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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** lypopolysaccharides  
**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<sub>CM</sub>** T cell central memory

**TCR** T-cell receptor  
**T<sub>EM</sub>** 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<sub>RM</sub>** T cell tissue-resident memory  
**T<sub>SCM</sub>** T stem cell memory  
**T<sub>TM</sub>** T cell transitional memory  
**Ub** Ubiquitin  
**Vp** Viral Particle  
**WB** Western Blot

## ABSTRACT

The Invariant chain (Ii) is a non-polymorphic type II integral membrane protein involved in the formation and cell surface display of the MHC-II/peptide complex. Fusion of antigen (Ag) to Ii within Adenovirus based genetic vaccines leads to a significant enhancement of the magnitude and breadth of CD8<sup>+</sup> T cell response. The effect of Ii as genetic vaccine enhancer was consolidated using human and murine Ii sequences linked to different antigens and in various animal models. However, despite the extensive characterization of the effect of Ii, its mechanism of action still remains unknown. The purpose of this study was to identify the molecular mechanism of Ii as “genetic adjuvant” of T cell-mediated responses and to identify the minimal domain(s) responsible for the enhancement of CD8<sup>+</sup> T cell response. To this end, we used ovalbumin (OVA) as a model antigen and linked it to the murine Ii (mIi); the mIi-OVA fusion was encoded by a replication defective adeno 5 viral vector. Tethering mIi to OVA markedly increased presentation of the OVA immunodominant epitope (SIINFEKL) in the context of MHC class I upon vector infection of bone marrow dendritic cells (BMDC). The involvement of the proteasome degradation pathway in this phenomenon was confirmed by using proteasome inhibitors and analysis of antigen expression upon infection of Hela cells. Proteasome inhibition revealed the presence of a large number of polyubiquitinated mIi-OVA species, and we could show that this massive antigen modification was occurring on Lys48 residues as predicted by the observed MHC-I increase in antigen presentation. Similar results were obtained using green fluorescent protein (GFP) instead of OVA, indicating that the mIi induced polyubiquitination is likely to be responsible for the ability of Ii to improve CD8<sup>+</sup> T cell response when physically linked to an antigen. Different protein domains were shown to play a role in the physiological function of Ii, such as the trimerization domain, the endosomal sorting signal, the transmembrane domain and the CLIP and KEY motifs. To verify if any of these domains is important for the adjuvant effect, we generated Ii deletion mutants targeting these previously characterized functional domains, fused them to the OVA antigen and encoded the fusion proteins in the Adeno vector. *In vitro* and *in vivo* studies with the resulting viruses showed that none of these protein fragments is essential for the Ii mediated increase of antigen immunogenicity, and that the minimal Ii region that retains the adjuvant effect corresponds to the protein fragment spanning amino acids 50 to 75. The identification of a short Ii variant capable of maintaining the adjuvant effect is likely to minimize the risk of inducing auto-immunity in human vaccine applications.

Perhaps most interestingly, we could show that a substitution mutant in the unique lysine residue present in the first 75 amino acids of mIi induced polyubiquitination of the downstream OVA antigen and increased CD8<sup>+</sup> T cell immunogenicity *in vivo*, suggesting that the mechanism of Ii mediated increase of T cell immunogenicity consists of increased ubiquitination and proteasome degradation of the linked antigen, thereby resulting into a more efficient presentation of antigen derived peptides.

# 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- $\alpha:\beta$  and  $\gamma:\delta$  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  $\alpha_2$  and  $\beta_2$  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  $\alpha$  and  $\beta$ , 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  $\alpha_3$  domain of MHC class I molecule and form interactions with the residues in the base of the  $\alpha_2$  domain.

The CD8 binding site to the MHC class I complex is formed by interaction between CD8  $\alpha$  and  $\beta$  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  $\alpha$  chain, is encoded in the MHC and is non covalently associated with a smaller chain,  $\beta_2$ -microglobulin, which is not polymorphic and is encoded on different chromosomes.

The complete molecule has four domains, three formed from the MHC-encoded  $\alpha$  chain and one contribute by  $\beta_2$ -microglobulin. The  $\alpha_3$  domain

and  $\beta_2$ -microglobulin closely resemble Ig-like domains in their folded structure. The folded  $\alpha_1$  and  $\alpha_2$  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  $\alpha$  and  $\beta$ , 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)

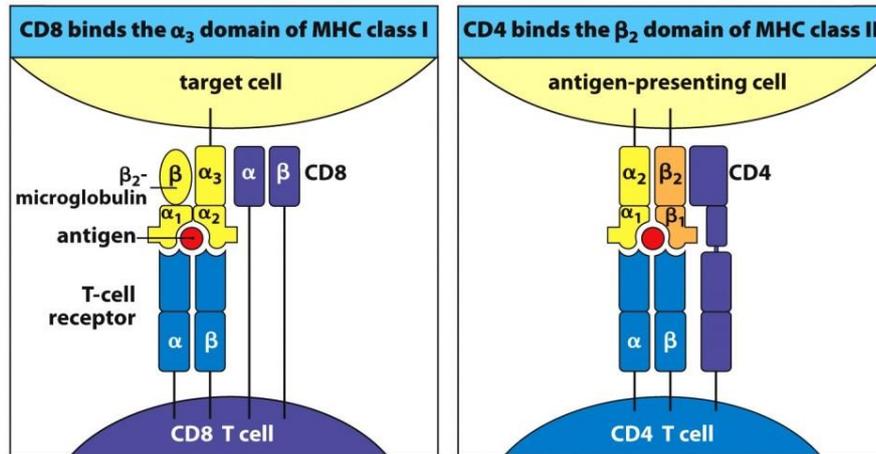
In both MHC class I and class II molecules, bound peptides are sandwiched between the two  $\alpha$ -helical segments of the MHC molecule. The peptide is bound as an integral part of the MHC molecule's structure and MHC molecule are unstable when peptides are not bound.

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  $\beta_2$  domain of an MHC class II molecule, in the hydrophobic crevice between the  $\beta_2$  and  $\alpha_2$  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  $\alpha_1$  and  $\alpha_2$  domains (from Janeway's Immunobiology 8<sup>th</sup> 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<sup>+</sup> T cells is granulysin, which is able to induce apoptosis.

However, most CD8<sup>+</sup> cytotoxic T cells also release the cytokines IFN- $\gamma$ , TNF- $\alpha$  and LT- $\alpha$  which contribute to host defense in several ways. IFN- $\gamma$  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- $\gamma$  also activates macrophages, recruiting them to the site of infection both as effector cells and as antigen-presenting cells. TNF $\alpha$  and LT $\alpha$  can synergize with IFN- $\gamma$  in macrophage activation (Prezzi et al., 2001).

In contrast with CD8<sup>+</sup> T cells, **CD4<sup>+</sup> T cells** differentiate into several subsets of effector T cells with a variety of different functions: T<sub>H1</sub>, T<sub>H2</sub>, T<sub>H17</sub> and regulatory T cells.

T<sub>H1</sub> cells help control bacteria that can set up intra-vesicular 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<sub>H1</sub> 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<sub>H2</sub> 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<sub>H1</sub> and T<sub>H2</sub> subsets are distinguished principally by their production of specific cytokines, such as interferon (IFN- $\gamma$ ) and IL-2 by T<sub>H1</sub> cells, and IL-4 and IL-5 by T<sub>H2</sub> cells.

T<sub>H17</sub> 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<sup>+</sup> 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<sup>+</sup> regulatory T cells with different phenotypes are thought to differentiate from naive CD4<sup>+</sup> 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- $\beta$  (TGF- $\beta$ ) 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<sub>EM</sub>)** that migrate to inflamed peripheral tissues and display immediate effector function. By contrast, reactive memory is mediated by **central memory (T<sub>CM</sub>)**. 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<sub>CM</sub> are CD45R0<sup>+</sup> and constitutively express CCR7 and CD62L, two receptors that are also characteristic of naïve T cells. Following TCR triggering, T<sub>CM</sub> produce mainly IL-2, but after proliferation they efficiently differentiate to effector cells and produce large amount of IFN $\gamma$  or IL-4.

Human T<sub>EM</sub> 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<sub>CM</sub>, T<sub>EM</sub> are characterized by rapid effector function. CD8<sup>+</sup> T<sub>EM</sub> cells carry large amounts of perforin and both CD4<sup>+</sup> and CD8<sup>+</sup> produce IFN- $\gamma$ , IL-4 and IL-5 within hours following antigenic stimulation. In vivo antigens stimulate T<sub>CM</sub> and T<sub>EM</sub> in different ways. T<sub>CM</sub> can be stimulated by

mature dendritic cells whereas T<sub>EM</sub> 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<sup>+</sup> and CD4<sup>+</sup> 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<sup>+</sup> 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  $\alpha$ -rings. The two inner rings of the 20S proteasome core are composed of constitutively expressed proteolytic subunits called  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 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  $\beta$  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- $\gamma$ . This is the binding to the proteasome of a protein complex called the

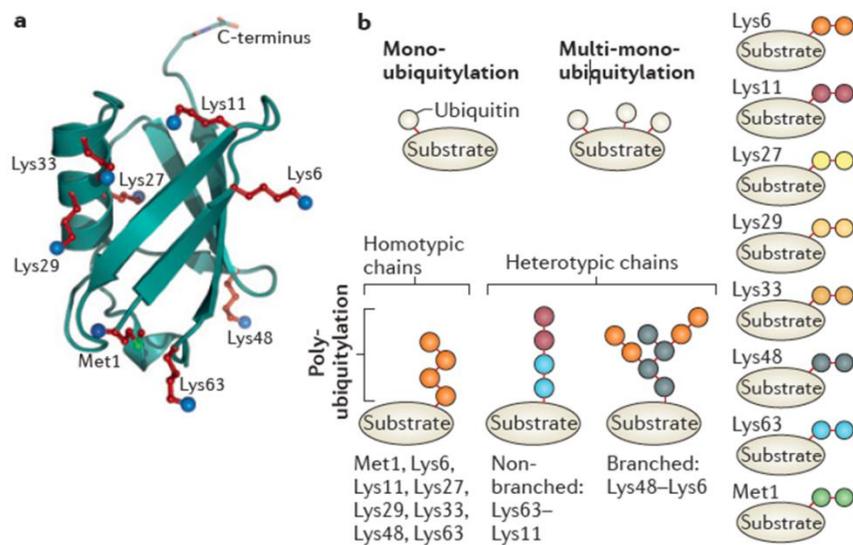
PA28 proteasome-activator complex. PA28 is a six or seven-membered ring composed of two proteins, PA28 $\alpha$  and PA28 $\beta$ , both of which are induced by INF- $\gamma$ . A PA28 ring can bind to either end of the 20S proteasome core in place of the 19S regulatory cap, and acts to increase the rate 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<sup>+</sup> T cell response.



**Figure 2: Forms of ubiquitination**

a) The seven Lys residues and Met1 of ubiquitin are shown 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

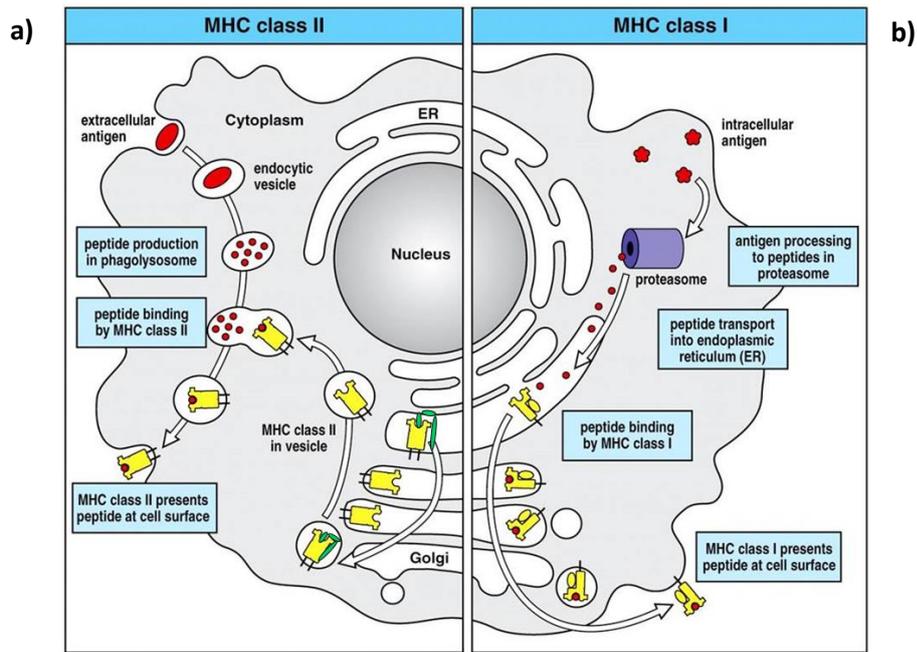
bacterial antigens have been taken up by an antigen-presenting cell such as macrophage or immature dendritic cell. In other case, the source of antigen may be bacteria or parasites that have invaded the cell to replicate in intracellular vesicles. In both cases the antigen-processing pathway is the same. 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-Do $\alpha$  chain and HLA-Do $\beta$  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-Do $\beta$  chain is not increased by IFN- $\gamma$ , whereas the expression of HLA-DM is. Thus, during inflammatory response, in which IFN- $\gamma$  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  $\alpha$  and  $\beta$  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<sup>+</sup> 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  $\beta$ 2microglobulin, 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 8<sup>th</sup> 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).

