University of Nova Gorica
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Cross-presentation and cancer immunotherapy

Dissertation

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List of abbreviations

ADCC:	Antibody-dependent cell-mediated cytotoxicity
AID:	Activation-induced (cytidine) deaminase
APC:	Antigen presenting cell
BCR:	B-cell receptor
CAR:	Chimeric antigen receptor
CD:	Cluster of differentiation
cDC:	Conventional dendritic cells
CDC:	Complement-dependent cytotoxicity
CEA:	Carcinoembrionic antigen
CFSE:	5- 6- carboxyfluorescein diacetate succinimidyl ester
CLR:	C-type lectin receptor
CMV:	Cytomegalovirus
CpG:	Cytidine-phoshate-guanosine
CSR:	Class-switch recombination
C-term:	Carboxy terminal
CTL:	Cytotoxic T-lymphocyte
DC:	Dendritic cell
ECD:	Extracellular domain
ECL:	Enhanced chemiluminescence
EGF:	Epidermal Growth Factor
ELISA:	Enzyme-linked immunosorbent assay
ER:	Endoplasmic reticulum
ERAAP:	ER-associated amminopeptidase
FACS:	Fluorescence-activated cell sorter
Fc:	Constant fragment
FcR:	Fc receptor
FCS:	Fetal calf serum
FDA:	Food and Drug Administration
GM-CSF:	Granulocyte-Macrophage colony stimulating factor
HEK:	Human epithelial kidney cell
HER:	Human epidermal growth factor receptor
huC_H3:	Third constant domain of human IgG1
ICAM:	Inter-cellular adhesion molecule
ICD:	Intracellular domain
IFN:	Interferon
Ig:	Immunoglobulin
IL:	Interleukin
ITAM:	Immune receptor tyrosine-based activation motif
ITIM:	Immune receptor tyrosine-based inhibitory motif
K14:	Keratin-14
LDL:	Low-density lipoprotein
LTR:	Long-terminal repeat
mAb:	Monoclonal antibody
MAP:	Mitogen-activated protein
MHC:	Major histocompatibility complex
MMTV:	Mouse mammary tumour virus
muC_H3:	Third constant domain of murine IgG1
NF-κB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK:	Natural Killer cells

NOP:	Neu ovalbumin protein
N-term:	Amino terminal
OVA:	Ovalbumin
PAGE:	Poly-acrylamide gel electrophoresis
PAMP:	Pathogen-associated molecular pattern
pDC:	Plasmacytoid dendritic cells
PRR:	Pattern-recognition receptor
PSA:	Prostate specific antigen
PVDF:	Polyvinylidene fluoride
RAG:	Recombinase activating gene
scFv:	Single chain variable fragment
SDS:	Sodium dodecyl sulphate
SH:	Somatic hypermutation
SIP:	Small immune protein
SR:	Somatic recombination
TAA:	Tumour-associated antigen
TAP:	Transporter-associated with antigen processing
TCR:	T-cell receptor
TGF:	Transforming growth factor
T_H:	T helper
TIL:	Tumour-infiltrating lymphocyte
TKI:	Tyrosine-kinase inhibitor
TLR:	Toll-like receptor
TM:	Transmembrane domain
TMB:	Tetramethyl benzidine
TSA:	Tumour-specific antigen
VEGF:	Vascular endothelium growth factor
V_H:	Variable region of heavy chain
V_L:	Variable region of light chain
WAP:	Whey acidic protein
WASP:	Wiskott-Aldrich syndrome protein

Abstract

Dendritic cells (DCs) are the most important and powerful antigen presenting cells (APCs) of the immune system. DCs exhibit distinctive morphological and physiological features related to their ability to uptake and process antigens. These cells are considered the main actors in orchestrating the immune response against all antigens, because they are able to prime T-cells and determine their fate by cell-to-cell interactions and also by providing the most appropriate signals for the activation of the adaptive immune response.

For these reasons, DCs have progressively acquired more importance in the clinical field, because it is possible to target specific tumour-associated antigens to these cells in order to obtain a vigorous and efficient immune response against malignancies.

To this aim, many different technical advances in the route and the way of administration of antigens have been developed, spanning from the administration of synthetic peptides to genetic immunisation, in some cases with encouraging results.

Among the different types of tumours where cancer immunotherapy strategies are focused, breast malignancies represented one of the most important. In one third of breast cancer patients there is a dysregulation of HER2, a receptor belonging to the family of epidermal growth factor receptors, which can be used as a tumour-associated antigen.

We implemented a genetic immunisation approach to induce an immune response against two model antigens, ovalbumin and HER2. A gene gun DNA immunisation strategy, based on administration of the antigen fused with a targeting unit for an internalising receptor displayed on the surface of DCs, was assessed for its ability to enhance tumour protection in both a non-self and a self (tolerogenic) environment. We demonstrated that with such an approach it is possible to orient antigens towards the cross-presentation pathway and to induce the activation of specific $CD8^+$ T-cells. We also showed that gene gun DNA immunisation is a valid strategy to induce a repertoire of antibodies qualitatively different from the one obtained by protein immunisation.

We finally showed that *in vivo* targeting of DC-receptors is a good strategy to overcome immune tolerance and is required for a proficient immunity against tumour development.

Povzetek

Dendritične celice (DC) so najučinkovitejše antigen predstavitvene celice (APC) v imunskem sistemu. Imajo značilne morfološke in fiziološke lastnosti, ki so povezane z njihovo sposobnostjo privzemanja in procesiranja antigenov. DC veljajo za glavne igralce pri koordiniranju imunskega odziva proti praktično vsem antigenom, saj so sposobne aktivirati limfocite T in vplivati na njihovo usodo z medceličnimi interakcijami, prav tako pa so tudi vir drugih signalov za aktivacijo pridobljenega imunskega odziva.

Zaradi vsega tega so DC postale pomembne tudi na kliničnem področju, saj jih lahko pripravimo tako, da predstavljajo specifične tumorske antigene in na ta način spodbudijo močan in učinkovit anti-tumorski imunski odziv. Obstajajo številni načini za vnos tumorskih antigenov v DC, ki segajo vse od uporabe sintetičnih peptidov do genske imunizacije. V nekaterih primerih so ti postopki tudi že precej uspešni.
Rak na dojkah je eden pomembnejših malignih obolenj, na katerega je osredotočen razvoj številnih imunoterapij. Pri tretjini bolnic z rakom na dojkah se pojavijo napake v regulaciji receptorja HER2 iz družine receptorjev epidermalnih rastnih faktorjev, ki je tudi eden izmed tumorskih antigenov.

V naši raziskavi smo za aktivacijo imunskega odziva proti dvema modelnima antigenoma, ovalbuminu in receptorju HER2, uporabili gensko imunizacijo. Imunizacija z gensko pištolo je temeljila na administraciji antigena, ki je bil sklopljen s tarčno enoto za internalizirajoči receptor, izražen na površini DC. V raziskavi smo ovrednotili učinkovitost tega pristopa pri vzpostavljanju anti-tumorske zaščite v heterolognem in v avtolognem (tolerogenem) okolju. Pokazali smo, da je s tovrstnim pristopom mogoče usmeriti antigene v pot navzkrižnega predstavljanja antigenov in posledično aktivirati specifične limfocite T CD8+. Prav tako smo pokazali, da je DNA imunizacija z gensko pištolo primerna metoda za generiranje repertoarja protiteles, ki so kvalitativno drugačna od protiteles pridobljenih s proteinsko imunizacijo. Naša raziskava dokazuje, da je moduliranje receptorjev DC in vivo dobra strategija za preseganje imunske tolerance in je hkrati učinkovita pri vzpostavljanju imunost, ki preprečuje nastanek in razvoj tumorjev.

Introduction

General aspects of immune system

The immune system is a network of cells, tissues, organs and molecules that work together to protect an organism against the myriad potentially pathogenic foreign substances and organisms.

This complex system is divided primarily into two branches that act synergistically and in cooperative fashion, the innate and the adaptive immunity.

Innate immunity is the first line of defence against dangerous substances and organisms. It is composed by physical and chemical barriers, that prevent the entry of these threats, as well as by cells with the ability to phagocytise these substances and to kill them. This primary response is not specialised: irrespective of the nature of these dangers, the action mechanisms are always the same and repeat equally to subsequent challenges.

The adaptive immunity is, in contrast, a specialised complex of cells and their products that specifically recognize and attack the different dangers with high efficiency. In addition, if the organism has to face with the same threat, adaptive immunity responds with more focused and efficient mechanisms that induce a faster eradication of the menace. The components of this second branch of the immune system are mainly the lymphocytes, which determine the specificity of the response, cooperating with other cells to orchestrate the most appropriate effector phase.

Lymphocytes are divided into two major groups, i.e. B- or T-cells, that derive from a common hematopoietic progenitors but whose maturation occurs in different districts of the body: B-cells mature in bone marrow whereas T-cell in the thymus.

B-lymphocytes

B-lymphocytes are the only cells able to secrete a particular type of immunological relevant products, the immunoglobulins.

Antibodies or immunoglobulins (Ig) are glycoproteins made by two polypeptide chains, called heavy and light chains, composed by a constant and a variable region. The N-terminus (N-term) of each pair of heavy-light chains consists of two variable immunoglobulin domains (V_L and V_H), that form a surface within which antigen binds. This association is responsible for antigen recognition, generating a collection of unique antigenic determinants called idiotype. The constant part (constant fragment or Fc) is responsible for the biological activity of the Ig, interacting with cell surface receptors called Fc receptors (FcR) and some proteins of the complement system. Depending on the characteristics of the Fc, immunoglobulins are divided into subclasses, called isotypes, with different localisations and functions.

Immunoglobulins can be soluble or membrane-bound: in this latter case, they serve as the receptor for the antigen recognition, and, together with other invariant proteins, composes the B-cell receptor (BCR).
In contrast to other cell types receptors, which are equal in all cells, individual B-cell express on their surface a particular BCR with unique specificity. This specificity is conferred by the particular membrane-bound immunoglobulin, which is encoded by a set of genes organised into three loci, one for the heavy chain and two for the light chain. These multigene complexes are formed by several gene segments, separated by non-coding regions: these gene segments are selected and joined together to drive the expression of functional heavy and light chains, which then pair to generate the mature immunoglobulin. This process is called V(D)J recombination and it is similar for both heavy and light chains, even though the two multigene complexes are independent and situated into different chromosomes (in human, heavy chain genes are in chromosome 14, and light chains ones in chromosomes 2 or 22 depending on the type of light chain, κ or λ).
Light chains contain V, J and C segments: the rearrangement of VJ segments encodes the variable region (V_L), while in the heavy-chains, the variable region (V_H) is encoded by V, D and J segments. Constant regions of both chains are encoded by the C segments (fig. 1) (Abbas, Lichtman *et al.* 2007).

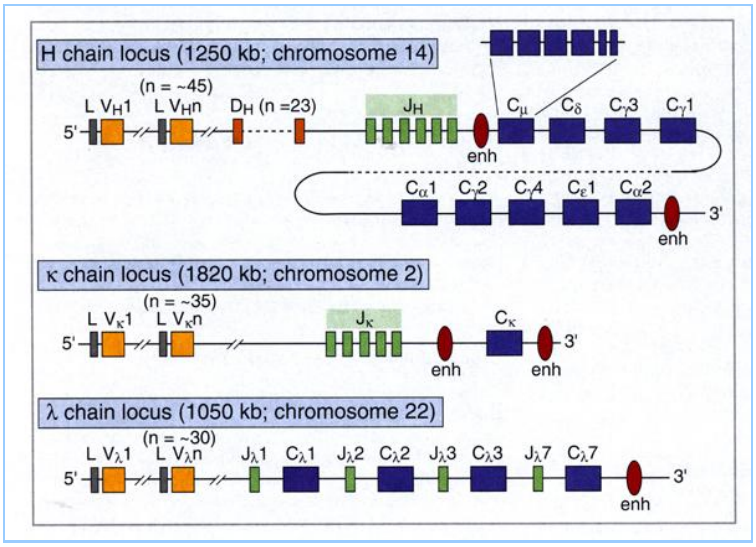

Fig. 1: Localisation and organisation of the V(D)J and C gene segments of human immunoglobulin (figure taken from (Abbas, Lichtman *et al.* 2007).

The recombination process mediates the selection and the joining of different gene segments and the removal of the sequences between them. Two lymphoid-specific factors, the recombinase activating gene (RAG-1 and RAG2) enzymes, which recognize specific recombination signals present at the 3' of V, 3' and 5' of D and 5' of J segments, are responsible and act during all the steps of the V(D)J recombination (Oettinger, Schatz *et al.* 1990).

The gene rearrangement is associated with the different stages of B-cell maturation (fig. 2). The first stage is represented by pro-B cells, where V(D)J recombination starts. The first event is the joining of D and J segments of heavy chain, followed by the recombination of V segment, with the generation of the entire variable region of the heavy chain, to which the constant μ region is then added, due to mRNA splicing. At this phase, cells are known as pre-B-cells. The μ chain is thus transiently displayed on the surface of the cells coupled with two proteins that mimic the presence of light chains, forming the pre-B-cell receptor (pre-BCR). Once the recombination of light chains genes has occurred in a similar mechanism, light chain assembles with the μ chain, originating a mature IgM that is transported to the cell surface in the form of a functional BCR complex. During this phase, alternative splicing events of constant regions can occur with the production of IgD, which can be also displayed as part of the BCR in alternative to IgM (Paul 1999; Abbas, Lichtman *et al.* 2007).

B-cells are now mature and enter into blood stream to reach lymph nodes, where they patrol for their respective antigens. If the BCR engages its specific antigen, a signalling pathway leads the cells to proliferate and to downregulate the expression of membrane-bound Ig in favour of its soluble version, which is secreted in the environment.

Mature B-cells can undergo other antigen-dependent differentiation events to improve the affinity and functionality of the antibodies after a second exposure to the antigen: these events occur at the level of single clonal mature B-cells and are named somatic hypermutation and class-switch recombination.

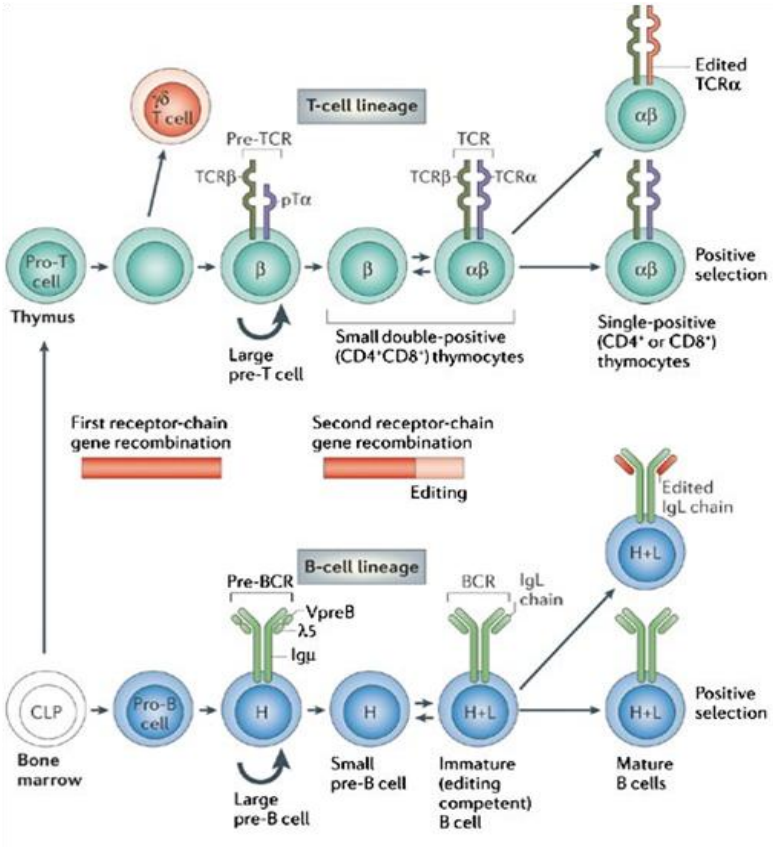

Fig. 2: T- and B-lymphocyte receptor origin

B- and T-cells share a common organisation of the genes encoding their receptors (BCR and TCR, respectively). Maturation consists of a series of events that consist of the rearrangement of the different gene segments in order to obtain the extremely variant repertoire of BCR and TCR (figure taken from (Nemazee 2006)).

Somatic hypermutation (SH) is the process by which point mutations (insertion, deletions and, mainly, substitutions) are introduced in the variable regions of the immunoglobulin. It is specific for each individual immune cell and it is devoted to produce immunoglobulins with higher affinity for the antigen (Di Noia and Neuberger 2007; Teng and Papavasiliou 2007).

Class-switch recombination (CSR) is another biological process that serves to change the antibody constant region (or isotype) expressed by B-cells (Honjo, Kinoshita *et al.* 2002). CSR involves recombination between two different switch (S) regions that are located upstream of each C region of the heavy chain locus, with deletion of the DNA sequence between these two switch regions. Replacement of the Cμ region by a C region of another class of immunoglobulin (Cγ1-4, Cα1-2 or Cε) results in the production of different antibody isotypes (IgG1-4, IgA1-2 or IgE). This is a complex mechanism that involves different enzymes, the most important of which is the activation-induced cytidine deaminase (AID), which is also responsible of SHM (McBride, Gazumyan *et al.* 2008). The resulting antibody retains the same antigen specificity but with different characteristics conferred by the constant part, for example half-life, ability to interact with FcRs or the location where these antibodies accumulate, thus mediating different biological effects.

T-lymphocytes

T-lymphocytes represent the second group of lymphocytes. These cells derive from bone marrow progenitors but, in contrast to the B-cells, their final maturation occurs in secondary lymphoid organ, i. e. in the thymus.

T-cells display on their surface an antigen specific T-cell receptor (TCR), an anchored-membrane proteins composed by two chains, which are encoded by a set of genes similar to the one of B-cells. The expression of TCR is a mechanism that requires a gene rearrangement process similar to the one needed for the generation of mature Ig (fig. 2). However, the maturation process does not produce a receptor able to directly recognise antigen, as the BCR does. The TCR requires that the antigen, represented by a short peptide, to be recognised is associated to another anchored-membrane protein, a major histocompatibility complex (MHC) molecule. There are

two main groups of MHC molecules, the MHC class I (MHC-I) and the MHC class II (MHC-II) molecules, with different functions (Paul 1999).
MHC-I are composed of a single anchored-membrane α chain, with three different domains, associated with the β2-microglobulin protein, while MHC-II are composed by two transmembrane chains, α and β, each of them constituted by two different domains. MHC molecules are expressed differently by distinct type of cells: MHC-I are found in every nucleated cell of the body, while MHC-II are expressed by only few specialised cells, the antigen presenting cells (APCs), among which there are dendritic cells (DCs), B-cells and macrophages.

Each T-cell specifically interacts with only one of the two types of MHC molecules depending on the presence of other co-receptors associated with the TCR. MHC-I molecules are recognised by T-cells expressing the CD8 co-receptor, while MHC-II by T-cells with the CD4 co-receptor. The expression of these co-receptor is the base for T-lymphocyte functions: $CD8^+$ T-cells, also called cytotoxic T-lymphocytes (CTLs), cause the direct killing of target cells by several mechanisms, such as the release of proteases, which upon their internalisation induce cell death, or the direct induction of apoptosis via the expression of the Fas ligand.
$CD4^+$ T-cells, also called T-helper cells (T_H), mediate and boost activation of either B-cells or CTLs, by the direct cell-to-cell contact, with the engagement of accessory interacting membrane protein, as well as by releasing soluble molecules that favour their survival and proliferation.

As already mentioned, T-cells derive from hematopoietic progenitors but, in contrast to B-cells, the final maturation process occurs in the thymus.
The initial requirement for T-cell maturation is that a functional TCR is present. Only those cells with both TCR α and β-chains correctly expressed and assembled survive. Otherwise, failure of a complete α/β TCR expression, usually because abortive rearrangement of Vα or Vβ genes, causes cell apoptosis (β-selection). T-cells must undergo other two rounds of selection, positive and negative, to obtain a repertoire of mature T-cells able to selectively recognise the antigen and mediate either killing ($CD8^+$ T-cells) or immune modulation ($CD4^+$ T-cells) efficiently. In the first round of selection (positive selection) precursors of T-lymphocytes are initially evaluated for the binding ability of TCR towards antigens. This requires the presence

of CD4 and CD8 co-receptors. Double positive ($CD4^+$ and $CD8^+$) T-cells proceed into lineage commitment process, where only one of the two co-receptors is selected by down-regulation of the other: if TCR interacts with MHC-II molecules, CD8 expression is down-regulated with generation of $CD4^+$ T-cells, while if there is interaction with MHC-I, CD4 down-regulates and $CD8^+$ T-cells arise.
Single positive T-cells (either $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ T-cells) undergo the second selection step (negative selection). Negative selection is required to prevent the random appearance of TCRs that can recognise with high affinity self-peptides bound to MHC. During negative selection, highly reactive T-cells are induced to up-regulate the expression of pro-apoptotic proteins, with the subsequent cell death (Paul 1999; Abbas, Lichtman *et al.* 2007).
Surviving cells (naive T-cells) are now ready to reach lymph nodes where they can interact with APCs, acquiring their effector characteristics.

Dendritic cells

T- and B-lymphocytes represent the two effector compartments of the adaptive immunity. For full activation of B-cells, T-cells are required, while for the activation of the latters, a third group of cells is indispensable, the APCs.

Dendritic cells (DCs) are the most important and powerful APCs of the immune system (Banchereau and Steinman 1998; Villadangos and Schnorrer 2007). DCs were described for the first time in the early 70's by Steinman and Cohn (Steinman and Cohn 1973), who observed a novel heterogeneous type of cells in a murine spleen primary cell line whose most conserved characteristic was the morphology (irregular shape with pseudopods).
From this initial report, many studies have demonstrated that DC family is composed by several different subsets, with specific molecular signature and biological functions, as it will be presented extensively later in a subsequent chapter. However, cells belonging to this heterogeneous family share common characteristics.
DCs reside in most peripheral tissues, particularly at sites of interface with the environment. They are able to link innate and adaptive immunity and to specifically trigger immunological pathways depending on the nature of the antigen.

DCs are critical for both recognition of the universe of antigens and shaping of the immune response, starting with their abilities in antigen uptake and processing.
The pioneer work of Steinmann and co-workers brought to the Langerhans cell paradigm, in which it has been proposed that DCs exist in two different functional states: immature and mature. In the immature state, peripheral DCs can take up antigens but their ability to prime T-cells is limited. Once they encounter a pathogen with simultaneous inflammation signals, they enter the maturation cycle in which they progressively lose their uptake capacity and migrate to the T-cell areas of lymph nodes, where they can stimulate T-cells thanks to the expression of high levels of MHC and costimulatory molecules.

Antigen uptake

As other APCs, the role of DCs is to uptake antigen and activate the immune system by the presentation of short peptides to T-cells. APCs are able to internalise antigens and cause their degradation into peptides that are displayed on the cell surface. These events are referred to as antigen processing and presentation and are required for the recognition of the antigens by the components of adaptive immunity, mainly T-cells.

According to their origin, antigens can be classified into two major groups, endogenous and exogenous.
Endogenous antigens are represented by molecules that are produced directly inside the cells, such as cellular and viral proteins.
On the contrary, exogenous antigens are those captured from outside by DCs, in order to enter them into the presentation pathways. Exogenous antigens can be captured by DCs by two different mechanisms, which depend on their characteristics: macropinocytosis and phagocytosis.

Macropinocytosis

Macropinocytosis (Sallusto, Cella *et al.* 1995) allows the sampling of large volumes of extracellular milieu. This mechanism involves the Rho-family of GTPases, with the triggering of an actin-dependent formation of membrane protrusions that collapse and fuse with the plasma membrane, forming large vesicles (0.5-3 μm) inside which

extracellular fluid is present. It is constitutively active in immature DCs and it is a non-saturable mechanism, even though the degree of antigen internalization and processing depends on the concentration of the molecule in the fluid. Macropinocytosis is of particular interest for the processing of soluble antigens released by pathogens or externally provided following intradermal, intraperitoneal or intravenous immunisations (Trombetta and Mellman 2005).

Phagocytosis

Phagocytosis is similar to macropinocytosis but more important for the uptake of particulate antigens. It represents the most common form of antigen uptake and normally starts by the specific engagement of a cell surface receptor. The consequence of this recognition is the activation of Rho-GTPases family with a remodelling of the cytoskeleton and the formation of protrusions that engulf the antigen (Conner and Schmid 2003). Depending on the receptor involved, differences in the strength of the protrusive activity and the subsequent signalling pathway are observed. Moreover, the presence of these receptors increases the ability of DCs in sensing antigens that are present at very low concentration.

In receptor-mediated endocytosis, there are several receptor families involved. In addition, there are different subsets of DCs that display distinct repertoires of these internalizing receptors, which allow these cells to trigger the most suitable immune response.

The C-type lectin receptor (CLR) family is one of the most interesting due to its ability to recognize a wide range of carbohydrate structures on antigens, both of self- and pathogen origins, in a Ca^{2+}-dependent manner. They are classified into two different subtypes depending on the orientation of their N-terminus (N-term): type I with extracellular N-term, and type II with cytoplasmatic N-term. Another classification of this family is based on the number of carbohydrate recognition domains present in their extracellular portion: normally only a single domain is present but other CLRs have more than one carbohydrate binding domain (for example ten in CD205).

One example of CLR of particular interest is CD205, also called DEC205 (Jiang, Swiggard *et al.* 1995). Characterised in the 90's, it is a type I transmembrane protein of 205 kDa with a cysteine-rich extracellular domain and a short cytoplasmic tail with many different conserved motifs. One of these is a tri-acidic cluster that targets internalised antigens to lysosomes and MHC-II endosomes. This protein is highly conserved between species (murine DEC205 shares more than 80% amino acid identity with its human counterpart). As other CLRs, it recognizes a broad range of carbohydrates, especially mannose- and fucose-containing structures.
A classical type II CLR is DC-SIGN. It has been shown to recognize a wide range of pathogens (bacteria and viruses (van Liempt, Bank *et al.* 2006)) but can also interact with ICAM-3 molecule on the T-cell surface (Geijtenbeek, Torensma *et al.* 2000). This receptor is used to internalise many pathogens and viruses: indeed, DC-SIGN functions as a receptor for several viruses, including Ebola virus, cytomegalovirus and Dengue virus. It happens, however, that some pathogens can interact with DC-SIGN and manipulate the immune response towards T_H2 or by inducing the production of anti-inflammatory cytokines, such the case of *Mycobacterium* (Geijtenbeek, Van Vliet *et al.* 2003), with the consequent immunological escape and the onset of chronic infections (Engering, Geijtenbeek *et al.* 2002; van Kooyk and Geijtenbeek 2003).

Other well characterised type II CLRs are dectin (Ariizumi, Shen *et al.* 2000; Ariizumi, Shen *et al.* 2000) and langerin (Valladeau, Ravel *et al.* 2000).

An additional class of receptors is the family of scavenger receptors (SRs). These trans-membrane receptors vary broadly in their structure but are all able to mediate endocytosis of polyanionic ligands, including modified low-density lipoproteins (LDLs) (Greaves, Gough *et al.* 1998). Their ligands include proteins, polysaccharides and lipids, both microbial and host-derived. Among the different subgroups of SRs, defined on the basis of their structures, three classes are expressed by DCs: CD36, the scavenger receptor A (SR-A) and Lox-1.
CD36 is an 88 kDa heavily N-glycosylated membrane protein expressed by the erythrocyte precursor, vascular endothelial cells, monocytes, DCs and many other cell types (Febbraio and Silverstein 2007). It is a double spanning (class B) SR receptor with short N-term and C-term cytoplasmic domains that are important for

the efficient membrane expression. CD36 is associated with lipid rafts and its localization is stabilized by palmytoilation of two cysteine residues in the cytoplasmic domain. CD36 plays a role in the uptake of apoptotic cells, oxidized LDLs (oxLDLs) and components of Gram-positive bacterium walls. Its role in immunity against Gram-positive bacteria requires the interaction with Toll-like receptors (TLRs) 2 and 6, with a subsequent internalization of the complex and its targeting to the Golgi apparatus for further processing. This broad ligand binding ability is reflected on the physiological roles of the receptor: it has been shown that CD36 has effects on cardiovascular diseases, on pathogen recognition and also in maintaining cellular homeostasis thanks to interactions with surface molecules of apoptotic cells. CD36 has been also shown to be an efficient receptor for cross-presentation of exogenous antigen in DCs (Tagliani, Guermonprez *et al.* 2008).

SR-A is a highly conserved type II trimeric transmembrane glycoprotein that contains five-six domains depending on the splicing variant. The α-helical coiled coil domain, that is present in the extracellular portion of the receptor, is important for the trimerisation and dissociation of the ligand from the receptor in the endosome, whereas the collagenous domain, located near the C-term, is supposed to be the ligand binding domain (Acton, Resnick *et al.* 1993). Apart from its ability to bind pathogens (Mukhopadhyay and Gordon 2004; Amiel, Nicholson-Dykstra *et al.* 2007), SR-A has been postulated to have a role also in modulating antitumor immunity through its interaction with heat-shock proteins (HSPs), thus inducing cross-priming events (Berwin, Hart *et al.* 2003; Wang, Facciponte *et al.* 2007).

The third SR receptor is Lox-1, a type II SR of about 50 kDa. It binds both Gram-positive and Gram-negative bacteria (Shimaoka, Kume *et al.* 2001) and acts in association with TLR-2 for the internalization of bacterial outer membrane components such as OmpA.

DCs can also uptake opsonised antigens via Fc receptors (FcRs) and complement receptors (CRs). The specificity of FcRs is based on the isotype matching between the receptor and the opsonising antibody, whereas CRs, mainly CR3 and CR4, mediate the internalisation of different ligands such as complement-opsonised apoptotic cells or pathogen molecules.

Antigen processing and presentation

Antigen processing is the progressive smashing of polypeptide chains into pieces that are loaded onto the MHC molecules.

There are two classical pathways to process and to present peptides depending on the source of the antigen. In the case of endogenous antigens, APCs and all other nucleated cells use MHC class I-restricted presentation; for exogenous antigens, the processing machinery of APCs leads to the MHC class II-restricted presentation. DCs, however, have also a third way for the processing of antigenic molecules, the so-called cross-presentation pathway, where exogenous antigens are presented bound to MHC-I (Heath, Belz *et al.* 2004) (fig. 3).

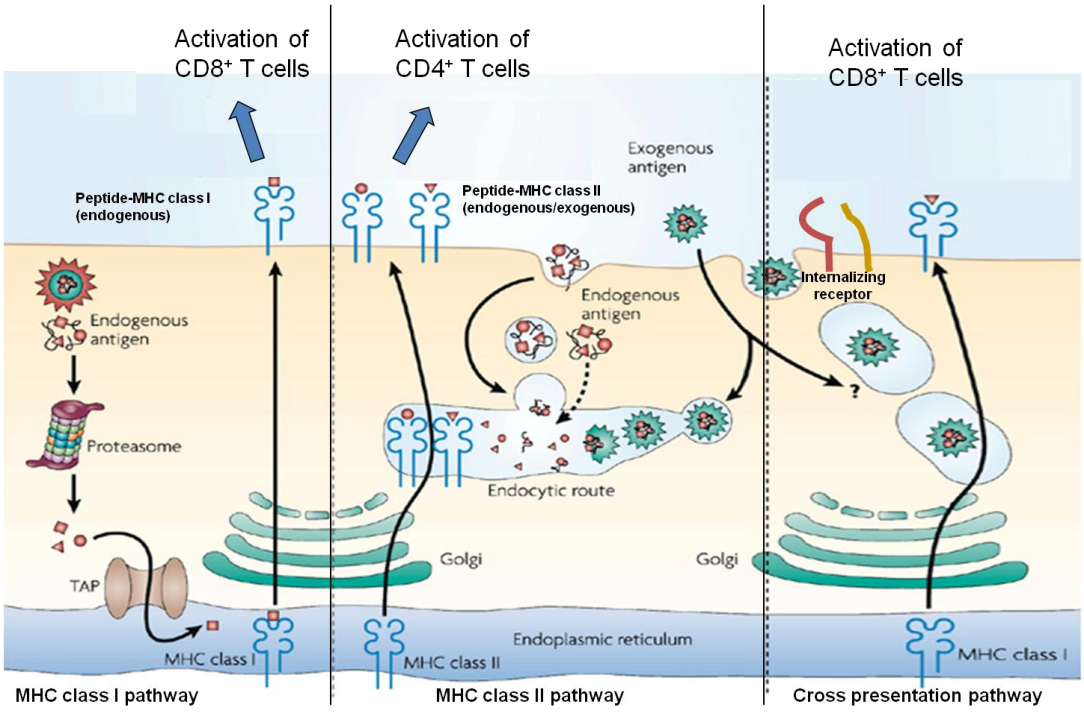

Fig. 3: The antigen presentation pathways in dendritic cells.

Endogenous antigens (proteins of either self or pathogenic origin) are mainly degraded by proteasome into peptides that are transported through TAP molecules into the endoplasmic reticulum for loading on MHC class I molecules. The so-formed complex is displayed and recognized by TCR of $CD8^+$ T-cells.

Exogenous antigens are endocytosed from the extracellular environment and degraded proteolytically in endosomal/lysosomal compartments. Peptides are then loaded on MHC class II molecules. The complex is transported to membrane surface, where it can be recognized by $CD4^+$ T-cells.

$CD8\alpha^+$ DCs have a unique ability to deliver exogenous antigens to the MHC class I (cross-presentation) pathway, although the mechanisms involved in this pathway are still poorly understood (figure modified from (Villadangos and Schnorrer 2007)).

