Dendritic Cell Maturation and Antigen Presentation

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Modulation of dendritic cell (DC) function and control of DC maturation are critical control points in the generation of antigen specific immune responses. In this thesis DC function was studied in three different settings: age-related development of functional competence during early life, interaction of DCs and polysaccharide and glycoconjugate vaccine antigens and the pre-clinical evaluation of novel protein vaccine antigens in a DC-based culture system.

Human DCs were generated from peripheral blood monocytes. Comparison of infant and adult DCs revealed that infant DCs underwent the phenotypic and morphological changes observed in adult DCs upon maturation. In contrast, DCs from infants at least up to two years of age were severely impaired in their capacity to produce IL-12p70. This defect is likely to limit the Th1-driving capacity of the infant DCs and may contribute to the increased susceptibility of infants to infection.

Infants are particularly susceptible to infection with encapsulated bacteria. Given the impaired functional capacities of early life DCs, it was important to investigate the interaction of DCs and polysaccharide and glycoconjugate vaccine antigens. Incubating DCs with such antigens in vitro resulted in uptake and processing of the antigens. This is the first report to show the direct interaction between human DCs and polysaccharide (pneumococcal) vaccine antigens. In the absence of inflammatory mediators, these antigens did not induce DC maturation. However, they were capable of modulating the response of the DCs to a second signal (LPS) by altering the cytokine balance. IL-10 was significantly increased and IL-12 reduced compared to LPS alone. DC-pneumococcal interaction may affect subsequent immune responses via an altered cytokine balance which may have a profound effect on DC-driven T cell priming.

Considering the limitations of conjugate vaccines, the search for novel vaccine antigens has focussed on bacterial outer membrane proteins and secreted virulence factors. Evaluating the potential of such vaccine candidates to induce efficient cellular immune responses and DC maturation in the absence and presence of
adjuvant as well as evaluating DC antigen presentation to T cells, is potentially an
important component of the Phase I evaluation of putative vaccine antigens. A DC-
based model system for the evaluation of cellular immune responses to potential
vaccine antigens during in vitro presentation was developed. A variety of microbial
proteins, which are all currently being considered as potential vaccine candidates for
otitis media in infants, were evaluated. Results showed that the capacity of an antigen
to induce DC maturation in vitro correlated well with the presence of T cell memory.
Such antigens are likely to be useful for future analysis as vaccine antigens.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALRIs</td>
<td>acute lower respiratory infections</td>
</tr>
<tr>
<td>AOM</td>
<td>acute otitis media</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>APRIL</td>
<td>a proliferation-inducing ligand</td>
</tr>
<tr>
<td>BDCA-2</td>
<td>blood DC antigen-2 (CLEC)</td>
</tr>
<tr>
<td>BLyS</td>
<td>B lymphocyte stimulator</td>
</tr>
<tr>
<td>BM</td>
<td>bacterial meningitis</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CIIV</td>
<td>class II vesicle</td>
</tr>
<tr>
<td>CPS</td>
<td>capsular polysaccharides</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>DC-specific ICAM-3-grabbing non-integrin</td>
</tr>
<tr>
<td>FHA</td>
<td>filamentous hemagglutinin</td>
</tr>
<tr>
<td>Hib</td>
<td><em>H. influenzae</em> type b</td>
</tr>
<tr>
<td>ITAMs</td>
<td>immunoreceptor tyrosine-based activation motifs</td>
</tr>
<tr>
<td>LAL</td>
<td>limulus amebocyte lysate</td>
</tr>
<tr>
<td>LAM</td>
<td>lipoarabinomannan</td>
</tr>
<tr>
<td>LAMP</td>
<td>lysosome-associated membrane protein</td>
</tr>
<tr>
<td>LM</td>
<td>Lyme meningoencephalitis</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>ManLAM</td>
<td>mannose-capped lipoarabinomannan</td>
</tr>
<tr>
<td>MIIC</td>
<td>MHC class II-positive compartment</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule-organising centre</td>
</tr>
<tr>
<td>NK cell</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NTHi</td>
<td>nontypeable <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PMA:</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PPS:</td>
<td>pneumococcal (capsular) polysaccharide</td>
</tr>
<tr>
<td>PRP:</td>
<td>polyribitolribosyl phosphate</td>
</tr>
<tr>
<td>SFC:</td>
<td>spot-forming cell</td>
</tr>
<tr>
<td>SMAC:</td>
<td>supramolecular activation complex</td>
</tr>
<tr>
<td>TD:</td>
<td>T-dependent</td>
</tr>
<tr>
<td>TfnR:</td>
<td>transferrin receptor</td>
</tr>
<tr>
<td>Th1/Th2:</td>
<td>T helper type 1/2</td>
</tr>
<tr>
<td>TI-2:</td>
<td>T-independent type 2</td>
</tr>
<tr>
<td>TLR:</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>Tr1 cell:</td>
<td>T regulatory cell 1</td>
</tr>
<tr>
<td>TT:</td>
<td>tetanus toxoid</td>
</tr>
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INTRODUCTION

1. INFECTIOUS DISEASE IN CHILDHOOD

Contrary to the optimistic beliefs held only a decade ago, infectious diseases continue to be a major burden for global health. Accounting for 17 million of 52 million total deaths a year (1995), they are the leading cause of premature death (World Health Organization 1996). This rate is even higher for children under 5 – of the 11 million deaths in total in 1995, 9 million children died of infectious diseases, many of them before their first birthday. Most of these deaths could have been prevented had appropriate existing vaccines and/or drugs been given.

Acute lower respiratory infections (ALRIs), i.e. infections of the lungs, are the main cause of death in this age group, responsible for 4.4 million deaths per year, 99% of which occur in developing countries. This amounts to one child dying every 8 seconds. Most of these children could be saved by antibiotics at a cost of only $0.20 per child. However, with 394,750,000 episodes occurring each year, even such a seemingly affordable treatment becomes very costly, requiring a total expenditure of $79 million in antibiotics alone. Episodes of ALRIs are also the reason for 30-50% of doctor visits of children worldwide, resulting in further heavy strain on health resources (World Health Organization 1996).

A common and severe lung infection is pneumonae, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b being the most frequent causative agents. These two bacteria are not only major causes of childhood pneumonae but are also responsible for many cases of middle ear infections, like otitis media, and meningitis. They are also becoming increasingly resistant to common and affordable antibiotics such as penicillin, cotrimoxazole and ampicillin that used to be extremely effective in treating these infections (McCormick et al 2003)

The marked susceptibility of very young children of less than 2 years of age to these infections indicates particular weaknesses of the infant immune system. One distinguishing feature shared by these pathogens and also *Neisseria meningitidis*, another common cause of bacterial meningitis in infancy, is their polysaccharide capsule. It is a major virulence factor, so much so that its presence determines for
some bacteria (e.g. *S. pneumoniae*) whether they are harmless or whether they cause fatal disease. The capsule is also the major antigenic determinant and immunity is dependent on generating antibodies against it (Prescott et al 1996).

Polysaccharides are often considered classic examples of T-independent type 2 (TI-2) antigens, i.e. they cannot elicit conventional T-cell help and the antibody response is generally limited in that there is no demonstrable affinity maturation, isotype switching or memory formation (for a more detailed review of TI-2 responses see below). In adults, the strength and rapidity of the polyclonal primary response is still sufficient to effectively combat infection but, for reasons that are not well understood, children less than two years of age are unable to respond like adults to polysaccharide antigens, be it in a natural infection or in a vaccine (Janeway, Jr. and Travers P. 1997). In the first months of life they are still protected by maternal antibodies, but as this wanes the incidence of infection rises.

This problem has been partly resolved by conjugating the polysaccharides to a protein carrier to form glycoconjugate vaccines. Conjugate vaccines induce good affinity maturation, isotype switching and the formation of memory cells. Most importantly they induce antibody responses in the very young (Goldblatt 2000).

In general, development of conjugate vaccines is well advanced and conjugate vaccines for *H. influenzae* type b (Hib), *N. meningitidis* serogroup C and *S. pneumoniae* are licensed (Goldblatt 2000). Despite the great success of conjugate vaccines, little is known about the molecular mechanisms underlying their improved immunogenicity. It is thought that the inclusion of a protein carrier converts the T independent response to a T dependent one. This would suggest that T helper cells are primed by antigen-presenting cells (APC), which have taken up and processed the conjugate or carrier protein. Both T cells responding to the carrier protein and the polysaccharide moiety can be found after immunization with a conjugate (Muthukkumar 2004). This may indicate that APC can present a polysaccharide-derived carbohydrate, maybe in conjunction with a protein-derived peptide. However, there is no experimental evidence to prove this so far and other mechanisms may function.
Despite the success of conjugate vaccines there are still some limitations to their use. They do induce memory and elicit production of protective antibody both in adults and – more importantly – also in infants. However, multiple doses are required in infants to achieve the antibody levels found in adults after only one injection. Some antibody isotypes are more difficult to elicit than others. In one study, infant IgA and IgG2 levels remained far below adult levels even after three primary immunizations and one booster (Vidarsson et al 1998). Pneumococcal vaccination poses special problems as there are over 90 serotypes of \textit{S. pneumoniae}, more than 20 of them of clinical importance. Achieving good antibody response to all the serotypes included in various multivalent pneumococcal conjugate vaccines has been difficult so far (Black et al 2000).

Acute otitis media (AOM) is an infection of the middle ear causing ear pain, temporary hearing loss and fever. It is the most frequent reason for pediatric office visits, resulting in about 20 million visits and costs of $2 to 3.8 million a year in the USA (Eskola et al 2001). Peak incidence is between 6 and 11 months of age and the three major causative agents of bacterial AOM are \textit{S. pneumoniae}, nontypeable \textit{Haemophilus influenzae} (NTHi) and \textit{Moraxella catarrhalis}, in order of importance.

Vaccines for \textit{S. pneumoniae} have been mentioned above. There are no vaccines currently available for NTHi or \textit{M. catarrhalis} but a number of antigens (mostly outer membrane proteins) have been identified as good vaccine candidates as judged by their ability to elicit functional antibodies that transudate into the middle ear cavity and mediate killing of the bacteria or interfere in some other way with the ability of the bacteria to survive in the host (McMichael 2000).

Development of good humoral responses with high titres of high-affinity antibodies requires T cell help. Thus, a good vaccine candidate antigen has to elicit both humoral and cell-mediated immunity. Pre-clinical studies in the search for vaccine candidates for NTHi and \textit{M. catarrhalis} have so far mainly relied on data from humoral immune responses, i.e. \textit{in vitro} assays assessing the functional capacity of antibody from human serum or from experimental animal immunizations.
Information from animal models is limited as both *M. catarrhalis* and NTHi are exclusive colonizers of humans and primates (Poolman et al 2000).

There are no studies investigating human cell-mediated immunity to vaccine candidates for otitis media. Dendritic cells (DCs) initiate T-cell responses and are therefore of utmost importance for cellular immunity. They are the main APC present at the sites of infection in otitis media, i.e. the middle ear mucosa and the adenoids (Ichimiya et al 1997). Their interaction with various antigens derived from outer membrane of *S. pneumoniae*, NTHi and *M. catarrhalis* will be examined in the course of this project in order to find those antigens which elicit not only good humoral but also strong cellular immune responses.

It is becoming more and more apparent that presentation of antigens, both in natural infections and during immunization, to effector B and T cells is central to the induction of effective immune responses. It is also becoming more and more evident just how delicately this process is regulated at its key point, the dendritic cell and how important it is that DC function is optimally adapted to each situation. This means in most cases their maturation into fully competent APCs, but for some, as for example the suppression of autoimmune responses, may require just the opposite.

One of the future goals will be to manipulate DC function to optimise vaccine responses. Trying to modify such a delicate process requires profound knowledge of the real biological events. An ever increasing number of studies of DC function in mice and adult humans have allowed elucidation of a large part of the life of a DC, however, very little is known about the development of DC function over the various stages of life, for example in old age or in the first weeks, months and years after birth.

Thus, this work set out to elucidate how DCs function in early life and in situations resulting in particular problems for the infant immune system, focussing on immune responses to encapsulated bacteria. A key question throughout was how DCs mature into fully competent APCs. This knowledge will hopefully provide a rational basis for modifying of DC function to our benefit.
1.2. IMMUNITY IN EARLY LIFE

The increased susceptibility of newborns and infants to a multitude of infectious diseases stems from a generalized immaturity of the immune system at birth and during infancy. The newborn transfers from a sterile environment where responsiveness must be avoided to a highly infectious environment where protection is vital. At birth, many components of both the innate and the adaptive immune system are defective in one way or another (Marshall-Clarke et al 2000, Kovarik and Siegrist 1998). After birth, the immune system goes through a process of learning, adapting and 'fine-tuning' until it reaches a state ready to combat most infections very effectively. In the first months after birth, the neonate is still protected passively by maternal antibodies. These, however, disappear before the infant's own immune system reaches maturity, creating a window of susceptibility.

Very little is known about this transition. Immune responses in infants have been studied but these studies are mostly related to vaccine efficacy and data are limited to antibody responses. Hardly anything is known about cellular responses in this age group. One of the reasons for the absence of more work in this area is that larger volumes of blood or tissue are required for these kinds of studies which are very difficult to obtain from this age group.

INNATE IMMUNITY

The early phases of the host response to infection depend on innate immunity, which represents the first barrier to the invading pathogen. Various components of innate resistance mechanisms are impaired in newborns. Neutrophils, the most abundant white blood cell in peripheral blood, are important phagocytes, engulfing and killing extracellular pathogens. Neonatal neutrophils have reduced adherence, chemotaxis and enzymatic activity, making containment of rapidly multiplying bacteria more problematic (Kovarik and Siegrist 1998). They have normal oxidative radical production but the dramatic increase in production seen in adult neutrophils upon
lipopolysaccharide (LPS) or TNF-α stimulation does not occur in cord blood neutrophils (Bortolussi et al 1993).

Coating of extracellular pathogens with complement proteins in the initial phases and antibodies in the later phases of an immune response greatly facilitates pathogen removal and killing by phagocytes such as neutrophils or macrophages. Levels of complement in neonatal serum are below those of adults, activation of the classical and alternative pathways and significant levels of the important component C’3 do not appear until around 2 years of age (Marshall-Clarke et al 2000). This may be particularly critical for the poor anti-polysaccharide responses observed in young infants until this age (see section 1.7).

Natural killer (NK) cells are very important in innate immunity to viruses and other intracellular pathogens, as they are able to kill infected host cells and release IFN-γ. This promotes TH1 T cell responses, which are vital during the adaptive phases of immune responses to intracellular pathogens. Neonatal NK cells have lower cytotoxic activity and produce lower levels of IFN-γ upon IL-2 stimulation than adult NK cells (Kovarik and Siegrist 1998, Cohen et al 1999). However, NK cell function can be restored by providing optimal stimulation e.g. by addition of exogenous IL-12. This implies that the defect in NK cell function may be related to defects in other cells rather than being intrinsic.

IL-2, IL-12 and IL-15 are critical cytokines regulating NK function. They can be provided by mononuclear cells. These, however, are also impaired in neonates and produce less IL-12 and IL-15 (Cohen et al 1999). Neonatal monocytes have reduced MHC class II and ICAM-1 expression (Roncarolo et al 1994, Kampalath et al 1998) but their phagocytic and bactericidal activities appear to be comparable to those of adult monocytes (Dretscher et al 1976, Orlowski et al 1976).
Table 1.1. Summary of main defects of various immune cells and components in early life.

<table>
<thead>
<tr>
<th>Cell/ component</th>
<th>Defect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>↓ adherence, chemotaxis and enzymatic activity, Defective oxidative burst</td>
<td>Kovarik 1998, Bortolussi 1993</td>
</tr>
<tr>
<td>Complement</td>
<td>Low serum levels, especially C3 Defective activation of classical and alternative pathway</td>
<td>Marshall-Clarke 2000</td>
</tr>
<tr>
<td>NK cells</td>
<td>Low cytotoxic activity, ↓ IFN-γ production</td>
<td>Kovarik 1998, Cohen 1999</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>Low IL-12 and IL-15 production, ↓ MHC class II and ICAM-1</td>
<td>Cohen 1999, Roncarolo 1994</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

**ADAPTIVE IMMUNITY**

Monocytes are precursor cells for the central antigen-presenting cell, the DC (Randolph et al 1999). The DC provides the link between innate and adaptive immunity. Recognition of invading pathogens activates local DCs, which initially produce chemokines and cytokines that enhance innate immunity at the site of infection. Activated DCs then leave this site and migrate to the local lymph nodes where they alert cells of the adaptive immune system to the presence of an infection. DCs have the unique ability to prime naïve T cells but they have recently also been implicated in initiation and maintenance of certain B cell responses directly through release of soluble factors, without the normally required intermediary T helper cell (Saeki et al 2000, Balazs et al 2002, Litinskiy et al 2002).

Adaptive immune responses after early life vaccination are characterized by slower and weaker IgM-dominated antibody responses and a bias towards T_{H2} responses, even though T_{H1} responses can be observed (Siegrist 2001). Analysis of early life B cells revealed that most B cells in neonates and infants are immature IgM^{hi}IgD^{lo} cells, whereas most B cells in adults are mature IgM^{lo}IgD^{hi} cells. Immature B cells are refractory to anti-IgM-induced proliferation and triggering of their B cell receptors results in apoptosis rather than proliferation (Chang et al 1991). In addition, other factors must be involved as even relatively mature B cells are prone to such negative signalling if they come from the neonatal environment (Marshall-Clarke et al 2000). Murine B cells have a reduced capacity for antigen presentation related to very low levels of MHC II and costimulatory molecules (Muthukumar et al 2000). These molecules are nearly absent at birth but increase steadily after that. Efficient class-switching normally requires CD40 ligation. Class switching appears to be impaired in neonatal B cells which is related to an inability of the B cells themselves to respond efficiently to CD40 ligation as well as an inability of neonatal T cells to provide this signal due to reduced CD40 ligand expression on their surface (Durandy et al 1995). Many but not all defects in neonatal B cells can be overcome by appropriate stimulatory signals as for example adult T cells *in vitro* (Chang et al 1991).
Neonates are particularly deficient in responses to T-independent type 2 antigens such as bacterial capsular polysaccharides. Reasons are multifactorial and likely to be related to immaturity of particular B cell subsets, the complement system and possibly APC function in neonates (see section 1.7). Responsiveness can be restored by appropriate stimulation, for example the IgM response of anti-Ig-dextran-activated neonatal splenic B cells can be restored to adult levels by costimulation with CD40L or LPS (Snapper et al 1997).

Macrophages and B cells can act as professional antigen-presenting cells but only DCs can prime naïve T cells (see section 1.6). DCs isolated from cord blood or generated from cord blood monocytic precursors have been found to be phenotypically and functionally different from their adult counterparts and less able to support effector responses. One of the most important findings is their apparent inability to secrete IL-12, vital for induction of Th1 effector responses (see section 1.6). This may be the key to the limited occurrence of such responses in neonates and infants.

T cells isolated from neonates have performed differently from adult T cells in many experimental settings. Neonatal T cells are significantly less antigen-experienced than adult T cells. This is reflected in the observation that the majority of cord blood T cells are CD45RA⁺ naïve cells whereas both CD45RA⁺ naïve cells and CD45RO⁺ activated/memory cells are represented in adults in equal numbers (Keever 1993). Neonatal T cells produce less cytokines of either the Th1 or the Th2 profile, which agrees with their naïve, non-polarized status but are particularly deficient in production of the Th1 cytokine IFN-γ (Wilson et al 1986, Demeure et al 1995, Cohen et al 1999).

They also seem to have lower proliferative capacity (Harris et al 1992). This may be due to the fact that they are mostly naïve cells and naïve T cells are known to require longer TCR triggering and/or stronger co-stimulation before they commit to proliferation (Iezzi et al 1998). However, functionally defective neonatal DCs are less able to provide such intensive stimulation. It has in fact been found that neonatal T cells can be induced to respond like adult cells in vitro upon optimal stimulation by
I. INTRODUCTION

provision of anti-CD3 and anti-CD28 stimuli or use of adult mature DCs providing all of these factors (Hunt et al 1994). Therefore, neonatal T cells have greater requirements for costimulatory signals than adult cells but can achieve adult-level function when optimal stimulation is provided (Adkins 1999).

Reasons for the increased stimulatory requirements of neonatal T cells may lie in their reduced expression of surface molecules such as TCR and adhesion molecules, which are vital for a productive dialogue between T cell and antigen-presenting cell (Adkins 1999).

Neonatal T cells are particularly poor at producing the TH1 cytokine IFN-γ. In addition, in mice they readily produce the TH2 cytokine IL-4. IL-4 production is not seen in humans but reduced TH1 responses indirectly lead to augmented TH2 responses due to their reciprocal negative regulation. This leads to a TH2 bias in neonatal effector responses, which is easily demonstrable in mice (Barrios et al 1996). It has also been assumed to occur in human neonates but strong evidence is still lacking. Strong TH1 responses can be observed in human newborns for example after natural Bordetella pertussis infection, exposure to mycobacterial antigens, and administration of strong TH1-driving vaccines such as DNA vaccines or CpG adjuvanted vaccines. However, unless such strong TH1 inducers are used, responses are skewed towards the TH2 type (Adkins 1999, Kovarik et al 1999).

The neonatal TH2 skew is thought to revert to TH1 over time through exposure to TH1-inducing stimuli. These were suggested to include the saprophytic members of the mycobacterial family, present in immense numbers in soil, and many childhood infections. This idea formed the basis of the hygiene hypothesis which states that reduced exposure to such stimuli in industrialized countries hinders the TH2 to TH1 switch of the infant immune system, thereby maintaining the TH2 bias and allowing TH2-based autoimmune manifestations and allergies to occur (Rook and Stanford 1998). However, this does not agree with the observation that TH1-related autoimmune manifestations have also been increasing steadily in industrialized countries over the last 20 years. The hygiene hypothesis also cannot account for the fact that there is a very low incidence of allergy in developing countries where
helminth infection – which also results in a $T_h2$-biased immune response – is endemic.

The increasing incidence of allergy is now attributed to increasingly limited immunologic exposure in general. Limited exposure to environmental mycobacteria, reduced breast-feeding and thus altered gut flora, increased consumption of processed foods, intermittent exposure to antibiotics and so-called $T_h2$-biased vaccination schedules may all play a role. Exposure to innocuous environmental antigens during early life has recently been shown to be pivotal in the establishment of peripheral tolerance against such antigens through development of regulatory T cells. Dysregulation of such tolerance promoting mechanisms may allow development of harmful effector responses, causing both $T_h1$- and $T_h2$-related autoimmune and allergic manifestations (Umetsu et al 2002). This idea is supported for example by the studies showing that intestinal helminth infection can protect against food allergy by inducing high levels of IL-10 (Bashir et al 2002). This immunosuppressive cytokine is implicated in the development and also the activity of certain regulatory T cells (see section 1.6). *Schistosoma haematobium* infection also reduces atopy in children, again at least in part mediated by parasite-induced IL-10 (van den Biggelaar et al 2000), lending further support to the concept that dysregulation of peripheral tolerance rather than defective $T_h1$ effector immunity is responsible for the increased incidence of autoimmune and allergic manifestations in industrialized countries.
1.3. DENDRITIC CELL BIOLOGY

THE LIFE CYCLE OF DENDRITIC CELLS

DCs are vital to the immune system as they are professional APCs and the only APC, which can stimulate naïve resting T cells. They are the most efficient APC and able to not only present antigen and induce de novo immune responses but also control their magnitude, quality and the generation of memory.

Initially, DC progenitors in bone marrow give rise to DC precursors which home to tissues as immature DCs where they take up their role in antigen surveillance. Here they constantly take up antigen and monitor the surrounding tissues for signs of infection or inflammation. In this state they are optimally adapted for antigen uptake but are poor at antigen presentation owing to high levels of endocytosis and low levels of MHC as well as costimulatory and adhesion molecules which are required for efficient interaction with T cells (Banchereau and Steinman 1998).

Upon activation DCs initially produce cytokines and chemokines which attract cells of the innate immune system to the site of infection, e.g. macrophages, NK cells, eosinophils and also other immature DCs. Maturing DC then leave the tissues and migrate to a lymphoid organ (Weinlich et al 1998). During this transit they dramatically change their phenotype and function to become optimally adapted for their new task of presenting the foreign antigens to rare antigen-specific effector cells (T and B cells) in the lymphoid organ and driving appropriate polarization of responding cells. Maturation is associated with downregulation of endocytic activity, upregulation of MHC class II molecules and their redistribution to the surface, upregulation of the co-stimulatory molecules CD40, CD58, CD80 and CD86, changes in morphology, in expression of adhesion molecules, chemokines and chemokine receptors, and upregulation of cytokine production (Banchereau et al 2000)

Activation and maturation stimuli, often called danger signals, are very diverse and range from ‘direct’ activation by pathogen-derived molecules (such as LPS, CpG
DNA, dsRNA) recognised by pattern-recognition receptors to 'indirect' activation by inflammatory mediators produced by the host cells during the course of the infection (Banchereau et al 2000, see also section 1.8).

DCs are always present in the afferent lymph entering the T cell areas but are not found in efferent lymph, indicating that DCs die in the lymphoid tissue (Banchereau and Steinman 1998).

DCs are optimally equipped for all of their tasks. They can efficiently capture antigens by phagocytosis, macropinocytosis and receptor-mediated endocytosis (Inaba et al 1993, Sallusto et al 1995, Sallusto and Lanzavecchia 1994). They employ mechanisms to arrest the processing before its complete breakdown into single amino acids and can thus create peptides for presentation. They delay antigen processing until presentation is actually required (Lutz et al 1997, Turley et al 2000). They not only express large amounts of MHC Class I and II molecules and co-stimulatory molecules when needed, but also express all isoforms of the CD1 family, a non-classical antigen-presenting molecule implicated in presentation of lipids and glycolipids (Sugita et al 2000) (For a detailed review of antigen uptake, processing and presentation by DCs see section 1.5).

The interaction between DCs and T cells is very complex. Activated, effector and memory T cells rapidly (0.5 – 2 hr) respond to low doses of antigen and short stimulation via the T cell receptor even in the absence of co-stimulation. These cells can also be stimulated by other professional antigen-presenting cells such as macrophages and B cells. In contrast, naïve T cells can only be primed by DCs. They need high doses of antigen and strong co-stimulation and it may take 6 to more than 30 hours for them to commit to proliferation (Lanzavecchia and Sallusto 2001a, see section 1.6).

Once primed, T cells differentiate into non-polarised or polarized (T_H1 or T_H2) effector cells or memory cells. DCs play a key role in determining the fate of an activated T cell (Banchereau et al 2000, see also section 1.6). They do so mainly via the production of IL-12, levels of which are crucial for driving T cell differentiation.
Low levels of IL-12 usually do not overcome the IL-4-induced 'default' development into T\(_{H}2\) effectors, high levels drive T\(_{H}1\) responses (Ohshima and Delespesse 1997). Generating the right class of immune response is crucial for the outcome of an immune response i.e. whether it will result in continued spread, chronic maintenance or resolution of the disease. One of the most well known examples is leprosy in which a T\(_{H}1\) type response to the parasite results in the less severe, tuberculoid form of the disease while a T\(_{H}2\) biased response leads to the often lethal lepromatoid form (Pulendran et al 2001).

Interaction with T cells is a reciprocal process that also provides signals to the DCs. Activated T cells trigger DCs via CD40 and TRANCE, which improves T cell stimulatory capacity, increases IL-12 production and prolongs the life span of DCs. Anergic or regulatory T cells on the other hand can provide inhibitory signals to DCs (Lanzavecchia and Sallusto 2001b).

DCs provide a critical link between innate and adaptive immunity. They respond to pathogen-derived signals but translate this information into signals understood by the adaptive system.

**DENDRITIC CELL POPULATIONS**

*In vivo*, DCs have been identified in most tissues, e.g. skin (Schuler and Steinman 1985), airways (Holt et al 1988), interstitial spaces of many organs (Hart and Fabre 1981), lymphoid tissues (Steinman and Cohn 1973) and blood (Kyewski et al 1986) with the exception of immune privileged brain, retina and testes (Banchereau et al 2000). Specialized DC subpopulations with distinctive features can be found in various tissues, e.g. skin epidermal Langerhans cells, respiratory tract DCs, liver DCs, thymic DCs or T-cell zone interdigitating DCs (Banchereau et al 2000).

A tremendous variety of different DC subsets have been described in recent years (Banchereau et al 2000, Liu 2001, Kelsall et al 2002). It is unclear whether these differences are due to different lineages or rather to a different stage in development
or a difference in the environment the cell encounters. Many researchers today believe that the *in vivo* DC system is a mixture of both, i.e. certain fundamentally different subsets exist in a limited number but the cells are still receptive to the exact environmental conditions they encounter and adapt according to the situation (Kelsall et al 2002, Manickasingham et al 2003). This would allow the development of a very dynamic yet finely adapted and highly specialized system of immune surveillance.

