EFFECT OF HYPOCHLOROUS ACID ON ANTIGEN PROCESSING AND PRESENTATION.

A thesis submitted by

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I, Zofia Maria Prokopowicz, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

ABSTRACT

Dendritic cells play a key role in both innate and adaptive immunity. Recognition and presentation of antigens on the major histocompatibility complex (MHC) provide an efficient response against infections. However, this response strongly depends on antigen presentation. During inflammation several oxidative reactions occur that lead to the production of oxidants. Under these conditions protein antigens are exposed to high concentrations of hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2) that conduct changes in antigen structure and enhance the specific T cells response. However, the mechanism of presentation of oxidized antigens is presently unknown.

In this thesis, we have focused on the mechanism involved in the enhancement of the T cell response to oxidized proteins. In particular, we have studied the uptake, processing and presentation of oxidized antigens in *in vitro* and *in vivo* mice models to MHC II-restricted T cells; we have investigated the potential receptor-mediated mechanism underlying the enhanced immunogenicity of oxidized antigens; and finally we have analyzed the connection between protein chemical modifications and the enhanced response to oxidized antigens.

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ABBREVIATIONS

AEP	Asparaginyl endopeptidase;
Ag	Antigen;
$AgNO_3$	Sliver nitrate
CLRs	C-type lectin receptors;
DAMP	Danger associated molecular pattern;
DCs	Dendritic Cells;
DMEM	Dulbecco's Modified Eagle's Medium;
DMSO	Dimethyl Sulfoxide;
DNPH	2,4-dinitrophenyl-hydrazine;
FCS	Fetal calf serum;
FITC	Fluorescein isothiocyanate;
H_2O_2	Hydrogen peroxide;
HBSS	Hank's buffered Salt Solution;
HDL	High density lipoprotein;
HOBr	Hypobromous acid;
HOCl	Hypochlorous acid;
IL-2	Interleukin 2;
IMDM	Iscove's modified Dulbecco's Medium;
LDL	Low density lipoprotein;
LOX-1	Lectin-like oxidized LDL receptor
LPS	Lipopolysaccharide;
LTA	Lipoteichoic acid;
MHC	Major histocompatibility complex
MIIC	MHC class II containing compartment
MPO	Myeloperoxidase;
MMR	Measles-mumps-rubella;

MR	Mannose receptor;
Myd88	myeloid differentiation factor-88;
NA	Neuraminidase;
Na_2CO_3	Sodium Carbonate
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase;
NaOCl	Sodium hypochlorite;
$Na_2S_2O_3$	Sodium Thiosulphate
NETs	Neutrophil extracellular traps;
$^1O_2$	Singlet oxygen;
·OH	Hydroxyl radical;
OVA	Ovalbumin;
PAMP	Pathogen associated molecular pattern;
PG	Peptidoglycan;
PMB	Polymyxin B
rGM-CSF	recombinant mouse granulocyte/macrophage-colony stimulating factor;
ROS	Reactive oxygen species;
RPMI-164	Roswell Park Memorial Institute;
RT	Room temperature;
SRs	Scavenger receptors;
SVV	Small Vessel Vasculitis;
TBS	Tris buffered saline;
TCA	Trichloro acetic acid
TLR	Toll-like receptor;
TNBS-	2,4,6-trinitrobenzenesulfonic acid;
TRIF	TIR domain-containing adaptor-inducing IFNβ;
TRITC	Tetramethylrhodamine B isothiocyanate;

CHAPTER 1 INTRODUCTION

This PhD project focused on studying a new link between innate and adaptive immunity and in this chapter some of the main aspects of both responses will be discussed; in particular this chapter deals with describing the connection between neutrophils, dendritic cells and macrophages.

Neutrophils recruited to the site of inflammation/injury produce a whole range of chemically active compounds that are often important danger signals recruiting further immune effector cells. In this study, the main interest is in the strong oxidant - hypochlorous acid (HOCl). HOCl reacts rapidly with proteins which results in different levels of chemical modifications. Therefore, one may assume high levels of exposure of protein antigens to HOCl in any innate immune/inflammatory site. It is likely that antigens – both exogenous and endogenous – will be modified by HOCl, and that adaptive immunity is generated either in the presence of, or directed against, those modified proteins.

Dendritic cells (DCs) and macrophages are specialized in effective capturing, processing and presentation of protein antigens in order to activate adaptive immunity, and are therefore likely to be the main consumers of HOCl modified protein antigens. Interestingly, neither of these cell types, themselves produce HOCl.

Therefore this study proposes a model where protein antigens undergo modification by HOCl produced by neutrophils and in this new chemical form are presented to adaptive immunity. There are two possible routes of protein modification. Firstly, that modification of protein antigens occurs inside the neutrophil, and macrophages or DCs then phagocytose dead neutrophils together with the modified protein antigens **(Fig 1.1A).** Secondly, that HOCl is released from the neutrophil (for example via leakage from the phagosome) and modification of protein antigens occurs in the immediate vicinity of neutrophils infiltration. These modified antigens are then taken up directly by macrophages/DCs **(Fig 1.1B)**.

The proposed model is based on earlier experimental studies that showed an enhanced adaptive immune response to HOCl modified antigens. For example, it has been demonstrated that lower concentrations of HOCl oxidized ovalbumin are required to stimulate cellular and humoral immune responses compared to the concentrations of native protein needed to achieve the same outcome. The modified form was processed more efficiently by the presenting cell (Marcinkiewicz, 1991, 1992) which in this instance was a B lymphoma cell line. Similar results have been obtained using other model proteins and other methods of oxidation. Oxidation of hen egg lysozyme and bovine α-lactalbumin, either via chlorination or via performic acid, induced higher T cell activation responses when presented by macrophages (Carrasco-Marin, 1998). The release of oxidants can lead to the formation of reactive advanced oxidation products (AOPP), cross-linked proteins

that may act as superantigens (Alderman et al., 2002) and will activate dendritic cells (Alderman et al., 2002). Similarly, aldehyde modifications of protein antigens by glycolaldehyde or by oxidation with $NaIO_4$ were found to render antigens highly immunogenic (Allison, 2000). This carbonyl group may be responsible for the T helper 2 immunogenicity of formaldehyde treated vaccines (Moghaddam, 2006).

Therefore, in this chapter I briefly review the role of the neutrophil and in more detail its unique ability to synthesize HOCl using molecular oxygen. I then briefly review DCs, and in more detail the role of the antigen presenting cells in mediating the interaction between innate and adaptive immunity (danger and pathogen associated molecular patterns).

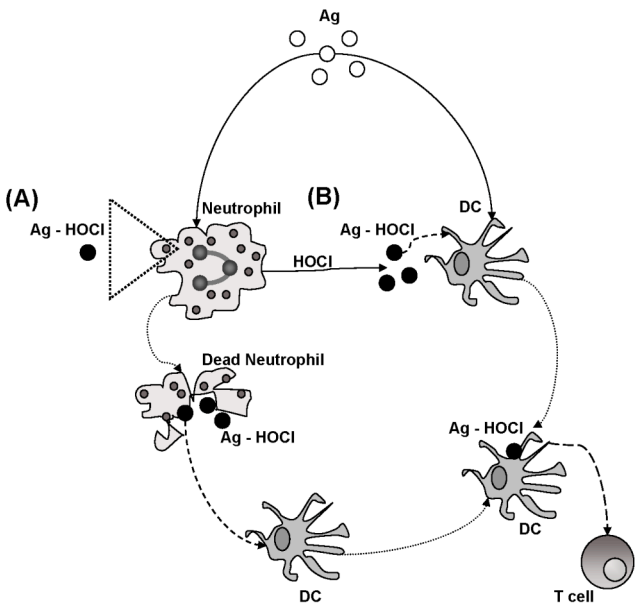


Figure 1.1 Model for the enhancement of adaptive immunity by MPO-derived HOCl.

Protein antigens are likely to be exposed to HOCl oxidation via two ways: (A) oxidation occurs within the neutrophil phagosome, dead neutrophil are then phagocytosed by antigen presenting cells that process and present HOCl-oxidized antigens; (B) oxidation occurs in the immediate vicinity of neutrophil infiltration, HOCl-oxidized antigens are directly taken up, processed and presented by antigen presenting cells.

1.1 NEUTROPHIL CHARACTERISTICS.

Neutrophils are phagocytic cells that mediate a first line of innate immune defense and therefore play a critical role in protection against pathogens. However, uncontrolled neutrophil infiltration may lead to host tissue damage as observed in pathological sites. Neutrophils are also an important link between innate and adaptive immunity. All these aspects are discussed in this chapter.

1.1.1 NEUTROPHIL HOMEOSTASIS.

Neutrophils are short-lived white blood cells with high rate of spontaneous apoptosis. In the presence of inflammatory stimuli however, cell death is altered. Neutrophils are characterized by a rapid turnover. On average 10^9 cells per kg of body weight leave the bone marrow per day in healthy humans; The half-life of neutrophils depends on the species e.g. for humans it is 7-10 hours and for mice 11 hours (von Vietinghoff and Ley, 2008).

Neutrophil proliferation and survival is mainly controlled by cytokine G-CSF; it has been shown, in mice, that lack of G-CSF or the G-CSF receptor leads to neutropenia (Lieschke, 1994).

In mice, most neutrophils are found in the bone marrow and only 1-2% of mature cells are in the circulation (Semerad et al., 2002). Retention of neutrophils in bone marrow is controlled by the interaction between SDF1 [stromal derived factor 1; CXCL12] and the CXCR4 molecule (Christopher, 2007). High levels of SDF1 in bone marrow and increased SDF1/CXCR4 signalling provides a key signal to accumulate

neutrophils in the bone marrow. On the other hand neutrophil mobilization to the peripheral blood occurs after treatment with G-CSF that leads to a rapid decrease in CXCR4 surface expression (Kim et al., 2006).

1.1.2 NEUTROPHIL RECRUITMENT.

The release of neutrophils usually takes place in response to infection or stress. There are many signals that can induce neutrophil mobilization and elicit an inflammatory exudate locally such as bioactive peptides (leukotrien-B_4), chemokines (IL-8) or other bacterial products and inflammatory mediators (C5a complement fragment) (Witko-Sarsat, 2000). In such conditions, the activated endothelium of the blood vessels allows extravasation of neutrophils into extravascular tissues where they migrate along the chemoatractant gradient to the site of inflammation (or injury).

Neutrophil recruitment is characterized by the multi-step leukocyte adhesion cascade divided into five steps: (1) slow rolling, (2) adhesion strengthening, (3) intraluminal crawling, (4) paracellular and transcellular migration and (5) migration through the basement membrane (Ley, 2007) **(Fig.1.2).** Selectins and integrins are the major molecules involved in the regulation of neutrophil rolling and adhesion. P-selectin, E-selectin (expressed by activated endothelium) and L-selectin (expressed on leukocytes) interact with PSGL1 (P-selectin glycoprotein ligand 1) expressed by most leukocytes. Interaction between those molecules allows leukocytes to roll properly on the endothelium. In fact, a knockout mice study confirmed that lack of binding between selectins and PSGL1 leads to severe

leukocytosis (Robinson, 1999).

Both β_1 and β_2 integrins are essential for neutrophil recruitment. Expressed by leukocytes, VLA4 (very late antigen 4 also known as $\alpha_4\beta_1$ integrin) interacts with VCAM1 (vascular cell-adhesion molecule 1). While, LFA1 (lymphocyte function associate antigen 1 also known as $\alpha_1\beta_2$ integrin) supports rolling on ICAM1 (intercellular adhesion molecule 1). Mice deficient in LFA-1 (α integrin chain), or in CD18 (β_2 integrin chain) develop a strong leukocytosis (like to mice deficient in selectins) (Ding et al., 1999; Scharffetter-Kochanek et al., 1998). In humans a heterogeneous mutation in CD18 leads to leukocyte adhesion deficiency type I disorder also characterized by leukocytosis, spontaneous skin infections and impaired leukocyte migration (Bunting, 2002).

The transendothelial migration step is preceded by neutrophils crawling inside the blood vessels; a process controlled by MAC-1 (macrophage receptor 1, also known as CD11b-CD18 and α_M β_2-integrin) and ICAM-1 interaction. Finally, migration through the endothelial basement is further support by $\alpha_6\beta_1$-integrin and other β_1-integrins together with cell-surface expressed elastase (Ley, 2007).

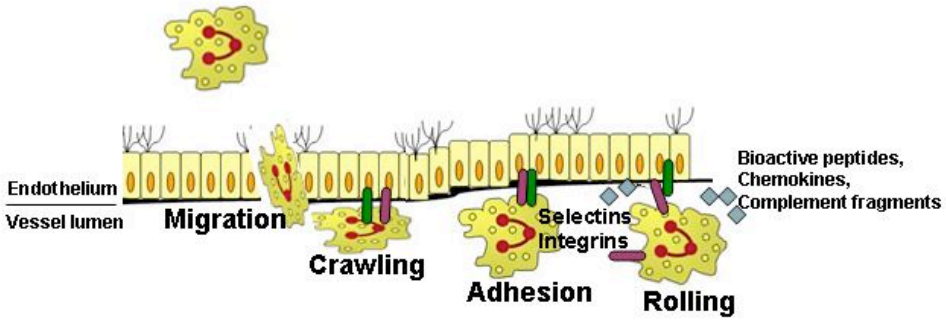


Figure 1.2 Neutrophil recruitment is characterized by a multi-step leukocyte cascade.

The neutrophil (green) is attracted to the site of inflammation by active biopeptides; Interaction between neutrophil and endothelium is characterized by a multi-step cascade that leads to neutrophil extravasation.

1.1.3 NEUTROPHIL PHAGOCYTOSIS.

Neutrophils can absorb both opsonized and non-opsonized particles. Opsonized particles are predominantly recognized by Fc receptors (via binding to immunoglobulin), or by β_2-integrin MAC1 (via binding to C3bi complement fragment). There are several types of Fc receptors. In mice, neutrophils express FcRIII and FcRIV receptors, while in humans, neutrophils express FcRIIA [CD32] and FcRIIIB [CD16] receptors (Warren, 2003). The signalling pathway is mediated by the ITAM-domain in case of mouse neutrophils and by the ITAM-domain together with a unique GPI domain (glycosyl-phosphatidyl-inositol) in human neutrophils (Tsuboi, 2008). In addition, in both species mouse (Jakus et al., 2008) and human (Tsuboi, 2008) neutrophil Fc receptors are critical in promoting immune-complex

mediated diseases.

After particle internalization, the phagosome undergoes a maturation process. This brings changes in phagosomal contents and membrane, leading to activation of bactericidal mechanisms. Contrary to other classic phagocytic cells such as macrophages, mature neutrophils do not undergo an active endocytosis. They do not form early and late endosomes, nor lysosomes, but instead, they possess, specialized granules that contain antimicrobial weapons.

1.1.4 NEUTROPHIL GRANULES.

Neutrophils contain four types of cytoplasmic granules synthesized during individual stages of neutrophil development. Azurophil granules are formed first at the promyelocytic stage; specific granule proteins are synthesized in the myelocytic stage; gelatinase granules appeared in the metamyelocyte stage; finally secretory vesicles are synthesized by endocytosis (Borregaard et al., 2007) **(Fig.1.3);** all four types display differential sensitivity to intracellular levels of Ca^{2+}, that results in a hierarchical pattern of degranulation (Cowburn, 2008). Sequential mobilization of vesicles and granules has been shown both *in vitro* (Sengelov, 1993) and *in vivo* (Sengelov, 1995) and occurs in order as follows: (1) secretory vesicles, (2) gelatinase granules, (3) specific granules, and (4) azurophil granules. Mobilization of secretory vesicles occurs when rolling neutrophils make contact with activated endothelium and when an interaction between selectins and their ligands take place (Borregaard and Cowland, 1997); the vesicle membrane then incorporates with the plasma membrane leading to decoration of neutrophils with a whole range of molecules

normally stored in the vesicles. Of particular interest are the MAC1 molecule, and flavocytochrome b_{558} (a key component of NADPH oxidase), IFN-R1, CD14 and several other proteins. Some of these, that have not yet been confirmed on a protein level but have been shown by gene expression profile during neutrophils development, examples are: TLR-1,-2,-4,-6,-8, TNFR-1 and -2, receptors for IL-(1,4,6,10,13,17,18), TGF-R2, Fc receptors for IgG, IgE and IgA (Borregaard et al., 2007). It is a critical step in neutrophil activation, because the contents of secretory vesicles transform the neutrophil into a highly responsive cell.

Subsequently, gelatinase granules are mobilized. These secrete high concentrations of gelatinase (also known as metalloprotease 9 (MMP9)) (Sengelov, 1993) and leukolysin (also know as MMP25) (Kang et al., 2001). Both proteases degrade laminin, collagen, proteoglycans and fibronectin hence they play a role in neutrophil recruitment (Faurschou, 2003).

Finally, specific granules and azurophil granules are mobilized (with the lowest potency to exocytosis). Both granules contain essential bactericidal proteins. Specific granules secrete lactoferrin (Cramer, 1985; Sengelov, 1995). Azurophil granules secrete: [1] serine proteases such as neutrophil elastase (Kjell and Inge, 1974), cathepsin G (Owen and Campbell, 1999) and proteinase 3 (Cramer, 1985), [2] azurocidin (inactive serine protease homolog) (Campanelli et al., 1990), [3] defensins (Ganz, 1985), [4] myeloperoxidase (Cramer, 1985; Sengelov, 1995).

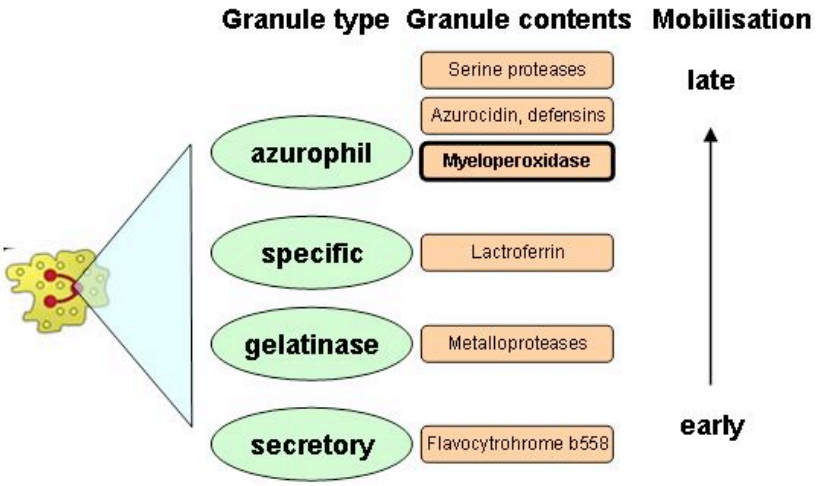


Figure 1.3 Mobilization of neutrophil granules.

Neutrophil granules (green) are mobilized sequentially; each type of granule contains a range of active proteins (examples in orange).

1.2 NEUTROPHIL ROLE IN HOST DEFENCE.

Neutrophils are crucial components of innate immunity and are in the first line of antimicrobial defence. Activated neutrophils recognize, engulf and kill pathogens; they secrete a diverse range of chemoattractants that attract other components of the immune system and help to overcome inflammation. Activated neutrophils show two characteristic features, firstly, they release a variety of proteases and secondly, they release bulk amounts of reactive oxygen species (ROS) into phagosomes. However, it remains open to debate, whether these two factors – oxidative (ROS) or, non-oxidative (proteases) play a critical role in the killing process.

1.2.1 OXIDATIVE PATHWAY.

Production of ROS occurs during the oxidative burst reaction – a process characterized by cyanide-insensitive, rapid oxygen consumption. The enzyme mediating oxidative burst is named nicotinamide adenine dinucleotide phosphate oxidase (NADPH). It is a multicomponent enzyme that is located in the plasma and phagosomal membrane in active cells (El-Benna et al., 2008).

NADPH oxidase activity moves an electron current across the membrane that would normally lead to massive depolarization. However, this process is balanced by a rapid movement of a positive current across the membrane. The initial discovery of NADPH reported an associated massive efflux of H^+ during respiratory burst (Henderson, 1987). Hence, it is very likely that the majority of charge is compensated by protons.

However, recently, Reeves et al (2002) proposed an alternative mechanism involving an additional K^+ flux. In their studies, the K^+ flux was not only compensating negative charge, but was also leading to degranulation and to the release of serine proteases via an increase in vacuolar pH, thus diminishing the role of ROS in bacterial killing. However, further studies done by this group brought incoherent data. Their statement that microbial killing is exclusively controlled by maxi-K^+ channels was later abrogated by several independent studies (Essin et al., 2009; Essin et al., 2007; Rada et al., 2004).

There is no doubt that the main products of the NADPH oxidase enzyme (ROS) are an important component of neutrophil antimicrobial activity. A defect in one of

the components of NADPH results in chronic granulomatous disease (CGD). CGD patients suffer from recurrent bacterial and fungal infections which if not treated may lead to death in childhood (Geiszt et al., 2001).

NADPH catalyses the reduction of oxygen to superoxide [**reaction 1.1**] that is an initial radical for further reactions (El-Benna et al., 2008).

$$2O_2 + NADPH \Rightarrow 2O_2^{-\bullet} + NADP^+ + H^+ \qquad \textbf{[reaction 1.1]}$$

Superoxide is then converted to hydrogen peroxide by spontaneous dismutation at acid pH, or by superoxide dismutase (SOD) catalysed dismutation [**reaction 1.2**].

$$2O_2^{-\bullet} + 2O_2^{-\bullet} + 2H^+ \Rightarrow H_2O_2 + O_2 \qquad \textbf{[reaction 1.2]}$$

Hydrogen peroxide can react further with other superoxide radicals, or is used as a substrate for the myeloperoxidase enzyme (which is discussed in more detail below).

One product of myeloperoxidase enzyme activity is hypochlorous acid (HOCl) **(Fig.1.4)**. In addition to HOCl, myeloperoxidase activity catalyses the reactions with other halogens such as Br^- and SCN^- that lead to production of highly toxic hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN). Due to the nature of this work, further discussion will focus on HOCl.

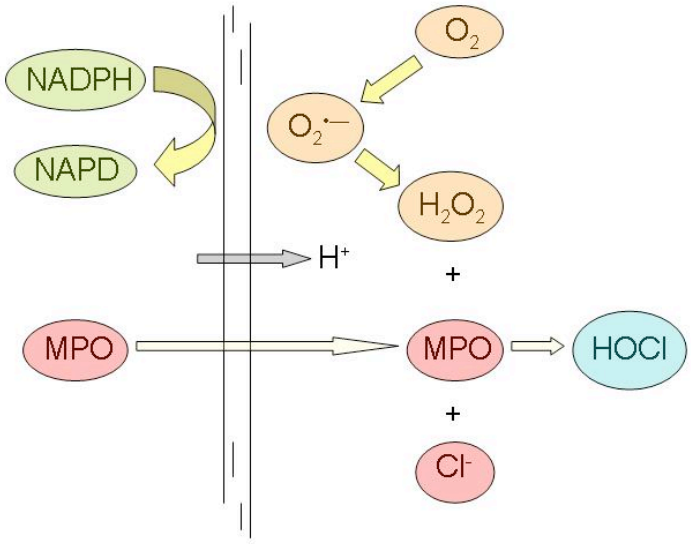


Figure 1.4 Mechanism of HOCl formation [taken from (Klebanoff, 2005)].

NADPH - reduced nicotinamide adenine dinucleotide phosphate; $O_2^{\bullet-}$ - superoxide anion; HOCl - hypochlorous acid; MPO – myeloperoxidase.

1.2.2 NONOXIDATIVE PATHWAY

Serine proteases released from granules are the key weapons used by neutrophils to kill pathogens in a nonoxidative manner. Using a knock out approach, several studies have demonstrated that serine proteases play a critical role in killing both Gram-positive and Gram-negative bacteria as well as fungi. Mice deficient in neutrophil elastase are more susceptible to infection with Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae* (Belaaouj, 1998), and to infection with enterobacteria *Shigella flexneri*, *Salmonella enterica* and *Yersinia enterocolitica* (Weinrauch, 2002). In a more detailed study on mice infected with *E. coli*, neutrophil elastase was shown to destroy the outer membrane protein A (OmpA),

a characteristic feature of Gram-negative bacteria (Belaaouaj et al., 2000). In additon, neutrophil elastase was shown to degrade a virulence factor of enterobacteria (Weinrauch, 2002). On the other hand mice deficient in cathepsin G are more sensitive to infection with Gram-positive bacteria *Staphylococcus areus* (Reeves, 2002). Finally, both enzymes are crucial for defence against fungal infection, deficient animals did have increased susceptibility to infection with *Aspergillus fumigatus* (Tkalcevic, 2000) and *Candida albicans* (Reeves, 2002).

It is important to note that serine proteases are not only responsible for the direct killing of bacteria but can also promote neutrophil activation and thus the general inflammatory reaction. A recent study on proteinase-3 and neutrophil elastase deficient mice (Kessenbrock, 2008) describes both enzymes to be involved in inactivation of anti-inflammatory factor progranulin. Active progranulin inhibits neutrophil oxidative burst, and was found in an active form only in mice deficient in both enzymes.

1.2.3 NEUTROPHIL EXTRACELLULAR TRAPS.

Recently described neutrophil extracellular traps (NETs) are chromatin structures that are coated with proteinase-3, neutrophil elastase, cathepsins G and myeloperoxidase released from neutrophils (Birnkmann, 2004). NETs are formed by activated neutrophils and play a role as traps for bacteria. Firstly, both Gram-positive (*S.aureus*) and Gram-negative (*S.flexneri*) bacteria were shown to associate with NETs. Secondly, the ability to degrade NETs is associated with increased virulence. Some streptococcus bacteria which can escape from NETs demonstrated

increased pathogenicity. It has been demonstrated that the DNase (Sda1) expressed by highly pathogenic Group A streptococcus (GAS) degrades NETs structure (Buchanan et al., 2006). Also Streptococcus pneumoniae was found not be killed by NETs due to expression of EndoA nuclease (Beiter et al., 2006). Thirdly, defects in NETs formation in term and preterm infants leads to impaired extracellular bacterial killing and may be critical determinants in severe neonatal infections such as sepsis and pneumonia (Yost et al., 2009).

A recent study has linked an inappropriate immune response to NETs with a chronic autoinflammatory disorder - small-vessel vasculitis (SVV). SVV is characterized by the presence of antineutrophil cytoplasmic autoantibodies (ANCAs) (Davies, 1982) directed against MPO (Falk, 1988), proteinase 3 (Goldschmeding, 1989) and lysosomal membrane protein-2 (Kain et al., 2008). Kessenbrock et al (2009) has shown that in the case of SVV patients, proteinase-3 and myeloperoxidase normally coating NETs are in fact the main targets for ANCAs autoantibodies; such MPO-ANCA and proteinse-3-ANCA complexes are one of the primary pathogenic factors in SVV.

1.3 MYELOPEROXIDASE

Myeloperoxidase (MPO) belongs to the mammalian haem peroxidase enzyme family. It is one of the key antimicrobial enzymes involved in oxygen – dependent killing of pathogens, and thus plays an important role in mounting an effective

innate response (Klebanoff, 1967). Like many glycoprotein enzymes, MPO biosynthesis is complex, and includes a series of proteolytic cleavage and processing events. The first of these is conversion of the 80kDa primary translation product to 90kDa apoproMPO. There are three distinct steps that are recognised: first, cleavage of a signal peptide; second, N-linked glycosylation; and third, limited deglycosylation of high mannose oligosaccharide side chains (Hansson et al., 2006). ApoproMPO is the enzymatically inactive haem – free form, and has a relatively long half-time in the endoplasmic reticulum (ER) (Hansson et al., 2006). The inactive form interacts with ER molecular chaperones calreticulin (Nauseef et al., 1995), calnexin (Nauseef et al., 1998) ERp57 (Goedken et al., 2007) via the oligosaccharides attached to the protein. These transit associations with apoproMPO promote proper folding, but it is unclear whether or not they are necessary for the final MPO maturation process.

In the ER the next important event which occurs is the insertion of the iron containing (haem) prosthetic group. This results in the formation of an enzymatically active form, proMPO (Nauseef et al., 1992). Although haem incorporation is essential for exit from the ER, and for proMPO reaching it's destination in the primary granules, the final maturation requires a further series of proteolytic events. First a short (125 amino acid) peptide is cleaved off from proMPO to form a 74kDa short-lived temporary product that can undergo secondary cleavage into a 59kDa heavy subunit and a 13.5kDa light subunit (Olsson, 1984). A combination of two heavy-light subunit pairs homodimerises (linked by a disulfide bond between the heavy chains) to form the mature MPO

with a molecular weight of about 150kDa (Andrews and Krinsky, 1981). It is this protein that is stored and can be released into the phagosome when the neutrophil is activated. Excess MPO is inactivated by a negative feedback loop via products of the respiratory burst (King et al., 1997) or is cleared from the extracellular fluid by macrophages via the macrophage mannose receptor (Shepherd, 1990).

1.3.1 BIOLOGICAL EFFECTS.

Several possible outcomes of MPO activation and resultant interaction with hydrogen peroxide have been investigated. These include the generation of hypochlorous acid, and resultant chloramines and aldehydes; of hydroxyl radicals; of singlet oxygen; and of ozone**.**

i. Hypochlorous acid. The unique feature of MPO amongst the mammalian peroxidases is that it is able to catalyse the oxidation of Cl^- to HOCl at neutral pH and physiological plasma concentrations of halide (Harrison and Schultz, 1976; Klebanoff, 1999). The H_2O_2 required for this reaction is produced as a result of superoxide dismutase activity, which is itself generated via NADPH oxidase activity as part of the respiratory burst (Babior, 2004). Approximately 45% of the H_2O_2 consumed by MPO is converted to HOCl (van Dalen, 1997). There are three potential redox intermediates that can be formed during this process **(Fig. 1.5)**. The first compound (I) is formed by the reaction of H_2O_2 with the haem component of MPO. This iron is present normally in ferric form, and is converted to oxy-ferryl haem. The resultant primary catalytic complex of MPO can react with halides to form hypohalous acid, with associated iron regeneration, or can react with excess

H_2O_2 to form the second compound (II), which retains the oxyl-ferryl centre. Reducing agents, including superoxide, can convert compound II back to native enzyme. The third alternative is direct interaction of superoxide with native MPO to form a short-lived compound (III) which has oxygen attached to the haem centre (Klebanoff, 2005).

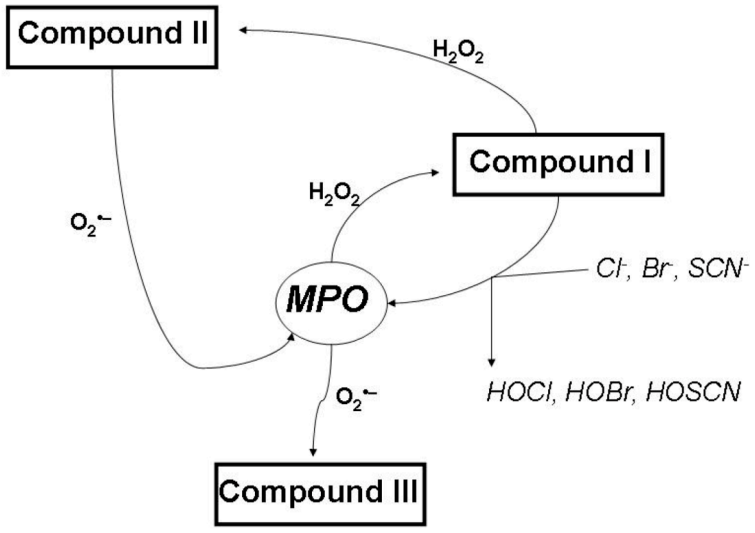


Figure 1.5 Formation of MPO complex.

MPO has the ability to catalyse oxidation of Cl^- to HOCl. There are three potential redox intermediates that can be formed during this process: Compound I, II and III.

The major product of MPO catalysis is hypochlorous acid, but there is some debate about whether or not the MPO-H_2O_2-Cl^- system produces free HOCl from Cl^- or prefers kinetically to catalyse reactions with other accessible halides like Br^-. This debate is based upon the quality of the scientific evidence showing free HOCl in

the system (Spalteholz et al., 2006). In early studies HOCl was detected by reactions with small molecules, such as taurine, that might have been chlorinated directly by HOCl. However, later kinetic studies suggested that taurine interacts at low pH with the enzyme bound intermediate, compound I, and that it is this intermediate that is responsible for taurine oxidation rather than HOCl (Marquez and Dunford, 1994). Other recent studies supported the original view: for example, Davies et al (Davies, 2008) observed that extracellular matrix polysaccharides, like heparin sulphate, despite their bulky size, are chlorinated efficiently by MPO-H_2O_2-Cl^-, where only HOCl can be the oxidizing agent. Thus it is likely that there are two possible routes: compound I may oxidize small molecules, and the final effect on larger proteins is due to HOCl.

ii. Chloramines and aldehydes. At physiological pH HOCl is in equilibrium with hypochlorite (OCl^-) at approximately equimolar concentrations. In lower, acidic, pH, as in phagosomes, HOCl is the dominant form and is in equilibrium with chlorine (Cl_2) which is also a powerful antimicrobial agent, and which damages host tissues (Hazen et al., 1998). In addition HOCl reacts with nitrogen – containing functional groups, particularly amines, which leads to the formation of mono- and dichloro-amines [**reaction 1.3**] (Hawkins, 1998).

