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DOCTORATE
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

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Optimized armed oncolytic adenoviral vaccines (PeptiCRAd) for an enhanced anti-cancer immune response
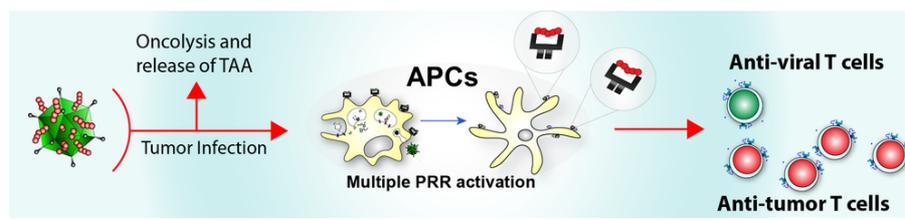
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"Gutta cavat lapidem, non bis sed saepe cadendo"
-Giordano Bruno-

…to beloved professor and mentor Imma Nunziata

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ABBREVIATIONS

Ad	Adenovirus
BMMC	Bone Marrow derived Mast Cells
CAR	Coxsackie and Adenovirus Receptor
CTA	Cancer Testis Antigens
CTL	Cytotoxic T-Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cells
DMEM	Dulbecco´s modified Eagle´s medium
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunospot
ER	Estrogen Receptor
ERDs	Estrogen Receptor Down regulators
FDA	Food and Drug Administration
Foxp3	Forkhead box P3
GM-CSF	Granulocytes Macrophages Colony Stimulating Factor
HER2	Human Epidermal growth factor Receptor 2
HLA	Human leucocytes antigens
HMGB1	High Mobility Group Box 1
HPV	Human Papilloma Virus
HSPGs	Heparan Sulfate ProteoGlycans
HSV1	Herpes Simplex Virus 1
IFN	Interferon
IL	Interleukin
LC	Liquid Chromatography
LPS	LipoPolySaccharide
mAb	Monoclonal antibody
MAGE-A4	Melanoma Associated antigen 4
MCC	Merkel Cell Carcinoma
MDSC	Monocytic Myeloid-Derived Suppressor Cell
MHC I	Major histocompatibility complex 1
MHC II	Major histocompatibility complex 2
mRNA	messenger RNA
MS	Mass Spectrometry
MSCs	Mesenchymal Stem Cells

MTOC	Microtubule Organizing Centre
Nab	Neutralizing Antibodies
NK	Natural Killer
NSCLC	Non-Small Cell Lung Cancer
OAd	Oncolytic adenovirus
OVs	Oncolytic Virus
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase chain reaction
PD-1	Programmed (cell) death 1
PD-L1	Programmed (cell) death ligand 1
PD-L2	Programmed (cell) death ligand 2
PeptiCRAd	Peptide-coated Conditionally Replicating Adenovirus
PgR	Progesterone Receptor
PRR	Pattern Recognition Receptors
Rb	Retinoblastoma
RPMI	Roswell Park Memorial Institute
SERMs	Selective Estrogen Receptor Modulators
T reg	Regulatory T cell
TAA	Tumour Associated Antigens
TIL	Tumour-infiltrating lymphocytes
TIM-3	T-cell immunoglobulin and mucin-domain containing
TLR	Toll like receptor
TLR	Toll Like Receptor
TNBC	Triple Negative Breast Cancer
TNF	Tumour Necrosis Factor
TP	Terminal Protein
TSA	Tumour Specific Antigens
VEGF	Vascular Endothelial Growth Factor
VP	Virus Particle
VSV	Vesicular Stomatitis Virus
WHO	World Health Organization

ABSTRACT

In according to the last available data, cancer is the second cause of death in many countries, following only cardiovascular diseases. As the risk of cancer increases in the elderly and also because of other factors such as tobacco, low vegetable and fruit intake, pollution and that develop countries are aging and are notorious for their "unhealthy lifestyle", soon the cancer will be the first "killer" in the world: new treatments approaches are needed in order to replace or combine the classical treatments such as surgery, radiotherapy and chemotherapy. Among the new therapeutic approaches, one emerging and promising field is the immunotherapy, which aim to elicit de novo anti-tumour response and/or boost the pre-existing anti-tumour immunity.

The cancer immunotherapy consists of different approaches such as oncolytic viruses, which are able to replicate only in the cancer cells, and the immune checkpoint inhibitors, which revert or prevents the T-cell exhaustion. Both approaches showed efficacy in the eliciting anti-tumour immune response, but there are solid tumours poor immunogenic and immunosuppressive that could benefit from a combination of these treatments, such as Triple-negative breast cancer.

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer currently resistant to available treatment approaches; therefore, in the present study, we decided to evaluate the efficacy of a tumour-specific vaccine platform based on peptide-coated oncolytic adenovirus (PeptiCRAd) that we had previously developed for different tumours.

PeptiCRAd is a versatile and rapid system to adsorb tumour-specific major histocompatibility complex class I (MHC-I) peptides onto the viral surface to drive the immune response toward the tumour epitopes. In fact, the combination in a single treatment of the adjuvancy of the virus with the immunological targeting of tumour-derived peptides converts the powerful anti-viral immune response obtained with viral vaccines into a more efficient anti-tumoural response.

In the present work, we adapted PeptiCRAd in a poor immunogenic and high immunosuppressive tumour model such as TNBC and for the first time we improved the PeptiCRAd platform adding on the same oncolytic vaccine tumour peptides restricted for both MHC-I and MHC-II in order to harm TCD8^{+} and TCD4^{+} lymphocytes and to obtain a more efficient and complete immune response.

Tumour cells evade immune recognition and destruction by down regulating MHC-I and up-regulating PDL-1. Thus, we chose and characterized human (MDA-MB-436) and mouse (4T1) triple negative breast cancer cell lines for the expression of MHC-I, MHC-II and PDL-1, demonstrating that they are a solid model for our immunotherapeutic approach. As PeptiCRAd relies on Ad-5-D24-CpG, we demonstrated that has similar cytopathic effect (CPE) to Ad-5/3-D24, already validated in human model of TNBC.

In the first set of *in vivo* experiments, we observed that oncolytic vaccines coated with a combination of MHC-I and MHC-II peptides induced a stronger response compared to those coated with either MHC-I or MHC-II peptides. Interestingly, we also observed that administration of mixture of equal concentrations of oncolytic vaccines coated with MHC-I or MHC-II peptides is less efficient compared to the double coated formulation: therefore, we concluded that MHC-I and MHC-II peptides have to be loaded on the same surface to maximize the effect.

Next, we evaluated the synergistic effect of administration of the PeptiCRAd-D.C. preparation with anti-PDL1 antibody in TNBC; our results clearly demonstrated a significant improvement of the oncolytic vaccine efficacy when administrated in combination with anti-PDL1.

Finally, we translated our treatment in relevant human model of TNBC, we performed a Cytotoxic T-lymphocytes (CTL) killing assay in a co-culture experiment with human tumours. *In vitro* we pulsed with our vaccine human peripheral blood mononuclear cell (PBMCs) HLA-matched with the tumours and we added them to the tumour sample; cancer cells viability was then evaluated. The tumour peptides selected in the above experiment for the PeptiCRAd preparation were selected

from well-known human triple negative breast cancer antigens. In addition, one tumour peptide was selected by using an improved version of ligandome analysis.

In conclusion, we have demonstrated for the first time the efficacy of PeptiCRAd technology based oncolytic vaccine in a challenging model of TNBC; in addition, we observe that vaccine coating with a combination of MHC-I and MHC-II restricted peptides is more effective than the previously used MHC-I restricted peptides coating, leading to a further improvement of the system.

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1. INTRODUCTION

1.1 Cancer: a global health problem

Tumour is the result of uncontrolled cell proliferation and can arise from any kind of cells of our body, so that we have at least 200 different type of tumours. We distinguish benign and malignant tumour.

The benign tumour is located at its original site, not invades the surrounding tissues either spreads in different part of the body; the malignant tumour is properly referred as cancer and owns both capacities of invading surround tissues and spreading in different parts of the body.

At cellular level, cancer is defined as a multistep process, in which the cells, because of genetic and epigenetic changes, mutate and a clone of tumour cells acquires selective advantages among the other cells such as evading growth suppressors. Other hallmarks of cancer consist of self-sustaining proliferative signalling, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, deregulating cellular energetics, genome instability, avoiding immune system and tumour-promoting inflammation (Hanahan and Weinberg 2011) . Therefore, the cancer is a complex disease and especially in the advanced stages difficult to eliminate.

According to the last data released by World Health Organization (WHO) (Ferlay et al. 2015) cancer is the second leading cause of death in the world, following only cardiovascular diseases; the principle risk factors are behaviour related and include smoking, alcohol, low vegetable and fruit intake, lack of physical activity and ageing; all these factors are being common, in particular, in the develop countries. In fact, the number of new cases of cancer is expected to rise by about 70% over the next 2 decades. Nevertheless, the cancer has a strong economic impact: in 2016, it has been estimated a total cost, comprehensive of oncology and supportive care, of 113 billion at a growth rate of 11.6% (imshealth.com/global oncology trends 2017).

It is clear that the cancer represents a public health emergency and in order to eradicate it new therapies are urgently needed; in fact, the actual treatment approach consists of surgery, radiotherapy and chemotherapy with their well know side effects; however, in the last years is emerging a new field with encouraging clinical results: the cancer immunotherapy.

1.2 Cancer Immunotherapy

The cancer immunotherapy is defined as the science that harness the immune system to fight the cancer. Even if the immunotherapy is attracting the public attention only in the last years, it goes back as far a 100 years ago.

The history of immunotherapy begins in 1891 with the Coley´s toxins; William Coley noticed that a 31-old man with a terminal sarcoma, had a completely cancer regression, as result of the erysipelas, a skin infection caused by streptococcus bacterium. Following this observation, Coley injected the erysipelas bacterium in the tumour of a man with a terminal neck cancer; the patient survived cancer free for eight years.

In the early 20 centuries, still little was known about the immune system and also less about the interplay with the cancer. The Coley´s toxins worked sporadically and no one knew "*how*", so that the Coley´s work was forgotten.

In 1957 Burnet and Thomas introduced for the first time the concept of the immune surveillance (Burnet 1957), concept developed later in immune editing (Dunn et al. 2002).

The immune surveillance is the process in which the immune system recognizes and eradicates cancer cells; however, this concept describes only the protecting host function of the immune system in the early stages of the cancer transformation; later, in order to include also the tumour sculpting activity on the immune system, the concept was developed from immune surveillance to immune editing.

The immune editing is described with the acronym "three E´s" (Elimination, Equilibrium and Escape).

The elimination phase coincides with the immune surveillance, in which the immune system recognizes and eliminate the cancer cells.

The equilibrium phase is the process by which the immune system controls but not fully extinguishes the tumour cells genetically unstable and highly mutated. New tumour clones arise during the process and are subject to a strong selection: the cells that survive to the immune attack will be able later to escape to the immune control. This is the longest phase of the immune editing and require years.

The escape phase is the process wherein the immunological sculpted tumour expands in uncontrolled manner (Dunn et al. 2002; Kim, Emi, and Tanabe 2007).

During the years, several breakthroughs have been achieved in the field of the cancer immunotherapy: discovery of the dendritic cells (DC) (Steinman and Cohn 1973), discovery of MHC-I restricted T-CD8 (Zinkernagel and Doherty 1974a) (Zinkernagel and Doherty 1974b), first administration of autologous T-cell (Rosenberg et al. 1985), first study with tumour necrosis factor (TNF) in melanoma and sarcoma (Lienard et al. 1992) until the first vaccine approved by the FDA to prevent the cancer caused by the human papilloma virus (HPV) (Kenter et al. 2009).

However, the cancer immunotherapy is still growing and the aim is inducing the anti-tumour response, stimulating the person´s immune defences. To this end two main approaches have been developed: 1. non-antigens specific and 2. antigens specific strategies.
The first approach is comprehensive of therapies such as non-specific immune stimulation (use of cytokine like TNF and interleukine2 (IL-2)) and inhibition of immune check point;
The second approach educates the immune system to recognize the tumour as non-self by using techniques like adoptive cell transfer and therapeutic vaccination (Lesterhuis, Haanen, and Punt 2011).

1.2.1 Cancer and Immune checkpoints

Immune checkpoints are molecules involved in the checking and in the balance of the immune system by controlling its activation. Under physiological conditions, the immune checkpoints maintain the self-tolerance, avoiding autoimmunity reaction and protecting the tissue from collateral damages when the immune system is activated in response to infections or other pathogens (Pardoll 2012).

The cancer cells dysregulate immune checkpoints, as one of the mechanism of resistance, in particular against the T-cells. In fact, T cells selectively recognize peptides derived from proteins in all cellular compartments: TCD8$^+$ cells, also known as CTLs, identify and kill antigen-expressing cells; TCD4$^+$, also known as helper T cells, orchestrate diverse immune responses, by integrating adaptive and innate effector mechanisms: hence, TCD8$^+$ and TCD4$^+$ are the main protagonists in the orchestrating the anti-tumour immune response (Pardoll 2012; Sharma and Allison 2015b, 2015a).

The blockade of immune checkpoints, by using monoclonal antibodies, is currently under investigation in order to unleash the T-cell mediated anti-tumour response. The immune checkpoint inhibitors work blocking the inhibitory pathway and are mainly focus on two immune check points: CTLA-4 and PD-1/PD-L1(table 1).