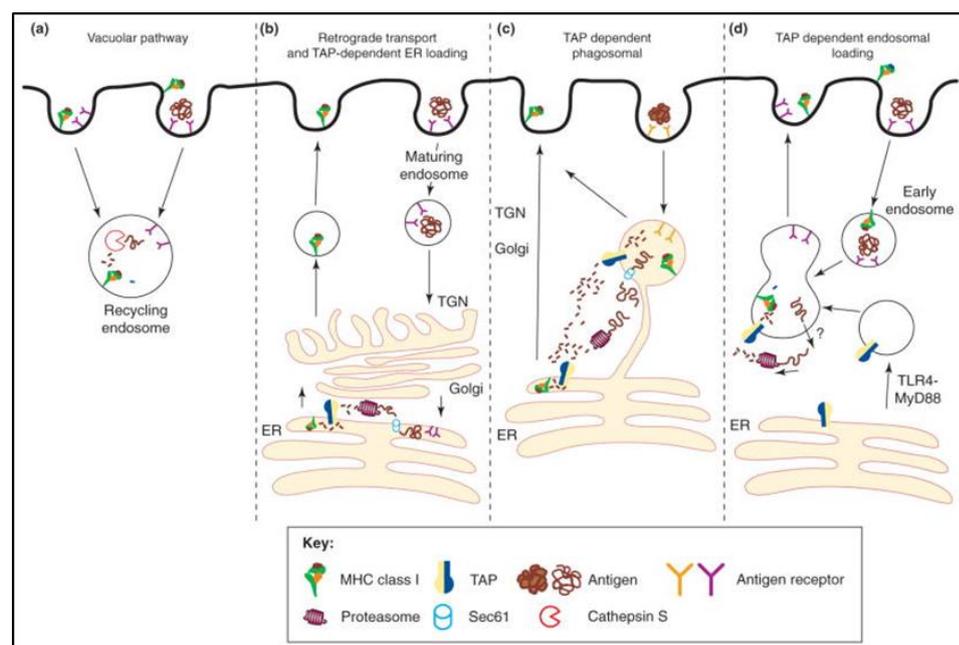
ERAD is a complex process through which folding-defective proteins are selected and ultimately degraded by the ubiquitin-proteasome system. It can be envisioned as encompassing four distinct, coupled steps: 1) substrate recognition; 2) dislocation across the lipid bilayer; 3) addition of poly-ubiquitin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

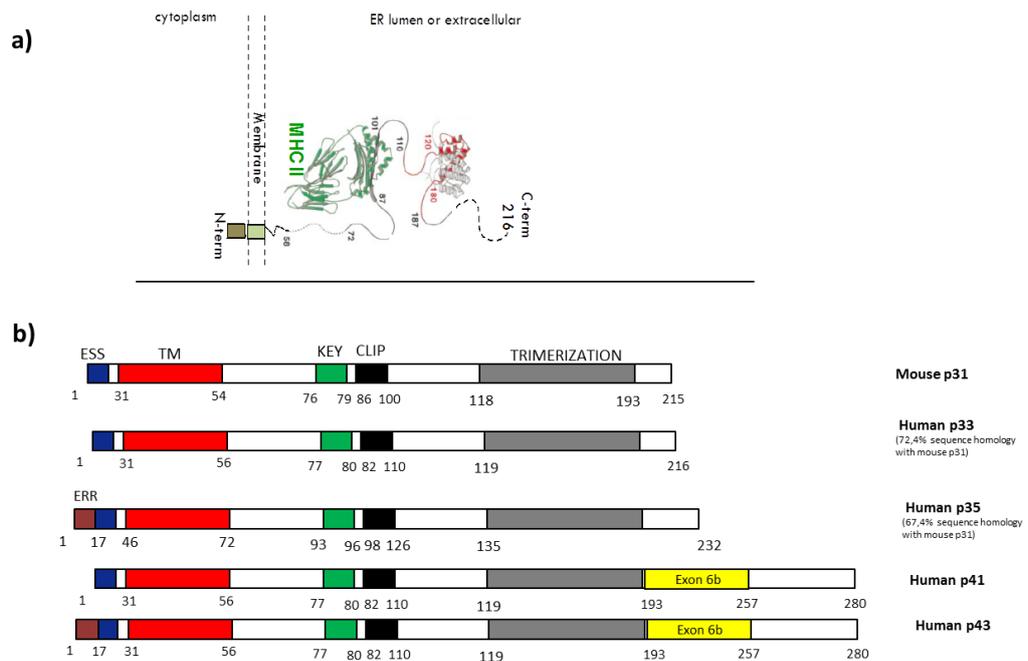
Ii is capable of assembling into homo-trimers (Roche et al., 1991). The region responsible for its self-association is mapped between 118 and 193 residues. This trimerization domain is organized in a  $\alpha$ -helical coiled structure, forming a scaffold that allows interaction with three MHC-II heterodimers ( $\alpha$ :  $\beta$ ) (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 Fc $\gamma$  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-Andr e et al., 2008). It also functions as a surface receptor for macrophage migration inhibitory factor (MIF) (Matza et al., 2002). The cytosolic domain of It is released upon binding MIF and it acts as an essential transcription factor in B-cell maturation and survival, by signaling through Nf kB and by upregulating anti-apoptotic markers such as BCL-Xl and BCL-2 (Starlets et al., 2006).

It 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). It 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.

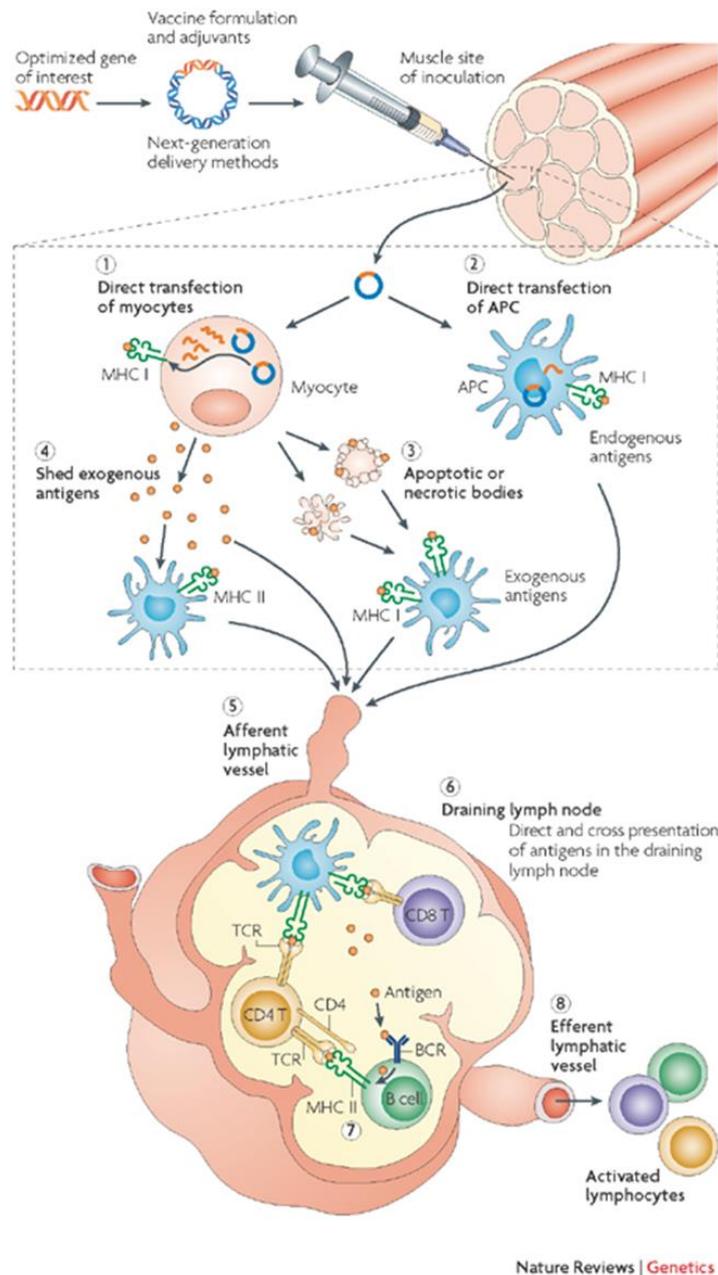
The use of DNA as a vaccine determines increased protein synthesis, augmented T cell help and MHC class I presentation and the production of specific cytokines and pathogen associated molecular patterns that increase innate immune activation leading to T-cell response as well as the production of antibodies 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<sup>+</sup> 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-synthesized 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).*

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)- $\gamma$  secreting antigen- specific T cell responses in humans to a pre-erythrocytic 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 parasitemia 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 Copenhagen 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 properties 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<sup>+</sup> CD95<sup>+</sup> memory CD4<sup>+</sup> 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<sup>+</sup> 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.

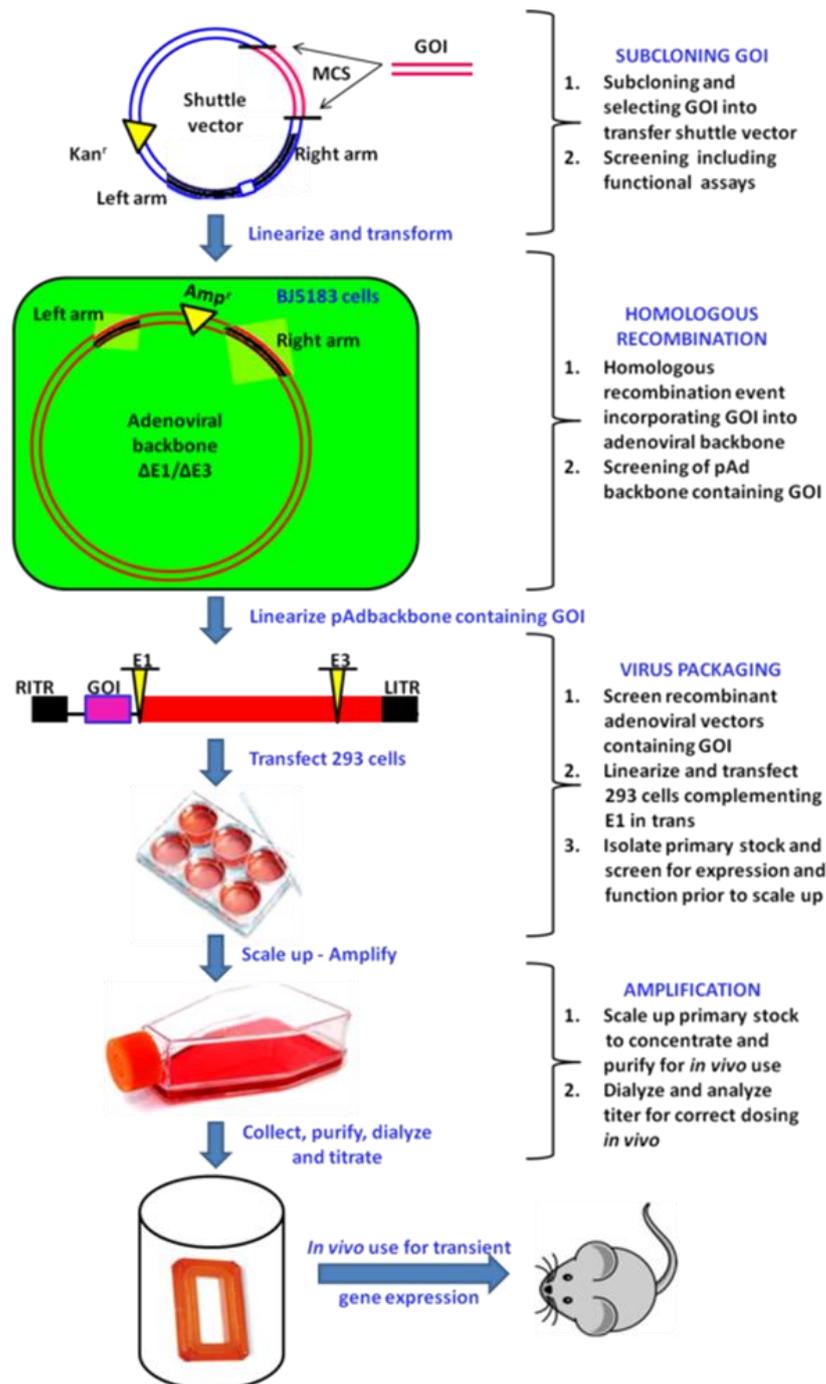
The E4 transcription unit encodes seven polypeptides through distinct open reading frames (ORFs), which affect viral transcription and a number of host cell functions including cell proliferation and apoptosis.

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 carries 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<sup>+</sup> T cells though low CD4<sup>+</sup> 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<sup>+</sup> T cell response against the encoded antigens (Casimiro et al., 2003).

