UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

Synthetic Pathogens for Integrated Biophysical and Genetic Dissection of Antigen Cross-Presentation

RUI PEDRO DA SILVA ALBUQUERQUE E FREITAS

Doutoramento em Ciências Biomédicas
Especialidade em Ciências Morfológicas

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Preface

"Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive"

Fernando Pessoa

One of the most important and difficult steps in a scientist short life is to decide start writing the PhD thesis, but even before, to be brave or naive enough to start a scientific career and to run the unknown and tricky road until the end. It is a kind of matrimonial relation, where almost all scientists begin to love science, adore and enjoy the idea of discover something new and interesting with the propose of finding a solution or eliminating something that was not so bright. Therefore, everything starts with conceptual dreamers without frontiers.

This thesis describes the work carried between January 2006 and July 2009 mainly at the Instituto de Medicina Molecular (Lisbon, Portugal). During this period, part of the research was done at Massachusetts General Hospital (Boston, USA), at MIT (Cambridge, USA) and at the Institute Currie (Paris, France). The main goal was to study how the biochemical and biophysical properties of specific particulate antigens influence the cross-presentation pathway(s) and try to dissect and indentify the mechanism(s) behind it.

This thesis was divided in 6 Chapters:

The introduction comprises a general overview of specific key immunology concepts, such as Innate Immunity, Dendritic Cell biology, and antigen presentation mechanism with emphasis on antigen cross-presentation.

The second chapter focuses on particulate antigen design and the goal of specific properties introduced in the particles; the shRNA lentiviral library production and its application in high-throughput approaches. It includes a summary description of my participation in the work done within this period and the resulting publications.

The third chapter is composed by the materials and methods used throughout my work, including the different particulate platforms design and biochemical and cellular techniques for antigen presentation studies.

Results of my main project are described on Chapter 4, where different platforms of particulate antigen were used to study antigen cross-presentation mechanism(s).

Discussion is presented on Chapter 5 and concluding remarks on Chapter 6.

The results presented in this thesis, in collaboration with Darrell Irvine's lab at MIT, are under preparation for publication.

"A person who never made a mistake never tried anything new"

Albert Einstein

ABSTRACT

The study of host-pathogen interactions is crucial to unveil the diversity of the immune response outcome. Dendritic Cells (DCs) play a central role in the initiation and regulation of T-Cell immunity, functioning as master switches that control whether the outcome of antigen presentation results in tolerance, or immunity. Antigen cross-presentation is a necessary mechanism to generate immunity against tumors, bacteria and viruses. In addition, it is extremely important to induce cytotoxic immunity by vaccination with antigens. Moreover, particulate antigens have been used in vaccine design tools as a platform to deliver different types of signals and in the modulation of DC-dependent immune responses. DCs express a series of different receptors that mediate the transfer of signals from the environment. Among them, Toll-Like Receptors (TLRs) play a critical role in the early innate immune response to invading pathogens. These receptors have the ability to recognize a broad range of pathogen-associated molecular patterns (PAMPs), turning them, key receptors in distinguishing between self/non-self antigens. The precise mechanisms underlying the crosstalk between TLRs and antigen presentation are not entirely understood. Therefore, the main goal of this project is to understand how TLR agonists coupled to particulate antigens influence antigen cross-presentation.

In our studies, we have used newly synthesized particle antigens, denominated as 'synthetic pathogens', coupled with a model antigen (Ovalbumin - OVA), and/or a model ligand (TLR agonist). These particle platforms have distinct, well-defined physical and biochemical properties, and function as a novel approach to elucidate the intrinsic mechanism(s) of antigen cross-presentation. In addition, they represent a valuable and powerful tool, which might be explored for therapy applications.

TLR4 is unique among TLRs as it can signal through both MyD88 and TRIF adaptors upon LPS stimulation, but mainly by the TRIF pathway when LipidA is the agonist. Our results revealed that when LPS is in the same cargo as the particle antigen, it impairs antigen cross-presentation and dictates a shift to MHC class- II presentation. This antigen cross-presentation abolishment is recovered on TLR4 deficient DCs and in the presence of the p38 MAPK pathway inhibitor, but not in the absence of the MyD88 adaptor. Moreover, LipidA reproduces the same phenotype as LPS, implicating the TLR4/TRIF-mediated signaling on particulate antigen cross-presentation impairment. Thus, here we describe a new mechanism of antigen selection in DCs for antigen cross-presentation that is dependent on the antigen based-environment. We show that the efficiency of presenting antigens from phagocytosed cargo is dependent on the presence of TLR ligands within the cargo. The influence of the compartmentalization on the crosstalk between the TLR-signaling and the antigen cross-presentation pathway(s), may constitute a tool used by DCs in order to discriminate the contents of phagosomes and present an appropriate immune response to specific stimuli. Therefore, DCs may have the "capacity" to decide which kind of destiny an antigen should have depending on the type and origin of the stimuli.

In order to dissect the mechanisms behind the cross-presentation phenotype, we have addressed the role of particle LPS on several antigen presentation key steps. Our data show that LPS-containing phagosomes enhance phagosome maturation (higher levels of colocalization with lysosomes) characterized by higher rates of phagosomal acidification and a decrease of phagosomal reactive oxygen species (ROS) production. The induction of phagosome maturation mediated by LPS signaling seems to shut down the machinery for antigen release into the cytosol, where the epitopes for MHC class-I are predominantly generated by the proteasome. Moreover, lower levels of antigen degradation occur when LPS is in

the same cargo as antigen, mainly in a proteasome-dependent manner. This phenotype mediated by particulate LPS stimulus seems to be related with lower levels of particle antigen cross-presentation.

Therefore, we propose that antigen cross-presentation is enhanced during the brief period of time when phagosomal acidification is "sustained" and an immature phenotype is predominant, where endoplasmic reticulum machinery important for MHC class-I presentation probably is available. In addition this phenotype allows antigen escape into cytosol and the generation of epitopes for MHC class-I by the proteasome. On the other hand, antigen cross-presentation is impaired when a stimulus that induces phagosome maturation/acidification is in the same cargo as the antigen, producing a mature phenotype, allowing the generation of epitopes on the endocytic pathway that is compromised for MHC class-II antigen presentation.

In order to address if the abolishment on antigen cross-presentation phenotype is transversal to others TLRs, studies were extended using different TLR specific agonists. When particle antigen contains TLR agonists that preferentially signal through MAPK/NF-kB pathways, antigen cross-presentation is induced. In contrast, in the presence of TLR agonists that preferentially signals through IFN-Type I pathway, particle antigen cross-presentation is inhibited. Therefore, a signaling pathway correlation may exist in the outcome of antigen presentation pathway(s) mediated by TLR agonist-containing particle antigens.

In sum, this work shows for the first time the inhibitory effect of TLR4 signaling on cross-presentation when agonists are delivered in the same cargo as particulate antigen. This phenotype is likely to be mediated by TRIF-dependent signaling, mainly by p38 MAPK activation. This knowledge could have a major impact in the dissection of the antigen cross-presentation mechanism, which will be highly valuable for novel vaccine design inducing T-Cell responses of the desired type and specificity.

RESUMO

O estudo das interações patogénio-hospedeiro é fundamental para a compreensão da diversidade da resposta imunitária e para o desenvolvimento de novas estratégias terapêuticas. As células dendríticas (DCs) desempenham um papel central na iniciação e regulação da imunidade mediada por linfócitos T, funcionando como "interruptores", que podem originar uma resposta de tolerância ou imunidade em relação a um determinado antigénio. O mecanismo de *cross-presentation* de antigénios tem sido descrito como necessário para gerar imunidade contra tumores, bactérias e vírus, e fudamental na indução de imunidade citotóxica mediada por vacinação. Por outro lado, os antigénios particulados têm sido utilizados como ferramentas no *design* de vacinas, possibilitando uma plataforma na qual se podem integrar diferentes tipos de estímulos.

As DCs expressam uma diversidade de receptores à superficie, o que permite uma detecção e transmissão eficazes dos vários tipos de "sinais" do meio ambiente. Entre eles, os *Toll-Like Receptors* (TLRs) desempenham um papel crucial, na resposta imune inata contra patogénios invasores. Estes receptores têm a capacidade de reconhecer uma ampla gama de padrões moleculares associados a patogénios (PAMPs), implicando-os como receptores-chave na distinção entre antigénios próprios e não-próprios. O mecanismo subjacente à *crosstalk* entre TLRs e a apresentação de antigénios não é totalmente conhecido. Por isso, um dos principais objetivos do meu trabalho foi compreender como é que os agonistas dos TLRs no mesmo contexto que antigénios particulados, influenciam a sua *cross-presentation*.

Neste projecto foram utilizadas partículas sintéticas – designadas por *synthetic pathogens* - na presença de um antigénio modelo (Ovalbumina) e/ou de um ligando

(agonista dos TLRs). Estas plataformas têm propriedades físicas e bioquímicas distintas e bem definidas, pelo que funcionam como uma nova abordagem para dissecar o mecanismo de *cross-presentation* de antigénios, bem como explorar o seu potencial para utilizaçao nas mais diversas terapias.

Os resultados demonstram que quando o Lipopolissacarídeo (LPS - agonista do TLR4) está presente no mesmo contexto que as partículas contendo o antigénio, ocorre uma redução nos níveis de *cross-presentation* de antigénios. Este fenótipo é acompanhado por uma mudança na via de apresentação de antigénios para MHC classe- II, que é induzida quando comparada com as partículas só com o antigénio. Este mecanismo foi demonstrado como sendo mediado pelo TLR4, onde a *cross-presentation* de antigénios é restabelecida em DCs deficientes nesse receptor .

A origem física dos estímulos (partícula *vs* solúvel) parece ser crucial para a regulação da via de *cross-presentation* de antigénios. Quando o LPS solúvel é co-incubado com partículas contendo o antigénio (dois estímulos físicos diferentes), verifica-se um aumento da activação/proliferação de células T em ambos os contextos de apresentação de antigénios - MHC classe-I e MHC classe -II. No entanto, quando o LPS é utilizado numa partícula diferente daquela que contém o antigénio, não se verificam diferenças significativas na eficiência das duas vias.

A influência da compartimentação no *crosstalk* entre a sinalização mediada pelos TLR e a via de *cross-presentation* de antigénios, pode constituir uma ferramenta importante que as DCs utilizam para discriminar o conteúdo dos fagossomas e iniciar uma resposta imune apropriada aos estímulos específicos. Esta observação é de extrema importância para compreender o papel de estímulos "patogénicos" no destino da apresentação de antigénios. Com o objectivo de compreender o mecanismo adjacente ao fenótipo observado da via da *cross-presentation* de antigénios particulados, o papel da activação do TLR4 foi estudado em vários processos importantes na apresentação de antigénios. Os resultados

obtidos indicam que fagossomas que contêm LPS têm uma indução na maturação (níveis mais elevados de colocalização com lisossomas), caracterizada por taxas mais elevadas de acidificação e uma diminuição da produção de espécies reactivas de oxigénio (ROS). A indução da maturação dos fagossomas mediada pela sinalização por LPS parece bloquear o mecanismo de libertação do antigénio dos fagossomas para o citosol, onde os epítopos para a apresentação em MHC classe -I são predominantemente gerados pelo proteassoma. Para além disso, níveis mais baixos de degradação do antigénio, mediada principalmente pelo proteassoma, ocorrem quando o LPS está no mesmo contexto. Este fenótipo é devido à sinalização mediada pelo LPS e parece estar relacionado com níveis baixos de *cross-presentation* do antigénio particulado.

Posto isto, sugerimos que a *cross-presentation* de antigénios é reforçada durante um breve período de tempo quando o pH dos fagossomas é mantido em valores próximos do estado basal, onde um fenótipo imaturo é predominante. Este estado imaturo é caracterizado pela existência de componentes do retículo endoplasmático (ER) importantes para a apresentação em MHC classe-I, permitindo o escape do antigénio para o citoplasma, onde os epítopos podem então ser gerados pelo proteassoma e apresentados no contexto MHC classe-I à superfície. Por outro lado, a *cross-presentation* de antigénios é diminuída quando um estímulo que induz a maturação dos fagossomas que contem o antigénio está no mesmo contexto. A formação de fagolisossomas leva à rápida acidificação e produz um fenótipo maduro, permitindo a geração de epítopos na via endocítica que é direccionada para a via MHC classe-II de apresentação de antigénios.

O TLR4 é singular entre os TLRs, uma vez que pode sinalizar tanto pelo adaptador MyD88 ou pelo TRIF quando estimulado por LPS, mas preferencialmente pelo adaptador TRIF quando LipidA é o agonista. Com o objectivo de estudar o impacto da sinalização do TLR4 na via de *cross-*

presentation dos antigénios particulados, o LipidA foi utilizado no mesmo contexto que o antigénio particulado. Observou-se uma reprodução do fenótipo de supressão da via de *cross-presentation* de antigénios obtido na presença do LPS. Além disso, a *cross-presentation* de antigénios particulados na presença de LPS não foi recuperada usando DCs deficientes no adaptador MyD88, ao contrário do que acontece quando se usa DCs deficientes no TLR4 e na presença de inibidores de activação da via das MAPK, principalmente a p38 MAPK. Estes resultados implicam a via TLR4/TRIF na inibição da *cross-presentation* de antigénios.

Com o objectivo de verificar se o efeito inibitório na via de *cross-presentation* de antigénios é transversal aos outros TLRs, os estudos foram alargados usando agonistas dos diversos TLRs no mesmo contexto que as partículas de antigénio. Verificou-se que, os agonistas dos TLR que sinalizam preferencialmente através da via MAPK/NF-kB induzem a *cross-presentation* de antigénios particulados. Em oposição, os agonistas dos TLR que sinalizam preferencialmente através da via do IFN Tipo-I, levam à inibição da *cross-presentation* destes antigénios. O TLR4 pode sinalizar através dos dois adaptadores (MyD88 e TRIF) em diferentes localizações, sendo preferencialmente via TRIF quando o TLR4 é internalizado nos endossomas. A inibição da via de *cross-preserntation* de antigénios mediada pelo LPS quando no mesmo contexto que o antigénio particulado, poderia indicar que o LPS em partículas sinaliza preferencialmente através da via TRIF, quando estas são internalizadas. Esta observação corrobora os dados obtidos com os outros agonistas de TLR que preferencialmente sinalizam através da via do IFN Tipo-I, como é o caso do TLR3, do TLR7 e do TLR9, que estão localizados em endossomas. Conclui-se assim que os vários TLRs estão envolvidos em mecanismos diferentes que levam a efeitos distintos nas vias de apresentação de antigénios. Além disso, um padrão da via de *cross-presentation* de antigénios

parece existir mediado por partículas contendo os vários agonistas dos diferentes TLRs.

Em colaboração com o grupo do Prof. Darrell Irvine do MIT, pretendemos alargar estes estudos para outras plataformas de antigénios. Partículas de poli(ácido lático-*co*-ácido glicólico), PLGA, e hidrogel têm sido usadas como plataforma para administrar drogas, assim como em aplicações de biomateriais e concepção de vacinas. De acordo com os dados obtidos para a plataforma "fixa" de antigénios (partículas utilizadas nos ensaios anteriores), o LipidA no mesmo contexto que o antigénio particulado inviabiliza a via de cross-presentation de antigénios, comprovando a sua acção como um inibidor de sinalização mediada pelo TLR4, por um mecanismo dependente do adaptador TRIF. Assim, podemos concluir que mesmo na presença de partículas com propriedades distintas, a via mediada pelo adaptador TRIF tem um papel importante na inibição da via de *cross-presentation* de antigénios quando estimulada pelos agonistas do TLR4.

Este trabalho mostrou pela primeira vez, o efeito inibitório da *crosstalk* entre a sinalização pelo TLR4 e a *cross-presentation* de antigénios quando os agonistas estão no mesmo contexto do antigénio particulado. Este fenótipo é susceptível de ser mediado pela via TLR4/TRIF, principalmente através da activação da via p38 MAPK. Estes resultados podem ter assim um impacto deveras importante na dissecção do mecanismo de *cross-presentation* de antigénios, assim como na concepção de novos protocolos de vacinação e na indução de respostas específicas mediadas por linfócitos T.

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LIST OF ABREVIATIONS

Ag	Antigen
APCs	Antigen Presenting Cells
BMDCs	Bone Marrow-derived Dendritic Cells
Cat.S	Cathepsin S
cDCs	Concentional Dendritic Cells
CFSE	Carboxyfluorescein diacetate Succinimidyl Ester
CpG	Cytosine-guanosine oligonucleotide
CTL	Cytotoxic T Lymphocyte
Cyt *c*	Cytochrome *c*
Cytcp	Particles loaded with Cyt *c*
Cytc=LPSp	Particles loaded with Cyt *c* and LPS
DCs	Dendritic Cells
DHR123	Dihydrorhodamine 123
DQ-OVAp	Particles loaded with DQ-OVA
DQ-OVA=LPSp	Particles loaded with DQ-OVA and LPS
ELISA	Enzyme-linked Immunosorbent Assay
ER	Endoplasmatic Reticulum
ERAD	ER-associated Degradation
FACS	Fluorescence Activated Cell Sorter
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
Hydrogel=OVAp	Hydrogel particles loaded with OVA
Hydrogel=OVA=MPLAp	Hydrogel particles loaded with OVA and MPLA
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IL-…	Interleukin-…
LPS	Lipopolysaccharide
MPAKs	Mitogen-activated protein kinases
MHC	Major Histocompatibility Complex
MPLA (LipidA)	Monophosphoryl Lipid A
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear Factor kappa B
OVA	Ovalbumin
OVA488	Ovalbumin–Alexa488
OVAp	Particles loaded with OVA
OVA=CpGp	Particles loaded with OVA and CpG
OVA=Flagellinp	Particles loaded with OVA and Flagellin
OVA=LPS	Particles loaded with OVA and LPS
OVA=LipidAp	Particles loaded with OVA and LipidA
OVA=Pam2p	Particles loaded with OVA and Pam2

OVA=Pam3p	Particles loaded with OVA and Pam3
OVA=PolyI:Cp	Particles loaded with OVA and PolyI:C
OVA=ssRNA40p	Particles loaded with OVA and ssRNAS40
OVA488p	Particles loaded with OVA488
OVA488=LPSp	Particles loaded with OVA488 and LPS
OVA=DHR123p	Particles loaded with OVA and DHR123
OVA=DHR123=LPSp	Particles loaded with OVA, DHR123 and LPS
PAMPs	Pathogen-Associated Molecular Patterns
pDCs	Plasmacytoid Dendritic Cells
PLGA	poly(lactic-co-glycolic acid)
PLGA=OVAp	poly(lactic-co-glycolic acid) particles loaded with OVA
PLGA=OVA=MPLAp	poly(lactic-co-glycolic acid) particles loaded with OVA and MPLA
PRRs	Pattern Recognition Receptors
RNAi	RNA interference
ROS	Reactive Oxygen Species
sDCs	Splenic Dendritic Cells
shRNA	short hairpin RNA
SIINFEKLp	Particles loaded with SIINFEKL
SIINFKL=LPSp	Particles loaded with SIINFEKL and LPS
TRIF	TIR-domain-containing adapter-inducing interferon-β
TAP	Transporter Associated with Antigen Processing
TCR	T-Cell Receptor
TLR	Toll-Like Receptor
u.v.	Ultraviolet radiation
WB	Western Blot

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1

Introduction

1. Immune System

The immune system is one of nature's most fascinating creations. It is composed of different cell types and uses almost all known proteins, with different "jobs" in fighting foreign invaders and to recognize and tolerate self. It protects against bacteria, viruses, and other parasites, but unfortunately some pathogens can bypass and establish diseases. In some cases, immune cells fail their function and progress to recognize self-antigens (autoimmune response). For these reasons, immunology is one of the most studied subjects, and its knowledge seems to be interminable and fascinating. The immune system has been "conceptually" subdivided in 2 sub-systems, innate and adaptive, each one with a different function and role. The main distinction relates to the mechanisms and receptors used for the immune recognition, which will be described bellow (Medzhitov and Janeway, 2000).

1.1. Innate Immune System

The idea of a generic host defence was introduced over 100 years ago by Eli Metchnikoff, who revealed the role of "phagocytes" in destroying invading microbes. In 1957, important components of innate immunity, named interferons, were discovered by Alick Isaacs. The interferons were recognized as important molecules for host defence mechanism activation with broad specificity. In 1973, Steinman and Cohn made a big step in immunology, by identification of Dendritic Cells (DCs) as a subtype of phagocytes, which were specialized for the capture and presentation of antigens. Nonetheless, the "big bang" in innate immunity was introduced by Charles Janeway Jr. in his seminal 1989 commentary. There he introduced the "immunologist´s dirty little secret", i.e. that most antigens would only elicit an adequate immune response when mixed with adjuvants containing microbial products.

The Innate Immune system is an evolutionarily ancient part of the host defense mechanisms and is found in all metazoans (the same molecular modules are found

in plants and animals, meaning that it arose before the split into these two kingdoms) (Hoffmann *et al.*, 1999). Innate immunity major functions are: *a)* inflammatory responses through the production of chemical factors (cytokines and chemokines); *b)* activation of the complement cascade that promote the clearance of pathogens, dead cells or antibody complexes; *c)* identification and removal of cellular debris, foreign particles or microorganism by phagocytosis; *d)* activation of the adaptive immune system through a process known as antigen presentation. Innate immune cells originate from pluripotent hematopoietic stem cells present in the bone marrow and include: Natural killer cells, Mast Cells, Eosinophils, Basophils and the phagocytic cells (Macrophages, Neutrophils and DCs) (Janeway and Medzhitov, 2002). There are hundreds of receptors involved in innate immune recognition and approximately 10^{14} and 10^{18} different somatically generated immunoglobulin receptors and T-Cell receptors respectively. However, microbes are extremely heterogenous and can mutate at a much higher rate than any of their hosts (Medzhitov and Janeway, 2000).

Therefore, the strategy of the innate immune response may not be to recognize every possible antigen, but rather to focus on a few, highly conserved structures present in large groups of microorganisms, that are structurally distinct from the host. These structures are referred to as Pathogen-Associated Molecular Patterns (PAMPs), and the receptors of the innate immune system that evolved to recognize them are called Pattern-Recognition Receptors (PRRs) (Janeway, 1989). These receptors allow the first line of defence: discrimination between self and non-self (Janeway, 1989). When Charles Janeway Jr. answered the questions: *"How does the immune system determine the origin of the antigen, and how does it decide whether to induce and immune response or not? Does innate immune system has a major role on that?"*, the PAMPs and their recognition by PRRs, emerged as essential components for the innate immune system to respond or not to a specific antigen (Janeway and Medzhitov, 2002).

1.2 Adaptive Immune System

Adaptive Immunity is present only in vertebrates and it was the "big bang" in immunity. It is composed of highly specialized, systemic cells: B Lymphocytes (B cells), T Lymphocytes (T-Cells) - $CD8^+$ T-Cell or Cytotoxic T-Cell, $CD4^+$ T-Cell or Helper T-Cells (Th1 or Th2) and gamma/delta T-Cells (Medzhitov and Janeway, 2000; Reis e Sousa, 2004a). Adaptive immunity is a relative newcomer on the evolutionary landscape. Because the mechanism of generating receptors in the adaptive immune system involves great variability and rearrangement of receptor gene segments, the adaptive immune system can provide specific recognition of foreign antigens, immunological memory of infection, and pathogen-specific adaptor proteins. However, the adaptive immune response is also responsible for allergy, autoimmunity, and the rejection of tissue grafts (Janeway and Medzhitov, 2002). This variability of receptors is due to Somatic hypermutation on two types of antigen receptors: T-Cell receptors (TCRs) on T-Cells and immunoglobulin receptors (IgR) on B cells. These antigen receptors are generated by random somatic gene rearrangement and are expressed in a clonal fashion on lymphocytes. Since each lymphocyte displays a single kind of structurally unique receptor, the repertoire of antigen receptors in the entire population of lymphocyte is extremely unique (each lymphocyte with a structurally unique receptor). Thus, these receptors are able to recognize almost all antigens that exist in nature. However, these receptors recognize not only pathogenic, but also environmental or self-antigens (Hoffmann *et al.*, 1999; Medzhitov and Janeway, 1998). Rarely, responses from these receptors to environmental or self-antigens could lead to allergies or autoimmune diseases. What adaptive immunity adds to the underlying innate immune system is specific recognition of proteins, carbohydrates, lipids, nucleic acids (Janeway, 1989). The signals induced on recognition by the innate immune system, in turn, control the activation of adaptive immune responses that respond to a pathogen only after it has been recognizing by the innate immune system

(Medzhitov and Janeway, 2000). DCs make the link between innate and adaptive immune system by presenting antigens to naive T-Cells and expressing molecules such as cytokines, chemokines, costimulatory molecules and proteases to initiate an immune response (Steinman, 1991). To generate an efficient immune response to a specific pathogen, it is critical that the recognition of a specific antigen by lymphocyte receptors could trigger its activation and proliferation. This process termed clonal selection is the basic property of the adaptive immune system. Unfortunately, these receptors cannot be passed on to the next generation, even if they give a survival advantage. Antigen Receptors have to be reinvented by every generation (Medzhitov and Janeway, 2000). This mechanism takes 3-5 days to produce enough number of clones and to differentiate into effector cells. For most pathogens, this period could be enough to damage the host and establish disease (Medzhitov and Janeway, 2000). However, the effector mechanism of innate immune system (antimicrobial peptides, phagocytosis and alternative complement pathway), is activated immediately after infection, which normally leads to a rapid control of the infection pathogen. "Retarding" the infection until the adaptive immune system is ready to deal with it, is one of the main functions of innate immunity (Janeway and Medzhitov, 2002).

2. Pathogen and Antigen Recognition

2.1. Pattern Recognition Receptors

The Pattern Recognition Receptors (PRRs) concept was introduced by Janeway 20 years ago, with the idea that immune system senses microbial infection by these receptors that are predominantly expressed on sentinels cells. Pathogen-associated molecular patterns (PAMPs) are the molecular signature recognized by PRRs, which are broadly expressed in pathogens but not in host cells. Therefore, PRRs are able to discriminate between self from non-self (Janeway, 1989). During the past years, different families of PRRs were identified. The most important are: Toll-like receptors (TLR), RIG-I-like receptors (RLR), NOD-like receptors (NLR) and C-Type Lectin Receptor (CLR).

Studies have shown the existence of a cytosolic detection system for intracellular PAMPs. These cytosolic PRRs include retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). RLRs belong to the RNA helicases family that specifically detects RNA species derived from viruses in the cytoplasm and coordinate anti-viral programs via type I IFN induction (Yoneyama and Fujita, 2008). NLRs constitute a large family of intracellular PRRs, where the NOD1, NOD2 and NALP3 are the most relevant (Ting *et al.*, 2008). NOD1 and NOD2 recognize intracellular bacterial cell products, and NALP3 responds to multiple stimuli to form a multi-protein complex termed the NALP3 inflammasome, which promotes the release of the IL-1 family of cytokines (Fritz *et al.*, 2006; Inohara *et al.*, 2005; Kanneganti *et al.*, 2007; Meylan *et al.*, 2006; Schweichel *et al.*, 2006; Yu and Finlay, 2008). CLRs, are diverse families of receptors containing one or more C-type lectin domains, identified as a carbohydrate-binding structure. The different groups vary widely in ligand recognition and function and only a few acts as innate PRR mediating the recognition of PAMPs by DCs (Robinson *et al.*, 2006). CLR

expressed by DCs included dectin-1, DEC-205 and DC-SIGN, which all belong to a separate group of CLR (Zelensky and Gready, 2005). Dectin-1 has been shown to act as PRR resulting in DC activation (Rogers *et al.*, 2005). It recognizes carbohydrate structures in the form of fungal β 1,3-glucans (Palma *et al.*, 2006). DEC-205 and DC-SIGN play a role in uptake, processing and presentation of antigens from pathogens, but does not induce DC activation (Jiang *et al.*, 1995; Mahnke *et al.*, 2000).

In addition to PAMPs, innate immunity has the potential to respond to endogenous molecules that are released by host cells as a result of necrosis, pathogen infection, damage, injury and certain pathological conditions, which are directly or indirectly recognized by TLRs, NLRs, RLRs or as-yet undefined sensors. The recognition of endogenous molecules by PRRs is tightly linked to the pathogenesis of autoimmune and inflammatory diseases (Kawai and Akira, 2009).

TLR family detects PAMPs either on the cell surface or the lumen of intracellular vesicles such as endosomes or lysosomes (Kawai and Akira, 2009). This work was focused on TLRs function in antigen cross-presentation; therefore they will be described more extensively as follows.

2.1.1. Toll-like Receptors: Overview

The first member of the Toll family, *Drosophila Toll*, was discovered as one of 12 maternal effect genes that function in a pathway required for dorso-ventral axis formation in fly embryos (Hashimoto *et al.*, 1988). Analysis of the sequence of the *toll* gene revealed that it encodes a transmembrane protein with a large extracellular domain containing leucine-rich repeats. The sequence of the cytoplasmic domain of the toll turned out to be similar to the cytoplasmic domain of the human interleukin-1 receptor (hIL-1R), and it became apparent that they had possible functional similarities (Gay and Keith, 1991). Both hIL1-R and toll in drosophila have homologous cytoplasmic TIR domains and signal through

homologous protein kinases (Pelle and IRAK). Their signal-transduction leads to activation of transcription factors of the nuclear factor-kB (NF-kB) family pathway (Anderson, 2000; Belvin and Anderson, 1996). Earlier studies done by Ruslan Medzhitov in 1997, lead to the identification of a family of membrane-bound receptors in mammalian similar to drosophila toll (Medzhitov and Janeway, 1997). Therefore, a family of pattern-recognition receptors homologues of drosophila toll have been identified in mammals and are referred as toll-like receptors (TLRs) (Medzhitov and Janeway, 1997; Rock *et al.*, 1998). Until now, there are described 13 members of mammalian TLR family (Akira *et al.*, 2006). TLR1-9 are conserved between humans and mice, TLR10 is not functional in mice because of a retrovirus insertion, and TLR11, TLR12 and TLR13 are lost in human genomes.

TLRs differ from each other in: *(1)* ligand specificities, *(2)* expression patterns and in *(3)* target genes they can induce (Akira *et al.*, 2006). The TLR family members can be conveniently divided into two subpopulations with regard to their cellular localization. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed exclusively on the cell surface and recognize microbial membrane components such as lipids, lipoproteins and proteins. TLR3, TLR7, TLR8 and TLR9 are localized in intracellular vesicles such as the endosomes or lysosome and the endoplasmic reticulum (ER) and predominantly recognize microbial nucleic acid species (Akira and Takeda, 2004).

Therefore, TLR were initially implicated in the recognition of bacterial, fungal patterns and viruses (Pasare and Medzhitov, 2005; Takeda and Akira, 2004) but the exact mechanism of PAMPs recognition has not yet been well characterized. Recent studies have revealed the crystal structure of TLR1, TLR2, TLR3 and TLR4 and suggest their mechanism of ligand recognition (Jin and Lee, 2008). The ligands for most TLRs were identified trough generation of mice deficient for each TLR. Genetic studies revealed that TLR are able to recognize a wide range of PAMPs including lipids, lipoproteins, proteins, glycans and nucleic acids and play

a central role in initiating innate immune responses (Akira *et al.*, 2006). Bacterial lipopeptide, a ligand for the TLR-1-TLR2 heterodimer, interacts with internal protein pockets, and hydrophobic interactions are responsible for ligand recognitions (Jin *et al*. 2007). On the other end, viral double-stranded RNA (dsRNA), a TLR3 ligand, interacts with both the N-terminal and the C-terminal sites on the lateral side of the convex surface of TLR3 by ionic and hydrogen bonds with the sugar-phosphate backbones of dsRNA (Choe *et al.*, 2005). TLR4 is responsible for bacterial LPS recognition but there are no direct interactions. TLR4 form a complex with another LRR protein known as MD-2 (LPS-binding component) by ionic and hydrogen bonds in two oppositely charged patches (Kim *et al.*, 2007; Ohto *et al.*, 2007). All these TLR ligands induce a homodimer or heterodimer of TLRs (TLR1-TLR2, TLR3-TLR3, TLR4-TLR4) showing an "m"-shaped complexes. This dimerization is necessary for triggering downstream signaling by recruitment the TIR domain-containing adapter protein (*fig.1*).

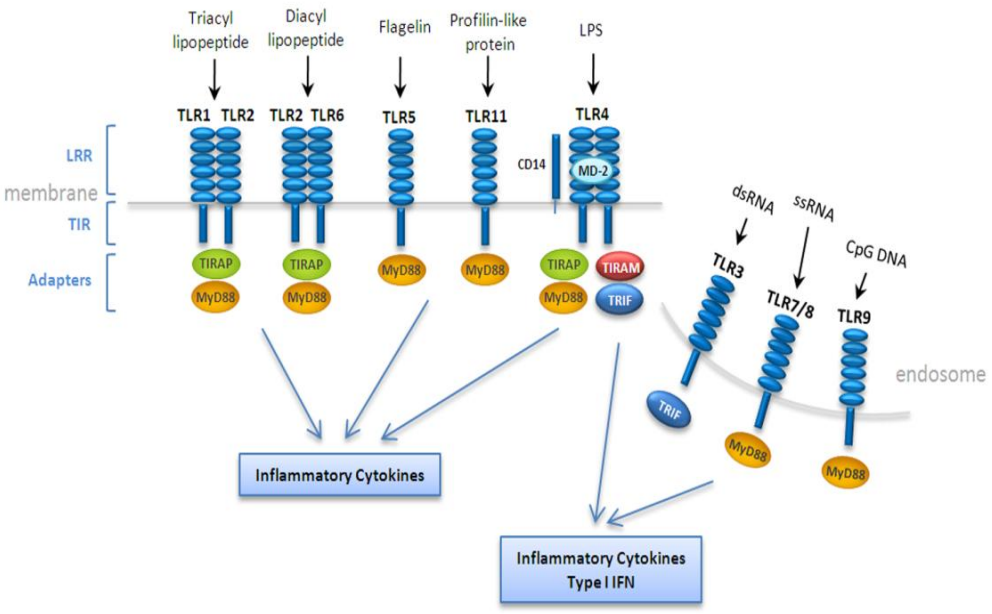


Fig.1: TLR-mediated immune responses. The TLR family can be divided into subfamilies: the TLR at the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR11) primarily detected bacterial, fungal and protozoan cell components, while intracellular TLR (TLR3, TLR7/8 and TLR9) recognize nucleic acid ligands in specific endosomal compartments. TLR2 in concert with TLR1 or TLR6 discriminates between the molecular patterns of triacyl and diacyl lipopeptide, respectively. TLR3 recognizes dsRNA. TLR4 recognizes bacterial LPS. TLR7/8 mediates recognition of imidazoquinolines and ssRNA. TLR9 recognizes CpG DNA of bacteria and viruses. TLR5 recognizes bacterial flagellin and mouse TLR11 recognizes components of uropathogenic bacteria and profilin like molecule of the protozoan parasite *Toxoplasma gondii*. TLR1/2 and TLR2/6 utilize MyD88 and TIRAP as essential adapters. TLR3 utilizes TRIF. TLR4 utilizes four adapters, including MyD88, TIRAP, TRIF and TRAM. TLR7/8, TLR9, TLR5 and TLR11 use only MyD88. The MyD88-dependent pathway controls inflammatory responses, while TRIF mainly mediates type I IFN responses. In addition, TLR7/8 and TLR9 induce type I IFN in a MyD88-dependent manner. Adapted from (Kawai and Akira, 2006).

2.1.2. Toll-like Receptors at cell surface

Toll-like Receptor 4: The first human TLR (hTLR), that was identified by Ruslan Medzhitov in 1997 is now referred to as toll-like receptor 4, and it was shown to activate, like its drosophila homolog, NF-kB signaling pathway (Medzhitov *et al.*, 1997). In 1998, further four TLR were reported (Rock *et al.*, 1998). Through NF-kB pathway, activation of TLR4 induces the expression of a variety of inflammatory cytokines and co-stimulatory molecules that are crucial to adaptive immune response (Medzhitov *et al.*, 1997). This evidence implies TLRs as receptors of the immune system (Medzhitov and Janeway, 1997). The first link arises when it was shown that TLR4 is the receptor for lipopolysaccharide (LPS) in mice. Mice with either spontaneous mutation or a target disruption of the *tlr4* gene, have no response to LPS and are thus resistant to endotoxin shock (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). Together, these studies demonstrated the essential role for TLR4 in recognition of LPS, a major component of gram-negative bacteria, which is a potent immunostimulatory molecule and cause septic shock.

TLR4 is not directly involved in LPS recognition. Soluble LPS molecules first interact with a serum protein called lipopolysaccharide-binding protein (LBP) that is present as a soluble protein or as a plasma membrane protein (Ulevitch and Tobias, 1995). At the plasma membrane, LBP binds CD14, a receptor that is

anchored to cell surface by a glycosylphosphoinositol tail (GPI-linked cell surface protein) and delivers LPS-LBP to the TLR4 (Miyake, 2007; Wright *et al.*, 1990; Wright *et al.*, 1989).

A small protein, MD-2, is also required for TLR4-mediated recognition of LPS, making the three components of LPS-recognition complex (TLR4, CD14 and MD-2). MD-2 lacks a transmembrane anchor but is constitutively associated with the extracellular region of TLR4, whereas CD14 is presumably recruited to the complex after binding LPS (Shimazu *et al.*, 1999). The cell-surface events that lead to LPS recognition are not clear, but important data indicate that a complex of TLR4/MD-2/CD14 directly binds LPS (da Silva Correia *et al.*, 2001; Lien *et al.*, 2000; Poltorak *et al.*, 2000). 'Smooth' LPS is composed of a polysaccharide O-antigen side chain and has complete core oligosaccharides, whereas 'rough' LPS lacks O-antigen and has shorter core oligosaccharides; both forms contain lipid A, a biologically active component of LPS. Cells lacking CD14 are unresponsive to smooth LPS; however, they still respond to rough LPS or lipid A (Dybdahl *et al.*, 2002; Vabulas *et al.*, 2002). TLR4 is known to activate two signaling pathways - the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway and the TIR-containing adapter inducing IFN-β (TRIF)-dependent pathway. Monophosphoryl lipid A (MPLA) is a low-toxicity derivative of LPS with useful immunostimulatory properties. MPLA is inefficient with respect to stimulation of TLR4/MyD88-induced gene expression, it has fortuitously retained TLR4/TRIF-associated activities, such as induction of type I interferon (Mata-Haro *et al.*, 2007).

These results suggest that the diversity of the structures of LPS among bacterial species may influence selective activation of these pathways. In addition to the detection of components of Gram-negative bacteria, TLR4 has been implicated as well, in the recognition of liptocheic acid (LTA), the heat shock protein hsp60, and the fusion protein of the respiratory syncytial virus (RSV) and mouse mammary

tumors virus (Kurt-Jones *et al.*, 2000; Ohashi *et al.*, 2000; Takeuchi *et al.*, 1999; Vabulas *et al.*, 2001). The physiological relevance of these putative TLR4 ligands remains to be demonstrated. However it is clear that mammalian TLRs do not discriminate between classes of pathogens (Janeway and Medzhitov, 2002).

TLR4 has recently been shown to signal from two locations (Kagan *et al.*, 2008). At the plasma membrane, TLR4 recruits the second TIR domain adapter to be described, termed MyD88-adapter-like protein (MAL), and MyD88, leading to NF-kB activation. It then appears to traffic to the endosome, where it recruits two other TIR domain adapters, translocating chain-associating membrane protein (TRAM) and TIR-domain-containing adapter-inducing interferon-β (TRIF). Consequently leading to activation of the protein kinase TANK-binding kinase-1 (TBK-1) and activation of interferon regulatory factor 3 (IRF3), the transcription factor required for induction of Type I interferons and many other genes that contain the interferon responsive response element. This capacity for signaling from two locations appears to be unique to TLR4 among the TIR domain-containing receptors (O'Neill, 2008).

Toll-like Receptor 2 and heterodimers: TLR2 recognizes a wide range of PAMPs derived from various pathogens, ranging from bacteria, fungi, parasites and viruses (Akira *et al.*, 2006). These ligands include triacyl lipopeptides from bacteria and mycobacteria, diacyl lipopeptides from mycoplasma, peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria, porin from Neisseria, lipoarabinomannan from mycobacteria, zymosan (containing b-glucan, mannans, chitin, lipid and protein) from fungi, Trypanosoma GPI-mucin (tGPI-mucin) and hemagglutinin protein from measles virus. TLR2 generally forms a heterodimer with TLR1, TLR6 or non-TLR molecules such as CD36, CD14 and dectin-1 to discriminate the molecular structure of the ligands. TLR2–TLR6

recognizes the mycobacterial diacylated lipopeptide, LTA and zymosan, whereas TLR2–TLR1 recognizes the bacterial triacylated lipopeptide.

The role of extracellular TLRs may be as sensors for danger signals. TLR2 and TLR4 are also implicated in the recognition of endogenous molecules. These include heat shock proteins (HSP60, HSP70, gp96 and HSP22), fibrinogen, the extra domain A of fibronectins, hyaluronic acid, heparan sulfate, fatty acids, high-mobility group box 1 (HMGB1), modified low-density lipoprotein and β-defensin 2, most of which are released during inflammation or tissue damages or by necrotic cells (Akira and Takeda, 2004).These endogenous ligands trigger production of TNF-α, IL-12 and nitric oxide (Miyake, 2007).

Toll-like Receptor 5: TLR5 recognizes a highly conserved central site of flagelin, a protein that is a component of bacterial flagella, which is required for protofilament formation and bacterial motility (Hayashi *et al.*, 2001). TLR5 is highly expressed on the basolateral surface of intestinal epithelial cells and moreover preferentially in $CD11c^+$ $CD11b^+$ lamina propria of DCs (LPDCs) in the small intestine (Uematsu *et al.*, 2006), which suggests a role of TLR5 in the detection of invasive flagellated bacteria in the gut. TLR5 on LPDCs plays a critical role in regulating both innate and adaptive immune response in the intestine (Uematsu *et al.*, 2008).

Toll-like Receptor 11: Mouse TLR11, which is a relative to TLR5, is highly expressed in the kidney and bladder. Because TLR11-deficient mice are susceptible to uropathogenic bacteria infection, is likely to sense its products, however a specific ligand has not been identified yet (Zhang *et al.*, 2004). TLR11 also recognizes a parasite component from *Toxoplasma gondii* tachyzoites known as soluble *Toxoplasma* antigen that is a potent inducer for IL-12. The active component is a profiling-like molecule that functions as an actin-binding protein

and involved in parasite motility and invasion (Plattner *et al.*, 2008) and is recognized by mouse TLR11 (Yarovinsky *et al.*, 2005).

2.1.3. Intracellular Toll-like Receptors

The intracellular TLRs, TLR3, TLR7, TLR8 and TLR9, are expressed in intracellular compartments. They are on the ER in resting cells and trafficked to the endosomal compartments such as endosomes and lysosomes, in response to PAMP-mediated stimulation (Latz *et al.*, 2004; Nishiya *et al.*, 2005). This intracellular localization is important for the recognition of viral nucleic acids that are delivered to TLR expressing intracellular vesicles through the endosomal pathway. Moreover, this is also important for discrimination of self from non-self nucleic acids since ectopic expression of TLR9 on the macrophage cell surface causes it to respond to DNA derived from self (Barton *et al.*, 2006). Intracellular TLRs appear to be sensors of foreign nucleic acids and trigger anti-viral innate immune responses by producing type I IFN and inflammatory cytokines.

Toll-like Receptor 3: TLR3 is a receptor for dsRNA. It recognizes a synthethic analogue of dsRNA polyinosinic-polycytidylic acid (poly:IC), genomic RNA purified by dsRNA viruses (such as reovirus) and dsRNA produced during the course of single-stranded RNA (ssRNA) viruses replication (such as RSV, encephalomyocarditis virus (EMCV) and West Nile virus (WNV) (Alexopoulou *et al.*, 2001; Wang *et al.*, 2004). TLR3 is also implicated in the recognition of small interfering RNA (siRNA) in a sequence independent manner (Kleinman *et al.*, 2008). TLR3 mRNA is expressed in conventional Dendritic Cells (cDCs) but mostly in $CD8\alpha^+$ DCs, which have high phagocytic activity for apoptotic bodies of virus-infected or dsRNA-loaded cells. This allows dsRNA to gain access to TLR3 compartments and signaling to produce IL-12p40 and IFN-β (Schulz *et al.*, 2005).

Toll-like Receptor 7: TLR7 is a natural receptor for ssRNA. It was originally identified to recognize imidazoquinoline derivatives (imiquimod and resiquimod (R-848)), and guanine analogues such as loxoribine. These compounds have anti-viral and anti-tumor properties (Hemmi *et al.*, 2002). Synthethic polyuridine ssRNA and some siRNAs as well as natural compounds such guanosine-rich and uridine-rich ssRNA from HIV or influenza virus were identified as ligands for TLR7 (Diebold *et al.*, 2004; Heil *et al.*, 2004). TLR7 is mostly expressed in a subset of DCs, plasmacytoid Dendritic Cells (pDCs), which are unique in their capacity to rapidly secrete vast amounts of type I IFN in response to viral infections. These response to influenza or VSV was impaired in TLR7-deficient pDCs and it is independent of envelope virus replication such influenza or herpes viruses. It seems that these virus are endocyted to intracellular compartments, were viral particles are degraded, allowing the viral RNA to engage TLR7 (Gilliet *et al.*, 2008).

Toll-like Receptor 8: TLR8 is related phylogenetically with TLR7. TLR8 is mostly expressed in monocytes, and is up-regulated upon bacterial infection. Human TLR8 recognizes R-848, ssRNA from virus (HIV, VSV and influenza A) and bacterial RNA, however, TLR8-deficient mice responds normally to these molecules suggesting a species-specific function of TLR8 (Heil *et al.*, 2004).

Toll-like Receptor 9: TLR9 was identified to recognize DNA motifs that are frequently present in bacteria, but are rare in mammalians - unmethylated 2´-deoxyribo cytidine-phosphate-guanosine (CpG) (Hemmi *et al.*, 2000). Synthethic CpG oligodeoxynuvleotides (ODNs) are TLR9 ligands. This recognition is independent of the base sequence, and the sugar backbone 2´-deoxyribose of DNA is sufficient for signaling (Haas *et al.*, 2008). TLR9 is highly expressed in pDCs as TLR3 and TLR7, which serve as sensor for virus infection. pDCs respond to DNA

virus infection (such as MCMV,HSV-1 and HSV-2) and to CpG ODNs by production high amounts of type I IFN and this response was totally dependent on TLR9 (Krug *et al.*, 2004a; Krug *et al.*, 2004b; Lund *et al.*, 2003). Recently, a compound derived from *Plasmodium falciparum* - hemozoin (Hz)– potentially activates, trough TLR9, macrophages and DCs to produce inflammatory cytokines and chemokines (Coban *et al.*, 2005; Parroche *et al.*, 2007; Pichyangkul *et al.*, 2004).

2.1.4. Toll-like Receptors signaling pathway(s)

TLRs are type I transmembrane proteins (N-terminal is outside de membrane) composed of three major domains. The ectodomain is responsible for PAMPs recognition and is characterized by Leucin-Rich Repeats (LRRs). There is a transmembrane domain and an intracellular domain homologous to that of the IL1-R known as Toll/IL1R (TIR) domain which is required for initiation of the downstream signaling pathways. The LRR domain is composed of 19-25 tandem copies of LRR motifs, 20-30 amino acids in length, that contain the 'xLxxLxLxx' motif as well as 'xUxxUxxxxUxxLx (U: hydrophobic)' sequences. LRR domain contains a beta-strand and an alpha-helix linked by loops, which leads to the prediction that the LRR has a horseshoe-like structure.

Differences in the TIR-domain-containing adaptors used for downstream signaling by TLRs have a crucial influence on the cytokine patterns induced in response to ligand recognition (*fig.2*). Except for TLR3 which exclusively signals via the TIR domain-containing adaptor inducing IFN-β (TRIF), all TLR share the adaptor molecule MyD88 (O'Neill and Bowie, 2007). While the TIR domain of MyD88 is recruited directly to the cytoplasmic TIR domain of most TLR, TLR2 and TLR4 require the MyD88-adaptor-like adaptor (MAL), also called TIR-domain-containing adaptor protein (TIRAP), as a bridge for MyD88 recruitment (Fitzgerald *et al.*, 2001; Horng *et al.*, 2002; Kagan and Medzhitov, 2006;

Yamamoto *et al.*, 2002). In addition to these two TIR-containing adaptor molecules, TLR4 also signals via TRIF and TRIF-related adaptor molecule (TRAM) (Fitzgerald *et al.*, 2003; Yamamoto *et al.*, 2003). These differences in adaptor molecule usage between the TLR leads to activation of various transcription factors in the downstream signaling cascade. Triggering of TLR2 homo and heterodimers and TLR5 lead exclusively to NF-kB activation, whereas TLR4 additionally induces interferon regulatory factor 3 (IRF3) in a TRIF/TRAM-dependent manner (Fitzgerald *et al.*, 2003). While IRF3 is crucial for the induction of interferon-β (IFN-β) in response to TLR4, NF-kB-mediated immune activation leads to the rapid induction of pro-inflammatory cytokines such IL-6, IL-12 and TNF-α. IRF5 was shown to play a crucial role in the induction of pro-inflammatory cytokines and IFN type-I in response to TLR activation in general (Takaoka *et al.*, 2005).

As a summary, the general consequence of TLR activation results in induction of mitogen activated protein kinases (MAPK) p38, ERK and JNK and transcription of nuclear factor kB (NF-kB) and interferon regulatory factor (IRF)-responsive genes pivotal to immunity (*fig.2*).

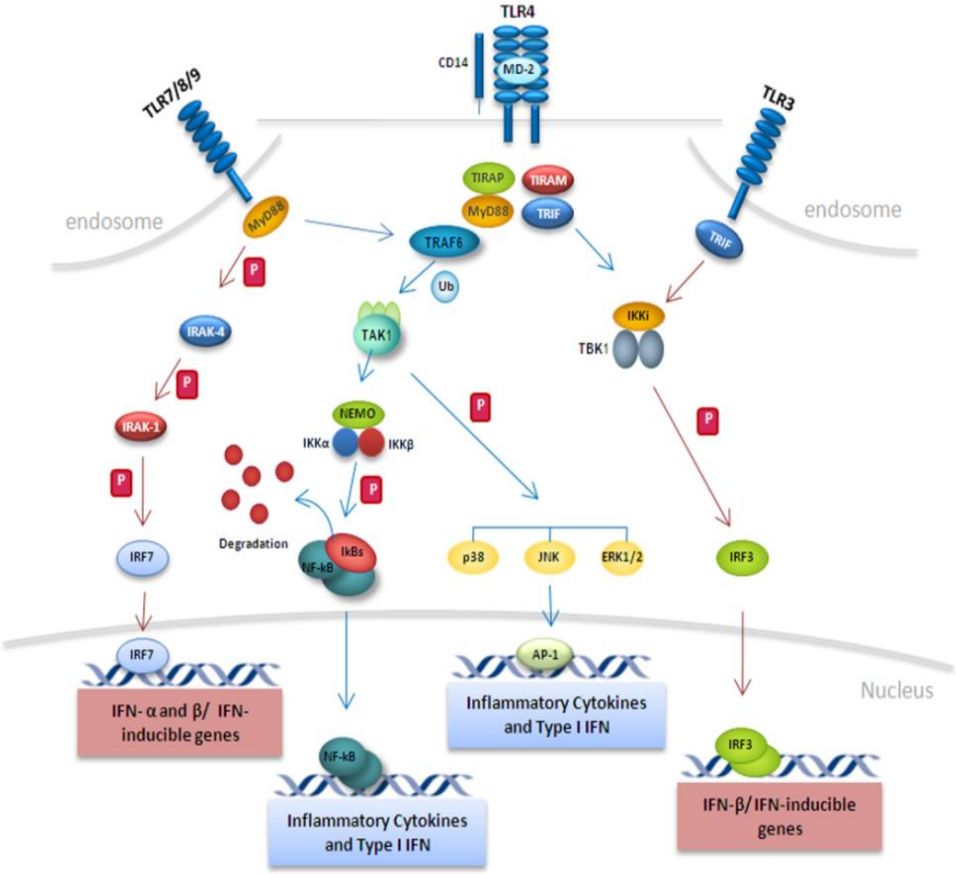


Fig.2: TLR signaling pathway(s). Lipopolysaccharide recognition by **TLR4** initiates both MyD88-dependent and TRIF-dependent pathways. The TLR4–MD-2 complex engages with LPS on the cell surface via LBP and CD14 and then recruits a TIR domain-containing adapter complex including TIRAP and MyD88. The TLR4–MD-2–LPS complex is subsequently trafficked to the endosome, where it recruits TRAM and TRIF adapters (not shown (Kagan *et al.*, 2008)). *TIRAP–MyD88* recruits IRAK family members and TRAF6 to activate TAK1. The TAK1 complex activates the IKK complex composed of IKKa, IKKb and NEMO (IKKc), which catalyze phosphorylation of IkB proteins. Phosphorylated IkB proteins are degraded, allowing NF-kB to translocate to the nucleus. TAK1 simultaneously activates the MAPK pathway that induces the activator protein-1 (AP-1). The activation of NF-kB and AP-1 results in induction of inflammatory cytokine genes (MyD88-dependent pathway). *TRAM–TRIF* recruits TRAF6 and RIP-1 for activation of TAK1 as well as TRAF3 for activation of TBK1– IKKi that phosphorylates and activates IRF3, in addition to NF-κB and AP-1. Whereas NF-kB and MAPK regulate expression of inflammatory cytokine genes in both pathways, IRF3 regulates expression of type I IFN in the TRIF-dependent pathway only. **TLR7 and TLR9** reside in the ER and interact with UNC93B and traffic to the endosome to recognize viral ssRNA and DNA, respectively. These TLRs recruit MyD88, IRAK4 and TRAF6, which in turn activates TAK1, IRF5 and TRAF3. TAK1 mediates activation of NF-kB and MAPK, which leads to the induction of inflammatory cytokine genes. TRAF3 activates IRAK1 and IKKα, which catalyze

the phosphorylation of IRF7 and induce type I IFN genes. **TLR3** signaling basically goes through the same pathway as the TLR4-TRIF-dependent pathway, without TRAM. TLR7 and -9 initiate only the MyD88-dependent pathway, but they induce IFNα expression by activating IRF7 via TNF receptor-associated factor 3 (TRAF3). Adapted from (Kawai and Akira, 2009; Lee and Kim, 2007).

