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Muna ALJIELI

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Toxoplasma gondii et *Neospora caninum* comme plateformes d'expression de fragments d'anticorps thérapeutiques contre la voie PD-1/PD-L1 : une nouvelle stratégie pour l'immunothérapie du cancer

THÈSE dirigée par :

Mme MEVELEC Marie-Noëlle	CR, HDR, Université de Tours
Mme DI TOMMASO Anne	IGR, HDR, Université de Tours
Mr MOHAMMED AHMED Elhadi	PU, Université de Gezira

RAPPORTEURS :

BEAUVILLAIN Céline	MCU-PH, HDR, Université d'Angers
BOISGERAULT Nicolas	CR, INSERM, HDR, Université de Nantes

JURY :

président du jury : Mr JOUBERT Nicolas	PU, HDR, Université de Tours
Mme BEAUVILLAIN Céline	MCU-PH, HDR, Université d'Angers
Mr BOISGERAULT Nicolas	CR, INSERM, HDR, Université de Nantes
Mme LABARRIERE Nathalie	DR, INSERM, HDR, Université de Nantes
Mme DI TOMMASO Anne	IGR, HDR, Université de Tours
Mr MOHAMMED AHMED Elhadi	PU, Université de Gezira

MEMBRE INVITE :

Mme MEVELEC Marie-Noëlle	CR, HDR, Université de Tours

To the soul of my mom, the greatest love of my life

To the soul of my dad, my hero

may they be in peace …

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Résumé

L'immunothérapie anticancéreuse est un domaine en plein essor qui cherche à exploiter le système immunitaire pour mieux combattre les tumeurs. Bien que diverses stratégies aient été explorées, les inhibiteurs de point de contrôle immunitaire (ICI), notamment ceux ciblant la voie PD-1/PD-L1, ont été une remarquable avancée. Toutefois, l'amélioration des patients dits « répondeurs » à ce type de thérapie et l'atténuation des effets secondaires induits sont des préoccupations qui subsistent, et pour lesquelles il est nécessaire de proposer de nouvelles alternatives thérapeutiques. L'utilisation de micro-organismes vivants, tels que les virus et les bactéries, a suscité un intérêt tout particulier du fait de leurs propriétés oncolytiques et de leurs effets immunostimulants. Alors que les virus et les bactéries ont fait l'objet de nombreuses études et certaines applications cliniques, les parasites ont reçu moins d'attention, nonobstant les preuves prometteuses de leur efficacité à induire des réponses immunitaires et à protéger des tumeurs. Parmi ceux-ci, *Toxoplasma gondii* et *Neospora caninum* ont montré un fort potentiel antitumoral, malgré la subsistance de certaines limites liées à la spécificité, au ciblage des tumeurs ou encore à l'optimisation de l'efficacité. Dans cette étude, nous avons exploré la possibilité de combiner une immunothérapie fondée sur l'utilisation de ces protozoaires avec des ICI, plus spécifiquement des protozoaires capables i) soit d'exprimer un anti-PD-L1 à leur surface afin de favoriser un ciblage antitumoral spécifique, ii) soit d'exprimer et de sécréter un anti-PD-L1 directement au site tumoral. La construction d'un fragment d'anticorps de type scFv anti-PD-L1 a été réalisée à partir des séquences de l'anti-PD-L1 humain l'Atezolizumab, et fusionné à la protéine membranaire SAG1 de *T. gondii* SAG1. Cet ancrage du scFv, à la membrane de *T. gondii*, a médié un ciblage spécifique du protozoaire aux cellules PD-L1+ (avec un taux de 93%) tout en diminuant drastiquement les liaisons aux cellules PD-L1-. Par ce ciblage, cette souche recombinante de *T. gondii* a démontré une meilleure proportion de cellules tumorales lysées en comparaison à la souche parentale. De plus, des essais de co-culture ont mis en exergue la capacité de cette souche recombinante à inhiber l'interaction PD-1/PD-L1 et à améliorer les réponses immunitaires médiées par les cellules T CD8+. Nous avons ensuite essayé de faire exprimer à la surface de *N. caninum* l'anti-PD-L1 sous format scFv, selon une stratégie d'ingénierie similaire à celle de *T. gondii*, ou encore de faire sécréter par *N. caninum* le scFv anti-PD-L1 via la voie sécrétoire des micronèmes. Si les nombreuses tentatives d'obtenir une souche de *N. caninum* capable d'exprimer à sa surface l'anti-PD-L1 se sont révélées infructueuses, l'expression et la sécrétion de ce fragment d'anticorps ont été un vrai

succès avec la mise en évidence *in vitro* d'un scFv anti-PD-L1 capable à la fois de cibler les cellules tumorales humaines et murines PD-L1+, mais aussi de bloquer l'interaction PD-1/PD-L1 et de favoriser l'activité des lymphocytes T CD8+.

L'ensemble de ces résultats ouvre la voie sur deux domaines thérapeutiques intéressants et appliqués pour la première fois à des protozoaires dans un contexte tumoral : le ciblage de *T. gondii* à la tumeur via l'ingénierie des fragments d'anticorps, et la vectorisation par *N. caninum* de fragments d'anticorps fonctionnels laissant suggérer de potentiels effets antitumoraux synergiques.

Abstract

Cancer immunotherapy is a rapidly evolving field seeking to harness the immune system to combat tumors. While various strategies have been explored, immune checkpoint inhibitors (ICIs) targeting the PD-1/PD-L1 pathway have been a pivotal advancement. Nonetheless, enhancing patient responses and minimizing toxicity remain priorities, driving the investigation of novel approaches. The use of live microorganisms, such as viruses and bacteria, has garnered attention due to their oncolytic properties and immunostimulatory effects. While viruses and bacteria have been extensively studied, parasites have received less attention despite promising evidence of their efficacy in inducing immune responses and tumor protection. Notably, the closely related intracellular parasites like *Toxoplasma gondii* and *Neospora caninum* have shown potential in cancer immunotherapy, though further research is warranted to address concerns regarding specificity, tumor targeting, and efficacy optimization.

In this study, we explored the prospect of enhancing and combining parasite-based immunotherapy with ICIs by engineering anti-PD-L1 i) to be displayed on parasite's surface to enhance tumor-specific targeting and (ii) to be secreted by the engineered parasites to allow direct delivery and secretion into tumor site.

The anti-PD-L1 construct was designed using the scFv region of the human anti-PD-L1 antibody Atezolizumab, fused with the membrane protein *T. gondii* SAG1 (TgSAG1) for membrane anchoring. The surface display of anti-PD-L1 on *T. gondii*, mediated its specific binding to targeted cells and markedly minimized off-target binding and retained tachyzoites on PD-L1 expressing cells (by 93%). The engineered parasite exhibited increased killing ability compared to the parental strain. Furthermore, co-culture assays revealed the ability of the anti-PD-L1-displaying parasites to inhibit PD-1/PD-L1 interaction and enhance CD8+ T cell-mediated immune responses.

We then attempted at expressing anti-PD-L1 scFv on *N. caninum* attached to the outer membrane using TgSAG1 protein or targeted to micronemes secretory pathway. Efforts to express anti-PD-L1 on the surface of *N. caninum* were unsuccessful; however, targeting the antibody fragment to the micronemes resulted in secretion of functional anti-PD-L1 scFv that is capable to bind PD-L1-expressing human and murine tumor cell lines. The secreted anti-PD-L1 scFv effectively blocked PD-1/PD-L1 interaction and promoted CD8+ T cell functionality in vitro.

These findings underscore the potential of engineering antibody fragments onto parasite surfaces as a promising strategy for targeted tumor therapy. Additionally, vectorizing *N. caninum* to secrete functional antibody fragments presents an avenue for synergistic anti-tumor effects.

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1. Introduction

1. Introduction

I. Cancer

1. General

Cancer- also known as malignant tumors or neoplasms- is a serious health problem characterized by normal cells transformed into abnormal cells which proliferate in unregulated manner (Mathur *et al*., 2015). These abnormal cells can invade adjoining tissues and organs and spread through blood and lymphatic vessels to form other tumors, a process which is referred to as metastasis. Metastasis is the major cause of cancer's morbidity and mortality and it accounts for around 90% of cancer-associated death (Guan, 2015).

2. Epidemiology

Globally, cancer is the second leading cause of death. In the year 2018, the World Health Organization (WHO) estimated 18.1 million new cancer cases around the world (Figure 1). Among this, 9.6 million died from the disease (representing 1 in 6 deaths) with 4.5 million were classified as premature deaths "death between ages 30 and 70 years". By the year 2040, these figures are expected to increase to nearly the double, with the greatest impact in low and middle-income countries (LMIC) (WHO Report on Cancer: Setting Priorities, Investing Wisely and Providing Care for All, 2020).

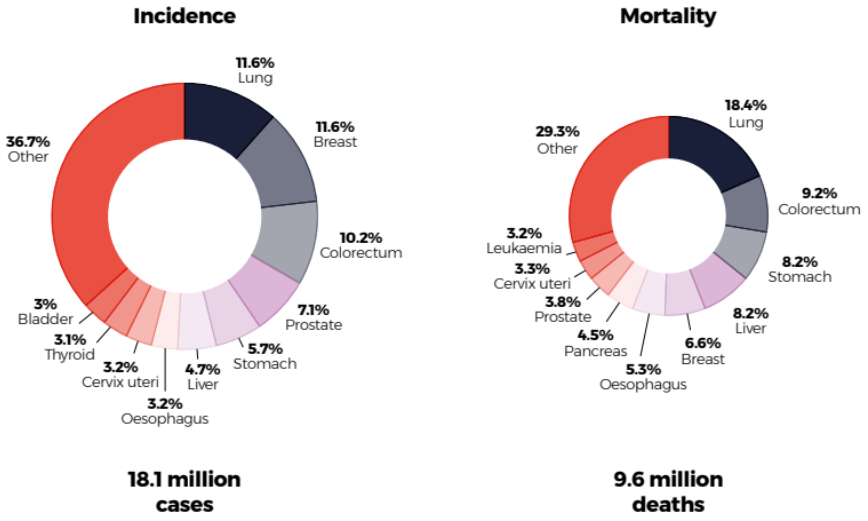


Figure 1. Distribution of cases and deaths by the leading 10 cancer types in 2018 for both sexes (WHO, 2020).

3. Risk Factors

The past decades witnessed enormous progress in the understanding of cancer etiology and extensive efforts have been directed on identifying cancer-associated risk factors. These risk factors have been classified based on their biological nature, modifiability and use in literature into intrinsic "non-modifiable" and non-intrinsic "modifiable" risk factors (Wu et al., 2018a). Two recent modelling studies suggested that considerable cancer burden might be due to unmodifiable intrinsic risk related to cell biology (Tomasetti & Vogelstein, 2015a). These intrinsic risks arise as a result of random errors in DNA replication which leads to unavoidable mutations, hence this risk is referred to as non-modifiable risk (Wu et al., 2018b). On the other hand, several environmental risk factors, referred to non-intrinsic, have been conclusively related to the etiology of various malignancies. These include factors that are related to exogenous exposure such as infectious agent, UV radiation, and drugs as well as lifestyle factors including tobacco, obesity, alcohol consumption and hormonal therapy. Additionally, many endogenous factors such as inflammation, hormones and growth factors, metabolic effects, reactive oxygen species, immune responses also contribute to tumors etiology. The non-intrinsic risk contributes in various percentages to the incidence of different type of cancers (reviewed in Arem & Loftfield, 2018; Stein & Colditz, 2004; Wu et al., 2018).

4. Cancer Immunoediting

The past few decades witnessed a great understanding in the role of the immune system in tumor dynamics. The hypothesis that host defense may prevent neoplastic cells from developing into tumors providing the cornerstone for the "cancer immunosurveillance" (Burnet, 1970; Dunn et al., 2002). The core of the cancer immunosurveillance hypothesis is that cancer cells are immunologically distinguishable from normal cells due to the expression of tumor specific-neoantigens which allow the immune system to identify them as non-self-antigens, therefore eliminating the established tumor (Burnet, 1964.; Finn, 2018). However, latent years uncovered the facts that immune system can also functions to promote or select tumor variants with reduced immunogenicity, thereby providing developing tumors with a mechanism to escape immunologic detection and elimination (Gubin & Vesely, 2022). Thus, the "immunoediting" concept emerged as a refinement of cancer immunosurveillance which in its most complex embodiment, proceeds sequentially through three distinct phases that were termed "elimination," "equilibrium," and "escape" which emerged as the three Es (Figure 2) (Shankaran, 2001).

a- Elimination phase

During the elimination phase, immune cells locate, identify, and destroy newly transformed cells and prevent the development of malignancy (Figure 2, lower part, left panel). This notion was confirmed by various research groups utilizing several immunocompetent versus immunocompromised mice models. For instance, it was shown that mice deficient in either adaptive immunity [RAG2−/− mice lacking T cells, B cells, and natural killer T (NKT) cells] or interferon gamma (IFN-γ) responsiveness (for example, gene-targeted mice lacking the IFN-γ receptor) were more prone to carcinogen-induced and spontaneous primary tumor formation. Similar results from different studies were reported, and collectively these findings demonstrated that the immune system can function as an extrinsic tumor suppressor (reviewed in Vesely et al., 2011).

During the first stages of growth, the proliferation of the transformed cells is associated with remodeling of the surrounding tissues together with angiogenesis and vascular proliferation. Both mechanisms contribute to the recruitment of innate immune cells (NKT, NK, macrophage, and dendritic cells) through the production of pro-inflammatory molecules and increased permeability of blood vessels, respectively (Liu & Zeng, 2012). In addition, the hypoxic environment in the tumor microenvironment (TME) induces cell apoptosis and necrosis, which is associated with release of Damage-Associated Molecular Pattern (DAMP) molecules. The later act as ligands for innate immune cells' toll-like receptors to recruit more immune cells. The recruited immune cells initiate an anti-tumor immune response featured by high production of interleukin (IL)-12 and IFN-γ. IFN-γ, in addition to its direct anti-proliferative effect via *Stat1* pathway, it also induces cytokine production such as CXCL9, CXCL10, and CXCL11, which in turn promote further immune activation and limit angiogenesis (Schreiber et al., 2011; Teng et al., 2013). The production of IFN-γ, particularly by NK cells, induces the activation of macrophages which contributes to the elimination of tumor cells through production of reactive oxygen species (ROS), nitric oxide (NO), granzymes and perforin-dependent digestion. Moreover, IFN-γ production lead to activation of signaling cascades to a pro-apoptotic, anti-proliferative and anti-angiogenic effects within the developing tumor (Castro et al., 2018). The tumor cells apoptosis mediated by the inhibition of angiogenesis and tumor growth results in generation of cellular debris and antigenic peptides that are ingested by host's dendritic cells (DCs). Subsequently, DCs migrate to draining lymph nodes to induce adaptive immune cells including CD4+ T cells and CD8+ effector T cells against further tumor growth.

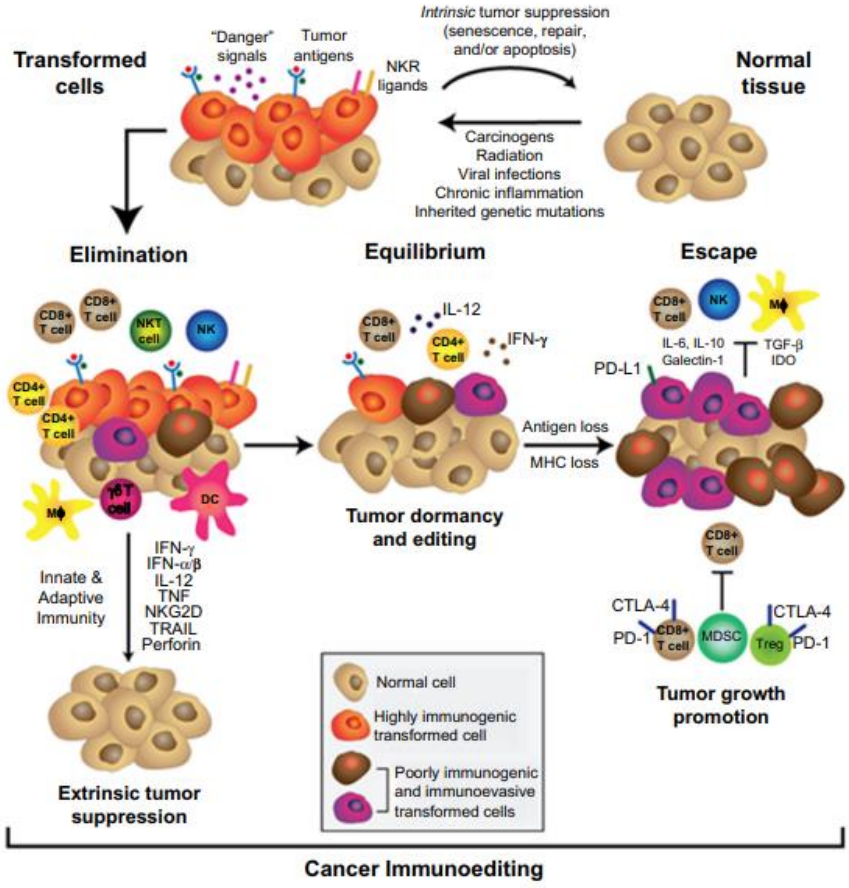


Figure 2. The cancer immunoediting process (the three Es phases). **(Upper part)** shows transformation from normal to transformed cell. **(Lower part, left panel)** the elimination phase, innate and adaptive immune components collaborate to eradicate growing tumors long before they become clinically apparent. **(Lower part, middle panel)** the equilibrium phase, T cells, IL-12, and IFN- γ are required to maintain tumor cells in an immune-mediated dormant state. Neither NK cells or molecules that participate in the recognition or effector function are required in this phase suggesting that equilibrium is a function of only adaptive immunity. **(Lower part, right panel)** the escape phase, the constant immune selection process may result in emergence of tumor variants that are (i) no longer recognized by immune cells, (ii) insensitive to immune effector mechanisms, (iii) or induce immunosuppressive status within the TME. These tumor cells may then enter the escape phase, in which their outgrowth is no longer blocked by immunity giving raise to the clinical manifestation of cancer (Teng et al., 2013).

Upon arrival to the tumor's site, local IFN-γ stimulates these migrated CD8+ T cells to specifically recognize and target tumor cells bearing an immunogenic antigen (reviewed in Desai et al., 2022). Tumor cells recognition by CD8+ cytotoxic lymphocytes (CTLs) is -probably- through the expression of the major histocompatibility (MHC) class I. Upon detecting MHC I, CTLs can induce cell lysis via perforin- and granzyme-dependent mechanisms.

Moreover, activated CD4+ T-helper cells work concomitantly to promote the proliferation of B cells into antibody secreting plasma cells which in turn participate in the anti-tumor immunity through complement- mediated lysis or by antibody-dependent cellular cytotoxicity (ADCC). Moreover, the secretion of IL-4, tumor necrosis factor (TNF), granulocyte- macrophage colony-stimulating factor (GM-CSF) and INF-γ by CD4+ T-helper cells is also essential in activating macrophages. Macrophages either phagocytize and eliminate tumor cells or function as antigen presenting cells (APCs) to represent tumor antigens to CD4+ T cells and dendritic cells (reviewed in Bhatia & Kumar, 2015). In the elimination phase of cancer immunoediting, tumor cells are eliminated faster by the host defense than the tumor can proliferate. Thus, tumor growth is halted and tumor progression to later phases is prevented by effective immune control during this phase.

b- Equilibrium

Cancer is capable of growing despite the integrated innate and adaptive immune responses initiated in the elimination phase, giving rise to a second phase named equilibrium phase (Figure 2, lower part, middle panel). In this process, as described by (Dunn et al., 2002), the host immune system and tumor cells enter a dynamic balance that is characterized by the co-existence of tumor cells and immune response without one of them dominating over the other. This balance can be owed to the several mutations that formed during the unregulated tumor growth which enhance tumor cell's ability to survive or circumvent tumor suppressor genes (reviewed in Bhatia & Kumar, 2015).

c- Escape

When tumors acquire the ability to avoid immune recognition and/or destruction, they enter into the escape phase and emerge as progressive visible tumors (Figure 2, lower part, right panel). Tumor outgrowth is facilitated by changes that either increase resistance to the cytotoxic effects of immunity (e.g., by inducing anti-apoptotic mechanisms involving persistent activation of pro-oncogenic transcription factors like STAT3 or expression of anti-apoptotic effector molecules like BCL-2) or reduce immune recognition (e.g., loss of antigens) (Khong & Restifo, 2002; Mittal et al., 2014). One of the most studied tumor escape mechanisms is evading the immune system through the loss of MHC I expression which makes it more difficult for CTLs to identify and eradicate malignancies (Dhatchinamoorthy et al., 2021).

Alternatively, immune evasion may evolve because of the establishment of an immunosuppressive tumor microenvironment. Tumor cells can promote the emergence of such a state through the production of immunosuppressive cytokines such as vascular endothelial

growth factor (VEGF), transforming growth factor-β (TGF-β), or galectin (Vesely et al., 2011). Moreover, tumor cells can recruit regulatory immune cells, which contribute to the suppression of anti-cancer immunity. Regulatory T cells (Treg cells) and myeloid-derived suppressor cells (MDSCs) are the two major key players in the inhibition of host-protective antitumor responses. Activated Treg induce immunosuppression status through the secretion of the immunosuppressive cytokines, IL-10 and TGF- β, expression of negative co-stimulatory molecules or depletion of IL-2 that is critical for CTLs functionality. MDSCs are a heterogeneous group of immature myeloid and myeloid progenitor cells. These cells suppress the activity of lymphocytes through a variety of mechanisms, including the induction of Treg cells, the production of TGF-β, the depletion or sequestration of amino acids necessary for T cells function (Vignali et al., 2008; Vinay et al., 2015).

Furthermore, one of the well-studied mechanism of immunosuppression is the expression of the negative co-stimulatory immune checkpoints such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death ligand 1 (PD-L1). They contribute to T cells suppression through binding to their respective receptors on T cells resulting in inhibition of both T cells proliferation and IL-2 secretion (H. Zhang et al., 2021).

5. Cancer Treatment Modalities: conventional therapy

Because the metabolic processes of cancer cells and host cells functionally overlap, it was difficult to develop treatments that primarily target malignant cells while sparing non-cancerous ones (Anand et al., 2023). With the growing knowledge and understanding of pathways and characteristics of different cancer entities, several cancer treatment modalities emerged. The conventional treatment modalities are relying on targeting tumors with surgery, radiotherapy and/or chemotherapy. Surgery and radiotherapy are considered standard treatments of accessible and localized solid tumors (Barazzuol et al., 2020; Zenga et al., 2018). Chemotherapy which describes the concept of using toxic chemical compounds to treat diseases has emerged in the twentieth century as a result of the increased understanding of the biochemistry and cellular metabolism of tumor cells. Chemotherapies comprise several groups of cytotoxic drugs which all aim at destroying cancerous cells through competing with macromolecule nutrients (such as folates) and altering the metabolic pathways or interfering with the DNA formation, thus interrupting DNA synthesis (Anand et al., 2023). Chemotherapy and radiotherapy are associated with severe side effects mainly due to the lack of specificity of the cytotoxic effect (Barazzuol et al., 2020; Nurgali et al., 2018). Recently, more novel

modalities in cancer treatment have been introduced including hormone therapy, and targeted therapy (Debela et al., 2021).

a- Hormonal therapy

Hormonal therapy is mainly applied in breast, ovarian and prostate cancers were hormones are believed to play important role in some of these cancers' development such as estrogens for certain breast cancers (Yue et al., 2010) and testosterone for certain prostate cancers (Michaud et al., 2015). Thus, the principle of hormonal therapies is to block or reduce the production and/or action of these hormones. Tamoxifen was the first FDA approved (in 1998) hormonal therapy for breast cancer (Buzdar & Hortobagyi, 1998), but the new gold standard treatment for estrogen receptors -positive breast cancer are the aromatase inhibitors, a group of drugs which block the enzyme aromatase responsible of estrogen synthesis (Drăgănescu & Carmocan, 2017). Currently, several hormonal therapies are approved, however, resistance is highly reported in all patients with metastatic breast and prostate cancer resulting in disease progression. Several and complex mechanisms can be attributed to developing resistance including selection of new resistant variants, increased sensitivity of hormone receptors and/or up-regulated downstream survival pathways (Abraham & Staffurth, 2016).

b- Targeted therapy

The concept of "targeted therapy" refers to group of therapies designed to interfere with a specific target protein (specific enzymes, growth factor receptors, and signal transducers) that is believed to have a critical role in tumor growth or progression (Tomasetti & Vogelstein, 2015). There are several different direct and indirect methods that are included in targeted therapy. Direct strategies encompass monoclonal antibodies or small-molecule medications that interfere with these target proteins by altering their signaling. Indirect strategies depend on tumor antigens expressed on the cell surface by antibody coupled to other effector molecule (cytotoxic, chemotherapeutic or radioactive molecules) (Wu et al., 2006). The key to this therapy's effectiveness is to minimize the off-target adverse effects to healthy tissues while targeting the release of therapeutic molecules to the tumor site (Padma, 2015).

One of the most studied targets are the protein kinases particularly, tyrosine kinases which are important mediators in intracellular signaling for cell division. Dysregulated increased tyrosine kinase activity has been linked to the development of numerous cancers (Knight & Shokat, 2005). Therefore, this enzyme family has become one of the most important drug targets and in 2001, the first selective tyrosine kinase inhibitor, Imitanib mesylate was approved for the

treatment of chronic myeloid leukemia (Capdeville et al., 2002). However, many patients rapidly developed high resistance following treatment with Imitanib mesylate which limited this efficacy. Nonetheless, the concept was still interesting for many researchers to investigate other tyrosine kinases as targets. These efforts led to launching of Gefitinib (in 2003), a selective oral epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that increases tumor cell apoptosis (Hartmann et al., 2009). Currently, more than 50 tyrosine kinase inhibitors are licensed for treatment solid tumors and nonsolid tumors (Roskoski, 2023).

The indirect approach of target therapy is based on antibody-drug conjugates (ADC) (Lambert & Berkenblit, 2017). The first ADC approved by the FDA was Gemtuzumab ozogamicin which consists of a semisynthetic derivative of calicheamicin (a cytotoxic antibiotic) linked to a recombinant monoclonal antibody directed against the CD33 antigen. This ADC was approved for treatment of acute myeloid leukemia (AML) in the year 2000 (Bross et al., 2001). However, it was recalled from the market due to the lack of efficacy and higher incidence of hepatotoxicity observed in a phase 3 clinical trial (Lö et al., 2010). Several other studies were conducted to optimize the doses and schedules using lower and fractionated doses. In 2017, Gemtuzumab ozogamicin was re-introduced for treatment of patients with $CD33^{+}$ AML. Other ADCs were developed and achieved remarked improvement in progression-free survival (PFS) compared to chemotherapy in various hematologic cancers. For instance, brentuximab vedotin is an ADC consisting of the cytotoxic molecule mono methyl auristatin E coupled to an antibody against CD30 (a surface marker that is extensively expressed by numerous lymphomas) received FDA approval in 2011 (Senter & Sievers, 2012).

ADCs were not exclusively designed for hematologic tumors, despite the notion that solid tumors are less amenable than “liquid” (hematologic) tumors to ADC approaches (Lambert & Berkenblit, 2017). Ado-trastuzumab emtansine (T-DM1), a conjugation of the maytansinoid DM1 to the antibody trastuzumab targeting human epidermal growth factor receptor-2, was the first ADC approved for solid tumors. Pivotal phase III trials in patients with HER2- positive breast cancer favored T-DMI arm over the comparators in term of overall response rate (ORR), overall survival (OS) and PFS (Carlson, 2016; Krop et al., 2014). Up to date, there are 14 ADCs have been launched and several are currently in clinical trials as monotherapy or combined with conventional chemotherapy (Z. Fu et al., 2022).

II. Cancer immunotherapy

Recently, advancements in understanding the complexity of the tumor microenvironment (TME) and the diverse roles of various immune cells have highlighted the TME and related immune components as potential targets for cancer therapy. This has ushered in a new era of cancer treatment known as cancer immunotherapy which has fundamentally changed the standard and concept of tumor treatment. Cancer immunotherapies manipulate the immune system to reactivate the antitumor immune responses and overcome the pathways leading to escape (C. Liu et al., 2022a). Currently, many immunotherapeutic approaches are being used including cytokines, monoclonal antibodies (mAbs), immune checkpoint inhibitors (ICIs), adoptive cell therapy, oncolytic viruses, and cancer vaccines (Figure 3) (Yang et al., 2022).

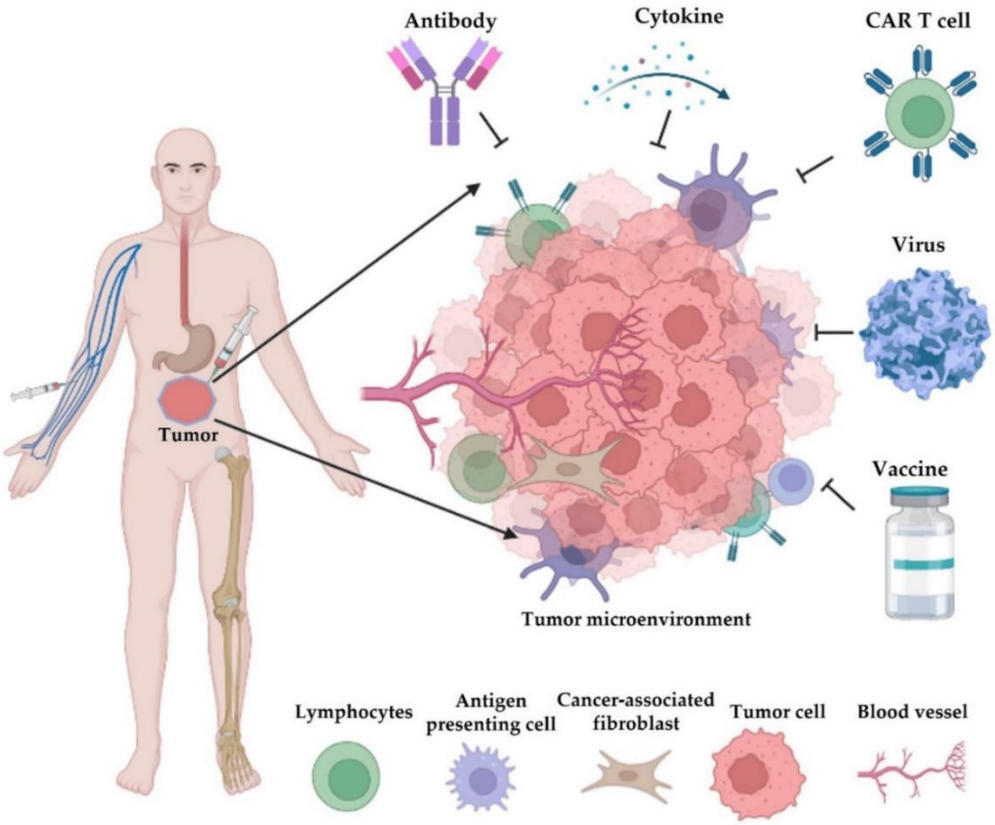


Figure 3. Different strategies of cancer immunotherapy. They mainly consist of antibodies blocking immune checkpoints, adoptive transfer of engineered cells (e.g., chimeric antigen receptor (CAR) T cells), cytokine therapy, infection of oncolytic viruses and cancer vaccines (Yang et al., 2022).

1. Cytokines

Cytokines are small polypeptides or glycoproteins with a molecular weight usually less than 30 kilo Dalton. They function to provide growth, differentiation, inflammation and non-inflammatory signals to different cell types (Berraondo et al., 2019). Several cytokines inhibit tumor cells growth directly by inhibiting proliferation or promoting apoptosis, or indirectly by inducing the cytotoxic activity of immune cells against tumor cells. Cytokines act on every phase of the cancer immunity cycle; improving antigen priming, increasing the number of effector immune cells in the TME and enhancing their cytolytic activity (Hamdan & Cerullo, 2023). Owing to this defined role in cancer immunity together with the advances in recombinant DNA technologies, several recombinant cytokines have been extensively studied in pre-clinical models for their potential as cancer immunotherapeutic agents. However, when implicated in clinical trials, the results did not live up to the expectations raised in the preclinical models. Various limitations are associated with cytokines-based therapy including: the short half-life of most of them and narrow therapeutic windows with only modest anti-tumor efficacy (Berraondo et al., 2019).

Thus, as a result of these limitations, up to date, only two cytokines, IL-2 and IFN-α, received the FDA approval for cancer treatment (Song, 2024a). IL-2 is a pluripotent cytokine that can activate the immune system in a variety of ways, nonetheless, it plays a vital role in the activation of T-cells and NK cells (Lehmann et al., 2011). A recombinant IL-2 has been approved by the FDA for the treatment of metastatic RCC (Fyfe et al., 1995) and metastatic melanoma (Wargo et al., 2015). IL-2 administration was associated with remarked increment in NK cells and T cells activation in most of the treated patients. However, the main negative effect of IL-2 is the stimulation of regulatory T cells (Tregs), which in contrast suppresses anticancer immunity. Furthermore, the systemic administration of IL-2 has been linked to several potentially fatal toxicities due to an increased inflammatory response (Pachella, et al., 2015). Currently, several clinical trials are ongoing to optimize IL-2-based therapy.

Type I IFNs including IFN-α and IFN-β play an essential role in the presentation of cancer antigens by mediating the maturation and activation of dendritic cells and inducing the expression of MHC I by tumor cells (Borden, 2019). IFN-α was the first cytokine to receive FDA approval for treatment of hairy cell leukemia in 1986 (Golomb et al., 1986). Following this approval, high doses of IFN-α has been used for treatment of several hematological malignancies and solid tumors including advanced follicular lymphoma (Solal-Celigny et al., 1993) and high-risk resected cutaneous melanoma (Kirkwood et al., 1996). In 2012, a

chemically modified variant of INF-α which has longer half-life was approved as an adjuvant therapy for melanoma (Herndon et al., 2012). Despite the successful application of IFN-α, the advent of targeted therapies and novel immunotherapies with superior safety and efficacy profiles has reduced the clinical use of IFN-α in onco-hematology.

Although only IL-2 and IFN-α were approved for clinical use, nonetheless, cytokines-based therapy is a promising immunotherapeutic approach that targets the TME through various means, including the regulation of the host immune response and activation of the immune system. However, several challenges should be overcome. To address these challenges, currently, several clinical trials are exploring cytokines-based anti-cancer immunotherapy either as monotherapy or in combination with other strategies (Figure 4). Additionally, through the integration of diverse technologies, such as the development of immune cytokines or fusion proteins, the anticancer efficacy of cytokines is increased, and side effects are minimized. These innovative approaches in cytokine anti-cancer immunotherapy hold the potential to successfully treat various blood cancers and solid tumors (Song, 2024b).

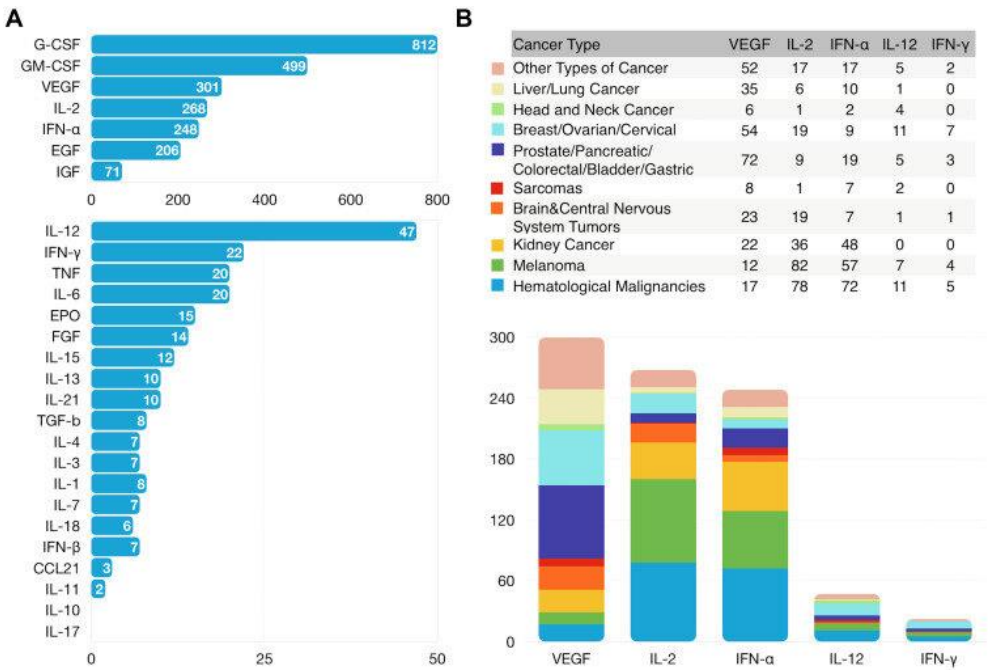


Figure 4. Clinical research status of cytokines. Number of clinical trials using cytokine-based therapy for treating **(A)** collectively for all cancer types or **(B)** classified according to each cancer type (Qiu et al., 2021).

2. Tumor-targeting monoclonal antibodies

The expression “tumor-targeting” refers to mAbs that specifically alter the signaling functions of receptors expressed on the surface of malignant cells, bind to, and neutralize, trophic signals produced by malignant cells or recognize tumor cells based on the expression of TAAs. Tumor targeting antibodies have functionally distinct variants: I) naked mAbs that inhibit signaling pathways required for tumor cell -but not normal cell- survival. The antibody directed against the epidermal growth factor receptor, Cetuximab has earned the FDA approval for the treatment of head and neck cancer (HNC) and colorectal carcinoma (CRC) (C. M. Lim et al., 2013; Rouge et al., 2007); II) naked mAbs that activate potentially fatal receptors present on the surface of cancer cells but not those of their non-transformed counterparts. Tigatuzumab, a mAb against tumor necrosis factor receptor superfamily 10B, is currently under clinical development (Forero-Torres et al., 2015); III) naked mAbs that alter tumor cells by binding to TAAs and subsequently trigger complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent cell-mediated cytotoxicity (ADCC). Rituximab, a CD20-specific mAb that is currently approved for the treatment of non-Hodgkin lymphoma and chronic lymphocytic leukemia (CLL) is an example for this group (Cavallo et al., 2007); IV) Bi-specific antibodies that contain two single chain fragment variables (scFv) one specific for TAA and the other is specific for T cell surface antigen, therefore called “bispecific T-cell engagers”. Among this group is blinatumomab, a CD19- and CD3 BiTE recently approved for the therapy of Philadelphia chromosome-negative precursor B-cell acute lymphoblastic leukemia (Hoffman & Gore, 2014; Walter, 2014).

Despite the fact that mAbs therapy has shown promise in the treatment of cancer, one major issue with these therapeutics is their persistent clinical resistance where the majority of patients develop refractory disease within a year. Resistance can be owed to the mutations of the antibody target and/or induction of alternative growth signaling pathways. Currently, treatment paradigms are shifting toward applying these mAbs in combination with other modalities providing encouraging avenues for maximizing the therapeutic benefits (Zahavi & Weiner, 2020).

3. Adoptive cell therapy (ACT)

Adoptive cell therapy refers to a variant of cell-based therapy that generally involves the injection of activated immune cells to patients (Rosenberg & Restifo, 2015). One form of ACT is the injection of tumor-infiltrating lymphocytes (TILs), which includes collection and *ex vivo*

expansion of TILs, then re-administration to patients most often after lymphodepleting pre-conditioning and in combination with immunostimulatory agents (Maus et al., 2014). Although the FDA has -recently- approved this application, numerous clinical investigations are still being conducted in order to optimize this therapy (Hamdan & Cerullo 2023).

Another approach to increase the armamentarium of T-cells is by genetically engineer them to express specific T cell receptors (TCRs) specific for tumor antigen. The first TCR T cell immunotherapy used in the clinic was tested against metastatic melanoma and utilized a TCR that bound a human lymphocyte antigen A2 (HLA-A2)–restricted peptide from a melanocytic differentiation antigen (Rosenberg & Restifo, 2015). Subsequently, a higher-avidity TCR targeting the MART-1 (melanoma antigen recognized by T cells 1) epitope was developed, with the aim of achieving enhanced recognition of malignant cells with lower MART-1 expression. Although an improved response rate was demonstrated, it came with a cost of also targeting normal melanocytes in the skin, eye, and cochlea (June et al.,2018). Other studies investigated the possibility of targeting cancer neo-antigens that are not expressed by normal cells. Currently, T cell engineered to express TCR against the cancer-testis antigen NY-ESO-1 is being evaluated in a clinical trial (NCT01343043).

Chimeric antigen receptor (CARs) is another strategy to optimize ACT to overcome some limitations of engineered TCRs, such as the need for MHC expression. A CAR combines antigen-binding domains (most commonly, a single-chain variable fragment (scFv) with signaling domains from the TCR chain and extra costimulatory domains from receptors such as CD28, OX40, and CD137. These types of synthetic receptor molecules enabled MHC independent target recognition by T cells. Since loss of MHC-associated antigen presentation by tumor cells is a significant mechanism of cancer immunoevasion, the CAR T cell's independence from MHC limitation confers a fundamental anticancer advantage (Garrido et al., 2016; Gross et al., 1989). Currently, six CAR-T cell treatments have been approved for clinical use including CAR-T cells targeting CD19 or BCMA for B-cell based malignancies or multiple myeloma, respectively.

4. Cancer vaccines

The concept of cancer vaccines relies on inducing immune response and protective immunological memory against cancer to prevent or treat cancer and preventing tumor relapse. Nevertheless, Cancer genomics, however, has demonstrated the difficulty in achieving this, as the majority of tumor antigens that are substantially expressed in tumors are also shared by healthy cells. Because of the potentially extremely toxic "off-target" consequences, this lack of

specificity is significant for patients (Blass & Ott, 2021). Despite these challenges, in 2010 FDA approved the first therapeutic cancer vaccine for asymptomatic or mildly symptomatic metastatic prostate cancer (Adis, 2011). This vaccine consists of DCs isolated from patient's PBMCs and expanded and activated *ex vivo* using a prostate cancer TAA named prostatic acid phosphatase (PAP). The cells are then infused back into the patient to stimulate the activation and expansion of cytotoxic T lymphocytes (CTLs). The DC vaccine, Ilixadencel was granted the FDA approval in 2021 for the treatment of patients with soft tissue sarcoma (C. Liu et al., 2022b). DC vaccines are plagued by a lack of cell sources, prolonged preparation time, and expensive costs. However, they still have a wide market potential due to their benefits, which include minimal adverse effects, high tolerance, and long-term immunological memory. In addition to DC vaccines, therapeutic vaccines include tumor cell vaccines, DNA/mRNA vaccines, and peptide vaccines (reviewed in Saxena et al., 2021).

The key for developing cancer vaccines is the need to identify the antigens that are exclusive to tumor cells. The instability of cancer cell genome and the frequent genetic modifications (such as mutations, deletions, duplications) results in the arise of neoantigens that is not found on normal cells. These antigens provide ideal targets for cancer vaccines. Vaccines that target neoepitopes have shown potential in early preclinical research. Neoepitope vaccinations (administered with different adjuvants) generated strong antigen-specific IFN-γ responses in CD4 and CD8 T cells associated with reduced tumor growth in mouse models of glioma, melanoma, sarcoma, and colorectal cancer (reviewed in Lee et al., 2021). However, these neoantigens are not very immunogenic and fail to induce a sustainable immune response. This field is still a hot topic. Over 100 clinical trials are currently utilizing neoepitope vaccines.

5. Immune checkpoint inhibitors (ICIs)

The tumor microenvironment (TME) is infiltrated with many types of innate and adaptive immune cells. Tumor cells tend to suppress the immune surveillance functions and the anti-tumor activity of immune cells by several mechanisms. One of the most studied and targeted immune evasion mechanisms is the expression of inhibitory surface proteins known as immune checkpoints referring to the molecules that act as gate keepers for the immune response (Binnewies et al., 2018; He & Xu, 2020). Under homeostatic condition, immune checkpoint proteins function to preserve self-tolerance and avoid exaggerated inflammatory responses. However, malignant cells disrupt this balance by overexpressing the inhibitory and/or downregulating the stimulatory molecules, therefore creating immunosuppressive status which encourages immune evasion and tumor growth (S. Lim et al., 2017). Blocking these inhibitory

pathways by immune checkpoint inhibitors (ICIs) (Figure 5), reinvigorate antitumor immune responses by interrupting co-inhibitory signaling pathways and promote immune-mediated elimination of tumor cells (Webb et al., 2018a). The development of ICIs particularly, those which target and reinvigorate the anti-tumor-T lymphocytes has been a game changer in the battle against cancer (Hargadon et al., 2018). T cells express a set of immune checkpoints (co-inhibitory receptors) which function to terminate the immune responses following antigen activation; an action that is required for the maintenance of peripheral tolerance and autoimmunity (Webb et al., 2018a). The two most investigated inhibitory receptors are the T lymphocyte antigen 4 (CTLA-4) and the programmed cell death protein 1/programmed cell death ligand 1 (PD-1/PD-L1) pathway. Thus, the use of monoclonal antibodies blocking CTLA-4 and PD-1/PD-L1 pathways is becoming increasingly prevalent in the treatment of a growing number of malignancies (Tan et al., 2022).