Bassett et al (2011) demonstrate that unlike most acute infections, the CD8<sup>+</sup> 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<sub>EFF</sub>; CD127<sup>-</sup> CD62L<sup>-</sup>) and effector memory (T<sub>EM</sub>; CD127<sup>+</sup>CD62L<sup>-</sup>) T cells with little evidence of central memory (T<sub>CM</sub>; CD127<sup>+</sup> CD62L<sup>+</sup>) 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 intracellular 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<sup>+</sup> 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<sup>+</sup> 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, IFN $\gamma$  and TNF $\alpha$  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 *Trypanosoma 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  $\beta 2$  microglobulin ( $\beta 2m$ ) 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  $\beta$ 2m 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  $\beta$ 2-microglobulin determines a CD8<sup>+</sup> T cell response that is faster and more consistent than the CD8<sup>+</sup> T cell response induced by the full length viral glycoprotein. The generated CD8<sup>+</sup> T cells provide long-term protection from virus infection, thus adding support to the role of CD8<sup>+</sup> 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<sup>+</sup> 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 BalbC17 (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<sup>+</sup> and CD4<sup>+</sup> 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<sup>+</sup> and CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 IFN $\gamma$  and interleukin IL-4 or IL-2 responses in enzyme-linked immunosorbent spot assay, epitope-specific CD4<sup>+</sup> 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- $\gamma$ /CD4<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells show increased cytotoxicity compared to Ad NS3. In addition, Ad li-NS3 elicited high quality memory cells characterized for being poly-functional (IFN $\gamma$ , TNF $\alpha$  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<sup>+</sup> T cells and maintenance of T cell memory (Mikkelesen et al., 2011).

Ii does not only enhance CD8<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 (thrombospondin 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<sup>+</sup> 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 effectively clear this infection, the host elicits an adaptive immune response involving both CD8<sup>+</sup> and CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 outbred 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<sup>+</sup> 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<sup>+</sup> 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% CO<sub>2</sub>/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-glutamine (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<sup>6</sup> 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% CO<sub>2</sub>. 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<sup>6</sup> 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% CO<sub>2</sub>. 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% CO<sub>2</sub>/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-adenovector 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-adenovector 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-adenovector backbone is:

```
5'GATCTATACATTGAATCAATATTGGCAATTAGCCATATTAGTCA
TTGGTTATATAGCATAAATCAATATTGGCTATTGGCCATTGCATAC
GTTGTATCTATATCATAATATGTACATTTATATTGGCTCATGTCCAA
TATGACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGT
AATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCC
GCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCC
AACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCAT
AGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAG
TATTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCAT
ATGCCAAGTCCGCCCCCTATTGACGTCAATGACGGTAAATGGCC
CGCCTGGCATTATGCCAGTACATGACCTTACGGGACTTTCCCTA
CTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGA
TGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTTGA
CTCACGGGGATTTCCAAGTCTCCACCCATTGACGTCAATGGG
AGTTTTGTTTTGGCACAAAATCAACGGGACTTTCCAAAATGTC
GTAATAACCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGT
ACGGTGGGAGGTCTATATAAGCAGAGCTC 3'
```

The sequence of HCMV promoter of the second pre-Adeno backbone is:  
5'CCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATT  
GGCTCATGTCCAACATTACCGCCATGTTGACATTGATTATTGACT  
AGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCA  
TATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCT  
GGCTGACCGCCCAACGACCCCCGCCATTGACGTCAATAATGA  
CGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGT  
CAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA  
TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATG  
ACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTA  
TGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCT  
ATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGG  
ATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTG  
ACGTCAATGGGAGTTTGT TTTGGCACCAAATCAACGGGACTT  
TCCAAAATGTCGTAACA ACTCCGCCCCATTGACGCAAATGGGC  
GGTAGGCGTG TACGGTGGGAGGTCTATATAAGCAGAGCTCTCC  
CTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGATCGTCGA  
CGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATC  
CACGCTGTTTGGACCTCCATAGAAGACACCGGGACCGATCCAG  
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  $3-5 \times 10^6$  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  $2 \times 10^9$  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  $\mu$ l per side (100  $\mu$ l final volume). The injected dose for all mouse experiments was  $3 \times 10^6$  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- $\gamma$ ELISpot**

Mouse splenocytes were plated in duplicate at two different densities ( $2 \times 10^5$  and  $4 \times 10^5$  cells per well) after blocking and washing of MSIP S4510 plates (Millipore, Billerica, MA) coated with 10  $\mu$ g/ml of anti-mouse IFN- $\gamma$  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 2  $\mu$ g/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. Plates were incubated with biotinylated anti-mouse IFN- $\gamma$  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- $\gamma$  per millions of splenocytes. To have a positive ELISpot response these following conditions must occur: IFN- $\gamma$  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-glutamine (GibcoBRL) at 100 MOI (Multiplicity Of Infection-vp/cell) for Western blot analysis ( $10^6$  cells), 50 MOI (vp/cell) for immunoprecipitation ( $3 \times 10^6$  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 (10 $\mu$ M Sigma), pepstatin (1 $\mu$ M)/ E64D (10 $\mu$ M) (ratio 1:1), chloroquine (500nM), radicicol (90 $\mu$ M) (Hsp90 inhibitor) and 17-allylamino-17-demethoxy-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 CO<sub>2</sub> incubator at 37°C and 5% CO<sub>2</sub>) and were infected in 48 well- plate with adenoviral vectors in RPMI containing 2% FBS and 2mM L-glutamine (GibcoBRL) at 200 MOI (infection units/cell) (2x10<sup>5</sup> 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 (1 $\mu$ M)/ E64D (10 $\mu$ M) (ratio 1:1) for 16 hours.

For immunofluorescence analysis HuH7, HEK293 and BMDC cells (10<sup>5</sup> 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) (2 $\mu$ g) 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  $\mu$ g clone Apu2 rabbit monoclonal Millipore) or with anti Ub-Lys63 antibody (10  $\mu$ g 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  $\mu$ g 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 (2 $\mu$ g mouse monoclonal clone B-2 Santa Cruz) or with anti HA (2 $\mu$ g rabbit polyclonal Sigma) for 16 hours at 4°C with rocking. After the supernatants were incubated with protein A sepharose CL-4B (500  $\mu$ g 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 Chemiluminescent 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 ( $2 \times 10^5$ ) were transfected with DNA encoded GFP or mli GFP (0.8 ug) or infected with adenoviral vectors encoding for GFP as described above. After 4 hours they were treated with MG-132 (10 $\mu$ M Sigma), chloroquine (1x) and pepstatin (1 $\mu$ M)/E64D (10 $\mu$ M) (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 mli (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 mli GFP and treated or not with MG-132 for 24 hours. Polyethylenimine (PEI) in water (1ug/ul) was used as 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  $\pm$  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.

## 4. RESULTS

### 4.1 mIi-OVA increases antigen processing via proteasome degradation pathway.

The fusion of Ii to a downstream antigen encoded by adenoviral vectors enhances both CD4<sup>+</sup> and CD8<sup>+</sup> T cells responses with a major effect on the latest (Holst et al., 2008). This strategy was found to accelerate the kinetics of the response, to enhance and prolong vaccine-induced protection against acute or chronic viral infection targeting both dominant and subdominants epitopes from several different antigens (Malcherek et al., 1998; Holst et al., 2008; Capone et al., 2014; Spencer et al.,). One of the postulated mechanisms for the Ii-mediated enhancement of CD8<sup>+</sup> T cell responses is the increase of antigen presentation in the context of MHC class I (Holst et al., 2008). To evaluate the effect of Ii on antigen presentation, we selected as model antigen OVA, given the availability of several tools developed to study antigen presentation, included an antibody recognizing the immunodominant CD8 epitope (SIINFEKL) presented on H2-Kb MHC class I haplotype. Indeed the adjuvant effect of Ii in enhancing CD8<sup>+</sup> T cell responses was confirmed *in vivo* also for OVA antigen. *In vitro*, a rapid and efficient assay to measure antigen presentation was set up by using mouse derived bone marrow dendritic cells (BMDC) (Muccioli et al., 2011). These cells were obtained from femurs of C57BL/6 mice and were differentiated *in vitro* by using GM-CSF. Differentiation was verified using the surface marker CD11c that is specific for dendritic cells (DC). BMDC were then infected with  $\Delta$ E1-E3Ad5 vector encoding OVA (Ad5 OVA) or OVA fused to the murine Ii (mIi OVA) at the amino-terminus. After 16 hours, presentation of the immunodominant OVA peptide SIINFEKL on CD11c<sup>+</sup> infected cells was evaluated using a fluorochrome-labeled antibody recognizing the SIINFEKL peptide presented in the context of the C57BL/6 H2-Kb class I molecule.

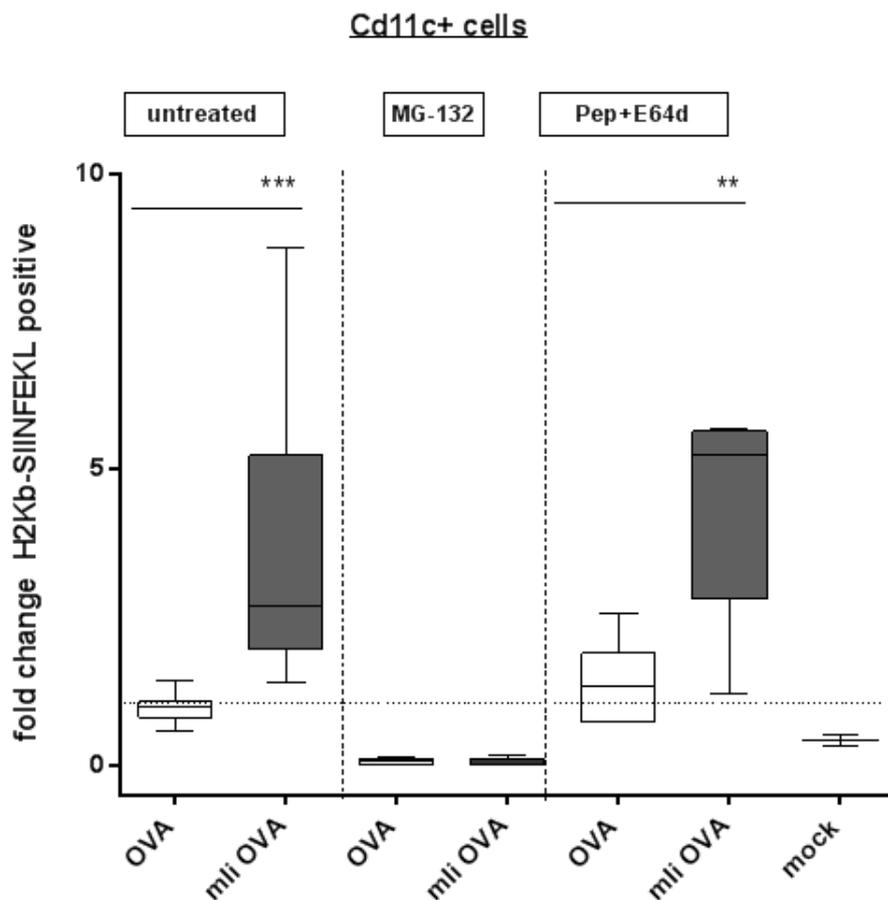
Tethering of mIi to OVA led to increased antigen presentation in class I as compared to OVA, confirming previous observations (Holst et al 2008; Fig 8).

To define the molecular pathway involved in the increased Ii-mediated antigen presentation, two inhibitors specific for MHC class I and class II pathways were used.

MG-132 is a proteasome inhibitor that acts as transition state analog and forms a hemiacetal adduct with the proteasome active sites (Rock et al.,

1994) and it is used as inhibitor for MHC class I pathway. Pepstatin (Pep), in combination with E64D, allows a broad spectrum inhibition of lysosomal cysteine proteases, which are implicated in the processing of antigens for presentation on MHC class II molecules (Manoury et al., 1998). After infection with OVA and mIi OVA, BMDC cells were treated with MG-132 and Pep/E64d for 16 hours. Uninfected BMDC (mock) were used as negative control.

The mIi-mediated enhancement of antigen presentation was abrogated by treatment with MG-132, while the inhibition of the class II pathway presentation with Pep/E64d did not affect antigen presentation (Fig 8), suggesting that the enhancement of antigen-specific CD8<sup>+</sup> T cells responses is a consequence of antigen degradation via the proteasome. Our results also suggest that the main physiological function of Ii which is related to the mechanism of MHC-II presentation does not play a role in the phenomenon of CD8<sup>+</sup> T cell potentiation.



**Figure 8: The effect of mIi OVA increased the surface antigen presentation and it was a proteasome-dependent process.**

*BMDC were infected with OVA and mIi OVA and 4 hours post infection were treated with MG-132 and Pep/E64d or left untreated. The level of SIINFEKL peptide bound to H2-Kb was evaluated on CD11c+ cells after 16 hours post infection. The data were shown as ratio of % SIINFEKL positive cells measured in BMDC infected with mIi OVA relative to OVA control. Statistical significance was determined by one-tailed unpaired Student's t-test (\*\* $p < 0,01$ , \*\*\* $p < 0,001$ ). Mock: not infected BMDC cells.*

#### **4.2 Linkage of mIi increases the antigen targeting to the proteasome via ubiquitin signal.**

To clarify the mechanism through which the invariant chain is able to increase antigen presentation, we performed western blot assays on whole cell extract from HeLa cells infected with Ad OVA and mIi OVA. HeLa cells were chosen because they are easy to manipulate and are efficiently infected by Adenoviral vectors.

Antigen expression was measured by using an antibody specific for a hemagglutinin tag (HA tag) present between mIi and OVA (for mIi OVA construct) or at the N-terminus of the OVA sequence (for OVA construct) (Fig 9a). GAPDH expression levels were used as internal control for normalization. No significant differences were observed between the levels of OVA and mIi OVA. However, when infected cells were treated with the proteasome inhibitor MG-132, Ii OVA levels were five-fold higher than OVA (Fig. 9b). The higher amount of mIi OVA may be due to higher expression levels or to a property of Ii to be insensitive to degradation pathways, others than the proteasome pathway, which don't lead to antigen presentation.