Upon PAMPs recognition, TLRs induce inflammatory responses and a variety of antimicrobial effector responses. In particular, TLR ligation on specialized antigen-presenting cells called Dendritic Cells (DCs) directly induces a differentiation program, called DC maturation, which is characterized by the induction of co-stimulatory molecules on the cell surface. The co-stimulatory signal "flags" the antigenic peptides as foreign and is required (along with the TCR ligand–MHC/peptide complex) for the activation of T lymphocytes. Thus, by recognizing microbial molecular patterns, TLRs couple recognition of infection with the induction of pathogen-specific adaptive immune responses. TLR have also been implicated in autoimmunity and their manipulation has been seen to be extremely important in immunotherapies (Janeway and Medzhitov, 2002).

The activation of DCs determines the ability to deliver three signals to naive T lymphocytes (*fig.3*). The three signal model is composed by: Signal 1: delivered to the T-Cell receptor by the engagement of peptide-MHC complex of DCs. Antigens internalized that are delivered to late endosomes compartments, could be processed and loaded onto MHC class-II molecules (Turley *et al.*, 2000). However, Blander and Medzhitov have shown that the response to peptide loading after phagocytosis by MHC class-II only occurs if TLR signals are triggered (Blander and Medzhitov, 2006b)**,** however little is known about the implication of TLR signaling on MHC class-I antigen cross-presentation. Signal 2: delivered to T-Cells through co-stimulatory molecules such, CD40, CD80, CD83 and CD86. T-Cell activation is determined by the expression of these co-stimulatory molecules on DCs (Banchereau and Steinman, 1998; Iwasaki and Medzhitov, 2004; Reis e Sousa, 2006) that is induced by triggering TLR signaling pathways (Iwasaki and Medzhitov, 2004). In the absence of co-stimulatory signal naive T-Cells are

tolerized (when receive signal 1 alone) and primed when signal 1 and signal 2 are both present. Signal 3: refers to DC-derived signals, such as cytokines, and determine the T-Cell differentiation fate and for consequence the outcome of immune response (Reis e Sousa, 2006). As initially postulated, many of these signals are controlled by TLR (Akira *et al.*, 2006; Amsen *et al.*, 2004; Reis e Sousa, 2006).

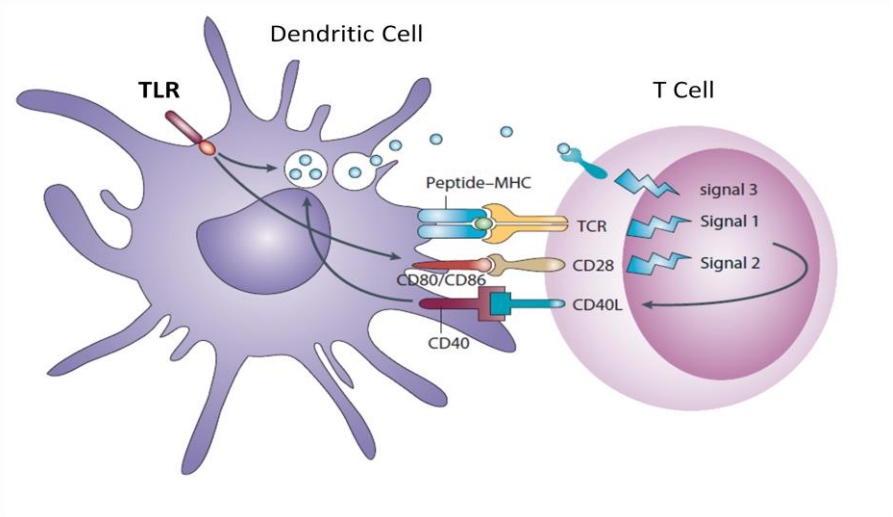


Fig.3: Interactions between Dendritic Cell and naive T-Cell. **Signal 1** is the antigen-specific signal that is mediated through T-Cell receptor (TCR) triggering by MHC class molecules peptides processed from pathogens after internalization through specialized pattern recognition receptors (PRRs). **Signal 2** is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86 that are expressed by DCs after ligation of PRRs, such as Toll-like receptors (TLRs) that are specialized to sense infection through recognition of pathogen-associated molecular patterns (PAMPs) or inflammatory tissue factors. **Signal 3** is the polarizing signal that is mediated by various soluble or membrane-bound factors, such as interleukins and chemokines. The nature of signal 3 depends on the activation of particular PRRs, such as TLRs by PAMPs. Optimal activation of DCs requires feedback stimulation by CD40 ligand (CD40L) expressed by T-Cells after activation by signals 1 and 2. Adapted from (Kapsenberg, 2003).

3. Antigen Presenting Cells (APCs)

3.1. The importance of being professional

Virtually any cell type expressing cell surface MHC class-I and II α/β heterodimers was able to engage T-Cells in an antigen-specific manner (Malissen *et al.*, 1984). However, different cell types can process and present antigens with different efficiencies (Mellman *et al.*, 1998; Robadey *et al.*, 1996; Schneider and Sercarz, 1997). This finding has led investigators to consider certain cells as antigen presenting cells (APCs), a group that typically includes B Lymphocytes (B Cells), Macrophages (MØ), and especially Dendritic Cells (DCs).

3.2. Dendritic Cells (DCs): The key players in antigen presentation

The historical, functional, and morphological definition of a DC is a veiled cell characterized by the presence of numerous membrane processes, that extended for up to hundreds micrometers in the form of dendrites, pseudopods or veils. Additional, morphological features of DCs included high concentrations of intracellular structures related to antigen processing such as endosomes and lysosomes. The fundamental characteristic is the unique ability to stimulate a naive T-Cell into cycle (Steinman, 1991). Phenotypically, DCs expressed in their surface large amounts of MHC class-II molecules, but are excluded of lineage markers including CD14 (monocyte), CD3 (T-Cell) CD19, 20, 24 (B cell), DCD56 (natural killer cell) and CD66b (Granulocyte) (Hart, 1997). Concerning their antigen presenting function, DCs also express various adhesion molecules: CD11a (LFA-1), CD11c, CD50 (ICAM-2), CD54 (ICAM-1), CD58 (LFA-3) and CD102 (ICAM-3), as well expressed in monocytes and macrophages (Hart and Prickett, 1993); and co-stimulatory molecules: such as CD80 (B7.1), CD86 (B7.2) and CD40. The adhesion molecules, co-stimulatory molecules and MHC class-II

molecules are up-regulated upon activation (Banchereau *et al.*, 1994; Fagnoni *et al.*, 1995).

DCs are the sentinels of the immune system, which play a critical role in the regulation of the adaptive immune response, making the link to adaptive immune system. These "professional" APCs function as key players as their primary function is to present antigens being unique among APCs for their critical role in stimulating naive T-Cells. DCs are rare cells present in blood, skin and all lymphoid organs, however, they are crucial for normal immune responses (Banchereau *et al.*, 2000; Banchereau and Steinman, 1998). Mice depleted of DCs display defective immune responses to viral (Ciavarra *et al.*, 2006), parasitic (Jung *et al.*, 2002; Liu *et al.*, 2006) and bacterial infections (Jung *et al.*, 2002).

DCs function as "master switches", being able to control whether the antigen gives a tolerance or a cellular immune response (Steinman *et al.*, 2003). They reside in immature state in peripheral tissues to "sample" the environment. When encounter bacteria, viral particles or apoptotic cells, DCs could enter in a mature stage. This maturation program leads to migration to secondary lymphoid organs, and consequently antigens could be presented directly in MHC class-II molecules, triggering $CD4^+$ T-Cells or cross-presented on MHC class-I molecules to $CD8^+$ T-Cells (Basta and Alatery, 2007; Bevan, 1976b; Rock and Shen, 2005). In this way, all systemically and peripherally expressed antigens can be presented to T-Cells.

The existence of distinct DC subsets is due to their inherent plasticity and to the changing microenvironment modulating their immunological properties. Because different DC types may play distinct roles in induction of immunity and tolerance (Belz *et al.*, 2002a; Belz *et al.*, 2002b; Chilton *et al.*, 2004; Heath *et al.*, 2004; O'Keeffe *et al.*, 2005) the outcome of the vaccination strategy in each scenario might vary (Corbett *et al.*, 2005). DCs form a heterogeneous cell population, which could be classified as plasmacytoid (pDCs) or conventional DCs (cDCs) (Shortman and Naik, 2007).

3.2.1. DCs sub-populations: Conventional Dendritic Cells (cDCs)

Within mouse lymphoid organs the cDCs can be recognized by the expression of high levels of CD11c and MHC class-II (Shortman and Liu, 2002). Further separation of the cDCs into subsets depends upon the organ in question and can be subdivided into migratory DCs and resident DCs (Villadangos and Schnorrer, 2007). Migratory DCs develop in peripheral tissues and migrate constitutively into lymph nodes, even in the absence of inflammatory stimuli (Walton *et al.*, 2006; Wilson *et al.*, 2008). These include Epidermal Langerhans cells (LCs) and different subsets of epidermal DCs: pulmonary $CD103^{+}CD11b^{-}$ and $CD103^{-}CD11b^{+}$ DC (Hintzen *et al.*, 2006) and their recently described dermal counterparts $CD103^{+}$ $CD11b^{-}langerin^{+}$ and $CD103^{-}CD11b^{+}langerin^{-}$ dermal DCs (Bursch *et al.*, 2007; Ginhoux *et al.*, 2007; Poulin *et al.*, 2007). Resident DCs differentiate in the lymphoid organs from blood-borne precursors (Liu *et al.*, 2007; Naik *et al.*, 2006) and can be divided by the CD8αα homodimer marker into three ‘‘conventional’’ ($CD11c^{high}$) DCs subsets: $CD8^{+}CD4^{-}$ DC, $CD8^{-}$ $CD4^{-}$ DC and DN DC. These subsets are discussed as follow.

3.2.1.1 Splenic Dendritic Cells (sDCs)

sDCs compose 1% of total splenocytes. Of these, approximately 80% are cDC and the remaining 20% are pDC (Asselin-Paturel *et al.*, 2003). The separation of spleen cDC into functionally distinct subsets is possible by the separation of $CD11c^{hi}$MHC II^{+} cells based on the expression of CD4 and the CD8αα homodimer (Vremec *et al.*, 2000).

$CD8^{+}$ Spleen DC: The $CD8^{+}CD4^{-}$ subset compose about 25% of total spleen cDC and resides in the T-Cell areas of spleen being the shortest lived cDCs subtype, with a turnover rate about three days (Kamath *et al.*, 2000). The $CD8^{+}$ DCs are important *in vivo* due to their ability to secrete extremely high levels of the pro-inflammatory cytokine IL-12p70 upon activation, lending these cells with a

Th-1-inducing profile (Maldonado-Lopez *et al.*, 1999). The $CD8^+$ cDCs also express other cytokines upon activation including IL-6, TNF-α, and low levels of chemokines including Mip-1α and β and RANTES (Proietto *et al.*, 2004). Under some circumstances the $CD8\alpha^+$ DCs also produce type I IFN (Hochrein *et al.*, 2001). The $CD8^+$ cDCs in all organs are unique amongst the cDCs as they have the exquisite ability to constitutively present exogenous cell-associated or soluble proteins very efficiently in the context of MHC class-I (den Haan *et al.*, 2000; Pooley *et al.*, 2001; Schnorrer *et al.*, 2006).

$CD4^+$ Spleen DC: The $CD4^+CD8^-$ cDCs of mouse spleen comprise about 50% of spleen cDCs and are located in non-T-Cell zones of the spleen. They require IRF4 and IRF2 for development and normal function (Ichikawa *et al.*, 2004). The $CD4^+$ cDCs stand out as the cDCs population that produces the highest levels of inflammatory-type chemokines: Mip-3α, Mip-3β, RANTES (Proietto *et al.*, 2004). However, the $CD4^+$ cDCs display a high capacity to stimulate $CD4^+$ and $CD8^+$ T -Cells in "direct" presentation assays. In fact the $CD4^+$ cDCs, together with the $CD4^-CD8^-$ cDCs are the most potent presenters of MHC class-II antigen complexes to $CD4^+$ T-Cells (den Haan and Bevan, 2002; Pooley *et al.*, 2001). Contrary to $CD8^+$ cDC, the $CD4^+$ cDCs and $CD4^-CD8^-$ cDCs have been shown to induce TH2 responses by responder T-Cells (Hammad *et al.*, 2004; Maldonado-Lopez *et al.*, 1999). When compared to the $CD8^+$ cDCs, the antigen cross-presentation capacity of $CD4^+$ cDCs in the steady state is poor.

$CD8^-CD4^-$ (DN) Spleen DC: The $CD4^-CD8^-$ DCs in mouse spleen comprise about 20–25% of spleen cDCs and closely resemble the $CD4^+$ DCs in function. Similarly, they also produce high levels of Mip-3α, Mip-3β and RANTES, although the levels produced in the steady state are considerably lower than those produced by the $CD4^+$ cDCs. The $CD4^-CD8^-$ cDCs are also poor at cross-presenting exogenous antigen, although they are as efficient as $CD4^+$ cDCs in direct MHC class-I and MHC class-II presentation (Schnorrer *et al.*, 2006).

Microarray data does suggest that at least the majority of cells that are identified as $CD4^{-}CD8^{-}$ DCs are extremely closely related to the $CD4^{+}$ cDCs (Edwards *et al.*, 2003; Lahoud *et al.*, 2006). Mice treated with fms-like tyrosine kinase 3 ligand (Flt3-L) have a large increase in $CD4^{-}CD8^{-}$ and $CD8^{+}$ cDCs, and, in contrast, the $CD4^{+}$ cDC are preferentially increased in a mouse treated with GM-CSF (O'Keeffe *et al.*, 2002). There are other cDCs: Thymic cDCs, Lymph Node cDCs and Immature cDCs of Blood and Bone Marrow (Hochrein and O'Keeffe, 2008).

Plasmacytoid DC (pDCs): The phenotype of pDCs is always $CD11c^{int}$MHC $II^{lo}CD11b^{-}$ $CD205^{-}$ and they defy the classical definition of a DC since in the steady state they completely lack any cytoplasmic protrusions or veils (plasma cell morphology) and lack the ability to stimulate naive T-Cells into cycle. However, upon activation by viruses, TLR7, 8, or 9 ligands they rapidly acquire the morphological and phenotypical characteristics of a cDC together with their trade mark high type I IFN production. There is no doubt that these cells have a major function in innate immune responses with their exceptional ability to produce rapid, high levels of type I IFN upon activation (Barchet *et al.*, 2005a; Barchet *et al.*, 2005b; Fuchsberger *et al.*, 2005; Liu, 2005; Naik *et al.*, 2005a; Soumelis and Liu, 2006). It remains unclear the real ability of pDC to stimulate naive T-Cells into cycle when compared to the potent stimulatory activity of cDCs. Several recent reports cite pDCs as capable and necessary of maintaining tolerance (Abe *et al.*, 2005; de Heer *et al.*, 2004; Ochando *et al.*, 2006). cDCs phenotype is summarized on following table and compared to their *in vitro* equivalent.

Table I: Mouse cDCs subsets, *in vitro* equivalent and respective phenotype.

	$CD8^+$ DC	**$CD8^-$ DC**	**DN DC**	**pDCs**	**Monocyte Derived**
CD11c	+++	+++	+++	+	+++
CD8	++	-	-	+/-	-
CD4	-	+	-	-	-
CD205	++	-	+/-	-	+/-
CD11b	-	++	++		++
Langerin	+	-	-	-	-
Ly6C	-	-	-	++	++
***In vitro* equivalent**	Bone-Marrow precursors plus FLT3-L				BM, spleen or blood percursosr plus GM-CSF

Adapted from (Villadangos and Schnorrer, 2007)

3.2.2. DC generation *in vitro* and their *in vivo* counterpart

At least two distinct pathways of DC development from pluripotent bone marrow stem cells have been identified in mice, myeloid and lymphoid, which differ in phenotype, localization, and function (Cella *et al.*, 1997; Steinman, 1991). Both subsets express high levels of CD11c, MHC class-II complex, and the co-stimulatory molecules CD86 and CD40. To date, the most reliable marker to distinguishing these two subsets is CD8α, which is expressed as a homodimer on the lymphoid DC, but is absent from the myeloid subset. Other markers such as DEC-205 and CD1d are expressed at higher levels on lymphoid DCs, but they can be up-regulated on myeloid DCs by in vitro culture or by LPS treatment (Maraskovsky *et al.*, 1996; Pulendran *et al.*, 1997; Vremec and Shortman, 1997; Wu *et al.*, 1996). The establishment of defined cell-culture systems to generate DCs *in vitro* has been instrumental in assessing the functional properties of these

cells. Granulocyte/macrophage colony stimulating factor (GM-CSF) preferentially expands the myeloid DC subset in vivo (Pulendran *et al.*, 1999). Several methods to generate mouse DCs *in vitro* have been described (Shortman and Naik, 2007). The most common involves culturing bone-marrow (primitive hematopoietic progenitors) or spleen precursors in medium that is supplemented with GM-CSF, which are able to fully function as a DC capable of priming antigen specific T-Cell responses, with or without interleukin-4 (IL-4) (Inaba *et al.*, 1992; Scheicher *et al.*, 1992). The DCs generated by this method resemble monocyte-derived DCs, which almost certainly do not correspond with any of the lymphoid-organ-resident DC subsets found *in vivo* (Shortman and Naik, 2007). DCs can also arise from lymphoid-committed precursors (Ardavin *et al.*, 1993; Saunders *et al.*, 1996) (Steinman *et al.*, 1997; Vremec *et al.*, 1992; Wu *et al.*, 1996). To generate these, bone-marrow precursors must be cultured with FMS-like tyrosine kinase 3 ligand (FLT3L) (Lyman and Jacobsen, 1998), and both lymphoid and myeloid DC numbers increase dramatically upon Flt3-L injection (Pulendran *et al.*, 1997; Shurin *et al.*, 1997). It is expected that this culture system will allow comparative studies among resident DC subtypes, and between these DCs and monocyte-derived DCs (Shortman and Naik, 2007).

4. The mechanism of Antigen Presentation

As described, DCs are the "professional" APCs, because they are able to degrade antigens in a "fine-tune" way, presenting the resulting fragments at cell surface and activate both naive $CD4^+$ T-Cells and $CD8^+$ T-Cells (Banchereau and Steinman, 1998). DCs process intracellular and extracellular antigens differently, using different pathways, which could outcome in a different immune response.

4.1. Antigen presentation pathways

4.1.1. Classical pathways

4.1.1.1 Extracellular antigens - MHC Class-II pathway

Extracellular antigens are internalized by endocytosis **(exogenous pathway)**. As the phagosome matures it acquires the machinery for antigen processing and loading. MHC class-II molecules (MHC-II) are assembled as dimmers in the endoplasmic reticulum (ER) with help of the specialized chaperone invariant chain (li), which occupied the peptide-binding groove. These MHC-II/li complexes are transported to the MHC class-II containing compartments (MIIC). Here the invariant chain is degraded by cathepsins and proteases until only the part that occupies the peptide-binding groove is left, which is called CLIP. In these compartments, MHC-II encounters antigenic peptides fragments, in size between 9-25 mer, derived from proteins degraded in the endocytic track. CLIP is then exchanged for one of these fragments with the help of the chaperone HLA-DM. The resulting peptide fragments associate with MHC class-II molecules presents within phagosomes and migrate to cell membrane to be presented to $CD4^+$ T helper cells that stimulate mainly the production of antibodies (Ramachandra *et al.*, 1999; Rocha and Neefjes, 2007).

4.1.1.2. Intracellular antigens - MHC Class-I pathway

Intracellular antigens from self or from pathogens, such as virus, are degraded in the cytoplasm or nucleus by the ubiquitin-proteasome pathway **(endogenous pathway)**, where the proteins are conjugated with a chain of ubiquitin molecules, a marker for rapid degradation (Goldberg and Rock, 1992; Hershko and Ciechanover, 1998; Rock *et al.*, 1994). The proteasome degrades proteins into oligopeptides ranging in size from 2-3 residues to >20 amino acids, and the majority of these residues are further hydrolyzed by cytosolic peptidases ultimately into amino acids that are re-utilized for protein synthesis or energy (Rock and Goldberg, 1999). However, a small fraction of resulting peptides >7 residues that survived to complete destruction are shuttled into the endoplasmatic reticulum (ER) through the transporter associated with antigen processing (TAP protein). In the ER, long peptides are trimmed by ER aminopeptidase-1 (ERAP1) to peptides of 8-9 residues (York *et al.*, 2002). 8-mer or 9-mer peptides with appropriate sequences, are loaded into newly synthesized MHC class-I molecules by loading complex (ER chaperones clanexin, calreticulin and tapasin). MHC class-I/peptide complexes are then transported to the cell surface, where it stimulates cytotoxic T lymphocytes (CTLs) that kill directly infected cells. The MHC class-I presentation of endogenous antigen by professional APCs to elicit an immune response is also called direct presentation.

$CD8^+$ cytotoxic lymphocytes are crucial for clearance of infected cells and provide the major defense against cancer. However, this pathway does not explain how can CTL responses be elicited against tumor cells of non-hematopoietic origin or against viruses that no not infect professional APCs? The first evidence was shown by Bevan in 1976, were CTL responses could be elicited against antigens derived from an exogenous source. He showed that minor histocompatibility antigens could be transferred from donor cells to host APCs which result in T-Cell priming, “cross-priming” (Bevan, 1976a, 1976b). Consequently, extracellular

antigens can have access into endogenous pathway by a mechanism not well known, and antigens could be present into MHC class-I complex. This mechanism is denominated antigen **cross-presentation** and it is almost restricted to DCs, which should have specialized machinery. The term "cross priming" is used when presentation results in activation and "cross tolerance" is used when there is tolerization of $CD8^+$ T-Cells (Heath and Carbone, 2001b; Touret *et al.*, 2005; Yewdell *et al.*, 1999). Antigen cross-presentation is teleologically attractive in that it serves three evolutionary purposes (Chen *et al.*, 2004b): *(1)* as a fallback mechanism for viruses that evade CTL activity by failing to replicate within APCs; *(2)* as a tumor surveillance mechanism and *(3)* as a means for inducing peripheral tolerance to self-antigens not synthesized within APCs (Lin *et al.*, 2008b).

4.1.2. Antigen cross-presentation

4.1.2.1. Internalization pathways for antigen cross-presentation

Immunity to microbial infections or tissue remodeling (eliminating self) depends on engulfment into intracellular vesicles by specialized phagocytic cells (Macrophages, Neutrophils and DCs). However, these two different processes usually give rises to a different immune response, where phagocytosis of microbes triggers inflammatory response but not engulfment and degradation of apoptotic cells. These inflammatory *vs* anti-inflammatory outcome depends on the type of receptors and signaling pathways that are engaged during recognition (Greenberg and Grinstein, 2002; Henson *et al.*, 2001; Underhill and Ozinsky, 2002).

The uptake of particles >0.5 μm in size is termed phagocytosis, whereas particles < 0.5 μm are taken up by receptor-mediated endocytosis or pinocytosis (Rejman *et al.*, 2004). Distinct types of phagocytosis tend to be ligand specific: bacteria (~0.5–3 μm) or yeast (~3–4 μm) are internalized by macrophages through scavenger receptors. Microorganisms can also be coated with serum components (for example, complement) or antibodies and then taken up through complement

(Underhill and Ozinsky, 2002; van Lookeren Campagne *et al.*, 2007) or Fc-receptors (Nimmerjahn and Ravetch, 2006; Swanson and Hoppe, 2004), respectively. Cells that undergo apoptosis, which can range in size from 5 to 50 μm, must also be removed (Kinchen and Ravichandran, 2008).

DCs acquire exogenous antigens through four major pathways. The nature of the antigen determines which internalization route is used. *(a)* Large particulate antigens (such as bacteria, cell debris, apoptotic cells and biologically inert particles) are internalized by phagocytosis**,** a clathrin-independent process trough phagosomes (a membrane-bound organelle formed when a phagocytic cell engulfs particulate material) (Brown, 1986). *(b)* Small particulate antigens enter the cell by endocytosis, the formation of vesicles of between 150-200nm formed at sites of membrane invaginations, termed coated pits, mediated by cells surface receptors (Mellman, 1996). *(c)* Pinocytosis describes the uptake of soluble antigens as part of the extracellular fluid present in the vicinity of the budding endosome. Uptake of endocytic vesicles can be both clathrin dependent and independent. *(d)* Large fluid volumes are internalized by macropinocytosis**,** by the formation of nonspecifically large vacuoles around 200-500nm at sites of membrane ruffling (Brode and Macary, 2004).

Antigen from endocytic vesicles is either retro-translocated into the cytosol for presentation via the classical pathway or loaded on MHC class-I molecules within the endocytic compartment for antigen cross-presentation, or in MHC class-II molecules in vacuolar pathway (Brode and Macary, 2004). This balance is not well understood so far as well the type of stimulus that favors the different pathways. However, physiologically, phagocytosis is probably a major route for antigen uptake and cross-presentation (Savina and Amigorena, 2007). Phagocytosis is the center of immune response by providing a route for destruction of pathogens and generation of antigenic ligands. This process could be divided in four stages: *(1)* particle attachment to cell surface and target recognition by surface receptors that

initiate cytoskeletal rearrangements and membrane trafficking; *(2)* particle internalization, characterized by the flow redistribution of the plasma membrane to surround the particle; *(3)* phagosome formation / maturation by exchange material initially with early endosomes and subsequently with late endosomes and lysosomes to form phagolysosomes; *(4)* fusion with plasma membrane leading to antigen presentation in MHC class molecules to T-Cells (Greenberg and Grinstein, 2002).

Work done in DCs, showed that particle antigens (i.e. Ovalbumin adsorbed to latex beads), that force internalization by phagocytosis, strongly increased the efficiency of antigen cross-presentation (Kovacsovics-Bankowski *et al.*, 1993; Shen *et al.*, 1997). Initially it had been suggested that the efficiency of antigen cross-presentation may be due to differences in phagocytic capacity (Albert *et al.*, 1998). Indeed, all DCs subsets have equivalent capacities of both soluble and particulate antigens. However they have a different ability to cross-present (den Haan *et al.*, 2000; Pooley *et al.*, 2001; Schulz *et al.*, 2002). It is important to note that uptake of particulate antigens may trigger a different pathway(s) than uptake of soluble antigens, which could lead to different efficiencies in antigen cross-presentation (Graham *et al.*, 2007). Therefore, whether receptors only selectively drive antigens to specific endocytic cross-presentation compartments or just accumulate antigens within DCs to favor antigen cross-presentation is still unknown (Guermonprez *et al.*, 2002). Once antigen is in the phagosome, how are presented peptides generated and how do they get to MHC class-I molecules?

4.1.2.2. Antigen loading into MHC class I pathway - Mechanism(s)

There are two main pathways for presentation of exogenous antigens via MHC class–I: *(1)* one requires escape of exogenous antigen via active transfer from phagosomes into the cytosol for proteasomal degradation – **Cytosolic Pathway** (Arnold *et al.*, 1995; Kovacsovics-Bankowski and Rock, 1995; Norbury *et al.*, 1995; Reis e Sousa and Germain, 1995); *(2)* the other involves the activity of lysosomal proteases for peptide generation within the phagosome itself – **Vacuolar Pathway** (Shen *et al.*, 2004). There is another interesting model proposed by J. Neefjes' lab, which implicates GAP junctions in the swapping of intracellular peptides to adjacent APCs (Neijssen *et al.*, 2005), but its relevance has not been addressed so far. The cytosolic pathway is considered the most important pathway under physiological conditions for antigen cross-presentation, while the "alternative" vacuolar pathway contribution *in vivo* is relatively insignificant (Rock and Shen, 2005).

4.1.2.2.1. The Vacuolar Pathway – TAP independent pathway

The vacuolar pathway model (*fig.4*) propose that MHC class-I molecules encounter and bind exogenously derived peptides in post-Golgi or endolysosomal compartments, in the same way as MHC class-II molecules, before being transported to the cell surface. Indeed, MHC class I-β2-microglobulin dimmers are present in the endosomes of immature DCs and can traffic rapidly to the cell surface upon encounter with a maturation signal (Ackerman and Cresswell, 2003; Kleijmeer *et al.*, 2001; MacAry *et al.*, 2001). How peptide-receptive MHC class-I molecules traffic to the vacuole is unknown, although there are several possible routes of entry. Specific sorting of MHC class-I molecules from the plasma membrane into endosomes that could fuse with phagosomal compartments (Chiu *et al.*, 1999; Reid and Watts, 1990), is mediated by a highly conserved tyrosine motif

within the cytoplasmic tail region (Lizee *et al.*, 2003). Intriguingly, deletion or mutation of this motif abrogates acquisition and antigen cross-presentation of exogenously derived peptides *in vitro* and attenuate T lymphocyte responses to immunodominant viral epitopes *in vivo* (Lizee *et al.*, 2003). Consistent with these possibilities, vacuolar antigen cross-presentation was reported to be insensitive to brefeldin A (BFA) which blocks exocytosis of proteins from the ER (Pfeifer *et al.*, 1993). This mechanism does not require TAP and was insensitive to proteasome inhibitors and therefore was clearly different from the phagosome-to-cytosol pathway (Song and Harding, 1996). Different types of antigens have been shown to be cross-presented, at least in part, by this vacuolar pathway: Proteins associated with *E.coli* (Campbell *et al.*, 2000; Pfeifer *et al.*, 1993; Song and Harding, 1996; Wick and Pfeifer, 1996), poly(lactic-coglycolic acid) (PLGA) particles (Shen *et al.*, 2004), viral proteins/virus-like particles (Bachmann *et al.*, 1995; Ruedl *et al.*, 2002; Stober *et al.*, 2002) or even soluble antigens (Chen and Jondal, 2004).

For vacuolar pathway presentation, antigens are not generated in the cytosol but instead within endocytic vacuoles. The proteases resident in these compartments may play an important role in peptide generation. Protease inhibitor leupeptin, several cathepsins resident in the endocytic compartments shown to be sensitive to leupeptin (Chapman *et al.*, 1997; Villadangos *et al.*, 1999), and DCs deficient on cathepsin S were unable to present Ovalbumin antigen by the vacuolar pathway but does not affect the phagosome-to-cytosol Ovalbumin antigen presentation pathway (Shen *et al.*, 2004). These indicate that the proteases involved in the two pathways were clearly distinct. However, an absence of cathepsin B, L or D had no effect on antigen cross-presentation by both pathways (Shen *et al.*, 2004). Cysteine Proteases, including cathepsin S, are able to generate peptides for MHC class-II presentation pathway. Shen and colleagues shown that chatepsin S could play a key and non-redundant role in the vacuolar pathway of antigen cross-presentation,

at least for several antigens, and was not substitute by others cathepsins (Shen *et al.*, 2004).

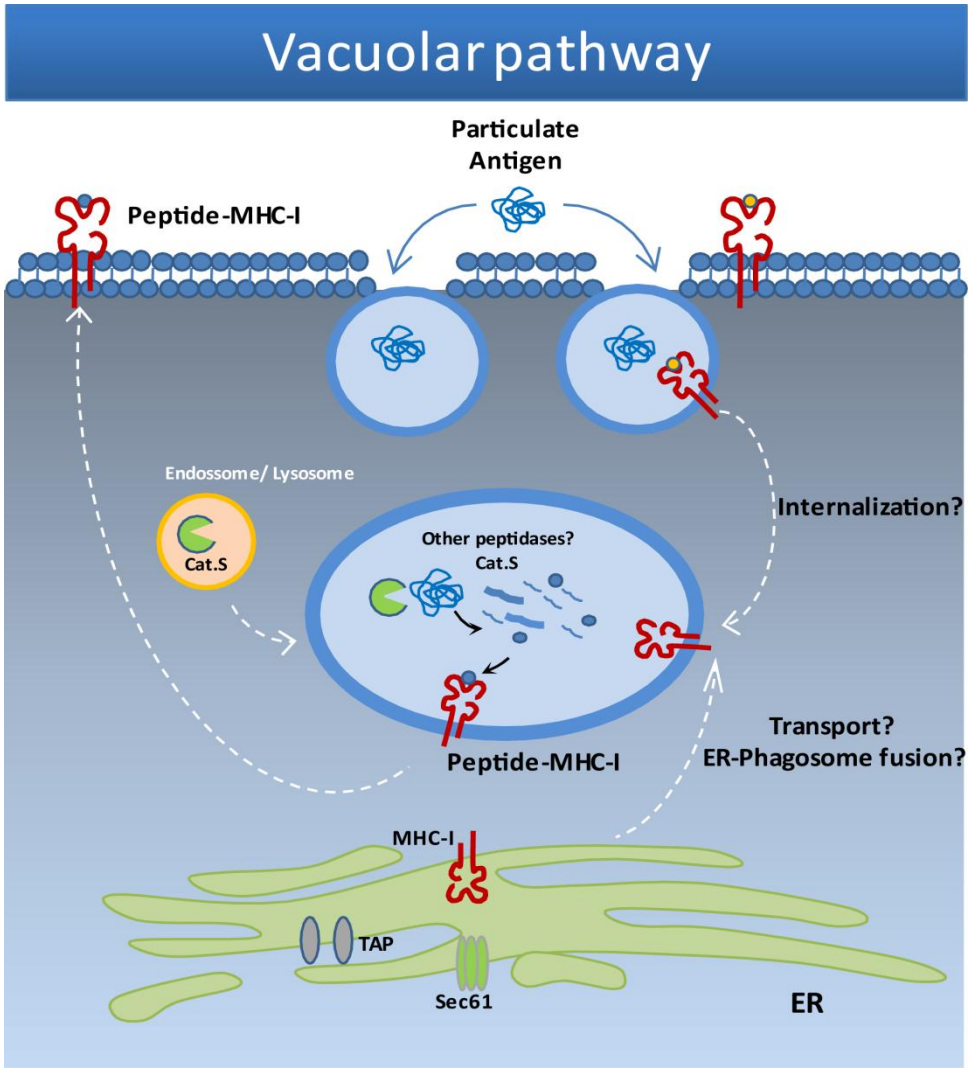


Fig.4: Vacuolar pathway of antigen cross-presentation: In the vacuolar pathway, antigen is internalized into phagosomes where it is degraded into oligopeptides by cathepsin S and possibly other endosomal proteases. The resulting peptides are probably loaded onto major histocompatibility complex class-I molecules (MHC-I) that have trafficked into the vesicle from the plasma membrane or from the endoplasmic reticulum (ER), either by internalization, transport or ER–phagosome fusion, and presented at cell surface. See text above for details. Adapted from (Rock and Shen, 2005).

4.1.2.2.2. Phagosome-to-cytosol pathway - TAP-dependent pathway

The Phagosome-to-cytosol pathway model (*fig.5*) proposes that the antigen internalized into endosomes is followed by release into the cytosol (translocation) of the APCs (Delamarre *et al.*, 2003; Rodriguez *et al.*, 1999). In the cytosol, proteins need to be degraded (Cresswell *et al.*, 2005) by proteasome before being transported into the lumen of the endoplasmatic reticulum (ER) via TAP. In the ER the antigens are loaded onto newly synthesized MHC class-I molecules, that are transported to the cell surface to be presented to $CD8^+$ T-Cells. At least, three observations support this model: Antigen cross-presentation is abrogated by the inhibition of proteasomal degradation using lactacystin (Rodriguez *et al.*, 1999); by inhibition of TAP or in TAP-deficient professional APCs (den Haan *et al.*, 2000); and by inhibition of the secretory pathway and the trans-golgi network using brefeldin A (Kovacsovics-Bankowski and Rock, 1995).

4.1.2.2.2.1. The ER-Phagosome Model

Recent revelations regarding the phagocytic process support an alternative molecular model for antigen cross-presentation. This may occur autonomously through an involvement of the ER in the generation of phagosome compartments but in a TAP - dependent manner, the **ER-phagosome fusion model**. This model proposes that during phagosome formation there is an ER recruitment, which allows the phagosomes to contain all MHC class-I loading machinery (Pierre, 2005) and other required ER-components important for antigen cross-presentation (Gagnon *et al.*, 2002; Houde *et al.*, 2003). This model is similar to the phagosome-to-cytosol model in that antigen retro-translocated to the cytosol for proteasome-mediated processing. However, it differs in that peptides are transported back into the phagosomes, instead of the ER, for the MHC class-I/peptide complexes formation. It has been a paradigm for more than three decades that the plasma

membrane, through invaginations, provides all of the membrane required to form complete phagosomes. Active phagocytes are capable of engulfing large numbers of particles without any apparent loss from their plasma membrane (Werb and Cohn, 1972). Membrane regeneration process alone is difficult to explain this observation. The ER-phagosome fusion model was originally suggested by proteomic analysis (mass spectrometry and two-dimensional gel electrophoresis) of latex bead phagosomes from mouse macrophages that shown presence of many ER-derived components, including the MHC-I loading machinery in phagosomes (Garin *et al.*, 2001). Further analysis showed that phagosomes fuse with the ER during particle engulfment, and that ER membranes constitute a large part of phagosomal membranes (Gagnon *et al.*, 2002). Three independent studies came out at same time with the evidence that phagosomes in both macrophages (Houde *et al.*, 2003) and DCs (Ackerman *et al.*, 2003; Guermonprez *et al.*, 2003) were competent organelles for antigen cross-presentation. Additionally, antigen cross-presentation studies via this route was only partially inhibited by brefeldin A (blocks exocytosis of proteins from the ER), suggesting that the ER-fused phagosomes are able to transport peptide-loaded MHC class-I complexes directly to the cell surface independent of Golgi-mediated transport (Yewdell and Haeryfar, 2005). Briefly, ER membranes fuse with the plasma membrane to form the complete phagosome with a large portion of ER-derived membranes and proteins including all the major elements of the MHC class-I loading complex such as TAP, tapasin, calnexin, MHC class-I heavy chain, ER chaperones, disulphide isomerases (Grp78, ERp57), ER-aminopeptidases associated with peptide trimming (ERAP) and the peptide translocation channel (sec61) (Gagnon *et al.*, 2002; Houde *et al.*, 2003). Despite much controversy regarding the purity of the phagosomal preparations in such studies (Touret *et al.*, 2005), subsequent studies confirmed the existence of endoplasmic reticulum–based proteins in phagosomes (Ackerman *et al.*, 2003; Guermonprez *et al.*, 2003; Houde *et al.*, 2003).

Phagosomes gradually progress through the endocytic pathway and acquire hydrolases such as cathepsins. Partial proteolysis by cathepsins generates polypeptides suitable for export into cytosol by Sec61. Cytosolic peptides are poly-ubiquitinated by the ubiquitinating enzyme complex (UBC) become substrates for proteasomal degradation. After proteasomal degradation, processed peptides are re-imported (translocated) by the TAP into the lumen of phagosomes compartments and trimmed by ER aminopeptidase (ERAP) into 8-9 residues. Indeed, the import of peptides is dependent on TAP, as demonstrated by: antibodies against TAP, the inhibitory peptide ICP47 derived from herpes simplex virus, and US6 a low-molecular weight cytomegalovirus protein (Ackerman and Cresswell, 2003). This 8-9 length ideal peptides are loaded into MHC class-I molecules by the MHC class-I loading complex. Phagosomes containing loaded MHC class-I molecules may recycle back to the plasma membrane by exocytosis to be present to $CD8^+$ T-Cell. The role of the cytosolic pathway in antigen cross-presentation was recently confirmed *in vivo*, by the selective suicide of DCs that are able to translocate exogenous proteins, using cytocrome *c* intravenously (Lin *et al.*, 2008a).

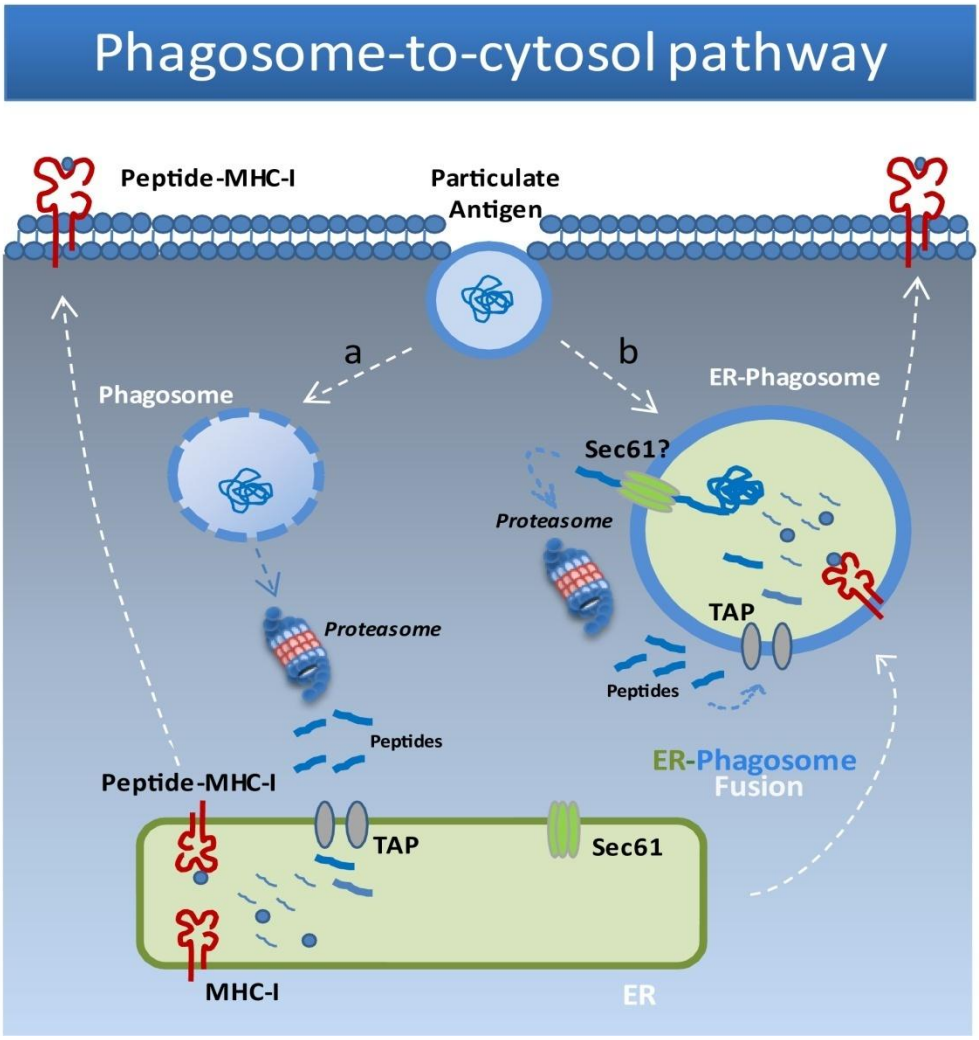


Fig.5: Antigen cross-presentation: Phagosome-to-cytosol pathway. In the phagosome-to-cytosol pathway, antigen is internalized into phagosomes or macropinosomes and then transferred into the cytosol. Recently, it was found that a subset of phagosome acquires transporter associated with antigen processing (TAP), MHC class-I, Tapasin, and Sec61 from the ER, and it is not presently clear to what extent these vesicles versus standard phagosomes participate in this pathway. The mechanism by which proteins are transferred from phagosomes into the cytosol is not understood, although it has been hypothesized that this export may occur through Sec61. Once in the cytosol, the antigen is hydrolyzed by proteasome into oligopeptides that are then transported by TAP and loaded onto MHC class-I molecules in the endoplasmic reticulum (ER) or the 'ER – phagosome' vesicles, and presented at cell surface. See text above for details. Adapted from (Rock and Shen, 2005).

4.1.2.3. Key steps in MHC class-I antigen cross-presentation

These exogenous antigens to be presented to $CD8^+$ T-Cells need to utilize the conventional MHC class-I presentation pathway, where exists three crucial steps: the release into cytosol (retro-translocation machinery) (Gagnon *et al.*, 2002; Houde *et al.*, 2003), the antigen processing mediated by proteasome complex and the transport associated (Brossart and Bevan, 1997; Kovacsovics-Bankowski and Rock, 1995) and the control of phagosomal maturation (Blander and Medzhitov, 2006a).

4.1.2.3.1. Antigen transport into the cytosol (retro-translocation)

For the antigens to be degraded by the proteasome the antigens should have access to the cytosol (Pierre, 2005). Therefore, the logical question to ask is, how are antigens internalized into endosomes/phagosomes transferred across membranes into cytosol? There is a simple explanation that antigens could passively egress through “leaky” phagosomal membranes (Reis e Sousa and Germain, 1995). However this observation is questioned by Amigorena´s lab, were they shown that immune complexes tracked microscopically by fluorescent dye retained their immunoglobulin portion within endosomal compartments after cytosolic translocation (Rodriguez *et al.*, 1999). In this work, they also proposed the existence of a selective size-specific process that permits retro-translocation of the phagosome into the cytosol. The nature of the channel or complex is not well defined (Lilley and Ploegh, 2004; Ye *et al.*, 2004), however there is some evidence from the interaction of Ovalbumin antigen with the ER-associated degradation (ERAD) pathway translocon, sec61 (Imai *et al.*, 2005). Another channel protein, Derlin1, another component of the ERAD pathway, was also implicated (Lilley and Ploegh, 2004; Ye *et al.*, 2004).

Sec61 is normally involved in the import of newly synthesized proteins from ribosomes into the endoplasmic reticulum. However, in ways that are incompletely understood, the direction of transport through Sec61 can be reversed (Tsai *et al.*, 2002). Sec61 is known to be involved in the retro-translocation of misfolded proteins from the endoplasmic reticulum to the cytosol for degradation, so it has been proposed that Sec61 might be involved in the translocation of proteins in phagosomes to the cytosol (Ackerman *et al.*, 2003). Sec61 has been isolated from purified phagosomes from DCs, but there is no crucial experiments showing mediated export of internalized antigens (apart from dextrans) (Rodriguez *et al.*, 1999) from phagosomes into cytosol. Recently, Cresswell's lab made use of exotoxin A, inhibitor of sec61 transporter, which results in inhibition of antigen cross-presentation of soluble Ovalbumin (Ackerman *et al.*, 2006). The exogenous antigens were rapidly found in cytosol within 1-2hr of internalization and sec61 appears to be involved (Guermonprez *et al.*, 2003; Houde *et al.*, 2003; Roy, 2002). This translocation was also observed with the colera toxin subunit 1 (CTA1), a known substrate for Sec61, suggesting the role of this complex in the translocation events observed (Schmitz *et al.*, 2000).

However, a major problem exists: the size of sec61 channel. A crystal structure for secY (homolog of sec61), showed that the diameter of this pore-like channel is around 5-8Å in diameter (Van den Berg *et al.*, 2004), which appear to be too small to accommodate large proteins as 30-60 KDa, dextrans, luciferase or horse radish peroxidase, with an expected radii of >30Å (Van den Berg *et al.*, 2004), that have been previously shown to egress from phagosomes into the cytosol (Norbury *et al.*, 1997; Norbury *et al.*, 1995; Rodriguez *et al.*, 1999). In a recent review, Lin and colleagues suggest that this could be due to: *1)* the sec61 channel has the capacity to widen, *2)* proteins are transferred out of phagosomes in an unfolded state and subsequently refolded in the cytosol or, *3)* additional pathways for cytosolic transfer are available (Lin *et al.*, 2008b; Rock, 2006).

4.1.2.3.2. Antigen processing /degradation

To be presented in MHC class-I molecules, antigenic peptides of defined length (usually 8–10 amino-acid residues long) and sequence have to be generated. Where and how are generated these peptides? Houde and colleagues have found that some of the antigens internalized into phagosomes are polyubiquitinated (Houde *et al.* 2003). Proteasome - an ATP dependent, multisubunit protease - is the central proteolytic machinery in the cell, involved in the turnover of proteins and plays a critical role in initiation protein breakdown to generate most antigenic peptide ligands for MHC class-I molecules (Rock *et al.*, 1994; York *et al.*, 1999). Proteasome are required to make the C-terminal cleavages that generate the presented peptide (Goldberg *et al.*, 2002). Proteasome tends to cleave after hydrophobic or basic residues — residues that are favored as carboxy-terminal anchor residues – this final N-terminal peptide generation is crucial for correct loading into MHC class-I molecules (Beninga *et al.*, 1998; Mo *et al.*, 1999). Other proteases and peptidases could contribute to the MHC class-I peptide pool, but in a minor extension (Schwarz *et al.*, 2000).

Because large percentage of antigenic peptides are not generated as 8 or 10-residue products that bind MHC class-I molecules, but precursors peptides of 10-12 residues, they required to be cleaved at N-terminal by cytosolic and ER amino peptidases (ERAAP or ERAP1) (Lauvau *et al.*, 1999; Mo *et al.*, 1999). This trimming should in some way controlled, a role develop by cytosolic chaperons who protect MHC class-I epitopes for total degradation in cytosol.

Polyubiquitylated proteins are marked for rapid degradation by 26S proteasome, composed of the 20S proteasome, representing the catalytic core, and two 19S regulator complexes that are responsible for the binding and unfolding of substrates (Kloetzel, 2001). The proteasome composition and expression of modulatory cofactors can vary accordingly the type of inflammatory environment, which will affect the repertoire of peptides produced (Naujokat *et al.*, 2007;

Visekruna *et al.*, 2006). However, the evidence that proteasomes are involved in cross-presenting of exogenous antigens remains indirect and only based on *in vitro* data using proteasome inhibitors (Ackerman and Cresswell, 2004; Kovacsovics-Bankowski and Rock, 1995; Norbury *et al.*, 1997; Norbury *et al.*, 1995; Shen *et al.*, 2004). Proteasomes are localized in the cytosol and are not found in the ER and endolysosomes, demonstrating that cross-presented proteins are processed by either form of proteasomes should intersect the cytosol during antigen cross-presentation. Typical proteasome are not always well suited for generating antigenic peptides. To process antigens more efficiently, the cell replaces some of its proteasomal subunits with more appropriate subunits. Exposure of cells to IFN-γ induces the synthesis of three proteolytic proteasome subunits (LMP2 (βli), LMP7 (β5i), and MECL-1(β2i)), which are incorporated into an alternative form of proteasome, called immunoproteasome, displacing the constitutive subunits β1, β2, and β5, respectively (Aki *et al.*, 1994; Kelly *et al.*, 1991; Martinez and Monaco, 1991; Ortiz-Navarrete *et al.*, 1991). Other stimuli like TNF-α and IFN-β as well as stimulation of DCs can influence the expression levels of the three immuno-subunits (Jamaluddin *et al.*, 2001; Kuckelkorn *et al.*, 2002; Loukissa *et al.*, 2000). It had been previously demonstrated that defined T-Cell epitopes are exclusively generated by immunoproteasomes and fail to be generated by the constitutive proteasomes (Cerundolo *et al.*, 1995; Chen *et al.*, 2001; Gileadi *et al.*, 1999; Toes *et al.*, 2001; Van Kaer *et al.*, 1994). Immunoproteasomes should generate more peptides with C-terminal hydrophobic or basic residues, the kinds of peptides that preferentially bind to TAP and MHC class-I molecules (Goldberg *et al.*, 2002).

