracellular matrix; GvHD, Graft-versus-host disease; hPDX, Humanized patient-derived xenografts; ICI, Immune checkpoint inhibitors; NOD-scid, Nonobese diabetic/severe combined immunodeficiency; NRG, NOD-Rag1<sup>null</sup>IL2rg<sup>null</sup>; NSG, NOD scid gamma mice; PBMC, Peripheral blood mononuclear cells; PDAC, Pancreatic ductal adenocarcinoma; PD-1, Programmed cell death protein-1; PD-L1, Programmed-death ligand 1; PDO, Patient derived organoids; PSC, Pancreatic stellate cells; OOC, Organ-on-a-chip; OTSC, Organotypic slice cultures; SRG, Sprague Dawley-Rag2<sup>em2hera</sup>IL2rg<sup>em1hera/HblCr1</sup>; TIL, Tumor infiltrating lymphocytes; T(I)ME, Tumor (immune) microenvironment; TME, Tumor microenvironment.

## Introduction

Immunotherapy has emerged as an important pillar in cancer therapy comprising multiple strategies, e.g. cell-based approaches as chimeric antigen receptor T cells (CAR T cells) (1–4) or tumor infiltrating lymphocytes (TIL) (5), immune checkpoint inhibitors (ICI) (6–13), oncolytic viruses (14) and tumor vaccines (15). However, despite promising preclinical data, only a very low percentage of oncological treatments reach phase III trials or even clinical application (16, 17) and even those strategies that have entered clinical routine often exert less pronounced anti-tumor effects than observed in model systems. In addition, clinicians are faced with great heterogeneity in terms of patient responses to therapy even if levels of predictive biomarkers (e.g. specific mutations or immunohistochemical staining of protein biomarkers) are comparable. This highlights the limitation of personalizing treatment strategies solely based on genomics and single biomarkers as well as the need for valid co-clinical testing systems. Functional drug testing in those co-clinical models representing the individual tumor biology of a patient as accurately as possible (also termed “patient avatars”) to predict the individual susceptibility to drugs appears as a desirable approach to truly personalize patient treatment (18). Increasing efforts are therefore made to improve preclinical tumor models in order to optimally represent the complex and dynamic interplay between tumor cells and the immune system, especially in the tumor microenvironment (TME) of solid and hematologic malignancies. Irrespective of whether the model system is used for tumor immunological studies or individualized therapy prediction, an optimal patient avatar needs to reflect intra- and intertumoral heterogeneity (19) and comprise the entire tumor (immune) microenvironment (T(I)ME) (20–22). Particularly, to test immunotherapeutic strategies, the whole spectrum of innate and adaptive immune cells should be present in the patient avatar to mimic the direct and indirect cellular interactions of tumor cells and all stromal (cell) components of the tumor.

## Tumor model systems and patient avatars

### 2D tumor cell models

Two-dimensional (2D) tumor cell models comprise established and often immortalized cell lines or primary cell cultures directly established from fresh tumor material. Established cancer cell lines derived from solid tumors, leukemias and lymphomas have been extensively used for basic cell biology experiments and drug discovery since the early 1950s (23). As these cells grow in monolayers, culture maintenance is comparatively simple, inexpensive and analyses (including imaging) are easy to perform due to limited complexity.

To improve the representation of the complex TME, monolayer cell cultures have been advanced into co-cultures enriched by coating with defined extracellular matrix (ECM) proteins or

addition of distinct stem, stroma or (allogeneic) immune cell populations to allow the study of direct cell-cell interactions of different cell types or paracrine interactions in indirect cultures mostly using transwell inserts. The presence of immune cell populations (e.g. peripheral blood mononuclear cells (PBMC) or purified effector cells) is a prerequisite to study the preclinical effect of immunotherapies that aim to activate present immune cell populations. Alternatively, the cellular therapy itself (e.g. CAR T cells) constitutes the immune cell component within the co-culture model. Immunotherapeutic strategies including ICI (12), CAR T cells (1), CD3-targeted bispecific antibodies (24, 25) or oncolytic viruses (14) have been tested within 2D co-cultures. Of note, the cellular composition, activation and fitness status of circulating and tumor infiltrating immune cells often differs between healthy donors and cancer patients as they often display signs of reduced effector function and increased levels of exhaustion (26–30). Therefore, the integration of immune cells isolated directly from tumor tissue or PBMC of cancer patients into 2D cell cultures (as well as 3D co-culture models) is of great interest to approximate the functional capacity of the patient’s immune system. However, the use of allogeneic co-cultures to test respective immunotherapeutics is limited to a short experimental period up to a few days to avoid MHC-mediated alloreaactions. Another critical point of this model system is that intratumor heterogeneity is not well reflected, as established tumor cell lines undergo clonal selection and genetic drift (31–33). Moreover, the complex tumor architecture with respect to spatial and cellular composition, ECM, gradients of oxygen, nutrients and other soluble factors including cytokines is obviously lacking (34, 35). Subsequently, these models have shown to have limited predictive value (36–38) as they do not optimally represent the complex tumor biology.

To improve the representation of patient’s tumors, primary tumor cells may be used instead. For example, Kodack et al. established mono cell cultures with primary cells isolated from tumor tissues of different tumor entities and advanced them into co-cultures with fibroblasts for drug testing of tyrosine kinase inhibitors (39). However, their success rate was limited to 26% with differing rates between tumor entities (39). Kornauth et al. demonstrated the potential of leukemia and lymphoma cell suspensions as a predictive tool for individualized treatment in aggressive hematologic malignancies (40). Within a clinical trial, single cell suspensions of tumor material (biopsies, blood or bone marrow aspirates) were generated and directly subjected to treatment with 139 drugs circumventing the time-consuming and failure-prone establishment of cancer cell lines. In this approach, the drug response of tumor cells within the cell bulk was determined by immunofluorescent microscopy and quantification of the surviving proportion of tumor cells in comparison to controls (40). Of note, 56 heavily pretreated patients were treated based on the results of this testing resulting in a clinical benefit in 54% (30 patients) including a relevant number of exceptional responses. Although these results are promising in terms of a co-clinical model, evaluation of immunotherapeutic strategies was not included in this trial and requires further advancement of this model by adding effector cell types or cellular therapies.

### 3D spheroids

A further improvement of the above mentioned 2D cultures are spheroids which are three dimensional aggregates of one or multiple cell types. Spheroids can be comprised of tumor cells (primary cells or cell lines) only or of mixtures of tumor and stroma/immune cells (41). Furthermore, ECM can be supplemented. The 3D structure results in formation of a hypoxic zone in the spheroid core as it is commonly observed in tumors where the tumor center is often hypoxic (42). Different culture techniques are used to generate spheroids, e.g. using low-adherent surface plates or the hanging drop method, but all of them are based on preventing attachment of tumor cells to the culture plate and promoting 3D cell-cell aggregation (43, 44). The fast and easy way to generate spheroids from established tumor cell lines along with established readout assays (45) allows high throughput drug screens which can be particularly beneficial for testing novel therapeutic approaches. Recently, 3D spheroids have been used to evaluate different immunotherapeutics, e.g. CAR NK cells against triple negative breast cancer (46), ICI targeting of PD-L1 in pancreatic ductal adenocarcinoma (PDAC) (41) or a strategy to activate tumor associated macrophages via CSF1R inhibition and CD40 activation in Her2-positive breast cancer (47).

However, besides most of the limitations mentioned for 2D cultures, the uncontrollable arrangement of the cells in the spheroids and the reduced complexity of the spheroids with regard to an incomplete cellular and acellular composition (42) limit the usage of 3D spheroids as co-clinical model particularly for testing immunotherapeutic strategies.

### Patient-derived organoids

Organoid technology has rapidly developed as a transformative 3D model since Clevers et al. established an intestinal 3D culture system from intestinal stem cells in 2009 (48). Organoid technology is now vastly used for modeling of physiological tissue but also of different cancers in patient-derived organoids (PDO). To generate organoids, small tissue fragments from surgical specimen or biopsies are dissociated into single cells and subsequently cultured, most often embedded in 3D matrices providing ECM support and in complex culture media enriched with multiple growth factors (49, 50). Today, PDO are available for multiple tumor entities, including prostate cancer (51), colorectal cancer (48, 52), or PDAC (53). Compared to 2D and spheroid cell cultures, PDO offer an improved insight into tumor biology as the heterogeneity of driver mutations and phenotypes of the primary tumor are better retained (53) and thus, tumor cell complexity, differentiation, and functionality are better represented (54, 55). Furthermore, PDO allow genetic engineering and genomic analyses that cannot be accurately modeled in animals (22, 56). However, major limitations of PDO remain the lack of vascularization and the complex TME (57) which sometimes constitutes the major compartment of a tumor, e.g. in cancers like PDAC (58, 59). Furthermore, time of establishment (currently weeks to months) is still time-consuming and the success rates are highly variable

(16% to > 90%) differing between patients and tumor entities (60–64). Despite these limitations, PDO have been constantly advanced and increasingly used for preclinical testing of immunotherapies including ICI (7–9), bispecific antibodies (65), CAR T cells (66) or TIL generation (67).

For co-clinical evaluation, co-culture models of PDO with autologous immune cells and additional components of the TME appear to be ideal (68–70). To this end, Forsythe et al. established co-culture PDO models of appendiceal cancer with autologous immune cell populations to evaluate the efficacy of ICI nivolumab and pembrolizumab and identified 10–20% of PDO to be susceptible to ICI therapy (71). PDO may also offer a cost-effective opportunity to select for and expand TIL or generate patient specific cellular therapies. Dijkstra et al. successfully enriched autologous tumor reactive T cells from peripheral blood of colorectal and lung cancer patients (67). Similarly, Parikh et al. used organoids derived from metastases of multiple solid cancers to identify and generate TIL directed against individual tumor neoantigens with highly effective anti-tumor activity (72). These TIL co-cultured PDO could be established within two months for 75% of resected samples (72). To test CAR T cell treatment strategies in solid cancers, Schnalzger et al. used colorectal cancer PDO for evaluation of tumor cell killing and established a protocol to test the tumor cell specificity in competition assays using spiked-in organoids derived from healthy intestinal tissue (73). Beyond preclinical testing of immunotherapeutics, PDO can be employed to produce tumor cell specific T cells from induced pluripotent stem cells. This strategy may enable the production of allogeneic “off-the-shelf” CAR T cells circumventing the laborious and expensive generation of autologous CAR T cells (74). Moreover, large drug screens were successfully conducted implementing automated organoid seeding using automated microscopy or destructive viability assays as read-outs for drug efficacy paving the way for applications within the highly regulated clinical setting (75, 76). Recent studies indicate that PDO can be also used as co-clinical models for the prediction of treatment responses (60, 77, 78) and clinical trials are underway using functional testing in PDO to guide treatment decisions (79). Hence, several smaller collectives have been already established indicating a moderate to good correlation of drug responses in organoid-based patient avatar models with clinical responses (60, 77, 80). Guillen et al. combined mouse PDX and matched PDO of treatment resistant metastatic breast cancer to improve accuracy of modeling and combination of *in vitro* and *in vivo* drug testing (81).

However, to incorporate PDO-based treatment prediction into clinical workflows, PDO need to be improved in terms of reducing establishment time and optimizing generation success rates (61–64), highly varying among cancer patients and entities (60) not ensuring PDO generation from every patient. Finally, to accelerate meaningful implementation of PDO-based patient avatars into clinical application, prospective and systematic evaluation of their accurate representation of biological properties of the disease of origin and their predictive properties need to be considered in translational programs accompanying prospective clinical trials. Additionally, the implementation of the TME requires further developments, PDO generation needs to be methodologically standardized following standard operating procedures and



predefined cut-offs for treatment response need to be defined to guide clinical decisions (82). Here, synthetic ECM substitutes have been already used to significantly reduce batch variability of ECM components ensuring a higher degree of standardization (83, 84). Hopes are high to use living PDO biobanks (52, 81, 85) for testing immunotherapies to build the translational bridge between basic research and patient care.

## Humanized patient-derived xenografts

To evaluate novel immunotherapies and identify biomarkers, humanized patient-derived xenografts (hPDX) are an important platform (86–92). Meanwhile, more than 45 PDX models are available including NSG, NOD-scid, NRG, BRGS, SRG and next-generation humanized mice (86, 89). Besides therapeutic responses, possible side effects as well as tumor progression and metastasis can be studied in these whole organism models. In hPDX, almost all histological, genetic, molecular, and immunological characteristics are at least represented at low passages (93, 94), fulfilling several key requirements of patient avatars (95–98). Particularly, testing of immunotherapies demands the patient's immune system which can be activated towards the patient's tumor. For this purpose, hPDX models require humanization of mice and full engraftment with the patient's immune system. However, it has not been possible to reconstitute mice with the complete operational immune system of cancer patients, yet (86, 90, 91, 99). For testing immunotherapies based on T cells, the engraftment with patient's PBMCs is of great interest. However, this is only feasible for short-term experiments due to the rapid onset of graft-versus-host disease (GvHD). This issue has been diminished by eliminating MHC-I and -II expression (100) or using mice lacking murine CD47 (101). Of note, PBMC-engrafted mice can undergo a switch in immune cellular composition within 7 days. As a result, T cells might dominate and concomitantly myeloid as well as B and NK cells are underrepresented (89) thereby not fully representing the immune system of patients. Alternatively, engraftment can be achieved by CD34+ human hematopoietic stem cells to study immunotherapies in hPDX (91, 102, 103).

An important limiting factor of hPDX as patient avatar is the generation duration of months up to a year (91) depending on tumor entity, technology and mouse strain (89). In most cases, this time frame is not feasible to establish a patient avatar as co-clinical model as patient's treatment must start within a short period of time (i.e. most often within a few weeks, in cases with high tumor burden even faster). Additionally, it is necessary to take into account potential effects of patient's pre-treatment in terms of acquired resistance mechanisms (104) or cumulative toxicity, which remains difficult to model in hPDX (105). Finally, even if tumors are transplanted with their respective human stroma, the TME in hPDX will be remodeled, e.g. by conversion from human to murine ECM (94).

Weighting the above-mentioned improvements and remaining limitations, good correlations between tumor responses in hPDX models and clinical responses of the corresponding patients were observed suggesting that this model is principally suitable as co-

clinical model for therapy prediction (106–108). Moreover, hPDX have been used as major models to study CAR T cell therapies (2–4), ICI (5, 6, 11) and TIL (5).

## Organ-on-a-chip

As a strategy to avoid animal experiments, organ-on-a-chip (OOC) models have been designed to mimic physiological functions of different organs or tissues (109, 110). OOC can be based on established cell lines or organoids co-cultured with immune cells, fibroblasts or endothelial cells (111, 112). Additionally, epithelial and endothelial linings as well as ECM proteins may be included. In contrast to a conventional direct co-culture, in OOC cells are assembled on a chip containing a chamber and channels allowing for medium influx and efflux. Adding microfluidics via constant pumping of media allows to maintain gradients (e.g. of growth factors) and micromechanics (e.g. shear stress) while ensuring culture conditions for multiple cell types simultaneously. Geyer et al. modeled the physical barrier formed by pancreatic stellate cells (PSC) that prevent PBMCs, especially T cells, to migrate towards PDAC cells in a PDAC OOC. This barrier was overcome by treatment with Halofuginon inducing PSC death thereby increasing PBMC migration (113). These findings again illustrate the importance to consider the TME in the model system to properly test immunotherapeutic strategies. An additional layer of functional complexity can be added by including cell types that mediate drug metabolism, i.e. hepatocytes allowing the study of prodrugs (114). Cui et al. used a patient specific OOC to analyze the efficacy of anti-PD1 immunotherapy in different glioblastoma subtypes (10), Nyen et al. examined the response to trastuzumab and the impact of the tumor stroma in a breast cancer OOC (115) and Paterson et al. evaluated a CAR T construct in another breast cancer OOC (116). These studies clearly indicate the potential of OOC as patient avatar for individual therapy response prediction. Although OOC is a promising model for this purpose as it allows the combination of different cell types in one system and microscopic analysis is enabled by transparent polymers (109, 110), the most critical point is again the time needed for model establishment. Tumor cell isolation, organoid formation, OOC generation and treatment are all time-consuming steps, limiting its potential application as a patient avatar particularly for fast progressing and advanced cancers.

## Organotypic slice culture

Finally, patient derived organotypic slice cultures (OTSC) have emerged as a sophisticated patient avatar with a great potential to reduce the number of animal experiments (117, 118). OTSC are derived from tumor tissues obtained during surgical resection or via core needle biopsy (118–120). Afterward, tissues are cut mostly using a vibratome into tissue slices (117, 119, 120). The slice thickness varies from 150–500 µm and depending on tissue origin and cultivation method, OTSC remain intact for distinct time periods. Thus, it has been shown that OTSC remain viable for 5–9 days for

PDAC (119–122), up to 10 days for non-small cell lung cancer (123), up to 6 days for breast cancer (124) and up to 16 days in glioblastoma (125). However, changes in the T(I)ME over time were not always characterized in detail (117, 119, 120, 122). Cultivation often takes place on inserts at the air-liquid-interface to ensure sufficient oxygenation and to prevent cell death due to hypoxia (120, 126). Here, the composition of the medium is a critical factor, as certain media can support growth of certain cell types and thus influence the original tissue composition by driving selection of certain cell clones and phenotypes (119, 127).

In contrast to organoids and other cell culture models, which often represent only a reconstruction of the original tumor cell compartment, OTSC preserve the tumor and stroma heterogeneity thereby representing the tumor in its native environment, comprising epithelial/tumor cells, entire ECM as well as stroma and immune cells (117, 121, 128). In this way, all cells retain their function (hormone secretion, vascular contractility, cytokine secretion) along with their proteome and secretome (e.g. for immunological functions), and neurons also remain viable due to the presence of nerve growth factor (117, 129). This high similarity to the original tumor tissue creates unique conditions for analyzing the interplay of tumor cells with their TME thereby providing improved insights into tumor biology. Embedding of glioblastoma spheroids in brain tissue slices, Decotret et al. showed that the brain TME has a decisive influence on glioblastoma cell invasion (130). Besides, OTSC also appear to be well suited for the development and testing of novel therapeutic approaches (117, 118, 131). Thus, a combination treatment targeting carcinoma associated fibroblasts (CAF) by CXCR4 blockade and immune cells by ICI, increased T cell migration and activation towards tumor cells was observed resulting in tumor cell apoptosis (118). In line with these results, ECM reduction in OTSC improved T cell invasion towards tumor cells and increases the efficacy of blockade of the immune checkpoint molecule PD-1 (13). As OTSC are the only model system preserving the entire patient's tumor contexture over a distinct time period, it can be considered the best patient avatar to date. Importantly first studies indicate that OTSC are suitable for testing immunotherapies (123), although data on the correlation between treatment responses in patients and corresponding OTSC is still scarce. Therefore, the predictive power of OTSC has to be proven yet.

Besides these important advantages of OTSC, some critical points still deserve optimization. As mentioned above, the medium composition impacts survival and proliferation of cells thereby selecting certain cell populations (127). Furthermore, despite cultivation at the air-liquid-interface, longer cultivation might lead to hypoxia resulting in culture-induced cell death in certain areas of the section (120, 126). The limited culture duration in turn also impedes long-term studies including studies analyzing long-term effects of applied treatments. Furthermore, long-term storage of viable OTSC for future analyses is not possible yet, and the limited number of OTSC which can be obtained from one patient limits high throughput drug screening (120). Finally, to properly assess treatment responses, appropriate and reliable readout parameters have to be identified and quantified. Here, (live cell) imaging might be difficult due to the thickness of the OTSC (117).

However, since the response to (immuno)therapies often varies among cancer patients, OTSC have a high potential to play a role in the development of patient tailored therapy. The rapid availability of OTSC after surgery or core needle biopsy allows for rapid drug testing and, at the same time, characterization of the entire tumor including its T(I)ME even in patients with advanced tumor diseases. This offers the great opportunity to allow a prompt therapy prediction for the patient.

## Discussion and future perspectives

Significant progress has been made advancing existing *in vitro*, *ex vivo* and *in vivo* tumor models into patient avatars containing the patient's T(I)ME thereby trying to mimic the patient's tumor characteristics in the best possible manner. These efforts have led to invaluable insights into tumor (immune) biology and the efficacy of immunotherapeutic strategies. However, as outlined above and summarized in Table 1, every model system bears its advantages and disadvantages which need to be carefully weighed in order to make the right choice of the patient avatar for research or co-clinical therapy testing and prediction. Accordingly, further efforts are needed to focus on the following two aspects: First, addressing remaining limitations in the representation of the T(I)ME in existing models and second, advancing existing models towards co-clinical patient avatars that support clinical decision making based on functional assays. Results from these assays may then complement

TABLE 1 Overview of key features of currently used patient avatars and their suitability as co-clinical models for testing of immunotherapies.

	<i>in vitro</i> – 2D		<i>in vitro</i> – 3D		<i>ex vivo</i>	<i>in vivo</i>
	Cell models	Spheroids	PDO	OOC	OTSC	hPDX
<b>Tumor cell heterogeneity</b>	limited for established cell lines	limited for established cell lines	improved	improved	high	high
<b>Microenvironment</b>	ECM has to be exogenously added, indirect & direct co-cultures with allogeneic immune or stroma cell populations possible	ECM has to be exogenously added, direct co-cultures with allogeneic immune or stroma cell populations possible	ECM has to be exogenously added, indirect & direct co-cultures with allogeneic (autologous) immune or stroma cell populations possible	ECM has to be exogenously added, Indirect & direct co-cultures with allogeneic (autologous) immune or stroma cell populations possible	completely preserved for distinct time (depending on tumor entity)	completely preserved for distinct time, conversion into murine stroma

(Continued)

TABLE 1 Continued

	<i>in vitro</i> – 2D	<i>in vitro</i> – 3D			<i>ex vivo</i>	<i>in vivo</i>
	Cell models	Spheroids	PDO	OOC	OTSC	hPDX
<b>Nutrient/oxygen gradient &amp; vascularization</b>	missing	hypoxic zone in spheroid core, lack of vascularization	missing	possible	nutrient & oxygen gradient observed, lack of vascularization	present
<b>Model establishment</b>	fast	fast	time consuming	time consuming	fast	time consuming
<b>Reproducibility</b>	high	variable	patient-dependent	variable	patient-dependent	patient-dependent
<b>High throughput screening</b>	possible	possible	possible	limited	limited	limited
<b>Testing of immunotherapies*</b>	ICI (12) Bispecific antibodies (24, 25) CAR T cells (1) Oncolytic viruses (14)	CAR-NK cells (46) ICI (41) Macrophage activation (47)	Therapy prediction (60, 77, 78) ICI (7–9, 71) Bispecific antibodies (65) CAR T cells (66, 73, 74) TIL (67, 72)	ICI (10) CAR T cells (116)	Drug testing & development (117, 118, 131) ICI (13, 118)	therapy prediction (81, 96, 106–108) CAR T cells (2–4) ICI (6, 11) TIL (5)

\*Only exemplary studies mentioned in the text are listed.

existing strategies to personalize tumor therapies based on genomics, transcriptomics and immunohistochemical tumor analysis.

Finally, to advance patient avatars towards clinical application, a critical and important point is the standardization, e.g. by using harmonized protocols for generation and maintenance, reducing batch variability in reagents, increasing throughput while reducing costs for generation and characterization and defining experimental endpoints that are clinically meaningful (82, 132). Organoid or OTSC-based models may then even serve to develop patient specific therapies such as TIL and CAR T cells.

The current dynamics of the field are reflected by a multitude of ongoing clinical trials set up to evaluate the power of organoid or PDX based-models to predict clinical outcomes in cancer patients (133). Results from these mostly observational clinical trials will provide novel insights into feasible strategies to advance and implement personalized functional assays based on patient avatars for evaluation of (immuno) therapeutics.

## 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 author.

## Author contributions

Conceptualization, CK, AB, AW, SS. Supervision, SS. Visualization, CK, AB. Writing – original draft, AB, CK, AW, SS. Writing – review and editing, AB, CK, AW, SS. All authors contributed to the article and approved the submitted version.

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## Conflict 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.

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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.2023.1237565/full#supplementary-material>

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Anne Caignard,  
Institut National de la Santé et de la  
Recherche Médicale (INSERM), France

## REVIEWED BY

Srinivas S. Somanchi,  
Independent Researcher, San Diego,  
United States  
Francisco Borrego,  
Biocruces Bizkaia Health Research  
Institute, Spain  
Paulo Rodrigues-Santos,  
University of Coimbra, Portugal

## \*CORRESPONDENCE

Javier G. Casado  
✉ jgarcas@unex.es  
Rafael Solana  
✉ rsolana@uco.es

†These authors have contributed  
equally to this work and share  
first authorship

‡These authors share senior authorship

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# Enhanced expression of natural cytotoxicity receptors on cytokine-induced memory-like natural killer cells correlates with effector function

Sofía Carreira-Santos<sup>1†</sup>, Nelson López-Sejas<sup>1†</sup>,  
Marina González-Sánchez<sup>1†</sup>, Eva Sánchez-Hernández<sup>1</sup>,  
Alejandra Pera<sup>2,3</sup>, Fakhri Hassounah<sup>2</sup>, Esther Durán<sup>4</sup>,  
Rafael Solana<sup>2,3,5\*‡</sup>, Javier G. Casado<sup>1,6,7,8\*‡</sup>  
and Raquel Tarazona<sup>1,8‡</sup>

<sup>1</sup>Immunology Unit, Department of Physiology, Universidad de Extremadura, Cáceres, Spain,

<sup>2</sup>Immunology and Allergy Group (GC01), Maimonides Biomedical Research Institute of Córdoba (IMIBIC), Córdoba, Spain, <sup>3</sup>Department of Cell Biology, Physiology and Immunology, Universidad de Córdoba, Córdoba, Spain, <sup>4</sup>Anatomy and Comparative Pathological Anatomy Unit, Department of Animal Medicine, Faculty of Veterinary Medicine, Universidad de Extremadura, Cáceres, Spain,

<sup>5</sup>Immunology and Allergy Service, Reina Sofia University Hospital, Cordoba, Spain, <sup>6</sup>Centro de Investigación Biomédica En Red (CIBER) de Enfermedades Cardiovasculares, Instituto de Salud Carlos III (ISCIII), Madrid, Spain, <sup>7</sup>RICORS-TERAV Network, Instituto de Salud Carlos III (ISCIII), Madrid, Spain,

<sup>8</sup>Institute of Molecular Pathology Biomarkers, Universidad de Extremadura, Cáceres, Spain

**Introduction:** Natural killer (NK) cells are a key component of the innate immune system, involved in defending the host against virus-infected cells and tumor immunosurveillance. Under *in vitro* culture conditions, IL-12/15/18 can induce a memory-like phenotype in NK cells. These cytokine-induced memory-like (CIML) NK cells possess desirable characteristics for immunotherapies, including a longer lifespan and increased cytotoxicity.

**Methods:** In this study, NK cells were isolated from peripheral blood of healthy donors and stimulated with IL-12/15/18 to induce a memory-like phenotype or with IL-15 alone as a control. After seven days of culture, multiparametric flow cytometry analysis was performed to evaluate the phenotypic and functional profiles of CIML and control NK cells.

**Results:** Our results showed a significantly higher expression of CD25, CD69, NKG2D, NKp30, NKp44, NKp46, TACTILE, and Granzyme B in CIML NK cells compared to control NK cells. In contrast, KIR2D expression was significantly lower in CIML NK cells than in control NK cells. Moreover, functional experiments demonstrated that CIML NK cells displayed enhanced degranulation capacity and increased intracellular IFN- $\gamma$  production against the target cell line K562. Interestingly, the degranulation capacity of CIML NK cells was positively correlated with the expression of the activating receptors NKp46 and NKp30, as well as with the inhibitory receptor TACTILE.

**Discussion:** In conclusion, this study provides a deep phenotypic characterization of *in vitro*-expanded CIML NK cells. Moreover, the correlations found between NK cell receptors and degranulation capacity of CIML NK cells allowed the identification of several biomarkers that could be useful in clinical settings.

#### KEYWORDS

NK cells, memory-like, cytokine-induced memory-like NK cells, NKG2D, natural cytotoxicity receptors, degranulation capacity, cancer immunotherapy

## 1 Introduction

Natural Killer (NK) cells are innate lymphocytes that are essential not only in host defense against virus-infected cells, but also in tumor immune surveillance (1, 2). The effector function of NK cells is regulated through a rigorous balance of signals that are mediated by the interaction between the activating and inhibitory receptors expressed on the surface of NK cells and their corresponding ligands on target cells (2–4).

The interaction between NK activating receptors, including NKG2D, NKG2C, DNAM-1, NCRs (NKp46, NKp44, and NKp30), and NKp80, with their corresponding ligands on tumor cells results in the activation of NK cells, leading to enhanced NK cell cytotoxicity (3–5). The ligands for these receptors, MICA/B (ligands for NKG2D), HLA-E (ligand for NKG2C), CD122, CD155 (both ligands for DNAM-1) (3–5), AICL (ligand for NKp80) (6, 7), and B7-H6 (ligand for NKp30) (8, 9), are frequently upregulated in tumor cells and can even be entirely absent in healthy cells, as is the case for B7-H6. In addition, the CD16 molecule is a low-affinity Fc receptor for IgG, enabling NK cell activation and triggering antibody-dependent cellular cytotoxicity (ADCC) without requiring additional receptor signals (10, 11).

It is interesting to note that CD112 and CD155 also serve as ligands for NK inhibitory receptors, such as TIGIT (which recognizes both CD112 and CD155) and TACTILE (which recognizes CD155). The interaction between these ligands and NK inhibitory receptors leads to a decrease in the cytotoxic capacity of NK cells while promoting tumor cell invasion and migration (12). More recently, several inhibitory receptors have been described in NK cells: LAG-3 (lymphocyte-3 activation gene), which binds to LSECtin (liver and lymph node sinusoidal endothelial cell C-type lectin) on tumor cells, TIM-3 (mucin domain-containing protein 3), which binds to multiple ligands such as galectin-9, HMGB1, Ceacam-1 and Phosphatidylserine, and PD-1, which binds to PD-L1 (programmed death-ligand) (3).

Traditionally, NK cells have been characterized as innate immune cells due to their rapid proliferation and effector function, including cytotoxicity, and cytokine production (IFN- $\gamma$  and TNF- $\alpha$ ), without prior sensitization or antigen specificity (13–16). However, recent studies have revealed that NK cells can undergo clonal expansion and give rise to long-lived memory-like NK cells which exhibit rapid degranulation and cytokine

production upon reactivation (15, 16). The development of this memory-like phenotype in NK cells can occur through three distinct scenarios: hapten-specific, virus-specific, and cytokine-induced (16–18).

Cytokine-induced memory-like (CIML) NK cells are a subset of NK cells that after a short *in vitro* stimulation with IL-12, IL-15, and IL-18, exhibit enhanced functional and phenotypic characteristics (14, 18, 19). Despite being antigen nonspecific, CIML NK cells retain memory of previous activation and persist within the host for long periods of time, exhibiting an enhanced functional and proliferative capacity (13, 19).

Their longer half-life, together with the maintenance of their highly cytotoxic and anti-tumor responses in the immunosuppressive tumor microenvironment, makes CIML NK cells a great candidate for future immunotherapies (13, 20, 21). In fact, multiple preclinical and clinical studies have demonstrated the therapeutic potential of CIML NK cells in various cancer models, including ovarian cancer, melanoma, acute myeloid leukemia (AML), and lymphoma (22–25). All these promising results, together with their functional and phenotypic characteristics, support the idea that CIML NK cells may have a key role in combating diverse types of malignancies.

In this study, we conducted a comprehensive analysis of the phenotype and functional capacity of *in vitro*-expanded CIML NK cells. Our findings revealed that, after 7 days of culture, CIML NK cells exhibited a significantly higher expression of CD25, CD69, NKG2D, natural cytotoxicity receptors (NCRs), and Granzyme B compared to control NK cells. Additionally, CIML NK cells showed significantly higher percentages of expression of the inhibitory receptors NKG2A and TACTILE (CD96), along with significantly lower expression of KIR2D. Furthermore, we found that CIML NK cells exhibited a greater degranulation capacity and higher production of IFN- $\gamma$  against the target cell line K562 than control NK cells. Moreover, to further explore the relationship between the phenotype and functionality of CIML NK cells, we conducted a multiple correlation analysis that revealed positive correlations between the degranulation capacity of CIML NK cells and the expression of activating receptors (NKp46, and NKp30), and inhibitory receptors (TACTILE). Here we suggest that the phenotypic analysis of these molecules prior to adoptive cell therapy may help to predict the functionality and therapeutic potential of CIML NK cells.



## 2 Materials and methods

### 2.1 Sample processing and NK cell culture

NK cells were isolated from the buffy coats of healthy donors ( $n = 8$ ) by a negative selection process using the NK cell enrichment cocktail RosetteSep<sup>TM</sup> (STEMCELL Technologies, Vancouver, BC, Canada) and density gradient centrifugation on Lymphoprep<sup>TM</sup> (STEMCELL Technologies). The cells were then cultured in HyClone RPMI-1640 (Cytiva, Marlborough, MA, USA) supplemented with 1% Sodium Pyruvate, 1% GlutaMAX<sup>TM</sup> (Gibco<sup>TM</sup>, ThermoFisher Scientific, Waltham, MA, USA), 1% Penicillin-Streptomycin (Lonza Ltd., Verviers, Belgium), and 10% Human Serum AB male (Biowest, Nuaille, France).

To generate CIML NK cells, the cells were seeded in 24 well plates at a density of  $1 \times 10^6$  cells/mL and stimulated overnight (~16h) with 10 ng/mL rhIL-12, 1 ng/mL rhIL-15 (PreproTech, Rocky Hill, NJ, USA), and 50 ng/mL rhIL-18 (MBL International, Woburn, MA, USA). In parallel, NK cells incubated overnight with 1 ng/mL rhIL-15 were defined as control NK cells. After the initial stimulation, the cells were washed twice and seeded again in the presence of 1 ng/mL rhIL-15. Cell culture medium supplemented with rhIL-15 was replaced on day 4.

The ethics committee of the University of Extremadura (Ref.: 118//2020) approved the study, and informed consent was obtained following the Declaration of Helsinki.

### 2.2 Cell lines

The human erythroleukemic cell line K562 and human melanoma cell line MaMel56 (from OISTER and ESTDAB projects) were cultured in HyClone RPMI-1640 supplemented with 10% Foetal Bovine Serum (Gibco<sup>TM</sup>, ThermoFisher Scientific, Waltham, MA, USA), 1% GlutaMAX<sup>TM</sup>, and 1% Penicillin-Streptomycin. A non-enzymatic cell dissociation solution (Sigma-Aldrich, St. Louis, MO, USA) was used to detach the MaMel56 cells from the culture flask.

### 2.3 Flow cytometry analysis

The phenotypic and functional profiles of both the CIML and control NK cells were analyzed by multiparametric flow cytometry using a panel of commercially available antibodies (Supplementary Tables 1-3). The cells were stained (Supplementary Table 1) with fluorochrome-conjugated antibodies and incubated for 30 min at room temperature. For intracellular staining, the cells were fixed and permeabilized with the IntraCell kit from Immunostep (Salamanca, Spain) following the manufacturer's instructions; intracellular antibodies (Supplementary Table 2) were incubated for 30 min at room temperature. The cells were washed and resuspended in PBS before flow cytometry analysis. Isotype-matched antibodies and fluorescence minus one (FMO) were used as controls to ensure proper gating.

Briefly, after doublet exclusion, lymphocytes were gated according to their size and granularity using forward (FSC) and side scatter (SSC) detectors, and NK cells were identified within the lymphocyte gate as CD3<sup>-</sup> CD56<sup>+</sup>. Individual gates were defined for antibodies included in the panel for CD3<sup>-</sup>CD56<sup>+</sup> cells. A detailed outline of the gating strategy is provided in the [Supplementary Material \(Supplementary Figure 1\)](#).

Flow cytometry was performed using MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using FlowLogic v.8.6. (Inivai Technologies, Mentone, Victoria, Australia).

### 2.4 NK cell degranulation and cytokine production assays

The functionality of CIML and control NK cells was determined as specific degranulation capacity after 7 days of culture. Degranulation capacity was estimated by CD107a and CD107b expression on NK cells, and specific degranulation capacity of NK cells against target cells was calculated after subtracting the basal degranulation in the absence of target cell from the degranulation in the presence of target cell. Briefly, CIML and control NK cells ( $5 \times 10^5$  cells) were stimulated with the target cells, the K562 and MaMel56 cell lines, at a 1:1 effector:target (E:T) ratio. In order to favor cell-to-cell contact, effector and target cells were transferred into sterile 5 mL polystyrene round-bottom tubes (Falcon<sup>®</sup>, Corning Inc., Corning, NY, USA) and centrifuged for 5 min at 200g. Then, cells were resuspended in 200  $\mu$ L of cell culture medium and stained with the FITC-conjugated anti-CD107a (H4A3; BD Biosciences, San José, CA, USA) and FITC-conjugated anti-CD107b (H4B4; BD Biosciences) monoclonal antibodies. Thereafter, the protein transport inhibitors GolgiStop<sup>TM</sup> and GolgiPlug<sup>TM</sup> (BD Biosciences), were added to each tube following the manufacturer's indications, and the cells were incubated at 37°C, 5% CO<sub>2</sub>, and 95% humidity for 6 hours. The cells were then stained with the appropriate antibodies (Supplementary Table 3) following the method explained above and analyzed by flow cytometry.

The assessment of intracellular cytokine production (IFN- $\gamma$  and TNF- $\alpha$ ) was conducted on a different group of donors ( $n = 7$ ). For this purpose, specific cytokine production of NK cells against target cells was calculated after subtracting the basal production in the absence of target cell. CIML and control NK cells were stimulated as previously described for degranulation assays, in the presence of protein transport inhibitors GolgiStop<sup>TM</sup> and GolgiPlug<sup>TM</sup>. Finally, after a 6h incubation, cells were stained with the appropriate and intracellular antibodies (Supplementary Table 3), as described in section 2.3, and analyzed by flow cytometry.

### 2.5 Statistical analysis and graphical representation

Prior to the statistical analysis, the normality of the datasets was checked using the Shapiro-Wilk normality test. The non-parametric Friedman test, followed by pairwise comparisons (Durbin-Conover test), was used for multiple comparison analysis, while the non-

parametric Wilcoxon signed-rank test was used to study paired samples. The statistical analysis of the samples was carried out in the open-source statistical software Jamovi v2.3.21 (Sydney, Australia). The correlation analysis between the different biomarkers was based on the Pearson correlation coefficient and conducted on R-Studio v2022.12.0 + 353.

The graphical representations were performed using GraphPad Prism v8.0.1 (San Diego, CA, USA), except for the correlation matrix, which was obtained through R-Studio.

Simplified Presentation of Incredibly Complex Evaluations (SPICE) software was used for pie chart representation and comparison. The statistical comparison of the distributions was accomplished by a non-parametric partial permutation test (26).

## 3 Results

### 3.1 *In vitro* expansion of CIML NK cells can be achieved with IL-12/15/18 cytokines

In our experimental conditions, NK cells were firstly enriched from peripheral blood lymphocytes using RosetteSep<sup>TM</sup>. In all cases, NK cell purity (defined as CD3<sup>+</sup> CD56<sup>+</sup> cells) resulted in a mean purity of 76.10% ± 9.66%. After 7 days of culture, CIML NK cells and control NK cells reached a purity of 82.26% ± 11.21% and 80.62% ± 12.78% respectively. Furthermore, while there were no statistically significant differences in the proliferation rates and absolute cell counts between CIML and control NK cells, it is worth noting that in 6 out of the 8 donors included in this study, CIML NK cells displayed higher absolute cell counts compared to control NK cells after 7 days of culture (Supplementary Figure 2).

In addition, the assessment of NK cell viability during the experimental phase indicated that on day 1, the viability of both CIML and control NK cells fell within the range of 85% to 90%. Subsequently, after 7 days of culture, a notable increase in cell viability was observed, with values ranging from 95% to 99% for both CIML and control NK cells.

### 3.2 The phenotypic profiles of CIML and control NK cells are significantly different

In order to study the changes in activating and inhibitory NK cell receptors, and the effect of stimulation with IL-12/15/18 on NK cells, the phenotype of both CIML and control NK cells was studied at different time points: right after the reception and processing of the samples (D0), 16 h after the initial stimulation with cytokines (D1), and after a week of expansion and culture with 1 ng/mL rhIL-15 (D7). The results presented herein correspond to those obtained at D7; for a more comprehensive understanding of the analyses conducted at D0 and D1, Supplementary Material has been provided (Supplementary Figures 3–6).

After 7 days of culture, expression of NK cell activation markers CD25 ( $p < 0.001$ ) and CD69 ( $p = 0.044$ ) was significantly higher in CIML NK cells than in control NK cells (Figure 1A). Furthermore, the phenotypic profile of activating NK cell receptors was also

studied at D7. In this analysis, NKG2D, NKp46, NKp44, and NKp30 receptors were significantly increased on CIML NK cells when compared to control NK cells ( $p < 0.001$  for NKG2D, NKp44, and NKp30;  $p = 0.011$  for NKp46). No statistical significance was observed in the expression of NKG2C, CD16, DNAM-1, NKp80, and CD8 (Figure 1B).

Regarding inhibitory receptors, CIML NK cells expressed significantly higher percentages of NKG2A and TACTILE ( $p < 0.001$ , respectively), and significantly lower levels of KIR2D ( $p < 0.001$ ) than control NK cells after 7 days of culture. No significant differences were observed in TIGIT, LAG-3, TIM-3, and PD-1 (Figure 1C).

Among the cytotoxic proteins, after 7 days of culture, CIML NK cells showed a significantly higher expression of Granzyme B than control NK cells ( $p = 0.005$ ). No significant differences were observed in Perforin and Granulysin (Figure 2).

### 3.3 Phenotypic profiling and subset analysis of CIML and control NK cells based on the co-expression of activating and inhibitory receptors

To further analyze the phenotypic profile of both CIML and control NK cells, and to perform a deep analysis of different NK cell subsets, the co-expression of activating and inhibitory receptors was determined using FlowLogic's Boolean gating and graphically represented with the SPICE 6.1 software.

Sixteen different combinations were analyzed for the following markers: CD69, NKG2D, NKG2A, and CD25. The in-depth phenotypic profiling of CIML and control NK cells revealed statistically significant differences ( $p = 0.0023$ ) in the distribution of NK cell subsets between CIML and control NK cells (Figure 3A). Eight different combinations were analyzed for DNAM-1, TIGIT, and TACTILE (Figure 3B), as well as for LAG-3, TIM-3, and PD-1 (Figure 3C), and NKp46, NKp44, and NKp30 (Figure 3D). The distribution of NK cell subsets according to NCRs was significantly different when comparing CIML and control NK cells ( $p = 0.0049$ ).

In order to identify differences between the different subsets in CIML and control NK cells, each combination of markers was further analyzed separately. The statistical analysis demonstrated that when compared to control NK cells, CIML NK cells had significantly higher percentages of the CD69+NKG2D+NKG2A+CD25+ subset ( $p = 0.008$ ) than control NK cells. Control NK cells, however, showed significantly higher levels of the CD69–NKG2D–NKG2A–CD25– subset ( $p = 0.008$ ) than CIML NK cells (Figure 4A). For a more comprehensive understanding of the evolution of these combinations of markers at the different times of culture (from D0 to D7), refer to the Supplementary Material (Supplementary Figure 7).

Moreover, after 7 days of culture, control NK cells showed significantly higher percentages of the DNAM-1–TIGIT+TACTILE+ subset ( $p = 0.008$ ) than CIML NK cells (Figure 4B). On another note, there were no significant differences between the different combinations of LAG-3, TIM-3, and PD-1 (Figure 4C). As for the NCRs, significantly higher percentages of the NKp46+NKp44+NKp30+ subset ( $p = 0.008$ )

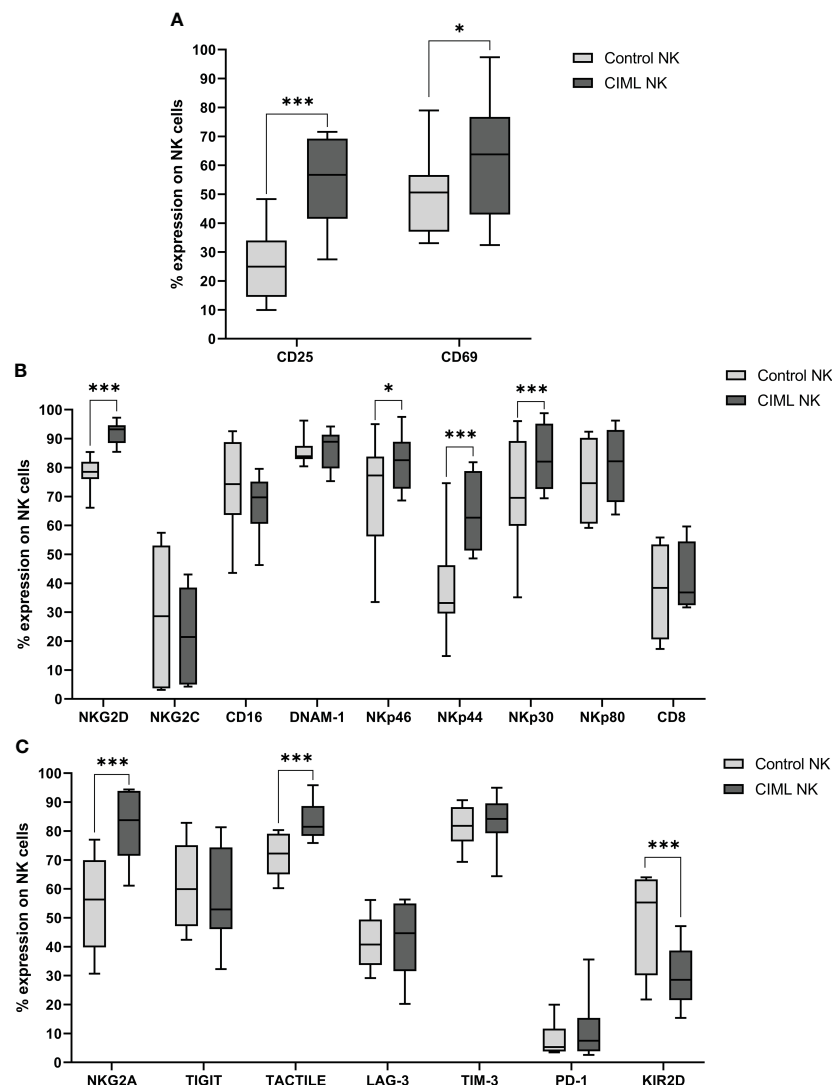


FIGURE 1

Phenotypic analysis of CIML and control NK cells after 7 days of culture. (A) Expression of the activating markers CD25 and CD69. (B) Expression of NK cell activating receptors and CD8. (C) Expression of NK cell inhibitory receptors.  $p$ -values were calculated by using the non-parametric Friedman test, followed by pairwise comparisons (Durbin-Conover test), \*  $p \leq 0,05$ , \*\*\*  $p \leq 0,001$ .

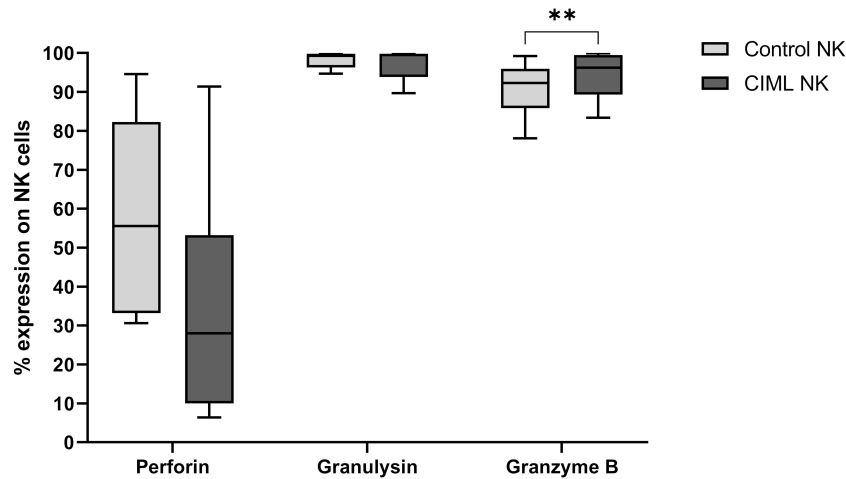
were detected for CIML NK cells when compared to CIML NK cells, while the percentages of the NKP46+NKP44–NKP30– ( $p = 0.008$ ) and NKP46+NKP44–NKP30+ ( $p = 0.039$ ) subsets were significantly higher in control NK cells than in CIML NK cells (Figure 4D).

### 3.4 CIML NK cells showed higher degranulation and intracellular IFN- $\gamma$ production against the target cell line K562 than control NK cells

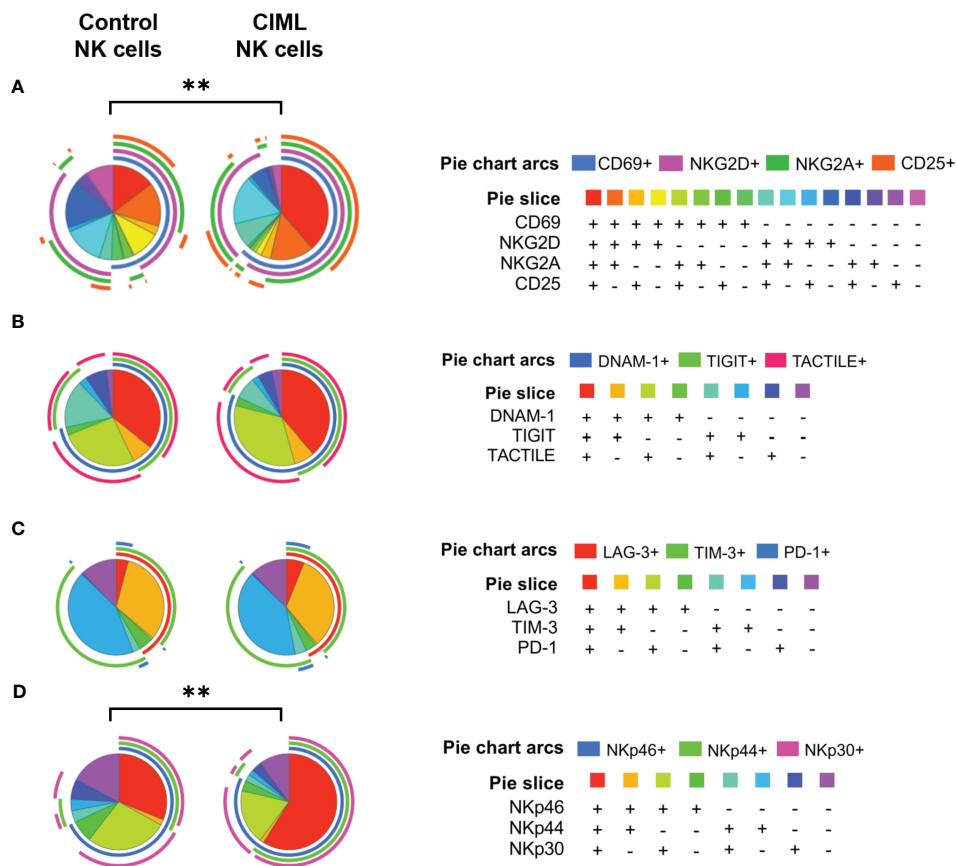
Determination of NK cell degranulation is frequently used as an indirect measure of NK cell cytotoxic activity (27). In order to evaluate the functionality of CIML and control NK cells, their

degranulation capacity was tested *in vitro* using K562 and MaMel56 cell lines as targets (E:T = 1:1). Our results demonstrated that CIML NK cells showed a significantly higher degranulation capacity against K562 cell line than control NK cells ( $p = 0.031$ ). The degranulation capacity of both CIML and control NK cells, however, was very low when targeting the melanoma cell line MaMel56 and did not show any statistical differences (Figure 5A).

In terms of intracellular cytokine production, our results indicated a significant increase in IFN- $\gamma$  production by CIML NK cells against the K562 cell line compared to control NK cells ( $p = 0.027$ ). However, no statistically significant differences were found in IFN- $\gamma$  production between CIML and control NK cells against the melanoma cell line MaMel56 (Figure 5B). Additionally, there were no statistically significant differences in TNF- $\alpha$  production between CIML and control NK cells (Figure 5C).



**FIGURE 2** Expression of the cytotoxic proteins Perforin, Granulysin, and Granzyme B in CIML and control NK cells after 7 days of culture. *p*-values were calculated by using the non-parametric Friedman test, followed by pairwise comparisons (Durbin-Conover test), \*\* *p* ≤ 0,01.



**FIGURE 3** Co-expression of activating and inhibitory receptors in CIML and control NK cells after 7 days of culture. **(A)** Pie charts represent the percentages of CIML and control NK cells expressing CD69, NKG2D, NKG2A, and CD25. **(B)** Pie charts represent percentages of CIML and control NK cells expressing DNAM-1, TIGIT, and TACTILE. **(C)** Pie charts represent percentages of CIML and control NK cells expressing LAG-3, TIM-3, and PD-1. **(D)** Pie charts represent percentages of CIML and control NK cells expressing NKp46, NKp44, and NKp30. *p*-values were calculated by a non-parametric partial permutation test, \*\* *p* ≤ 0,01.



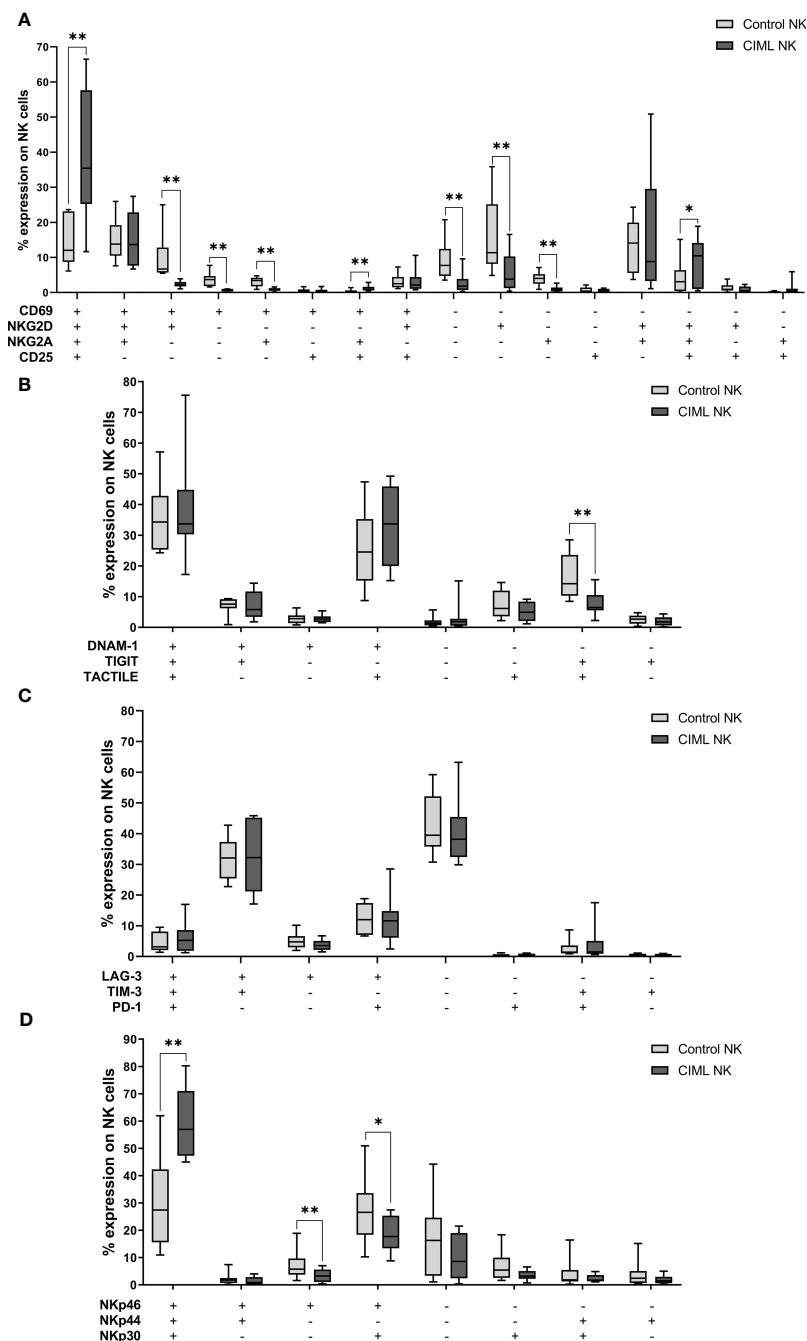


FIGURE 4

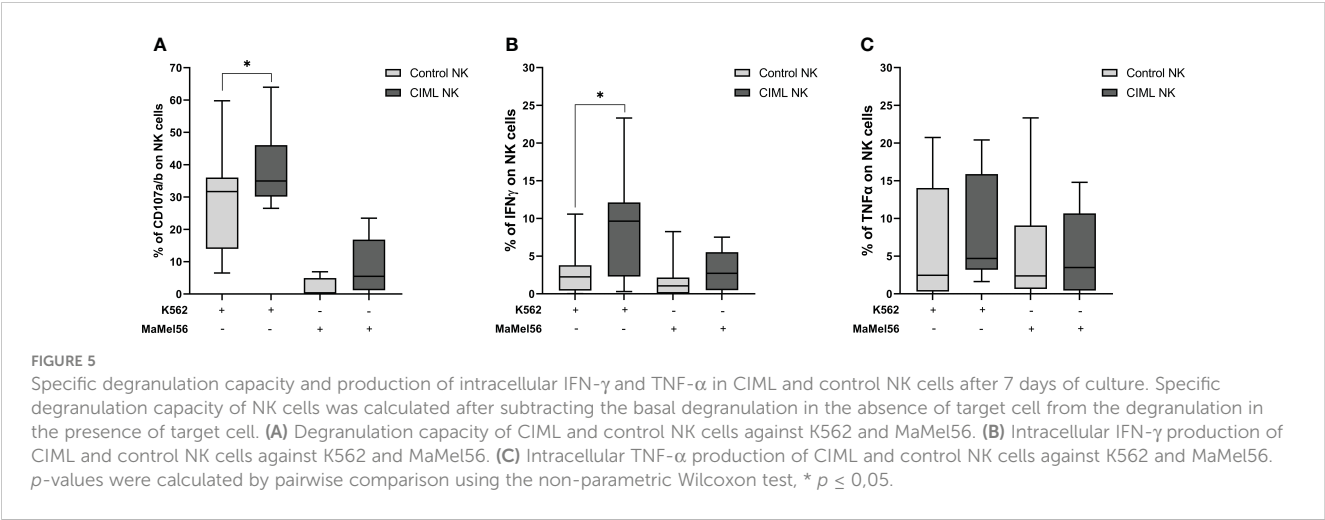
Co-expression of activating and inhibitory receptors in CIML and control NK cells after 7 days of culture. **(A)** Expression of CD69, NKG2D, NKG2A, and/or CD25. **(B)** Expression of DNAM-1, TIGIT, and/or TACTILE. **(C)** Expression of LAG-3, TIM-3, and/or PD-1. **(D)** Expression of NKp46, NKp44, and/or NKp30. *p*-values were calculated by pairwise comparison using the non-parametric Wilcoxon test, \*  $p \leq 0,05$ , \*\*  $p \leq 0,01$ .

### 3.5 Multiple correlation analysis revealed positive correlations between the degranulation capacity of NK cells and the expression of activating and inhibitory receptors

To establish a correlation between the functional and phenotypic profile of CIML NK cells a multiple correlation assay was conducted, analyzing the relationship between the

degranulation capacity and the expression of activating and inhibitory receptors (Figure 6).

Positive correlations were observed between the degranulation capacity and the expression of the activating receptors NKp46 and NKp30, and the inhibitory receptor TACTILE. Moreover, the multiple correlation analysis revealed additional positive correlations between various surface markers and cytotoxic proteins. For instance, the activating receptor DNAM-1 showed a strong positive correlation with the cytotoxic proteins Granzyme B

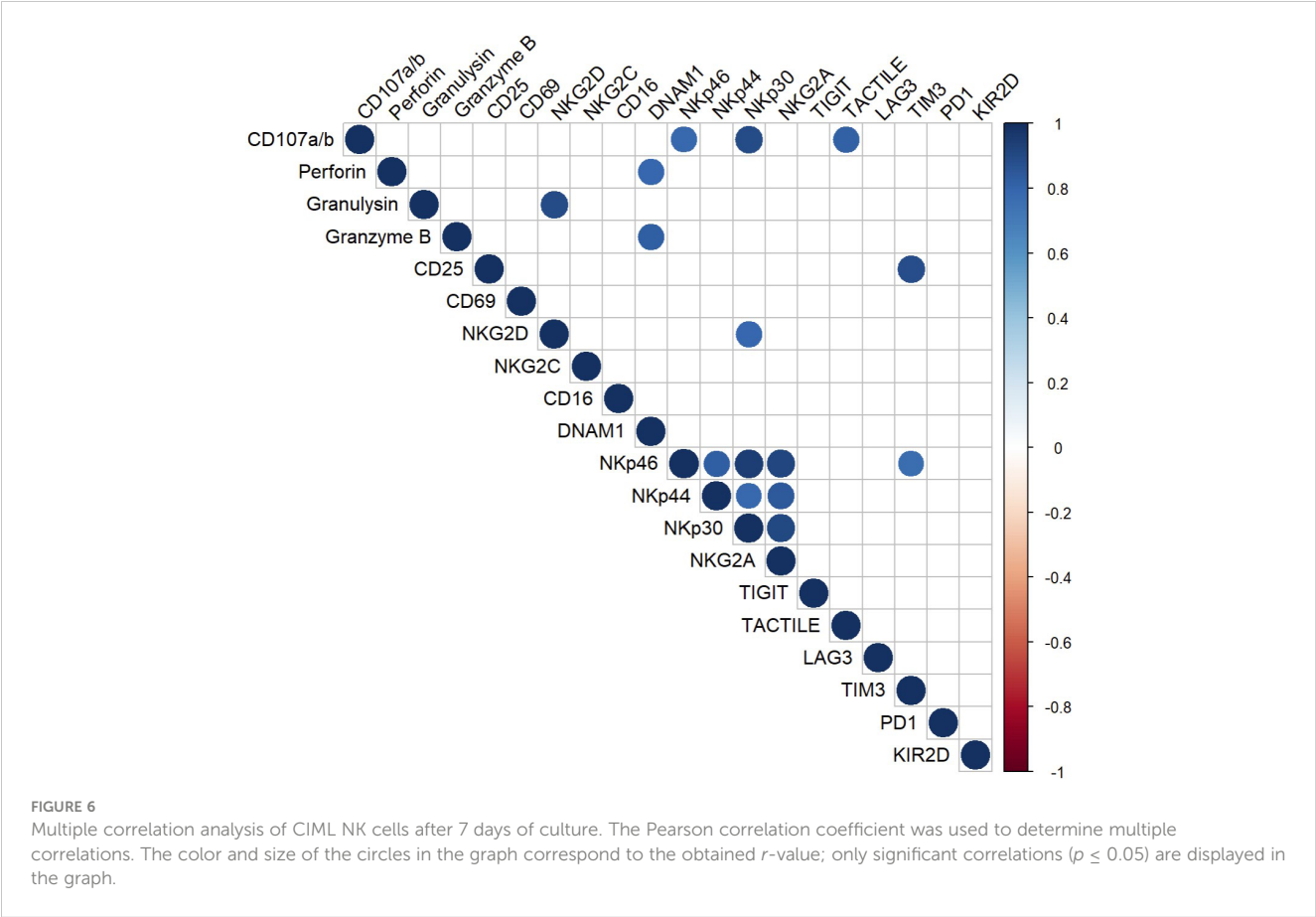


and Perforin; the activating receptor NKG2D, on the other hand, appeared to be strongly correlated with the cytotoxic protein Granulysin. Moreover, strong positive correlations were observed between the activating receptors NKG2D and NKp30, among the NCRs, and between the NCRs and the inhibitory receptor NKG2A.

The results from the multiple correlation analysis are represented in [Figure 6](#). Only those correlations with *p*  $\leq$  0.05 are displayed. A full compilation of the *r*-values and *p*-values can be found in the [Supplementary Material \(Supplementary Tables 4, 5, respectively\)](#).

#### 4 Discussion

Natural killer cells play a critical role in innate immunity, as they are able to rapidly recognize and eliminate tumor-transformed and virus-infected cells without previous sensitization ([1, 2, 13–16](#)). In recent years, there has been an increasing interest in a distinct subset of NK cells with memory-like properties. Owing to their heightened effector function and longer half-life, CIML NK cells have become a promising tool in immunotherapy for various types of tumors ([13, 20–24](#)). In this study, we aimed to characterize the



phenotypic and functional differences between *in vitro*-expanded CIML and control NK cells. Using multiparametric flow cytometry, we examined the impact of cytokine stimulation on NK cell functionality and surface marker expression, highlighting the distinct features of CIML NK cells and contributing to a deeper understanding of their biology and therapeutic potential.

Our phenotypic and functional studies provide compelling evidence for the distinguishable nature of CIML NK cells compared to control NK cells. After a short stimulation with IL-12, IL-15, and IL-18, surface marker analysis showed that CIML NK cells displayed a more activated phenotype than control NK cells, as evidenced by the increased expression of CD25 and CD69. As expected, these results are in agreement with previous reports showing the expression of these receptors in CIML NK cells (20, 24, 28, 29).

Phenotypic characterization of activating receptors revealed enhanced expression of NKG2D, NKp46, NKp44, and NKp30 in CIML NK cells after 7 days of culture, whereas the expression of other activating receptors (NKG2C, CD16, DNAM-1, and NKp80) was similar between CIML and control NK cells. Some of these results, such as the increase in NKG2D, NKG2C, and NCRs have previously been described in research articles and reviews (23, 29). Notably, both NKG2D and NCRs play crucial roles in NK cell effector responses by recognizing their ligands on tumor cells and mediating the production of cytokines and cytotoxic molecules (30, 31). Therefore, the upregulation of these activating receptors on CIML NK cells suggests an augmented capacity for target cell recognition and cytotoxicity in this subset of memory-like NK cells.

Moreover, in our comprehensive analysis of NK cell surface markers, we observed the presence of CD8 expression in about one third of both CIML and control NK cells. However, our statistical analysis did not reveal any statistically significant differences in CD8 expression between CIML and control NK cells after 7 days of culture. Notably, previous studies employing CIML NK cells in leukemia treatment have suggested a potential unfavorable connection between CD8 expression and clinical outcomes following *in vivo* adoptive cell transfer (32). Given these findings and the fact that the physiological role of CD8 on human NK cells is not yet fully understood, additional research is necessary to unveil the implications of CD8 expression and its potential impact on future immunotherapies (33).

In addition to activating receptors and CD8, the effect of cytokine stimulation on the expression profile of inhibitory receptors was also assessed, revealing heightened expression of NKG2A and TACTILE, and significant downregulation of KIR2D in CIML NK cells after 7 days of culture. However, the expression of TIGIT, LAG-3, TIM-3, and PD-1, was unaffected. Moreover, the upregulation of NKG2A (22, 23, 29) and TACTILE (34), as well as the downregulation of KIR2D (13, 35), have previously been observed by other authors. The upregulation of NKG2A and TACTILE observed in *in vitro*-expanded memory-like NK cells may negatively influence the functionality of adoptively transferred cells. This idea is supported by the fact that TACTILE binding to CD155 on human hepatocellular carcinoma cells contributes to immune escape by inducing NK cell exhaustion and reducing cytotoxicity and cytokine production (36). In contrast, the

downregulation of KIR2D expression found in *in vitro*-expanded CIML NK cells may enhance their anti-tumor efficacy, as it has previously been demonstrated (35).

A comprehensive analysis of NK surface markers, such as the one presented in this study, could be of great value to identify more effective NK cell subsets prior to cell infusion in patients as suggested by Schwab et al. (37). Moreover, our phenotypic study has relevant and meaningful implications for identifying the surface markers that could be targeted in immunotherapeutic strategies, such as immune checkpoint inhibition. Many monoclonal antibodies (mAbs) are currently used to block the inhibitory pathways of NK cells and prevent immune evasion by allowing NK cells to efficiently recognize and eliminate tumor cells (38). The efficacy and safety of anti-NKG2A (monalizumab) and anti-KIR (lirilumab) mAbs have been demonstrated in various clinical trials (38–41), and they have shown promising results against hematological malignancies. TACTILE blockade has shown promising results in inhibiting metastatic progression in three different mouse tumor models (42) and could be a compelling candidate for future immunotherapies.

Following IL-12/15/18 stimulation, NK cells undergo a series of phenotypic changes characterized by the increased expression of CD69, NKG2D, NKG2A, and CD25 (14, 23). Our investigation also focused on the co-expression patterns of these markers, revealing a significantly higher abundance of the CD69+NKG2D+NKG2A+CD25+ subset in CIML NK cells than in control NK cells. In contrast, the CD69–NKG2D–NKG2A–CD25– subset was significantly more abundant in control NK cells than in CIML NK cells. Moreover, the co-expression analysis of NCRs revealed that the NKp46+NKp44+NKp30+ subset was significantly more abundant in CIML NK cells than in control NK cells. These findings demonstrate that CIML NK cells are defined by the co-expression of activating receptors, which is in agreement with previous studies describing the enhanced activation state of NK cells after cytokine stimulation (23). In addition, these marker combinations could potentially be useful in establishing guidelines for distinguishing memory from naïve NK cell populations.

Furthermore, analysis of the co-expression patterns of DNAM-1, TIGIT, and TACTILE revealed a decrease of DNAM-1–TIGIT+TACTILE+ subset in CIML NK cells compared to control NK cells. This phenotype is related to decreased effector functions and cytotoxicity of NK cells in AML patients (43). The lower abundance of this subset in CIML NK cells and the higher expression of the activating receptor DNAM-1 could suggest higher DNAM-1-mediated degranulation and cytotoxicity.

Notably, we observed a significant increase in the cytotoxic protein Granzyme B in CIML NK cells after 7 days of culture, while the percentage of Granulysin+ cells was similar between CIML and control NK cells. However, we observed a slight, albeit not significant, decrease in Perforin+ cells in CIML NK cells when compared to control NK cells, which has also been detected by other authors (44, 45) and could be explained by the inter-donor variability in Perforin expression observed in our samples. In addition, the higher expression of Granzyme B in CIML NK cells has previously been reported by other authors who have described increased lysis of leukemia target cells *in vitro* (23, 46).

Additionally, along with the phenotypic characterization of NK cell surface markers, we evaluated the degranulation capacity and intracellular cytokine production of CIML NK cells against K562, an NK-sensitive target cell line. Our findings are consistent with previous studies demonstrating that CIML NK cells exhibit enhanced degranulation capacity and higher IFN- $\gamma$  production against the K562 cell line (23, 29, 47). Nonetheless, it is important to note that cytokine production displayed substantial donor-to-donor variability among the participants included in our study.