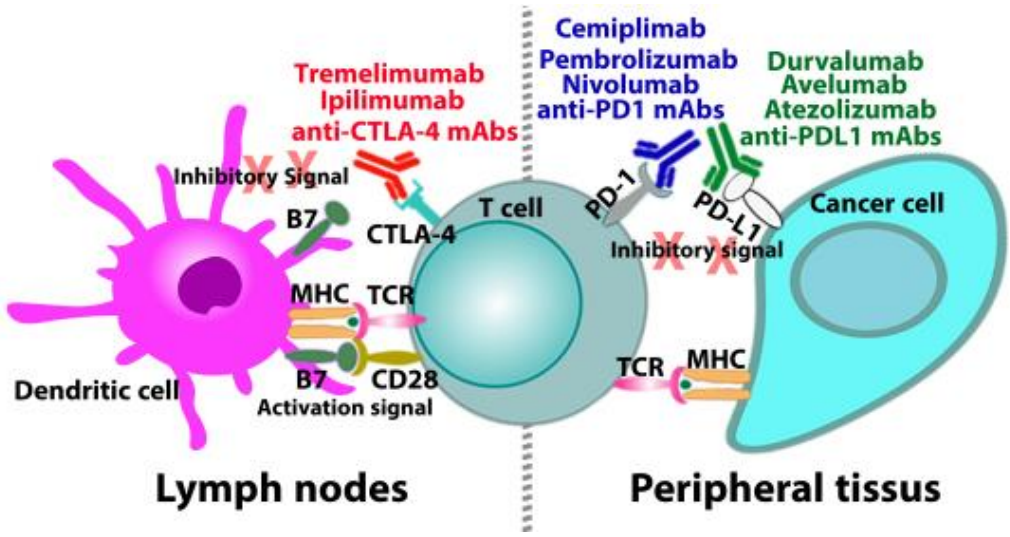


Figure 5. Mechanism of CTLA-4 and PD-1/PD-L1 blockade. Activation of T cells is mediated by binding to MHC II and B-7 located in the APC. CTLA-4 expressed on the surface of T cell delivers negative signals by competing with CD28 for binding to B-7. These inhibitory signals can be blocked by anti-CTLA-4 antibodies. Negative T cells regulation induced by interaction between PD-1 and PD-L1 on the surface of tumor cells can be reversed by PD-1 and PD-L1 inhibitors (Banday and Abdalla, 2023).

a. The CTLA-4 axis

CTLA-4, dubbed “godfather of checkpoints”, is the first immune-checkpoint receptor to be clinically targeted (Pardoll, 2012). It is exclusively expressed on T cells where it mainly controls

T cells function by inhibiting CD28, a co-stimulatory receptor. The latter is a surface-expressed protein that upon recognition of an antigen, it potentiates TCR signaling, thus activating T cells. CD28 and CTLA-4 share the same ligands, CD80 (also known as B7.1) and CD86 (known as B7.2) (Figure 6). However, CTLA-4 exerts much higher affinity for these ligands, therefore its expression on the surface of T cells dampens their activation through disputing with CD28 for binding to CD80 and CD86 (Hosseini et al., 2020; Shiravand et al., 2022). The major inhibitory role of CTLA-4 is through two subsets of CD4+ T cells: downmodulating helper T cells activity and augmenting regulatory T_{reg} cells immunosuppressive activity. Therefore, blockade of CTLA-4 is associated with wide augmentation of helper T cells-mediated immune responses (Peggs et al., 2009).

In the tumoral context, CTLA-4 overexpression has a prognostic role and reported to correlate with tumor progression in a number of cancers including non-small cell lung cancer (NSCLC), breast cancer, clear cell renal cell carcinoma (ccRCC) and colorectal cancer (CRC) (Angulo-Aguado et al., 2023; Paulsen et al., 2017; Kern & Panis, 2021; S. Liu et al., 2020). In 2011, the FDA approved the use of the first human immunoglobulin G (IgG) monoclonal anti-CTLA-4, Ipilimumab for the treatment of advanced melanoma (Lipson & Drake, 2011). Although as monotherapy, Ipilimumab is approved only for treatment of melanoma, however, several clinical trials are currently investigating its therapeutic benefits in other types of cancer including RCC, NSCLC, prostate cancer, and others. Nonetheless, a combination of Ipilimumab with Nivolumab, another ICI that block PD-1, has been approved as the first line treatment of unresectable malignant pleural mesothelioma (MPM), metastatic NSCLC and unresectable, advanced, recurrent or metastatic esophageal squamous cell carcinoma (ESCC), as well as approval for treatment of patients with hepatocellular carcinoma (HCC) (Nakajima et al., 2022; Saung et al., 2021; Vellanki et al., 2021). In addition to Ipilimumab, a second CTLA-4 blocking mAb, Tremelimumab is being developed (by AstraZeneca, under license from Pfizer). In October 2022, it was approved in the USA in combination with Durvalumab (another ICIs blocking PD-1) for the treatment of adult patients with unresectable hepatocellular carcinoma (uHCC) and in December, 2022 this combination received the approval of the EU Committee for Medicinal Products for Human Use as a first line treatment of adults with advanced or unresectable HCC (Keam, 2023).

b. PD-1/PD-L1 axis

Programmed cell death protein 1 (PD-1) -also known as CD279- is a surface co-inhibitory protein that belongs to the immunoglobulin superfamily and is encoded by the PDCD1 gene in

human (Ishida et al., 1992). It is expressed by activated T cells and B cells as well as NKT cells, NK cells, monocytes and subsets of dendritic cells (Keir et al., 2008a). PD-1 has two ligands, PD-L1 (B7- H1) and PD-L2 (B7-DC) both expressed on APCs, including DCs, however, PD-L1, thought to be the principal mediator of PD-1-dependent immunosuppression (Keir et al., 2008b; Ohaegbulam et al., 2015a). Upon binding of PD-1 to one of its ligands, it triggers a dual mechanism of stimulating apoptosis in PD-1 expressed T-cells while simultaneously reducing apoptosis in regulatory T-cells, resulting in down-regulation of the immune system, thus maintaining immune-hemostasis (Mahoney et al., 2015; Riella et al., 2012a).

In the TME, PD-L1 can be markedly upregulated on tumor cells specifically in the presence of IFN-γ while the expression of PD-1 is significantly lifted on tumor-infiltrating lymphocytes (Dong et al., 2018a). Engagement of PD-L1 to PD-1 suppresses T cells functionality by promoting T-cell tolerance, inhibiting T cells proliferation, and reducing their cytokine production which favors tumors progression and proliferation (Dong et al., 2018a; Han et al., 2020a). In addition to PD-1/PD-L1 mediated inhibition of T cells, PD-L1 can also suppress T cells functionality through inhibition of CD80/CD28 pathway. CD80 is another B7 stimulatory membrane protein expressed by APCs and binds to CD28 on T cells. This engagement mediates stimulatory signaling pathway provoking T cells activation. PD-L1 competes with CD80 for binding to CD28 and block its T-cells-mediated activation (Butte et al., 2007a). Thus, blocking these inhibitory pathways by targeting PD-1 or PD-L1, can greatly reinvigorate the antitumor immune responses (Webb et al., 2018a).

Following several clinical trials evaluating the impact of blocking PD-1 on tumor regression and patient's survival (Topalian et al., 2014), the FDA approved the anti-PD-1 monoclonal antibodies Pembrolizumab (in 2014), Nivolumab (in 2016), and Cemiplimab (in 2018) for the treatment of non-small cell lung cancer, melanoma, renal cell carcinoma, bladder cancer and squamous cell skin cancer (Kazandjian et al., 2016a; Migden et al., 2018). Concomitantly, monoclonal antibodies that block the PD-L1 arm have been also developed and launched including Avelumab (in 2015), Durvalumab (in 2016), and Atezolizumab (in 2017) for treatment of a variety of solid tumors including NSCLC, urothelial carcinoma, Merkel cell carcinoma and renal cell carcinoma (reviewed in Vaddepally et al., 2020; Wilky, 2019).

The use of ICIs changed the paradigms of cancer therapy and the miraculous improvements in patient's overall survival and progression-free survival in several tumors inspired further clinical trials to evaluate monotherapy and combination strategies in a variety of solid and hematologic malignancies (Wilky, 2019). Randomized clinical trials of Ipilimumab-Nivolumab

combination showed an improved PFS over either ipilimumab or nivolumab monotherapy, including PD-L1-negative tumors which influenced the FDA approval for this combination for treatment of hepatocellular Carcinoma (HCC) and metastatic NSCLC (Saung et al., 2021; Vellanki et al., 2021).

Despite the revolutionary impact of ICIs on treatment outcomes, their use is faced by many obstacles. One major challenging issue is the severe immune-related adverse effects (irAEs) which can affect nearly all organs and systems resulting -in some cases- in permeant health conditions such as diabetes and neurological disorders. Developing irAEs is a leading cause for terminating ICIs therapy and -in many cases- patient's hospitalizations (Lozano et al., 2022; Morad et al., 2021). Additionally, the complex immune evasion mechanisms, the abundance of immunosuppressive cells in the TME together with the diminished infiltrating T cells (cold tumors) are factors that contribute to low response rates in many patients (Labani-Motlagh et al., 2020). Therefore, novel measures are critically needed to alleviate the safety concerns and potentiate the efficacy of ICIs therapy. In this context, combinatorial treatment strategies of ICIs with chemotherapy, radiotherapy, T-cell-based therapies, and targeted agents seem promising and currently being investigated in pre-clinical models and clinical trials (Banday & Abdalla, 2022).

III. Microorganisms-based cancer immunotherapy

1. Oncolytic virotherapy

In addition to the immune checkpoint inhibitors, another ground-breaking development in the field of cancer immunotherapy was the use of live viruses to destroy tumor cells. Since the early 1900s, many studies have noted observable tumor regression linked to viral infections and in a few cases, inoculated patients with viruses produced long-lasting remission (Sinkovics and Horvath, 1993). This area witnessed an increasing research interest that reached fiver pitch during in the middle twentieth century when it became feasible to model human cancers using mice models. This allowed for the pre-clinical study of the oncolytic activity of many viruses (Kelly & Russell, 2007). After decades of research, the term "oncolytic viruses (OVs)" has emerged that identifies viruses which can selectively infect and damage tumor cells while sparing normal tissues (Russell & Peng, 2007). Normally, each virus has a natural tropism for specific cells (for example, hepatitis B virus infects hepatocytes, the influenza virus damages the epithelium of the airways and so on). It has been observed that many naturally occurring

viruses (including, reovirus, Newcastle disease virus, mumps virus and Moloney leukemia virus) possess preferential tropism for tumor cells (Russell et al., 2012). Taken together, these results marked the beginning of the exploration of viruses as possible cancer-treating agents.

Similar to other immunotherapies, OVs have a multimodal mechanism of action with both direct and indirect toxic effects on tumor cells (Figure 6) (Tian *et al*., 2022). Direct cell lysis from traditional viral machinery is one method of toxic injury. Moreover, OVs can activate various forms of immunogenic cell death (ICD) leading to release of hallmark immunostimulatory damage-associated molecular patterns (DAMPs). DAMPs together with the viral elements known as pathogen associated molecular patterns (PAMPs) become exposed to the host immune system where they establish a proinflammatory microenvironment by stimulating the production of proinflammatory cytokines (such as type I IFNs, interleukins, GM-CSF) and chemokines (such as CCL2, CCL3, CCL5, and CXCL10). Oncolysis of tumor cells initiates the release of tumor-associated antigens and neoantigens (TANs) which are presented by APCs in complex with MHC molecules. APCs then cascade to CD4+ and CD8+ T-cell activation leading to the transformation of immunologically "cold" tumors into "hot" tumors. Subsequently, CD4+ and CD8+ T-cell responses can mediate global anti-tumor effects at distant loci and direct tumor cell killing (Ahmed & Tait, 2020; Raja et al., 2018; Tian et al., 2022).

Many viral species have been investigated for potential application in immuno-oncology. The majority of these OVs are modified by using various strategies to generate tumor-targeted viruses with tumor specific tropism (Raja *et al*., 2018). Among the strategies applied to reprogram OVs, is the use of soluble bispecific binder molecules (bispecific scFv or scFv fused to a ligand binding motif) recognizing both the virus and a target on the tumor cell. For example, a scFv directed against EGFR fused either to a scFv anti-adenovirus fiber or the gD-binding V domain of nectin-1 markedly enhanced the infection efficiency of adenoviral vectors and Herpes simplex virus in EGFR-expressing cells (Haisma et al., 2000; Nakano et al., 2005). Ligand binding motifs may also be directly covalently attached to or expressed by the virus. Affilin, 98 amino acids small ubiquitin-based Affilin molecule with specific binding to EGFR, covalently attached to the adenoviral capsid was shown to strongly enhanced transduction of EGFR-positive tumor cancer cells in vitro (Wienen et al., 2023a). Also, ankyrin repeat protein (DARPin) binding domains specific for EGFR fused to the surface hemagglutinin glycoprotein of measles virus were able to target the recombinant measles virus (Hanauer et al., 2019).

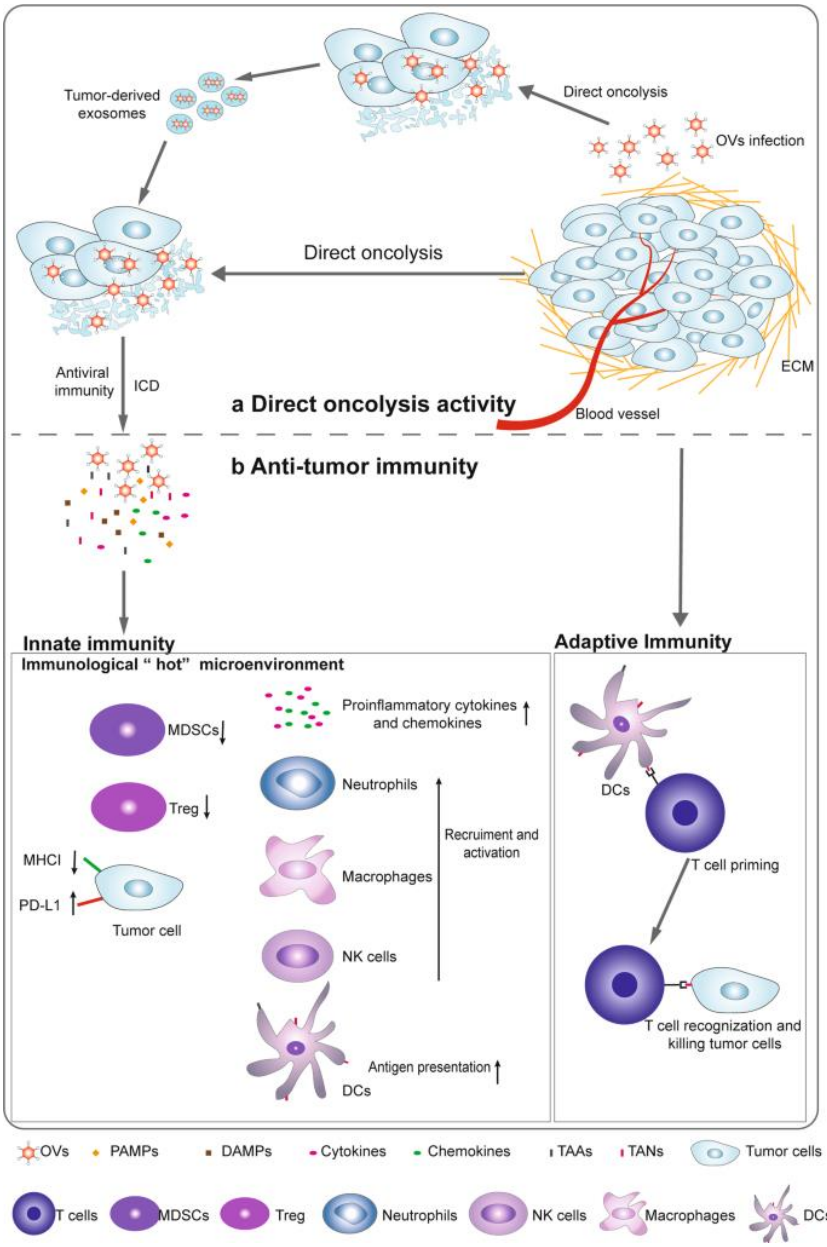


Figure 6. Mechanism of OVs anti-tumor activity. **(a)** direct oncolysis: new viral particles are released from infected tumor cells and infect adjacent tumor cells. Exosomes derived from OV-infected tumors exhibit high tumor tropism. **(b)** Antitumor immunity: immunogenic cell death (ICD) leads to the release of pathogen-associated molecular pattern molecules (PAMPs), damage-associated molecular pattern molecules (DAMPs), tumor-associated antigens (TAAs) and tumor-associated neoantigens (TANs). Identification of PAMPs/DAMPs through pattern recognition receptors (PRRs) triggers the expression of proinflammatory cytokines (which stimulate innate immune cells such as NK cells and DCs) and chemokines (which recruit neutrophils and macrophages). The released TAAs and TANs are processed and presented on the APCs surface. Multiple cytokines and chemokines contribute to the recruitment and activation of antitumor $CD8^+$ T cells and B cells (Tian et al., 2022).

Another strategy is based on fragment antibodies including scFvs, nanobodies (single heavy variable domain of camelid antibodies) and affibodies (small alpha-helical polypeptide ligands that function as inert antibody mimetics) expressed by the virus, with scFv being the most studied targeting entity. A nanobody-based retargeting has been applied to an oncolytic herpesvirus for targeting CXCR4 positive GBM cells by insertion of an anti-human CXCR4 nanobody in glycoprotein D of an attenuated HSV-1. This CXCR4-retargeted virus specifically infected CXCR4 positive cells and was able to limit the tumor progression and to significantly improve mice survival in an orthotopic xenograft model of GBM (Sanchez Gil et al., 2022). Her2/neu-reactive Affibody- molecules (ZH) were inserted into the HI loop of an adenovirus receptor binding-ablated fiber. The ZH-containing vectors were specific for their target, resulting in efficient infection and killing of Her2/neu-positive cancer cells in vitro (Magnusson *et al*, 2012).

HSV was retargeted to selectively bind and replicate in HER-2 and EGFR positive tumors by generating recombinant strains into which anti-HER-2 or anti-EGFR /EGFRvIII scFv were inserted in the gD protein. These recombinant strains demonstrated enhanced tumor regression and increased median survival time in tumor-bearing mice models of breast and ovarian cancers (Nanni et al., 2013a) and glioblastoma (Appolloni et al., 2021). Retargeted measles viruses to EGFR or EGFR mutant vIII using specific scFv inserted in the H protein (hemagglutinin) efficiently entered cells through their respective targeted receptors and demonstrated specific receptor-mediated anti-tumor activity in mice bearing EGFR-positive human tumor xenografts (Nakamura et al., 2005). Another investigated target is PSMA which is abundantly expressed on high-grade and metastatic prostate cancer as well as neovasculature of human non-prostate cancers. PSMA-retargeted HSV, through insertion of an anti-PMSA scFv in the gD protein achieved remarkable inhibition of primary tumor growth through establishing of long-term T cells immune response, immune heating of the microenvironment, de-repression of the anti-tumor immune phenotype and increased sensitization to checkpoint blockade (Vannini et al., 2021). Similarly, PSMA-retargeted recombinant measles virus specifically infected PSMA cells and had potent antitumor activity against LNCaP and PC3-PSMA human prostate tumor xenograft models (C. C. Liu et al., 2010). Other targets such as the cell markers CD20, CD38 and CD46 and their associated scFv, have also been investigated as targets notably for modified Measles viruses (measles scFv CD20 Bucheit et al., 2003; Peng et al., 2003; Muñoz-Aliá et al., 2021; Peng et al., 2003 and reviewed in Mühlebach, 2020);

Another rationale for genetic modification of OVs is to invest them as drug-carriers due to their appreciated tumor specificity. Using viruses as drug delivery platforms provides an avenue for reducing the clinical toxicity associated with systemic therapy by concentrating the immunomodulatory payload at the site of tumor (Rahman & McFadden, 2021). Thus, burgeoning number of OVs have been emerged as expression platforms for therapeutic immune modulators such as cytokines, chemokines and ICIs. Virus-based cytokine expression using OVs armed to express IL-2, IL-12, IL-15 and IL-24 used to treat a variety of tumors in murine-bearing tumor models and showed enhanced tumor regression, improved survival and improved immune effector functions (T and NK cell priming and activation) [reviewed in (Pearl et al., 2019)]. Additionally, Chemokines such as CCL2, CCL5, CLL19, CCL20, CCL21, and CXCL11 have been expressed using OVs to enhance the migration of immune cells into TME [(reviewed in (J. G. Pol et al., 2020)].

The first (and up to date the only) virotherapy approved by the FDA is an attenuated HSV encoding human GM-CSF (T-VEC) licensed in 2015 for the treatment of melanoma patients with injectable but nonresectable lesions (J. Pol et al., 2016a). This approval represented viruses as trustworthy cytokine-secreting vectors and paved the way for other viruses to be explored in the same context. Recently, a third generation (triple-mutated) recombinant oncolytic HSV1 Teserpaturev/G47Δ has been approved in Japan for intratumoral treatment of malignant glioma. Teserpaturev/G47Δ is also currently being evaluated in clinical trials phase II for prostate cancer, phase I for malignant pleural mesothelioma and recurrent olfactory neuroblastoma (Frampton, 2022). Furthermore, Teserpaturev/G47Δ armed with IL-12 (expressing), combined with anti-PD-1 and anti CTLA-4 demonstrated better antitumor efficacy as opposed to only Teserpaturev/G47Δ armed with IL-12 in a murine GBM (Saha et al., 2017).

Oncolytic viruses (OVs) virotherapy has been shown to be associated with the upregulation of PD-L1 in the tumor microenvironment (TME), likely mediated by IFN-γ. This process turns immunologically 'cold' tumors into 'hot' ones, which are more responsive to immune checkpoint inhibition therapy. Therefore, combining OVs with PD-1/PD-L1 blockade has emerged as a promising approach to increase anti-tumor efficacy (Lv et al., 2021). One of the most interesting combinatorial modalities is engineering OVs to express anti-PD-1 and anti-PD-L1 (most probably in scFv format) directly in the tumor bed. For example, recombinant strains of herpes simplex virus (HSV), measles virus, myxoma virus, Newcastle disease virus, and retroviruses secreting anti-PD-L1 or anti-PD-1 scFvs have demonstrated enhanced anti-tumor immune responses. These enhancements include increased T cell infiltration, upregulated IFN pathway

and antigen processing and presentation genes, and downregulated angiogenesis and cell adhesion genes. All these factors contributed to tumor inhibition and durable anti-tumor responses with reduced systemic concentrations of the secreted antibody in various pre-clinical mice models (Bartee et al., 2017a; C. Lin et al., 2020a; Mitchell et al., 2019; Passaro et al., 2019a; Vijayakumar et al., 2020). Moreover, recent studies have shown that PD-L1 can be used as a target to direct adenoviruses to PD-L1 expressing tumors and combine the immune checkpoint blockade with virotherapy. Lv *et al*. developed oncolytic Adenovirus encapsulated into bioengineered cell membrane nanovesicles with PD-1 expression. Following intravenous injection of this construct, significant accumulation on PD-L1 expressing tumors was observed. This model effectively activated tumor-infiltrating T cells and elicited a strong anti-tumor immune response (Lv et al., 2021). In another study, a full-length bispecific antibody recognizing an inserted epitope in the VP capsid and PD-L1was used to redirect Adeno-associated virus (AAV). The bispecific antibody/AAV complex was able to selectively transduce receptor positive cells (Kuklik et al., 2021a).

In summary, oncolytic virotherapy is a rapidly growing field that catches a lot of interest and encompasses several investigation avenues including the many approaches of genetic modification and several possibilities for combination therapy with other treatment modalities. Currently, there are over 300 ongoing clinical trials evaluating OVs as mono or combined therapy (D. Lin et al., 2023a).

2. Bacteria-based cancer immunotherapy

During the late nineteenth century, William B. Coley pioneered the use of bacteria to treat cancers when he treated sarcomas patients with heat-killed *streptococcal* bacteria with *Serratia marcescens* (together named "Coley's Toxins") and obtained tumor regression (Coley, 1891). These findings paved the way for researchers to investigate several bacteria as potential immunotherapeutic agents. It has been reported that certain obligate and facultative anaerobes could preferentially colonize the hypoxic and necrotic areas in solid tumors after systemic administration. Accumulative evidences suggesting that the high infiltration of bacteria to tumor site is inseparable of the hypoxic nature and the increased availability of metabolic nutrients in the TME (Forbes et al., 2003; Lambin et al., 1998; Taniguchi et al., 2010). In addition to the predominate infiltration and colonization in tumors, bacteria immunogenicity contributes to inducing robust immune response against tumors mediated by bacterial components including peptidoglycan, lipopolysaccharide (LPS), lipoteichoic acid (LTA),

flagellum, DNA, RNA, etc., (Huang et al., 2021). LPS for example, is a potent immunogenic microbial-associated molecular patterns (MAMPs). Bacterial flagella can also induce innate immune response via TLR5 pathway (Sfondrini et al., 2006). Immunogenicity of un-flagellated bacteria can be owed to peptidoglycan and polysaccharide which can activate macrophages, NK cells, DCs and B lymphocytes. All mentioned mechanisms eventually lead to production of immune active substances, such as IL-1, IL-6, IL-12, TNF-α, IFN-γ by effector cells and thereby enhancing anti-tumor immunity (reviewed in Zhou et al., 2023).

Currently, several bacterial strains have been identified as tumor-suppressors, such as Clostridium, Bifidobacterium, Listeria, Salmonella, and Lactobacillus with Salmonella being the most extensively studied (Guo et al., 2024a). The major concern of this bacteria-based approach was the potential risk associated with using potentially pathogenic organism. Therefore, efforts have been made to develop attenuated strains in a way that improve safety while maintaining the therapeutic efficacy (Kaimala et al., 2018). Intratumor administration of live attenuated Salmonella inhibited tumor growth by inducing apoptosis, severe necrosis, recruitment of inflammatory cells and the cross-presentation of tumor antigens, which allowed for the induction of tumor-specific immune response in tumor-bearing mice (Avogadri et al., n.d.; S. Zhou et al., 2016). Furthermore, a study by Chen *et al*. showed that, Salmonella -in addition to the discussed mechanisms- could also break the TME immune tolerance by downregulating the immune checkpoint PD-L1 expression which resulted in significant inhibition of tumor growth together with increased infiltration and reduced apoptosis of T cells in mice (M. C. Chen et al., 2020).

Although so far limited, few clinical trials have evaluated attenuated bacterial strains in human patients. Results from phase I clinical trial in which an attenuated Salmonella strain was administered intravenously to patients with metastatic melanoma, reported tolerability and safety with the administered doses, however, differently from results in mice, no tumor colonization nor regression was reported (Toso et al., 2002). In a more recent phase I trial, administration of a single intratumor injection of attenuated *Clostridium novyi* to patients with refractory solid tumors produced robust local tumor destruction and stimulated inflammatory and tumor-specific immune responses. Still, significant toxicities were detected, and the authors identified several factors to be modified and considered for future trials (Janku et al., 2020).

In the following years, many researchers attempted to potentiate tumor targeting and invasion of *Salmonella* -as well as other species- through ligand targeting tumor specific antigens

displayed on bacteria's outer membrane. Massa *et al*. developed engineered attenuated *Salmonella* strain expressing single domain antibody (VHH) against CD20 tumor antigen which is highly expressed by several tumors. The anti-CD20 on the bacteria surface mediated bacterial entry and accumulation on only CD20+ tumor cells while significantly diminished dissemination to other tissues in mice- bearing CD20+ tumor (Massa et al., 2013a). Similarly, Salmonella was engineered to target the tumor antigen alpha v beta 3 (αvβ3) integrin, which is widely overexpressed on cancer cells and blood vessels during cancer angiogenesis, through surface display of arginine-glycine-aspartate (RGD) (a well-studied tumor peptide that binds to αvβ3) on the outer membrane protein A (Park et al., 2016). Their results showed that RGD-displaying *Salmonella* showed strong targeting efficiency, resulting in the regression in αvβ3-overexpressing cancer xenografts, and prolonged survival of mouse models of human breast cancer and human melanoma models.

One of the most interesting strategies that has shown promise in pre-clinical and clinical trials is combining bacteria-based therapy with immune checkpoint blockade. Moreo et al. demonstrated that treating mice bearing invasive bladder tumors with a live attenuated Mycobacterium strain (MTBVAC) in combination with anti-PD-L1 maximized protection and significantly improved survival compared to each treatment as monotherapy (Moreo et al., 2022). Similarly, co-administration of low doses of attenuated Salmonella typhimurium significantly improved the outcomes of anti-PD-L1 therapy in a mouse immunogenic model of colon cancer, increasing the inhibition of tumor growth from 30% to 75% compared to monotherapy (Al-Saafeen et al., 2022). The ability of these bacteria to colonize tumors, along with their engineerability, has led to a more novel strategy combining bacteria-based therapy and immune checkpoint inhibitors (ICIs) by engineering bacteria to function as expression vectors for specified fragments. For instance, a probiotic-based ICI delivery system was developed by utilizing E. coli (strain Nissle 1917) engineered to express anti-PD-L1 and anti-CTLA-4 nanobodies. In syngeneic animal models, these modified systems showed improved therapeutic responses compared to clinically relevant antibodies, leading to tumor regression and elevations in systemic T cell memory populations (Gurbatri et al., 2020). The promise of this approach encouraged the first-in-human study evaluating the administration of Novyi-NT spores in combination with the anti-PD-1 antibody pembrolizumab in patients with injectable, treatment-refractory solid tumors. Preliminary results concluded that a single intratumor injection of C. novyi-NT in combination with pembrolizumab achieved encouraging anti-cancer activity with a manageable toxicity profile (NCT03435952).

Numerous challenges still stand in the way of the clinical application of bacterial-based immunotherapies, particularly biosafety concerns and the risk-benefit balance between safe doses and clinical outcomes cancer (Guo et al., 2024b). Nonetheless, with further advancements and investigations, bacteria-based cancer immunotherapy holds promise as an important weapon in the fight against.

3. Parasites in cancer immunotherapy

In the context of cancer immunotherapy, while viruses and bacteria have been extensively explored, parasites as immunotherapies are relatively less explored. However, many experimental data have shown that Protozoa or their components can stimulate the immune system which -in the presence of cancer- results in overcoming cancer immunosuppressive microenvironment and coordinating different immune cells and cytokines to prevent the development, spread, and recurrence of tumors. These findings hint at the potential of strategic use of parasites as promising immunotherapeutic agents in cancer. Indeed, a considerable number of parasites such as *plasmodium, Trypanosoma cruzi*, *Toxoplasma gondii*, *Leishmania*, and *Eimeria* have been explored for their anti-tumor properties in pre-clinical models (Darani & Yousefi, 2012; Zheng et al., 2023a) .

The earliest protozoa to be explored for its anti-tumor potential is *T. cruzi* whose investigation dates back to the mid-20th century when significant anti-tumor response was reported with parasite's extracts (Klyueva and Roskin,1946). In a more recent study, mice were chronically subcutaneously infected with *T. cruzi* then they were subjected to DMH (1,2 dimethylhyadrazine), a drug that is known to stimulate the development of colon adenocarcinoma. *T. cruzi* infection significantly protected mice form developing colon cancer compared to control groups (Oliveira et al., 2001). Moreover, researchers investigated the possibility of using lysates rather than live parasite to induce anti-tumor response. For this purpose, Batmonkh *et al*. inoculated Ehrlich adenocarcinoma cells into mice for one week before treating them with lysed epimastigotes of *T. cruzi.* Treated mice had 3-fold inhibition in tumor growth compared to control (Batmonkh et al., 2006). However, significant side effects were reported from these treatments. Others also indicated that *T. cruzi* epimastigotes lysates exhibited strong protection against DMH induced colon cancer and N-nitroso-N-methylurea (NMU) induced breast cancer in rats. This protection was featured with activation of both CD4+ and CD8+ T cells (Ubillos et al., 2016). Another explored protozoan is *Leishmania* which in a recently conducted study comparing tow species of *Leishmania* in mice-bearing breast cancer

models, have shown remarkable induction of the immune response against 4T1 breast cancer which was more remarkable with live-attenuated *L. tropica* than *L. infantum* (Caner et al., 2020).

Among the widely investigated parasites for their potential in immune oncology, are the apicomplexan parasites; *Plasmodium*, *Toxoplasma gondii*, and more recently *Neospora caninum*. It has been well known today that *Plasmodium* infection is associated with robust innate and acquired immune responses in hosts (Chandley et al., 2023). Two separate studies have shown that treatment of mice-bearing Lewis lung cancer (LLC) model with non-lethal *P. yoelii* strain revealed effective activation of both the innate and adaptive immune system featured by increased secretion of IFN-γ and TNF-α, higher numbers of activated NK cells, elevated levels of tumor-specific CD8+ T cells and prolonged survival together with the reduction in the proportion of suppressive immune cells [myeloid-derived suppressor cells (MDSCs) and Tregs] (Adah et al., 2019; L. Chen et al., 2011; D. Zhou et al., 2019). Similar results were obtained from a study using triple-negative breast cancer mouse model where a non-lethal *P. yoelii* strain injected intraperitoneally to mice promoted the infiltration of CD8+ T cells into the TME and the secretion of granzyme B (Pan et al., 2021). However, while the engineered *P. yoelii* was non-lethal and was rapidly cleared, clinical symptoms were observed, and safety was a concern (Q. Liu et al., 2017).

The clinical translation of parasite-based immunotherapy is still limited, however, China has started the first clinical trials evaluating parasites as immunotherapeutic agents by initiating three single-arm clinical trials based on *Plasmodium* for advanced cancers (NCT03375983), advanced lung cancer (NCT03375983) and advanced breast and liver cancers (NCT03474822). While these findings provide solid evidence that protozoa can be effectively invested in the field of cancer immunotherapy, yet, this area is still an uncharted territory that needs further research and exploration. Herein, another two apicomplexan parasites *T. gondii* and *N. caninum* (which are the core of this thesis) are going to be more thoroughly reviewed.

IV. *Toxoplasma gondii* and *Neospora caninum*

1. *T. gondii* and *N. caninum* shared characteristics

Apicomplexans are a diverse group of unicellular protozoans, of which, the majority are obligate, intracellular parasites infecting a wide range of vertebrate hosts. *T. gondii* and *N.*

caninum are closely related unicellular Apicomplexan parasites, both with obligate intracellular lifestyles (Morrissette & Sibley, 2002a). While *T. gondii* was identified as early as the beginning of the 20th century, *N. caninum* on the other hand was relatively recently identified in the year 1984 (Bjerkfis et al., 1984a). Molecular analyses have determined that *N. caninum* is very closely related to *Toxoplasma gondii*, and consistent with their recent divergence, many of the biological characteristics that have been described for *T. gondii* also apply to *N. caninum* (Howe & Sibley, 1999)

These parasites possess three infectious forms (Figure 7): (i) tachyzoite (Figure 7-a), which represent the rapid multiplication form. Tachyzoites can enter all nucleated cells by active penetration and form a cytoplasmic vacuole. Following several rounds of replication, the host cells disrupt and dye, and tachyzoites spread and infect neighboring cells. (ii) Bradyzoite (Figure 7-b) is a form of slow multiplication that is characteristic of the chronic infection and that originates the tissue cysts. (iii) Sporozoite (Figure 7-c), which is produced only in the definitive hosts during the sexual reproduction and released in the oocysts via the feces (Attias et al., 2020; Dubey, 1999; Dubey et al., 1998).

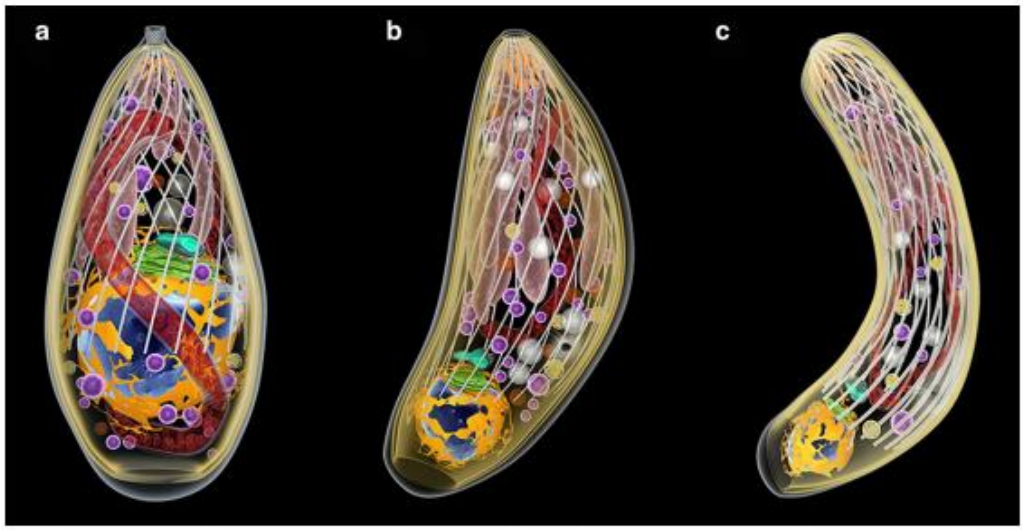


Figure 7. The three infective stages of *T. gondii*. (a) Tachyzoite, (b) bradyzoite and (c) sporozoite. The nucleus (blue) is surrounded by the rough endoplasmic reticulum (yellow). Above it, The Golgi complex (green) and the apicoplast (blue green). The single mitochondrion spreads through the cytosol (red). Dense granules (magenta) and amylopectin granules (white) are dispersed in the cytosol. The apical complex is composed by the cylindrical conoid. Below, the secretory organelles: micronemes (orange) and rhoptries (pink). The cell body is limited by three membrane units (the pellicle) and below it a set of subpelicular microtubules (Attias et al., 2020).

In order to survive within their host, apicomplexan parasites have evolved mechanisms that allow them to evade and/or modulate the host immune response. Apicomplexans invade the host cell by an active process through secretion of a large number of unique proteins. When they invade the host cells, *they* forcibly indent the host cell plasma membrane and create the parasitophorous vacuole (PV) which keeps the tachyzoites away from the endocytic trafficking pathway of its host while interfacing for nutrient acquisition (Howe & Sibley, 1999).

The unique set of proteins that provide these parasites with their characteristic invading capabilities come from three dedicated secretory organelles that release their contents during invasion; small, elongated micronemes (or microspheres, depending on their shape), the club-shaped rhoptries and the spherical dense granules (Figure 8) (Hakimi et al., 2017a). Micronemes and rhoptries are found with the conoid in the apical end of the parasite forming the apical complex from where this phylum derives its name, while dense granules are evenly distributed throughout the parasite cytoplasm (Ravindran & Boothroyd, 2008). Numerous proteins from each of these secretory organelles have been identified. Secretion of microneme proteins (perforins, lipases, proteases, adhesins) facilitates the egress of the tachyzoite from the infected cell and guarantees the gliding motility by which the tachyzoites move within extracellular space (Dubois & Soldati-Favre, 2019).

Also, micronemal proteins containing adhesive domains such as epidermal growth factor (EGF)-like, thrombospondin (TSP)-like, lectin-like, Apple/PAN and MAR (microneme adhesive repeat) domains (for example, TgMIC1- 4, NcMIC1- 4) play important role in targeting of the tachyzoite toward the noninfected cells and contribute to the attachment of *Toxoplasma* (Figure 8-A) *or Neospora* to new host cells (Cowper et al, 2012). This suggests that microneme secretion is dosed as long as parasites are between host cells. Although it occurs at a basal rate in the absence of host cells, however, it appears to be transiently up regulated upon contact with the host cell. This suggests that micronemal proteins are secreted in greater abundance in the extracellular space at the time of attachment (V. Carruthers & Boothroyd, 2007a; Y. Zhang et al., 2019).

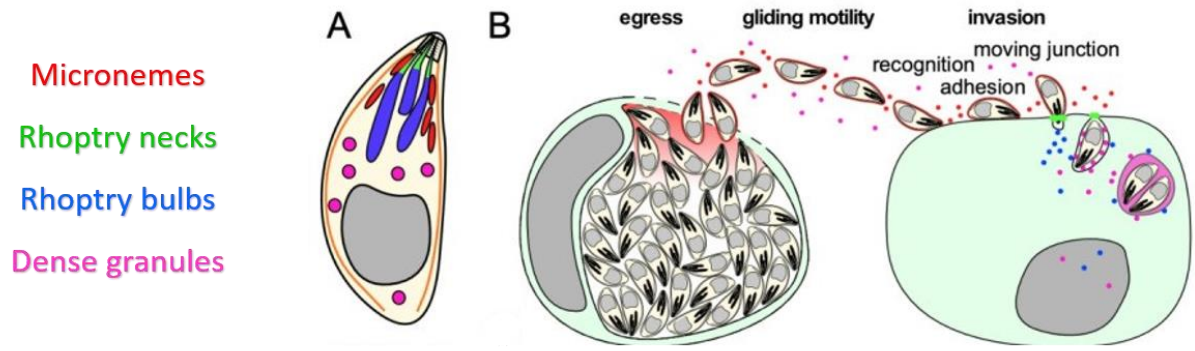


Figure 8. Schematic representation of the function and timing of secretion proteins in the *T. gondii* lytic cycle. **(A)** The three secretory organelles in the tachyzoite form of the parasite. **(B)** Schematic representation of secretion events throughout the lytic cycle (Tagoe et al., 2021).

Rhoptry neck proteins (RONs) are released first to work in collaboration with some micronemal proteins -AMA1- to form a 'moving junction' that migrates down the length of the invading parasite during invasion which help to direct the parasite into the host cell (Figure 8-B). This moving junction-dependent invasion structure is highly conserved within Apicomplexa (Sardinha-Silva et al., 2019) and AMA1 has been identified as a conserved antigenic protein in *T. gondii* and *N. caninum* (Y. Zhang et al., 2019)**.** Upon apical attachment of the tachyzoite to host's cells, rhoptry contents from the bulb portion are discharged immediately into the host cell (Bradley et al., 2005; Ravindran & Boothroyd, 2008). Rhoptry proteins reshape the host cell membrane for invasion and modulate host cell signaling. For both *T. gondii* and *N. caninum*, some ROP proteins have been identified as potential virulence factors (Marugan-Hernandez, 2017).

After host cell invasion and PV formation, dense granule proteins (GRA) are released within the PV lumen from where they traffic to PV membrane (PVM), intravacuolar network (IVN), host cell cytoplasm and even to the host cell's nucleus (Figure 8-B) (Mercier & Cesbron-Delauw, 2015). GRA proteins have been implicated in several functions from interference with host cell signaling, transfer of small molecules between host cells and PV, manipulation of host cell immune response and even parasite egress (Tagoe et al., 2021).

In addition to the secreted proteins, *T. gondii* and *N. caninum* possess a number of glycophosphatidylinositol (GPI)-anchored surface antigens (SAGs) that play a role in initial host cell recognition. For *T. gondii*, the major tachyzoite surface antigen is TgSAG1, a 30 kDa

protein encoding 336 amino acids, which has been isolated and described by Burg et al. in 1988 (Y. Wang & Yin, 2015). For *N. caninum* tachyzoites, two major antigens have been identified: NcSAG1 (which displays 53% homology to TgSAG1) and NcSRS2 (Hemphill · B. Gottstein, 1996). Specific antibodies against TgSAG1 or NcSAG1have been shown to inhibit the invasion of either *T. gondii* or *N. caninum* to host cells suggesting their implication in host cell recognition (Abdelbaky et al., 2023; Mineo and Kasper, 1994).

While sharing theses similarities, these parasites somehow differ in their life cycle, hosts and - in this context of immunotherapy- induced immune responses and application in the field of cancer immunotherapy. These differences will be discussed separately for each parasite.

2. Toxoplasma gondii

2.1. Life cycle

T. gondii is globally distributed and can infect humans and all warm-blooded animals and birds. However, only the members of the family Felidae including domestic cats are considered definitive host where the sexual stage of the life cycle takes place. Other mammals are considered intermediated hosts because only asexual stages occur in them (Dubey, 2009). During the life cycle of *T. gondii* (Figure 9). The sporozoites replicate in the definitive host, during the acute infection phase and form oocysts which are released in the faeces (Figure 9 a-b). Following shedding, sporulation takes place and when intermediate hosts (mammals including humans) consume vegetables or water contaminated with oocysts, they become infected (Figure 9 c-e). Infection can also be acquired for both definitive and intermediate hosts by consumption of tissues of chronically infected animals. After ingestion, *T. gondii* sporozoites or bradyzoites infect gut epithelial cells and then differenciate into tachyzoites which under pressure from the immune response convert to the bradyzoite stage and form tissue cysts. Within humans, infection can be transmitted via blood transfusion, maternofetal transmission through placenta or organ transplant (Figure 9 h-j) (Montoya and Liesenfeld, 2007)

2.2. Toxoplasmosis

In humans, primary infection with *T. gondii* (Toxoplasmosis) in immune-competent individuals is asymptomatic in more than 80% of individuals or manifesting as flu-like symptoms. However, tachyzoites can disseminate to the brain and skeletal muscles and form bradyzoite cysts (representing the chronic phase of the disease) (Daher et al., 2021a). Toxoplasmosis can be of concern during pregnancy due to the placental transmission of the parasite to the fetus

(Lindsay & Dubey, 2011). Fetal infection during the first trimester often leads to abortion, stillbirth, or a child born with severe brain and/or eyes abnormalities (Singh, 2016).

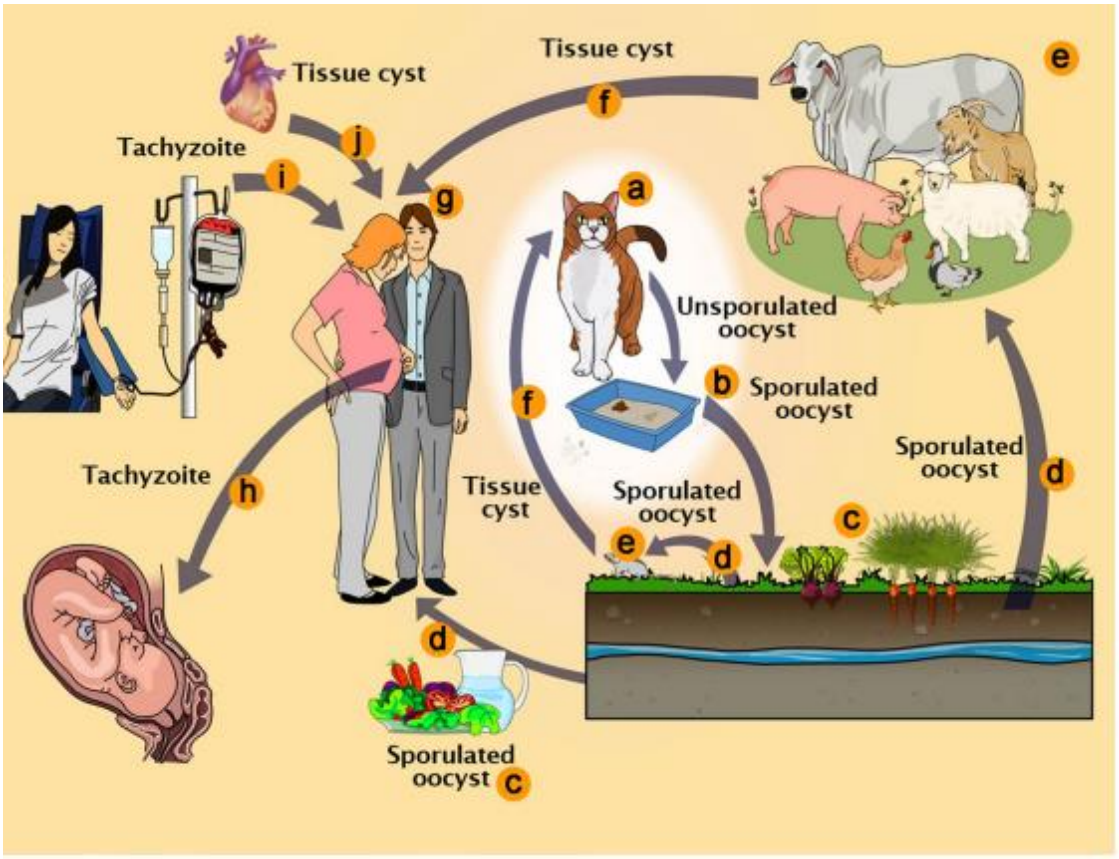


Figure 9. *Toxoplasma gondii* pathways of transmission. **(a)** Definitive host (cat). **(b)** Unsporulated oocysts in cat feces. **(c)** Food contaminated with sporulated oocysts. **(d)** Oocysts may be ingested by intermediate hosts via water or raw vegetables. **(e)** Intermediate hosts (e.g. cattle, sheep, poultry and swine). **(f)** Ingestion of tissue cysts in uncooked meat. **(g)** Intermediate hosts (humans). **(h)** Tachyzoites transmitted through the placenta to the fetus. **(i)** Transmission by blood transfusion and **(j)** organ transplant. (Attias et al., 2020).