**IN VITRO GENERATION OF DENDRITIC CELLS**

DCs can be isolated from peripheral tissues such as skin (in the form of Langerhans cells, typical immature DC) and also from the blood where they circulate in low numbers. However, the study of human DCs is confounded by the low yield from all tissues and the difficulty in isolating DCs at each differentiation stage. Therefore, many researchers have isolated more ubiquitous precursors cells, which can then be grown into DCs *in vitro* in various conditions. Precursors can be for example CD34⁺ cells from cord blood (Caux et al 1996) or blood-derived CD14⁺ monocytes (Sallusto and Lanzavecchia 1994). Cells obtained by these different methods exhibit phenotypic and functional features of DCs but the exact nature of DCs *in vivo* is still unclear, as is the exact relationship between *in vivo* DCs and *in vitro* generated cells (Banchereau et al 2000).

Sallusto and Lanzavecchia (Sallusto and Lanzavecchia 1994) described an *in vitro* culture system that yields large numbers of DCs that can be maintained in culture in their immature state. In this new culture system, monocytes are purified from peripheral blood and grown in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Over the course of about 5 days, the cells lose their CD14 expression (monocyte marker) and differentiate into immature DCs characterised by the typical dendritic morphology, high endocytic capacity, intermediate levels of MHC class I and II, expression of CD1, CD11c, CD40, CD86, ICAM-1 and FcγRII. These cells can be stimulated to activate and mature using LPS, TNF-α, CD40 ligand, dsRNA and a variety of other stimuli. They
become typical mature DCs with low endocytic capacity but very potent T cell stimulatory capability. These cells correspond morphologically, phenotypically and functionally to well-defined populations of mature DCs in vivo or ex vivo, e.g. cultured epidermal Langerhans cells, cutaneous DCs obtained from skin explants and blood DCs after short-term in vitro culture (Romani et al 1996).

Good yields can be obtained using the peripheral blood CD14+ monocytic precursors in this manner. Therefore this culture system is optimal for the isolation of DCs from infant blood samples where only very small quantities can be obtained. Validation for the use of monocyte-derived DCs also comes from the fact that they are being used in many cancer trials and thus extremely well characterised.
1. INTRODUCTION

1.4. DENDRITIC CELLS IN NEWBORNS AND INFANTS

Current knowledge about DCs in neonates and infants stems mainly from studies in mice and from studies relying on the use of human cord blood. The difficulty of obtaining large volumes of blood from infants has limited the study of DCs beyond the early neonatal period.

MURINE EARLY LIFE DCs

In mice, DCs are sparse or undetectable in the spleen and do not acquire antigen processing and presentation capacity until later in ontogeny (Lu et al 1980). Also Ridge and co-workers (Ridge et al 1996) reported a quantitative deficiency in DCs in neonatal mice. Muthukkumar and co-workers (Muthukkumar et al 2000) compared murine B cells and DCs from the neonatal period (0 and 3 days) through infancy (7, 14, 21 days) to adulthood (8-12 weeks old). At birth both DCs and B cells were very poor (~10% of adult response on day 3) at supporting T cells responses to a protein (tetanus toxoid, TT) and a conjugate vaccine antigen (MCPS: N. meningitidis group C polysaccharide-TT conjugate). A steady increase in antigen presentation capacity was seen until near adult levels were reached at 4 weeks of age. Deficient antigen presentation was attributed to low levels of critical surface molecules such as CD80, CD86 or MHC class II which were nearly absent at birth but steadily increased to near adult levels at four weeks of age. MHC class I levels were high from birth. The infant DCs were morphologically similar to adult DCs but had higher expression of the monocyte marker F4/80 and the granulocyte marker Ly6G and lower expression of the DC marker CD11c.

In a contrasting report, Dadaglio and colleagues show that splenic DCs purified from 7-day-old infant mice expressed normal levels of MHC molecules and were functionally competent and able to induce CTL responses after ex vivo pulsing and re-administration. Discrepancies with the above studies were suggested to be due to differences in selection procedures and isolation of different DC subpopulations (Dadaglio et al 2002).
HUMAN EARLY LIFE DCs

In humans, DCs directly isolated from cord blood are functionally inferior to the corresponding cell population isolated from adult blood. They express lower levels of MHC molecules and ICAM-1 and are less efficient at supporting a mixed leukocyte reaction (Hunt et al 1994). In contrast, Sorg and colleagues reported that DC population they isolated from cord blood was very potent at stimulating allogeneic T cells (Sorg et al 1999). This discrepancy may have resulted from different isolation procedures yielding mainly myeloid DCs in the first case (Hunt et al 1994) whereas most of the DCs isolated by Sorg and colleagues expressed the CD123 marker, were CD11c' and poorly endocytic, hallmarks of plasmacytoid DCs (Facchetti et al 1999). These findings are reinforced by a recent publication reporting elevated numbers of plasmacytoid DCs in cord blood decreasing with age whereas the size of the myeloid DC population remains stable (Teig et al 2002).

Three recently published studies revealed similar defects in DCs generated from cord blood monocytes. Cord blood monocytes cultured with IL-4 and GM-CSF develop into immature DCs that are morphologically and phenotypically similar to adult immature DCs. They seem to have slightly lower levels of MHC class II, but expression of CD11c and CD86 is not impaired. However, the data show that these cells are unable to respond to inflammatory stimuli as well as their adult counterparts. Addition of LPS, TNF-α or Poly I:C, a synthetic surrogate of viral double-stranded RNA, causes strong upregulation of HLA-DR, CD83, CD86 or CD40 expression in adult cells. This was completely abrogated in neonatal DCs (Liu et al 2001, Goriely et al 2001, Langrish et al 2002).

Adult DCs maintained this mature phenotype stably for at least 78 hours after stimulation whereas cord DCs did not upregulate and even lost expression of these surface markers over this time period. CD14, in contrast, increased over time instead of decreasing possibly suggesting a return of the cord DCs to their monocytic origins (Langrish 2002).
Other hallmarks of impaired maturation include continued high levels of endocytic uptake, no switch in chemokine receptor expression and no increase in the capacity of DCs to prime allogeneic naïve T cells (Langrish et al 2002, Liu et al 2001). This confirmed earlier reports showing that cord blood-derived human DCs are unable to efficiently prime neonatal or adult T cells and adult or cord blood T cells responded much less well to mitogens when cord blood-derived DCs rather than adult DCs were used (Petty and Hunt 1998).

Murine neonatal splenic accessory cells are deficient in the production of IL-12 when stimulated with LPS (Bondada et al 2000) as are human neonatal DCs (Goriely et al 2001, Langrish et al 2002). This has important consequences, as DCs exert their effect in driving CD4^ T effector cell development mainly via the production of IL-12. Goriely et al and Langrish et al showed that adult DCs produced high levels of this cytokine with a burst in secretion between 8 and 12 hours after addition of LPS and reached a peak of IL-12 p70 levels at approximately 18 hours. Levels then remain constant for at least a further 30 hours. Cord DCs failed to produce IL-12p70 at all time points. However, the LPS response was not completely abrogated in cord DCs as they produced IL-10 and TNF-α at comparable levels and with comparable kinetics to adult DCs (Langrish et al 2002).

Further experiments indicated that impaired LPS response was not due to an inherent inability to respond to LPS. The main receptor for *E.coli* LPS, TLR4 in association with MD2, was shown to be expressed on both adult and cord DCs at equivalent levels before and after stimulation. The experiments also showed that the impaired response was not due an increased requirement for stimulation of the cord DCs as the failure to produce IL-12 was observed at LPS concentrations ranging from 10ng/ml to 1000ng/ml (Langrish 2002).

If LPS-receptor signalling is intact in monocyte-derived cord DCs, the defect is presumably found downstream in the signalling pathway. Goriely and colleagues (Goriely et al 2001) have indeed shown that the reduced expression of the IL-12 p35 subunit is responsible for the failure to produce IL-12 p70.
The defect in IL-12 production, while levels of other cytokines such as the potent immunosuppressive cytokine IL-10 remain constant, may have profound consequences and may be key to the reduced Th1 responsiveness in infants. Immunosuppressive cytokines such as IL-10 or TGF-β have also been suggested to play an important role in the induction of peripheral tolerance, particularly at the mucosal surfaces in the respiratory and gastrointestinal tract (Weiner 1994, Akbari et al 2001, see section 1.6).

IL-12 has been shown to enhance antibody responses to various TI-2 antigens including DNP-Ficoll as well as meningococcal and pneumococcal vaccine antigens (Buchanan et al 1998). This may suggest that deficient IL-12 production plays a role in impaired TI-2 responses in neonates and infants. The enhancing effect of IL-12 was unexpectedly found to be independent of the presence of NK cells and T cells, indicating that DCs may also directly influence B cell activation (see also section 1.7).

Studies on the ontogeny of DCs and age-related development of their functional competence have so far been performed only in mice due to the difficulties in obtaining material from human infants.

No studies on human infant DCs have been published until recently. Bickham and colleagues reported in a meeting abstract (Bickham et al 2000) that they found normal levels of surface molecules such as MHC class II, CD83 and CD86 on immature DCs isolated from pediatric samples. These DCs matured and supported T cells responses as well as adult DCs. This study only included six samples from a very wide range of ages (11 months to 14 years). However, children of less than 2 years of age are specifically immunocompromised and this age group will be the focus of our work.

A recent report from Upham and colleagues (Upham et al 2002) showed that mononuclear cells from cord blood as well as from peripheral blood of children aged 5 and 12 years are impaired in their capacity to produce IL-12 p70. IL-12 levels
steadily increase with age but are still below adult levels at the age of 12 (difference was highly significant with p<0.0001).

As mentioned above, children of less than 2 years of age are specifically immunocomprised in their responses to encapsulated bacteria, the focus of this work, and will be the target group. However, in the light of the above-mentioned findings by Upham and colleagues, our studies were extended to include children older than 2 years.
1.5. ANTIGEN UPTAKE, PROCESSING AND PRESENTATION IN DENDRITIC CELLS

Macrophages can take up large quantities of particulate and soluble antigens, which are then processed inside the cell extremely rapidly. Processing normally leads to full degradation of the protein into single amino acids but some peptides are rescued and presented in the normal fashion on MHC molecules. The entire process from uptake to presentation takes as little as 20 minutes (Harding and Geuze 1992).

Dendritic cells also take up antigens efficiently and rapidly. They are able to internalize quantities of extracellular fluid equal to their own volume (~2.5 x 10^{-13} l) in approximately 1 hour (Sallusto et al 1995). However, processing is rather different to that seen in macrophages due to the different functional demands made on DCs. The main task for macrophages is to remove free antigen from the circulation and destroy it while DCs take up antigen to generate immunogenic peptides for presentation to effector cells (T and B cells) and for the initiation of immune responses (Roitt et al 1997). Therefore they employ mechanisms to arrest proteolytic degradation of proteins before complete breakdown into single amino acids (Lutz et al 1997).

ANTIGEN UPTAKE

Antigens are internalized by DCs via one of several mechanisms: phagocytosis (Inaba et al 1993), macropinocytosis (Sallusto et al 1995) and receptor-mediated endocytosis (Sallusto and Lanzavecchia 1994). Mechanisms of uptake are developmentally regulated in DCs, i.e. macropinocytosis and phagocytosis are reduced upon DC maturation whereas receptor-mediated endocytosis is not affected by maturation (Garrett et al 2000).

Endocytosis correctly refers to the three different methods of uptake, but is most commonly used to describe receptor-mediated endocytosis in particular. In phagocytosis, large particles bind to surface receptors capable of triggering their own
uptake during which F-actin-driven pseudopods engulf the particle and form a vacuole. Pinocytosis, or fluid-phase uptake, is the constitutive formation of small vesicles carrying extracellular fluid. It is based on membrane ruffling activity and reflects the passive capture of fluid when rims of membrane folds fuse back with the plasma membrane (Mellman 1996). Unlike receptor-mediated endocytosis, both macropinocytosis and phagocytosis are processes dependent on the restructuring of the actin cytoskeleton (Mellman 1996).

Immature DCs are specialized for antigen uptake and can interact with antigens through a diverse range of antigen receptors expressed on its surface. Many function primarily as receptors that bind antigens for uptake, such as Fc receptors, CR3 (Mac-1) or a wide variety of different C-type lectins such as the MR, DC-SIGN, DCIR, dectin-1 and 2, langerin, MGL, CLEC-1 and -2 and BDCA-2. Some of these such as DC-SIGN, CLEC-2 and dectin-1 contain cytoplasmic tyrosine residues that are part of immunoreceptor tyrosine-based activation motifs (ITAMs) (Engering 2002b, Figdor et al 2002). Such motifs generally allow delivery of an intracellular signal and initiation of signalling.

**ANTIGEN PROCESSING AND PRESENTATION**

Dendritic cells employ both receptor-mediated endocytosis and macropinocytosis for internalisation of soluble antigenic ligands, such as those vaccine antigens this work is focussed on. DCs can also efficiently internalise particulate antigens and pathogens through phagocytosis.

After uptake, antigens are passaged through vesicles and progressively processed until complete degradation or rescue of immunogenic peptides for presentation. Events described in detail in the following. A summary is presented in Figure 1.1.
Figure 1.1. Representation of intracellular events during endocytosis after uptake of antigen via receptor-mediated endocytosis (A) or macropinocytosis (B).
Receptor-mediated endocytosis

Receptor-mediated endocytosis is initiated when ligands bind to specific receptors on the surface and is much faster than phago- or pinocytosis. When a ligand binds to a receptor, a clathrin-coated pit is formed and internalised as a small vesicle, which then fuses with an early sorting endosome. In macropinocytosis, membrane folds fuse with each other, trapping extracellular fluid and with it soluble antigens. Again, small vesicles are formed which fuse with early endosomes.

Early endosomes are part of a dynamic network of tubules and vesicles dispersed throughout the cytoplasm. Their pH is slightly acidic allowing for dissociation of ligands from their receptors. Receptors and other membrane proteins pass into the tubular endosomal extensions, which bud off and form recycling vesicles. These will either carry the receptors directly back to the plasma membrane or move along microtubule tracks to the perinuclear cytoplasm where they accumulate close to the microtubule-organising centre (MTOC) and form an intracellular pool of recycling receptors (Mellman 1996).

Ligands accumulate in the vesicular regions of the early endosomal structures. These also bud off and migrate to the perinuclear cytoplasm to fuse with late endosomes. The endosomal membrane invaginates and the late endosomes take on their classic multivesicular or multilamellar appearance. They have a lower pH and already contain active hydrolases, which initiate the degradative process. This process is continued in lysosomes where the pH drops even further.

Late endosomes and lysosomes are collectively termed late endocytic compartments or MHC class II-positive compartments (MIICs) and the distinction between them is very vague. There seems to exist a constant cycling between the two as antigens are progressively degraded and more carrier vesicles bring hydrolytic enzymes from the trans-Golgi network. Once all the contents have been digested the lysosome is in a resting state containing only non-digestible material until it again fuses with vesicles bringing hydrolases from the trans-Golgi network or vesicles from the early endosomal network with new material destined for degradation.
I. INTRODUCTION

**Class II vesicles**

In APCs, some material is rescued before degradation is completed and bound as a peptide to MHC molecules. Peptide-MHC complexes are removed from the degradative climate of the lysosome and transported in class II vesicles (CIIVs) to the surface for presentation (Mellman 1996). These intracellular vesicles are distinct from other endocytic organelles (Turley et al 2000). They are MHC class II-positive, nonlysosomal, peripheral vesicles that only transiently exist in DCs when those are in an intermediate state between 'immature' and 'mature'. CIIVs are negative for the lysosomal markers H-2M and LAMP. They contain the peptide-MHC II complexes and appear to mediate the transfer of the complexes from lysosomes to the plasma membrane where the complexes appear about 20 hours after the activation stimulus was given. The lysosomes left behind after the removal of peptide-MHC II complexes become MHC II-negative and accumulate in a small cluster associated with the MTOC (Turley et al 2000).

CIIVs not only carry peptide-MHC complexes to the surface but also contain other molecules, which are involved in the interaction with T cells, as e.g. the co-stimulatory molecule CD86. Late in the intermediate stage (10 to 12 hours after activation) peptide-MHC II complexes and CD86 appear on the cell surface where they are expressed in a punctate pattern for long periods of time. This clustering may facilitate or enhance synapse formation with the T cell (Turley et al 2000).

**Table 1.2. Markers for endocytic organelles (from a variety of sources):**

<table>
<thead>
<tr>
<th>Endocytic Organelles</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early endosomes:</td>
<td>rab4⁺, rab5⁺, recycling receptors, lamp⁻, pH 6.0-6.8</td>
</tr>
<tr>
<td>Recycling vesicles:</td>
<td>rab5⁺, rab4⁺, recycling receptors, lamp⁻, pH 6.4-6.5</td>
</tr>
<tr>
<td>Late endosomes:</td>
<td>rab7⁺, rab9⁺, rab11⁺, MPR⁺, lamp⁺, LBPA⁺</td>
</tr>
<tr>
<td></td>
<td>(lysobisphosphatidic acid), CD63⁺, cathepsinD⁺, pH 5.0-6.0</td>
</tr>
<tr>
<td>Lysosomes:</td>
<td>MPR⁺, lamp⁺, acid hydrolases, pH 4.5-5.5</td>
</tr>
<tr>
<td>Class II vesicles (CIIV):</td>
<td>rab7⁻, neutral or mildly acidic, MHC class II⁺, lamp⁻</td>
</tr>
</tbody>
</table>
Regulation of endocytosis in DCs

Antigens usually go through this process very rapidly. However, in DCs this would pose a great problem, as there can be a delay of several days between DC activation due to uptake of a foreign antigen and presentation of the processed peptide in the lymphoid organ. The ability to delay processing for such extended periods of time is unique to DCs. B cells and macrophages for example do not retain intact protein antigens for more than 4 hours. Freshly isolated Langerhans cells in comparison retained the same antigen in its native form for at least 2 days (Lutz et al 1997).

The molecular mechanisms underlying this process are not fully understood but it is believed that DCs can regulate acidification of their endocytic system. Lutz and colleagues identified a novel type of endocytic vesicle, which is different from the MIICs and stably non- or mildly acidic (Lutz et al 1997). These so-called retention compartments seem to be the storage vesicles for intact antigen during DC migration. Retention of antigen in this form was not seen in cell lines representative of monocytes, macrophages, B cells or in freshly isolated monocytes or B cells.

Turley and colleagues (Turley et al 2000) showed that antigen processing in DCs is indeed tightly controlled. The model protein antigen hen egg lysozyme (HEL) could be retained for at least 3 days in its native form and could then still be processed and presented. Antigen processing was arrested in immature DCs and only started upon receipt of a strong activation stimulus (in this case LPS). Shortly thereafter, antigen processing began in the lysosomes and immunogenic HEL peptide-MHC II complexes were being formed.

Maturation of DCs not only results in increased transport of lysosomal MHC class II molecules to the surface but also in a redirecting of newly synthesised MHC II molecules. In immature DCs, most are transported to lysosomes where they are degraded if they are not loaded with peptide. In mature DCs however newly synthesized molecules are sent to the plasma membrane directly, devoid of the invariant Ii chain that usually occupies the peptide-binding groove until it is replaced by a peptide derived from a foreign antigen (Cella et al 1997, Davoust and Banchereau 2000).
I. INTRODUCTION

Cross-presentation in DCs

The antigens mentioned so far were all exogenous antigens taken up by DCs and presented on MHC class II to CD4^+ T helper cells. However, DCs also interact with and present antigen to CD8^+ cytotoxic T lymphocytes (CTL). Antigens for presentation to CTL are normally derived from the cytosol and thus these are antigens that are actually synthesized inside the cell. They are processed in the MHC class I-specific pathway in the proteasome. However, most viral and tumour antigens presented on MHC class I by DCs are never synthesized inside them.

To resolve this problem, DCs employ molecular mechanisms that allow cross-presentation, i.e. the presentation of exogenous antigen on MHC class I. Cross-priming is very important in immune responses against transplantation antigens, tumours, particulate antigens and viruses. This pathway also exists, though to a lesser extent and with different capabilities, in macrophages. Antigen derived from apoptotic cells or antigen-immunoglobulin immune complexes for example are presented to specific CTL only by DC but not by macrophages or B cells (Watts 1999). DCs can also present exosomes derived from tumour cells and peptides carried on heat-shock proteins on MHC class I (Banchereau et al 2000).

Internalized antigen gains access to the cytosol and therefore the MHC class I pathway by an endosome-to-cytosol pathway. Rodriguez and colleagues showed that in D1 cells (a splenic-derived DC cell line representative of immature DCs) or primary bone marrow-derived mouse DC, soluble antigens such as ovalbumin, horseradish peroxidase, immune complexes and dextrans are transported from lysosomes to the cytosol, processed in the proteasome and presented to MHC class I-restricted T cell lines. This transport is slow, specific for the internalized antigens, does not result from general leakage of lysosomal contents and is size selective as only small molecules (e.g. 3K and 40K dextrans as opposed to large 500K or 2000K dextrans) reached the cytosol. In this case, cytosolic staining was only seen in DC but not in macrophages (Rodriguez et al 1999). Other groups have published similar findings (Brossart and Bevan 1997, Shen et al 1997, Machy et al 2000).
I. INTRODUCTION

**CD1-mediated antigen presentation**

A novel group of non-classical antigen-presenting molecules (in addition to MHC class I and II) has been identified recently. The CD1 family is specialised for the presentation of lipids and glycolipids such as mycobacterial lipoarabinomannan. DCs express all three group I CD1 molecules (CD1a, CD1b and CD1c) whereas B cells in comparison express only CD1c (Sugita et al 2000), highlighting the key role of DCs in antigen presentation.

It is hypothesized that the different CD1 isoforms evolved for complementary functions as they localise to different endocytic compartments and present antigens sampled from distinct stages of the endocytic pathway (Briken et al 2000). CD1-restricted T cells have been found in all major T cells subsets, i.e. CD4⁺ helper cells, CD8⁺ cytotoxic cells, CD4⁺CD8⁻ double negative cells, T cells bearing α/β as well as γ/δ TCRs. Emerging evidence suggests that these T cells show restricted diversity in their TCR gene usage (Schaible and Kaufmann 2000).

CD1a is found in early and recycling endosomes and excluded from the late endocytic compartments. CD1b shows a reverse expression pattern, being localised mostly in late endosomes and lysosomes and weakly staining the plasma membrane. CD1c has a very broad distribution on the plasma membrane, in early endosomes, in MIICs and also recycling vesicles. It is often expressed in the absence of other CD1 molecules, e.g. on B cells or epidermal Langerhans cells, and its broad distribution may allow comprehensive survey of the endocytic system under those circumstances (Schaible et al 2000, Sugita et al 2000, Briken et al 2000).

The ligands for CD1 molecules found so far have mainly been of mycobacterial origin, one of the most studied being lipoarabinomannan (LAM) (Schaible and Kaufmann 2000). In its soluble form it is internalised by DCs via the macrophage and dendritic cell mannose receptor (MR), trafficks through early and late endosomes and lysosomes. Usually the MR releases its ligand in early endosomes and recycles back to the membrane but experimental evidence supports the possibility that the MR carries the LAM molecule through to the MIICs where the
LAM would be released, bound to CD1b molecules and on those transported to the plasma membrane for presentation to CD4⁺CD8⁺αβ T cells (Prigozy et al 1997).

In contrast to group I CD1 molecules, no microbial ligand has been described yet for the group II CD1 molecule, CD1d. It is recognized by NK T cells that can respond directly to CD1d, seemingly even in the absence of any antigen. NK T cells release a burst of IL-4 upon activation, following by rapid, IL-12 or IL-18-induced production of IFN-γ (Schaible and Kaufmann 2000). It has been proposed to view the NK T cell/CD1d system as a primitive system of antigen recognition involved in the very early, innate phase of immune responses (Schaible and Kaufmann 2000).

CD1-dependent presentation of antigens to CD1-restricted T cells may generally be a biological phenomenon specifically adapted to early phases of immune responses. Cao and colleagues recently showed that none of the CD1 isoforms undergo dramatic changes in expression and localization as seen for MHC molecules. Consistent with this, DCs efficiently presented CD1b-restricted lipid antigens to specific T cells with similar efficiency in immature and mature cells (Cao et al 2002).
1.6. DC-DRIVEN T CELL DIFFERENTIATION

T CELL ACTIVATION AND MEMORY FORMATION

Dendritic cells can be extremely efficient at priming T cells. Under conditions that force DC-T cell interactions, one DC can drive up to 20 naïve T cells to clonal expansion (Langenkamp et al 2002). T cells compete for access to DCs in vivo resulting in intraclonal competition. This is very low in the initial phases of a primary immune response due to paucity of antigen-specific precursor T cells but increases as the responding T cells proliferate. This may drive functional diversification: those T cells with high avidity TCRs achieve longer and stronger TCR stimulation and differentiate into effector and effector memory cells while those receiving only short stimulation remain in an intermediate state giving rise to non-polarized effector and central memory cells (Lanzavecchia and Sallusto 2001b).

Once activated, naïve T cells differentiate in a step-wise fashion into effector cells, central memory cells and effector memory cells (Sallusto et al 1999). How far they move along this pathway is determined by the strength of stimulation, i.e. the ligand dose, DC number, duration of the DC-T cell interaction and TCR avidity (Langenkamp et al 2002, Sallusto et al 1999). These parameters also influence the polarization into distinct types of effector and memory cells, e.g. $T_H1$ versus $T_H2$.

Development of two different memory compartments, central and effector memory, allows for division of labour in the memory response and occurs both in the CD4$^+$ and the CD8$^+$ compartments in both mice and humans (Iezzi et al 2001, Langenkamp et al 2000).

Effector memory cells lose expression of the chemokine receptor CCR7, which allows them to leave the secondary lymphoid organs and patrol the peripheral tissues ready for immediate polarized action upon antigen recognition thereby rapidly containing pathogens. Central memory cells retain CCR7 expression and are able to reach secondary lymphoid organs. These clonally expanded, non-polarized cells have lower thresholds for proliferation but do not yet produce effector cytokines. Upon
antigenic challenge they can efficiently stimulate DCs via CD40L expression, help B cells and rapidly generate a new wave of effector and effector memory cells (Sallusto et al 1999). The simultaneous generation of effector and memory CD4\(^+\) T cells after antigenic stimulation has been visualized in whole bodies of mice (Reinhardt et al 2001).

When a T cell encounters a DCs, it migrates over the surface until its TCR binds an agonist peptide-MHC complex, steps which are facilitated by adhesins and integrins. MHC-peptide complexes and TCRs aggregate in a supramolecular activation complex (SMAC) and several such small TCR clusters merge to form a TCR synapse where TCRs and MHC molecules are enriched in a central position surrounded by a ring of integrins and adhesion molecules (Dustin and Cooper 2000). These form within minutes of initial cellular contact and allow efficient interaction.

Ligation of the TCR results in translocation of the complex into a specialized region of the membrane called a raft. TCR synapse rafts contain molecules, which are crucial for signalling transduction and the phosphorylation cascade. TCR signalling can be amplified up to 100-fold by the engagement of CD28 by co-stimulatory molecules on the antigen presenting cell. Co-stimulation does not actually affect the extent or the kinetics of TCR triggering but amplifies the signal by recruiting new rafts to the synapse. CD28 engagement also excludes negative regulators such as the phosphatases CD45 and SHP-1 from the synapse (Lanzavecchia and Sallusto 2000).

Naïve T cells have low levels of rafts on their surface and depend on co-stimulation to allow efficient signalling. Effector and memory T cells already express high levels of membrane rafts and hence do not need co-stimulation to amplify signalling (Lanzavecchia and Sallusto 2000). Therefore, memory cells can be activated by cells other than DCs e.g. macrophages and B cells but DCs are the only antigen presenting cells able to prime naïve T cells due to their strong expression of antigen presenting and co-stimulatory molecules.

As activated T cells proliferate and differentiate, they upregulate an additional CD80/CD86 receptor, CTLA-4 (cytotoxic T-lymphocyte antigen 4). It resembles
CD28 closely but binds the co-stimulatory molecules about 20 times more avidly than CD28 and delivers a negative signal to the activated T cell resulting in reduction of T cell proliferation. This is an important mechanism involved in the termination of immune reactions (Walunas et al 1994).

The interaction between DCs and T cells is not a one-way process but leads to mutual stimulation. Both cells rely on signals from the other cell for function, survival and death. Activated T cells stimulated DCs via CD40 ligand and TRANCE, improving their T cell stimulatory capacity, boosting IL-12 production and prolonging their life span (Lanzavecchia and Sallusto 2001b). In fact, it has been observed that LPS-matured DCs rapidly die by apoptosis once they have entered the T cell areas of the secondary lymphoid organs unless they receive survival signals from activated T cells (De Smedt et al 1998).

