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"Mechanism of action of MHC class II-associated invariant chain as an adjuvant of Adenovirus based genetic vaccines"

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LIST OF ABBREVIATIONS

Ab Antibody
Ad Adeno
APC Antigen presenting cells
CD Cluster of differentiation
CD74 MHC class II associated Invariant chain
ChAd Chimpanzee adenovirus
CMV Cytomegalovirus
CLIP Class II-associated invariant chain peptide
CTL Cytotoxic T lymphocyte
BCR B-cell receptor
BMDC Bone marrow dendritic cells
DC Dendritic cells
DMSO Dimethyl sulfoxide
ER Endoplasmic reticulum
ERAD Endoplasmic reticulum associated degradation
FCA Freud's complete adjuvant
GFP Green Fluorescent Protein
GM-CSF Granulocyte-macrophage colony-stimulating factor
GP Glycoprotein
GOI Gene of interest
HCV Hepatitis C virus
hIi human MHC class II associated Invariant chain
HIV Human immunodeficiency virus
HLA Human leukocyte antigen
Ii MHC class II associated Invariant chain
IFN Interferon
IL Interleukin
IP Immunoprecipitation
IU Infection units
LAMP1 Lysosomal-associated membrane protein
LCMV Lymphocytic Choriomeningitis Virus
LPS lypopolysaccarides
Lys lysine
mIi murine MHC class II associated Invariant chain
MIF Migration inhibitory factor
MDP Muramyldipeptide
MyD88 Myeloid differentiation primary response gene 88
MHC Major Histocompatibility Complex
MOI Multiplicity Of Infection
MVA Modified Vaccinia Ankara
NHP Non-human primates
OVA Ovalbumin
ORF Open reading frame
PBMC Peripheral blood mononuclear cells
PEP Pepstatin
PRF Perforin
TAP Transporter associated with antigen processing
T_{CM} T cell central memory

TCR T-cell receptor
T_{EM} T cell effector memory
TIR cytosolic toll-interleukin 1 receptor
TLR Toll like receptor
TM Transmembrane
TNF Tumor Necrosis Factor
TRIF TIR domain containing adapter-inducing interferon
T_{RM} T cell tissue-resident memory
T_{SCM} T stem cell memory
T_{TM} T cell transitional memory
Ub Ubiquitin
Vp Viral Particle
WB Western Blot

1. BACKGROUND

1.1 Adaptive immunity and antigen presentation

1.1.1 Adaptive immunity

The immune system is a host defense system comprising many biological structures and processes within an organism that protects against disease. The immune system can be classified into subsystem such as **innate immune system**, that provides the first line of defense against infection and **adaptive immune system**.

The innate responses call the adaptive immune responses into play, and both work together to eliminate the pathogens. Unlike innate immune responses, the adaptive responses are highly specific to the particular pathogen that induced them. They can also provide long-lasting protection. The function of adaptive immune responses is to destroy invading pathogens and any toxic molecules they produce. Because these responses are destructive, it is crucial that they are made only in response to molecules that are foreign to the host and not to the molecules of the host itself. The ability to distinguish what is foreign from what is self in this way is a fundamental feature of the adaptive immune system.

The adaptive immune response creates immunological memory after an initial response to a specific pathogen and leads to an enhanced response upon subsequent encounters with that pathogen.

The cell types mediating adaptive immunity are **B** and **T lymphocytes**.

B lymphocytes develop in the bone marrow from hematopoietic precursor cells. When immature B cells binds antigen through B cell receptor, the lymphocytes will proliferate and differentiate in plasma cells that produce antibodies. They are glycoproteins that neutralize viruses or bacteria by activating the complement system through the classical pathway. Each immunoglobulin molecule is made up of two heavy chains and two light chains joined by disulfide bonds and generally recognizes a small region on the surface of a large molecule, named epitope (Padlan et al., 1994).

1.1.2 Cell-mediated immune responses: T lymphocytes

T lymphocytes derive from the multipotent hematopoietic stem cells in the bone marrow. The progenitor cells migrate from the bone marrow via blood to the thymus where they mature.

Developing T cells, named thymocytes, undergo different changes in the status of T cell receptor genes, in the expression of T cell receptor or cell-surface proteins such as CD3 complex and co-receptor CD4 and CD8. Two distinct lineages of T cells-α:β and ɣ:δ which have different types of T cell receptors chains, are produced early in T-cell development.

When progenitor's cells first enter the thymus from the bone marrow, they lack most of the surface molecules characteristic of mature T cells and their receptor genes are not rearranged. Interactions with the thymic stroma trigger an initial phase of differentiation, but T cells do not express any of the three cells surface markers (CD3, CD4 or CD8). For this reason these cells are called double negative thymocytes.

These cells undergo different rearrangements up to be double positive thymocytes (Pang et al.,2010).

Lymphoid precursors are triggered to proliferate and become thymocytes committed to the T-cell lineage through interactions with the thymic stroma. The different stages of development are characterized by the expression of particular cell-surface proteins, which change until the development of CD8 or CD4 positive single cells.

Once T cells have completed their development in the thymus, they enter the bloodstream.

On reaching a peripheral lymphoid tissue, they leave the blood to migrate through the lymphoid tissue, returning via the lymphatics to the bloodstream to recirculate between blood and peripheral lymphoid tissues. Mature recirculating T cells that have not yet encountered their specific antigens are known as naïve T cells. When a naïve T cell recognizes its specific antigen on the surface of mature dendritic cell, it ceases to migrate and differentiate in **effector T cell**.

After expansion and differentiation, the effector T cells exit the efferent lymphatics and reenter the bloodstream, through which they migrate to the sites of infection.

These effector cells can be divided into two classes: one carrying the **co-receptor CD8** on its surface and the other bearing the **co-receptor CD4**. CD8 is carried by **cytotoxic T cells** which recognize peptides loaded on the MHC class I complex, via their heterodimeric receptor whereas CD4 is carried by T cells whose function is to activate other cells of the

immune system and recognize peptides loaded on the MHC class II complex through their specific receptors.

CD4 is a single polypeptide, folded into four external immunoglobulin-related domains. It has a unique strand topology between domains 1 and 2 (D1 and D2) and between domains 3 and 4 (D3 and D4). The MHC binding region on CD4 is located mainly on the lateral face of the D1 domain and CD4 binds to a hydrophobic crevice formed in the junction of the α_2 and β_2 domains of the MHC class II molecule (Zamoyska et al., 1998). The D1 and D2 domains of CD4 are intimately connected. The G-strand of D1 extends directly into the A-strand of D2, and a substantial interface is buried between these two domains. The crystal structure of fragment of rat CD4 consisting of the third and fourth extracellular domains (D3D4) reveals that this fragment is strikingly similar to the D1D2 fragment (Leaky, 1995). The intracellular portion of CD4 interacts strongly with a cytoplasmic tyrosine kinase called Lck and brings Lck close to the intracellular signaling components associated with the T cell receptor.

The structure of **CD8** is quite different. It is a disulfite-linked dimer of two different chains, called α and β, each containing a single Ig-like domain linked to the membrane by a segment of extended polypeptide. This protein segment is extensively glycosylated, to maintain it in an extended conformation and protect it by proteolytic cleavage.

CD8 binds weakly to an invariant site in the α_3 domain of MHC class I molecule and form interactions with the residues in the base of the α_2 domain.

The CD8 binding site to the MHC class I complex is formed by interaction between CD8 α and β chains.

The binding strength of CD8 to MHC I molecule is influenced by the glycosylation state of the CD8 molecule. Indeed**,** an increased number of sialic acid residues added to the CD8 carbohydrate structure decrease the strength of the interaction. Given that the pattern of sialylation of CD8 changes during the maturation of T cells and upon activation, studies suggest that this process may have a role in modulating antigen recognition (Moody ., 2003).

Antigen recognition by T cells receptors happens when peptides are bound to MHC molecules (Fig 1).

MHC class I and MHC class II molecules are closely related in overall structure but differ in their subunit composition.

MHC class I molecules consist of two polypeptides chains. One chain, the α chain, is encoded in the MHC and is non covalently associated with a smaller chain, β_2-microglobulin, which is not polymorphic and is encoded on different chromosomes.

The complete molecule has four domains, three formed from the MHC-encoded α chain and one contribute by β_2-microglobulin. The α_3 domain and β_2-microglobulin closely resemble Ig-like domains in their folded structure. The folded α_1 and α_2 domains form the walls of a cleft on the surface of the molecule; this is where the peptide binds and is known as the peptide-binding cleft or peptide- binding groove (Bouvier et al., 2003).

MHC class II molecules consists of a non-covalent complex of two chains α and β, both of which span the membrane. Each chain has two domains and the two chains together form a compact four-domain structure similar to that of the MHC class I molecule. (Fremont et al., 1996)

In both MHC class I and class II molecules, bound peptides are sandwiched between the two α-helical segments of the MHC molecule. The peptide is bound as an integral part of the MHC molecule's structure and MHC molecule are unstable when peptides are not bound.

Peptides that bind to MHC class I molecules are usually 8-10 amino acids long.

These interactions give MHC class I molecules a broad peptide-binding specificity. In addition MHC molecules are highly polymorphic. There are hundreds of different versions, or alleles, of the MHC class I genes in the human population. The main differences between allelic MHC variants are found at certain sites in the peptide-binding cleft, resulting in different amino acids in the key peptide-interaction sites. In consequence, the different MHC variants preferentially bind different peptides (Bouvier et al., 1994).

The peptide binding for the MHC class II molecules is different from MHC class I. Natural peptides that bind to MHC class II molecules are at least 13 amino acids long and can be much longer.

The peptide lies in an extended conformation along the peptide-binding cleft. It is held (1) by peptide side chains that protrude into shallow and deep pockets lined by polymorphic residues (2) by interactions between the peptide backbone and side chains that protrude into shallow and deep pockets lined by polymorphic residues (3) by interactions between the peptide backbone and side chains of conserved amino acids that line the peptide-binding cleft in all MHC class II molecules (Conant et al., 2003).

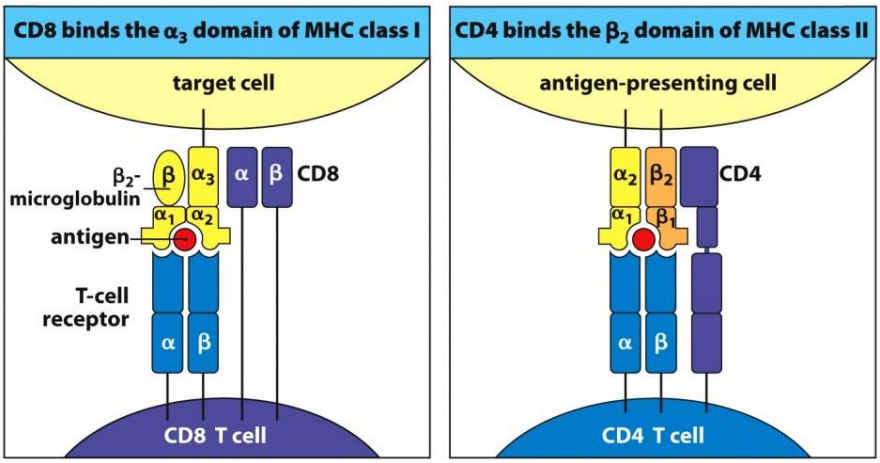

Figure 1: The binding sites for CD4+ and CD8+ on MHC class II and class I molecules.

The binding site for CD4+ lies at the base of the β_2 domain of an MHC class II molecule, in the hydrophobic crevice between the β_2 and α_2 domains. The binding site for CD8+ lies in a similar position to that of CD4+ in the MHC class II molecule, but CD8+ binding also involves the base of α_1 and α_2 domains (from Janeway's Immunobiology 8th edition 2012).

CD8+ cytotoxic cells (CTLs) are important in the defense against intracellular pathogens, especially viruses. Virus-infected cells display fragments of viral proteins, on the surface of MHC class I complex which are then recognized and then killed by cytotoxic T lymphocytes. Perhaps because the effector action of these cells is so destructive, naïve CD8+ T cells require more co-stimulatory activity to become activated. The activation provides the involvement of mature dendritic cells, which have high intrinsic co-stimulatory activity. In some viral infections, dendritic cells become sufficiently activated to directly induce CD8+ T cells to produce the IL-2 required for their proliferation and differentiation, without the support of CD4+ effector cells. In the majority of viral infections, however, CD8+ T cell activation requires additional help, which is provided by **CD4+ effector T cells**. Effector CD4+ T cells that recognize related antigens presented by the antigen-presenting cells can amplify the activation of naïve CD8+ T cells by further activating the antigen presenting cell. CD4+ T cells also produce abundant IL-2 and thus help drive CD8+ T cell proliferation (Weninger et al., 2002).

