UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXIV CYCLE

Cinzia Vetrei

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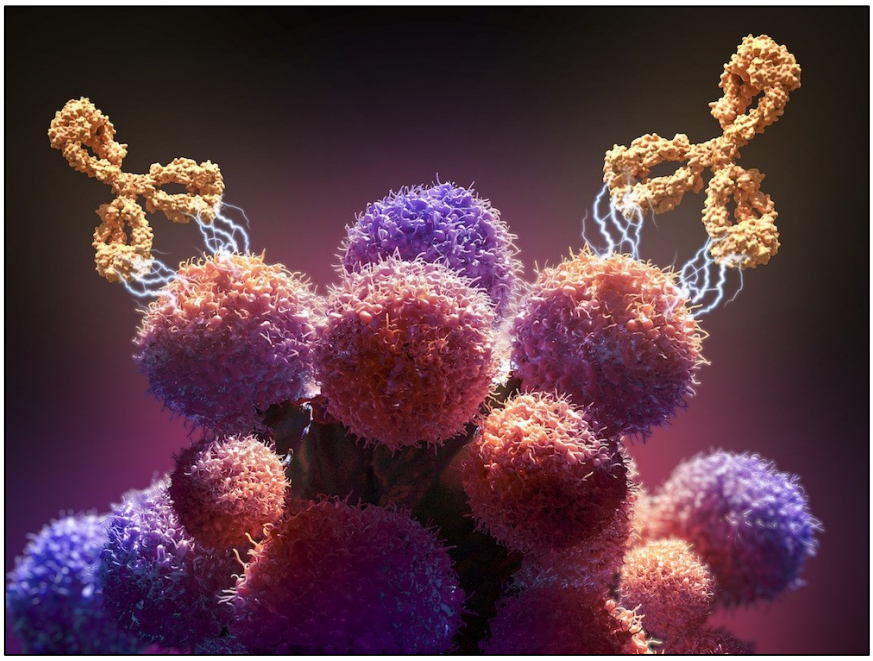

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COMBINATORIAL APPROACHES BASED ON NOVEL HUMAN IMMUNOMODULATORY ANTIBODIES AND APTAMERS FOR SAFE CANCER THERAPY

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2018-2022

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LIST OF ABBREVIATIONS

TSA: Tumor-Specific Antigens

TAA: Tumor-Associated Antigens

MAbs: Monoclonal Antibodies

NK: Natural Killer cells

PD-1: Programmed Cell-Death 1

PD-L1: Programmed Death-Ligand 1

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

LAG-3: Lymphocyte Activation Gene 3

ICs: Immune Checkpoints

ICIs: Immune Checkpoint Inhibitors

APC: Antigen Presenting Cells

TCR: T-Cell Receptor

MHC: Major Histocompatibility Complex

HAMA: Human Anti-Mouse Antibody

ADCC: Antibody-Dependent Cell-mediated Cytotoxicity

CDC: Complement-Dependent Cytotoxicity

RTK: Tyrosine Kinase Receptor

MAPK: Mitogen Activated Protein Kinase

PI3K: PhosphatidylInositol 3-Kinase

FDA: Food and Drug Administration

ScFv: Single-chain variable Fragment

TNBC: Triple Negative Breast Cancer

ER: Estrogen Receptor

PR: Progesterone Receptor

NSCLC: Non-Small-Cell Lung Cancer

HER2: Human Epidermal Growth Factor Receptor 2

EGFR: Epidermal Growth Factor Receptor

TKI: Tyrosine Kinase Inhibitor

C5N5: COP9 signalosome subunit 5

mTOR: mammalian Target Of Rapamycin

IL-6: Interleukin 6

IL-2: Interleukin 2

IFN-γ: Interferon γ

hPBMCs: Human Peripheral Blood Mononuclear Cells

SEB: Staphylococcal Enterotoxin B

PHA: Phytohemagglutinin

ABSTRACT

Cancer Immunotherapy consists of multiple approaches aimed at improving immune responses against cancer cells. Among them, we focused on the use of monoclonal antibodies (mAbs) specifically targeting either tumor associated antigens (TAA), overexpressed on tumor cells, or immunecheckpoints (ICs), key regulators of the immune system, for activating T lymphocytes against cancer cells.

In our laboratory, novel fully human monoclonal antibodies targeting the ICs Programmed death-ligand 1 (PD-L1) or Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) were successfully generated by massive parallel screening of phage antibody libraries, by using for the first time a selection strategy on human activated lymphocytes to generate large collections of single-chain variable fragments (scFvs) recognizing multiple ICs. In order to identify human-mouse cross-reactive mAbs to be used in future *in vivo* studies, parallel panning rounds were performed in this thesis project on purified mouse and human CTLA-4 proteins. The selection for cross-reactive mAbs was guaranteed by a high throughput sequencing to identify the clones commonly enriched by the two parallel pannings on human and mouse CTLA-4.

We identified two clones, named ID-1 and ID-8, able to bind with high affinity and specificity to both human and mouse CTLA-4 antigen, either expressed on lymphocytes (in its native conformation) or as purified recombinant proteins, showing nanomolar or sub-nanomolar Kd values. They were found able to activate both human and mouse PBMCs, as confirmed by the high levels of IL-2 and IFN-γ cytokines secretion induced by their treatments. Interestingly, ID-1 and ID-8 activated also Natural Killer (NK) cells and efficiently inhibited cancer cell growth.

Since recent studies showed the expression of ICs also on tumor cells, we treated them with the clinically validated Ipilimumab and Nivolumab and the novel human antibodies targeting CTLA-4 (ID-1 and ID-8) and PD-L1 (PD-L1_1 and 10_12) mAbs, previously produced in our laboratory, to further investigate on their effects on intracellular pathways. Unexpectedly, we found that they are able to inhibit ERK phosphorylation and to activate NF-kB transcription factor, with following upregulation of IC expression. On the contrary, treatments with agonistic PD-L1 and Programmed Cell Death 1 (PD-1) recombinant proteins showed opposite effects, suggesting also the probable existence of a crosstalk in tumor cells among multiple ICs.

Since anti-ICs mAbs show their highest anti-tumor efficacy by activating lymphocytes against cancer cells, we also set up *in vitro* systems based on co-cultures of lymphocytes with tumor cells or human fetal cardiomyocytes, in order to identify the most efficient combinations of immunomodulatory mAbs

associated with the lowest cardiac adverse events. We found that novel combinations involving the immunomodulatory mAbs, PD-L1_1 and ID-1, have a more potent anti-cancer activity than the clinically validated Ipilimumab and Nivolumab combination, while lacking their cardiotoxic side effects. We think that these co-cultures-based assays represent useful tools to test also other combinatorial treatments of emerging immunomodulatory mAbs against different ICs to early screen combinatorial therapeutic regimens for both anti-tumor potency and safety.

Finally, we also combined the novel antibodies with other drugs, such as aptamers, since these oligonucleotides have been recently proposed as promising drugs due to their low size, and lack of immunogenicity. In particular, we decided to combine an anti- epidermal growth factor receptor (EGFR) aptamer (CL4) with anti-tumor mAbs, in order to create bi-specific molecules overcoming aptamers' limits, such as short half-life and lack of effector functions. Here, we constructed, for the first time, three novel bispecific conjugates, made up of CL4 aptamer linked either with an immunomodulatory (anti-PD-L1 or anti-CTLA-4) antibody or with an anti-epidermal growth factor receptor 2 (ErbB2) compact antibody. These three novel immuno-conjugates retain the targeting ability of both the parental moieties and acquire a more potent cancer cell killing activity by combining the anti-tumor properties of the two parental moietes. Furthermore, the conjugation of the anti-EGFR aptamer with the anti-PD-L1 (10_12) or anti-CTLA-4 (Ipilimumab) antibody allowed for efficient redirection and activation of T cells against cancer cells, leading to a dramatic enhancement of the cytotoxicity of the two conjugated partners.

SOMMARIO

L'immunoterapia del cancro è basata sull'impiego di diversi approcci che hanno lo scopo comune di potenziare la risposta immunitaria contro le cellule tumorali. Nel nostro laboratorio erano stati generati anticorpi monoclonali (mAbs) in grado di riconoscere specificamente sia antigeni associati ai tumori (TAA), iperespressi sulla superficie delle cellule tumorali, che *immunecheckpoints* (ICs), recettori regolatori espressi sui linfociti T con ruolo chiave nella modulazione del sistema immunitario.

Alcuni nuovi anticorpi umani anti-ICs, utili per attivare i linfociti T contro le cellule tumorali, erano stati isolati con successo nel nostro laboratorio, mediante tecnologia del *phage display* con selezione di repertori fagici su linfociti umani precedentemente attivati. E' stato così generato un repertorio di frammenti anticorpali (scFvs) in grado di riconoscere numerosi antigeni di superficie sui linfociti tra cui gli ICs *Programmed death-ligand 1* (PD-L1) e *Cytotoxic T Lymphocyte Antigen 4* (CTLA-4).

Allo scopo di identificare mAbs cross-reattivi per la specie murina, utili per futuri studi *in vivo*, in questo progetto di tesi sono stati effettuati successivi cicli paralleli di selezione sulle proteine ricombinanti umane e murine di CTLA-4. La selezione degli anticorpi cross-reattivi è stata garantita da un sequenziamento massiccio volto ad identificare i cloni comunemente arricchiti nei due cicli di selezione paralleli sulla proteina CTLA-4 umana e murina.

Sono stati identificati due cloni, chiamati ID-1 e ID-8, capaci di legare con elevata affinità e specificità l'antigene CTLA-4 sia umano che murino. In particolare, tali anticorpi riconoscono sia la proteina ricombinante purificata che quella nella sua conformazione nativa, quando espressa sui linfociti, mostrando affinità di legame (Kd) nanomolare o sub-nanomolare. Tali anticorpi hanno mostrato la capacità di attivare sia linfociti umani che murini, come confermato dagli alti livelli di secrezione di citochine IL-2 e IFN-γ indotti dai loro trattamenti. E' interessante notare che ID-1 e ID-8 sono in grado di attivare anche le cellule Natural Killer (NK) e di inibire efficacemente la crescita delle cellule tumorali.

Recenti studi avevano mostrato l'espressione di ICs anche su cellule tumorali, pertanto queste ultime sono state da noi trattate con gli anticorpi in uso clinico, Ipilimumab e Nivolumab, ed i nuovi anticorpi umani specifici per CTLA-4 (ID-1 e ID-8) e PD-L1 (PD-L1_1 e 10_12) , generati precedentemente nel nostro laboratorio, per studiare più approfonditamente i loro eventuali effetti su *pathways* intracellulari. E' stato così evidenziato che essi sono in grado di inibire la fosforilazione di ERK e di attivare il fattore di trascrizione NF-kB, che a sua volta induce l'espressione degli ICs. Inoltre, studi in parallelo con le proteine ricombinanti PD-L1 e Programmed Cell Death 1 (PD-1), hanno mostrato effetti opposti a quelli degli anticorpi, suggerendo anche la probabile esistenza di un crosstalk tra i diversi ICs nelle cellule tumorali.

Tuttavia, considerando che gli anticorpi che riconoscono ICs mostrano la loro maggiore efficacia attivando i linfociti contro le cellule tumorali, è stato messo a punto un sistema *in vitro*, basato su co-colture di linfociti con cellule tumorali o con cardiomiociti fetali umani, per identificare le combinazioni di anticorpi immunomodulatori più efficienti ad inibire la crescita tumorale, ma allo stesso tempo dotati di ridotti effetti cardiotossici. Le combinazioni dei nuovi anticorpi immunomodulatori, come PD-L1_1 e ID-1, hanno mostrato una più potente attività anti-tumorale e ridotti o assenti effetti cardiotossici, rispetto alle combinazioni degli anticorpi in uso clinico, Ipilimumab e Nivolumab. Questi saggi *in vitro* possono rappresentare strumenti utili per testare anche altri trattamenti combinatoriali di anticorpi immunomodulatori emergenti diretti contro differenti ICs, consentendo di effettuare analisi precoci di efficacia e di eventuali effetti collaterali.

Infine, tali anticorpi sono stati impiegati in combinazione anche con un aptamero, chiamato CL4, che riconosce il recettore del fattore di crescita epidermico (EGFR), per valutare eventuali effetti sinergici e creare molecole bispecifiche in grado di superare i limiti degli oligonucleotidi, come la ridotta emivita in circolazione e la mancanza di funzioni effettrici. Per la prima volta, sono stati costruiti tre nuovi immuno-coniugati bispecifici costituiti dall'aptamero CL4 legato o ad un anticorpo immunomodulatore, in grado di riconoscere PD-L1 o CTLA-4, o ad un anticorpo compatto, che riconosce il recettore del fattore di crescita epidermico 2 (ErbB2). Questi tre nuovi immuno-coniugati conservano le proprietà funzionali delle molecole originali, ma mostrano una più potente attività contro le cellule tumorali, combinando le proprietà anti-tumorali di entrambi i componenti da cui derivano. Inoltre la coniugazione dell'aptamero anti-EGFR con gli anticorpi immunomodulatori anti-PD-L1 (10_12) e anti-CTLA-4 (Ipilimumab) ha mostrato un più efficiente reclutamento dei linfociti T contro le cellule tumorali, portando ad un drammatico aumento della citotossicità dei due componenti originali.

INTRODUCTION

1.1 Immune surveillance theory and the concept of cancer immunoediting

At the beginning of the 19th century Ehrlich already hypothesised a role of the host immune defense in the prevention of tumor developement. Some years later, in 1957 Burnet highlighted the immune system importance in the biology of cancer by experimentally testing its role for the first time, by using tumor transplantation models **[Burnet F.M. 1957]**. However, the idea of the immune system eradicating transformed cells, before they are clinically manifested, remained a controversial topic for several years. According to Burnet's "immune surveillance theory", the recognition by immune soldiers of neo-antigens expressed on tumor cells induces an immunological reaction against them. Later, Dunn and Schreiber proposed the concept of "cancer immunoediting" **[Dunn G.P., Old L.J., Schreiber R.D. 2004]**, a process composed of three phases: elimination, equilibrium, and escape (Figure 1). In the first phase nascent transformed cells are recognized and killed by Natural Killer (NK), CD4+ and CD8+ T cells; in the second phase an equilibrium is reached when original tumor cells are destroyed. However, their elimination determines a selection of tumor cells carrying mutations that make them more resistant to immunologic attacks. Since the immune system results unable to destroy these tumor variants, in the third phase the tumor develops and becomes clinically detectable.

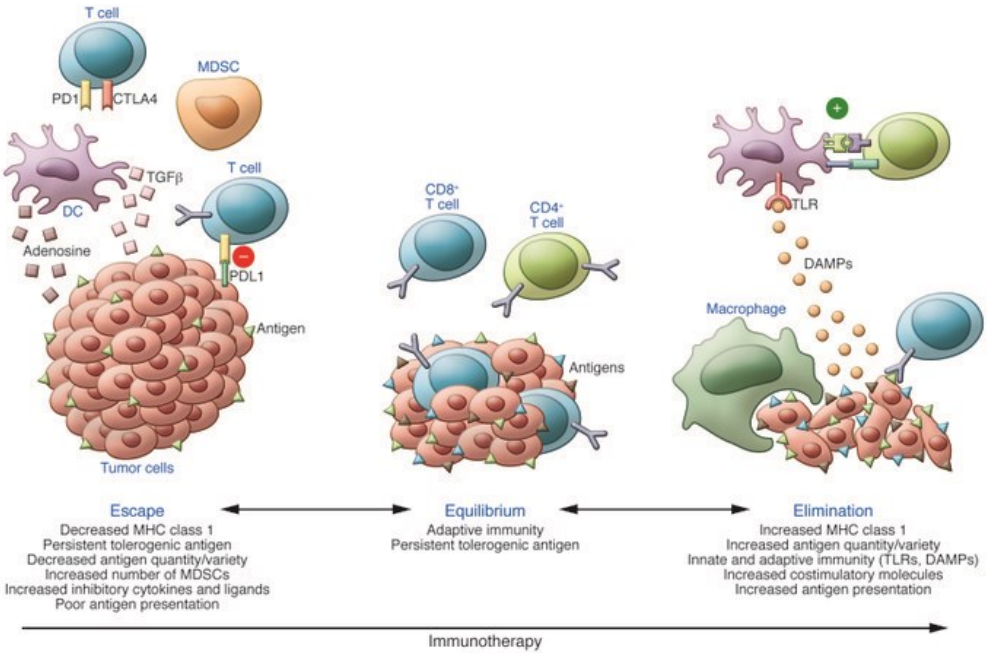

Figure 1. **Cancer immunoediting process: a schematic representation of three phases. [Anusha Kalbasi, *J Clin Invest.* 2013]**

Tumor microenvironment usually contains infiltrates of innate immune cells, such as NK cells and macrophages, and adaptive immune cells, such as T cells. During an inflammatory response tumor cells killing induces APC cells to capture and present antigens, through the exposure of peptides associated with the major histocompatibility complex (MHC) to T cells, in order to activate them, in tumor draining lymph nodes (priming phase) **[Schumacher T.N., Schreiber R.D. 2015.; Mueller DL, *et al.* 1989]**.

The tumor antigens can be categorized into two classes: tumor-specific antigens (TSA), specifically expressed on tumor cells, arised either from mutations associated with the process of carcinogenesis or from oncogenic viruses, and tumor-associated antigens (TAA), normally expressed at lower levels on healthy cells but overexpressed or aberrantly expressed in cancer cells **[Sharma P. *et al.* 2015; Szabo S.J., *et al.* 2003]**. Nevertheless, additional costimulatory signals are required in order to fully activate naïve T cells, because the recognition of antigen-MHC complex is not sufficient and may render them anergic (Figure 2) . The interaction between CD28, constitutively expressed on CD4+ and CD8+ T cells surface, and its ligands B7-1 (CD80) or B7-2 (CD86) expressed on the APCs **[Janelle V., *et al.* 2020; Schumacher T.N., Schreiber R.D. 2015]**, induces downstream signals leading to clonal expansion, survival and differentiation of T cells, through the production of interleukin-2 (IL-2).

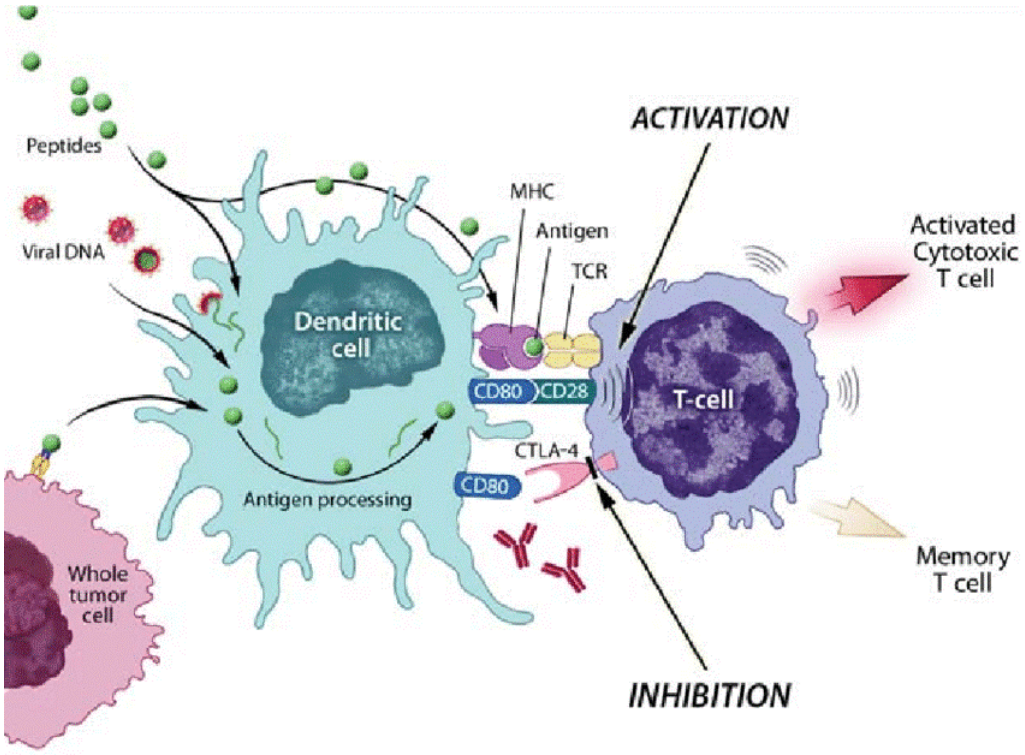

Figure 2. Antigen presentation and activation of T cells through costimulatory signals [Tarassoff C.P., *Oncologist.* , 2006]

In advanced stages, tumor microenvironment becomes more complex including suppressive immune cells, such as regulatory T cells **[Uyttenhove C., *et al.* 2003]**, inhibitory cytokines **[Hanahan D., Weinberg R.A., 2011]** or inhibitors of T cell responses **[Fife BT, *et al.* 2008]**, attenuating immune pressure and evading classic mechanisms of tumor suppression, thus supporting cancer cells escape from immune surveillance. This capacity was defined by Hanahan and Weinberg in 2011 as the seventh hallmark of cancer **[Leach D.R., *et al.* 1996]**.

Nevertheless, indeed tumor suppression can lead to immune escape of tumor cell variants characterized by a dampened immunogenicity or the ability to attenuate immune response by developing some mechanisms such as the secretion of immunosuppressive factors or the reduced expression of Major Histocompatibility Complex I (MHC I) and co-stimulatory ligands **[Koebel C.M., *et al.* 2007; Papaioannou N.E., *et al*. 2016; Hanahan D., Weinberg R.A., 2011]**, can be exploited by tumor cells for inhibiting immune responses.

Furthermore, a set of molecules called "Immune Checkpoint" (ICs), were initially identified as regulators of T cells activation at multiple steps, protecting tissues from damage caused by an extensive immune response and maintaining tolerance to self-antigens **[Hirano F., *et al.* 2005]**.

1.2 Cancer immunotherapy

Cancer immunotherapy is aimed at improving immune responses against cancer cells by activating its components. Immunotherapy of cancer can be classified into two main types of intervention: active and passive (Figure 3) **[Papaioannou N.E., *et al.* 2016]**. Passive protocols are widely used in cancer therapy particularly in patients with an unresponsive immune system. This approach comprises the infusion of *ex vivo*-activated immune cells or molecules, such as antibodies, in order to compensate for missing or deficient immune functions. Examples are the adoptive transfer of T cells or dendritic cells preactivated *in vitro* to induce tumor killing *in vivo* or the use of recombinant cytokines and tumor-specific antibodies recognizing TAAs, used mainly for the targeted therapy. On the contrary, active approaches are aimed at stimulating effector funtions *in vivo*, involving anti-cancer vaccines (peptides, dendritic cell-based and allogeneic whole cell vaccines), as well as immune checkpoint inhibitors. **[Papaioannou N.E., *et al.* 2016]**.

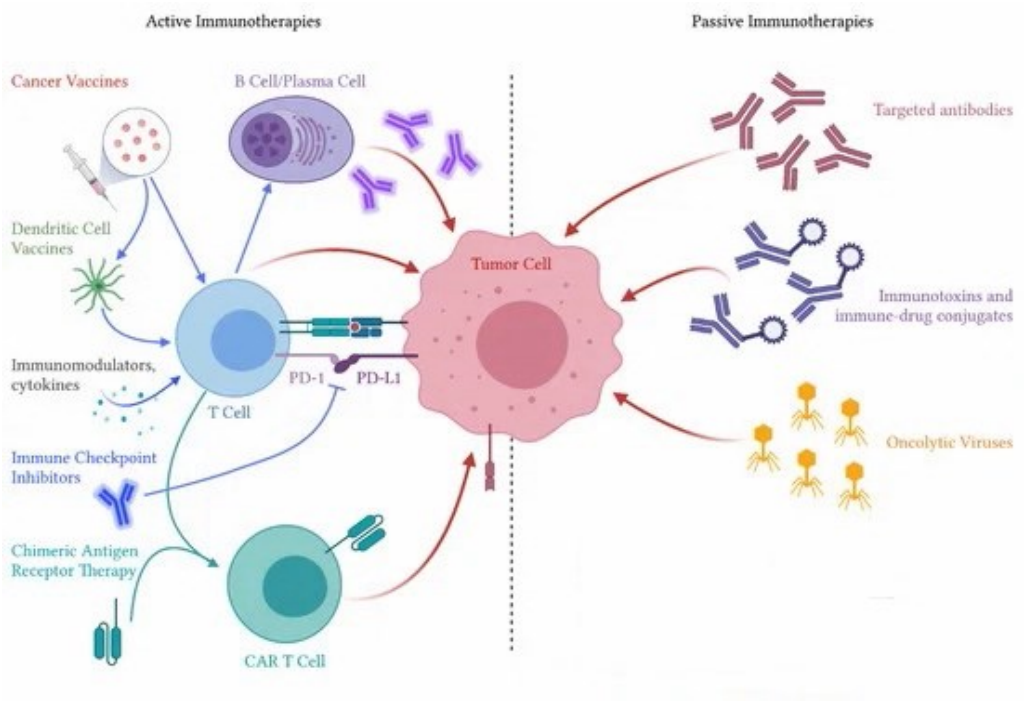

Figure 3. Classification of active and passive cancer immunotherapy. The passive cancer immunotherapy approach is based on the infusion of tumor specific mAbs, immunotoxins and oncolytic viruses. The active immunotherapy approach includes vaccines, immunomodulators cytokines and immune checkpoint inhibitors **[Pegna G.J., *Cancers*, 2021].**

In the last decades, preclinical and clinical investigations led to the approval by FDA of several antibodies targeting immune checkpoints for clinical treatment of melanoma and other cancer types **[Galluzzi L., *et al.* 2014]**.

The combinations of different immunotherapeutic interventions could be wisely organized by boosting anti-cancer responses, on one hand, and by neutralizing negative immune regulators, on the other hand (Figure 4).

Furthermore, the combination of different cancer treatment modalities (e.g., chemotherapy and checkpoint inhibitors) and the improvement of targeted drugs (associated with the discovery of new targets and the development of new drugs and new molecules) could help in targeting simultaneously multiple molecular compensatory pathways (e.g., pan-tyrosine kinase inhibitors) **[Papaioannou N.E., *et al.* 2016]**.

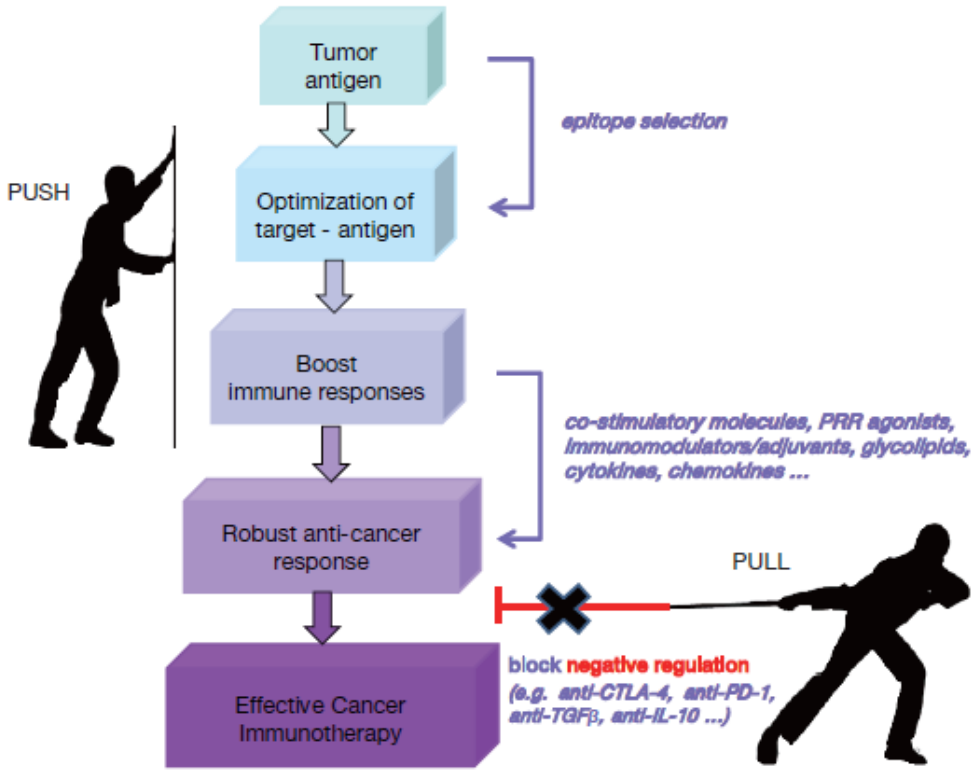

Figure 4. The synergy between different cancer treatments. The combination of different immunotherapeutic approaches to simultaneously induce immune anti-cancer responses (PUSH) and inhibit negative immune regulators (PULL) **[Papaioannou N.E., *Ann Transl Med.* 2016].**

1.3 Antibody-based immunotherapy against Tumor Associated Antigens

The main limits of conventional anti-cancer treatments are represented by the absence of selectivity for tumor cells and by non-specific side toxic effects for normal tissues. For this reason, the scientists have worked for years with the aim of developing biological weapons able to specifically induce immune responses against cancer, overcoming these limits **[Wurz G.T., *et al.* 2016; Kirkwood J., *et al.* 2012; Galluzzi L., *et al.* 2014]**. Immunotherapy based on the use of novel mAbs, endowed with anti-tumor activity, is a very useful targeted therapy as it allows to specifically recognize antigens on tumor cells to either inhibit their oncogenic function or to specifically deliver toxic compounds. The targets of these therapies comprise two big classes of antigens: Tumor Specific Antigens (TSAs), expressed only on tumor cells, and TAAs, overexpressed by tumors but present also at low levels on other normal tissues **[Wurz G.T., *et al.* 2016; Savage P., *et al.* 2014]**, such as some growth factor receptors.

The excitement for these new weapons grew over the years leading scientists to further investigations aimed at the generation of the first therapeutic mAbs of mouse origin. Unfortunately, the human anti-mouse antibody (HAMA) response represented an obstacle to the success of this approach and induced further progresses to avoid this problem **[Thorpe S.J., *et al.* 2003]**. In order to reduce the immunogenicity, the mouse sequences recognized by human immune system, responsible for HAMA response, were replaced by the constant human sequences, by using DNA recombinant tecniques, thus creating chimeric molecules retaining only the variable regions of mouse origin **[LoBuglio A.F., *et al.* 1989]**.

Further improvements for reducing immunogenicity were obtained with the humanized mAbs generated by inserting only the mouse hypervariable loops of an antibody in the human V-region frameworks **[Carter P., *et al.* 1992]**.