MHC class I-restricted antigen presentation

Endogenous molecules, whose origin can be viral or self-cellular products such as misfolded proteins, are targeted to the proteasome. Proteasomes are multisubunit protease complexes that perform most of the non-lysosomal ATP-dependent proteolysis in the cytosol of eukaryotic cell. Thus, membrane and secretory molecules, which are located in the endoplasmic reticulum (ER), in order to be processed by the proteasome need to be transported back to the cytosol, a process called retrotranslocation.

Proteasome cleavage is the crucial step for the correct loading of peptides on MHC-I: it causes the definition of the C-term of the peptides that are translocated via TAP to the ER lumen, where other proteases (ER-associated aminopeptidase or ERAAP) define the N-term. Once peptides are generated, they cross the nascent MHC-I heavy chain and β2-microglobulin. This trimeric complex is ideal for the optimal folding and delivery to the cell surface (Vyas, Van der Veen *et al.* 2008).

MHC class II-restricted antigen presentation

Extracellular antigens are taken from the environment by the different mechanisms above described and confined in a delimited compartment, the phagosome. The phagosome undergoes different structural modifications (Stuart and Ezekowitz 2008), with the recruitment of other proteins, such as RAB-5 (Kinchen, Doukoumetzidis *et al.* 2008), and the subsequent fusion with the lysosomal compartment (Blander and Medzhitov 2006), a process called phagosome maturation. Lysosomal enzymes cause the degradation of the proteins in small peptides, which are thus loaded in a *de novo* synthesised MHC-II αβ dimer. This molecule assembles in the ER with the chaperon molecule invariant chain (Ii) that drives the localization of this precursor to the endosomal-lysosome compartment and prevents the loading of proteasome-derive peptides present in the ER. Once in this endosome-lysosome niche, acidic proteases cut Ii producing a small fragment known as class II-associated invariant chain peptide or CLIP, which remains associated to the MHC class II peptide binding groove, stabilizing the structure. CLIP is finally displaced from the peptide binding site and replaced by peptide antigen in a process

favoured by a chaperone protein, the HLA-DM (Denzin and Cresswell 1995; Sloan, Cameron *et al.* 1995).

In recent studies, it has been demonstrated that some cytosolic products can also be targeted to the MHC-II-restricted pathway, with most of them derived from the LAMP-2-mediated phenomenon of autophagy (Li, Gregg *et al.* 2005), but this mechanism is not well yet understood.

One crucial point in MHC-II antigen presentation is phagosome maturation: many pathogens have evolved mechanisms to interfere with this process, in order to avoid the immune response. Some examples are the blocking of phagosome maturation by *Mycobacterium tuberculosis* (van der Wel, Hava *et al.* 2007), the impairment of phagosome-lysosome fusion by *Legionella pneumophyla* (Kagan and Roy 2002) and other types of survival mechanisms (for example inhibiting the acidification of phagosome-lysosome) compartment as the ones of *Salmonella typhimurium* (Alaniz, Cummings *et al.* 2006) and *Toxoplasma gondii* (Mordue and Sibley 1997).

Further control mechanisms of MHC-II presentation pathway are based on the action of TLRs: it has been demonstrated that only the concomitant costimulation of TLRs leads to an effective delivery of peptide-MHC-II complexes to the surface of APCs (Blander and Medzhitov 2006), allowing a discrimination between self vs. non-self antigens.

Cross-presentation

There are several cases where DCs cannot produce the antigen by their own (for instance when a virus does not infect DCs directly), with the consequent absence of a CTL response. However, to eradicate cells infected by virus or parasites, or tumour cells, DCs possess the unique ability of taking up exogenous antigens and processing them via MHC class I presentation pathway. Therefore, exogenous antigens can also be presented to $CD8^+$ T-cells, inducing a cellular-mediated cytotoxic response. This phenomenon was firstly described by Bevan in the 1976 (Bevan 1976) and is generally referred to as “cross-presentation”.

The mechanism by which internalised exogenous antigens are transferred into MHC class I loading pathway remains unclear. Two alternatives have been proposed. In the

first one, the proteins are retrotranslocated to cytosol by a mechanism that probably involves Sec 61 complex, processed by the proteasome and targeted back to the ER via TAP and thus associated with MHC class I molecules. The second hypothesis claims that cross-presentation is mediated by a process where phagosomes fuse with ER-derived vesicles, in which all the components required for the correct loading of antigen on MHC-I are present (Guermonprez, Saveanu *et al.* 2003; Houde, Bertholet *et al.* 2003).

One of the major open questions is which mechanism targets internalised antigens either to the MHC-II presentation pathway or to the cross-presentation one. It has been proposed that the amount of antigen must overcome a threshold in order to enter the cross-presentation pathway (Kurts, Miller *et al.* 1998). However, there is an open debate on this assumption because an obvious objection is that the efficiency in the degradation of the antigen and not its abundance makes the difference. Other groups affirm that also the characteristics of the cells themselves, i.e. their type and physiological conditions, could have important effects on this behaviour (Heath and Carbone 2001). Some still controversial hypothesis imply exosome (Utsugi-Kobukai, Fujimaki *et al.* 2003), HSP-driven (Ichiyanagi, Imai *et al.* 2010) or receptor-mediated internalization: in fact, some DC-internalising receptors have been shown to be very efficient in delivering antigen to the cross-presentation pathway.
Apart from DCs, other APCs, for example macrophages, have the ability of cross-presenting antigens, but the efficiency of this process is lower than in immature DCs.

Despite the poor understanding of the molecular basis that regulate direct vs. cross-presentation pathways, it is clear that many molecules can enter this last pathway: there are reports of cross-presented soluble proteins, bacteria, virus and also tumour-associated antigens (TAAs).

Activation of DCs

Displaying of peptide-MHC molecules on the membrane of DCs is not sufficient to initiate a competent immune response. Indeed, DCs must migrate from periphery to draining lymph nodes, where they can interact and stimulate specific T-cells. This chemotactic event requires the full maturation of DCs, which change their

physiological characteristics by progressively losing the antigen uptake ability and increasing the expression levels of MHC and other costimulatory molecules, such as the B7 protein family (CD80 and CD86) and CD40, which are required for the activation of T-cells.

For this reason, the maturation process is crucial and tightly regulated. It is initiated with the recognition of inflammatory signals or pathogen-associated molecular patterns (PAMPs) by many different receptors called pattern-recognition receptors (PRRs).
The most characterised family of PRRs is represented by the TLR family (Takeda and Akira 2005). All TLRs are type I transmembrane glycoproteins, formed by an ER-luminal/extracellular domain containing different leucine-rich repeat motifs and a cytoplasmic Toll1/IL-1R homology domain. The signalling pathways triggered by TLRs are essentially the JNK and NF-κB pathways, which provoke the production of different pro-inflammatory cytokines, for example TNF-α and IFN.
In mammals, there are at least twelve different TLRs, with different cellular localizations, recognition ability and downstream signalling pathways. Some of them are expressed on the surface of DCs and are specific for bacterial products (TLR-1, 2, 4, 5 and 6): for example, TLR-4 and TLR-2 are probably the most important ones for the recognition of bacteria, being able to bind LPS and lipoproteins. Other TLRs are on endocytic vesicles or organelle membranes (TLR-3, 7, 8 and 9): for example, TLR-9 recognises unmethylated CpG dinucleotides, a common feature of bacterial genome.
However, both of these PAMPs can also interact with other surface receptors such as CLRs or SRs, increasing the complexity and the specificity of the response.

Other receptors are cytosolic and belong to the NOD-like receptors (NLRs) and RIG-1 like receptors (RLRs), which recognize bacterial and virus components, like double-strand RNA (dsRNA), inducing the maturation program via MAP kinases and NF-κB, as TLRs do (Takeda and Akira 2005).

The engagement of the FcR family also induces DC maturation. This process requires the FcR-associated γ-chain that contains an immunoreceptor tyrosine-containing activation motif (ITAM) recognised by Src-kinase family (Nimmerjahn and Ravetch 2007): in general, FcγRIII and FcγRIV are activatory receptors. In

contrast, FcRIIb, contains inhibitory motifs (ITIMs), and thus is an inhibitory receptor. These two families are present on the membrane of DCs and modulate their activation status by balancing the immune response through two alternative signalling pathways with different outcomes on the DC maturation program.
It has been shown that also Dectin-1, a CLR, uses a similar mechanism to induce the maturation process, due to the presence in its structure of an ITAM-like motif that is recognized by Src-kinases.

Other maturation signals operating in DCs comprise mediators of inflammation (for example, TNF-α, IL-1 or prostaglandin E), or the sensing of cellular components released by necrotic cells (Sauter, Albert *et al.* 2000), as well as the interaction with $CD4^+$ T-cells (both CD40-dependent and independent).

Activation of adaptive immunity

The maturation of B- and T-lymphocytes is the basic requirement for the starting of the adaptive immune response, with the participation of APCs.
$CD4^+$ T-cells (T helper, T_H cells) have a pivotal role in this process because they are able to mediate the activation of both humoral and cell-mediated responses (fig. 4).

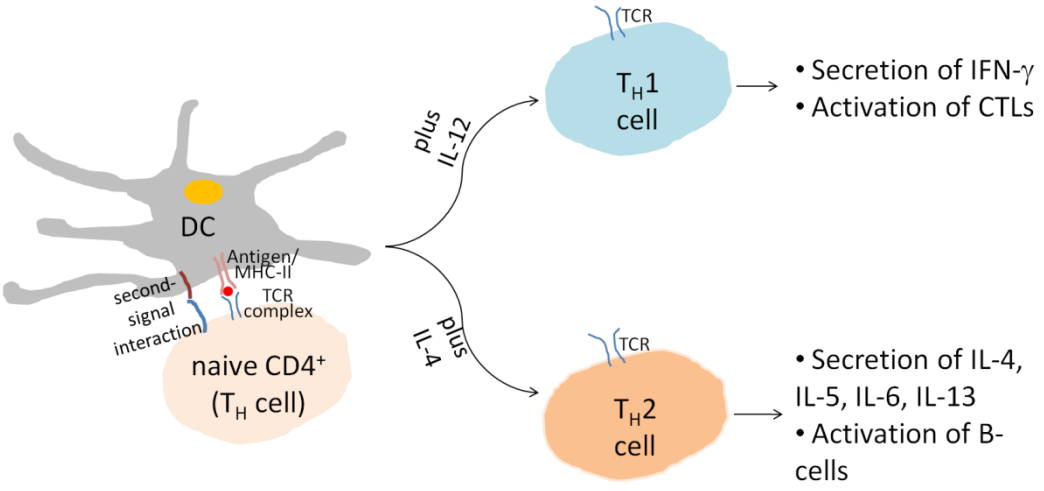

Fig. 4: Activation of T_H cells and their polarisation into T_H1 or T_H2 cells induce activation of CTL and B-cells, respectively.

The ability to stimulate either CTLs or B-cells derives from two different subset of T_H cells, respectively T_H1 and T_H2. They both derive from common progenitors, naive $CD4^+$ T-cells migrating from the thymus to the peripheral immune organs. Here, they are activated by the contemporary presence of two signals. One is the engagement of the TCR recognising the antigen-derived peptide bound to MHC-II molecule on the membrane of APCs. This start a signalling cascade originating from the CD3 complex, a group of cell surface molecules associated with the TCR.
A second 'co-stimulus' signal is conferred by cell surface molecules on the APC that interact with corresponding T-cell co-receptors, for instance the CD40 (on APC) with CD40L (on T-cells) or the CD80 and CD86 (on APCs) with CTLA-4 (on T-cells).

T_H cell polarisation is determined mainly by two cytokines (Glimcher and Murphy 2000; O'Garra and Arai 2000): IL-12 induces the differentiation into T_H1 cells while IL-4 causes the TH2 phenotype. Other cytokines act synergistically, such as IL-18 and IFN-γ (for T_H1) or IL-13 (for T_H2). As a consequence, mature T_H cells proliferate and differentiate into immunocompetent primed T-cells.
The hallmarks that distinguish these two subsets are the types of cytokines produced: T_H1 cells exclusively release IFN-γ while T_H2 cells exclusively produce IL-4, IL-5, IL-6 and IL-13.

$CD8^+$ T-cells (CTLs) are activated in a similar fashion: their TCRs interact with peptides bound to MHC-I molecules displayed on the surface of APC. Cytokines, especially IFN-γ, released by T_H1 cells boost and prolong the activation status of these cells.

APCs, in particular DCs, are considered to be the link between innate and adaptive immunity: they sense environmental signals and activate specific pathways in order to present antigens and to provide the most appropriate second signal to induce activation of both CTL and T_H lymphocytes (fig. 4). DCs indeed secrete several cytokines such as IL-12 and IFN, which drive the differentiation of naive T-cells into effector cells (Haring, Badovinac *et al.* 2006; Smith-Garvin, Koretzky *et al.* 2009). This cytokine milieu represents a third signal for T-cell activation, influencing their activation and functional status.

Moreover, mature DCs increase expression of surface adhesion molecules that are responsible for the stability and the duration of the immunological synapse, that is the contact zone between DCs and T-cells (Benvenuti, Lagaudriere-Gesbert *et al.* 2004). The duration of immunological synapse is responsible for an efficient T-cell priming and, although debated, the generation of immunological memory (Hugues, Fetler *et al.* 2004; Tadokoro, Shakhar *et al.* 2006; Scholer, Hugues *et al.* 2008).

The key role of DC functionality for a proficient but controlled immunity has been demonstrated in some diseases, such as the Wiskott-Aldrich syndrome: in this disease, where there is a defect in the Wiskott-Aldrich protein (WASP), an impairment of actin remodelling causes defects in both cytoskeleton remodelling and release of second signal, with an impairment in the activation of T-cells (Pulecio, Tagliani *et al.* 2008; Bouma, Mendoza-Naranjo *et al.* 2011).

DCs are also able to modulate T-cell activation and to cause the appearance of peripheral tolerance. Central tolerance is the process by which clonal deletion of self-reactive T-cells is induced in the thymus upon interaction with APCs that display self-antigens. However, even though efficient, this is also an incomplete process, due to the existence of self-antigens that cannot be presented in the thymus, for example antigens that are expressed after puberty.
Recent work has demonstrated that DCs are possible players in the induction of peripheral tolerance by causing anergy of T-cells that escape from central tolerance. It has been shown that the maturation status of DCs is the main mechanism by which peripheral tolerance is induced: indeed, immature DCs do not express the second signals needed for T-cell activation, with the consequent triggering of T-cell apoptosis (Hawiger, Inaba *et al.* 2001).
Another possibility is the existence of a subset of DCs that is responsible for inducing T-cell apoptosis directly by triggering the Fas/Fas-ligand pathway (Suss and Shortman 1996).

All these findings indicate that DCs have a pivotal role in shaping immunity by several different mechanisms, both immunostimulatory and tolerogenic (fig. 5).

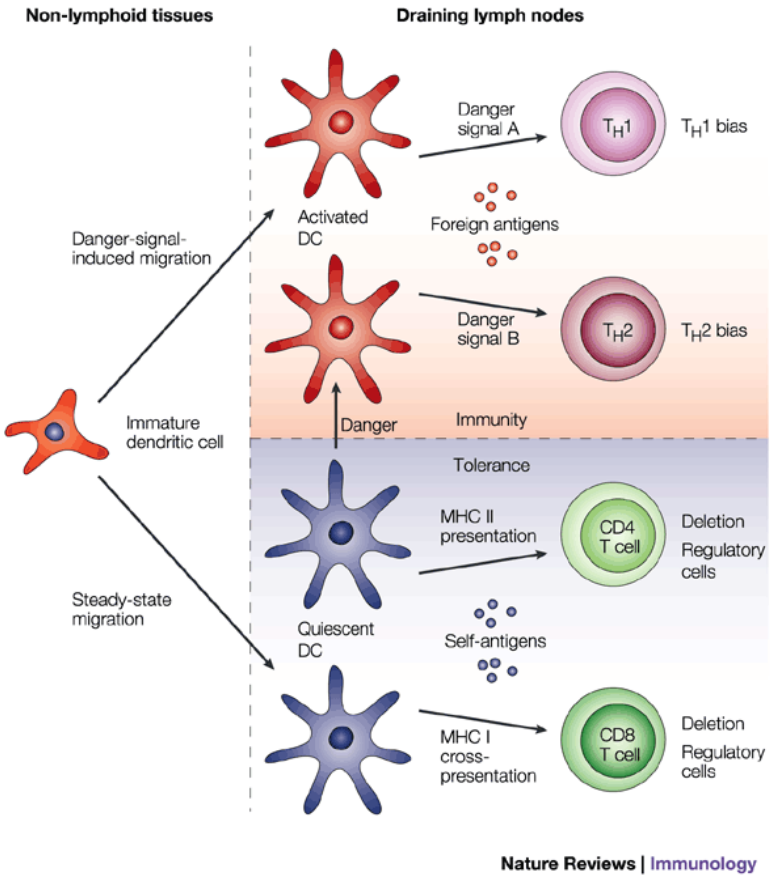

Fig. 5: Dendritic cells modulate T-cell activation.

DCs migrate from non-lymphoid tissues to draining lymph nodes. If DCs are fully maturate, they are able to prime T-cells and lead to the activation of immunocompetent T-cells.

Immature DCs induce, on the contrary, peripheral tolerance, with apoptosis of T-reactive cells or the appearance of regulatory T-cells (figure taken from (Shortman and Liu 2002)).

DC subsets and functions

DCs have a great impact on immunity, by modulating the immune response both qualitatively and quantitatively. This is possible also because DCs are a heterogeneous family of cells.

Phenotypic and functional analyses of DCs have revealed a great complexity and heterogeneity. They all are capable of antigen uptake, processing and presentation but the different DCs subtypes differ in their origin, localisation and function.

Mouse and human DCs can be primarily divided into two major subgroups, with distinctive origin, phenotype and function: “conventional” DCs (cDCs), with dendritic shape and DC function, and “plasmacytoid” DCs (pDCs).

Among cDCs, two major categories can be distinguished: migratory DCs and lymphoid tissue-resident DCs.

Migratory DCs comprise interstitial or dermal DCs and epidermal DCs (called also Langerhans cells). Langerhans cells are easily discriminated from the other migratory DCs for the selective expression of high levels of the C-type lectin langerin, CD45 and intracellular organelles known as Birbeck granules (Merad, Ginhoux *et al.* 2008), as well as the expression of other surface markers, such as CD11b. They migrate from peripheral tissues and traffic to the lymph nodes in response to danger signals.

The second major subgroup of cDCs is represented by lymphoid tissue-resident DCs. They represent half of all the lymph node DCs and all the spleen and thymus DCs. They are further subdivided into three different subsets that have a differential distribution in the spleen and lymph nodes. The main difference among the lymphoid tissue-resident DCs is in the expression of CD4 and CD8α markers: lymphoid tissue-resident DCs can be $CD4^+CD8\alpha^-$, $CD4^-CD8\alpha^+$ or $CD4^-CD8\alpha^-$ (Pooley, Heath *et al.* 2001).

$CD4^+CD8\alpha^-$DCs are preferentially located in the marginal zone and are more effective in the presentation of exogenous antigens on MHC-II molecules.

On the other hand, $CD4^-CD8\alpha^+$ DCs, which are preferentially found in the T-cell zone, seem to be extremely efficient in cross-presenting cell-associated and soluble antigens to T-cells. A third subgroup, which does not express either CD4 or CD8α markers, has been also described, even though its role is still not well defined.

pDCs constitute a small subset of circulating blood DCs. They lack most cDC lineage markers and selectively express the immunoglobulin-like transcripts 7 (ILT-7) (Cao, Rosen *et al.* 2006), the IL-3 receptor and the C-type lectin receptor BDCA2 (Dzionek, Sohma *et al.* 2001; Ju, Hacker *et al.* 2004) and have the unique ability to sense and respond to viral infections mainly by secreting large amounts of type I IFN. pDCs constitutively express the IFN regulatory factor 7 (IRF-7) for a rapid secretion of large amounts of IFN-α upon the engagement of TLRs 7 and 9 (Izaguirre, Barnes *et al.* 2003). Recent works demonstrate that pDCs cooperate with other DCs in inducing not only anti-viral immunity but also anti-tumour immunity (Lou, Liu *et al.* 2007).

Cancer immunotherapy

The immune system can recognize precursors of cancer cells and in many cases block the progression towards malignancy. Indeed, several evidences have shown that defects in different components of the immune system have the consequence of higher predisposition to spontaneous or induced carcinogenesis (Shankaran, Ikeda *et al.* 2001; Smyth, Swann *et al.* 2005; Zerafa, Westwood *et al.* 2005). On the contrary, immunostimulatory regimens have a great impact on tumour protection (Smyth, Wallace *et al.* 2005; Taieb, Chaput *et al.* 2006).

Despite the "immune surveillance" to control proliferation of cancer cells, tumours display several different strategies to counteract the immune system action, a phenomenon called "immunological escape", A frequent mechanism is represented by the downregulation or loss of expression of MHC-I molecules and/or the TAP transporter (Campo, Graham *et al.*; So, Takenoyama *et al.* 2005). In both cases, presentation of peptides to CTLs is compromised and, thus, cancer cells become insensitive to the killing by these cells. In addition, cancer cells can secrete inhibitory soluble factors such as TGF-β (Flavell, Sanjabi *et al.* 2010) and IL-10 (Kawamura, Bahar *et al.* 2002), to down regulate the activation of immune response.

The first report of an efficient cancer immunotherapy strategy occurred in the late years of the nineteenth century: a surgeon, William Coley, injected streptococcus into a cancer patient in order to cause erysipelas and stimulate the immune system (reviewed in (McCarthy 2006)). This experiment was a proof-of-concept for immunotherapy, even though the mechanism of tumour eradication was far to be clear and elucidated.
Current immunotherapy approaches are now more focused and efficient, also because the knowledge on the molecular mechanism of carcinogenesis has increased and many different aspects of cancer progression have been elucidated.
In addition, immunological studies have highlighted the presence of molecules that are strongly associated with tumours.

The majority of this tumour associated markers were discovered by serological screening of cDNA expression libraries from tumours, using serum of cancer patients. However, this is a limited approach because it can only find antigens

recognized by antibodies. A further improvement was the screening of libraries using tumour-reactive T-cells, with the necessity of established T-cell lines.

Currently, more than 3,000 of these markers that could be used to induce specific anti-tumour immunity have been proposed and assayed.

These tumours markers can be subdivided into main subgroups depending on their origins, functions and expression, even though the distinction between them is sometimes not so-well defined (Van den Eynde and van der Bruggen 1997):

- antigens encoded by genes that are normally silent in most normal tissues but are activated in most tumours. Among these, the prototypal example are antigens encoded by PIA in the mouse and by the MAGE genes in humans;
- tumour-specific antigens (TSA), which are present in tumour cells but not in normal tissues and can be shared among cancer patients. They are present for example in tumours caused by oncogenic virus, such as the cervical cancer caused by HPV (Scheffner, Werness *et al.* 1990);
- differentiation antigens, that correspond to normal proteins with highly restricted tissue distribution. Examples of this category are melanoma-associated antigens such as TRP-2, tyrosinase, Melan-A^{MART1} (Beatty and Vonderheide 2008; Forgber, Trefzer *et al.* 2009), as well as carcinoembrionic antigen (CEA) (Gold and Freedman 1965), prostate-specific antigen (PSA) or lymphoma-associated idiotype;
- tumour-associated antigens (TAA), that is proteins that are normally present in normal tissues but overexpressed by tumour cells. In this group, one of the best known is HER2/neu;
- antigens derived from mutations of proteins in tumour cells. The most known is represented by the gene Bcr-Abl, produced by the translocation between chromosome 9 and 22 with the production of a fusion protein that causes acute lymphoblastic leukemia (ALL) (Nowell and Hungerford 1960);
- ubiquitous antigens, that are expressed by normal and malignant cells.

Nowadays, two fundamentally different strategies aiming to stimulate anti-cancer immunity and to overcome immunological escape have been developed, namely passive or active immunotherapy.

Passive immunotherapy

The term "passive immunotherapy" refers to an approach consisting of the passive administration of different components of the immune system. Often, it has only a transitory effect, because immunological memory is not induced. There are different mediators used in clinical treatments, the most important of which are monoclonal antibodies (mAbs).

Passive immunotherapy with mAbs has been employed with success in many types of cancer, among which there are B-cell malignancies. In those diseases, cells express on their surface a clonal repertoire of idiotopes (epitopes in the idiotype), which can be considered TSA and, as such, targets for antibodies. Making mAbs specific for the malignant idiotype, expressed by the clonal repertoire of each patient, can be time-consuming and expensive, despite the increasing research on personalized medicine. Therefore, current strategies focus on surface molecules expressed by most B lymphocytes, as the CD20 molecule, which is expressed by mature B-cells and the vast majority of patients with B-cell lymphoma (Anderson, Bates *et al.* 1984). Anti-CD20 mAb Rituxan was the first mAb approved by the United States Food and Drug Administration (FDA) for the treatment of cancer (James and Dubs 1997).

Different mAbs targeting specific TAAs or factors required for carcinogenesis are currently being used, for example mAbs targeting HER2 (e.g. Trastuzumab and Pertuzumab) or the vascular endothelium growth factor (VEGF) receptor (e.g. Bevacizumab) among others (Pegram, Lipton *et al.* 1998; Hurwitz, Fehrenbacher *et al.* 2004).

Other possible target of mAbs are stroma components required for tumour growth (Hofmeister, Schrama *et al.* 2008). For example, interfering with the activity of VEGFR impairs neo-angiogenesis, blocking the supply of metabolic factors, such as nutrients and oxigens, and thus causing cell death (Shojaei, Wu *et al.* 2007).

mAbs are currently widely used in the clinic for the treatments of some malignancies. Their efficacy is sometimes limited due to the heterogenous expression of target antigens by tumour cells. Moreover, therapeutic antibodies frequently exhibit poor tumor penetration and are often restricted to perivascular spaces, limiting their efficacy in solid tumour therapy (Baker, Lindquist et al. 2008; Rudnick, Lou et al. 2011).

mAbs have different biological mechanisms of action, among which there are antibody-dependent cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). ADCC is the process by which antibodies mediate the recognition of their target by a cell component of innate immunity, the natural killer cells (NK cells), through the FcRγ/CD16 interaction. These cells secrete cytotoxic factors, such as perforins and granzymes, to eliminate the target cells. In CDC, killing of cancer cells is mediated directly by the activation of the complement system.

mAbs also modify or interfere with signalling pathways, leading to direct apoptosis of malignant cells. It has been demonstrated, for example, that a mAb targeting theganglioside GD2, which is associated with neuroblastomas and small cell lung cancer (SMLC), induces dephosphorylation of focal adhesion kinase-1 (FAK) and activation of p38, which results in cell death (Aixinjueluo, Furukawa et al. 2005). More recently, a direct induction of apoptosis was demonstrated with a mAb targeting the tyrosine kinase receptor ROR1, recently identified to be overexpressed in chronic lymphocytic leukemia (CLL) (Daneshmanesh, Hojjat-Farsangi et al. 2012). Another possibility is the direct engagement of receptors that trigger apoptosis, e.g. Fas or receptors for TRAIL: targeting of TRAIL death receptors on cancer cells with agonistic mAbs has shown its potential as therapeutic strategy (Buchsbaum, Zhou et al. 2003; de Vries, Gietema et al. 2006). There is also a small group of mAbs that are able to induce a complement-independent cytotoxicity not related to apoptosis of cells expressing N-glycolyl-GM3 ganglioside (Fernandez-Marrero, Roque-Navarro et al. 2011).

Another class of molecules that are used in passive immunotherapy are cytokines. Cytokines modulate the activation and function of immune cells. Among the plethora of cytokines, there are members of the IFN family and IL-2, which have been

approved by regulatory agencies as first line or adjuvant therapies for different types of cancer.

IFNs are a family of glycoproteins whose primary role is to block viral replication. At the same time, these molecules have been widely used in anti-cancer therapies due to their ability to favour activation of NK cells and T-cell cytotoxicity, promotion of T_H1 response, and up-regulation of MHC class I molecules (Kirkwood, Strawderman *et al.* 1996; Vlock, Andersen *et al.* 1996; Pfeffer, Dinarello *et al.* 1998; Shankaran, Ikeda *et al.* 2001; Ferrantini, Capone *et al.* 2007).

IL-2 stimulates the activation, expansion and survival of T-cells (Waldmann 2006). It is widely used in the treatment of melanoma patients (Atkins, Lotze *et al.* 1999).

The administration of these and other cytokines (e.g. TNF-α and GM-CSF), presents several side effects potentially dangerous for the health of the patients. This is due to unspecific stimulation of the immune system, with a consequent status of general inflammation that can be life-threatening.

Table A: FDA-approved molecules for passive immunotherapy of human malignancies

Type of therapy			**Disease**
Cytokines (Tagawa 2000; Dranoff 2004)	**Name**		
	IL-2		Melanoma, renal cell carcinoma
	IFN-α		Melanoma, chronic myeloid leukemia
	TNF-α		Melanoma, sarcoma
	GM-CSF		Prostate cancer, melanoma
Monoclonal antibody (Oldham and Dillman 2008; Eisenbeis and Grau 2012)	**Name**	**Target**	
	Rituximab Ofatumumab	CD20	Non-Hodking lymphoma, chronic lymphocytic leukemia
	Trastuzumab Pertuzumab	ErbB2	Breast cancer
	Ipilimumab	CTLA-4	Melanoma, prostate, lung cancer
	Bevacizumab	VEGF	Colorectal cancer
	Cetuximab Panitumumab	EGFR	Colorectal cancer
	Girentuximab	Carbonic anhydrase IX	Renal cell carcinoma

Active immunotherapy

Active immunotherapy refers to all those treatments where the immune system is stimulated to respond against antigens contained in immunizing formulations. It is the base of all vaccine approaches.
Initially designed for the induction of an immune response against pathogens, vaccines are currently used also in the cancer immunotherapy field.
In analogy to what happens in the vaccination against pathogenic organisms, there are two types of cancer vaccines: therapeutic, which acts after the onset of the neoplasm, and prophylactic, which on the contrary prevents the onset of the tumours.

Immunogens used in cancer vaccines are represented by the different TSAs and TAAs, which are given in order to stimulate an immune response focused on the cells expressing these molecules.

Antigen administration for efficient therapy: genetic immunisations

Despite the nature of the antigen, an important issue in the vaccination field is the mode of administration of antigens in vivo. The state-of-the-art approach is protein immunisation: protein formulations are prepared *ex vivo*, a process that requires optimisation for the production of high amounts of pure and stable immunogens, with the general requirement of maintaining the cold-chain in all manufacturing steps. Proteins are then administered with different modalities (injections or oral administration among the others), with the activation of immune system. Unfortunately, TAAs are normally week immunogens, often requiring adjuvants in order to provide the inflammatory signals for an efficient and complete activation of all components of immunity.
An alternative approach is represented by genetic vaccination, that is the transfer of nucleic acids (DNA plasmids, RNA, replicons or virus) to the organism. First demonstrations of the feasibility of *in vivo* gene transfer to induce the production of foreign products and the induction of an immune response go back to the early ‘90s. Wolff *et al.* demonstrated that injection of DNA and RNA constructs in the mouse muscle causes a persistent expression of the gene product (Wolff, Malone *et al.*

1990) and the appearance of an immune response against it. Few years later, Tang *et al.* demonstrated that the transfer of plasmid DNA to the mouse skin induced the appearance of a specific immune response (Tang, DeVit *et al.* 1992).

Among the different types of nucleic acids that can be administered, DNA plasmid vaccines offer a precise but flexible strategy for delivering antigens to the immune system. Indeed, DNA vaccines, irrespective of the way of administration, are able to stimulate the immune response mimicking naturally occurring infections. DNA molecules are uptaken by tissue-resident APCs and the antigen is produced directly by APCs and processed via MHC-I pathway, with the activation of $CD8^+$ T-cell response. If DNA molecules reach non-APCs cells, antigens is released in the environment where it can be either captured by APCs and processed via MHC-I pathways upon antigen cross-presentation, with CTL response, as well as via MHC-II pathway, or in alternative sensed by the BCR of B-cells, with a consequent humoral response.

Despite the possibility that DNA molecules reach APCs and other cells randomly, inducing both humoral and cell-mediated response, it has been demonstrated that the main source of antigen in intradermal DNA immunisation is due to expression by non-immune cells, especially keratinocytes, and cross-priming is the predominant mechanism for inducing CTLs (Corr, von Damm *et al.* 1999; Cho, Youn *et al.* 2001) (fig. 6).