Finally, we performed a multiple correlation analysis to identify correlations between the phenotype and degranulation capacity of CIML NK cells. Our results revealed strong positive correlations between the degranulation capacity of CIML NK cells and the expression of a number of activating molecules (NKP46 and NKP30) and inhibitory receptors (TACTILE). These findings support the crucial role of NKP46, and NKP30 in NK cell function. Previous research has shown that antibody blockade of these receptors would result in a reduction of NK cell degranulation against target cells (48). Additionally, the expression of NCRs has been strongly associated with the ability of NK cells to degranulate and kill tumor cells (49, 50). Furthermore, although the exact function of TACTILE is not yet fully understood (12), this receptor is known to possess both activating and inhibitory motifs, suggesting its involvement in mediating positive and negative signals in NK cells (51). Moreover, it has been observed that TACTILE expression in NK cells directly inhibits IFN- $\gamma$  production but does not affect NK cell degranulation (52). In addition, K562 cells express high levels of CD155 ligand (53), and it has been reported that TACTILE expression facilitates NK cell adhesion to CD155-expressing cells, thereby stimulating NK cell cytotoxicity (54).

Our study also identified positive correlations between activating receptors (NKG2D and DNAM-1) and cytotoxic proteins (Granulysin, Perforin, and Granzyme B). Both NKG2D and DNAM-1 are known to be involved in NK-cell mediated cytotoxicity by recognizing their ligands on target cells, triggering downstream signaling pathways that result in the degranulation of cytotoxic proteins (55–58).

Additionally, our multiple correlation analysis revealed strong positive correlations between NKG2D and NKP30 on CIML NK cells, as well as among the NCRs. The simultaneous expression of these receptors may synergistically contribute to tumor cell lysis (59–62). Furthermore, our findings also revealed positive correlations between the activating receptors CD25 and NKP46, and the inhibitory receptor TIM-3 in CIML NK cells. Although our understanding of the role of TIM-3 in NK cell biology remains limited, it is known that this inhibitory receptor is constitutively expressed on resting NK cells and can negatively impact NK cell cytotoxicity (63). In addition, TIM-3 has been associated with NK cell maturation and exhaustion, phenomena observed in human NK cells following continuous exposure to IL-15 or stimulation with IL-12/15/18 *in vitro* (64, 65).

Overall, our findings broaden the general understanding of *in vitro*-expanded CIML NK cells and their potential as antitumor effector cells. Our comprehensive characterization of the phenotype and functional profile of memory-like NK cells provides valuable

insights into optimizing the expansion protocols of CIML NK cells and for the selection of potent NK cell subsets that could help improve the efficacy of immunotherapies. While further research is necessary to investigate the clinical applications of CIML NK cells and fully understand the underlying mechanisms driving their enhanced functionality, we believe that our biomarker panel holds significant potential for practical use in clinical settings and adoptive cell therapies.

## Data availability statement

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

## Ethics statement

The studies involving humans were approved by The ethics committee of the University of Extremadura (Ref.: 118//2020). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because blood samples were obtained from anonymous healthy adult donors under the supervision of the Blood Donation Center of Extremadura (Mérida, Spain).

## Author contributions

SC-S: Formal Analysis, Investigation, Methodology, Writing – review & editing, Conceptualization, Data curation, Writing – original draft. NL-S: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. MG-S: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. ES-H: Methodology, Writing – review & editing. AP: Formal Analysis, Writing – review & editing. FH: Methodology, Writing – review & editing. ED: Writing – review & editing, Supervision. RS: Writing – review & editing, Conceptualization, Funding acquisition. JC: Supervision, Writing – review & editing, Formal Analysis, Investigation, Methodology, Writing – original draft. RT: Conceptualization, Funding acquisition, Writing – review & editing, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft.

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## Conflict 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.

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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.2023.1256404/full#supplementary-material>

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## EDITED BY

Chiara Porta,  
University of Eastern Piedmont, Italy

## REVIEWED BY

Belén Blanco,  
Research Institute Hospital 12 de Octubre,  
Spain  
Stefan Barth,  
University of Cape Town, South Africa  
Jaroslav Zak,  
The Scripps Research Institute,  
United States

## \*CORRESPONDENCE

Matthias Peipp  
✉ m.peipp@med2.uni-kiel.de

<sup>†</sup>These authors have contributed  
equally to this work and share  
last authorship

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# Novel NKG2D-directed bispecific antibodies enhance antibody-mediated killing of malignant B cells by NK cells and T cells

Sebastian Lutz<sup>1,2</sup>, Katja Klausz<sup>2</sup>, Anca-Maria Albici<sup>2</sup>,  
Lea Ebinger<sup>2</sup>, Lea Sellmer<sup>2</sup>, Hannah Teipel<sup>2</sup>, André Frenzel<sup>3</sup>,  
Anna Langner<sup>2</sup>, Dorothee Winterberg<sup>2</sup>, Steffen Krohn<sup>2</sup>,  
Michael Hust<sup>3,4</sup>, Thomas Schirrmann<sup>3</sup>, Stefan Dübel<sup>4</sup>,  
Regina Scherließ<sup>5</sup>, Andreas Humpe<sup>1</sup>, Martin Gramatzki<sup>2</sup>,  
Christian Kellner<sup>1†</sup> and Matthias Peipp<sup>2\*†</sup>

<sup>1</sup>Department of Transfusion Medicine, Cell Therapeutics and Hemostaseology, University Hospital, Ludwig Maximilians University (LMU) Munich, Munich, Germany, <sup>2</sup>Division of Antibody-Based Immunotherapy, Department of Medicine II, Kiel University, Kiel, Germany, <sup>3</sup>YUMAB GmbH, Braunschweig, Germany, <sup>4</sup>Technische Universität Braunschweig, Institut für Biochemie, Biotechnologie und Bioinformatik, Abteilung Biotechnologie, Braunschweig, Germany, <sup>5</sup>Department of Pharmaceutics and Biopharmaceutics, Kiel University, Kiel, Germany

The activating receptor natural killer group 2, member D (NKG2D) represents an attractive target for immunotherapy as it exerts a crucial role in cancer immunosurveillance by regulating the activity of cytotoxic lymphocytes. In this study, a panel of novel NKG2D-specific single-chain fragments variable (scFv) were isolated from naïve human antibody gene libraries and fused to the fragment antigen binding (Fab) of rituximab to obtain [CD20×NKG2D] bibodies with the aim to recruit cytotoxic lymphocytes to lymphoma cells. All bispecific antibodies bound both antigens simultaneously. Two bibody constructs, [CD20×NKG2D#3] and [CD20×NKG2D#32], efficiently activated natural killer (NK) cells in co-cultures with CD20+ lymphoma cells. Both bibodies triggered NK cell-mediated lysis of lymphoma cells and especially enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) by CD38 or CD19 specific monoclonal antibodies suggesting a synergistic effect between NKG2D and FcγRIIIA signaling pathways in NK cell activation. The [CD20×NKG2D] bibodies were not effective in redirecting CD8+ T cells as single agents, but enhanced cytotoxicity when combined with a bispecific [CD19×CD3] T cell engager, indicating that NKG2D signaling also supports CD3-mediated T cell activation. In conclusion, engagement of NKG2D with bispecific antibodies is attractive to directly activate cytotoxic lymphocytes or to support their activation by monoclonal antibodies or bispecific T cell engagers. As a perspective, co-targeting of two tumor antigens may allow fine-tuning of antibody cancer therapies. Our proposed combinatorial approach is potentially applicable for many existing immunotherapies but further testing in different preclinical models is necessary to explore the full potential.

## KEYWORDS

bispecific antibody, phage display, antibody therapy, NKG2D, FcγRIIIA, CD20, lymphoma

# 1 Introduction

Since approval of rituximab in 1997 antibody-based immunotherapy has become the fourth pillar in the treatment of cancer besides surgery, radiation and chemotherapy. However, despite this success story not all patients benefit and relapse is still a serious issue (1). Therefore, further development and optimization of antibody therapy is a major objective in current translational research. Recruitment of effector cells plays a crucial role for the efficacy of therapeutic antibodies as revealed in murine tumor models and by observations in patients (2–5). Thus, approaches improving this effector function are very attractive.

This mechanism of action is based on the interaction of the antibody fragment crystallizable (Fc) domain and activating Fcγ receptors expressed on effector cells, resulting in antibody-dependent cellular phagocytosis (ADCP) or antibody-dependent cell-mediated cytotoxicity (ADCC). Especially in the treatment of minimal residual disease antibody therapy is promising, since at low tumor burden high effector-to-target cell ratios (E:T ratios) can be expected. However, recruitment of effector cells through therapeutic antibodies is often insufficient in patients (6). To overcome this limitation various strategies have been developed to improve effector cell engagement, for example by optimizing the Fc-domain of immunoglobulin G (IgG) antibodies by Fc-engineering (7), combining monoclonal antibodies (mAbs) with immune stimulatory molecules (8), or using bispecific antibodies (bsAbs) (9).

BsAbs for effector cell recruitment represent a promising class of therapeutic agents in cancer immunotherapy. These molecules combine at least two antigen binding moieties of different specificity, the first to target an antigen on tumor cells and the second to trigger an activating receptor on immune cells. Especially natural killer (NK) cells and T cells can be activated by these agents efficiently, when targeting e.g. Fcγ receptor (FcγR) IIIA (CD16a), CD3 or the γδ T cell receptor (TCR), respectively (9–11). Besides chimeric antigen receptor (CAR) T cells, CD3 bsAbs constitute the most powerful agents for induction of major histocompatibility complex (MHC)-independent T cell responses against cancer (12). In 2015, the bispecific T cell engager (BiTE) blinatumomab, a [CD19×CD3] bsAb received marketing approval in both the US and the EU in the treatment of relapsed or refractory B cell precursor acute lymphoblastic leukemia (13, 14). More recently, with teclistamab and mosunetuzumab-axgb two additional T cell engagers received approval in the treatment of multiple myeloma and B cell lymphoma, respectively (15, 16). Yet, NK and T cells express various receptors with stimulatory or co-activating functions, which also have great potential to act as trigger molecules for bsAbs.

One candidate in this aspect is the activating receptor natural killer group 2 member D (NKG2D; CD314), a C-type lectin-like receptor, that plays a key role in immunosurveillance of tumors and pathogens (17, 18). In humans, NKG2D is expressed by NK cells and T cells and recognizes “induced-self proteins”, which are frequently expressed at the cell surface after viral infection or malignant transformation (19, 20). Human NKG2D ligands include major histocompatibility complex (MHC) class I-related

chain (MIC) A and B as well as UL16-binding proteins (ULBP) 1–6. Recognition of these danger signaling antigens results in cell activation through an intracellular signaling pathway via the NKG2D-associated adapter protein DNAX-activating protein of 10 kDa (DAP10) (21). In NK cells, this signal leads to induction of natural cytotoxicity without further co-stimulation (22). In addition to NK cells, in humans NKG2D is also expressed on CD8<sup>+</sup> αβ T cells, γδ T cells, NKT cells as well as subsets of CD4<sup>+</sup> T cells and mediates stimulating or co-activating signals. Previous studies have shown that co-stimulation by NKG2D regulates priming, proliferation and function of cytotoxic T cells (23, 24). After prolonged stimulation with IL-2 or IL-15, T cells were also able to kill target cells TCR-independent via NKG2D activation (25, 26). Diefenbach and colleagues showed that the expression of NKG2D ligands triggered adaptive immune responses, which were dependent on activation of NK cells and T cells (27).

During cancer progression many tumors escape this immunosurveillance mechanism through downregulation or proteolytic shedding of NKG2D ligands (19, 28, 29). Recently, leukemic stem cells have been described to lack NKG2D ligand expression, thereby evading destruction by NK cells (30). Therefore, different strategies were pursued to restore NKG2D-mediated recognition of malignant cells. In a recent study, anti-MICA and anti-MICB antibodies were used to inhibit shedding of these ligands, resulting in enhanced NK cell cytotoxicity through NKG2D and additional FcγRIIIA activation (31). As NKG2D is expressed on NK cells as well as on T cell subsets, it may also represent a promising target for antibody-based immunotherapy. Fusion proteins of antibody fragments and NKG2D ligands were employed to coat tumor cells with the danger signal. Thus, a bispecific immunoligand, which consists of the natural NKG2D-ligand ULBP2 fused to a scFv targeting CD138 expressed on multiple myeloma (MM) cells, showed promising results *in vitro* and in a xenograft mouse model (32). With a similar construct targeting CD20, we have shown that bispecific immunoligands engaging NKG2D trigger NK cell cytotoxicity and synergistically enhance NK cell-mediated ADCC by therapeutic antibodies (33, 34). In addition, ULBP2 containing immunoligands were demonstrated to promote CD8<sup>+</sup> T cell activation when combined with a bispecific CD3 T cell engager *in vitro* (35). Similar approaches have been described using MICA-based fusion proteins (36). Furthermore, a bsAb with specificities for NKG2D and SLAM family member 7 (SLAMF7; CS-1; CD319) was shown to exert therapeutic effects in preclinical models of MM (37). In recent studies, bispecific antibodies targeting Her2 and NKG2D were generated using scFv or VHH antibodies and were shown to trigger efficient target cell killing (38, 39). Interestingly, such bispecific molecules were also able to redirect genetically modified T or NK cells engineered to express a NKG2D-based chimeric antigen receptor against Her2 expressing cancer cells (39).

In this study, novel human NKG2D antibodies were isolated by phage display (40) and used to develop costimulatory bsAbs targeting CD20<sup>+</sup> lymphoma cells. The use of antibodies as compared to natural ligands as triggering device may circumvent potential production issues related to a complex glycosylation profile present on natural ligands. The molecules were



biochemically characterized and screened for their potential to activate effector cells. Promising candidates were utilized to further improve the cytotoxic potency of NK cells in combination with tumor specific monoclonal antibodies as well as a bispecific T cell engager, respectively. This strategy could improve existing clinically used antibody therapies by enhancing the cytotoxicity of both NK and T effector cell populations in a co-stimulatory approach.

## 2 Materials and methods

### 2.1 Phage display

Phage display experiments were performed as described previously (41). Naïve antibody gene libraries HAL7 and HAL7b were used for bio-panning against a recombinant human NKG2D-Fc fusion protein. NKp30-Fc was employed as a control for negative selection and competition (34). A total of 276 clones from HAL7 and 230 clones from HAL7b were repackaged with M13K07 helper phage and then screened by a monoclonal phage ELISA (42). Briefly, a total of 50 ng/well NKG2D-Fc and NKp30-Fc antigens were coated in 96-well MTPs (High Binding, Costar) in PBS over night at 4°C. Subsequent blocking was performed with 2% (w/v) skim milk powder in PBS with 0.05% Tween-20 (M-PBST) for 1 h at room temperature. All following washing steps were performed three times with PBST using an ELISA washer. A total of 100 µL/well of diluted antibody-phage carrying different scFv clones from the panning against NKG2D-Fc were prepared in M-PBST and incubated on the antigen-coated wells. Detection was performed with a mouse anti-M13 antibody HRP conjugate (GE Healthcare) followed by goat anti-mouse Fc specific secondary antibody horseradish peroxidase (HRP) conjugate (Sigma). Finally, substrate TMB (3,3',5,5'-tetramethylbenzidine) was added, and the color reaction was stopped by adding 100 µL 1 N sulfuric acid. Absorbances were measured at 450 nm (with 620 nm reference wavelength) using an ELISA reader (SUNRISE, Tecan).

### 2.2 Sequencing, sequence analysis

Sanger sequencing was used to identify different NKG2D-specific scFvs and to verify DNA sequences. Sequences were analyzed using VBASE2 (43).

### 2.3 Cell culture

Raji cells (DSMZ) were maintained in RPMI 1640 Glutamax-I medium (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS; Thermo Fisher Scientific), 100 U/mL penicillin and 100 mg/mL streptomycin (Thermo Fisher Scientific). GRANTA-519 (DSMZ) and Lenti-X 293T cells (Takara Bio Europe/Clontech) were cultured in Dulbecco's modified Eagle medium-Glutamax-I medium (Thermo Fisher Scientific) supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL

streptomycin. Chinese hamster ovary (CHO)-S, suspension-adapted CHO cells (Thermo Fisher Scientific) were kept in CD CHO-Medium (Thermo Fisher Scientific) containing 1% GlutaMax-I (200 mM L-Ala-L-Gln, Gibco/Thermo Fisher Scientific) and 1% HT Supplement for maintenance and in CD OptiCHO (Thermo Fisher Scientific) supplemented with 1% Pluronic-F68, 1% GlutaMax-I and 1% HAT-Supplement (CHO production medium) for antibody production.

### 2.4 Cloning, expression and purification of bsAbs and antibody derivatives

For construction of the heavy chain derivatives of bibodies, DNA sequences for the different anti-NKG2D scFvs were ligated as NcoI/NotI cassettes into expression vector pΔIRES-RTX-VH-CH1 (unpublished). This is a derivative of vector pIRES-ZSK Green, in which both the internal ribosomal entry site and the GFP coding sequence had been replaced by sequences coding for the rituximab VH leader, rituximab VH chain, the IgG1 CH1 domain and the antibody's upper hinge region. For production of small quantities for [CD20×NKG2D] bibody screening, Lenti-X 293T cells were transiently co-transfected with expression vectors encoding either the bibodies' heavy chain derivative or the rituximab light chain (44) by the calcium phosphate method as described earlier (10). Selected clones were also expressed transiently in CHO-S cells by electroporation using MaxCyte STX electroporation system (MaxCyte) (45). Transfected cells were cultured in CD OPTiCHO medium containing 1% pluronic-F68 (Thermo Fisher Scientific), 1% Glutamax-I (Thermo Fisher Scientific) and 1% HT supplement (Thermo Fisher Scientific) at 32°C, 5% CO<sub>2</sub> and 143 rpm. After 24 h, sodium butyrate (Sigma) was added to a final concentration of 15 µM and 3.5% (v/v) feed stock solution, which contained 70% CHO CD Efficient Feed A Stock Solution (Thermo Fisher Scientific), 14% Yeastolate TC UF (Becton Dickinson), 3.5% GlutaMax-I (200 mM) and 12.5% Glucose (450 g/L, Sigma), was supplemented daily. The production was terminated when cell viability decreased below 50% and the cell culture supernatant was collected. Bibodies were purified by affinity chromatography with CaptureSelect IgG-CH1 affinity matrix (Thermo Fisher Scientific) following manufacturer's instructions. BiTE-like constructs [CD19×CD3] and [HER2×CD3], which both are based on the CD3 scFv moiety from blinatumomab (WO2005/040220), were expressed and purified as described previously (11). Fusion proteins NKG2D-Fc, NKp30-Fc were produced as previously published (33). After extensive dialysis against phosphate-buffered saline (PBS, Invitrogen) the molecules were stored at 4°C until usage. For selected experiments multimers were removed by size exclusion chromatography (Supplementary Figure 1).

### 2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Separation and detection of recombinant bsAbs were performed by SDS-PAGE under reducing or non-reducing conditions,

according to standard procedures. Proteins were analyzed by Coomassie staining (Coomassie brilliant blue G250 solution, Carl Roth GmbH). The concentration of purified bsAbs was estimated against a standard curve of rituximab (Roche).

## 2.6 Size exclusion chromatography

Size exclusion chromatography was performed on an ÄKTA purifier (GE Healthcare) using PBS as running buffer at a constant flow rate of 1 ml/min. Thyroglobulin (669 kDa, Cytiva), aldolase (158 kDa; Cytiva) and ribonuclease A (13.7 kDa; Cytiva) were used for calibration.

## 2.7 Flow cytometry

Flow cytometry experiments were performed on a Navios flow cytometer (Beckman Coulter). Three hundred thousand cells were washed in PBS supplemented with 1% bovine serum albumin (Sigma-Aldrich) and 0.1% sodium-azide. Simultaneous binding was demonstrated by incubating Raji cells with the [CD20×NKG2D] bibodies (50 µg/mL), followed by a second incubation step with either NKG2D-Fc (100 µg/mL) or the control protein NKp30-Fc on ice for 60 minutes. Finally, the surface-bound complex was visualized by staining with polyclonal FITC-coupled anti-human IgG-Fc F(ab')<sub>2</sub> fragments (Beckman Coulter). Isolated NK or T cells were characterized by flow cytometry using FITC- or Pacific Blue-conjugated CD3 or CD8, APC-coupled CD56, PE-conjugated CD16 antibodies (Beckman Coulter) and corresponding isotype controls according to the manufacturer's recommendations. CD19, CD20 and CD38 expression on target cells was analyzed analogously using PE or FITC-conjugated antibodies (Beckman Coulter).

## 2.8 Preparation of mononuclear cells and isolation of NK and T cells

All experiments were authorized by the Ethics Committee of the Kiel University (Kiel, Germany). Blood from donors was drawn after having received written informed consent. Preparation of MNC from peripheral blood of patients and healthy volunteers or from leukocyte reduction system chambers was performed via Ficoll-Paque PLUS density gradient (GE Healthcare). After centrifugation, MNC were collected at the Serum/Ficoll interface and remaining erythrocytes were removed by hypotonic lysis. NK cells and CD8<sup>+</sup> αβ T cells were isolated from MNC by MACS technology via negative selection using NK cell isolation kit and CD8<sup>+</sup> T cell isolation kit (Miltenyi), respectively, following the manufacturer's protocols. In CD8<sup>+</sup> T cell preparations any potentially remaining NK cells were removed in a secondary depletion step using CD56 MicroBeads (Miltenyi). Purified MNC were directly employed in functional assays. NK cells were cultured overnight at a density of 2×10<sup>6</sup> cells/mL in RPMI 1640 Glutamax-I medium supplemented with 10% FCS, 100 U/mL penicillin and 100 mg/mL streptomycin. CD8<sup>+</sup> T cells were

kept at a density of 1×10<sup>6</sup> cells/mL in RPMI 1640 Glutamax-I medium supplemented with 10% FCS, 100 U/mL penicillin and 100 mg/mL streptomycin and stimulated with IL-2 (300 U/mL) for 48 h prior to functional analysis.

## 2.9 Analysis of NK cell activation

One hundred thousand NK cells were co-incubated with equal numbers of GRANTA-519 cells in microtiter plates in a volume of 200 µL. The [CD20×NKG2D] bibodies at the indicated concentrations or PBS were added. After 4 h cells were stained with antibodies against CD69 (PE-conjugated, Beckman Coulter), CD56 (APC, Beckman Coulter), CD19 (FITC, Beckman Coulter) and CD3 (Pacific Blue, Beckman Coulter) and analyzed by flow cytometry. CD56-positive, CD3- and CD19-negative NK cells were gated and the expression levels of CD69 were determined.

## 2.10 Analysis of NK cell and T cell cytotoxicity

Cytotoxicity was analyzed in standard 4 h <sup>51</sup>Cr release experiments, which were performed in 96-well microtiter plates in a total volume of 200 µL as described previously (10). Human NK cells, CD8<sup>+</sup> T cells or MNC were used as effector populations at the indicated E:T ratios. The bispecific [CD20×NKG2D] bibodies, the antibodies daratumumab (Janssen), CD19-DE or trastuzumab (Roche) as a non-binding IgG1 control and the BiTE molecules [CD19×CD3] or [HER2×CD3] were analyzed at the indicated concentrations.

## 2.11 Statistical analysis and data processing

P-values were determined using repeated measures ANOVA and the Bonferroni post-test. The null hypothesis was rejected for  $p < 0.05$ . Statistical and graphical analyses were performed with GraphPad Prism 5.0 software. Synergy was analyzed by interpolating required antibody doses at distinct effect levels using GraphPad Prism 5.0 software and calculating combination index (CI) values using the formula  $CI_x = D_A/D_{xA} + D_B/D_{xB}$  ( $D_{xA}$  and  $D_{xB}$ , dose of drugs A and B alone producing x% effect;  $D_A$  and  $D_B$ , doses of drugs A and B in combination producing equal effects) (46). Synergistic effects were classified into strong synergy (CI = 0.1 – 0.3), synergy (CI = 0.3 – 0.7), moderate synergy (CI 0.7 – 0.85), slight synergy (CI = 0.85 – 0.95), additivity (CI = 1) and antagonism (CI > 1).

# 3 Results

## 3.1 Isolation of human NKG2D antibodies by phage display

To generate novel human NKG2D-specific antibodies the two naïve human scFv antibody libraries HAL7 and HAL7b (41) were

screened by bio-panning against a recombinant fusion protein consisting of the extracellular domain of human NKG2D and the human IgG1-Fc part. Binding analyses of isolated clones revealed that 38 different phage clones bound NKG2D-Fc, but not a control molecule consisting of the extracellular domain of natural killer protein 30 (NKp30-Fc, **Figure 1A**). Sequence analysis by aligning the variable (V) regions of the different antibodies revealed that the clones clustered in distinct groups according to primary sequence diversity. In addition, the individual clones could be assigned to different families of germline V gene segments, had various combinations of different variable heavy ( $V_H$ ) and variable light ( $V_L$ ) chains and could be divided into three groups (**Figure 1B**). The majority of all clones contained IGHV3, which has been reported to display the highest thermodynamic stability and yield of soluble protein (48). The largest group had combinations of IGHV3/IGLV3 (25 clones) which interestingly separated in two groups, followed by IGHV3/IGLV1 (9 clones). Furthermore, rare combinations of IGHV1/IGLV3 (2 clones), IGHV1/IGLV6 and IGHV5/IGLV1 (1 clone each) were identified.

### 3.2 Generation of bispecific antibodies

The 38 isolated scFvs were processed into bispecific [CD20×NKG2D] antibodies. To ensure an efficient screening process we used the heterodimeric bibody format (49), which in this case consists of the fragment antigen binding (Fab) derived from the CD20 specific mAb rituximab, genetically fused to the different anti-NKG2D scFvs via a flexible glycine-serine-linker (**Figures 2A, B**). The resulting bibodies were transiently expressed and purified from cell culture supernatants via affinity chromatography. Thirty-six of the 38 individual anti-NKG2D scFvs were successfully produced in the bibody format. Two

constructs did not show any expression and were not feasible for unknown reasons. Integrity and purity of the proteins were analyzed by Coomassie-stained SDS-PAGE under reducing and non-reducing conditions (**Figures 2C, D**, respectively).

### 3.3 Antigen binding and activation of NK cells

The binding abilities of the different [CD20×NKG2D] bibodies were analyzed by flow cytometry. In particular, the capacity of simultaneous binding to both antigens was determined, which is essential to achieve the crosslinking between target and effector cells. Therefore, CD20<sup>+</sup> lymphoma cells were first incubated with the [CD20×NKG2D] bibodies, and then with soluble human NKG2D-Fc or the control protein NKp30-Fc. Cell-bound NKG2D-Fc fusion protein was subsequently detected with a secondary antibody conjugate directed towards the human Fc domain. Detection of the bibody/NKG2D-Fc complex was only possible when the [CD20×NKG2D] bibody bound both, cellular CD20 and soluble NKG2D-Fc simultaneously. The mean value of fluorescence intensity from experiments without adding bibody was calculated and clones demonstrating mean fluorescence values increased by at least a factor of 1.5 above that value were rated as binders. As indicated by shifts in mean fluorescence intensity, the different NKG2D-specific bibodies reacted with both CD20 and NKG2D, except one clone (**Figures 3A, B**). Interestingly, varying fluorescence intensity values were obtained with different clones, which may reflect different affinities to NKG2D. In contrast, after incubation with the control molecule NKp30-Fc no binding was detectable confirming the specificity of the [CD20×NKG2D] bibodies.

An important function of NK and T cell engagers is their capacity to activate the redirected immune effector cell population.

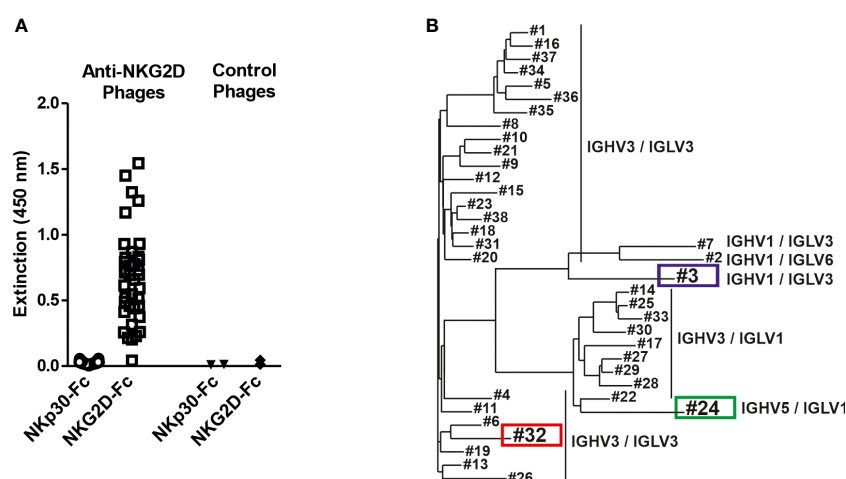


FIGURE 1

Isolation of NKG2D-specific human scFv antibodies and sequence analysis of the V regions. (A) ScFv phage, which had been isolated from a naïve antibody library by panning against the human NKG2D antigen, were analyzed for specific antigen binding by phage ELISA using an NKG2D-Fc fusion protein and the analogously constructed control protein NKp30-Fc. ScFv phage binding an irrelevant antigen were used as controls. (B) The isolated NKG2D-specific scFvs were grouped via sequence analysis into 3 different groups according to different germline gene segment families as well as their  $V_H/V_L$  combinations (detailed sequence information is available (47)). The further characterized clones #3 (blue) and #32 (red) and the later used control scFv #24 (green) are highlighted.

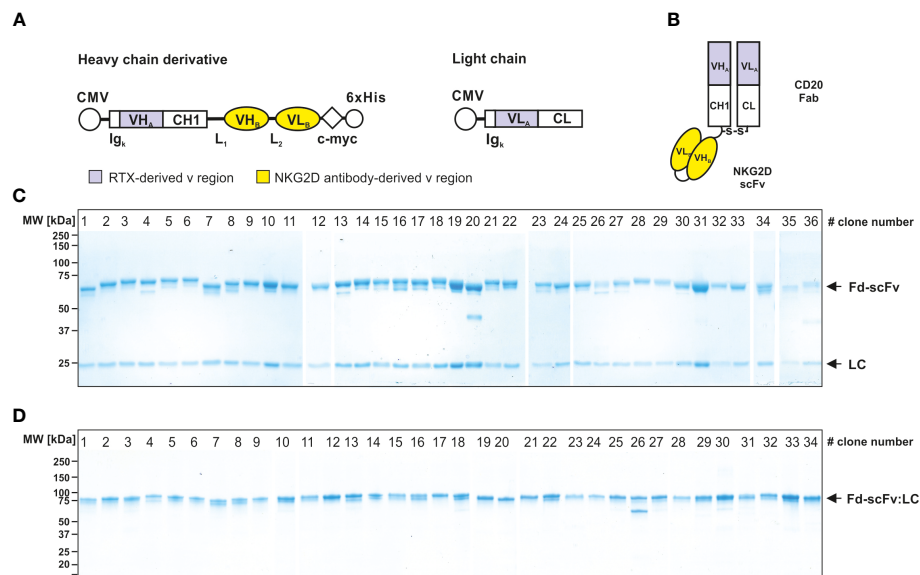


FIGURE 2

Generation and characterization of bispecific [CD20×NKG2D] bibodies. **(A)** Schematic illustrations of the expression cassettes of bispecific [CD20×NKG2D] antibodies in the bibody format (Fab-scFv). CMV, cytomegalovirus promoter; Ig<sub>k</sub>, human Ig kappa secretion leader; VH<sub>A</sub>, VL<sub>A</sub>, sequences coding for the variable regions of the immunoglobulin heavy and light chains of the CD20 antibody rituximab (RTX), respectively; CH1, CL, sequences coding for the human immunoglobulin heavy chain constant region 1 and the human immunoglobulin kappa-light chain constant region, respectively; VH<sub>B</sub>, VL<sub>B</sub>, cDNA sequence coding for the variable heavy and light chain regions of the NKG2D-specific scFv; L<sub>1</sub>, L<sub>2</sub>, sequence coding for a linker peptides; c-myc, 6xHis, sequence coding for the c-myc epitope and a hexahistidine tag, respectively. **(B)** Block structure of the produced bispecific antibodies in the bibody format. The NKG2D-specific scFvs were fused to a CD20 directed Fab. S-S, disulfide bridge. Purity and integrity of purified bispecific antibodies, consisting of a light chain (LC, approx. 25 kDa) and a heavy chain derivate (Fd-scFv, approx. 60 kDa), were analyzed by Coomassie stained SDS-PAGE under reducing (10% PAA) **(C)** and non-reducing conditions (4 – 15% PAA) **(D)**. Of note: Only 36 of the initially sequenced 38 NKG2D scFvs could be successfully expressed as recombinant protein. Bibodies 35 and 36 expressed at very low levels and were not analyzed in all assay conditions. The numbering of the lanes represents the clone numbers #1 - #36 introduced in Figure 1. One representative experiment out of three is shown.

Hence, the remaining 36 [CD20×NKG2D] bibodies were analyzed for their ability to activate human NK cells. Therefore, NK cells and lymphoma cells were incubated in the presence of the bibodies. Expression of the early activation marker CD69 on NK cells was only significantly induced by four [CD20×NKG2D] bibody constructs (Figure 3C). These data demonstrate that NKG2D engagement-induced activation is not a common feature of all NKG2D antibodies in the bibody format. In the following experiments we focused on bibodies containing anti-NKG2D scFv clones #3 (in the following figures indicated with blue color) and #32 (red color), which showed the highest NK cell activation efficiency. Clone #24 (green) was chosen as representative control for bibodies with low activation profile.

### 3.4 Cytotoxic capacity and synergistic activity in combination with non-engineered and Fc-engineered antibodies

In previous studies, we have shown that bispecific immunoligands engaging NKG2D trigger NK cell cytotoxicity and enhance NK cell-mediated ADCC by therapeutic antibodies (33, 34). To investigate whether the novel [CD20×NKG2D] bibodies exerted this function, the two bibodies with the highest activatory activity were either analyzed as single agents or were

combined with the CD38 antibody daratumumab, and cytotoxicity was analyzed with both MNC or purified NK cells. CD38<sup>+</sup>/CD20<sup>+</sup> mantle cell lymphoma (MCL) GRANTA-519 cells or lymphoma cells freshly isolated from two MCL patients were used as target cells. Both, bibody [CD20×NKG2D#3] and [CD20×NKG2D#32] used as single agents, induced lysis of GRANTA-519 MCL cells and primary lymphoma cells with MNC and purified NK cells as effector cell population, although to a moderate extent (Figures 4A, B). Importantly, the combination of the CD38 antibody and CD20-directed bibodies [CD20×NKG2D#3] or [CD20×NKG2D#32] was, with 33.0% and 45.2%, significantly more effective in triggering effector cell killing of tumor cells than the single agents. This was the case both when the cell line GRANTA-519 (Figure 4A) or isolated tumor cells from MCL-patients were analyzed (Figure 5B). Interestingly, the [CD20×NKG2D#24] bibody with low NK cell activation capacity in terms of CD69 induction was not able to increase tumor cell lysis in combination with monoclonal antibodies (Supplementary Figure 2).

Fc-engineering of mAbs by increasing their affinity to FcγRIIIA is a powerful method to augment their cytotoxic potential (7). To analyze whether the cytotoxic capacity of Fc-optimized antibodies could be enhanced by our novel agents, the Fc-engineered CD19 antibody (CD19-DE), which was modified for enhanced binding to activating FcγR (50), was tested in combination with the novel NK cell activating [CD20×NKG2D] bibodies in ADCC reactions. The



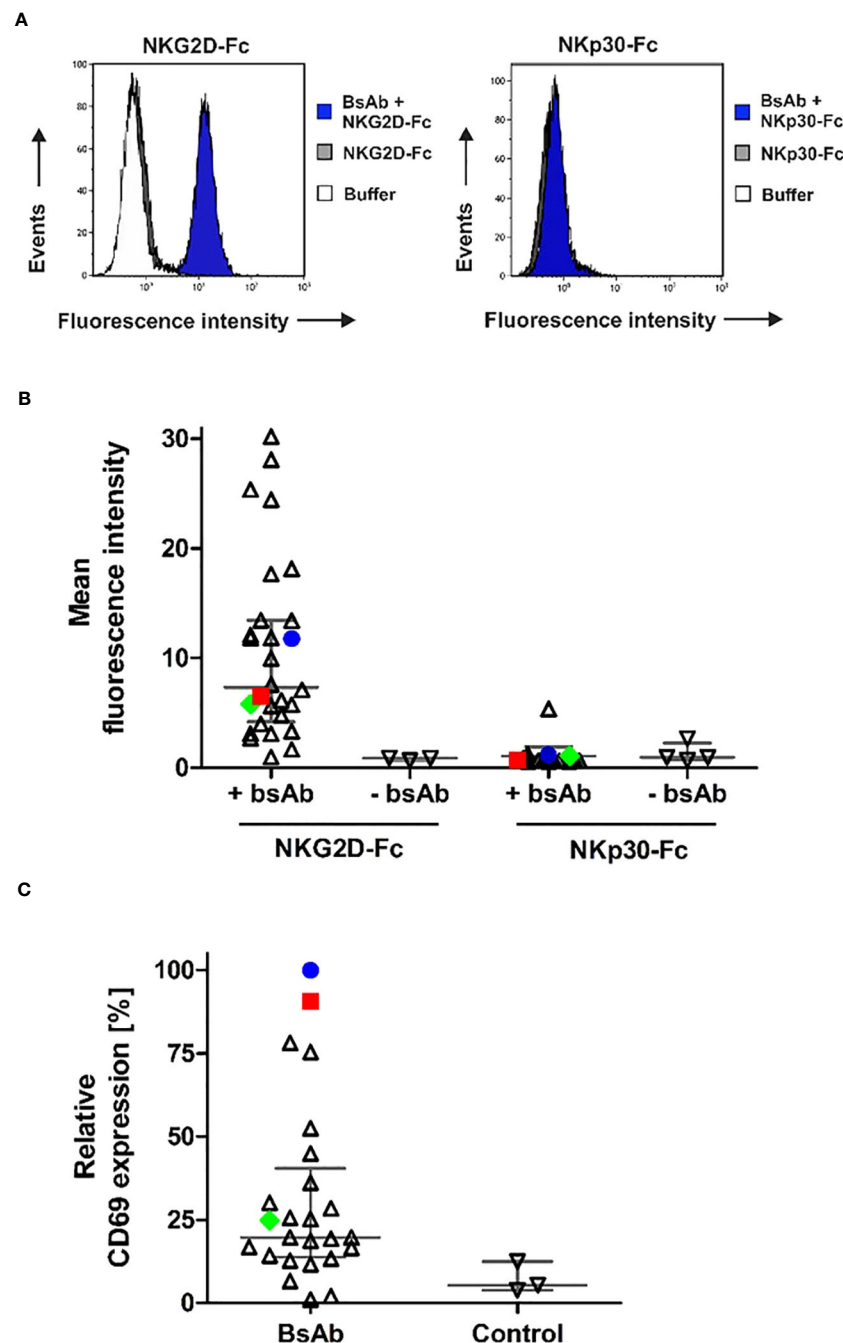


FIGURE 3

Simultaneous antigen binding of [CD20xNKG2D] bibodies and induction of NK cell activation. **(A)** CD20<sup>+</sup> Raji lymphoma cells were first incubated with the different [CD20xNKG2D] bibodies and then reacted with NKG2D-Fc or with the control protein NKp30-Fc. Dual antigen binding of the bibodies was visualized by a FITC-coupled antibody against human Fc via flow cytometry. As a control, cells were incubated with either NKG2D-Fc or NKp30-Fc (control) in absence of the [CD20xNKG2D] bibodies or with the FITC-coupled detection antibody alone (buffer). The exemplary results are shown for the bibody [CD20xNKG2D#3], which specifically interacts with CD20 and NKG2D-Fc but not with NKp30-Fc. Note: in the left panel buffer control and NKG2D-Fc stainings are superimposed. **(B)** Abilities of various individual [CD20xNKG2D] bibody constructs containing different NKG2D scFv clones to simultaneously bind CD20 and NKG2D. Each data point represents an individual construct and indicates the mean fluorescence intensity value from three independent experiments. Horizontal lines show medians with interquartile range. The further characterized clones #3 (blue) and #32 (red) and the later used control scFv #24 (green) are highlighted. **(C)** NK cells were incubated with the [CD20xNKG2D] bibodies (10 µg/ml) in the presence of GRANTA-519 mantle cell lymphoma cells. As a control, NK cells and lymphoma cells were incubated in absence of a bibody. After 4 h, the induced expression of the activation marker CD69 was analyzed on CD56<sup>+</sup>/CD3<sup>+</sup> NK cells via flow cytometry and mean fluorescent intensities were determined. Data points were normalized to CD69 expression induced by the bibody [CD20xNKG2D#3] and indicate mean values from 3 independent experiments. Horizontal lines indicate medians with interquartile range. The further characterized bibody constructs based on clones #3 (blue) and #32 (red) and the later used control construct #24 (green) are highlighted.

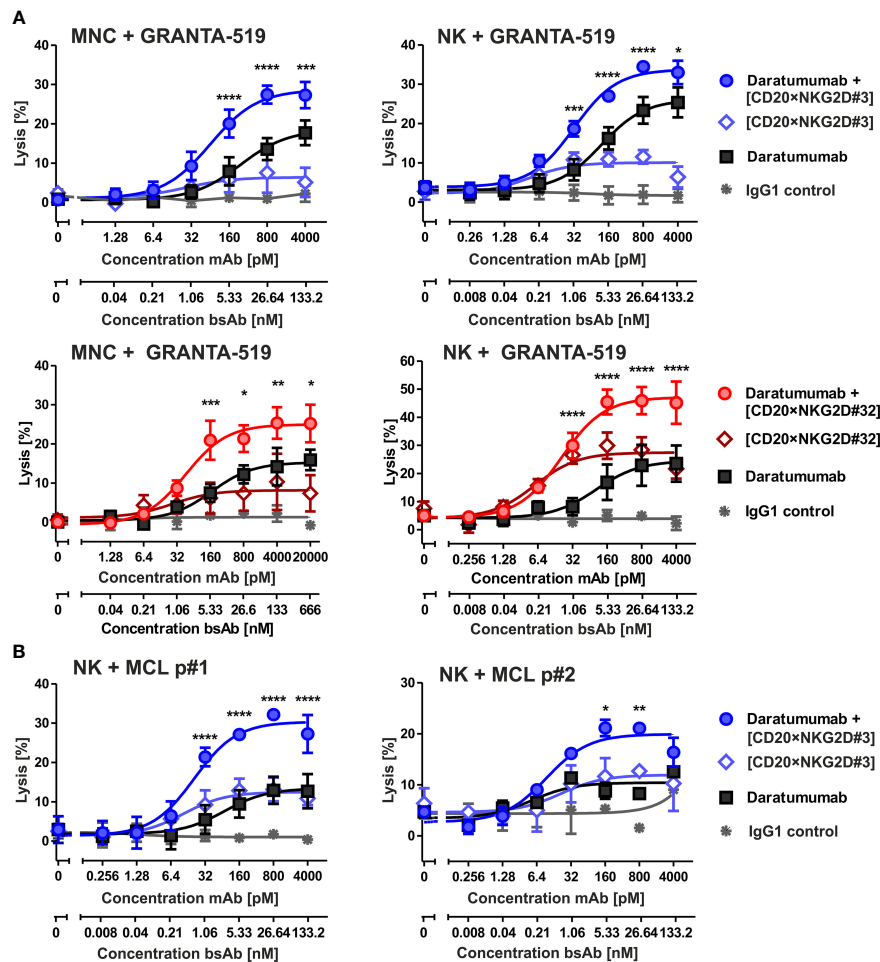


FIGURE 4

Cytotoxicity of the bibodies [CD20×NKG2D#3] and [CD20×NKG2D#32] and synergy with the CD38 specific mAb daratumumab. **(A)** CD20<sup>+</sup>/CD38<sup>+</sup> GRANTA-519 MCL cells were incubated either with daratumumab, with the bispecific [CD20×NKG2D] antibodies or with their combinations, respectively, in presence of mononuclear cells (MNC; E:T ratio: 40:1) or NK cells (E:T ratio = 10:1) as effector population. After 4 h lysis of target cells was analyzed. The data points represent mean values of three independent experiments  $\pm$  SEM. (\*, statistically significant differences to treatment with daratumumab only;  $p \leq 0.05$ ). **(B)** [CD20×NKG2D#3] enhances ADCC triggered through daratumumab against tumor cells derived from two different MCL patients (p). NK cells were used as effector population. Data points represent the mean value from two independent experiments  $\pm$  SEM (\*, statistically significant differences to treatment with daratumumab only;  $p \leq 0.05$ ). \*\*, P values between 0.001 and 0.01; \*\*\*, P values between 0.0001 and 0.001; \*\*\*\*, P values less than 0.0001.

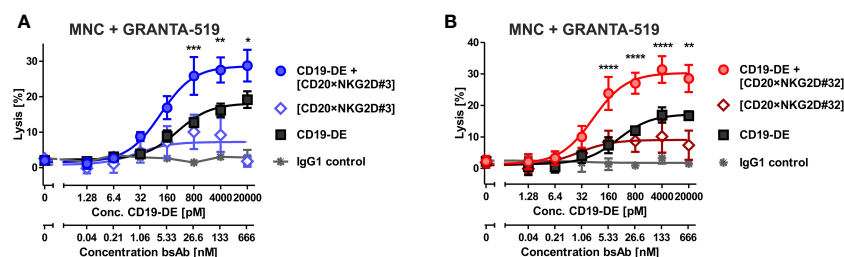


FIGURE 5

Cytotoxicity of combinations of bibody [CD20×NKG2D#3] and [CD20×NKG2D#32] with an Fc-engineered CD19 mAb (CD19-DE). The cytotoxic function of the bispecific antibodies [CD20×NKG2D#3] **(A)** and [CD20×NKG2D#32] **(B)** alone, or in combination with the Fc-engineered CD19-DE mAb, was analyzed in 4 h <sup>51</sup>Cr release assays. GRANTA-519 MCL cells (CD19<sup>+</sup>, CD20<sup>+</sup>) were used as target cells and MNC isolated from healthy donors were applied as effector population (E:T ratio = 40:1). A non-binding monoclonal IgG1 Ab was used as a control. The data points represent the mean value of four independent experiments  $\pm$  SEM. (\*, statistically significant differences against the treatment with CD19-DE only;  $p \leq 0.05$ ). \*\*, P values between 0.001 and 0.01; \*\*\*, P values between 0.0001 and 0.001; \*\*\*\*, P values less than 0.0001.

TABLE 1 CI values for combinations of monoclonal antibodies with the [CD20×NKG2D] bibodies.

Combination	Targets	Effectors	CI values at lysis of	
			5%	10%
Dara + [CD20×NKG2D#3]	GRANTA-519	MNC	0.31	n.a.
Dara + [CD20×NKG2D#3]	GRANTA-519	NK cells	0.68	0.17
Dara + [CD20×NKG2D#3]	MCL p#1	NK cells	0.59	0.24
Dara + [CD20×NKG2D#3]	MCL p#2	NK cells	2.47	0.22
Dara + [CD20×NKG2D#32]	GRANTA-519	MNC	0.42	n.a.
Dara + [CD20×NKG2D#32]	GRANTA-519	NK cells	0.49	1.52
CD19-DE + [CD20×NKG2D#3]	GRANTA-519	MNC	0.58	n.a.
CD19-DE + [CD20×NKG2D#32]	GRANTA-519	MNC	0.45	n.a.

Combination index (CI) values were calculated from dose response curves using the indicated target and effector cells for two different effect levels using GraphPad Prism 5.0 software. Not available values (n.a.), could not be calculated since not all single agents reached the required threshold (Dara, daratumumab).

co-stimulation of NKG2D with these bibodies enhanced CD19-DE-mediated ADCC against CD19<sup>+</sup>/CD20<sup>+</sup> GRANTA-519 MCL target cells significantly and synergistically, even beyond the dose independent cytotoxicity plateau of single CD19-DE treatment (Figure 5, Table 1). Thus, in comparison to CD19-DE as single agent, the maximum lysis was increased from 18.2 ± 1.1% to 28.7 ± 1.9% by [CD20×NKG2D#3] and from 17.2 ± 1.7% to 30.3 ± 2.1% by [CD20×NKG2D#32], respectively. In conclusion, NKG2D co-stimulation synergistically enhanced mAb-mediated ADCC throughout all combination experiments (Table 1).

### 3.5 Co-stimulation of bispecific T cell engagers

NKG2D is also expressed on CD8<sup>+</sup> αβ T cells. We demonstrated in previous studies, that NKG2D-directed bispecific immunoligands were able to induce lysis of lymphoma cells by γδ T cell lines (51), but had low activity levels with αβ T cells (34). To investigate the potential T cell stimulatory function of the novel bibodies, T cell-mediated tumor cell killing triggered by bibodies [CD20×NKG2D#3] and

[CD20×NKG2D#32] alone or in combination with a [CD19×CD3] bsAb was analyzed. Interestingly, both [CD20×NKG2D] bibodies significantly increased T cell-mediated lysis of GRANTA-519 MCL target cells from 26.3% ± 3.2 to 34.7 ± 3.3% and from 22.4 ± 3.0% to 33.0 ± 2.8% respectively, in combination with the [CD19×CD3] bsAb. As single agents the bibodies did not stimulate cytotoxicity of CD8<sup>+</sup> αβ T cells (Figure 6).

In conclusion, bispecific antibodies, based on two novel anti-NKG2D human scFv antibodies were able to trigger NK cell cytotoxicity as single agents at moderate levels. Importantly, the bibodies were able to enhance ADCC of other tumor specific mAbs with non-engineered or engineered Fc as well as tumor cell killing by CD8<sup>+</sup> cytotoxic T cells in combination with T cell engagers beyond the maximum level mediated by FcγRIIIA or CD3 triggering, respectively.

## 4 Discussion

In recent years, combination strategies of antibodies or their derivatives with either blockade of inhibitory immune checkpoint

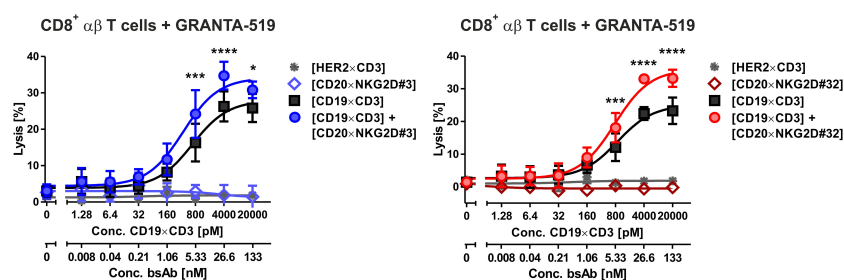


FIGURE 6

Cytotoxic activity of bispecific [CD20×NKG2D] antibodies with CD8-positive αβ T cells as effector population. CD8<sup>+</sup> αβ T cells were isolated via MACS. The purity was determined by flow cytometry using CD3, CD8, CD16 and CD56 antibodies labelled with appropriate fluorescent dyes. Purified T cells were stimulated with IL-2 (300 U/ml) for three days and were tested as effector cells (E:T ratio: 20:1) for the bispecific antibodies [CD20×NKG2D#3] (left graph) and [CD20×NKG2D#32] (right graph) as well as their combinations with a [CD19×CD3] BiTE in a 4 h <sup>51</sup>Cr release assay. GRANTA-519 MCL cells were used as target cells. The data points represent the mean value of three independent experiments ± SEM. (\*, statistically significant differences against the treatment with [CD19×CD3] only; p ≤ 0.05). A [HER2×CD3] BiTE construct that does not bind to the target cells was used as a negative control. \*\*\*, P values between 0.0001 and 0.001; \*\*\*\*, P values less than 0.0001.

receptors or addition of a co-stimulatory signal through activation of a second trigger molecule on effector cells have gained particular interest in cancer immunotherapy. In this regard, co-stimulation of FcγRIIIA and NKG2D was reported to be an attractive strategy for the improvement of NK cell-mediated ADCC (33, 52). In this study, we isolated two novel NKG2D-specific bibodies and used them in combination with an approved non-engineered antibody. We further demonstrated synergistic action of these bibodies to enhance the cytotoxic capacity of Fc-engineered mAbs and a [CD19×CD3] T cell engager.

NKG2D and its ligands have received growing attention in immunotherapy of cancer (53). Current approaches include strategies to increase the expression of NKG2D ligands on tumor cells (54) or to trigger NKG2D signaling with antibody-derivatives. Moreover NKG2D ligands are investigated as target structures on tumor cells for different formats of therapeutic antibodies or CART cells (55–58). In this study, we identified 38 fully human NKG2D-specific scFv antibodies by phage display selection from the human antibody libraries HAL7 and 7b. These antibodies covered six different combinations of V<sub>H</sub> and V<sub>L</sub> families with high sequence diversity in their CDRs. The derived bibodies bound simultaneously to CD20 and NKG2D confirming their functional dual antigen binding. However, there were huge differences in the binding intensities measured by flow cytometry, which indicates different affinities of the scFv moieties to cell-presented NKG2D. These differences likely result from the NKG2D-specific scFvs, since all bibodies were constructed in the same format using the same CD20-specific Fab. Interestingly, the level of measured binding was not associated with the capacity to induce NK cell activation. The bibodies containing one of the two different NKG2D specific scFv antibody clones #3 and #32 induced high levels of NK cell activation, whereas NKG2D binding was only average. Previous studies demonstrated that affinity and avidity of bispecific NK cell engagers to their corresponding tumor antigen play a crucial role in terms of their efficacy (59, 60). Although speculative and not directly proven, our findings related to high NK cell activation by NKG2D scFvs with only average binding activity may be in line with a “hit rate” model of activation proposed for T cell engagers. Therefore, high affinity of the arm targeting the activating NKG2D receptor on NK cells may not enhance cytolytic activity above a certain maximum level and may even be detrimental as described for CD3-directed T cell engagers (61–63). Furthermore, the topology of the recognized NKG2D epitope and resulting orientation of the bispecific antibody may be different between the different clones which could also influence the bispecific cross-linking and NK cell activation. Nevertheless, bibodies based on clones #3 and #32 were able to induce NK cell-mediated tumor cell lysis by triggering NKG2D as single agents. Interestingly, [CD20×NKG2D#32] was more efficient in inducing NK cell-mediated tumor cell lysis than the bibody [CD20×NKG2D#3], despite its slightly lower activation levels.

Of note, we observed that both [CD20×NKG2D#3] and [CD20×NKG2D#32] bibodies enhanced NK cell-mediated ADCC of tumor specific mAbs - promoting the “dual-dual-targeting” concept, which we already proposed in the context of NKG2D-

specific immunoligands (33). This approach based on re-targeting immune effector cells by linking two different activating or co-stimulatory receptors on effector cells with two different antigens on tumor cells may enhance tumor selectivity and cytotoxicity. We chose CD19 or CD38 as second tumor target, because both antigens are co-expressed with CD20 on a variety of B cell lymphomas or leukemias making these tumor antigen combinations ideal for the dual-dual-targeting approach. In our study, we showed that two novel [CD20×NKG2D] bibodies enhanced the ADCC of mAbs targeting CD19 or CD38 on the same tumor cells, thus, demonstrating the synergy of the NKG2D and FcγRIIIA mediated dual NK cell activation and the dual targeting of the CD20<sup>+</sup>/CD19<sup>+</sup> or CD20<sup>+</sup>/CD38<sup>+</sup> tumor cell lines.

Moreover, the bibodies [CD20×NKG2D#3] and [CD20×NKG2D#32] were able to even enhance the NK cell-mediated ADCC by the Fc-engineered antibody CD19-DE, which was optimized for FcγRIIIA binding and enhanced ADCC activity (50). This is an important finding, because in previous studies ADCC mediated by Fc engineered antibodies could not be enhanced above a certain threshold by further increasing the affinity of the engineered Fc to FcγRIIIA (64). However, here we could show, that simultaneous NKG2D activation may overcome this maximum level of cytotoxicity triggered by the FcγRIIIA receptor. Moreover, both bibodies were also able to enhance the cytotoxicity of re-targeted CD8<sup>+</sup> T cells when applied in combination with a [CD19×CD3] BiTE (12, 13). Although the increase in cytotoxic activity was moderate further investigation of this combinatorial approach may be interesting because our actual test system may not reveal the whole spectrum of T cell effector functions triggered by the antibody combination. For example, analysis of cytokine release will be interesting since the level and profile of cytokine production may not directly correlate with cytotoxic activity, as recently shown by our group for NK cell engagers triggering two different receptors in the effector cell (65). Together, these findings suggest that the “dual-dual-targeting approach” may not only be applicable to monoclonal antibody therapy to enhance FcγRIIIA-mediated NK cell cytotoxicity but also to cytotoxic T cells when combined with CD3 targeting T cell engagers. Whether bispecific NKG2D antibodies will also be able to provide co-stimulatory signals to enhance TCR-mediated activation and thereby promote adaptive T cell responses as described for natural NKG2D ligands (27) remains to be determined.

Our data indicate that NKG2D and its different signaling cooperates synergistically with FcγRIIIA in NK cells and CD3 in T cells. As NKG2D contains an immunoreceptor tyrosine based activation motif (ITAM)-independent intracellular signal cascade by pairing with DAP10 (21), we assume that the activation of two distinct intracellular pathways leads to the synergistic effects between NKG2D and either FcγRIIIA or CD3, which both signal via ITAM motifs of the associated FcεRIγ and/or CD3ζ polypeptides (66). This conclusion is supported by previous studies in which NKG2D enhanced FcγRIIIA mediated effects, whereas another NK cell receptor, natural killer protein 46 (NKP46), which signals through the same intracellular signal cascade as FcγRIIIA, did not (67). However, recently trifunctional



NK cell engagers, which consisted of two antibody binding domains, one directed to a tumor associated antigen and one directed to NKP46, and the human Fc domain, showed enhanced cytotoxic properties, which suggests also a cooperation between NKP46 and FcγRIIIA and may be caused by additional factors, which remain to be uncovered (68).

Both T cells and NK cells are regulated by inhibitory receptors which may be employed as target structures for immune modulation. Thus, specific blockade of the immune checkpoints cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or programmed cell death protein 1 (PD-1) has shown potential in the treatment of different types of cancer by promoting T cell responses in patients. Also in NK cells a number of candidate inhibitory receptors have been identified as potential targets for immune checkpoint blockade (69). Besides human killer cell immunoglobulin-like receptors (KIR), T cell-activated increased late expression (TACTILE), T cell immunoreceptor with Ig and ITIM domains (TIGIT) and others, in particular natural killer group 2 member A (NKG2A) may represent an interesting target, since its blockade enhanced NK cell-mediated ADCC (70, 71). However, the impact of these inhibitory receptors on the proposed “dual-dual-targeting” approach relying on concomitant activation of FcγRIIIA and NKG2D in NK cells or CD3 and NKG2D in T cells has not been analyzed yet. It would be interesting to assess whether cytotoxicity may be enhanced even further by simultaneous co-blockade of such inhibitory receptors.

In conclusion, screening of human antibody libraries led to the identification of a panel of NKG2D-specific scFv antibodies. Two scFvs showed favorable characteristics when used as NK and T cell activating or co-stimulatory moieties in dual specific [CD20×NKG2D] bibodies. The novel bibodies enhanced cytotoxicity of NK and T cells, particularly in combination with mAbs or bispecific T cell engagers targeting the same tumor and effector cell type. This novel dual-dual targeting approach of two different cytotoxic signal mechanisms in NK and T cells in combination with targeting two different tumor antigens led to a higher maximum level of cytotoxicity. This maximum was not achieved with higher doses of the single agent. Bispecific antibodies as described in this study can potentially offer a broad application to potentiate immunotherapies employing mAbs or NK/T cell engagers which trigger cytotoxicity via FcγRIIIA or CD3, but especially studies in preclinical animal models are required to explore their full potential.

## Data availability statement

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

## Ethics statement

The studies involving humans were approved by Ethics committee of the medical faculty of the Christian Albrechts University of Kiel; Haus U 27 Schwanenweg 20; D - 24105 Kiel. The studies were conducted in accordance with the local legislation

and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

SL, A-MA, LE, HT, AL, DW, SK, KK, RS designed and performed the experiments and/or analyzed data and discussed the data. AF, MH, TS, SD generated and provided essential reagents, contributed to the design of the study and contributed to writing the manuscript. CK, SL, MP, MG, TS outlined the research project. All authors contributed to writing, editing and discussing the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

AF, MH, SD and TS are co-founders and shareholders of YUMAB GmbH. SL, CK, MG, MP and YUMAB GmbH submitted a patent application related to the described antibodies.

The remaining 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.

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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.2023.1227572/full#supplementary-material>

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## EDITED BY

Virginie Lafont,  
Institut National de la Santé et de la  
Recherche Médicale (INSERM), France

## REVIEWED BY

Azam Roohi,  
Tehran University of Medical Sciences, Iran  
Jian Yu,  
University of Southern California,  
United States

## \*CORRESPONDENCE

Ana Carolina Martínez-Torres  
✉ ana.martinezto@uanl.edu.mx  
Daniel Scott-Algara  
✉ daniel.scott-algara@pasteur.fr

<sup>†</sup>These authors share first authorship

<sup>‡</sup>These authors share senior authorship

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# Immunotherapies inducing immunogenic cell death in cancer: insight of the innate immune system

Kenny Misael Calvillo-Rodríguez<sup>1†</sup>,  
Helen Yarimet Lorenzo-Anota<sup>1,2†</sup>, Cristina Rodríguez-Padilla<sup>1</sup>,  
Ana Carolina Martínez-Torres<sup>1\*†</sup> and Daniel Scott-Algara<sup>3\*‡</sup>

<sup>1</sup>Laboratorio de Inmunología y Virología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, NL, Mexico, <sup>2</sup>The Institute for Obesity Research, Tecnológico de Monterrey, Monterrey, NL, Mexico, <sup>3</sup>Département d'Immunologie, Unité de Biologie Cellulaire des Lymphocytes, Pasteur Institute, Paris, France

Cancer immunotherapies include monoclonal antibodies, cytokines, oncolytic viruses, cellular therapies, and other biological and synthetic immunomodulators. These are traditionally studied for their effect on the immune system's role in eliminating cancer cells. However, some of these therapies have the unique ability to directly induce cytotoxicity in cancer cells by inducing immunogenic cell death (ICD). Unlike general immune stimulation, ICD triggers specific therapy-induced cell death pathways, based on the release of damage-associated molecular patterns (DAMPs) from dying tumour cells. These activate innate pattern recognition receptors (PRRs) and subsequent adaptive immune responses, offering the promise of sustained anticancer drug efficacy and durable antitumour immune memory. Exploring how onco-immunotherapies can trigger ICD, enhances our understanding of their mechanisms and potential for combination strategies. This review explores the complexities of these immunotherapeutic approaches that induce ICD, highlighting their implications for the innate immune system, addressing challenges in cancer treatment, and emphasising the pivotal role of ICD in contemporary cancer research.

## KEYWORDS

onco-immunotherapy, immunogenic cell death, innate immune system, monoclonal antibodies, cytokines, oncolytic virus, cellular therapies, immunomodulators

## 1 Introduction

Cancer immunotherapy aims to harness the patient's own immune system to target and eliminate malignant cells. In 2013, cancer immunotherapy was named as "Breakthrough of the Year" by the journal Science for its promising potential in the field of oncology (1). Immunotherapy is often combined with other cancer treatments such as



chemotherapy, surgery, radiotherapy, and targeted therapies, which aim to eliminate cancer cells by killing them. The combination of immune system activation with cancer cell-killing not only triggers the immune response but also precisely targets and eliminates cancer cells. This fusion demonstrates a markedly enhanced antitumor effect. In this way, cancer cells are eliminated by direct killing, while at the same time the immune system is activated.

Recently, ICD has been described as a promising form of therapy-induced anti-tumour immune system activation, and it is now considered to play a central role in various cancer treatment modalities. Although ICD targets cancer cells and mediates tumour-specific immune responses, it occurs in the precise context of cell death induced by a specific therapy, making it conceptually distinct from cases of immune stimulation or inflammatory responses that do not rely on a therapy capable of inducing a specific modality of cell death (2). Thus, although ICD could be considered a form of immunotherapy, it has not been classified as such because it depends on a specific treatment that must be able to induce a specific cell death pathway that ends with a dying cancer cell that has sufficient antigenicity and adjuvanticity able to activate the immune system. ICD is characterized by the exposure and release of DAMPs from dying tumour cells that confer adjuvanticity. DAMPs are recognised by innate PRRs, resulting in the activation of innate cells and the subsequent activation of adaptive cells that mediate tumour-specific immune responses. ICD is a promising strategy because it induces long-term efficacy of anticancer drugs through the combination of the direct cancer cell killing and the activation of the antitumour immune system, leading to an anti-tumour immunological memory (3).

Some chemotherapies, radiotherapies, and targeted therapies have been shown to induce immunogenic cell death (4–6). Such ICD inducers have been successfully combined with different types of immunotherapies to promote better outcomes (7, 8). However, some immunotherapies, in parallel with their immunomodulatory effect that targets the immune system, can also be directly cytotoxic to the cancer cell and induce ICD. This article will review these specific types of immunotherapies, with an additional focus on their impact on the innate immune system.

## 2 Immunotherapies for cancer treatment

Oncology met immunology in 1891 when William B. Coley noticed that cancer patients who got infections after surgery seemed to do better than those who didn't. So he tried immunotherapy for cancer by using erysipelas on a patient with inoperable sarcoma (9). He then created a filtered mixture of bacterial lysates called "Coley's Toxins" to treat tumours. His first patient, John Ficken, with a large inoperable tumour (probably a malignant sarcoma) had a complete remission that lasted until his death from a heart attack 26 years later. Coley's toxins may have stimulated the immune system to attack cancer cells. Thereafter, clinical interest in onco-immunotherapy waned, with research focusing on radiotherapy

and chemotherapy (10–12), until 2013, when immunotherapy of cancer was named "Breakthrough of the Year" by Science (1).

Cancer is characterised by a number of features including activation of oncogenes, inactivation of tumour suppressor genes, resistance to cell death, angiogenesis, maintenance of proliferative signalling, immune suppression and avoidance of immune destruction (13). Even during cancer immunosurveillance, the most immunoevasive or highly mutagenic cancer cells may acquire the ability to evade immunosurveillance and thus generate a clinically relevant tumour. In this sense, cancer cells in an established tumour can evade anti-tumour immunity. In addition to the immunosuppressive microenvironment within the tumour, cancer cells can use several mechanisms for immunoevasion, which include (1): reducing their immunogenicity through the downregulation of tumour-associated antigens (TAAs) and major histocompatibility complex (MHC) class I expression (2), inducing tolerance by suppressing T cells (CD4+ and CD8+) through the promotion of immunosuppressive cytokines (e.g. IL-10 or TGFβ) or immune-checkpoints (e.g. regulated cell death 1, regulated cell death-ligand, cytotoxic T lymphocyte-associated protein-4), and (3) avoiding the immune cell-mediated lysis by overriding cell death pathways (14, 15), among others.

Pharmacological induction of cell death is the basis of almost all non-invasive cancer therapies. One of the major challenges in cancer treatment is to restore an anti-tumour immune response. In this sense, immunotherapies have transformed cancer treatment in recent years and have revitalised the field of tumour immunology (16). Cancer immunotherapy aims to stimulate the immune system in a controlled manner to eliminate cancer cells and prevent uncontrolled autoimmune inflammatory responses that lead to contraindications and therapeutic limitations (17). The main goals are to increase the quality or quantity of immune cells (especially effector cells), to generate tumour antigens and to eliminate mechanisms associated with immunosuppression, while minimising off-target effects. In addition, immunotherapies seek to induce long-lasting and durable responses in several cancer subsets, including solid and haematological malignancies. In this sense, several types of cancer immunotherapies have been developed with the aim of promoting cancer remission (18). These onco-immunotherapies are very vast and include (1) monoclonal antibodies (2), cytokines (3), oncolytic viruses (4), cellular therapies (5), and other biological and synthetic immunomodulators.

Activation of the anti-tumour immune system requires treatment strategies that can overcome the physiological barriers that control immune responses against tumour cells. Accordingly, immunotherapy uses strategies that target specific immunoregulatory processes to enhance anti-tumour immunity. However, cancer immunoediting can occur in response to immunotherapy as well as during tumour development. In this sense, immunotherapy may induce secondary (acquired) resistance that manifests as a clinical response followed by cancer progression (secondary escape) (19).

In general, the resistance of most cancers to immunotherapies and the lack of anti-tumour memory underline the need to

overcome the immunosuppressive microenvironment, improve the immunogenicity of tumour cells and promote the induction of anti-tumour memory, rather than focusing only on stimulating broad and untargeted immune responses (20). In this sense, a novel strategy to induce immune system activation, antitumour memory and tumour microenvironment remodelling is the induction of a specific form of cell death called immunogenic cell death.

### 3 Immunogenic cell death in cancer

The immunogenicity of cancer cells has been identified as an essential factor in the development of anti-cancer therapies. Therefore, new research has focused on understanding the immunobiology of tumours in order to overcome the immunosuppressive function of the tumour microenvironment (TME) and increase the immunogenicity of cancer cells (21). In this sense, ICD is characterised by the increased immunogenicity of the cells (acting as a tumour vaccine) and the release of DAMPs, leading to the generation of immunological memory (21).

ICD is a type of cell death that can promote the antitumour immune response and induce immunological memory against endogenous (cellular) or exogenous (viral) antigens. The ability of ICD to stimulate adaptive immunity comprises two main parameters: antigenicity and adjuvanticity. Antigenicity is the ability of a molecule, such as a protein, to be recognised as an antigen and to promote an inflammatory response. This is provided by the production and presentation of antigens in the context of central tolerance in a given host that do not lead to clonal deletion, indicating that the host has naive T cell clones that can recognise such antigens. Adjuvanticity is mainly provided by the release or exposure of danger signals such as DAMPs due to cell damage or stress, and by pathogen-associated molecular patterns (PAMPs) in pathogen-derived ICD, which promote the recruitment and maturation of dendritic cells (DCs). These molecules have non-immunological effects within the cell, but, their exposure on the cell surface or their release into the extracellular space due to cellular stress allow their binding to receptors in immune cells (14, 21).

Cancer research has undergone a significant paradigm shift in recent years, with increasing emphasis on the importance of ICD in the context of cancer therapy. Both preclinical and clinical data have converged to support the notion that the way cancer cells undergo cell death in response to treatment carries is more important for long-term disease outcome than the proportion of cells that die. Given the challenge posed by the inability of current cancer therapies to achieve the utopian goal of eradicating 100% of cancer cells, there is a growing consensus among scientists for a strategic shift in focus. Rather than seeking cell death in isolation, the forefront of cancer research is now centred on the development of innovative combination therapeutic regimens designed to stimulate the antitumour immune system and induce cancer cell death (22–24).