However, fetus infection during the second or third trimester is less likely to cause severe clinical manifestations but may result in subclinical disease (Daher et al., 2021a). Contrasting the case in immunocompetent individuals, toxoplasmosis can be life threatening in immunocompromised patients. As the host immune response plays a key role in the control of *T. gondii*, severely immunocompromised individuals (such as HIV patients, patients taking immunosuppressive therapies or cancer chemotherapy) are exposed to the reactivation of chronic toxoplasmosis as well as to primary infections (Robert-Gangneux & Dardé, 2012).

T. gondii disease severity is determined by many factors, including the genotype of the parasite and host species (reviewed in (Mukhopadhyay et al., 2020). In Europe and North America, the population structure of *T. gondii* is dominated by four main clonal types (I, II, III, and XII) whereas non-archetypal (atypical) strains, with highly diverse genotypes are found in South America. In Europe and North America, the majority of human and livestock infections are caused by type II strains, the infection being usually inapparent, whereas severe cases of acquired toxoplasmosis in immunocompetent patients has been observed in South America due to atypical strains (Galal et al., 2019). Disease development also depends on the type of host. Several species seem to be naturally resistant to *T. gondii* infection (for example rats, cows), while others are very susceptible (for example wallabies, squirrel monkey). In mouse models, mice are natural hosts for *T. gondii*, type I *T. gondii* strains are highly virulent whereas type II and type III strains are less virulent.

2.3. Immune response against *T. gondii*

Toxoplasma gondii infection initiates strong innate immune responses, which in turn induce acquired immune responses dependent on a typical T helper type I (Th1) effector response. This involves IFN-γ-producing CD4+ T cells, cytotoxic CD8+ T cells, and a specific humoral response. A robust IFN-γ-dependent adaptive immune response is crucial for controlling chronic infection in both mice and humans. As the first line of defense, innate immune sensors detect *T. gondii* invasion, leading to the production of chemokines, interleukins, and growth factors. In mice, recognition of *T. gondii* via PRRs such as Toll-like receptors (TLRs) results in the production of type 1 IFN and proinflammatory cytokines, including TNF-α and IL-12. For instance, *T. gondii's* profilin interacts with TLR11/12, and tachyzoite-derived GPIs interact with TLR2 and TLR4, inducing Myd88-dependent IL-12 production and TNF-α secretion, respectively. (Debierre-Grockiego et al., 2003; Yarovinsky et al., 2005a). Additionally, micronemes proteins (TgMIC1 and TgMIC4) can stimulate IL- 12 secretion by bone-marrow derived DCs through stimulation of TLR2/4 (Sardinha-Silva et al., 2019). Other TLRs such as TLR7 and TLR9 have been shown to be recognized *T. gondii* RNA and DNA (Melo et al., 2011). The parasite can also be detected by C-C motif chemokine receptor 5 (CCR5) on dendritic cells, with the cyclophilin 18 acting as the ligand that stimulates IL-12 expression. (Aliberti et al., 2000). Furthermore, *T. gondii* triggers inflammasome activation via the inflammasome sensors NLRP1 and 3 in a caspase-1-dependent manner resulting in IL-1b and IL-18 production (Gorfu et al., 2014; López-Yglesias et al., 2019).

Unlike in mice, *T. gondii* infection in humans does not result in the production of TLR11- and TLR12- dependent IL-12 because these receptors are absent in humans. Human monocytes infected with *T. gondii* tachyzoites released Alarmin S100A11, leading to the induction of the chemokine C-C Motif Ligand 2 (CCL2) a major chemokine required for monocyte-mediated immunity (Safronova et al., 2019). Additionally, in infected monocytes the inflammasome sensor NLRP3 (Gov et al., 2017) is induced leading to IL-1b production. Moreover, the inflammasome sensor NLRP1 was shown to contribute to control parasite growth (Witola et al., 2011). Human monocytes and dendritic cells (CD1c+) are the major myeloid cells in human peripheral blood that produced IL-12 and TNF-a in response to phagocytic uptake of live parasites and that these cells do not produce IL-12 when exposed to soluble tachyzoite products (Tosh et al., 2016). However, neutrophils produced both IL-12 and TNF-a in response to *T. gondii* antigen stimulation (Bliss et al., 1999).

The cytokine IL-12 controls the infection through initiation of IFN-γ production and is a key cytokine that links innate and adaptive immune system (Gazzinelli et al., 1994; Yap et al., 2000). Using bicistronic IL-12- yellow fluorescent protein reporter mice, it was shown that CD11c+MHCII+F4/80- dendritic cells, F4/80+ macrophages, and Ly6G+ neutrophils were the dominant cellular sources of IL-12 in the peritoneal cavity of mice infected intraperitoneally with *T. gondii* tachyzoites (Mercer et al., 2020). IL-12 drives the expansion of NK cells, CD4+ T cells, and CD8+ T cells resulting in massive IFN-γ production. IFN-γ is also supplied by neutrophils and IFN-γ production by neutrophils is IL-12 independent but is regulated by TNF and IL-1 (Sturge et al., 2013). IFN-γ promotes multiple effectors mechanisms against *T. gondii* infection in a host species-specific and cell type-specific manner (reviewed in Lüder, 2024). *T. gondii* counteracts the host immune response. It has developped strategies, partially strain-dependent, to manipulate host immunity through secretion of mainly ROP and GRA proteins that modify host transcriptional programs or signaling pathways (reviewed in Mukhopadhyay et al., 2020)**.**

3.4. *T. gondii* in cancer immunotherapy

Based on this identified ability to stimulate strong and durable immune responses, *T. gondii* has been one of the most extensively studied protozoa for its anti-tumor activity. As early as 1977, *T. gondii* infection has been linked to a notable increase in necrotic areas within central nervous system tumors, thereby prevented tumor progression (Conley & Remington, 1977). These findings paved the way for exploring the anti-tumor potentials of *T. gondii* in a number of pre-

clinical studies. For instance, the injection of live *T. gondii* tachyzoites inhibited tumor growth in murine Lewis lung cancer ((Kim, 2007) and murine melanoma (Hunter et al., 2001).

However, as with other pathogenic organisms, the major concern in using *T. gondii* is the safety issues due to the virulence of this parasite. Therefore, efforts were to be shifted toward the generation of attenuated strains. The first created attenuated strain was CPS, a RH (type I) uracil auxotroph strain in which the Carbamoyl Phosphate Synthetase gene is disrupted (Fox and Bzik, 2002). CPS strain was applied in several antitumor experimental studies. More recently, other attenuated *T. gondii* strains lacking either lactate dehydrogenases, dense granule 17 (GRA17), dense granule 5 (GRA5) or double mutant by lacking two lactate dehydrogenases (ldh1 and ldh2) were developed (M. Chen et al., 2023a; Y. Li et al., 2021a; Zhu et al., 2021a). These attenuated strains were shown to stimulate a wide range of cellular potent immune responses (Figure 10) resulting in eradication of several solid tumors in pre-clinical mice models (reviewed in J. Chen et al., 2022).

Macrophages and dendritic cells concomitantly boosted their MyD88-dependent IL-12 production. Treated mice also showed increased tumor infiltrated CD4+ and CD8+ T-cell elevated IFN-γ production, together with reduced number of suppressive macrophages and Treg cells (Sanders et al., 2015a). Then, a ssubsequent research by the same group, evaluated the protective role of CPS against disseminated pancreatic cancer. They showed that mice inoculated with primary pancreatic tumor developed long lasting antitumor immunity and gained protection against developing disseminated tumors. This long-lasting immunity was featured by tumor-specific IgG as well as potential CD4+ T cell memory (Sanders et al., 2016). Similarly, Baird *et al*. evaluated the therapeutic potential of CPS in mice-bearing aggressive xenograft model of murine ovarian carcinoma. Intraperitoneal injection of CPS reactivated the ability of the immunosuppressive CD11c+ cells to efficiently cross-present antigen and prime CD8+ T cell responses (Baird, Fox, et al., 2013). For instance, in the study by Sanders *et al*. using CPS strain in mouse pancreatic and ovarian cancer, they showed that CPS intraperitoneal treatment significantly decreased tumor-associated macrophages and markedly increased dendritic cell infiltration. Moreover, CPS increased expression of co-stimulatory molecules CD80 and CD86 in tumor resident. Intratumor injection of CPS regressed also established dermal B16F10 melanoma by immune mechanisms (Baird et al, 2013).

Depletion with antibodies of CD8+ T cell and NK1.1 expressing cells, but not CD4+ T cells, abrogated the antitumor efficacy. CPS treatment efficacy required IL-12 and IFN-γ production. No effect on the tumor was observed in either IL12p35 or IFN-γ knockout mice. CXCR3

blockade reduced the antitumor efficacy suggesting a role of the CXCR3 axis in the recruitment of effector T cells. In a more recent study, intratumor injection of *T gondii* GRA17 knockout RH (type I) strain (RH ΔGRA17) to mice challenged with B16F10 murine melanoma, MC38 murine colon adenocarcinoma or LCC murine lung cancer, induced systemic immune responses in injected and non-injected distant tumors (Zhu et al., 2021a). This tumor regression was

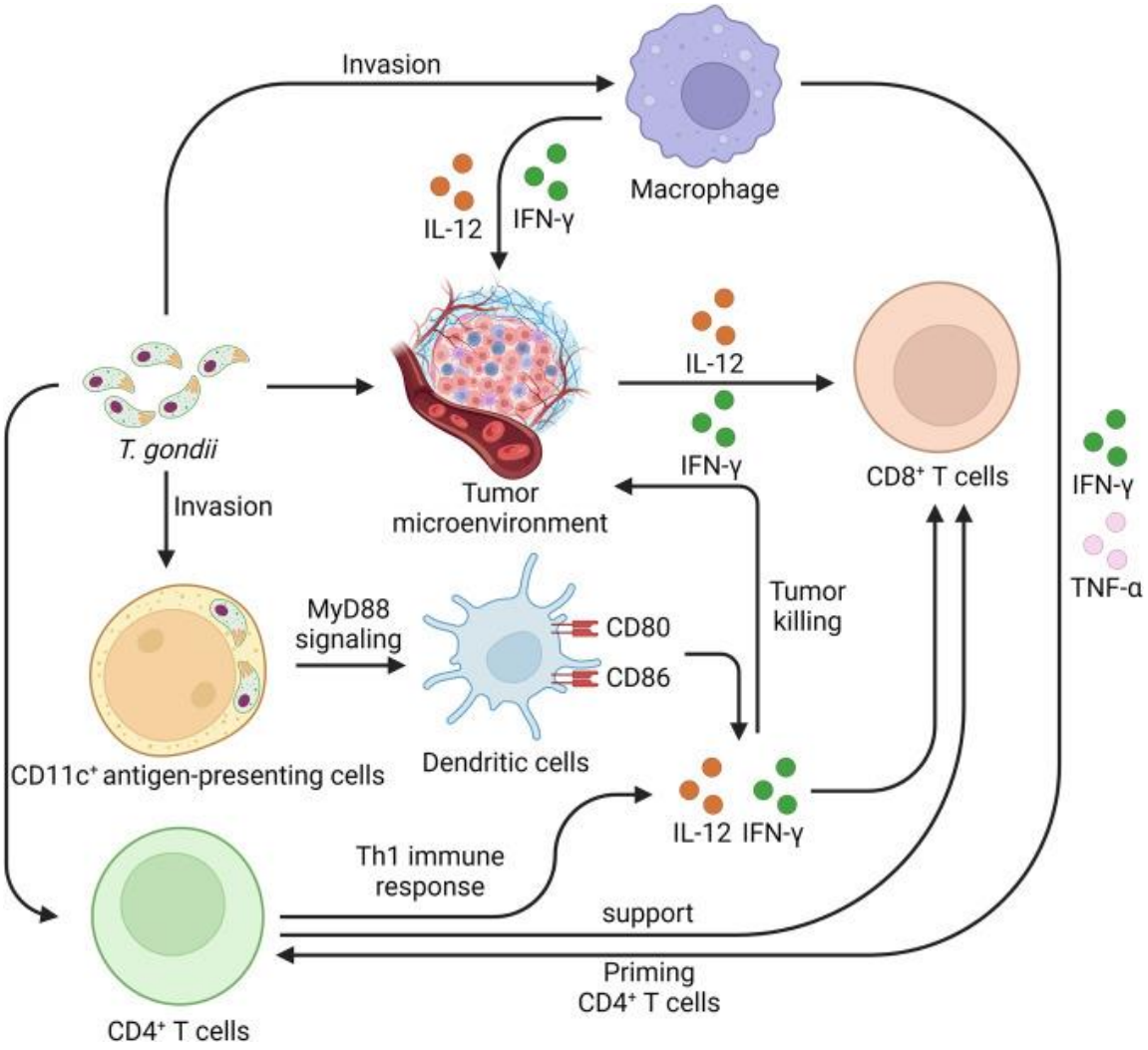


Figure 10. Mechanisms of reversing tumor-associated immunosuppression with *T. gondii*. *T. gondii* recruits macrophages that produce proinflammatory cytokines (IFN-γ and TNF-α) which in turn, enhance CD4+ T-cell priming. In addition, *T. gondii* invades CD11c+ APCs and converts DCs to a CD80- and CD86-expressing immunostimulatory phenotype via MyD88 signaling. IL-12 and IFN-γ production restricts tumor growth through enhancement of CD8+ T-cell infiltration. In addition, T. gondii infection significantly enhances infiltrating CD4+ T-cell activation and helps maintain CD8+ T-cell activation. Finally, macrophages, CD4+ T cells, and CD8+ T cells are recruited to the TME to restrict tumor growth synergistically via secreted IL-12 and IFN-γ (Yoon et al., 2024).

mediated with CD8+ and CD4+ T cells and elevated levels of IFN-γ and TNF-α. Interestingly, treatment with RH GRA17 also increased the upregulation of PD-L1 in the TME. Therefore, combining RH GRA17 treatment with 250 µg of anti-PD-L1 demonstrated notable synergistic activity with a long-term persistence of anti-tumor memory. This robust anti-tumor response led to improved survival without recurrence of cancer in treated mice (Zhu et al., 2021a). Similarly, Bahwal *et al*. evaluated the attenuated non-replicating *T. gondii* (RH strain) uracil auxotroph (NRTUA) (uridine phosphorylase and orotidine-5'-monophosphate decarboxylase genes knocked out), in pancreatic mice model as monotherapy or in combination with anti-PD-1 antibody. Combination treatment was superior in inhibiting tumor growth and markedly reduced the immunosuppressive myeloid-derived suppressor cell population in treated mice, indicating a synergistic activity between *T. gondii* and PD-1 blockade (Bahwal et al., 2022a).

Moreover, *in situ* administration of the GRA5 knockout type II strain (ME49Δgra5) to mice inoculated with murine breast cancer 4T1 inhibited tumor growth and prevented lung metastasis (M. Chen et al., 2023a). ME49Δgra5 treatment increased tumor-infiltrating lymphocytes (CD4+ and CD8+) and increased IFN-γ and IL-12 both in serum and tumor microenvironment. Intratumoral administration of another dual-mutant *T. gondii* strain lacking two lactate dehydrogenases (ME49 Δldh1-Δldh2) suppressed the growth of established tumors and helped to inhibit lethal tumor development in the mice with murine melanoma. The sera of parasite-treated mice had high levels of TNF-α and INF-γ, which likely contributed to the tumor-repressing activity (Y. Li et al., 2021b).

Taken together, these experimental data demonstrate that *T. gondii* can boost immune responses against cancer in a multimodal manner representing *T. gondii* as promising immunotherapeutic agent that can be added to the arsenal of fight against cancer.

3. Neospora caninum

3.1. Life cycle

Neospora caninum has been identified for the first time in the year 1984 when it was isolated from the central nervous system and skeletal muscle of dogs in Norway (Bjerkfis et al., 1984b) and it has been described and characterized in 1988 (Dubey et al., 1988). Like other Apicomplexan parasites, *N. caninum* can infect a wide range of domestic and wild animals including dogs, cattle, horses, goats and others. The definitive host, however, is dogs and wild canines which are considered as both intermediate and definitive hosts (Dubey, 1999). When

canines ingest food contaminated with *N. caninum* cysts, these cysts release their load of bradyzoites in the host's intestine where the sexual stage of the life cycle occurs (Figure 11). Oocysts are then shed in the feces and forms spores. Intermediate hosts become infected when they consume food or water contaminated with these spore-forming oocysts. Subsequently, oocysts release tachyzoites, the infectious proliferative form. Tachyzoites can replicate and migrate in the host and reach the central nervous system where they encyst as bradyzoites. Tachyzoites and bradyzoites, allow the asexual reproduction of *N. caninum* in the intermediate host. Furthermore, *N. caninum* can be transmitted through placenta (vertical transmission). Fetal infection results in fetal death and abortion or survive to have congenital neosporosis (Dupuy, 1999).

3.2. Neosporosis

Neosporosis, the infectious disease associated with *N. caninum* infection, is a serious health problem for livestock and the main cause of congenital calve diseases and abortion in cattle (Dubey, 1989). Although Neosporosis is not considered a zoonotic disease, yet, data from many screening studies confirmed that humans have been exposed to *N. caninum* (Dubey et al, 2007). For instance, antibodies to *N. caninum* have been identified in the serum of small percentage (6.7%) of screened human individuals (Tranas et al., 1999a). However, higher *N. caninum* seropositivity (38%) have been identified in a population of immunocompromised individuals (Lobato et al., 2006a). Due to the similarity between *N. caninum and T. gondii*, in a study by Petersen et al, women with recurrent abortion were enrolled and screened for *N. caninum* antibodies. Results revealed that there was no link between abortion and *N. caninum* since no antibodies were detected (Petersen et al, 1999). Moreover, to identify if *N. caninum* infection can be identified in human's fetus, umbilical cord blood samples were analyzed. 24% of samples were shown seropositive and *N. caninum* DNA was detected in only 1% with no corresponding detection in the placenta (Duarte et al., 2020a). These data indicate that, regardless the recorded exposure to *N. caninum*, no evidences indicated the occurrence of Neosporosis in human indicating that it is not pathogenic in humans.

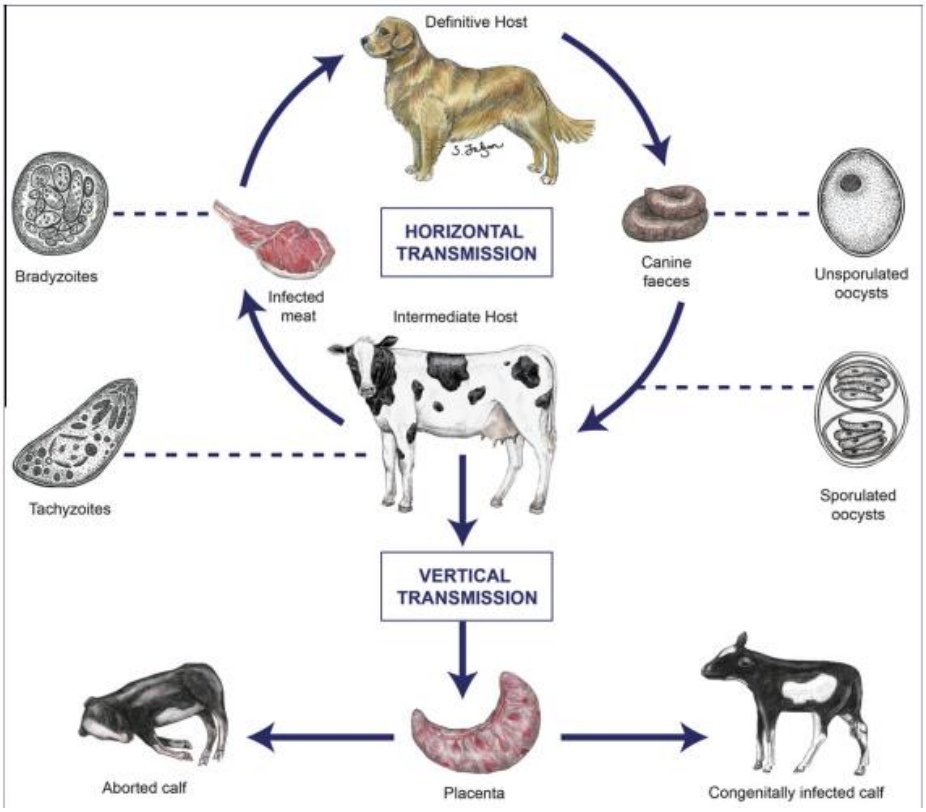


Figure 11. life cycle of *Neospora caninum*. The complete life cycle includes both sexual replication (in the canid definitive host) and asexual replication in the intermediate host (e.g. cattle). Horizontal (or postnatal) transmission (usually via oocysts and tissue cysts). Canids become infected by eating contaminated meat. Oocysts are excreted and persist in the environment then, intermediate host infected by ingesting faecally contaminated pasture or water oocysts. Vertical (or transplacental) transmission occurs when tachyzoites are transmitted transplacentally from mother to fetus (Goodswen et al., 2013).

3.3. Immune response against *N. caninum*

N. caninum infection, evokes a strong innate immune response in the infected hosts initiated via activation of many TLRs (Fereig & Nishikawa, 2020). In mice, Myeloid differentiation factor 88, a crucial adaptor protein for the IL-1 receptor and most of the TLRs, is required for resistance to *N. caninum* infection. Impaired innate responses observed after infection of mice genetically deficient for TLR2 was followed by downregulation of adaptive immune responses. These results confirmed that, TLR2 signaling strongly participates in the generation of effector immune responses against *N. caninum* (Mineo et al., 2010). *N. caninum* extravesicular vesicles are recognized by TLR-2 and have the ability to enhance the production of cytokines by BMDMs from mice (S. Li et al., 2018). Furthermore, *N. caninum* GPIs induced stimulation of both TLR2 and TLR4 from murine macrophages and DC cells (Débare et al., 2019).

Unlike *T. gondii*, TLR3 also plays a role in the immune response against *N. caninum* through recognizing the RNA and subsequently inducing type I IFN responses (Beiting et al., 2014). This has been found to enhances *N. caninum* infection control in mice via TLR3-TRIF pathway (Dos Santos Miranda et al., 2019). Moreover, as *T. gondii'*s profilin induces potent TLR11-dependent IL-12 response, similarly, *N. caninum*'s profilin -which shares over 95% amino acid identity with *T. gondii* profilin- elicited systemic IFN-γ and IL-12 responses after injection into BALB/c mice (Jenkins et al., 2010; Yarovinsky et al., 2005b). However immediate systemic IFN-γ is only partially dependent on TLR11 in infected mice (Coombs et al., 2020). *N. caninum*, as *T. gondii* is detected by CCR5 where a recombinant *N. caninum* cyclophilin (NcCyp) protein has been shown to induce the CCR5-dependent migration of murine and bovine cells (Kameyama et al., 2012). In *N. caninum*-infected CCR5 -/- mice, poor migration of DCs and NKT cells to the site of infection, associated with higher mortality were observed compared to wild type mice (Abe et al., 2015). Moreover, in murine BMDMs infected with *N. caninum*, activation of NLPR3 was associated with the release of IL-1B and IL-18, cleavage of caspase-1 and cell death (X. Wang et al., 2017).

Based on this potent immune response, *N. caninum* infection in mice is tolerable. Coombs et al. showed that mice infected with up to one million tachyzoites of *N. caninum* strains do not exhibit any observable morbidity (Coombs et al., 2020), while *T. gondii* infection on the other hand is much more pathogenic. Pathogenicity depends on the strain, *T. gondii* type I strain are much more virulent, than type II and III (Sibley, 1992). Each of the three major *T. gondii* clonal lineages has a distinct repertoire of virulence factor alleles. Mice infected with even 1 tachyzoite of virulent *T. gondii* strains will become morbid (Khan et al., 2009).

Using bioluminescence imaging, Coombs *et al*., compared the kinetic of *N. caninum and T. gondii* proliferation in BALB/c mice. They selected a high virulent (TgS23: Luc) and a low virulent (TgS1T: Luc) *T. gondii* strain for comparison with the *N. caninum* strain Nc1:Luc. After intraperitoneal injection both *T. gondii* strains and *N. caninum* proliferated at similar rates during the first 20 hours, then both *T. gondii* strains continued to grow while *N. caninum* was controlled. Within the first 24 h of infection, *N. caninum* induced significantly higher levels of proinflammatory cytokines than the two *T. gondii* strains. For instance, IL-12 and IFN-γ production occurred as early as 4 hours post infection in response to *N. caninum* infection, but IFN-γ was not detectable in *T. gondii*-infected mice during the first 24 h of infection. This early IFN-γ production was dependent upon MyD88 and IL-12 and was not detected in TLR11-/- mice. TLR11-/- mice produced serum IFN-γ beginning at 24 hours, suggesting that TLR11 is

not the only MyD88-dependent recognition mechanism capable of contributing to the production of critical IFN-γ required to control *N. caninum*. These results indicate that *T. gondii* is capable of evading host detection during the first hours after infection, while *N. caninum* is not (Coombs et al., 2020).

3.4. *N. caninum* in cancer immunotherapy

While the anti-tumor activity of *T. gondii* is reported in many studies, *N. caninum* has been newly introduced to the field of cancer immunotherapy. The high similarity between the two parasites provided the assumption that *N. caninum* may induce -as well- strong immune responses against tumors. BioMAP team (UMR ISP 1282 University of Tours – INRAe) pioneered the use of *N. caninum* to treat solid tumors in pre-clinical mice models. In an initial study by Lantier *et al*., a syngeneic preclinical model of EG7 thymic lymphoma was used, with the tumor inoculated subcutaneously in the flank of C57BL/6 mice. The mice received intratumor or remote subcutaneous injections of live *N. caninum* strain NC1 tachyzoites on days 4 and 7 post-tumor inoculation. The treatment inhibited tumor growth and provided lasting protection, preventing tumor development when mice were re-challenged on the opposite flank. Crucially, heat killed NC1 tachyzoites did not induce such protection, highlighting the necessity of the live parasite for anti-tumor activity. Further studies demonstrated that *N. caninum* could induce strong tumor regression in a NOD/SCID mouse model of human Merkel Cell Carcinoma. (Lantier et al., 2020a). Furthermore, it has been demonstrated that *N. caninum* can be engineered to express functional IL-15 associated with the alpha-subunit of the IL-15 receptor. The IL-15 secreting recombinant strain showed an added value over the wild type strain in treating solid mouse thymoma tumors and metastatic melanoma in mice models (Battistoni et al., 2023a; Lantier et al., 2020a). Similarly, a more recent study evaluated the therapeutic effect of *N. caninum* in a subcutaneous preclinical model of B16F10 melanoma in C57BL/6 mice. Results showed that intratumor and distant treatment of the tumor induced an inhibition of tumor development, associated with an increase in the number of macrophages and CD8+ T cells and higher expression at the transcriptomic level of IL-12, IFN-γ, TNF-α and IL-2 (X. Li et al., 2022a).

Neospora caninum has demonstrated significant potential as a novel agent in cancer immunotherapy. Its ability to induce strong immune responses and inhibit tumor growth, coupled with the rapid clearance of the parasite, positions it as a promising candidate for further research and development in cancer treatment.

2. Objectives

2. Objectives

Cancer immunotherapy is a rapidly evolving field seeking to harness the immune system to combat tumors. The use of live microorganisms, such as viruses and bacteria and more recently protozoan parasites, has garnered attention due to their oncolytic properties and immunostimulatory effects. In addition to their natural oncolytic activity, these microorganisms can also be engineered to potentiate their anti-tumor activity and promote safety. In particular, strategies to improve tumor tropism or to block the immunosuppressive PD-1/PD-L1 pathway by immune checkpoint inhibitors (ICIs) have shown to induce better therapeutic effects against cancer.

In the context of parasite-based immunotherapy, the closely related intracellular parasites *Toxoplasma gondii* and *Neospora caninum* have shown potential in cancer immunotherapy by several teams, including ours. Thus, in this research project, we explored the prospect of enhancing and combining the potential oncolytic activity of these two parasites with ICIs through engineering anti-PD-L1 (i) to be displayed on parasite's surface to enhance tumor-specific targeting and (ii) to be secreted by the engineered parasites to allow direct delivery and secretion into tumor site for restoration of T cells functionality.

I. *Toxoplasma gondii* retargeted to tumor cells through expression of surface anti-PD-L1 scFv

1. Previous work

Previously within BioMAP team, the immunogenic *T. gondii* SAG1 protein was attached to a single chain variable Fragment (scFv) against the murine DC-restricted antigen-uptake receptor (DEC205). This strategy was developed to improve the efficiency of vaccination against *T. gondii* by targeting *T. gondii* antigen to dendritic cells thereby improving antigen presentation and enhancing the immune response. Generally, for construction of scFv, the variable regions of light (VL) and heavy (VH) immunoglobulin chains encoding antigen-binding domains are engineered into a single polypeptide joined by a flexible linker sequence (Figure 12). To construct the anti-DEC205 scFv, VH and VL were derived from NLDC-145 (anti-DEC205 antibody) in VH-VL orientation and connected with flexible linker. *T. gondii* SAG1 was attached to the scFv sequence via a short spacer. This construction (named DC2-SAG1) proved

functional and its administration by intranasal and subcutaneous routes improved the protection against chronic *T. gondii* infection in mice (Lakhrif et al., 2018).

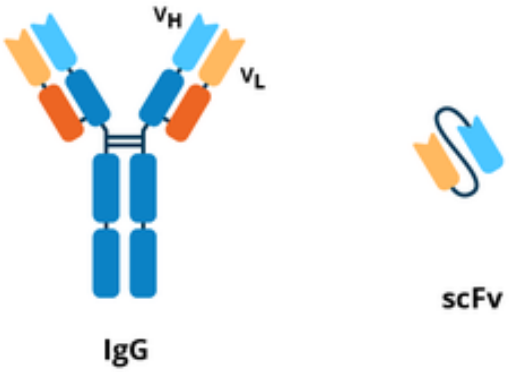


Figure 12. Intact full-length immunoglobulin G (IgG) and the single chain fragment variable (scFv).

To generate recombinant *T. gondii or N. caninum* tachyzoites, BioMAP team previously constructed two plasmids (pUC5 and pUC8 derived from a pUC18 backbone plasmid) containing two expression cassettes. One cassette was designed to express a CAT-GFP (chloramphenicol acetyl transferase - green fluorescent protein) fusion protein to allow drug selection (chloramphenicol) of stably transfected parasites, the second was designed to express proteins of interest. The expression of CAT-GFP is driven by the promoter of the *T gondii* α-tubulin gene (αTUB5) and the 3 'untranslated region of the *T. gondii* SAG1 gene. The expression of the protein of interest is driven by the promoter of the *T. gondii* α-tubulin gene (α TUB5) in which a five-repeat element was inserted upstream of the transcriptional start site for high-level expression of the protein of interest (Soldati and Boothroyd, 1995). In pUC5 the two cassettes are in the same orientation, while in pUC8 they are in opposite side.

Gene expression requires generally a genus-specific promoter. *T. gondii* and *Neospora caninum* are closely related, though phenotypically distinct (Holmdhl *et al.,* 1994). As the feasibility of using DNA vectors developed for *T. gondii* to transfect and transform *Neospora caninum* has been demonstrated (Beckers et al., 1997; Howe et al., 1997), the same constructs were used for both parasites.

2. General objective

In this research work, first we investigated the possibility of engineering *T. gondii* to express functional scFv on its surface using the skeleton of the previously constructed DC2-SAG1. This approach was adopted for targeting *T. gondii* to tumors expressing PD-L1 through engineering anti-PD-L1 scFv on *T. gondii's* surface, and the functionality of this recombinant parasite was evaluated *in vitro*. By this approach, we aimed at developing a novel strategy for safely applying invasive strain of *T. gondii* through targeting them to tumors and providing multimodal tumor inhibition mechanisms through: (i) direct lysis of tumor cells by the invasive *T. gondii*, (ii) stimulation of anti-tumor immune response, and (iii) reactivating suppressed T cells through blocking PD-1/PD-L1 immunosuppressive axis.

3. Specific objectives

3.1. The possibility of engineering scFv on the surface of *T. gondii*

The first objective was to confirm the possibility of anchoring antibody fragment on the surface of *T. gondii*. For this, the previously constructed DC2-SAG1 was used as a model to proof the concept. Thus, for the first objective, anti-DEC205 was constructed to be anchored in the surface of *T. gondii* via two strategies: either using glycosylphosphatidylinositol (GPI) signal sequence or GPI signal sequence in addition to SAG1 fusion protein. To allow for detection and characterization of the expressed protein, human influenza hemagglutinin tag (tag HA) was added. Then, we aimed at generating two recombinant *T. gondii* strains expressing anti-DEC205-GPI or anti-DEC205-GPI-SAG1 (Figure 13). For this, the sequence encoding either scFv anti-DEC205-GPI or scFv-anti-DEC205 SAG1-GPI where cloned into pUC55. Parasites electroporated with linearized plasmid containing the sequence of the antibody fragment of interest were subjected to chloramphenicol selection and confirmed when GFP positive vacuoles were observed with EVOS immunofluorescent microscope. Stable recombinant parasites were then cloned by limited dilution.

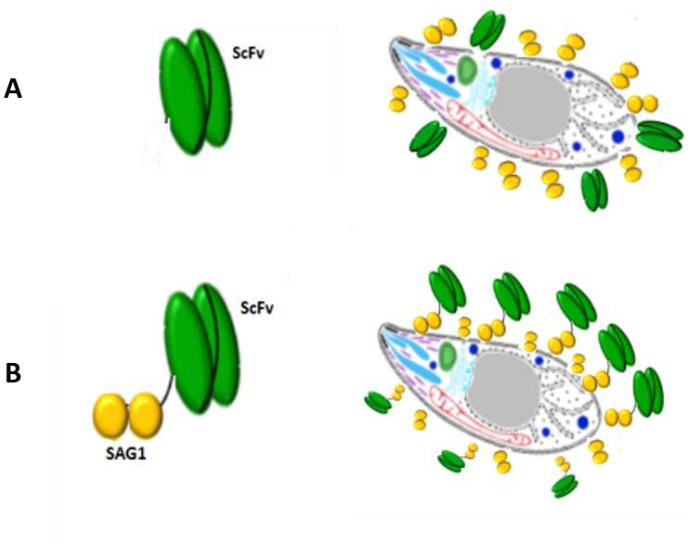


Figure 13. Schematic representation of **(A)** scFv and recombinant *T. gondii* expressing surface scFv, and **(B)** scFv fused to SAG1 protein and expressed on *T. gondii's* surface via SAG1 anchoring.

3.2. To analyze the expression and the *in vitro* functionality of the recombinant *T. gondii* expressing anti-DEC205

The second objective was to analyze the expression of the anti-DEC205 scFv displayed on the surface of *T. gondii* using immunoblotting techniques and to determine the impact of each anchoring strategy on the level of expression and functionality of the expressed protein. Next, the aim was to analyze if the expression of anti-DEC205 scFv on *T. gondii*'s surface will induce increased interaction with dendritic cells expressing the targeted receptor, DEC205. Our results indicated that fusion of SAG1 protein resulted in a better functionality.

3.3. Generation of *T. gondii* targeted to tumors expressing PD-L1 through expression of surface anti-PD-L1 scFv

Next, following the confirmation of the possibility of engineering functional scFv on the surface of *T. gondii* and determining the best anchoring strategy, the objective was to invest this approach in tumor targeting through engineering *T. gondii* strain expressing anti-PD-L1 scFv targeted to PD-L1- expressing tumors. First, with the aim of constructing anti-PD-L1 scFv to be stably expressed on *T. gondii*'s surface and to have the possibility of testing the construct in mice models, the human anti-PD-L1 Atezolizumab was selected which is known to block both human and murine PD-L1. Thus, the aim was to construct scFv VH and VL chains of Atezolizumab connected with the flexible linker and fused to SAG1 protein via the short spacer. Advances in structural studies of complexes formed between Atezolizumab and PD-L1 have

revealed that all three complementary determining regions (CDRs) from the heavy chain of Atezolizumab are involved, whereas only two from the light chain (LCDR1 and LCDR3) form partial contacts. In the light of this theory, inserting a tag HA coding sequence attached to the N-terminus region of VH chain, may affect the functionality of this fragment. As the localization of tag HA can impact the functionality of the expressed anti-PD-L1, another objective was to examine two possibilities of localizing tag HA through designing of two configurations, one with tag HA in the N terminus HA and the other with tag HA in the C-terminus of the scFv. Subsequently, the following objective was to generate two recombinant *T. gondii* strains corresponding to the two anit-PD-L1 scFv configurations.

3.4. Analysis of the expression and functionality of the surface displayed anti-PD-L1 scFv

Next, the objective was to analyze the expression and functionality of the anti-PD-L1 on both recombinant strains using different immunoblotting, immunostaining and flow cytometry techniques and to determine the impact of the tag HA localization on the protein's level of expression and functionality. Both constructions were adequately expressed but only the construct with HA in the C terminus has been shown to be functional.

3.5. The *in vitro* biological and anti-tumor activities of *T. gondii* expressing anti-PD-L1 scFv

Following confirmation of the expression and functionality, the next objective was to evaluate the impact of the surface display of anti-PD-L1 scFv on targeting tumors expressing the corresponding ligand. First, the capacity of specific binding to PD-L1-expressing human tumor cells was examined in human breast cancer cell line. Then, the objective was to confirm if the *T. gondii* expressing human anti-PD-L1 is also capable of binding to murine tumor cells expressing PD-L1 and the impact of the level of PD-L1 expression on directing the parasite-tumor cell binding. Another objective was to use a mixture of targeted (PD-L1 positive cells) and non-targeted cells to evaluate if this recombinant *T. gondii* would selectively target PD-L1 expressing cells even when they are co-existing with non-targeted cells. In order to investigate the impact of surface expression of anti-PD-L1 on the oncolytic activity of *T. gondii* in targeted tumor cells, the objective was then to treat PD-L1 expressing human and murine tumor cells *in vitro* with either the recombinant *T. gondii* or the parental unmodified strain and to measure the induced tumor lysis. The final objective was to develop an *in vitro* system of PD-L1 positive tumor cells (as target cells) and PD-1-expressing effector immune cells and to use this system to evaluate whether the surface displayed anti-PD-L1 is capable to block PD-1/PD-L1

interaction and reverse immunosuppression. Murine B16F10 tumor cells incubated with MHCI-restricted peptide SIINFEKL, were used as target cells. Murine B3Z CD8+ T cells, which recognized the SIINFEKL bound to H-2Kb MHC-I were used as antigen-specific CD8+ effector T cells. Activated B3Z cells also express β-galactosidase under the control of the IL-2 promoter, thus, the activity of B3Z can be measured by β-galactosidase and IL-2 expression. In addition to the ability of specifically target tumor cells with negligible off-target binding, the engineered parasite exhibited increased killing ability compared to the parental strain. Furthermore, co-culture assays revealed the ability of the anti-PD-L1-displaying parasites to inhibit PD-1/PD-L1 interaction and enhance CD8+ T cell-mediated immune responses.

II. Arming *Neospora caninum* with anti-PD-L1 scFv

1. Previous work

BioMAP team initiated the investigation of the anti-tumor therapeutic effect of *N. caninum* in solid tumors modeled in mice. Treating mice-bearing EG7 murine lymphoma with intratumor or distant injection of *N. caninum* live tachyzoites elicited significant tumor regression mediated by induction of immune response. Furthermore, to improve the protective effect of *N. caninum*, a recombinant strain expressing secreted form of interleukin 15 (IL-15) was developed. To construct recombinant IL-15 to be secreted by *N. caninum*, IL-15 encoding sequence was targeted to micronemes secretory organelle through fusion of the N-terminal signal peptide and predomain motif of microneme protein 5 (MIC5). This engineering strategy proved functional and substantial quantities of IL-15 was secreted in the recombinant strain (NC-IL-15hRec) culture supernatant. The secreted IL-15 resulted in reinforcing the immunostimulatory properties of *N. caninum* both *in vitro* and *in vivo.* Treating mice with EG7 tumor or metastatic B16F10 melanoma with either wild type strain or NC-IL-15hRec clearly indicated that recombinant strain has an added value in tumor growth inhibition and stimulation of the immune response (Lantier et al., 2020; Battistoni et al., 2023).

2. General objective

As it has been previously discussed, combination of oncolytic microorganism therapy with PD-1/PD-L1 blockade, highly potentiates the anti-tumor responses and increases the sensitivity to anti-PD-L1 or anti-PD-1 therapy. Hence, in this axis of the research work, we aimed at augmenting the anti-tumor activity of *N. caninum* by coupling it to PD-1/PD-L1 blockade through arming *N. caninum* with anti-PD-L1 scFv to be displayed on the surface or to be

secreted by the engineered parasite. As it has been shown in the previous work that *N. caninum* can be safely administered to treat tumors in mice, we hypothesized that engineering the anti-PD-L1 scFv on the surface would lead to increased accumulation of injected parasite in targeted tumors and reduce dissemination to other tissue, a strategy that would be beneficial in treating non-accessible tumors distantly.

The second approach was to arm *N. caninum* with secreted anti-PD-L1 scFv to allow for its secretion directly in tumor bed. This strategy would offer the benefits of (i) restoration of T cells functionality and anti-tumor immune response due to PD-1/PD-L1 blockage, (ii) targeted drug delivery of the expressed therapeutic molecules into the tumors which will increase intra-tumor concentrations of the therapeutic fragments and reduce systemic toxicity.

3. Specific objectives

3.1. Generation of recombinant *N. caninum* strain expressing surface anti-PD-L1 scFv

As it has been previously shown that *T. gondii* SAG1 protein could be expressed by *N. caninum* (Zhang *et al.,* 2010), the first objective was investigating the possibility of expressing anti-PD-L1 scFv on the surface of *N. caninum* using the same previously constructed plasmid to express anti-PD-L1 on *T. gondii*. Then, to analyze the level and stability of the expression and the functionality of the expressed protein. Nevertheless, for this objective, efforts to express functional anti-PD-L1 on the surface of *N. caninum* were unsuccessful.

3.2. Generation of recombinant *N. caninum* strain secreting anti-PD-L1 scFv

The second objective was to construct anti-PD-L1 scFv secreted by the recombinant engineered strain. For this objective, the same strategy used to construct the secreted IL-15 was adopted by using the anti-PD-L1 scFv fragment previously designed, attached to sequence encoding the N-terminal signal peptide and predomain TgMIC5. Then the aim was to engineer *N. caninum* with this constructed antibody to generate the recombinant strain. Another objective was to produce a recombinant purified anti-PD-L scFv using the Chinese Hamster Ovary (CHO) cells system to be used as a reference antibody during characterization experiments.

3.3. Analysis of the expression and functionality of anti-PD-L1 scFv secreted by recombinant *N. caninum*

After generating the recombinant strains, the following objective was to see if this strain is capable of secreting functional anti-PD-L1 scFv. This objective was fulfilled through analyzing culture supernatants of recombinant *N. caninum* for the presence of anti-PD-L1 fragment using different immunoblotting techniques. Then the functionality of the secreted scFv was analyzed by binding to its target protein.

3.4. Quantification of PD-L1-scFv secreted in culture supernatant

Another critical point in designing secreted form of antibodies, is the amount of the antibody secreted by the expression system since this amount will determine the biological activity. Thus, the next objective was to measure the concentration of the anti-PD-L1 scFv secreted by the recombinant *N. caninum* tachyzoites. For this, the amount of scFv in culture supernatants collected from different culture conditions and by using different doses of parasites were analyzed by immunoblotting for estimated quantification. For the more precise quantification, culture supernatants were subjected to ELISA using the antibody purified from the CHO system as a reference.

3.5. *In vitro* biological functionality of the secreted anti-PD-L1 scFv

Following verifying that recombinant *N. caninum* could secret functional and measurable quantities of antiPD-L1 scFv, then the objective was to analyze the biological activity of this secreted scFv. First, the ability of the secreted scFv to bind to PD-L1 expressing tumor cell was analyzed in human and murine tumor cell lines. Then the co-culture system established earlier (Murine B16F10 tumor cells and Murine B3Z CD8+ T cells) was used to evaluate if the secreted anti-PD-L1 scFv was capable of blocking PD-1/PD-L1 indicated by increased cytokine secretion by T cell.

3. Article

Specific Cell Targeting by *Toxoplasma gondii* displaying Functional Single Chain Variable Fragment as a Novel Strategy; a Proof of Principle

Objectives:

The aim of this study was to investigate the possibility of engineering *Toxoplasma gondii* to display functional single chain variable fragments (scFv) on its surface as a strategy to increase specific cell targeting. First the aim was to target dendritic cells by engineering *T. gondii* displaying scFv targeting the mouse dendritic cells endocytic receptor, DEC205. Next the objective was to engineer *T. gondii* targeting tumors expressing protein death ligand PD-L1 by displaying surface anti-PD-L1 scFv.