Cytotoxic CD8+ T cells lead to the death of the target cells by apoptosis. The killing steps require the receptor binding and the directed release of cytotoxic granules. Such proteins are stored in these granules in an active

form, but conditions within the granules prevent them from functioning until after their release. One of these cytotoxic proteins, known as perforin, act in the delivery of the contents of cytotoxic granules to target-cell membranes.
Another class of cytotoxic proteins comprises a family of serine protease, called granzymes. Still, another class of cytotoxic CD8+ T cells is granulysin, which is able to induce apoptosis.
However, most CD8+ cytotoxic T cells also release the cytokines IFN-ɣ, TNF-α and LT-α which contribute to host defense in several ways. IFN-ɣ inhibits viral replication directly and induces an increased expression of MHC class I molecules and of other proteins that are involved in the peptide loading of these newly synthesized MHC class I molecules in infected cells. This increases the chance that infected cells will be recognized as target cells for cytotoxic attack.
IFN-ɣ also activates macrophages, recruiting them to the site of infection both as effector cells and as antigen-presenting cells. TNFα and *LTα* can synergize with IFN-ɣ in macrophage activation (Prezzi et al., 2001).

In contrast with CD8+ T cells, **CD4+ T cells** differentiate into several subsets of effector T cells with a variety of different functions: T_H1, T_H2, T_H17 and regulatory T cells.

T_H1 cells help control bacteria that can set up intra-vescicular infections in macrophages, such as the mycobacteria. These bacteria are taken up by macrophages in the usual way but can evade the killing mechanism. If a T_H1 cell recognizes bacterial antigens displayed on the surface of an infected macrophage, it will interact with the infected cell to activate it, stimulating the macrophage's micro-biocidal activity thereby enabling to kill its resident bacteria.

T_H2 cells help to control infections by parasites through promoting responses mediated by eosinophils, mast cell and the IgE antibody isotype by promoting an immunity barrier on the mucosal surfaces (Bluestone et al., 2003).

The T_H1 and T_H2 subsets are distinguished principally by their production of specific cytokines, such as interferon (IFN-ɣ) and IL-2 by T_H1 cells, and IL-4 and IL-5 by T_H2 cells.

T_H17 cells secrete IL-17 family cytokines that induce local epithelial and stromal cells to produce chemokines that recruit neutrophils to sites of infection early in the adaptive immune response. (Littman et al., 2010)

All effector T cells are involved in activating their target cells to make responses that help clear pathogen from the body. The other CD4+ T cells found in the periphery have a different function. These are the regulatory T cells, whose function is to suppress T-cell responses. Two main groups of regulatory T cells are recognized. One subset becomes committed to a regulatory fate in the thymus; they are known as the natural regulatory T cells. Other subsets of CD4+ regulatory T cells with different phenotypes are thought to differentiate from naive CD4+ T cells in the periphery under the influence of particular environmental conditions. This group is known as induced regulatory T cells (Bluestone et al., 2003). These cells are distinguished by expression of transcription factor FoxP3 and cell-surface CD4 and CD25 and are produced when naïve T cells are activated in the presence of cytokine transforming growth factor-β (TGF-β) alone and in the absence of IL-6 and other pro-inflammatory cytokines.

The hallmark of the acquired immune system is memory. It results from the clonal expansion and differentiation of the antigenic-specific lymphocytes that confer immediate protection in peripheral tissues and mount recall responses to antigens in secondary lymphoid organs (Sallusto et al., 2004). Protective immunity is mediated by **effector memory T cells** (**T_{EM}**) that migrate to inflamed peripheral tissues and display immediate effector function. By contrast, reactive memory is mediated by **central memory** (**T_{CM}**). Although these cells which home to lymphoid organs, have little or no effector function, they readily proliferate and differentiate to effector cells in response to antigenic stimulation (Sallusto et al.,1999).

Human T_{CM} are CD45R0+ and costitutively express CCR7 and CD62L, two receptors that are also characteristic of naïve T cells. Following TCR triggering, T_{CM} produce mainly IL-2, but after proliferation they efficiently differentiate to effector cells and produce large amount of IFNɣ or IL-4.

Human T_{EM} are memory cells that have lost the constitutive expression of CCR7, are heterogeneous for CD62L expression and display characteristic sets of chemokine receptors and adhesion molecules that are required for homing to inflamed tissues. When compared to $T_{CM,}$ T_{EM} are characterized by rapid effector function. CD8+ T_{EM} cells carry large amounts of perforin and both CD4+ and CD8+ produce IFN-ɣ, IL-4 and IL-5 within hours following antigenic stimulation. In vivo antigens stimulate T_{CM} and T_{EM} in different ways. T_{CM} can be stimulated by

mature dendritic cells whereas T_{EM} can be stimulated by antigen presented by non-professional APC cells (Gunzer et al., 2000).

1.1.3 Antigen processing and presentation

MHC class I and class II molecules are similar in function: they present peptides at the cell surface to CD8+ and CD4+ T cells, respectively. These peptides originate from different sources: intracellular for MHC class I molecules and exogenous for MHC class II molecules and they are obtained by different pathways.

The classical pathway for presenting short peptides to CD8+ T cells in the context of MHC class I molecule involves cytosolic digestion of proteins to optimal length (8-10 amino acids) or N terminal extended proteins by the proteasome (Donoso et al., 2005; Tsvetkov et al., 2010). A typical proteasome is composed of one 20S catalytic core and two 19S regulatory caps, one at each end; both the core and the caps are multi-subunit complexes of proteins. The 20S core is a large cylindrical complex of some 28 subunits, arranged in four stacked rings of seven subunits each. It has a hollow core linked by the active site of proteolytic subunits. The 19S regulatory cap serves to recognize ubiquitinated client proteins and is thought to play a role in their unfolding and translocation into the interior of the 20S CP, which contains catalytic threonine residues on the surface of a chamber formed by two –rings. The two inner rings of the 20S proteasome core are composed of constitutively expressed proteolytic subunits called β1, β2, and β5 which form the catalytic chamber. These constitutive subunits are sometimes displaced by three alternative catalytic subunits, called LMP2, LMP7 and MECL-1 and are induced by interferons (Cascio et al., 2002).

The proteasome comprises two forms: constitutive proteasome present in all cells and the immunoproteasome present in cells stimulated with interferons. The replacement of the β subunits by their interferon-inducible counterparts alters the enzymatic specificity of the proteasome such that there is increased cleavage of polypeptides after hydrophobic residues and decreased cleavage after acidic residues. This produces peptides with carboxyl-terminal residues, which are preferred anchor residues for binding to most MHC class I molecules and are also preferred structures for transport by TAP(transporter associated with antigen). The production of antigenic peptides of the right length is increased by a modification of the proteasome induced by interferon-Ɣ. This is the binding to the proteasome of a protein complex called the

PA28 proteasome-activator complex. PA28 is a six or seven-membered ring composed of two proteins, PA28α and PA28β, both of which are induced by INF-Ɣ. A PA28 ring can bind to either end of the 20S proteasome core in place of the 19S regulatory cap, and acts to increase the rate at which peptides are released. In addition to simply providing more peptides, the increased rate of flow will allow potentially antigenic peptides to escape additional processing that might destroy their antigenicity.

Proteins are often tagged for degradation by the attachment of the ubiquitin molecule. Ubiquitination is a post-translational modification in which the 76 amino acid protein ubiquitin is covalently attached via its carboxyl terminus to Lys residues in target proteins. Ubiquitin can be attached to proteins as a single entity on one or multiple sites, yielding mono and multi-monoubiquitylated proteins. Poly-ubiquitylation is the formation of a ubiquitin chain on target proteins. The formation of poly-ubiquitin chains involves isopeptide bond connection between the carboxyl-terminal glycine residue of ubiquitin and an internal K residue or the amino-terminal (M1) of another ubiquitin. Different lysine residues could be involved such as Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63 thereby creating a large variety of ubiquitin chains (Fig 2).

Ubiquitin chains are assembled by a three-step enzymatic cascade comprising E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-ligating enzyme. The involvement of one lysine rather than another can address different subcellular processes.

Indeed, Lys48-linked ubiquitin chains are identified on proteasome substrate and Lys63- linked ubiquitin chains have a role in DNA repair, DNA damage response and cytokine signaling (Kulathu and Komander ., 2012).

Ubiquitination regulates different immune functions focusing on PRR (pattern-recognition receptor) signaling in innate immunity inflammation, DC maturation, T cell differentiation and tolerance. Especially, it is highly important to mark proteins that can be presented by MHC class I complex. In this way these target proteins can be processed by proteasome and elicit a CD8+ T cell response.

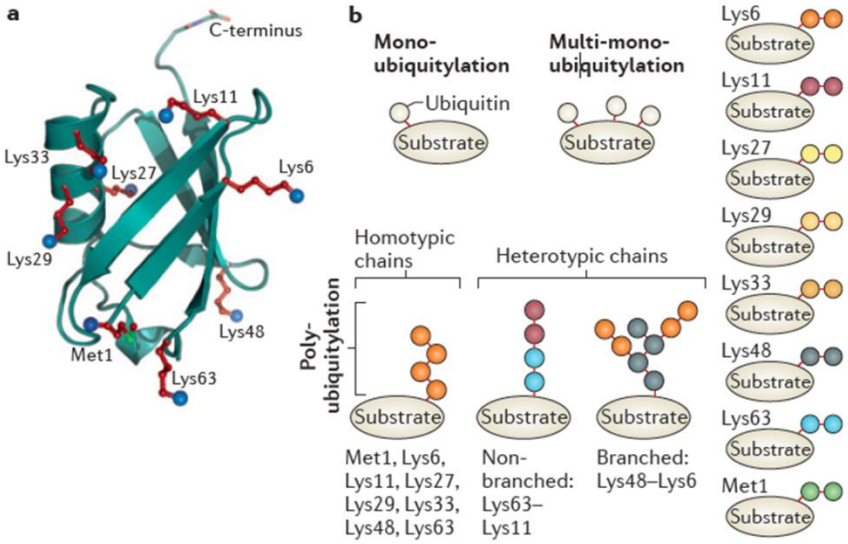

Figure 2: Forms of ubiquitination

a) The seven Lys residues and Met1 of ubiquitin are show in stick representation, and amino groups that are modified with ubiquitin during chain formation are shown as blue spheres. b) Overview of different forms of ubiquitination. A substrate can be modified by mono-, multi-mono or polyubiquitin (from Kulathu and Komander ., 2012).

The main substrates for the proteasome are cytosolic endogenous proteins and viral proteins for the classical MHC class I pathway and exogenous proteins re-trotranslocated from phagosomes or endosomes to the cytosol, for cross presentation (Raghavan et al., 2008; Blum et al., 2013). The peptides are transported into the ER by TAP (transporter associated with antigen processing) and where needed N terminal cleaved to optimal length peptides by endoplasmic reticulum aminopeptidase 1(ERAP1; ERAAP in mice) and ERAP2. These peptides associate with the protein-loading complex (PLC) which differs slightly between HLA allotypes, but usually involves: tapasin, endoplasmic reticulum protein 57 (Erp57), calnexin (CAL), calreticulin (CRT) and the MHC-I heterodimer. Loading of high affinity peptides stabilize the MHC-I molecule and releases PLC, allowing MHC-I: peptide complexes to traffic through the Golgi to the cell surface for presentation to CD8+ T cells (Hammer et al., 2006) (Fig 3).

The antigens presented by class II peptides are derived from extracellular proteins. These extracellular foreign antigens, such as bacteria or

bacterial antigens have been taken up by an antigen-presenting cell such as macrophage or immature dendritic cell. In other case, the source of antigen may be bacteria or parasites that have invaded the cell to replicate in intracellular vesicles. In both cases the antigen-processing pathway is the same. The pH of the endosomes containing the engulfed pathogens decreases progressively, activating proteases within the vesicles to degrade the engulfed material. At some point on their pathway to the cell surface, newly synthesized MHC class II molecules pass through such acidified vesicles and bind peptide fragments of the antigen, transporting the peptides to the cell surface.

The biosynthetic pathway for MHC class II molecules starts with its translocation into ER. However, they must be prevented from binding prematurely to peptides transported to ER lumen or to the cell's own newly synthesized polypeptides.

Binding is prevented by the assembly of newly synthesized MHC class II molecules with a membrane protein known as the **MHC class II associated invariant chain** (**Ii, CD74**).

When antigen peptides are ready to be loaded, the proteases cleave the Ii in the acidic vesicles, leaving the CLIP (class II-associated invariant chain peptide) bound to the MHC class II molecule. Pathogens and their proteins are broken down into peptides within acidified endosome, but these peptides cannot bind to MHC class II molecules that are occupied by CLIP. The class II-like molecule HLA-DM binds to MHC class II: CLIP complexes, catalyzing the release of CLIP and the binding of antigenic peptides.

A second atypical MHC class II molecule, called HLA-DO is produced in thymic epithelial cells and dendritic cells. This molecule is a heterodimer of the HLA-Doα chain and HLA-Doβ chain. HLA-DO acts as negative regulator of HLA-DM, binding to it and inhibiting both the HLA-DM- catalyzed release of CLIP and the binding of other peptides to MHC class II molecules. Expression of HLA-Doβ chain is not increased by IFN-Ɣ, whereas the expression of HLA-DM is. Thus, during inflammatory response, in which IFN-Ɣ is produced by T cells and NK cells, the increased expression of HLA-DM is able to overcome the inhibitory effects of HLA-DO (Fig 3).