Anti-CTLA-4 is the first immune checkpoint inhibitor used for the treatment of cancer, discovered and tested by Allison and colleagues (Leach, Krummel, and Allison 1996); biologically CTLA-4 counteracts the activity of the T cell co-stimulatory receptor, CD28, with which shares the ligands CD80 (B7.1) and CD81 (B7.2) in the lymphoid compartment. Thus, CTLA-4 regulates the T-cell activation.

Unlike CTLA-4, the pathway PD-L1/PD1 regulate the effector T- cells; hence, PD1/PD-L1 occur in the late stages, when the T-cells are already in the tumour. In the present work, we focused our attention on this pathway.

Name	Target	Indication
Yervoy (ipilimumab) from Bristol Myer Squibb	CTLA-4	Advanced melanoma
Keytruda (pembrolizumab) from Merck	PD-1	Advanced melanoma, NSCLC, Hodgkin´s lymphoma and head and neck cancer
Opdivo (nivolumab) from Bristol Myer Squibb	PD-1	Advanced melanoma, advanced NSCLC, advanced renal cell cancer, bladder cancer, Hodgkin's Lymphoma and squamous cell carcinoma of the head and neck
Tecentriq (atezolizumab) from Genentech	PD-L1	Advanced bladder cancer and NSCLC
Bavencio (avelumab) from Pfizer	PD-L1	MCC and bladder cancer
Imfinzi (durvalumab) from AstraZeneca	PD-L1	Advanced bladder cancer

Table 1 Source:
https://www.drugs.com/slideshow/immunecheckpoint-inhibitors-1249

1.2.2 PD-1/PD-L1: immunosuppressive pathway in the tumour bed

The pathway PD-1/PD-L1 fine tunes the immune response, maintaining the self-tolerance, mainly regulating the T-cell activity and it is made of PD-1(CD279) and its ligand PD-L1(B7-H1, CD274) and PD-L2 (B7-DC, CD273) (Riley 2009).

PD-1, encoded by the PDCD1 gene, is a receptor and, in particular, it is a type I transmembrane protein of 268 amino acids, member of the extended CD28/CTLA-4 family T-cell regulators (He et al. 2015).

The structure includes an extracellular IgV domain, a transmembrane region and an intracellular tail. This latter consists of two regions: the immune receptor tyrosine-based inhibitory motif (ITIM) and the immune receptor tyrosine-based switch motif (ITSM). The ITSM is involved in the T-cell inhibitory function. PD-1 is mainly expressed on the surface of T-cells, B-cells, activated monocytes, natural killer (NK), DC and TILs (He et al. 2015; Ceeraz, Nowak, and Noelle 2013). In addition is also expressed on T reg, facilitating their proliferation and the inactivation of immune system (Francisco et al. 2009).

PD-L1 was described for the first time at Maya Clinic and identified as B7-H1 (Dong et al. 1999); later, it was discovered to be a ligand of PD-1, from which take the actual name PD-L1.

PD-L1 is a transmembrane protein of 290 amino acids and consists of extracellular domain (signal sequence, IgV-like domain, IgC-like domain), transmembrane domain and intracellular domain. This latter is made of only 30 amino acids conserved across diverse species and without a known function (Keir et al. 2008). The same structure has been described for PD-L2.

The two ligands of PD-1 differ in their expression pattern; PD-L1 is constitutively express on T and B cells, DC, macrophages,

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mesenchymal stem cells (MSCs), bone marrow derived mast cells (BMMC) and also on a wide range of non-hematopoietic cells; instead PD-L2 expression is only inducible on DCs, macrophages, BMMC and resting peritoneal B1 cells(Keir et al. 2008).

PD-L1 is induced by pro inflammatory molecules such as types I and II IFN-γ, TNF-α, lipopolysaccharide (LPS), granulocyte macrophage colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), IL-10 and IL-4;

Activated type 1 T cells produce IFN-γ and TNF-α, on the other hand cancer stromal cells release GM-CSF and VEGF; hence, the tumour microenvironment upregulates PD-L1 expression, creating an effect called "adaptive immune resistance", since the tumour protects itself by inducing PD-L1 in response to IFN-γ produced by activated T cells and in response to GM-CSF and VEGF produced by the cancer stromal cells (He et al. 2015).

The pathway PD-L1/PD-1 modulate the immune response in physiological condition and in cancer, by diverse mechanisms (Fig.1):

1. Apoptosis: the PD-L1 engagement to its receptor PD-1 induces the phosphorylation of two intracellular tyrosines, recognized and bind by two phosphatases: SH2-domain containing tyrosine phosphatase 1 (SHP-1) and 2 (SHP-2). The binding of SHP-1 is weaker than SHP-2: in general, the signalling is modulated through SHP-2. The SHP-2 inhibits PI3K that in turn cannot activate AKT. This latter cannot induce Bcl-xL, anti-apoptotic gene (Keir et al. 2008; He et al. 2015).

2. Anergy and Exhaustion: anergy and exhaustion occur when the effector T cell lose its effector function during chronic infections and cancer. The exact mechanism is not completely understood, but it has been observed that they occur in response to high level of PD-L1(Pauken and Wherry 2015) that inhibits the expression of GATA3, Tbet and Eomes, all transcription factor associated to the effector T cell (Nurieva et al. 2006).

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3. Treg induction: The PD1/PD-L1 interaction induces the down-regulation of AKT/mTor, inducing the expression of transcription factor Foxp3, characteristic of Treg cells. Furthermore, PD1/PD-L1 downregulates the phosphorylation of p42/ERK, hence MAP kinase cascade signalling, promoting the Treg development (Francisco et al. 2009).

4. Anti-proliferative effect: PD1/PD-L1 interaction induces the down-regulation of AKT/PI3K and Ras/MEK/ERK signalling with accumulation of p27 and p15 that restrain the T cell in the G1 phase of the cell cycle (Patsoukis, Sari, and Boussiotis 2012). The PD1 engagement reduces also the phosphorylation of PCK-theta that is essential for IL-2 production (Sheppard et al. 2004).

5. Inhibition of T cell activation: PD1/PD-L1 blocks also the signalling downstream triggered by the interaction antigen (Ag)/MHC; in fact, PD1 ligation induces the de-phosphorylation of ZAP70/CD3ζ, blocking the downstream signalling activation pathway CD3 mediated (Parry et al. 2005).

The cancer environment produces immunosuppression in the tumour bed by inducing PD-L1; therefore, cancer characterized by immune infiltration benefit from treatments with immune check point inhibitors. Nevertheless, as another resistance mechanism, the cancer can loss the immune infiltration, and in this case of "cold" tumour, the immune check point inhibitors results ineffective.

Nowadays, the main aim is converting the tumour from "cold" (lack of immune infiltration) to "hot" (presence of immune infiltration) and to this end it's necessary to identify which is the best standard care therapy to combine with immune checkpoint inhibitors and how;

Among the several combination approaches proposed, oncolytic viruses result the best way to stimulate the T cell infiltration in the tumour bed; in fact, talimogene laherperepvec (T-VEC), oncolytic herpes virus, is currently studied in combination with ipililmumab

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(anti-CTLA4) in a phase II trial (NCT01740297) and with pembrolizumab (anti-PD1) in a phase Ib/III trail (NCT02263508) in melanoma patients (Swart, Verbrugge, and Beltman 2016).

T-VEC (trade named Imlygic, Amgen) is a herpes virus modified to express GM-CSF and approved by FDA in October 2015 to treat melanoma patients: T-VEC represents the first drug, belonging to oncolytic viruses' class, approved in West to treat cancer patients (https://www.fda.gov/newsevents/newsroom/pressannouncements/uc m469571.htm).
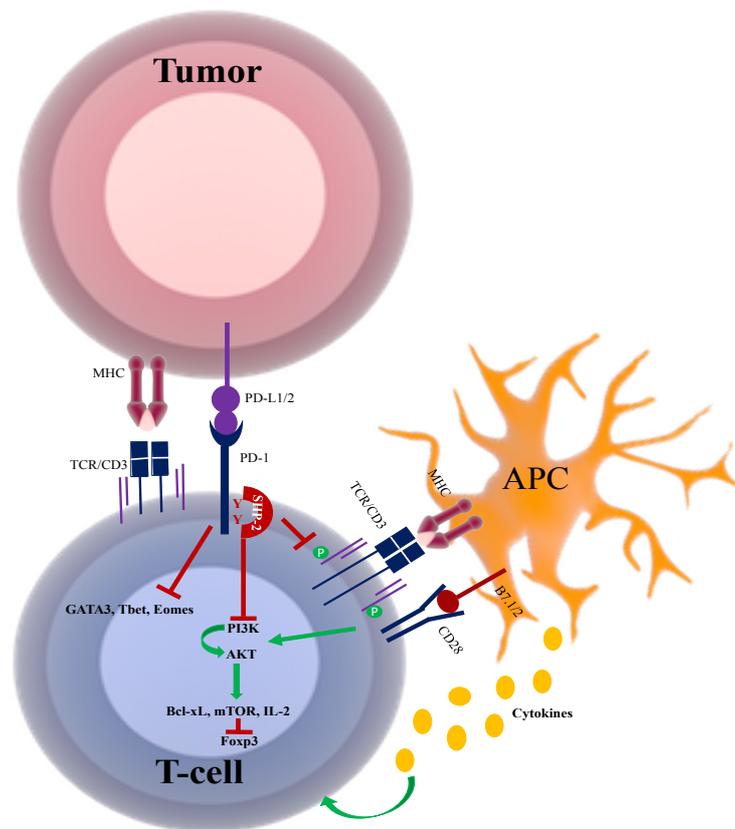
Figure 1 PD1/PD-L1 interaction decreases the TCR signalling. The T-cell activation requires three signals: contact peptide/MHC complex, CD28/B7.1/2 and cytokines, all signals from APC. The PD-1 engagement leads to the downregulation signalling pathway mediated by TCR in different ways; one of the mechanisms PD-1 mediated is the de-phosphorylation of PI3K with the AKT downregulation pathway.

1.3 Oncolytic Viruses

Oncolytic viruses (OVs) refer to a wide range of natural or genetically modified viruses that are able to induce cancer cells lysis by different mechanisms during their life cycle, except the retrovirus, that can be rendered lytic by toxic transgene expression (Lawler et al. 2017).

The wild type OVs in clinical are few: reovirus and coxsackievirus, both with human host and OVs with animal host such as Newcastle disease virus (avian), parvovirusH1 (rat) and vesicular stomatitis virus (VSV) (insects, horses, cows and pigs). On the other hand, most of the OVs are engineered to improve the cancer cell selectivity; for instance, Herpes simplex virus 1 (HSV1) brings the deletion in ICP6 gene; this deletion allows the HSV1 replication in the cells with inactivation of the p16INK4A, a tumour suppressor, often mutated in cancer cells; similarity, OAd brings a deletion of 24 base pairs in E1A gene, that allows the virus replication only in the cells with inactivation in oncosuppressor retinoblastoma (Rb), commonly mutated in the cancer cells (Lawler et al. 2017).

Even if during the years most of the researches were focused on the identification and/or engine viruses selectively replicative in cancer cells, nowadays it is often appreciated an immune component in their action mechanism, so that OVs are currently considered immunotherapy agents, not anymore components of the virotherapy, but of the immune virotherapy.

In fact, OVs induce systemic innate and tumour-specific adaptive immune responses; following the cancer cell lysis, tumour associated antigens (TAA) are released; TAA promote the tumour-specific adaptive immune response, by inducing tumour regression also in sites far from virus infection. The lysis cause also the release of viral Pathogen Associated Molecular Pattern (PAMP) such as LPS, dsRNA, CpG islands, recognized by receptor of innate immune response called Pattern Recognition Receptors (PRR), present in APC.

In addition, Danger Associated Molecular Pattern (DAMP) (for example high mobility group box 1 (HMGB1), ATP, uric acid, calreticulin) and cytokines (IFN I, IFNγ,TNFα, IL-12) are also released, inducing the maturation and the activation of the APCs. These activate antigen specific TCD4$^+$ and TCD8$^+$ responses; upon antigen specific activation, the TCD8$^+$ can expand in CTLs and migrate to the sites with established tumour growth, in which they start the anti-tumour specific immune response (Kaufman, Kohlhapp, and Zloza 2015).

Hence, OVs infect and lysis the cancer cells, with release of TAA, PAMP, DAMP and cytokines; in particular, TAA in combination with cytokines and DAMP are able to induce and sustain anti-tumour immune response.

1.3.1 Adenoviruses

The Adenoviruses are the most common vectors used in the gene therapy and we also used them in the present work for our experimental approaches.

The Ad were described for the first time by Rowe and colleagues during a study of the growth of human adenoid tissue; they observed the presence of a transmissible agent, that was able to induce tissue and cell degeneration; because of that, they called this agent the "Adenoid Degeneration agent " "A.D. agent" (Rowe et al. 1953), from which take the actual name "Adenovirus".

The Ads belong to the genus *Mastadenovirus* and are subdivided in seven species (A-G), based on their ability to agglutinate erythrocytes (Rosen 1960); in turn, the species consist of 68 serotypes; the classification of 1-51 Ads serotypes relies on the traditional serological methods in composition and pathogenicity, whereas the classification of 52-68 Ads serotypes is based on genomic sequencing and bioinformatics analysis.

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Typical adenoviral infections occur in children, are self-limiting and consist of different clinical events, including conjunctivitis, gastroenteritis, hepatitis, myocarditis and pneumonia (Ghebremedhin 2014).

As Ads infect dividing and not-dividing cells, the most common platforms based on oncolytic viruses use Ad; in particular serotype 5 Ad (Ad5) is a popular vector in the oncolytic immunotherapy, since its biology is well known and it is relatively harmless; Furthermore, Ad5 is classified as specie C and among the other viruses, it is the more effective in the binding of the coxsackie and adenovirus receptor (CAR) (Shashkova, May, and Barry 2009); finally, the Ad5, being genetically well characterized, can be easily manipulated and modified to express immune-stimulatory molecules, for example cytokines (Choi et al. 2012).