Internalized antigens into phagosomes, follow normal maturation process, enter the endocytic pathway and are processing for presentation in MHC class-II molecules. This pathway is able to generated suitable peptides for MHC class-I molecules (Shen *et al.*, 2004). During phagosome maturation process (vesicular interactions with endosomes and lysosomes) phagosomes acquired molecules

involved in generation of toxic reactive oxygen species (ROS), lysosomal proteases (e.g., cathepsins S, L, B, D, and AEP) and proton pumps (e.g., V-ATPase) (Delamarre *et al.*, 2005; Lennon-Dumenil *et al.*, 2002). The acidic environment (drop in pH) that is coupled to phagosome maturation, creates an optimal environment for catabolitic protease activity, that contributes to both killing of microbes and proteolytic processing of antigens (Ramachandra *et al.*, 2009). Chatepsin S could play a key and non-redundant role in the vacuolar pathway of antigen cross-presentation, at least for several antigens, and is not substitute by others cathepsins. An explanation could be the differences in their cleavage site or be the predominat protease on this vacuolar compartments (Lennon-Dumenil *et al.*, 2002), but most important the unique ability to cathepsin S to be catalytically active at neutral pH (Chapman *et al.*, 1997; Shi *et al.*, 1992). In contrast all others cathepsins require acid environment for catalytic activity, which may be crucial, because at low pH, class-I molecules may not be able to stably bind peptides (Chapman *et al.*, 1997; Villadangos *et al.*, 1999). Another possibility is that peptides generated in endocytic compartments could be loaded onto class-I molecules in another location in the cell for antigen cross-presentation. It is reported that peptides from phagosomes could traffic to MHC class-I molecules in the ER through a retrograde transport mechanism (Day *et al.*, 1997). The precise site(s) of processing and peptide loading was not well identified; however lysosomes (or late endosomes) are obvious candidates, by their capacity of digestion of internalized antigens were loading machinery accumulates (Trombetta and Mellman, 2005).

However, a fine balance should be achieved between proteolytic function to process antigens and complete destruction, for the antigens to be present by MHC molecules. This are regulated at phagosomal acidification level as well as at protease activity during different stages of APCs activation or maturation (Delamarre *et al.*, 2005; Trombetta *et al.*, 2003; Trombetta and Mellman, 2005).

DCs have adapted their intracellular machinery to focus on antigen presentation rather than microorganism killing (antigen degradation) that is best played by macrophages and neutrophils (Savina *et al.*, 2006). This type of mechanism by DCs can generate long-lived peptide-MHC complexes leading to "antigenic memory" which result in high efficiency in antigen presentation (Trombetta and Mellman, 2005; Villadangos *et al.*, 2005).

4.1.2.3.3. Regulation of phagosomal maturation

Regulated activation of lysosomal acidification appears to be an important element controlling antigen presentation during DC maturation. The control of lysosomal pH depends on many factors and is primarily due to the influx of H^+ into the lysosome (Mellman *et al.*, 1986) mediated by the vacuolar ATPase (V-ATPase) (Nishi and Forgac, 2002; Stevens and Forgac, 1997). After internalization, a gradual remodeling of the phagosomal membrane and contents occurs through a finely coordinated sequence of fusion events with vesicular components of the endocytic and possibly also secretory pathways (Beron *et al.*, 1995; Desjardins *et al.*, 1997; Mayorga *et al.*, 1991; Pitt *et al.*, 1992). This process was described as "phagosome maturation" (*fig.6*) (Vieira *et al.*, 2002). Despite multiple rounds of fusion, the surface area of the phagosome remains approximately constant (Bajno *et al.*, 2000; Hackam *et al.*, 1998; Holevinsky and Nelson, 1998) by virtue of concomitant fission events that contribute to remodeling. Maturing phagosomes ultimately fuse with lysosomes for terminal degradation of the cargo and killing of internalized microorganisms. This process is accompanied by a progressive decrease in phagosomal pH, and could reach lower values such pH 4.5 in lysosomes (Hayashi *et al.*, 1973). Hydrolytic enzymes resident in late endosomes and lysosomes have low pH optimum, which ensures that their activities are confined to a particular stage in the endocytic pathway (Blander and Medzhitov, 2006a). Acidification of the phagosomal lumen is generated by the vacuolar

ATPase (V-ATPase) (composed by V1 domain that bind and hydrolyze ATP and V0 domain that serves as the pore for protons transport) delivered via fusion with membranes of the endocytic pathway (Forgac, 1998; Nishi and Forgac, 2002).

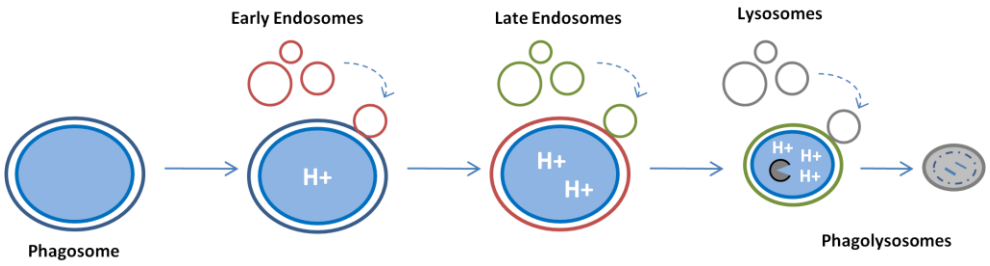


Fig.6: The sequential incorporation of early endosomes, late endosomes and lysosomes to phagosomes drives phagosome maturation. These vesicles (identities indicated by different colors) are recruited to the phagosomal surfaces and then fuse with phagosomes, providing the phagosome with a variety of protein and lipid materials composition. Phagosome luminal pH starts to decrease after the completion of engulfment and reaches the lowest level when a phagosome evolves into a phagolysosome. A phagolysosome gradually decreases in size and eventually disappears. Adapted from (Zhou and Yu, 2008).

In addition, as found for the endocytic and secretory systems, there is mounting evidence suggesting that acidification is not only a consequence but also a determinant of phagosomal maturation (Gordon *et al.*, 1980) and phagosome-endosome fusion (Gordon *et al.*, 1980; Hart and Young, 1991). The control and regulation of phagosome maturation is not yet fully understood. Phagosome acidification seems to be tailored to the functions of the particular cell type. The outcome of phagosome maturation in macrophage is the killing of pathogens and complete degradation and clearance of phagosomal cargo. Instead, in DCs, it serves to prevent complete degradation of cargo antigens such MHC molecules can present the right epitope to T-Cells. DCs seems to actively maintain a more alkaline pH within their phagosomes (Savina *et al.*, 2006). A progressive decrease in phagosomal pH occurs over time in macrophages whereas no significant acidification seems to occur in DCs (Janssen *et al.*, 2006). The steps in phagosomal

maturation seems to be conserved among different cell types, however phagosomes are not created equal and there is a significant degree of phagosome heterogeneity and individuality that is dictated primarily by the cargo contained in phagosomes as well as by external signals and the activation/or differentiation state of phagocytic cells (Griffiths, 2004) .

Recent proteomic studies, revealed phagosomal complexity and association with hundreds of proteins that differ upon stimulation (Desjardins *et al.*, 1994; Garin *et al.*, 2001; Griffiths and Mayorga, 2007). The rate of phagosome maturation is: *i)* dependent on its contents, illustrated by the ability of some intracellular pathogens to arrest phagosome development to aid their survival (Vergne *et al.*, 2004) and *ii)* mediated by signaling pathways activated during engulfment as suggested by studies inhibiting Toll-like receptor signaling (Blander and Medzhitov, 2004; Shiratsuchi *et al.*, 2004). At the phagosomal level, receptor-ligand interactions during phagocytosis could have an important role.

4.1.2.4. Antigen cross-presentation: State-of-the-art

4.1.2.4.1 Antigen cross-presentation *in vivo* - Differential contribution of different mechanisms

The different mechanisms for antigen cross-presentation have been characterized *in vitro*. Are similar mechanisms operative *in vivo*? Ken Rock´s lab has shown that the same particulate antigens that are cross-presented by APCs *in vitro* are similarly acquired by these cells when injected *in vivo* and stimulate strong CTL immunity (Falo *et al.*, 1995; Kovacsovics-Bankowski *et al.*, 1993). In contrast, injection of soluble antigen fails to prime $CD8^+$ T-Cell response. Therefore *in vivo*, as *in vitro* phagocytosis and phagosomes plays an important role in antigen cross-presentation (Rock and Shen, 2005). Some studies using wild type irradiated mice reconstituted with TAP-deficient bone marrow macrophages and DCs, have shown that TAP-dependent antigen cross-presentation is active *in vivo*

and plays a major role in cross-priming of $CD8^+$ T-Cells to viruses, tumors (Huang *et al.*, 1996; Sigal *et al.*, 1999) and transplant cells (Shen *et al.*, 2004). In contrast, in TAP-deficient bone marrow chimeras, $CD8^+$ T-Cell response was primed to PLGA-OVA, high-dose influenza virus, and also weakly to transplant cells (Shen *et al.*, 2004). Therefore, the cathepsin S-dependent pathway is operative *in vivo*, although its contribution is less than the TAP-dependent pathway. TAP-independent pathway can be up-regulated during DC maturation, either by LPS or CpG, suggesting that under certain conditions it can play a more significant role (Chen and Jondal, 2004; Robson *et al.*, 2003). It potentially plays a major role in situations where the TAP-dependent pathway is inhibited (due to infection with virus encoding immune evasion molecules or with antigens that are unable to access the cytosolic pathway).

4.1.2.4.2 Are all DC sub-types able to cross-present antigens?

Not all DCs are created equal, as some subsets may have a greater capacity to cross-present antigens to $CD8^+$ T-Cells (Lin *et al.*, 2008b; Villadangos and Heath, 2005). Bevan´s group was the first to show differences in antigen cross-presentation ability to cell-associated antigens between the various DC subsets, reporting that all subsets were capable of antigen uptake, but only the $CD8^+$ DC subset are able to cross-present *in vivo* (den Haan *et al.*, 2000). Further studies confirmed the same observation, shown that cell-associated antigen was cross-presented by $CD8^+$ but not $CD8^-$ DC (Iyoda *et al.*, 2002; Naik *et al.*, 2005b; Schulz and Reis e Sousa, 2002). The capacity of the $CD8^+$ DC in uptake of dead cells was in the origin of the unique ability to cross-presenting this form of antigen (den Haan *et al.*, 2000; Iyoda *et al.*, 2002; Kerksiek *et al.*, 2005; Schulz and Reis e Sousa, 2002; Valdez *et al.*, 2002). Moreover, antigens in immunocomplexed form (OVA/Ig complexes), or associated to bacteria, were reportedly cross-presented by both $CD8^+$ and $CD8^-$ DC (den Haan and Bevan, 2002; Iyoda *et al.*, 2002; Schulz

and Reis e Sousa, 2002). Thus, the context of antigen with additional activator ligands, if in an immune complex (via the FcR-γ), or additionally if seen together with Toll-like receptor ligands (associated with bacteria), may endow cDC, at least in spleen, with the ability to cross-present antigens (Maurer *et al.*, 2002). Therefore, both spleen $CD8^-$ cDC subsets, under certain circumstances, are able to cross-present antigen at least as well as $CD8^+$ cDC.

These findings led to the hypothesis that all DCs can cross-present and that the role of each DC subset in cross-presentation of a given antigen is dictated simply by their ability to capture that antigen. However, work done with soluble antigen by Pooley *et al*., shown that both $CD8^+$ and $CD8^-$ DC captured this form of antigen *in vivo*, but only the $CD8^+$ DC cross-presented it efficiently (Pooley *et al.*, 2001). Recent work done by Schnorrer *et al*., support the notion that $CD8^+$ DC possess specialized machinery to deliver different forms of antigen to the cross-presentation pathway and that this machinery is largely absent in the other splenic DC subsets (Schnorrer *et al*., 2006). Indeed, the $CD8^+$ DC capacity for antigen cross-presentation was extended to soluble antigen (Pooley *et al.*, 2001), viral (such as herpes simplex virus, vaccinia and influenza) (Belz *et al.*, 2004a; Smith *et al.*, 2003), bacterial (such as *Listeria monocytogenes*) (Belz *et al.*, 2005) and apoptotic cells (Iyoda *et al.*, 2002). In contrast, $CD8^-$ DCs generally appear to be required for $CD4^+$ T-Cell immunity to soluble antigens (Pooley *et al.*, 2001), HSV-2 infections (Zhao *et al.*, 2003) and cutaneous *Leishmania* (Filippi *et al.*, 2003). pDCs have been implicated in antigen cross-presentation at least *in vitro* (Hoeffel *et al.*, 2007). However, in most studies, the efficacy of antigen cross-presentation by pDCs is modest compared with cDCs (Salio *et al.*, 2004; Schnurr *et al.*, 2005). However the route of administration (such as intravenous, lung or intragastric delivery) of cross-presented antigen may also determine the DC subset involved. The general conclusion of different *in vivo* studies using different routes of administration is that resident $CD8^+$ DC in spleen and peripheral lymph nodes are

potent at cross-presenting, whereas migratory $CD8^-$ DCs have a small capacity to cross-present (Belz *et al.*, 2004b; Chung *et al.*, 2005; Iwasaki and Kelsall, 2001; O'Connell *et al.*, 2003; Pillarisetty *et al.*, 2004; Stoitzner *et al.*, 2006; Vremec *et al.*, 2000; Waithman *et al.*, 2007). Therefore, in a per cell basis the $CD8^+$ DCs have higher antigen cross-presentation capacity. A summary of $CD8^+$ and $CD8^-$ DCs antigen presentation capacities are represented in the following table.

Table II: Summary of $CD8^+$ and $CD8^-$ DCs antigen presentation and uptake abilities mediated by the source of the antigen.

Antigen	$CD8^+$ DCs			$CD8^-$ DCs		
	Uptake	MHC-I	MHC-II	Uptake	MHC-I	MHC-II
Endogenous	ø	++	++	ø	++	++
Phagocytosed						
Cells-antigen	++	++	+	+/-	-	+/-
Beads-antigen	++	++	+/-	++	-	++
Pinocytosed	++	++	++	++	+/-	++

Adapted from (Villadangos and Schnorrer, 2007)

4.1.2.4.3 Antigen cross-presentation in tolerance and immunity

During these past years has been clear that immune system uses antigen cross-presentation to monitor tissues for the presence of foreign antigens in cells. However it is not clear exactly how these proteins are acquired by the APCs, by released of cellular proteins by secretion or cell death or even by material "sequestration" of living cells by APCs (Rock and Shen, 2005). The cross-presented antigens can be acquired in several different forms including DNA or RNA, peptides, peptide-HSP complexes (Freigang *et al.*, 2003; Suto and Srivastava, 1995; Udono and Srivastava, 1993). However, they play a minor role (Wolkers *et al.*, 2004) where cellular proteins appear to be the major source of cross-presented antigens in vivo (Shen and Rock, 2004). The aggregation or

association of antigen with cellular debris may promote the internalization of the exogenous antigens into the antigen cross-presentation pathways of the APCs (Carbone and Bevan, 1990; Li *et al.*, 2001).

Cross-tolerance requires the constitutive presentation of self-antigens to cause deletion of self-reactive CTLs (Davey *et al.*, 2002; Kurts *et al.*, 1997). Several studies have reported that pancreatic β-cell expression of model self-antigens leads to cross-tolerance by host bone marrow-derived DCs (Kurts *et al.*, 1997; Kurts *et al.*, 1999; Morgan *et al.*, 1999), and later identified as $CD8^+$ DC (Belz *et al.*, 2002a). A model to study antigen cross-presentation of apoptotic cells *in vivo* was developed by Steinman and co-workers (Liu *et al.*, 2002). In this study deletion tolerance of OVA-transgenic $CD8^+$ T-Cell was induced following antigen cross-presentation of apoptotic cells carrying osmotically loaded OVA by the $CD8^+$ DC subset (Liu *et al.*, 2002). Thus, together these data indicate that $CD8^+$ DCs plays an important role in constitutively antigen cross-presentation in the steady state, resulting in deletion of naïve peripheral T-Cells and antigen-specific tolerance. $CD8^-$ DCs were recently implicated in cross-tolerance to intestinal soluble OVA (Chung *et al.*, 2005) and to OVA expressed in keratinocytes (Waithman *et al.*, 2007) as well as $CD4^+$ T-Cell tolerance to gastric (Scheinecker *et al.*, 2002) and pancreatic autoantigens (Hugues *et al.*, 2002).

Adjuvants, most of them from microbial origins, play an essential role in the generation of immunity, and have been described to enhance immune responses. In the absence of these stimuli, antigen can lead to tolerance instead of immunity (Dresser, 1961; Heath *et al.*, 1998; Hunter, 2002). Adjuvants are thought to exert their effects at least in part by stimulating DCs to fully mature and express costimulatory molecules. Therefore, tissue antigens that are internalized by DCs, in absence of these stimuli will be predicted to induce cross-tolerance (Heath *et al.*, 1998; Kurts *et al.*, 1997). However, the release of adjuvants from cells provides a

mechanism by which a dying cell's antigen can stimulate immunity instead of tolerance (Kurts *et al.*, 1998; Shi *et al.*, 2003; Shi *et al.*, 2000).

4.1.2.4.4 Antigen cross-presentation in therapy

Antigen cross-presentation can be the dominant pathway for vaccinia-induced CTL responses. There are a number of infectious diseases for which vaccines are unavailable or only stimulate suboptimal immunity (Gasteiger *et al.*, 2007). The evidence for the role of antigen cross-presentation of certain cellular antigens *in vivo* (such as minor histocompatibility antigens, protein-coated cells, intracellular bacteria, intracellular protozoa, certain virus like HSV, influenza and vaccinia) appears convincing (Heath *et al.*, 2004). Moreover, there are non-infectious indications, such as cancer, that could be potentially treated with vaccines (Rock and Shen, 2005; Thomas *et al.*, 2004; Valmori *et al.*, 2007). Antigen cross-presentation of dying target cells may be important not only in the pathogenesis of a CTL-mediated autoimmune disease like type 1 diabetes (Liadis *et al.*, 2005) but also in epitope spreading in this disease (Krishnamurthy *et al.*, 2006; Yamanouchi *et al.*, 2003). Most vaccines consist of non-living components of pathogens (killed or subunit vaccines). However, they normally fail to elicit $CD8^+$ T-Cell immunity, which is extremely important in most of viral infections and cancer, because antigens in these preparations do not get presented on the MHC class-I molecules of APCs. The major problem could be the way that antigen is delivered. Particulate form of the antigen are taken up efficiently by APCs, and presented on both MHC class-I and class -II molecules (Falo *et al.*, 1995; Raychaudhuri and Rock, 1998; Rock and Clark, 1996). Immature DCs avidly take up particulate antigens, which are precisely the cells that can most efficiently stimulate immune responses. Many studies have been done with particulate preparations of biocompatible and biodegradable materials such polylactide-*co*-glycolide (Desai *et al.*, 1997; Fu *et al.*, 2000; Kaiser-Schulz *et al.*, 2007; Newman *et al.*, 2002; Newman *et al.*, 1998;

Raychaudhuri and Rock, 1998; Waeckerle-Men *et al.*, 2006; Waeckerle-Men *et al.*, 2004; Walter *et al.*, 1999; Wang *et al.*, 1999). One advance is the possibility to target particulate antigens in ways to elicit $CD4^+$, $CD8^+$ T-Cell immunity or both responses to better mount a specific immune response to a specific disease. This could be done *in vivo*, by directing this particles to DCs or introduce antigens into the antigen cross-presentation pathways of DCs *ex vivo* and then inject these APCs back in vivo as a cellular vaccine (Celluzzi and Falo, 1998; Moron *et al.*, 2004; Nestle *et al.*, 2005).

4.1.2.4.5 TLRs in DCs subsets and antigen cross-presentation

The separation of DCs into multiple subsets based on phenotype normally correlates with a difference in function. These functional differences could lead to understand and manipulate the immune response to pathogens, tumors and self (autoimmune diseases). The outcome of immune responses depends on the state of DC differentiation or maturation. During the steady state, DCs reside in an immature form, and can promote immune tolerance (Davis *et al.*, 1999). Exposure to stimuli such as pathogens activates or matures DCs and initiates immunity. Therefore, the type of immunity depends the upon the particular maturation stimulus that the DC encounters (Dhodapkar *et al.*, 2008; Granucci *et al.*, 2003).

TLRs, the ancient and highly conserved family of receptors, have been implicated in the immune responses to pathogens and many of these could lead to many autoimmune pathology. Activation of TLRs by agonist, leads to DC to enter into a maturation process, undergoing a number of phenotypical and functional changes (Janeway and Medzhitov, 2002). The maturation process, in general, involves a redistribution of MHC molecules from intracellular endocytic compartments to the DC surface, down-regulation of antigen internalization, an increase in expression of co-stimulatory molecules (CD80, CD86, CD40) and MHC class-II molecules at cell surface. It is normally characterized by

morphological changes (e.g. dendrites formation), cytoskeleton re-organization, surface expression of adhesion molecules and chemokine receptors and secretion of chemokines, proteases and cytokines (Akira, 2006). A wide variety of cytokines may be expressed (not necessarily simultaneously) by mature DCs: IL-12, IL-1α, IL1-β, IL-15, IL-18, IFN-α, IFN-β, IL-4, IL-10, IL-6, IL-17, TNF-α and MIF (Morelli *et al.*, 2001). The qualitative and quantitative composition of the cytokine pattern induced in response TLR stimulation depends on the receptor that are triggered, the ligand that are recognized, and the cell type that are activated and their maturation stage (Reis e Sousa, 2004a, 2004b).

Several populations of DC have been described (Shortman and Liu, 2002; Villadangos and Heath, 2005) with different capacities of antigen cross-presentation (den Haan *et al.*, 2000; Heath *et al.*, 2004; Iyoda *et al.*, 2002; Pooley *et al.*, 2001; Schnorrer *et al.*, 2006; Schulz and Reis e Sousa, 2002) and different expression of TLR family members (Boonstra et al., 2003; Iwasaki and Medzhitov, 2004). However, how this differential expression is related with different capacities of DCs substes to cross-present antigens is not well understood. TLR expression on different sDCs and *in vitro* generated DCs are summarized in the following table.

Table III: TLR expression by mouse splenic DCs subsets and *in vitro* generated DCs.

Mouse DC	**TLR1**	**TLR2**	**TLR3**	**TLR4**	**TLR5**	**TLR6**	**TLR7**	**TLR8**	**TLR9**
Spleen $CD4^+$	++	++	+	+	++	+++	++	++	++
Spleen $CD8^+$	++	++	+++	+	**low**	++	-	++	++
Spleen $CD4^-CD8^-$	++	++	++	+	+	++	+	++	++
Spleen pDC	++	++	**low**	+	+	++	+++	++	+++
in vitro DCs (IL-4 + GM-CSF)				++					++

Adapted from (Boonstra et al., 2003; Iwasaki and Medzhitov, 2004)

4.1.2.4.5.1 How do TLRs influence antigen cross-presentation?

TLR signaling has been shown to have influence in the efficiency of antigen processing and presentation (Apetoh *et al.*, 2007; Blander and Medzhitov, 2006b; West *et al.*, 2004; Yarovinsky *et al.*, 2006; Zaru *et al.*, 2007). TLRs have been implicated in antigen cross-presentation efficiency of different "forms" of antigen (Bevaart *et al.*, 2004; Chen *et al.*, 2005; Datta and Raz, 2005; Datta *et al.*, 2003; Heit *et al.*, 2003; Lin *et al.*, 2008a; Schulz *et al.*, 2005; Weck *et al.*, 2007).

TLR ligands were shown to acutely stimulate antigen macropinocytosis, leading to enhanced presentation by MHC class-II and MHC class-I (West *et al.*, 2004). Soluble antigens are poor substrates for antigen cross-presentation and their uptake and traffic differs from particulate antigens (Shen *et al.*, 1997). It has been shown that cross-priming in mice and even in primates is more efficient if an adjuvant like CpG oligonucleotide (ODNs) or other immunostimulatory DNA sequences were chemically linked to antigen in a TLR9-dependent manner (Cho *et al.*, 2000; Heit *et al.*, 2005; Schirmbeck *et al.*, 2003; Wille-Reece *et al.*, 2005). More recently, Christian Kurts and colleagues demonstrated that efficient antigen cross-presentation of soluble antigen required TLR-MyD88 signaling and appeared biased towards antigens containing microbial molecular patterns (Burgdorf *et al.*, 2008). TLR3 stimulation resulted in cross-priming in two vaccine models: vaccination with either virally infected cells or isolated Ovalbumin proteins (Schroder and Bowie, 2005; Schulz *et al.*, 2005). Reis e Sousa and colleagues showed that cross-priming of cell-associated antigens is more potent when the cells were either virus infected or loaded with poly I:C (Schulz *et al.*, 2005).

Studies from Laurence Zitvogel's laboratory demonstrated that activation of tumor antigen-specific T-Cell immunity requires secretion of the high-mobility-group box 1 (HMGB1) protein by necrotic tumor cells and the action of HMGB1 on TLR4 expressed by DCs. DCs required signaling through TLR4 and MyD88 for efficient processing and cross-presentation of antigen from dying tumor cells

(Apetoh *et al.*, 2007). In humans, there is evidence that addition of CpG ODNs to a melanoma vaccine resulted in a CTL response (Speiser *et al.*, 2005).

Using biodegradable microspheres, Marcus Groettrup and colleagues found that the co-encapsulation of TLR ligands and antigen onto this microspheres, compared with co-injection of antigen with TLR ligands, enhance cytotoxic T-Cell activation in vaccinated mice (Schlosser *et al.*, 2008).

The "communication" between TLR signaling pathways and the phagosome/ lysosome pathway leads to the control of phagosome maturation and MHC class-II presentation (Blander and Medzhitov, 2006b). Whether cross-presentation of phagocytosed antigens is also dependent on TLR signaling, as shown for MHC class-II presentation, is not clear. In the absence of TLR signaling, antigen cross-presentation may occur constitutively to ensure that $CD8^+$ T-Cells, with auto-reactivities to endogenous cellular proteins, encounter autoantigens in the absence of inflammation and become tolerant. Alternatively, concerning the physiological role of antigen cross-presentation in antiviral immunity (Heath and Carbone, 2001a; Rock and Shen, 2005), only infected apoptotic cells may successfully be cross-presented, where viral nucleic acids within these cells could trigger TLRs. Conflicting studies exist showing both outcomes (Chen *et al.*, 2004a; Datta and Raz, 2005; Datta *et al.*, 2003; Delamarre *et al.*, 2003; Hamilton-Williams *et al.*, 2005; Heit *et al.*, 2003; Palliser *et al.*, 2004; Salio and Cerundolo, 2005; Schulz *et al.*, 2005; Tabeta *et al.*, 2006; Wagner *et al.*, 2004; Wilson *et al.*, 2006; Winau *et al.*, 2006). The general consensus is that constitutive antigen cross-presentation does occur at steady state (Heath *et al.*, 2004). A direct comparison, however, in the presence or absence of TLR signals has not been enlightened.

Therefore, we propose to identify whether TLRs also control antigen cross-presentation of phagocytosed antigens and the intrinsic mechanism(s).

2

Objectives and Integrated Research Plans:

Synthetic Pathogen Platform &
shRNA genetic tools

1. Integrated research plans

Particles carrying antigen have been extensively applied to study antigen cross-presentation (Kovacsovics-Bankowski *et al.*, 1993; Kovacsovics-Bankowski and Rock, 1994; Reis e Sousa and Germain, 1995) and for the design of improved vaccines (Jain *et al.*, 2005; Langer *et al.*, 1997; Marx *et al.*, 1993; Singh and O'Hagan, 1999). In a few studies, these particle carriers have also been modified with ligands to improve uptake by target cells (Keegan *et al.*, 2003; Kempf *et al.*, 2003) or to modulate APCs function. Thus, we proposed to study the internalization, traffic and processing of 'synthetic pathogens'- model particles with distinct, well-defined physical and biochemical properties - by using a novel approach. It may allow us to understand how the biophysical nature of particulate antigens influences their uptake and fate in APCs. Signals from pathogen structure/composition itself, which modulate phagocytosis, can be compared and subsequent immunity in the context of a single well-defined particle platform. Previous results from our lab using particle antigen co-delivered with a **phagocytic ligand** (circulating opsonins: complement and IgG) led to higher levels of $CD8^+$ T-Cell responses comparable to those induced by Ovalbumin coated particles (*unpublished data*). These results might be explained by enhanced phagocytic levels or/and re-routing the particles to specific compartments for efficient antigen cross-presentation. In order to understand how the signals from pathogens integrate and influence antigen presentation outcome, and how parasites can subvert the endocytic traffic/antigen presentation pathways for evasion and disease establishment, particle antigen coupled with a **modulation signal** (TLR agonists) were used. Making use of the RNAi technology to generate loss of function phenotypes, we proposed to determine the signaling events that regulate this process and to probe the role of both known and newly identified genes on antigen cross-presentation of model particle antigens, complementing with biochemical

approach to characterize each step in detail. This work could have an important role on elucidating how pathogen structure and chemistry dictates signaling, intracellular traffic, antigen processing, immune responses and pathogen survival or elimination.

2. Synthetic Pathogen Platform & shRNA genetic tools

2.1 Why to use particle antigens?

DCs are able to cross-present exogenous proteins in soluble form only when they are incubated with very high concentration (Kovacsovics-Bankowski *et al.*, 1993; Norbury *et al.*, 1995; Rock *et al.*, 1990). This finding suggested that antigens are taken up by fluid-phase endocytosis and access the antigen cross-presentation pathway inefficiently, which may explain why immunization with soluble protein antigens generally fails to stimulate CTL immunity. Ken Rock and co-workers found that when a soluble protein was "made" particulate (adsorption to particles of 1-5µm) antigen cross-presentation occurs at 10^3-10^4 fold lower concentrations of antigen (Harding and Song, 1994; Kovacsovics-Bankowski *et al.*, 1993; Kovacsovics-Bankowski and Rock, 1994). As, immature DCs are highly phagocytic, they avidly ingest particulate antigens. The strong CTL response was also observed *in vivo* when Ovalbumin coated particles are inject into animals (Falo *et al.*, 1995; Harding and Song, 1994; Kovacsovics-Bankowski *et al.*, 1993) and when another particulate antigens, bacterial antigens, were used (Pfeifer *et al.*, 1993). This phenomenon generally explains why cellular antigens are cross-present *in vivo* efficiently, because cell-associated antigens are essentially particulate in nature (Carbone and Bevan, 1990; Li *et al.*, 2001). Thus, why is particulate antigen cross-presented much more efficiently than soluble antigens? It seems that the amount of antigen internalized by phagocytosis is much greater comparing when

soluble proteins are internalized by fluid-phase pinocytosis. However, when the amount of antigen internalized is kept constant, phagocystosis is more efficient than endocytosis in antigen cross-presentation (Reis e Sousa and Germain, 1995). Therefore, it seems that the pathway by which particulate antigens are internalized gives access to a type of compartment with an "easy access" to the antigen cross-presentation pathway.

2.2 Applications in vaccine design

The ultimate goal of the vaccine field is to develop effective immunity after a single vaccine injection. Therefore, strategies for preventive and therapeutic vaccines have focused on the ability to deliver antigen to DCs in a target and prolonged manner. DCs have important properties for vaccination as they controlling adaptive immune response by internalization and processing antigen through MHC class-I and class-II pathways, presenting antigenic peptides to $CD8^+$ and $CD4^+$ T Lymphocytes respectively (Banchereau and Palucka, 2005; Banchereau and Steinman, 1998; Nestle *et al.*, 2001). This highlights the need to develop technologies that effect the robust and simple targeting of DCs, using biomaterial vectors. The most promising biomaterials for drug vehicles are biodegradable polymer microparticles and nanoparticles (Lutolf and Hubbell, 2005; Peppas and Langer, 1994). These polymer particles are suitable for conjugation or loading with antigens and adjutants, protecting the antigen from complete degradation *in vivo* during particular steps of the cell-internalization pathways. From these particles, antigen could be released intracellularly, in a manner that can activate both antigen presentation pathways and consequently $CD4^+$ and $CD8^+$ T-Cell immunity. Moreover, the surface of these biomaterial vehicles can be conjugated with DC-specific antibodies or ligands (danger signals) to increase targeting specificity or activation, enhancing the adjuvant effects on

DCs (Reddy *et al.*, 2006). These strategies demonstrate how biomaterials can be designed to respond specifically to the intracellular environment for efficient antigen release and specific processing and presentation and elicit an ideal immune response to a specific antigen.

2.3 What is the effect of TLR agonists on these particles?

Activation of the immune system by a vaccine requires: *(i)* the delivery of a sufficient amount of antigen to antigen presenting cells (Macrophages and DCs*)*, *(ii)* the controlled presentation of antigen molecules to target immune cells ($CD4^+$ and/or $CD8^+$ T-Cells), *(iii)* the proliferation of effector cells such as cytotoxic T lymphocytes and plasma B cells, and *(iv)* the maintenance of an activated immune system for the desired period of time. Adjuvants could play an important role on those requirements (Reddy *et al.*, 2006).

Successful immunization results in activation of adaptive immunity, which could be performed for example by TLR agonists, at least by up-regulation the expression of MHC molecules and co-stimulatory molecules (Iwasaki and Medzhitov, 2004). TLRs have been implicated in particle antigen uptake and antigen presentation. A recent work showed that TLR ligands differentially affect uptake and presentation of cellular antigens (Weck *et al.*, 2007). Blander has shown that TLRs are also implicated in antigen uptake of particulate antigens and MHC class-II presentation (Blander, 2007; Blander and Medzhitov, 2004).

PLGA microspheres have been used as a vaccine platform (Acharya *et al.*, 2009; Cleland *et al.*, 1994; Singh *et al.*, 2006; Sun *et al.*, 2003). It has been shown that surface coating or micro-encapsulation of an adjuvant and an antigen yields better antibody titers in vaccine animals (Hunter *et al.*, 2001; Kazzaz *et al.*, 2006; Singh *et al.*, 2004) or better T helper cell proliferation and cytokine secretion *in vitro* (Westwood *et al.*, 2006). More recently, was reported that strong CTL

response to Ovalbumin could be elicited with PLGA microspheres containing both Ovalbumin and CpG oligonucleotide (ODNs) than when antigen and adjuvant are loaded into separately microspheres (Heit *et al.*, 2007; Schlosser *et al.*, 2008) or co-injected (Storni *et al.*, 2004). The same phenotype was observed for Poly I:C but less stronger comparing to CpG oligonucleotide (Schlosser *et al.*, 2008). Another study has shown that immunization of mice with PLGA microspheres containing recombinant prion protein and CpG oligonucleotide was able to induce antibodies, T helper- and CTL responses to the prion protein, showing that the paradigm of co-encapsulation is not valid only for vaccination with one model antigen (Kaiser-Schulz *et al.*, 2007).

As such, antigen cross-presentation is an important mechanism leading to effective vaccine response against intracellular pathogens that required CTL-mediated immunity (Heath and Carbone, 2001; Touret *et al.*, 2005; Yewdell *et al.*, 1999). All these observations have important consequences for the design of microparticulate vaccines, specifically if they can be extended to cross-priming CTLs. Some studies described above, implicated TLR agonists in antigen cross-presentation but the question whether an adjuvant and antigen must be co-localized within one and the same particle in order to optimal elicit cross-priming is not completely solved, being the principal aim of my PhD work.

Part I: Synthetic Pathogen Platform design and characterization

TLR agonists have been implicated as important molecules to elicit and induce efficient immune response and have been focus of innumerous studies in the vaccine field for anti-tumor treatment and infectious diseases (Erard and Ryffel, 2008; Ishii and Akira, 2007; Pulendran, 2007; Wolska *et al.*, 2009). To better study the interaction of particle antigens with DCs (host-pathogen interaction) we focused on TLRs to design our synthetic pathogen platform. Understanding whether the capacity to cross-present is dictated by antigen capture or handling is important for the design of vaccination strategies based on antigen targeting *in vivo* (Bonifaz *et al.*, 2002; Corbett *et al.*, 2005; Wille-Reece *et al.*, 2005a; Wille-Reece *et al.*, 2005b). To address de question of whether an adjuvant and antigen must be co-localized in the same particle in order to optimally elicit cross-priming, or to evaluate the role of a single ligand in a particulate antigen, or even to study de dynamic of antigen and ligand exposure, we have proposed to create 3 types of model particles that would deliver antigen and cell-modulating ligands (TLR agonists) to DCs in a different way:

(1) **Fixed-ligand particles (Polystyrene)**, which have covalently immobilized antigen and ligand on their surface;
(2) **Mobile-ligand particles (PLGA)**, where antigen and ligands are tethered to a lipid bilayer coated on the surface of the poly (lactic-*co*-glycolic acid) particle;
(3) **Sequestered dynamic-ligand particles (Hydrogel)**, which have antigen and ligands 'masked' by a thin polymer shell which is designed to dissolve at phagolysosomal pH (lower pH), allowing staged delivery of signals to DCs.

1. Polystyrene particles (fixed-ligand particles)

Commercially available monodisperse carboxylated polystyrene microspheres from Polysciences were used as the basis of our fixed-'Synthetic Pathogens'. Particles with sizes range from 50 nm to 6.0 µm were applied, in order to cover a relevant range to intact microbes. Ovalbumin protein was used as a model antigen to be delivered by these particles. In order to provide a 'universal' platform, TLR agonists were co-attached to the particle antigen. These particles allow the creation of a simple model, where the influence of a specific TLR agonist in antigen cross-presentation can be addressed.

1.1 Two-step coupling of Protein and TLR ligands to Carboxylated Microspheres

Carbodiimide chemistry was the basis for these model particles syntheses. EDC/NHS coupling chemistry was used to attach antigen and TLR agonist to the surface of carboxylated polystyrene particles. The principle of this chemistry is based on: *N*-hydroxysulfosuccinimide Sulfo-NHS (Pierce - Sulfo-NHS is water-soluble, but not membrane-permeable. NHS is membrane-permeable but not water-soluble) was used to modify a carboxyl group to an amine-reactive ester. This is accomplished by mixing Sulfo-NHS with a carboxyl-containing molecule and a dehydrating reagent such as EDC (Pierce). The addition of EDC will cause a dehydration reaction between the carboxyl and the NHS hydroxyl group, giving rise to a NHS-ester-activated molecule. The NHS-ester-containing molecule can then react spontaneously with a primary amine-containing molecule (*fig.7*). While the carboxyl-containing molecule can be made to react directly with the amine-containing molecule by the addition of EDC, the reaction is much more efficient with Sulfo-NHS present because a stable intermediate is created (Grabarek and Gergely, 1990; Staros *et al.*, 1986).

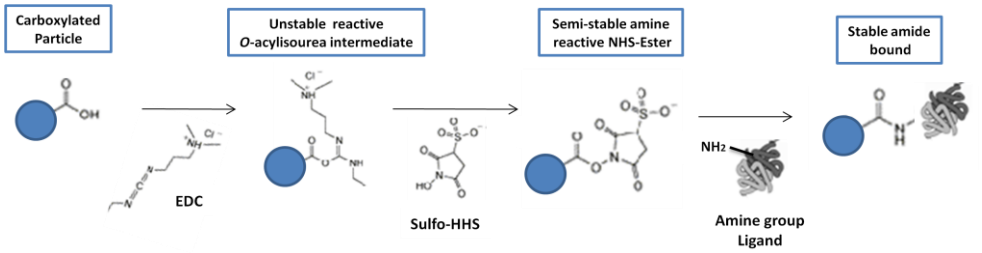


Fig.7: Activation and cross-linking mechanism of NHS/EDC. EDC reacts with a carboxyl group on carboxylated particle, forming an amine-reactive *O*-acylisourea intermediate. This intermediate may react with an amine on amine group ligand yielding a conjugate of the two molecules joined by a stable amide bond. However, the intermediate is also susceptible to hydrolysis, making it unstable and short-lived in aqueous solution. The addition of Sulfo-NHS stabilizes the amine-reactive intermediate by converting it to an amine-reactive Sulfo-NHS ester, thus increasing the efficiency of EDC-mediated coupling reactions. The amine-reactive Sulfo-NHS ester intermediate has sufficient stability to permit two-step cross-linking procedures, which allows the carboxyl groups on amine group ligand to remain unaltered. *Adapted from* http://www.piercenet.com

The reaction should be quenched by adding a solution with BSA and sodium azide to remove any un-reacted NHS present in solution. This method of quenching causes hydrolysis to occur with any un-reacted NHS present, very quickly. This is recommended if the proteins are not susceptible to high pH extremes. Carboxylate microspheres will be covalently linked to a mixture of the protein and ligand-modified, via carbodiimide coupling of the core particle's carboxy groups and exposed amines of ligands. As model ligands LPS (TLR4 agonist) and unmethylated CpG (TLR9 agonist) were used as extracellular and intracellular agonists respectively.

The LPS has phosphatidylethanolamine functional groups, so the most efficient and direct route is to couple LPS to carboxylic acid functionalized microspheres by carbodiimide crosslinker as used for Ovalbumin stimulation. In any event, using these available amines is by far the easiest way of achieving covalent conjugation. A ninhydrin test for amines could be used, so that the moles of primary amine per milligram LPS could be determine. This will be helpful in situations where we may want more control over the amount of LPS that are loaded onto the

particles. Through knowing the moles of amine, we are able to titrate in a competing amine, such as glycine or ethanolamine so that we saturate the particles' functional groups while controlling LPS content. In some cases, a non-chemical approach that would leave LPS structurally intact was used. This reaction would be to simply mix a solution of LPS with Ovalbumin-coated microspheres. Ovalbumin has a very high affinity for fatty acids and binds LPS very efficiently. We will use this approach in other different type of approaches when conformational alteration of protein is not appropriate.

The CpG ODN **(**synthetic cytosine-guanosine oligonucleotide) ligand can be coupled to the surface of carboxylate particles by making use of 3´ amino modified unmethylated CpG with the objective to perform EDC/NHS coupled chemistry in the way to use the free amine group to couple directly to free carboxylated at particles surface. The oligo was made by customization by sigma-genosys as follow: CpG 1826 (mouse specific) 5′- TCC AT*G ACG TT*C CT*G ACG TT*-3′ (-NH_2 with a C12 linker at 5´ and Phosporothioate ligations between bases – protected from DNases digestion).

For detailed Protocol see *Chapter 3*

1.2 Coupling reaction efficiency

In order to check the coupling reaction efficiency, the relative quantity of antigen, as well of the model agonists (LPS and CpG) on particles surface, fluorescent microscopy for fluorescent forms of antigen (OVA-Alexa594) and agonists (LPS-FITC and NH_2-CpG-FAM) were used (*fig.8*).

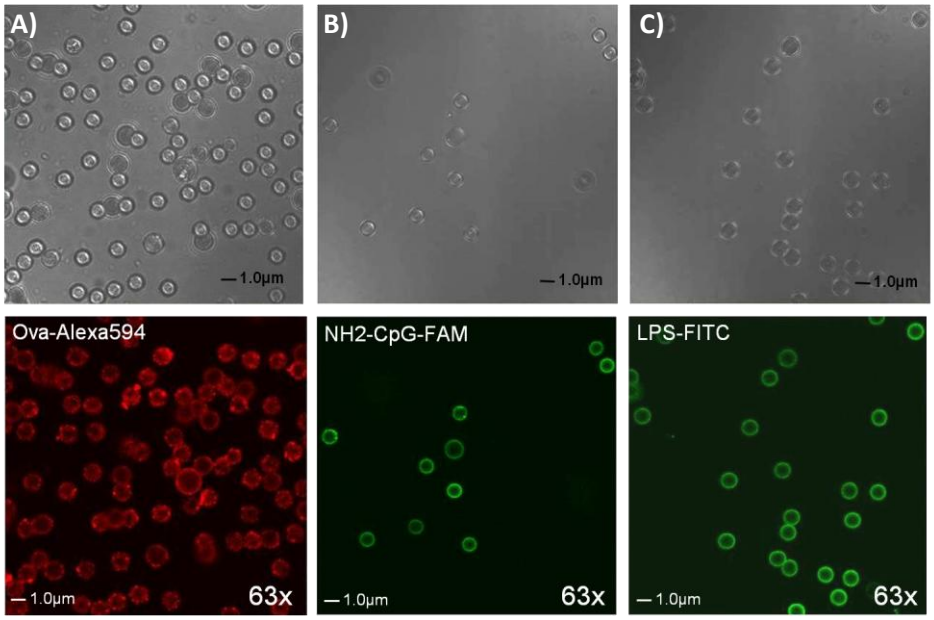


Fig.8: Model particles antigen loading efficiency by CLSM images. Images of 1.0 µm polystyrene particles loaded with (A) 200µg of OVA-Alexa594 (red) or (B) 20µg of NH_2-CpG-FAM (green) or (C) 20µg of LPS-FITC (green). Bright-field images are shown in upper panels and fluorescence images in lower panels. Objective of magnification 63x was used (Scale bar 1.0µm).

These images show that either the protein or the model agonists form a well-defined layer at the surface of the polystyrene particles, which confirms the efficiency of the coupling reaction. However, a question arises: Does the presence of agonists on coupling reaction affect the binding efficiency of the model antigen between the different type of particles used (OVAp, OVA=LPSp and OVA=CpGp)? To address this, antibodies were used against Ovalbumin to measure the amount of antigen at particle surface by FACS, as follows:

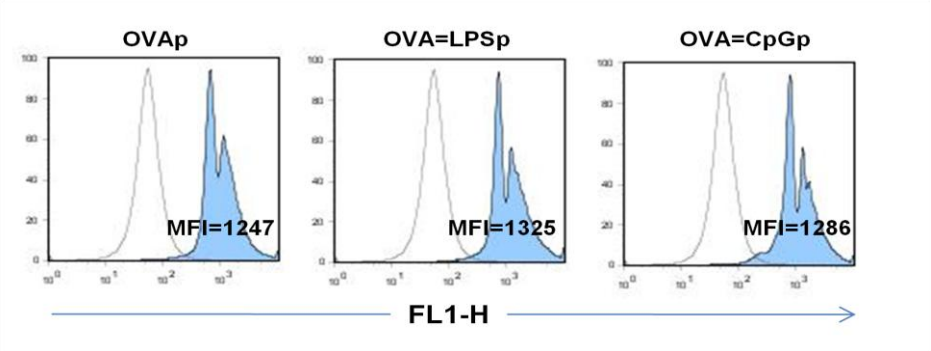


Fig.9: Ovalbumin-loaded particles efficiency by FACS based assay. Ovalbumin measurement at 1.0 µm polystyrene particles surface (OVAp, OVA=LPSp and OVA=CpGp) using Rabbit polyclonal antibody for Ovalbumin (Abcam) and 2nd anti-Rabbit conjugated to Alexa488 (Abcam). The white filled plot represents correspondent particles loaded only with 2nd antibody and the blue filled plot represents Ovalbumin staining. Quantitative coupling of Ovalbumin was examined by FACS by analyzing the Mean of fluorescence intensity (MFI) in the FL1-H channel. Numbers represent the MFI in the FLH-1 channel of stained particles. The graph is representative of at least three independent experiments.

These plots show that the loading efficiency (values of MFI) was similar in model particles either in the presence or absence of agonists (LPS and CpG) during the coupling reaction.

1.3 Particles Quantification

To better study particulate antigen presentation, the number of particles should be measured. Particles were titrated by absorbance at 600nm. An example of particle quantification is shown in *figure 10* using 1.0µm particles as a model. Using a calibration curve, the estimate number of particles in each condition could be measured indirectly by replacing the y value with the absolute value of absorbance at 600 nm (*fig.10*). The remaining particle sizes (50 nm to 6µm) were measured in a concentration range where absorbance is linear.

Table IV: Calibration curve: Values of absorbance at 600nm for the different particles concentration.

Particles/100µl	$9{,}1x10^{7}$	$4{,}55x10^{7}$	$2{,}28x10^{7}$	$1{,}52x10^{7}$	$1{,}14x10^{7}$	$9{,}10x10^{6}$	0
Abs 600nm	0,340	0,210	0,157	0,133	0,120	0,111	0,080

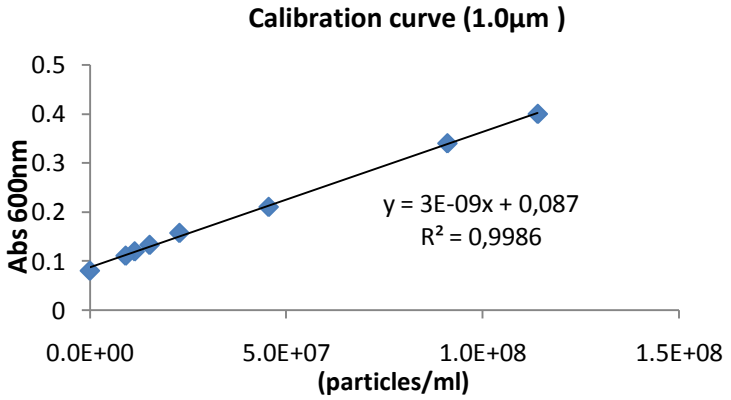


Fig.10: Particles Titration: Calibration curve of model particles with 1.0µm in size: particles concentration *vs* Abs at 600nm.

1.4 Coupling TLR ligands using a sonication protocol

To study the influence of other TLR agonists, we made use of an adapted protocol from Yates *et al.*, to couple different TLR mouse agonist for TLR (TLR1-9 Agonist Kit from InvivoGen). This kit contains: TLR1/2 agonist - **Pam3CSK4,** TLR2 agonist – **HKLM,** TLR3 agonist - **Poly(I:C),** TLR4 agonist - **LPS-EK,** TLR5 agonist - **ST-FLA,** TLR6/2 agonist - **FSL1,** TLR7 agonist - **ssRNA40**, TLR9 agonist - **ODN1826** were adsorbed onto the surface of OVA-coated particles by sonication with 100ng /µl of ligand in PBS for 20 minutes at 40°C. The particles were washed extensively in PBS prior to use (Yates and Russell, 2005). The conjugation efficiency can be assessed by measuring inflammatory cytokine production after challenging DCs. In order to confirm the efficiency of this

method, we made use of available fluorescent ligands (LPS-FITC, NH2-CpG-FAM) and compared with data from EDC/NHS coupling reaction with the same fluorescent ligands (*fig.11*).

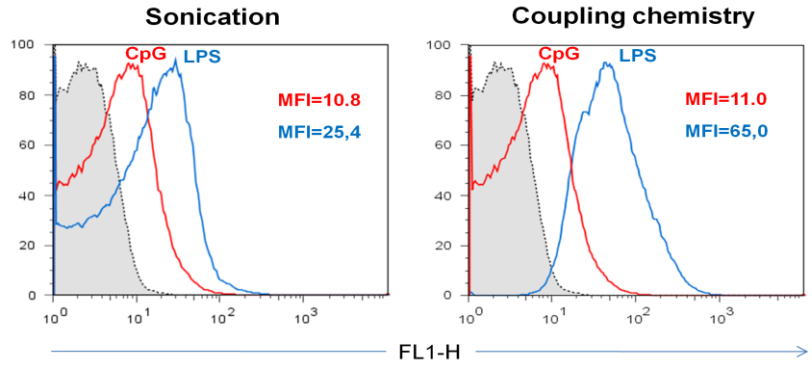


Fig.11: Adsorption of model agonists to Ovalbumin particles by sonication. Fluorescent LPS and CpG were adsorbed onto the surface of the 1.0 µm polystyrene Ovalbumin-coated particles by sonication of the particles in 100ng/ml of ligand in PBS for 20 minutes at 40°C. OVAp alone (grey line) and adsorbed with LPS-FITC (blue line) and NH2-CpG-FAM (red line). Quantitative coupling of LPS and CpG were examined by FACS by analyzing the Mean of Fluorescence Intensity (MFI) in the FL1-H channel. Numbers represent the MFI in the FLH-1 channel. The graph is representative of at least three independent experiments.

The loading efficiency of LPS and CpG by sonication method was similar as for EDC/NHS coupling reaction. This approach can be very useful, and allow the use of another conjugation strategy to study model particles antigen presentation.

1.5 Relative antigen coupling estimation for different particles size

Particles size from 50nm to 6µm was used to cover a broad range of pathogens ranging from virus to bacteria to address the question if size influences antigen cross-presentation, using the same particle ratio per DCs. In order to estimate the amount of antigen loaded into different particles size the following equation was used to predict the maximum protein loaded on particles surface (summarized in table V).

Table V: Expected maximum protein at close packing on microspheres surface for the different particles size. The equation was adapted from Prof. Darrell Irvine.

