UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXX CYCLE

AFFINITY MATURATION OF NOVEL HUMAN ANTIBODIES FOR CANCER IMMUNOTHERAPY, BY YEAST SURFACE DISPLAY TECHNOLOGY

Tutor
Prof. Alfredo Nicosia

Candidate
Biancamaria Cembrola

COORDINATOR

Prof. Vittorio Enrico
Avvedimento

Academic Year 2016/2017

INDEX

ABSTRACT

The affinity engineering is a key step to increase the therapeutic efficacy of monoclonal antibodies (mAbs). Yeast surface display (YSD) is the most widely used and powerful affinity maturation approach, allowing for the achievement of low picomolar antibody binding affinities. A great number of mAbs approved for clinics are used in cancer immunotherapy, for the targeting of either tumor neoantigens or immune checkpoint components; this second approach aims to re-activate the T cell-mediated anti-tumor immunity, which is often impaired by cancer cells through several immune escape mechanisms. In this study, we describe an optimization of the YSD methodology, applied to the generation of potentially therapeutic high affinity single chain antibody fragments (scFvs) targeting PD-L1, an immune checkpoint component which is often upregulated on cancer cell surface. We generated two different yeast libraries with high mutant frequency and diversity, by multi-step random mutagenesis of the heavy chain variable region CDR3 of an anti PD-L1 scFv. By panning the libraries against soluble PD-L1 antigen and through few sequential rounds of fluorescence-activated cell sorting (FACS), we quickly isolated mutated yeast clones with conserved mutation hotspots. Among these scFv-yeast clones, 6 of them were enriched and showed a 6,3- to 9,8-fold affinity improvement compared with the parental one. These scFvs maintained some binding improvement also when converted into IgGs and tested on PD-L1 protein showed on the plasma membrane of human activated lymphocytes. For this reason, these novel antibodies could be good candidates for an antibody-based, PD-L1-targeted cancer immunotherapy.

1. INTRODUCTION

1.1. Antibodies as therapeutics

Monoclonal antibodies (mAbs) are widely used as therapeutics in various kinds of medical applications, such as autoimmune diseases (Chan et al, 2010), infectious diseases (Casadevall et al, 2004; Hey, 2015), post-transplantation immunosuppressive regimens (Mahmud et al, 2010) and cancer (Carter, 2001).
Since 1986, 74 mAbs have been approved by Food and Drug Administration (FDA) (https://www.accessdata.fda.gov/scripts/cder/daf/) and hundreds are in clinical trials with 50 of them undergoing late-stage clinical studies (Reichert, 2017).
Although most of the therapeutic mAbs are whole antibodies (murine, chimeric, humanized or fully human), alternative antibody formats consisting in antibody fragments or single antibody domains are emerging; thanks to their smaller size, these non-canonical antibodies overcome some pharmacokinetic issues such as tissue penetration. This class of molecules includes single chain variable fragments (scFv), formed by an heavy and a light chain variable domain (VH and VL respectively), covalently linked to each other; derivatives of scFvs (diabodies, tribodies, tetrabodies); antigen-binding fragments, (Fab, mono- or bi-specific); minibodies, i.e. immunoglobulins lacking the first and second constant domains of heavy chains (CH1 and CH2) or even single VH domains (Holliger et al, 2005).
Whatever the antibody format, all these molecules can be engineered to reach the desired pharmacological effect by improving various properties, such as affinity, solubility and stability in buffer formulations suitable for injection in humans, pharmacokinetic properties, immunogenicity and effector functions (if the molecule retains any effector domain). To improve the binding characteristics of an antibody, the variable region is usually engineered and it is not unusual that manipulating this region has an impact on all the other above properties too; on the contrary, changes in the constant region or crystallizable fragment (Fc) usually affect only the antibody effector functions (Igawa et al, 2011).

1.2. In vivo antibody affinity maturation

The binding of an antibody to its antigen is a reversible process in which the speed of association/dissociation depends on two kinds of forces. The first one is called affinity and it measures the binding strength of a single antibody

to its ligand partner; the affinity is measured through the equilibrium dissociation constant (Kd) that is a ratio of the antibody dissociation rate (k_{off}) to the antibody association rate (k_{on}) and it is inversely correlated with affinity. The other force is called avidity and plays a key role in the formation of great immunocomplexes in which many antibodies bind simultaneously to many antigen molecules; for this reason, avidity measures the overall binding strength resulting from the contribution of each antibody binding site (Rudnick et al, 2009; Oda et al, 2004).
Antibodies developed for clinics should have very high affinities for their targets, ensuring a more effective therapy (for example the ability of the antibody to recognize very low concentrations of its target) and allowing for a reduction in the dosage or in the number and frequency of administrations, mainly thanks to longer dissociation rates. Thus, a newly discovered antibody (called "lead antibody") is frequently engineered to improve its affinity through a process called affinity maturation. The principles underlying this process are the same as those used *in vivo* by B cells during the immune response (Chowdhury et al, 1999).
Each naïve B lymphocyte, resident in lymph node follicles, expresses a different B cell receptor (BCR) also called immunoglobulin (Ig), able to recognize a specific and unique foreign antigen. The diversification of the B cell receptor *repertoire* is achieved during B cell differentiation through somatic recombination of three different kinds of genic segments, called V, D and J respectively, each present in many different copies in the human genome. Once rearranged in a specific combination, these fragments form the complete and unique sequence of the variable region of an antibody, enabling it to specifically recognize a single antigen (Roth, 2014). All these Igs share the same Fc region (M isotype) and, upon antigen stimulation, they are produced in a soluble format in order to neutralize the foreign antigen circulating in the blood. At the beginning of the B cell response, IgMs show a very weak binding strength for their own antigens (their affinities range between 10^-4 and 10^-6 mol/L), but when secreted, they are grouped into pentameric complexes having a high avidity for the antigen that is sufficient to ensure a first-line and effective protection. In the meantime, the B cell receptor locus undergoes various somatic rearrangements, called somatic hypermutation (SHM) and class switch recombination, aimed to produce Igs with the same antigen specificity, but improved affinity and different effector functions (IgG, IgA, IgE) (Stavnezer et al, 2008), allowing for a more effective and long lasting protection.
The main responsible of the genetic alterations at the B cell receptor locus is the DNA-editing enzyme activation-induced cytidine deaminase (AID). This protein, whose expression is induced in B cells by antigenic stimulation, converts deoxycytidine to deoxyuridine, generating mismatches between

deoxyuridine and guanine that activate the error-prone mechanisms of DNA repair. In this way, point mutations or indels are introduced in the site of deamination during B cell division with a frequency that is 10^5-10^6-fold higher than the mutation rate which normally occurs during DNA replication. These mutations fall in the sequences encoding for the heavy and light chain variable regions, with mutation hotspots in the complementarity-determining regions (CDRs), i.e. the short amino acid loops directly contacting the antigen (Di Noia et al, 2007; Maul et al, 2010). Only a small fraction of these mutations (about 20%) affects affinity; the B cells expressing Igs with lower affinity for the antigen die for apoptosis (a process called negative selection) due to the lack of antigen binding, which represents the stimulus for their survival and proliferation. On the contrary, the B cells in which a high affinity Ig has been generated by SHM are positively selected for proliferation and differentiate into antibody-secreting plasma cells or long-living memory B cells; alternatively, they undergo another cycle of mutagenesis in order to further increase Ig affinity as the immune response goes on (Gatto et al, 2010). The *in vivo* affinity maturation is able to generate antibodies with affinities in the picomolar range (10^{-10} mol/L) (Batista et al, 1998; Foote et al, 1995) (fig. 1).

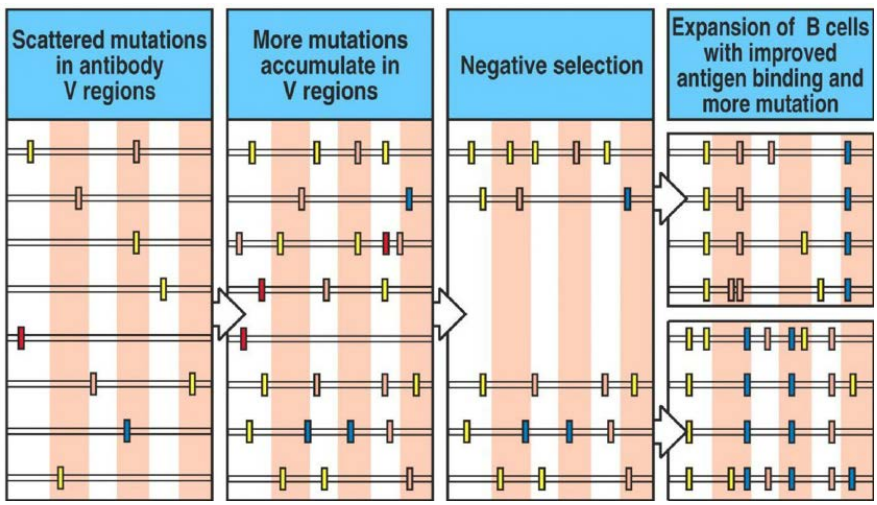

Fig. 1: schematic representation of *in vivo* antibody affinity maturation. Pink stripes represent the CDRs of the VH or VL, whereas colored bars indicate point mutations. At the beginning of the B cell response, they are interspersed throughout the variable region; while the immune response goes on, more mutations accumulate in this locus, but only B cells in which they

increase the antibody affinity for the antigen can survive. Mutations are more frequent in the CDRs.