T CELL POLARIZATION

Following the first division, T cells proliferate rapidly and over time differentiate into effector cells. Dendritic cells regulate this process at various points. One is the strength of TCR stimulation delivered by the DCs: depending on the levels of co-stimulation, a short or weak TCR stimulation may just allow expansion of non-polarized effector cells, Th0, whereas long or strong TCR triggering will result in the development of polarized populations (Langenkamp et al 2002). Polarization is strongly influenced by the secretion of polarizing cytokines by the DCs.

The best-known example of T cell polarization is the Th1/Th2 paradigm, first described in mice by Mosmann and Coffman in 1986 (Mosmann et al 1986). T helper cell polarization is best observed in long-term immune responses such as parasitic diseases or chronic inflammation as most T cell responses start as Th0 responses and then slowly become biased towards one or the other or remain a mixture (Janeway, Jr. and Travers P. 1997).
T\textsubscript{H}1 cells are characterized by production of IFN-\(\gamma\) and promote cell-mediated immunity by activating macrophages and assisting in the development of cytotoxic T cells. T\textsubscript{H}2 cells are specialized to promote humoral immunity via the release of IL-4, IL-5 and IL-13, which help B cells to produce appropriate antibodies and activate eosinophils (Banchereau and Steinman 1998). T\textsubscript{H}1 responses protect mainly against intracellular pathogens whereas T\textsubscript{H}2 responses protect against extracellular pathogens such as gastrointestinal bacteria and helminths. Both subsets can also mediate detrimental immune reactions such as diabetes or allergic and atopic manifestations, respectively (Murphy and Reiner 2002).

T helper cell polarization is driven by a variety of factors most of which are related to DC function, e.g. duration of TCR signalling, ligand dose, DC number (Tanaka et al 2000), particular co-stimulatory molecules and most importantly cytokines (Kalinski et al 1999).

The best-explored factor is IL-12, which plays very potent role in driving T\textsubscript{H}1 polarization \textit{in vitro} (Hilkens et al 1997, Ohshima and Delespesse 1997) and \textit{in vivo} (Magram et al 1996). High levels are released by DCs in response to maturation stimuli such as TNF-\(\alpha\), double-stranded RNA, bacterial CpG DNA or LPS (Sallusto et al 1995, Cella et al 1999; Hartmann et al 1999, Hilkens et al 1997). IL-12 production can be boosted potently by activated T cells through CD40 ligation (Schulz et al 2000) and it can be inhibited by immunosuppressive factors such as prostaglandin E\(_2\) (Kalinski et al 1997) or IL-10 (De Smedt et al 1997). Strong T\textsubscript{H}1 responses are also induced by plasmacytoid DCs via the release of IFN-\(\alpha\) in response to viruses (Siegal et al 1999, Cella et al 2000).

It is much more controversial how T\textsubscript{H}2 responses are initiated. IL-4 can potently drive T\textsubscript{H}2 polarization (Janeway, Jr. and Travers P. 1997) but the major cellular source \textit{in vivo} is less clear. It is produced by T cells themselves and by NK T cells, a specialized T cell subset belonging to the innate arm of the immune system.

Due to the apparent lack of strong T\textsubscript{H}2 polarization factors released by mature DC, it is often suggested that T\textsubscript{H}2 responses are initiated as a default pathway when T\textsubscript{H}1-
driving factors are absent. DCs can be conditioned to mature but not produce IL-12, e.g. by maturation in the presence of PGE$_2$ (Kalinski et al 1998), or they may have exhausted their IL-12- or IFN-α-producing capacity (Langenkamp et al 2000).

Production of inflammatory cytokines such as TNF-α, IL-6 and IL-12 by DCs is restricted to a narrow temporal window (8 to 16 hours) after induction of maturation. As the production of these cytokines wanes, secretion of the anti-inflammatory cytokine IL-10 increases and is maintained for about 24 hours after induction of maturation (Langenkamp et al 2000). Therefore it has been proposed that IL-12 producing myeloid DCs might preferentially prime Th1 responses during the early phase of an infection when recently stimulated DCs enter the T cells areas in large numbers. This may be followed by priming of Th2 and non-polarized T cells at later time points when the influx of DCs ceases and the DCs surviving in the T cell areas have exhausted their cytokine-producing capacity (Langenkamp et al 2000).

Requirements for differentiation of CD8$^+$ T cells are much less stringent than for CD4$^+$ T cells. Once they commit to proliferation, they can divide autonomously and acquire cytotoxic function in the absence of antigen presenting cells and antigenic stimulation (Lanzavecchia and Sallusto 2001b). Type 1 and 2 effector cells have also been described for this compartment, as well as for NK T cells, but in contrast to CD4$^+$ cells, CD8$^+$ T cells have a strong preference for differentiating into type 1 effectors. The factors driving their polarization are very similar to those involved in T helper cell differentiation, i.e. IFN-γ and IL-12 induce type 1 responses whereas IL-4 drives development of type 2 cells (Mosmann et al 1997).

**DCs IN THE ESTABLISHMENT OF TOLERANCE**

Random rearrangements of the T cell receptor genes produce an immense diversity in receptor specificities including many autoreactive receptors. Tolerance limits harmful responses against self by tolerizing these autoreactive T cells by mechanisms such as deletion, anergy or suppression.
Central tolerance is established in the thymus and the role of DCs in the negative selection and deletion of T cells responding to self-antigens is well known (Banchereau et al 2000). However, 25-40% of T cells escape selection and persist as circulating cells in the periphery (Bouneaud et al 2000). In addition, many self-antigens never gain access to the thymus due to restricted tissue-specific distribution. Others are released only in specific situations such as cell death during infection or normal cell turnover. Others enter the body as foreign but innocuous antigens, such as environmental inhaled or ingested antigens. In these situations, mechanisms of peripheral tolerance are employed to limit or prevent harmful immune reactions.

The role of DCs in establishing and maintaining peripheral tolerance was controversial at first as DCs are the key cells in the initiation of immunity and had in fact been used to break T cell tolerance against a variety of antigens, including soluble, viral, tumour-derived and transplant antigens and also in the neonate (Banchereau et al 2000).

The solution to this apparent paradox is intricately connected to the maturation state of the DC and their central role is now widely accepted (Roncarolo et al 2001, Mahnke et al 2002, Steinman et al 2003). The exact mechanisms by which they exert their tolerogenic function are still unclear, particularly in vivo, but include the deletion of T cells, the induction of anergic T cells and T cells with regulatory properties mediated via direct cell-cell contact or secretion of immunomodulatory cytokines.

Tolerogenic DCs are often divided into cells that induce tolerance due to their immature state and cells that have been modulated to convert from being immunogenic to being tolerogenic, e.g. by production suppressive cytokines such as IL-10 and TGF-β (Jonuleit et al 2001).

As described above, T cells activation requires at least two signals: triggering of the TCR by MHC-bound peptide (signal 1) indicating the identity of the antigen and co-stimulatory signals such as CD28 ligation (signal 2) conveying the DC-stimulating (i.e. pathogenic) potential of the antigen. Signal 1 and 2 result in antigen-specific
activation of naïve T cells (Kalinski et al 1999). The requirement that the same cell presents both signals is vital in preventing destructive immune responses to self antigens. It has long been known that TCR triggering in the absence of co-stimulation not only fails to activate the cells but leads to a state called anergy in which the T cell is functionally inactive and becomes refractory to further stimulation (Mueller and Jenkins 1995).

Anergic T cells are poorly proliferative, produce little IL-2, do not secrete effector or immunomodulatory cytokines, fail to lyse target cells (in the case of CD8^+ anergic cells) and have reduced expression of the IL-2 receptor α-chain (CD25). They are unable to respond to re-challenge with antigen, even when given with a strong adjuvant such as complete Freund's adjuvant (Jonuleit et al 2001). Anergy can be partially reversed by high doses of IL-2 (Jonuleit et al 2000, Steinbrink et al 2002).

Taams and co-workers have suggested that different levels of T cell anergy exist, determined by the antigen dose. Low (suboptimal levels) resulted in development of hyporesponsive cells; higher (optimal) levels induced anergic cells with some immunomodulatory functions and high (supraoptimal) levels induced anergic cells with potent immunosuppressive capacities (Taams et al 1999). Cells with such modulatory or suppressive activity are now often called regulatory T cells. Suppression or regulation can be antigen-specific or antigen-nonspecific.

In the absence of infection or inflammation, often called the steady state, immature DCs migrate at a rapid rate between tissue sites and lymphoid organs (Kamath et al 2000). They continuously capture antigen and – in the absence of maturation stimuli – present these antigens at low density and in the absence of costimulation, inducing anergy in naïve T cells and may also allow the DCs to induce the development of regulatory T cells.

The best-characterized regulatory T cell subset so far is CD4^+CD25^+ T cells. This population comprises 5-10 % of all peripheral T cells in mouse and human (Groux et al 1997, Taams et al 2001). They are anergic cells producing high levels of IL-10 and, despite this, exert their suppressive function in a strictly contact-dependent
fashion. As shown recently, the suppressive signal delivered by membrane-bound TGF-β (Chen and Wahl 2003). It is regulated by multiple factors including CD40-CD40L interaction (Kumanogoh et al 2001), IL-2, GITR (glucocorticoid-induced TNF receptor-family related gene) and CTLA-4 (Chen and Wahl 2003). Substantial levels of CTLA-4 are only found on this T cell subset (Taams et al 2001). CD4^CD25^ regulatory cells require activation via the TCR, i.e. antigen-specific, to become suppressive, but intriguingly their suppressor function, once activated, is completely antigen-nonspecific (Thornton and Shevach 2000).

The importance of this subset in vivo is highlighted by the fact that its eradication results in the development of many autoimmune diseases such thyroiditis, gastritis, insulitis and polyarthritis (Sakaguchi et al 1995).

CD4^CD25^ regulatory T cells are the best characterized so far, but subsets with regulatory functions have also been found among γδ T cells, NK T cells, CD8^, other CD4^ compartments and CD4^CD8^ double-negative cells (Hayday and Tigelaar 2003, Wilson and Delovitch 2003, Francois 2003).

Human CD4^CD25^ regulatory T cells have the CD45RO^CD45RB^ phenotype and short telomeres indicative of highly differentiated primed/memory T cells that have experienced repeated episodes of antigen-specific stimulation in vivo. It has been suggested that they are primed and maintained by repeated interaction with non-professional antigen-presenting cells, the major candidate being immature DCs (Taams et al 2001).

Work by Helmut Jonuleit and co-workers has indeed shown that repetitive stimulation of allogeneic CD4^ T cells with human immature, monocyte-derived DCs leads to the development of such regulatory T cells (Jonuleit et al 2000). This also seems to occur in vivo, as injection of immature DCs pulsed ex-vivo with antigen (influenza matrix peptide) into two human volunteers resulted in functional inactivation of antigen-specific CD8^ effector cells and the appearance of antigen-specific IL-10-, but not IL-4-producing cells (Dhodapkar et al 2001).
In vivo, many self-antigens are captured by DCs after cell death, potential ligands for many autoreactive T cells. However, uptake of apoptotic cells does not deliver any maturation signal to DCs and they remain in their immature state (Gallucci et al. 1999, Sauter et al. 2000). Ingestion of apoptotic cells in fact impaired their response to inflammatory stimuli and their T cell stimulatory capacity (Stuart et al. 2002). These observations are in accordance with the suggestion that the primary function of immature DCs in the steady state is the generation and maintenance of tolerance against self-antigens (Roncarolo et al. 2001).

The establishment and maintenance of peripheral tolerance by immature DCs has been viewed as a requisite to allow them to function correctly in the induction of immunity. When mature, immunocompetent DCs present antigen during an infection, they present pathogen-derived antigens as well as self-antigen at the same time on the same cell. Autoreactive cells that recognize self-antigens will have been deleted or anergized by immature DCs during the establishment of peripheral tolerance. However, mature DCs overcome anergy and may therefore reactivate such anergized autoreactive T cells.

A second level of peripheral tolerance

Suppressive T cells acting in an antigen-nonspecific fashion are not able to suppress effector T cells that have already received a full activation signal (Taams et al. 1998). This may require more potent regulatory or suppressive cells that can down-regulate responses in an antigen-specific fashion and even overcome resistance of fully activated effectors.

Groux and co-workers reported the generation of a different CD4+ subset, termed T regulatory cells 1 (Tr1), generated in the presence of IL-10, which could suppress effector cells in an antigen-specific manner and actively downregulate pathological responses (colitis) in vivo suggesting that IL-10 may be important for the induction of regulatory cells (Groux et al. 1997). Martin and colleagues showed recently that IL-10-secreting regulatory cells were even able to suppress previously primed immune responses in an antigen-specific manner (Martin et al. 2003).
Modulation of DCs during maturation to induce high level IL-10 production is a common immune escape mechanism used by pathogens and also tumors to prevent or suppress immune reactions directed against them (see section 1.8). It is being more and more commonly also used by scientist to achieve the same goals.

Steinbrink and colleagues used IL-1G-treated human DCs to induce the development of anergic T cells which suppressed effector cells of the same antigen specificity in a strictly cell-contact dependent manner (Steinbrink et al 1999, Steinbrink et al 2002).

Many of these regulatory cells produce IL-10 or TGF-β. IL-10 is also produced by Th2 cells but at much lower levels. IL-10 has very potent immunosuppressive effects and low levels may allow development Th2 responses whereas high levels result in immunosuppression (Enk et al 1993).

IL-10 is the prototype immunosuppressive cytokine with suppressive effects not only on DCs but on many different cell types. It prevents accumulation of DCs in vivo (Qin et al 1997), hinders their development into DCs from monocytic precursors when presents during development (Buelens et al 1997a) and inhibits maturation when present during exposure to maturation stimuli. Such IL-10-exposed DCs remain immature cells with low levels of MHC and costimulatory molecules, low IL-12 production and low T cell stimulatory capacity (Caux et al 1994, Morel et al 1997). Production by low, but sizeable amounts of IL-10 by monocyte-derived immature DCs prevents spontaneous maturation, limits, but not prevents, LPS- and CD40-mediated maturation and increases IL-10 production in a positive feedback loop (Corinti et al 2001).

Microenvironmental factors present at mucosal surfaces, e.g. in the respiratory or gastrointestinal tract, also modulate DC function resulting in a strong bias towards Th2 responses or induction of tolerance (Stumbles et al 1998, Constant et al 2000).

Akbari and colleagues reported that respiratory exposure to the innocuous antigen ovalbumin results in the rapid migration of pulmonary DCs to the draining lymph
node. DCs mature during transit but produce IL-10 rather than IL-12. Again, antigen-specific T cells undergo a rapid expansion followed by deletion and residual anergy. In vitro, the IL-10-producing DCs induced the production of IL-4 and IL-10 in naïve T cells. This cytokine combination is usually associated with T_\text{H}2 cells but the very large amounts of IL-10 produced in this setting are more characteristic of regulatory cells (Akbari et al 2001). The same group showed later that not only DC-derived IL-10 but also ICOS-ICOS ligand interaction is required for induction of airway regulatory cells (Akbari et al 2002).

TGF-\beta is another immunosuppressive cytokine of importance in the induction of tolerance, as it appears particularly in models of oral tolerance. It is produced by DCs isolated from the mesenteric lymph nodes after oral administration of antigen and enables such DCs to direct the development of TGF-\beta-producing regulatory T cells (Chen et al 1994, Akbari et al 2001). In similarity to IL-10, TGF-\beta inhibits activation and maturation of DCs in response to inflammatory stimuli. Intriguingly, however, it is critically required for the development of precursor cells into Langerhans cells (Geissmann et al 1999, Strobl and Knapp 1999).

In conclusion, it is hypothesized that two broad types of regulatory cells exist with different but complementary functions in peripheral tolerance. So-called natural regulatory cells suppress immune responses in a contact dependent manner and function in general homeostasis to block the actions of autoimmune cells in non-inflammatory settings. They constitutively express high levels of CD25, probably owing to ongoing engagement of their TCRs by self-antigens in the periphery. Their maintenance seems to rely on the delivery of low levels of co-stimulatory molecules, as are present on immature DCs (Salomon et al 2000). The so-called adaptive regulatory cells enhance suppression in an inflammatory milieu, mostly by release of cytokines such as TGF-\beta and IL-10 (Sakaguchi 2000, Bluestone and Abbas 2003). Adaptive tolerance seems to be a temporal phenomenon, with numbers of antigen-specific effector cells reaching a minimum after several weeks to months and improving thereafter (Dhodapkar et al 2001). In contrast, natural regulatory cells appear to be always present in normal individuals. The mechanisms inducing and
maintaining tolerance may not be as robust as other immune responses as infection often induces autoimmunity (Horwitz and Sarvetnick 1999).
1.7. **T-INDEPENDENT TYPE 2 RESPONSES**

**TYPES OF ANTIGENS**

Antigens can be divided into three groups based on their capacity to elicit immune responses without T cell help. T dependent antigens such as diphtheria toxin, viral haemagglutinin, purified protein derivative (PPD) and *Mycobacterium tuberculosis* require antigen-specific, activated T helper cells in order to elicit antibody responses, whereas T independent (TI) antigens elicit antibody production in the absence of T cells. TI antigens type 1 can activate B cells polyclonally, i.e. in an antigen-nonspecific manner, because they act as B cell mitogens. Examples are bacterial LPS or *Brucella abortus* (Janeway, Jr. and Travers P. 1997).

TI-2 antigens such as dextran, hapten-conjugated ficoll or pneumococcal capsular polysaccharides usually are very large molecules consisting of orderly arrays of repeating antigenic epitopes. This structure allows them to crosslink receptors on the B cell surface and thus activate the B cell to produce antibody, mainly low-affinity IgM (Janeway, Jr. and Travers P. 1997). Antibody production to TI-2 antigens can be greatly enhanced by the presence of various cytokines (Snapper and Mond 1996, Vos et al 2000).

**POLYSACCHARIDE VACCINES**

The T cell-independency of polysaccharides is a major problem for vaccination against encapsulated bacteria such as *Streptococcus pneumoniae*, *Neisseria meningitidis* or *Haemophilus influenzae* type b. Vaccination with pure capsular polysaccharides induces secretion of low-affinity IgM and there is no demonstrable affinity maturation, isotype-switching or memory formation. In fact, some studies report an immunological hyporesponsiveness after polysaccharide vaccination (Borrow et al 2001, Richmond et al 2000, MacDonald et al 2000, Borrow et al 2000, Sverremark and Fernandez 1998, Wuorimaa et al 2001). In adults, the strength and the rapidity of the polyclonal response is still sufficient to effectively combat
infection but children of less than two years of age are unable to respond like adults or older children to polysaccharide antigen, be it in a natural infection of in a vaccine (Janeway, Jr. and Travers P. 1997).

THE SPLENIC MARGINAL ZONE AND TI-2 RESPONSIVE B CELLS

The inability of infants to respond to polysaccharides is generally attributed to the immaturity of various components of the immune response, one of the most important being the spleen. Its marginal zone is one of the main sites for antibody production against TI-2 antigens and its absence or immaturity predisposes individuals to overwhelming infection with encapsulated bacteria (Timens et al 1989). It is absent in neonates and then slowly develops to reach maturity at around 2 years of age, coinciding with the appearance of anti-polysaccharide responses. However, significant number of marginal zone CD4⁺ T cells also did not appear until 20 months of age (Timens et al 1989).

The presence of an intact marginal zone is important but not sufficient for competent TI-2 responses. The function of the polysaccharide-responsive B cell itself seems to be more important than splenic architecture. TI-2-nonresponsive CBA/N mice have a well-defined marginal zone but their B cell pool fails to mature and is remarkably similar to the IgM⁺, IgD⁻ immature B cells found in neonates and infants (Liu et al 1988).

Two particular B cell subsets are specially adapted to respond to TI-2 antigens, B1-a cells and marginal zone B cells. The spleen plays a key role for both. It is critical for the generation and maintenance of the B1-a pool as well as home to the marginal zone B cells. Both subsets are required for the production of antibodies to different carbohydrate antigens, as proven in elegant experiments using mice with a specific defect in marginal zone B cell or B1 cell development (Guinamard et al 2000, Prior et al 1994). It has been proposed that marginal zone B cells may be more important in the later phases of an infection when pathogens are found in the blood and filtered into the marginal zone. B1-a cells play a key role in the limitation of early bacterial growth at the site of infection through the production of natural antibodies and

When injected into wild-type mice, model TI-2 antigens such as TNP-Ficoll as well as bacterial TI-2 antigens such as pneumococcal capsular polysaccharides rapidly localize to marginal zone B cells and follicular dendritic cells in both mice and humans (Harms et al 1996, Peset Llopis et al 1996). This is dependent on opsonization of the polysaccharides by complement fragment C3d that in turn binds to CR2/CD21 present at a high density on marginal zone B cells. Localization of polysaccharides to marginal zone B cells via C3d-CD21 occurred in the absence of specific antibody (Peset Llopis et al 1996). This has the advantage that even at first encounter with a TI-2 antigen, close contact is possible with B cells, which are particularly well adapted to rapid activation and response (Oliver et al 1997).

In neonatal and infant spleens there is a striking lack of C3d receptor CD21 (CR2) expression (Timens et al 1989, Peset Llopis et al 1996). Consistent with this observation, pneumococcal polysaccharides were unable to bind neonatal or infant spleens in the presence of normal human serum (Peset Llopis et al 1996).

These studies also showed – in both mice and humans – that germinal centres formed after immunization with pure pneumococcal polysaccharide. Germinal centre formation in response to the TI-2 antigen dextran B512 is dependent on T cells (Sverremark and Fernandez 1998).

In humans, B cells with the morphology and phenotype of marginal zone B cells have also been found in other organs such as the tonsils and Peyer’s patches in the gut. Here, as in the marginal zone of the spleen, the cells are optimally placed at sites of first encounter with microbial challenge. Sequence analysis of the V region genes suggested that the majority of those cells are memory cells (Spencer et al 1998).

This distinction is very important as crosslinking of receptors on immature B cells leads to apoptosis and clonal deletion instead of activation, which is seen in mature B cells (Chang et al 1991). Most of the B cells in infants are immature, which may
explain why infants cannot respond to polysaccharides (Janeway, Jr. and Travers P. 1997).

**REGULATION OF TI-2 RESPONSES**

Irrespective of B cell subpopulation, cross-linking of surface receptors on mature B cells will stimulate the cell to proliferate but will not be sufficient to induce the secretion of cytokines or the production of antibody. Secondary signals are needed which can be provided by a wide variety of sources (Mond et al 1995a).

They could be provided by the pathogens themselves. Many bacterial components are potent polyclonal B cell mitogens (mostly TI-1 antigens) that deliver costimulatory signals directly to the polysaccharide-specific, membrane-Ig-actived B cells (e.g LPS, lipoproteins from Gram-positive and Gram-negative bacteria, neisserial porin proteins and bacterial DNA) (Snapper and Mond 1996).

In this case, the immune response would completely circumvent the antigen-presenting cell and would be independent of any complex cell-to-cell interactions and therefore very rapid (Roitt et al 1997). This could be of evolutionary advantage in infections where rapid production of antibodies is crucial to slow the spread of the bacteria. In fact, responses to TI-2 antigens appear within 48 hours of exposure, too soon for the generation of antigen-specific T cells (Janeway, Jr. and Travers P. 1997).

Secondary signals could also be provided by other components of the innate immune system, without the need to activate adaptive immunity. NK cells can become activated in a TI manner during bacterial infections and release cytokines such as IFN-γ and GM-CSF. These mediators can act synergistically to induce antibody secretion in B cells activated by TI-2 but not T dependent (TD) antigens (Snapper 1996). GM-CSF is also produced by macrophages, endothelial cells, mast cells and even B cells themselves. γδ T cells and CD1-restricted αβ T cells can recognize
microbial components directly, an event shown to lead to release of IFN-γ early during bacterial infections (Snapper and Mond 1996).

An immune response that only involves B cells and the pathogens would be very rapid but it would also be short-lived and very restricted, as B cells need specific signals from other cells to optimize the antibody secreted via affinity maturation and isotype switching and to develop into memory cells. In the case of protein antigens, these signals are provided by T helper cells (Janeway, Jr. and Travers P. 1997).

Responses to TI-2 antigens can definitely occur \textit{in vivo} in the absence of T cells but T cells may still play a regulatory role the extent of which is still unclear. There is a large body of literature indicating that T cells or T cell derived factors are involved. For example, rigorous depletion of T cells does result in markedly reduced \textit{in vitro} responses to TI-2 antigens (e.g. Letvin et al 1981, Nordin and Schreier 1982). Some studies suggested a role for T cells because T-cell derived factors, as e.g. IFN-γ and GM-CSF were required for antibody production by B cells activated by a TI-2 antigen. However, as mentioned above, many of these factors could also be produced by other cells of the immune system (Snapper and Mond 1996).

\textbf{THE ROLE OF APCs IN TI-2 RESPONSES}

Responses to TI-2 antigens are assumed to be completely independent of APCs based on the fact that polysaccharides generally do not associate with MHC Class II molecules (Harding et al 1991). However, a growing body of evidence suggests a role for APCs in TI-2 responses.

One possibility is the indirect involvement in TI-2 responses through transport of the antigens to the responding B cell. Antibody production by MZB in response to TI-2 antigens has shown to come from static and not recirculating cells (Gray et al 1982). Antigen is carried passively in the blood and captured by the FDC in the spleen. Activated complement components and specific antibody greatly facilitates this
pathway. However, antigens are transported actively from the site of infection to the secondary lymphoid organ.

The crucial role of dendritic cells in the transport of protein antigens is well established. It is less clear for TI-2 antigens. Macrophages take up model TI-2 antigens such as dextran (Kato et al. 2000) and they migrate to the marginal zone but their main task is to destroy the antigen. They do not transfer antigen to B cells or FDC or present antigen to B cells.

Dendritic cells strongly express the macrophage mannose receptor, which binds polysaccharides and mediates their internalisation (Sallusto et al. 1995, Kato et al. 2000 [dextran], Zamze et al. 2002 [pneumococcal capsular polysaccharides]). In addition, DCs are covered in a wide variety of different lectins, carbohydrate-binding surface molecules (Figdor et al. 2002, Ariizumi et al. 2000, Geijtenbeek et al. 2000a, Colonna et al. 2000, Valladeau et al. 2000, Nicoll et al. 1999, Bates et al. 1999, Swiggard et al. 1995). Many of these have been shown to act as receptors for antigen uptake, e.g. Langerin (Valladeau et al. 2000), Dectin-2 (Ariizumi et al. 2000) or DCSIGN (Engering et al. 2002a).

DCs have been shown to move to B cells areas, interact directly with the B cells to induce antibody secretion and isotype switching through release of soluble factors without the need for helper T cells as an intermediate (Saeki et al. 2000, Balazs et al. 2002, Litinskiy et al. 2002). DCs can store naïve antigen for long periods of time and then transfer it to B cells which can then still process the antigen (Wykes et al. 1998, Dustin and Dustin 2001).

A second possibility is the direct involvement of dendritic cells in TI-2 responses through internalisation and possibly presentation of the antigen and priming of effector cells.

Two very early studies suggested that supernatants from a cloned, mature DC cell line promote responses to TNP-ficoll (and TNP-dextran) and that injection of TNP-ficoll can prime T cells in vivo. A critical requirement for Ia-bearing (a murine MHC
class II molecule) dendritic accessory cells was shown (DeKruijf et al 1985, Letvin et al 1981).

Bondada and colleagues (Bondada et al 2000) have shown that the combination of IL-1 and IL-6 is required for B cells to respond to TI-2 responses and that neonatal accessory cells (dendritic cells and macrophages) are deficient in their production of IL-1 upon stimulation with TI-2 antigens. Addition of exogenous IL-1 restored the responses of neonatal B cells to various TI-2 antigens and the avidity of the antibodies produced were similar to those produced by adult cells, suggesting that neonatal splenic B cells can respond normally to TI-2 antigens if given the right signals by accessory cells which in turn may be deficient in neonates.

Model TI-2 antigens like dextran were shown to be taken up by dendritic cells and other APC and to be trafficked through the endocytic pathway, as would TD antigens (Kato et al 2000, Prigozy et al 1997, Schaible et al 2000, Sallusto et al 1995). However, these studies did not show whether TI-2 antigens could be processed or presented by DCs.

The dogma that TI-2 do not bind the MHC molecules and therefore do not prime T cells has been challenged recently by the finding that zwitterionic polysaccharides carrying both positive and negative charge, isolated from strains of Bacteroides fragilis, Staphylococcus aureus and Streptococcus pneumoniae type 1, do indeed elicit potent CD4+ T cell responses. Induction of T cell responses is dependent on direct contact with MHC class II-bearing dendritic cells (Kalka-Moll et al 2002).

**RESPONSES TO GLYCOCONJUGATES**

The T-independent response to polysaccharides can be converted into a T-dependent response by chemically linking the carbohydrate to a protein carrier.