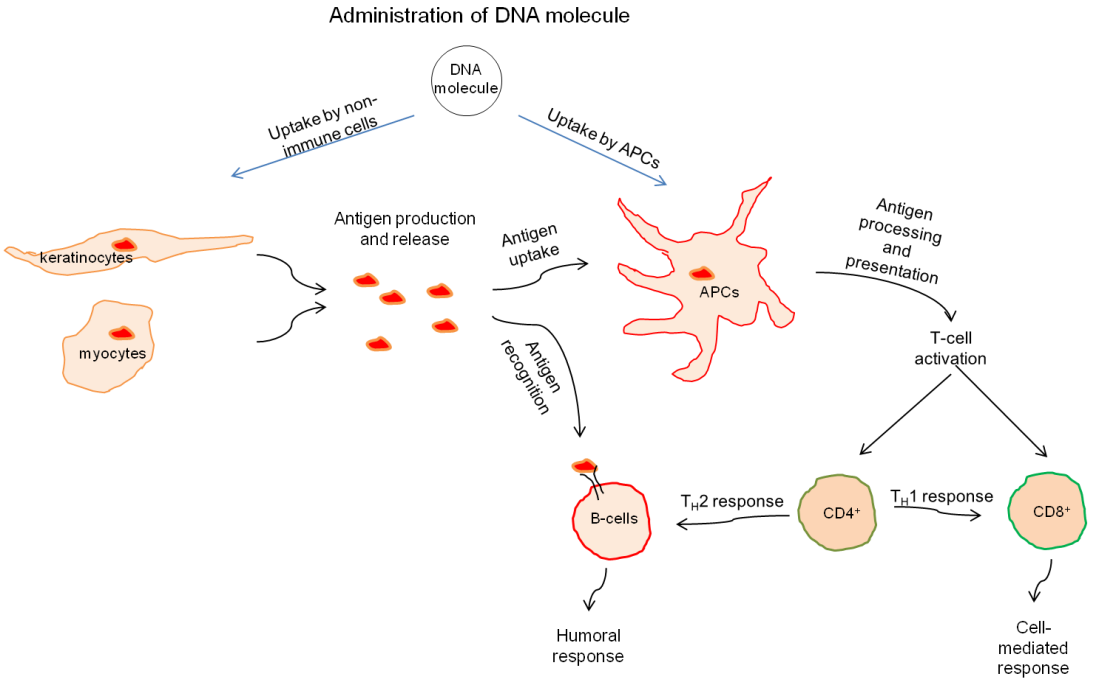

Fig. 6: Possible mechanisms of activation of the immune response by DNA vaccines
Administered DNA molecules can reach APCs as well as other cell types (mainly keratinocyte or myocytes). In the first case, antigen of interest is produced and processed directly by APCs. On the contrary, in the second case antigen is produced by non-APCs and released in the environment, where it can be recognized by the BCR, activating B-cells, or uptaken, processed and presented by APCs, both via MHC-II and via cross-presentation pathways. The T-cell compartment is thus activated, with the induction of the effector phases of both $CD4^+$ and $CD8^+$ T-cells.

The plasmid DNA vaccination approach consists of the administration of a bacterial plasmid containing an eukaryotic promoter, the sequence (for example a gene or cDNA) of interest and a polyadenylation/transcriptional termination sequence.

There are many advantages of using plasmids, the most important of which is their extreme versatility. With the development of molecular biology techniques, it is possible to manipulate the gene of interest, to limit the expression of the protein to certain tissues or cell types by using specific promoters and to add costimulatory molecules.

With respect to the engineering of the gene of interest, several optimisation strategies can be adopted. It has been shown, for example, that there is a codon bias in several species, thus the use of particular codons in the genes has an impact on the expression levels of the gene products. Moreover, it is possible to modify the gene of

interest by adding particular localisation signals to enhance the immune response (Boyle, Koniaras *et al.* 1997): indeed, antigen secretion favour its availability to APCs and B-cells, with the consequent boosting of the immunity.

As mentioned before, a common characteristic of plasmid backbone is the presence of a promoter and a polyadenylation/transcriptional termination sequence. By manipulating these elements, it is possible to enhance the availability of antigens to particular subsets of cells (Norman, Hobart *et al.* 1997): ubiquitously working promoters, such as the CMV immediate early enhancer-promoter, can be substituted with others with tissue-specific or time-specific activity, for example promoters of genes that are expressed only by particular cells, such as keratin, modulating the expression of the antigen. Also, the choice of terminator sequences can influence the expression levels of the antigen.

Plasmid backbones have also other advantages that enhance their ability to fully activate immune compartments. One of these is the presence of immunostimulatory molecules, such as hypomethylated CpG motifs of bacterial plasmids. The mechanism by which hypomethylated CpG motifs act as "built-in adjuvant" was recently discussed: these short sequences are recognized by TLR-9 as a danger signal, with the consequent activation of DCs (Hemmi, Takeuchi et al. 2000). However, a published work demonstrated that the magnitude of the response induced upon DNA vaccination is similar in the absence of engagement of TLR-9 by CpG (Spies, Hochrein *et al.* 2003). A new hypothetical mechanism to explain the high immunogenicity of DNA constructs is the presence of double strand DNA with the engagement of non-classical IκB kinase (IKK) cascade (Ishii, Coban *et al.* 2006). Even though the mechanism of action of CpG motifs has not been clearly elucidated, the presence of these sequences induces immunity also in the case of poorly immunogenic molecules such as self-antigens, whose administration normally evokes tolerance (Gurunathan, Klinman *et al.* 2000; Miller, Ozenci *et al.* 2005; Facciabene, Aurisicchio *et al.* 2006; Klinman 2006).

DNA constructs have no concern for biological safety: they do not contain any functional eukaryotic replication origin, which prevents their multiplication upon administration. To limit safety issues due to the antibiotic-resistance cassette, which

is needed for the selection during bacterial culture, kanamicyn resistance is preferred instead of ampicillin.

Another possibility to enhance the immune response, in addition to the intrinsic properties of bacterial DNA, is the inclusion in the vectors of genes encoding proteins able to activate or target APCs. As described earlier, APC status has a great impact on the quality of immune response. Cytokine sequences can be added to the DNA plasmids, in order to favour the appearance of particular subsets of T-cells or immunoglobulins. For example, fusion of the antigen with CTLA-4 induces higher amounts of specific immunoglobulins with a bias towards IgG1 isotype with respect to IgG2a (Boyle, Brady *et al.* 1998). Other examples are cytokines or growth factors that mediate DC precursor recruitment or their maturation in the site of antigen expression, such as Mip1α, granulocyte and macrophage-colony stimulating factor (GM-CSF) and Flt3L (Biragyn, Surenhu *et al.* 2001; Kutzler and Weiner 2004; Stevenson, Rice *et al.* 2004; Applequist, Rollman *et al.* 2005; Liu, Wahren *et al.* 2006; Yo, Hsu *et al.* 2007).

There are several possibilities for the administration of optimised plasmid vectors, among which the simplest is the direct injection of naked DNA into the host. This approach requires high amounts of DNA plasmids (typically between 10 and 100 μg), which last for a short period of time in the host (Kawabata, Takakura *et al.* 1995).

Different methods have been developed to increase the stability and the persistence of the vector in the host, as well as its ability in reaching cells.
Electroporation consists of producing a transient electric field to facilitate the entry of plasmids inside the cells by increasing the cell membrane permeability. It is normally used in molecular biology laboratories to introduce genetic material into mammalian cells, bacteria and yeasts. It has been demonstrated that it is a suitable method to enhance the uptake of DNA plasmids *in vivo* (Rosati, Valentin *et al.* 2008; Chen, Wen *et al.* 2011; Provinciali, Barucca *et al.* 2011).
An alternative method is the biolistic delivery, also called gene gun (Sasaki, Takeshita *et al.* 2003). Originally developed for the transfer of genetic material in plants, it consists of the bombardment of cells with DNA-coated gold particles. It is able to promote the transfer of DNA plasmids towards both Langerhans and dermal

resident DCs, as well as keratinocytes (Porgador, Irvine *et al.* 1998; Gaffal, Schweichel *et al.* 2007).

The efficacy of DNA vaccines can be increased by means of prime-boost vaccination protocols, where priming is performed with plasmid DNA and boosting with DNA delivered via a virus. It has been described that this regimen increases the efficacy of vaccines both in animals and humans (Woodland 2004) and it is associated with a higher immune-response (Cesco-Gaspere, Benvenuti *et al.* 2005).

There are several clinical trials in which DNA vaccination is tested for its efficacy and safety. Among these, there are different DNA vaccines against melanoma-associated antigens. In 2007, Wolchok and collaborators demonstrated that genetic immunisation with a tyrosinase-encoding sequence induced the appearance of a specific T-cell response in half of the treated patients with a concomitant higher survival rate when compared to non-responders (Wolchok, Yuan *et al.* 2007). Other ongoing clinical trials target melanA (Weber, Boswell *et al.* 2008) or gp100 (Rosenberg, Yang *et al.* 2003), with contradictory results.

DNA vaccines are being also used in the treatment of other cancer types. Among these, lymphoma is of particular interest because the clonality of the immunoglobulin expressed by tumour cells allows its isolation and use in a genetic immunisation treatment as demonstrated by different groups (Hawkins, Zhu *et al.* 1994; Timmerman, Singh *et al.* 2002).

Other examples are DNA immunisations against PSA for the treatment of prostate cancer (Low, Mander *et al.* 2009), HPV-associated protein (Kim, Gambhira *et al.* 2008) and for the treatment of liver or breast cancer (Curigliano, Spitaleri *et al.* 2006; Ladjemi, Jacot *et al.* 2010) (ClinicalTrials.gov website, identifiers NCT00093548 and NCT00363012, respectively).

Cell immunotherapy

As mentioned above, the immune system is able to identify and impair the expansion of tumour cells, especially in the early phase of tumour progression. Indeed, immune cells are recruited to tumour tissue, as demonstrated by the presence of tumour-infiltrating lymphocytes (TILs). Moreover, the presence of TILs is normally

associated with a more favorable prognosis in different types of malignancies (Zhang, Conejo-Garcia *et al.* 2003; Sharma, Shen *et al.* 2007). These cells can be isolated, expanded *in vitro* and re-infused into the donors in order to enhance their frequency in the organism (Rosenberg, Yannelli *et al.* 1994). However, this approach requires of pre-existing patient tumor-reactive cells expandable *ex vivo*: in some cases, these cells are difficult to isolate, especially in tumours with not well-characterised TAAs. More often, it happens that T-cells are anergic due to tolerance mechanisms. To overcome these problems, a recent work from the Rosenberg group demonstrated the feasibility of creating tumour-specific functional T-cells from autologous lymphocytes collected from the peripheral blood of melanoma patients (Morgan, Dudley *et al.* 2006). This and other studies (Clay, Custer *et al.* 1999; Zhao, Wang *et al.* 2009) demonstrated that T-cells can be genetically engineered, by the addition of a chimeric antigen receptor (CAR) that is normally composed by the antigen binding site of a mAb with the signal activating machinery of a T cell. The presence of this CAR frees the requirement of recognition of the antigen bound to MHC for the activation of T-cells. It thus enhances the possibility of using T-cell immunotherapy even in cancer cells where MHC-I expression was down-regulated for immunological escape.

Another possibility of cell therapy is based on the use of DCs, taking into account their central role in the orchestration of the immune response (Tacken, de Vries *et al.* 2007).

DCs can be isolated from patients from blood stream, *ex vivo* loaded with the antigen of interest and reinfused back into the host. A first issue is the isolation of enough amounts of viable cells from the blood. The alternative is the isolation of hematopoietic precursors of DCs: several different combinations of stimuli have been used in order to orient maturation of the precursors into particular subsets of DCs, such as culturing of these precursors with GM-CSF and TNF-α, or in alternative GM-CSF, IL-4 and IL-13 (Tuyaerts, Aerts *et al.* 2007). Furthermore, the maturation process can be optimised to avoid tolerance instead of immunity, worsening the disease. Thus, TLR agonists and inflammatory cytokines are added to the medium in order to ensure the entrance of DCs in the maturation process (Shimizu, Thomas *et al.* 2001).

The next step in the DC-based immunotherapy is the antigen-loading. Depending both on the type of antigens and on the modality of loading, the MHC-I or MHC-II presentation pathways are preferred. The simplest way to select preferentially one of the two presentation pathways is not to load the whole TAA but as a mixture of short peptides that are predicted to be presented bound to MHC-I or MHC-II molecules, activating respectively mainly CTL or $CD4^+$ T-cells. In this approach, the MHC haplotype of the patients must be considered: indeed, different peptides have different affinity for the various MHC alleles. Moreover, this characteristic is not sufficient to predict the efficacy of the treatment, because there are several haplotypes whose peptide binding preferences is not yet characterised and clearly elucidated.

Optimisation approaches are required also to determine the amount of antigen required for an efficient presentation to T-cells and also to enhance the persistence of the peptide-MHC complex on the surface of the DCs.

Ex vivo loading of the antigen can also be performed with genetic approaches: DCs can be transduced with viral vectors or transfected with nucleic acids coding the TAA with high efficiency. By this approach, it is possible to favour intracellular trafficking of the antigen by fusing specific localisation signals, and thus influencing the display of the antigen bound to MHC-I or MHC-II molecules, with activation of $CD8^+$ or $CD4^+$ T-cells (Wu, Guarnieri *et al.* 1995; Leifert, Rodriguez-Carreno *et al.* 2004). The major concern of these strategies is the safety issue, because transfected nucleic acids can integrate into the host genome and large amounts of viral particles must be produced (Tuyaerts, Aerts *et al.* 2007).

To avoid the *ex vivo* manipulation of DCs, it is possible to take advantage of the different types of internalising receptors on DCs with an *in vivo* targeting. The endocytic receptors enhance the ability of DCs to capture low amounts of antigens and, furthermore, their capacity in presenting such molecules to evoke the immune response. Targeting these receptors can improve the uptake and processing of the TAA of interest. Since targeting of specific receptors can lead to different types of immunity, it is possible to choose and modulate the most suitable processing pathway for the antigen simply by changing the targeting strategy.

Initial studies identified the CLR DEC-205 as the most promising candidate receptor for an efficient in vivo targeting strategy. It is expressed by $CD8\alpha^+$ $CD4^-$ DCs of the

T-cell area of lymphoid tissues, the proper localization to prime naive T-cells (den Haan, Lehar *et al.* 2000). The Steinman group and others demonstrated that the administration of antigens by targeting them to DEC-205 improved the vaccination outcome by enhancing both humoral and cellular responses (Bonifaz, Bonnyay *et al.* 2004; Johnson, Mahnke *et al.* 2008; Flacher, Sparber *et al.* 2009). However, it was shown that it is necessary to add some costimulatory signals to the vaccination protocol to avoid the appearance of tolerance, such as CD40L or antibody against CD40 (Hawiger, Inaba *et al.* 2001; Bonifaz, Bonnyay *et al.* 2002).

Apart from DEC-205, induction of antigen-specific T-cell responses has been achieved by targeting antigens to DC-SIGN (Kretz-Rommel, Qin *et al.* 2007), dectin-1 (Carter, Thompson *et al.* 2006; Carter, Thompson *et al.* 2006) langerin (Idoyaga, Cheong *et al.* 2008) and other such as Clec9A (Sancho, Mourao-Sa *et al.* 2008) or CD11c (Castro, Tutt et al. 2008).

Recently, Tagliani *et al.* (Tagliani, Guermonprez *et al.* 2008) demonstrated that CD36 is a good candidate for inducing both $CD4^+$ and $CD8^+$ T-cells responses against soluble antigens, resulting in an anti-tumour response. In that work, the effect on the immune response of DC targeting, achieved by fusing the model antigen to an antibody fragment (single chain fragment variable, scFv) that specifically recognizes the CD36 receptor was assessed. Using as model a $CD8^+$ T-cell clone specific for an ovalbumin (OVA)-derived MHC class I immunodominant peptide, it was shown that CD36 is able to internalise antigens and lead them to the cross-presentation pathway without the necessity of other costimulatory molecules, making it a good alternative to DEC-205 targeting.

The human epidermal growth factor receptor family

The human epidermal growth factor (EGF) receptor family comprises four membrane proteins called HER1 (EGFR, ErbB1), HER2 (Neu, ErbB2), HER3 (ErbB3), and HER4 (ErbB4).

Among the different receptors, HER1 and HER3 show the highest similarity (around 70%), whereas HER1 and HER2 the lowest (around 53%). Despite their differences, all these proteins have a common structure with an extracellular ligand-binding domain (ECD) of about 600 amino acids, a single membrane-spanning region and an intracellular cytoplasmatic protein tyrosine kinase domain (ICD) (fig. 7).

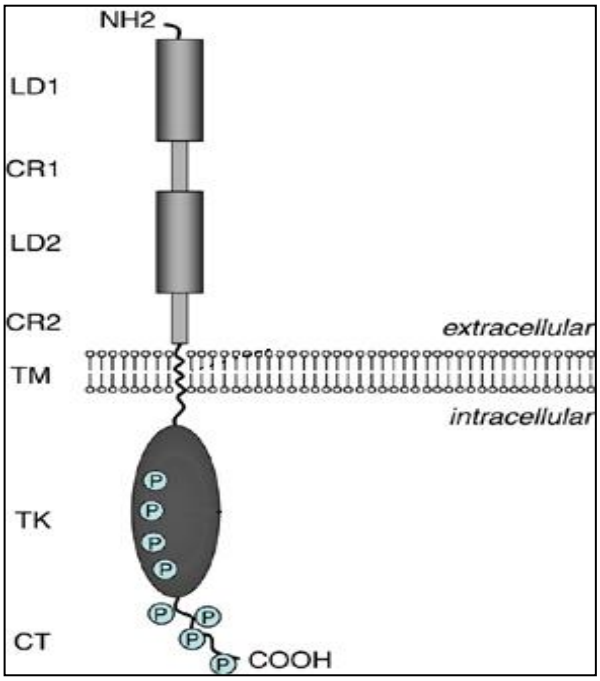

Fig. 7: Schematic representation of EGFR receptors structure.

EGFRs are composed by an extracellular domain (ECD), a short transmembrane domain (TM) and an intracellular domain (ICD) with tyrosine kinase activity (figure modified from (Moasser 2007)).

A deeper analysis has highlighted that the highest sequence identity (average 59–81%) resides precisely into the ICD, with the exclusion of the C-term domain, which exhibits the highest dissimilarity (average 12–30%) (Jorissen, Walker *et al.* 2003).

The ECD of ErbB receptors comprise four domains: there are two ~190-amino acid ligand-domain (LD1 and LD2) and two ~120-amino acid cysteine-rich domains (CR1 and CR2), similar to the one of the insulin growth factor. These domains are

arranged as a tandem repeat of one LD-domain followed by one CR-domain (Cho and Leahy 2002; Cho, Mason *et al.* 2003).

The ICD can be subdivided into three different parts: a region with the typical bi-lobed architecture of the tyrosine kinase domains and two autophosphorylation sites near the C-term, the nearest of which has been shown to have an important regulatory effect, because its absence or mutation causes a constitutive activation of the receptor (Carpenter and Cohen 1990; Jorissen, Walker *et al.* 2003).

In the absence of ligand, HERs predominantly exist in a monomeric form, in which the kinase domain is auto-inhibited by the C-term tail of the receptor. Several ligands, such as EGF and NDF families, have been demonstrated to interact with the HER family members but with different affinity: this contributes to activate well-defined signalling pathways, as describe later. Up to now, twenty different pairing have been demonstrated depending on the type of ligand bound to the ECD, with a subsequent different signalling cascade.

The initial step in HER signalling is the ligand binding which induces a conformational change. The ligand interacts with only LD1 and LD2, but its binding has an effect also in the two CR domains. Upon growth factor binding, domains LD1 and LD2 get closer favouring the freeing of the domain CR1 (Ferguson, Berger *et al.* 2003; Jorissen, Walker *et al.* 2003). The ICD is thus poised to form receptor dimers between two identical receptors (homodimerisation) or between two different ones (heterodimerisation) where one of the two partners acts as buttress. A particular case is represented by HER2, whose ligand is unknown and which is always in a active conformation. The buttress receptor causes phosphorylation of intracellular tyrosine residues, which cluster in the C-term tail, with the consequent activation of the signalling cascade.

The pattern of phosphorylated residues determine the recruitment of a subset of Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain-containing molecules and, consequently, the downstream pathways activated (Olayioye 2001). This process explains the multiplicity of signalling pathways that are activated by these receptors: the mitogen-activated protein kinase (MAPK) pathway; the phospholipase C gamma (PLC-γ) pathway; the phosphatidylinositol-3 kinase stimulation (PL3K) of antiapoptotic AKT kinase signalling; and finally the signal transducer and activator of transcription (STAT) signalling as the most direct route to transcriptional control

of genes involved in cell survival and proliferation (Levkowitz, Klapper *et al.* 1996) (fig. 8).

Ligand binding is not the only way for the receptor to be activated: for example, HER3 has ligand binding capacity but it is defective in its tyrosine-kinase activity due to a mutation in the ATP-binding motif of its ICD: it can thus serve as a catalytically inactive activator of the kinase domain in the heterodimerisation process (Guy, Platko *et al.* 1994) (fig. 8).

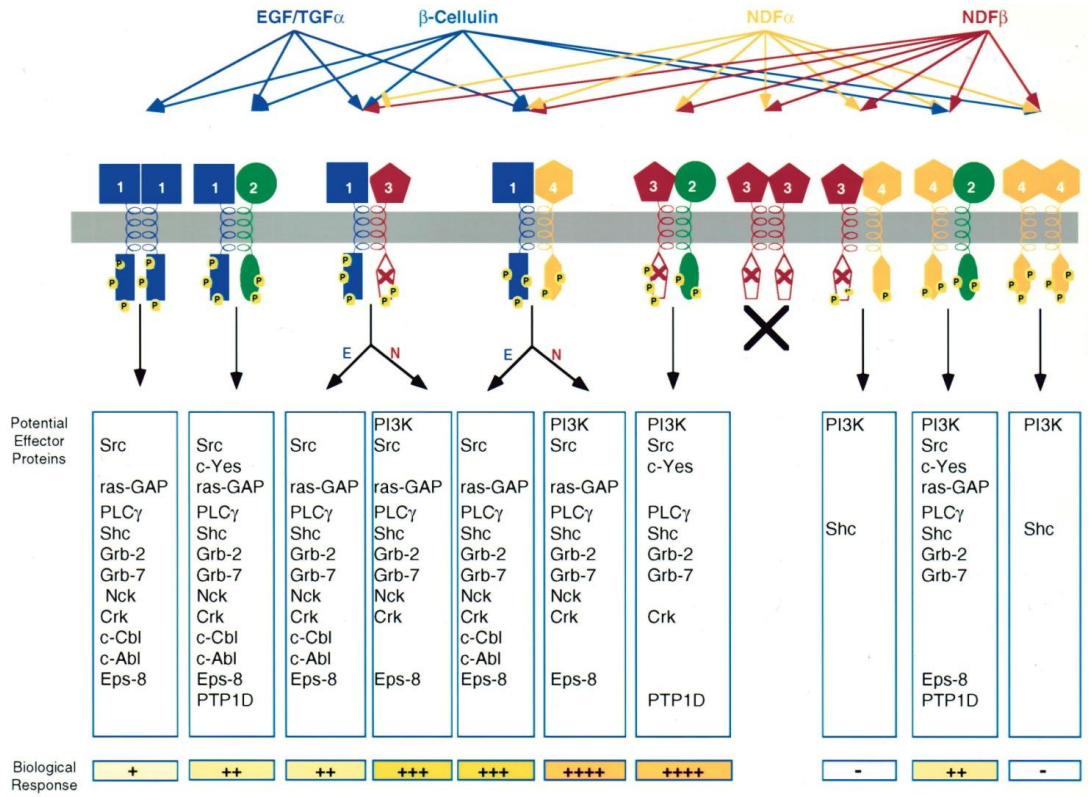

Fig. 8: Schematic representation of HER signalling network

Ligand binding induces conformational changes in the receptor with the subsequent homo or heterodimerization. This leads to the phosphorylation of specific tyrosine residues of the ICD, with the recruitment of specific adaptor proteins. Depending on those adaptors, different signalling cascades are induced (figure taken from (Alroy and Yarden 1997))

The members of HER family have become an attractive target for cancer therapy (Rosen, Ashurst et al. 2010). Existing therapeutic approaches have largely focused on two classes of agents. The first one is represented by mAbs that, by binding to the ECD of the receptors, interfere with their function. A very well known example is Trastuzumab, which is used for the therapy of HER2-overexpressing tumours (Cobleigh, Vogel et al. 1999), as discussed more extensively in the next chapter. Another one is Cetuximab, which targets EGFR (HER1) and has been approved by FDA to treat patients with advanced colorectal cancer, as monotherapy (Saltz, Meropol et al. 2004) or in combination with other drugs (Cunningham, Humblet et al. 2004). It inhibits the binding of activating ligands to the receptor, thus preventing ligand-dependent activation of EGFR. Moreover, it mediates ADCC, favouring the apoptosis of cancer cells (Kurai, Chikumi et al. 2007).

The second approach is represented by small tyrosine kinase inhibitors (TKIs), which inhibit enzyme function by targeting the conserved ATP binding site in the ICD of HER family members. Their mechanism of action is not clearly elucidated: they could act either by direct inhibition of signalling or by preventing the dimerization to occur. TKIs have excellent oral bioavailability and are generally well tolerated, with diarrhoea and skin rash as most frequent toxicities (Arora and Scholar 2005).

One TKI currently used in clinic is Lapatinib (Rusnak, Affleck et al. 2001; Xia, Mullin et al. 2002), which was approved as second line therapy for the treatment of advanced and metastatic breast cancer. It is a dual HER1/HER2 tyrosine kinase inhibitor which has been recently proposed to favour the appearance of unphosphorylated, thus inactive, HER dimers (Vazquez-Martin, Oliveras-Ferraros et al. 2010).

Another example is Gefitinib, approved by FDA for the treatment of non-small lung cancer (NSLSC) (Cohen, Williams et al. 2004). Other TKIs are currently under evaluation in phase I-II clinical trials as both monotherapy and in combination with other targeted therapies (Rosen, Ashurst et al. 2010).

HER2

One among these tyrosine kinase receptors is HER2, also called ErbB2 (the human isoform) or Her2/neu (the rodent one). Its role was revealed in the early 80's by Shih *et al.* (Shih, Padhy *et al.* 1981) and its ligand has not been yet identified. In this receptor, the ECD does not pivot between active and inactive conformations but it exists only in an activated conformation, with no need of ligand binding for its activity. HER2 normally does not homodimerize, while it is the preferential binding partner for the other members, mediating their trans-activation.
HER2 is expressed by different tissues of epithelial origin and is involved in cellular proliferation and differentiation during development. In tissues, its gene is present in a single copy in normal cells.

HER2 has a strong transforming potential, both *in vitro* and *in vivo*, mediated by two possible mechanisms, overactivity and overexpression.
Overactivity is an increase in the tyrosine kinase activity due to somatic mutations that mimic the presence of phosphorylated residues (Weiner, Liu *et al.* 1989; Wang, Narasanna *et al.* 2006). This is the case of Her2/neu-mediated tumourigenesis in rodents, where a single point mutation, located in the TM domain (V655E), favours receptor dimerization and enhanced tyrosine kinase activity. This has been confirmed in transgenic mice, with the appearance of different types of carcinoma: activated neu (the rat isoform of HER2) induces adenocarcinomas in mouse mammary tissues (Muller, Sinn *et al.* 1988), as well as in prostate (Li, Szabolcs *et al.* 2006). Overexpression of Her2/neu induces tumours also in other mouse tissues such as skin (Kiguchi, Bol *et al.* 2000).
Mutations in HER2 are able to induce tumourigenesis also in humans but they are extremely unfrequent. The tumourigenesis is on the contrary manly mediated by overexpression of HER2. This overexpression has a double effect: the increase in HER2 levels changes the composition of HER family dimers, significantly increasing HER2 homodimerization and heterodimerization, especially with HER3. Moreover, HER2 overexpression has an impact on the trafficking of HER2-containing heterodimers. These heterodimers have a different turnover: the ligand binding is highly stabilised and, even though the receptor is internalised, lysosomal degradation is impaired in favour of recycling of the heterodimer. This causes the display of a

still active form of the receptor on the surface of the cells, resulting in a stronger and long-lasting signalling (Worthylake, Opresko *et al.* 1999).
It has been shown that in about 30% of all human breast malignancies there is an amplification of the HER2 gene with the subsequent overexpression of the protein (Yarden 2001), which in turn causes a deregulation of the normal control mechanisms (Hynes and Stern 1994). This type of deregulation has been also found in other tumours such as ovarian (Slamon, Godolphin *et al.* 1989) and colon (Ross and McKenna 2001) cancers, and correlates with poor prognosis (Menard, Tagliabue *et al.* 2000).

The ECD of HER2 is also cleaved and shed from the receptor by an actively regulated proteolytic cleavage (Codony-Servat, Albanell *et al.* 1999). The presence of this soluble version of HER2 has clinical significance, because it is associated to a worse prognosis and insensitivity to endocrine and chemotherapeutic treatments. It is thought that the absence of the ECD enhances the tyrosine kinase activity of the truncated version and its transforming potential, as demonstrated by a recent work where deletion mutants with only ICD are able to promote the growth of tumours in nude mice, in a process that is strictly dependent on their tyrosine kinase activity (Anido, Scaltriti *et al.* 2006).
Histological and serological characterization of breast tumours, aimed to identify HER2 overexpression and the presence of soluble ECD, are currently performed in the clinics.

Different research groups have demonstrated that high titers of anti-HER2 antibodies can be found in the sera of patients with early-stage breast cancer (Disis, Calenoff *et al.* 1994; Disis, Pupa *et al.* 1997). Also, different T-cell epitopes able to induce CTL responses have been identified (Ercolini, Machiels *et al.* 2003; Baxevanis, Sotiriadou *et al.* 2006; Jacob, Radkevich *et al.* 2006; Conrad, Gebhard *et al.* 2008).
These studies suggest that HER2 might be an ideal candidate for cancer immunotherapy, because it is able to induce an immune response. Moreover, it is strongly associated with tumour onset and progression.

A further confirmation for the possibility of blocking HER2-associated cancer progression is the efficacy of the treatment with a monoclonal antibody (mAb) approved by the FDA, Trastuzumab (Herceptin). This humanized mAb binds a β-

hairpin region of domain CR1 in the ECD. Trastuzumab has a preferential binding with HER2-homodimers (Ghosh, Narasanna *et al.* 2011) and its mechanism of action is not well elucidated. Several mechanisms have been proposed, including induction of ADCC (Gennari, Menard *et al.* 2004), impairment of ECD shedding (Molina, Codony-Servat *et al.* 2001), inhibition of the PI3K pathway (Delord, Allal *et al.* 2005) or of angiogenesis.

Trastuzumab, and more recently a different mAb which also targets HER2 (Pertuzumab), have been demonstrated to confer enhanced survival to patients with HER2-overexpressing tumours. A major side reaction of Trastuzumab is its cardiotoxicity. Although Pertuzumab and Lapatinib are as Trastuzumab able to inhibit ErbB2 signalling, which is essential for cardiomyocyte development and function (Lee, Simon et al. 1995), the adverse effects of the formers on cardiac function are marginal, which suggests that Trastuzumab affects unique intracellular events that lead to this dysregulation (Force, Krause et al. 2007).

Moreover, despite the different possible mechanisms of action of Trastuzumab, several reports indicate that many HER2-overexpressing breast tumours can acquire resistance to this treatment (Vogel, Cobleigh *et al.* 2002; Wang and Greene 2008). For example, some human polymorphisms present in the Fc receptor impair the strength of the interaction with Trastuzumab, with a progressive loss of efficacy (Musolino, Naldi et al. 2008). Trastuzumab resistance is also associated with an increase in the signalling from other HER family members or other related growth factor receptors such as the insulin-like growth factor-I receptor.

Trastuzumab is often used in combination with other therapies, e.g. TKI administration. It has been proposed that Trastuzumab treatment alone enhances HER2 ubiquitination and degradation, even though this finding is still matter of debate (Austin, De Maziere et al. 2004; Valabrega, Montemurro et al. 2005). Recently, it has been suggested that the TKIs can prevent receptor downregulation by inhibiting kinase activation, a required step for the HER2 ubiquitination and degradation; therefore, in combination with Trastuzumab these molecules could enhance the clinical benefit of the mAb (Scaltriti, Verma et al. 2009).

Following these clinical evidences, important efforts are been made to clearly identify responsive patients and the most appropriate therapeutic regimen in terms of type, dosage and treatment duration (Gutierrez and Schiff 2011).

Although mAbs are currently used in many cancer therapies, they are not always sufficient for tumour eradication. A common clinical observation in patients with HER2-overexpressing tumours as with other unrelated types of cancer, is that even though specific antibodies against the TAA are present, they are not sufficient for cancer protection. Indeed, TAAs are normally recognized as self proteins, to which high affinity T-cells, both $CD4^+$ and $CD8^+$, may have been tolerized (Ambrosino, Spadaro *et al.* 2006).
Alternative strategies are currently being investigated, among which there are several forms of Her2/neu-based vaccines.

In a preventive immunotherapy approach, DCs have been employed in order to confer tumour protection. DCs were induced to express HER2 antigen in several ways (*ex vivo* loading, transfection or viral transduction) and transferred to mice. This approach conferred tumour protection (Ercolini, Machiels *et al.* 2003; Mossoba, Walia *et al.* 2008; Edlich, Hogdal *et al.* 2010; Fu, Wu *et al.* 2011).
Other pre-clinical studies have demonstrated that DNA vaccination is a good strategy to induce immunity against HER2 (Smorlesi, Papalini *et al.* 2006). Recently, Orlandi *et al.* used a combined approach of active and passive immunotherapy. They modified the HER2 sequence in order to favour the recognition of immunologically active epitopes ("epitope enrichment" strategy) This strategy introduces point mutations in low-affinity epitopes in order to favour their binding onto MHC-I molecules, thus increase MHC-class I occupancy of these peptides and the stimulation of $CD8^+$ T cells with a lower avidity for the original peptide antigen. A plasmid encoding the "enriched HER2" was administered by gene gun biolistic delivery in association with the injection of Trastuzumab. They obtained an improvement in the immune response and in tumour protection upon challenge (Orlandi, Guevara-Patino *et al.* 2011).

Active immunotherapy for HER2-associated cancer treatments is still a relatively new and not well-defined approach that requires further studies to elucidate the best alternative. All this preclinical studies are possible thanks to the increased availability of mouse models where particular aspects of carcinogenesis, not only HER2-mediated, were evaluated in order to develop new possible therapeutic approaches.