Furthermore, novel strategies recently developed, such as phage display, have allowed the generation of human antibody libraries, in order to perform an *in vitro* selection of human single-chain variable fragments (scFvs), to be then converted into fully human antibodies specifically targeting tumor cells **[Frenzel A., *et al.* 2016]**. Some of the therapeutic mAbs approved by FDA for clinical use in cancer therapy are directed against either growth factors, such as vascular endothelial growth factor A, or growth factor receptors, such as human epidermal growth factor receptors EGFR and HER2/ErbB2. These therapeutic mAbs not only have specific and direct inhibitory effects on the signal transduction activated by these receptors expressed on tumor cells, but they can also induce Antibody Dependent Cellular Cytotoxicity (ADCC) and Complement Dependent Cytotoxicity (CDC). Futhermore, they can be used as vehicles to deliver towards tumor cells toxic payload, such as chemoterapeutics, toxins or radioisotopes. More recently, bispecific antibodies able to recognize two antigens have been created to retarget cytotoxic T lymphocytes to any cell of choice **[Wurz G.T., *et al.* 2016]**.

1.4 ErbB2 and EGFR receptors as validated TAAs for breast cancer therapy

Breast cancer was one of the most common disease in women, with 1.9 million new cases (2017), second only to nonmelanoma skin cancer (NMSC), the leading cause for cancer death in men and women in 2017 **[Fitzmaurice C., *et al.* 2019]**.

About 30% of breast cancers overexpress ErbB2, a Tyrosine Kinase Receptor (RTK), which is considered an attractive and useful TAA target in cancer immunotherapy, as it plays a key role in the development of malignancy **[Slamon D.J., *et al.* 2001; Tagliabue E., *et al.* 1991; Lohrisch C., Piccart M., 2001; Gravalos C., Jimeno A., 2008]**.

ErbB2 receptor belongs to the ErbB family of RTK, but, unlike the other members, it lacks its own natural ligand, acting preferentially as the heterodimerization partner with the other receptors of the family. Its overexpression on breast cancer cells, due to gene amplification, can induce the activation of downstream signaling pathways involved in cell growth, differentiation and survival **[Yarden Y., Sliwkowski M.X. 2001; Klapper L.N., *et al.* 2000; Busse D., *et al.* 2000; Baselga J., *et al.* 2009]**, such as those of mitogen-activated protein kinases (MAPK) and phosphatidylinositol-3-kinase (PI3K) (Figure 3), with a consequent dysregulated proliferation (Figure 5).

Trastuzumab, a humanized anti-ErbB2 antibody approved by the FDA in 1998, is an example of well estabilished breast cancer therapy, resulting very effective in the treatment of ErbB2-positive breast carcinoma either at early or metastatic stages. However, a high fraction of patients show resistance to the treatment and some of treated patients can develop cardiac dysfunction, frequently leading to heart failure in particular in the presence of other risk factors **[Stebbing J., *et al.* 2000; Sparano, J.A., *et al.* 2001; Natha R., *et al.* 2006; Riccio, G., *et al.* 2016]**.

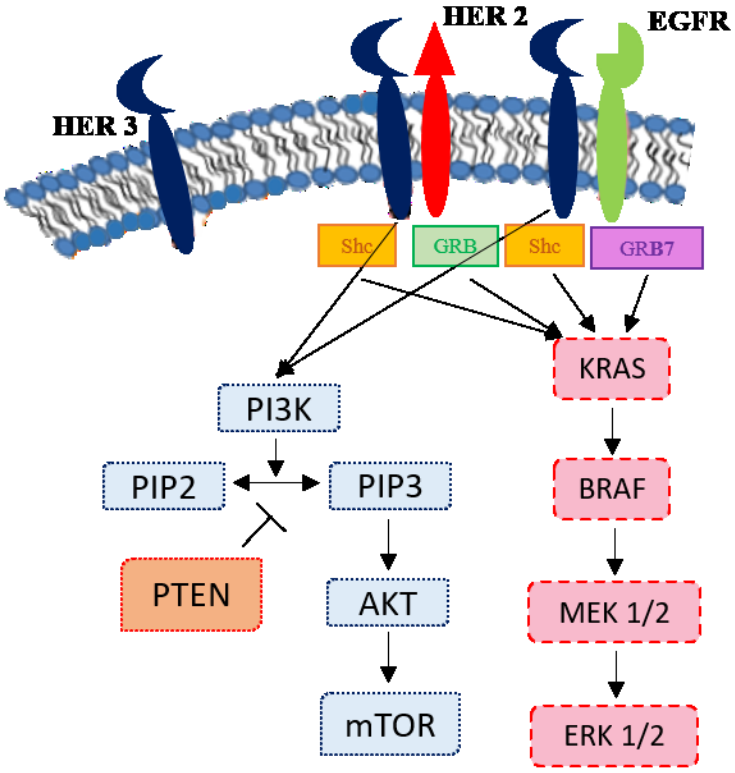

Figure 5. EGF receptor family. Schematic representation of the Tyrosine Kinase Receptors EGFR, ErbB2/HER2 and HER3 and their downstream pathways and inhibitors.

Antitumor strategies based upon biologic molecules have been further successfully developed for ErbB2 RTK, leading to the recent clinical approval of Pertuzumab another humanized mAb used for combinatorial treatments with Trastuzumab and Docetaxel **[Baselga J., Swain S.M. 2010; Chung, C.; Lam M.S. 2013]**.

Despite the common target, these mAbs are characterized by different mechanisms of action, presumably due to the recognition of different epitopes localized in the domain II (Trastuzumab) and in the domain IV (Pertuzumab) of ErbB2. Unfortunately, the clinical efficacy of these drugs is still limited by resistance and cardiotoxicity issues **[Seidman A., *et al.* 2002; Maurea, N.; *et al.* 2016; De Lorenzo C., *et al.* 2018]**.

In order to overcome the limits of Trastuzumab, a novel immunoagent was constructed in our laboratory, starting from the isolation through phage display technology of a human anti-ErbB2 single-chain variable fragment (scFv). In particular this anti-ErbB2 scfv, named Erbicin, not only is able to selectively bind ErbB2-positive tumor cells and inhibit tumor growth, but also to recognize an epitope of ErbB2 different from that of Pertuzumab and Trastuzumab **[De Lorenzo C., *et al.* 2002; Troise F., *et al.* 2011]**.

Furthermore, the Fc region of a human IgG1 was genetically fused with Erbicin leading to the construction of a new immunoagent, called Erb-hcAb (human compact antibody) for its compact size (100 kDa) if compared with the full size (155 kDa) of a natural IgG (Figure 6) **[De Lorenzo C., *et. al.* 2004]** . This new antibody retains the immune effector functions of a full size IgG, but due to its reduced molecular size it can diffuse more easily into the tumor masses with respect to a conventional IgG. Erb-hcAb, endowed with the ability to selectively bind to ErbB2-positive breast tumor cells, was found to be a powerful cytotoxic agent able to inhibit the growth of tumor cells *in vitro* and *in vivo*, by inducing also ADCC and CDC **[De Lorenzo C., *et al.* 2004]**. A more interesting feature of Erb-hcAb is represented by the lack of cardiotoxic effects in *in vitro* and *in vivo* models, differently from the clinically validated Trastuzumab and Pertuzumab **[Riccio G., *et al.* 2009; Fedele C., *et al.* 2012]**.

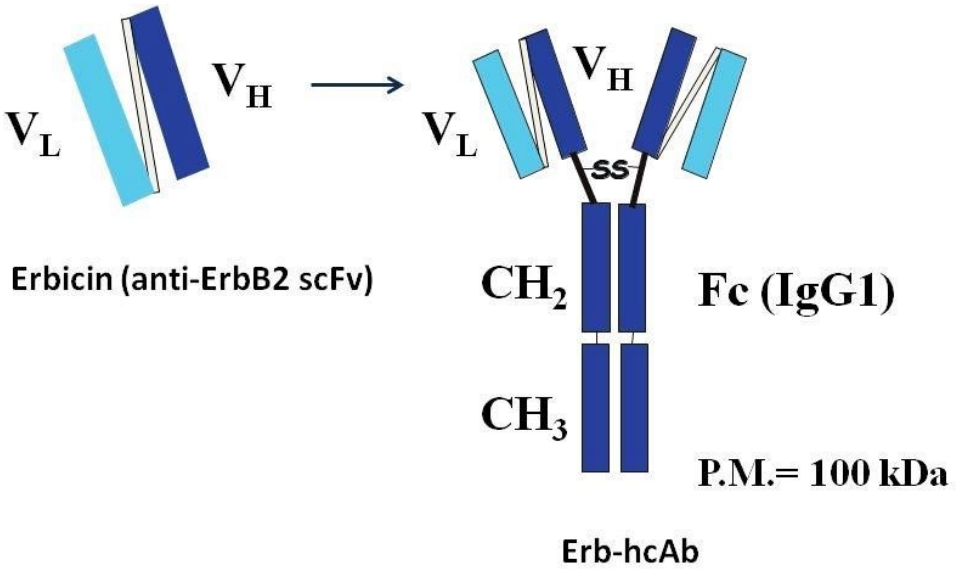

Figure 6. Erb-hcAb. Schematic representation of Erbicin and its derived compact antibody (Erb-hcAb).

Unfortunately, a large fraction of breast tumors lacks the overexpression of ErbB2 used for the targeted therapy, as in the case of Triple Negative Breast Cancer (TNBC). TNBC is a particularly aggressive subtype of breast cancer, defined by the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). TNBC accounts for about 10-20% of breast cancer cases and the affected patients show high risk of recurrence with a death rate higher then any other subtype. Overexpression of EGFR has been reported in ~60% of TNBC and correlates with poor outcome, thus it could be considered as a potential target for TNBC therapy.

EGFR is another Tyrosine kinase receptor of c-erbB receptor family members. Once activated it initiates myriad of signaling pathways, such as Ras/MAPK and PI3K/AKT pathways. High EGFR expression levels may influence multiple aspects of tumor biology including cell proliferation, motility, invasiveness and resistance to treatment **[Stommel J.M., *et al.* 2007; Greenall S.A., *et al.* 2015; Haynes H.R., *et al.* 2014]**.

In parallel other biological drugs with affinities and specificities comparable to those of antibodies have been recently developed. These novel drugs, called aptamers, are single strand oligonucleotides that can form structures acting as scaffolds for molecular interactions with either proteins or small-molecule targets. The relevance of these novel molecules is mainly due to their low molecular weight and the lack of immunogenicity **[Keefe A.D., *et al.* 2010]**.

A nuclease resistant RNA-aptamer, named CL4, able to recognize with high affinity and inhibit human EGFRwt, has been recently generated in the laboratory of our collaborators, by an *in vitro* selection procedure called SELEX (Figure 7) (Systematic Evolution of Ligands by Exponential Enrichment) **[Tuerk C., Gold L. 1990; Camorani S., *et al.* 2018; Camorani S., Cerchia L. 2015]**. The CL4 oligonucleotide aptamer was also found able to bind to and inhibit the EGFRvIII mutant, expressed on the surface of Glioblastoma Multiforme (GBM) cancer cells and characterized by a truncated extracellular domain **[Esposito C.L., *et al.* 2011; Camorani S., *et al.* 2015]**. Indeed, CL4, by interacting with domain IV of the receptor present in the EGFRvIII variant, can inhibit the autophosphorylation of the receptor and its downstream ERK1/2 and STAT3 pathways, thus affecting migration and invasion of cancer cells **[Camorani S., *et al.* 2015]**. CL4 was also found able to prevent the EGFR-integrin αvβ3 interaction, thus inhibiting the vasculogenic mimicry (VM) and tumor growth in TNBCs resistant to both Erlotinib and Cetuximab (EGFR-TK inhibitors) **[Camorani S., *et al*. 2017]**, proving to be a valuable tool to overcome the tumor resistance to EGFR Tyrosine Kinase Inhibitors (TKIs).

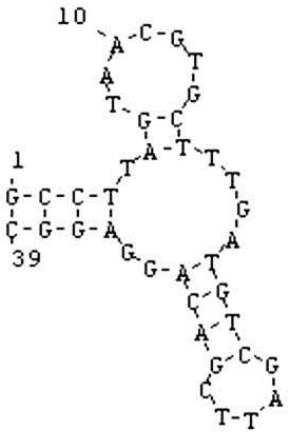

Figure 7. CL4 aptamer. Structure of the anti-EGFR CL4 aptamer **[Esposito C.L., *PLoS One* 2011].**

1.5 Antibody-based immunotherapy targeting immune regulatory checkpoints

The alternative antibody-based approach can regulate specific T-cell responses, thus enhancing the protective role of the immune system against cancer **[Peggs K.S., *et al.* 2009; Nurieva R.I., *et al.* 2011, Pardoll D.M. 2012, Wei, S.C., *et al.* 2018]**.

Multiple stimulatory and inhibitory pathways, involved in the regulation of immune activation, can be targeted by agonistic or antagonistic mAbs in order to improve immune response against cancer. Due to their direct effects on cancer

cells, effector T cells were considered as the primary soldiers, but as reported in literature also other immune populations, such as NK and T reg cells, might become targets.

As mentioned above, the single engagement of the MHC associated with antigenic peptides by the TCR is not sufficient for the full activation of T cells. A series of stimulatory or inhibitory receptor-ligand pairs can determine the fate of the T cell response, such as their activation with the subsequent differentiation into effector T cells, their deletion or anergy **[Chen L., Flies D.B. 2013; Davis M.M., Bjorkman P.J. 1988]**.

To translate this concept into the clinic, researchers worked hard to generate agonistic mAbs for co-stimulatory receptors or antagonistic ones for those with inhibitory activity, with the aim to exploit ICs to amplify immune responses against cancer cells (Figure 8) **[Pardoll D.M. 2012]**.

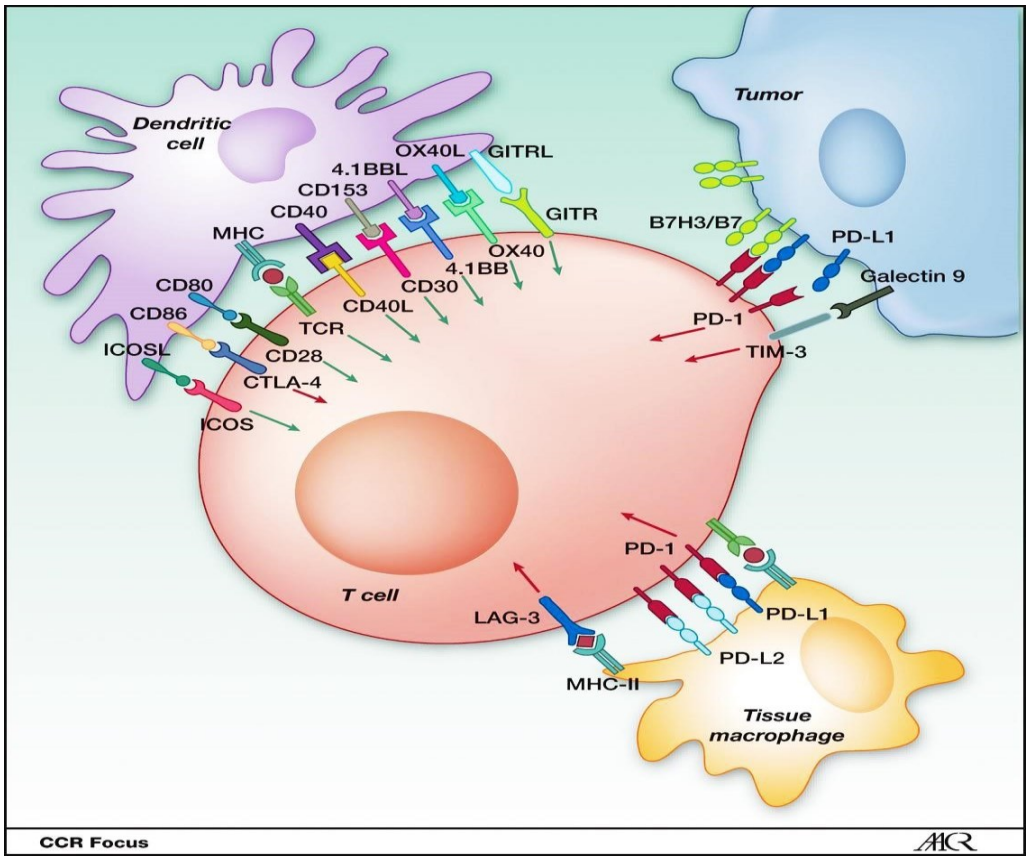

Figure 8. Potential targets for immunotherapy of cancer based on monoclonal antibody. The interaction between inhibitory receptors, such as CTLA-4, PD-1, LAG-3, TIGIT, TIM-3 with their ligands leads to an inhibition of the T cell activation (red arrows). Co-stimulatory receptors such as CD28, ICOS, 4-1BB, OX40, CD27, on the contrary, enhance the effector function of T cells (green arrows).

1.6 Immune checkpoints and their relative antibodies in clinical use or development

The first Immune Checkpoint to be characterized and reported in literature is called Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) **[Leach D.R., *et al.* 1996]**. CTLA-4, also known as CD152, is a B7/CD28 family member sharing 30% sequence identity with CD28, even though it plays a crucial role in T cell inhibition (Figure 9) **[Zang X., Allison J.P. 2007]**.

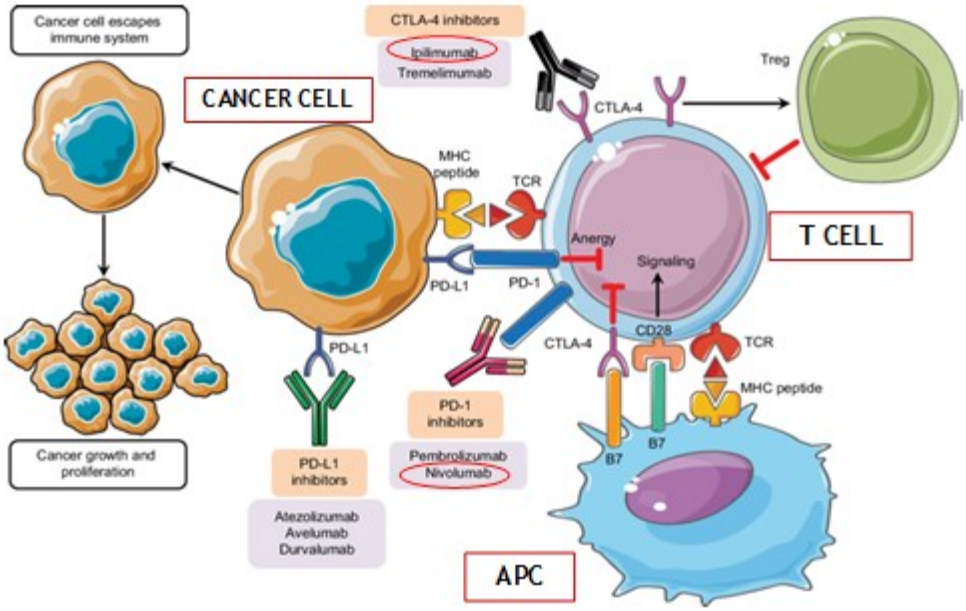

Figure 9. Role of CTLA-4 and its interaction with CD80 or CD86 in lymphnodes and binding of PD-1 with PD-L1 or PD-L2 in peripheral tissues exploited by cancer cells **[Ayoub N.M., *Breast cancer (Dove Medical Press).* 2019]**

Its regulation depends on its localization within the cell: in resting naive T cells, CTLA-4 is mainly located in the intracellular compartment, but after TCR stimulation, a member of SRC kinases family, Lck, phosphorylates a tyrosine of YVKM motif inducing its delocalization and retention on cell surface **[Linsley P.S., *et al.* 1996]**. Indeed, CTLA-4 expression is upregulated within 24–48 hours after T cell activation **[Brunet J.F., *et al.* 1987; Anagnostou V.K., Brahmer J.R. 2015]**. Once displayed on T cells surface, it competes with T cell-associated CD28 receptor, by binding to B7-1/B7-2 (CD80/ CD86) ligands **[Linsley, P.S. *et al.* 1991]** with higher affinity with respect to CD28 **[Fife B.T., Bluestone J.A. 2008, Krummel M.F., Allison J.P. 1995]**. The binding of CTLA-4 to these ligands, expressed on APC **[Aruffo, A., Seed B. 1987, Linsley, P.S. *et al.* 1990]**, blocks the co-stimulatory CD28 signal, thus inhibiting the activation of ERK and JNK pathways downstream the TCR necessary for full T-cell activation (Figure10). These effects lead to the downregulation of

transcription factors, such as NF-κB, AP-1 and N-FAT, which negatively affect IL-2 secretion and cell cycle progression **[Krummel M.F., Allison J.P. 1996]**. The association between CTLA-4 and SHP-2 might induce the dephosphorylation of the CD3 complex associated with TCR, whereas the association with PP2A might inhibit PI3K signaling (Figure 10) (even though the mechanism is not yet clear) **[Legat A., *et al.* 2013, Alegre M.L., *et al.* 2001]**.

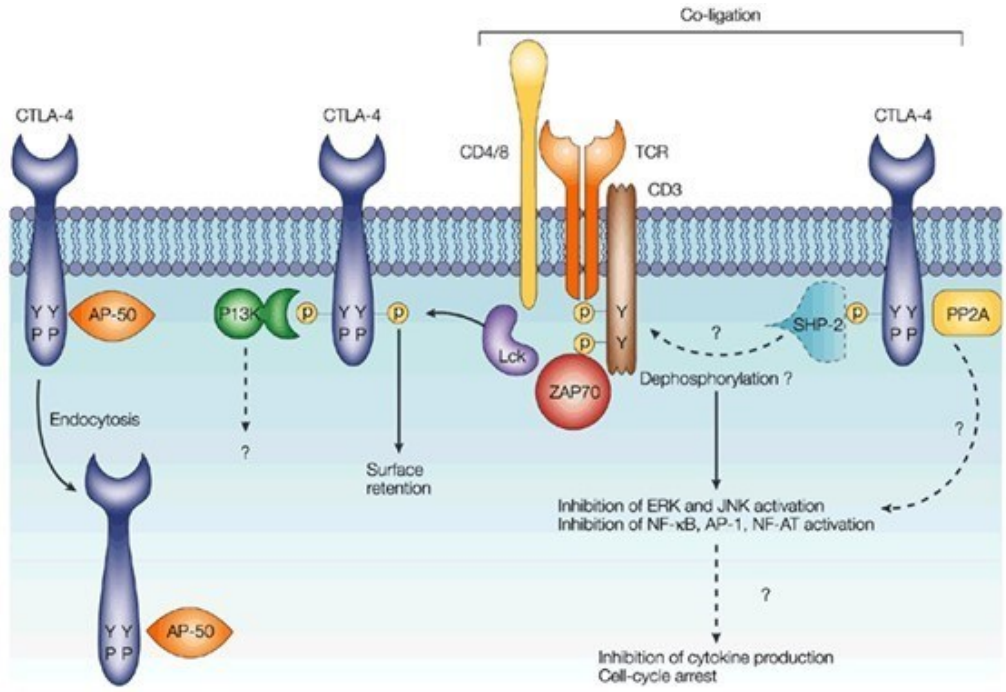

Figure 10. Model of CTLA-4 signaling pathways in T cells **[Alegre M.L., *Nat Rev Immunol.* 2001]**

CTLA-4 is also constitutively expressed on T regulatory cells (T reg), which can impair immune response by mediating the removal of B7-1 and B7-2 through trans-endocytosis, thus reducing their stimulatory function **[Gombos, R.B. *et al.* 2018, Egen, J.G., *et al.* 2002]**. Recently, it has been reported that effector and T reg cells are not the only populations of the immune system exhibiting this antigen, as also NK cells display CTLA-4 on their surface, where it seems involved in the proliferation of this immune population **[Stojanovic, A. *et al.* 2014; Passariello M. *et al.* 2020]**. Furthermore, recent studies reported that this protein is also expressed on non-lymphoid cells including leukemia and solid tumor cells **[Mao, H., *et al.* 2010; Contardi E., *et al.* 2005; Wang, X.B., *et al.* 2002]** and although its role in tumor cells has not yet been fully elucidated, some reports indicate that it can affect tumor cell survival. The recognition of the important role of CTLA-4 led to the development of inhibitory antibodies

blocking its function, as an attractive strategy for cancer treatment. After preliminary positive outcomes in mice models, the fully human anti-CTLA-4 mAb, Ipilimumab, has been developed from Allison's research group in 1996 **[Leach, D.R., *et al.* 1996]**. Ipilimumab was approved by the U.S. FDA in 2011 for the treatment of metastatic melanoma **[Cameron, F., *et al.* 2011, Patel V., *et al.* 2011]**, and several clinical trials have been started for other solid tumors, such as non-small-cell lung cancer (NSCLC), and renal cell and prostate carcinomas **[Cabel L., *et al.* Cancer 2017]**. Nevertheless, the monotherapy approach seemed to be not effective in the case of poor immunogenic tumors, thus inducing studies and trials focused on the combination of Ipilimumab with other anti-tumor drugs, such as IL-2, peptide vaccines, chemotherapy drug decarbazine **[Patel S.P., Woodman S.E. 2011; Savoia P., *et al.* 2016; Attia P.J., *et al.* 2005]**, or with other ICIs, such as anti-PD-1 mAbs.

PD-1, also known as CD279, is an immune checkpoint belonging to the B7/CD28 family and firstly identified in lymphoid cell lines undergoing programmed cell death. PD-1 is expressed on activated T-cells, B-cells, dendritic cells (DCs) and monocytes. The intracellular domain of PD-1 contains an immunoreceptor tyrosine inhibitory motif (ITIM) and an immunoreceptor tyrosine switch motif (ITSM) which binds to the inhibitory phosphatases SHP-2 and PP2A **[Liotti F., *et al.* 2021]**. SHP-2 engagement inhibits TCR signaling by suppressing PI3K/AKT, MAPK and mTOR pathways, and thus reducing IFN-γ, TNF-α, and IL-2 secretion. Furthermore, PD-1 activation reduces T-cell proliferation and survival by inhibiting the antiapoptotic protein BCL-XL **[Legat A. Front Immunol. 2013, Liotti F., *et al.* 2021; Pardoll D.M. 2012]**. Thus, the main role of PD-1 on T-cells is to limit the activity of T-cells in peripheral tissues during inflammatory responses and to inhibit autoimmune responses. Once T cells become activated, PD-1 expression is induced; then, when engaged by one of its ligands (Figure 11), such as PD-L1, it inhibits downstream signaling involved in T cell activation **[Pardoll D.M. 2012]**. The inhibition mechanism mediated by PD-1 can be exploited by the tumor cells expressing its ligand to neutralize the antitumor response of T cells **[Haile S.T., *et al.* 2013]**. Surprisingly, in recent studies, PD-1 expression has been found expressed in several tumor types, such as thyroid carcinoma cells **[Liotti F., *et al.* 2021]**, melanoma, hepatocarcinoma and lung carcinoma **[Kleffel S., *et al.* 2015; Du S., *et al.* 2018, Li H., *et al.* 2017]**. It has been proposed that PD-1 signaling sustains cancer cell proliferation through the mammalian target of rapamycin (mTOR)/ribosomal protein S6 Kinase (S6K1) pathway in melanoma **[Kleffel S. Cell. 2015]** and hepatocarcinoma **[Li H. *et al.* 2017]**, or through BRAF/MEK/MAPK cascade in thyroid carcinoma, promoting proliferation and migration **[Liotti, F., *et al.* 2021]** (Figure 11). PD-1 can bind to SHP-2 through the latter SH2 domain and directly or indirectly induce Ras activation (via GRB2/SOS complex) **[Liotti, F., *et al.* 2021]**. Moreover, it seems that regulatory T cells can create an immunosuppressive tumor environment by expressing PD-1 on their surface **[Alsaab, H. O., *et al.* 2017]**.

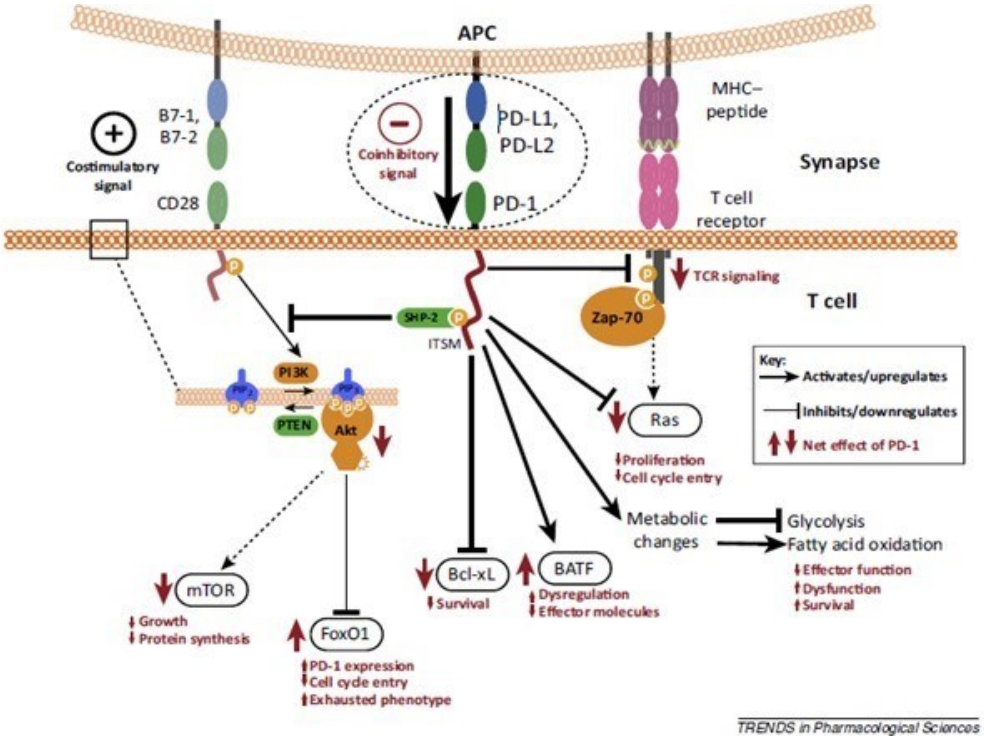

Figure 11. PD-1/PD-L1 signaling in T cells **[Pardoll D.M., *Nat Rev Cancer*. 2012]**

PD-1 ligands are PD-L1 (B7-H1/CD274) and PD-L2 (B7-CD/CD273), but the binding affinity of PD-1 for PD-L1 is 3-fold higher than the affinity for PD-L2. Furthermore, PD-L1 is expressed in a wide range of hematopoietic and non-hematopoietic cells following the upregulation exerted by IFN-γ and TNF-α; PD-L2 expression, on the other hand, is restricted to macrophages, dendritic cells and mast cells **[Alsaab, H. O., *et al.* 2017]**. The interaction between PD-1 and PD-L1 induces an inhibitory signal that limits T cell activation during the effector phase of the immune response in peripheral tissues (Figure 11) **[Fife B.T., *et al.* 2008]**. Cancer cells can avoid immune surveillance by PD-L1 overexpression which, in turn, can downregulate T cell activation **[Chinai J.M., *et al.* 2015]**. Furthermore, in glioblastomas the expression of PD-L1 is linked to the activation of PI3K/AKT signaling, whereas in some lymphomas and lung cancers is linked to the upregulation of STAT3 (modulated, in turn, by IL-6) and ALK signaling **[Pardoll D.M. 2012]**. Finally, the involvement of PD-L1 signaling in tumor cell proliferation and survival has been associated also to MAPK pathway **[Passariello M., *et al.* 2019]**.