1.3.1.1 Structure

Adenoviruses have a size in range from 65-80nm in diameter and a molecular weight of ~150MDa; the virions are non-envelop (no outer lipid layer) with an icosahedral protein capsid, containing a linear duplex DNA genome of approximately 36,000 base pairs and internal proteins (Wold and Toth 2013).

The capsid consists of 252 capsomeres, subdivided in 240 hexons and 12 pentons, corresponding to the twelve icosahedral vertices ; the base of the penton is made up of a fiber, which length varies among the serotypes; the fiber consists of three domains: a short N-terminal tail that attaches the fiber to the penton base, a shaft domain comprised of a repeating triple β-spiral motif and a globular knob domain, required for the receptor binding; in particular, the binding occur at lateral surface of the knob rather than at its extreme distal portion (Campos and Barry 2007; Smith et al. 2010).

In detail, the icosahedral capsid contains seven different proteins: II, III, IIIa, IV, VI, VIII and IX; in particular, the icosahedron is composed by twenty facets, made up of 240 trimers of hexon (protein II), whereas

the penton is a complex of proteins III and IV. The virion stabilization is mediated by IIIa, VI, VIII and IX, which are proposed to be the "cement" of the structure (Smith et al. 2010).

The viral genome inside the capsid encodes 35 proteins and it is structurally associated and condensed with the proteins V, VII and X (µ) and a two copies of a 55KDa terminal protein (TP) is covalently linked to each 5´ends of Ad DNA (Smith et al. 2010) (Wold and Toth 2013; Campos and Barry 2007) (Figure 2).
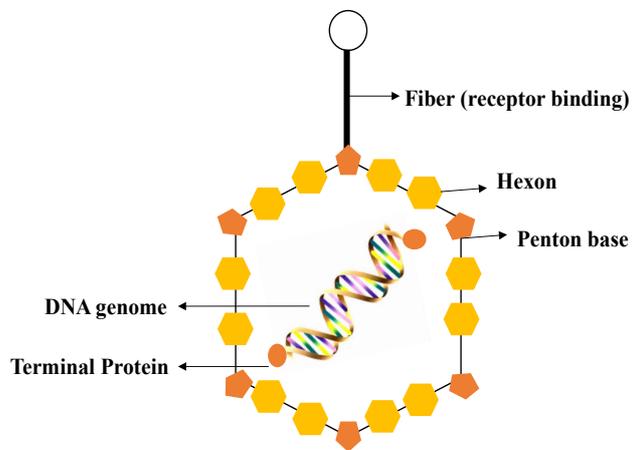
Figure 2 Adenovirus virion structure: capsid and core.

1.3.1.2 Life cycle

The viruses are defined obligate intracellular parasites: they completely lack the machinery needed for their reproduction, so that they depend on cellular machinery of the host for viral gene expression and for synthesis of proteins. The Ad are not an exception.

In fact, he adenoviral life cycle starts with the cellular infection, mediated by the binding of distal knob domain of adenoviral fiber on the host cells receptors; during their evolution, the Ad have exploit diverse cell-surface molecules interaction, which determined the actual adenoviral tropism. Most of Ad subgroups recognize the CAR receptor, a type 1 transmembrane protein, belonging to the immunoglobulin family, involved in the formation of the cellular thigh junction and localized at level basolateral (Campos and Barry 2007).
Instead, the adenoviral subgroup B, which includes Ad3, uses either CD46, CD80/CD86, an unidentified glycoprotein receptor or a combination of these. In addition, it has been observed that Ad5 interaction with certain cellular types can be favourite by the presence of heparan sulfate proteoglycans (HSPGs) (Campos and Barry 2007).

After the binding to the primary receptor, the motif Arg-Gly-Asp (RGD) on the adenoviral base penton interact with the cellular αv $\beta 3$ and αv $\beta 5$ integrins; this binding triggers the signalling cascade and the clathrin-mediated endocytosis (Russell 2009).

Following the internalization, the virions, inside the vesicles, enter in the endosomal pathway; the acid endosomal environment induces irreversible structural changes in the penton and capsid: the virus became uncoated (in this stage the virus is surrounded only by hexons). The modified conformation allows the endosomal escape, in which the virus passes from the vesicles in the cytoplasm. Here, the partial adenoviral capsid binds the dynein, a cellular motor protein, that bring the virion to the microtubule organizing centre (MTOC) close to the host cellular nucleus. By an unknown mechanism, the virion enters through the nuclear pores in the nucleus, where it initiates the transcription of the early genes (E1-E4), and late genes (L1-L5), respectively before and after the DNA replication.

E1A is the first protein expressed and it is involved in the modulating the cellular metabolism to facilitate the virus reproduction; then, it influences the proteins that control the cell cycle; for example, E1A binds Rb and makes free the transcription factor E2F, favouring the enter of the host cell in S phase of the cell cycle. In sequence are expressed the following genes products: E1B that control the late virus messenger RNA (mRNA) transcription, stabilize p53 and function as important effector of inflammation *in vivo*; E2 that provide the machinery for the DNA replication; E3 inhibits the anti-viral host defence response; E4 that facilitate the virus mRNA metabolism and the transcription of the late genes. After the DNA replication, the L1-L5 are expressed: they are structural proteins that allow the virion formation.

The adenoviral life cycle ends with the lysis of the host cells and the release of the new virions. The early phase requires 6-8 hours and the late phase 4-6 hours (Russell 2000).

1.3.1.3 Modifications for oncolytic strategies

As previously mentioned, Ads infect their target cells, replicate inside and, in the end, induce the lysis of the host cells; during the years, this lytic property has been exploited to kill the cancer cells.

In order to use Ads as oncolytic agents, two main strategies have been used: promoting the virus-replication restricted to cancer cells and enhancing tropism towards the tumour, modifying the structural components (trasductional targeting) or the gene expression (transcriptional targeting) (Barnett et al. 2002).

One approach to generate a replication-selective Ad is the deletion of viral genes needed for the correct replication in normal cells, but not in cancer cells. Oncorine (H101) in China and ONYX-015 (dl/1520) (Kirn 2001) in USA are the first oncolytic Ad developed following this strategy: in both cases the OAd has been generated by deleting the E1B gene. This modification allows the OAd to infect and kill p53-deficient cancer cells; however, only 50% of the cancer cells own p53 mutation

and the lack of E1B reduces the replication and production of Ad, that in turn reduces the anti-tumour efficacy (Choi et al. 2012).

Since E1A is the first gene expressed, after the virus infection, one of the most useful modification to generate the OAd is the 24-base pair deletion (D24) in the pRb binding site of the E1A region (Fueyo et al. 2000). In normal cells, the Ad-D24, because of that deletion, is unable to bind Rb, that in turn form a complex with E2F, resulting in the inhibition of E2F-mediated activation genes involved in the viral replication and cell cycle progression. Instead, in cancer cells pRB/p16 pathway is often inactivated (Knudsen and Wang 2010) and this allow Ad-D24 to replicate in malignant cells (Fueyo et al. 2000).

In addition to genetic manipulations, several approaches have been used to enhance the immune-stimulation OAd mediated, such as Ad5D24 engineered with 18 immunostimulatory islands (Ad5D24CpG) (Cerullo et al. 2012) or engineered with GM-CSF (Ad5/3D24-GM-CSF, Oncos 102) (Kuryk et al. 2017). Then, another approach is the generation of chimeric Ad-5/3-D24, in which the knob domain belongs to Ad3 and tail and shaft domains of Ad5; this chimeric Ad is able to infect a large spectrum of cancer cells compare to Ad5: the primary binding is mediated via Ad3 receptor and the following internalization achieved by domain of the penton base of Ad5 (Krasnykh et al. 1996). Finally, the inclusion motif such as RGD and/or poli-lysines (pK7) in the adenoviral capsid, increase the infection, making the virus able to infect positive and negative CAR cells (Wu et al. 2002; Pesonen et al. 2012).

1.3.1.4 Anti-viral and anti-tumour immune responses

The infection Ad or OAd mediated induces innate and adaptive anti-viral immune response, being virus recognized as "non-self" by DCs and macrophages, the frontline against the viral infection (Akira, Uematsu, and Takeuchi 2006). Following the virion internalization, the adenoviral un methylated CpG dsDNA (member of PAMP family) in the endosomal compartment binds Toll Like Receptor 9 (TLR9, member of the PRR family); this interaction leads via Myd88 and/or

TRIF to the production of type I IFNs and to other pro-inflammatory cytokines (for example IL-6, IL-12, TNF-α) (Nociari et al. 2007).

In particular, type I INFs play a pivotal role in the innate and adaptive anti-viral immune response: type I INFs promote DCs maturation, the survival of activated anti-viral T cell and the production of neutralizing antibodies (NAbs) in response to adenoviral infection, activating B-cells and TCD4$^+$ (Zhu, Huang, and Yang 2007; Huang and Yang 2009) The cytokines released function as chemoattractant for macrophages, neutrophils and NK, that are recruited at infection site and can directly engulf the virus (Thaci et al. 2011).

Furthermore, as DCs and macrophages are professional APC, they uptake, process and cross-present adenoviral components in the context of MHC-I and MHC-II, activating the TCD8$^+$ and TCD4$^+$, respectively. Whereas TCD8$^+$ directly kill the cell infected with the virus, the TCD4$^+$ collaborate with B-cells for the antibodies production (Heath and Carbone 2001).

In addition to anti-viral immune response, OAds trigger a strong anti-tumoural immune response. Indeed, OAds induce cancer cell lysis, enhancing the availability of TAA and unclosing hidden tumour antigens to APCs (for example DCs); then, the lysis releases DAMPs (uric acid, HSP) that being danger signals, activate DCs (Prestwich et al. 2008; Pesonen, Kangasniemi, and Hemminki 2011).

Hence, DCs take up the TAA, integrate the danger signals and present the antigen in a correct costimulatory context to activate the harm of adaptive immune response, in particular effector TCD8$^+$. Although TAA are exogenous antigens and should be present in the context of MHC II to activate TCD4$^+$ immune response, however the DCs can present these antigens in the context of MHC I, activating TCD8$^+$; this biological event is termed "cross-presentation" and the viral signal seems a strong inducer of the cross-priming (Schulz et al. 2005).

OAds induce the anti-viral innate and adaptive immune response plus an anti-tumour specific response. In order to increase the OAd immunostimulatory properties, several genes coding cytokine such as IL23 (Choi et al. 2013), INF-α (LaRocca et al. 2015), CD40L (Iida et al. 2008) or GM-CSF (Kuryk et al. 2017) have been introduced in the

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adenoviral back-bone; also OAds coding tumour antigens have been designed (Sorensen et al. 2009) in order to induce a stronger anti-tumour specific response. The activity of genomic modified OAds relies on efficient transcription, often limited by the presence of Nab; furthermore, the genetic manipulation requires time and it is not compatible with the growing demand of fast personalized medicine.

So that, in our laboratory we developed PeptiCRAd, a simply and fast system to adsorb the TAA on the OAd (Ad-5-D24-CpG) capsid in order to elicit a powerful TCD8+ anti-tumour specific activity (Capasso et al. 2016); in the present work, we proposed an improvement of the system, by using TAA to induce TCD8$^+$ and TCD4$^+$ immune response, in order to have a more complete response. In addition, we are also developing an innovative and fast technology (PeptiCHIP) for the identification of TAA, in order to respond to the new horizon of personalized medicine.

Since OAds increase the immune infiltration in the tumour bed, they represent powerful tool to induce the anti-tumour immune response; nevertheless, the tumour protect itself generating immunosuppressive microenvironment, for example over-expressing immune check point molecules such as PD-L1; but the combination immune checkpoint inhibitors (to control the immune-suppressive environment) and OAd (to recall the anti-tumour response) represent nowadays one of the most promising treatment of poor immunogenic tumours such as TNBC.

1.4 Triple Negative Breast Cancer

Triple negative breast cancer (TNBC) is still one of the most aggressive cancer form with no approved systemic treatment approach (Jitariu et al. 2017); TNBC is associated with younger age and advanced stage at diagnosis, increased risk of visceral metastasis and poorer outcome than the other breast malignant forms (Wahba and El-Hadaad 2015; Zeichner, Terawaki, and Gogineni 2016). As TNBC lacks of the expression of therapeutic targets (Foulkes, Smith, and Reis-Filho 2010), patients do not benefit from the already approved therapy for the treatment of other form of breast cancer (Mousavi et al. 2013).

In fact, TNBC is characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PgR) and human epidermal

growth factor receptor 2 (HER2) (Bianchini et al. 2016). Therefore, TNBC is not responsive to hormonal therapies, including aromatase inhibitors, SERMs (selective estrogen receptor modulators) and ERDs (estrogen receptor down regulators) or to anti-HER2 monoclonal antibody (Herceptin), all therapies approved by FDA (Wahba and El-Hadaad 2015). In absence of an approved systemic treatment approach, TNBC is still treated with a combination of radiotherapy, surgery and chemotherapy (Bianchini et al. 2016; Wahba and El-Hadaad 2015). Nevertheless, TNBC represents 15-20% of total breast cancers and tends to be more aggressive than the others (Stagg and Allard 2013).

Because of that, TNBC is still an important challenge in the research as in the clinical fields (Narod, Dent, and Foulkes 2015), highlighting the needed of a new therapeutic approach.