We used different inhibitors of intracellular processing to verify if other known degradation pathways were involved. In particular, chloroquine, radicicol and 17-AAG were tested.

Chloroquine is a well-known inhibitor of the MHC class II pathway as it blocks acidic proteases present in the endosomal compartment (Lombard-Platlet et al., 1993). Indeed, it is known that MHC-II molecules generally bind peptides derived from internalized exogenous proteins degraded by lysosomal proteases, which are inhibited by chloroquine (Huotari et al., 2011). Heat shock proteins (Hsp70 and Hsp90) are chaperones that transfer antigenic peptides to MHC class I and class II molecules (Lie et al., 2002). They were proposed to act as carriers for the cross-presentation of cellular polypeptides, (Li et Srivastava., 1993). Radicicol is an inhibitor of Hsp90 while 17-AAG inhibits Hsp70/90.

HeLa cells were infected with mIi OVA and treated with each of the above indicated compounds for 16 hours. None of these molecules influenced the total amount of OVA or mIi OVA (Fig 9c).

The majority of the protein fragments which accumulated after MG-132 treatment had a high molecular weight indicative of poly-ubiquitination. Indeed, protein degradation through the proteasome is a highly specific process that implies as crucial step the covalent attachment of one or more ubiquitin molecules to the protein to be degraded.

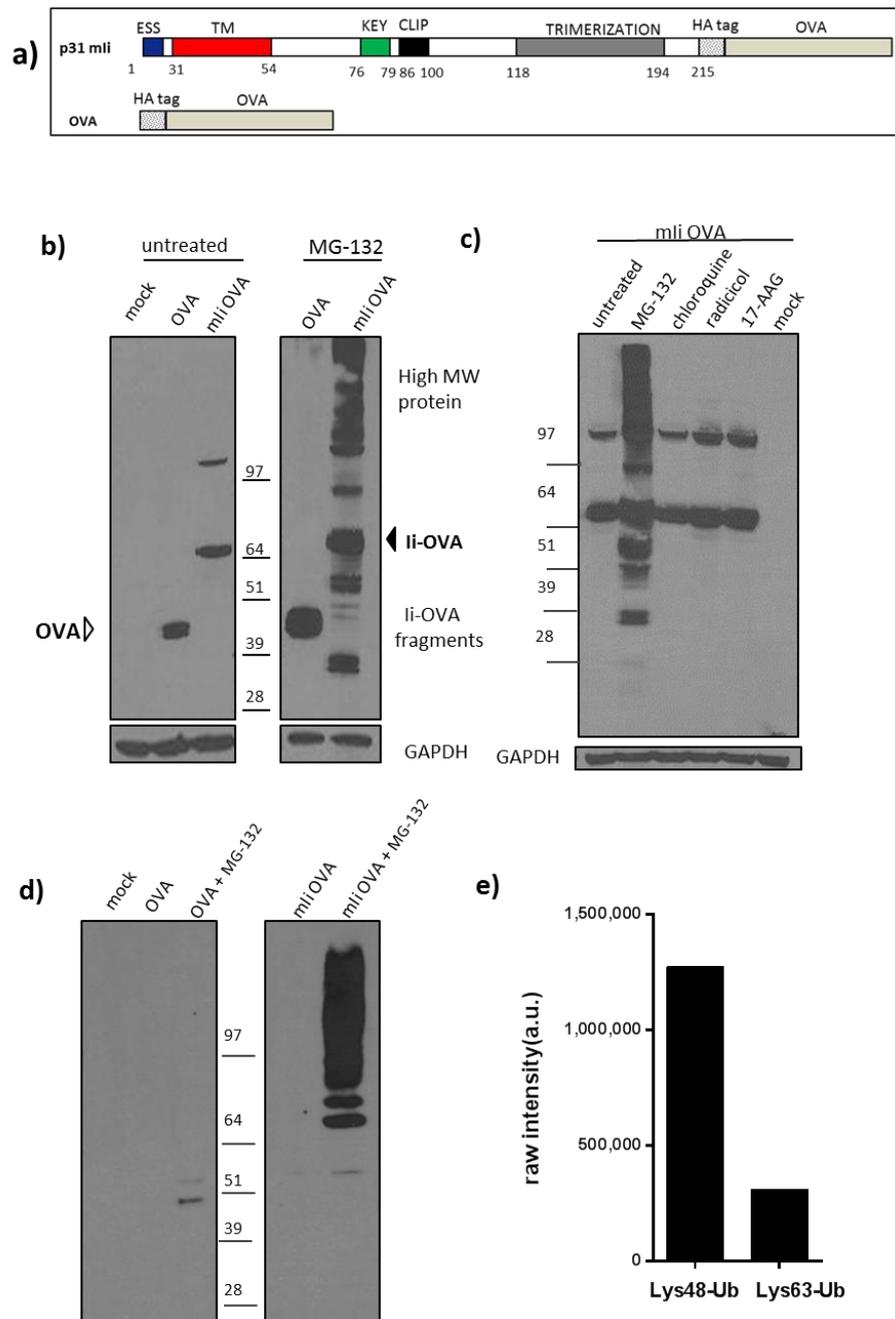
To more specifically address whether the ubiquitination is signaling for mIi OVA degradation via the proteasome, HeLa cells were transfected with a plasmid encoding for ubiquitin and then infected with OVA or mIi OVA in presence of MG-132.

The protein lysates were immunoprecipitated using an anti-ubiquitin Lys48 antibody and the detection was performed using an anti HA antibody in a Western blot assay. The inhibition of proteasome degradation, through MG-132 treatment, led to a higher recovery of OVA antigen in comparison to untreated state, underlying that there was a percentage of antigen OVA that underwent ubiquitination and subsequent degradation.

The fusion of mIi to antigen determined an accumulation of poly-ubiquitinated fragments post proteasome inhibition in comparison to OVA (Fig 9d). Thus, Invariant chain determined the presence of a greater amount of protein in the cytosol that was efficiently poly-ubiquitinated and degraded by the proteasome generating larger amount of antigenic peptides which were more proficiently presented in class I.

To confirm that poly-ubiquitin chains were linked through a specific lysine, Lys48, that addresses mIi linked to OVA to the proteasome, another antibody, anti-ubiquitin Lys63 has been used in an immunoprecipitation assay.

Lys63-linked poly-ubiquitin tagging is involved in a variety of cellular events that do not rely on degradative signaling via proteasome, but involves post-replicative DNA repair, ribosome function, endocytosis, mitotic checkpoint and inheritance of mitochondrial DNA (Weissman et al., 2001). Most of the poly-ubiquitin chains that were present on the mIi OVA fusion after proteasome blocking were linked via Lys48 and not Lys63, further supporting the involvement of the proteasome in the phenomenon of increased antigen processing of the mIi OVA fusion (Fig 9e).



**Figure 9: li enhanced the targeting of the antigen to the proteasome via ubiquitin signaling.**

a) Schematic representation of Ad5 vectors encoding for full length murine p31 invariant chain fused to OVA antigen. ESS: endolysosomal sorting motif; TM: transmembrane domain; CLIP: class II-associated Ii chain peptide; HA tag: human influenza hemagglutinin. b-c) HeLa cells were infected with OVA or mli OVA in absence or in presence of MG-132, chloroquine (lysosome inhibitor), radicicol (Hsp90 inhibitor) and 17 AAG (Hsp70/90 inhibitor). Expression of mli OVA versus OVA in untreated and treated cells was evaluated by Western Blot using anti HA antibody and normalized on GAPDH. d) HeLa transiently transfected

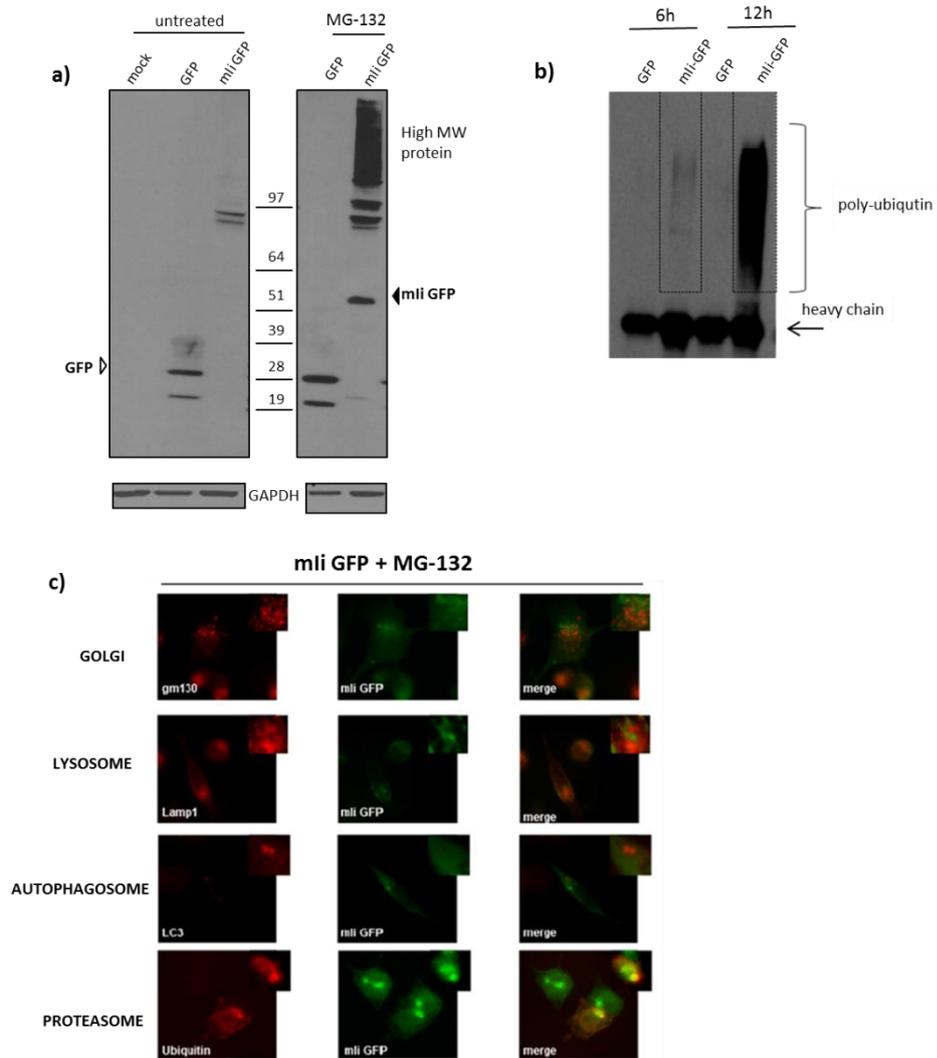
*with ubiquitin plasmid were infected with OVA or mli OVA and in absence or in presence of MG-132. Cells extracts were immunoprecipitated with anti-Lys48 antibody and analyzed by WB with an anti HA Ab detecting OVA. e) Levels of ubiquitination on Lys48 and Lys63 measured by densitometry analysis using NIH image program.*

#### **4.3. The effect of Invariant chain on antigen processing is also confirmed by fusion to GFP.**

To verify if the Ii-induced poly-ubiquitination and increased proteasome degradation are also observed with proteins different from OVA, a second antigen was investigated. GFP (Green Fluorescent Protein) was selected for these studies given its fluorescence property which offers the possibility to run co-localization studies aiming to identify the subcellular structures where the Ii-fused antigens accumulate and undergo degradation.

HeLa cells were infected with Ad5 GFP and Ad5 mli GFP and then treated or not with MG-132 for 16 hours. The protein lysates were analyzed by Western blot assay using an anti-GFP antibody.

GFP and mli GFP fusion displayed similar levels of expression; however, the levels of mli GFP fusion were significantly higher than those of the GFP protein when HeLa cells were treated with the MG-132 proteasome inhibitor (Fig 10a). These results confirmed the effect of mli linkage on antigen expression and degradation as previously seen for OVA antigen. To confirm that also in this case the high molecular weight species would correspond to poly-ubiquitinated GFP, HeLa cells were transfected with FLAG-tagged ubiquitin and then infected with GFP or mli GFP in presence of MG-132. Immunoprecipitation of GFP followed by western blot with anti-FLAG antibody showed a high molecular weight smear in cells infected with mli GFP that became detectable just after 6 hours post infection but it was more visible after 12 hours post infection (Fig 10b), suggesting that the mli linked GFP undergoes poly-ubiquitination. Consistent with the proteasome-mediated degradation of mli GFP (and mli OVA), a physical interaction of mli GFP with the proteasome but not with other subcellular structures such as the Golgi, the lysosome or the autophagosome, was found in co-localization experiments (Fig 10c).



**Figure 10: mli GFP co-localizes with proteasome and induces proteasomal degradation via ubiquitin signal.**

a) Expression of GFP and mli GFP was evaluated by W.blot in HeLa cells (untreated and MG-132 treated) and normalized with GAPDH. b) HeLa transiently transfected with FLAG-tagged-ubiquitin were infected with Ad5 GFP or mli GFP and then treated with MG-132 for 6h or 12 h. Cell extracts were immunoprecipitated with anti-GFP Ab and analyzed by W.blot with anti-FLAG. c) Localization of GFP in HeLa cells with mli GFP in the Golgi, lysosome, autophagosome, proteasome was evaluated post MG-132 treatment.