Therefore, the use of immunotherapies that can both stimulate the immune system and induce ICD is of great interest because they can enhance the immune system's ability to fight cancer while

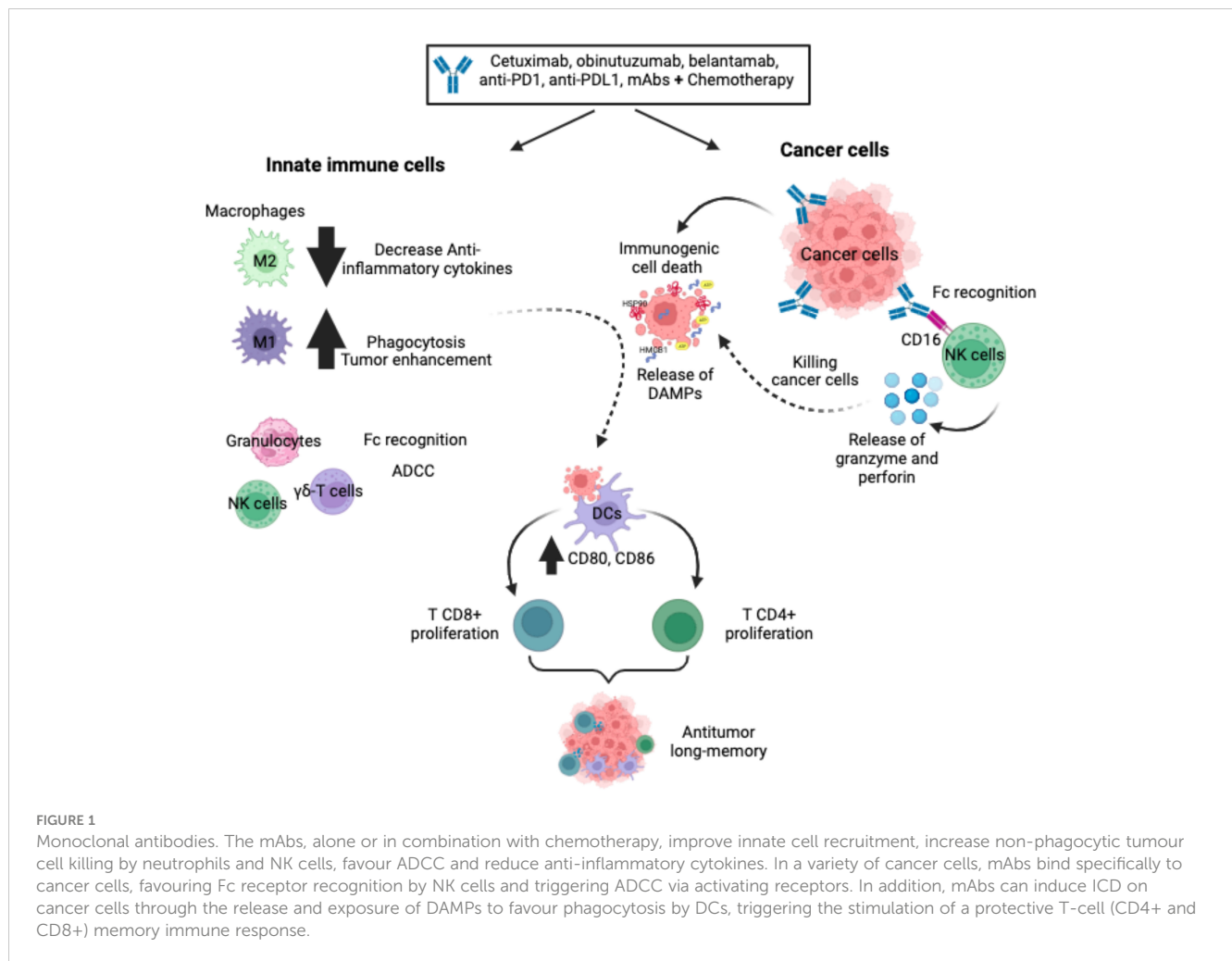
killing cancer cells. In the next section, we will focus on describing the role of the main onco-immunotherapies (monoclonal antibodies, cytokines, oncolytic viruses, cellular therapies, and other biological or synthetic immunomodulators) in immunogenic cell death induction and their role in modulating the innate immune system.

## 4 Dual action of immunotherapy: inducing immunogenic cell death and stimulating the innate immune response

### 4.1 Monoclonal antibodies

Monoclonal antibody (mAb)-based immunotherapies have recently emerged as one of the most important components of cancer therapy compared to surgery, radiation, and chemotherapy. Novel mAbs have been developed against neoantigens or overexpressed antigens in cancer cells that favour a variety of cell death mechanisms, including ICD (25, 26). The main clinically relevant mechanisms of action induced by mAbs on cancer cells are: antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), which involve the activation of innate cells such as natural killer (NK) cells, dendritic cells, and macrophages (27). As the aim of this review is to focus on immunotherapies that induce ICD, we will describe the principal mAbs that induce ICD and their role in innate immune responses (Figure 1).

Some types of monoclonal antibodies can induce ICD as monotherapy. For example, belantamab mafodotin is a humanised mAb that targets B-cell maturation antigen (BCMA) in multiple myeloma and other B-cell malignancies. The anti-BCMA is afucosylated and linked to the microtubule polymerization inhibitor, MMAF via a protease-resistant maleimidocaproyl linker. After binding to the cell surface, anti-BCMA is internalised, leading to cell-cycle arrest and apoptosis. This type of cell death triggers cell surface exposure of calreticulin (CRT), heat shock protein 70 and 90 (HSP70, HSP90), and the release of the high mobility group box protein 1 (HMGB1), adenosine triphosphate (ATP), HSP70, and HSP90, triggering activation and maturation of DCs. This leads to host innate and adaptive immune responses through tumour recruitment of cytotoxic T lymphocytes, NK cells and DCs (28). In addition, the afucosylation favours binding to FcγRIIIa receptors on the surface of immune effector cells, which promotes immune cell recruitment, activates ADCC and ADCP and generates long-term immune memory (28). Obinutuzumab (GA101), the second generation of rituximab (anti-CD20 mAb) induces ICD, which is characterised by the release of DAMPs, such as HSP90, HMGB1 and ATP, which induce DCs maturation (enhancing CD86 and CD83 expression) and subsequent T-cell proliferation (29). In addition, GA101 potentiates cellular immune responses by binding to NK cells, promoting their activation and triggering ADCC more effectively than rituximab (30). It is also able to activate γδ T-cells and



potentiate killing of lymphoma cells (31), and strongly engage monocytes and M1 macrophages, leading to high levels of nitric oxide and the elimination of CD20-expressing tumour cells (32).

Although some mAbs can induce ICD as a single treatment, most reports include their combination with other immunotherapies or chemotherapies to induce ICD on multiple cancer cells. For example, cetuximab has been shown to induce increased ICD in colorectal cancer when used in combination with leucovorin calcium (folinic acid), fluorouracil, and irinotecan hydrochloride (FOLFIRI). Cetuximab induced endoplasmic reticulum (ER) stress and CRT and ERp57 expression on the cell surface, favouring phagocytosis by DCs of dying cancer cells, which triggered the stimulation of a protective T-cell (CD8+) memory immune response observed alone and in combination with FOLFIRI (33). In addition to its direct ICD induction, cetuximab is able to repolarise tumour-associated macrophages (TAMs) from M2-like to M1-like phenotypes, mainly by suppressing IL-6 expression through NF $\kappa$ B and STAT3 pathways (34).

Among the various antibodies used in combination, anti-PD-1 and anti-PD-L1 are the most used. For example, dinaciclib, a CDK1, -2, 5 and -9 kinase inhibitor, is a bona fide ICD inducer that, when combined with anti-PD1 mAbs, enhances DCs activation and favours antitumour response in a variety of murine syngeneic

tumour models (35). Also, photodynamic therapy (PDT) enhances antitumour effects of the anti-PD-L1 mAb, inducing ICD in SCC7 cells by stimulating DCs maturation. In fact, the combination of PDT-DC vaccine and anti-PD-L1 mAb synergistically triggered an antitumour immune response and inhibited tumour progression (36). The PD-L1 mAb in combination with doxorubicin improved the immunosuppressive tumour microenvironment and promoted NK and T cell activation and proliferation. It also increased infiltrating CD8+ T cells through the secretion of CRT and HMGB1, and promoted tumour necrosis factor alpha (TNF- $\alpha$ ) and interferon gamma (IFN- $\gamma$ ) production in tumour tissue in a hepatocarcinoma model (37). In addition to these effects, anti-PD-1 and anti-PD-L1 therapies increase the levels of M1-like macrophages markers and promote macrophage polarization towards the pro-inflammatory phenotype (38–40), improve NK cell anti-tumour efficacy and promote NK cell persistence and retention of their cytotoxic phenotype (41, 42). The principal antibodies that were related to ICD induction are summarized in Table 1.

Monoclonal antibodies can also activate cells of the innate immune system, such as NK cells, and trigger ADCC via NK cell-activating receptors, such as CD16. Even polymorphonuclear granulocytes such as eosinophils, neutrophils, macrophages, and

TABLE 1 Monoclonal antibodies related to ICD induction.

mAbs	Cancer model	DAMPs	Cell death modality/ characteristic	Key Result	Ref
Cetuximab alone or in combination with FOLFIRI	Panel of BRAF WT colorectal cancer cell lines	CRT and Erp57 exposure to the cell surface	Apoptosis through ER stress	Induces phagocytosis of tumour dying cells by DCs and the induction of a protective CD8+ T cell memory immune response.	(33)
Obinituzumab	Human lymphoma cell lines (Raji, Daudi and SU-DHL4)	Release of HSP90, HSP60, HMGB1 and ATP	Non-apoptotic programmed cell death	Induces DCs maturation (enhancing CD86 and CD83 expression) and subsequent T-cell proliferation.	(29)
Belantamab	Multiple Myeloma (NCI-H929 cells)	Exposure and release of CRT, HSP70, HSP90, HMGB1 and release of ATP	Apoptosis	Induces cell-cycle arrest and apoptosis and promotes the recruitment of immune cells leading to ADCC and ADCP.	(28)
The PD-L1 mAb in combination with doxorubicin	Mouse hepatocarcinoma cell lines Hepa1-6 and H22	Release of CRT and HMGB1, TNF- $\alpha$ and IFN- $\gamma$ production in tumour tissues	Apoptosis through cell cycle arrest	Improves tumour immunosuppressive microenvironment and promotes the activation and proliferation of NK and T cells. Also, increased CD8+ T cells infiltration	(37)
Anti-PD1 mAb combined with Dinaciclib	Mouse colon adenocarcinoma in MC38 cell line	Release of CRT, HMGB1 and ATP	Not described	Enhances DCs activation and antitumour activity in several murine syngeneic tumour models.	(35)
Anti-PD-L1 mAb combined with Photodynamic therapy	Squamous cell carcinoma SCC7 cells	Release of CRT, HMGB1 and ATP	Not described	Stimulates DCs maturation, induces antitumour immunity, and suppresses tumour progression.	(36)

monocytes can engage in Fc-mediated effector functions against antibody-opsonized tumour cells through multiple mechanisms (43, 44).

Several antibody therapeutics approved by the Food and Drug Administration (FDA) for various haematological and solid cancers have been reported (45), and have shown to activate innate immune cells. Among these, Rituximab was the first FDA approved mAb against B-cell lymphomas; its therapeutic activity in combination with chemotherapy improves innate cell recruitment (46). Human anti-CD47 antibodies enhance nonphagocytic tumour cell killing by neutrophils and NK cells in an acute myeloid leukaemia (47). Enavatuzumab is a humanized IgG1 antibody that exerts potent ADCC on TweakR positive tumour cells by monocytes and NK cells *in vitro* (48). Monalizumab, a humanized anti-NKG2A antibody, increased NK cell activity against cancer cells and established CD8+ T cell function in BALB/c mice bearing B cell lymphoma A20 cells *in vivo* (49).

The potential of mAbs to induce innate antitumour immune responses suggests that it may only be a matter of time before we fully exploit their capabilities to orchestrate a collaborative effort between innate and adaptive immune responses against cancer, ultimately generating long-term antitumour memory (Figure 1).

## 4.2 Cytokines

Cytokines are molecules that play a central role in cellular autocrine or paracrine signals that are released or produced in response to various stimuli, leading to differentiation, proliferation,

activation, cell death and other effects. Cytokines also regulate innate and acquired immune responses such as pro- and anti-tumour effects. Thus, cytokine-based immunotherapies are a promising therapeutic approach that can be used to promote, enhance, maintain or regulate the establishment of an anti-tumour immune system (50).

In general, it has been reported that IL-2, IL-7, IL-12, IL-15, IL-18, and IL-21 induce the expansion and enhance the cytotoxicity of NK, NKT and T lymphocytes, whereas granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) promote the expansion and activation of DCs, and most of them have recently been evaluated in various clinical trials (50, 51). However, most of the cytotoxic or antitumour evaluations of cytokines are in combination with other agents and the direct cytotoxic effect of cytokines in tumour cells is poorly evaluated.

In this sense, the combination of TNF- $\alpha$  and secondary mitochondria-derived activator of caspases (SMAC) mimetics has been reported to induce immunogenic cell death in fibrosarcoma, melanoma, liposarcoma, synovial sarcoma and patient-derived cutaneous squamous cell carcinoma. *In vivo*, the combination of TNF- $\alpha$ , SMAC mimetics and melphalan induced tumour shrinkage, promoted the activation of CD8+ T cells as well as NK cells and prolonged survival in a rat model of liposarcoma (52).

On the other hand, a cytokine-triggered inflammatory cell death pathway involving crosstalk between the machinery of pyroptosis, apoptosis and necroptosis cell death, termed PANoptosis, has recently been reported. Subbarao et al. showed that a cocktail of pro-inflammatory cytokines including TNF- $\alpha$ ,



IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-18, IL-6, IL-8, and IL-15 induced cell death in NCI-60 colon cancer cells, while individual treatments or a cocktail lacking TNF- $\alpha$  and IFN- $\gamma$  did not induce cell death, suggesting a synergistic effect of these cytokines signalling to induce cell death. The unique combination of TNF- $\alpha$  and IFN- $\gamma$  induced PANoptosis in different cancer cell types such as colon, melanoma, lung cancer and leukemic cell lines, highlighting the robust cell death induction by TNF- $\alpha$  and IFN- $\gamma$  in a wide range of cancer cells. Interestingly, the intratumorally administration of a combination of TNF- $\alpha$  and IFN- $\gamma$  suppresses the tumour growth in a human colon cancer model (53), while independent treatment does not induce these effects. However, although pyroptosis and necroptosis are associated with immunogenicity, immunogenic cell death has not been assessed, but these reports shed light on the possibility that these combinations could lead to ICD.

### 4.3 Oncolytic viruses

Oncolytic viruses (OVs) are a novel immunotherapy strategy using competent or genetically modified oncolytic viruses that selectively infect, replicate, and induce cell death in tumour cells. OVs have a unique mechanism of action, combining direct tumour cell death, tumour-specific immune response, and antiviral immune system activation. OVs induce oncolysis through the production of viral particles that spread to surrounding tumour cells and promote immune system activation through the release of PAMPs, DAMPs, viral particles and neoantigens (54, 55) (Figure 2). OVs have been reported as ICD inducers and, depending on the type of virus

(adenovirus, herpes simplex, semliki forest virus, vaccinia virus, reovirus, among others), they can induce cell death by different mechanisms. These cell death mechanisms include apoptosis, necroptosis, pyroptosis and autophagic cell death, but in general they all induce the exposure and release of DAMPs and PAMPs (54, 56).

Currently, a diverse array of OVs has undergone extensive evaluation of their ability to induce ICD across a wide spectrum of tumour models. Notable examples include adenovirus OBP-702 in pancreatic cancer (57), adenovirus dl922-947 in mesothelioma (58), talimogene laherparepvec, and measles virus in melanoma (59), adenovirus serotype 5, semliki forest virus, and vaccinia virus in osteosarcoma (60), reovirus type 3 Dearing strain in lymphoma and prostate cancer (61), adenoviruses, Ad884 and Ad881 in colon cancer (62), oncolytic Newcastle disease virus (NDV) in human lung cancer (63), while herpes virus H-1PV, RH2, and VC2 in pancreas, squamous carcinoma, and melanoma cancer cells (64–66) (Table 2).

These viruses have demonstrated their capacity to trigger the release of critical DAMPs, such as ATP, HMGB1, calreticulin, HSP70, and HSP90 from dying cancer cells. This release enhances the phagocytosis and maturation of DCs, facilitating the infiltration of cytotoxic T cells, and bolstering the NK cell's antitumor activity, particularly notable in the case of the measles virus, reovirus type 3 Dearing strain, and HSV-P10 (59, 61, 70). This immunogenic response leads to the secretion of pro-inflammatory cytokines such as IFN- $\gamma$ , TNF- $\alpha$ . Table 2 offers a comprehensive compilation of various research studies delving into the utilization of oncolytic viruses as potent inducers of immunogenic cell death.

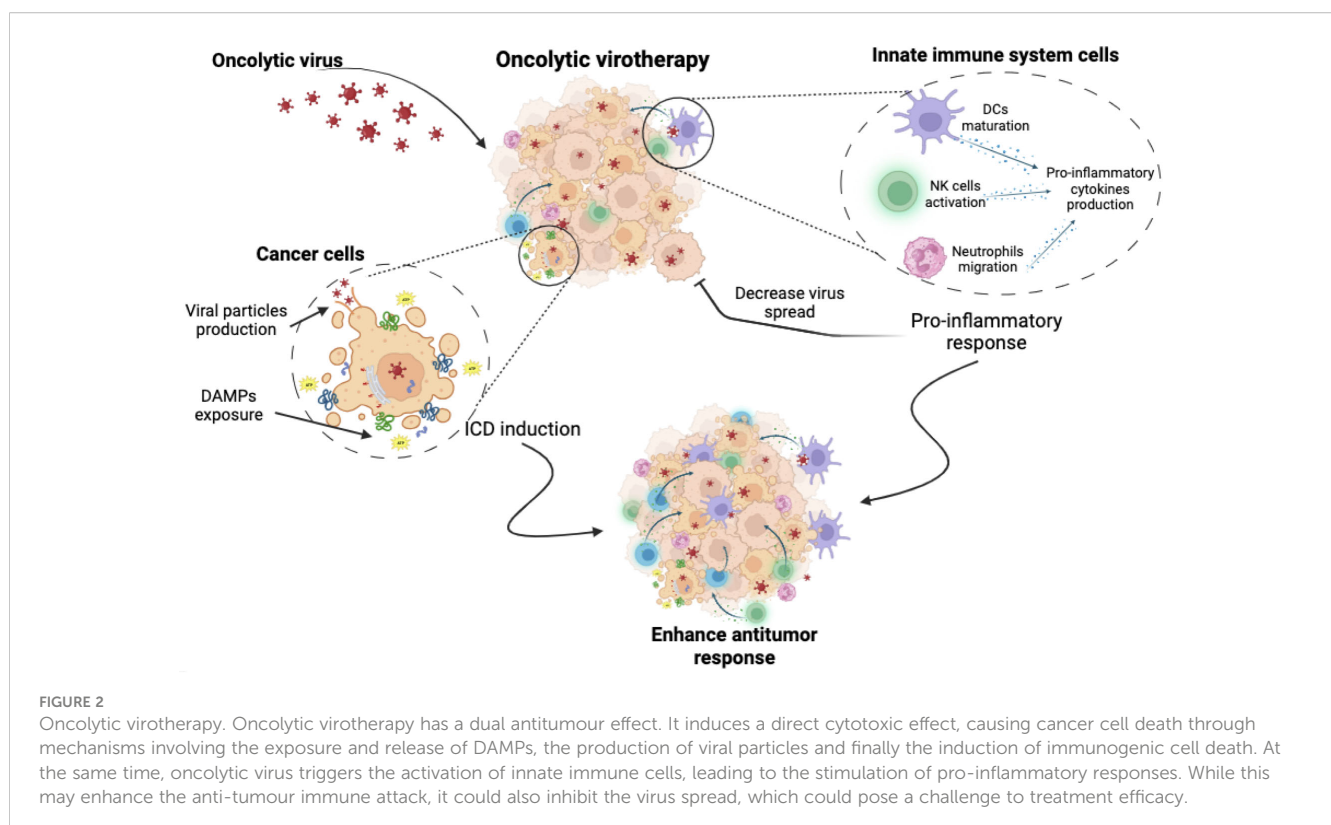


TABLE 2 Oncolytic virus eliciting immunogenic cell death.

Virus	Cancer model	DAMPs	Cell death modality/characteristic	Antitumour effect and immune system involvement	Ref
Adenovirus OBP-702	Human Pancreatic ductal adenocarcinoma cells (PDAC) with different p53 status (Capan-2, PK-59, PK-45H, Capan-1, MIA PaCa-2, BxPC-3) and murine PDAC cells (PAN02)	ATP and HMGB1	Increased the expression of p53, cleaved PARP, decreased the expression of p62	Tumour infiltration of CD8+ T cells and CD11c dendritic cells	(57)
dl922-947	Malignant mesothelioma cell lines MSTO-211H and NCI-H28	ATP, HMGB1 and Calreticulin	Necroptosis	Inhibits tumour growth and reduces the tumour micro-vessel density (TMD)	(58)
Talimogene laherparepvec (T-VEC)	Human melanoma cell line SK-MEL-28	ATP, HMGB1 and Calreticulin	Cleaved caspase-3 and PARP	Increased of CD3+ and CD8+ T cells, and induces a systemic pro-inflammatory gene signature	(67)
Wild-type human Adenovirus serotype 5 (Ad)	Human bone osteosarcoma cell line HOS and human lung carcinoma cell line A549	ATP, HMGB1, Calreticulin and HSP90	RIP3 and MLKL activation, Inflammasome assembly and mature IL-1 $\beta$ , autophagosome formation	Increased DCs phagocytosis and maturation, activation of antigen specific T cells	(60)
Semliki Forest virus (SFV) strain4	Human bone osteosarcoma cell line HOS and human lung carcinoma cell line A549	ATP, HMGB1, Calreticulin and HSP90	Cleaved caspase-3/7 and caspase-8, autophagosome formation	Increased DCs phagocytosis and maturation, activation of antigen specific T cells	(60)
Vaccinia virus (VV) Western Reserve strain	Human bone osteosarcoma cell line HOS and human lung carcinoma cell line A549	ATP, HMGB1, Calreticulin and HSP90	Activation of MLKL	Increased DCs phagocytosis and maturation	(60)
Measles virus	Human melanoma cell lines Mel888, Mel624, SkMel28 and MeWo	Not determined	Not determined	Increased the activation marker CD69 and degranulation marker CD107a in NK cells. Promoted DCs maturation and T CD8+ cells priming.	(59)
Reovirus type 3 Dearing strain	B cell lymphoma (Daudi) and bladder (EJ) tumour cell lines	Not determined	Not determined	Promoted DCs maturation and proliferation of T cells and enhance NK cells anti-tumour-cytotoxicity	(61, 68)
	Prostate cancer-derived cell lines PC-3, DU145 (human), and TRAMP-C2 (Murine)	ATP, HMGB1 and Calreticulin	Not determined	Promoted the survival of TRAMP-C2-bearing C57BL/6 mice, and increased the CD4+ expressing IFN- $\gamma$ cells and promotes antitumour memory	
Reovirus type 3 Dearing strain-mutant jin-3	Human prostate cancer cell lines PC-3M-Pro4luc2, DU145, and 22Rv1	Not determined	Cleaved caspase-3	Decreased tumour burden and tumor volume. Increased the expression of the inflammatory cytokines CXCL10, TNF- $\alpha$ , and IL-1 $\beta$ .	(69)
HSV-P10	Murine breast cancer cell lines DB7, Met-1, and MVT-1 and human MDA-MB-231, SK-BR-3, MCF-7, and MDA-MB-468	Not determined	Not determined	Increased mice survival and induced antitumour immune memory of mice bearing breast cancer brain metastases. Induced intratumoral infiltration of macrophage, DCs, NK and CD8+ cells.	(70)

In addition to their primary role in killing cancer cells, OV also serve as potent activators of the innate immune system (71). This is because OV are pathogens specifically designed to infect and destroy cancer cells. When OV infect tumour cells, they elicit an inflammatory response, leading to the localized production of cytokines and chemokines that promote the stimulation of the innate immune response through different mechanisms. These mechanisms include the activation and recruitment of neutrophils

(72), macrophages, and NK cells (71). Furthermore, OV can initiate the activation of PRR in innate cells, triggering the activating receptors such as toll like receptors (TLRs).

Thus ICD-induction by OV plus the antiviral immune response activated by OV, promote an immune-stimulatory environment, leading to the uptake of TAAs and neo-antigens by PRR stimulated antigen presenting cells (APCs). Altogether, these events result in a dual immune system activation, on the one hand

the generation of immune responses against virally infected cancer cells, and in the other hand immune responses against TAAs and neo-antigens of un-infected cancer cells. Thus, the ‘indirect’ effects of the antiviral immune response within the tumour site, including the release of pro-inflammatory cytokines and the cytotoxicity of infected tumour cells, can reverse the immunosuppressive TME. This in turn may enhance ICD-related properties, including stimulation of innate and adaptive immune cells, release of pro-inflammatory cytokines, and recruitment of immune cells into tumours (63, 73). The anti-viral immunity triggered against viral antigens from the resultant infection is also a key player during OV-based therapies, as tumour cell infection promotes the antiviral immune response, which can be seen as a negative response triggered against OVs, but it helps to settle an inflammatory site that turns “cold” tumours “warm” (74).

However if unbalanced, this immune response could induce premature clearance of OVs and compromise their antitumour efficacy (75), as it has been observed in herpes simplex virus (oHSV) therapy, where activated NK cells reduce the anti-tumour efficacy of HSV in glioblastoma cells (76). In this regard, the combination of transforming growth factor beta (TGF $\beta$ ) and oHSV therapy inhibits NK cell recruitment and function, resulting in enhanced viral replication in glioblastoma mouse models (77). In addition, the downregulation of the NK cell-activating ligand CD155 inhibited NK cell recruitment *in vitro*, enhancing viral replication in a rat model of hepatocellular carcinoma (78). IFN- $\gamma$  and TNF- $\alpha$  secretion also induce virotherapy resistance in different animal models (79, 80), being IFN- $\gamma$  signalling modulators/inhibitors a strategy used to improve the success of OV treatment (81).

In summary, oncolytic viruses exert a wide range of direct and indirect antitumour effects, including tumour oncolysis, induction of tumour-specific immune responses and activation of the antiviral immune system. These effects culminate in the effective generation of neoantigens and the release of DAMPs and PAMPs, which in turn promote the recruitment of neutrophils, granulocytes, NK cells and APCs to the site of viral replication (56, 72, 81). This in turn enhances the activation of the T- and B-cell-mediated adaptive immune response (56). Finally, these combined effects can potentiate the anti-tumour immune response, which is further enhanced by the induction of ICD, ultimately leading to the establishment of anti-tumour memory.

## 4.4 Cellular immunotherapies

Cellular immunotherapy, or adoptive cell therapy, is a form of treatment that involves the infusion of live cells into a patient’s body to eliminate cancer. Some of these approaches involve the direct isolation and subsequent expansion of immune cells (T cell-based, NK cell-based, macrophage cell-based, and DC-based), while others use genetic engineering techniques, such as gene therapy, to enhance their cancer-fighting potential (such as chimeric antigen receptor (CAR)-based immunotherapies). CAR-based immunotherapies involve the genetic modification of the cells to

confer tumour specificity and include CAR T cells, which comprise the innate T cell subsets such as the  $\gamma\delta$  T cells ( $\gamma\delta$ CAR T cells) and natural killer T (CAR NKT), CAR NK, and CAR macrophages, which together enhance the potential of cellular immunotherapies (82, 83). In this section, we will focus on the role of cellular immunotherapies in ICD and the activation of innate immune cells.

### 4.4.1 CAR-based immunotherapies

#### 4.4.1.1 CAR T-cell therapies

CAR T-cell therapy is a highly promising and rapidly advancing treatment approach primarily for haematologic malignancies (84, 85). CAR T cells eliminate cancer cells by binding to target cell surface antigens without the need for MHC restriction. Many articles have reviewed broadly their use, limitations and potential strategies (86–89), while in this review we will principally approach their possibility as ICD inducers, and CAR innate T cell subsets.

Although T cells predominate in CAR-based immunotherapies, innate T cell subsets can also be used for CAR redirection, such as  $\gamma\delta$ CAR T cells and CAR NKT cells. It has been proposed that CARs in innate cells may be preferable than CAR T cells, because of a reduced cytokine release storms (CRS) and graft versus host disease (GvHD). CAR NKT cells simultaneously express the invariant TCR in addition to the CAR, thereby preserving their responsiveness to glycolipid antigens (90). CAR NKT cells in addition to presenting effects on liquid tumours like B-cell lymphoma and multiple myeloma, through CD19, CD38/BMCA, CEA, or HER2 targeting (91, 92), they can also target solid tumours like GD2 in neuroblastoma (93), CSPG4 in melanoma (94) due to their unique capabilities, like high infiltration into TME (91). On the other hand,  $\gamma\delta$ CAR T cells can respond to CD19-positive and -negative tumour cells, suggesting that CD19-directed  $\gamma\delta$ CAR T cells can target leukemic cells even after antigen loss (95). In addition to CD19 targeting,  $\gamma\delta$ CAR T cells have also shown interesting results when targeting glypican-3 in hepatocellular carcinoma (96). In addition,  $\gamma\delta$ CAR T cells produce less IFN- $\gamma$  and other inflammatory cytokines when compared to conventional  $\alpha\beta$  CAR T-cells, which may result in a lower risk of CRS (97).

CAR T cells mediate their anti-tumour effects using similar mechanisms as native T cells, such as granular exocytosis and expression of death ligands (98). These mechanisms lead, in native T cells, to different cell death modalities that include apoptosis, necroptosis, pyroptosis, and ferroptosis (99), and can lead to immunogenic cell death in target cancer cells (100, 101). T lymphocytes promote calreticulin exposure, HMGB1 and IL-1 $\beta$  release, leading to DC uptake and cross presentation, providing a mechanism for amplification and self-perpetuation of the immune response against cancer neoantigens (100, 101). They also release cytokines that sensitise the tumour stroma and promote inflammatory signalling, such as IFN- $\gamma$  which has also the ability to sensitize to cell death and directly trigger cell death alone or combined with other inflammatory molecules, such as TNF- $\alpha$  (99). Although there are no reports specifically describing whether CAR T,  $\gamma\delta$ CAR T or CAR NKT therapies can induce ICD on cancer cells, it is likely that this could occur, as they share common mechanisms of cytotoxicity.

#### 4.4.1.2 CAR NK Cells

Natural killer cells are a vital component of the innate immune system, serving as the frontline defence against infected, transformed, and stressed cells. NK cell activation is initiated by stimulation of an activating receptor, often NK46 encoded by NCR1 (102, 103). Upon activation, NK cells release cytotoxic granules that contain perforin and granzymes to directly lyse cells (104, 105), or regulate the adaptive immune responses by releasing chemokines and cytokines such as IFN- $\gamma$  and TNF- $\alpha$ . Importantly, NK cells are critical for tumour immunosurveillance, as increased cancer susceptibility and metastasis have been reported in mouse models and clinical trials with low NK activity (106, 107). Due to their inherent ability to target and destroy cancer cells, NK cell-based immunotherapies have been investigated for cancer treatment for decades, including therapies such as CAR NK cell therapy.

A wide range of tumour antigens have been targeted by CAR NK cells in pre-clinical studies for haematological malignancies and solid tumours (108). As for CAR T therapies CD19 is the most common target in CAR NK cells in both preclinical and clinical studies. Also, molecules such as CD20 and Flt3, have been developed as specific targets of CAR-NK against B-cell tumours (109), while CD38, CD138, B-cell maturation antigen, and signalling lymphocytic activation molecule family member 7 have been developed against acute myeloid leukaemia, and CD3, CD5 and CD7 for the cases of T-cell malignancies (110–113). Interestingly, in another therapeutic approach, CAR NK cells seek to eliminate myeloid-derived suppressor cells (MDSCs) (114) and M2TAMs (115) to reverse the immunosuppressive tumour microenvironment.

While detailed analyses of cell death induced by CAR NK cells have not been conducted, it is plausible that they share similar mechanisms with conventional NK cells, which execute cellular cytotoxicity through granule exocytosis and death ligands. NK cells possess the versatile ability to activate diverse cell death pathways, including apoptosis, necroptosis, and pyroptosis, and they are also capable of mediating immunogenic cell death by enhancing dendritic cell uptake of dying cells and facilitating antigen cross-presentation, ultimately leading to the development of immunogenic memory (101, 116). Similar to CAR T cells, there are no reports describing whether CAR NK therapies can induce ICD on cancer cells, but it is likely that this mechanism could also be applicable.

#### 4.4.1.3 CAR macrophages

CAR macrophages (CAR M) are widely recognised as a potential treatment for solid tumours due to their prominent functions in immune regulation and their ability to infiltrate solid tumours. They are currently under clinical investigation as they retain phagocytic and M1 functions while migrating to both primary and metastatic tumours (117). Tumour antigen-specific CARs show significantly enhanced cytotoxicity against tumour antigen-expressing cells and have the potential to remodel the tumour microenvironment (118). CAR constructs used in CAR M cells principally include CD19 in non-solid tumours (119), HER

2 in breast (120) and ovarian cancer cells (121), and mesothelin in ovarian cancer cells (121).

The molecular mechanisms underlying the anti-tumour activity of macrophages are not fully understood. It has been established that macrophages have the ability to eliminate cancer cells through multiple mechanisms, including (1) indirect killing by recruiting cancer cell-killing immune cells such as innate (NK) and adaptive (T) cells (2), cytotoxicity through antibody (Ab)-dependent cellular cytotoxicity, and (3) direct cancer cell killing by releasing oxygen radicals such as nitric oxide, reactive oxygen species, IL-1 $\beta$ , and TNF- $\alpha$  (122). Although nitric oxide (NO), reactive oxygen species (ROS), IL-1 $\beta$  and TNF- $\alpha$  mediated cell death has been studied in cancer cells and may be associated with ICD induction, this cell death mechanism has not yet been elucidated in macrophage mediated cell death.

#### 4.4.2 Dendritic cells

Dendritic cells are the major APCs that form the link between the innate and adaptive immune systems. These cells efficiently process and present antigens via histocompatibility complex I and II molecules to both innate and adaptive immune cells, thereby triggering the activation of both cellular and humoral immune responses (123).

In addition, DCs play a central role in the activation of the antitumour response during immunogenic cell death. Following the induction of cancer DAMPs exposure or release by various treatments (anthracyclines, oncolytic viruses, anticancer peptides, among others), DCs can be stimulated by different pathways (Table 3).

Due to the immunostimulatory effects of DCs, and their crucial role in the presentation of TAAs, DCs are an excellent means of enhancing the body's natural anti-tumour responses. Therefore, DC-based immunotherapy focuses on harnessing the potential of

TABLE 3 The impact of DAMPs in Dendritic cells.

DAMPs	Cell receptor	Effect in DCs	Ref
ATP	P2X7, P2Y2	Intracellular Ca <sup>2+</sup> increase, actin rearrangement, chemotaxis, migration, activation of NLRP3 inflammasome and release of IL-1 $\beta$	(124–126)
Calreticulin	CD91	Promotes the phagocytic activity and the release of pro-inflammatory cytokines	(125–128)
HSP70 and HSP90	CD91, TLR4	Promotes the antigen cross presentation, and enhances the processing and presentation of antigens	(129, 130)
HMGB1	TLR2, TLR4 and RAGE	Stimulates the generation of pro-inflammatory cytokines while simultaneously aiding in effective antigen presentation and promotes cross presentation	(131–133)



DCs to effectively present tumour antigens and induce targeted anti-tumour immune responses.

In DC-based immunotherapy, the source of the DCs (peripheral blood monocytes, haematopoietic precursors, peripheral blood enriched DCs, etc.) and the stimulation with the antigen are crucial steps for the efficacy of the therapy. In addition, different sources of TAAs such as: whole tumour lysates, synthetic peptides, purified tumour antigens, genetically engineered DCs, among others, and different antigen-loading methods are used for stimulation (134). Interestingly, a very important source of TAAs for DC vaccines are cancer cells killed by ICD inducers or strategies. In particular, whole tumour vaccines are crucial for the stimulation of long-term anti-tumour immune responses by DC vaccines, as they serve as a potential reservoir of tumour antigens and could lead to enhanced anti-tumour T-cell responses (135). DCs vaccines loaded with doxorubicin-treated tumour cells are effective in a prophylactic application by reducing tumour development in neuroblastoma (NXS2) and melanoma (B16F10) cell-bearing mice in a prophylactic and therapeutic setting, respectively (136, 137). Also, a DCs vaccine loaded with shikonin (an ICD inducer) treated melanoma cells significantly promoted tumour reduction and improved survival of mice in a therapeutic application (137). Additionally, DCs stimulated with shikonin-treated breast cancer (4T1) cells suppressed metastasis and increased survival of breast cancer cells in an orthotopic tumour resection model (138).

On the other hand, although ICD was initially conceived as a form of chemotherapy-induced tumour cell death, physical anticancer approaches (radiotherapy, photodynamic therapy, among others) have demonstrated the capacity to generate an immune response that can be exploited in DC-based vaccine strategies (125). In this sense, it has been reported that DCs vaccines stimulated with killed squamous cell carcinoma cells by PDT promote tumour reduction and increase the survival of mice showing a better response than the application of tumour cell lysate (139), indicating that the use of DCs enhance the antitumour response. In addition, PDT-based DCs vaccine inhibits the growth of mesothelioma tumours and increases the survival of mice (140).

Furthermore, immunotherapy with DCs is currently being used in combination with ICD inducers. Mice treated with DCs and doxorubicin show an increase in CD8<sup>+</sup> T lymphocytes within metastatic tumours and inhibition of metastatic growth (141). Similarly, an increase in serum IL-2, IL-12 and IFN- $\gamma$ , as well as the proportion of IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells, was observed in a randomized trial of oesophageal cancer patients treated with DCs vaccine and radiotherapy (142).

Finally, despite the diverse reports of DCs vaccine effects, most of the DCs activities in immunotherapy focus on the activation of the adaptive immune system. However, there are reports on the effect of DCs in different cells of the innate immune system. DCs activate and potentiate the cytotoxic activity of NK cells via IL-12, IL15 and IFN- $\gamma$  (143). In addition, DCs can activate NKT cells through the expression of invariant CD1 molecules and the presentation of glycolipids (144). Thus, DCs play a critical role in the activation of both innate and adaptive immune responses. However, the activation of innate immune cells by DCs is poorly

understood and further evaluation in the context of ICD induction is needed to expand the knowledge of the effect of DCs and to propose more efficient combinatorial treatments.

## 4.5 Other immunomodulators

### 4.5.1 Biological immunomodulators

Biological immunomodulators, also called biological therapies are a subset of immunotherapies obtained from biological entities, such as bacillus Calmette-Guérin (BCG), an attenuated *Mycobacterium bovis* derivative, and dialyzable leukocyte extracts, obtained from immune cells, among others. They are used in several diseases such as autoimmune diseases, viral and bacterial infections and recently in cancer (145, 146).

Bacille-Calmette-Guerin (BCG) is a live attenuated tuberculosis vaccine that is widely used in neonates to induce long-term immunity against pathogens such as *Mycobacterium tuberculosis*, *Candida albicans* and *Staphylococcus aureus* (147). Few reports have described its involvement in the cancer immune response. In this sense, it has been reported that it induces caspase-independent cell death with the release of HMGB1 into the extracellular space in a dose-dependent manner in urothelial carcinoma (UC) T24 and 253J cell lines. The authors also found urinary levels of HMGB1 in patients diagnosed with UC at 24 hours after BCG therapy (148). In other hand, BCG vaccination triggers innate immune training in several types of immune cells, including monocytes, neutrophils, NK cells and dendritic cells. This training occurs through the interaction of various PRRs with PAMPs present in the bacterial cell wall (149). Immediately, innate cells respond by secreting pro-inflammatory cytokines, including IL-6 IL-1 $\beta$ , TNF- $\alpha$ , monocyte chemoattractant protein-1 (MCP-1), and IL-8 (150). Consequently, cellular infiltration of T cells (CD3<sup>+</sup>), monocytes (CD14<sup>+</sup>), but predominantly CD15<sup>+</sup> neutrophils occur at the vaccination site (151). *In vitro* studies show that human blood neutrophils obtained from BCG vaccination sites cooperate with dendritic cells to enhance antigen-specific T-cell responses (152). Indeed, BCG enhances innate immunity in the context of pathogen protection, however the implication of BCG vaccination and anti-tumour immunity is not yet described (148).

Other types of biological immunomodulators are animal extracts derived from the immune system. This group includes substances produced by immune system cells, also known as dialysable leukocyte extracts (DLE). DLE are a diverse mixture of low-molecular weight compounds derived from blood or lymphoid tissue with immunomodulatory properties (153). Several reports have shown that DLE derived from human blood or lymphoid tissue from different animals (crocodile, porcine or bovine) can regulate numerous molecular targets, thereby facilitating immunomodulatory effects in conditions such as autoimmune diseases, immunodeficiencies, asthma, bacterial infections and certain types of cancer (153–156). The bioactive peptides contained in DLE, irrespective of their source species, have displayed analogous effects on both mouse and human leukocytes, involving the activation of comparable signalling pathways associated with their immunomodulatory properties (155, 157).

Recently, DLE have been shown to induce cytotoxicity in several cancer cell lines (156, 158–161).

Immunopotent CRP (I-CRP) is a DLE obtained from bovine spleen (bDLE), which has a wide range of applications in humans. Several studies have shown that it can modulate human and murine immune cells, while inducing cytotoxicity against human and murine tumour cell lines. In particular, its cytotoxic effect has been demonstrated in lung cancer (161), breast cancer (156, 159, 160), murine lymphoma (146), cervical cancer (160, 162) and leukemic cell lines (163). Currently, in a murine melanoma model, I-CRP has been shown to increase the release of DAMPs and the immunogenicity in combination with oxaliplatin (164). It also induces ICD in a murine breast cancer model, involving the DCs maturation in lymph nodes and the increase of CD8<sup>+</sup> T cells in lymph nodes, peripheral blood and tumour site, favouring long-term memory (156). On the other hand, in human PBMC, ICRP increased the CD56Dim CD16<sup>+</sup> subset and modulated NKp30, NKp44, NKp46, NKG2D, NKG2C and KIR receptors, whereas there were no significant differences in CD160, CD85j and CD226 in human NK cells. These alterations revealed increased antitumour cytotoxic activity due to changes in the receptor repertoire of NK cells (155) (Figure 3).

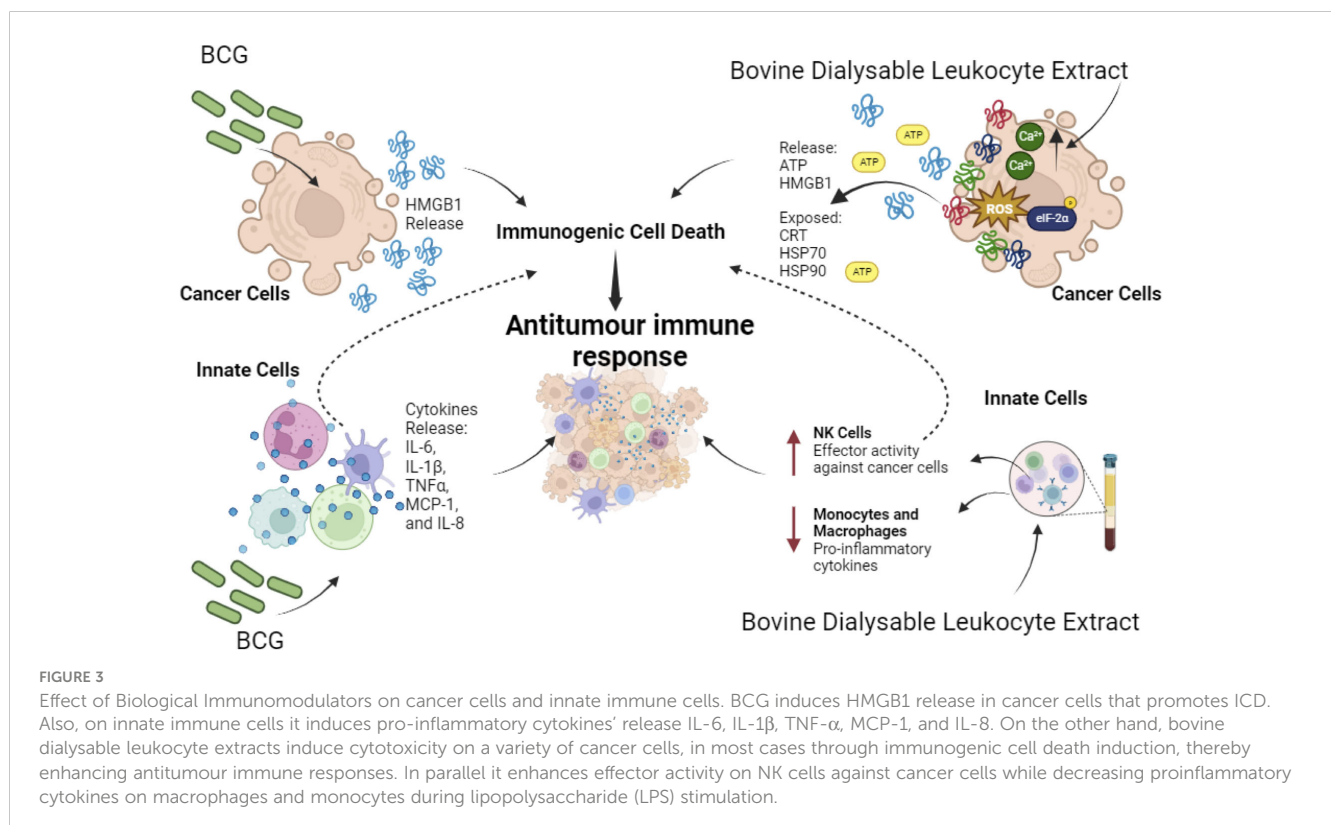
Another type of DLE, derived from human blood cells (Transferon), suppressed tumour growth and promoted the differentiation of haematopoietic stem/progenitor cells into CD56<sup>+</sup>CD16<sup>+</sup>CD11c<sup>+</sup> NK-like cells capable of eliminating tumour cells and stimulating the proliferation of  $\gamma\delta$  T lymphocytes (165). Besides, it decreased metastatic dissemination of intracardiac prostate epithelial cells and prevented tumour

establishment of subcutaneous isografts. This effect has been associated with high levels of IL-12 and CXCL1, diminution of VEGF levels and changes in tumour infiltration of mononuclear cells and neutrophils (158). Also, Immodin, another human DLE, in combination with manumycin A suppressed tumour growth and prolonged survival in mammary tumour-bearing mice. This combination increased the infiltration of neutrophils and eosinophils into the TME, while independent treatments increased the phagocytic activity of monocytes and neutrophils (166). However, the immunogenicity of cell death has not been evaluated. In Figure 3 we can depict the effect of biological immunomodulators in cancer cells and innate immune cells.

#### 4.5.2 Synthetic immunomodulators

Synthetic immunomodulators are chemical agents that can be derived from diverse sources and can modulate biological responses by interacting with specific cellular targets (167, 168). Synthetic immunomodulators, which may encompass peptides and small molecules, have been used for decades (167–170). They are now being employed in cancer immunotherapy, with a focus on targeting specific surface molecules on cancer cells. These compounds can directly influence signalling pathways and modulate immune cells to selectively target specific types of cancer cells (168, 170). Additionally, some of them show a cytotoxic effect and their role as ICD inducers has recently been explored.

One of these molecules is imiquimod (IMQ), the first member of the immune response modifier family to be approved by the FDA in 1997, for the treatment of external genital and perianal warts



(171, 172). IMQ activates toll like receptor 7 (TLR7), which is overexpressed in different types of cancer (173), it also has potent antiviral and antitumour effects as shown in preclinical and clinical studies. Specifically, in human peripheral blood mononuclear cells (PBMC), IMQ has been shown to increase cytokine production including IFN- $\gamma$ , TNF- $\alpha$ , IL-1, IL-6 and IL-12 by macrophages and monocytes. IMQ also stimulates NK cell activity against skin-infected cells and the activation of macrophages to produce nitric oxide (171, 174). Also, in acute and chronic infectious diseases it promotes anti-inflammatory molecules such as IL-10, and indoleamine 2,3-dioxygenase (IDO) (175–177).

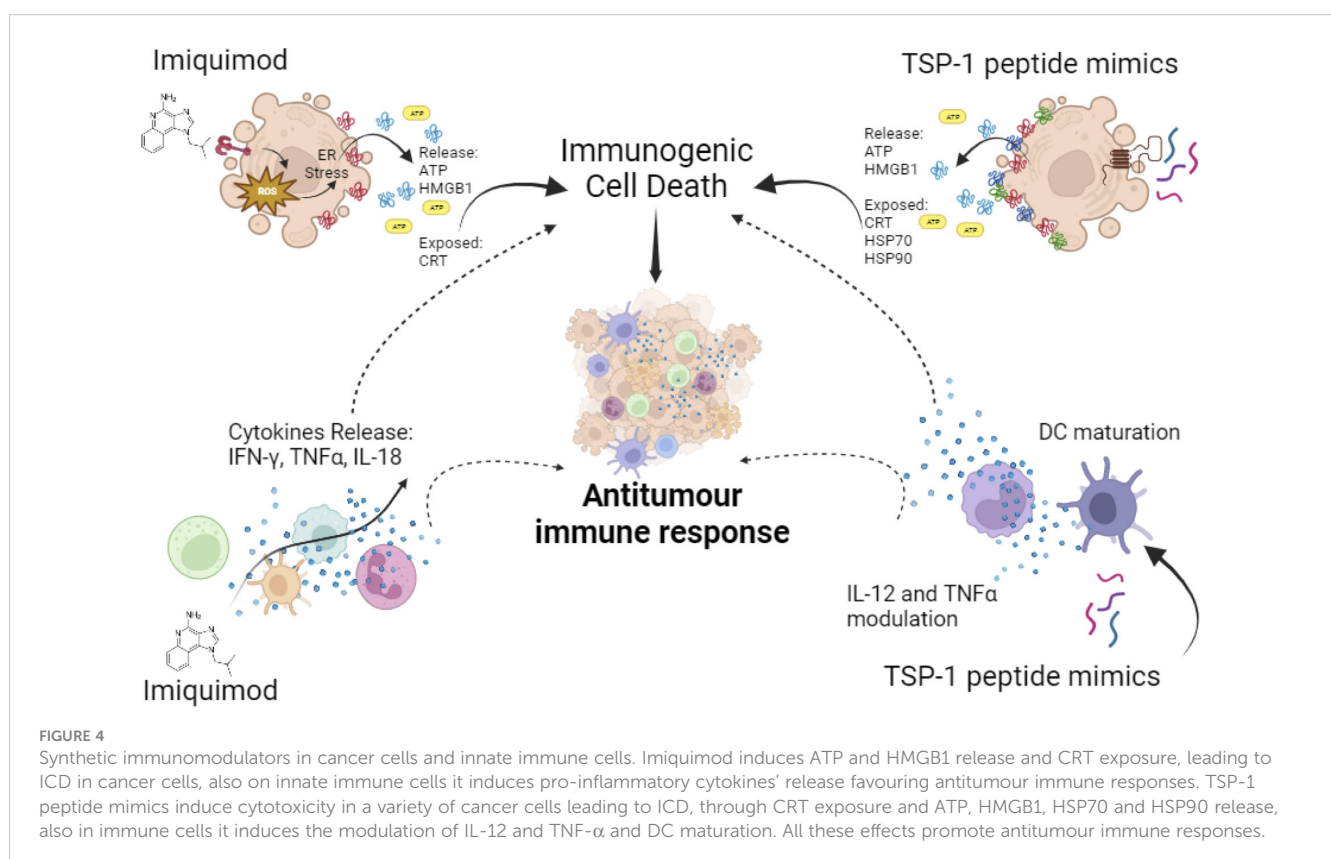
In the context of cancer cells, it can directly induce tumour autophagic cell death in melanoma (178), breast cancer (179) and colorectal cancer (180). It has also been shown that IMQ induces ICD by promoting ROS production, which triggers ER stress followed by surface-exposed CRT, ATP secretion and HMGB1 release (Figure 4), in BCC/KMC-1, AGS, HeLa and B16F10 cancer cells (181). Vaccination with IMQ-killed cancer cells also increased T lymphocyte proliferation, cytotoxic killing and immune cell infiltration into the tumour lesion in an *in vivo* melanoma model (181). In transgenic mice IMQ promoted breast cancer tumour regression, which progressed at the end of treatment due to CD4+ cells augmentation that enhanced IL-10 levels (182). On the other hand, IMQ could also improve the antitumour immune response by MAA peptide-pulsed DC immunotherapy (183). These effects are due to the stimulation of TLR7 in tumour cells and seem to depend on the type of cancer, the level of TLR7 expression, the downstream function of TLR7 signalling, or chemotaxis of

suppressive cells into the tumour (173). Thus, although IMQ has promising cell death inducing and immunomodulatory effects, caution should be taken about these contrary effects reported.

Other synthetic molecules such as thalidomide, lenalidomide and pomalidomide have demonstrated cytotoxicity in a variety of cancer subsets, however their activity as ICD inducers have not been described (184, 185). Recently, clinical trials have reported improved antitumour responses in multiple myeloma when used alone or in combination with other immunomodulatory agents (186–188). Thalidomide, in particular, was originally synthesised in the late 1950s as a non-addictive, non-barbiturate sedative (189). It is clinically useful in a number of cancers because of its antitumour activity, which is related to the secretion of various cytokines, including IL-2 and IFN- $\gamma$  as well as inducing T-cell costimulatory and antiangiogenic activities (189, 190). These reports suggest that these molecules can provide satisfactory stimulation of innate immune cells and contribute to cancer elimination through ICD induction.

#### 4.5.2.1 Peptide-based immunotherapies

Peptides are short-chain molecules, typically consisting of less than 50 amino acids. They have applications in the treatment of various conditions such as allergies, infections, tumours, and other diseases. Some peptides can induce cell death in bacteria, fungi, and tumour cells. In particular, peptides are gaining prominence in the field of immunotherapy due to their significant impact on the immune system (191). Therapeutic peptides have found application in immunotherapy, serving various purposes such as



cancer vaccines, blocking or inhibiting agents, and inducers of cell death, among others (192). As this review focuses on immunotherapies that induce ICD, we aimed to describe immunotherapeutic peptides that are capable of inducing ICD.

#### 4.5.2.2 Host defence peptides

An important source of ICD-inducing peptides are the host defence peptides (HDP), also known as antimicrobial peptides (AMPs), which are a conserved component of the innate immune system of a wide range of organisms (193), and they have specific physicochemical properties, such as a net positive charge and a specific distribution of cationic and hydrophobic amino acids (194), which enable their electrostatic interaction with cell membranes, membrane proteins or intracellular targets to promote cell lysis or regulated cell death.

Host defence peptides have immunomodulatory properties, such as modulation of inflammatory responses, chemokine expression, activation and differentiation of leukocytes, stimulation of antigen presentation, among others (193). Many HDP also have antitumour activities, most of them related to overcoming the immunosuppressive microenvironment, including: reduction of immunosuppressive cells, migration of phagocytic cells, reduction of pro-tumour molecules, recruitment of antitumour cytotoxic cells, among others (193). Among the HDP, some have been reported as ICD inducers, such as LTX-315 which, in addition to its effect in reducing pro-tumour immune cells, has also been reported to induce the emission of DAMPs (calreticulin, HMGB1 and ATP) and to induce *in vivo* myeloid and T lymphocyte tumour infiltration (195). The oncolytic peptides DTT-205 and DTT-304 induced calreticulin exposure and HMGB1 release, promoting tumour remission and the development of long-term immune memory against sarcoma and lung cancer cells *in vivo* (196). In addition, the peptide LTX-401 induced the release of ATP and HMGB1, and induced tumour remission with abscopal effect and promoted the establishment of antitumour memory against hepatocellular carcinoma cells *in vivo* (197). Taken together, the diverse effects of HDP could enhance their ICD properties to promote the antitumour immune system activation.

#### 4.5.2.3 Thrombospondin-1 peptide mimics

Thrombospondin-1 (TSP-1) mimic peptides are synthetic sequences (natural or modified) designed to mimic the functions of the different motifs in the TSP-1. In this sense, two sequences with a VVM motif were identified within the C-terminal cell-binding domain (CBD) of TSP-1 (198, 199). This led to the generation of 7N3 (1102-FIRVVMYEGKK-1112) and 4N1 (1016-RFYVVMWK-1024) peptides. Then, a modified version of 4N1, called 4N1K (K-RFYVVMWK-K) was developed, containing two lysine (K) residues flanking the 4N1 sequence, to increase the peptide solubility (198, 200).

4N1K was found to induce cell death in leukemic cells in addition to the modulation of cytokines in DCs and microglial cells (201–204). Thus, to improve these effects a structure-activity relationship study led to the synthesis of the first serum-stable analogue of 4N1K, called PKHB1. PKHB1 is recognized as an ICD

inducer in leukemic and breast cancer cells. This peptide induces a calcium-dependent and caspase-independent cell death mechanism. Furthermore, PKHB1-induced cell death exhibits key molecular hallmarks of ICD, including the exposure of calreticulin, HSP70, HSP90, and the release of ATP and HMGB1 (Figure 4) in various leukemic and breast cancer cell lines (205–207). Furthermore, PKHB1-treated cells promote DC maturation and stimulate the antitumour response of T-cells *ex vivo*. In a prophylactic context, PKHB1-treated cells prevent the tumour establishment in leukemic and breast cancer tumour bearing mice. PKHB1 also induce tumour shrinkage, increasing cytotoxic T-cell counts in blood and tumours, while reducing MDSCs and regulatory T-cells (T-regs) in breast cancer tumour-bearing mice (207). Notably, PKHB1 possess antiviral properties by triggering ICD in cases of infectious corneal disease caused by Herpes simplex virus type II. This event triggers an antiviral immune response, that reduces viral levels and mitigates the severity of the infection (208). Finally, PKHB1 also promotes the elimination of inflammatory macrophages in models of subretinal and peritoneal inflammation (209). Despite the evaluation of the immunogenicity and the antitumor effect of PKHB1, its impact on innate immune system cells and their role in the antitumor activity of PKHB1 have not been evaluated to date.

#### 4.5.2.4 Other peptides as ICD-inductors

F-pY-T is a mitochondria-targeting peptide that has been reported as an ICD inducer, triggering calreticulin exposure (*in vitro* and *in vivo*), ATP and HMGB1 release. F-pY-T *in vivo* induced DC maturation and promoted the intratumoral infiltration of CD8+ cells, and inhibited tumour growth (210).

The recombinant human milk peptide lactaptin RL2 induced calreticulin exposure, ATP and HMGB1 release in breast cancer cells and promoted the phagocytosis of dying-cancer cells by macrophages. *In vivo*, vaccination with RL2-treated cells also increased the survival of mice (211).

The calmodulin binding peptide CBP501 has been reported as an ICD inducer, which promotes calreticulin exposure and HMGB1 release, and increases *in vivo* mouse survival in vaccination experiments (in combination with cisplatin). Also, the combination of cisplatin and CBP501 also reduces tumour growth and increases intratumoral CD8+ cell infiltration (212).

Other peptides that have been demonstrated to possess immunomodulatory proprieties and induce immunogenic cell death are peptide-based proteasome inhibitors. Proteasome inhibitors are a class of drugs whose main mechanism is to inhibit the multi-protease subunits of the proteasome, leading to the accumulation of undegraded proteins, affecting different cellular processes which lead to cell death (213). Bortezomib is a dipeptide boronic acid derivative that acts as a reversible inhibitor of the 26S proteasome and is the first FDA approved proteasome inhibitor (214). It has been shown to have various immunomodulatory effects in allogeneic stem cell transplantation, antibody-mediated graft rejection and various inflammatory diseases (215). Furthermore, it is considered an ICD inducer, as it (1) prevents breast cancer tumour establishment (216) (2); promotes HSP90 exposure, DC



maturation and antitumour T-cell response against myeloma cells from patients (217) (3); triggers calreticulin exposure, induces DCs maturation (increase of CD83 and CD86) and the antitumour T-cell response, increasing the number of effector memory CD4+ and CD8+ cells (4); the *in vivo* vaccination with bortezomib-treated cells prevents tumour establishment and promotes long-term antitumour memory against multiple myeloma cells (218).

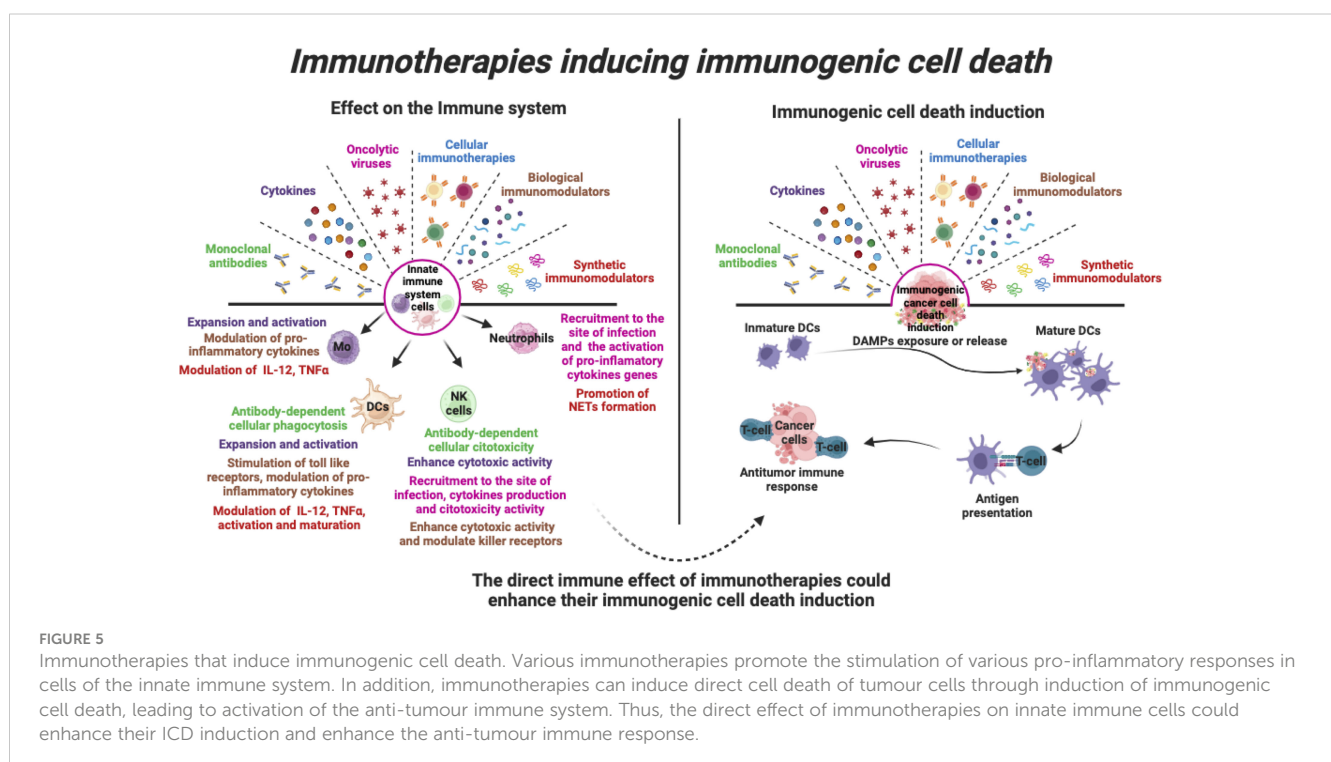
## 5 Discussion and concluding remarks

Since 1891, when Coley used the first immunotherapies, there have been tremendous advances and discoveries that have revealed the promising potential of immunotherapies for the prevention and treatment of cancer. Although these are usually combined with other cancer treatments capable of killing cancer cells to attack cancer cells from different perspectives, some immunotherapies have the capacity to be cytotoxic to cancer cells through immunogenic cell death induction. Although few in number, these ICD-inducing immunotherapies represent a promising and innovative approach in the fight against cancer, with the innate immune system playing a key role in their success.

ICD has the potential to induce a robust antitumor immune response (219). However, the principal challenge is associated with treatment resistance, which could hamper its therapeutic efficacy. This may be related with the ICD induction which depends on the host (for example immune perception of ICD), the tumour (for example DAMPs' exposure), the ICD inducer (for example, its immunosuppressive effects), or the specific cancer microenvironment (the specific immunosuppressive cells present in the TME) (220). Cell death resistance could be addressed by

combination regimens of therapeutic alternatives that could attack from different sources. For example, it has been demonstrated that bortezomib improves adoptive T cell therapy by sensitizing cancer cells to FasL cytotoxicity (221). Also, oncolytic viruses provide potent antitumor effects against brain tumours when combined with adoptive T-cell therapy (222). While, bovine dialyzable leukocyte extract, which induces ICD in breast cancer, when combined with cyclophosphamide induces synergic cell death (223). The combination of chemotherapy with immunotherapies is a primary approach evaluated to overcome cancer cell resistance (224–226). However, these combinations mostly look for ICD inducing chemotherapies with non-necessarily ICD inducing immunotherapies, yet combination of different ICD-inducing agents might promote better responses. Especially if combining immunotherapeutic and chemotherapeutic agents that possess ICD potential and immunomodulatory properties, as it has also been described for certain types of chemotherapies (227, 228). Immunotherapies may help to surpass tumour resistance mechanisms, as immunotherapies that stimulate the innate immune system may augment ICD by enhancing the effect triggered by DAMPs, thereby promoting a robust immune response (229). Additionally, they may activate DCs, enhancing antigen presentation and promoting the recruitment and activation of effector immune cells (125), like NK cells, which in turn can efficiently trigger cellular cytotoxicity, potentially leading to ICD (101). This synergy is a promising alternative to overcome ICD resistance, providing an interesting avenue for enhanced antitumor effects and improved therapeutic outcomes.

The intricate interplay between ICD and the innate immune response opens new avenues for the development of more effective and durable cancer treatments with promising potential. These



onco-immunotherapies, including monoclonal antibodies, cytokines, oncolytic viruses, cellular immunotherapies, and other biological or synthetic immunomodulators, have clearly demonstrated their potential to harness the body's natural defences against cancer cells. By triggering the ICD, these treatments also facilitate the release of tumour antigens and danger signals, stimulating innate immune cells such as dendritic cells, natural killer cells and macrophages. Activation of these innate first-line defence cells is critical for mounting a potent and sustained anti-cancer response, which involves the durable long-term memory of the adaptive immune system (Figure 5).

As these potential actions of immunotherapies have not been the primary focus, several challenges remain. For example, uncovering the potential role of immunotherapies in inducing immunogenic cell death is a significant challenge, given that only a few of them have been studied as ICD inducers. This is particularly striking when compared to the large body of evidence highlighting their role in the immune system. Another aspect is not only to elucidate their role in ICD induction, but also to propose combinations that enhance this dual action for a more comprehensive approach against a wide range of cancers. In conclusion, we recommend that these strategies be emphasised, as addressing these aspects will undoubtedly contribute to a deeper understanding, more effective use and further development of the enormous potential offered by these immunotherapies.

## Author contributions

KC-R: Data curation, Investigation, Methodology, Validation, Writing – original draft, Writing – review and editing. HL-A: Data curation, Investigation, Methodology, Writing – original draft, Writing – review and editing. CR-P: Writing – review and editing. AM-T: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – original draft, Writing – review and editing. DS-A: Conceptualization, Validation, Writing – review and editing.

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## Glossary

ICD	Immunogenic cell death
DAMPs	Damage-associated molecular patterns
PRRs	Pattern recognition receptors
TAA	Tumour-associated antigens
MHC	Major histocompatibility complex
TME	Tumour microenvironment
PAMPs	Pathogen-associated molecular patterns
DCs	Dendritic cells
mAb	Monoclonal antibody
ADCC	Antibody-dependent cellular cytotoxicity
CDC	Complement-dependent cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
NK	Natural killer
BCMA	B-cell maturation antigen
CRT	Calreticulin
HSP	Heat shock protein
ATP	Adenosine triphosphate
HMGB1	high mobility group box protein 1
FOLFIRI	5-fluorouracil, irinotecan and leucovorin
ER	Endoplasmic reticulum
TAMs	Tumour associated macrophages
PDT	Photodynamic therapy
TNF- $\alpha$	Tumour necrosis factor alpha
IFN- $\gamma$	Interferon gamma
FDA	Food and Drug Administration
GM-CSF	Granulocyte macrophage colony-stimulating factor
G-CSF	Granulocyte-colony stimulating factor
SMAC	Secondary mitochondria-derived activator of caspases
PANoptosis	Pyroptosis apoptosis and necroptosis cell death
OVs	Oncolytic viruses
NDV	Newcastle disease virus
TLRs	toll like receptors
APCs	antigen presenting cells
oHSV	herpes simplex virus
TGF $\beta$	transforming growth factor beta
PDAC	Human Pancreatic ductal adenocarcinoma cells
TMD	tumour micro-vessel density
CAR	chimeric antigen receptor

(Continued)

### Continued

CRS	cytokine release storms
GvHD	graft versus host disease
MDSCs	myeloid-derived suppressor cells
CAR M	CAR macrophages
NO	nitric oxide
ROS	reactive oxygen species
BCG	Bacille-Calmette-Guerin
MCP-1	Monocyte chemoattractant protein-1
UC	urothelial carcinoma
DLE	dialysable leukocyte extracts
I-CRP	Immunopotent CRP
bDLE	dialysable leukocyte extract obtained from bovine spleen
LPS	Lipopolysaccharide
IMQ	Imiquimod
TLR7	toll like receptor 7
PBMC	peripheral blood mononuclear cells
IDO	indoleamine 2, 3-dioxygenase
HDP	host defence peptides
AMPs	antimicrobial peptides
TSP-1	Thrombospondin 1
CBD	C-terminal cell-binding domain
T-regs	regulatory T-cells





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## EDITED BY

Mary Poupot-Marsan,  
INSERM U1037 Centre de Recherche en  
Cancérologie de Toulouse, France

## REVIEWED BY

Richard Lopez,  
Duke University, United States  
Emmanuel Scotet,  
Institut National de la Santé et de la  
Recherche Médicale (INSERM), France

## \*CORRESPONDENCE

Cindrilla Chumduri  
✉ cindrilla.chumduri@bce.au.dk  
Dieter Kabelitz  
✉ Dietrich.Kabelitz@uksh.de  
Thomas F. Meyer  
✉ t.meyer@ikmb.uni-kiel.de;  
✉ meyer@mpiib-berlin.mpg.de

†These authors have contributed equally to  
this work and share first authorship

†These authors have contributed equally to  
this work and share last authorship

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# $\gamma\delta$ T cell-mediated cytotoxicity against patient-derived healthy and cancer cervical organoids

Junxue Dong<sup>1,2†</sup>, David Holthaus<sup>1†</sup>, Christian Peters<sup>3</sup>,  
Stefanie Koster<sup>2</sup>, Marzieh Ehsani<sup>1</sup>, Alvaro Quevedo-Olmos<sup>1</sup>,  
Hilmar Berger<sup>1,2</sup>, Michal Zarobkiewicz<sup>3,4</sup>, Mandy Mangler<sup>5,6</sup>,  
Rajendra Kumar Gurumurthy<sup>2</sup>, Nina Hedemann<sup>7</sup>,  
Cindrilla Chumduri<sup>1,2,9\*†</sup>, Dieter Kabelitz<sup>3\*†</sup>  
and Thomas F. Meyer<sup>1,2\*†</sup>

<sup>1</sup>Laboratory of Infection Oncology, Institute of Clinical Molecular Biology, Christian-Albrechts-Universität zu Kiel and University Hospital Schleswig-Holstein, Kiel, Germany, <sup>2</sup>Department of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany, <sup>3</sup>Institute of Immunology, Christian-Albrechts-Universität zu Kiel and University Hospital Schleswig-Holstein, Kiel, Germany, <sup>4</sup>Department of Clinical Immunology, Medical University of Lublin, Lublin, Poland, <sup>5</sup>Department of Gynaecology and Obstetrics, Vivantes Auguste Viktoria-Klinikum, Berlin, Germany, <sup>6</sup>Department of Gynaecology, Charité University Medicine, Berlin, Germany, <sup>7</sup>Department of Gynaecology and Obstetrics, University Hospital Schleswig-Holstein, Kiel, Germany, <sup>8</sup>Laboratory of Infections, Carcinogenesis and Regeneration, Medical Biotechnology Section, Department of Biological and Chemical Engineering, Aarhus University, Aarhus, Denmark, <sup>9</sup>Chair of Microbiology, University of Würzburg, Würzburg, Germany

Cervical cancer is a leading cause of death among women globally, primarily driven by high-risk papillomaviruses. However, the effectiveness of chemotherapy is limited, underscoring the potential of personalized immunotherapies. Patient-derived organoids, which possess cellular heterogeneity, proper epithelial architecture and functionality, and long-term propagation capabilities offer a promising platform for developing viable strategies. In addition to  $\alpha\beta$  T cells and natural killer (NK) cells,  $\gamma\delta$  T cells represent an immune cell population with significant therapeutic potential against both hematologic and solid tumours. To evaluate the efficacy of  $\gamma\delta$  T cells in cervical cancer treatment, we generated patient-derived healthy and cancer ectocervical organoids. Furthermore, we examined transformed healthy organoids, expressing HPV16 oncogenes E6 and E7. We analysed the effector function of *in vitro* expanded  $\gamma\delta$  T cells upon co-culture with organoids. Our findings demonstrated that healthy cervical organoids were less susceptible to  $\gamma\delta$  T cell-mediated cytotoxicity compared to HPV-transformed organoids and cancerous organoids. To identify the underlying pathways involved in this observed cytotoxicity, we performed bulk-RNA sequencing on the organoid lines, revealing differences in DNA-damage and cell cycle checkpoint pathways, as well as transcription of potential  $\gamma\delta$  T cell ligands. We validated these results using immunoblotting and flow cytometry. We also demonstrated the involvement of BTN3A1 and BTN2A1, crucial molecules for  $\gamma\delta$  T cell activation, as well as differential expression of PDL1/CD274 in cancer, E6/E7+ and healthy organoids. Interestingly, we observed a significant reduction in cytotoxicity upon

blocking MSH2, a protein involved in DNA mismatch-repair. In summary, we established a co-culture system of  $\gamma\delta$  T cells with cervical cancer organoids, providing a novel *in vitro* model to optimize innovative patient-specific immunotherapies for cervical cancer.