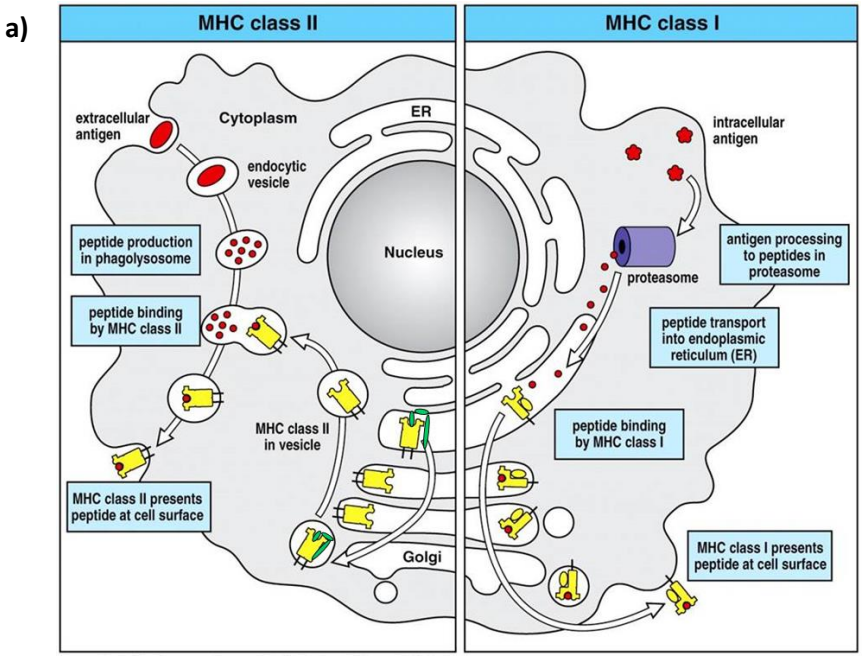

Figure 3: Antigen presentation on MHC class II and MHC class I molecules.

a) ***MHC class II pathway****: MHC-Ii α and β chains are translated by ER-associated ribosomes, they are brought together and stabilized into MHC II heterodimers by the Invariant chain. It forms a trimer and stabilizes three heterodimers to form a nonamer. Ii provides the endolysosomal sorting sequence, which leads the MHC II-Ii complexes via the trans-Golgi network to mature endosomes. MHC II-Ii can also reach the cell surface and be transported back into endosomes. In the acidic environment of the late endosome, MHC class II compartment (MIIC) and phagolysosomes cathepsins cleave Ii sequentially, leaving the CLIP peptide in the peptide-binding groove of MHC II molecules. HLA-DM associates with MHC II-CLIP, causing a conformational change that releases CLIP and allows cathepsin cleaved peptides to bind to MHC II. Protein substrates for cathepsins and other endosome resident proteases are delivered to the late endosomes by phagocytosis, endocytosis, and autophagy, allowing both exogenous and endogenous proteins to be presented on MHC-II. Binding of high affinity peptides releases HLA-DM and frees MHC II-peptide molecules to traffic to the cell surface for presentation to CD4+ T cells.*

b) ***MHC class I pathway****: MHC-I molecules present short peptides which are derived from native proteins, defective ribosomal products (DRiPs), viral proteins and retro-translocated exogenous proteins (for cross-presentation). The main source of peptides for MHC-I*

loading are the degradation products of the proteasome, which cleaves proteins in the cytosol to peptides that are transported via TAP to the ER. When in the ER peptides can be trimmed by ERAP-1 and ERAP-2 or they can bind directly to MHC-I heterodimers in protein loading complexes (PLCs). Initially MHC-I heterodimers in the ER are stabilised by interactions with calnexin (CXN), which allows the binding of β2microglobulin, and then association with calreticulin (CRT), tapasin, and ERp57. Tapasin stabilizes MHC-I heterodimers in the PLC in close proximity with TAP, which supplies cytosolic peptides. Only on binding of high affinity peptides are tapasin and the PLC released from MHC-I-peptide molecules, which are then allowed to traffic to the cell surface for presentation of peptides to CD8+ T cells (Janeway's Immunobiology 8th edition 2012).

An interesting link, termed cross-presentation, exists between the two pathways. In this process extracellular antigens, which are normally loaded on MHC class II complex, are instead presented by MHC class I molecules. The proposed pathway for cross-presentation could be divided into two main categories: those dependent on TAP and proteasome and those relatively independent of these factors (Raghavan et al., 2008). The latter, termed vacuolar pathway is where cathepsin S is the principal protease that generates antigenic peptides in the endo-lysosome and binds to MHC class I (Fig 4a).

For TAP and proteasome-dependent presentation, the antigen must reach the cytosol. It is possible that some antigens are transported in the cytosol after their uptake in DCs cells through the endoplasmic reticulum associated protein degradation (ERAD) by Sec61, a putative translocon channel (Fig 4b) (Ackerman et al., 2006).

ERAD is a complex process through which folding-defective proteins are selected and ultimately degraded by the ubiquitin-proteasome system. It can be envisioned as encompassing four distinct, coupled steps: 1) substrate recognition; 2) dislocation across the lipid bilayer; 3) addition of poly-ubiquitin adducts; 4) degradation by the 26 S proteasome.

At first molecular chaperones and lectins within the ER lumen interact with incompletely folded or unassembled clients. These factors link substrate recognition to the dislocation machinery by binding to membrane-embedded adaptors. Then, substrates are dislocated across the bilayer presumably through proteinaceous pores (dislocons), via a process coupled to the energy derived from ATP hydrolysis by VCP/p97. On gaining access to the cytosol, substrates are polyubiquitinated by E3

ligases, especially on Lys11 and these ubiquitinated substrates are degraded by cytosolic 26S proteasomes (Olzmann et al.,2013).

Another mechanism that allows the transport of antigen to the cytosol takes place from the phagocytic, endocytic or macropinocytic compartments. Gagnon et al (2003) demonstrated that the phagosomal membranes are formed in part from the ER membrane and the ERAD factors and components of MHC class I peptide loading machinery are found together within these membranes (Fig 4c).

Other possible pathway provides that soluble exogenous antigens are retro-translocated in the cytosol from the endosomal compartment. Then after proteasome processing, these peptides are re-imported back into endosomes via endosomal TAP that can be recruited to early endosome in a TLR4-Myd88 dependent manner, where peptide loading of recycling MHC class I occurs (Fig 4d) (Raghavan et al., 2008).

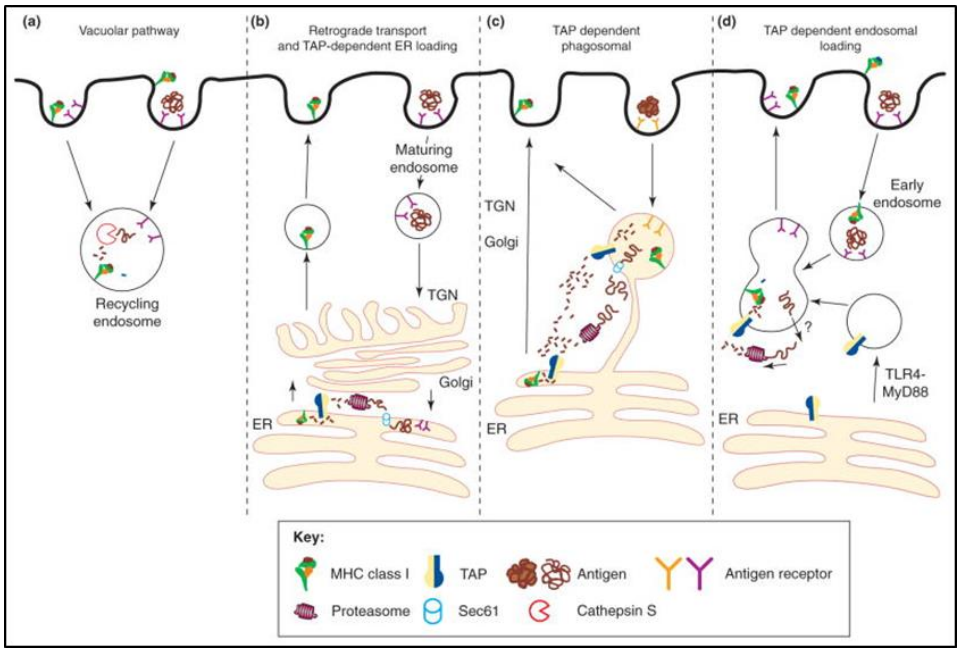

Figure 4: Different cellular pathways for antigen cross- presentation

a) Vacuolar TAP independent pathway: endocytosed antigens are proteolytically processed by cathepsin S and loaded on MHC class I into endosome.

b) *Retrograde translocation model: Once in the ER, the soluble antigens retro-translocate into the cytosol by ERAD machinery and process similarly to endogenous proteins for MHC class I presentation*
c) *TAP-dependent phagosomal pathway is supported by the presence of ER components on phagosomes. Phagocytosed antigens use Sec61 channel to exit from the phagosome, are processed by proteasome and are reimported into the phagosome for loading into MHC class I molecules within the phagosome.*
d) *TAP-dependent endosomal pathway: antigen egress from the endosome by an unknown transporter and after proteasomal proteolysis, processed fragments are ferried back into endosome by recruited TAP and loaded into recycling MHC class I molecules (Raghavan et al.,2008)*

1.2 Invariant chain (Ii) and its function

Ii is a non-polymorphic type II trans-membrane protein, first identified as the chaperone for MHC class II molecules (Blum et al., 2013). The main function of Ii is to stabilize the interaction of the α and β chains as they are formed in the endoplasmic reticulum (ER) and to occupy the peptide-binding groove of the MHC class II molecules. Ii inhibits peptide loading within the ER and targets MHC class II to the endosomal network via its endolysosomal targeting sequence signal peptide (ESS; Sandoval et al., 1994). After its synthesis, Ii is inserted in the ER thanks to its internal transfer peptide and its transmembrane domain. The complex Ii-MHC class II goes through Golgi and reaches the peripheral endocytic pathway where Ii is sequentially cleaved by cathepsins to CLIP peptide, which is then displaced by exogenous peptides for presentation to CD4+ T cells. In humans Ii is expressed as four different isoforms named according to their alternative molecular weights in kilodalton: Ii p33, Ii p35, Ii p41 and Ii p43. The isoforms are produced from a single gene through a combination of alternative splicing and alternative translation (Sand et al., 2014). The short isoforms, denoted p33 and p35, differ from the long isoforms, denoted p41 and p43, by a 64 amino acid insertion encoded by the alternatively spliced exon 6 in the C terminal luminal domain which has been shown to regulate the activity of cysteine proteases (Sand et al., 2014). In addition to this splicing variation, an alternative initiation codon gives rise to a 16 amino acid N-terminal extension present in the p35 and the p43 isoforms of human Ii. This additional segment confers retention in the ER consisting of three successive arginine residues (Fig 5).

The predominant form is the p33 form in humans that in mice is is termed p31.

Several post-translational modifications of the CD74 protein have been identified. N- and O-glycosylation has been reported for murine and human CD74. Furthermore, modification of a specific serine residue with glycosaminoglycans has been described (Miller et al., 1988). Human invariant chain is phosphorylated within its cytoplasmic domain.

Ii is capable of assembling into homo-trimers (Roche et al., 1991). The region responsible for its self-association is mapped between 118 and 193 residues. This trimerization domain is organized in a α-helical coiled structure, forming a scaffold that allows interaction with three MHC-II heterodimers (α: β) (Cresswell et al., 1996)

Other domains in the Ii structure are also important for its function.

The cytoplasmic tail of Ii contains two endosomal targeting sequences within amino acid residues 12-29 and the deletion of this segment reveal the presence of a second endosomal targeting sequence, located within the first 11 amino acid residues (Pieteres et al., 1993). The transmembrane domain might contribute to stable homotrimer formation (Stumptner-Cuvelette and Benaroch., 2002) and is also required for efficient delivery to endocytic processing compartment containing a signal distinct from the Ii cytoplasmic tail (Odorizzi et al., 1994).

This domain has long been implicated in both correct folding and function of the MHC class II complex. Specific mutations in the transmembrane domain have been shown to disrupt MHC class II functions such as mature complex formation and antigen presentation.

CLIP domain (amino acids 86-100) is important to prevent the premature binding of antigenic peptides while KEY (amino acids 76-79) plays an important role in CLIP loading on MHC class II peptide binding groove (Chen et al., 2012).

