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# Antigen-antibody complex stimulation of dendritic cells

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## **SUMMARY**

Dendritic cells are the critical link between innate and adaptive immunity. These cells are specialized in the capturing and processing of antigens, stimulation of B and T lymphocytes and activation of antigen-specific T cells, among other functions. Monocyte-derived dendritic cells are the most used DC lineages in immunotherapy. Furthermore, antigen-antibody immune complexes (ICs) have demonstrated to improve antigen presentation by DCs 100-fold compared to soluble antigens and additionally, have showed a possible application in cancer therapy. This study shows an increased stimulation of MoDCs by two different antigen-antibody immune complexes. DCs were derived from monocytes isolated from PBMCs and cultured with IL-4 and GM-CSF for 6 days before incubation with immune complexes. Ovalbumin, used as a protein model, triggered higher stimulation of DCs when forming immune complexes in 36.3% of the donors, in comparison to soluble antigen. The second construct examined, termed [MTTE]<sub>3</sub>-SLP, consists of several synthetic peptides (B cell epitopes) derived from the tetanus toxin that has been linked to a T cell epitope. [MTTE]<sub>3</sub>-SLP-ICs elicited higher stimulation of DCs from 66% of the donors. This represents the first study of human MoDCs stimulation by ICs based on synthetic long peptides. These findings demonstrate that antigen-antibody immune complexes are more efficient than soluble antigens to trigger functional activation of human MoDCs, which can improve vaccine development. Additional studies are needed to understand how immune complexes can stimulate different cell subsets, their effect on T cell priming and its application in the clinic.

**Key words:** Immune complexes, dendritic cells, synthetic peptides, DCs stimulation.

## **ABBREVIATIONS**

<b>APC</b>	Antigen-presenting cell
<b>CTL</b>	Cytolytic T lymphocyte
<b>CMV</b>	Cytomegalovirus
<b>DC</b>	Dendritic cell
<b>FcR</b>	Fc receptor
<b>GM-CSF</b>	Granulocyte-macrophage colony-stimulating factor
<b>IC</b>	Immune complex
<b>IL-4</b>	Interleukin-4
<b>MAC</b>	Membrane-attack complex
<b>MHC</b>	Major histocompatibility complex
<b>MoDC</b>	Monocyte-derived dendritic cell
<b>OVA</b>	Ovalbumin
<b>PBMC</b>	Peripheral blood mononuclear cell
<b>SLP</b>	Synthetic long peptide
<b>TLR</b>	Toll-like receptor

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# 1. INTRODUCTION

The immune system uses an elaborate network of proteins and cells to protect the organism from internal and external danger (Cziri and Wyss-Coray, 2012). According to Matzinger (1994) the driving force for the immune system is the need to recognize danger and potential destruction, rather than the distinction between self and non-self.

In vertebrates, two types of immunity, innate and adaptive, protect the host from infections (Iwasaki and Medzhitov, 2010).

## 1.1 Innate immune system

The innate immune response provides the first line of defense against pathogenic microorganisms (Luster, 2002). Innate immune cells include macrophages, monocytes, NK cells and dendritic cells (DCs) among others (Janeway and Medzhitov, 2002). Innate immunity also comprises soluble components, as the complement system, which includes more than 30 proteins present in plasma as well as associated to cellular surfaces (Walport, 2001).

As opposed to the adaptive system, the innate host defense systems are present in all multicellular organisms although in different forms (Fearon and Locksley, 1996). The recognition of “danger” or infection in the innate immune system is mediated by pattern recognition receptors (PRRs), a set of germline encoded non-clonal receptors that detect relatively invariant molecular patterns present in pathogens. The molecular structures recognized by the PRRs are called pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997), as well as endogenous molecules released from damaged cells, called damage-associated molecular patterns (DAMPs) (Takeuchi and Akira, 2010). Among the conserved molecules recognized as PAMPs, which are only produced by pathogens, are lipopolysaccharides (LPS), lipoteichoic acid (LTA), mannans, glycans and bacterial CpG DNA (Janeway and Medzhitov, 2002). When PAMPs are recognized by immune cells, they elicit a strong immune response that can lead to direct activation of effector mechanisms by innate immune cells (Medzhitov and Janeway, 1998).

The pattern recognition receptors are expressed as transmembrane proteins, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) families, as well as cytoplasmic proteins, such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs). PRRs are expressed in some nonprofessional immune cells, including tumor cells, as well as in dendritic cells and macrophages (Jego et al., 2006; Takeuchi and Akira, 2010). They can also be secreted in the bloodstream and tissue fluids, as the mannan-binding lectin and C-reactive protein (CRP) (Janeway and Medzhitov, 2002). An effective detection of PAMPs by the PRRs rapidly triggers host immune responses through signaling pathways, including complement cascade activation, and secretion of inflammatory responses as cytokines and chemokines (Kumar et al., 2011).

### 1.1.1 The complement system

The complement system, a key component of innate immunity, is involved in 3 principal activities: the defense against pyogenic bacterial infection, as a bridge between innate and adaptive immunity and the disposing of immune complexes and products of inflammatory injury (Walport, 2001). There are three different routes of activation of the complement system: the classical, the mannanose-binding lectin and the alternative pathway. All these cascades lead to the cleavage of the third complement component,

C3, followed by the lytic pathway (Fujita, 2002), in which the membrane-attack complex (MAC) is formed, a lipophilic complex in cell membranes from plasma proteins (Walport, 2001) (Figure 1).

The classic component pathway is activated when the C1 complex binds antigen-antibody immune complexes (ICs). The C1q protein, which belongs to the C1 complex, plays a crucial role in the clearance of immune complexes, an important method to eliminate antibody-coated bacteria (Alegretti et al., 2012).

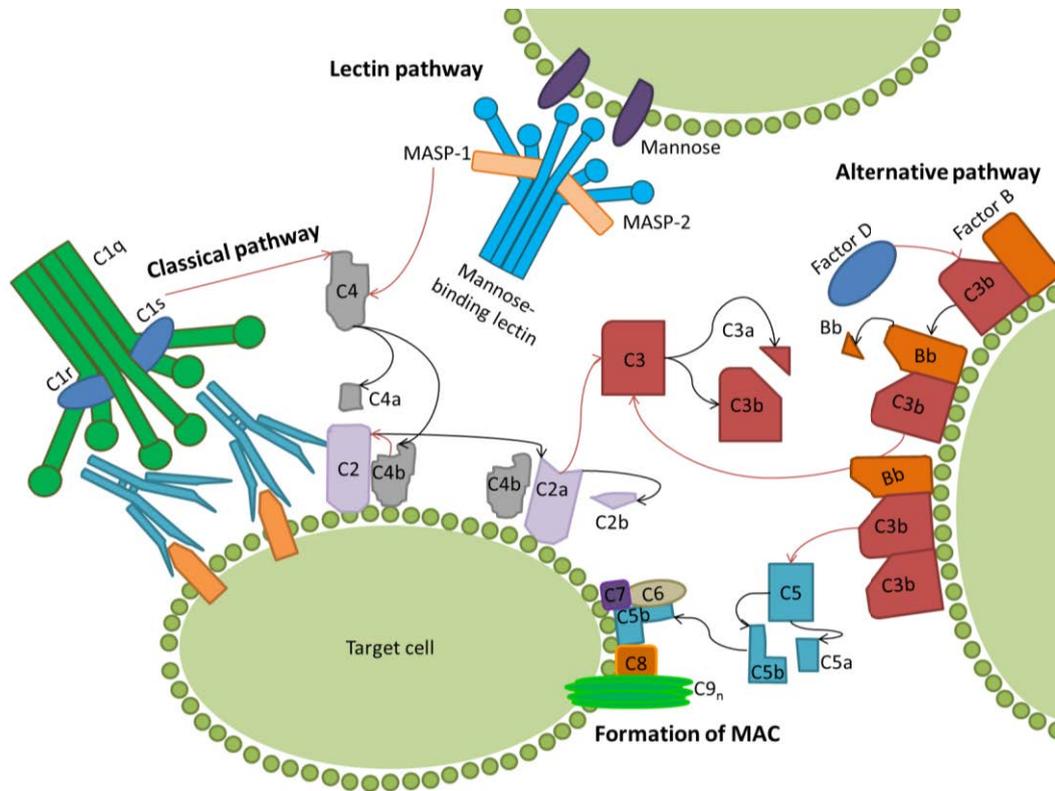


Figure 1. **The three complement activation pathways and formation of the membrane-attack complex (MAC).** The classical pathway initiates by the binding of the C1 complex to ICs. C1q cleaves C4, which subsequently cleaves C2, leading to the formation of a C4b2a complex, the convertase of the classical pathway. Similarly, the mannose-binding lectin and MASP-1 and MASP-2 (Mannose-binding lectin-associated proteins) when bound to mannose groups on the surface of bacterial cells leads to formation of C3 convertase enzyme in the mannose-binding lectin pathway. The alternative pathway starts with the bound of C3b to the cell surface of the target cell which elicits the binding of factor B, which is cleaved by factor D, driving the formation of the complex C3bBb. C3b acts as a receptor for C5 and cleaves it to C5a and C5b, which leads to the binding of C6, C7, C8 and then several units of C9 to form the lytic pore, the membrane-attack complex (MAC). Adapted from Walport (2001), Ricklin et al. (2010).