A mouse model to study HER2-expressing breast cancer immunotherapy

Different mouse models have been developed to clarify in preclinical studies the carcinogenesis associated to the HER2 receptor. The majority of the transgenics generated have employed either the mouse mammary tumour virus long terminal repeat (MMTV-LTR) or the whey acidic protein (WAP) promoter to express the HER2 in mammary tissues.

MMTV is a retrovirus that was first characterised in the ’30s as a milk-transmitted agent associated with mammary tumors in mice. MMTV-induced mammary tumourigenesis is mediated by proviral integration into different sites nearby cellular oncogenes (Matsuzawa, Nakano *et al.* 1995).
MMTV has a 9kb-long genome with two extremely long LTRs, which encodes one of the accessory viral proteins and many transcription factor binding sites that determine tissue specificity. MMTV-LTRs are active throughout mammary development and are able to drive high levels of viral expression in a hormone-regulated fashion, even though the virus is able to infect also B- and T-lymphocytes. For these characteristics, MMTV-LTRs have been extensively used to drive the expression of oncogenes in transgenic mice.

WAP is the major milk protein found in several mammalian organisms. Its promoter has been used to drive the expression of proteins of interest mainly in mammary glands of pregnant and lactating rodents. The specificity of its activity resides in the presence of a series of consensus sequences for transcription factors associated with mediation of mammary-specific gene expression (Ozturk-Winder, Renner *et al.* 2002).

In 2008, Wall *et al.* (Wall, Milne *et al.* 2007) developed a transgenic mouse where the rat HER2 (Her2/neu) receptor was fused to two well-characterised T-cell epitopes of OVA and expressed in mammary glands under the control of the MMTV promoter. To further increase the frequency of tumours, a dominant negative mutant of the p53 gene whose expression is under the control of WAP was also inserted. This transgene induced the spontaneous development of breast tumours in about 90% of the female mice within six months of age, with characteristics similar to the

human carcinogenesis process. The rationale upon this transgenic model was to tag a known TAA, the Her2/neu, with the T-cell epitopes derived from OVA, a well-known model antigen, to be used for the evaluation of T-cell compartment with exogenously provided specific T-cells.

OVA is a soluble secreted protein that is normally present in the egg white. It is a widely used model antigen because of the presence of two dominant T-cells epitopes that are recognized by $CD8^+$ and $CD4^+$ T-cell clones from two transgenic mice, called OT-I and OT-II, respectively.

OT-I mice contain transgenic inserts for mouse TCRα-V2 and TCRβ-V5 genes. The association of these two sequences with CD8 coreceptor induces the appearance of a monoclonal repertoire of $CD8^+$ T-cells able to recognize OVA residues 257-264 in the context of the H2-Kb MHC-I molecule (Hogquist, Jameson *et al.* 1994). Clarke *et al.* demonstrated that in this transgenic mouse, there is a strong skewing towards $CD8^+$ both in periphery and in thymus. Moreover, they showed that TCR is positively selected only by K^b molecules, as demonstrated by thymic negative selection in the presence of mutant Kb molecules (Clarke, Barnden *et al.* 2000).

The other transgenic mouse strain, called OT-II, expresses a particular TCRα and β-chains paired with the CD4 coreceptor. All $CD4^+$ T-cells are specific for OVA 323-339 peptide which is presented in the context of the MHC class II I-A b allele (Barnden, Allison *et al.* 1998).

These two transgenic mouse strains are frequently used to evaluate the $CD4^+$ and $CD8^+$ T-cell compartments after immunisation with OVA.

Using the above described model of Her2/neu-OTI/II, Wall *et al.* showed that $CD8^+$ T-cells were crucial for tumour eradication. Nevertheless, specific $CD4^+$ T-cells acted synergistically to enhance the benefits of $CD8^+$ T-cell administration.

The results obtained in cancer immunotherapeutic studies are encouraging but still far to be exhaustive. Further approaches can try to enhance the immune response by specific DC-targeting, in order to evaluate the direct contribution of the humoral and cellular responses in the delay of the tumour progression process or in tumour rejection. Modulating the different antigen processing pathways, for example by specific DC receptor targeting, can also provide new experimental tools to regulate the immune response.

Aiming to design a new active immunotherapy approach, we investigated the effect of stimulating the cross-presentation pathway for the TAA HER2 on the immune response and tumor protection activity of a novel DNA vaccine. Different recombinant version of HER2 were used in order to demonstrate their ability to confer tumour protection in a tolerogenic environment both in a prophylactic and in a therapeutic vaccination approach.

Materials and Methods

Cell culture, transfection and selection

HEK 293, HEK293T/17 and HeLa cells from American Type Culture Collection (Rockville, MD, numbers CRL-1573, CRL-11268 and CCL-2, respectively) were cultured in Dulbecco's modified Eagle's medium 4,5 g/l glucose (DMEM, Gibco-Invitrogen, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (FCS) (Gibco), 50 µg/ml gentamycin (Gibco) and 2 mM L-glutamine.

Transfections of HEK 293T/17 and HEK293 cells were performed essentially as described by Sambrook *et al.* (Sambrook, Fritsch *et al.* 1989), using circular or linearized plasmids, respectively. Briefly, cells were seeded in 6-well plates (for transient transfections) or 10-mm Petri dishes (for stable transfections). Fresh medium was added 4 h before transfections. For transient transfections, 5 µg of circular plasmid was resuspended in 50 µl of 0,1x TE (10mM Tris, 1mM EDTA). A mix was prepared by addition of 169 µl of deionized water, 5 µl of $CaCl_2$ 2 M and, drop by drop, plasmidic DNA, followed by 26 µl of $CaCl_2$ 2 M. This mix was added, drop by drop, to 250 µl of 2x HBS (280 mM NaCl, 10 mM KCl, 1,5 mM NaH_2PO_4, 12 mM dextrose and 50 mM Hepes). Total mix was added to cells, drop by drop. After an overnight (o/n) incubation, medium was replaced. The day after, cell and/or supernatants were collected and analyzed. For stable transfections, selection of transfectants was done by addition of 0,4 mg/ml geneticin (G418, Gibco) to the medium 24 h after transfection. Single clones were then individually selected, expanded to 6-wells plates and screened for their production of molecules of interest by ELISA, Western blotting or flow cytometry (FACS) analysis.

HeLa cells were transfected using circular plasmid and Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer instruction.

Sp2/0-Ag14 (ATCC: CRL-1581) cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat inactivated FCS, 50 µg/ml gentamycin, 2 mM L-glutamine and 1 mM sodium piruvate.

Stable transfections of Sp2/0-Ag14 cells were performed by electroporation of 10e6 cells with 10 μg of linearized plasmids at 960 μF and 250 V with the Gene Pulser device (Bio-Rad, Hercules, CA). Cells were subsequently seeded in 96-well plates. Twenty-four hours later, 0,4 mg/ml geneticin was added to the medium, which was replaced after 7 days. Stable transfectant clones were screened for production of molecules of interest by ELISA, Western blotting or FACS analysis.

NOP6 cell line was derived from a spontaneous breast tumour (Wall, Milne *et al.* 2007). It was cultured in D-MEM 4,5 g/l glucose supplemented with 10% heat-inactivated FCS, insulin-transferrin-sodium selenite supplement (ITS, Sigma, St. Louis, MO) and 50 μg/ml gentamicyn.

E.G7-OVA (ATCC: CRL-2113) cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 50 μg/ml gentamycin, 2 mM L-glutamine, 1 mM sodium piruvate and 50 μM 2-mercaptoethanol.

Mice

C57BL/6j mice were purchased from Harlan (Milan, Italy). OVA-specific, TCR transgenic OT-I animals were purchased from the Jackson Laboratories (Bar Harbor, ME). CD45.1 congenic C57BL/6j were bred with OT-I mice to obtain OT-I/CD45.1 animals.

Her2/neu-OTI/II transgenic mice (Wall, Milne *et al.* 2007) were purchased from Jackson Laboratories and bred with C57BL/6j mice. To identify transgenic animals, genomic DNA extraction from tails of 20 days-old animals was performed. Briefly, tails were clipped and digested by addition of proteinase K (Invitrogen) 100 μg/ml in lysis solution (Tris-HCl 50 mM pH 8, EDTA 100 mM pH 8, SDS 0,5%), followed by o/n incubation at 57°C. Genomic DNA precipitation was performed with alcoholic extraction and genotyping was done by PCR with specific oligonucleotides (Table 1) and PCR Taq Gold enzyme (Roche, Basel, Switzerland).

Mice were bred and maintained at animal house of ICGEB in conformity with institutional guidelines and in compliance with national and international laws and policies.

Table 1: List of the oligonucleotides used for the genotyping of animals and cycling conditions

Transgene	Primers	Sequence	Cycling condition
Neu-OT	Neu-OT for	5'-TGTCCGGCCTGCTGGTGCTACTCTAGAAAG-3'	1. 94°C for 2' 2. 94°C for 30'' 3. 60°C for 1' 4. 72°C for 1' 5. 72°C for 7' 6. 4°C hold 7. Repeat step 2-4 for 35 times
	Neu-OT rev	5'-TCACACTCCAGCCTCTGCTGACCCTACCAC-3'	
DNp53	DNp53 for	5'-CCGTCGACGGCCACAGTGAAGACCTCCGGCCAG-3'	1. 94°C for 2' 2. 94°C for 30'' 3. 55°C for 30'' 4. 72°C for 1,30' 5. 72°C for 7' 6. 4°C hold 7. Repeat step 2-4 for 30 times
	DNp53 rev	5'-GCGTGACACCCTGCTGGGAAGGAGGAGGATGAG-3'	

Construction of plasmids

scFv-SIP-OVA plasmids were previously described and available in the Molecular Immunology laboratory at ICGEB (Tagliani, Guermonprez *et al.* 2008).

To generate the Her2^{ECD}-containing constructs, different strategies were adopted.

pcDNA3-Her2^{ECD}-muC$_H$3, Her2^{ECD}-huC$_H$3 and Her2^{ECD}-εSIP plasmids were obtained by amplification of the extracellular domain (ECD) of the Her2/neu from pSE280-NT00 plasmid (a kindly gift of Dr. Brad Nelson, Deeley Research Center, Victoria, BC, Canada) with the oligonucleotides Her2-SIP-for and Her2-SIP-rev (the sequences of all the oligonucleotides are listed in table 2). The resulting fragment was cloned into pUT-sec vector (Li, Pedraza *et al.* 1997) digested *Apa*LI-*Spe*I, originating the pUT-sec-Her2^{ECD} plasmid. A fragment *Hin*dIII-*Age*I comprising the Her2^{ECD} fragment with the secretory signal sequence from a mouse immunoglobulin heavy chain was then transferred into the SIP pcDNA3-based vector digested *Hin*dIII-*Bsp*EI (Li, Pedraza *et al.* 1997; Bestagno, Vangelista *et al.* 2001), where a self-dimerizing domain derived from immunoglobulin heavy chain is present at the C-term of a scFv to obtain the dimeric version of the Her2^{ECD}.

To generate the Her2^{ECD}-GPI construct, the fragment *Hind*III-*Bsp*EI from pUT-sec-Her2^{ECD} plasmid was transferred into a pcDNA3-SV5-GPI available in the lab.

The same fragment above described was used to generate pcDNA3-Her2^{ECD}-BAP bigenic plasmid: it was transferred into the bigenic vector pcDNA3-scFv-SV5-BAP-BirA described elsewhere (Predonzani, Arnoldi *et al.* 2008).

The OVA-containing plasmids were constructed from the intermediate vectors pcDNA3-Her2^{ECD}-OVA-NS and pcDNA3-scFv^{CD36}-Her2^{ECD}-OVA-NS.

pcDNA3-Her2^{ECD}-OVA-NS was created by PCR amplification of Her2^{ECD} sequence from pSE280-NT00 (oligonucleotides Her2-SIP-for and Her2-*Kpn*I-rev) and subsequent cloning into pUT-sec vector digested *Apa*LI-*Kpn*I. The fragment *Hin*dIII-*Kpn*I comprising the Her2^{ECD} fragment with the secretory signal sequence was then transferred into a pcDNA3 modified (pcDNA3-mod) by the insertion of the *Cla*I site with two overlapping oligonucleotides (oligonucleotides *Kpn*I-*Cla*I-*Xho*I for and rev), originating pcDNA3-mod-Her2^{ECD} plasmid. The fragment *Kpn*I-*Bst*BI from the pcDNA3-scFv^{CD36}-huC$_H$3-OVA plasmid, comprising the full-length sequence of mature OVA, was taken and transferred into pcDNA3-mod-Her2^{ECD} plasmid digested *Kpn*I-*Cla*I.

pcDNA3-scFv^{CD36}-Her2^{ECD}-OVA-NS was generated by PCR amplification of Her2^{ECD}-OVA from pcDNA3-Her2^{ECD}-OVA-NS (oligonucleotides *Nhe*I-3'-scFv^{CD36} for and Her2-OVA-*Xba*I rev) and subsequent cloning of *Nhe*I-*Xba*I fragment into pcDNA3-scFv^{CD36}-huC$_H$3-OVA equally digested.

The pcDNA3-Her2^{ECD}-muC$_H$3-OVA plasmid was derived from the pcDNA3-Her2^{ECD}-muC$_H$3 construct: to remove the stop codon downstream the C$_H$3 sequence, pcDNA3-Her2^{ECD}-muC$_H$3 was modified by PCR amplification (oligonucleotides Her2-SIP-for and C$_H$3-*Kpn*I-rev), transferred into pUT-sec and the fragment *Hin*dIII-*Kpn*I finally cloned into pcDNA3-Her2-OVA-NS vector equally digested.

pcDNA3-scFv^{CD36}-Her2^{ECD}-muC$_H$3-OVA was obtained by taking *Afe*I-*Xba*I fragment from pcDNA3-Her2^{ECD}-muC$_H$3-OVA plasmid and subsequently cloned into pcDNA3-scFv^{CD36}-Her2-OVA-NS equally digested.

Plasmids containing the sequence encoding the scFv^{CD36} were digested *Hin*dIII-*Bsp*EI and the fragment was replaced with an equally digested scFv fragment deriving from the pcDNA3-scFv^{DEC205}-huC$_H$3-OVA or the scFv^{Irr}-huC$_H$3-OVA plasmids.

The pcDNA3-Her2^{ECD}-muC$_H$3-H6 construct was generated by amplifying the Her2^{ECD} from pcDNA3-Her2^{ECD}-muC$_H$3 plasmid by PCR (oligonucleotides 3'ECD-*Bst*EII for and muC$_H$3-*Nhe*I-TAA-*Xba*I rev) to insert an *Nhe*I site required for the introduction of two complementary oligonucleotides (H6 for and rev).

In all the plasmids where the CMV promoter was replaced with the K14 one, a *Bgl*II-*Hin*dIII fragment, containing the CMV sequence, was excised and replaced with the sequence encoding the K14 promoter, taken from a plasmid available in the lab and elsewhere described (Vassar, Rosenberg *et al.* 1989).

All DNA restriction and modification enzymes were purchased from Promega (Madison, WI) and New England Biolabs (NEB; Ipswich, MA), and kits for DNA purification from Euroclone S.p.A. (Milan, Italy) and Qiagen (Hilden, Germany). PCRs for vector construction were performed with KOD Hot Start DNA polymerase (Novagen, Merck, Darmstadt, Germany).

Table 2: List of the oligonucleotides used for the generation of the different plasmids.

The annealing portion of each oligonucleotide is underlined and the used restriction site sequences are shown in lower case characters.

Construct name	Oligonucleotides	Sequence
$Her2^{ECD}$-muC_H3 $Her2^{ECD}$-huC_H3 $Her2^{ECD}$-εSIP $Her2^{ECD}$-BAP $Her2^{ECD}$-GPI	Her2-SIP-for	5'-AGGTgtgcacTCTCAAGTGTGTACCGGCACAG-3'
	Her2-SIP-rev	5'-CAGAGAGCCAGCCCGGTGACAaccggtactagtGGTA-3'
pcDNA3-$Her2^{ECD}$-OVA-NS	Her2-SIP-for	5'-AGGTgtgcacTCTCAAGTGTGTACCGGCACAG-3'
	Her2-*Kpn*I-Rev	5'-CAGAGAGCCAGCCCGGTGACAGGAggtaccGGAG-3'
pcDNA3-mod	*Kpn*I-*Cla*I-*Xho*I for	5'-cGCTAGCatcgatTAAc-3'
	*Kpn*I-*Cla*I-*Xho*I rev	5'-gtaccGCTAGCatcgatTAActcga-3
$scFv^{CD36}$-$Her2^{ECD}$-muC_H3-OVA	*Nhe*I-3'-$scFv^{CD36}$ for	5'-TCAgctagcTCCGGAGGCTCTGGGCAAGTGTGTACCGGCACAGACA-3'
	Her2-SIP-rev	5'-CAGAGAGCCAGCCCGGTGACAaccggtactagtGGTA-3'
pcDNA3-$scFv^{CD36}$-Her2-OVA-NS	*Nhe*I-3'-$scFv^{CD36}$ for	5'-TCAgctagcTCCGGAGGCTCTGGGCAAGTGTGTACCGGCACAGACA-3'
	Her2-OVA-*Xba*I rev	5'-TCGATTAACTCGAGCATGCAtctagaGGG-3'
pcDNA3-$Her2^{ECD}$-muC_H3-OVA	Her2-SIP-for	5'-AGGTgtgcacTCTCAAGTGTGTACCGGCACAG-3'
	CH3-*Kpn*I-rev	5'-ATggtaccTTTCCCGGGAGAGTGGG-3'
$Her2^{ECD}$-muC_H3-H6	3'-ECD-*Bst*EII for	5'-ACATggttaccGTACATCTCA-3'
	muC_H3-*Nhe*I-TAA-*Xba*I rev	5'-TCTCCCACTCTCCCGGGAAAgctagcTAAtctagaATA-3'
	H6 for	5'-ctagcCATCACCATCACCATCACTAAt-3'
	H6 REV	5'-cCATCACCATCACCATCACTAATctag-3'

Production of mono-biotinylated Her2^{ECD}

Mono-biotinylated Her2^{ECD} was recovered from culture supernatants from Her2^{ECD}-BAP-stably transfected HEK293 cells. Cells were cultured as parental cell line. When 80% of confluence was reached, cells were gently washed with PBS twice and medium replaced with DMEM supplemented with 50 μg/ml gentamycin (Gibco), 2 mM L-glutamine and 100 μM biotin (Fluka, Sigma). After 48 h, medium was collected and dialyzed o/n against PBS at 4°C.

Production and purification of Her2^{ECD}-muC$_H$3-H6 protein

Her2^{ECD}-muC$_H$3-H6-transfected HEK293 cells were grown in EX-CELL® 293 Serum-Free Medium (Sigma) in stirrer bottles at 37°C and 5% CO_2. Medium was collected, concentrated by ultrafiltration using Omega™ Ultrafiltration Membrane Disc Filters (Pall Corporation, Port Washington, NY) and dialyzed extensively against PBS. PolyH6 tag protein purification was performed using Ni-NTA agarose (Qiagen, Venlo, Netherlands): concentrated and dialyzed supernatant was added to an equilibrated Ni-NTA column (10x column volume of PBS pH 8, 10 mM imidazole) and incubated for 3 h at 4°C. Column was then emptied by gravity flow and washed with PBS pH 8. Bound molecules were eluted by addition of PBS pH 8, 300 mM imidazole and extensively dialyzed against PBS.

To check the purity of the eluted protein, standard sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) followed by Coomassie (Bio-Rad) or silver staining (Invitrogen) was performed. HPLC analysis was done in collaboration with the Protein Structure laboratory at ICGEB with standard procedures. Protein was stored at -20°C until use.

Gene gun cartridge preparation and gene gun DNA vaccination

Gene gun cartridges were prepared using Helios Gene gun system (Bio-Rad) according to manufacturer instructions. Briefly, 50 μg of plasmid DNA were adsorbed on 1 μm gold particles and the excess of DNA removed by $CaCl_2$ precipitation. Gold particles were then transferred into a Teflon tube and let them to adhere to inner surface. Pieces of 1 cm of length were cut and used for gene delivery. Mice abdominal area was shaved and the content of one cartridge, carrying 1 μg of DNA, was shot at 400 psi using the Bio-Rad gene delivery device.
Mice were given one shot every two weeks for three times. Sera were collected ten days after the last shot.

Protein vaccination

C57BL6/j mice (6-7 weeks-old) were immunized by subcutaneous injection with 50 μg of purified $Her2^{ECD}$-muC_H3-H6 protein with or without Freund's adjuvant, complete in the first dose, or incomplete in the two subsequent injections, every two weeks. Protein was eventually denatured before use by 10 min of boiling. Sera were collected ten days after the last immunisation.

Adoptive transfer of OT-I cells

OT-I cells were isolated freshly for each experiment from OT-I-transgenic mice. Lymph nodes (LNs) were collected and crushed through a 100 μm filter to prepare a single-cell suspension. Cells were washed with PBS/bovine seroalbumin (BSA) 0,1% and incubated with 400 μl of the same buffer supplemented with 5 μM 5- 6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Invitrogen) for 10 min at 37°C. Labelling was stopped by addition of 10 ml of RPMI 1640 supplemented with 10% heat-inactivated FCS. Cells were counted and resuspended at 10e7 cells/ml. *In vivo* OT-I activation experiments were performed by injecting 10e6 cells into tail vein. Twenty-four hours later, mice were vaccinated and

OT-I proliferation was assessed three days post-immunisation by the CFSE dilution assay, with the cells taken from the inguinal LNs.

Flow cytometry analysis

FACS analysis was performed with standard protocols. Cells were blocked for 30 min on ice with PBS-BSA 1% and then labelled with appropriate amount of antibodies diluted in the same buffer for at least 30 min on ice. Then, cells were washed, resuspended in PBS and analyzed with BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). Data were processed with CellQuest software (Becton Dickinson).

For FACS analysis, the following goat anti-mouse antibodies were used: PE-conjugated anti-CD8α (Caltag, Invitrogen), biotin-conjugated anti-CD45.1 (BD Pharmingen), DyeLight^{488}-conjugated anti-IgG (γ-chain specific) (Jackson ImmuneResearch, Suffolk, UK). QDot^{655}-streptavidin was purchased from Molecular Probes (Invitrogen).

Enzyme-linked immunoadsorbent assay (ELISA)

96-well Maxisorp ELISA plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml purified OVA, 5 μg/ml purified human IgG (Sigma) or 5 μg/ml purified avidin (Sigma). Plates were washed and blocked with PBS/BSA 1%, Tween 20 0,05%. For the evaluation of anti-Her2^{ECD} response, dialyzed supernatant containing mono-biotinylated Her2^{ECD}-SV5-BAP was diluted 2x in PBS/BSA 1% and added to avidin-coated plates. Sera were diluted in blocking solution and binding was detected using an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody (Jackson Laboratory) followed by tetramethylbenzidine peroxidase substrate (TMB, Sigma). All incubation steps were done at 37°C for at least 2 h or o/n at 4°C.

For the analysis of the quality of antibodies, mono-biotinylated Her2^{ECD} was denatured by boiling for different times before its use.

Western blotting analysis

Cell supernatants were resolved in a SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) previously activated in methanol. After blotting, membrane was blocked with PBS-milk 5% for 45 min at room temperature and incubated with the appropriate amount of primary antibody for 1 h. Two mouse primary antibodies were used, the mAb anti-SV5 (Invitrogen) and the mAb 7.16.4 (Calbiochem-Merck, Darmstadt, Germany), and a rabbit one, the mAb anti-OVA (Abcam, Cambridge, UK). Membranes were washed and incubated with an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody (Jackson Laboratory) or an HRP-conjugated goat anti-rabbit IgG (Thermo Scientific, Waltham, MA). Binding was revealed with the enhanced chemoluminiscence (ECL) Detection Reagent (Pierce, Rockford, IL, USA).

Gel retardation assay was performed as previously described (Predonzani, Arnoldi *et al.* 2008). Briefly, dialyzed supernatants were incubated with 1 μg of purified streptavidin (Sigma) for 1 h at 4°C. Complexes formed by biotinylated $Her2^{ECD}$ and streptavidin were resolved by SDS-PAGE as above described.

Tumour protection experiments

Vaccinated mice were subcutaneously injected into their mammary fat pad with 10e6 NOP6 cells. After three days, ultrasound imaging was performed according to the protocol from Tilli et al (Tilli, Parrish *et al.* 2008). Briefly, mice were anesthetized by inhalation of isofluorane with 1–3% oxygen and flank hair was removed using a mild depilatory cream. Mice were placed on a thermostatically-controlled heating pad to help maintain body temperature. Images were taken with the Visualsonics Vivo 660 High-Resolution Imaging System for small animal ultrasound (Toronto, Ontario, Canada), with 60 MHz transducer. Mammary fat pad was screened for the presence of less echogenic area in the injection site.

Mice were evaluated for the presence of tumours every week and animals with tumours were sacrificed. Tumours were dissected and weighed.

For E.G7-OVA tumour protection experiments, vaccinated mice were subcutaneously injected into their right flank with 2x10e5 cells. Tumour size was

measured with a calliper ruler 21, 28 and 32 days after the challenge. Average size is expressed in cubic millimetres using the formula $V=4/3 \times \pi \times (\text{length} \times \text{width})$

Statistical analysis

Statistical analysis was performed using GraphPad Prism suite. Differences in tumour weight were evaluated with the U-Mann Whitney test.

Results

Immune response induced by gene gun DNA immunisation

The key point for every immunotherapy strategy is the activation of both humoral and cell-mediated immune responses. This is also true in the physiological control of tumour cell appearance and proliferation, where both antibodies and cytotoxic T-cells act synergistically.

It is believed that to obtain relevant clinical results, the stimulation of both arms is necessary. There are many factor that should influence the efficacy of this induction: the nature of the given stimulus (for example viral or parasite molecules), route of administration and the concomitant presence of other adjuvant molecules all together shape both quantitatively and qualitatively the immune response.

In our laboratory, there is a large experience in the biolistic delivery of DNA molecules to induce an effective immune response against different types of antigens: in particular, this strategy was efficiently applied in a model of B-cell lymphoma, where a strong antibody response against the immunoglobulin expressed by malignant cells was obtained. This approach was also able to confer tumour protection (Benvenuti, Burrone *et al.* 2000).

Despite this strong expertise, it is still unclear which should be the best regime to eradicate solid tumours. To this aim, we investigated gene gun DNA immunisation to induce tumour protection against a well-known tumour-associated antigen, such as the HER2 receptor, in a tolerogenic environment.

Before moving towards this TAA, it was necessary to set up all the procedures with an antigen that allows an easy read out of the outcome, to minimize variability ascribable to technical problems. We thus started our studies with a well characterised model antigen, the ovalbumin (OVA).

OVA-derived peptides, as all others, should be presented by major histocompatibility complex (MHC) class I or II molecules of antigen presenting cells (APCs) depending on their origin: if peptides are produced directly by the APC itself, they will be processed and displayed in association with MHC-I. On the contrary, soluble and

exogenous peptides will be displayed, upon their uptake, associated with MHC-II. There is a third possibility in DCs: the cross-presentation pathway. In this case, circulating antigens are uptaken, processed and displayed with MHC-I. Cross-presentation could therefore be rendered more efficient by redirecting antigens to defined DC-surface internalising receptors.

Among the different OVA-peptides present in the protein, there are two strong immunodominant epitopes. These two epitopes are processed and presented by APCs in association with MHC class I or II, with the subsequent activation of $CD8^+$ or $CD4^+$ T-cells, respectively. The immunological consequence of this knowledge led to the creation of two different transgenic mice with transgenic TCRs specific for each of these two complexes. One strain is called OT-I, where the transgenic TCR expressed in all $CD8^+$ T-cells is able to recognize exclusively the OVA peptide SIINFEKL, which is presented in association with the H2-Kb haplotype of MHC-I. In the second mouse strain, called OT-II, all $CD4^+$ T-cells express a transgenic TCR that can recognize only the OVA-derived peptide on the MHC-II. Moreover, it is possible to breed these two strains with other transgenic animals expressing on the surface of T-cells a different cluster-differentiation (CD) marker, for instance the CD45.1 or CD45.2 alleles. These specific T-cells can be transferred into syngenic recipients and easily identified, for example by using specific antibodies that recognize CD markers. All these features allow the analysis of the activation of both $CD8^+$ and $CD4^+$ T-cells by APC in an easier way with respect to the analysis of the endogenous repertoire of T-cells, where the frequency of specific T-cells is very low and whose specificity is generally not well characterised for most antigens.

In a context of cancer immunotherapy, antigen targeting to a DC-internalising receptors should be very useful and productive to induce both antibody and cell-mediated tumour protection.

In our laboratory, antigen targeting to specific DC-internalising receptor was able to mediate cross-presentation of OVA when administered as purified protein (Tagliani, Guermonprez *et al.* 2008).

We wanted to expand this finding with a tumour-relevant antigen using the gene gun DNA immunisation instead of the protein administration.

In the initial set-up, we used three plasmids (fig. 9), all containing the sequence encoding the full-length OVA. Two different targeting moieties were used to demonstrate the possibility to reorient our model antigen towards the cross-presentation pathway. They derived from two monoclonal antibodies (mAbs) that specifically recognize two different internalising receptors, DEC205 and CD36, which have been previously shown by our group and others to efficiently mediate cross-presentation. Instead of using full-length antibodies, which could be detrimental for different reasons (steric hindrance, low ability to reach tumour tissues, etc), we preferred to use the single chain fragment variable (scFv) format, where the variable region of light (V_L) and heavy (V_H) chains are connected by a flexible short linker. As a control, we used a third antibody that does not recognize any DC-internalising receptor ($scFv^{Irr}$), thus allowing the evaluation of the cross-presentation pathway induced by the direct targeting of the antigen to DCs.

In our constructs, between scFv and OVA sequences, there is a third domain, the C_H3 domain of IgG. There are three reasons for the presence of this domain: 1- this moiety has the ability to induce self-dimerization of the molecule, resulting in a molecule (Li, Pedraza *et al.* 1997) with prolonged half-life in circulation and increased avidity when compared with a monomeric scFv; 2- from previous studies, we knew that scFv dimerization allows cross-linking of the receptor, which is needed for the internalisation and the induction of cross-presentation pathway; 3- it has been shown that, in certain cases of poor immunogenic self-antigens, the xenogenic C_H3 (for example human in mouse) is essential to increase the immune response.

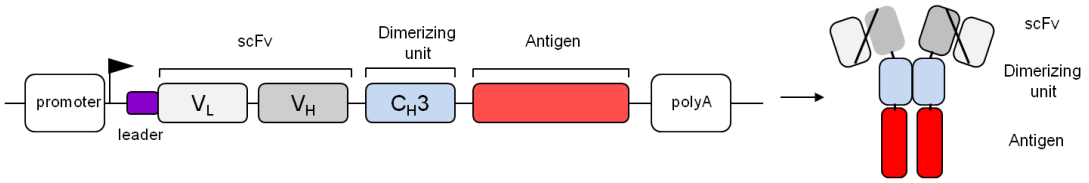

Fig. 9: Schematic representation of DNA plasmids.

DNA plasmids are composed by three different parts: 1- a N-term receptor targeting unit; 2- a C_H3 dimerizing domain and 3- a model antigen (OVA) at the C-term.

T-cell response

The OVA antigen allowed us to analyze both humoral and cellular responses. By gene gun DNA immunisation it is possible to induce antigen expression in well-defined tissues by using different tissue-specific promoters. We first chose the CMV promoter, due to its high efficiency of transcription in all the possible tissues where plasmid DNA arrives.
The first objective was to assess the possibility to determine T-cell responses in wild type animals following immunisation.

To evaluate the activation of specific $CD8^+$ T-cells, OVA-specific OT-I cells obtained from lymph nodes (LNs) of OT-I mice were labelled *in vitro* with CFSE, a non-toxic and fluorescent dye, which is unable to diffuse out across cell-membrane after its cellular uptake through its esterification by a cellular enzyme. These cells were transferred into syngenic recipient animals, which were vaccinated twenty-four hours later with one of the three different plasmids. After three days, LNs of recipient mice were collected and analyzed for the presence of fluorescent T-cells. If immunisation was efficient, specific T-cells would be activated and proliferate, with a concomitant reduction (50%) of intracellular dye. By FACS analysis, it is possible to appreciate this phenomenon determining a decrease in the intensity of labelled T-cells. As shown in fig. 10, a strong proliferation of these cells was demonstrated, in groups receiving both $scFv^{DEC205}$- and $scFv^{CD36}$-encoding plasmids. Surprisingly, in contrast to what had been observed with the purified protein, activation of the cells was also achieved with the construct containing the $scFv^{Irr}$. We hypothesised that this effect could be due to direct transfection of tissue-resident APCs, because by gene gun delivery, all the cells that are present in the epidermis could receive the plasmid and start to produce the protein due to the absence of any transcription restriction (fig. 10).