Methods:

The scFv targeting DEC205 was constructed using the heavy (VH) and light (VL) chains of the anti-DEC205 NLDC-145 connected with a flexible linker in VH-linker-VL orientation. An HA tag was added via short spacer to the N-terminus of the scFv sequence. Anchoring on parasite's surface was targeted either by using the anchoring signal of the surface anchor glycosylphosphatidylinositol (GPI) or by GPI plus the surface protein SAG1 (GPI-SAG1). Anti-PD-L1 scFv was generated using the heavy (VH) and light (VL) chains of the anti-PD-L1 Atezolizumab in VH-linker-VL orientation with GPI and SAG1 fusion protein. An HA tag was also incorporated either in the N-terminus of the scFv sequence or attached to the C-terminus via short spacer.

Results:

The anti-DEC205 scFv was correctly displayed on *T. gondii*'s surface and showed pronounced functionality following both anchoring techniques (GPI and GPI-SAG1). However, fusion of SAG1 protein resulted in a better functionality as indicated by binding to recombinant DEC205 protein and DCs expressing the DEC205 receptor. The *T. gondii* strains expressing the both configurations of anti-PD-L1 scFv showed adequate expression. However, the strain engineered with the construct with the HA tag attached to the C-terminus showed remarkable functionality on binding to the target cells and recombinant protein. In mixed cell populations, anti-PD-L1 expressing *T. gondii* bound specifically to PD-L1 overexpressing cells with minimal off-target binding. This recombinant strain exhibited superior killing ability of PD-L1 positive tumor cells and significantly increased CD8+ T cell immune response to tumors compared to the parental strain *in vitro*.

Conclusion

Engineering *T. gondii* to display functional scFv on its surface is a viable strategy for specific cell targeting, with potential applications in targeted tumor therapy.

Specific Cell Targeting by *Toxoplasma gondii* displaying Functional Single Chain Variable Fragment as a Novel Strategy; a Proof of Principle

(Article Accepted in Cells)

Muna Aljieli[1,2], Clément Rivière[1], Louis Lantier[1], Nathalie Moiré[1], Zineb Lakhrif[1], Anne-France Boussemart[1], Thomas Cnudde[1], Laurie Lajoie[1], Nicolas Aubrey[1], Elhadi. M. Ahmed[2], Isabelle Dimier-Poisson[1], Anne Di-Tommaso[1,*], Marie-Noëlle Mévélec[1]

[1]BioMAP, UMR ISP 1282 INRAe – Université de Tours, 37200 Tours, France

[2] Faculty of Pharmacy, University of Gezira, Wad Madani, Sudan

Correspondence: anne.ditommaso@univ-tours.fr

Abstract: *Toxoplasma gondii* holds significant therapeutic potential; however, its nonspecific invasiveness results in off-target effects. The purpose of this study is to evaluate whether *T. gondii* specificity can be improved by surface-display of scFv directed against Dendritic cells endocytic receptor, DEC205 and immune checkpoint PD-L1. Anti-DEC205 scFv was anchored to the *T. gondii* surface either directly via glycosylphosphatidylinositol (GPI) or by fusion with the SAG1 protein. Both constructs were successfully expressed but the binding results suggested that the anti-DEC-SAG1 scFv had more reliable functionality towards recombinant DEC protein and DEC205-expressing MutuDC cells. Two anti-PD-L1 scFv constructs were developed that differed in the localization of the HA tag. Both constructs were adequately expressed but the localization of HA tag determined the functionality by binding to PD-L1 protein. Co-incubation of *T. gondii* displaying anti-PD-L1 scFv with tumor cells expressing/displaying different levels of PD-L1 showed strong binding depending on the level of available biomarker. Neutralization assays confirmed that binding was due to the specific interaction between anti-PD-L1 scFv and its ligand. A mixed cell assay showed that *T. gondii* expressing anti-PD-L1 scFv predominately targets the PD-L1 positive cells, with negligible off-target binding. The recombinant RH-PD-L1-C strain showed increased killing ability on PD-L1+ tumor cell lines compared to the parental strain. Moreover, a co-culture assay of target tumor cells and effector CD8+ T cells showed that our model could inhibit PD1/PD-L1 interaction and potentiate T cell immune response. These findings highlight surface display of antibody fragments as a promising strategy of targeting replicative *T. gondii* strains while minimizing nonspecific binding.

Keywords: *Toxoplasma gondii*; surface display; targeting; scFv; cancer immunotherapy; immune checkpoint; PD-L1.

1. Introduction

Toxoplasma gondii is an obligate intracellular apicomplexan protozoan with a natural ability to infect all nucleated mammalian cells [1]. In humans, primary infection with *T. gondii* in immunocompetent individuals is predominantly asymptomatic. However, it becomes a significant concern in cases of materno-fetal transmission or in severely immunocompromised individuals[2]. It has been reported that *T. gondii* infection can stimulate a cascade of potent immunological responses characterized by the generation of specific CD4+ and CD8+ T cells that produce interferon gamma (IFN-γ) [3]. The potential therapeutic benefits of *Toxoplasma gondii* infection in various diseases, including cancer, have been reported decades ago. Chronic infection and even parasite extracts have been shown to confer resistance in mice to numerous pathogens, including viruses [4], bacteria [5], parasites [6], yeast [7] and tumors [8, 9].

It is noteworthy that, in the context of tumors, immunotherapy based on live microorganisms such as viruses and bacteria has emerged as a promising treatment strategy. Several microorganisms are currently undergoing preclinical and clinical trials to investigate their oncolytic potential [10,11] . Moreover, the advances in genetic engineering allowed for application of several strategies to enhance oncolysis and immune stimulation or to counteract the tumor neovascularization [12,13] Additionally, several attenuated viruses and bacteria strains have been generated and widely investigated as vaccine vectors to elicit tumor antigen-specific CD8+ T cell responses and induce protective immunity against tumor development [14,15]. In 2015, the U.S Food and Drug Administration (FDA) has approved the use of the recombinant attenuated herpes simplex virus encoding human granulocyte macrophage colony-stimulating factor (GM-CSF) for treatment of melanoma patients with injectable but non-resectable lesions in the skin and lymph nodes [16].

In the context of microorganisms-based immunotherapy, *T. gondii* merges as a promising candidate for immunotherapeutic applications. However, to ensure its safe implementation, significant challenges must be addressed. Indeed, uncontrolled parasite reproduction, inflammatory processes, and damage to healthy tissues might all contribute to undesirable adverse effects. To address this challenge, attenuated strains have demonstrated considerable utility, facilitated by advancements in genetic manipulation tools developed since 1993 [17]. The first recombinant attenuated strain was a live, non-replicating parasite, named CPS (in which Carbamoyl Phosphate Synthetase gene is disrupted) [18]. This strain had successfully achieved tumor regressions in murine models of melanoma [19], ovarian cancer [20], and pancreatic cancer [21] following intratumoral injections of the parasite. The live parasites activate the immune system, counteracting immunosuppression and thereby reactivating the tumor's specific adaptive immunity. Recently, more attenuated strains have been generated including those lacking lactate dehydrogenases, dense granule 17 (GRA17), or dense granule 5 (GRA5). These strains demonstrated effective anti-tumor effects in mice models [22,23,24].

Despite the successful application of attenuated strains, nonetheless, the invasiveness of the live *T. gondii* remains crucial for its anti-tumor activity [25]. Therefore, developing novel strategies allowing the use of replicative strains is also essential to enhance oncolysis and

overcome the limitations of attenuation. One of these strategies is targeting tumor-associated antigens that are uniquely expressed or significantly overexpressed in tumor cells. This approach has been widely applied to several viruses [26] and bacteria [27] notably through expression of antibody fragments directed against tumor specific surface antigens. Targeting strategies have emerged as promising avenues for future vaccines and therapies. In the context of vaccination, dendritic cells (DCs) represent one of the most optimal targets due to their potent ability to prime immune responses [28]. Among the various receptors suitable for targeting on DCs is the DEC205 receptor which has been extensively studied for antigen delivery. We previously employed this strategy to improve vaccine efficiency against *T. gondii* where we showed that a *T. gondii* antigen, SAG1 targeted to DCs via single chain variable fragment (scFv) against DEC205 (NLDC-145) by intranasal and subcutaneous administration improved the protection against chronic *T. gondii* infection [29]. Interestingly, in a previous study, Michon *et al*. (2015) successfully expressed NLDC-145 scFv on the surface of *Lactobacillus plantarum* using different anchoring techniques [30]. Depending on the surface anchoring method, surface display of anti-DEC205 scFv efficiently enhanced *L. plantarum* internalization. Taken together, these findings suggest that anti-DEC205 scFv would be a suitable choice for assessing *T. gondii's* capacity to produce functional membrane-anchored antibodies.

Therefore, to test our hypothesis, we sought to engineer *T. gondii* strain displaying anti-DEC205 NLDC-145 scFv on its surface. The scFv comprises the heavy (VH) and light (VL) chains of an antibody linked with a flexible peptide [(Gly4Ser)3] in the VH-linker-VL orientation. An HA tag was incorporated in the N-terminus of the heavy chain for immune detection and characterization. Proteins displayed on the *T. gondii* tachyzoite's surface are glycosylphosphatidylinositol (GPI)-anchored antigens known as SAGs (surface antigens). Among these surface proteins, SAG1, also referred to as SRS29B, is the most abundant antigen [31,32]. In this study, we investigated two possible strategies for anchoring anti-DEC scFv on the surface of *T. gondii*: direct GPI anchoring or fusion of SAG1 protein to the scFv sequence. Our results indicated that fusion of SAG1 protein resulted in a better functionality.

Next, to further explore if this approach can be expanded to construct other scFvs, we aimed to target tumor cells expressing the programmed death ligand 1 (PD-L1) by expressing anti-PD-L1 scFv on the surface of *T. gondii*. For this purpose, we used the scFv fragment of Atezolizumab, a monoclonal antibody against human PD-L1, which has been approved by the FDA for the treatment of advanced urothelial carcinoma and metastatic non-small cell lung cancer [33,34]. Structural studies of complexes formed between Atezolizumab and PD-L1 have revealed that all three complementary determining regions (CDRs) from the heavy chain of Atezolizumab are involved, whereas only two from the light chain (LCDR1 and LCDR3) form partial contacts [35,36]. In consideration of this hypothesis, inserting a HA tag coding sequence attached to the N-terminus region of the VH chain may impact the functionality of this fragment. Therefore, we compared a construct with the HA tag positioned outside the scFv, in the N-terminal part of SAG1 and linked via a GGGAS spacer to the C-terminus of the VL, to another construct where the HA tag is positioned at the N-terminal part of the VH. Our results indicate that indeed, in our model, the position of the HA tag may affect the interaction with

PD-L1.

The aim of this study was to investigate in vitro, whether *T. gondii* engineered to express antibodies, in the scFv format displayed at the surface, enhances selective interaction with cells that express the antigen recognized by these scFvs. To the best of our knowledge, this is the first study to evaluate the functionality of antibodies expressed by *T. gondii* and to evaluate replicative *T. gondii* strains in tumor targeting.

2. Materials and Methods

2.1. Parasites

Tachyzoites of the RH strain of *T. gondii* were cultivated in Human Foreskin Fibroblasts (HFFs; ATCC CRL-1634) in Dulbecco's Modified Eagle Medium 2 mM L-glutamine (DMEM, Gibco, Thermo Fisher Scientific) with 10% Fetal Bovine Serum (FBS; Dutscher), 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco, Thermo Fisher Scientific) and 1% HEPES (Biowest). Cells were cultured at 37 °C in 5% carbon dioxide (CO_2) and infected with tachyzoites when they were 90% confluent. Tachyzoites were collected when they were freshly egressed.

2.2. Cell Lines

Human breast cancer cell lines MDA-MB-231 (kindly provided by Professor Emilie Allard-Vannier, EA 6295 Nanomedicaments et Nanosondes, University of Tours, France) were cultured in DMEM 2 mM L-glutamine supplemented with 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin and 1% Non-Essential Amino Acid (NEAA, PAN-Biotech).

Two murine melanoma cell lines were also used, B16F10 transfected with mCherry fluorescent protein (kindly provided by Dr. Mehdi Khaled, UMR 1299, Gustave Roussy Institute, Paris-Saclay University, France), and B16K1 (kindly provided by Dr. Laurent Gros, IRCM, INSERM, U896; University of Montpellier, France). These cells were grown in Roswell Park Memorial Institute medium 2 mM L-glutamine (RPMI 1640, Gibco, Thermo Fisher Scientific) containing 10% FBS and 50 U/ml penicillin and 50 µg/ml streptomycin.

The murine dendritic cell line MutuDC-1950 (kindly provided by Dr. Hans Acha-Orbea, University of Lausanne, Epalinges, Switzerland) were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Thermo Fisher Scientific) supplemented with 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin, 10 mM HEPES and 50 µM β-mercaptoethanol (Sigma-Aldrich). The B3Z reporter hybridoma, specific for the MHCI-restricted peptide SIINFEKL (OVA257-264) in association with Kb class I MHC molecules, that express β-galactosidase under the control of the IL-2 promoter (kindly provided by Dr. Nicolas Blanchard, Center for Pathophysiology Toulouse-Purpan (CPTP), INSERM, CNRS, University of Toulouse, Toulouse, France) was grown in RPMI containing 2 mM L-glutamine, 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin, 1% sodium pyruvate (Gibco, Thermo Fisher Scientific) and 50µM β-mercaptoethanol. All cells were cultured at 37 °C in 5% CO_2. For all experiments, cells were collected when they were in the logarithmic growth phase.

2.3. Antibodies Construct

Two expression cassettes were constructed to constitutively express proteins in *T. gondii* using 5′ αTub promoter and 3′ SAG1 UTRs . These sequences are available from GenBank, with accession numbers M20024 and X14080 respectively. One expression cassette was designed to express a CAT-GFP fusion protein to allow drug selection of stably transfected parasites [37,38], the second was designed to express proteins of interest. In the latter, a five-repeat element was inserted in the *T. gondii* α-tubulin gene, upstream of the transcriptional start site (leading to promoter α-TUB8) for high-level expression of the protein of interest [39]. Two enzymes restriction sites *PmeI* and *NotI* were also included between the α-TUB8 and 3'UTR SAG1 sequences to allow insertion of the sequence encoding the protein of interest. The two cassettes (File S1) were cloned into pUC18, in the same orientation (pUC5) or in the reverse orientation (pUC8). In this study, all proteins of interest are membrane bound proteins fused or not to the *T. gondii* surface antigen SAG1 (Genbank, accession number X14080), the major GPI-anchored protein of tachyzoites [40,41]. To achieve proper targeting, the proteins contain the following elements: the N terminal signal sequence of SAG1 (MFPKAVRRAVTAGVFAAPTLMSFLRCGVMASD) including the Kozak sequence and the ATG start codon, the sequence encoding the scFv of interest, fused or not to the SAG1 sequence (D1D2 domains, [29]), the gpi anchor signal sequence of SAG1 (AAGTASHVSIFAMVIGLIGSIAACVA) and the stop codon.

The sequence encoding the scFv of interest comprised, a variable heavy chain (VH), a (GGGGS)x3 linker, a variable light chain (VL) derived from an anti-DEC205 (NLDC-145) [42] or an anti-PD-L1 (Atezolizumab available on IMGT information system) and a linker (GGGAS) at the C terminus. A HA tag (YPYDVPDYA) was incorporated at the N terminus of either the scFv or the SAG1 protein. The sequences for all newly constructed genes were confirmed by DNA sequencing (GATC Online).

2.4. Generation of Recombinant *Toxoplasma gondii* Strains

Transfections were performed with 10^7 tachyzoites resuspended In cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4: KH_2PO_4, 25 mM HEPES, 2 mM EDTA, 5 mM $MgCl_2$, 3 mM ATP and 3 mM glutathione, pH 7.6) along with 50 μg of linearized plasmid (using a Biorad Gene PulserII Electroporator (Bio-Rad, Hercules, CA) at settings of 2000 V, 50 ohms and 25 μF. Tachyzoites were then transferred to a fresh culture of HFF cells. After overnight growth, transfectants were selected with 20 μM chloramphenicol for three passages before cloning by limiting dilution in 96 well plates. Stably transfected clonal parasite lines were designated : RH-DC2 (RH expressing an anti-DEC205 with N-terminal HA tag [DC2]), RH-DC2-SAG1 (RH expressing an anti-DEC205 fused to SAG1 with N-terminal HA tag [DC2-SAG1]), RH-PD-L1-N (RH expressing an anti-PD-L1 fused to SAG1 with N-terminal HA tag [anti-PD-L1-N]) and RH-PD-L1-C (RH expressing an anti-PD-L1 fused to SAG1 with C-terminal HA tag [anti-PD-L1-C]).

2.5. Recombinant DEC205 Protein (CF14)

The sequence coding for the ligand binding activity of DEC205 (UniProt ID : Q60767) including, the N terminal cysteine-rich domain (CR), followed by the fibronectin type II domain (FN), and four C-type lectin-like domains (CTLD 1-4) was generated by gene synthesis (GeneArt). The synthetic gene was inserted in the plasmid vector pMT/BiP/V5 (Invitrogen) using *Bgl*II and *Nhe*I restriction enzymes and a Twin StrepTag (SAWSHPQFEK(GGGS)2GGSAWSHPQFEK) nucleotide-coding sequence was introduced at the C-Terminal end using *Nhe*I and *Xho*I restriction enzymes. Schneider 2 cells (S2 cells) were transfected with the generated plasmid, using the Drosophila expression system (DES) purchased from Invitrogen. CF14 was purified from the supernatant of stably transfected cells, using a Strep-Tactin® Sepharose® column (2-1202-101, IBA) following the manufacturer's protocol. The purified protein was analyzed by SDS-PAGE with coomassie blue staining.

2.6. Analysis of HA-tagged Proteins Expression

2.6.1. ELISA Assay

ELISA was performed on whole tachyzoites of the recombinant *T. gondii* strains. In brief, 2×10^5 parasites/well were coated on a flat-bottom 96-well plate (Maxisorp Nunc, Roskilde, Denmark) and fixed with 0.5% glutaraldehyde for 5 min at room temperature. The assay plates were blocked with phosphate-buffered saline (PBS) containing 4% Bovine Serum Albumin (BSA, Sigma) for 2 h at 37 °C. Following saturation, tachyzoites were incubated with rabbit anti-HA polyclonal antibody (Invitrogen) followed by goat anti-rabbit IgG, alkaline phosphatase conjugate (Sigma). Bound phosphatase activity was measured with p –nitrophenylphosphate (PNPP, Sigma) (1 mg/ml in DEA-HCl 1 M buffer, pH 9.8). The OD at 405 nm of each well was then read using micro-plates reader (BioTek).

2.6.2. Immunoblot Analysis

Electrophoresis and immunoblotting of *T. gondii* tachyzoites were performed as previously described [43]. Briefly, freshly released tachyzoites (5×10^6 tachyzoites/20 μL) were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing dithiothreitol (50 mM) and loaded on homogenous 10% gels. After electrophoresis, proteins were transferred onto a nitrocellulose membrane, and probed with rabbit anti-HA polyclonal antibody followed by anti-rabbit secondary antibody conjugated to alkaline phosphatase (Sigma). Alkaline phosphatase activity was detected using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) liquid substrate system (Promega). Prosieve QuadColor Protein Markers (Lonza) was used to determine the protein molecular weights.

2.6.3. Dot Blot Assay

To compare the relative abundance of the HA-tagged proteins expressed in different recombinant *T. gondii* strains, 1 μL of 2-fold serially diluted tachyzoite crude lysates (prepared in SDS-PAGE sample buffer) was applied dot-wise to a dry nitrocellulose membrane. The

membrane was then left to dry for 15 min before proceeding with the detection process using rabbit anti-HA polyclonal antibody followed by anti-rabbit secondary antibody conjugated to Horseradish peroxidase (Invitrogen) as described for Immunoblot analysis. Dots were visualized using luminol chemiluminescent substrate (SuperSignal West Pico PLUS Chemiluminescent Substrate, ThermoFisher Scientific).

2.6.4. Immunofluorescent Assay

For localization of the scFv to the surface of recombinant *T. gondii*, approximately, 1×10^5 freshly egressed tachyzoites were collected from infected HFF cells and washed in cold PBS. Tachyzoites were fixed in PBS containing 4% paraformaldehyde (PAF, Thermo Fisher Scientific) followed by incubation with rabbit anti-HA polyclonal antibody and a mouse anti-SAG1 (1E5) monoclonal antibody [44], simultaneously overnight at 4 °C. Following incubation, tachyzoites were double stained with Alexa flour 488-conjugated goat anti-rabbit IgG and biotin goat anti-mouse IgG followed by Alexa flour 594-conjugated streptavidin for 30 min at room temperature. After washing in PBS, nuclei of tachyzoites were stained using Hoechst. Slides were mounted using Immu-Mount (Thermo Scientific) and images were captured with Olympus IX73 fluorescent microscope using cellSens Dimension software. Except for the mouse anti-SAG1 monoclonal antibody, all antibodies and reagents were purchased from Thermo Fisher Scientific.

2.7. Characterization and Functionality of Recombinant *T. gondii* Expressing Murine anti-DEC205 scFv

2.7.1. Binding to DEC205 Protein

The functionality of the expressed anti-DEC205 (DC2 or DC2-SAG1) was tested by binding to the CF14. In an ELISA-based assay, Maxisorp 96-well plates were coated with 5 μg/ml of CF14 diluted in PBS and incubated overnight at 4°C. After blocking of the coated wells with the saturation buffer for 2 h at 37 °C, tachyzoites of the selected RH-DC2 or RH-DC2-SAG1 clones were incubated with CF14 (5×10^5 /well) for another 2 h at 37°C. Binding of the parasites to CF14 was assayed using *T. gondii* polyclonal antibody from infected rabbit serum followed by alkaline phosphatase conjugated mouse monoclonal anti-rabbit IgG (Sigma). Bound phosphatase activity was revealed with 1 mg/ml PNPP (Sigma) and quantified by determining the absorbance at 405 nm using micro-plates reader (BioTek).

2.7.2. Binding to MutuDC Cells

Binding of the selected clones of RH-DC2 and RH-DC2-SAG1 to the murine dendritic cell line -MutuDC-1950- which is known to express DEC205 [45] was determined by flow cytometry. MutuDC cells were harvested and 10^6 cells were mixed with $2x10^6$ parasites at multiplicity of infection (MOI) of 2 in cold PBS containing 5% FBS and incubated for 1 h on ice. Unbound parasites were removed by three washes at 100 g for 5 min, then non-specific binding was blocked by anti-FcγR monoclonal antibody (clone 2.4G2, eBioscience). Parasites binding was detected by incubating cells with mouse monoclonal antibody T4 2EI2, specific for *T. gondii*

tachyzoite surface glycoprotein, gp23 for 30 min on ice [31] followed by APC-conjugated anti-mouse IgG, for 30 min on ice. The fluorescence intensity of the cells was determined by flow cytometry (MACS Quant, Miltenyi Biotec) and data was analyzed using Flow-logic software (Miltenyi Biotech).

2.8. Characterization and Functionality of Recombinant *T. gondii* Expressing Human anti-PD-L1 scFv

2.8.1. Binding to Human PD-L1 Protein

To test the functionality of the expressed anti-PD-L1 by binding to human PD-L1, tachyzoites of the chosen clones were coated and fixed with glutaraldehyde on a flat-bottom P96-well plate. Assay plates were saturated for 2 h at 37 °C then, incubated with 10 μg/ml of a histidine-tagged recombinant human PD-L1 protein (Sino Biological) for 1 h at 37 °C. Binding of PD-L1 protein to parasites was detected using mouse monoclonal anti-poly-histidine (Sigma) followed by alkaline phosphatase conjugated anti-mouse IgG monoclonal antibody (Sigma). Bound phosphatase activity was revealed as previously described.

2.8.2. Cell Stimulation and Flow Cytometry Analysis of PD-L1 Expression

The expression of the surface PD-L1 on tumor cell lines was detected by flow cytometry. For MDA-MB231 and HFF cell lines, the cells were collected, washed in cold PBS containing 5% FBS and 5×10^5 cells were stained with PE-labeled human anti-PD-L1 (Invitrogen) for 30 min on ice. PD-L1 overexpression on B16F10 cells was induced using recombinant murine interferon gamma (IFN-γ Gibco, Thermo Fisher Scientific). Briefly, 5×10^5 B16F10 cells per well were seeded on a 6-well plate and incubated overnight to allow for adherence. Following adherence, cells were treated with 20 ng/ml of IFN-γ for 24 h. PD-L1 expression in IFN-γ-stimulated B16F10 cells as well as rested (non-stimulated) B16F10 and B16K1 cells was analyzed by staining 5×10^5 cells with APC-conjugated murine anti-PD-L1 (Invitrogen) for 30 min on ice.

2.8.3. Binding to PD-L1-Expressing Tumor Cells

Human cells, MDA-MB231 tumor cells and HFF cells and murine tumor cells, IFN-γ-stimulated B16F10, rested B16F10 and rested B16K1 cells (5×10^5) were mixed with freshly collected tachyzoites of either RH-PD-L1-C or RH-DC2-SAG1 at MOI 5 for 2 hours at 4 °C in complete cell's medium. Following incubation, cells were washed at 100 g, 5 min to remove unbound parasites. Pellets were re-suspended in PBS and adhesion was measured by the percentage of GFP positive cells using flow cytometry. For the neutralization assay, 5×10^5 cells MDA-MB231 cells were incubated with saturating concentration of Atezolizumab (20 μg/ml) and 5×10^5 IFN-γ-stimulated B16F10 cells were incubated with increased concentrations of Atezolizumab (ranging from 1-1000 ng/ml) for 2 h at 4°C. Following incubation with Atezolizumab, RH-DC2-SAG1 or RH-PD-L1-C tachyzoites were added for another 2 h. Adhesion was measured as previously described.

2.9. In Vitro Oncolytic Activity Assay

For the *in vitro* oncolytic activity experiments, MDA-MB-231 and IFN-γ-stimulated B16F10 cells were applied onto flat-bottomed 96-well culture plates at densities of 10^4 cells/well and let overnight to attach. Cells were then either left uninfected or infected with RH wild type or RH-PD-L1-C at MOI 3 and incubated for 24, 48 or 72 h. After each time point, medium was discarded and 100 µL of medium containing 10% of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide reagent (MTT, Invitrogen) were added and incubated for another 4 h. The dark purple crystals that formed in intact cells were dissolved in DMSO and viable cells were quantified by measuring the absorbance at 490 nm using micro-plates reader (BioTek). Cells viability calculated as a percentage of the control uninfected cells.

2.10. Mixed Cell Assay

Mixed cell assay was performed using a mixture of targeted and non-targeted cells. For this purpose, IFN-γ-stimulated B16F10 (targeted) and rested B16K1 (non-targeted) cells were collected and mixed together at a ratio of 1:1. Cell mixture was incubated with RH-PD-L1-C at MOI 5 for 2 hours at 4 °C. Cell mixture incubated with RH-DC2-SAG1 was used as a control. After washing at 100 g, 5 min to remove unbound parasites, cells were analyzed using flow cytometry. The two cell populations were distinguished by mCherry expression and the percentage of GFP-positive cells among each of the two cell groups indicated the percentage of parasite's binding.

2.11. Co-culture of Recombinant RH-PD-L1-C with B16F10 Tumor Cells and B3Z CD8+ T cells

B16F10 cells were pretreated with recombinant IFN-γ (20 ng/ml) for 24 h, then washed with medium to remove IFN-γ before plating on 96-wells culture plate at densities of 5×10^4 cells/well. Cells were then incubated with 10 ng/ml of ovalbumin peptide MHC1 (OVA 257-264, Invivogen) for 4 h at 37 °C. Following incubation, cells were washed with RPMI medium without red phenol to remove ovalbumin peptide and red phenol. Cell surface PD-1 expression on B3Z cells was confirmed by flow cytometer using FITC-conjugated mouse anti-PD-1 (Invitrogen). Subsequently, B3Z cells were co-cultured with B16F10 cells (ratio 1:1) alone or in the presence of the wild type RH or RH-PD-L1-C recombinant tachyzoites at MOI 3 in medium without red phenol (total volume of 200 µL/well). These co-cultures were incubated for 16 h at 37 °C, and then 150 µL of supernatants were harvested and assessed for interleukin-2 (IL-2) using an ELISA kit (Invitrogen) according to manufacturer's protocol. For measurement of β-galactosidase activity, cells mixtures were lysed by adding 50 µL of 0.01% Triton 100X (Sigma) then, 100 µL of β-galactosidase substrate [CRPG (Chlorophenol red-β-D-galactopyranoside), Roche] in HEPES 0.1 M was added to each well. Plates incubated for 1 h at 37 °C and β-galactosidase activity was revealed by measuring absorbance at 570 nm.

2.12. Statistics

All analysis was performed using Graph-Pad Prism 8.00 Software. Due to the non-normal distribution of the data, statistical significance was analyzed by the non-parametric Mann-Whitney and Kruskal-Wallis tests. Estimation of the Gaussian distribution of the data was performed using Shapiro–Wilk and D'Agostino Pearson normality tests. All the data are displayed as medians and interquartile range. Statistical differences were indicated with asterisks: * $p < 0.05$, ** $p < 0.01$.

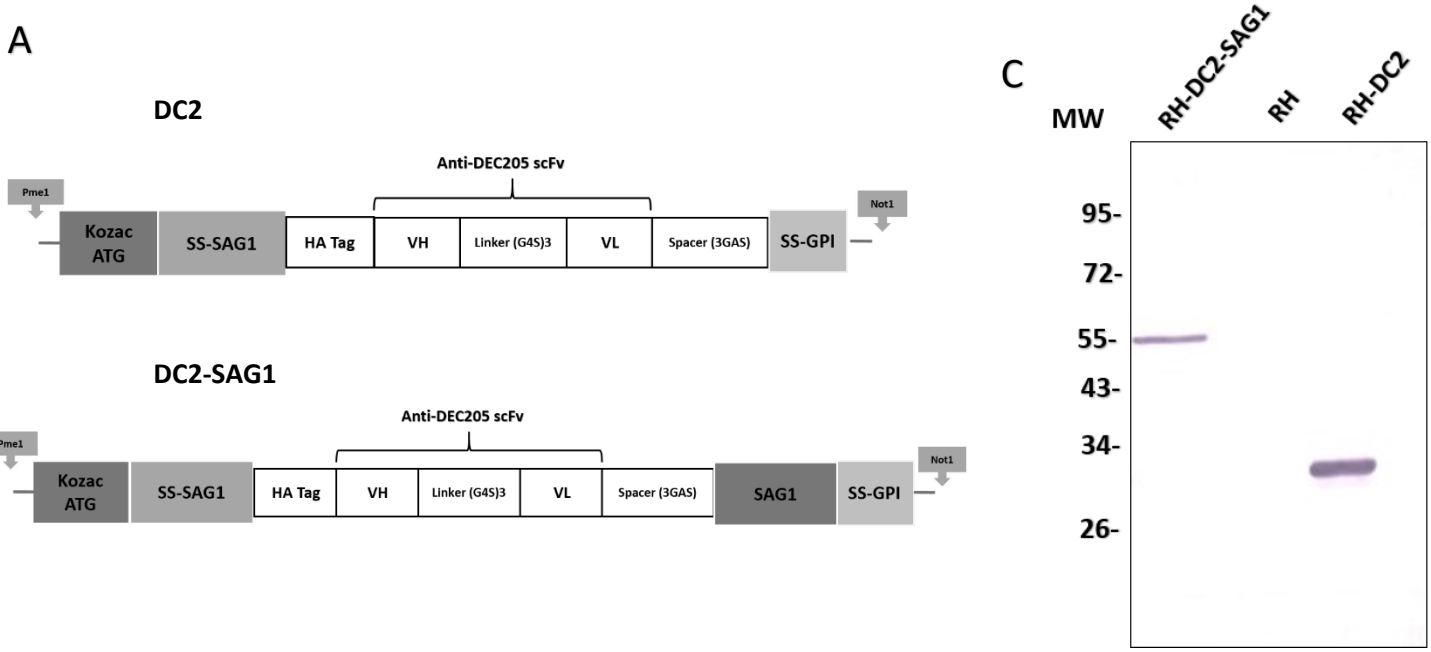
3. Results

3 .1. Design of anti-murine DEC-205 Single Chain Variable Fragment (scFv)

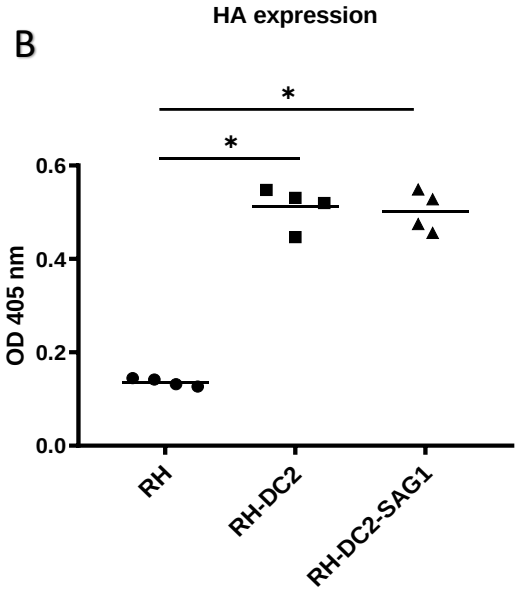
In our previous work by Lakhrif *et al*. [29], scFv directed against DEC205 was constructed by using the heavy (VH) and light (VL) chain variable regions of the monoclonal antibody NLDC145, in VH-linker-VL orientation. A sequence encoding SAG1 protein was added to the C-terminus of the scFv via a GGGAS spacer. Moreover, a similar anti-DEC205 scFv construct was successfully engineered on the surface of *L. plantarum* by using different anchoring strategies to increase targeting and internalization in DCs [30]. In their construct, Michon *et al*. added HA tag sequence in the N –terminus of the scFv for immune detection and characterization. Hereby, and in the light of this information, we hypothesized that *T. gondii* can be engineered to express surface anti-DEC205 scFv tagged with HA tag in the N-terminus . The previously constructed recombinant scFv sequence [29] was utilized, preceded by Kozak sequence, the ATG start codon, the sequence encoding the N terminal signal sequence of SAG1 and HA tag for immune detection. For attachment of the scFv to the tachyzoite's membrane, GPI anchor was used by adding sequence encoding SAG1 anchor signal (GPI). Using this skeleton, two anti-DEC205 configurations were developed (Figure 1A). In the first construct (DC2), the SAG1 anchor signal (GPI) with a stop codon was attached to the C-terminus of the scFv by a short spacer (GGGAS). In the second construct (DC2-SAG1), a sequence encoding SAG1 was introduced after the spacer (at the C-terminus of the scFv) followed by the SAG1 anchor signal (GPI) and the stop codon. Both sequences were flanked in 5' by a *PmeI* site and in 3' by a *NotI* site. Consequently, the sequences of DC2 and DC-SAG1 were cloned into pUC8 plasmid using *PmeI* and *NotI* sites to produce pUC8DC2GPI and pUC8DC2GPISAG1 respectively. Next, the plasmids were transfected into tachyzoites of *T. gondii* strain RH to generate the recombinant strains RH-DC2 and RH-DC2-SAG1 respectively.

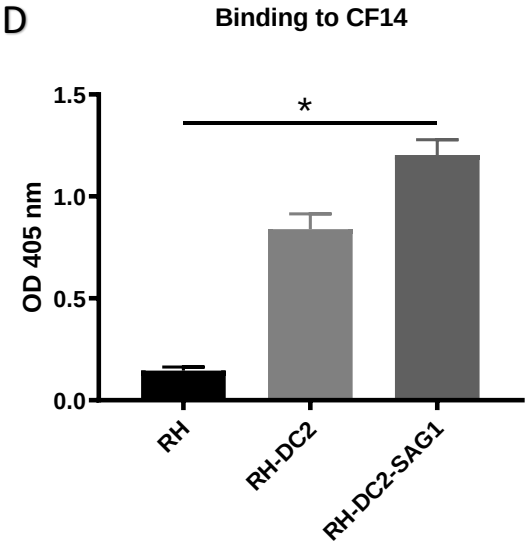
3.2. RH-DC2 and RH-DC2-SAG1 Recombinant Tachyzoites Express Functional anti-DEC205 scFv

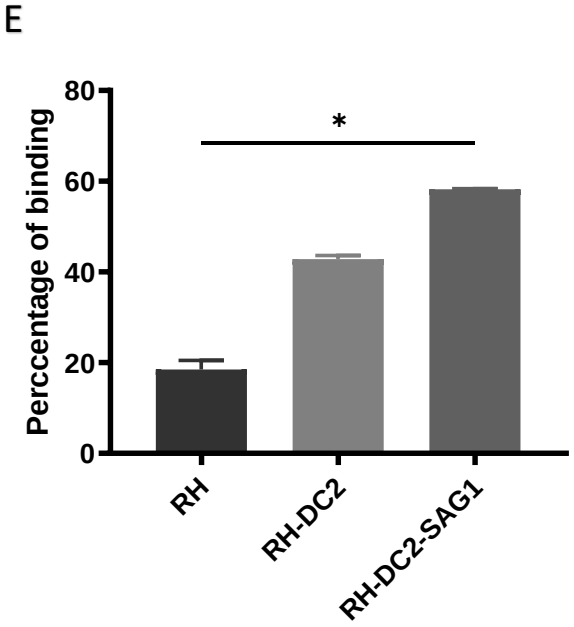
A set of ELISAs were conducted to screen the surface display of the anti-DEC205 (DC2 and DC2-SAG1), in various chosen clones from RH-DC2 and RH-DC2-SAG1 strains, respectively. Tachyzoites from the selected clones were collected from infected HFF cells and incubated with anti-HA antibody. As shown in Figure 1B, all examined clones exhibited significant levels of expression compared to wild type RH, with no discernible variation in the expression levels.


A
DC2
Anti-DEC205 scFv
Pme1
Kozac ATG
SS-SAG1
HA Tag
VH
Linker (G4S)3
VL
Spacer (3GAS)
SS-GPI
Not1
DC2-SAG1
Anti-DEC205 scFv
Pme1
Kozac ATG
SS-SAG1
HA Tag
VH
Linker (G4S)3
VL
Spacer (3GAS)
SAG1
SS-GPI
Not1

C
MW
RH-DC2-SAG1
RH
RH-DC2
95-
72-
55-
43-
34-
26-


B
HA expression
OD 405 nm
0.0
0.2
0.4
0.6
*
*
RH
RH-DC2
RH-DC2-SAG1


D
Binding to CF14
OD 405 nm
0.0
0.5
1.0
1.5
*
RH
RH-DC2
RH-DC2-SAG1


E
Perccentage of binding
0
20
40
60
80
*
RH
RH-DC2
RH-DC2-SAG1

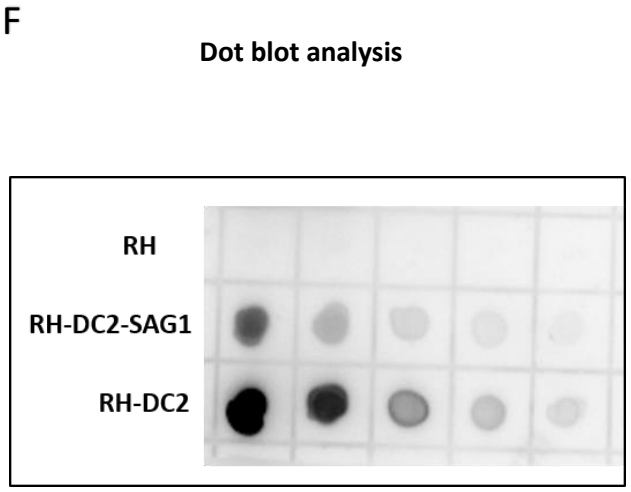

F
Dot blot analysis
RH
RH-DC2-SAG1
RH-DC2

Figure 1. Engineering and characterization of recombinant *T. gondii* expressing a single chain variable fragment directed against the murine DEC205 (anti-DEC205 scFv). (A) Schematic representation of the anti-murine DEC205 scFv (DC2) and the anti-murine DEC205 scFv fused with SAG1 (DC2-SAG1). The locations of Kozac sequence and start codon (ATG), signal sequences of the N terminus surface antigen 1 (SS-SAG1) and of glycosylphosphatidylinisotol (GPI), human influenza hemagglutinin (HA) tag, antibody variable domains (heavy [VH] and light [VL]), peptide linkers ([Gly4Ser]3) and (Gly3AlaSer), and surface antigen 1 sequence (SAG1) are indicated. (B) Screening based on HA expression by ELISA of different selected clones of RH-DC2 and RH-DC2-SAG1, using rabbit anti-HA followed by AP-conjugated anti-rabbit IgG. RH tachyzoites were used as control (n= 3 replicates). (C) Western blot from parasite cell lysates of RH-DC2-SAG1 (line 1), RH wild type (line 2) and RH-DC2 (Line 3) revealed using rabbit anti-HA followed by AP-conjugated anti-rabbit IgG. MW: molecular weight marker in kilo Dalton. (D) Binding of RH, RH-DC2 and RH-DC2-SAG1 tachyzoites to CF14 recombinant protein assessed by ELISA using *T. gondii* antibody from infected rabbit serum. (n= 3 replicates) (E) Binding of RH (control), RH-DC2 and RH-DC2-SAG1 tachyzoites to murine dendritic cells MutuDC expressing DEC205 assessed by flow cytometer using mouse monoclonal antibody specific for *T. gondii* gp23 glycoprotein, followed by APC-conjugated anti-mouse IgG. Binding was measured as the percentage of GFP positive cells (n= 4 replicates) (F) Dot blot to compare the relative abundance of the HA-tagged proteins expressed by RH-DC2 and RH-DC2-SAG1. Serial 2-fold dilutions of tachyzoite crude lysates were loaded onto nitrocellulose membrane and probed with rabbit anti-HA followed by HRP-conjugated anti-rabbit IgG and detection with chemiluminescent HRP substrate. Statistical significance is indicated by *$p < 0.05$. In all ELISA experiments, optical densities were read at 405 nm. All experiments were repeated at least three times. AP: Alkaline Phosphatase. HRP: Horseradish peroxidase.

For further investigations, one clone from each recombinant strain was chosen. To analyze if DC2 and DC2-SAG1are correctly expressed by both engineered recombinant strains, whole parasite cell lysates were analyzed by Western-blotting. Wild type RH was used as control. As revealed in Figure 1C, the DC2 and DC2-SAG1 proteins migrate at the expected size under reducing conditions (around 30 kDa and 55 kDa respectively). Next, the functionality of DC2 and DC2-SAG1 displayed at the surface of *T. gondii* was validated by their binding to the CF14. This recombinant mouse DEC205 protein, represented by the N-Terminal part of the murine DEC205 receptor which binds NLDC145 [46], was produced in the Drosophila Schneider 2 cell line. The ability of RH-DC2 and RH-DC2-SAG1 to recognize and bind to the recombinant DEC205 protein was assayed by ELISA (Figure 1D). Both engineered strains showed efficient binding to CF14, though, RH-DC2-SAG1 exhibited increased binding compared to RH-DC2.

The feasibility of binding to targeted cells was also assessed using the murine dendritic cell line, MutuDC-1950. Tachyzoites of RH-DC2 and RH-DC2-SAG1 were incubated with MutuDC cells at 4°C to allow parasites attachment to cells without internalization. Binding was demonstrated by immunostaining of bound parasites followed by flow cytometer analysis. The results were presented as percentages of binding (Figure 1E). Both RH-DC2 and RH-DC2-SAG1 demonstrated higher ability to bind to MutuDC compared to the wild type strain. In agreement with the previous results, higher binding capacity was seen with RH-DC2-SAG1

than RH-DC2 (58% and 45% respectively, compared to 20% for RH). On the basis that surface expressed DC2-SAG1 showed higher binding to recombinant DEC205 protein and MutuDC cells than DC2, protein quantities expressed by RH-DC2-SAG1 and RH-DC2 has been investigated. As a mean of semi-quantification, dot blot analysis was performed using enhanced chemiluminescence (ECL) substrates (Figure 1F). Interestingly, in parasite cell lysates, RH-DC2 tended to show higher protein expression than RH-DC2-SAG1 (roughly three-fold). Nonetheless, RH-DC2-SAG1 demonstrated the strongest binding to the targets. Taken together, these results suggest that fragments, vectorized in *T. gondii*, have been correctly expressed and were able to recognize DEC205 protein. Fusion of SAG1 encoding sequence to the anti-DEC205 scFv resulted in enhanced functionality in term of binding to targets.

3.3. Design of Human anti-PD-L1 scFv

The results obtained from the successful surface display of anti-DEC205 were encouraging for broadening the uses of this innovative approach for further applications. Thus, targeting PD-L1-expressing tumors through surface display of anti-PD-L1 scFv was chosen to be investigated. For this purpose, the heavy- and light-chain variable regions of the human monoclonal antibody, Atezolizumab connected with a flexible linker in VH-linker-VL orientation were utilized. Furthermore, to investigate the impact of HA tag localization on the functionality of the scFv, two anti-PD-L1 configurations were developed (Figure 2A). In one construct, as for scFv anti-DEC205, HA tag encoding sequence was incorporated in the N-terminus region of VH chain (anti-PD-L1-N), while in the second construct it was inserted in the N-terminus of SAG1 (attached to the C-terminus of the VL chain by the GGGAS spacer) (anti-PD-L1-C). These sequences were then cloned into the plasmid pUC5 using *PmeI* and *NotI* cloning sites to generate pUC5PD-L1-N and pUC5PD-L1-C vectors respectively. Subsequently, *T. gondii* strain RH tachyzoites were transfected with either of the two vectors to generate the recombinant strains RH-PD-L1-N and RH-PD-L1-C respectively.

3.4. Anti-PD-L1 scFvs are Correctly Expressed in *T. gondii*

Next, three clones from each recombinant strain were selected to assess anti-PD-L1 surface display in ELISA-based assays (Figure 2B). The results indicated that anti-PD-L1 scFv was markedly expressed in all the selected clones compared to the wild type strain, which was used as control. The clone from each strain that displayed the highest expression (the highest OD measurement) was, then, chosen for the subsequent characterization and evaluation experiments. In order to confirm that the anti-PD-L1 scFv is correctly expressed at the expected size, whole parasite cell lysates of each of the selected clones were analyzed by immunoblotting. Figure 2C demonstrates that both RH-PD-L1-N and RH-PD-L1-C were equally able to generate a molecule with an apparent size corresponding to a scFv linked to SAG1. The bands obtained were comparable to that obtained by RH-DC2-SAG1. However, a faint band (at around 27 kDa) was observed with the migration of anti-PD-L1-N.