The complement system is strictly regulated by different mechanisms: a) the cleavage of deposited C3b and C4b by factor I, CD46 and complement receptor 1 (CR1), or by factor H and C4b-binding protein; b) the decay-accelerating activity process, which comprises the inhibition of activation of the alternative complement pathway by CD55, factor H, the C4b-binding protein and binding of CR1g to C3b, thus abolishing the interaction of C3 and C5; and c) inhibition of the formation of the membrane-attack complex by CD59 (Kemper and Atkinson, 2007; Kim and Song, 2006; Walport, 2001; Wiesmann et al., 2006).

### 1.1.2 Dendritic cells (DCs)

Adaptive immunity is subordinated to innate immunity, relying on antigen presentation, a function performed by antigen-presenting cells (APCs), which includes dendritic cells (DCs) (Hoebe et al., 2004).

Dendritic cell maturation is the critical link between innate and adaptive immunity (Steinman, 2012). DCs are the initiators of immune responses: act as sentinels *in vivo*, capturing and processing antigens, migrating to the lymph nodes and activating antigen-specific T cells, stimulate naïve and memory B and T lymphocytes and additionally, potentiate the stimulation of T cells. Moreover, DCs induce tolerance of T cells to self-antigens, thus avoiding autoimmunity (Banchereau and Steinman, 1998).

Dendritic cells are a heterogeneous group of cells that were initially described by Steinman and Cohn (1973) as large “stellate” cells. There are different subtypes of dendritic cells, four main types of cells have been categorized: classical or conventional DCs (cDCs), plasmacytoid dendritic cells (pDCs), Langerhans cells and monocyte-derived dendritic cells (MoDCs) (Satpathy et al., 2012).

Classical DCs are short-life specialized antigen-processing and antigen-presenting cells. When these cells encounter microbial products or inflammatory stimuli, upregulation of costimulatory molecules takes place, as well as increased presentation of peptide-MHC complexes (Satpathy et al., 2012). High diversity is present among the cDC populations. Human cDCs have been separated in two subsets, BDCA-1<sup>+</sup> and BDCA-3<sup>+</sup> (Dzionek et al., 2000; Reizis, 2012).

Monocyte-derived dendritic cells generated by culturing monocytes with GM-CSF and IL-4 were initially developed by Sallusto and Lanzavecchia (1994). These cells showed the characteristic phenotype and functional properties of immature DCs, such as their elevated antigen uptake capacity and high expression of CD1 and MHC class I and II molecules, as well as the stimulatory capacity of naïve T cells (Sallusto and Lanzavecchia, 1994). Nevertheless, the cross-presentation efficiency of the MoDCs differs from conventional DCs depending on the antigen internalization pathway, being similarly effective when antigens are captured by receptor-mediated or bulk phase endocytosis, but almost inactive when the same antigen is phagocytized (Kamphorst et al., 2010). Moreover, it is relevant to mention that the *in vivo* physiologic equivalent of this widely used *in vitro* system has not yet been identified (Satpathy et al., 2012).

Moreover, as a part of the innate immunity, DCs perceive stimuli through germline-encoded pattern recognition receptors, of which TLRs are the best characterized. The different dendritic cell subtypes express different groups of TLRs, which is related to their roles in innate and adaptive immunity (Zanoni and Granucci, 2010). The engagement of TLRs in dendritic cells induces different effects, including chemokine secretion, migration and cytoskeletal rearrangements (Reis e Sousa, 2004a). It also induces increased expression of major histocompatibility complex (MHC)-peptide complexes, thus inducing antigen presentation to T cells (Reis e Sousa, 2004b).

## 1.2 Adaptive immunity

The adaptive immune responses, in contrast to innate immunity, can recognize and respond to an extensive array of antigens (Van den Berg et al., 2004). Adaptive immunity is mediated by immunoglobulin (Ig) B cell receptors (BCRs) and T cell receptors (TCRs), expressed on B and T lymphocytes, and it is only present in jawed vertebrates (Pancer and Cooper, 2006). B and T cells express a large repertoire of receptors, which are generated by V(D)J recombination of gene segments of the Ig and TCR genes (Thompson, 1995). V-, D- and J-gene segments are selected from a substantial number of variant genomic sequences, which added to the imprecise joining,

generates a large diversity of receptors (Van den Berg et al., 2004). The theoretical diversity of the receptors could reach approximately  $10^{18}$  for immunoglobulins and  $10^{20}$  for TCRs, however is not reached due to constraints as biased gene segment usage and recombination failures (Borghesi and Milcarek, 2007).

### *1.2.1 B lymphocytes*

B cells play a key role in the adaptive humoral immunity, producing antibodies in response to stimuli from other cell types as T cells or dendritic cells (Bonilla and Oettgen, 2010). The development of B cells starts with hematopoietic stem cells located in the fetal liver or adult bone marrow (Kaileh and Sen, 2012). Mature naïve B cells are subdivided into different classes: B1, located especially in body cavities, and B2 or conventional B cells found in secondary lymphoid organs and are further subclassified into marginal zone B cells or follicular B cells. The latter are involved in T-cell dependent responses while marginal zone B cells express polyreactive B-cell receptors (Browne, 2012).

Antibody molecules are formed by 4 chains, 2 light and 2 heavy, identical to one another. The high variability between antibodies relies on the recombination of the Ig heavy and light chain genes. Constant and variable portions are located in the light and heavy chains, determining in the latter the main immunoglobulin isotypes:  $\gamma$ ,  $\mu$ ,  $\alpha$ ,  $\delta$ ,  $\epsilon$ , which will form IgG, IgM, IgA, IgD and IgE, respectively (García Merino, 2011).

### *1.2.2 T lymphocytes*

The effector cells of cellular immunity, T cells, develop in the thymus from hematopoietic stem cells from the bone marrow. Even though T cells can be experimentally generated from a diverse set of stem and progenitor populations, the exact lineage commitment for the development of T cells is controversial and depends from the experimental model used (Koch and Radtke, 2011).

There is also a big diversity in the effector functions carried out by T cells. The larger group of T lymphocytes in the body is the  $CD4^+$   $\alpha\beta$  TCR population, termed helper cells ( $T_H$ ). Alternatively,  $CD8^+$  T cells, denominated cytolytic T lymphocytes (CTLs), are involved in clearance of cells infected with intracellular pathogens or transformed cells. Moreover, there are also small subsets of lymphocytes as  $\gamma\delta$  T cells and natural killer T (NKT) cells, which belong to innate immunity and recognize nonpeptide antigens, as microbial pyrophosphates and lipids, presented by nonclassical MHC molecules, members of the CD1 antigen-presenting protein family, and participate in controlling microbial and viral infections, respectively, among other roles as tumor surveillance.

## **1.3 Antigen presentation. MHC molecules**

The T lymphocytes need the antigen to be processed and presented to them by an APC. The TCRs recognize peptides bound to MHC molecules that are located on the surface of APCs (Banchereau and Steinman, 1998). The major histocompatibility complex (MHC) is a genomic region located on chromosome 6 that contain the genes that encode for the polymorphic class I and II molecules. The MHC proteins capture peptides from pathogens and display them on the cell surface (Martínez-Borra and López-Larrea, 2012).

The antigen processing in antigen-presenting cells, such as macrophages, DCs and B cells, occurs through two major pathways: an endosomal or exogenous pathway and an endogenous or proteosomal pathway (Lipscomb and Masten, 2002).

MHC class II molecules present exogenous antigens which are processed through an endosomal pathway before being loaded on MHC class II molecules and

presented to CD4<sup>+</sup> T cells, which have an essential role in adaptive immune response regulation (Van den Hoorn et al., 2011). MHC class II molecules are primarily expressed by professional antigen-presenting cells (Neefjes et al., 2011) (Figure 2).