1.3. In vitro antibody affinity maturation

In vitro affinity maturation mimics the events that happen *in vivo*: the sequence of the lead antibody variable region is randomly mutated to generate a highly diversified *repertoire* of antibodies with different affinities for the same antigen; then the antibodies showing the highest affinity for the target are selected among this heterogeneous population. These *in vitro* technologies have successfully generated antibodies with low picomolar or even femtomolar affinities for their target (from 10^{-10} to 10^{-15} mol/L) (Boder et al, 2000). The most common approaches to generate these antibody repertoires starting from the parental sequence are random mutagenesis, randomization of targeted residues using degenerate oligonucleotides, chain shuffling and *in silico* approaches.

The random mutagenesis allows the generation of a broad range of variants of the parental antibody in which each residue could be potentially mutated; mutations are introduced through error-prone PCR or *E. coli* mutator bacterial strains, which are defective in DNA repair (Rasila et al, 2009).

The error-prone PCR (ep-PCR) has the advantage to select for a specific antibody region in which mutations have to be introduced; the mutagenesis can be targeted to the whole variable region or only to VH or VL or to more restricted sequences, most commonly one or more CDRs. The ep-PCR system is based on an error-prone Taq polymerase which, lacking proofreading activity, randomly inserts point mutations in the target sequences with high frequency. Nevertheless, at the amino acid level not all the substitutions have the same chance to occur at any position; this happens when two or even all the three nucleotides of the same codon have to be changed simultaneously to encode for a given amino acid (Neylon, 2004).

Randomization of targeted residues exploits degenerated oligonucleotides and restricts the mutagenesis only to specific amino acid residues. The target positions, generally identified by alanine-scanning, are modified with a series of *ad-hoc* PCR primers, in order to generate all possible amino acid substitutions (Ko et al, 2015).

Chain shuffling consists in combining a *repertoire* of VH with a fixed VL (or *vice versa*), but chain shuffling of the CDRs only are also described (Yoshinaga et al, 2008; Marks, 2004). In more recent works, *in silico* approaches have also been successfully used; these methods are based on computational design and structure predictions of antibody-antigen interactions (Clark et al, 2006; Barderas et al, 2008).

Once new *repertoires* have been generated, the antibodies with improved affinity can be selected by means of display methods, which are grouped into cell-based and cell free systems.
In cell-based approaches, the sequence variants of the parental antibody are showed (displayed) on the cell surface of yeasts (yeast surface display), phages (phage display), bacteria *(E. coli* surface display) or mammalian cells (commonly HEK293, CHO or B-lineage cells) (Doerner et al, 2014). The antibodies are fused to a proper protein of the cell surface and subsequently panned against the antigen of interest, provided in the format of soluble protein or in the mammalian cell surface context (whole cells or detergent-solubilized cell membranes) (Tillotson et al, 2013), if it is a membrane protein. Various cycles of panning are usually performed (up to 4 or 5), increasing the stringency of selection at each round in order to isolate and enrich the clones displaying the antibodies with the improved affinity; the stringency can be typically increased by a reduction of the antigen concentrations (Chao et al, 2006;). However, in some cases only one selection step has been sufficient to isolate high affinity variants.
The scFvs are the most commonly displayed antibody format due to their little size and the absence of post-translational modifications, which allows the production and display both in prokaryotes and eukaryotes. Fab fragments or full length Igs can be also displayed and in the case of whole antibodies, which need for post-translational modifications, eukaryotic systems are necessarily required (Doerner et al, 2014).
In cell free systems, which include ribosome display and mRNA display, the library generation is achieved by *in vitro* translation of the DNA, using ribosome preparations instead of the cellular translation machinery; chaperones and other enzymes are added to the reaction to help the proper polypeptide folding (Lipovsek et al, 2004).

1.4. Affinity maturation by yeast surface display (YSD)

YSD is the most widely used affinity maturation platform, combining a lot of advantages compared with the other methods (Chao et al, 2006; Gera et al, 2013). Firstly, unlike phages and bacteria, yeasts are eukaryotic cells, consequently the displayed proteins are properly folded by the endoplasmic reticulum chaperones and receive all the post-translational modifications necessary for their function.
In addition, the generation of the libraries is easier compared to all the other systems because it is achieved by *in vivo* yeast recombination, which is more efficient than *in vitro* cloning by ligation, used in the other display systems. Once generated by mutagenesis, the sequence variants are co-transformed

with a proper yeast expression plasmid, which remains episomal after recombination. By optimization of the vector-insert ratio, a maximum transformation efficiency of about 10^9 colony forming units can be easily obtained routinely (Benatuil et al, 2010), meaning that at most 10^9 different sequences can be found in a yeast library. Although, a higher theoretical diversity can be obtained by other approaches (up to 10^{11} by phage display or even 10^{14} by cell-free systems), a repertoire of 10^9 is considered more than enough when performing affinity maturation (Chao et al, 2006; Gera et al, 2013; Boder et al, 2000).

However, the main advantage of YSD is represented by the possibility to use flow cytometry to select for clones with improved affinity. The use of fluorescence allows a real-time quantification of the protein expression level and the antigen binding strength directly during the screening process. This detection method is very powerful to discriminate even little differences in the binding properties of the antibody variants.

Saccharomyces cerevisiae and, more recently, *Pichia pastoris* are the most commonly used species for YSD. Various yeast cell wall proteins are suitable as anchors for display, in particular the a- and α- agglutinins, the flocculin Flo1p and the Pir family proteins (Pir1-4) (Pepper et al, 2008). The most frequently used yeast strain is called EBY100; it has been generated through the genetic engineering of the chromosome of *S. Cerevisiae* BJ5465 strain and its display system is based on the a-agglutinin Aga (Chao G et al, 2006). Aga is a yeast mating protein with a dimeric structure, in which one subunit (Aga1p) is covalently linked to the cell wall through a glycosylphosphatidylinositol (GPI) anchor, while the other one (Aga2p) is linked to Aga1p by two disulfide bonds (fig. 2). Aga1p sequence is stably integrated into the yeast genome, whereas Aga2p gene is encoded by the episomal display plasmid and it is fused in frame with the antibody encoding sequences, at either their N or C-terminus; both Aga1p and Aga2p-antibody expression is driven by a galactose-inducible promoter; this system allows a fine tuning of the display level, up to $5x10^4$ molecules/cell. EBY100 genome has also been modified through the insertion of mutations in some genes involved in the synthesis of certain nucleotides and amino acids, rendering this strain unable to grow in absence of these substrates (the so-called auxothrophic strains). These missing proteins are provided by the plasmid used for recombination, consequently only yeasts in which the recombination has occurred are positively selected for growth in a medium deprived of the above nutrients.

The yeast-antigen complexes are detected using a two-color labeling with fluorescent antibodies (fig. 2 and 3) (Chao et al, 2006). One antibody (fluorescence 1, FL1 in fig. 2 and 3) recognizes a tag located at the N or C-terminus of the Aga2p fusion proteins expressed on yeast surface; therefore,

this antibody is used to distinguish the displaying yeasts from the non-displaying ones that will appear as two distinct cell populations (a positive and a negative one, respectively) in a typical flow cytometry dot plot (lower right and lower left quadrant, respectively, in fig. 3). As result, the fluorescence intensity of the displaying yeasts depends on how many Aga-antibody fusion proteins are showed on their surface. The advantage of using a C-term tag is that only yeasts displaying full length molecules can be detected and then included into the selection. The second antibody (fluorescence 2, FL2 in fig. 2 and 3) recognizes the soluble antigen or a surface marker of the antigen expressing cells. As a result, the displaying yeasts that bind the antigen are positive for both the fluorescences (upper right quadrant in fig. 3). Also in this case, the number of antigen molecules bound to each cell determines its fluorescence intensity in FL2 channel.
Fluorescence-activated cell sorting (FACS) is used to isolate the best binder yeasts. The cells of interest are identified directly on the plot before the isolation, by drawing a diagonal gating in the quadrant of the double positive fluorescent population (Gera N et al, 2013) and then sorted for the separation from the library. The diagonal gate should include those yeast clones that, for each level of scfv display along the abscissa, bound the highest number of antigen molecules (fig. 3).
The enrichment of the yeasts with a better antigen binding compared with the parental antibody is obtained through repeated sortings, typically three to five, in which the selection stringency is progressively increased using lower antigen concentrations at each selection step (Chao et al, 2006).

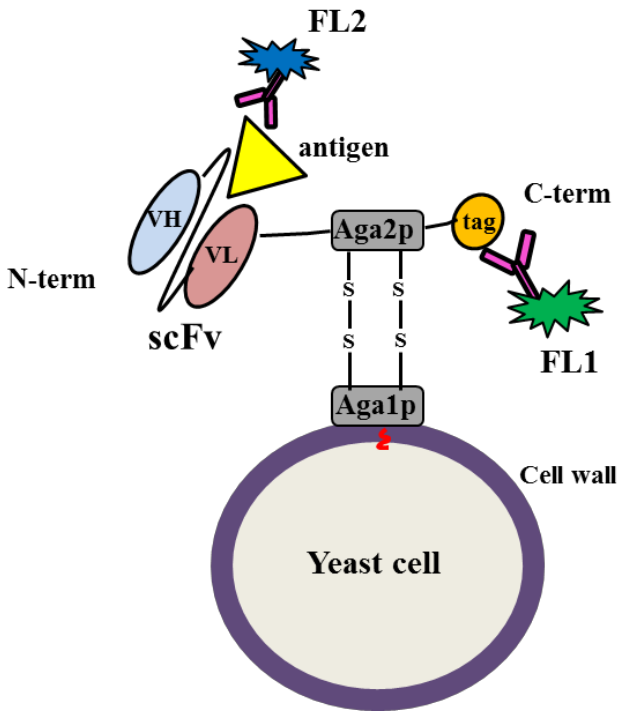

Fig. 2: Schematic representation of the detection system of the scfv-antigen complex by flow cytometry. The scfv is fused to the Aga2p subunit (at its N-terminus in this case) and this fusion protein carries a terminal tag (an example of a C-term tag is shown, in orange). The chimeric protein is covalently linked to Aga1p through two disulfide bonds and anchored to the yeast cell wall (purple) thanks to Aga1p GPI anchor (showed in red). The protein display is detected by an anti-tag fluorescent antibody (FL1). If the scfv binds to the antigen (in yellow), the complex is labelled with a second antibody (FL2), giving double fluorescent signal that can be detected by flow cytometry.