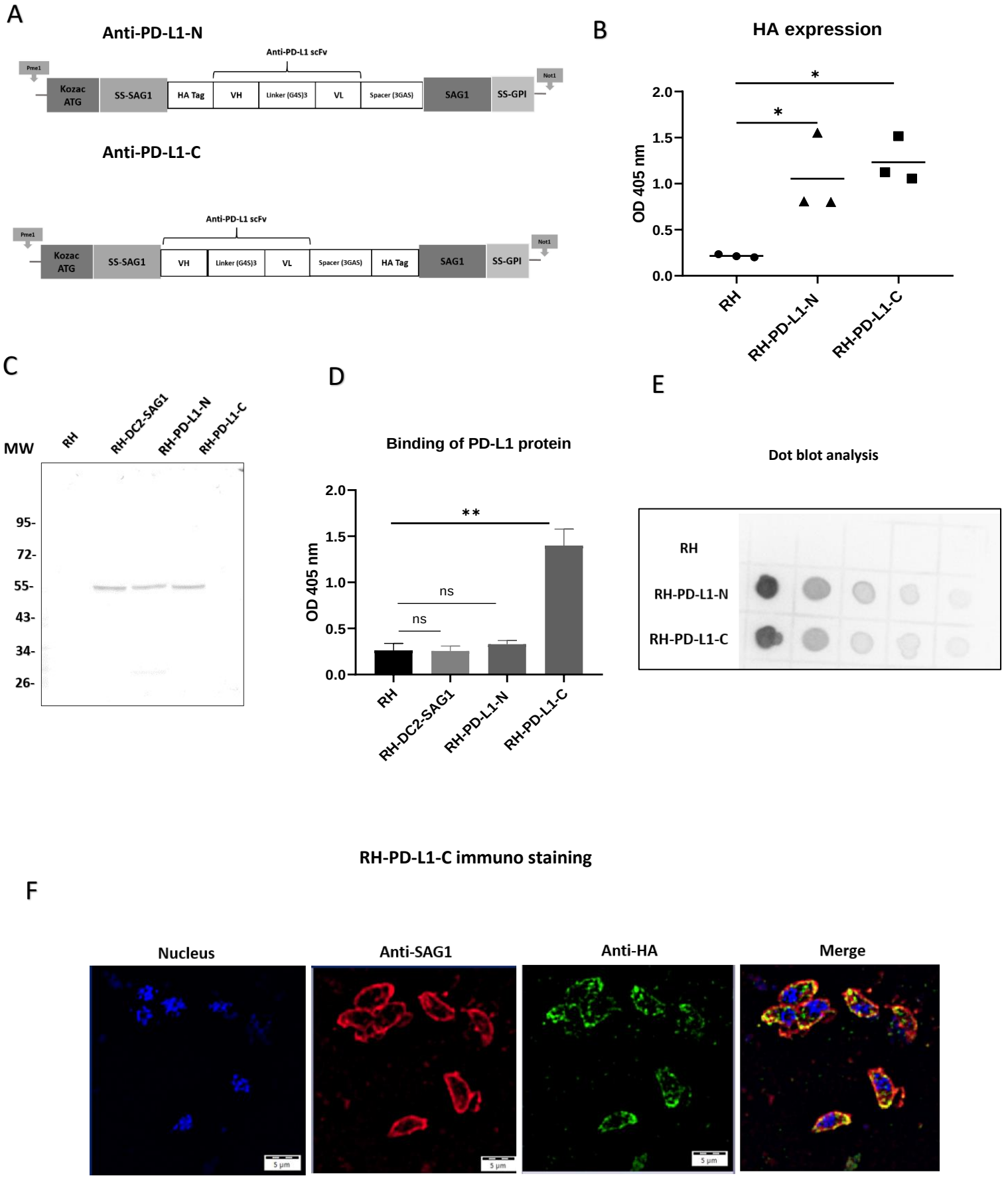


Figure 2. Engineering and characterization of recombinant *T. gondii* expressing a single chain variable fragment directed against the human programmed death-ligand 1 (anti-PD-L1 scFv). **(A)** Schematic

representation of the anti-human PD-L1 scFv with HA tag in the N terminus (anti-PD-L1-N) and the anti-human PD-L1 scFv with HA tag in the C terminus (anti-PD-L1-C). The locations of Kozac sequence and start codon (ATG), signal sequences of N-terminus of surface antigen 1 (SS-SAG1) and of glycosylphosphatidylinisotol (GPI), human influenza hemagglutinin (HA) tag, antibody variable domains (heavy [VH] and light [VL]), peptide linkers ([Gly4Ser]3) and (Gly3AlaSer), and surface antigen 1 sequence (SAG1) are indicated. **(B)** HA expression in three selected clones of RH-PD-L1-N and RH-PD-L1-C analyzed by ELISA using rabbit HA polyclonal antibody followed by anti-rabbit IgG AP-conjugated. RH tachyzoites were used as control (n= 3 replicates). **(C)** Western blot from parasite cell lysates RH (Line 1), RH-DC-SAG1 (line 2), RH-PD-L1-N (line 3) and RH-PD-L1-C (line 4) revealed using rabbit anti-HA followed by anti-rabbit IgG AP-conjugated. MW: molecular weight marker in kilo Dalton. **(D)** Binding of histidine-tagged PD-L1 recombinant protein to RH-PD-L1-N and RH-PD-L1-C tachyzoites assessed by ELISA using anti-His tag antibody. RH and RH-DC2-SAG1 tachyzoites were used as control (n= 5 replicates). **(E)** Dot blot to compare the relative abundance of the HA-tagged proteins expressed by RH-PD-L1-C and RH-PD-L1-N tachyzoites. Serial 2-fold dilutions of tachyzoite crude lysates were loaded onto nitrocellulose membrane and probed with rabbit anti-HA followed by HRP-conjugated anti-rabbit IgG and detection with chemiluminescent HRP substrate. **(F)** Localization of anti-PD-L1 scFv display to the membrane of *T. gondii*. Free tachyzoites of RH-PD-L1-C recombinant strain were incubated with mouse SAG1 mAb followed by biotin- anti-mouse IgG and AF-594-conjugated streptavidin (red) or rabbit anti-HA followed by anti-rabbit IgG-AF-488 (green). Nucleus was stained using Hoechst (blue). Images were captured at x600 with Olympus IX73 fluorescent microscope. Scale bars represent 5 μm**.** Statistical significance is indicated by *$p < 0.05$, **$p < 0.01$. ns: not significant. In all ELISA experiments, optical densities were read at 405 nm. All experiments were repeated at least twice. AP: Alkaline Phosphatase. HRP: Horseradish peroxidase. AF: Alexa Flour.

3.5. RH-PD-L1-C but not RH-PD-L1-N Strain Expresses a Functional anti-PD-L1 scFv

To explore whether the membrane-expressed anti-PD-L1 scFv could bind to the immune checkpoint PD-L1, the binding of recombinant tachyzoites to a commercial recombinant human PD-L1 with a C-terminal polyhistidine tag (His tag) was analyzed by ELISA (Figure 2D). RH-PD-L1-C tachyzoites exhibited strong binding to the recombinant human PD-L1 indicating that the expressed surface anti-PD-L1 scFv can recognize and bind to its target. Conversely, RH-PD-L1-N tachyzoites could not demonstrate similar functionality since no binding to the recombinant human PD-L1 was observed. To verify this finding, a set of ELISA assays was performed to screen all the chosen clones of RH-PD-L1-C and RH-PD-L1-N for binding to the recombinant human PD-L1 (Figure S1). As per the earlier findings, all RH-PD-L1-C clones showed significant binding to PD-L1 protein, nonetheless, none of the RH-PD-L1-N clones shown any binding.

To further analyze these findings, dot blotting of whole parasite cell lysates of RH-PD-L1-C and RH-PD-L1-N using ECL substrates was performed (Figure 2E). As the dot blot is revealing, the anti-PD-L1 scFv was adequately detected in both recombinant parasite cell lysates. Interestingly, the dot intensities indicate that anti-PD-L1 scFv was expressed in higher quantities in RH-PD-L1-N lysates. These findings imply that, despite the increased expression of anti-PD-L1 scFv by RH-PD-L1-N, when whole parasites were employed, only RH-PD-L1-C

has been shown to be functional. Accordingly, we selected RH-PD-L1-C strain for further characterization and investigation.

3.6. Surface Display of the anti-PD-L1-C scFv

In order to guide the expression of the anti-PD-L1 to the membrane of *T. gondii*, the sequence encoding the N-terminal sequence signal of SAG1 that guides the surface transport of the protein was incorporated. Additionally, to promote anchoring and fixation on the *T. gondii* membrane, the anchoring GPI sequence signal with the entire sequence of the mature SAG1 was also added (Y. Wang & Yin, 2014). To confirm that the anti-PD-L1 expression is localized in the *T. gondii* membrane as it is intended to be, an immuno-fluorescence microscopy was performed. To do so, RH-PD-L1-C tachyzoites were collected and the membrane was stained in red using mAb against SAG1 while the anti-PD-L1 fragment was stained in green by targeting HA tag. As illustrated in Figure 2F, upon merging of the two images together, the orange color that results from the green-red merge is concentrated in the membrane, indicating clearly that the protein is successfully targeted to the parasite's membrane.

3.7. RH-PD-L1-C Strain Exhibits Strong Binding to Human PD-L1-Expressing Cells *In Vitro*

Next, the aim was to investigate whether anti-PD-L1 display on the surface of *T. gondii* would direct the parasites to cells expressing PD-L1. The human breast cancer cell line, MDA-MB231 which is known to express PD-L1 on the cell membrane was used whereas HFF cells were used as PD-L1 negative control. RH-DC2-SAG1, as a recombinant, GFP positive strain expressing an irrelevant surface scFv was used as negative control. Surface PD-L1 display on MDA-MB231 and HFF cell lines was analyzed by flow cytometry (Figure 3A). This analysis verified that MDA-MB231 cells express substantial PD-L1 levels while HFF cells were PD-L1 negative. Then, using HFF cells as non-targeted (PD-L1 negative) cells, RH-PD-L1-C or RH-DC2-SAG1 tachyzoites were incubated with either of the cells for 2 h at 4°C. Using flow cytometer, cell binding was measured as a percentage of GFP-positive cells. As shown in Figure 3B, RH-PD-L1-C exhibited more pronounced binding to MDA-MB231 cells compared to the non-targeted, HFF cells (33% versus 7 % respectively). While, on the other hand, RH-DC2-SAG1 demonstrated comparable binding between the two cell lines regardless to the PD-L1 expression (5% and 7%). To verify that this enhanced binding of RH-PD-L1-C to MDA-MB231 cells was induced by PD-L1 expression; cells were incubated with saturating concentrations of Atezolizumab to block PD-L1 binding sites. Following saturation, RH-PD-L1-C or RH-DC2-SAG1 were added to MDA-MB231 cells and binding was assessed by flow cytometry (Figure 3C). RH-PD-L1-C binding to MDA-MB231 cells was significantly decreased when PD-L1 was blocked with Atezolizumab, whereas RH-DC2-SAG1 binding remained unaffected, confirming that binding was in fact dependent on PD-L1 expression.

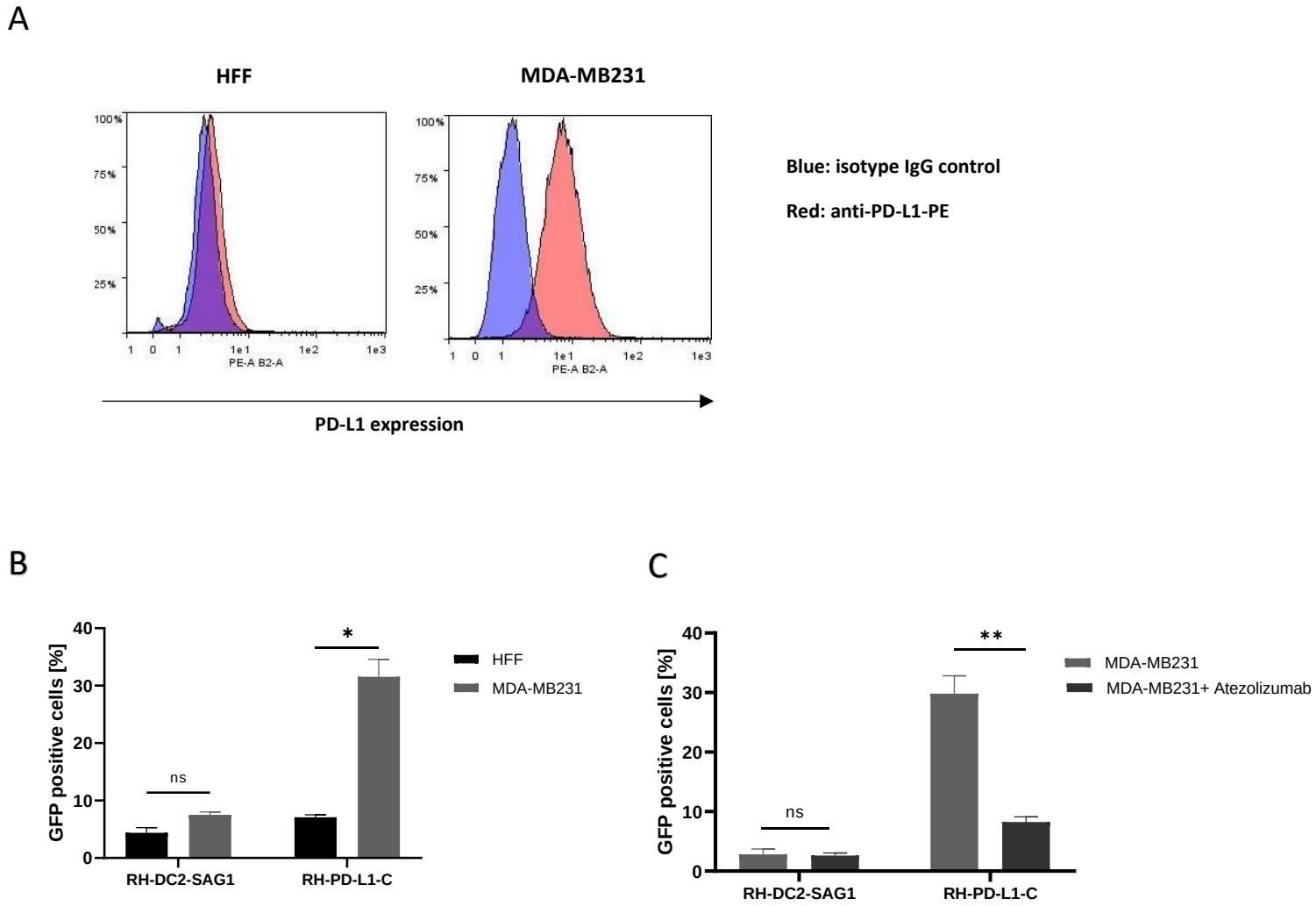


Figure 3. Binding of RH-PD-L1-C and RH-DC2-SAG1 (irrelevant scFv control) recombinants tachyzoites to human cells expressing or not PD-L1. **(A)** PD-L1 expression on HFF and MDA-MB231 cells. Cells were stained with PE-conjugated anti-human PD-L1 and analyzed by flow cytometry. Blue area depicts the profile of cells stained with isotype control IgG and red area depicts the profile of cells stained with anti-PD-L1. (**B)** Binding quantification of *T. gondii* recombinants tachyzoites to HFF or MDA-MB-231 cells analyzed by flow cytometry. Human cells were incubated with either RH-DC2-SAG1 or RH-PD-L1-C at MOI 5 and binding was indicated by percentages of GFP positive cells (n = 3 replicates) **(C)** Binding competition with Atezolizumab. After incubating MDA-MB-231 cells with saturating concentration of Atezolizumab followed by incubation of the RH-PD-L1-C or RH-DC2-SAG1 strain, binding was quantified by flow cytometry. (n = 5 replicates). Statistical significance is indicated by *p < 0.05, **p <0.01, ns: not significant. All experiments were repeated at least twice. MOI: multiplicity of infection.

3.8. RH-PD-L1-C Strain Exhibits Strong Binding to Murine PD-L1-expressing Cells *In Vitro*

Studies showed that the human anti-PD-L1, Atezolizumab can efficiently bind to murine PD-L1 [47,48]. Subsequently, to test if the recombinant RH-PD-L1-C strain would bind to murine tumor cells expressing PD-L1, the murine melanoma cell lines B16K1 and B16F10 were employed. The basal PD-L1 expression on these cell lines, B16K1 and B16F10 is low to moderated respectively. However, this moderate PD-L1 expression on B16F10 can be upregulated by stimulating cells with IFN-γ. First, the basal and induced expression of PD-L1 was confirmed on the rested (un-stimulated) B16K1 and B16F10 cells and IFN-γ-stimulated

B16F10 cells by flow cytometry. As illustrated in Figure 4A, the three cell populations showed three different levels of PD-L1 expression. Subsequently, to evaluate the impact of different PD-L1 expression levels on binding, rested B16K1 and B16F10 cells and IFN-γ-stimulated B16F10 cells were incubated with RH-PD-L1-C or the control strain, RH-DC2-SAG1 for 2 h at 4°C. Binding was analyzed by flow cytometry and represented by the percentages of GFP positive cells (Figure 4B). Recapitulating the results obtained in human cell line, RH-PD-L1-C strain, similarly, exhibited increased binding to PD-L1 expressing murine tumor cells. Interestingly, the binding was shown to be increased in accordance with the increased PD-L1 expression levels with the IFN-γ-stimulated B16F10 cells showing the highest level of binding. Furthermore, to negate the possibility that this increased binding was induced by any other factors than PD-L1 expression, a neutralization assay was performed by incubating IFN-γ-stimulated B16F10 cells with increasing concentrations of Atezolizumab to block PD-L1 binding sites. The indicated parasites, were then, added for 2 h and binding (GFP positive cells) was analyzed by flow cytometer. As illustrated in Figure 4C, neutralization with Atezolizumab led to inhibition of RH-PD-L1-C binding to B16F10 cells in a concentration dependent manner. Maximal inhibition was achieved with saturating concentrations of Atezolizumab (1 μg/ml) where binding of RH-PD-L1 was equivalent to the binding of the control RH-DC2-SAG1. When taken together, these findings suggest that RH-PD-L1-C could efficiently bind to both human and murine tumor cells and this cells binding is dependent upon surface display of PD-L1.

3.9. RH-PD-L1-C selectively Binds to Targeted Cells in Mixed Cell Population

Next, we asked if the recombinant RH-PD-L1-C strain would specifically target PD-L1-overexpressing cells when they are in a mixed cell population. To answer this question, the targeted, IFN-γ-stimulated B16F10 cells that also expressed mCherry fluorescent protein, were mixed with non-targeted B16K1 cells at a ratio of 1:1. Cell mixture was then, incubated for 2 h at 4°C with either RH-PD-L1-C or RH-DC2-SAG1 strain and targeting was analyzed by flow cytometry. As shown in Figure 4D, the two cell populations in the cell mixture were distinguished by the mCherry expression (left dot blot). Then, binding was indicated according to the percentages of GFP positive cells in each group of cells. The control strain, RH-DC2-SAG1, maintained almost 50-50 % targeting (around 3% binding) on both cell groups with no respect to PD-L1 expression (middle dot blot). In contrast, RH-PD-L1-C tachyzoites predominantly bound to the PD-L1-overexpressing cells, with a negligible number of tachyzoites showing off-target binding (right dot blot).

3.10. Oncolytic Activity of RH-PD-L1-C *in Vitro*

To correlate the enhanced binding of RH-PD-L1-C strain to tumor cells expressing PD-L1 to cellular toxicity, the infectious and oncolytic ability was compared to those of the wild type strain in human (MDA-MB231) and murine (B16F10) tumor cell lines. MDA-MB231 and IFN-γ-stimulated B16F10 were infected with either RH wild type or RH-PD-L1-C strain at MOI 3 or left uninfected, and cells viability was assessed after 24, 48 and 72 hours. As shown in Figure

5A, results demonstrated that RH-PD-L1-C could efficiently infect and kill both human and murine tumor cells with a significantly higher capacity than the parental *T. gondii* strain.

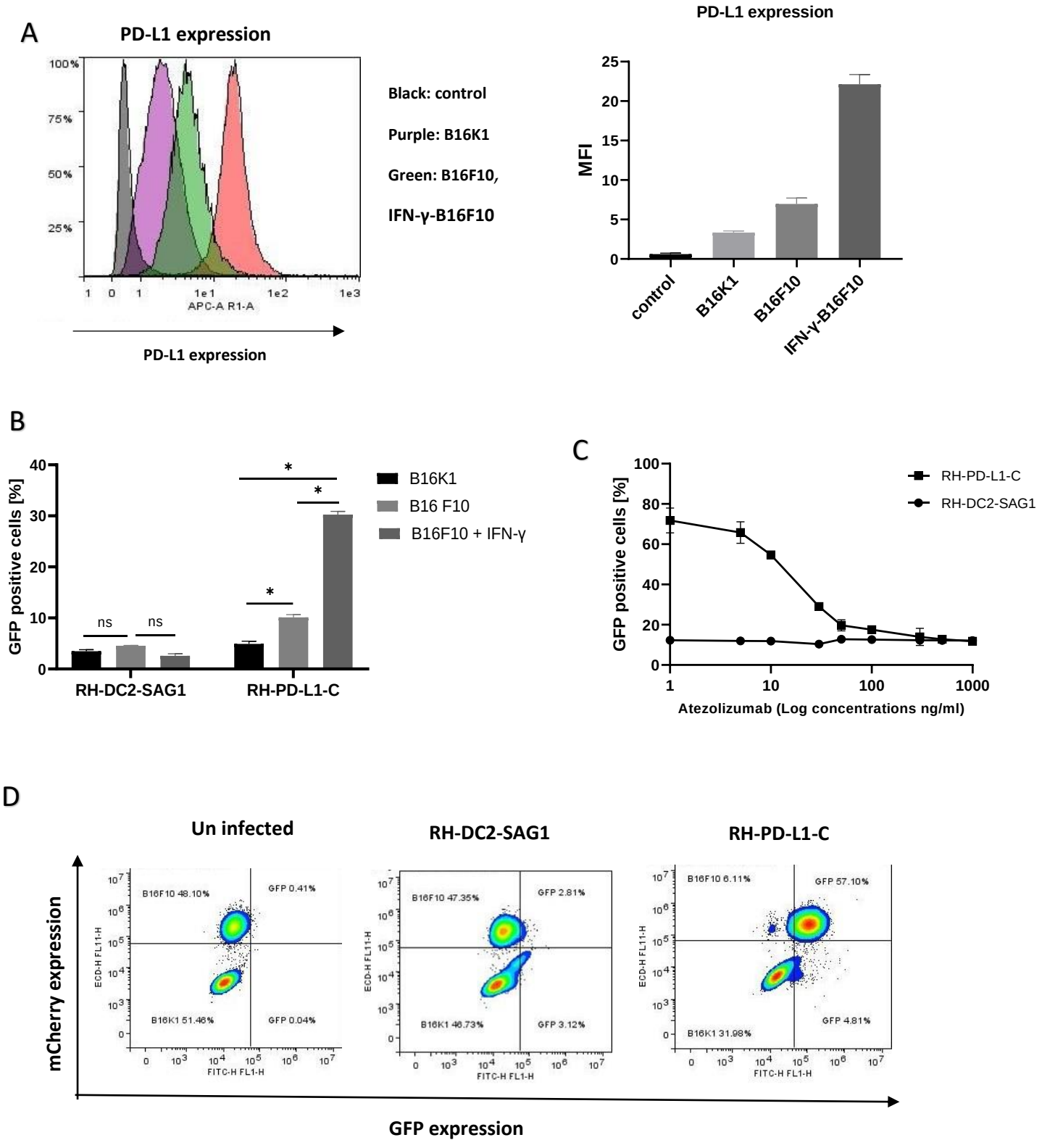


Figure 4. Binding of RH-PD-L1-C and RH-DC2-SAG1 (irrelevant scFv control) recombinants tachyzoites to murine tumor cell lines expressing or not PD-L1. **(A)** Surface display level of PD-L1 on IFN-γ-stimulated B16F10 (B16F10 + IFN- γ), non-stimulated B16F10 (B16F10) and B16K1 cells. Cells were stained with APC-conjugated mouse anti-PD-L1 before flow cytometry analysis. Black area indicates the profile of cells incubated with isotype control IgG. Profile of cells stained with anti-PD-L1 are indicated in; Purple area (B16K1), green area (B16F10) and red area (IFN-γ-stimulated B16F10). **(B)** Murine cell lines were incubated with either RH-DC2-SAG1 or RH-PD-L1-C tachyzoites at MOI 5 and the percentage of GFP positive cells was quantified by flow cytometer. (n = 3 replicates). **(C)** Competitive binding assay. IFN-γ- stimulated B16F10 cells were incubated with increased concentrations of Atezolizumab followed by addition of either RH-DC2-SAG1 or RH-PD-L1-C tachyzoites at MOI 5. Binding was assessed as a percentage of GFP positive cells by flow cytometry. (n = 3 replicates). **(D)** Selective targeting of PD-L1 over-expressing cells by RH-PD-L1-C in a mixed cell assay. B16K1 and IFN-γ-stimulated B16F10 cells were mixed together at a ratio of 1:1 and the cells mixture were incubated with RH-PD-L1-C or the control strain, RH-DC2-SAG1 at MOI 5. The two cell populations were discriminated by mCherry expression by gating on mCherry positive (B16F10) and mCherry negative (B16K1) cells. Binding was indicated by the GFP positive cells among each group. Statistical significance is indicated by *$p < 0.05$, ns: not significant. All experiments were repeated at least twice. MOI: multiplicity of infection.

3.11. RH-PD-L1-C strain Inhibits the Binding of PD-1/PD-L1 and Promotes T cells Activation

The ability of the anti-PD-L1 scFv displayed on RH-PD-L1-C tachyzoite surface to interfere with PD-1/PD-L1 interaction and to promote T-cell responses was evaluated in *in vitro* co-culture assay. Murine B16F10 tumor cells incubated with MHCI-restricted peptide SIINFEKL OVA257-264), were used as target cells. Murine B3Z CD8+ T cells, which recognized the SIINFEKL bound to H-2Kb MHC-I and OVA-peptide, were used as antigen-specific CD8+ effector T cells. Activated B3Z cells also express β-galactosidase under the control of the IL-2 promoter, thus, the activity of B3Z cells can be measured by β-galactosidase and IL-2 expression. For the co-culture assay, first, surface PD-1 display on B3Z was confirmed by flow cytometry (Figure 5B). Then, B16F10 cells were stimulated to over-express PD-L1 by IFN-γ before co-culturing with B3Z in the presence of RH-PD-L1-C or RH wild type (control) tachyzoites. B3Z cells response (measured as β-galactosidase activity) to B16F10 was significantly enhanced in the presence of RH-PD-L1-C tachyzoites in respect to wild type RH (Figure 5C). To obtain further evidence, IL-2 secretion was measured. As illustrated in Figure 5D, RH-PD-L1-C strain was able to stimulate more IL-2 secretion compared to RH wild type. These findings indicate that surface display of anti-PD-L1 on the surface of *T. gondii* not only increased the targeting of PD-L1 positive cells, but also proved effective in blocking PD-1/PD-L1 pathway and potentiating T cells response.

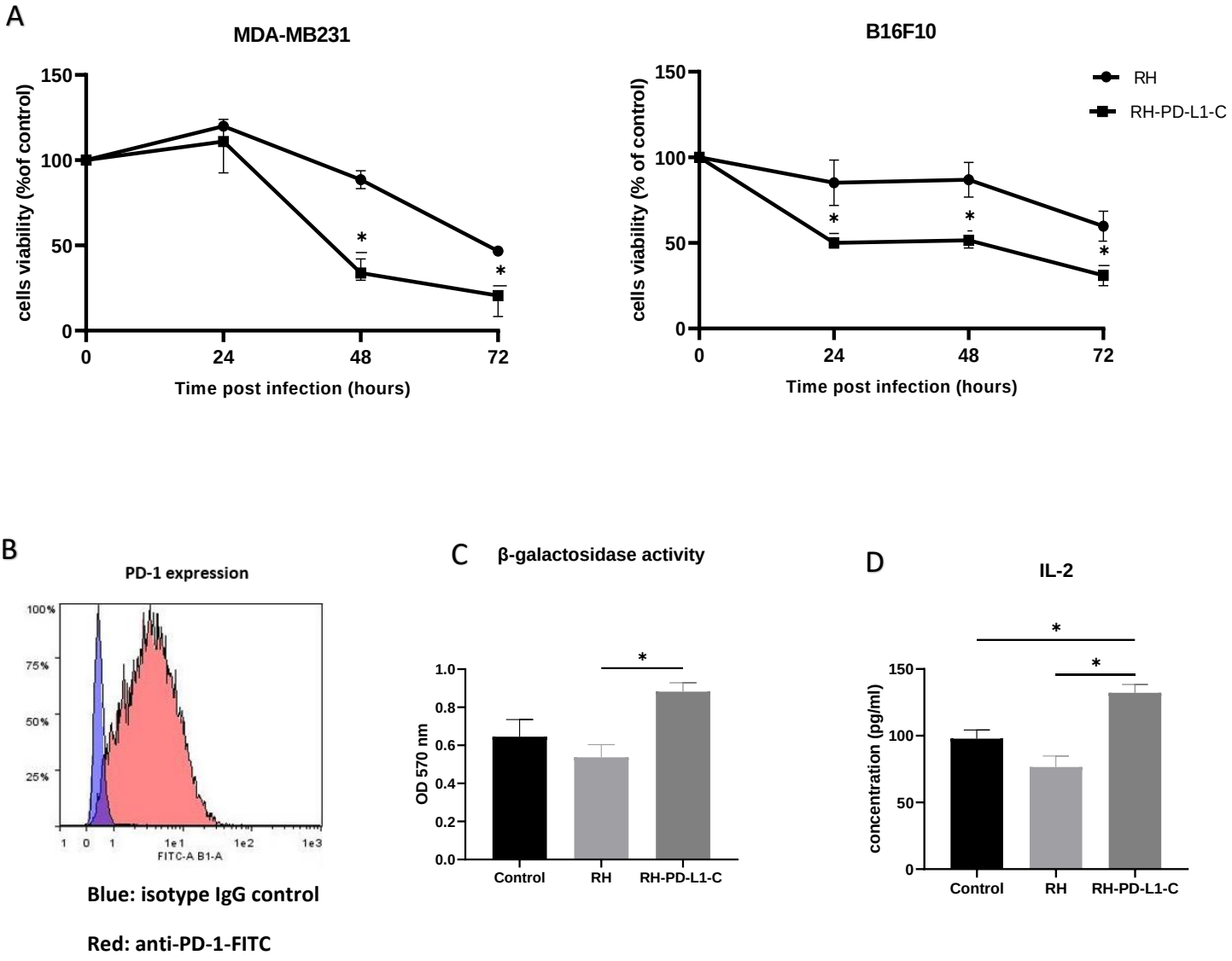


Figure 5. In vitro biological activity. (A) In vitro oncolytic activity. MDA-MB231 and IFN-γ-stimulated B16F10 cells were left un-treated or infected with either the wild type *T. gondii* RH strain or RH-PD-L1-C recombinant strain at MOI 3. Cells viability was measured 24, 48 and 72 h post infection using MTT test. Results are represented as percentages of viable cells in respect to the control un-infected cells. Optical densities were read at 490 nm. Asterisks indicate statistically significant differences between RH wild type-treated and RH-PD-L1-C-treated cells (*$p < 0.01$). All experiments were repeated at least twice. (B) PD-1 expression on B3Z cells was analyzed by flow cytometer using FITC-labeled mouse anti-PD-1. Blue area indicates the profile of cells incubated with isotype control IgG while profile of cells stained with anti-PD-1 are indicated in red area (C-D) Activation of the MHC class I-restricted T cell line B3Z in the context of presentation of the SIINFEKL OVA peptide or Presentation of the soluble OVA peptide SIINFEKL to B3Z cells. B3Z cells were co-cultured for 20 h with IFNγ-stimulated B16F10 cells loaded with OVA peptide in presence of RH (control) or RH-PD-L1-C recombinant tachyzoites. The B3Z response was measured as β-galactosidase activity (C) by measuring absorbance at 570 nm after incubation with β-galactosidase substrate (CRPG) and Il-2 quantification (D) in co-culture supernatants by ELISA. Statistical significance is indicated by *$p < 0.05$. All experiments were repeated at least twice.

4. Discussion

We hereby present, for the first time, the generation and characterization of replicative *Toxoplasma gondii* strains displaying surface single chain variable fragments as a novel strategy for targeting specific cells. The strategic use of *T. gondii* in the context of several diseases offers a promising therapeutic approach. Given *T. gondii*'s ability to invade all nucleated cells, one approach to reduce nonspecific cell invasion and promote selectivity is by targeting parasites to receptors that are unique or overexpressed by the targeted cells [49]. Therefore, we aimed to determine whether engineering *T. gondii* to express surface antibody fragments would induce specific cell targeting. To test this hypothesis, we selected Dendritic Cells (DCs) as the target by employing a single chain variable fragment (scFv) against the murine DC-restricted antigen-uptake receptor DEC205. Our research group [29] and others [50] investigated DCs targeting as a strategy to deliver vaccines to DCs, thereby enhancing antigen presentation and improving the immune response. Previously, Michon *et al*. demonstrated the feasibility of targeting DCs through membrane display of anti-DEC205 scFv on the surface of recombinant *Lactobacillus plantarum* strains[30]. Building upon this approach, we hypothesized that we could engineer *T. gondii* to express anti-DEC205 scFv on its surface. Hereby, we report the generation and characterization of recombinant *T. gondii* strains displaying surface mouse anti-DEC205 scFv. Two strategies were tested to generate *T. gondii* stable transfectants expressing surface scFv: direct GPI anchoring or GPI anchoring in addition to fusion of SAG1 protein to the scFv sequence. Subsequently, two recombinant strains were generated named RH-DC2 and RH-DC2-SAG1, respectively In previous studies, a few heterologous proteins have been expressed on the surface of *T. gondii*. Anchoring the protein of interest by GPI has been successfully achieved for both alkaline phosphatase and Cryptosporidium Glycoprotein Antigen Cpgp40/15 [51,52]. However, membrane display of heterologous surface proteins by T. gondii through fusion with SAG1 has been reported to be unsuccessful, resulting in either undetectable or short truncated protein products expressed in the cytoplasm of recombinant parasites, as observed for the *Plasmodium yoelii* circumsporozoite protein [53]. Furthermore, in a study by Gregg *et al*. aimed at targeting Ovalbumin to various *T. gondii* oragnelles, GPI-membrane anchored Ovalbumin was indeed targeted to the surface, however, unstable expression was observed appeared as degraded bands on Western blot analysis with some amount being shed in the culture supernatant. In contrast to these findings, our results indicate that both strategies were effective in expressing stable membrane scFv as illustrated in the immune-blotting figures. urthermore, our dot blot analysis data demonstrated higher quantities of the anti-DEC205 scFv detected in RH-DC2 parasite lysates. Nonetheless, in subsequent functionality assays for binding to the recombinant DEC205 protein using intact tachyzoites, RH-DC2-SAG1 showed higher binding ability than RH-DC2. The lower binding capacity of RH-DC2, despite the protein being highly expressed, is most likely due to limited accessibility to the receptor, potentially resulting from its structural assembly.

T. gondii possesses a natural ability to bind to and invade all nucleated mammalian cells. A Previous work by Swee *et al*. (2015) demonstrated that the addition of a single-domain

antibody against CD19 to the *T. gondii* surface via sortagging significantly increased parasite targeting to B cells while decreasing binding to non-B cells [54]. Although effective for targeting, sortagging requires the production of recombinant proteins, conjugation for targeted tachyzoites, and purification steps. Surface engineering of *T. gondii* offers a solution to these challenges. Thus, our study aimed to investigate whether surface display of an antibody against the DCs receptor DEC205 would enhance parasite targeting to these cells compared to the parental parasite. To address this question, we utilized a DCs cell line known to express DEC205 on its surface. Consistant with our hypothesis, the adhesion assays showed that the surface display of anti-DEC205 increased the binding of RH-DC2-SAG1 and, to a lesser extent, RH-DC2 to DCs. Taken together, these findings suggest that *T. gondii* can be successfully engineered to express functional surface scFv, and this surface display results in increased binding to the targeted cells compared to the wild type strain. When the SAG1 encoding sequence was fused to the anti-DEC205 scFv, this construct exhibited enhanced functionality in terms of binding to targets.

These findings offer the possibility of further exploration for surface engineering applications of *T. gondii*. Therefore, we aimed to investigate the potential of this novel approach for tumor targeting. Generally, the reprogramming of oncolytic microorganisms to target tumor cells while sparing normal cells has been widely researched. One efficient way to generate tumor-specific microorganisms is by targeting them to cell surface receptors or tumor-associated antigen specifically expressed or significantly overexpressed on tumor cells. This approach has been successfully applied to various oncolytic bacteria and viruses through surface modification via the attachment of antibody fragments targeting various tumor receptors or antigens. Various targets have been explored, with HER2 [55,56] and CD20 [57,58] being among the most investigated targets.

In this study, we investigated the possibility of targeting programmed cell death ligand 1 (PD-L1). PD-L1 is an immune checkpoint protein that is overexpressed on many solid tumors [59]. This ligand interacts with the receptor programmed cell death-1 (PD-1), expressed on the cell surface of T cells, B cells, monocytes, and natural killer (NK) T cells [60]. Activation of the PD-1/PD-L1 pathway negatively regulates T cell-mediated immune responses and induces T cell exhaustion. Several solid tumors upregulate PD-L1 expression as an immune evasion mechanism, making the inducible PD-L1 expression at the tumor site a selective target for antitumor therapy [61]. Furthermore, earlier studies on mice-bearing melanoma have demonstrated that treatment with *T. gondii* increases PD-L1 upregulation, rendering these tumor cells more sensitive for PD-L1 [23]. Targeting oncolytic microorganisms to PD-L1-positive cells has been described recently, primarily through modification of oncolytic viruses. This modification involves either using bispecific antibodies as adaptors between the adenovirus and the cell surface receptor [62], or coating the virus with bioengineered cell membrane nanovesicles expressing programmed cell death protein 1 (PD-1) [63].

or the construction of the anti-PD-L1 single-chain variable fragment (scFv), we utilized the heavy (VH) and light (VL) chain variable regions of the commercially available human anti-PD-L1 antibody, Atezolizumab. Building upon the successful fusion of the membrane protein

SAG1 to the scFv in our previous anti-DEC205 constructs, we employed the same design approach for the anti-PD-L1 scFv. In the effort to generate a recombinant *T. gondii* expressing functional anti-PD-L1 scFv, we searched scientific literature for characterized PD-L1 antibody fragments (scFvs) to select the most appropriate design and insertion sites of HA tag. Emerging evidence indicates that, for binding of anti-PD-L1 monoclonal antibodies, the VH is essential rather than VL chains [64], suggesting that HA tag at the N-terminus of VH may negatively impact the functionality ot the expressed fragment. However, in contrast to this theory, others have constructed a functional anti-PD-L1 scFv with the GFP encoding sequence at in the N-terminus of the VH [65]. Thus, we examined both strategies by constructing two anti-PD-L1 scFv configurations: one with the HA tag placed at the N-terminus of the VH chain (anti-PD-L1-N) and the other with the HA tag positioned outside the scFv, at the N-terminus of SAG1 (anti-PD-L1-C). Consequently, two recombinant strains were generated, named RH-PD-L1-N and RH-PD-L1-C, respectively. After confirming the display of anti-PD-L1 on the surface of both recombinant strains by ELISA and Western blotting, functionality was assessed by binding to recombinant PD-L1 protein. The strain engineered with the anti-PD-L1 scFv featuring the HA tag at the C-terminus exhibited robust binding to PD-L1 protein. Conversely, the strain engineered with the HA tag at the N-terminus of the scFv failed to recognize and bind to PD-L1, despite adequate protein expression. Consistent with the observations of Zhang et al. (2017), our data indicate that positioning the HA tag at the very N-terminus of the scFv might interfere with its binding to PD-L1 protein, validating the crucial role of the VH chain in binding to PD-L1 protein..

We further evaluated the ability of RH-PD-L1-C to bind to tumor cells expressing PD-L1 by flow cytometry. For this adhesion analysis, we utilized the human breast cancer cell line MDA-MB-231 which is known to express high levels of PD-L1. In this analysis, we relied on GFP expression by the recombinant parasites where binding was indicated by the percentage of GFP-positive cells. As a control, we used the recombinant parasite RH-DC2-SAG1 which is also surface modified, expressing an irrelevant scFv. Firstly, we confirmed that both surface-modified strains demonstrated low replication compared to the control in non-targeted HFF cells (Figure S2). Similarly, in the previous study by Michon *et al*. (2015) on Lactobacillus displaying surface anti-DEC205 scFv, they showed that the recombinant strains had a substantially lower growth rate than the wild type bacteria still, all strains showed reasonable growth [30]. However, when targeted PD-L1 was expressed on the cell surface, significantly higher binding was observed with RH-PD-L1-C to MDA-MB-231 cells. To further verify that this increased binding was induced by PD-L1/anti-PD-L1 interaction, a neutralization assay was performed. The neutralization assay with Atezolizumab confirmed that this binding was due to the specific interaction of RH-PD-L1-C with PD-L1 expressed by MDA-MB-231 cells.

In a study by Magiera-Mularz *et al.,* they demonstrated that PD-L1 of both mouse and human origins share high structural similarity [48]. They indicated that Atezolizumab can bind to mouse PD-L1 and mice bearing MC38 cells were successfully treated with Atezolizumab. Based on this study, we theorized that our recombinant *T. gondii* expressing surface anti-PD-L1 scFv derived from Atezolizumab would bind to murine PD-L1-expressing tumor cells. To

test this theory, we employed murine tumor cell lines, B16K1 and B16F10 which show low and moderate levels of PD-L1 respectively. It is well known now that PD-L1 expression can be upregulated by the IFN-γ receptor II signaling pathway. Therefore, by stimulating B16F10 cells with recombinant IFN-γ, we generated three levels of PD-L1 expression, low (B16K1), medium (B16F10) and high (IFN-γ-stimulated B16F10). The ligand-receptor interaction is generally influenced by the affinity of the ligand for the receptor and the expression and surface accessibility of the receptor. As expected, the surface-engineered *T. gondii* bound to cells in the order IFN-γ-stimulated B16F10 > B16F10 > B16K1, showing a strong correlation between binding and PD-L1 expression levels. This correlation was further verified by neutralizing PD-L1 binding sites after it had been upregulated with IFN-γ with increasing concentrations of Atezolizumab. The inhibition of binding by atezolizumab in a concentration dependent manner confirmed that the adhesion is mainly achieved via the interaction between scFv on the *T. gondii* surface and its ligand on the cell surface, rather than via the nonspecific interactions.

Thus far, we have confirmed that the surface display of anti-PD-L1 by *T. gondii* resulted in a high capacity to bind both human and murine PD-L1-positive tumor cells. However, these recombinant strains retain the capability to infect and replicate in non-targeted cells. Here, we investigated whether these recombinant parasites would specifically target and bind to PD-L1 overexpressing cells within a mixed cell population. To address this assumption, we studied targeting in a cellular mixture consisting of PD-L1-overexpressing (IFN-γ-stimulated B16F10) cells and cells expressing low levels of PD-L1 (B16K1 cells). Our results showed that binding was primarily restricted to the cells that overexpressed the surface marker, with very few tachyzoites exhibited off-target binding. Such data has been achieved before by using retargeted viruses or bacteria where invasion genes are deleted. In our model, we achieved targeting by a replicative strain. Selective targeting according to surface display of markers normally associated with diminished nonspecific cell targeting is of relevance to potential therapeutic applications.

In addition to immune system induction, the direct oncolytic activity achieved through intracellular multiplication within cancer cells is an argument in favor of using replicative rather than attenuated *T. gondii* strains. Therefore, we evaluated the capacity of our model to induce direct tumor cell death by invading and infecting PD-L1 positive tumor cells. Our results confirmed the superior killing ability of the recombinant replicative strain, RH-PD-L1-C compared to the parental RH strain. These findings suggest that RH-PD-L1-C is capable of directly eradicating tumor cells -*in vitro*- in the absence of immune system influence, by infecting cells and causing their death.

PD-L1 expressed on tumor cells interacts with PD-1 on effector T lymphocytes resulted in T cell exhaustion. Exhausted CD8 T cells lose their effector function, evidenced by their inability to secrete pro-inflammatory cytokines, such as IL-2, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-a) [60,66]. Therefore, we evaluated the ability of our model to inhibit PD-1/PD-L1 interaction in a co-culture *in vitro* system of B16F10 tumor cells and B3Z CD8+ T cells to mimic tumor microenvironment where T cells activity will be inhibited by

tumor cells. Our results showed that the addition of RH-PD-L1-C recombinant tachyzoites to the co-culture potentiated the T cells activity measured by β-galactosidase and IL-2 secretion (by 1.7 and 1.6 fold respectively) compared to the wild type strain. These results suggest that anti-PD-L1 scFv expressed at the surface of *T. gondii* tachyzoites could achieve a combination of immune checkpoint blockade and efficient targeting of tumor cells. In addition, up-regulation of PD-L1 by *T. gondii* would improve the efficacy of this strategy by presenting more targets. Interestingly, in a similar approach, adenoviruses coated in a bioengineered cell membrane expressing PD-1 to target PD-L1 were shown to effectively reactivate exhausted tumor-specific CD8+ T cells, inhibit tumor cell proliferation, and improve the anti-tumor efficacy in vitro and in vivo [63]. As a next step, future studies should be conducted to evaluate these recombinant tachyzoites in 3D cell culture systems and appropriate *in vivo* animal models to confirm their ability to target PD-L1+ tumor cells, leading to better antitumor activity.

5. Perspectives

The results presented here demonstrate that displaying antibody fragments on the surface of *T. gondii* effectively induces a targeted effect. Our strategy paves the way for the use of replicative strains of *T. gondii* that potentiate tumor oncolysis while localizing the therapeutic effect at the target site. This approach is promising in the context of various tumors.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, File S1: sequences of CAT-GFP expression cassette and the cassette for the expression of the protein of interest; Figure S1: Binding of RH-PD-L1-N and RH-PD-L1-C clones to PD-L1 protein; Figure S2: Replication of RH-PD-L1-C and RH-DC2-SAG1 recombinant tachyzoites in non-targeted cells.