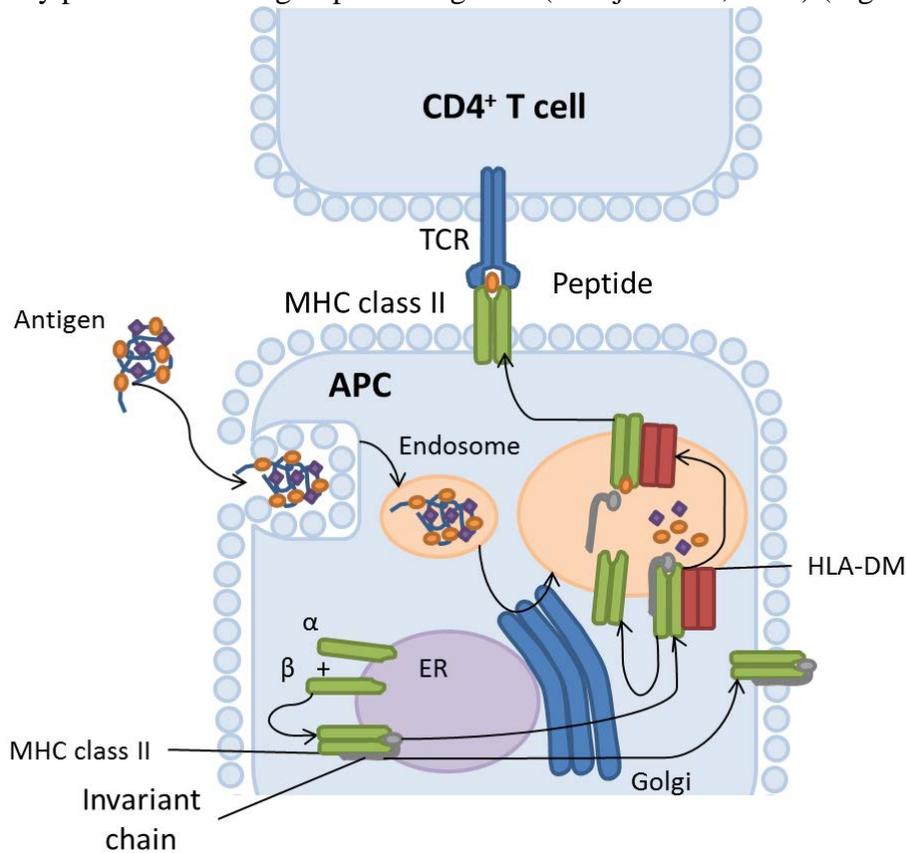


Figure 2. **Basic MHC class II antigen presentation pathway.** MHC class II  $\alpha$ - and  $\beta$ -chains are assembled in the endoplasmic reticulum (ER) and form a complex with the invariant chain (Ii) and transported through the Golgi to a MHC—II-rich endosomal compartment (MIIC). Exogenous antigens are internalized into the endosomal pathway and subjected to different proteases. Peptides are transported to MIICs. The invariant chain is exchanged for an antigenic peptide with the help of the chaperone human leukocyte antigen-DM (HLA-DM). MHC class II molecules are then transported to the plasma membrane to present antigenic peptides to CD4<sup>+</sup> T cells. Adapted from Purcell et al. (2007), Neefjes et al. (2011).

MHC class I molecules are expressed by all nucleated cells. MHC class I proteins present peptides derived from the degradation of endogenous proteins in the cytosol (Joffre et al., 2012). To ensure that only infected cells are killed, the MHC class I pathway within most of the cells is limited to endogenous proteins (Heath et al., 2004). However in some antigen-presenting cells, exogenous antigens are also presented by MHC class I molecules, and their processing after being internalized runs through different mechanisms before being loaded onto class I MHC molecules. MHC class I proteins are recognized by CD8<sup>+</sup> T cells, which are activated upon engagement of TCRs with class I MHC molecules and become effector cytotoxic T lymphocytes (Joffre et al., 2012). The term ‘cross-presentation’ refers to the capacity of antigen-presenting cells to process exogenous antigens, to load them onto class I MHC molecules, to present the antigen and to subsequently activate CD8<sup>+</sup> T cells, which may lead to T cell priming (cross-priming) or T cell tolerance (cross-tolerance) (Heath et al., 2004; Lin et al., 2008) (Figure 3).

Dendritic cells are specialized in cross-presentation and have a crucial role in the *in vivo* priming of CD8<sup>+</sup> T cells (Jung et al., 2002). Specific mechanisms in regards of antigen uptake, degradation and transport of the antigens from endosomes or

phagosomes to the cytosol have been developed by dendritic cells which benefit cross-presentation, thus playing a key role in CTL response against tumor cells or pathogens (Lin et al., 2008).

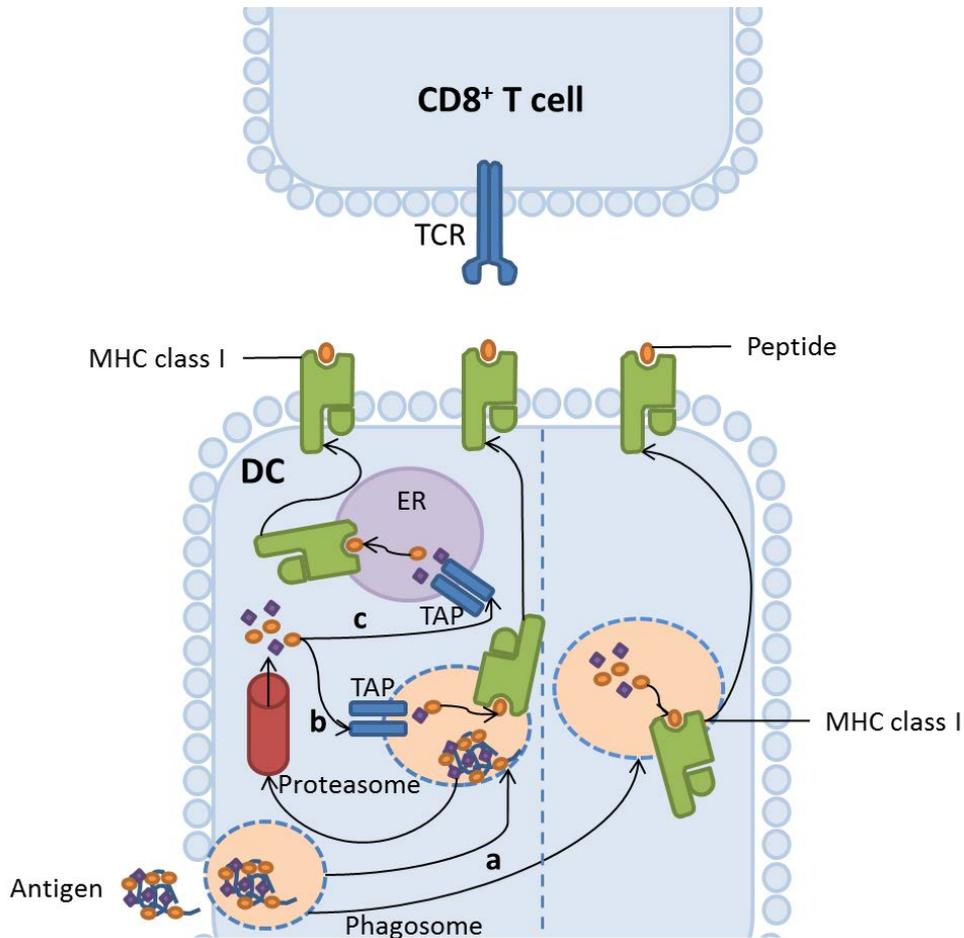


Figure 3. **Cross-presentation in dendritic cells.** After exogenous antigens are phagocytized, they can be processed by two different pathways: a) The vacuolar pathway involves degradation of the antigen into peptides in the phagosome, where they are directly loaded on MHC class I molecules. Alternatively, in the cytosolic pathway exogenous antigens are exported to the cytosol where they are degraded by the proteasome. The peptides can b) be re-imported into the phagosome through the transporter associated with antigen processing (TAP) where they are loaded onto the MHC class I molecules or c) be loaded on MHC class I molecules in the endoplasmic reticulum (ER). Adapted from Lin et al. (2008), Joffre et al. (2012).

#### 1.4 Antigen-antibody Immune complexes

Prior to the presentation of the antigens by MHC molecules, they must be internalized by the cells. Dendritic cells can internalize antigens through fluid-phase pinocytosis or by receptor-mediated endocytosis and present them on MHC class II molecules (Sallusto et al., 1995). Furthermore, exogenous antigens can also be processed and presented on MHC class I molecules, thus being involved in cross-presentation (Brossart and Bevan, 1997). The receptors that mediate endocytosis in dendritic cells include Fc receptors (FcRs) (Manca et al., 1991), and C-type lectin receptors (Sallusto et al., 1995). Fc receptors can interact with the Fc domain of immunoglobulins. FcεRs bind to IgE, while FcγRs interact with IgG (Ravetch, 1994). Of the latter, high-affinity receptors have been identified, as FcγRI (CD64), as well as low-affinity receptors, as FcγRII (CD32) and FcγRIII (CD16). High- and low-affinity

FcγRs can bind antigen-antibody immune complexes, but only high-affinity FcγRs can interact with monomeric IgG (Daëron, 1997).