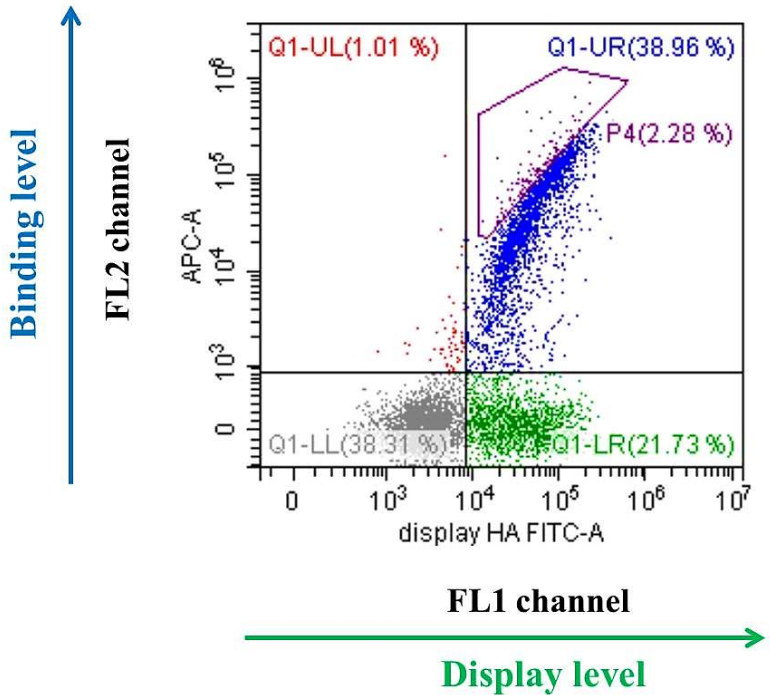

Fig. 3: typical flow cytometry dot plot in a YSD experiment. This example refers to a yeast library labelled with the two antibodies indicated in fig. 2 (FL1 and FL2) and shows the different cell populations (here represented by different colors), distinguished on the basis of their fluorescence level. Antigen binding cells are in the double positive quadrant (blue, Q1-UR quadrant) and are sorted by a typical diagonal gating strategy, here represented by the diagonal P4 gate (purple). In Q1-LR (single positive for FL1) displaying cells unable to bind the antigen are located, while the non-displaying yeasts appear in the double negative quadrant (grey, Q1-LL).

1.5. Antibodies with potential therapeutic application, isolated by YSD

A lot of examples of antibody affinity engineering through yeast display are reported in literature. The most surprising result is that obtained by Boder et al in 2000. They isolated some anti-fluorescein antibody fragments with an affinity of 48 fM, starting from a parental scFv with an affinity of 0,7 nM. These scFvs were isolated after four subsequent cycles of affinity maturation from libraries with complexities between 10^5 and 10^7. Their affinity is the highest so far reported for an engineered protein, but they are not for clinical use.

One of the most recent studies regarding antibodies for potential therapeutic application was performed in 2012 by Tillotson et al to improve the affinity of a scFv targeting the transferrin receptor. Since this is a plasma membrane protein, detergent-solubilized membranes of HEK293 cells, ectopically expressing this receptor, were used as source of the antigen. A library of about 5x10^7 yeast cells was generated by random mutagenesis of the whole parental scFv and its screening led to the identification of various scFvs with 3- to 7-fold improved affinities (from 0,38 to 0,18 nM) compared with the starting one.

By yeast display, antibodies against some toxins have been engineered too, such as botulinum neurotoxin type A (Siegel et al, 2005). After a single cycle of error prone mutagenesis of two previously identified anti-toxin scFvs (Kd values of 0,8-0,9 nM), two mutants were identified with 45- and 37-fold improved affinities, respectively (20 pM).

In 2005 Rajpal et al succeeded in generating improved versions of adalimumab (Kd 1 nM), an anti tumor necrosis factor-α (TNF-α) antibody already approved for the treatment of rheumatoid arthritis. They generated yeast libraries by applying a sort of high-throughput mutagenesis involving all the residues of the six CDRs; in this way, they isolated novel variants whose affinities were between 500- and 870-fold higher than adalimumab. Remarkably, these scFvs showed a 15- to 30-fold improvement of *in vitro* TNF neutralization.

Yeast display has been successfully used to improve affinity of a lot of proteins other than antibodies, such as T cell receptors (Buonpane et al, 2007; Buonpane et al, 2005), integrin I domain (Jin et al, 2006), epidermal growth factor (Cochran et al, 2006), natural killer cell receptor (Dam et al, 2003) and interleukin-2 (IL-2) (Rao et al, 2004). Affinity maturation is not the only application of YSD: its versatility has been also demonstrated in *de novo* selection of binders with specificity for a given target, epitope mapping, screening of cDNA libraries, identification of protein variants with improved production and stability (Pepper et al, 2008).

1.6. Antibodies in cancer treatment

Among all the FDA approved mAbs, many are used for cancer treatment, where they offer a variety of therapeutic solutions, being administered alone ("naked antibodies") or as immunoconjugates (Carter, 2001, Chiavenna et al, 2017).
Naked antibodies are used to mask the ligand-binding site or the dimerization site of oncogenic receptors, thus preventing their activation. In addition, some antibody isotypes (IgG1 and IgG3) are also able to activate the antibody-dependent cellular cytotoxicity (ADCC) and the complement-dependent cytotoxicity (CDC). These mechanisms involve natural killer (NK) cells and complement components, which are recruited by the antibody Fc effector domain on the cancer cell surface, inducing cell lysis (Clynes et al, 2000).
The immunoconjugates are widely used to deliver other therapeutic molecules or compounds only to cancer cells, limiting or avoiding the off-target effects of traditional chemotherapies which often damage healthy cells too (fig. 4). The immunoconjugates commonly vehicle cytotoxic molecules (toxins or radioactive isotopes), cytokines, killer cells or liposomes loaded with other drugs. They often carry enzymes which convert a prodrug in a toxic compound directly on cancer cell surface; alternatively, they are streptavidin-conjugated and used to capture a biotin-conjugated radioisotope only at the tumor site (multistep targeting).
A similar principle is used for the retargeting of oncolytic viruses; through genetic manipulation, some molecules of the viral envelope, which are involved in the recognition of normal cells, are replaced by tumor-specific antibody fragments, enabling the viruses to infect and lysing only tumor cells (Campadelli-Fiume et al, 2016).
Besides all these approaches, aimed to a direct destruction of cancer cells, an innovative antibody-based approach has emerged, whose purpose is to "awaken" the patient's immune system to fight cancer. A deeper understanding of cancer biology has brought to light that tumor cells have developed a lot of strategies to block the immune surveillance, resulting in tumor growth and progression and so explaining the resistance to the traditional therapies (Dunn et al, 2002).

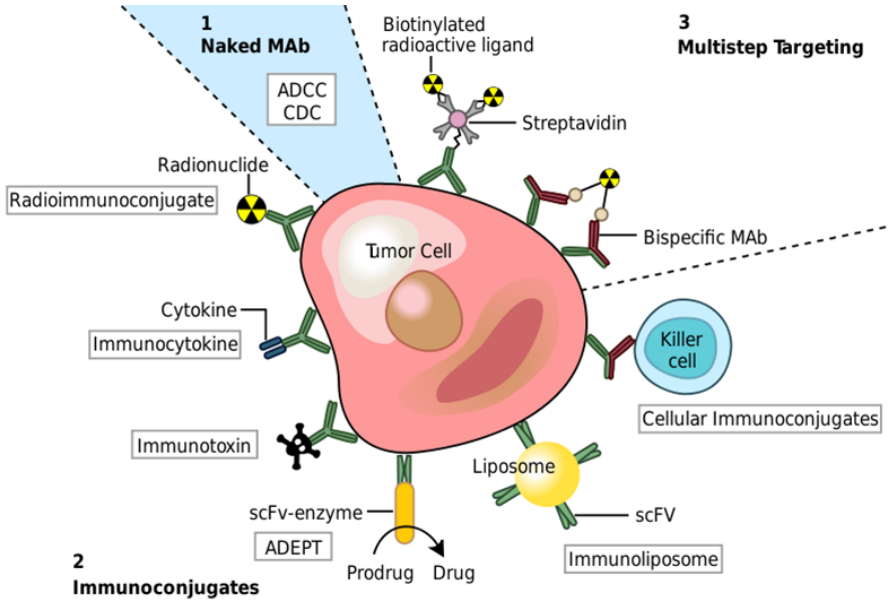

Fig. 4: applications of monoclonal antibodies in cancer treatment. In conventional anti-cancer treatments, mAbs are used alone (naked mAbs), to block oncogenic receptors and to activate antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Alternatively, mAbs are used to carry a variety of toxic compounds with non-specific activity only to tumor cells. ADEPT: Antibody-directed enzyme prodrug therapy.