#### KEYWORDS

cervical cancer, human papillomavirus, ectocervix, organoids, immunotherapy,  $\gamma\delta$  T cells

## 1 Introduction

According to latest WHO data, cervical cancer is the fourth common cancer among women worldwide (1). The majority of cervical cancers are strongly associated with persistent infection with high-risk oncogenic human papilloma virus (HR-HPV) (2, 3). Among HR-HPVs, HPV16 and HPV18 account for about 70% of cervical cancers (4). Yet, additional infectious agents have also been implicated as co-drivers of cervical cancer (5). Considering that only a quarter of cervical cancer patients respond to chemotherapy, the development of more personalized therapies is urgently needed. Recent advances in organoid technology have enabled the culture of human ectocervical organoids that faithfully replicate healthy and cancerous tissues (6). These organoids serve as a promising *in vitro* model, preserving original cellular heterogeneity and accurately recapitulating epithelial architecture and functionality. Recognizing the absence of *in vitro* models for HPV, we have earlier reported the transformation of healthy cervical organoids with HPV-derived oncogenes E6 and E7 via lentiviral transfer (5). These transformed organoids exhibit significant deviations from their corresponding healthy controls (5).

A numerically minor yet important subset of T lymphocytes in the peripheral blood endowed with anti-cancer activity is  $\gamma\delta$  T cells (7, 8).  $\gamma\delta$  T cells differ from conventional  $\alpha\beta$  T cells in at least two important aspects: (i)  $\gamma\delta$  T cells do not recognize peptides presented by MHC class I or class II molecules but rather recognize phosphorylated intermediates (“phosphoantigens”) of the cholesterol synthesis pathway and additional unconventional ligands; and (ii)  $\gamma\delta$  T cells do not require MHC molecules for antigen recognition, which opens the possibility of applying  $\gamma\delta$  T cells across HLA barriers (9, 10).  $\gamma\delta$  T cells express CD3-associated T cell receptors (TCR) composed of  $\gamma$  and  $\delta$  chains (11, 12). Like other cytotoxic effectors,  $\gamma\delta$  T cells directly participate in the elimination of tumour cells, but they also indirectly control the tumour immune response by modulating the activity and functions of other immune cells (7). In healthy adults, approximately 1–5% of circulating T lymphocytes express V $\delta$ 2 paired with V $\gamma$ 9; the proportion of such V $\delta$ 2 T cells can rapidly increase during the acute phase of several infectious diseases (11, 12). Recent studies have shed light on the mode of recognition of phosphoantigens (pAg) by  $\gamma\delta$  T cells. In this context, an indispensable role of members of the butyrophilin (BTN) family of transmembrane molecules has been discovered. According to the current model, microbe- or tumour-derived pyrophosphates bind to the

intracellular B30.2 domain of BTN3A1 which then interacts with BTN2A1 to induce a conformational alteration of the extracellular BTN3A1/2A1 complex which is recognized by the  $\gamma\delta$  TCR (13–16).

$\gamma\delta$  T cells have well-established protective roles in cancer (8, 17, 18). Multiple mouse models have demonstrated their protective role in cancer progression (17, 19). However, previous studies so far have only investigated the effect of  $\gamma\delta$  T cells on cervical cancer-derived cancer cell lines and tissue slices (17, 20) or utilized NK cells (21), while studies comparing their effects on patient-derived organoids have not yet been reported. In this study, we provide a model to assess  $\gamma\delta$  T cell-mediated cytotoxicity by live cell imaging and flow cytometry-based assays.

## 2 Methods

### 2.1 Isolation of cervical tissue and cervical organoid culture

Human cervical specimens were obtained from volunteers undergoing standard surgical procedures at the Department of Gynaecology, Charité University Hospital, and August-Viktoria Klinikum, Berlin (Ethics Approval EA1/059/15 from local authorities), informed consent was obtained from all donors. Samples were processed within 2–3 hours after removal.

Organoids were derived from tissue resection as previously described (5, 6, 22). In short, biopsies were washed and cells were isolated by mincing and enzymatic digestion with 0.5 mg/mL collagenase type II for 2.5 h at 37°C. Afterwards, cells were pelleted at 100g for 5 min (4°C) and then again digested with TrypLE Express Enzyme (Gibco, 12604013) for 15 min at 37°C. Cells were then expanded in collagen-coated T25 tissue culture flasks in cervical organoid medium (Advanced DMEM/F-12 (Gibco, 12634) supplemented with 10 mM HEPES (Capricorn, HEP-B), 1% GlutaMax (Gibco, 35050061), 1x NCS21 Neuronal Supplement (Capricorn, C21-H), 1x N2 (Capricorn, N2-K), 10 ng/mL human EGF (Peprotech, AF-100-15), 0.5  $\mu$ g/mL hydrocortisone (Sigma, H0888), 100 ng/mL human noggin (Peprotech, 120-10C), 100 ng/mL human FGF-10 (Peprotech, 100-26-25), 1.25 mM N-acetyl-L-cysteine (Sigma, A9165), 2  $\mu$ M TGF- $\beta$  receptor kinase Inhibitor IV (A83-01, Cayman Chemicals, Cay9001799), 10  $\mu$ M ROCK inhibitor (Y-27632, Cayman Chemicals, Cay10005583), 10 mM nicotinamide (Sigma, N0636), 10  $\mu$ M forskolin (Sigma, F6886)) until reaching 70–80% confluency.

After successful establishment, cells were harvested and ~20,000 cells were seeded into 50  $\mu$ l Matrigel domes (Corning, 11543550).

Organoids were maintained by passaging every 1–2 weeks at a 1:2–1:5 ratio using enzymatic digestion with TrypLE followed by mechanical singularization of cells using a syringe with an 18G needle (BD medical, BDAM303129) as described earlier (22).

## 2.2 Cell lines and 3T3-J2 irradiation

AGS0 cells (AGS cell line devoid of *parainfluenza virus 5* infection, kindly provided by Richard E. Randall, School of Biology, St. Andrews University) were maintained in RPMI 1640 medium (Capricorn, RPMI-HA) supplemented with 1 mM Sodium pyruvate (Capricorn, NPY-B), 1x Penicillin/Streptomycin and 10% fetal calf serum (Sigma, F7524). HeLa (ATCC CCL-2; RRID : CVCL\_0030) and 3T3-J2 cells (kindly provided by Craig Meyers; Howard Green laboratory, Harvard University; RRID : CVCL\_W667) were maintained in DMEM (Capricorn, DMEM-HPSTA) supplemented with 1x Penicillin/Streptomycin (Gibco, 15070063), 10 mM HEPES and 10% fetal calf serum (Sigma, F7524). The 3T3-J2 cells were irradiated with 30 Gy in a Gammacell 40 Exactor. After irradiation,  $1 \times 10^6$  irradiated 3T3-J2 were seeded per T25 Flask and incubated overnight until all cells attached to the surface.

## 2.3 2D cervical stem cell lines culture

Passage 0 epithelial cells were cultured in plastic cell culture vessels without an irradiated 3T3-J2 monolayer and seeded in a Collagen I (1:100, Fisher Scientific, A1064401) pre-coated T25 flask. After most epithelial cells attached to the surface, the medium was changed every 2–3 days until confluence reached 80–90%. The cells were then passaged at a ratio of 1:2. For the passage 0, the medium was removed and the cells were washed three times with PBS (Capricorn, PBS-1A) and dissociated with 1 mL TrypLE (for each T25 flask) incubating 10 min at 37°C. The cells were detached from the flask surface and the cell suspension was collected with 10 mL Advanced DMEM/F12. After centrifugation at 400g for 4 min at 4°C, the cell pellet was resuspended with cervical organoid medium and seeded on irradiated 3T3-J2 monolayers.

From passage 1, ectocervical epithelial cells were cultured with an irradiated 3T3-J2 layer and the medium was refreshed every 2–3 days. When it reached 80%–90% confluency, the cells were enzymatically detached twice. The first time, 0.5 mL TrypLE was used for each T25 flask for 1 min to detach the 3T3-J2 feeder cells, and then the cervical epithelial cells were detached with another 1 mL TrypLE and incubated for 10 min.

## 2.4 Whole mount immunofluorescence assays (IFAs) and microscopic analyses

Phase contrast and brightfield images were taken using an IX50 (Olympus) microscope with a 4x or 10x objective. Images were

contrast adjusted and scale bars were added using FIJI (ImageJ; RRID : SCR\_002285) (23).

For fluorescent microscopy, organoids were processed as described before (24). In short, organoids were harvested and washed once in PBS, then incubated in cell-recovery solution (Corning, 47743-696) for 30 min, followed by an additional wash with PBS (Capricorn, PBS-1A), and fixed for 20 min in 4% para-formaldehyde (PFA; Carl Roth, P087). Cells were permeabilized with 0.1 M glycine (Carl Roth, 3908), 0.2% Triton-X100 (Carl Roth, 3051) in TRIS-buffered saline (TBS; 50 mM Tris-Cl (Carl Roth, 9090), pH 7.5, 150 mM NaCl (Carl Roth, 3957)) and incubated in blocking buffer consisting of 3% bovine serum albumin (Carl Roth, 3854), 1% normal goat serum (Capricorn, GOA-1A), 0.2% Triton-X-100, and 0.1% Tween-20 (Sigma, P9416) in TBS for 2 h at RT. Primary antibodies were added for incubation at 4°C overnight in blocking buffer and organoids were washed the next day four times with 0.2% Triton-X100, 0.1% Tween-20 in TBS. Secondary antibodies and 10  $\mu$ g/mL Hoechst 33342 (Thermo Fisher, H1399) as nuclear stain were added, kept for 1 h at RT in the dark, followed by three additional washing steps with TBS. After a final wash with deionized water, organoids were mounted with Fluoromount-G (Southern Biotech, 0100-01) on glass slides. Images were taken using a cLSM 880 microscope (Zeiss), equipped with Plan-Apochromat 20x/0.8 M27 and C-Apochromat 40x/1.20 water M27 objectives and analysed with ZEN blue software (v3.5; RRID : SCR\_013672) and FIJI. Antibodies and dilutions are listed in [Supplementary Table 1](#).

## 2.5 Isolation of gDNA and real-time quantitative-polymerase chain reaction

For nucleic acid extraction, organoids were harvested, pelleted, and washed with ice-cold PBS. Matrigel was removed by incubation with cell-recovery solution for 30 min at 4°C. Genomic DNA (gDNA) was isolated with the Quick-DNA Miniprep (Zymo, D3024) according to manufacturer's instructions. Purity of DNA was assessed by A260/A280 absorbance ratio using an Infinite M200 Pro plate reader (Tecan). RT-qPCR was performed with a StepOnePlus (Agilent) using the Luna Universal qPCR Master Mix (NEB, M3003), and included initial enzyme activation for 3 minutes at 95°C, followed by 40 cycles of 20s at 95°C, 30s at 60°C and 20s at 72°C. A minimum of 30 ng gDNA was used per well. Melting curve analysis was performed to verify amplicon specificity. Relative expression was calculated using the  $\Delta$ CT method, and HPV status was assessed by electrophoresis of the amplified products. For that, amplicons were separated on a 1.5% agarose gel containing SYBR Safe Nucleic Acid Gel Stain (Invitrogen, S33102) in 0.5x TRIS-Borate-EDTA (TBE) -buffer (40 mM Tris-Cl, 45 mM boric acid (Carl Roth, P010), 1 mM EDTA (Carl Roth, X986)). The following primers were used:

HPV16-for 5'-AGCTGTCATTTAATTGCTCATAACAGTA-3' (25);

HPV16-rev 5'- TGTGTCCTGAAGAAAAGCAAAGAC-3' (25);

HPV18-for 5'-CGAACCACAACGTCACACAAT-3' (25);

HPV18-rev 5'-GCTTACTGCTGGGATGCACA-3' (25);  
 GAPDH-for 5'-GGTATCGTGAAGGACTCATGAC-3' (5);  
 GAPDH-rev 5'-ATGCCAGTGAGCTTCCCGTTTCAG-3' (5).

Signal was recorded using a ChemiDoc MP Imaging System (Biorad).

## 2.6 V $\gamma$ 9V $\delta$ 2 T cell expansion

V $\gamma$ 9V $\delta$ 2 T cells were expanded from peripheral blood mononuclear cells (PBMCs) isolated from leukocyte concentrates of healthy blood donors using a Ficoll-Hypaque (Biochrom, Biocoll L 6113/5) density gradient according to the manufacturer's protocol. Leukocyte concentrates were provided by the Institute of Transfusion Medicine UKSH Campus Kiel and were used in an anonymized fashion and Ethics Approval D 434/22 was provided by local authorities. The cell pellet was resuspended in RPMI medium (Capricorn, RPMI-STA) containing 10% FCS (Sigma, F7524). Before expansion, the initial V $\gamma$ 9V $\delta$ 2 T population among CD3+ T cells was checked by staining PBMCs with anti-V $\delta$ 2-FITC and anti-CD3-APC. Samples with a V $\gamma$ 9V $\delta$ 2 T cell proportion over 2% were deemed acceptable and the sample was further processed. V $\gamma$ 9V $\delta$ 2 T cells were selectively activated by stimulating PBMCs with 5  $\mu$ M zoledronic acid (Novartis) and 50 IU/mL IL-2 (Novartis) over the expansion period of 14 days essentially as described (26). IL-2 was supplemented every other day. Purity of V $\gamma$ 9V $\delta$ 2 T cell lines was checked with anti-V $\gamma$ 9-FITC and anti-CD3-APC. Lines were used for experiments when V $\gamma$ 9V $\delta$ 2 T cells represented more than 90% of the total cell population. Antibodies and dilutions are listed in [Supplementary Table 1](#).

## 2.7 V $\gamma$ 9V $\delta$ 2 T cell co-culture with cervical cells

The B-cell lymphoma Daudi cell line (ATCC, CCL-213; RRID : CVCL\_0008) with known sensitivity to V $\gamma$ 9V $\delta$ 2 T cell killing (27) was included as positive control. All lines were washed with PBS and pelleted in 15 mL falcon tubes by centrifuging at 1400 rpm for 5 min. Cells were stained with PKH67 (Sigma, PKH67GL) green dye was prepared according to manufacturer protocol. After incubation at RT for 4 min in the dark, 7 mL RPMI medium containing 10% FCS was added and incubated for further 1 min to stop the staining. Cells were used at a ratio of 10:1 (T cells: epithelial cells). Co-culture was performed for 4 hours with the respective surface blocking mAb, if appropriate, and thereafter cells were washed in PBS, and taken up in sample buffer containing 0.2  $\mu$ g/mL propidium iodide (PI) for 20 min at 4°C and analysed by flow cytometry. Data was then analysed with FlowJo v10 (RRID : SCR\_008520). The absolute number of viable epithelial cells (FITC+PI-) was calculated with the formula derived from Sacchi et al. (28):

$$\text{Cytotoxicity} = 100 - [(\% \text{ of living epithelial cells in co-culture with } \gamma\delta \text{ T cells} / \% \text{ of living epithelial cells without } \gamma\delta \text{ T}) \times 100]$$

## 2.8 CD107a degranulation assay

50  $\times$  10<sup>3</sup> effector cells were cultured alone or together with 5  $\times$  10<sup>3</sup> tumour cells for 4 h at a 10:1 ratio in the presence of anti-CD107a-PE mAb (clone: H4A3; BD Biosciences) and monensin (3  $\mu$ M; added after 1 h). Thereafter cells were washed twice in PBS and analyzed by flow cytometry. Where appropriate, the synthetic pAg bromohydrin pyrophosphate (BrHPP; Innate Pharma) was added at 300 nM to activate V $\gamma$ 9V $\delta$ 2 T cells. Cells were immediately acquired on a BD FACSCanto I flow cytometer. Data was then analysed with FlowJo v10.

## 2.9 Live cell imaging

Prior to co-culture, a flat-bottom 384 well-plate (black with clear bottom, Corning) was coated with Poly-2-hydroxyethyl methacrylate (poly-HEMA; Sigma, P3932) in 100% ethanol as described earlier (29). 30  $\mu$ l poly-HEMA was added in each well and the plate was left to dry overnight under the culture hood. The plate was washed with PBS immediately before use.

The imaging protocol was adapted from previous studies (30, 31). Organoid lines were cultured for around seven days and V $\gamma$ 9V $\delta$ 2 T cells were expanded for 14 days as described above. Matrigel was removed by incubation with cell-recovery solution for 30 min at 4°C. A part of the suspension of organoids was taken and digested into single cells using TrypLE then the number of single cells was counted to estimate organoid equivalents. The organoids were washed with cold PBS and thereafter organoids were collected and stained with CellTrace CFSE Cell Proliferation Kit (Thermo, C34554) according to the supplier's protocol. Then, V $\gamma$ 9V $\delta$ 2 T cells were added again at a ratio 10:1 (T cells: epithelial cells). NucRed Dead 647 ReadyProbe Reagent (Thermo, R37113) was added to distinguish dead and live cells. In the respective conditions,  $\gamma\delta$  T cells were activated with 300 nM BrHPP, "Dead cells" were boiled for 10 min at 95°C before the start of the experiment, and 1 mg/mL Puromycin (InVivoGen, ant-pr-1) treated cells served as death control over time. Measurements were performed using the CELLVISTA 4 automated cell imager in combination with the SYBOT X-1000 with CYTOMAT 2 C-LiN system (all SYNENTEC GmbH). Wells were imaged every hour for a total of 8 hours and afterwards fluorescence data and images were extracted with YT-Software (SYNENTEC GmbH) using the Real Cytoplasm (2F) application. The settings were modified to detect all organoids in the green channel and subsequently, the average intensity of the yellow and red signal within each spheroid area was analysed. The following settings were used for different channels. Exciter: Blue (475/28) - Emissionfilter: Green Filter 530nm (530/43), Exciter: Green (529/24) - Emissionfilter: Amber Filter 580nm (607/70), Exciter: Red (632/22) - Emissionfilter: Far Red Filter 670nm (685/40). Scatter plots were assembled in R (v4.1.0) with ggplot2 (v3.4.0).

## 2.10 Live/dead cytotoxicity assay

1x10<sup>4</sup> cervical organoid cells were seeded into Collagen I pre-coated 96-well plates without feeder cells. On the next day, cells



were co-incubated with  $1 \times 10^5$   $\gamma\delta$  T cells (EC : TC = 1:10) for four hours in Opti-MEM (Gibco, 11058021). To quantify dead/live cells, the MultiTox-Glo Multiplex Cytotoxicity Assay kit (Promega, G9272) was used according to manufacturer's instructions. The values were normalized to the respective untreated control, and the ratio of dead/live cells was then normalized to the healthy organoids to obtain fold changes.

## 2.11 SDS-PAGE and immunoblotting

For SDS-PAGE analysis, organoids were harvested, pelleted, and washed with ice-cold PBS. Matrigel was removed by incubation with cell-recovery solution (Corning) for 1 h at 4°C. Cells were then lysed in RIPA-buffer (50 mM Tris-HCl pH 8.0, 1% w/v NP-40 (Sigma, 492018), 0.5% w/v sodium deoxycholate (Sigma, D6750), 0.1% w/v SDS (Carl Roth, 0183), 150 mM NaCl, and 5 mM EDTA). After assessing the protein content using the Pierce Rapid Gold BCA Protein Assay Kit (Thermo, A53225) samples were diluted with 6x Laemmli buffer (70% v/v Tris HCl pH 6.8, 10% w/v SDS, 30% (v/v) glycerol (Carl Roth, 3783) and 0.01% w/v Bromophenol blue (Sigma, B0126) containing 10% v/v  $\beta$ -mercaptoethanol (Carl Roth, 4227). Samples were boiled at 95°C for 5 minutes. 25  $\mu$ g of protein per condition was blotted onto Amersham Protran nitrocellulose membranes (Fisher Scientific, 10600016) using standard techniques and transfer quality was validated by Poinceau S (Sigma, P3504) total protein staining. The membranes were blocked for 1 hour in 1x RotiBlock (Roth, A151) and incubated with primary antibodies overnight. After three washing steps, membranes were incubated with respective peroxidase-conjugated secondary antibodies and signals were detected using chemiluminescence with a ChemiDoc MP Imaging System (Biorad). Antibodies and dilutions are listed in [Supplementary Table 1](#).

## 2.12 Analysis of bulk RNA sequencing

Quality of raw reads was assessed by FastQC. Reads were mapped to the human reference genome (assembly GRCh37) using the splice aware aligner STAR (v2.7) (32) in two-pass mode. Quality GTF files downloaded from Gencode (v28) (33), were used to map reads into genes with FeatureCounts in order to obtain raw counts. From this matrix of raw counts, genes with no expression across samples were filtered out, and differential gene expression was performed using DESeq2 (v1.32.0) (34) in R (35). Volcano plots were generated using ggplot2 (v3.4.0). Genes were ranked based on their log<sub>2</sub> fold change compared to healthy controls, and gene set enrichment analysis (GSEA) was performed using the fgsea implementation from the clusterProfiler R package (v4.0.5) (36). Gene sets from Hallmark (H), Curated Gene Sets (C2) and GO Biological Process (C5-BP), retrieved from MsigDB (37) using msigdb (v7.5.1) were studied. The gene expression heatmaps of the TPMs were generated using the pheatmap package (v1.0.12). Principal Component Analysis (PCA) was performed on the TPM normalized counts and the plots were generated using factoextra (v1.0.7) on the result of the pca

function in base R. All the analyses were performed in R (v4.1.0). RNA-seq DGE and GSEA analysis results are included in [Table 1](#).

## 2.13 Statistical analysis and panel composition

Basic calculations were performed using MS EXCEL 2016 (Microsoft). Figures were plotted using Prism 10 (GraphPad) or R (v4.1.0). P values  $\leq 0.05$  were considered statistically significant. Asterisks indicate statistical significance values as follows: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . The panel composition and annotations were created using Affinity Designer 2.1.1 (Serif).

# 3 Results

## 3.1 Ectocervical organoids accurately represent the phenotype of the parental tissue

To establish a suitable model mimicking ectocervical tissue, we have established patient-derived organoids (PDOs) cervical tissues in our earlier studies (5, 6, 22). These lines were grown in T25 tissue flasks on irradiated 3T3-J2 mouse fibroblast feeder cells and subsequently embedded in Matrigel to generate 3D organoids ([Figure 1A](#)). In order to create paired HPV E6E7-positive lines (referred to as HPV+), the healthy organoid lines were transformed with a construct containing human papilloma virus (HPV) oncogenes E6 and E7 as described earlier (5, 22). We were able to passage all lines for approximately 20 passages prior to growth arrest. Visual inspection under brightfield microscopy revealed no discernible phenotypic difference between healthy, HPV+ and cancer lines ([Figure 1B](#)). Amplification of integrated HPV16 E6E7 oncogenes was detected by PCR of genomic DNA in the HPV+ and cancer organoids ([Figure 1C](#)). Since HPV16 and 18 are the most common causes of cervical cancers, we also assessed the HPV18 status in our organoids. HPV18 was detected in one of the lines (Cancer D3), also reflecting the naturally occurring ratio of HPV16 and HPV18 infections of two to one (38). HPV18 primer sensitivity was verified using HeLa cells as a positive control ([Figure 1C](#)). We have summarized the characteristics of the organoid lines in [Table 1](#).

To validate the ectocervical nature of our organoids, we next characterized the organoid lines for marker gene expression and localization by whole mount immunofluorescence assays. All lines were positive for cytokeratin 5 (KRT5), a marker of ectocervix, in the outer layers of the organoids resembling the *in vivo* stratified squamous epithelium of the parental tissue. As the basal layer is responsible for proliferation *in vivo*, marker of proliferation KI67 (KI67)-positive cells are expected in this layer. Interestingly, KI67-positive cells were indeed detected in the parabasal layers of healthy PDOs, while they were located throughout the whole organoid for HPV+ and cancer-derived lines ([Figure 1D](#)). A more detailed characterization of ectocervical organoid cultures has been previously provided in our studies (5, 6). Thus, the organoids

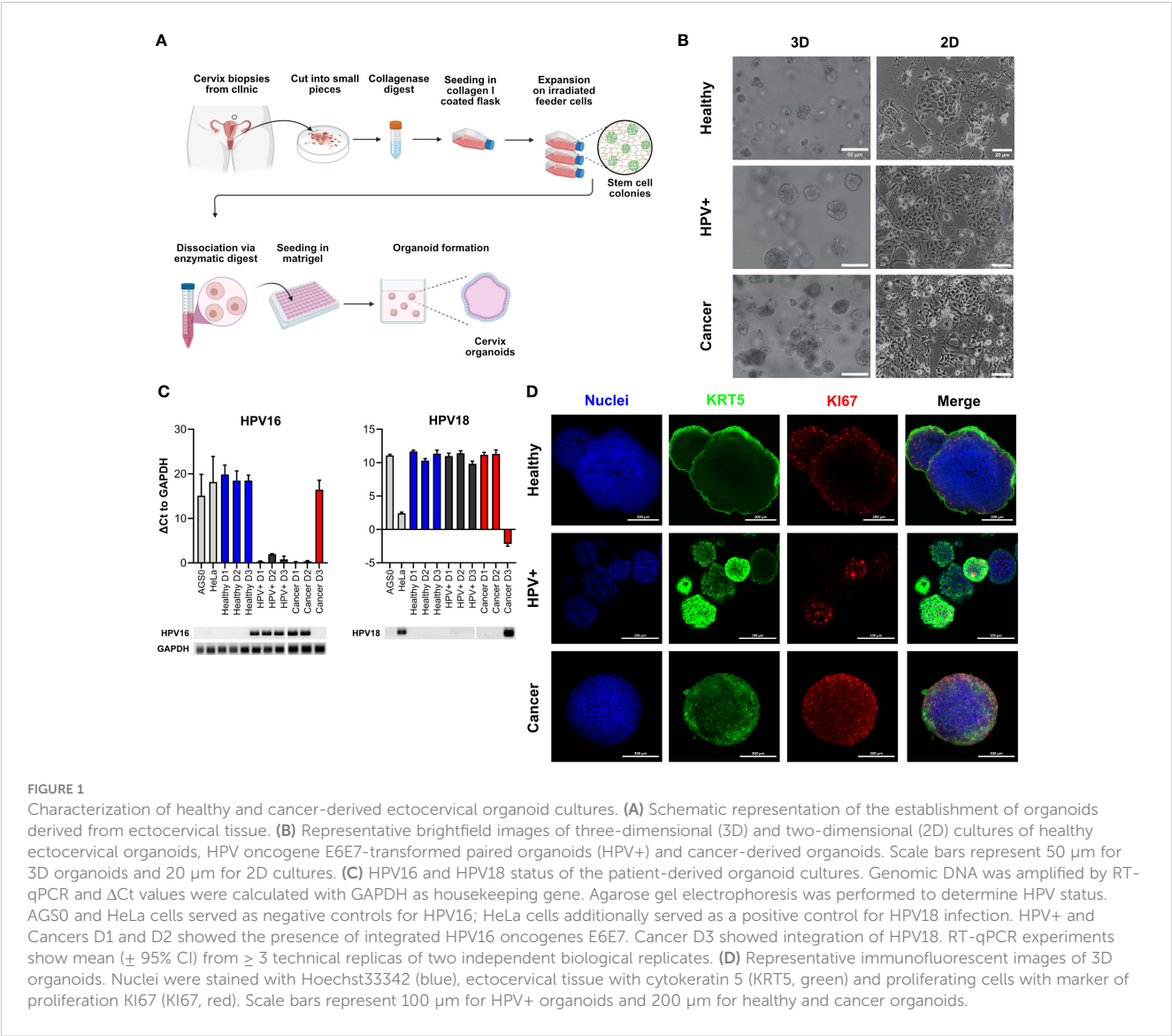


TABLE 1 Characteristics of the organoid lines.

ID	Age at surgery	Disease	HPV16/18 status
Healthy Donor 1	58	not applicable	HPV16/18 free
Healthy Donor 2	51	not applicable	HPV16/18 free
Healthy Donor 3	48	not applicable	HPV16/18 free
Cancer Donor 1	35	Squamous cell carcinoma	HPV16+
Cancer Donor 2	54	Squamous cell carcinoma	HPV16+
Cancer Donor 3	61	Squamous cell carcinoma	HPV18+

HPV+ are derived from the respective healthy lines.

reflect the *in vivo* phenotypes of healthy and cancerous ectocervical tissues.

### 3.2 $\gamma\delta$ T cells exhibit enhanced killing of HPV+ and cancer-derived organoids

$\gamma\delta$  T cells are an important subset of T lymphocytes involved in anti-tumour immunity. They can kill tumour target cells by the secretory pathway via the release of perforin and granzymes as well as by death receptor pathways (e.g. Fas/Fas-ligand) (11). To first assess whether the tissue identity is also reflected in the immune response of tumour-killing T cell subsets, we co-incubated *in vitro* expanded V $\gamma$ 9V $\delta$ 2 T cells with healthy, HPV+ and cancer-derived organoid lines. Before co-incubation, the purity of  $\gamma\delta$  T cell lines was assessed; only cultures with > 90%  $\gamma\delta$  T cells were used. Representative examples of purity determinations as analysed by flow cytometry are presented in [Supplementary Figure 1](#). To determine  $\gamma\delta$  T cell-mediated cytotoxicity, organoids were stained

with NucRed Dead 647 (Yellow: general stain, Red: dead cells) as previously described for breast cancer-PDOs (31). This staining enabled the detection of an increasing number of dead cells within the organoid population by automatized live cell imaging. Live cell imaging revealed distinct responses of  $\gamma\delta$  T cells to HPV+ and cancer-derived organoid lines after four hours of co-incubation (Figures 2A–C). The average accumulation of red stained dead cells in these groups was higher in comparison with healthy organoids, as shown by an increased ratio of Dead/Live signal (Figures 2A–C). When visualizing individual organoid's Dead/Live (Red/yellow) ratios as scatter plots, a clear shift to the Red/Dead signal was observable in cancer and HPV+ organoids when co-incubated  $\gamma\delta$  T

cells, even in the absence of the pAg BrHPP (Figure 2B). We observed a significant increase in the ratio of red/dead to yellow/live signal in cancer and HPV+ organoids in comparison to healthy organoids after 4 hours after analysing single organoids (Figure 2C). Consistently, additional activation of  $\gamma\delta$  T cells by BrHPP resulted in enhanced cytotoxicity in all organoid lines (Figure 2B; Supplementary Figure 2). Unmerged scatters plots for untreated, unstained, puromycin-treated and dead cells are provided in Supplementary Figure 2. To further confirm the findings, organoid-derived cells were co-incubated with  $\gamma\delta$  T cells and cytotoxicity was assessed by an alternative, protease-based cytotoxicity assays after 4h. Higher cytotoxicity was again

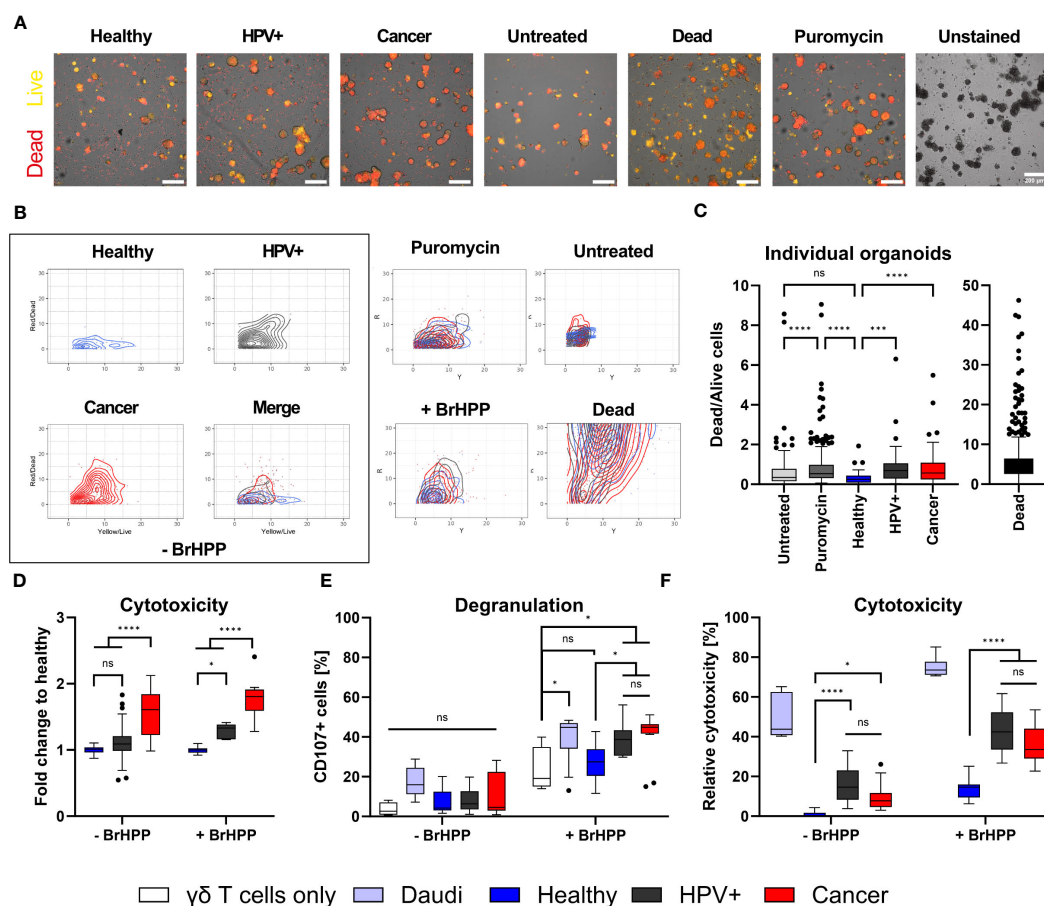


FIGURE 2

$\gamma\delta$  T cells specifically target HPV-positive and cervical cancer organoids. (A) Representative fluorescent images of ectocervical organoids either untreated or co-incubated with  $\gamma\delta$  T cells for four hours.  $\gamma\delta$  T cells were activated with 300 nM BrHPP, "Dead cells" were boiled for 10 min at 95°C before start of the experiment, and Puromycin-treated cells served as death control over time. Cells were stained with NucRed Dead 647 Ready Probe Reagent. Yellow marks viable cells, red marks dead cells. Scale bars represent 200  $\mu$ m. The experiment was performed at least twice with similar results. (B) Scatter plots of dead/live (red/yellow) cell staining in individual organoids show the increasing shift of yellow to red staining of HPV+ and cancer organoids. Merge of all conditions for organoids incubated with  $\gamma\delta$  cells in the absence of 300 nM BrHPP is seen in the box at the lower right. Merges of the control conditions are given. Unmerged control conditions are depicted in Supplementary Figure 2. (C) Ratio of dead/live (red/yellow) cell staining of individual organoids after four hours. Organoids with a ratio > 10 were excluded from the graph for visual purposes. Data shows  $\geq 40$  organoids per condition. Statistical significance between conditions was determined using a Kruskal-Wallis test with Dunn's correction for multiple testing. \*\*\* $p < 0.001$  \*\*\*\* $p < 0.0001$  (D) Quantification of cytotoxicity measured by MultiTox-Glo Multiplex Cytotoxicity Assay after four hours of co-incubation of organoids with  $\gamma\delta$  T cells in the absence or presence of 300 nM BrHPP as indicated. Data was normalized to untreated or BrHPP-treated controls and the ratio of dead/live cells was calculated; fold-change to healthy control was assessed. Data is derived of 12–20 replicates per condition from two independent experiments. (E) Quantification of degranulation of  $\gamma\delta$  T cells by CD107a staining with and without additional activation of  $\gamma\delta$  T cells by BrHPP using flow cytometry. The B lymphoma cell line Daudi was included as a positive control. Data shows four independent experiments. (F) Quantification of cytotoxicity induced by  $\gamma\delta$  T cells by propidium iodide (PI) staining with and without activation of  $\gamma\delta$  T cells by BrHPP using flow cytometry. Data shows  $\geq 3$  independent experiments run in triplicates. All box plots show Median with Tukey whiskers. Statistical significance in (D–F) was determined using a Two-Way ANOVA with Tukey's correction for multiple testing. ns not significant \* $p < 0.05$  \*\*\*\* $p < 0.0001$ .

observed in cancer PDOs after co-incubation with unstimulated  $\gamma\delta$  T cells (Figure 2D, left), while treatment with BrHPP additionally resulted in a significant increase in  $\gamma\delta$  T cell-mediated cytotoxicity against HPV-transformed organoids and cancer lines (Figure 2D, right).

To confirm the differences in  $\gamma\delta$  T-mediated cytotoxicity induced by  $\gamma\delta$  T cells against healthy and HPV+ and cancer-derived cervical organoids, we determined degranulation/Lamp-1 mobilization of  $\gamma\delta$  T cells by flow cytometry. As positive control we included the B lymphoblast cancer cell line Daudi. As expected, we could show that the addition of BrHPP significantly enhanced degranulation in all conditions (Figure 2E, right). However, the addition of BrHPP also resulted in a significant increase in degranulation of HPV+ and cancer organoids in comparison with healthy controls (Figure 2E). Confirming our previous results using an alternative cytotoxicity read-out, we demonstrated that unstimulated  $\gamma\delta$  T cells already exhibit significantly increased cytotoxic activity against cancer and HPV+ cells in comparison to healthy controls as measured by propidium iodide (PI) staining to detect dead cells (Figure 2F). Again, the addition of BrHPP increased the overall response and significantly enhanced the killing of HPV+ and cancer organoids as compared to healthy cells (Figure 2F).

### 3.3 Transcriptomic and protein analysis reveals differences in molecules relevant for $\gamma\delta$ T cell activation

We have observed significant differences in  $\gamma\delta$  T cell-mediated cytotoxicity against distinct organoid lines. To identify putative underlying factors facilitating increased cytotoxicity on cancer and HPV+ organoids, explorative bulk RNA-sequencing (RNAseq) was conducted. Gene expression analysis revealed differences between conditions and also between patient isolates (Figure 3A; Table 1). Cancer lines exhibited comparable expression of genes that were distinguishable from the other conditions, as shown by the first principal component (Figure 3A; Supplementary Figure 3). Isolates transformed with HPV oncogenes E6E7 grouped as intermediates between healthy and cancer organoids (Figure 3A; Supplementary Figures 3, 4). HPV+ isolates generally moved into the cancer direction on the first principal component. Interestingly, the transformation of HPV+ D3 resulted in a clear shift in the second principal component from the other lines. Differential gene expression analysis showed a strong upregulation of cell proliferation-associated genes in the HPV transformed isolates such as *CDC7*, *MCM6/7*, and *RFC3* (Figure 3B; Supplementary Material Table 1). It is notable that there is a clear bias of upregulation of genes in the HPV+ condition in comparison with healthy organoids (Figure 3B). Cancer organoids showed an upregulation of pathways involved in DNA-damage, cell cycle checkpoint, and DNA-binding/modification pathways and downregulation of pathways involved in differentiation of squamous epithelia (Figure 3B; Supplementary Figure 4; Table 1). No significant elevation of antiviral/IFN-associated pathways was detected.

Having gained insights into the differential expression of transcripts between conditions we further explored the possible underlying mechanisms of enhanced killing of HPV+ and cancer organoids. For this, we investigated the expression of putative  $\gamma\delta$  T cell ligands and molecule involved in  $\gamma\delta$  T cell activation (Figure 3C) as summarized in Kabelitz et al. (11). As expected, we observed pronounced differences in expression patterns between the conditions. Upon transformation with E6E7 gene expression patterns of healthy organoids shifted more towards the cancer direction. Some molecules associated with  $\gamma\delta$  T cell activation such as the *Butyrophilin (BTN)*-family, *UL16 Binding Protein (ULBP)*-family, *MHC Class I Polypeptide-Related Sequence A (MICA)* and *B (MICB)*, *Annexin A2 (ANXA2)*, and *Apolipoprotein A1 (APOA1)* were lower in cancer organoids. In contrast, others such as *protein C receptor (PROCR)*, and *ATP Synthase Peripheral Stalk-Membrane Subunit B (ATP5F1/BP)* were expressed in a higher amount than healthy controls (Figure 3C). CD1d was only highly expressed in Cancer D3.

In addition to the well characterized genes involved in  $\gamma\delta$  T cell activation, we also investigated the regulation of other genes such as members of the *human MutS homologue (MSH)*-family, *heat shock protein family D (Hsp60) member 1 (HSPD1)* and *Killer Cell Lectin Like Receptor G2 (KLRG2)*. These genes have been described to play a role in DNA mismatch repair or are involved in stress signalling of cells (39, 40). Interestingly, ectopically expressed *MSH2* has been previously identified as a target for human V $\gamma$ 9V $\delta$ 2  $\gamma\delta$  T cells (11, 39, 41, 42). We found these genes to be upregulated in HPV+ and cancer organoids (Figure 3C). Another highly upregulated gene was found to be *Butyrophilin-Like Protein 9 (BTNL9)* which has been implicated to be of prognostic significance in various cancer types (43, 44). Its potential involvement in the regulation of  $\gamma\delta$  T cell mediated killing of tumour cells is unknown but deserves further investigation in light of our current findings with HPV-transformed and cancer organoids. Additionally, *programmed cell death ligand 1 (PD-L1/CD274)* was significantly downregulated in cancer organoids. The PD-L1 receptor PD1 conveys a negative signal on T cells. (45). PD-L1 downregulation might therefore support the enhanced killing of cancer cells by  $\gamma\delta$  T cells.

To confirm whether the regulation of transcripts could be translated into protein expression, we analysed the protein expression of selected differentially expressed genes by western blotting (Figure 3D). We confirmed that KI67 was elevated in cancer-derived organoids, consistent with earlier reports (46) and as was observed in immunofluorescence (Figure 1D). Additionally, expression of *MSH2* was upregulated in HPV+ and cancer organoids and PD-L1 was downregulated in cancer organoids in comparison with healthy controls, pointing to possible functional differences in  $\gamma\delta$  T cell-mediated killing. As PD-L1 needs to be expressed on the cell surface to fulfil its function, we also conducted flow cytometry to confirm our western blotting and RNA-sequencing results, which, indeed, confirmed decreased surface expression of PD-L1 in cancer organoid isolates (Figure 3E).

Next, we addressed the functional relevance of some of the above molecules for  $\gamma\delta$  T cell-mediated killing of HPV+ and cancer organoids. To this end, we co-incubated organoids with expanded  $\gamma\delta$  T cells with BrHPP in the absence or presence of blocking



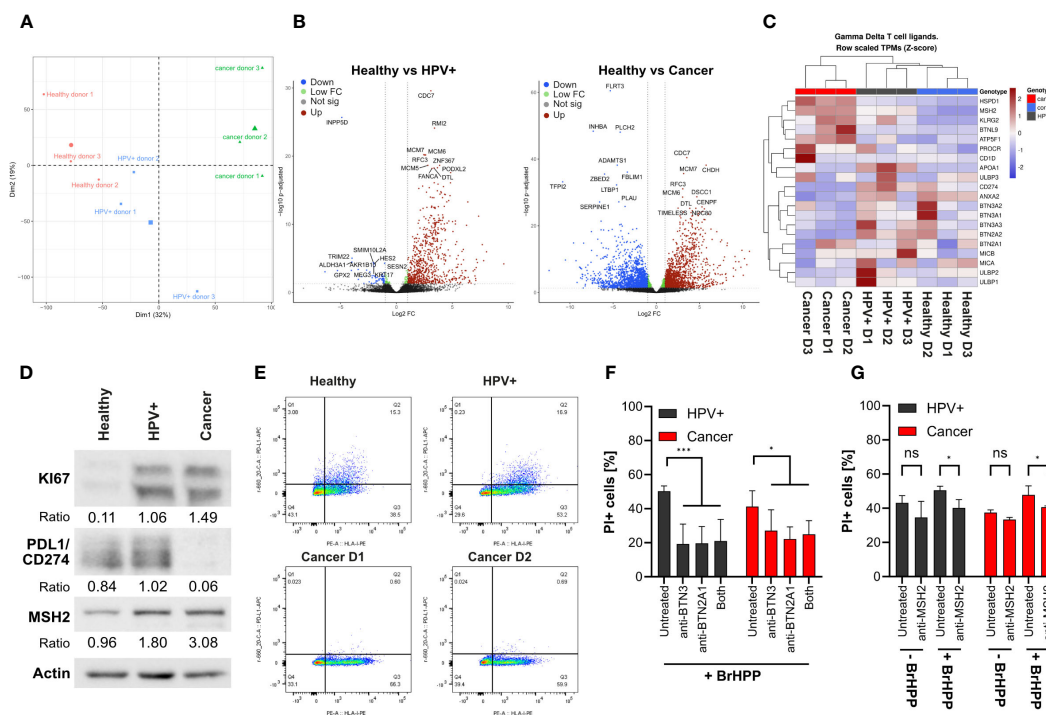


FIGURE 3

$\gamma\delta$  T cell mediated killing is partially mediated by BTN family members and MSH2. (A) PCA analysis of bulk RNA sequencing of the organoid lines. (B) Volcano plots of differential gene expression analysis, showing mean Log2 fold-change vs  $-\log_{10}$  adjusted p values. HPV16 integration alters the organoid transcriptome. RNA sequencing was performed for 3 independent patient organoid isolates per condition. Downregulated genes are marked in blue, differentially regulated genes with higher p values in green, not significantly regulated genes in grey, and upregulated genes in red. Top10 most significantly up- and downregulated genes are highlighted. (C) Heatmap of the gene expression of  $\gamma\delta$  T cell ligands highlighting the downregulation of BTN family members and the upregulation of DNA damage associated molecules in the cancer isolates. (D) Confirmation of RNAseq results by Western blotting highlighting the differential expression of selected proteins in cancer organoids. (E) Dot plot of PDL1/CD274 (Y axis) and HLA class I (X axis) expression analysed by flow cytometry on organoids as indicated, confirming protein expression results in (D). (F) Quantification of cytotoxicity in organoid lines by propidium iodide staining with and without blocking antibodies for BTN2A1/3 by BrHPP activated  $\gamma\delta$  T cells using flow cytometry. Data shows mean ( $\pm$  95% CI) of three independent experiments. (G) Representative quantification of a flow cytometry experiment using anti-MSH2 antibody (2-4 replicates per condition). The percentage of propidium iodide-positive cells is shown. Statistical significance in F and G was determined using a Two-Way ANOVA with Tukey's correction for multiple testing. ns not significant \* $p < 0.05$  \*\*\* $p < 0.001$ .

antibodies against BTN2A1 and BTN3A1 (Figure 3F). While the inhibitory anti-BTN3 antibody used here (clone 103.2) does not discriminate between isoforms BTN3A1/A2/A3 (47), the isoform critically involved in  $\gamma\delta$  T cell activation is BTN3A1 (48). We found that blockade of BTN2A1 or/and BTN3A1 significantly reduced killing in all conditions as was in line with earlier reports (49). However, blocking in cancer organoids seemed to be slightly less affected than in HPV+ organoids (Figure 3F). In view of the results shown in Figures 3C, D, we also addressed the potential role of MSH2 which has been identified by Dai et al. (39) as a  $\gamma\delta$  T-cell ligand when ectopically expressed on the surface of tumour cells. Blocking of MSH2 by antibodies led to an overall decrease of  $\gamma\delta$  T cell-mediated cytotoxicity, most notably against cancer organoids in the presence of BrHPP (Figure 3G). Blocking could decrease  $\gamma\delta$  T cell-mediated cytotoxicity of BrHPP activated  $\gamma\delta$  T cells to the level of unstimulated  $\gamma\delta$  T cells (Figure 3G). While the inhibitory effect of anti-MSH2 antibody blocking was much lower than the effect of anti-BTN2A1/3A1 antibodies, our results suggest that multiple ligand-receptor interactions are involved in the organoid- $\gamma\delta$  T-cell interaction.

In summary, we have identified multiple possible target molecules and pathways that may contribute to enhanced the killing of HPV-infected cervical tissue by  $\gamma\delta$  T cells. While the precise role of some molecules (including BTNL9) requires further investigation, the currently established organoid-  $\gamma\delta$  T cell co-culture system should prove useful for testing additional modulators (e.g. small molecules, antibodies, tumour sensitizers, etc.) with the ultimate goal of improving the efficacy of  $\gamma\delta$  T cell immunotherapy for cervical cancer.

## 4 Discussion

The association between HPV infection and the onset and the development of cancer has been extensively studied since almost 50 years (50). However, there has been a lack of suitable models that accurately represent and maintain the characteristics of cervical tissue. Previous studies have relied on immortalized cell lines or tissue slices (20). Here, we present a model to study the interaction of tumour-reactive T lymphocyte populations with healthy,

matched HPV16 E6E7 transformed, and cancer-derived primary ectocervical tissues. To achieve this, we utilized an earlier established human ectocervical organoid model including paired HPV16 E6E7 transformed organoids (5, 6). We detected infection-specific differences in proliferation patterns of healthy and HPV-infected organoids by staining with KI67 antibody. Healthy controls exhibited proliferating cells only in the parabasal regions of organoids, cancer organoids showed a wider distribution of proliferation while HPV+ showed an intermediate phenotype. Similar was observed in HPV+ tissue and cancer biopsies confirming the preservation of tissue origin characteristics (46, 51).

$\gamma\delta$  T cells play a distinct role in the immune surveillance against virally infected (including HPV) cells and tumour cells (3, 8, 11, 52). They are believed to be important in the cervical defence against HPV infection (3, 53). To establish an *in vitro* model for in-depth analysis, we co-incubated organoid-derived cells with *in vitro* expanded V $\gamma$ 9V $\delta$ 2  $\gamma\delta$  T cells isolated from healthy donors. In view of the HLA independence of  $\gamma\delta$  T cells, the use of allogeneic  $\gamma\delta$  T cells from healthy donors is a valuable approach also from a translational perspective. In fact, the adoptive transfer of allogeneic  $\gamma\delta$  T cells from healthy donors has already been applied in phase I studies with no major adverse reactions (10). It was thus possible to compare responses of  $\gamma\delta$  T cells to healthy, HPV-transformed as well as tumour organoids. Our results demonstrated elevated responses of unstimulated and phosphoantigen-activated  $\gamma\delta$  T cells to HPV-infected cells in comparison to healthy controls, independently of the type of assay used. Live cell imaging, protease-dependent cytotoxicity assays, and flow cytometry-based cytotoxicity and degranulation assays demonstrated elevated responses to HPV+ and/or cancer organoids. However, the observed differences in the potency of the assays might reflect differential involvement of the secretory (perforin and granzyme-dependent) and death receptor pathways (e.g., TRAIL, Fas/CD95). It is well known that  $\gamma\delta$  T cells can utilize both pathways to kill tumour targets (54, 55). The organoid co-culture system established in our current study will allow us to dissect the precise mechanisms of killing of cancer versus HPV-infected organoids by  $\gamma\delta$  T cells in future studies. This will also include the analysis of soluble mediators such as TRAIL, Fas-ligand, granzyme B, IFN $\gamma$ , and others.

To unravel possible mechanisms behind the responses of  $\gamma\delta$  T cells to HPV16-infected organoids, we conducted bulk RNA sequencing. The RNAseq revealed differences between HPV infected vs healthy controls. Importantly, HPV-transformed organoids displayed an intermediate state between healthy and cancer organoids with replicating some, but not all patterns of cancer tissue. Consistent with earlier studies we detected evidence for HPV16-induced DNA damage (5, 56). Interestingly, HPV-induced antiviral responses including the type I interferon pathway were negligible in the pathway analysis. Dekkers et al. (31) have earlier reported a high association between cancer cell killing and the preservation of tumour-specific inflammatory features in breast cancer organoids. In their study, sensitivity to cancer metabolome-sensing engineered T cells (TEGs) was highly associated with the expression of antiviral genes such as *MX1*, *IFIT1*, *OASL* and *XAF1* (31).

Our pathway analysis demonstrated some overlap in DNA-modification pathways and  $\gamma\delta$  T cell ligands or associated molecules

between cancer and HPV+ conditions. We reproduced earlier reports (57, 58) of altered transcription and expression of genes such as *TTK protein kinase (TTK)*, *maternal embryonic leucine zipper kinase (MELK)*, *forkhead box M1 (FOXM1)*, *mismatch repair enzyme as mutS homolog 2 (MSH2)*, *matrix metalloproteases (MMPs)* and *programmed cell death ligand 1 (PD-L1/CD274)*. While up-regulation of *PD-L1* is typically associated with poorer outcomes in chemotherapy treatment (58), it was almost absent in our cancer isolates. While exhaustion the role of *Programmed Cell Death 1 (PD-1/CD279)*, *Hepatitis A Virus Cellular Receptor 2 (TIM3/HAVCR2)*, *Lymphocyte Activating 3 (LAG3)* and other checkpoint molecules has been less studied in  $\gamma\delta$  T cells compared to conventional  $\alpha\beta$  T cells (59), the low to absent PD-L1 expression on cancer organoids may favour persistent effector activity of  $\gamma\delta$  T cells. Interestingly, the expression of BTN-family members including *BTN2A1/3A1*, crucial in pAg-mediated  $\gamma\delta$  T cell activation was largely down-regulated in the cancer isolates. However, our blocking studies using inhibitory antibodies against *BTN2A1* and *BTN3A1* clearly indicated the significance of these BTN molecules in triggering the cytotoxic activity of  $\gamma\delta$  T cells against HPV+ and cancer organoids, in line with previous reports (49). While not directly measured in our study, it can be assumed that HPV+ and cancer organoids produce increased amounts of endogenous pAg isopentenyl pyrophosphate (IPP), leading to *BTN2A1/3A1*-dependent  $\gamma\delta$  T cell activation and subsequent tumour cell killing (8, 11, 60). It is noteworthy, however, that blocking of *BTN2A1/3A1* pathways by inhibitory antibodies only partially reduced the killing of organoids. Given that such antibody blockade completely wipes out all phosphoantigen-mediated activation of human  $\gamma\delta$  T cells, this strongly suggests that other pathways are involved in organoid- $\gamma\delta$  T cell interaction, one of which might be *MSH2*. Additionally, transcripts of *BTNL9* and cellular stress molecules *HSPD1*, *KLRG2*, and DNA damage associated molecule *MSH2* were upregulated in cancer organoids. While the roles of *HSPD1* and *KLRG2* play a role in regulating  $\gamma\delta$  T cell-mediated cytotoxicity in cancer have been described (61, 62), there is limited information about the involvement of *BTNL9* in  $\gamma\delta$  T cell-mediated killing of cancer cells. Given our results, further investigation is warranted. Moreover, the upregulation of *MSH2* may be functionally relevant. *MSH2* ectopically expressed on the surface of tumour cells has been identified as a ligand for the human V $\gamma$ 9V $\delta$ 2 TCR (39, 41, 42). Our study demonstrated that the killing of HPV+ and cancer organoid cells by BrHPP-activated  $\gamma\delta$  T cells was reduced in the presence of anti-*MSH2* antibody, supporting the hypothesis that the increased levels of *MSH2* contribute to sensitizing of HPV+ and cancer organoid cells to cytotoxicity mediated by  $\gamma\delta$  T cells. Taken together, our findings suggest that *BTN2A1/3A1* and *MSH2* are involved in triggering the cytotoxic activity in  $\gamma\delta$  T cells towards HPV+ and cancer organoids, but additional pathways might be involved.

In summary, we have established a suitable model to study cellular immune responses to healthy, HPV-transformed and cancerous ectocervical tissue using organoids in combination with short-term expanded  $\gamma\delta$  T cells. Co-incubation of organoid-derived cells with  $\gamma\delta$  T cells resulted in enhanced killing of HPV-transformed and cancer organoids. We demonstrated the

involvement of BTN-family members BTN2A1/3A1 in  $\gamma\delta$  T cell-mediated cytotoxicity and obtained evidence for the additional involvement of human MutS homologue (MSH)-family member MSH2. The presented *in vitro* model will be highly valuable for in-depth analysis of additional pathways and mechanisms of how to enhance  $\gamma\delta$  T cell effector function with the ultimate goal of improving  $\gamma\delta$  T cell immunotherapy of cervical carcinoma. Such strategies might include (but are not restricted to) sensitizing cancer cells with  $\gamma\delta$  T cell activating drugs like zoledronate (63), bispecific  $\gamma\delta$  T cell engagers (64), or agonistic anti-BTN3A1 antibodies (65).

## Data availability statement

The original contributions presented in the study are publicly available. The data can be found under GSE246571 in GEO at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE246571>.

## Ethics statement

The studies involving humans were approved by the ethical committees of the Charité Berlin and the Medical Faculty of the Christian-Albrechts-Universität zu Kiel. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

JD: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. DH: Data curation, Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. CP: Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – review & editing. SK: Methodology, Resources, Writing – review & editing, Validation. ME: Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. AQ-O: Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – review & editing. HB: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review & editing. MZ: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. MM: Methodology, Resources, Writing – review & editing. RKG: Conceptualization, Methodology, Resources, Writing – review & editing. NH: Methodology, Project administration, Supervision, Writing – review & editing. CC: Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. DK: Methodology, Project administration, Supervision, Writing – review & editing. TFM: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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## Conflict 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.

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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.2023.1281646/full#supplementary-material>

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## EDITED BY

Virginie Lafont,  
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## REVIEWED BY

Julien Faget,  
INSERM U1194 Institut de Recherche en,  
Cancérologie de Montpellier (IRCM),  
France  
Xue-Yan He,  
Washington University in St. Louis,  
United States  
Steven Edwards,  
University of Liverpool, United Kingdom

## \*CORRESPONDENCE

Wence Zhou  
✉ zhouwc129@163.com

<sup>†</sup>These authors have contributed equally to  
this work

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# Neutrophil extracellular traps regulating tumorimmunity in hepatocellular carcinoma

Weixiong Zhu<sup>1,2†</sup>, Chuanlei Fan<sup>3†</sup>, Shi Dong<sup>1,2</sup>, Xin Li<sup>1,3</sup>,  
Haofei Chen<sup>1,2</sup> and Wence Zhou<sup>1,2\*</sup>

<sup>1</sup>The Second Clinical Medical College, Lanzhou University, Lanzhou, China, <sup>2</sup>Department of General  
Surgery, The Second Hospital of Lanzhou University, Lanzhou, China, <sup>3</sup>The First Clinical Medical  
College, Lanzhou University, Lanzhou, China

As a component of the innate immune system, there is emerging evidence to suggest that neutrophils may play a critical role in the initiation and progression of hepatocellular carcinoma (HCC). Neutrophil extracellular traps (NETs) are web-like chromatin structures that protrude from the membranes during neutrophil activation. Recent research has shown that NETs, which are at the forefront of the renewed interest in neutrophil studies, are increasingly intertwined with HCC. By exploring the mechanisms of NETs in HCC, we aim to improve our understanding of the role of NETs and gain deeper insights into neutrophil biology. Therefore, this article provides a summary of key findings and discusses the emerging field of NETs in HCC.

## KEYWORDS

hepatocellular carcinoma (HCC), neutrophil extracellular trap (NET) formation, tumorigenesis and progression, liver transplantation, liver ischemia-reperfusion injury (liver I/R injury)

## 1 Introduction

Primary liver cancer is the third leading cause of cancer-related death worldwide (1). Hepatocellular carcinoma (HCC), the most common form of primary liver cancer, often develops in the setting of chronic liver disease and cirrhosis (2, 3). The progression of HCC involves the evasion of immune surveillance and the establishment of an immunosuppressive tumor microenvironment (TME), thereby inhibiting cytotoxic immune cells (4). HCC is characterized by a high infiltration of leukocytes from multiple immune cell lineages, which negatively affects effector lymphocyte activity and correlates with poor outcomes (5–7). Therefore, the current focus in HCC research lies not only on lymphocytes but also on exploring the interactions of multiple immune cells (4).

Neutrophils, one of the phagocytes, as the first line of defense against pathogen invasion by employing their potent antimicrobial arsenal and also contribute to the activation of adaptive immunity (8, 9). In addition, neutrophils play a critical role in tumor progression and tumorigenesis through the release of various mediators. Tumor-related inflammation, in contrast to conventional inflammation, promotes tumor initiation

and growth by facilitating the evasion of immune surveillance (10). Neutrophils, as representatives of conventional inflammation, show distinct effects on the malignant phenotype of tumors, as supported by numerous lines of evidence (4, 11, 12).

Previous studies have focused primarily on the role of neutrophils as pathogen scavengers during conventional inflammation (13). However, it is now recognized that neutrophils have multiple and diverse functions (13). In humans, neutrophils represent 50–70% of all circulating leukocytes, whereas in mice they make up 10–25% (14, 15). Notably, neutrophils play a crucial role in chronic inflammation, specifically in chronic liver disease and malignancies, facilitating immune infiltration (16, 17). The challenge at hand is that tumor immunotherapy has shown limited efficacy in many patients with HCC, which represents a significant obstacle in the field (18–21). For certain subgroups of liver cancer patients, a combination of targeted therapy and immunotherapy targeting infiltrating immune cells (except for T lymphocytes), may prove more effective than immune checkpoint inhibitors such as atezolizumab plus bevacizumab. By modulating the functional characteristics of neutrophils, it may be possible to enhance the sensitivity of HCC patients to systemic therapy by altering the immune microenvironment (4).

In addition to producing oxidants, proteins, and granular enzymes, neutrophils also have the ability to generate NETs (9, 22, 23). NETs consist of aggregated DNA that serves as a backbone that interacts with various molecules either positively or negatively (24). By forming NETs, activated neutrophils can ensnare a wide range of microorganisms, including bacteria (24, 25), fungi (26, 27), and viruses (28–31), enabling an effective response and subsequent clearance through the action of effector molecules (23). Numerous studies have provided evidence for the involvement of NETs in tumor initiation, progression, and angiogenesis (32–34). Interestingly, NETs also play a similar role in HCC (35, 36).

In this article, we review evidence for the presence of NETs in HCC which may support the hypothesis that NETs play a pivotal role in the pathogenesis of liver cancer. We discuss the proposed mechanisms by which NETs may drive tumor progression. Furthermore, considering that research on NETs in HCC is still at an early stage, we examine studies conducted on NETs in other types of cancers and discuss emerging areas of interest.

## 2 NETs discovery, origin and diversity

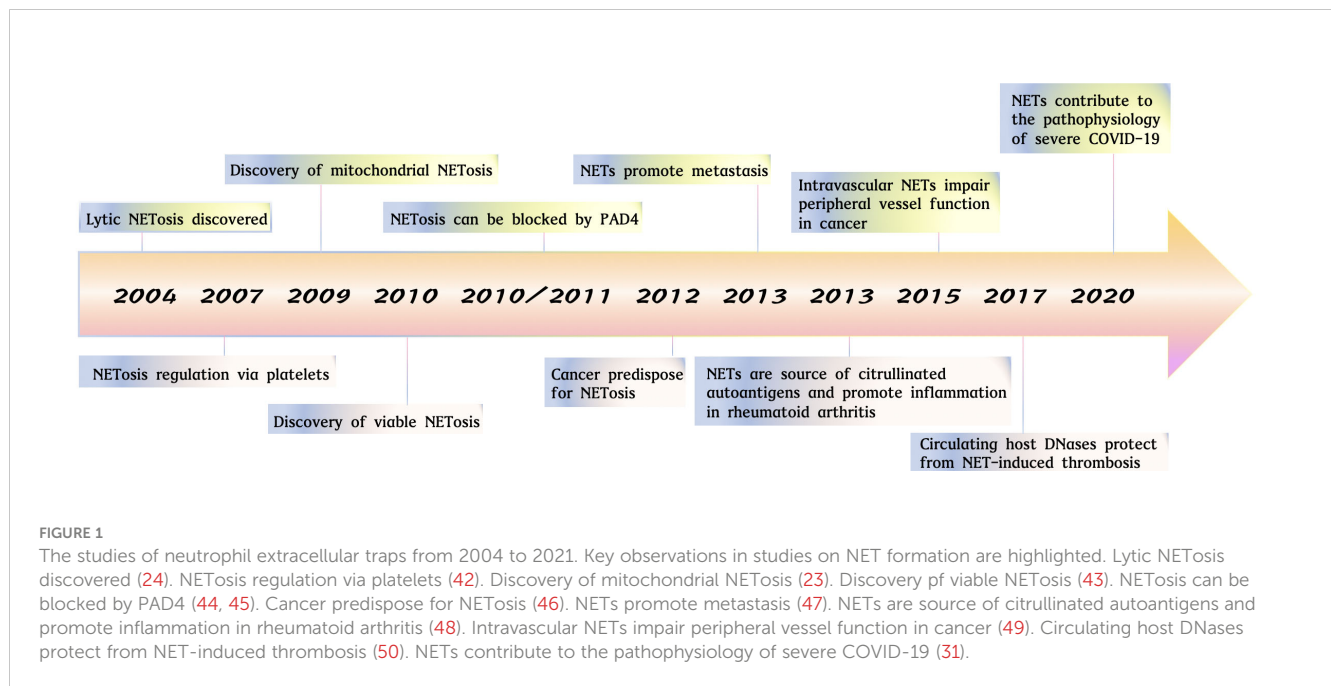
### 2.1 The historical and current existence of NET

NET formation, the process responsible for the formation of NETs to eliminate invaders through the release of granular proteins and chromatin, can be classified into two subtypes: lytic and vital (24, 37). Lytic NET formation, also known as suicidal NET formation, involves a slow, active cell death that occurs over several hours, distinguishing it from other forms of cell death such as necrosis or apoptosis (37). In addition to suicidal NET formation, another rapid process known as “vital NET formation”, which rapidly expels DNA from the nucleus or mitochondria of

living cells within a few minutes while maintaining cell functionality and viability.

Phorbol 12-myristate 13-acetate (PMA), a classic nonphysiological stimulus, induces a distinct form of neutrophil death that is fundamentally different from apoptosis and necrosis (38). PMA promotes cell death by increasing chromatin loosening and incorporating the nuclear envelope into organelles, a process that occurs within a few hours (39, 40). This process involves four key steps: (1) enhanced plasma membrane permeability, (2) accelerated disintegration of the nuclear envelope and cytoskeleton, (3) assembly of antimicrobial proteins onto chromatin scaffolds, and (4) decondensation of chromatin (41). *In vitro* experiments using isolated neutrophils treated with PMA have provided valuable mechanistic insights into lytic NET formation (Figure 1), demonstrating that neutrophil activation is accompanied by the assembly and activation of a multicomponent nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and ROS (51–53). ROS can be generated by both NADPH oxidase and mitochondrial metabolism (53). In addition, G protein-coupled receptors (GPCRs) (54), CXC chemokine receptors (CXCRs) (55), toll-like receptors (TLRs) (56, 57), and cytokine receptors contribute to this process. Hydrogen peroxide ( $H_2O_2$ ) is a robust oxidant generated by NADPH oxidase during the reduction of molecular oxygen through electron transfer, and it plays a crucial role in both NET and ROS generation (41). The correlation between neutrophil metabolism and ROS generation has been highlighted in mechanistic studies (58, 59). In NADPH oxidase-deficient neutrophils, PMA compensates for the lack of NADPH oxidase and, in combination with protein kinase C (PKC), triggers calcium release and subsequent activation of the Raf-MEK-ERK pathway (60) and ROS-dependent p38MAPK (61). However, in mice, NET formation may not be associated with ROS generated by NADPH oxidase (62). In addition to the classical pathway requiring ROS, two recent papers reported a novel pathway of NETosis mediated by a pore-forming protein, gasdermin D (GSDMD), that is independent of ROS (63, 64). Actin cytoskeletal dynamics plays a critical role in neutrophil activation, and during the first half hour after stimulation, limiting actin polymerization reduces NET formation (65).

Peptidyl-arginine deiminase 4 (PAD4), an intracellular calcium-dependent enzyme, activated by intracellular calcium levels. It converts arginine to citrulline. Upon translocation to the nucleus, PAD4 deaminates histones H2A, H3, and H4, resulting in decreased chromatin compactness (66, 67). Notably, mouse model studies indicate that NET formation explicitly relies on PAD4 and neutrophil elastase (NE) (62). However, recent research suggests that PAD4 is mainly required for NET formation in response to ionomycin and immune complexes, whereas it is dispensable for NET formation induced by cholesterol crystals, fungi, or PMA in human neutrophils (44, 68, 69). Inhibition of PAD2, another member of the PAD family, can reduce NET formation and inflammatory cytokine production (45). NE, which is released from intracellular granules into the cytoplasm during NET formation, may then degrade linker histones to promote chromatin decondensation. Interestingly, NE and myeloperoxidase (MPO), released from azurophilic granules and



translocated to the nucleus, cooperate in facilitating chromatin decondensation independently of their enzymatic activities (70, 71).

In 2009, pivotal research identified a distinct form of NET formation known as vital NET formation, which is distinct from lytic NET formation. It is induced by granulocyte-macrophage colony-stimulating factor (GM-CSF), followed by stimulation with lipopolysaccharide (LPS) or C5a, and can occur either a ROS-dependent or independent manner (23, 72, 73). The entire process of vital NET formation proceeds by a unique and remarkably rapid mechanism, that takes only 5–60 minutes and being independent of oxidants (72). Notably, the existence of lytic NET formation upon LPS treatment suggests that different modes of NET formation under different circumstances synergistically contribute to immune defense (74). Importantly, there is heterogeneity in the propensity of neutrophils to undergo NET formation, with studies reporting a range of 10% to 60% of cells capable of NET formation at a given moment (37, 70, 75, 76).