Furthermore, different IgG subclasses interact differently with FcγRs, as shown by Bruhns et al. (2009): complexed IgG1 and IgG3 interact with all human FcγRs; complexed IgG2 and IgG4 can bind to FcγRIIA, IIB and IIC, and also FcγRIIIA; and, the inhibitory receptor FcγRIIB has a lower affinity for complexed IgG1, IgG2 and IgG3 in comparison to the other FcγRs.

Antigen-IgG ICs can interact with FcγRs in the surface of macrophages, DCs and B cells (Amigorena and Bonnerot, 1999). This pathway of internalization has been demonstrated by Sallusto and Lanzavecchia (1994) to be 100-fold more efficient in the presentation of the antigens on MHC molecules, compared to soluble antigen.

#### *1.4.1 Previous studies on antigen-antibody immune complexes*

Ovalbumin, a glycoprotein that comprises around 60-65% of the total protein content in avian egg white (Huntington and Stein, 2001), has been used as a model protein to study antigen uptake and presentation.

Regnault et al. (1999) showed on immature mouse DCs that OVA-ICs induced a significant increase in the expression of MHC class II molecules and CD86 in comparison to cells treated with the free OVA antigen. Furthermore, Regnault et al. (1999) were able to demonstrate that the uptake of immune complexes lead to efficient MHC class I-restricted antigen presentation, resulting in T cell activation.

Thereafter, Schuurhuis et al. (2002) indicated that the uptake of OVA-ICs was superior to free soluble OVA in mouse DCs and this could improve the MHC class I antigen-presentation of OVA-ICs by DCs. Schuurhuis et al. (2002) also observed an enhanced CTL response due to IC-activated dendritic cells and induce OVA-specific CTLs *in vivo*.

Moreover, Van Montfoort et al. (2007) have observed in mice that C1q complement protein enhances the uptake of immune complexes mediated by dendritic cells, and thus facilitate the presentation of antigens derived from immune complexes to T CD8<sup>+</sup> cells.

Additionally, immune complexes are known to enhance the antibody response to this antigen and that this ICs can drive immunological memory (Diaz de Ståhl and Heyman, 2001). Furthermore, IgG2a/Ag enhanced uptake by Fcγ receptors can be followed by efficient presentation to CD4<sup>+</sup> T helper cells (Getahun et al., 2004).

#### *1.4.2 Synthetic long peptides*

Therapeutic vaccination has been a capital approach in the immunotherapy of infectious diseases and in cancer treatment. Different strategies have been applied in that regard, including viral vectors encoding tumor-associated or viral antigens, antigen-loaded dendritic cells and synthetic peptides (Harrop et al., 2006; Gilboa, 2007; Melief and van der Burg, 2008).

Synthetic peptides have several benefits, particularly in the safety, preparation and storage, as the absence of infectious materials and of risk of genetic integration or recombination, when compared to conventional vaccines (Purcell et al., 2007).

In this regard, epitope-based vaccines, with peptides of 8-10 amino acids have been previously used. These short peptides do not require processing and can be loaded on any MHC class I molecules on APCs (Quakkelaar and Melief, 2012). Nevertheless, APCs will not express costimulatory molecules, required by CD8<sup>+</sup> T cell responses, after treatment with short peptides, therefore this therapy will lead to tolerance, rather than to the development of memory CTLs (Melief and Van der Burg, 2008). In most

studies carried out with short peptides only a limited immune response has been noted, which is associated with a low treatment efficacy (Quakkelaar and Melief, 2012).

In this respect, extended synthetic peptides, with a length of 25 to 50 amino acids have been developed (Bijker et al., 2008). In contrast to the short peptides, synthetic long peptides (SLP) have to be internalized and processed before antigen-presentation, hence they are primarily processed by immature dendritic cells (Quakkelaar and Melief, 2012). Maturation of dendritic cells is associated with upregulation of costimulatory molecules, which are essential for the induction of T cell responses, therefore leading to the development of immunity (Melief and Van der Burg, 2008; Satpathy et al., 2012). Clinical studies have been performed to analyze the T-cell response induced by synthetic long peptides on patients with human papilloma virus, melanoma, colorectal cancer, among others, with promising results as therapeutic vaccines (Kenter et al., 2008; Ebert et al., 2009; Speetjens et al., 2009).

Synthetic long peptides can also contain CD4<sup>+</sup> and CD8<sup>+</sup> T cell epitopes, which have proved to elicit strong immune responses in patients with human papillomavirus, indicating the potential in immunotherapy of this vaccine (Welters et al., 2008).

Previous studies have focused on the benefits that antigen-antibody immune complexes provide in regards of antigen uptake, cross-presentation and subsequent T cell activation in mice. Nevertheless, the number of studies analyzing these aspects in human dendritic cells has been limited. Monocyte-derived dendritic cells are one of DC lineages most widely used, and have demonstrated to possess similar capacity of antigen uptake and cross presentation, among other characteristics, of conventional DCs. Therefore, an improved understanding of their antigen uptake capacity and stimulation index is essential for a better understanding of the therapeutic potential of an IC based therapy.

## **1.5 Aim of the project.**

### *1.5.1 General aim of the project:*

Analyze the stimulation of monocyte-derived dendritic cells by antigen-antibody immune complexes.

### *1.5.2 Specific aims of the project:*

Evaluate the stimulation of monocyte-derived dendritic cells by OVA-antiOVA or [MTTE]<sub>3</sub>-SLP\* immune complexes.

Determine the uptake capacity of free soluble antigen and immune complexes of monocyte-derived dendritic cells.

Examine the role of complement in the antigen uptake and stimulation of monocyte-derived dendritic cells.

## **2. MATERIALS AND METHODS**

### **2.1 Reagents**

Ovalbumin was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Rabbit anti-OVA IgG antibody was obtained from ICN Biomedicals (Aurora, OH). Ovalbumin-Alexa Fluor 488 conjugate (OVA-Alexa488), [MTTE]<sub>3</sub>-SLP and [ETTM]<sub>3</sub>-SLP, anti-MTTE IgG fractions from human and rabbit anti-MTTE serum were provided by our collaborators (STW; Jan Wouther Drijfhout and Ferry Ossendorp, Leiden University Medical Center, Netherlands). MTTE and ETTM were 18-mer synthetic peptides. Each SLP construct contained 3 peptides linked to a CMVpp65 epitope = NLVPMVATV, which can bind HLA-A2. RPMI 1640 medium, FBS, PeSt, HEPES, B-mercaptoethanol and Trypan Blue were obtained from Gibco (Life Technologies, Grand Island, NY). The cell culture medium was supplemented with the cytokines IL-4 and GM-CSF purchased from Gentaur (Brussels, Belgium). BSA and carbonate-bicarbonate buffer were obtained from Sigma-Aldrich (St. Louis, MO).

Surface phenotypic analysis was carried out using the following human monoclonal antibodies: CD14-FITC, CD1a-APC, CD86-APC and HLA-DR-PE were purchased from BioLegend (San Diego, CA).

Human IL-12p40 and biotinylated anti-IL12-p40 antibodies were purchased from BioLegend (San Diego, CA).  $\alpha$ -rabbit anti-human IgG conjugated to HRP, Avidin/HRP and TMB substrate were obtained from Dako (Copenhagen, Denmark).

### **2.2 Isolation of PBMCs and monocytes.**

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from the blood bank of the university hospital of Uppsala (Akademiska Sjukhuset, Uppsala, Sweden). Blood samples were diluted 1:1 with PBS and PBMCs were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation (400xg). The PBMCs were resuspended in 5 mL of MACS buffer (PBS, 2 mM EDTA, 2% FBS). The monocytes were isolated from PBMCs by using MACS CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) with an LS column, according to manufacturer's instructions. The unlabeled cells that passed through were also collected. The CD14 magnetically labeled cells were eluted with R cell medium (87.5% RPMI 1640 medium, 10% FBS, 1% PeSt, 1% HEPES and 0.2% B-mercaptoethanol)

### **2.3 Generation of monocyte-derived dendritic cells (MoDCs).**

Approximately 20 million peripheral blood monocytes were cultured at a density of 10<sup>6</sup> cells/ml in R cell medium containing hGM-CSF (150 ng/ml) and hIL-4 (50 ng/ml) in T75 flasks at 37°C in 5% CO<sub>2</sub>. Half of the medium was changed on day 2 and 5.