1.7. Role of the immune surveillance in cancer progression and development of immune escape mechanisms

The cancer immune surveillance is the mechanism used by the immune system to recognize and remove cancer cells from our body; it is activated by tumor-associated neoantigens that result from the accumulation of mutations in various genes, due to the high genomic instability of cancer cells (Lawrence et al, 2013). The neoantigens are presented on the cell surface in complex with the Major Histocompatibility Complex-class I (MHC I) and then recognized by T cells as non-self-antigens (Snyder et al, 2015; Sensi et al, 2006); as result, the adaptive immune system is activated in order to kill cancer cells.

Among the several strategies developed by tumors to escape the immune surveillance, the one arousing the greatest therapeutic interest is the upregulation of molecules that activate the immune checkpoints (Pennock et al, 2015), a group of pathways which suppress T cell functions. Considering that these molecules are showed on cancer cell surface, they can be easily blocked by mAbs and so they have become an attractive therapeutic target.
This is not the only strategy adopted by tumors to evade immunity. Another common escape mechanism is the impairment of the MHC I antigen presentation or of the endoplasmic reticulum and Golgi epitope processing, through genetic and epigenetic alterations (Fruci et al, 2012; Seliger et al, 2002). In fact, a reduced expression of proteins associated with the immunoproteasome, such as the subunits low-molecular-weight protein 2 and 7 (LMP-2 and LMP-7), or with the endoplasmic reticulum, for example the transporter associated with antigen presentation (TAP), has been reported in some tumors and cancer cell lines (Seliger et al, 1996; Alpan et al, 1996; Pandha et al, 2007).
At last, tumors can evade the immune system by secreting immunosuppressive mediators that are able to inhibit the maturation and/or activation of tumor infiltrating immune cells. These effects are mainly due to the constitutive activation of the signal transducer and activator of transcription 3 (STAT3) pathway observed in many cancers (Wang et al, 2004), which leads to the production of vascular endothelial growth factor (VEGF), IL-10, IL-6 and transforming growth factor-β (TGF-β) (Johnson et al, 2007; Liu et al, 2012). These molecules block NK and granulocyte effector functions, impair dendritic cell (DC) differentiation (Wang et al, 2004) and promote the commitment of immature myeloid cells into myeloid suppressor cells, i.e. a subset of cells that inhibit T lymphocyte functions (Gabrilovich et al, 2009; Gabrilovich et al, 2012).

1.8. The immune checkpoints - the PD-1/PD-L1 pathway and its role in the regulation of T cell functioning

The activity of T cells, which have a central role in the adaptive immunity against cancer, is regulated through a balance between activating and inhibitory stimuli generating from two different groups of surface receptors. The activating stimuli are also called co-stimulatory signals; they are necessary for activation of naïve T cells after the binding of the T cell receptor (TCR) to the MHC I-peptide complex. The co-stimulatory receptors are grouped into two main families. The first is the B7/CD28 family, which includes CD28, the inducible costimulator (ICOS) and the B- and T-lymphocyte attenuator (BTLA); among them, the best characterized receptor

is CD28, which is activated upon binding of B7-1 (CD80) and B7-2 (CD86) expressed on antigen presenting cells (APCs). CD28 co-stimulation leads to cell cycle progression and increases cell metabolism through the activation of PI3K/AKT pathway.
The other main group is the tumor necrosis factor receptor (TNFR) family. The members of this group, which includes CD40, CD27, CD30, OX40, 4-1BB and the glucocorticoid-induced TNFR-related gene (GITR), share a common T cell activation mechanism that is the stimulation of the nuclear factor-kB (NF-kB), with the consequent increase in expression of pro-inflammatory genes.
The signaling pathways that negatively regulate T cell function are called immune checkpoints and play a key role in two main mechanisms. The first is the peripheral tolerance (Fife et al, 2008), a process that blocks the self-reactive T cells that escape from negative selection in the thymus; the second is the switching-off of T lymphocyte effector functions that normally occurs during an immune response. This group of receptors includes the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), the programmed death-1 (PD-1) receptor, the lymphocyte-activation gene 3 (LAG-3) and the T cell immunoglobulin 3 (TIM-3); among them, CTLA-4 and PD-1 pathways are the best characterized ones.
CTLA-4 is expressed on T cells as a response to T cell activation and competes with the co-stimulatory receptor CD28 for the binding to the B7 ligand. CTLA-4 signaling is mediated by the intracellular phosphatase PP2A, which switches-off the Akt pathway, previously activated by co-stimulatory receptors.
PD-1 (also known as CD279) is a monomeric transmembrane protein of the Ig superfamily and its sequence shares 64% of homology with its murine orthologue (Lin et al, 2008). PD-1 extracellular region contains an IgV-like domain that interacts with the Ig domains of its ligands, programmed death ligand-1 and -2 (PD-L1 and PD-L2); the cytoplasmic tail has no intrinsic enzymatic activity but contains two tyrosine-based signaling domains, called immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM), whose tyrosine residues are phosphorilated and recruit PD-1 downstream effectors to the plasma membrane (Riley, 2009).
PD-L1 (also called B7-H1 or CD274) has 70% sequence identity with its murine orthologue, which allows *in vitro* cross reactivity between murine and human PD-1 and PD-L1 (Lin et al, 2008). Unlike its receptor, PD-L1 extracellular region contains two Ig domains, an N-terminal IgV-like domain that binds to PD-1 and an IgC-like one. It is a monomeric protein, but crystallographic analyses showed that it can exists in solution as dimeric complex too, although it is not clear the role of this complex *in vivo* in

immune system functioning (Chen et al, 2010). The other ligand PD-L2 (B7-DC or CD273) was discovered more recently than PD-L1 (Latchman et al, 2001). These two ligands have the same structure and share 40% amino acid identity.

All downstream effects of PD-1 are mediated by the Src homology phosphatase 2 (SHP-2), which is recruited *via* a Src homology 2 **(**SH-2) domain to the phosphorylated ITIM motif (Zhang et al, 2004). Among SHP-2 substrates, we find the lymphocyte-specific protein tyrosine kinase (Lck) and the zeta-chain-associated protein kinase 70 (ZAP-70), which are the main effectors of the TCR; they are inactivated through dephosphorilation by SHP-2, blocking the TCR signaling and consequently downregulating the target genes that promote T cell effector functions (Sheppard et al, 2004). PD-1 activation also impairs the PI3K/Akt and Ras/MAPK pathways (Parry et al, 2005), (fig. 5). The blockade of the PI3K/Akt pathway mainly affects glucose metabolism, by reducing the glycolytic enzyme activity and the expression of glucose transporters on T cell membrane; as a consequence, T cells are deprived of the main source of energy necessary for their growth and cell division. These metabolic changes also alter their differentiation (Chang et al, 2013; Patsoukis et al, 2015), promoting the switch from a T effector to a Treg and T-exhausted phenotype, which are responsible for the suppression of the immune response. On the other hand, the PD-1 mediated inhibition of the Ras/MAPK signaling, which is involved in the positive regulation of the cell cycle, stimulates T cells to enter G0 phase (Patsoukis et al, 2012); as a result, T cell proliferation is blocked.

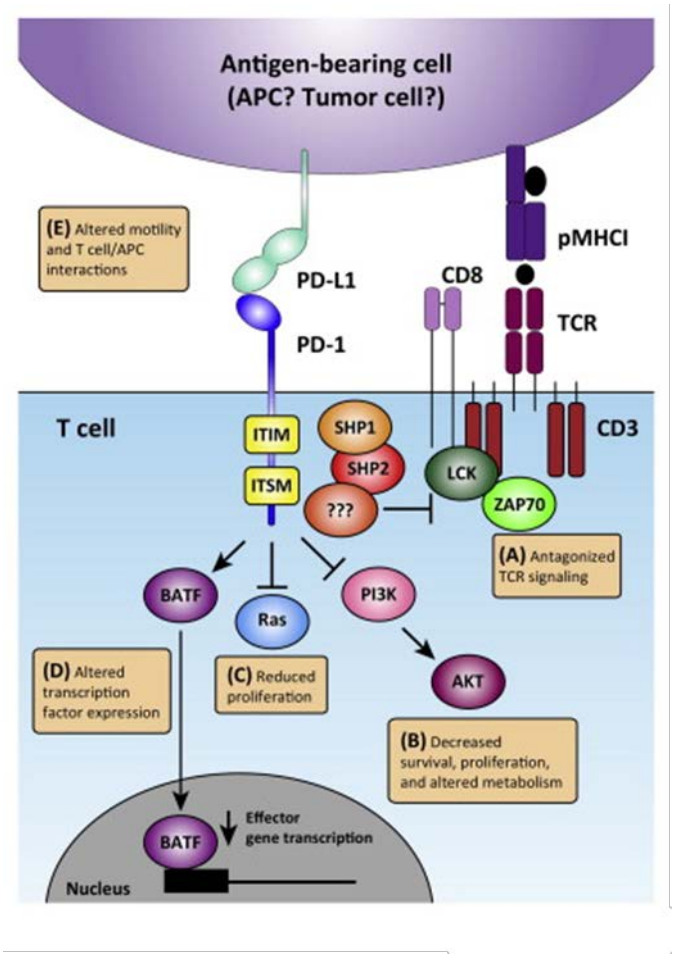

Fig. 5: schematic representation of PD-1 intracellular pathway. Upon engagement of PD-1 (in blue, on T cell surface) by PD-L1 (in light blue on APC or tumor cell surface), the SHP1/SHP2 phosphatases are recruited to PD-1 effector domains (in yellow) and then activated. These proteins affect the function of several targets: the complex of the T cell receptor (TCR, on the right), the Ras/MAPK and PI3K pathways and the basic leucine zipper transcriptional factor ATF-like (BATF). Boxes A, B, C, D and E summarize the resulting molecular and cellular alterations.