It could be supposed that PD-L1 expression on cancer cells is directly modulated by NF-κB, a master transcription factor of inflammation, as it, in macrophages, binds to PD-L1 promoter by inducing its transcription **[Mulero M.C., *et al.* 2019]** and can improve its post-translational stabilization by inducing the expression of proteins such as COP9 signalosome subunit 5 (CSN5). Moreover, oxidative- and stress-induced signals as well as inflammatory cytokines and chemotherapeutic drugs-deriving signals can

activate NF-κB, by indirectly controlling PD-L1 up-regulation **[Walmsley S.R., *et al.* 2005, Antonangeli F., *et al.* 2020]**.

Since, the interaction of PD-1, expressed on the surface of activated T cells, and PD-L1 on the surface of tumor cells results in immunosuppression, the interference mediated by an antibody specific for PD-1 or PD-L1 could be a suitable strategy to improve T cells activation. A first humanized mAb targeting PD-1, Pembrolizumab (Lambrolizumab), has been approved by the FDA in 2014 firstly for the treatment of patients with unresectable or metastatic melanoma after treatment with Ipilimumab, and then approved in 2017 for the treatment of locally advanced or metastatic urothelial carcinoma **[Robert C., *et al.* 2014]**. A second fully human anti-PD-1 mAb, named Nivolumab, has been approved for the therapy of Hodgkin lymphoma, unresectable or metastatic melanoma, metastatic renal cell carcinoma and NSCLC **[Raedler L.A. 2015; Kazandjian D., *et al.* 2016; Hazarika M., *et al.* 2017]**. A number of antibodies targeting PD-L1 are in clinical development for several types of advanced cancer, but at the moment only the humanized anti-PD-L1 IgG1 Atezolizumab has been approved by the FDA for NSCLC in October 2016 and in 2019 for TNBC **[Weinstock C., *et al.* 2017; Narayan P., *et al.* 2020]**, whereas it is currently undergoing evaluation in clinical trials for patients with other types of cancer, including renal cell carcinoma, bladder cancer, and small-cell lung cancer **[Krishnamurthy A., Jimeno A. 2017]**.

Drug	Trade name	Year	Target	Location	Indications*
Ipilimumab	Yervoy®	2011	CTLA-4	T-lymphocyte	Melanoma, RCC, colorectal carcinoma
Nivolumab	Opdivo®	2014	PD-1	T-lymphocyte	Melanoma, NSCLC, SCLC, RCC, Hodgkin lymphoma, SCC of H&N, urothelial carcinoma, colorectal carcinoma, HCC
Pembrolizumab	Keytruda®	2014	PD-1	T-lymphocyte	Melanoma, NSCLC, Hodgkin lymphoma, SCC of H&N, urothelial carcinoma, gastric tumors, bladder cancer, head and neck cancer, esophageal cancer, cervical cancer, HCC, RCC, Merkel cell carcinoma, breast cancer, colorectal carcinoma
Atezolizumab	Tecentriq®	2016	PD-L1	Tumor cell	NSCLC, urothelial carcinoma, SCLC, breast cancer
Durvalumab	Imfinzi®	2017	PD-L1	Tumor cell	Urothelial carcinoma, NSCLC
Avelumab	Bavencio®	2017	PD-L1	Tumor cell	Merkel cell carcinoma, urothelial carcinoma, RCC
Cemiplimab	Libtayo®	2018	PD-1	T-lymphocyte	Cutaneous SCC

Table 1. FDA-approved immune checkpoint inhibitors (mAbs) and their indications **[Chhabra N., *J. Med. Toxicol.* 2021]**

Despite the success of clinically validated mAbs targeting ICs, their average response is still limited, as they result effective in only about 20-30% of the patients **[Lee C.S., *et al.* 2013; Buqué A., *et al.* 2015; Pardoll D.M. 2012]**.

New emerging targets belonging to the class of co-inhibitory receptors have been identified in the last years, such as lymphocyte activation gene 3 (LAG-3), which is an immunosuppressive receptor expressed on activated T lymphocytes and T-regulatory lymphocytes. Other examples of co-inhibitory receptors are the T cell immunoglobulin and mucin-3 (TIM3) and T cell immunoglobulin and ITIM domain (TIGIT), which are expressed on exhausted CD8+ T cells in tumors **[Anderson A.C., *et al.* 2016; Goldberg M.V., Drake C.G. 2011; Huang C.T., *et al.* 2004]**. Another co-inhibitory receptor, the B- and T-lymphocyte attenuator (BTLA) is expressed during the activation of T cells, leading to inhibition of human CD8+ cancer specific T cells **[Watanabe N., *et al.* 2003]**.

Combinations of antibodies targeting different immune checkpoint receptors, in order to achieve additive or synergistic activity, can potentially translate into a better anti-tumor activity. Proof of concept for this approach was provided by the finding of increased efficacy of the Ipilimumab and Nivolumab combination versus monotherapy in the treatment of metastatic melanoma **[Mahoney K.M., *et al.* 2015; Larkin J., *et al.* 2015]**. Thus, combinatorial treatments to achieve higher therapeutic index with respect to monotherapies have been tested by several clinical trials, demonstrating several benefits in terms of overall survival **[Ribas A., *et al.* 2018],** but also increased side immune-related adverse events (irAEs) **[Lee, C.S., *et al.* 2013; Hamilton, G., *et al.* 2017; Kourie, H.R., *et al.* 2016; Ventola C.L., *et al.* 2017; Haanen, J.B.A.G., *et al.* 2017]**. Furthermore, a number ofclinical trials involving approved or novel antibodies against immune checkpoints used in monotherapy or in combination with other biologics or small molecules are being carried out worldwide.

The generation of a complete repertoire of fully human antibodies specific for all these T cell checkpoint modulators is a great goal that researchers are pursuing with the aim of testing them in multiple possible combinations **[Sasso E., *et al.* 2018]**.

1.7 Phage Display Technology for the generation of a human repertoire of mAbs for cancer immunotherapy

An innovative strategy based on the well-known phage display technology was performed in our laboratory in order to generate a repertoire of fully human immunomodulatory mAbs against several targets to be used in monotherapy or in combinatorial treatments for cancer therapy.

Through this technique, a gene encoding a protein of interest can be inserted into a phage coat protein gene, inducing the phage to "display" the protein on the capsid thus allowing for its interaction with a specific target.

Large libraries of scFvs can be screened in a process called *in vitro* selection, which is analogous to natural selection.

The scFv is the smallest portion of the immunoglobulin, which retains the antigen-binding ability. Indeed, an antibody in the scFv format consists of the variable region of heavy (VH) and light (VL) chains, joined together by a flexible peptide linker, with a molecular weight of 27 kDa **[Ahmad Z.A., *et al.* 2012]**.

The phage display technology allows for the generation of libraries containing up to 10^{10} different variants, that can be used in a screening process with the aim to select antibodies against an almost unlimited array of biological (including human self-antigens) and non-biological targets (including toxic molecules). The gene encoding each scFv is cloned in phagemids packed inside the virion as a fusion construct with the gene encoding the coat protein, and the corresponding product is expressed as a fusion protein with the pIII protein of the phage coat (Figure 12).

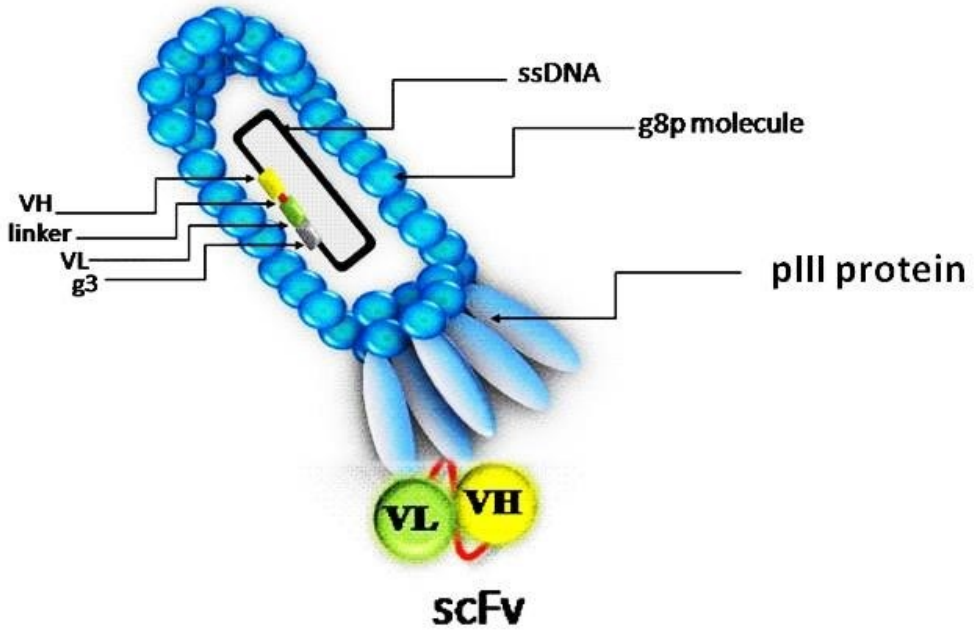

Figure 12. Schematic representation of scFv expressed as a fusion product with the pIII protein of the phage coat.

Then, the scFv-phages can be panned either on targets immobilized on solid support or expressed on the cell surface, selecting the binder clones from the whole repertoire by using affinity selection techniques on the target.

In our laboratory a large number of scFvs targeting tumor or other receptors have been successfully isolated by panning human scFv libraries either on purified proteins or on live cells expressing the targets on their surface **[De Lorenzo C., *et al.* 2002; Palmieri D., *et al.* 2015; Paciello R., *et al.* 2016; Sasso E., *et al.* 2018]**.

A novel strategy was defined by our research group for rapid parallel screening of phage displayed antibody libraries by directly panning on activated human lymphocytes. This novel approach allowed for the productive selection in one single panning of several phage clones recognizing, multiple targets as they are displayed on the live cell membrane, in their native conformation. Subsequent panning rounds performed on human purified proteins allowed for the isolation of scFvs specific for the binding of each IC. As a proof of concept, fully human IgGs from three phage clone collections specific for PD-1, PD-L1 and LAG-3 were successfully characterized **[Sasso E., *et al.* 2018]**. Among them some antibodies targeting PD-1, PD-L1 and LAG-3 were found able to specifically bind and activate T cells *in vitro* more efficiently than the clinically validated Nivolumab.

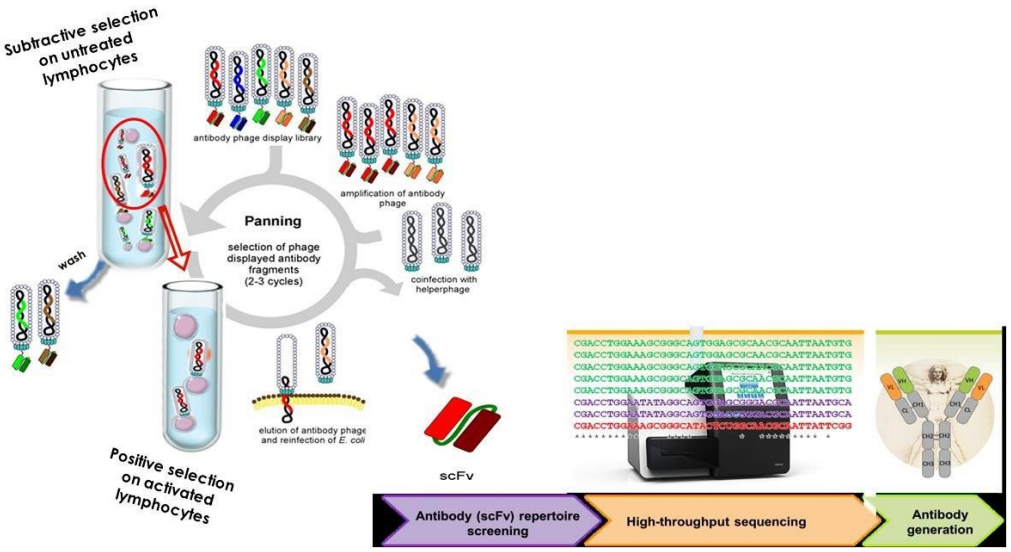

Figure 13. Strategy of scFvs selection by phage display against immune checkpoints. The phages were incubated with activated lymphocytes after a subtractive step on untreated lymphocytes in order to select specific phages for receptors expressed upon activation. The screening of enriched clones was performed by Next Generation Sequencing, and the identified scFvs were then converted into IgG4.

1.8 *Novel combinatorial approaches*

The combination of different treatments involving ICIs or other targeting anti-tumor agents is considered a suitable strategy to achieve additive or synergistic activity. Indeed, the combination of Nivolumab and Ipilimumab has been used in patients with metastatic melanoma **[Nomura M., *et al.* 2017]**, advanced renal cancer cell **[Cetin B., *et al.* 2019]**, metastatic colorectal cancer **[Ciardiello D., *et al.* 2019]** and recurrent small-cell lung cancer **[Cope S., *et al.* 2019]** leading to encouraging clinical benefits in some malignant tumors, especially for advanced melanoma **[Larkin J., *et al.* 2019]**.

Since both ErbB2-positive tumors and TNBC express high levels of PD-L1 **[Turcotte M., *et al.* 2017; Stovgaard E.S., *et al.* 2018]** and a high proportion of PD-L1 positive tumors are co-infiltrated with PD1+ lymphocytes a new combinatorial strategy could also include the combination of anti-ErbB2 or anti-EGFR agents with novel anti-PD-L1 mAbs. Combination of Trastuzumab and Pembrolizumab, anti-ErbB2 and anti-PD-1 mAbs respectively, are under investigations **[De Melo Gagliato D., *et al.* 2017**] for the therapy of breast cancer **[De Melo Gagliato D., *et al.* 2017; Turcotte M., *et al.* 2017; Stovgaard E.S., *et al.* 2018]**.

Furthermore, the IMpassion130 Trial **[IMpassion130 ClinicalTrials.gov number, NCT02425891]** combined nab-paclitaxel with the anti-PD-L1 Atezolizumab, evidencing a remarkably prolonged progression-free survival (PFS) in patients with metastatic TNBC, and leading to FDA approval for patients with unresectable advanced PD-L1-positive TNBC.

Despite the great success in terms of OS and PFS, these combinatorial treatments are characterized by more marked immune-related adverse events (irAEs), such as cytokine release syndrome, dermatologic toxicities (occurring in 40-50% of patients treated with Ipilimumab and 30-40% of patients treated with ICIs which target the PD-1 axis), endocrine dysfunction, gastrointestinal and hepatic adverse effects, neurologic (occurring in 6 to 12% of patients and are generally of low grade), ocular and renal irAEs **[Chhabra N., *et al.* 2021].**

Recently, some cases of ICI-related cardiotoxicity, which mainly occurs in the early stage after treatment, have been reported, such as cases of myocarditis, dilated cardiomyopathy, pericarditis, arrhythmias and heart failure **[Yang S., *et al.* 2018]**. In particular combinatorial ICIs treatment with Nivolumab plus Ipilimumab have been associated to a 5-fold higher risk of cardiotoxicity when compared to monotherapies.

Thus, there is urgent need for the identification of novel combinatorial treatments that could overcome these limits and allow for effective and safe therapy.

AIMS

The main goal of my PhD project was the development of novel therapeutic approaches based on the generation of fully human mAbs to be combined in order to increase their anti-tumor potency.

Considering the rare but severe cardiotoxic side effects observed with the combination of Ipilimumab and Nivolumab, we aimed at setting up cell-based assays to early identify those combinations of mAbs with high efficacy against cancer cells, but devoid of harmful side effects.

The first aim was represented by the generation of novel fully human antibodies recognizing both human and mouse CTLA-4 immune checkpoint in its native conformation, to be used in future for *in vivo* studies on mouse models. In this regard, the phases of the research activity were the following:

- set up of an innovative strategy based on phage display to perform a parallel selection on both human and mouse chimeric recombinant CTLA-4 protein;
- identification of the most enriched scFvs clones in the parallel selections by NGS and bioinformatics;
- conversion of the selected scFvs into fully human IgG1;
- purification and characterization of the anti-CTLA-4 antibodies.

The leader candidates were tested in comparison with anti-PD-L1 antibodies, previously generated in our laboratory, for their binding affinity by ELISA assays on lymphocytes and on purified proteins; for their ability to activate T cells in co-cultures with tumor cells, and for their antitumor effects on intracellular pathways.

An additional aim was the identification of the best combinatorial treatments of immunomodulatory mAbs by testing them on both tumor and cardiac cells, in order to identify those endowed with synergistic effects on tumor cells but ineffective on cardiomyocytes.

The final aim was represented by the development of novel immunoconjugates made up of mAbs and aptamers, in order to create bispecific molecules that could combine the properties of these drugs overcoming the limits of small aptamers, such as rapid clearance and lack of effector functions.

MATERIALS AND METHODS

3.1 Cell cultures

MDA-MB-231 breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technologies, Paisley, UK). BT-549, SK-BR-3, LNCaP, MDA-MB-453 and mouse CT26 cancer cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI 1640, Gibco, Life Technologies, Paisley, UK). A-549 lung cancer cells were cultured in Kaign's Modification of Ham's F-12 Medium (F-12K, American Type Culture Collection, Manassas, VA, USA). MCF-7 cells were cultured in Modified Eagle's Medium (MEM, Gibco, Life Technologies, Grand Island, NE, USA). Human fetal cardiomyocytes were cultured in Cardiac Myocyte Medium (CMM, Innoprot, Derio-Bizkaia, Spain).

All the cell lines were purchased from the American Type Culture Collection (ATCC) and cultured in humidified atmosphere containing 5% CO^2 at 37 °C. The media were supplemented with 10% (vol/vol) (20% in the case of HuT-78 cells) heat-inactivated fetal bovine serum (FBS, Sigma, St Louis, MO, USA) and were used after addition of 50 U/mL penicillin, 50 µg/mL streptomycin, 2 nM L-glutamine (all from Gibco, Life Technologies, Paisley, UK).

3.2 Antibodies and Human Recombinant Proteins

The following antibodies were used, as reported: anti-EGFR polyclonal antibody; Erb-hcAb human anti-ErbB2 compact antibody (Biotecnol); anti-human PD-1 human mAb Nivolumab (Opdivo®); anti-CTLA-4 mAb Ipilimumab (Yervoy, Bristol Myers Squibb, NY, USA); anti-human PD-L1 human mAb (G&P Biosciences, Santa Clara, CA, USA); commercial Human anti-human CTLA-4 Antibody (R&D Systems, Minneapolis, MN, USA); HRP-conjugated antibody anti-human IgG (H+L) (Promega, Madison, WI, USA); HRP-conjugated anti-human IgG (Fab')2 goat monoclonal antibody (Abcam, Cambridge, UK); anti-human p-Erk rabbit polyclonal antibody, anti-human Cleaved Caspase-3 rabbit polyclonal antibody, anti-phospho-(Ser/Thr) Akt and anti-EGFR polyclonal antibody (all from Cell Signaling, Danvers, MA, USA); NF kappa B p65 C-20 polyclonal antibody, p-NF kappa B p65 A-8 monoclonal antibody and anti-vinculin monoclonal antibody (all from Santa Cruz Biotechnology, Inc. Dallas, TX, USA); anti-actin antibody (Sigma-Aldrich, Darmdstadt, Germany); HRP-conjugated anti-goat IgG (R&D Systems, Minneapolis, MN, USA); HRP-conjugated anti-human IgG (Fc-specific), HRP-conjugated anti-Mouse IgG and HRP-conjugated anti-rabbit IgG (all from Sigma); anti-human IgG antibody (Fluorescein (FITC) AffniPure Goat Anti-Human IgG (H + L) (Jackson ImmunoResearch Laboratories Inc., Madison, WI, USA).

The following recombinant proteins were used: human PD-1/Fc, human PD-L1/Fc and human IgG1 Fc Protein (all from R & D Systems, Minneapolis, MN, USA); human CTLA-4 Protein (His & Fc Tag); mouse CTLA-4 Protein (Fc Tag) (both from Sino Biological, Wayne, PA, USA); Streptavidin-HRP (BioRad, Hercules, CA, USA).

ID-1 and ID-8 (anti-CTLA-4), PD-1_1 (anti-PD-1), PD-L1_1 and 10–12 (anti-PD-L1) monoclonals were produced taking advantage from the enhanced cell line HEK293_ES1 expressing a long non-coding SINEUP RNA **[Sasso E., *et al.* 2015]**

3.3 Aptamers

2' Fluoro-pyrimidines (2' F-py) RNA aptamers (CL4, its corresponding versions containing a C6-NH_2 or a biotin 5' terminal modification, and CL4Sc representing the scrambled from of CL4 used as a negative control) were purchased from TriLink Biotechnologies (Tebu-bio, San Diego, CA, USA) with purity above 95% (PAGE analysis, short wave) **[Passariello M., *et al.* 2019; Passariello M., *et al.* 2020]**.

Sequence of CL4:

5' GCCUUAGUAACGUGCUUUGAUGUCGAUUCGACAGGAGGC 3'

[Esposito C.L., *et al.* 2011, Camorani S., *et al.* 2015 , Camorani S., *et al.* 2017]

Sequence of CL4Sc:

5' UUCGUACCGGGUAGGUUGGCUUGCACAUAGAACGUGUCA 3'

[Esposito C.L., *et al.* 2011, Camorani S., *et al.* 2015, Camorani S., *et al.* 2017]

Before each treatment, the aptamers were subjected to a short denaturation-renaturation step (85°C for 5 minutes, snap-cooled on ice for 2 minutes, and allowed to warm up to 37°C for 10 minutes). For cell treatments longer than 24 hours, the aptamer was renewed each day and the RNA concentration was determined to ensure the continuous concentration of 200 nM, taking into account the 6 hours-life of the aptamer in 10% (vol/vol) serum **[Esposito C.L., *et al.* 2011, Camorani S., *et al.* 2015]**.

3.4 Antibody-Oligonucleotide Conjugation

The conjugation of Erb-hcAb, 10_12 or Ipilimumab mAb with CL4 aptamer was obtained by using an Antibody-Oligonuclotide Solulink Conjugation Kit (TriLink Biotechnologies). Briefly, in the first step, the amino-terminated CL4 aptamer was labeled at 5’ with an aromatic aldehyde functional group (formylbenzamide, 4FB), by reaction with an amine-reactive NHS ester (S-

4FB), following the manufacturer's recommendations. In the second step the antibody was modified with HyNic functional groups by incubating it with S-HyNic reagent at RT for 2 h. Then, 4FB-oligonucleotide was incubated with HyNic-modified antibody for 2 h at RT to allow for conjugation. After the incubation, the reaction mixture was buffer-exchanged by centrifugation at 1500x g for 2 min in a spin column (Solulink kit). To obtain the conjugate, the reaction mixture was transferred into a tube containing magnetic beads and incubated for 40 min at RT, taking care to mix the beads every 10 min during the incubation period. The beads were then placed on a magnetic stand for discarding the supernatant. After several washes, the beads were resuspended into the Elution Buffer (Solulink kit) and incubated for 15 min, taking care to resuspend them every 5 min. After the last resuspension, the beads were placed on the magnet stand to elute the immunoconjugate. The final antibody-oligonucleotide conjugate concentration was determined by using a BCA protein assay Kit (Pierce, Perbio, Rockford, IL, USA).

3.5 Isolation of Human and Mouse Peripheral Blood Mononuclear Cells

Human PBMCs were isolated from blood samples by using Greiner Leucosep® tube (Sigma-Aldrich, 227,288) following the manufacturer's instructions and frozen in a solution containing 90% FBS and 10% dimethyl sulfoxide (DMSO) until use. Mouse PBMCs were isolated from the spleen of sacrified animals, by mashing the organ into the cell strainer. Splenocytes were isolated by lysing red blood cells by using ACK lysis buffer and then frozen in the same solution used for hPBMCs.

Before their use, the samples were thawed out by resuspending them in RPMI medium supplemented with 2 nM L-glutamine, 1_ CTLWash Supplement 10_ (Cellular Technology Limited, Shaker Heights, OH, USA) and 100 U/mL Benzonase (Merck Millipore, Burlington, USA). PBMCs were incubated for overnight resting at 37°C in R10 medium made up of RPMI 1640 medium, supplemented as described above, with the addition of 10m MHEPES. For mouse lymphocytes 50 mM β-Mercaptoethanol (both from Gibco Thermo Fisher Scientific, Paisley, Scotland, UK) was also added. All the PBMCs were then counted by using the Muse cell analyzer (Merck Millipore, 0500-3115, Darmdstadt, Germany).

3.6 Isolation of NK Cells

Human NK cells were isolated from hPBMCs by using the NK cell isolation kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's guidelines. Briefly, the sample of hPBMCs was incubated with NK Cell Biotin-Antibody Cocktail for five minutes at 4 °C. NK cell microbead cocktail was then added and incubated for 10 min at 4 °C, diluted with buffer,

and placed on a magnetic separator. The cells in the flow-through, corresponding to the enriched fraction of NK cells, were collected by centrifugation at 1200 rpm for eight minutes at 25°C. The cell pellet was then resuspended in R10 medium and counted by using a Muse cell analyzer (Merck Millipore, 0500–3115, Darmdstadt, Germany).

3.7 Selection of scFv-Phage Clones

Phagemid particles, recovered from the cells infected with the library by using the M13-K07 helper phage (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA 92008, USA) were isolated by precipitation with PEG, as previously described **[Sasso E., *et al.* 2018]**. In a first round the phages were incubated with human PBMCs, previously activated with Human T-Activator CD3/CD28 Dynabeads (ThermoFisher Scientific, Baltics UAB, Lithuania) for 96 h. The recovered phages were amplified by infecting E. Coli TG1 cells to prepare phages for the following second round performed on coated Recombinant Human CTLA-4 Fc protein (20 μg/mL), followed by two parallel rounds of selection on coated Recombinant Human or Mouse CTLA-4 Fc proteins. Before each round of selection on the chimeric proteins, the phages were submitted to two subsequent rounds of negative selection on Recombinant Human IgG1 Fc Protein to remove phage-scFv recognizing the Fc domain, as previously described **[Sasso E., *et al.* 2018**.

3.8 Preparation of DNA Fragments and Generation of Libraries for High-Throughput Screening

Phagemid double-strand DNAs containing the scFvs were prepared by Endo free Plasmid Maxi Kit (Qiagen, 12362) from the three sub-libraries. The variable heavy chains were extracted in a two-step restriction process by BamHI (R3136) and HindIII (R3104) followed by NcoI (R3193) and XhoI (R0146) (New England Biolabs, Ipswich, Massachusetts, USA), as previously reported **[Sasso E., *et al.* 2018]**. Libraries were prepared by TruSeq ChIP sample prep kit (Illumina, 15,023,092) and sequenced to a final concentration of 10 pM with 2 X 300 nt SBS kit v3 on an Illumina MiSeq apparatus. The sequencing was performed at the Center for Translational Genomics and Bioinformatics, Hospital San Raffaele, Milano, Italy. Paired end 2 X 300 sequencing was performed and FastQC software was used to examine quality of fastq files. Reads were joined by using fastq-join and Fastqc was used for quality checks on joined sequences. Raw counts were normalized to the total number of counts within each sub-library, obtaining counts per million values. Joined sequences were translated considering the correct open reading frame.

3.9 ScFv Reconstitution and Antibodies Production

The full length sequences of scFvs of interest were isolated from human_Cycle3 by overlapping PCR. Briefly, using clone-specific primers designed within the heavy CDR3 region, VH and VL fragments were obtained separately in two PCR reactions by Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, F530S,Waltham, Massachusetts, USA). The two obtained fragments were overlapped by CDR3 sequence and extended to get the full scFvs **[Sasso E., *et al.* 2015]**. The scFvs of interest were converted into whole human IgG1 and IgG4 antibodies by cloning the corresponding VHand VL cDNAs in SINEUP-competent, Fc encoding vectors by In-FusionHDcloning kit (Clontech Laboratories, 639,692. Mountain View, California, USA). PEUVL4.2_SA was used to subclone the VL sequences. PEUVH1.2 and PEUVH8.2 were respectively used to generate the selected antibodies in IgG1 and IgG4 isotypes **[Sasso E., *et al.* 2018]**. The vectors encoding the heavy and light chains of interest were prepared with an endotoxin-free system (EndoFree Plasmid Maxi Kit, Qiagen, 12,362) and were co-transfected in HEK293EBNA SINEUP (HEK293ES_1) cells by using Lipofectamine Transfection Reagent (Life Technologies, Inc. 11,668,019. Carlsbad, California, USA) and grown up for about 10 days at 37°C in chemical defined CD CHO medium (Gibco, Life Technologies, Inc. 10,743,029) complemented with 5 mL of L-glutamine 200 mM (Gibco, Life Technologies, A2916801), 5 mL of Penicillin-Streptomicyn 10,000 U/mL (Sigma-Aldirch, Cancers 2020, 12, 2204 19 of 24 P0781) in 150 mm Corning® tissue-culture treated culture dishes. The antibodies were purified from the conditioned media by using Protein A HP SpinTrap30 (GE Healthcare Life Sciences, 28–9031–32. Chicago, Illinois, USA). The quality of antibodies preparation was evaluated by SDS-PAGE NuPAGE 4–12% Bis-Tris Protein Gels (Thermo Fisher Scientific, NP0321BOX,Waltham, Massachusetts, USA) followed by Coomassie blue staining (Biorad, 1,610,786. Hercules, California, USA). Purified antibodies were desalted and buffer-exchanged by PD-10 Columns (GE Healthcare Life Sciences, 17085101). The antibody preparations were sterilized by filtration with 0.22 μm durapore hydrophilic filters (Millipore, SLGS033SS. Burlington, Massachusetts, USA) and stored in aliquots at -80°C.

3.10 ELISA Assays

To measure the ability of the mAbs or aptamers to bind to receptors displayed on the cell surface, human or mouse lymphocytes (4 x 10^5 cells/well) previously activated with anti-CD3/CD28 beads for 48–72 h, or cancer cells (2 x 10^5 cells/well) were plated on round-bottom 96-well plates and incubated with the mAbs or aptamers in PBS/BSA 3% buffer solution for 2 hours at Room Temperature, by gently shaking. After extensive washes with PBS 1X, the plates were incubated with an anti-human IgG (H+L) HRP-conjugated antibody or with HRP-conjugated Streptavidin (to detect the binding of CL4 aptamer) for 1 hour at room temperature.