In general, TILs have been used to evaluate the immune responsiveness to the tumour peptides; recent studies have evaluated the TILs in TNBC, demonstrating the presence of reactive T-cell to new peptides (named neoantigens), arising from the tumour (Garcia-Teijido et al. 2016); the presence of TILs is considered a prognostic factor (Loi et al. 2014): TNBC patients without treatment and positive for TILs, own improved overall survival, increased metastasis-free survival and decreased distant recurrence (Loi et al. 2014; Garcia-Teijido et al. 2016).
In addition, TNBC have been extensively studied for PD-L1 expression; unlike other breast cancers, TNBC is characterized by high expression level of PD-L1 and also high level of TILs positive for PD-1, making the tumour environment strongly immunosuppressive (Mittendorf et al. 2014; Tung et al. 2016).

This line of evidence underlines the rationale of immunotherapy in the TNBC; in fact, several recent clinical trials based on anti-PDL1, have been performed in patients with metastatic TNBC, showing real promising results (Emens et al. 2015; Nanda et al. 2016).

Following these researches, in the present work we propose to combine the anti-PDL1 approach with OAd, in order to elicit a specific anti-tumour TILs infiltration and break the immunosuppressive TNBC

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behavior. In order to have a human and clinical relevant model of TNBC, in the present work have been extensively used PBMCs.

1.5 Peripheral Blood Mononuclear Cells

Peripheral Blood Mononuclear Cells, isolated from peripheral blood, are any blood cells with a round nucleus from innate and adaptive immune system (de Mello et al. 2012); in detail, PBMCs include lymphocytes (T cell, B cell and NK), monocytes and DCs in according to the following percentage: lymphocytes 70-90%, monocytes 10-20% and DCs 1-2%; in turn, the lymphocytes consist of 70-85% TCD3$^+$ cells, 5-10 % B-cells and 5-20% NK cells. The TCD3+ compartment is composed mainly of two subpopulations: TCD4$^+$ and TCD8$^+$ approximately in a 2:1 ratio. The T cells present in PBMCs population are mainly naïve or without effector functions (resting) and the percentage of T cell reactive to a specific antigen is low (Kleiveland 2015)

The PBMCs are extensively used as *in vitro* tool to predict the immune-situation *in vivo*, showing to be a valid and predictive method (Vissers et al. 2013; Cribier et al. 1995); in particular, the cytokines production from PBMCs *in vitro* is one of the most used methodology to assess the influence on the immune-system; is common evaluating level of cytokines in the supernatant of cell medium by using ELISA or ELISPOT assay (Dias et al. 2012) or cytokines inside the cells by using intracellular staining (Muris et al. 2012).

1.6 New frontier: Personalized Cancer Immunotherapy

In addition to immune editing, the cancer escapes from immune system recognition by using several mechanisms, that change from patient to patient; consequently, a personalized cancer immunotherapy is urgently needed in order to overcome this issue, to identify the rate-limiting steps in any patients, to decide the strategies and finally to start the correct immunotherapeutic approach (Kakimi et al. 2017). In fact,

cancer cells, unlike their normal counterparts, undergo to several epigenetic and genetic alterations that result in production of aberrant protein termed "tumour antigens" (Gubin et al. 2015). The mutational burden differs among the individual cancers and can be studied by using a combination of next-generation sequencing and bioinformatics tools, allowing the identification of the most suitable targets for the treatment of each malignant (Bethune and Joglekar 2017; Yadav et al. 2014).

1.6.1 Tumour antigens

The capacity of the immune system to fight cancer cells relies on presence of antigens from malignant cells and identified by T cell as "non-self".
In particular, T cells recognize peptide epitopes in the context of MHC-I and MHC-II on the surface of cancer cells (Schumacher and Schreiber 2015).
These antigens are called "cancer rejection antigen" and are subdivided in three categories: tumour associated antigens (TAA), tumour specific antigens (TSA) and cancer-germline/cancer testis antigens (CTAs) (Gubin et al. 2015).

The TAA belong to the cell genome and are overexpressed compare to the normal condition, for example HER2/neu in breast cancer (Baselga et al. 1996); even if they are self-protein, they are subject to incomplete central T cell tolerance, allowing the presence of reactive T cell (Gilboa 1999).

The TSA are completely absented in the cell genome and arise either from oncogenic viral infection or from genomic alteration that result in the formation of new protein sequence, called "neo-antigens"; because of that, TSA are not affected by central T cell tolerance, resulting more visible target for the immune system (Gilboa 1999) and are not shared among the patients.

The CTA are normally expressed in the male germ in the testis and occasionally in fetal ovaries and trophoblast, but are not present in adult tissues in physiologically condition; the CTA regulation is altered in

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cancer, resulting in their expression (Scanlan et al. 2002). Melanoma-associated antigen (MAGE-A1) have been the first CTA isolated, able to elicit a strong T cell response (Boon and van der Bruggen 1996). Because of their restricted tissue expression, the CTA result strong immunogenic.

Hence, TSA and CTA represents attractive targets in the field of the immunotherapy; in particular the "neo-antigens" are attracting the major attention, because as Dr. Schumacher said "The genetic damage that on the one hand leads to oncogenic outgrowth can also be targeted by the immune system to control malignancies (Schumacher and Schreiber 2015)."

1.6.2 Identification of tumour antigens

Among the tumour antigens, the "neo-antigens", because of their immunogenicity, are attracting growing interest in the cancer-immunotherapy. Nowadays, the challenge is the fast and reliable identification of neo-antigens, in order to design an efficient and personalized therapy; to this end several methods have been developed such as exome-sequencing, predicting peptide binding tools and ligandome analysis.

The *next-generation sequencing* RNA extracted from tumour patients is converted in DNA and only the coding part (exome) is sequenced. This technology is called exome-capture and it is based on probes that "capture" the exon sequences; these are released, amplified, quantized and sequenced. This approach has been used for the identification of neo-antigens (Kalaora et al. 2016).

The procedure requires three days, but often the quality/quantity of DNA/RNA from biopsy is not sufficient for an accurate analysis (Gubin et al. 2015).

The *Predicting Peptide binding tools* Algorithms that predict the binding of peptides to MHC I and MHC II rely on bioinformatics and biochemical aspects extensively reviewed, that have been used to generated prediction binding programmes. First examples of these were SYFPEITHI, Rankpep and BIMAS; now some more accurate prediction algorithms are available, that resulted in the notorious

Immune Epitope Database and Analysis Resource (IEDB)(Soria-Guerra et al. 2015).

Since MHC I alleles are polymorphic (in human nearly 2500 MHC I allelic sequences) and the number of potential self and foreign peptides processed by normal, infected, or transformed cells is very large, the accurate prediction of which tumour-derived mutant peptide will bind a particular MHCI is still uncertain (Gubin et al. 2015), but still these programs are actually used for the design vaccination (Gubin et al. 2014)

The *Ligandome analysis* This method, known also as "immunopeptidome", allows the characterization of natural peptides recognized by class I restricted cells, through the direct isolation of ligands from MHC I(Rammensee, Falk, and Rotzschke 1993). Briefly, The MHC I precipitation is performed from biopsy and next the complex MHC I/bound peptides are eluted, in order to collect only the peptides; these are resolved by liquid chromatography coupled to mass spectrometry in tandem (LC-MS/MS). This technology actually identifies about 30% of total eluted proteins and further improvements are needed (Kowalewski and Stevanovic 2013; Rammensee and Singh-Jasuja 2013).

The next-generation sequencing in combination with bioinformatics tools and ligandome analysis identify antigens to generate epitope-based vaccine; however, fast and reliable method are needed for the accurate identification of neo-antigens.

2. AIM

The main aim of the work described in this thesis is eliciting the anti-tumour response in a murine model of Triple Negative Breast Cancer, combining vaccine based oncolytic adenovirus (PeptiCRAd) and anti-PD-L1.
In particular, the goal is

1. Evaluating approaches to enhance the anti-tumor efficacy PeptiCRAd mediated, exploiting MHC-I and MHC-II peptides.

2. Improving the response rate to checkpoint blocking antibodies in the context of a poor immunogenic tumor model (TNBC).

3. Testing the efficacy of the co-treatment anti-PDL1 and PeptiCRAd in a co-culture human system of TNBC.

3. MATERIALS AND METHODS

3.1 Cell lines and reagents

Mouse breast cancer cell line (4T1) and human breast cancer cell line (MDA-MB-436) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) The cells were cultured in RPMI-1640 high glucose and DMEM low glucose respectively and supplemented with 10% FBS ,1% antibiotics and 1% of L-Glutamine.

The human chronic myeloid leukaemia cell line (K562) and the human colon cancer cell line (CACO2) were purchased from the ATCC and cultured in RPMI-1640 low glucose and DMEM low glucose respectively and supplemented with 10% FBS (20% FBS for CACO-2), 1% antibiotics and 1% of L-Glutamine.
All cells were grown in 37°C, 5% CO_2 in a humidified atmosphere.

PBMC were purchased from immune Spot (Bonn, Germany) matched with HLA-A, HLA-B and HLA-DRB1 of MDA-MB-436

Murine and human interferon γ used in the vitro experiment were purchased from Sigma Aldrich and used both at final concentration of 10ng/mL.

The anti-mouse PDL-1(B7-H1) monoclonal antibody was purchased from Bio X Cell and used in animal experiment at 200μg per tumor. The treatment was performed every two days until the end of the experiment.

3.2 Viability assay

MTS assay was performed according to the manufacturer´s protocol (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Nacka, Sweden). Spectrophotometric data were acquired with Varioskan Flash Multimode Reader (Thermo Scientific, Carlsbad, CA, USA).

3.3 Animal Experiments and ethical permits

All animal experiments were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.
Female BALB/cOlaHsd mice 4-6 weeks old were obtained from Envigo (Harlan, USA) and used as a syngeneic mouse tumour model, inoculated subcutaneously with $3x10^5$ 4T1 cells in the right flank.
The treatment was performed on established tumours.

3.4 Flow Cytometry analysis

Flow cytometry analysis was performed using Gallios (Beckman Coulter) flow cytometer, and FlowJo software v10 (Ashland, Oregon, USA). The antibodies used are the following: TruStain Fc block anti-mouse and anti-human CD16/32 (BioLegend); FITC anti-mouse CD8 (ProImmune); APC anti-mouse CD4 (BioLegend); PE-anti-mouse and anti-human CD279/PD1 (BioLegend); PE/Cy7 anti-mouse TIM3 (BioLegend); Percp/Cy5.5 anti-mouse CD3ε (BioLegend); APC anti-mouse H2Kd (BioLegend); APC anti-mouse I/A-I/E (BioLegend); PE-anti-human and mouse CD274 B7-H1 (PDL-1), PE anti-human HLA-A, B, C (BioLegend).

All procedures were performed according to the manufacturer´s recommendation.

3.5 Intracellular staining for FOXP3

The intracellular staining in mice tumour samples were performed according to the manufacturer´s protocol (eBioscience) and data acquired using BD-LSRF Fortessa (BD cell analyzer).
The staining for T_{reg} was performed using the following antibodies: PE-Foxp3 (eBioscience), Percp/cy5.5-CD3ε (BioLegend) and Pe-

Cyanine7-CD4 (eBioscience) and the data were analyzed using FlowJo software v10 (Ashland, Oregon, USA).

3.6 MDSCs panel analysis

Mice tumour samples were staining using FITC-Cd11b (BD Pharmingen), PE-Ly6G (BD Pharmingen) and APC-Ly6C (BD Pharmingen) according to the manufacturer´s recommendation. The data were acquired using BD Accuri 6C plus (BD) and the data analyzed using FlowJo software v10 (Ashland, Oregon, USA).

3.7 PeptiCrad complex formation

Oncolytic adenovirus and polyK epitope (Ontores, Zhejiang, China) were mixed to prepare the PeptiCRAd complex. We mixed polyK epitope with Ad-5-D24-CpG for 15 minutes at room temperature and after that, we use this complex to treat the mice.
For the *in vivo* experiments, we combined 7 µg for each epitope with $1x10^9$ vp for each tumour.

In the group called PeptiCRAd-D.C. to obtain the same final amount of epitope, we combined 3.5 µg for each epitope with $1x10^9$ vp for each tumour;

In the group called PeptiCRAd-S.C., we combined 7 µg for each epitope to induce TCD4 subtype response with $1x10^9$ vp for each tumour in one eppendorf and 7 µg for each epitope to induce TCD8 subtype response $1x10^9$ vp for each tumour in another eppendorf. After the 15 minutes, we took the half amount of each eppendorf and we combined them together.

3.8 HLA genotyping

The DNA extraction was performed according to the manufacturer´s protocol (QIAamp DNA Mini Kit 50 Qiagen); the Finnish Red Cross

analyzed the HLA-A, B and DRB1 on high resolution (4digit Sanger´s sequencing).

3.9 Elisa IL-2

The IL-2 level in the cell culture supernatant from PBMCs were measured by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer´s directions.

3.10 Statistical Analysis

Statistical analysis was performed using Graphpad Prism 6.0 software (Graphpad Software Inc., La Jolla, CA USA). 2way ANOVA with Tukey´s multiple comparisons test was used and $P<0.05$ was considered statistically significant.
All results are expressed as the mean ± standard error of the mean (SEM).

4. RESULTS

4.1 4T1 and MDA-MB-436 represent solid model for an immunotherapeutic approach.

Cancer cells escape the immune recognition and destruction by several mechanisms. One of these is the down regulation of MHC-I (HLA-A, B and C in human) (Campoli and Ferrone 2008) and the up-regulation of PD-L1 (Robainas et al. 2017). To assess the feasibility of our immunotherapeutic approach, we needed solid model of TNBC cell line.