#### **4.4 Mutagenesis of different Invariant chain domains to assess their role in the adjuvant effect.**

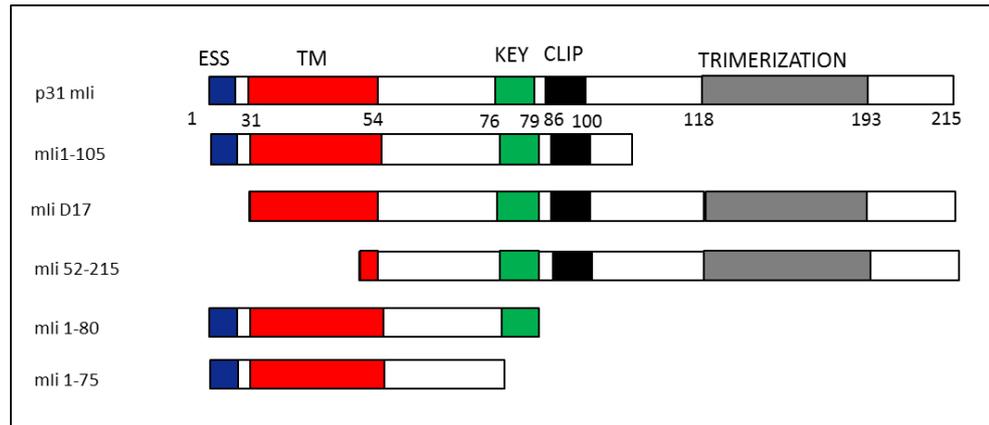
The Invariant chain is a type II transmembrane protein initially identified as a chaperone for MHC class II molecules (Blum et al., 2013). The Invariant chain performs different functions, including i) stabilization of the interaction of  $\alpha$  and  $\beta$  chains of the MHC class II molecules in the endoplasmic reticulum (ER); ii) prevention of class II molecules from prematurely binding peptides; and iii) targeting the MHC class II complex to the endosome through Golgi thanks to its endolysosomal sorting signal (ESS; Sandoval et al., 1994).

After synthesis, the Invariant chain is inserted in the ER membrane by virtue of its internal start transfer peptide and its transmembrane domain (TM domain). Upon translocation in the ER, Ii is anchored to the membrane with its N terminus protruding into the cytoplasm and the rest of the protein facing the ER lumen (Villadangos et al., 1997).

The transmembrane domain, 31aa-54aa (Ashman and Miller., 1999), has been implicated in anchoring Ii to the ER membrane (Parks et al., 1991), ensuring correct folding and function of the MHC class II complexes (Dixon et al., 2006), as well as in contributing to stable homotrimer formation (Stumptner-Cuvelette and Benaroch., 2002). The Invariant chain transmembrane domain has also been implicated in efficient delivery to endocytic processing compartment containing a signal distinct from the Ii cytoplasmic tail (Odorizzi et al., 1994).

A trimerization domain, 118aa-194aa (Ashman and Miller., 1999), is responsible for homotrimerization of the Invariant chain and subsequent association with MHC II  $\alpha$  and  $\beta$  chains leading to a stable conformation which is required for effective post ER transport. The MHC class II-Ii complex, goes through the Golgi, and reaches the peripheral endocytic pathway where exposure to acidic pH and/or proteolytic enzymes results in the cleavage of Ii. Only a small Ii fragment, CLIP (class II-associated Ii chain peptide) remains tied to MHC class II groove to prevent premature binding of antigenic peptides. In this function CLIP is helped by an invariant chain sequence called KEY that plays an important role in CLIP loading in the MHC class II peptide binding region (Chen et al., 2012). When endocytosed antigens are degraded to peptides in the endosomes, CLIP is removed and peptides are loaded on MHC class molecules. After peptide binding the MHC II complex is then translocated to the plasma membrane and displayed on the cell surface for interaction with CD4+ T cell receptor and immune activation (Bikoff et al., 1993).

To better dissect the mechanism responsible for the adjuvant effect of Ii on CD8<sup>+</sup> T cell responses, we generated deletion mutants targeting known and well characterized Ii functional domains involved in the MHC class II pathway. OVA was used again as a model antigen encoded by Ad5 and we generated mutants of mIi deleted of the trimerization region (mIi 1-105 OVA), the endolysosomal motif (ESS; mIi D17 OVA), the transmembrane domain (mIi 52-215 OVA), the CLIP (mIi 1-80 OVA) and KEY (mIi 1-75 OVA) sequences (Fig 11).



**Figure 11: Ii variants fused to OVA** Schematic representation of Ad5 vectors encoding for full length murine p31 Invariant chain (mIi) and trimmed variants of mIi fused to Ovalbumin antigen (OVA). ESS: endolysosomal sorting signal; TM: transmembrane domain; CLIP: class II-associated Ii chain peptide.

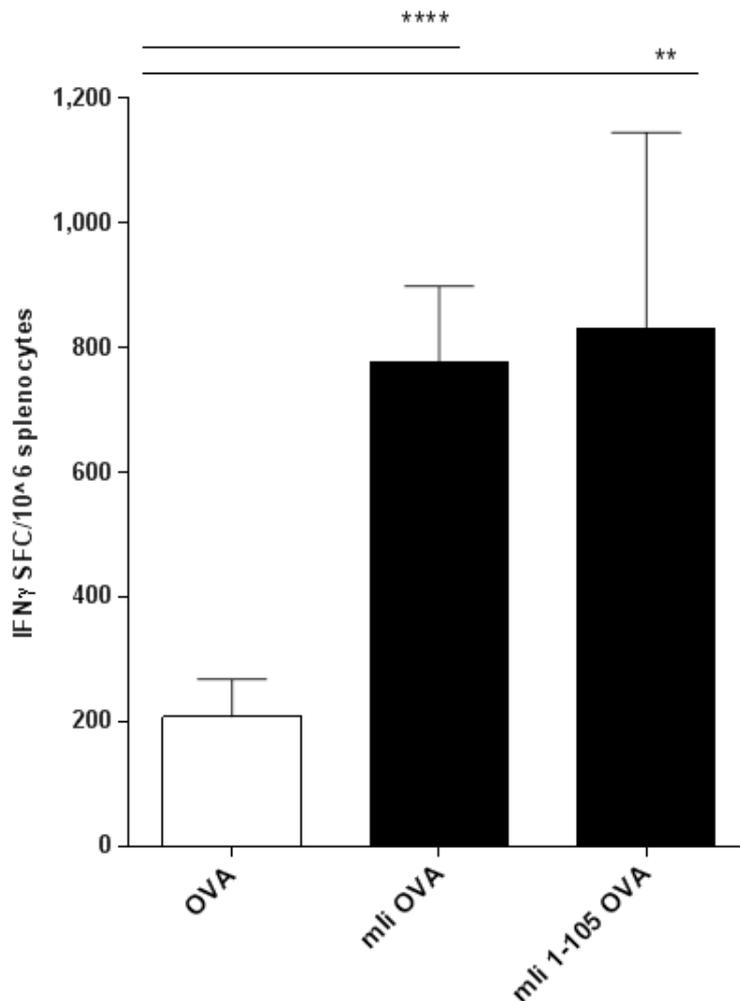
As a first step, we tested these mutants for their ability to improve immunogenicity of mIi-mutants linked antigens upon Adeno delivery in the mouse muscle.

#### 4.4.1 Deletion of the trimerization domain does not affect antigen-specific CD8<sup>+</sup> T cell enhanced response.

We first generated an Ii variant deleted of the trimerization domain (mIi 1-105; Fig 11). As described previously, this domain ensures the Invariant chain to adopt the right conformation allowing interaction with three MHC-II heterodimers ( $\alpha$ :  $\beta$ ; Cresswell et al., 1996).

C57black6 mice were vaccinated with Ad5 OVA and Ad5 mIi OVA as negative and positive controls respectively, and with Ad5 mIi 1-105 OVA. After 2 weeks, an IFN $\gamma$  ELISpot assay was performed on splenocytes of vaccinated mice to measure the level of anti-OVA T cell immunity.

The results in Figure 15 showed that deletion of the trimerization domain did not affect Ii-mediated enhancement of CD8+ T cell response.



**Figure 12:** *The removal of trimerization domain didn't affect Ii effect* C57 mice were vaccinated with OVA, mli OVA and mli 1-105 OVA. T cell responses were evaluated 2 week after single intramuscular immunization by interferon- $\gamma$  ELISpot on splenocytes and expressed as number of T cells producing IFN $\gamma$  per millions of splenocytes. Statistical significance was determined by one-tailed unpaired Student's t-test (\*\* $p < 0,01$ ; \*\*\* $p < 0,001$  ).

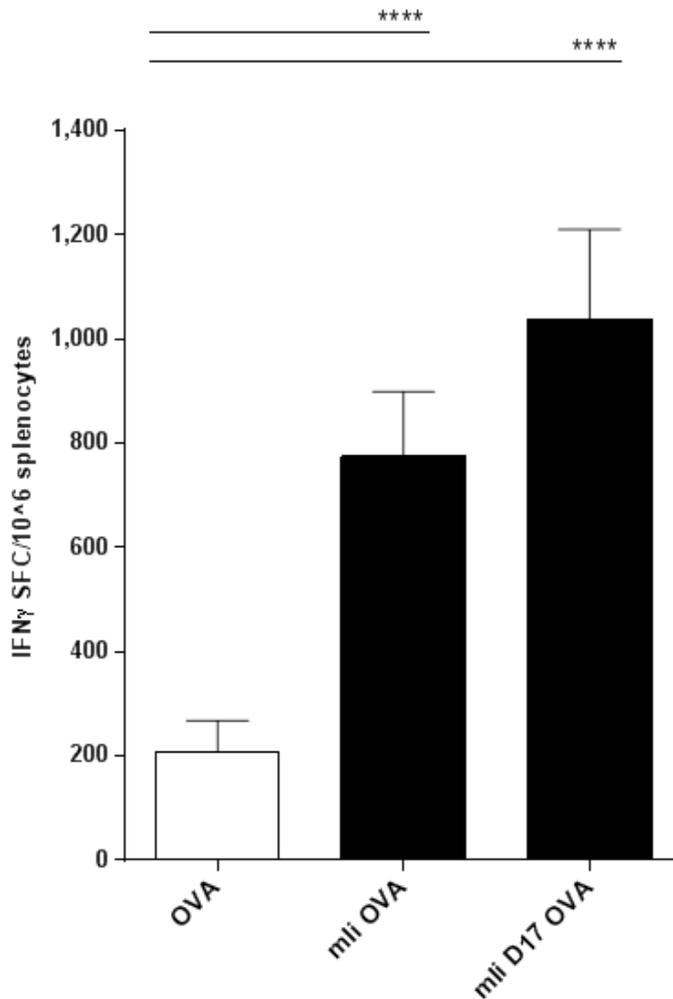
#### **4.4.2 Removal of the endolysosomal sorting signal and of the transmembrane domain does not impact the increased antigen immunogenicity due the linkage of Ii.**

The endolysosomal sorting motif (ESS) of Ii, mediates the targeting of the MHC class II-Ii complex to the endosomal pathway, and promotes

antigen cross-presentation to CD8<sup>+</sup> T cells (Basha et al., 2012). In addition, it has been demonstrated that the expression of a form of Ii devoid of the ESS motif, failed to promote DC cross-presentation and activation of CTL response. The reconstitution with a CD74 molecule, containing ESS motif, restored cross-presentation (Powis et al., 2006; Basha et al., 2012).

To verify if the ESS motif is important for the Ii mediated increase of antigen specific CD8<sup>+</sup> T cell response, possibly due to cross-presentation, we generated an mIi mutant deleted of the first 17 amino acids at its N terminus and linked it to the OVA sequence (mIi D17; Fig 11).

Mouse immunization studies with an Adenovirus vector encoding for this variant showed that deletion of the ESS motif does not impair the ability of the Invariant chain to improve immunogenicity (Fig 13), further supporting the hypothesis that the Ii-mediated adjuvant effect is not dependent on the previously described role of the invariant chain in cross-presentation.



**Figure 13:** *The removal of ESS motif didn't affect Ii effect* C57 mice were vaccinated with OVA, mli OVA and mli D17 OVA T cell responses were evaluated 2 week after single intramuscular immunization by interferon- $\gamma$  ELISpot on splenocytes and expressed as number of T cells producing IFN $\gamma$  per millions of splenocytes. Statistical significance was determined by one-tailed unpaired Student's t-test (\*\*\*\* $p < 0,001$ ).

The transmembrane region ensures the anchoring of Ii to the ER membrane. Insertion of Ii into the ER membrane is co-translational and allows stabilization and correct folding of newly synthesized MHC class II molecules that are translocated into the ER.