This strategy was and is successfully being employed to overcome the impaired response to polysaccharide vaccines in infants. Glycoconjugate vaccines induce a T-
dependent response with good affinity maturation, isotype switching and the 
formation of memory cells. Most importantly they induce antibody responses in the 
very young (Borrow et al 2001, O'Brien et al 1996, Goldblatt 2000). They also 
overcome the immunological hyporesponsiveness induced by some polysaccharide 
vaccines (Borrow et al 2001, Richmond et al 2000, MacDonald et al 2000, 

As with other vaccines, adjuvants can greatly enhance responses to glycoconjugate 
vaccines. CpG oligonucleotides for example strongly activate B cells, DCs and NK 
cells and enhance responses to polysaccharides but only when these are conjugated 
to a protein carrier (e.g. in the pneumococcal and Hib conjugates). It has no effect on 
responses to plain polysaccharides (Kovarik et al 2001, Chu et al 2000).

An intriguing alternative explanation for the enhanced anti-polysaccharide response 
in adults compared to infants has been suggested recently (Baxendale et al 2000). 
Adult volunteers were vaccinated with pure pneumococcal polysaccharide or 
conjugate. B cell receptor sequence analysis of polysaccharide-specific B cells 
showed high levels of somatic hypermutation that were inconsistent with a primary 
response. As these individuals had never been vaccinated before, it was suggested 
that wild-type infection or nasopharyngeal carriage of *Streptococcus pneumoniae* in 
adults may induce memory and the response to polysaccharide or conjugate 
vaccination in adults is in fact a secondary response. Therefore the relative lack of 
exposure to naturally conjugated polysaccharide in infants may be one of the reasons 
for their poor response to plain polysaccharides (Baxendale et al 2000). Similar 
findings were reported by Hougs and colleagues who found that a primary 
vaccination with a Hib conjugate elicited a secondary type response (Hougs et al 
1999).

It is widely accepted that conjugate vaccines induce a T-dependent response. Using 
specifically deficient mice, Guttormsen and colleagues showed that MHC class II-
antigen-TCR, B7(CD80/86)-CD28 costimulatory and CD40-CD40 ligand 
interactions are critical for an immune response to glycoconjugates *in vivo* 
(Guttormsen et al 1999) thereby confirming that APC-T cell interactions are
involved. The exact molecular mechanisms of T cell and dendritic cell involvement, however, are still unclear, i.e. whether the polysaccharide component is 'accidentally' presented or can actually be processed and be bound to antigen-presenting molecules for presentation.

In vivo injection of naturally conjugated polysaccharides (e.g. of encapsulated Streptococcus pneumoniae bacteria) resulted in activation of polysaccharide-specific marginal zone and B1 B cells in the spleen. This was dependent on critical survival signals from CD11c^lo blood dendritic cells, which were singularly responsible for initiating and supporting the TI immune response. These DCs take up bacteria in the blood stream, move to the splenic marginal zone and secrete the soluble ligands BLyS and APRIL, crucial signals required for plasma cell generation in TI immune responses (Balazs et al 2002). BLyS and APRIL are upregulated on DCs upon exposure to IFN-α, IFN-γ, CD40L or LPS and, in the presence of IL-4, IL-10 or TGF-β, induce CD40-independent immunoglobulin class switching (Litinskiy et al 2002).

A variety of studies have shown that various glycoproteins are presented by APCs and are recognized by T cells as glycopeptides bound to MHC class I or II. The T cells respond to the glycopeptide but not the corresponding non-glycosylated peptide, suggesting that the carbohydrate moiety has some influence on the interaction between the MHC-glycopeptide complex and the TCR. Such glycoproteins have been shown to elicit activation of T helper as well as cytotoxic T cells, T cells bearing α/β as well as γ/δ TCRs (Michaelsson et al 1996, Harding et al 1993, Speir et al 1999, Vlad et al 2002).

There seems to be some controversy as to whether the carbohydrate components of the glycoproteins are altered in any way during processing inside DCs (Vlad et al 2002, Prigozy et al 2001, Porcelli 2001) and whether the carbohydrate moiety of the glycopeptide is actually specifically recognized by T cells (Vlad et al 2002, Michaelsson et al 1996) or its influence is determined by size and position than chemical structure (Speir et al 1999, Harding et al 1993).
The recent discovery of a non-classical system for antigen presentation, the CD1 system, offers an alternative to MHC-restricted presentation of carbohydrate antigens. Experimental evidence strongly suggests that CD1 molecules have evolved to specialize in presentation of lipidated proteins and carbohydrates. The lipid component is bound in the CD1 molecules and the peptide or carbohydrate moiety is presented to and recognized by CD1-restricted subsets of T cells (Fairhurst et al 1998a). Many naturally occurring polysaccharides, such as the capsular polysaccharides of *Haemophilus influenzae* type b, *Neisseria meningitidis* groups A-C and several K serotypes of *Escherichia coli*, are lipidated and structurally very similar to the well-known CD1b ligand lipoarabinomannan (LAM) (Arakere et al 1994). It was suggested that the palmitic acid residue present in many lipidated polysaccharides anchors diverse covalently attached polysaccharides in the hydrophobic groove of CD1 (Fairhurst et al 1998a).

In fact, Fairhurst and co-workers (Fairhurst et al 1998b) showed that non-peptide antigens derived from *H. influenzae* (most likely to be capsular polysaccharides) were presented on CD1 to donor-derived T cell lines. The T cell phenotype mirrored other, mycobacterium-specific, CD1-restricted T cell populations (CD4^+CD8^-, CD56^+, α/β TCR).

Four different isoforms of CD1 exist in humans, which seem to have evolved to present lipidated antigens sampled from different subcellular compartments to allow for a wide variety of antigens to be presented. Many DC subsets express all four isoforms. B cells in peripheral blood, tonsil, spleen and lymph node express only CD1c with CD1c expression being particularly prominent among marginal zone B cells (Mond et al 1995b). Based on this observation, it was hypothesized that a CD1c^+ B cell population might be able to process and present lipidated polysaccharides to T cells (Fairhurst et al 1998a).

No studies have so far reported any data regarding processing and presentation of pure polysaccharides in dendritic cells.
1.8. DENDRITIC CELL MATURATION AS KEY TO COMPETENT IMMUNE RESPONSES

The DC has a crucial role in the decision over induction of tolerance or immunity. Modulation of its maturation state is an indispensable tool for the DC to relay its decision to the effector arm of the immune system: ‘Antigen presentation sets the stage for antigen-specific recognition but maturation controls the T cell response’ (Steinman and Pope 2002). With this realization has come a flood of publications examining how pathogens and host-derived molecules modulate DC maturation to direct the induction of immune responses favourable to their survival, propagation or pathology.

INDUCTION OF DC MATURATION

Immature DCs are specialized for antigen uptake and can interact with antigens through a diverse range of antigen receptors expressed on its surface. Many function primarily as receptors that bind antigens for uptake, such as Fc receptors, CR3 (Mac-1) or a wide variety of different C-type lectins such as the MR, DEC-205, DC-SIGN, DCIR, dendin-1 and 2, langerin, MGL, CLEC-1 and -2 and BDCA-2. Some of these (e.g. DC-SIGN, CLEC-1, -2, MGL and dendin-1), but not all, care capable of delivering an intracellular signal and initiate signalling due to the presence of immunoreceptor tyrosine-based activation motifs (ITAMs) (Rescigno et al 1999, Figdor et al 2002).

A second class of receptors found on DCs is formed by the toll-like receptors (TLRs). TLRs recognize evolutionary conserved molecular patterns in microbial components (pathogen-associated molecular patterns, PAMPs) and activate signalling pathways that induce antimicrobial effector responses and inflammation (Wright 1999, Takeda et al 2003). The different TLRs recognize particular PAMPs.
### Table 1.3. Toll-like receptors (TLRs) and their ligands

<table>
<thead>
<tr>
<th>TLR</th>
<th>Microbial ligand</th>
<th>Host ligand</th>
</tr>
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<tbody>
<tr>
<td>TLR2</td>
<td>Peptidoglycan, lipoproteins, glycolipids from <em>Staphylococcus</em>, <em>Mycobacteria</em>, <em>Spirochetes</em>, <em>Listeria</em>, <em>Porphyromonas</em>, <em>Klebsiella</em>, <em>N. meningitidis</em></td>
<td>Hsp60</td>
</tr>
<tr>
<td></td>
<td>Parasites e.g. <em>Trypanosoma cruzi</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yeast-derived zymosan</td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>Viral dsRNA</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS from Gram⁺-bacteria</td>
<td>Hsp60</td>
</tr>
<tr>
<td></td>
<td>Lipoteichoic acid from Gram⁻-bacteria</td>
<td>Hsp70</td>
</tr>
<tr>
<td></td>
<td>Plant-derived taxol</td>
<td>Hyaluronan</td>
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<tr>
<td></td>
<td>F protein from RSV</td>
<td>Fibronectin components</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin from Gram⁺- and Gram⁻-bacteria</td>
<td></td>
</tr>
<tr>
<td>TLR6</td>
<td>Diacylated lipoproteins (with TLR2)</td>
<td></td>
</tr>
<tr>
<td>TLR7/8</td>
<td>Viral ssRNA</td>
<td></td>
</tr>
<tr>
<td>TLR9</td>
<td>Unmethylated CpG motifs in bacterial DNA</td>
<td></td>
</tr>
<tr>
<td>TLR11</td>
<td>Uropathogenic bacteria</td>
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</tr>
</tbody>
</table>

The fact that some of the TLRs do not only bind microbial components but also self-antigens, released by cells and tissues during stress such as injury or inflammation has led to the idea that perhaps TLRs originally evolved as receptors for injury-related signals and that microbes subsequently evolved mechanisms to use these receptors for their own benefit (Matzinger 2002).

Another class of stimulatory receptors comprises of TNF-type signals delivered to the DC by other host cells such as T and B lymphocytes, NK cells, NK-T cells, platelets and mast cells. These signals include TNF, CD40 ligand, Fas ligand and
TRANCE (RANKL). The receptors for the T cell-derived signals are upregulated on DCs upon maturation. As a result, when DCs reach the lymphoid organs, T cells are able to influence DC function (Steinman and Pope 2002). Full expression of some DC functions e.g. IL-12 production may require both signals (Schulz et al 2000).

**DCs AND VACCINATION**

Traditionally, vaccines are based on live attenuated or inactivated organisms or inactivated toxins. These, where available, have been very successful in the past, however, there are limitations to their use. Many raise concerns regarding safety, especially in immunocompromised individuals; some are difficult or impossible to grow making vaccine preparation problematic. Therefore, novel vaccine design has concentrated recently on using non-replicating antigens and vectors, such as recombinant protein subunits, synthetic peptides and plasmid DNA (Singh and O'Hagan 1999). Depending on its nature and purity, the antigenic component itself can have a substantial influence on immune responses but generally such vaccines are poorly immunogenic and rely on co-administered adjuvants for induction of immunity (Schijns 2003).

Adjuvants can enhance immunogenicity of weak antigens, increase the speed and duration of the immune response, modulate antibody avidity, isotype and subclass distribution, stimulate cell-mediated immunity, induce immune responses in immunologically immature or senescent individuals, reduce the antigen dose and thereby vaccine costs (Schijns 2003). Aluminium-based mineral salts (generically called alum) are most commonly used but many novel adjuvants are under development and many are being used in clinical trials or even included in licensed vaccines already introduced onto the market (Singh and O'Hagan 1999). Despite their impressive success, the mechanisms underlying their activity are still poorly understood.

Some adjuvants, in particular oil-based emulsions, aluminium or liposomes, assist in vaccination by acting as a depot allowing the prolonged or repeated release of
antigen over a sufficient period of time. Others have direct immunostimulatory properties resulting in provision of the co-stimulation necessary for induction of immunity. Numerous experimental adjuvants contain microbial components that may act as ligands for pathogen recognition receptors such as TLRs on DCs and thereby induce DC maturation. Such activity is found in unmethylated CpG motifs in bacterial DNA, LPS, Lipid A analogs, recombinant yeast vaccines, mycobacterium or other fragments of mostly bacteria (Schijns 2003, Singh and O'Hagan 1999).

Many well-known adjuvants evoke tissue damage and cell stress, thus releasing endogenous signals from necrotic cells (Schijns 2003), which are known to potently activate and mature DCs (Sauter et al 2000, Gallucci et al 1999). This mechanism may have been exploited inadvertently in the attenuated smallpox and measles vaccines. These both infect and kill DCs, yielding infected dead cells which can be internalised and presented efficiently by other DCs (Steinman and Pope 2002).

Lipid particles and microparticulate adjuvants such as liposomes or microspheres have also been successfully targeted to specific immune cells such as the M cells of mucosal associated lymphoid tissue (MALT) by incorporation of M cell-specific lectins (Singh and O'Hagan 1999). Such an approach involving specific lectins and antibodies also shows great promise for direct delivery of antigens to APCs, especially DCs. As expected considering the role of immature DCs in the establishment of tolerance, co-delivery of stimulatory signals was of utmost importance for the induction of competent immune responses (Singh and O'Hagan 1999, Mahnke et al 2003, Bonifaz et al 2002).

Most vaccines induce secretion of neutralizing antibodies which is necessary and/or sufficient for many infectious diseases but not for all. Some of the more challenging diseases such as HIV, tuberculosis, malaria require induction of strong CTL responses which has been difficult to achieve so far with the existing vaccine formulations. Also other viral infections e.g. herpes simplex, papilloma, Epstein Barr and hepatitis C viruses, would require a vaccine that induces cell-mediated rather than humoral immunity (Steinman and Pope 2002).
DC maturation is critical for both the development of high-titre, high affinity antibody as well as strong CTL responses. Many of the novel adjuvants currently undergoing clinical trials are capable of directing DCs to induce potent CTL responses. Examples are monophosphoryl Lipid A (MPL), a lipopolysaccharide from *Salmonella minnesota*, the saponin derivative QS21 and CpG oligonucleotides (Singh and O'Hagan 1999).

CpGs also appear to have significant potential as mucosally administered adjuvants. Induction of strong immune responses at mucosal surfaces is a highly desirable goal for many vaccines as it is at these sites where many pathogens will first be encountered. Access to mucosal DCs for vaccine antigens may be indispensable for establishment of mucosal immunity and topical application would have further important advantages such as easier administration, reduced side effects, better compliance in communities where access to health care professionals is difficult and would avoid the risks stemming from re-use of needles.

However, at the moment hardly any vaccine is given by this route. This is not surprising given the propensity of mucosal DCs in the gastrointestinal or genital tract for tolerance induction (see section 1.6). Overcoming the tolerogenic role of DCs may therefore be especially important in the establishment of mucosal immunity. Genetically modified toxins from *Vibrio cholerae* (CT) and particularly from *Escherichia coli* (LT) are the most potent mucosal adjuvants available. In the past, they have been shown to induce protective immunity in mice against bacterial challenge and to induce potent CTL responses to intranasally delivered HIV-1 p24 gag protein (Singh and O'Hagan 1999).

Both CpGs and LT mutants have shown potential for the modulation of existing immune responses in e.g. chronic infections, cancer, autoimmune diseases or allergies. Novel adjuvanted vaccines have shown some therapeutic benefit in subjects infected with herpes simplex virus 2 and could eradicate established infection with *Helicobacter pylori* in mice (Singh and O'Hagan 1999).
IMMUNE EVASION VIA ALTERED DC MATURATION

TLR signalling potently induces DC maturation and release of pro-inflammatory cytokines, further enhanced by TNF-type signals, resulting in the induction of antigen-specific effector responses and elimination of the invading pathogen. Many pathogens have evolved mechanisms that interfere with DC function in general and DC maturation in particular in order to avoid effector responses and elimination. A common theme is emerging in that many pathogen-derived molecules either prevent DC maturation completely or alter it so that the DC matures but loses its capacity to prime effector cells and instead becomes a tolerogenic cell that releases large amounts of IL-10 and is able to directly anergise antigen-specific cells or prime suppressive regulatory cells.

Pathogens employ a multitude of different strategies to interfere with or take advantage of DC function including antigen uptake, migration, maturation and antigen presentation. They can interfere with antigen presentation by inhibiting phagosome/lysosome fusion and pathways for both MHC class I and MHC class II presentation, e.g. modulating MHC synthesis, trafficking or expression, antigen processing (mycobacteria, Salmonella, Yersinia). They may compromise progenitor cells and impair their development into DCs (lymphocytic choriomeningitis virus, LCMV). During chronic *Leishmania donovani* infection, DCs are produced in abundance but, as a result of CCR7-deficiency, they do not migrate properly. Also *Schistosoma mansoni* egg antigens suppress migration of Langerhans cells (Schoppet et al 2000, Harding et al 2003, Iwasaki and Kelsall 1999). Vaccinia produces cytokine receptor homologues in the early cycle. These ‘mop up’ inflammatory cytokines produced by infected tissues, thereby avoiding activation of local DC and induction of immunity (Engelmayer et al 1999).

HIV appears to use a ‘Trojan horse’ strategy, i.e. it does not productively infect DCs but is carried by maturing DCs to lymph nodes where it is efficiently transferred to its final target, T cells, during DC-T interaction. Upon binding to DC-SIGN on DCs, the virus is taken up into nonlysosomal compartments where its stability and infectivity appears to become enhanced instead of reduced. Other pathogens, such as Ebola (Simmons et al 2003), dengue virus (Tassaneetrithep et al 2003), Helicobacter
pylori, Leishmania mexicana, Schistosoma mansoni and Mycobacterium tuberculosis (Appelmelk et al. 2003, Geijtenbeek et al. 2003) can also bind to DC-SIGN. C-type lectins appear to be particularly amenable for pathogens to evade immunity through modulation of DC function (see below).

Plasmodium falciparum (Urban et al. 1999), measles virus (Fugier-Vivier et al. 1997), human cytomegalovirus (Grigoleit et al. 2002, Moutaftsi et al. 2002), Ebola and Lassa virus, herpes simplex virus and LCMV (Steinman and Pope 2002) block DC maturation directly, thus impairing T cell activation. Expression by DCs of the viral determinant Nef (HIV and SIV) allows production of inflammatory cytokines and chemokines upon activation but inhibits phenotypic maturation. This strategy facilitates virus dissemination to newly recruited cells at the site of infection but avoids development of virus-specific immunity (Messmer et al. 2002). Measles virus infection of DCs induces expression of TNF-related apoptosis-inducing ligand (TRAIL), rendering the infected DCs cytotoxic for T cells with which they interact (Vidalain et al. 2001).

In contrast to simply blocking DC maturation to avoid any immune response, a variety of microbial pathogens directly modulate DC maturation to either stimulate the incorrect type of effector responses, i.e. T\textsubscript{H}1 versus T\textsubscript{H}2, or convert immunogenic DC into tolerogenic cells thereby evading and actively suppressing specific immunity. A variety of pathogen-derived molecules bind to antigen receptors on DCs which appear to negatively influence TLR signalling and alter the cytokine balance produced by DCs upon activation, i.e. IL-12 levels are reduced and IL-4 or IL-10 production are dramatically enhanced. Such tolerogenic DCs induce the development of adaptive regulatory T cells with antigen-specific suppressor activity instead of effector cells. The Bordetella pertussis adhesin FHA (filamentous hemagglutinin) binds to CR3 on DCs and induces IL-10 and inhibits LPS-driven IL-12 production. Such DCs contribute to the severe suppression of T\textsubscript{H}1 effector responses observed in the acute phase of infection by stimulating the development of IL-10-producing suppressive regulatory cells (McGuirk et al. 2002). Also erythrocytes infected with blood-stage plasmodium (Ocana-Morgner et al. 2003), the mycobacterial cell wall antigen ManLAM (mannose-capped lipoarabinomannan) (Geijtenbeek et al. 2003),
HIV-1 envelope glycoprotein gp120 (Geijtenbeek et al 2000a) or schistosomal lysophosphatidylserine (van der et al 2002) induce dysregulated cytokine production in DCs. Binding of ManLAM to DC-SIGN results in leads to high levels of IL-10 production by DCs upon challenge with LPS and suppression of effector immunity (Geijtenbeek et al 2003). DC-SIGN not only functions in negative regulation of TLR signalling but normally serves as an antigen receptor that internalises antigens for targeting to late endosomal compartments and efficient presentation to T cells (Engering et al 2002a) and also as an adhesion molecule involved in tethering and rolling of immature DCs from blood vessel to inflammatory sites (Geijtenbeek et al 2000b).

Also other antigen receptors seem capable of modifying TLR signalling. Binding of the ligands mannose, ManLAM or a monoclonal antibody to the mannose receptor inhibits LPS-induced IL-12 production by human DCs (Nigou et al 2001). The C-type lectin BDCA-2 is expressed specifically by plasmacytoid DCs which release very high levels of Type I IFN upon TLR ligation. When BDCA-2 is engaged simultaneously, IFN production is reduced dramatically indicating that also BDCA-2 can deliver a negative signal and interfere with TLR signalling (Engering et al 2002b).

Immune evasion and/or profound immune suppression are also found in cancer. Just as the pathogens mentioned above, tumours employ a variety of strategies to actively suppress DC function, modulate their maturation to maintain the immature state or convert them into tolerogenic DCs allowing efficient suppression of tumour-specific immunity. Supernatants from HeLa-cell cultures enhance DC migration but inhibit their maturation (Kudela et al 2001). The prostate cancer microenvironment impairs DC development from precursors and inhibits their maturation. It also kills newly arriving mature DCs (Aalamian et al 2001). Enk and colleagues studied DCs from progressing and responding metastases in melanoma patients. Melanoma cells from progressing metastases produced large amount of IL-10 and metastasis-derived DCs induced antigen-specific anergy. In contrast, supernatants from responding metastases contained IL-2, IFN-γ and IL-12 and metastasis-derived DCs had potent T
cell stimulatory capacity and did not induce anergy (Enk et al 1997). IL-10 is produced by many tumours.

In summary, numerous infections as well as many different types of tumours progress because impaired DC maturation hinders the development of specific immunity. Therefore, restoring competent DC maturation is a highly desirable goal for immunotherapy. A variety of different approaches are being taken to solve this problem.

In chronic infections such as leishmaniasis, DCs engineered to constitutively produce IL-12 might be used to revert harmful Th2 to protective Th1 responses. In HIV-1 vaccine trials mature DCs pulsed with antigen are injected for active vaccination. This approach has also been used for cancer vaccination. Antigen can be targeted to DCs in vivo directly, but the most common clinical protocol involves generation of large numbers of DCs from a patient ex vivo, loading cells with tumour antigens and exposure to a maturation stimulus ex vivo before re-administration to the patient to enhance or induce tumour-specific cell-mediated immunity.

DCs IN AUTOIMMUNITY AND TRANSPLANTATION

Maturation of DCs is generally a crucial requisite for generation of immunity. However, it can sometimes also lead to responses, which are detrimental for the host. DCs are implicated as the major players in various autoimmune diseases and complications arising from transplantation, such as graft-versus-host disease (Lechler et al 2001). Autoimmune disease may be triggered during an infection as a result of presentation of self-antigens or cross-reactive pathogen-derived molecules by mature DCs. This may lead to ‘accidental’ activation of autoreactive T cells and the development of autoimmune disease (Horwitz and Sarvetnick 1999).

Recently it has become clear that in addition to initiating protective immunity, DCs also play a major role in the establishment of peripheral tolerance. This has led to the hope that active immunization with tolerogenic DCs might be used to dampen
autoimmune responses and prolong graft survival after transplantation. Immature DCs have been shown to prime regulatory cells that can suppress effector responses in an antigen-nonspecific manner in vitro and in vivo both in mice and humans (see section 1.6). However, the use of immature DCs entails the risk that the cells may inadvertently be activated and matured in situ. One solution to this problem is to use cells that have been manipulated in vitro before injection to differentiate into modulated DCs with stably reduced immunogenicity and enhanced tolerogenic potential. This can be achieved by genetically engineering DCs to constitutively express IL-10, TGF-β, FasL or CTLA-4. All of these factors are used by DCs in various settings to induce differentiation into regulatory and suppressive cells (Lechler et al 2001). Tolerogenic DCs and regulatory cells can also be generated by exposure to various immunosuppressive drugs, such as dexamethasone or vitamin D₃ (de Jong et al 1999, Verhoeven et al 2000, Rea et al 2000, Berer et al 2000, Lechler et al 2001, O'Garra and Barrat 2003).

The tolerogenic role of DCs may compromise vaccine efficiency. It may, however, be useful in the design of a new class of 'drugs' for suppressing immunity in autoimmune diseases, allergy and transplantation. The propensity of mucosal DCs in the gut and the respiratory tract for tolerance induction has been exploited in models of experimental autoimmune diseases where feeding mice proteins from target tissues, such as insulin or myelin basic protein, protected them against autoimmune diabetes or experimental allergic encephalomyelitis (EAE) respectively. Disappearance of autoreactive $\text{T}_{\text{H}1}$ cells was inversely correlated with appearance of TGF-β-producing regulatory CD4⁺ T cells (Hartmann et al 1999, Janeway, Jr. and Travers P. 1997).
It is clear from the evidence in this review that there is great potential for using the tremendous potency of DCs in vaccination and immunotherapy. The ultimate challenge is to design prophylactic and therapeutic vaccines that induce optimally effective immune responses in different clinical settings. Modulation of DC function and controlling DC maturation are critical control points. I set out to study DC function in three different settings where DC maturation is key to competent immune responses: the age-related development of functional competence during early life, the role of DCs in the response to polysaccharide and glycoconjugate vaccine antigens and the pre-clinical evaluation of novel protein vaccine antigens in a DC-based culture system.

The first question I addressed was that of DC function in early life. While studies on cord blood DCs have highlighted important functional deficiencies of neonatal DCs, it is not known whether these deficiencies persist beyond the first days of life where they may play an important role in the altered immune responsiveness observed in infants. A particularly important example of altered responsiveness in early life is the deficient immune response to capsular polysaccharide antigens of encapsulated organisms such as *S. pneumoniae*. DCs were generated from infants during this period and compared to DCs derived from adults with regard to their ability to undergo maturation in response to inflammatory stimuli.

While it is clear that a number of components deficient in early life play a role in the impaired responses to polysaccharide antigens, the role of DCs in such responses in general and in early life is unclear. Therefore in the second part of this thesis, I set out to examine the interaction between DCs and such antigens *in vitro*. I hypothesized that DCs are indirectly or directly involved in such responses and impaired DC maturation and function in early life may contribute to the deficient responses to polysaccharide antigens in infants.

Continuing difficulties in the development of a conjugate vaccine for *S. pneumoniae* has focussed much recent attention on the identification of potential protein-based
vaccines. Induction of DC maturation is imperative for induction of efficient cellular immune responses. Most antigens, however, do not themselves induce DC maturation and must therefore be given in conjunction with adjuvants. Evaluating the potential of vaccine candidates to interact with DC's in the absence and presence of adjuvant as well as evaluating DC antigen presentation to T cells, is an important component of the Phase I evaluation of putative vaccine antigens.

In this thesis I have utilised this approach in the development of vaccines against common diseases of very young infants, such as otitis media. Assays of this kind are currently lacking in vaccine development. I addressed this issue by establishing a DC-based model system for the evaluation of cellular immune responses to potential vaccine antigens during *in vitro* presentation. I included assays detecting T cell memory in unvaccinated adults in order to assess the potential of such vaccine candidates to induce cellular immune responses during natural exposure. Proof of concept was provided by assessing a variety of microbial proteins, which are all currently being considered as potential vaccine candidates for otitis media in infants.
Chapter II

Materials and Methods
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II. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. General reagents

General buffers and reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated. Antibodies were generally purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

2.1.2. Tissue culture reagents

Culture media was purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA) unless otherwise stated. Low endotoxin plasticware was purchased from Falcon (BD, Bedford, MA, USA) and pre-packaged endotoxin-free pipette tips from Gilson (Middleton, WI, USA). The limulus amebocyte lysate (LAL) quantitative chromogenic assay (Chromogenix, Sweden) was used for the determination of any endotoxin contamination in the reagents.

Complete medium comprised of RPMI-1640 with 25mM HEPES and L-Glutamine supplemented with 1% penicillin-streptomycin and 10% foetal calf serum (FCS). A specialised batch of FCS with no/low endotoxin contamination (Invitrogen/Life Technologies [Carlsbad, CA, USA]) was used to minimise unwanted maturation of the generated DCs. AB medium comprised of RPMI-1640 with 25mM HEPES and L-glutamine supplemented with 1% penicillin-streptomycin and 10% human serum type AB (Sigma, St. Louis, MO, USA).