Size	Group	Quantity	Surface Area	Nº Protein	Protein (x/1.0µm)
0.05µm	-COOH	$3{,}64x10^{14}$ p./ml	$7{,}8x10^{-3}$ µm^2	307	$3{,}0x10^{-3}$
0.10µm	-COOH	$4{,}55x10^{13}$ p./ml	0,031 µm^2	1097	$1{,}1x10^{-2}$
0.20µm	-COOH	$5{,}68x10^{12}$ p./ml	0,126 µm^2	4141	$4{,}2x10^{-2}$
0.50µm	-COOH	$3{,}64x10^{11}$ p./ml	0,785 µm^2	24971	$2{,}53x10^{-1}$
0.75µm	-COOH	$1{,}08x10^{11}$ p./ml	1,766 µm^2	55734	$5{,}65x10^{-1}$
1.0µm	-COOH	$4{,}55x10^{10}$ p./ml	3,14 µm^2	98683	1,0
2.0µm	-COOH	$5{,}68x10^{9}$ p./ml	12,56µm^2	392342	3,97
3.0µm	-COOH	1.68 x 10^{9}p./ml	28,26µm^2	880981	8,93
6.0µm	-COOH	$2{,}10x10^{8}$ p./ml	113,04µm^2	3516776	35,64

Using Alexa Fluor 488 Ovalbumin (OVA488) the loading efficiency at the surface of different particles size can be measured by FACS (*fig.12*).

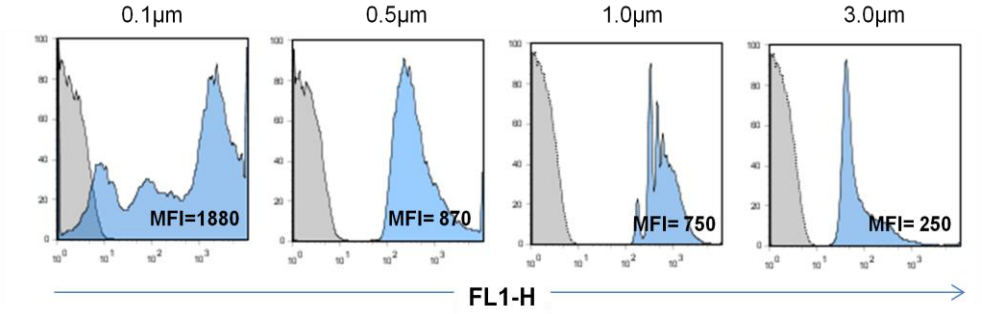


Fig.12: Antigen loading efficiency to particles with different sizes. Fluorescent Ovalbumin (Alexa Fluor 488 Ovalbumin - OVA488) was covalently linked to model particles of 500nm, 1.0µm and 3.0µm in size. Quantitative coupling of fluorescent particles antigen was examined by FACS and the mean of fluorescence intensity (MFI) in the FLH-1 channel were analyzed. Numbers represent the MFI in the FLH-1 channel of fluorescent particles. The graph is representative of at least three independent experiments.

2. PLGA particles (Biodegradable particles)

Biodegradable particles made of the polymer Poly D,L-lactic-*co*-glycolic acid (PLGA), can be used as antigen delivery devices for macrophages and DCs (Gander, *et al*., 2005). PLGA particles of about 0,5-5µm in diameter are actively phagocytosed by human and murine DCs, and can be used to bind to or encapsulate proteins and peptides, in addition to adjuvants such as DNA or RNA (Newman *et al.*, 2002; Newman *et al.*, 1998; Wang *et al.*, 1999). The PLGA particles by themselves do not trigger DC maturation (Waeckerle-Men *et al.*, 2004). PLGA polymer hydrolyzes slowly in aqueous environments, and releases encapsulated peptides and proteins into the processing pathways for presentation on either MHC class-I and class-II pathways (Otten *et al.*, 2003; Partidos *et al.*, 1997; Waeckerle-Men *et al.*, 2006). PLGA has been successful as a biodegradable polymer because it undergoes hydrolysis in the body to produce the original monomers, lactic acid and glycolic acid. These two monomers under normal physiological conditions are by-products of various metabolic pathways in the body. Since the body effectively deals with the two monomers, there is very minimal systemic toxicity associated with using PLGA for drug delivery or biomaterial applications (Waeckerle-Men *et al.*, 2004). As an example, a commercially available drug delivery device using PLGA is Lupron Depot® used in the treatment of advanced prostate cancer. State-of-the-art of PLGA particles: *i)* clinically proven biocompatibility for poly(D,L-lactide-*co*-glycolide); *ii)* promising candidate technique for vaccinations (delivery of PLGA complexes by various routes; including oral, nasal and subcutaneous); *iii)* protected protein antigen and increasing delivery efficiency (Acharya *et al.*, 2009). The illustrated protocol for PLGA micro-particles synthesis with and without a lipid layer will be described as follows. For detailed protocol see chapter 3. This work has been done in collaboration with Prof. Darrell Irvine´s group at MIT.

2.1. PLGA microspheres loaded with protein mixed in the core

Illustrative representation of PLGA microspheres loaded with protein mixed in the core (*fig.13*). For detailed protocol see *Chapter 3*.

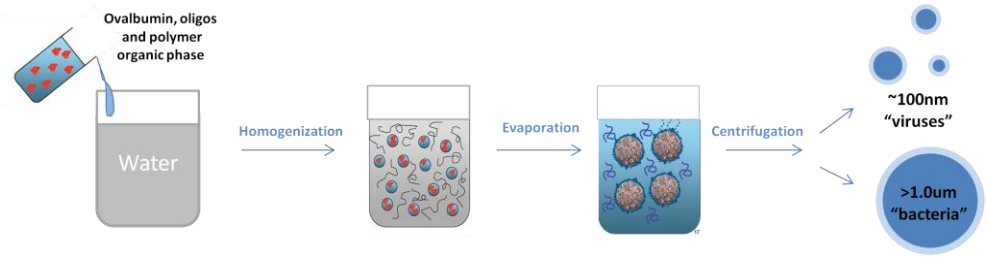


Fig.13: Schematic illustrating the process of PLGA particles synthesis. Particles were formed by homogenization of polymer-containing organic phase into water, followed by evaporation of the organic solvent overnight and centrifugation for 5 minutes at 2.000xg. Scanning electron images of the pellets and supernatants (not shown) indicate that micron scale particles, mimicking bacteria, were separated from 100 nm-scale particles, mimicking viruses. (Adapted from Irvine´s Group – unpublished data).

In order to address the oligonucleotide (oligo) and protein conjugation to PLGA particles, fluorescent equivalents (oligo-Texas red and OVA-Alexa594) were used (*fig.14*).

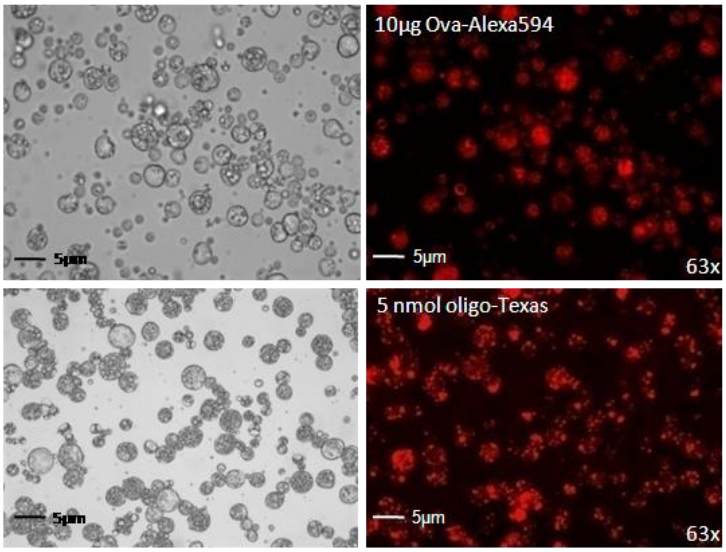


Fig.14: Fluorescence microscopy of PLGA particles. Bright-field images (left panels) and fluorescent (right panels) of PLGA particles loaded with 5nmol oligo-Texas Red and 10µg Ova-Alexa594. Objective of magnification 63x was used (Scale bar 5µm).

This image shows that both antigen and oligos were widely distributed on PLGA particles due to surfactant effect. Confocal microscopy was employed using OVA-FITC loading PLGA particles (*fig.15*), confirming the widely pattern distribution of Ovalbumin on PLGA particles as follows in next figure.

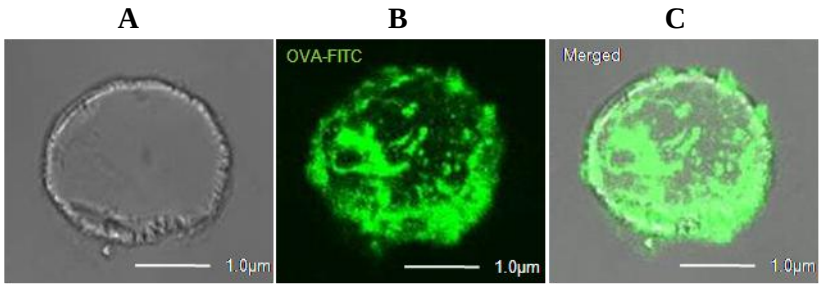


Fig.15: CLSM images of PLGA particles loaded with fluorescent antigen. (A) bright-field images, **(B)** fluorescence images of PLGA particles loaded with OVA-FITC and **(C)** Merged images. Objective of magnification 63x was used with FITC filters (Scale bar 1.0µm).

2.2 PLGA microspheres with lipid layer to mimic pathogens and allow protein and ligand conjugation

Illustrative representation of PLGA microspheres synthesis with lipid layer and loaded with protein and MPLA (*fig.16*). For detailed protocol see *Chapter 3*.

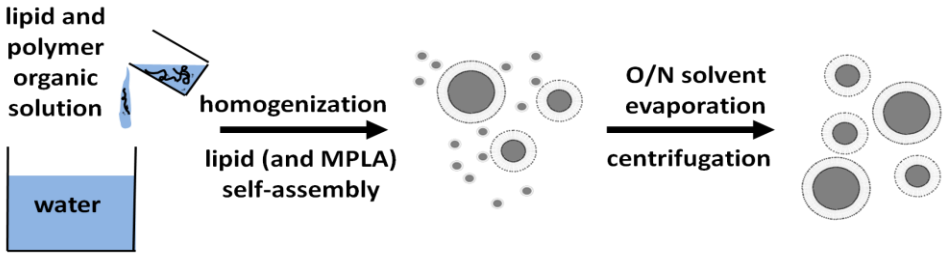


Fig.16: Schematic illustrating the process of PLGA microspheres synthesis with lipid layer. Particles were formed by homogenization of a lipid- and polymer-containing organic phase into water, followed by evaporation of the organic solvent overnight and centrifugation for 5 minutes at 2.000xg. Scanning electron images of the pellets and supernatants (not shown) indicate that micron scale particles, mimicking bacteria, were separated from 100 nm-scale particles, mimicking viruses. (Adapted from Irvine´s Group – unpublished data).

To analyze PLGA particles synthesis and morphology by this method, scanning electron microscopy (SEM) were performed. The antigen conjugation efficiency was addressed by conjugating Alexa Fluor 488 Ovalbumin with maleimide-modified PLGA particles via this method and analyzing by CLSM (*fig.17*).

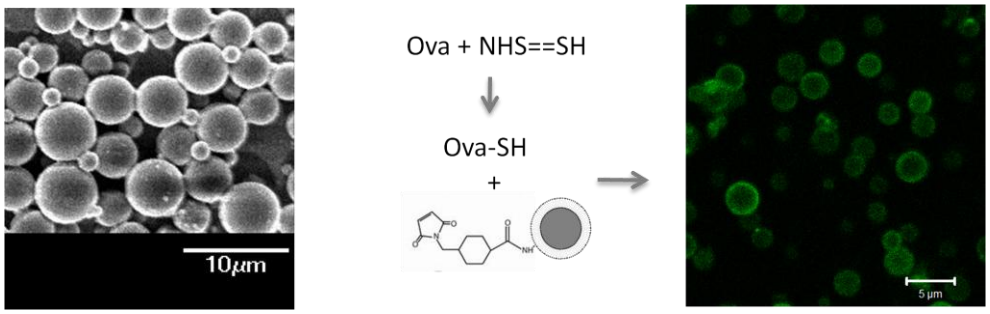


Fig.17: Schematic illustrating the process of particle synthesis and morphology of particles observed by scanning electron microscopy (SEM). Particles were formed by homogenization of a lipid- and polymer-containing organic phase into water, followed by evaporation of the organic solvent overnight and centrifugation for 5 minutes at 2.000xg. Left: Scanning electron images of the particles indicate that micronscale particles, mimicking bacteria, were separated from 100 nm-scale particles, mimicking viruses. Scanning electron micrograph showing 1-5 µm particle diameter (scale bar 10 µm) Right: Confocal micrograph of Alexa Fluor 488 Ovalbumin conjugated to maleimide-modified PLGA particles via this method (scale bar 5 µm). (This image was kindly provided by Anna Bershteyn from Irvine´s lab at MIT).

In order to address the Lipid bilayer formation and Ovalbumin distribution/ conjugation onto PLGA particles, a fluorescent dye DiI (1,1´-dioctadecyl 3,3,3´,3´-tetramethylindo- carboxycyanate perchlorate from invitrogen) Red labeling (which fluorescence was readily detected after binding to phospholipid bilayer membranes), and Alexa Fluor 488 Ovalbumin were used (*fig.18*).

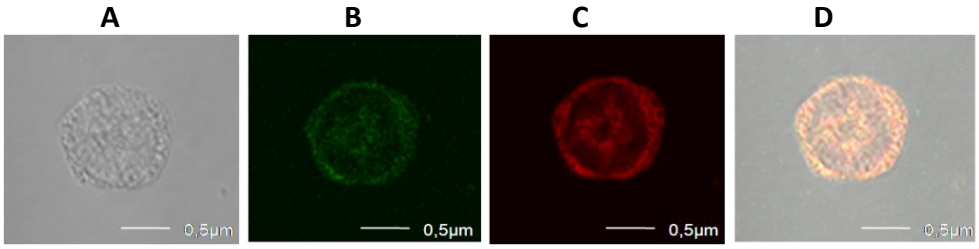


Fig.18: CLSM images of PLGA particles loaded with fluorescent antigen and lipids. **(A)** bright-field images and **(B)** fluorescence images of maleimide-modified PLGA particles conjugated to Alexa Fluor 488 OVA (green) and **(C)** DiI Lipophilic tracer (red) by method previously described. **(D)** Merged image of the three previous ones. Colocalization appears in yellow. Images were obtained with 63x objective amplification with respective filters (Scale bar 0.5µm).

These images showed that both Ovalbumin and lipids are widely distributed on particles surface and in some extend in the core of particles. By this process, the lipid bilayer keeps most of the antigen at the cell surface. To visualize the morphology of the lipid layer at particles surface and address if it mimics a cellular bilayer, Cryo-TEM were used (*fig.19*).

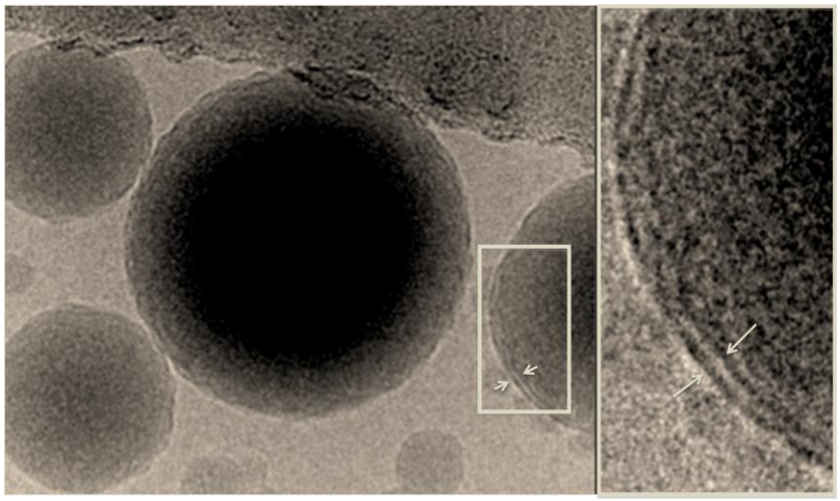


Fig.19: Cryo-TEM micrographs of lipid-coated particles. Particles synthesized with a 1:25 weight ratio of DMPC to PLGA were enveloped by single shells of lipid resembling previous Cryo-TEM studies of lipid-coated silica nanoparticles. PLGA particles made of the same materials but smaller, to allow us to visualize the lipid surface. (This image was kindly provided by Anna Bershteyn from Irvine´s lab at MIT).

Cryo-TEM images show a well defined lipid bilayer formed at PLGA particles surface, which support this method of PLGA particles synthesis as an ideal system to use it to mimic pathogens and to better study host-pathogen interactions.

3. Hydrogel particles (Sequestered dynamic-ligand particles)

During the past few years, many potentially powerful therapeutic strategies for the treatment of diseases require improvement of delivery of drugs into the cytosol or nuclear compartments of cells. Examples include therapeutic protein delivery (Determan *et al.*, 2006; Schweichel *et al.*, 2006), anti-tumor toxin delivery (Borghouts *et al.*, 2005; Devalapally *et al.*, 2007; Son *et al.*, 2003), gene therapy mediated by plasmid DNA (Medina-Kauwe *et al.*, 2005; Putnam, 2006), RNA/DNA that trigger potent anti-viral immune responses (Diebold *et al.*, 2003) and gene silencing via RNA interference (Tagami *et al.*, 2007). Internalized compounds/macromolecules by cells are confined to closed vesicles (endosomes or phagosomes), where the pH is progressively lowered by fusion with lysosomes. The pH could reach lower values such pH 4.5 and where the degradation machinery of the cell are activated (Akinc and Langer, 2002; Asokan and Cho, 2002). This process could lead to rapid destruction of therapeutic molecules with little or no release to cytosol. PLGA particles have been used as polymer-based delivery systems to encapsulate DNA and provide sustained release as the polymer degrades. However the major issue is to avoid the DNA degradation by low pH and enzymes from lysosomes before it can be successfully releases to the cytosol (Fu *et al.*, 2000; Walter *et al.*, 1999). In addition, delivery system using PLGA possess a very poor ability to escape from endolysosomes (Cui and Mumper, 2002). Therefore, endosomal escape is a major and critical point of current intracellular delivery systems and synthetic carrier materials that respond to changes in pH could be useful for drug or antigen release from these compartments before

degradation. The idea of using this type of particles platform that allows the endosomal particles escape contents could have different applications as follows:

3.1. Genesis of Hydrogel pH-responsive particles: Mechanism of exogenous antigens delivery into MHC class-I pathway

Antigen presentation to cytotoxic T-Cells is greatly induced (up to 1000-fold) by delivery antigens to the cytosolic of DCs, to allow the intracellular machinery to load the right peptide onto MHC class-I molecules (Zarei *et al.*, 2003). The biomaterial vehicle must release the antigen intracellularly in a manner that will enable processing by MHC class-I, class-II or both pathways. To deliver exogenous antigens to MHC class-I pathway, they have to bypass the rapidly traffic of phagosomes to lysosomes where antigens are then degraded enzimatically, preventing the antigen to be processed and presented intracellularly (Banchereau, *et al*. 2000). To avoid lysosomal trafficking, Murphy and colleagues have designed smart polymers that use acid-degradable acetal bonds to disrupt endosomes in a pH-dependent manner (Murthy *et al.*, 2003). From an extracellular pH of 7.4, particles that were internalized will eventually fuse with lysosomes and achieve a pH ~5-5.5 (Underhill *et al.*, 1999). These particles allow antigen and adjuvants to release into cytosol as the endosomes are acidified before lysosomal fusion, which enhance the processing by the MHC class-I pathway instead of MHC class-II (Murthy *et al.*, 2003).

Our approach is based on de-constructible hydrogen-bonded multilayers, a concept originally demonstrated by Sukhishvili and colleagues (Sukhishvili *et al.*, 2000, 2002). Hydrogen-bonded multilayers of neutral and polyacid chains assembled on the surface of colloidal particles have been reported (Kozlovskaya *et al.*, 2005). Here we extended this concept to the assembly of neutral and polybase chains assembled on our functionalized particles, to create an acid-responsive

protective shell (illustrated in *Fig.21*). Polymers bearing hydrophilic groups such as –OH, CONH, -COOH, -SO_3H and NH_2 can be crosslinked to form hydrogels. The swelling properties of ionic hydrogels are unique due to the ionization of their pendant functional groups, and the equilibrium degree of swelling can be changed suddenly by several orders of magnitude near the pKa or pKb of the hydrogels. Taking advantage of the swelling ability of cationic gels, they can be applied for endosomal disruption at low pH (Khare and Peppas, 1993). It has been shown that cationic hydrogels made from diethyl aminoethyl methacrylate (DEAEMA) and poly (ethylene glycol) monomethacrylate (PEGDMA) have a pKb ~7 which is the ideal pKb to respond to endosomal pH (Podual *et al.*, 2000). At pH below 7 a fraction of the tertiary amine groups of core on the poly (DEAEMA-co-PEGDMA) were protonated and thus positively charged, while the net surface (shell) charge is negative due to primary amines that remain charged at all moderate pH, allowing electrostatically-driven adsorption (*fig.21*). Thus, siRNA, oligos or antigen could be electrostatically bound to the surface of the particles. At pH higher than 7.0, poly (DEAEMA-co-PEGDMA) are largely uncharged and capable of strong hydrogen bonding. In near-neutral extracellular conditions, the polymer multilayer coating will remain hydrogen-bonded and prevent access of the 'masked' ligands to cells. On internalization, the drop in pH within the phagocytic pathway will induce ionization of the poly (DEAEMA-co-PEGDMA) chains (due to the pK_a of the tertiary amino groups in the polymer, which is near neutral pH (Schwarte *et al.* 1998), leading to loss of hydrogen bonding, dissolution of the coating, and exposure of the masked ligand (*fig.20*). Antigen and/or selected ligands could be adsorbed to poly (DEAEMA-co-PEGDMA) core-shell nanoparticles. Our research was thus to investigate the use of synthetic pH-sensitive hydrogel nanoparticles as a novel intracellular antigen delivery system to cytosol, bypassing the requirement of retro-translocation machinery in antigen cross-presentation.

Because the surface membrane or envelope of some pathogens is not compromised until fusion of the phagosome with lysosomes, internal components of these pathogens may not be exposed to antigen processing machinery until late stages of the phagolysosomal processing pathway. With this approach, TLR agonists and antigen could be selectively exposure to endosome environment as the pH drops during phagosome maturation. This hypothesis is consistent with the localization of certain TLRs that recognize internal components of pathogens to phagolysosomal compartments (Latz *et al.*, 2004; Oshiumi *et al.*, 2003), rather than at surface of phagocytes. To determine whether sequential encounter of antigen or activating signals impacts the response of phagocytes to pathogens, hydrogel particles with 'masked' antigen or TLR agonist layers could be synthesized, which can be selectively exposed based on the pH of the particle microenvironment as illustrated in the next figure.

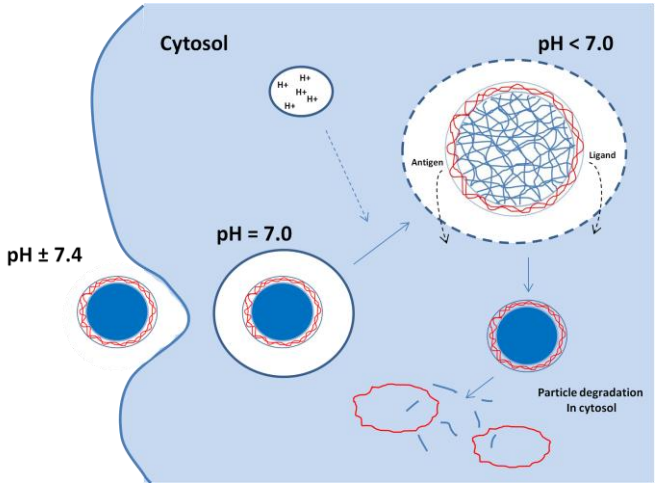


Fig.20: Hydrogel pH-responsive particles cellular mechanism. Particles are internalized along with an agonist and a model antigen into endosome. During internalization, endosomes matures and become acidic (pH<7.0) to break down internalized molecules. The particles begin to be protonated at this pH, absorbing protons that are pumped into the endosome. As protons are absorbed, anions are also pumped into the endosome to maintain charge neutrality.This causes an osmotic pressure buildup that will drive water into the endosome, eventually disrupting or rupturing the membrane and causing release of the ligand, antigen and particle into the cytosol. Adapted from (Hu *et al.*, 2007).

3.2. New application system: RNAi delivery on same context as antigen

Up regulation of MHC class-II and CD86 were adopted as a surrogate marker of DC maturation with the assumption that this always correlates with immunogenicity (Finkelman *et al.*, 1996). DCs were later found to also induce tolerance, and it was suggested that tolerance and immunity were mediated by immature and mature DCs, respectively (Finkelman *et al.*, 1996; Steinman and Nussenzweig, 2002). Most researchers interpreted 'immature tolerogenic DCs' to refer to MHC class $II^{low}CD86^{low}$ DCs. Besides this, many studies have used this terminology while relying on naive T-Cell proliferation as a correlate of immunogenicity, which is not correct because T-Cell proliferation can lead to tolerance as well as shown by 'phenotypically mature' DCs were found to induce tolerance (Albert *et al.*, 2001) or at least not to induce immunity (Sporri and Reis e Sousa, 2005). Therefore, an interesting approach could be explored for the efficient delivery of RNAi for regulatory molecules of antigen presentation in same context as a particular antigen in order to amplify or suppress adaptive immune response for vaccines or immunotherapy (Greenland *et al.*, 2007). An ideal delivery system: *(1)* be able to bind RNAi in a reversible manner as to ensure the subsequent release of the RNAi; *(2)* escape from endosomal compartment; and *(3)* be biocompatible. Therefore, pH-sensitive core-shell nanoparticles have recently been proved to be a good delivery system for RNAi (Blackburn *et al.*, 2009; Hu *et al.*, 2009). The promise of RNAi will only be a clinical reality when safe and efficient delivery systems become well established.

3.3 Synthesis and characterization of pH-sensitive core-shell nanoparticles

Illustrative representation of Hydrogel pH-responsive core-shell nanoparticles synthesis and chemical composition (*fig.21*). For detailed protocol see *Chapter 3.*

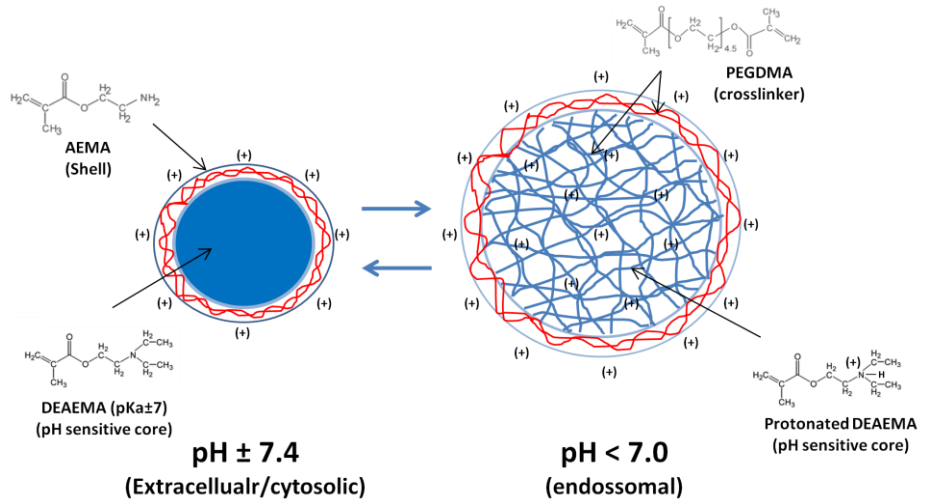


Fig.21: Schematic structure and chemical composition of pH-responsive core-shell nanoparticles. At extracellular/cytosolic pH, tertiary amines of DEAEMA repeat units in the particle cores are largely uncharged, and the particles are collapsed; at endolysosomal pH, the core tertiary amines ionize, and the particles swell. Surfactant-free polymerization of DEAEMA formed the core structure of hydrogel nanoparticles, crosslinked by PEGDMA. AEMA was polymerized in a second stage to form a thin shell structure rich in primary amines. Particles can swell ~2-fold (8- fold volume change) in response to pH drop below ~7. Adapted from (Hu *et al.*, 2007).

In order to show physiologic properties of Hydrogel particles comparing to PLGA particles, calcein, a membrane-impermeant fluorophore, was used as a model drug molecule and tracer to monitor the stability of endo/phagosomes following particle uptake (*fig.22*). This work has been done in collaboration with Prof. Darrell Irvine´s group at MIT.

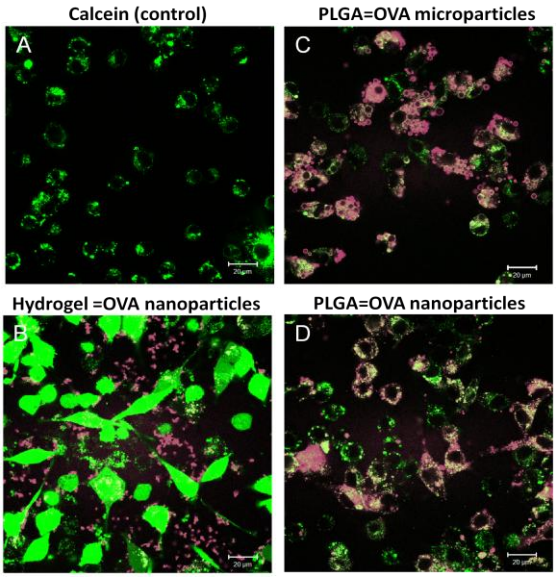


Fig.22: Endosomal escape of the membrane-impermeable dye molecule calcein into the cytosol of DCs in the presence of pH-responsive core-shell nanoparticles comparing to PLGA nanoparticles and microparticles. CLSM images at 40x - Fluorescence overlays (red, nanoparticles; green, calcein). sDCs were co-incubated with 1µM of LysoTracker Red DND-99 (to label endolysosomes), 0.24 mM of calcein, and 1:20 (DCs:particles) ratio. **(A)** Cells were treated with calcein alone. Cells were co-incubated with **(B)** calcein and Hydrogel=OVA nanoparticles **(C)** with calcein and PLGA=OVA microparticles **(D)** with calcein and PLGA=OVA nanoparticles (Scale bar 20µm). (This image was kindly provided by Anna Bershteyn from Irvine´s lab at MIT).

Hydrogel nanoparticles exhibited calcein fluorescence throughout the cytosol and nucleus (*fig. 22-B*). Calcein entry into the cytosol is triggered by the presence of nanoparticles required at the pH-sensitive core (*fig. 22-B*), as calcein remained in an endosomal distribution in cells co-incubated with calcein and PLGA nano and microparticles (*fig.22-C and D*). Therefore, hydrogel particles are able to delivery components into cytosol upon internalization by DCs, but not PLGA nanoparticles. These results implicate hydrogel pH-responsive particles as a good delivery vehicle into cytosol for antigen cross-presentation studies.

Part II: shRNA Library: New tools for the genetic dissection of antigen cross-presentation pathway(s)

1. Technology overview and design

The information resulting from genome-sequence increased the need for tools that allow genome-scale functional studies. In model organisms such *Caenorhabditis elegans* and *Drosophila melanogaster*, the recognition that RNA interference (RNAi) can be used to suppress gene expression (Fire *et al.*, 1998; Kennerdell and Carthew, 1998), has lead to identification of the genes underlying many biological processes through loss-of-functions screens (Bettencourt-Dias *et al.*, 2004; Boutros *et al.*, 2004; Fraser *et al.*, 2000; Kamath *et al.*, 2003; Kiger *et al.*, 2003; Lum *et al.*, 2003). Chemically synthesized RNAi also suppresses gene expression in mammalian cells and become essential tool for biological studies (Elbashir *et al.*, 2001). RNAi screen have been done with commercially available libraries (Aza-Blanc *et al.*, 2003; MacKeigan *et al.*, 2005; Pelkmans *et al.*, 2005). As many mammalian cell types are resistant to transfection methods, an alternative approach has to be used to introduce synthetic siRNA into cells. In 2002 emerged a new "transfection" technology based in transduction mammalian cells with viruses carrying expression cassettes that encode short hairpin RNAs (shRNAs) to generate gene-specific siRNAs in cells. This approach produces stable and highly effective gene suppression in a variety of mammalian cell types (Abbas-Terki *et al.*, 2002; Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Stewart *et al.*, 2003).

2. The RNAi consortium (TRC)

The RNAi Consortium (TRC) is a collaborative group of 11 world-renowned academic and corporate life science research groups whose mission is to create comprehensive tools for functional genomics research. The RNAi Consortium

emerged with the objective of generating genome-scale shRNA libraries in viral vectors to target almost all human and mouse gene, that allows gene silencing in most dividing and non-dividing cell types.

2.1 Lentiviral shRNA library production

The TRC lentiviral shRNA library contains now constructs targeting all human and mouse genome, with ~5 distinct shRNA constructs per gene. The inclusion of five different shRNA constructs targeting each gene increases the opportunity to achieve strong knockdown, often offers a gradation of knockdown and provides the means to rapidly evaluate the gene specificity of a phenotypic hit. Constructs were designed using a siRNA rules based algorithm consisting of sequence, specificity and position scoring for optimal hairpins, that attempt for maximize knock-down and minimize off-target effects, as well as to ensure that most genes in the library contain shRNAs that target both the 3´ unstranlated region (UTR) and coding sequence (CDS) of their transcripts (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). For each shRNA, they designed a 21 base stem for the target transcript and an intervening 6 base loop consisting of a XhoI site. The hairpins were cloned into the pLKO.1 vector, which carries the puromycin-resistance gene and drives shRNA expression from a human U6 promoter (*fig.24*). The pLKO1 lentiviral vector enables efficient transduction of primary and non-dividing cells making it easy to perform RNAi studies in these hard to transfect cell lines (Federico, 2003). Typically, 3-5 shRNA constructs are created for each target gene to provide varying levels of knockdown and to target different regions of mRNA transcript. One in five clones will typically provide at least 70% knockdown of the gene target. Sense and anti-sense hairpin oligonucleotide pairs for 90 hairpin sequence were annealed separately and ligated into pLKO.1 at AgeI and EcoRI restriction sites, and the ligations were transformed into competent bacteria in a 96-well plate.

The 90 transformations in each plate were then pooled and plated onto a large agar plate. A total of 672 colonies were selected robotically for growth, plasmid purification, and sequencing. This process yields 94% of the designed clones, each gene has an average of 4.7 unique shRNA constructs, and 96% of the genes have four or more different constructs (Moffat *et al.*, 2006). The TRC creates a production pipeline to generate a library of sequence verified shRNAs in pLKO.1. In summary, the production of the library comprises several steps: *1)* Hairpin Design; *2)* Vector Preparation; *3)* Oligo Pair Annealing, Ligation and Transformation; *4)* Colony Picking and Sequencing Validation; *5)* Quality Assessment of Library Glycerol Plates; *6)* Tests of Recombination; *7)* High-Throughput DNA Production; *8)* Lentiviral Production; *9)* Lentiviral infections; *10)* Quantitative RT-PCR and *11)* Titering Assay (Moffat *et al.*, 2006).

2.2 High-Throughput lentiviral particle production

A high-throughput (HT) method to generate high-titer lentiviruses was generated by TRC shRNA library consortium. Although preparation of transfection-quality plasmid DNA and subsequent packaging of the viral plasmids into viruses is quite straightforward for individual samples, performing this process efficiently in a high-throughput 96-well format is more challenging; furthermore, high-throughput screening demands high and uniform viral titers. TRC consortium has developed protocols for DNA and viral production for the TRC library. These protocols are frequently updated with improvements and the latest versions can be found online (http://www.broad.mit.edu/genome_bio/trc/protocols.html).

A semi-automated procedure was optimized in 96-well plates in which HEK293T cells were transfected with library and packaging plasmids (pCMV-dR8.91) and envelope plasmid (pMD2.G). pCMV-dR8.91 and the envelope plasmid pMD2.G are available from Addgene. Transfection-quality DNA was

prepped using 96-well PureLink kits (Invitrogen) with average yields of 4 µg DNA/well, quantified using a PicoGreen assay (Molecular Probes), and normalized robotically in each plate. Lentiviruses were made in 96-well format by transfecting packaging cells (HEK 293T) with a three-plasmid lentivirus packaging system (Naldini *et al.*, 1996; Zufferey *et al.*, 1997). 300 µl of transfected cell supernatants containing VSV-G pseudotyped lentiviruses are collected over 36–60 hrs and aliquoted and stored these lentivirus containing supernatants at 80ºC. With current HT methods of viral production in 96-well plates, viral titters with average of $3x10^7$ infectious units (I.U)/ml in A549 cells infected were obtained. Typically, 50% of wells fall within a twofold range of viral titer. Thus, the procedure above yields sufficient volumes of lentiviral supernatants (300µl) from a single 96-well plate, is sufficient to provide virus for several hundreds shRNA infectious, depending as well on the transducibility of the target cells (Root *et al.*, 2006). One consequence of using lentiviral vectors with high titer and broad tropism is the requirement for adequate biosafety procedures for manipulation. The three plasmid–based lentivirus productions of self-inactivating viruses nearly eliminates the possibility of recombination to create replication-competent viruses, and the rate of recombination is several orders of magnitude lower than that of comparable amphotropic retroviral systems. Many institutions apply biosafety level 2 practices to work with third-generation lentiviruses. TRC consortium used biosafety level 2^+ precautions for all lentiviral shRNA library work with appropriate containment and decontamination procedures. An example of institutional guidelines for lentiviral work is available online (http://www.ohsu.edu/research/rda/ibc/protocols.shtml) (Root *et al.*, 2006).

2.3 Subsets compilation

During the first year of my PhD I have been in Boston at Nir Hacohens' lab (Harvard/MGH/Broad institute), one of the main collaborative groups that compose the TRC consortium, where the UBCSI lab was originated. The objective was to learn about the state-of-art shRNA library its compilation and how to transfer specific subsets of the shRNA library to perform loss-of-function screens, in order to perform the rapid identification of the genes underlying many biological processes such as antigen presentation and inflammation that are the two main areas of interest for the UBCSI lab. With this powerful tool we are able to generate and study loss-of-function phenotypes of genes that compose specific protein functional families. As a summary, we organized different subsets of shRNA for mice and human genes, in order to produce bacteria, DNA and lentivirus (*fig.23*):

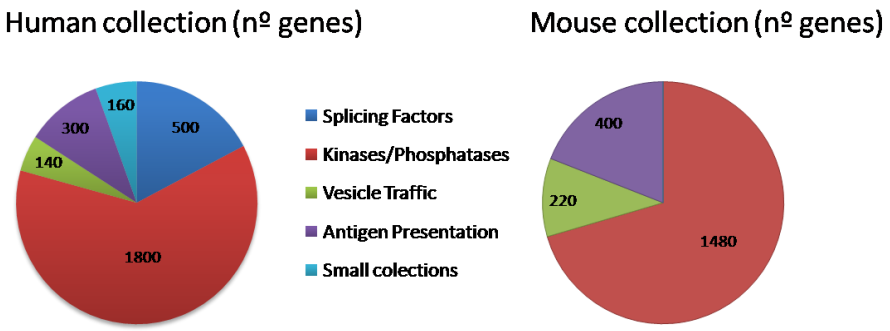


Fig.23: Relative representation of different shRNA bacterial glycerol stock library collections for Human and Mouse genome. Numbers represent the sets of shRNA target genes in each collection: Splicing factors, Kinases/Phosphatases, vesicle traffic, antigen presentation and others small collections.

2.4 Library production and use

The next figure shows a schematic represenation of shRNA lentiviral library production, lentiviral infection and phenotype assay (*fig.24*).

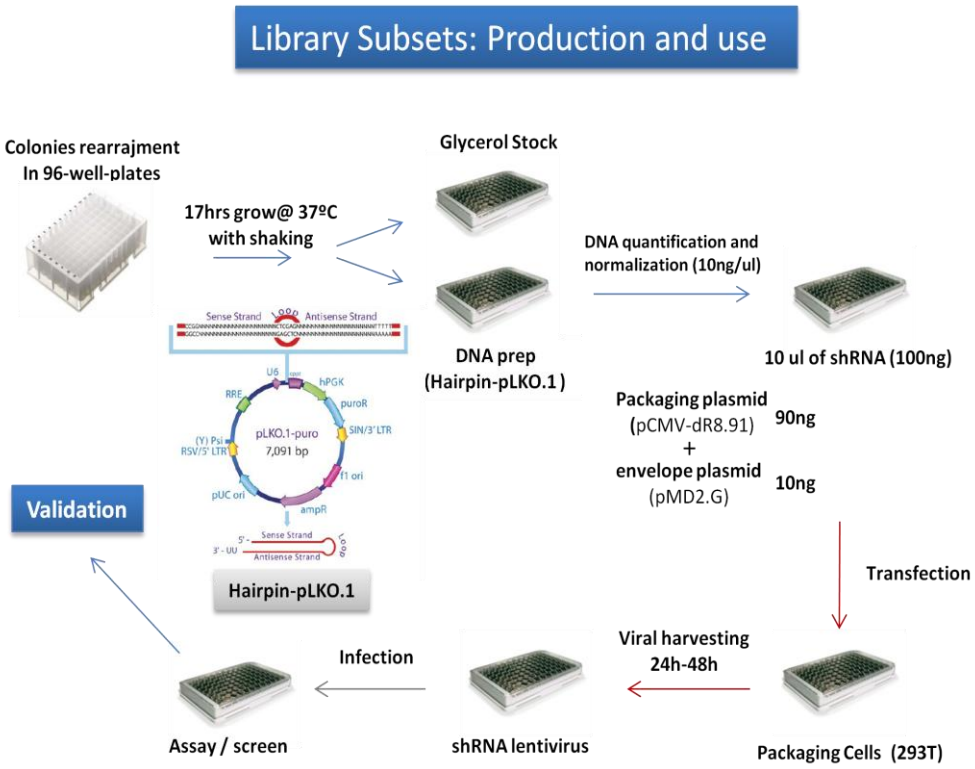


Fig.24: Scheme for library production and use. **Bacterial glycerol production**, **pLKO.1shRNA constructs DNA prep, viral production and transduction method**. **Vector map for the pLKO.1 lentiviral vector**: The self-inactivating lentiviral vector backbone contains elements for efficient viral packaging and shRNA expression. Expression of the shRNA is driven by the human U6 promoter (hU6). The lentiviral vector also contains the mammalian selection marker puromycin resistance gene (*PAC*) and the bacterial ampicillin resistance gene (AmpR). **Part I: Inoculation, growth, and duplication of glycerol stocks in 96-well plates:** To create the different shRNA library families, different colonies were re-organized from the master TRC library collection into new 96-well plates. Colonies were inoculated one by one, and grown for 17hrs at 37ºC in Terrific Broth supplemented with 100ng/µl of Carbenicillin with constant shaking at 300 rpm in an appropriated shaker for 96-well plates. After, glycerol stocks were prepared using 40µl autoclaved 50% glycerol and 80µl of culture from deep well growth plate into each destination plate to make replicate copies. These plates were freeze immediately and store at -80ºC. **Part II: Preparation of Transfection-Quality Plasmid DNA in 96-well Plates**: The rest of bacterial culture was used to prepare of transfection-quality plasmid DNA using TRC library protocols for glycerol and plasmid preparation. See detailed protocol: https://www.broadinstitute.org/genome_bio/trc/protocols/trcGlycerolStockPlasmidPrep.pdf. **Part III: lentiviral Production:** Packaging Cells (HEK 293T) were transfected with the 3 lentivirus plasmids (hairpin-pLKO.1 vector, packaging plasmid and envelope plasmid). At 18 hours post-transfection: medium were removed and replaced with fresh high-serum media (30% FBS). At 24 hours viruses were harvest by replacing the medium with C10. At 48hr afterwards, viruses were harvested again and packaging cells were discarded according to TRC library protocols for lentiviral production. See

detailed protocol: http://www.broadinstitute.org/genome_bio/trc/protocols/trcLentiVirusProd.pdf.**Part IV: Lentiviral infection**: High titer of lentiviruses was used **(**10µl) to transduce target cells by spinoculation (2200rpms at 37ºC during 90 min). Polybrene were used as 8µg/ml as final concentration. After 2 days at 37ºC, cells were selected with an optimal concentration of puromycin (concentration should be optimized for each cell line; typical concentrations range from 2-5 µg/ml). Puromycin selection requires at least 48hrs. Incubated periods are highly dependent on the post-infection assay. **Part V**: **Phenotypic assay**: could be performed 3+ days after puromycin incubation. **Validation** by mRNA Knockdown (qPCR) or protein Knockdown (Western/FACS) could be performed 2+ days or 3+ days respectively.
Adapted from http://www.broadinstitute.org/genome_bio/trc/publicProtocols.html and (Moffat *et al.*, 2006).

2.5. Publications

The ultimate objective with this tool was to create a specific collection of shRNA, which we called as "antigen presentation collection", to generate loss-of-function of specific genes involved in different key steps of antigen presentation pathways. Using this powerful tool, we initially proposed to dissect and clarify the antigen cross-presentation mechanism(s) mediated by our platform of synthetic model particles. In addition to the knowledge of the technology behind shRNA platform, another proposed was to generate important subsets of families of genes crucial for the development of different projects that were occurring in the lab (UBCSI at Instituto de Medicina Molecular) and with collaboration of different groups abroad:

i) One of the collaborations was done with Anjana Rao´s lab in Cambridge at Harvard Medical School, with the aim to identify splicing factors required for the activation-induced switch from $CD45RA^{+}$ isoforms to the short isoform CD45RO (exclusion of exons 4-6 (A-C) of CD45 transcripts). As the transition from naïve to activated T-Cells is marked by alternative splicing of pre-mRNA encoding the transmembrane phosphatase CD45, it is of great importance to understand how this regulation occurs. From this work using the Splicing Factors shRNA library, we identified a single factor, heterogeneous ribonucleoprotein L-like (HNRPLL), which is up-regulated in response to PMA stimulation and whose depletion

eliminated stimulation-dependent CD45RO expression. HNRPLL is necessary and sufficient to induce CD45RO expression in B and T-Cell lines and primary T-Cells. We conclude that HNRPLL is a crucial regulator of CD45 alternative splicing in activated T-Cells. This work was published in ***Science.*** 2008 Aug 1; 321 (5889):686-91).

ii) Another project developed in collaboration with Sebastian Amigorena´s lab in Paris at Curie Institute, proposed to identify vesicle traffic proteins involved on the control of different steps of the Exosome secretion pathway. Exosomes are secreted by several cell types and can be involved in intercellular communication and in the pathogenesis of infectious and degenerative diseases. The molecular mechanisms of their biogenesis and secretion are, however, poorly understood. Using a shRNA interference screen for vesicle traffic proteins, we identified 5 small GTPases of the Rab family involved in exosome secretion in HeLa cells, were the two Rab27, Rab27a and Rab27b, play a major role but different and complementary in the exosomal pathway. By showing that major inhibition of exosome secretion is associated with alterations of late endocytic compartments, we demonstrate that exosomes originate mainly from MVEs. This work was published in ***Nature Cell Biol.*** 2010 Jan;12(1):19-30.

iii) Caetano Reis e Sousa´s lab in London at Cancer Research Institute was interested in fungal response and the mechanism behind that. Caetanos´s lab has shown that with Dectin-1, a PRR for fungi, a novel innate signaling pathway involving Syk kinase and the adaptor CARD9 is defined, which is critical for inducing Th17 responses to fungal infection. We demonstrated that another C-type lectin, Dectin-2, also signals via Syk indirectly trough association with FcRγ, and CARD9, and contributes to Dendritic Cell activation by fungal particles**.** We concluded from this work that Dectin-2 constitutes a major fungal PRR that can couple to the Syk–CARD9 innate signaling pathway to activate DCs and regulate

adaptive immune responses to fungal infection. This work was published in ***Journal of Experimental Medicine*** 2009 Aug 206: 2037-2051.

iv) In collaboration with Prof. João Gonçalves´ lab in Lisbon at Faculdade de Farmácia, we proposed to identify novel HIV-1 replication of druggable targets identified by a shRNA-based screen enriched in kinases/Phosphatases in T lymphocytes. HIV-1 is a complex retrovirus that uses host machinery to promote its replication. Understanding cellular proteins involved in the multistep process of HIV-1 infection may result in the discovery of more adapted and effective therapeutic targets. Kinases and phosphatases are a druggable class of proteins critically involved in regulation of signal pathways of eukaryotic cells. Here, we have focused in the discovery of kinases and phosphatases that are essential for HIV-1 replication but dispensable for cell viability. We have performed an iterative screen in Jurkat T-Cells with a short-hairpin-RNA (shRNA) library highly enriched for human kinases and phosphatases. We identified new 14 proteins essential for HIV-1 replication that do not affect cell viability. These proteins were described to be involved in MAPK, JNK and ERK pathways, vesicular traffic and DNA repair. Moreover, we have shown that most proteins do not affect viral integration but rather affect viral transcription/translation. This study brings new insights for the complex interplay of HIV-1/host cell and opens new possibilities for antiviral strategies. This work was published in ***PLoS ONE*** 2010 Feb 17;5(2):e9276.

The results presented in this thesis, in collaboration with Darrell Irvine's lab at MIT, are under preparation for publication:

Freitas RP, Bershteyn A, Moita C, Irvine DJ, Moita LF. *The particle antigen cross-presentation is impaired by a crosstalk effect of TLR4 signaling in a phagosome dependent manner.* **In preparation.**

3

Materials and Methods

Mice

We have used 6- to 12-week-old C57BL/6 Wild Type, MyD88KO, TLR4KO, OT-I and OT-II mice. All mice were bred and maintained under specific pathogen-free conditions at Instituto de Medicina Molecular animal breeding facility according to institutional guidelines.

Cells Isolation/Preparation

- **Bone Marrow-derived Dendritic Cells (BMDCs)**

DCs differ in developmental origin and most DCs are considered to be of myeloid origin; the main evidence for this comes from studies of DC development in cultures stimulated by Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Inaba *et al.*, 1993). The principle method for generating BMDCs with GM-CSF was adapted from previous publications (Inaba *et al.*, 1992a; Inaba *et al.*, 1992b; Inaba *et al.*, 2001; Scheicher *et al.*, 1992). Culture of DCs *in vitro* was carried out in RPMI medium 1640 (GIBCO) supplemented with 10% heat-inactivated and filtered (0.22 µm, Milli-Pore) Fetal Bovine Serum (endotoxin-free, BIOWEST), β-mercaptoethanol (50µM Sigma- GIBCO) L-glutamine 100 units/ml (2mM, GIBCO), penicillin (100 U/ml, GIBCO) streptomycin (100µg/ml GIBCO) and GM-CSF (15-30% J558 supernatant, depending on GM-CSF concentration, tested previously in culture) - Complete Medium (C10). The BMDCs were differentiated in 96 round-well plates (corning) or large Petri dish (non treated dish) depending the number of DCs and the type of experiment. Bone-Marrow derived Dendritic Cells (BMDCs) isolation from mice bone-marrow was done as follow:

Day 0: Femurs and tibiae of female, 6–12 weeks old female C57BL/6 or TLR4KO, were removed and purified from the surrounding muscle tissue. Then

both ends were cut with scissors and the marrow flushed with BMDCs Medium using a Syringe with a 25G needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting and filtered. After spin down cells were resuspended in 2 ml of TAC buffer to lysis Red blood cells (8.32 g NH4Cl; 0.82 g NaHCO3; 0.043 g EDTA in 1L of miliQ water) for 2 minutes. This reaction was stopped with 8 ml of complete medium. The cells were pelleted and counted. 15-30% J558 supernatant (depending on GM-CSF concentration) was added. BMDCs were plated in 96 well plates at $5x10^4$/well in 200µl or alternatively in 150 mm petri dishes (non treated dish) at $10\text{-}12x10^6$/petri dish in 20ml of medium and incubated at 37ºC. For 96 well plates, BMDCs care is performed every two days until BMDCs are ready to harvest on day 6 or 7. Remove old medium - Carefully aspirate in circular fashion ¾ of medium. This sucks up nutrient depleted medium and non-adherent-non DCs. Prepare new medium - add J5 (1:30 dilution) to C10 and replace with ¾ of volume.

Day 3: For the petri dishes cultured BMDCs, take the supernatant with floating cells and transfer it into a 50 ml tube. Add 10 ml of phosphate-buffered saline (BS) to the dish and swirl gently trying to detach some cell clusters (avoiding bubbles). Mix this PBS containing cells with the floating cells supernatant. Add 4ml RT - trypsin to the dish and let it for 2 minutes; stop by adding 4 ml BMDC medium and swirl gently. Transfer this volume and mix with the rest of the cells. After this short trypsin treatment many cells remain attached to the bottom of the dish. Do not try to take them; most of them have a macrophage-like phenotype. Centrifuge the cells and resuspend them in some volume of BMDC medium; count them and plate again into 150 mm dishes ($10\text{-}12 .10^6$ cells in 20ml/Petri dish).

Day 7: Repeat as described for day3.