$RCH_2NH_2 + HOCl \Rightarrow RCH_2NHCl + H_2O$ [**reaction 1.3**]

This reaction is restricted primarily to His, Lys and Arg side chains, together with N-terminal amino groups (Hawkins et al., 2003). Chloramines formed at the N – terminus of small peptides (Teresa Stelmaszynska, 1978) and at the α-amino

site of free amino acids decompose to form aldehydes. Long-lived chloramines and aldehydes are powerful bactericidal MPO-H_2O_2-Cl^- end-products within the phagosome.

iii. Hydroxyl radical. The original suggestion about formation of ·OH was that this was a result of a Haber-Weiss iron catalysed interaction between H_2O_2 and superoxide [**reaction 1.4**] (Haber, 1934).

$$H_2O_2 + O_2^{-\bullet} \Rightarrow O_2 + OH^- + \bullet OH \qquad \textbf{[reaction 1.4]}$$

This reaction requires the presence of free iron which is present at low concentrations in biological fluids, implying that formation of ·OH via this mechanism is limited. There is a second, (and physiologically more likely) mechanism which has been suggested (Ramos et al., 1992). This is that HOCl, produced via the MPO-H_2O_2-Cl^- system, reacts directly with superoxide to form ·OH [**reaction 1.5 and 1.6**].

$$H_2O_2 + Cl^- \Rightarrow HOCl + OH^- \qquad \textbf{[reaction 1.5]}$$

$$HOCl + O_2^{-\bullet} \Rightarrow \bullet OH + O_2 + Cl^- \qquad \textbf{[reaction 1.6]}$$

However, ·OH is a major product only if there are no proteins or other biological targets for HOCl within the phagosome. Therefore it has been suggested (Winterbourn et al., 2006) that if there is ongoing protein release from either the neutrophil or the ingested bacterium then ·OH generation via reaction with HOCl will be maintained at a very low level that is not likely to be important either

physiologically or pathologically.

iv. Singlet oxygen. Singlet oxygen ($^1O_2$) is a highly reactive form of oxygen. Several groups have shown that activated neutrophils generate this product in vitro. During the first minutes of an antimicrobial response the pH within the phagosome increases from ~5.7 to 7 (Segal, 1981) and the rate of formation of $^1O_2$ rises, while chloramines formation decreases and eventually is abolished (Fumio Arisawa, 2003). These findings have been correlated with observations that within two minutes of engulfment by *S. aureus* into a phagosome almost all bacteria are killed (Segal, 1981). However, this pathway, too, has been linked to the MPO-H_2O_2-Cl^- system, in that an important route to the production of $^1O_2$ is via direct interaction of H_2O_2 with HOCl (Allen et al., 1972). Furthermore, later kinetic studies on the potential targets of HOCl within the phagosome have shown that $^1O_2$ generation cannot compete with other reactions and thus $^1O_2$ levels within the phagosome in vivo never reach high concentrations (Winterbourn et al., 2006).

v. Ozone. There has been considerable debate about generation of ozone (O_3) by activated neutrophils (Babior, 2003; Wentworth et al., 2002). This was based upon the assumption that $^1O_2$ is present at high concentrations within the phagosome (Babior, 2003). As outlined above, this is improbable, and therefore, despite the detailed studies using sensitive traps, O_3 is not an important MPO – generated product.

1.3.2 HOCL – INDUCED PROTEIN OXIDATION.

Amongst the products of the MPO-H_2O_2-Cl^- system, it appears that HOCl is the most important kinetically, competing with (as well as contributing to) other mechanisms of antimicrobial activity, and involved in tissue damage.

HOCl reacts rapidly with proteins, DNA (Prutz, 1996), lipids (Winterbourn et al., 1992), cholesterol (Carr et al., 1996) and free thiols and disulfides (Prutz, 1996). However, proteins are likely to be the main target and therefore the consequences of this process have been studied in most detail. HOCl is a selective oxidant that modifies particular amino acid residues preferentially (Hawkins et al., 2003). Recently second order rate constants have been determined for the reactions of HOCl at physiological pH with all of the potential reactive sites within a protein (Pattison and Davies, 2001). The order of reactivity for the side chain was Met > Cys >> Cystine ~ His ~ α-amino >Trp > Lys >> Tyr > Arg > Gln ~Asn. Reaction with some of these groups is complex and contains unstable intermediates. For example, interaction with Cys forms an unstable compound sulfenyl chloride that reacts either with water to form cysteic acid, or with other Cys groups to form cystine. Amines, especially Lys side chain and terminal α-amino groups are first converted to mono- and dichloramines that can decompose to yield aldehydes. Met is oxidised directly to stable methionine sulfoxide, and Tyr side chains either react at the ~ α-amino group to form p-hydroxyphenylacetaldehyde or via the aromatic ring to give 3-chlorotyrosine (Hawkins et al., 2003).

HOCl is a selective oxidant, and several groups have shown that it inactivates target groups by side-chain specific oxidation. This has been shown for several different proteins: for example, Met and Cys groups in the GroEL chaperone (Khor et al., 2004), Met and Trp-Gly groups in matrilysin matrix metalloproteinase 7 (MMP-7) (Fu et al., 2003), Cys groups in the tissue inhibitor of metalloproteinase 1 (TIMP-1) (Wang et al., 2007), Met groups in cathepsin G (Shao et al., 2005) and lysozyme (Hawkins and Davies, 2005) are all susceptible. HOCl was also shown (together with chloramines) to oxidize glutathione disulfide (GSSG). The reaction was demonstrated to be irreversible; the two products were resistant to reduction by glutathione reductase (Yuan et al., 2009).

The HOCl modification leads to deregulation of protein balance between active and inactive forms and this is often observed when HOCl is implicated in the pathogenesis of chronic diseases.

1.3.3 THE "PATHOPHYSIOLOGICAL" ROLE OF MPO IN BACTERIAL KILLING.

The contribution of MPO, and of the MPO-H_2O_2-Cl^- system, to bacterial killing has been explored more than any other aspect of this pathway. This has included both *in vitro* and *in vivo* experiments, and both murine and human studies.

It is important to note that in this particular system the mouse model may not mirror the human accurately. Apart from the general issues such as accessibility (mouse tissue studies versus human peripheral blood samples), mice have only 10-20% of the MPO activity that is found in humans. Furthermore in the mouse the MPO gene lacks the AluRRE element in the promoter that is seen in primates

(Hansson et al., 2006). In humans an allelic polymorphism in this region (-463G/A) affects the binding of the SP1 transcription factor (Piedrafita et al., 1996). Individuals with two copies of the G-allele have a 25-fold greater rate of MPO transcription than those with -463A/A (Piedrafita et al., 1996).

The original studies *in vivo* demonstrated that purified MPO, in the presence of H_2O_2 and halide ions, can kill bacteria effectively (Klebanoff, 1967) Two different approaches were used to follow-up on these studies. In one approach the phagosome content was subjected to careful scrutiny and HOCl was shown to be present, to be active in chlorination, and to be effective in *S. aureus* killing (Chapman et al., 2002). Interestingly using isotopically labelled tyrosine they also showed that vast majority of chlorination occurs on neutrophil rather than on bacterial proteins. The other approach used mice with impaired HOCl synthesis due to deficiency of MPO. These MPO deficient animals were more susceptible to infection and death following *Klebsiella pneumoniae* infection. (Hirche et al., 2005) However, organisms differ in their susceptibility to MPO-dependent antimicrobial killing. In a more detailed study MPO-knockout mice did have increased susceptibility to infections caused by *Candida albicans* (Aratani et al., 1999), *Candida tropicalis*, *Trichosporon asahii* and *Pseudomonas aeruginosa* (Aratani et al., 2000) whereas responses to *S.aureus*, *S. pneumoniae*, *Candida glabrata* and *Cryptococcus neoformans* were comparable to those seen in wild-type mice (Aratani et al., 2000). In addition the relative killing defects do not translate necessarily into spread of infection and disease: e.g. the consequences of failure

to kill *Candida albicans* did not seem to be as serious as expected (Lanza, 1998).

1.3.4 MPO DEFICIENCY.

In humans MPO-deficiency is a relatively common disease, with an incidence of between 1:2,000 and 1: 4,000 in Europe and the United States (Parry, 1981). There are two forms: primary and secondary. Primary deficiency is genetic in origin with complete absence of MPO activity in all white blood cells, as well as in bone marrow myeloid precursors (Lanza, 1998). Secondary deficiency occurs due to concomitant underlying diseases and can be corrected successfully by treatment of these diseases (Lanza, 1998).

Since MPO is involved in killing pathogens, MPO deficiency of the enzyme should result in severe impairment of host response, but this is not the case. Most MPO-deficient patients are not susceptible particularly to persistent or severe infections. Thus, it is probably true that in MPO-deficiency in humans impairment of antimicrobial activity depends on the pathogen species. Moreover, there are several MPO-independent mechanisms that can compensate for absence of the peroxidase in the phagosomes, such as prolongation of the respiratory burst (Cramer, 1982; Kitahara et al., 1981).

1.3.5 THE ROLE OF MPO IN INFLAMMATORY PATHOPHYSIOLOGY.

The correlation between the presence of abnormal levels of MPO and the risk of pathological disorders has been only studied in the last 10 years. One of the main reasons was a lack of good screening methods that would allow validating patients for MPO deficiency or MPO polymorphism and would detect *in vivo* presence

of MPO in pathological tissues. However, increasing evidence that MPO is in fact a risk factor in several disorders ranging from autoimmune disorders to cancer, led to the development of new methods of MPO detection and to increasing numbers of population studies. Some of the major evidence implicating a role of MPO in disease processes is summarized in **Table 1.1**.

One recent study by Gross *et al.* (2009) proposed tracking active MPO in inflamed tissue using a bioluminescence imaging technique. They described three aspects that make luminol a promising diagnostic tool: (1) luminol is non-toxic; (2) *in vivo* studies demonstrated that luminol activity depends on MPO activity and that was demonstrated for a range of diseases associated with MPO; (3) using this method a new role for MPO was identified in early tumorgenesis.

Magnetic resonance imaging (MRI) was also proposed as a useful non-invasive method to track MPO activity. An enzyme-activatable MRI agent was detected by active MPO. This has been shown on a mice model with cerebral ischemia, where level of MPO correlated with size of infarct (Breckwoldt et al., 2008).

Disease	MPO implications
Atherosclerosis	• Oxidation of LDL (Hansson, 1999; Pattison, 2006; Podrez et al., 2000) • Oxidation of HDL (Undurti et al., 2009)
Pulmonary disease	• Cystic fibrosis - Lung damage - Chronic lung infections (Kettle et al., 2004; Reynolds, 2006)
Renal disease	• Up-regulation of genes involved in ROS metabolism, stress and tissue remodeling (Porubsky et al., 2004) • Present in inflamed glomeruli (Groene, 2002)
Arthritis and vasculitis	• Cartilage damage (Schiller, 2003) • Pathogenic factor in Small Vessel Vasculitis (SVV) (Kessenbrock, 2009)
Neurological disease	• Alzheimer's disease - present in brain aggregates - inhibition of metabolic pathways (Jeitner, 2005; Reynolds et al., 1999) • Multiple sclerosis - present in plaque lesions - damaging the myelin sheath (Nagra et al., 1997)

Neoplastic disease	• Damaging cellular DNA (Prutz, 1996) • Pro-inflammatory risk factor (Reynolds et al., 1997)
Polymorphism **- 463G>A**	• Increased risk of gastric cancer (Hsu et al., 2008) • Increased risk of bronchial asthma (Polonikov et al., 2009) • Increased risk of Alzheimer's disease (Maki et al., 2009)

Table 1.1 Role of MPO in pathophysiology.

LDL - low density lipoprotein, HDL – high density lipoprotein, ROS - reactive oxygen species.

1.4 MOUSE DENDRITIC CELLS.

Studies of DCs subsets have been performed over several years and there is a vast body of literature covering this topic, an overview of work from Carbone (Heath and Carbone, 2009), Villadangos (Villadangos and Schnorrer, 2007) and Ardavin (López-Bravo and Ardavín, 2008) is described below.

In general it is accepted (both in mouse and human models) that DCs are subdivided into two main groups: **myeloid DCs** (also referred as **conventional DCs**, cDCs) and **plasmacytoid DCs** (pDCs) The cDCs are further subdivided into at least three subgroups: two found in the steady-state conditions named lymphoid-tissue-resident and migratory and a third found during inflammation named monocyte derived or inflammatory DCs.

The pDCs are placed apart from cDCs and their characteristics and role will not be discussed in detail here; pDCs in the steady-state condition circulate in the blood and lymphoid tissues; while activated pDCs are the major producers of type I interferon (Colonna et al., 2004).

Lymphoid-tissue-resident DC. They develop from bone marrow precursors and are resident in lymphoid organs. They do not migrate through peripheral tissues but respond to the antigen present in the lymphoid organs. The group includes DCs from thymus and spleen. Resident DCs enter lymphoid organs in an immature state and remain immature in steady-state conditions until they take up and process antigen.

The first discovery of different subsets of DCs in the mouse spleen was published by Shortman's group; they identified two subsets of splenic DCs depending on their surface expression of the CD8α marker; $CD8\alpha^-$ and $CD8\alpha^+$ (Vremec, 1992). More detailed studies have shown that $CD8\alpha^-$ can be further divided into $CD4^-$ and $CD4^+$ subsets. Thus there are three major subsets of splenic DC: $CD8^+$, $CD8^-CD4^+$, $CD8^-CD4^-$ (double negative).

Migratory DC. Migratory DCs derive from the earlier precursors in the peripheral tissue; their life cycle can be divided into (1) antigen capture, (2) migration, (3) maturation, (4) antigen presentation. The subdivision of migratory DCs is much more complex than resident DCs but three major groups are identified; Langerhans cell (LC), classic dermal cells characterized by high expression of CD11b and dermal cells expressing langerin and low levels of CD11b.

Monocyte-derived DC. Monocyte-derived DC emerge only at the site of inflammation and are derived from recruited monocytes.

The phenotype of discussed subsets of DCs are summarized in **Table 1.2.**

	Steady-state						Inflammation
	Resident DC			Migratory DC			
Surface markers	$CD4^+$	$CD8^+$	$CD4^-CD8^-$	Langerhans	Classic dermal	Dermal $langerin^+$	Mono-DC
CD11c	+++	+++	+++	+++	+++	+++	+++
CD11b	++	+/-[a]	++	+++	++	-	++
CD8	-	+++	-	++	-	-	-
CD4	+	-	-	-	-	-	-
Langerin	-	+	-	++	-	++	-
MHC II	++	++	++	+++	+++	+++	++

Table 1.2 Phenotype of mouse dendritic cell subsets.

Markers expression "-" null; "+" low, "++" intermediate, "+++" high. [a] *Low expression (López-Bravo and Ardavín, 2008), null expression (Villadangos and Schnorrer, 2007).*

1.5 MECHANISMS CONTROLING IMMUNE RESPONSE.

1.5.1 RECOGNITION OF DANGEROUS SIGNALS.

The hypothesis that there is a link between neutrophils and DCs is based on the fact that neutrophils produce a range of chemically active compounds that may lead to modification of protein antigens. These proteins are then recognized by DCs that differentially handle modified antigen compared to native equivalents leading to an altered immune response.

The function of the link between innate and adaptive immunity is to accurately recognize dangerous signals, typically associated with infection, that if not controlled, may lead to fatal consequences. The innate immune system can recognize infection either indirectly as a consequence of tissue damage (danger

associated molecular patterns (DAMPs)) or directly via pathogen associated molecular patterns (PAMPs). Recognition occurs via germline-encoded pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) proteins (Nod-like receptors = NLRs). In addition response to PAMPs can be mediated by C-type lectin receptors (CLRs) and scavenger receptors (SRs).

The role of DAMPs and PAMPs is to guide the innate immune response system to recognize pathogens or, more generally microbes. It is a key task that if not properly regulated may lead to un-controlled inflammation and death or chronic autoimmune disorders. The relationship between DAMPs and PAMPs is complex and still not fully understood. Studies on PAMPs have been performed over several years and only more recently has attention focused on understanding the mechanisms controlling responses to DAMPs.

Since DAMPs and PAMPs belong to two separate categories of dangerous signals they will be described separately. Firstly, examples of PAMPs in the context of TLRs and NLRs activation will be described. Secondly, some examples of DAMPs and the brief explanation of the recent models of DAMPs signalling will be discussed.

1.5.1.1 PAMPs

PAMPs are microbial components and have three characteristic features: (1) they are present among different microorganisms, (2) they are products of pathways that are unique for microogranisms and (3) they play an important role in

the physiology of microorganisms.

TLRs are the major receptors recognizing PAMPs. TLRs are type I transmembrane proteins characterized by an extracellular domain containing leucin-rich-repeat (LRR) motifs and a cytoplasmic signalling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain. TLRs are expressed on numerous immune cells including neutrophils, macrophages, dendritic cells, B cells and specific types of T cells as well as on nonimmune cells like fibroblasts and epithelial cells. There are several members of the TLRs family that recognize a variety of microbial-specific elements, these are summarized in **Table 1.3**. TLRs can be expressed both extra- and intracellularly. TLRs expressed extracellularly include TLRs 1, 2, 4, 5, and 6. TLRs expressed intracellularly include TLRs 3, 7, 8 and 9.

TLR signalling is initiated by binding of an adaptor protein to the Toll/Interleukin-1 receptor (TIR) domain of TLRs. Four adaptor proteins have been so far identified, myeloid differentiation factor-88 (Myd88), Myd88 adaptor-like (MAL), TIR domain-containing adaptor-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). In general Myd88 mediates signalling of all TLRs except of TLR3 that is controlled by TRIF protein. TRIF protein also mediates Myd88-independent signalling of TLR4. The MAL adaptor protein was demonstrated to act only for TLR4 and for low ligand concentration for the TLR2 signalling cascade (Kenny et al., 2009; Yamamoto et al., 2002), while TRAM was shown to be involved in the TLR4 signalling cascade (Oshiumi et al., 2003).

TLRs	LIGAND	SELECTED ORGANISM
TLR2 (Bieback, 2002; Takeuchi, 1999)	Lipoteichoic acid (LTA)	Gram(+) bacteria
	Peptidoglycan (PG)	Gram(+) bacteria
	Hemagglutinin protein	Measles virus
TLR2-TLR1 (Takeuchi et al., 2002)	Triacylated lipoproteins	Bacteria and mycobacteria
TRL2-TLR6 (Takeuchi et al., 2001)	Diacylated lipoproteins	*Mycoplasma*
	Lipoteichoic acid (LTA)	Group B *Staphylococcus*
TLR3 (Alexopoulou, 2001)	dsRNA	Viruses
TLR4 (Hoshino et al., 1999; Kurt-Jones, 2000; Poltorak et al., 1998)	Lipopolysaccharide (LPS)	Gram(-) bacteria
	Envelope proteins	RSV
TLR5 (Uematsu, 2006)	Flagellin	Flagellated bacteria
TLR7, TLR8 (Diebold et al., 2004; Heil et al., 2004)	ssRNA	RNA viruses
TLR9 (Coban et al., 2005; Hemmi, 2000)	CpG-DNA	Bacteria and mycobacteria
	Hemozin	*Plasmodium*
TLR11 (Yarovinsky et al., 2005; Zhang et al., 2004)	Profilin	*Toxoplasma gondi*
		Uropathogenic bacteria

Table 1.3 Characteristic of Toll-like receptors.

Another group of receptors mediating signalling from PAMPs are **NLRs**. NLRs are intracellular receptors. They contain three domains: (1) at the N terminus pyrin N-terminal homology domain (PYD) or, CARD, or Baculoviral IAP repeat (BIR) domain; (2) at the intermediary region NOD or, NACHT or; dinucleotide (NAD) domain and (3) a C-terminal LRR domain (Ishii, 2008). There are five groups of NLRs classified according to N-terminal domain: NLRA, NLRB, NLRC3-5 (and NOD1 and NOD2), NLRP1-14, NLRX (Ting, 2008). NLRs recognize specific elements of bacterial PGN such as γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), which activate NOD1 and NOD2 respectively (Chamaillard et al., 2003; Girardin et al., 2003a; Girardin et al., 2003b). In addition NAIP5 (NLRB) and IPAF (NLRC4) are activated by flagellin from *Legionella pneumophila* and *Salmonella typhimurium* (Franchi, 2006; Miao, 2006; Ren et al., 2006).

1.5.1.2 DAMPS

DAMPs include molecules such as proteins, lipids and nucleic acids or smaller molecular weight chemicals that originate, for example, from cells undergoing the process of necrosis. Therefore, one would expect to find high concentrations of DAMPs during tissue injury or as a response to cancer. DAMPs can either activate TLR/NLRs signalling directly or are released as a consequence of TLRs/NLRs signalling activation.

Heat shock proteins (HSPs) are a good example of DAMPs that are recognized by the TLR pathway. In DCs, HSPs were shown to promote antigen presentation, for example Gp86 was shown to activate DCs via TLR4 and TLR2 signaling

(Warger, 2006).

The protein, high mobility group box 1 (HMGB1) is another example of a DAMP. HMGB1 was studied firstly in the context of gene transcription. However, later studies have shown HMGB1 is involved in inflammatory responses (Abraham et al., 2000) and released from necrotic cells (Scaffidi, 2002). In addition, more recent studies demonstrated a release of HMGB1 in response to hypoxia (Tsung et al., 2007). Studies done on cultured hepatocytes exposed to hypoxic conditions demonstrated that the release of HMGB1 requires TLR4-dependent production of ROS and the activation of calcium/calmodulin-dependent protein kinases (CaMKs).

Although DAMPs and PAMPs signal through the same receptors they belong to two separate groups of danger signals. Moreover, DAMPs are self-derived molecules and thus continous strong responses in their presence could potentially lead to severe autoimmunity. Therefore, a mechanism must exist that allows immune cell firstly to distinguish PAMPs from DAMPs and secondly to repress the host response to DAMPs. In a recent study, Chen at al (2009) proposed a mechanism where recognition of DAMPs by TLRs/NLRs requires the presence of CD24 – Siglec G (for mouse) or – Siglec 10 (for humans) complex. DAMPs interact with CD24-Siglec G/10 complex, the process leads to the cross-linking with TLRs/NLRs and that allows Siglec to associate with SHP-1 phosphates and further NF-κB signalling.

1.5.2 OTHER IMMUNE RECEPTORS.

CLRs are a large and diverse group of receptors that were demonstrated to bind sugars. However, it is not clear if all CLRs are lectins. Sugar residues can be found in

two common structures, polysaccarides that are a polymeric carbohydrate structures and glycoproteins that are proteins containing oligosaccharide chain attached to protein via O- or N-glycosylation. Fungal cell walls, for example, are made up predominantly of polysaccharides and thus CLRs have been shown to be involved in protection against fungal infections. For example, the dectin-1 receptor was shown to specifically recognize soluble and particulate β(1→3) and/or β(1→6) linked glucans. It was also shown to recognize zymosan, a stimulatory cell-wall extract of *Saccharomyces cerevisiae* that is composed mainly of β-glucan (Brown, 2006). Dectin-1 was found to be expressed only by two subsets of DC double negative $CD8\alpha^{-}CD4^{-}$ and dermal DCs (Carter et al., 2006), the last group obviously plays an important role in the recognition of skin pathogens such as fungi.

Varieties of CLRs are expressed on all antigen presenting cells, but in particular they are expressed on immature DCs that use CLRs to sense and capture antigens. Thus CLRs very often play a role of antigen uptake receptors that can specifically target antigens on MHC class I or class II presentation systems. Some important examples are described below.

Studies on mannose receptor (MR) deficient mice demonstrated a MR role in antigen cross presentation and the activation of the $CD8^{+}$ T cell response (Burgdorf, 2006). Antigen (or peptide) coupled with DC-SIGN antibody against human DC-SIGN was shown to elicit strong MHC class I and II responses by human DCs (Tacken et al., 2005), in other studies keyhole limpet hemocyanin (KLH) coupled to anti-DC-SIGN-antibody was shown to increase KLH adjuvant properties which led

to the inhibition of cell tumor growth (Kretz-Rommel et al., 2007). Because the biology of human and mouse DC-SIGN is different, in this study they used a mouse model reconstituted with human immune cells. Finally, bone marrow and splenic DCs derived from human DC-SIGN transgenic mice, when exposed to ovalbumin modified with glycan that targets DC-SIGN (such as Lewis X and B oligosaccharides), were demonstrated to induce a strong $CD4^+$ T cell response (Singh et al., 2009).

Also, targeting through other CLRs e.g. DEC-205 was demonstrated to induce a strong and specific T cell response. Ovalbumin-αDEC-205 conjugate used in a form of protein vaccine and delivered s.c. to a mouse was successfully processed by DCs that induced a specific $CD8^+$ T cell response (Bonifaz et al., 2004).

A recently described new type of CLR is mouse DC-immunoactivating receptor-1 (mDCAR1). Its expression is restricted to a $CD8^+$ DC subset in the spleen and thymus and on a subpopulation of $CD11b^+$ myeloid cells in the bone marrow and spleen, which makes it an attractive candidate to use in vaccination; antigen delivered via mDCAR1 is presented by both MHC class I and class II molecules and induces $CD4^+$ and $CD8^+$ T cell responses (Kaden et al., 2009).

Other examples of receptors with restricted expression are MGL and mouse MGL2. MGL is expressed by immature DCs and was demonstrated to bind GalNAc-polymers. In contrast, mouse MGL2 receptors are restricted to dermal DCs and may lead to the induction of contact hypersensitivity reactions

(Kumamoto et al., 2009).

SRs are a separate large family of receptors that are expressed by APCs and some endothelial cells. SRs are divided into eight classes SR-A, SR-B, SR-C, SR-D, SR-E, SR-F, SR-G and SR-H. Although originally identified as the receptors for modified low-density lipoprotein (LDL) (oxidized or acetylated but not native), they are also involved in microbial recognition of both Gram-negative and Gram-positive bacteria and uptake of apoptotic cells.

The first study on the involvement of SRs in apoptotic cell recognition was based on competition experiments using monoclonal antibodies against oxLDL. Recognition of apoptotic cells by macrophages was blocked by monoclonal antibodies that bind specific epitopes on oxLDL, indicating that apoptotic cells express oxidation specific epitopes on their surface which are recognized by the same receptor as oxLDL (Chang et al., 1999). Later, other studies confirmed that the uptake of apoptotic cells is mediated by a lectin-like oxidized LDL receptor LOX-1 (SR-E) (Oka et al., 1998), CLA-1 (homolg of rodent SR-BI) (Murao et al., 1997) and CD36 (SR-B) (Ren et al., 1995). Moreover, it was demonstrated that CD36 is expressed preferentially on immature DCs that can present apoptotic antigens via the mechanism of cross-presentation on MHC class I complexes (Albert et al., 1998).

More recently SRs have been identified directly as being involved in innate immune recognition. The class A SRs bind LPS from the Gram-negative bacteria and also the lipoteichoic acid (LTA) component of Gram-positive bacteria. More importantly SR-A has been shown to mediate non-opsonic phagocytosis of several bacteria, for

example *E.coli* (Peiser et al., 2000), *Neisseria meningitides* (Peiser et al., 2002) and *Staphylococcus pneumoniae* (Arredouani et al., 2006).

Interesting studies were also done on the class B CD36 receptor. Hoebe *et al*. (2005) demonstrated that during an innate immune response SRs interact with TLRs; CD36 was shown to play a role as a co-receptor for TLR2 in response to bacterial LTA. CD36 was also shown to be involved in malaria pathogenesis and the uptake of erythrocytes infected with *Plasmodium falciparum* (Patel et al., 2004); and very recently it has been discovered that CD36 and SCARF1 (SR-F) bind the β-glucan component of *Cryptococcus neoformans* and *Candida albicans* and thus play a role in the response to fungal pathogens (Means et al., 2009).

Other members of the SR family also control innate immunity; as mentioned above, class F, SCARF1 and class E, LOX-1 can recognize bacterial surface proteins, such as outer membrane proteins from *Klebsiella pheumonia* (KpOmpa) (Jeannin et al., 2005). While class G, SR-PSOX (also known as CXCL16) can recognize bacterial CpG motifs (Gursel et al., 2006).

The importance of SRs in pathogen recognition is emphasised by the fact that some pathogens have evolved systems to escape from SRs recognition. For example, MARCO (SR-A) is known to be very potent in the uptake of *E.coli*. But, recent studies by Pinheiro da Silva *et al.* (2007) demonstrated a mechanism where binding of *E.coli* to FcRIIIγ receptor led to negative regulation of MARCO. The process was shown to depend on the tyrosine phosphatase SHP-1 that was binding MARCO.

Finally, SRs like TLRs and CLRs can modulate the adaptive immune response, leading to enhanced T cell responses. DCs treated with SR-A ligand, fucoidan were shown to mature and to enhance the specific T cell response (Jin et al., 2009). Also, binding the antigen with 2-macroglobulin (2M) (in active form it binds to scavenger receptor CD91/LRP), was demonstrated to increase antigen uptake and the T cell response (Hart et al., 2004). SRs are promising targets for cancer immunotherapy; a lectin-like oxidized LDL receptor (LOX-1) expressed on DCs mediates both *in vitro* and *in vivo* effective antigen cross-presentation (Delneste et al., 2002); while CD36-mediated endocytosis of soluble Ag was shown to enhance peptide delivery on MHC class II and via the cross-presentation on MHC class I (Tagliani et al., 2008).

1.6 ANTIGEN UPTAKE, PROCESSING AND PRESENTATION.

1.6.1 ANTIGEN UPTAKE AND PROCESSING.

Antigens, depending on their size and form (soluble or particulate), can be taken up via endocytosis (soluble antigen) or phagocytosis (particulate antigen). Soluble antigens destined for MHC class II complex are directed into processing endosomes and are then rapidly degraded. Alternatively, soluble antigens can also be destined for cross-presentation on MHC class I complex, in this case they are taken up by a distinct endocytosis mechanism that keeps antigens in early endosomes and protects them from complete degradation. Finally, particulate antigens can be directed either on MHC class I or class II after entering phagosomes.

Endocytosed antigens are targeted to the endocytic pathway which is composed of three compartments: early endosome, late endosome and lysosome **(Fig 1.6).**

Each of the compartments is characterized by a progressive acidification and the presence of specific RAB-family proteins. For example, early endosomes are positive for Rab5 while late endosmes are positive for Rab7 protein.

In macrophages and DCs antigens are also taken up via phagocytosis which is a receptor-mediated, actin driven process of particle internalization. Phagosomes are formed de novo at the plasma membrane and undergo a series of sequential fusions with endosomes that lead to phagosome maturation and final fusion with lysosomes (Kinchen and Ravichandran, 2008). In macrophages, the process is characterized by a rapid decrease in phagosomal pH from 5.5 to 4.5. Acidification is essential for the activation of lysosomal acidic proteases that are responsible for both protein degradation and the activation of MHC II. Interestingly, as compared to macrophages, immature DCs are capable of keeping phagosomes at an alkaline pH ranging between 7 and 7.5 for several hours until they receive a maturation signal (Trombetta et al., 2003). Two membrane complexes were found to control this specific feature: NADPH oxidase (described earlier in neutrophils) and ATPase. It has been demonstrated that phagosomes from mice lacking the NADPH subunit gp91*phox* are more acidified than phagosomes from wild type mice (Savina et al., 2006). Thus, the presence of NADPH seems to mediate active alkalization of DC phagosomes. However increased acidification in *gp91phox* knockout mice was still not as efficient as in macrophages. Hence this finding indicates that another protein, perhaps the vacuolar ATPase (V-ATPase) is important (Trombetta et al., 2003).

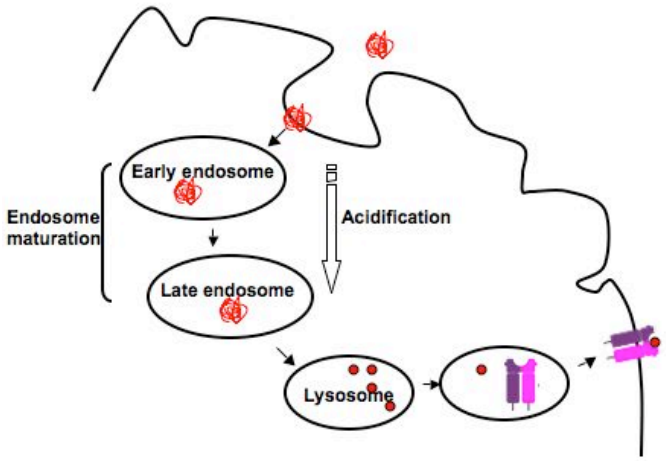


Figure 1.6 Endocytosis of soluble antigens.

Antigens entering the cell are directed into the early endosome that undergoes the process of endosome maturation characterized by progressive acidification. Antigens are degraded in the lysosomes where they are loaded on the MHC class II complex.

In DCs there are three groups of proteases which have been directly implicated in class II MHC antigen processing: (1) the cysteine proteases such as cathepsin S, B, H and L; (2) the aspartate proteases such as cathepsin D and E; and (3) the asparagine endopeptidases (AEP) (Honey and Rudensky, 2003). Enzymes involved in the antigen presentation are summarized in the **Table 1.4**. Each enzyme has a defined range of pH for an optimal activity that allows DCs to control the level of antigen proteolysis.

It has also been demonstrated that DCs, despite having a comparable amount of lysosomes to macrophages, contain significantly lower levels of proteases than

macrophages. Hence, DCs demonstrate limited proteolysis capacity which was shown to favor antigen presentation (Chain, 1986; Delamarre et al., 2005). Indeed, some further studies have suggested that antigens less susceptible to proteolysis are more immunogenic (Delamarre et al., 2006).

Enzyme	Protease type	Expression	Knockout phenotype
Lysosomal proteases			
Cathepsin B	Cysteine	B cells, DCs, MΦ	No marked immune-system phenotype reported
Cathepsin D	Aspartic	B cells, DCs, MΦ	No immune-system phenotype reported; die at 21 days
Cathepsin F	Cysteine	MΦ, epithelial cells	No data
Cathepsin K	Cysteine	MΦ, osteoclasts	No marked immune-system phenotype reported
Cathepsin L	Cysteine	Activity: cortical TECs, MΦ, thymocytes; Protein expression B	Decreased $CD4^+$ T cell and NKT cell selection; epidermal hyperplasia; hair-follicle deficiencies; dialed cardiomyopathy

Enzyme	Protease type	Expression	Knockout phenotype
Cathepsin S	Cysteine	B cells, DCs, MΦ, epithelial cells	Decreased MHC II presentation of exogenous Ag by B cells and DCs; decreased numbers of NKT cells; deficient germinal-center formation; impaired class switching to IgG2a and IgG3 isotypes
Asparaginyl endopeptidase (AEP)	Cysteine	B cells, DCs	Hemaphagocytic syndrome phenotype; lower NK cell activity
Non-lysosomal proteases			
Cathepsin E	Aspartic	DCs, Langerhans cells, microgilia, lymphocytes	Increased bacterial infections and decreased cell-surface expression of TLR1 and TLR2; lysosomal storage disorder in MΦ; accumulation of mast-cell carboxypeptidase A.

Table 1.4 Proteases implicated in antigen presentation taken from (Honey and Rudensky, 2003), updated from (Chan et al., 2009; Zaidi et al., 2008).

1.6.2 ANTIGEN PRESENTATION.

In general, captured antigens can be presented by two distinct types of molecules: major histocompatibility complex class I (MHC I), or class II (MHC II). However, because of the focus of this thesis only presentation on MHC class II complex will be described further.

Expression of the MHC class II complex is restricted to professional antigen presenting cells (APCs) such as macrophages, B cells and DCs. Peptides loaded on MHC class II are generated from exogenous proteins and stimulate $CD4^+$ T cells.

Peptide presentation on MHC class II can be divided into five main steps **(Fig 1.7).** (1) Exogenous antigens are endocytosed and directed to endosomes. (2) Fragmented antigens are transported to an MIIC compartment containing MHC class II molecules that are being recycled from cell surface and transported by the endoplasmic reticulum (ER) system. The MHC class II peptide binding groove is occupied by the invariant chain (Ii) that also docks MHC class II to MIIC (3) The Ii region is enzymatically removed and only CLIP Ii is left in the peptide binding groove. (4) Under the support of H-2M (in humans HLA-DM) chaperon antigen is exchange with CLIP and loaded on MHC class II. (5) MHC class II together with loaded peptide is transported to on the cell surface (Rocha, 2008).