1.9. Impairment of PD-1/PD-L1 pathway in cancer

PD-1 expression is limited to immune cells, both lymphoid and myeloid lineages (T and B lymphocytes, NK cells, monocytes and DCs) (Keir et al, 2008), in which it is expressed at a low basal level. During the immune response, it is upregulated by the cytokines IL-2, IL-7, IL- 15, IL-21 and interferon-α (IFN-α) (Kinter et al, 2008; Cho et al 2008) and by a prolonged stimulation of T and B cell receptors (Freeman et al, 2006).
On the contrary, PD-L1 expression is not restricted to immune cells; in healthy conditions, it is expressed in many other tissues to protect themselves from immune attacks; PD-L1 is found at high levels in thymus cortex, lung, heart muscle, placenta, liver, mesenchymal stem cells and vascular endothelium; lower PD-L1 levels are found in pancreatic islets, astrocytes, neurons, keratinocytes (Keir et al, 2008), cornea and retina (Hori et al, 2006; Sugita et al, 2009). A lot of cytokines produced during the immune response, such as IL-2, IL-10, IL-7, IL-15, IL-21 and IFN-γ, upregulate PD-L1 in immune cells.
Interestingly, PD-L1 is expressed at high levels in many kinds of hematologic and solid tumors, due to the constitutive activation of some oncogenic pathways that positively control its transcription, in particular STAT3 (Marzek et al, 2008), PI3K/Akt and PI3K/mTOR pathways (Crane et al, 2009). Moreover, the IFN-γ present in the tumor microenvironment can induce PD-L1 expression too. IFN-γ is secreted by tumor infiltrating T lymphocytes activated by tumor antigens during the early stages of cancer development; in addition to its pro-inflammatory activity, this cytokine upregulates PD-L1 in tumor cells and so it is responsible for the suppression of T cell antitumor activity (fig. 6) (Taube et al, 2012). High PD-L1 levels have been detected not only on cancer cells, but also on the surface of the immune and non-immune cells that form the tumor microenvironment, that is macrophages, DCs, stromal fibroblasts and the endothelium of tumor neovasculature (fig. 7).

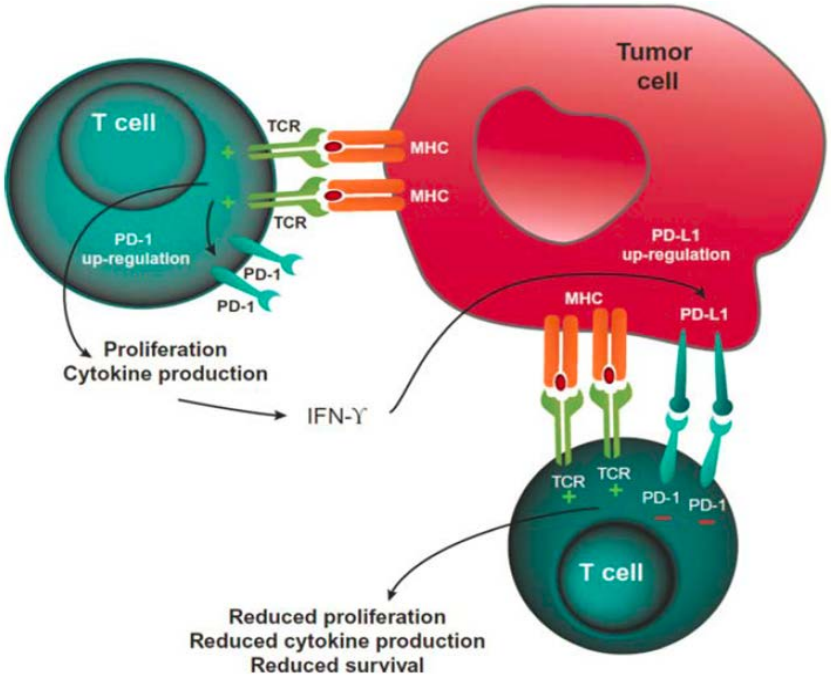

Fig. 6: mechanism of PD-L1 mediated inhibition of T cells by tumors. T cells activated by tumor- specific antigens, proliferate and produce cytokines. These inflammatory stimuli upregulate PD-L1 in tumor cells; its binding to PD-1 expressed on the same T cells negatively regulate their proliferation and effector functions, preventing cancer cells from killing.

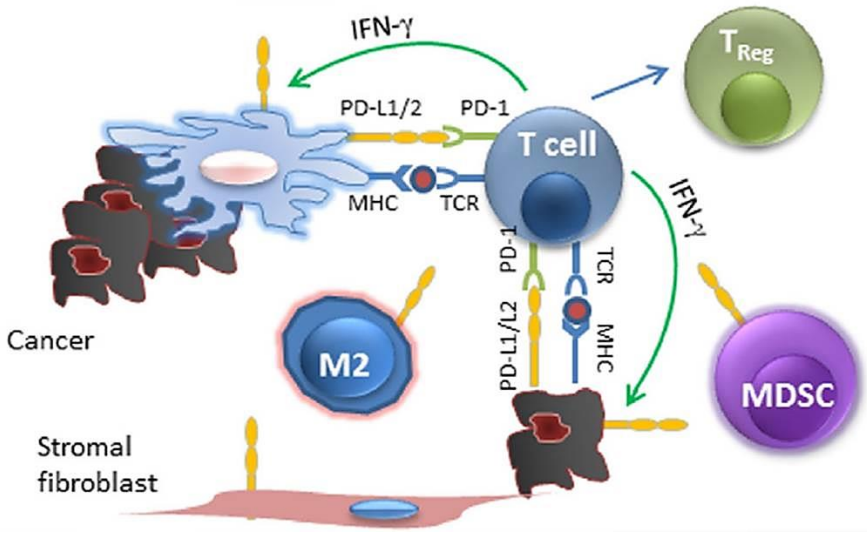

Fig. 7: PD-L1 and PD-L2 expression in the tumor microenvironment. Cancer cells (in black), stromal fibroblasts, tumor-infiltrating myeloid-derived suppressor cells (MDSC) and macrophages (particularly M2 subtype) show PD-1 ligands on their surface (in orange). This promotes the engagement of infiltrating T lymphocytes and their differentiation into Treg cells, with immunosuppressive activity.

PD-L2 is expressed only on stromal cells of thymus medulla, macrophages, bone marrow derived mast cells, peritoneal B1 cells and activated DCs (Zhong et al, 2007). In DCs its expression is positively regulated by IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Selenko-Gebauer et al, 2003). PD-L2 is also expressed in various types of human tumors (Rozali et al, 2012); however, it has not received as much attention as PD-L1 and its role in modulating antitumor immunity is less clear.

The overexpression of PD-L1 on cancer cells (or cells of the tumor stroma) is used by tumors as a mechanism of escape of the immune response, to protect themselves from killing by T cells. The binding of PD-L1 to its receptor PD-1 expressed on the tumor infiltrating lymphocytes activates the PD-1 checkpoint, blocking the downstream T cell pathways involved in cytotoxic response against the tumor.

The binding of PD-L1 to PD-1 is able to transduce signals not only in T cells, but in cancer cells too (Azuma et al, 2008). In fact, PD-L1 can act as PD-1 receptor in tumor cells, transmitting them anti-apoptotic signals which

enhance their resistance to apoptosis induced by both immune effectors and proapoptotic drugs.

1.10. Targeting of immune checkpoints with mAbs

Considering that the activation of immune checkpoints is a common mechanism used by tumors to escape the immune surveillance, there has been a great interest in developing therapeutic strategies that blocked these pathways. A great promise for cancer treatment is represented by the monoclonal antibodies that recognize the checkpoint receptors or ligands, preventing them to bind to each other. In this way, the inhibitory signals transduced to immune cells are blocked, so that they are reactivated to recognize and kill tumor cells (fig. 8).

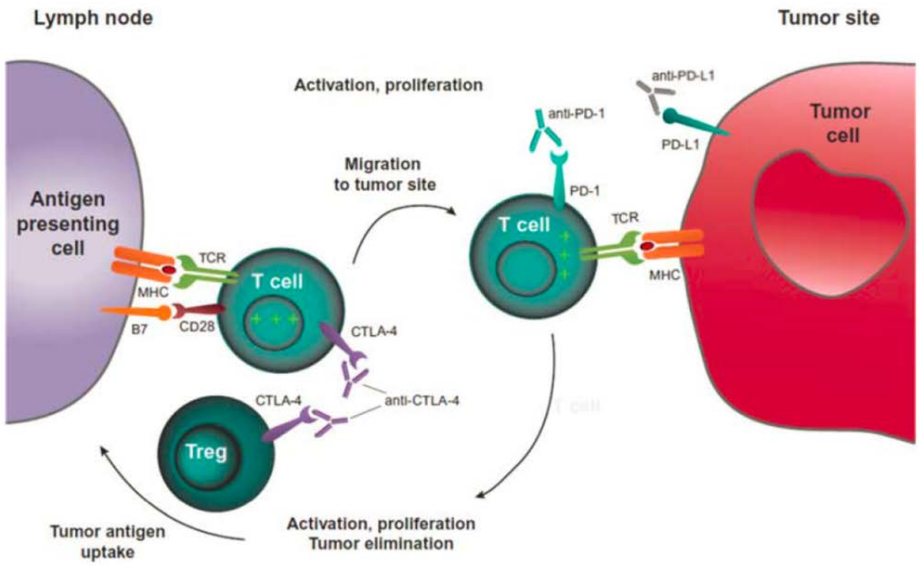

Fig. 8: effects of the immune checkpoint blockade by monoclonal antibodies. The binding of therapeutic mAbs to immune checkpoint components (anti CTLA-4, anti PD-1 and anti PD-L1, in purple, light blue and grey, respectively) removes the negative stimuli to T cell proliferation. In this way, T cells are re-activated to proliferate and recognize cancer cells. If used in combination, these antibodies can have a sinergistic effect.