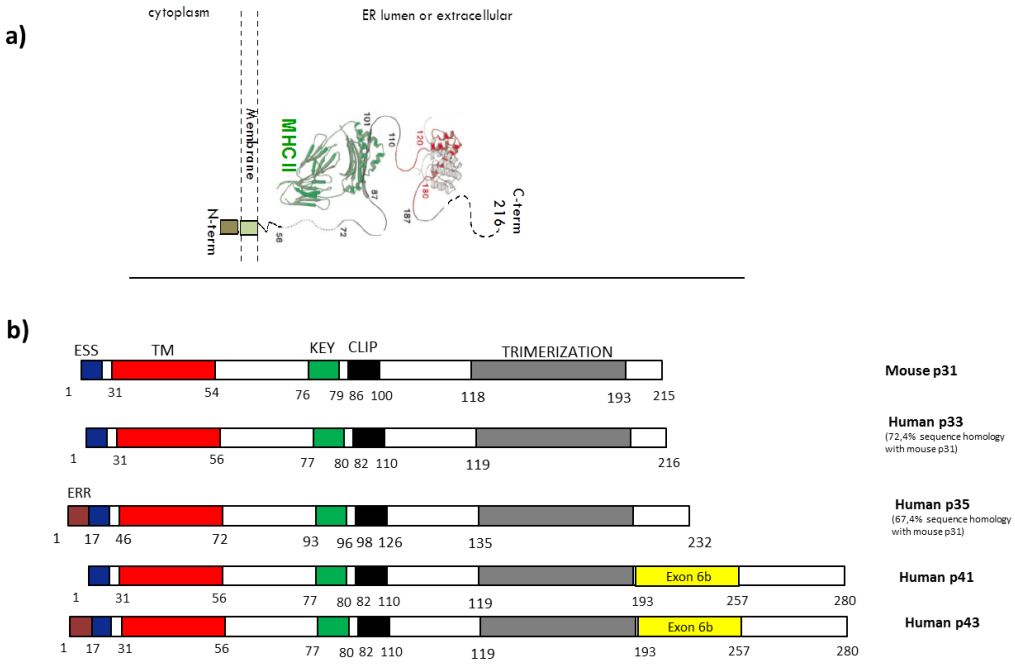

Figure 5: The known functional domains of Ii

a) *Ii is represented interacting with MHC class II (green) and with another Ii chain via its trimerisation domain (red).* ***(b)*** *Aligned representation of murine Ii and the main human isoforms of Ii. The key domains are labeled and numbered according to their amino acid position along the murine Ii sequence. The 30 amino acid cytosolic tail at the N-terminal of the Ii encodes the endolysosomal sorting sequence (ESS; a di-leucine based signal). Attached to this are a type-II transmembrane region (amino acids 31-54) and a large luminal domain, which contains the KEY motif and CLIP (class II-associated invariant chain peptide) - the residues that interact with MHC Class II molecules - and a trimerisation domain (aminoacids 118-193). CLIP is the section of Ii that sits directly within the binding groove of MHC class II molecules and KEY has a role in peptide exchange within this binding groove. Human p35 and p43 isoforms of Ii (numbered according to their molecular weight) have an additional 17 amino acids at the beginning of Ii, which encodes an ER retention sequence (ERR). Human p41 and p43 isoforms contain an additional exon.*

Ii is a complex protein with many functional domains and roles within the cell, not all of which are involved in antigen processing. In much the same way as it interacts with and controls the trafficking of MHC-II molecules, it acts as a chaperone for CD1d (Kang and Cresswell, 2002) and for the neonatal Fcγ receptor molecules (Ye et al., 2008), both of which are structurally related to MHC-II. Ii has been shown to associate

with CD70 (a CD27 ligand), by mediating its delivery to the immunological synapse (Zwart et al., 2010), and it binds to myosin-II, thereby limiting cell migration in DCs (Faure-Andrè et al., 2008). Ii also functions as a surface receptor for macrophage migration inhibitory factor (MIF) (Matza et al., 2002). The cytosolic domain of Ii is released upon binding MIF and it acts as an essential transcription factor in B-cell maturation and survival, by signaling through NfκB and by upregulating anti-apoptotic markers such as BCL-Xl and BCL-2 (Starlets et al., 2006).

Ii regulates the trafficking of additional molecules, such as angiotensin II type I receptor (AT1). It associates with AT1 and impedes its intracellular trafficking (Szaszak et al., 2008). Ii regulates the B cell development, DC motility and thymic selection.

1.3 Genetic vaccines and how to improve immune response

1.3.1 Why genetic vaccines?

Vaccines have been undeniably successful at inducing immune responses, most notably neutralizing antibodies that prevent viral or bacterial infections. However, to protect against more complex pathogens such as HIV, hepatitis C, malaria, tuberculosis (TB), it might be necessary to elicit also CD8 T cells of sufficient magnitude and effector function to recognize and rapidly clear infected cells before the pathogen establishes a chronic infection.

Pre-clinical and clinical evidence supports the role of T cell immunity and in particular $CD8^+$ T cells in the control and/or clearance of these diseases (Kim and Ahmed., 2010). Therefore, a rapidly expanding field in vaccinology is the development of so-called genetic vaccines. These are designed to induce antigen-specific $CD4^+$ and $CD8^+$ T cells of sufficient magnitude and necessary phenotype or effector function that directly contribute to pathogen clearance, rather than only $CD4^+$ T cell help for B cells leading to protective antibody responses.

One way to induce a T cell response against a given antigen is to express that antigen intracellularly, along with suitable pathogen-derived innate activators, through gene delivery; genetic or gene-based vaccines attempt to use physiological antigen processing and MHC class I presentation to activate a $CD8^+$ T cell response.

DNA vaccines were initially thought to be the ideal way to induce T cell responses.

1.3.2 DNA vaccination

DNA vaccine consists of a plasmid encoding a pathogenic gene and all elements needed to transcribe this gene in mammalian cells.
The use of DNA as a vaccine determines increased protein synthesis, augmented T cell help and MHC class I presentation and the production of specific cytokines and pathogen associated molecular patterns that increase innate immune activation leading to T-cell response as well as the production of antibodies against the encoded protein (Carvalho et al., 2010).
Once the sequence of the gene of interest has been optimized, inserted into the multiple cloning region of a plasmid backbone and delivered to the inoculation site through intramuscular or intradermal injection, the plasmid enters in the nucleus of transfected monocytes and of resident antigen presenting cells (APCs) by using the host cellular machinery.
APCs have a dominant role in the inducing the immunity of DNA vaccines by presenting vaccine-derived endogenous peptides on MHC class I molecules. APC cells can do this by a direct transfection by plasmid vaccine, by cross presentation of cell-associated exogenous antigens or lastly by engulfment of apoptotic transfected cells.
Antigen-loaded APCs travel to the draining lymph node via afferent lymphatic vessel where they present peptide antigens to naïve T cells via MHC and T cell receptor (TCR) in combination with costimulatory molecules. In response to peptide-bound MHC molecules and co-stimulatory secondary signals, activated CD4 T helper cells secrete cytokines during cell-to cell interaction with B cells.
In this way the two arms of the immune system are elicited by DNA immunization (Fig 6).

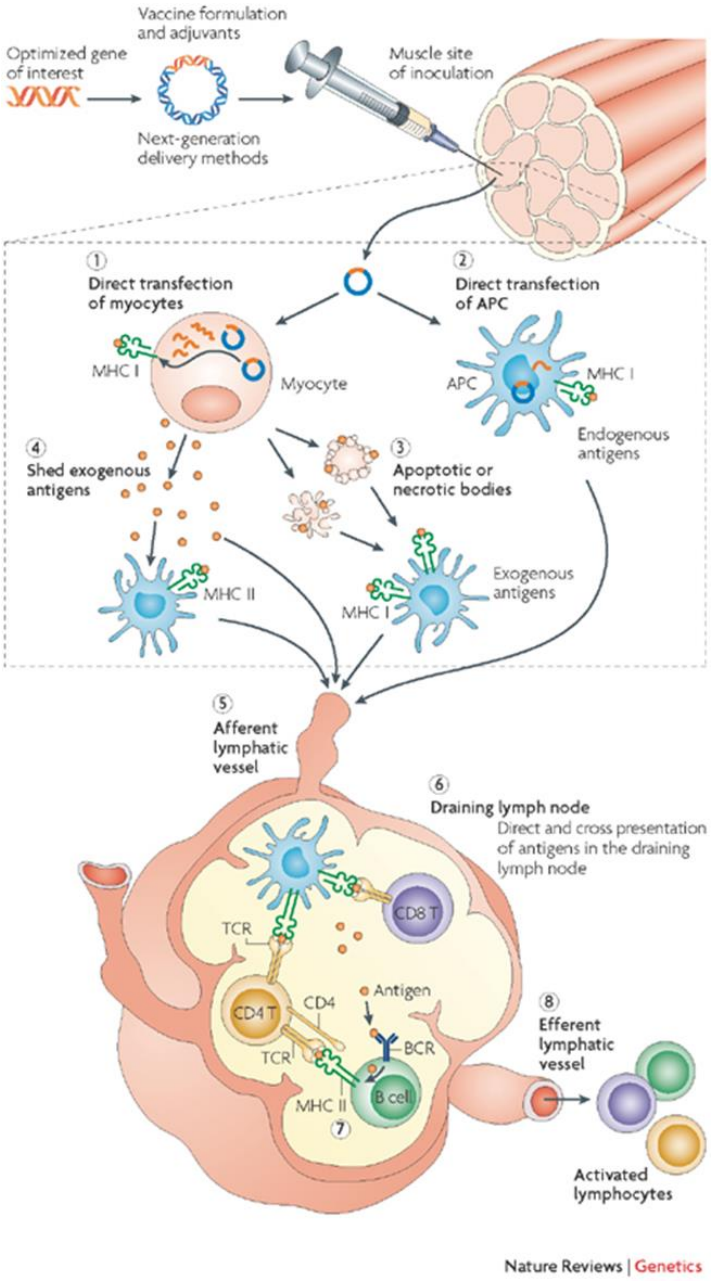

Figure 6: Cellular and humoral immunity after DNA vaccines

The optimized sequence of the gene of interest is inserted in a plasmid backbone, purified and delivered to the inoculation site. Using the host cellular machinery, the plasmid enters the nucleus of transfected monocytes (1) or APC cells (2). The plasmid components initiate gene transcription, which is followed by protein production in the cytoplasm. These host-synthetized antigens could be elicited immune system through MHC class I or class II complexes. APCs have an important role in the induction of immunity of DNA vaccines by presenting vaccine endogenous peptide to MHC class I or cross-presentation of cell-associated exogenous antigens: for example owing to APC engulfment of apoptotic transfected cells (3).

APC mediate the display of peptides on MHC class II molecules after secreted protein antigens that have been shed from transfected cells are processed by endocytic pathway(4). Antigen-loaded APC travel to the draining lymphnode (DLN) via the afferent lymphatic vessel (5) where they present antigens to naïve T cells via MHC and TCR (6). In response to peptide-bound MHC molecules, CD8+ T cells and CD4+ T helper cells (7) are activated. The latter ones activate also B cells. Now activated cells can travel through the efferent lymphatic system (from Kutzler and Weiner., 2008).

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A series of trials of prophylactic and therapeutic DNA vaccines in humans against various pathogens, including HIV, HCV and malaria have been tested.

Indeed, McConkey et al (2003) have demonstrated that a heterologous prime-boost vaccination regime of DNA administered either intramuscularly or epidermally, followed by intradermal recombinant modified vaccinia virus Ankara (MVA) induces high frequencies of interferon (IFN)-ɣ secreting antigen- specific T cell responses in humans to a pre-erytrocytic malaria antigen, thrombospondin-related adhesion protein (TRAP).

This vaccination regime determines responses that are five to ten folds higher than the T cell responses induced by DNA vaccine or MVA vaccine alone. In addition it produces a partial protection through a delayed parassitemia after sporozoite challenge with a different strain of Plasmodium falciparum.

The use of a DNA vaccine presents a series of pros and cons. The pros are that, compared to conventional approaches the plasmids can be manufactured by generic processes, including standardized quality control and storage conditions that are independent of the encoded sequence (Carvalho et al., 2010). They can be manipulated to co-express cytokines or other molecules to enhance immune response.

The cons are that the generated T cell responses are very low magnitude and after incorporation of immunizing DNA, various oncogenes could be activated along anti-DNA antibodies.

Different strategies have used an adjuvant to enhance immunogenicity of DNA vaccines in humans, but the improvement has been far too modest (Baden et al., 2011).

Other possible problems regard a suboptimal delivery, poor antigen expression and the lack of a localized inflammatory response, all essential for antigen presentation and an effective immune response to the immunogens (Li et al., 2012).

1.3.3 Viral vector based vaccines

An alternative powerful technology for delivering heterologous antigens is based on the use of viruses as vaccine vectors. It is possible to delete crucial region of viral genome to render the virus as replication-defective (Tatsis et al., 2004). Virus-derived vectors offer different advantages including the efficient delivery of exogenous gene into target cells, high level of production of proteins antigens within cells of the immunized host and the adjuvant effects derived from the use of viruses as vectors exploiting their own propriety. A number of organisms have been used for vector vaccines, including poxviruses, alpha viruses, and lentiviruses.