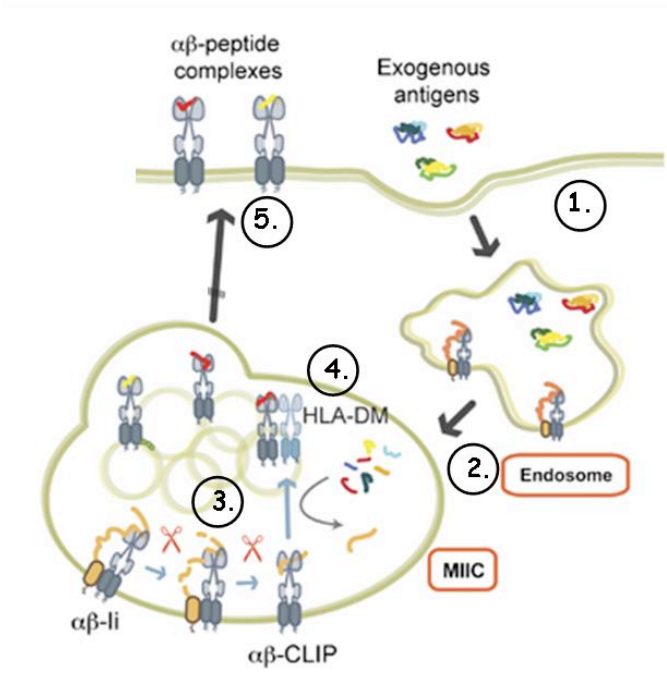


Figure 1.7 Peptide presentation on MHC class II complex taken from (Rocha, 2008).

Antigen presentation by MHC class II. (1) antigen uptake and fragmentation; (2) antigen transport to MIIC compartment; (3) degradation of Ii, CLIP blocks peptide binding groove; (4) exchange of CLIP to antigen peptide with a support of HLA-DM chaperon; (5) transport of peptide loaded MHC class II on the cell surface.

There are several factors that can regulate efficient MHC II – peptide complex presentation. One of the crucial factors is a low, acidic pH. As already mentioned, it has been demonstrated that during DCs maturation, lysosomal pH decreases while in immature DCs it is kept alkaline (Trombetta et al., 2003). Therefore, only mature DCs are capable of expressing high levels of peptide-loaded MHC II molecules on the cell surface. DC maturation has also been recently linked with caspase activity. Protein transport between the *trans*-Golgi and the endosomal compartment is

controlled by the adaptor protein AP-1 and, notably, AP-1 activity is controlled by caspase. Indeed, it has been shown that caspase inhibition leads to DCs maturation (Santambrogio et al., 2005).

DCs also possess another unique mechanism to control MHC II expression. It has been demonstrated that in immature DCs, the MHC class II β-chain cytoplasmic tail is ubquitinated, which prevents proper MHC II sorting. However, in the presence of a DC activator factor, such as LPS, ubiquitination is suppressed and MHC class II is fully functional (Shin et al., 2006; van Niel et al., 2006).

1.6.3 ENHANCING ANTIGEN IMMUNOGENICITY.

Understanding the mechanism controlling enhanced antigen immunogenicity is of particular interest and several groups worldwide conduct extensive studies. One of the reasons for this attention on the subject is the need to improve vaccination strategies and to be able to obtain strong and long-lasting immune responses. A few examples of new strategies are presented here both in the context of MHC class II and MHC class I presentation.

For MHC class II presentation, the delivery of an antigen in a complex with a mediator of a maturation signal such as TLR-4 ligand LPS (Blander and Medzhitov, 2006) led to significantly enhanced antigen presentation.

For MHC class I presentation an enhanced response was demonstrated, for example, for antigens coupled with anti-DEC-205 antibodies (Dudziak et al., 2007), for antigens combined with a trafficking signal molecule that directed antigen into

compartments containing MHC class I (Kreiter et al., 2008), for peptides derived from tumor antigens complexed with Hsp70 heat shock protein (Bendz et al., 2007), and for tumor antigens oxidized with HOCl and used in a form of vaccine (Chiang et al., 2006; Chiang et al., 2008).

Finally, the biochemical structure of the antigen also seems to play an important role. As already described, antigens resistant to proteolysis were shown to be more immunogenic (Delamarre et al., 2006). In addition, the chemical introduction of aldehyde groups; or direct chlorination of proteins were shown to significantly improve T cell responses (Allison, 2000; Marcinkiewicz, 1991).

1.7 AIMS OF STUDY.

The aim of this project is to investigate the mechanism by which oxidized antigens enhance immune responses. So far, previous studies have shown that HOCl modified proteins may show enhanced immunogenicity both *in vitro* and *in vivo* (Marcinkiewicz, 1991, 1992). However, the mechanisms behind these effects remain poorly understood.

To investigate this question the processing and presentation of HOCl-treated antigen was examined and compared to native antigen ovalbumin. Since HOCl is a strong oxidant capable of completely destroying proteins, the first objective was to establish optimal conditions for antigen oxidation, and to determinate the HOCl-induced chemical modifications critical for antigen immunogenicity.

The next objective was to validate the immunogenicity of modified protein. Bone marrow derived DCs or splenic DCs were used, together with T cell lines or TCR transgenic cells that recognize known, specific ovalbumin epitopes.

Based on these findings the next objective was to explore the outcome of HOCl modification both *in vitro* and *in vivo*. The contribution of several variables such as, TLR ligands, response to different epitopes, antigen uptake, involvement of sugar groups and scavenger receptors were assessed.

Finally the outcome of the experiments has led to a possible mechanism which is discussed in the last chapter.

CHAPTER 2 MATERIAL AND METHODS

2.1 ANIMALS

6-8 week old C57BL/6 and Balb/c mice were obtained from Harlan, UK and maintained by Biological Services, UCL. Transgenic mice expressing the OT-II anti-OVA receptor were a kind gift from Prof. M. Collins (UCL). Myd88/TRIF deficient mice were obtained from Cancer Research UK. All experiments were carried out under UK Animal Project Licence authorization.

2.2 CELLS AND TISSUE CULTURE

2.2.1 MEDIA

Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI)-164 and Iscove's modified DMEM (IMDM) were purchased from Invitrogen, U.K. Complete DMEM, RPMI-164 and IMDM media were made by supplementing with 10% fetal calf serum (FCS) (PAA laboratories GmbH, Haidmannwerg, Austria) and 100 U/ml penicillin/streptomycin (Sigma-Aldrich, U.K.)

2.2.2 CELLS

2.2.2.1 CHINESE HAMSTER OVARY (CHO) CELLS

The CHO cells were cultured in DMEM supplemented with 5% FCS and 100 U/ml penicillin/streptomycin at 37ºC, 5% CO_2. The CHO cells expressing LOX-1 receptor (CHO-LOX-1) were a kind gift from Dr. Yves Delneste (Center d'Immunologie Pierre Fabre, France) and maintained in the same media as the CHO cells and

supplemented with 400 µg/ml hygromycin B (HygroGold, InvivoGen) used as a selection antibiotic.

2.2.2.2 T CELL HYBRIDOMAS

T cell hybridomas DO11.10, 3DO18.3, MF2.2D9 (kind gift from Dr. K. Rock, University of Massachusetts) were cultured in complete DMEM at 37ºC and 5% CO_2. T cell hybridoma Ad71 was cultured in complete RPMI-1640 supplemented with 50µM 2-mercaptoethanol (Invitrogen) 37ºC, 5% CO_2. All cells were passaged every three days and were maintained at a density of between $3x10^5$ and $5x10^5$ cells/ml. The MHC haplotype and epitope specificity is shown in **Table 2.1.**

	Hybridoma	Sequence	MHC II haplotype
OVA	**DO11.10**	P.323-339 ISQAVHAAHAEINEAGR	I-A^d
OVA	**3DO18.3**	P.273-288 MEERKIKVYLPRMKME	I-A^d
OVA	**MF2.2D9**	P.257-278 SIINFEKLTEWTSSNVMEERKI	I-A^b
HEL	**Ad71**	P.71-85 YGILQINSRWWCNDG	I-A^d

Table 2.1 Characterization of T cell hybridomas.

Epitope specificity and MHC II haplotype of the ovalbumin (OVA) and hen-egg lysosome (HEL) specific T cell hybridoma used in this thesis.

2.2.2.3 CTLL-2

CTLL-2 cells were cultured in complete IMDM supplemented with 10ng/ml IL-2 (Peprotech, U.K.) and 50µM 2-mercaptoethanol at 37ºC in 5% CO_2.

2.2.3 GENERATION OF OT-II CELLS.

OT-II cells were cultured from spleens of mice expressing the OT-II T cell receptor (Barnden, 1998). Single cell suspensions of spleen cells were cultured for 24 hours in complete RPMI-164 supplemented with 250nM 323-339 ovalbumin peptide and then expanded for 6 days in complete RPMI-164 supplemented with 10ng/ml of IL-2. The cells were counted, aliquoted and stored at -80ºC until required. Before stimulation, the cells were thawed rapidly, washed and used immediately.

2.2.4 GENERATION OF DENDRITIC CELLS.

2.2.4.1 PREPARATION OF BONE MARROW DERIVED DENDRITIC CELLS.

Bone marrow cells were isolated from the femurs and tibias. The bones were disinfected with 70% ethanol and one end of the bone was cut with scissors, the bone marrow was removed using IMDM with a 1ml syringe and 25G needle. The cells were washed once with IMDM and cultured in complete IMDM media supplemented with 50µM 2-mercaptoethanol (Invitrogen) and 20ng/ml of rGM-CSF (Peprotech, U.K.). The rGM-CSF was renewed on day 4 of culture. On day 7 of culture cells were harvested and purified by positive selection using magnetic $CD11c^+$ beads according to the manufacturer's instructions (MircoBeads, MiltenyiBiotec). Purified cells were stained for a CD11c marker and analyzed on FACScan flow cytometry. The sorted population was > 85% pure CD11c positive (**Fig 2.1**).

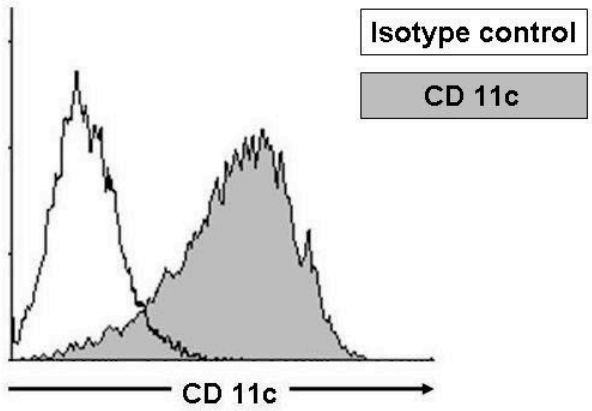


Figure 2.1 DCs purified by positive selection using $CD11c^+$ beads.

In grey cells positive for CD11c, in white staining for isotype control.

2.2.4.2 PREPARATION OF SPLENIC DENDRITIC CELLS.

Fresh spleens were obtained from mice previously injected with FITC-OVA, FITC-OVA_{CI}^{I}, or HBSS. Spleen fragments were incubated in 5ml of complete RPMI-164 media containing 1ml of 200 µg/ml collagenase type II (Sigma) and 1ml of 500 µg/ml DNase I (Sigma) for 30 minutes at room temperature. Collagenase activity was stopped by adding 1ml of 100mM EDTA (Sigma) for 5min. To obtain a single cell suspension spleen pieces were gently mashed with a sterile 1ml syringe plunger in a 40 µm cell strainer (BD Falcon, Bedford, MA, USA). Then the cells were washed once with HBSS and incubated with Red Blood Cell Lysing Buffer Hybri-Max (Sigma) for 3 minutes at RT. Following this, cells were washed twice with HBSS and purified by positive selection using magnetic $CD11c^{+}$ beads according to the manufacturer's instructions (MircoBeads, MiltenyiBiotec).

2.2.5 GENERATION OF MACROPHAGES

Bone marrow cells isolated as above were cultured in complete DMEM supplemented with 10% supernatant from confluent L929 fibroblast cells. On day 3 of culture, non-adherent cells were removed and the media was replaced with fresh complete DMEM supplemented with 10% supernatant from confluent L929 fibroblast cells. On day 6 of culture the media was replaced with fresh complete DMEM supplemented with 10 ng/ml IFNγ (Peprotech, U.K.). After a further 2 days of culture, the cells were harvested and used immediately.

2.3 PROTEIN ANTIGEN MODIFICATION.

2.3.1 HYPOCHLOROUS ACID PROTEIN MODIFICATION.

OVA (2mg/ml) (grad V, SigmaAldrich, Pool, UK), endotoxin free OVA (2mg/ml) (EndoGrad, Profos, Germany) or HEL (2mg/ml) (Boehringer Mannheim, Germany) were dissolved in HBSS and mixed with three different dilutions of 1.5M sodium hypochlorite solution NaOCl (Sigma) at 37ºC for 1 hour at pH 9:

0.45µmoles/mg protein — this antigen will be referred to as **OVA_{Cl}^{L}, HEL_{Cl}^{L}**

4.5µmoles/mg protein — this antigen will be referred to as **OVA_{Cl}^{I}, HEL_{Cl}^{I}**

45µmoles/mg protein — this antigen will be referred to as **OVA_{Cl}^{H}, HEL_{Cl}^{H}**

The NOCl concentration was determined by absorbance measurements at 292nm using a molar extinction coefficient of 350 M^{-1} cm^{-1}. The modified protein was purified and transferred into complete IMDM by passage over a PD-10 Sephadex G-25 column (GE Healthcare) according to the manufacturer's instructions. Control OVA and HEL was prepared in the same way but HBSS buffer was used instead of NaOCl. These antigens will be referred to as OVA and HEL. In some experiments, the oxidized protein was reduced by the addition of 50mM $NaBH_4$ (Sigma) and further incubation for one hour at 37ºC. Alternatively, protein chloramines were converted back to free amines by the addition of 30mM L-methionine (Sigma) and incubation for 30 min at room temperature. Samples were stored at -20°C.

2.3.2 HYDROGEN PEROXIDE PROTEIN MODIFICATION.

OVA (2 mg/ml) was dissolved in HBSS and mixed with excess of hydrogen peroxide solution H_2O_2 (Sigma):

10x — this antigen will be referred to as **OVA_{H2O2}^{10}**

100x — this antigen will be referred to as **OVA_{H2O2}^{100}**

1000x — this antigen will be referred to as **OVA_{H2O2}^{1000}**

The reaction was carried out at 37ºC for 1 hour at pH 9. For the quantification of carbonyl groups, OVA (3mg/ml) was dissolved in HBSS and mixed with 290µM H_2O_2 and optionally with 290µM ferrous sulfate heptahydrate ($FeSO_4 \bullet 7H_2O$) (2mM stock was made up fresh). The modified protein was purified and transferred into complete IMDM by passage over a PD-10 Sephadex G-25 column (GE Healthcare) according to the manufacturer's instructions. Samples were stored at-20°C.

2.3.3 PREPARATION OF FLUOROCHROME LABALLED ANTIGENS

OVA and OVA_{Cl}^{I} were transferred to 0.1M sodium carbonate buffer by passage over a PD-10 sephadex G-25 column. FITC (2 mg/ml) (Sigma) and TRITC (6 mg/ml) (Fluka) in anhydrous dimethyl sulfoxide (DMSO) (Acros organic, Geel, Belgium) were prepared fresh. Fluorochromes were added slowly to the protein (50µl per mg protein) and left for four hours at room temperature in the dark. The unconjugated fluorochromes were removed by passage over PD-10 sepadex G-25 column to HBSS buffer. FITC-OVA, TRITC-OVA, FITC-OVA_{Cl}^{I} and TRITC-OVA_{Cl}^{I} were stored at -20C.

2.3.4 CALCULATION OF DYE:PROTEIN MOLAR RATIO

Dye:protein molar ratio was determined by absorbance measurements at 493nm using a molar extinction coefficient of 72,000 M^{-1} cm^{-1} for FITC and at 555nm using a molar extinction coefficient of 65,000 $M^{-1}cm^{-1}$ for TRITC.

$$\text{moles dye / mole protein} = \frac{A_{max} \text{ of the labeled protein}}{\varepsilon \text{ x protein concentration (M)}} \text{ x dilution factor}$$

A_{max} – absorbance of a dye solution measured at the wavelength maximum for the dye molecule.

ε – protein molar extinction coefficient

dilution factor – the extent to which the sample was diluted for absorbance measurement

2.3.5 PREPARATION OF BEAD-CONJUGATED ANTIGENS

OVA and OVA_{Cl}^{I} were conjugated with fluorescent beads (FluoSpheres sulfate microspheres, 1μm, red fluorescent 580/605, Molecular Probes, Invitrogen) according to the manufacture's instruction. Briefly 1μm beads ($5x10^9$) in polystyrene tube were mixed with the protein (1mg/ml) dissolved in HBSS. Samples were shaken gently in the dark at room temperature for 18 hours, washed three times in HBSS (3500g, 20 minutes) and re-suspended in the HBSS to a final volume of 1ml. The presence of the protein on the beads was confirmed by SDS-PAGE analysis. Samples were stored at 4°C.

2.3.6 QUANTIFICATION OF CARBONYL GROUPS

Carbonyl groups were quantified by reaction with 2,4-dinitrophenyl-hydrazine (DNPH) (Camlab Chemicals, Cambridge) (Dalle-Donne et al., 2003). Briefly, protein (1mg/ml in HBSS buffer) and a blank control (HBSS buffer) were mixed (1:1,v/v) with 10mM 2,4-dinitrophenyl-hydrazine in 2N HCl. Samples were mixed gently in the dark at room temperature for 1 hour and centrifuged (3 minutes at 15,500g). The supernatants were discarded and the protein pellets were washed three times with 1ml of ethanol/ethyl acetate (1:1, v/v), each time allowing the sample to stand for 10 minutes before centrifugation. Finally, the pellets were dissolved in 6M guanidine hydrochloride in 2N HCl at 37ºC for 15 minutes. Carbonyl groups were determined from the absorbance at 370nm using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

2.3.7 QUANTIFICATION OF AMINO GROUP.

Amino groups were quantified by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Fluka). Protein (2mg/ml in 0.05M $Na_2B_4O_7$ pH 9.5) or a blank control (0.05M $Na_2B_4O_7$ pH 9.5) was mixed with 100µl of 0.02M TNBS in water. The samples were incubated in the dark at room temperature for four hours. Amino groups were determined from the absorbance at 367nm using a molar absorption coefficient of 11,000 M^{-1} cm^{-1}.

2.4 ANTIGEN PRESENTATION ASSAY.

2.4.1 OVA PRESENTATION ASSAY

OVA-specific T cell hybridomas (2 x 10^4 /well) were cultured together with purified DCs (5 x 10^3 /well) or with macrophages (10^4 /well) in the presence of the antigen at the indicated concentrations. For some experiments DCs (10^5) were pre-cultured with antigen in polypropylene tubes for different times. The excess antigen was removed by washing twice in HBSS and the DCs were fixed with 0.05% glutaraldehyde (Sigma-Aldrich) for 30 seconds. Excess glutaraldehyde was removed by two more washes and different numbers of fixed DCs were co-cultured with T cell hybridomas (2 x 10^4 /well). After 24 hours, the supernatants were harvested and frozen at -20°C. The T cell response was measured indirectly by measuring IL-2 release, using IL-2 sensitive CTLL-2 indicator cells. CTLL (5 x 10^3 /well) were incubated in 100µl of test supernatant or recombinant IL-2 standards. An example of such a standard curve is shown in **Fig 2.2**. After 24 hours one µC [^{3}H]thymidine (MP Biomedicals, Irvine, CA) was added to each well and the cells were incubated for a further 18 hours. Incorporation of radioactivity into DNA was measured by harvesting the DNA on a harvester (Tomtec) and counting using a Microbeta ß-counter (Trilux). Triplicate cultures were set up for each experimental condition.

TCR transgenic T cells thawed, washed once in fresh complete IMDM and used immediately (2 x 10^4/well) were cultured with purified DCs (5 x 10^3 /well) in the presence of the antigen at indicated concentrations. For some experiments, antigen was preincubated for 30min at room temperature with 1µg/ml of polymixin B

(InvitroGen, San Diego CA, USA). After 18 hours one µC [^{3}H]Thymidine (MP Biomedicals, Irvine, CA) was added. T cell proliferation was measured after further 18 hours of incubation by harvesting the cells and measuring the incorporation of radioactivity into DNA using the DNA harvester (Tomtec) and Microbeta β-counter (Trilux).

2.4.2 HEL PRESENTATION ASSAY

HEL-specific T cell hybridomas (5 x 10^4 /well) were cultured together with purified bone marrow-derived DCs (5 x 10^4 /well) in the presence of the antigen at the indicated concentrations. After 24 hours, the supernatants were harvested and frozen at 20°C. The T cell response was measured indirectly by measuring IL-2 release, using IL-2 sensitive CTLL-2 indicator cells. CTLL-2 cells (5 x 10^3 /well) were incubated in 50µl of test supernatant or recombinant IL-2 standards. After 24 hours, one µC [^{3}H]thymidine (MP Biomedicals, Irvine, CA) was added to each well and the cells were incubated for a further 18 hours. Incorporation of radioactivity into DNA was measured by harvesting the DNA on a harvester (Tomtec) and counting using a Microbeta ß-counter (Trilux). Triplicate cultures were set up for each experimental condition.

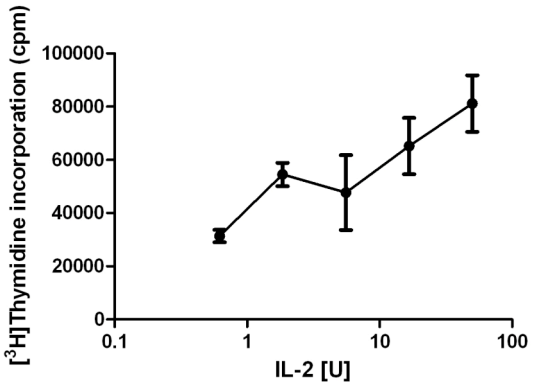


Figure 2.2 Validation of antigen presentation assay on the IL-2 dependent cell line CTLL-2.

CTLL-2 cells constitutively express IL-2 receptor and depends completely on the presence of IL-2 in media. CTLL-2 cells were incubated with a range of IL-2 concentrations for 24 hours. Cells were cultures for an additional 16 hours with one µC [3H]Thymidine. The resulting cpm was plotted against IL-2 concentrations. Results are expressed as the average [^{3}H]-thymidine incorporation from triplicate cultures and it is one representative of several experiments.

2.5 ACTIVATION OF BONE MARROW DERIVED DCS.

2.5.1 DENDRITIC CELL STIMULATION

Day 7 purified DCs (8 x 10^5 /well) were stimulated with 20µg/ml EndoGrad OVA (Profos, Germany) or 20µg/ml EndoGrad OVA_{Cl}^{I}, or 100ng/ml LPS (Salmonella abortus equi, Sigma) for 18 hours at 37°C, 5% CO_2. As a negative control, DCs were treated with media only. The surface phenotype was analyzed by flow cytometry.

2.5.2 ANTIBODIES

CD11c-PE (clone N418), CD11c-APC (clone N418), CD11c-PE-Cy7 (clone N418), CD8a-PE-Cy7 (clone 53-6.7) CD86-FITC (B7-2), CD54-FITC (ICAM-1) and MHC II I-A/I-E – FITC (2G9) were purchased from BD Bioscience.

2.5.3 FLOW CYTOMETRY

Cells (10^5 /sample) were incubated in ice-cold **blocking buffer** (HBSS, 10% rat serum. 0.1% sodium azide) for 15 min on ice, washed in **washing buffer** (HBSS, 5% FCS, 0.1% sodium azide) and incubated with an appropriate dilution of directly conjugated primary antibodies for 30 min on ice. The cells were washed twice in washing buffer and then fixed with 3.8% formaldehyde (BDH, U.K.). Samples were analyzed on a FACScan flow cytometer using CellQuest or FlowJo software.

2.6 ANTIGEN UPTAKE ANALYSIS.

2.6.1 IN VITRO STUDIES

Purified bone marrow derived DCs; or CHO and CHO-LOX-1 cells were incubated with different concentrations of FITC-OVA or FITC-OVA_{CI}^{I} for the time indicated. Excess antigen was removed by washing the samples twice in ice-cold HBSS. Cells were fixed with 3.8% formaldehyde and analyzed on a FACScan flow cytometer using CellQuest software, or on a confocal microscope using Leica confocal software.

2.6.2 CONFOCAL MICROSCOPY

For confocal microscopy, cells were cultured on glass coverslips and incubated with

FTIC-Ag as above. Cells were fixed with 3.8 % paraformaldehyde for 15 minutes, washed with ice-cold HBSS and blocked with 10% donkey serum for CHO and CHO-LOX-1 staining. CHO and CHO-LOX-1 cells were stained with polyclonal anti-LOX-1 sheep antibody (a kind gift from Dr. Sreenivasan Ponnambalam, Leeds University U.K.) in 1:100 dilution, followed by secondary conjugate donkey anti-sheep IgG (Alexa Fluor 555, Invitrogen) in 1:500 dilution. The cells were washed and stained with the DNA stain 4'6-diamidino-2-phenylindole (DAPI) (2µg/ml) for 5 minutes and mounted on slides. Samples were stored at 4°C in the dark.

2.6.3 IN VIVO STUDIES

C57BL/6 mice were injected intravenously with 1mg FITC-OVA, or FITC-OVA_{Cl}^{I}, or an equal volume of HBSS. For the injection, a BD Micro-Fine U-100 sterile 1ml insulin syringe (Becton Drive, NJ, USA) was used. Spleens were collected at various time points, and $CD11c^{+}$ DCs were purified as described above. DCs were stained for the CD8a (clone 53-6.7) marker. Cells were analyzed on FACSan flow cytometer using CellQuest software, or on a confocal microscope using Leica confocal software.

2.7 ANTIGEN ENZYMATIC DIGESTION.

2.7.1 TRYPSIN DIGESTION

OVA, or OVA_{Cl}^{I} (20 µg) was incubated with sequencing grade modified trypsin (1 µg) (Promega, Madison, WI, USA) at 37°C for 18 hours. Digestion was performed in digestion buffer (50mM Tris-HCl, 10mM $CaCl_2$ pH 7.8).

A total reaction volume of 20µl was made by adding 2 µl of trypsin (1µg), 10µl of protein (20 µg) and 8µl of digestion buffer.

2.7.2 CATHEPSIN E DIGESTION.

OVA, or OVA_{Cl}^{I} (10µg) was digested with 10ng of cathepsin E (R&D Systems, UK) at 37°C for 18 hours. Digestion was carried in 0.1M NaOAc, 0.1M NaCl pH 3.5. A total reaction volume of 15µl was made by adding 10µl of cathepsin E (10ng) and 5µl of protein (10µg).

2.7.3 PNGASE F DIGESTION.

OVA, or OVA_{Cl}^{I} (10µg) was digested with 1000 units of PNGase F (BioLabs, UK) at 37°C for 18 hours. A total reaction volume of 20µl was made by adding 2µl of 10x G7 reaction buffer (BioLabs, UK), 2µl of PNGase F, 5µl of protein (10µg) and 11µl of water.

2.7.4 NEURAMINIDASE DIGESTION.

OVA, or OVA_{Cl}^{I} (2 µg) was digested with 400 units of neuraminidase (BioLabs, UK) at 37°C for 18 hours. As a positive control, 1µg of fetuin (Sigma, UK) was digested with 200 units of neuraminidase in the same reaction conditions. Digestion was performed in a reaction buffer G1 (BioLab, UK) of total volume of 15µl.

2.8 SEPARATION OF PROTEINS BY SDS-PAGE.

Proteins were separated using 4-12% NuPage Novex Bis-Tris mini gels (Invitrogen). Protein samples were added to an equal volume of **reducing sample buffer** containing: 100% glycerol (BDH,UK), ß-mercaptoethanol (Sigma), 10% sodium

dodecyl sulfate (Sigma), 0.5M Tris HCl pH 6.8 (Calbiochem, san Diego, CA, USA) and 1% bromophenol blue (Sigma). Before loading, the samples were reduced by heating at 95°C for five minutes. Gels were run at 200V in MES running buffer (Invitrogen) for 45 minutes at room temperature. Prestained protein ladder PageRuler plus (Fermentas, UK) was used as the molecular marker. Bands were visualized using silver staining (**Table 2.2**) (Nesterenko, 1994) or comassie blue staining. ImageJ software was used for densitometry analysis.

STEP	SOLUTION	INCUBATION TIME
Wash	dH_2O	2x 1sec
Fix Proteins	60ml acetone 1.5ml TCA 25µl formaldehyde	5min
Wash	dH_2O	3x 5sec, 5min, 3x 5sec
Pre-treat (1)	60ml acetone	5min
Pre-treat (2)	60ml dH_2O 100µl $Na_2S_2O_3$	1min
Wash	dH_2O	3x 5sec
Prove	60ml dH_2O 0.8ml $AgNO_3$ 0.6ml formaldehyde	8min
Wash	dH_2O	2x 5sec
Develop	60ml dH_2O 25µl formaldehyde 25µl $Na_2S_2O_3$ 1.2g Na_2CO_3	10-60sec
Stop	Acetic acid	>30sec

Table 2.2 Protocol of sliver stain of SDS gels.

Acetone stock solution (50% v/v in dH_2O); formaldehyde 37%; TCA-trichloro acetic acid stock (50% w/v in dH_2O); $Na_2S_2O_3$ stock (10% w/v in dH_2O, prepared fresh); acetic acid (1% in H_2O).

2.9 WESTERN BLOT ANALYSIS.

2.9.1 IMMUNOBLOT

Protein samples resolved as above were transferred to Hybond-ECL nitrocellulose membrane (Amersham Bioscience, UK) at 30V for 1 hour at room temperature. After transfer, the membrane was incubated for 1 hour at room temperature in **blocking buffer** (5% skimmed milk powder [Tesco, UK] in Tris buffered saline [TBS] plus 0.1% Tween 20 [BDH,UK]). OVA was detected using polyclonal anti-OVA (prepared in our laboratory) at a 1:500 dilution. The following day the membrane was washed four times (15 minutes each with shaking) in **washing buffer** (TBS with 0.1% Tween 20) and incubated with horseradish peroxidase (HRP) conjugated swine anti-rabbit IgG (Dako) at a 1:3000 dilution for 1 hour at room temperature. The membrane was washed four times in washing buffer and an additional four times in TBS only (15 minutes each with shaking). Protein detection was carried out using ECL reagent (Amersham Pharmacia Biotech) according to the manufacturer's guidelines.

2.9.2 LECTINBLOT

Protein samples resolved as above were transferred to a Hybond-ECL nitrocellulose membrane (Amersham Bioscience, UK) at 30V for 1 hour at room temperature. After transfer, the membrane was incubated at 4°C overnight in **blocking buffer** (3% bovine serum albumin [Sigma] in Tris buffered saline [TBS] plus 0.1% Tween 20 [BDH, UK]). Next the membrane was incubated for 1 hour at room temperature with the relevant lectin-biotin solution as follows: 10 µg/ml MAL-Biotin

(Maackia amurensis lectin) (Galab, Germany) dissolved in 10ml of blocking buffer. It was then washed four times (15 minutes each with shaking) with washing buffer (TBS with 0.1% Tween 20) and incubated with horseradish peroxidase (HRP) conjugated streptavidin (R&D System, Minneapolis, MN, USA) at a 1:200 dilution for 1 hour at room temperature. The membrane was washed four times with washing buffer and an additional four times with TBS only (15 minutes each with shaking). Protein detection was carried out using ECL reagent (Amersham Pharmacia Biotech), or SuperSignal WestFemto reagent (Thermo Scientific, Rockford, Il, USA) according to the manufacturer's guidelines.

2.10 STATISTICAL ANALYSIS

Where appropriate, the paired groups were analyzed by a 2-tailed Student's t test or the two-way ANOVA test using GraphPad Prism software.

CHAPTER 3 HOCL MODIFICATION FACILITATES PROCESSING AND PRESENTATION TO $CD4^+$ T CELLS.

3.1 INTRODUCTION.

Previous studies have shown that HOCl modified proteins may show enhanced immunogenicity both *in vitro* and *in vivo* (Marcinkiewicz, 1991, 1992). However, the mechanisms behind these effects remains poorly understood. To address this question we have compared the processing and presentation of HOCl-treated antigen with that of native antigen ovalbumin (OVA).

OVA is a glycoprotein (MW 42,881Da) consisting of 386 amino acids. The sequence includes four sulfhydryl groups with a single disulfide bridge linking position 74 and 121. OVA has an acetylated N-terminus glycine, C-terminus proline and a single heterogeneous carbohydrate chain covalently linked to the amide nitrogen of Asn293. There are three main forms of OVA containing two, one and zero phosphate groups per molecule at Ser69 and Ser345.

Importantly, in the old literature the starting codon for Met was not included in sequencing and so OVA was described as consisting of only 385 amino acids. Therefore, according to the present method of sequencing where starting Met is included, the commonly known OVA epitope p.323-339 should in fact be named p.324-340. However, because OVA epitopes are named as they were firstly described in most literature, the historic names are used in this thesis.

As a source of HOCl, we have used sodium hypochlorite (NaOCl). Briefly, in aqueous solution, this forms sodium hydroxide (NaOH) and HOCl [**reaction 3.1**]. HOCl is in equilibrium with the hypochlorite ion (OCl^-) [**reaction 3.2**].

$NaOCl + H_2O <-> NaOH + HOCl$ [**reaction 3.1**]

$HOCl <-> H^+ + OCl^-$ [**reaction 3.2**]

3.2 OBJECTIVES.

- To optimize the HOCl-triggered oxidation reaction, and determine changes in aldehyde and amino group formation in oxidizing conditions.
- To compare the immunogenicity of native and HOCl-treated OVA in the functional assay using bone marrow derived DCs or, macrophages and T cells recognizing different OVA epitopes.
- To test another neutrophil derived oxidizing agent H_2O_2 using the functional assay.

3.3 RESULTS.

3.3.1 HOCL INDUCES FORMATION OF ALDEHYDES GROUPS AND LOSS OF FREE AMINES GROUPS.

The HOCl treatment of proteins is known to result in the generation of a variety of covalent modifications (Hawkins et al., 2003). In particular, the HOCl reaction with free alpha and epsilon amino side chains leads to the formation of unstable chloramines that, when situated at the alpha-amino site, decompose to yield aldehydes. OVA contains no free terminal amino group (Steven, 1958), hence only epsilon groups must be involved in the formation of chloramines. On the other hand the formation of aldehyde groups can result from backbone chain cleavage.

OVA was treated with different concentrations of HOCl: low (OVA_{Cl}^{L}), intermediate (OVA_{Cl}^{I}) or high (OVA_{Cl}^{H}). Protein modification was monitored by measuring the formation of aldehyde groups and disappearance of amino groups as described in Material and Methods. Increasing amounts of HOCl induced a dose dependent decrease in number of free amino groups (**Fig 3.1A**) and a dose dependent increase in number of aldehyde groups (**Fig 3.1.B**).