To this end, we characterized 4T1 and MDA-MB-436, respectively a murine and human TNBC cell line, for the expression of MHC-I, MHC-II and PD-L1.

First, we performed a flow cytometry analysis in 4T1 cell line stimulated and not stimulated overnight with IFN-γ ,to evaluate the levels of MHC-I, MHC-II and PD-L1.

The use of IFN-γ is justified to recreate the tumour microenvironment, in which it has an antitumor potential by inducing MHC, but at same time induces the expression of PD-L1 (Parker, Rautela, and Hertzog 2016) . As attended, in presence of IFN-γ the expression of MHC I **(Fig.1A)**, MHC II **(Fig.1B)** and PDL-1 **(Fig.1C)** is higher compared to the cells without stimulation, but it is interesting that also in absence of IFN-γ the cells showed basic levels of MHC-I, MHC-II and PD-L1; thus, the 4T1 cells resulted a valid model for an immunotherapeutic treatment.

Because the *in vitro* levels of MHC-I, MHC-II and PD-L1 in 4T1 cells may not reflect the *in vivo* situation, we decided to detect the level of MHC-I and PDL-1 in cells from mice bearing 4T1 tumours, confirming the *in vitro* data previously described **(Fig. 1D and 1E)**.
Furthermore, the *in vivo* analysis in 4T1 showed higher TILs PD1$^+$ in tumours compared to spleens and lymph nodes **(Fig. 1F and 1G)**.

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Hence, in according to the data aforementioned, 4T1 cell line represents a valid model to evaluate the efficacy of an immunotherapeutic approach.

The next step of all the experiments described in this thesis is the clinical translation; because of that, we selected MDA-MB-436 and also in this case, we analysed and confirmed the expression of HLA-A, B, C and PD-L1. **(Fig. H and I)**. These first set of experiments defines 4T1 and MDA-MB-436 as rationale model for the following experiments.

A

B

C

D

E

36

F CD8 cells

G CD4 cells

H

MDA-MB-436

I
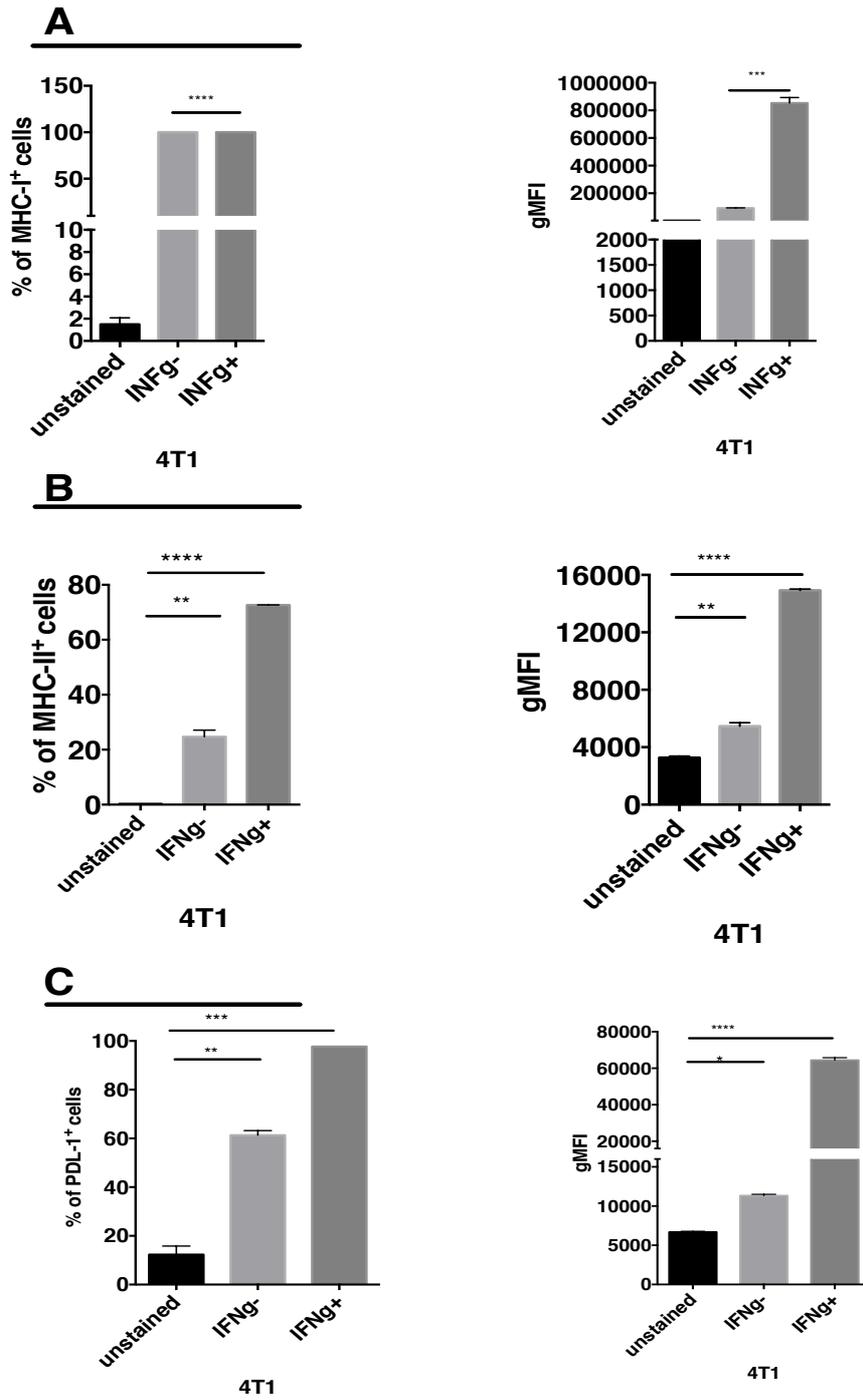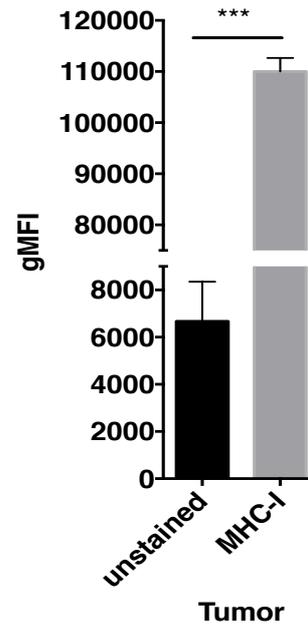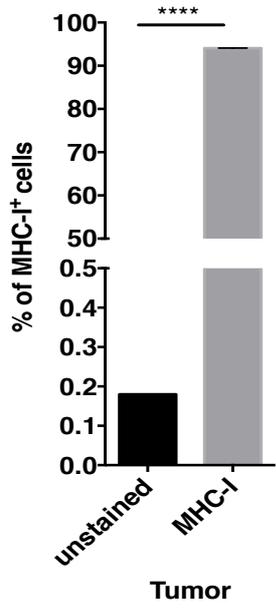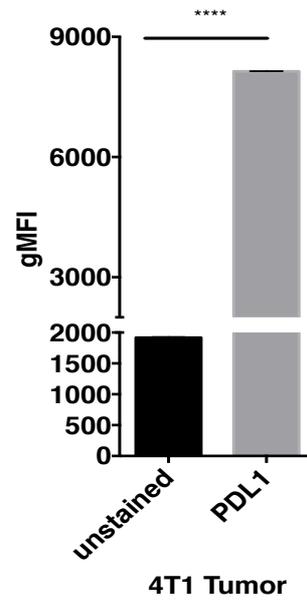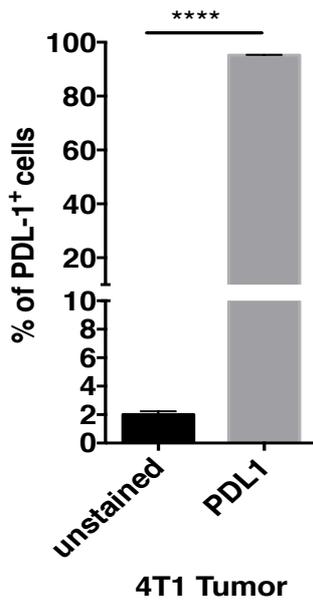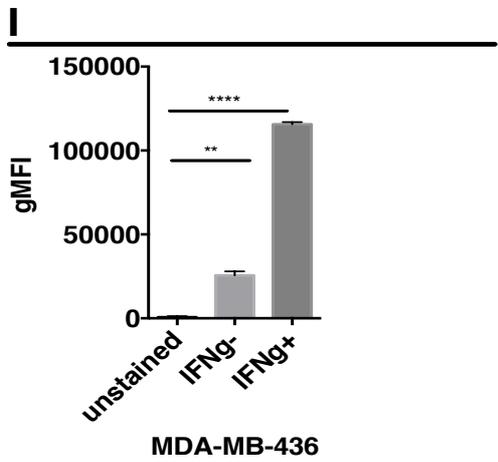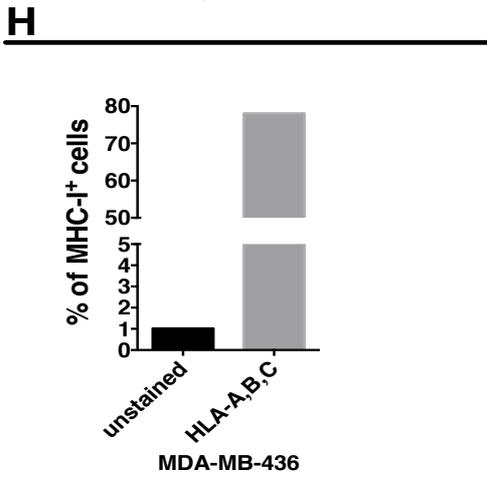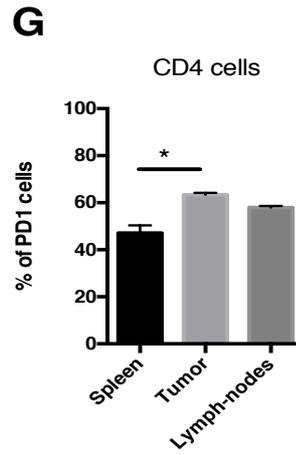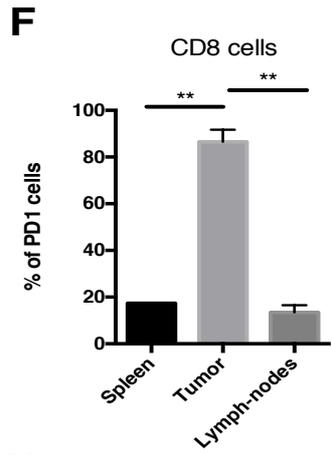
MDA-MB-436

Figure1 4T1 and MDA-MB-436 immune characterization. MHC-I **(A)**, MHC-II **(B)** and PD-L1 **(C)** in 4T1 cell line are described. The cells were seeded at final number of $3x10^5$ in 5mL and the day after IFN-γ was added to medium at final concentration of 10 ng/mL overnight. The following day we performed flow cytometry analysis using anti-mouse APC-H2Kd, anti-mouse APC-MHCII and anti-mouse PE-PDL1

Expression levels of MHC-I **(D)** and PD-L1 **(E)** in tumors from mice bearing 4T1 tumors and T cell infiltration PD1$^+$ in tumors, spleens and lymph nodes from mice bearing 4T1 tumor are presented **(F and G)**. We performed a flow cytometry analysis in tumors, spleen and lymph node samples collected from mice and frozen. We used anti-mouse PE-PD1, anti-mouse Percp/Cy5.5 CD3ε, anti-mouse FITC-CD8 and anti-mouse APC- CD4.

MDA-MB-436 showed high level of HLA-A, B, C **(H)** and PD-L1 **(I)**. We seeded the cells at final number of $4x10^5$ cells and the day after we stimulated the cells over night with human INF-γ at final concentration of 10 ng/ml. We performed the flow cytometry analysis using anti-human PE-HLA-A, B, C and PE-CD274 B7-H1 (PD-L1) (statistical analysis one-way Anova and unpaired t test $P<0.05$).

4.2 Ad-5-D24-CpG cytotoxic effect is identical to Ad-5/3-D24 in murine and human TNBC cell line.

PeptiCRAd is based on Ad5-D24-CpG, that bears a 24bp deletion in E1A gene and CpG rich islands to increase the immunogenicity, trought the Toll Like Receptor 9 (TLR9) stimulation (Cerullo et al. 2012).

First, we investigated the cell-killing efficacy of Ad5-D24-CpG in 4T1 and MDA-MB-436; we compared its efficacy to Ad-5/3-D24, already used and validated in human model of TNBC (Bramante et al. 2016).

Since human adenovirus not produce infection viral progeny by using murine cells line (Zhang et al. 2015), as expected Ad-5-D24-CpG and Ad-5/3-D24 at 1 vp/cell and 10 vp/cell showed killing activity in human MDA-MB-436 **(Fig.2A)** , but not in 4T1 **(Fig.2B)**.

In the future, when we will translate this treatment in clinical, we will take advantages by combining the boosting of immune system induced by PeptiCRAd with the intrinsic viral oncolytic activity.