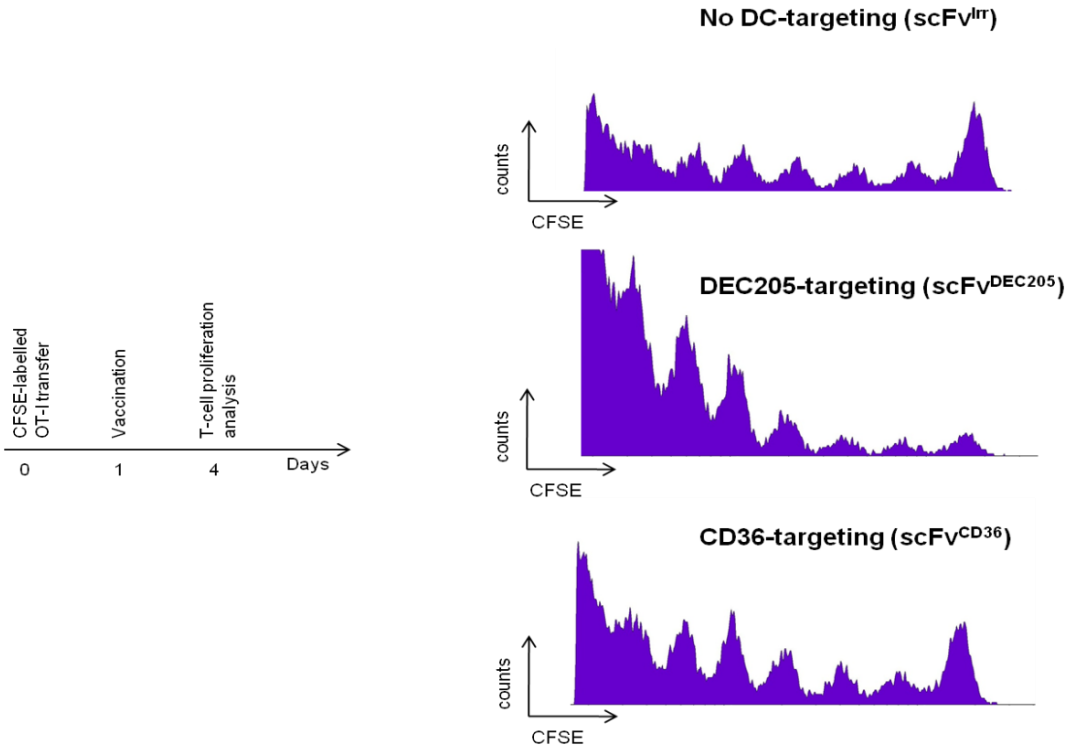

Fig. 10: Gene gun DNA immunisation induces specific T-cell activation.

10e6 OT-I cells were labelled with CFSE and adoptively transferred into syngenic recipients (C57Bl/6j mice). The day after mice were immunised with the plasmids encoding the indicated scFv fusion constructs and three days later, cells were collected from inguinal lymph nodes and evaluated by FACS. The result with one representative mouse is shown for each construct.

To better characterise the persistence of the activated T-cells, we evaluated the frequency of adoptively transferred OT-I cells from peripheral blood of recipient mice at different time points. For this purpose, we used cells from OT-I mice expressing the CD45.1 surface marker, which is absent in the recipient mice that express the CD45.2 allele.

As shown in fig. 11, the presence of these cells was detected up to 30 days after their injection, without any need of other stimuli, in all the vaccination groups.

However, the frequencies of circulating specific T-cells reflect a trend similar to that observed at early time points in the LNs: there is a differential ability of the DC-targeting scFvs to stimulate the immune response, i. e. $scFv^{DEC205}$ is more effective than $scFv^{CD36}$, even though this difference is quite reduced. Also in this case, adoptively transferred T-cells were found in mice immunised with the plasmid encoding the $scFv^{Irr}$ plasmid, but their frequency was lower than in the other two groups.

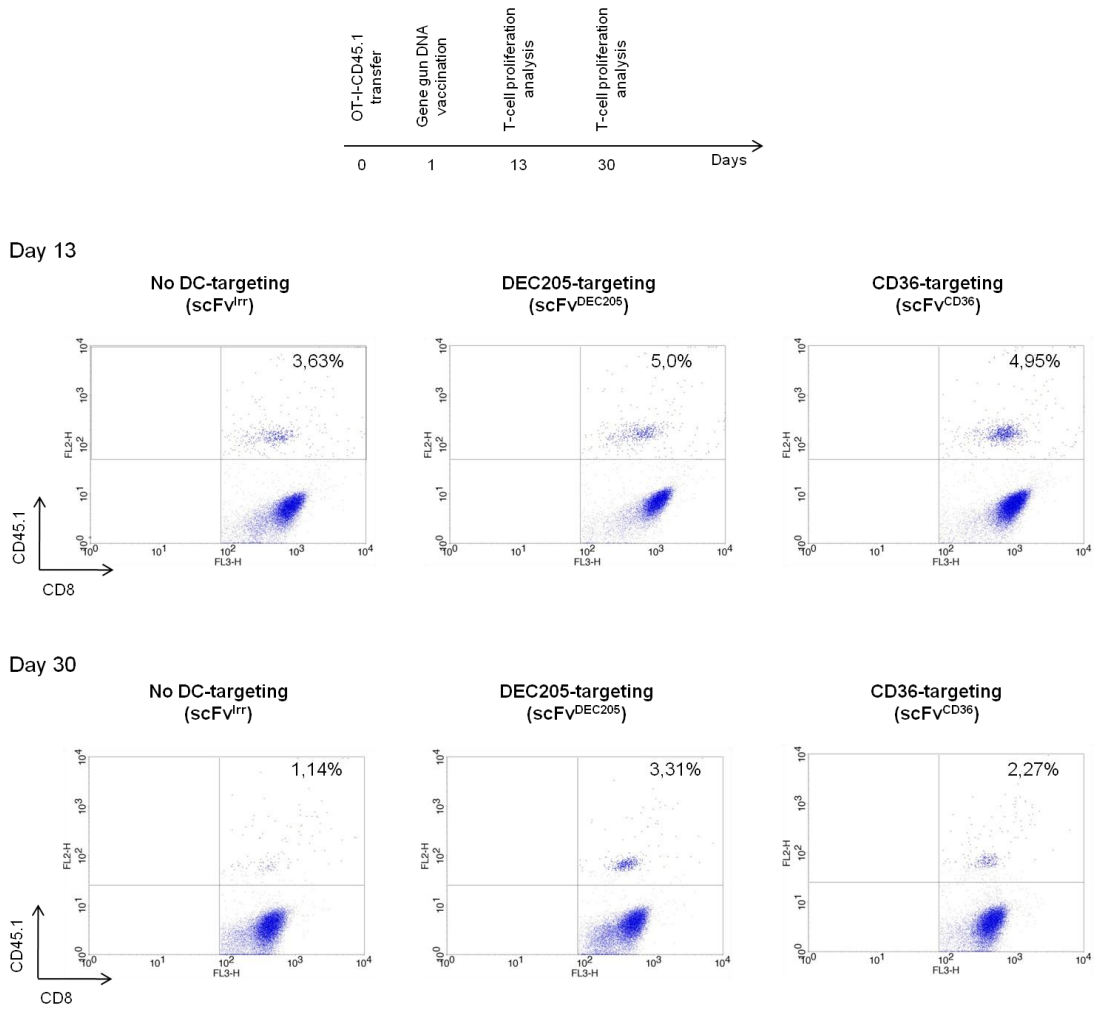

Fig. 11. OT-I persistence after DNA immunisation

10e6 OT-I - CD45.1 cells were labelled with CFSE and adoptively transferred to C57Bl/6j CD45.2 recipients. The day after mice were immunised with the indicated scFv fusion constructs. Peripheral blood samples were collected at day 13 (upper panels) or day 30 (lower panels) after the adoptive transfer. The presence of OT-I – CD45.1 was detected by FACS with PE-conjugated anti-CD8 and biotinylated anti-CD45.1 plus $QDot^{655}$-conjugated streptavidin. Each dot blot represents the result with one representative mouse out of three evaluated.

The proliferation of specific $CD8^+$ T-cells achieved in the case of the recombinant protein containing a non-DC targeting scFv ($scFv^{Irr}$), although at reduced levels, was attributed to the transfection of resident APCs by the DNA-coated particles administered by gene gun, without the involvement of the cross-presentation pathway. To prove this hypothesis, we constructed a plasmid where the fusion protein was under the transcriptional control of a tissue-specific promoter. The main tissue target of the biolistic delivery is the epidermis, which is mainly composed by keratinocytes. We reasoned that the use of a keratinocyte-restricted promoter should be useful to prevent expression of the antigen in transfected resident DCs, while still being able to drive the production of similar antigen levels.

The CMV promoter was thus replaced by the promoter of the keratin-14 (K14) gene. In order to test the transcription activity of this new promoter, we transfected HEK293T and HeLa cells with plasmids containing the enhanced green fluorescent protein (EGFP) sequence under the transcriptional control of both CMV and K14 promoters and evaluated the presence of fluorescent cells by FACS or immunofluorescence.

As shown in fig. 12, the construct with CMV promoter were functional in both HEK293T (fig. 12a) and HeLa (fig. 12b) cell lines, as demonstrated by the expression of EGFP. On the contrary, the K14 promoter was active only in epithelial-derived cell lines, as HeLa.

To confirm that K14 promoter was able to drive the expression of our model antigen, the CMV promoter was replaced in all three antigen plasmids with the K14 one. The culture supernatants of transfected cells were then analyzed by Western blotting. As expected, K14 was not able to drive the production and secretion of the fusion protein in HEK293T (fig. 12c), but only in HeLa (fig. 12d) cells. Interestingly, as observed in FACS analysis, where the EGFP signal from cells transfected with CMV-containing plasmid was higher than the one obtained with K14-containing plasmid, the amount of protein produced under the transcriptional control of CMV promoter was about four times higher than the one obtained with K14, as comparable signals were obtained when using the undiluted supernatant from HeLa cells transfected with the K14-containing plasmid versus the 4-fold diluted one from the cells transfected with the CMV-containing plasmid (fig. 12d).

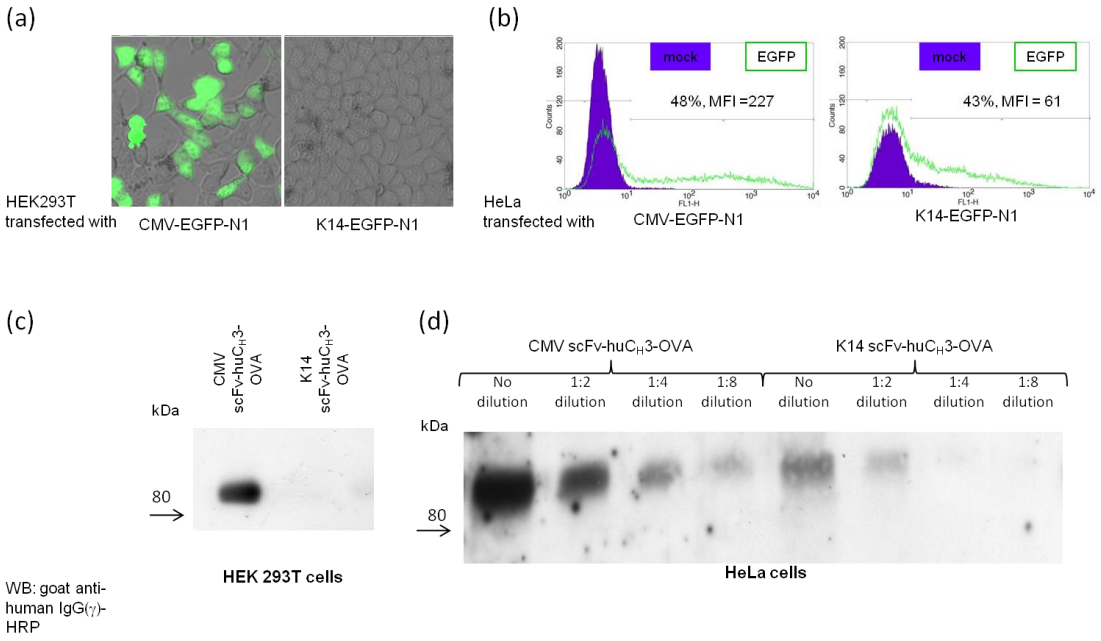

Fig. 12: Production of model protein under transcriptional control of different promoter.
(a) Immuno-fluorescence of HEK293T and (b) FACS analysis of HeLa cells transiently transfected with plasmids encoding EGFP under the transcriptional control of either CMV or K14 promoter. (c-d) Western blotting analysis of supernatants of transiently transfected HEK293T (c) or HeLa (d) cells with plasmids encoding scFv-huC$_H$3-OVA protein. The presence of the protein was assessed with an HRP-conjugated goat anti-human IgG (γ-chain specific) antibody.

Once demonstrated that plasmids with the K14 promoter were well secreted, we tested them in *in vivo* experiments. As previously done with the CMV promoter, we evaluated the T-cell activation by CFSE dilution assay of adoptively transferred OT-I cells in mice immunised by gene gun. As expected, a strong OT-I proliferation was observed upon immunisation with the plasmids with the DC-internalising receptor moieties (scFv^{DEC205} and scFv^{CD36}). Similar to the experiment with CMV-containing plasmid, the OVA targeting via DEC205 receptor was more efficient than via CD36, as demonstrated by the lower numbers of fluorescent cells in this latter case. In contrast to what we observed with the CMV promoter, in the absence of specific DC-internalising receptor targeting scFv (scFv^{Irr}), OVA was not cross-presented by DCs and OT-I cells did not proliferate (fig. 13).

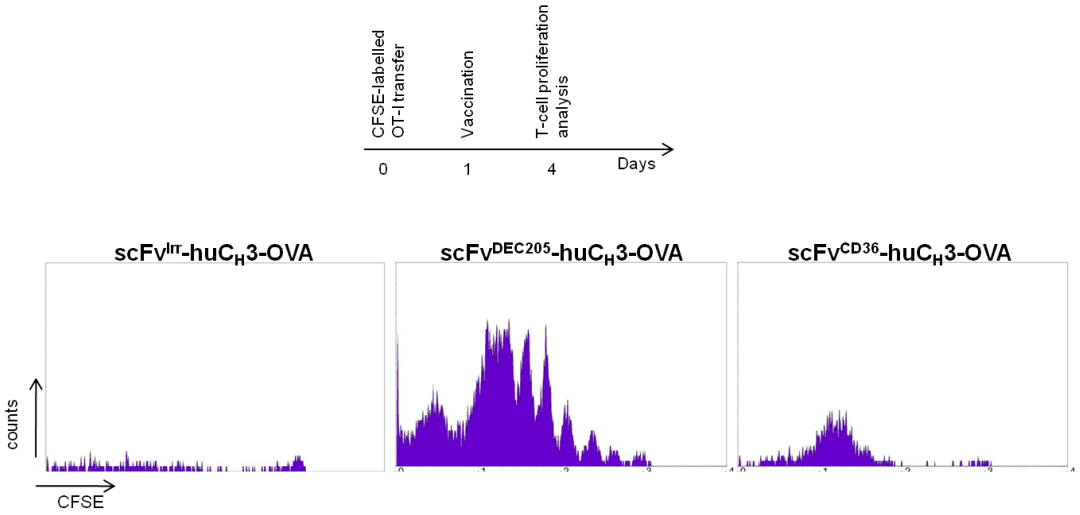

Fig. 13: K14-restricted expression of OVA-containing molecules induces T-cell activation and proliferation by cross-presentation pathway only in the presence of specific DC-targeting scFvs.

10e6 OT-I cells were labelled with CFSE and adoptively transferred into syngenic recipients (C57Bl/6j mice). The day after mice were immunised with the different plasmids containing the keratinocyte-restricted K14 promoter and three days later cells were collected from inguinal lymph nodes and evaluated by FACS. The result with one representative mouse is shown for each construct.

These data confirmed the hypothesis that the proliferation of T-cells observed in animals immunised with the scFv^{Irr} fusion protein under the transcriptional control of the CMV promoter was due to the direct presentation of the antigen driven by direct transfections of resident DCs.

More importantly, these data allowed us to conclude that our design of antigen, comprising a DC-targeting moiety, is also efficient in inducing cross-presentation by DCs, following expression of the antigen by keratinocytes upon gene gun DNA immunisation.

Antibody response

The above described data demonstrated that by gene gun DNA immunisation there was a strong and long-lasting activation of CD8$^+$ T-cells. We next determined the induction of the antibody response. Anti-OVA antibodies were detected by ELISA.

As shown in figure 14a, similar antibody levels were generated irrespective of the specificity of the scFv moiety.

The presence of antibodies specific for the xenogenic human C_H3 domain was also demonstrated (Fig 14b).

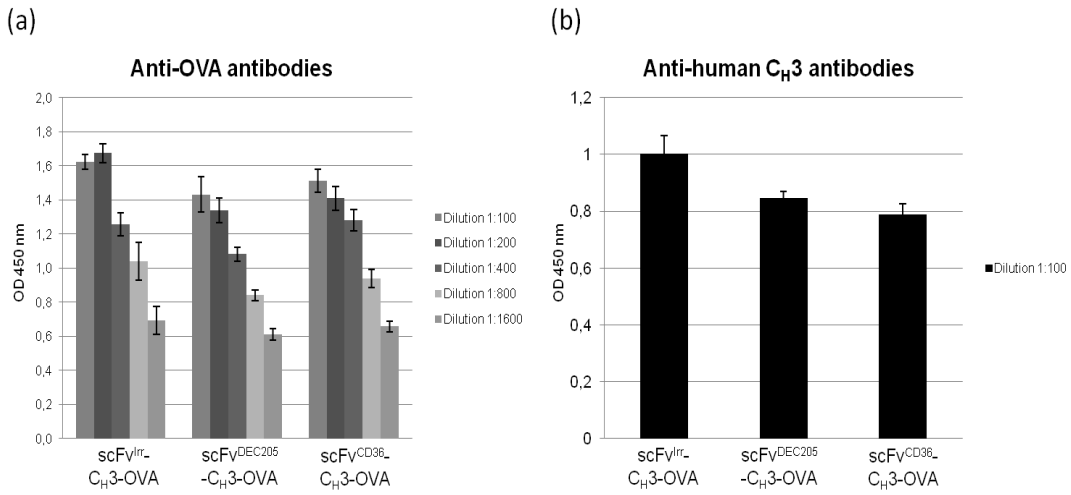

Fig 14: Antibody response against OVA and human C_H3 by gene gun DNA immunisation. C57Bl/6j mice were immunised three times by gene gun with $scFv^{DEC}$-, $scFv^{CD36}$- or $scFv^{Irr}$-C_H3-OVA-encoding plasmids. Ten days after last shot sera were collected and analyzed by ELISA. Plates were coated with purified OVA (a) or purified human IgG (b). Reactivity was determined using an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody. Each bar represents the mean value obtained with sera from two different mice, each analyzed in triplicate. Standard errors are indicated.

As previously done for T-cell activation, impact on the antibody response of the substitution of an ubiquitously working promoter, such as CMV, with the tissue specific K14 promoter was investigated. We evaluated the presence of antibodies specific for the recombinant protein, using ELISA assays as described above. A strong antibody response against both OVA (fig. 15a) and the xenogenic domain human C_H3 (fig. 15b) was confirmed without significant differences due to antigen internalisation mediated by the scFvs. This was expected because B-cell stimulation requires availability of exogenous antigen to be recognized by the B-cell receptor (BCR) as well as priming by $CD4^+$ T-cells. These cells are activated upon engagement of their TCRs with MHC-II-antigen complexes, a mechanism that always takes place as a consequence of uptake of exogenous antigen by DCs via the endosomal-lysosomal pathway.

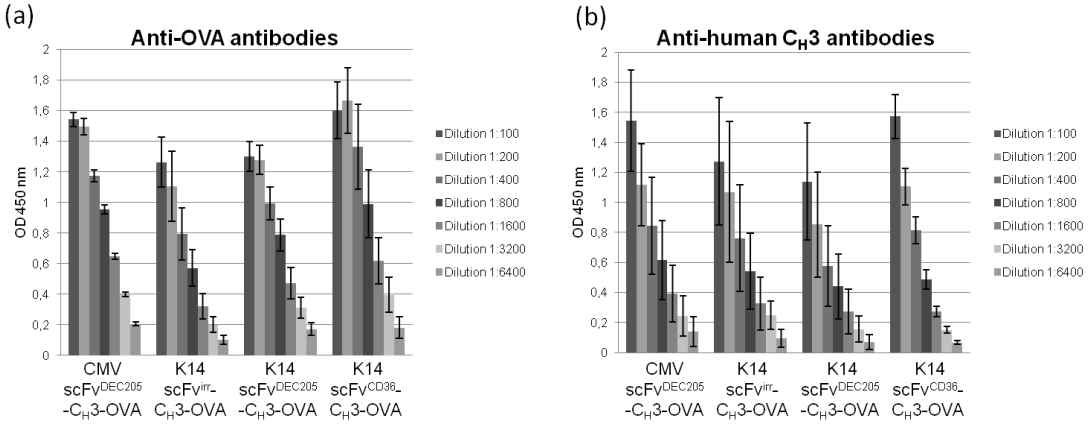

Fig. 15: Antibody response against OVA and the human C_H3 domain by gene gun DNA immunisation with plasmids containing the K14 promoter.

C57Bl/6j mice were immunised three times by gene gun with, $scFv^{DEC205}$-, $scFv^{CD36}$- or $scFv^{Irr}$-C_H3-OVA-encoding plasmids. Ten days after last shot sera were collected and analyzed by ELISA. Plates were coated with purified OVA or human IgG. Reactivity was determined using an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody. As reference, signals obtained with sera from animals immunised with $scFv^{DEC}$-C_H3-OVA under the transcriptional control of CMV promoter are included. Each bar represents the mean value obtained with sera derived from three different mice. Standard errors are indicated.

Altogether these data confirmed that gene gun DNA immunisation was able to activate both T- and B-cell immune compartments, in a similar way as observed with protein immunisation.

Gene gun DNA immunisation for cancer immunotherapy

Once we demonstrated that gene gun DNA immunisation was able to induce an immune response against a model antigen, we moved to a clinically relevant antigen, the HER2 receptor.

We investigated the effect of gene gun DNA immunisation on both humoral and cellular responses against its extracellular domain ($Her2^{ECD}$), either in a non-self or in a tolerogenic environment. For this purpose, we generated a set of plasmids, all encoding the $Her2^{ECD}$ of the rat isoform (Her2/neu). We used Her2/neu because it is expressed in the mammary glands of a transgenic mouse model. With this transgenic model, differences between the immune responses (humoral and T-cell-mediated) in a non-self with respect to a tolerogenic environment could be evaluated, also in terms of ability to induce tumour protection.

In analogy to what we did with the OVA constructs, a self-dimerizing $Her2^{ECD}$ molecule was obtained by fusing it to either a xenogenic (human) or syngenic (murine) C_H3 domain (fig. 16a). This format also should have the advantage of increased secretion of the molecule of interest and reduced clearance from the blood circulation, in analogy of what we have seen with other molecules in the lab.
Expression and secretion of these two versions of $Her2^{ECD}$ were analyzed by Western blotting (fig. 16b): HEK293T cells were transiently transfected with $Her2^{ECD}$-muC_H3 and $Her2^{ECD}$-huC_H3 constructs and supernatants analyzed by non-reducing SDS-PAGE followed by Western blotting with a specific mAb (7.16.4) that binds $Her2^{ECD}$. As shown in fig. 16b, both molecules were well expressed and secreted in a dimeric form, as confirmed by the appearance of a band of an apparent molecular weight around 150 kDa. The dimerization was confirmed by exposing the samples at high temperature to perturb the strong non-covalent association between two C_H3 domains, which resulted in the appearance of a band of about 90 kDa, the expected molecular weight of the monomer.

Moreover, this procedure decreased the reactivity of the mAb 7.16.4 with $Her2^{ECD}$, indicating that full denaturation impaired the recognition of the epitope by this antibody.

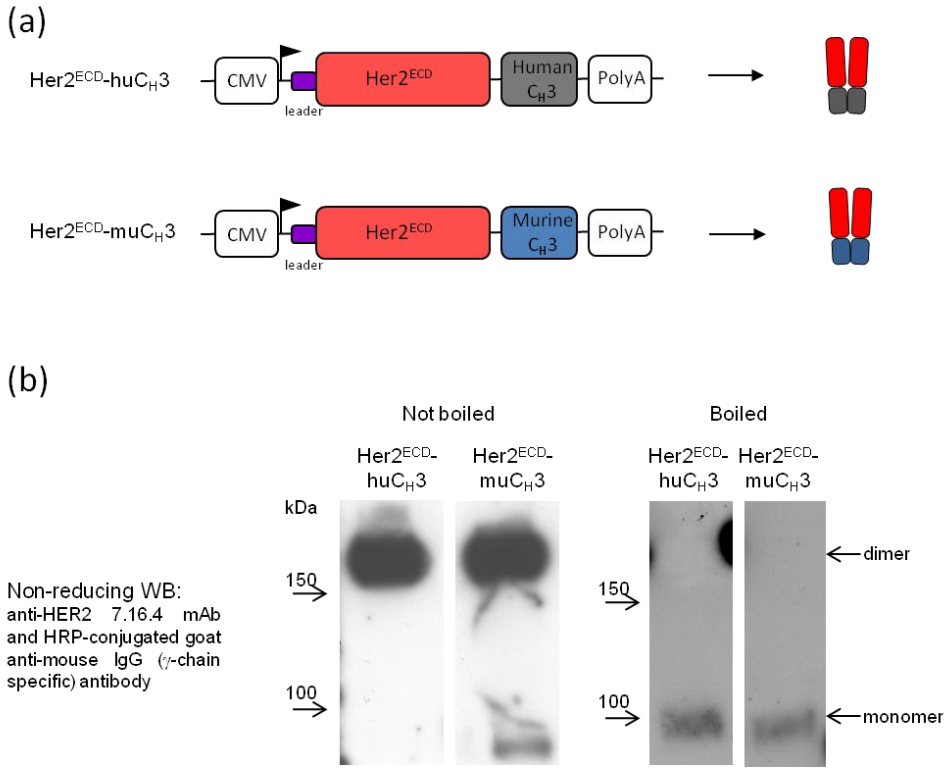

Fig. 16: Dimeric version of Her2^{ECD}.

(a) Schematic representation of plasmids encoding soluble dimeric version of Her2^{ECD}. All the plasmids have a secretion signal before the sequence encoding the Her2^{ECD}. Downstream to this antigen, two different dimerization domains are present. (b) Analysis of culture supernatants of HEK293T cells transiently transfected with the indicated plasmids. Secreted Her2^{ECD}-molecules were visualized by non-reducing SDS-PAGE followed by Western blotting analysis with anti-HER2 mAb (7.16.4) and an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody.

In order to have tools for the analysis of the antibody response in FACS analysis, the $Her2^{ECD}$ was fused with two different membrane anchor sequences to generate $Her2^{ECD}$-displaying cell lines (fig. 17a). In the first one, the C-term region of a human IgE heavy chain was used. This region has been previously validated to display other molecules on the cell membrane and consists of the dimerizing C_H4 domain, followed by the external membrane proximal domain (EMPD) and the transmembrane/cytosolic domain (TM/cyto), obtaining a dimeric cell surface $Her2^{ECD}$ ($Her2^{ECD}$-εSIP). The second sequence, also previously validated, was the glycosylphosphatidylinositol (GPI)-anchor motif ($Her2^{ECD}$-GPI). In both cases, there is also a linear tag (SV5) that allows the detection of the molecule with a mAb.

The two membrane-anchored fusion proteins-encoding plasmids were stably transfected into the non-immunoglobulin-secreting myeloma cell line Sp2/0, which grows in suspension and therefore is very convenient for FACS analysis. These cells were analyzed by labelling with the mAb 7.16.4 and the expression of the $Her2^{ECD}$-membrane-tethered-molecules assessed by FACS analysis. As shown in fig. 17b, both membrane fusion proteins were well expressed.

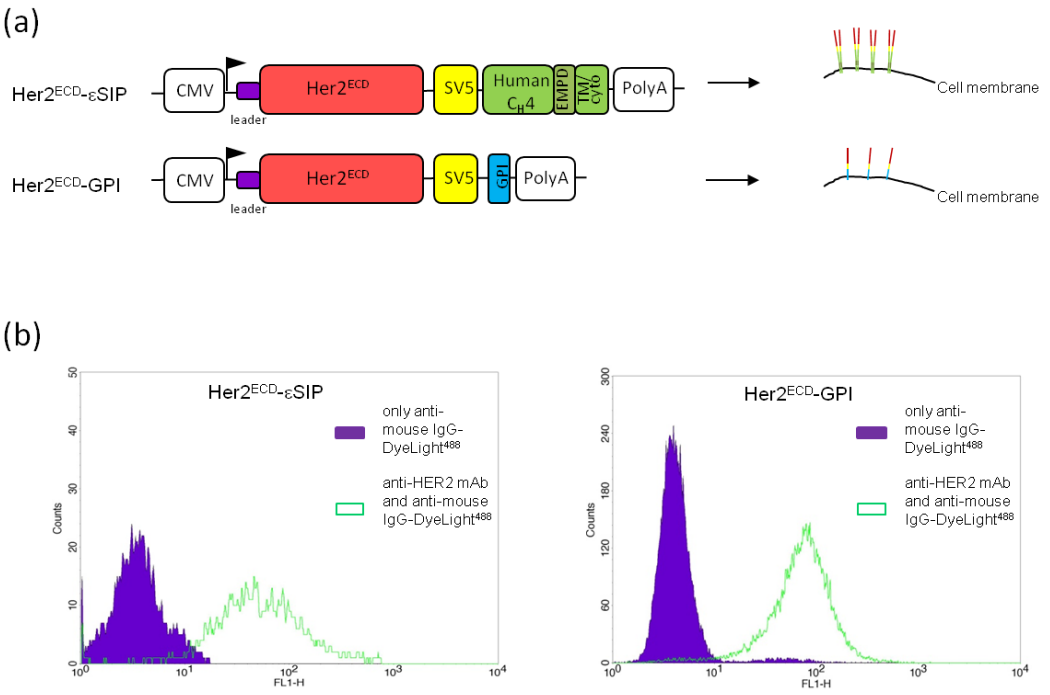

Fig. 17: Expression of Her2^{ECD}-membrane-anchored molecules.

(a) Schematic representation of plasmids encoding Her2^{ECD}-membrane-anchored molecules. Downstream Her2^{ECD}, which is followed by the SV5 tag, two different membrane anchor motifs are present: the C_H4 dimerizing region, the EMPD and the TM/cyto domains of the heavy chain of human IgE in Her2^{ECD}-εSIP, or the GPI motif in Her2^{ECD}-GPI construct. (b) FACS analysis of Sp2/0 cells transfected with plasmids encoding the membrane-bound versions of recombinant Her2^{ECD}. Expression was revealed with 7.16.4 mAb followed by a DyeLight^{488}-conjugated goat anti-mouse IgG (γ-chain specific) antibody.

Her2^{ECD} is able to induce an antibody response in wild type animals

After demonstrating that all constructs were well expressed and secreted, we performed a preliminary vaccination experiment to determine the ability of Her2^{ECD} in inducing a humoral response and the possible effect of the addition of the xenogenic human C_H3 domain on the immunogenicity of the molecule. We vaccinated wild type mice with three shots of the plasmids encoding Her2^{ECD} fused with either the xenogenic or the syngenic dimerizing unit. After ten days, sera were collected and the presence of anti-HER2 antibodies analyzed by FACS with the cells expressing Her2^{ECD}-εSIP. As shown in fig. 18, gene gun DNA immunisation with both plasmids was effective in inducing an antibody response. Moreover, the presence of the xenogenic carrier seemed to have no effect on the antibody titers, as higher dilutions of sera gave similar signals (data not shown). This indicates that the Her2^{ECD} T-cell epitopes are efficiently recognized by the immune system and there is no need for other adjuvant moiety. For this reason, we decided to perform all the next experiments with the constructs containing the syngenic C_H3 domain, in order to simplify the read out and to focus the immune response only in the therapeutically relevant domain (Her2^{ECD}).

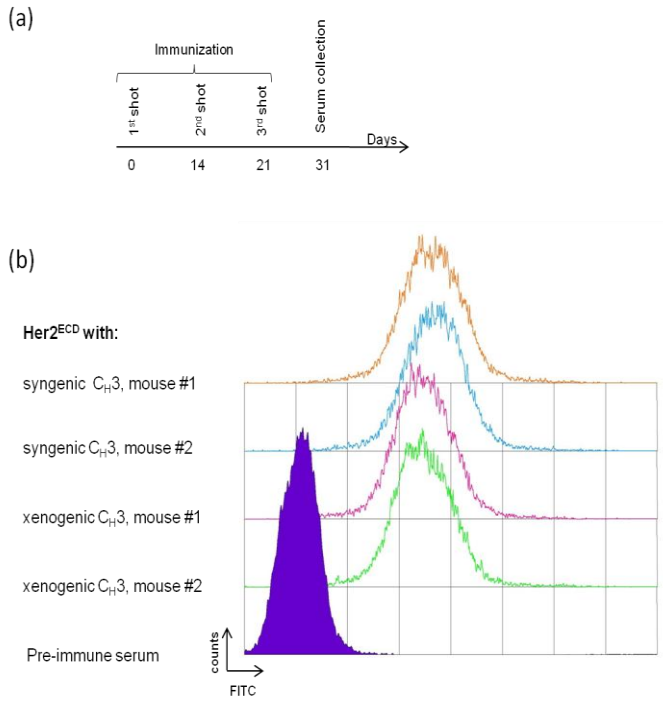

Fig. 18: Gene gun DNA immunisation with Her2^{ECD}-containing plasmids is able to induce strong antibody responses.

(a) C57Bl/6j mice were immunised three times with plasmids encoding Her2 ^{ECD}-huC$_H$3 or Her2 ^{ECD}-muC$_H$3. Ten days after the last shot, sera were collected and analyzed by FACS. (b) FACS analysis of binding activity of antibodies in sera from vaccinated mice to Her2^{ECD}-εSIP-displaying Sp2/0 cells. Binding was revealed with the anti-HER2 7.16.4 mAb followed by a DyeLight^{488}-conjugated goat anti-mouse IgG (γ-chain specific) antibody.