Day 9-11: Repeat as described for day3. Perform CD11c staining for Fluorescent Activated Cell Sorter **(**FACS) analysis. The level of $CD11c^+$ population should be more than 70% from day 10-11 and increase over time. It is important to verify if $CD11c^+$ cells are immature and how is their capacity of maturation. Treat some cells during at least 20 hours with 10 µg/ml LPS. Perform staining of MHC-II, CD40, CD86 in treated and not treated cells and analyze by FACs. The levels of these 3 markers should be low in non-treated cells and importantly increased in LPS treated DCs.

Once the population is 80-90% $CD11c^+$, cells can be platted at higher concentration (around $15\text{-}20x10^6$ cells in 20ml/Petri dish). They can be used for 7-10 days depending on maturation markers. CD11c staining for FACs analysis was performed (*fig.25*). The level of $CD11c^+$ population should be more than 70% from day 6 and increase over time. To verify if $CD11c^+$ cells are immature and how is their capacity of maturation, BMDCs were stained with anti-CD86, anti-CD40 and anti-MHC-II treated and not treated with LPS stimulation (10ng/ml) for at least 20 hours and analyzed by FACs for the surface expression of maturation markers. The levels of these 3 markers should be low in non-treated cells and increased in LPS treated DCs (*fig.26*).

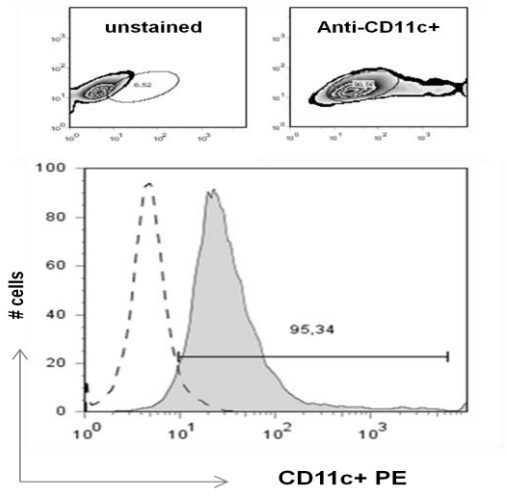


Fig.25: BMDCs staining at day 9 with anti-$CD11c^+$-PE antibody. Upper left panel unstained BMDCs population *vs* upper right panel stained BMDCs with anti-$CD11c^+$-PE antibody (Abcam). Lower graph shows histogram of BMDCs unstained (dot curve) and stained with $CD11c^+$-PE (filled curve). Number represents the percentages of the positive $CD11c^+$ cells, analyzed on PE channel. These data are representative from one experiment repeated at least three times with similar results.

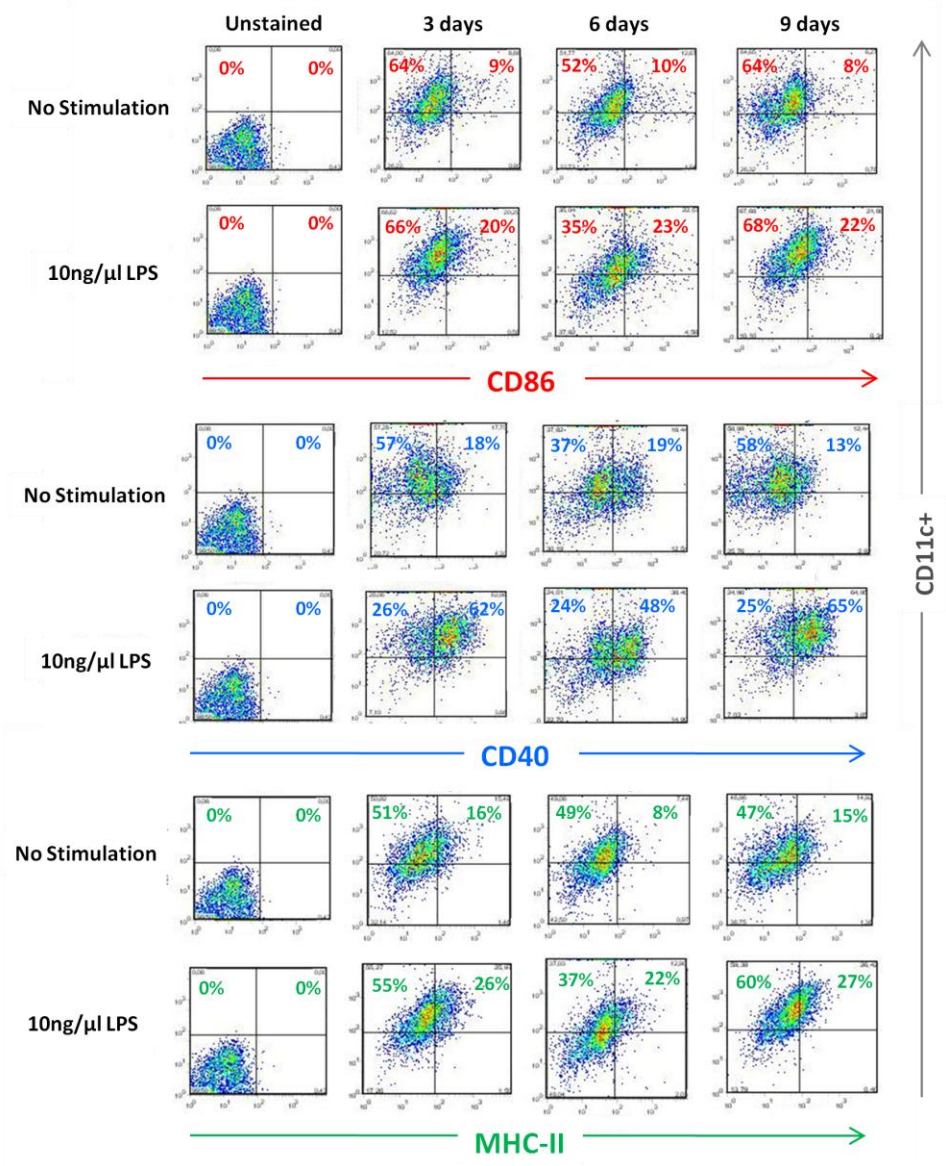


Fig.26: BMDCs maturation with GM-CSF, staining at day 3, 6 and 9 in culture. BMDCS were stained with anti-CD86, anti-CD40, anti-MHC-II and anti-CD11c antibodies (Abcam), with and without LPS stimulation (10ng/ml) for at least 20 hours. BMDCs were analyzed by FACS and plots represent surface expression of CD86, CD40 and MHC class-II *vs* DC marker ($CD11c^+$). Numbers show the percentage of cells on $CD11c^+$ quadrants. Antibodies were used with 1:200 dilution. These data are representative from one experiment repeated at least three times with similar results.

- **Splenic Dendritic Cells (sDC)**

DCs form lymphoid origin were isolated form mouse spleen as previously described (Vremec *et al.*, 2000), making use of Immunomagnetic bead purification kit (CD11c MicroBeads mouse - AutoMACS; Miltenyi Biotec) (Ing *et al.*, 2006; Williamson *et al.*, 2002). The spleen was cut into small fragments and left for 30 minutes for enzymatic disaggregation in medium with Collagenase D (1mg/ml) and *DNaseI* (0,02mg/ml) at 37ºC to generate single cell suspension. After, the fragments were resuspended and filtered through 30µm nylon mesh, to remove cell clumps that may clog the column. To prevent DC maturation during the isolation protocol, the procedure was performed on ice or at 4ºC. Cell number was determined and cells were recovered from the digestion by centrifugation for 200xg for 5 minutes. Cell pellet was resuspended in 400µl of MACS buffer (solution containing PBS pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution 1:20 with autoMACS™ Rinsing Solution per 10^8 total cells (keep buffer cold,4−8 °C). 100µl of CD11c MicroBeads were added per 10^8 of total cells. The cells were mixed with the beads and incubate for 15 minutes in the refrigerator (2-8ºC). Cells were washed by adding 1 ml of buffer per 10^7 cells and centrifuge at 200xg for 5 minutes. Cells were resuspended up to 10^8 cells in 500µl of MACS Buffer. Next magnetic separation was performed: Place MS column in the magnetic field separator and equilibrate with 500µl of MACS Buffer. Apply cell suspension onto the column and collect flow-through containing unlabeled cells. Always wait until the column reservoir is empty before proceeding to next step. Wash column 3x with 500µl of Buffer and collect again the flow-trough. Remove column from the separator and placed in a 15ml falcon tube. Elute the $CD11c^+$ cells by pippeting 500µl of MACs buffer onto the column and immediately flush out the magnetically labeled cells by firmly

pushing the plunger into the column. This protocol excluded $B220^+$ ‘‘plasmacytoid DC’’ from the DC preparation.

- **T-Cells ($CD8^+$ Cytotoxic T-Cells and $CD4^+$ T Helper Cells)**

$CD8^+$ Cytotoxic T-Cells and $CD4^+$ T helper cells were isolated from spleen of OT-I and OT-II mice respectively, using two different T-Cell isolation Kits from MACS Miltenyi Biotec. T-Cell Isolation Kit is an indirect magnetic labeling system for the isolation of untouched T-Cells from single-cell suspensions of lymphoid organs. Consequently, $CD8\alpha^+$ T-Cells were isolated by depletion of non-$CD8\alpha^+$ T-Cells and $CD4^+$ T-Cells were isolated by depletion of non-$CD4^+$ T-Cells (negative selection), as described elsewhere (Gruber and Brocker, 2005). Highly pure untouched $CD8\alpha^+$ T-Cells and $CD4^+$ T-Cells are isolated respectively using this approach. Non-$CD8\alpha^+$ T-Cells, i.e. T helper cells, B cells, NK cells, DCs, macrophages, granulocytes, and erythroid cells, are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies against CD4 (L3T4), CD45R (B220), CD49b (DX5), CD11b (Mac-1), and Ter-119, as well as anti-Biotin MicroBeads. Non-$CD4^+$ T-Cells i.e. cytotoxic T-Cells, B cells, NK cells, DCs, macrophages, granulocytes, and erythroid cells, are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies against CD8a (Ly-2), CD45R (B220), CD49b (DX5), CD11b (Mac-1), and Ter-119, as well as Anti-Biotin MicroBeads. Isolation of T-Cells was achieved by depletion of the magnetically labeled cells. The protocol is the same for $CD8^+$ T- Cell or $CD4^+$ T-Cell isolation, using however different magnetic beads cocktail described previously.

Sample preparation: The spleen was dissociated mechanically, between 2 slides sterilized with ethanol 70%. The resulting tissues were resuspended with a pipette and the cell suspension was filtered. Cells were recovered by centrifugation at 300xg during 5 minutes. 2 ml of TAC were added for 5 minutes at RT to remove

erythrocytes. Complete medium were added to stop the reaction and the pellet was recovered by centrifugation at 300xg during 5 minutes. Pellet was resuspended in 1 ml MACS buffer and cell number was determined.

Magnetic labeling: Cell suspension was centrifuge at 300xg during 5 minutes and pellet was resuspended in 40µl of MACS buffer per 10^7 of total cells. 10 µl of Biotin-Antibody Cocktail **(**Cocktail of biotin-conjugated monoclonal antibodies), were added per 10^7 cells and incubated at 4ºC during 15 minutes (mixture were mixed every 5 minutes). 30µl of MACs Buffer and 20 µl of Anti-Biotin Microbeads (monoclonal antibodies – secondary labeling reagent) were added per 10^7 of total cells. Resuspension was mixed and refrigerated for 15 minutes at 4ºC. Cells were washed by adding 1 ml per 10^7 cells and centrifuged at 300xg for 5 minutes. Pellet was resuspended up to 10^8 cells in 500µl (for higher cell number buffer volume was scale up).

Magnetic Separation: MS columns were placed in the magnetic field of MACS separator. Cell column were equilibrated by adding 500µl of MACS buffer. Cell suspension was applied onto the column (Cells were allowed to pass through and collect effluent as fraction with unlabeled cells, representing the enriched T-Cell fraction). Column was washed 3x with 500µl of MACS Buffer and the effluent was collected in the same tube. The purity of the enriched $CD8\alpha^+$ T-Cells or $CD4^+$ T-Cells were evaluated by FACS with a fluorochrome-conjugated antibody against CD8α (CD8α-FITC) or CD4 (CD4-FITC) respectively.

Synthetic Pathogen Platform

- **Fixed-ligand particles – Polystyrene particles**

  - **Two-step coupling to Carboxylated Microspheres**

Method of preparation:

A two-step coupling reaction of protein and TLR agonists was performed using carboxylated microspheres. 100µl of 1.0 µm microspheres (4.55×10^{10} particles/ml from Polysciences) from stock suspension were pelleted at 13.000 rpm, 5 minutes and washed 2x with 1ml of PBS. Particles were resuspended in 1,5ml of *Activation Buffer* (0.1 M MES 0.5 M NaCl, pH 6.0). Pre-solution of 0,1g/ml of EDC (Pierce) and Sulfo-NHS (Pierce) were prepared in activation buffer. 2mg of EDC (~6,7mM) and Sulfo-NHS (6mM) were added to beads suspension, vortexed, and incubated covered on shaker for 15 minutes at RT. 2µl of 2-Mercaptoethanol (Pierce), at final concentration of ~20mM, were added to quench EDC. Particles were pelleted and washed 1x with activation buffer. Particles were resuspended in 100µl of *Coupling Buffer 2x* (0.2 M MES 1M NaCl, pH 7.0) and divided into 3 batches (50 µl each): 1- Antigen only and 2- Antigen and LPS and 3- Antigen and CpG, as shown in following table.

Table VI: Covalent conjugation of OVA and TLR agonists (LPS and CpG) to polystyrene particles.

	OVAp	**OVA=LPSp**	**OVA=CpGp**
Coupling Buffer 2x	50µl	50µl	50µl
Ovalbumin endograde (10mg/ml)	20µl	20µl	20µl
LPS endo-toxin free (1mg/ml)	-	20µl	-
CpG-NH_2 (100ng/µl)	-	-	20µl
MQ water (endotoxin free)	30µl	10µl	10µl
Total volume	100µl	100µl	100µl

This reaction was mixed by vortex and incubated on a rotator covered for 3 hours at RT. Beads were pelleted and 1ml of *Quenching Buffer* (1%BSA, 50mM Tris, 0,1%NaN_3, pH7,4 in PBS) was added to stop the coupling reaction and incubated on a rotator covered for 1 hour. Beads were pelleted and washed 1x with 1ml of quenching buffer and 3x more with 1ml of PBS 1x (if they form "clusters" beads were washed 1x PBS 0.05%Tween). Resuspended in 100µl of PBS 1x and stored at 4ºC. For particles with a size range inferior to 0.5µm, micro vectaspin columns were used in centrifugation steps (Whatman).

The optimal Ovalbumin and agonists' concentrations were titrated and used in saturation conditions. Different concentration ranges have been used with no significance differences in loading efficiency as measured by FACS (*data not shown*). Quantitative methods could be applied to measure the conjugation efficiency (amount of Ovalbumin and agonists at particle surface), based either on the amount of the compound that remains in supernatant (Jain *et al.*, 2005) or a more specific method based on a FACS assay using calibrated fluorescent particles labeled with the same dye as the compounds. However, the aim of this work was not to quantify the precise amount of antigen or agonists on model particles, but instead a more qualitative approach.

- **Mobile-ligand particles – PLGA particles**

  - **PLGA microspheres loaded with protein mixed in the core**

Method of preparation:

Depending on the ratio of lactide to glycolide used for the polymerization, different forms of PLGA can be obtained. These are usually identified in regard to the monomers' ratio used (PLGA 50:50 - identifies a copolymer whose composition is 50% lactic acid and 50% glycolic acid - from Lakeshore

Biomaterials). PLGA particles loaded with protein mixed in the core were prepared as described: Weigh 100mg of PLGA in Kimble glass tube (from Laboratory Disposable Products, Inc) with cap, and then add 1ml of dichloromethane, cover with cap and vortex at highest speed (or sonicate less than 30 seconds) until it dissolves- [solution 3]. Weigh 20mg of BSA in a 1.5ml eppendorf tube and add 100µl 2% PVA (use pipette and gently pipette up and down so we can prevent bubbles- [solution 4]. If incorporating protein, then add this BSA solution (BSA is used to protect the protein encapsulated in the PLGA particle) as the carrier protein to the lyophilized Ovalbumin protein vial (20µg), gently pipette up and down, and this will be our solution 4 in this case. Add solution 4 to solution 3 and emulsify by homogenization originally at speed 1, then immediately turn up to speed 3 (10.000 rpm) for 1 minute. Add 5 nmol (estimated quantity) of oligo and or 20µg of LPS and repeat last step. Then place homogenizer tip in the solution 1 beaker (2% PVA/50ml Water MQ) and start at speed 1, then add (use blue pipette) the above emulsion (from (3), ~1.1ml) to solution 1 and turn up to speed 3 to homogenize, since this is in a beaker, so move the homogenizer tip around once while homogenizing so can make it more homogenously homogenized, then place in the middle of the solution for 1 minute. Place solution 2 (1%PVA/ 100ml Water MQ) on stir plate and start stirring at speed 4, then add above emulsion (from (3), total ~51,1ml) to solution 2 and stir for 3-4 hours at speed 4 without homogenization (1.000rpm at RT to evaporate DCM) on stir plate (VT= 150ml). At the end of stirring, dispense it in three 50ml conical tubes and centrifuge at 10.000rpm for 20 minutes. Pellet the particles and collect in one 50ml conical tube. Wash 2x more. After the last centrifugation, decant supernatant; add 2-3ml of water. Particles could be separated by size, using vectaspin micro 0.02µm and 0.2µm (from whatman) and stored at 4ºC for a couple of days. For a long storage, they could be

place in liquid nitrogen and then cover the tube with Kimwipe and place in lyophilizer.

- **PLGA microspheres with lipid layer to mimic pathogens -allow protein and ligand conjugation**

With this technology, it is possible to create an improved dynamic system, where particles with surface lipid layers mimicking the composition of lipid-enveloped pathogens. Our collaborators described the self-assembly of different components of biological membranes or lipid-like tracer molecules at the surface of PLGA particles (Bershteyn *et al.* 2008). We employed the emulsion approach to fabricate lipid-enveloped polymer microparticles and nanoparticles.

Method of preparation:

a) Stock solutions: 80 mg of PLGA were pre-aliquot into eppendorfs and stored at -20^{o}C. This is to avoid moisture entering the polymer stock due to numerous freeze-thaw cycles. Lipid stocks were resuspended in chloroform and can be pre-mixed and stored in aliquots.

DOPC, *1,2-Dioleoyl-sn-Glycero-3-Phosphocholine,18:1PC(cis)* (Avanti; 11.7 mg/ml stock in chloroform). DOPG,*1,2-Dioleoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)](SodiumSalt)18:1 PG* (Avanti; 3.00 mg/ml stock in chloroform). DSPE-PEG-maleimide,*1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N [Maleimide (Polyethylene Glycol)2000] (Ammonium Salt)* (Avanti; 5.53 mg/ml stock in chloroform). MPLA *Lipid A, monophosphoryl from Salmonella enterica serotype minnesota Re 595 (Sigma;* 3.32 mg/ml stock in chloroform).

These stocks were stored at -20ºC or -80ºC ideally with Teflon tape wrapped around the seal to keep solvent in, or at least with parafilm to keep moisture out. When possible, the containers were purged with nitrogen gas to remove oxygen before storage.

*b) Preparation of organic phase***:** The stocks of lipid and polymer should be leave at room temperature for a few minutes, until the vials are no longer cold to touch. This will keep condensation from entering the stocks. Add 250 µl of DOPC, DOPG, and PEG-maleimide stock. For MPLA-containing beads, also add 250 µl of MPLA stock. Use a steady stream of nitrogen to dry the lipid in the vial, removing the chloroform solvent or a vacuum chamber with a bump trap to remove residual chloroform. Add 80 mg PLGA to each vial and add 1 ml dichloromethane. Let stand, swirling or pipeting occasionally, until polymer has dissolved. Add 4 ml more dichloromethane.

c) Synthesis of microparticles: Clean the homogenizer (*Ika T25 Homogenizer)* with acetone, ethanol, and water, in this sequence. Pour 40 ml pure water into an Erlenmeyer flask and begin homogenizing at speed 3. Use a 5 ml pipet to slowly pour the organic solution along the homogenizer tip. Polymer and lipid co-dissolved in dichloromethane are emulsified into ultrapure water with a homogenizer; slow evaporation of the organic solvent led to formation of solid particles. After dispensing all organic solution, homogenize for an additional 2 minutes. Add stir bar and stir at ~400 rpm at room temperature overnight. Clean the homogenizer by blotting away excess solution, squirting with acetone, and cleaning with acetone, ethanol, and water. Repeat for each sample.

After 12-24 hours, strain particles through a 40 µm cell strainer. The polydisperse products of this synthesis can be separated by centrifugation (5 minutes at 2.000xg) into bacterial-sized and virus-sized populations. Place particles into a capped container for storage at 4ºC.

d) Conjugation of model antigen – Ovalbumin:

i) Modification of OVA and quenching: Use Frozen aliquots of SAT(PEO)4 at 250 mM in DMSO. Ovalbumin dissolved in sterile PBS at 4.5 mg/mL (0.1 mM) should be added a 10-fold molar excess of a freshly thawed

aliquot of SAT(PEO)4 (1:250 for 1 mM final). Incubate Ovalbumin on rotator for 30 minutes. (When 1 batch of plain and 1 batch of MPLA beads, started with 130 µl of OVA and add 0.52 µl SAT(PEO)4.). Add 1:40 quenching buffer (75 mg/ml or 1 M glycine in PBS stock for final 25 mM) and rotate for another 15 minutes.

ii) Deprotection of -SH groups on ova: Prepare a Zeba desalt column according to the instructions, spinning 4 additional times for buffer exchange with PBS. After Ovalbumin reaction is complete, desalt OVA into PBS. (Any excess modified OVA can be stored at 4ºC at this point.) Measure the volume of eluted OVA and add 1 part deacetylation solution (0.5 M hydroxylamine, 25 mM EDTA in PBS pH 7.2-7.5) per 10 parts of OVA (~20 µl deacetylation solution to ~200 µl OVA). Incubate on rotator for at least 2 hours to deprotect reactive groups.

iii) Reaction with microparticles: Coupling buffer (10 mM EDTA, 0.01mM TCEP in PBS pH 7.2-7.5) must use a fresh addition of 0.01-0.02 mM TCEP due to the short half-life of this reagent in PBS. Weigh a minimal but accurate amount of TCEP and dissolve at 1 mg/ml in pure water. Incubate on rotator to dissolve. Meanwhile, spin down 1 ml beads at 1.100xg for 1 minute to remove small particles. Resuspend in 50 µl PBS, add 950 µl PBS, and spin again at 13.000 xg for 5 minutes. Resuspend beads in 50 µl coupling buffer (no TCEP).
At this point, mix coupling buffer with 2x TCEP by adding 14.3 µl of 1 mg/ml TCEP per 2.5 ml coupling buffer precursor (Remember that the TCEP will have a short half-life.). Solvent exchange a Zeba desalt column into this solution. Desalt the OVA from deacetylation solution into coupling buffer with TCEP. Finally, add 50% of the OVA/coupling buffer/TCEP solution to a batch of plain beads, and 50% to a batch of MPLA-containing beads. Let the reaction for at least 3 hours, and then wash beads 3x with PBS at 13.000xg for 5 minutes before quantifying using hemocytometer and OD600.

- **Sequestered dynamic-ligand particles – Hydrogel particles**

  - **Synthesis and characterization of pH-sensitive core-shell nanoparticles**

Reagents:

2-diethylamino ethylmethacrylate (DEAEMA), 2-aminoethyl methacrylate hydrochloride (AEMA 90%) and ammonium peroxodisulfate (APS) were purchased from Sigma-Aldrich Co. Poly (ethylene glycol) dimethacrylate (PEGDMA, MWPEO = 200g/mol - cross-linker) were purchased from Polysciences Inc.

Method of preparation:

Disperse DEAEMA (1ml, 4.97mmol) premixed with PEGDMA 200 (10µl, 0.03mmol) in water (9ml) with stirring and equilibrated at 70°C for 15 minutes. DAEMA is hydrophobic, thus surfactants are not necessary. Ovalbumin (100µg) and TLR agonists (LipidA – 20µg) could be pre-mixed with this solution, before adding APS (50µl of 200mg/ml freshly made solution) as the initiator - the solution becomes white in ~ 1 minute. The emulsion polymerization occurs at 70°C for 3 hours to grow the particle core, followed by injection of AEMA (50µl of 800mg/ml freshly made solution, 0.24mmol) to grow the particle shells for an additional 1.5 hours.

The nanoparticles were purified by dialysis (10,000 MWCO Slide-A-Lyzer® Dialysis Cassettes, Pierce Chemical Co.) in deionized water pH~ 5-6 (membrane of 8-12kDa) for three days followed by ultrafiltration 3x (10.000 MWCO PLGC Ultrafiltration Membrane, Millipore Co.) and centrifugation 3x with PBS (pH 7.4) at 15.000xg. Purified particles can be stored in PBS at 4°C. Another approach used to couple Ovalbumin and TLR agonists is to pre-mix with core-shell nanoparticles for 5 minutes to allow electrostatic adsorption of the protein and agonists to the cationic surfaces of the core-shell particles. The majority of free

Ovalbumin and agonists could be removed by centrifugation. Ovalbumin and agonists are tightly absorbed on the surface of the core-shell nanoparticles due to the positively charged amine groups in the PAEMA shell.

Antigen Presentation to Naive T Cells *in vitro*

- **Antigen Presentation Model**

To study antigen presentation we made use of an *ex vivo* model using primary APCs (either BMDCs or sDCs), model particles covalently loaded with model antigen (Ovalbumin) and/or TLR agonist. Primary T-Cells isolated either from OT-I mice ($CD8^+$ T-Cells with a transgenic T-Cell receptor (TCR) K^b/SIINFEKL-specific) and OT-II mice (H-2b-restricted OVA class-II epitope OVA4 ($OVA_{323-339}$) were co-incubated with DCs to measure MHC class-I and MCH class-II antigen presentation respectively. T-Cell proliferation/activation can be measured by:

1) Fluorescence-activated cell sorting (**FACS)** using the FACSCaliber (BD instruments), following T-Cell division.

2) ELISPOT assay (**ELISA)** for detection of IFN-γ or IL-2 released by activated antigen-specific T-Cells.

The antigen presentation model is schematically represented in the following figure:

Antigen Presentation Model

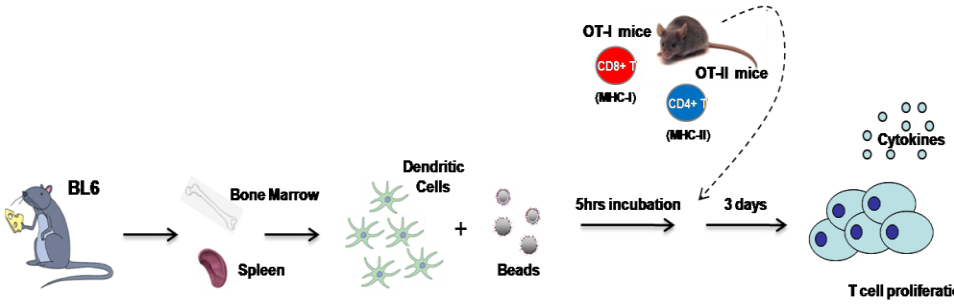


Fig.27: Antigen Presentation Model: C57BL/6 mice (in some cases TLR4KO and MyD88KO mice were used) DCs are isolated either from Spleen (sDCs) or from Bone Marrow (BMDCs) and incubated with antigens during 5 hours. Primary T-Cells from OT-I ($CD8^+$ T-Cells with a transgenic T-Cell receptor (TCR) K^b/SIINFEKL-specific – $OVA_{257-264}$:K^b) or OT-II (transgenic CD4 TCR specific for the MHC class II–restricted OVA peptide aa323–339 - $OVA_{323-339}$:$I\text{-}A^b$) mice were isolated from spleen and co-culture with DCs. T-Cell activation are follow at day 3, by measuring T-Cell proliferation by FACS or by Cytokine production (IL-2/IFN-γ) by ELISA.

1) FACS Proliferation assay - CFSE

T-Cell activation can be follow by FACS, using a Fluorescent T-Cell staining dye (carboxy-succinimidyl-fluorescein-ester - CFSE; Molecular Probes, Eugene, OR). During each round of cell division, relative fluorescence intensity of the dye is decreased by half, thus cell division can be follow by looking to the clearly defined peaks following division in FACS plot.

Individual DCs populations (BMDCs or sDCs) were plated in 96-well round bottom plates (Costar-Corning) at $2.5x10^4$ cells per well and challenged with the indicated number/concentration depending on the type of experiment:

- Soluble antigen OVA (endotoxin free Ovalbumin from PROFOS AG endotoxin concentration < 1 EU/mg; and BSA fraction V from Sigma as a negative control.
- Soluble peptides: K^b epitope $pOVA_{257-264}$ – *SIINFEKL* from NeoMPS; H-2b epiope $pOVA_{323-339}$ – *OVA4* from NeoMPS

- Synthetic Particles (Fixed-ligand particles: Particles loaded with OVA- OVAp; OVA particles coupled with TLR ligands- OVA=LPSp, OVA=CpGp, OVA=LipidA, OVA=Pam2p, OVA=Pam2p, OVA=PolyI:C, OVA=ssRNA40 and OVA=Flagellinp. Mobile-ligand particles: PLGA particles loaded with OVA- PLGA=OVA; and PLGA=OVA particles loaded with MPLA. Sequestered dynamic-ligand particles: Hydrogel particles loaded with OVA- Hydrogel=OVA; and Hydrogel=OVA particles loaded with MPLA).
- Soluble TLR agonists (LPS, CpG, PolyI:C and LipidA) .
- Fixed OVA-expressing *E. coli* (a kind gift from Darren Higgins' lab),
- OVA-loaded dying cells were prepared by osmotic shock as described previously (Liu *et al.*, 2002).

DCs were challenged for at least 2 hours at 37°C in complete medium followed by extensive washes in PBS. DCs were allowed to internalize soluble OVA and OVA particles for the indicated times. Alternately, DCs were loaded with the specific OVA peptide as a control of surface exposure of MHC class-I molecules and BSA as a negative control. For TLR4 inhibition pathway assay, DCs were pre-incubated for 2 hours before antigen pulse with 10µM of drugs for the tree main MAPK pathway. Drugs were kept during the entire pulse period. The drugs used were: P38 inhibitor – SB203580 from Promega; MEK inhibitor- PD98059 from Cell Signaling and JNK inhibitor- SP600125 from Promega. DCs were washed 3x and resuspended in 200 µl of complete medium containing $5x10^4$ CFSE-labeled OT-I or OT-II cells. T-Cells were labeled as follow: T-Cells were resuspended in medium without serum at 10^6 cell/ml. 2µl of CFSE (invitrogen) (5mM) were added to each 10^6 cell/ml, and incubated 10 minutes at 37°C. Cells were pellet at 200xg for 5 minutes and resuspend in complete medium in manner to add the same number of T-Cells as initial number of DCs. T-Cell proliferation was analyzed after 60–65 hours of culture by FACS as described elsewhere (Wilson *et al.*, 2003).

Supernatants form 60–65 hours of culture was stored at -20ºC to further cytokines analysis by ELISA. Each determination was performed in triplicate.

2) ELISA assay (Enzyme Linked Immuno Sorbent Assay)

- **IFN-γ and IL-2**

Elisa 96-well plates (Nunc) were coated with 50µl/well of the respective capture antibody diluted in PBS and incubate at 4ºC overnight. Plate were washed 3x with 0.01% Tween20 in PBS. 100µl/well of Blocking buffer were added and plate were incubated 1 hour at RT. Plate were washed 3x with 0.01% Tween20 in PBS. Samples and standards (50 µl/well) were added and incubated 1 hour at RT or at 4ºC O/N. Plates were washed 3x with 0.01% Tween 20 in PBS. 50 µl/well of 2^{nd} antibody diluted in 5 ml of blocking buffer (each antibody as an appropriate dilution) were added and incubated 1 hour at RT. Plates were washed 3x with 0.01% Tween20 in PBS. 50µl/well of streptavidin diluted in PBS (1:200) were added and incubated for 30 minutes at RT. Plates were washed 3x with 0.01% Tween20 in PBS. 50 µl/well of TMB (5 ml) were added and disclosed in the dark. When a difference between all the standards and sample were observed, reaction was stopped by the addition of 50 µl/well of H_2SO_4. The absorbance at 450nm was measured in TECAN infinite® 200 plate reader.

3) B3Z assay:

After challenge for 6-12 hours with antigen and ligands, sDCs were fixed with 0.08% glutaraldehyde during 5 minutes, and stopped in glycine 0.2M in 96 flat-well plates. Cells were washed and co-cultured (10^5 per well in 96 flat well plates) for 18 hours with the B3Z $CD8^+$ T-Cells, a T-Cell hybridoma specific for the$H\text{-}2K^b/OVA_{257–264}$ complex (10^5 per well) (Karttunen *et al.*, 1992). B3Z activation was

monitored by measuring the induction of lacZ reporter under NF-AT elements using 100 µl of 0.15mM the CPRG substrate (Roche) in PBS/0.5% NP-40. The absorbance of wells was read after 4 hours incubation at 37ºC, at 595nm (Karttunen *et al.*, 1992).

4) Antibody staining - H-2K^b/OVA

For labeling H-2K^b/OVA complexes on DCs surface we made use of Phycoerythrin (PE) anti-K^b/OVA 25-D1.16 (eBioscience). The 25-D1.16 monoclonal antibody reacts with the Ovalbumin-derived peptide SIINFEKL bound to H-2Kb of MHC class-I, but not with unbound H-2Kb, or H-2Kb bound with an irrelevant peptide. DCs were pulsed for 30 minutes and chased for 2 to 16 hours with model particles. Cells were washed extensively with PBS. Staining with 25-D1.16 antibody**,** or Mouse IgG1 isotype matched control, were performed during 30 minutes on ice and extensively washed in PBS. Staining was analyzed by FACS in the FLH-2 channel. Data were analyzed against control without antigen stimulation.

ELISA assay for pro-inflammatory and anti-inflammatory cytokines__

- **IL-6, IL-12 and TNF-α**

Protocol was the same as for IFN- γ and IL-2 as described previously.

- **IFN-β**

To measure IFN- β, we made use of Mouse Interferon Beta Single Plate (96 Well) ELISA Kit from R&D. The protocol was done as described in Product Data sheets for Mouse Interferon Beta ELISA Kit v.1.4.

Phagocytosis Assay

Phagocytosis was determined using particles covalently coupled with OVA-Alexa488 by EDC/NHS chemistry. sDCs (2×10^5 cells/250 µl) were incubated for 1 hour at 37°C in the presence of fluorescent particles. The cells were then extensively washed with cold PBS and immediately analyzed by FACS. The percentage of phagocyting cells (phagocytic index) was calculated by FACS measuring the MFI in the FLH-1 channel. A control at 4°C and other with a 2 hours pre-incubation with 10 M of cytochalasin D, potent inhibitor of actin polymerization, (Sigma), was done to calculate the proportion of particles that are associated with but not phagocytosed by DCs. As an additional experiment control, cells were washed 3x in PBS, distributed on to poly-l-lysine coated slides, and fixed in 4% paraformaldehyde before imaging by confocal microscopy. Cells were visualized on a confocal microscope equipped with LSM image analysis software (Carl Zeiss, Inc.). Images were acquired using a 60X objective lens with a 10X ocular lens. Image processing was performed in Image J and LSM Image Browser confocal software.

Antigen Degradation Assay

To measure antigen degradation we used different approaches: antigen processing of DQ-Ovalbumin visualized by FACS (Daro *et al.*, 2000; Santambrogio *et al.*, 1999) and levels of Ovalbumin by Western Blot (Savina *et al.*, 2009).

- **Self-quenched fluorescent Ovalbumin (DQ-Ovalbumin)**

DQ-Ovalbumin (molecular probes) is a self-quenched conjugate of Ovalbumin that exhibits bright green fluorescence upon proteolytic degradation. This substrate, which is labeled by BODIPY® FL a pH insensitive dye in a range of 3-9, is designed especially for the study of antigen processing and presentation.

The coupling of DQ-Ovalbumin to polystyrene carboxylated particles was performed by passive adsorption, instead of coupling conjugation, to avoid protein unfolding or desnaturation. Passive adsorption was performed as described by Amigorena´s lab (Savina *et al.*, 2006). Briefly, 100µl of 1.0µm carboxylate polystyrene particles from stock solution were pellet (4 minutes at 13.000 rpm), washed 2x in PBS and resuspended in a solution of DQ-Ovalbumin in PBS at 10mg/ml. The reaction was performed on a rotator overnight at 4°C. Particles were centrifuged during 4 minutes at 13.000 rpm to remove soluble DQ-Ovalbumin and 4 washes were performed in PBS. The last resuspension was done in PBS in the same volume as the stock solution (100µl or more) and stored at 4ºC. Polystyrene particles are ready to use. LPS was adsorbed to DQ-Ovalbumin particles as describe previously (20µg during 2 hours shaking at RT followed by extensive wash in PBS). DCs were pulsed and chased at the indicated times with the coupled polystyrene particles. DCs (5×10^5 /200 µl DME) were pulsed with DQ-Ovalbumin coupled particles(1:20) for 15 minutes at 37°C, washed extensively with cold PBS and resuspended in 500µl of complete medium. They were cultured for the indicated time points. When indicated, sDCs were incubated with inhibitors for proteases, Z-FL-COCHO (calbiochem), highly specific inhibitor of Cathepsin S, and MG132- Z-Leu-Leu-Leu-CHO (calbiochem), a highly specific, fully reversible inhibitor of proteasomal proteolytic activity. OVA-processing was followed by measuring the MFI in the FLH-1 channel by FACS.

- **Ovalbumin antigen manipulation for Western Blot assay**

To measure antigen processing we made use of Ovalbumin loaded particles or biotinylated Ovalbumin loaded particles. The biotinylation was performed as follows: Primary amines of Ovalbumin were biotinylated using the EZ-Link NHS-PEO_4-Biotin Biotinylation Kit a polyethylene glycol (PEG)-containing reagent. The proteins at a concentration of 5 mg/ml in PBS were incubated at room temperature for 30 minutes with 50x molar excess of NHS-PEO_4-Biotin. The reaction was stopped by adding excess amount of ethanolamine–HCl (pH 8.5). The reaction mixture was loaded onto Zeba Desalt Spin Columns to remove un-reacted biotin and recover the final product. The number of biotin molecules incorporated into protein molecules could be determined using a standard HABA assay according to the manufacturer's protocol. Molar ratios of the biotin moieties/proteins could range from 0.8 to 13. The biotinylated Ovalbumin were incubated with streptavidin-coated particles for 1 hour at RT and washed extensively. LPS was adsorbed to biotinylated Ovalbumin particles as describe previously (20µg during 2 hours shaking at RT followed by extensive wash in PBS). DCs were pulsed and chased at the indicated times with the coupled polystyrene particles. Dendritic cells (5×10^5/200 µl DME) were pulsed with particles at 1:20 (DC:particles) ratio for 15 minutes at 37°C. DCs were washed extensively with cold PBS and resuspended in 500µl complete medium, and cultured for the indicated time points. When indicated, sDCs were incubated 2 hours previously with inhibitors for proteases (Z-FL-COCHO (calbiochem), highly specific inhibitor of cathepsin S, and MG132 - Z-Leu-Leu-Leu-CHO (calbiochem), a highly specific and fully reversible inhibitor of proteasomal proteolytic activity) and kept during the incubation time points.

Western Blot:

sDCs from the different experiments were collected and washed 2x with cold PBS solution. Cells were lysed for 15 minutes at 4ºC using RIPA buffer (50 mM Tris-HCl at pH=7.4, 1% NP-40, 0.25% Sodium Deoxicholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF in the presence of proteases inhibitor cocktail from Roche). Lysed cells were centrifuged at 14.000 rpm for 15 minutes at 4ºC, and supernatants kept at -20ºC.

Protein concentration in whole cell lysates was determined with RC/DC Protein Assay (Bio-Rad, Hercules, CA). Cell lysates were denatured at 95ºC for 10 minutes in the presence of Laemli buffer (Biorad) and 30 μg separated by 10% Sodium Dodecyl Sulfate (SDS)-PAGE gels. Electrophoresis was performed at 110 V for 2 hours, and gels transferred to nitrocellulose membranes (Whatman) at 220 mA for 1 hour. Membranes were blocked in a solution containing 5% Milk 0.05% Tween20 in PBS for 1 hour. To detect Ovalbumin antigen, the membrane was incubated with anti-Ovalbumin rabbit IgG from Abcam (1:1000). Membranes were washed 3x with 0.1% PBS-Tween20 (Sigma-Aldrich) and incubated with the respective HRP conjugated secondary antibodies (Molecular Probes, Eugene, OR) for 1 hour at room temperature, followed by 3x 10 minutes washes with 0.1% PBS-Tween20, before developing with ECL plus reagent (GE Healthcare). Membranes were developed in anAGFA Curix 60® equipment. To detect biotinilated Ovalbumin antigen, the membrane was incubated with HRP-Sav (1:500) (elisa kit from R&D), washed 3x with 0.1% PBS-Tween20 before developing with ECL plus reagent (GE Healthcare). Membranes were developed in an AGFA Curix 60® equipment. To control loadings, the membrane was stripped using Restore WB Stripping Buffer (Pierce, Rockford, IL) and incubated with anti-*β*-Actin.

- **Antigen available in cytosol (apoptosis mediated by Cytocrome *c*)**

Cyt *c* from horse heart (Sigma) was adsorbed to carboxylated polystyrene particles in water at 4ºC O/N at 10mg/ml. Particles were extensively washed in PBS. LPS was adsorbed to cyt *c* particles as describe previously (20µg during 2 hours shaking at RT followed by extensive wash in PBS). BMDCs were pulsed and chased at the indicated times with the coupled polystyrene particles. BMDCs (5×10^5/200 µl DME) were pulsed with particles at 1:20 (BMDCs:particles) ratio for 15 minutes at 37°C, washed extensively with cold PBS and cultured for the indicated time points in 500µl of complete medium (C10). BMDCS were analyzed for apoptosis by FACS using an Annexin-V-FITC Kit (Pierce). Briefly, After 18 hours of chase, BMDCs were washed 2x in cold PBS and resupended in Binding Buffer. $1x10^6$ BMDCs were incubated with 1:200 of Annexin-V-FITC for 15 minutes at RT in dark and analyzed immediately by FACS in the FLH-1 channel. PI can be used as the viability marker (5 µl of a 50 µg/ml stock solution).

Western Blot for p38-P and IKb-α (TLR pathway signaling)

sDCs were pulsed and chased at the indicated time points with the Ovalbumin particles (OVAp) and Ovalbumin particles containing LPS (OVA=LPSp).sDCs (5×10^5/200 µl DME) were pulsed with particles at 1:20 (sDCs:particles) ratio for 15 minutes at 37°C, washed extensively with cold PBS and cultured for the indicated time points in 500µl of complete medium. sDCs from the different experiments were collected and washed twice with cold PBS. Cells were lysed for 15 minutes at 4ºC using RIPA buffer (50 mM Tris-HCl at pH=7.4, 1% NP-40, 0.25% Sodium Deoxicholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF in the presence of proteases inhibitor cocktail - Roche). Lysed cells were centrifuged at 14.000 rpm for 15 minutes at 4ºC, and supernatants kept at -20ºC.

Protein concentration in whole cell lysates was determined with RC/DC Protein Assay (Bio-Rad, Hercules, CA).Cell lysates were denatured at 95ºC for 10 minutes in the presence of Laemli buffer (Biorad) and 30 μg separated by 10% Sodium Dodecyl Sulfate (SDS)-PAGE gels. Electrophoresis was performed at 110 V for 2 hours, and gels transferred to nitrocellulose membranes (Whatman) at 220 mA for 1 hour. Membranes were blocked in a solution containing 5% Milk 0.05% Tween20 in PBS for 1 hour. Primary antibodies incubation for p38-P (38KDa) (cell signaling) and IkB-α (39KDa) (cell signaling) were performed for 1 hour at room temperature. Membranes were washed 3x with 0.1% PBS-Tween20 (Sigma-Aldrich) and incubated with the respective HRP conjugated secondary antibodies (Molecular Probes, Eugene, OR) for 1 hour at room temperature followed by 3x 10 minutes washes with 0.1% PBS-Tween20, before developing with ECL plus reagent (GE Healthcare). Membranes were developed in an AGFA Curix 60® equipment. To control loadings, the membrane was stripped using Restore WB Stripping Buffer (Pierce, Rockford, IL) and incubated with anti-*β*-Actin.

Phagosome maturation assay

- **Measurement of Phagosomal pH**

The phagosomal measurements were done as previously described (Savina *et al.*, 2006). Three micrometers of amino polybeads previously loaded with Ovalbumin, were covalently coupled with FITC (pH sensitive) and FluoProbes 647 (pH insensitive) in the presence of sodium hydrogen carbonate buffer at pH 8 for 2 hours at room temperature. After extensively washing with glycine 100 mM, the particles were suspended in PBS. BMDCs were pulsed with the coupled particles for 10 minutes and then extensively washed in cold PBS. The cells were then incubated at 37°C (chase) for the indicated times and immediately analyzed by

FACS, using a FL1 (FITC)/FL4 (FluoProbes 647) gate selective for cells that have phagocytosed one latex particles. The ratio of the mean fluorescence intensity (MFI) emission between the two dyes was determined. Values were compared with a standard curve obtained by resuspending the cells that had phagocytosed beads for 2 hours at a fixed pH (ranging from pH 5.5 to 8) and containing 0.1% Triton X100. The cells were immediately analyzed by FACS to determine the emission ratio of the two fluorescent probes at each pH. To certify that the decrease in FITC fluorescence observed at low pH was not due to loss of FITC from the beads, the pH 5.5 buffer was neutralized after measurement using NaOH. In some cases, to evaluate the effect on pH of blocking V-ATPase activity, 200nM of Bafilomycin A was added to WT DCs 30 minutes before pulse, and kept during chase.

- **ROS measuring using DHR123**

Ovalbumin loaded particles with 1µm (prepared as described previously) were covalently coupled with dihydrorhodamine 123 (DHR123) from Invitrogen, in the presence of sodium hydrogen carbonate buffer at pH 8 for 2 hours at room temperature. After extensively washing with glycine 100 mM, particles were resuspended in PBS. BMDCs were pulsed with the DHR123-coupled particles during 20 minutes in CO2-independent medium and then extensively washed in cold PBS. After incubation with the particles, the cells were chased for the indicated times and immediately analyzed by FACS using a FCS/SSC gating selective for cells that have phagocytosed DHR123 particles. The variation of the MFI emission in the FL-1channel of DHR123 was determined.

- **Confocal Microscopy**

BMDCs were seeded on poly-L-lysine-coated glass coverslips (12 mm) for 15 minutes at room temperature (RT). The coverslips were washed, and complete

medium was added to further incubate the attached cells at 37°C and 5% CO_2 for 1 hour. Attached cells were pulsed with OVA-Alexa488 particles at 1:20 (BMDCs:particles) ratio during 15 minutes at 37ºC in RPMI and extensively washed with cold PBS. Completed medium was added subsequently and cells were chased for 30 minutes, 1 hour and 2 hours at 37ºC an atmosphere of 5% CO_2. When indicated, as a control for phagocytosis, 10nM of Cytochalasin D was used 30 minutes before incubation and kept during chasing. 10 minutes before each time point, 15mM of lysotracker was added in RPMI. The cells were then washed and fixed in 2% paraformaldehyde (PFA) for 10 minutes at RT followed by 3x washes with 1 mM glycine PBS. When indicated, Actin was labeled using 1U/ml of Phalloidin Red (Invitrogen), which binds specifically to F-Actin skeleton, for 30 minutes, and washed 3x with 1 mM glycine PBS. Coverslips were mounted on glass slides using vectashield.

All immunofluorescence images were acquired on a Zeiss LSM 510 META inverted confocal laser scanning microscope (Carl Zeiss, Jena, Germany) using a PlanApochromat 63×1.4 oil immersion objective. DAPI fluorescence was detected with a violet 405 nm diode laser (30 mW nominal output) and a BP 420-480 filter. Alexa Fluor 488 fluorescence was detected using the 488 nm laser line of an Ar laser (45 mW nominal output) and a BP 505-550 filter. Phalloidin Red fluorescence was detected using a 561 nm DPSS laser (15 mW nominal output) and a LP 575 nm filter. Sequential multi-track/frame imaging sequences were used to avoid any potential bleed-through from the different fluorophores. All confocal images were acquired with a frame size of 1024x1024 pixels and with the pinhole aperture set to 1 Airy unit. Fluorescence intensity around phagosomes and colocalization of Alexa488 and Lysotracker Red stainings were assessed using ImageJ (http://rsbweb.nih.gov.ij) to perform image processing and quantification. Briefly, each image was thresholded in the green and red channels before

colocalization was determined for each pixel, using the ImageJ plugin RG2B colocalization (http://rsbweb.nih.gov/ij/plugins/rg2bcolocalization.html) which creates a binary mask for colocalizing pixels. This image mask was further processed with a median filter (radius = 1 pixel) to remove noise. For each image, the number of beads with positive colocalization events where then counted.

shRNA Lentivirus Production

Plate cells: 2.2 x 10^4 HEK 293T cells/well were splited on a 96 Flat well plate in 100 µl DMEM + 10%FBS, without P/S, at 37ºC and incubated for 24h.

Transfecion: 100ng of viral vector DNA (10ng/µl) were transferred to 96 well plates. 100ng/well (10ng/µl) of viral FuGW vector DNA and 100ng/well (10ng/µl) viral siSCRAM vector DNA are used as controls. 24.26µl/well of Optimem and 0,6ul/well of transfection reagent (transit-LT1) are incubated for 5 minutes at RT. The packaging and envelope plasmids were mixed, 0.133µl/well of Δ8.9 (0.75 µg/ul) and 0.01µl of VSV-g (1µg/µl) at a total volume of 25µl/well. The total volume (25µl) of the mix were added immediately on DNA plate and incubated for 30 minutes at RT. The transfection mixture (35µl/well) was pipetted very gently onto cells (do not mix). Cells were incubated at 37ºC overnight. On the next 24 hours, all media were removed and replaced with 180 µl medium with 30% FBS, 1% L-glut, 1% P/S.

Lentivirus harvest: 150µl of virus were collected after another 24 hours and medium were replaced with C10. After another 24 hours, all medium were collected and plate was trashed with bleach. Supernatants with Lentiviral particles were aliquoted and stored at -80ºC.

Lentiviral Infection_____

Day 0: plate $1x10^5$ BMDCs/well in 200 µl of BMDCs medium (C_{10}) + GM-CSF (or J5) in round bottom 96 well plates.

Day 2: All media were removed carefully remove without disturbing the cells in the bottom; 10 µl/well of virus were added to transduced BMDCs and resuspended ~5x; 40 µl/well of C_{10} + polyB (1:1000 final concentration) were added. Spinoculation protocol for infection was used: Centrifugation at 2200 rpm, 37ºC, 90 minutes. Plates were wrapped in Saran wrap or equivalent to avoid evaporation. The media were removed (50 µl/well) and 200ul/well C10 + GM-CSF (or J5) were added. BMDCs were incubated during 2 days @ 37ºC.

*Day 4***:** 100µl of culture medium were removed and 150 µl of puromycin added to each well to a final concentration of 5 µg/ml using C10 + GM-CSF (or J5).

Day 6: Harvest and proceed with experiments (Puromycin was kept in medium when they are used for long time points).

Note: The tips were discarded into a recipient with bleach for 24 hours (trashed normally afterwards).

Knock Down phenotype validation _____

- **FACS staining for TLR4 and TLR9**

At day 6 (4 days after puromycin selection) of BMDCs transduction with shRNAs lentivirus for TLR4 and TLR9 and siSCRAM, cells were collected and washed 3x in PBS. Primary Antibodies for mouse TLR4 and TLR9, anti-TLR4 and anti-TLR9 from Abcam, were used with a 1:200 dilution and incubated with cells in PBS at 4ºC for 30 minutes. Cells were washed 3x with PBS and incubated with secondary antibody at 4ºC for 30 minutes on dark. BMDCs were washed and analyzed by FACS.

- **Real-time PCR (qPCR) for TLR4 and TLR9**

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Following treatment with 2U/sample of RQ1 DNase, in the presence of 50 U/sample of RNase inhibitor (Invitrogen), for 30 minutes at 37°C, 1 μg of RNA was reverse transcribed, using Superscript reverse transcriptase (Invitrogen), following normalized against the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the relative expression of each gene was calculated using *Pfaffl*'s method (for details see ABI PRISM 7700 – User Bulletin #2) (Pfaffl, 2001). The primers were designed using different online software, MGH/Harvard Primer bank (http://pga.mgh.harvard.edu/primerbank/). The oligonucleotides used were for GAPDH, TLR4 and TLR9**.** Primers reverse and forward sequences are shown. Expression levels were evaluated by quantitative real-time PCR (qRT-PCR) with the ABI PRISM 7700 instrument (Applied Biosystems, Forster City, CA, USA) using 1x SYBR Green PCR Master Mix (Applied Biosystems).