2.1.3. Buffers and other solutions

- MACS CD14 selection
  MACS buffer: PBS with 0.5% BSA
II. MATERIALS AND METHODS

- **ELISA**
  ELISA coating buffer: 0.1 M sodium carbonate (8.4g NaHCO₃ and 3.56g Na₂CO₃ per 1 L), pH 9.5
  ELISA wash buffer: PBS with 0.1% TWEEN-20
  ELISA assay diluent: PBS with 10% FBS
  ELISA stop solution: 2N H₂SO₄

- **ELISPOT**
  Elispot wash buffer: PBS with 0.05% TWEEN-20

- **Intracellular FACS staining**
  FACS buffer: PBS with 0.1% sodium azide and 1% FCS
  Perm buffer: FACS buffer with 0.1% saponin

2.1.4. Antigens

- LPS (from *E.coli* 026:B6, Sigma, St. Louis, MO, USA). Used at 0.5 μg/ml unless stated otherwise.
- Dextran: FITC-labelled 3K and 500K dextran, TexasRed-labelled 70K dextran, obtained from Molecular Probes (Eugene, OR, USA), used at 1mg/ml unless stated otherwise.
- PPS: Purified capsular polysaccharides derived from *Streptococcus pneumoniae* (serotypes 1, 6B, 9N, 14, 19F and 23 F), obtained from the American Type Culture Collection (Rockville, USA) and diluted in distilled water to a concentration of 1mg/ml. Concentrations used in cell cultures were between 4.4 and 6.4 μg/ml, unless stated otherwise, adjusted for each serotype to match the concentration of the polysaccharide component in 10 μg/ml conjugate.
- FITC-labelled PPS: Fluorescently labelled pneumococcal capsular polysaccharide (serotypes 14 and 19F), kindly provided by Dr. Chris Jones, NIBSC, UK, used at 60μg/ml.
II. MATERIALS AND METHODS

- **PPS-TT**: Above pneumococcal capsular polysaccharides (serotypes 1, 6B, 9N, 14, 19F and 23F) conjugated to a tetanus toxoid carrier, used at a concentration of 10 μg/ml for the carrier component, obtained from Jean Michel Chapsal at Pasteur Merrieux, Lyon, France.
- **TT**: Control antigen tetanus toxoid, used at 10 μg/ml, obtained from Jean Michel Chapsal at Pasteur Merrieux, Lyon, France.
- **S. pneumoniae**: Whole heat-killed *S. pneumoniae* bacteria, prepared as described in section 2.3, used at a ratio of 100:1 in DC cultures.
- **Haemophilus influenzae** capsular polysaccharide polyribitolribosyl phosphate (PRP) and its conjugate PRP-TT, both obtained from Jean Michel Chapsal at Pasteur Merrieux, Lyon, France.
- **Streptococcus pneumoniae** antigens PhtD, N-ter PhtD, C-ter PhtD and N-ter CbpA, kindly provided by Jan Poolman at GlaxoSmithKline, Rixensart, Belgium.
- Nontypeable *Haemophilus influenzae* PD and LB1, both produced by Professor Lauren D Bakaletz, Department of Pediatrics, The Ohio State University, College of Medicine and Public Health, Columbus, Ohio, provided subject to agreement with SmithKline Beecham Biologicals.
- **Moraxella catarrhalis** antigens CopB, CD and UspA, provided by John McMichael, Wyeth Lederle Vaccines and Pediatrics, Rochester, NY.

2.1.5. Blood samples

Peripheral blood was venisected from healthy adult volunteers. Infant peripheral blood was collected by anaesthetic staff at the time of induction in children attending Great Ormond Street Hospital for routine minor surgical procedures. Prospective children were selected by a pediatric registrar from planned general and ENT surgical lists. The child was recruited to the study if there was no history of problems with infection, no recent viral infection and no documented or suspected chromosomal or genetic abnormality. Informed consent was gained from the mothers prior to venisecion, with approval by the Great Ormond Street Hospital and the Institute of Child Health Research Ethics Committee. The blood was collected into heparinized tubes and used as soon as possible to avoid activation, usually within one hour.
II. MATERIALS AND METHODS

2.2. CELL CULTURE

2.2.1. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from healthy adults and infants by standard Ficoll-Paque method using Lymphoprep (Nycomed, Roskilde, Denmark). Initially the blood was diluted 1:1 in RPMI-1640, carefully layered over the ficoll and centrifuged at 2200 rpm (~680g) for 25 minutes. Acceleration was low and the brakes switched off for this spin to avoid disturbing the layers as this reduced yield. The opaque interface containing mainly B and T lymphocytes, NK cells and monocytes was then removed, diluted at least 1:1 with RPMI-1640 and spun at 1400 rpm (~380g) for 10 minutes. The cell pellet was washed again in RPMI-1640 and spun at 1100 rpm (~250g) for 5 minutes. This slower spin removed the majority of platelets which otherwise would have interfered with subsequent procedures. The cells were then resuspended in complete medium for counting using a haemacytometer.

2.2.2. Generation and culture of dendritic cells

For DC preparations, CD14⁺ monocytes were positively selected from PBMCs using magnetic anti-CD14 microbeads, MiniMACS columns and magnet (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMCs were resuspended in 100μl/50x10⁶ cells ice-cold MACS buffer and incubated with 1μl/1x10⁶ cells anti-CD14 microbeads for 15 minutes in a fridge. The tube was flicked repeatedly during incubation to mix. All reagents were kept ice-cold throughout the procedure to minimise phagocytic uptake of beads. Cells were resuspended in 15ml MACS buffer and spun at 1400 rpm (~380g) to wash. A MiniMACS column was placed into the appropriate magnet and 500μl MACS buffer passed through it. The cell pellet was resuspended in 1 ml MACS buffer and passed through a MiniMACS column. The column was washed three times with 500μl MACS buffer and the CD14⁺ cells retained inside the column eluted with a 1ml of MACS buffer. The CD14⁺ fraction containing mostly B and T lymphocytes and NK cells was washed, frozen slowly to -80°C in a Cryobox (Nalgene) in freezing mix comprising of 90%FCS with 10% dimethylsulphoxide (DMSO) and stored for co-culture experiments.
II. MATERIALS AND METHODS

The CD14\(^+\) monocytic fraction was cultured in RPMI-1640 with 25mM HEPES and L-glutamine supplemented with 1% penicillin and streptomycin (10,000 IU/ml, Invitrogen/Life Technologies) and 10% LPS-low fetal calf serum (Myoclone Superplus, Invitrogen/Life Technologies) in the presence of 100 ng/ml recombinant human granulocyte-macrophage colony stimulating factor (Leucomax [Sandoz Pharmaceuticals, Camberley, UK]) and 125 ng/ml recombinant human interleukin-4 (gift from Professor D. Katz, University College London, UK).

On day 6, nonadherent, immature DCs were harvested, i.e. they were gently washed from the wells, pooled and spun at 1000 rpm (~200g) for 10 minutes. The cell pellet was resuspended in complete medium for phenotyping (see section 2.4) or AB medium for co-culture experiments (see section 2.2.3). This procedure gave pure preparations of immature DCs as assessed routinely by FACS analysis of a panel of antibodies including CD11c, Lin-1, CD14, HLA-DR, CD83, CD86, CD40, CD1a,b,c,d and appropriate isotype controls (see section 2.4).

Immature DCs were re-plated on 24-well or 48-well plates at 200,000 to 400,000 cells/ml depending on experimental requirements and cell numbers. DCs were stimulated for 24 hours with a variety of antigens at concentrations as listed in section 2.1.-Antigens and/or activated and matured by addition of 0.5\(|\mu|g/ml LPS.

2.2.3. DC-PBMC co-culture

DCs were generated as described above and stimulated with a variety of antigens and/or LPS for 24 hours. Frozen CD14\(^+\) PBMCs were defrosted rapidly, diluted in at least 1:15 RPMI-1640, spun at 1400rpm (~380g) to wash off the DMSO and resuspended in AB medium. DCs washed three times in RPMI-1640 to remove free antigen and co-cultured with PBMCs for 7 to 8 days at a ratio of 1:10 in round-bottom 96-well-plates at 10,000 DC/well for Elispots, 20,000 DC/well for intracellular cytokine staining and 30,000 DC/well for measurement of proliferation by CFSE staining. For the latter, PBMCs were stained with CFSE before addition to the DCs, see section 2.12.
2.3. PREPARATION OF WHOLE, HEAT-KILLED STREPTOCOCCUS PNEUMONIAE

2.3.1. Preparation of bacteria

Cultures of *S. pneumoniae* serotypes 1, 6B, 9N, 14, 19F and 23F (American Type Culture Collection) were streaked onto blood agar plates and incubated overnight at 37°C/5% CO₂. Small quantities of the pure cultures were inoculated into Todd Hewitt broth containing 0.5% yeast extract and 5% heat-inactivated, filtered human AB serum (both Sigma, St. Louis, MO, USA) to give a change in OD of approx. 0.05 at 660nm. Bacterial suspensions were incubated at 37°C/5% CO₂ until the pneumocci were at log phase, after approximately 4 to 5 hours, giving an OD of 0.5 to 0.6 at 660nm. Cultures were heat-inactivated for one hour at 60°C and a small quantity streaked onto blood agar plates to confirm that bacteria are non-viable. Suspensions were centrifuged, brought to an OD of 1.0 (approximately 1 x 10⁹ bacteria/ml), split into aliquots of 1 ml each, washed three times with ice cold 1% BSA-HBSS, resuspended in 1ml 1%BSA-HBSS, aliquoted into 100μl aliquots and stored at -70°C until use.

2.3.2. The Quellung reaction

The Quellung reaction was performed to confirm that all strains were highly encapsulated. In brief, 5 μl of the bacterial suspension were spread out on a glass slide, left to dry, mixed with 5 μl of serum containing irrelevant or relevant capsule-type specific antibody (Statens Serum Institute, Copenhagen, Denmark) and 5 μl of 0.3% methylene blue, covered with a coverslip and observed under x100 oil immersion lens. The bacterial cells stained blue and were surrounded by a clear halo, confirming the presence of a polysaccharide capsule.
2.3.3. Agglutination tests

Agglutination tests were used to corroborate the results of the Quellung reaction and to verify the specificity of the strains. For each strain, 10μl of bacterial suspension were mixed with either irrelevant or relevant pool serum or suspected type serum (Statens Serum Institute, Copenhagen, Denmark) on a glass slide and gently rocked to mix liquids slowly. Formation of small but distinct aggregates was a positive result indicating that the capsular antigen had bound to specific antibody.
2.4. PHENOTYPING OF DCs

DCs were resuspended in their wells, transferred to FACS tubes (100μl/tube) containing various combinations of antibodies shown in table 2.1. and incubated in the dark for 30 minutes on ice. 2ml of cold PBS were added and samples spun at 1400rpm (350g) for 5 minutes. The cell pellets were resuspended in 300μl 1% paraformaldehyde (PFA). Marker expression was analysed on a Beckman Coulter XL flow cytometer with EXPO2 software. Relevant isotype control antibodies were used, with gating arbitrarily set between 1% and 2% positive. All antibodies were used at 1:20 dilution.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone</th>
<th>Source^a)</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c (PE)</td>
<td>S-HCL-3</td>
<td>BD</td>
<td>mouse IgG2b</td>
</tr>
<tr>
<td>CD25 (FITC)</td>
<td>M-A251</td>
<td>BD</td>
<td>mouse IgG1</td>
</tr>
<tr>
<td>CD45 (FITC)</td>
<td>2D1</td>
<td>BD</td>
<td>mouse IgG1</td>
</tr>
<tr>
<td>CD83 (FITC)</td>
<td>HB15e</td>
<td>BD</td>
<td>mouse IgG1</td>
</tr>
<tr>
<td>CD86 (FITC/PE)</td>
<td>2331</td>
<td>BD</td>
<td>mouse IgG1</td>
</tr>
<tr>
<td>CD14 (FITC)</td>
<td>M5E2</td>
<td>BD</td>
<td>mouse IgG2a</td>
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<tr>
<td>CD40 (PE)</td>
<td>5C3</td>
<td>BD</td>
<td>mouse IgG1</td>
</tr>
<tr>
<td>HLA-DR (PE)</td>
<td>L243</td>
<td>BD</td>
<td>mouse IgG2b</td>
</tr>
<tr>
<td>CD1a (CY)</td>
<td>HI149</td>
<td>BD</td>
<td>mouse IgG1</td>
</tr>
<tr>
<td>CD1b (FITC)</td>
<td>M-T101</td>
<td>BD</td>
<td>mouse IgG1</td>
</tr>
<tr>
<td>CD1c (purified)</td>
<td>11.86</td>
<td>BD</td>
<td>mouse IgG2a</td>
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<td>2° IgG2a (FITC)</td>
<td>G155-78</td>
<td>BD</td>
<td>goat IgG2a</td>
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<td>CD1d (PE)</td>
<td>CD1d42</td>
<td>BD</td>
<td>mouse IgG1</td>
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<td>Lin1^b) (FITC)</td>
<td>Mixture</td>
<td>BD</td>
<td>mouse IgG2a</td>
</tr>
<tr>
<td>IgG1 (FITC/PE)</td>
<td>-</td>
<td>BD</td>
<td>isotype control</td>
</tr>
<tr>
<td>IgG2 (FITC/PE)</td>
<td>-</td>
<td>BD</td>
<td>isotype control</td>
</tr>
</tbody>
</table>

^a) Becton Dickinson (BD).

^b) Contains antibodies against CD3, CD14, CD16, CD19, CD20 and CD56
II. MATERIALS AND METHODS

2.5. ELISA

Cytokine production was analysed from lymphocyte culture supernatants with commercial kits for IL-12 (BD Biosciences Pharmingen, San Diego, CA, USA) and IL-10 (eBioscience, San Diego, CA, USA) using reagents and instructions supplied by the manufacturer. In brief, 96-well plates were coated with capture antibody diluted 1:250 and incubated overnight at 4°C. Prior to use, the wells were washed three times with ELISA wash buffer and blocked for 1 hour with assay diluent. Samples and standards were defrosted and diluted as desired in assay diluent. A range of standards (7.8pg/ml to 500pg/ml for IL-12 ELISA and 3.1pg/ml to 200pg/ml for IL-10 ELISA) was prepared by serial dilution. Wells were washed three times with ELISA wash buffer and samples and standards added in duplicate. After 2 hours incubation at room temperature, binding was visualized by incubation with the relevant biotinylated detection antibody at 1:250 dilution for 1 hour, then avidin-horse radish peroxidase (HRP) conjugate detection enzyme at 1:250 dilution for 30 minutes. Wells were washed five times with 200μl ELISA wash buffer after each incubation step. Wells were then incubated with substrate solution (tetramethylbenzidine and hydrogen peroxide) incubated until colour had developed sufficiently and the reaction stopped by adding stop solution (2N H₂SO₄). Developed and stopped plates were read immediately at 450nm with correction at 570nm, using a Dynatech MRX plate reader with revelation software. Samples were used neat, as 1:5 or 1:50 dilutions depending on the experiment. Results are expressed as the concentration of cytokine (pg/ml) in the culture supernatant of 0.4 x 10⁶ cells, incubated at this concentration/ml in 24-well plates.

2.6. IMAGING OF HOMOTYPIC DC CLUSTERS

Immature DCs were plated in 24-well tissue culture plates at a concentration of 1-4 x 10⁵/ml cells and were left unchallenged or incubated for 24 hours with antigen. After 20 hours incubation, the DCs were imaged using a Hamamatsu digital camera attached to a Zeiss Axiovert 135 microscope with x10 and x40 Hoffman lenses. The software was Improvision Openlab, version 3.03, run on an Apple Macintosh computer.
2.7. CONFOCAL MICROSCOPY

1-2 x 10^4 DCs were plated onto 18x18mm coverslips (BDH) coated with 10μg/ml human fibronectin (Sigma, St. Louis, MO, USA) and allowed to adhere and polarize at 37°C for 30 min to 2 hr. They were fixed for 15 minutes PBS with 4% PFA/3% glucose at room temperature and washed six times with 1 ml PBS. Cells were then permeabilised for 5 minutes in 0.5% TRITON X-100, washed three times with 1 ml PBS and blocked for at least 20 minutes with PBS plus 1% BSA. Cells were stained for 30 minutes with 2U/ml rhodamine-labelled phalloidin for localisation of filamentous actin and with 2μM TO-PRO 3-iodide (both from Molecular Probes, Eugene, OR, USA) for visualization of the nucleus.

For visualisation of other structures, permeabilized cells were stained with unconjugated, monoclonal, mouse anti-human antibodies against CD71 (transferrin receptor, TfnR, specific for early endosomes), CD107a (lysosomal-associated membrane protein-1, LAMP-1, specific for late endosomes and lysosomes) or HLA-DR. HLA-DR localised to late endosomes and lysosomes in immature DCs and to the cell surface in mature DCs. Alexa488- or Alexa546-conjugated anti-mouse antibodies were used as the second layer. All antibodies were obtained from Molecular Probes (Eugene, OR, USA). Antibodies were diluted in PBS to 1:40 (primary antibodies) or 1:100 (secondary antibodies). Cells were washed in PBS and dH₂O, mounted on glass microscope slides with a drop of Citifluor and sealed with nail varnish.

In endocytosis experiments, adhered, polarized cells were fed fluorescently labelled antigen for the indicated period of time before fixation, permeabilization and staining as described above.

Confocal images were obtained using a confocal laser scanning microscope system (TCS NT, Leica, Switzerland) fitted with appropriate filter sets. To combine a three-dimensional z series into a two-dimensional image, 10 to 20 optical sections (0.5 μm) spanning the entire cell were projected and superimposed. In double-labelling experiments, bleed-through corrections were done according to the manufacturer’s
instructions. To visualise co-localisation of two fluorochromes, the images in the two channels were merged. A yellow signal was interpreted as co-localization. Images were processed with the TCS Leica Start and Adobe Photoshop 5.0 software.

2.8. FLOW CYTOMETRIC ENDOCYTOSIS ASSAY

DCs (approx. 25,000 cells in 100 µl complete medium) were incubated for the indicated times with FITC-labelled antigen in FACS tubes in the dark. Samples were set up in duplicate and incubated either at 37°C to allow uptake or on ice to quantify background staining. In some experiments, cells were pre-incubated with 10 nM cytochalasin D or 100 nM wortmannin (both Sigma, St. Louis, MO, USA), concentrations that had previously been shown to strongly inhibit receptor-mediated endocytosis and macropinocytosis (Hiltbold et al 2000). Surface-bound antigen was quenched with 75 µl quenching solution (ORPEGEN Pharma, Heidelberg, Germany), cells were washed in PBS and analysed immediately on a Beckman Coulter XL flow cytometer with Expo2 software.
2.9. PBMC ELISPOT

Sterile 96-well polyvinylidene difluoride (PVDF) filter plates (Millipore, Billerica, MA, USA) were coated with 15μg/ml capture antibody (Mabtech, Sweden) for 2 hours at 37°C, washed 8 times with 200μl Elispot buffer and blocked with 200μl complete medium. Freshly purified PBMCs were plated in pre-coated and blocked Elispot plates and incubated for 24 hours with antigens directly added to the cell suspensions in the wells. Cells were then washed off (8x200μl Elispot buffer). Wells were incubated with 1μg/ml biotinylated detection antibody (Mabtech, Sweden) and, after a further 8 washes, with 4 μg/ml ExtrAvidin alkaline phosphatase conjugate (SIGMA, St. Louis, MO, USA). Captured cytokine was visualised after addition of 100μl of the BCIP/NBT substrate (Bio-Rad Laboratories, Hercules, CA, USA), diluted 1:100 in the substrate buffer provided by the manufacturer. Plates were washed under tap water when the colour of the spots was sufficient and dried in the hood. Spots were counted using a Bio-Sys ELISpot reader (Bio-Sys, Karben, Germany).

Table 2.2. Elispot antibodies.

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ</th>
<th>IL-5</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capture antibody, purified</td>
<td>Clone 1-D1-K</td>
<td>Clone TRFK5</td>
<td>Clone 9D7</td>
</tr>
<tr>
<td>Detection antibody, biotinylated</td>
<td>Clone 7-B6-1</td>
<td>Clone JES1-5A10</td>
<td>Clone 12G8</td>
</tr>
</tbody>
</table>

c) All antibodies were obtained from Mabtech, Nacka, Sweden.

2.10. DC-PBMC ELISPOT

DCs and PBMCs were co-cultured as described in section 2.2.3. for 7 to 8 days. Plates were spun at 1400 rpm (~380g) and supernatants taken off and stored for further analysis. Cells were transferred to an Elispot plate, pre-coated and blocked as described for the PBMC Elispot in section 2.9. Controls were unstimulated DCs, stimulated DCs, PBMCs alone, PBMCs activated for 24 hours with 25ng/ml phorbol 12-myristate 13-acetate (PMA) and 2.5μg/ml ionomycin (both Sigma, St. Louis, MO, USA) and co-cultures of unpulsed DCs and PBMCs at a ratio of 1:10. Captured cytokine was visualized after 24 h as described in section 2.9.
2.11. INTRACELLULAR CYTOKINE STAINING

DC-PBMC co-cultures were set up as described in section 2.2.3. and incubated for 7 to 8 days. 25μg/ml brefeldin-A (Sigma, St. Louis, MO, USA) were added for the final 16 hours of culture. Cells were resuspended in their wells, transferred to 15ml plastic tubes, diluted with 1 ml cold FACS buffer and spun at 1400 rpm (~350g) to wash. All reagents were kept cold throughout the procedure. Cell pellets were resuspended in FACS buffer and cells transferred to a 96-well-plate at 100μl/well. The plate was centrifuged at 1200 rpm (~200g) for 7 minutes, the supernatant flicked off and the plate vortexed to resuspend the pellets. These were then fixed in 100μl/well 4% Pfa for 10 minutes at room temperature. Cells were washed in 50μl/well FACS buffer, then 150μl/well Perm buffer and then resuspended in 25μl/well Perm buffer containing the required antibodies. Antibodies were diluted as shown in table 2.3. The plate was incubated for 30 to 60 minutes in the dark at 4°C. Cells were washed twice in Perm buffer and once in FACS buffer and finally resuspended in 200μl FACS buffer and transferred to FACS tubes for analysis on a Beckman Coulter XL flow cytometer with EXPO2 software.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone</th>
<th>Source</th>
<th>Isotype</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 (FITC)</td>
<td>Q4120</td>
<td>Sigma</td>
<td>mouse IgG1</td>
<td>1:50</td>
</tr>
<tr>
<td>CD4 (PE)</td>
<td>MT310</td>
<td>DAKO</td>
<td>mouse IgG1</td>
<td>1:40</td>
</tr>
<tr>
<td>IFN-γ (PE)</td>
<td>4S.B3</td>
<td>Pharmingen</td>
<td>mouse IgG1</td>
<td>1:25</td>
</tr>
<tr>
<td>IL-5 (PE)</td>
<td>JES1-39D10</td>
<td>Pharmingen</td>
<td>mouse IgG1</td>
<td>1:25</td>
</tr>
<tr>
<td>IL-10 (PE)</td>
<td>JES3-9D7</td>
<td>Pharmingen</td>
<td>mouse IgG1</td>
<td>1:25</td>
</tr>
<tr>
<td>IgG1 (PE)</td>
<td>-</td>
<td>Becton Dickinson</td>
<td>isotype control</td>
<td>1:25</td>
</tr>
</tbody>
</table>
2.12. MEASUREMENT OF PROLIFERATION BY CFSE STAINING

DC-PBMC co-cultures were set up as described in section 2.2.3. However, PBMCs were labelled with carboxyfluorescein diacetate, succinimidy ester (CFDA, SE [Molecular Probes, Eugene, OR, USA]) before addition to the DCs.

CFDA, SE spontaneously penetrates cell membranes and is converted to carboxyfluorescein succinimidy ester (CFSE) by intracellular esterases. CFSE then couples irreversibly to intracellular proteins producing uniform and stable fluorescent labelling that can be analyzed by flow cytometry using excitation at 488 nm and the FL1 detection channel. When cells divide, CFSE labelling is distributed equally between the daughter cells. Successive generations can therefore be distinguished as populations undergoing successive halving of fluorescence (Hodgkin et al 1996).

PBMCs were washed in RPMI-1640 and resuspended in 6 to 10 ml RPMI-1640. CFDA, SE was added to a final concentration of 1µM and cells incubated for 10 minutes at 37°C. The reaction was stopped by diluting the cell suspension in 20 to 30 ml RPMI-1640 supplemented with 10% FCS. Cells were spun at 1400 rpm (~380g), the pellet washed again in RPMI-1640+10%FCS and then in RPMI-1640 without added FCS. Labelled cells were then resuspended in AB medium and an aliquot taken out and fixed in 1% PFA as a baseline control. The remaining cells were incubated with DCs for 7 to 8 days as described in section 2.2.3.

As a positive control, 5ng/ml phytohaemagglutinin (PHA, Sigma, St. Louis, MO, USA) were added for the final 4 days of culture. Cells were then resuspended in their wells, stained with CD4 PE using the same procedure as described in section 2.11 and transferred to FACS tubes for analysis on a Beckman Coulter XL flow cytometer with EXPO2 software. The baseline control cells were used to determine the maximum fluorescence of undivided cells (Figure 2.1.A). Then gates were set to distinguish successive generations of dividing cells as shown in Figure 2.1. B making sure that the median fluorescence value halves for each successive division.
Figure 2.1. Gating on CFSE labelled cell populations. PBMCs were stained with CFSE, fixed in 1% PFA as a baseline control (A) or stimulated with 5n/ml PHA for 4 days and then fixed in 1% PFA and analysed on a Beckman Coulter XL flow cytometer with EXPO2 software.
Chapter III

Maturation of adult and infant DCs
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3.1. INTRODUCTION

DC maturation is a key event in the DC life cycle. It is intricately linked to dramatic changes in DC phenotype and biology, which in turn are critical for DC function in initiating adaptive immune responses. Morphology, surface markers, chemokine and chemokine receptor expression, cytokine production, antigen uptake, processing and presentation are all affected during the maturation of DCs. Functionally they become the most potent antigen-presenting cells, able to prime even naïve T cells, via the expression of high levels of antigen-presenting and co-stimulatory molecules. Blocked or impaired DC maturation may therefore have severe consequences for the ensuing interactions with effector cells.

Impaired DC maturation has recently been found to be a prominent feature of cord blood DCs (Goriely et al 2001, Langrish et al 2002). Cord blood DCs were generated in the same manner as adult DCs from peripheral blood monocytes through culture with GM-CSF and IL-4. This procedure yielded populations of immature DCs derived from adult and cord blood that were phenotypically and functionally very similar (Liu et al 2001, Goriely et al 2001, Langrish et al 2002). However, When DCs were stimulated with inflammatory stimuli such as LPS, TNF-α or Poly I:C, cord blood DCs were unable to mature like adult DCs. Addition of such stimuli to adult DCs causes strong upregulation of HLA-DR, CD83, CD86 or CD40. These antigen-presenting and co-stimulatory molecules are critically important for priming of naïve T cells. This phenotypic maturation was completely abrogated in neonatal DCs (Langrish et al 2002).

Maturation also results in a number of other changes in the DCs. The cells change shape and develop an abundance of fine dendrites and membrane ruffles thereby increasing the surface area available for interaction with lymphocytes. Expression of adhesion molecules changes, which results in increased adhesion to substrates and other cells, including other DCs, and leads to formation of homotypic clusters in mature DC populations and heterotypic clusters in mixed DC-lymphocytes cultures. Langrish and colleagues reported that cord blood DCs exhibited a markedly reduced tendency to form such clusters both with each other and with T cells (Langrish
I. MATURATION OF INFANT AND ADULT DCs

2002). This is likely to have significant consequences for the effectiveness of interaction with effector cells.

Vital in the control and regulation of such interactions with effector cells is the production of cytokines by the DC. One key cytokine produced by DCs is IL-12, which is crucial for driving T cell differentiation. Low levels of IL-12 usually do not overcome the IL-4-induced 'default' development of T cells into T<sub>H</sub>2 effectors, while high levels drive T<sub>R</sub>I responses (Ohshima and Delespesse 1997).

Adult DCs produce significant amounts of IL-12 in response to LPS. Langrish and colleagues reported that a burst in secretion could be observed between 8 and 12 hours after the addition of LPS and total IL-12 p70 levels peak at approximately 18 hours. Levels then remain constant for at least a further 30 hours. In contrast, cord blood DCs did not produce any IL-12 at any of the time points (Langrish et al 2002), corroborating data published by Goriely and colleagues (Goriely et al 2001).

The LPS response was not completely abrogated in cord DCs as they produced IL-10 and TNF-α at comparable levels and with comparable kinetics to adults DCs (Langrish et al 2002). The defect in IL-12 production by cord DCs, balanced by their conserved ability to produce other cytokines such as the potent immunosuppressive cytokine IL-10, may have profound consequences and may result in altered immune responses in neonates.

Impaired immune responses to natural infection and vaccination are seen not only in the neonatal period but also throughout infancy. It is not known whether defective DC maturation persists into childhood when it may be key to altered immunity. To address this question, in this chapter, monocyte-derived DCs from infants ranging from 0 to 2 years of age were compared to monocyte-derived DCs from healthy adult controls. The two groups were compared with regard to their ability to phenotypically and morphologically mature, cluster and produce bioactive IL-12 in order to determine the time in development when DCs gain full functional competence.
3.2. OPTIMISATION OF DC GENERATION AND MATURATION

Immature DCs were generated by culturing adult peripheral blood monocytes in IL-4 and GM-CSF for 5 to 7 days and were activated and matured by addition of LPS. Phenotypic maturation was assessed at 24 hours after the addition of 0.5 µg/ml LPS by FACS analysis of the expression of the surface markers shown in Table 3.1.