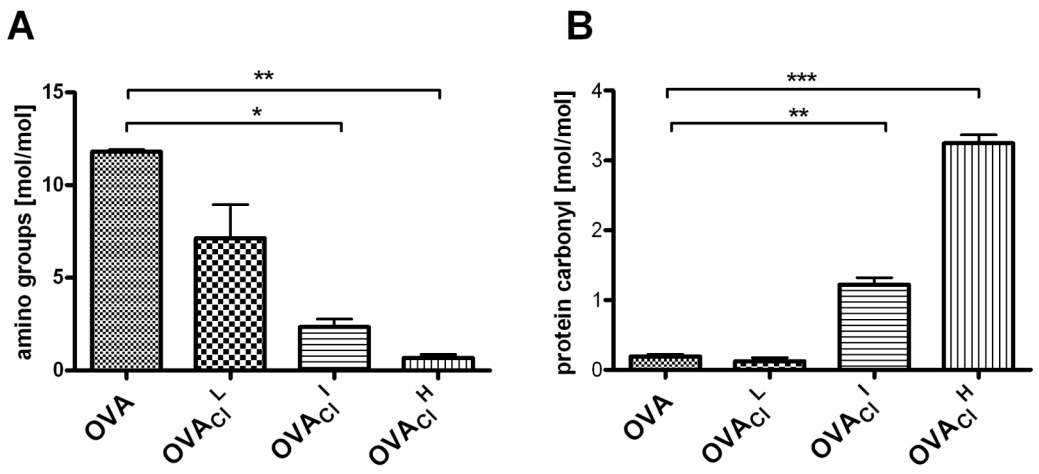


Figure 3.1 The reaction of HOCl with OVA causes a dose dependent loss of free amines and the formation of aldehyde groups.

HOCl was incubated with OVA at different concentrations of HOCl (L –low, I – intermediate, H –high). ***A.*** *Loss of free amine groups and* ***B.*** *formation of aldehyde groups were quantified as described in Material and Methods using TNBS and DNPH, respectively. Results are presented as the average of three independent experiments ± SEM. Statistical analysis was performed using a 2-tailed Student's t test. * P<0.05, **P<0.01, ***P<0.001.*

3.3.2 INTERMEDIATE CONCENTRATIONS OF HOCL ARE OPTIMAL TO ENHANCE OVA IMMUNOGENICITY.

The immunogenic properties of OVA oxidized with three different concentrations of HOCl were analyzed in the functional assay. Low concentrations of HOCl may not be sufficient to modify the protein. Conversely, high concentrations of HOCl may lead to protein fragmentation and generation of peptide fragments. This can result in an unwanted, direct T cell response to the peptides, to the loss of the protein fragments during the purification step, or to protein cross-linking.

The optimal degree of modification of free amines was assessed in the functional experiment and the response of DO11.10 T cell hybridomas to OVA_{Cl}^{L}, OVA_{Cl}^{I}, and OVA_{Cl}^{H} was tested (**Fig 3.2.A**). DO11.10 responds poorly to native OVA, except at very high concentrations (> 20 µM). In contrast, OVA_{Cl}^{I} induced a DO11.10 response even at concentrations of 0.2 µM. However, both OVA_{Cl}^{L} and OVA_{Cl}^{H} were poorly presented. The same functional experiment was repeated using T cells from the TcR transgenic mouse OT-II, which recognize the same sequence of OVA as DO11.10 (p.323-339), but presented by $I\text{-}A^{b}$ instead of $I\text{-}A^{d}$, and with 200-greater sensitivity. In this case, native OVA processing was detected at concentrations of 2µM. However, as for DO11.10, processing of OVA_{Cl}^{I} was at least 10-fold more efficient (**Fig 3.2.B**).

The degree of modification of free amines was not the only factor influencing the optimal antigen modification. Protein oxidation reaction was routinely carried out at pH 9. However even a slight increase of this parameter led to a failure in

enhancing T cells response. OVA_{Cl}^{I} prepared at pH 9.8 did not induce DO11.10 response (**Fig 3.3**).

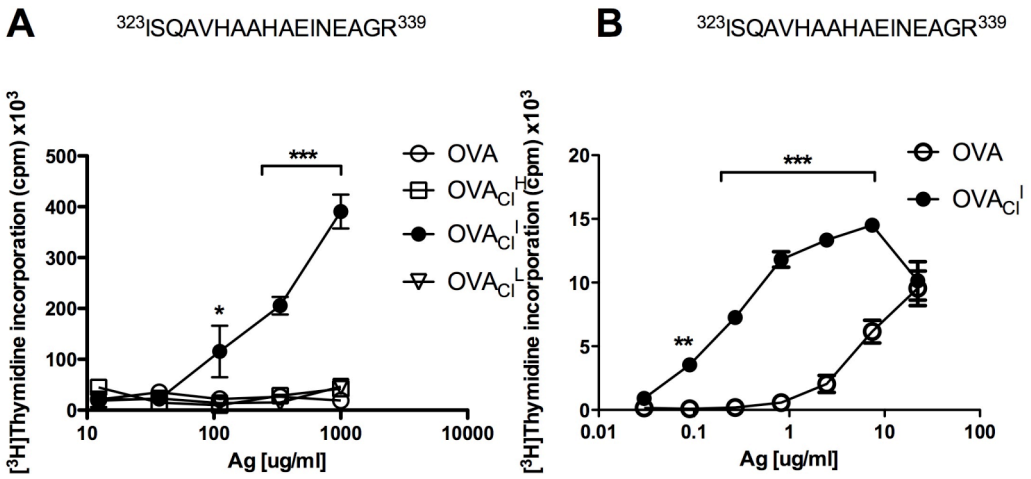


Figure 3.2 Intermediate level of OVA modification with HOCl enhances the T cell response.

A. *DO11.10 hybridoma cells (2x10^4/well) (which recognize the epitope sequence shown above the graph) were incubated with different concentrations of OVA or, with different concentrations of OVA modified with low OVA$_{Cl}^{L}$, intermediate OVA$_{Cl}^{I}$, or high OVA$_{Cl}^{H}$ concentrations of HOCl as described in Material and Methods and purified bone marrow derived DCs (5x10^3/well). Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line.* ***B.*** *TCR transgenic OT-II T cells (2x10^4/well) were incubated with different concentrations of OVA or OVA$_{Cl}^{I}$ and purified bone marrow derived DCs (5x10^3/well). The graph shows OT-II proliferation measured as thymidine incorporation. Results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. Experiments were performed three times. Statistical analysis was performed using the two-way ANOVA test. *P<0.05, **P<0.01, ***P<0.001.*

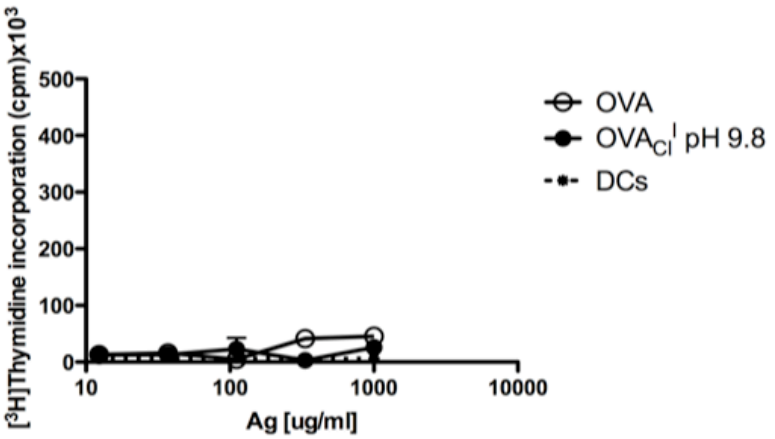


Figure 3.3 Protein modification in pH 9.8 does not enhance antigen immunogenicity.

DO11.10 hybridoma cells ($2x10^4$/well) were incubated with different OVA concentrations or, with different OVA_{Cl}^- concentrations prepared at pH9.8 as described in Material and Methods and purified bone marrow derived DCs ($5x10^3$/well). Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line. Results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures.

3.3.3 PRESENTATION OF OVA_{CL}^{I} IS EFFICIENT AT EARLY TIME POINTS.

In previous experiments DCs were co-cultured continuously with the antigen and T cells for 24 hours. In order to study the antigen processing and presentation at earlier time points, we have modified the functional assay by three additional steps. Firstly, DCs were co-cultured with antigen for indicated time points. Secondly, antigen was removed by extensive washing with HBSS. Thirdly, DCs were fixed with 0.5% glutaraldehyde which blocked further antigen processing.

DCs were co-cultured with OVA_{Cl}^{I} for one and three hours and the response of DO11.10 T cells to the antigen was tested. OVA_{Cl}^{I} was efficiently presented at the concentration of 5µM at both time points but no response was observed for native antigen (**Fig 3.4 A**).

Similar enhancement of presentation using OVA_{Cl}^{I} was observed for TCR transgenic OT-II T cells. DCs were co-cultured with OVA_{Cl}^{I} for two hours. OT-II T cells response moderately to 5µM of native OVA but like DO11.10 T cells response to OVA_{Cl}^{I} was significantly greater (**Fig 3.4.B**). None the less the response to the antigen after two hours is different from the response following the over night incubation.

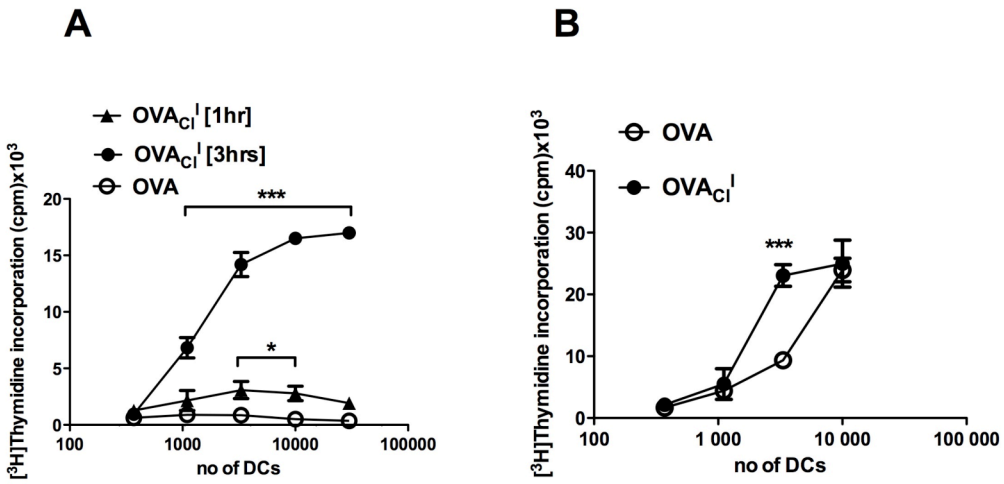


Figure 3.4 HOCl modified OVA, co-cultured with DCs for one, two or three hours enhances the T cell response.

A. *Purified bone marrow derived DCs (10^5/sample) were incubated for 1 or 3 hours with OVA (5μM) or OVA$_{Cl}^{l}$ (5μM). Antigen was removed by washing cells 2x in HBSS. Cells were fixed with 0.05% glutaraldehyde. Glutaraldehyde was removed by washing cells 2x in HBSS. 3-fold serial dilutions were made and DCs were co-cultured in 96-well plates with DO11.10 hybridomas ($2x10^4$/well). As the positive control DCs were incubated with synthetic Ova p.323-339 (1μg/ml). Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line.* ***B.*** *Purified bone marrow derived DCs (10^5/sample) were incubated for 2 hours with OVA (5μM) or, OVA$_{Cl}^{l}$ (5μM). Antigen was removed by washing cells 2x in HBSS. Cells were fixed with 0.05% glutaraldehyde. Glutaraldehyde was removed by washing cells 2x in HBSS. 3-fold serial dilutions were made and DCs were co-cultured in 96-well plates with TCR transgenic OT-II T cells ($2x10^4$/well). The graph shows OT-II proliferation measured as thymidine incorporation. Results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. Experiments were performed three times. Statistical analysis was performed using the two-way ANOVA test. *P<0.05, ***P<0.001.*

3.3.4 THE EFFECTS OF HOCL ARE EPITOPE SPECIFIC.

The epitope selectivity of the HOCl enhancement was explored using additional OVA specific T cell hybridomas described in Material and Methods**.** Two T cell hybridomas, MF2.D9 specific for OVA p. 273-288 and 3DO18.3 specific for OVA p. 257-278 were tested. In contrast to DO11.10 and TCR transgenic OT-II T cells which showed a great increase in response to OVA_{Cl}^{I}, the response of two other T cell hybridomas was almost completely abrogated by HOCl-induced oxidation (**Fig 3.5 A**). Similarly when antigen was removed from the culture after two hours, and DCs processing and presentation was blocked, MF2.D9 and 3DO18.3 did not respond to OVA_{Cl}^{I} (**Fig 3.5 B**).

3.3.5 ANTIGEN IMMUNOGENICITY IS ENHANCED BY HOCL BUT NOT BY H_2O_2.

Previous studies have demonstrated that proteins are selectively inactivated by HOCl but not by H_2O_2 (Khor et al., 2004; Shao et al., 2005). H_2O_2 oxidation, also known as the Fenton reaction requires the presence of the metal iron (Fe^{2+}), that is necessary to break the oxygen-oxygen bond of peroxide (H:O — O:H).

The H_2O_2 oxidation reaction was performed in the presence, or absence of the metal iron. The level of protein oxidation was monitored by measuring the appearance of aldehyde groups. For both experimental conditions (with, or without metal iron), H_2O_2 treatment did not induce aldehyde formation (**Fig 3.6A**). Furthermore over a wide range of concentrations, 10-, 100- and 1000- molar excess of the H_2O_2, neither DO11.10 (**Fig 3.6 B**) nor OT-II (**Fig 3.6 C**) T cells respond to OVA_{H2O2}.

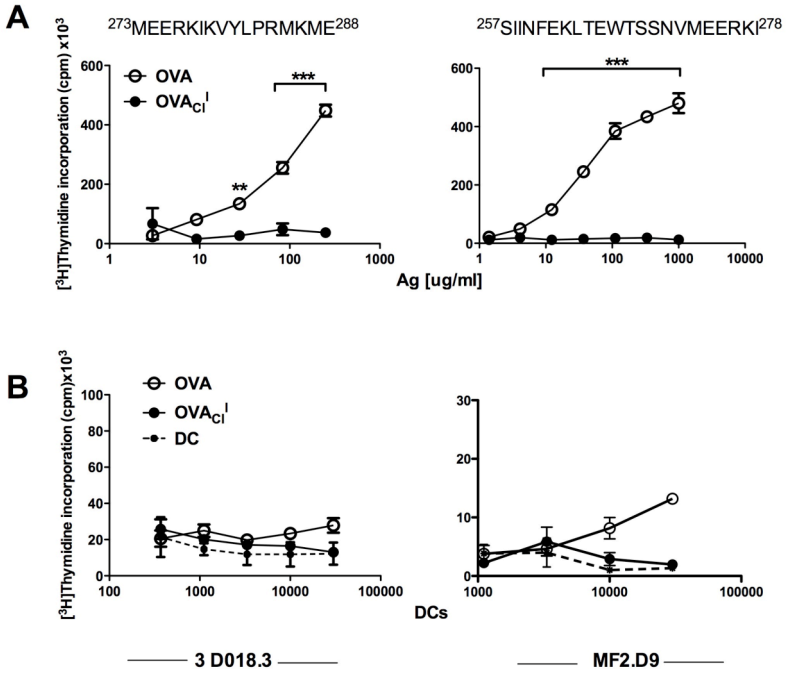


Figure 3.5 The effects of HOCl are epitope specific.

A. *3DO18.3 hybridomas (left) or MF2.D9 hybridomas (right) (2x10^4/well) (which recognize the epitope sequence shown above the graph) were incubated with different concentrations of OVA or, OVA$_{Cl}^{l}$ and purified bone marrow derived DCs (5x10^3/well). Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line.* ***B.*** *Purified bone marrow derived DCs (10^5/sample) were incubated for 2 hours with OVA (5μM) or, OVA$_{Cl}^{l}$ (5μM). Antigen was removed by washing cells 2x in HBSS. Cells were fixed with 0.05% glutaraldehyde. Glutaraldehyde was removed by washing cells 2x in HBSS. 3-fold serial dilutions were made and DCs were co-cultured in 96-well plates with 3DO18.3 (left) or, MF2.D9 (right) T cell hybridomas (2x10^4/well). Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line. Results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. Experiments were performed three times. Statistical analysis was performed using the two-way ANOVA test. **P<0.01, ***P<0.001.*

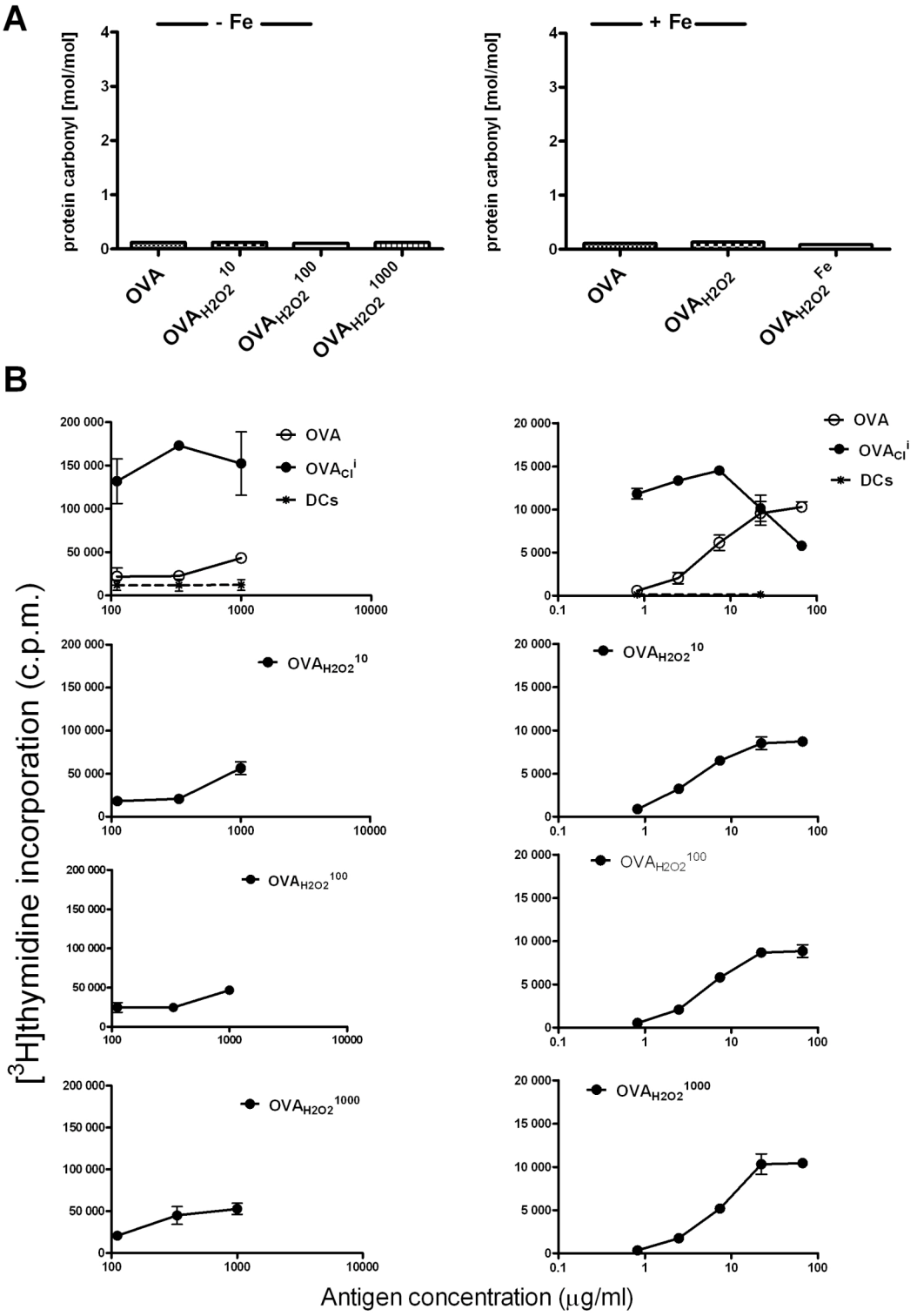

A
- Fe
+ Fe
protein carbonyl [mol/mol]
OVA
OVA$_{H2O2}$ 10
OVA$_{H2O2}$ 100
OVA$_{H2O2}$ 1000
OVA$_{H2O2}$ Fe
B
OVA
OVA$_{Cl}^{i}$
DCs
OVA$_{H2O2}^{10}$
OVA$_{H2O2}^{100}$
OVA$_{H2O2}^{1000}$
[^{3}H]thymidine incorporation (c.p.m.)
Antigen concentration (μg/ml)

Figure 3.6 Antigen immunogenicity is enhanced by HOCl but not by H_2O_2.

A. *H_2O_2 was incubated with OVA at different molecular ratio (10:1, 100:1 and 1000:1) in the presence (right) or, absence (left) of iron. Formation of aldehyde groups was quantified as described in Material and Methods using DNPH. Results are representative of two independent experiments.* ***B.*** *DO11.10 hybridomas (left) or TCR transgenic OT-II T cells (right) ($2x10^4$/well) were incubated with different concentrations of OVA or, OVA_{Cl}^{I} or, OVA modified with increasing molecular ratios of H_2O_2 to the protein (OVA_{H2O2}^{10}, OVA_{H2O2}^{100}, OVA_{H2O2}^{1000}) and purified bone marrow derived DCs ($5x10^3$/well). Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line (left), or OT-II proliferation measured as thymidine incorporation (right). Results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. Each experiment was performed twice.*

3.3.6 ENHANCED PRESENTATION OF BEAD-ASSOCIATED OVA_{CL}^{I} BY MACROPHAGES.

Antigen presenting cells can utilize several mechanisms of antigen uptake and processing. Whereas, the uptake of soluble antigens is mediated by constitutive macropinocytiosis or clathrin-mediated endocytosis, the uptake of particulate antigens, requires receptor-mediated phagocytosis. Particulate antigens are also processed via different pathways from soluble antigens. In addition, previous studies have demonstrated that only bead-associated OVA in the presence of LPS induces strong $CD4^{+}$ T cell responses (Blander and Medzhitov, 2006).

The influence of HOCl induced oxidation was therefore explored using a model in which OVA or, OVA_{Cl}^{I} were first coated on 1µm beads. Western Blot analysis confirmed that equal amounts of OVA or, OVA_{Cl}^{I} were bound to the beads (**Fig 3.7).** In order to facilitate removal of excess beads before addition of T cells, these experiments were carried out with adherent macrophages, rather than the non-adherent DCs. As with the previous experiments using soluble OVA, OVA on beads without the presence of LPS was presented very inefficiently, by the macrophages (**Fig 3.8A**). In contrast, bead-associated OVA_{Cl}^{I} was presented very efficiently by the macrophages, but only when cells were incubated with at least 10^{8} beads. According to the microscopy analysis both bead-associated OVA and OVA_{Cl}^{I} were taken up by macrophages (**Fig 3.8C**). Furthermore these experiments confirmed that enhanced processing and presentation of HOCl-treated antigen is not DC specific. The OVA_{Cl}^{I} presented by macrophages also enhanced response of DO11.10 T cells (**Fig 3.8B**).

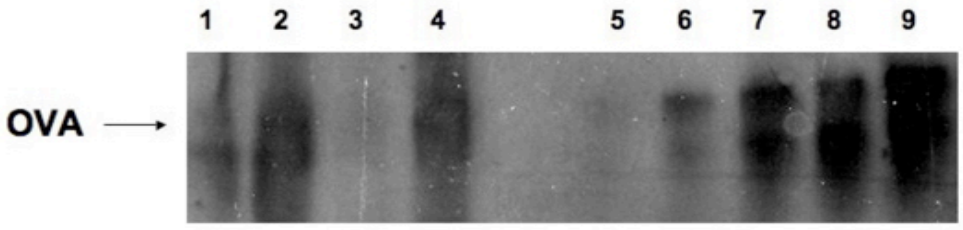


Figure 3.7 OVA and OVA_{Cl}^{I} coated on fluorescent beads.

OVA (1mg/ml) or OVA_{Cl}^{I} (1mg/ml) were coated on fluorescent beads (1μm). Samples were fractionated by PAGE and analyzed by Western blot using OVA specific polyclonal antibody. Line 1: 10^6 beads with OVA, line 2: 10^7 beads with OVA, line 3: 10^6 beads with OVA_{Cl}^{I}, line 4: 10^7 beads with OVA_{Cl}^{I}, line 5: OVA (100ng), line 6: OVA (500ng), line 7: OVA (1μg), line 8: OVA (1.5μg), line 9: OVA (2μg). Experiment was repeated three times.

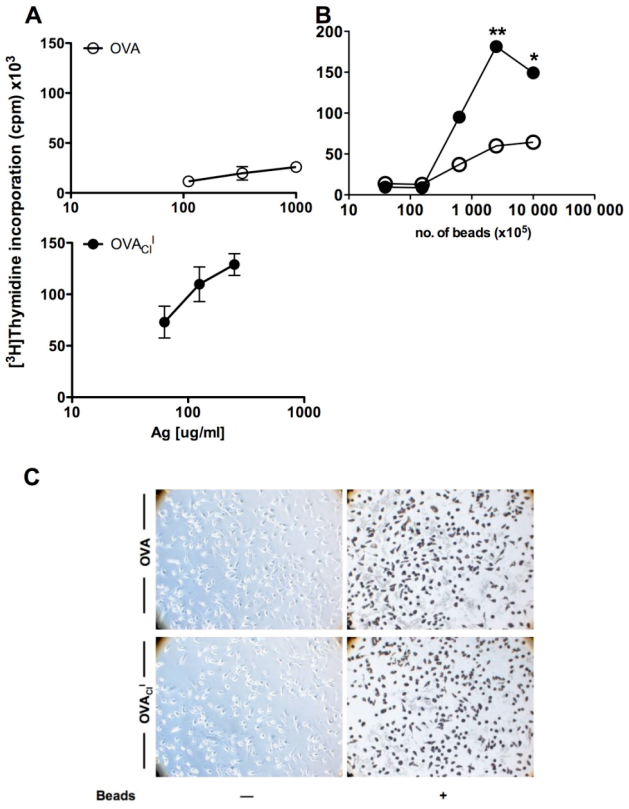


Figure 3.8 Soluble and particulate HOCl modified OVA is effectively processed and presented by macrophages.

A. *DO11.10 hybridomas (2x10^4/well) were incubated with different concentrations of OVA or OVA$_{Cl}^{I}$. Supernatants were collected after 24 hours. The graphs shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line.* ***B.*** *DO11.10 hybridomas (2x10^4/well) were incubated with different numbers of 1μm beads coated with OVA or OVA$_{Cl}^{I}$ and bone marrow derived macrophages (10^4/well). A. and B. Results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures.* ***C.*** *Bone marrow derived macrophages were incubated with or, without 10^8 of 1μm beads coated with Ova or OVA$_{Cl}^{I}$. After 2 hours cells were washed twice with HBSS and fixed with p-formaldehyde. Samples were analyzed using a light microscope. Experiments were repeated twice (A, B) and once (C). Statistical analysis was performed using the two-way ANOVA test. *P<0.05, **P<0.01.*

3.4 DISCUSSION

HOCl can reach a high concentration at inflammatory foci and efficiently modifies a broad range of molecules leading to changes in their functions and properties. However, proteins are likely to be major targets for reaction with HOCl. Thus, under inflammatory conditions, it is likely that extensive changes in the protein structure will occur that, in consequence, may alter protein antigen immunogenicity.

In this study we have focused on comparing the immunogenicity of native OVA to HOCl-modified OVA in different experimental conditions using both bone marrow derived DCs and macrophages. We have obtained an experimental model in which oxidation of OVA with HOCl leads to at least a 10-fold higher T cell response for the same OVA and OVA_{Cl}^{I} concentrations.

In the first step we have focused on obtaining the optimal conditions for protein modification. That required a monitoring system to measure the level of the oxidation. The most general indicator used as a marker of protein oxidation is the content of protein carbonyl groups. Protein carbonyls are very stable and easy to measure via their reaction with DNPH (see Chapter 2 Material and Methods). Product of the reaction, yellow hydrazone can be measured spectrophotometrically. Alternatively, DNPH derivatives can be quantified using antibodies to DNPH, that can be detect by both ELISA and Western blotting.

It is well known that HOCl reacts readily with amines to form N-chloramines that can undergo further reactions to form carbonyls (**reactions 3.3 and 3.4**)

(Hawkins et al., 2003).

$R\text{-}CH_2\text{-}NHCl \rightarrow R\text{-}CH{=}NH + HCl$ [**reaction 3.3**]

$R\text{-}CH{=}NH + H_2O \leftrightarrow R\text{-}CH{=}O + NH_3$ [**reaction 3.4**]

However, formation of aldehydes was only observed for chloramines formed from free amino groups on the α-carbon and not from chloramines formed on the ε-amino group. As already mentioned OVA α-carbon is blocked via acetylation of N-terminus glycine. Therefore, we observed the disappearance of free amino groups (formation of chloramines cause a loss of side amino group) and appearance of carbonyl groups regulated by two independent reactions.

Formation of chloramines groups can be detected spectrophotometrically via the specific reaction with 5-thio-2-nitrobenzoic acid (TNB).

Formation of carbonyl groups can be explained by three different mechanisms (Berlett, 1997):

- oxidation of protein side chains like: proline to 2-pyrrolidone, arginine to glutamic semialdehyde, lysine to α-aminoadipic semialdehyde and threonine to 2-amino-3-ketobutyric acid.
- oxidative cleavage of proteins by either the alfa-amidation pathway or, by oxidation of glutamyl side chains, both reactions block N-terminal amino acid by an α-ketoacyl derivative.

- secondary reaction of nucleophilic side chains of cysteine, histidine and lysine residues with aldehydes produced e.g. during lipid peroxidation.

Another chemical group that can be used as the indicator for protein oxidation is the cysteine residue. Oxidation of the thiol group of cysteine leads to formation of different products such as disulfides. These can be detected spectrophotometrically using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) or, fluorometrically using ThioG1. In our model, however, we focused on measurements of free amines and carbonyl groups.

Protein oxidation depends on a number of experimental parameters, which we control in our experiment. The primary factor is the concentration of HOCl but pH, temperature and light also play a role. In aqueous solution HOCl partially dissociates into the hypochlorite anion (OCl^-) that exists in equilibrium with free HOCl [**$OCl^- + HOH \leftrightarrow HOCl + OH^-$**]. The oxidizing power of OCl^- depends on pH and in the form of free acid is greater in acid solution than alkaline solution (redox potential Cl^-/ClO^- in alkaline solution pH=14 +0.885V, redox potential Cl^-/HOCl in acid solution pH=0 +1.495V) (Wiberg E., 2001).

In the presence of strong light HOCl decomposes quickly to hydrochloric acid (HCl) [**$2HOCl \rightarrow 2HCl + O_2$**]. However, in the dark the process is slow. In addition, in basic solution HOCl decomposes (via disproportionate reactions) almost completely to hydrochloric acid (HCl) and chloric acid ($HClO_3$), or chloride and chlorate anion [**$3HOCl \rightarrow 2HCl + HClO_3$**]. Moreover moderate amounts of HOCl are converted into

dichlorine monoxide (Cl_2O) [**$2HOCl \leftrightarrow Cl_2O + H_2O$**].

In our experimental model, the oxidation reaction was carried out at pH 9, in the dark, at 37°C. Therefore, the key agents responsible for protein modification are probably HOCl, and $HClO_3$. The exact concentration of oxidizing agents is difficult to calculate. However, it seems that the proportion of each compound in the reaction mixture play a critical role in antigen modification. Indeed, when the oxidation reaction was carried at a higher pH 9.8, enhanced immune response was not observed, even though the antigen was exposed to the same concentrations of HOCl. This may be explained, at least in part, by the change in the chemical species at equilibrium induced by an increase in pH.

Since, over time HOCl decomposes and loses its oxidizing power, we determined the HOCl concentration each time prior to preparation of the modified antigen. However, as discussed above, HOCl can form other chemical species that also possess oxidizing properties, and which seem to strongly influence the overall oxidizing power of HOCl. For example, we found that fresh stocks of NaOCl are more powerful than old stocks (opened for couple of months), even when according to mathematical calculations the same concentration of NaOCl were used. Hence, it is important that dilutions of new stocks of NaOCl are checked for functional activity in the T cell activation assay.

We did not observe an enhanced T cell response when OVA was modified with high concentrations of HOCl. This may be explained by three possible mechanisms.

Firstly, high concentrations of HOCl and its derivatives induce different chemical modification than moderate concentrations of HOCl (similarly, too low concentrations of HOCl did not modify protein to an extent required to enhance immune response). Pattison and Davies (2001) predicted the reactivity of various molar excess of HOCl with human serum albumin (HSA) and apo-A1 protein. In proposed model, HSA (molecular weight 67kDa) and apo-A1 (molecular weight 31kDa) show different behavior to HOCl. However, the relative order of consumption of each residue was the same for each protein and thus these data can be applied to other proteins.

The data predict that in general at low molar excess of HOCl, the majority of HOCl reacts with methionine and cysteine residues while for very high concentrations of HOCl backbone amides and tyrosine residues are modified. The detail of modifications for both proteins is presented on **Fig 3.9,** taken from (Pattison and Davies, 2001). Similarly, more recent data on bovine serum albumin (BSA) (molecular weight 66.4kDa), done by the same group again predicted that the majority of HOCl reacts with methionine and cysteine residues, while at high HOCl concentrations histidine and lysine residues become the target (Pattison et al., 2009).

By combining these data with known OVA structure we can speculate that, high concentrations of HOCl lead to oxidation of tryptophan (3 per OVA), lysine (20 per OVA) and tyrosine (10 per OVA) residues and backbone amides and that modification of these groups, or some of these groups do not enhance antigen

immunogenicity, or may reduce it by modifying the required T cell epitope. This could be further studied by spectrophotometrical measurements of dichlorotyrosine, bityrosine and tryptophan contents.

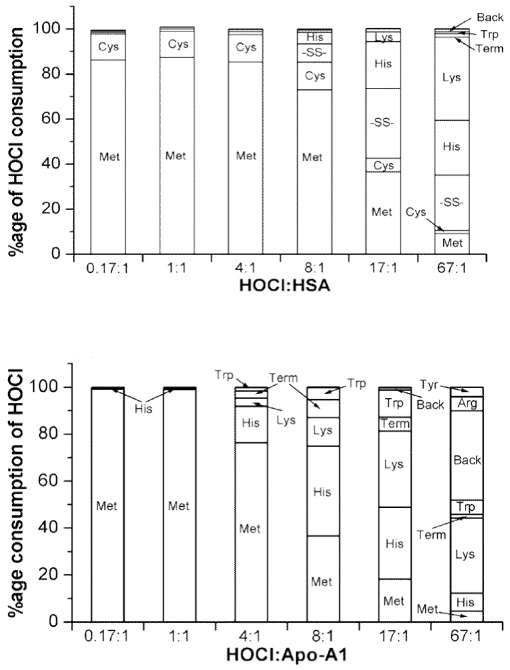


Figure 3.9 Predicted reactivity of various molar excess of HOCl, taken from (Pattison and Davies, 2001).