A

MDA-MB-436

B

4T1
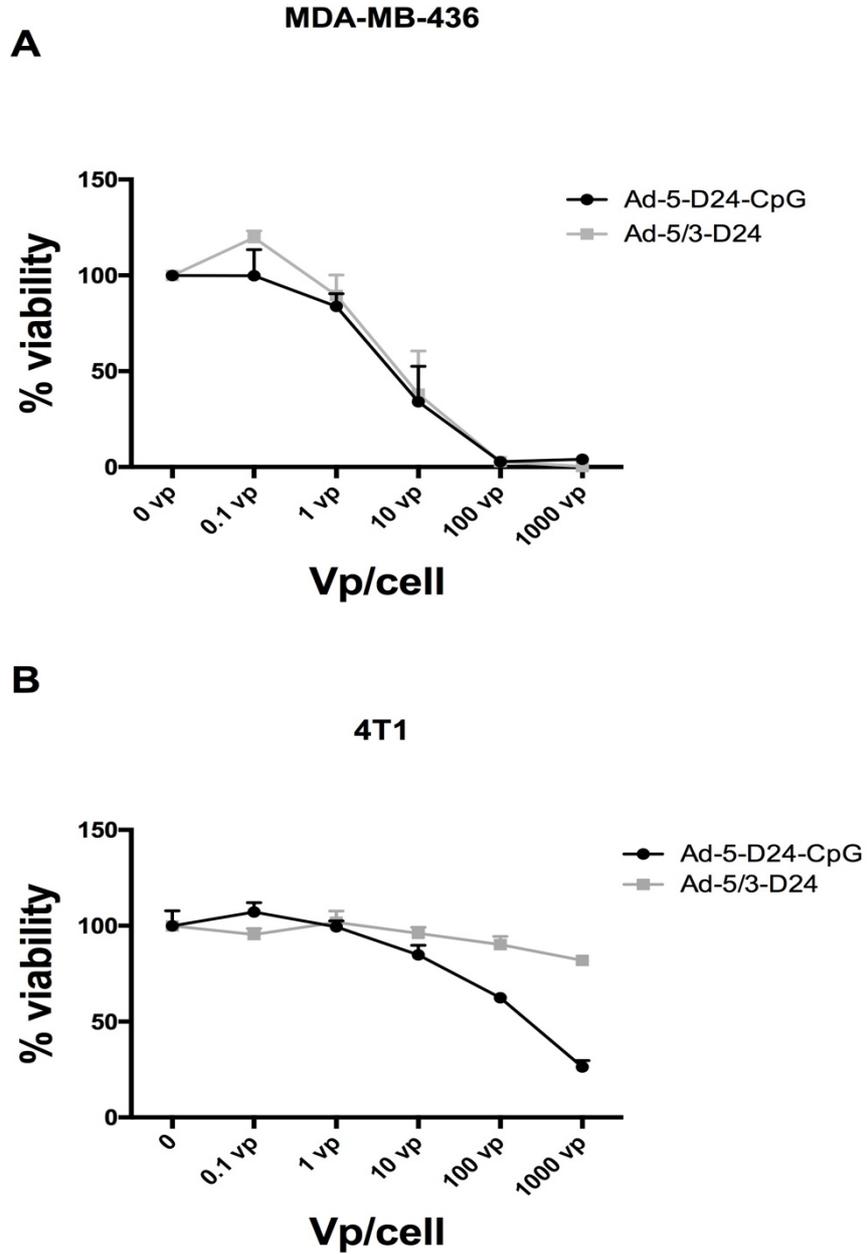
Figure2 Ad-5-D24-CpG and Ad-5/3-D24 showed identical killing-cell activity. We tested both viruses in human MDA-MB-436 and murine 4T1 cell line, by using 0.1vp/cell, 1 vp/cell, 10vp/cell, 100 vp/cell and 1000 vp/cell concentration; in addition, we included one condition without virus as control. **(A)**Six days post-infection, already at 1 and 10 vp/cell both viruses showed killing activity in MDA-MB-436. **(B)** As expected, the same killing is not observed in 4T1 cell line. The data are shown as mean $\pm$ SEM (n=3).

4.3 The TCD4 and TCD8 subtypes responses synergistically elicit antitumor response in early stage of TNBC.

In these set of *in vivo* experiments, we evaluated the anti-tumour efficacy of different PeptiCRAd preparations in TNBC.

First, we selected and analysed a list of peptides from literature (Kreiter et al. 2015; Singh et al. 2013; Gravekamp et al. 2008; Song et al. 2014). Indeed, we used the bioinformatics on-line platform IEDB to predict the binding affinity for H2Kd (MHC-I) and MHC-II **(Table1)**. Next, we prepared diverse PeptiCRAd complexes coated either with peptides MHC-I (*i.e.,* **PeptiCRAD-CD8)** or MHC-II (*i.e.,* **PeptiCRAd-CD4)** restricted. Furthermore, we coated PeptiCRAd with both peptides MHC-I and II restricted or loading them on the same adenovirus (*i.e.,* **PeptiCRAd-D.C.)** or by mixing equal concentration of adenovirus covered with the peptides (*i.e.,* **PeptiCRAd-S.C.**).

Balb/c immunocompetent mice engrafted with 4T1 received PeptiCRAd intratumourally at 9, 11 and 13 days after tumour implantation; at day 15 we observed significantly difference in the tumour's growth in the PeptiCRAd-D.C. group compared to the Mock **(Fig.3A and B)**; interestingly, PeptiCRAd-D.C. was more effective also compared to PeptiCRAd-S.C. **(Fig.3C)**. Since the previously analysis showed a strong immune suppressive component in 4T1, as expected, at the end of the experiment the average volume of the tumours in all group was not significantly lower than the Mock.

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Next, we evaluated the immunological background in lymph nodes and tumours from the treated groups. In particular, we analysed the exhausted phenotype of T-cell, using PD1 and TIM3 as marker to verify possible difference among the groups. In the lymph nodes **(Fig. 3D)** and in tumours **(Fig.3C)** the antigen experienced T cells (PD1$^+$ TIM3$^-$) were similar, confirming that the immunosuppressive environment overcome the benefit PeptiCRAd induced.

These results are coherent with the widely characterization of the pathway PD1/PD-L1 in TNBC and highlight the beneficial use of PD-L1 blockade in this cancer disease.

GENE	Reactive T cell subtype	SEQUENCE	CHARGE (net charge at pH7 without poly K tail)	CHARGE (net at pH7 with poly K tail)
Tmtc2	T-CD8	KKKKQGVTVLAVSAVYDIFVFHR LKMKQILP	2.1	6.1
Wdr11	T-CD8	KKKKKKKKKKNDEPDLDPVQELI YDLRSQCDAIRVTK	-4.1	6.1
Zfr	T-CD4	KKAHIRGAKHQKVVTLHTKLGKP IPSTEP	4.3	6.3
Adamts9	T-CD4	KKKKKKKDYTAAGFSSFQKLRLDL TSMQIITTD	0	6
Pan HLA-DR reactive epitope (PADRE)	T-CD4	KKKKAKFVAAWTLKAAA	2	6
Mageb-1/Mageb-2	T-CD8	KKKKKKAGTSIQHTLKDPI	0.1	6.1

Table 1 We added poly-lysine (polyK) chain to the amino acidic sequence to increase the net charge of the peptide to +6 mV at neutral pH, in order to allow electrostatic interaction with adenovirus capsid and the formation of the PeptiCRAd complex.

E

F

43
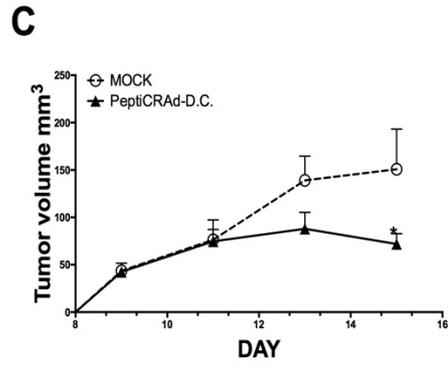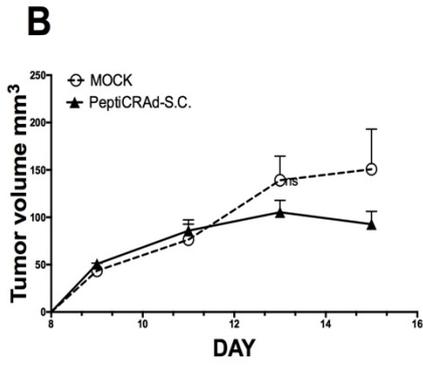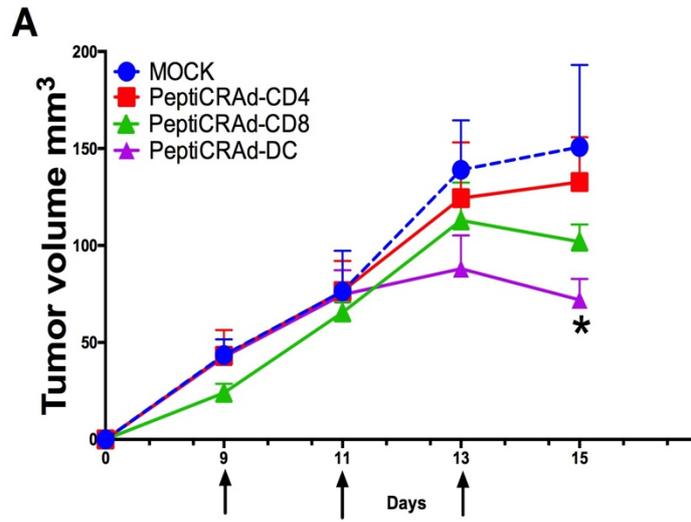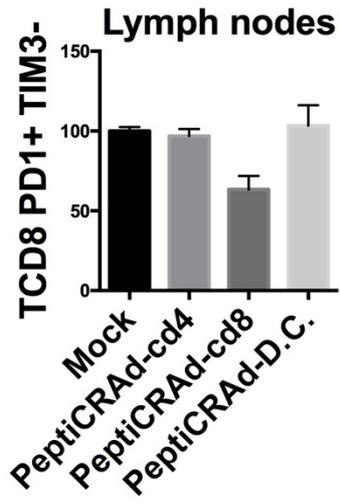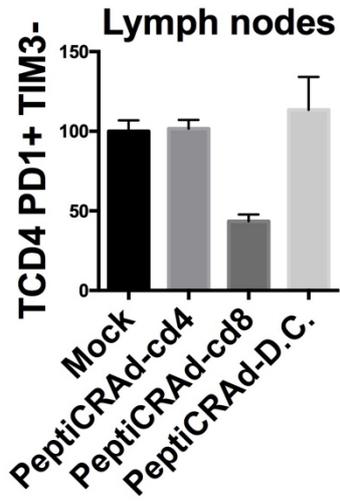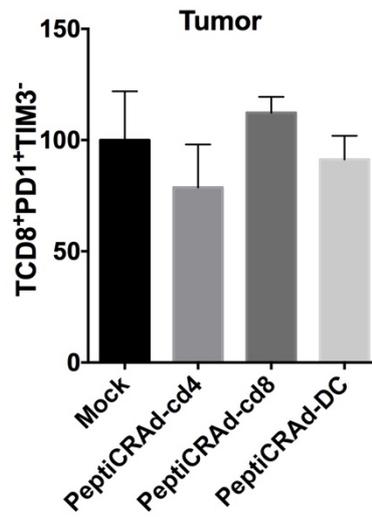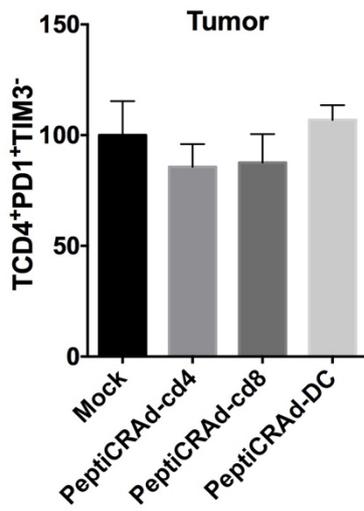
Figure 3 Antitumor activity of different PeptiCRAd formulations and immunological background analysis. (A) Balb/c mice (n=7) received 3x105 4T1 cells in the right flank. Treatments were initiated on established tumors (9 days after implantation) and the mice were treated three times (on days 9, 11 and 13, black arrows). The average volume is represented in the tumour´s growth curve as mean ± SEM (statistical analysis 2way ANOVA with Tukey´s multiple comparisons test, P<0.05). After 15 days, the tumor volume in the group PeptiCRAd-D.C. was significantly lower compared to Mock. **(B and C)** Oncolytic adenovirus double coated efficiently control tumor growth compared to the mixture of equal concentration of oncolytic adenovirus with MHC-I and MHC-II peptides. The average volume is represented in the tumour´s growth curve as mean ± SEM (statistical analysis 2way ANOVA with Tukey´s multiple comparisons test, P<0.05). **(D)** At the end of the experiments the organs were collected and analysis were performed. Per each group, five draining lymph nodes were pulled together and the analysis executed in duplicate. The antigen experienced T-cells (PD1$^+$ TIM3$^-$) is showed (graphs represented as median). **(E)** Tumors were also analyzed (tumors n. 4-5, graphs represented as median) for the T cell PD1$^+$TIM3$^-$.

4.4 PD-L1 blockade and PeptiCRAd synergistically enhance anti-tumour efficacy against TNBC by modulating the immunosuppressive environment.

The PD-1/PD-L1 blockade usually reverts or prevents the T-cell exhaustion, since PD-L1 modulate the active immune response in the "tumour bed" (Chen and Mellman 2013). To further investigate the anti-tumour efficacy of PeptiCRAd in combination with anti-PDL1, we implanted $3x10^5$ 4T1 cells in the right flank of Balb/c mice and we started the treatments on the established tumours. The treatment groups included anti-PDL1 in absence (*i.e.*,a-PDL1) or in presence of PeptiCRAd double coated (*i.e.*, PeptiCRAd-D.C. + a-PDL1).