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Supplementary Data

File S1

CAT GFP expression cassette

Cassette, bordered by LoxP sites, to express the fusion protein CAT-GFP driven by the promoter of the *T gondii* -tubulin gene (TUB) and the 3 'untranslated region of the *T. gondii* SAG1 gene (3'UTR SAG1)

Color code:

HindIII LoxP PromotorTUB CAT AvrII GFP 3'UTR SAG1 loxP XbaI

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Expression cassette

Cassette to express a protein of interest driven by a modified promoter of the *T gondii* -tubulin gene (TUB8) and the 3 ‘ untranslated region of the *T. gondii* SAG1 gene (3’UTR SAG1)

Length: 1018 bp

5 repeat motifs underlined (Soldati et al, 1995)

Promotor TUB8: XbaI/PmeI (509 bp)

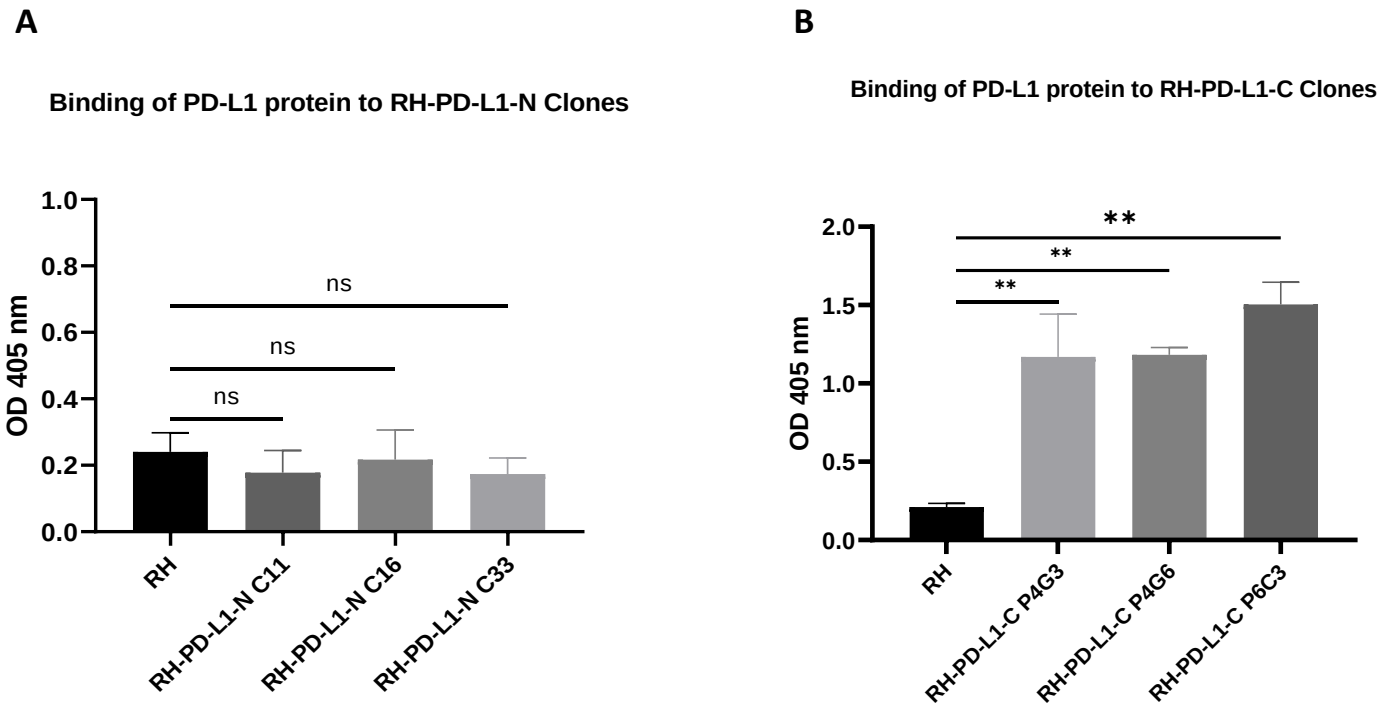
3’UTR SAG1 : NotI/AvrII (345 bp)

Cloning sites : PmeI (510), NotI (532)

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XbaI PmeI NotI AvrII

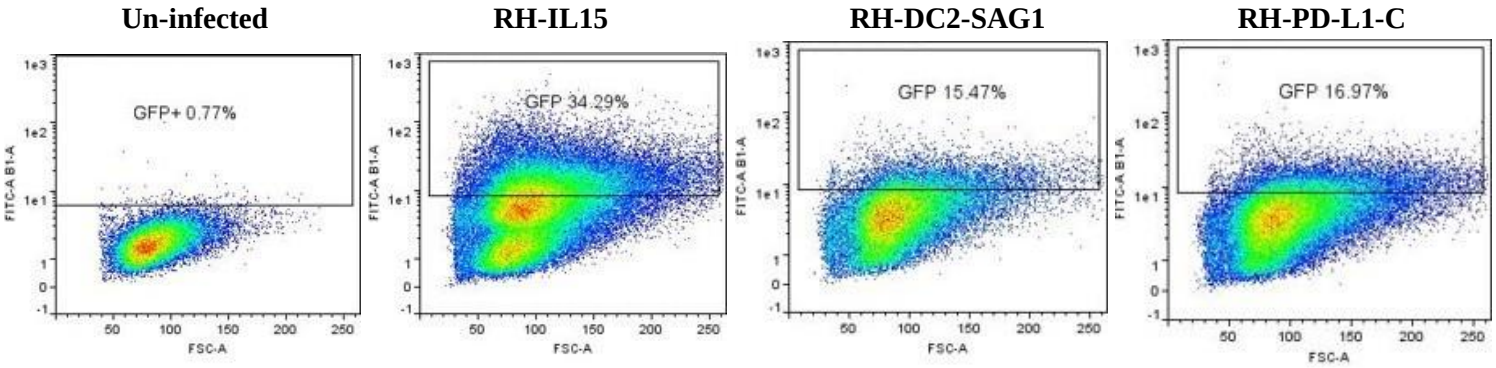
Figure S1



Supplementary Figure 1. Binding of RH-PD-L1-N and RH-PD-L1-C clones to PD-L1 protein. Tachyzoites of the selected clones of RH-PD-L1-N **(A)** and RH-PD-L1-C **(B)** recombinant strains were incubated with histidine-tagged PD-L1 recombinant protein and bound protein was assessed by ELISA using anti-His tag antibody (n= 3 replicates). Optical densities were read at 405 nm. Statistical significance is indicated by **p < 0.01. ns: not significant. All experiments were performed at least three times.

Figure S2

A



B

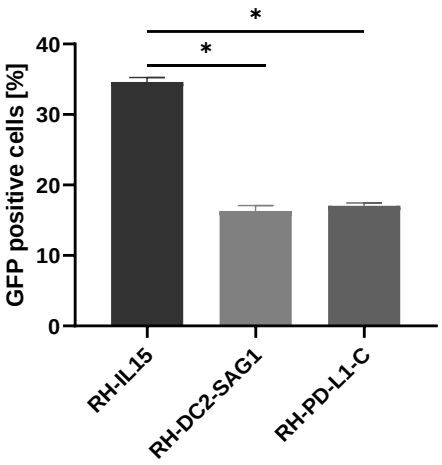


Figure S2. Replication of RH-PD-L1-C and RH-DC2-SAG1 recombinant tachyzoites in non-targeted cells. The replication of RH-PD-L1-C and RH-DC2-SAG1 tachyzoites in HFF cells was compared to the recombinant RH strain engineered to secret IL-15 (as a surface-non-modified, GPF positive strain). HFF cells were infected with each of the indicated strain at MOI 1, then, incubated for 1 h at 37 °C to allow for invasion. Following incubation, cells washed to remove extracellular tachyzoites and incubated for 24 h. Invasion was analyzed by flow cytometry. **(A)** Representative flow cytometry dot blots showing the infected cells indicated by the GFP positive cells. **(B)** Quantification of invasion in 3 replicates. Statistical significance is indicated by *$p < 0.05$. Experiments was performed three times.

4. Unpublished work

Arming *Neospora caninum* with a functional single chain fragment variable antibody against PD-L1

Objectives:

This study aimed at optimizing the antitumor efficacy of *Neospora caninum* by engineering recombinant strains expressing anti-PD-L1 single-chain variable fragments (scFv), either displayed on the parasite's surface or secreted through one of the protein secretory pathways.

Methods:

The anti-PD-L1 scFv was constructed using the heavy and light chains of the anti-PD-L1 Atezolizumab coupled via a flexible linker in VH-linker-VL orientation. The scFv was targeted to parasite’s membrane by glycosylphosphatidylinositol (GPI) anchor signal and SAG1 fusion protein. For secreted scFv, the sequence was targeted to micronemes secretory pathway by adding N-terminal signal peptide propeptide domain of *T. gondii* microneme protein 5 (TgMIC5).

Results:

Surface display of anti-PD-L1 scFv using SAG1 protein was unsuccessful resulting in transient expression followed by loss of expression. In contrast, recombinant *N. caninum* strain secreting anti-PD-L1 scFv was able to secret functional and stable scFv through microneme secretory pathway.

Conclusion:

The study demonstrates the feasibility of engineering *N. caninum* to secrete functional anti-PD-L1 scFv, opening new avenues to enhance its antitumor efficacy. The preliminary *in vitro* results encourage further exploration of more complex antibody formats, such as scFv fused with the crystallizable fragment (Fc), to potentially enhance the therapeutic benefits of this novel immunotherapeutic strategy.

Arming *Neospora caninum* with a functional single chain fragment variable antibody against PD-L1

(On going work)

1. Introduction

For several decades, cancer treatment has relied on targeting tumor strategies such as surgical intervention, cytotoxic chemotherapy, and radiation (Santos Guimares et al., 2013). Since the beginning of the 19th century, the role of the immune system in cancer development and control has become increasingly evident, positioning the immune system as an efficient tool for combating cancer (Esfahani et al., 2020; Oiseth & Aziz, 2017). Following intensive research, the concept "cancer immunotherapy" has emerged (Dobosz & Dzieciątkowski, 2019). Unlike conventional therapeutic strategies, cancer immunotherapies aim to reactive the immune system to restore the antitumor immune response and overcome the mechanisms of immune escape (Liu et al., 2022). The last decade has witnessed a major breakthrough in cancer immunotherapy with the development of immune checkpoint inhibitors (ICIs) (Basudan, 2023). These monoclonal antibodies (mAb) targeting cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed death-1 (PD-1) and its ligand programmed death-1 ligand 1 (PD-L1) have significantly improved patient survival in multiple high-incidence cancer indications (Emens et al., 2017). PD-1 is an inhibitory receptor that is mainly expressed in various immune cells, including macrophages, B lymphocytes, dendritic cells (DCs), monocytes, tumor-specific activated T cells including CD8+ cytotoxic T lymphocytes and CD4+ T-helper lymphocytes, myeloid cells and natural killer (NK) cells (Ohaegbulam et al., 2015). PD-1 inhibits T cell functions through binding to its ligands: PD-L1 or PD-L2 expressed on the surface of antigen presenting cells (APCs) (Riella et al., 2012) which many tumor cells overexpress to suppress T cells functionality and evade immune response. This promotes T-cell tolerance, inhibits T cells proliferation, and reduces their cytokine production resulting in tumors progression and proliferation (Dong et al., 2018; Han, Liu and Li, 2020).

Moreover, as antigen presenting cells (APCs) express CD80/B7.1 which is recognized by CD28 on T cells and mediates T cell activation, PD-L1 can also bind to CD80/B7.1 and therefore prevents binding of CD80/B7.1 to CD28 and blocks its T-cell-mediated activation (Butte et al., 2007). Thus, blocking these inhibitory pathways by monoclonal antibodies (mAbs) targeting PD-L1 or PD-1, revives the antitumor immune responses by interrupting co-inhibitory signaling pathways and promote immune-mediated elimination of tumor cells (Webb et al., 2018).

Although the first approved immune checkpoint inhibitor was a mAb blocking CTLA-4 pathway (Lipson & Drake, 2011), shortly thereafter, monoclonal antibodies blocking PD-1 and PD-L1 followed (Kazandjian et al., 2016; Raedler, 2015). In 2016, the U.S Food and Drug Administration (FDA) approved Atezolizumab, the first monoclonal antibody targeting PD-L1, for the treatment of advanced urothelial carcinoma and metastatic non-small cell lung cancer (Ning et al., 2017; Weinstock et al., 2017). However, despite the spectacular success of ICIs during the past decade, patient's response rates range from 15 to 30% (in most solid tumors) to 45–60% (in melanomas) and accumulating evidences suggest that tumors can develop different resistance mechanisms in addition to immune related adverse effects of this group of medicines (Das & Johnson, 2019). Undoubtedly, there is constant need for developing novel therapeutic strategies to optimize ICIs therapy and expand its application.

The next revolutionary wave in cancer immunotherapy involves the use of live microorganisms as an interesting strategy to combat cancer. Oncolytic microorganisms can identify, infect, and kill tumor cells, and /or stimulate the immune system, thereby promoting an antitumor response. In this context, several viruses and bacteria have been investigated as potential immunotherapeutic agents (D. Lin et al., 2023; Yarahmadi et al., 2024; Zhou et al., 2018). These microorganisms can either possess a natural tropism for tumor cells or they can be modified, through genetic engineering, to target tumors (Fu et al., 2019; Nguyen et al., 2023). With advances in genetic engineering tools, burgeoning number of oncolytic platforms armed with therapeutic immune modulators has emerged, which enhanced their anti-tumoral activity and allowed for intratumor delivery of immune therapeutics (reviewed in Gurbatri, 2022; Arpaia and Danino, no date; de Graaf et al., 2018). Notably, in 2015, T-VEC, a recombinant attenuated herpes simplex virus encoding human granulocyte macrophage colony-stimulating factor (GM-CSF) earned the FDA approval for the treatment of melanoma patients with injectable but non-resectable lesions in the skin and lymph nodes (Pol et al., 2016). Although the majority of research is focusing on the lower order microorganisms, protozoa have also shown potential as cancer immunotherapeutic agents. The link between parasitic infections and beneficial therapeutic outcomes in cancer was reported in numerous researches (reviewed in Yousefi et al., 2023). Protozoa can stimulate the immune system, reversing the immunosuppressive TME and coordinating immune responses to prevent tumor development, spread and recurrence (Zheng et al., 2023).

Our research team is focusing on two apicomplexan parasites: *Toxoplasma gondii* and *Neospora caninum*. The efficacy of *T. gondii* to eradicate growing tumors has been reported

since 1977 when *T. gondii* infection resulted in regression of central nervous system tumors (Conley and Remington, 1977). Since then, *T. gondii* attracted a lot of attention and several other researches highlighted its ability to inhibit tumor growth in different mice tumor models (Baird et al., 2013; Hunter, 2001.; Kim, 2007). While *T. gondii* has been investigated in the context of cancer immunotherapy for several years now, *N. caninum* -which is the focus of this research work- is a relatively recently discovered parasite that receives increasing research interest.

Neospora caninum is an obligate intracellular parasite belonging to the phylum Apicomplexa (Morrissette & Sibley, 2002). *N. caninum* is closely related to *T. gondii* and until recent time it was misidentified as *T. gondii* before it was first isolated and identified by Bjerkfs in 1984 from the central nervous system and skeletal muscle of dogs in Norway (Bjerkfis et al., 1984). Like *T. gondii*, it can infect a wide range of livestock and wild animals, but canines are the definitive hosts (Dubey et al., 2003). Serosurveys were conducted to evaluate human's exposure to *N. caninum* and reported low levels of antibodies against *N. caninum* in human sera (Graham 1999; Tranas et al., 1999; Lobato et al., 2006; Duarte et al., 2020). Nevertheless, there is no evidence that *N. caninum* can cause zoonotic disease. *N. caninum* secretes a unique set of proteins from three dedicated secretory organelles that release their contents during host's cell invasion. Micronemes, which is found in the apical end of the parasite, secret their proteins during egress from infected cells, gliding motility, and host cell invasion (Dubois & Soldati-Favre, 2019). Rhoptries, which with micronemes form the apical complex from where this phylum derives its name, secret proteins directly into the cytosol of host cells before and during cell invasion (Boothroyd & Dubremetz, 2008). Dense granules, which are evenly distributed throughout the parasite's cytoplasm, secrete proteins after when the parasite resides inside the intracellular parasitophorous vacuole (PV) (Hakimi et al., 2017).

Being nonpathogenic in humans, *N. caninum* has been proposed as a safe microbial immunotherapeutic agent with potential implications in cancer immunotherapy. Within our research group, in a previous work by Lantier *et al*., they showed that both distant and intratumor administration of live *N. caninum* tachyzoites inhibited tumor development and induced potent anti-tumor immunity in mice-bearing EG7 mouse lymphoma. *N. caninum* also elicited significant tumor regression in early and established human Merkel cell carcinoma in mice models (Lantier et al., 2020). Furthermore, *N. caninum* was further engineered to secret interleukin 15 (IL-15) associated with the alpha-subunit of the IL-15 receptor (IL15hRec) as a strategy to potentiate the induced immune responses and to improve its anti-tumor efficacy. IL-

15 was engineered by targeting it to the micronemes secretory pathway through fusion of the N-terminal signal peptide and propeptide domain of *T. gondii* microneme protein 5 (TgMIC5). The recombinant *N. caninum* strain secreting IL-15 showed enhanced anti-tumor activity in mouse model of EG7 lymphoma and B16F10 melanoma lung metastases (Battistoni et al., 2023; Lantier et al., 2020). Thus, following this approach, in this research work we aimed at investigating the possibility of expressing stable and functional human anti-PD-L1 scFv in *N. caninum* by targeting it to microneme secretory pathway using the same strategy. Moreover, we previously demonstrated that engineering *T. gondii* to display single chain fragment variable (scFv) against PD-L1 on its **s**urface increased targeting of PD-L1-expressing tumor cells and potentiated T cell response via inhibiting PD-1/PD-L1 binding *in vitro*. As we -in our previous work- verified the possibility of targeting live replicative parasites through surface engineering, herein, in this research work we also investigated the possibility of adopting this strategy in *N. caninum*.

Therefore, the aim of this study is to investigate the possibility of engineering *N. caninum* expressing a functional human anti-PD-L1 scFv targeted to the parasite's membrane through GPI signal peptide and SAG1 fusion protein or targeted to microneme secretory pathway through TgMIC5 propeptide and to evaluate these recombinant strains *in vitro*. Our results showed that anchoring of anti-PD-L1 scFv via TgSAG1 resulted on unstable expression and lack of functionality. However, we demonstrated the feasibility of arming *Neopora* with a functional secreted single chain variable fragment antibody against PD-L1, contributing to the development of innovative cancer immunotherapies.

2. Materials and Methods

2.1. Parasites

The *N. caninum* NC-1 strain was obtained from ATCC (50843) and grown in Human Foreskin Fibroblasts (HFFs; ATCC CRL-1634) in Dulbecco's modified Eagle medium 2 mM L-glutamine (DMEM, Gibco, Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (FBS; Dutscher), 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco, Thermo Fisher Scientific) and 1% HEPES (Biowest). Cells were cultured at 37 °C in 5% carbon dioxide (CO_2) and infected with tachyzoites when they were 90% confluent. Supernatants were collected when all cells were infected and lysed.

2.2. Cell Lines

Human breast cancer cell line MDA-MB-231 (kindly provided by Professor Emilie Allard-Vannier, EA 6295 Nanomedicaments et Nanosondes, University of Tours, France) were cultured in DMEM 2 mM L-glutamine containing 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin and 1% Non-Essential Amino Acid (NEAA, PAN-Biotech). Murine melanoma cell line B16F10 (kindly provided by Dr. Mehdi Khaled, UMR 1299, Gustave Roussy Institute, Paris-Saclay University, France) were grown in Roswell Park Memorial Institute medium 2 mM L-glutamine (RPMI 1640, Gibco, Thermo Fisher Scientific) with 10% FBS and 50 U/ml penicillin and 50 µg/ml streptomycin.

2.3. Generation of recombinant *N. caninum displaying* surface anti-PD-L1 single chain variable fragment (scFv)

N. caninum NC1 wild type strain (10^7 tachyzoites) were collected from infected HFF cells and transfected with 50 µg of linearized pUC5PD-L1-C plasmid vector (previously used to transfect *T. gondii* displaying surface anti-PD-L1 scFv) in cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2 HPO_4: KH_2 PO_4, 25 mM HEPES, 2 mM EDTA, 5 mM $MgCl_2$, 3 mM ATP and 3 mM glutathione, pH 7.6) using a Biorad Gene PulserII Electroporator (Bio-Rad, Hercules, CA) at settings of 2000 V, 50 ohms and 25 µF. Tachyzoites were then transferred to a fresh culture of HFF cells. After overnight growth, transfectants were selected with 20 µM chloramphenicol for at least 10 passages before cloning by limiting dilution in 96 well plates. Stably transfected clonal parasite lines were designated: NC-PD-L1mb.

2.4. Expression and functionality of the surface protein

2.4.1. Enzyme-linked immunosorbent assay *(ELISA)*

ELISA was performed on whole tachyzoites of the wild type and recombinant *N. caninum* strains. In brief, 2×10^5 tachyzoites/well were coated on a flat-bottom 96-well plate (Maxisorp Nunc, Roskilde, Denmark) and fixed with 0.5% glutaraldehyde for 5 min at room temperature. The assay plates were blocked with *Phosphate-buffered saline* (PBS) containing 4% Bovine Serum Albumin (BSA, Sigma) for 2 h at 37 °C. Expression of surface anti-PD-L1 was assessed through the expression of the tag HA by incubating the tachyzoites with rabbit anti-HA polyclonal antibody (Invitrogen) followed by goat anti-rabbit IgG, alkaline phosphatase (AP) conjugate (Sigma). Bound phosphatase activity was measured with p–nitrophenylphosphate (PNPP, Sigma) (1 mg/ml in DEA-HCl 1 M buffer, pH 9.8). OD were measured at 405 nm.

2.4.2. Flow cytometry analysis

Recombinant *N. caninum* (NC-PD-L1mb) and *T. gondii* (RH-PD-L1-C, used as positive control) tachyzoites expressing surface anti-PD-L1 scFv were collected from infected HFF cells, washed in cold PBS containing 5% FBS and incubated with rabbit anti-HA polyclonal antibody for 1 h followed by APC-conjugated anti-rabbit IgG (Invitrogen) for 30 min at 4 °C. Wild type NC1 and RH strains were used as negative controls. Surface expression of anti-PD-L1 scFv was analyzed by flow cytometry (MACS Quant, Miltenyi Biotec) and data were analyzed using Flow-logic software (Miltenyi Biotech).

2.4.3. Western blot analysis

Electrophoresis and immunoblotting of *T. gondii* and *N. caninum* tachyzoites were performed as previously described (Bassuny Ismael *et al.*, 2003). Briefly, freshly released tachyzoites of the indicated strain (5×10^6 tachyzoites/20 µL) were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing dithiothreitol (50 mM) and loaded on homogenous 10% gels. After electrophoresis, proteins were transferred onto a nitrocellulose membrane, and probed with rabbit anti-HA antibody followed by AP-conjugated anti-rabbit secondary antibody (Sigma). Alkaline phosphatase activity was detected using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) liquid substrate system (Promega). Prosieve QuadColor Protein Markers (Lonza) was used to determine the protein molecular weights.

2.4.4. Functionality of recombinant *N. caninum* displaying surface anti-PD-L1 scFv

The functionality of the surface expressed anti-PD-L1 was analyzed by binding to human PD-L1 protein. Recombinant *N. caninum* (NC-PD-L1mb) and *T. gondii* (RH-PD-L1-C, used as positive control) tachyzoites were collected, washed in cold PBS containing 5% FBS and incubated with 10 µg/ml of histidine tagged-recombinant human PD-L1 protein (Sino Biological) for 1 h at 4 °C. Binding of PD-L1 protein to parasites was analyzed by flow cytometry using mouse monoclonal anti-poly-histidine (Sigma) followed by PE-conjugated anti-mouse IgG (Invitrogen).

2.5. Generation of recombinant *N. caninum* secreting human anti-PD-L1 scFv

2.5.1. Construction of the secreted human anti-PD-L1 scFv

For the secreted anti-PD-L1 scFv construct, the sequence encoding the scFv part of the human anti-PD-L1 Atezolizumab (available on IMGT information system) was used. The sequence

comprised of the variable heavy chain (VH), $(G_4S)_3$ linker, and the variable light chain (VL). For targeting to micronemes, the scFv sequence was linked in the N-terminus with the signal sequence (MLRPTVRSSLLSLGLTVILYLALTGSADA) and propeptide (LASHLRSRHMEAGR) of TgMIC5 (Brydges *et al.*, 2000) including, Kozak sequence and the ATG start codon. A sequence encoding HA tag (YPYDVPDYA) was incorporated in the C-terminus of the scFv using a short spacer (GGGAS) followed by a stop codon.

2.5.2. Cloning strategy and generation of recombinant *N. caninum* strain

Two expression cassettes were constructed to constitutively express proteins in *N. caninum* and *T. gondii* using previously described functional sequences: 5' αTub promoter and 3' UTR SAG1 (Kim et al., 1993; Striepen et al., 1998). Sequences are available from GenBank, with accession numbers M20024 and X14080 respectively. One expression cassette was designed to express a CAT-GFP fusion protein to allow drug selection of stably transfected parasites, the second was designed to express protein of interest. In the latter, a five-repeat element was inserted in the *T. gondii* α-tubulin gene, upstream of the transcriptional start site (leading to promoter α-TUB8) for high-level expression of the protein of interest (Soldati & Boothroyd, 1995). Two enzymes restriction sites *PmeI* and *NotI* were also included between the 5' α-TUB8 and 3'UTR SAG1 sequences to allow insertion of the sequence encoding the protein of interest. The two cassettes were then cloned into pUC18 plasmid in the same orientation to generate the plasmid pUC5. The anti-PD-L1 scFv sequence was cloned into pUC5 using *PmeI* and *NotI* restriction sites producing the vector pUC5PD-L1-HA. Subsequently, *N. caninum* strain NC1 tachyzoites were transfected with the generated vector as it has been described earlier (with *N. caninum* expressing surface anti-PD-L1 scFv). The stably transfected clonal parasite lines were designated NC-PD-L1sec and the secreted protein designated scFv-PD-L1.

2.6. Production of recombinant anti-PD-L1 scFv from Chinese hamster ovary cells

The sequences coding for the human anti-PD-L1 scFv and HA tag (without the signal sequence and propeptide of TgMIC5) was cloned into plasmid pcDNA3.4 to be expressed under the control of pCMV promoter. The plasmid was then used to transfect ExpiCHO cells using ExpiFectamine™CHO reagent (Thermo Fisher Scientific) and the supernatant of the transfected cells was collected 7 days post transfection. The protein was purified by affinity chromatography on PpL agarose (Life Technologies, 20512) as previously described (Lakhrif et al., 2016) and designated scFv-PD-L1-CHO.

2.7. Expression and characterization of the secreted HA-tagged protein

2.7.1. ELISA

The secretion of scFv-PD-L1 in culture supernatants was screened in selected clones of the recombinant NC-PD-L1sec strain. Briefly, HFF cells in 6-well culture plates were infected with 10^6 tachyzoites of the wild type NC1 strain or several NC-PD-L1sec clones. When all cells were infected and lysed, supernatants were collected and kept at -20 °C to be used for characterization and functionality tests. A flat-bottom P96-well plate was coated with 1µg/ml of recombinant *Peptostreptococcus Protein L* (*PpL, GE Healthcare*) [which can recognize the naturally occurring IGKV12-46 motif in the anti-PD-L1 scFv (Lakhrif et al., 2016) overnight at 4°C. Coated wells were then blocked with PBS with 4% BSA for 2 h at 37 °C. 100 µL of the indicated supernatants were added to the coated *PpL* and incubated for 2 h at 37°C. Bound scFv-PD-L1 was measured using rabbit anti-HA antibody followed by AP-conjugated anti-rabbit IgG. Phosphatase activity was revealed as stated earlier.

2.7.2. Immunofluorescence assay

To visualize the expressed scFv-PD-L1 intracellularly, HFF cells were infected with NC-PD-L1sec tachyzoites at multiplicity of infection (MOI) of 3 and incubated for 24 h at 37 °C. Following incubation, cells were washed with cold PBS and fixed in PBS containing 4% paraformaldehyde (PAF, Thermo Fisher Scientific) for 10 min. Cells were then permeabilized using PBS containing 0.1 % Triton 100X (Sigma) for 10 min and incubated with polyclonal rabbit anti-HA antibody for 2 h followed by anti-rabbit IgG-PE conjugate (Invitrogen) to stain the anti-PD-L1 scFv. After washing in PBS, nuclei were stained using Hoechst. Slides were mounted using Immu-Mount (Thermo Scientific) and images were captured with Olympus IX73 fluorescent microscope using cellSens Dimension software. All incubation steps were carried out at room temperature.

2.7.3. Binding of secreted scFv-PD-L1 and scFv-PD-L1-CHO to PD-L1 protein

To assess binding of scFv-PD-L1 to PD-L1 protein, histidine tagged-recombinant human PD-L1 protein 10 µg/ ml was coated in Maxisorp 96-well plate overnight at 4°C. Following incubation, wells were blocked with PBS with 4% BSA for 2 h at 37°C. Then, the previously collected culture supernatants of NC1 or NC-PD-L1sec tachyzoites were serially diluted (by 2-fold) and incubated with the coated PD-L1 protein (100 µl/well) for 2 h at 37°C. Bound scFv-PD-L1 was revealed using anti-HA as previously described. To assess the binding of scFv-PD-L1-CHO to PD-L1 protein, 100 nM of scFv-PD-L1-CHO or Atezolizumab (used as control)

were added to coated PD-L1 protein and incubated for 2 h at 37°C. Following incubation, bound scFv-PD-L1-CHO was revealed using rabbit anti-HA followed by alkaline phosphatase-conjugated anti-rabbit IgG and bound Atezolizumab was revealed by alkaline phosphatase conjugated anti-human IgG (Sigma). Phosphatase activity was revealed as previously detailed.

2.7.4. Western blot analysis

For production of supernatant containing scFv-PD-L1, to generate detectable bands on Western blot, HFF cells in T75 culture flasks were infected with NC-PD-L1sec tachyzoites. Once mature vacuoles were formed, the medium was replaced with 2 ml FBS-free medium and flasks were incubated back at 37°C to allow for the secretion of the scFv-PD-L1. Indicated concentration of the purified scFv-PD-L1-CHO protein, culture supernatant collected from NC1 or supernatant of NC-PD-L1sec culture were electrophoresed in reducing conditions then transferred to nitrocellulose membrane and probed with rabbit anti-HA antibody followed by HRP-conjugated anti-rabbit secondary antibody (Sigma). Bands were visualized using luminol chemiluminescent substrate (SuperSignal West Pico PLUS Chemiluminescent Substrate, ThermoFisher Scientific).

2.8. Induction of microneme proteins secretion by ethanol

HFF cells in T75 culture flasks were infected with NC-PD-L1sec tachyzoites and when mature vacuoles were formed, medium was replaced with medium with 1% FBS and cells were scraped and passed through syringe 27 g to release the tachyzoites. Cells were removed by centrifugation at 200 g for 5 min then, tachyzoites were collected by centrifugation at 800 g for 10 min and washed in 10 ml medium containing 1% FBS. Pellet containing 1.5×10^7 tachyzoites was resuspended in 100 µL medium containing 1% FBS and 1% ethanol and incubated at 37°C for 5 min to allow for micronemes proteins secretion. Secretion was stopped by incubation on ice for 5 min. Secretion in medium without ethanol was performed as control. Tachyzoite-free supernatant was collected from both conditions (assay and control) and subjected to Western blot analysis as described above. Temperature was maintained at 4°C throughout the experiment.

2.9. Quantification of the secreted scFv-PD-L1

To produce concentrated supernatants, tachyzoite excreted/secreted antigen (ESA) was produced by infecting HFF cells in T75 culture flasks with NC1 and NC-PD-L1sec tachyzoites until mature vacuoles were formed. Tachyzoites were then scraped and allowed to secret the protein extracellularly for 4 hours in low volume of FBS-free culture medium (10^8 parasites/100

µl). The produced scFv (scFv-PD-L1-ESA) or the supernatants which were previously collected from NC-PD-L1sec culture in P6 well plates were subjected to set of ELISAs for quantification using *PpL* protein as previously described. Purified scFv-PD-L1-CHO was used to generate the standard curve.

2.10. *In vitro* oncolytic activity of recombinant NC-PD-L1sec tachyzoites

MDA-MB231 cells were plated in P96-well plates at densities of 1×10^4 cells/well and let for few hours to attach. Cells were then let uninfected or infected with either NC1 wild type strain or NC-PD-L1sec tachyzoites at the indicated MOIs and incubated for 48 h. Following incubation, cells viability was measured by excluding dead cells using trypan blue staining. Percentage of cells viability was calculated in respect to control uninfected cells.

2.11. Binding of the secreted scFv-PD-L1 to PD-L1-expressing tumor cells

MDA-MB231, B16F10 and IFN-γ-treated B16F10 cells (5×10^5) were collected, washed in cold PBS containing 5% FBS and mixed with 100 µL of either NC1 culture supernatants or supernatant containing 40 ng/ml scFv-PD-L1 and incubated for 1 h. Cells were then washed and incubated with rabbit anti-HA for 1 h followed by APC-conjugated anti-rabbit IgG for 30 min. Binding of scFv-PD-L1 to cells was then analyzed by flow cytometry (MACS Quant, Miltenyi Biotec) and data were analyzed using Flow-logic software (Miltenyi Biotech). Standard human or mouse anti-PD-L1 IgG (Invivogen) were used as positive control. All incubations were carried out at 4 °C.

2.12. Statistics

All analysis was performed using Graph-Pad Prism 8.00 Software. Due to the non-normal distribution of the data, statistical significance was analyzed by the non-parametric Mann-Whitney and Kruskal-Wallis tests. Estimation of the Gaussian distribution of the data was performed using Shapiro–Wilk and D'Agostino Pearson normality tests. All the data are displayed as medians and interquartile range. Statistical differences were indicated with asterisks: * $p < 0.05$, ** $p < 0.01$.

3. Results

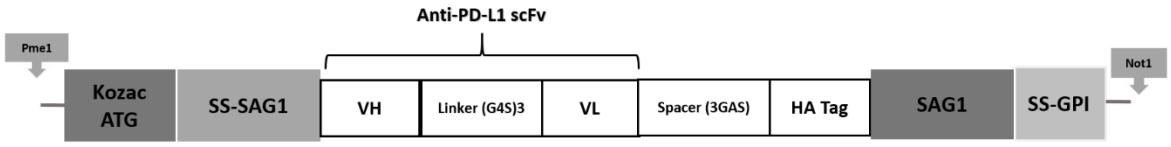
3.1. Generation and characterization of recombinant *N. caninum* strain expressing surface anti-PD-L1 scFv

To generate recombinant *N. caninum* tachyzoites displaying surface anti-PD-L1 scFv, we used a construct in which anti-PD-L1 scFv was fused to SAG1 protein with an HA tag at the C-terminus of SAG1 (anti-PD-L1-C) (Figure 1A). This construct was cloned into the plasmid

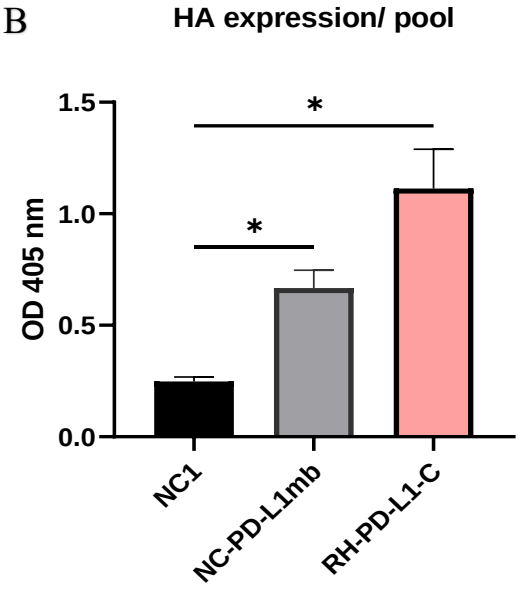
pUC5PD-L1-C and used to transfect *N. caninum* strain NC1 tachyzoites. Following transfection and a few culture passages of the transfected tachyzoites, recombinant tachyzoites were obtained and confirmed through immunofluorescent microscopy based on GFP expression. The surface expression of the anti-PD-L1 scFv indicated by HA tag expression and functionality through binding to PD-L1 protein was assessed in the pool of the recombinant tachyzoites (Figure 1B). The recombinant tachyzoites pool was passaged in culture for 6 weeks before cloning. After successful cloning, four clones were selected for further analysis by ELISA assays to assess surface expression of anti-PD-L1 scFv in comparison to the recombinant *T. gondii* strain expressing surface anti-PD-L1 scFv (RH-PD-L1-C). Two of the selected clones demonstrated significant expression of anti-PD-L1 compared to the wild type strain, although the expression level was still lower than observed with RH-PD-L1-C. The other two clones showed slightly higher OD readings than the wild type NC1 strain, but these differences were not statistically significant (Figure 1C). To evaluate the stability of this surface expression, the two clones with significant expression were cultured and passaged for five additional passages, after which surface display of anti-PD-L1 scFv was reassessed (Figure 1D). Interestingly, HA expression was markedly lost after these additional passages. Flow cytometry analysis was condicted to verify these results by assessing HA-surface expression in comparison to RH-PD-L1-C with results were represented as mean fluorescence intensity (MFI) (Figure 1E)

In accordance with ELISA results, flow cytometry analysis did not show any detectable signals of surface expression, confirming significant loss of HA tag expression. To determine if the loss of HA-tag expression correlated with loss of anti-PD-L1 scFv protein, the ability of these recombinant tachyzoites to bind PD-L1 protein was tested using flow cytometry. As shown in Figure 1F, the two clones of NC-PD-L1-C failed to bind PD-L1 protein compared to the positive RH-PD-L1-C strain. In order to further analyze and verify these results, recombinant tachyzoites from NC-PD-L1-C clone B11 were subjected to Western blot analysis using RH-PD-L1-C tachyzoites as positive control and NC1 wild type tachyzoites as negative control. As shown in Figure 1G, one major and intact protein's band was detected in RH-PD-L1-C line which corresponds to the estimated size of the scFv heavy and light variable regions linked to SAG1 protein. Conversely, NC-PD-L1-C showed degraded bands, none of them matched the expected size range.

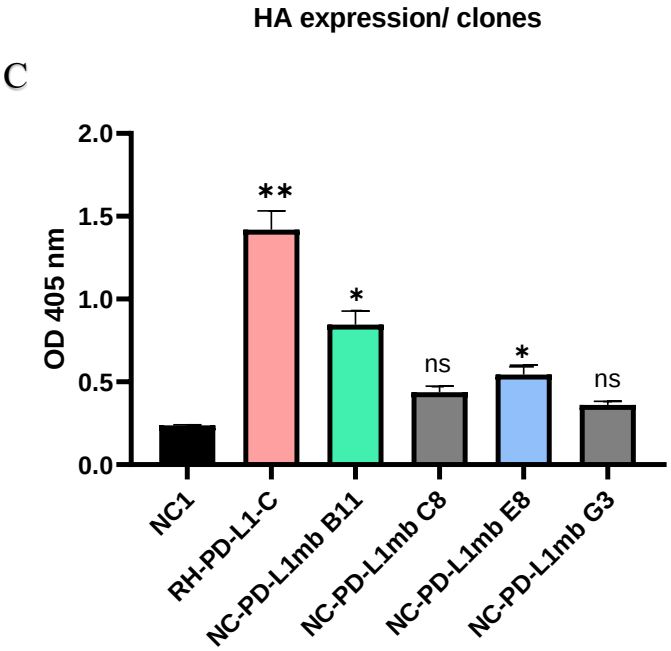
A


Anti-PD-L1 scFv
Pme1
Kozac ATG
SS-SAG1
VH
Linker (G4S)3
VL
Spacer (3GAS)
HA Tag
SAG1
SS-GPI
Not1

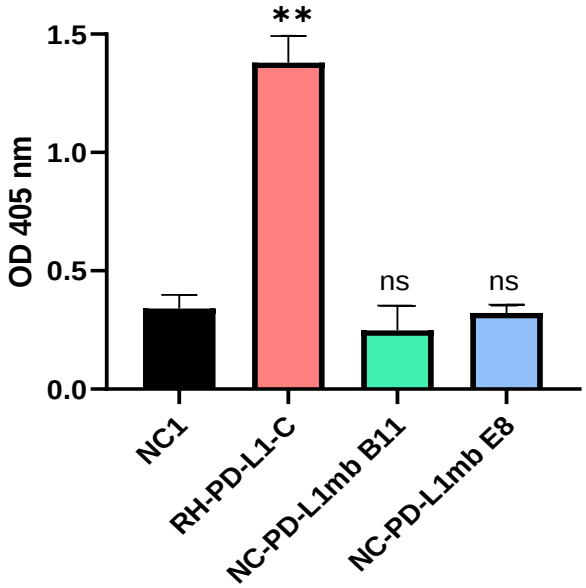
B


HA expression/ pool
OD 405 nm
1.5
1.0
0.5
0.0
*
*
NC1
NC-PD-L1mb
RH-PD-L1-C

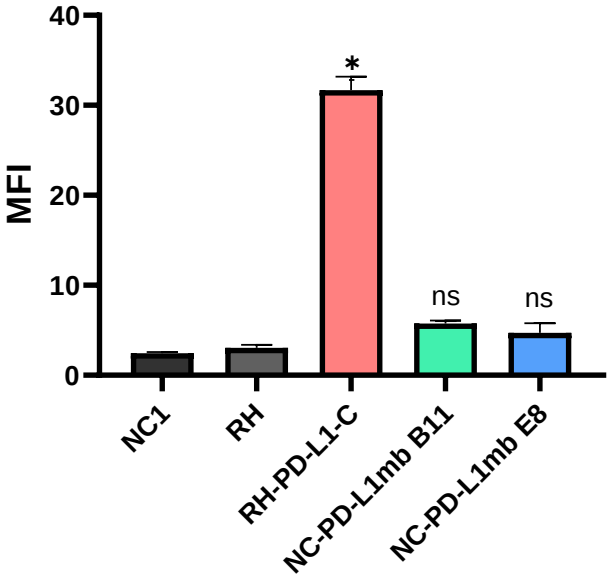
C


HA expression/ clones
OD 405 nm
2.0
1.5
1.0
0.5
0.0
**
*
ns
*
ns
NC1
RH-PD-L1-C
NC-PD-L1mb B11
NC-PD-L1mb C8
NC-PD-L1mb E8
NC-PD-L1mb G3

D


HA expression after 5 passages
OD 405 nm
1.5
1.0
0.5
0.0
**
ns
ns
NC1
RH-PD-L1-C
NC-PD-L1mb B11
NC-PD-L1mb E8

E


HA expression/ MFI
MFI
40
30
20
10
0
*
ns
ns
NC1
RH
RH-PD-L1-C
NC-PD-L1mb B11
NC-PD-L1mb E8

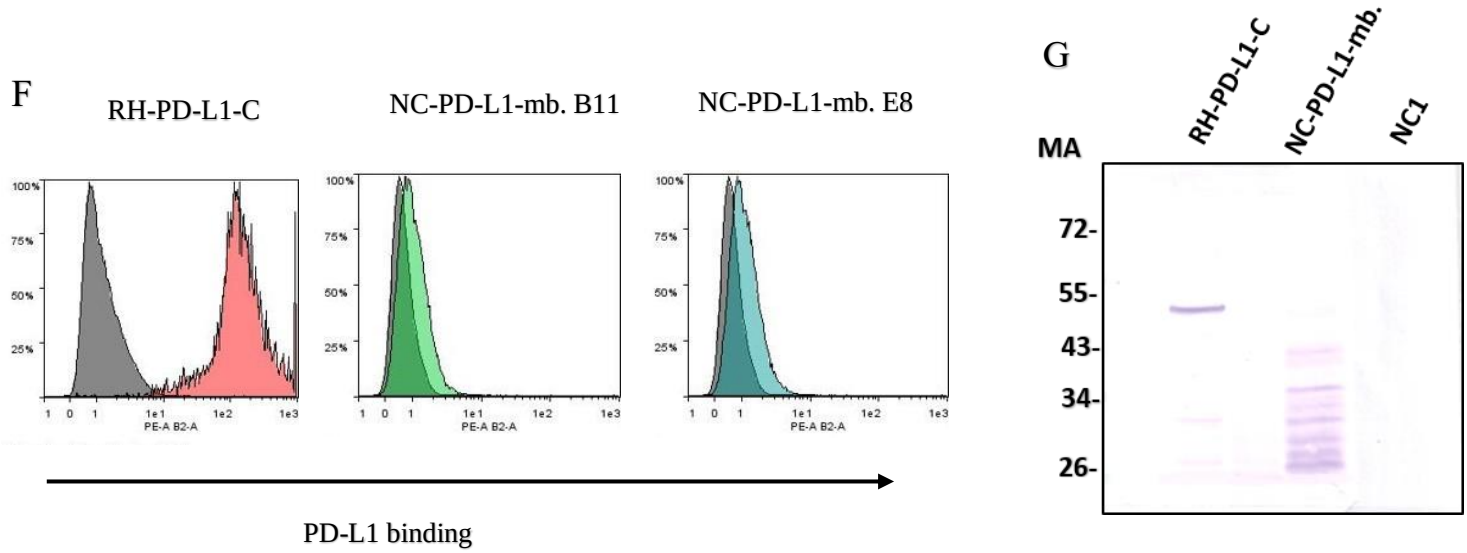


Figure 1. Generation and characterization of recombinant *N. caninum* expressing surface single chain variable fragment directed against the human programmed death-ligand 1. **(A)** Schematic representation of the anti-human PD-L1 scFv. The locations of Kozac sequence and start codon (ATG), signal sequences of N-terminus of surface antigen 1 (SS-SAG1) and of glycosylphosphatidylinisotol (GPI), antibody variable domains (heavy [VH] and light [VL]), peptide linkers ([Gly4Ser]3) and ([Gly3AlaSer]), human influenza hemagglutinin (HA) tag, and surface antigen 1 sequence (SAG1) are indicated **(B)** anti-PD-L1 scFv expression indicated by HA expression in pool of recombinant tachyzoites analyzed by ELISA using rabbit anti-HA polyclonal antibody followed by anti-rabbit IgG AP-conjugated (n= 3 replicates). **(C)** anti-PD-L1 scFv expression indicated by HA expression in four selected clones analyzed by ELISA using rabbit anti-HA polyclonal antibody (n= 3 replicates). **(D)** Clones NC-PD-L1mb B11 and NC-PD-L1mb E8 were passaged for two weeks (five passages) and surface expression of anti-PD-L1 scFv was analyzed again by ELISA (n= 3 replicates). **(E)** Confirmation of HA expression in the two clones (B11 and E8) by flow cytometry using rabbit anti-HA antibody followed by APC-conjugated anti-rabbit IgG. Results represented as the mean fluorescence intensity (MFI) (n= 3 replicates). **(E)** Binding of PD-L1 recombinant protein to NC-PD-L1mb tachyzoites. Recombinant tachyzoites were incubated with histidine-tagged PD-L1 protein and binding was assessed by flow cytometry using mouse anti-His tag antibodies followed by PE-conjugated anti-mouse IgG. Data presented are representative histograms of three independent experiments. **(G)** Western blot from parasite cell lysates of RH-PD-L1-C (Line 1), NC-PD-L1mb B11 (line 2) and NC1 (line 3) revealed using rabbit anti-HA polyclonal antibody followed by anti-rabbit IgG AP-conjugated. MW: molecular weight marker in kilo Dalton**.** In all experiments, NC1 tachyzoites were used as negative control and *T. gondii r*ecombinant strain expressing surface anti-PD-L1 scFv (RH-PD-L1-C) was used as positive control. All experiments were repeated at least three times. Statistical significance is indicated by *$p < 0.05$, **$p < 0.01$., ns: not significant. In all ELISA experiments, optical densities were read at 405 nm. AP: Alkaline Phosphatase.