Poxviruses have been used as vaccines for infectious organisms and tumors because they are high immunogenic and high readily engineered for their large capacity to insert foreign genes (at least 25kb), thus enabling multivalent vaccines to be created. Further they are safe in humans and had a series of properties including: broad tropism of the virus in mammalian cells, localization of the virus in the cytoplasm thus avowing integration risk; several routes of administration and the ability to induce both antibody and cytotoxic T cell response after a single inoculation (Folgori and Capone; 2012). Vaccines based on poxviruses are derived from vaccinia virus or members of the avipox genus. Indeed, non-replicating vectors, such as avipox or fowlpox, which confer extended expression times and enhanced immunogenicity, are now undergoing clinical trials (Mastrangelo et al., 2000). Two main types of approaches have been taken to enhance the safety of vaccinia virus. The former consists, based on scientific knowledge, to delete viral genes which are involved in nucleic acid metabolism, host interactions, and extracellular virus formation. The latter consists in successive passage of the virus in an unnatural host or in tissue culture, and the isolation of virus variants. Both approaches have led to safe vaccinia virus strains.
The safety of vaccinia virus can also be enhanced by multiple deletions. This has been demonstrated by the engineering of the NYVAC strain of vaccinia virus (Tartaglia et al., 1992) derived from the Copenaghen strain of vaccinia and rendered replication incompetent by 18 specific engineered deletions.
Successive passage of the virus in an unnatural host or in tissue culture, and the isolation of virus variants, has also lead to safer strains of vaccinia virus. The most notorious example is the modified vaccinia virus Ankara (MVA) strain which was isolated after more than 500 passages in chicken embryo cells. MVA has lost the ability to replicate in mammalian cells, is a pathogenic even for immunodeficient animals, and

was administered without apparent incident to about 120,000 humans including many who were considered a poor risk for the conventional smallpox vaccine. MVA replication cycle is blocked at a late stage of morphogenesis in mammalian cells and an examination of the MVA genome revealed six major deletions totaling approximately 31 Kb. Marker transfer experiments indicate that multiple gene defects need to be corrected for efficient replication of MVA in mammalian cells. The safety of MVA has been demonstrated in pre-clinical studies of immunodeficient mice and immunosuppressed macaques (Wyatt et al., 2004; Stittelaar et al., 2001) and in Phase I clinical trials evaluations of MVA as a next generation smallpox vaccine (Parrino et al., 2007).

Another viral- vectored based vaccine entails the use of alpha viruses. Alpha viruses are positive-stranded RNA viruses that have a broad host range and therefore are capable of replicating in the cytoplasm of infected cells.

The single-stranded alpha virus genome is divided into two ORFs. The first ORF encodes the nonstructural proteins that are translated upon entry of the virus into the cytoplasm and are responsible for transcription and replication of viral RNA. The second ORF is under the control of a sub-genomic promoter and normally encodes the structural proteins, which are responsible for encapsidation of viral RNA and final assembly into enveloped particles. Expression vectors have been engineered replacing structural genes with heterologous genes up to 5kb while structural proteins are provided *in trans* from two helper transcripts that lack a packaging signal. These RNA vectors, known as replicons are single cycle vectors incapable of spreading from infected to non-infected cells. Alpha virus replicon vectors are being developed as a platform vaccine technology for numerous viral, bacterial, protozoan and tumors antigens where they have been shown to be efficient inducers of both humoral and T cell responses. In addition, as the alpha virus structural proteins are not expressed in vaccine recipients, anti-vector immune responses are generally minimal, allowing for multiple effective immunizations of the same individual (Rayner et al., 2002).

The new alpha viruses that are being developed as vaccine vectors include Venezuelan equine encephalitis virus (VEE), Sindbis virus (Sin), Semliki forest virus (SFV) and VEE-SIN chimaeras (Thornburg et al., 2007). Several features of alpha viruses make them useful for vaccine development: (1) they infect a broad range of animals, including humans, often with mild or no symptoms.; (2) the seroprevalence to alphaviruses is low, thus reducing the probability of interference with immune responses to vectored gene products; (3)they have or may be engineered

to have lymph node tropism that results in effective antigen presentation and induction of a strong and balanced immune response (Rayner et al., 2002). Further the immune responses are enhanced because as the self-amplification of the vector RNA through a double-stranded RNA intermediates which stimulate activation of interferon cascade and multiple innate signaling pathways (Naslund et al., 2011). The vectors also induce apoptosis in some cells types and the release of apoptotic bodies that are efficiently taken up by antigen-presenting cells can result in enhanced immune cross-priming (Perri et al., 2003). There are three main approaches described for the use of alpha virus as vaccine vectors: virus-like particles, layered DNA-RNA vectors and replication-competent vectors. Virus-like particles are non-replicating vectors and are able to undergo only one cycle of expression. Layered DNA-RNA vectors express SFV replicon from a cDNA copy via a cytomegalovirus promoter. Replication-competent vectors contain as the gene of interest as the structural genes important for replication machinery. Packaged SFV-, SIN- and VEE-based replicon vectors have been demonstrated to induce robust humoral, mucosal and cellular immune responses in animals. At the same time, there aren't neutralizing anti-vector antibodies to ensure the use of these vectors as booster (Durso et al., 2007).
The chimera VEE/SIN has been developed exploiting the replication proprieties of VEE and the envelope glycoprotein packaging component of SIN. In mice this chimera ensured a potent immune response as VEE its self, being it is a pathogenic virus. Also it is superior than SIN or SIN-VEE chimera (Perri et al., 2003). Other studies in macaques showed that the VEE/SIN chimera elicited more potent systemic and mucosal immune responses to an inserted HIV envelope gene product compared to the SIN vector alone (Gupta et al., 2006).

A specific T cell response can be also induced using recombinant lentiviral vectors (LV) as vaccines. LV are able to transduce non-dividing cells, such as dendritic cells, at high transduction efficiencies and to allow persistent antigen presentation through high level expression of transgenes and low interfering anti-vector immune responses (Breckpot et al., 2003). It has been shown that LV encoding HIV-1 polyepitopes induces CD8+ T cell response in mice (Iglesias et al., 2007). Beignon and colleagues (2009) showed that a prime/boost vaccination strategy using lentiviral vectors pseudotyped with a glycoprotein G from two non-cross-reactive vesicular stomatitis virus serotypes elicited robust and broad cellular immune responses against the vector-encoded antigen, simian immunodeficiency virus (SIV) GAG, in cynomolgus macaques. Vaccination conferred strong protection against a massive intrarectal

challenge with SIVmac251, as evidenced both by the reduction of viremia at the peak of acute infection and by the full preservation of the CD28+ CD95+ memory CD4+ T cells during the acute phase.
Despite the use of lentivirus as vectors ensures the advantage to deliver the transgenes into dendritic cells, vector integration in the host cell genome has provoked safety concerns. For this reason Wanisch et al (2009) generated an integration-deficient lentiviral vectors (IDLVs) by interrupting the function of integrase or its attachment sites in the vector backbone. Despite the integration is inhibited, the vectors continued to infect dividing cells and maintained durable transgene expression in non-dividing cells (Philippe et al., 2006). Preliminary experiments provide the single injection of IDLV encoding the envelope protein of either HIV-1 (Negri et al., 2007) or West Nile virus (Coutant et al.,2008) resulted in significant and prolonged immune responses against inserted antigen. Other reports showing that after immunization in mice with IDLV encoding viral or tumor antigens, antigen-specific immune responses were elicited (Karwacz et al., 2009).

One of the most potent technologies demonstrated to induce CD8+ T cell response in mice, primates, and humans is based on adenovirus vector (Ad).
Adenoviruses are double-stranded DNA viruses with a genome of 34-43 kb. The linear genome flanked by two origins for DNA replication (ITRs) has eight units for RNA polymerase II-mediated transcription. The genome carries five early units (E1A, E1B, E2, E3,E4 and E5), two units that are expressed with a delay after initiation of viral replication (IX and Iva2) and one late unit(L) that is subdivided into L1-L5.
The E1A unit activates transcription of the other viral genes. Deletion of E1A renders the virus replication-defective. E1A stimulates viral DNA synthesis, deregulates cell-cycle control by increasing the stability of p53 and promote apoptosis unlike E1B that has anti-apoptotic activity.
The E2 unit encodes DNA-binding proteins and a polymerase and is essential for viral replication. The E3 unit is nonessential for virus replication. E3 proteins allow the virus to escape immunosurveillance by reducing expression of major histocompatibility complex (MHC) class I determinants.
The E4 transcription unit encodes seven polypeptides through distinct open reading frames (ORFs), which affect viral transcription and a number of host cell functions including cell proliferation and apoptosis.
The late transcription units, divided into five subunits (L1-L5) encode as many as 45 different species of RNA. Products of the late transcription unit form the viral capsid (Rux et al., 1999).

Generation of adenoviral vectors where it is possible to clone the gene of interest (GOI) entails three steps. The first step entails the cloning of GOI into the shuttle vector. Shuttle vectors are commercially available. The most common shuttle vector is pshuttle-CMV. The second step entails the homologous recombination in vivo in bacteria. In particular, the shuttle vector containing GOI is linearized and introduced into highly electro competent BJ5183 bacterial cells pre-transformed with supercoiled backbone plasmid vector containing most of the viral genome flanked by inverted terminal repeat (ITR). The third and final step entails virus production in packaging cell lines. In particular, PacI-digested linearized recombinant adenoviral DNA containing GOI is transfected into packaging cell lines, *viz*, HEK-293 cells or PER.C6 cells. Viruses are then harvested 14-20 days later (Luo et al., 2007).

The basic shuttle vectors have–1) resistance cassettes (Ampicillin or kanamycin), 2) multiple cloning sites with highest capacity to accommodate a transgene, 3) a promoter (usually CMV promoter) and a polyadenylation signal, and 4) stretches of sequence homologues to the backbone vectors to facilitate homologous recombination.

The backbone vectors, instead, retain the majority of human adenovirus serotype 5 (Ad5) with deletions of E1 and E3 genes which create space for transgenes and eliminate self-replication capacity. Depending on the size of the gene of interest, there is the possibility to delete the E4 gene to have more space. It also carriers ampicillin resistance, which is lost after recombination with shuttle vector (Fig 7).

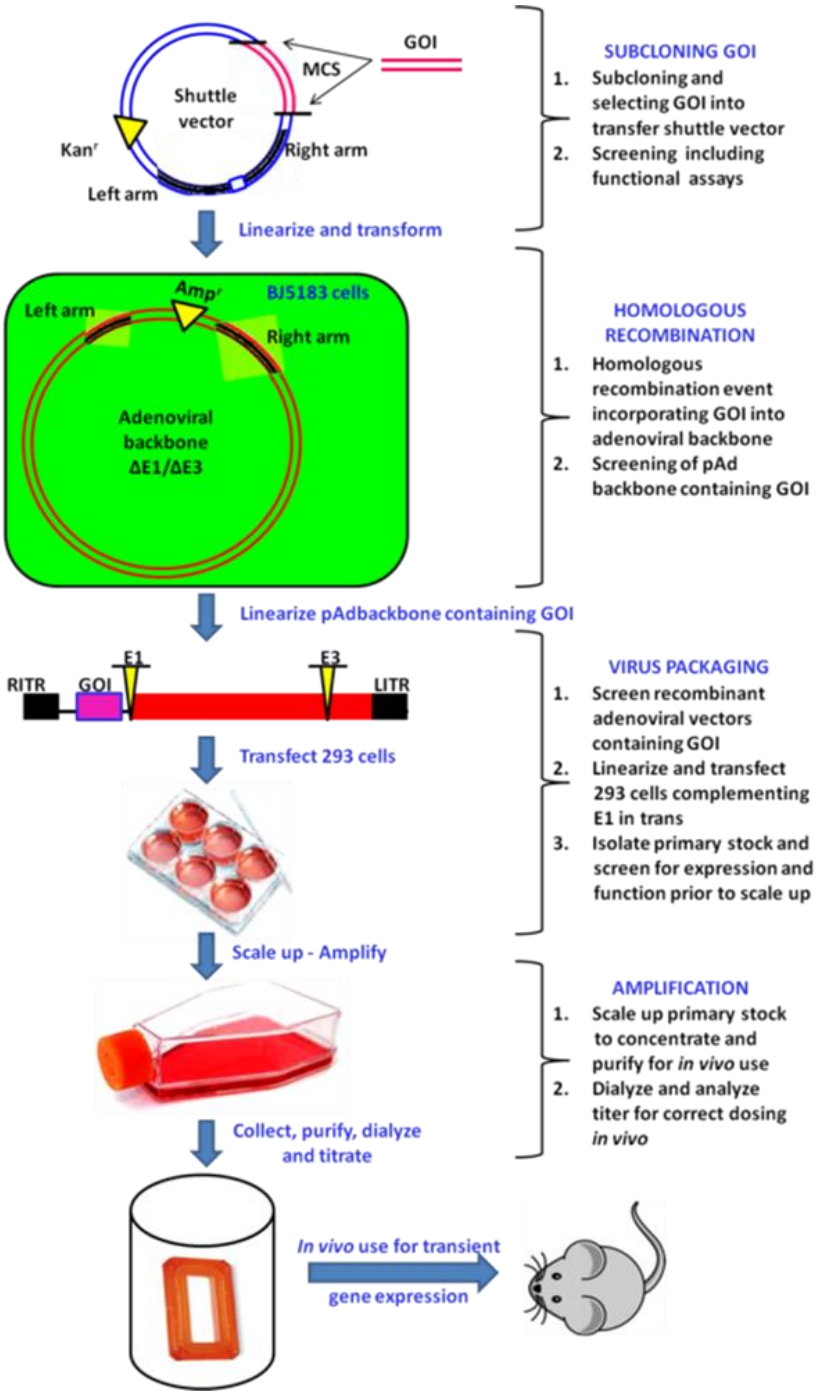

Figure 7: Schematic illustration of generating recombinant adenoviruses

Three independent steps are important for recombinant adenoviruses production: 1) subcloning GOI into a shuttle vector which transfers the GOI into the pAd plasmid containing the adenoviral backbone 2) during homologous recombination in bacterial system, and 3) packaging recombinant adenoviral DNA containing GOI in 293 cells complementing E1 in trans. Recombinant adenoviruses are collected, amplified, concentrated and titrated suitable for in vivo application (from Luo et al., 2007).