To address if the Ii transmembrane domain and its trafficking to the ER are important for the Ii mediated increase of antigen immunogenicity, we generated a mli mutant deleted in both the ESS and the transmembrane

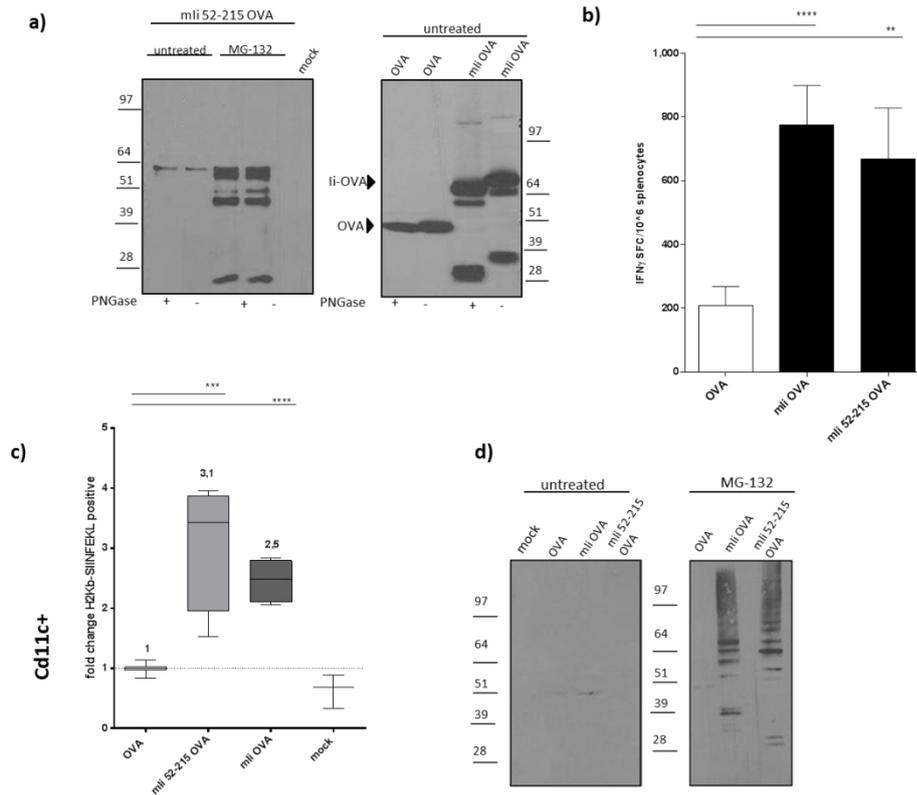
domain and linked it to the OVA antigen (mIi 52-215 OVA; Fig 11). This variant should stay in the cytosol and not be able to reach the ER. To verify this, we analyzed the glycosylation state of the variant and of the OVA fusion to the full length form of the Invariant chain.

The glycosylation state of a protein is an important tool for studying its trafficking through the secretory pathway given the fact that this process occurs in the ER. We used the enzyme PNGase F which is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins. Treatment with PNGase F was performed on immunoprecipitated mIi 52-215 OVA or full length mIi OVA using an anti HA antibody. After digestion with this enzyme, while mIi OVA fusion displayed a shift in migration in SDS PAGE, no change in the molecular weight of the mIi 52-215 OVA was observed, confirming that the mutant was not glycosylated and didn't access the ER (Fig 14a).

We then tested the mIi 52-215 OVA variant *in vivo* in mice and compared it to full length mIi OVA and OVA. The results of this experiments showed that removal of the transmembrane domain did not reduce the adjuvant effect of the Invariant chain (Fig 14b).

Consistently, the ESS and transmembrane region deleted “soluble mutant” showed the same level of increased antigen presentation as the full length mIi when assessed in the BMDC presentation assay of the immunodominant epitope SIINFEKL bound to H2-Kb (Fig 14c) (Muccioli et al., 2011).

To control if also for this soluble variant, the mechanism of enhanced presentation and immunogenicity is due to ubiquitination and increased proteasome targeting and degradation, we performed ubiquitin Lys48 immunoprecipitation in presence or absence of MG132 treatment. Results show that both full length mIi OVA and mIi 52-215 OVA were efficiently ubiquitinated (Fig 14d).



**Figure 14: The transmembrane motif of Ii was not essential for Ii adjuvant effect.** a) HeLa cells were infected with OVA or mli OVA or mli 52-215 OVA and left treated with MG-132. Cells extracts were immunoprecipitated with anti-HA antibody, treated with PNGase enzyme and analyzed by WB with an anti-HA Ab detecting OVA. b) C57 mice were vaccinated with OVA, mli OVA and mli 52-215 OVA. T cell responses were evaluated 2 week after single intramuscular immunization by interferon- $\gamma$  ELISpot on splenocytes and expressed as number of T cells producing IFN $\gamma$  per millions of splenocytes. c) BMDC were infected with OVA, mli OVA and mli 52-215 OVA. The level of SIINFEKL peptide bound to H2-Kb of MHC class I was evaluated on CD11c+ cells after 16 hours post infection. The data were shown as ratio in terms of %H2-kb SIINFEKL of OVA positive cells normalized on BMDC cells infected with Ad5 OVA. Statistical significance was determined by one-tailed unpaired Student's t-test (\*\*p<0, 01, \*\*\*p<0, 001, \*\*\*\*p<0, 0001). Mock: not infected BMDC cells. d) HeLa cells transiently transfected with ubiquitin plasmid were infected with OVA or mli OVA or mli 52-215 OVA and left treated with MG-132. Cells extracts were immunoprecipitated with anti-Lys48 antibody and analyzed by WB with an anti-HA Ab detecting OVA.

#### **4.4.3 CLIP and KEY motifs are not essential for enhanced immunogenicity.**

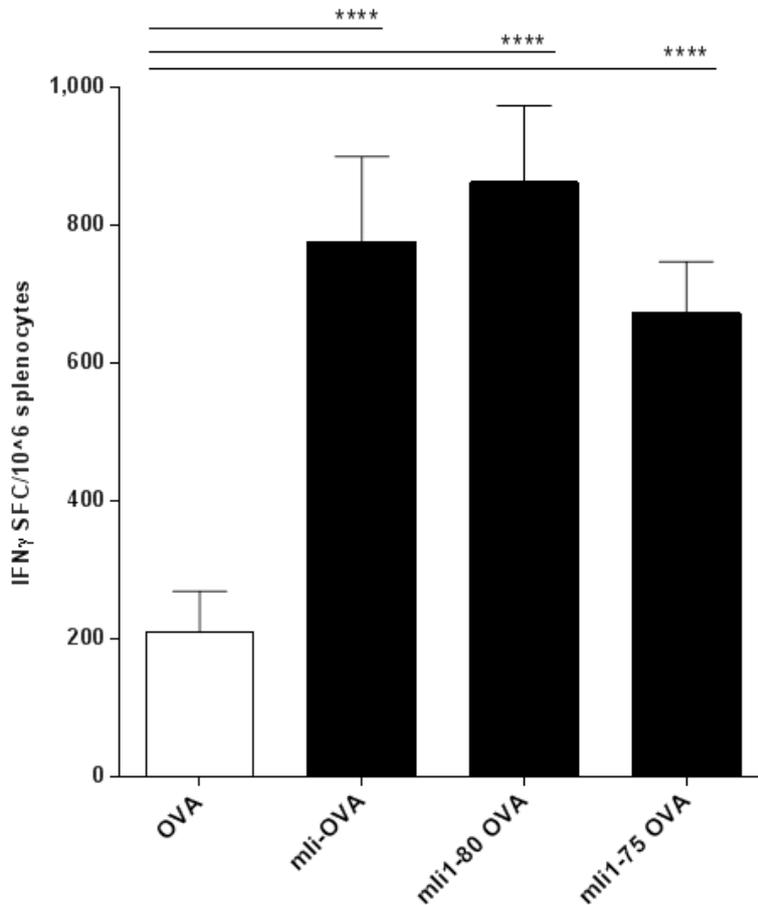
The CLIP domain prevents the premature loading of antigenic peptides on the MHC class II molecules and the KEY motif assists CLIP in this function (Chen et al., 2012). Recently, CLIP was also shown to be involved in the association of Ii with MHC class I molecules for induction of cross-presentation (Powis et al., 2006).

Therefore, we evaluated if deletion these protein sequences could impact on the Ii adjuvant activity on CD8<sup>+</sup> T cells.

We generated two new variants of Ii: mIi 1-80 OVA, lacking the CLIP region and mIi 1-75 OVA where both CLIP and KEY were removed (Fig 11).

Mice were vaccinated with Ad5 vectors encoding for these variants and with Ad5 OVA and Ad5 mIi OVA as negative and positive controls, respectively.

The results of this experiment showed that the removal of CLIP and KEY motifs did not have any effect on the Invariant chain adjuvant activity, indicating that the physiological role of Ii is not involved in the enhancement of CD8<sup>+</sup> T cell response and that cross presentation cannot explain this novel Ii function (Fig 15).



**Figure 15: CLIP and KEY are not necessary for CD8+ T cell enhancement.**

*C57 mice were vaccinated with OVA, mli OVA, Ad5 mli 1-80 OVA and mli 1-75 OVA T cell responses were evaluated 2 week after single intramuscular immunization by interferon- $\gamma$  ELISpot on splenocytes and expressed as number of T cells producing IFN $\gamma$  per millions of splenocytes. Statistical significance was determined by one-tailed unpaired Student's *t*-test (\*\*\*\* $p < 0, 0001$ ).*

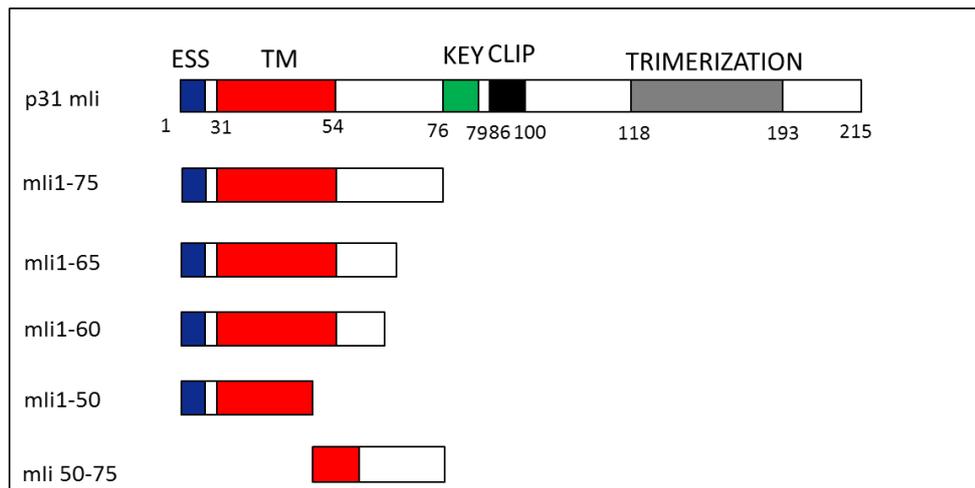
#### **4.5 Mapping the minimal domain of Invariant chain that retains the adjuvant effect.**

Our data showed that the Ii-mediated enhancement of CD8+ T cell response does not depend on the physiological role of the Ii because the deletion of important domains all implicated in the MHC class II pathway, did not impact on the adjuvant effect.

We then aimed at defining the minimal region that could retain the Ii adjuvant effect.

To this end, we generated C-terminal deletion mutants starting from the mIi 1-75 fragment that still retained full activity (mIi 1-65, mIi 1-60 and mIi 1-50, Fig 16) fused to OVA antigen and measured resulting T cell immune response in mice.

These *in vivo* experiments showed clear loss of the adjuvant effect, confirming that the minimal fragment of mIi retaining the full adjuvant effect is mIi1-75 (Fig 17a).



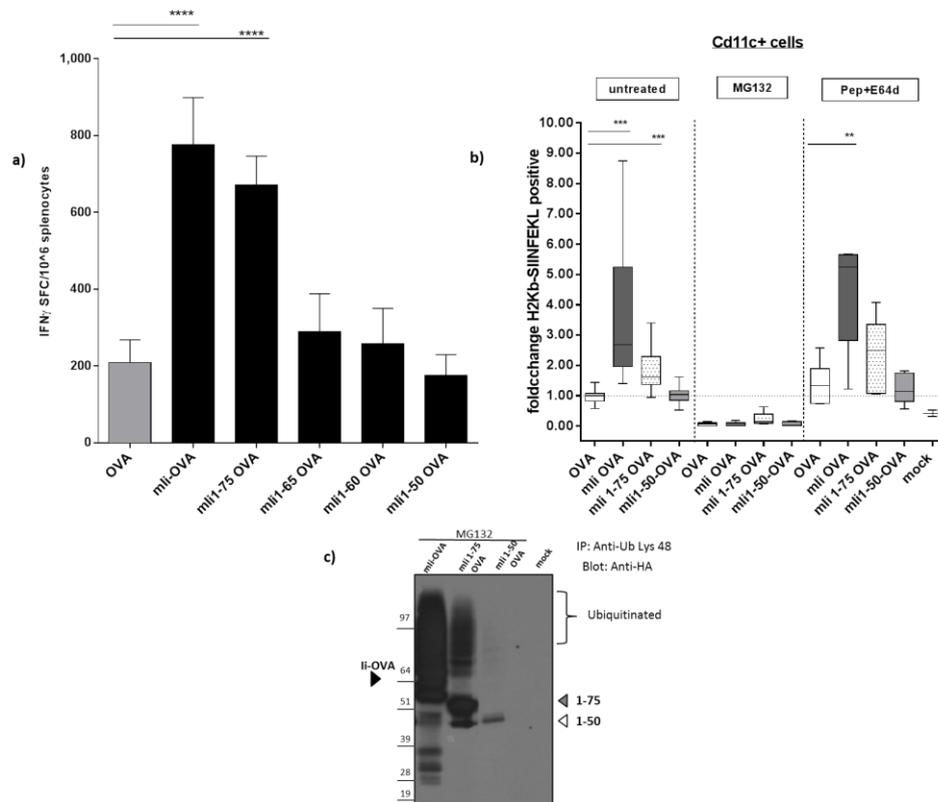
**Figure 16: Short mIi variants fused to OVA** Schematic representation of Ad5 vectors encoding for full length murine p31 Invariant chain (mIi) and trimmed variants of mIi fused to Ovalbumin antigen (OVA).