Secondly, high concentrations of HOCl can lead to partial OVA protein fragmentation (Olszowski, 1996). Similarly, exposure of plasma proteins to excess of HOCl results in loss of the parent plasma protein (Hawkins, 1999). We were not able to obtain a clear SDS page profile of OVA exposed to high concentrations of HOCl. The samples formed smears along the whole lane, probably due to the unspecific cross-reaction with SDS. Therefore we can only speculate that strong oxidation leads to protein fragmentation and that some of these fragments were then

removed on the desalting column we used for removal of excess of HOCl.

Thirdly, p.323-339 is situated inside the OVA molecule (**Fig 3.10**) and therefore be protected from direct exposure to HOCl. High concentrations of HOCl may lead to a partially open OVA structure and exposure of p.323-339 to HOCl cleavage. Our experiments showed that only OVA exposed to intermediate concentrations of HOCl at the pH 9, induces enhanced T cell responses to the OVA p.323-339 presented by dendritic cells and by macrophages.

HOCl is a selective oxidant that preferentially modifies particular amino acids residues especially methionine and cysteine residues as described in chapter 1. Therefore, we compared the T cell response to epitopes derived from OVA_{Cl}^{I} that contain or, lack methionine residues:

^{323}ISQAVHAAHAEINEAGR^{339}

^{273}**M**EERKIKVYLPR**M**K**M**E^{288}

^{257}SIINFEKLTEWTSSNV**M**EERKI^{278}

We observed an enhanced T cells response exclusively to epitopes lacking methionine residues; whereas HOCl treatment inhibited the T cell response to methionine containing epitopes (recognized by MF2.D5 and 3DO.18 T cell hybridomas). HOCl may modify methionine residues directly to form methionine sulfoxide derivatives and therefore block recognition of these epitopes by the T cell. This hypothesis could theoretically be explored using mass spectrometry, although we and others have encountered technical difficulties in analyzing OVA by

this method, due at least partly to the presence of several phosphate groups per OVA molecule, and partly to the presence of the glycan.

Moreover, mapping of the different epitopes on the three-dimensional structure of OVA also suggested an additional explanation of the differential effects of HOCl on different epitopes (**Fig 3.10**). Epitope p.323-339, whose presentation is enhanced, is localized within the core of the protein, almost buried completely from solution, while the two inhibited epitopes are localized in the same region of the protein, closer to the surface of the protein. HOCl oxidation may lead to partial degradation of OVA, this could then lead to differential uptake of different fragments, or differential enzyme digestion pattern within the lysosomal compartment, leading to altered peptide generation (discussed in detail in later chapter).

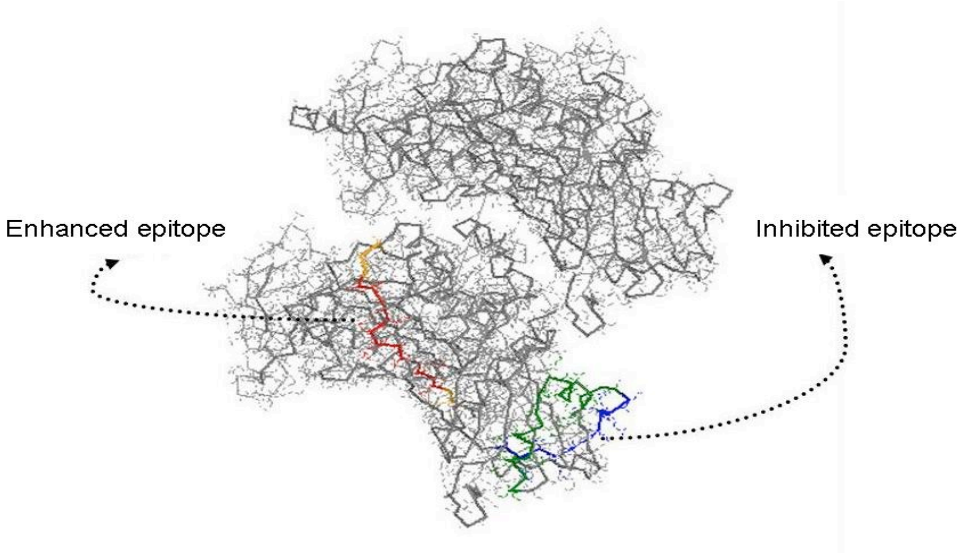


Figure 3.10 Enhancement of T cells response depend on peptide localization.

Jmol: an open-source Java viewer for chemical structures in 3D http://www.jmol.org.

The enhancement of processing seems to be a selective feature of HOCl, and not of oxidation in general, since treatment of OVA with different concentrations of H_2O_2 did not alter the T cell response. The lack of effect of H_2O_2 may have three possible explanations.

Firstly, H_2O_2 may not be a strong enough oxidant to modify OVA significantly. In other words it is not as reactive agent as HOCl. In fact, a lot of studies have shown the powerful oxidizing effect of HOCl but not H_2O_2 in the context of protein modification (Bergt et al., 2004; Fu et al., 2003; Hawkins and Davies, 2005; Szuchman-Sapir et al., 2008; Wang et al., 2007). Moreover as already mentioned HOCl , but not H_2O_2 can selectively induce loss of enzymatic activity (Khor et al., 2004; Shao et al., 2005). Nevertheless, recent, data has confirmed that H_2O_2 can indeed act as a protein oxidant. Manzanares et al (Manzanares et al., 2007) have shown that H_2O_2 via Fenton oxidation is more potent in altering surfactant protein B activity than HOCl. In addition the significance of H_2O_2 oxidizing power had been shown by studying methionine sulfoxide reductase (Msr), precisely the MsrA enzyme (Le et al., 2009). The author demonstrated that MsrA enzyme can be inactivated by high doses of H_2O_2 and the probable mechanism underlying this reaction is via the susceptibility to oxidation of catalytic cysteine domain.

Secondly, although both oxidants modify OVA, only HOCl produces the specific oxidative modifications required to enhance the T cell response. Unfortunately, this hypothesis is difficult to validate. The best method that can precisely identify single amino acid modifications - mass spectrometry, has proved problematic in

the case of OVA protein. The other option would be to quantify thiol and amino groups in HOCl or, H_2O_2 treated samples. Potential differences in the amount of each group might explain the lack of an effect of H_2O_2 on OVA immunogenicity. For the future, it would be interesting to investigate these protein modifications further in detail.

Thirdly, no oxidation reaction may have occurred at all. This again requires to be established by more extensive validation experiments.

Finally, in the presented study we described the model where protein antigen is directly exposed to H_2O_2 activity. In other words there is a direct interaction between the protein antigen and the oxidant. However, when the whole cells were exposed to H_2O_2 activity, it has been demonstrated that the high concentrations of H_2O_2 altered DCs phenotype and also induced DCs apoptosis (Handley, 2005). Therefore the final outcome of the H_2O_2 activity may depend on used experimental conditions.

Both the macrophages and dendritic cells used here are bone marrow derived cells that process antigen, but the nature of the processing is different in the two cell types.

Macrophages are a first line of defense and recognize and capture a very broad diversity of soluble and particulate antigens. DCs selectively express some phagocytic receptors and antigens are taken up in a more selective manner.

Macrophages also have higher concentrations of proteolytic enzymes and their

phagosomal pH decreases rapidly after antigen uptake. This results in total degradation of the antigen. DCs have lower concentrations of proteases (Delamarre et al., 2005) and have an active system of alkalinization of phagosomes (Savina et al., 2006). This results in much less proteolysis and only partial degradation of antigens.

The alkalinization mechanism was shown to be particularly important when antigens are cross-presented by MHC class I complex to $CD8^+$ T cells (Savina et al., 2006). On the other hand, loading peptides on the MHC II complex is more efficient at acid pH and occurs later when pahgosomal pH is more acidic. Moreover according to Savina et al, DCs actively keep alkaline pH in the first 3 hours of phagocytosis. Interestingly in our model, OVA_{Cl}^{I} was efficiently presented on MHC class II complex even after 1 hour of co-culture with DCs (although we only showed it for soluble OVA that is taken up to endosomes). In addition like DCs, macrophages also induced enhanced DO11.10 T cells response when co-cultured with OVA_{Cl}^{I}. In this context, we can speculate that the enhanced immunogenicity of OVA_{Cl}^{I} is independent of the pH of the endosome/phagosome within the antigen presenting cell.

In general, the presented data suggests that the mechanism for enhanced immunogenicity of HOCl-modify antigen applies to both types of antigen presenting cells, DCs and macrophages and to both types of antigen, soluble and particulate.

3.5 CONCLUSIONS

- **HOCl** enhances antigen processing/presentation to T cells in a concentration dependent manner.
- **Dendritic cells and macrophages** process and present HOCl-modified antigen with a higher efficiency than native equivalent.
- The effects of HOCl are **epitope specific**.
- Both **particulate and soluble** HOCl-modified antigens enhance the T cell response.
- Hydrogen peroxide does not enhance antigen processing/presentation to T cells.

CHAPTER 4 ENHANCED IMMUNOGENICITY OF HOCL MODIFIED OVA IS NOT MEDIATED VIA TLRS.

4.1 INTRODUCTION

DCs patrol the body tissues sensing dangerous signals such as presence of pathogens. Recognition of microbial antigens is mediated by pattern recognition receptors (PRRs) for example, the intensively studied TLRs (discussed in detail in Chapter 1). TLRs mediated signaling leads to upregulation of many molecules on DCs involved in antigen presentation including MHC and co-stimulatory molecules. The role of PRRs in antigen presentation has been emphasized in many recent studies. In addition an important study suggested that antigen processing might also absolutely require TLR ligation (Blander and Medzhitov, 2006).

In this thesis we describe a novel mechanism of antigen presentation that is independent of TLRs mediated signaling. We used several methods to exclude TLR-4 ligand LPS. Proteins can be purified to remove LPS contamination, or residual LPS can be rendered inactive by addition of specific inhibitors, for example polymyxin B, which is a natural antibiotic that binds and neutralizes Lipid A (a major component of LPS).

Further evidence can be obtained from knockout studies. In the case of TLRs, signalling is mediated either by Myd88 or, by TRIF signal adaptor molecule. Myd88 is an adaptor molecule utilized by all TLRs apart from TLR3 which mediate signal via TRIF adaptor domain (Yamamoto et al., 2003) (**Fig 4.1**). In addition, TRIF can

mediate signalling from TLR4. Hence, knockout of both molecules is an excellent model to study antigen processing in the absence of TLRs.

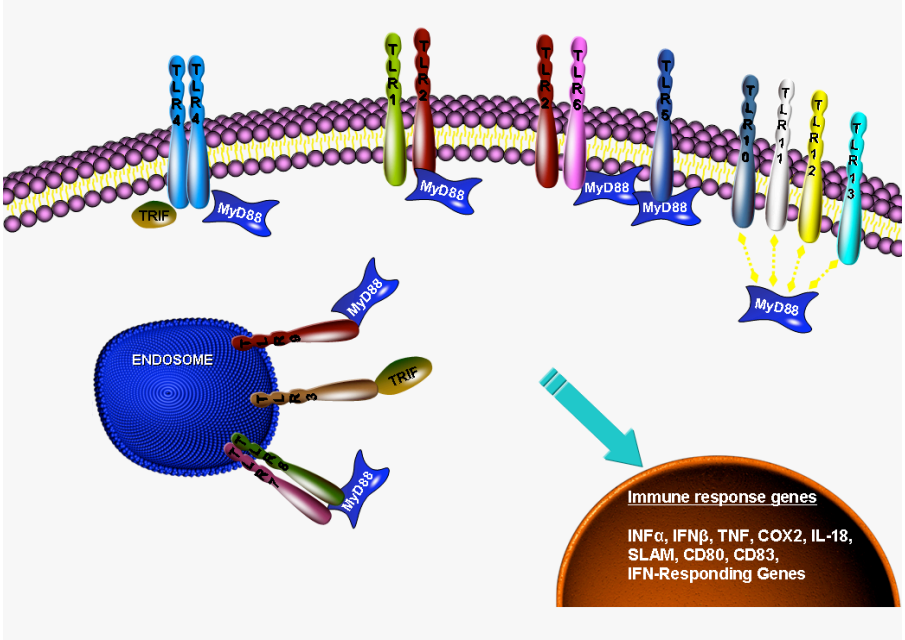


Figure 4.1 Myd88 or TRIF are common adaptor molecules for all TLRs signaling pathways.

Signalling of all TLRs except TLR-3 is mediated by MyD88 adaptor molecule. Signalling of TLR-3 is mediated by TRIF adaptor molecule. Signalling of TLR-4 can be mediated by both adaptor molecules MyD88 and TRIF. Modify from SA Bioscience.

4.2 OBJECTIVES

- to test the purity of OVA samples in the context of LPS contamination.
- to test the OVA_{Cl}^{I} potency to activate bone marrow derived DCs.
- to test the contribution of TLRs in enhanced presentation of OVA_{Cl}^{I}

4.3 RESULTS

4.3.1 HOCL MODIFIED OVA DOES NOT INDUCE DCS MATURATION.

It is known that exposure to TLR ligands such as LPS results in DCs maturation. Therefore, we characterized the ability of OVA_{Cl}^{I} to mature DCs. We analyzed the expression of maturation markers CD86, CD54 and MHC class II in response to endotoxin free EndoGrad OVA (20 µg/ml) or, EndoGrad OVA_{Cl}^{I} (20 µg/ml) (**Fig 4.2**). As a positive control DCs were co-cultured with LPS (100ng/ml) and as a negative control media only were used. Up-regulation of all three maturation markers was observed for LPS treated cells only. However, neither OVA, nor OVA_{Cl}^{I} induced DCs maturation confirming the absence of any biologically active TLR-4 ligand in the preparation.

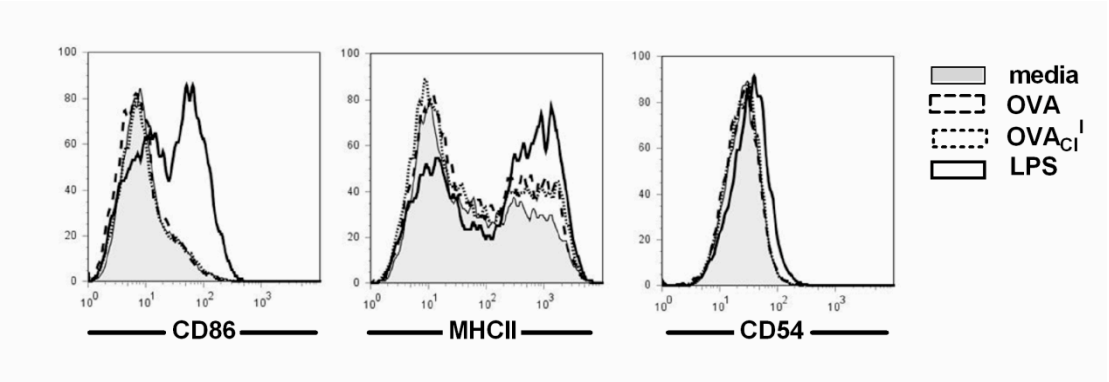


Figure 4.2 OVA_{Cl}^{I} does not induce DCs maturation.

Bone marrow derived DCs were incubated for 18 hours with EndoGrad OVA (20 µg/ml) or EndoGrad OVA_{Cl}^{I} (20 µg/ml, endotoxin free) or LPS (100 ng/ml) or media only and then analyzed for CD86, MHCII and CD54 expression by flow cytometry. One representative experiment from three independent experiments is shown.

4.3.2 ENHANCED IMMUNOGENICITY IS NOT ASSOCIATED WITH LPS CONTAMINATION.

Contamination of protein preparations with LPS would result in an increase in immunogenicity of OVA_{Cl}^{I}. In order to ensure that LPS is not present in tested samples we have repeated the functional experiment using endotoxin free OVA (EndoGrad). TCR transgenic OT-II T cells were co-cultured with increasing doses of EndoGrad OVA or, EndoGrad OVA_{Cl}^{I} and bone marrow derived DCs (**Fig 4.3 A**). In parallel, as a control, cells were incubated with standard OVA or OVA_{Cl}^{I} Grad V purchased from Sigma (**Fig 4.3 B**)**.** The enhancement of T cell responses by HOCl was comparable for both samples: EndoGradOVA_{Cl}^{I} and standard OVA_{Cl}^{I}.

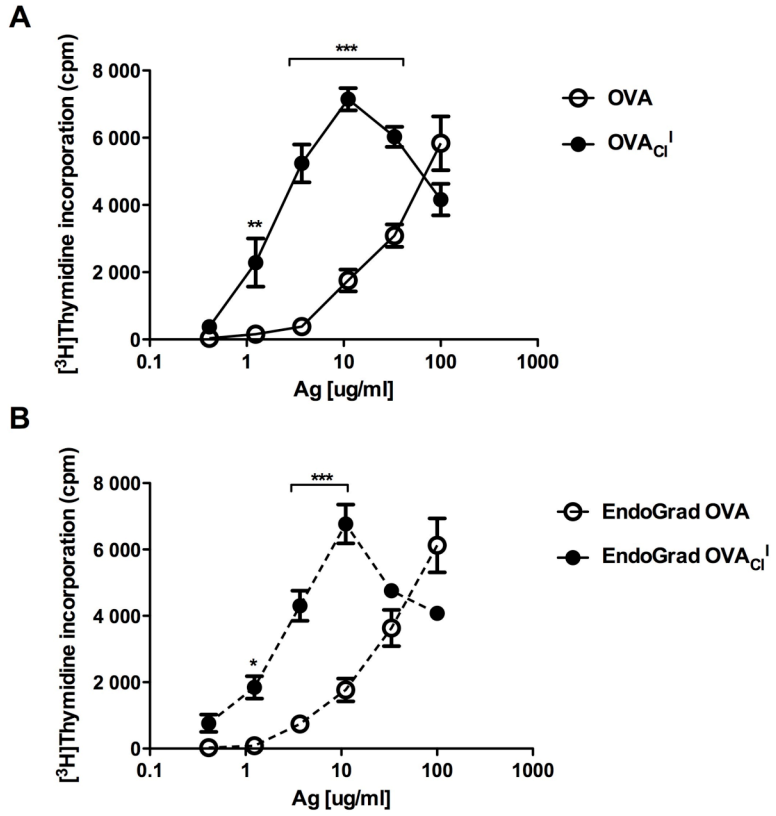


Figure 4.3 Standard and endotoxin free OVA_{Cl}^{I} induce the enhanced T cell response.

*TCR transgenic OT-II cells ($2x10^4$/well) were incubated with different concentrations of **A.** OVA or OVA_{Cl}^{I} (Grad V from Sigma) or, **B.** endotoxin free EndoGrad OVA or EndoGrad OVA_{Cl}^{I} and purified bone marrow derived DCs ($5x10^3$/well). The graph shows OT-II proliferation measured as thymidine incorporation. The results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiment was repeated three times. Statistical analysis was performed using the two-way ANOVA test. *P<0.05, **P<0.01, ***P<0.001.*

4.3.3 POLYMYXIN B DOES NOT INHIBIT THE ENHANCED T CELL RESPONSE INDUCED BY OVA_{CL}^{I}.

Specific LPS inhibitors such as polymyxin B (PMB) can efficiently block LPS. Therefore, we have tested if pre-incubation of OVA_{Cl}^{I} with PMB leads to the elimination of the observed enhancement of the T cell response. TCR transgenic OT-II T cells were co-cultured with OVA_{Cl}^{I} pre-incubated for 30 minutes with PMB (PMB-OVA_{Cl}^{I}) and bone marrow derived DCs. Blocking of LPS did not affect enhanced presentation and PMB-OVA_{Cl}^{I} was presented efficiently even at 20nM (**Fig 4.4D**). Similarly, there was no difference in T cells response to OVA with or, without PMB (**Fig 4.4A**). However, pre-incubation with LPS led to an enhanced response to native OVA (LPS-OVA) (**Fig 4.4B**). Nevertheless, the response was much lower than the response to the same concentration of OVA_{Cl}^{I} and was slightly reduced after addition of PMB (LPS-PMB-OVA) (**Fig 4.4C**).

On the other hand OVA_{Cl}^{I} pre-incubated with LPS induced a lower T cell response (**Fig 4.4E**) than without addition of LPS or, following blocking with PMB (LPS-PMB-OVA_{Cl}^{I}) (**Fig 4.4F**). Inhibitory effect of LPS may be due to the early activation of DCs and in consequence decrease in antigen uptake.

PMB alone does not have any toxic effect on T cells. Peptide pre-incubated with PMB induced as good a T cell response as peptide in media only (**Fig 4.5**).

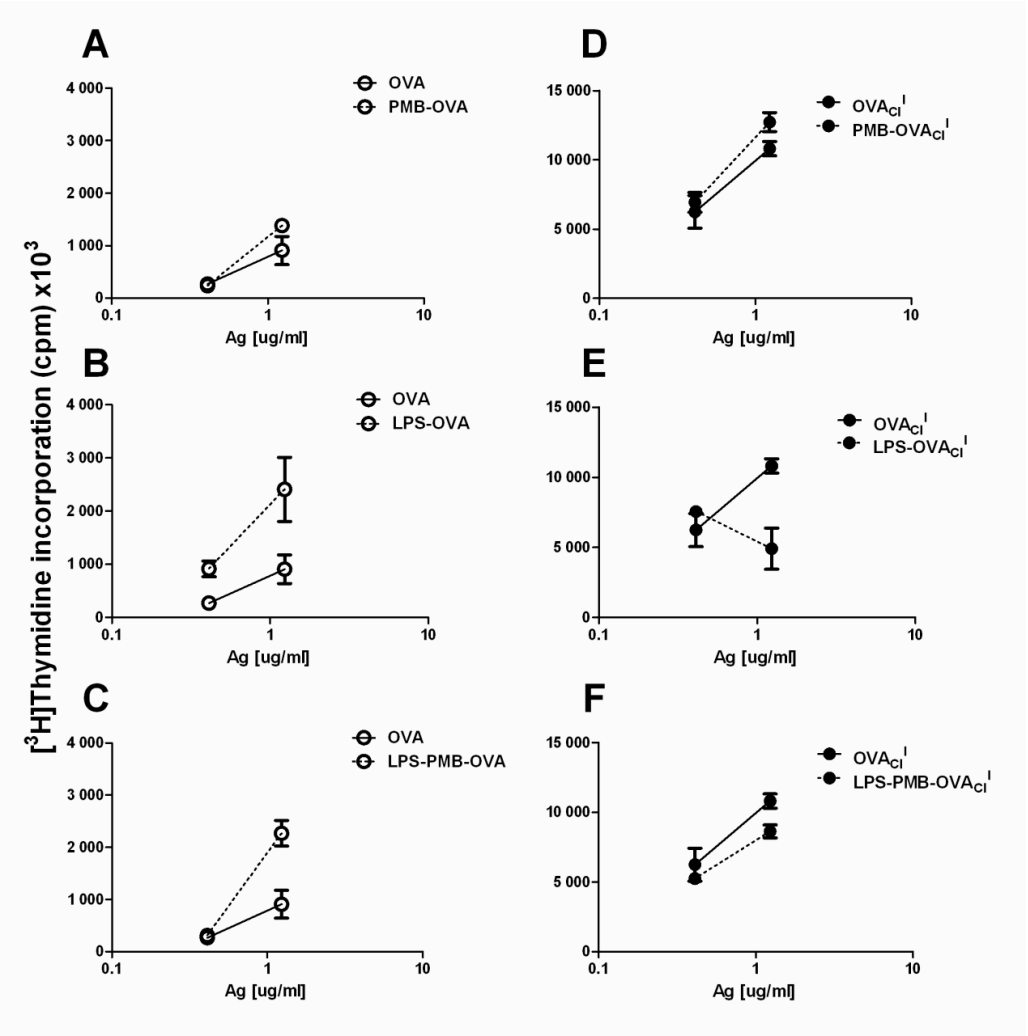


Figure 4.4 Polymyxin B treated modified OVA induce the enhanced T cell response.

TCR transgenic OT-II T cells were incubated with different concentrations OVA or OVA_{Cl}^{I} and bone marrow derived DCs. ***A.*** *and* ***D.*** *cultures were performed in the presence or absence of polymyxin B (100 ng/ml).* ***B.*** *and* ***E.*** *cultures were performed in the presence or absence of LPS (1 µg/ml).* ***C****. and* ***D****. cultures were performed in the presence or, absence of LPS (1 µg/ml) followed by incubation with polymyxin B (100 ng/ml). The graph shows OT-II proliferation measured as thymidine incorporation. The results are presented as the average ± SEM [*^{3}*H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiments were performed twice.*

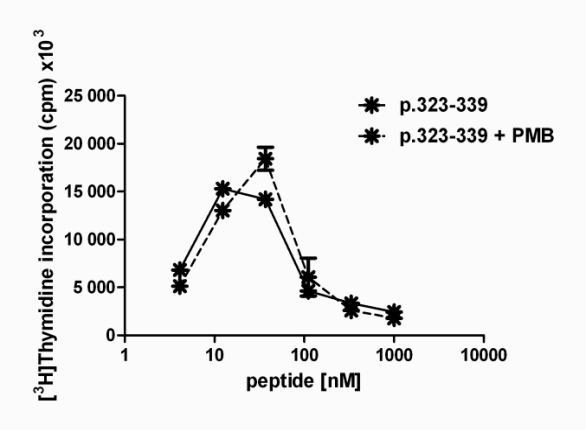


Figure 4.5 Polymyxin B does not block T cell responses.

TCR transgenic OT-II T cells were incubated with p.323-339 in the presence or absence of polymyxin B (100 ng/ml). The graph shows OT-II proliferation measured as thymidine incorporation. The results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiment was done twice.

4.3.4 TLR SIGNALING PATHWAY IS NOT INVOLVED IN PROCESSING AND PRESENTATION OF HOCL MODIFIED OVA.

Blander and Medzhitov (Blander and Medzhitov, 2006) proposed that presentation of non-self antigens by the MHC class II complex is efficient only when in parallel presenting cells receive TLRs stimulation signal such as ligand for TLR4, LPS. In other words, TLRs induced stimulation is required to distinguish self-antigen from non-self one.

As shown above OVA_{Cl}^{I} is LPS free, however in order to exclude TLRs contribution completely, we used mice deficient in major TLR signalling molecules Myd88 and TRIF.

OVA and OVA_{Cl}^{I} were co-cultured with TCR transgenic OT-II T cells and bone marrow derived DCs isolated from Myd88/TRIF deficient mice (**Fig 4.6**). The absence of these two key TLR downstream signalling adaptor molecules did not affect the enhanced processing and presentation of OVA_{Cl}^{I} that was presented even at concentration of 20nM.

Those data confirmed that the HOCl effect observed by us is independent from TLRs activation.

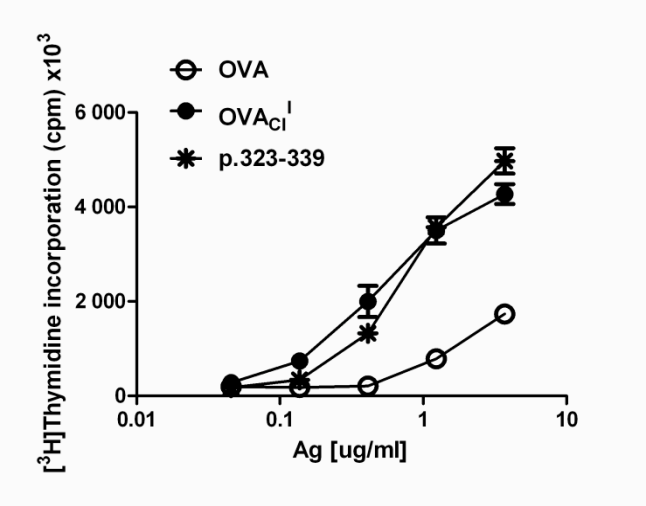


Figure 4.6 Immunogenic effect of HOCl is independent of TLR signaling.

TCR transgenic OT-II T cells were incubated with different concentrations of OVA or, OVA$_{Cl}^{l}$ and bone marrow derived DC from Myd88/TRIF double knockout mice. The graph shows OT-II proliferation measured as thymidine incorporation. The results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiment was done twice.

4.4 DISCUSSION

It is known that DCs stimulation with TLRs ligands (TLR-L) leads to their activation and enhanced antigen presentation on both MHC class I and II complexes (West et al., 2004). We have demonstrated in chapter 3 that antigen modification with HOCl leads to enhanced $CD4^+$ T cell responses. We postulate that it is a novel, unknown mechanism independent from DCs stimulation via TLRs. Therefore in this context it is important to exclude completely the possibility that the effect observed by us is related to contamination with TLR ligand such as LPS.

Two routes of contamination of OVA with LPS are possible: first, LPS may become incorporated into the OVA preparation during the commercial isolation and purification of the protein from eggs; second, LPS could be introduced in our laboratory during laboratory preparation of HOCl-modified and mock-modified samples. Since these samples are prepared in endotoxin free conditions (i.e. using endotoxin free plastics and water and buffers) the risk of contamination is minimal.

Nevertheless we used four independent approaches to test the purity OVA_{Cl}^{I}:

- T cell activation assay using endotoxin-free OVA.
- T cell activation assay using OVA preincubated with the LPS inhibitor polymyxin B.
- Test OVA_{Cl}^{I} potential to mature bone marrow derived DCs.
- T cell activation assay using bone marrow DCs from Myd88/TRIF double knockout mouse.

All four experiments have proven that OVA_{Cl}^{I} is not contaminated with LPS in addition the last experiment has shown that the effect of enhanced immunogenicity occurs independently of TLRs signaling. However, we did not find any strong differences between experiments using endotoxin free OVA and standard OVA (from Sigma). This is an important finding, since many of the other experiments in this thesis were done using only standard OVA.

The TLRs association with antigen processing and presentation has been studied extensively.

In the case of MHC class II restricted antigens, the presence of LPS has been shown to be essential for generation of stable MHC class II – peptide complexes (Cella et al., 1997). Moreover more recent studies have shown that LPS induced DCs maturation leads to activation of a vascular proton pump that enhances lysosomal acidification required for activation of proteases. This leads to enhanced antigen proteolysis and as a consequence efficient presentation on MHC-II complexes (Trombetta et al., 2003). Since we have shown that DCs co-cultured with OVA_{Cl}^{I} do not induce cell maturation, lysosomal acidification may also not occur. In this context it would be interesting to look in greater detail at the DCs lysosomal compartments after uptake of OVA_{Cl}^{I} or native OVA, and in particular to analyze the pH, types of active proteases and the level of antigen proteolysis. This last issue is discussed further in chapter 6.

Another example of a link between antigenicity, processing/presentation and TLR activation is the MHC class II restricted antigen profilin, a protein from

Toxoplasma gondii that was demonstrated to be a direct target for TLR11 recognition. This protein is the immundominant antigen in $CD4^+$ T cell responses to Toxoplasma (Yarovinsky et al., 2006). Similarly antigens conjugated with CpG (TLR9-L), were shown to promote a specific Th1 response (Shirota et al., 2001). In these cases, however, it is unclear whether the TLR promotes antigen processing/uptake of the antigen, or simply enhances the ability of the antigen presenting cells to activate T cells. In contrast, as already mentioned, antigen non-covalently bound to LPS, delivered in a form of microspheres bound with antigen and covered with LPS was demonstrated to be very efficiently loaded onto MHC class II molecules (Blander and Medzhitov, 2006). These authors propose that the link between TLR ligation and processing may contribute to the mechanisms of selection between self and microbial antigens, since antigens taken up by phagocytosis will only be efficiently presented when delivered simultaneously with a TLR ligand.

In agreement with Blander and Medzhitov we found (chapter 3) that native OVA coated on beads was presented inefficiently and failed to induce T cell responses. However, OVA_{Cl}^{I} on beads was presented efficiently by MHC class II molecules and induced enhanced T cell responses. Using other T cells (e.g. OT-II instead of DO110) however, we showed that native OVA as well as OVA_{Cl}^{I} could be presented in the absence of LPS. This study therefore suggests that, contrary to Blander and Medzhitov's conclusions, antigen processing of OVA is not exclusively TLR dependent.

At least three aspects in their experimental model differ from the experimental model used in this study. Firstly, Blander and Medzhitov used high concentrations of LPS to coat microspheres loaded with antigen, 1000 times higher than we and others have used as soluble LPS to induce DC maturation. Obviously, not all LPS is absorbed on the microspheres but it still seems to be far from concentrations we can expect to observe *in vivo*. Secondly, the microspheres they used are significantly bigger (10 and 4.5 μm) than used by us (1 μm), and have different surface functional groups. Carboxylate-modified microspheres, used by Blander and Medzhitov bind to proteins covalently, while sulfate-modified microspheres used in the present study passively adsorb proteins. Thirdly, as antigen presenting cells they used DCs while in our experiments with beads we used macrophages. However, as already discussed we did not observe any difference between macrophages and DCs response to OVA_{Cl}^{I}.

In conclusion, it is apparent that association between antigen and TLR ligands can result in more efficient antigen presentation and T cell responses. In some cases, at least, this increased immunogenicity may result from more efficient antigen processing. Linking antigens to TLR ligands may therefore provide a good strategy for improving vaccine efficacy. As one example MPL, a synthetic LPS analogue is incorporated into the commercially available vaccine for cervical cancer that has been shown to efficiently prevent HPV type 16 and 18 infections (Paavonen et al., 2007; Paavonen et al., 2009).

Nevertheless, the suggestion that antigen processing is absolutely dependent on TLRs ligation is likely to be an oversimplification. In particular, HOCl modification enhances antigen processing/presentation via a TLRs independent mechanism. We proposed that this novel mechanism could also be used as a new, TLR-independent vaccination strategy. In fact, studies already done in our laboratory have shown that the HOCl-modified tumor line SK-OV-3 can stimulate specific T cells isolated from ovarian cancer patients (Chiang et al., 2008). Further studies exploring this immunotherapeutic approach are in progress.

Our HOCl-model may also shed new light on mechanisms triggering responses to autoantigens as observed in chronic autoimmune disorders. It has already been shown that oxidized antigens are present in autoimmune diseases such as systemic lupus erythematosus (Hal Scofield et al., 2005). The autoantigens were demonstrated to cluster in a close proximity to the endoplasmic reticulum (ER) and nuclear membranes, compartments where extensive generation of reactive species takes place (Casciola-Rosen, 1994). Thus it is tempting to speculate that strong recognition of such antigens occurs in the same manner as observed by us for HOCl-modified OVA, and may contribute to the pathogenesis of the autoimmune response.