Table 3.1. Surface markers used to phenotype immature and mature DCs

<table>
<thead>
<tr>
<th>Surface marker</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin-1</td>
<td>Non-DC lineage marker</td>
</tr>
<tr>
<td>CD25</td>
<td>IL-2 receptor, activation marker</td>
</tr>
<tr>
<td>CD11c</td>
<td>αβ subunit of integrin CR4, binds fibrinogen</td>
</tr>
<tr>
<td>CD14</td>
<td>Receptor for complex of LPS and LPS-binding protein (LBP)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Antigen presentation (MHC class II)</td>
</tr>
<tr>
<td>CD83</td>
<td>DC maturation marker</td>
</tr>
<tr>
<td>CD86</td>
<td>Co-stimulatory molecule, ligand for CD28 and CTLA-4</td>
</tr>
<tr>
<td>CD40</td>
<td>Binds CD40L, delivers stimulatory signal</td>
</tr>
</tbody>
</table>

Lin-1 is an antibody cocktail containing antibodies against CD3, CD14, CD16, CD19, CD20 and CD56. It was used to evaluate purity of DC cultures by staining for T cells (CD3), B cells (CD19, CD20), monocytes (CD14), macrophages (CD14, CD16), neutrophils (CD16) and natural killer cells (CD16, CD56).

A representative example of the expression profiles of immature and LPS-matured DCs is shown in Figure 3.1. These expression profiles were highly reproducible. Cells were always CD11c positive, Lin-1 and CD14 negative or low, and strongly upregulated CD25, HLA-DR, CD83, CD40 and CD86 in the presence of LPS.

DCs are highly sensitive to LPS, a ubiquitous component of the outer membrane of Gram-negative bacteria. LPS is often found as contamination in reagents and antigen preparations, which could have dramatic effects on DCs. In order to determine the
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effect of very low doses of LPS on DC phenotype, dose experiments using LPS dose ranges from 100pg to 500ng/ml were performed. As shown in Figure 3.2, 100 pg/ml had some effect on surface marker expression and 500 pg/ml LPS were sufficient to induce full upregulation of surface markers. Therefore, everything was done to ensure that LPS levels in reagents, media and antigen preparations used with the DCs were as low as possible.
Figure 3.1. Phenotype of immature versus mature DCs. Adult immature DCs were left unchallenged (hatched lines) or incubated with 0.5 μg/ml LPS for 24 hours (filled profiles) and surface marker expression analysed by FACS. Cells were selected by gating according to forward and side scatter first (A). Isotype controls (dotted lines) are shown for comparison.

Figure 3.2. Sensitivity of DCs to very low doses of LPS. Immature DCs (dotted lines) were incubated with 100 pg/ml (hatched lines) or 500 pg/ml (filled profiles) LPS for 24 hr and assessed by FACS for expression of HLA-DR, CD83 and CD86.
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3.3. PHENOTYPIC MATURATION OF INFANT AND ADULT DCs

CD14+ monocytes were isolated from the peripheral blood of adults and infants (aged 0 to 2 years) and used to generate DCs as described in section 3.1. All blood samples were processed within 2 hours of collection to minimize activation of the cells. As determined in preliminary experiments (data not shown), time periods of up to 6 hours between collection and processing of the blood do not affect the phenotypic characteristics of the resulting DC populations.

In order to assess their ability to undergo phenotypic maturation, adult and infant immature DCs were activated with 0.5 µg/ml LPS and cell cultures were imaged after 6 hours and 24 hours. After 24 hours, supernatants were collected and stored for cytokine measurements by ELISA. Cells were used for confocal microscopy or assessment of their phenotypic maturation status by FACS using a panel of antibodies including CD11c, Lin-1, CD14, CD40, HLA-DR, CD83, CD86 and relevant isotype controls.

As shown before (Figure 3.1.), adult DCs were CD11c positive cells that increase surface expression of HLA-DR, CD83, CD86 and CD40 when activated with LPS (Figure 3.3. A). Infant DCs underwent the same process of phenotypic maturation, i.e. they strongly upregulated HLA-DR, CD83, CD86 and CD40 expression. Shifts in surface marker expression similar to adult DCs were seen in DCs from infants as young as 6 weeks (Figure 3.3. C). No difference was found in the extent of upregulation between adult and infant DCs with the exception of CD86 expression, which was found to be consistently and significantly higher in immature DCs from infants compared to adults (Figure 3.3. D)
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Figure 3.3. Phenotypic analysis of adult and infant DCs. Immature adult (A) and infant DC (B: 1.5 months old infant, C: 22 months old infant) were incubated in the absence (empty profiles) or presence (filled profiles) of 0.5 µg/ml LPS for 24 hr and surface markers expression analysed by FACS. D: Pooled data from 7 independent experiments. *p=0.04, **p<0.001 using Student’s paired T test.
3.4. MORPHOLOGY AND CLUSTERING OF INFANT AND ADULT DCs

Maturing DCs with LPS not only results in a change in phenotype but also changes the behaviour of DCs in culture and their morphology. In order to investigate the ability of infant DCs to undergo such changes indicative of maturation, adult and infant DCs were exposed to LPS or left unchallenged and their morphological features visualised by light and confocal microscopy. Light microscopy of the cells allowed us to determine their characteristics when in culture, i.e. general shape and cluster formation. Confocal microscopy was used to further investigate changes of morphological features, in particular of adhesive structures called podosomes. These allow the cells to establish new contact sites at the leading edge and, by retraction of the cell body, to achieve translocation (Burns et al 2001). Podosomes and the actin cytoskeleton were readily visible under the confocal microscope after staining with fluorescently labelled phalloidin.

Microscopic analysis of adult DCs revealed that immature DCs were very large cells with irregular shapes and few dendrites, which usually extended from one side of the cell only. They were single cells and did not attach to plastic in culture (Figure 3.4). When adhered to fibronectin-coated coverslips, they spread and formed a leading edge with abundant membrane protrusions such as lamellipodia and multiple podosomes clustered just behind the leading edge (Figure 3.4a and Figure 3.5).

In culture, cells flattened, polarized and adhered strongly to the substrate after only a few hours of LPS stimulation. They formed large complexes of overlapping projections and cell bodies from multiple DCs (Figure 3.4). After 24 hours of stimulation, the majority of cells had become much smaller, round and covered in membrane ruffles and abundant dendrites. By this time they had lost all podosomes, formed large, tightly packed aggregates (homotypic DC clusters) and had detached again from the substrate (Figure 3.4 and 3.5). They adhered poorly to fibronectin and had little tendency to spread.

When infant DCs were stimulated with LPS and stained with phalloidin for confocal microscopy, changes in morphology identical to those in adult DCs were observed.
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(Figure 3.5). Again, the majority of cells adopted the typical appearance of mature DCs. They became small, round, covered in dendrites and membrane ruffles, and podosomes were no longer detectable.

When cells were imaged in culture, similar changes occurred in the first 4 to 8 hours of stimulation in both cell populations, i.e. both adult and infant DCs attached strongly to the plastic wells, flattened out and polarized (Figure 3.4). However, 24 hours after stimulation differences between adult and infant DCs became apparent. Where adult DCs had formed the typical clusters of small, round, spiky cells, most infant DC samples remained as single, polarized or irregularly shaped immature-like cells that did not form clusters.
Figure 3.4. Clustering of adult and infant DCs. Immature adult (A) and infant DC (B: 2.5 months old infant) were incubated in the absence or presence of 0.5 μg/ml LPS for 24 hr and visualized at the indicated times using an inverted light microscope (x32, phase contrast).
Figure 3.5. Morphology of adult and infant DCs. Immature and LPS-matured adult (A) and infant DC (B, 8 months old infant) were adhered to coverslips and stained with TRITC-phalloidin (red) and TOPRO (blue, infant cells only) to visualise the actin cytoskeleton and the nucleus respectively. TRITC-phalloidin also labels podosomes, seen as brightly stained focal structures at the leading edge of the immature cells. Confocal micrographs were obtained using a confocal laser scanning microscope system (TCS NT, Leica).
3.5. **IL-12 PRODUCTION BY INFANT AND ADULT DCs**

Abrogated IL-12 production is one of the most important consequences of the impaired maturation of cord blood-derived DCs. It is not known how long this reduced production of IL-12 manifests. Therefore, infant and adult DCs were stimulated with LPS and culture supernatants removed after 24 hours and stored at -80°C for analysis. Bioactive IL-12 p70 in the supernatants was then measured using standard cytokine ELISA kits.

Figure 3.6 shows that there appeared to be at least two distinctive populations among adult DCs with regard to IL-12 production. 40% (9/23) of adults tested produced comparatively low levels of IL-12 (mean: 90 pg/ml), whereas 60% (14/23) adults secreted much higher levels of around 720 pg/ml. In contrast to relatively equal distribution in adults, only one of the seven infants tested produced high levels of IL-12. The remaining six infants all fell into the group of low producers, their mean IL-12 secretion being even lower than that of the adult low producers (mean: 61 pg/ml).

These data did not follow the typical Gaussian bell-shaped distribution of statistically normal data, even after transformations such as squaring or cubing. Therefore, the non-parametric Mann Whitney test was used to analyse the data. Applying this test to the IL-12 data from infant and adult DCs (including both high and low producers) gave a $p$ value of 0.0471. Given the lower statistical power of non-parametric test, in particular when sample sizes are so small, this $p$ value indicates that there may be a real difference in IL-12 production by infant and adult DCs.
Figure 3.6. IL-12 production by adult and infant DCs in response to LPS. Immature adult and infant DC (n=7, ages as shown on x axis) were incubated in the absence or presence of 0.5 µg/ml LPS for 24 hr and IL-12 levels in culture supernatants analysed by standard ELISA.
3.6. DISCUSSION

In the first part of this thesis I examined the ability of infant monocyte-derived DCs to undergo full morphologic, phenotypic and functional maturation in response to inflammatory stimuli.

**Phenotypic maturation**

I found that DCs from infants as young as 6 weeks were able to fully upregulate HLA-DR and CD86 (Figure 3.3). High levels of these antigen-presenting and co-stimulatory molecules are essential for efficient antigen presentation allowing the DCs to prime even naïve T cells. In contrast to memory T cells, naïve T cells require much higher levels of co-stimulation before they commit to proliferation (Lanzavecchia and Sallusto 2000). Expression of such adult-like levels of surface markers on infant DCs therefore suggests that DCs develop the ability to initiate secondary and even primary immune responses very early on in life.

To our knowledge, only one other group has investigated the phenotype and function of immature infant DCs (Bickham et al 2000 et al). This group only studied six children over a very large age range (11 months to 14 years) and this work has not been published in a peer-reviewed journal to date. However, my data corroborate their findings that mature pediatric DCs express high levels of surface HLA-DR and CD86, enabling them to support recall responses to influenza and tetanus toxoid as effectively as adult DCs.

**Morphological changes**

I have shown that infant DCs can undergo the typical changes in morphology seen in adults upon stimulation with LPS (Figure 3.5). The cells become round, small and compact. Despite this apparent shrinking in size, a large surface area for interaction with effector cells is maintained as a consequence of the appearance of abundant membrane ruffles and dendrites. DCs derived from infants were also shown to lose their podosomes on maturing. These adhesive structures are a very prominent feature of immature DCs and are always lost upon maturation of DCs derived from adult
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blood. Loss of podosomes thus showed that DCs derived from infants can respond to LPS stimulation with at least some of the characteristic changes seen in maturing adult DCs.

**Clustering**

One characteristic change of mature adult DCs that was not demonstrated by the majority of infant derived DCs was the ability to cluster. A visually striking feature associated with adult DC maturation is the dramatic change in the shape of the cells and in their behaviour in culture. Over a short period of time after exposure to LPS (< 24 hours) adult DCs first polarize to form networks of extremely elongated cells and then shorten and round up to become very small, compact cells (Figure 3.4). Immature DCs attach strongly to the substrate and do not attach to each other, whereas in mature DCs the opposite is true implicating that maturation also leads to changes in the pattern of adhesion molecules expressed on the surface.

All infant DC samples polarised like the adult control cells after about 4 hours of LPS stimulation, but only a few then went on to attach to each other and form the large homotypic DC-DC clusters seen in adult DC cultures. Only three out of 10 samples available for this experiment clustered to the extent seen in the adult control samples while the other infant samples remained single, immature-like cells (Figure 3.4).

DC-DC clustering has been observed by other groups *in vitro* (Corinti et al 1999) and *in vivo* (Lukas et al 1996, Weinlich et al 1998). The physiologic relevance of homotypic clustering currently remains unanswered but has been proposed as a mechanism to stimulate DC function, by mutual delivery of maturation signals and by the transfer of antigen between cells (Knight et al 1998, Delemarre et al 2001). Cluster formation has also been associated with maturation status of the DC (Delemarre et al 2001).

Mature DCs do not only attach to each other but also to other cells. This close association may assist in their interaction with effector cells, in particular T cells.
Cluster formation with T cells has not yet been investigated in the infant samples as small blood volumes severely limit the extent of experimentation that is possible on each sample. However, cluster formation has been examined in more detail by Langrish et al using cord blood-derived DCs (Langrish 2002). These exhibited a markedly reduced tendency to form either DC-DC or DC-T clusters. This defect is possibly related to their failure to up-regulate CD54 (ICAM-1), which was found to be essential for efficient clustering.

Efficient antigen presentation is achieved by the generation of stable synapses between DCs and T cells allowing TCR triggering and subsequent T cell activation. Synapses are initiated and stabilised by adhesion molecules (Dustin and Cooper 2000). The reduced tendency of cord and infant DCs to form clusters may have important consequences for their ability to form stable interactions with T cells and may potentially hinder antigen presentation.

**IL-12 production in response to LPS**

DCs not only function in the priming of T cells but also influence their differentiation into distinct effector subtypes. IL-12 is the main factor driving T_{H1} polarisation. While adult DCs produce sizeable amounts of this cytokine in response to LPS stimulation, cord DCs are intrinsically unable to secrete bioactive IL-12 p70 (Goriely et al 2001, Langrish et al 2002). The data presented in this chapter indicate that the specific defect in IL-12 production observed at birth does in fact persist into infancy and possibly childhood. Levels were low but detectable which is in contrast to the complete absence of IL-12 production by DCs at birth suggesting a slow rise in LPS responsiveness with age.

Figure 3.6. shows that adults appeared to fall into at least two groups with regard to IL-12 production, low producers and high producers. Adults distributed almost equally between the two groups whereas nearly all of the children fell into the low producer group. In addition, the mean and peak IL-12 levels measured in that group were below those observed in the corresponding adult group. Pooling data from both groups revealed a statistically significant difference in IL-12 production between
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infant and adult DCs. The figure also shows that the markedly reduced capacity to produce IL-12 appears to persist throughout infancy. Release of IL-12 does not appear to increase over the period studied (up to 2 years of age).

The small number of samples tested is of course a considerable limitation in the interpretation of the data. Access to peripheral blood from healthy children in this age range is very limited and obtaining sufficient volumes of blood for in vitro generation of DCs is very difficult. This study is ongoing, however, and increasing sample size will allow more definite conclusions in future.

Our findings are in agreement with a recent report by Upham and colleagues. This group evaluated the development of the IL-12-producing capacity by PBMCs over age, from birth (cord blood) to childhood (5-year-olds and 12-year-olds) to adult life (Upham et al 2002). Their data show that IL-12 production by PBMCs is markedly impaired at birth, slowly rises throughout infancy and childhood but even at 12 years of age still does not reach adult levels. Depletion experiments indicated that the major source of IL-12 in adult peripheral blood are HLA-DR⁺, CD14⁺, CD19⁻ putative DCs suggesting that the defect observed in vitro may reflect physiologic situation. The intriguing observation that IL-12 secretion by PBMCs is still below adult levels at 12 years of age prompted us to extend the age range for our study to include older children. This is currently ongoing work.

**IL-12 production in response to LPS and IFN-γ**

IL-12 production in response to LPS can be boosted dramatically by addition of IFN-γ, which does not induce IL-12 on its own but acts potently in synergy with LPS. IFN-γ can also be substituted with CD40 ligand in order to enhance the effect of LPS on IL-12 production (Snijders et al 1998). This two-signal requirement is unique to secretion of IL-12 which may reflect the potent Th1 driving capacity of this cytokine and the stringent control mechanisms it must underlie in order to avoid potentially harmful responses.
Addition of IFN-γ overcomes the block in IL-12 production in cord DCs suggesting that appropriate signals may also allow infant DCs to function fully. Only one infant sample has so far yielded enough cells to compare IL-12 secretion in response to LPS alone and in response to LPS and IFN-γ. This 6 week-old infant produced 31 pg/ml upon stimulation with LPS and 1077 pg/ml when exposed to both LPS and IFN-γ. The adult control DCs released 481 pg/ml and 12,500 pg/ml respectively. Even though the total level of IL-12 in the infant sample does not reach the total level in the adult sample, levels increase by 35-fold in the infant as compared to 26-fold in the adult. This suggests that IFN-γ may indeed overcome the impaired LPS responsiveness in infants. If this holds true in more samples, it may suggest that early life DCs are like most other components of the early life immune system in that they are not completely unable to perform their functions but they need stronger signals.

This is supported by the finding that, despite complete lack of IL-12 production, cord blood DCs secrete normal levels of other immune mediators such as IL-10 and TNF-α. (Langrish et al 2002). The resulting imbalance between IL-12 and the potent immunosuppressive cytokine IL-10 may allow the preferential development of TH2-type effector cells or even regulatory cells. However, given the appropriate signals, early life DCs should be able to induce TH1 development.

This may be the reason why TH1 responses are possible, e.g. after BCG vaccination (Vekemans et al 2001), but TH2 responses are more readily induced in infants. It may also explain why the induction of TH1 responses relies on the use of agents that deliver very strong activation signals to DCs, such as live replicating agents or CpG DNA-containing vaccines (Kovarik et al 1999).

**Conclusion**

In conclusion, infant DCs do undergo the morphological and phenotypic changes typically associated with DC maturation. However, they display a markedly reduced tendency to cluster and their IL-12-producing capacity is severely diminished. Therefore, infant 'mature' DCs should be able to induce proliferation of naïve T cells but may not be able to support their differentiation into TH1-type effector cells.
allowing the preferential development of TH2-type effector cells or even regulatory cells.

The work in this chapter also shows that the various aspects of the LPS response may be regulated in a differential manner throughout ontogeny. While some features such as morphology and phenotype are the first to achieve adult-like competence, others such as clustering and IL-12 production may undergo a slow process of development reaching full competence only much later in life. There may not be one specific point in ontogeny when impaired cord-like cells completely switch to fully competent adult-like cells.
Chapter IV

Interaction of DCs with polysaccharide antigens
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IV. Interaction of DCs with polysaccharide antigens

4.1. INTRODUCTION

In the first part of this thesis we have shown that certain components of the LPS-induced maturation of infant DCs are impaired, which may explain altered immune responsiveness in early life. Th2 and humoral immune responses are generally more easily achieved in early life than Th1 biased cellular immune reactions. This may be related to the diminished IL-12-producing capacity of infant DCs.

Despite the generally good induction of humoral immunity in early life, young infants are particularly susceptible to infections with encapsulated bacteria. The basis for their susceptibility to these pathogens is that they are unable to mount protective antibody responses to the capsular polysaccharides (CPS) surrounding these bacteria. Bacterial CPS have long been considered typical examples of so-called T-independent type 2 responses. Originally thought to occur in the absence of T cell help, recent evidence indicates that antigen-presenting cells may be of importance in the establishment of such responses.

While classical antigen presentation of CPS is not thought to occur (see comments about T independence above) there are a number of ways that DCs may interact with CPS antigens. DCs express an abundance of different lectins, carbohydrate-binding surface molecules, which may allow the DCs to bind CPS and it is possible that in this way DCs bind CPS and, in the absence of internalisation, transport CPS to the appropriate B cells in germinal centres. However, it is known that many of the lectins found on the surface of DCs act as receptors for antigen uptake and thus the binding of CPS to lectins on the surface of DCs may enable the DCs to internalise CPS. A number of reports have shown that model polysaccharide antigens such as TNP-Ficoll or dextran are indeed internalised by DCs (Sallusto et al 1995, Prigozy et al 1997, Kato et al 2000, Schaible and Kaufmann 2000). In addition, there is some evidence that DCs can also take up pathogen-derived polysaccharides (Fairhurst et al 1998b).

It is unclear which mechanisms of uptake and which specific receptors may be involved in the internalisation of polysaccharides. Dextrans have been shown to rely
IV. Interaction of DCs with polysaccharide antigens

both on macropinocytosis and receptor-mediated endocytosis for uptake (Sallusto et al 1995, Kato et al 2000). A number of C-type lectins may serve as receptors but the mannose receptor has been shown to be of particular importance for dextran uptake by monocyte-derived DCs (Kato et al 2000, Sallusto et al 1995). This receptor has also been suggested to bind pneumococcal CPS (Zamze et al 2002).

After internalisation, dextrans are transported to different intracellular locations depending on their size. Small molecules such as dextran 3K and 40K are delivered from endosomal vesicles to the cytosol via as yet unknown mechanisms. Larger dextran of 500K or 2000K are trafficked through the endocytic compartments similar to protein antigens, i.e. they go through early endosomes, then late endosomes and then lysosomes (Rodriguez et al 1999).

Based on these observations, we hypothesized that DCs may be directly involved in responses to CPS through internalisation and possibly presentation of the antigen. In order to investigate this hypothesis, we studied the interaction of human monocyte-derived DCs and pneumococcal CPS focussing on uptake and intracellular processing.
4.2. INTERNALISATION OF POLYSACCHARIDES BY DCs

To investigate whether DCs can internalise polysaccharides, we incubated immature DCs with pneumococcal CPS serotypes 9N and 14 (PPS9N and PPS14) as well as two different sizes (3K and 500K) of the model antigen dextran. All antigens were FITC-labelled to allow assessment of uptake by confocal microscopy and flow cytometry.

For confocal microscopy, cells were fed labelled antigen, fixed after 1 hour and stained with TRITC-phalloidin to highlight the actin cytoskeleton and the cellular outline. As shown in Figure 4.1, all four antigens were taken up by immature DCs after 1 hr incubation at 37°C. Their intracellular localization, however, showed different patterns. As expected, FITC-labelled dextran 500K localized to large perinuclear vesicles (Figure 4.1. A and B) whereas dextran 3K (Figure 4.1. C and D) was found distributed throughout the cytoplasm in all cells. This may have been in very small vesicles or indeed in the cytosol, as suggested by the findings of Rodriguez and colleagues (Rodriguez et al 1999). Pneumococcal PS serotype 14 was also taken up and localized to vesicles mostly in the perinuclear area (Figure 4.1. E and F). PPS9N was detected in vesicles in the perinuclear area and some was distributed throughout the cytoplasm (Figure 4.1. G and H). Z-sections show that the antigens were indeed found internally and not attached to the cell surface.

Incubation of DCs with the different antigens also seemed to have an effect on the morphology of the cells, e.g. cells incubated with dextran 3 K and PPS14 appeared to have lost their podosomes to some degree, an indication of activation and onset of maturation as described in Chapter III.

Internalisation of FITC-labelled antigens by DCs was confirmed using a flow cytometric method (Figure 4.2.). Again, DCs were incubated with the four different FITC-labelled antigens for 1 hour. Duplicate cultures were incubated either at 37°C or left on ice to assess internalisation and background staining respectively. This technique confirmed that all four antigens were taken up by DCs.
Flow cytometry also allowed quantitative analysis of uptake over time. As shown in Figure 4.3., both pneumococcal polysaccharides were internalised continuously and steadily, slowing down after 4 hours but still on-going after 24 hours. As the antigens were not removed during the experiment, the apparent slowing down of uptake after 4 hours may in fact not be due to reduced uptake but due to onset of intracellular processing resulting in reduced intensity of the fluorescent signal.
IV. Interaction of DCs with polysaccharide antigens

Figure 4.1. Internalisation of pneumococcal and model polysaccharides – Confocal microscopy. Immature DCs were incubated for 1 hr with 1 mg/ml FITC-labelled (green) dextran 500 K (A and B), 3 K (C and D) or 60 μg/ml PPS14 (E and F) and PPS9N (G and H), fixed and stained with rhodamine-phalloidin (red) to visualize filamentous actin. Two different cells for each condition are shown. Images are average projections of 10 to 15 optical sections of 0.5 μm each. Scale bar represents 10 μm. Side panels are z-sections through the cells.
IV. Interaction of DCs with polysaccharide antigens

Figure 4.2. Internalisation of pneumococcal and model polysaccharides – flow cytometry. Immature DCs were incubated with 2 mg/ml FITC-dextran 500K (A) or 3K (B), 60 μg/ml PPS14 (C) or PPS9N (D). Cells were either incubated at 37°C for 30 min to allow uptake (filled profiles) or left on ice to measure background staining (empty profiles). Surface-bound antigen was quenched and cells were immediately analysed by flow cytometry. Similar results were obtained in three independent experiments.

Figure 4.3. Kinetics of uptake of pneumococcal polysaccharides type 9N and 14. DCs were incubated with 60 μg/ml PPS14 or PPS9N for periods of time indicated. Surface-bound antigen was quenched and cells were immediately analysed by flow cytometry. Background uptake on ice was negligible. Results are representative of three independent experiments.
IV. Interaction of DCs with polysaccharide antigens

4.3. MECHANISMS OF POLYSACCHARIDE UPTAKE

Two major mechanisms of uptake are receptor-mediated endocytosis and macropinocytosis, both of which are employed by DCs for uptake of the model polysaccharide dextran (Kato et al 2000, Sallusto et al 1995). They can be blocked by using specific inhibitors, such as cytochalasin D and wortmannin (Hiltbold et al 2000).

Cytochalasin D is a cell permeable fungal toxin (isolated from *Zygosporium mansonii*), which acts as a potent inhibitor of actin polymerisation. Actin-based cytoskeletal rearrangements are critically important for pit formation during the initiation of receptor-mediated endocytosis but also involved in the formation of membrane folds during macropinocytosis.

Wortmannin is an antifungal antibiotic isolated from *Penicillium fumiculosum*. It inhibits phosphatidylinositol-3-kinase (PI3-kinase), a signal transduction molecule critically involved in ruffling and the fusion of membrane folds during macropinocytosis. Due to its role in the fusion of membranes, wortmannin also hinders the closure of the membrane after pit formation during receptor-mediated endocytosis.

I used both cytochalasin D and wortmannin to inhibit the different mechanisms of uptake and investigate their relative contribution to internalisation of pneumococcal polysaccharides. FACS analysis showed that 23.4% of cells stain positive for intracellular polysaccharide after 2 hours of incubation (Figure 4.4). When DCs were pre-incubated with inhibitory concentrations of cytochalasin D or wortmannin (Hiltbold et al 2000), uptake was reduced dramatically in both cases. These data suggest that both actin-based cytoskeletal rearrangements as well as PI3-kinase activation may be required for internalisation of pneumococcal polysaccharides by DCs.
IV. Interaction of DCs with polysaccharide antigens

2.4% to <0 e

FITC

23.4% to o

FITC

3.5%

6.0%

Figure 4.4. Inhibition of uptake of pneumococcal polysaccharide type 14. Immature dendritic cells were incubated with 60 μg/ml PPS14 for 2 hours on ice (A) or at 37°C (B) to determine background staining and internalisation respectively. Cultures run in parallel were pre-incubated for 30 min with 10 nM cytochalasin D (C) or 100 nM wortmannin (D). Similar results were obtained in two further experiments and for PPS9N.
IV. Interaction of DCs with polysaccharide antigens

4.4. INTRACELLULAR PROCESSING OF POLYSACCHARIDES

After internalisation, protein antigens are generally transported through early and late endosomes to lysosomes and progressively degraded to generate peptide for loading onto MHC class II (Mellman 1996). No studies have so far investigated the intracellular fate of pathogen-derived polysaccharides. However, the processing of the model polysaccharide dextran is well characterized and follows the same endocytic pathway as described for protein antigens.

Endocytic organelles can be visualized by staining organelle-specific molecules. We used antibodies against the transferrin receptor (TfnR) to label early endosomes, against the lysosome-associated membrane protein (LAMP) to stain late endosomes and lysosomes and against MHC class II to visualize late endosomal compartments in immature DCs and the surface in mature DCs. An example of such staining is shown in Figure 4.5.