At particular times in their lifespan, a subset of neutrophils expresses Olfactomedin 4 (OLFM4) and CD177, two molecular markers associated with NET biology, regardless of their maturation or activation stage (77, 78). Approximately 25% of circulating neutrophils in healthy individuals express OLFM4 [73]. This expression of OLFM4 in a subset of neutrophils is conserved in mice, and it is secreted and colocalized with NETs (79, 80). Clinical studies suggest that high numbers of OLFM4<sup>+</sup> neutrophils in patients with septic shock are associated with risk of organ failure and death (81). This is consistent with the findings that OLFM4<sup>−</sup> neutrophils, in contrast to their OLFM4<sup>+</sup> counterparts, provide protection against sepsis-induced mortality in OLFM4<sup>−/−</sup> mice following LPS stimulation (79). These studies suggest that OLFM4 may reflect NET-induced toxicity rather than serve as a marker of NET formation. Another neutrophil protein associated with NET formation is CD177, which is expressed by

approximately half of the neutrophils in peripheral blood. Research has shown that CD177<sup>−</sup> neutrophils do not respond to LPS stimulation via NET formation (82, 83). Therefore, the regulation of OLFM4 and CD177 in NET formation may be a promising avenue for therapeutic intervention in NET-associated diseases, whether inflammatory or non-inflammatory.

Recently, a significant discovery had been found in the process of NETosis (84). They found that NETosis is triggered by mitogens and results in the upregulation of cell cycle markers, such as the phosphorylation of retinoblastoma proteins and laminin, disruption of the nuclear membrane, and replication of centrosomes. Further investigations revealed that CDK6, and possibly CDK4, are necessary for the signaling of NETosis. Interestingly, inhibition of CDK4/6 did not affect ROS production, leading to the hypothesis that CDK might function downstream of ROS activation. On the other hand, the translocation of NE to the nucleus relies on the activity of CDK4/6. However, the connection between CDK activity and ROS activation remains unclear, particularly since CDK6 also translocates to the nucleus during NETosis (84). CDK activation during NETosis occurs downstream of MAPK, although further experiments are required to confirm this. Future studies should focus on identifying the substrates of CDK4/6 involved in the NETosis pathway, as well as the mechanisms enabling neutrophils to respond to CDK4/6 activation, ultimately resulting in cell death instead of cell proliferation. Notably, investigating the role of CDKs in neutrophils using transfection or gene editing is challenging due to the short lifespan of neutrophils in culture (1–2 days). However, Amulic et al. ingeniously synthesized a peptide that mimics the inhibitory structural domain of p21 associated with CDKs (85). This peptide was coupled with a cell-penetrating sequence, providing an innovative approach for researchers facing difficulties in studying neutrophils due to their short half-life.



## 2.2 NETs content

The structure of NETs consists primarily of loosely packed chromatin composed of DNA and RNA, together with proteins composed predominantly of positively charged histones (86). The positive charge of these proteins facilitates the adsorption of small negatively charged foreign particles, while histones and nucleic acids themselves have bactericidal effects (87). In addition to histones, NET-associated proteins include cytoplasmic, granule, and cytosolic proteins, as well as neutrophil-derived metabolic enzymes (70, 88). Previous studies have shown that NETs not only contain their own effector molecules but also trap and release cytokines from other cells (89). Due to the diverse environmental conditions, both the structure and proteome of NETs exhibit considerable variation (52, 86). However, the core NET proteome, primarily derived from neutrophils, consists of histones, NE, proteinase 3, MPO,  $\alpha$ -defensins, and cathepsin G (90, 91). Therefore, it is essential to identify disease-specific NETs (Figure 2).

## 2.3 Neutrophil and NETs

A growing body of research suggests that not all neutrophils possess the ability to secrete NETs, which have been observed in diverse tissues, organs, and species. It is becoming increasingly clear that the molecular properties of NETs secreted by neutrophils varies

across different stages of neutrophil maturation and among different neutrophil subtypes. Traditionally, neutrophils have been viewed as a homogeneous population of cells, but recent studies have revealed their plasticity, allowing them to adapt their phenotype to the environment and fulfill different functional requirements (11, 13). This adaptability is exemplified by their differential capacity to release NETs. The significant involvement of NETs in disease pathogenesis is underscored by a considerable increase in research efforts over the past decade (Figure 3).

Recent studies have shown that Only about 30% of mice neutrophils produce NETs whereas up to 60% of human neutrophils can produce NETs (70, 75). These observations suggest that not all types of neutrophils exhibit the same ability to release NETs under identical stimulation, though the underlying reasons for these differences remain unclear. Previous research has indicated that the increased capacity for NET formation corresponds to the transition from the immature stage to the mature stage in bone marrow-derived neutrophils of mice (92). However, circulating neutrophils, while still capable of releasing NETs, exhibit a reduced capacity for NET formation during their immature stage (93). The variation in the ability of neutrophils to generate NETs at different stages of disease in different tissue environments remains unclear and requires further investigation. It remains to be determined whether there are changes in the components of NETs under these circumstances. Investigation of such changes may provide insight into the initiation of NETs in different diseases and the subsequent progression of these diseases.

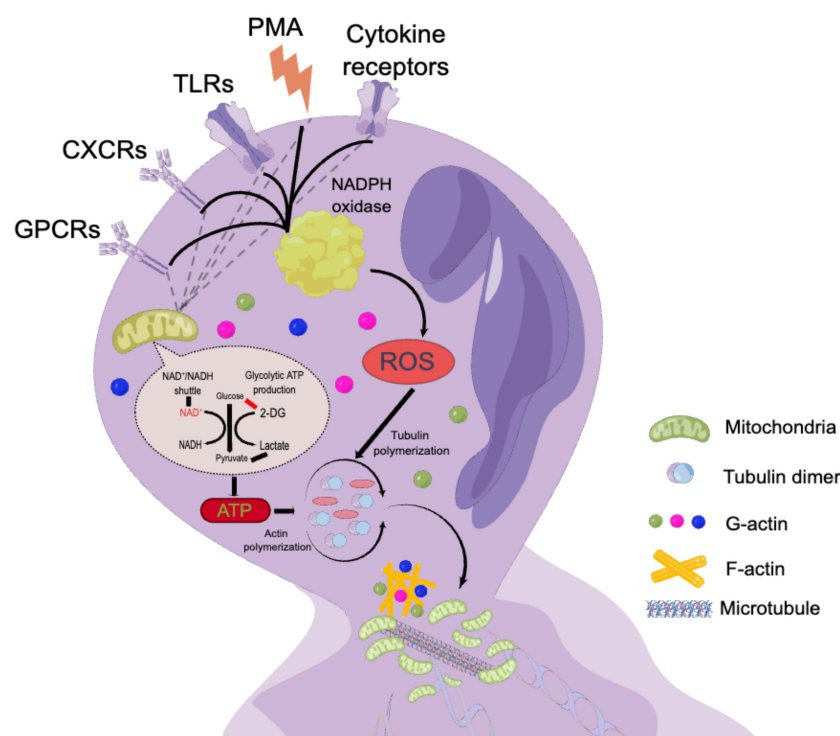
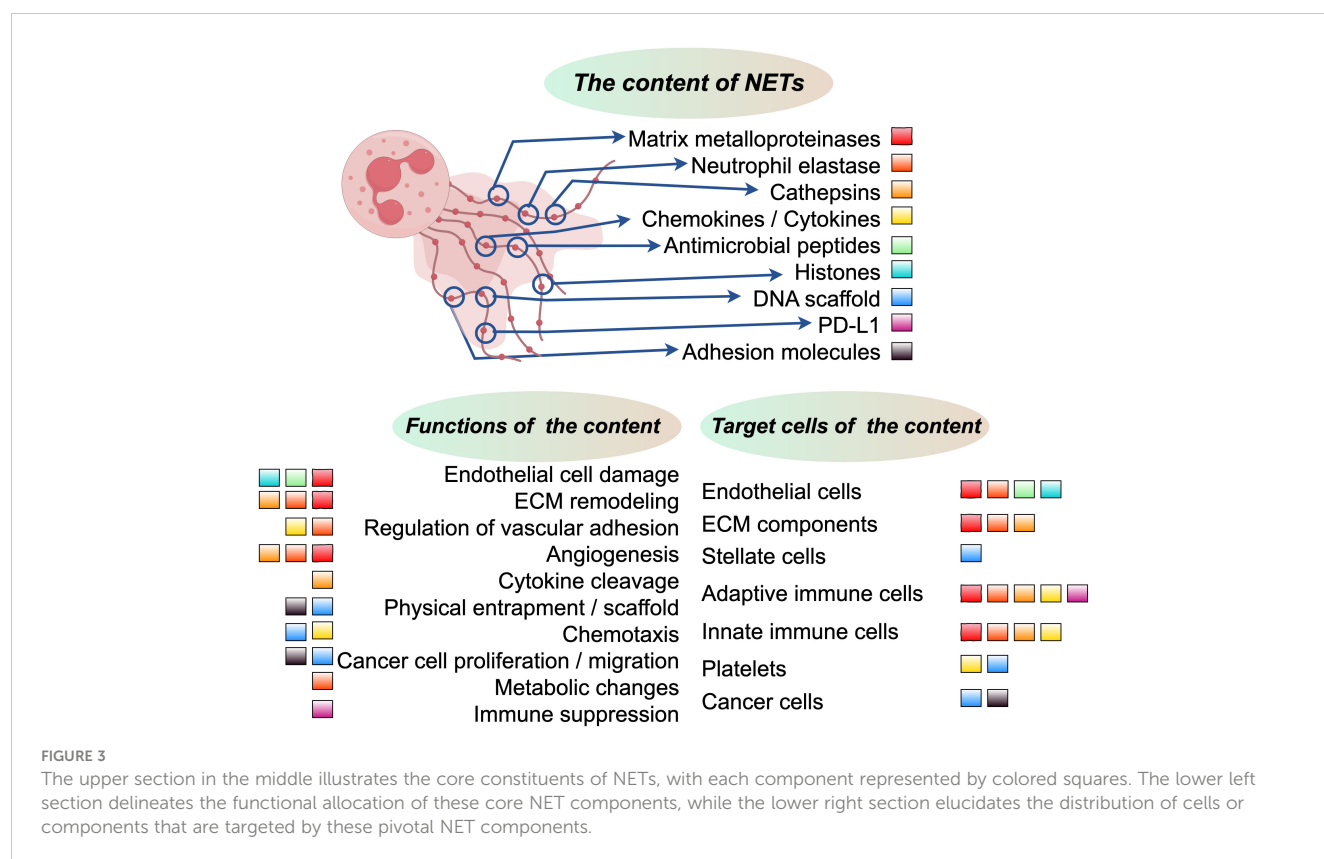


FIGURE 2

The mechanism of lytic NET formation. By processing isolated neutrophils with PMA in neutrophil, which in turn lead to the production of multicomponent NADPH oxidase and ROS (52–54). ROS can be produced by either NADPH oxidase or mitochondrial metabolism (54). GPCRs (55), CXCRs (56), TLRs (57) and cytokine receptors were also involved in this process.



NET formation is subject to circadian regulation that is influenced by the contents of neutrophil, including proteolytic enzymes, antimicrobial peptides, and adhesion molecules (94). Intriguingly, degranulation has been observed under both acute activation and steady-state conditions. In support of this notion, CXCR2-mediated autocrine signals can induce degranulation, with mouse neutrophils exhibiting reduced content of primary granules during daylight hours after being mobilized into the circulation at night (55). Temporal degranulation which is tightly regulated by the circadian machinery, suggests a significant temporal dependence of NET formation, specifically within the 24-hour cycle. This finding is particularly relevant as recent studies have demonstrated remarkable discrepancies in NET formation *in vivo* for neutrophils isolated at different times, observed in both mice and humans, and replicated in cases of acute lung injury or liver ischemia-reperfusion injury (53). Disturbed circadian patterns have been linked to several diseases, including tumors (95–97). However, the mechanisms underlying the association between NETs and circadian pattern-related diseases are not yet fully understood, and further research is required in this area. It is also unclear whether NETs can serve as a clinically reliable marker to predict the impact of circadian pattern disruption on the development of specific diseases.

Recent studies have highlighted the critical role of the microbiome in regulating neutrophil aging (98). In inflammatory conditions, an overactive subset of aged neutrophils significantly enhances NET formation (98). Aging neutrophils, despite limited evidence for circadian regulation, exhibit a bias toward activation and NET formation in the context of stress-induced inflammation

and vascular occlusion in mouse models of sickle cell disease (98–100). The quantity of NETs associated with *Haemophilus* sp. is elevated in the sputum of patients with chronic obstructive pulmonary disease (101). Accordingly, elimination of gastrointestinal bacteria in mice reduces NET formation; nevertheless, this strategy may have side effects (98). It is important to note that the absence of gut microbiota is correlated with increased NET formation in mouse models of acute mesenteric ischemia-reperfusion injury (102). Based on these observations, it can be concluded that the composition of the microbiome may have different effects on NET formation. Segmented filamentous bacteria promote NET formation in neutrophils, whereas in human neutrophils, *Lactobacillus rhamnosus* strain GG, a member of the probiotic system, inhibits NET formation by attenuating ROS production *in vitro* (98, 103). Interestingly, researchers have discovered that microorganism-derived metabolites can influence NET production in multiple and potentially deleterious ways. The regulation of NET production may depend on diurnal variations in the location, function of the microbiome, and the control of intestinal permeability (104).

The regulation of NET formation at different age and maturation stages is an important aspect to consider. A recent study has demonstrated that neutrophils egress from the bone marrow follows a circadian rhythm, with peak levels occurring in the morning (100). This observation implies that particular subsets of neutrophils, rather than only neutrophils of a certain age, are able to produce NETs. In addition, circadian fluctuations drive increased neutrophil output from the peripheral blood into tissues at night through the regulation of chemokine CXCL2 expression by the

circadian clock gene *Bmal1* and subsequent CXCR2-dependent signaling (100). Interestingly, NET formation in neutrophils also follows circadian rhythms, with recently released neutrophils being more susceptible to these rhythms. The *Bmal1*-CXCL2-CXCR2 axis plays a crucial role in NET formation, and any disruption to its components will impede this process (55). Another study found age-related variability in the ability of neutrophils to generate NETs, which may explain the increased susceptibility of the elderly to infection and age-related inflammation (105). This diminished capacity for NET formation was observed not only in mice (average age of 18 months) but also in humans (average age of ~70 years) (106, 107).

These factors may be attributed, among others, to the age-related increase in neutrophils within the bone marrow and the degranulation of circulating neutrophils. These processes have been linked to a decline in the capacity for NET formation (55). Aside from age, the distribution of neutrophils can also influence NET formation and exhibit significant associations with various diseases. In conditions such as sterile neutrophilia and septicemia, intravascular NETs can form clots in the circulation, obstructing blood vessels and causing organ damage in the absence of host DNases (50, 108). Similarly, the significant susceptibility to thrombosis and inflammatory injury in the liver and lungs may be attributed to the intensive ability of relatively immature neutrophils, which have not been completely cleared from the circulation, to produce NETs (49, 55, 109). In the context of persistent inflammation, neutrophil stimulation at various locations may lead to excessive NET formation, as observed in

the lungs of SARS-Cov-2 patients (43), autoimmune diseases (52, 110), and mouse models of atherosclerosis (111, 112).

### 3 NETs in hepatocellular carcinoma

HCC typically begins insidiously and progresses rapidly. Unfortunately, by the time it is diagnosed, the majority of cases have already progressed to the intermediate and late stages, which are often associated with a grim prognosis. It has been observed that an elevated neutrophil-lymphocyte ratio (NLR) and a high infiltration of tumor-associated neutrophils are associated with worse outcomes in HCC (5, 7, 113). Nevertheless, the precise mechanisms through which neutrophils, particularly NETs, contribute significantly to the progression of HCC have yet to be thoroughly investigated. Apart from their well-known functions in innate immunity, NETs also play a crucial role in various stages of tumor initiation and progression. They promote tumor angiogenesis and growth, facilitate tumor expansion, and provide a protective shield for tumor cells against antitumor immunity (76, 114, 115). Notably, high expression of NETs has been observed in both the blood serum and tissue specimens of HCC patients, as well as in individuals with liver diseases such as nonalcoholic steatohepatitis and liver cirrhosis. This suggests a potential involvement of NETs in the development and progression of HCC (35, 46). Nevertheless, the specific molecular mechanism of NETs in HCC have yet to be fully elucidated. This review aims to summarize and provide further insights into these functions (Figure 4).

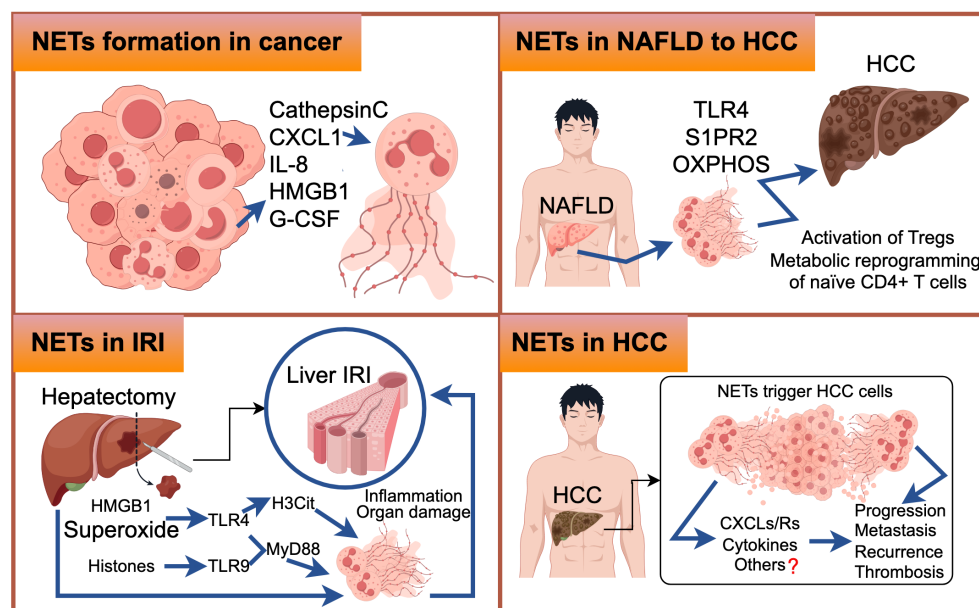


FIGURE 4

The upper-left figure illustrates selected molecular mechanisms underlying the formation of Neutrophil Extracellular Traps (NETs) in cancer. Similarly, the upper-right figure elucidates the molecular pathways through which NETs are generated during the transition from Non-Alcoholic Fatty Liver Disease (NAFLD) to Hepatocellular Carcinoma (HCC). In the lower-left figure, we delineate the molecular events that lead to NET formation in the context of Ischemia-Reperfusion Injury (IRI). The lower-right figure comprehensively outlines the intricate molecular processes associated with NET formation in HCC, with question marks indicating areas necessitating further exploration.

### 3.1 NETs in HCC initiation and growth

Non-alcoholic fatty liver disease (NAFLD) encompasses a range of chronic hepatic disorders characterized by the accumulation of fat in hepatocytes, known as steatosis. It is commonly associated with obesity, hyperlipidemia, and insulin resistance. Notably, more than 10% of NAFLD patients progress to nonalcoholic steatohepatitis (NASH) (116), which represents the most significant risk factor for HCC development (117). In the livers of STAM mice (induced by neonatal streptozotocin and a high-fat diet), early infiltration of neutrophils and the formation of NETs were observed, followed by the production of inflammatory cytokines, the influx of monocyte-derived macrophages, and the progression of HCC (36). NETs formation, triggered by S1P receptor 2 signaling, accelerates inflammation and maintains disease progression in the early stages of NASH (118), as well as playing a crucial role in the transition from NASH to HCC and HCC metastases in later stages (36). It is worth noting that while the accumulation of fat in liver cells is independent of NET formation, NETs have an initiating role in the pathogenesis of fully developed NASH by modulating the inflammatory environment and promoting the activation of novel monocyte-derived macrophages (36). Treatment with DNase therapy or the use of PAD4<sup>-/-</sup> mice did not affect the progression of hepatic steatosis but altered the subsequent hepatic inflammation pattern, ultimately resulting in reduced tumor growth (36). Inhibiting NETs *in vivo*, either through

PAD4-deficient mice or treatment with DNase I, leads to reduced activity of regulatory T cells (Tregs) in NASH, and depletion of Tregs significantly inhibits the initiation and progression of HCC (119). RNA sequencing data revealed that NETs have an impact on gene expression profiles of naïve CD4<sup>+</sup> T lymphocytes, particularly genes involved in oxidative phosphorylation in mitochondria (119). Enhancing mitochondrial respiration can promote the differentiation of regulatory T cells through the facilitation of NET formation (119) (Figure 5A). Future research should focus on investigating the specific components of NETs' structure that interact with TLR4 on CD4<sup>+</sup> T cells, as well as elucidating the properties of downstream signaling pathways. Moreover, further *in vivo* studies are necessary to understand the effect of NETs on Treg suppressive function. Targeting the interaction between neutrophils and Tregs or inhibiting Treg activity may enhance cancer immunosurveillance and prevent HCC formation. In HBV-infected HCC patients, NETs have been found to promote HCC proliferation both *in vitro* and *in vivo*, primarily through NETs-mediated cell entrapment, EMT-associated cellular migration, and MMPs-induced degradation of the extracellular matrix (ECM) (46).

The presence of NETs in the inflammatory microenvironment associated with liver cancer promotes tumor growth (120). *In vitro* experiments neutrophils from HCC patients showed a higher tendency to release NETs (35). A study revealed that plasma NET formation levels are increased in patients with liver cirrhosis and/or HCC, and these levels correlate with the severity of hepatic

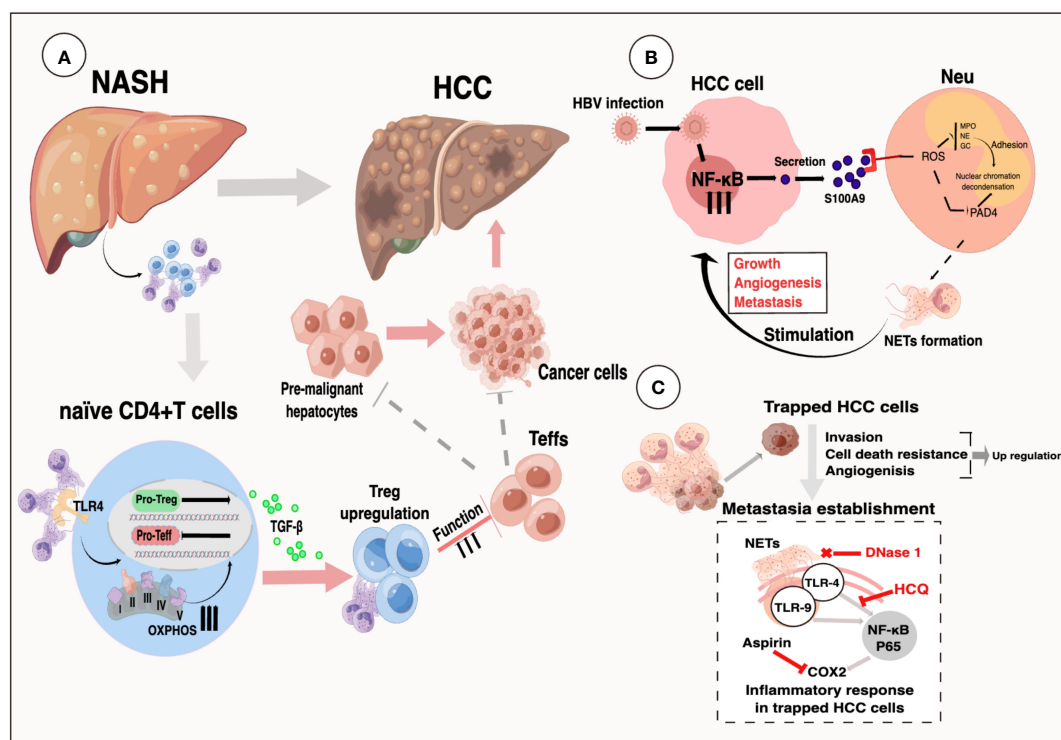


FIGURE 5

(A) Selectively increased intrahepatic Tregs can promote an immunosuppressive environment in NASH livers. NETs link innate and adaptive immunity by promoting Treg differentiation via metabolic reprogramming of naïve CD4<sup>+</sup> T-cells (119). (B) Activation of RAGE/TLR4-ROS signaling by HBV-induced S100A9 resulted in abundant NETs formation, which subsequently facilitated the growth and metastasis of HCC cells (116). (C) System illustration of NETs to trap and fuel HCC metastasis potential through triggering an inflammatory response through TLR4/9-COX2 signaling (35).



dysfunction (121). Elevated levels of MPO-DNA (NET serum markers) were associated with increased mortality after hepatectomy in HCC patients (122). There is growing evidence, albeit preliminary, suggesting that NET formation plays a crucial role in HCC development and proliferation. However, further research is needed to investigate the specific mechanisms of NET formation in HCC recurrence after surgical intervention, portal vein tumor thrombus, and systemic therapeutic resistance.

Liver transplantation offers benefits to patients with partial liver cancer. However, the occurrence of liver ischemia-reperfusion injury (IRI) is strongly associated with procedural failure in liver transplantation (123). Inflammation plays a significant role in the pathogenesis of liver IRI, with neutrophils being a key component of hepatic injury after reperfusion. Excessive activation and recruitment of neutrophils in the reperfusion tissue contribute to the development of ischemia-reperfusion injury. Neutrophils perform various functions throughout this intricate process, including activation, transport through the vasculature, and migration (124–126). A growing body of research has indicated that NETs could contribute to liver IRI. Released from damaged liver cells, HMGB1 proteins are associated with tissue damage and can trigger NET formation through the activation of TLR4 and TLR9 (127). In a study, extracellular superoxide treatment induced the release of NET DNA, citrullination of histone H3 (H3Cit) (128), and elevated levels of MPO-DNA complexes in neutrophils in a TLR-4 dependent manner. Pretreatment with allopurinol and N-acetylcysteine attenuated the formation of NETs and liver injury following ischemic injury in mice (56). Both neutrophils and NETs negatively correlate with the expression of histidine-rich glycoprotein (HRG) in mouse models of liver IRI. Overexpression of HRG in mice suppressed neutrophil infiltration and NET formation in the liver, thus reversing hepatic IRI (129). Another study demonstrated that hydroxychloroquine inhibits NET formation and alleviates liver IRI in mouse models of liver ischemia-reperfusion (48). Acrolein triggers neutrophil chemotaxis and exacerbates NET release both *in vitro* and *in vivo*. Inhibition of acrolein-induced NET release was observed *in vitro*, while suppression of inflammatory cytokine expression, P38MAPK-ERK activation, and apoptotic signals in the liver of mice subjected to IR resulted in a slower recovery rate of the liver after surgery (130). TMP, a compound derived from the plant *Ligusticum wallichii* Franchet, inhibited NET formation in rats after hepatic transplantation, improved hepatic function, and alleviated liver IRI (131). These studies have shown that antioxidant treatment can reduce NET formation and protect against hepatic IRI. However, when considering the therapeutic efficacy of inhibiting NETs, it is important to take into account the underlying preoperative disease and potential complications in immunocompromised individuals following transplantation.

### 3.2 NETs in hepatocellular carcinoma on the metastatic cascade

The previous study discovered that NETs sequester tumor cells in circulation and promote metastasis (132). Recently, researchers

revealed that patients with liver cancer, particularly those with metastatic hepatocellular carcinoma, exhibit enhanced NET formation in neutrophils (133). NETs ensnare HCC cells, inducing resistance to cell death and enhancing invasiveness, thereby triggering metastatic potential. This process is mediated by the internalization of NETs into trapped HCC cells and activation of the TLR4/9-COX2 signaling pathway. In mouse models, a combination therapy involving DNase 1-mediated degradation of NETs and anti-inflammatory drugs such as aspirin/hydroxychloroquine effectively mitigates liver cancer metastasis (35). In HBV-infected HCC patients, the promotion of HCC metastasis by NETs was demonstrated both *in vitro* and *in vivo*, primarily through NETs-mediated cell entrapment, cell migration associated with epithelial-mesenchymal transition (EMT), and the degradation of ECM induced by MMP (46). Moreover, the induction of S100A9 by HBV accelerates the generation of NETs through the activation of TLR4 and the receptor for advanced glycation end products (RAGE), along with the signaling cascade involving ROS. Furthermore, the formation of NETs in the circulation was significantly associated with TNM stage and metastasis in HBV-induced liver cancer, and the presence of NETs was shown to have predictive value for extrahepatic metastasis (46) (Figure 5B). Deficient DNASE1L3 (an extracellular DNase) facilitates HCC invasion through NET formation by activating GMP-AMP synthase in circulation and the non-canonical pathway of NF-kappa B in diabetic HCC (47). Elevated levels of NET release have been observed in patients diagnosed with HCC, particularly those with portal vein tumor thrombosis (PVTT) (134). IL-8 produced by liver cancer cells triggers the formation of NETs via an NADPH oxidase-dependent pathway, and NET-associated cathepsin G (cG) facilitates hepatocellular carcinoma metastasis both *in vitro* and *in vivo* (134). The metabolic shift of tumor-associated neutrophils towards glycolysis and the pentose phosphate pathway, induced by tumors, promotes the formation of NETs in a ROS-dependent manner. Subsequently, NETs trigger tumor cell migration and suppress tight junction molecules on adjacent endothelial cells, thereby promoting tumor intravasation and metastasis (133). Activated neutrophils play a crucial role in the generation of ROS through their oxidative bursts (135). CXCL1-triggered hepatic impairment was found to be dependent on p47 phox, which mediates oxidative burst and is a constituent of the NADPH oxidase 2 complex (136, 137). In mice fed a high-fat diet, the absence of PAD4 or NE genes failed to abolish the capacity of elevated CXCL1 levels to induce NASH. This suggests that NETs or NE are not indispensable for the onset of CXCL1-induced NASH (138). Neutrophils in patients with liver cancer exhibit elevated levels of mitochondrial ROS (mitoROS) and generate NETs that are enriched with oxidation products of mitochondrial DNA (mtDNA) in a manner dependent on mitoROS (139). NET formation and oxidized mtDNA have clinical relevance. When bound to NET-associated proteins, oxidized mtDNA exhibits a heightened ability to elicit inflammatory mediators that promote metastasis in HepG2 cells (139). Targeting oxidized mtDNA with metformin can reduce the inflammatory state that facilitates metastasis, thereby undermining the metastatic capacity of hepatocellular carcinoma (139). Furthermore, high levels of NET infiltration have also been observed in patients with colon and breast cancers who have

developed hepatic metastasis. Additionally, the level of NETs in serum can serve as a predictive marker for the incidence of liver metastases in early-stage breast cancer patients (17).

Previous research has revealed that soluble factors derived from tumors, such as hyaluronan fragments, metabolites, and cytokines, can have significant effects on the regulation of phenotypes and functions in tumor-infiltrating neutrophils. However, it is still uncertain which of these components, or if any other factors in the model, could be related to triggering the metastatic switch and leading to the release of NETs. In addition, DNase I has commonly been employed to eliminate NETs. However, a lingering question remains regarding whether DNase I eliminates the entire molecular structure of NETs or selectively targets specific components within the molecular structure. This uncertainty raises concerns about its potential impact on the specific mechanisms underlying NETs in HCC. Therefore, further investigations are warranted to address these inquiries thoroughly.

### 3.3 The role of NETS on hepatocellular cancer recurrence

Metastasis is the primary cause of carcinoma mortality, and neutrophils play a critical role in this process (140). Several studies have shown that NETs capture circulating tumor cells and release proteases, leading to tumor proliferation and metastasis (62, 141, 142). The capture of NETs has been associated with an increased incidence of microscopic liver metastases within 48 hours and a higher burden of gross metastatic disease at 2 weeks after tumor cell inoculation (132). One study suggests that elevated NET formation is linked to shorter recurrence-free survival and overall survival, making it a potential pre-surgery biomarker in serum for identifying patients at higher risk of recurrence (143). Another study demonstrated that HCC harbors NETs characterized by the enrichment of oxidized mitochondrial DNA, which promotes inflammation and metastasis (139). Enhanced circulating NET markers are also associated with an increased risk of recurrence in patients with advanced colorectal cancer and liver metastasis who have undergone major hepatic resection (34). Further study has found that NET formation may play a pivotal role in impeding emboli during metastasis, protecting them from NK cell attacks while traversing the bloodstream and infiltrating affected organs (144). Subsequently, another study combined this with engineering techniques: the application of an injectable hemostatic gel adhesive containing a neutralizer and NETs lyase has been shown to enhance adoptive natural killer cell immunotherapy and prevent liver cancer recurrence after resection (145). Additionally, NETs may act as a protective barrier, shielding cancer cells from the cytotoxicity of CD8<sup>+</sup> T cells and NK cells. However, NETs are not the sole factor contributing to the impediment of antitumor immunity mediated by T cells and NK cells (144). The aforementioned studies indicate a positive correlation between elevated levels of NETs and an increased likelihood of HCC recurrence. However, the precise role of NETs in the specific mechanisms of HCC recurrence remains unclear. Additionally, it is yet to be determined whether the influence of NETs on HCC recurrence is influenced by circadian regulation, patient age, preoperative

neoadjuvant therapy, postoperative chemotherapy, or other interactions that may impact HCC recurrence differently. Therefore, further investigations are needed to explore in-depth the specific mechanisms underlying the involvement of NETs in HCC recurrence.

### 3.4 The role of NETs in hepatocellular carcinoma on thrombosis

NETs are widely recognized for their crucial role in the pathogenesis of thrombosis and coagulation disorders, particularly in cancer (115, 146). Heparins, anticoagulant polyanions released by mast cells at pathogen entry sites, modify fibrin structure and stabilize fibrin clots through size-dependent modulation and kringle-dependent inhibition of plasmin-mediated fibrinolysis. All polyanions mechanically fortify the clots; however, smaller P45, P100, and low molecular weight heparin (LMWH) decrease the pliability of fibrin, while larger UFH and P700 increase the maximum tolerable deformation of blood clots (147). Administration of LMWHs inhibits the activation-induced autophagy of neutrophils and NET formation. Research has shown that LMWHs profoundly alter the capacity of neutrophils to produce NETs and mobilize primary granule contents in response to non-related inflammatory stimuli, such as HMGB1, PMA, and IL-8 in humans (148). Additionally, research has found that heparin and polyphosphate variants, procoagulant polyanions released by platelets and microorganisms, modulate the susceptibility of fibrin-histone clots to lysis and their mechanical stability. The size and charge of these variants play a crucial role in this process (42). Lu et al. developed a micellar nanoparticle loaded with doxorubicin, LMWH, and astaxanthin (LMWH-AST/DOX, LA/DOX NP) to prevent the occurrence of these loops and inhibit hepatic metastasis by inhibiting NET formation. These nanoparticles exhibited dual effects by not only inhibiting metastasis to the lungs but also mitigating the inflamed and immunosuppressed microenvironment within tumors (149).

Research has demonstrated that the interplay between neutrophils and platelets is especially significant in driving the formation of NETs and maintaining the process of diffuse coagulation (150–152). While this concept is substantiated in the context of sepsis, it necessitates further exploration in the context of HCC. This is especially pertinent for patients with HCC combined with portal vein thrombosis, as well as for those experiencing postoperative complications related to disseminated intravascular coagulation (DIC) in the context of HCC. Moreover, neutrophils primarily induce the formation of NETs through serine proteases, thereby activating both extrinsic and intrinsic coagulation pathways (109). A study investigating hepatic surgery observed that NETs and platelet-rich microthrombi contribute to microvascular alterations in injured organs. However, inhibiting NETs formation using DNase reduced immunothrombosis and organ damage (153). Another study demonstrated that the combined action of tissue plasminogen activator (tPA) and DNase I effectively triggers thrombolysis (154). Administering DNase I to rats with thrombosis proved successful in preventing myocardial infarction, recurrent cerebrovascular accidents, and deep vein

thrombosis (155). Nevertheless, further research is needed to determine whether the degradation of NETs by DNase I could increase the risk of thrombosis and inflammation.

### 3.5 Approaches to targeting NETs in hepatocellular carcinoma

NETs have emerged as key contributors to the pathogenesis of various diseases, prompting extensive research into potential therapeutic targets. The presence of NETs has been linked to cancer patient survival (156, 157). However, it is crucial to carefully assess the risks associated with targeting NET formation. While NETs offer protection against severe infectious diseases, inhibiting NETs could potentially increase susceptibility to infections (44, 158). For instance, mice lacking the PAD4 gene (-/-) exhibited heightened vulnerability to bacterial diseases due to impaired NET formation, in contrast to their PAD4(+/+) counterparts (44). Conversely, a separate study found that PAD4 deficiency had no discernible impact on polymicrobial sepsis with concurrent bacteremia and the mitigation of endotoxemic shock (159). Notably, tumor cells, shielded from cytotoxicity by NET formation, play a pivotal role in the successful metastasis of cancer in mice. Moreover, the combination of PAD4 inhibitors and immune checkpoint inhibitors has demonstrated immunotherapeutic synergy by inhibiting the process of NET formation (144). Therefore, the risks associated with targeting NETs as therapeutic interventions may vary depending on the presence of specific disorders and the immunological state of the organism. Furthermore, the degradation of NET formation presents another significant hazard, as it leads to the release of DNA and histones derived from NETs, which have the potential to trigger inflammation. Currently, therapies aimed at modulating NET formation can be categorized into two distinct groups: inhibition of NET formation and destabilization/degradation of existing NETs.

Extensive research has already been conducted on the inhibition of NET formation. Inhibiting the expression of PAD4 can prevent NET formation (160). Another study demonstrated that suppressing the enzymatic function of PAD4 disrupts NET formation in both mice and humans, although it does not seem to affect bacteremia in the context of polymicrobial sepsis (161). Recent investigations have identified BMS-P5, GSK199, and GSK484 as inhibitors of NET development, capable of suppressing associated diseases in both *in vivo* and *in vitro* settings (161, 162). NE inhibitors, which hold great promise as therapeutic targets, play a pivotal role in suppressing NET formation (132). For instance, sivelestat, an NE inhibitor, was found to suppress NET formation in mice (163). Additionally, antibodies have also been recognized for their ability to impede NET formation. One such antibody, tACPA, restrains the development of NETs in neutrophil-mediated inflammatory conditions (164). However, the approach of targeting pre-existing NETs in HCC has not been thoroughly explored. NETs play a crucial role in the pathogenesis of HCC, and focusing on inhibitors or modulators of NETs in HCC may introduce a novel therapeutic approach for managing this malignancy.

An alternative approach to target NETs involves inducing their degradation. DNase I has been shown to possess the ability to partially lyse NETs (154). The suppression of NET production by DNase I effectively eliminates the promotion of HCC growth and metastasis induced by NET formation (46). In a murine model, the direct destruction of NET formation by DNase I, along with the administration of agents possessing anti-inflammatory properties such as hydroxychloroquine or aspirin, effectively attenuates liver cancer metastasis (35). Compelling evidence demonstrates that neutrophil-derived NET formation impedes the cytolytic activity of both cytotoxic T lymphocytes and NK cells during their interactions with cancer cells (144). Moreover, the presence of NETs and an acidic tumor microenvironment significantly counteract the potential benefits of cancer therapy involving NK cell infusion.

In experimental model, we consistently observed a concurrent occurrence of autophagy and the formation of NETs. The use of autophagy inhibitors, such as wortmannin and 3-MA, effectively hindered the formation of NETs (148, 165). Furthermore, the study found that pretreatment of neutrophils with low-molecular-weight heparin (LMWH) *in vitro* significantly impaired their ability to initiate autophagy. In a separate study involving healthy volunteers, a single administration of parnaparin as a preventive measure rendered neutrophils incapable of triggering autophagy and generating NETs (148) (refer to Table 1 for details).

## 4 Discussion, concluding marks and key questions

### 4.1 NETs in other cancers

The recent advancement in our comprehension of cancer-associated NET formation is the identification of the ability of carcinomas to enhance neutrophil production of thrombogenic NETs. This discovery was first reported in 2012 (115). Neutrophil extracellular traps have now been associated with various non-infectious diseases, such as cancer (166), diabetes (169), autoimmunity (167), cardiovascular disease (168), and systemic lupus erythematosus (170), all of which have an inflammatory component. In the following section, we present and review the current understanding of cancer-associated NET formation, including the diverse mechanisms underlying NET formation in different types of cancers.

Despite significant advancements in our understanding of the mechanism of NETs in tumors, they still remain enigmatic. In 2013, a groundbreaking discovery was made, demonstrating the correlation between NET formation and malignancy. This finding suggested that the presence of intratumoral NETs may be associated with an unfavorable prognosis in a subset of patients diagnosed with Ewing sarcoma (171). Subsequently, the potential of NETs has been extensively investigated across diverse malignancies, revealing their oncogenic effect in the majority of cancers, although not in all conditions. Notably, mice with tumors exhibited an increase in plasma levels of NET formation compared to control mice. Neutrophils in this context tended to spontaneously form NETs,

TABLE 1 The approaches to targeting NETs.

Target	Parameter assessed	Content	Disease	References
<b>1. Inhibition of NETs</b>				
<b>PAD4 inhibitors</b>				
GSK199	Western blot and H3Cit imaging assays	Inhibit activity of PAD4 subsequently lead to the damage of mouse and human NET formation	–	(165)
GSK484	Western blot and H3Cit imaging assays	Inhibit activity of PAD4, subsequently lead to the damage of mouse and human NET formation	–	(165)
BMS-P5	Western blot and fluorescent microscopy	Block formation of NETs and delays progression of multiple myeloma	Multiple myeloma	(166)
<b>NE inhibitors</b>				
Sivelestat	Intravital video microscopy and ELISA	Primary tumors induce NETs with targetable metastasis-promoting effects, and blocking NETosis with sivelestat significantly inhibits spontaneous metastasis to the lung and liver.	Advanced esophageal, gastric, and lung cancer. Cancer-associated liver metastasis	(133)
<b>Antibody</b>				
tACPA	FACS analysis and IF microscopy	tACPA reduced NET release and potentially initiated NET uptake by macrophages <i>in vivo</i>	IA, IBD, pulmonary fibrosis, and sepsis.	(167)
<b>2. Degradation of NETs</b>				
DNase 1	Western blot, ELISA and IF staining	Inhibition of NETs generation by DNase 1 effectively abrogated the NETs-aroused tumor growth and metastasis.	Hepatocellular carcinoma; NASH thrombosis;	(37, 115, 117, 119, 158)
DNase 1 and aspirin/hydroxychloroquine	Serum MPO-DNA level, H3Cit and Ly6G imaging assays	A combination of DNase 1 with aspirin/hydroxychloroquine effectively reduced HCC metastasis in mice model.	Hepatocellular carcinoma	(36)
<b>3. NETs and others</b>				
NETs and CTLs	Immunohistochemistry and flow cytometry	NETs inhibit immune cell cytotoxicity by impeding contact with tumor cells	Human cancer	(147)
NETs and acidic TME	Western blot and immunohistochemistry	Injectable adhesive hemostatic gel with tumor acidity neutralizer and NETs lyase for enhancing adoptive NK cell therapy prevents post-resection recurrence of HCC	Hepatocellular carcinoma	(146)
<b>Autophagy inhibitors</b>				
3-MA	Western blot, flow cytometry and scanning electron microscopy	Inhibition of autophagy by 3-MA alleviated the ROS burst and subsequent NETosis caused by diphenyl phosphate	–	(151, 168)
Wortmannin	ELISA, flow cytometry and confocal microscopy	Using of wortmannin can prevent NET generation	–	(151, 168)
NETs and LMWH	ELISA, flow cytometry and confocal microscopy	LMWH prevent the induction of autophagy of activated neutrophils and the formation of NETs	–	(151)

leading to thrombus formation and a pro-coagulant state (115, 172). Moreover, it was found that tumor cells stimulated the formation of NETs, thereby promoting breast cancer lung metastasis (172).

Low-density neutrophils (LDNs), which are a subset of activated neutrophils with distinct phenotypic and functional characteristics, exhibit differences compared to normal-density neutrophils (NDNs) in human peripheral blood polymorphonuclear neutrophils (PMNs) (173). In a murine model of orthotopic pancreatic adenocarcinoma, suppression of PAD4 resulted in decreased levels of circulating NET formation, leading to reduced tumor growth and improved survival rates. Furthermore, NETs stimulated pancreatic stellate cells, which were found to promote tumor proliferation (174). Similar findings

were observed in an experimental melanoma model, where NETs accumulated in the TME and promoted cancer growth (175). Interestingly, the researchers also observed the presence of NETs in ulcerated tissue of melanoma patients, which were negatively associated with the patients' prognostic outlook. However, co-incubation of NET formation and melanoma cells disrupted melanoma cell migration and viability (176). This observation suggests that the potential factors *in vivo* may block the anti-tumor effects of NETs in melanoma, and further exploration in this regard could be a promising direction. While the presence of tumor-associated and circulating NETs has been linked to a worse prognosis in patients with diffuse large B-cell lymphoma, the exact



correlation remains to be fully elucidated in humans. Both *in vivo* and *in vitro* studies have also demonstrated that NETs promote the proliferation and migration of tumor cells (177).

In recent studies by Yang et al., the role of NETs in promoting cancer metastasis, as well as the association between NETs and neutrophil DNA, has been strongly supported (17). They investigated specific biomarkers, such as MPO and H3Cit, to identify distinct neutrophils and their associated NETs. The results revealed that liver metastases exhibited the highest level of NET infiltration. Interestingly, higher levels of serum MPO-DNA were found to be independently associated with subsequent liver metastasis, while no such correlation was observed in other organs. These findings suggest that excessive hepatic NET formation may occur prior to the detection of metastases in breast cancer patients, thereby promoting subsequent hepatic metastasis (17). Furthermore, experiments conducted on PAD4-deficient C57BL/6 mice demonstrated a significant attenuation in the formation of NETs and hepatic metastases. Conversely, NET-DNA extracted from PMA-treated neutrophils exhibited an opposite effect. Additionally, tumor cells exhibited enhanced chemotaxis towards the DNA component of hepatic NETs through high-affinity binding with a transmembrane protein called CCDC25, which is expressed on carcinoma cells. The interaction between NET formation DNA and CCDC25 promotes cancer cell migration and initiates metastasis by triggering intracellular signaling pathways (17).

It is evident that the formation of NETs plays a crucial role in cancer metastasis, particularly in the context of liver metastases. Targeting the components of hepatic NETs, such as MPO and H3Cit, may hold significant therapeutic potential for the treatment of HCC. Moreover, exploring the interplay between immune cells and cancer cells, as well as the potential of injectable hydrogels in enhancing immunotherapy, and the impact of senescence-associated secretory phenotype (SASP) on the tumor microenvironment, could provide valuable insights for future research. Ultimately, by targeting heparin as a potential therapeutic intervention and modulating the tumor immune microenvironment through the interaction of cancer cells and immune cells, novel strategies for HCC treatment may be developed.

Metastasis, the spread of cancer cells to distant tissues, is the leading cause of mortality in cancer patients, surpassing the impact of the original primary tumor. The process of metastatic initiation can persist for an extended period, potentially due to the protective effects of immune cells or inadequate angiogenic capacity. This leads to a state of uncertainty between cell proliferation and apoptosis (10, 12, 178). The factors that disrupt this delicate balance remain elusive. D. Barkan and B.L. Pierce proposed an intriguing theory suggesting that low-grade inflammation may trigger the transition from dormancy to proliferation in metastatic cells (179, 180). Recently, a study found that the recruitment and activation of neutrophils occur prior to treatment with LPS in rats with dormant lung tumor cells (142, 181). Depleting neutrophils eliminated the awakening of dormant cancer cells induced by LPS. The presence of NETs in the lung was rapidly (within 4 hours) induced by LPS instillation and persisted for 24 hours. Similar observations were made using a PAD4 inhibitor. These findings were also confirmed in a prostate cancer

model exposed to tobacco smoke, a proinflammatory stimulus. Therefore, various rat models exposed to multiple inflammatory stimuli induce the awakening of dormant cancer cells through NET formation. The authors also discovered that the reactivation of quiescent carcinoma cells relies on ECM remodeling and laminin cleavage, which require matrix metalloproteinases 9 (MMP9) and NE, an enzyme associated with NETs. The appearance of a novel epitope in laminin, resulting from proteolytic remodeling, promotes cancer cell proliferation through the  $\alpha 3 \beta 1$  integrin pathway. The presence of NETs prompts quiescent cancer cells to initiate proliferation. However, when these disseminated cells embark on their proliferation journey, they become susceptible to recognition by T cells and NK cells (178, 182, 183). The specific impact of the adaptive immune system in mouse models remains unclear. Nevertheless, lipopolysaccharide exposure elevates glucocorticoid levels (184) while simultaneously suppressing adaptive immune cells. Consequently, the combination of lipopolysaccharide and smoking may facilitate the growth of dormant cells. This is achieved by triggering signaling pathways through NETs to initiate proliferation and concurrently disrupting immune control through glucocorticoids.

## 4.2 Conclusions

Over the past decade, the crucial role of neutrophils in both inflammatory and non-inflammatory diseases has been firmly established. One of the contributing factors is NET. In the context of cancer, particularly HCC, NET formation may play a significant role in disease progression. However, studying NETs presents challenges, as primary human neutrophils cannot be transfected, and inhibiting NET formation pathways *in vivo* is difficult. Furthermore, the heterogeneity between human and mouse neutrophils raises questions about the relevance of NET formation in tumors, as most studies rely on mouse models (13, 98, 185–187).

Neutrophils can polarize into two subtypes: N1, which exhibits anti-tumorigenic properties (188, 189), and N2, which displays pro-tumorigenic characteristics (190–192). Both subtypes have the capacity to generate NETs. Additionally, other matrix cells in the TME, such as basophils (193–195), eosinophils (27, 195–198), mast cells (199, 200), and macrophages (201), can also release extracellular DNA traps. However, the specific components of NETs or extracellular DNA traps that possess anti-tumorigenic or pro-tumor effects remain unclear. Therefore, it is crucial to inhibit NET formation without compromising other effective constituents or generating detrimental degradation products of neutrophil extracellular traps. This requires a comprehensive understanding of the mechanisms involved in NET formation and degradation. Furthermore, targeting NETs and related enzymes may hold promise as a therapeutic strategy for combating cancer metastasis.

The concentration of NETs is elevated in both blood serum and tissue specimens from patients with HCC at different stages of liver cancer, including HCC patients with HBV, those who have undergone liver surgery (including liver IRI), NASH, and liver cirrhosis. While most research has focused on the role of NETs in regulating tumor progression and proliferation, further

investigation is needed to understand the specific components of NETs that interact with signaling pathways. Additionally, more research is required to identify the mechanisms by which NETs are regulated *in vivo*. Although previous *in vivo* or *in vitro* studies have primarily induced NET formation using LPS or PMA, the detailed mechanisms underlying the interplay between NETs and other immune cell components, such as macrophages, CD4<sup>+</sup> T cells, and Tregs, remain largely unexplored. Moreover, various risk factors associated with disease progression, including diabetes, age, and the presence of advanced cirrhosis or fibrosis, have been identified through clinical research. However, there are still gaps in our understanding of the pathobiology of HCC.

The formation of NETs has been identified as a valuable biomarker for diagnostic, predictive, and prognostic purposes in various human cancers, including HCC. Abundant evidence supports the notion that tumor-associated NET formation plays a pivotal role in facilitating the unchecked proliferation of malignant cells, creating a permissive environment for cancer development, expediting tumor progression during systemic infection, and promoting cancer-associated thrombosis in HCC. Furthermore, the presence of NETs in HCC patients has been shown to enhance metastatic potential and contribute to disease recurrence. The potential of NETs as therapeutic targets for HCC holds great promise, as targeting NETs could potentially prevent the progression of liver cancer, inhibit metastasis, mitigate cancer-associated thrombosis, and reduce post-operative recurrence. Clinical trials investigating anti-NET therapies, either alone or in combination with immune checkpoint inhibitors (ICI), are currently underway. It is hoped that combination therapy will yield a synergistic effect, effectively treating HCC patients by counteracting NET-mediated immunosuppression in the presence of ICI.

### 4.3 Key questions for further research

It is necessary to determine the immunomodulatory properties of NETs, such as their antibacterial activity, which play a crucial role in triggering or suppressing inflammation, in order to further elucidate their mechanisms. Conversely, changes in the composition and structure of NETs may result from variations in proteomic and transcriptomic profiles among neutrophil subpopulations, as well as activation by diverse stimuli. These aspects warrant further investigation. Moreover, the quantity, timing, and site of NET release exert a profound influence on the balance between detrimental and beneficial outcomes. Given the inherent heterogeneity of neutrophils within the hepatocellular carcinoma TME (11), exploring the involvement of distinct neutrophil subpopulations in the timing and quantity of NET release within the context of hepatocellular carcinoma (secondary dysregulation induced by disease initiation and other factors) may represent a promising research avenue. Furthermore, NETs have been identified in various human diseases, prompting the need to investigate their potential utility as markers of therapeutic efficacy. Additionally, further investigate the mechanisms observed in animal models, including those related to HCC, and determine their applicability and relevance in clinical settings is crucial. Nonetheless, it is worth noting that most current NET research remains mechanistic in nature, with clinical translation remaining a distant goal. Nevertheless, a more comprehensive understanding of the molecular mechanisms underlying NETs holds the potential to bridge this gap. Lastly, as strategies for neutrophil clearance are often unviable in the context of most human conditions, the field faces the challenge of identifying pathogenic cell subpopulations that can be effectively targeted during therapy (Figure 6).

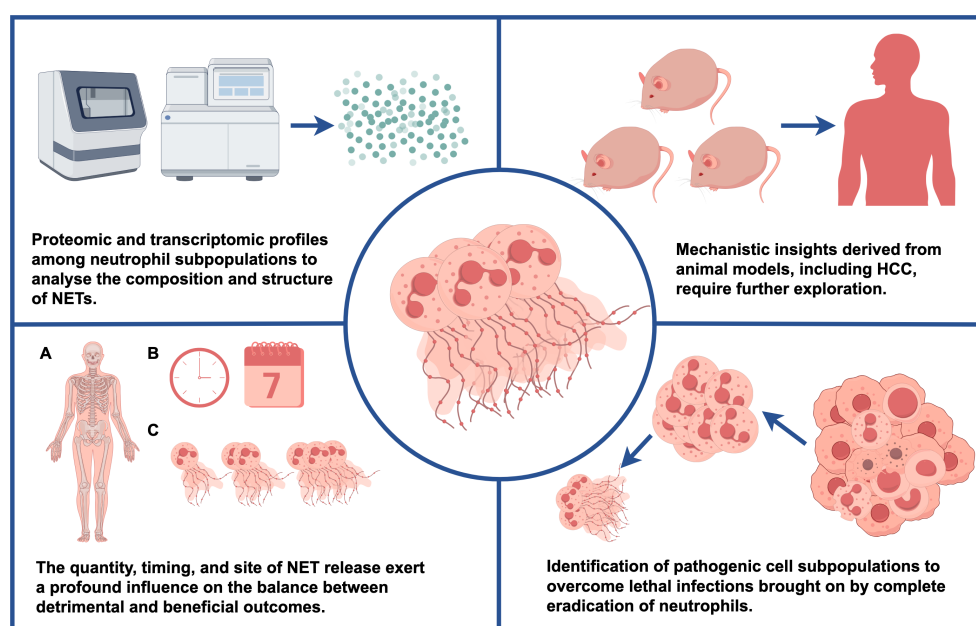


FIGURE 6

This figure illustrates potential future research directions and key questions.

## Author contributions

WXZ: Data curation, Writing – original draft, Writing – review & editing, Conceptualization, Formal Analysis, Investigation, Software, Validation. CF: Writing – original draft. SD: Conceptualization, Investigation, Software, Writing – original draft. XL: Methodology, Supervision, Writing – original draft. HC: Data curation, Writing – original draft. WCZ: Writing – review & editing.

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## Conflict 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.

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## EDITED BY

Anne Caignard,  
Institut National de la Santé et de la  
Recherche Médicale (INSERM),  
France

## REVIEWED BY

Michał Zarobkiewicz,  
Department Of Clinical Immunology, Poland  
Ahmet Emre Eskazan,  
Istanbul University-Cerrahpasa, Türkiye

## \*CORRESPONDENCE

Ali G. Turhan  
✉ turviv33@gmail.com

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# Chimeric antigen-receptor (CAR) engineered natural killer cells in a chronic myeloid leukemia (CML) blast crisis model

Jusuf Imeri<sup>1</sup>, Paul Marcoux<sup>1</sup>, Matthias Huyghe<sup>1</sup>,  
Christophe Desterke<sup>1</sup>, Daianne Maciely Carvalho Fantacini<sup>2</sup>,  
Frank Griscelli<sup>1,3,4,5</sup>, Dimas T. Covas<sup>2,6</sup>,  
Lucas Eduardo Botelho de Souza<sup>2,6</sup>,  
Annelise Bennaceur Griscelli<sup>1,3,4,7</sup> and Ali G. Turhan<sup>1,3,4,7\*</sup>

<sup>1</sup>INSERM UMR-S-1310, Université Paris Saclay, Villejuif, France and ESteam Paris Sud, Université Paris Saclay, Villejuif, France, <sup>2</sup>Blood Center of Ribeirão Preto/Ribeirão Preto School of Medicine/University of São Paulo, Ribeirão Preto, SP, Brazil, <sup>3</sup>INGESTEM National iPSC Infrastructure, Villejuif, France, <sup>4</sup>CITHERA, Centre for iPSC Therapies, INSERM UMS-45, Evry, France, <sup>5</sup>Université Paris Descartes, Faculté Sorbonne Paris Cité, Faculté des Sciences Pharmaceutiques et Biologiques, Paris, France, <sup>6</sup>Biotechnology Nucleus of Ribeirão Preto/Butantan Institute - Ribeirão Preto, Ribeirão Preto, SP, Brazil, <sup>7</sup>APHP Paris Saclay, Department of Hematology, Hopital Bicetre & Paul Brousse, Villejuif, France

During the last two decades, the introduction of tyrosine kinase inhibitors (TKIs) to the therapy has changed the natural history of CML but progression into accelerated and blast phase (AP/BP) occurs in 3-5% of cases, especially in patients resistant to several lines of TKIs. In TKI-refractory patients in advanced phases, the only curative option is hematopoietic stem cell transplantation. We and others have shown the relevance of the expression of the Interleukin-2-Receptor  $\alpha$  subunit (IL2RA/CD25) as a biomarker of CML progression, suggesting its potential use as a therapeutic target for CAR-based therapies. Here we show the development of a CAR-NK therapy model able to target efficiently a blast crisis cell line (K562). The design of the CAR was based on the scFv of the clinically approved anti-CD25 monoclonal antibody (Basiliximab). The CAR construct was integrated into NK92 cells resulting in the generation of CD25 CAR-NK92 cells. Target K562 cells were engineered by lentiviral gene transfer of CD25. *In vitro* functionality experiments and *in vivo* leukemogenicity experiments in NSG mice transplanted by K562-CD25 cells showed the efficacy and specificity of this strategy. These proof-of-concept studies could represent a first step for further development of this technology in refractory/relapsed (R/R) CML patients in BP as well as in R/R acute myeloblastic leukemias (AML).

## KEYWORDS

CAR-NK, NK92, CD25, leukemia, blast crisis CML



# 1 Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by the reciprocal translocation t(9;22) (q34;q11.2) (1). As a result, a *BCR::ABL* hybrid gene is formed on the derivative Ph chromosome and is characterized by a dysregulated tyrosine kinase (TK) activity (2, 3). The use of tyrosine kinase inhibitors (TKIs) has dramatically modified the therapy of CML, generating durable remissions and prolonging survival in TKI responders. TKI therapies allow, albeit with different efficiencies, deep molecular responses in the majority of patients but TKI-cessation attempts show that despite these long-lasting deep responses, rapid molecular recurrence occurs in approximately 50% of cases. The highly quiescent leukemic stem cells (LSCs) are known to be resistant to TKIs (4, 5) and responsible for relapse after TKI treatment cessation (6–9). Therefore, most patients require the administration of life-long TKI therapies and a large fraction develop resistance to these drugs. TKI resistance and disease progression into an advanced phase/blast phase (AP/BP) remains problematic especially in this last group of patients as the only curative approach remains stem cell transplantation. This therapy is difficult to apply in patients lacking donors and especially in patients with significant co-morbidities. Hence, the development of new therapies based on targets expressed in AP/BP CML remains an unmet medical need even in the era of TKI therapies.

CD25 (also known as the  $\alpha$  subunit of IL2R) is one of the three subunits that constitute the receptor to IL-2, the two others are the  $\beta$  and the  $\gamma$  subunit or CD122 and CD132 respectively (10). In normal conditions, CD25 is highly expressed in activated T cells and regulatory T cells, which plays an important role in the homeostasis of T cell activity and immune tolerance (11, 12). CD25 is also shown to be expressed in, BP-CML, BP-CML transformed in acute myeloid leukemia (AML) but also in *de novo* AML with dismal prognosis (13–19). More interestingly, CD25 is shown to be aberrantly expressed in the LSC of CML rendering it a very interesting target for the eradication of the LSC and the CML treatment (20).

We have previously shown that CD25 is overexpressed in BP-CML model generated from patient-specific induced pluripotent stem cells (iPSC) and confirmed the increase of its expression during CML progression (21). These findings prompted us to evaluate the possibility of targeting this receptor using chimeric antigen receptor (CAR)-based therapy in the setting of CML.

CARs are fusion proteins introduced into lymphocytes (T and NK) and recently into macrophages to confer them specific recognition skills. During the last decade, cell therapies based on CAR-T technology have shown groundbreaking results in R/R hematological malignancies leading to their clinical licensing in acute lymphoblastic leukemia (ALL) and lymphomas, by essentially targeting the B-cell marker CD19 (22). Recently, CAR-NK has been introduced as a solution to the limitations of allogeneic transplants which are associated with side effects such as graft versus host disease (GvHD) of CAR-T cells (23, 24). In the context of CML, the use of CAR-NK cells has a double interest because NK cells are known to have also a CAR-independent anti-leukemic effect inherent to their antitumoral properties (25, 26).

Here we show the experimental development of a CAR-NK therapy strategy against the IL2RA/CD25 which as described above has been previously reported as being overexpressed, in advanced CML (21). This strategy is based on the use of a single chain variable fragment (scFv) of the clinically approved monoclonal humanized antibody, Basiliximab recognizing specifically CD25 and targeting K562 cells that we have genetically engineered to overexpress CD25. We therefore use the intrinsic antileukemic potential of NK92 cells and the increased specificity and activation through the CAR constructs to target K562-CD25 cells. We demonstrate an increased cytotoxic activity of CD25 CAR-NK92 cells against leukemic cells expressing CD25 and their efficiency *in vivo*.

# 2 Materials and methods

## 2.1 Cell culture

The Lenti-X 293T cell line (a kind gift from Dr. Lucas Botelho de Souza) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, 11560636) and 100 U/mL of penicillin-streptomycin (PenStrep) solution (Gibco, 11548876). K562 were cultured in RPMI 1640 (Gibco, 11875093) supplemented with 10% of FBS and 100 U/mL of PenStrep. NK92 were cultured in RPMI 1640 supplemented with 20% of FBS, 100 U/mL of PenStrep, and 150 U/mL of IL-2 (Miltenyi Biotec, 130-097-745). NK92 and K562 cells were passaged every 3 to 4 days at a dilution of  $3 \times 10^5$  cells/mL and Lentix were plated at  $3.5 \times 10^4$ /cm<sup>2</sup>.

## 2.2 Lentiviral production and viral transduction

In order to produce IL2RA expressing lentiviruses, we used Lenti-X 293T as a packaging cell line and ps-PAX2.2, and pMD2.G as packaging vector and envelope vector, respectively. Briefly, the Lentix-293T cells were cultured in a T150 mm dish as described above and co-transfected by lipofectamine 3000 reagent (ThermoFisher, L3000015) with 20  $\mu$ g packaging vector of ps-PAX2.2 (Addgene, USA), 10  $\mu$ g envelope vector of pMD2.G (Addgene, USA) and 30  $\mu$ g transfer vector (EF1a-hIL2RA). The supernatant was collected at 24h and 48h. K562 cells were transduced by the use of unconcentrated virus at three different dilutions. Transduced cells were spinoculated at 1200 rpm for 90 minutes at RT and 8  $\mu$ g/mL of Polybrene was added. After 48 hours, the virus was removed and transduced cells were selected with 5  $\mu$ g/mL Blastidine S (Gibco, A1113903). Resistant cells were analyzed by FACS and sorted for CD25.

For CD25 CAR lentivirus production, Lentix-293T cells have been transfected with 3<sup>rd</sup> generation lentiviral system using lipofectamine 3000 with the following plasmids: pLP1 (HIV-1 gag and pol), pLP2 (HIV-1 rev), pLP VSV-G (vesicular stomatitis virus G glycoprotein) and the backbone with the following ratios 5: 5: 3: 7. Transduction was performed as described above.

## 2.3 Molecular constructs

The EF1a-hIL2RA lentivirus plasmid was purchased from VectorBuilder (Guangzhou, China). CD25 CAR lentivirus expression plasmids were purchased from Creative Biolabs (New York, USA). In this construct, Basiliximab scFv was used under EF1a promoter followed by the CD8a hinge domain, CD28 transmembrane domain, 4-1BB co-stimulatory domain, and DAP12 for NK cell activation. GFP and Puromycin resistance were used as selection markers (Figure 1A).

## 2.4 RNA extraction and qRT-PCR

Total RNA was extracted using RNeasy Mini Kit (74104; Qiagen, Germany) and 1 µg was reverse transcribed using a reverse transcription (RT)-PCR kit (Superscript III 18080-44; ThermoFisher Scientific). An aliquot of cDNA was used as a template for qRT-PCR analysis using a fluorescence thermocycler (ThermoFisher Scientific QuantStudio 3™) with FastStart Universal SYBR Green (04913914001, Roche) DNA dye. For the amplification of the housekeeping gene (Actin B), the following primers have been used, Forward: “GTGGCCGAGGACTTTGATTG” and Reverse: “TGGACTTGGGAGAAGGACTGG” and for the CAR sequence, Forward: “GGCCCCAGAAGTCGAAGTAG” and Reverse:

“ACCAGAAGTTCGAGGGCAAG”. Relative expression was normalized to the geometric mean of housekeeping gene expression and was calculated using the  $2^{-\Delta\Delta Ct}$  method.

## 2.5 Flow cytometry

For extracellular staining, cells were washed with DPBS and incubated with antibodies in a volume of 100 µL of DPBS with an adequate amount of antibody (Table 1). For intracellular staining, cells were thereafter fixed with BD Cytofix™ (BD, 554655) for 10 minutes at room temperature, protected from light, washed, and permeabilized with BD Perm/Wash™ (BD, 555028) together with intracellular antibodies for 20 minutes at 4°C. Flow cytometry was performed with a BD FACS LSRFortessa™ and data were analyzed using FlowJo.

## 2.6 Cell sorting

Cells were stained with extracellular antibodies as described above and were resuspended in their culture media at a concentration of  $5 \times 10^6$  cells/mL. Thereafter, they were sorted with a BD FACSARIA™ SORP (BD) and sorted cells were immediately resuspended at the adequate concentration and cultured at 37°C, 5% CO<sub>2</sub>.

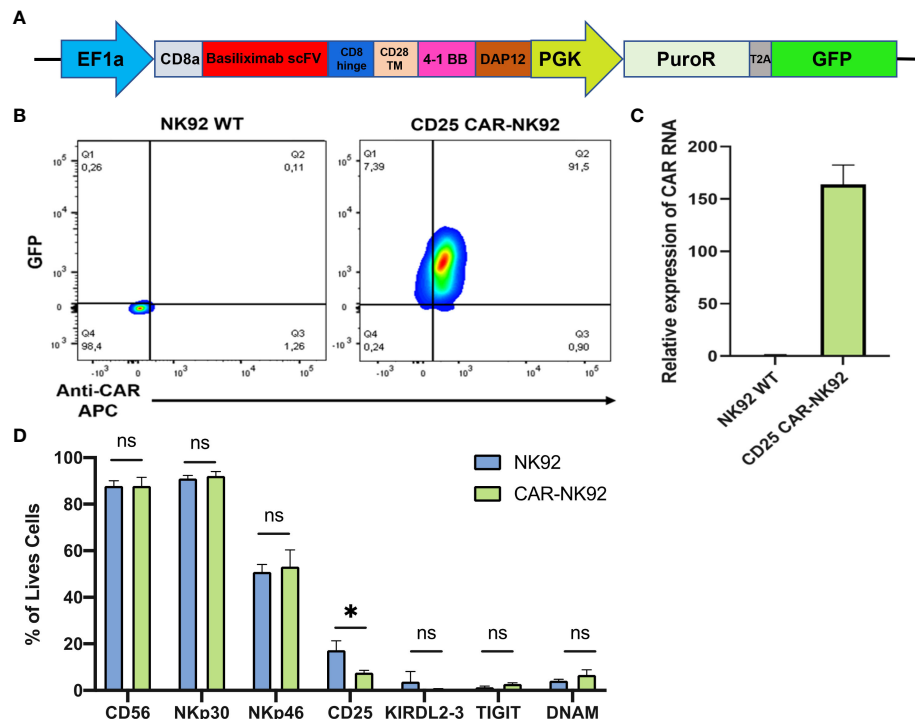


FIGURE 1

Generation of CAR-NK cells from NK92 cells. (A) Constitutive CAR expression construct under EF1a promoter followed by puromycin resistance and GFP under PGK promoter. (B) CAR and GFP expression level of NK92 transduced cells gated on NK92 WT. (C, D) Main NK markers expressed on CAR-NK cells (in pink) and in NK-92 WT cells (in blue). Means and SD are represented. Experiments have been performed 3 times. P-values were calculated using a 2-tailed Student's t-test. ns, not significant; \*P < 0.05.

TABLE 1 Antibodies used for FACS and their respective references.

Antibody and Clone	Reference
AffiniPure F(AB) Anti-mouse IgG Alexa Fluor 647 Polyclonal	115-606-072-Jackson ImmunoResearch
CD56-APC B159	55518-Becton Dickinson
CD25-APC REA945	130-115-535 Miltenyi Biotec
CD107a-PE-Vio 770 REA792	130-111-622 - Miltenyi Biotec
Granzyme B-BV421 GB11	563389-Becton Dickinson
KIRDL2/3-PE-Vio 770 REA1006	130-116-835-Miltenyi Biotec
NKp30-PE-Vio 770 RE823	130-112-432-Miltenyi Biotec
NKp46-PE-Vio 770 REA808	130-112-123-Miltenyi Biotec
Perforin-BV421 $\delta$ G9	563393-Becton Dickinson
TIGIT-PE-Vio 770 REA1004	130-116-817-Miltenyi Biotec

## 2.7 CD107a expression and IFN $\gamma$ expression

Wild type NK92 (NK92 WT) and CD25 CAR-NK92 cells were co-cultured with target cells in a 96 well plate. Antibody was added to each well at a dilution of 1:100 and incubated for 1 hour. Golgistop and GolgiPlug (554724 and 555029, BD) were thereafter added for 2 additional hours. After the incubation cells were washed with FACS buffer and stained with CD56 surface marker for 25 minutes at 4°C protected from light. Following this last incubation, cells were washed with FACS buffer and analyzed by FACS.