## **2.4 Preparation of antigen-antibody complexes.**

OVA or OVA-Alexa488 immune complexes were prepared in RPMI 1640 medium without supplements performing serial 1:2 dilutions in a round bottom 96-well plate (Corning Inc., Lowell, MA). 50 µg/ml rabbit anti-OVA was added to half of the antigen samples, mixed at 1:1 ratio. Rabbit anti-OVA (50 µg/ml) and LPS (1 µg/ml) were added to the plate as controls. The plate was incubated 30 minutes at 37°C. Then the plates were sealed with parafilm and stored at 4°C overnight.

SLP immune complexes were similarly prepared. Titrations of MTTE<sub>3</sub>-SLP or ETTM<sub>3</sub>-SLP were performed in RPMI 1640 medium. 100 µg/ml of human or rabbit Anti-MTTE were added. Both Anti-MTTE antibodies (100 µg/ml) and LPS (1 µg/ml) were used as controls. The plate was incubated 30 minutes at 37°C, and stored at 4°C overnight.

## **2.5 DC incubation with antigen-antibody immune complexes.**

After 6 days of culture, MoDCs were counted and resuspended in R cell medium. 50000 cells/well were cultured in 96-well flat bottom plates (3595, Corning Inc.) and incubated at 37°C for 1 hour.

The previously prepared plate with the immune complexes was mixed at a 1:1 ratio with the serum or medium samples in a 96-well round bottom plate (Corning Inc.). The mixture of the immune complexes and serum samples was added to the cells in a 2:1 ratio followed by incubation at 37°C for time intervals as indicated.

## **2.6 Flow cytometry.**

Cells were stained with 5 µg/ml of the corresponding antibodies diluted in PBS (1% BSA) and incubated for 15 minutes at RT. 100µl of PBS (1% BSA) was added followed by two washing steps with PBS (1% BSA). Cells were resuspended with 200µl of PBS (1% BSA) and stored at 4°C until the flow cytometry was carried out. Data acquisition was performed on FACS Canto II (BD Biosciences, San Jose, CA). The subsequent data analysis was carried out with FlowJo 6.5 software (Treestar, Ashland, OR).

For the uptake analysis, following 24 hours incubation, the cells with OVA-Alexa488-antiOVA were analyzed by flow cytometry. The plates were centrifuged at 250g for 5minutes. The supernatant was discarded and 100 µl of PBS (with 1% BSA) was added to the wells. The duplicates were pooled and transferred to polystyrene round tubes. Trypan blue was added to one set of cells at 1:1 ratio immediately before the FACS analysis was performed. Another set of cells was analyzed without any additional staining. Cells were analyzed by flow cytometry on FACS Canto II (BD Biosciences, San Jose, CA) to detect Alexa488 levels. Data was subsequently analyzed with FlowJo 6.5 software.

## **2.7 Enzyme-linked immunosorbent assay (ELISA).**

Detection of human IL-12: 96-well round bottom plates (Corning Inc.) were coated with 100 µl of IL-12p40 antibody and incubated overnight at 4°C. Plates were washed three times with PBS-0.05% Tween. Plates were incubated 1 hour at 37°C with

100  $\mu$ l PBS (1% BSA)/well for blocking and followed by three washing steps with PBS-0.05% Tween. 100  $\mu$ l of supernatant collected from cell stimulation assays was added to each well and followed by 2 hours incubation at RT. Three washing steps with PBS-0.05% Tween were done and 100  $\mu$ l/well of 1  $\mu$ g/ml biotinylated antibody anti-IL12-p40 (PBS, 1% BSA, 0.05% Tween) were added, followed by 1 hour incubation at 37°C. Plates were subsequently washed three times with PBS-0.05% Tween and 50  $\mu$ l/well of Avidin/HRP diluted 1:4000 (PBS, 0.05% Tween, 1% BSA) followed by 1 hour incubation at RT in the dark. Plates were washed three times with PBS-0.05% Tween. Subsequently, 100  $\mu$ l/well of TMB (Dako) was added as a substrate and incubated at RT in the dark for approximately 10-15 minutes, before 50  $\mu$ l/well of stop solution was added (1M H<sub>2</sub>SO<sub>4</sub>). Absorption was read at 450nm.

### **2.8 Human blood sera heat inactivation**

Venous blood samples were taken from healthy donors after they had given informed consent. Blood was directly drawn into Z/serum clot activator-tubes. Samples were kept at room temperature for 50 minutes to let the blood to clot and centrifuged at 3376g for 8 minutes. The serum was isolated and stored in polystyrene and eppendorf tubes. Sera samples aliquoted in eppendorf tubes were kept at 56°C for 30 minutes for heat inactivation. All the samples were then stored at -80°C.

### 3. RESULTS

#### 3.1 Dendritic cells were generated from monocytes

Monocyte-derived dendritic cells have been commonly used *in vitro* as a dendritic cells model in immunotherapy, since they were generated by Sallusto & Lanzavecchia (1994). In this study, MoDCs were originated from monocytes after 6-days culture in R medium supplemented with IL-4 and GM-CSF. Following a 6-days incubation period, MoDCs expressed high levels of CD1a, and a clear reduction of CD14. The expression of CD86 and HLA-DR was also examined, observing a slight increase in the expression of these molecules after 6-days culture (see figure 1).

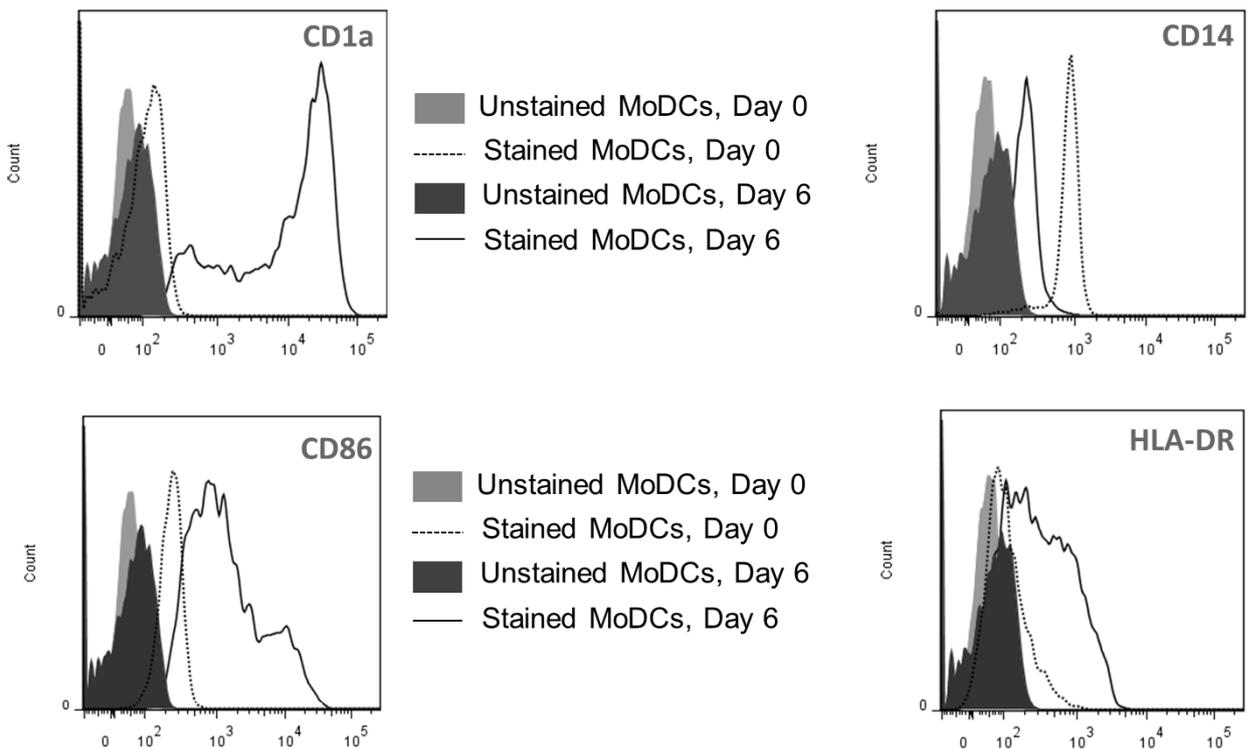


Figure 1. **Monocyte-derived DCs cell surface phenotype at day 0 and day 6 of culture.** CD14, CD1a, HLA-DR and CD86 expression was evaluated by flow cytometry. Unstained cells from each day are also represented. Representative figures presented correspond to one donor.