In order to follow the intracellular trafficking of polysaccharides, immature DCs were incubated with FITC-labelled pneumococcal polysaccharide type 14 or FITC-labelled dextran for 24 hours and fixed. The cells were then permeabilized, stained with the various organelle-specific markers and visualized by confocal microscopy. Our results show, that PPS14 co-localized with LAMP but not TfnR after 24 hours of incubation indicating that this polysaccharide reached late endosomal compartments (Figure 4.6a). The cells remained immature as seen by localisation of MHC class II in late endosomal compartments in the perinuclear area. PPS14 also co-localized with MHC class II confirming the results obtained with LAMP and TfnR. The same pattern was observed for dextran (Figure 4.6b).
IV. Interaction of DCs with polysaccharide antigens

**Figure 4.5. Intracellular localisation of endocytic organelles.** Immature DCs adhered to fibronectin-coated coverslips were permeabilized and stained with markers for the various stages in the endocytic pathway to show intracellular localization of the various organelles. Primary antibodies were unconjugated; alexa488 was used as the secondary antibody.
IV. Interaction of DCs with polysaccharide antigens

Figure 4.6a. Intracellular processing of pneumococcal polysaccharide type 14. Immature dendritic cells were incubated with FITC-labelled PPS14 for 24 hr, fixed and stained with specific markers for various stages in the endocytic pathway as indicated (alexa568-labelled - red) and a DNA label (blue). Yellow staining indicates co-localization of alexa568 and FITC. Insets in the merged images are close-ups of the region of interest.
Figure 4.6b. Intracellular processing of the model polysaccharide dextran. Immature dendritic cells were incubated with FITC-labelled dextran 500K for 24 hr, fixed and stained with specific markers for various stages in the endocytic pathway as indicated (alexa568-labelled - red) and a DNA label (blue). Yellow staining indicates co-localization of alexa568 and FITC. Insets in the merged images are close-ups of the region of interest.
IV. Interaction of DCs with polysaccharide antigens

4.5. DISCUSSION

The results presented in this chapter provide evidence that pneumococcal capsular polysaccharides can indeed be internalized by DCs and that uptake appears to involve both actin-based cytoskeletal rearrangements and PI3-kinase activation. These are of major importance in receptor-mediated endocytosis and macropinocytosis respectively suggesting that both of these mechanisms may play a role in uptake of pneumococcal polysaccharides. No other studies have investigated the interaction between DCs and bacterial CPS although several report the uptake of model polysaccharides such as dextrans, ficoll and sucrose (Sallusto et al 1995, Prigozy et al 1997, Kato et al 2000). Our results regarding uptake and mechanisms of uptake agree with those described for other, similar antigens.

The finding that pneumococcal CPS can enter DCs via receptor-mediated endocytosis suggests the existence of a specific receptor. One candidate may be the mannose receptor (MR). Zamze and colleagues have recently shown that this receptor can bind most pneumococcal CPS serotypes in vitro in both surface-bound and soluble forms (Zamze et al 2002). While their affinity is not as strong as that of the classical MR ligand yeast mannan, pneumococcal CPS of serotypes 3, 9V, 23F, 19A, 9N, 6B, 2, 14 and 19F (in order of increasing binding) all bound soluble MR. Intriguingly, none of the pneumococcal CPS have the structural features associated with known mannose receptor specificities, as for example multiple mannose residues. The authors therefore suggest that criteria other than the primary polysaccharide structure must be taken into account and that recognition possibly relies more on secondary structure and conformational epitopes (Zamze et al 2002).

Monocyte-derived DCs display not only the mannose receptor but also a wide variety of other carbohydrate-binding molecules that may allow them to bind polysaccharides. DEC-205 and DC-SIGN are two other likely candidates. Both are C-type lectins that are expressed on myeloid DCs. They bind glycosylated antigens and deliver those to intracellular compartments for degradation and loading onto classical and non-classical antigen-presenting molecules (Mahnke et al 2000, Prigozy et al 1997).
IV. Interaction of DCs with polysaccharide antigens

Many of the C-type lectin antigen receptors are capable of intracellular signaling as suggested by the presence activation and inhibitory motifs in their intracellular domains (Colonna et al 2000, Yokota et al 2001, Engering et al 2002a and Bates et al 1999 respectively). Therefore binding of pneumococcal CPS to such receptors may influence the induction of immunity by such antigen-loaded DCs. In chapter 5 this possibility will be explored further.

In this chapter, intracellular trafficking of pneumococcal CPS was followed. The data show that pneumococcal polysaccharide type 14 reaches late endosomal compartments in DCs after a prolonged period of incubation. These findings could be confirmed and extended in future by co-incubating DCs with pneumococcal polysaccharide and dextran labelled with different fluorophores.

The intracellular processing of dextran is well characterized. Prigozy and colleagues showed that dextran is taken up rapidly and is easily detectable in early endosomes after 5 to 15 minutes (Prigozy 1997). After two hours of incubation it labels late endosomes in the peripheral and the perinuclear area. A one-hour pulse followed by an overnight chase in antigen-free medium finally allows concentration of dextran in late endocytic compartments including lysosomes and MHC class II compartments in the perinuclear area. Therefore co-incubation of pneumococcal CPS and dextran and visualisation at early, medium and late time points would allow us to further dissect the intracellular processing of the pneumococcal CPS.

Proteins antigens are progressively degraded in late endosomes and lysosomes to generate immunogenic peptides. It is not known whether polysaccharide antigens undergo the same process. In macrophages, polysaccharides are transported to lysosomes where they accumulate and finally interfere with the processing of protein antigens (Gonzalez-Fernandez et al 1997). These inhibitory effects of carbohydrates on protein processing are not seen in DCs and B cells suggesting that these cells may have the capacity to degrade polysaccharides.
IV. Interaction of DCs with polysaccharide antigens

We have performed preliminary experiments assessing the intracellular degradation of polysaccharides. DCs were pulsed with fluorescently labelled polysaccharides, washed extensively to remove free antigen and chased for prolonged periods of time. Flow cytometry was used to measure fluorescence intensity over time. Preliminary data show that the intensity of fluorescence does decrease over time indicating degradation of the labelled antigen (data not shown). However, it is unclear whether this was due to loss of signal from the fluorophore rather than degradation of the entire antigen.

Digestive enzymes are contained in lysosomal vesicles, which originate in the Golgi apparatus and fuse with the late endosomal compartments containing material destined for enzymatic breakdown. Presence of the pneumococcal polysaccharides in late endosomal compartments implies that these antigens are exposed to digestive enzymes and if the appropriate enzymes are present may undergo processing.

Prigozy and colleagues have recently shown that such a carbohydrate-processing pathway exists and is required for efficient presentation of certain glycolipids (Prigozy et al 2001). These have oligosaccharide head groups that are trimmed intracellularly to allow binding of the glycolipid to CD1 and recognition of the carbohydrate moiety by carbohydrate-specific CD1-restricted T cells. By using specific inhibitors, Prigozy and colleagues showed that enzymatic processing was due to activity of lysosomal hydrolases, in particular α-galactosidase A. These findings raise the possibility that antigen-presenting cells may also be able to process pure carbohydrate antigens.

In conclusion, the work in this chapter shows that DCs can internalise pneumococcal polysaccharides, possibly via a specific receptor, and traffic such antigens through the endocytic pathway. This supports the hypothesis that DCs may be directly involved in responses to pneumococcal polysaccharides. This possibility will be investigated further in Chapter V.
Chapter V

Maturation of DCs to polysaccharide and conjugate vaccine antigens
V. Maturation of DCs to polysaccharide and conjugate antigens

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5.1. INTRODUCTION

In the previous chapter I have shown that pneumococcal polysaccharides are indeed taken up by DCs and undergo intracellular trafficking. It is still unclear however, whether this results in processing of the antigens and their presentation to effector cells. It is also unclear whether uptake of polysaccharide antigens can induce DC maturation, which in the case of protein antigens is pivotal to efficient antigen presentation.

The dogma that polysaccharides cannot be presented because they do not bind classical MHC molecules has recently been challenged. Kalka-Moll and colleagues showed that at least certain polysaccharides could elicit potent CD4⁺ T cell responses (Kalka-Moll et al 2002). This depended on direct contact with MHC class II-bearing DCs. In this case, the potent T cell stimulatory capacity was ascribed to the particular chemical structure of the polysaccharide. The polysaccharide used was pneumococcal polysaccharide serotype 1 which has both positively and negatively charged groups. Presence of both groups was required for the T cell proliferative response.

The chemical structure of most other polysaccharides is unfavourable for binding to MHC class I or II. However, they may still bind non-conventional antigen-presenting molecules such as the CD1 family. Capsular polysaccharides in their naturally occurring, lipidated form have long been suggested to bind to CD1 molecules, in particular CD1b (Fairhurst et al 1998a). CD1b as well as CD1c localise to late endosomes and lysosomes in DCs. As I have shown in the previous chapter, pneumococcal polysaccharides also localise to late endosomes and lysosomes and thus the polysaccharides might gain access to CD1b and CD1c.

No other studies have so far investigated the involvement of DCs in responses to pure polysaccharide vaccines and this is generally regarded as unlikely. However, the data presented in the previous chapter in this thesis indicate that DCs may in fact directly interact with such antigens. This prompted me to investigate this possibility further. The findings are presented in this chapter.
While the involvement of DCs in responses to pure polysaccharides is still uncertain, it has been clearly shown that polysaccharide-protein complexes induce typical T-dependent immune responses and result in secretion of high-affinity, isotype-switched antibodies and generation of memory. Most efficient help for B cells is generally provided by T cells that have been primed by DCs. The importance of DCs in such responses has also been demonstrated more directly using knockout mice. These experiments have shown that responses to glycoconjugates critically rely on MHC-antigen-TCR, B7-CD28 and CD40-CD40ligand interactions (Guttormsen et al 1999).

In addition, DCs appear to be able to present glycopeptides derived from glycosylated proteins to glycopeptide-specific T cells. These data provide strong evidence for an involvement of DCs in responses to glycoconjugate antigens. However, the direct interaction between DCs and pneumococcal conjugates has never been evaluated and will be investigated in the following chapter.

As with other vaccines, adjuvants enhance responses to glycoconjugate vaccines (Chu et al 2000, Kovarik et al 2001). This prompted us to hypothesise that conjugates may not by themselves provide strong enough signals to induce DC maturation and that co-delivery of immunostimulatory adjuvants might induce DC maturation thereby greatly increasing intracellular processing of the antigen and enhancing the T cell stimulatory capacity of the DC.

In order to shed light on the role of DCs in responses to pure polysaccharides and polysaccharide-protein complexes, we set out to investigate the interaction between capsular polysaccharides and their corresponding glycoconjugate complexes and human monocyte-derived DCs. We used several different serotypes that were chosen on the basis of the frequency with which they cause disease in humans (James et al 2003).

Presentation of pneumococcal polysaccharides in combination with a protein occurs not only during the immune response to the glycoconjugate vaccine but also during natural exposure to the encapsulated bacteria. Therefore, whole, heat-killed bacteria
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of the same serotypes as the polysaccharides and conjugates were included in this study. Access to a *Haemophilus influenzae*-derived capsular polysaccharide and its conjugate allowed us to also include these antigens in order to get a broader overview of the interactions between DCs and polysaccharide and conjugate antigens.

In the following chapter we focussed on the ability of the different antigens to induce and modulate DC maturation as judged by changes in phenotype and cytokine production. We also present data regarding the presentation of polysaccharides by DCs.
5.2. EFFECT OF PPS/PPS-TT ON DC PHENOTYPE AND FUNCTION

The level of phenotypic maturation of the DC is key in priming of naïve T cells, with high activation state favouring priming and low activation state likely to induce anergy or tolerance (Steinman et al 2003). Therefore, I examined the effect of polysaccharides and conjugates on DC phenotype.

I incubated immature DCs with the pneumococcal polysaccharides type 1, 6B, 9N, 14, 19F and 23F and the corresponding conjugates for 24 hours. Concentrations were adjusted for the different reagents so that the TT components of the conjugates was at 10 µg/ml and the concentration of the pure polysaccharides was the same as the concentration of the polysaccharide component in the conjugates (i.e. 4.4 µg/ml PPS1, 6.4 µg/ml PPS6B, 5 µg/ml PPS9N, 5.3 µg/ml PPS14, 4.5 µg/ml PPS19F and 5.8 µg/ml PPS23F). Tetanus toxoid (TT) was included as a control antigen and used at 10µg/ml.

After overnight incubation of DCs with these antigens, expression of critical surface markers such as HLA-DR, CD86 and CD40 was analysed by flow cytometry. Figure 5.1. shows that LPS induced strong upregulation of these important antigen-presenting and co-stimulatory molecules. LPS also upregulated CD83, a DC maturation marker, indicating that DCs underwent full maturation. In contrast, neither PPS, PPS-TT nor the control antigen TT had any effect on surface markers.

T cell activation and differentiation by DCs is critically influenced not only by phenotype but also the secretion of cytokines. IL-12 and IL-10 are particularly important due to their potent inflammatory and immunosuppressive activities. Therefore we tested DC culture supernatants for production of these key cytokines in response to polysaccharides, conjugates or LPS. As shown in Figure 5.2, immature DCs produced no or very little IL-12 or IL-10 after overnight incubation with PPS or PPS-TT. In contrast, they produced significant amount of both cytokines after stimulation with LPS.
Figure 5.1. Effect of PPS/PPS-TT on DC phenotype. A) Immature DCs were incubated overnight with PPS or PPS-TT (types 1, 6B, 9N, 14, 19F or 23F) or 0.5μg/ml LPS and expression of HLA-DR, CD40 and CD86 analysed by flow cytometry. As there were no differences between the various serotypes in surface marker expression, data were pooled and represent means of 6 different serotypes and of 5 independent experiments. B) FACS plots (representative of 3 experiments) of DC surface expression of HLA-DR, CD83 and CD86 after overnight incubation with 10 μg/ml TT (filled profiles). Empty profiles are immature DC. *p<0.001, using Student’s paired T test.
Figure 5.2. Effect of PPS/PPS-TT on DC cytokine production. Immature DCs were incubated for 24 hr with LPS, PPS or PPS-TT or left unchallenged and levels of IL-12 and IL-10 in culture supernatants measured by standard ELISA. As there were no differences between the various serotypes in surface marker expression, data were pooled and represent means of 6 different serotypes and of 5 independent experiments.
5.3. MODULATION OF LPS-INDUCED DC MATURATION BY PPS/PPS-TT

The previous experiments showed that pneumococcal polysaccharides or conjugates do not induce DC maturation in the absence of other inflammatory stimuli. However, in vivo, DCs are likely to encounter polysaccharide antigens or conjugates in the presence of immunostimulatory molecules, be it other bacterial components during natural infection or adjuvants during vaccination. There are numerous different inflammatory stimuli that have diverse effects on DCs. *E. coli* LPS is one of the most widely used maturation stimuli and LPS-induced DC maturation has been very well characterized (Verhasselt et al 1997, Buelens et al 1997b, De Smedt et al 1998, Langrish et al 2002).

I therefore incubated DCs with pneumococcal polysaccharides or conjugates for 24 hours in the absence or presence of LPS in order to determine whether presence of such antigens modulates LPS-induced DC maturation. When LPS was used, it was added at the same time as the antigens. Tetanus toxoid was used as a control antigen. After 24 hours incubation, the phenotype of the cells was analysed by flow cytometry.

FACS analysis showed that incubation with PPS, PPS-TT or TT and LPS resulted in slightly reduced levels of HLA-DR, CD86 and CD40 as compared to LPS alone but the distinct upregulation of these markers was still seen (Figure 5.3 A). These differences were not statistically significant. In contrast, changes in CD14 expression were statistically significant (Figure 5.3 B). CD14, a marker of monocytes and macrophages, is present at low levels on immature DCs and is downregulated upon maturation. When DCs were incubated with PPS or PPS-TT and LPS upregulation of CD14 was noted. When PPS or PPS-TT were added 4 hours after addition of LPS, i.e. when DCs were just undergoing maturation, PPS and PPS-TT did not affect the LPS-induced downregulation of CD14.

Supernatants of these cultures had been stored and were used for measurement of cytokines by ELISA. This revealed striking differences. As shown in Figure 5.4, DCs produced considerable levels of IL-10 upon challenge with LPS. When they were co-
incubated with PPS or PPS-TT, however, IL-10 secretion increased significantly
\( (p=0.008 \text{ and } 0.07 \text{ respectively}) \). IL-12 secretion tended to be reduced as IL-10
increased but differences were not significant.
Figure 5.3. Effect of PPS/PPS-TT on LPS-induced phenotypic maturation of DCs.
A) Immature DCs were incubated with medium alone (dotted lines), with LPS alone (hatched lines) or with PPS, PPS-TT or TT in the presence of LPS (filled profiles) for 24 hours. Surface marker expression was analysed by flow cytometry. FACS plots of HLA-DR, CD86 and CD40 expression are representative of 9 independent experiments.

B) Pooled data of CD14 expression are shown as bars representing means and standard errors. This graph also shows CD14 expression by control cells stimulated with LPS first and then antigen ('control'), allowing a total of 24 hours exposure to LPS as all other LPS-exposed cells. p values were calculated using Student's paired T test (n=9) and are indicated.
Figure 5.4. Effect of PPS/PPS-TT/TT on LPS-induced DC cytokine production. Immature DCs were incubated with PPS, PPS-TT or TT in the absence or presence of 0.5 µg/ml LPS and IL-12 and IL-10 levels in the culture supernatant measured by ELISA after 24 hours of incubation. Two different serotypes were used (14 and 19F). As there were no differences between the various serotypes in cytokine production, data were pooled and represent means of 2 different serotypes and of 9 independent experiments.
5.4. PRESENTATION OF PPS/PPS-TT BY DCs

The previous experiments have shown that uptake of pneumococcal polysaccharides does not result in preparation of the DC for antigen presentation, i.e. there is no upregulation of MHC class II and co-stimulatory molecules and no production of cytokines. I have also demonstrated that while pneumococcal polysaccharides do not induce maturation themselves, they can modulate the LPS-induced DC maturation. These observations raise the questions of whether these antigens can be presented by DCs to T cells and whether high-level IL-10 production after exposure to PPS in the presence of LPS would alter the polarization of responding T cells.

5.4.1. Initial screening

In pilot experiments, we screened adult volunteers for the presence of effector memory using PBMC Elispots. PBMCs were incubated overnight on a pre-coated Elispot plate with PPS or PPS-TT or the control antigen TT directly added to the wells. Each of these was stimulated either with antigen alone or antigen and LPS. IFN-γ, IL-5 and IL-10 were selected for this assay as these cytokines may provide most information as to the subtype of any responding T cell population, i.e. Th1 versus Th2 versus regulatory cells.

The results from these experiments are presented in Figure 5.5. The IFN-γ and IL-10 values exhibited a normal distribution and are therefore shown as bars representing means and standard errors. The data from the IL-5 were highly skewed and there was only one donor that responded at all. Therefore, these data are shown as individual values in a scatterplot.

As shown in Figure 5.5, all donors produced IFN-γ in response to TT. There was no response to the polysaccharide and a small but significant response to the conjugate, presumably due to recognition of the TT component. However, this was below the response to TT alone, which is surprising as both were used at the same concentration for the TT component. Addition of LPS significantly enhanced responses in all cases. There was no difference in IFN-γ production between LPS-
stimulated PBMCs and PBMCs exposed to polysaccharide and LPS or conjugate and LPS.

IL-5 production was very low in all donors but detectable. The positive control response to the mitogenic stimulus PMA/ionomycin was approximately 20 spots/200,000 cells (median, data not shown). Figure 5.5. shows that only one donor produced IL-5 at all. This donor responded to TT and conjugates by production of both IL-5 and IFN-γ, indicating effector memory of a mixed Th1/Th2 profile. As expected, addition of LPS reduced the IL-5 response in this donor.

IL-10 production was also very low, the mean positive control response to PMA/ionomycin being 16 spots/200,000 cells (not shown). As shown in Figure 5.5, we detected a small IL-10 response after stimulation with TT. Again, the response to PPS-TT was lower than that to TT alone. Addition of LPS enhanced all responses, but not all to statistical significance. There was no increase in IL-10 production by PPS+LPS-stimulated PBMCs as compared to LPS-stimulated PBMCs. The donor that produced IL-5 in response to TT and PPS-TT also produced the highest levels of IL-10 in response to these antigens (data not shown).

After the initial screening, we performed the more labour-intensive and time-consuming co-culture experiments, initially for the polysaccharides only. Here, we pulsed DCs with PPS or PPS and LPS and co-cultured them with PBMCs for 7 days to allow T cells activation and expansion. We measured proliferation by CFSE labelling and used Elispots and intracellular cytokine staining in combination with surface staining to further characterize the responding T cells. IFN-γ, IL-5 and IL-10 were again selected as most representative of different T cell subtype profiles. DCs were loaded with PPS in the absence and presence of LPS to evaluate the influence of antigen loaded, immature versus antigen-loaded, mature, IL-10 producing DCs on differentiation of the responding T cells.
Figure 5.5. IFN-γ production by PBMC in response to PPS/PPS-TT and LPS stimulation. Freshly isolated adult PBMC were stimulated with medium alone, PPS, PPS-TT or TT, each in the absence (filled bars or squares) or presence of LPS (empty bars or squares) Cells were incubated 24 hours on an Elispot plate pre-coated with anti-cytokine capture antibody before visualisation. SFC is the number of spot-forming cells, i.e. the number of cells secreting IFN-γ. Some of the statistically significant differences are indicated (*). *p values for these differences were between 0.01 and 0.04, calculated using Student’s paired T test (n=7).
5.4.2. Proliferation of PBMCs in response to antigen-loaded DCs

For measurement of proliferation, DCs were pulsed with PPS or PPS + LPS and co-cultured with CFSE-labelled autologous PBMCs. After 7 days of culture, cells were stained for CD4 expression and analysed by flow cytometry. Gates for successive generations of dividing cells were set as explained in section 2.12.

Figure 5.6. shows results from three independent experiments. In all plots cells with the fewest divisions had the highest CFSE staining and cells with the most divisions had the lowest CFSE staining. In all three donors, a subpopulation of the PBMCs had proliferated vigorously in response to antigen-loaded or LPS-stimulated DCs whereas the remaining cells did not undergo any or only one division after 7 day culture. The histograms in Figure 5.6.A clearly show two widely separated groups and there were no smaller populations between the two groups. This indicates that the activation occurred very early during the co-culture and that the proliferating cells went through successive cycles of division collectively.

As expected, only a few cells divided when stimulated with immature DCs whereas a large proportion of PBMCs proliferated vigorously in response to LPS-matured DCs. Intriguingly, more cells proliferated in cultures with PPS-loaded DCs than those with immature DCs, as seen by the higher percentage of cells that had divided in the presence of PPS. DCs incubated with PPS and LPS also stimulated more cells to multiply than did DCs matured with LPS alone.

In an attempt to further characterize this putative PPS-responsive cell population, CFSE staining was analysed in conjunction with CD4 expression. The scatterplots in Figure 5.6.B show that the undivided cells comprised of CD4+ and CD4- cells in roughly equal numbers. However, in two out of three donors the group of proliferating cells contained many more CD4+ cells than CD4- cells. This demonstrated that, while some CD4- cells did proliferate in these co-cultures, CD4+ cells did so much more vigorously. The few proliferating cells in the CD4- compartment are likely to contain mostly CD8+ T cells.
### Figure 5.6. Proliferation by PBMCs in response to antigen-loaded DCs.

Immature DCs were incubated overnight with PPS or PPS and LPS and co-cultured for 7 days with CFSE-labelled autologous PBMCs at a ratio of 1:10. Cells were then surface-stained for CD4 and analysed by flow cytometry. Both histograms and scatterplots from 3 independent experiments (A, B, C) are shown. Histograms depict the gates for successive generations and scatterplots show CFSE staining of CD4⁺ and CD4⁻ populations.
5.4.3. Cytokine production of PBMCs in response to antigen-loaded DCs

The previous experiments suggested the presence of a putative PPS-responsive population that can be primed to proliferate by antigen-loaded DCs. In the following experiments we attempted to further characterise this cell population by assessing cytokine production both by the Elispot technique and by intracellular cytokine staining in combination with surface staining.

DCs were loaded with PPS or PPS + LPS and co-cultured with autologous PBMCs. After 7 days of culture, some of cells were transferred to pre-coated Elispot plates for a further 24 hours incubation. Brefeldin A was added to the remaining cells for the final 16 hours of culture to block cytokine secretion. The following day, the Elispot plates were developed and the brefeldin A-exposed cells stained for intracellular cytokine and for surface CD4 expression. In both cases, IFN-\(\gamma\), IL-5 and IL-10 production were measured.

Figure 5.7. shows images obtained from the Elispot plates of one experiment. IFN-\(\gamma\)-producing cells were easily detectable by this technique. There was some background staining of the wells and the spots were very diverse in size and in intensity, making accurate counting very difficult. The images show that IFN-\(\gamma\) is readily produced in response to the mitogenic stimulus PMA-ionomycin and in response to LPS. PBMCs co-cultured with immature or PPS-pulsed DCs did not produce any IFN-\(\gamma\). The response to DCs incubated with PPS in the presence of LPS appeared to be not different from the response to DCs stimulated with LPS alone. However, the inaccuracy of counting made it difficult to draw conclusions from these data.

IL-5 was produced by very few cells. However, there was no background staining of the well and the spots were very defined and homogenous, facilitating accurate counting. There appeared to be no IL-5 production by PBMCs in response to immature, mature or PPS-loaded DCs. The small response to PPS+LPS-loaded DCs in the donor depicted in Figure 5.7. was also seen in the other two donors. However,
the very small numbers of responding cells makes interpretation of such observations difficult.

IL-10 Elispots always led to relatively high background staining, presumably due to more non-specific binding of this antibody as compared to the IL-5 antibody. Nevertheless, spots were clearly defined, of homogenous size and easily detectable and countable. PBMCs did not produce IL-10 in response to any of the DC populations.

The co-cultures were then analysed by intracellular cytokine staining in combination with CD4 surface staining. Gates were set using appropriate isotype controls. Scatterplots of the results are presented in Figure 5.8.

As in the Elispot, IFN-γ production was detected easily. Both the CD4+ and the CD4− fraction of the PBMCs produced IFN-γ equally well after stimulation with PMA/ionomycin with 24% and 23% of cells respectively staining positive. The fluorescence intensity of positive cells was spread out over more than two logs demonstrating that IFN-γ production at the single-cell level is very variable. This corresponded to the variability in spot size and intensity seen in the IFN-γ Elispot.

When PBMCs were stimulated with immature DCs, about 7% of cells produced some IFN-γ with more CD4+ than CD4− cells staining positive. This increased 3-fold to 19.8% positive cells when DCs had been matured with LPS. Use of DCs incubated with PPS+LPS reduced the number of IFN-γ-producing cells to 8.5%. These trends were observed in all donors. DCs loaded with PPS appeared to induce some IFN-γ production in one donor (shown in Figure 5.8).

In all three donors, the total CD4+ fraction, both cytokine-secreting and not secreting, was slightly larger than the total CD4− fraction (approximately 60% and 40% of total PBMCs respectively). This difference may have accounted for the difference observed for the PPS condition (8.6 versus 4.8%). For all the other conditions, differences in IFN-γ production between the two populations were larger than could
be accounted for by differences in cell number. This would suggest that immature, LPS- or PPS and LPS-loaded DCs preferentially induced IFN-γ production by CD4^+ cells.

None of the donors produced any IL-5 in response to immature, mature, PPS- or PPS and LPS-loaded DCs. Also IL-10 production was very low in all cases. The small number of cells secreting IL-5 or IL-10 made interpretation of the values very difficult. A further problem was the lack of a good positive control for IL-5 and IL-10 production.
### Figure 5.7. IFN-γ, IL-5 and IL-10 production by PBMC in response to antigen-loaded DCs - Elispot.

DCs were incubated with PPS, LPS or PPS+LPS and then co-cultured with autologous PBMCs for 7 days at a ratio of 1:10. Control PBMCs were cultured alone for 7 days. Cells were transferred to Elispot plates pre-coated with anti-cytokine capture antibody and incubated for a further 24 hours before visualisation. PMA and ionomycin were added to unstimulated PBMCs for the last 24 hours as a positive control. Images of representative wells are shown for each condition and each cytokine. Numbers of spot-forming, i.e. cytokine-secreting, cells were counted using a Bio-Sys ELISPOT reader.
**Figure 5.8. IFN-γ, IL-5 and IL-10 production by PBMC in response to DCs - Intracellular cytokine staining.** Immature DCs were incubated with LPS, PPS or PPS+LPS and co-cultured for 7 days with autologous PBMCs at a ratio of 1:10. Control PBMCs were cultured alone for 7 days and stimulated with PMA/ionomycin for the final 16 hours of culture. Brefeldin A was added to all cells for the final 16 hours to block cytokine secretion. Cells were then stained for intracellular expression of IFN-γ, IL-5 or IL-10 and surface expression of CD4 and then analysed by flow cytometry. Scatterplots from 1 of 3 independent experiments are shown. Gates were set arbitrarily to approximately 1% for the isotype control antibody.
5.5. **EXPRESSION OF NON-CLASSICAL ANTIGEN PRESENTING MOLECULES BY DCs**

Presentation of polysaccharides by DCs might occur in the context of classical or non-classical antigen-presenting molecules. Efficient antigen presentation on MHC class II requires upregulation of this molecule. We have shown before that pneumococcal CPS do not alter expression of MHC class II. Therefore we next wanted to explore their effect on expression of the non-classical antigen presenting molecules of the CD1 family, all of which are expressed on DCs.