Adenoviruses are highly immunogenic. They activate the innate immune system presumably by expressing so-called pathogen-associated molecular patterns (PAMPs), induce maturation of dendritic cells (Medzhitov et al., 2000), but principally enhance cellular immunity.

In preclinical models, these vectors are shown to induce potent transgene-specific T and B cell response. T cells are mainly CD8+ T cells though low CD4+ T cell responses of the Th1 are also induced (He et al., 2000).

Most importantly, head to head comparisons with other genetic vaccines (i.e. poxviruses, lentiviruses, alpha virus-based vectors and naked DNA) in animal models and the results obtained in human clinical trials, including vaccines against Ebola, SARS, HIV and Anthrax clearly show that Ad5-based vectors represent the most potent currently available delivery system for eliciting a CD8 T cell response against the encoded antigens (Casimiro et al., 2003).

Bassett et al (2011) demonstrate that unlike most acute infections, the CD8+ T cell memory population, elicited by persistent, low-level transgene expression from recombinant human adenovirus serotype 5, shows an effector memory phenotype.

The memory population is composed of effector (T_{EFF}; CD127- CD62L-) and effector memory (T_{EM}; CD127+CD62L-) T cells with little evidence of central memory (T_{CM}; CD127+ CD62L+) T-cell accumulation (Yang et al., 2006).

Moreover, Adeno vectors have a natural aptitude for increasing innate immunity, thanks to the activity of their structural viral proteins.

Different pathways, belonging to innate immunity, are implicated in the response against Adeno, including Toll-like receptors (TLRs).

Indeed, Appledorn et al (2009) have evaluated the role of TLR pathway signaling in Ad-specific inductions of innate and adaptive immunity and have identified different TLR proteins that contribute to immune response.

TLRs are type 1 transmembrane pathogen recognition receptor proteins that contain leucine-rich repeat extracellular domains and a cytosolic toll-interleukin-1 receptor (TIR) homology domain that is essential for intra-cellular signaling. Upon activation, TLRs recruit TIR containing adaptor molecules, including myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-(TRIF, also known as TICAM1).These two adaptor molecules have a critical role in mediating TLR signaling in a temporal manner and determine the production of different cytokines.

Using MyD88 and TRIF double knockout mice, Applendorn et al (2009) demonstrated that these adaptor proteins can play either additive or redundant roles in mediating certain aspects of Ad vector-induced immune responses.
The improved ability of Adeno vectors to elicit immune response with respect to other vaccine formulations has been demonstrated by Shiver et al (2002). They have compared the immunogenicity of several vaccine vectors in three different formulations: DNA vector, modified vaccinia Ankara vector (MVA) virus and a replication incompetent adenoviruses type 5 (Ad5) vector expressing the SIV gag protein belong to simian immunodeficiency virus of rhesus monkey. They tested the vaccines either as a single modality or in combined modality regimens. They found that replication-incompetent Ad5 vector, used alone or as a booster inoculation after priming with a DNA vector induced the most potent response. Moreover, they showed that challenging animals previously immunized with Ad5 vector with a pathogenic HIV-SIV hybrid virus (SHIV), promoted a highly pronounced attenuation of the virus infection.

Steffensen et al. (2012), also, demonstrated that a DNA-primed immune response can be effectively boosted by the Ad35 vector and that this response can be further boosted by Ad5 vector. This series of vaccinations leads to a memory CD8+ T cell population characterized by low CD27 expression and high CD127 and killer cell lectin-like-receptor subfamily G member 1 (KLRG1) expression. These memory CD8 T cell are able to proliferate in response to viral challenge and to protect against infection.
Human adenoviruses vectors are attractive for several reasons.
First, they possess a stable virion, wide cell tropism and the transferred information remains epichromosomal, avoiding the risk of insertional mutagenesis. Second, they are safe and can be propagated easily with good yields in complementing cells lines. Finally, compared to DNA vaccines, adenoviral vectors more efficiently enter in the transduced cells, especially APC cells and induce high level of protein expression.
However, the majority of the human population is exposed to Ad5 in first years of life and develops high titer of anti-Ad5 neutralizing antibodies (nAb) against the adenovirus capsid. These pre-existing Ad5 nAb impair the immunogenicity of Ad5-based vaccines in animal models and humans and may also potentially compromise their safety.
To overcome this drawback, several strategies are used including rare human adenovirus serotypes such as Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50. All of these serotypes are rarely neutralized by antibodies present in humans and are currently being evaluated in a

number of pre-clinical and clinical studies (Abbink et al., 2007). However adeno vectors from different serotypes have lower immunological potency than Ad5 in mice and in non-human primates (Colloca et al., 2012). Another approach is to encapsulate adeno vectors in inert polymer or to use nonhuman adenoviruses, such as simian and porcine vectors. Indeed, Colloca et al., (2012) have generated a large collection of replication defective vectors based on Ad isolated from chimpanzees (ChAds). These types of vectors are strongly related to human Ads, showing high degree of DNA homology with human Ads belonging to the same species and similar genomic structure. All ChAds which reveal significant diversity in the hypervariable regions in the hexon protein from the highly sieroprevalent Ad5, are not neutralized in vitro and in vivo by anti-Ad5 antibodies and some of these have an equivalent immunological potency of Ad5 such as ChAd3,ChAd63,ChAd83,PanAd1,PanAd2 and PanAd3. Indeed two of the most potent ChAd vectors have been selected for clinical studies as carriers for malaria and hepatitis C virus (HCV) genetic vaccines (Colloca et al, 2012).

ChAd63 encoding for pre-erythrocytic insert multiple epitope thrombospondin-related adhesion protein for malaria ME-TRAP, was tested in a phase I clinical study from 2007 to 2010. It was administered alone or in a prime-boost regime with a modified vaccinia virus Ankara (MVA) ME-TRAP 8 weeks later. At the end of the study, ChAd63 resulted safe and highly immunogenic (O'Hara et al., 2012).

In a recent study, two other adenoviral vector expressing NS proteins from HCV genotype 1b were constructed based on rare serotypes such as human adenovirus 6 (Ad6) and chimpanzee adenovirus 3 (ChAd3). They were tested in a phase 1 study of healthy human volunteers (Barnes et al., 2012). Both vectors primed T cell responses against HCV proteins, by targeting multiple proteins and were able to recognize heterologous strains (genotypes 1A and 3A). HCV-specific T cell consisted of CD4 and CD8 T cell, secreted IL2, IFNɣ and TNFα and was sustained for at least 1 year after boosting with heterologous adenoviral vector.

Swadling et al (2014) assessed the immunogenicity of ChAd3 vector encoding for NS3, NS4, NS5A and NS5B proteins of HCV genotype 1b in a prime-boost regimen with MVA virus in healthy human volunteers. The specific T cell response, elicited after prime, was optimally boosted with MVA, generating high level of CD4+ and CD8+ T cells targeting multiple HCV antigens. Furthermore, elicited T cells showed a effector memory phenotype that evolved over time with improvement of quality after boost.

Altogether, these preclinical studies highlight that an adenovirus prime followed by an MVA boost is a powerful strategy to induce a strong and durable T cell response.

1.3.4 Improve immunogenicity of viral vector-based vaccines

Different strategies had been explored to improve immune response against antigens inserted in DNA and viral-vectored vaccines. Improved responses might be needed to reduce the dose of the vaccine or the number of injections; and to improve the efficacy of vaccines in newborns, the elderly, and immunocompromised people.

Strategies employed to enhance immunogenicity of DNA vaccines included: 1) addition of CpG sequences in the vector to increase its immunological propriety; 2) maximize the antigen presentation using strong constitutive promoters by adding regulatory elements that work as transcriptional enhancers or by optimizing the codon usage of the antigen sequence; 3) co-administration of DNA with cytokine such as IL-2, IL-23, or GM-CSF to boost –immunization with genes encoding protective antigens much more effective; 4) co-administration of DNA with lipid complexes, micro particles or other delivery formulations to increase transfection and expression efficacy (Cheung et al., 2004; Barouch et al.,2005).

Another approach to improve immune response after DNA vaccine is to use signal sequences that target the antigen to intracellular compartments. For example, the invariant surface glycoprotein (ISG) and the N-terminal trans-sialidase (nTSA) of *Trypanosma brucei* were used as an antigen model by Silva et al., 2009. In their study, the authors fused three different targeting sequences to the antigens such as secretion signal and lysosomal-associated membrane protein (LAMP-1) sequence (Ahlen et al., 2007; Ulmer et al., 2006).

It is also possible to co-express the immunogen protein and the cytolytic protein, perforin (PRF), a protein of 67kda which upon degranulation, that leads to a non-apoptotic cell death. This strategy is adopted for human immunodeficiency virus-1 (HIV) Gag protein, a model antigen luciferase or HCV proteins after intradermal DNA vaccination (Gargett et al., 2014; Gummow et al., 2015).

Bartholdy et al (2003) demonstrated that the linkage of MHC class I restricted epitopes of lymphocytic choriomeningitis virus covalently linked to human β2 microglobulin (β2m) in a DNA vaccine results in a major presentation of the inserted epitope covalently fused to the MHC complex. Mice vaccinated with the DNA vaccine encoded for epitope

linked to β2m show stronger recall response than mice vaccinated with a conventional mini-gene construct that codifies for an immunodominant peptide from a given pathogen.

The same results were obtained by Holst et al (2007) using recombinant replication deficient adenovirus to express either full-length protein or minimal MHC class I restricted epitopes from lymphocytic choriomeningitis virus (LCMV). These authors demonstrated that minimal immunodominant epitope covalently linked to β2-microglobulin determines a CD8+ T cell response that is faster and more consistent than the CD8+ T cell response induced by the full length viral glycoprotein. The generated CD8+ T cells provide long-term protection from virus infection, thus adding support to the role of CD8 T cell-memory in protection from lethal infection.

This strategy uses an Ad5 vector encoding for a TLR agonist derived from Eimeria tenella (EA) as an adjuvant linked to HIV-Gag. The expression of rEA elicits TLR mediated innate immune responses by evaluating the production of chemokines or cytokines and activation of the innate immune system in mice (Appledorn et al., 2010).

Other approaches are based on linking to the antigen the herpes virus VP22 protein and calreticulin which have been tested in Sindbis virus replicon particles (Cheng et al., 2002) and vaccinia vectors (Hsieh et al., 2004), and the herpes viral glycoprotein D, which has been tested using Ad vectors (Lasaro et al., 2008).

Since viral vectors are good inducers of CD8+ T cell response and the ubiquitination has an important role in the processing of antigens loaded in MHC class I molecules, several vaccine strategies could exploit it to increase antigen presentation.

Rodriguez et al (1997) set out a DNA transfection experiment using a plasmid encoded LCMV viral antigen linked to ubiquitin molecule at its N terminus. They showed an enhanced intracellular degradation of LCMV nucleoprotein (NP) thanks to the linkage of ubiquitin. The ubiquitinated NP is hardly detectable at steady state, but is easily visualized after proteasome inhibition using ALLN inhibitor, suggesting a rapid turnover of ubiquitinated protein and an important role of proteasome in degradation of the ubiquitin-tagged proteins. In vitro cytotoxicity assay, using MC57 (MHC haplotype b) and BalbCl7 (MHC haplotype d) cells transfected with DNA plasmids encoding for NP and Ub-NP and splenocytes derived by mice infected with LCMV virus as effector cells showed that the presence of ubiquitin-NP products led to an improvement of MHC class I presentation, since transfected tissue culture cells are more sensitive to CTL lysis. Further mice immunized with plasmid DNA encoding this rapidly degraded protein are able to

mount an enhanced antiviral CTL induction and protective antiviral immunity. Bazhan et al (2010) designed DNA vaccines encoding for ten selected HLA-A2 epitopes from the major HIV antigens evaluating also different approaches to improve their immunogenicity through the linkage of ubiquitin molecule to the N terminus of poly-epitope to increase the targeting to proteasome, or use spacer sequences between epitopes to optimize proteasome liberation and TAP transport. After a first in vitro analysis, they assessed the immunogenicity of these constructs through double DNA prime and single vaccinia virus boost immunization of HLA-A2 transgenic mice. They showed that most immunogenic vaccine construct contained the N-terminal ubiquitin for targeting the poly-epitope to the proteasome and included both proteasome liberation and TAP transport optimized spacer sequences that flanked the epitopes within the poly-epitope construct.

Reguzova et al (2015) designed novel HIV poly-epitope T cell immunogens based on conserved natural CD8+ and CD4+ T cell epitopes from different HIV strains and restricted by most major HLA alleles.