4.5 CONCLUSION

- OVA_{Cl}^{I} is not contaminated with LPS.
- OVA_{Cl}^{I} does not induce DCs maturation.
- Enhanced immunogenicity of HOCl-modified antigen is triggered by a **TLR-independent mechanism**.

CHAPTER 5 IN VITRO AND IN VIVO STUDIES ON OVA_{CL}^{I} UPTAKE AND PROCESSING.

5.1 INTRODUCTION.

The chemical modification of OVA, and specifically of its carbohydrate side chain, might alter the handling of antigen by the antigen presenting cell. Therefore we decided to study the antigen uptake in both *in vitro* and *in vivo* models.

So far we used bone marrow derived DCs differentiated in media containing GM-CSF. Those cells are equivalents of monocyte derived DCs located at the site of inflammation (Villadangos and Schnorrer, 2007). As shown in **Chapter 4,** bone marrow derived DCs are phenotypically immature and are characterized by high expression of the CD11c marker.

Another group of DCs subtypes we studied were isolated from mouse spleen: $CD8^{+}$ and $CD8^{-}$ DC (Proietto et al., 2008). $CD8^{+}$ DC are involved in antigen cross-presentation on MHC class I complexes, while $CD8^{-}$ were not as efficient (Pooley et al., 2001). $CD8^{-}$ DCs are the major population that presents antigen on MHC class II complex (Dudziak et al., 2007).

In order to study *in vivo* antigen uptake by splenic DCs, fluorescently labelled antigen was injection into a mouse. There are several routes of *in vivo* antigen administration, the most popular methods are summarized in **Table 5.1**, and the first three are frequently used for mouse. In this study we used intravenous injections.

Route	Advantage	Disadvantage
Intravenous	Ag is delivered primarily to the spleen; good for soluble Ag without adjuvants	Adjuvant cannot be used; poor response to primary immunization; risk of tolerance or anaphylactic reaction
Subcutaneous	Large volume can be used; low rate of absorption; does not induce anaphylactic reaction	Local irritation, difficult to control rate of absorption.
Intraperitoneal	Rapid uptake of the Ag that is transferred to the draining lymph nodes; different types of adjuvant can be used	Risk of anaphylactic reaction.
Intradermal	Ag taken up to the lymphatic system by Langerhan's cells.	Injection site can ulcerate; special preparation of the site of injection necessary.
Intramuscular	Ag uptake into to blood stream and lymphatics; good for small Ag; large volume can be used;	Injection site cannot be monitored

Table 5.1 Advantages and disadvantages of different routs of *in vivo* antigen administration in mice.

5.2 OBJECTIVES.

- To label OVA protein antigen with fluorescent dyes and follow antigen uptake using flow cytometry and confocal microscopy tools.
- To study *in vitro* uptake of OVA and OVA_{Cl}^{I} by bone marrow derived DCs and to investigate which receptor mediates this uptake.
- To study *in vivo* uptake and processing of OVA and OVA_{Cl}^{I} by splenic DCs.

5.3 RESULTS.

5.3.1 LABELING WITH FLUOROCHROMES DOES NOT INTERFERE WITH IMMUNOGENIC PROPERTIES OF OVA_{CL}^{I}.

In order to visualize antigen uptake by DC, we labeled OVA and OVA_{Cl}^{I} with two distinctive fluorescent dyes fluorescein isothiocyanate (FITC) that emits green fluorescence and tetramethylrhodamine isothiocyanate (TRITC) that emits red fluorescence (as described in Material and Methods). The ratio between protein and dye was calculated (as described in Material and Methods) and was only slightly lower for OVA_{Cl}^{I}.

Because the fluorochromes are destroyed by oxidation, OVA_{Cl}^{I} was modified firstly with the appropriate amount of HOCl before labeling with fluorescent dye.

In addition before labeling, sample was treated with L-methionine that reacts with free chloramines. The step was important because, both dyes bind to free amino

group of lysine and arginine predominantly and this can be blocked by chloramines.

The response to the fluorescent OVA derivatives was assessed in a functional assay, the DO11.10 T cell hybridoma response to OVA labeled with FITC (OVA-FITC), OVA labeled with TRITC (OVA-TRITC), OVA_{Cl}^{I} labeled with FITC (OVA_{Cl}^{I} - FITC) and OVA_{Cl}^{I} labeled with TIRTC (OVA_{Cl}^{I} – TRITC) was tested (**Fig 5.1**). DO11.10 T cells respond poorly (> 2 μM) to OVA-FITC and OVA-TRITC. However, similarly to what has been already shown, OVA_{Cl}^{I}-FITC and OVA_{Cl}^{I}-TRITC induced the DO11.10 T cell responses even at the antigen concentration of 0.2 μM. Therefore, the response to fluorescent derivatives of native and HOCl-modified OVA corresponds to response obtained to the unlabelled equivalents, indicating that introduction of fluorescent dyes does not interfere with the antigen properties.

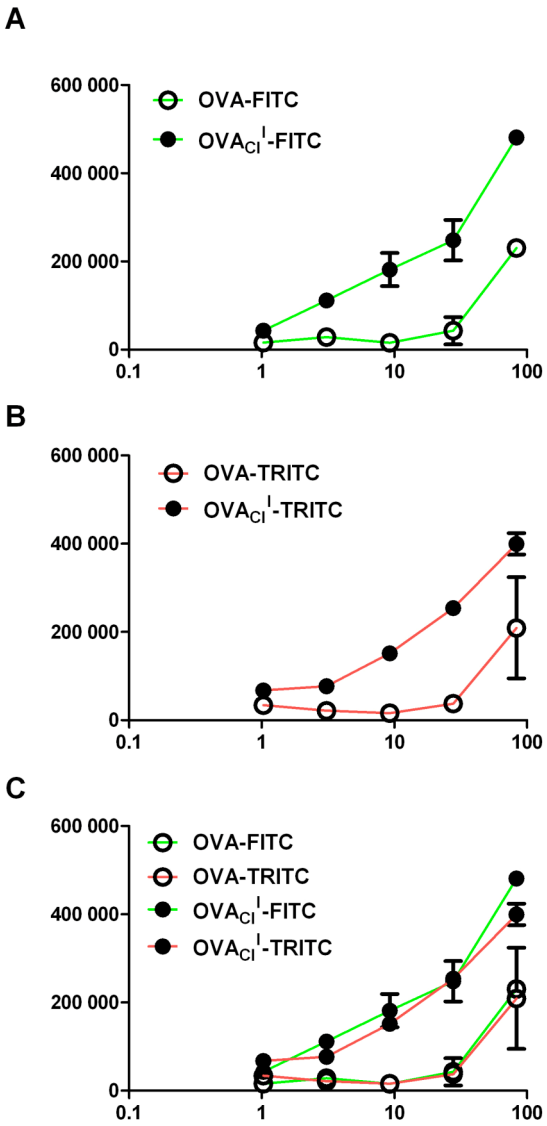


Figure 5.1 Immunogenic activity of OVA and OVA$_{Cl}^{I}$ FITC or TRITC derivatives.

*DO11.10 hybridoma cells (2x10^4/well) were incubated with different concentrations of **A.** OVA-FITC and OVA$_{Cl}^{I}$-FITC or **B.** OVA-TRITC and OVA$_{Cl}^{I}$-TRITC and purified bone marrow derived DC (5x10^3/well). Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line. The results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiment was repeated twice.* ***C.*** *Overlay of panel A and B.*

5.3.2 HOCL MODIFICATION ENHANCES ANTIGEN UPTAKE. *IN VITRO* STUDIES.

The uptake of OVA and OVA_{Cl}^{I} labeled with FITC or TRITC fluorescent dye was assessed by flow cytometry analysis. Bone marrow derived DCs were co-cultured for two hours with different concentrations of OVA-FITC, OVA-TRITC, OVA_{Cl}^{I}-FITC or, OVA_{Cl}^{I}-TRITC (**Fig 5.2**). OVA_{Cl}^{I}-FITC was taken up with almost 100-fold higher efficiency than native equivalent (**Fig 5.2A**). The observed effect was even more significant for lower concentrations of antigen. For example 100 ng/ml OVA_{Cl}^{I}-FITC were taken up as efficiently as 10 µg/ml OVA-FITC. Similar results were obtained for OVA-TRITC and OVA_{Cl}^{I}-TRITC (**Fig 5.2B**). No uptake was observed for samples incubated at 4°C (**Fig 5.3**).

Since flow cytometry cannot distinguish between antigen bound to the surface of the cell, and antigen taken up into the cell, the enhanced uptake of OVA_{Cl}^{I} was also analyzed by confocal imaging (**Fig 5.4**). Bone marrow derived DCs were co-cultured with the same concentration of OVA-FITC and OVA_{Cl}^{I}-TRITC. Antigens were administered simultaneously. Most of the DCs were observed to take up OVA_{Cl}^{I}-TRITC exclusively. However, some of the cells were found to take up both labeled OVA and OVA_{Cl}^{I} into the endocytic vesicles inside the cells. Images showed co-localization of the two fluorochromes in the same compartment, suggesting that HOCl-modification enhances the magnitude of antigen uptake, but does not alter intracellular routing.

Unexpectedly, further analysis also showed a competition between uptake of OVA and OVA_{CI}^{I} (**Fig 5.5A**). OVA-FITC (100 µg/ml) was incubated with decreasing concentrations of unlabelled OVA_{CI}^{I}. The uptake of OVA-FITC was inhibited by 60% for the 100 µg/ml OVA_{CI}^{I} and gradually decreased to 10% for 100 ng/ml OVA_{CI}^{I}. The competition effect was not detectable for the reverse experiment where OVA_{CI}^{I} -FITC (100 µg/ml) was mixed with decreasing concentrations of unlabeled OVA (**Fig 5.5B**).

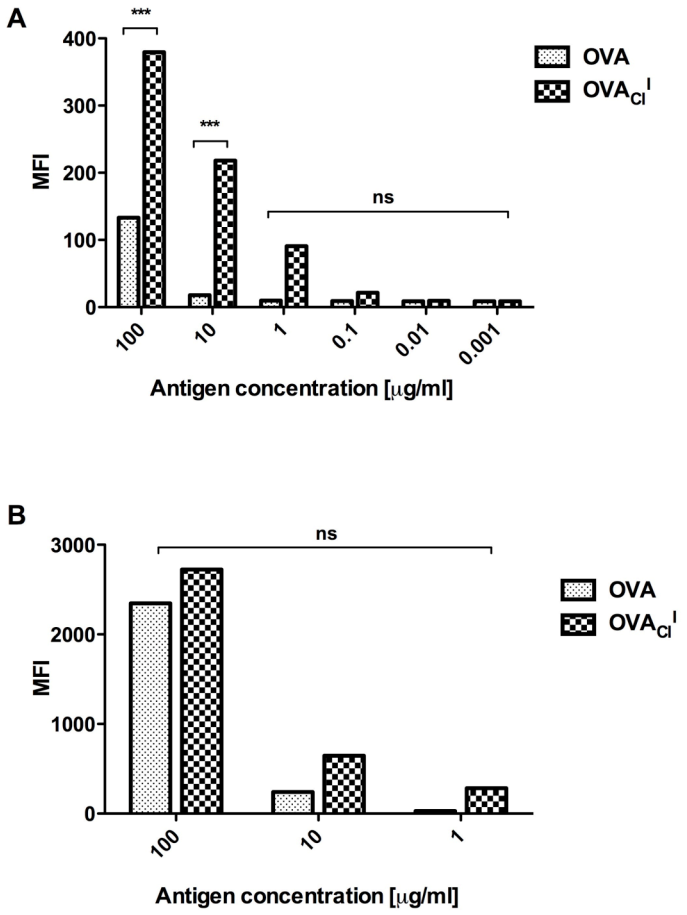


Figure 5.2 HOCl modification enhances antigen uptake *in vitro.*

Bone marrow derived DCs were cultured for 2 hours at 37°C with decreasing concentrations of ***A.*** *OVA-FITC and OVA_{Cl}^{I}-FITC or* ***B.*** *OVA-TRITC and OVA_{Cl}^{I}-TRITC. Excess antigen was removed by washing the cells twice with ice-cold HBSS and the cells were fixed with 3.8% formaldehyde. The figures shows the mean fluorescent intensity of A. FITC-positive or B. TRITC-positive cells and are one representative experiment from at least three independent experiments. Statistical analysis was performed using the two-way ANOVA test. ***P<0.001, ns non-significant.*

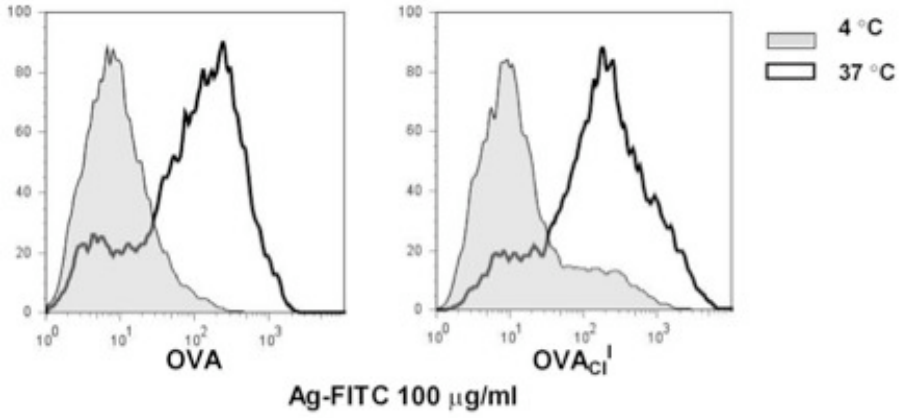


Figure 5.3 The uptake of OVA and OVA$_{Cl}^{I}$ by DCs is blocked at 4° C

Bone marrow derived DCs were cultured for 2 hours at 4°C (filled grey) or at 37°C (open black) with 100µg/ml OVA-FITC or, OVA$_{Cl}^{I}$-FITC 100µg/ml. Excess antigen was removed by washing the cells twice with ice-cold HBSS and the cells were fixed with 3.8% formaldehyde. The figure shows histograms of the DC population in a FITC channel and it is one representative experiment from three independent experiments. Statistical analysis was performed using the Student's t test, for OVA P<0.05, for OVA$_{Cl}^{I}$ P<0.01.

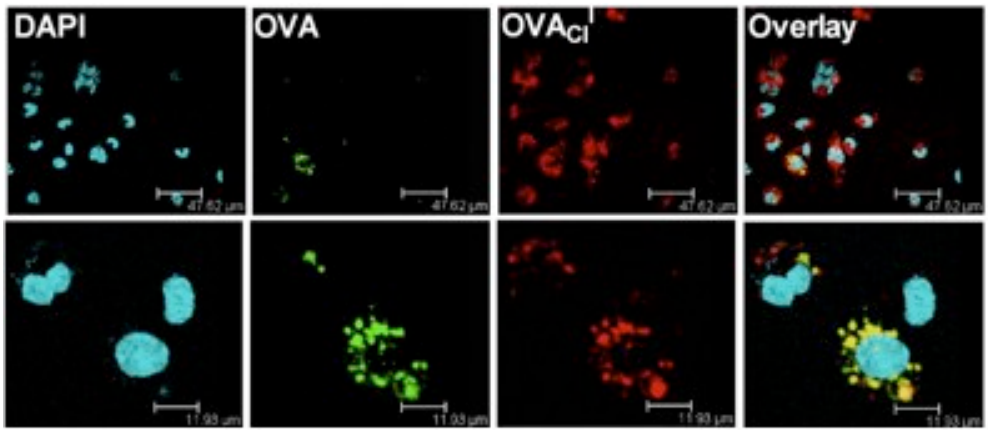


Figure 5.4 OVA and OVA_{Cl}^{I} co-localized inside the same DCs compartment.

Bone marrow derived DCs were incubated with OVA-FITC and OVA_{Cl}^{I}-TRITC (100 µg/ml) for 2 hours at 37^{o}C. Excess antigen was removed, and the cells were fixed in 3.8% formaldehyde. Fluorescence was analyzed by confocal microscopy. The figure shows a representative low power (upper row) image, and a high power image (lower row) of a cell, which has taken up both antigens. The experiment was repeated three times.

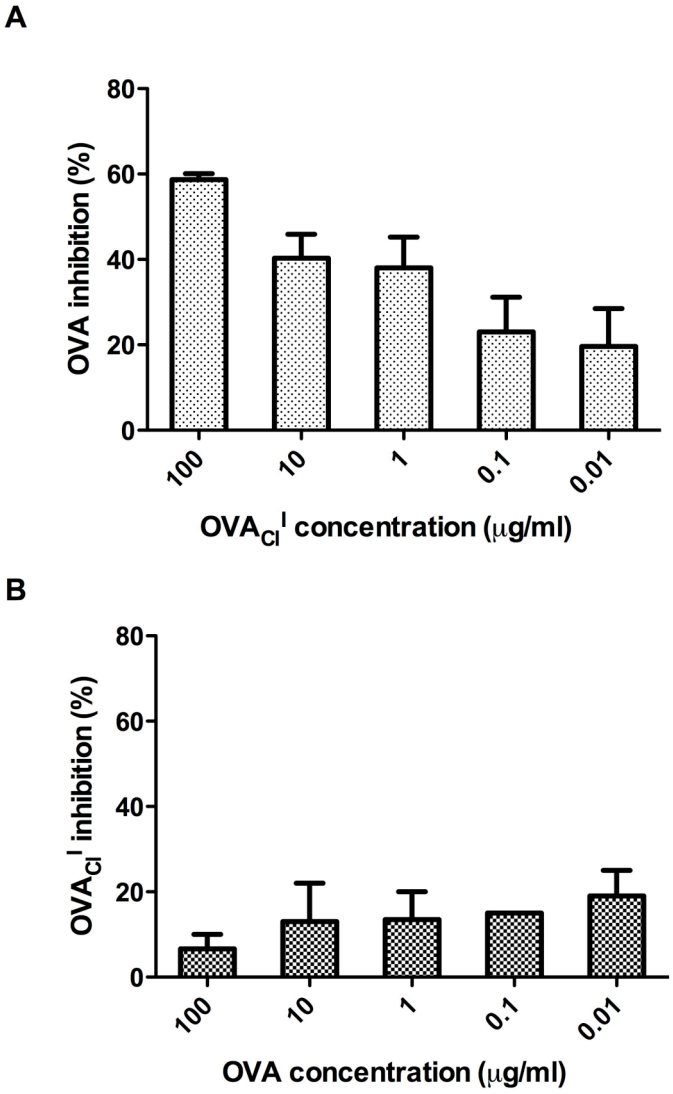


Figure 5.5 OVA$_{Cl}^{I}$ inhibits OVA uptake *in vitro.*

*Bone marrow derived DCs were incubated for 2 hours at 37°C with **A.** 100µg/ml of OVA-FITC and with decreasing concentrations of OVA$_{Cl}^{I}$ or, with **B.** 100µg/ml OVA$_{Cl}^{I}$-FITC and decreasing concentrations of OVA. Excess of antigen was removed and the cells were fixed with 3.8% formaldehyde. The figure shows the percentage of uptake inhibition relative to DC incubated without a competitor. Error bars represent SEM. The experiment was repeated three times.*

5.3.3 HOCL MODIFICATION ENHANCED ANTIGEN UPTAKE. IN VIVO STUDIES.

The uptake of OVA and OVA_{Cl}^{I} was also investigated *in vivo*. The same amounts (1 mg) of OVA or, OVA_{Cl}^{I} labeled with FITC were administered intravenously into a mouse. After the indicated time splenic $CD11c^{+}$ DC were isolated (**Fig 5.6**) and stained for CD8 surface marker. The amount of the antigen within the DCs was assessed by flow cytometry analysis (**Fig 5.7A**). Both OVA and OVA_{Cl}^{I} could be detected within splenic $CD11c^{+}$ DC. There was a moderate enhancement in the uptake of OVA_{Cl}^{I} at 30 minutes post injection in both $CD8^{+}$ and $CD8^{-}$ DCs subpopulations. Interestingly, 18 hours post injection, the situation reversed and the amount of OVA_{Cl}^{I} was closer to the background level (i.e. DCs with no OVA injected). Both antigens were confirmed to be present inside the DCs by confocal image analysis (**Fig 5.7B**).

The immunogenic properties of OVA_{Cl}^{I}-primed DCs were then tested *ex vivo*. TRC transgenic OT-II T cells were co-cultured with splenic $CD11^{+}$ DCs isolated from mice injected with 1 mg of OVA or, OVA_{Cl}^{I}. As a control OT-II T cells were co-cultured with splenic $CD11c^{+}$ DCs from a control mouse (injected with HBSS only) together with exogenously added OVA or, OVA_{Cl}^{I}**.** No difference in the T cell response was observed for decreasing number of splenic DCs isolated from mice injected with OVA or, OVA_{Cl}^{I} isolated after 30 minutes (**Fig 5.8A**) or, 18 hours (**Fig5.8C**). However, splenic DCs from a control mouse isolated after 30 minutes (**Fig 5.8B**) or, 18 hours (**Fig 5.8D**) co-cultured with decreasing concentrations of OVA_{Cl}^{I} induced good T cell responses, while native OVA was presented very poorly

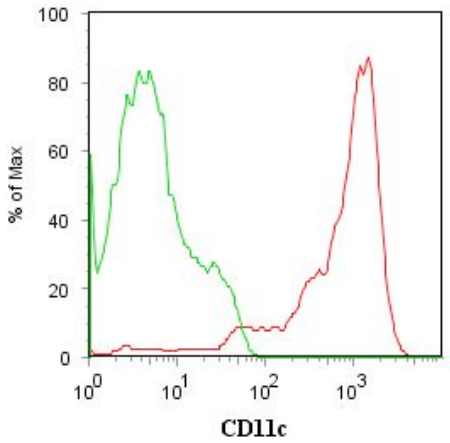


Figure 5.6 Purification of CD11c$^+$ splenic DCs.

Splenic DCc were isolated as described in the Material and Methods section. After enrichment with CD11c$^+$ beads, DCs were stained for CD11c surface marker and analyzed by flow cytometry. DCs were >90% positive for CD11c (red) compared to the isotype control (green).

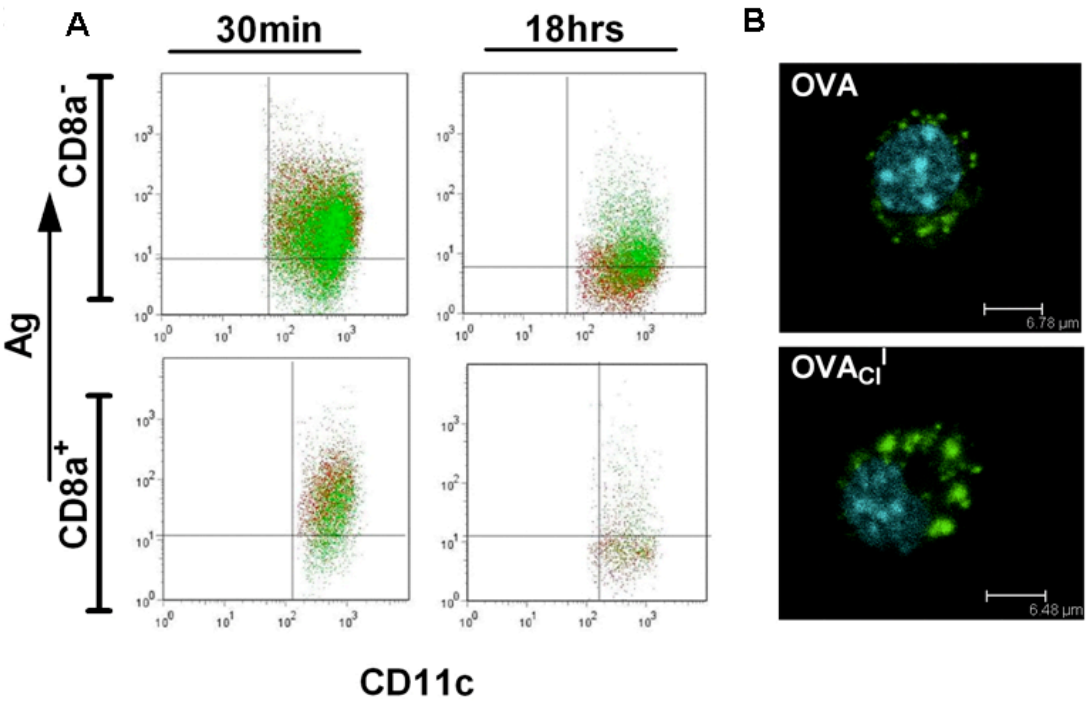


Figure 5.7 Modification with HOCl facilitates uptake and processing of OVA *in vivo.*

A. *C57Bl mice were injected intravenously with OVA or OVA_{Cl}^{I} conjugated with FITC (1 mg/mouse). Spleen cells were collected after 30 minutes or 18 hours, and $CD11c^{+}$ DCs were isolated by magnetic bead enrichment. The splenic DCs were stained for CD11c and CD8a cell surface marker. The plots show an overlay of OVA-FITC (green) and OVA_{Cl}^{I}-FITC (red) for $CD8a^{+}$ and $CD8^{-}$ DCs.* ***B.*** *Confocal analysis of cells from the 30 minutes time point to demonstrate intracellular localisation of OVA and OVA_{Cl}^{I} (gree) and nucleus (blue). The figure is the representative data obtained from one mouse. The experiment was done three times each time three mice per group were used.*

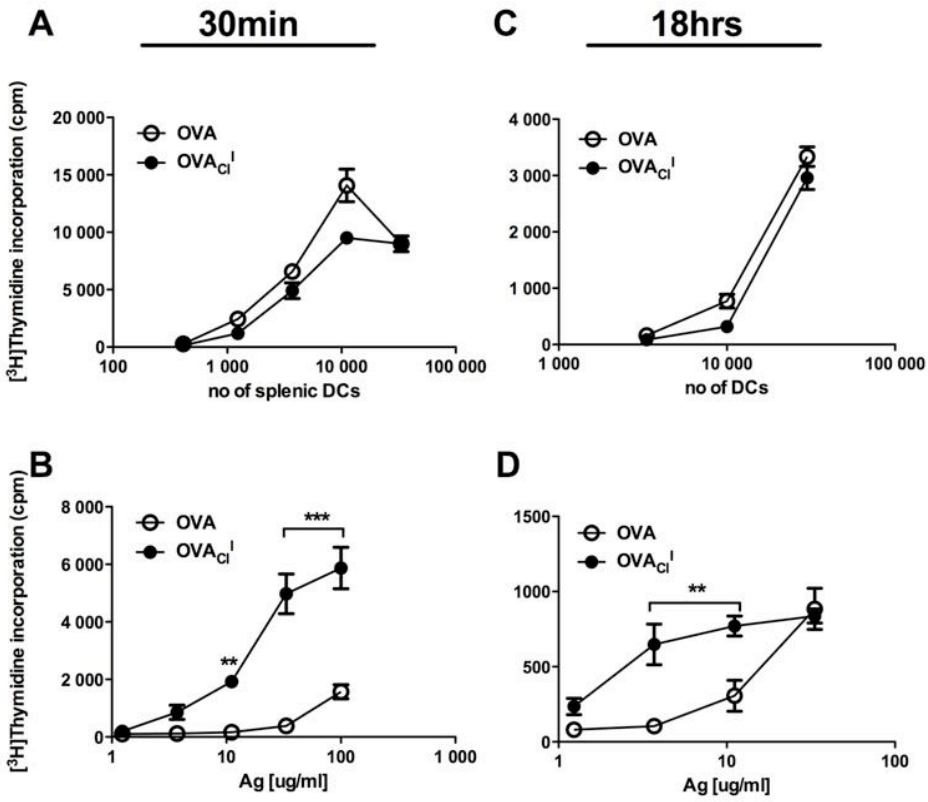


Figure 5.8 Splenic DCs process and present HOCl modified OVA.

HOCl modification enhances T cells response in an antigen dose dependent manner. C57Bl mice were injected intravenously with OVA or OVA_{Cl}^{I} conjugated with FITC (1 mg/mouse) or an equal volume of HBSS. Spleen cells were collected after 30 minutes or 18 hours, and CD11c DCs were isolated by magnetic bead enrichment. ***A.*** *and* ***C.*** *purified splenic DCs from mice injected with OVA-FITC or, OVA_{Cl}^{I}-FITC were plated in 96-well plates ($3x10^4$/well), 3-fold serial dilutions were made. DCs were co-cultured with TCR transgenic OT-II T cells ($2x10^4$/well).* ***B.*** *and* ***D.*** *purified splenic DCs ($5x10^3$/well) from a mouse injected with HBSS were co-cultured with decreasing concentrations of OVA or OVA_{Cl}^{I} and TCR transgenic OT-II T cells ($2x10^4$/well). The graphs show OT-II proliferation measured as thymidine incorporation. The results are presented as the average ± SEM [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiment was repeated three times. Statistical analysis was performed using the two-way ANOVA test. **P<0.01, ***P<0.001.*

5.4 DISCUSSION

In this chapter we have focused on one possible mechanism that may contribute to HOCl induced enhanced antigen immunogenicity. Following the studies on oxLDL that showed the presence of a receptor for the oxidised form, as compared to un-oxidised (Sawamura, 1997) we can speculate that: firstly. OVA_{Cl}^{I} uptake is receptor-mediated; and secondly, that there is a specific receptor recognizing HOCl-modified proteins. In this chapter we discuss the first aspect only.

First of all, in order to follow antigen uptake we labeled both proteins with either FITC or TRITC fluorescent dye. We chose these two dyes because of their distinctive excitation and emission spectra (FITC 488/520nm, TRITC 544/572nm) that allowed combined use to follow the uptake of both antigens. They are also both relatively simple to couple to ovalbumin via free amino groups on the target protein side chain.

As fluorescent dyes are hydrophobic, and therefore may bind non-covalently to the protein, we removed all non-specifically bound dye by gel filtration using a sephadex-25 buffer exchange column.

Despite the similar chemical structure the ratio of dye to the protein was lower for TRITC than for FITC dye. Therefore in case of experiments where both antigens were delivered simultaneously, we have used both combinations of colors (OVA-FITC and OVA_{Cl}^{I}-TRITC or OVA-TRITC and OVA_{Cl}^{I}-FITC). Thus we were able to ensure that the results we obtained were not attributable to the differences between

the specific labels.

Importantly, the immune recognition properties were unchanged after labeling and antigens induced comparable T cells response to unlabeled equivalents.

It has already been shown, by our laboratory that cells treated with HOCl are taken up more efficiently by DCs than non-treated cells (Chiang et al., 2006). In our studies, we demonstrated the enhanced uptake of HOCl-modified glycoprotein antigen.

We have explored the nature of antigen uptake using three independent approaches: firstly, antigen uptake competition between native and modified antigen measured by flow cytometry; secondly, antigen uptake at low temperature measured by flow cytometry; thirdly, direct measurements of binding strength between antigen and cell evaluated by atomic force microscopy.

Firstly, antigen uptake was quantified by flow cytometry. DCs were incubated with decreasing concentration of antigen. Both native and oxidized antigens were taken up by DCs but native OVA uptake fell sharply at 10µg/ml while OVA_{Cl}^{I} uptake was detectable at concentrations as low as 0.1µg/ml (similar to the active concentrations observed in the functional assay, chapter 3).

Secondly, in order to test the nature of OVA uptake we have carried out experiments at 4°C. It has been already shown that at low temperature DCs endocytic activity is blocked and thus also the ability to take up antigens (Sallusto, 1995). Indeed, at 4°C we have observed significantly reduced antigen

uptake. Native OVA binding was completely inhibited, while OVA_{Cl}^{I} binding was significantly inhibited compared to 37°C. These results suggest that the measured signal for OVA/OVA_{Cl}^{I} uptake at 37°C is due primarily to internalized OVA/OVA_{Cl}^{I}, a hypothesis supported by the confocal images. However, from these experiments we cannot rule out that cell surface binding is also temperature dependent. In future, it would also be interesting to determine the extent of cell surface versus internalised protein uptake at 37°C. One approach, for example, would be to compare the antigen uptake of DCs treated with trypsin to non-treated cells. Trypsin at low concentrations can be used to strip off cell surface proteins from the cell, and thus distinguish between OVA/OVA_{Cl}^{I} that is and is not internalised inside the DCs.

Thirdly, in order to investigate in detail the nature of OVA_{Cl}^{I} binding to the cell we have used atomic force microscopy (AFM) tool, which allows direct measurement of single molecule binding events. The AFM measurement was done by our collaborators Dr. S. Zapotoczny and Prof. M. Nowakowska from Jagiellonian University Krakow.

AFM allows the measuring of forces between single molecules. The principle of AFM is a use of a sharp tip at the end of flexible cantilever that probes the surface of the sample (**Fig 5.9**). The precise measurements are achieved by a computer-controlled piezotube.

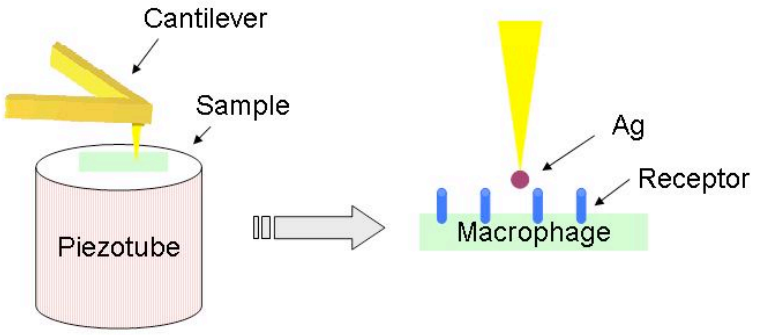


Figure 5.9 Principle of the AFM measurement.

OVA protein molecules were immobilised on the tip of cantilever, and macrophages were immobilised on the glass surface. Force distance measurements were performed by cycling the sample on a piezotube in the vertical direction.

AFM is used in structural biology, immunology and pharmacology; it is an excellent tool to study topological features and mechanistic characteristics of the sample, but is also provides information about binding properties of biological systems. For example it has been used in the characterisation of RAG synaptic complex (Shlyakhtenko et al., 2009); in study of the Syk kinase dependent signalling in DC mediated via crystals binding to lipid membrane (Ng et al., 2008); and in studies on the interaction of human rhinovirus interaction with cell surface receptors (Rankl et al., 2008).

In our model, a direct measurement using AFM showed that enhanced antigen uptake is in fact associated with stronger binding to the cell membrane (**Appendix A**). Our collaborators analyzed the binding of OVA, OVA_{Cl}^{I} or, OVA_{Cl}^{H} to fixed macrophages and observed that only HOCl-modify OVA bound strongly to

the cell surface**.** The data also showed enhanced ligand/receptor binding (expressed as adhesion probability) for OVA_{Cl}^{H}. Since we have not observed enhanced T cell responses to OVA_{Cl}^{H} in the functional assay, it is difficult to explain these data. However, as already mentioned in chapter 3 we have postulated that the lack of response to OVA_{Cl}^{H} is due to HOCl induced modification of amino acid residues that do not facilitate enhanced antigen immunogenicity. Thus one possible explanation is that in the functional assay OVA_{Cl}^{H} is processed inside the cell and because of induced amino acid modification the T cell response is altered. In other words the lack of response to OVA_{Cl}^{H} is due to altered processing machinery. But in AFM measurement we analyze direct interaction between antigen and receptor omitting the antigen processing machinery (OVA_{Cl}^{H} is bound to the tip of cantilever) and therefore "facilitating" the interaction between the ligand (OVA_{Cl}^{H}) and the receptor on macrophages.