The binding ability of the mAbs was also tested on the purified recombinant human or mouse CTLA-4/Fc, human PD-L1/Fc or the isolated Fc portion used as a negative control in parallel assays. After coating on NuncTM flat-bottom 96-well plates at a concentration of 5 μg/mL and blocking with PBS/milk 5% at 37°C for 1 hour, the immobilized chimeric proteins were incubated with the indicated mAbs in PBS/BSA 3% buffer solution for 2 hours at room temperature. After extensive washes with PBS1X solution, plates were incubated with HRP-conjugated anti-human IgG (Fab')2 goat monoclonal antibody in PBS/milk 3% buffer solution for 1 hour at room temperature. After the second incubation, the plates were washed again with PBS 1X and incubated with TMB (Sigma-Aldrich, St. Louise, USA) reagent for 10 min, before quenching with an equal volume of 1 N HCl. Absorbance at 450 nm was measured by the Envision plate reader (Perkin Elmer, 2102, San Diego, CA, USA). The Kd values were calculated by elaboration of ELISA binding curve analyses by Prism (GraphPad Prism 5) tool.

3.11 Western Blotting Analysis of Cell Extracts

Cancer cells were plated at a density of 6 x 10^5 cells/well in six-well plates in the absence or in the presence of drugs under treatment for 72 h. The population enriched in NK cells were plated at a density of 1 x 10^6 cells/well in 48-well plates in the absence or in the presence of drugs under treatment for 66 h. Cells were scraped and collected by centrifugation at 1200 rpm for 10 min. The cell pellets were resuspended in a lysis buffer of 10 mM Tris-HCl pH 7.4, 0.5% Nonidet-P-40, 150 mM NaCl, containing 1 mM Sodium orthovanadate (Sigma-Aldrich, St. Louise, MO, Cancers 2020, 12, 2204 21 of 24 USA) and protease inhibitors (Roche, Indianapolis, IN, USA). After lysis, the protein concentration of cell extracts was determined by the Bradford colorimetric assay (Sigma-Aldrich, St. Louise, MO, USA) and analyzed by SDS-PAGE followed by Western blotting analyses performed by incubating the nitrocellulose filters with the indicated commercial primary antibodies, followed by specific HRP-conjugated secondary antibodies used for the detection. The analysis of signal intensity was performed by using ChemiDoc Imaging System (Bio-Rad Laboratories, Inc. Hercules, California, USA) and the quantification of the bands was performed by ImageLab software.

3.12 Confocal Microscopy

SK-BR-3, MDA-MB-453 and MCF-7 cells (10^5 cells/well in 24-well), previously seeded on a coverslip for 24 h, were incubated for 10 minutes with FAM-labeled CL4 or for 1 h with 10_12, Ipilimumab, 10_12-CL4 or CL4-ipilimumab conjugates in BlockAid™ Blocking Solution (Life Technologies) at RT. Then, cells were washed three times in PBS and fixed in PBS/PFA 4% for 20 min. For the fluorescence visualization of mAbs and conjugates, cells were

incubated with FITC-labeled anti-human IgG antibody AffniPure Goat Anti-Human IgG (H + L) 1:300, Jackson ImmunoResearch Laboratories Inc., Madison, WI, USA) for 1 h at RT and then washed three times with PBS. Finally, cells were incubated with 1.5 μM 4',6-Diamidino-2-phenylindole (DAPI, D9542, Sigma-Aldrich) and mounted with glycerol/PBS. Samples were visualized by Zeiss LSM 700 META confocal microscopy equipped with a Plan-Apochromat 63x/1.4 Oil DIC objective.

3.13 Cell Growth Inhibition Assays

In order to evaluate the effects induced by the different drugs, their combinations or derived conjugated molecules, cancer cells were plated at a density of 5 x 10^3 cells/well, whereas HFC were plated at a density of 1 x 10^4 cells/well in 96-well flat-bottom plates for 16 h. Then, they were incubated in the absence or in presence of the specific drugs or an unrelated IgG control for 72 or 48 h, respectively. Viable cells were counted by the trypan blue exclusion test and cell survival was expressed as percent of viable cells with respect to the untreated cells used as negative control.

3.14 Cytotoxicity in co-cultures Assays and LDH Detection

Co-cultures of cells with hPBMCs or immune population enriched in NK cells were used to test the cytotoxic effects of different molecules used as single agents or in combination. Tumor cells were plated in 96-well flat-bottom plates at the density of 1 x 10^4 cells/well, whereas HFC cardiac cells were plated at a density of 1.5 x 10^4, for 16 h. Then, immune cells were added in the absence or presence of mAbs, aptamer or conjugates, used alone or in combination at 37 °C for 24 or 48 h. Untreated cells and cells incubated with an unrelated IgG control were used as negative controls. Tumor and cardiac cell lysis were evaluated by measuring the release of lactate dehydrogenase (LDH) in the supernatant of co-cultures described above by LDH detection kit (Thermofisher Scientific, Rockford, IL, USA), following the manufacturer's recommendations. Cell lysis was analyzed by measuring the fold increase of LDH in the presence of each treatment, with respect to the amount present in the supernatant of co-cultures untreated or treated with an unrelated mAb. Cytolysis values were obtained from at least three independent values.

3.15 Cytokine Secretion Assays

The secretion of Interleukin 6, Interleukin 2 and IFN-γ by hPBMCs or NK cells treated with the mAbs alone or in co-cultures with tumor or cardiac cells were evaluated by ELISA assays. Briefly, after treatments culture supernatants were centrifuged and analysed by ELISA for quantification of human IL-6 (ELISA MAXTM Deluxe Set Human IL-6, BioLegend, San Diego, CA, USA), or IL-2 and IFN-γ (DuoSet ELISA, R&D Systems, Minneapolis, MN, USA), according to the producer's recommendations.

3.16 In vivo studies on mouse models

Six-weeks old female BalBC mice (Envigo, USA) were used for in vivo studies. Mice were challenged with a subcutaneous injection of 2 x 10^5 CT26 cells (day 0). Three days after, mice were left untreated (control) or treated with PD-L1_1 (200 μg ip) or anti-mouse PD-L1 (200 μg ip, clone 10F.9G2, BioXcell) administered at day 3, 6 and 10. Tumor growth for individual mice was monitored over time using a digital caliper every 3–4 days up to day 21. Tumor volume was calculated by using the formula: 0.5 × length × width2. Tumors from control group and mice treated with PD-L1_1 were harvested at day 21, subjected to three homogenization cycles (3 minutes at 30 Hz) by using RIPA buffer, containing Protease inhibitors and Na3VO4, and centrifuged. For the analysis of tumor cells in the absence of infiltrating lymphocytes, tumors from control group and mice treated with α-mPD-L1 were also cut into small pieces and digested at 37°C with Collagenase I. Cell suspension was filtered through a 70 μm cell strainer and incubated with ACK Lysis solution (Gibco, Grand Island, USA). After a last filtration, the cell suspension was placed in a T75 flask at 37°C, overnight. The day after, adherent cells were washed with PBS, trypsinized, collected and centrifuged. Cell pellets were stored at −80°C until protein extraction and Western Blotting analyses were performed as described above. Experiments involving animals were approved by the Italian Ministry of Health (Authorizations 213/2016 PR) and have been done in accordance with the applicable Italian laws (D.L.vo 26/14 and following amendments), the Institutional Animal Care and Use Committee of CEINGE and Allevamenti Plaisant SRL.

3.17 Statistical Analyses

All the *in vitro* experiments were performed in triplicates and the reported values were obtained as the mean of at least three determinations. Error bars were calculated by considering the results obtained by at least three determinations obtained in three independent experiments. Error bars depict means ± SD. Statistical analyses were assessed by Student's t-test (two variables). Statistical significance was established as *** $p \leq 0.001$; ** $p < 0.01$; * $p < 0.05$.

RESULTS

4.1 Selection of human antibodies cross-reactive for human and mouse CTLA-4

The phage display strategy used for the isolation of anti-CTLA-4 scFvs consisted in alternate panning rounds of phage antibody libraries either on live activated hPBMCs expressing the target protein or on recombinant purified argets, as performed in previous studies **[Sasso E., *et al.* 2018]**. This approach guaranteed an efficient selection of a large number of clones with a high specificity for CTLA-4 antigen in its native conformation, as that presented on the cell membrane. To this aim, phage particles from a Phagemid library of up to 10^{10} different clones were prepared as described in Materials and Methods. In the first selection round, human PBMCs, previously activated with Dynabeads Human T-Activator CD3/CD28 for 96 h, were used as antigen-positive cells. Then, the subsequent panning rounds used as bait for parallel selections the recombinant human or mouse chimeric CTLA-4-Fc protein. Subtractive selection rounds consisted in two successive pannings on untreated lymphocytes, to remove from the repertoire all the non-specific phages recognizing common antigens on the cell surface of hPBMCs used in the first panning round, or on the Fc portion, to subtract the phages that recognize the Fc domain present in the CTLA-4/Fc chimeric proteins, used in the following two parallel rounds.

In each selection round, the tubes either containing CTLA-4 positive lymphocytes or coated with the recombinant chimeric proteins were incubated overnight at 4°C with th phage library. After extensive washes, phages bound to the cell surface or to the coated protein were eluted by using a low pH buffer, and used to infect *Escherichia coli* TG1 for amplification and further selection rounds **[Passariello M., *et al.* 2020]**. The screening of scFvs cross-reactive for human and mouse CTLA-4 proteins was performed by high throughput sequencing as shown in Figure 15. Briefly, in collaboration with the group of Prof. Zambrano and Prof. Nicosia, after each selection round on human or mouse protein, the cDNA encoding the VH region of the scFvs was extracted from each sub-library by restriction enzyme digestion, rather than by PCR amplification, to preserve the differences in relative representativeness. Three different barcodes were incorporated, respectively, for human-cycle_2, human-cycle_3 and mouse-cycle_3 sub-libraries. The fragments were pooled into a single run of sequencing on MiSeq Illumina platform (San Diego, CA, USA) to obtain at least 1.5 X 10^6 sequences from each sample.

After the analysis of the resulting data, the abundance of each encoded protein sequence was normalized within the proper sub-library according to count per million (cpm) values, and the sequences without a significant

abundance (<10 cpm) were discarded. The four scFv clones more enriched by the third cycle on the human protein were identified as potential binders and named ID-1, ID-4, ID-5, and ID-8 according to their ranking against the human protein (Figure 15).

To predict their cross-reactivity to murine CTLA-4, the ranking of ID-1, ID-4, ID-5, and ID-8 was analyzed in the sub-library obtained from the panning performed on mouse protein. Two out of the four clones resulted significantly enriched in the sub-library from mouse CTLA-4 selection and were found to be respectively ID-1 and ID-8. Interestingly, ID-1 resulted the most enriched clone in both human and murine sub-libraries, suggesting its potential recognition of a conserved region of CTLA-4 in the two different species. The enrichment of ID-4 and ID-5 clones in the murine sub-library was not significant and predictive for weak or no binding to the mouse protein. On the basis of the analysis of parallel sequencing data, ID-1 and ID-8 clones were considered as potential binders for both mouse and human CTLA-4 and were thus selected for additional characterization. To this aim, the corresponding scFvs were rescued from the library by overlapping PCR, and the cDNAs encoding the variable heavy and light regions were used to generate full IgG1 antibodies **[Passariello M., *et al.* 2020]**.

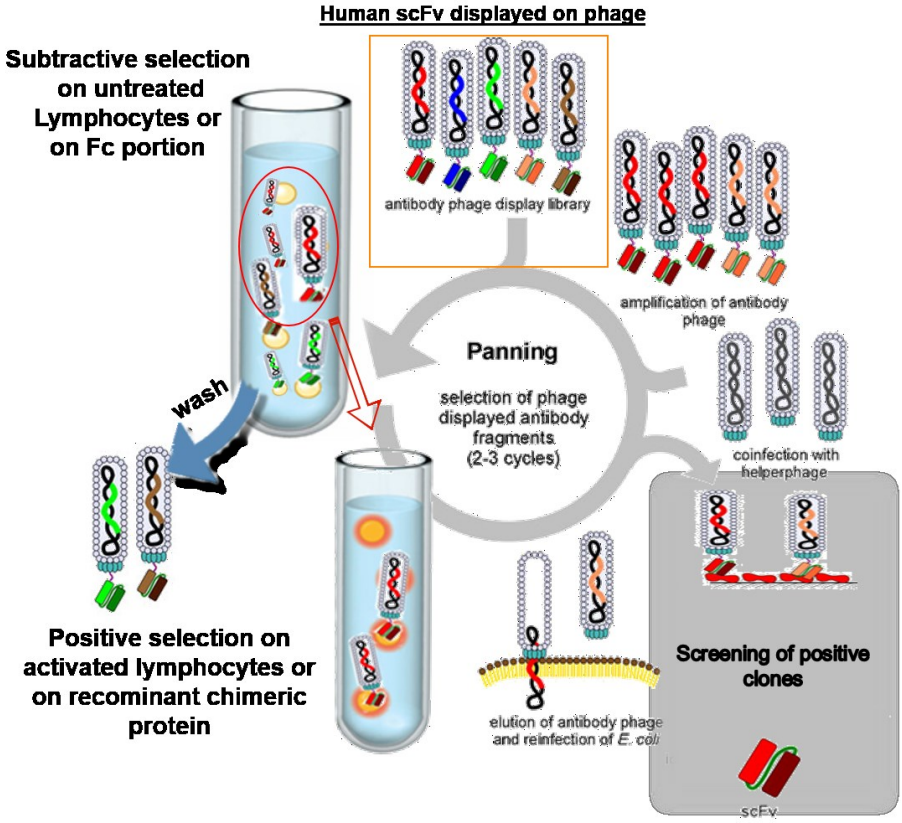

Figure 14. Representative scheme explaining the selection panning rounds to isolate scFvs.

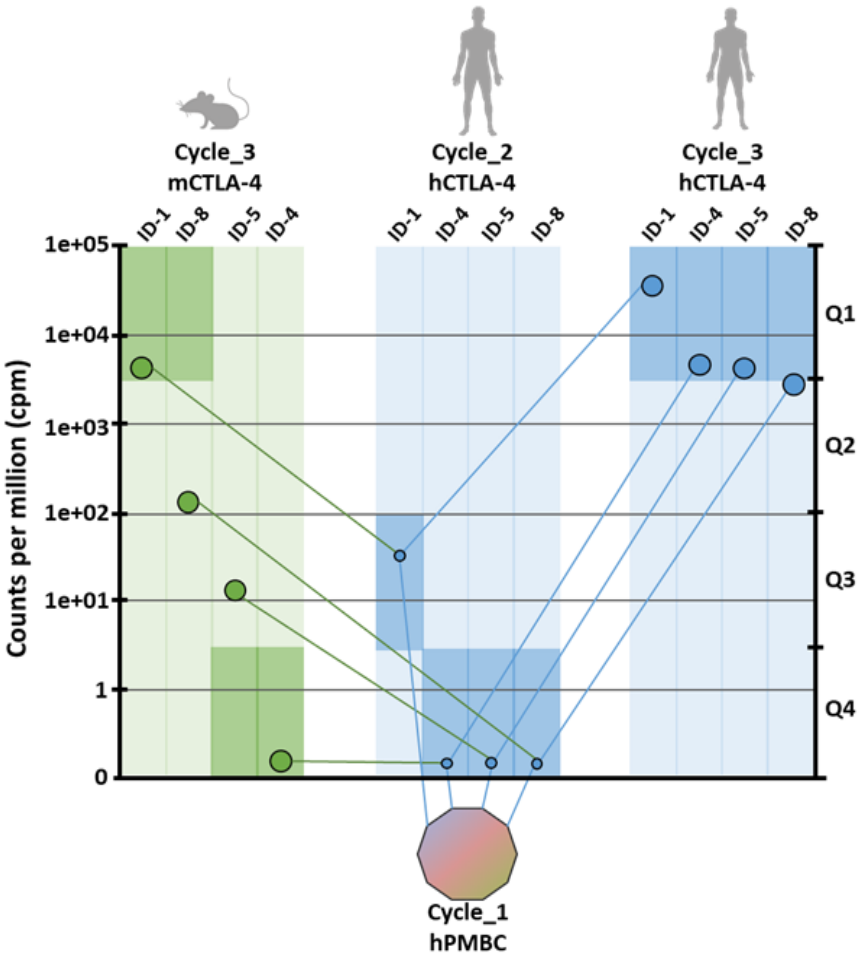

Figure 15. Screening strategy and next generation sequencing data analysis. The screening was carried out starting from the first panning round on hPBMC indicated as colored decagon**.** The human recombinant cytotoxic T lymphocyte-antigen 4 (CTLA-4) protein was used as bait in the second selection cycle and the relative enrichment of indicated clones was represented as small circles. Human and murine CTLA-4 recombinant proteins were used to perform the third parallel panning rounds. The count per million (cpm) values for each clone are depicted in the corresponding side of the figure (left side in light green for murine; right side in light blue for human) as large circles. The ranking of ID-1, ID-4, ID-5, and ID-8 clones was also determined according to the belonging quartile (Q1, Q2, Q3, Q4) in each sub-library as indicated by dark green (in murine sub-library) and dark blue (in human sub-library) rectangles. **[Passariello M., *et al.* *Cancers* 2020]**

4.2 Binding of the Converted Immuno-modulatory mAbs to Human or Mouse Lymphocytes and Purified Recombinant Protein targets

The strategy used for the isolation of scfvs-phages recognizing CTLA-4 was previously used to identify scfvs-phages that recognize PD-L1 IC with high affinity. After the efficient selection of a large number of clones able to recognize with high specificity the targets, some of the best scFv binders were converted into full size IgG-.

Hence, the converted monoclonal antibodies, named PD-L1_1, PD-L1_2, PD-L1_3 (in the case of anti-PD-L1 mAbs) or ID-1, ID-5, ID-8 (anti-CTLA-4 mAbs), were firstly analysed by ELISA assays on both untreated and activated hPBMCs, using the mAbs at increasing concentrations, to confirm their ability to bind to their specific targets. As shown in figure 16A, the three novel anti-PD-L1 antibodies were found capable of specifically binding to the activated hPBMCs, whereas only poor binding was observed on untreated lymphocytes. Among the novel anti-CTLA-4 mAbs, ID-1 and ID-8 were found able to bind to their target expressed on activated lymphocytes, with a higher affinity than that observed on the untreated lymphocytes, thus confirming the validity of the strategy used for the selection. On the contrary, ID-5 did not show a highly specific binding to activated lymphocytes. Moreover, since anti-CTLA-4-scFvs were isolated by performing a parallel panning round on both human and mouse recombinant CTLA-4 protein, ELISA assays were carried out also on mouse PBMCs (see figure 16B) to determine their cross-reactivity for mouse CTLA-4. As shown in figure 16B, ID-1 and ID-8 were found able to bind with high specificity to the mouse protein, whereas ID-5, a selected clone with low specificity, used as negative control, was not cross-reactive for mouse CTLA-4 protein.

To further investigate the binding specificity of the novel antibodies, we also tested their ability to recognize the recombinant purified human and mouse target proteins. The ELISA assays performed on human purified PD-L1-Fc chimeric protein showed that all the three anti-PD-L1 mAbs were able to bind to the target with high affinity, whereas only a poor binding was found on Fc protein, used as a negative control (Figure 17A). As shown in Figure 17, also the anti-CTLA-4 ID-1 and ID-8 mAbs were found able to bind with high affnity and specificity to both human (Figure 17B) and mouse (Figure 17C) CTLA-4-Fc chimeric proteins, whereas only a poor binding was observed on the Fc portion, used as negative control. These data confirm the binding specificity of the two selected antibodies, ID-1 and ID-8, for their targets and the efficiency of the strategy designed for the selection of mouse-human cross-reactiv mAbs. As shown in tables 2 and 3, the calculated Kd values, corresponding to the half-saturating concentrations, resulted in the low nanomolar range for both the anti-CTLA-4 and the anti-PD-L1 mAbs. In addition the Kd values of ID-1 and ID-8

for human CTLA-4 were quite identical to those obtained for the mouse counterpart.

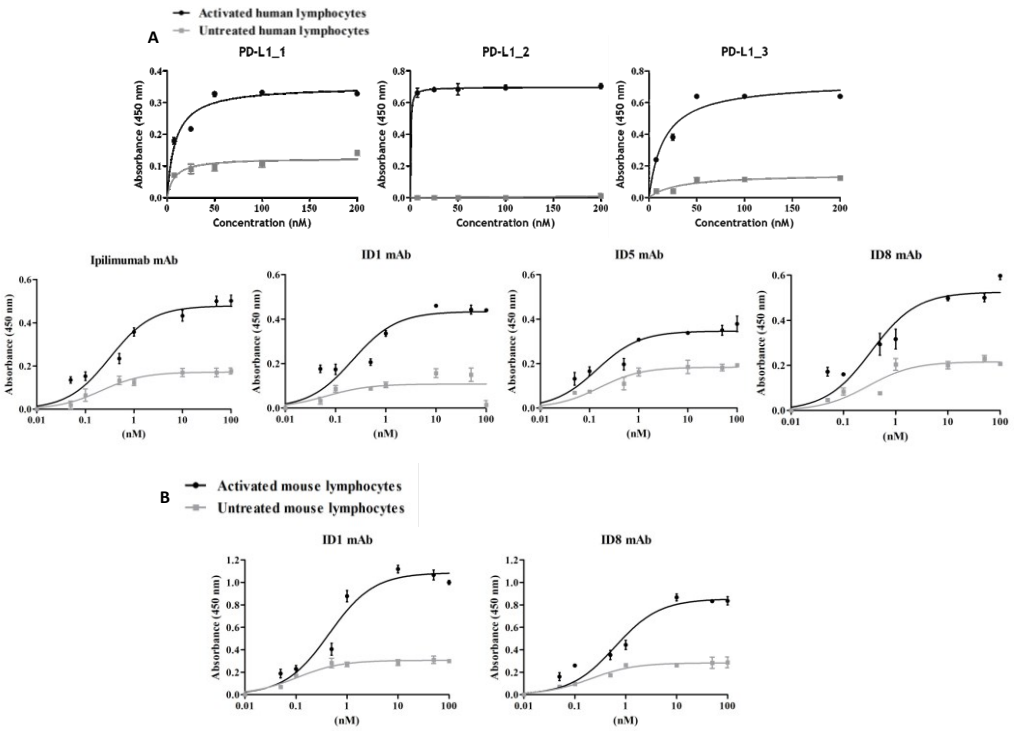

Figure 16. Binding curves of the novel generated anti-PD-L1 or anti-CTLA-4 mAbs to human or mouse lymphocytes. Cell ELISA assays on human (**A**) or mouse (**B**) lymphocytes untreated (grey curves) or activated (black curves) for 48-72 hours by testing PD-L1_1, PD-L1_2, PD-L1_3, ID-1, ID-8, and ID-5 at increasing concentrations. Ipilimumab was used as positive control. Error bars depict means ± SD. **[Passariello M., *et al. Cancers* 2020]**

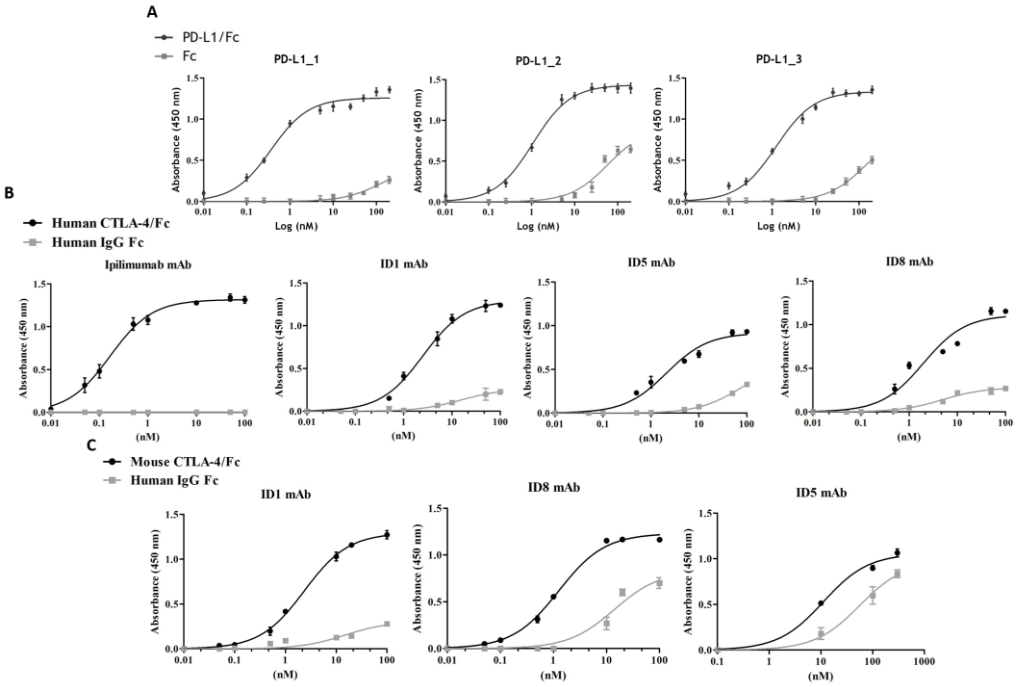

Figure 17. Binding curves of the novel generated anti-PD-L1 and anti-CTLA-4 mAbs to human or mouse purified PD-L1-Fc or CTLA-4-Fc. (**A**) Binding curves of PD-L1_1, PD-L1_2 and PD-L1_3 to immobilized human PD-L1-Fc (black curves) purified protein or to the corresponding Fc portion (grey curves). Binding curves of ID-1, ID-8 and ID-5 mAbs to the human (**B**) or mouse (**C**) purified CTLA-4-Fc (black curves) or to the corresponding Fc portion (grey curves), used as a negative control. Ipilimumab was used in parallel assays as a control. Error bars depict means ± SD. **[Passariello M., *et al. Cancers* 2020]**

Kd values of the selected anti-PD_L1 mAbs		
	PD-L1/Fc	Activated hPBMCs
PD-L1_1	0,3 nM	9 nM
PD-L1_2	1 nM	0,4 nM
PD-L1_3	1,2 nM	16 nM

Table 2. Binding affinity of the selected mAbs for PD-L1 purified or expressed on lymphocytes. The Kd values obtained from the binding curves of the novel isolated anti-PD-L1 mAbs on human PD-L1-Fc recombinant protein and on activated human lymphocytes are reported in the table.

Kd values of the selected anti-CTLA-4 mAbs				
mAbs	**Human CTLA-4 protein**	**Human activated lymphocytes**	**Mouse CTLA-4 protein**	**Mouse activated lymphocytes**
Ipilimumab	0.17 nM	0.32 nM	N. D.	N. D.
ID1	2.5 nM	0.22 nM	2.35 nM	0.45 nM
ID8	1.95 nM	0.34 nM	1.25 nM	0.62 nM

Table 3. Binding affinity of the selected mAbs for CTLA-4 purified or expressed on lymphocytes. The Kd values obtained from the binding curves of the novel isolated anti-CTLA-4 mAbs on human or mouse CTLA-4-Fc recombinant protein and on activated human or mouse lymphocytes are reported in the table.

4.3 Effects of the Novel immunomodulatory mAbs on lymphocytes activation

Antibodies targeting ICs are aimed at inhibiting these key regulators of T cells in order to potentiate immune response against cancer cells. Thus, the novel mAbs were firstly tested for their ability to induce the activation and proliferation of immune cells. To this aim, we evaluated the levels of IL-2 and IFN-γ released by unfractionated human or mouse PBMCs stimulated with Staphylococcal Enterotoxin B (SEB) or Phytohaemagglutinin (PHA) at the concentrations of 50 ng/mL and 2.5 µg/mL respectively, in the absence or presence of the novel anti-PD-L1 or anti-CTLA-4 mAbs for 66 h. In parallel assays, as positive controls, we treated the stimulated lymphocytes with the clinically validated anti-PD-1 (Nivolumab), anti-PD-L1 (Atezolizumab), anti-CTLA-4 (Ipilimumab) antibodies or an unrelated IgG. As shown in Figure 18, PD-L1_1 was found to strongly activate T-cell proliferation and to induce cytokines secretion more efficiently than the clinically validated antibodies Nivolumab and Atezolizumab. Similarly, ID-8 activated the hPBMCs more efficiently than Ipilimumab (Figure 19A), whereas ID-1 showed a similar activity to that of Ipilimumab. As expected, ID-5, used as negative control, showed a poor activity on the stimulation of hPBMCs compared to that of the other two novel mAbs, thus it has been discarded and not used for further characterization. Interestingly, both ID-1 and ID-8 antibodies efficiently activated the IL-2 and IFN-γ cytokines secretion also by stimulated mouse PBMCs (see Figure 19B), confirming their cross-reactivity for the mouse receptor and the possibility to use them in both human and mouse species**[Passariello M., *et al.* 2020]**. Since previous data reported the expression of CTLA-4 not only on CD3+ T cells but also on NK cells, we further investigated on the effects of the novel mAbs on this immune cell population, by using them separately in distinct assays. To this aim, we analysed the biological efficacy of ID-1, ID-8, or Ipilimumab, used as a positive control, on a fraction

of lymphocytes enriched in NK cells isolated from hPBMCs by using the NK Cell Isolation Kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) by following the manufacturer's guidelines. The NK cells were stimulated with SEB (50 ng/mL) for 66 h in the absence or in the presence of the mAbs (0.5–50 nM). As shown in Figure 19C, the novel mAbs led to a slightly stronger activation of the NK cell population than Ipilimumab, by inducing a more efficient secretion of both IL-2 and IFN-γ cytokines, in particular when ID-8 was compared with Ipilimumab. Furthermore, the effects of the novel mAbs and those of Ipilimumab on NK cells are comparable to those observed on unfractionated hPBMCs, confirming the important role of NK cells in the immune responses mediated by anti-CTLA-4 mAbs, such as Ipilimumab**[Passariello M., *et al.* 2020]**.