## 2.8 Annexin V apoptosis assay

Apoptosis induced by NK cells was evaluated by staining Annexin V on the surface of target cells previously stained with CellTrace™ Yellow (C34567, ThermoFisher Scientific). For CellTrace staining, cells were incubated with 1  $\mu$ L of the dye for  $1 \times 10^6$  cells/mL in PBS for 20 min at 37°C. Thereafter, the free dye was removed by adding five times the original staining volume media containing 10% FBS. After 2h of co-culture as described above, cells were stained for Annexin V according to the manufacturer's instruction. Briefly, cells were washed with 1X binding buffer, centrifuged and resuspended in binding buffer containing AnnexinV-APC (88-8007-74, ThermoFisher Scientific). After 15 minutes incubation, cells were washed again and analyzed by FACS.

## 2.9 In vivo assays

NSG (NOD-Prkdc<sup>scid</sup> IL2rg<sup>tm1</sup>/Bcgen) mice were intraperitoneally injected with K562-CD25 cells expressing Luciferase at Day -3 ( $3 \times 10^6$  cells/mouse, n = 30). At Days 0, 3, and 7, mice were injected intraperitoneally with either 10 Gy irradiated CD25 CAR-NK92 cells

( $10 \times 10^6$ /mouse; n=10) or irradiated wild-type NK92 cells (n=10). The clinical evolution of transplanted mice was followed by luminescence (IVIS 200, Caliper Life Sciences).

## 2.10 Statistical analysis

Two-tailed Student's t-tests were performed using GraphPad Prism version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA, [www.graphpad.com](http://www.graphpad.com). Means and SD are represented on the graphs. All experiments have been performed 3 times. ns, not significant; \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001. Survival analyses were performed in R software environment version 4.2.1. Overall survival stratified on experimental groups was fitted with survival R-package version 3.5-7. Kaplan-Meier plot and log-rank test analysis were done with survminer R-package version 0.4.9.

## 3 Results

### 3.1 Generation of K562 cells overexpressing ILRA/CD25

As the native K562 cells lack CD25 receptor expression, our initial step involved generating a CD25 positive cell line to serve as a proof-of-concept for our CAR-CD25 strategy. Therefore, a CD25 transgene was introduced into K562 leukemic cells via lentiviral transduction. The CD25 gene was under the control of the CMV promoter and Blasticidin S resistance was used as a selection marker (Figure 2A). Following antibiotic selection and CD25<sup>+</sup> cell sorting, we successfully acquired a highly purified population with CD25 expression levels approaching 100% (K562-CD25) (Figure 2B, blue). Therefore, these cells serve as an ideal CML model expressing CD25, making them the optimal choice for assessing the effectiveness of CD25 CAR-NK92 cells.

### 3.2 Generation of NK92 cells expressing CD25 CAR

The anti-CD25 CAR-NK was designed as a polycistronic construct, incorporating the scFv from the clinically approved monoclonal antibody Basiliximab, driven by the potent EF1a promoter. CD8a was employed as a signal peptide to address the CAR protein to the cell surface. Two costimulatory domains, 4-1BB as well as the NK-specific DAP12 were incorporated, followed by puromycin resistance and GFP, with both genes being regulated by the PGK promoter (Figure 1A). Following lentiviral transduction of NK92 cells with the CAR construct, transduced cells were evaluated based on their GFP expression and the level of CAR expression at their cell surface. Approximately 90% of cells were found to be double-positive (GFP<sup>+</sup> and CAR<sup>+</sup>) while no GFP or CAR expression was detected in NK92 WT (Figure 1B). The level of CAR expression was also evaluated by qRT-PCR and we observed a 150-fold higher

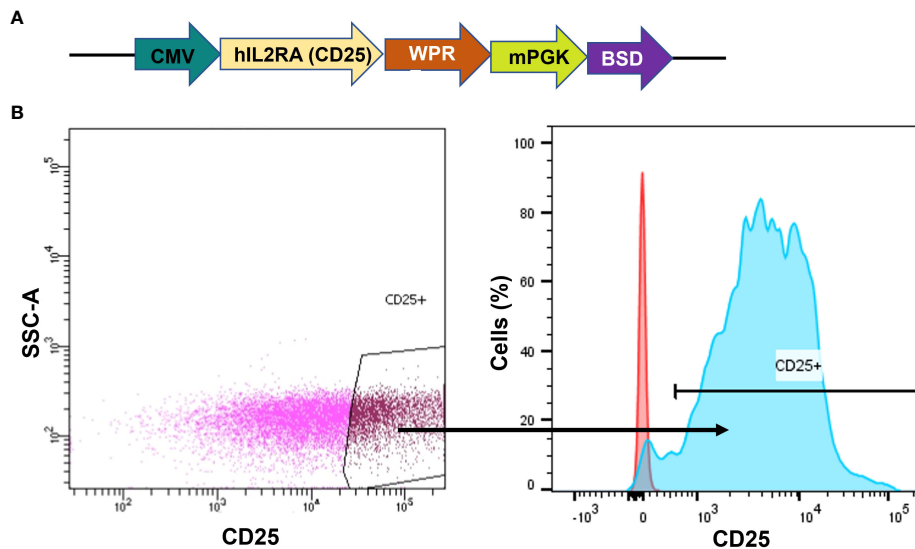


FIGURE 2

Generation of K562 cells expressing CD25. (A) hCD25 expression vector under the control of CMV promoter and followed by Blasticidin S resistance. (B) CD25 expression on K562 cell line after lentiviral transduction. The gate shows the sorted population (left). CD25 expression on sorted cells (in blue) as compared to K562 WT (in red) (right).

relative expression of CAR mRNA in CD25 CAR-NK92 cells as compared to untransduced cells (Figure 1C). In terms of phenotype, there was no significant distinction in the expression of NK activating and inhibitory signals between CD25 CAR-NK92 and NK92 WT. This suggest that the transduction did not affect the phenotype of the cells. Intriguingly, we observed a reduction in the level of CD25 expression in CD25 CAR-NK92 when compared to NK92 WT (Figure 1D).

### 3.3 CD25 CAR-NK92 cells exhibit a higher activated profile and cytotoxicity as compared to NK92 WT

In order to assess the functionality of the CD25 CAR-NK92 cells, we evaluated the level of Granzyme B and Perforin expression following their co-cultivation with K562-CD25 cells. We observed a remarkable increase in Granzyme B expression in CD25 CAR-NK92 cells as compared to NK92 WT (Figure 3A). The levels of Perforin were assessed in the same conditions and were found to be significantly higher in CD25 CAR-NK92 cells as compared to NK92 WT (Figure 3B).

The degranulation potential was assessed by CD107a staining of NK92 WT and CD25 CAR-NK92 cells cocultured with or without K562-CD25. As can be observed in Figure 3C, CD25 CAR-NK92 cells show a much higher degranulation profile as compared to NK92 WT when stimulated with K562-CD25 (Figure 3C).

IFN $\gamma$  production after stimulation of CD25 CAR-NK92 and NK92 WT was assessed by ELISA. We observed a strong increase of IFN $\gamma$  levels in the CD25 CAR-NK92 condition co-cultured with K562-CD25 as compared to NK92 WT ( $p < 0.0001$ ) (Figure 3D). This suggests a much stronger activation of CD25 CAR-NK92 cells when co-cultured with K562-CD25 as compared to NK92 WT co-

cultured with K562-CD25. We also observed a statistical difference ( $p < 0.01$ ) between the levels of IFN $\gamma$  produced by CD25 CAR-NK92 and NK92 WT when co-cultured with K562-WT showing a higher inherent activity of CAR cells as compared to WT (Figure 3D).

In order to evaluate the cytotoxic potential of CD25 CAR-NK92 cells, the number of apoptotic cells resulting from a co-culture with K562-CD25 at different ratios was assessed. We observed a much higher percentage of apoptotic K562-CD25 cells (Annexin V<sup>+</sup>) in the CD25 CAR-NK92 condition as compared to NK92 WT for all ratios. This is coherent with the high level of activation of CD25 CAR-NK92 cells observed above (Figure 3E).

### 3.4 CD25 CAR-NK92 cells show enhanced *in vivo* antitumoral effect against K562-CD25 as compared to NK92 WT cells

We next assessed the anti-leukemic activity of CD25 CAR-NK92 *in vivo* by using a noncurative mouse model of K562-CD25 cells. NSG (NOD-Prkdc<sup>scid</sup> IL2rg<sup>tm1</sup>/Bcgen) mice were injected intraperitoneally at D-3 with K562-CD25 Luciferin expressing (Luc) leukemic cells. The mice were treated by the injection of either NK92 WT or CD25 CAR-NK92 at D0, D3 and D7. Mice were followed for two months and imaged regularly by bioluminescence (BLI) (Figure 4A). As shown in Figure 4B, untreated mice exhibited a fast-developing cancer, mostly isolated at the beginning but later spreading in the peritoneum. Mice treated with NK92 WT cells had a delayed development of leukemia as compared to the untreated cohort which is related to the natural antileukemic activity of NK92 cells. On the other hand, the cohort treated with CD25 CAR-NK92 cells showed much slower cancer progression resulting in mice that were completely tumor-free after one week of treatment (Figure 4B). This result is shown with the Kaplan-Meier curve in



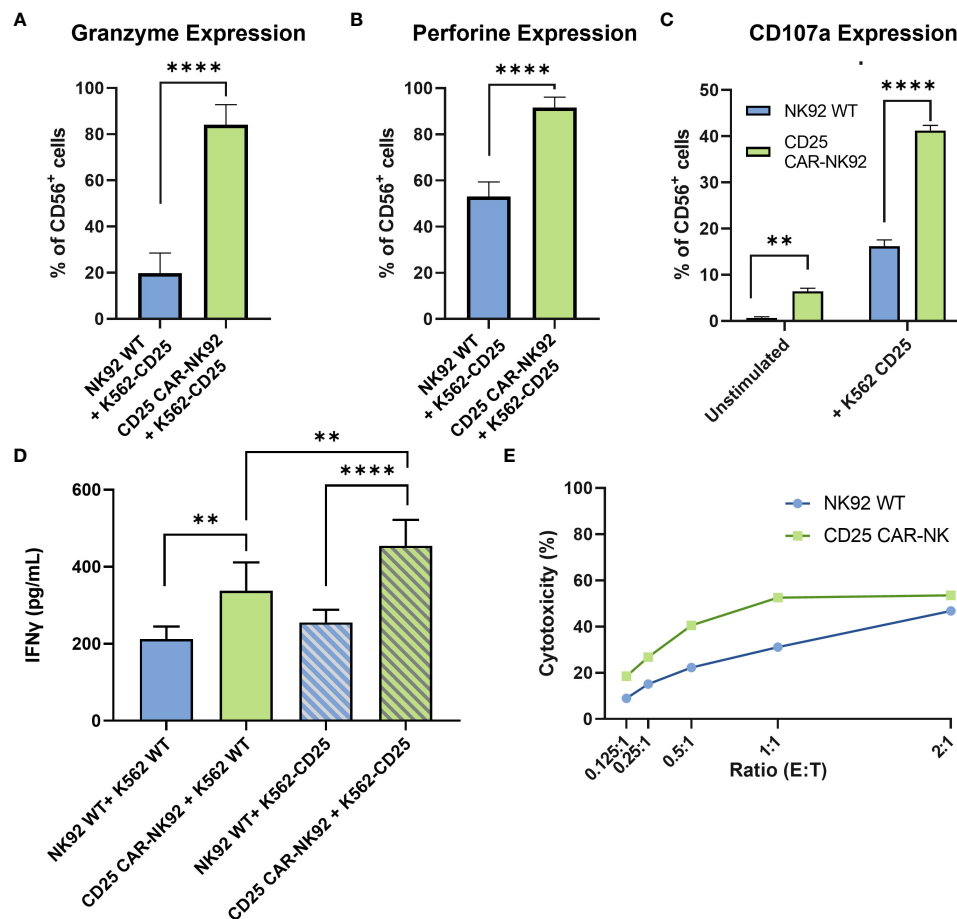


FIGURE 3

Functional and cytotoxic assessment of CD25 CAR-NK92 cells. (A, B) Granzyme B and Perforin expression levels of CD25 CAR-NK92 and NK92 WT cells after co-culture with K562-CD25 cells. (C) Degranulation assay of NK92 WT and CD25 CAR-NK92 cells after co-culture with K562-CD25. (D) IFN $\gamma$  levels assessment of CD25 CAR-NK92 and NK92 WT after stimulation either with K562-CD25 or K562-CD25. (E) Cytotoxic assay at different Effector (E) Tumor (T) ratios for NK92 WT: K562-CD25 in blue and CD25 CAR-NK92 in green. Means and SD are represented. Experiments have been performed 3 times. P-values were calculated using a 2-tailed Student's t-test. \*\*P < 0.01; \*\*\*\*P < 0.0001.

Figure 4C, in which the efficiency of CD25 CAR-NK92 therapy appears clearly significant in the survival rate as compared to the untreated and NK92 WT treated cohorts. Indeed, the majority of untreated mice died between D20 and D30. In the NK92 WT treated cohort, 6 mice survived at D40 which is coherent with the delay due to the NK92 antileukemic activity observed also previously. At day 60, only 4 mice were still alive in the NK92 WT treated cohort. While in the CD25 CAR-NK92 condition, two mice died around D20 and the others remained tumor-free without rejection for more than two months (Figure 4C). This shows the major interest of targeted therapy via CD25 CAR-NK92 cells against K562 cells expressing CD25, validating the potential of this strategy for future clinical use in blast-phase CML and AML.

## 4 Discussion

Although the use of TKI therapies against CML has allowed tremendous progress during the past twenty years, the development of TKI resistance and CML progression remains still a challenging

issue because of limited therapeutic solutions at this stage of the disease. Developing new therapeutic tools against AP/BP-CML is therefore an unmet medical need in this life-threatening step of CML. We and others have shown previously the involvement of CD25 as a biomarker detected in primary BP-CML (14, 20) as well as its expression in *in vitro* BP-CML models (21). These findings prompted us to design CAR-NK to target CD25 as an off-the-shelf immunotherapy for CML blast crisis. The advantages of NK cells over T cells for CAR-based therapies are multiple. Firstly, NK cells have the innate ability to kill cancer cells or virally infected cells (27). Additionally, they have the advantage to induce cytotoxicity in an HLA-independent manner and do not give rise to graft-versus-host-disease (GvHD) which is the major therapeutic issue in the setting of allogeneic stem cell transplantation. Other advantages of NK cells over T cells include the absence of cytokine release syndrome or neurotoxicity (23, 24), as well as their antibody-dependent cellular cytotoxicity (ADCC) and their ability to kill tumor cells in a CAR-independent manner (25). The advantage of using a CAR-based strategy over existing monoclonal antibodies (mAb) is the fact that CAR-NK and CAR-T cells directly lyse the

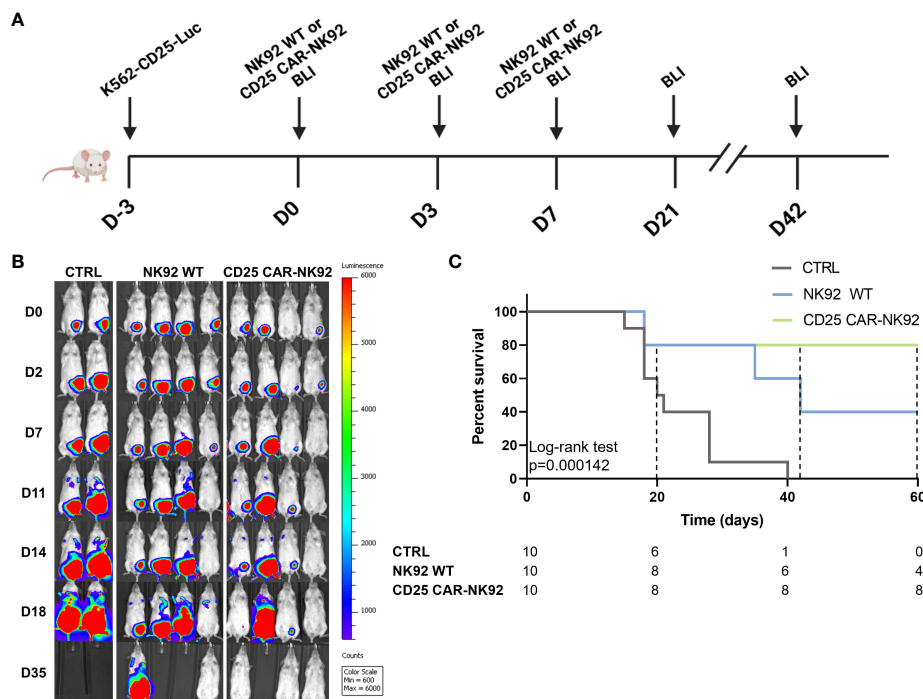


FIGURE 4

*In vivo* antitumoral activity assessment of CD25 CAR-NK92 cells. (A) Schematic representation of the experimental procedure, (BLI – Bioluminescence imaging) (B) BLI obtained by IVIS of three groups of mice, untreated (CTRL), NK92 WT treated in the middle and CD25 CAR-NK92 treated at the left (C) Kaplan-Meier and log-rank p-value of the overall survival stratified on experimental groups. survival curve of the three groups.

tumor cell upon engaging the antigen using the physiologic cytotoxic machinery of killer cells (28). Moreover, CAR cells have the ability to target cells with low antigen expression which can escape to mAb (28). In the context of CML progression, it has been shown that NK cells have a central role in the control of the disease and the anti-leukemic activity of NK cells inversely correlates to disease progression (29). For that reason, restoring the activity of NK cells in CML patients has been widely studied and the CAR-NK approach is of particular interest because it combines specificity and functional NK activity (29).

Basiliximab is a chimeric mouse-human monoclonal antibody to the  $\alpha$  chain of the IL-2 receptor. It is indicated for the prophylaxis of acute organ rejection in *de novo* allogeneic renal transplantation (30). To the best of our knowledge, its scFv domain has never been used against CD25 in anti-tumoral approaches. The other anti-CD25 monoclonal antibody, Daclizumab, has been withdrawn from the market.

In order to generate CD25 CAR-NK92, we transduced the NK92 cell line with CAR-expressing lentivector. Phenotypic analyses showed no loss of the main NK markers. Interestingly, the loss of CD25 expression in the CD25 CAR-NK92 condition was not related to fratricide because no increased apoptosis was observed (Data not shown). The levels of Granzyme B and Perforin after stimulation with K562-CD25 cells in CD25 CAR-NK92, were coherent with the increased activity and degranulation of these cells. Consequently, we observed higher levels of IFN $\gamma$  and apoptosis *in vitro*. *In vivo* assay showed a clear difference between

the NK92 WT and CD25 CAR-NK92 where tumor-free mice survived without rejection for more than three months.

NK92 cells have an inherent anti-leukemic activity against K562 (31) and this is observed in all experiments with K562-CD25. Nevertheless, the CD25 CAR introduction conferred an increased antileukemic activity against K562-CD25 cells.

Although CAR-NK therapies exhibit a major clinical potential due to their safer profile in terms of GvHD as compared to CAR-T cells as well as to their HLA independence and allogeneic utility, some questions remain still to be raised. One of these is the source of NK cells for therapy. Heterogeneity and batch-to-batch differences for peripheral blood-NK (PB-NK) and umbilical cord blood (UCB-NK) as well as the accessibility suggest the need for new NK sources. NK92 cell line, even though largely used in therapy after irradiation, remains a tumorigenic cell line with potential risks for the patients.

Immunotherapies based mostly on CAR-T and more recently in CAR-NK cells against myeloid malignancies especially AML are giving hope for the treatment of these diseases (32). In BP-CML, recent work has shown the feasibility of targeting CD38<sup>+</sup> blast cells using autologous CAR-T cells (33). The interest of a CD25 CAR-NK approach among existing CAR-T therapies against myeloid malignancies is the combination of the specific targeting (CD25) of blast cells with NK cells whose advantages over T cells are described above. Moreover, CD25 is also shown to be expressed in the leukemic stem cell compartment of CML, suggesting an additional potential beneficial effect of this approach for the eradication of CML stem cells.

It would be also of great interest to associate Ponatinib with the CD25 targeting approach by CAR-NK strategy. Indeed, Ponatinib is used alone or in combination with other agents in patients with BP-CML (34) but relapses and or resistance are common with dismal prognosis in the absence of stem cell transplantation.

In healthy adults, the CD25 molecule is expressed in regulatory T cells (Tregs) playing a crucial role in the maintenance of immunological self-tolerance (35). These cells function throughout life by suppressing the activity of autoreactive T cells and represent therefore a major cell population against the development of autoimmunity (36). Tregs have also been shown to play a role in cancer and the depletion of Tregs could be beneficial in cancer therapy (37). Interestingly, numbers and frequencies of Tregs in peripheral blood and bone marrow are increased in CML patients at diagnosis (38–40). Moreover, Tregs are shown to be involved in the immune escape of leukemic stem cells (41) and the depletion of CD25<sup>+</sup> Tregs has been shown to enhance effector activation and antitumor immunity (42). Therefore, a potential depletion of this increased Treg population in CML by CD25 CAR-NK92 might be of interest.

As opposed to CAR-T cells the adverse effects of which are well-established (43) CAR-NK cells are expected to have less toxicity with a high level of GVL effect and the absence of GVHD effects (44). Further experiments are needed to determine the off-side activity and the ability of CD25 CAR-NK92 cells to target non-cancerous CD25-expressing cells. The determination of the expression of stress markers in Tregs and other tissues would be important to predict potential off-target activity.

Altogether, these results pave the way for a new potential way of fighting BP-CML by targeting together the leukemic blast cell mass as well as LSCs, while potentially inhibiting by the same approach the immune escape of the leukemic cells.

## 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 author.

## Ethics statement

The animal study was approved by Inserm Animal Ethical Committee. The study was conducted in accordance with the local legislation and institutional requirements.

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## Author contributions

JJ: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Formal analysis, Methodology. PM: Formal analysis, Investigation, Methodology, Writing – review & editing. MH: Investigation, Methodology, Writing – review & editing. CD: Data curation, Investigation, Methodology, Validation, Writing – review & editing. DMC: Investigation, Methodology, Writing – review & editing. FG: Investigation, Resources, Supervision, Writing – review & editing. DTC: Resources, Supervision, Writing – review & editing, Project administration. LD: Investigation, Methodology, Conceptualization, Data curation, Resources, Validation, Writing – review & editing. AB: Resources, Supervision, Conceptualization, Investigation, Writing – review & editing. AT: Investigation, Resources, Supervision, Conceptualization, Project administration, Writing – original draft, Writing – review & editing.

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## EDITED BY

Jennifer D. Wu,  
Northwestern University, United States

## REVIEWED BY

Gabriel Marseres,  
The Netherlands Cancer Institute  
(NKI), Netherlands  
Ana Vuletić,  
Institute of Oncology and Radiology  
of Serbia, Serbia

## \*CORRESPONDENCE

Hans-Heinrich Oberg  
✉ Hans-Heinrich.Oberg@uksh.de

<sup>†</sup>These authors share first authorship

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# Vdelta1 T cells are more resistant than Vdelta2 T cells to the immunosuppressive properties of galectin-3

Jan Schadeck<sup>1†</sup>, Hans-Heinrich Oberg<sup>1\*†</sup>, Matthias Peipp<sup>2</sup>,  
Nina Hedemann<sup>3</sup>, Wolfgang W. Schamel<sup>4,5</sup>, Dirk Bauerschlag<sup>3</sup>  
and Daniela Wesch<sup>1</sup>

<sup>1</sup>Institute of Immunology, University Medical Center Schleswig-Holstein, Christian-Albrechts University, Kiel, Germany, <sup>2</sup>Division of Antibody-Based Immunotherapy, University Medical Center Schleswig-Holstein, Christian-Albrechts University, Kiel, Germany, <sup>3</sup>Department of Gynecology and Obstetrics, University Medical Center Schleswig-Holstein, Kiel, Germany, <sup>4</sup>Signalling Research Centre Biological Signalling Studies (BIOSS) and Centre of Integrative Biological Signalling Studies (CIBSS), Faculty of Biology, University of Freiburg, Freiburg, Germany, <sup>5</sup>Centre for Chronic Immunodeficiency (CCI), Medical Centre Freiburg, and Faculty of Medicine, University of Freiburg, Freiburg, Germany

Ovarian carcinomas have the highest lethality amongst gynecological tumors. A problem after primary resection is the recurrence of epithelial ovarian carcinomas which is often associated with chemotherapy resistance. To improve the clinical outcome, it is of high interest to consider alternative therapy strategies. Due to their pronounced plasticity,  $\gamma\delta$  T cells are attractive for T-cell-based immunotherapy. However, tumors might escape by the release of lectin galectin-3, which impairs  $\gamma\delta$  T-cell function. Hence, we tested the effect of galectin-3 on the different  $\gamma\delta$  T-cell subsets. After coculture between ovarian tumor cells and V $\delta$ 1 or V $\delta$ 2 T cells enhanced levels of galectin-3 were released. This protein did not affect the cytotoxicity of both  $\gamma\delta$  T-cell subsets, but differentially influenced the proliferation of the two  $\gamma\delta$  T-cell subsets. While increased galectin-3 levels and recombinant galectin-3 inhibited the proliferation of V $\delta$ 2 T cells, V $\delta$ 1 T cells were unaffected. In contrast to V $\delta$ 1 T cells, the V $\delta$ 2 T cells strongly upregulated the galectin-3 binding partner  $\alpha$ 3 $\beta$ 1-integrin after their activation correlating with the immunosuppressive properties of galectin-3. In addition, galectin-3 reduced the effector memory compartment of zoledronate-activated V $\delta$ 2 T cells. Therefore, our data suggest that an activation of V $\delta$ 1 T-cell proliferation as part of a T-cell-based immunotherapy can be of advantage.

## KEYWORDS

gammadelta T cells, Vdelta1, galectin-3, tumor-infiltrating T cells, ovarian cancer, integrins, TIGIT, PD-1

**Abbreviations:** bsTCE, Bispecific T-Cell Engager; EOC, epithelial ovarian tumors; CI, cell index; KI-OCp, KIEL-Ovarian Cancer primary cells; EpCAM, epithelial cell adhesion molecule; HER-2, human epidermal growth factor receptor-2; MFI, Median fluorescence intensity; MUC16, Mucin 16; OC, ovarian cancer; PDAC, pancreatic ductal adenocarcinoma; PD-1, programmed cell death protein-1; TIGIT, T cell immunoreceptor with Ig and ITIM domains; Treg, regulatory T cells; TCR, T-cell receptor; TIL, tumor infiltrating cells.

## 1 Introduction

Human  $\gamma\delta$  T cells can be classified in at least three major subsets, defined by the variable domain of the  $\delta$  chain (1). Alternatively, they can be classified by the variable domain of the  $\gamma$  chain in six major subsets (2, 3). V $\delta$ 1 T cells with a variable  $\gamma$  chain are present in the skin and mucosa, while V $\delta$ 3 T cells with a variable  $\gamma$  chain constitute a main population in skin and liver (4). Their antigens are so far not defined in detail (5, 6). V $\delta$ 2 T cells which coexpress V $\gamma$ 9 are mainly found in the peripheral blood. V $\gamma$ 9V $\delta$ 2 T cells recognize with their canonical T-cell receptor prenyl pyrophosphates that are enhanced in many tumor cells due to a dysregulated mevalonate pathway and are recruited into the tumor via chemokine receptors (7–9). All three human  $\gamma\delta$  T-cell subsets infiltrate in tumors including colorectal cancer, Merkel cell carcinoma and ovarian cancer and have been implicated in cancer immunosurveillance (3, 10, 11). A prognostic significance of  $\gamma\delta$  T cells in a broad range of human tumor entities, a correlation with patient outcome together with their high plasticity and their HLA-independent recognition of antigens offers interesting perspectives for  $\gamma\delta$  T-cell-based immunotherapy (12–14). Otherwise, the high functional plasticity of  $\gamma\delta$  T cells can promote  $\gamma\delta$  T-cell differentiation into an immunosuppressive phenotype (15–17).

Our recently published data revealed that the activation of V $\gamma$ 9V $\delta$ 2 T cells cocultured with pancreatic ductal adenocarcinoma (PDAC) cells induced an enhanced release of the lectin galectin-3 by PDAC cells (18). Galectin-3 binds to  $\beta$ -galactoside and thus to glycosylated Natural killer (NK) and T-cell surface receptors thereby inducing impairment of NK cell activity and anergy of tumor infiltrating CD8 lymphocytes (CD8 TIL) in cancer (19–22). Therefore, galectin-3 is regarded as an intrinsic tumor escape mechanism (17, 23). Our previous results demonstrated that galectin-3 did not influence V $\gamma$ 9V $\delta$ 2 T-cell cytotoxicity against PDAC cells but inhibited their proliferation by interacting with the glycosylated receptor  $\alpha$ 3 $\beta$ 1 integrin (CD49/CD29) on the cell surface, which is of high relevance for  $\gamma\delta$  T-cell-based immunotherapy (18).

Here, we were interested whether other tumor entities such as epithelial ovarian tumors (EOC) cocultured with V $\gamma$ 9V $\delta$ 2 T cells release comparable amounts of galectin-3 as the cross talk of PDAC and V $\gamma$ 9V $\delta$ 2 T cells did. Recently, others described a negative correlation with the overall survival rate, a platinum resistance and a correlation with pathologic grading in EOC patients which highly express galectin-3 and p65 (24, 25). As shown in experimental animal tumor models, targeting the interaction of galectin-3 with N-glycosylated ectodomain MUC16 expressed on serous ovarian cancer cells by high-affinity antibody is suggested as a potential cancer therapeutic strategy (26, 27).

More interestingly, the effector function of V $\gamma$ 9V $\delta$ 2 T cells was examined after cross talk with ovarian cancer cells in comparison to V $\delta$ 1 T cells, since the number of tumor infiltrating V $\delta$ 1 T cells (V $\delta$ 1 TIL) is increased within pancreatic and ovarian tumor tissue. A different sensitivity of both  $\gamma\delta$  T-cell subsets towards immunosuppressive properties of galectin-3 could have consequences for  $\gamma\delta$  T-cell-based immunotherapy.

## 2 Materials and methods

### 2.1 Patient cohort

Leukocyte concentrates from healthy adult blood donors were provided by the Department of Transfusion Medicine of the University Medical Center Schleswig-Holstein (UKSH) in Kiel, Germany. EDTA blood and tumor tissue from patients were obtained from the Department of Gynecology and Obstetrics of the UKSH in Kiel, Germany. Written informed consent was obtained from all donors in accordance with the Declaration of Helsinki, and the research was approved by the relevant institutional review boards (Ethic Committee of the Medical Faculty of the CAU Kiel, code number: D 445/18).

### 2.2 Established and freshly isolated tumor cell lines and their culture conditions

The human PDAC cell line PancTu1 was kindly provided by Dr. C. Röder and Prof. Dr. A. Trauzold, Institute for Experimental Cancer Research, Kiel, Germany. Esophageal adenocarcinoma OE-33 cell line, UM-UC-3 bladder cancer cell line, non-small cell lung adenocarcinoma NCI-H1693 cells, breast cancer cells (MCF-7, MDA-MB-231) and ovarian cancer cells (OVCAR-3, BG-1 and SKOV-3) were ordered from ATCC, Manassas, VA, USA. Freshly isolated KIEL-Ovarian Cancer primary (KI-OCp) cells derived from tumor tissue (Gynecology Department, UKSH, Kiel) were prepared as described elsewhere (3). Briefly, tumor tissues were minced and treated with components A, H, and R of the Tumor Dissociation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) for 1 h at 37°C in 5 mL PBS in a gentle MACS (Miltenyi Biotec). Whereas KI-OCp1, 012 and 15 were passaged over several times, freshly isolated ovarian tumor cells KI-OCp79, 88 and 91 were used directly after tumor dissociation. All tumor cells were cultured in complete medium under regular conditions (5% CO<sub>2</sub>, humidified, 37°C). For dissociation of the adherent tumor cell lines from flasks, 0.05% trypsin/0.02% EDTA (Sigma Aldrich/Merck, Darmstadt, Germany) was used. The cells were then collected, centrifuged and individual amounts of tumor cells were disseminated with medium in flasks again. Absence of mycoplasma was routinely confirmed by RT-PCR (Venor R GEM classic, Minerva Biolabs GmbH, Germany) and genotype by short tandem repeat analysis.

### 2.3 Isolation of peripheral blood mononuclear cells and tumor-infiltrating lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from the leukocyte concentrates or from EDTA blood of ovarian patients by Ficoll-Hypaque<sup>TM</sup> PLUS (Cytiva, Uppsala, Sweden) density gradient centrifugation. Cells were washed in PBS, and resuspended in RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 2 mM L-glutamine, 25 mM Hepes, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin (PanReac AppliChem,

Darmstadt, Germany), 10% FCS (Thermo Fisher Scientific, Langensfeld, Germany) (complete medium). Tumor-infiltrating cells (TIL) were isolated from the dissociated tumor tissue described under 2.2. Digested cell suspension was passed after the gentle MACS through a 100 µm cell strainer (Falcon, BD Biosciences, Heidelberg, Germany), and centrifuged at 481 ×g for 5 min. TIL and tumor cells were separated by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation followed by an adherence step for several hours.

The percentage of Vδ1 T cells within PBMC ranged between 0.1 and 3% (median 0.5%), and in TIL between 0.5 and 5% (median 1%), whereas the percentage of Vδ2 T cells within PBMC ranged between 0.1 and 10% (median 1.7%), and in TIL between 0.1 and 2.5% (median 0.9%).

## 2.4 Establishment of γδ T-cell lines out of PBMC or TIL

To expand Vγ9Vδ2-expressing T cells, 1x10<sup>6</sup> PBMC or TIL/mL were stimulated with 2.5 µM aminobisphosphonate (n-BP) zoledronate (Novartis, Basel, Switzerland), which induces selective activation and proliferation. The expansion of Vδ1-expressing γδ T cells was induced by coating 24-well plates with 250 µL/well of 10 µg/mL anti-Vδ1 mAb clone R9.12 (Beckmann Coulter, Krefeld, Germany) overnight at 4°C and washing the wells afterwards. 1x10<sup>6</sup> PBMC or TIL/well were cultured in the coated wells with soluble 1 µg/mL anti-CD28 mAb clone CD28.2 (BioLegend, San Diego, USA). In one patient Vγ2,3,4-expressing Vδ1 T cells were expanded by incubating PBMC with anti-Vγ2,3,4 mAb clone 23D12 (28) and cross-linking via rabbit-anti-mouse polyclonal Ab (Dianova, Hamburg, Germany) for a period of 30 minutes each. After washing, 1x10<sup>6</sup> PBMC/well were cultured with 50 IU/mL rIL-2 and 1 µg/mL anti-CD28 mAb clone CD28.2 for a period of 14 days. Since resting, initially stimulated γδ T cells produced only low amounts of IL-2, 50 IU/mL of recombinant IL-2 was added every 2 days over a culture period of 14 days.

After 14 days, the short-term activated γδ T-cell lines were stained with AF700-labeled anti-CD3 clone SK7 (BioLegend), AF488-labeled anti-Vγ9 clone 7A5 (29), AF647-labelled anti-Vγ2,3,4 clone 23D12 (28) PE-Cy7-labeled anti-T-Cell Receptor (TCR) pan γδ clone 11F2, PE-labeled anti-Vδ2 clone B6 (both BD Biosciences, Heidelberg, Germany) and with VioBlue-labeled anti-Vδ1 clone REA173 (Miltenyi Biotec, Bergisch Gladbach, Germany), and analyzed by flow cytometry to determine the purity. At a purity of >95%, γδ T cells were used for functional assays or preserved in liquid nitrogen, while they were subjected to a positive magnetic separation by using anti-Vγ2,3,4 mAb clone 23D12 or anti-Vδ1 mAb clone R9.12 followed by anti-Mouse IgG MicroBeads (Miltenyi Biotec) at a purity <95%. After positive selection, cells were restimulated in rIL-2-supplemented medium with 0.5 µg/mL phytohemagglutinin (Thermo Fisher Scientific), and irradiated PBMC (20x10<sup>6</sup> cells) and/or EBV-transformed B cell lines (2x10<sup>6</sup> cells) as feeder cells for 20x10<sup>6</sup> γδ T cells. Dead feeder cells were removed 3–4 days after restimulation by Ficoll-Hypaque density gradients. Purity of γδ T cells was >95% as analyzed by flow cytometry.

## 2.5 Functional cell culture assay

To analyze the effect of galectin-3 (BioLegend) on the proliferation of γδ T cells, the percentage of γδ T cells was determined and accordingly adapted to 2x10<sup>4</sup> γδ T cells per well. Therefore, the number of PBMC and TIL ranged between 1.5 to 7x10<sup>5</sup> PBMC or TIL per 24-well. PBMC or TIL were plated in complete medium with 50 IU/mL rIL-2. These cells were selectively activated by either 2.5 µM zoledronate or by coating the wells with 250 µL/well of 10 µg/mL anti-Vδ1 mAb clone R9.12 together with soluble 1 µg/mL anti-CD28 mAb clone CD28.2 in the absence or presence of different concentrations (0.01, 0.1, 1, 10, 1000 ng/mL) of galectin-3 or 100 nM galectin-3 inhibitor TD-139 (Selleck Chemicals, Planegg, Germany) daily. After 6, 7 and/or 9 days, the Vδ1 and Vδ2 T-cell proliferation was analyzed as described in the 'absolute cell number analysis by Flow Cytometry' section.

When coculturing tumor cells to determine absolute cell numbers or perform galectin-3 ELISA, 20x10<sup>3</sup> PDAC cells (PancTu1) or different ovarian cancer cells were plated in 24-well plates in complete medium. After 24 hours, a calculated amount of PBMC were added to reach an effector/target (E/T) ratio of 1:1 (Vδ1 or Vδ2 T cells/tumor cells) PBMC were either coculture in 50 IU/mL rIL-2 in complete medium only, with 2.5 µM zoledronate or with bispecific T-Cell Engagers (bsTCE) selectively targeting human epidermal growth factor receptor (HER)-2 expressing ovarian tumor cells to Vγ9Vδ2 (30) or Vδ1 T cells (Oberg et al., manuscript in preparation).

To determine the expression of differentiation (naïve, central and effector memory, TEMRA), activation (CD25, CD69) and immune check point markers (TIGIT, PD-1) or galectin-3 binding partner α3β1 (CD49c/CD29), 5x10<sup>5</sup> PBMC were cultured in complete medium, and stimulated with 2.5 µM zoledronate or with coated anti-Vδ1 mAb clone R9.12 (10 µg/mL) together with soluble anti-CD28 mAb clone CD28.2 (1 µg/mL).

For CD49c/CD29 expression, 5x10<sup>5</sup> PBMC were additionally cocultured with 5x10<sup>4</sup> OVCAR-3 cells in the presence of bsTCE selectively targeting HER-2 expressing ovarian tumor cells to Vγ9Vδ2 or Vδ1 T cells.

Cells were stained as described in flow cytometry section after time points indicated in the figures.

## 2.6 Flow cytometry

In total, 1x10<sup>6</sup> PBMC from healthy donors or cancer patients, TIL from ovarian cancer patients and generated γδ T-cell lines were stained by multicolor flow cytometry approach to distinguish between diverse γδ T-cell subsets within different CD45<sup>+</sup> leukocyte populations for usage in functional assays. The color panel included the following backbone mAb: PerCP-labeled anti-CD45 clone 2D1, PE-Cy7-labeled anti-TCR pan γδ clone 11F2 (both BD Biosciences), AF700-labeled anti-CD3 clone SK7, BV510-labeled anti-CD4 clone OKT4 (both BioLegend), and additional mAbs: VioBlue-labeled anti-Vδ1 clone REA173 (Miltenyi Biotec), PE-labeled anti-Vδ2 clone B6 (BD Biosciences), AF488-labeled

anti-V $\gamma$ 9 clone 7A5 (29), AF647-labelled anti-V $\gamma$ 2,3,4 clone 23D12 (28), PEeFluor610-labeled anti-CD56 clone CMSSB (Thermo Fisher Scientific), APC-Cy7-labeled anti-CD8 clone SK1 and BV605-labeled anti-CD19 clone HIB19 (both Biolegend).

To determine activation, immune check point and differentiation marker, we combined backbone mAbs with PE-labeled anti-CD25 mAb clone REA945 (Miltenyi Biotec), APC-labeled anti-CD69 mAb clone FN50 (Biolegend), BV711-labeled anti-TIGIT mAb clone 741182 (BD Biosciences), BV785-labeled anti-PD-1 (CD279) mAb clone EH12.2H7 (Biolegend), BV605-labeled anti-CD45RA mAb clone HI100, PE-Dazzle594-labeled anti-CD27 mAb clone O323 (both Biolegend), APC-Vio770-labeled anti-V $\delta$ 2 mAb clone REA771 and VioBlue-labeled anti-V $\delta$ 1 clone REA173 (Miltenyi Biotec).

To analyze expression of CD49c and CD29, cells were stained with the backbone mAb together with APC-Vio770-labeled anti-V $\delta$ 2 mAb clone REA771, VioBlue-labeled anti-V $\delta$ 1 mAb clone REA173, PE-labeled anti-CD49c mAb clone C3 II.1 (BD Biosciences) and AF647-labeled anti-CD29 mAb clone TS2/16 (Biolegend).

Alternatively, a color panel described in the absolute cell number section was used for staining the PBMC, TIL and  $\gamma\delta$  T-cell lines at d0, d6, d7 and d9 of the functional assays.

To determine the expression of tumor-associated antigens such as epithelial cell adhesion molecule (EpCAM) and HER-2,  $1 \times 10^5$  of tumor cells were stained with the following mAbs: PerCP-labeled anti-CD45 clone 2D1 (BD Biosciences), PE-Vio770-labeled anti-HER-2 clone 24D2 and APC-labeled anti-EpCAM clone HEA-125 (both from Miltenyi Biotec) and corresponding isotype controls (BD Biosciences or Miltenyi Biotec). All tumor cells were also intracellularly stained with AF647-conjugated anti-galectin-3 mAb clone M3/38 or AF647-conjugated isotype rat IgG2a mAb clone RTK2758 (both BioLegend). For the intracellular staining,  $2.5 \times 10^5$  cells were washed with staining buffer, fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences), for 20 min following the procedures outlined by the manufacturer. Thereafter, the cells were washed twice with Perm/Wash by centrifugation and stained with anti-galectin-3 or isotype control mAb for 25 min, washed again twice and measured. All samples were analyzed on LSR-Fortessa flow cytometer (BD Biosciences) using Diva 9 software.

## 2.7 Absolute cell number analysis by flow cytometry

After culturing PBMC or TIL in the presence or absence of tumor cells, Trucount Tubes (#340334 from BD Biosciences) were used to measure the absolute cell number of viable V $\delta$ 1, V $\delta$ 2 and tumor cells. Therefore, supernatant was collected from the wells to determine galectin-3 release, and the remaining cells were transferred from the wells into 1.5 mL reaction tubes. To dissociate and collect the adherent cells, 0.05% trypsin/0.02% EDTA was used. After a washing step, the cells were stained with PerCP-labeled anti-CD45 mAb clone 2D1, PE-Cy7-labeled anti-TCR pan  $\gamma\delta$  mAb clone 11F2 (both BD Biosciences), AF700-labeled anti-CD3 mAb clone SK7 (BioLegend), APC-Vio770-labeled anti-

V $\delta$ 2 mAb clone REA771, VioBlue-labeled anti-V $\delta$ 1 mAb clone REA173, APC-labeled anti-EpCAM mAb clone HEA-125 (all three Miltenyi Biotec) and then washed again. For the differentiation between viable and dead cells, SYTOX<sup>TM</sup> Green dead cell stain with a dilution of 1:4000 (Thermo Fischer Scientific, order number S34860) was added to the probes with washing buffer. These were transferred into the Trucount tubes and analyzed on LSR-Fortessa flow cytometer (BD Biosciences) using Diva 9 software. Because each Trucount Tube contains a defined amount of beads the absolute cell number of the different cell populations can be calculated by dividing the total amount of beads by the measured beads with the flow cytometer and using this digit to multiply the various cell populations (Supplementary Figure 1).

## 2.8 Enzyme-linked immunosorbent assay

The quantification of galectin-3 released by tumor cells, PBMC and TIL alone or after coculture of these subsets was measured by sandwich DuoSet ELISA kit (#DY1154 from R&D System, Wiesbaden, Germany) in duplicates following the procedures outlined by the manufacturer. For this measurement 500  $\mu$ L of the supernatants were collected after different incubation times (24 hours to 9 days), centrifuged and  $2 \times 200 \mu$ L were stored at  $-20^\circ\text{C}$  until use.

## 2.9 Real-time cell analyzer

The cytotoxicity of the  $\gamma\delta$  T-cell lines against adherent cancer cell lines was analyzed in triplicates by using an RTCA (x-Celligence, Agilent Technologies, Inc., Santa Clara, CA, USA). The tumor cells were added to a 96-well micro-E-plate (10,000 cells per well) with complete medium to monitor the impedance via electronic sensors every 5 minutes for up to 24 hours, which can be equated with the adherence of the tumor cells. After the tumor cells have reached a linear growth phase,  $\gamma\delta$  T-cell lines with 50 IU/mL rIL-2 were added to the 96-well micro-E-plate. The cells were cultured in medium or stimulated with bsTCE such as [(HER2) $_2$   $\times$  V $\gamma$ 9] and [HER2  $\times$  CD3] described elsewhere (30) or an unpublished bsTCE targeting HER2-expressing ovarian tumor cells to V $\delta$ 1 T cells (manuscript in preparation). In addition, galectin-3 or titrated galectin-3 inhibitor TD-139 (Selleck Chemicals, Planegg, Germany) were added. Impedance variations are shown in an arbitrary unit called cell index (CI) which correlates with adherence and spreading, cell proliferation and cell death (in this case the tumor cells). To compare the ability of the different  $\gamma\delta$  T-cell lines to lyse the tumor cells and due to slight differentiation before adding the substances and T cells, the CI was normalized to 1. Triton X-100 with the final concentration of 1% per well was added to 3 wells with tumor cells only as a positive control for tumor cell death. The mean of Triton-X-100 samples was calculated and defined as 100% lysis after 4, 12 and 24 hours. The raw data files were exported from the RTCA software 2.0 to Microsoft Excel to calculate the cytotoxic potential of the  $\gamma\delta$  T-cell lines towards tumor cells. The ratio of each sample to spontaneous lysis of tumor cells alone was calculated and the ratio was normalized to maximal inducible lysis by Triton-X-100.



## 2.10 Statistical analysis

GraphPad Prism (GraphPad Software, LLC., La Jolla, CA, USA) was used for statistical analysis. The Shapiro-Wilk test was applied to determine the normal distribution assumption. For parametric data of matched datasets, a paired, two-tailed t-test was used. For non-parametric data of matched datasets, a Wilcoxon matched-pairs signed rank test was used. All statistical tests were two-sided, and the level of significance was set at  $\alpha \leq 5\%$ . The appropriate tests are indicated in the figure legends.

## 3 Results

### 3.1 Cross talk of tumor cells with activated V $\delta$ 2 T cells induces enhanced release of galectin-3

Recently, we demonstrated that galectin-3 produced by PDAC cells inhibited V $\gamma$ 9V $\delta$ 2 T-cell proliferation (18). Therefore, we asked whether the expression and release of galectin-3 is a common tumor escape mechanism of different tumor cells. Comparable to PDAC cells (PancTuI), esophageal adenocarcinoma OE-33 cell line, the UM-UC-3 bladder cancer cell line, non-small cell lung adenocarcinoma NCI-H1693 cells, breast cancer cells (MCF-7, MDA-MB-231) and ovarian cancer cells (OVCAR-3, BG-1 and SKOV-3) expressed intracellular galectin-3 (Figures 1A, B).

OVCAR-3 and SKOV-3 cells were established from ascitic fluid of different ovarian cancer patients, while BG-1 cell line was derived from a poorly differentiated stage III solid primary ovarian tumor. We investigated whether galectin-3 expression of these three commercially available ovarian cell lines (Figure 1B) is comparable to the expression of ovarian cell lines established out of primary serous ovarian tumors in our laboratory (KI-OCp1, 11 and 012, stage IIIc) and freshly isolated serous ovarian tumor cells (KI-OCp79, 88 and 91, stage III) (Figure 1C). We observed that the different established ovarian tumor cell lines expressed intracellular galectin-3 comparable to freshly isolated ovarian tumor cells.

As shown by a time kinetic over 72 hours, galectin-3 was released only in small amounts by either pancreatic and ovarian tumor cells (PancTuI, KI-OCp012 or OVCAR-3 cells) or by peripheral blood mononuclear cells (PBMC) (Figures 2A, C, E). In contrast, the coculture of the tumor cells combined with the selective activation of V $\gamma$ 9V $\delta$ 2 T cells within the PBMC by zoledronate increased galectin-3 release within 72 hours (Figures 2B, D, F). We further compared the commercially available ovarian cell lines BG-1 and SKOV-3 with our established serous ovarian cell line KI-OCp1 and our mucinous ovarian cell line KI-OCp15 by coculturing them with PBMC of three different donors in the absence (Figures 2G, J, L) or presence of zoledronate (Figures 2H, K, M) for 48 and 72 hours. We confirmed that PBMC and tumor cells released small amounts of galectin-3 (Figures 2G, H), which were significantly increased when coculturing PBMC with ovarian cancer cell lines (KI-OCp1 and 15,

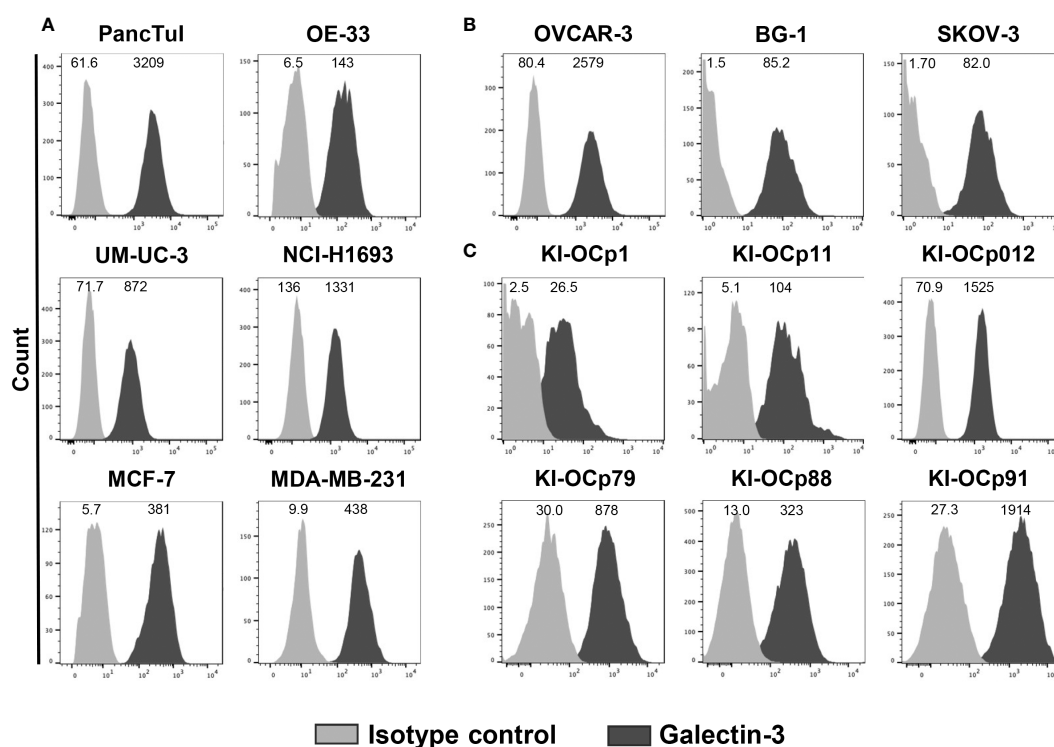


FIGURE 1

Intracellular galectin-3 expression in different tumor cells. (A–C) Median fluorescence intensity (MFI) ( $n = 13$ ) of galectin-3 expression (clone M3/38) and isotype control is shown for the indicated cell lines measured by LSR-Fortessa. The grey histograms represent the isotype control and the black histograms the galectin-3 expression.

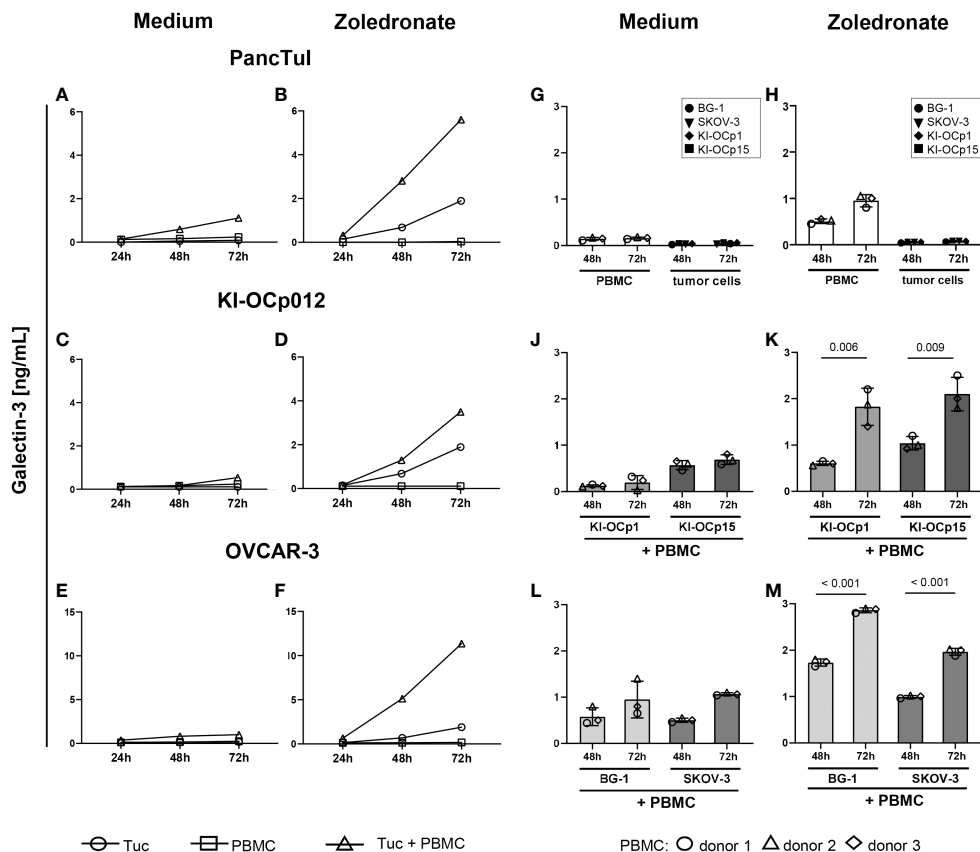


FIGURE 2

Coculture of PDAC and ovarian cells with zoledronate-activated PBMC induces the highest galectin-3 release. (A–M)  $3 \times 10^5$  freshly isolated PBMC containing  $2 \times 10^4$   $\gamma\delta$  T cells from four healthy donors were cultured with the indicated tumor cells (Tuc) with a calculated E/T ratio of 1:1 ( $\gamma\delta$  T cells to tumor cells) or each of them cultured separately. Cells were either cultured in medium (A, C, E, G, I, K, L) or stimulated with  $2.5 \mu\text{M}$  zoledronate (B, D, F, H, J, M).  $50 \text{ IU/mL}$  rIL-2 was added to each coculture. Cell culture supernatants were collected after 24, 48 and 72 hours and released galectin-3 was determined by ELISA. Statistical comparison was carried out parametrically by using paired, two-tailed *t*-test. Indicated P-values are shown.

BG-1 and SKOV-3) in the presence of zoledronate after 72 hours (Figures 2K, M). In the absence of zoledronate galectin-3 was slightly increased in the presence of tumor cells after 72 hours compared to 48 hours (Figures 2J, L).

The results demonstrated that all analyzed tumor cells express galectin-3 and that the coculture with  $\gamma\delta$  T cells enhanced the galectin-3 release.

### 3.2 Galectin-3 did not influence the cytotoxic activity of different $\gamma\delta$ T-cell subsets

Since an enhanced galectin-3 release is suggested as an intrinsic tumor escape mechanism, we investigated the influence of galectin-3 on the cytotoxicity, proliferation, activation and differentiation of the two major  $\gamma\delta$  T-cell subsets, V $\delta$ 1 and V $\delta$ 2 T cells.

By firstly focusing on  $\gamma\delta$  T-cell cytotoxicity, we cocultured a V $\delta$ 1 and a V $\delta$ 2 T-cell line established from a healthy donor together with ovarian tumor cells (KI-OCp012, OVCAR-3, SKOV-3) and PDAC PancTul cells in the absence or presence of bispecific T-Cell Engagers (bsTCE) targeting V $\delta$ 1 and V $\delta$ 2 T cells and HER-2 expressing tumor

cells (Figure 3). The results revealed that V $\delta$ 1 T cells have a superior capacity to lyse SKOV-3 and PancTul cells, whereas V $\delta$ 2 T cells are more effective in killing KI-OCp012 and OVCAR-3. Independently of this observation, the cytotoxic capacity of both  $\gamma\delta$  T-cell subsets can be increased by an enhanced effector/target ratio (10:1 versus 5:1) and/or the addition of bsTCE (Figure 3). Since the lysis of KI-OCp012 and OVCAR-3 differed, we analyzed whether the effector cells or galectin-3 release is crucial for the difference in lysis.

Therefore, we generated diverse  $\gamma\delta$  T-cell lines from PBMC of healthy donors and ovarian cancer patients as well as an autologous one and investigated their efficacy to lyse these both ovarian tumor cells (Figure 4). The  $\gamma\delta$  T-cell cytotoxicity of V $\delta$ 1 as well as of V $\delta$ 2 T cells was impaired against KI-OCp012 cells compared to OVCAR-3 cells (Figure 4A, med). Obviously, the V $\delta$ 1 T-cell line generated out of patient OCp012 has a low cytotoxicity against the autologous KI-OCp012 cells and the allogeneic OVCAR-3 cells. Besides, the addition of different concentrations of TD-139, a potent small-molecule inhibitor of galectin-3, did not improve  $\gamma\delta$  T-cell cytotoxicity against ovarian cancer cells after 24 hours (Figure 4A) or at earlier time points (Supplementary Figure 2).

The results were substantiated by experiments adding different galectin-3 concentrations (ranging from 0.01 to 10 ng/mL) to the

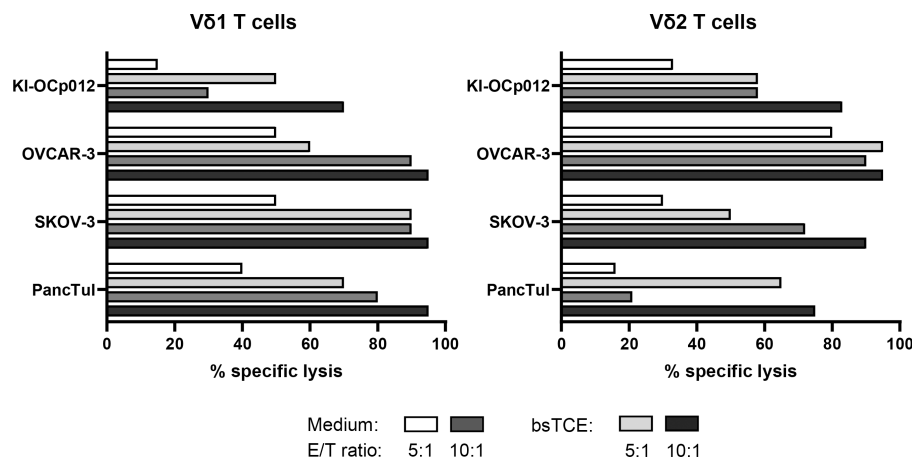


FIGURE 3

Cytotoxicity of Vδ1 and Vδ2 T cells against tumor cells can be enhanced by bispecific T-Cell Engagers (bsTCE). A total of  $10^4$  pancreatic (PancTul) or ovarian tumor cells (KI-OCp012, OVCAR-3, SKOV-3) per well were cultured in triplicates in complete medium overnight. Impedance of these adherent tumor cells expressed as cell index (CI) was analyzed in 5 minutes steps over ~24 hours in a RTCA system. After reaching the linear growth phase, tumor cells were cultured with medium alone (spontaneous lysis) or cocultured with Vδ1 and Vδ2 T-cell lines generated out of peripheral blood from one healthy donor. 12.5 IU/mL rIL-2 was added together with Medium (white and middle grey bars) or 1 µg/mL of bsTCE (light and dark grey bars) at an E/T ratio of 5:1 (white and light grey bars) or 10:1 (middle and dark grey bars). The loss of tumor cell impedance and thus a decrease of CI correlated with lysis of tumor cells. Specific lysis of tumor cells was calculated in comparison to spontaneous lysis and maximal lysis (100%) by Triton-X-100 24 hours after adding the γδ T cells.

cocultures (Figure 4B). Our results revealed that the cytotoxic capacity of the γδ T cell lines generated out of PBL or TIL is very similar in the absence of an immunosuppressive tumor microenvironment. Further, the different galectin-3 concentrations did not influence the γδ T-cell-mediated cytotoxicity towards the ovarian cancer cells after 24 hours (Figure 4B) or after earlier time points (Supplementary Figure 3).

In sum, comparable to Vδ2 T cells, cytotoxicity of Vδ1 T cells against tumor cells is not influenced by galectin-3.

### 3.3 Proliferation of Vδ2 T cells but not of Vδ1 T cells was inhibited by galectin-3 producing ovarian tumor cells

Previously, we found that galectin-3 released from PDAC cells inhibits Vδ2 T-cell proliferation (18). Therefore, we asked whether other tumor entities such as ovarian cancer cells have the same capacity, and whether Vδ1 T-cell proliferation is influenced by galectin-3.

We determined the percentage (Figure 5A) or the absolute cell number of Vδ2 T cells (Figure 5B) within PBMC and added a specific amount of PBMC to the culture to provide an E/T ratio of 1:1 of Vδ2 T cells and tumor cells. After 9 days of coculture, we analyzed the percentage (Figure 5A) or absolute cell number of viable proliferating Vδ2 T cells (Figure 5B) and tumor cells again. As a control PBMC were cocultured without tumor cells (Figures 5A, B; without KI-OCp012 or none). A vigorous selective Vδ2 T-cell growth after stimulation with zoledronate in the absence of the indicated ovarian tumor cells compared to the control was observed after 9 days (Figures 5A, B). In the presence of ovarian tumor cells (KI-OCp012, KI-OCp15, BG-1, SKOV-3), the

proliferation of Vδ2 T cells was significantly inhibited after stimulation with zoledronate in comparison to cultures without tumor cells (Figures 5A, B).

An increased release of galectin-3 was observed when coculturing the PBMC with KI-OCp012 cells after stimulation with zoledronate for 6 to 9 days (Figure 5C). A decrease of the absolute cell number of viable Vδ2 T cells in the presence of ovarian tumor cells together with zoledronate is shown for these two time points (Figure 5D). In contrast, a 46-fold increase of Vδ2 T cells within the PBMC in the absence of ovarian tumor cells is demonstrated in the same figure. In parallel, the absolute cell number of viable EpCAM (CD326)-positive ovarian tumor cells is reduced compared to day 0 (Figure 5E), which underline the observation that Vδ2 T-cell cytotoxicity is not influenced by galectin-3 (Figure 4).

Following the assumption that Vδ2 TIL are in a pre-activated stage, we analyzed the proliferative capacity of freshly isolated Vδ2 TIL cocultured in medium without or with freshly isolated autologous ovarian tumor cells (E/T ratio 1:1) in further experiments. Comparable to PBMC, an inhibition of the Vδ2 T-cell outgrowth was observed after stimulation with zoledronate in the presence of autologous ovarian tumor cells in comparison to the absence of tumors. This is shown for the absolute cell number of viable Vδ2 TIL of seven different donors (Figure 5F). The daily supplementation of galectin-3 inhibitor TD-139 to three different patients (closed symbols) restored the tumor cell mediated inhibition of Vδ2 T-cell proliferation (Figure 5F). An increase of galectin-3 was measured when Vδ2 TIL were cocultured with freshly isolated autologous ovarian tumor cells compared to the culture without tumor cells (Figure 5G).

Since the antigens for other γδ T-cell subsets than Vδ2 T cells are not well defined, we used plate-coated anti-TCR Vδ1 mAb

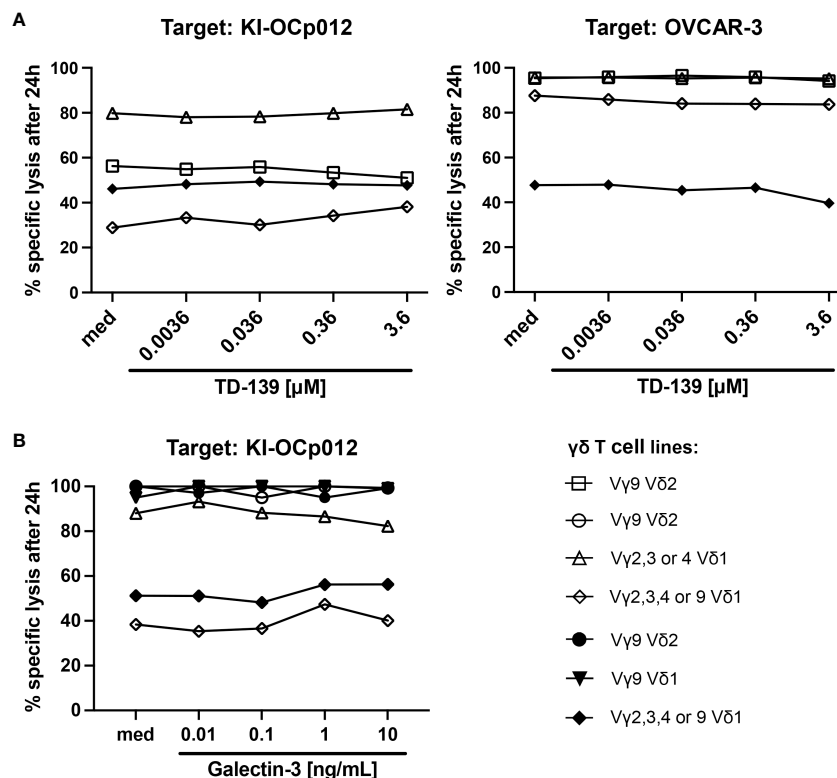


FIGURE 4

Galectin-3 does not influence  $\gamma\delta$  T-cell cytotoxicity against ovarian tumor cells. (A, B) A total of  $10^4$  indicated ovarian tumor cells per well were cultured in triplicates in complete medium overnight. Impedance of these adherent tumor cells expressed as cell index (CI) was analyzed in 5 minutes steps over ~24 hours in a RTCA system. After reaching the linear growth phase, tumor cells were cultured with medium (spontaneous lysis) or cocultured with different  $\gamma\delta$  T-cell subset lines isolated out of peripheral blood from healthy donors (open symbols,  $n = 4$ ) or ovarian cancer patients (closed symbols,  $n=3$ ) at an E/T ratio of 5:1. The V $\delta$ 1 T-cell line marked with a closed rhombus is autologous to KI-OCp012 tumor cells. 12.5 IU/mL rIL-2 was added to the cultures and cells were stimulated with 1  $\mu$ g/mL of bsTCE in the absence (med) or presence of the indicated concentrations of galectin-3 inhibitor TD-139 (A) or galectin-3 in distinct concentrations (0.01, 0.1, 1, 10 ng/mL) (B). The loss of tumor cell impedance and thus a decrease of CI correlated with lysis of tumor cells. Lysis of tumor cells was measured after normalization to 1 in 3 minutes steps for additional 24 hours and compared to maximal lysis (100%) by Triton-X-100. Specific lysis of tumor cells was calculated in comparison to spontaneous lysis and maximal lysis (100%) by Triton-X-100 24 hours after adding the  $\gamma\delta$  T cells.

together with soluble anti-CD28 mAb to stimulate V $\delta$ 1 T cells within PBMC and TIL in several of the experiments. However, the coating of plates with anti-TCR V $\delta$ 1 mAb was not possible when coculturing PBMC or TIL with adherent tumor cells. Therefore, we stimulated V $\delta$ 1 and V $\delta$ 2 T cells with our bsTCE. These both bsTCE selectively stimulated the different  $\gamma\delta$  T-cell subsets within PBMC or TIL and significantly enhanced the  $\gamma\delta$  T-cell mediated lysis against tumor cells (3) (manuscript in preparation). Since these bsTCE are not developed to induce  $\gamma\delta$  T-cell proliferation, our results with bsTCE stimulation revealed only a slight proliferation of V $\delta$ 1 or V $\delta$ 2 T cells within PBMC (closed symbols) or TIL (open symbol, autologous situation) cocultured with freshly isolated ovarian tumor cells (KI-OCp79, 88 and 91). Nevertheless, the proliferation was enough to determine a different effect of the galectin-3 inhibitor TD-139 on V $\delta$ 1 versus V $\delta$ 2 T cells. While the daily supplementation of TD-139 over 9 days of culturing restored the tumor cell mediated inhibition of V $\delta$ 2 T-cell proliferation, V $\delta$ 1 T cells are not influenced by TD-139 (Figures 6A, B).

To test whether the concentration of galectin-3 released by the freshly isolated tumor cells was not sufficient to inhibit V $\delta$ 1 T-cell proliferation, we added different concentrations of galectin-3 to

PBMC either stimulated with zoledronate or anti-TCR V $\delta$ 1/anti-CD28 mAbs as illustrated in Figures 6C, D. V $\delta$ 2 and V $\delta$ 1 T cells expanded 9 days after their selective activation compared to day 0. After stimulation, V $\delta$ 2 T cells expanded by 45-fold and V $\delta$ 1 T cells by 11-fold increase (Figures 6C, D). While the addition of increasing concentrations of galectin-3 inhibited V $\delta$ 2 T-cell proliferation, the V $\delta$ 1 T-cell proliferation was not impaired and slightly enhanced in the presence of 1-10 ng/mL recombinant galectin-3 (Figures 6C, D).

Taken together, galectin-3 inhibits the V $\delta$ 2 T-cell proliferation but not the V $\delta$ 1 T-cell proliferation.

### 3.4 Different effects by galectin-3 on the differentiation and activation of V $\delta$ 1 versus V $\delta$ 2 T cells

Since we observed different effects of galectin-3 on the proliferation of V $\delta$ 1 and V $\delta$ 2 T cells, we asked whether other features such as differentiation, activation and expression of immune check point markers differ between V $\delta$ 1 and V $\delta$ 2 T cells after their exposure to galectin-3.