The other interesting observation we have obtained from AFM measurement was that pre-incubation of macrophages with increasing concentrations of OVA_{Cl}^{I} led to reduction of the adhesion probability factor (**Appendix A**). This demonstrates the saturation effect where the receptor which mediates OVA_{Cl}^{I} uptake is dose dependently engaged by increasing concentrations of OVA_{Cl}^{I}. In conclusion AFM data strongly suggest that enhanced binding of OVA_{Cl}^{I} to macrophages is predominantly receptor-mediated.

Finally, we further explored antigen uptake in the competition experiment. When both antigens were added simultaneously we observed strong competition

between OVA_{CI}^{I} and OVA, resulting in a dose dependent inhibitory effect on OVA uptake. Such competitive effects are usually interpreted to imply a specific receptor mediated process.

We also analyzed the samples by confocal imaging. However, this analysis was qualitative rather than quantitative. We used this technique to resolve two issues. Firstly, whether the antigen is in fact inside the cell, and secondly, whether both antigens (native and modified) localize inside the cell together or separately.

The first issue is important because, as discussed above, by analysing antigen uptake by flow cytometry only, we cannot exclude the possibility that antigen we measure is bound to the cell surface. The aim of this experiment was to explore if enhanced uptake could explain the enhanced immunogenicity of modified OVA, and therefore we are only interested in the antigen that is taken up and processed by the DCs and not remaining at the cell surface.

We have observed that both antigens localize in close proximity to the nucleus within cytoplasm organelles (endosome-like) confirming the intracellular localization of antigen. However, for future work it will be necessary to determine in which compartments the antigens are more exactly and if both antigens are processed with the same kinetics. Specific antibodies against such proteins like EEA1 (Mu et al., 1995) and Rab5 (Zerial and McBride, 2001) can be used to visualize early endosomes and against Rab7 (Feng et al., 1995) and Lamp1 (Fukuda, 1991) to visualize late endosomes and lysosomes. Moreover, additional information about the endocytic pathway that controls antigen uptake can be obtained from some

already well characterized proteins. Two examples are, AcLDL that are taken up via scavenger receptors and transferrins taken up via transferrin receptors. Simultaneous incubation of OVA/OVA_{Cl}^{I} with one of the proteins (both labeled with fluorochrome) can tell us if both proteins co-localize within the same cell compartment and thus probably go through the same endocytic pathway; and can also tell as if they are processed with the same kinetic (that is if within the time both proteins are in the same compartment).

An interesting alternative would be also to track captured antigen using quantum dots. These small molecules are semiconductor nanocrystals that emit photostable fluorescence (spectrum depends on the dot size) and can be conjugated with proteins. Because of their optical properties, two-photon microscopy can be used to visualize quantum dot-protein conjugates. A recent study has shown DCs in the lymph nodes containing quantum dots captured after subcutaneous injection (Sen et al., 2008). However, this prospective technique has a disadvantage. OVA conjugated with quantum dots induced enhanced T cell responses comparing to OVA alone. Therefore it is possible that quantum dot binding mimics the mechanism observed using HOCl-modified antigen.

Previous experiments were done *in vitro* using bone marrow derived DCs. Since this experimental model does not mirror the process that occurs *in vivo* entirely we have focused on two new experimental approaches. First we examined *in vivo* antigen uptake by splenic DCs, and second we examined the *ex vivo* capability of splenic DCs to stimulate T cells. We have focused on splenic DCs because the

antigen was injected intravenously and via this route soluble antigen is delivered through the bloodstream to the spleen. However, in future it would be interesting to inject antigen via other routes, for example intraperitoneally or subcutaneously, and to isolate DCs from draining lymph nodes.

Work with splenic mouse DCs leads to two important problems. Firstly, the procedure used to isolate DCs can favor a particular subtype of DCs. We have isolated cells by the preparation of single cell suspension using the enzymatic digestion method (collagenase); cells were enriched by incubation with CD11c beads, followed by positive selection. The enrichment step was required to avoid difficulties with FACS analysis.

Several other isolation procedures are described: isolation of low-density DCs after centrifugation in metrizamid, isolation of transiently adherent DCs after short-term culture (Walton et al., 2006), negative selection by lineage-specific monoclonal antibodies (Pulendran et al., 1997; Vremec, 2000). The method we have chosen has several advantages, it is fast and easy and we have obtained high numbers of fresh $CD11c^+$ cells. However, we have not fractionated the several sub-populations of DCs described earlier.

The second important problem is the presence of non-lymphoid, autofluorescent cells in the preparation. According to the published literature their surface markers expression (high F4/80 and Cd11b and low CD11c) and morphology resemble macrophages (Vremec, 2000). Macrophages are well known potent phagocytic cells and hence, will also contribute to antigen uptake that may result in diminished

availability of antigen for DCs.

However, as we have already mentioned, DCs were enriched on $CD11c^+$ beads resulting in a >90% $CD11c^+$ population. Hence, the contamination with autofluorescent cells in analyzed samples should be in fact minimal. Indeed, we have observed the presence of very small subpopulation of autofluorescence cells that could be detected in all fluorescence channels.

There are two possible options to eliminate this signal. First is to gate out the autofluorescent cells using a channel, which is not used for analysis. However, this can only eliminate the problem partially; the autofluorescent signal is usually strong enough to overlap with other channels leading to significant problems with color compensation. The second option is to sort the autofluorescent cells. This method is much more effective but it is time consuming. Since we had several samples to analyze it was technically impossible to sort the cells. However, we were able to significantly eliminate the autofluorescence using the extra channel.

We analyzed *in vivo* antigen uptake and *ex vivo* capability to stimulate T cells, at two different time points, early after 30 minutes and late after 18 hours. We also focused on two major DCs subtypes $CD8^+$ and $CD8^-$ (including $CD4^+$ and $CD4^-$). As described in chapter 1, $CD8^+$ and $CD8^-$ DCs are specialized differently. That is, $CD8^+$ are more efficient in antigen presentation on MHC class I complex, while $CD8^-$ are specialized in presentation on MHC class II complex (Dudziak et al., 2007). Moreover, several studies have already demonstrated *in vivo* antigen uptake by both DCs subtypes. Just to mention few examples, uptake of necrotic cells material

(Sancho et al., 2009), apoptotic cells (Morelli et al., 2003), tumor cells (Iyoda et al., 2002) and malaria-infected red blood cells (Ing et al., 2006) was demonstrated to be a selectively property of $CD8^+$ DCs subtype. While for example uptake of stomach self-antigen (Scheinecker et al., 2002) and air-derived allergens (Wikstrom et al., 2006) was shown to be triggered by $CD8^-$ DCs subtype.

We found that within the first minutes post injection, both antigens were taken up very efficiently by splenic $CD8^+$ and $CD8^-$ DCs. However, in contrast to what we have observed *in vitro* OVA_{Cl}^I uptake was only slightly more efficient than OVA. This difference can be explained by the different antigen concentration used for *in vitro* and *in vivo* assays. We observed significantly enhanced OVA_{Cl}^I uptake *in vitro* for the antigen concentration ranging from 1 to 10 µg/ml. However, in order to follow the antigen uptake *in vivo*, we have injected as much as 1mg of the antigen. Thus, it is probable that the lack of difference in the antigen uptake *in vivo* is due to too high concentration of injected antigen. Nevertheless, when we used lower antigen concentrations, the antigen uptake was not detected (data not shown). The second, more plausible explanation is the possibility that OVA_{Cl}^I was taken up by other types of phagocytic cells e.g. macrophages that have not been screened by flow cytometry.

Longer time points gave us even more unexpected results. After 18 hours post injection we observed a rapid disappearance of OVA_{Cl}^I, while native OVA was still present within DCs at high levels. OVA_{Cl}^I uptake by both $CD8^+$ and $CD8^-$ DCs was almost at the level of background (DCs from mouse injected with HBSS).

Two mechanisms may contribute to these effects. Firstly, that OVA_{Cl}^{I} is processed and degraded faster than native OVA; and secondly, that the FITC molecule is removed/quenched more rapidly from OVA_{Cl}^{I}. Both mechanisms may occur and this question requires further studies on antigen proteolysis.

Enhanced antigen susceptibility to proteolysis has been already discussed in the literature. Delamarre et al (2006) have studied *in vivo* how antigen's susceptibility to proteolysis correlates with antigen's immunogenicity. They have used two different antigens (each in a more stable or more susceptible form to lysosomal proteolysis) and different routs of injections (intraperitoneal, intradermal and intramuscular) and demonstrated that more stable forms of both antigens induce stronger IgG response and stronger T cells priming.

However, when we studied the potential of splenic DCs isolated from mice injected with OVA or OVA_{Cl}^{I}, to stimulate *ex vivo* TCR OT-II T cell, we did have not observe significant difference between oxidised and native antigens, although splenic DCs behaved like bone marrow DCs in *in vitro* assays. Both induced a good T cell response. A number of parameters, such as the route of immunisation, the precise dose of antigen, or the subpopulation of DCs processing/presenting antigen may explain these discrepancies. For example, it has been demonstrated that DEC-205 is expressed by $CD8^{+}$ subtype DCs but not by $CD8^{-}$ subtype (Henri et al., 2001), while dectin-1 is specifically expressed by $CD8^{-}CD4^{-}$ subtype (Carter et al., 2006).

These variables may be analysed in future studies.

5.5 CONCLUSIONS.

- Labeling of OVA and OVA_{Cl}^{I} with FITC or, TRITC dye does not change protein antigenicity.
- HOCl modification leads to **enhanced receptor-mediated antigen uptake *in vitro.***
- **OVA and OVA_{Cl}^{I} co-localize** within the same compartment inside the DCs.
- HOCl modification leads to early **enhanced antigen uptake *in vivo.***
- OVA_{Cl}^{I} is taken up by both splenic DC subtypes $CD8^{+}$ and $CD8^{-}$.

CHAPTER 6 MECHANISMS THAT MEDIATE THE ENHANCED IMMUNOGENICITY OF A HOCL-MODIFIED PROTEIN ANTIGEN.

6.1 INTRODUCTION

HOCl treatment of proteins results in the formation of aldehydes and disappearance of chloramines (discussed in chapter 3). Previous studies have proposed that insertion of carbonyl groups into proteins results in enhanced immunogenic properties (Allison, 2000; Moghaddam, 2006).

The treatment of proteins with high concentrations of HOCl is known to result in the alteration of amino acid side-chains together with some protein fragmentation and aggregation (Hawkins et al., 2003) that may lead to altered proteolysis. As discussed in chapter 3 and chapter 5 the difference in susceptibility of OVA and OVA_{Cl}^{I} to proteolysis is potentially an important factor that can mediate the action of HOCl. Briefly, the 323-339 sequence recognized by the DO11.10 and OT-II T cells is buried within the hydrophobic core of the protein and thus extensive proteolysis is required to release this sequence for further processing. Enhanced proteolysis could therefore result in enhanced processing and presentation of this epitope. In addition the *in vivo* studies, which demonstrated disappearance of OVA_{Cl}^{I}-FITC after 18 hours post-injection, could also be explained by faster processing and degradation of the antigen.

OVA is a glycoprotein that has one carbohydrate side chain attached to asparagine (Asn293) residue (**Fig. 6.1**). The side chain is composed mainly of mannose (Man) and N-acetylglucosamine (GlcNAc) residues. The end of the chain is terminated by sialic acid moieties (SA) (Lattova et al., 2004) although the extent of sialation remains unclear.

Components of the side chain may be important in immunogenicity. For example mannose residues are recognized by the mannose receptor that has been demonstrated to play an important role in uptake of antigen for presentation on MHC class II (Sallusto, 1995) and cross-presentation on MHC class I (Burgdorf, 2006).

Another example of the role of the side chain is GlcNAc. It was demonstrated that GlcNA was a target for HOCl modification and this leads to the production of a whole range of oxidative products (Rees et al., 2005) that can interfere with a protein structure.

The sialic acid component has also been implicated; presence of sialic acid prevents protein recognition by scavenger receptor (Thomas et al., 2008), and thus may mask for example recognition of mannose structures by the mannose receptor.

In chapter 5 we demonstrated that OVA_{Cl}^{I} uptake is mediated by a receptor. There are many potential candidates that are known to be involved in antigen presentation (discussed in detail in chapter 1). In this project we have tested one, the scavenger receptor lectin-like oxidized low-density lipoprotein receptor 1

(LOX-1). LOX-1 is expressed on DCs and macrophages (Delneste et al., 2002) and has been shown to be involved not only in the recognition of oxLDL but also in antigen cross-presentation leading to protective anti-tumoral response (Delneste et al., 2002).

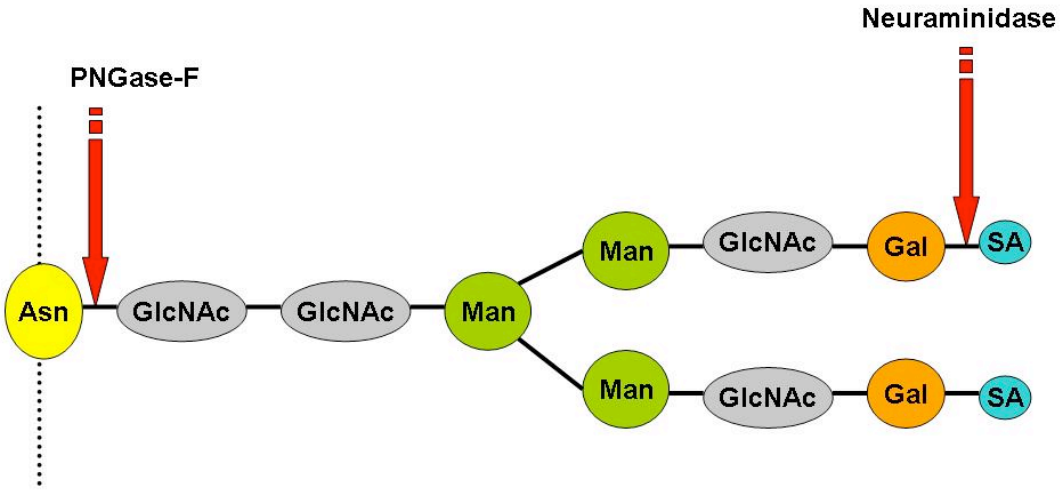


Figure 6.1 Schematic representation of OVA carbohydrate side chain.

Asn *– asparagines residue;* ***Man*** *– mannose;* ***GlcNAc*** *- N-acetylglucosamine;* ***Gal*** *- galactose;* ***SA****- sialic acid.*

6.2 OBJECTIVES

Based upon this range of possibilities, there were several objectives of this part of the investigation:

- To test possible methods to reverse the enhanced immunogenicity of oxidized OVA, specifically by reduction of carbonyl groups and removal of free chloramines.
- To test susceptibility of OVA and OVA_{Cl}^{I} to proteolysis by trypsin as an example of serine protease and cathepsin E as an example of aspartic protease.
- To test the processing of OVA_{Cl}^{I} in the presence of the cathepsin D/E inhibitor, MPC6.
- To test the T cell response to oxidative modification of OVA_{Cl}^{I} after removal of the carbohydrate side chain and of sialic acid.
- To test the involvement of the LOX-1 scavenger receptor in OVA_{Cl}^{I} recognition.

6.3 RESULTS

6.3.1 ENHANCED PROCESSING DOES NOT REQUIRE ALDEHYDE GROUPS, OR CHLORAMINES.

To investigate the role of aldehydes, oxidation of OVA with HOCl was followed by reaction with excess of reducing agent - sodium borohydride ($NaBH_4$), which reduces any aldehydes to alcohols (**Fig 6.2A**). The number of aldehyde groups in the $NaBH_4$ treated sample was comparable to native OVA (and almost zero). Oxidation, as expected induced the formation of aldehydes. However, reduction of aldehydes in the OVA_{Cl}^{I} sample did not reverse the enhancement in the T cell response. Bone marrow derived DCs co-cultured with decreasing concentrations of $NaBH_4$-treated OVA_{Cl}^{I} (redOVA_{Cl}^{I}) induced strong DO11.10 T cell hybridoma responses (**Fig 6.2C**). Hence, in contrast to previous studies aldehydes groups were not required for the HOCl induced enhancement of the response.

Chloramines are another major product of protein oxidation and thus their importance was also examined. Chloramines were reduced back to free amines by reaction with methionine (**Fig 6.2B**). The number of free, accessible amines in OVA_{Cl}^{I} sample after methionine treatment was even greater that in native OVA, suggesting that oxidation altered protein conformation allowing access to previously inaccessible amine side chains. Like aldehyde groups, chloramines were also not required for HOCl induced enhancement of the response, since reduction with methionine did not reverse enhanced T cell responses (**Fig 6.2C**). OVA peptide 323-339 was used as a control (**Fig 6.2D**).

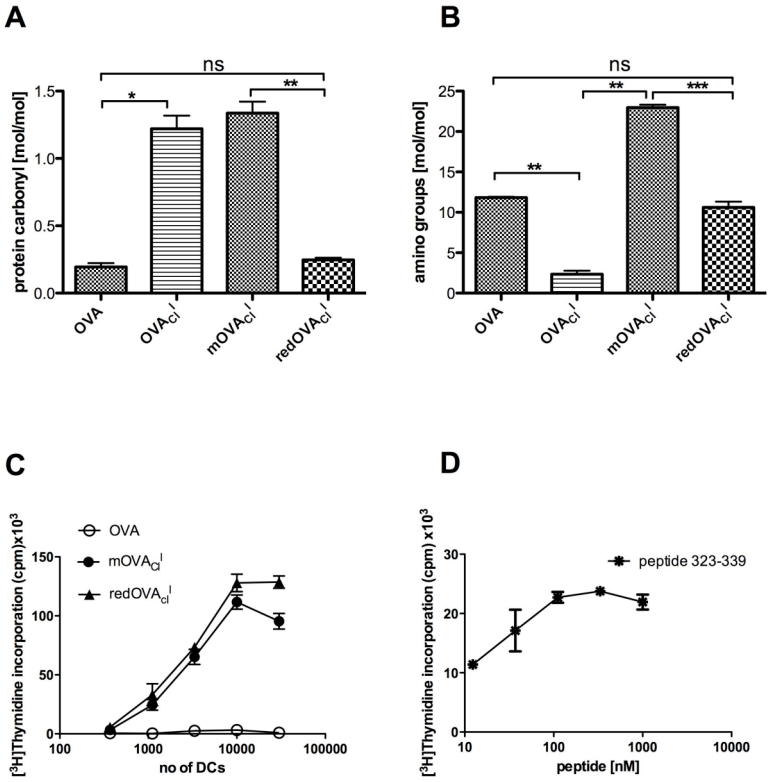


Figure 6.2 Aldehyde groups and chloramines do not contribute to the enhanced immunogenicity of OVA_{Cl}^{I}.

OVA was incubated with HOCl at an intermediate ratio (OVA_{Cl}^{I}). Aldehyde groups were reduced with 50mM $NaBH_4$ ($redOVA_{Cl}^{I}$). Free chloramines were removed with methionine (50mM) ($mOVA_{Cl}^{I}$). ***A.*** *Aldehyde groups and* ***B.*** *free amine groups were quantified as described in the Material and Methods section using DNPH and TNBS, respectively. The results are presented as the average of three independent experiments ±SEM.* ***C.*** *and* ***D.*** *DO11.10 hybridoma cells ($2x10^4$/well) were incubated with decreasing concentrations of OVA, $mOVA_{Cl}^{I}$, $redOVA_{Cl}^{I}$ or p.323-339 and purified bone marrow derived DC ($5x10^3$/well). Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of CTLL2 indicator cell line. The results are presented as the average ±SD [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiments were performed three times. Statistical analysis was performed using the Student's t test. *P<0.05, **P<0.01, ***P<0.001, ns non-significant.*

6.3.2 HOCL OXIDATION DOES NOT INDUCE PROTEIN FRAGMENTATION BUT ENHANCES OVA SUSCEPTIBILITY TO PROTEOLYSIS.

To explore whether HOCl induced OVA fragmentation and therefore bypassed the requirement for antigen processing, two methods were used.

Firstly, OVA_{Cl}^{I} presentation was tested using fixation to abolish DCs processing. Bone marrow derived DCs were fixed by brief exposure to glutaraldehyde (Shimonkevitz et al., 1984) before being exposed to decreasing concentration of antigen. DO11.10 T cell responses was assessed in the functional assay. Presentation of both OVA and OVA_{Cl}^{I} was abolished completely and only synthetic peptide 323-339 stimulated specific T cell responses (**Fig 6.3A**).

Secondly, the fragmentation of OVA_{Cl}^{I} was analyzed directly by SDS Page. No small molecular weight protein fragments could be detected below the main OVA band (45kDa), even when silver staining was used (**Fig 6.3B**). Thus, intermediate concentration of HOCl does not induce protein fragmentation.

Native OVA is highly resistant to proteolytic degradation (Tani, 1997). Therefore the susceptibility of OVA_{Cl}^{I} to two human proteolytic enzymes, trypsin and cathepsin E was tested. Trypsin is a serine protease that specifically cleaves at the carboxylic side of lysine and arginine residues. Cathepsin E is an aspartic protease required for OVA processing by DC (Chain et al., 2005). In contrast to native protein, OVA_{Cl}^{I} was found to be digested readily by both enzymes (**Fig 6.4A** and **B**).

Even though protein oxidation with HOCl induced increased susceptibility to proteolysis by cathepsin E, OVA_{Cl}^{I} processing and presentation was efficiently blocked by the cathepsin D/E inhibitor MPC6 that was shown to inhibit OVA (**Fig 6.5**)

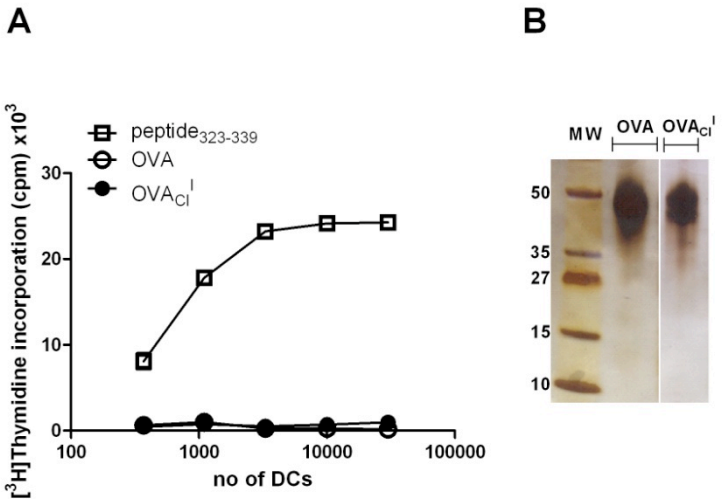


Figure 6.3 An intermediate concentration of HOCl does not induce protein fragmentation.

A. *Purified bone marrow derived DCs were fixed with 0.05% glutaraldehyde. Glutaraldehyde was removed by washing cells twice in HBSS. DC (10^5/sample) were incubated for 1 hour with OVA (5μM), OVA_{Cl}^{I} (5μM) or synthetic OVA peptide p.323-339 (1μg/ml). The antigen was removed by washing cells twice in HBSS. 3-fold serial dilutions of the cell were made and the DCs were co-cultured in 96-well plates with DO11.10 hybridomas ($2x10^4$/well). Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line. The results are presented as the average ± SD [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiment was repeated twice.* ***B.*** *OVA and OVA_{Cl}^{I} (5 μg) were fractionated by 4-12% gradient PAGE, and the gel was stained with silver stain as described in Material and Methods. No small molecular weight fragments were visible.*

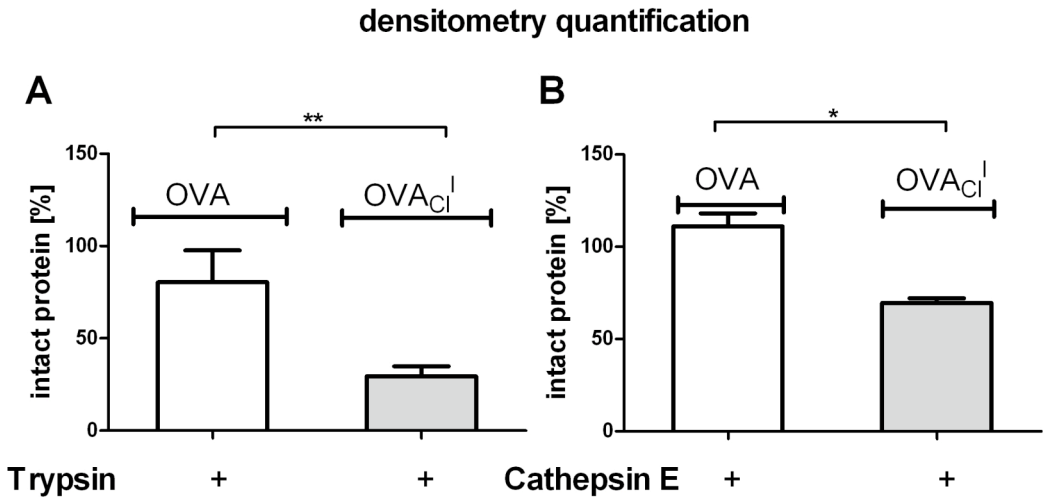


Figure 6.4 Modification with HOCl increases the proteolytic susceptibility of OVA.

*OVA and OVA$_{Cl}^{I}$ were digested with trypsin **A.** and cathepsin E **B.** as described in Materials and Methods, and analyzed by 4-12% gradient denaturing PAGE. Histograms show the degree of proteolysis quantified by comparing the intensity of the OVA bands before and after digestion using densitometry as described in Material and Methods. Statistical analysis was performed using a 2-tailed Student's t test. * P<0.05, **P<0.01.*

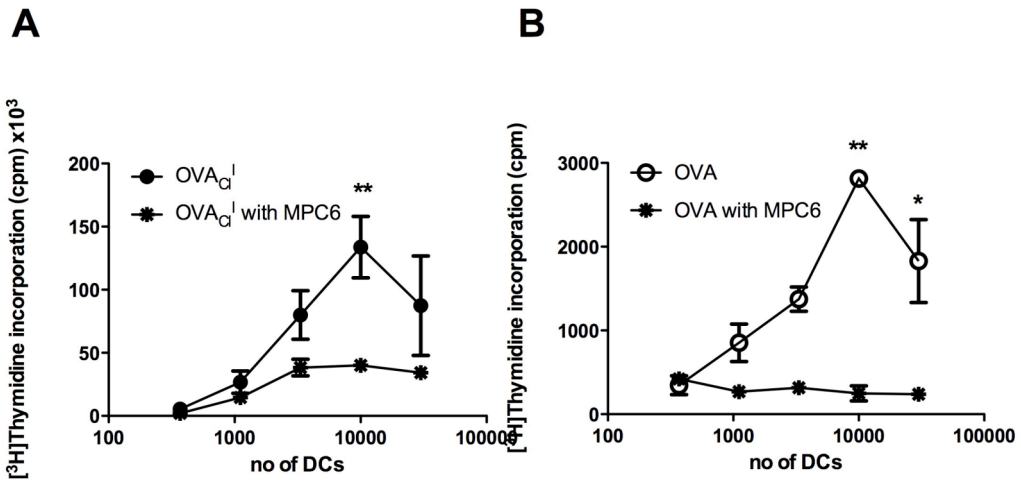


Figure 6.5 HOCl-modified OVA still requires processing by aspartic proteinases digestion.

*OVA or OVA_{Cl}^{l} (4µM) were incubated with purified bone marrow DCs in the presence or absence of the aspartic proteinase inhibitor MPC6 (10µM) for 2 hours. Excess antigen and inhibitor were removed by washing cells twice in HBSS and the DCs were fixed in gluteradehyde. Fixed DCs (10^5/sample) were co-cultured with DO11.10 T cell hybridomas ($2x10^4$/well). Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line. Results are presented as the average ± SD [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiment was repeated three times. Statistical analysis was performed using the two-way ANOVA test. *P<0.05, **P<0.01.*

6.3.3 ENHANCEMENT OF HOCL-MODIFIED OVA REQUIRES THE CARBOHYDRATE SIDE CHAIN.

6.3.3.1 REMOVAL OF SIALIC ACID RESIDUES.

The OVA protein contains a single N-linked carbohydrate side chain attached to the asparagine residue at position 293. The carbohydrate side chain may be capped by sialic acid residues. The T cell response in the absence of either the whole carbohydrate side chain or, only the sialic acid moiety was studied.

The presence of sialic acid moieties on OVA and OVA_{Cl}^{I} was confirmed using MAL (Maackia amurensis) lectin, specifically recognizing 2,3-sialic acid residues (**Fig 6.6A**) Increasing concentrations of OVA and OVA_{Cl}^{I} were tested. Sialic acid was detected for all samples however; a slightly lower amount was detected for OVA_{Cl}^{I}.

In order to selectively remove sialic acid moieties, OVA and OVA_{Cl}^{I} were treated with neuraminidase (NA). Because the molecular weight of NA (43kDa) is similar to OVA (45kDa), and NA is also recognized by MAL lectin, the removal of sialic acid was difficult to confirm by western blot. Therefore NA enzymatic activity was tested using as a positive control fetuin (68kDa) (**Fig 6.6B**).

The TCR transgenic OT-II T cell response to OVA treated with NA (OVA + NA) and OVA_{Cl}^{I} treated with NA (OVA_{Cl}^{I} + NA) was tested. Treatment with NA had no effect on the presentation of either OVA or, OVA_{Cl}^{I} (**Fig 6.6C**).

6.3.3.2 REMOVAL OF CARBOHYDRATE SIDE CHAIN.

OVA and OVA_{Cl}^{I} were both treated with endoglycosidase F (PNGase-F), which removes the entire carbohydrate side chain (**Fig 6.1**). Protein deglycosylation was

confirmed by SDS PAGE; the protein without the carbohydrate chain had a reduced molecular weight compared to the native protein (**Fig 6.7A**).

The contribution of the carbohydrate side chain in HOCl-induced enhanced antigen immunogenicity was assessed in the functional assay. Bone marrow derived DCs were incubated with decreasing concentrations of OVA treated with PNGase-F (OVA + PNGase-F) or, OVA_{Cl}^{I} treated with PNGase-F (OVA_{Cl}^{I} + PNGase-F) or, with their equivalents without PNGase-F and cultured with TCR transgenic OT-II T cells. Enzyme treatment had no effect on the presentation of OVA, but abolished completely the enhancement observed after HOCl-treatment (**Fig 6.7B**). PNGase-F had no toxic effect on the cells; p.323-339 pre-treated with PNGase-F or the reaction buffer induced as good T cell response as non-treated control (**Fig 6.7C**).

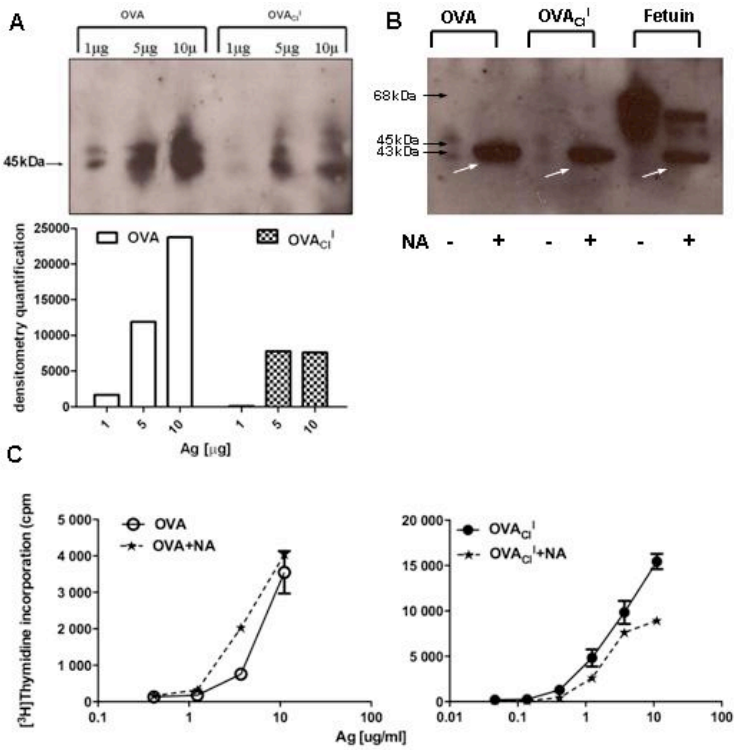


Figure 6.6 Sialic acid moieties do not contribute to the enhanced immunogenicity effect of HOCl.

A. *The presence of sialic acid moieties was tested. OVA and OVA$_{Cl}^{I}$ were fractionated by 4-12% gradient denaturing PAGE and analyzed by Lectin blot using MAL lectin specific for the 2,3-sialic acid residue. The histogram shows the amount of sialic acid, quantified by the intensity of the OVA and OVA$_{Cl}^{I}$ bands using densitometry.* ***B.*** *Proteins were treated with neuraminidase (NA) as described in the Material and Methods section. Enzyme treated (+) and untreated (-) samples were analysed by 4-12% gradient denaturing PAGE (1µg/well) and analyzed by Lectin blot using MAL lectin. White arrows show bands for NA (43kDa).* ***C.*** *TCR transgenic OT-II cells (2x10^4/well) were incubated with decreasing concentrations of OVA or OVA$_{Cl}^{I}$ pre-treated with NA and purified bone marrow derived DCs (5x10^3/well). Proliferation of OT-II cells was measured after 18 hours as thymidine incorporation. The results are presented as the average ± SD [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiments were repeated three times.*

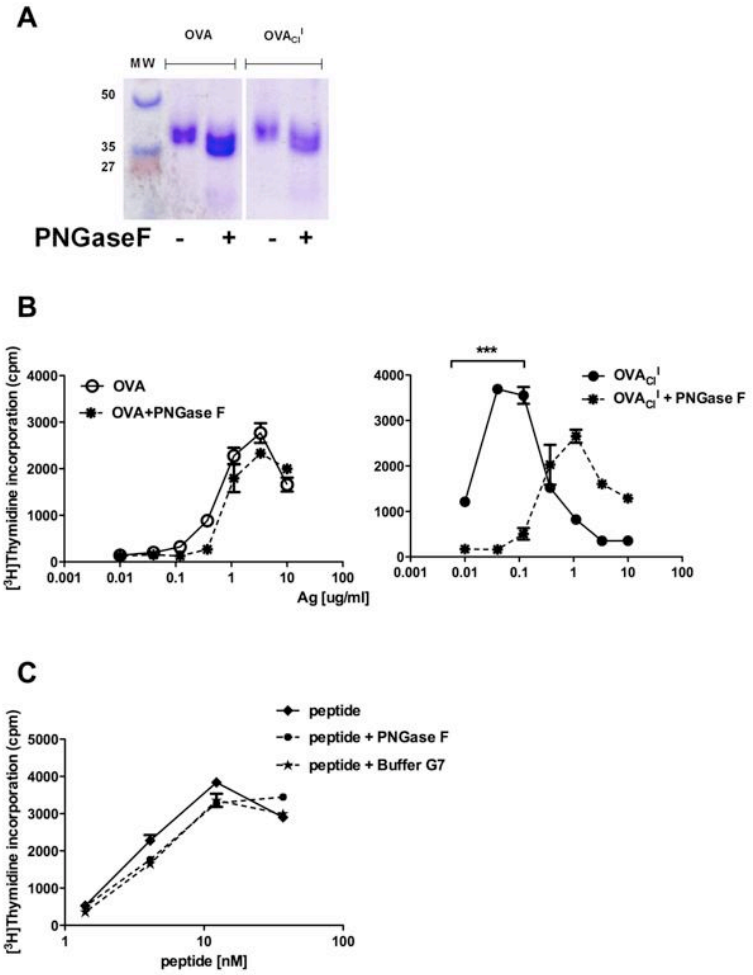


Figure 6.7 Protein deglycosylation abolished enhanced immunogenicity effect of HOCl.