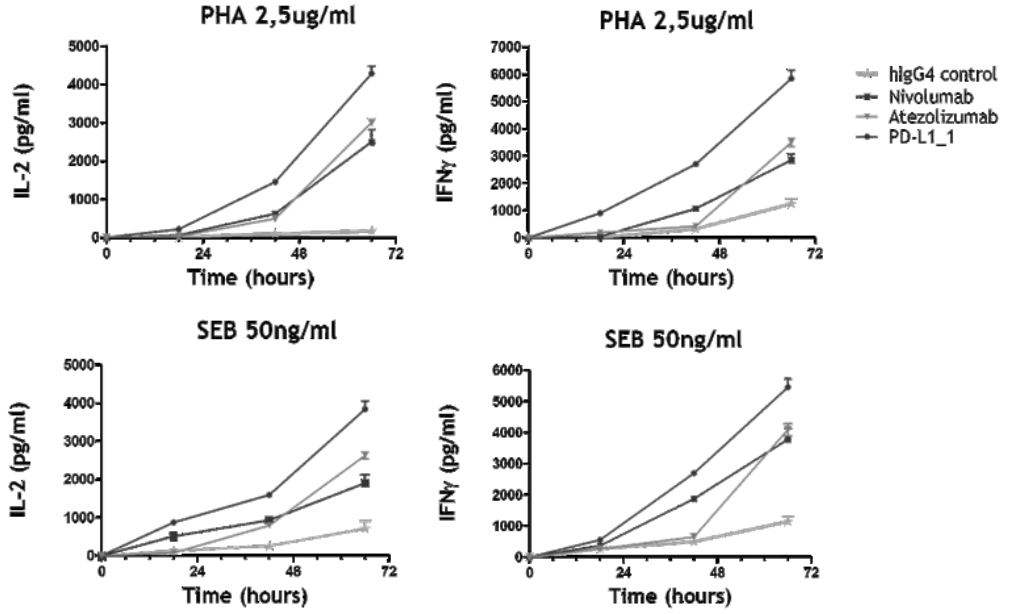

Figure 18. Effects of the novel PD-L1_1 mAb on IL-2 and IFN-γ cytokines secretion by stimulated hPBMCs. Unfractioned human PBMCs were incubated with PD-L1_1 (circles), the clinically validated Atezolizumab (triangles) or Nivolumab (rhomboids), used as positive controls, or an unrelated human IgG4 mAb (negative control) at increasing concentrations, in the presence of SEB (50 ng/mL) or PHA (2.5 g/mL), for 66 h at 37 °C. The levels of cytokine secretion were evaluated by ELISA assays on supernatants of the treated lymphocytes. Error bars were calculated by considering the results obtained by at least three determinations obtained in three independent experiments. Error bars depicted means ± SD. p-values for the indicated treatments relative to untreated cells, or to the treatment with Ipilimumab when indicated with vertical bars, are: *** $p \leq 0.001$; ** $p < 0.01$; * $p < 0.05$.

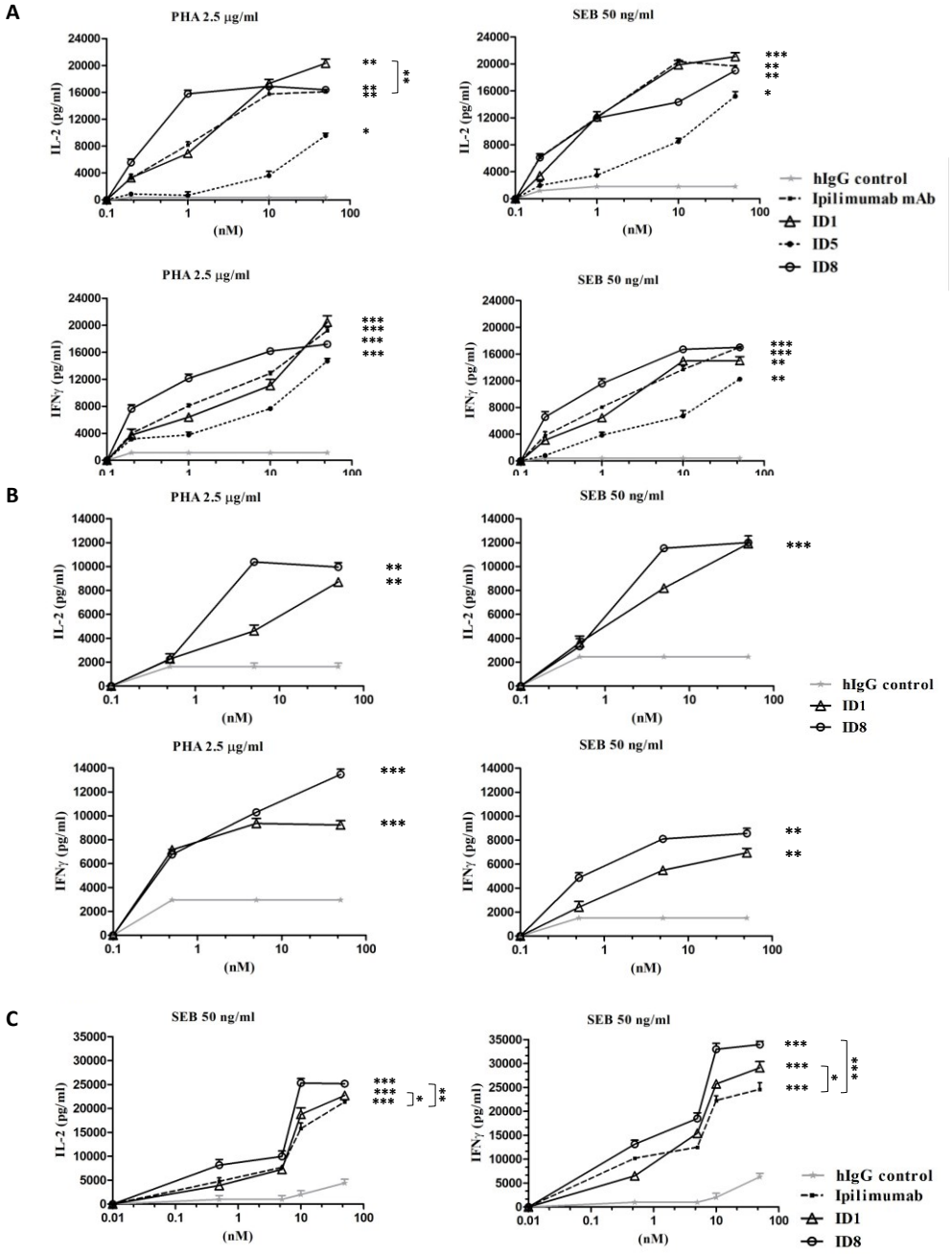

Figure 19. Effects of the novel anti-CTLA-4 mAbs on IL-2 and IFN-γ cytokines secretion by stimulated human or mouse PBMC or NK cells. Unfractioned human (**A**) or mouse (**B**) PBMCs or natural killer (NK) cells (**C**) were incubated with ID-1 (triangles), ID-8 (circles), or ID-5 (rhomboids) mAbs at increasing concentrations (0.5–50 nM), in the presence of SEB (50 ng/mL) or PHA (2.5 g/mL), for 66 h at 37 °C. The levels of cytokine secretion were evaluated by ELISA assays on supernatants of the treated lymphocytes. Ipilimumab or an unrelated IgG antibody were used as positive or negative controls, respectively. Error bars were calculated by considering the results obtained by at least three determinations obtained in three independent experiments. Error bars depicted means ± SD. p-values for the indicated treatments relative to untreated cells, or to the treatment with Ipilimumab when indicated with vertical bars, are: *** $p \leq 0.001$; ** $p < 0.01$; * $p < 0.05$. **[Passariello M., *et al. Cancers* 2020]**

4.4 Binding and effects of the novel immunomodulatory mAbs on tumor cells

Due to the ability of the novel isolated PD-L1_1 mAb to recognize with high affinity and specificity PD-L1 IC, not only as a purified protein but also in its native conformation, we tested its binding to PD-L1 displayed on breast tumor cells and its effects on cancer cell viability. To this aim, we used both TNBC MDA-MB-231 and ErbB2-positive breast cancer SK-BR-3 cell lines expressing satisfactory levels of PD-L1 on their surface. The MCF-7 mammary cell line, expressing low levels of PD-L1 protein, was used as a negative control. As shown in Figure 20A, PD-L1_1 selectively binds to PD-L1-positive tumor cells with an affinity comparable to that previously observed on lymphocytes, whereas only a poor binding was observed on MCF-7 cells, thus confirming its binding specificity for the target cells with a positive correlation between the level of expression of PD-L1 on the cells and the extent of binding of PD-L1_1 to those cells **[Passariello M., *et al.* 2019]**. In order to investigate the *in vitro* effects of this mAb on breast tumor cells, PD-L1_1 was tested at increasing concentrations (50–200 nM) on mammary SK-BR-3 and MDA-MB-231 cells for 72 hours at 37 °C in the absence of lymphocytes. As a control, PD-L1_1 was also tested in parallel, in the same conditions, on PD-L1-negative MCF-7 breast cancer cells. As shown in Fig. 20A , PD-L1_1 significantly inhibited the growth of both the PD-L1-positive cell lines in a dose dependent-manner, whereas no effects were observed on the viability of MCF-7 cells, thus confirming the specificity of its biological effects**[Passariello M., *et al.* 2019]**. In order to compare the biological anti-tumor activity of PD-L1_1 with that of the FDA approved anti-PD-L1 mAb Atezolizumab, we tested them in parallel assays at the dose of 100 nM on the indicated breast cancer cells (Fig 20B), by using as a negative control an unrelated IgG4 isotype antibody. As a further positive control, two variants of PD-L1_1 with higher affinity for PD-L1, called 10_3 and 10_12 were tested in parallel assays. These affinity-matured anti-PD-L1 antibodies were obtained by yeast surface display FACS-based methodology coupled with a CDR-targeted mutagenesis protocol applied to a single CDR in the heavy chain of PD-L1_1 **[Cembrola B., *et al.* 2019]**. Accordingly, they were found able to strongly inhibit the growth of both the tumor cell lines, with effects even more potent than those of the parental PD-L1_1 and Atezolizumab**[Passariello M., *et al.* 2019]**.

Similarly, since recent reports have evidenced the expression of CTLA-4 not only on immune cells but also on the surface of tumor cells **[Mao H., *et al.* 2010; Contardi E., *et al.* 2005; Zhang H., *et al.* 2019]**, we investigated the effects of the human anti-CTLA-4 mAbs on tumor cell lines expressing different levels of this antigen to verify whether they showed also a direct anti-tumor cell activity independent from the immune response. Firstly, we analyzed by ELISA assays the expression levels of CTLA-4 on mammary and prostate tumor by measuring the corresponding binding of the antibodies. As shown in Figure 21A, all the three anti-CTLA-4 mAbs, ID-1, ID-8, and Ipilimumab bound to SK-BR-3 and LNCaP cells, thus confirming CTLA-4 expression on these cells, whereas

no significant binding was observed on MCF-7 cells, used as a negative control (data not shown). Interestingly, ID-1 showed a higher binding affinity than Ipilimumab for the CTLA-4-positive tumor cells.We then tested the effects of the novel mAbs in comparison with Ipilimumab on tumor cell viability of SK-BR-3 and LNCaP cells when incubated at increasing concentrations for 72 h. As shown in Figure 21B, the three antibodies inhibited the growth of both CTLA-4-positive tumor cells also independently from the immune system. As expected, the novel antibodies and Ipilimumab showed no significant effects on MCF-7 tumor cells expressing very low levels of the two antigens, and thus used as negative control (data not shown)**[Passariello M., *et al.* 2020]**.

These results clearly indicate that the ICs PD-L1 and CTLA-4 are expressed also on tumor cells and thus likely exert an additional role in tumor cell survival, as the antibodies directed against them can inhibit tumor growth also in the absence of immune cells.

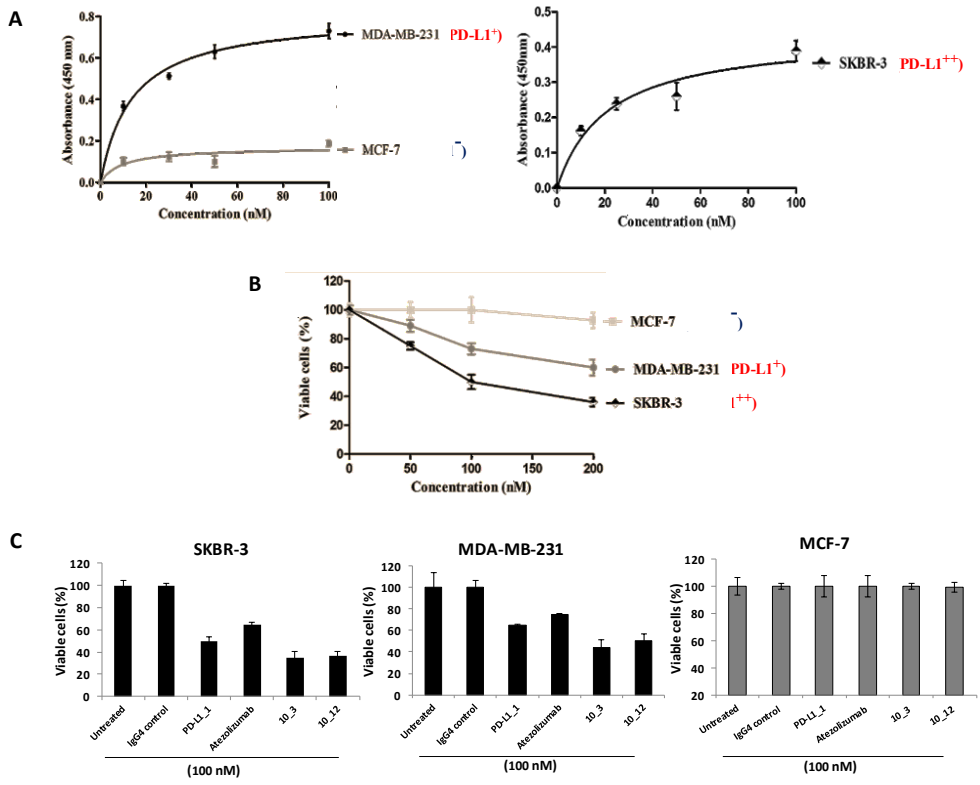

Figure 20. Binding of the novel isolated anti-PD-L1 mAb to tumor cells and its effects on tumor growth. (**A**) Binding curves of PD-L1_1 mAb tested by ELISA assays at increasing concentrations on SK-BR-3, MDA-MB-231 and MCF-7 tumor cells. (**B**) Anti-tumor effects of PD-L1_1 on SK-BR-3, MDA-MB-231 and MCF-7 tumor cells treated with increasing concentrations of the mAb for 72 h at 37 °C. (**C**) Anti-tumor effects of PD-L1_1 and its high affinity variants 10_12 or 10_3 mAbs on SK-BR-3, MDA-MB-231 and MCF-7 tumor cells treated at 100 nM for 72 h at 37 C. The percentage of viable cells is expressed with respect to untreated cells. Cells treated with an unrelated IgG are the negative control, whereas Atezolizumab was used as positive control. Error bars depict means ±SD. p-values for the indicated treatments relative to untreated cells are: ** $p < 0.01$; * $p < 0.05$.

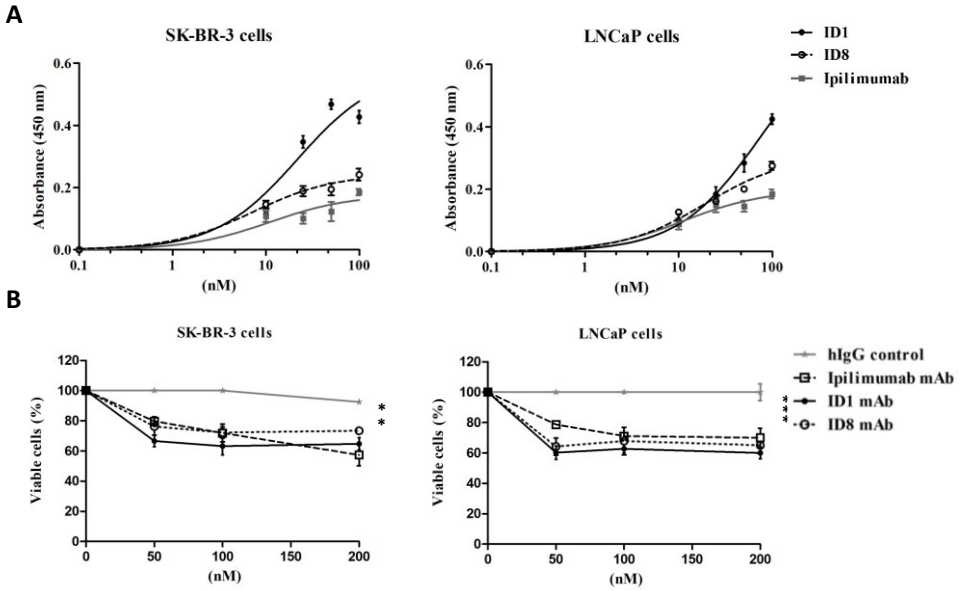

Figure 21. Binding of the novel isolated anti-CTLA-4 mAbs to tumor cells and their effects on tumor growth. (**A**) Binding curves of ID-1, ID-8, or Ipilimumab mAbs tested by ELISA assays at increasing concentrations on SK-BR-3 and LNCaP tumor cells. (**B**) Anti-tumor effects of ID-1, ID-8 or Ipilimumab mAbs on SK-BR-3 and LNCaP cells treated with increasing concentrations for 72 h at 37 °C. The percentage of viable cells is expressed with respect to untreated cells. Cells untreated or treated with an unrelated IgG were used as negative controls. Error bars depict means ± SD. p-values for the indicated treatments relative to untreated cells are: ** $p < 0.01$; * $p < 0.05$. **[Passariello M., *et al. Cancers* 2020]**

4.5 Tumor growth inhibition by anti-PD-L1 mAbs directly affect intracellular pathways downstream PD-L1

In order to shed light on the intracellular pathways affected by PD-L1_1 antibody involved in the inhibition of tumor cell proliferation, we performed Western blotting analyses of extracts from breast SK-BR-3 and triple negative MDA-MB-231 tumor cells treated for 72 hours at 37 °C in the absence or in the presence of PD-L1_1, used at the concentration of 200 nM. As shown in Figure 22, the phosphorylation of Erk, P38 and JNK proteins significantly decreased when both SK-BR-3 and MDA-MB-231(data not shown) cells were treated with PD-L1_1 compared to untreated cells. No significant effects were observed on the total amount of Erk, P38 and JNK (data not shown), as well as no significant effects were observed on both the levels of p-Akt and total Akt (data not shown).

Atezolizumab, used as a positive control on SK-BR-3 tumor cells, showed similar effects on p-Erk, but, differently from the novel anti-PD-L1 mAb, did not affect the level of p-JNK, and showed only a slight effect on p-P38 (Fig.22). As additional controls, the two high affinity variants of PD-L1_1, 10_3

and 10_12, were tested in parallel assays on SK-BR-3 or MDA-MB-231 tumor cells. After treatments with 10_3 and 10_12, the phosphorylation levels of Erk, P38 and JNK were found to be strongly reduced by these variants (Fig. 22), that showed a more potent effect than the parental PD-L1_1 mAb in both tumor cell lines**[Passariello M., *et al.* 2019]**.

When tested on MCF-7 cells, used as a negative control, PD-L1_1 and its high affinity variants did not show significant effects (data not shown), as expected. However, the effects obtained with the two variants of PD-L1_1 were more marked on SK-BR-3 cells compared to those observed on MDA-MB-231 cells, in line with the results on tumor cell viability **[Passariello M., *et al.* 2019]**, in which 10_3 and 10_12 inhibited tumor cell growth of SK-BR-3 tumor cells more efficiently compared to MDA-MB-231 tumor cells. Furthermore, we demonstrated here for the first time that in cancer cells the intracellular pathways downstream PD-L1 IC, involving MAPKs, are inhibited by anti-PD-L1 mAbs, even though further investigations are still needed to better clarify their mechanisms of action. To confirm this hypothesis on the role of PD-L1 on tumor cells, we tested also the effects of PD-L1_1 in parrallel with a commercially available anti-mouse PD-L1 mAb (clone 10F.9G2, BioXcell), previously validated *in vivo*, on these pathways in PD-L1-positive colon CT26 tumors *in vivo*. To this aim, mice were implanted with CT26 cells (day 0) and then treated with PD-L1_1 or an anti-mouse PD-L1 antibody (200 μg ip, clone 10F.9G2, BioXcell) reacting against murine PD-L1 (day 3, 6, 10). While the growth rate of tumors in untreated mice was very fast and uncontrolled, with the majority of tumors reaching sizes of >650 mm^3 at day 21, a drastic reduction in tumor volume was found in mice treated with α-mPD-L1 ($p = 0.02$), and similar effects were observed in mice treated with PD-L1_1, as expected (Fig 23A.). During the period of treatment, the animals did not show significant changes of weight or other visible signs of toxicity**[Passariello M., *et al.* 2019]**.

We then investigated on the effects of PD-L1_1 and α-mPD-L1 treatments by analyzing the activation of MAPK proteins in treated tumors. Cell extracts, obtained from tumors removed at the end of the experiment on day 21, were processed as described in Methods and then analyzed by Western Blotting. As shown in Fig. 23B, the anti-PD-L1 antibodies not only inhibited the phosphorylation/activation of MAPK and JNK, but also induced the cleavage of caspase-3 more efficiently if compared to untreated tumor cells. Hence, these results confirmed the previous association of PD-L1 protein to these pathways already observed *in vitro*, and mentioned above.

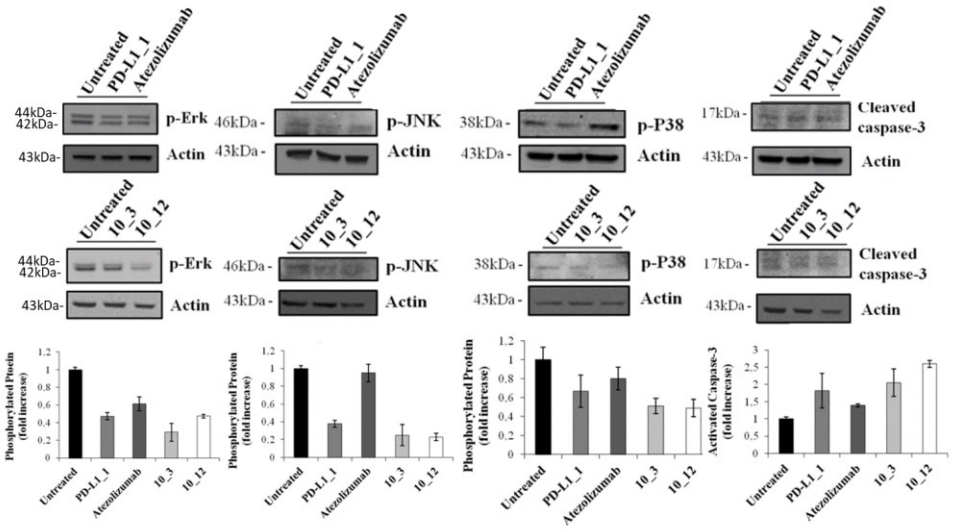

Figure 22. Effects of PD-L1_1 on the pathways associated with PD-L1 in tumor cells. Western blotting analyses with the specific antibodies for the indicated proteins of extracts from SK-BR-3 tumor cells treated in the absence or in the presence of PD-L1_1 or its high affinity variants. Atezolizumab was used as a positive control. Protein levels are also expressed as fold increase with respect to those observed in untreated cells and normalized to actin. Error bars depicted means ± SD. P values for the indicated mAbs relative to untreated cells, are: ***P ≤ 0.001; **P < 0.01; *P < 0.05. **[Passariello M., *et. al. Sci. Rep.* 2019]**

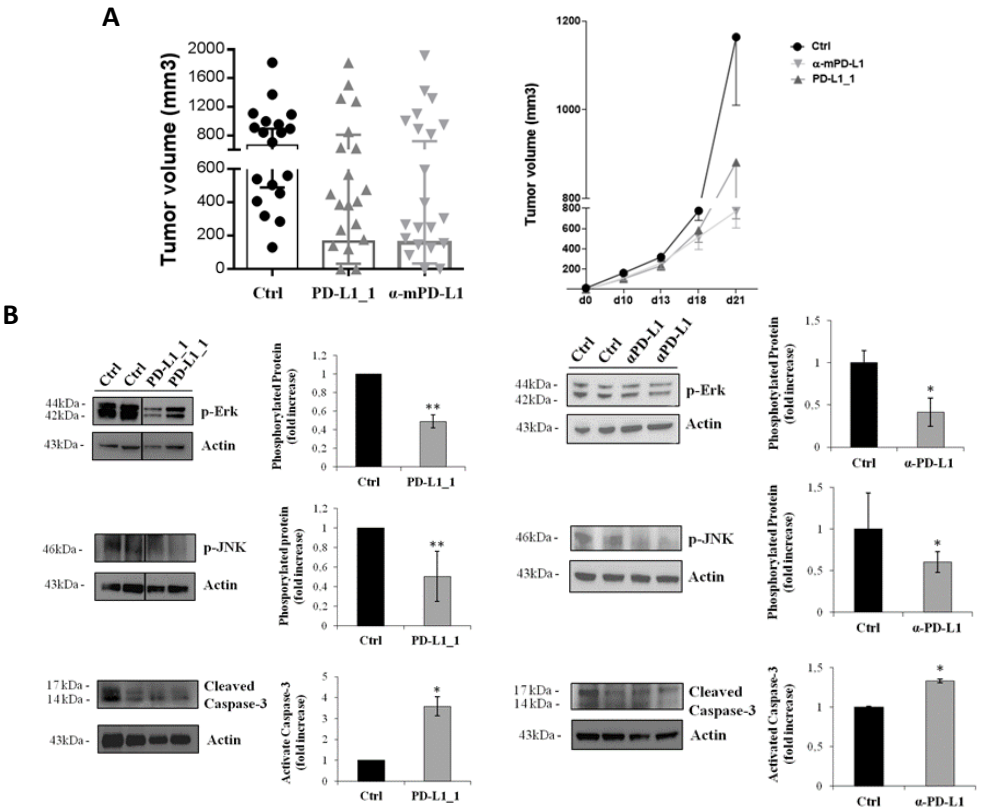

Figure 23. Effects of anti-PDL1 mAbs on tumor growth and signal transduction in CT26 tumors in vivo. (**A**) Tumor growth in mice inoculated sc with CT26 cells at day 0 and left untreated (black) or treated with PD-L1_1 (grey) or α-mPD-L1 (light grey). Shown is tumor volume for individual mice (n = 20) at day 21 (left panel) and mean of tumor volume for each group over time (right panel), *p = 0.02. (**B**) Western blotting was performed on cell extracts from tumor specimens of two mice killed on day 21 from untreated groups or from responder groups treated with PD-L1_1 or with α-mPD-L1, as described. Protein levels are also expressed as fold increase with respect to those observed in untreated mice and normalized to actin. Error bars depicted means ± SD. P values for the indicated mAbs relative to cell extracts from untreated groups, are: ***P ≤ 0.001; **P < 0.01; *P < 0.05. **[Passariello M., *et al. Sci. Rep.* 2019]**

4.6 Effects of the novel anti-CTLA-4 mAbs on intracellular pathways downstream CTLA-4 in tumor and NK cells

Considering the *in vitro* anti-tumor effects of the novel isolated anti-CTLA-4 mAbs described above, we hypothesised that these mAbs could act directly on cancer cells by affecting intracellular pathways similarly to the anti-PD-L1 mAbs. In order to investigate the molecular basis of these effects, we analyzed the intracellular pathways downstream CTLA-4 in SK-BR-3 and LNCaP treated cells by Western blotting analyses of cell extracts with anti-CTLA-4, anti-pTyr, anti-pAkt, and anti-caspase 3 antibodies. As shown in Figure 24, both Ipilimumab and ID-1 are able to induce a marked phosphorylation of the monomeric form of CTLA-4 (25 kDa) probably acting

on downstream intracellular pathways, but ID-1 mAb also inhibits the phosphorylation of Akt and induces the cleavage of caspase 3 more effciently than Ipilimumab. We cannot exclude that Ipilimumab affects other unknown downstream pathways involved in the cell growth inhibition observed in Figure 21. These results suggest that the novel mAb can directly affect the CTLA-4 function on tumor cells by inhibiting the downstream survival pathways, such as that of PI3K, and by inducing apoptosis through Akt downregulation, thus acting as an agonistic antibody on tumor cells **[Passariello M., *et al.* 2020]**. Indeed, ID-1 has a similar effect of apoptosis induction exerted by the CD-80 and CD-86 ligands on these tumor cells (data not shown), accordingly with previous results reported in literature **[Contardi E., *et al.* 2005]**.

Similar results were obtained also with ID-8 (data not shown), which in addition induces a more marked homodimerization of CTLA-4 (Figure 25, the arrows indicate the corresponding dimeric and monomeric forms in the blots), thus increasing the receptor level on the cell surface, in line with the hyperphosphorylation of the monomeric form of CTLA-4, which is required to avoid its internalization and the subsequent degradation **[Bradshaw J.D., *et al.* 1997]**. The resulting increase of the dimeric form of CTLA-4 could be responsible for the inhibition of ERK due to the recruitment of phosphatases such as PP2A **[Passariello M., *et al.* 2020]**.

Considering that CTLA-4 is mainly expressed on immune cells and that anti-CTLA-4 mAbs can affect the proliferation of lymphocytes, we decided to investigate on the intracellular pathways of a subpopulation of lymphocytes enriched with NK cells. To this aim, NK activated cells were treated with the anti-CTLA-4 mAbs for 66 h and then cell extracts were analysed by Western blotting. As shown in Figure 26, we found that the three antibodies induce the phosphorylation of Erk and Akt, and inhibit the cleavage of caspase 3, thus indicating that on NK cells they have a completely opposite effect with respect to those observed on cancer cells, activating the proliferation and survival pathways and inhibiting apoptosis. The novel ID-1 and ID-8 mAbs induce on NK cells a more marked phosphorylation of the monomeric form (25 kDa) of CTLA-4, with respect to Ipilimumab, thus suggesting that this event could likely induce the activation of the pathways downstream CTLA-4**[Passariello M., *et al.* 2020]**. Indeed, Ipilimumab did not affect the level of pAkt and cleaved caspase and showed only slight effects on the activation of Erk, thus suggesting that the novel antibodies could have a different mechanism of action with respect to the clinically validated anti-CTLA-4 mAb.