GAPDH_F 5′ GAGTCAACGGATTTGGTCGT 3′
GAPDH_R 5′ TTGATTTTGGAGGGATCTCG 3′

TLR4_F 5′ ATGGCATGGCTTACACCACC 3′
TLR4_R 5′ GAGGCCAATTTTGTCTCCACA 3′

TLR9_F 5′ ATGGTTCTCCGTCGAAGGACT 3′
TLR9_R 5′ GAGGCTTCAGCTCACAGGG 3′

Statistical analysis

To test the significance of the differences observed the Student's T-test was used. In all tests the statistical significance was 2-sided and considered at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Data are displayed as mean +/± 1SD.

4

Results

The main objectives of this work were to understand and to reveal intrinsic mechanism(s) of antigen cross-presentation to specific physical and biochemical forms of antigen. In order to achieve those goals we have made use of particle antigens with different biophysical properties.

Antigen cross-presentation model characterization

To study antigen cross-presentation we made use of an *ex vivo* model, where DCs and T-Cells were isolated from mice and cultured *in vitro*. We started by characterizing this model using particulate antigens (OVA particles- OVAp), soluble antigens (OVA protein and SIINFEKL peptide) and as a negative control BSA protein. Antigen presentation by DCs (either sDCs or BMDCs) has been measured in the context of MHC class-I and II. In order to measure MHC class-I antigen presentation, $CD8^+$ T-Cells were isolated from OT-I mice ($CD8^+$ T-Cells with a transgenic T-Cell receptor (TCR) -K^b/SIINFEKL, restricted class I epitope - $OVA_{257-264}$) and assays for T-Cell proliferation/activation were performed. In the case of MHC class-II antigen presentation, a similar approach was used, although $CD4^+$ T-Cells were isolated from OT-II mice ($CD4^+$ T-Cells with a transgenic T-Cell receptor - H-2b/ISQAVHAAHAEINEAGR-OVA4, restricted MHC class-II epitope - $OVA_{323-339}$). T-Cell proliferation could be followed by Fluorescent Activated Cell Sorter **(FACS**), using a Fluorescent T-Cell staining dye (Carboxy-Succinimidyl-Fluorescein-Ester - CFSE; Molecular Probes, Eugene, OR) – where during each round of cell division, relative fluorescence intensity of the dye is decreased by half. Consequently cell division could be followed by looking to the well defined peaks, representing each division, observed in the FACS plot (*fig.28*). Therefore, proliferation was quantified by determining the geometric mean of fluorescence of CFSE using Flowjo (Treestar, Inc.) or by analyzing the percentage of proliferation relative to the steady state (i.e. cells that do not divide). In addition,

T-Cell activation could be followed by ELISPOT assay (**ELISA)**, detecting the IFN-γ released into the supernatants of activated antigen-specific T-Cells.

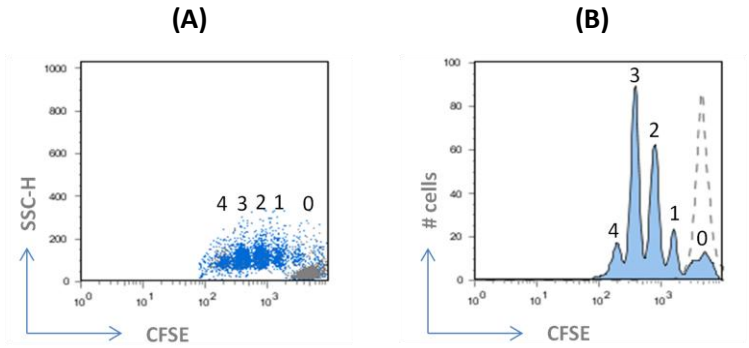


Fig.28: Pilot antigen cross-presentation FACS-based assay: OT-I T-Cell proliferation. sDCs ($2.5x10^4$) from C57BL/6 mice were incubated with OVAp at 1:10 (sDC:particles) ratio. OT-I T-Cells ($1x10^5$) stained with CFSE were co-incubated with sDCs. T-Cell proliferation was measured by FACS at day 3. Graphs represent T-Cell population gated on SSC *vs* CFSE plots. **(A)** FACS plot gated on OT-I T-Cells labeled with CFSE. **(B)** Histogram representing the same population of OT-I T-Cells. Open grey line plot represents control OT-I T-Cells that do not divide and blue filled plot represents OT-I T-Cells proliferation under OVAp stimulus. The numbers correspond to cell cycles, thus each peak corresponds to one cell division.

In order to validate the *ex vivo* antigen presentation model, both soluble and particulate antigens were used. As described before, Ken Rock and co-workers have shown that when a soluble protein was "made" particulate (adsorption to particles of 1-5µm) antigen cross-presentation occurs at 10^3-10^4 fold lower antigen concentrations (Kovacsovics-Bankowski *et al.*, 1993; Kovacsovics-Bankowski and Rock, 1994). This observation is one of the starting points for the use of particulate antigens to dissect and study antigen cross-presentation mechanism with a potential therapeutic target as the main goal. Antigen presentation by sDCs was measured by T-Cell proliferation FACS-based assay using soluble antigens at different concentrations (soluble OVA, soluble BSA and soluble OVA peptide -SIINFEKL)

and particulate antigens at different ratios (i.e. 1:10– ten particle antigens per one DC), as shown in *fig.29*.

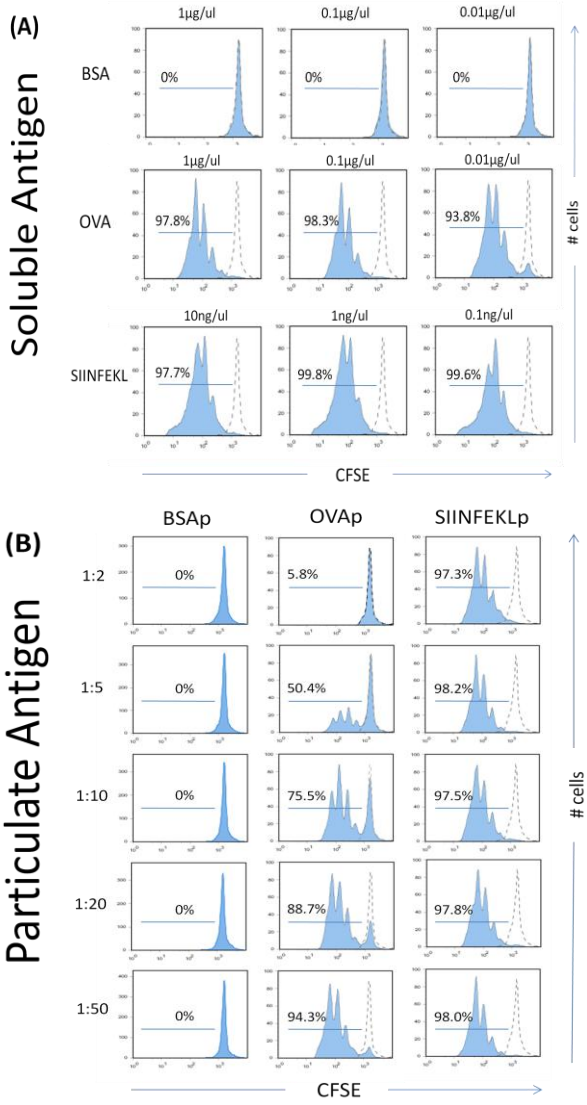


Fig.29: Antigen cross-presentation FACS-based assay: Soluble and particulate antigens.

(A) sDCs ($2.5x10^4$) from C57BL/6 mice were incubated with a broad range of soluble model antigens concentration: OVA (10ng/µl -1µg/µl), SIINFEKL peptide (0,1ng/µl - 10ng/µl) and BSA (10ng/µl - 1µg/µl) as a control. **(B)** sDCs ($2.5x10^4$) from C57BL/6 mice were incubated with model particles. Model antigens, OVA, BSA and SIINFEKL were covalently conjugated to 1.0µm polystyrene particles by a coupling reaction (see protocol for details). Model particles were incubated with sDCs at different ratios (sDC:particles - 1:2, 1:5, 1:10, 1:20, 1:50). OT-I T-Cells ($1x10^5$) stained with CFSE were co-incubated with sDCs. T-Cell proliferation was measured by FACS at day 3. Histograms represent T-Cell population gated on SSC *vs* CFSE plots. Open grey line plots represent control OT-I T-Cells that do not divide and blue filled plots represent OT-I T-Cells proliferation under specific stimulus. Numbers represent the percentages of the proliferating cells of total OT-I T-Cells. These data are representative from one experiment repeated at least three times with similar results.

As expected, the OT-I T-Cell proliferation is antigen dose-dependent. Soluble OVA and SIINFEKL peptide induced high levels of T-Cell proliferation even at low concentrations (0.01µg/µl and 0.1ng/µl respectively - *fig.29)*. However, lower amounts of soluble antigen should be used if one wants to characterize antigen-dose concentration on T-Cell responses. BSA protein was used as a negative control, as BSA processing by DCs does not generate the correct peptide for OT-I T-Cell receptor (TCR) transgenic mice recognition. Clonal expansion of OT-I $CD8^+$ T-Cells is specific for K^b-SIINFEKL generated by OVA processing.

To compare the "immunogenicity" of soluble and particulate antigen, the amount of proteins that are covalently attached to particles surface were estimated. Therefore, we made use of the following function that relates the surface area of microspheres with the size of the loading antigen.

Equation: Estimated maximum number of proteins molecules at particle surface:

Adapted from Prof. Darrell Irvine

Comparing soluble OVA and particulate OVA, similar levels of OT-I T-Cell proliferation were achieved when particulate antigen has used at 1:50 (sDC:OVAp) ratio or 0.01µg/µl of OVA soluble (2µg on total) were incubated with DCs (94.3% and 93.8% respectively). In total, $2.5x10^9$ OVA molecules exist when a ratio of 1:50 particles is used to stimulate $2.5x10^4$ sDCs. In a soluble condition, $2.68x10^{13}$ OVA molecules exist in 200µl for a soluble concentration of 0.01µg/µl. The relative ratio: $2.68x10^{13} / 2.5x10^9$ $1x10^4$.
Moreover, in our model, when comparing the proliferation index (percentage of cells that divide at least once), a particle antigen is presented at least ~10^4 times more efficiently than soluble antigen. These results are in line with the previous

studies performed by Ken Rock' lab (Falo *et al.*, 1995; Kovacsovics-Bankowski *et al.*, 1993). However, for more detailed conclusion, the relative amount of OVA molecules at the particle surface can be quantified, using fluorescent OVA (OVA-FITC) and calibrated particles with FITC dye. Our data revealed that the OVA measured at particles surface is majorly lower than expected theoretically, highlighting previous observation (*data not shown*).

Size-dependent antigen cross-presentation

It was reported that particles size itself (devoid of ligand) can determine the uptake pathway (Rejman *et al.*, 2004). Given the strict dependency of the immune response on the size of the microorganisms, from virus to bacteria, the possible role of particle size in antigen presentation has emerged. Thus, we set to determine the "best" particle size for antigen cross-presentation studies. To observe the effect of particle size on antigen cross-presentation efficiency, sDCs were exposed to OVA-loaded synthetic particles with sizes ranging from 0.05µm to 6.0µm, covering sizes from virus to bacteria. EDC/NHS coupling chemistry was employed to covalently attach OVA to polystyrene particles. For all particle sizes, different ratios (DC:particles) were used as described in *fig.30*.

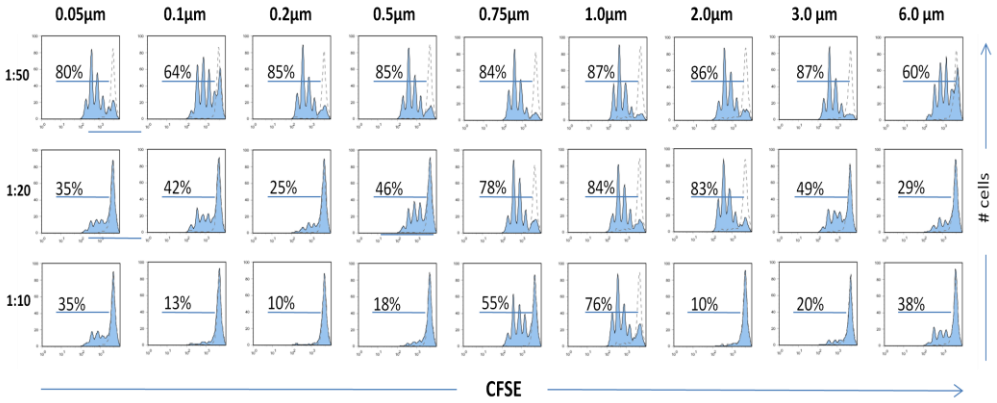


Fig.30: OVA particles size dependent antigen cross-presentation FACS-based assay. sDCs ($2.5x10^4$) from C57BL/6 mice were incubated at different ratios (sDC:particles -1:10, 1:20, 1:50) with a broad range of polystyrene particle size (0.05µm – 6.0µm), covalently coupled with OVA. OT-I T-Cells ($1x10^5$) stained with CFSE were co-incubated with sDCs. T-Cell proliferation was measured by FACS at day 3. Histograms represent T-Cell population gated on SSC *vs* CFSE plots. Open grey line plots represents control OT-I T-Cells that do not divide and blue filled plots represent OT-I T-Cells proliferation under OVAp stimulus. Numbers represent the percentages of the proliferating cells in total number of OT-I T-Cells. These data are representative from one experiment repeated at least three times with similar results.

Preliminary data revealed changes in the magnitude of OVA antigen cross-presentation by sDCs according to the size of the synthetic particles used. These data showed that 1.0µm particles are cross-presented more efficiently by DCs, being evident at lower ratios (*Fig.30*). Since particles size also affects the amount of antigen taken up by cells (Desai *et al.*, 1997), the differences in antigen cross-presentation could simply be attributed to the total amount of antigen internalized by DCs. Thus, to be able to address other type of conclusions, the amount of OVA covalently attached to the surface of the particles should be normalized, as well as the amount of internalized OVA using fluorescent-labeled OVA. The uptake of particles > 0.5 µm in size is termed phagocytosis, whereas particles < 0.5 µm are

taken up by receptor-mediated endocytosis or pinocytosis (Rejman *et al.*, 2004). To address if there were differences in the uptake by phagocytosis of the different particles size used, the amount of antigen internalized by phagocytosis was measured. Polystyrene particles of various diameters (0.5, 1.0 and 3.0μm) covalently coupled with fluorescent OVA-Alexa488 (Molecular Probes) were used. Phagocytosis was determined by a FACS based assay. Quantification was performed by determining the geometric mean of fluorescence in the FLH-1 channel, using Flowjo software (Treestar, Inc.).

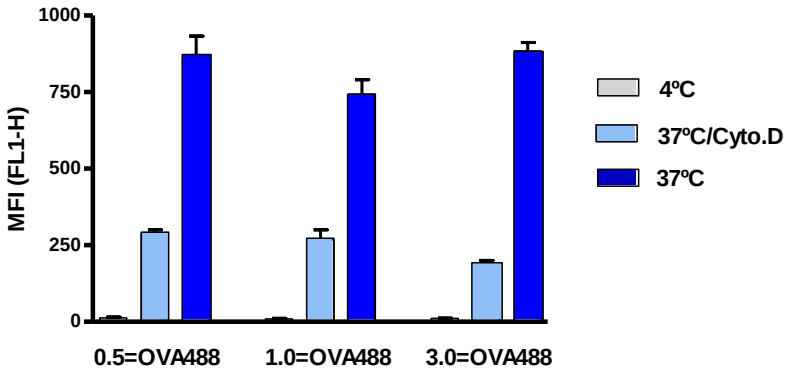


Fig.31: Size dependent quantitative uptake of fluorescent particles antigen. Fluorescent OVA (OVA-Alexa Fluor 488 - OVA488) was covalently coupled to model particles of 0.5 μm (0.5=OVA488), 1μm (1.0=OVA488) and 3.0μm (3.0=OVA488) in size. Particles were incubated with sDCs ($5x10^4$) from C57BL/6 at 1:10 (sDC:particles) ratio for initial 10 minutes of pulse and 2hrs of chase (phagocytosis). Cyto. D (10nM), a phagocytosis inhibitor, was incubated 1hr previously to particle addition. Quantitative uptake (Mean fluorescence intensity–MFI) of green fluorescent particles antigen by sDCs was examined by FACS in the FLH-1 channel. The graph represents the average + 1SD of three independent experiments. No statistically significant differences (P>0.05) were observed between amounts of fluorescent OVA of different size particles internalized by sDCs.

There was no significant difference in the amount of antigen of the different size particles that were phagocyted by DCs *(fig.31)*. This result supports the

evidence that the difference in antigen cross-presentation efficiency observed mediated by LPS is not due to the difference on the amount of antigen internalized. To determine whether the difference in the efficiency on particles size antigen cross-presentation is related to antigen processing/loading or transport to cytosol, we made use as antigen, SIINFEKL peptide (MHC class I–restricted OVA peptide) instead of OVA protein. A similar approach was performed for SIINFEKL peptide antigen presentation as used for OVA protein.

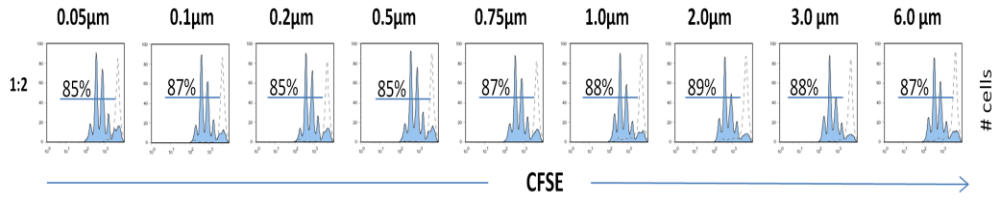


Fig.32: **SIINFEKL particles size dependent antigen cross-presentation FACS-based assay**. sDCs ($2.5x10^4$) from C57BL/6 mice were incubated with a broad range size of polystyrene particles (0.05µm – 6.0µm) covalently coupled with SIINFEKL, at a low ratio (sDC:particles – 1:2). OT-I T-Cells ($1x10^5$) stained with CFSE were co-incubated with sDCs. T-Cell proliferation was measured by FACS at day 3. Equimolar molecules of SIINFEKL peptide (comparing to OVA protein used on previous assays), were coupled to particles by covalent chemistry. Histograms represent T-Cell population gated on SSC *vs* CFSE plots. Open grey line plots represent control OT-I T-Cells that do not divide and blue filled plots represent OT-I T-Cells proliferation under SIINFEKLp stimulus. Numbers represent the percentages of the proliferating cells of total OT-I T-Cells. These data are representative from one experiment repeated at least three times with similar results.

SIINFEKL particles (SIINFEKLp) induced higher and similar levels of T-Cell proliferation / activation, even at low ratios of sDC:particles (1:2) and among the different range of particle sizes used (0.05µm-6.0µm) (*fig.32*). These data support the assumption that the different efficiency on particle antigen cross-presentation, due to different forms of uptake, may not be related to the amount of antigen internalized but instead on intracellular processing/loading mechanism of different

antigen particles size. Data obtained with different OVA particles size showed that the 1.0 µm particle-containing phagosomes are the most efficient on antigen cross-presentation (*fig.30*). We can hypothesize that the size of antigen carriers plays a critical role in directing antigen to the MHC class-I antigen presentation pathway. From now on, 1.0µm particles were used as a model size for further studies to better dissect the antigen cross-presentation mechanism.

TLR signaling on antigen cross-presentation

As the cellular mechanism of antigen cross-presentation is not well understood, as well the possible specificity of a specific stimulus, we have proposed to understand how TLR agonists in particulate antigens may influence their cross-presentation. To address the role of pathogen-like particles in antigen cross-presentation, model agonists for TLR4 and TLR9 were used, representing these receptors exogenous and endogenous pathogen "sensors" respectively. CpG oligo (a TLR9 agonist) or LPS (a TLR4 agonist) were covalently attached to Ova-conjugated particles using the EDC/HNS coupling reaction. Antigen model particles and a FACS-proliferation based antigen cross-presentation assay were used.

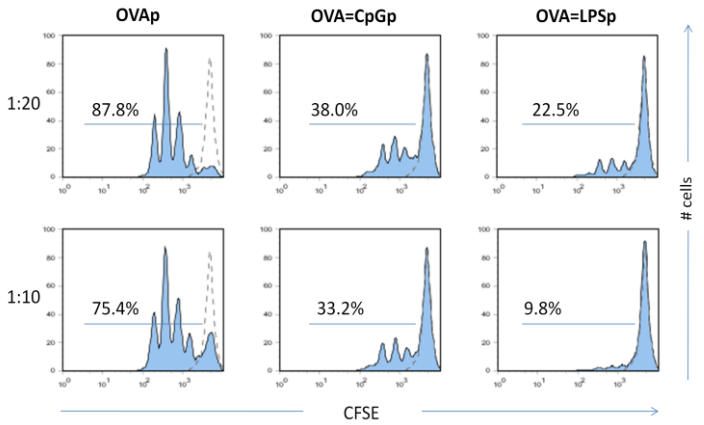


Fig.33: TLR model particles antigen cross-presentation FACS-based assay. sDCs ($2.5x10^4$) from C57BL/6 mice were co-incubated with model particles (OVAp, OVA=CpGp and OVA=LPSp) at 1:20 and 1:10 (sDC:particles) ratios, and with OT-I T-Cells ($1x10^5$) for 3 days. T-Cell proliferation was measured using CFSE staining by FACS. Histograms represent T-Cell population gated on SSC *vs* CFSE plots. Open grey line plots represent control OT-I T-Cells that do not divide and blue filled plots represent OT-I T-Cells proliferation under specific stimulus. Numbers represent the percentages of the proliferating cells of total OT-I T-Cells. These data are representative from one experiment repeated at least three times with similar results.

Surprisingly, these preliminary data revealed that antigen cross-presentation of OVA particles is almost abolished when TLR agonists (CpG and LPS) are in the same cargo (*fig.33*). However, this phenotype was not as evident at higher ratios as 1:50 (*data not shown*), which could be due to "saturation" of the DCs antigen presentation machinery. As a result, proliferation of $CD8^+$ T-Cells was significantly hampered in cells incubated with OVA=CpGp or OVA=LPSp conjugated particles at different ratios (1:10 and 1:20), when compared to cells incubated with "naked" OVAp (*fig.33*). Similar results were obtained, in a minor level, when BMDCs were used as model DCs instead of sDCs (*data not shown*).

Antigen cross-presentation phenotype using TLR KD models

To address if this phenotype is TLR dependent or due to other different mechanism or even an "artifact", sDCs from TLR4KO mice and BMDCs Knocked-Down (KD) for TLR4 or TLR9 by shRNA lentivirus were used. sDCs were isolated from WT and TLR4KO C57BL/6 mice as described previously, and model particles (OVAp and OVA=LPSp) were used to measure OT-I T-Cell proliferation, as shown in *fig.34*.

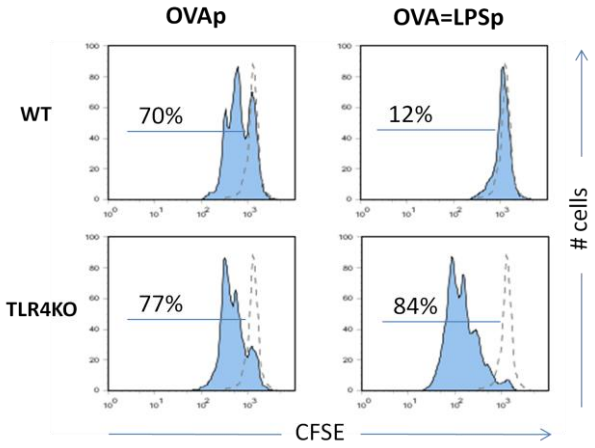
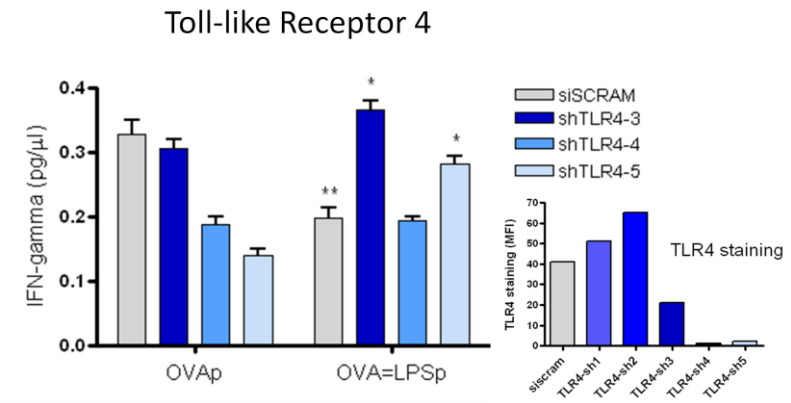


Fig.34: TLR model particles antigen cross-presentation FACS-based assay: WT *vs* TLR4KO DCs. sDCs ($2.5x10^4$) from C57BL/6 WT and TLR4KO mice were co-incubated with model particles (OVAp and OVA=LPSp) at 1:10 (sDC:particles) ratio and with OT-I T-Cells ($1x10^5$) for 3 days. T-Cell proliferation was measured using CFSE staining by FACS. Histograms represent T-Cell population gated on SSC *vs* CFSE plots. Open grey line plots represent control OT-I T-Cells that do not divide and blue filled plots represent OT-I T-Cells proliferation under specific stimulus. Numbers represent the percentages of the proliferating cells of total OT-I T-Cells. These are representative data from one experiment repeated at least three times with similar results.

As expected by previous data, antigen cross-presentation of WT sDCs was abolished when LPS is in the same cargo as OVA particles. However, antigen cross-presentation of OVA=LPSp by TLR4KO sDCs was not impaired, instead,

relative higher proliferation rates of OT-I T-Cells were observed *(fig.34)*. Moreover, OVAp antigen cross-presentation phenotype (OT-I T-Cell proliferation) was recovered for OVA=LPSp in sDCs deficient on TLR4. These data showed that the effect of particulate LPS in antigen cross-presentation phenotype is TLR4 dependent, suggesting the specificity of the LPS agonist through TLR4 signaling pathway. The TLR4KO proliferation data, where OVA=LPSp induce higher levels of T-Cell proliferation comparing to OVAp, suggest that particulate LPS in the absence of TLR4 could signal through other type of receptor, thus increasing (even in a low magnitude) antigen cross-presentation (*fig.34- lower panels*). In order to extend these studies to other type of DCs, BMDCs and shRNA lentivirus were used to Knock-Down TLR4 and TLR9.



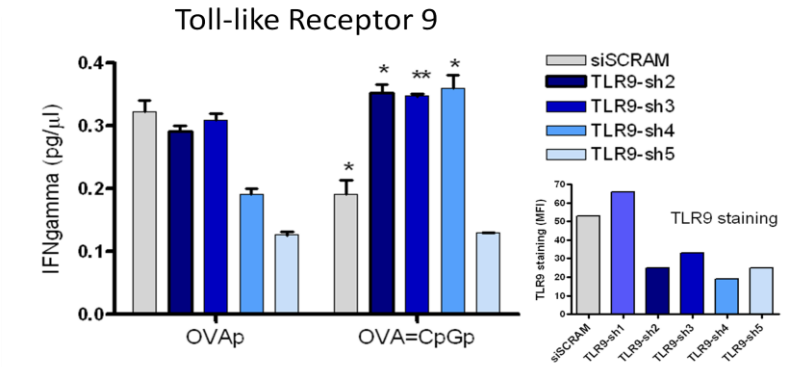


Fig.35: TLR model particles antigen cross-presentation FACS-based assay: TLR4 and TLR9 KD in DCs using shRNA. BMDCs ($5x10^4$) from C57BL/6 mice were transduced with 5 different lentivirus encoding shRNAs targeting TLR4 (upper graph) and TLR9 (lower graph). A random sequence (siSCRAM) was used as control. After selection at day 2 with puromycin, BMDCs were co-incubated at day 6 with model particles (OVAp, OVA=CpGp and OVA=LPSp) at 1:10 (BMDCs: particles) ratio and OT-I T-Cells ($1x10^5$) for 3 days. At day 6, BMDCs were analyzed by FACS staining for TLR4 and TLR9 to evaluate the KD of TLR4 and TLR9. T-Cell activation was measured by ELISA for IFN-γ, using supernatants at 60-65 hrs. The graphs represent the average + 1SD of three independent experiments. The asterisks represent statistically significant differences between OVAp and OVA=LPSp and OVAp and OVACpGp for the same shRNA (*P<0.05;**P< 0.01).

As expected, the levels of T-Cell activation (IFN-γ production) by BMDCs were lower comparing to sDCs (*see next figure- fig.36*). These data in BMDCs shown that T-Cell activation was decreased when OVA=LPSp and OVA=CpGp were used as particle antigen comparing to "naked" OVAp (*fig.35*), reproducing the same results obtained with sDCs (*fig.33*). The results may suggest that this phenotype is "transversal" to DCs populations, therefore proving BMDCS to be a good model for the use of shRNA interference tool in antigen cross-presentation phenotype characterization. Using two or more shRNAs constructs to target TLR4 and TLR9 on BMDCs (where KD efficiency was previously confirmed), in the presence of OVA=LPSp or OVA=CpGp respectively, the antigen cross-presentation phenotype was recovered (levels of OT-I T-Cell activation were similar as observed for "naked" OVAp), suggesting the specificity effect of the TLR agonists (LPS and CpG) through their TLR signaling pathway (*fig.35*).

MHC class-II antigen presentation pathway

LPS was used as the "model" TLR agonist aiming to better characterize the antigen cross-presentation phenotype and to try to dissect the underlying mechanism. We decided to use LPS for different reasons, mainly because: *1)* it is one of the most used TLR agonists and TLR4 is the usual target of immunologists and vaccinologists, *2)* it is easy to conjugate to different type of model particles, *3)* the nature of its receptor, TLR4 is unique among TLRs, because signals through two different adaptors, MyD88 and TRIF.

As MHC class-II is the classical pathway for extracellular antigen presentation and in order to have a mechanistic control comparing to antigen cross-presentation, we next addressed the role of LPS on MHC class-II presentation. Therefore, activation of OT-II Th1 Cells ($CD4^+$) and OT-I cytotoxic T-Cells ($CD8^+$) by DCs was addressed by measuring their IFN-γ production.

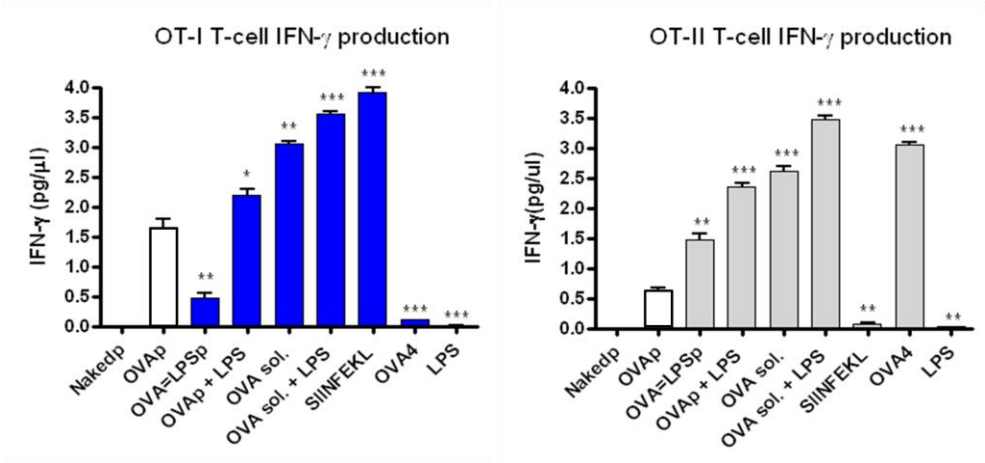


Fig.36: Antigen presentation: INF-γ based T-Cell activation assay. T-Cell activation was addressed by ELISA for INF-γ secretion at 60-65hrs by OT-I T-Cells (left graph) and OT-II T-Cells (right graph) in response to sDCs ($2.5x10^4$) from C57BL/6 mice given diverse stimuli. Particulate antigens: Naked particles, OVAp and OVA=LPSp at 1:10 (sDC:particles) ratio; soluble antigen: OVA endograde (100ng/µl), SIINFEKL peptide (1ng/µl) and OVA4 peptide (1ng/µl) were used.

Soluble LPS was used at 10ng/µl. The graph represents the average + 1SD of three independent experiments. The asterisks represent statistically significant differences comparatively to OVAp (*$P<$ 0.05; **$P<$ 0.01 *** $P<$ 0.001).

The ELISA data for IFN-γ produced by OT-I T-Cells, reproduced the previous results obtained for OT-I T-Cell proliferation (*fig.33*). Thus, in the presence of LPS in the same cargo as particle antigen, OT-I T-Cells activation (IFN-γ) was decreased approximately 3 times comparing to OVAp. Moreover, OVA=LPSp induced OT-II T-Cell proliferation, approximately 3 times, comparing to OVAp (*fig.36*). The induction observed on MHC class-II antigen presentation pathway mediated by LPS on same cargo as particle antigen corroborates previous data obtained by Blander *et.al* (Blander and Medzhitov, 2006b). It is interesting to notice that the relative antigen presentation for OVAp in absence of LPS stimulus is higher in MHC-class I when comparing to MHC class-II. When soluble LPS was co-incubated with OVAp (two different stimuli) there was an increase in T-Cell activation (IFN-γ) and proliferation (FACS analysis - *data not shown*) in both MHC class-I and MHC class-II contexts. Furthermore the magnitude was much higher for MHC-class-II (~ 4.5x) when compared to MHC class-I (~1.25x) (*fig.33*). These data suggest that LPS when in same cargo as antigen impairs antigen cross-presentation and dictates a shift to MHC class-II antigen presentation.

TLR signaling in a different physical form

Concerning the physical nature of stimulus (particulate *vs* soluble), we next addressed if the efficiency of presenting antigens from phagocytosed particles is dependent on the presence of TLR4 agonist within the antigen cargo. T-Cell activation was measured using IFN-γ ELISA assay.

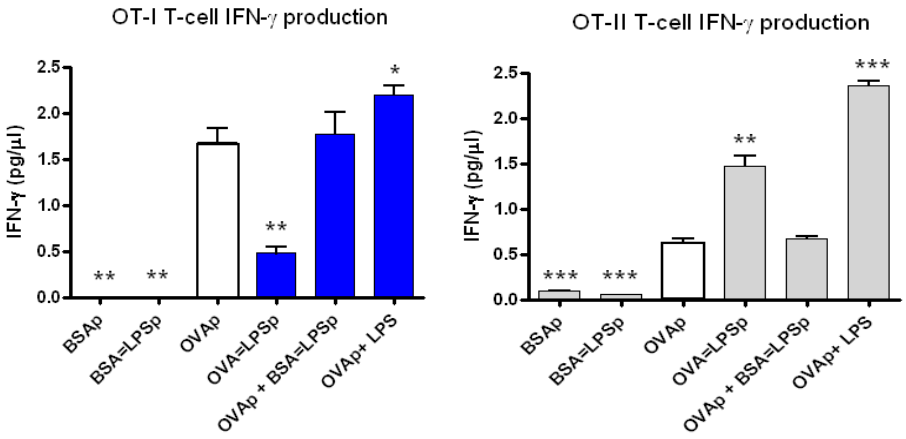


Fig.37: INF-γ based T-Cell activation assay: Particle antigen presentation using different physical LPS stimuli. T-Cell activation was addressed by ELISA for INF-γ secretion at 60-65hrs by OT-I T-Cells (left graph) and OT-II T-Cells (right graph) in response to sDCs from C57BL/6 mice given particulate antigens: BSAp, BSA=LPSp, OVAp and OVA=LPSp. Particles were used at 1:10 (sDC:particle) ratio. Soluble LPS was used at 10ng/µl. The graph represents the average + 1SD of three independent experiments. The asterisks represent statistically significant differences comparatively to OVAp (**P*< 0.05; ***P*< 0.01 *** *P*< 0.001).

These data suggest that when LPS is in a particulate form, but in a different cargo as particle antigen, there is no significant difference between these two stimuli (OVAp and OVAp + BSA=LPSp), either in MHC class-I and MHC class-II antigen presentation (*fig.37*). In opposition, when LPS is present in a soluble form, (*fig.36 and 37*), antigen presentation is induced in both MHC class-I and MHC class-II context, with higher magnitude for MHC class-II presentation. As a control, model particles with OVA labelled with a fluorescent dye were used, to ensure that the amount of antigen internalized was the same when sDCs where co-cultured with OVAp alone or with OVAp and BSAp. However, no significant differences were observed (*data not shown*). Our results suggest that soluble LPS is able to activate all the antigen presentation machinery (up-regulation of

molecules involved in antigen presentation – MHC class-I and II and co-stimulatoy molecules). However, when LPS is in particulate state, relies the concept of *"phagosome autonomous maturation"* in antigen presentation (Blander and Medzhitov, 2006a), where distinct phagosomes are processed individually on the same cell. Thus, the TLR agonist must be in same cargo as antigen to signal a specific pathogen-mediated antigen fate.

To better characterize the antigen cross-presentation phenotype, some "key" steps in antigen presentation should be dissected. We have proposed to characterize important mechanisms, such as uptake ratio, antigen processing and phagosome maturation mediated by antigen model particles. To control if the efficiency on antigen cross-presentation is due to different capacities on particle antigen internalization, we addressed the role of phagocytic ability on DCs mediated by LPS stimulus.

Phenotype characterization I - Uptake

It has been suggested that the efficiency of antigen cross-presentation is due to differences at phagocytic capacity level (Albert *et al.*, 1998). As the amount of antigen that was internalized by DCs could influence the antigen presentation, the question if TLR agonists (more specifically LPS) influenced this process should be addressed. The implication of TLR signaling in phagocytosis and antigen uptake has been described but is quite controversial (Diwan *et al.*, 2003; Khan *et al.*, 2007; Weck *et al.*, 2007). In order to address the possible involvement of TLR signaling in antigen uptake, phagocytosis of antigen model particles using OVA labeled with alexa 488 (OVA488) as model antigen was measured using a FACS-based assay.

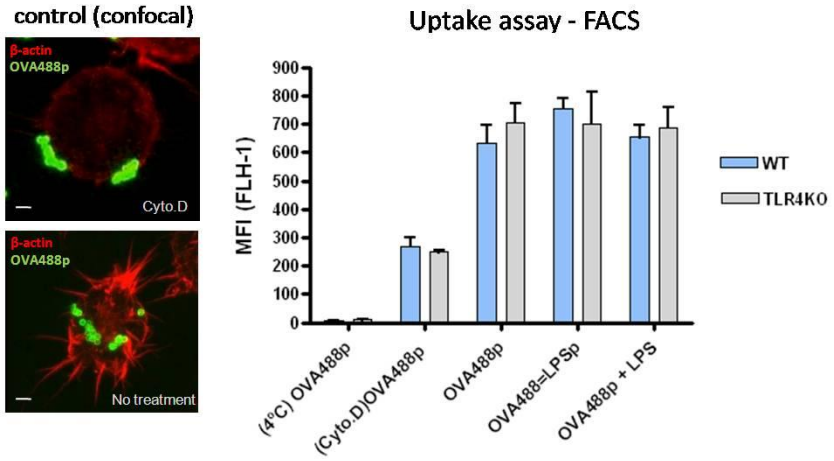


Fig.38: Uptake assay of model particles: FACS: Measurement of antigen uptake by sDCs from C57BL/6 WT and TLR4KO mice using green fluorescent OVA (OVA488) loaded particles (OVA488p and OVA488=LPSp). Particles were co-incubated with sDCs at 1:10 (sDC:particles) ratio for initial 10 min of pulse and 2hrs of chase (phagocytosis). Soluble LPS was used at 10ng/µl. Cyto. D (10nM) and LPS (10ng/ml) were incubated 1hr previously to particle addition. MFI in the FLH-1 channel was calculated for phagocytic cells. The graph represents the average + 1SD of three independent experiments. No statistically significant differences (P>0.05) were observed between amounts of fluorescent OVA488 model particles internalized by sDC from C57BL/6 WT and TLR4KO mice. Confocal images: Particles were co-incubated with BMDCS (plated in cover slips 12hrs before to allow adhering) from C57BL/6 mice at 1:10 (sDC:particles) ratio during 10 min (pulse). After 2 hrs of incubation (chase), PFA1% was added during 5 min and cells were washed with PBS. Cyto. D (10nM) was incubated 1hr previously to OVA=488p addition. Phalloidin red was added at 1:40 dilution during 20 min, and BMDCs mounted in coverslips with vectashield medium for confocal analysis with a 63x objective (Scale bar, 2 µm).

The FACS assay showed that the uptake capacity of sDCs was similar for OVA model particles (OVAp, OVA=LPSp and for OVAp + soluble LPS) and between WT and TLR4KO sDCs populations (*fig.38*). Therefore, TLR4 signaling does not affect in a significant way particle antigen internalization, when LPS is present either in same cargo as antigen or in a soluble form. As such, we may assume that the amount of antigen that reached the phagosomes by model particles is

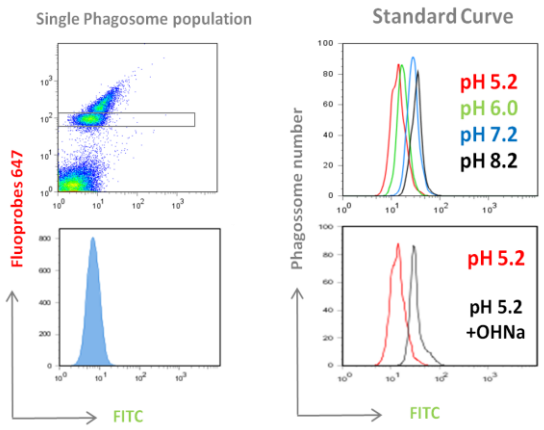
approximately the same. Therefore, the differences observed in antigen presentation studies should not be due to differences in phagocytosis uptake of model particles used.

Phenotype characterization II – Phagosome maturation (pH)

Efficient antigen processing in phagosomes requires limited and controlled antigen degradation, generating the correct array of peptides to be loaded on MHC molecules and to avoid total destruction by unspecific lysosomal proteases (Savina *et al.*, 2006). Endosomal pH regulation is one of the most direct ways to control the lysosomal proteases activity (Delamarre *et al.*, 2005) and it has been described to be involved in antigen presentation (Blander and Medzhitov, 2006a).

In order to address the role of LPS on pH of phagosome containing particle antigen, we proposed to measure phagosomal pH in DCs accurately by a FACS assay, using pH-sensitive particles, developed and already published by Amigorena´s lab (Savina *et al.*, 2006). Briefly, latex 3.0µm amino particles adsorbed with OVA were coated with pH-sensitive (Fluorescein isothiocyanate – FITC) and pH-insensitive (FluoProbe647) fluorescent dyes. After different time points of particles phagocytosis, the fluorescence intensity of the two dyes was quantified using a FACS-based assay. Population of DCs with an average of one particle internalized was selected on FluoProbe647 channel (FL4). The differences in pH was measured by deviations on FITC channel (FLH-1), reflecting the pH in the phagosomal environment. The absolute value of phagosomal pH was calibrated with a standard curve established in cells permeabilized with 0.1% Triton X100 and immersed in buffers of fixed calibrated pH (*Fig. 39-A*).

(A)



(B)

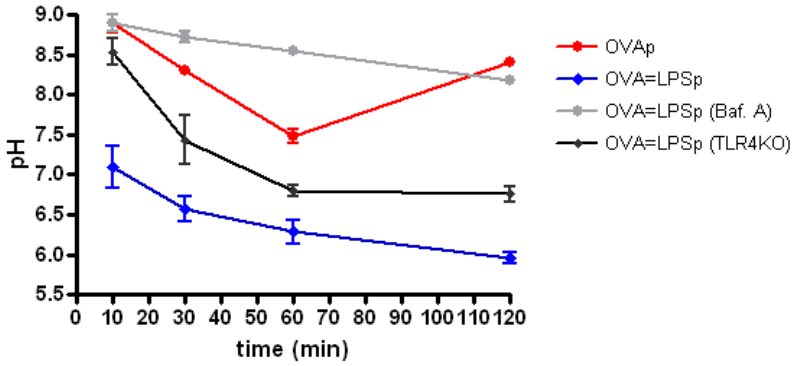


Fig.39: Phagosomal pH measurement FACS-based assay. (A) pH measurement method: BMDCs ($1x10^6$) from C57BL/6 WT and TLR4KO mice were allowed to phagocytose latex particles coated with pH sensitive (FITC) and insensitive (Fluoprobes647) fluorescent probes for 10 min of pulse and 120 min of chase (see experimental procedures for details). Upper left graph: Histograms represent BMDCs population gated on FLH-1 (FITC) *vs* FL4 (Fluoprobes647) plots. Population of cells with an average of one particle per cell was selected (same FL4 fluorescence) and FLH-1 shift measured. Upper right graph: Histograms represent BMDCs population gated on SSC *vs* FLH-1 plots. Calibration curve was achieved by using BMDCs permeabilized with 0.1% Triton X100 for 1min in buffer solutions with specific pH ranging from 5.2 to 8.2. Representative FACS analysis for phagosomal pH calibration, showing MFI of the pH-sensitive probe at different pH (after 10 min pulse + 120 min chase). NaOH were applied in BMDCs permeabilized with 0.1% Triton X100 in

buffer pH5.2, in order to neutralize the phagosomal pH and show the dynamic recover of fluorescence. **(B) pH phagosome assay**. Model particles covalently coupled with OVA were adsorbed with FITC (pH sensitive dye) and Fluoprobe647 (pH insensitive dye) in equimolar amounts for 1hr. LPS was adsorbed to OVAp loaded with pH dyes for 2hrs. BMDCs ($1x10^6$) from C57BL/6 WT and TLR4KO mice were allowed to phagocytose model particles (OVAp and OVA=LPSp) coated with pH sensitive and insensitive fluorescent probes for 10 min of pulse and 120 min of chase. Population with an average of one particle per cell was selected (same FL4 fluorescence) for each chase time and FLH-1 shift measured. 2µM Bafilomycin A1 (inhibitor of V-ATPase) was added 1hr previously. In contrast to others strategies for phagosomal pH measurements, the one used here analyzes independently 10.000 BMDCs, which means that at least 10.000 phagosomes were measured per condition in each experiment. The graph represents the average ± 1SD of three independent experiments.

As showed in pH phagosome assay (*fig. 39*), by reporting the mean fluorescence intensity in the different conditions to a standard curve (*fig. 39-A*), the "real time" pH values in phagosomes were determined. After 10 minutes of phagocytosis pulse a general pH decreasing phenotype during the chase time was observed. The pH in BMDCs phagosomes with OVAp after 10 minutes pulse followed by 10 minutes chase, was relatively alkaline (below 9.0), higher than the extracellular medium (pH~7.4). This means that during the 20 minutes after particles incubation, there was an active and sustained mechanism of phagosome alkalinization, probably due to NOX2 activity as described previously (Savina *et al.*, 2006).

Concerning the phagosomal pH with OVAp during the chase time, it drops as the phagosome matures, acidified until pH 7.5 in 60 minutes. It rose after 60 minutes, keeping phagosomes at higher pH values in an immature stage, recovering to the initial stage (pH~8.5) within 120 minutes (*fig. 39*).

The rate of phagosome acidification was significantly enhanced in phagosomes containing OVA=LPSp. Phagosomal pH~7.2 was achieved earlier in 10 minutes after chase and acidified further over a 120 minutes chase, reaching values around pH 6.0. Lower pH values as pH~6.5 were achieved in early 30 minutes of chase. The kinetic of phagosome maturation changed around after 30 minutes of chase,

and was kept at slower rate until longer time points of incubation (120 minutes), decreased only 0.5 units from pH 6.5 to pH 6.0 (*fig. 39*).

In the presence of Bafilomycin A, a V-ATPase inhibitor, the higher phagosomal acidification kinetics induced by OVA=LPSp was blocked. The pH decreased only 0.5 units from pH~8.9 – pH~8.4, during the entire chase time (120 minutes). This result could imply V-ATPase in phagosomal pH acidification mediated by LPS signaling. In the absence of TLR4 (BMDCs from TLR4 KO), the rate of phagosome maturation induced by OVA=LPSp particles was decreased, with a 0.8 pH units of difference at the end of chase time. A slower rate of phagosome maturation mediated by OVA=LPSp was observed, from pH 8.5 at 10 minutes to pH 7.0 in 120 minutes of chase. After 30 minutes, the pH achieved a "plateau" (lowest value - pH~6.8) and remained quite stable during the rest of chase. However, the phagosomal pH did not rise again to the initial stage as observed in absence of LPS stimulus on WT BMDCs, within 120 minutes (*fig. 39*). Therefore, the phagosome acidification related to OVA=LPSp may not totally dependent on TLR4-mediated signaling. Moreover, this observation implies TLR4 signaling on phagosomal acidification mediated by LPS

The control and regulation of phagosomal pH is mediated in majority by the function of two complexes, V-ATPase and NADPH Oxidase NOX2 (Savina *et al.*, 2006). Amigorena´s lab described NOX2 as a specific adaptation of DCs endocytic pathway to the antigen presentation function by causing an active and sustained phagosome alkalinization, keeping pH at relative high values (Savina *et al.*, 2006). The NOX2 generates ROS, causing transient phagosome alkalinization, in part through the consumption of protons in the phagocytic lumen (Lee *et al.*, 2003; Segal, 2005). To better address the role of LPS signal within the cargo on phagosome maturation, we proposed to measure the phagosomal ROS production in order to correlate with phagosomal pH data.

Phenotype characterization II – Phagosome maturation (ROS)

To address ROS production, specifically in phagosomes containing OVAp and OVA=LPSp, dihydrorhodamine 123 (DHR123), a dye that only emits fluorescence under oxidative conditions (Vowells *et al.*, 1995), was covalently linked to OVAp and OVA=LPSp. DHR123 is a pH stable molecule, thus the possibility of DHR123 degradation in phagosomal lumen could be excluded (Savina *et al.*, 2006).

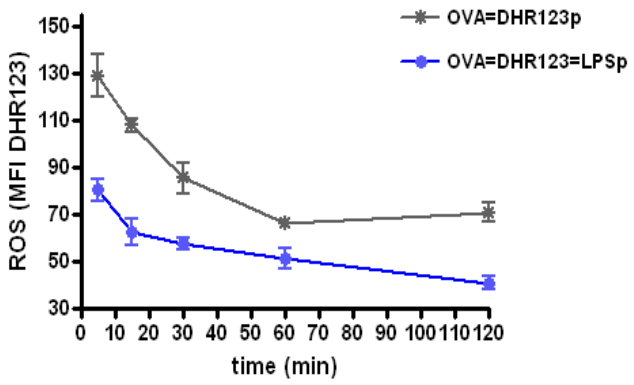


Fig.40: Phagosomal ROS measurement based assay. Measurement of phagosomal ROS production using DHR123 coupled to model particles (OVA=DHR123p and OVA=DHR123=LPSp). MFI of FLH-1 channel was calculated for phagocytic cells. $2x10^5$ BMDCs per time point were pulsed for 10 min in CO2 independent medium using 1:10 (BMDCs:particles) ratio and chased for 5 min, 15 min, 30 min, 60 min and 120 min. The graph represents the average ± 1SD of three independent experiments.

As shown by FACS analysis, DHR-coated particles became fluorescent after phagocytosis, showing the production of ROS in DCs phagosomes. Moreover, ROS production in phagosomes containing model particles decreased during the chase time (*fig.40*). This means that phagosomal environment in DCs is oxidative due to ROS generation. ROS production in phagosomes containing OVAp was higher comparing to phagosomes containing OVA=LPSp, even at earlier chase time points (5 minutes) and during the entire chase time (120 minutes) (*fig.40*). It seems that the production of ROS decreased during the phagosome maturation mediated by model particles and it was more evident in phagosomes containing

OVA=LPSp. After 60 minutes of chase, the ROS production on phagosomes containing OVAp seemed to stabilize, with a tendency to recover even with low rate. However, ROS production in OVA=LPSp phagosomes appeared to decrease continually until the end of chase, with a higher rate (*fig.40*). As a result, these data showed that when LPS is in same cargo as OVA particles mediates the reduction of ROS production in phagosomes, which could be related to the higher rate of phagosome maturation/acidification induced by OVA=LPSp during the experimental time points (*fig. 39*). These data support the evidence showed by the pH results, which suggested that "naked" OVAp kept the phagosomes in a immature stage comparing to OVA=LPSp which enhance phagosome maturation (*fig.39 and 40).*

Phenotype characterization III – Phagosome maturation (Confocal)

DCs have been shown to have lower contents of lysosomal proteases and acidification mechanism (Delamarre *et al.*, 2005). In order to link phagosomal maturation with the pH regulation and ROS production profiles obtained above with model particles (*fig.39 and 40*), the intracellular localization of model particles was examined by confocal microscopy. Phagosome maturation ratios with different cargos was assessed by fluorescent microscopy, by qualitatively merging the phagosomes containing OVA488 fluorescent particles (OVA-Alexa488 - pH stable fluorescence) and lysosomes labeled with Lysotracker Red (Invitrogen). This colocalization could be quantified by determining the total colocalization area (pixel quantification using the ImageJ plugin RG2B colocalization which creates a binary mask for colocalizing pixels). Lysotracker probes have high selectivity for labeling and tracking acidic organelles in live cells, as the result of protonation, results in a pH dependent increase of fluorescent intensity (Shiratsuchi *et al.*, 2004).