#### 3.2 MoDCs stimulation by OVA immune complexes

It has been previously shown that Ag-Ab immune complexes can be more efficient in cross-presentation and activation of APCs than free soluble antigen *in vivo* and *in vitro* in mice (Regnault et al., 1999; Schuurhuis et al., 2002, 2006). This represents the first study where stimulation of human DCs by ICs is addressed. Therefore, we aimed to establish the activation capacity, and reproducibility, of OVA-ICs on multiple human MoDC cultures. For this assay, dendritic cells were generated from monocytes isolated from 11 donors and incubated with titrated amounts of OVA and a fixed concentration of rabbit anti-OVA (raOVA) for 48 hours. Optimal formation of the immune complexes was obtained at 0.625  $\mu\text{g/ml}$  OVA and a fixed antibody concentration of 50  $\mu\text{g/ml}$ , therefore this concentration was used during the study. The expression of CD86 was examined by flow cytometry, and a 1.5-fold change or higher

was observed in 36.3% of the donors, comparing cells incubated with OVA-ICs versus free soluble OVA. High variation in the CD86 expression was found among these 4 donors that showed increase stimulation with OVA-ICs, with a fold-increase ranging between 2.1 and 14.7 (Figure 2).

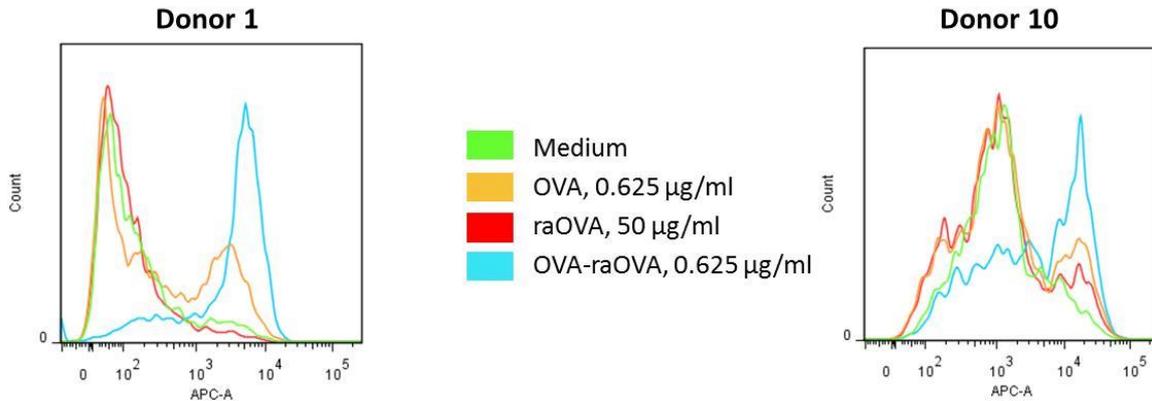


Figure 2. **CD86 expression of MoDCs after 48 hour incubation with OVA-ICs.** CD86 expression was examined by flow cytometry. Representative figures from 2 donors are presented.

Furthermore, the production of IL-12p40 by MoDCs after stimulation with OVA-ICs was detected by ELISA. Higher IL-12p40 levels were released by MoDCs stimulated with OVA forming ICs respect to soluble OVA. The IL-12p40 detected levels present a similar pattern to CD86 expression, therefore suggesting altogether a functional activation of DCs by OVA immune complexes (Figure 3).

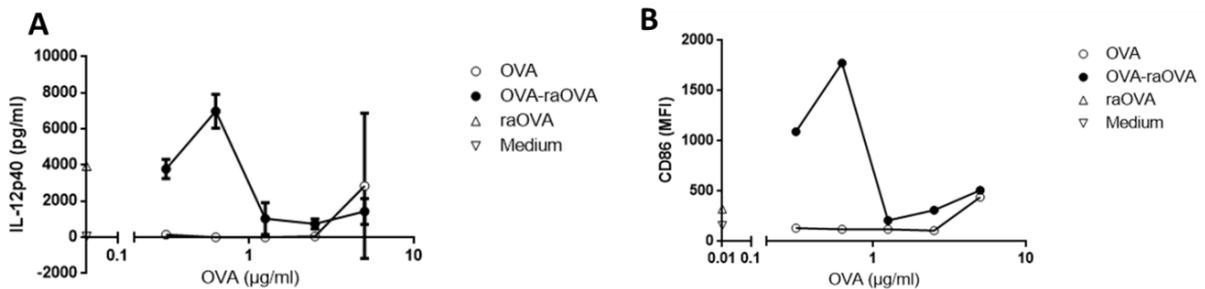


Figure 3. **IL-12p40 production and CD86 expression of MoDCs after incubation with OVA-ICs.** (A) IL-12p40 levels were detected by an ELISA in the supernatant of MoDCs cultured for 48 hours with OVA-ICs. (B) CD86 cell surface expression on MoDCs incubated with different OVA concentrations was determined by flow cytometry. Figures correspond to 1 donor.

### 3.3 OVA-ICs uptake by monocyte-derived DCs

Moreover, the uptake capacity of OVA immune complexes by MoDCs was investigated. In this regard, dendritic cells from a donor which showed increased stimulation with OVA-ICs were incubated for 24 hours with OVA conjugated to the fluorescent dye Alexa488 (OVA-Alexa488), a system that can be directly detected by flow cytometry. The role of complement in the uptake was also analyzed in this assay; therefore dendritic cells were incubated with 5% complement intact (CI) human serum, 5% heat-inactivated (HI) serum, where complement is inactivated, and with R medium only. OVA uptake increased with the concentration of the antigen and higher values were detected in cells incubated without serum and in 5% serum with intact

complement. A slightly higher uptake was observed in cells incubated with soluble OVA than with OVA forming immune complexes (see figure 4A, C and E).

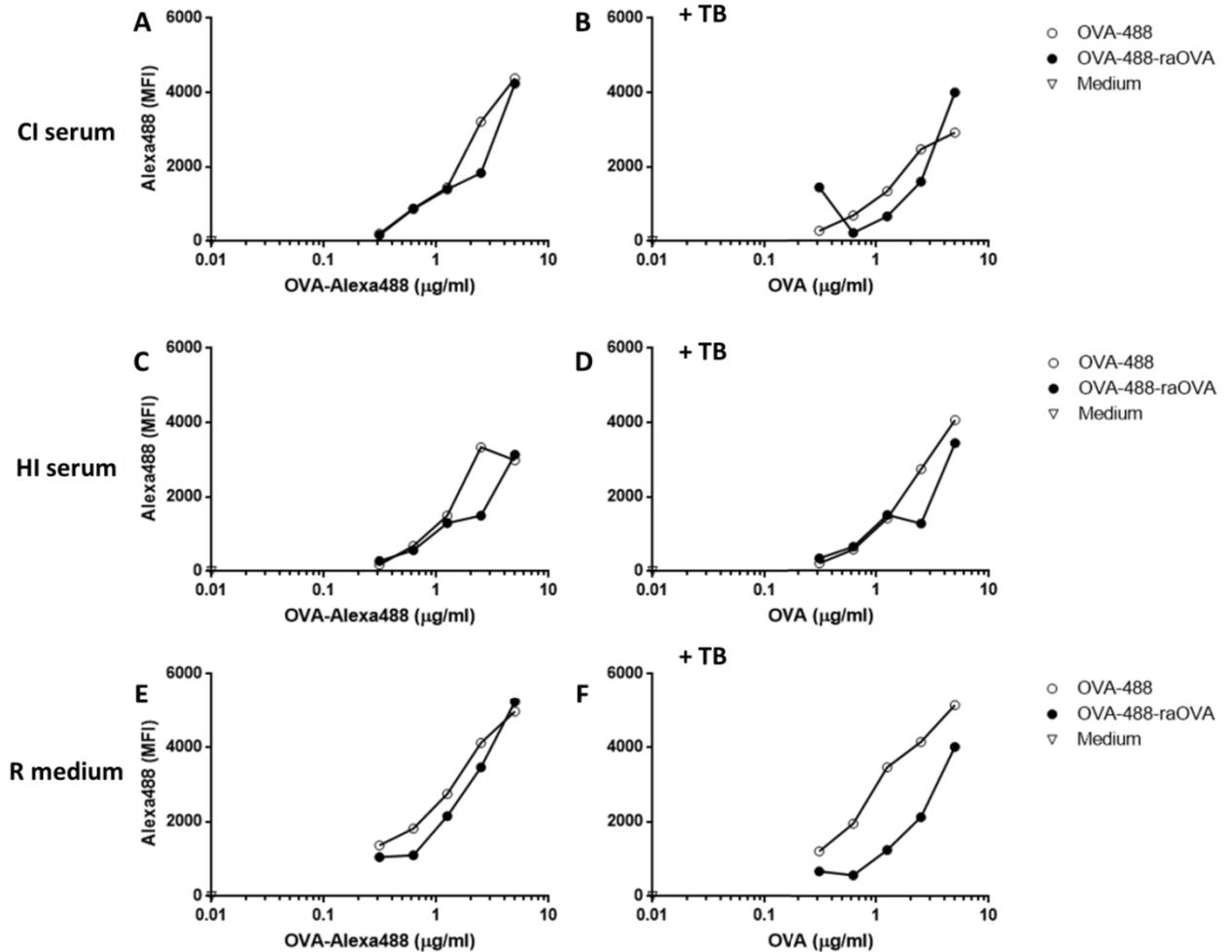


Figure 4. **OVA uptake by monocyte-derived DCs.** MoDCs were incubated with OVA-Alexa488 or OVA-Alexa488-IC in different culture conditions: (A, B) 5% complement intact (CI) human serum; (C, D) 5% heat-inactivated serum; and (E, F) R medium only. After 24 hours incubation cells were harvested, resuspended and Alexa488 levels were detected by flow cytometry in DCs in the absence (A, C, E) or presence (B, D, F) of trypan blue (TB).