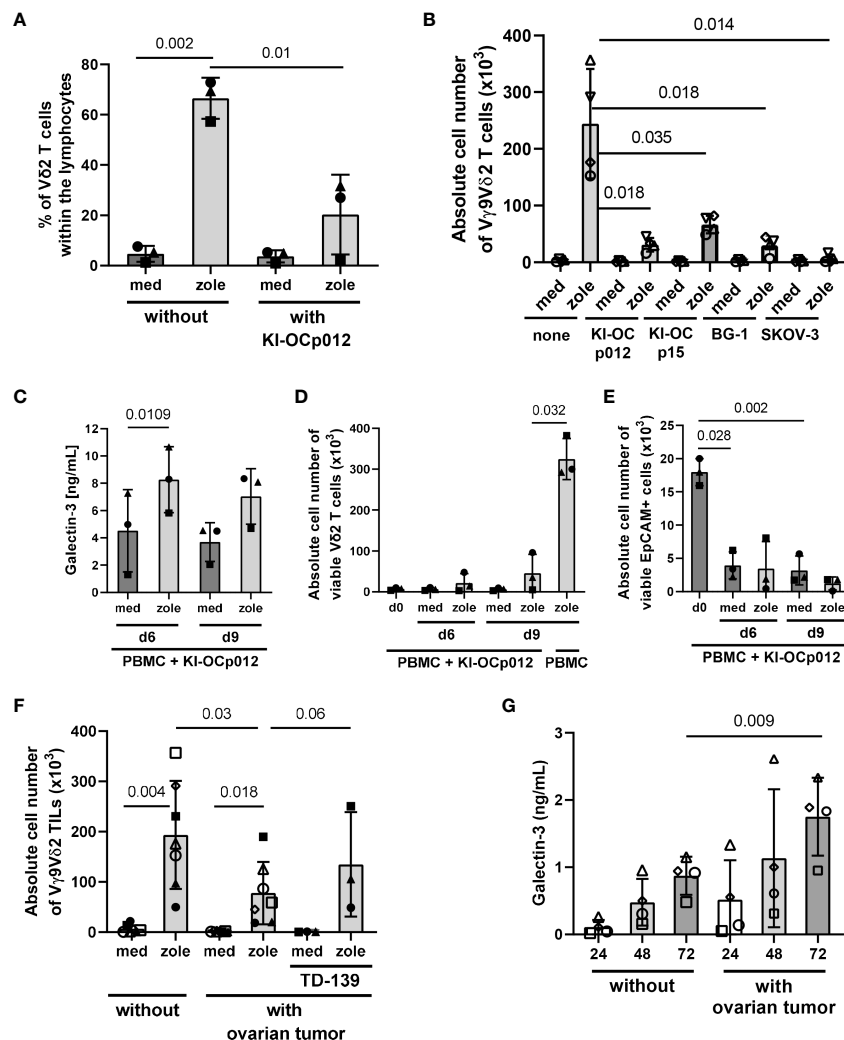


FIGURE 5

Coculturing ovarian cancer cells with PBMC or TIL leads to a decreased Vδ2 T-cell proliferation and increased amounts of released galectin-3. A total of  $3\text{--}7 \times 10^5$  freshly isolated PBMC from healthy donors (each  $n = 3$  in (A) and (C–E)), ( $n = 4$  in (B)) and  $1.5\text{--}7 \times 10^5$  freshly isolated TIL from an ovarian cancer patients ( $n = 7$  in (F, G)) were cultured for 6 (C–E); 7 (F, G) or 9 days (A–E) with or without KI-OCp012 (A–E), the indicated tumor cells (B) or autologous tumor cells (F, G). The E/T ratio was 1:1 calculated with  $2 \times 10^4$  γδ T cells within PBMC or TIL and the same amount of tumor cells. (A–G) Cells were either cultured in medium or stimulated with  $2.5 \mu\text{M}$  zoledronate.  $50 \text{ IU/mL}$  rIL-2 was added to the PBMC or TIL. (F)  $100 \text{ nM}$  of the galectin-3 inhibitor TD-139 was added daily as indicated. Proliferation of Vδ2 T cells was measured and expressed (A) in percentage after 9 days or (B, D, F) the absolute cell number  $\pm$  SD was determined after the 9 days (B), the indicated time points (D) or after 7 days (F). (E) After 6 and 9 days, absolute cell number  $\pm$  SD of EpCAM-expressing ovarian tumor cells was measured by LSR-Fortessa. (C, G) Cell culture supernatants were collected from coculturing (C) PBMC or (G) TIL with allogeneic or autologous tumor cells, respectively, after 24, 48 and 72 hours and released galectin-3  $\pm$  SD was determined by ELISA. Statistical comparison was carried out parametrically by using paired, two-tailed *t*-test. Indicated P-values are shown.

Vδ1 T cells initially (d0) comprised less central memory (CM) T cells and more T effector memory cells re-expressing CD45RA (TEMRA cells) than Vδ2 T cells from the same donors (Figure 7, d0). The expression of the activation marker CD69 and of immune check point T cell immunoreceptor with Ig and ITIM domains (TIGIT) and programmed cell death protein (PD)-1 was initially enhanced on Vδ1 T cells in comparison to Vδ2 T cells (Figure 7, d0). After culturing the Vδ1 T cells in  $1 \mu\text{g/mL}$  galectin-3 for 5 days, the percentage of Vδ1 CM T cells was significantly diminished and the expression of CD25, CD69, TIGIT and PD-1 was increased compared to the culture without galectin-3. This was concentration

dependent, since Vδ1 T cells were activated with high galectin-3 concentrations (Figure 7, Medium) but not with low galectin-3 concentrations (Supplementary Figure 4, Medium). In contrast, Vδ2 T cells were not affected by any galectin-3 concentration (Figure 7; Supplementary Figure 4, Medium).

The stimulation of Vδ1 T cells with coated anti-Vδ1 and soluble anti-CD28 mAb and of Vδ2 T cells with zoledronate, induced significant alterations in the differentiation status and an enhanced expression of activation and immune check point markers. More importantly, only the combination of zoledronate and  $1 \mu\text{g/mL}$  galectin-3 stimulation, significantly enhanced the percentage of

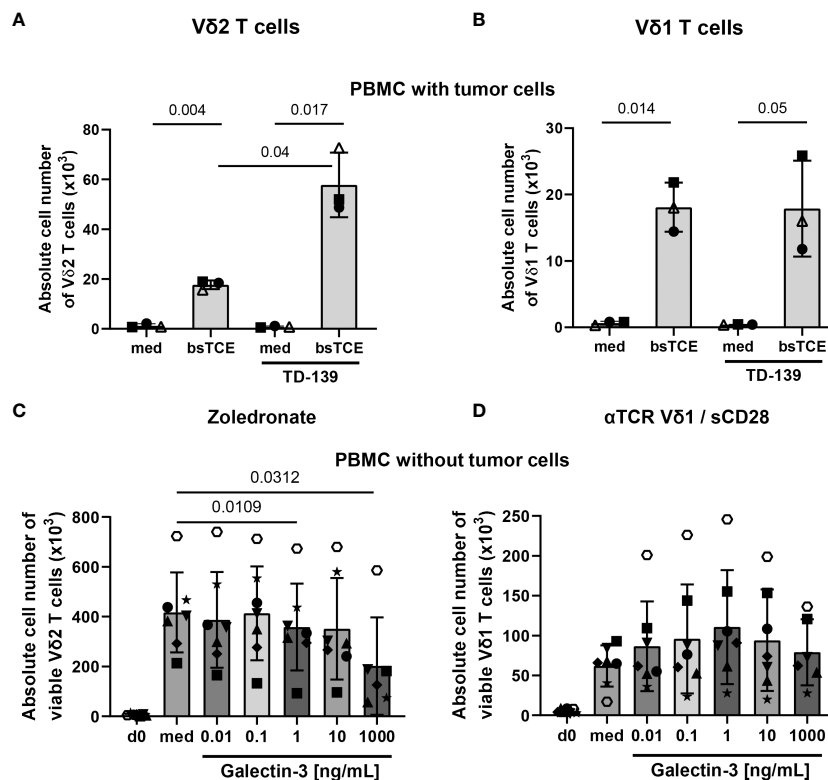


FIGURE 6

Different effects of galectin-3 on Vδ1 and Vδ2 T cells. (A–D) A total of  $3\text{--}7 \times 10^5$  freshly isolated PBMC from healthy donors (closed symbols,  $n = 2\text{--}6$ ) or from ovarian cancer patients (open symbols,  $n = 1$ ) were either stimulated (A) with  $1 \mu\text{g/mL}$  bsTCE targeting Vδ2 T cells, (B) with  $1 \mu\text{g/mL}$  bsTCE targeting Vδ1 T-cells, (C) with  $2.5 \mu\text{M}$  zoledronate for Vδ2 T-cell proliferation or (D) plate-coated anti-TCR Vδ1 and soluble CD28 mAb for Vδ1 T-cell proliferation (each  $2 \times 10^4$   $\gamma\delta$  T cells per well).  $50 \text{ IU/mL}$  rIL-2 was added to the PBMC and either complete medium (med) or (A, B) supplemented daily with  $100 \text{ nM}$  galectin-3 inhibitor TD-139 or (C, D) distinct concentrations of galectin-3 (0.01, 0.1, 1, 10 and  $1000 \text{ ng/mL}$ ). After 9 days absolute cell number  $\pm$  SD of viable Vδ2 and Vδ1 T cells was measured by LSR-Fortessa. Statistical comparison was carried out parametrically by using paired, two-tailed *t*-test or non-parametrically by using a Wilcoxon matched-pairs signed rank test. Indicated P-values are shown.

naïve Vδ2 T cells and significantly reduced the percentage Vδ2-expressing effector memory (EM) T cells (Figure 7; Supplementary Figure 4, right panel).

A reduction of Vδ2-expressing effector memory cells after zoledronate stimulation together with  $1 \mu\text{g/mL}$  galectin-3 could explain the significant galectin-3 mediated reduction of Vδ2 T-cell proliferation.

A further explanation for the differential sensitivity of the  $\gamma\delta$  T-cell subsets towards galectin-3 can be found in the expression of the galectin-3 binding partner. Our previous results suggest that the binding of galectin-3 to  $\alpha 3\beta 1$  integrin prevents the proliferation-promoting effect of CD49c/CD29 on Vδ2 T cells (18). Before stimulation, CD49c and not CD29 is nearly similar expressed on Vδ2 T cells compared to Vδ1 T cells determined within PBMC (closed symbols) and TILs (open symbols) (Figure 8, 0 h). After stimulation, CD49c and CD29 are significantly upregulated in Vδ1 T cells and in Vδ2 T cells. However, an up-regulation of both integrins was more pronounced in Vδ2 T cells than in Vδ1 T cells, and significant in the absence of tumor cells. The superior expression of CD49c and CD29 on Vδ2 T cells compared to Vδ1 T cells is shown already after 20 hours of stimulation

(Supplementary Figure 5) and is further increased 96 hours after stimulation (Figure 8).

The enhanced CD49c and CD29 expression on Vδ2 T cells after activation explain the different susceptibility of Vδ2 T cells to galectin-3.

## 4 Discussion

This study demonstrated that the coculture of stimulated  $\gamma\delta$  T cells with different tumor cells significantly enhanced the galectin-3 release, which did not influence  $\gamma\delta$  T-cell cytotoxicity against tumor cells. More importantly, the Vδ2 T-cell proliferation was inhibited in the presence of galectin-3, whereas the Vδ1 T-cell proliferation was slightly increased. The data are of great interest for an *in vivo* application of Vδ2 T-cell stimulating antigens such as zoledronate, which induces a selective Vδ2 T-cell outgrowth. A main problem of the repetitive *in vivo* application of zoledronate together with rIL-2 is the exhaustion of the Vδ2 T cells (31–33). Our data suggests that Vδ2 T cells infiltrating in tumors are inhibited in their proliferation if galectin-3 concentrations are increased since activation of Vδ2

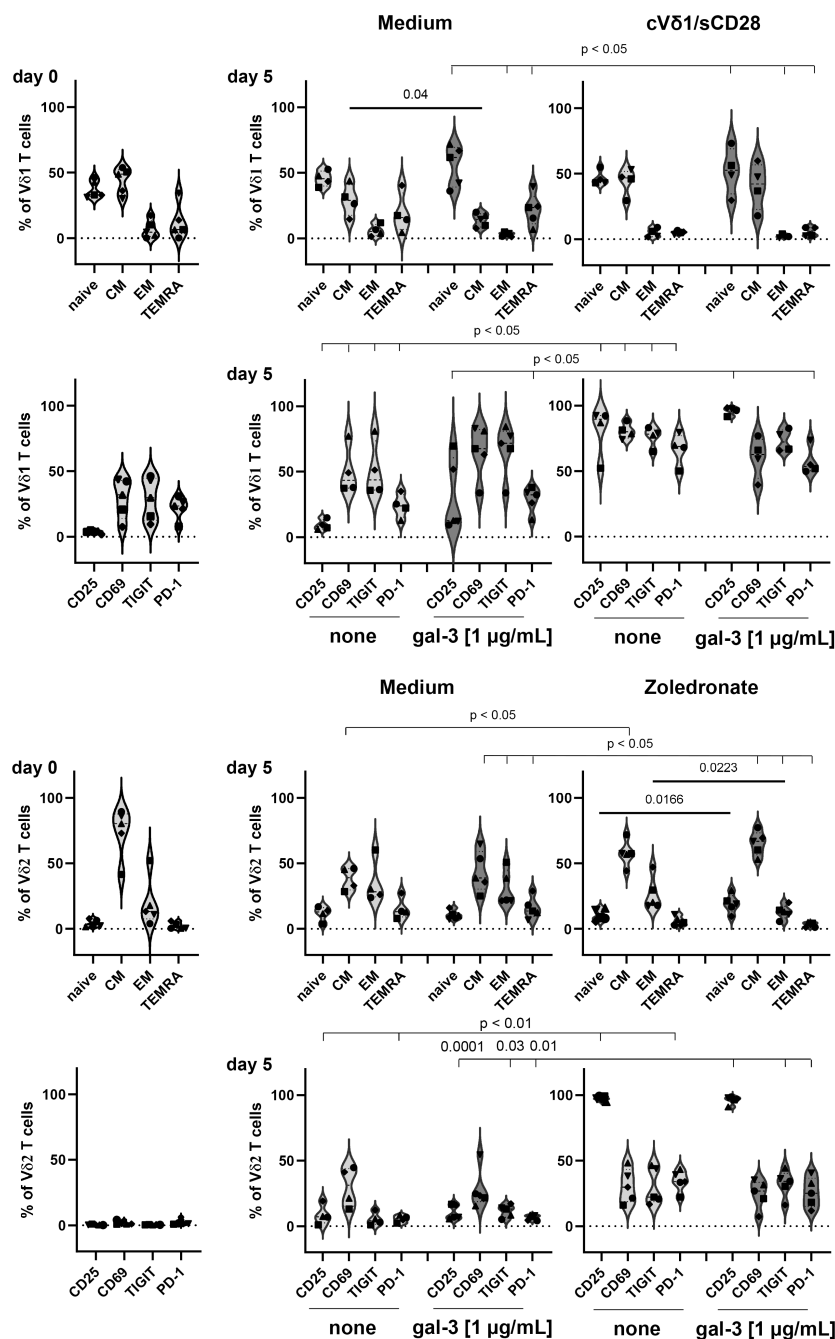


FIGURE 7

Expression of differentiation, activation and immune check point markers on Vδ1 and Vδ2 T cells.  $5 \times 10^5$  PBMC ( $n = 5$ ) were stained with anti-CD45RA and anti-CD27 mAbs to determine naïve, central and effector memory (CM and EM) or TEMRA cells of Vδ1 and Vδ2 T cells at day 0. Activation (CD69 and CD25) and immune check point (TIGIT and PD-1) markers were analyzed at day 0. Residual cells ( $5 \times 10^5$  cells/well) were cultured in complete medium, stimulated with 2.5  $\mu$ M zoledronate or with 10  $\mu$ g/mL coated anti-Vδ1 and 1  $\mu$ g/mL soluble anti-CD28 mAbs. Medium or 1  $\mu$ g/mL galectin-3 (gal-3) was added as indicated. After 5 days, cells were stained with the same mAbs as on day 0 and measured by LSR-Fortessa. A gate was set on CD45, CD3, TCR $\gamma\delta$  and Vδ1 or Vδ2 T cells to determine naïve, CM, EM T cells and TEMRA cells and the activation and immune check point markers on both  $\gamma\delta$  T-cell subsets. Statistical comparison was carried out parametrically by using paired, two-tailed *t*-test or non-parametrically by using a Wilcoxon matched-pairs signed rank test. Indicated P-values are shown.

TIL cocultured with tumor cells inhibited Vδ2 T-cell expansion and reduced effector memory activation. Moreover, Vδ2 T cells within PBMC, which grow out selectively after stimulation with zoledronate and can migrate to the tumor site, can also be inhibited in their proliferation after cross talk with tumor cells.

Beside tumor cells, other cells in the immunosuppressive tumor microenvironment (TME) produce galectin-3. For instance, in a lung adenocarcinoma tumor sphere-model, which mimic an immunosuppressive TME, galectin-3 is released in the TME and modulated the tumor infiltrating immune cells such as regulatory T

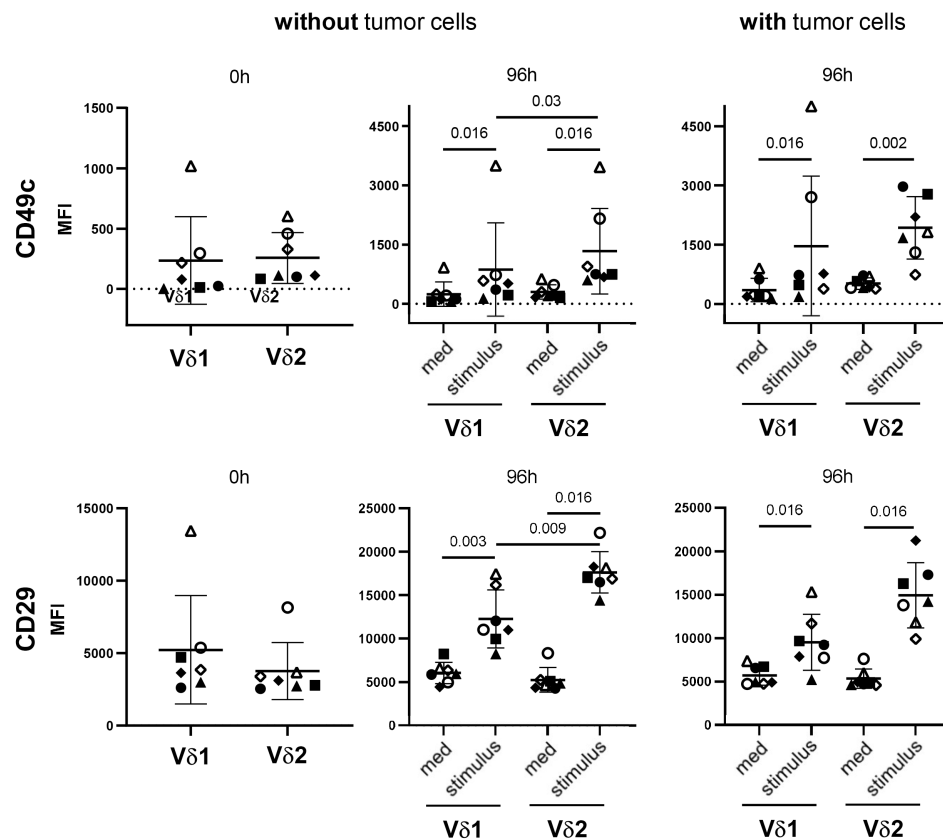


FIGURE 8

Differential expression of CD49c/CD29 on Vδ1 and Vδ2 T cells.  $5 \times 10^5$  PBMC (closed symbols,  $n = 4$ ) and TIL (open symbols,  $n = 3$ ) were stained after isolation (0 h) with anti-CD49c and anti-CD29 mAb. Residual cells ( $5 \times 10^5$  cells/well) (without tumor cells) were cultured in complete medium, stimulated with  $2.5 \mu\text{M}$  zoledronate or with coated anti-Vδ1 mAb ( $10 \mu\text{g/mL}$ ) together with soluble anti-CD28 mAb ( $1 \mu\text{g/mL}$ ) (stimulus). In parallel,  $5 \times 10^5$  PBMC (closed symbols) or TIL (open symbols) were co-cultured with  $5 \times 10^4$  OVCAR-3 cells (with tumor cells) in the presence of bispecific T-Cell Engagers (stimulus) selectively targeting HER-2 expressing ovarian tumor cells to Vγ9Vδ2 or Vδ1 T cells. After 96 hours, cells were stained and measured by LSR-Fortessa. A gate was set on CD45, CD3, TCRγδ and Vδ1 or Vδ2 T cells to determine the CD49c and CD29 expression on both γδ T-cell subsets after 0 and 96 hours (h). Statistical comparison was carried out parametrically by using paired, two-tailed *t*-test or non-parametrically by using a Wilcoxon matched-pairs signed rank test. Indicated P-values are shown.

cells (Treg) (34). These authors demonstrated that the patients with high soluble galectin-3 levels had more Treg cells, which can inhibit T cells (34). Treg are also suggested to inhibit γδ T-cell proliferation (35). In addition, galectin-3 is described to advance macrophage infiltration, M2-polarization and immunosuppressive effects of myeloid derived suppressor cells and Treg on cytotoxic CD8 T cells (36).

Our previous results demonstrated that the galectin-3 binding to glycosylated α3β1 integrin (CD49c/CD29) prevents the Vδ2 T-cell proliferation-promoting effect of CD49c/CD29. Since Vδ1 T cells are enriched at the tumor site of pancreatic and ovarian tumor cells (3, 18), the different impact of galectin-3 on Vδ1 T cells is of high interest and makes them attractive for γδ T-cell-based immunotherapy. One explanation for the different susceptibility to galectin-3 treatment of Vδ1 and Vδ2 T cells is due to the lower expression of CD49c/CD29 on Vδ1 T cells compared to Vδ2 T cells after their activation. CD49c/CD29 expressed on endothelial cells is described to bind galectin-3 producing metastatic cells thereby stabilizing tumor/endothelial cell adhesion (37). Chen and

colleagues reported that type I collagen (Col1) homotrimer is produced by pancreatic cancer cells and binds to α3β1 integrin thereby promoting oncogenic signaling and cancer cell proliferation. The deletion of Col1 homotrimers increases T-cell infiltration and improved anti-PD-1 immunotherapy (38). Tribulatti and colleagues demonstrated that galectin-3 impaired antigen-specific T-cell responses in murine CD4 T cells (39). Others demonstrated that galectin-3 is an inhibitory regulator also of human conventional T-cell activation and promotes TCR down-regulation, failure of TCR and CD8 colocalization and T-cell anergy (19, 40, 41). While an exact role of CD49c/CD29 interaction with galectin-3 is not described in human CD4 and CD8 αβ T cells, these interaction partners are responsible for the failure of Vδ2 T-cell proliferation in the presence of galectin-3 producing cells such as tumor cells. CD49c/CD29 is already described to be expressed on γδ T cells (42). Additionally, our results revealed an obvious and significant difference between Vδ2 and Vδ1 T cells after their activation which explains the different susceptibility of galectin-3 on the proliferation of these γδ T-cell subsets.



An increased V $\delta$ 1 T-cell infiltration in tumor tissue compared to the blood in ovarian cancer patients is described by us (3) and other groups (43–46). Intra-tumoral CD73-expressing V $\delta$ 1 TIL in breast cancer patients are suggested to have immunoregulatory properties which often suppress anti-tumor response (47). As shown in Figure 7, V $\delta$ 1 T cells isolated of PBMC from healthy donors or ovarian cancer patients are mainly naïve, CM, TEMRA T cells, which highly expressed immune check point inhibitors and exhaustion markers such as TIGIT and PD-1. Upon activation of the V $\delta$ 1 T cells, the CM T-cell population increased after 5 days (Figure 7) and EM population after 14 days (data not shown). This is in line with our own unpublished data demonstrating that the percentage of CM and EM V $\delta$ 1 TIL is increased. The expression of PD-1 and TIGIT on V $\delta$ 1 TIL is drastically enhanced compared to V $\delta$ 2 T cells generated out of blood or tumor tissue (Figure 7 and unpublished data). However, PD-1 and TIGIT are transiently increased in zoledronate or bsTCE activated V $\delta$ 2 T cells after 5 days (Figure 7) and decreased after 14 days (data not shown). Weimer and colleagues demonstrated an exhausted phenotype of V $\delta$ 1 TIL in ovarian cancer patients (45). On V $\delta$ 1 TIL, PD-1 was increased on CM, while ectonucleoside triphosphate diphosphohydrolase-1 (CD39) was enhanced on EM. Interestingly, ecto-5'-nucleotidase (CD73) was not expressed on ovarian  $\gamma\delta$  TIL (45). CD39 and CD73 are enzymes which mediate a gradual hydrolysis of danger signals of ATP and ADP to anti-inflammatory adenosine, which induce exhaustion of cells (48–50).

Although V $\delta$ 1 TIL seem to be in an exhausted stage in several advanced ovarian cancer patients, we were able to stimulate and expand V $\delta$ 1 TIL and PBMC in the absence of autologous tumor cells. After expansion, V $\delta$ 1 T cells cocultured with ovarian cancer cells exert a high cytotoxicity which was not influenced by galectin-3 release of tumor cells. In addition, V $\delta$ 1 T-cell proliferation was not influenced by galectin-3 which is probably an advantage for V $\delta$ 1 T-cell based immunotherapy. Since V $\delta$ 2 T cells are the predominant  $\gamma\delta$  T-cell subset in the blood of Caucasian population, in contrast to Asian and African population, almost all human  $\gamma\delta$  T-cell research is focused on V $\delta$ 2 T cells. However, Fisher and colleagues demonstrated that V $\delta$ 1 T cells and V $\delta$ 1/V $\delta$ 2-negative T cells within PBMC possess many characteristics, which recommend them for T-cell based immunotherapy instead of V $\delta$ 2 T cells. These characteristics include an enhanced cytotoxic activity of V $\delta$ 1 T cells per se, a reduced differentiation to a CD27, CD45RA and CD62L pattern, a long persistence in patients and a decreased PD-1 expression after their activation (51). Here, we described a resistance of V $\delta$ 1 T cells against galectin-3 mediated inhibition of proliferation, which is regarded as an additional advantage for V $\delta$ 1 T-cell-based immunotherapy. In addition, we observed an enhanced percentage of ovarian V $\delta$ 1 TIL coexpressing V $\gamma$ 9 and expressing PD-1 (unpublished observation). These cells are EM V $\delta$ 1 TIL with a high cytotoxic activity towards different ovarian cancer cells (Figure 7). The enhanced cytotoxic activity was supported by the slight expression of PD-L1 on ovarian cancer cells (3) suggesting that a certain V $\delta$ 1 T cell-subset could be suitable for a V $\delta$ 1 T-cell based immunotherapy.

## 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 author.

## Ethics statement

The studies involving humans were approved by Ethic Committee of the Medical Faculty of the CAU Kiel, code number: D 445/18. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

JS: Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. H-HO: Conceptualization, Investigation, Methodology, Supervision, Visualization, Writing – review & editing. MP: Resources, Writing – review & editing. NH: Methodology, Resources, Writing – review & editing. WS: Funding acquisition, Resources, Writing – review & editing. DB: Resources, Supervision, Writing – review & editing. DW: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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## Conflict 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.

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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.2023.1286097/full#supplementary-material>

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## EDITED BY

Daniela Wesch,  
University of Kiel, Germany

## REVIEWED BY

Prashant Sharma,  
University of Arizona, United States  
Wenwei Tu,  
The University of Hong Kong,  
Hong Kong SAR, China

## \*CORRESPONDENCE

Andy Hee-Meng Tan

✉ andy\_tan@bti.a-star.edu.sg

Chelsia Qiuxia Wang

✉ chelsia\_wang@bti.a-star.edu.sg

<sup>†</sup>These authors contributed  
equally to this work and share  
first authorship

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# Gamma/delta T cells as cellular vehicles for anti-tumor immunity

Chelsia Qiuxia Wang <sup>1\*†</sup>, Pei Yu Lim <sup>1†</sup>  
and Andy Hee-Meng Tan <sup>1,2\*</sup>

<sup>1</sup>Immune Cell Manufacturing, Bioprocessing Technology Institute (BTI), Agency for Science,  
Technology and Research (A\*STAR), Singapore, Singapore, <sup>2</sup>Food, Chemical and Biotechnology  
Cluster, Singapore Institute of Technology (SIT), Singapore, Singapore

Adoptive cellular immunotherapy as a new paradigm to treat cancers is exemplified by the FDA approval of six chimeric antigen receptor-T cell therapies targeting hematological malignancies in recent years. Conventional  $\alpha\beta$  T cells applied in these therapies have proven efficacy but are confined almost exclusively to autologous use. When infused into patients with mismatched human leukocyte antigen,  $\alpha\beta$  T cells recognize tissues of such patients as foreign and elicit devastating graft-versus-host disease. Therefore, one way to overcome this challenge is to use naturally allogeneic immune cell types, such as  $\gamma\delta$  T cells.  $\gamma\delta$  T cells occupy the interface between innate and adaptive immunity and possess the capacity to detect a wide variety of ligands on transformed host cells. In this article, we review the fundamental biology of  $\gamma\delta$  T cells, including their subtypes, expression of ligands, contrasting roles in and association with cancer prognosis or survival, as well as discuss the gaps in knowledge pertaining to this cell type which we currently endeavor to elucidate. In addition, we propose how to harness the unique properties of  $\gamma\delta$  T cells for cellular immunotherapy based on lessons gleaned from past clinical trials and provide an update on ongoing trials involving these cells. Lastly, we elaborate strategies that have been tested or can be explored to improve the anti-tumor activity and durability of  $\gamma\delta$  T cells *in vivo*.

## KEYWORDS

$\gamma\delta$  T cell, Gamma/delta T cell, chimeric antigen receptor (CAR), anti-tumor immunity, cancer immunotherapy, Unconventional T cells, non-HLA-restricted T cells, cellular immunotherapy

## 1 Introduction to $\gamma\delta$ T cells

Recent advances in genomic editing of cells (1–3) have propelled cellular immunotherapy as a new paradigm to treat cancers, which is rapidly gaining traction with the FDA approval since 2017 of six therapies involving T cells engineered with different chimeric antigen receptors (CARs) targeting primarily B cell malignancies [summarized in (4, 5)]. These approved therapies, and many others undergoing investigation in pre-clinical studies and clinical trials, have largely utilized conventional



$\alpha\beta$  T cells which are limited to autologous applications. If infused in a recipient patient with mismatched human leukocyte antigen (HLA),  $\alpha\beta$  T cells will recognize as foreign and attack the patient's tissues that results in potentially life-threatening graft-versus-host disease (GvHD). One approach to circumvent the occurrence of GvHD is to use innate or innate-like immune cells such as  $\gamma\delta$  T cells, which possess characteristics rendering them appropriate for allogeneic therapy.

$\gamma\delta$  T cells represent a small population of total leukocytes in umbilical cord blood (UCB) and peripheral blood (PB), comprising approximately 0.0045–0.035% of UCB and 0.5–5% of PB (6–9). Despite their low abundance, these cells play crucial roles in immune defense against bacterial and viral infections, as well as in immune surveillance of cancer.  $\gamma\delta$  T cells are poised to recognize intracellularly stressed cells, such as infected and tumor cells, and respond by directly eliminating such cells (10). The infected and tumor cells convey their intracellular stress to  $\gamma\delta$  T cells via a myriad of molecules.  $\gamma\delta$  T cells sense these dysfunctional cells by recognizing tumor-associated metabolic byproducts such as butyrophilins (BTNs) on tumor cells in the peripheral circulation or stress-associated proteins like MHC class I-related chain A or B (respectively MICA or MICB) upregulated on stressed cells in both PB and tissues. Engagement of these ligands by their receptors on  $\gamma\delta$  T cells activate direct killing mechanisms via granzyme B and perforin rapidly without prior exposure to pathogen- or tumor-associated antigens (Figure 1). They also stimulate secretion of effector molecules such as interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$ .

There are several advantages in employing  $\gamma\delta$  T cells for immunotherapy (Figure 1). Firstly,  $\gamma\delta$  T cells express a wide repertoire of cell surface receptors conferring the ability to broadly recognize a diversity of tumor ligands and thereby target multiple tumor types, unlike HLA-restricted tumor recognition by  $\alpha\beta$  T cells. This is particularly useful for tumors which have downregulated HLA class I expression to evade immune

recognition by  $\alpha\beta$  T cells. Secondly, the cytotoxic function of  $\gamma\delta$  T cells is therefore activated independently of HLA which drastically reduces their chance of provoking GvHD and allows for their allogeneic use. Thirdly,  $\gamma\delta$  T cells are the major early producers of pro-inflammatory IFN- $\gamma$  which triggers their anti-tumor response and orchestrates  $\alpha\beta$  T, B and dendritic cells in a cascade of adaptive immune responses that further amplify tumor killing (11). The cross-talk between  $\gamma\delta$  T cells and other immune cells are described in a recent review (12). Such interactions in the tumor microenvironment (TME) allow  $\gamma\delta$  T cells to shape its immediate environment into a tumor-suppressing one. Moreover,  $\gamma\delta$  T cell subtypes characterized by certain rearrangements of their  $\gamma\delta$  T cell receptor (TCR) intrinsically populate specific tissues, namely skin, large intestine, spleen and liver. It is thought that such tissue tropism may enhance the capacity of  $\gamma\delta$  T cell subtypes to infiltrate the TME of diverse solid tumors consisting tissues which are the physiological habitats for the respective cell subtypes. Furthermore, as engineered  $\gamma\delta$  T cells exhibit similar anti-tumor efficacy but generally secrete lower levels of cytokines compared with their similarly modified  $\alpha\beta$  counterparts,  $\gamma\delta$  T cell therapy harbors a potentially lower risk of cytokine release syndrome (CRS) (13–15).

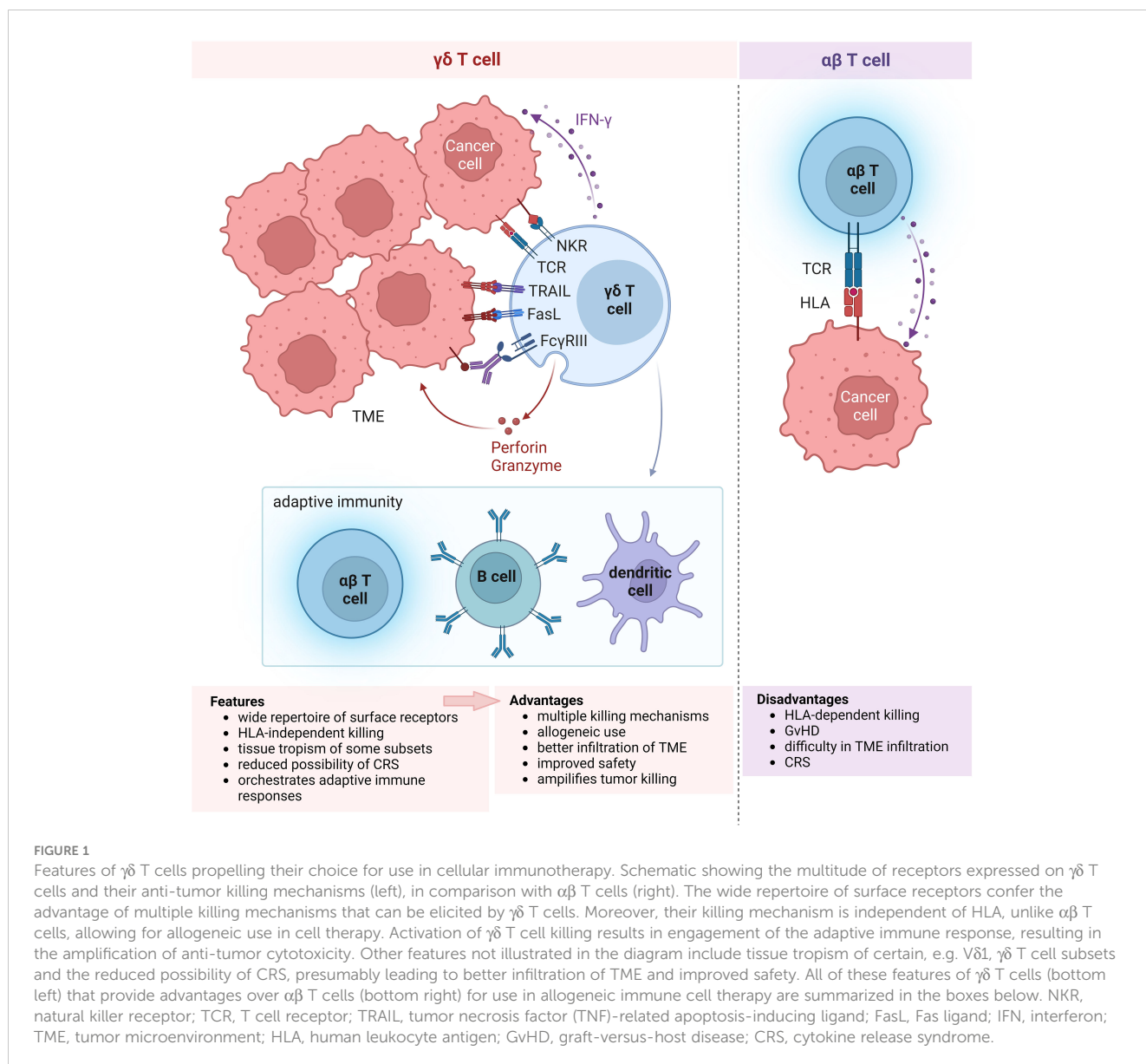
In this review, we summarize fundamental concepts underlying the biology of  $\gamma\delta$  T cells, as well as recent developments related to their role in cancer prognosis and survival revealed by multiple lines of research evidence which will be elaborated in the following sections. We discuss the gaps in knowledge that can improve ways to harness  $\gamma\delta$  T cells for cellular immunotherapy. We also take stock of the current outlook of clinical trials relating to  $\gamma\delta$  T cell therapies that have been carried out thus far and discuss what we can learn from these trials. Lastly, we review current or propose new strategies to improve the anti-tumor efficacy of  $\gamma\delta$  T cell therapies.

## 2 $\gamma\delta$ T cells: what are the gaps to be filled?

### 2.1 Refinement of $\gamma\delta$ T cell subtypes and their associated ligands

Human  $\gamma\delta$  T cells can be divided into several subtypes, including V $\delta$ 1 and V $\delta$ 2 subtypes based on their expression of TCR $\delta$  chain variant, contrasting with murine  $\gamma\delta$  T cell subsets which are categorized according to their  $\gamma$  chain expression. While V $\delta$ 2 cells are predominantly found in blood circulation, V $\delta$ 1 cells are localized mainly in mucosal epithelial tissues. There also exist less well studied subtypes such as V $\delta$ 3 cells that reside in the liver. Regardless of their subtype based on TCR $\delta$  chain variant expression,  $\gamma\delta$  T cells can be distinguished in terms of functional potency based on their expression of cell surface receptors, including CD56 (16, 17), NKG2A (18), the SCART scavenger receptors (SCART1 and SCART2) (19), CD27 (20) and CD161 (21), signatures of which correlate with cytokine secretion and anti-tumor cytotoxicity. Interestingly, V $\delta$ 1 and V $\delta$ 2 subtypes can each be functionally differentiated by the expression of CD56. While

**Abbreviations:** CAR, chimeric antigen receptor; HLA, human leukocyte antigen; GvHD, graft-versus-host disease; UCB, umbilical cord blood; PB, peripheral blood; BTN, butyrophilins; MICA, MHC class I-related chain A; MICB, MHC class I-related chain B; IFN, interferon; TCR, T cell receptor; TME, tumor microenvironment; CRS, cytokine release syndrome; TNF, tumor necrosis factor; BrHPP, bromohydrin pyrophosphate; EphA2, ephrin receptor A2; EPCR, endothelial protein C receptor; ULBP, UL16-binding protein; IL, interleukin; Treg, regulatory T cell; TNBC, triple-negative breast cancer; CRC, colorectal cancer; TEM, effector memory T cell; TEMRA, terminally differentiated T cell; Tnaïve, naïve T cell; TCM, central memory T cell; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; TIL, tumor-infiltrating lymphocyte; PDA, pancreatic ductal adenocarcinoma; MDSC, myeloid-derived suppressor cell; BM, bone marrow; PD-1, programmed cell death protein 1; COX-2, cyclooxygenase-2; DOT, V $\delta$ 1-enriched delta one T cell; iPSCs, induced pluripotent stem cells; BiTE, bispecific T cell engager; scFv, single chain variable fragment; CCR, chimeric co-stimulatory receptors; HCC, hepatocellular carcinoma; TAC, T cell Antigen Coupler; NSCAR, non-signaling CAR; SAR, synthetic agonistic receptor; taFv, tandem scFv; ECM, extracellular matrix; MMP14, matrix metalloproteinase 14; IRE, irreversible electroporation; TKIs, tyrosine kinase inhibitors.



CD56<sup>+</sup> V $\delta$ 2 T cells have greater anti-tumor effector function compared with their CD56<sup>-</sup> counterparts, the opposite is observed with V $\delta$ 1 T cells for which positive expression of CD56 is associated with lower anti-tumor potency. It should be noted that the latter finding was based on tumor-infiltrating V $\delta$ 1 T lymphocytes derived from a single patient and hence requires further validation. Delineation of the spectrum of cytotoxic properties within each  $\gamma\delta$  T cell subset will yield added insight into the functional roles of  $\gamma\delta$  T cells (Figure 2, right, points 1 and 2).

Binding of their TCR ligands activates  $\gamma\delta$  T cells to secrete IFN- $\gamma$ , TNF- $\alpha$  and other cytotoxic effector molecules that act against tumor cells (22) (Figure 2, left). The activated  $\gamma\delta$  T cells also secrete granzyme B and perforin which aid in their cytolytic function. While many ligands remain currently unidentified (Figure 2, right, point 3), metabolites of the isoprenoid pathway, also known as phosphoantigens, or pharmacological agents that promote their

accumulation have been found to efficiently activate and expand V $\gamma$ 9V $\delta$ 2 T cells. Physiologically, upregulation of the mevalonate pathway in tumor cells results in the accumulation of phosphoantigens, such as isopentenyl pyrophosphate, which induce conformational changes of BTN3A1 in these cells (23). In turn, such a conformational change mediates interactions between BTN2A1 and BTN3A1 and leads to the subsequent binding of TCR V $\gamma$ 9 to BTN2A1. An example of a synthetic phosphoantigen that has been assessed in clinical trials is bromohydrin pyrophosphate (BrHPP). Identification of *de novo* biomolecules that can preferentially stimulate other  $\gamma\delta$  T cell subsets will facilitate their *ex vivo* and *in vivo* expansion for therapeutic purpose. In addition, V $\delta$ 1 T cells can recognize ephrin receptor A2 (EphA2) (24) and MHC-related protein 1 (25), while V $\delta$ 3 T cells are activated by annexin A2 (26) on tumor cells and V $\delta$ 5 T cells bind endothelial protein C receptor (EPCR) on cytomegalovirus-infected and

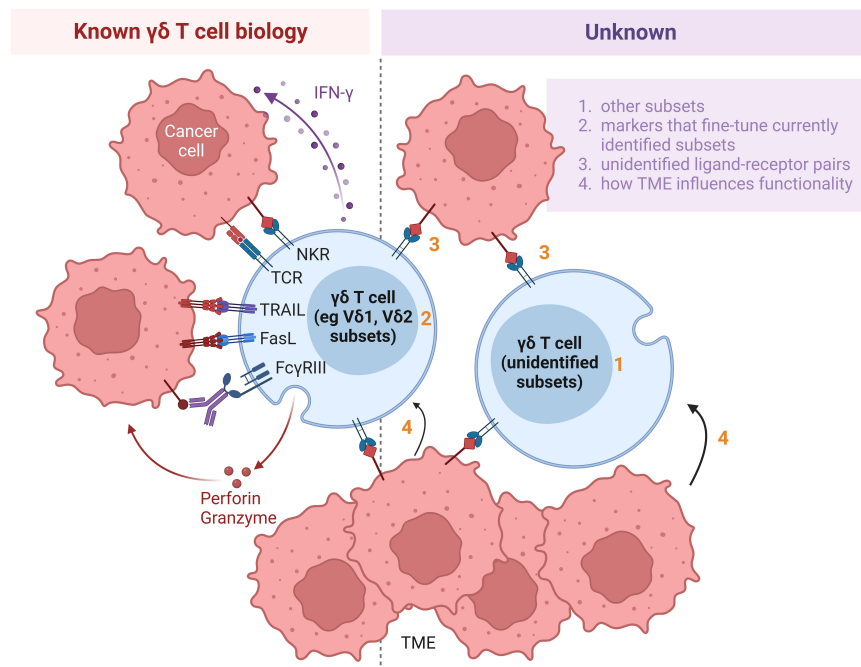


FIGURE 2

Hurdles impeding application of  $\gamma\delta$  T cells for cell therapy. Whilst increasing amount of information is being discovered about  $\gamma\delta$  T cell biology (left panel), much remains to be further elucidated (right panel). Firstly, various subsets, including V $\delta$ 1 and V $\delta$ 2, have been identified. However, presence of substantial V $\delta$ 1 and V $\delta$ 2 populations suggest that continuous identification of additional subsets is warranted (point 1). Secondly, within each subset, the  $\gamma\delta$  T cells can be delineated by additional markers, such as CD56, CD27 and NKG2A. Identification of such additional markers will yield added insight into the functional roles of  $\gamma\delta$  T cells (point 2). Thirdly, while several molecules have been identified as ligands to the multitude of receptors on  $\gamma\delta$  T cells, many ligands recognized by TCR $\gamma\delta$  or other surface receptors remain currently unidentified (point 3). Furthermore, the influence of the TME on the functions of  $\gamma\delta$  T cells has been established, but the exact conditions which tune  $\gamma\delta$  T cells towards pro- versus anti-tumor subsets are not yet well defined (point 4). Research delving to uncover the unknown aspects of  $\gamma\delta$  T cell biology and their interactions with cells in the TME will improve the effectiveness of adoptive transfer of  $\gamma\delta$  T cells in immunotherapy. NKR, natural killer receptor; TCR, T cell receptor; TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand; FasL, Fas ligand; IFN, interferon; TME, tumor microenvironment.

epithelial tumor cells (27) through their respective TCRs. Due to the tissue tropism of non-V $\delta$ 2 T cells, the ligand-receptor recognition pathways involved in the activation of non-V $\delta$ 2 T cells presumably play a more important role in  $\gamma\delta$  T cell activation in the context of solid tumors. Apart from TCR ligands, ligands induced on epithelial and tumor cells via stress or structural damage, including MICA, MICB and UL16-binding proteins (ULBPs), are recognized by NKG2D on both intraepithelial V $\delta$ 1 and circulating V $\delta$ 2 cells (14). The aforementioned ligands, among others, could be engineered in feeder cells to support *ex vivo* expansion of  $\gamma\delta$  T cells to attain clinically relevant numbers of  $\gamma\delta$  T cells which are estimated to be  $10^8$  to  $10^{11}$  cells per infusion.

Although most  $\gamma\delta$  T cell subsets exhibit cytotoxicity against tumor cells, there exist pro-tumorigenic interleukin (IL)-17-producing (28) and PD-L1-overexpressing  $\gamma\delta$  T cells (29). These broadly termed regulatory  $\gamma\delta$  T cells ( $\gamma\delta$  Tregs) antagonize the therapeutic efficacy of cytotoxic  $\gamma\delta$  T cells and therefore suppress host immune responses (30). Interestingly, prior exposure or not to ligands during development in the murine thymus programs the effector fate of  $\gamma\delta$  T cells into respectively IFN- $\gamma$  or IL-17-producing cells (31). The divergent roles of  $\gamma\delta$  T cell subsets in anti-tumor immunity have to be carefully delineated in order for their innate properties to be harnessed for immunotherapy.

## 2.2 Association of $\gamma\delta$ T cells with prognosis and survival outcomes

### 2.2.1 $\gamma\delta$ T cells are frequently associated with positive prognosis and survival

Notwithstanding their dual nature imprinted by thymic development, tumor-infiltrating or circulating  $\gamma\delta$  T cells are generally correlated with positive clinical outcomes or prognoses (32). Evidence from representative studies on various tumor types are described in this section (Table 1). For example, intratumoral V $\delta$ 1 T cells harvested from melanoma patients exhibited convincing anti-tumor function *in vitro* and when infused into patients (17). Intratumoral V $\delta$ 2 cell frequencies were found to correlate inversely with the stage of melanoma disease, with high V $\delta$ 2 frequencies observed in patients lacking cancer metastases and negligible frequencies in patients bearing advanced stage and metastatic melanomas (33). Increased intratumoral infiltration of  $\gamma\delta$  T cells was associated with overall survival benefit of gastric cancer patients (34). Moreover, Wu et al. reported that V $\delta$ 1<sup>+</sup> cells were more abundant within triple-negative breast cancer (TNBC) vis-à-vis paired healthy tissues, especially when the cancer is in remission (44). Another study by Janssen et al. showed that the predominant population in TNBC was V $\delta$ 2<sup>+</sup> cells eliciting a

**TABLE 1** Association of tumor-infiltrating and circulating  $\gamma\delta$  T cells with prognosis or survival of patients with different cancers.

Cancer type	TILs or circulating lymphocytes	$\gamma\delta$ T cell subtype	References
<b>Studies supporting positive correlation with prognosis or survival</b>			
melanoma*	TIL	V $\delta$ 1 <sup>+</sup> , V $\delta$ 2 <sup>+</sup>	(17, 33)
gastric cancer	TIL	unknown	(34)
breast cancer*	TIL	V $\delta$ 1 <sup>+</sup> , V $\delta$ 2 <sup>-</sup>	(35)
colorectal cancer*	TIL	V $\gamma$ 9V $\delta$ 2	(36)
prostate cancer	circulating	V $\gamma$ 9V $\delta$ 2	(37)
acute myeloid leukemia, acute lymphoblastic leukemia	circulating	V $\delta$ 1 <sup>+</sup>	(38)
lymphoma	circulating	V $\gamma$ 9V $\delta$ 2	(39)
B cell chronic lymphocytic leukemia	circulating	V $\delta$ 1 <sup>+</sup>	(40)
<b>Studies supporting negative correlation with prognosis or survival</b>			
breast cancer*	TIL	V $\delta$ 1 <sup>+</sup> Tregs	(41)
pancreatic ductal adenocarcinoma	TIL	V $\gamma$ 9V $\delta$ 2 <sup>-</sup>	(29)
colorectal cancer*	TIL	V $\delta$ 1 <sup>+</sup> Tregs	(28)
squamous cell carcinoma	TIL	V $\delta$ 1 <sup>+</sup> and V $\delta$ 2 <sup>+</sup> Tregs	(42)
melanoma*	circulating	V $\delta$ 1 <sup>+</sup>	(43)

Asterisks (\*) highlight cancer types in which  $\gamma\delta$  T cells are associated with both positive and negative prognosis or survival. TIL, tumor-infiltrating lymphocytes; Tregs, regulatory T cells.

proinflammatory rather than an IL-17-expressing signature (35). Interestingly, their tumor reactivity is prescribed by the diverse TCR $\gamma$  and TCR $\delta$  chains and less characterized by the more “generic” anti-tumor response achieved via innate receptors such as NKG2D. Whichever the case, these findings support the observation that higher  $\gamma\delta$  T cell infiltration correlated with better survival of TNBC patients (45). Contrary to a prior study reporting the polarization of IL-17-producing V $\delta$ 1<sup>+</sup> T cells that promote colorectal cancer (CRC) pathogenesis (28), a recent study by Meraviglia S et al. found that tumor-infiltrating  $\gamma\delta$  T cells expressing the TCRGV9-encoding gene were not the major producers of IL-17 in the CRC TME and their higher frequencies were associated with significantly longer disease-free survival rate (36). Notably, the latter study provided indirect evidence that mediators secreted by CRC cancer stem cells likely inhibited  $\gamma\delta$  T cell function in TME.

Late-stage prostate cancer patients who were treated with zoledronate and IL-2 had superior clinical outcomes compared with zoledronate alone, as the former combination resulted in

greater frequencies and more pronounced activation of peripheral  $\gamma\delta$  T cells (37). Combined zoledronate and IL-2 therapy elevated populations of  $\gamma\delta$  T cells bearing effector memory (T<sub>EM</sub>) and terminally differentiated phenotypes (T<sub>EMRA</sub>), with concomitant decrease in cell populations of naïve (T<sub>naïve</sub>) and central memory (T<sub>CM</sub>) phenotypes in all seven patients examined (37).

Separately, a long-term study demonstrated enhanced leukemia-free and overall survival of patients who received allogeneic hematopoietic stem cell transplantation for treatment of acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) when their levels of donor-derived, circulating and predominantly V $\delta$ 1<sup>+</sup>  $\gamma\delta$  T cells were high (38).  $\gamma\delta$  T cells which were responsive to proliferative stimulation by pamidronate and low-dose IL-2 contributed to effective anti-lymphoma responses *in vivo* while lack of  $\gamma\delta$  T cell proliferation correlated with poor objective tumor responses (39). Disease progression in patients suffering from B cell chronic lymphocytic leukemia was associated with low numbers of circulating V $\delta$ 1<sup>+</sup> cells. Reciprocally, patients who had higher V $\delta$ 1<sup>+</sup> cell counts maintained stable disease (40). Taken together, these studies strongly suggest that  $\gamma\delta$  T cells exert cytotoxic effects against majority of cancer types.

## 2.2.2 $\gamma\delta$ T cells are occasionally associated with negative patient prognosis and survival

As earlier alluded, certain  $\gamma\delta$  T cell types are known to be tumor-promoting (Table 1) given pre-programming of different functional subsets during development and dependence on tumor context in activating selective subsets (30). For instance, V $\delta$ 1<sup>+</sup> Tregs were found to be the dominant tumor-infiltrating lymphocyte (TIL) population in breast cancer tissues examined in 11 patients (41). These V $\delta$ 1<sup>+</sup> Tregs, most being subsequently identified to express CD73, potentially suppressed dendritic cell maturation and function, as well as cytokine secretion by CD4<sup>+</sup> helper T cells and CD8<sup>+</sup> effector T cells (46). The inhibitory function of V $\delta$ 1<sup>+</sup> Tregs can be abrogated by Toll-like receptor 8 ligand engagement to enhance anti-tumor immunity (41). It was observed that  $\gamma\delta$  T cells infiltrating pancreatic ductal adenocarcinoma (PDA) overexpressed checkpoint ligands PD-L1 and Galectin-9 to directly suppress  $\alpha\beta$  T cells, hence creating an immunosuppressive TME (29). V $\gamma$ 9<sup>+</sup> cells were noticeably absent, implying that TILs were V $\gamma$ 9V $\delta$ 2<sup>-</sup> cells. The frequency of IL-17-secreting V $\delta$ 1<sup>+</sup>  $\gamma\delta$  Tregs present in CRC positively correlated with advanced clinicopathological features of the disease (28). These pro-tumorigenic  $\gamma\delta$  Tregs were shown to promote the migration, proliferation and accumulation of myeloid-derived suppressor cells (MDSCs) via production of IL-17A, IL-8, GM-CSF and TNF- $\alpha$ . In both PDA and CRC,  $\gamma\delta$  TILs manifested a T<sub>EM</sub> phenotype, whereas normal healthy tissue counterparts possessed a T<sub>CM</sub> phenotype (28, 29). Furthermore, patients in the advanced stages of a type of skin cancer called squamous cell carcinoma harbored more V $\delta$ 1<sup>+</sup> and V $\delta$ 2<sup>+</sup> IL-17-producing  $\gamma\delta$  T cells in contrast to those in the early stages of cancer which had more IFN $\gamma$ -producing cells (42). Elevated frequencies of PB V $\delta$ 1<sup>+</sup> T cells in patients with metastatic melanoma were correlated with poorer clinical prognoses, unlike those of PB V $\delta$ 2<sup>+</sup> counterparts which lack association (43).



While human  $\gamma\delta$  Tregs have been less studied than their murine counterparts (47), it is recognized that pro-tumorigenic  $\gamma\delta$  Tregs do not exert direct effects on tumor cells but are able to shape the TME via other cell types to become an immune suppressive one, thereby promoting oncogenic progression. Collectively, the aforementioned studies, albeit non-exhaustive, serve as a timely reminder of the opposing roles that  $\gamma\delta$  T cell subsets play in tumor immunity.

## 2.3 The gaps to fill for the roles of $\gamma\delta$ T cells in anti-tumor immunity

In some studies,  $\gamma\delta$  T cells were not clearly distinguished based on subsets defined by  $\gamma\delta$  TCR usage, which could affect the interpretation of results, since the anti-tumor properties of different subsets and even populations within the same subset vary with tumor context. To account for population variations going forward, researchers should proactively include  $\gamma\delta$  T cell subset analyses in their studies. Investigating the effector phenotypes of  $\gamma\delta$  T subsets offers important insight into their recruitment patterns to tumor sites (33, 42). While identifying the wide range of ligands recognized by  $\gamma\delta$  T cells continues to pose a challenge to researchers, of greater pertinence is the choice of a specific antigen or antigens that can be used either for *ex vivo* activation and expansion of  $\gamma\delta$  T cells or direct administration to expand the cells *in vivo*. This is exemplified by the use of BrHPP or zoledronate to expand V $\gamma$ 9V $\delta$ 2 T cells. Furthermore, whether  $\gamma\delta$  T cells play tumor-suppressive or promoting roles in a particular

cancer type is possibly influenced by the specific TME. This can be assessed *in vitro* by co-incubating  $\gamma\delta$  T cells with supernatants derived from the culture of specific cancer cell types (36, 42). Whether  $\gamma\delta$  T cells are associated with good or poor prognosis for the same cancer type, such as breast cancer, CRC or melanoma (Table 1) may be dependent on the stage of cancer (42). Clearly, identification of specific molecules secreted by cancer cells in the culture supernatant that impact the “fate commitment” of  $\gamma\delta$  T cells will shed light on possible mechanisms educating the pro- or anti-tumor behavior of these cells in a given TME. Further insights into the interaction between  $\gamma\delta$  T cell biology and the TME will inform strategies of employing  $\gamma\delta$  T cells as an effective oncotherapy (Figure 2, right).

## 3 Harnessing $\gamma\delta$ T cells for anti-tumor immunotherapy

### 3.1 Lessons learnt from past clinical trials

Several  $\gamma\delta$  T cell immunotherapy clinical trials have been carried out. In Table 2, we focus on summarizing the completed and on-going  $\gamma\delta$  T cell immunotherapy clinical trials that specifically utilized direct  $\gamma\delta$  T cell administration to provide an overview of their status, phase of trial, types of cells administered, target cancer types, and their clinical outcomes. From the accumulating number of clinical trials, we have gained invaluable insight and herein discuss the important lessons we can learn from

TABLE 2 Ongoing and past clinical trials involving direct cellular administration of unmodified and modified  $\gamma\delta$  T cells, including study outcome (if available).

ClinicalTrials.gov Identifier/reference	Status	Cell type (s) infused	Donor source	Cell source	Modification of cells, if applicable	Trial phase	Condition/disease	Outcome
(48)	Completed	Enriched in V $\gamma$ 9V $\delta$ 2 T cells (Innacell <sup>TM</sup> ; single BrHPP stimulation followed by 2-week expansion in presence of IL-2 <i>in vitro</i> ); infused with IL-2	Autologous	PB	nil	1	Metastatic RCC	n = 10 <u>Efficacy</u> 6 SD: 60% 4 PD: 40% PFS: 25.7 weeks (5-111 weeks) <u>Safety and toxicity</u> DLT: 1 out of 3 patients treated at 8 x 10 <sup>9</sup> cells
(49)	Completed	Activated by 2-methyl-3-butenyl-1-pyrophosphate and expansion in the presence of IL-2 until day 14	Autologous	PB	nil	Not applicable	Advanced RCC	n=7 <u>Efficacy</u> 3 PR: 43% <u>Safety and toxicity</u> No serious adverse events observed.
(50)	Completed	Expanded using IL-2 and zoledronate	Autologous	PB	nil	1	NSCLC	n=10 <u>Efficacy</u> 3 SD: 30% 5 PD: 50% <u>Safety and toxicity</u> No serious adverse events observed.

(Continued)

TABLE 2 Continued

ClinicalTrials.gov Identifier/reference	Status	Cell type (s) infused	Donor source	Cell source	Modification of cells, if applicable	Trial phase	Condition/disease	Outcome
(51)	Completed	Enriched in V $\gamma$ 9V $\delta$ 2 T cells (zoledronate stimulation followed by 2-week expansion in presence of IL-2 <i>in vitro</i> ); infused with zoledronate	Autologous	PB	nil	1	Breast cancer, cervical cancer and other solid tumors	n=18 <u>Efficacy</u> 1 CR: 6% 2 PR: 11% 3 SD: 17% PR and CR achieved with co-treatment. <u>Safety and toxicity</u> No DLT observed.
NCT02418481	Completed	$\gamma\delta$ T cells with or without DC-CIK cells	Autologous	PB	nil	1 & 2	Breast cancer	
NCT02425735 (52)	Completed	V $\gamma$ 9V $\delta$ 2 T cells with or without DC-CIK cells	Autologous	PB	nil	1 & 2	Hepatocellular liver cancer (including CCA)	1 case study published (allogeneic). <u>Efficacy</u> Positively regulated peripheral immune functions of the patient, depleted tumor activity, improved quality of life, and prolonged his life span. <u>Safety and toxicity</u> No adverse effects.
NCT02425748	Completed	$\gamma\delta$ T cells with or without DC-CIK cells	Autologous	PB	nil	1 & 2	Non small lung cancer (without EGFR mutation)	No published results.
NCT03180437 (53)	Completed	V $\gamma$ 9V $\delta$ 2 T cells with or without IRE surgery	Allogeneic	PB	nil	1 & 2	Locally advanced pancreatic cancer	n=62 <u>Efficacy</u> Median OS: 14.5 months compared to 11 months without $\gamma\delta$ T infusion Median PFS: 11 months compared to 8.5 months without $\gamma\delta$ T infusion <u>Safety and toxicity</u> 14 serious adverse events (grade 3 and 4) observed that were likely due to IRE treatment and not $\gamma\delta$ T cells
NCT03183206, NCT03183219, NCT03183232 (54)	Completed	V $\gamma$ 9V $\delta$ 2 T cells expanded using zoledronate, IL-2, IL-15 and vitamin C for 12-14 days	Autologous	PB	nil	1 & 2	Breast cancer, liver cancer and lung cancer, respectively	n=132 <u>Efficacy</u> 18 patients (13.6%) showed response and prolonged survival Median OS (liver cancer patients):

(Continued)

TABLE 2 Continued

ClinicalTrials.gov Identifier/reference	Status	Cell type (s) infused	Donor source	Cell source	Modification of cells, if applicable	Trial phase	Condition/disease	Outcome
								23.1 months compared to 8.1 months in control group Median OS (lung cancer patients): 19.1 months compared to 9.1 months in control group <u>Safety and toxicity</u> No significant adverse events (immune rejection, GvHD or CRS) observed.
NCT03790072 (55)	Completed	Ex vivo expanded V $\gamma$ 9V $\delta$ 2 T cells (OmnImmune®) using zoledronate and IL-2	Allogeneic (matched or haploidentical family donors)	PB	nil	1 & 2	AML	n=7 <u>Efficacy</u> 1 CR: 14% 1 SD: 14% (eventually progressed) 1 MLFS: 14% <u>Safety and toxicity</u> No DLT and significant adverse effect (GvHD or neurotoxicity) observed. 1 patient suffered possible grade 1 CRS.
NCT04696705	Recruiting	Ex-vivo expanded $\gamma\delta$ T cells	Allogeneic (blood-related donor)	PB	nil	Early phase 1	NHL, PTCL	No published results.
NCT04702841	Recruiting	CAR $\gamma\delta$ T cells	Autologous	PB	CD7 CAR	Early phase 1	R/r CD7 <sup>+</sup> T cell-derived malignant tumors	No published results.
NCT03533816	Recruiting	Expanded/activated $\gamma\delta$ T cell, followed by depletion of $\alpha\beta$ T-cells (INB-100)	Allogeneic (haploidentical donors)	PB	nil	1	AML, CML, ALL, MDS	n=7 <u>Efficacy</u> 7 CR: 100% PFS: 2.6 - 36 months <u>Safety and toxicity</u> No DLT observed. All patients experienced low grade (1–2) GvHD
NCT04165941	Recruiting	$\gamma\delta$ T cells (activated and gene modified) (INB-200)	Autologous	PB	MGMT-gene modified to be drug resistant	1	Glioblastoma multiforme	n=8 <u>Efficacy</u> Cohort 1 (single dose) PFS: 7.4-11.9 months OS: 9.6-17.7 months Cohort 2 (3 doses) PFS: 19.4-23.5 months <u>Safety and toxicity</u> No DLT and

(Continued)

TABLE 2 Continued

ClinicalTrials.gov Identifier/reference	Status	Cell type (s) infused	Donor source	Cell source	Modification of cells, if applicable	Trial phase	Condition/disease	Outcome
								serious adverse events (CRS and ICANS) observed. Some grade 1-2 treatment emergent adverse events observed.
NCT04990063	Recruiting	Tumor killer cells: mixed cocultures of NK cells & $\gamma\delta$ T cells	Autologous	PB	nil	1	Advanced NSCLC	No published results.
NCT05015426	Recruiting	$\gamma\delta$ T cells (Artificial Antigen Presenting Cell-expanded donor T cells)	Allogeneic	Not stated	nil	1	AML	No published results.
NCT04735471, NCT04911478	Recruiting	Ex vivo activated and expanded V $\delta$ 1 T cells, followed by depletion of $\alpha\beta$ T cells (ADI-001)	Allogeneic	PB	Anti-CD20 CAR (3H7-CD8 HTM-BBz)	1	Follicular lymphoma, MCL, MZL, burkitt lymphoma, mediastinal lymphoma, DLBCL, NHL	N=16 <u>Efficacy</u> 6 CR: 38% 1 PR: 6% 2 SD: 13% 5 PD: 31% <u>Safety and toxicity</u> No DLT, GvHD, Grade 3 or higher CRS or ICANS reported.
NCT05400603	Recruiting	$\gamma\delta$ T cells in combination with dinutuximab, temozolomide, irinotecan and zoledronate (V $\delta$ 2 T cells)	Allogeneic	PB	nil	1	R/r neuroblastoma (pediatric)	No published results.
NCT05653271	Recruiting	V $\delta$ 2 T cells (ACE1831) or ACE1831 and obinutuzumab	Allogeneic	PB	anti-CD20 antibody conjugated	1	B cell lymphoma, NHL, DLBCL, primary mediastinal large B cell lymphoma, MZL, follicular lymphoma	No published results.
NCT04764513	Recruiting	Ex vivo expanded $\gamma\delta$ T cells (expansion from same donors as HSCT)	Allogeneic	PB	nil	1 & 2	Hematological malignancies after allogeneic HSCT: AML, ALL, MDS, lymphoma	No published results.
NCT04765462	Recruiting	Ex vivo expanded $\gamma\delta$ T cells (expansion from same donors as HSCT)	Allogeneic	Not stated	nil	1 & 2	Malignant solid tumour	No published results.
NCT05554939	Recruiting	CAR $\gamma\delta$ T cells	Allogeneic	PB	anti-CD19 CAR	1 & 2	R/r B cell NHL	No published results.
NCT05886491	Recruiting	Enriched for V $\delta$ 1+ $\gamma\delta$ T cells (GDX012) after lymphodepleting	Allogeneic	PB	nil	1 & 2	AML	No published results.

(Continued)



TABLE 2 Continued

ClinicalTrials.gov Identifier/reference	Status	Cell type (s) infused	Donor source	Cell source	Modification of cells, if applicable	Trial phase	Condition/disease	Outcome
		chemotherapy (fludarabine/cyclophosphamide)						
NCT03849651	Recruiting	TCR $\alpha\beta$ -depleted hematopoietic cell transplantation with additional memory cell DLI and selected use of blinatumomab	Allogeneic/haploidentical	PB	nil	2	ALL, AML, MDS, NK cell Leukemia, Hodgkin lymphoma, NHL, JMML, CML	No published results.
NCT05358808	Recruiting	V $\delta$ 2 T cells (TCB-008)	Allogeneic	PB	nil	2	AML	No published results.
NCT05686538	Recruiting	Innate donor lymphocyte infusion enriched in NK and $\gamma\delta$ T cells	Allogeneic	PB/BM	nil	2 & 3	AML, MDS	No published results.
NCT05388305	Recruiting	CAR $\gamma\delta$ T cells	Allogeneic	Not stated	anti-CD123 CAR	Not applicable	R/r AML	No published results.
NCT05302037	Not yet recruiting	CAR $\gamma\delta$ T cells	Allogeneic	PB	NKG2DL-targeting CAR	1	Advanced solid tumours or haematological malignancies	No published results.
NCT03939585	Not yet recruiting	NK/ $\gamma\delta$ T cell-enriched product (donor lymphocytes depleted of TCR- $\alpha\beta$ T cells and B cells)	Allogeneic (HLA matched sibling donors or partially related, related haploidentical donors)	PB	nil	1	Allogeneic stem cell transplant candidate AML, ALL, MDS, MPN, LPD	No published results.
NCT04806347	Not yet recruiting	TCR $\alpha\beta$ +/CD19+ depleted HSC graft	Allogeneic (closely matched unrelated donors or haploidentical related donors)	PB	nil	1	Blood disease	No published results.
NCT05664243	Not yet recruiting	$\gamma\delta$ T cells (DeltEx) (INB-400)	Allogeneic	PB	genetically-modified (drug resistance immunotherapy)	1 & 2	Recurrent or newly diagnosed glioblastoma	No published results.
NCT00562666	Terminated	$\gamma\delta$ T cells	Autologous	PB	nil	1	HCC	No published results.
NCT05001451	Terminated (business decision, not related to safety)	Enriched for V $\delta$ 1+ $\gamma\delta$ T cells (GDX012)	Allogeneic	PB	nil	1	AML	No published results.
NCT05628545	Withdrawn (COVID Pandemic)	$\gamma\delta$ T cells (GDKM-100)	Allogeneic	Not stated	nil	1 & 2	Advanced HCC	No published results.
NCT02459067	Terminated	$\gamma\delta$ T cells (ImmunCell®)	Autologous	PB	nil	2	Malignant melanoma, NSCLC, RCC	No published results.
NCT04700319	Unknown	CAR $\gamma\delta$ T cells	Autologous	PB	CD19/CD20 CAR	Early phase 1	Advanced CD19/CD20 <sup>+</sup> B cell line recurrent or	No published results.

(Continued)

TABLE 2 Continued

ClinicalTrials.gov Identifier/reference	Status	Cell type (s) infused	Donor source	Cell source	Modification of cells, if applicable	Trial phase	Condition/disease	Outcome
							refractory haematological malignancies	
NCT04028440	Unknown	$\gamma\delta$ T cells	Autologous	PB	nil	Early phase 1	NHL, r/r B cell NHL, CLL, PTCL	No published results.
NCT04518774	Unknown	Ex-vivo expanded $\gamma\delta$ T cells	Allogeneic (blood-related donor)	PB	nil	Early phase 1	HCC	No published results.
NCT02656147	Unknown	CAR $\gamma\delta$ T cells	Allogeneic	Not stated	Anti-CD19-CAR	1	Leukemia, lymphoma	No published results.
NCT04008381	Unknown	Ex-vivo expanded $\gamma\delta$ T cells	Allogeneic (blood-related donor)	PB	nil	1	AML	No published results.
NCT04107142	Unknown	CAR $\gamma\delta$ T cells	Allogeneic/haploidentical	PB	NKG2DL-targeting CAR	1	Colorectal cancer, TNBC, sarcoma, NPC, prostate cancer, gastric cancer	No published results.
NCT02585908	Unknown	$\gamma\delta$ T cells with or without CIK cells	Autologous	PB	nil	1 & 2	Gastric cancer	No published results.
NCT04796441	Unknown	CAR $\gamma\delta$ T cells	Allogeneic	PB	anti-CD19 CAR	Not applicable	Relapsed AML	No published results.
NCT03885076	Unknown	CAR V $\delta$ 2 T cells	Autologous	PB/BM	anti CD33 CAR	Not applicable (observational study)	AML (except M3)	No published results.