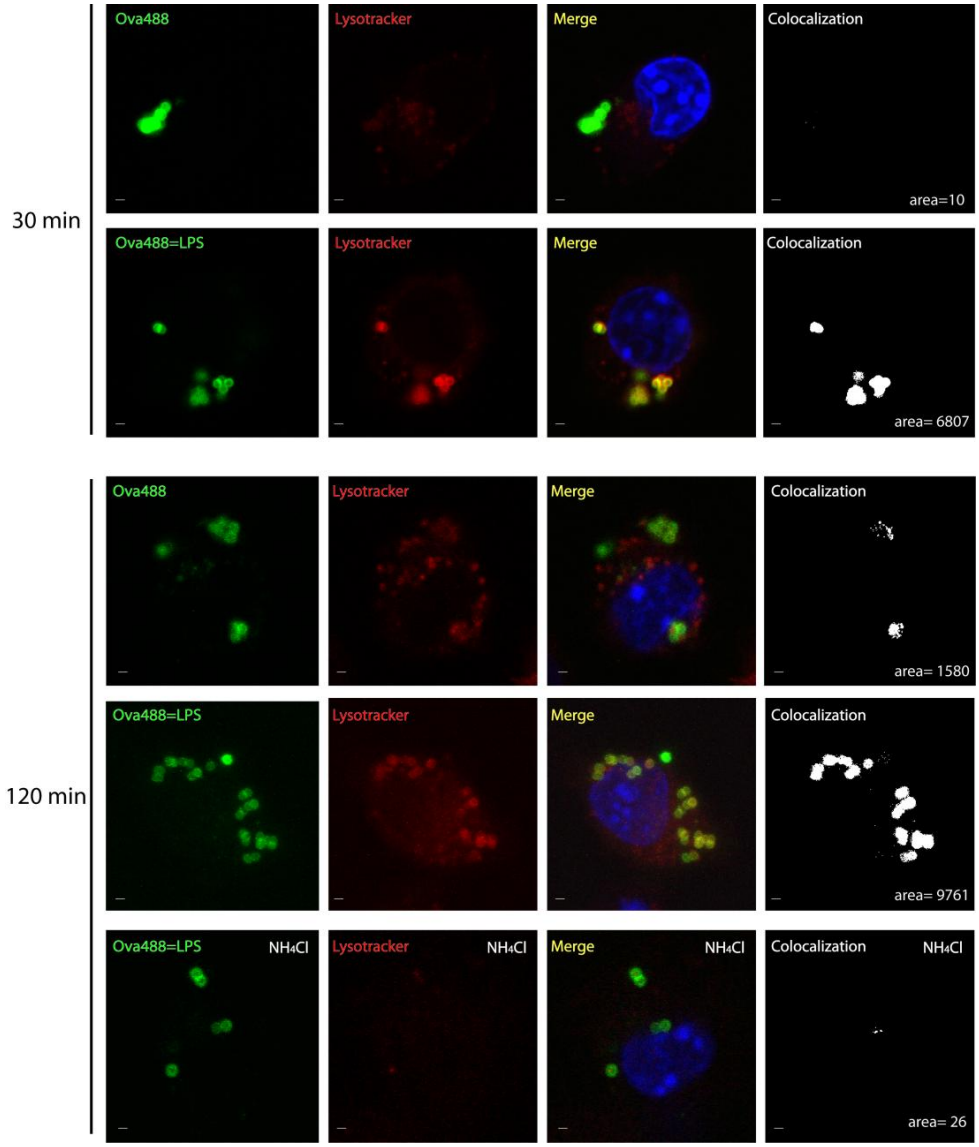


Fig.41: Confocal imaging based phagosomal maturation assay. Representative fluorescent micrographs showing phagocytosis of OVA488 fluorescent particles (green) and lysosomes (red). BMDCs from C57BL/6 mice were incubated with 1µm OVA488 fluorescent polystyrene model particles (OVA488p and OVA488=LPSp) at a ratio (BMDCs:particles) of 1:10 for 15 min, washed, and further incubated for either 30 min or 120 min. BMDCs were stained with Lysotracker Red to label lysosomes and DAPI (blue) to label nuclei and fixed with 4% of paraformaldehyde. BMDCs

were washed and mounted in coverslips with vectashield medium for confocal analysis with a 63x objective. Merged images are shown in yellow and colocalization in white with values of total pixel colocalization. Slides were examined by confocal microscopy to determine the intracellular localization. The color images represent particles (green), lysosomes (red), lysosomes fused with phagosomes containing particles (yellow). NH_4Cl (50mM) were used as a weak base that raises the pH of acidic compartments to disrupt the low pH at lysosomes and worked as a loading control. Images are representative at least from three independent experiments (Scale bar, 1µm).

The confocal data showed that phagosomes containing OVA488=LPSp mature with higher ratio than those containing OVA488p (*fig.41*). This observation is independent of the phagocytic index, which is not different for OVAp and OVA=LPSp and not markedly agonist specific (*fig.38*). After 30 minutes of incubation, OVAp did not colocalize with lysosomes and phagosomes exhibited an alkaline environment where the pH is around 8.5 (*fig.39*). Whereas the majority of OVA=LPSp colocalize with a higher extend (*Fig.41*) showing an average pH of 6.5 (*fig.39*). Even after 120 minutes incubation, phagosomes containing OVAp had reduced values of colocalization with lysosomes (*Fig.41*), with higher pH values around 8.5 (*fig.39*). In contrast, phagosomes containing OVA=LPSp completely merged with lysosomes, indicating that internalized particles were routed into lysosomes (*Fig.41*), where phagosomal pH reaches lower values as pH 6.0 (*fig.39*). These data indicate that LPS-containing phagosomes completely mature into phagolysosomes. As a phagosomal maturation control, NH_4Cl was used as a weak base that raises the pH of acidic compartments to disrupt the low pH. Therefore, the colocalization between OVA488=LPSp and lysosomes was abolished in the presence of NH_4Cl (*fig.41*), suggesting that the fusion was mediated by lower pH values. In addition, OVA488=LPSp and lysosomes appears to be concentrated on the periphery of the cell nucleus (labeled blue by DAPI), a feature of late endosomal and lysosomal compartments (Tran and Shen, 2009). Moreover, these results are reliable with the phagosomal pH profiles obtained before (*fig.39*).

Phenotype characterization IV – Antigen Processing / Degradation

Phagosomal pH regulation (either by V-ATPase or NOX2) is the main mechanism used by DCs to "protect" peptides from complete degradation, with the ultimate goal being to generate T-Cell epitopes (Savina *et al.*, 2006; Trombetta *et al.*, 2003). Therefore, we next sought to evaluate the role and functional consequences of such a drop in phagosomal pH and ROS production mediated by LPS in antigen processing/degradation.

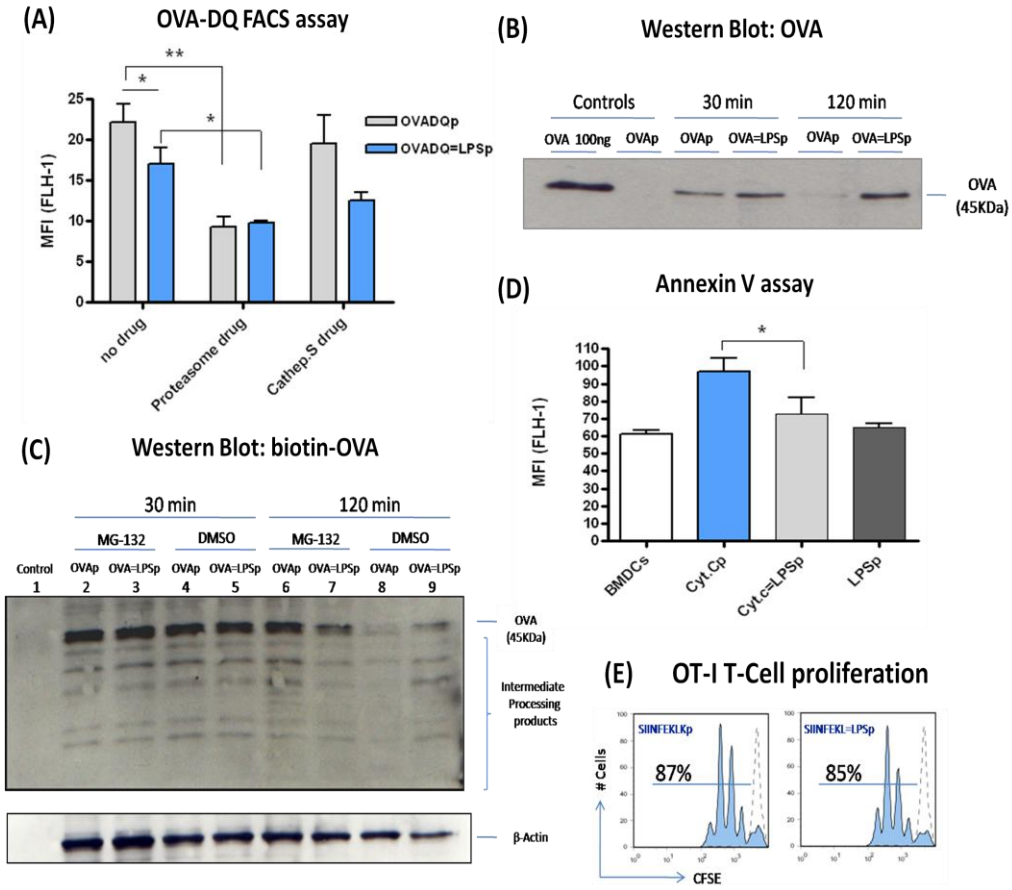


Fig.42: Antigen processing/degradation based assays. A) Quantitative antigen processing/ degradation was examined by FACS by measuring the MFI of DQ-OVA in the FLH-1 channel. sDCs ($1x10^5$) from C57BL/6 mice were challenged with particles containing DQ-OVA (DQ-OVAp) and DQ-OVA + LPS (DQ-OVA=LPSp) at 1:10 (sDC:particles) ratio during 2hr. DQ-OVA (100ng/ml) and LPS (10n/ml) were adsorbed to 1.0 µm polystyrene particles during 2hr. Proteasome inhibiting drug, MG-132 (Z-leu-Leu-Leu-CHO) and the inhibitor of Cathepsin S (Z-FL-COCHO), were added 1hr previously. The asterisks represent statistically significant differences (**P*< 0.05; ***P*< 0.01). **B)** sDCs ($1x10^5$) from C57BL/6 mice were incubated with model particles (OVAp and OVA=LPSp) at 1:10 (sDC:particles) ratio during 30 and 120 min. OVA were detected using anti-OVA monoclonal antibody (Abcam). Bradford normalization was performed. The chase with particles was performed without washing the remaining particles after pulse (long course kinetic). 30 μg of total protein extracts from sDCs were separated by 10% SDS-PAGE. **C)** sDCs ($1x10^5$) from C57BL/6 mice were pulsed with streptavidin particles coupled with OVA-biotin (OVAp) and OVA-biotin + LPS (OVA=LPSp) at 1:10 (sDC:particles) ratio for 10 min, washed extensively and chased during 30 min and 120 min. OVA-biotin (100ng/ml) and LPS (10ng/ml) were adsorbed to 1.0 µm polystyrene particles during 2hr. OVA-biotin from whole cell lysates after particles phagocytosis was detected by Western blot using Sav-HRP from ELISA kit. As a loading control β-actin staining was performed. Lanes: *1)* Control: only sDCs *2)* OVAp 30 min + MG132 *3)* OVA=LPSp 30 min + MG132 *4)* OVAp 30 min *5)* OVA=LPSp 30 min *6)* OVAp 2hrs + MG132 *7)* OVA=LPSp 2hrs + MG132 *8)* OVAp 2hrs *9)* OVA=LPSp 2hrs. 30 μg of total protein extracts from sDCs were separated by 10% SDS-PAGE. **D)** Antigen retro-translocation assay (availability in cytosol) using cyt *c* particles (apoptosis assay). Horse Heart cyt *c* and LPS was adsorbed to 1.0µm polystyrene particles during 2hr. BMDCs ($1x10^5$) from C57BL/6 mice were challenged with particles loaded with cyt *c* (Cyt.cp) and particles loaded with cyt *c* and LPS (Cyt.c=LPSp) at 1:10 (sDC:particles) ratio during 30 min and washed extensively. The amount of free cyt *c* in cytosol was measured by apoptosis assay using Annexin V-FITC kit at 18hrs. Apoptosis was examined by FACS measuring the MFI in the FLH-1 channel. The asterisk represents statistically significant differences (**P*< 0.05). **E)** sDCs ($2.5x10^4$) from C57BL/6 mice were co-incubated with model particles (SIINFEKLp, SIINFEKL=LPSp) at 1:2 (sDC:particles) ratio, and OT-I T-Cells ($1x10^5$) for 3 days. T-Cell proliferation was measured using CFSE staining by FACS. Histograms represent T-Cell population gated on SSC *vs* CFSE plots. Open grey line plots represent control OT-I T-Cells that do not divide and blue filled plots represent OT-I T-Cells proliferation under specific stimulus. Numbers represent the percentages of the proliferating cells of total OT-I T-Cells. These data are representative from one experiment repeated at least three times with similar results.

Using the performed assays, we are not able to distinguish between antigen processing (peptide epitope generation) and degradation (random cleavage). DCs have reduced expression levels and low recruitment of proteolytic enzymes to phagosomes, which leads to low lysosomal proteolysis (Delamarre *et al.*, 2005; Jancic *et al.*, 2007) and a limited phagosomal acidification by V-ATPase (Savina *et al.*, 2006; Trombetta *et al.*, 2003). Therefore, we next sought to address the role of proteasome as an important protease catalytic complex in particle antigen

processing/degradation and cross-presentation in DCs. Thus, we have addressed the role of LPS on particle antigen processing/degradation using different molecular approaches as follows:

i) As starting point, antigen degradation was followed by FACS analysis using DQ-OVA™ (a self-quenched conjugate of OVA that exhibits bright green fluorescence upon proteolytic degradation) as a model antigen. The data showed that after 2 hrs of chase, the overall particle antigen processing/degradation was reduced when LPS (similar results were obtained in the presence of CpG – *data not shown*) was in same cargo as antigen (*fig.42-A*). To address the role of proteasome, as a key step in antigen processing for antigen cross-presentation (Guermonprez and Amigorena, 2005; Houde *et al.*, 2003; Vyas *et al.*, 2008), we used a proteasome inhibitor, MG-132 (Z-leu-Leu-Leu-CHO), at lower concentrations as possible to avoid unspecific inhibition of other proteases. MG-132 is highly specific, fully reversible inhibitor of proteasomal proteolytic activity (bind specifically 20S subunit). When proteasome activity was inhibited, at 120 minutes of incubation, there was a significant reduction of antigen processing/degradation, either on OVAp and OVA=LPSp for similar levels (*fig.42-A*). This suggests that proteasome plays an important and crucial role on particle antigen processing/degradation. In the presence of Z-FL-COCHO (Cathepsin S - Cat.S - inhibitor that do not block the activity of non-proteasomal serine proteases) antigen processing/degradation was reduced but not as evident as observed upon proteasome inhibition (*fig. 42-A*). These results could indicate a minor role of Cat.S in particle antigen degradation, either when an LPS stimulus occurs. Moreover, "blocking" an important endoprotease of vacuolar pathway degradation (Cat.S), the role of proteasome degradation on particulate antigen degradation was highlighted, existing at higher level when LPS stimulus was not present (*fig.42-A*).

ii) As a simple approach, particle antigen degradation (OVAp and OVA=LPSp) was followed by immunobloting detection, using 30 minutes and 120 minutes for the time course experiment without pulse (antigen was continuously internalized by DCs) (*fig.42-B*). In agreement with results obtained previously by DQ-OVA measurement (*fig.42-A*), western blot for OVA antigen showed a higher antigen processing/degradation for OVAp comparing to OVA=LPSp already at 30 minutes, being clearer at 120 minutes of incubation (*fig.42-B*). These differences observed on the antigen levels were not due to differences in antigen uptake, as it was shown previously (*fig.38*).

iii) To support previous data and to better address the role of proteasome complex in particulate antigen processing/degradation, biotinylated OVA was conjugated to model particles. Consequently products of antigen degradation could be followed by immunobloting. Proteasome inhibitor was used to reveal the role of this protease complex in particle antigen processing/degradation. Using OVAp and OVA=LPSp, in combination with MG-132, during the first 30 minutes of chase there was no significant differences on antigen processing/degradation (*fig.42-C*). However, they were observed using a different immunobloting approach described previously (*fig.42–B*). Therefore, this could be due to OVA biotinylation that could influence their cleavage, or simply due to experimental differences. When a continuously challenge with particle antigen was used (*fig.42-B*), the amount of antigen internalized was higher and the differences on antigen processing/ degradation between OVAp and OVA=LPSp could be better addressed. At 120 minutes, almost total antigen degradation was observed for OVAp but in a lesser extended for OVA=LPSp (*fig.42–B*). Analyzing the immunobloting data on the end of chase (120 minutes), it was evident that OVA protein and intermediate processing products occurred at higher levels compared to those observed when LPS was in same cargo as antigen (*fig.42-C*), confirming our previous results (*fig.*

42-B). However, in the presence of MG-132, OVA processing/degradation was inhibited significantly, but in a lower level when LPS was in same cargo. The degradation of OVAp was apparently totally reverted but in a minor extend for OVA=LPSp (*fig. 42-C*).

This could highlight the role of proteasome on particle antigen processing/ degradation when a "bacterial" signal such as LPS is absent. Moreover, particle LPS may have a role on the control of proteasome-independent antigen processing/degradation, such as vacuolar endoproteases.

iv) Reduced antigen processing/degradation in the presence of LPS may result from a decrease in proteasome activity. Alternatively, it may result by a reduction of antigen escape from the phagosome into the cytosol (retro-translocation), either by channel recruitment or kinetic function. The nature of this phagosomal "pore" remains controversial, although it appears to have a size limited. Internalized antigens with molecular masses of <40 kDa have been reported to gain access to the cytosol rapidly (Rodriguez *et al.*, 1999), making small proteins ideal to study the amount of antigen that was available in cytosol. To relatively quantify the amount of internalized antigen available in cytosol, which results from the egress from phagosomes, cytochrome *c* (cyt *c*) a soluble 13-kDa mitochondrial protein was used. As proof of principle, when cyt *c* is available in the cytosol apoptosis was induced (Cai *et al.*, 1998). As this assay was not suitable for sDCs (due to the short half-life in culture) BMDCs were employed as a model. Given the strict dependence of efficient antigen cross-presentation mechanism on the capacity to transfer exogenous antigens to the cytosol, exogenous cyt *c* would preferentially induce apoptosis when the mechanism of retro-translocation is more efficient (Hao *et al.*, 2005; Schafer and Kornbluth, 2006). Our data showed that when LPS is in same cargo as cyt *c* particles, lower amounts of cyt *c* were available in the cytosol (less apoptosis), comparing to cyt *c* particles (*fig. 42-D*). Lower levels of apoptosis

could be due either higher degradation or to lower rates of cyt *c* escape from phagosomes into cytosol. Supporting previous data, where antigen degradation occurs at higher efficiency in the absence of LPS (*fig.42- A, B and C*) and the inhibition of proteasome almost completely abolished antigen degradation (*fig.42- A and C*), we have observed less cyt *c* in cytosol (apoptosis) in LPS-containing particles (*fig. 42-D*). In addition, our previous data showed that phagosome maturation occurs at a higher rate in the presence of LPS containing particles (*fig.39 and 41*). Therefore we hypothesized that fewer antigen amounts could escape from phagosomes to cytosol, due to maturation kinetics, when LPS is present. These results are then in accordance with the low apoptosis level observed in this assay, when LPS is in same cargo as cyt *c* (*fig. 42-D*). Thus, we propose that reduced antigen processing/degradation mediated by LPS could be due not only to low proteasomal activity but also to low retro-translocation efficiency.

v) Using SIINFEKL peptide as a model antigen, the retro-translocation step to the cytosol could be bypassed, as well as the requirement of the proteasome processing for antigen cross-presentation. Our data showed that the particle antigen cross-presentation is similar either in the presence or absence of LPS (*fig. 42- E*). LPS in same cargo as antigen did not impair SIINFEKL peptide cross-presentation, whereas in same cargo as OVA did (*fig. 33*). The influence of LPS in antigen retro-translocation and/or proteasome activity was thus supported by this assay.

Phenotype characterization V – The role of p38 in cross-presentation

Blander and Medzhitov have found that activation of the p38 mitogen-activated protein (MAP) kinase downstream of TLRs was needed for accelerated phagosome maturation and MHC class-II antigen presentation (Blander and Medzhitov, 2004, 2006a). As a downstream signal molecule of TLRs, and specifically TLR4, it makes sense to further address their role on the antigen cross-presentation

phenotype observed, using a p38 MAP kinase inhibitor, as other inhibitor drugs for the other MAPKs, ERK and JNK.

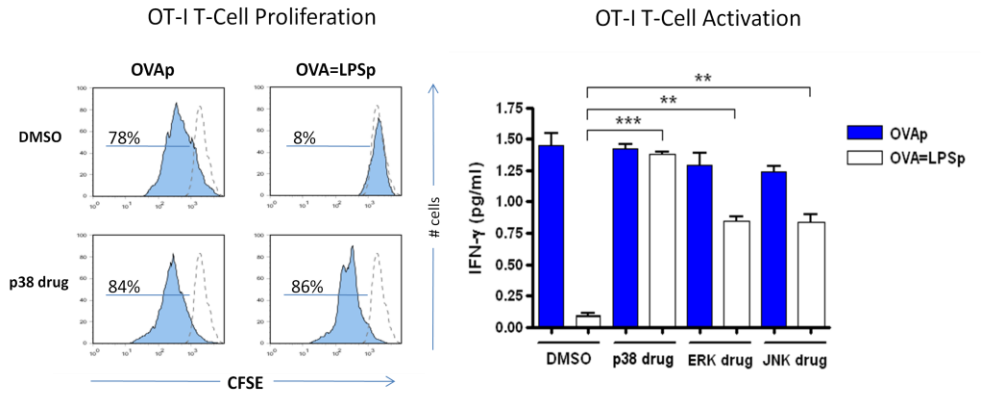


Fig.43: Particle antigen cross-presentation based assay using MAPKs inhibiting drugs. sDCs ($2.5x10^4$) from C57BL/6 mice were incubated previously with inhibiting drugs for major MAPK pathway (p38, ERK and JNK) and DMSO as negative control for 2hr. Model particles (OVA and OVA=LPS) were incubated at 1:10 (sDC:particles) ratio. Left graph: OT-I T-Cells ($1x10^5$) were co-cultured with sDCs for 3 days. T-Cell proliferation was measured using CFSE staining by FACS. Histograms represent T-Cell population gated on SSC *vs* CFSE plots. Open grey line plots represent control OT-I T-Cells that do not divide and blue filled plots represent OT-I T-Cells proliferation under specific stimulus. Numbers represent the percentages of the proliferating cells of total OT-I T-Cells. These data are representative from one experiment repeated at least three times with similar results. Right graph: T-Cell activation was addressed by ELISA for IFN-γ secretion at 60-65hrs by OT-I T-Cells. The graph represents the average + 1SD of three independent experiments. The asterisks represent statistically significant differences comparatively to OVA=LPSp in absence of inhibiting drugs (*P< 0.05; **P< 0.01; *** P< 0.001).

These data showed that in the presence of an inhibitor of p38 phosphorilation (SB203580), similar levels of OT-I T-cell proliferation / activation were achieved for both OVAp and OVA=LPSp (*fig. 43*), reversing the inhibitory effect on antigen cross-presentation mediated by LPS on same cargo as OVA particles. Therefore, this result indicates that p38 activation might have a major role on antigen cross-presentation impairment mediated by LPS signaling. Additionally, inhibiting drugs

for the others MAPK, JNK (SP600125) and ERK (PD98059), have also been used, but the effect on recovering of the phenotype was not as evident as shown with the p38 inhibiting drug (*fig.43*). Upon activation of TLR4, p38 becomes phosphorylated and IkB-α is degraded, players of two different main pathways (Fitzgerald *et al.*, 2003). Therefore we decided to evaluate p38 phosphorylation state and IkB-α expression by protein immunoblot, as a manner to measure downstream TLR4 signaling activation mediated by particle LPS stimulus.

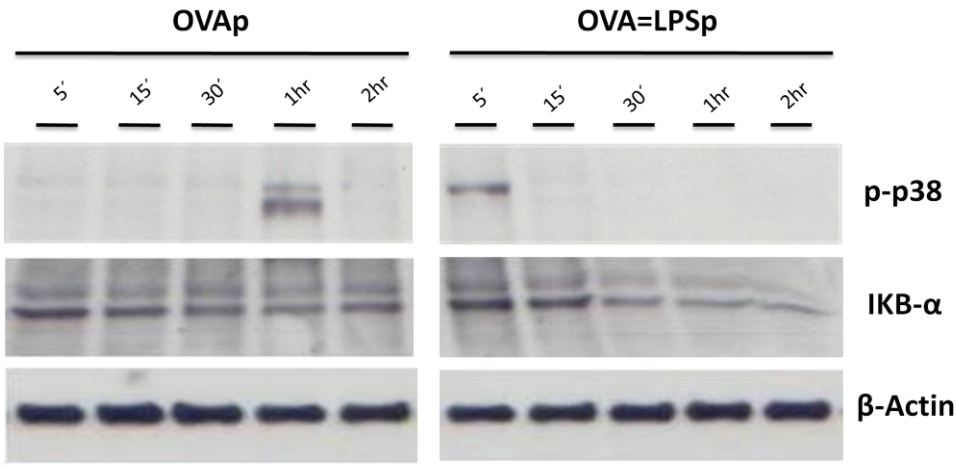
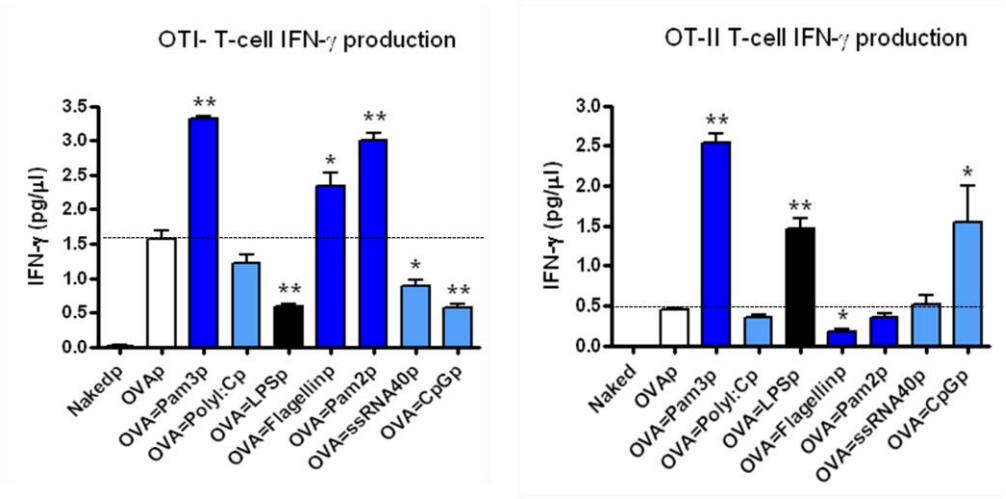


Fig. 44: Western blot for p-p38 and IKB-α upon model particles stimulation. sDCs ($1x10^5$) from C57BL/6 were pulsed with 1:10 (sDC:particles) ratio with OVAp and OVA=LPSp for 15 min, and chased for indicated time points (5 min, 15 min, 30 min, 1hr and 2hr). Whole cell lysates were analyzed for p38 phosphorylation and NF-kB activation (degradation of IkB-α) by WB using 1:200 dilution of anti-p-38 and IKB-α antibodies (Abcam). 30 μg of total protein extracts from sDCs were separated by 10% SDS-PAGE. β-actin staining was used as loading control (bottom).

These data showed that in the presence of OVA=LPSp, p38 phosphorylation and IKB-α degradation occurs with higher kinetics comparing to OVAp (*fig. 44*). Indeed, LPS on particulate state, effectively induce TLR4-mediated signaling, as showed by p38 and NFk-B signaling transduction pathways activation.

Phenotype characterization VI – The role of other TLRs agonists

To address if the effect was "transversal" to other TLRs, agonists for different TLRs (Pam3, PolyI:C, LPS, Flagellin, Pam2, ssRNA40 and Cpg) were used coupled to OVA particles, using a sonication protocol as described by Yates *et.*al, (Yates and Russell, 2005).



Ligand	TLR	Pathway	MHC-I	MHC-II
Pam3	TLR1/2	NF-kB / MAPK	+	+
PolyI:C	TLR3	IFN type I	-	±
LPS	TLR4	NF-kB/ MAPK – IFN type I	-	+
Flagellin	TLR5	NF-kB / MAPK	+	±
Pam2	TLR2/6	NF-kB / MAPK	+	±
ssRNA40	TLR7	IFN type I	-	+
CpG	TLR9	IFN type I	-	+

Fig.45: INF-γ based T-Cell activation assay: TLR agonist model particles antigen presentation. T-Cell activation was addressed by ELISA for INF-γ secretion at 60-65hrs by OT-I T-Cells (left graph) and OT-II T-Cells (right graph) in response to sDCs ($2.5x10^4$) from C57BL/6 mice given model particles. The different TLR agonists were adsorbed to OVAp by a sonication protocol during 20 min. Naked particles, OVAp, OVA=Pam3p, OVA=PolyI:Cp, OVA=LPSp, OVA=Flagellinp, OVA=Pam2p, OVA=ssRNA40p and OVA=CpGp were used. Model particles were incubated at 1:10 (sDC:particles) ratio. The graph represents the average + 1SD of three independent experiments. The asterisks represent statistically significant differences comparatively to OVAp (**P*< 0.05; ***P*< 0.01). The table represents the principal pathways activated by engagement of a specific TLR agonist, and the relative induction of MHC class-I presentation *vs* MHC class-II presentation.

These data showed that different TLR might have different roles and lead to different outcomes in antigen presentation pathways. Moreover, it seems that there is a pattern in the pathways induced by particulate TLR agonist and the outcome of antigen presentation (*fig.45*). Thus, TLR agonist that preferentially signals through MAPK/NF-kB pathways, such as Pam3, Flagellin and Pam2 seems to induce antigen cross-presentation. In opposition, TLR agonists that preferentially signal through IFN-Type I pathway, such as ssRNA40, polyI:C and CpG seem to inhibit antigen cross-presentation (*fig.45*). TLR4 is unique among TLRs, as it can signal through both MyD88 and TRIF adaptors. Recent work done by Kagan and colleagues has shown that TLR4 could signal through the two adaptors when in different locations and TRIF pathway is preferentially induced when TLR4 is internalized into endosomes (Kagan *et al.*, 2008). This could indicate that LPS-containing particles signal preferentially through TRIF dependent pathway when it are internalized. This hypothesis correlates with our results obtained for other agonists that preferential signal trough IFN type-I pathway, where antigen cross-presentation is inhibited by (*fig. 45*). To further clarify the previous results, *E.coli* expressing OVA and apoptotic cells loaded with OVA were used to address the effect of physiological stimuli based on different sources of ligands.

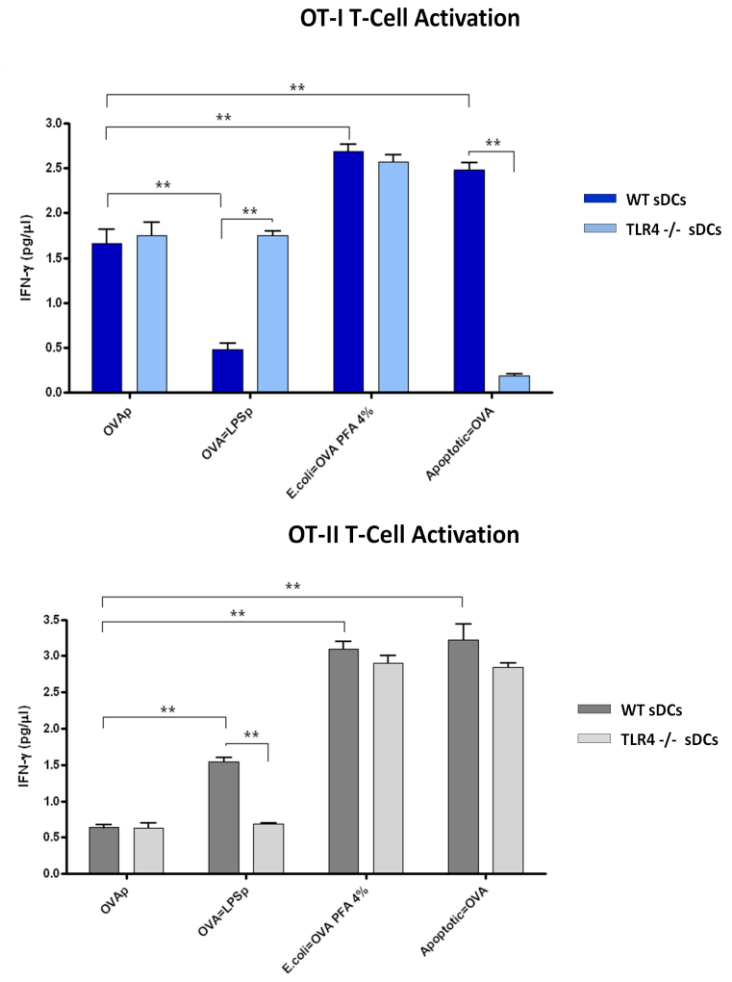


Fig.46: INF-γ based T-Cell activation assay using: Antigen presentation of model particles, *E.coli* expressing OVA and apoptotic cells OVA-loaded by WT and TLR4 KO DCs. T-Cell activation was addressed by ELISA for INF-γ secretion at 60-65hrs by OT-I T-Cells (upper graph) and OT-II T-Cells (lower graph) in response to sDCs ($2.5x10^4$) from C57BL/6 WT and TLR4KO mice stimulated with OVAp, OVA=LPSp, *E.coli* expressing OVA (*E.coli*=OVA*)* and apoptotic cells OVA-loaded (Apoptotic=OVA). Model particles, *E.coli* and apoptotic cells were incubated at 1:10 (sDC:particles) ratio. The graph represents the average + 1SD of three independent experiments. The asterisks represent statistically significant differences (**P*< 0.05; ***P*< 0.01).

As shown previously by the proliferation data (*fig. 34*), similar levels of OT-I T-Cell activation were achieved for both OVAp and OVA=LPSp using sDCs from TLR4KO mice (*fig.46*). Thus the inhibitory effect of LPS on same cargo as OVA particles was abolished. In the case of MHC class-II presentation, in the absence of TLR4, the OVA=LPSp induced similar levels of antigen presentation as for OVAp (*fig.46*). Therefore, the inducible effect of LPS observed on MHC class-II particle antigen presentation is abolished in absence of TLR4. Using *E. coli* expressing OVA, antigen presentation was induced at higher levels in both MHC class-I and MHC class-II comparing to OVA particles. Moreover, there are not significantly changes on antigen presentation mediated by *E. coli* when sDCs deficient on TLR4 were used (*fig.46*). It is possible that *E.coli* could induce a different pattern of receptors, resulting in a general inductor effect of antigen presentation.

When *u.v.* irradiated cells loaded with OVA were used, antigen presentation was induced in both MHC class-I and MHC class-II comparing to OVA particles. Surprisingly, in the absence of TLR4, antigen cross-presentation of dead cells loaded with OVA was inhibited but not the MHC class-II presentation (*fig.46*). This could be due to specific danger signals that are produced during *u.v.* irradiation, which may stimulate TLR4 in a way, inducing antigen cross-presentation.

As mentioned before, TLR4 could signal through two adapters, therefore the effect of LPS in antigen cross-presentation could be MyD88 dependent, TRIF dependent or both. Our previous data indicate that the inhibitory mechanism could be mediated mainly by a TRIF dependent mechanism (*fig.45*). To address this hypothesis, sDCs from MyD88KO mice were used to confirm the role of this adaptor in antigen cross-presentation phenotype, mediated by particulate LPS in same cargo as antigen.

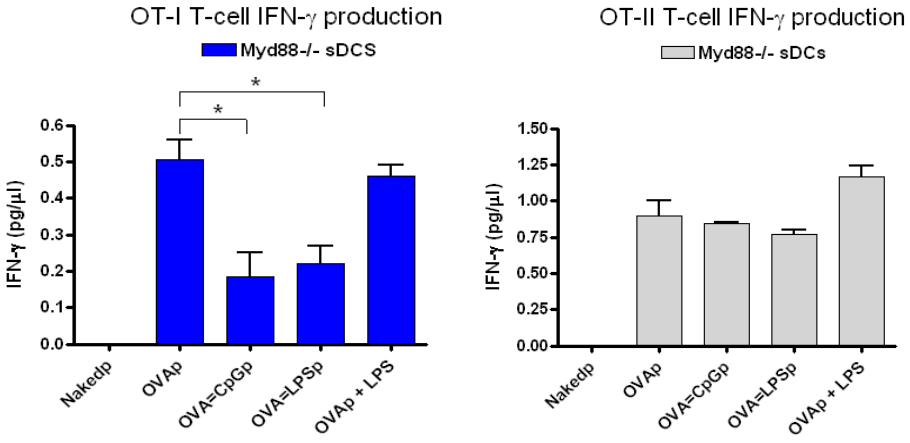


Fig.47: INF-γ based T-Cell activation assay: Antigen presentation of model particles by MyD88 KO DCs. T-Cell activation was addressed by ELISA for INF-γ secretion at 60-65hrs by OT-I T-Cells (left graph) and OT-II T-Cells (right graph) in response to sDCs ($2.5x10^4$) from MyD88KO mice in the presence of different particulate antigens: Naked particles, OVAp, OVA=LPSp and OVA=CpGp. Model particles were incubated at 1:10 (sDC:particles) ratio. The graph represents the average + 1SD of three independent experiments. The asterisks represent statistically significant differences comparing to OVAp (*P< 0.05).

The basal antigen cross-presentation capacity of OVA particles by sDCs from MyD88KO mice is lower when compared with WT sDCs (~3 times less), not verified for MHC class-II antigen presentation (*fig.46 and 47*). These data showed that the enhanced effect of particulate LPS and CpG in MHC class-II presentation (*fig.45*) is abolished in MyD88 deficient sDCs (*fig.47*). The antigen cross-presentation phenotype of model particles mediated by MyD88 deficient sDCs is comparable with WT sDCs. Indeed, similar inhibitory phenotype of antigen cross-presentation were observed for OVA=LPSp and OVA=CpGp (*fig.45*), even in absence of MyD88 adapter (*fig.47*). Therefore, these results suggest that signaling through MyD88 adapter by TLR4 is not responsible for the abolishment effect on antigen cross-presentation mediated by LPS-containing particles. Unexpectedly, in absence of MyD88 adaptor, CpG impairs particle antigen cross-presentation. As

MyD88 is the only adaptor known for TLR9, this effect on antigen cross-presentation could be due to signaling through another adaptor not described so far. The impairment on antigen cross-presentation mediated by CpG-containing particles be through other receptor is excluded by previous data, where KD of TLR9 revert the abolishment of antigen cross-presentation (*fig.35*).

Phenotype characterization VII – Cytokines

Cytokines has been described as an important signal on the outcome of immune response, mainly by controlling of T-Cell activation (Macagno *et al.*, 2007). In order to address the effect of our model particles on cytokine production, Inflammatory cytokines such as IL-6, IL-12, TNF-α, and IFN type I cytokines such as IFN-β, were measured upon stimulation with model particles, by ELISA assay.

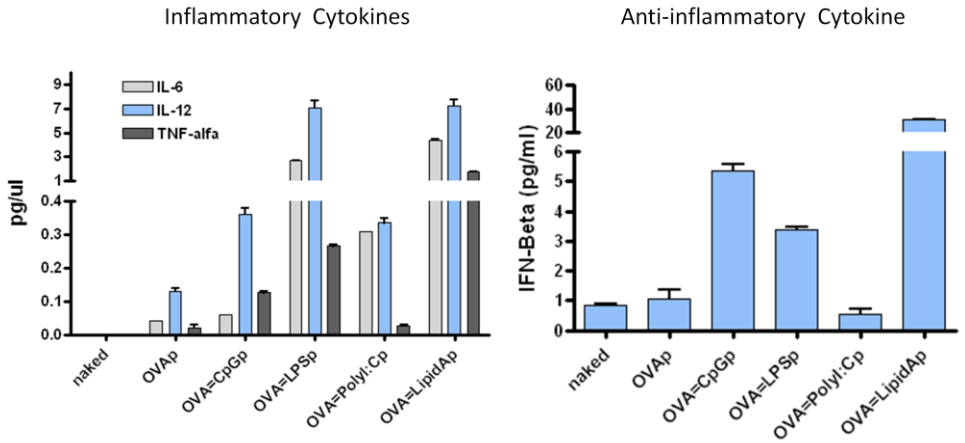


Fig.48: Inflammatory and anti-inflammatory cytokine secretion by DCs in response to model particles. ELISA measurement of cytokine secretion by sDCs ($2.5x10^4$) from C57BL/6 mice at 12 hrs after challenged with model particles. Left graph: Inflammatory cytokines (IL-6, IL-12 and TNF-α); Right graph: Anti-Inflammatory cytokines (IFN-β). Model particles: Naked particles, OVAp, OVA=CpGp, OVA=LPSp, OVA=PolyI:Cp and OVA=LipidAp were co-incubated at 1:10 (sDC:particles) ratio. The graph represents the average + 1SD of three independent experiments.

As expected, OVA particles loaded with TLR agonists induced higher levels of co-stimulatory cytokines (IL-6, IL-12 and TNF-α) when compared with "naked" OVAp. However, TNF-α levels were not induced by OVA=PolyI:Cp (*fig.43*). IFN-β, one of the major cytokines produced upon IFN-type I pathway activation, was also induced by particulate TLR agonists, but with a higher extent for OVA=LipidAp (*fig.48*). As described, monophosphoryl LipidA (MPLA) is an agonist of TLR4 that induces particularly TLR4/TRIF-dependent pathway (Mata-Haro *et al.*, 2007). To address the possible effect of IFN-β on particle antigen cross-presentation impairment mediated by TLR4 agonists (more specifically LipidA), we made use of soluble rIFN-β in combination with OVAp.

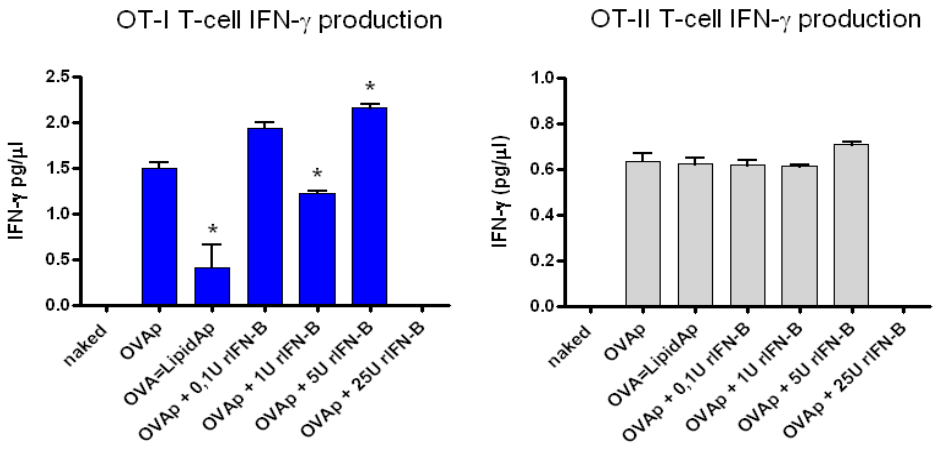


Fig.49: INF-γ based T-Cell activation assay: Antigen presentation of model particles using soluble rIFN-β. T-Cell activation was addressed by ELISA for INF-γ secretion at 60-65hrs by OT-I T-Cells (left graph) and OT-II T-Cells (right graph) in response to sDCs ($2.5x10^4$) from C57BL/6 mice given particulate antigens: Naked particles, OVAp and OVA=LipidAp. In some conditions, OVAp were co-delivered with a range (0.1–25U) of soluble rIFN-β concentration. 1U of rIFN-β corresponds to ~50 pg/ml. Model particles were incubated at 1:10 (sDC:particles) ratio. The graph represents the average + 1SD of three independent experiments. The asterisks represent statistically significant differences comparing to OVAp (*P< 0.05).

These data showed that OVA=LipidAp have a similar antigen cross-presentation phenotype as verified for OVA=LPSp (*fig.36*), leading to an inhibition on OT-I T-Cell activation when compared to OVAp. However, in the case of MHC class-II presentation no significant differences were observed between OVAp and OVA=LipidAp (*fig.49*). Instead, an increase in MHC class-II presentation occurred when LPS was in same cargo as OVA particles (*fig.36*). This might suggest, in accordance with MyD88KO data (*fig.47*), that the inhibitory effect of antigen cross-presentation could be mainly due to a TRIF-dependent pathway.

To address if this phenotype was related with IFN-β secretion by DCs upon model particles stimulation, soluble rIFN-β was used. Using lower concentrations of soluble rIFN-β, in a range observed by ELISA measurement of IFN-β produced upon stimulation with model particles (0.1-25 U), there was no effect in MHC class-II antigen presentation (*fig.49*). Concentrations as high as 25U, appear to be toxic to the cells as antigen presentation on both MHC class-I and MHC class-II were complete abolished (*fig.49*). Concerning to antigen cross-presentation, there was not a general inhibitory effect due to soluble rIFN-β, but slight tendency on antigen cross-presentation inhibition, only when IFN-β was present around 1U of concentration (*fig.49*). On contrary, and increase on antigen cross-presentation occurs, when IFN-β was present around 5U of concentration. Nevertheless, the rINF-β used could be not in an "ideal" conformation/processing state to signal properly through the IFN-R, as it was known to be critical to IFN-β signaling.

However, we cannot exclude the hypothesis that soluble IFN-β could have a role on antigen cross-presentation inhibitory effect due to particle LPS/LipidA stimulation, although, further characterization should be done as other mechanism may also occur.

Phenotype characterization VIII – PLGA and Hydrogel particle platform

PLGA biodegradable particles have been used as *in vivo* carriers, and could be used as a dynamic system for antigen delivery (Acharya *et al.*, 2009). Thus, a different platform of particulate antigen was used to address the role of TRIF-dependent pathway mediated by TLR4. As described, MPLA (Monophosphoryl LipidA) is a low-toxicity derivative of LPS with useful immunostimulatory properties that trigger exclusively the TRIF-dependent pathway and has been used as *in vivo* modulation ligand (Mata-Haro *et al.*, 2007). In fact, MPLA is in regulatory approval for use as a human vaccine adjuvant. Therefore, we next sought to evaluate the role of PLGA particles loaded with model antigen in the presence of MPLA on both MHC class-I and MHC class-II antigen presentation pathways.

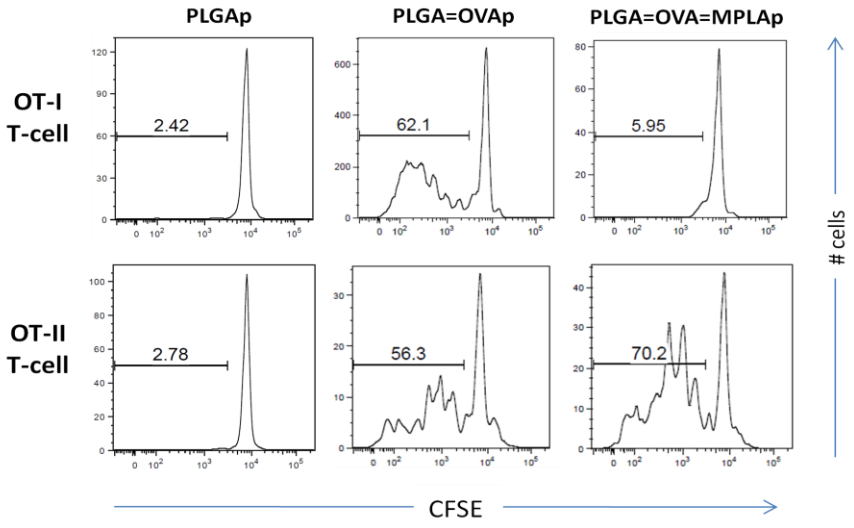


Fig.50: PLGA model particles antigen cross-presentation FACS-based assay. sDCs ($2.5x10^4$) from C57BL/6 mice were incubated with model particles (PLGAp, PLGA=OVAp and PLGA=OVA=MPLAp) at 1:40 (sDC:particles) ratio. Similar results were obtained with lower ratios 1:20 and 1:10. $1x10^5$ OT-I T-Cells (upper panel) and OT-II T-cells (lower panel) were co-incubated

for 3 days. Histograms represent T-Cell population gated on SSC *vs* CFSE plots**.** T-Cell proliferation was measured using CFSE staining by FACS. Numbers represent the percentages of the proliferating cells of total OT-I T-Cells. These are representative data from one experiment repeated at least three times with similar results.

The results obtained with the PLGA model particles were in total accordance with previous ones obtained using fixed ligand (polystyrene) particles (*fig.49*), supporting the effect of TLR4 agonists as an inhibitory signal for antigen cross-presentation when present in same context as particle antigen (*fig. 50*). Therefore, when MPLA is on same cargo as PLGA=OVAp, there was an inhibitory effect on antigen cross-presentation and a slightly increase MHC class-II presentation (*fig.50*). As TRIFKO mice were not available, TRIF/MyD88 double KO mice were used to address the direct role of TLR4/TRIF dependent pathway on antigen cross-presentation phenotype inhibition mediated by MPLA-containing particles.

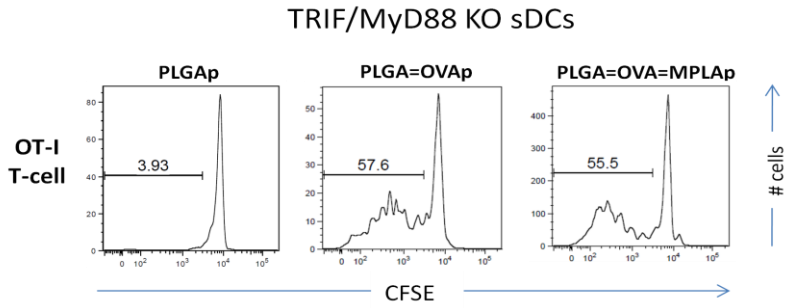


Fig.51: PLGA model particles antigen cross-presentation FACS-based assay by TRIF/MyD88 double KO DCs. sDCs ($2.5x10^4$) from TRIF/MyD88 double KO mice were co-incubated with model particles (PLGAp, PLGA=OVAp and PLGA=OVA=MPLAp) at 1:40 ratio (similar results with lower ratios 1:20 / 1:10) and OT-I T-Cells ($1x10^5$) for 3 days. T-Cell proliferation was measured using CFSE staining by FACS. Histogram represents T-Cell population gated on SSC *vs* CFSE plots. Numbers represent the percentages of the proliferating cells of total OT-I T-Cells These are representative data from one experiment repeated at least three times with similar results.

As expected, in the absence of TRIF-dependent pathway, the inhibitory effect of MPLA-containing PLGA particles on antigen cross-presentation was abolished and similar levels of OT-I T-Cell proliferation were obtained for PLGA=OVAp

and PLGA=OVA=MPLAp *(fig.51)*. This result support, even using different particle system, that the impairment on antigen cross-presentation phenotype mediated by TLR4 agonists could be due to a TRIF dependent pathway, as MPLA signals through TLR4 specifically using this adaptor (Mata-Haro *et al.*, 2007).

As Hydrogel pH-responsive particles are able to disrupt the phagosomes and to force the release of their contents into cytosol (Hu *et al.*, 2007), we next sought to address the hypothesis if the MPLA mediated abolishment on antigen cross-presentation could be in some extent related to the control of the mechanism of antigen escape to the cytosol as hypothesized previously (*fig.42*).