Until the end of the experiment, the co-treatment PeptiCRAd-D.C./ anti-PDL1 induced statistically reduction of the tumour growth **(Fig.4A)** as showed also by the single tumour growth per each mouse treated **(Fig.4B).**

At the end of the experiment, tumours were collected and analysed for immunological studies. PD-L1 alone was not able to induce T cell infiltration; instead, the combination PD-L1/PeptiCRAd-D.C. drove TCD8^{+} and TCD4^{+} in the tumour tissue **(Fig. 4C).**

Previous works highlight the strong immunosuppressive environment in 4T1 mammary carcinoma model in which, among the other mechanisms, myeloid-derived suppressor cells (MDSCs) are involved (Ali et al. 2014; Bunt et al. 2006; Youn et al. 2008). In according to these studies, we decided to verify the MDSCs infiltration in the tumour samples from treated mice; the MDSCs are a heterogeneous population of bone marrow-derived myeloid cells that co-express the CD11b and Gr1 surface markers. Gr1 is made of Ly6C^{+} and Ly6G^{+} and the MDSCs are subdivided in two populations: M-MDSC (Ly6C^{+}), the monocyte population and PMN-MDSC (Ly6G^{+}), the polymorph nuclear population.

In a first set of analysis, the absence or presence of Ly6C and Ly6G, identified two sub populations: Cd11b^{+}Ly6C^{+} (M-MDSC) and Cd11b^{+}Ly6G^{+} (PMN-MDSC). Interestingly, the PMN-MDSCs

population in the group PeptiCRAd-D.C.+aPDL1 is statistically predominant compared to Mock **(Fig.4D)**.

In the second step, we isolate the neutrophil population from the PMN population. Following previous analysis (Ali et al. 2014), we designed the neutrophil as Ly6G^{high} and Ly6C $^{intermediate/high}$ and the PMN population as Ly6G^{high} and Ly6C$^{-/low}$.

The neutrophil population in tumours from PeptiCRAd-D.C.+aPDL1 group was statistically predominant compared to the Mock **(Fig.4E)**. Knowledge surrounding neutrophils are still in progress and needs clarification; however, it has been demonstrated that they can oppose the tumour growth through their cytotoxic activity in mice (Fridlender et al. 2009).

Finally, we analysed the Treg population (TCD4+Foxp3+) in these tumour samples. The PD-L1 blockade in presence of PeptiCRAd-D.C caused a depletion of Treg population compared to the group treated with only anti-PDL1 and Mock **(Fig.4F)**.

These data demonstrate that the combination PeptiCRAd/PDL1 blocking enhances the tumour immune infiltration and allows controlling the strong immune suppressive environment in 4T1 tumour.

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C

Mock	aPDL1	PeptiCRAd-DC+aPDL1

- 11.67% TCD4
- 88.33% TCD8

- 9.39% TCD4
- 90.61% TCD8

- 3.14% TCD4
- 96.86% TCD8

D

E

Neutrophil

F

MOCK	aPDL1	PeptiCRAd-DC+aPDL1
62.46% TCD4	67.72% TCD4	70.72% TCD4
37.54% Foxp3	32.28% Foxp3	29.28% Foxp3

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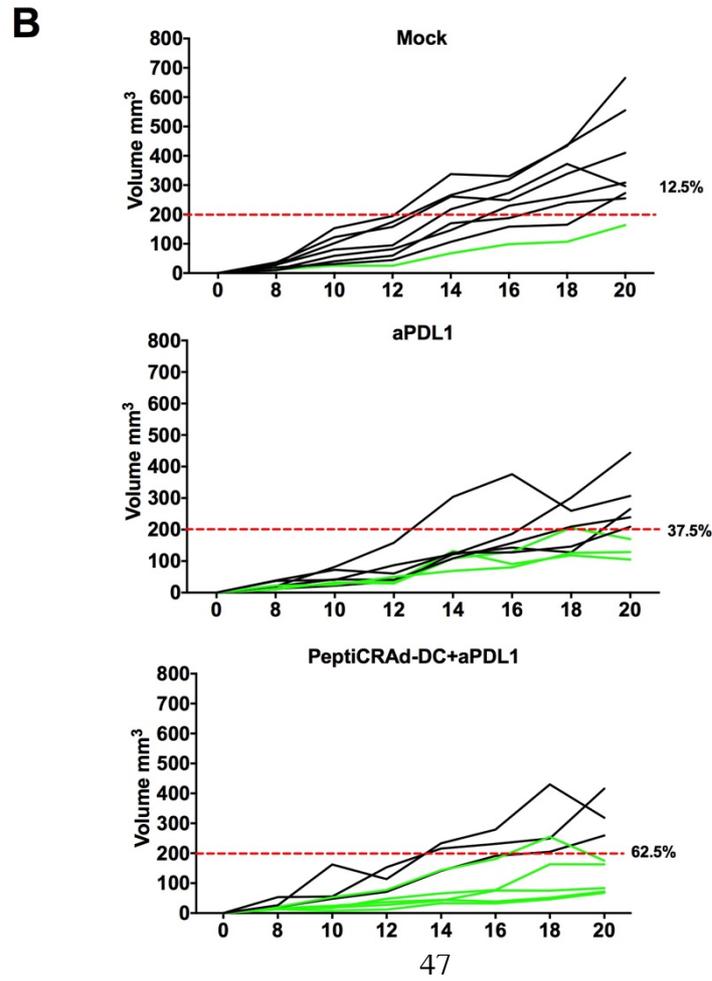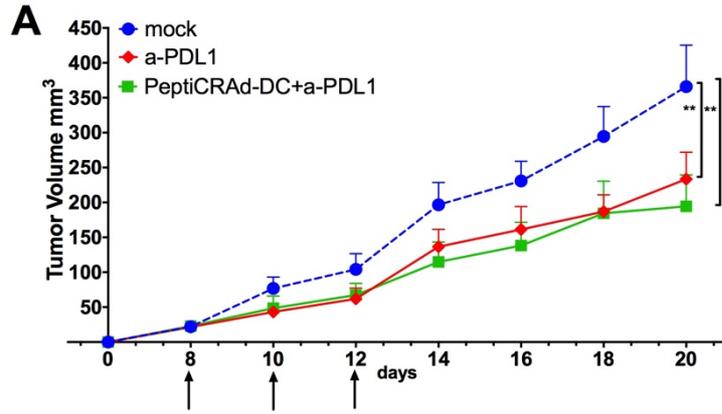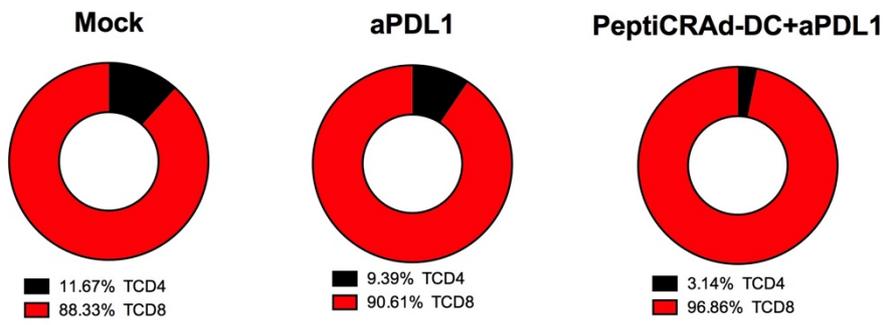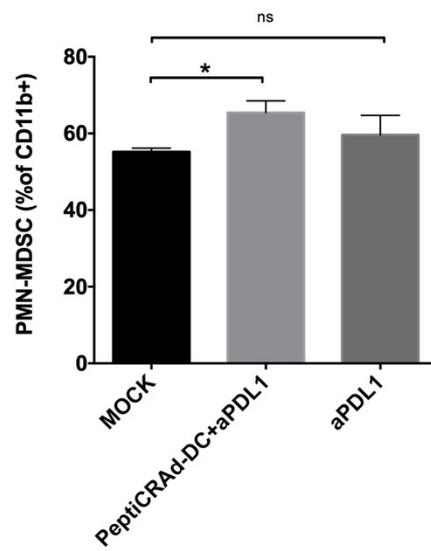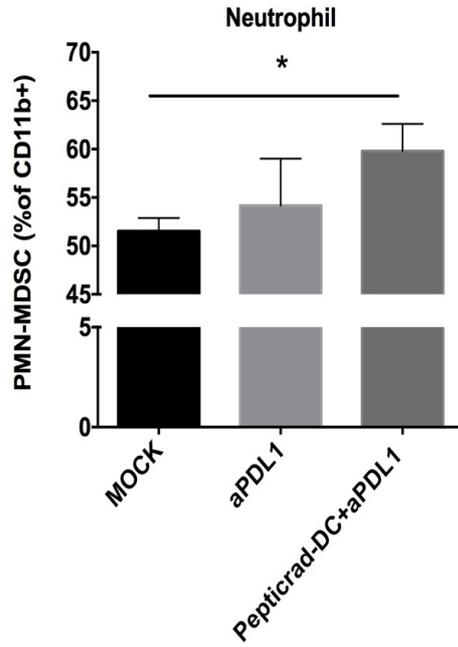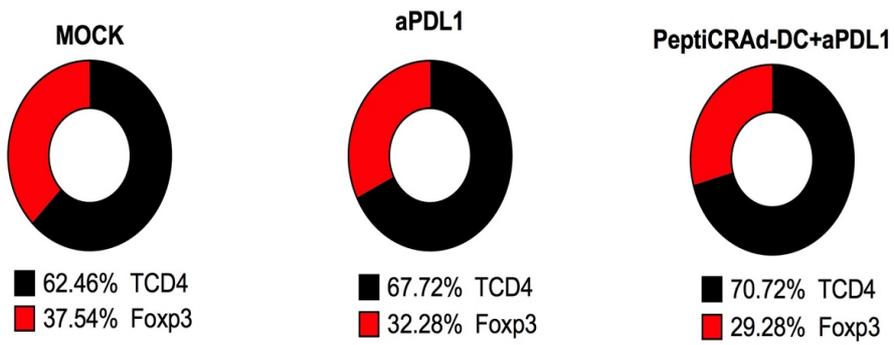
Figure 4 Anti-tumour activity PD-L1 blockade and PeptiCRAd mediated, and immune modulation in tumour microenvironment. (A) Balb/c (n=8 per group) received 3×10^5 4T1 cells in the right flank and the treatment started on established tumour. The tumour´s growth is represented as mean ± SEM (statistical analysis 2way ANOVA with Tukey´s multiple comparisons test, P<0.05). At the end of the experiment, the tumour's growth in PeptiCRAd+aPDL1 group was statistically lower compared to Mock. **(B)** The single tumour growth curves for single mouse and one graph for each group are presented. Responders are defined in percentage (displayed next to each graph) as mice that show an absolute volume lower than 200 mm3. **(C)** Flow cytometry analysis of CD4 and CD8 T cells in tumour samples from the animal groups is presented. **(D)** At the end of the experiment the tumours were collected and analysed for the PMN-MDSCs infiltration. The data are showed as bar (mean ± SEM) and median (statically analysis unpaired t-test P<0.05). **(E)** The analysis was repeated considering the intermediate value of Ly6C+ signal, identifying the neutrophil population. The data are showed as bar (mean ± SEM) and median (statically analysis unpaired t-test P<0.05). **(F)** Relative percentage of subset of TCD4 FoxP3 for each group is described.

4.5 PeptiCRAd pulsed PBMCs enhance human TNBC killing in a co-culture system.

To move forward in a relevant clinical model, we performed an *in vitro* T cell killing assay, in which we used peripheral blood mononuclear cells (PBMCs).
First, we needed PBMCs with HLA A, B and DRB1 matched with the MDA-MB-436 genotype.

To this end, genomic DNA from MDA-MB-436 was isolated and analysed **(Fig.5A-B)**.
Next, we stimulated the T-cells in PBMCs HLA matched versus specific and well know tumour associated antigens (TAA) from human TNBC (Cabezon et al. 2013; Mathe et al. 2015; Stagg and Allard 2013).

It is well know the difficult to obtain the T-cell proliferation *in vitro* in PBMCs in response to an exogenous antigen (Kennell, Gould, and Salaman 2014); in order to overcome the problem, we cultured all the PBMCs in this work with human IL-2 plus PeptiCRAd in according to the following groups:
PBMCs pulsed with either MAGEA4-PeptiCRAd or with NYBR1-PeptiCRAd or PeptiCRAd-P001; furthermore, we had a group pulsed with MAGEA4+NYBR1-PeptiCRAd. In addition, we used as control PBMCs pulsed with OAd-5-D24-CpG (group called AD) only and the PBMCs without PeptiCRAd stimulation (group called IL2).

The peptide called P001 is the result of the ligandome analysis, in an improved version set up in our laboratory.

After three days of stimulation, we added the PBMCs to MDA-MB-436 at ratio 1:10. At day 6 MTS assay was performed to assess MDA-MB-436 viability **(Fig.5C)**. Statistically killing T-cell activity was observed in the MDA-MB-436 from the groups MAGE-A4 PeptiCRAd, NYBR1+MAGEA-4 PeptiCRAd and P001 PeptiCRAd compared to the controls.

Finally, in order to demonstrate the T-cell activation in response to PeptiCRAd, we measured the IL-2 levels by ELISA in the cell

supernatant. The results shown higher IL-2 level in all the conditions compared to the control groups, confirming the T-cell activation antigen specific. **(Fig.5D)**.

Our data demonstrate that PeptiCRAd prime T-cell population in response to TAA from TNBC, a poor immunogenic cancer.