DC, dendritic cells; CIK, cytokine-induced killer cells; IRE, irreversible electroporation; HSCT, hematopoietic stem cell transplantation; HSC, hematopoietic stem cell; HLA, human leukocyte antigen; PB, peripheral blood; BM, bone marrow; CAR, chimeric antigen receptor; HCC, hepatocellular carcinoma; CCA, cholangiocarcinoma; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; RCC, renal cell cancer; r/r, relapsed or refractory; NHL, non-Hodgkin lymphoma; PTCL, peripheral T cell lymphoma; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; TNBC, triple-negative breast cancer; MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasm; LPD, lymphoproliferative disorders; MCL, mantle-cell lymphoma; MZL, marginal zone lymphoma; DLBCL, Diffuse large B cell lymphoma; NPC, nasopharyngeal carcinoma; JMML, Juvenile myelomonocytic leukemia; EGFR, epidermal growth factor receptor; TAC, T cell antigen coupler; CR, complete response; PR, partial response; SD, stable disease; MLFS, morphologic leukemia-free state; PFS, progression-free survival; OS, overall survival; DLT, dose-limiting toxicity; CRS, cytokine release syndrome; GvHD, graft-versus-host disease; ICANS, immune effector cell-associated neurotoxicity syndrome.

these trials. We also put forth several strategies to advance  $\gamma\delta$  T cell immunotherapy.

Strategies to utilize  $\gamma\delta$  T cells for cancer immunotherapy are summarized in recent reviews (56–59). These include the activation or stimulation of endogenous  $\gamma\delta$  T cells via exogenous aminobisphosphonates and anti-CD3/anti-tumor antigen bispecific antibodies as well as *ex vivo* expansion of peripheral blood-derived  $\gamma\delta$  T cells (60). Despite their purported capability to target diverse tumor cell types,  $\gamma\delta$  T cells have performed poorly in clinical trials, yielding largely disappointing clinical outcomes exemplified by low objective tumor response rates and almost no complete responses, with the exception of IN8bio's trial (NCT03533816) and Adicet Bio's trial (NCT04735471) which reported 100% and 69% complete responses respectively (59) (Table 2; refer to Supplementary Table for additional fields of information). Long-term outcome data are currently limited as many of the clinical trials are still on-going and many of them are in the early phases, which focus on establishing safety profile and dose limiting toxicity. Nevertheless,  $\gamma\delta$  T cell therapy has shown to increase the overall survival and progression-free survival of

patients in a limited number of studies (NCT03533816, NCT04165941, NCT03180437, NCT03183206, NCT03183219, NCT03183232) (48, 53, 54), with the longest survival outcomes observed in IN8bio's trial in which one patient had progression-free survival for at least 3 years. This is remarkable considering that patients treated in this trial had high-risk AML or failed multiple treatments before receiving  $\gamma\delta$  T cell therapy. While we can only speculate the reasons why these trials showed exceptional  $\gamma\delta$  T cell efficacy compared with the rest of the trials, we noted that the therapy targeted hematological malignancies for which patient outcomes are typically more favorable compared with those for solid tumors. In IN8bio's trial, patients underwent haploidentical bone marrow (BM) transplantation followed by cyclophosphamide treatment prior to  $\gamma\delta$  T cell infusion. The regime preceding  $\gamma\delta$  T cell infusion could have synergized with the latter's therapeutic effects. In Adicet Bio's trial,  $\gamma\delta$  T cells were programmed with anti-CD20 CAR which likely increased their efficacy to recognize and kill B lymphoma cells. The company's proprietary expansion process may also have enriched for the subset of cytotoxic V $\delta$ 1 T cells. Other factors to consider are discussed in the following subsections.

### 3.1.1 Factors affecting tumor-infiltration of $\gamma\delta$ T cells must be considered

Even though  $\gamma\delta$  T cells are one of the major populations found in solid tumors (32), not many studies have extensively characterized their infiltration when human clinical trials are carried out. Of all the completed trials, only Nicol and colleagues reported the migration of  $\gamma\delta$  T cells after infusion. They observed that the adoptively transferred  $\gamma\delta$  T cells migrated rapidly to lungs within a few hours before travelling to the liver and spleen. However, only a small number of  $\gamma\delta$  T cells were found to traffick to tumor sites (51). More studies are needed to understand the infiltration capabilities of adoptively transferred  $\gamma\delta$  T cells in solid tumors. Knowledge on the phenotypes of  $\gamma\delta$  T cells that have successfully migrated to tumor sites will also shed light as to why patient outcomes are generally poor for solid tumors compared to hematological malignancies. One can then devise potential solutions to overcome some of the hurdles impeding solid tumor immunotherapy. For detailed discussion on tumor infiltrating  $\gamma\delta$  T cells and their clinical relevance in cancer patients, we refer readers to other review papers (61, 62). Even if  $\gamma\delta$  T cells manage to infiltrate solid tumors, another immediate hurdle that they must overcome is the hostile conditions they are subjected to within the TME.

### 3.1.2 The tumor microenvironment inhibits anti-tumor immune responses

$\gamma\delta$  T cells are subjected to signals within the TME, which can drive their differentiation into different functional subsets (63). Cells in the TME comprise of immunosuppressive tumor associated macrophages, MDSCs, cancer-associated fibroblasts and tumor cells themselves, among others. These cells can secrete immunoinhibitory molecules, such as TGF- $\beta$  (64), which in turn promote the pro-tumorigenic polarization of  $\gamma\delta$  T cells. In addition,  $\gamma\delta$  T cells can become exhausted and dysfunctional in the TME of certain tumors. For example, programmed cell death protein 1 (PD-1), LAG-3 and TIM3 were shown to be upregulated in  $\gamma\delta$  T cells infiltrating multiple myeloma, and together with the increased expression of the cognate ligands on tumor cells, result in their anergy (65, 66).

Cells in the TME can also directly inhibit the anti-tumor cytotoxicity of  $\gamma\delta$  T cells (67). It has been shown that PDA cells upregulate cyclooxygenase-2 (COX-2) expression in response to IFN- $\gamma$  and TNF- $\alpha$  secreted by  $\gamma\delta$  T cells (68). COX-2 leads to an increase in PGE2 in tumor cells as a result of increased enzymatic action. As a consequence of PGE2 binding to their receptors on  $\gamma\delta$  T cells, TCR signaling is inhibited and this in turn causes the dampening of  $\gamma\delta$  T cell cytotoxic function. Elevated Cox-2 expression was also observed in breast cancer (69). Recently, it was demonstrated that IL-10 secreted by EBV-transformed lymphoblastoid B cell lines reduced the cytotoxicity of V $\gamma$ 9V $\delta$ 2 T cells (70). In addition, Tregs have been shown to inhibit the proliferation of  $\gamma\delta$  T cells (71).

The anti-tumor activity of  $\gamma\delta$  T cells is also highly suppressed by tumor hypoxia in various cancers (72–74). Even if  $\gamma\delta$  T cells could infiltrate solid tumors, their cytotoxicity can be suppressed by

hypoxic conditions in the TME due to apoptosis via PD-1 and reduced expression of NKG2D. In brain tumors, the use of metformin, a repurposed drug that has been shown to elicit an anti-tumor effect (75, 76), reduced hypoxia and rescued the anti-tumor effect of  $\gamma\delta$  T cells (72). In oral cancers, blockade of PD-1 or targeting hypoxia-inducible factor-1 $\alpha$  could also help to overcome tumor hypoxia (73). On the other hand, in the case of breast cancer, cancer cells may also evade detection by  $\gamma\delta$  T cells by shedding MICA under hypoxia (74). Therefore, strategies for  $\gamma\delta$  T cells to prevail under TME conditions should be catered for specific tumor types. Taken together, more studies are required to characterize both  $\gamma\delta$  T and cancer cells, and their interactions in the TME.

### 3.1.3 Culture conditions during *ex vivo* expansion influences $\gamma\delta$ T cell functionality

Besides understanding what happens *in vivo*, the process of *ex vivo* expansion can affect  $\gamma\delta$  T cell phenotypes and cytotoxicity. Despite the great success achieved by the two trials mentioned earlier in treating liquid tumors, we noted that the clinical outcomes in a study (NCT03790072) that also targeted liquid tumor pale in comparison, with a complete response rate of 14%. The media and/or expansion method employed could have affected the quality, quantity and ultimately the efficacy of  $\gamma\delta$  T cells produced. Xu et al. examined the effect of  $\gamma\delta$  T cells grown in the presence of different media supplements and infused into patients on the patients' overall survival (54). Eighteen patients that were administered with V $\gamma$ 9V $\delta$ 2 T cells grown in their newly formulated media supplemented with zoledronate, IL-2, IL-15 and vitamin C, were found to have better overall survival compared with patients that were administered with  $\gamma\delta$  T cells grown in media supplemented with zoledronate and IL-2. When expanded with the new formula, the authors obtained higher cell yield and observed less cell death corroborated by RNAseq results showing downregulation in expression of apoptosis-related genes. In addition, there was an increase in V $\gamma$ 9V $\delta$ 2 T cells harboring terminally differentiated effector memory (CD45RA<sup>+</sup>CD27<sup>-</sup>) phenotype which were previously found to express homing receptors such as CCR5 and CXCR3 (77), and a decrease in cell populations with naïve (CD45RA<sup>+</sup>CD27<sup>+</sup>) and central memory (CD45RA<sup>-</sup>CD27<sup>+</sup>) phenotypes, although there were no significant changes in counterparts bearing effector memory (CD45RA<sup>-</sup>CD27<sup>-</sup>) phenotype. The cells also more highly expressed co-stimulatory molecules such as CD80, CD86 and MHC-II. Collectively, these data suggest that appropriate media supplements can prime  $\gamma\delta$  T cells to migrate to tumor sites and exert cytotoxic effects, thus leading to better clinical outcomes.

### 3.1.4 $\gamma\delta$ T cell subtypes variably affect the clinical outcome

Another possible reason for the dismal failure is that a significant proportion of trials focused on harnessing V $\gamma$ 9V $\delta$ 2  $\gamma\delta$  T cells for therapy (Figure 3A) as they can be readily expanded *ex vivo* to large numbers using zoledronate and IL-2. However, V $\gamma$ 9V $\delta$ 2 cells are naturally abundant in PB and do not typically home to tissues which may partially explain their limited cytotoxic

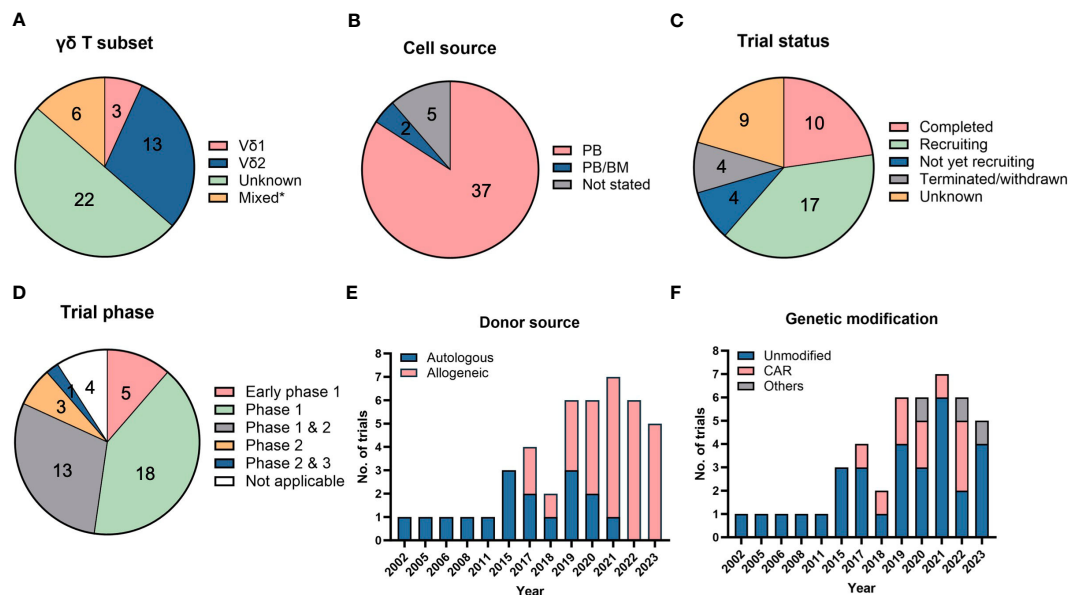


FIGURE 3

Clinical trials using direct cellular administration of  $\gamma\delta$  T cells. Pie charts showing the (A)  $\gamma\delta$  T subsets that were infused into patients, (B) cell sources from which  $\gamma\delta$  T cells were obtained, (C) trial status and (D) different clinical trial phases. \*\*Mixed\* in (A) refers to the use of other cell types, namely natural killer (NK), dendritic cell-cytokine-induced killer (CIK) or CIK cells, that were infused together with  $\gamma\delta$  T cells. "Unknown" in (A) refers to trials in which details on  $\gamma\delta$  T subsets were not available. The category "not applicable" in (D) is used for trials without FDA-defined phases, according to clinicaltrials.gov, and includes observational studies. The number of trials in each category is listed within the pie charts in (A–D). Bar graphs depicting the (E) donor sources from which  $\gamma\delta$  T cells are derived and (F) types of modifications in  $\gamma\delta$  T cells. A total of 44 trials were analyzed in (A–F).

capacity against solid tumors. Vδ1-enriched delta one T (DOT) cells (60), polyclonal  $\gamma\delta$  T cells comprising multiple subsets or other non-Vδ2 subsets (6) have been or could be explored as potential alternatives that demonstrate greater potency against such tumors. For example, Vγ4<sup>+</sup> TCRs have been shown to bind butyrophilin like 3 expressed by gut epithelial cells and EPCR expressed mostly by endothelial cells (27, 78) to facilitate immunosurveillance of virus-infected and tumor cells. Such ligands are thought to mediate homing of  $\gamma\delta$  T cells to and their killing of tumors. Moreover, UCB-derived Vδ2<sup>+</sup> T cells were shown to be more cytotoxic than their Vδ2<sup>+</sup> counterparts (8). Other tumor-associated ligands recognized by non-Vδ2 TCRs are summarized in a recent review by Dong R et al. (79) As such, the relative importance amongst the various  $\gamma\delta$  T cell subsets and which ones should be used in the application for  $\gamma\delta$  T cell therapy should be considered. In addition, incorporation of a step to specifically deplete pro-tumorigenic subsets prior infusion could improve clinical outcome.

### 3.1.5 Cell source used for *ex vivo* expansion can influence the properties of $\gamma\delta$ T cell product

Clinical trials typically rely on adult PB as a source to harvest and expand  $\gamma\delta$  T cells with a restricted TCR repertoire (Figure 3B). Expansion from UCB will generate "younger" cells equipped with a more polyclonal TCR repertoire able to recognize a broader diversity of tumor ligands but are not yet endowed with distinct homing properties characteristic of adult, tissue-resident  $\gamma\delta$  T cells (59). In-depth characterization of the functional profiles, such as cytokine secretion and varying TCR affinities towards different

ligands, of polyclonal  $\gamma\delta$  T cells will be important to ascertain advantages of their therapeutic use.

### 3.1.6 $\gamma\delta$ T cell therapy is safe, but its anti-tumor potency requires improvement

The clinical safety of unmodified  $\gamma\delta$  T cells has been confirmed largely by the paucity of serious adverse events following either dose escalation of aminobisphosphonates and IL-2 to stimulate their *in vivo* expansion in patients or *ex vivo* expansion and subsequent adoptive transfer to patients in multiple trials (80, 81).  $\gamma\delta$  T cell therapy is relatively safe and accompanied by low-grade adverse events such as fever, fatigue, or gastrointestinal disorder, some of which can self-resolve in a few days (49, 51). However, naturally occurring  $\gamma\delta$  T cells in most of these trials failed to promote substantial tumor regression and enforce remission, highlighting the need for targeted engineering to (1): instruct commitment of  $\gamma\delta$  T cells towards cytotoxic and not regulatory lineage and (2) restore their metabolic fitness compromised by the immunosuppressive TME.

Addressing the former challenge would entail utilizing culture conditions and specific antigens to expand and enrich  $\gamma\delta$  T cells with anti-tumor properties, i.e. cytotoxic T cells, while minimizing or depleting those with pro-tumorigenic properties, i.e.  $\gamma\delta$  Tregs. In addition, tackling the issues of T cell infiltration and immunosuppressive TME would require strategies such as rejuvenation of T cells via immune checkpoint inhibition. More details are described in section 3.3. Improving anti-tumor potency of  $\gamma\delta$  T cell therapy is a pressing issue because these relatively newer therapies will no doubt be continuously compared to currently approved CAR-T therapies for lymphoma and myeloma.



## 3.2 Clinical trials involving administration of unmodified or engineered $\gamma\delta$ T cells

The strategy to engineer and expand  $\gamma\delta$  T cells *in vitro* followed by their *in vivo* infusion compared with combinatorial administration of stimulatory  $\gamma\delta$  T ligands and cytokines to selectively expand these cells *in vivo* likely enables more robust and precise improvement of  $\gamma\delta$  T anti-tumor efficacy. Here, we summarize in **Table 2** clinical trials which implemented or are in process of implementing a regimen of *ex vivo* expansion followed by infusion of unmodified or CAR-modified  $\gamma\delta$  T cells into cancer patients, providing evidence that engineered  $\gamma\delta$  T cells are increasingly preferred to unmodified counterparts for tumor immunotherapy.

Out of the 44 clinical trials in **Table 2**, 10 are completed trials, all of which involved unmodified  $\gamma\delta$  T cells (**Figure 3C**). Seventeen clinical trials are currently recruiting patients. A broad range of cancers are targeted in these clinical trials with a trend towards trials targeting liquid or blood (24 trials) compared to solid malignancies (21 trials). Among blood cancers, AML is the most common cancer targeted whereas lung, liver and breast cancers are the most commonly targeted solid tumors.

Interestingly, we note that most of these trials utilized PB or BM as their cell sources and there are currently no trials utilizing  $\gamma\delta$  T cells expanded from UCB and induced pluripotent stem cells (iPSCs) (**Figure 3B**), although there is ongoing work in the field to generate  $\gamma\delta$  T cells from these sources (8, 82). iPSCs could potentially be an unlimited cell source for allogeneic treatment, which requires a large number of cells for scale-up. iPSCs-derived  $\gamma\delta$  T cells have been successfully generated and shown to illicit cytotoxicity effect on several cancer cell lines (83).

While majority (91%) of the clinical trials are in their early phases (Phase 1 or 2) (**Figure 3D**), an allogeneic treatment study using TCR $\alpha\beta$ /CD19-depleted innate donor lymphocyte infusion has been approved for Phase 2 & 3 clinical trial in 2023 (NCT05686538) (**Table 2**). Besides the clinically proven advantageous safety profile of  $\gamma\delta$  T cells in not causing GvHD, another reason favoring adoption of allogeneic therapy is the difficulty in recovering and expanding sufficient numbers of autologous  $\gamma\delta$  T cells of high quality from diseased patients. This is exemplified by trials conducted by Fuda Cancer Hospital, China (NCT03183206, NCT03183219, NCT03183232) which initially planned to use patients' own PB-derived  $\gamma\delta$  T cells but the investigators encountered challenges in expanding the cells, prompting them to source cells from allogeneic donors. Similar manufacturing obstacles in some patients were recorded by Vydra et al. when they conducted a trial using autologous  $\gamma\delta$  T cells (55). In recent years, the number of clinical trials for the allogeneic use of  $\gamma\delta$  T cells has surpassed those for autologous treatments (**Figure 3E**).

To further improve the specificity and efficacy of  $\gamma\delta$  T cells for targeting tumors, several strategies have been explored by others, which are discussed in Section 3.3. Equipping  $\gamma\delta$  T cells with CAR is one of the earliest and most common strategies, given the success witnessed in CAR-modified  $\alpha\beta$  T cells. Anti-CD7, anti-CD19/20, anti-CD33, and anti-CD123 CAR have been designed to target

liquid cancers, while NKG2DL-targeting CAR is constructed for targeting solid tumors. Since 2017, the number of trials conducted with genetically modified  $\gamma\delta$  T cells has been increasing (**Figure 3F**). As of 2023, there are 10 trials that involved CAR-modified  $\gamma\delta$  T cells. Besides engineering CAR,  $\gamma\delta$  T cells have also been modified to be resistant to chemotherapy drug, temozolomide, which is useful to treat glioblastoma using combination therapy (NCT04165941, NCT05664243).

## 3.3 Strategies to augment anti-tumor cytotoxicity of $\gamma\delta$ T cells

In this section, we describe how various strategies have been applied or can be adapted to improve anti-tumor efficacy of  $\gamma\delta$  T cells. Such strategies include those that were used to modify conventional  $\alpha\beta$  T cells or can complement their therapy via non-genetic engineering approaches. *In vitro* and pre-clinical data, where applicable, are discussed.

### 3.3.1 Non-genetic engineering approaches

As a first illustration, a bispecific T cell engager (BiTE) antibody construct, AMG 330, administered to leukemic patients yielded encouraging safety and anti-leukemic outcomes (84). A BiTE is a synthetic fusion protein which is designed based on linking the antibody-binding domains of two antibodies. In this example, AMG 330 simultaneously binds CD33 antigen on leukemic blasts and CD3 co-receptor on T cells, placing T cells in close proximity to CD33<sup>+</sup> leukemic cells and ultimately mediating destruction of the latter by the former cells. In similar fashion, a bispecific tribody which recognizes V $\gamma$ 9 on  $\gamma\delta$  T cells and ERBB2 (HER2/neu) on pancreatic cancer cells enhanced  $\gamma\delta$  T cell cytotoxicity against PDA *in vitro* and *in vivo* (85). Recombinant immunoligands comprising an anti-CD20 single chain variable fragment (scFv) linked to a NKG2D ligand, MICA or ULBP2, activated specific elimination of CD20<sup>+</sup> but not CD20<sup>-</sup> lymphoma cells by *ex vivo* expanded V $\delta$ 1 and V $\delta$ 2  $\gamma\delta$  T cells, a therapeutic result which could be further augmented by concurrent agonistic stimulation of the cells with BrHPP (86). Hence, exogenous application of BiTE or similarly designed molecules can be employed in combination with  $\gamma\delta$  T administration.

Another example obviating non-genetic modification involves programming  $\gamma\delta$  T cells with various combinations of cytokines to enhance their tumor killing capacity (87). Schilbach and colleagues demonstrated that the combination of IL-2, IL-12 and IL-18 synergize to significantly induce both IFN- $\gamma$  and TNF- $\alpha$  secretion in the presence of TCR stimulus (88). The increase in TNF- $\alpha$  was observed even in the absence of a TCR signal. The authors also showed that in IL-2/IL-12/IL-18 stimulated  $\gamma\delta$  T cells, granzyme B and perforin protein expression was upregulated to a similar extent compared to TCR stimulation. Interestingly, the expression of FasL was increased under conditions of IL-2/IL-12/IL-18 stimulation, but not TCR stimulation. Together, these mechanisms mediate the increased anti-tumor killing capacity of cancer cells by the stimulated  $\gamma\delta$  T cells. More recently, Liu and colleagues showed

that  $\gamma\delta$  T cells pre-treated with a combination of IL-12, IL-18 and IL-21 led to their enhanced inhibition of tumor growth not only *in vitro*, but also *in vivo* after adoptive transfer (89). They showed that such a pre-activation cocktail promoted the proliferation of  $\gamma\delta$  T cells and their secretion of IFN- $\gamma$  and TNF- $\alpha$ , which can promote the anti-tumor function of endogenous CD8<sup>+</sup> T cells *in vivo*. Therefore, in the cytokine pre-treatment strategy,  $\gamma\delta$  T cells can be stimulated *ex vivo* by cytokine combinations to boost their anti-tumor activity. Potent cytokine-activated V $\delta$ 1<sup>+</sup> DOT cells have been generated and appears promising for clinical use (60).

Based on newly acquired knowledge on the mechanism of V $\gamma$ 9V $\delta$ 2 T cell activation, agonistic antibodies directed against BTN3A1 and BTN2A1 (90, 91) can be used to heighten sensitivity of tumor cells to  $\gamma\delta$  T cell killing and offers a promising therapeutic strategy to enhance  $\gamma\delta$  T cell cytotoxicity. An anti-BTN3A monoclonal antibody (ICT01) is currently in phase 1/2a clinical trial (NCT04243499).

Other possible strategies are targeted at overcoming the immunosuppressive effects of the TME on  $\gamma\delta$  T cells. For instance, CD137 costimulation using a recombinant CD137L protein was found to reduce the expression of IL-10 receptor, IL-10R1, thereby reducing the sensitivity of the  $\gamma\delta$  T cells to the immunosuppressive effects of endogenous IL-10 (70).

Interestingly, it was shown that acute systemic  $\beta$ -adrenergic receptor activation was largely responsible for the exercise-augmented mobilization, *ex vivo* expansion and anti-tumor activity of V $\gamma$ 9V $\delta$ 2 T cells from healthy donors (92). Administration of an antagonist inhibiting both  $\beta$ 1- and  $\beta$ 2-adrenergic receptors abrogated these exercise-induced effects. This finding suggest that  $\beta$ -adrenergic receptors are potential targets to improve the potency of *ex vivo* expanded  $\gamma\delta$  T cells.

Novel methods of  $\gamma\delta$  T cell delivery other than the traditional intravenous infusion route could be designed to improve their efficacy in solid tumors. When CAR-T cells were delivered to tumor sites directly using biopolymer scaffolds, they were able to migrate to and kill tumor cells more effectively as compared to systemic delivery method (93). Treatment of glioblastoma has been notoriously challenging due to the difficulty in reaching the blood-brain barrier by immune cells. This could be overcome by stereotactic injection of  $\gamma\delta$  T cells directly into the brain (94, 95). These direct intratumoral delivery methods could be applied to treat solid tumors that are known to be difficult to infiltrate by immune cells.

Beyond delivering  $\gamma\delta$  T cells *per se*, it is noteworthy that newer strategies using cell-free extracellular vesicles, such as exosomes, confer a safety advantage over cell-based therapies and have shown promising anti-tumor efficacy. The small size (20-200 nm) of exosomes renders easy infiltration into solid tumor sites and they are resistant to the immunosuppressive TME. Such  $\gamma\delta$  T cell-derived vesicles were shown to control tumor progression of and elicit anti-tumor responses against Epstein-Barr virus-associated B-cell lymphoma, gastric carcinoma and nasopharyngeal carcinoma (96, 97). These exosomes derived from activated V $\delta$ 2 T cells were not only positive for NKG2D, which is responsible for their uptake by tumor cells, but were also positive for FasL and TRAIL, which facilitate their death-inducing properties. More recently,  $\gamma\delta$  T

extracellular vesicles were used as carriers to deliver tumor-associated antigens, and the extracellular vesicles-based cancer vaccines were successful in controlling tumors *in vivo* (98).

### 3.3.2 Genetic engineering approaches

CAR-modified  $\gamma\delta$  T cells were first explored as effector cells of tumor-directed immunity in a 2004 study which demonstrated these cells efficiently recognized CAR antigen-expressing neuroblastoma and malignant B cell tumour cells as assessed by their upregulation of CD69 and secretion of IFN- $\alpha$  (99). Consistent with operating multiple mechanisms of cytotoxicity, CD19 CAR  $\gamma\delta$  T cells were found to exert not only CAR-directed activity against CD19<sup>+</sup> leukemia cells but also CAR-independent activity against CD19<sup>-</sup> leukemia cells or cells which have lost expression of CD19 antigen (100), highlighting the advantage of using  $\gamma\delta$  T vis-à-vis  $\alpha\beta$  T cells. Although arming  $\gamma\delta$  T cells with CARs endows tumor specificity, CAR signaling components can be optimized to increase  $\gamma\delta$  T efficacy against hematological malignancies and solid tumors. Firstly, transduction with second-generation CARs bearing CD3 $\zeta$  activation domain is known to elicit tonic signaling and exhaustion marked by PD-1 and TIM-3 upregulation in  $\alpha\beta$  and  $\gamma\delta$  T cells. Modifying the endodomain of the chimeric co-stimulatory receptors (CCRs) that replace CD3 $\zeta$  with DAP10 domain in  $\gamma\delta$  T cells led to effective activation of cytotoxic responses in the presence of CCR-specific stimuli or cognate tumor cells (101). V $\delta$ 1 T cells that were genetically modified to express 4-1BB/CD3 CAR targeting the oncofetal antigen glypican-3 and a constitutively secreted form of IL-15 exhibited superior proliferation and anti-tumor activity against hepatocellular carcinoma (HCC) lines and HCC subcutaneously engrafted in immunodeficient mice compared with their non-cytokine secreting counterparts (102). Such armored CAR design which allows release of transgenic cytokine(s) of interest upon CAR signaling had previously been employed successfully in  $\alpha\beta$  T cells to counteract the inhibitory cytokine milieu of and recruit innate effector cells into the TME (103, 104). Therefore, continued innovation of CAR designs is warranted.

Beyond CAR, introduction of a tumor-specific  $\alpha\beta$  TCR and the corresponding CD4 or CD8 co-receptor for recognition of HLA-restricted tumor antigen in  $\gamma\delta$  T cells led to their pronounced cytokine secretion and cytolytic effects against leukemia (105). One obvious drawback using  $\alpha\beta$  TCR is the requirement for additional CD4 and CD8 co-receptors. The advent of CRISPR/Cas technology has opened new avenues for genetic, including TCR, modification of  $\gamma\delta$  T cells. Such targeted TCR editing enables controlled replacement of the endogenous TCR with the transgene, thereby allowing for transgene TCR to be expressed at homogeneous, physiological levels on the T cells, and consequently less functional variability compared to virus-mediated transgene integration (106). In this respect, Immatics, a clinical-stage biopharmaceutical company, has entered into a research collaboration and licensing agreement with Editas Medicine, a genome editing company, to advance off-the-shelf adoptive  $\gamma\delta$  T cell therapy platform. Reciprocally, V $\gamma$ 9V $\delta$ 2 TCR was shown to effectively reprogram both CD4<sup>+</sup> and CD8<sup>+</sup>  $\alpha\beta$  T cells to kill a broad

diversity of cancer but not normal cells, and substantially diminished but did not completely abrogate alloreactivity (107). Recently, a clinical stage immune-oncology company, Triumvira Immunologics, developed proprietary T cell Antigen Couplers (TACs) for incorporation in T cells (108). TAC consists of 3 components: a tumor antigen binding domain, a CD3 binding domain which interacts with and co-opts the native TCR and a CD4 co-receptor transmembrane and intracellular domain. When bound to its target antigen, TAC triggers the native TCR signaling cascade by recruiting downstream kinases and thereby activating T cell killing in an HLA-independent manner. TAC-modified  $\alpha\beta$  T cells are currently undergoing Phase 1 & 2 clinical trials for autologous treatment of HER2<sup>+</sup> solid tumors (NCT04727151). HER2-targeting TAC-modified  $\gamma\delta$  T cells are similarly being developed and preclinically evaluated. TAC  $\gamma\delta$  T cells were observed to exhibit cytotoxicity against tumor xenografts that are resistant to unmodified  $\gamma\delta$  T cells (109), suggesting modifications of  $\gamma\delta$  T cells need not be restricted to CAR. However, the safety of their use requires further evaluation.

Despite the established clinical safety profile of unmodified  $\gamma\delta$  T cells, the enhanced anti-tumor efficacy achieved by modification of  $\gamma\delta$  T cells may correspondingly increase their off-tumor, on-target toxicity, resulting in undesirable side effects. To address this potential challenge, non-signaling CARs (NSCARs) lacking signaling/activation domains but retaining tumor-specific targeting capability were expressed in  $\gamma\delta$  T cells. CD5- and CD19-targeting NSCARs significantly elevated the intrinsic, HLA-independent cytotoxicity of  $\gamma\delta$  T cells against T cell and B cell ALL but expectedly did not enhance the antigen-specific cytotoxicity of  $\alpha\beta$  T cells (110). An alternative T cell therapy platform involving the concept of a synthetic agonistic receptor (SAR) originally applied in  $\alpha\beta$  can be potentially extended to  $\gamma\delta$  T cells. SAR-transduced T cells are directed by an engineered tandem scFv construct (taFv) to antigen-expressing tumor cells in a manner similar to BiTEs (111). The taFv construct comprises two scFvs, one binding the artificial antigen receptor composing an extracellular EGFRvIII domain fused to intracellular T cell-activating domains transduced in T cells and another binding a specific antigen on the surface of cancer cells, thus juxtaposing T and cancer cells. Unlike the BiTE approach which activates pan-T cells, this system specifically activates SAR-transduced T cells and is able to terminate SAR T cells via antibodies clinically approved by FDA should adverse toxicity events arise.

Strategies to improve anti-tumor cytotoxicity of  $\gamma\delta$  T cells need not be confined to improving recognition of tumor antigens. Other options include boosting the infiltration of  $\gamma\delta$  T cells into solid tumor by expressing surface proteins that can aid its migration through the extracellular matrix (ECM) surrounding the tumor whilst harnessing the diverse HLA-independent receptors of  $\gamma\delta$  T cells to target tumors, particularly those which have escaped antigen targeting. When modified to express matrix metalloprotease 14 (MMP14) enzyme that can digest the ECM,  $\gamma\delta$  T cells were able to more efficiently migrate in the tumor milieu (112). However, despite being able to kill TNBC cells effectively *in vitro* and showing an improved migration profile, MMP14-engineered  $\gamma\delta$  T cells could not eliminate TNBC tumors *in vivo* due to down

regulation of  $\gamma\delta$  T cell ligands Fas, MICB and intercellular adhesion molecule 1 (ICAM-1) on breast cancer stem cells. Pre-treatment using zoledronate recovered some cancer stem cell killing by  $\gamma\delta$  T cells, suggesting that prior activation of  $\gamma\delta$  T cells may be necessary for TNBC eradication.

### 3.3.3 Combination therapies

In addition to the aforementioned strategies, supplementing  $\gamma\delta$  T cell therapy with immune checkpoint inhibitors, such as those targeting PD-1/PD-L1 and CTLA4 pathways (65), or novel cancer stem cell-targeting strategies may further bolster the effectiveness and durability of engineered  $\gamma\delta$  T anti-tumor responses. Rossi et al. demonstrated that  $\gamma\delta$  T cells infiltrating follicular lymphoma highly express PD-1 and anti-PD1 blockade consequently increased their cytotoxicity (113).

Another combination treatment that has shown better efficacy is the treatment of locally advanced pancreatic cancer using irreversible electroporation (IRE) with  $\gamma\delta$  T cells infusion (NCT03180437). The median overall survival of patients treated with IRE alone was 11 months but with  $\gamma\delta$  T infusions, the overall survival increased to 14.5 months, showing the potential of combination treatment in prolonging patient's life (53).

Taking advantage of the cross-talk between  $\gamma\delta$  T cells and other immune cells in the TME, anti-tumor responses in  $\gamma\delta$  T cell therapies could be further enhanced by boosting the anti-tumor cytotoxicity mediated by other immune cells. For instance, CD137 (4-1BB) co-stimulation with recombinant human CD137L has been shown to increase NKG2D expression on NK cells, which is directly responsible for tumor cell killing (114). An added mechanism of action by these NK cells is the killing of dendritic cells which would otherwise promote inflammation and tumor growth (115).

Other plausible therapies involve targeting the tumor cells within TME, some of which have demonstrated promising preclinical results. These include the use of COX inhibitors which ameliorate the effects of the immunosuppressive TME (116) and celastrol which upregulates death receptor expression on tumor cells (117).

Similarly, patients may also develop resistance with other treatments that could be rescued by co-treatment with  $\gamma\delta$  T cells. For instance, tyrosine kinase inhibitors (TKIs) have been successful in treating various cancers such as advanced or metastatic renal cell carcinoma, non-small-cell lung cancer and HCC (118–120). However, many patients eventually develop resistance against treatment with TKIs (121). When used alone,  $\gamma\delta$  T cell therapy also showed some efficacy against these cancers (as summarized in Table 2). Therefore, these two complementary therapies potentially add to or synergize with each other in treating cancer patients (48).

## 4 Concluding remarks

$\gamma\delta$  T cells are a highly promising immune subset that can be harnessed for “off-the-shelf”, allogeneic immunotherapy (Figure 1) and additionally engineered to amplify their anti-tumor efficacy. There exist several hurdles which need to be overcome in order that  $\gamma\delta$  T cells can be employed as an effective oncotherapy (Figure 2). Firstly, the challenge of translating the preclinical finds into clinical

trials require extensive knowledge on  $\gamma\delta$  T cell infiltration and their plasticity within the TME. Learning how to effectively deliver  $\gamma\delta$  T cells to solid tumor sites by exploiting context-dependent mechanisms which drive  $\gamma\delta$  T cells to adopt anti- rather than pro-tumor function is absolutely crucial. Secondly, elucidating hitherto unknown ligands that activate and expand specific populations, as defined by TCR usage, of  $\gamma\delta$  T cells which play important roles in anti-tumor immunity will help to activate  $\gamma\delta$  T cell in the settings of *in vivo* administration or *ex vivo* expansion. Thirdly, culture conditions and cell source undeniably moulds the  $\gamma\delta$  T cell final product during the manufacturing process. Identifying the most optimal parameters to adopt in  $\gamma\delta$  T cell expansion *ex vivo* should be incorporated as part of  $\gamma\delta$  T cell therapy process development. The relative importance amongst the various  $\gamma\delta$  T cell subsets and specific depletion of pro-tumorigenic subsets prior infusion should also be considered in the application for  $\gamma\delta$  T cell therapy. Finally, innovations in modular engineering of  $\gamma\delta$  T cells and combination strategies will be crucial in improving their *in vivo* anti-tumor cytotoxicity and persistence to prevent tumor relapse whilst minimizing likelihood of detrimental alloreactive responses.

## Author contributions

CW: Conceptualization, Data curation, Investigation, Project administration, Visualization, Writing – original draft, Writing – review & editing. PL: Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing. AT: Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

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## Conflict 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.

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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.2023.1282758/full#supplementary-material>

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EDITED BY  
Daniela Wesch,  
University of Kiel, Germany

REVIEWED BY  
Selena Sheng Yan,  
The University of Hong Kong, Hong Kong  
SAR, China  
Ana Carolina Martinez-Torres,  
Autonomous University of Nuevo León,  
Mexico

\*CORRESPONDENCE  
Andrew Zloza  
✉ anzloza@utmb.edu  
Kajal H. Gupta  
✉ KajaL.Gupta@rush.edu

<sup>†</sup>These authors have equally contributed to this work

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# Seasonal influenza vaccines differentially activate and modulate toll-like receptor expression within the tumor microenvironment

Kajal H. Gupta<sup>1,2\*†</sup>, Eileena F. Giurini<sup>1†</sup> and Andrew Zloza<sup>3,4\*</sup>

<sup>1</sup>Division of Surgical Oncology, Department of Surgery, Rush University Medical Center, Chicago, IL, United States, <sup>2</sup>Division of Pediatric Surgery, Department of Surgery, Rush University Medical Center, Chicago, IL, United States, <sup>3</sup>Division of Surgical Oncology, Department of Surgery, The University of Texas Medical Branch, Galveston, TX, United States, <sup>4</sup>Division of Translational and Precision Medicine, Department of Internal Medicine, Rush University Medical Center, Chicago, IL, United States

Toll-like receptors (TLRs) are well-known for their role in cancer development as well as in directing anti-tumor immunity. Because TLRs have also been implicated in the innate recognition of the influenza virus, it was of great interest to investigate the potential TLRs' contribution to the reduction in tumor growth following intratumoral injection of an unadjuvanted influenza vaccine and the lack of antitumor response from an adjuvanted vaccine. In our previous publication, we showed that the unadjuvanted flu vaccine modulates TLR7 expression leading to anti-tumor response in a murine model of melanoma. Here, we show that the unadjuvanted and adjuvanted flu vaccines robustly stimulate different sets of TLRs, TLR3 and TLR7, and TLR4 and TLR9, respectively. In addition, the reduction in tumor growth and improved survival from intratumoral administration of the unadjuvanted vaccine was found to be diminished in TLR7-deficient mice. Finally, we observed that both vaccines have the capacity to modulate TLR expression on both innate and adaptive immune cells. Our findings add to the mechanistic understanding of the parameters that influence tumor outcomes in unadjuvanted and adjuvanted influenza vaccines.

## KEYWORDS

toll like receptor (TLR), innate immunity, flu vaccine, tumor microenvironment (TME), cancer vaccine

## Introduction

The tumor immune microenvironment (TIME) encompasses innate immune factors that possess both pro-tumor and anti-tumor properties. Tumor progression, using the innate immune system, can be facilitated through two extremes: an immune-suppressed or chronically inflamed tumor microenvironment (TME) (1–3). A lack of immune infiltration



in the TME is in part perpetuated through suppressive innate immune cell populations and the secretion of immunosuppressive cytokines, while the chronically inflamed TME is primarily mediated by sustained activation of pathogen recognition receptors (PRRs) and production of pro-inflammatory cytokines (2–4). Maintenance of these TME profiles culminates in tumor growth, angiogenesis, and invasion (2, 3). Though the extremes of immune activation within the TME are shown to promote tumorigenesis, an acute, potent inflammatory response induced within the TME can drive anti-cancer effects (5, 6). Activation of the innate immune system can convert suppressive tumor-associated macrophages (TAMs) to M1 macrophages and shape anti-tumor adaptive responses (7, 8).

Within the innate immune system are a set of PRRs that also possess a dual role in tumor outcomes, one such PRR is Toll-like receptors (TLRs). Upon recognition of pathogen-associated molecular patterns (PAMPs) by TLRs, dendritic cells (DCs) upregulate co-stimulatory molecules as well as major histocompatibility complex (MHC) I and II, leading to DC activation (9). Accordingly, TLR-activated DCs can induce anti-tumor T cell responses (10, 11). Additionally, TLR stimulation can shift suppressive immune cells to a pro-inflammatory or tumoricidal phenotype to initiate innate immune-mediated tumor eradication (12–14). However, TLR stimulation in an inappropriate context or with an inappropriate ligand can promote tumor progression. TLR-driven tumorigenesis primarily occurs through inducing tumor-promoting cytokine production and dysregulating TLR signaling (15, 16). Consequently, metastasis and resistance to apoptosis have been found to occur in a TLR activation-dependent manner (15–17).

In a previous study, we established that the seasonal influenza vaccine without adjuvants, as opposed to the adjuvanted vaccine, diminishes the growth of melanoma tumors. This is due to an increase in the CD8<sup>+</sup> T cell population and a decrease in regulatory B cells in the TME (18). While prior research has shed light on the function of adaptive immunity in the intratumor effects of the two influenza vaccinations, the role of innate immune sensors remains unknown. In this study, we conduct a thorough analysis to understand the complex interplay of innate immune components in the context of unadjuvanted and adjuvanted influenza vaccinations, identifying a dualistic TLR profile associated with each formulation. Our research interests include determining the implications of these disparate TLR profiles in tumor outcome modification. We hope that by doing this systematic investigation, we will be able to provide a more comprehensive and nuanced understanding of the complex immunological landscape dictating the efficacy differences between unadjuvanted and adjuvanted influenza vaccinations in the setting of melanoma tumor dynamics.

## Methods

### Animals

B6 (C57BL/6J) and TLR7<sup>-/-</sup> (B6.129S1-Tlr7<sup>tm1Flv</sup>/J) mice were purchased from The Jackson Laboratory at 6–10 weeks of age.

Both male and female mice were used in this study. All animals were housed in specific-pathogen-free facilities and all experimental procedures were by policies approved by the Institutional Animal Care and Use Committee (IACUC) and Rush University Medical Center.

### Seasonal influenza vaccines & adjuvant

Two 2020–2021 FDA-approved seasonal influenza vaccines were purchased for these studies: Fluarix Quadrivalent (GlaxoSmithKline) and Fluad Quadrivalent (Seqirus). Addavax (Invivogen), a squalene-based oil in water adjuvant, was utilized to mimic adjuvant MF59 (Novartis). 50 µl of influenza vaccine or a PBS control were administered to mice via intratumoral injection. Removal of MF59 was completed using Amicon Ultra-0.5 centrifugal filter units with 30 kDa cutoff and a regenerated cellulose membrane. 500 µl of Fluad was added to the filter unit and washed with 250 µl acetone (3 times) followed by 250 µl PBS (3 times). The vaccine concentrate was subsequently collected and resuspended in 500 µl PBS.

### Cell culture

HEK-Blue TLR reporter cell lines (Invitrogen) transfected with plasmids containing a murine TLR and an NF-κB inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene, were used to determine TLR stimulation. mTLR2, mTLR3, mTLR4, mTLR5, mTLR7, mTLR8, mTLR9, and mTLR13, and non-TLR expressing parental cell lines Null1, Null1k, Null1v, Null2, and Null2k (Invitrogen) were cultured using DMEM (Gibco), supplemented with 10% FBS (Corning), 100 units/mL penicillin (Gibco), 100 mg/mL streptomycin (Gibco), and 100 µg/mL normocin (Invivogen). Expression of TLR and SEAP plasmids were maintained with the addition of selective antibiotics blasticidin (10 µg/mL or 30 µg/mL, Invivogen), zeocin (100 µg/mL, Invivogen), or 1X HEK-Blue Selection (Invivogen). Murine melanoma cell line B16-F10 was cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 mg/mL streptomycin, and 100 µg/mL normocin.

### TLR activation assay

For evaluating TLR stimulation, 20 µl of each treatment was plated in triplicate in 96-well plates. TLR agonist positive controls were run simultaneously: TLR2 (Pam3CSK4, 1 µg/mL), TLR3 (Poly I: C, 1 µg/mL), TLR4 (LPS-EK, 100 ng/mL), TLR5 (FLA-ST, 2.5 µg/mL), TLR7 (CL264, 50 µg/mL), TLR8 (Poly(dT)/Imiquimod 10 µM), TLR9 (CpG ODN 2395, 5 µM), TLR13 (Sa19, 200 µg/mL). 1X PBS was used as a negative control. After plating the treatments, 50,000 mTLR or Null cells suspended in 180 µl HEK-Blue Detection (Invivogen) medium were added to the respective wells. All cells were incubated for 24 hours at 37°C and 5% CO<sub>2</sub>. Following incubation, TLR stimulation was

quantified using a Cytation 3 plate reader (BioTek) measuring absorbance at 620<sub>nm</sub>.

## Tumor challenge

B6 and TLR7<sup>-/-</sup> mice were anesthetized with isoflurane and challenged with 100,000 B16-F10 melanoma cells in 100 µl PBS via intradermal injection. Initiation of tumor treatment occurred when tumors ranged from 9–25 mm<sup>2</sup> in size. Tumor development was monitored using Vernier calipers, where tumor area was determined by two measurements in perpendicular directions. To comply with IACUC policies, tumor-bearing mice were humanely sacrificed upon tumor measurements reaching 15 mm in either direction.

## Flow cytometry

Tumor-bearing mice were humanely sacrificed via carbon dioxide inhalation. For IL-10 staining, mice were administered (i.v., via retro-orbital injection) 50 µg of monensin (Sigma-Aldrich) dissolved in a diluted ethanol solution six hours before sacrifice. Tissues were processed as previously described (18). Extracellular staining for flow cytometry was performed with antibodies targeting CD3, CD11b, CD11c, CD20, CD45, F4/80, Ly6C, Ly-6G/Gr-1, MHCII, and TLR4. All antibodies were purchased from BioLegend, BD, eBioscience, or R&D Systems. An extracellular stain cocktail comprised of 1–5 µl/test in a total volume of 100 µl (made in PBS) was added to each sample and subsequently incubated in a dark environment at room temperature for 30 minutes. 0.25 µl/test of Live/Dead Fixable Aqua Dead Cell Stain Kit (405 nm excitation) (Invitrogen) was also added to the extracellular stain cocktail. Following extracellular staining, samples were washed twice with PBS before intracellular staining. For optimal intracellular staining, samples were permeabilized and fixed with 100 µl of Cytofix/Cytoperm (BD) for 15 minutes at 4°C. Samples were subsequently washed with 100 µl of 1X Perm/Wash Buffer (BD). Antibodies targeting IL-10, TLR3, TLR7, and TLR9 were used at 1–5 µl/test in a total volume of 100 µl (made in 1X Perm/Wash Buffer). Samples were incubated with intracellular stains for 30 minutes at 4°C, protected from light. Samples were subsequently washed with 200 µl of 1X Perm/Wash Buffer twice, followed by two 200 µl PBS washes. Flow cytometry was performed on the BD LSRFortessa. Flow cytometry analysis was completed using FlowJo (BD, version 10).

## Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 9.1.2). For studies involving more than two groups, a 1-way ANOVA with Tukey correction was used to determine

statistical significance. A two-way ANOVA was performed to determine statistical significance for studies with multiple time points. A Mantel-Cox log-rank test was used to determine the statistical significance of survival curves. For all studies, a p-value <0.05 was considered to be statistically significant.

## Results

### Unadjuvanted and adjuvanted seasonal influenza vaccines activate different sets of TLRs

Host recognition of influenza virus infection begins with the detection of PAMPs by the innate immune system (19–21). Given that single-stranded RNA (ssRNA) from the influenza virus is a recognized PAMP, we sought to determine the TLR stimulatory potential of an unadjuvanted seasonal influenza vaccine (FluVx), as it contains inactivated influenza virus, and importantly, we have previously established its antitumor effects (18). Following FluVx treatment of a murine TLR reporter panel comprising cell lines for TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, and TLR13, both TLR3 and TLR7 were significantly stimulated compared to a non-TLR expressing Null cell line, (Figure 1A). Following these findings that FluVx activated several TLRs, the TLR stimulatory profile of an adjuvanted seasonal influenza vaccine (AdjFluVx) was evaluated. AdjFluVx was found to stimulate TLR2, TLR4, TLR5, TLR8, and TLR9, but most notably TLR4 and TLR9 (Figure 1B) compared to Null cell lines. To further elucidate the TLR-modulatory effects of the addition or removal of a squalene-based MF-59-like adjuvant (Adj) to the vaccines, TLR activation by Adj was evaluated. Adj was found to activate many of the same TLRs as AdjFluVx, and similarly most robustly stimulating TLR4 and TLR9, (Supplementary Figure S1) thus confirming that the TLR stimulatory profile observed with AdjFluVx is driven by its inclusion of Adj. To further confirm these findings, we sought to identify a baseline activation status of the cells. Both TLR and Null cell lines were treated with a PBS control and demonstrated no significant changes in activation between the TLR and Null cell lines (Supplementary Figure S2). After confirming our findings that FluVx and AdjFluVx stimulate different sets of TLRs, the next step was to ascertain any difference in TLR activation upon removal of Adj (MF59) from AdjFluVx. Treatment of TLR3 and TLR7 cell lines with AdjFluVx was consistent with prior experiments, no significant TLR stimulation was observed. However, when Adj was removed from AdjFluVx, significant TLR3 and TLR7 stimulation was uncovered, more akin to that of FluVx (Figures 1C, D).

Concurrently, the removal of the adjuvant from AdjFluVx yielded loss in its ability to activate TLR4 and TLR9 (Figures 1E, F). As expected, addition of Adj to FluVx diminished its ability to stimulate TLR3 and TLR7, while increasing TLR4 and TLR9 stimulation (Supplementary Figure S2). Altogether, these data

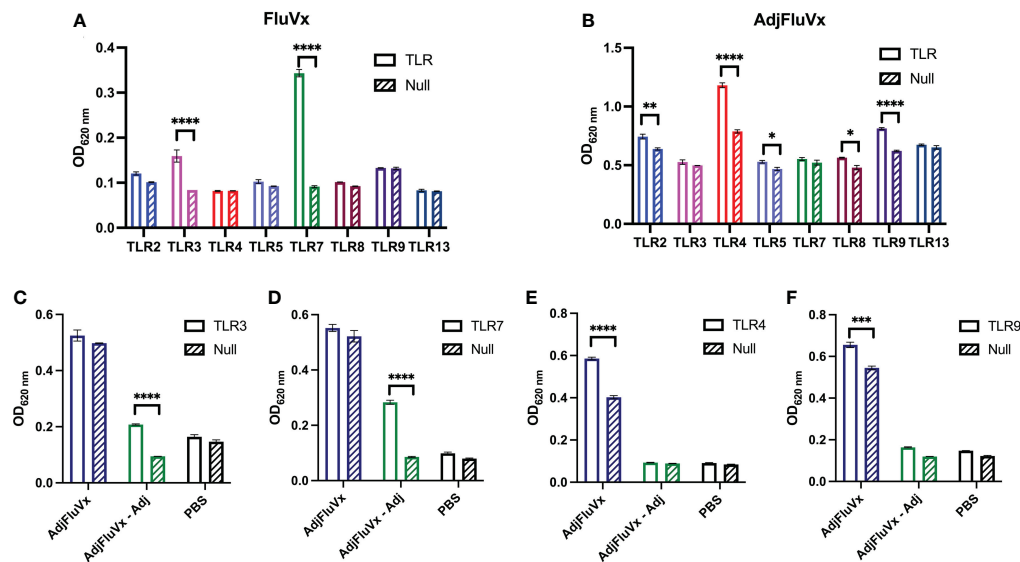


FIGURE 1

Unadjuvanted and adjuvanted seasonal influenza vaccines stimulate different sets of TLRs. (A, B) Activation of a murine TLR reporter panel and non-TLR expressing (Null) cells treated with an unadjuvanted (FluVx) or adjuvanted influenza vaccine (AdjFluVx). (C, D) Stimulation of TLRs previously not activated by AdjFluVx with an adjuvant-removed influenza vaccine. (E, F) Stimulation of TLRs previously activated by AdjFluVx with an adjuvant-removed influenza vaccine. Data are representative of three independent experiments run in triplicate. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ; two-way ANOVA with Sidak's correction for multiple comparisons. Values represent mean  $\pm$  S.E.M.

indicate that FluVx and AdjFluVx stimulate discrete sets of TLRs, determined by the absence or presence of Adj.

## TLR7 contributes to improved tumor outcomes from the intratumoral unadjuvanted seasonal influenza vaccine

To determine any association between *in vitro* TLR activation and the impact of TLRs in the antitumor effects of FluVx, we conducted an experiment with TLR7-deficient mice. TLR7-deficient (TLR7<sup>-/-</sup>) mice bearing B16 melanoma tumors treated with FluVx did not experience reduction in tumor progression which was found in TLR7-competent, C57BL/6J (WT) mice (Figures 2A, B). This finding suggests that the observed *in vitro* activation of TLR7 from FluVx is also important in facilitating its antitumor immunity. To further examine the role of TLR7 in tumor outcomes from FluVx treatment, we observed survival of FluVx-treated WT and TLR7<sup>-/-</sup> mice. Not only was reduction in tumor growth abrogated in TLR7<sup>-/-</sup> mice after FluVx treatment, but the prolonged survival was also diminished (Figure 2C). In accordance with our *in vitro* findings that AdjFluVx did not stimulate TLR7, intratumoral administration of AdjFluVx in WT and TLR7<sup>-/-</sup> mice bearing B16 melanoma tumors did not experience any change in tumor progression (Figures 2D, E). Accordingly, no significant change in survival was observed between WT and TLR7<sup>-/-</sup> treated with AdjFluVx (Figure 2F). These data indicate that TLR7 is an important mediator in the antitumor immune response from FluVx.

## Seasonal influenza vaccines modulate TLR expression on innate and adaptive immune cells within the tumor

In several contexts, TLR expression is upregulated in the presence of its respective stimulating ligand (22–26). Accordingly, we assessed the modulatory capacity of AdjFluVx and FluVx on TLR expression within the tumor by flow cytometry (Figure 3A), gating strategy summarized in Supplementary Figure S3. Changes in TLR expression on regulatory B cells (B<sub>regs</sub>) was of particular interest, as TLR expression on B<sub>regs</sub> has been previously recognized (27–29). After AdjFluVx treatment, intratumoral B<sub>regs</sub> were found to have significantly upregulated both TLR4 (Figures 3B, C). Unexpectedly, B<sub>regs</sub> from AdjFluVx treatment also decreased TLR7 expression on intratumoral B<sub>regs</sub> (Figures 3D, E), suggesting AdjFluVx has the ability to upregulate the TLRs it stimulates that were observed *in vitro*. Similarly, TLR9 expression on B<sub>regs</sub> was increased in the AdjFluVx treated groups compared to PBS and FluVx treated groups (Figures 3F, G). In addition, TLR expression profiling was conducted for dendritic cells (DCs). FluVx treated tumors were found to significantly upregulate TLR7 on DCs and TLR3 on neutrophils (Figures 3H–K), suggesting that FluVx is also capable of modulating the expression of the TLRs it activates. Finally, intratumoral administration of AdjFluVx was found to significantly upregulate TLR4 expression on tumor localized myeloid derived suppressor cells (MDSCs) (Figures 3L, M). These data indicated that both FluVx and AdjFluVx can modulate the TLRs expressed on immune cells that are encompassed within the tumor.

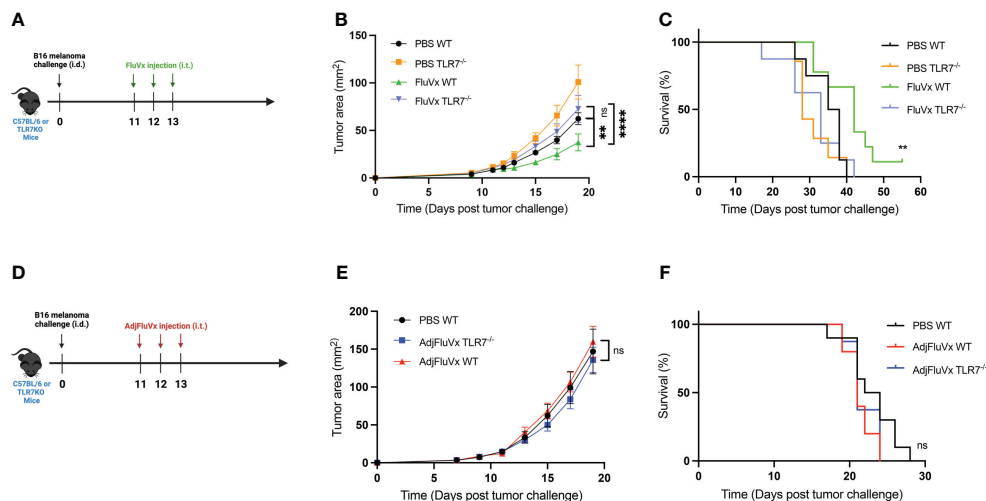


FIGURE 2

Reduction in tumor growth and improved survival from intratumoral administration of seasonal influenza vaccine is mediated by TLR7.

(A) Experimental design. n = 8–10 mice per group. (B) Tumor growth curves of experiment described in (A). (C) Survival curves from experiment described in (A). (D) Experimental design. n = 10 mice per group. (E) Tumor growth curves of experiment described in (D) n = 10 mice per group.

(F) Survival curves from experiment described in (D) Data are representative of at least two independent experiments. ns, not significant, \*\* $P < 0.01$ , \*\*\*\*  $P \leq 0.0001$ ; 2-way ANOVA with Tukey correction, Mantel-Cox log-rank test. Values represent mean  $\pm$  S.E.M.

## Discussion

TLR activation emerges as a critical determinant in the complex landscape of the TME, with the dualistic ability to either promote or restrict tumor progression. Given the disparities in responses reported after intratumoral delivery of FluVx and AdjFluVx, as well as influenza's inherent TLR activation potential, research into the *in vivo* influence of TLRs on these effects became critical.

Our research found that FluVx and AdjFluVx had distinct TLR activation profiles, with no overlap in TLR activation between the two vaccine formulations. TLR4 and TLR9 were shown to be the most responsive to the adjuvanted vaccine, AdjFluVx. Previous research has linked TLR4 and TLR9 activation to cancer growth in a variety of situations (30–32). As a result, our previously documented maintenance of tumor development following AdjFluVx treatment could be attributed to TLR4 and TLR9 activation, supporting an immunosuppressed tumor microenvironment.

FluVx, on the other hand, was found to activate TLR3 and TLR7. These TLRs have gotten a lot of interest because of their therapeutic potential in cancer (33, 34). Notably, the TLR7 agonist imiquimod is used to treat basal cell carcinoma and encouraging results have been observed by simultaneously targeting TLR3 and TLR7. TLR3 and TLR7 identification of viral-based PAMPs inside the tumor microenvironment is thought to initiate antitumor immunity following intratumoral FluVx therapy.

Following the discovery that seasonal influenza vaccinations activate TLRs *in vitro*, we focused on establishing TLR7's role in B16 melanoma tumor development after therapy. Using a TLR7-deficient mouse model, we observed that TLR7 deficiency promotes tumor growth and decreases survival rates. However, intratumoral FluVx treatment reduced tumor development and increased survival rates. This attenuation is most likely owing to a

lack of downstream signaling caused by the interaction of FluVx and TLR7.

Concurrently, the lack of observable effects on tumor progression following intratumoral injection of AdjFluVx in TLR7-deficient animals highlights the insignificant role of TLR7 activation in influenza vaccines containing a squalene oil-in-water adjuvant.

FluVx and AdjFluVx were found to affect the expression of certain TLRs inside the tumor microenvironment, supporting the idea that TLR stimulatory ligands can upregulate target TLRs. The elevation of TLR4 and TLR9 expression on regulatory B cells following AdjFluVx therapy was particularly notable, matching with previous findings that TLR4 and TLR9 activation stimulates the release of immunosuppressive cytokines such as IL-10 from immune cells, including regulatory B cells (28). Consistent with our previous findings that IL-10 blockade allows the adjuvanted influenza vaccine to suppress tumor growth, these findings suggest that AdjFluVx treatment upregulates and activates TLR4 and TLR9 on regulatory B cells in a feed-forward manner, likely contributing to sustained IL-10 production and impeding any antitumor response (35).

TLR7 expression on regulatory B cells within the tumor was also reduced following AdjFluVx therapy. TLR7 activation, despite being expressed on regulatory B cells, has been identified as a negative regulator for these cells (1), decreasing IL-10 production and reducing splenic regulatory B cell numbers (29). TLR7 downregulation by AdjFluVx may contribute to the maintenance of regulatory B cell populations and IL-10 production within the tumor, encouraging tumor growth (30).

Furthermore, the elevation of TLR7 expression on tumor dendritic cells following FluVx therapy is significant. Given that FluVx treatment increases dendritic cell populations within the tumor (18), this finding suggests a possible mechanism in which



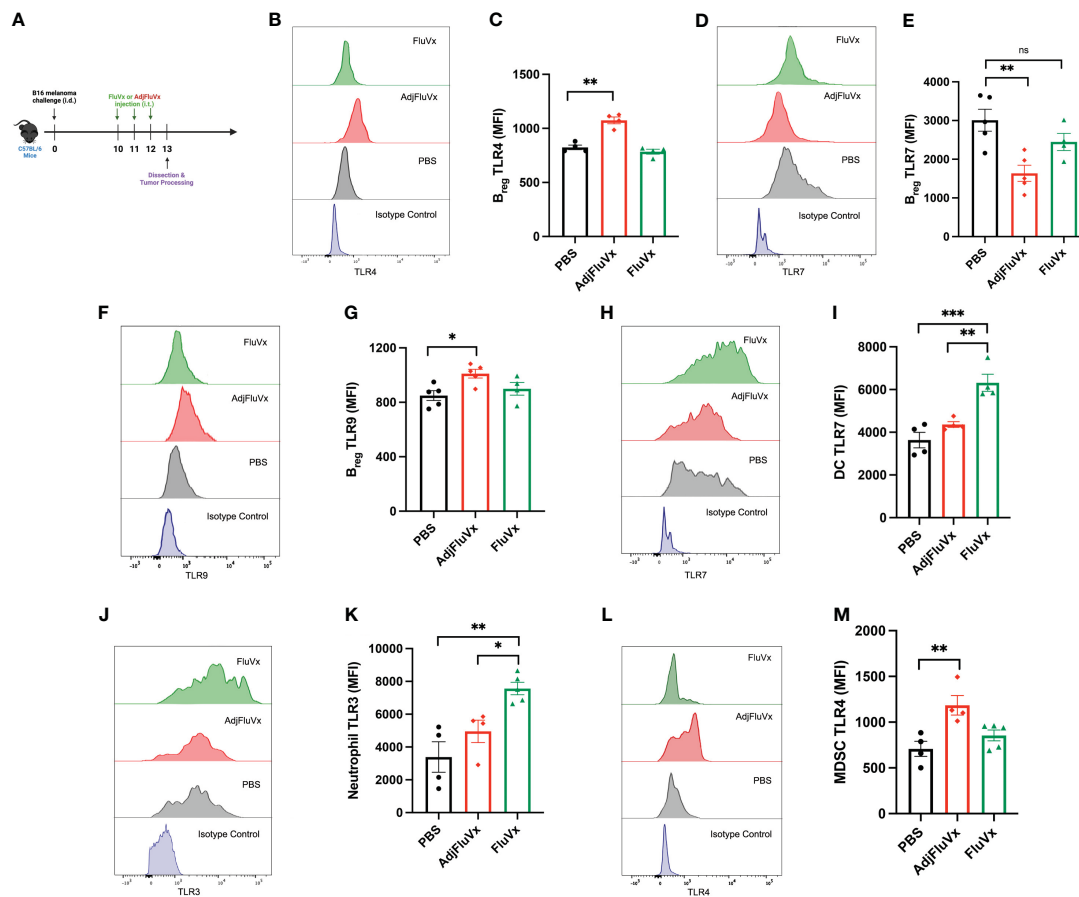


FIGURE 3

Intratumoral injection of unadjuvanted and adjuvanted seasonal influenza vaccines modulates TLR expression within the tumor microenvironment. TLR expression was measured by mean fluorescence intensity (MFI). (A) Experimental design.  $n = 3-5$  tumors per group. (B) Representative flow cytometry histogram of TLR4 expression on regulatory B cells ( $B_{regs}$  (CD45+ CD20+ IL-10+)) each treatment group. (C) TLR4 expression from intratumoral  $B_{regs}$  following experimental conditions described in (A). (D) Representative flow cytometry histogram of TLR7 expression on  $B_{regs}$  from each treatment group. (E) TLR7 expression on intratumoral  $B_{regs}$  following experimental conditions described in (A). (F) Representative flow cytometry histogram of TLR9 expression on  $B_{regs}$  from each treatment group. (G) TLR9 expression on intratumoral  $B_{regs}$  following experimental conditions described in (A). (H) Representative flow cytometry histogram of TLR7 expression on dendritic cells (DCs), DCs were defined as (CD45+ CD11c+ MHCII+). (I) TLR7 expression on intratumoral DCs following experimental conditions described in (A). (J) Representative flow cytometry histogram of TLR3 expression on neutrophils, neutrophils were defined (CD45+ CD11b+ Ly6G/Gr-1+ F4/80-). (K) TLR3 expression on intratumoral neutrophils following experimental conditions described in (A). (L) Representative flow cytometry histogram of TLR4 on myeloid-derived suppressor cells (MDSCs), MDSCs were defined as (CD45+ CD11b+ Ly6G+ Ly6G/Gr-1- F4/80+). (M) TLR4 expression on intratumoral MDSCs following experimental conditions described in (A). ns, not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; 1-way ANOVA with Tukey's multiple comparisons test. Values represent mean  $\pm$  S.E.M.

FluVx upregulates and activates TLR7 signaling on dendritic cells, subsequently activating downstream targets such as NF- $\kappa$ B or MAPK/ERK pathways that regulate cell proliferation. TLR7 signaling may also activate dendritic cells, boosting tumor antigen presentation and promoting tumor-targeting CD8+ T cell responses. TLRs are expressed on both immune and tumor cells in the TME and play a dual role, triggering both anti-tumor (innate and adaptive immunity) and pro-tumor (cell proliferation, migration, invasion, and cancer stem cell maintenance), and have been linked to a variety of cancers including glioblastoma (31), breast cancer, melanoma, and brain tumors (32). TLR7 has been targeted as a potential therapeutic for hepatocellular carcinoma (33).

In conclusion, our data highlight the multifaceted and dualistic role of TLRs in cancer. Intratumoral FluVx therapy reveals the anticancer potential of TLR signaling, with TLR7

identified as a factor to tumor progression reduction. TLRs activated by AdjFluVx, on the other hand, increase the generation of immunosuppressive cytokines, sustaining an environment favorable to tumor growth. The minimal adverse effects and strong safety profile of FluVx established by the FDA (34) in combination with these findings giving light to intriguing immunotherapeutic targets, position FluVx as a promising and well-tolerated cancer immunotherapy.

## 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.

## Ethics statement

All the experiments were conducted in accordance with procedures approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee at Rush University Medical Center.

## Author contributions

KG: Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. EG: Formal analysis, Investigation, Methodology, Writing – review & editing. AZ: Funding acquisition, Resources, Writing – review & editing.

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## Conflict 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.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2024.1308651/full#supplementary-material>

### SUPPLEMENTARY FIGURE 1

Squalene-based adjuvant found in adjuvanted influenza vaccine stimulates TLR2, TLR4, TLR5, TLR8, and TLR9. (A) Activation of a murine TLR reporter panel and parental, non-TLR transfected Null cell lines with squalene oil-in-water adjuvant (Adj). (B) Detection of baseline activation of murine TLR reporter panel and Null cell lines with PBS control. Data are representative of three independent experiments run in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.001$ ; two-way ANOVA with Sidak's correction for multiple comparisons. Values represent mean  $\pm$  S.E.M.

### SUPPLEMENTARY FIGURE 2

The addition of squalene oil-in-water adjuvant to unadjuvanted seasonal influenza vaccine diminishes intrinsic TLR stimulation and promotes activation of other TLRs. Ratio of TLR reporter cell stimulation to parental Null cell stimulation when treated with unadjuvanted influenza vaccine (FluVx) or adjuvant (Adj) added to unadjuvanted influenza vaccine (FluVx + Adj). Data are representative of three independent experiments run in triplicate.

### SUPPLEMENTARY FIGURE 3

Flow cytometry gating strategy of intratumoral immune cell populations. (A) Flow cytometry gating of regulatory B cells (Bregs). Bregs were defined as CD45<sup>+</sup> CD20<sup>+</sup> CD3<sup>-</sup> IL-10<sup>+</sup>. (B) Flow cytometry gating of dendritic cells (DCs). DCs were defined as CD45<sup>+</sup> CD11c<sup>+</sup> MHCII<sup>+</sup>. (C) Flow cytometry gating of myeloid derived suppressor cells (MDSCs). MDSCs were defined as CD45<sup>+</sup> CD11b<sup>+</sup> Ly6C<sup>+</sup> Ly6G<sup>-</sup>. (D) Flow cytometry gating of neutrophils. Neutrophils were defined as CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>+</sup> F4/80<sup>-</sup>. (E) CD45 isotype control. (F) CD45<sup>+</sup> cells stained with CD20 or CD3 isotype control. (G) CD45<sup>+</sup> cells stained with CD3 or CD20 isotype control. (H) CD45<sup>+</sup> CD20<sup>+</sup> cells stained with IL-10 isotype control. (I) CD45<sup>+</sup> MHCII<sup>+</sup> cells stained with CD11c isotype control. (J) CD45<sup>+</sup> cells stained with MHCII isotype control. (K) CD45<sup>+</sup> cells stained with F4/80 isotype control. (L) CD45<sup>+</sup> cells stained with Ly6G isotype control. (M) CD45<sup>+</sup> cells stained with CD11b isotype control. (N) CD45<sup>+</sup> cells stained with Ly6C isotype control.

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