A lot of monoclonal antibodies have been designed to target components of both activating and inhibitory pathways for the treatment of many solid tumors and hematologic malignancies (Peggs et al, 2009) (fig. 9). Many of these mAbs are in different phases of clinical development, few others have already been approved by FDA. The most targeted checkpoints are CTLA-4 and PD-1.

Ipilimumab, an anti CTLA-4 IgG1, was the first checkpoint inhibitor approved by FDA; it entered clinics in 2011 in USA and Europe for the treatment of unresectable or metastatic melanoma (Wolchok et al, 2013). Other clinical trials are ongoing for the evaluation of its therapeutic efficacy in combination regimens with other checkpoint inhibitors, in advanced melanoma cases which had not received any previous treatment (Larkin et al, 2015; Hodi et al, 2016). Ipilimumab, in combination with chemotherapy or other mAbs, is in advanced clinical trial also for the first line treatment of other solid tumors, particularly small and non-small cell lung cancer (SCLC and NSCLC) (Lynch et al, 2012; Reck et al, 2013; Antonia et al, 2016).

Given the therapeutic success of CTLA-4 blockade, other checkpoints have been targeted, particularly the PD-1/PD-L1 checkpoint. Among the antibodies developed to target this pathway, pembrolizumab and nivolumab have already been approved by FDA, while the others are in advanced clinical trials.

Pembrolizumab, a humanized IgG4, was the first anti PD-1 mAb to be approved by the FDA in 2014 for the treatment of metastatic melanoma that was not responsive to previous treatment with ipilimumab or with BRAF inhibitors (in tumors with BRAF mutations) (Robert et al, 2015). In 2015, the FDA extended its approval also to NSCLC (Garon et al, 2015).

Nivolumab is a fully human IgG4 in clinical use for the treatment of melanoma since 2014, with the same therapeutic indications as pembrolizumab (Weber et al, 2015). It demonstrated a good response rate in both untreated and previously treated metastatic melanoma cases. Very good results were obtained when nivolumab was used in combination with ipilimumab; in fact, in a phase III trial, the group of advanced melanoma patients treated with both the antibodies showed a 5-fold higher response rate than the group treated with ipilimumab alone (Postow et al, 2015). The clinical efficacy of nivolumab was also proven in squamous and metastatic NSCLC, renal cell carcinoma (RCC), head and neck cancer and Hodgkin lymphoma; in this last case the response rate was even 87% (Ansell et al, 2015).

Interestingly, a higher clinical response rate to these two anti PD-1 mAbs was observed when tumors expressed high levels of its ligand PD-L1 (Weber et al, 2015). The same result was also obtained after treatment with MPDL3280A (atezolizumab), a human anti PD-L1 IgG1 that was tested on

277 patients affected by various types of advanced incurable cancer (NSCLC, melanoma, RCC, colorectal cancer, gastric cancer and head and neck squamous cell carcinoma) (Herbst et al, 2014). Tumors that upregulated PD-L1, especially on tumor infiltrating immune cells, showed a better response than those tumors in which no or very low PD-L1 levels were detected. This studies support the hypothesis that PD-L1 expression within the tumor could be considered as a predictive biomarker of good outcomes upon treatment with PD-1/PD-L1 inhibitors.

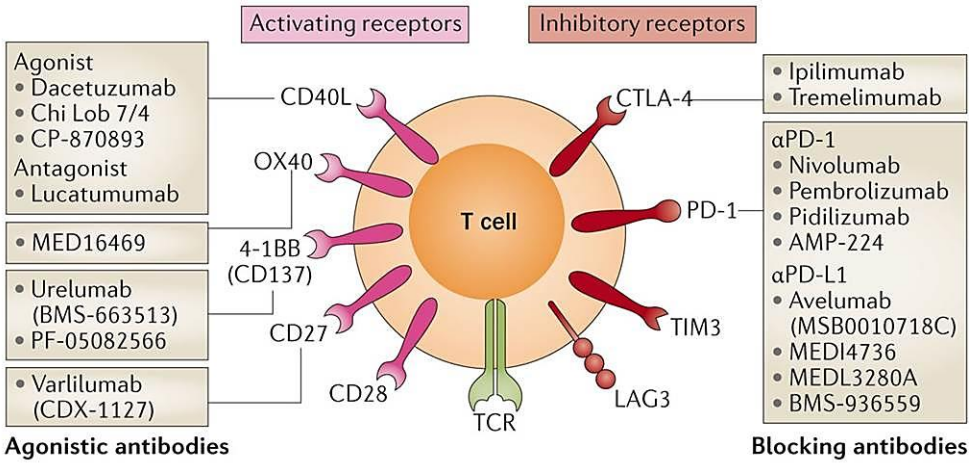

Fig. 9: spectrum of mAbs developed against activating or inhibiting T cell surface receptors to reactivate the antitumor immune response.

4. REFERENCES

Alpan RS, Zhang M, Pardee AB. Cell cycle-dependent expression of TAP1, TAP2, and HLA-B27 messenger RNAs in a human breast cancer cell line. *Cancer Research* (1996) 56(19):4358–4361.

Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, Schuster SJ, Millenson MM, Cattry D, Freeman GJ, Rodig SJ, Chapuy B, Ligon AH, Zhu L, Grosso JF, Kim SY, Timmerman JM, Shipp MA, Armand P. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *New England Journal of Medicine* (2015) 372(4):311–9.

Antonia SJ, López-Martin JA, Bendell J, Ott PA, Taylor M, Eder JP, Jäger D, Pietanza MC, Le DT, de Braud F, Morse MA, Ascierto PA, Horn L, Amin A, Pillai RN, Evans J, Chau I, Bono P, Atmaca A, Sharma P, Harbison CT, Lin CS, Christensen O, Calvo E. Nivolumab alone and nivolumab plus ipilimumab in recurrent small-cell lung cancer (CheckMate 032): a multicentre, open-label, phase 1/2 trial. *Lancet Oncology* (2016) 17(7):883-95.

Azuma T, Yao S, Zhu G, Flies AS, Flies SJ, Chen L. B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. *Blood* (2008) 111(7): 3635–43.

Barderas R, Desmet J, Timmerman P, Meloen R, Casal JI. Affinity maturation of antibodies assisted by in silico modeling. *Proceedings of the National Academy of Sciences USA* (2008) 105:9029-34.

Batista FD, Neuberger MS. Affinity dependence of the B cell response to antigen: a threshold, a ceiling and the importance of off-rate. *Immunity* (1998) 8:751-9.

Benatuil L, Perez JM, Belk J, Hsieh CM. An improved yeast transformation method for the generation of very large human antibody libraries. *Protein Eng Des Sel* (2010) 23(4):155-9.

Boder ET, Midelfort KS, Wittrup KD. Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proceedings of the National Academy of Sciences USA* (2000) 97:10701-5.

Buonpane RA, Churchill HR, Moza B, Sundberg EJ, Peterson ML, Schlievert PM, Kranz DM. Neutralization of staphylococcal enterotoxin

B by soluble, high-affinity receptor antagonists. *Nature Medicine* (2007) 13(6):725-9.

Buonpane RA, Moza B, Sundberg EJ, Kranz DM. Characterization of T cell receptors engineered for high affinity against toxic shock syndrome toxin-1. *Journal of Molecular Biology* (2005) 353(2):308-21.

Campadelli-Fiume G, Petrovic B, Leoni V, Gianni T, Avitabile E, Casiraghi C, Gatta V. Retargeting Strategies for Oncolytic Herpes Simplex Viruses. *Viruses* (2016) 8(3):63.

Carter P. Improving the efficacy of antibody-based cancer therapies. *Nature Reviews Cancer* (2001) 1(2):118-29.

Casadevall A, Dadachova E, Pirofski LA. Passive antibody therapy for infectious diseases. *Nature Reviews Microbiology* (2004) 2(9):695-703.

Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. *Nature Reviews Immunology* (2010) 10:301–16.

Chang CH, Curtis JD, Maggi LB Jr, Faubert B, Villarino AV, O'Sullivan D, Huang SC, van der Windt GJ, Blagih J, Qiu J, Weber JD, Pearce EJ, Jones RG, Pearce EL. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* (2013) 153(6):1239–51.

Chao G, Lau WL, Hackel BJ, Sazinsky SL, Lippow SM, Wittrup KD. Isolating and engineering human antibodies using yeast surface display. *Nature Protocols* (2006) 1(2):755-68.

Chen Y, Liu P, Gao F, Cheng H, Qi J, Gao GF. A dimeric structure of PD-L1: functional units or evolutionary relics? *Protein and Cell* (2010) 1(2):153-60.

Chiavenna SM, Jaworski JP, Vendrell A. State of the art in anti-cancer mAbs. *Journal of Biomedical Science* (2017) 24:15.

Cho HY, Lee SW, Seo SK, Choi IW, Choi I, Lee SW. Interferon-sensitive response element (ISRE) is mainly responsible for IFN-alpha-induced upregulation of programmed death-1 (PD-1) in macrophages. *Biochimica and Biophysica Acta* (2008) 1779(12):811–9.

Chowdhury PS, Pastan I. Improving antibody affinity by mimicking somatic hypermutation in vitro. *Nature Biotechnology* (1999) 17(6):568 – 572.

Clark LA, Boriack-Sjodin PA, Eldredge J, Fitch C, Friedman B, Hanf KJM, Jarpe M, Liparoto SF, Li Y, Lugovskoy A, Miller S, Rushe M, Sherman W, Simon K, Van Vlijmen H. Affinity enhancement of an in vivo matured therapeutic antibody using structure-based computational design. *Protein Science* (2006) 15:949-60.

Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nature Medicine* (2000) 6(4):443-6.

Cochran JR, Kim YS, Lippow SM, Rao B, Wittrup KD. Improved mutants from directed evolution are biased to orthologous substitutions. *Protein Eng Des Sel* (2006) 19(6):245-53.

Crane CA, Panner A, Murray JC, Wilson SP, Xu H, Chen L, Simko JP, Waldman FM, Pieper RO, Parsa AT. PI(3) kinase is associated with a mechanism of immunoresistance in breast and prostate cancer. *Oncogene* (2009) 28(2):306–12.

Dam J, Guan R, Natarajan K, Dimasi N, Chlewicki LK, Kranz DM, Schuck P, Margulies DH, Mariuzza RA. Variable MHC class I engagement by Ly49 natural killer cell receptors demonstrated by the crystal structure of Ly49C bound to H-2K(b). *Nature Immunology* (2003) 4(12):1213-22.

Di Noia JM, Neuberger MS. Molecular mechanisms of antibody somatic hypermutation. *Annual Review of Biochemistry* (2007) 76:1–22.

Doerner A, Rhiel L, Zielonka S, Kolmar H. Therapeutic antibody engineering by high efficiency cell screening. *FEBS Letters* (2014) 588: 278–287.

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: From immunosurveillance to tumor escape. *Nature Immunology* (2002) 3:991–998.

Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunological Reviews* (2008) 224:166–182.

Foote J, Eisen HN. Kinetic and affinity limits on antibodies produced during immune responses. *Proceedings of the National Academy of Sciences USA* (1995) 92:1254-6.

Freeman GJ, Wherry EJ, Ahmed R, Sharpe AH. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *Journal of Experimental Medicine* (2006) 203(10):2223–7.

Fruci D, Benevolo M, Cifaldi L, Lorenzi S, Lo Monaco E, Tremante E, Giacomini P. Major histocompatibility complex class I and tumour immuno-evasion: how to fool T cells and natural killer cells at one time. *Current Oncology* (2012) 19(1):39–41.

Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nature Reviews Immunology* (2009) 9:162–74.

Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nature Reviews Immunology* (2012) 12(4):253–268.

Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, Patnaik A, Aggarwal C, Gubens M, Horn L, Carcereny E, Ahn MJ, Felip E, Lee JS, Hellmann MD, Hamid O, Goldman JW, Soria JC, Dolled-Filhart M, Rutledge RZ, Zhang J, Lunceford JK, Rangwala R, Lubiniecki GM, Roach C, Emancipator K, Gandhi L. Pembrolizumab for the treatment of non-small-cell lung cancer. *New England Journal of Medicine* (2015) 372(21):2018–28.

Gatto D, Brink R. The germinal center reaction. *Journal of Allergy and Clinical Immunology* (2010) 126(5):898 – 907.

Gera N, Hussain M, Rao BM. Protein selection using yeast surface display. *Methods* (2013) 60(1):15-26.

Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, Sosman JA, McDermott DF, Powderly JD, Gettinger SN, Kohrt HEK, Horn L, Lawrence DP, Rost S, Leabman M, Xiao Y, Mokatrin A, Koeppen H, Hegde PS, Mellman I, Chen DS, Hodi FS. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* (2014) 515(7528):563–7.

Hey A. History and Practice: Antibodies in Infectious Diseases. *Microbiology Spectrum* (2015) 3(2):AID-0026-2014.

Hodi FS, Chesney J, Pavlick AC, Robert C, Grossmann KF, McDermott DF, Linette GP, Meyer N, Giguere JK, Agarwala SS, Shaheen M, Ernstoff MS, Minor DR, Salama AK, Taylor MH, Ott PA, Horak C,

Gagnier P, Jiang J, Wolchok JD, Postow MA. Combined nivolumab and ipilimumab versus ipilimumab alone in patients with advanced melanoma: 2-year overall survival outcomes in a multicentre, randomised, controlled, phase 2 trial. *Lancet Oncology* (2016) 17:1558–68.

Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. *Nature Biotechnology* (2005) 23(9):1126-36.

Hori J, Wang M, Miyashita M, Tanemoto K, Takahashi H, Takemori T, Okumura K, Yagita H, Azuma M. B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts. *Journal of Immunology* (2006) 177(9):5928–35.

https://www.accessdata.fda.gov/scripts/cder/daf/

Igawa T, Tsunoda H, Kuramochi T, Sampei Z, Ishii S, Hattori K. Engineering the variable region of therapeutic IgG antibodies. *MAbs* (2011) 3(3):243-52.

Jin M, Song G, Carman CV, Kim YS, Astrof NS, Shimaoka M, Wittrup DK, Springer TA. Directed evolution to probe protein allostery and integrin I domains of 200,000-fold higher affinity. *Proceedings of the National Academy of Sciences USA* (2006) 103(15):5758-63.

Johnson BF, Clay TM, Hobeika AC, Lyerly HK, Morse MA. Vascular endothelial growth factor and immunosuppression in cancer: current knowledge and potential for new therapy. *Expert Opinion in Biological Therapy* (2007) 7(4):449-60.

Kabat EA. Sequences of Proteins of Immunological Interest (1991) National Institutes of Health Publication No. 91-3242, 5th ed., United States Department of Health and Human Services, Bethesda, MD.

Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annual Reviews of Immunology* (2008) 26:677–704.

Kinter AL, Godbout EJ, McNally JP, Sereti I, Roby GA, O'Shea MA, Fauci AS. The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands. *Journal of Immunology* (2008) 181(10):6738–46.

Ko BK, Choi S, Cui LG, Lee YH, Hwang IS, Kim KT, Shim H, Lee JS. Affinity Maturation of Monoclonal Antibody 1E11 by Targeted

Randomization in CDR3 Regions Optimizes Therapeutic Antibody Targeting of HER2-Positive Gastric Cancer. *PLoS ONE* (2015) 10(7): e0134600.

Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, Schadendorf D, Dummer R, Smylie M, Rutkowski P, Ferrucci PF, Hill A, Wagstaff J, Carlino MS, Haanen JB, Maio M, Marquez-Rodas I, McArthur GA, Ascierto PA, Long GV, Callahan MK, Postow MA, Grossmann K, Sznol M, Dreno B, Bastholt L, Yang A, Rollin LM, Horak C, Hodi FS, Wolchok JD. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *New England Journal of Medicine* (2015) 373(1):23–34.

Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, Greenfield EA, Bourque K, Boussiotis VA, Carter LL, Carreno BM, Malenkovich N, Nishimura H, Okazaki T, Honjo T, Sharpe AH, Freeman GJ. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nature Immunology* (2001) 2(3):261–8.

Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, Carter SL, Stewart C, Mermel CH, Roberts SA, Kiezun A, Hammerman PS, McKenna A, Drier Y, Zou L, Ramos AH, Pugh TJ, Stransky N, Helman E, Kim J, Sougnez C, Ambrogio L, Nickerson E, Shefler E, Cortés ML, Auclair D, Saksena G, Voet D, Noble M, DiCara D, Lin P, Lichtenstein L, Heiman DI, Fennell T, Imielinski M, Hernandez B, Hodis E, Baca S, Dulak AM, Lohr J, Landau DA, Wu CJ, Melendez-Zajgla J, Hidalgo-Miranda A, Koren A, McCarroll SA, Mora J, Crompton B, Onofrio R, Parkin M, Winckler W, Ardlie K, Gabriel SB, Roberts CWM, Biegel JA, Stegmaier K, Bass AJ, Garraway LA, Meyerson M, Golub TR, Gordenin DA, Sunyaev S, Lander ES, Getz G. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* (2013) 499(7457):214–218.

Lin DY, Tanaka Y, Iwasaki M, Gittis AG, Su HP, Mikami B, Okazaki T, Honjo T, Minato N, Garboczi DN. The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of antibodies and T cell receptors. *Proceedings of the National Academy of Sciences USA* (2008) 105(8):3011–6.

Lipovsek D, Plückthun A. In-vitro protein evolution by ribosome display and mRNA display. *Journal of Immunological Methods* (2004) 290(1-2):51-67.

Liu C, Zhang L, Chang X, Cheng Y, Cheng H, Ye X, Fu T, Chen J, Cui H. Overexpression and Immunosuppressive Functions of Transforming Growth Factor 1, Vascular Endothelial Growth Factor and Interleukin-10 in Epithelial Ovarian Cancer. *Chinese Journal of Cancer Research* (2012) 24(2): 130–137.

Lynch TJ, Bondarenko I, Luft A, Serwatowski P, Barlesi F, Chacko R, Sebastian M, Neal J, Lu H, Cuillerot JM, Reck M. Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non-small-cell lung cancer: Results from a randomized, doubleblind, multicenter phase II study. *Journal of Clinical Oncology* (2012) 30(17):2046–2054.

Mahmud N, Klipa D, Ahsan N. Antibody immunosuppressive therapy in solid-organ transplant Part I. *MAbs* (2010) 2(2):148–156.

Marks JD. Antibody affinity maturation by chain shuffling. *Methods in Molecular Biology* (2004) 248:327-43.

Marzec M, Zhang Q, Goradia A, Raghunath PN, Liu X, Paessler M, Wang HY, Wysocka M, Cheng M, Ruggeri BA, Wasik MA. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). *Proceedings of the National Academy of Sciences USA* (2008) 105(52):20852–7.

Maul RW, Gearhart PJ. AID and somatic hypermutation. *Advances in Immunology* (2010) 105:159–91.