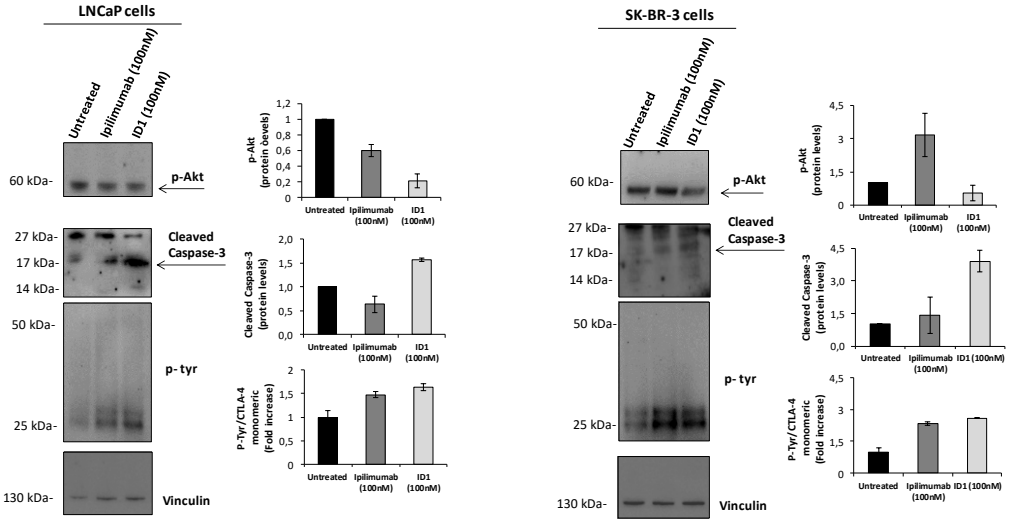

Figure 24. Effects of ID-1 on intracellular pathway downstream CTLA-4 in tumor cells. Western blotting analyses with the antibodies specific for the indicated proteins of cell extracts from LNCaP and SK-BR-3 tumor cells, treated in the absence or in the presence of Ipilimumab or ID-1 mAb for 72 h. Protein levels are expressed as fold increase with respect to untreated cells and normalized to vinculin. **[Passariello M., *et al. Cancers* 2020]**

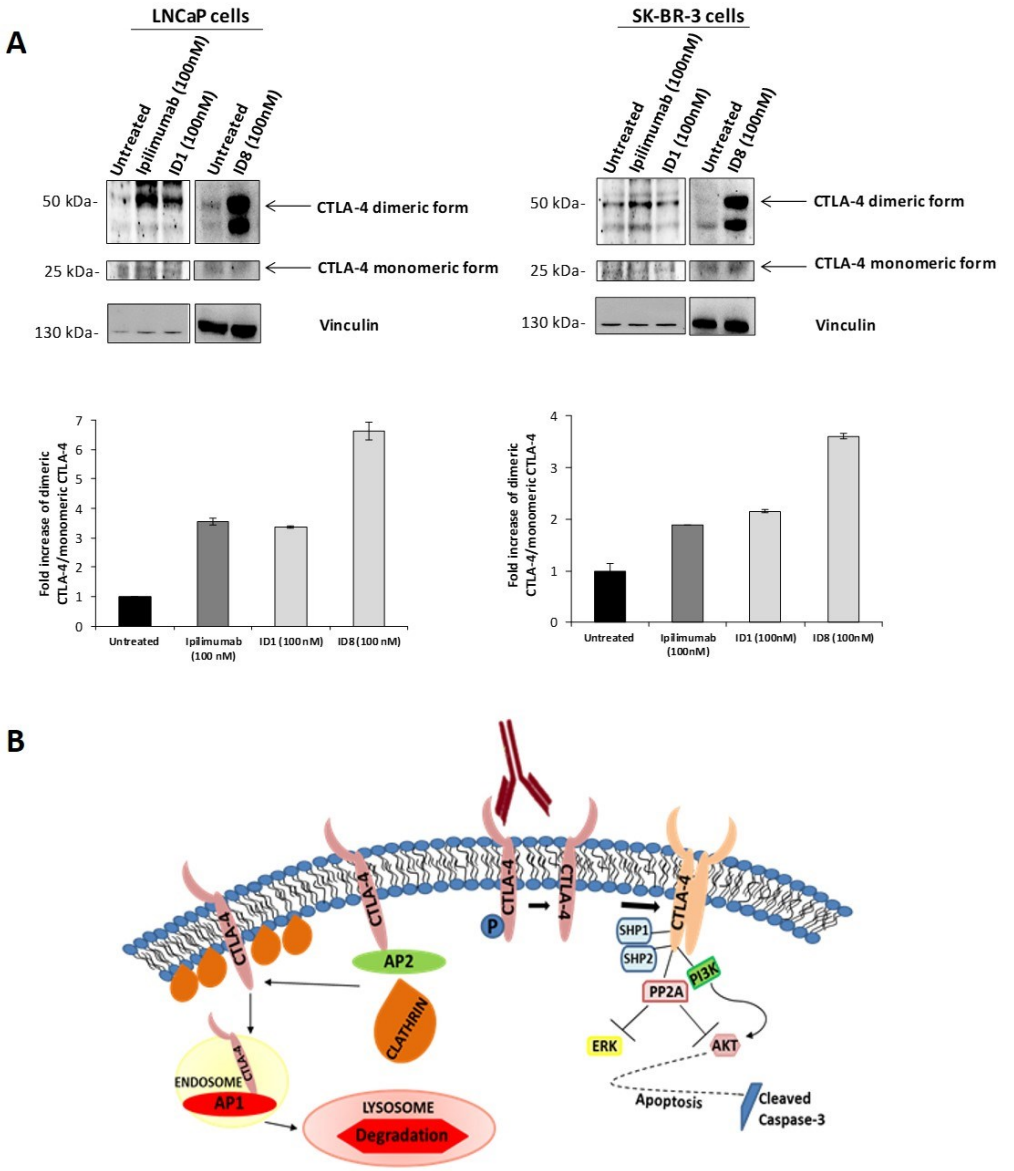

Figure 25. Effects of anti-CTLA-4 mAbs on monomeric or dimeric CTLA-4 form in tumor cells. (**A**) Western blotting analyses with the antibodies specific for CTLA-4 protein of cell extracts from LNCaP and SK-BR-3 tumor cells, treated in the absence or in the presence of Ipilimumab, ID-1 or ID-8 mAb for 72 h. Protein levels are expressed as fold increase of dimeric vs. monomeric form (see arrows) with respect to untreated cells and normalized to vinculin. (**B**) Model proposed to explain the effects of the novel mAbs on CTLA-4 expressed on tumor cells and its downstream intracellular pathways. **[Passariello M., *et al.* *Cancers* 2020]**

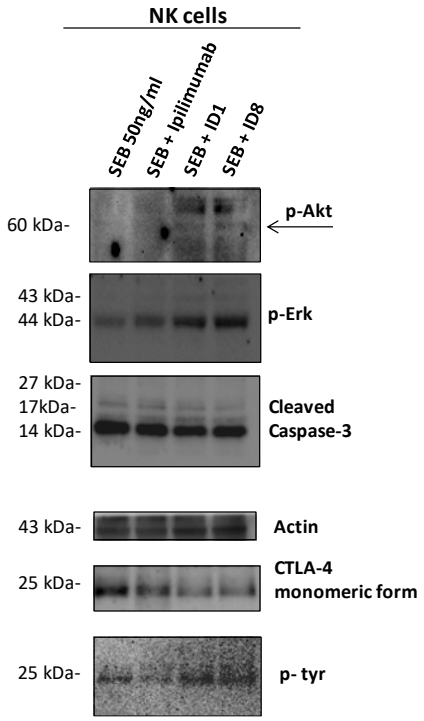

Figure 26. Effects of the novel anti-CTLA-4 mAbs on intracellular pathways downstream CTLA-4 in NK cells. Western blotting analyses of cell extracts from NK cells, treated with ID-1, ID-8, or Ipilimumab, under stimulation with SEB (50 ng/mL) for 66 h. The staining was performed with the antibodies specific for p-Akt, p-Erk, caspase 3 and actin or CTLA-4 and p-tyr. Protein levels are expressed as fold increase with respect to untreated cells and normalized to actin. **[Passariello M., *et al. Cancers* 2020]**

4.7 Effects of immunomodulatory mAbs targeting PD-L1, PD-1 and CTLA-4 on the expression of ICs and their crosstalk on tumor cells

Since many ongoing clinical trials are evaluating the efficacy of combinatorial treatments involving immunomodulatory mAbs on different types of cancer, we decided to investigate the effects of anti-PD-1, anti-PD-L1 and anti-CTLA-4 immunomodulatory mAbs and their combinations not only on breast but also on lung cancer cell lines. To this aim, we first checked the levels of expression of PD-1, PD-L1 and CTLA-4 ICs on MDA-MB-231 and BT-549 breast cancer cells, and A-549 lung cancer cells by cell ELISA to measure the levels of these ICs exposed on the cell surface in order to choose those suitable for combinatorial treatments. We also analyzed by Western blotting the total

amounts of the proteins by using the commercial anti-PD-1, anti-PD-L1 and anti-CTLA-4 mAbs (see Figure 27A and B).

These tumor cell lines express satisfactory levels of the selected ICs, in particular the expression levels of PD-L1 are comparable among the three indicated cell lines, whereas the levels of PD-1 are much higher in A-549 cells with respect to the other two cell lines (Figure 27). MDA-MB-231 cells express the highest level of CTLA-4, which is present in both dimeric and monomeric forms, as previously reported **[Passariello M., *et al.* 2020]**, whereas A-549 cells seem to express the lowest levels of CTLA-4 on the cell surface (Figure 27A) **[Vetrei C., *et al.* 2021]**. Once confirmed the significant expression of the ICs of interest in two out of three cell lines, we analyzed the effects of the human monoclonal antibodies, currently in clinical use for cancer treatment, Nivolumab, Ipilimumab (anti-PD-1 and anti-CTLA-4 respectively) and of the novel human anti-PD-L1 (10_12 and PD-L1_1), anti-PD-1 (PD-1_1) or anti-CTLA-4 (ID1) immunomodulatory mAbs, previously generated in our laboratory. Considering the previous results on tumor cell growth inhibition induced by anti-PD-L1 and anti-CTLA-4 mAbs, we treated the three cancer cell lines with the novel generated PD-L1_1, 10_12, PD-1_1, or ID-1 for 72 h at 100 nM, to compare their biological activity to those of the clinically validated Nivolumab or Ipilimumab. As expected, all the tested mAbs significantly affected tumor cell viability. The strongest effect was observed on BT-549 where PD-1_1, ID-1, 10_12 and PD-L1_1 reached more than 50 % tumor cell growth inhibition with respect to the untreated cells or the cells treated with an unrelated antibody (see figure 28A).

We decided to further investigate on the molecular bases of these anti-tumor effects induced by the novel immunomodulatory antibodies, even in the absence of immune cells. Thus, we analyzed cell lysates, after treatments of 72 hours with the indicated mAbs at a concentration of 200 nM, focusing on the intracellular pathways and on the eventual crosstalk of PD-1, PD-L1 and CTLA-4 ICs. As negative controls we used in parallel assays untreated cells or cells treated with an unrelated mAb. The anti-CTLA-4 mAbs (Ipilimumab and ID1) were not tested on A-549 cells as this cell line does not seem to express sufficient levels of cell surface CTLA-4, as observed by Cell ELISA (Figure 27B). Surprisingly, we found for the first time an enhanced expression of PD-1 receptor when the tumor cells were treated with anti-PD1, anti-PD-L1 or anti-CTLA-4 mAbs (Figure 28B), whereas it was confirmed a decreased phosphorylation of Erk (Figure 28B), that could partially explain the reduction of cell viability, found after treatment with all of them (Figure 28A). No significant effects were observed on Akt phosphorylation or cleavage of caspase-3 (Figure 28B and 29A) by the treatments with mAbs, with the exception of 10_12 (the anti-PD-L1 mAb) which already showed effects on caspase 3 cleavage in MDA-MB-231, as mentioned above **[Vetrei C., *et al.* 2021]**.

To better clarify the role of the increased expression of PD-1, found especially when the cells were treated with the antibodies against PD-1 (Nivolumab) and PD-L1 (10_12), we decided to analyze the cell extracts of tumor cells treated with PD-1 or PD-L1 agonists (PD-1/Fc or PD-L1/Fc chimeric proteins) for 72 h, in order to compare their effects to those obtained by their respective antagonists, Nivolumab or 10_12 mAbs. As shown in Figure 29A, the agonists show opposite effects on PD-1 receptor levels by significantly reducing its expression in treated tumor cells. The antagonistic mAbs induced again not only an increased expression of PD-1 but also an increased phosphorylation of NF-kB transcription factor, which is reported in the literature **[Antonangeli F., *et al.* 2020]** to be involved both directly and indirectly in PD-L1 expression on tumor cells (Figure 29A) **[Vetrei C., *et al.* 2021]**.

These results led us to hypothesize that in tumor cells could occur a crosstalk between PD-1 receptor and its ligand PD-L1 to support tumor cell survival and, in case the PD-1/PD-L1 cis-interaction is blocked, a compensatory increased expression of both the proteins occurs. Indeed, when the PD-L1 or PD-1 induced signaling is affected, as in the case of treatment with antagonistic mAbs, the tumor cells seem to perceive this ligand/receptor unavailability and respond by activating NF-kB hyperphosphorylation, provoking its translocation into the nucleus and likely PD-1, PD-L1 or CSN5 transcription (see Figure 29B) **[Vetrei C., *et al.* 2021]**. NF-kB activity seems to coordinate the expression of both these two ICs, PD-1 and its ligand PD-L1. To test the eventual involvement of mammalian target of rapamycin (mTOR) kinase protein, we further checked the effects of PD-1/PD-L1 agonists and antagonists on its phosphorylation and level of expression, but no relevant effects were observed. Unexpectedly, in parallel to the enhanced expression of PD-1 receptor and its ligand, we also observed a significant increase of CTLA-4 expression on the indicated tumor cell lines, after treatment with the antagonists Nivolumab and 10_12. Again an opposite effect (decreased expression of CTLA-4) was found under treatments with PD-1/PD-L1 agonists (see Figure 29A). These findings suggest the additional involvement of CTLA-4 receptor in the crosstalk existing between PD-1 and PD-L1 ICs, like a common thread of coordination to respond to the effects of the antagonistic or agonistic compounds of PD-1/PD-L1 axis also in tumor cells.

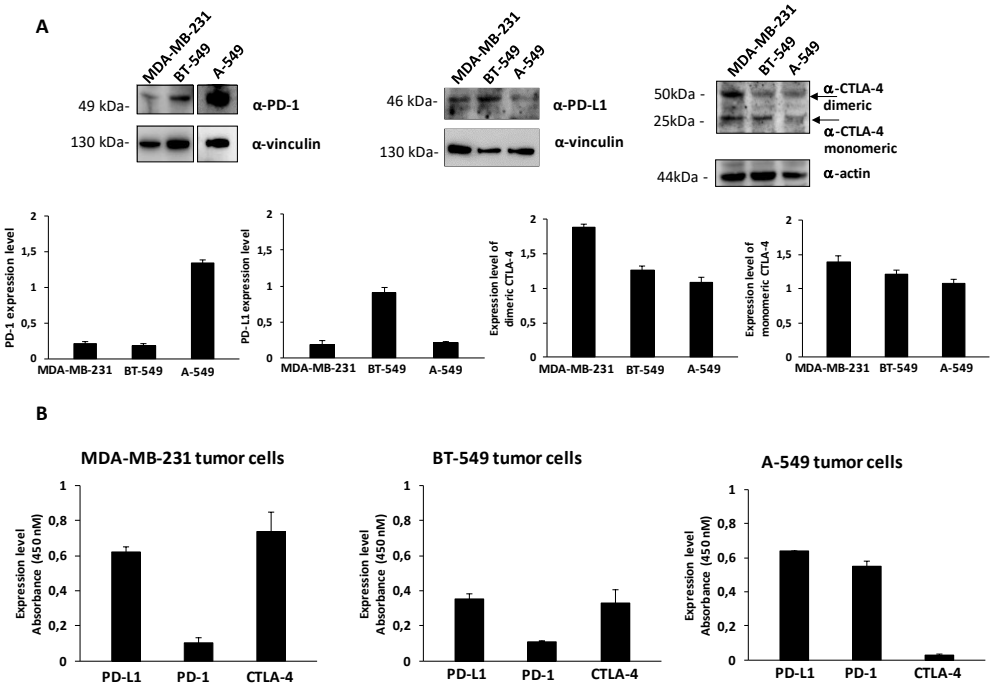

Figure 27. Expression of PD-1, PD-L1 and CTLA-4 ICs on cancer cells. (**A**) Western Blotting analyses of extracts from MDA-MB-231, BT-549 and A-549 tumor cells, by using the commercial anti-PD-L1, anti-PD-1 or anti-CTLA-4 mAbs; the arrows indicate the dimeric and monomeric forms of CTLA-4. The intensity of the bands corresponding to ICs was normalized to actin or vinculin and their ratio is reported in the graphics as protein expression levels. (**B**) Cell ELISA assays were performed on whole cells to measure the cell surface expression of ICs with commercial anti-PD-1, anti-PD-L1 or anti-CTLA-4 mAbs on MDA-MB-231, BT-549 or A-549 tumor cells. Binding values were reported as the mean of at least three determinations obtained in three independent experiments. Error bars depicted means ± SD. **[Vetrei C., *et al. Cancers* 2021]**

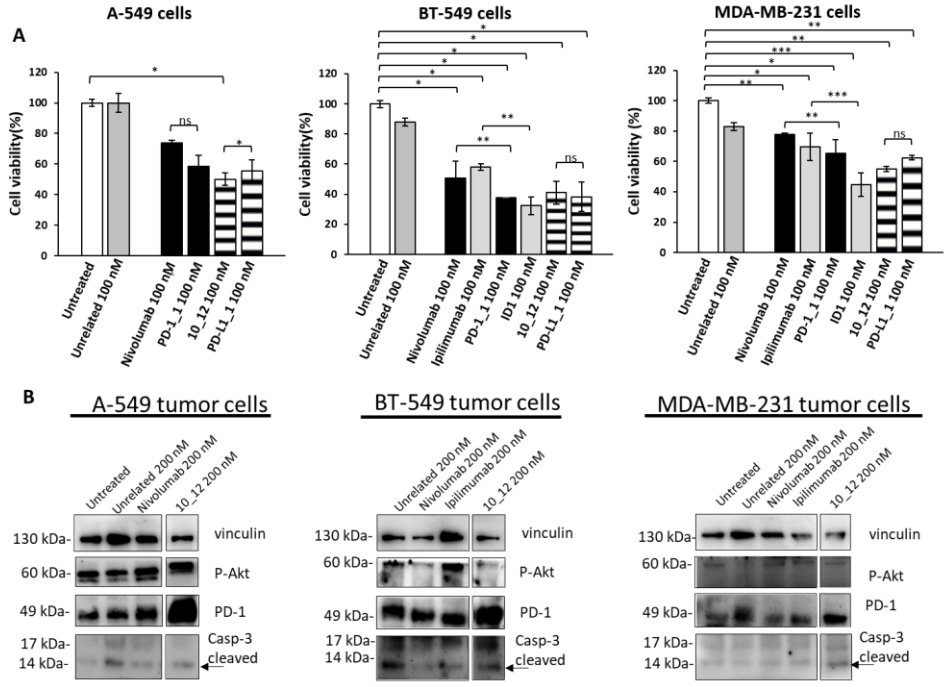

Figure 28. Imunomodulatory mAbs reduce tumor cell viability by affecting intracellular pathways. (**A**) A-549, BT-549 and MDA-MB-231 tumor cells were treated for 72 h with anti-PD-1, anti-PD-L1, anti-CTLA-4 or unrelated (anti-SRB1 or anti-Claudin 1) mAbs at the indicated concentrations. Cell survival is expressed as percentage of viable cells with respect to untreated ones. Cells were counted before and after Trypan Blue exclusion test. The values were reported as the mean of at least three determinations obtained in three independent experiments. Error bars depict means ± SD *** $p \leq 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, not significant. (**B**) Western blotting analyses of extracts from MDA-MB-231, BT-549 or A-549 tumor cells treated for 72 h as indicated. **[Vetrei C., *et al. Cancers* 2021]**

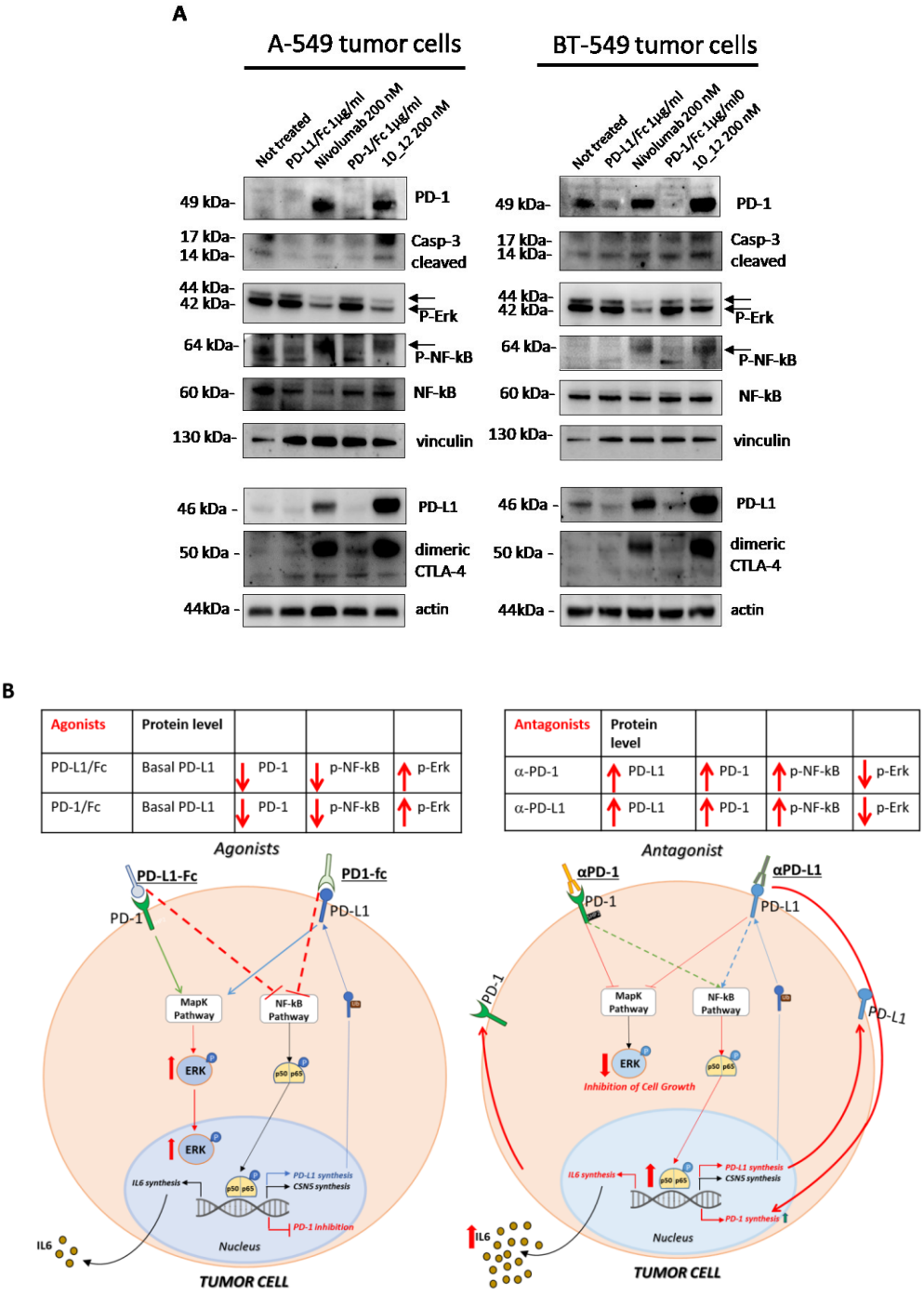

Figure 29. Figure 3. Effects of agonists and antagonists of PD-1 and PD-L1 on tumor cells. (**A**) Western blotting analyses of cell extracts from A-549 or BT-549 tumor cells, treated for 72 h with PD-1/PD-L1 agonists (PD-1/Fc or PD-L1/Fc) or antagonists (Nivolumab or 10_12 mAbs). The intensity of the bands was normalized to vinculin or actin. (**B**) Model proposed to explain the effects of agonists or antagonists of PD-1 and PD-L1 on tumor cells and on their intracellular pathways. **[Vetrei C., *et al. Cancers* 2021]**

4.8 Evaluation of cytotoxic effects of immunomodulatory mAbs and their combinations on co-cultures of hPBMCs and tumor cells

Considering that the most potent anti-tumor activity of immunomodulatory mAbs is obtained by activating tumor infiltrating lymphocytes against cancer cells, we also investigated the anti-tumor effects of the novel anti-PD-1, anti-PD-L1 or anti-CTLA-4 mAbs, used alone or in combination on tumor cells co-cultured with hPBMCs. Thus, BT-549 and MDA-MB-231 TNBC cells, that were found to be the most sensitive to the previous treatments, were cultured in the presence of hPBMCs (Effector:Target cells ratio of 5:1) for 48 h in the absence or presence of single agent treatments or their combination, at a concentration of 100 nM. In parallel assays, co-cultures treated with the commercial anti-CTLA-4 and anti-PD-1 mAbs (Ipilimumab and Nivolumab), or their combination, were used as positive controls, whereas cells untreated or treated with an unrelated human IgG1 mAb were used as negative controls.

The cytotoxic effects induced by the mAbs were detected by measuring the Lactate Dehydrogenase (LDH) release in the supernatant of co-cultures. As shown in Figures 30 and 31, the PD-1_1, PD-L1_1 and ID-1 mAbs, when used as single agents (black and grey bars), showed stronger cytotoxic effects than the clinically validated Nivolumab and Ipilimumab, by inducing an increase of LDH release (~30%) from tumor cells. More interestingly, the cytotoxic effects obtained with the combinations of ID-1 with PD-1_1 or with PD-L1_1 mAb (striped bars) on both the indicated cell lines, were significantly higher than the single agent treatments (see Figures 30A and 31A). These combinations seem to significantly improve not only the anti-tumor effects of each mAb, but they also show more potent anti-tumor effects with respect to the Nivolumab-Ipilimumab combinatorial treatment, especially on BT-549 cell line. Moreover, when we combined Nivolumab with the novel anti-CTLA-4 ID-1 mAb, we observed an enhanced effect on LDH release raising up to 50 and 60% of cell lysis, respectively on MDA-MB-231 and BT-549 tumor cell lines (Figures 30A and 31A), thus indicating the higher efficiency of this combination, compared to the lower effect (about 30%) observed when Nivolumab was combined with Ipilimumab **[Vetrei C., *et al.* 2021]**.

In order to evaluate whether the immunomodulatory antibodies induce tumor cell lysis through the activation of lymphocytes, we then examined the release of IL-2 and IFN-γ. Thus, the supernatant, collected from the co-cultured cells treated as indicated above, was analyzed by ELISA for the detection of IL-2 and IFN-γ. As reported in Figures 30B and 31B, all the immunomodulatory mAbs used alone or in combination, increased the secretion of both cytokines, with respect to co-cultures untreated or treated with an unrelated control mAb (white and light grey bars), thus confirming their ability to stimulate hPBMCs, improving immune responses against cancer. The highest release of IL-2 and

IFN-γ was observed on both the cell lines with the combination of PD-L1_1 and ID-1 (striped bars), at the concentration of 100 nM, which reached levels of IL-2 up to 6000 pg/mL and of IFN-γ up to 4500 pg/mL **[Vetrei C., *et al.* 2021]**.