Designed immunogens contained optimized core poly-epitope sequence and additional sequences such as N terminal ubiquitin or N-terminal signal peptide, and C-terminal tyrosine motif of LAMP-1 protein that increase epitope processing and antigen presentation. So far they engineered three DNA vaccines, by encoding novel T cell immunogens and different combination of signal sequences.
All constructs have been tested in vivo and the attachment of either ubiquitin or ER signal/LAMP1 sequences increases both CD8+ and CD4+T cell response in comparison with poly-epitope immunogen without any additional sequences, but the linkage of ubiquitin to immunogen core determined an highest magnitude of T cell responses.

1.3.5 Invariant chain as genetic adjuvant

The generation of high quality memory cells together with the induction of a substantially expanded CD8+ T cell population is possible by linking the major histocompatibility complex class II associated invariant chain (Ii) to an antigen encoded by an adenoviral vector.
Holst et al (2008) have demonstrated that an adenovirus expressing the glycoprotein (GP) of the lymphocytic choriomeningitis virus (LCMV) tethered to Ii increases the kinetics, magnitude and durability of T cell specific responses in vivo.

Ii was initially used with the aim to improve CD4+ T cell response when linked to an antigen. Malcherek et al (1998) have engineered a Ii vector by inserting T cell epitopes of tetanus toxin or acetylcholine receptor in the CLIP region. When peripheral blood mononuclear cells (PBMC) are pulsed with Ii hybrids T cell response is increased compared to stimulation after priming with native or recombinant proteins. Fujii et al (1998) also designed a mutated human form p33 in which they substituted CLIP with streptococcal M12 p55-68 and evaluated the peptide presenting function through a T cell proliferation assay. Mouse L transfectants expressing mutated form of Ii with HLA-DR4 can process and present M12 p55-68 to the peptide specific and DR4 restricted CD4+ T cell clone.

Another method to increase antigen-specific stimulation of T-helper cells entails the use of the Ii hybrids, in which a four-amino-acid sequence (LRMK), the KEY motif of Ii, is linked to T-helper epitopes. The mechanistic hypothesis states that the Ii-KEY binds initially to an allosteric site just outside the MHC class II binding groove at the cell surface (Voutsas et al., 2007). This induces a conformational change in the trough, facilitating antigenic epitope charging, and a concomitant increase in the potency of antigen presentation compared with the unmodified class II epitope. As vector, Ii-KEY and Ii enhanced the interferon IFNγ and interleukin IL-4 or IL-2 responses in enzyme-linked immunosorbent spot assay, epitope-specific CD4+ T cell activation, or specific antibody production (Chen et al., 2012).

In addition, Mwangi et al (2007) have designed a DNA-construct encoding for the major surface protein 1a of *Anaplasma marginale* fused with the lysosome-targeting motif of bovine Ii. A single inoculation with this construct primed immune response seen as a potent proliferation of IFN-Ɣ/CD4 T cells and production of IgG. A single injection of this construct induced antigen specific memory cells, which formed the basis for an accelerated response to repeated doses of the antigen.

The linkage of Ii to the N terminus of glycoprotein of LCMV virus encoded by Adenovirus-based vaccines induced enhanced proliferation of naïve CD4+ and CD8+ T cells determining an accelerated, enhanced and prolonged vaccine-induced protection against acute or chronic viral infection. Additionally, this vaccine provides efficient protection against viral escape variants due to an increased breadth of the vaccine-induced CD8+ T cell response (Holst et al., 2008).

Likewise, the adjuvant effect of Ii is obtained when Ii is linked to NS3, a nonstructural protein from HCV and the fusion is encoded by Adeno vector. Ad-murine-Ii NS3-induced CD8+ T cells show increased cytotoxicity compared to Ad NS3. In addition, Ad li-NS3 elicited high quality memory cells characterized for being poly-functional (IFNɣ, TNFα and IL2) central memory T cells expressing the phenotypic cell surface markers CD27 and CD127. These markers are associated with long-term survival of Ag-specific memory CD8+ T cells and maintenance of T cell memory (Mikkelesen et al., 2011).

Ii does not only enhance CD8+ T cell responses when tethered to antigen within Ad vectors but has been shown to enhance responses when included in DNA plasmids, lentiviral vectors and MVA.

Indeed, Rowe et al (2006) constructed lentiviral vaccine vectors carrying Ii sequence linked to chicken ovalbumin, as model antigen. The Ii-OVA vector was the most efficient at inducing cytokine-secreting CD4+ and CD8+ T cells in mice and at protecting mice from challenge with the OVA-expressing tumor, EG7.OVA.

The enhanced T cell response is visible also when murine p31 Ii is linked to naked DNA encoded antigens. Grujic et al (2009) compared two DNA vaccines, pACCMV.pLpA expressing either the glycoprotein (GP) of LCMV virus (DNA-GP) or GP linked to the p31 Ii chain (DNA Ii-GP) after gene immunization through FACS analysis. The insertion of the murine Ii chain at the N terminus of GP antigen results in increased priming of antigen specific CD8+ T cells directed against both dominant and subdominant epitopes as previously described by Holst et al (2008) using Adeno5 vaccine vector. Further they demonstrated that DNA mIi-GP vaccinated mice were significantly better protected against systemic infection than DNA-GP vaccinated animals.

The enhanced protection derived by Ii has also been demonstrated in a murine cancer model where the vaccination becomes a tool to improve cancer specific T cell responses and vaccine-induced tumor control. In brief, C57BL/6 mice are challenged with B16.F10 melanomas expressing the dominant epitope (GP33) of LCMV glycoprotein and vaccinated with Ad5 Ii GP. Vaccination with Ad5 Ii GP leads to a sustained rejection of the tumor in mice vaccinated prior to tumor challenge and a delay of tumor growth in therapeutically vaccinated mice compared to Ad5 GP (Sorensen et al., 2009).

The effect of Ii was seen for both murine and human form of Ii and was observed in inbred and outbred mice (as described previously) and in

cynomolgus macaque as described by Capone et al (2014). They demonstrated that fusion to murine Ii in CD1 mice and NHP improves the magnitude and the breadth of CD8+ T-cell response to vaccine-encoded homologous and heterologous HCV NS antigen in a prime-boost regime using a chimpanzee Adeno vector (ChAd3) as primer and MVA virus as booster. Since HCV virus exists in six different genotypes and accumulates mutations, during its replication, this poly-specific elicited response facilitates targeting multiple epitopes from divergent HCV virus genotypes. Like the murine version of Ii, the human form of Ii accelerated a CD8+ specific T cell response in both animal models. Despite macaque Ii has very high amino acid sequence homology with human p35 form of Ii, no self-reactive T cells of Ii were detected. This demonstrates that the enhancement, derived by Ii, is not species specific and translates from mice to NHP.

The same results were obtained by Spencer et al (2014) when they tested in a regime of prime boost, the adjuvant effect of murine and human Ii linked to ME-TRAP, an antigenic construct encoded for full length *Plasmodium falciparum* TRAP (thrombospodin related adhesion protein) fused to ME, a string of 20 malarial T and B cell epitopes. In particular, after prime-boost vaccination with ChAd63 and with a subsequent MVA boost (given 8 weeks later), they observed a significantly higher antigen specific CD8+ T cell response in mice and macaques than in those expressing unmodified ME-TRAP.

In other studies, Ii shows protection not only by challenge with virus but also by intracellular bacteria, such as *Listeria monocytogenes* Gram positive bacteria that cause chorioamnionitis in pregnant women and septicemia and meningitis in immunocompromised individuals.. To effective clear this infection, the host elicits an adaptive immune response involving both CD8+ and CD4+ T cells. For this reason, Jensen et al (2013) tested two Adeno vectors expressing the glycoprotein of LCMV virus (GP) with and without the Ii linkage. After vaccination with Ad5 Ii GP and Ad5 GP, mice are challenged with a recombinant strain of *Listeria monocytogenes* modified to secrete part of LCMV GP. The tethering of Ii to antigen conferred protection in mice vaccinated with Ad5 Ii GP than with Ad GP.

Despite the extensive description of the adjuvant effect of Ii, the molecular mechanisms underlying this effect have not yet been elucidated.

2. AIM OF THE STUDY

My research activity, during the last three years, focused on identification of the mechanism of action of Invariant chain as genetic adjuvant in the T cell response induced by adenoviral vectors used as vaccines.

Ii is a non-polymorphic type II trans-membrane protein, first identified as the chaperone for MHC class II molecules (Blum et al., 2013) and prevents the premature binding of antigen peptides in the ER on class II molecules. Although Ii plays a role to ensure a CD4+ T cell response, its genetic fusion of Ii to antigens encoded by human adenoviral vectors (such as Ad5) increases the kinetics, magnitude and durability of CD8 T cell response (Holst et al., 2008).

The adjuvant effect of Ii has been demonstrated linking as the murine as the human forms of Ii to several antigens, such as malaria, Ebola, HCV and LCMV viruses in inbred and outbreed mice and non-human primates (NHP) (Malcherek et al., 1998; Holst et al., 2008; Capone et al., 2014; Spencer et al., 2014).

Despite the extensive characterization of the effect of Ii as genetic enhancer of CD8+ T cell response, its mechanism of action still remains unknown.

For this reason my research activity was devoted to identify the functional domains of Ii involved in enhanced CD8+ T cell response and meanwhile to define the minimal region of the Ii that could retain adjuvant effect.

Trimming of Ii to very short functional sequences might offer a better candidate for evaluation of this technology in humans reducing possible off target effects within transduced cells or improve the chances of breaking tolerance.

3 MATERIALS AND METHODS

3.1 Cell line cultures

Hela cells (ATCC) were cultured in Dulbecco's Modified Essential Medium (DMEM) (GibcoBRL) supplemented with 10% Fetal Bovine Serum (FBS) (heat inactivated 56°C 30 min Hyclone) and 2mM L-glutamine (GibcoBRL) at 37°C in a 5% CO2/95% air atmosphere.

BMDC (Bone Marrow Dendritic Cells) were obtained from femurs of 6-10 week old female CB6F1 mouse. The protocol for generation and purification of BMDC was adapted from Muccioli et al., 2011. The ends of the bones were cut off and the inside of the bones infused with RPMI using a sterile syringe inside a petri dish. Bone marrow leukocytes clumps were broken up and the cell suspension washed twice in RPMI (GibcoBRL) containing with 10% FBS (Hyclone), 2mM L-glutammine (GibcoBRL), 10mM Hepes (GibcoBRL), 55µM 2-mercaptoethanol (GibcoBRL) (1200 rpm for 5 minutes) after collection. Red blood cells were lysed using ACK lysis buffer (5 minutes RT) and washed with RPMI and counted. 3 $x10^6$ cells were cultured in 10ml R10% in a T75 flask (BD 353136) with 10 ng/ml of recombinant murine granulocyte-macrophage colony-stimulating factor (rGM-CSF; Invitrogen PMC2015) at 37°C with 5% CO2. On day 3 cultures were supplemented with 10mls of R10 with 10 ng/ml rGM-CSF. On day 6 of culture the media was harvested, cells were pelleted (1200 rpm for 5 minutes) and suspended in 20ml of fresh R10 supplemented with 10 ng/ml rGM-CSF until 10 days.

For the last part of experiments it was used another protocol to differentiate BMDC cells in suspension (Zanoni et al., 2011). 7 $x10^6$ cells were cultured in 10ml R10% in a T75 flask (Corning 3814) with 10 ng/ml of recombinant murine granulocyte-macrophage colony-stimulating factor (rGM-CSF; Invitrogen PMC2015) at 37°C with 5% CO2. On days 4 and 7 5 ml of R10 with 10 ng/ml rGM-CSF were added and cells grew until day 8/9.

The new protocol was introduced to achieve a better differentiation and viability of BMDC cells in vitro and to collect a high number of cells for each experiment. The results obtained with two protocols were comparable.

HuH7 and HEK293 (ATCC) were grown in DMEM supplemented with 10% FBS (Hyclone) and 2mM L-glutamine (GibcoBRL) at 37°C in a 5% CO2/95% air atmosphere

3.2 Adenoviral vectors: construction, amplification and purification

The first adenoviral vectors were made by Peter Holst (University of Copenhagen). Murine Ii (mIi) insert (accession number NM_1042605.1) was obtained from plasmid murine Ii opt.

All Ad constructs were E1E3 deleted and all full and truncated Ii sequences were cloned at the N-terminus of the transgene under HCMV and SV40pA.

For the second part of the work, the new short variant mIi 50-75 OVA , the mutated vectors in the full length and in the short forms and related controls such as Ad5 OVA, Ad5 mIi OVA or Ad5 1-75 mIi OVA were generated by me using another preAdeno shuttle.

GeneArt (Life Technologies, Paisley, UK) had synthesized pvj plasmid encoded for mutated and short variants of mIi using pvj shuttle.

All full and truncated Ii sequences were cloned at the N-terminus of the transgene OVA under HCMV and BGHpA control and then transferred into the pre-adeno vector by homologous recombination in BJ5183 cells. All Ad constructs were E1E3 deleted and therefore non-replicative except in E1 supplemented cell lines.

The first pre-adeno backbone and the last one differed for the poly A signal and also for the length of the HCMV promoter.