Antigen targeting to DC enhances T-cell response

The previous experiments demonstrated that the extracellular domain of the Her2/neu is by itself capable to induce an antibody response.

To evaluate activation of T-cells by the $Her2^{ECD}$ constructs, the OVA sequence was fused to the $Her2^{ECD}$, downstream of the C_H3 domain. Also, the addition of the two DC-targeting moieties ($scFv^{CD36}$ or $scFv^{DEC205}$) at the N-term of the fusion protein allows to reorient the uptake of the antigen to DC internalising receptors and to determine the contribution of cross-presentation for T-cell activation.

The expression of these molecules from CMV promoter-plasmids was assessed in the culture supernatants of transiently transfected cells by non-reducing Western blotting with an anti-HER2 mouse serum and an anti-OVA mAb. Serum was used instead of the anti-$Her2^{ECD}$ 7.16.4 mAb because full denaturation of the molecule impairs the binding of this mAb, as showed before (fig. 16) and confirmed extensively later. In contrast, the anti-OVA mAb was able to recognize the fully denatured antigen.
As shown in fig. 19, all the new engineered molecules were well expressed and secreted.

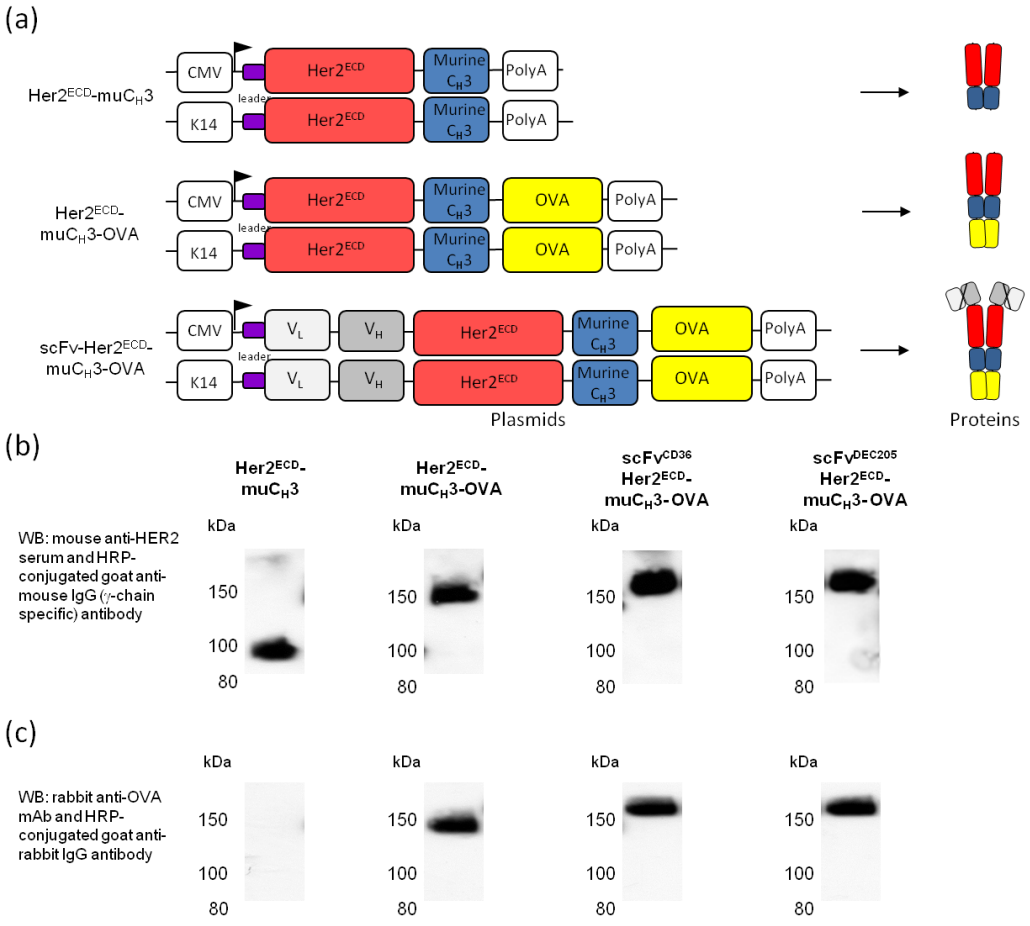

Fig. 19: Recombinant proteins containing Her2^{ECD} and OVA

(a) Schematic representation of plasmids and recombinant molecules containing Her2^{ECD}.

(b) Western blotting analysis with anti-HER2 mouse serum of culture supernatants from transiently transfected HEK293T cells. Binding was revealed with an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody.

(c) Western blotting analysis with anti-OVA mAb of culture supernatants from transiently transfected HEK293T cells. Binding was revealed with an HRP-conjugated goat anti-rabbit IgG antibody.

Since we wanted to evaluate whether these constructs were still able to activate T-cells via cross-presentation upon antigen targeting to DCs, the same constructs with K14 promoter were obtained and used to evaluate OT-I activation by the CFSE dilution assay.

As shown in fig. 20, both DEC205 and CD36 targeting resulted in efficient T-cells activation. Therefore, in these new constructs the presence of the $Her2^{ECD}$ did not interfere with OVA presentation, allowing efficient activation of specific $CD8^{+}$ T-cells.

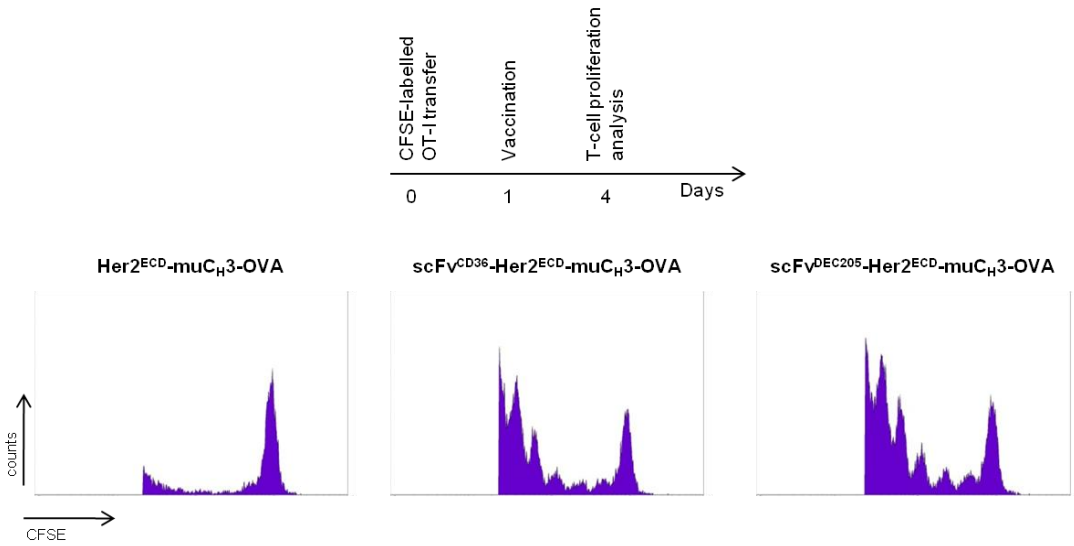

Fig. 20: Specific T-cell proliferation by activation of cross-presentation pathway by DC targeting scFvs in fusion proteins containing $Her2^{ECD}$.

10e6 OT-I cells were labelled with CFSE and adoptively transferred into syngenic recipients (C57Bl6/j mice). The day after mice were immunised with the different plasmids containing the keratinocyte-restricted K14 promoter and three days later cells were collected from inguinal lymph nodes and evaluated by FACS. The result with one representative mouse is shown for each construct.

Antibody response against Her2^{ECD} in a non-self environment

Development of the "conformational ELISA"

Once analyzed T-cell compartment, the humoral response against the two antigens was evaluated.

Standard ELISA assays, where antigen is directly coated to plates, may induce the appearance of non-native epitopes, which are likely rarely presented upon gene gun DNA immunisation: indeed, previous work has demonstrated that gene gun DNA immunisation induce the appearance of antibodies mainly against conformational epitopes (Benvenuti and Burrone 2001). Although FACS analysis with cells displaying antigen should preserve conformational epitopes, the assay might be not sensitive enough to discriminate and quantify differences among the different treatments.

For these reasons, an ELISA strategy was developed, where the folding of the antigen were not preserved as produced by mammalian cells.

The assay is based on the *in vivo* biotinylation of the antigen produced by mammalian cells. The antigen Her2^{ECD} was genetically modified by the addition of a 15-aa long peptide, called biotin acceptor peptide (BAP) and co-expressed with the E. *coli* derived biotin ligase BirA. This enzyme catalyzes the covalent attachment of a single biotin to a lysine residue within the BAP sequence. We previously demonstrated that with this system different mono-biotinylated molecules can be produced *in vivo* in mammalian cells, directly recovered from the culture supernatant and used for many different biological assays (Predonzani, Arnoldi *et al.* 2008).

A bigenic DNA construct coding the Her2^{ECD} with the BAP sequence fused to its C-term and the engineered secretory version of BirA (Her2^{ECD}-BAP) was obtained and used to stably transfect HEK293 cells (fig. 21a).

A stable clone secreting fully biotinylated Her2^{ECD}, as demonstrated by the Western blotting retardation assay analysis (fig. 21b), was obtained. This clone was used for the production of large quantities of mono-biotinylated Her2^{ECD} to be used as coating antigen for ELISA.

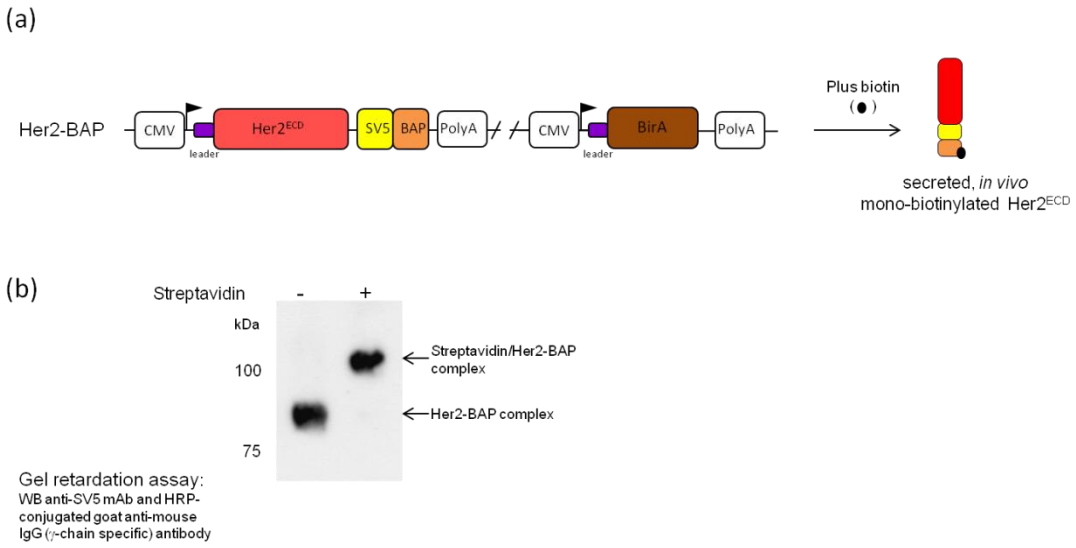

Fig. 21: Expression of mono-biotinylated Her2^{ECD}

(a) Schematic representation of the Her2^{ECD}-BAP plasmid and the encoded protein; (b) HEK293 cells were stably transfected with the Her2^{ECD}-BAP plasmid. To discriminate the amount of biotinylated Her2^{ECD} with respect to the total amount of the protein, a gel retardation assay was performed: culture supernatants were incubated with streptavidin and thus resolved by SDS-PAGE and Western blotting analysis with anti-SV5 mAb and HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody as elsewhere described (Predonzani, Arnoldi *et al.* 2008).

Mono-biotinylated Her2^{ECD} was added to avidin-precoated ELISA plates to capture antigen in native conformation, henceforth called "conformational ELISA" (fig. 22a).

When compared with the classical coating directly with the antigen, capturing the same amount of mono-biotinylated Her2^{ECD} via avidin resulted in up to 30% increase in antigen detection of sera with anti-Her2^{ECD} activity (fig. 22b), while the increase of reactivity of the anti-HER2 mAb 7.16.4 was only 10% (fig. 22c). These results indicate that this strategy increases the availability of conformational epitopes from the coating antigen, thus enhancing the sensitivity of the assay when testing antibodies specific for the native molecule.

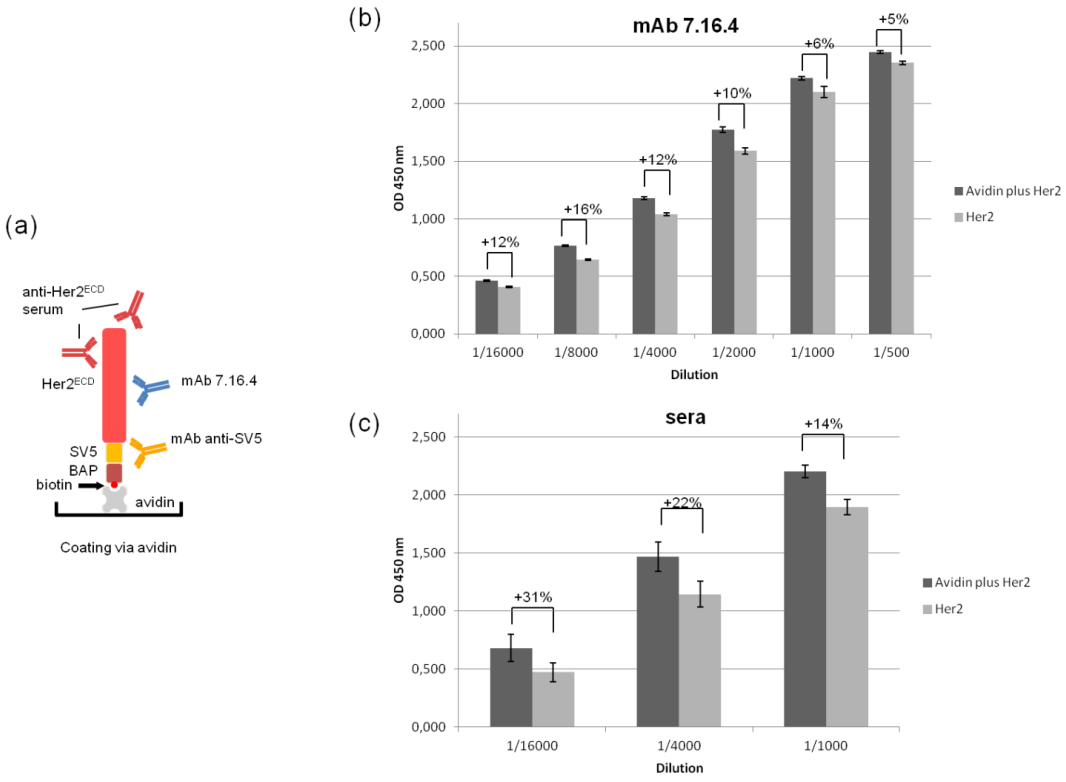

Fig. 22: Optimization of the coating conditions for the evaluation by ELISA of the antibody response against Her2^{ECD}.

(a) ELISA plates were coated with the same amount of biotinylated Her2^{ECD} directly or by preadsorbed avidin. (b) mAb 7.16.4 (specific for the Her2^{ECD}) or (c) sera obtained from animals immunised by gene gun with the Her2^{ECD}-muC$_H$3 plasmid were added. Reactivity was determined using an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody. Each bar represents the mean value obtained from samples analyzed in triplicate. Standard errors are indicated.

With this ELISA, the antibody responses in wild type animals following gene gun DNA immunisation were analyzed. Strong antibody responses against Her2^{ECD} (fig. 23a) and OVA (fig. 23b) were generated, irrespective of the presence of DC-targeting units. Interestingly, for both antigens it was observed a trend towards lower titers when the scFv^{DEC205} was used, not attributable to the amount of recombinant protein produced (see fig. 19).

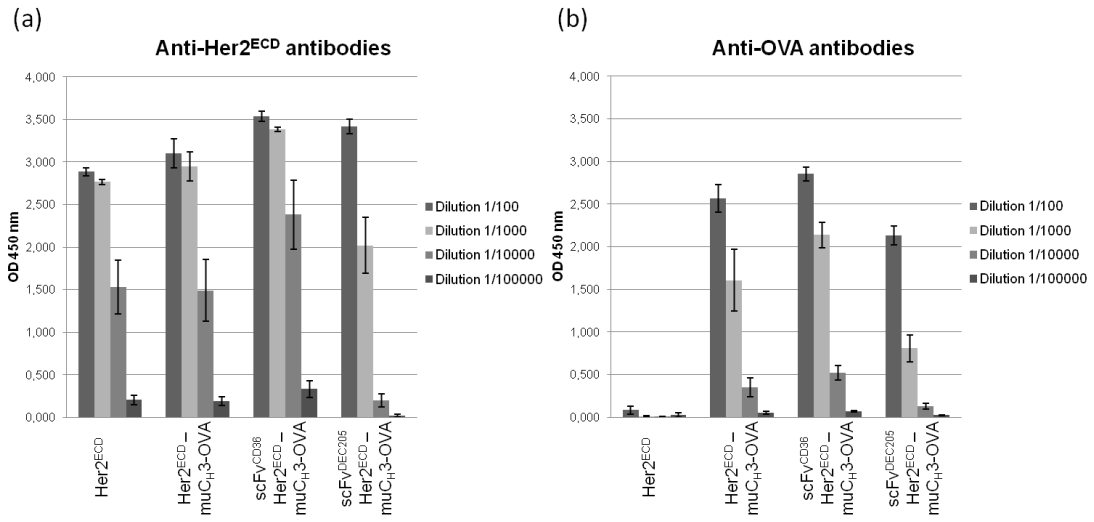

Fig. 23: Antibody response against Her2^{ECD} and OVA by gene gun DNA immunisation with plasmids encoding the fusion proteins.

C57Bl/6j mice were immunised three times by gene gun DNA immunisation with plasmids encoding the indicated constructs. Ten days after last shot, sera were collected and analyzed by ELISA. Plates with were coated with avidin and biotinylated Her2^{ECD} or with purified OVA. Reactivity was determined using an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody. Each bar represents the mean value obtained with sera from five different mice, each analyzed in triplicate. The standard errors are indicated.

Characterization of the antibody response induced by gene gun DNA immunisation

As mentioned before, gene gun DNA immunisation was able to induce a strong antibody response. However, the quality and specificity of this antibody repertoire was not determined. Our group has previously demonstrated that gene gun DNA immunisation was able to induce antibody responses with distinct characteristics as compared to protein immunisation. In particular, in an anti-idiotype model, upon gene gun DNA immunisation most of the antibodies were directed against conformational epitopes formed by assembling the two idiotype domains (V_L and V_H) (Benvenuti and Burrone 2001; Lopez-Requena, Mateo de Acosta *et al.* 2007). Similarly, other unpublished evidences of immunisation against viral proteins indicated that immunisation with protein was able to elicit a strong antibody response yet not associated to epitopes present in the native protein, while the response obtained with gene gun DNA immunisation was exquisitely directed against native conformational epitopes.

This was also observed on a pivotal experiment with $Her2^{ECD}$ as antigen. Gene gun DNA immunisation induced mainly anti-$Her2^{ECD}$ antibodies that recognized conformational epitopes (fig. 24). The same amount of mono-biotinylated $Her2^{ECD}$ was progressively heat-denatured and used in the "conformational ELISA". A pool of sera from vaccinated mice was then tested and compared to the 7.16.4 mAb, which requires a conformational epitope. Signals from both sera and mAb progressively decreased in contrast to the anti -SV5 mAb, which recognizes a linear epitope. The progressive increase of this latter signal indicated a better accessibility of the SV5 tag, due to unfolding of the protein caused by heat-denaturation.

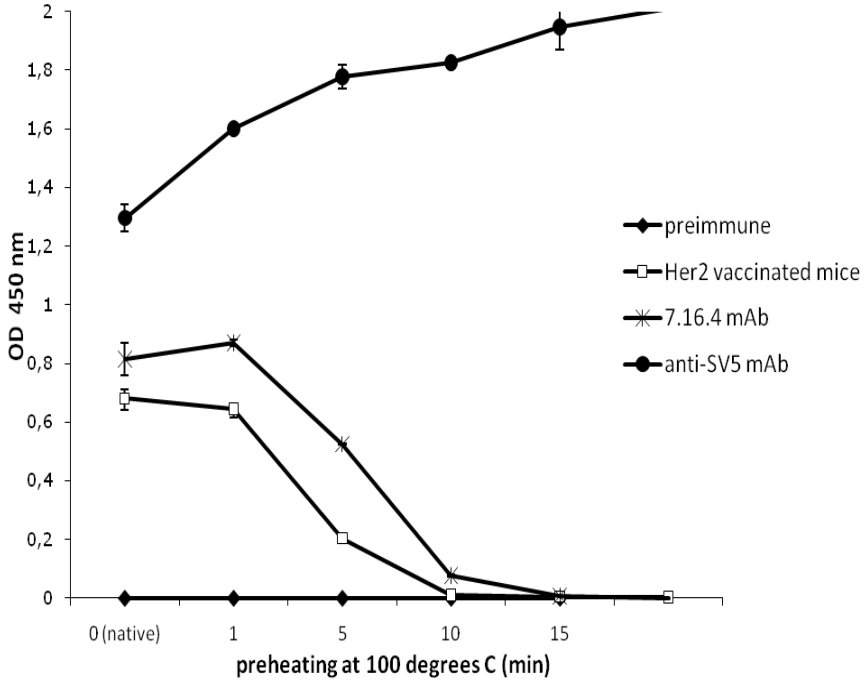

Fig. 24: Gene gun DNA immunisation induces anti-conformational epitope antibodies.
C57Bl/6j mice were immunised three times by gene gun DNA immunisation with Her2-muC_H3 plasmid. Ten days after last shot, sera were collected and analyzed by ELISA. Plates with preadsorbed avidin were coated with progressively heat-denatured biotinylated $Her2^{ECD}$ and sera or indicated mAbs were added. Reactivity was determined using an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody. Each bar represents the mean value obtained with sera from three different mice, each analyzed in triplicate. The standard errors are indicated.

Purification of recombinant Her2^{ECD}-muC$_H$3-H6 protein

The result shown in fig. 24 clearly indicated that serum antibodies are mainly directed against conformational epitopes, with a behaviour similar to the mAb. A question still open was whether this anti-conformational epitope antibody repertoire was induced also by protein immunisation.

To investigate this aspect with the HER2 model, the dimeric Her2^{ECD}-muC$_H$3 protein was purified from culture supernatants of mammalian cells to be used as immunogen. A six histidine (H6) tag was added downstream the Her2^{ECD}-muC$_H$3-coding sequence. HEK293 cells were stably transfected with this plasmid and culture supernatants analyzed by ELISA for the presence of the secreted protein. A clone secreting up to 5 μg of protein per 10e6 cells was obtained and expanded (data not shown). The Her2^{ECD}-muC$_H$3-H6 protein was purified in nickel columns and the purification efficiency checked by electrophoresis. As shown in fig. 25a, a good recovery of the protein was obtained in the first three elution fractions. We did not detect protein either in the flow through or in the washing fractions. By Coomassie staining we estimated an amount of about 2 mg/ml of recovered protein, confirmed by Bradford quantification. Purity of the protein was confirmed by silver staining and high pressure liquid chromatography (HPLC) analysis. As depicted in fig. 25b, silver staining confirmed that there was neglectable amounts of contaminants. By HPLC (fig. 25c), a main peak representing more than 70% of all species was obtained. A peak corresponding to about the 20% of all the purified molecules, located just after the major peak, was also observed. Due to the chemical characteristics of the Her2^{ECD}, we hypothesised that this last peak did not represent contaminants but aggregates or differently glycosylated molecules.

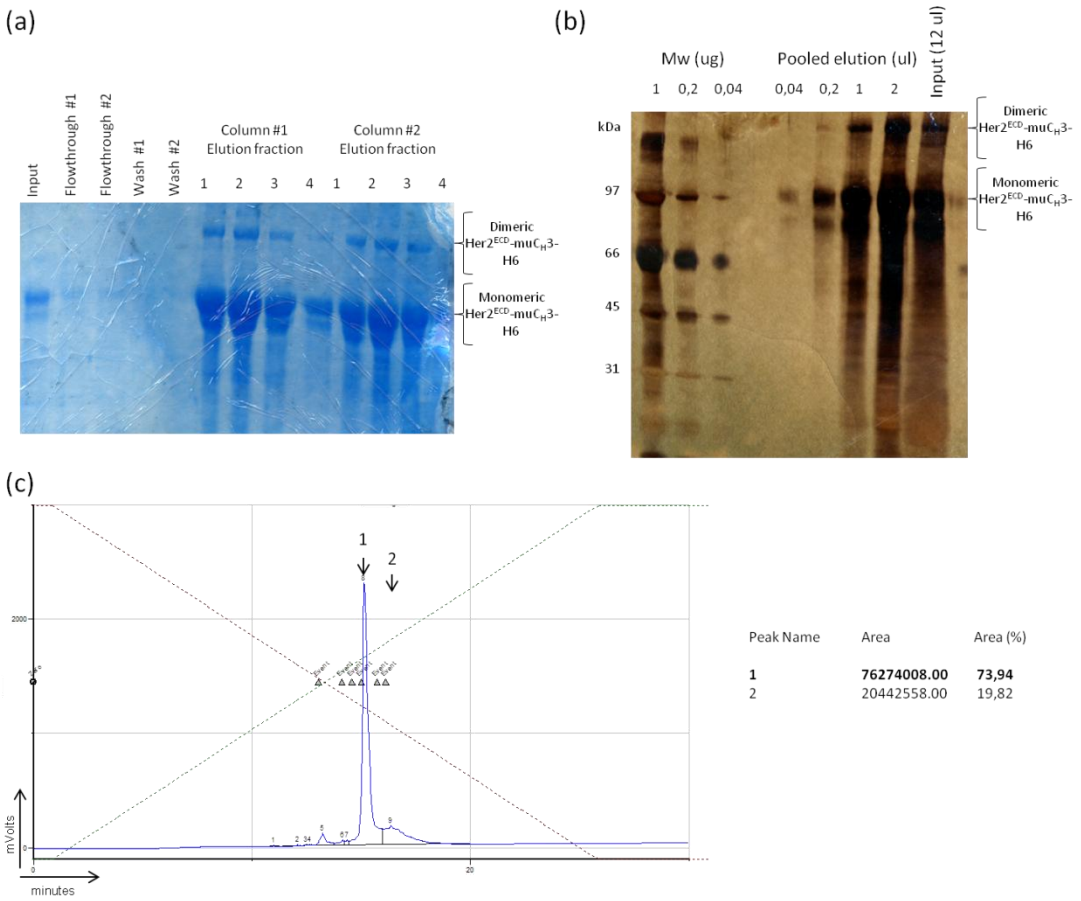

Fig. 25: Purification and purity assessment of the recombinant Her2^{ECD}-muC$_H$3-H6 protein.
Her2^{ECD}-muC$_H$3-H6 protein was purified from culture supernatant of stably transfected HEK293 cells by Ni-NTA agarose column and the recovery and purity of the protein was verified. (a) SDS-PAGE and Coomassie staining of purification process fractions. (b) SDS-PAGE and silver staining of pooled elution fraction. (c) HPLC profile of pooled elution fraction.

Quality of the antibody response induced by gene gun DNA immunisation versus protein immunisation

The highly pure and native protein obtained was used to immunise animals (n = 5 for each group) by subcutaneous injection. The purified native protein was given in the presence or absence of Freund's adjuvant to animals. Two other groups were immunised with the heat-denatured protein. In all cases, mice were immunised subcutaneously three times with two week intervals. Gene gun DNA vaccination with the $Her2^{ECD}$-muC_H3-H6 plasmid was included as comparison group.

Ten days after the third immunisation, we collected sera and analyzed antibody titers by the "conformational ELISA".

As shown in fig. 26, high levels of specific antibodies were obtained in all groups but the group that received the denatured protein in saline solution, probably due to the absence of the dominant conformational epitopes. By gene gun DNA immunisation, slightly higher titers were obtained when compared with immunisation of the native protein in saline solution, while the highest titers corresponded to the group immunised with the native protein plus adjuvant.

To confirm that the denatured protein was able to induce antibodies against non-conformational epitopes, we analyzed the sera using heat-denatured mono-biotinylated $Her2^{ECD}$ as coating antigen. In this case, we confirmed the presence of such antibodies only in the sera of animals immunised with the denatured protein, irrespective of the presence of adjuvant (fig. 26b).

We further confirmed these data by FACS analysis on cells that display on their membrane the $Her2^{ECD}$. As shown in fig. 26c, sera from animals immunised by gene gun showed higher signals with respect to those from animals immunised with protein without adjuvant. Also in this case, we did not appreciate any binding to the cells of sera from animals vaccinated with the heat-denatured protein. This corroborated that in this antigen the vast majority of the epitopes are conformational and responsible for the onset of a humoral response able to recognize its native structure. These results were also confirmed in a cell line derived from spontaneous breast cancer, which express the full length receptor (data not shown).

To assess the quality of the antibody response generated by protein versus gene gun DNA immunisation, we repeated the similar "conformational ELISA" as in fig. 22),

but with partially denatured $Her2^{ECD}$ (fig. 26d). With this approach, we were able to detect differences between native protein without adjuvant immunisation with respect to the gene gun DNA immunisation. In fig. 26d, the data reported corresponded to signals obtained with dilutions of sera within the linear range (i.e. 1:10000, 1:2000 and 1:400 for 0', 2.5' and 5' of heat-denaturation, respectively). Upon heat-denaturation, sera from both DNA and native protein-immunised mice showed lower reactivity. However, the group of native protein was less sensitive than the gene gun DNA one, showing higher signals at the 2.5' and 5' heat-denaturation time points. Positive and negative controls were represented by the mAb 7.16.4 and the sera from denatured protein-immunised mice. These differences were clarified by plotting the same signals relative to the ones obtained with DNA-immunised mice, taken as 100% (fig. 26e): signals from the native protein group were about 70% of those from gene gun and increased more than two times after 5' of heat-denaturation of coating antigen. This trend was clearly more evident with sera obtained from animals immunised with the denatured protein. As a control, the mAb 7.16.4, which completely loose reactivity following heat-denaturation, was used.

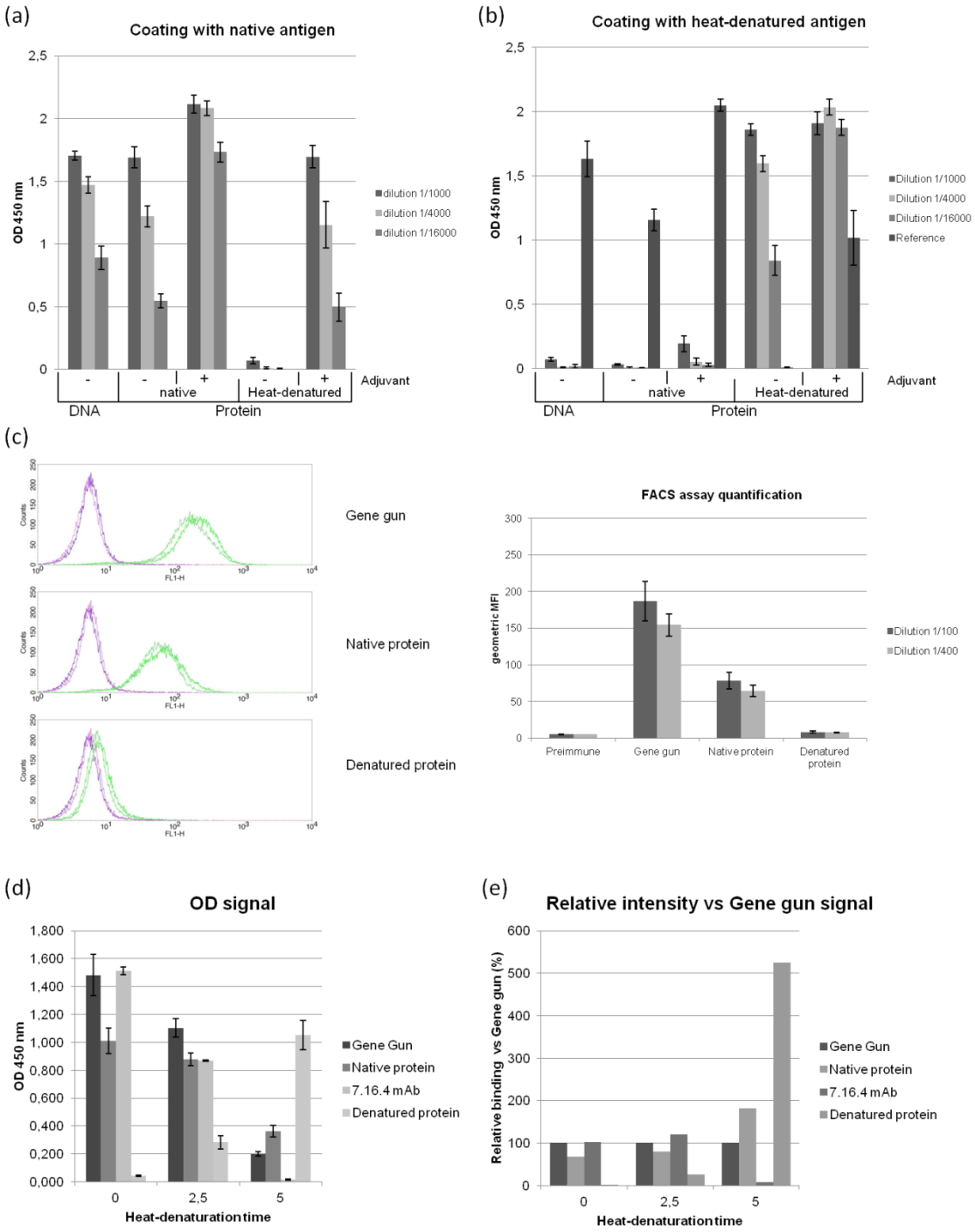

Fig. 26: Different antibody repertoire induced by gene gun DNA or protein immunisation.