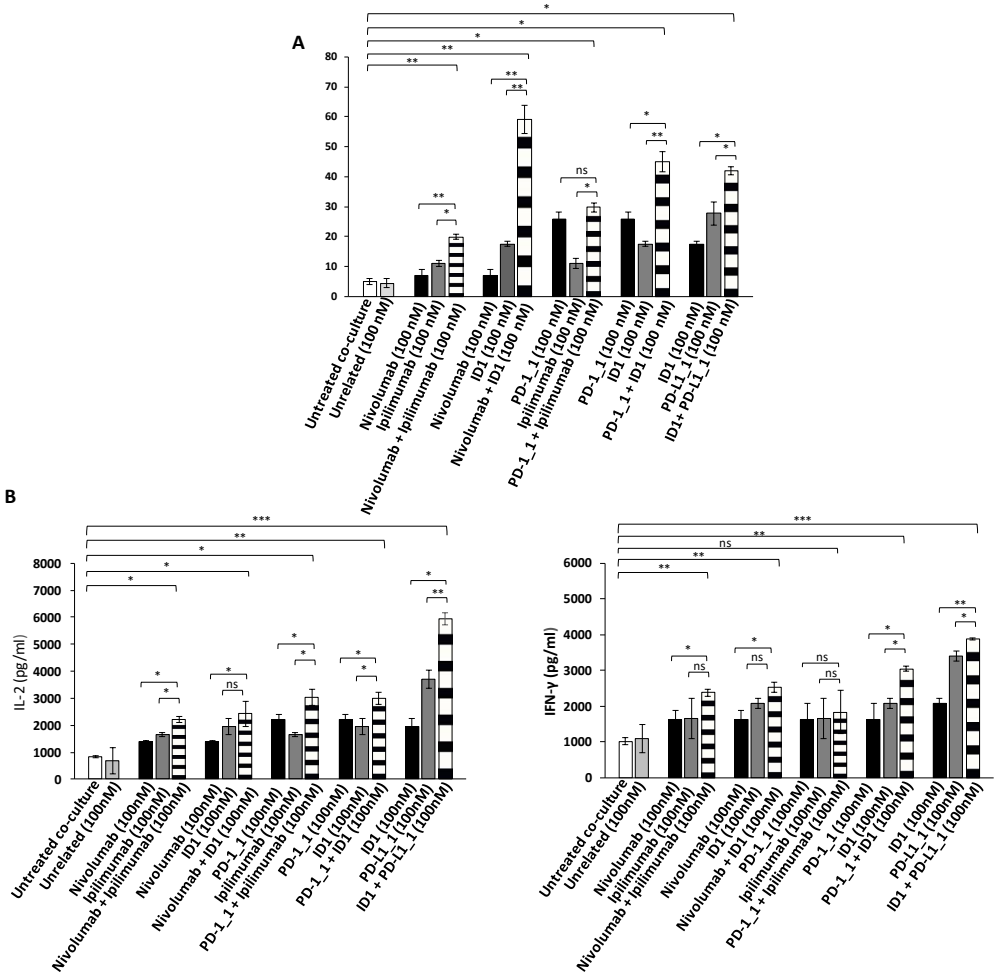

Figure 30. Anti-tumor effects of immunomodulatory mAbs and their combinations on BT-549 tumor cells co-cultured with hPBMCs. (**A**) Cell lysis was measured by evaluating the release of Lactate dehydrogenase (LDH) in the supernatants by BT-549 tumor cells co-cultured with hPBMCs for 48 h in the absence or presence of immunomodulatory mAbs, used alone (black and dark grey bars) or in combination (striped bars), at the indicated concentrations. (**B**) The levels of secreted IL-2 and IFN-γ were measured by ELISA on supernatants of co-cultures treated as indicated. Cytokines are expressed as pg/mL. The values were reported as the mean of at least three determinations obtained in three independent experiments. Error bars depict means SD *** $p \leq 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, not significant. **[Vetrei C., *et al. Cancers* 2021]**

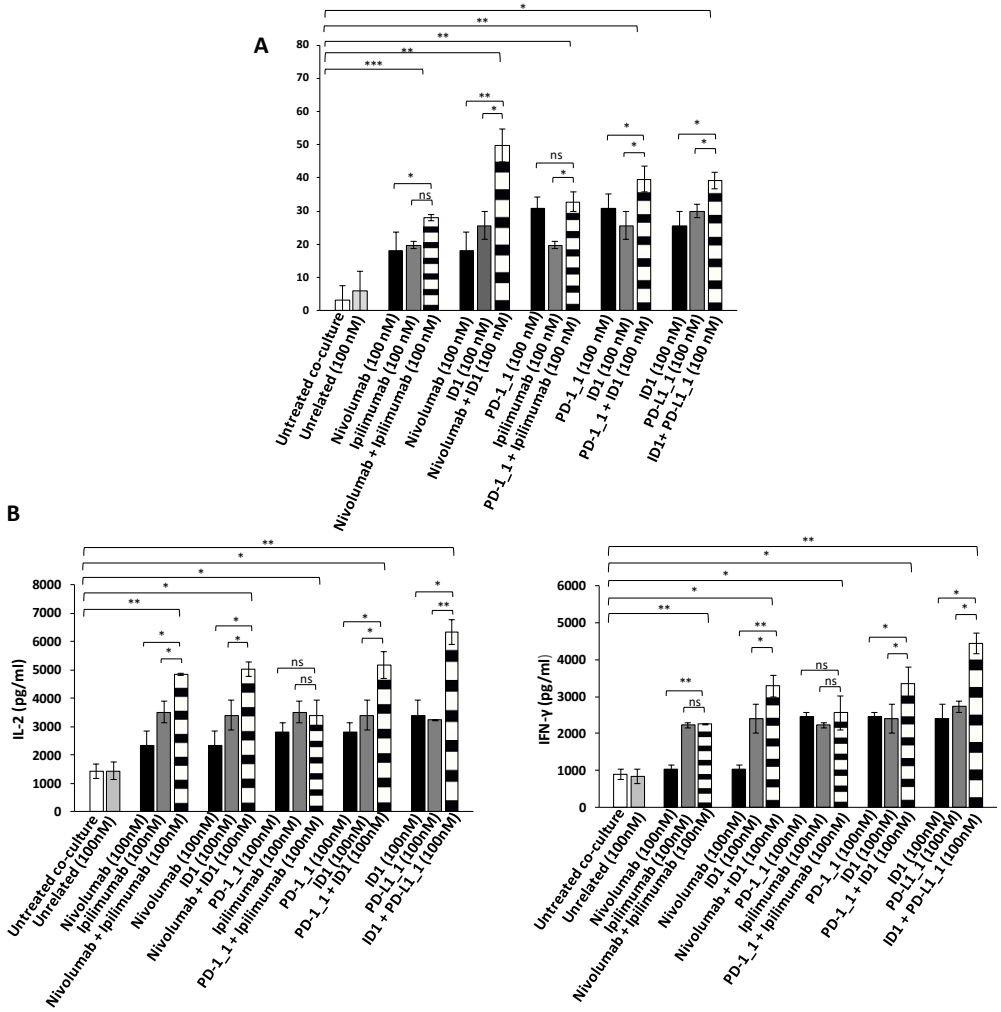

Figure 31. Anti-tumor effects of immunomodulatory mAbs on MDA-MB-231 tumor cells co-cultured with hPBMCs. (**A**) Evaluation of tumor cell lysis by detecting LDH release from MDA-MB-231 tumor cells co-cultured with hPBMCs, for 48 h in the absence or presence of immunomodulatory mAbs, used alone (black and dark grey bars) or in combination (striped bars), at the indicated concentrations. (**B**) Activation of lymphocytes by anti-PD-1, anti-PD-L1 or anti-CTLA-4 mAbs was analyzed by evaluating the secretion of IL-2 and IFN-γ in the supernatant of the treated co-cultures. Cytokine levels in the supernatant are expressed as pg/mL. The values were reported as the mean of at least three determinations obtained in three independent experiments. Error bars depict means ± SD *** $p \leq 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, not significant. **[Vetrei C., *et al. Cancers* 2021]**

4.9 Comparison of the cardiotoxic side effects of novel mAbs with those of the clinically validated Ipilimumab and Nivolumab

Despite the considerable advantages of cancer immunotherapy with respect to conventional therapies, still a wide spectrum of side effects has been reported. Among the more severe adverse events, some cases of myocarditis and pericarditis have been evidenced especially in combinatorial treatments of anti-ICs mAbs**[Larkin J., *et al.* 2019]**. In order to evaluate the safety of the novel PD-1_1, PD-L1_1 and ID-1 mAbs, we investigated their effects on Human Fetal Cardiomyocytes (HFC) co-cultured in the presence of hPBMCs, and compared their anti-tumor effects with eventual cardiac side effects. We firstly measured the levels of expression of PD-1, PD-L1 and CTLA-4 on HFC and then we tested the effects of the mAbs on their growth (Figure 32). As shown in Figure 32B, even though the ICs are expressed also on these cells, no significant effects were observed on HFC cell viability in the absence of immune cells.

To mimic the *in vivo* environment where immunomodulatory mAbs exert their most potent anti-tumor effects activating infiltrating lymphocytes, HFC were co-cultured with hPBMCs (Effector:Target cells ratio 5:1) and treated with PD-1_1, PD-L1_1, ID-1 or their combination for 24 hours at the concentration of 100 nM. In parallel assays we tested the cardiotoxic effects of the FDA approved mAbs specific for the same targets (Nivolumab and Ipilimumab), used as positive controls. Co-cultures untreated or treated with an unrelated human IgG1 mAb were used in parallel assays as negative controls. As shown in Figure 32C, treatments with PD-1_1, PD-L1_1 or ID-1 mAbs induced much lower cardiac cell lysis with respect to Nivolumab or Ipilimumab mAbs. Moreover, the levels of IL-6, a proinflammatory cytokine frequently associated with cardiac injury **[Quagliariello V., *et al.* 2019, Quagliariello V., *et al.* 2020]**, resulted to be lower when the cells were treated with the novel immunomodulatory mAbs with respect to those observed in treatments with the corresponding clinically validated mAbs (Figure 32D). Accordingly, the combination of Nivolumab and Ipilimumab shows the highest IL-6 pro-inflammatory cytokine secretion (~9700 pg/mL), thus confirming the cardiotoxic side effects of this combination. On the contrary, the combination of the other anti-CTLA-4 mAb ID-1 with Nivolumab, PD-1_1 or PD-L1_1, even though equally or more effective on tumor cells than the combination of Ipilimumab and Nivolumab (see figure 32), showed much lower toxicity for HFC, as highlighted by reduced LDH release (Figure 32C) and lower levels of secreted pro-inflammatory IL-6 (Figure 32D) **[Vetrei C., *et al.* 2021]**.

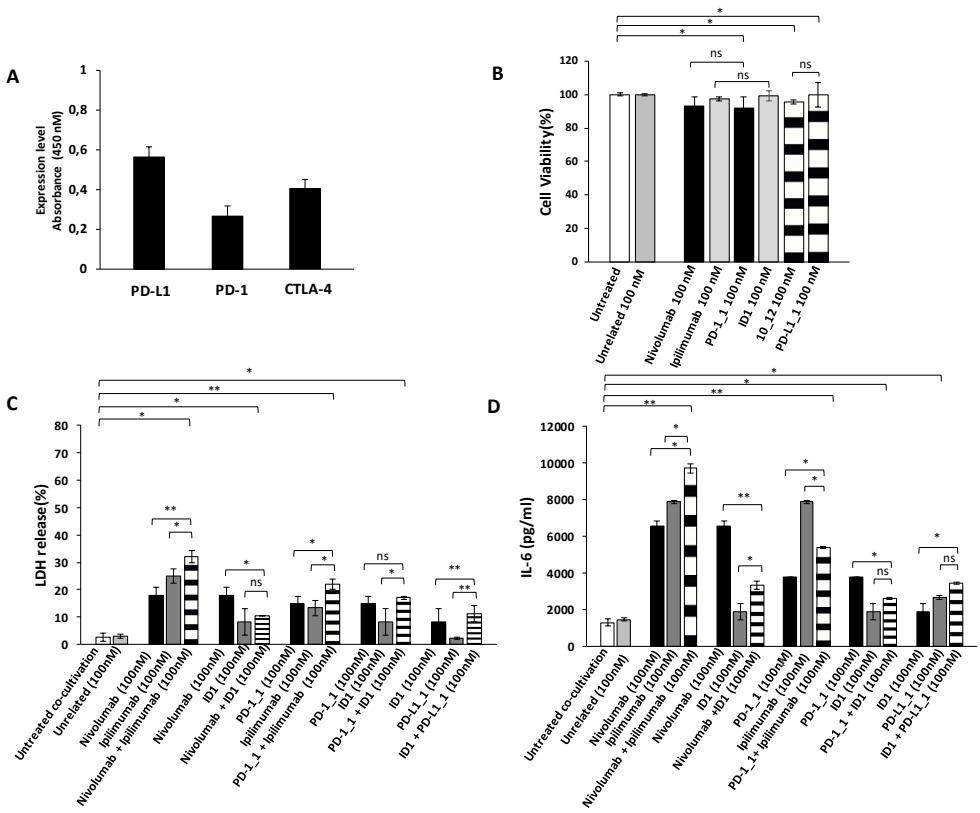

Figure 32. Cardiotoxic and pro-inflammatory effects induced by immunomodulatory mAbs on human fetal cardiomyocytes. (**A**) Cell ELISA assay performed on HFC cardiac cells by using commercial anti-PD-1, anti-PD-L1 or anti-CTLA-4 mAbs (200 nM). (**B**) Cell viability assay on human fetal cardiomyocytes, treated for 48 h with anti-PD-1(black bars), anti-PD-L1 (striped bars) or anti-CTLA-4 (grey bars) mAbs. Cell viability is expressed as percentage of viable cells with respect to control untreated ones. Cells were counted by Trypan Blue exclusion test. (**C**) Cardiotoxic effects of immunomodulatory mAbs were analyzed by evaluating the LDH release on the supernatants of HFC cells co-cultured with hPBMCs, treated for 24 h with single mAbs (black and grey bars) or their combinations (striped bars). (**D**) Pro-inflammatory effects of immunomodulatory mAbs were analyzed by evaluating the secretion of IL-6 in the supernatant of the treated co-cultures. IL-6 concentation in the supernatant is expressed as pg/mL. The values were reported as the mean of at least three determinations obtained in three independent experiments. Error bars depict means ± SD, ** $p < 0.01$; * $p < 0.05$; ns, not significant. **[Vetrei C., *et al. Cancers* 2021]**

PRODUCTION OF IMMUNO-CONJUGATES MADE UP OF IMMUNOMODULATORY MABS AND AN ANTI-EGFR APTAMER

4.10 Evaluation of the effects on tumor cells of combined treatments of Erb-hcAb, PD-L1 or Ipilimumab mAbs with anti-EGFR aptamer CL4

In the last decade the antibody-based immunotherapy has revolutionized the cancer therapy, improving the outcomes of several types of tumors, but monoclonal antibodies are not the only promising drugs developed in the last years. More recently, oligonucleotide aptamers have risen increasing attention for cancer therapy thanks to their low size (effcient tumor penetration) and lack of immunogenicity, even though their short half-life and lack of effector functions still hinder their clinical applications **[Keefe A.D., *et al.* 2010; Camorani S., *et al.* 2018]**. Thus, since several pre-clinical and clinical studies have been focused on combinatorial treatments to maximize therapeutic benefits, we decided to analyse the effects of novel combinations including either anti-TAA or immunomodulatory mAbs with an aptamer.

It has already been reported that combinatorial treatment of human cancers with anti-ErbB2 and anti-EGFR antibodies leads to more effective growth inhibition than either treatment alone **[Zheng L., *et al.* 2014; Zhou Y., *et al.* 2006]**. Considering these observations, we have investigated the possibility to combine the anti-ErbB2 compact antibody (Erb-hcAb) **[De Lorenzo C., *et al.* 2004]** with the anti-EGFR aptamer (CL4) **[Esposito C.L., *et al.* 2011]** to ascertain whether this combinatorial treatment allows for more effective and lower therapeutic doses of these drugs. To this aim, EGFR- and ErbB2-positive cancer cells, including SK-BR-3 and MDA-MB-453 breast and LNCaP prostate cancer cell lines, were treated with a combination of Erb-hcAb and CL4 at increasing concentrations to test whether these two biomolecules together affect cell viability more effciently than when they are used as single agents. As shown in Figure 33A, the combined treatment led to a stronger inhibition of tumor cell growth compared to single receptor blocking (60% growth inhibition on all the three cell lines after combinatorial treatments with respect to about 20–30% after their use in monotherapy). As a negative control, we used in parallel assays mammary MCF-7 cells (Figure 33A) expressing very low levels of EGFR and ErbB2 and no significant effects were observed either under single or combinatorial treatments, thus confirming the binding specificity of the compounds for their targets **[Passariello M., *et al.* 2019]**.

In addition, as several clinical studies combining PD-1/PD-L1 pathway inhibitors with EGFR inhibitors in cancer patients are on-going **[Gainor J.F., *et al.* 2016]** and PD-L1 expression has been found to be upregulated by EGFR overexpression in several types of cancer cells, we investigated on a dual EGFR

and PD-L1 targeting strategy. To this aim, we first tested the effects on cancer cell viability of the anti-EGFR CL4 aptamer in combination with the PD-L1_1 affinity variant, named 10_12, to verify whether a bi-specific construct made up of these two moieties could be considered beneficial for anti-cancer treatment. We chose SK-BR-3 and LNCaP cancer cell lines as models since they display both EGFR and PD-L1 on their surface. The MCF-7 mammary cell line, expressing low levels of EGFR and PD-L1 on their surface, was used as a negative control. As shown in Figure 33B , the anti-PD-L1 antibody significantly inhibited the growth of both the PD-L1-positive cell lines tested and, importantly, the combined treatment with CL4 led to additive effects, whereas no significant effects were observed on MCF-7 cells for both single and combined treatments **[Passariello M., *et al.* 2019]**. The immune independent antitumor activity of anti-PD-L1 mAb was previously ascribed to its ability to affect the mitogen-activated protein kinases (MAPKs) pathway in tumor cells **[Passariello M., *et al.* 2019]**.

Furthermore, previously mentioned studies reporting on the expression of CTLA-4 on tumor cell surface and highlighting a potential tumor growth inhibition by using CTLA-4 inhibitors, also in the absence of immune cells, suggested us to analyse the effects of Ipilimumab and of the anti-EGFR aptamer CL4 on tumor cell viability (Figure 33C).

Firstly, we analysed the effects of the single Ipilimumab on cancer cells, observing a reduction of tumor growth of about 30% in SK-BR-3 and 20% in LNCaP cells when incubated at a concentration of 100 nM for 72 h, suggesting that it directly inhibits the growth of prostate CTLA-4-positive tumor cells also independently from the immune system. In parallel, we tested again the effects of the anti-EGFR CL4 aptamer on these tumor cells and, according to previous findings, we observed a significant inhibition of tumor cell growth when used at the dose of 200 nM for 72 h, whereas no effect was observed with a scrambled aptamer (CL4Sc), used as a negative control. As expected, both the antibody and the aptamer showed no significant effects on MCF-7 tumor cells, used as negative control as it expresses low levels of the two antigens.

On the basis of these results, we decided to evaluate the effects of Ipilimumab combined with the anti-EGFR CL4 aptamer (Figure 33C). The combination of the two drugs reduced the cell growth of the double antigen-positive tumor cells (50%–60% inhibition), more efficiently than single-agent treatments, whereas no significant effects were observed on the cell line used as negative control, thus confirming the specificity of these drugs for their targets **[Passariello M., *et al.* 2020]**.

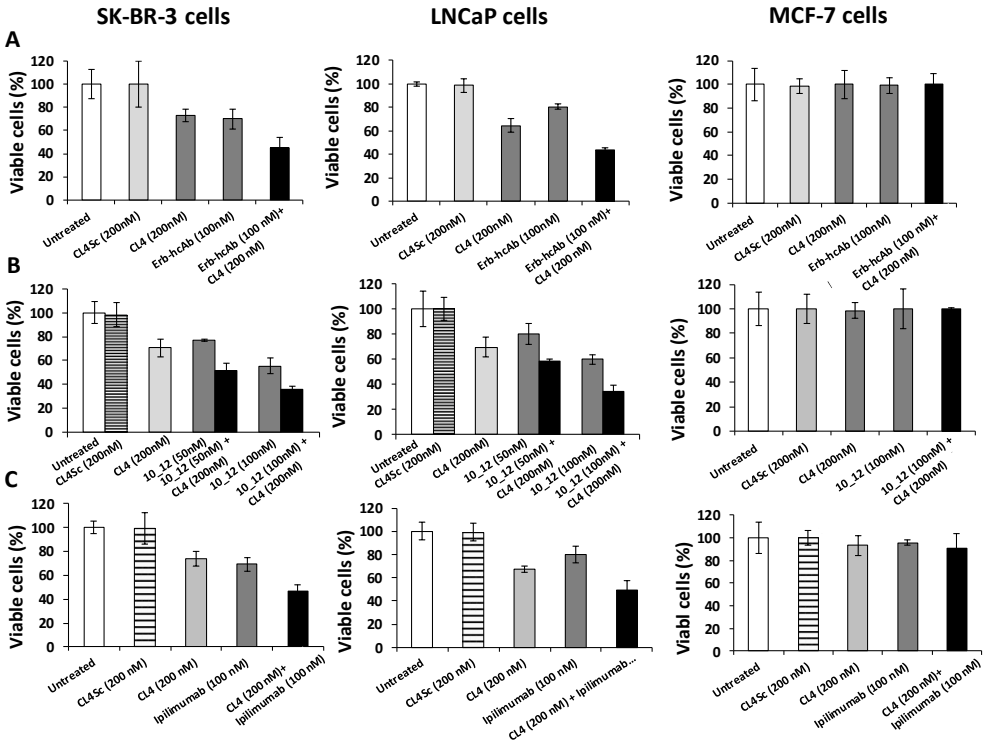

Figure 33. Combined treatments of anti-ErbB2, anti-PD-L1 or anti-CTLA-4 mAbs and CL4 aptamer efficiently inhibit tumor cell survival. SK-BR-3, LNCaP, and MCF-7 cells were treated for 72 h with CL4, (**A**) Erb-hcAb, (**B**) 10_12 or (**C**) Ipilimumab, alone or in combination, at the indicated concentrations. Cell survival is expressed as percent of viable treated cells with respect to untreated cells. CL4Sc was used in parallel as a negative control. Error bars depict means ± SD.

4.11 Construction and purification of new bi-specific constructs made up of a compact antibody or a mAb and the anti-EGFR aptamer

Considering the promising results obtained with combinatorial treatments, we decided to generate novel bi-specific immunoconjugates made up of an antibody moiety and an aptamer. In order to generate the antibody-aptamer constructs, we conjugated the constant region of the antibody with the aptamer through a free aminogroup attached to the 5'end of the 2'FPy-modified RNA aptamer by exploiting the well-known coupling chemistry of formylbenzamide (4FB). With the aim to minimize the steric hindrance between the two moieties of the chimeric construct, we inserted a six-carbon spacer arm in the aptamer. Specifically, the amino-terminated 2′FPy RNA aptamer was conjugated with the bifunctional succinimidyl-4-formylbenzamide linker (S-4FB), which incorporated an aromatic aldehyde functional group (formylbenzamide, 4FB) at the 5′-end of the oligonucleotide, and then reacted with the S-HyNic-modified antibody **[Passariello M., *et al.* 2019]**. The conjugation was allowed to proceed for 2 h by gentle rotation at room temperature (RT) following the manufacturers

recommendations. Briefly, the antibodies were modified by using the S-HyNic reagent and mixed with the 4FB-modified aptamer in the presence of a reaction catalyst (aniline) to form the conjugate through a covalent bond. The conjugates obtained from the conjugation of Erb-hcAb, 10_12 or Ipilimumab and CL4, named Erb-hcAb-CL4, 10_12-CL4 and CL4-Ipilimumab, were then purified by a magnetic affinity matrix and eluted following the manufacturers recommendations.

4.12 Analysis of binding ability of the new constructs to their target cells

First we assessed whether the chimeric bi-specific molecules preserve the ability to recognize the targets of the parental drugs. To this aim Erb-hcAb-CL4, 10_12-CL4 and CL4-Ipilimumab conjugates (50 nM) were incubated for 75 min at RT onto SK-BR-3 cells (ErbB2- and PD-L1-positive cells) and human lymphocytes (PD-L1-positive). Binding was measured by ELISA assays, and compared to that of unconjugated antibodies. As shown in Figure 34B, a statistically significant binding was observed with the conjugates Erb-hcAb-CL4 and 10_12-CL4 on SK-BR-3 cells and human lymphocytes, respectively.

CL4-Ipilimumab conjugate not only retains the binding ability of both the parental aptamer and antibody for their targets expressed on the surface of SK-BR-3 tumor cells and activated lymphocytes (CTLA-4-positive), but it also acquires a much higher avidity for double antigen-positive SK-BR-3 tumor cells **[Passariello M., *et al.* 2020]**.

These results indicate that the covalent attachment of the aptamer to the antibody, in the frame of the three conjugates, did not affect the antibody targeting function, on the contrary it was beneficial for higher binding ability.

Notably, the Erb-hcAb-CL4 and 10_12-CL4 conjugates, differently from their antibody counterparts, were also able to bind to MDA-MB-231 and MDA-MB-453 cells, that lack ErbB2 and PD-L1 expression, respectively, thus indicating that the conjugates' binding is mediated by the aptamer moiety, which retains the ability to bind to EGFR expressed on both the cell lines (Figure 34B). As a control, we also tested the combination of unconjugated CL4 aptamer and 10_12 mAb on MDA-MB-453 cells and, as expected, no significant binding to the cell line tested was detected with the anti-human IgG H+L, thus further confirming that the binding of 10_12 mAb to the EGFR-positive cell line occurs only when the molecule is covalently conjugated to the aptamer, as expected. Accordingly, confocal microscopy analyses showed that the 10_12-CL4 conjugate efficiently localized on the membrane of both SK-BR-3 and MDA-MB-453 cells, whereas the parental antibody recognized only PD-L1-positive

SK-BR-3 cells (Figure 34C) **[Passariello M., *et al.* 2019]**. Moreover, the specific binding of the CL4-Ipilimumab conjugate to the cell surface of SK-BR-3 cells was further shown by confocal microscopy (Figure 34C): the conjugate-associated signal on the cell surface had a higher intensity with respect to those obtained with the single parental aptamer or antibody, whereas, as expected, no signals were observed on negative control MCF-7 cells (Figure 34C). These results demonstrated that the antibody and aptamer moieties maintain their targeting functions in the chimeric proteins **[Passariello M., *et al.* 2020]**.

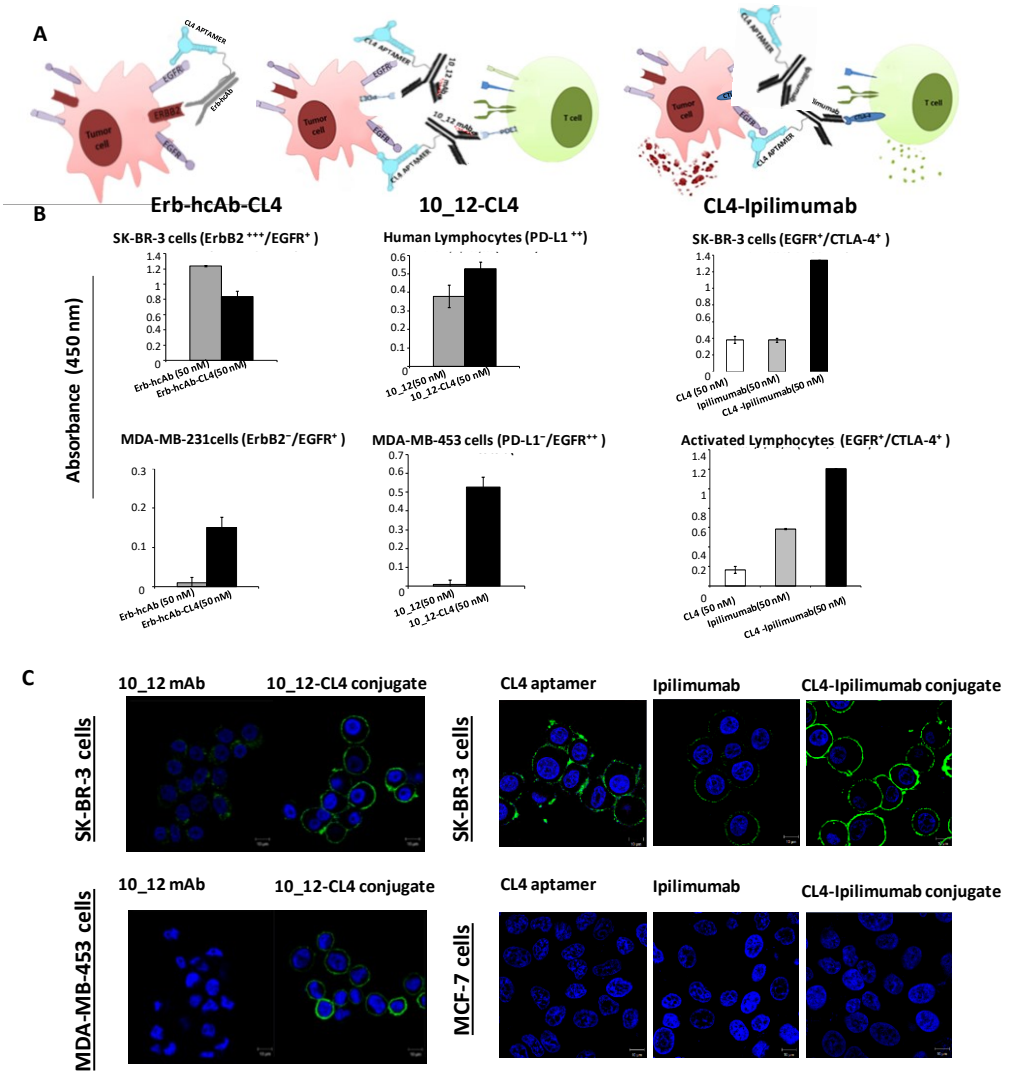

Figure 34. Schematic representations and binding of the three conjugates. (**A**) Schematic representations of Erb-hcAb-CL4, 10_12-CL4 and CL4-Ipilimumab conjugates, generated by the fusion of CL4 amino-oligonucleotide (anti-EGFR aptamer) with the Fc region of Erb-hcAb (anti-ErbB2 compact antibody), 10_12 (anti-PD-L1 monoclonal antibody) or Ipilimumab (anti-CTLA-4 monoclonal antibody). (**B**) Binding by ELISA of Erb-hcAb-CL4 conjugate (black bars) to SK-BR-3 or MDA-MB-231 cells. As control, unconjugated Erb-hcAb antibody (grey bars) was tested in parallel by ELISA assays at the same concentration. Binding of 10_12-CL4 (black bars) to MDA-MB-453 cells and human activated lymphocytes. As a control, unconjugated 10_12 antibody (grey bars) was tested in parallel by ELISA assays at the same concentration. Binding of CL4-ipilimumab conjugate (black bars) on SK-BR-3 or activated lymphocytes. Unconjugated CL4 aptamer (white bars), or Ipilimumab mAb (grey bars) were used as controls. Error bars depict means ± SD. In B and C, p-values for the indicated mAbs relative to untreated cells, are: *** $p \leq 0.001$; ** $p < 0.01$; * $p < 0.05$. (**C**) Representative confocal microscopy images of SK-BR-3, MDA-MB-453, MCF-7 cells incubated with 10_12, 10_12-CL4, CL4, Ipilimumab or CL4-Ipilimumab as indicated. The aptamer, mAbs and conjugates are visualized in green; nuclei are visualized in blue. Magnification 63x, 1.0x digital zoom. Scale bar = 10 μm.

4.13 Cytotoxic effects of the novel aptamer–antibody conjugates on tumor cells

The finding that the novel immunoconjugates are endowed with both binding specificities for their targets led us to investigate whether they could also inhibit the growth of cells overexpressing both the targets more efficiently than the parental antibody or aptamer. To this aim, SK-BR-3 or LNCaP cells (expressing all the three targets) were plated in the absence or in the presence of the immunoconjugates, used at increasing concentrations, and incubated for 72 h at 37°C. Cell survival was measured by counting Trypan blue-excluding cells. In parallel experiments, the effects on cell survival of the parental antibodies and anti-EGFR aptamer were also tested. As shown in Figure 35, Erb-hcAb-CL4, 10_12-CL4 and CL4-Ipilimumab conjugates were found to selectively inhibit tumor cell growth for both the cell lines. The IC50 values were found to be lower than those obtained for CL4 aptamer and the parental antibodies used as single agents, thus confirming that the novel immunoconjugates have more potent cytotoxic effects than the single antibodies and aptamer. These results strongly indicate that bi-specific constructs made up of antitumor antibody and aptamer could become valuable tools for increasing the antitumor efficacy **[Passariello M., *et al.* 2019; Passariello M., *et al.* 2020]**.

The efficacy of 10_12-CL4 was also tested on SK-BR-3 cells co-cultured with human hPBMCs to exploit also the inhibitory effects of 10_12 mAb in the PD-1/PD-L1 interaction between tumor cells and T cells, which leads to T cells activation, by inducing IL-2 and IFN-γ cytokines secretion. To this aim, SK-BR-3 cells were treated with the immunoconjugate (50 nM) or the unconjugated CL4 aptamer and 10_12 mAb (50 nM), used alone or in the presence of hPBMCs (effector:target ratio 10:1) for 24 h at 37 °C.We used lower concentrations (down to 50 nM) of the conjugate with respect to those (200–400 nM) used for the aptamer in combinatorial treatments, reported above, to verify whether the doses could be lowered for eventual therapeutic applications. As shown in Figure 36A, the immunoconjugate 10_12-CL4 induced the death of tumor cells more efficiently than both the parental moieties, significantly increasing the secretion of IL-2 and IFNγ cytokines (Figure 36C) by lymphocytes, as well as LDH release by tumor cells (Figure 36B)**[Passariello M., *et al.* 2019]**. Similarly, we decided to verify whether also CL4-Ipilimumab anti-tumor effects could be improved in the presence of lymphocytes. Since novel interesting data from our group reported an effect of Ipilimumab not only on hPBMCs but also on a fraction of human lymphocytes enriched in NK cells, we decided to test this mAb, the aptamer and the derived immunoconjugate on co-cultures of tumor cells with the above mentioned immune cell populations. To this aim, SK-BR-3 cells were co-cultured with hPBMCs or its fraction enriched in NK cells (Effector:Target ratio: 3:1) and treated for 24 h in the absence or in the presence of the immunoconjugate and its parental compounds used either as single agents or in combinatorial treatments at the same concentrations.

As shown in Figures 37A and 38A, the immunoconjugate induced the death of tumor cells in a dose-dependent fashion and more efficiently than both the parental moieties, by significantly increasing the secretion of IL-2 and IFN-γ cytokines by both hPBMCs and NK populations (Figures 37C and 38C) , thus leading to increased cell lysis with a higher lactate dehydrogenase (LDH) release by tumor cells (Figures 37B and 38B). Noteworthy, in these conditions the effects exerted by the antibody and its derived immunoconjugate on the two co-cultures of tumor cells and hPBMCs or NK cells are comparable, thus suggesting that NK cells are responsible for most of the immune responses mediated by hPBMCs and modulated by Ipilimumab and its derived immunoconjugate **[Passariello M., *et al.* 2020]**.