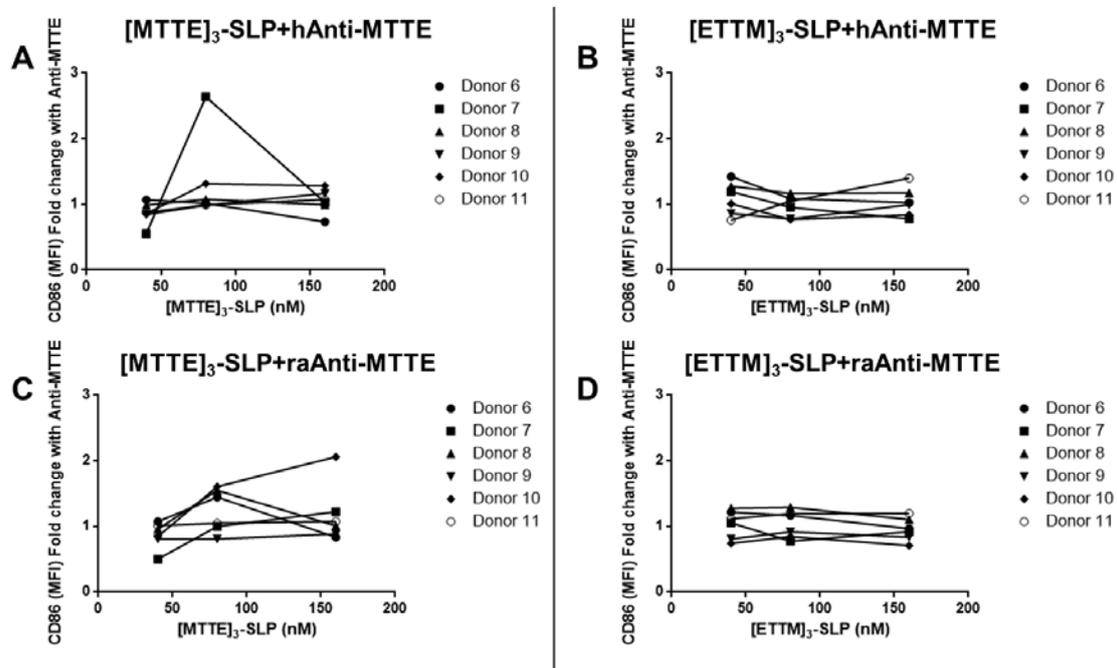
In addition, trypan blue, which quenches extracellular, but not intracellular fluorescence (Van Strijp et al., 1989), was used to examine if these DCs had internalized the OVA antigen. Following 24 hours incubation with OVA-Alexa488, cells were harvested and resuspended and trypan blue was added before FACS analysis. Dendritic cells cultured in presence of complement intact human serum or in R medium only and incubated with OVA-Alexa488-ICs presented a 50% to 75% reduction of the fluorescent signal when treated with this staining. In contrast, soluble OVA-Alexa488 could not be quenched by trypan blue. This suggests that most of the antigen captured by the cell is bound to the cell surface when is administered as Ag-Ab immune complexes, but when the antigen is free it is internalized with limited binding to the cell surface. However, no reduction of the fluorescent signal was observed in cells cultured with 5% HI serum, suggesting the involvement of complement in the antigen endocytosis mediated by receptors. For the CI serum data at the highest Ag

concentration (B) there appears to be increased IC internalization compared to Ag alone indicating that the assay might have been performed in the wrong Ag and/or Ab range to establish the potency of Ab-mediated enhanced uptake of OVA.

### 3.4 Stimulation of MoDCs by MTTE-SLP immune complexes

We further studied the stimulation mediated by a synthetic construct on MoDCs. In this assay, dendritic cells were stimulated with a construct termed  $[MTTE]_3$ -SLP (synthetic long peptide), that contains 3 peptides derived from the tetanus toxin linked to a peptide of choice (T cell antigen). The 3 MTTE peptides are linked and conjugated to a long CMVpp65 derived peptide, which binds HLA-A2 molecules and can be used to stimulate antigen-specific T cells. Moreover, a scrambled MTTE peptide, termed ETTM, was used as a control. This peptide does not bind to the Anti-MTTE antibodies. The constructs were administered in combination with anti-MTTE antibodies obtained from human or rabbit, to examine the stimulation of dendritic cells.

Dendritic cells generated from monocytes obtained from 6 donors were incubated for 48 hours with  $[MTTE]_3$ -SLP-ICs, with a stimulation efficiency of 50% of ICs formed with rabbit Anti-MTTE while  $[MTTE]_3$ -SLP complexed with human Anti-MTTE antibodies triggered increased stimulation in 2 donors (see figure 5).  $[MTTE]_3$ -SLP immune complexes triggered high stimulation of DCs, which was not observed in DCs incubated with the  $[ETTMM]_3$  construct. However, high variation between donors was obtained when the  $[MTTE]_3$ -SLPs formed immune complexes with human or rabbit Anti-MTTE.



**Figure 5. Stimulation of MoDCs by  $[MTTE]_3$ -SLP immune complexes. Fold-change comparison between donors.** CD86 cell expression was evaluated by flow cytometry following 48 hours incubation with the preformed ICs. Fold-change of CD86 MFI values of the DCs after incubation with the immune complexes versus CD86 expression of DCs incubated with  $MTTE_3$  or  $ETTMM_3$  were calculated and are presented for 6 donors: (A)  $[MTTE]_3$ -SLP + human Anti-MTTE; (B)  $[MTTE]_3$ -SLP + rabbit Anti-MTTE; (C)  $[ETTMM]_3$ -SLP + human Anti-MTTE; (D)  $[ETTMM]_3$ -SLP + rabbit Anti-MTTE.

Additionally, MoDCs from one of the previous donors showed increased stimulation with immune complexes formed with human and, especially, rabbit anti-MTTE, presenting a 2.2-fold increase in CD86 expression versus free MTTE<sub>3</sub> (see figure 6).

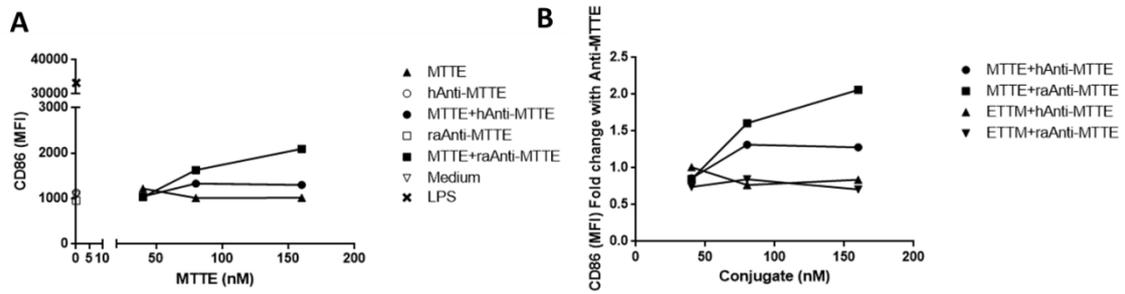


Figure 6. **[MTTE]<sub>3</sub>-SLP immune complexes trigger stimulation of MoDCs.** MoDCs were stimulated for 48 hours with [MTTE]<sub>3</sub>-SLP and [ETTM]<sub>3</sub>-SLP titrations and 100 µg/ml Anti-MTTE. CD86 expression was examined by flow cytometry and MFI values from one donor are presented. (A) CD86 expression of MoDCs incubated with [MTTE]<sub>3</sub>-SLPforming ICs with human or rabbit Anti-MTTE. (B) Fold-change comparison of CD86 expression on MoDCs incubated with Ag-Ab complexes versus the free soluble antigen. Figures presented correspond to one donor.

However, no increase in CD86 expression was observed when ETTM-ICs were given to the cells, indicating no stimulation of DCs by the scrambled peptide and specific binding of the antibodies to the MTTE constructs.

## 4. DISCUSSION

This study shows that preformed immune complexes can be more efficient in stimulating monocyte-derived dendritic cells than soluble antigens however data also indicate that there is great variation in responses depending on the donor. The reason for this has to be investigated further.