A. *Proteins were treated with PNGaseF as described in the Material and Methods section. Enzyme treated (+) and untreated (-) samples were analysed by 8% PAGE and stained with Coomassie blue.* ***B.*** *TCR transgenic OT-II cells (2x10^4/well) were incubated with decreasing concentrations of OVA or OVA$_{Cl}^{I}$ pre-treated with PNGase F and purified bone marrow derived DCs (5x10^3/well). Proliferation of OT-II cells was measured after 18 hours as thymidine incorporation. The results are presented as the average ± SD [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures.* ***C.*** *as for B. but cells were incubated with OVA p.323-339 pre-treated with PNGaseF (1000 units) or G7 reaction buffer (2µl). The experiments were repeated three times. Statistical analysis was performed using the two-way ANOVA test. ***P<0.001.*

The role of the carbohydrate side chain in mediating enhanced immunogenicity was tested further using a naturally non-glycosylated protein HEL. HEL, like OVA was treated with three different molar rations of HOCl to protein: low (HEL_{Cl}^{L}), intermediate (HEL_{Cl}^{I}) and high (HEL_{Cl}^{H}). The immunogenic property of oxidized HEL was assessed in the functional assay and the response of Ad71 T cell hybridomas (specific for HEL) was tested. Bone marrow derived DCs were incubated with increasing concentration of HEL, HEL_{Cl}^{L}, HEL_{Cl}^{I} and HEL_{Cl}^{H} (**Fig 6.9**). AD71 responded to native HEL, HEL_{Cl}^{L} and HEL_{Cl}^{I} at the moderate concentration (30-300 μg/ml), and in contrast to OVA_{Cl}^{I}, no enhancement in T cell responses was observed. Similarly to OVA_{Cl}^{H}, HEL_{Cl}^{H} was presented poorly.

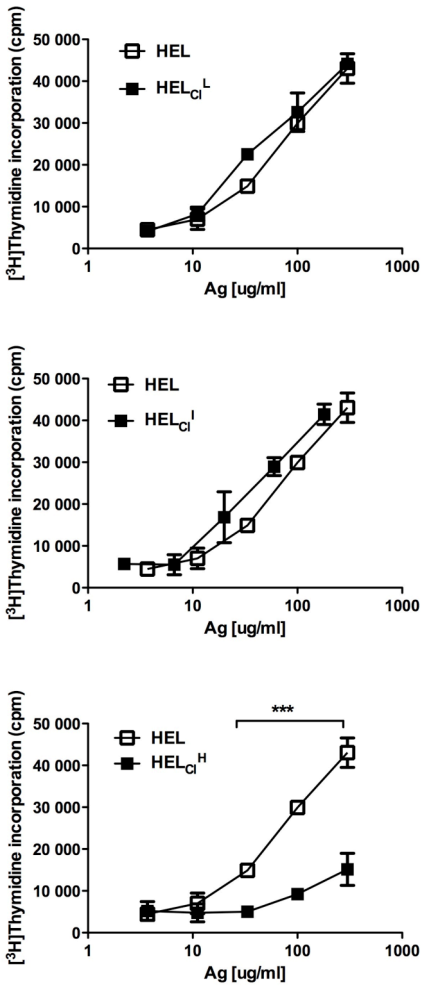


Figure 6.8 Oxidation of nonglycosylated protein antigen does not induce an enhanced T cell response.

*HEL was oxidized with low (HEL_{Cl}^{L}), intermediate (HEL_{Cl}^{I}) and high (HEL_{Cl}^{H}) HOCl concentrations. Ad71 T cell hybridomas ($5x10^4$/well) were incubated with decreasing concentrations of HEL, HEL_{Cl}^{L}, HEL_{Cl}^{I} or HEL_{Cl}^{H} and purified bone marrow derived DCs. Supernatants were collected after 24 hours. The graph shows IL-2 release measured as thymidine incorporation of the CTLL2 indicator cell line. The results are presented as the average ± SD [^{3}H]Thymidine incorporation (c.p.m.) of the triplicate cultures. The experiment was repeated three times. Statistical analysis was performed using the two-way ANOVA test. ***P<0.001.*

6.3.4 ENHANCED UPTAKE OF OVA_{CL}^{I} IS MEDIATED BY LECTIN-LIKE OXIDIZED LDL (LOX-1) SCAVENGER RECEPTOR.

The contribution of LOX-1 in mediating enhanced OVA_{Cl}^{I} uptake was explored using CHO cells stably transfected with the LOX-1 receptor.

CHO-LOX-1 cells were incubated with different concentrations of FITC labeled OVA or FITC labeled OVA_{Cl}^{I}, excess antigen was removed and antigen uptake was assayed by flow cytometry. As a control CHO cells were used. The expression of LOX-1 enhanced uptake of both OVA and OVA_{Cl}^{I}, but OVA_{Cl}^{I} was taken up more efficiently (**Fig 6.9A**).

The samples were also analyzed by confocal imaging. CHO-LOX-1 cells or, CHO cells were incubated with 100 µg/ml of OVA-FITC or OVA_{Cl}^{I}-FITC for 30 minutes and stained for LOX-1 receptor (**Fig 6.9B**). Both antigens are taken up by CHO-LOX-1 cells (green) that expressed high levels of LOX-1 receptor (red). No uptake was observed by CHO cells.

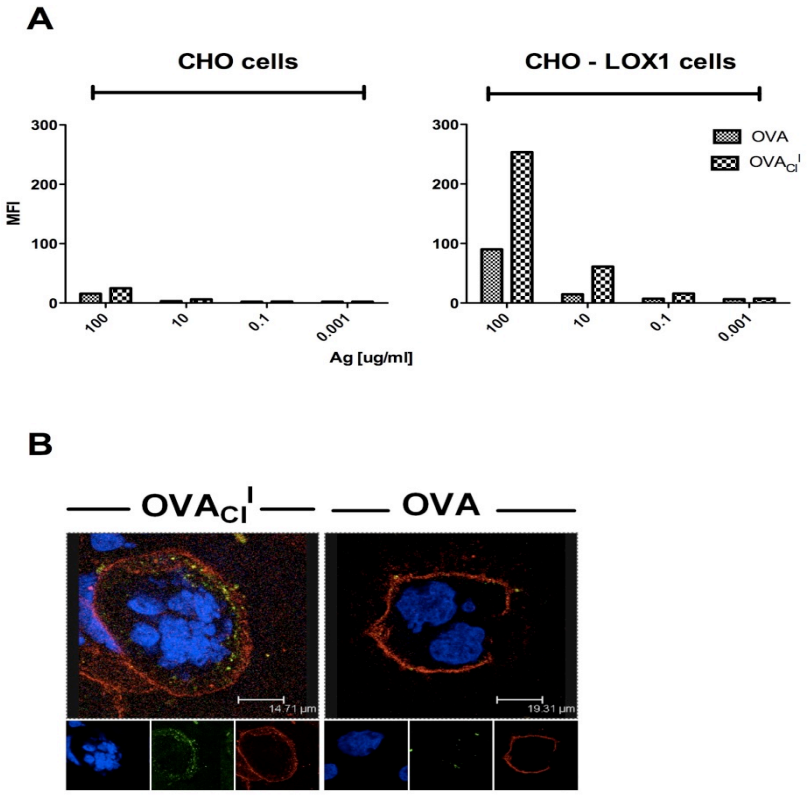


Figure 6.9 Scavenger receptor LOX-1 contribute to enhanced OVA$_{Cl}^{I}$ uptake.

CHO cells expressing the LOX-1 scavenger receptor, or control untransfected cells were incubated with the indicated concentrations of OVA-FITC or OVA$_{Cl}^{I}$-FITC. After 30 minutes the excess antigen was removed, and the cells were fixed in 3.8% formaldehyde and analyzed by flow cytometry. The results are expressed as median fluorescent intensity in the FITC channel and are one representative from three independent experiments performed. ***B.*** *CHO cells expressing LOX-1 were incubated with OVA-FITC or OVA$_{Cl}^{I}$–FITC (100µg/ml) for 30 minutes. Excess antigen was removed; the cells were fixed in 3.8% formaldehyde and stained for LOX-1 (red) and DAPI (blue). Fluorescence was analyzed by confocal microscopy. The figure shows a representative high power image of a cell which has taken up antigen (green). The experiment was repeated three times.*

6.4 DISCUSSION

In this final chapter we focused on the identification of possible mechanisms that mediate the enhanced immunogenicity of a HOCl-modified protein antigen. We studied four possible alternatives, discussed in detail below.

Firstly, we studied the immunological outcome of chemical modification induced by oxidation, in particular contribution of aldehyde groups. In chapter 3 we demonstrated the dose-dependent formation of aldehyde groups after treatment with HOCl.

In the context of previous work it has been demonstrated that insertion of carbonyl groups (for example by treatment with glycolaldehyde) into the protein results in its enhanced immunogenic properties compared to native protein (Allison, 2000). In addition, more recent studies by Moghaddam et al (2006) have shown that treatment of proteins by formaldehyde, which has been used in preparation of vaccines for many decades, can enhance the immune response via introduction of aldehyde groups into the protein. In order to test the contribution of aldehyde in our model, we have compared the T cell response to oxidized OVA (containing aldehyde) with the response to reduced OVA (aldehyde free). Reduced OVA was first oxidized with HOCl and then treated with reducing agent. Thus the only difference between these two samples was the presence of aldehyde groups. The samples were reduced with sodium borohydride ($NaBH_4$). The reaction leads to formation of alcohols as shown.

R-CHO + $NaBH_4$ → R-OH + NaH_2BO_3 [reaction 6.1]

In contrast to the formaldehyde and glycolaldehyde-treated proteins, in our studies aldehyde groups do not mediate enhanced immunogenicity effect of HOCl since both oxidized and reduced OVA enhanced the T cell response to the 323-339 epitope. As we discussed in chapter 5 OVA_{Cl}^{I} induces both enhanced T cell responses and *in vivo* humoral responses; while the study on glycolaldehyde treated proteins was mainly focused on antibody response and hence may relate to different mechanism. In addition, the previous studies have focused on protocols, which add multiple aldehyde groups to proteins; in contrast, reaction with HOCl induced the formation of only approximately one aldehyde group per protein molecule. The importance of the aldehyde groups under these conditions may be limited.

Secondly, a number of recent studies have demonstrated an association between antigen susceptibility to proteolysis and antigen immunogenicity. This subject was discussed briefly in chapter 5.

We demonstrated that OVA_{Cl}^{I} still requires proteolytic digestion inside the cell in order to be presented since treatment with HOCl does not lead to protein fragmentation. In addition OVA_{Cl}^{I} processing is similar to native OVA (since processing of both was blocked effectively by cathepsins D/E inhibitor).

Enhanced susceptibility to proteinases is not associated invariably with increased immunogenicity. For example Delamarre et al. (2006) showed that antigens which

are more resistant to proteolysis are in fact more immunogenic. Earlier studies from another group showed that aspartyl-protease activity leads to release of epitopes for T cell recognition but that antigen presentation was in fact improved when a major aspartyl protease, Cathepsin D, was inhibited (Catherine et al., 2005). Moreover, DCs were shown to be less active proteolytically than macrophages (Chain, 1986). One possible mechanism that may play a role is an active alkalisation system in the phagosomal compartments of DCs that increases pH and leads to deactivation of some proteases (Delamarre et al., 2005). Finally, a recent study has demonstrated that short-course treatment of mice with chloroquine improved $CD8^+$ T cell priming against soluble antigen (Garulli et al., 2008). Chloroquine is a lysosomotropic agent that inhibits lysosomal acidifications and de facto inhibits protease activity. Hence, these studies support the view that limiting proteolysis can promote T cell responses.

However, many examples showing the requirement for proteolysis in antigen presentation have been published. For example, studies on processing and presentation of tetanus toxin C fragment (TTCF) showed that TTCF is cleaved by asapragine endopeptidase (AEP) and that there are a limited number of residues that when cleaved by AEP lead to optimal TTCF presentation. In a more recent paper Moss et al (2007) further explore this observation. They found that low concentrations of AEP lead to cleavage in three residues while high concentrations cleaved at six residues and that this correlates with enhanced antigen presentation. Moreover, when TTCF was firstly treated with AEP, and then AEP activity was

blocked and pre-digested TTCF was treated with either cathepsin L or S or D, they observed differences in the digestion pattern between TTCF treated with low and high concentrations of AEP. Finally they found that single enzymes frequently dominate antigen processing.

In this study OVA_{Cl}^{I} was digested by trypsin and cathepsin E enzymes more readily than native OVA. As shown in chapter 5 after 18 hours post i.v. injection OVA_{Cl}^{I}-FITC was not detected in splenic DC. From these two experiments one cannot conclude that enhanced immunogenicity of OVA_{Cl}^{I} is caused by enhanced antigen proteolysis, since a direct causal link between proteolysis and presentation is not shown. In addition, as discussed in chapter 5, FITC may be quenched/removed from the antigen. Furthermore enhanced digestion was tested only for two examples of proteolytic enzymes.

However, based on the literature discussed above, this is a possible mechanism that can explain this data. OVA_{Cl}^{I} (in contrary to native OVA) may be more accessible for digestion by, for example AEP, that "unlocks" (a term taken from (Moss et al., 2007)) the OVA_{Cl}^{I} antigen, and this initiates the possibility of further proteolytic events which lead to enhanced OVA_{Cl}^{I} presentation and T cell activation. There are two reasons why OVA_{Cl}^{I} is more accessible for digestion. Firstly, after treatment with HOCl the structure is more "relaxed" and "open" and thus more cleavage residues are accessible for the enzyme. Secondly, the pattern of N-glycosylation has been shown to regulate protein proteolysis (Cheng-Chih et al., 2009). Since we demonstrated that HOCl modifies the carbohydrate side chain, it is possible that

altered N-glycosylation leads to altered accessibility to proteolysis.

Thirdly, the contribution of protein sialation was investigated. Sialation is a known mechanism that can control ligand-receptor interactions and this happens by blocking an interaction between the two molecules. Two examples, the endothelial hyaluronan receptor LYVE-1 was silenced functionally (*in vitro* and *in vivo*) by reversible terminal sialation which led to inhibition of hyaluronan binding (Nightingale et al., 2009). In another study on pathogenesis of Haemophilus influenzae infection sialation of LPS from H. influenzae protects from killing by human sera (Derek et al., 1999).

Using specific lectin staining demonstrated the presence of sialic acid residues however, less were observed on OVA_{Cl}^{I} than OVA. Therefore, we hypothesised that oxidation with HOCl leads to removal of sialic acid residues and hence make the OVA_{Cl}^{I} carbohydrate chain more accessible for DCs recognition and as a consequence enhanced antigen uptake. This hypothesis could be tested by specific removal of sialic acid residues from native OVA, which would be expected to induce enhanced T cell responses. Response to OVA_{Cl}^{I} treated with enzyme should not be changed.

Native OVA was treated with neuraminidase (NA) that cleaves off α2-3 and α2-6 linked sialic acid residues. Removal of sialic acid can be confirmed by Western blot, isolectric focusing or mass spectrometry. Western blotting was used, but because OVA and NA molecular weights are very similar, the data obtained were

unclear. Nevertheless, as shown for the control protein fetuin, even 10 times less NA than was used for OVA and OVA_{Cl}^{I} successfully removed sialic acid residues. Further studies using isolectric focusing would be required to fully confirm the efficiency of sialic acid removal from the OVA.

The processing/presentation of NA-treated samples was examined. Neuraminidase treated and control native OVA induced comparable T cell responses. Thus, even though reduced amounts of sialic acid residues for OVA_{Cl}^{I} were observed the loss of this chemical moiety is not associated with enhanced immunogenicity.

Fourthly, we decided to test whether the carbohydrate side chain contributes to HOCl induced immunogenicity. As shown in **Fig 6.1** one of the major components of the side chain is N-acetylglucosamine (GlcNAc), which is also an important target for HOCl (Rees et al., 2003; Rees et al., 2005). The reaction leads to formation a whole range of oxidized products, such as N-chlorosulphonamides and chloramides. Thus the carbohydrate side chain in OVA_{Cl}^{I} may contain a very high density of oxidation products, which may play a key role in uptake, processing and presentation of modified antigen.

Two experimental approaches were used to test this hypothesis. Firstly, the entire carbohydrate side chain from OVA_{Cl}^{I} was enzymatically removed (the response to deglycosylated antigen was measured); and secondly we tested the T cell response to a naturally non-glycosylated protein antigen treated with HOCl.

Carbohydrate residues can be specifically removed from proteins by endoglycosidase PNGase F. PNGase F cleaves between the asparagine residues and inner GlcNAc as shown on **Fig 6.1** (Maley et al., 1989) leading to complete removal of carbohydrate from the protein. The T cell response to OVA and OVA_{Cl}^{I} after treatment with PNGase F showed that removal of the carbohydrate residues from native OVA did not have an effect compared to the non-treated control. However, removal of carbohydrate residue from oxidized OVA abolished the enhanced T cell response completely. Deglycosylated OVA_{Cl}^{I} induced comparable T cell responses to native OVA.

This result suggests strongly that protein glycosylation plays a critical role in the HOCl effect. Thus, we hypothesis that treatment with HOCl enhances antigen immunogenicity only when modified protein antigen is glycosylated.

To support this view T cell responses to a naturally unglycosylated protein antigen, HEL were tested. HEL is a smaller protein (~14kD) than OVA but is also commonly used as a model antigen. There are several available T cell hybridomas that recognize different HEL epitopes (Drakesmith et al., 1998). In our study, we tested the response to HEL epitope 71-85 recognized by AD71 T cell hybridomas. Importantly, the epitope we chose did not contain methonine residues and therefore the possibility that lack of enhanced T cell responses is due to oxidized methonine residues could not be excluded (discussed in chapter 3).

HEL was oxidized with the same HOCl ratios as OVA: low, intermediate and high. Samples were tested in the functional assay. Oxidation (in all three HOCl ratios) did not induce enhanced T cell responses compared to native OVA.

Thus these results substantiate that (1) the **carbohydrate side chain** is an important target for HOCl and (2) its **oxidative derivatives do enhance the immunogenic effect of protein antigens.**

The effect of oxidation of the carbohydrate side chain can be mediated by both extrinsic as well intrinsic processes. An example of an extrinsic process could be, when the modified carbohydrate chain mediates antigen uptake by a specific, potentially new receptor, or by the same receptor but with increased affinity. An example of an intrinsic process could be altered glycosylation that influences proteolysis (or another other step of antigen processing) inside the cell.

Glycosylated antigens are recognized specifically by C-type lectin receptors (CLRs) and scavenger receptors (SRs), leading to processing and presentation of antigens on MHC class I and II molecules. CLRs are a large and diverse group of receptors, expressed by several types of cells, characterized by a large variety of functions (in detail discussed in chapter 1.). In **table 6.1** we summarized some that are expressed by DCs and are known to mediate enhanced T cell responses.

CLR	Function	Reference
Mannose receptor	Antigen cross-presentation and activation of $CD8^+$ T cells	(Burgdorf, 2006)
Human DC-SIGN (expressed in transgenic mouse)	Strong $CD4^+$ T cell response to OVA modified with glycan that targets DC-SIGN (Lewis X and B oligosaccharides)	(Singh et al., 2009)
MGL	Specifically expressed on immature DCs; binds GalNAc-polymers	(Denda-Nagai et al., 2002)
Mouse MGL2	Specifically expressed on dermal DCs; induction of contact hypersensitivity reaction	(Kumamoto et al., 2009)
Mouse DCAR1	Strong $CD8^+$ and $CD4^+$ T cell response	(Kaden et al., 2009)
Dectin-1	Enhanced $CD4^+$ T cell response	(Carter et al., 2006)

Table 6.1 CLRs mediate an enhance T cell response.

The list of potential candidates that recognize HOCl-modified glycoprotein antigens is long. In addition some of these receptors recognize similar or, even the same carbohydrate structures. For example mannose receptor and DC-SIGN both recognize mannosylated proteins. Thus, inhibition or competition studies cannot be used in this model because blocking OVA_{Cl}^{I} uptake with mannose could be due to competition for either the mannose or the DC-SIGN receptor. Therefore, a better experimental approach would be a transgenic approach using receptor-knock out mouse and combing this with detail proteomics studies.

The second group of receptors that were shown to bind glycosylated products are scavenger receptors (SRs). The majority of studies focus on SRs functions in atherogenesis, but there is much evidence that SRs are involved in the uptake of both apoptotic cells and pathogens (see chapter 1). There are also several examples where SRs mediate enhanced antigen uptake and induce enhanced T cell response. (1) Maleylated proteins were shown to be more immunogenic than unmodified equivalents (Abraham et al., 1995); (2) Enhance uptake of AGE-modified OVA was demonstrated to be mediated by SR type A I/II (Ilchmann et al.); (3) CLA1 and CLA2 (human analog of rodent CD36, SB-BI/II) increased by several fold uptake of various bacteria (Vishnyakova et al., 2006); (4) Increased uptake and enhanced specific $CD8^{+}$ T cell response was observed for antigens bound to α2-macroglobulin, a ligand of scavenger receptor CD91/LRP (Binder et al., 2001; Chu and Pizzo, 1993; Hart et al., 2004); (5) LOX-1 SR was demonstrated to mediate antigen cross-presentation (Delneste et al., 2002).

The LOX-1 receptor is expressed by both murine and human DCs and was shown to be involved in recognition of oxidized products, oxidized LDL. The CHO cells stably transfected with LOX-1 receptor were incubated with fluorescently labelled antigen and showed that LOX-1 can mediate enhanced OVA_{Cl}^{I} uptake, compared to native OVA. This result, however, does not demonstrate that LOX-1 is important for the enhanced presentation of oxidized OVA by DCs.

Taken as whole, the data suggest that the scavenger receptor LOX-1 contributes to enhanced immunogenicity of HOCl-treated OVA, but further studies are required to extend these findings and define the full set of molecules mediating uptake and processing of oxidized proteins.

6.5 CONCLUSIONS

- Neither aldehyde nor chloramine groups are essential for the enhanced T cell response to OVA_{Cl}^{I}.
- Oxidation of OVA at intermediate levels of HOCl does not produce OVA fragments, which could be loaded directly on MHC class II molecules.
- OVA_{Cl}^{I} is digested more readily by both serine proteases (e.g. trypsin) and aspartic proteases (e.g. cathepsin E enzymes), but the contribution of this effect to enhanced presentation remains unclear.
- Enhancement of immunogenicity by HOCl requires the presence of a carbohydrate side chain, and is not mediated by sialic acid destruction.
- The scavenger receptor LOX-1 can contribute to enhanced uptake of OVA_{Cl}^{I} although that does not exclude involvement of other receptors.

CHAPTER 7 SUMMARY.

This study demonstrates the immunogenic effect of HOCl on protein antigens. It shows that HOCl enhances the T cell response to the model antigen OVA and thus facilitates the processing and presentation of HOCl-modified protein via the MHC class II pathway. The mechanism of enhancement is shown to be independent from TLRs signaling and does not lead to DCs maturation. On the other hand, enhancement is mediated by the N-linked carbohydrate side chain of OVA. HOCl-modified protein is taken up more efficiently by DCs and is also digested more readily by serine and aspartic proteases. Uptake is receptor mediated and the scavenger receptor LOX-1 is one of the potential candidates that could be involved in this mechanism.

7.1 IMMUNOGENIC EFFECT OF HOCL.

This study was based on earlier findings (Marcinkiewicz, 1991, 1992) which demonstrated that protein chlorination facilitates recognition by APCs and thus enhances the immune response.

The data presented in chapter 3 shown that, indeed optimal concentration of HOCl enhanced immunogenicity of model antigen OVA. Both DCs and macrophages processed and presented oxidized OVA to DO11.10 and TCR transgenic OT-II cells with increased efficiency. However, the effect was strictly epitope specific and the response to other T cell hybridomas MF2.D9 and 3DO18.3 was completely abolished.

For DO11.10 hybridoma cells, HOCl treatment was necessary to obtain antigen responses in a physiological concentration range. Therefore, presence of HOCl *in vivo* at the side of inflammation may be an important factor mediating immune responses to both foreign- and self-antigen.

The immunogenic effect of HOCl is concentration dependent and hence antigen treated with high ratio of HOCl reduces responses to all tested T cells. The mechanism controlling HOCl reaction with antigen is unknown but data presented in chapter 3 raise several possibilities, for example high concentrations of HOCl may lead to protein fragmentation, destruction of more accessible epitopes or modification of different amino groups.

The immunogenic effect of HOCl is independent of TLRs signaling. These findings with HOCl modified antigen differ from published earlier data (Blander and Medzhitov, 2006) indicating a requirement of TLRs pathway in efficient antigen processing of unmodified OVA.

Our study has at least two important implications. Firstly, treatment with HOCl may be useful as a new vaccination strategy. Secondly, HOCl may be involved in triggering or enhancing the response to autoantigens as observed in chronic autoimmune disorders.

The second part of this study focused on identification of potential mechanisms contributing to the immunogenic effect of HOCl. As shown in chapter 5 the uptake of HOCl-modified OVA is more efficient than that of native OVA. Moreover AFM

measurements together with competition studies have shown that the uptake is receptor mediated and is due to stronger protein binding to the cell surface. Scavenger receptor LOX-1 has been identified as one of the specific receptors recognizing HOCl-modified OVA. Nevertheless other receptors cannot be excluded, in particular receptors recognizing components of carbohydrate side chains. Indeed, the immunogenic effect of HOCl seems to depend on the type of modified protein, and thus it has been demonstrated that presence of the N-linked carobohydrate side chain attached to the protein is critical.

Finally, HOCl enhances the sensitivity of OVA to proteolysis by serine and aspartic proteases. Because other recent study presented an opposite hypothesis (Delamarre et al., 2006), that is that enhanced antigen proteolysis decreases immunogenicity, the observation reported here needs to be further studied.

7.2 FUTURE WORK

The studies presented in this thesis suggest further future work that in general can be subdivided into three, main categories (1) biochemical studies, (2) *in vivo* studies and (3) antigen processing studies.

The biochemical studies would include identification of the amino acid modifications caused by HOCl in OVA and their relationship to immunogenicity. One approach would be spectrophotometrical studies of tyrosine and tryptophan groups of OVA epitopes that enhance T cell responses. Also the question of methionine groups needs to be resolved. That would require extensive screening of epitopes

with and without methionine residues. Furthermore in this study the HOCl effect was compared only to H_2O_2, but obviously other oxidants should be used for example products of nitrogenation.

The second groups of studies that can be further explored are *in vivo* studies. study demonstrated that we can observe an *in vivo* immunogenic effect of HOCl but there are open questions regarding the subtype of DCs controlling recognition and presentation of modified antigen and the kinetics of modified antigen processing. Moreover, only one route of injection was tested here. In the context of target vaccination this question is specifically important.

HOCl seems also to be a possible candidate as a vaccine adjuvant. It fulfills the major characteristic of adjuvants such as to boost specific immune response to antigens and low cost in use. For example in case of BCG vaccine, HOCl may be use as a killing agent for Mycobacterium, exposure of bacteria proteins to HOCl would lead to their modification and enhanced immunogenicity. A similar approach may be used in the case of virus vaccines where whole inactived virus is used. One of the methods to inactive virus is treatment of the live virus with chemicals for example with formaldehyde. HOCl may be use as an alternative. Moreover, inactived vaccines may lead to an increase risk of allergies, firstly due to high amounts of antigens that has to be used, and secondly because formaldehyde may lead to a contact allergy. However, the cause of the allergic reaction may vary. Antibiotics, egg proteins or gelatin often causes allergic reactions. Antibiotics are used in some vaccines, for example, polio vaccine includes streptomycin, neomycin and

polymyxin B. Egg proteins are components of influenza vaccine. Gelatin is found in measles-mumps-rubella (MMR), varicella and yellow fever vaccines. In this context HOCl is a very promising target, its strong immunogenic effect decreases the amount of antigen used; and since it is naturally present in humans (as a product of neutrophils) allergic side effects should be minimal.

The uptake studies are obviously related to identification of specific receptors, which bind HOCl-modified OVA. Here only the LOX-1 receptor was tested but broader receptor screening is required. This study would include *in vitro* screening of stably transfected cell lines with selected candidates, but also *in vivo* studies using knockout mice, for example mice, which do not express the mannose receptor. In order to obtain a list of selected candidates detailed studies of the carbohydrate side chain have to been done. As shown in chapter 6, the HOCl enhanced effect is true only for glycosylated proteins like OVA. Detail information about glycoproteins can be obtained for example by mass spectrometry using Matrix Assisted Laser Desorption Ionisation (MALDI) technique, or by less sophisticated methods combining lectin blot analysis followed by enzymatic treatment (as shown in chapter 6).

Finally, a lot of information could come from further studies on the cell biology of antigen processing. HOCl alters uptake and sensitivity of the antigen to proteolysis. Hence firstly studies should focus on clarifying the proportion of cell surface bound and internalized antigen; secondly, analysis of the lysosomal compartment containing internalized antigen in the context of active proteases. Other work

would include a comparison of native and modified OVA localization inside the different endocytic compartments and analysis of the kinetics with which antigens are processed through these compartments. Finally the processing of modified OVA *in vivo* requires further studies. For example, it remains unclear whether HOCl modification really leads to faster antigen processing or whether the disappearance of signal is merely the disappearance of the fluorescent tracking dye.

In this study only one glycosylated and one non-glycosylated antigen was tested. Because the immunogenic effect of HOCl seems to depend on several overlapping mechanisms, the final effect of HOCl modification may vary between antigens. Thus other protein antigens need to be tested.

7.3 CONCLUDING REMARKS.

The immunogenic effect of HOCl was firstly described more than 10 years ago but this early finding did not receive much attention. This study is a continuation of this earlier work and deals in detail with possible mechanisms controlling the immunogenic effect of HOCl. The study was done at different experimental levels including biochemical studies, *in vitro* analysis and *in vivo* analysis. HOCl or its derivatives may be a possible candidate for a new approach to adjuvanticity in the absence of TLR or NLR ligands. Further studies will be required to fully understand the role of HOCl in the modulation of adaptive immunity, and its potential for improving vaccination and immunotherapy. Hopefully this study will lead to greater interested in understanding the immunogenic effect of HOCl.

7.4 PUBLICATION.

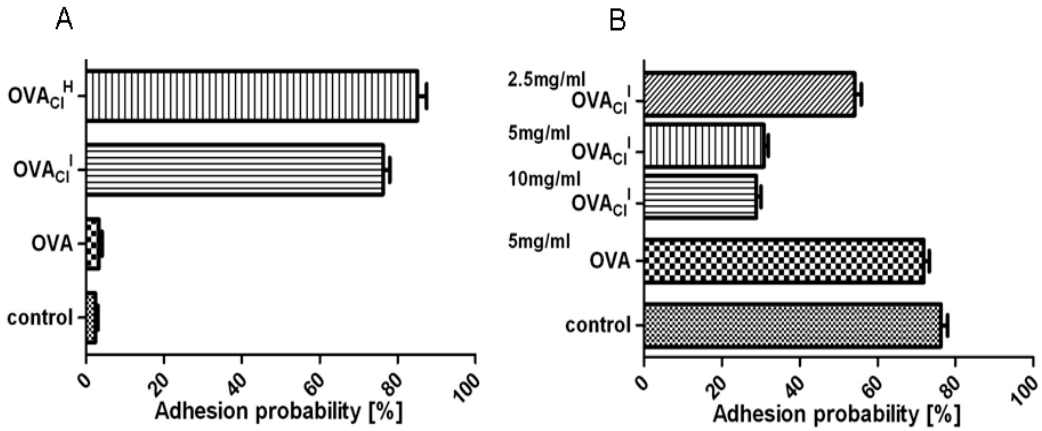
Data and concepts in this thesis have contributed to the following publication.

Prokopowicz, Z.M. et al. (2010) Hypochlorous acid: a natural adjuvant that facilitates antigen processing, cross-presentation, and the induction of adaptive immunity. J.Immunol. 184, 824-35.

APPENDIX

APPENDIX A – ATOMIC FORCE MICROSCOPY MEASUREMENT.

Experiments done by Dr. S. Zapotoczny and Prof. M. Nowakowska from Jagiellonian University Krakow.



HOCl modified OVA binds preferentially to receptors on the cell surface.

(A) *The interaction of OVA, OVA$_{Cl}^{I}$ and OVA$_{Cl}^{H}$ bound to the surface of a probe, with the surface of macrophages was measured by AFM (AFM tips were modified with a mixture of sulfonate-terminated thiol (sodium 2-mercapto-1H-benzo[d]imidazole-5-sulfonate) and OVA or OVA$_{Cl}^{I}$. Force–distance measurements were performed using Picoforce AFM (Veeco) by cycling the sample on a piezo scanner over 500 -1000 nm in vertical direction varying the speed of the movement (loading rate) and the time tips stay in contact with the surface (contact time). The final measurements were performed for the optimized loading rate (1000 nm/s) and contact time (1s). The measurements were performed in phosphate buffered saline. The curves were measured on different areas of the macrophages immobilized on glass and on the glass surface itself as negative controls. 100-300 curves were collected on each spot; the data were processed and averaged for number of spots. The measurements for each condition were performed at least twice changing the tip and the set of*

cells. For the given set of the curves the adhesion probability (AP) was determined, where: AP = number of curves with adhesion / number of all curves. ***(B)*** *The binding of* OVA_{Cl}^{I} *was measured by AFM as in (A), but cells were preincubated in OVA (5 mg/ml) or different concentrations of* OVA_{Cl}^{I}

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