Altogether these results confirm that CTLA-4 is indeed expressed on tumor cells and on NK cells and it plays a critical role in NK cell anti-tumor activity. Hence, taking this into consideration, the use of this novel immunoconjugate could improve the anti-tumor effects of Ipilimumab by activating different lymphocytes populations with a higher potency than Ipilimumab and by cross-linking tumor cells with immune cells.

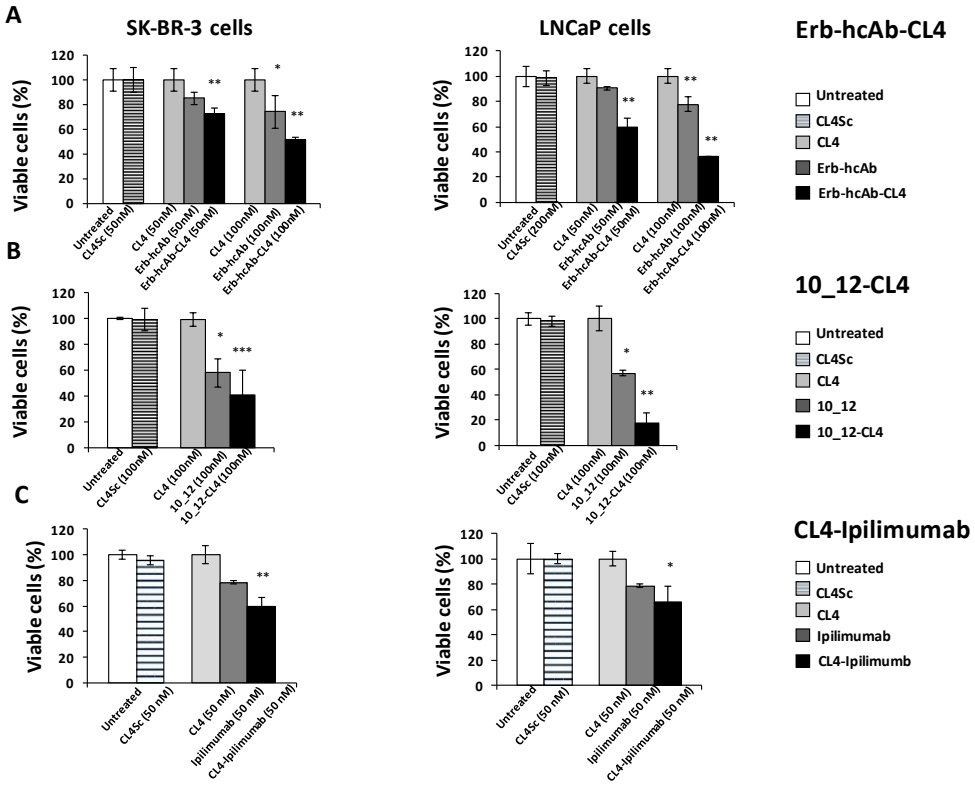

Figure 35. Effects of Erb-hcAb-CL4, 10_12-CL4 and CL4-Ipilimumab conjugates on tumor cell survival. SK-BR-3 and LNCaP tumor cells were incubated with: (**A**) CL4, Erb-hcAb or Erb-hcAb-CL4; (**B**) CL4, 10_12 mAb or 10_12-CL4 conjugate; (**C**) CL4, Ipilimumab or CL4-Ipilimumab, at the indicated concentrations. Cell survival is expressed as percentage of viable treated cells with respect to untreated cells. The scrambled aptamer was used in parallel as a negative control. Error bars depict means ± SD. p-values for the indicated mAbs relative to untreated cells are: *** $p \leq 0.001$;** $p < 0.01$; * $p < 0.05$.

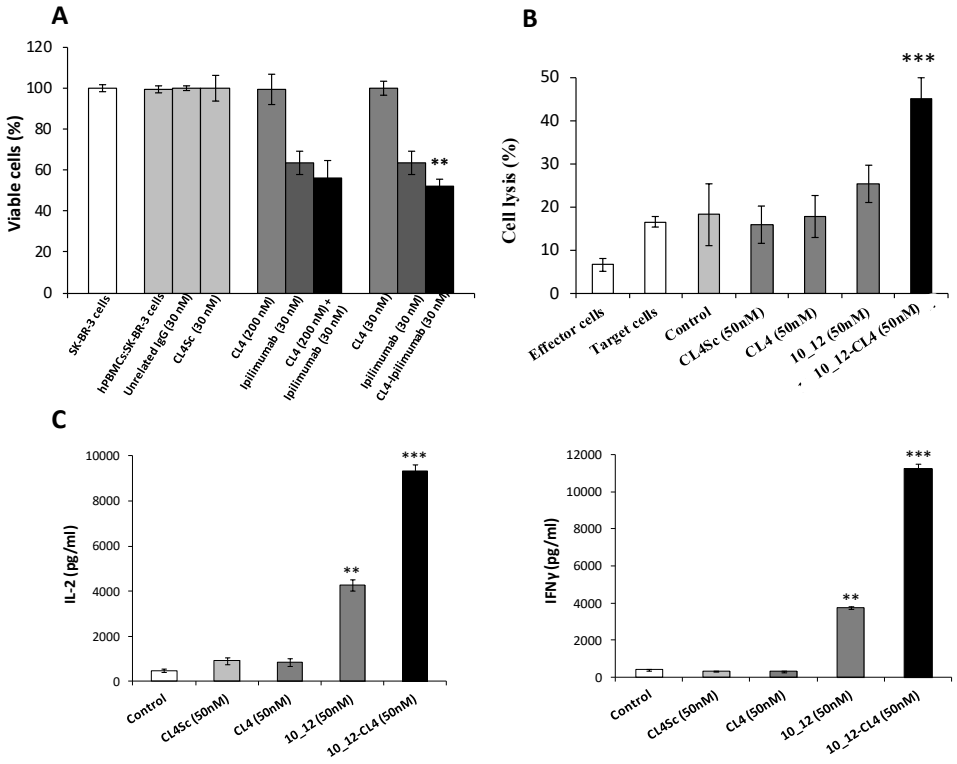

Figure 36. Cytotoxic effects of 10_12-CL4 conjugate on breast cancer cells co-cultured with lymphocytes. (**A**) SK-BR-3 cells were co-cultured with lymphocytes (effector:target ratio 10:1) and treated for 24 h with CL4 aptamer, 10_12 mAb, or 10_12-CL4 conjugate at the indicated concentrations. SK-BR-3 cell survival is expressed as percentage of viable treated cells with respect to untreated cells. (**B**) SK-BR-3 cell lysis, measured by the LDH release after the incubation with the indicated compounds, used at the concentration of 50 nM. The levels of LDH are expressed as percentage of lysis of treated cells with respect to the effects observed in co-cultures of tumor cells and lymphocytes in the absence of the drugs, used as controls. (**C**) IL-2 and IFN-γ cytokine secretion levels (pg/mL) were measured by ELISA assays performed on cell supernatants. Untreated or treated cells with scrambled CL4 in the presence of lymphocytes were used as negative controls. Error bars depict means ± SD. p-values for the indicated mAbs relative to untreated cells are: *** $p \leq 0.001$; ** $p < 0.01$; * $p < 0.05$.

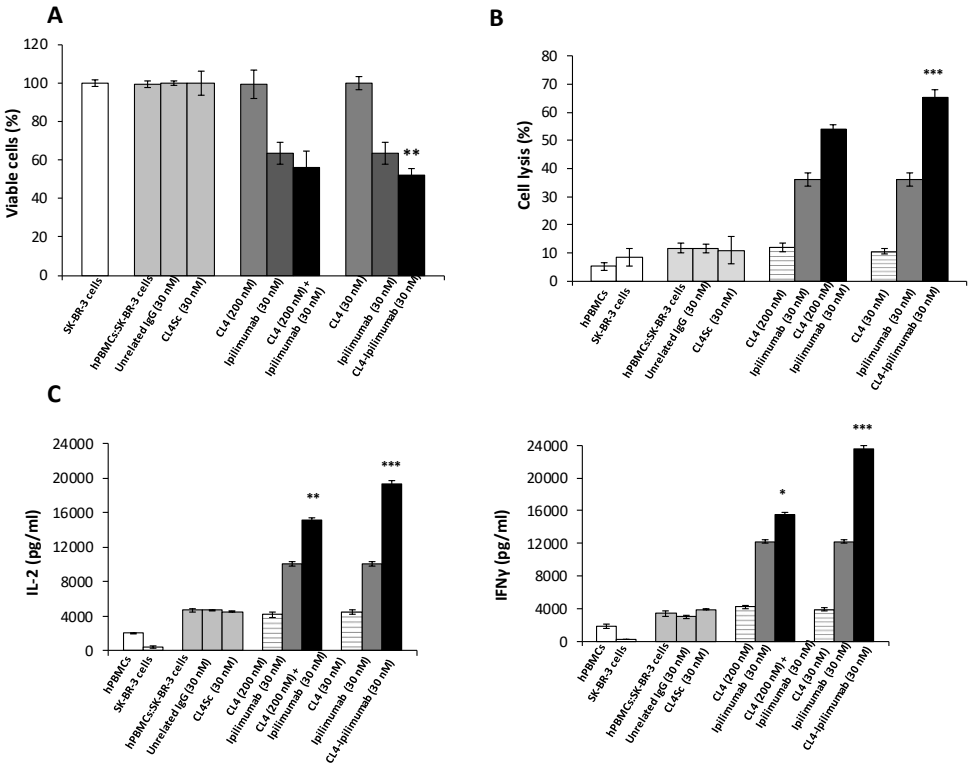

Figure 37. Cytotoxic effects of CL4-Ipilimumab on SK-BR-3 tumor cells co-cultured with hPBMCs. (**A**) SK-BR-3 cells were co-cultured with hPBMCs (Effector:Target cells ratio 3:1) and treated for 24 hours with CL4 aptamer (striped bars), ipilimumab mAb (grey bars), their combination or CL4-Ipilimumab conjugate (black bars) at the indicated concentrations. SK-BR-3 cell survival is expressed as percentage of viable treated cells with respect to untreated cells. (**B**) SK-BR-3 cell lysis, measured by the LDH release after the incubation with the indicated compounds. The levels of LDH are expressed as percentage of lysis of treated cells with respect to the effects observed in co-cultures of tumor cells and lymphocytes in the absence of the drugs, used as controls. (**C**) IL-2 and IFN-γ cytokine secretion levels (pg/mL) were measured by ELISA assays performed on supernatants of cells treated ad indicated. Untreated or treated cells with scrambled CL4 in the presence of lymphocytes were used as negative controls. Error bars depict means ± SD. p-values for the indicated mAbs relative to untreated cells are: *** $p \leq 0.001$; ** $p < 0.01$; * $p < 0.05$.

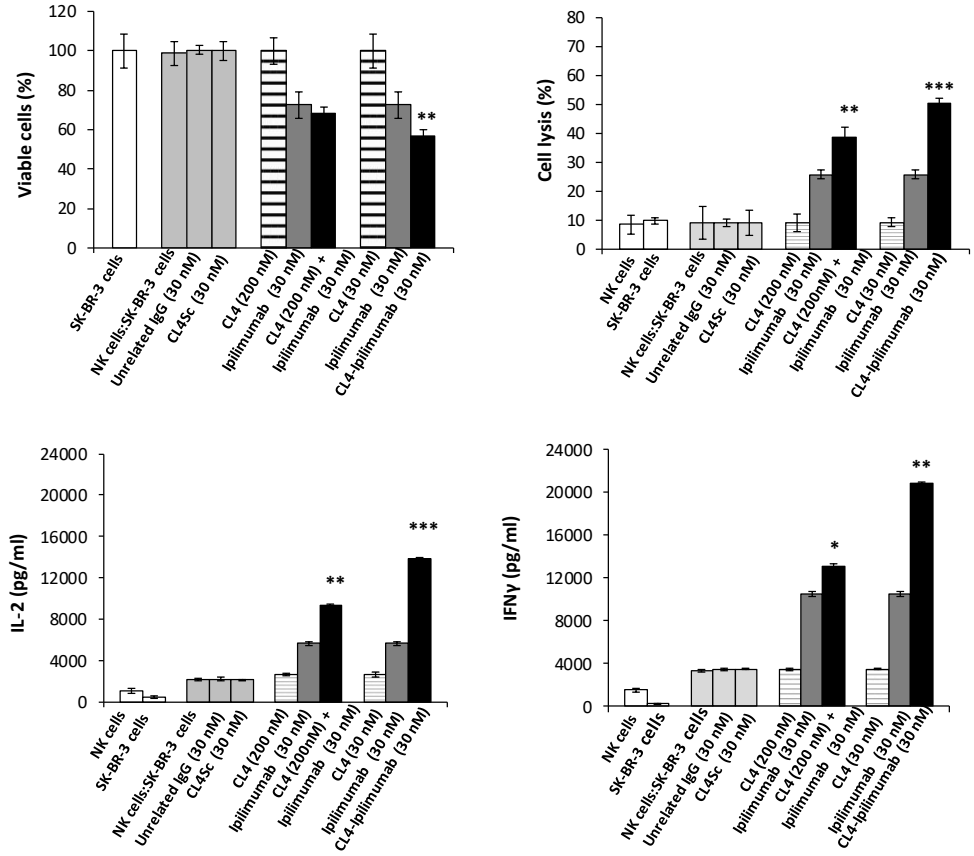

Figure 38. Cytotoxic effects of CL4-ipilimumab conjugate on SK-BR-3 tumor cells co-cultured with NK cells. (**A**) SK-BR-3 cells were co-cultured with NK cells (effector:target cells ratio: 3:1) and treated for 24 h with CL4 aptamer (striped bars), Ipilimumab mAb (grey bars), their combination, or CL4-Ipilimumab conjugate (black bars), as indicated. SK-BR-3 cells separately treated with the immunoconjugate, the scrambled aptamer, or with NK cells were included as controls. The percentage of viable cells is expressed with respect to untreated cells. (**B**) The release of lactate dehydrogenase was tested for measuring % cell lysis as described in Materials and Methods. (**C**) The levels of secreted IL-2 and IFN-γ were measured by ELISA on supernatants of cells treated, as indicated. Error bars depict means ± SD. p-values for the indicated treatments are: *** $p \leq 0.001$; ** $p < 0.01$; * $p < 0.05$.

DISCUSSION

During the last three decades, cancer immunotherapy has become an encouraging therapeutic approach and several immunotherapeutic drugs have been approved by FDA for clinical use against cancer. Indeed, many innovative therapeutic regimens, involving the combination of new drugs, such as monoclonal antibodies, with conventional treatments, have been tested in clinical trials with interesting positive results, leading to their approval for therapeutic applications **[Galluzzi L., *et al.* 2014]**.

In particular, combinatorial treatments of clinically validated mAbs targeting immune-regulatory checkpoints significatively improved their anti-tumor effects. Unfortunately, a marked increase of efficacy sometimes corresponds to a worsening of adverse events associated to anti-ICs drugs **[Larkin J., *et al.* 2019].** Therefore, the development of preclinical models to early detect unwanted side effects are needed to identify combinations of mAbs with the highest anti-tumor activity associated with no or low side effects **[Vetrei C., *et al.* 2021]**.

5.1 Generation and characterization of novel human antibodies targeting ICs

Among the cancer immunotherapy approaches, the use of antibodies able to improve the activity of immune effector cells, has become a precious strategy to overcome the limits of conventional therapies. The comprehension of the role of ICs in the mechanisms underlying the proliferation and regulation of T-cells **[Wurz G.T., *et al.* 2016]** improved the efficacy of the therapeutic approaches through the development of antibodies agonistic for co-stimulatory or antagonistic for inhibitory receptors. In the last decade a number of monoclonal antibodies directed against ICs were approved by FDA for the treatment of cancer, such as Ipilimumab (anti-CTLA-4), Nivolumab (anti-PD-1) and Atezolizumab (anti-PD-L1). To further increase their efficacy, these mAbs were used in combination with other mAbs or with conventional anti-tumor treatments.

For instance, Ipilimumab, the first anti-CTLA-4 antibody approved by FDA for melanoma **[Cameron F., *et al.* 2011; Patel V., *et al.* 2011]** has been succesfully combined with the anti-PD-1 mAb Nivolumab, in clinical trials for other types of cancers, such as renal cell carcinoma or non-small-cell lung cancer (NSCLC) **[Ribas A., Wolchok J.D. 2018; Postow M.A., *et al.* 2015]**. Despite the successful results in terms of overall survival (OS), these combinatorial treatment led also to significant immune-related adverse effects (irAEs) **[Kourie H.R., *et al.* 2016; Ventola C.L., *et al.* 2017; Haanen J.B.A.G., *et al.* 2017]**. The lack of cross-reactivity of these mAbs for mouse hortologs was often the reason

for the reduced preclinical studies in mouse models that could have been useful to predict toxic side effects.

In order to obtain cross-reactive anti-CTLA-4 mAbs useful for future *in vivo* studies, we performed an innovative strategy of phage display and high throughput screening to identify antibody sequences commonly enriched in two parallel selections of a large antibody library on human and mouse CTLA-4 protein.

The novel identified ID-1 and ID-8 mAbs were found able to bind with high affinity and specificity to both human and mouse purified proteins and lymphocytes. More importantly, they were found able to induce the activation of human and mouse PBMCs, by strongly increasing the IL-2 and IFN-γ cytokine secretion, thus confirming the efficiency of the strategy used for the selection of cross-reactive mAbs. Interestingly, both the anti-CTLA-4 mAbs showed also the ability to induce cytokines secretion from an immune population enriched in NK cells, in line with recent findings highlighting the expression of CTLA-4 also on this specific cell population **[Passariello M., *et al.* 2020]**. Specifically, ID-8 was able to stimulate NK cells more efficiently than Ipilimumab, showing a more potent anti-tumor activity by an efficient redirection of NK lymphocytes against cancer cells when co-cultured, thus strongly enhancing the ADCC effects.

Since recent results showed the expression of ICs, such as PD-L1 or CTLA-4, not only on T-cells but also on cancer cells, they have been considered as potential co-targets for cancer treatments **[Passariello M., *et al.* 2019; Passariello M., *et al.* 2019; Wimberly H., *et al.* 2015; Velcheti V., *et al.* 2014]**.

Thus, ID-1 and ID-8 were characterized in parallel to anti-PD-L1 mAbs, for their effects on tumor cells.

The anti-PD-L1 mAbs, previously generated in our laboratory, were found able to bind with high affinity and specificity to human purified proteins and lymphocytes. In particular, PD-L1_1 mAb, endowed with the highest affinity for human PD-L1 IC, induced cytokine secretion by treated lymphocytes more efficiently than the clinically validated Nivolumab and Atezolizumab.

Previous studies on cultures of tumor cells showed the ability of the novel anti-PD-L1 isolated antibodies to inhibit cell growth by acting on intracellular pathways even in the absence of T-cells , thus suggesting an additional role of ICs in promoting tumor cell proliferation.

Indeed, the novel anti-PD-L1 mAb was able to affect the phosphorylation of Erk, JNK and P38 in tumor cells, thus confirming findings previously reported in literature **[Massi D., *et al.*. 2014]**.

Similarly, when tested on cultures of tumor cells, ID-1 and ID-8 anti-CTLA-4 mAbs were found able to inhibit cell growth, affecting intracellular pathways differently from Ipilimumab.In particular, ID-1 and ID-8 induced a marked phosphorylation of CTLA-4, inhibited the phosphorylation of Akt and induced caspase activation. These results support the hypothesis that ID-1 and ID-8 directly affect the CTLA-4 function on tumor cells, by inhibiting the downstream survival pathways, such as that of PI3K, and by inducing apoptosis,

in agreement with previous reports indicating that the binding of ligands to CTLA-4 expressed on tumor cells can induce the sequential activation of both caspase-8 and caspase-3 **[Contardi E., *et al.* 2005]**. Further investigations revealed that the novel mAbs induced a marked homodimerization of CTLA-4, thus increasing its level on the cell surface, in line with the hyperphosphorylation of the monomeric form of CTLA-4, needed to avoid its internalization and the subsequent degradation **[Bradshaw J.D., *et al.* 1997; Shiratori T., *et al.* 1997]**. More interestingly, ID-1, ID-8 and Ipilimumab showed opposite effects on NK cells by inducing the phosphorylation of Erk and Akt, and inhibiting the activation of caspase 3. These findings suggest for the first time that CTLA-4 could have differential roles in different lymphocytes subsets and cell populations, as it can regulate diverse downstream pathways.

Furthermore, since ID-1 and ID-8 do not induce the degradation of CTLA-4, they could provide more safe and efficient anti-tumor activity, differently from those mAbs that trigger degradation of CTLA-4, leading to an increased toxicity and a reduced anti-tumor efficacy **[Zhang Y., *et al.* 2019]**. Indeed, an important feature for anti-CTLA-4 antibodies would be represented by the uncoupling of their therapeutic effects from irAEs. Up to date the immunotherapeutic effects have been achieved through the antagonistic activity of the anti-CTLA-4 antibodies, i.e., blocking the checkpoint that can prevent autoimmunity and immune responses against cancer **[Seidel J.A., *et al.* 2018]**, thus making it difficult to achieve cancer immunity without irAEs. The novel antibodies, lacking the full antagonistic activity but still endowed with potent effects on NK cells, could provide efficient anti-tumor effects without the irAEs associated with the antagonistic activity of Ipilimumab. This aspect could be even more important in case of combinatorial treatments such as the successful combination, previously reported **[Rotte A., *et al.* 2019; Buchbinder E.I., *et al.* 2016]**, of anti-PD-1 and anti-CTLA-4 antibodies resulting in a longer progression-free survival and a higher rate of response, but leading to increased autoimmune reactions representing the major toxic side effects of this class of therapeutic antibodies.

Thus, we decided to further test the novel ID-1 and PD-L1_1 mAbs to shed light on potential beneficial effects of the novel combinations of mAbs in comparison with the clinically validated Nivolumab and Ipilimumab.

We firstly tested them for their effects on intracellular pathways of tumor cells in the absence of immune cells highlighting for the first time a possible reciprocal modulation of ICs mediated by these mAbs, thus suggesting the existence of a crosstalk also in tumor cells involving not only PD-L1 and its receptor PD-1, but also CTLA-4.

Interestingly, the increased expression of ICs, induced by treatment with anti-PD-L1 and anti-PD-1 antagonistic mAbs, was associated with a marked hyperphosphorylation of the NF-kB transcription factor, in line with previous findings reporting on the ability of NF-kB to induce the expression of genes

encoding ICs in other cells, such as NSCLC cells and macrophages **[Antonangeli F., *et al.* 2020; Bally A.P., *et al.* 2015].**

5.2 Set up of a strategy to identify effective and safe combinatorial treatments

Then, we tested these novel mAbs for their effects on immune cells when cultured with tumor or cardiac cells. The latter choice derived from the observation that inhibition of the T cell co-inhibitory pathways or activation of the co-stimulatory pathways mediated by mAbs might generate serious risk of cardiovascular adverse events, such as myocarditis and pericarditis, even though in a low percentage of treated patients **[Larkin J., *et al.* 2019; Simons K.H., *et al.* 2019; Varricchi G., *et al.* 2018; Palaskas N.L., *et al.* 2020; Haratani K., *et al.* 2018; Moslehi J.J., *et al.* 2018; Mahmood S.S., *et al.* 2018; De Luca G., *et al.* 2018]**.

Thus, we decided to set up a preclinical model to identify the most efficient combinations of anti-ICs mAbs for achieving potent anti-tumor efficacy associated with the lowest adverse side effects. To this aim, we used novel simple and predictive *in vitro* models based on co-cultures of hPBMCs with tumor cells or human fetal cardiomyocytes. As a proof of concept, we firstly tested the treatment of Nivolumab and Ipilimumab, characterized by higher anti-tumor efficacy but associated to cardiac injuries in cancer patients **[Larkin J. N., *et al.* 2019]**. This combination was found to induce a significant release of LDH (more than 30%) in co-cultures of cardiomyocytes and lymphocytes associated with significant secretion of pro-inflammatory IL-6, which has been previously reported to be involved in the etiopathogenesis of myocarditis **[Quagliariello V., *et al.* 2020; Quagliariello V., *et al.* 2019; De Luca G., *et al.* 2018; Maier H.J., *et al.* 2012]**.

On the contrary, combinations including PD-1_1 or PD-L1_1 with ID-1 **[Sasso E., *et al.* 2018; Passariello M., *et al.* 2020]**, showed a more potent anti-tumor activity, inducing increased tumor cell lysis and secretion of IL-2 and IFN-γ, with lower cardiotoxic effects on cardiomyocytes, with respect to the combination Nivolumab–Ipilimumab. Indeed, we observed a reduced cell lysis on cardiac cells and the secretion of lower levels of IL-6 pro-inflammatory cytokine, thus suggesting that the combination including ID-1 and PD-L1_1 is endowed with the highest anti-tumor cytotoxicity and the lowest cardiotoxicity **[Vetrei C., *et al.* 2021]**.

Thus, we propose these co-cultures system-based assays to test also other combinatorial treatments of novel immunomodulatory mAbs against different ICs (such as those specific for Lag-3, TIM-3, ICOS and others) to early predict not only their anti-cancer activity but also their toxic side effects, allowing for the early screening of most potent and safe combinatorial therapeutic regimens.

5.3 Construction of new bi-specific conjugates made up of mAbs and aptamers

Oligonucleotide aptamers have raised attention in oncological scientific research due to their specific binding to tumor associated targets, easy chemical synthesis, convenient modification, small size and lack of immunogenicity **[Keefe A.D., *et al.* 2010]**. However, their short *in vivo* half-life and lack of effector functions have been the main limitations for their approval for cancer therapy and clinical use. On the other hand, monoclonal antibodies have shown to be successful drugs in the treatment of cancer patients, due to their efficacy and safety, but despite their longer half-life in circulation, could sometimes have limited biodistribution due to their large molecular size which may hinder tumor tissue penetration, in particular if conjugated with other large size molecules, such as toxins **[Chames P., *et al.* 2009]**.

In order to overcome these limits, we have considered to test the chemical conjugation of a small size anti-TAA aptamer (12 kDa) with an antibody targeting a different antigen to obtain novel bi-specific constructs with an acceptable molecular size for good penetration in tumor masses and endowed with effector functions. More importantly, the presence of the antibody in the construct increases the molecular size of the aptamer, thus allowing to overcome the rapid clearance by renal filtration and short half-life in circulation of the aptamers.

To this aim, we have constructed three different bi-specific immunoconjugates, made up of the anti-EGFR aptamer (CL4), endowed with anti-tumor activity, fused either with an anti-TAA (anti-ErbB2) compact antibody (Erb-hcAb), or an immunomodulatory anti-PD-L1 mAb (10_12) or with an anti-CTLA-4 mAb (Ipilimumab). We successfully obtained for the first time these mAb-aptamer immunoconjugates, that were called Erb-hcAb-CL4, 10_12-CL4 and CL4-Ipilimumab, respectively.

We demonstrated that all the these three immunoconjugates retained the biological functions of both the parental moieties and acquired a more potent cytotoxic activity against tumor target cells by combining the biological properties of the two different targeting agents **[Passariello M., *et al.* 2019, Passariello M., *et al.* 2020]**.

Furthermore, the conjugation of the anti-EGFR aptamer with the immunomodulatory 10_12 mAb or Ipilimumab mAb allowed for an efficient redirection and activation of T cells against cancer cells, enhancing the cytotoxicity of the two conjugated compounds. In particular, CL4-Ipilimumab also induced the activation of another specific subpopulation of immune cells, such as NK cells, more efficiently than the parental mAb by inducing a more efficient secretion of cytokines in co-cultures with tumor cells, thus strongly increasing the cytotoxicity of the two partners in the chimeric construct.

The interesting biological properties shown by this novel bi-specific conjugates suggest that the novel proposed methodology has the potential to become a universal platform that can be expanded to all the desired antibodies and aptamers. Indeed, once conjugated, these bi-specific constructs could acquire other optimal biological features for therapeutic applications, such as increased specificity for tumor cells displaying both the targets, crosslinking of immune and cancer cells and improved pharmacokinetic and pharmacodynamic properties due to the combination of advantages of both aptamers and antibodies.

However, limitations to our studies were represented by the low yields of production and purification of the immunoconjugates and by the lack of *in vivo* studies in animal models, needed to confirm the extended *in vivo* half-life of the immuno-conjugates. Thus, our future plans will include the optimization of the conjugation and purification protocols for larger scale productions that will allow to get higher yields of pure chimeric constructs to be administered in mice, in order to confirm their anti-tumor efficacy and improved pharmacokinetic properties.

CONCLUSIONS

An innovative phage display strategy, consisting in panning a large human antibody library on live lymphocytes and on both human and mouse proteins, allowed the successful isolation of fully human mAbs cross-reactive for mouse targets, thus useful for future *in vivo* studies in mouse models. Indeed, the novel generated mAbs were found able to recognize with high affinity and specificity both human and mouse recombinant proteins or PBMCs. More importantly, the novel mAbs showed their ability to activate PBMCs or other immune subpopulations, such as NK cells, by increasing IL-2 and IFN-γ cytokines secretion. We used them also as tools to investigate on the effects on IC downstream pathways in tumor cells, shedding light on the possible additional role of PD-L1 and CTLA-4 in cancer cells. Notably, we evidenced for the first time the potential cis-interaction between some immune checkpoints in cancer cells.

As a further goal, we set up an *in vitro* model, based on parallel co-cultures of hPBMCs with tumor or cardiac cells, to early predict the efficacy and eventual cardiotoxic side effects of combinatorial treatments of immunomodulatory mAbs. By using this *in vitro* systems we showed the efficacy of PD-L1_1 and ID-1 and their combination as well as their lack of cardiotoxicity, thus suggesting that they can become powerful and safe anti-tumor weapons to be used alone or in combination, to achieve more potent anti-tumor effects. Hence, we think that this strategy of simple *in vitro* co-cultures-based assays could be useful to test in the future also combinatorial treatments of other immunomodulatory mAbs against different ICs, thus allowing for the early screening of most potent and safe combinatorial therapeutic regimens.

Finally, we also combined some of the novel antibodies with other drugs, such as aptamers, and generated, for the first time, novel bispecific aptamer–antibody conjugates combining the advantageous properties of the two different targeting anti-tumor agents, thus improving their anti-tumor efficacy and specificity. The strategy, based on easy conjugation protocols, could become a universal platform to extend this novel methodology to many different antibodies and aptamers in order to create the desired bispecific molecules endowed with higher anti-tumor potency.

ACKNOWLEDGMENTS

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