The sequence of HCMV promoter of the first pre-adeno backbone is:

5’GATCTATACATTGAATCAATATTGGCAATTAGCCATATTAGTCA
TTGGTTATATAGCATAAATCAATATTGGCTATTGGCCATTGCATAC
GTTGTATCTATATCATAATATGTACATTTATATTGGCTCATGTCCAA
TATGACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGT
AATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCC
GCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCC
AACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCAT
AGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAG
TATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCAT
ATGCCAAGTCCGCCCCCTATTGACGTCAATGACGGTAAATGGCC
CGCCTGGCATTATGCCCAGTACATGACCTTACGGGACTTTCCTA
CTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGA
TGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTGA
CTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGG
AGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTC
GTAATAACCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGT
ACGGTGGGAGGTCTATATAAGCAGAGCTC 3’

The sequence of HCMV promoter of the second pre-adeno backbone is:
5’CCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATT
GGCTCATGTCCAACATTACCGCCATGTTGACATTGATTATTGACT
AGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCA
TATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCT
GGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGA
CGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGT
CAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA
TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATG
ACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTA
TGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCT
ATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGG
ATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTG
ACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTT
TCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGC
GGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCC
CTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGATCGTCGA
CGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATC
CACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAG
CCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGT
GCCAAGAGTGAGATCTTCCGTTTATCTAGGTACCAGATA 3’

The second pre-adeno backbone had a longer promoter than the first one. Probably this justified difference in the vaccination dosages.
The change of backbone had been useful to verify if the phenotype that it is visible in vivo between OVA and mIi OVA, is independent from transcriptional regulation signals such as promoter and polyA.
Pre-adeno plasmids were first digested with PmeI to release the viral ITRs then 3-5×106 HEK293/PER.C6 cells grown in DMEM, 10% fetal bovine serum (FBS), 1% Penn-Strept in T25 flasks, were transfected with 10 micrograms of cloned viral vector. DNA transfection was performed using Lipofectamine 2000 (Invitrogen). Vectors were then expanded up to a production scale of 2×10^9 cells. Purification was performed by two step Cesium Chloride gradient (Colloca et al., 2012). All viruses were controlled by restriction and sequencing analysis.

3.3 Animal and vaccination

All experimental procedures were approved by the local animal ethics council and were performed in accordance with national and international laws and policies (EEC Council Directive 86/609; Italian Legislative Decree 116/92). The ethical committee of the Italian Ministry of Health

approved this research. Animal handling procedures were performed under anesthesia, and all efforts were made to reduce animal numbers and minimize suffering. Six-week-old female C57 mice were purchased from Charles River (Como, Italy). All day-to-day care of the mice was performed by trained mouse house staff at CEINGE. Viral vectors were administered with intramuscular injection in the quadriceps by delivering a volume of 50 µl per side (100 µl final volume). The injected dose for all mouse experiments was 3×10^6 viral particles (vp) for Ad vectors. For the second part of the project the dose of injection was 10^6 vp.

3.4 Ex vivo IFN-γ ELISpot

Mouse splenocytes were plated in duplicate at two different densities (2x 10^5 and 4 x 10^5 cells per well) after blocking e washing of MSIP S4510 plates (Millipore , Billerica, MA) coated with 10 µg/ml of anti- mouse IFN-γ antibody (U-CyTech Utrecht, The Netherlands) overnight at 4°C. The cells were stimulated overnight at 37°C with immunodominant OVA peptide 257-264 (SIINFEKL) at a final concentration of 2µg/ml on each well. Two controls were used in this assay: the peptide diluent dimethyl sulfoxide (Sigma- Aldrich, Milan, Italy) as negative control and concanavalin A (Sigma- Aldrich, Milan, Italy) as positive control. Plated were incubated with biotinylated anti-mouse IFN-γ antibody (U-CyTech Utrecht, The Netherlands) conjugated streptavidin-alkaline phosphatase (BD Bioscience, San Jose, CA) and at the end with 5-bromo-4-chloro-3-indoyl-phosphatase/nitro blue tetrazolium 1-step solution (Thermo Fisher Scientific, Rockford , IL). An automated ELISA –spot assay video analysis system automated plate reader was used to analyze plates. The ELISpot data were expressed as number of T cells producing IFN- γ per millions of splenocytes. To have a positive ELISpot response these following conditions must occur: IFN- γ spots present in wells stimulated with Con-A; at least 50 specific spots/million splenocytes to immunodominant peptide; the number of spots of positive wells was three times the number identified in the mock control wells (dimethyl sulfoxide) and responses diminished with cells dilutions.

3.5 Infection

Hela cells were stripped from the plate using pre-warmed trypsin (0.05% GibcoBRL 25300-054) and were infected with adenoviral vectors in DMEM containing 2% FBS and 2mM L-glutammine (GibcoBRL) at 100 MOI (Multiplicity Of Infection-vp/cell) for Western blot analysis (10^6 cells), 50 MOI (vp/cell) for immunoprecipitation ($3x10^6$ cells) and FACS analysis (10^6 cells) and 50 MOI for immunofluorescence (10^5 cells).

After 1 hour the cells were added with DMEM with 10% FBS. After 3 hours the cells were treated with appropriate inhibitors for 16 hours such as MG-132 (10µM Sigma), pepstatin (1µM)/ E64D (10µM) (ratio 1:1), chloroquine (500nM), radicicol (90µM) (Hsp90 inhibitor) and 17-allylamino-17demethoxy-geldanamycin (17-AAG 170nM) (Hsp70/90 inhibitor).
BMDC cells were stripped from the plate using pre-warmed trypsin (0.25% GibcoBRL 25200-056) and cell dissociation solution (Sigma, C5789-100ml) 1:1 mix (incubated 7 minutes in a CO2 incubator at 37°C and 5% CO2) and were infected in 48 well- plate with adenoviral vectors in RPMI containing 2% FBS and 2mM L-glutammine (GibcoBRL) at 200 MOI (infection units/cell) ($2x10^5$ cells). After 1 hour the cells were added with RPMI with 10% FBS. After 3 hours the cells were treated with MG-132 inhibitor and pepstatin (1µM)/ E64D (10µM) (ratio 1:1) for 16 hours.
For immunofluorescence analysis HuH7, HEK293 and BMDC cells (10^5 cells) were infected with a dose escalation of MOI (5-10-50 MOI).

3.6 Immunoprecipitation for ubiquitination analysis

For the ubiquitination analysis Hela cells were transfected with Ub-FLAG plasmid (Invitrogen) (2µg) for 16 hours with Lipofectamine 2000 (Invitrogen) according to the manufacture instruction. After change of medium, the cells were infected with adenoviral vectors for 16 hours and treated with MG-132 for 24 hours. The Hela cells were lysed and the protein lysates were immunoprecipitated with anti-UbLys48 antibody (10 µg clone Apu2 rabbit monoclonal Millipore) or with anti Ub-Lys63 antibody (10 µg clone Apu3 rabbit monoclonal Millipore) for 16 hours at 4°C with rocking. After the supernatants were incubated with protein A sepharose CL-4B (500 µg GE Healthcare) for 45 minutes at 4°C with rocking. The samples were analyzed by Western blot.

3.7 Analysis of PNGase activity

Protein lysates were immunoprecipitated with anti GFP (2µg mouse monoclonal clone B-2 Santa Cruz) or with anti HA (2µg rabbit polyclonal Sigma) for 16 hours at 4°C with rocking. After the supernatants were incubated with protein A sepharose CL-4B (500 µg GE Healthcare) for 45 minutes at 4°C with rocking. The samples were treated with 0,2% SDS in 75mM sodium phosphate buffer pH7.5, boiled (95°C for 5 minutes) and reduced with DTT. After they were treated with 2% Tryton in 75 mM sodium phosphate buffer (pH 7.5) and

inhibitors protease and digested with PNGase enzyme (1000U for each sample Biolabs) for 3 hours at 37°C. This enzyme is an amidase which cleaves between the innermost GlcNAc and asparagine residues of high mannose,hybrid and complex oligosaccharides from N-linked glycoproteins. At the end of incubation lysates were resolved with Western blot analysis.

3.8 Western blot analysis

The Hela cells were lysed in 20mM Tris-HCL pH7.5, 150mM NaCl and 1mM EDTA pH8 and cell debris was removed by centrifugation (13000 rpm for 30 minutes). Lysates were resolved by 4-12% SDS PAGE and transferred to nitrocellulose membrane. The membrane was probed with anti GFP-HRP (mouse monoclonal anti –Green Fluorescent Protein-Horseradish Peroxidase Miltenyi Biotec 1:5000), anti HA –HRP(mouse monoclonal anti –Hemmagglutinin-Horseradish Peroxidase Miltenyi Biotec 1:5000), anti GFP (mouse monoclonal clone B-2 Santa Cruz 1:1000), anti-FLAG (mouse monoclonal clone M2 Sigma 1:5000) anti GAPDH (mouse monoclonal Sigma-Aldrich 1:3000) and peroxidase-conjugated anti-mouse(A3682, Sigma-Aldrich, 1:5000). The blot was visualized using ECL (SuperSignal West Pico Chemiluminscent substrate Thermo scientific). To quantify the relative amounts of the immunolabeled bands, different exposures of the blot were analyzed with the NIH image program.

3.9 Antigen presentation assay

After transduction BMDC are harvested as above, washed in RPMI twice, and suspended in PBS. BMDC are pelleted and suspended in PBS-Fc blocked (1:25 BD Bioscience) at 4°C for 20 minutes. After wash in PBS, the pellet was suspended in violet fixable live/dead (1:50 by 1:20 dilution Life Technologies) and stained at room temperature for 20 minutes. Cells were washed with PBS and incubated with CD11c-Pecy7 (1:100 BD Bioscience) and H2-Kb-SIINFEKEL (1:100 Biolegend) in FACS buffer (1% FBS in PBS) for 30 minutes at room temperature. After centrifugation at 1200 rpm for 5 minutes, the pellet was washed in FACS buffer and suspended in PBS. Stained cells were acquired on a FACS Canto flow cytometer and analyzed using DIVA software (BD Biosciences). Voltages and gating were checked with BMDC infected with Ads containing full length mIi and unstained and not infected cells.

3.10 FACS analysis

Hela cells ($2x10^5$) were transfected with DNA encoded GFP or mIi GFP (0.8 ug) or infected with adenoviral vectors encoding for GFP as described above. After 4 hours they were treated with MG-132 (10µM Sigma), chloroquine (1x) and pepstatin (1µM)/E64D (10µM) (ratio 1:1), for 16 hours. Cells were harvested and suspended in violet fixable live/dead (1:50 by 1:20 dilution) at room temperature for 20 minutes. After incubation cells were washed with PBS. Stained cells were acquired on a FACS Canto flow cytometer and analyzed using DIVA software (BD Biosciences). Voltages and gating were checked with Hela infected with Ads containing full length mIi (or transfected with the same DNA form) and unstained and not infected cells.

3.11 Immunofluorescence and colocalization evaluation

Hela, BMDC, HEK29 and HuH7 cells (10^5 cells) were infected with 50MOI or dose escalation of MOI (5-10-50 MOI) with Ad5 GFP or Ad5 mIi GFP and treated or not with MG-132 for 24 hours. Poliethylenimmine (PEI) in water (1ug/ul) was used at transfecting agent. Briefly 2 ug of DNA were mixed with 5 ug of PEI in 150 mM NaCl to be then added after 30 minutes of incubation to a 10 cm dish of the cells in complete fresh medium. After detaching with 0,05% of trypsin, the cells were plated on glass coverslips and were fixed in 4% Formaldehyde dissolved in PBS for 30minutes. Formaldehyde was quenched by incubating the coverslips for 30minutes in 0,1M Glycine dissolved in PBS. Cells were permeabilized in 0, 1% TritonX100 for 10minutes at RT to be then incubated with primary and secondary antibody diluted in PBS for 1hour and 30minutes, respectively. The fluorescence of GFP was evaluated with confocal microscopy

For co-localization study the antibodies used were: rabbit polyclonal anti GM130 (Sigma 1:100), rabbit polyclonal anti Lamp1 (Thermo fisher 1:200), rabbit polyclonal anti LC3 (Abcam 1:50) and mouse monoclonal anti FLAG (Sigma 1:100) that recognizes the FLAG tag linked to Ubiquitin FLAG plasmid, transfected in the cells (as described above). The secondary antibody was a rabbit Texas-Red conjugated polyclonal antibody (1:400 Sigma). The immunofluorescence intensity in the Texas-Red channel (depending only on the PM localized CB1) was measured using NIH ImageJ Biophotonic programs and normalized to one of the GFP channel. The results are given as mean +/− s.d.m. Immunofluorescence images were taken by a Leica DFC320 video-camera (Leica, Milan, Italy) connected to a Leica DMRB microscope

equipped with a 100 X objective and the Image J Software (National Institutes of Health, Bethesda, MD) was used for analysis.

3.12 Statistical evaluation

Statistical analysis was performed using GraphPad Prism (version 6). Because populations were more or less normally distributed, a parametric one-tailed unpaired Student's t-test was used to evaluate statistical significance (*p <0,05; **p <0,01;***p <0,001; ****p <0,0001).

One-tailed tests are appropriate when testing a specific, directional hypothesis. Because our a priori predictions, supported also by a lot of works presented in literature, were directional towards an improvement of T cell response thanks the presence of Ii, one-tailed tests were used.

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