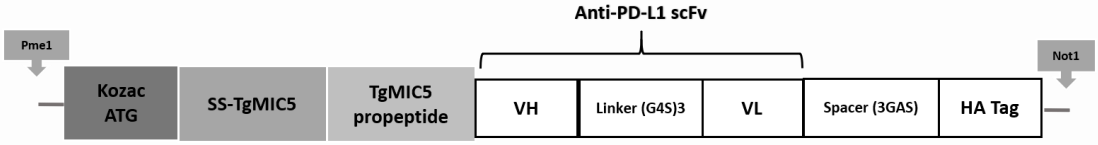
The results presented herein, are representative and consistent with the results of three independent experiments (results are shown in Supplementary Figure 1) that were conducted to generate a recombinant *N. caninum* strain displaying surface anti-PD-L1 scFv. Taken all together, we concluded that anchoring anti-PD-L1 scFv in *N. caninum* membrane by using *T. gondii* SAG1 resulted in unstable protein expression and lack of functionality.

3.2. Generation and characterization of recombinant *N. caninum* strain secreting anti-PD-L1 scFv

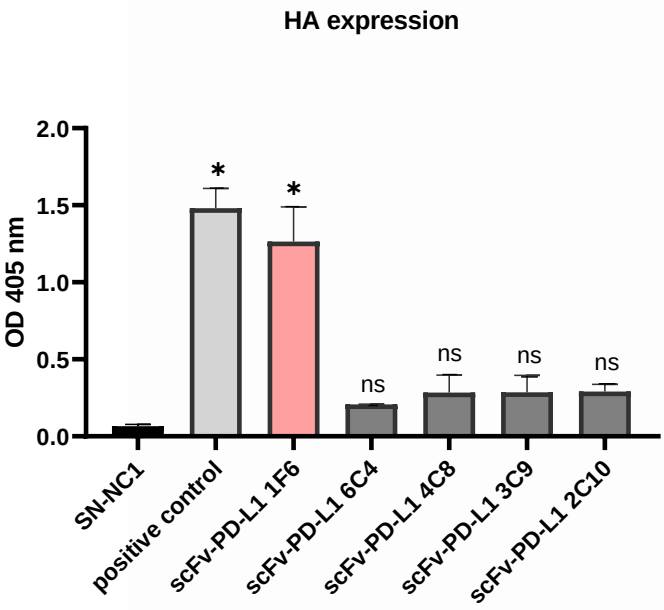
In parallel to our efforts to display anti-PD-L1 scFv on the membrane display, we also investigated the possibility of engineering *N. caninum* to stably secrete functional anti-PD-L1 antibody fragment. Previously in our laboratory, a *N. caninum* recombinant strain was successfully developed to secret functional interleukin 15 (IL-15) by targeting IL-15 encoding sequence to the microneme secretory pathway (Lantier et al., 2020). Building on the functionality of this construct, we aimed at constructing recombinant *N. caninum* strain secreting anti-PD-L1 scFv. Thus, as previously described, the scFv sequence of the human monoclonal antibody, Atezolizumab was constructed using the heavy and light chain variable regions connected with a flexible linker and attached to HA tag. To target the scFv to micronemes, a sequence encoding the N-terminal signal peptide and propeptide domain of *T. gondii* MIC5 was added. This construct cloned into pUC5 was then transfected into *N. caninum* NC1 strain and the generation of the recombinant tachyzoites was indicated by the GFP expression. Subsequently, recombinant clones were obtained and scFv-PD-L1 secretion was analyzed in culture supernatants of a number of selected clones by ELISA (Figure 2B). Compared to culture supernatant of NC1 tachyzoites, one clone exhibited significant secretion of scFv-PD-L1. This clone was used for subsequent characterization and *in vitro* evaluation. Having confirmed the protein expression, we next evaluated the ability of the secreted scFv-PD-L1 to bind to the immune checkpoint PD-L1 protein. The culture supernatant containing secreted scFv-PD-L1 was serially diluted (2-fold dilution) and tested for binding to the recombinant PD-L1 protein by ELISA (Figure 2C). Although the concentration in the supernatant is undefined, the secreted scFv-PD-L1 demonstrated strong dose-dependent binding to the human PD-L1 protein.

To visualize the expressed protein intracellularly, HFF cells were infected with NC-PD-L1sec tachyzoites for 24 h, then stained using anti-HA to visualize the scFv-PD-L1. As illustrated in Figure 2D, the red color indicating the scFv staining was located in the apical part of the tachyzoites.

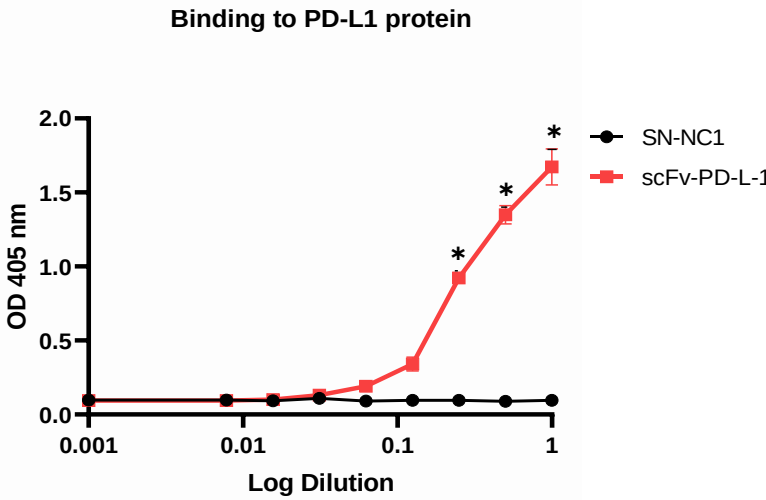
A



B



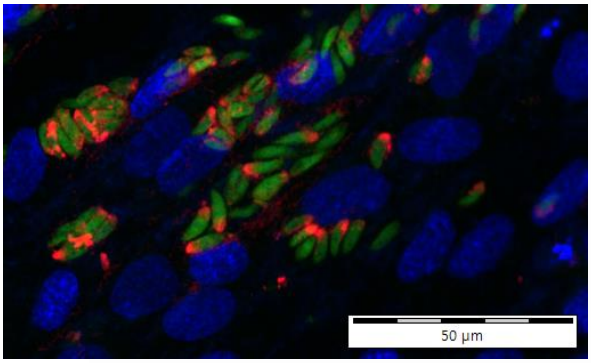
C



Ethanol enhanced secretion of scFv-PD-L1

D

Immunofluorescence staining of NC-PD-L1sec



E

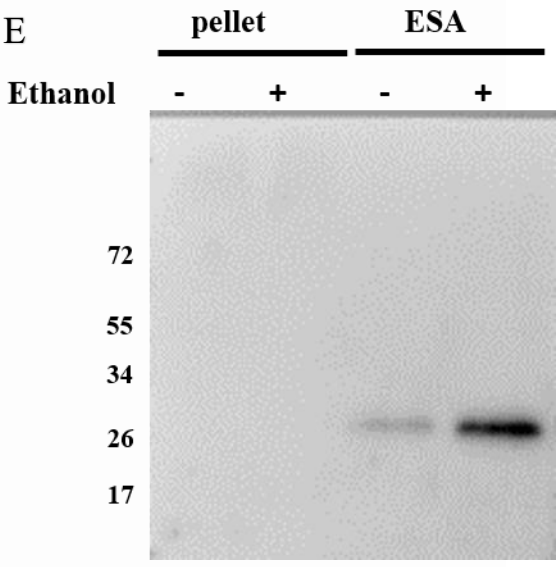


Figure 2. Generation and characterization of recombinant *N. caninum* secreting single chain variable fragment against the human programmed death-ligand 1. **(A)** Schematic representation of the sequence encoding the secreted scFv-PD-L1. The locations of Kozac sequence and start codon (ATG), signal sequences and propeptide of TgMIC5, antibody variable domains (heavy [VH] and light [VL]), peptide linkers ([Gly4Ser]3) and ([Gly3AlaSer]), and human influenza hemagglutinin (HA) tag are indicated. **(B)** scFv-PD-L1 secretion in culture supernatants. Five clones of NC-PD-L1sec. were selected and used to infect HFF cells at MOI 5. Culture supernatants were collected 72 h post-infection and scFv-PD-L1 secretion was analyzed by ELISA using rabbit anti-HA polyclonal antibodies followed by anti-rabbit IgG AP-conjugated. Culture supernatant of NC1 strain was used as control (n= 3 replicates). **(C)** Binding of scFv-PD-L1 to PD-L1 recombinant protein. Serially diluted culture supernatant collected from NC-PD-L1sec clone 1F6 (2-fold dilution) was analyzed for binding to histidine-tagged PD-L1 protein by ELISA. Bound scFv-PD-L1 was assessed by anti-HA antibody. Culture supernatant of NC1 strain was used as control (n= 3 replicates). **(D)** Visualization of scFv-PD-L1 expression by immunofluorescence. HFF cells infected with NC-PD-L1sec clone 1F6 for 24 h then permeabilized and stained with rabbit anti-HA followed by anti-rabbit IgG-PE (red). Nucleus were stained using Hoechst (blue). Images were captured at x600 with Olympus IX73 fluorescent microscope. Scale bars represent 50 μm**. (E)** Microneme secretion assay. Tachyzoites (1.5×10^7) were incubated for 5 min at 37 °C with or without 1% ethanol (induced versus constitutive secretion). After centrifugation, aliquots of pellets and supernatants were analysed by western blot probed with anti-HA antibody and subsequent incubation with HRP-conjugated anti-rabbit secondary antibody. All experiments were repeated at least three times. Statistical significance is indicated by *$p < 0.05$, ns: not significant. In all ELISA experiments, optical densities were read at 405 nm. MOI: multiplicity of infection.

While immunostaining indicates clearly the targeting of the scFv in the apical part of the tachyzoites, this does not necessarily confirm secretion via micronemes pathway. To verify if the scFv is properly targeted to and secreted via micronemes, ethanol enhanced secretion assay was performed. Ethanol is a potent stimulator for extracellular secretion of micronemes proteins (Carruthers et al., n.d.-a; Lovett et al., 2002). Accordingly, NC-PD-L1sec tachyzoites were collected from HFF infected cells and allowed to secret their proteins extracellularly in medium with or without ethanol 1%, then supernatants were analyzed by Western blot (Figure 2E). The protein bands obtained corresponded to the expected size of the heavy and light chains of the scFv. Moreover, ethanol stimulation was associated with a higher concentration of the secreted protein compared to medium without ethanol. On the other hand, analysis of tachyzoite pellets revealed no discernible bands indicating no or low protein. Together, these results strongly suggest that the secretion of anti-PD-L1 scFv occurrs through the micronemes secretory pathway.

3.3. Quantification of PD-L1-scFv secreted in culture supernatant

To determine the quantities of secreted anti-PD-L1 fragment in culture supernatant, we first needed a reference protein with a defined concentration. Sequences coding for scFv-PD-L1 (excluding the N terminal parts necessary for targeting to the micronemes) were cloned into pcDNA3.4 plasmid and transfected into CHO cells. After protein production and purification, 100 ng of the purified, recombinant anti-PD-L1 protein (designated scFv-PD-L1-CHO) was analyzed by Western blot to verify that the scFv is expressed at the predicted size. As demonstrated in Figure 3A, the obtained band around 28 kDa corresponds to the predicted size of a heavy and light chain of the scFv. To confirm the functionality of the purified scFv-PD-L1-CHO, its ability to bind to human PD-L1 protein was analyzed by ELISA and compared to Atezolizumab (Figure 3B). Comparable to Atezolizumab, the scFv indicated substantial binding to its target.

Using the successfully produced and characterized scFv-PD-L1-CHO as a reference, we quantified the scFv-PD-L1 secreted in the culture supernatant. Both secreted and CHO-purified proteins were analyzed by Western blotting. Due to uncertainty about the quantities secreted by the NC-PD-L1sec tachyzoites, we aimed at producing concentrated culture supernatant with - relatively high quantities of scFv to generate detectable bands. To this aim, HFF cells in a T75 culture flask were infected with NC-PD-L1sec tachyzoites and, once mature vacuoles formed, the medium was replaced with 2 ml medium to concentrate the scFv-PD-L1 in low volume of culture supernatant. The collected supernatant was then analyzed by Western blot in comparison to increased quantities of the scFv-PD-L1-CHO. As shown in Figure 3C, according to the intensities of the generated bands, the band generated from the scFv in culture supernatant was roughly equivalent in intensity to the band corresponding to 25 ng of scFv-PD-L1-CHO. Furthermore, for more precise quantification, secreted scFv-PD-L1 was analyzed by ELISA using CHO-purified anti-PD-L1 as a standard. To analyze the quantities secreted by different number of tachyzoites, HFF cells in 6-well plates were maximally infected with NC-PD-L1sec tachyzoites (MOI 10) and allowed to secret the anti-PD-L1 scFv in a volume of 2 mL of culture medium.

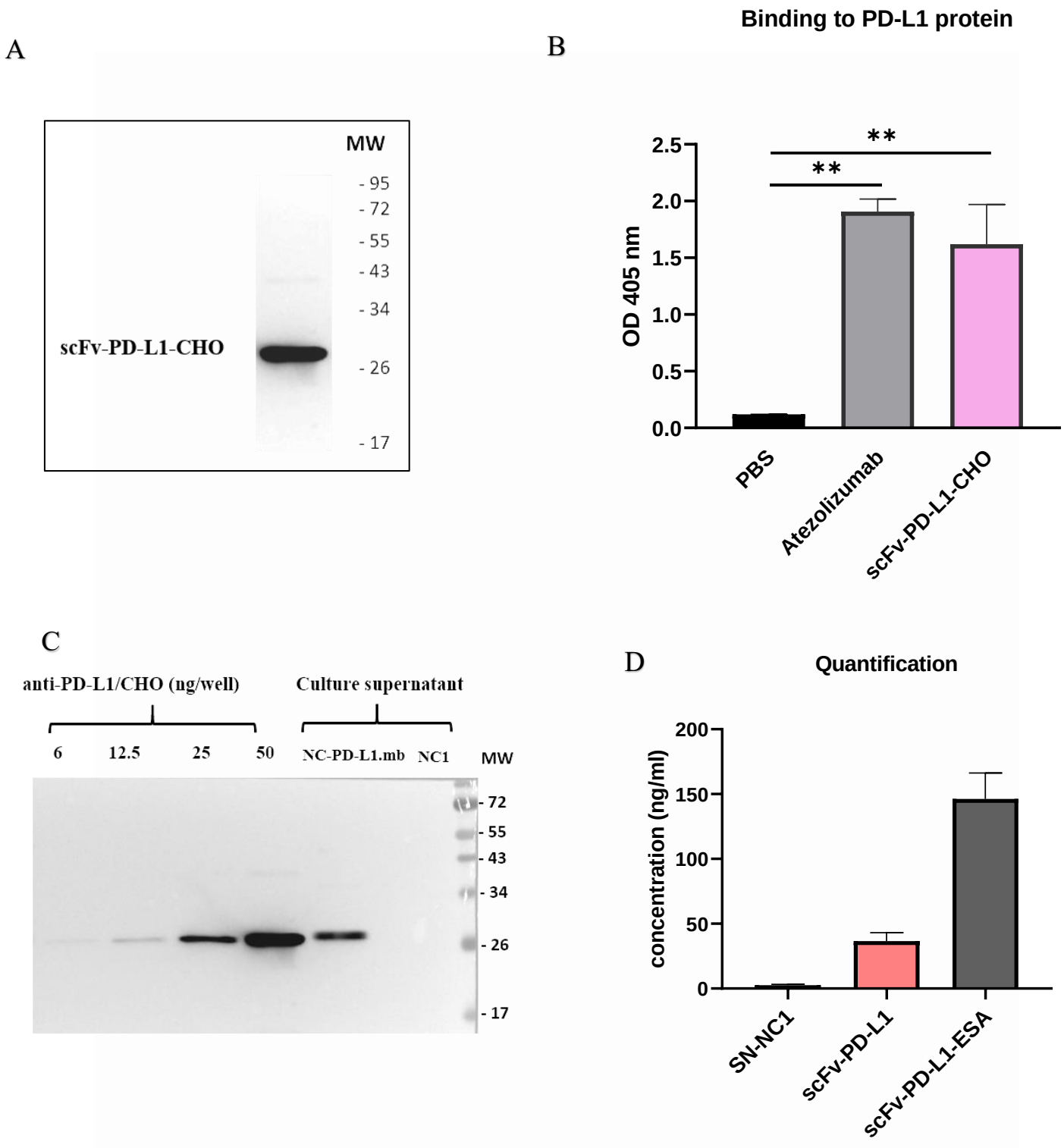


Figure 3. Quantification of the secreted scFv-PD-L1 in culture supernatants of recombinant NC-PD-L1sec tachyzoites **(A)** Generation of recombinant anti-PD-L1 scFv from Chinese Hamester Ovary (CHO) cells. CHO cells were transfected with the plasmid encoding for anti-PD-L1 scFv, recombinant protein (scFv-PD-L1-CHO) was purified, and 100 ng of the purified protein was analyzed by Western blot. The anti-PD-L1 scFv was detected using rabbit anti-HA antibody followed by anti-rabbit IgG HRP-

conjugated. MW: molecular weight marker in kilo Dalton. **(B)** Binding of the scFv-PD-L1-CHO to PD-L1 recombinant protein. scFv-PD-L1-CHO (100 nM) was analyzed for binding to histidine-tagged PD-L1 protein by ELISA. Bound scFv-PD-L1-CHO was assessed by rabbit anti-HA antibody followed by anti-rabbit IgG AP-conjugated. Atezolizumab (100 nM) was used as control and its binding to PD-L1 protein was measured by AP-conjugated anti-human IgG (n= 3 replicates). **(C)** Western blot of scFv-PD-L1 secreted by NC-PD-L1sec tachyzoites and scFv-PD-L1-CHO**.** Line 1: scFv-PD-L1-CHO 6 ng, line 2: scFv-PD-L1-CHO 12.5 ng, line 3: scFv-PD-L1-CHO 25 ng, line 4: scFv-PD-L1-CHO 50 ng, line 5: culture supernatant of HFF cells (in T75 culture plate) infected with NC-PD-L1sec tachyzoites (MOI 10) and collected in 2 mL of culture medium. Line 6: culture supernatant of NC1 (negative control). Protein bands were revealed using rabbit anti-HA antibody followed by anti-rabbit IgG HRP-conjugated. MW: molecular weight marker in kilo Dalton. **(D)** Quantification of the secreted scFv-PD-L1 by ELISA. NC1 (black column), scFv-PD-L1 secreted by 10^7 tachyzoites/mL (red column) and scFv-PD-L1 secreted by 10^9 tachyzoites/ mL (purple column) were quantified by ELISA using scFv-PD-L1-CHO as standard. All experiments were performed at least three times. Statistical significance is indicated by **$p < 0.01$. In all ELISA experiments, optical densities were read at 405 nm. AP: Alkaline Phosphatase. MOI: multiplicity of infection.

Additionally, to maximally concentrate the secreted scFv, tachyzoites were collected from mature but not lysed vacuoles of infected HFF cells and allowed to secret the protein extracellularly in limited volume (10^8 tachyzoites/ 100 uL). Subsequently, these two collected supernatants were analyzed by ELISA (Figure 3D). In accordance with the number of tachyzoites used to produce each supernatant, the concentrated supernatant (designated scFv-PD-L1-ESA) showed 10-fold increased secretion (about 150 ng/ml) compared to the regular supernatant (scFv-PD-L1) which contained about 40 ng/ml. By linking these estimated quantities to the number of parasites used to produce the evaluated supernatants, the quantities secreted can be estimated to be between 1 to 4 ng /10^6 tachyzoites.

3.4. Binding to PD-L1-expressing human and murine tumor cells

To evaluate the biological functionality of the secreted scFv-PD-L1 *in vitro*, we further evaluated its capacity to recognize and bound to PD-L1 expressed on the surface of tumor cells. To this aim, the triple negative human breast carcinoma cell line MDA-MB231 was used. Binding was evaluated by incubating 100 µL of culture supernatant containing scFv-PD-L1 (40 ng/ml) with MDA-MB231 cells (Figure 4A). Compared to culture supernatant of NC1 wild type strain, scFv-PD-L1 showed pronounced binding to MDA-MB231 cells. Human anti-PD-L1 IgG conjugated with APC was used as control. Moreover, considering that Atezolizumab can efficiently bind to murine PD-L1 (Magiera-Mularz et al., 2021), we examined the ability of human scFv-PD-L1 to bind to murine PD-L1 expressed on the surface of tumor cells using the

murine melanoma cell line B16F10. The B16F10 cell line shows moderate basal PD-L1 expression that can be upregulated by stimulating cells with IFN-γ.

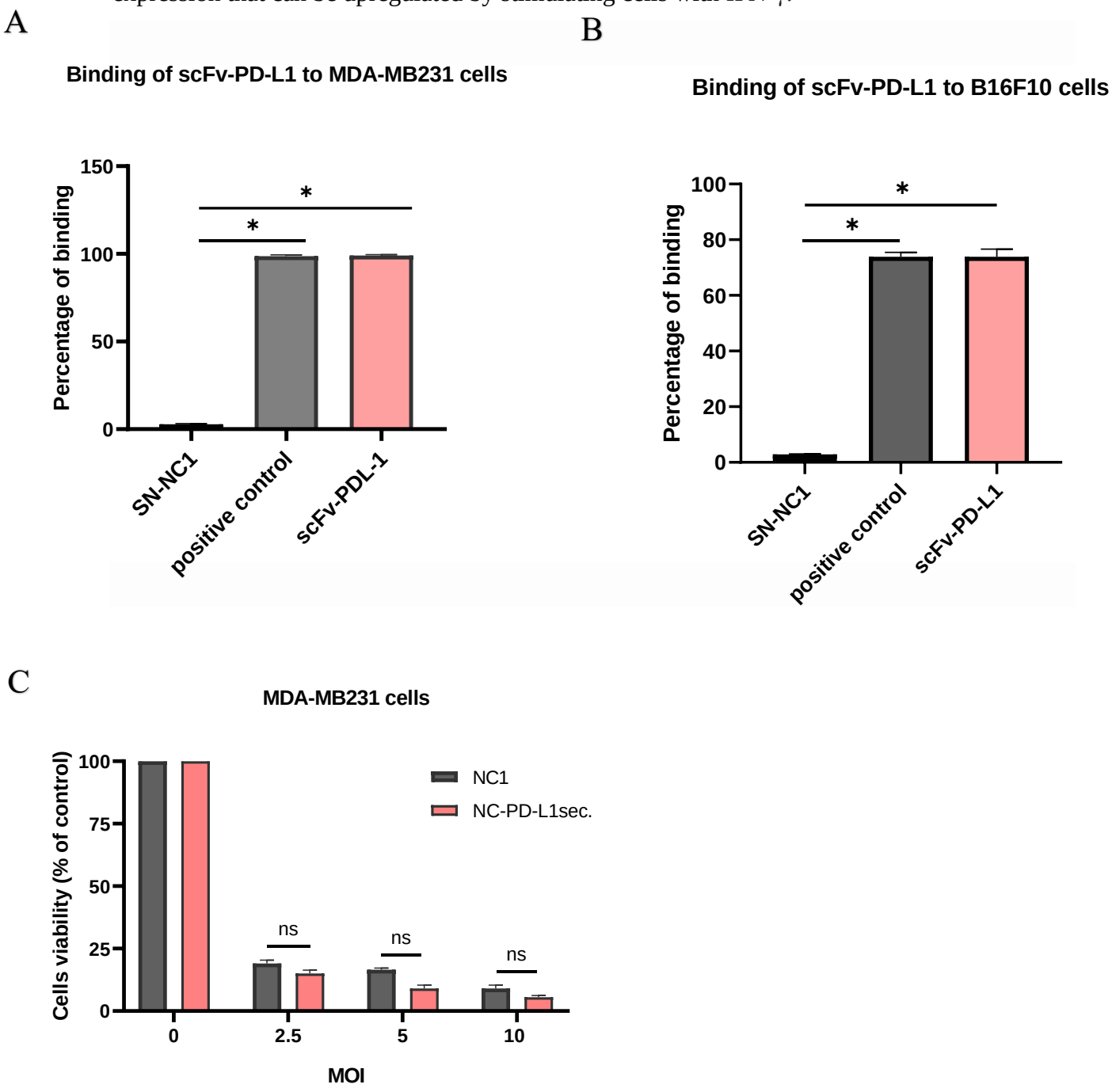


Figure 4. Biological functionality of the secreted scFv-PD-L1. **(A)** Binding of scFv-PD-L1 to human MDA-MB231 tumor cells. MDA-MB231 cells were incubated with 100 µL of scFv-PD-L1 and binding was analyzed by flow cytometry using rabbit HA antibody followed by anti-rabbit IgG APC-conjugated. Anti-human PD-L1-APC conjugated was used as positive control and culture supernatant of NC1 was used as negative control (n = 3 replicates) **(B)** Binding of scFv-PD-L1 to murine B16F10 tumor cells. 100 µL of scFv-PD-L1 was incubated with IFN-γ- stimulated B16F10 cells and bound protein was

analyzed by flow cytometry using rabbit anti-HA antibody. Anti-mouse PD-L1-APC conjugated was used as positive control and culture supernatant of NC1 was used as negative control (n = 3 replicates) **(C)** In vitro oncolytic activity. MDA-MB231 cells were left un-treated or infected with either the wild type NC1 tachyzoites or NC-PD-L1-sec recombinant tachyzoites at the indicated MOIs. 48 h post infection, cells were counted, and dead cells were excluded by trypan blue staining. Results are represented as percentages of viable cells in respect to the control un-infected cells. Statistical significance is indicated by *p < 0.05. All experiments were repeated at least twice. MOI: multiplicity of infection.

Following 24 h stimulation with 20 ng/ml of IFN-γ, both rested and IFN-γ-treated cells were incubated with 100 µL culture supernatant of either NC1 wild type strain or scFv-PD-L1 (40 ng/ml) and binding was evaluated by flow cytometry (Figure 4B) using mouse anti-PD-L1 APC-conjugated antibody as control. The results for binding to human and murine PD-L1-expressing cells were reproduced, as scFv-PD-L1 also showed significant binding to B16F10 cells that is increased in accordance to the PD-L1 expression level. These results indicated that the recombinant human anti-PD-L1 scFv secreted by NC-PD-L1sec recombinant strain can efficiently recognize and bind to both human and murine PD-L1.

3.5. *In vitro* oncolytic activity

Additionally, the ability of the engineered NC-PD-L1sec strain to infect and kill tumor cells was compared to that of the wild type strain. MDA-MB231 cells with its high basal PD-L1 expression represented a suitable model. Therefore, MDA-MB231 cells were left uninfected or infected with different MOIs of either NC1 or NC-PD-L1sec tachyzoites and cell viability was measured 48 h post-infection. Results in Figure 4C show that NC-PD-L1sec strain retains the typical cytopathic effect of NC1 with slightly enhanced killing ability that fell below the statistical significance. At MOI of 10, only 5 % of cells survived compared to 9 % with NC1.

4. Discussion

Neospora caninum has emerged as a promising immunotherapeutic agent that can be added to the realm of cancer immunotherapy. Recent studies in mice-bearing solid tumor models have demonstrated that *N. caninum* can inhibit tumor growth by directly infecting and lysing the tumors and through the stimulation of potent immune responses (Lantier et al., 2020; X. Li et al., 2022). A more recent study has shown that recombinant *N. caninum* strain engineered to secret IL-15 associated with the alpha-subunit of the IL-15 receptor significantly potentiated immune responses and improved antitumoral efficacy compared to the wild type strain (Battistoni et al., 2023). These findings were encouraging to investigate further strategies to

potentiate the protective effect of *N. caninum* and broaden its application to more challenging tumors. Thus, hereby in this research work, we aimed at investigating the possibility of arming *N. caninum* with anti-PD-L1 scFv to be either displayed on the parasite's membrane or by targeting it to one of the secretory organelles to be secreted by the engineered parasite.

Combining PD-1/PD-L1 blockade with oncolytic microorganism therapy has gained more interest in the recent years. Oncolytic microorganism therapy is generally featured by increased infiltration of immune cells to the TME which can turn "cold" tumors into "hot" ones making the TME more sensitive to PD-1/PD-L1 therapy. In a study by Zhu et al., they showed that treating mice-bearing murine melanoma B16F10 with *T. gondii* -closely related parasite to *N. caninum*- led to up regulation of PD-L1 and made the tumors more sensitive to anti-PD-L1 therapy (Zhu et al., 2021). Similarly, Bahwal *et al*. showed that co-administration of *T. gondii* and anti-PD-1 elicited a significant antitumor immune response and synergistically controlled tumor growth in mice pancreatic tumor models (Bahwal et al., 2022). Treatment with *N. caninum* was also found to upregulate PD-L1 expression in mice-bearing B16F10 murine melanoma (X. Li et al., 2022). Taken together, these findings indicate that combination between *N. caninum* and anti-PD-L1 might provide an interesting approach for optimizing the anti-tumor therapeutic outcomes.

First, based on our previous approach where *T. gondii* displaying surface anti-PD-L1 scFv was successfully generated and proved efficient in selectively targeting PD-L1 expressing tumors, here, we aimed at adopting a similar strategy by engineering the anti-PD-L1 scFv on the surface of *N. caninum*. Lantier *et al*. showed that distant treatment of mice-bearing solid tumors with *N. caninum* led to less inhibition of tumor growth compared to intratumor injection. This finding suggest that further strategies are needed to improve parasite targeting to tumor site when distantly injected (Lantier et al., 2020). Targeting of viruses and bacteria through expression of antibodies specific to tumor antigens has been shown to promote selective accumulation at tumor sites in various pre-clinical tumor models (Bereta et al., 2007; Nanni et al., 2013; Wienen et al., 2023). For example, Bereta et al. (2007) demonstrated that targeting an antibody specific to the epidermal growth factor receptor (EGFR) improved the selectivity of an oncolytic virus for EGFR-expressing tumors. Similarly, Nanni et al. (2013) showed that bacteria engineered to express tumor-targeting antibodies selectively accumulated in tumors, enhancing therapeutic outcomes. More recently, Wienen et al. (2023) confirmed that expressing antibodies specific to tumor antigens could guide therapeutic microorganisms to tumor sites, increasing their antitumor efficacy.

To engineer recombinant *N. caninum* strain displaying surface anti-PD-L1 scFv, we used the previously employed plasmid encoding the anti-PD-L1 scFv fused to TgSAG1 protein with an tag HA in the C-terminus designed to be expressed by *T. gondii.* Indeed, two previous studies, demonstrated that TgSAG1 protein could be correctly exported and displayed on the surface of *N. caninum* (Howe et al., 1997; Zhang et al., 2010). Thereof, the same plasmid was used to generate recombinant *N. caninum* strains. The generation of recombinant parasites was indicated by GFP expression seen under fluorescent microscope and initial characterization of expression revealed detectable level of scFv expression measured by ELISA in a pool of recombinant parasites and in two of the selected clones following 4-5 weeks of transfection. However, upon passaging these clones in culture for an additional two weeks, the surface expression was significantly diminished as it appeared in ELISA and flow cytometry analysis. Western blot analysis revealed the presence of degraded protein bands, none of them is corresponding to the expected band size. These recombinant tachyzoites also failed to recognize or bind to recombinant PD-L1 protein. Three independent attempts for transfecting *N. caninum* with this construct showed almost the same results (Supplementary Figure 1). Indeed, anchoring heterologous proteins using TgSAG1 has not always been successful. For instance, in a previous study by Charest *et al*., attempts to express the heterologous *Plasmodium yoelii* circumsporozoite protein on the surface of *T. gondii* through fusion with TgSAG1 was reported to be unsuccessful (Charest et al., 2000). Similarly, an effort to engineer the OVA protein on *T. gondii* by targeting it to different organelles, including surface expression via GPI-SAG1, revealed that membrane-bound OVA was significantly shed from the parasite surface and degraded bands appeared alongside the major band in Western blot analysis (Gregg et al., 2011). In line with these findings, we can presume that the unsuccessful expression of the anti-PD-L1 scFv might be linked to the use of TgSAG1 for anchoring.

To further analyze this assumption, we used the previously constructed antibodies DC2 and DC2-SAG1 which were designed to be displayed on the *T. gondii* membrane using either GPI or GPI-SAG1 respectively. To compare the two anchoring strategies and determine if the previously reported unsuccessful expression was influenced by TgSAG1, we transfected *N. caninum* tachyzoites with either the DC2 or DC2-SAG1 expression vectors. Western blot analysis, after 4 weeks culture under chloramphenicol selection, showed that while *N. caninum* tachyzoites transfected with the DC2 expression vector displayed a protein band corresponding to the expected size, those transfected with DC2-SAG1 did not successfully express the protein and exhibited the same degraded pattern previously seen with anti-PD-L1 scFv (Supplementary

Figure 2). However, the band intensity of DC2 indicated a low level of expression compared to the corresponding *T. gondii* recombinant strain. Thus, we concluded that, using TgSAG1 for displaying proteins on the surface of *N. caninum* is not the best strategy. Other strategies should be investigated to successfully express membrane bound proteins in *N. caninum*.

While membrane expression was not successful in this case, our attempt to generate *N. caninum* recombinant strain secreting anti-PD-L1 scFv was successful. Many researchers investigated the possibility of engineering oncolytic microorganism to express anti-PD-L1 or anti-PD-1 antibodies directly within the tumor bed. This strategy offers an added benefit of tumor-specific delivery of therapeutic antibodies, thus, potentially reducing the systemic toxicity associated with immune checkpoint inhibitors (ICIs) therapy. Supporting this approach, many viruses have been engineered to function as vector carriers for the intratumor delivery of anti-PD-1 or anti-PD-L1, demonstrating enhanced therapeutic responses (Passaro et al., 2019; Engeland et al., 2014; Bartee, Dunlap and Bartee, 2017).

Here, we describe the first generation and characterization of recombinant *N. caninum* strain secreting anti-PD-L1 scFv. For this purpose, we used the scFv comprising the heavy and light chain from the anti-PD-L1 Atezolizumab. To target this antibody to be secreted by one of the secretory organelles, we selected micronemes. Micronemes secret their proteins mainly on the extracellular space during egress from infected cells, gliding motility, and host cell invasion (Dubois & Soldati-Favre, 2019). Thus, targeting micronemes allows for the protein not only to be secreted in the infected cells but also for Bystander trans-binding by binding to adjacent non infected cells.

Previous attempts for targeting proteins to *T. gondii* rhoptries and micronemes indicated that proteins were correctly targeted but not secreted (Striepen et al., 2001). In a previous work by our research team, interleukin 15 targeted to microneme through TgMIC5 targeting signal sequences was successfully secreted by *N. caninum* and proved functional *in vitro* and *in vivo* (Lantier et al., 2020). Following the same approach, we constructed the anti-PD-L1 scFv incorporating N-terminal signal peptide and prodomain motif of MIC5 in the *N terminus* and tag HA sequence in the C-terminus. This protein was transfected into *N. caninum* tachyzoites and recombinant strains was generated as indicated with GFP expression.

Upon obtaining a positive clone, the secreted scFv-PD-L1 was analyzed and found to effectively recognize and bind to the recombinant immune checkpoint PD-L1 protein, indicating the functionality of this construct. The stability of expression has been preserved as long as recombinant parasites were cultured and no reduction was observed in the expression.

It has been reported that *N. caninum* recognizes *T. gondii* GRA and ROP targeting signals (Karsten et al, 1997). To our knowledge, *T. gondii* MIC targeting signals have not been investigated. However, given the high degree of homology between the two parasites secretory specific organelles, we can assume that *T. gondii* MIC targeting signals would function as well in *N. caninum*. Indeed, by immunofluorescence analysis, scFv anti-PD-L1 protein is localized to the apical end of the parasite, suggesting proper targeting to the micronemes. However, despite correct targeting, to either ROP or MIC *T. gondii* organelles, heterologous proteins expressed by *T. gondii* may not be secreted during invasion (Striepen et al, 2001). To further verify that the secretion was indeed occurring through the micronemes pathway, an ethanol enhanced secretion assay was performed. Ethanol is known to trigger microneme secretion in the absence of the host cells (Carruthers, 1999; Lovett et al., 2002). Indeed, Western blot analysis indicated an obvious increase in the amount of secreted anti-PD-L1 protein following ethanol stimulation suggesting that the scFv is secreted via micronemes.

To quantify the anti-PD-L scFv secreted by the recombinant *N. caninum* strain, the scFv was first produced in a CHO system to generate purified protein with a known quantity. This protein was then applied as standard. Quantification by two techniques revealed that the quantities secreted can be estimated to be around 1-4 ng for each one million parasites. These quantities are consistent with the IL-15hRec secreted by the recombinant NC-IL15hRec, which was also targeted to micronemes and produced in the range of 1-2 ng per 10^6 tachyzoites.

With these secreted quantities, IL-15-secreting *N. caninum* showed enhanced anti-tumor efficacy in mouse models of melanoma and thymoma (Battistoni et al., 2023; Lantier et al., 2020). Studies have shown that IL-15 quantities required to generate anti-tumor activity in mouse models have been shown in many studies to be between 2-3 µg. For instance, the intraperitoneal administration of 2 µg/mouse of human IL-15 linked to the human IL-15Ra sushi+ domain in mice bearing CT26 colon adenocarcinoma was associated with a strong proliferation of CD8+ T cells (Desbois et al., 2016). Similarly, Ng *et al*. demonstrated that the intraperitoneal administration of 3 µg/dose of heterodimeric IL-15 to mice with B16F10 melanoma, in combination with adoptive cell transfer, strongly promoted the infiltration and persistence of both adoptively transferred cells and endogenous CD8+ T cells into the tumor (Ng et al., 2017). Although the anti-PD-L1 scFv secreted by the recombinant *N. caninum* is in the same range as IL-15, the quantities used in pre-clinical studies to achieve an efficient anti-tumor response with anti-PD-L1 are much higher than those with IL-15. For instance, in the study by Zhu et al., to produce a synergistic effect between *T. gondii* and anti-PD-L1, mice bearing B16F10 melanoma were treated with the *T. gondii* ΔGRA17 strain along with an

intraperitoneal injection of 250 µg anti-PD-L1 antibody (Zhu et al., 2021). Similarly, Bahwal *et al*. demonstrated the synergistic activity between *T. gondii* and anti-PD-1 therapy by treating mice bearing Pan02 pancreatic tumors with an intraperitoneal injection of *T. gondii* strain NRTUA plus 205 µg of anti-PD-1 antibody (Bahwal et al., 2022). Another pre-clinical study evaluating the combination between Atezolizumab and anti-TGF-β treated mice with colon carcinoma MC38 tumors expressing Hu-PD-L1 by intraperitoneal injection of 2 mg/kg of Atezolizumab alone or in combination with anti-TGF-β (10 mg/kg) (Lim et al., 2021). These data raise questions about the efficacy of our model using *N. caninum* secreting anti-PD-L1 scFv in vivo. Therefore, it was essential to first develop in vitro systems to evaluate the functionality of this construct and to determine if the secreted quantities could be sufficient to generate the desired antitumor activity when locally delivered.

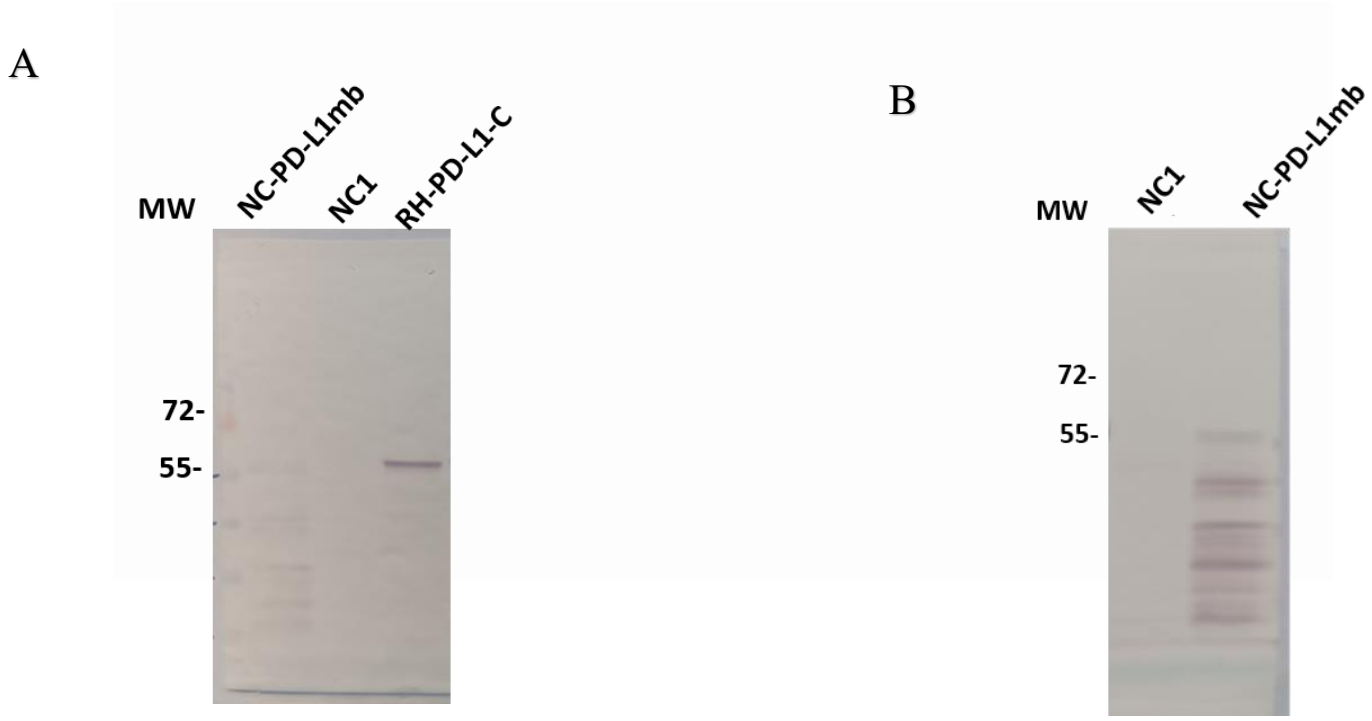
One powerful platform to study the interaction between immune cells and tumor cells in vitro is the co-culture model. The simplest forms of which are two-dimensional (2D) *in vitro* cell cultures comprising effector cells (immune cells) and target cells (could be tumor cells) (Jeong & Kang, 2023). This model can be used to generate preliminary results before proceeding for animal models. Thus, we aimed at developing a co-culture system to evaluate the oncolytic functionality of the recombinant *N. caninum*. In a previous study evaluating novel anti-PD-1 mAb, *in vitro* evaluation was carried out using co-culture system of jurkat T cells simulated to express PD-1 by phytohemagglutinin (PHA) as effector cells and hepatoma cell lines as target cells. This system proved effective to reflect the activity of their therapeutic compounds (Z. Li et al., 2020). A similar system composing of jurkat T cells and NCI-H460 lung cancer or MDA-MB231 breast cell lines was used to evaluate a plant extract claimed to have PD-1/PD-L1 inhibition activity (Jing et al., 2021). Following the same approach, we aimed at developing a system composing of jurkat T cells and MDA-MB231 cell line. First to validate this system, we used the anti PD-L1 Atezolizumab and anti-PD-1 Nivolumab to show if these antibodies can block PD-1/PD-L1 interaction and promote jurkat T cells activity. Despite the several attempts and different stimulation protocols to upregulate PD-1 expression and to optimize the system, neither addition of Atezolizumab nor Nivolumab showed any change in the jurkat T cells activity as it was measured by IL-2 and IFN-γ secretions.