C57Bl/6j mice (n = 5 for each group) were immunised three times every two weeks by gene gun DNA immunisation with the $Her2^{ECD}$-muC_H3-H6 plasmid or with subcutaneous native or heat-denatured protein injection, with or without Freund's adjuvant. Ten days after the last vaccination, sera were collected and analyzed. (a-b) Antibody response against $Her2^{ECD}$ measured by ELISA: plates with preadsorbed avidin were coated with native (a) or heat-denatured (b) biotinylated $Her2^{ECD}$. Mouse sera were added at different dilutions and reactivity was determined using an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody. Each bar represents the mean value obtained with sera from five different mice, each analyzed in triplicate. Standard errors are indicated. (continue...)

Fig. 26: (...continue).

(...) (c) FACS analysis of sera on Her2-GPI-displaying Sp2/0 cells. Sera were diluted 100x (darker) or 400x (lighter) and binding was assessed with a DyeLight^{488} conjugated goat anti-mouse IgG (γ-chain specific)-antibody. As negative control, preimmune sera were used (purple). A representative plot obtained from one mouse out of five evaluated is depicted (left) and the average geometric MFI is shown (right). (d-e) Antibody response against partially denatured Her2^{ECD} measured by ELISA. Plates with preadsorbed avidin were coated with biotinylated Her2^{ECD} boiled for different periods of time. (d) Optical density observed with progressively less diluted sera. (e) Relative intensity of the specific signals with respect to the ones obtained with sera from gene gun DNA immunisation group.

Further confirmation was obtained by analysing the reactivity of the different antibodies in Western blotting, with the Her2^{ECD} in reducing and non-reducing conditions.

As shown in fig. 27, sera from animals immunised with the denatured protein, with or without adjuvant, were able to detect the Her2^{ECD} under reducing conditions, while sera from the gene gun DNA or the native protein groups did not. In contrast, both gene gun DNA and native protein immunisation induced antibodies able to recognize the Her2^{ECD} only under non-reducing conditions, where folding of the Her2^{ECD} was less compromised. As observed in ELISA, DNA immunisation induced a higher response than native protein. In all cases, the use of adjuvant had two major effects: 1- it increased the response and 2- induced a partial denaturation of the native protein, reflected in the reactivity observed under reducing conditions (fig. 27).

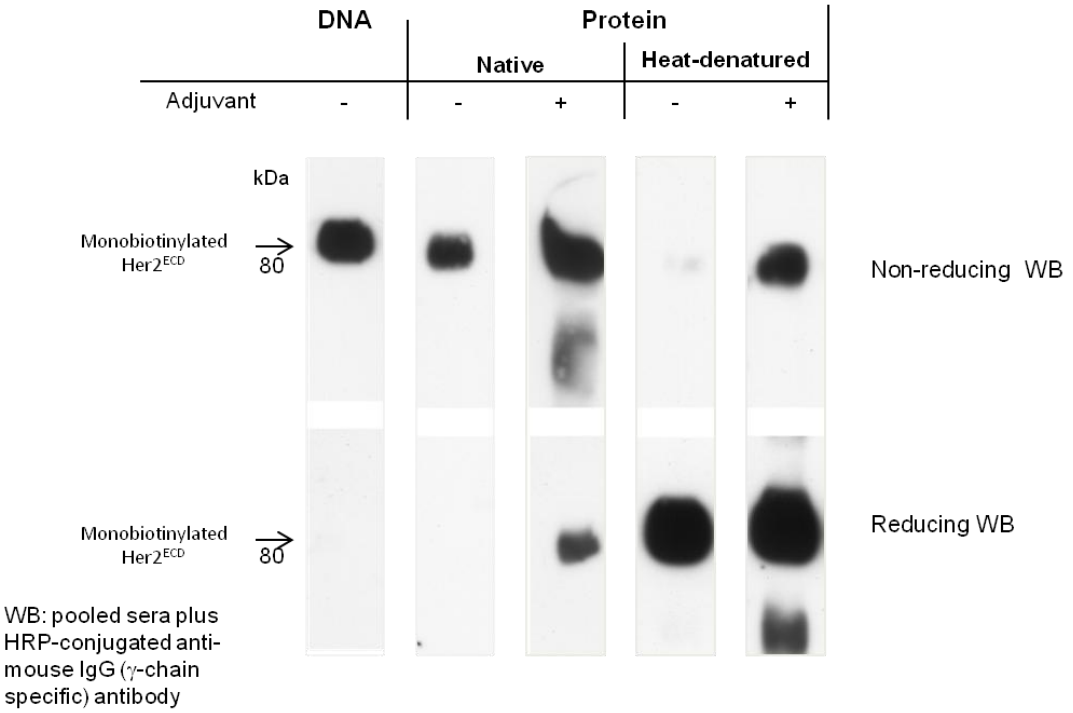

Fig. 27: Protein immunisation induces antibodies which recognizes different form of folding

C57Bl/6j mice were immunised three times every two weeks by gene gun DNA immunisation with the $Her2^{ECD}$-muC_H3-H6 plasmid or with subcutaneous native or heat-denatured protein injection, with or without Freund's adjuvant. Ten days after the last vaccination, sera were collected and analyzed. Culture supernatant containing biotinylated $Her2^{ECD}$ was separated by SDS-PAGE and the presence of the molecule was assessed by Western blotting with pooled sera from animals immunised by gene gun DNA vaccination or protein administration, followed by an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody.

All these data suggest that gene gun DNA immunisation is particularly appropriate to induce high titers of anti-conformational epitopes antibodies, which are more likely to be physiologically relevant.

Cancer immunotherapy in a tolerogenic environment

The mouse model

All the *in vivo* experiments described until now were performed in an environment where the antigen of interest was recognized as non-self. However, under physiological conditions, tumour progression is in part also the consequence of immunological tolerance, a mechanism by which the immune system is impaired to eliminate cancer cells. To investigate cancer immunotherapy in a context where target antigen is expressed as a self antigen, and thus the immune system is tolerized against it, we selected a mouse model where the HER2 is expressed as a self antigen. We chose the mouse model recently developed by Dr. Brad Nelson group (Deeley Research Center, Victoria, Canada (Wall, Milne *et al.* 2007)): in this mouse, the full-length rat isoform Her2/neu was genetically modified by fusing the two OVA-derived class I and II epitopes to the cytosolic C-term of the protein. This transgene was expressed under the control of the mouse mammary tumour virus (MMTV) promoter (fig. 28). Thus, in all the transgenic animals, Her2/neu-OTI/II transgene is expressed only in the mammary gland and, as consequence, tolerance to this antigen is induced. This mouse model develops spontaneous tumours with an incidence that is increased by the presence of a second transgene, represented by a variant of p53, also expressed by a mammary gland-restricted promoter (Whey acidic promoter, WAP).

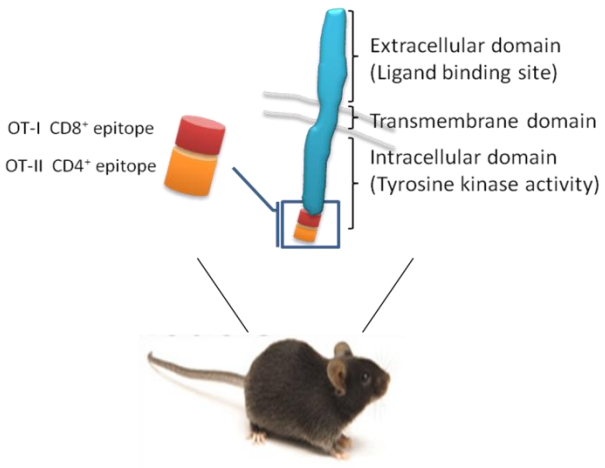

Fig. 28: Schematic representation of the Her2/neu-OTI/II-expressing transgenic mouse model.

The Her2/neu-OTI/II mouse expresses the rat isoform of HER2 with the OVA epitopes fused to its intracellular domain. The transcription of this fusion protein is under the control of the mouse mammary tumour virus (MMTV) promoter (figure modified from (Wall, Milne *et al.* 2007)).

This mouse model allowed us to study prophylactic gene gun DNA vaccination: by immunising mice before they develop tumours, it should be possible to compare different treatments and their impact on tumour appearance and progression.

This initial aim was not pursued due to some problems with this mouse model.
Indeed, by breeding double transgenic mice with syngenic counterparts, differences in the mendelian frequencies were obtained: about 50% of animals were positive for one of the two transgenes, but only 20% were positive for both transgenes.
We obtained a skew toward double transgenic males (60% vs 40%), which impaired the possibility to obtain a large cohort of age and sex-matched mice (only 8% of female double positive mice).
Yet, all the positive female mice were evaluated for the appearance of tumours in absence of any treatment for more than one year (the oldest animals were two years-old) and in none of these animals palpable mammary tumours developed.
This finding was confirmed by the group of Dr. Nelson, corroborating the hypothesis that the phenotype of these animals changed for unknown reasons.
This issue, i.e. the absence of spontaneous tumours in female animals, reoriented our aim: instead of prophylactic immunotherapy on spontaneous tumours, gene gun DNA immunisation was assayed in a tumour challenge setting.
This new approach still allowed us to examine the immune response in a tolerogenic environment. Several tumour cell lines (called NOP, from Neu OT Protein) have been previously derived from spontaneous tumours developed by the original mice (Wall, Milne et al. 2007). These cells can grow only in these transgenic animals, due to the presence of the xenogenic receptor transgene. In wild type mice, NOP cells are completely rejected, probably because a strong T-cell response more likely against the OVA.

Immune response upon gene gun DNA immunisation in a tolerogenic environment

In order to assess the generation of an immune response against the self antigen Her2/neu-OTI/II, we performed gene gun DNA immunisation in the transgenic animals with the same constructs previously described.

With respect to the antibody response, we observed that in all the immunised groups, specific antibodies recognizing both $Her2^{ECD}$ and OVA were obtained. However, the antibody titers were lower than those obtained with the same DNA constructs in wild type animals. DC-targeting via CD36 had a small detrimental effect on the antibody levels, while targeting via DEC205 was unfavourable: indeed, the antibody levels detected in sera from animals immunised with this construct were about half those observed with anti-CD36 scFv-containing protein (fig. 29).

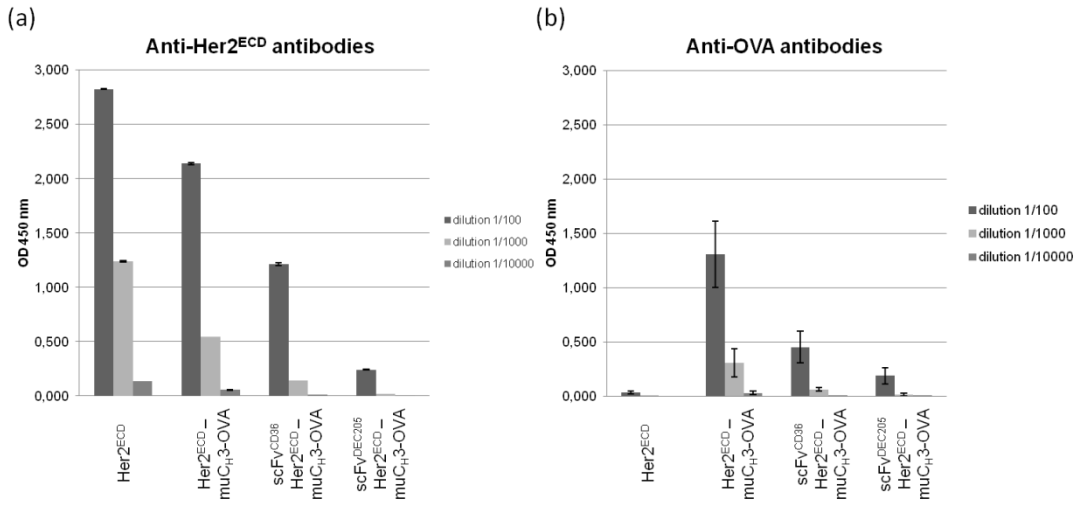

Fig. 29: Antibody response against $Her2^{ECD}$ and OVA upon gene gun DNA immunisation in a tolerogenic environment.

Her2/neu-OTI/II-expressing transgenic mice (n = 3 for each group) were immunised three times by gene gun DNA immunisation with plasmids encoding the indicated constructs. The days after last shot sera were collected and analyzed by ELISA. Plates were coated with (a) biotinylated $Her2^{ECD}$ (with preadsorbed avidin) or (b) purified OVA. Reactivity was determined using an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody. Each bar represents the mean value obtained with sera from three different mice, each analyzed in triplicate. Standard errors are indicated.

In order to assess the ability of gene gun DNA immunisation to induce tumour protection, we challenged immunised mice with NOP cells. NOP6 cells were subcutaneously injected into mammary fat pad of female mice. In mock (empty pcDNA3)-vaccinated mice, these cells formed palpable tumours after 7-10 days post-injection. We took advantage of a method previously described to monitor the onset of tumours at earlier stages by ultrasound imaging (Tilli, Parrish *et al.* 2008). By this technique, we detected tumours after 5-7 days (fig. 30). We observed differences both in tumour appearance and development among the groups vaccinated with the different $Her2^{ECD}$-containing constructs.

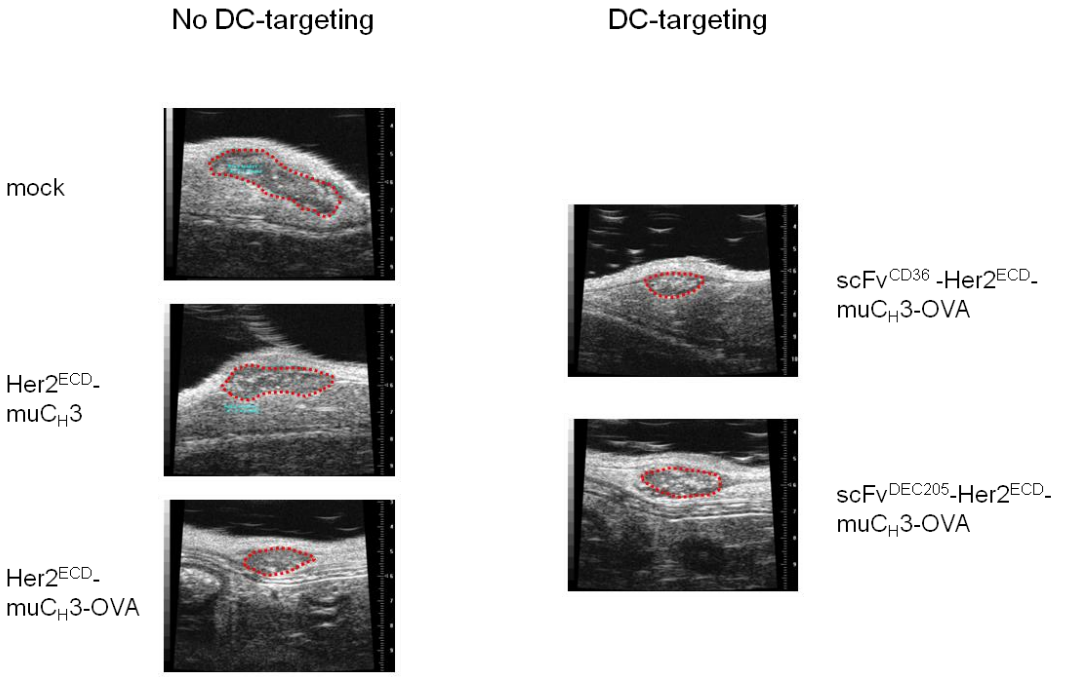

Fig. 30: Echografic appearance of tumours in gene gun DNA immunised transgenic mice expressing Her2/neu-OTI/II challenged with Her2/neu-OTI/II-expressing NOP cells.

Her2/neu-OTI/II-expressing transgenic mice (n = 6 mice for each group) were immunised three times every two weeks by gene gun DNA immunisation with the plasmids encoding the indicated fusion proteins. Fifteen days after the last shot, mice were subcutaneously injected with 10e6 Her2/neu-OTI/II-expressing NOP cells. Tumour development was evaluated by ultrasound imaging. Representative images correspond to measurements obtained a week after tumour challenge. Tumour area is surrounded by the red dashed line.

Tumour challenge followed immunisation with $Her2^{ECD}$ alone induced the appearance of tumours similar to the ones observed in mock-immunised group. In contrast, OVA impaired tumour growth, as demonstrated by the appearance of a differentially echogenic area smaller than the one observed in control animals. Targeting DCs, especially via CD36, seemed to be extremely efficient, because the dimensions of subcutaneous tumours were smaller than the ones observed both in control and in non-DC-targeting groups.

Only animals with palpable tumours at day 15 post cell-injection were sacrificed and tumour growth evaluated by determining their weights.

Tumour-free animals were further evaluated for the onset of tumours and were eventually sacrificed at day 30 (if tumours detected after day 15) or at day 37 for all the others.

Differences in the onset of tumours between the different immunisation groups were observed (fig. 31a): at day 15 post-challenge all the mice mock- or $Her2^{ECD}$-vaccinated developed tumours. In the case of mice vaccinated with the $Her2^{ECD}$ constructs containing also the full-length OVA ($Her2^{ECD}$-muC_H3-OVA), we observed a partial protection, as demonstrated by the absence of tumours in some of the animals (two out of six mice). This delay further increased in animals vaccinated with the DC-targeting unit fused to the $Her2^{ECD}$-muC_H3-OVA. In addition, some differences between the two scFvs were observed: targeting DEC205 induced a longer latency in the development of the tumour than targeting CD36.

Similar differences were observed for tumour growth at different time points (fig. 31b). $Her2^{ECD}$ immunisation seemed to have no effect: the weight of tumours were similar to those from mock-immunised animals (0,2937±0,082g vs 0,3920±0,1038g). In contrast, the presence of the full-length OVA had an impact not only in the time of tumour detection but also in their growth: the average weight was half the one observed in the control group (0,1821±0,0972g). Similar dimensions, however, were observed in tumours collected from the DEC205 group (0,1724±0,0584g) when compared to animals immunised with the non-DC targeting construct, despite the fact of higher delay in the onset. In mice vaccinated with CD36 targeting, tumour progression was impaired, resulting in the smaller tumours among all vaccination groups (0,07387±0,0218g).

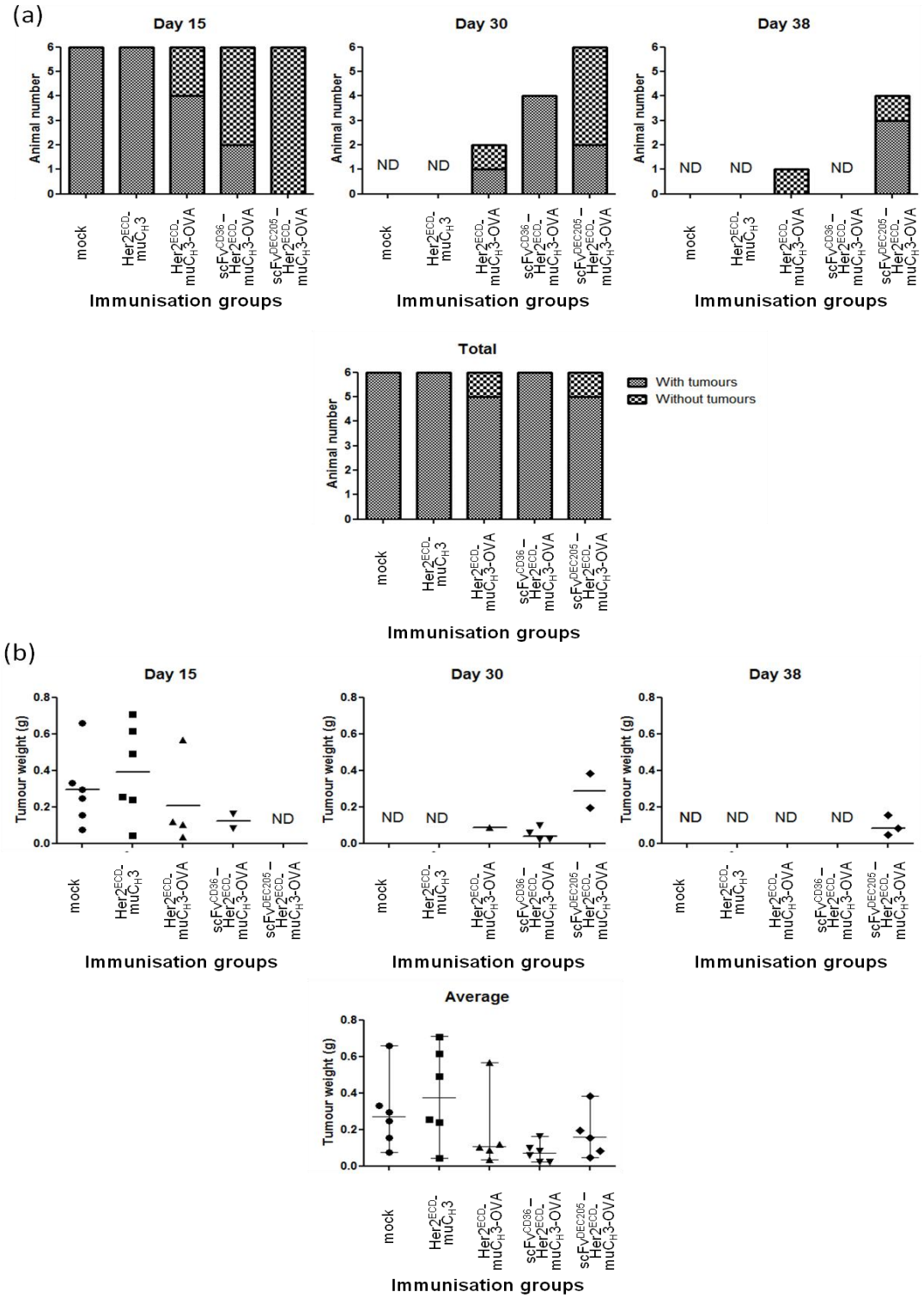

Fig. 31: Anti-tumour effect of gene gun DNA immunisation in Her2/neu-OTI/II-expressing transgenic mice.

Her2/neu-OTI/II-expressing transgenic mice (n = 6 for each group) were immunised three times every two weeks by gene gun DNA immunisation with the indicated plasmids. Fifteen days after the last shot, mice were subcutaneously injected with 10e6 Her2/neu-OTI/II-expressing NOP cells. (a) Animal with or without tumour 15, 30 and 38 day after tumour challenge . (b) Mice with palpable tumours at indicated day were sacrificed and tumour weights were determined. Bars indicate the median with range for each group.

Taken together, these results suggest two main conclusions: 1- anti-OVA T-cell responses were essential, since the construct containing $Her2^{ECD}$ alone did not protect, despite inducing antibody response, and 2- DC-targeting played an important role in boosting the anti-tumour effect. This latter result is consistent with activation of T-cells via antigen cross-presentation.

The contribution of the anti-OVA T-cell response was thus investigated. Two additional plasmids were generated: in both of them, the CD36 targeting unit was present, in order to obtain a fusion protein efficiently uptaken and cross-presented by DCs. The constructs encoded respectively the OVA or the $Her2^{ECD}$ alone (fig. 32a), in order to determine whether the anti-tumour effect was mediated by only the anti-OVA T-cell response or there was also a contribution by the concomitant anti-$Her2^{ECD}$ humoral and cellular responses.

Transgenic mice (n = 5 for eache group) were vaccinated with these plasmids three times and sera collected for antibody analysis. As expected, an antibody response against $Her2^{ECD}$ was generated, without any significant difference with respect to the antibody titers observed in mice vaccinated with a plasmid encoding both antigens, $Her2^{ECD}$ and OVA (fig. 32b).
On the contrary, a stronger antibody titer against OVA was observed in the absence of $Her2^{ECD}$ antigen (fig. 32c).

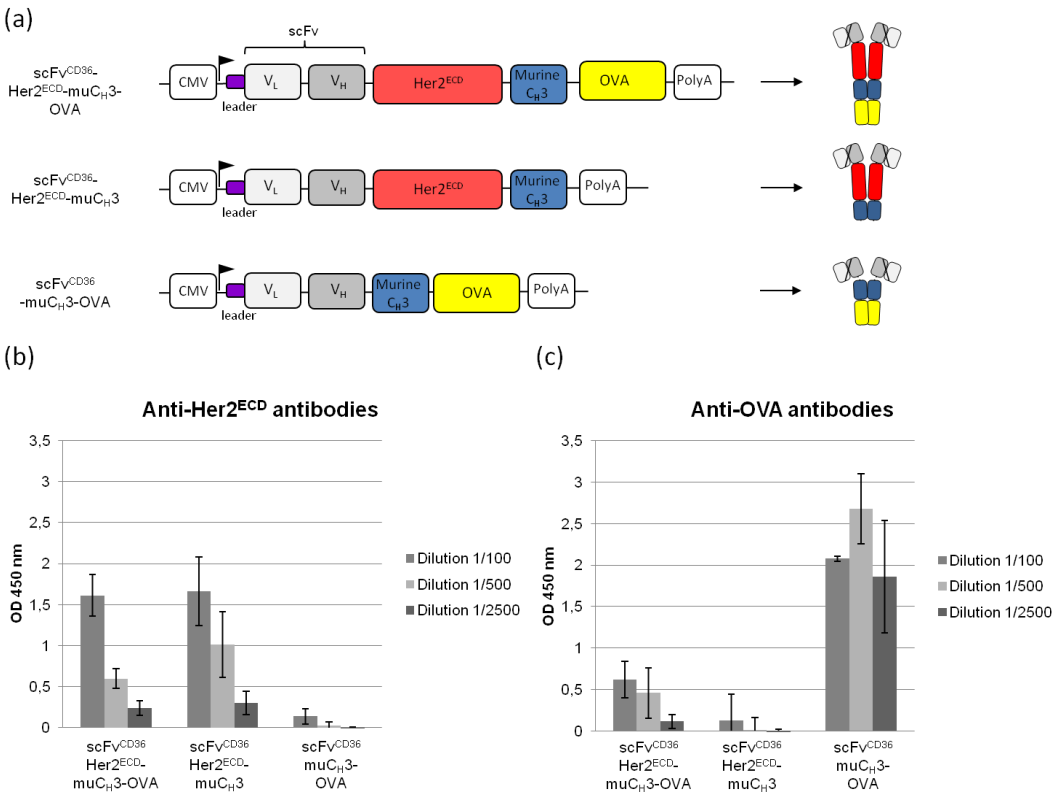

Fig. 32: Antibody response against Her2^{ECD} or OVA in Her2/neu-OTI/II-expressing transgenic animals immunised with constructs containing DC-targeting unit fused to the antigen.

(a) Schematic representation of the plasmids and the respective encoded proteins used to evaluate the contribution of anti-OVA T-cell and anti-Her2^{ECD} humoural and cellular responses. (b-c) Her2/neu-OTI/II-expressing transgenic mice (n = 5 mice for each group) were immunised three times by gene gun DNA immunisation with plasmids encoding the indicated constructs. Ten days after last shot sera were collected and analyzed by ELISA. Plates were coated with (b) biotinylated Her2^{ECD} (with preadsorbed avidin) or (c) purified OVA. Reactivity was determined using an HRP-conjugated goat anti-mouse IgG (γ-chain specific) antibody. Each bar represents the mean value obtained with sera from five different mice, each analyzed in duplicate. Standard errors are indicated.

The same mice were subsequently challenged with NOP6 cells in order to evaluate tumour protection after vaccination. After twenty-one days, mice were all sacrificed and macroscopic detectable tumours were weighed.

As shown in fig. 33, all animals vaccinated with $Her2^{ECD}$ alone developed tumours, while if OVA sequence was present in the DNA plasmids, some degree of protection was achieved. Indeed, three out of five transgenic animals immunised with OVA alone did not carried any visible tumours. The contemporaneous presence of both $Her2^{ECD}$ and OVA induced an intermediate situation: two out of five animals did not exhibit macroscopic tumours and another developed only a very small one, while in the other two animals tumour weights were similar to the average of the $Her2^{ECD}$ vaccinated group (fig. 33). This result indicated that anti-OVA T-cell response was responsible to induce tumour rejection and the activation of this compartment was achieved more likely by reorienting the cross-presentation of the OVA via internalising receptor.

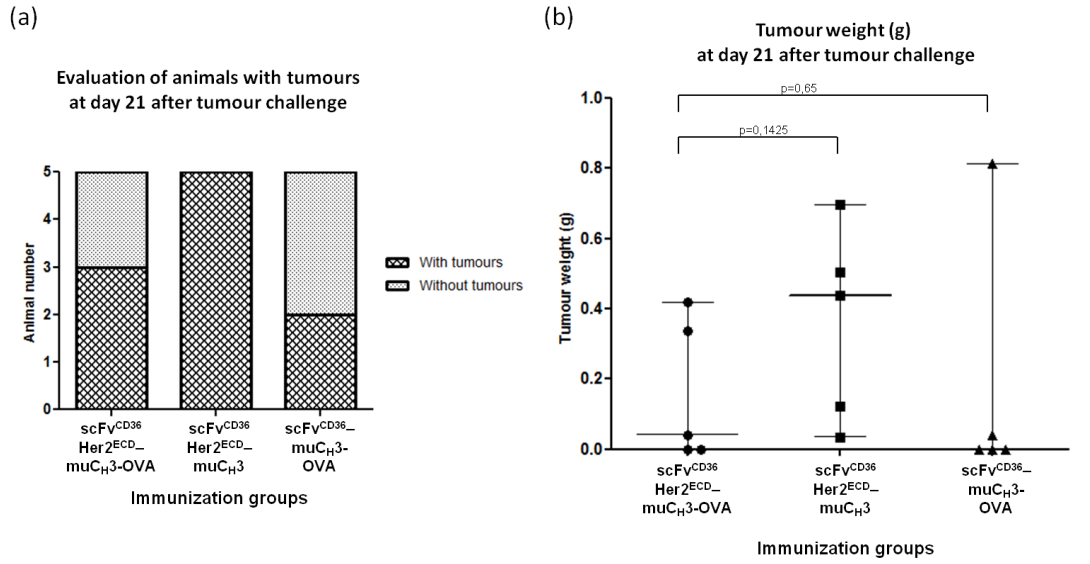

Fig. 33: Anti-OVA T-cell response is required for anti-tumour effect of gene gun DNA immunisation in Her2/neu-OTI/II-expressing transgenic mice.

Her2/neu-OTI/II-expressing transgenic mice (n = 5 for each group) were immunised three times by gene gun DNA immunisation with plasmids encoding the indicated constructs. Fifteen days after the last shot mice were subcutaneously injected with 10e6 Her2/neu-OTI/II expressing-NOP6 cells and sacrificed twenty-one days after. Tumour presences (a) and weight (b) were evaluated after necropsy. Statistical analysis was calculate with U-Mann-Whitney test. Bars indicate the median with range for each group.

This conclusion was further supported with an independent and preliminar experiment performed in wild type animals. In this approach, a model cell line for studying MHC-class I-restricted responses of cytotoxic T lymphocytes in mice was used, that is the E.G7-OVA. These cells are a mouse thymoma transfected with OVA, and have been previously selected to confirm the cross-presentation mediated by CD36 targeting upon protein immunisation (Tagliani et al, unpublished data) because they insensitivity to NK cell-mediated lysis and to anti-OVA-mediated complement-dependent lysis (Carbone and Bevan 1989; Zhou, Rouse *et al.* 1992).
Wild type animals (n = 3 for each group) were immunised with the same DNA constructs from the experiment described in fig. 13, those used to demonstrate cross-presentation of OVA. After three shots, mice were subcutaneously challenged with the tumour cells and tumour onset and development was assessed (fig. 34).
As expected, the control group, i.e. non-vaccinated mice, developed tumours that became palpable after 20 days. Vaccination with OVA fused with DC-targeting units strongly impaired the tumour growth, delaying their onset (day 28).
With respect to tumour volumes, also strong differences were observed between vaccinated and non-vaccinated mice (fig. 34b): at day 32, control mice exhibited tumours that were ten times bigger than those detected in vaccinated animals ($1159\pm214 mm^3$ vs $112\pm112 mm^3$ or $95\pm47 mm^3$ respectively).
No appreciable differences between the two targeting units were observed, probably due to the small number of animals examined.

These data further pointed out that by gene gun DNA immunisation OVA cross-presentation was achieved and the T-cell response arisen was enough to confer partial protection against OVA-expressing tumours.