A
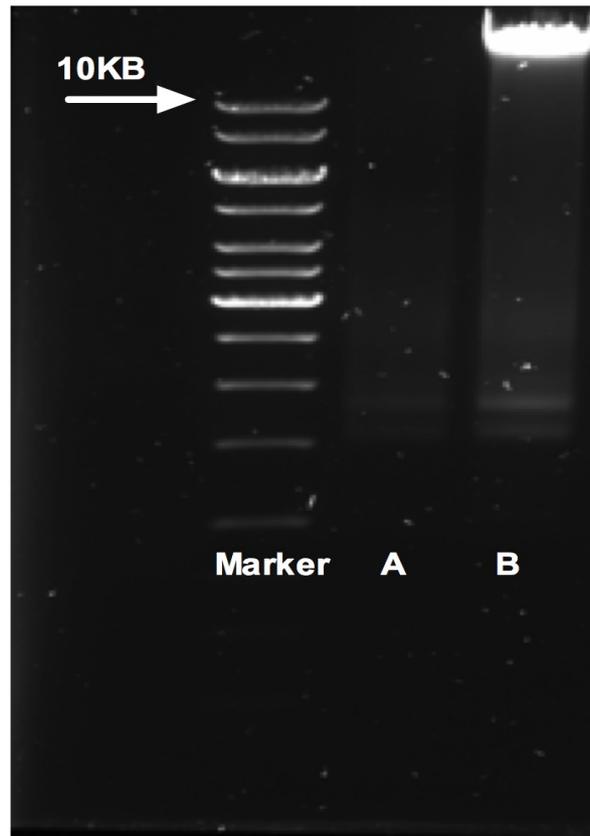
B

Test / Method	Parameter	Result
HLA-A typing, high resolution	A	01:01
HLA-B typing, high resolution	B	08:01
HLA-DRB typing, high resolution	DRB1	03:01

53

C

D

MDA-MB-436

MDA-MB-436+PBMCs
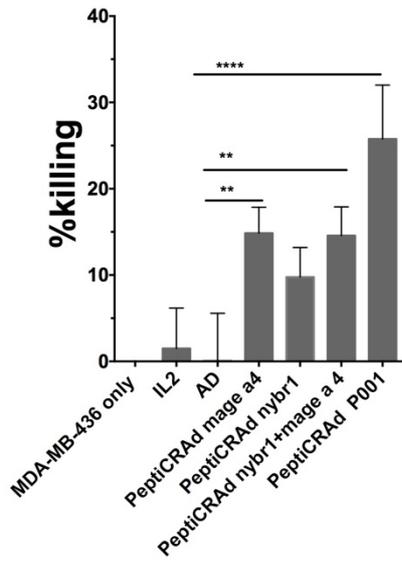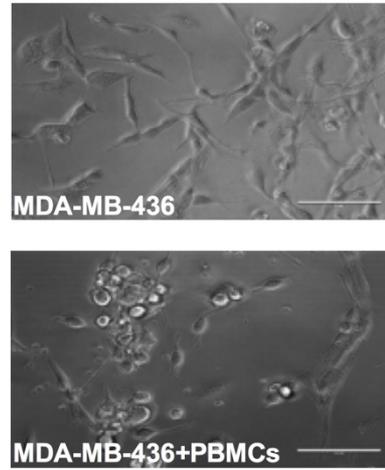
E
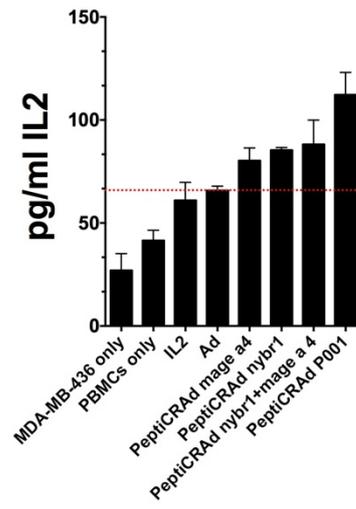
Figure 5 Genomic DNA isolated from MDA-MB-436 using and T-cell killing assay in co-culture system. (A) DNA quality was verified by running the DNA genomic digested with DNAse (lane A) and without DNAse (lane B). As attended the DNA genomic (lane B) is about 30-50Kb. **(B)**The Finnish Red cross performed the Sanger´s sequencing on high resolution (4 digit) for HLA-A, B (MHC-I) and DRB1(MHC-II) **(Fig.5B)**. **(C)**T-cell *in vitro* killing assay using PBMCs pulsed with PeptiCRAd. PBMCs were pulsed with PeptiCRAd as described and percentage of the killing is represented as bar of mean ± SEM. **(D)** Representative imagines of MDA-MB-436 and MDA-MB-436+PBMCs are reported. **(E)** IL-2 level from supernatant are shown and are represented as bar of mean ± SEM.

5.DISCUSSION

The TNBC is still one of the most aggressive cancer form with no approved systemic treatment approach (Jitariu et al. 2017). TNBC is associated with younger age and advanced stage at diagnosis, increased risk of visceral metastasis and poorer outcome (Wahba and El-Hadaad 2015; Zeichner, Terawaki, and Gogineni 2016). Because TNBC lack of the expression of therapeutic targets(Foulkes, Smith, and Reis-Filho 2010), patients do not benefit from the already approved therapy for the other form of the breast cancers (Mousavi et al. 2013). Therefore, TNBC is still an important challenging in the research as in the clinical field (Narod, Dent, and Foulkes 2015).

Recent studies underline that patients positive for tumour infiltrating lymphocytes respond to PD-L1 blockade. (Garcia-Teijido et al. 2016; Castaneda et al. 2016; Adams et al. 2014). Nevertheless, many patients fail to respond, because of the immunosuppressive environment and the absence of T cell infiltration, suggesting that patients can benefits from strategies that enhance immune response (Dushyanthen et al. 2015). The boosting of the immune system and the blocking of the immunosuppressive signal in the tumour environment represent nowadays one of the most promising treatment (de la Cruz-Merino et al. 2013).

To this end, we decided to combine PeptiCRAd with anti-PDL1. PeptiCRAd is a platform based on peptide-coated oncolytic adenovirus, already tested in other tumour forms (Capasso et al. 2016). The platform combines the adjuvancy of the virus with the immunological targeting of tumour-derived peptides, converting the powerful anti-viral immune response obtained with viral vaccines into a more efficient anti-tumoural response. In this study, for the first time, we used the platform in a cancer model traditionally considered poor immunogenic: TNBC; furthermore, we explored not only the TCD8$^+$ subtype response but also the TCD4$^+$, testing different PeptiCRAd peptides preparations.

Tumour escape the immune system down-regulating MHC-I and up-regulating PD-L1.

Thus, we first chose and characterized 4T1 and MDA-MB-436, respectively a murine and human cell line, for the expression of MHC I, MHC II and PD-L1 to verify their use as a rational model for an immunotherapeutic approach. Both cell lines showed high expression level of MHC-I, MHC II and PD-L1 and furthermore were solid model for an immunotherapeutic approach.

Next, we demonstrated that the oncolytic activity of Ad5-D24-CpG in 4T1 and MDA-MB-436 cell lines was similar to the already validated Ad-5/3-D24 in human model of TNBC. As expected, in the murine cell line we did not observe the cytopathic effect. Thus, all the effects described in the murine TNBC model were associated to the delivery action of OAd and the boosting of the immune system for the peptides loaded on the viral capsid. In clinical, the therapy could benefit also from the oncolytic activity of the OAd.

To characterize the anti-tumour activity of PeptiCRAd *in vivo*, we used mice engrafted with aggressive TNBC cell line 4T1 and we targeted antigens already validated in the literature (Kreiter et al. 2015; Singh et al. 2013; Gravekamp et al. 2008; Song et al. 2014). To further investigate TCD8^{+} and TCD4^{+} subtype responses, the "two harms" of the immune system, we selected antigens recognized in MHC complex I and II. The IEDB analysis helped us to selected the epitopes with the best predict binding capability to the MHC I and II. Mice treated with a combination of Ad5-D24-CpG covered with antigens to induce TCD8^{+} and TCD4^{+} subtype responses showed a significantly decreased tumour growth, but at the end of the experiment there were no difference among the groups in the size. The analysis of immunological background highlighted the strong immune- suppressive environment.

These results confirm TNBC as a poor immunogenic cancer model and are consistent with current knowledge about the pathway PD1/PD-L1, in particular with the widely characterization of PD-L1 in the TNBC patients (Chen et al. 2013). In fact, a preliminary and successful clinical trials with anti-PDL1antibody, called MPDL3280A and already used also in a successful clinical trial with metastatic bladder cancer(Powles et al. 2014) , was performed in patients with a metastatic form of TNBC (Emens et al. 2015), confirming that TNBC is a good candidate for an immunotherapy approach.

Because of that, we decided to combine PeptiCRAd-D.C. with monoclonal antibody anti-PDL1. We found that targeting tumour antigens to induce TCD4$^+$ and TCD8$^+$ subtype responses in combination with anti-PDL1 decreases the tumour growth rate; next, the immunological analysis performed by using tumour samples revealed a higher frequency of TCD8$^+$ in tumour sample from PeptiCRAD+aPDL1 compared to the Mock and to aPDL1.

We decided to further investigate the immunological status of the tumour samples. In particular, we focused the attention on myeloid-derived suppressor cells (MDSCs), a population that block the activation of anti-tumour TCD4$^+$ /TCD8$^+$ activity (Srivastava et al. 2010) and described in different tumour types and positively associated to the metastatic tumour phenotype (Ouzounova et al. 2017). In particular the 4T1 tumour have been previously reported as a tumour model characterized by MDSCs infiltration. (Youn et al. 2008; Danilin et al. 2012). Following these studies, we analyzed in our tumour samples the MDSCs population and we observed that the PMN-MDSCs population resulted statistically higher in the group PeptiCRAd+aPDL1 compared to the Mock. The PMN-MDSCs are the predominant population in the MDSCs population and it is associated to poorer diagnosis in the patient (Wynn 2013; Binsfeld et al. 2016) .
However, analysing the low/intermediate expression level of Ly6C$^+$ cells in the PMN population, in according to literature (Ali et al. 2014) we identified the neutrophils population that resulted higher in the PeptiCRAd+aPDl1 group compare to the Mock.
The role of neutrophils in cancer is still unclear, but it has been demonstrated that they can oppose the tumour growth through their cytotoxic activity in mice (Fridlender et al. 2009).

Finally, in order to validate PeptiCRAd in a relevant clinical model of human TNBC, we designed an *in vitro* T-cell killing assay; in particular, we selected PBMCs matched with the same HLA-A, B and DRB1 genotype of the human cell line MDA-MB-436. When we added the PBMCs pulsed with PeptiCRAd on the top of MDA-MB-436, we observed tumour cell killing, demonstrating that the use of PeptiCRAd created specific T-cell anti-tumour response, also confirmed by the IL2

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level in the supernatant of the killing assay. One peptide, used in this experiment, has been chosen by using the ligandome analysis, method for identifying epitopes MHC I restricted and improved in our laboratory.

Nowadays, the identification of new epitopes to use in cancer vaccine is limited by the available technology; for example, the actual genomic and proteomic technologies allow to isolate the over- /under- expressed proteins, but are unable to detect low proteins, often processed and presented in the MHC-I complex that induces a strong T cell response (Comber and Philip 2014). The most promising method to identify truly relevant tumour antigens is the direct analysis of the MHC-I peptides complex. To this end, we set up and improved the ligandome analysis for the direct isolation of the peptides from MHC-I.

6.CONCLUSIONS

In conclusion, PeptiCRAd is a versatile and rapid system to adsorb tumour-specific major histocompatibility complex class I (MHC-I) peptides onto the viral surface to drive the immune response toward the tumour epitopes (Capasso et al. 2016). Already validated in other tumour models, in this study we adapted the same platform in a challenging model of TNBC and for the first time we explored also the use of tumour specific MHC-II restricted peptides, observing that vaccine coating with a combination of MHC-I and -II-restricted peptides is more effective than the previously that used MHC-I restricted peptides coating, leading to a further improvement of the system. Therefore, PeptiCRAd has a clear advantage: it can harness both arms of the immune system and as oncolytic agent induce lysis of the human tumour cells.

Moreover, we successfully associate the antigen-specific T cell responses PeptiCRAd mediated with the PD-L1 blockade in a poor immunogenic tumor, thus providing a strong rationale for a combinatorial approach.

In the future, the advance in the ligandome and RNAseq for identifying neo-antigens and their application in versatile platform, such as peptiCRAd, will open new possibilities in the co-treatment with checkpoint inhibitors to treat cancer patients.

7.APPENDICS

FDA approves first-of-its-kind product for the treatment of melanoma:
https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm469571.htm

Immune Epitope Database and Analysis Resource
http://www.iedb.org/

8.ACKNOWLEDGMENTS

Thanks Manlio for the funny hours that we spend in the lab and for the help that you gave me in this year.
Thanks Bea for your precious technical support and your vegan/veggy theory that I always found really interesting.

Helsinki, October 3, 2017
(rainy days...)

Sara Feola

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10. LIST OF PUBLICATIONS

A novel *in silico* framework to improve MHC-I epitopes and break the tolerance to melanoma

Cristian Capasso, Aniket Magarkar, Victor Cervera-Carascon, Manlio Fusciello, **Sara Feola**, Martin Muller, Mariangela Garofalo, Lukasz Kuryk, Siri Tähtinen, Lucio Pastore, Alex Bunker & Vincenzo Cerullo
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Oncolytic Vaccines increase the response to PD-L1 blockade in immunogenic and poorly immunogenic tumors.

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