Neylon C. Chemical and biochemical strategies for the randomization of protein encoding DNA sequences: library construction methods for directed evolution. *Nucleic Acids Research* (2004) 32(4):1448–1459.

Oda M, Sato-Nakamura N, Azuma T. Molecular characterization of monovalent and multivalent hapten–protein conjugates for analysis of antigen-antibody interaction. *Analytical Biochemistry* (2004) 333(2):365–71.

Pandha H, Rigg A, John J, Lemoine N. Loss of expression of antigen-presenting molecules in human pancreatic cancer and pancreatic cancer cell lines. *Clinical and Experimental Immunology* (2007) 148(1):127–135.

Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, Linsley PS, Thompson CB, Riley JL. CTLA-4 and PD-1

receptors inhibit T-cell activation by distinct mechanisms. *Molecular and Cellular Biology* (2005) 25(21):9543–9553.

Patsoukis N, Bardhan K, Chatterjee P, Sari D, Liu B, Bell LN, Karoly ED, Freeman GJ, Petkova V, Seth P, Li L, Boussiotis VA. PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. *Nature Communications* (2015) 6:6692.

Patsoukis N, Brown J, Petkova V, Liu F, Li L, Boussiotis VA. Selective effects of PD-1 on Akt and Ras pathways regulate molecular components of the cell cycle and inhibit T cell proliferation. *Science Signaling* (2012) 5(230):ra46.

Peggs KS, Quezada SA, Allison JP. Cancer immunotherapy: co-stimulatory agonists and co-inhibitory antagonists. *Clinical and Experimental Immunology* (2009) 157(1):9–19.

Pennock GK, Chow LQ2. The Evolving Role of Immune Checkpoint Inhibitors in Cancer Treatment. *Oncologist* (2015) 20(7):812-22.

Pepper LR, Cho YK, Boder ET, Shusta EV. A decade of yeast surface display technology: where are we now? *Combinatorial Chemistry and High Throughput Screening* (2008) 11(2):127-34.

Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, Linette GP, Meyer N, Giguere JK, Agarwala SS, Shaheen M, Ernstoff MS, Minor D, Salama AK, Taylor M, Ott PA, Rollin LM, Horak C, Gagnier P, Wolchok JD, Hodi FS. Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. *New England Journal of Medicine* (2015) 372(21):2006–17.

Rajpal A, Beyaz N, Haber L, Cappuccilli G, Yee H, Bhatt RR, Takeuchi T, Lerner RA, Crea R. A general method for greatly improving the affinity of antibodies by using combinatorial libraries. *Proceedings of the National Academy of Sciences USA* (2005) 102(24):8466–8471.

Rao BM, Driver I, Lauffenburger DA, Wittrup KD. Interleukin 2 (IL-2) variants engineered for increased IL-2 receptor alpha-subunit affinity exhibit increased potency arising from a cell surface ligand reservoir effect. *Molecular Pharmacology* (2004) 66(4):864-9.

Rasila TS, Pajunen MI, Savilahti H. Critical evaluation of random mutagenesis by error-prone polymerase chain reaction protocols,

Escherichia coli mutator strain, and hydroxylamine treatment. *Analytical Biochemistry* (2009) 388(1):71–80.

Reck M, Heigener DF, Mok T, Soria JC, Rabe KF. Management of non-small-cell lung cancer: recent developments. *Lancet* (2013) 382(9893):709-19.

Reichert JM. Antibodies to watch in 2017. *Mabs* (2017) 9(2):167–181.

Riley JL. PD-1 signaling in primary T cells. *Immunological Reviews* (2009) 229(1): 114–125.

Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, Daud A, Carlino MS, McNeil C, Lotem M, Larkin J, Lorigan P, Neyns B, Blank CU, Hamid O, Mateus C, Shapira-Frommer R, Kosh M, Zhou H, Ibrahim N, Ebbinghaus S, Ribas A. Pembrolizumab versus ipilimumab in advanced melanoma. *New England Journal of Medicine* (2015) 372(26):2521–32.

Roth DB. V(D)J Recombination: Mechanism, Errors, and Fidelity. *Microbiology Spectrum* (2014) 2(6).

Rozali EN, Hato SV, Robinson BW, Lake RA, Joost Lesterhuis W. Programmed Death Ligand 2 in Cancer-Induced Immune Suppression. *Clinical and Developmental Immunology* Volume 2012, Article ID 656340, 8 pages.

Rudnick SI, Adams GP. Affinity and Avidity in Antibody-Based Tumor Targeting. *Cancer Biotherapy and Radiopharmaceuticals* (2009) 24(2):155–161.

Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 9.16 (1989).

Selenko-Gebauer N, Majdic O, Szekeres A, Höfler G, Guthann E, Korthäuer U, Zlabinger G, Steinberger P, Pickl WF, Stockinger H, Knapp W, Stöckl J. B7-H1 (programmed death-1 ligand) on dendritic cells is involved in the induction and maintenance of T cell anergy. *Journal of Immunology* (2003) 170(7):3637–44.

Seliger B, Cabrera T, Garrido F, Ferrone S. HLA class I antigen abnormalities and immune escape by malignant cells. *Seminars in Cancer Biology* (2002) 12(1):3–13.

Seliger B, Hohne A, Knuth A, Bernhard H, Ehring B, Tampe R, Huber C. Reduced membrane major histocompatibility complex class I density and stability in a subset of human renal cell carcinomas with low TAP and LMP expression. *Clinical Cancer Research* (1996) 2(8):1427–1433.

Sensi M, Anichini A. Unique tumor antigens: evidence for immune control of genome integrity and immunogenic targets for T cell-mediated patient-specific immunotherapy. *Clinical Cancer Research* (2006) 12(17):5023-32.

Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, Qiu Y, Jussif JM, Carter LL, Wood CR, Chaudhary D. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKC theta. *FEBS Letters* (2004) 574(1–3):37–41.

Siegel RW, Feldhaus M, Marks JD. Molecular evolution of antibody affinity for sensitive detection of botulinum neurotoxin type A. *Journal of Molecular Biology* (2005) 351(1):158–169.

Snyder A, Chan TA. Immunogenic peptide discovery in cancer genomes. *Current Opinion in Genetics & Development* (2015) 30:7-16.

Stavnezer J, Guikema JE, Schrader CE. Mechanism and regulation of class switch recombination. *Annual Reviews of Immunology* (2008) 26:261-92.

Sugita S, Usui Y, Horie S, Futagami Y, Aburatani H, Okazaki T, Honjo T, Takeuchi M, Mochizuki M. T-cell suppression by programmed cell death 1 ligand 1 on retinal pigment epithelium during inflammatory conditions. *Investigative Ophthalmology and Visual Science* (2009) 50(6):2862–70.

Sundberg EJ, Mariuzza RA. Molecular recognition in antibody-antigen complexes. *Advances in Protein Chemistry* (2002) 61:119–60.

Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, Chen S, Klein AP, Pardoll DM, Topalian SL, Chen L. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Science Translational Medicine* (2012) 4(127):127ra37.

Tillotson BJ, Cho YK, Shusta EV. Cells and cell lysates: A direct approach for engineering antibodies against membrane proteins using yeast surface display. *Methods* (2013) 60(1):27-37.

Tillotson BJ, de Larrinoa IF, Skinner CA, Klavas DM, Shusta EV. Antibody affinity maturation using yeast display with detergent solubilized membrane proteins as antigen sources. *Protein Engineering, Design and Selection* (2013) 26(2):101–112.

Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S, Bhattacharya R, Gabrilovich D, Heller R, Coppola D, Dalton W, Jove R, Pardoll D, Yu H. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nature Medicine* (2004) 10(1):48–54.

Wang XX, Cho YK, Shusta EV. Mining a yeast library for brain endothelial cell-binding antibodies. *Nature Methods* (2007) 4(2):143-5.

Wang XX, Shusta EV. The use of scFv-displaying yeast in mammalian cell surface selections. *Journal of Immunological Methods* (2005) 304(1-2):30–42.

Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, Hoeller C, Khushalani NI, Miller WH Jr, Lao CD, Linette GP, Thomas L, Lorigan P, Grossmann KF, Hassel JC, Maio M, Sznol M, Ascierto PA, Mohr P, Chmielowski B, Bryce A, Svane IM, Grob JJ, Krackhardt AM, Horak C, Lambert A, Yang AS, Larkin J. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. *Lancet Oncology* (2015) 16(4):375–84.

Wolchok JD, Hodi FS, Weber JS, Allison JP, Urba WJ, Robert C, O'Day SJ, Hoos A, Humphrey R, Berman DM, Lonberg N, Korman AJ. Development of ipilimumab: A novel immunotherapeutic approach for the treatment of advanced melanoma. *Annals of the New York Academy of Sciences* (2013) 1291:1–13.

Yoshinaga K, Matsumoto M, Torikai M, Sugyo K, Kuroki S, Nogami K, Matsumoto R, Hashiguchi S, Ito Y, Nakashima T, Sugimura K. Ig L-chain shuffling for affinity maturation of phage library-derived human anti-human MCP-1 antibody blocking its chemotactic activity. *Journal of Biochemistry* (2008) 143(5):593-601.

Zhang X, Schwartz JC, Guo X, Bhatia S, Cao E, Lorenz M, Cammer M, Chen L, Zhang ZY, Edidin MA, Nathenson SG, Almo SC. Structural and functional analysis of the costimulatory receptor programmed death-1. *Immunity* (2004) 20(3):337–47.

Zhong X, Tumang JR, Gao W, Bai C, Rothstein TL. PD-L2 expression extends beyond dendritic cells/macrophages to B1 cells enriched for V(H)11/V(H)12 and phosphatidylcholine binding. *European Journal of Immunology* (2007) 37(9):2405–10.