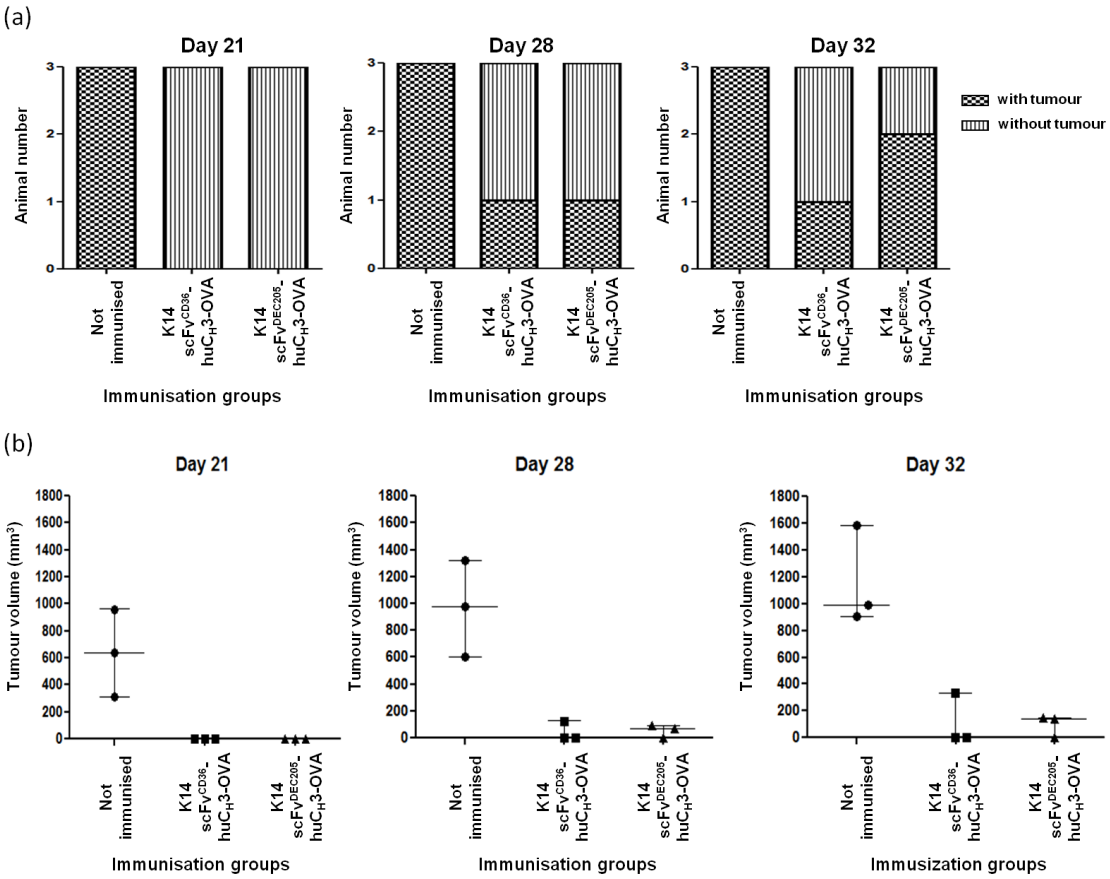

Fig. 34: Anti-OVA T-cell response is enough for anti-tumour effect of gene gun DNA immunisation also in the absence of humoral response

Wild type mice (n = 3) were immunised three times by gene gun DNA immunisation with plasmids encoding the indicated constructs. Fifteen days after the last shot mice were subcutaneously injected with 2x10e5 OVA-expressing E.G7 cells. (a) Animal with or without tumour 15, 30 and 38 days after tumour challenge. (b) Palpable tumours were measured with a caliper ruler and volume calculated. Bars indicate the median with range for each group.

Discussion

The transformation from normal cells to malignant cancer cells is a multistep process by which cells acquire different biological capabilities, becoming insensitive to the normal cellular homeostasis (Hanahan and Weinberg 2011).
Even though the role of the immune system in avoiding tumour progression is still debated, strategies aiming to stimulate an immune response against cancer cells are increasingly becoming part of conventional treatments, as chemotherapy and radiotherapy.

Several molecules expressed only by tumour cells (tumour-specific antigen, TSA) or overexpressed with respect to normal tissues (tumour-associated antigen, TAA) have been identified (Van Der Bruggen, Zhang *et al.* 2002; Wang 2002). These molecules can be used as target for mAb-based treatments (Weiner, Surana *et al.* 2010). Although immunotherapy with mAbs has demonstrated to be an effective strategy, it also faces limitations, such as production costs, limited tissue penetration and, in some cases, unclear mechanism of action when administered *in vivo* (Chames, Van Regenmortel et al. 2009).
TSA or TAA can also employed as immunogens, in order to provoke both humoral and cell-mediated responses able to impair tumour growth and to eliminate tumour cells in an active immunotherapy, i. e. vaccines, with the stimulation of a specific and long-lasting immunity (Gallwitz, Schutzbank *et al.* 2003; Kang, Yoo *et al.* 2009).

Shaping the immune response with genetic vaccine

In this work, we designed a vaccination strategy to demonstrate the feasibility of using DNA vaccination to confer tumour protection, with a plasmid encoding a defined antigen.

Activation of cytotoxic T-cells (CTLs) is generally mandatory for a complete eradication of tumours (Varghese, Widman *et al.* 2009; Steer, Lake *et al.* 2010). The activation of these cells requires that processed antigens are presented associated with MHC-I molecules on the surface of antigen presenting cells (APCs). Genetic

immunisation is a vaccine approach able to deliver antigen-derived peptides into MHC-I to activate CTLs, while maintaining its capability to stimulate also T helper (T_H) response via presentation of peptides bound to MHC-II and antibody production via stimulation of B-cells (Gurunathan, Klinman *et al.* 2000).
In genetic immunisation, the delivery of peptides to MHC-I can be the consequence of direct expression of antigens by APCs, but also through the cross-presentation pathway by a particular subset of APCs, the $CD8\alpha^+$ dendritic cells (DCs). In the cross-presentation pathway, immunogens not directly produced by APCs are uptaken and processed by DCs and finally displayed bound to MHC-I (Abdulhaqq and Weiner 2008). This unique ability of DCs has been exploited in several immunotherapy approaches. For instance, DCs can be isolated, loaded *ex vivo* with the antigen of interest and reinfused into the host in order to mediate specific T-cell responses (Gilboa 2007).

An alternative approach that does not require any manipulation of cells, takes advantage of the presence of receptors on the surface of $CD8\alpha^+$ DCs, which are able to mediate cross-presentation. These receptors recognise different types of ligands, such as carbohydrate motifs (Zelensky and Gready 2005) or lipid structures (Calderwood, Theriault *et al.* 2007) present on self proteins as well as on pathogens, triggering an immune response against them (Delneste 2004). Several preclinical studies have shown that targeting antigens to some of these receptors favours the presentation of the molecule of interest, consequently inducing a strong cytotoxic response (Flacher, Sparber *et al.* 2009; Tacken, Ginter *et al.* 2011).
Another possibility is to enhance the efficacy of the vaccine approach by targeting MHC-II, expressed on all DCs but also on B-cells and macrophages. It has been shown that antigens fused to recombinant antibody specific for the MHC-II molecule enhanced activation of $CD4^+$ T-cells and also tumour protection (Lunde, Western *et al.* 2002; Fredriksen, Sandlie *et al.* 2006).
These findings have been further confirmed in the clinic: for example, a recent phase I trial demonstrated that targeting a poorly immunogenic antigen to the mannose receptor along with the contemporaneous stimulation of Toll-like receptors (TLRs) was able to mediate a more potent immune response in patients with advanced epithelial malignancies (Morse, Chapman *et al.* 2011).

Following this reasoning, two receptors were chosen for this work: the C-type lectin receptor CD205, also called DEC205 (Jiang, Swiggard *et al.* 1995), and the scavenger receptor CD36, whose ability to mediate cross-presentation was recently characterised by our group (Tagliani, Guermonprez *et al.* 2008).

Two published works clearly demonstrated that these two internalising receptors can be employed to activate specific T-cells. Bonifaz *et al.* demonstrated that targeting ovalbumin (OVA) to DEC205 induces CTL activation (Bonifaz, Bonnyay *et al.* 2004), although it requires costimulatory signals (adjuvant) for DCs to avoid the appearance of peripheral tolerance, because the engagement of DEC205 is unable to induce maturation of DCs (Bonifaz, Bonnyay *et al.* 2002), a finding further demonstrated by Grossmann and colleagues in a prime-boost vaccination approach: OVA antigen was targeted via DEC205 and, in the absence of TLR agonist, a decrease in specific $CD8^+$ T-cell response was observed (Grossmann, Tenbusch et al. 2009). In contrast, Tagliani *et al.* demonstrated that targeting the same antigen to the CD36 receptor does not require adjuvants (Tagliani, Guermonprez *et al.* 2008).

We therefore decided to confirm that it was possible to induce cross-presentation of OVA via gene gun DNA immunisation, as those experiments were done with purified proteins. Furthermore, we decided to evaluate the efficacy of this targeted DNA vaccination approach in cancer therapy, as it has been demonstrated to be proficient for viral proteins (Nchinda, Kuroiwa et al. 2008).

Full-length OVA sequence was fused at the C-term of two scFvs, which specifically recognise DEC205 ($scFv^{DEC205}$) or CD36 ($scFv^{CD36}$) internalising receptors. By injecting OVA-specific $CD8^+$ T-cells (OT-I) to immunised animals, the activation of these cells was demonstrated. In the first set of experiments, the activation of OT-I was obtained irrespective of the presence of the DC-receptor targeting unit, in contrast to what was observed with protein administration. This finding was attributed to the transfection of dermal resident APCs, which directly produced and then processed the antigen via MHC-I pathway. To impair the production of the antigen by transfected APCs and to evaluate only the cross-presentation pathway, the expression of the molecule of interest was restricted to the main cellular component of the epidermis, that is keratynocytes, by using a keratinocyte-specific promoter (K14). In this latter case, cross-presentation of antigen, and thus OT-I activation, was shown to depend on the targeting of the DC receptors.

Another observation that contrasted to protein immunisation was that gene gun DNA immunisation with DEC205 targeting did not require any other immunostimulatory molecule to activate a long-lasting response, further supporting the intrinsic adjuvant properties of plasmid backbone (Klinman 2006).

The experiments performed previously with protein and now with gene gun DNA immunisation indicated that targeting via CD36 seemed to be as efficient as via DEC205 in inducing CTL activation, especially after more than twenty days from OT-I transfer. The choice of CD36 targeting might induce a sustained inflammatory status, with the consequent recruitment of other immune cells and thus favouring the complete activation of APCs. Indeed, several studies have demonstrated that CD36 activity can lead to the recruitment of immune cells, such as macrophages (Harb, Bujold *et al.* 2009), which in turn boost T-cells.

With regard to the B-cell compartment, a strong antibody response against OVA was obtained. Moreover, no differences attributable to the presence of the DC-targeting unit were appreciated. This is in contrast to the work of Nchinda *et al.* (Nchinda, Kuroiwa *et al.* 2008), who demonstrated that targeting OVA to DEC205 in a strategy of genetic immunisation with naked DNA and electroporation enhanced the antibody response. This effect progressively diminished as increasing amounts of immunising plasmid were used, which could indicate that both in this case and ours the high immunogenicity of OVA explains up to a certain point the insensitivity of the humoral response to the effect of antigen internalisation via receptors. However, despite this possibility, we did not observe any enhancement on the humoral response due to DEC205 targeting but rather a detrimental effect on a less immunogenic antigen, which will be discussed later.

Summarising, with our strategy of gene gun DNA immunisation we were able to activate both antibody and T-cell responses. In this latter case the analysis was performed with exogenously provided T-lymphocytes, while the activation of the endogenous repertoire is currently under investigation. However, a strong indication that the endogenous anti-OVA CTL repertoire was activated upon genetic immunisation, was obtained in experiments with a thymoma cell line expressing OVA (E.G7-OVA cells). The rejection of this tumour largely relies on the T-cell response (Zhou, Rouse *et al.* 1992) and vaccination of animals with a DNA plasmid

encoding OVA fused to the DC-targeting unit induced a strong delay in tumour onset, suggesting that CTL activation was taking place.

Targeting the HER2 with DNA immunisation

OVA is a widely used model antigen but with no clinical relevance. Taking into account our results with the designed vaccination approach, we moved to a TAA, the Her2/neu.

Her2/neu is a tyrosine kinase receptor belonging to the EGFR family (Schechter, Stern *et al.* 1984; Hynes and Stern 1994). Its overexpression is linked with many different malignancies (Menard, Tagliabue *et al.* 2000; Ross and McKenna 2001), especially breast cancer, and is associated with a bad prognosis (Slamon, Clark *et al.* 1987). Breast tumours with HER2 overexpression are currently treated with a combination of surgery and passive immunotherapy with Trastuzumab (Vogel, Cobleigh *et al.* 2002; Moasser 2007). However, this mAb is a high molecular weight compound, which does not effectively cross the blood–brain barrier: this characteristic impacts negatively on the beneficial effects on breast tumours because a higher incidence of brain metastasis in treated patients were observed with no efficient therapeutic options (Bendell, Domchek et al. 2003). Moreover, many HER2-overexpressing tumours become insensitive to this mAb by different mechanisms, such as the activation of alternative tumour proliferation-promoting signalling pathways, as well as downregulation or mutation in the $Her2^{ECD}$ (Wong, Leung *et al.* 2011). Another shortcoming of Trastuzumab is its adverse effect on cardiac function (Seidman, Hudis et al. 2002), even though the molecular mechanisms involved is not clearly elucidated (Bria, Cuppone et al. 2008).

Several different active immunotherapy strategies are being evaluated to provide new therapeutical options. Emens' group is investigating a whole cell-vaccine approach in phase I and II clinical trials (Emens, Asquith *et al.* 2009). They administer human HER2-expressing cells together with co-stimulatory molecules, such as GM-CSF, to overcome tolerance in patients with metastatic breast cancer. These clinical trials are still in recruiting phase. In another preclinical study, peripheral blood mononuclear cells (PBMCs) and APCs were stimulated *in vitro* with a recombinant protein composed by the ECD and the ICD of HER2 fused to GM-CSF and then

administered to patients, achieving a clinically relevant anti-HER2 cellular response (Park, Melisko *et al.* 2007).

Alternative active immunotherapy approaches consist of protein vaccines. A recent phase I clinical trial has demonstrated that a protein vaccine, composed by the ECD and part of the ICD of HER2, in combination with administration of TKI Lapatinib was well tolerated but felt short of expectations (Hamilton, Blackwell et al. 2012). Other examples employed peptide-based vaccines, where HER2-derived peptides were administered with TLR agonists or other cytokines (Peoples, Holmes *et al.* 2008; Disis, Wallace *et al.* 2009; Thomann, Heurtault *et al.* 2011), as well as DC-based vaccines (Brossart, Wirths *et al.* 2000; Edlich, Hogdal *et al.* 2010). In the latter case, the clinical trials are all using autologous DCs loaded *ex vivo* by adding HER2-derived peptides, with encouraging results (Brossart, Wirths *et al.* 2000; Czerniecki, Koski *et al.* 2007). Another example consisted of *ex vivo* generated DCs loaded with the ICD of HER2, with clinical benefits observed in all seven treated patients (Morse, Hobeika et al. 2007).

New HER2-vaccine approaches have now entered the clinical phase and are based on genetic immunisation with HER2-encoding DNA. Several preclinical studies have demonstrated that DNA vaccines encoding HER2 are able to inhibit or delay carcinogenesis. Wei et al. vaccinated animals with a modified human HER2 protein without tyrosine kinase activity, obtaining efficient humoral and cellular responses (Wei, Shi et al. 1999). Similarly, a DNA vaccine encoding only the ECD and the TM domains of HER2 was reported to be effective in inhibiting carcinogenesis in a transgenic mouse model (Rovero, Amici et al. 2000). Also, a HER2 DNA vaccine, consisting of the HER2 ECD and TM, induced a higher T-cell immunity than a whole cell-based vaccine (Whittington, Radkevich-Brown et al. 2009).

Other vaccination approaches are based on the targeting of HER2 to APCs directly in the host. Slots *et al.* (Sloots, Mastini *et al.* 2008) performed intramuscular injection of a DNA plasmid encoding the first 222 amino acids of the ECD of human HER2 fused to the ECD of human CTLA-4, which is the ligand for the B7 protein complex on APCs. B7 is not an internalising receptor and has no impact on antigen uptake and processing but its engagement only enhances the ability of APCs to upregulate costimulatory signals. They demonstrated that the contemporaneous presence of

CTLA-4 and HER2 induced better antibody and $CD8^+$ T-cell responses. Moreover, this strategy conferred tumour protection upon challenge.

More recently, a plasmid encoding a human HER2-derived sequence (aa. 364–391) fused to the Fc domain of a human IgG1 was administered to mice. This strategy was demonstrated to favour antigen internalisation and cross-presentation, thus resulting in a higher $CD8^+$ T-cell activation and a longer tumour latency (Zizzari, Veglia et al. 2011). In a previous work, the same effect was observed when combining a Her2/neu-specific mAb and a Her2/neu-expressing whole tumour cell vaccine, due to Fc-mediated activation of DCs (Kim, Armstrong et al. 2008).

In our strategy of gene gun DNA immunisation, plasmids encoded the ECD of the rat Her2/neu alone or fused to a scFv moiety targeting the DEC205 or CD36 receptors, in order to favour internalisation and cross-presentation of $Her2^{ECD}$. In contrast to Slots *et al.*, instead of using the costimulatory ligand CTLA-4, we performed a direct targeting of antigen to internalising receptors of DCs, to favour not only the production of costimulatory signals but also the uptake and processing of the $Her2^{ECD}$. In contrast with the experimental approach by Kim and Zizzari, we decided to focus on DC receptors mediating cross-presentation. In our experiments, we used rat Her2/neu ($Her2^{ECD}$) because this isoform is expressed by the transgenic mouse model that we used in several experiments (Wall, Milne *et al.* 2007).

A dimerisation unit, represented by the C_H3 domain of IgG, was present between the scFv and the antigen. The addition of this dimerization unit intended to favour secretion of the antigen, as previous unpublished works from our group have demonstrated that some antigens need such unit to be efficiently secreted. However, we did not observe any influence of the C_H3 on the amount of the secreted protein, because $Her2^{ECD}$ was easily secreted also as a monomer (data not shown). Nevertheless, the presence of the dimerisation unit should also confer the benefit of increasing the persistence of the molecule in circulation by reducing its clearance (Seymour, Miyamoto *et al.* 1995; Noguchi, Wu *et al.* 1998).

Moreover, the C_H3 dimerising unit allows the cross-linking of the receptors, a requirement for the internalisation of the antigen via CD36 as demonstrated in our lab (Tagliani *et al.* unpublished data).

In addition, the dimerisation unit could also increase the immunogenicity of the $Her2^{ECD}$, which can be further enhanced if using a domain of xenogenic origin, as it has been observed with other antigens used in our (unpublished data) and other laboratories (Sutherland, McKenzie *et al.* 2001). In our experiments, no differences in the antibody response were observed when wild type animals were vaccinated with $Her2^{ECD}$ fused to the human domain with respect to the mouse counterpart, indicating that $Her2^{ECD}$ is *per se* immunogenic enough.

A similar DC-targeting design was developed by Wei *et al.* (Wei, Wang *et al.* 2009). They performed a genetic immunisation approach with naked plasmids encoding a scFv moiety targeting the CD11c receptor of DCs fused to the human or rat $Her2^{ECD}$. In contrast to the results obtained by Wei and collaborators, where the antibody response was achieved only with the CD11c targeting, our gene gun DNA immunisation induced an antibody response irrespectively of the presence of the DC-targeting unit, even though targeting via DEC205 seemed to be marginally detrimental. Moreover, we targeted $Her2^{ECD}$ to two internalising receptors, able to mediate cross-presentation, expressed only by $CD8\alpha^{+}$ DCs while the function of CD11c, even though be a surface marker expressed by most of DCs, is not well understood (Sadhu, Ting *et al.* 2007).

Another important difference between the two approaches resides in the fact that Wei *et al.* required the contemporaneous injection of a high amount of CpG oligonucleotides to achieve a humoral response (ten times the amount of DNA plasmid), while our gene gun DNA immunisation showed to be more efficient in inducing antibodies without any additional immunostimulatory molecule.

Immunisation routes influence the quality of antibody response

We then decided to study deeper the antibody response induced by our strategy of genetic immunisation in comparison to immunisation with the purified antigen. DNA immunisation preserves the natural conformation and post-translational modifications of the antigen since it is directly expressed by cells of the host. Previous works from our group clearly sustained this feature: gene gun DNA immunisation induced mainly antibodies directed against conformational epitopes (Benvenuti and Burrone 2001), which were required for tumour protection in a

context of an anti-idiotype vaccination approach against a murine B-lymphoma (Cesco-Gaspere, Benvenuti *et al.* 2005). Moreover, the presence of PAMPs, such as hypomethylated CpG motifs, with an intrinsic adjuvant effect (Hemmi, Takeuchi *et al.* 2000), could confer a further advantage of DNA immunisation with respect to protein or peptide vaccination, to obtain an immune response against the properly folded protein. In a recent work, where DNA immunisation, protein immunisation or a prime-boost regimen for human immunodeficiency virus (HIV) gp120 were compared, the generated antibodies differed in terms of functionality, specifically in their ability to recognise the immunodominant V3 loop of gp120 and also other two epitopes outside this loop (Vaine, Wang *et al.* 2010).

We thus compared the antibody responses against $Her2^{ECD}$ obtained upon DNA or protein immunisation. Using a novel ELISA assay, we demonstrated that the antibodies induced by DNA immunisation had qualitative differences with respect to protein immunisation. For the analysis of mouse sera, a mono-biotinylated version of $Her2^{ECD}$, directly produced by eukaryotic cells as previously described by our group (Predonzani, Arnoldi *et al.* 2008), was immobilised on ELISA plates pre-coated with avidin, in order to preserve the tertiary structure of antigen and thus the accessibility to all possible conformational epitopes. With this method, an increased reactivity of both a mAb specific for the $Her2^{ECD}$ and of sera from immunised animals was observed when compared to the antigen immobilised directly on the plates. This increment, however, was dependent on the specificity of the antibody: indeed, the reactivity of the mAb, which recognises only a single epitope, showed lower increase than the polyclonal antibodies present in the sera, validating this strategy for a better accessibility to all conformational epitopes.

We also proved that polyclonal antibodies from mice immunised with gene gun practically behaved as the mAb in their reactivity against the heat-denatured antigen. Mice immunised with the purified protein without adjuvant, also developed antibodies recognising conformational epitopes, but still displayed higher reactivity against the denatured antigen when compared to those generated upon genetic immunisation. This result was consistent with the known structure of $Her2^{ECD}$, which is folded into a compact globular protein, where immunodominant B-epitopes are conformational. Interestingly, we demonstrated by Western blotting that the use of adjuvants (Freund's adjuvant) can induce some degree of denaturation in the

immunising protein, with the generation of antibodies recognising non-native conformation of the antigen. In addition, no significant differences were observed in antibody titers between the gene gun and protein strategies. In conclusion, gene gun DNA immunisation demonstrated to be effective for inducing high titers of antibodies specific for the native antigen, without the need of adjuvants, which are normally used in protein immunisation of humans with common safety concerns (Garcon, Segal *et al.* 2011).

In spite of these differences in the repertoire of antibodies, we have not yet proved any functional consequence. This may however be of relevance not only for $Her2^{ECD}$ but for all other antigens, for example viral ones. It was previously demonstrated in our lab that only antibodies against the native conformation of a murine lymphoma idiotype were able to confer tumour protection (Cesco-Gaspere, Benvenuti *et al.* 2005). Also Vaine *et al.* demonstrated that DNA vaccination was more efficient in inducing virus-neutralising antibodies (Vaine, Wang *et al.* 2010). Further studies will thus address this important issue. The capability of the generated antibody repertoire to directly kill tumour cells or inducing ADCC is under investigation, as well as the analysis of possible differences in the T_H polarisation.

All these results obtained in a non-self environment confirm that gene gun DNA immunisation is a good approach to deliver antigen to the host, activating efficiently both B- and T-cell compartments against native epitopes.

Immunotherapy of HER2-expressing tumours using genetic vaccination and dendritic cell targeting

To prove that this is also true in a tolerant environment, we used transgenic animals with constitutive expression but restricted to mammary glands of the Her2/neu-OTI/OTII transgene. It consists of the rat isoform of HER2 (Her2/neu) tagged in its ICD with the two dominant OVA-derived epitopes (Wall, Milne *et al.* 2007). The presence of this transgene causes the recognition of the OVA OTI/II-tagged Her2/neu molecule as a self-antigen, which is thus tolerised. This is similar to the situation found in humans, where TAAs can escape immune surveillance mainly due the negative selection of T-reactive cells at the thymic level (Smyth, Godfrey *et al.* 2001). Implementing a vaccination approach in such a tolerogenic environment

would thus allow the evaluation of strategies to overcome tolerance and to induce an efficient immune response.

This transgenic mouse strain should develop spontaneous breast tumours with an increased frequency in the presence of a dominant negative mutant version of p53, a key mediator in the control of cell proliferation whose mutation is a major event in the carcinogenesis process (Vogelstein 1990; Hollstein, Sidransky *et al.* 1991).
Despite the phenotype attributed to this mouse model, where it is claimed that in more than 90% of female animals expressing both OVA OTII/I-tagged Her2/neu and mutant p53 spontaneous tumours arise, we were unable to detect them. This change in the expected phenotype was also confirmed by Dr. Nelson's group. There may be several different reasons to explain the loss of the tumourigenic phenotype.
The first one is the silencing of the expression of either of the two transgenes due to epigenetic mechanisms (Garrick, Fiering *et al.* 1998; Calero-Nieto, Bert *et al.* 2010). Even though the presence of the two genes was assessed in the animals at the genomic level, the expression of their products could not be assured. This hypothesis, however, was indirectly refuted in the case of Her2/neu by the analysis of the antibody titers observed in vaccinated animals, as a strong reduction was observed when compared with non-tolerant wild type animals. Moreover, OVA OTI/II-tagged Her2/neu-expressing tumour-derived cells (Yang, Martin *et al.* 2009) grew in these transgenic animals while were rejected in wild type mice, indicating the expression and thus tolerisation of the transgene protein.
The second possibility is the requirement of hormones to activate the transcription of mutant p53, as the p53 transgene is under the transcriptional control of WAP promoter. As confirmed by the donating investigator group, however, two rounds of pregnancy seemed to be not enough to recover the original phenotype. Perhaps, increasing the number of pregnancy and lactation events could provide enough stimuli for the expression of this protein and the appearance of spontaneous tumours.
Other possibility is reduced expression of the transgenes, below the threshold for inducing tumours, yet sufficient to induce tolerance.

This transgenic model, however, was still useful for the evaluation of DNA vaccination. Albeit in our hands the OVA OTI/II-tagged Her2/neu was not able to induce spontaneous tumours by itself, its expression led to the presence of a

tolerogenic environment, where the contribution of humoral and CTL responses to tumour rejection could be evaluated in challenge experiments with antigen-expressing tumour cell lines (Yang, Martin *et al.* 2009).

Gene gun DNA immunisation was thus implemented in this transgenic mouse model. An anti-Her2^{ECD} antibody response was generated, even though a strong decrease in the titers was observed in the transgenic animals with respect to wild type mice, as mentioned before. Additionally, in contrast to wild type animals, antigen targeting to DEC205 strongly impaired the induction of antibodies, while this detrimental effect was less pronounced in the case of CD36 targeting. This could be due to a different accessibility of the recombinant molecule containing the scFv moiety: it is possible that, despite a similar *in vitro* expression, their persistence in the blood stream might be reduced with respect to the Her2^{ECD} alone, becoming less accessible to the recognition by circulating B-cells.

Several studies have demonstrated that the presence of antibodies against HER2 is not sufficient to a complete eradication of tumours, which requires of CTL activity (Orlandi, Venanzi *et al.* 2007; Orlandi, Guevara-Patino *et al.* 2011). With the model antigen OVA, we provided indirect evidences that specific CTL responses were induced by cross-presentation via CD36 or DEC205 upon gene gun DNA immunisation. We thus investigated whether targeting Her2^{ECD} to cross-presentation pathway could confer tumour protection. The ability of DNA immunisation has been proved to be enhanced by targeting antigen to the MHC-I processing pathways, as shown for the human papilloma virus E7 protein. Hauser and Chen in 2003 fused E7 to the heat shock protein 70 (HSP-70). This fusion protein was internalised through a classical mechanism but once inside the cytosol, the presence of HSP-70 oriented it towards the cross-presentation pathway (Hauser and Chen 2003).

We then assessed the suitability of our gene gun DNA immunisation strategy in inducing tumour protection in experiments of tumour challenge, favouring the internalisation of the antigen via targeting of DC receptors. In analogy to what has been previously seen by others (Amici, Smorlesi *et al.* 2000), immunisation with Her2^{ECD} alone was not sufficient to prevent tumour growth despite the generated antibody response.

Orienting the $Her2^{ECD}$ antigen towards cross-presentation did not modify this situation. The apparent lack of a specific anti-$Her2^{ECD}$ CTL response might be ascribable to the absence or low-immunogenicity of T-cell epitopes in the $Her2^{ECD}$ in such genetic background. Indeed, a recent work demonstrated that CTL response against HER2 was dependent on the genetic background, and thus on the MHC-I haplotypes, of animals. In this study, the efficacy of a HER2 DNA vaccine was tested in human HER2-transgenic mice with C57Bl/6, BALB/c and the hybrid (C57Bl/6 x BALB/c) backgrounds. C57Bl/6 animals showed the lowest antibody titers and specific CTL numbers (Radkevich-Brown, Jacob *et al.* 2009).

The second possible reason for the failure of $Her2^{ECD}$ cross-presentation to confer tumour protection is that the induced T-cell response was not efficient enough to destroy cancer cells.

In contrast, the presence of OVA in the immunising proteins had an impact both on tumour onset and growth. A T-cell response against OVA would target tumour cells, which express the OVA immunodominant T-epitopes. Alternatively, it might behave as a xenogenic carrier, which increases the immunogenicity of the $Her2^{ECD}$, in analogy to what has been shown with a xenogenic C_H3 domain in anti-idiotype response (Benvenuti, Cesco-Gaspere *et al.* 2002). However, since OVA alone induced tumour protection, this was unlikely to be the case.

Targeting antigens containing OVA to DC-internalising receptors to favour CTL activation caused a strong impairment of tumour growth. Differences were observed between targeting via DEC205 with respect to via CD36. DEC205 seemed to be more effective in the early phase of tumour progression but unable to sustain a prolonged immune response, while CD36 seemed to induce a less potent CTL response yet more prolonged. A possible explanation might be ascribed to the differences in the expression of these receptors by different cells: DEC205 is primarily expressed by $CD8\alpha^+$ DCs (Kronin, Wu *et al.* 2000), while CD36 is present on the surface of $CD8\alpha^+$ DCs but also on several other types of cells, such as endothelial cells, erythrocytes, monocytes and B-cells (van Schravendijk, Handunnetti *et al.* 1992). The secreted recombinant molecules could be thus internalised not only by DCs but also by macrophages, boosting the activation of specific $CD8^+$ clones by their MHC-I presentation pathways.

Another possible explanation derives from a recent work that demonstrated that tumour regression is dependent on B-cell presence, even though the exact mechanism is still far to be elucidated (Guo, Li *et al.* 2011). DC-targeting, especially via DEC205, impaired the production of antibodies, that should impact also on the sustained activation of CTL, and we cannot exclude this being one of the reasons for a less prolonged anti-tumour response.

This hypothesis should be addressed trying to enhance antibody production, implementing administration of T_H2-promoting cytokines, to counteract the detrimental effect of DC-targeting.

Future perspectives

We have designed a genetic immunisation strategy based on orienting defined antigen to cross-presentation pathway to induce immunity against tumours. The humoral response against both OVA and HER2 was demonstrated and characterised in a non-self as well as in a self (tolerant) environment. The quality of the antibodies against HER2 was further analysed by comparing two alternative routes of immunisation, protein administration or gene gun DNA immunisation. We also provided indirect evidences of the activation of endogenous CTL response, at least against one model antigen, the OVA. Further studies will aim to a deeper study of the functionality of the generated antibodies. Moreover, a deep characterisation of T-cell response will be pursued to address the enhancement of the T-cell responses induced by DC-targeting. We implemented the gene gun DNA immunisation approach in a tolerant environment, represented by a OVA OTI/II-tagged Her2/neu transgenic mouse to demonstrate that DC-targeting approach is a proficient way to confer tumour protection in a prophylactic scenario.

Optimisation studies will be further conducted to enhance the anti-tumour response. Implementing passive administration of antibodies or other immunostimulatory molecules as well as prime-boost with viral-mediated genetic immunisation will be explored in light of our encouraging results on DC-targeting as a profitable strategy to overcome naturally occurring immunological tolerance against tumour associated antigens.

Conclusions

We investigated the induction of a specific immune response both in a non-self and in a tolerogenic environment using a strategy of genetic immunisation. We used a model (OVA) and a clinically relevant (Her2/neu) antigen, administered in different recombinant versions by gene gun. In order to enhance activation of specific CTL responses, we designed fusion proteins to target DC-internalising receptors.

The results obtained indicate that:

- Targeting antigen to the DEC205 or CD36 DC-internalising receptor with gene gun DNA immunisation induces T-cell activation by cross-priming without the need of additional costimulatory molecules;
- Targeting CD36 is more effective than targeting DEC205 to confer tumour protection;
- The presence of the DC-targeting unit impact negatively on the antibody response, even though this detrimental effect is more evident in a tolerant environment;
- The antibody response against $Her2^{ECD}$ is not enough to confer tumour protection;
- Gene gun DNA immunisation is more effective than protein immunisation to induce high titers of conformational antibodies specific for the native antigen.

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