To verify if the mIi 1-75 variant would still be able to increase MHC class I presentation we tested it in the BMDC presentation assay (Fig 20b) (Muccioli et al., 2011).

Consistently with the *in vivo* immunogenicity data, both full length and 1-75 mutant displayed increased SIINFEKL peptide presentation with respect to the immunologically inactive 1-50 mutant and the unlinked OVA antigen. Also in this case the involvement of the proteasome versus endocytic pathway was demonstrated evaluating antigen presentation after inhibition with MG-132, and with the class II pathway inhibitor Pep/E64d (Fig 17b). Finally, the 1-75 variant was efficiently ubiquitinated on Lys48 while this feature was significantly impaired for the 1-50 mutant (Fig 17c).

Therefore the minimal C-terminal deletion Ii mutant that retains the same adjuvant effect of the full length Invariant chain *in vivo* and that shows

increased ubiquitination and proteasome-dependent increased MHC class I presentation in vitro, is the mli 1-75 fragment.



**Figure 17: mli 1-75 OVA enhanced antigen presentation and promoted proteasome degradation.**

a) C57 mice were vaccinated with OVA, mli OVA and short mli variants. T cell responses were evaluated 2 week after single intramuscular immunization by interferon- $\gamma$  ELISpot on splenocytes and expressed as number of T cells producing IFN $\gamma$  per millions of splenocytes. b) Bone marrow derived DC (BMDC) were infected with OVA, mli OVA and short variants and treated or not with class I and class II inhibitors. The level of SIINFEKL peptide bound to H2-Kb of MHC class I was evaluated on CD11c<sup>+</sup> cells after 16 hours post infection. The data were shown as ratio in terms of %H2-kb SIINFEKL of OVA positive cells normalized on BMDC cells infected with OVA. Statistical significance was determined by one-tailed unpaired Student's t-test (\*\* $p < 0,01$ , \*\*\* $p < 0,001$ , \*\*\*\* $p < 0,0001$ ). c) HeLa transiently transfected with ubiquitin plasmid were infected with mli OVA, mli 1-75 OVA and mli 1-50 OVA and left treated with MG-132. Cells extracts were

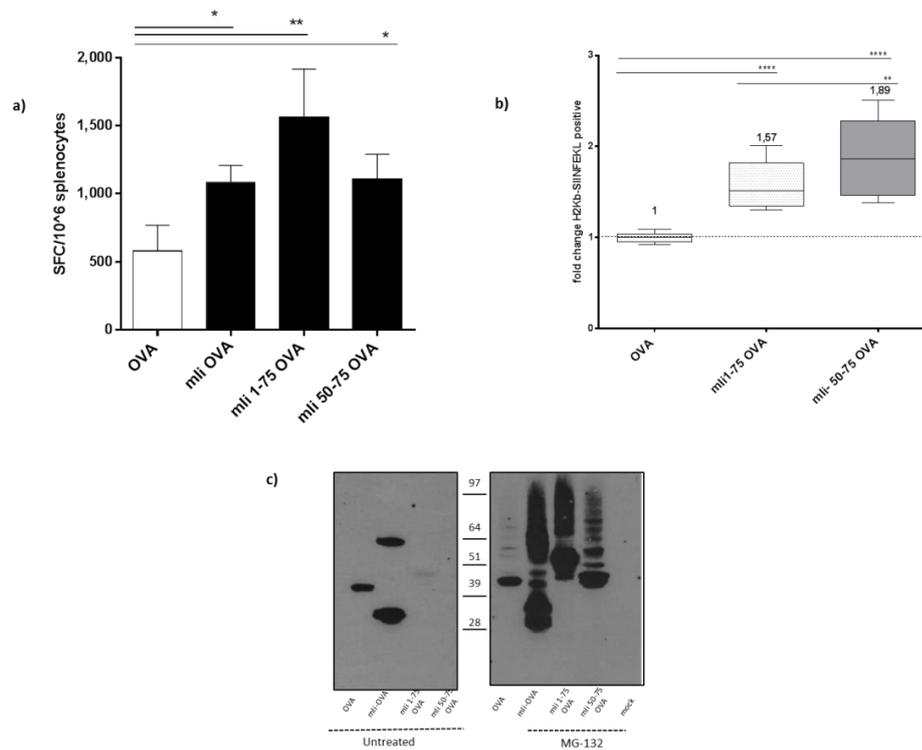
*immunoprecipitated with anti-Lys48 antibody and analyzed by WB with an anti-HA Ab detecting OVA.*

#### **4.5.1 The mIi 50-75 variant retains adjuvant effect in vivo, increases MHC class I antigen presentation via ubiquitination and proteasome dependent processing.**

Our in vivo and in vitro analyses have demonstrated that two shortened variants mIi 1-75 and mIi 52-215 retained the same adjuvant effect of full length Invariant chain. We therefore hypothesized that the amino acids between residues 50 and 75 of mIi may be sufficient to improve antigen-specific CD8+ T cell responses when linked to an antigen and delivered by Adenoviral vectors.

The mIi 50-75 sequence was fused to the OVA antigen (Fig 16) and encoded into an Ad5vector. Mouse immunization experiments confirmed that the short variant mIi 50-75 was indeed able to increase anti-OVA CD8 + T cell response (Fig 18a), despite being quite different to full length Ii and the 1-75 variant. In agreement with the in vivo data, the, mIi 50-75 fragment was able to induce increased OVA peptide presentation on BMDC (Zanoni et al., 2011) (Fig 18b) and was shown to be efficiently poly-ubiquitinated (Fig 18c).

These results indicated that as few as 25 amino acids of the Invariant chain are sufficient to reproduce the in vivo adjuvant effect, the increased ubiquitination, targeting for proteasome degradation and MHC class I presentation of the full length form.



**Figure 18: The new short variant mli 50-75 OVA retains the same phenotype of functional variants.**

- a) C57 mice were vaccinated with OVA, mli OVA and short variants mli 1-75 OVA and mli 50-75 OVA. T cell responses were evaluated 2 weeks after single intramuscular immunization by interferon- $\gamma$  ELISpot on splenocytes and expressed as number of T cells producing IFN $\gamma$  per millions of splenocytes. b) Bone marrow derived DC (BMDC) were infected with OVA, mli 1-75 and 50-75 OVA. The level of SIINFEKL peptide bound to H2-Kb of MHC class I was evaluated on CD11c<sup>+</sup> cells after 16 hours post infection. The data were shown as ratio in terms of %H2-kb SIINFEKL of OVA positive cells normalized on BMDC cells infected with OVA. Statistical significance was determined by one-tailed unpaired Student's t-test (\* $p < 0,05$ , \*\* $p < 0,01$ , \*\*\* $p < 0,0001$ ). c) HeLa transiently transfected with ubiquitin plasmid were infected with OVA, mli OVA, mli 1-75 OVA and mli 50-75 OVA and left treated with MG-132. Cells extracts were immunoprecipitated with anti-Lys48 antibody and analyzed by WB with an anti-HA Ab detecting OVA.

#### **4.6 The 1-75 fragment of Ii mediates ubiquitination of the C-terminally linked antigen.**

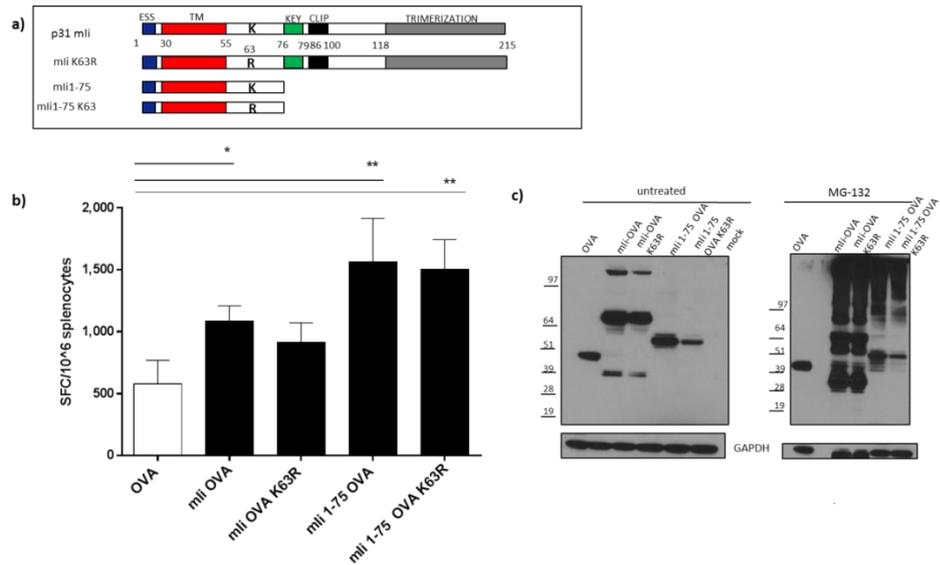
The above described results indicate that a key feature of Ii-mediated adjuvant effect is efficient ubiquitination of the fusion protein. We therefore set out to determine if lysine residues within the Ii sequence itself are required to accept ubiquitin moieties or if the Invariant chain can mediate ubiquitination of lysine residues present in the fused antigen.

To this end, we took advantage of the 1-75 deletion mutant which retains full adjuvant effect, enhanced ubiquitination and proteasome degradation. This Ii protein fragment contains a single lysine at position 63. We therefore substituted this specific lysine with an asparagine residue both in the context of full length Ii (mIi OVA K63R) and 1-75 short variant (mIi 1-75 OVA K63R) and tested the resulting mutants (Fig 19a) for their ability to induce ubiquitination of the corresponding OVA fusion and to increase immunogenicity in mice.

Immunogenicity experiments showed that mutation of the lysine 63 into arginine did not affect the adjuvant activity of both full length and the 1-75 fragment (Fig 19b).

Consistently both variants with mutated K63 lysine showed clear poly-ubiquitination of the corresponding fusion proteins in the presence of proteasome inhibitor, although at various degree (Fig 19c).

The results of this experiment demonstrated that the 1-75 K63R Ii mutant could induce ubiquitination of lysine residues present in the linked antigen thereby resulting in increased proteasome degradation, MHC class I presentation and CD8<sup>+</sup> response.



**Figure 19: The mutation of lysine 63 in arginine in the full and short form didn't influence the adjuvant effect in vivo and proteasome processing.**

a) Schematic representation of Ad5 vectors encoding for new short variant of mIi linked to OVA antigen. b) C57 mice were vaccinated with OVA, mIi OVA, mIi OVA K63R and short variants mIi 1-75 OVA and mIi 1-75 OVA K63R. T cell responses were evaluated 2 week after single intramuscular immunization by interferon- $\gamma$  ELISpot on splenocytes and expressed as number of T cells producing IFN $\gamma$  per millions of splenocytes. . Statistical significance was determined by one-tailed unpaired Student's t-test (\* $p < 0,05$ , \*\* $p < 0,01$ ) c) HeLa cells were infected at 100 MOI with OVA or mIi OVA or full length and short mutant in absence or in presence of MG-132. Expression of mIi OVA versus OVA in untreated and treated cells was evaluated by Western Blot using anti HA. Densitometry analysis using NIH image program.

## 5. DISCUSSION

Genetic fusion of Ii to the N terminus of an antigen encoded by Adenovirus-based vaccines was shown to consistently increase magnitude, breadth and longevity of induced T cell responses in different animal models. Despite the prevalent role of Ii as a chaperone for MHC class II molecule, CD8<sup>+</sup> T cells restricted by MHC class I are mostly enhanced. The experimental work described in this thesis was aimed at dissecting the mechanism through which Ii-fusion to antigen encoded by viral vector vaccines enhances mostly CD8<sup>+</sup> T cell response. To this aim we selected a well characterized Adenoviral- based vaccine vector, Ad5, encoding OVA for which many immunological tools are available. Dissecting the mechanism of action might lead to the design of improved “genetic adjuvant” sequences, support the evaluation of Ii-based vaccines in clinical studies and unveil unknown Ii functions.

Tethering of Ii to OVA markedly increased presentation of the OVA immunodominant epitope (SIINFEKL) in the context of MHC class I with respect to OVA alone in infected BMDC, as shown originally by Holst and collaborators (Holst et al, 2011) and confirmed by our own experiments. The involvement of MHC class I pathway was reinforced by experiments in TAP knock-out mice (Holst et al., personal communication). TAP transporter (Transporter Associated with Antigen processing) allows the translocation of peptides generated by the proteasome from the cytosol into the lumen of the ER. In the ER, peptides are loaded into the newly synthesized MHC class I molecules (Lankat-Buttgereit et al., 2002). The effect of Ii in enhancing T cell response was abolished in TAP knock-out mice after immunization with Ad5 encoding mIi OVA.