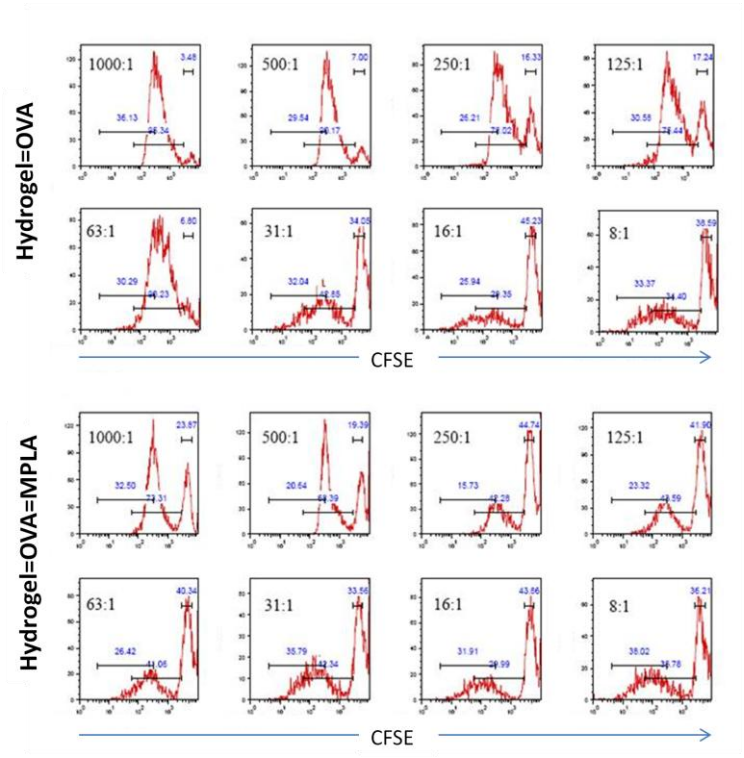


Fig.52: Hydrogel pH-responsive model particles model particles antigen cross-presentation FACS-based assay. sDCs ($2.5x10^4$) from C57BL/6 mice were co-incubated with Hydrogel particles (Hydrogel=OVAp and Hydrogel=OVA=MPLAp) from 8:1 to 1000:1 (particles:sDC) ratio and OT-I

T-Cells ($1x10^5$) for 3 days. T-Cell proliferation was measured using CFSE staining by FACS. Histogram represents T-Cell population gated on SSC *vs* CFSE plots. Numbers represent the percentages of total OT-I T-Cells. These are representative data from one experiment repeated at least three times with similar results.

These data showed that even using a particle system that forces the escape of antigen to cytosol, the inhibitory effect of MPLA on particles was evident even when antigen was present at higher ratios of concentration (*fig.52*). These data support the role of TLR4 agonists-containing particles on antigen cross-presentation impairment. From these data we could hypothesize that antigen cross-presentation inhibitory effect mediated by TLR4 agonists is not only due to decreased antigen availability on cytosol and processing by the proteasome (*fig.42*) or phagosome maturation (*fig.39 and 41*), but also to another intrinsic mechanism. However, hydrogel particles were at a range size of ~0.5µm (smaller than polystyrene and PLGA) and could reach different niches of internalized phagosomes that potentially have different antigen cross-presentation abilities (Kutomi *et al.*, 2009; Reinicke *et al.*, 2009; Tran and Shen, 2009). Further studies should be done in order to characterize this assumption.

In sum, all these results showed that the impairment of particle antigen cross-presentation of phagocytosed cargo is dependent on the presence of TLR4 agonists within the cargo (*summarized in Table VII*). All these data will be discussed in the following chapter.

Table VII: Representative illustration of principal model particles antigen presentation on both MHC class-I and MHC class-II pathways. LPS-mediated phenotype and influence on antigen presentation major key steps.

	Antigen Presentation					
	CD8+ (MHC-I)	CD4+ (MHC-II)	Uptake	"Release" to Cytosol	Processing (proteasome)	Phagosome Maturation
OVAp 	+	-	=	+	+	-
OVA=LPSp 	-	+	=	-	-	+

5

Discussion

Antigen presentation is at the basis of the $CD8^+$ T-Cell immune response against tumours or viral infections but also in self-tolerance mechanisms. Cross-presentation of antigens in DCs, is a key pathway to elicit effective $CD8^+$ T-Cell responses of exogenously-delivered antigens (Heath and Carbone, 2001; Touret *et al.*, 2005; Yewdell *et al.*, 1999). However, little is known about its mechanistic basis and how it responds to different types of foreign and self-antigens. Classical antigen presentation studies have focused on soluble extracellular antigens, but much evidence suggests that particulate antigens, such as bacteria, fungi and microparticles, are processed much more efficiently to stimulate $CD8^+$ T-Cells (Houde *et al.*, 2003; Kovacsovics-Bankowski *et al.*, 1993; Stuart and Ezekowitz, 2005). DCs express a series of different receptors that mediate the transfer of such signals from the environment. Toll-Like Receptors play a critical role in the immune response to invading pathogens by sensing microorganisms (Akira, 2006). As the role of these receptors in the outcome of the immune response and antigen presentation is not well understood, here we discuss how TLR agonists in same cargo as particulate antigens influence cross-presentation and its possible intrinsic mechanism.

Particles size influences OVA antigen cross-presentation

As a starting point and given the strict dependency of the immune response on the size of the microorganisms, we first looked at the role of particle size on antigen presentation. It was described that the size of ligand-devoid particles can determine their pathway(s) of entry (Rejman *et al.*, 2004). Our data revealed a size-dependent efficiency of particulate antigen cross-presentation by DCs (**Chapter 4**). Since particle size also affects the amount of antigen uptake (Desai *et al.*, 1997) the differences in antigen cross-presentation could be attributed to the total amount of antigen taken up by DCs. However, there were no significant differences in the

amount of antigens of particles with different sizes that were internalized by DCs. In addition, particulate MHC class-I OVA epitope (SIINFEKL) was efficiently cross-presented and not size related (**Chapter 4**). This evidence supports the idea that antigen cross-presentation size dependency is not due to different amounts of OVA antigen internalized, but instead, it can be related to different intracellular processing/loading mechanisms of antigens resulting from different uptake mechanisms. One possible explanation for these differences is suggested by the observation that after internalization, different kinds of particles localize to different niches of phagosomes (Brewer *et al.*, 2004; Cervi *et al.*, 2004; Oh and Swanson, 1996). Consequently, it is possible that the different OVA particles end up in different phagosomes with different antigen cross-presentation capabilities (Belizaire and Unanue, 2009; Tran and Shen, 2009). Recently, it was shown that particle size influences the phagosomal pH which is directly related with antigen cross-presentation efficiency. It was proposed that antigen bounded to 50 nm particles is shuttled rapidly to an acidic environment leading to its rapid and unregulated degradation and inefficient cross-presentation. In contrast, antigen bounded to 500 nm and 3 µm beads remained in a more neutral environment, which preserved the majority of antigens, leaving it available for the generation of peptides to be loaded onto MHC class-I molecules (Tran and Shen, 2009). Altogether, these observations suggest that the size of antigen carriers plays a critical role in directing antigens to the MHC class-I pathway. Therefore, enhancing phagocytic levels and/or re-route the particles to specific compartments could improve the efficiency of antigen cross-presentation.

"Pathogen-like" stimulus impairs particulate antigen cross-presentation

Previous studies showed that particulate antigens do not elicit a significant T-Cell response in the context of MHC class-II. Moreover, these same studies revealed that co-stimulation of APCs with pathogen "sensors", like TLR agonists, boost MHC class-II antigen presentation pathway (Blander, 2007a; Blander and Medzhitov, 2006). In addition, it was also described that antigen cross-presentation can be induced by TLR agonists (Bevaart *et al.*, 2004; Chen *et al.*, 2005; Datta and Raz, 2005; Datta *et al.*, 2003; Heit *et al.*, 2003; Schulz *et al.*, 2005; Weck *et al.*, 2007). Unexpectedly, our data revealed that cross-presentation of OVA particles is almost abolished when the TLR4 agonist LPS (same observation for the TLR9 agonist CpG) is present in same context as the antigen. On contrary, we observed an induction of the MHC class-II antigen presentation. The decrease in antigen cross-presentation mediated by LPS in same cargo as OVA particles was recovered in DCs deficient on TLR4 signaling, using both TLR4KO DCs and shRNA lentivirus mediated TLR4 gene knockdown (and TLR9 gene knockdown in case of OVA particles containing CpG).Targeting an important downstream pathway, MAPK pathway of TLR signaling, using inhibitors for JNK, ERK and p38, the levels of antigen cross-presentation of OVA particles containing LPS were recovered (higher extent for p38 MAPK inhibition), suggesting the LPS-mediated effect is through TLR4 signaling pathway (**Chapter 4**). This important discovery reveals that, when LPS is in same cargo as particle antigen, antigen cross-presentation is impaired and MHC class-II presentation is increased, mediated by a TLR4-dependent signaling crosstalk. This impairment effect on antigen cross-presentation mediated by TLR agonists on same cargo as particle antigen was never described before. However, when soluble LPS is co-incubated with OVA particles (two different physic stimuli) an increase of T-Cell activation/ proliferation occurs in both MHC class-I and MHC class-II (**Chapter 4**). It seems

that a strong soluble stimulus, such as LPS, is able to activate and reprogram all the antigen presentation machinery (up-regulation of molecules involved in antigen presentation – MHC class-I and class-II, co-stimulatory molecules, as well as other important cellular processes) leading to improved capacity to present an antigen in both classical pathways. The potential impact of this assumption, could justify its application on vaccine design projects, as TLR4 agonists were never described or used as a negative regulators of antigen cross-presentation response to a specific protein vaccine *in vivo*.

The origin of pathogen-like stimuli (particulate *vs* soluble) seems to be critical for cross-presentation pathway(s) regulation (**Chapter 4**). When LPS is used in a different particle of that containing the antigen, no significant differences on the efficiency of MHC class-I and MHC class-II antigen presentation are observed (**Chapter 4**). Altogether, these data suggest that when in a soluble form, LPS stimulus could be able to activate all the machinery for antigen presentation, on contrary, when LPS is present in a particulate state, one can apply the concept of *"phagosome autonomous maturation"* in antigen presentation described by Blander *et. al* (Blander and Medzhitov, 2006a). According to this assumption, DCs distinguish between self and non-self antigens by selectively maturing phagosomes that contain the TLR agonist, as the TLR4 agonist LPS. This may constitute a regulatory mechanism to avoid the recognition of self antigens by the immune system (Blander and Medzhitov, 2006a). Our results with the TLR4 agonist and the antigen located on different particles, seem to support the finding that two phagosomes in the same DCs are "processed" individually (Blander 2008; Blander and Medzhitov, 2004, 2006b).This assumption is of great relevance to understand the role of "pathogen" stimuli on antigen fate and the capacity of an antigen-presenting cell to decide the antigen presentation outcome of a specific antigen based-environment. The influence of the compartmentalization on the crosstalk

between the TLR-signaling and the antigen cross-presentation pathways may constitute a tool that the DCs use to discriminate between the contents of phagosomes and to better mount an appropriate immune response to a specific stimulus. In sum, here we describe a new mechanism of antigen selection in DCs for cross-presentation that is based on the origin of the antigen. We show that the efficiency of presenting antigens from phagocytosed cargo is dependent on the presence of TLR agonists within the cargo.

Antigen uptake is not regulated by particulate TLR stimulus

As the amount of antigen that is internalized by DCs may influence the antigen presentation, and moreover, TLRs have been implicated on phagocytosis (Blander, 2007a), we addressed whether a TLR4 agonist influences the particulate antigen uptake. Our results revealed that the uptake capacity of DCs is similar for particle antigen, when LPS is present in a soluble form or in the same context as antigen (**Chapter 4**). As such, the amount of antigen that reaches the phagosomes is almost the same, thus, the differences observed in antigen presentation for particle antigen and particle antigen containing LPS do not appear to be due to differences in phagocytic uptake.

Phagosome maturation mediated by LPS impairs antigen cross-presentation

The maturation of phagosomes containing antigens has been described as an important process related to antigen presentation (Blander, 2007a; Blander and Medzhitov, 2006a; Russell and Yates, 2007). It was previously reported that bacterial LPS enhances phagosome maturation/acidification (Blander and Medzhitov, 2004, , 2006a; Trombetta *et al.*, 2003) and improves DC performance in antigen presentation (Blander and Medzhitov, 2006a; Trombetta and Mellman, 2005). In agreement with the published studies, our data showed that, if present in

the same context as particulate antigens, LPS stimulus induces higher levels of phagosome maturation (phagosome colocalization with lysosomes). Moreover, in the absence of signals from TLR4, phagosomes failed to mature efficiently (**Chapter 4**).

Our data link phagosome maturation enhancement with a phagosomal acidification (decreased pH) mediated by LPS-containing phagosomes. In the absence of LPS stimulus, phagosomal pH is maintained at higher pH values, indicative of an immature stage (**Chapter 4**), which enhances the fusion between phagosomes and early endosomes (Hart and Young, 1991; Kjeken *et al.*, 2004; Pais and Appelberg, 2004). This blockade on phagosome maturation (phagolysosome formation) leads to phagosomal enrichment on ER machinery important for antigen cross-presentation (Ackerman *et al.*, 2003; Claus *et al.*, 1998; Houde *et al.*, 2003; Howland and Wittrup, 2008) and supported the observation that only early phagosomes are able to contribute to antigen cross-presentation in DCs (Ackerman *et al.*, 2003; Houde *et al.*, 2003).This is supported by the evidence that antigen cross-presentation may occur in a time-dependent manner, preferentially early after phagocytosis (Howland and Wittrup, 2008). Additionally, antigen cross-presentation is described to be enhanced in the presence of a lysosomotropic agent (cause an increase in lysosomal pH) (Accapezzato *et al.*, 2005; Howland and Wittrup, 2008) as low pH values perturb the loading of peptides onto MHC class-I molecules (Ackerman and Cresswell, 2004; Ackerman *et al.*, 2003) and favor antigen degradation mediated by vacuolar endoproteases (Claus *et al.*, 1998; Delamarre *et al.*, 2005; Manoury *et al.*, 1998).

Taken together our results with others, we hypothesize that the effect of LPS stimulus on phagosome maturation and pH regulation is related with particle antigen cross-presentation impairment.

The pH variation induced by LPS is blocked in the presence of Bafilomycin A (V-ATPase inhibitor), which implicates the V-ATPase complex on phagosomal pH regulation mediated by LPS. However, the TLR4KO pH phenotype mediated by LPS does not resemble completely the immature stage, suggesting that phagosomal pH kinetics mediated by LPS is not totally dependent on TLR4 mediated signaling, but seems to plays a major role (**Chapter 4**). V-ATPase is the principal responsible for phagosomal pH acidification and its function could be regulated by the control of phagosomal efflux of protons (Nishi and Forgac, 2002; Stevens and Forgac, 1997). This process is important to maintain phagosomal electrochemical balance, allowing its acidification and maturation (Nishi and Forgac, 2002). P2x4 receptor can play a role on this balance, as it is induced on phagosomes upon stimulation (Raouf *et al.*, 2007). Knockdown of P2x4 receptor enhanced particulate antigen cross-presentation. This effect was not observed following P2x7 receptor knockdown as a control (*data not shown*). As its function was described to be enhanced by LPS (Raouf *et al.*, 2007), P2x4 receptor could have an important role on phagosome maturation and consequently on particle antigen cross-presentation. A direct link between LPS signaling and V-ATPase control/regulation was not shown, however we can hypothesize the existence of such a link and that P2x4 receptor could have an important role on that. Further studies should be done in order to support this assumption based on preliminary data.

A recent study reported the DCs phagosomal pH to range between 7.0-7.5 upon challenge with pH sensitive particles (Savina *et al.*, 2006). However, our results show higher pH values at the starting point (pH~8.8) and also different kinetics of pH variation following the addition of particles. These differences might be due to the fact that, unlike the previous study that use plain amino particles as backbone to coupled the fluorescent dyes, we have used particles loaded with OVA, similar to the ones used during the antigen presentation studies. It was previously shown

that OVA is able to target specific receptors on DCs for efficient uptake and antigen presentation (Burgdorf *et al.*, 2007; Hao *et al.*, 2007; Idoyaga *et al.*, 2008). Studies performed with soluble OVA, implicated Scavanger Receptores, DEC-205 and most importantly Mannose Receptors (MR) in re-routing endocytosis pathways to introduce soluble antigens into separate intracellular compartments (Burgdorf *et al.*, 2007). The MR was also described to supply an early endosomal compartment distinct from lysosomes, which was committed to cross-presentation (Burgdorf *et al.*, 2007). A similar mechanism may also explain the different phagosomal pH values obtained in DCs in the presence of particles loaded with OVA. In this case, phagocytosis might end up on more alkaline compartments giving rise to higher pH values when using particles loaded with OVA. This would then explain the different observations made previously regarding the controversial effect (negative or neutral effect) of LPS on phagosomal maturation (Shiratsuchi *et al.*, 2004; Yates and Russell, 2005) and its relation with antigen cross-presentation.

LPS plays a negative role on ROS production – a step required for efficient cross-presentation

The control of phagosomal pH in DCs relies on the activity of the V-ATPase complex but also on the activity of NOX2 (Savina *et al.*, 2006), and its regulation results from a delicate balance between the recruitment and activity of these two complexes (DeCoursey *et al.*, 2001; DeCoursey *et al.*, 2003; Nanda *et al.*, 1994; Savina *et al.*, 2006). NOX2 has been described as a major player, limiting "blunt" phagosomal acidification in DCs, providing a sustained alkaline phagosomal environment (pH 7-7.5) (Savina *et al.*, 2006). Moreover, ROS production by NOX2 activity is required for efficient antigen cross-presentation (Mantegazza *et al.*, 2008; Savina *et al.*, 2006).

We showed that LPS-containing particles stimulus may reduce ROS production into phagosomes (**Chapter 4**). Thus, the lower rate of phagosomal maturation observed in the absence of LPS stimulus, maybe due in part to higher levels of ROS production. Increased ROS levels could consume the protons generated by V-ATPase, therefore able to block phagosome acidification. This regulation could occur due to the control of the activity and/or recruitment of NOX2 to LPS particles-containing phagosomes. Moreover, V-ATPase and NOX2 activities could be regulated by signals from TLRs, either by controlling the density and assembly of these complexes on the phagosomal membrane or by regulating the activity of associated proteins (Nishi and Forgac, 2002). Although, the effect of TLR activation on NOX2 recruitment to phagosomes has not been studied directly, it was shown that soluble LPS enhances NOX2 activity and expression in DCs (Vulcano *et al.*, 2004). This finding is in opposition to our results; however a simple explanation could be based on a different experimental set up, the physical nature of the LPS stimulus. Where others have used soluble LPS during a long time (~24h), and subsequently challenged with PMA, we used a LPS-particulate stimulus that could signal in a different time window or activation state of NOX2 regulation/recruitment. Besides the pH regulation, ROS production in phagosomes may also modify molecular antigen conformation by oxidation, which could affect the antigen sensitivity to different proteases or influence the peptide export to the cytosol (Amigorena and Savina, 2010), and consequently antigen cross-presentation. We suggest that higher levels of phagosome maturation of LPS-containing phagosomes should be due to a regulatory mechanism mediated by LPS stimulus that leads to a higher rate of phagosomal acidification but also to a decrease of phagosomal ROS production (**Chapter 4**). Taken together these data, we hypothesize that antigen cross-presentation is enhanced during the period of time when pH is maintained at near alkaline values, where phagosomal

acidification is "sustained" and occurs and active ROS production which resembles an immature phenotype. On the other hand, antigen cross-presentation is impaired when a stimulus induces phagosome maturation, acidification and decreased ROS production, producing a mature phenotype that is compromised for MHC class-II antigen presentation. In sum, we propose that phagosomal maturation mediated by LPS stimulus impairs antigen cross-presentation, due to enhanced acidification and decreased ROS production, two compensatory mechanisms.

LPS plays a role on particle antigen processing/degradation which is important for efficient cross-presentation

DCs have adapted their intracellular machinery to focus on antigen presentation rather than on antigen degradation (Savina *et al.*, 2006). As described, phagosomal pH regulation (either by V-ATPase or NOX2) is the main mechanism used by DCs to "protect" peptides from complete degradation, with the ultimate goal being to generate T-Cell epitopes (Savina *et al.*, 2006; Trombetta *et al.*, 2003).

Antigen processing/degradation during antigen presentation by DCs is mediated either by the vacuolar pathway, compromised with the MHC class-II epitope generation or by the proteasome (cytosolic) pathway, that plays a major role on MHC class-I epitope generation (Belizaire and Unanue, 2009; Trombetta and Mellman, 2005). Our results show a major role of the proteasome on particulate antigen processing/degradation, as this process is almost abolished when a proteasomal inhibitor is used. In the presence of LPS, lower levels of particulate antigen processing/degradation were observed, which could imply LPS as a negative regulator of the proteasome activity. In contrast, inhibition of Cathepsin S (a major endoprotease of the vacuolar degradation pathway) has a small effect on particulate antigen processing/degradation (**Chapter 4**).

Exogenous antigen processing by the proteasome is described as a key step and required for their cross-presentation (Houde *et al.*, 2003). As we have previously shown, LPS-containing particles impairs antigen cross-presentation which could be in part explained by their negative regulation in proteasome-dependent degradation (**Chapter 4**), leading to less MHC-I epitopes generation. Further experiments should be then performed to analyse MHC-I/SIINFEKL complexes at cell surface, to show if LPS influences the amount of the first signal on antigen cross-presentation. By using the methods available at the moment, either an anti-mouse H-2Kb OVA agonist peptide antibody or a B3Z T-Cell hybridoma based assay (Guermonprez *et al.*, 2003; Kwon *et al.*, 2005), we observed no differences in the MHC-I/SIINFEKL complexes at the cell surface, when LPS was in same cargo as particle antigen (data not shown). Nevertheless, other authors considered these methods with no sensitivity enough to evaluate particulate antigen presentation (Amigorena and Savina, 2010). Therefore, new and more efficient methods are required to accurately ascertain this question.

In addition to proteasome, proteolytic activity of endosomal proteases has also been described to be important for an efficient antigen cross-presentation (Chapman, 2006). Antigen cross-presentation is favoured by a neutral pH in DC phagosomes (Savina *et al.*, 2006). The Cathepsin S (Cat.S) endoprotease has been shown to have an optimal pH around 7.0 (Claus *et al.*, 1998) and to be able to generate the correct OVA peptide (SIINFEKL). This fulfils the requirements for the OVA antigen to be loaded in MHC class-I molecules (Shen *et al.*, 2004), favoring its cross-presentation. Our data don´t address a direct role for Cat.S activity on particle antigen cross-presentation. However, particulate LPS stimulus seems to have an important role on the control of proteasome-dependent antigen processing/degradation, but not on endosomal proteases activity (**Chapter 4**). On the other hand, it has been shown that certain antigens such as different bacterial

antigens do not require a cytosolic step for their cross-presentation (Belizaire and Unanue, 2009). Therefore, the proteasome and endoproteases may play important but different roles on particulate antigen processing for presentation on different pathways, depending on the type of signal in same context as antigen. We believe that depending on the context of antigen or the nature of the stimuli, endoproteases, may also generate MHC class-I restricted peptides that can be loaded in phagosomes or endosomes and presented to $CD8^+$ T-Cells.

A possible role of LPS on the retro-translocation of antigen to the cytosol during cross-presentation

The decreased antigen processing observed for particulate antigens in the presence of LPS may result exclusively from reduced proteasomal activity but may also result from a decreased antigen escape from the phagosome to the cytosol (retro-translocation) (Guermonprez and Amigorena, 2005; Houde *et al.*, 2003; Vyas *et al.*, 2008).

In order to address this assumption, an indirect measurement of antigen availability on the cytosol was performed. We showed that LPS on same cargo as particulate antigen reduces the antigen availability on the cytosol (as demonstrated by lower levels of apoptosis) (**Chapter 4**). This could be due to: *1)* lower levels of retro-translocation; *2)* higher levels of phagosomal degradation; *3)* rapid shuttling to the proteasome degradation machinery. The 3^{rd} hypothesis could be excluded by data showing that degradation of OVA occurs at higher efficiency in the absence of LPS and was almost abolished in the presence of proteasome inhibitor (**Chapter 4**). Concerning the other two hypotheses, and assuming that phagosome maturation is enhanced in particles with LPS, we hypothesize that particle antigen degradation takes place in acidified compartments at later stages of maturation rather than in the cytosol mediated by the proteasome activity. This would ultimately result in

reduced levels of antigen in the cytosol and is in accordance with our results showing that LPS in the same cargo as particulate antigen reduces the antigen availability on cytosol (**Chapter 4**). However, inhibition of Cathepsin S (a major endoprotease of the vacuolar degradation pathway) has a small effect on particulate antigen processing/degradation even in the presence of LPS (**Chapter 4**). Recent studies have shown that the transport of antigens to cytosol is a key step for antigen cross-presentation and could also require high pH in the lumen of the endocytic pathway (Mantegazza *et al.*, 2008). Our results show that in the presence of LPS, phagosomes loose the immature stage (pH>7.0), therefore unfavorable for retro-translocation (**Chapter 4**). This observation suggests that retro-translocation could be directly impaired by LPS stimulus. In the future, the ubiquitination state of cytoplasmic OVA should be addressed, in order to dissect the real value of LPS signaling on retro-translocation step on particulate antigen cross-presentation mediated by proteasome.

In order to bypass the retro-translocation step as well the requirement of the proteasome or/and endoproteases processing for cross-presentation, SIINFEKL particles were used as a model antigen. On contrary to what was observed when using OVA particles, our results showed that LPS does not influence antigen cross-presentation of particle antigen peptide (**Chapter 4**). Thus, the influence of LPS signaling in decreasing antigen retro-translocation and proteasome processing is once more supported by this assay.

We propose that the higher rate of phagosome maturation mediated by particulate LPS, could give rise to less antigen escape to cytosol and consequently lower levels of antigen loading into MHC class-I presentation pathway. Taken together our results and other published studies (Howland and Wittrup, 2008; Kovacsovics-Bankowski *et al.*, 1993; Palliser *et al.*, 2005), allow us to speculate that phagolysosome formation mediated by LPS signaling may shut down the

machinery necessary for antigen egress to the cytosol. Alternatively, phagosome maturation mediated by LPS stimulus may avoid the recruitment of vesicles with specific cargos important for retro-translocation mechanism. These processes may impose another limit on the time window for an efficient antigen release and cross-presentation. It seems that there is a LPS-dependent "check point" on phagosome maturation that blocks protein release to cytosol – *"the point of no return"*. Several groups have reported that the time of antigen persistence and degradative environment of exogenously-delivered antigen in phagosomes is critical to preserve peptides available for loading onto MHC class-I molecules (Howland and Wittrup, 2008; Savina *et al.*, 2006), that was shown to occur efficiently in early endosomes (Burgdorf *et al.*, 2007). In sum, we showed that the release of antigens from phagosomes to the cytosol was higher during early stages of phagosomal maturation, which is a crucial step for antigen cross-presentation. Taking all these observations together, we hypothesize that the epitopes for MHC class-II presentation are generated in phagolysosomes and should be retained on the endocytic pathway. In opposition, the MHC class-I epitopes should be generated predominantly in a proteasome dependent manner upon egress, during early stages of phagosome maturation.

It is generally accepted that the Sec61 complex plays a role in ER-associated degradation (ERAD) in phagosome-cytosol export and in cross-presentation mechanism, however little is still yet known about this process (Guermonprez *et al.*, 2003; Houde *et al.*, 2003; Roy, 2002). Preliminary data, using shRNA lentivirus for Sec61g in BMDCs showed that Sec61 translocon is not the most important transporter when we made use of particulate antigen, as no significant differences on antigen cross-presentation were observed when sec61g was KD (*data not shown*). In fact, this was challenged by others that have implicated Derlin-1 in ER retro-translocation (Lilley and Ploegh, 2004; Ye *et al.*, 2004) which

could have a role on particulate antigen cross-presentation. Nevertheless, we cannot exclude the importance of Sec61complex for other type and physical forms of antigens and further validations should be done in our system, as a compensatory effect, such as unspecific transport, could also occur. Even though all data obtained so far suggest a role of ERAD in antigen cross-presentation, the mechanisms and origins of antigen export involved in this antigen presentation pathway are not completely understood.

TLR4 signaling mediated by p38 MAPK plays a major role on particle antigen cross-presentation impairment

The role of TLR signaling on antigen cross-presentation is not clear and remains quite controversial. The TLR-p38 signaling pathway was implicated on the inducible mode of phagosome maturation and MHC class-II presentation (Blander, 2007b; Blander and Medzhitov, 2006a). Moreover, a simple event such as phosphorylation of p38 MAPK molecules by signals from TLRs can induce dynamic changes in the composition of the phagosome proteome (Cavalli *et al.*, 2001). Our data showed that the abolishment in antigen cross-presentation mediated by LPS in same cargo as particulate antigen was recovered when p38 phosphorylation was inhibited, and in a lesser extend for others MAPKs (**Chapter 4**). Integrating our data with others (Blander and Medzhitov, 2006a), it may suggest that a link between phagosome maturation and the impairment of antigen cross-presentation mediated by particulate LPS, could be played in major part by p38 MAPK activation. However, further studies should be done in order to address the precise role of p38 MAPK in particle antigen cross-presentation by analyzing the regulatory proteins upstream and downstream on the signaling pathway. This result implicates for the first time p38 MAPK activation mediated by TLR4 as a major negative regulator on antigen cross-presentation. However the precise

mechanism is not known. A recent work reports that TLR4 coordinates recruitment and signaling through TIRAP-MyD88 and TRAM-TRIF sequentially rather than simultaneously (Kagan *et al.*, 2008). In addition, these signals seem to be separated compartmentally but also temporally (Kagan *et al.*, 2008). The p38 activation could be mediated either by MyD88 and TRIF-dependent signaling pathways (Cekic *et al.*, 2009; Oda and Kitano, 2006). Indeed, it was reported that endocytosis of TLR4 terminates an initial phase of MyD88-dependent signaling at cell surface leading to an early activation of NF-kB and p38 MAPK. Sequentially, endocytosis leads the start of a second phase of TRIF-dependent signal transduction from TLR4 located in endosomes inducing a late activation of NF-kB, p38 MAPK and IFN type I cytokine production such as IFN-β (Kagan *et al.*, 2008). We have demonstrated that phospho-activation of p38 and degradation of IkB-α, occurs with higher kinetics in the presence of particulate LPS stimulus. The earlier activation of p38 at 5 minutes of chase, should be then due to signaling from cell surface mediated by MyD88 or from phagosomes mediated by TRIF, as DCs were allowed to uptake particles before chase. There is no evidence of a second wave of signaling within 2 hours of chase probably mediated by TRIF, which could occur later on (**Chapter 4**). However, we cannot exclude that the MyD88 and TRIF pathways could crosstalk in a time and spatial dependent way. As shown recently, synthetic derivate of LipidA, an agonist of TLR4 that tigers exclusively the TRIF-dependent pathway (Mata-Haro *et al.*, 2007), induces strong p38 MAPK but weak JNK activation (Cekic *et al.*, 2009). Moreover, p38 phosphorylation and NF-kB activation is delayed in cells lacking MyD88 (Hoebe *et al.*, 2003; Yamamoto *et al.*, 2003), which could be due to TRIF-dependent signaling. Our data using MyD88KO DCs, could exclude the MyD88 adapter as having a role in particle antigen cross-presentation impairment. In fact, in the absence of MyD88, the inducible effect on MHC class-II and abolishment of cross-presentation mediated

by particle antigen containing LPS does not occur (**Chapter 4**). These observations support the effect of TRIF-dependent pathway later on time, after particles internalization, on antigen cross-presentation impairment mediated mainly by p38 MAPK. Therefore, these data suggest that MyD88 adaptor is not responsible for the abolishment of particle antigen cross-presentation, instead, it´s responsible for MHC class-II induction when LPS is in the same context as particle antigen. Interestingly, particulate CpG could indeed mediate the same phenotype either in MHC class-I and MHC class-II antigen presentation, by signaling through TLR9 as shown by using shRNA for TLR9 (**Chapter 4**). Another interesting observation was observed in the absence of MyD88, as an inhibitory effect on particle antigen cross-presentation containing CpG occurs. This effect implies that the impairment on particle antigen cross-presentation mediated by TLR9 occurs by another adaptor beside MyD88. Our previous results exclude in part the impairment of particle antigen cross-presentation mediated by CpG through another receptor, as in absence of TLR9 the phenotype is reverted (**Chapter 4**). This assumption has not been addressed so far, and further studies should be done to clearly show this new observation. A further step should be done, using shRNA lentiviral to perform KD for all four main adapters known (MyD88, TIRAP, TRIF and TRAM- which mediate TLR signaling and share significant amino-acid sequence similarity within their TIR domains) (O'Neill *et al.*, 2003), in order to dissect a possible role of different adapters beside MyD88 in TLR9 signaling upon CpG-containing particle antigen stimulation.

TRIF/IFN-β pathway may have a role on particle antigen cross-presentation abolishment mediated by TLR4 agonists

It is well accepted that cytokine production by DCs sensing pathogens can have a role on T-Cell response (Macagno *et al.*, 2007). Moreover, a link between

antigen presentation pathway(s) and the expression cytokines could occur. As we made use of particles, internalization could lead to a second wave of signaling either amplifying or be suppress the first one originated from plasma membrane. The levels of inflammatory cytokines produced such as, IL-6, IL-12 and TNF-α, were induced by antigen particles containing TLR agonists. This induction was similar for particles containing LPS and LipidA but in a less extend for CpG containing particles (**Chapter 4**). These cytokines did not show a significant contribution in the antigen cross-presentation phenotype mediated by particulate antigen. In the presence of cytokines, in a physiological range induced by particulate stimulus, no differences on antigen cross-presentation were observed (*data not shown*). Thus we propose that particulate LPS and mostly LipidA could signaling through phagosomes and are able to activate IFN type-I pathway leading to IFN-β production, probably by a TRIF-dependent/MyD88-independent pathway (**Chapter 4**).

We have described that the TRIF-dependent pathway may be responsible for the particle antigen cross-presentation abolishment. The main evidence emerges by the observation that LipidA-containing particle impairs antigen cross-presentation in same extend as observed for LPS, which implies the TRIF-dependent pathway in the antigen cross-presentation impairment mediated by TLR4 signaling (**Chapter 4**). However, the TRIF/IFN-β pathway has been implicated in response to viral infection (Yamamoto *et al.*, 2002). The direct influence of IFN-β on antigen presentation was not described. However, the impairment on particulate antigen cross-presentation mediated by LipidA and LPS could be related in some extend to the stimulation mediated by soluble IFN-β. Our data shown that, there was not a general inhibitory effect due to soluble IFN-β, but slight tendency on antigen cross-presentation inhibition, only when IFN-β was present on a specific range of concentration (~1U- maximum produced by particle antigens under

physiological conditions). Nevertheless, the soluble INF-β used could not be in an "ideal" conformation/processing state to signal properly through the IFN-R, as it was none to be critical to IFN-β signaling. Moreover, soluble IFN-β at the same concentrations has no influence on MHC class-II (**Chapter 4)**. Indeed, this subtle effect should not be the major responsible for the mechanism of particulate antigen cross-presentation abolishment mediated by TLR4 pathway. However, this evidence implies again the TLR4/TRIF-dependent pathway on the inhibitory effect of particle antigen cross-presentation. Concerning the cytokine pattern mediated by particulate TLR agonists, signaling from MyD88 adaptor could cooperate with TRIF adaptor for synergistic induction of a set of target genes and activation events. A crosstalk between the MyD88 and TRIF pathways for a robust TLR-mediated activation of the immune system could occur (Ouyang *et al.*, 2007) and be important on particulate antigen presentation mediated by LPS/LipidA. A time-dependent activation of these two signaling pathways mediated by particulate LPS and LipidA should be further dissected.

Antigen cross-presentation abolishment is "transversal" to other TLR/TRIF mediated signaling

Different TLR signaling pathways may have different roles on particle antigen cross-presentation (Datta *et al.*, 2003; Weck *et al.*, 2007). Therefore, our data indicates that when in same cargo as particulate antigen, TLR agonist that preferentially signals through MAPK/NF-kB pathways seems to induced cross-presentation, instead, TLR agonists that preferentially signals through IFN Type-I pathway seems to inhibit cross-presentation (**Chapter 4**). However, TLR4 is singular as it could signal through the two adaptors at different locations, and TRIF pathway is preferentially induced when TLR4 is internalized into endosomes (Kagan *et al.*, 2008). This is one more evidence showing that the abolishment on

cross-presentation is due to the signaling of particulate LPS preferentially through TRIF-dependent pathway when it is internalized, correlating with other TLR agonists that signals preferentially through IFN type-I pathway, such as - TLR3, TLR7 and TLR9, localized in endosomes. Thus, these data support the TLR4/TRIF-dependent inhibitory mechanism on particle antigen cross-presentation mediated by TLR4 agonists. Therefore, different TLR could have different roles and lead to different outcomes in antigen presentation pathways. Moreover, a pathway pattern seems to exist in the outcome of antigen presentation mediated by a specific particulate TLR agonist.

"Integrated" stimuli affect differently antigen cross-presentation when compared to specific stimulus by "synthetic pathogens"

Our results clearly suggest that TLR cooperation seems to have a role on the outcome of antigen presentation. *E.coli* expressing OVA and OVA loaded Apoptotic cells increases antigen presentation in both MHC class-I and class-II context when compared to particulate OVA antigen (**Chapter 4**). In absence of TLR4, no major effect was observed on antigen presentation mediated by *E.coli;* surprisingly, there was a significant decrease on cross-presentation but not on MHC class-II antigen presentation mediated by apoptotic cells (**Chapter 4**). Therefore, *E.coli* could induce a different pattern of receptors beside TLR4, which results in a general enhancing effect on antigen presentation. Furthermore, there is a hypothesis that apoptotic cells, during *u.v* irradiation could generate specific "danger" components that signals through TLR4 in a way that results in antigen cross-presentation induction when compared in the absence of this receptor (**Chapter 4**). It has been described that OVA from dead opsonised *Toxoplama gondii* is not able to be cross-presented, while OVA expressed in other pathogens such as *E.coli* and *Salmonella* is cross-presented (Pfeifer *et al.*, 1993; Svensson and

Wick, 1999). The explanation claimed by the authors is that the amount of OVA in dead parasites is limiting (even if it is sufficient for presentation on MHC class-II molecules) and that the recruitment of ER components by the live *Toxoplama gondii* to the resident vacuole makes the cross-presentation mechanism more efficient (Goldszmid *et al.*, 2009). Further studies should be done to address the role of cross-presentation mechanism against intracellular pathogens, by using model particles, with different molecules from several pathogens. Co-deliver antigen with a phagocytic ligand and a modulation signal allow the possibility to determine how uptake and inflammatory signals integrate to be cross-presented.

LipidA has the same inhibitory effect on particulate antigen cross-presentation even in other particles platforms: PLGA and Hydrogel

In collaboration with Darrel Irvine´s group of Bioengineering at MIT, we proposed to extend our studies to other platforms of antigen delivering with the aim to use in *in vivo* studies for a vaccine application. Properties such as *in vivo* biodegradability, an adjustable and dynamic system for delivery and the very high encapsulation capacity are strong arguments to explore PLGA microspheres as antigen delivery systems for vaccination for more than 20 years (Acharya *et al.*, 2009; Cleland *et al.*, 1994; Shi *et al.*, 2002; Singh *et al.*, 2006; Sun *et al.*, 2003; Waeckerle-Men *et al.*, 2005). As expected, when LipidA was in same cargo as PLGA particles containing antigen, an inhibitory effect on cross-presentation and a slightly increase on MHC class-II presentation occurs. This effect was abolished in the absence of TRIF-dependent pathway when MyD88/TRIF double KO was used (**Chapter 4**). Thus, these data highlight previous results obtained using fixed ligand (polystyrene) particles, supporting the inhibitory outcome of TRIF-dependent pathway on particulate antigen cross-presentation and an induction on MHC class-II presentation pathway mediated by TLR4 agonists.We suggest that

TRIF-dependent pathway mediated by LipidA has an inhibitory role on antigen cross-presentation even when antigen is forced to "run away" from the specific phagosomal signaling cargo, achieved when using Hydrogel particles as antigen carriers (**Chapter 4**). Based only on these data, we can hypothesize that, antigen cross-presentation inhibitory effect is not only due to decreased antigen availability on cytosol and processing by the proteasome or by enhanced phagosomal maturation (**Chapter 4**), but also to another mechanism mediated by TLR4 agonist signaling . However, these results could be related to a size-dependent phenotype. Hydrogel particles were used at a range of size < 0,5µm (smaller than polystyrene and PLGA) and could reach different "niches" of endocytic compartments, comparing to other different size/nature OVA particles,(Brewer *et al.*, 2004; Cervi *et al.*, 2004; Harding *et al.*, 1991; Oh and Swanson, 1996) that potentially have different abilities or mechanisms for cross-presentation (Kutomi *et al.*, 2009; Reinicke *et al.*, 2009; Tran and Shen, 2009). Moreover, we can speculate that this type of niches could be "chosen" by signals present on same context as antigen such as TLR agonists.

In sum, our data suggest that cross-presentation inhibitory effect mediated by TLR4/TRIF-dependent pathway upon LPS/LipidA-containing particle antigen stimulation is reproducible by using different particle systems. In order to achieve a good vaccine response against pathogens/tumor antigens, cross-presentation is required (Heath and Carbone, 2001; Touret *et al.*, 2005; Yewdell *et al.*, 1999). LipidA have been used in *in vivo* studies and vaccine design because of its lower toxicity (Persing *et al.*, 2002). *In vivo* studies should be performed with our model particles in order to address the direct role of TLR4 agonist on antigen cross-presentation impairment. Its knowledge could be of outstanding interest on vaccine research and have tremendous impact unveiling a key for an efficient immune response upon vaccination.

6

Concluding Remarks

Using different particle platforms carrying antigen (Polystyrene, PLGA and Hydrogel) with different properties, we have shown that particulate antigen cross-presentation is impaired when co-signaling through the TLR4 pathway. However, besides antigen cross-presentation impairment, MHC class-II antigen presentation is induced. This negative regulation is triggered by the TLR4 agonists, LPS or LipidA, in same cargo as antigen. This is supposed to be mediated by TLR4/TRIF-dependent pathway, instead of TLR4/MyD88-dependent pathway. This result implicates for the first time TLR4 signaling as a negative regulator on antigen cross-presentation. As TLR4 signals preferentially through TRIF-dependent pathway when located in the endosomes, the LPS on same context as particle antigen is suggested to target TLR4/TRIF signaling after particles internalization, in a spatial and temporal manner. These findings support the effect of compartmentalization on TLR4 negative signaling crosstalk with antigen cross-presentation pathway, mediated by agonists when present in same cargo as the antigen. Moreover, this phenotype is reproducible with the different particle platforms studied, highlighting the role of the TLR4/TRIF-dependent pathway on the impairment of particle antigen cross-presentation. The crosstalk effect of TLR4 signaling and particle antigen cross-presentation impairment is proposed to be mediated by p38 MAPK-dependent activation. This kinase is known to play a major role on phagosome maturation, and consequently on MHC class-II presentation. For the first time p38 MAPK activation mediated by TLR4 signaling was described as a negative regulator on antigen cross-presentation. However, we could not show how this negative regulation occurs, and further studies should be done to better address this assumption, and associate this phenotype with phagosomal maturation. To address a direct link between the effect of phagosomal maturation/pH and particle antigen cross-presentation mediated by LPS, drugs for phagosome maturation/pH inhibition should be used in further antigen presentation

assays. Additionally we have shown that TLR agonists that preferentially signal through the MAPK/NF-kB pathway have an inducible effect on particle antigen cross-presentation. In opposition TLR agonists that preferentially signal through IFN-Type I pathway seem to inhibit cross-presentation. Therefore, this observation supports that the inhibitory mechanism of cross-presentation mediated by TLR4 agonist-containing particle antigen is mostly TRIF-dependent. Different TLRs could play different roles and lead to different outcomes in antigen presentation pathways. Moreover, a relation seems to exist in the outcome of antigen presentation mediated by particulate TLR agonist. This knowledge could be of extreme importance to address future questions in different fields, such as the antigen presentation response against tumours and pathogens. To dissect the specific pathway mediated by TLR4 and other TLRs, shRNA for the main adaptors (TIRAP, MyD88, TRAM and TRIF) and downstream signaling proteins should be then used.

Concerning the mechanism, we propose that the impairment of cross-presentation mediated by particulate antigen containing TLR4 agonist is related with phagosome maturation, retro-translocation and antigen processing, which are major steps in antigen presentation. Therefore, LPS in particulate state induces phagosome maturation, either by decreasing pH and ROS production or enhancing the inducible rate of fusion with lysosomes; decreasing the amount of antigen available in cytosol; or/and decreasing antigen processing mediated by proteasome. In order to integrate these data and to evaluate the importance of TLR4/TRIF signaling pathway on these key processes in antigen cross-presentation phenotype, TRIF and p38 KO DCs should be used to analyse each step in detail, mediated by particulate antigens in presence of TLR4 agonists: phagosome maturation, phagosomal pH and ROS regulation, antigen processing and retro-translocation should be addressed.

Experimental observations imply an endocytic selective sorting of antigen cargo, where particles antigens are targeted to a specific pathway depending of the origin of stimuli present and the type of receptor engaged during internalization. Concerning antigen presentation, there are two different compartments that mature with different kinetics acquiring different phenotypes. One is compromised to antigen cross-presentation and the other one to MHC class-II presentation. Endosomes that would be "arrested" in an early stage, in an alkaline environment avoiding fusion with lysosomes favour cross-presentation. Early compartments are enriched in ER components, with high pH and low proteolytic environment that may allow antigen escape to cytosol and MHC-I peptide loading. Engagement of TLR4 would drive the cargo to MHC class-II compartments, which in contrary to immature ones are competent to fuse with lysosomes. As phagosome matures, the presence of ER components decreases as well the pH and proteolysis levels are enhanced. Therefore, these comportments become incompetent for cross-presentation but specialized for MHC class-II restricted presentation (*fig.54*). Phagosome purification approach should be performed to address the biochemical alterations in phagosome proteomics during maturation steps in the presence of LPS stimulus. Important traffic proteins such as RABs, SNAREs and Syntaxins, pH regulatory complexes and transporter channels should be the first targets of study. A FACS analysis for antigen degradation of purified phagosomes should be done to better dissect the role of LPS stimulus on proteasome-dependent antigen particulate degradation and its direct role on particle antigen cross-presentation. A next logical and crucial step should be done in order to confirm antigen cross-presentation phenotype *in vivo,* by analyzing $CD8^+$ cytotoxic T-Cell response to particles antigen as well memory long-term response establishment. PLGA model particles should be on the first line, as they have been used as *in vivo* carriers due to their biocompatibility properties and potential for vaccine design.

As a conclusion, the compartmentalized nature on the crosstalk between the TLR-signaling and the antigen cross-presentation phenotype, could suggest the existence of a mechanism of selective maturation by which DCs may use to discriminate the contents of phagosomes and better mount an appropriate immune response. Therefore, DCs may have the "capacity" to decide which kind of destiny an antigen should have depending on the type and origin of the stimuli. This assumption was of great relevance to understand the role of "pathogen" stimuli on antigen fate and the capacity of an antigen-presenting cell to decide the outcome of a specific antigen based-environment. According to our data, we hypothesised that depending the nature of antigen, tumour/viral *vs* bacterial, DCs may use different cross-presentation pathways with different strength, depending on the antigen context and environment. In the absence of a pathogen-like stimulus, a cross-presentation mechanism appears to be preferred, in detriment of MHC class-II which is the "classical" antigen presentation pathway against pathogens. Together our data suggest that cross-presentation might not be the preferred pathway against pathogens but can, notwithstanding, have a dramatic impact in challenges deprived of TLR agonists, namely against tumor cells or in self/altered-self recognition.

The dissection and knowledge of the mechanism behind TLR signaling mediated by agonists in same context as antigen could be of extreme importance to the design of novel therapies. Our hypothesis could be highly valuable to design novel vaccination methodologies to induce T-Cell responses of the desired type and specificity, unveiling a key for an efficient immune response upon vaccination.

Therefore, synthetic well-defined TLR agonist-antigen particles conjugates could be designed for optimal DCs activation and specific T-Cell induction to better study and elucidate how pathogen structure and chemistry dictates signaling, intracellular traffic, antigen processing, immune responses and pathogen survival or elimination.

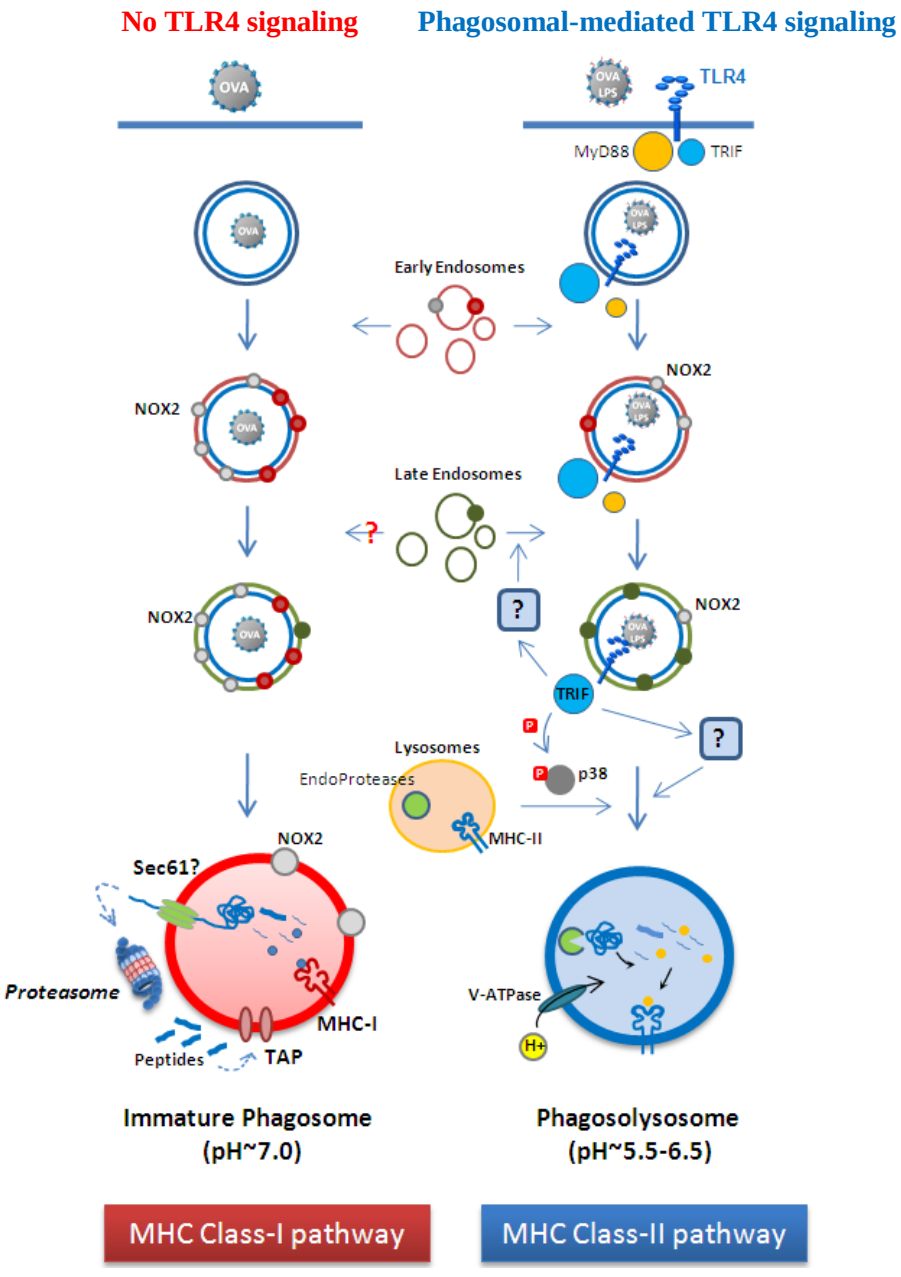


Fig.54: Proposed model of crosstalk between TLR4 signaling and particle antigen presentation pathway(s). Early phagosomes are enriched in certain ER compounds and display high pH and a low

degradation environment due to sustained ROS production by NOX2. These are most probably the compartments that allow antigen escape to the cytosol probably through Sec61. After proteasome processing in the cytosol, the resulting peptides can either be translocated into the ER and follow the endogenous route for MHC class-I molecules or be re-imported to the endocytic compartments for loading on MHC class-I molecules. As phagosomes mature through a TLR4-mediated signal from LPS-containing phagosomes, the presence of ER-derived proteins decreases, and the pH drops. The compartments become incompetent for antigen cross-presentation, but acquire the environment required for MHC class-II antigen presentation. This phenotype is supposed to be mediated by a TLR4/TRIF/p38-dependent mechanism. See Chapter 5 and text above for details.

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