As an alternative system, given that the scFv-PD-L1 effectively binds to murine PD-L1 positive tumor cells, we developed a model using the murine melanoma cell line B16F10 and the murine B3Z CD8+ T cells. B3Z is a T cell hybridoma expressing a TCR that specifically recognizes OVA (257–264) (SIINFEKL) in the context of H-2Kb. These cells are also express beta-galactosidase under the control of the IL-2 promoter and can recognize the SIINFEKL bound

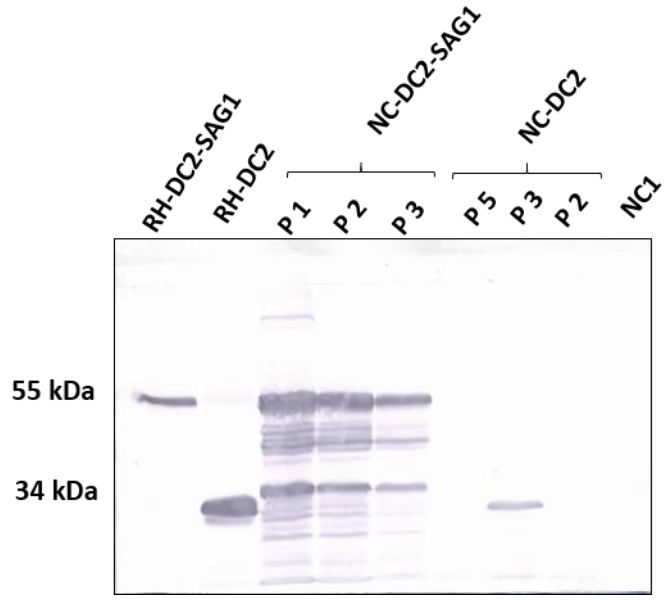
to H-2Kb MHC-I and OVA-peptide. The value of B3Z over the jurkat T cells is that they spontaneously express PD-1 with no need to stimulation. A previous study applied a co-culture between B3Z and B16F10 transfected with OVA to study the effect of hypoxic culture condition in activating B3Z by MHC I expression (Kajiwara et al., 2016). Thereof, B16F10 was loaded with ovalbumin peptide MHC1 and used as target cells and B3Z cells as effector cells. By using Atezolizumab, we verified that this system is capable of reflecting the PD-1/PD-L1 blockade and Atezoliaumab addition to the co-culture increased the concentration of the secreted IL-2 by B3Z (Supplementary Figure 3A). Next, we applied this system using the supernatant containing scFv-PD-L1 from the recombinant *N. caninum* culture (Supplementary Figure 3B). The addition of scFv-PD-L1 caused a modest 1.5-fold increase in IL-2 secretion compared to the supernatant from wild-type tachyzoite culture. This modest activity aligns with other studies evaluating the *in vitro* activity of blocking PD-1/PD-L1 interaction. For instance, Lin *et al*. evaluated an anti-PD-1 scFv delivered by oncolytic HSV-1 in a co-culture assay of splenocytes, purified T cells, and Hepa1-6 cells. They found that 3 µg of anti-PD-1 scFv resulted in a modest potentiation of T cell activity, which significantly increased at a higher quantity of 30 µg (C. Lin et al., 2020). Similarly, Orafaie *et al*. evaluated synthesized anti-PD-L1 peptides using the Lovo colorectal cell line co-cultured with Jurkat T cells. Addition of these peptides to co-culture, showed a 1.5 to 2-fold increase in IL-2 secretion by jurkat T cells (Orafaie et al., 2021) These preliminary results confirm the feasibility of arming *N. caninum* with functional antibody fragment against PD-L1 using the single chain variable fragment, the simplest antibody format. Encouraged by these results, we are now exploring the potential of arming *N. caninum* with more complex antibody formats to broaden the application of this system. Currently, our research group is developing and characterizing a recombinant *N. caninum* strain that secretes an anti-PD-L1 scFv fused with the crystallizable fragment (Fc) of mouse IgG. This approach aims to enhance the functionality and therapeutic potential of the antibody by leveraging the additional benefits conferred by the Fc region, such as improved stability and effector functions. This next step in our research will help determine whether more complex antibody formats can be effectively utilized in *N. caninum*-based immunotherapy.

In conclusion, our work establishes a novel platform for the localized delivery of immune checkpoint inhibitors, potentially offering a safer and more effective cancer treatment by reducing systemic toxicity. The continued development and characterization of these recombinant strains will be crucial in determining their efficacy and potential for clinical application.

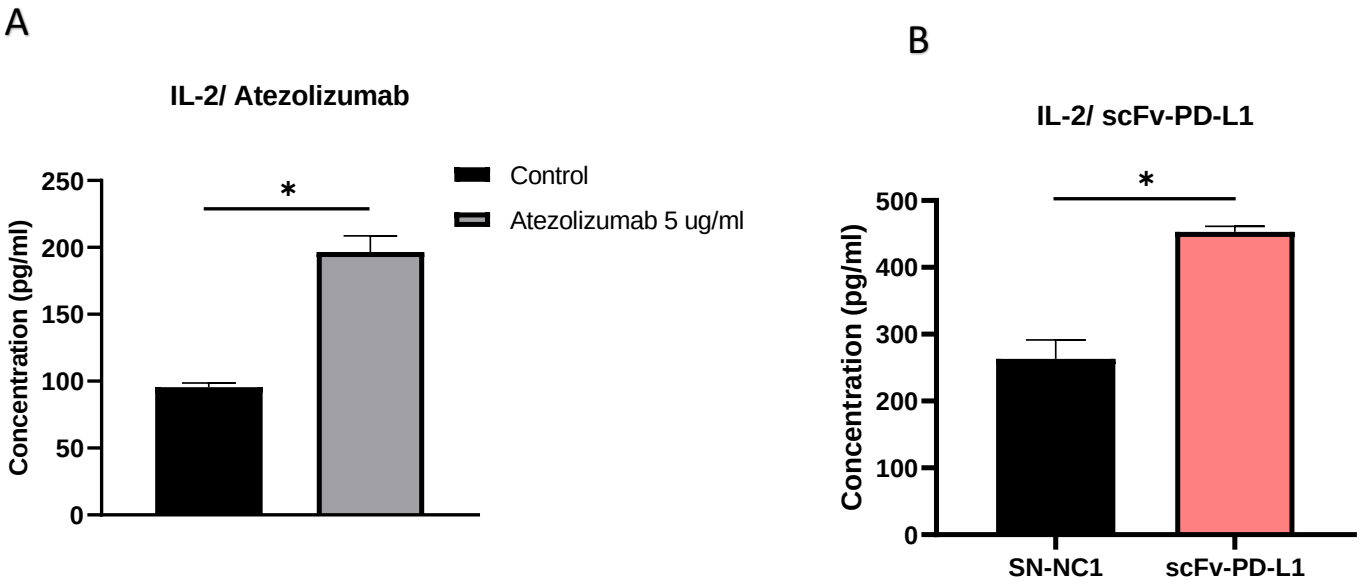
Supplementary Figures



Supplementary Figure 1. *Neospora caninum* engineered with human anti-PD-L1 scFv with SAG1 fusion protein and HA tag in the C-terminus (NC-PD-L1mb). Two independent transfection attempts were performed and transfected tachyzoites were subjected to Western blot analysis. Previously constructed *T. gondii* expressing anti-PD-L1 scFv with SAG1 fusion protein and tag HA in C-terminus (RH-PD-L1-C) was used as control. *N. caninum* wild type strain, NC1 was used as negative control. **(A) First experiment**. NC-PD-L1mb (Line 1), NC1 (line 2) and RH-PD-L1-C (line 3). **(B) Second experiment**. NC1 (line 1) and NC-PD-L1mb (Line 2). In both experiments, **p**rotein bands were revealed using rabbit anti-HA antibody followed by anti-rabbit IgG Alkaline phosphatase conjugated. MW: molecular weight marker in kilo Dalton.



Supplementary Figure 2. *Neospora caninum* engineered with murine anti-DEC205 scFv with GPI signal (NC-DC2) or with GPI signal and SAG1 fusion protein (NC-DC-SAG1). Transfected tachyzoites from each strain were divided into 3 pools and maintained in culture for 4 weeks under selection with CAT. Parasite cell lysates of these pools were analyzed by Western blot. Previously constructed *T. gondii* expressing DC (RH-DC2) or expressing DC2-SAG1 (RH-DC2-SAG1) were used as control. *N. caninum* wild type strain, NC1 was used as negative control. Lines represent: RH-DC2-SAG1 (Line 1), RH-DC2 (line 2), the three pools of NC-DC2-SAG1 (line 3-5), the three pools of NC-DC2-SAG1 (6-8), NC1 (Line 9). Protein bands were revealed using rabbit anti-HA followed by anti-rabbit IgG Alkaline phosphatase conjugated.



Supplementary Figure 3. Co-culture assay. The B3ZT cell line, specific for the MHCI-restricted peptide SIINFEKL (OVA257-264) in association with Kb class I MHC molecules were co-cultured for 20 h with IFNγ-stimulated B16F10 cells (over expressing PD-L1) loaded with OVA peptide (A) with Atezolizumab 5 µg/ml. Medium alone without Atezolizumab was used as control. (B) with NC1 culture supernatant (control) or scFv-PD-L1 secreted by NC-PD-L1sec tachyzoites. The B3Z response was measured by IL-2 secretion in co-culture supernatants by ELISA. Cell surface PD-1 expression on B3Z cells was confirmed by flow cytometer. Statistical significance is indicated by *$p < 0.05$. Experiments were repeated at least twice.

5. Discussion

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Cancer immunotherapy has emerged as a revolutionary approach to treat various types of cancers by harnessing the body's immune system to recognize and destroy tumor cells. One particularly intriguing and innovative strategy involves the use of microorganisms to stimulate or modulate anti-tumor immune responses offering a promising and multifaceted approach to cancer treatment. A wide range of bacteria and viruses have been explored as direct tumor killing tools due to their tropism for tumors (Yarahmadi *et al.*, 2024). However, accumulating evidence suggests their long-term benefits lie in inducing potent innate and adaptive immune responses and modulating the tumor microenvironment (Hamdan and Cerullo, 2023). Parasites have also shown promise in cancer treatment and recently being focused on as potent immunotherapeutic agents (Zheng *et al*., 2023). A growing body of evidence has demonstrated that parasites can achieve tumor regression in many solid tumors mediated through robust immune responses. In addition to their natural tropism, a key factor in applying microorganisms as immunotherapeutic agents is the possibility of arming them with multiple therapeutic molecules to enhance the anti-tumor effects and promote the safe application of these microorganisms. Ongoing research continue to explore the full potential of these biological agents. Several microorganisms, particularly viruses, are currently in clinical trials to investigate their anti-tumor potential as monotherapies or in combination with various other cancer treatment modalities (Wang et al., 2022).

In addition to microorganism-based therapy, the field of cancer immunotherapy encompasses several strategies including immune checkpoint inhibitors (ICIs), adoptive cell therapy, cancer vaccines and cytokines. While immunotherapy has significantly changed the paradigm of cancer therapy, many challenges and limitations remain. One key strategy to overcome these challenges is through combining different treatment modalities to optimize and enhance treatment efficacy, as well as achieving a durable anti-cancer effect. In this context, combinations between PD-1/PD-L1 blockade and oncolytic viruses (Lin et al., 2020; Mitchell et al., 2019) bacteria (Gurbatri *et al*., 2020) and parasites (Zhu et al., 2021; Bahwal et al., 2022) have shown promise in many pre-clinical studies. Current clinical trials are investigating the combination of oncolytic viruses and PD-1/PD-L1 blockade therapy. For instance, T-VEC, an FDA-approved herpes simplex virus engineered with GM-CSF, is being evaluated in combination with the anti-PD-1 pembrolizumab for patients with refractory head and neck

squamous cell carcinomas (Harrington et al., 2020) and advanced melanoma (Chesney et al., 2022). Additionally, the combination of pembrolizumab with oncolytic bacteria is also being investigated. An attenuated non-toxic strain of *Clostridium novyi* is currently in phase one first-in-human testing in combination with the anti-PD-1 pembrolizumab for treatment of patients with injectable, treatment-refractory solid tumors (NCT03435952).

Thus, in this research work, we investigated the possibility of combining the oncolytic activity of two apicomplexan parasites: *Toxoplasma gondii* and *Neospora caninum* with the immune checkpoint inhibition by targeting PD-1/PD-L1 pathway. Our primary objective was to optimize the use of *T. gondii*-based immunotherapy. The unique immunogenic properties of *T. gondii* make it an intriguing candidate for enhancing anti-tumor immune responses. However, its non-specific invasiveness and pathogenicity may limit its application. Researchers have attempted at overcoming these limitations by using attenuated strains which have shown promise in some solid tumor models in pre-clinical studies (Baird *et al*., 2013; Zhu *et al*., 2021; Chen *et al*., 2023). Nonetheless, the invasiveness of living *T. gondii* is essential for its anti-tumor activity. A study by Fox *et al.* demonstrated that a noninvasive heat killed uracil auxotroph strain of *T. gondii* induced early IL-12 production but did not stimulate IFN-γ secretion in the tumor microenvironment. Since CD4+ and CD8+ T cells are the major producers of IFN-γ in *T. gondii* infection, this result suggested that T cell populations were not highly activated in the absence of parasite invasion (Fox et al., 2016). Thus, using invasive *T. gondii* strains may offer an added value over attenuated strains by inducing a more potent immune response and allowing tachyzoites to replicate in tumor cells causing their lysis. To alleviate safety concerns associated with applying invasive strains, therapy based on *T. gondii* must be targeted to be tumor specific while sparing healthy tissues. This targeted approach could maximize the therapeutic benefits while minimizing potential risks, ensuring a more effective and safe cancer treatment strategy.

Specific tumor targeting has emerged as an approach to minimize non-specific biodistribution and enhance retention of oncolytic microorganisms within tumor sites. To this end, several researchers have investigated the feasibility of reprogramming oncolytic viruses by de-targeting them from their natural cell entry receptors through knockout deletion or mutation of specific viral genes and re-targeting them by incorporating new ligands or antibodies. These modifications facilitate binding to surface-expressed receptors, ligands, or antigens that are exclusively or abundantly expressed on cancer cells. For example, the measles virus (MV) naturally enters the cells through its H and F proteins, which together form a fusogenic

membrane glycoprotein complex. The H protein mediates attachment to either of the viral receptors CD4613 or SLAM14 on the cell surface and signals the F protein to trigger cell fusion (Lamb et al., 2006). Mutations in the amino acid residues involved in binding to CD46 and SLAM in the H protein resulted in generation of detargeted MV that is incapable of entering normal cells. To further retarget this MV to be tumor specific, an scFv against carcinoembryonic antigen (CEA) or EGFR was introduced into the C-terminus of H protein to mediate viral entry on receptor positive cells. Each modified virus induced cell death exclusively in cells expressing the relevant targeted receptor (Nakamura et al., 2004). Another example of detargeting-retargeting involves modifying the herpes simplex virus to achieve tumor specificity. HSV enters cells via the interaction of the virion envelope glycoprotein gD with the nectin1 receptor. A modified HSV strain, in which the gD Ig-core (amino acids 61–218) critical for nectin1 interaction was deleted and replaced with a single-chain variable fragment (scFv) against HER-2, which is overexpressed in many breast and ovarian cancers. This construct selectively infected cells expressing HER-2 receptors and demonstrated efficient tumor growth inhibition *in vivo* in mice bearing HER-2 overexpressing ovarian cancer (Menotti et al., 2009).

Targeting strategy through surface modification has also been applied to oncolytic bacteria to potentiate tumor colonization and reduce systemic infection and toxicity. For instance, an oncolytic *Salmonella* strain was engineered to express surface single chain antibody fragments (scFv) against the carcinoembryonic antigen (CEA) which is overexpressed in many tumors. The CEA-scFv surface expression led to increased accumulation in CEA positive tumors in mice which were inoculated with MC38 colon carcinomas on the left flank and CEA-transfected MC38 carcinomas (MC38CEA) on the right flank. Following the intravenous injection of *Salmonella* expressing CEA-scFv, a significantly higher numbers of bacteria was observed within the MC38CEA tumors over MC38 tumors ($P < 0.01$) with few clones detected in the spleen and liver. Then, treating mice bearing established MC38CEA tumor with either unmodified parental strain (as control) or *Salmonella* expressing CEA-scFv showed superior inhibition of tumor growth and increased survival with the engineered strain (Bereta *et al*., 2013). These examples showcase the potential of surface modification in enhancing the specificity and efficacy of oncolytic viruses and bacteria offering a targeted method to improve therapeutic outcomes while minimizing off-target effects.

Although surface modifications proved effective, this approach has not been applied in oncolytic parasites before. Thus, we investigated the feasibility of engineering *T. gondii* to display a fragment antibody on its surface to target a tumor over-expressed protein. As a target,

we selected the immune checkpoint protein, PD-L1. The combination of *T. gondii* with PD-L1 or PD-1 blockade has shown a synergistic effect, as *T. gondii* has been found to upregulate PD-L1 expression in the TME making it more responsive to anti-PD-L1 or anti-PD-1 therapy (Zhu *et al*., 2012; Bahwal *et al*., 2022). Targeting PD-L1 overexpressed on tumors to increase tumor localization and combining oncolytic microorganism therapy with immune checkpoint blockade has been previously applied to viruses using various surface modification techniques. For instance, encapsulating virus into bioengineered cell membrane nanovesicles with PD-1 displayed on the surface led to significant accumulation of the virus complex in PD-L1 expressing tumors and was associated with tumor growth inhibition and immune response induction (Lv *et al*., 2021). Similarly, nanoparticles were applied to bacteria by simultaneous conjugating tumor-specific antigen OVA and anti-PD-1 and performing in situ polymerization of dopamine onto the surface of *Escherichia coli Nissle* 1917. In mice-bearing OVA overexpressing CT26 and MC38 colon cancer, this decorated bacterial strain achieved synergistically improved tumor regression and animal survival following a single injection (J. Li et al., 2022). Another innovative approach involved a knob-into-hole based bispecific antibody recognizing PD-L1 and a short linear epitope introduced into the virus capsid. This bispecific antibody/ adeno-associated virus complex showed selective accumulation in PD-L1 positive tumors *in vitro* (Kuklik *et al*., 2021). These examples highlight the potential of surface modification techniques to enhance the specificity and efficacy of oncolytic microorganisms. By applying similar strategies to *T. gondii*, we aim to increase its tumor localization and synergize its oncolytic activity with immune checkpoint blockade, potentially leading to more effective and targeted cancer therapies.

To investigate our hypothesis, we selected the type I strain RH of *Toxoplasma gondii*. The RH strain, first cultured in laboratories *in vitro* since 1939, has become the reference *T. gondii* strain (Ajzenberg, 2010). This strain is highly replicative in mice having lost its cyst-forming capacity, keeping the parasites in replicative form (Asgari et al., 2013; Frenkel et al., 1976). The feasibility of engineering this strain has been demonstrated earlier. For instance, Nishikawa *et al*. successfully engineered the RH strain to secrete functional mouse IFN-γ (Nishikawa et al., 2003). Additionally, heterologous protein has been previously engineered to be displayed on the RH surface through glycophosphatidylinositol anchor attachment. An example of this is the expression of the *Cryptosporidium* Glycoprotein antigen, Cpgp40/15 gene on the RH surface, creating a surrogate system using *T. gondii* for the study of *Cryptosporidium* biology (O'Connor *et al*., 2003). Given its replicative nature and proven engineerability, the RH strain represents a

suitable replicative and engineerable model to investigate the potential of surface modification to enhance the targeting and therapeutic efficacy of *T. gondii* in cancer treatment.

Next, as a proof of concept for our theory, we began with an antibody previously constructed by our research group, targeting the dendritic cell-restricted antigen-uptake receptor DEC205 with a single-chain fragment variable (scFv). This antibody was initially developed to target the immunogenic *T. gondii* SAG1 protein to increase antigen presentation as a vaccination strategy against *T. gondii* in mice. The scFv consists of the variable domains of the heavy and light chains of an anti-DEC205 antibody in a VH-VL orientation, connected by a linker and attached to SAG1 via a short spacer. This construct has been proven functional *in vitro* and *in vivo* (Lakhrif et al., 2018). Furthermore, a previous study by Michon *et al*. reported that an anti-DEC205 scFv was successfully expressed on the surface of *Lactobacillus plantarum* bacteria. To target and anchor the scFv to the surface of *L. plantarum*, the authors explored different anchoring strategies and found that the fragment was detectable when coupled to cell wall anchors (Michon et al., 2015). Building upon this approach, we used the anti-DEC205 scFv as a model to be displayed on the surface of *T. gondii* using two anchoring techniques. The first technique involved direct glycosylphosphatidylinositol (GPI) anchor attachment of the scFv and the second technique involved adding the *T. gondii* membrane SAG1 between the scFv and the GPI (GPI anchored scFv-SAG1 fusion protein). Both strategies have been described before to display heterologous proteins on *T. gondii* surface (O'Connor et al., 2003; Charest et al., 2000). By implementing these anchoring techniques, we aimed to enhance the tumor-targeting capabilities of *T. gondii*, leveraging the specificity of the anti-DEC205 scFv to achieve more precise and effective targeting of tumor cells. This approach not only provides a proof of concept for our hypothesis but also paves the way for developing targeted oncolytic parasite therapies that can be combined with immune checkpoint blockade for enhanced anti-tumor efficacy.

In our effort for proper integration and selection for recombinant *T. gondii* strains, plasmids were constructed to encompass two expression cassettes one for expression of the desired protein and the other for the selection of positive transfected strains. *T. gondii* has a large array of selectable markers available, including those originally developed to establish stable transfection, such as DHFR-TS, bacterial chloramphenicol acetyl transferase (CAT) and bleomycin (BLE) selection (Limenitakis & Soldati-Favre, 2011). Chloramphenicol shows a potent but delayed parasiticidal effect, making the use of *E. coli* chloramphenicol acetyltransferase (CAT) as a reliable selectable marker gene (Kim et al., 1993). Striepen *et al*.

showed that using CAT fused with green fluorescent protein (CAT-GFP) for the selection of transfected *T. gondii* parasites resulted in 75% of population being fluorescent. Moreover, all parasites within each clone exhibited comparable fluorescent intensities as analyzed by flow cytometry analysis. Hybridization analysis of total DNA from low, intermediate, and bright subpopulations of stable CAT transgenics isolated by FACS confirmed the correlation between fluorescence intensity and gene copy number (Striepen *et al*., 1998). Thus, we identified CAT-GFP as a suitable system for selection of the recombinant strains. The other expression cassette in the plasmid was designed for expression of the desired protein under the *T. gondii* TUB1 (α-tubulin) promoter. Striepen and Soldati reported that a constitutive level of expression can be achieved using vectors derived from the TUB1 promoter (α-tubulin) as well as other promotors such as DHFR (dihydrofolate reductase), ROP1 (rhoptry protein 1), MIC2 (microneme protein 2) and several GRA (dense-granule proteins) (Striepen and Soldati 2007).

Accordingly, plasmids were constructed encoding the anti-DEC205 scFv with GPI or GPI-SAG1 and used to transfect RH tachyzoites, generating the recombinant RH strains: RH-DC2 and RH-DC-SA1 respectively. Although, Charest *et al*. reported that fusions of the heterologous protein with SAG1 resulted in either undetectable, or truncated protein products expressed in the cytoplasm of recombinant parasites (Charest *et al*., 2000), our results indicated successful expression of the anti-DEC205 scFv following both anchoring strategies. The addition of the SAG1 fusion protein resulted in a better exposure of the expressed protein as evidenced by its ability to target DEC205 protein and DCs expressing DEC205 receptor. These results verified the possibility of engineering functional single-chain fragment variables on the surface of *T. gondii*. In this specific case, the addition of SAG1 protein enhanced the functionality of the expressed scFv.

Encouraged by these results, we moved forward to apply this approach to promote tumor targeting by *T. gondii* through engineering anti-PD-L1 scFv to be displayed on the *T. gondii* surface. PD-L1 is overexpressed on many solid tumors and immunosuppressive myeloid cells in the TME. In the study by Zhu *et al*., they have shown that treatment with *T. gondii* in mice models of solid tumor resulted in upregulation of PD-L1 expression which increased the sensitivity of tumor to anti-PD-L1 therapy and synergistically improved the PD-1/PD-L1 blockade therapy outcomes (Zhu et al., 2021). We hypothesized that *T. gondii* engineered to display an anti-PD-1 on its surface could offer a multifaceted therapeutic strategy by: increasing accumulation on the tumor site following intra-tumoral injection, reducing dissemination in the organism, preferentially infecting tumor PD-L1 positive cells leading to tumor cell lysis.

Moreover, the anti-PD-L1 displayed on the surface on *T. gondii* may contribute to block PD-1/PD-L1 interaction and to reactivate the T cells mediated immune response. This strategy combines direct oncolytic effects with immune modulation, potentially leading to more effective and targeted cancer therapies.

To test this hypothesis, we constructed an anti-PD-L1 single-chain variable fragment (scFv) using the heavy and light chain variable regions of the human anti-PD-L1 antibody Atezolizumab. The selection of Atezolizumab was based on its efficient binding to and neutralization of murine PD-L1, making it suitable for evaluating the oncolytic activity in mouse tumor models (Magiera-Mularz et al., 2021). Following the same strategy used for designing the anti-DEC205 scFv, and given that fusion with the SAG1 enhances functionality, we constructed the anti-PD-L1 scFv in a VH-VL orientation, linked with a short linker, and attached it to the SAG1 protein via a short spacer. One more challenge was the localization of HA tag incorporated in the construct for immune detection and characterization. Previous studies have shown that the heavy chain is more critical for binding to PD-L1 than the light chain (Zhang *et al*., 2017). Therefore, adding an HA tag to the VH chain may negatively impact the functionality of the constructed antibody. To explore the impact of HA tag localization on the functionality of the expressed antibody fragment, two configurations of the anti-PD-L1 scFv were constructed: one with HA tag fused to the VH N-terminus and the other with HA tag attached to VL C-terminus with a short spacer. Consistent with Zhang *et al*., our results indicated that placing the HA tag at the VH N-terminus resulted in a correctly expressed but non-functional fragment. Conversely, the configuration with the HA tag at the VL C-terminus resulted in a correctly expressed and functional fragment. Thus, the recombinant strain named RH-PD-L1-C has been proved functional in binding to PD-L1 protein and PD-L1 expressing cells. The different experiments which were performed to investigate the oncolytic functionality and targeting feasibility of this construct indicated applicability of this approach to target *T. gondii* to the target positive tumor cells.

In addition to targeting, RH-PD-L1-C strain was shown to be able to potentiate T cell immune response to B16F10 melanoma cells *in vitro.* This phenomenon is suggested to be through blocking of PD-1/PD-L1 interaction. Currently, our research group is working on developing three-dimensions tumor models using various cell lines and tumor xenografts to develop complex in vitro models to evaluate our constructed immunotherapeutic agents. Thus, our next step is to evaluate the anti-PD-L1-expressing *T. gondii* in a model of PD-L1 expressing tumors to study the effect of targeting and blocking of PD-1/PD-L1 interaction on tumor growth

inhibition and to evaluate the immune response by analyzing different chemokines and cytokines productions.

The *in vitro* mixed cell assay revealed that the recombinant tachyzoites preferentially bound to cells overexpressing PD-L1, with negligible off-target binding (only 7%). However, it's crucial to address the *in vivo* behavior of this recombinant strain to fully understand its potential for targeted tumor therapy. Further experiments in mouse models of PD-L1-positive tumors are needed to evaluate the targeting efficiency and to assess if the surface display of anti-PD-L1 helps retain the parasites within tumors and reduces *in vivo* biodistribution.

While it is applicable with viruses to completely detarget them from their cell entry receptors, this notion is less applicable in parasites due to the more complex and not fully described cell entry machinery. Thus, obtaining *T. gondii* that is fully retargeted is challenging. In *T. gondii*, gliding motility and host cell invasion depend on an actin–myosin system and on the release and capping of proteins from apical secretory vesicles. Microneme proteins (MICs) contain modules that are homologous to adhesive domains from higher eukaryotic proteins and have often been shown to bind receptors on host cell. Upon secretion and redistribution on the parasite surface, transmembrane MICs are thought to connect external receptors to the submembranous acto–myosin motor that provides the power for parasite gliding and host cell invasion (Ménard, 2001). Thus, a modified *T. gondii* RH strain with disrupted MIC1 and MIC3 genes (mic1-3KO strain), has been shown to be less virulent in mice while remaining replicative (Cérède et al., 2005; Moiré et al., 2009). This strain could represent a suitable model to adopt the targeting strategy to reduce the virulence in non-targeted healthy tissue and reduce the parasite dissemination. Mic1-3KO strain can be further optimized to induce stronger immune response. This optimization may include deleting ROP 16 and inserting GRA15 gene from *T. gondii* type II strain. ROP16 type I activates signal transducer and activator of transcription (STAT3/STAT6) signaling pathways and deletion of ROP16 converts type I parasites from low to high inducer of IL-12 (Butcher et al., 2011). Furthermore, ROP16 type I has been shown to control the macrophage polarization towards a M2 state following infection with type I parasites (Jensen et al., 2011). GRA15 type II activates the NF-kB pathway and is responsable for a large part of the IL-12 response by infected macrophages in vitro (Rosowski et al., 2011). *N. caninum* could be also engineered to express TgGRA15 type II. as it has been shown to up-regulate the NF-kB pathway and to induce increased IL-12 production in macrophages (Mota et al., 2017).

The research presented herein demonstrates the feasibility of engineering single chain antibody fragments for display on *T. gondii's* surface, showcasing the potential application of this

strategy in inducing specific tumor targeting. This approach holds promise for further investigation across various *T. gondii* strains offering opportunities to explore the potential anti-tumor activity of this parasite in a safe context.

The second arm of this thesis research focuses on optimizing the anti-tumor efficacy of *Neospora caninum* by arming it with anti-PD-L1 scFv. Our research group has previously demonstrated the potential anti-tumor applicability of *N. caninum* in mouse models of solid tumors. These results indicated that intratumor treatment of mice-bearing murine thymoma and human Merkel cells tumors resulted in tumor growth inhibition and induction of potent anti-tumor immune response. However, distant treatment did not completely eradicate implanted tumors. As discussed, surface expression of antibodies targeting tumor-specific or overexpressed proteins resulted in specific tumor targeting and localization even with distant tumors. Thereof, we hypothesized that *N. caninum* could be directed to distant tumors through the expression of surface anti-PD-L1 scFv.

N. caninum shares similar morphological and genomic similarities (85%) with *T. gondii* albeit with differences in their disease pathologies and host range. While virulent strains of *T. gondii* are known to cause disease worldwide in all animals, *N. caninum* is restricted by a comparatively smaller host range (Coombs *et al*., 2020). Moreover, it has been previously reported that *N. caninum* can be engineered to express surface *T. gondii* SAG1 protein under the control of *T. gondii* promotor (Howe et al., 1997; Zhang et al., 2010). Thus, we hypothesized that the previously constructed membrane anti-PD-L1 scFv with SAG1 fusion protein to be expressed by *T. gondii* can be transfected into *N. caninum.* However, repetitive attempts to express this construct on *N. caninum* resulted in transient expression followed by loss of expression appeared as degraded protein bands. Failure to anchor heterologous proteins using *T. gondii* SAG1 fusion protein has been previously reported even on *T. gondii* (Charest *et al*., 2000). Thus, to further verify the link between using SAG1 protein and the unstable expression, we utilized the previously constructed proteins DC2 and DC2-SAG1 to compare the expression of the same protein with and without SAG1 fusion. The *N. caninum* tachyzoites transfected to express DC2-SAG1 showed degraded protein bands or, at best, transient expression that was lost within two months after transfection. Conversely, transfection of *N. caninum* with DC2 without SAG1 protein resulted in a single protein band corresponding to the expected size of scFv, although the band intensity was significantly lower than that of the corresponding *T. gondii* strain expressing the same construct, indicating low protein expression levels. This experiment was performed once, therefore, further experiments are needed to verify the

feasibility of expressing functional proteins with adequate levels using GPI anchoring. An alternative approach would be using membrane protein from *N. caninum* such as NcSAG1 rather than TgSAG1 or a different anchoring technique such as using transmembrane domain. In a previous study by Seeber et al., *T. gondii* was engineered to express TgSAG1 anchored in the membrane by human transmembrane domain (SAG1-TM) instead of its natural GPI anchor. The results showed that SAG1-TM is correctly folded and targeted to the surface of the parasite with no significant difference was seen in any aspect of the growth between the engineered and the wild type parasite (Seeber et al., 1998). This approach could also be explored in *N. caninum* to express functional surface proteins.

While targeting scFv to *N. caninum* surface through SAG1 anchoring was unsuccessful, targeting the scFv to secretory pathway resulted in the secretion of functional anti-PD-L1 fragments. In a previous work by our research group, *N. caninum* was successfully engineered to secrete IL-15 associated with the alpha-subunit of the IL-15 through targeting the protein sequence to micronemes secretory pathway. Micronemes secret their proteins mainly on the extracellular space during egress from infected cells, gliding motility, and host cell invasion (Dubois and Soldati-Favre, 2019). Thus, targeting micronemes allows for the protein to be secreted in extracellular space. Although a previous study by Striepen *et al*. showed that proteins targeted to *T. gondii* rhoptries and micronemes were correctly expressed and targeted but not secreted (Striepen et al., 2001). Yet, our previous model with *N. caninum* secreting IL-15 targeted to micronemes through MIC5 N-terminal signal resulted in a secretion of a functional protein. Thus, following the same approach, anti-PD-L1 scFv was constructed and recombinant *N. caninum* strain was successfully generated. The secreted protein was confirmed to be correctly expressed through various immunoblotting techniques and functional by binding to its target protein on human and mouse tumor cells expressing the targeted receptor.

The estimated quantity of the secreted anti-PD-L1 scFv by recombinant *N. caninum* was estimated to be around 1-4 ng per million tachyzoites. This prompted the question of whether these quantities secreted by the recombinant *N. caninum* are sufficient to elicit therapeutic outcomes with an added value to the wild type strain. The IL-15 secreted by the recombinant *N. caninum* via microneme pathway was produced in almost same quantities and exerted pronounced functionality both *in vitro* and *in vivo.* Yet, only 2-3µg/dose of IL-15 with intraperitoneal administration is needed to achieve therapeutic outcomes as it has been indicated in many pre-clinical studies. Many pre-clinical studies indicated that the quantities needed for

anti-PD-L1 or anti-PD-1 antibodies to achieve therapeutic outcomes in mice models are between 200-250 µg/dose administered systemically by intraperitoneal route. Yet, no data are available about the final concentrations of these antibodies on the tumor site. Thus, we hypothesized that intratumor injection of these recombinant parasites may result in higher secretion of antibodies in the tumor with a bystander effect on non-infected tumor cells, potentially reducing the quantities needed of the anti-PD-L1. However, a complex *in vitro* system is, firstly, needed to verify this assumption and to investigate the possibility of achieving bystander effect by binding of the secreted scFv to noninfected cells. A three-dimensional co-culture mode using PD-L1 positive tumor spheroids with effector immune cells would be used to evaluate the added value/ limitations of the secreted anti-PD-L1 scFv on blocking the PD-1/PD-L1 interaction and potentiating the immune response. This approach would help assess its efficacy in blocking the PD-1/PD-L1 interaction and potentiating the immune response, providing valuable insights into the therapeutic potential of re combinant *N. caninum* in cancer treatment.

Optimizing the functionality of the secreted anti-PD-L1 scFv could significantly enhance its therpautic potential. One promising approach is to increase the affinity of the scFv for its binding target, PD-L1. The study by Parkinson et al. demonstrated the feasibility of this approach by engineering mutants in the heavy chain sequence of Atezolizumab, resulting in a 17-fold improvement in the KD of the antibody. Implementing similar affinity-enhancing mutations in the engineered scFv could potentially improve its binding efficiency, even with relatively low secreted quantities (Parkinson et al., 2023).

Additionally, combining the targeting of micronemes pathway with GRAs secretion represents another intriguing avenue for investigation. A previous study by Nishikawa *et al*. showed that targeting IFN-γ to dense granules for secretion by *T. gondii* resulted in secreted quantities comparable to those achieved through micronemes targeting (Nishikawa *et al*., 2003). Targeting the protein to both GRA and micronemes may result in a higher secreted quantity, thereby enhancing its therapeutic efficacy. This dual-targeting approach warrants further exploration in future studies, as it could offer a promising strategy to improve the functionality of the secreted scFv and enhance its anti-tumor activity.

Combining immune checkpoint inhibitors (ICIs) with cytokines has emerged as a promising strategy for cancer treatment and integrating this approach with oncolytic microorganism-based therapy holds considerable potential. Clinical trials, such as the investigation of T-VEC in

combination with anti-PD-1 therapy, demonstrate the effectiveness of this combination strategy. Furthermore, preclinical research has explored engineering oncolytic microorganisms with multiple gene insertions to enhance their anti-tumor activity. For instance, ONCR-177, a gene-engineered recombinant herpes simplex virus (HSV), carries five transgenes, including IL-12, FLT3LG extracellular structural domain, CCL4, and antagonists of immune checkpoints PD-1 and CTLA-4. This construct is currently being evaluated in clinical trials either alone or in combination with a PD-1 antibody for the treatment of various advanced solid tumors (NCT04348916). This approach underscores the potential of combining oncolytic microorganisms with immunotherapy to provide multifaceted anti-tumor effects. Considering our models, there are numerous future avenues for generating new recombinant strains of *T. gondii* and *N. caninum*. These may involve combining the secretion of anti-PD-L1 and IL-15, as well as exploring strategies to simultaneously express therapeutic molecules on the surface and secrete them to promote tumor targeting and intratumoral delivery of therapeutic agents. By leveraging the unique capabilities of these microorganisms, such as their ability to replicate within tumor cells and modulate the tumor microenvironment, we can potentially enhance the efficacy of immunotherapy in cancer treatment. Continued research in this area holds promise for the development of innovative and effective cancer therapies

Despite the challenges, by this work, we demonstrated the possibility of arming *N. caninum* with a secreted antibody fragment in scFv format, an approach with promising applications in potentiating the antitumor activity of this potential immunotherapeutic parasite and broadening its applications. Encouraged by these results, we are currently developing and characterizing recombinant *N. caninum* engineered to secrete anti-PD-L1 scFv with an Fc region to explore the feasibility of using *N. caninum* as a platform for therapeutic molecule delivery. The functionality and added values of these two constructs would be evaluated in *ex vivo* PD-L1 expressing tumor models before proceeding to *in vivo* experimentations.

Our research demonstrates exciting progress in harnessing *N. caninum* as a potential immunotherapeutic platform for cancer treatment. By arming *N. caninum* with a secreted antibody fragment in single-chain variable fragment (scFv) format, we have shown promise in enhancing its antitumor activity. Moving forward, our plans to develop and characterize recombinant *N. caninum* engineered to secrete anti-PD-L1 scFv with an Fc region represent a significant step toward exploring the potential of *N. caninum* as a platform for therapeutic molecule delivery. Conducting *ex vivo* studies using PD-L1 expressing tumor models will provide valuable insights into the functionality and efficacy of these constructs before

advancing to *in vivo* experiments. This systematic approach ensures thorough evaluation and optimization of our immunotherapeutic strategy, paving the way for potential clinical applications in cancer treatment. Overall, our research holds promise for advancing the field of cancer immunotherapy and underscores the importance of exploring innovative platforms, such as *N. caninum*, for therapeutic molecule delivery in cancer therapy.

6. Conclusion and Perspectives

6. Conclusion and Perspectives

6.1. Conclusion

The exploration of microorganisms as immunotherapeutic agents in cancer treatment presents a promising approach in immune oncology. This research highlights the potential of integrating parasite-based immunotherapy with existing immunotherapy strategies to enhance anti-tumor activity. Specifically, the study investigated the combination of the oncolytic activity from *Toxoplasma gondii* and *Neospora caninum* with immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway.

T. gondii's unique immunogenic properties make it a compelling candidate for cancer immunotherapy. However, its non-specific invasiveness poses a significant challenge. In this study, we demonstrated and described the engineering and anchoring strategies to display functional single-chain variable fragment (scFv) antibodies on *T. gondii*'s surface. This strategy promotes selective cell targeting and minimizes off-target binding. The recombinant *T. gondii* displaying surface anti-PD-L1 scFv exhibited functional binding to PD-L1-expressing human and murine tumors and showed enhanced killing capacity for these tumors compared to the parental unmodified strain. Additionnally, this research demonstrated that engineered *T. gondii* expressing anti-PD-L1 scFv could inhibit PD-1/PD-L1 interaction and potentiate T cell response to tumor. This approach offers a promising strategy to enhance targeted cancer treatments by leveraging the natural oncolytic properties of *T. gondii* and the blockade of PD-L1 induced immunosuppression.

Neospora caninum, with its similarities to *T. gondii* but lower virulence and non-pathogenicity in humans, was also investigated for its potential in cancer treatment. The study explored the potential of arming *Neospora caninum* with anti-PD-L1 scFv antibody to be displayed on the surface or to be secreted. Although the strategy to anchor scFv on the surface of *N. caninum* using TgSAG1 was not successful, alternative strategies remain promising. This study described and demonstrated the feasibility of engineering *N. caninum* to secrete functional anti-PD-L1 scFv which efficiently bound to its target protein on PD-L1-expressing tumor cells and inhibited PD-1/PD-L1 interaction. This innovative approach combines direct oncolytic effects with immune checkpoint blockade, offering a synergistic strategy to enhance tumor-specific immune responses.

Pre-clinical studies to evaluate the efficacy and safety of these engineered *T. gondii* and *N. caninum* strains in animal models could provide valuable insights into their therapeutic potential and pave the way for clinical translation. In conclusion, leveraging the natural properties of microorganisms and enhancing them through genetic engineering holds significant promise for advancing cancer immunotherapy. The integration of microbial-based therapies with immune checkpoint inhibitors offers a novel and multifaceted approach to cancer treatment, potentially leading to more effective and durable anti-tumor responses.

6.2. Perspectives

Further experiments are still needed to validate the applicability and anti-tumor activity of these models:

1- Evaluate the targeting efficiency of *T. gondii* displaying surface anti-PD-L1 scFv in complex three-dimension models of PD-L1 expressing tumors and various effector immune cells to study the capacity of these engineered parasite to selectively target tumors and to analyze the impact on the immune response.

2- Analyze the tumor targeting and biodistribution of recombinant *T. gondii* displaying surface anti-PD-L1 *in vivo* in mice models to assess the impact of the surface expression on reducing the biodistribution of parasites and retaining them within the tumor site. Mice-bearing solid PD-L1 positive tumors will be a suitable model to investigate this hypothesis. Such experiment will also help determine the lethality and tolerable dose of this strain in mice and whether surface expression reduces the lethality by retaining the parasites within tumors.

3- Finally, if targeting is achieved and biodistribution is reduced *in vivo*, the anti-tumor activity of this model should be evaluated *in vivo* in solid tumor models with high PD-L1 expression such as murine B16F10 melanoma with sub-cutaneous inoculation to allow for intratumor administration.

4- For arming *Neospora caninum* with anti-PD-L1 scFv, alternative anchoring strategies will be investigated for engineering *N. caninum* to display surface anti-PD-L1 scFv to overcome the challenges related to protein stability. To proof the concept, further experiments will be performed to verify the possibility of expressing surface anti-DEC205 scFv anchored by GPI and to study the functionality and the level of expression. If not successful, different anchoring techniques such as using SAG1 protein from *N. caninum* rather than *T. gondii* will be explored.

5- For *N. caninum* secreting anti-PD-L1 scFv, evaluate whether the quantities secreted are adequate to provide an added value over the parental strain. Use *ex vivo* tumor models of PD-L1 expressing tumors and effector immune cells to assess the potential and limitations for blocking the PD-1/PD-L1 interaction and potentiating the immune response.

7. References

7. References

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Muna ALJIELI

Toxoplasma gondii and *Neospora caninum* as expression platforms for therapeutic fragment antibodies against PD-1/PD-L1 pathway: A new strategy for cancer immunotherapy

Résumé en français :

Dans un contexte tumoral, l'immunothérapie vise à exploiter le système immunitaire. Elle englobe plusieurs stratégies, dont celle des anticorps inhibiteurs de checkpoints inhibant la liaison du ligand PD-L1 au récepteur PD-1, ou encore celle fondée sur l'utilisation de micro-organismes oncolytiques, tels que des virus, des bactéries et des protozoaires. L'objectif de cette étude est d'évaluer la possibilité d'associer deux approches immunothérapiques via l'expression par des parasites d'un fragment variable à chaîne unique (scFv) contre le ligand PD-L1. Ce scFv a été développé de telle sorte qu'il était soit exprimé à la surface du parasite pour un ciblage spécifique à la tumeur, soit sécrété pour être délivré directement aux sites tumoraux. Nous avons conçu, à partir de l'anticorps humain anti-PD-L1, un scFv anti-PD-L1 fusionné à la protéine membranaire SAG1 pour favoriser son ancrage à la surface de *T. gondii*. *In vitro*, cette construction a montré un ciblage prédominant du parasite modifié aux cellules exprimant PD-L1, réduisant de manière significative les liaisons « hors cible ». Elle a également démontré une capacité accrue de lyse cellulaire des cellules tumorales PD-L1 positives, tout en renforçant les réponses immunitaires médiées par les cellules T CD8+.
Si les tentatives d'expression du scFv anti-PD-L1 par *N. caninum* à la surface du micro-organisme ont toutes échoué, la sécrétion de ce même scFv, via la voie de sécrétion des micronèmes, a été couronnée de succès. Ce fragment d'anticorps est capable de se lier efficacement aux cellules tumorales PD-L1 positives, de bloquer l'axe PD-1/PD-L1 et de favoriser la fonctionnalité des cellules T CD8+ *in vitro*.

Ainsi, ces résultats soulignent le potentiel des fragments d'anticorps exprimés à la membrane des micro-organismes pour une thérapie tumorale ciblée, mais aussi l'utilisation de *N. caninum* pour sécréter des fragments d'anticorps fonctionnels aux effets antitumoraux synergiques.

Mots clés : Protozoaire apicomplexe, *Toxoplasma gondii*, *Neospora caninum*, inhibiteurs de point de contrôle immunitaire, immunothérapie de cancer

Summary :

Cancer immunotherapy aims at harnessing the immune system to combat cancer. It encompasses several strategies including antibodies against PD-1/PD-L1 immune checkpoint pathway and the use of oncolytic microorganisms including viruses, bacteria and protozoa. The aim of this study is to investigate the possibility of combining parasite-based immunotherapy with anti-PD-L1 by engineering anti-PD-L1 single chain variable fragment (scFv) to be displayed on the parasite's surface for tumor-specific targeting or to be secreted by the engineered parasites for direct delivery into tumor sites.
We designed an anti-PD-L1 construct using the scFv region of the human anti-PD-L1 antibody Atezolizumab, fused with the SAG1 membrane protein for anchoring *T. gondii's* membrane. This construct displayed specific binding to PD-L1 expressing cells, significantly reducing off-target binding and retaining tachyzoites on these cells. The engineered *T. gondii* showed increased killing ability and enhanced CD8+ T cell-mediated immune responses by inhibiting PD-1/PD-L1 interactions *in vitro*.

Attempts to express anti-PD-L1 scFv on *N. caninum* using SAG1 for surface anchoring were unsuccessful. However, targeting the antibody fragment to the micronemes secretory pathway resulted in the secretion of functional anti-PD-L1 scFv. This secreted scFv effectively bound to PD-L1-expressing tumor cells, blocked PD-1/PD-L1 interaction, and promoted CD8+ T cell functionality *in vitro*.

These findings highlight the potential of engineering antibody fragments onto parasite surfaces for targeted tumor therapy and using *N. caninum* to secrete functional antibody fragments for synergistic anti-tumor effects.

Key words: Apicomplexa protozoa, *Toxoplasma gondii*, *Neospora caninum*, immune checkpoint inhibitors, cancer immunotherapy