In agreement with this first set of data, we observed that proteasome inhibition by the specific inhibitor MG-132 led to loss of Ii-mediated enhancement of antigen presentation in “in vitro” BMDC infection experiments. The specific effect of MG-132 on MHC class I antigen presentation was described by Rock and Goldberg (1994) that showed a significant decrease in presentation of ovalbumin peptide, introduced in murine B lymphoblastoid cells, after treatment with MG-132. Indeed, inhibition of the proteasome led to a lower level of H2-Kb complexes containing SIINFEKL detected on the cell surface by an antigen specific T-cell hybridoma. They assumed also that MG-132 inhibitor could penetrate in the living cells inhibiting the bulk of intracellular proteolysis and reversibly block the generation of antigenic peptides without affecting other steps in class I pathway.

When inhibitors of the MHC class II antigen presentation pathways, such as pepstatinA/E64d, were used in the same “in vitro” infection system no impact on the Ii-mediated enhancement of SIINFEKL epitope presentation was observed. These results indicate that at variance with its physiological role, Ii induces degradation of the antigen via the proteasome, leading to increased MHC-I presentation on the cell surface.

Our results are supported by data generated in MHC class II deficient mice where an Adenoviral vaccine encoding for Ii fused to the glycoprotein of LCMV virus induced stronger CD8<sup>+</sup> T cell response and a robust short term protection in the absence of MHC class II pathway involvement and of CD4<sup>+</sup> T cell help (Holst et al., 2011).

To better evaluate intracellular trafficking of Ii-fused antigen we investigated antigen expression in HeLa cells. This cell line is easy to handle and it is efficiently infected by Adenovirus. Interestingly, no difference in the level of expressed OVA antigen was observed after infection with Ad5 encoding OVA or mIi OVA unless cells were treated with MG-132. In this setting, Ii-OVA led to five fold higher levels of the antigen with respect to OVA. The effect was specific to the proteasome inhibitor since no changes in expression were seen after treatment with inhibitors targeting different degradation pathways such as chloroquine, radicicol, and 17-AAG. Importantly, the data collected with the model antigen OVA were confirmed with another antigen, GFP that allowed direct antigen visualization and co-localization with subcellular organelles. GFP levels were seven fold higher when fused to Ii and when the proteasome was blocked by MG-132. Co-localization studies underlined the crucial role of the proteasome and not of other subcellular structures such as Golgi, lysosome and auto-phagosome when the antigen was fused to Ii.

The larger amount of Ii-OVA with respect to OVA observed in the presence of proteasome inhibition could be due to increased mRNA transcription or translation of the fusion (which is currently under investigation). Alternatively, it could be hypothesized that fusion of Ii to an antigen prevents its degradation by a “non immunogenic” pathway, while re-routing it to the proteasome for rapid degradation and increased MHC class I restricted presentation. The rapid targeting to the proteasome may be also a consequence of antigen misfolding that fusion to Ii may cause.

Most of the protein fragments which accumulated after MG-132 treatment with both OVA and GFP had a high molecular weight

suggesting poly-ubiquitination. Proteasome degradation of proteins is a highly specific process that implies as a crucial step, the covalent attachment of one or more ubiquitin molecules to the protein to be processed.

In a poly-ubiquitin chain, ubiquitin molecules can be linked through one of its seven lysines: Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63 (Kulathu et al., 2012). The resulting polyubiquitinated proteins have distinct fates depending upon the nature of the ubiquitin linkage through which they are conjugated; K48-linked poly-ubiquitin chains mainly target proteins for proteasome degradation while K63-linked poly-ubiquitin typically regulates protein function, subcellular localization, and protein-protein interactions. Indeed, by immunoprecipitation experiments using anti Lys48 antibody, we showed an accumulation of poly-ubiquitinated fragments for mli OVA and not for the OVA after MG-132 treatment. Lys-63-linked ubiquitination was not involved in this process.

This finding is in line with previous results that underline the role of ubiquitination in immune response and its use to design improved genetic vaccines to more effectively induce CD8<sup>+</sup> T cell responses. Rodriguez et al (1997) fused an ubiquitin molecule to the N terminus of the nucleoprotein (NP) of LCMV encoded by plasmid DNA and showed an enhanced intracellular degradation of LCMV NP in vitro when linked to ubiquitin (Ub-NP). Moreover, mice immunized with plasmid DNA encoding this rapidly degraded protein were able to mount an enhanced antiviral CTL response and more effective protection against viral challenge. Bazhan et al (2010) evaluated different approaches to improve vaccine immunogenicity, including the design of a poly-epitope HIV DNA vaccine based on the genetic attachment of ubiquitin sequence to the N-terminus of the poly-epitope for proteasome targeting. Immunogenicity studies in mice showed that the construct with the N-terminal ubiquitin was among the most immunogenic ones.

More recently, Zhuo et al 2015 demonstrated that vaccination with ubiquitin-hepatitis B core antigen fused to cytoplasmic transduction peptide enhances the hepatitis B virus-specific cytotoxic T-lymphocyte immune response and inhibits hepatitis B virus replication in transgenic mice.

Despite all of the above, other authors have not observed any improvement in the T-cell responses by linking Ubiquitin to an antigen (Vidalin et al 1999). In contrast, we and others have consistently shown

that genetic fusion of Ii to an antigen, and delivery of the fusion by an Adeno vector is a very efficient strategy to improve CD8 T cell responses against many different antigens and in different animal models opening the way to its evaluation in clinical studies (PEACHI, EU, FP7; [www.peachi.eu](http://www.peachi.eu)).

Taking advantage of the well-known and mapped functional domains of Ii, we generated several “deletion” mutants and evaluated the effect of the deletions both “in vitro” and “in vivo” with the aim of identifying which functional domains of Ii are essential for its role as a genetic enhancer. We also intended to identify the minimal sequence of Ii preserving the ability to enhance antigen-specific T cell response. Such shorter sequence might be useful to facilitate vector construction due to reduced size of the fusion and to minimize the risk of inducing an autoimmune response when autologous Ii sequence is used as genetic enhancer in clinical studies.

After synthesis, the nascent Ii protein is inserted in the ER membrane by virtue of its internal start transfer peptide and its transmembrane domain and it binds to MHC class II molecules in the ER. Ii-MHC class II complex then reaches the endolysosomal pathway through the Golgi thanks to the ESS motif where exposure to acidic pH and/or proteolytic enzymes results in the cleavage of Ii. An interaction between MHC class I and Ii has been also demonstrated (Powis et al., 2006) and recent evidence suggest a role for Ii in TAP-independent cross presentation. Similarly to Ii-MHC class II interaction, Ii binds to and stabilize MHC class I molecules in the ER and via the ESS signal targets them to the endolysosome where exogenous peptides can be loaded (Basha et al 2012). Our experiments with Ii mutants exclude the involvement of the classical MHC class II presentation pathway in the mechanism of enhanced CD8<sup>+</sup> T-cell responses as we could show that none of the ESS, CLIP and KEY motifs are required to enhance CD8<sup>+</sup> T cell responses.

Consistently, we have shown that pH neutralization of endosomes or inhibition of lysosomal proteases does not abrogate the Ii adjuvant effect. Interestingly, CLIP was shown to be involved in the association of Ii with MHC class I molecules (Powis et al., 2006). The results with the Ii variant lacking CLIP suggest that the formation of the complex Ii-MHC class I is not required for the enhancement of antigen presentation and improvement of CD8<sup>+</sup> T cell immunogenicity.

Trimming of the mIi sequence also led to the identification of a minimal sequence of 75 amino acids that might be used in place of the full length.

“In vitro” characterization of the mIi 1-75 variant showed the same pattern of antigen accumulation and poly-ubiquitination. We mutated the specific single lysine of Ii contained in this short variant, and found no impact of the mutation on the Ii activity both “in vitro” and “in vivo”, suggesting that Ii can induce polyubiquitination of candidate Lys residues present in the antigen, thus leading to improved proteasome processing and CD8<sup>+</sup> T cell immunogenicity.

Further C-terminal trimming of mIi led to the identification of a 26 aa long sequence spanning aa 50 to 75 of the mIi that is still able to enhance T cell response when fused to the antigen, via Lys48 poly-ubiquitination and proteasomal degradation. Thus, we have identified a novel functional domain of Ii which can be used as an immunological enhancer.

One question which remains to be clarified is whether the adjuvant effect obtained by tethering Ii to an antigen requires the fusion protein to enter the ER membrane and be retro-translocated to the cytosol by the ERAD or whether the whole process leading to enhanced T cell response occurs in the cytosol.

Evidences showing that linkage of a ER targeting signal sequence (ERTS) or calreticulin, a Ca<sup>2+</sup> binding protein located to ER, to an antigen in a DNA vaccine could enhance a CTL response by increasing intracellular antigen presentation efficiency (Xu et al., 2005; Cheng et al., 2001) support a potential important role of trafficking into the ER and ERAD for the Ii-mediated enhancement of antigen presentation and immunogenicity. To investigate a potential role of ERAD in the mechanism of Ii mediated enhancement of CD8<sup>+</sup> T cell immunogenicity, we performed experiments with inhibitors of this pathways such as Eeyarestatin I that inhibits the anterograde and retrograde transport to ER. Unfortunately, no consistent data were obtained because of toxicity of the inhibitors.

The glycosylation state of a protein is an important tool to study its trafficking through the secretory pathway and it specifically occurs in the ER. Our analysis of glycosylation confirmed that full length Ii fused to the antigen entered ER. It is known that Ii interacts within the ER with calnexin, a chaperone protein involved in refolding of transmembrane proteins and targeting to ERAD (endoplasmic reticulum associated degradation). Augmented Ii degradation occurs when calnexin binding residues are mutated or with truncated variants of Ii that lack calnexin binding domains (Romagnoli et al., 1995) supporting the idea that calnexin acts primarily to retain Ii in the ER and to inhibit its

degradation. Linking Ii to the N terminus of antigens might hinder the interaction site with calnexin and potentially lead to a rapid translocation from the ER and subsequent cytosolic degradation. Furthermore, Ii folding is regulated within cells and misfolded proteins or non-trimerized Ii is rapidly recognized and targeted by ERAD machinery for translocation to the cytosol for degradation (Sevilla et al., 2004).

To better address the role of ER, we generated a variant of Ii (mIi 52-215 OVA) lacking the transmembrane domain which allows the targeting and anchoring of the complex Ii-MHC to the ER membrane (Parks et al., 1991) and demonstrated its cytosolic localization by analyzing its glycosylation state. This variant showed a similar enhancement of specific anti-OVA CD8<sup>+</sup> T cell response to the full-length sequence as well as similar level of antigen presentation and poly-ubiquitination. Therefore, routing of Ii linked antigens to the ER and ERAD is not required for poly-ubiquitination and targeting to the proteasome leading to enhanced MHC class I presentations and stronger CD8<sup>+</sup> T cell response.

## 6. CONCLUSIONS

Tethering of Ii to the N terminus of an antigen within Adenovirus based genetic vaccines was shown to increase magnitude and breadth of T cell-response as well as accelerate kinetics while retaining the phenotype and functionality of Ad-induced T cells. The effect of Ii as a genetic enhancer was consolidated with antigens from several pathogens, using different viral vectors and in different animal models where responses targeting dominant and subdominant epitopes were induced.

This technology seems therefore widely applicable to genetic vaccination and understanding its mechanism of action should provide with important insights toward the design of effective vaccines against cancer or chronic infections. For example, it has been shown that a candidate vaccine for HCV, where a ChAd encodes for a Ii-fused HCV polyprotein, was able to improve the breadth of the induced T cell response and the cross-reactivity of the induced T cells to heterologous and distant HCV genotypes (Capone et al., 2014). For malaria, the linkage of Ii to full length form of TRAP linked to ME, a string of malarial T and B cell epitopes in a ChAd vector, followed by MVA boosting, determined an increase of antigen specific CD8<sup>+</sup> T cell response (Spencer et al., 2014). In LCMV infection, Ii-fused antigen prevented T cell escape and loss of viral control (Holst et al., 2008).

Fusion of an antigen to Ii also accelerates the kinetics of T cell response after a single injection of an Adenovirus vaccine possibly avoiding the need for a booster vaccination even in the absence of CD4<sup>+</sup> T cell help such as HIV infection.

The project of my thesis consisted in the identification and characterization of the molecular mechanism by which Ii is able to improve CD8<sup>+</sup> T cell immunogenicity and in the mapping of the minimal domain responsible for the enhanced CD8<sup>+</sup> T cell response.

The enhanced immunogenicity obtained by tethering Ii to antigens was due to an augmented MHC class I antigen presentation that was specifically dependent on the proteasome activity. Inhibition of antigen degradation highlighted the role of poly-ubiquitination in targeting Ii-fused antigen to the proteasome. This mechanism was demonstrated not only for the full Ii sequence but also with short variants lacking known functional domains, and the minimal Ii protein fragment retaining the adjuvant effect was mapped down to a sequence of only 26 aa. Trimming Ii to very short functional sequences might offer a better candidate for

evaluation of the technology in humans because of potentially reduced off-target activity being Ii a self-molecule.

It is still unclear whether trafficking into the ER and retro-translocation in the cytoplasm via ERAD pathway has a pivotal role in enhancement of the CD8<sup>+</sup> T cell responses. Nevertheless, our data with a fully cytosolic Ii variant capable to enhance T cell response suggest that increased antigen poly-ubiquitination, degradation and presentation on MHC-I can occur independently on the antigen routing toward the ER and ERAD.

In summary, our data suggest that Ii hijacks the physiological MHC class I antigen presentation pathway to enhance CD8<sup>+</sup> T cell response at variance with its role as a chaperone for MHC class II presentation.

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