Dendritic cells are professional antigen-presenting cells, essential in cross-presentation and activation of T cells. When immature, dendritic cells are specialized in antigen capture, processing and form MHC peptide complexes. Fully matured DCs express higher levels of MHC class II, and present increased expression of adhesion and costimulatory molecules, as CD54 and CD86, respectively, (Banchereau and Steinman, 1998; Di Nicola and Lemoli, 2000). In this project dendritic cells derived from monocytes were used, which is a frequently used model in *in vitro* studies. After 6-days of culture expression of surface markers on cells from all donors were analyzed, observing increased levels of CD1a, a DC marker, as well as a clear reduction in CD14, an LPS receptor highly expressed on monocytes, which indicates differentiation to DCs (Di Nicola and Lemoli, 2000).

Herein it is demonstrated that immune complexes can be more efficient in stimulation of dendritic cells than free soluble antigen however with a great donor dependent variation. Initially, increased stimulation triggered by ovalbumin immune complexes with respect to free soluble antigen was observed. This protein has been previously used as a model to analyze the stimulation of murine dendritic cells *in vitro* and *in vivo* (Regnault et al., 1999; Bonifaz et al., 2002). This represents the first study where it has been analyzed on human DCs. Furthermore, high variation in the CD86 expression of DCs after stimulation was observed between donors, which might be associated to preexisting differences between individuals regarding FcγR expression. Polymorphisms in the genes coding for different subclasses of FcγRII and FcγRIII have been reported previously (Osborne et al., 1994; Wang et al., 2006; Bruhns et al., 2009; Marques et al., 2010). Moreover, as shown by Tada et al., (2012), a polymorphism of FcγRIIA have increased signal transduction activity, which might have relevance for antibody responses mediated by FcγRIIA. Additionally, these polymorphisms have been associated with autoimmune diseases as SLE and rheumatoid arthritis (Chu et al., 2004; Marques et al., 2010).

The uptake of soluble OVA has been shown to occur by fluid phase endocytosis or mediated by receptors, as the mannose receptor (Burgdorf et al., 2006). Additionally, it has been demonstrated that MoDCs, which express high levels of this receptor compared to other sets of DCs, are very efficient in the capture of soluble OVA (Andersson et al., 2012). However, OVA immune complexes with IgG antibodies can be internalized by Fcγ receptors (Amigorena and Bonnerot, 1999) which increases cross-presentation efficiency on DCs. The results obtained in this study indicate that soluble OVA and OVA-ICs were captured at similar levels by the MoDCs, which

suggests that the *in vitro* uptake capacity of ICs does not determine stimulation of the DCs. Previously, a study performed by Schuurhuis et al., (2002) with a splenic DC cell line derived from mice, D1, showed that OVA-ICs were more efficiently captured than soluble OVA. Conversely, Bergtold et al., (2005) detected higher OVA levels on bone marrow DCs from mice when incubated with free OVA antigen rather than OVA-ICs. This demonstrates the importance of the model used to analyze ICs-mediated uptake and stimulation and that the study of these aspects by human DCs is needed to ponder the future clinical applications of immune complexes. Finally, in this sense, Andersson et al., (2012) have showed that MoDCs and myeloid DCs from humans present different properties regarding antigen uptake. I also acknowledge that the assay may have been performed potentially out of the concentration range of where optimal Ag/Ab formation occurs to really establish the role of Abs for improved Ag uptake.

Furthermore, the uptake analysis of OVA suggested that capture and internalization of the ICs was dependent on the complement system. DCs incubated in heat-inactivated serum, where the complement is inactive, showed reduced uptake of the soluble antigen compared to cells incubated in complement intact human serum. The highest uptake was observed by MoDCs cultured without serum, which could be due to additional antigens present in the serum that compete with the OVA-Alexa488 complex to be captured. Additionally, treatment with trypan blue allowed determining the proportion of OVA internalized by the cell. Interestingly, the signal could not be quenched for soluble OVA, indicating that internalization occurs with limited binding on the cell surface, as indicated by Schuurhuis et al. (2002). Additionally, reduction in the fluorescent signal was observed in cells incubated with OVA-Alexa 488 forming ICs in presence of complement intact serum, which suggests that the complement system might affect the internalization of OVA immune complexes. In this sense, Van Montfoort et al., (2007) showed opposite results in murine DCs *in vitro* and *in vivo*, that C1q, a protein of the classical complement pathway, is essential for uptake of OVA immune complexes in murine DCs, *in vitro* and *in vivo*, and that this protein is necessary for cross-presentation and T cell activation. However, the Ag-Ab range analyzed in this study might not be the appropriate where optimal Ag/Ab formation occurs, to adequately examine the uptake capacity of DCs. Altogether, stimulation of MoDCs by OVA-ICs did not showed to be correlated with their uptake capacity of Ag-Ab immune complexes. However, additional studies are required on human myeloid DCs to examine this relation, and should consider analyzing the role of C1q, since this protein might be crucial to trigger functional activation of human DCs *in vivo*.

The effective stimulation mediated by OVA-ICs might be associated with a stronger preservation of the antigen. Van Montfoort et al., (2009) have indicated that Ag-Ab immune complexes are more protected from intracellular degradation than free soluble antigen and suggested that antigens captured through receptor-mediated uptake were stored in specialized compartments in DCs. This would lead to a time-extended cross-priming capacity of the DCs which could represent an important benefit in vaccine development.

Additionally, it is important to mention that the cross-linking of Fc $\gamma$  receptors on DCs triggers activating and inactivating signaling pathways, which depending on their magnitude, will determine if DCs are functionally activated (Nimmerjahn and Ravetch, 2007). Boruchov et al., (2005) have indicated that MoDCs express both, the activating Fc $\gamma$ RIIA, and the inhibitor Fc $\gamma$ RIIB. Therefore, higher binding of the ICs to Fc $\gamma$ RIIA will lead to activating signals on DCs which might be the cause of the increased stimulation mediated by preformed immune complexes rather than the soluble antigen. As suggested by Boruchov et al., (2005), targeting to activating specific receptors can be beneficial in therapy.

Alternatively, synthetic long peptides have been used in therapy with promising results in the treatment of HPV (Kenter et al., 2008). Additionally, considering the increased stimulation mediated by Ag-Ab immune complexes, the fusion of both strategies could represent a very attractive therapy for the clinic. These results show that immune complexes formed with MTTE triggered higher stimulation in comparison to the free conjugate. In addition, no increase in stimulation was elicited by a peptide with a scrambled sequence, which confirms the Ab dependent response triggered by the MTTE<sub>3</sub>-SLP immune complexes. It is important to mention that high variation was found among donors, as occurred with OVA-ICs. Such variation could be related to polymorphisms of Fc $\gamma$  receptors as mentioned previously, that can affect the stimulation of the dendritic cells.

Synthetic long peptides can be formed based on any peptide, which converts it in a very attractive strategy for therapy, especially for cancer. In this sense, downregulation of HLA class I molecules have been reported in many cancer types and also lack HLA class II expression (Van Luijn et al., 2011; Bukur et al., 2012). The lack of presentation of tumor-associated antigens makes tumor cells invisible to the immune system and to be recognized by cytolytic T lymphocytes, thus avoiding CTL-mediated killing (Maeurer et al., 1996). Therefore, the development of specific tumor-associated antigens might, in conjunction with antigen-antibody immune complexes, improve antigen uptake, trigger higher DCs stimulation and cross-presentation to elicit a specific T cell response against a particular antigen.

However, additional studies are needed to be performed on different cell subsets from the immune system to understand how immune complexes can be used in the clinic. It is important to remember that although MoDCs are one of the most used DCs models *in vitro* in immunotherapy, no biological equivalent has been found (Satpathy et al., 2012). Therefore, further studies should consider the use of unmodified natural cells to examine how antigen-antibody ICs are internalized and can trigger activation of DCs and T cells. Additionally we need to establish why donors respond with such variation to these immune complexes or if different activation markers are required to study this.

## **5. CONCLUSIONS**

This data indicate increased stimulation, however donor-dependent, of monocyte-derived dendritic cells by preformed antigen-antibody immune complexes compared to free soluble antigen. In this study synthetic long peptides were, for the first time, used as antigens forming immune complexes on MoDCs, which showed a higher stimulation than with the free conjugate. Additionally, the uptake of the OVA immune complexes was analyzed, suggesting a possible effect of the complement system in antigen uptake and internalization. These results show that immune complexes can improve vaccine development and should be evaluated together with synthetic long peptides as an immunotherapy approach. Nevertheless, given the reported differences between the different DCs subsets in human and in mice regarding immune complexes and free antigen uptake, additional studies should be performed on different cell types to get a better understanding of ICs effects in the development of an immune response.

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