At first, we examined patterns of CD1 expression on immature DCs and determined how these pattern change upon maturation with LPS. Thus, we incubated immature DCs overnight with LPS and then stained with monoclonal antibodies against CD1a, CD1b, CD1c and CD1d.

As shown in Figure 5.9, the CD1a profile of immature DCs shows a biphasic distribution, which was observed in all donors tested (n=12). CD1b and CD1c are expressed at significant levels by immature DCs, whereas CD1d was not detected in any of the donors. In contrast to the dramatic effects on MHC expression, LPS had little effect on CD1 profiles. CD1a levels decreased slightly but consistently and significantly. CD1b, CD1c and CD1d did not undergo any changes in expression when DCs were stimulated with LPS.

We then examined the effect of pneumococcal CPS (serotypes 1, 6, 9N, 14, 19F, 23F) and the corresponding glycoconjugates on CD1 expression. As shown in Figure 5.10, none of these antigens induced any changes in the expression of any of the CD1 molecules.
Figure 5.9. Changes in CD1 expression upon LPS activation. Immature DCs were stimulated with 0.5 μg/ml LPS for 24 hr and stained for surface expression of CD1a, b, c and d. FACS profiles shown (A) are representative of 12 independent experiments. B) Data for CD1a in 12 different experiments were pooled; bars show means and standard errors. p was calculated using Student’s paired T test.
Figure 5.10. Effect of PPS/PPS-TT on CD1 expression. Immature DCs were incubated with PPS or PPS-TT for 24 hr or left unchallenged (iDC) and stained for surface expression of CD1a, b, c and d. As there were no differences between the various serotypes in surface marker expression, data were pooled and represent means and error bars of 6 different serotypes and of 8 independent experiments.
5.6. MATURATION OF DCs IN RESPONSE TO S. PNEUMONIAE BACTERIA

As previously shown in this chapter, neither pure pneumococcal polysaccharides nor conjugates induced DCs maturation. However, both were able to modulate the response of DCs to the inflammatory stimulus LPS. Bacterial infection may be one situation where DCs encounter polysaccharide antigens in such an inflammatory context.

Therefore we wished to investigate the effect of encapsulated bacteria in our DC culture system. Bacteria were grown from frozen ATCC cultures as described in section 2.3, heat-killed and confirmed to be pure and non-viable. The Quellung reaction was performed to confirm that all strains were highly encapsulated. Agglutination tests were used to corroborate the results of the Quellung reaction and to verify the capsular type of the strains. Immature DCs were then incubated overnight with bacteria at a ratio of 1:100. DCs maturation was then assessed by staining of the surface markers HLA-DR, CD86 and CD40 and by measuring IL-12 and IL-10 in the culture supernatants.

As shown in Figure 5.11, challenge of immature DCs with bacteria did induce phenotypic maturation to some extent. HLA-DR, CD86 and CD40 were all upregulated, however, upregulation was not as strong as that achieved with LPS. When assessing culture supernatants for cytokine production, we found that the bacteria generally induced only very low levels of IL-12 and IL-10, with the exception of type 6B and type 19F (Figure 5.12). Type 6B stimulated low level IL-12 production but IL-10 was not different compared to the other serotypes. In contrast, type 19F was able to induce significant levels of both IL-12 and IL-10. IL-12 production in particular was high and not significantly different from that observed after challenge with LPS. IL-10 production was also slightly higher than observed with the other serotypes, but was significantly lower than LPS-induced IL-10 levels.
V. Maturation of DCs to polysaccharide and conjugate antigens

Figure 5.11. Effect of S. pneumoniae on DC phenotype. Immature DCs were incubated with LPS or whole, heat-killed S. pneumoniae at a ratio of 1:100 for 24 hr and surface marker expression (HLA-DR, CD40 and CD86 shown) analysed by flow cytometry. Bars represent means of 3 independent experiments and standard errors. *p<0.001, using Student’s paired T test.
Figure 5.12. Effect of *S. pneumoniae* on DC cytokine production. Immature DCs were incubated with whole, heat-killed *S. pneumoniae* bacteria, LPS or left unchallenged. After 24 hours incubation, IL-12 and IL-10 levels in the culture supernatant were measured by ELISA. Data are means of 3 independent experiments and standard errors. *p=0.3, **p=0.02, using Student’s paired T test.
5.7. SUMMARY OF RESULTS FOR PNEUMOCOCCAL ANTIGENS

- PPS/PPS-TT do not induce DC maturation.

- PPS/PPS-TT modulate LPS-induced DC maturation to induce high-level IL-10 production.

- Presence of PPS in the co-cultures increases T cell proliferation, both in the absence and in the presence of LPS.

- Loading DCs with PPS in the presence of LPS reduces IFN-\(\gamma\) production as compared to LPS alone.

- Neither PPS nor PPS-TT alter expression of CD1 molecules.

- \textit{S. pneumoniae} bacteria induce phenotypic DC maturation to some extent and some serotypes can stimulate IL-12 and IL-10 production.
5.8. HAEMOPHILUS INFLUENZAE-DERIVED ANTIGENS

In order to obtain a broader overview over interactions between human DCs and polysaccharide and conjugate antigens, we investigated the effects of *H. influenzae*-derived capsular polysaccharide (polyribosylribositol phosphate, PRP) and its conjugate (PRP-TT) on DC maturation.

5.8.1. Effect of PRP/PRP-TT on DC phenotype

Immature DCs were incubated overnight with PRP, PRP-TT or LPS and their phenotype analysed by flow cytometry. Figure 5.13 shows that PRP did not induce any changes in expression of HLA-DR, CD83 or CD86, whereas addition of PRP-TT resulted in strong upregulation of all three markers to levels comparable to those induced by LPS. Only the median fluorescence intensity of CD86 after incubation with PRP-TT remained somewhat below the LPS-induced level.

LPS is a frequent contaminant of reagents and antigenic preparations and DCs are highly sensitive to LPS. Thus, we wanted to exclude the possibility that upregulation of surface markers was due to LPS contamination in the antigenic preparation rather than the antigen itself. We determined LPS content in the preparations using the limulus amebocyte lysate (LAL) endotoxin test kit. Levels were found to be <4.4 pg/ml (the detection limit of the assay) for PRP and 41 pg/ml for PRP-TT. This is well below the level of 100 pg/ml which, as shown in Figure 3.2., did cause some upregulation but this was far lower than that induced by PRP-TT. We therefore believe that the effect of PRP-TT on the DCs was due to properties of the antigen itself and not due to LPS contamination.

While surface marker expression on PRP-pulsed DCs was not significantly different from that of immature DCs, there was a trend towards increased expression on PRP-pulsed DCs, in particular of HLA-DR. This trend correlated well with the extent of DC clustering observed in the cultures. As shown in Chapter III, DC clustering is a very consistent feature of DC maturation.
V. Maturation of DCs to polysaccharide and conjugate antigens

We imaged DCs on an inverted phase microscope after overnight incubation with PRP or PRP-TT. Representative images are shown in Figure 5.14. In agreement with the flow cytometry results, DCs incubated with PRP did cluster to some extent and many of the cells adopted the polarised form of maturing DCs. As expected, incubation with PRP-TT resulted in dramatic changes in DC morphology and formation of very large clusters.
Figure 5.13. Effect of PRP/PRP-TT on DC phenotype. Immature DCs were incubated for 20 hr with PRP, PRP-TT or LPS and analysed by flow cytometry for expression of HLA-DR, CD83 and CD86. Both values for % positive cells (hatched bars) and mean channel fluorescence (empty bars) are shown. Bars represent means and standard errors (n=5).
Figure 5.14. Effect of PRP and PRP-TT on DC clustering. Immature DCs were incubated for 24 hr with PRP or PRP-TT. Images were taken on a Zeiss Axiovert inverted microscope with x10 (top) and x40 (bottom) phase lenses using Improvision Openlab software and were processed using Adobe Photoshop 5.0.
5.8.2. Presentation of PRP/PRP-TT by DCs

In order to investigate whether this polysaccharide and its conjugate could be presented by DCs, I performed DC-T cocultures and evaluated T cell responses using IFN-γ Elispots.

In pilot experiments, we screened adult volunteers for possibly existing memory to the antigens using IFN-γ PBMC Elispots. Here, PBMCs were incubated overnight on a pre-coated IFN-γ Elispot plate with PRP or PRP-TT or the control antigen TT directly added to the wells. As shown in Figure 5.15, 3 out of 7 individuals produced some IFN-γ in response to the polysaccharide, in particular at high concentrations. The same donors also showed a response to the conjugate, with more cells secreting IFN-γ in response to the conjugate than with the polysaccharide. Again, IFN-γ production was antigen dose-dependent.

After the initial screening, we used cells from volunteers who showed a positive response for the more labour-intensive and time-consuming co-culture experiments. Here, DCs were pulsed with PRP or PRP-TT overnight and then co-cultured with autologous PBMC for 6 days. Cells were transferred to a pre-coated IFN-γ Elispot plate and incubated a further 24 hours before visualization. As shown in Figure 5.16, PBMCs did produce some IFN-γ in response to immature DCs. In contrast to the data from the PBMC Elispots, there was no antigen-specific response to PRP-pulsed DCs, while PBMCs responded strongly to stimulation with PRP-TT-loaded DCs. IFN-γ production in response to such DCs was not different from that induced by TT-pulsed DCs.
Figure 5.15. IFN-γ production by PBMC in response to PRP/PRP-TT stimulation. Freshly isolated adult PBMC were stimulated for 24 hr with PRP, PRP-TT, TT or left unstimulated (NS) on an Elispot plate pre-coated with anti-IFN-γ capture antibody. IFN-γ SFC is the number of IFN-γ spot-forming cells, i.e. the number of cells secreting IFN-γ. Lines of the same colour in the two graphs represent the same individual (n=7).
V. Maturation of DCs to polysaccharide and conjugate antigens

Figure 5.16. IFN-γ production by PBMCs in response to PRP/PRP-TT-pulsed DCs. Immature DCs were incubated with medium alone, PRP, PRP-TT or TT and then co-cultured with autologous PBMCs for 7 days at a ratio of 1:10. Cells were transferred to Elispot plates pre-coated with anti-cytokine capture antibody and incubated for a further 24 hours before visualisation and counting on a Bio-Sys ELISpot reader. IFN-γ SFC is the number of spot-forming cells, i.e. cells secreting IFN-γ. Control cultures of T cells, unpulsed and pulsed DCs alone were included and always showed between 0 and 5 spots (data not shown).
5.9. DISCUSSION

5.9.1. DC maturation in response to pneumococcal polysaccharides and conjugates

In this chapter we have investigated the interaction of DCs and pneumococcal polysaccharide antigens (PPS) or the corresponding polysaccharide-protein complexes. We show that neither PPS nor glycoconjugates are able to induce DC maturation, as judged by upregulation of surface markers and cytokine production. To our knowledge, this is the first report examining the interactions between PS or conjugates and DCs directly. There are some reports where the type of immunity in response to vaccination with conjugate vaccines was examined (McCool et al 1999, Guttormsen et al 1999). These publications show that helper T cells are directly involved in responses to conjugates, thereby providing indirect evidence for involvement of DCs.

The maturation of DCs is a pre-requisite for efficient antigen presentation. However, even if not optimal, immature DCs are still able to present antigen. In fact, presentation of self-antigens by immature DCs has been strongly implicated in the establishment of peripheral tolerance, in particular in the development of so-called natural regulatory cells. Therefore, presentation of PPS and conjugate antigens by such antigen-loaded, immature DCs may have serious consequences for the ensuing immune response.

5.9.2. Classical and non-classical antigen presentation of pneumococcal polysaccharides

If polysaccharides or conjugates are presented by DCs, it may be in the context of classical or non-classical antigen presenting molecules. In this chapter, we have shown that neither pneumococcal polysaccharides nor conjugates induce upregulation of MHC class II or CD1 in the absence of inflammatory stimuli. By comparison with protein antigens, this would suggest that presentation of PPS in the context of MHC class II would be very inefficient. The failure to alter CD1 expression is unlikely to be of consequence for the efficiency of presentation as CD1
molecules have been shown to present antigen in immature DCs as efficiently as in mature DCs suggesting a role for CD1-restricted presentation during the functions of immature DCs, i.e. maybe in the early phases of immune responses, innate-type responses or in the induction and maintenance of peripheral tolerance (Cao et al 2002).

Thus, CD1 or even as yet unidentified non-classical antigen-presenting molecules may play a role in the induction of polysaccharide-specific immune responses. A number of publications have indeed shown that immunization of mice with glycopeptides elicited MHC class I- as well as MHC class II-restricted T cells that specifically and only recognised the carbohydrate moiety (Michaelsson et al 1996, Speir et al 1999).

5.9.3. Modulation of DCs maturation by pneumococcal polysaccharides and conjugates

We have further demonstrated in this chapter that, while unable to induce DC maturation themselves, pneumococcal CPS and conjugates are able to modulate the maturation of DCs in response to LPS. We demonstrate that exposure to polysaccharides or conjugates before addition of LPS still allows phenotypic maturation but pre-disposes DCs to release high amounts of IL-10. IL-12 secretion appears slightly reduced and CD14 expression is upregulated. This is likely to be due to the presence of increased quantities of IL-10 as autocrine IL-10 has been shown to have inhibitory effects on maturing DCs (Corinti et al 2001).

Modulation of DC maturation to convert immunogenic into tolerogenic cells is a common immune escape mechanism used by many pathogens and also tumours to specifically prevent or suppress immune reactions directed towards them (see section 1.8). Such tolerogenic DCs produce reduced levels of IL-12 and/or dramatically increased levels of IL-10 upon activation and prime the development of antigen-specific regulatory cells. Such so-called adaptive regulatory cells are potent suppressors able to even down-regulate fully activated effector cells. Considering the high amount of IL-10 released by DCs upon incubation with polysaccharide or
conjugates and LPS, pneumococcal CPS may also manipulate DCs to induce antigen-specific regulatory cells that prevent or suppress immune reactions directed against them.

Pashenkov and colleagues recently published an intriguing observation regarding IL-10 production in response to pneumococcal antigens. They compared the cerebrospinal fluid (CSF) from patients with non-inflammatory neurological diseases or inflammatory conditions such as bacterial meningitis (BM) or Lyme meningoencephalitis (LM) (Pashenkov et al 2002). They assessed cytokines present in the CSF and the effect of these CSF on DCs in vitro. DCs are present in substantial numbers in the meninges and the choroid plexus and human cerebrospinal fluid contains myeloid and plasmacytoid DCs. In inflammatory LM and BM, the CSF contains a variety of microbial and endogenous DCs maturation factors and antigens and DCs are actively recruited from the blood. The study showed that CSF from both BM and LM contained some IFN-γ and IL-10 but only CSF from patients with bacterial meningitis contained very high levels of IL-10. BM was caused by *S. pneumoniae* (n=3), *N. meningitidis* (n=1) and *H. influenzae* (n=1). The source for the IL-10 production may be the DCs which would fit in with our observations.

5.9.4. Presentation of pneumococcal polysaccharides by DCs

I have shown in this chapter that human peripheral blood does contain a cell population that can be stimulated to proliferate by PPS-loaded DCs. In the absence of inflammatory stimuli, such DCs are phenotypically immature and do not produce cytokines, suggesting they may induce this population to develop into natural regulatory cells such as the CD4⁺CD25⁺ suppressor cells. Intriguingly, our experiments showed that in two out of three donors proliferation in response to PPS was concentrated in the CD4⁺ compartment.

In future experiments it will be interesting to further characterise this population with regard to their surface phenotype and study expression of CD25, CTLA-4 and TGF-β. Both CTLA-4 and TGF-β have been implicated in mediating suppression by natural regulatory cells. CTLA-4 binds with very high affinity to CD80 and CD86,
replacing the usual ligand CD28, and delivers inhibitory signals. It is upregulated upon T cell activation and proliferation and usually serves to terminate immune reactions. It is constitutively expressed on natural regulatory cells, the only cell population to show this constitutive expression. The role of TGF-β in tolerance induction was previously discounted due to lack of this cytokine in culture supernatants from regulatory cells. However, recent evidence shows that TGF-β is indeed released by regulatory cells but then binds to their surface and has suppressive action when in this membrane-bound form (Chen and Wahl 2003).

In the presence of inflammatory stimuli, in our experiments represented by LPS, PPS-loaded DCs are phenotypically mature, IL-10-producing cells that also appear to increase the proliferation of a cell population, which may be related to suppressive or regulatory cells. Many of the earlier studies of suppressor T cells showed that such cells do not proliferate in response to mitogenic or antigenic stimulation. However, Yamazaki and colleagues have recently shown that CD4⁺CD25⁺ suppressor cells can proliferate in vitro if stimulated with immature or mature DCs (Yamazaki et al 2003). Walker and colleagues have also demonstrated proliferation of such cells in vivo (Walker et al 2003).

A prominent feature of regulatory cells is their failure to produce effector cytokines such as IFN-γ or IL-4/IL-5. In our DC-T cell co-culture experiments we did not observe production of IL-5 or IL-10 by PBMCs stimulated with PPS-loaded, immature DCs. IFN-γ was produced by a fraction of PBMCs in one donor out of three. When PPS-loaded, LPS-matured DCs were used to activate PBMCs we still did not observe significant IL-5 or IL-10 production but unexpectedly did see some IFN-γ production.

In the study of CSF from bacterial meningitis patients mentioned above, the effect of the CSF on monocyte-derived DCs and their ability to stimulate T cells was also assessed in vitro (Pashenkov et al 2002). DCs exposed to BM CSF stimulated allogeneic T cells to produce only very little IFN-γ, IL-4 or IL-10. This is in agreement with our findings. However, there were also differences as BM-CSF
exposed DCs remained phenotypically and functionally immature with low levels of HLA-DR and CD86 and low production of IL-12 p40 presumably due to the suppressive effects of IL-10 present in the CSF.

When we compared IFN-γ production in response to PPS+LPS-pulsed DCs to that after co-culture with DCs matured with LPS alone, we observed a reduction in IFN-γ production. This may indicate the development of a population of regulatory cells that could suppress IFN-γ production by activated effector cells.

The reduction in IFN-γ production after pre-incubation with PPS was only seen in the intracellular cytokine staining. This highlights technical advantages and disadvantages of the two different techniques. The large variability in IFN-γ production by single cells makes counting cytokine-secreting cells in the Elispot technically challenging and unlikely to be accurate. The data from the intracellular cytokine staining appear much more reliable. In contrast, IL-5 and IL-10 are produced by so few cells and at such a low level, that this is either not detectable by intracellular cytokine staining or differences are very hard to detect. The Elispot technique, however, detects cytokine production at the single cell level and therefore allows detection of even very small numbers of cytokine-secreting cells.

IL-10 is not only implicated in the differentiation of regulatory cells but – at much lower levels – also in the polarisation of Th2 effector cells. Thus, one could also reason that PPS+LPS-pulsed DCs might induce the development of Th2 effector cells. This appears unlikely as we observed low levels of IFN-γ and no IL-5 in the cultures. However, the failure to detect IL-5 may be due to technical problems. Th2 type cytokines such as IL-4 or IL-5 are generally very hard to detect in this type of autologous system and their detection normally requires expansion of responding T cells with IL-2. However, IL-2 has also been reported to overcome the suppressive activity of regulatory cells (Thornton and Shevach 2000). The possible presence of such cells in our cultures meant we wanted to avoid addition of exogenous IL-2.
The data may allow a further interpretation, in particular for the proliferation in response to PPS. Pulsing with PPS alone results in DCs that are phenotypically immature, likely to have low levels of antigen-class II complexes, if any, and do not produce cytokines. Such features may be sufficient for the DCs to allow induction of central memory cells. After activation, naïve T cells differentiate into effector cells, central memory cells or effector memory cells depending on the strength of stimulation which in turn depends on ligand dose, DC number, duration of DC-T cell interaction and TCR avidity (Langenkamp et al 2002, Sallusto et al 1999).

Central memory cells are clonally expanded, non-polarized cells that have lower threshold for proliferation but do not yet produce effector cytokines. They are distinguished from effector cells by expression of the chemokine receptor CCR7 allowing them to reach secondary lymphoid organs. Therefore, in future it would be interesting to examine CCR7 expression by the responding cells in our co-culture system.

5.9.5. Presentation of pneumococcal conjugates by DCs

The pneumococcal conjugates were similar to the polysaccharides in that they did not induce DC maturation but pre-disposed DCs to release high levels of IL-10 upon challenge with LPS. This might suggest that they would have a similar effect on the response induced by such antigen-loaded, mature, IL-10 producing DCs. While in the PBMC Elispot we could not detect any responses to the polysaccharides, we did detect memory responses to the conjugates. These were presumably due to recognition of the TT component in the conjugates.

When comparing responses to TT and conjugates containing the same concentration of TT, IFN-γ production by PBMCs was consistently lower in response to the conjugates. The reasons for this are unclear. It is possible that the presence of the polysaccharide negatively affected the processing of the TT component, thereby reducing the number of available TT peptide-MHC complexes on the surface. Such an inhibitory effect of polysaccharides has been shown for processing of proteins in
macrophages (Gonzalez-Fernandez et al 1997). However, presence of polysaccharides did not affect processing of proteins in DCs and B cells suggesting that the reduced IFN-γ response might not be due to defective processing. However, this does not exclude the possibility that the covalently-linked polysaccharides present on the TT protein influenced processing as to alter the way the protein is cut into immunogenic peptides, thereby providing fewer or less diverse peptides for presentation thereby activating fewer T cells.

5.9.6. Presentation of H.influenzae-derived polysaccharides and conjugates by DCs

In this chapter, we have also investigated the ability of PRP and PRP-TT to induce DC maturation and elicit memory responses. Similar to the pneumococcal polysaccharides, PRP does not appear to induce DC maturation and PRP-loaded DCs were not able to induce IFN-γ production by PBMCs in vitro. However, despite this we did observe some memory IFN-γ responses to the pure polysaccharides in some individuals. In contrast to the pneumococcal conjugates, PRP-TT was a very potent stimulus for DC maturation and PRP-TT-pulsed DCs were able to elicit IFN-γ production at levels as seen with TT-loaded DCs, indicating the induction of Th1-type effector cells.

These results suggested that PRP-TT as a vaccine antigen may have very good immunogenicity on its own. This hypothesis is supported by the fact that, in contrast to pneumococcal conjugates and many other vaccines as e.g. against tetanus, PRP-TT is administered in the absence of adjuvant.

Good levels of IFN-γ production in response to PRP-TT were also observed in the PBMC Elispot, indicating presence of memory Th1-type effector cells. The volunteers used in this study are adults that have not been vaccinated against Hib. The responses to the polysaccharide and conjugate detected in some of them are therefore likely to arise from previous wild-type infection or carriage. This hypothesis is supported by the observation that vaccination of adults, who had never
previously been vaccinated, with Hib conjugate vaccines actually reactivates memory B cells (Hougs et al 1999).

The detection of IFN-γ producing cells upon stimulation with pure PRP was unexpected. The exact cellular source of this IFN-γ was unclear. PBMC isolated by centrifugation over Ficoll contain mostly T and B cells, monocytes but also NK cells. Of these, effector T or NK cells are the most likely to produce IFN-γ. NK cells have polysaccharide receptors and can be directly stimulated by such antigens to release cytokines including IFN-γ and GM-CSF (Mond 1995). These cells comprise about 5% of the PBMCs and could possibly be responsible for the IFN-γ production seen.

However, comparing responses to PRP and PRP-TT in our experiments showed that the same individuals that recognized the polysaccharide also responded to the conjugate, to the same extent or stronger than to TT alone. This suggests the source of the IFN-γ in response to polysaccharides may be effector T cells.

Presence of effector cells implies that DCs are able to induce such cells. This stands in contrast to the failure of the polysaccharides to induce DC maturation in vitro. A likely explanation for this discrepancy may be that the pure polysaccharide itself does not have any effect on DCs in vitro but in a natural infection or carriage may be presented in a different context, e.g. as a ‘natural conjugate’ associated with a cell-wall derived protein or in the presence of pathogen-derived inflammatory stimuli such as LPS. In contrast to the Gram-positive streptococci, the outer membrane of the Gram-negative Haemophilus contains the potent inflammatory stimulus lipopolysaccharide (Munson, Jr. 1990).

The capsular polysaccharides of encapsulated Gram-negative bacteria such as H. influenzae are very similar to the well-known CD1-ligand LAM. Thus, CD1-restricted presentation may be important for responses to such bacteria. Fairhurst and colleagues have in fact isolated a number of T cell lines that proliferated in response to nonpeptide Hib antigen (Fairhurst et al 1998b).
**5.9.7. Interaction between S. pneumoniae and DCs**

*S. pneumoniae* and *H. influenzae* are fundamentally different in that *S. pneumoniae* is Gram-positive and *H. influenzae* is Gram-negative. The potent inflammatory stimulus LPS is only present in the Gram-negative outer cell membrane. This is likely to have a significant impact on the interaction with DCs, as these are highly sensitive to such stimuli.

We have shown in this chapter that whole, encapsulated *S. pneumoniae* bacteria did induce some upregulation of surface markers and some cluster formation, indicative of onset of DC maturation. However, generally they were not able to induce production of IL-12 or IL-10. Secretion of such cytokines by DCs is vital for the induction of polarised effector immunity. Therefore, *S. pneumoniae* bacteria may be comparatively weak immunogens, as compared with other encapsulated bacteria such as *H. influenzae* or *Neisseria meningitidis*. To our knowledge, there are no studies that have directly evaluated the effect of *H. influenzae* bacteria on either human or murine DCs. However, a number of groups have studied the interaction between human DCs and *N. meningitidis*, another encapsulated Gram-negative bacterium that, like *S. pneumoniae* and *H. influenzae*, is a major pathogen for children under 2. This bacterium is well-known to have very potent DC maturation activity, with the meningococcal lipooligosaccharide shown to be the main mediator of the proinflammatory response in DCs (Unkmeir et al 2002).

In conclusion, the work presented in this chapter provides evidence that different pathogen-derived polysaccharides in the pure or conjugated form have very different effects on DC maturation resulting in populations of immature DCs, mature stimulatory DCs or mature, immunosuppressive DCs. This may have important consequences for the ensuing immune response.
Chapter VI

Maturation of DCs to protein vaccine antigens
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6.1. INTRODUCTION

The previous chapters focussed on polysaccharide vaccines and conjugates designed to prevent the major invasive diseases in childhood. However, in terms of the number of infectious episodes in childhood and GP consultations in childhood, otitis media remains the most important reason for such visits. Vaccines for otitis media are thus highly desirable and actively being pursued.

Acute otitis media (AOM) is an infection of the middle ear causing ear pain, temporary hearing loss and fever. It is the most frequent reason for pediatric office visits, resulting in about 20 million visits and costs of $2 to 3.8 million a year in the USA (Eskola et al 2001). Peak incidence is between 6 and 11 months of age and the three major causative agents of bacterial AOM are \textit{S. pneumoniae}, nontypeable \textit{Haemophilus influenzae} (NTHi) and \textit{Moraxella catarrhalis}, in order of importance. Different approaches are used in the development of vaccines against all of these.

The main focus of pneumococcal vaccination at the moment is the development of a polysaccharide-protein conjugate vaccine. Generally, conjugate vaccines have overcome many of the problems associated with polysaccharide-based vaccination. They induce good affinity maturation, isotype switching and the formation of memory cells. Most importantly they induce antibody responses in the very young (Goldblatt 2000, Eskola 2000). A successful conjugate that can prevent invasive disease is licensed and used routinely in the USA. Two more are undergoing clinical trials (Eskola 2000, Kayhty and Eskola 1996, Klein 1999). These contain 7 to 11 of the most prevalent serotypes covering 60-95\% of pneumococcal pneumonia and otitis media, at least in the developed world (Eskola 2000).

Pneumococcal vaccines appear promising but several limitations to their widespread use remain. The current formulations do not cover all the serotypes, which may cause disease, and different regions of the world have different distributions of prevalent serotypes (Eskola 2000). The use of a vaccine with limited specificity may also increase carriage of serotypes not represented in the formulations thereby increasing disease caused by such strains. This has in fact been observed in some clinical trials
(Eskola et al 2001) and may represent a major obstacle to large-scale vaccination with pneumococcal conjugates. Several injections and a booster dose are required to achieve strong and long-lasting protective immunity. Repeated use of the same carrier protein can reduce the immunogenicity of the conjugate (Dagan et al 1998). In addition, production of the conjugates is technically complex, thus limiting mass production, and very expensive, making them unaffordable for universal use in most countries (Russell and Mulholland 2002).

An alternative strategy receiving a lot of attention at the moment is the design of protein vaccines. One of their advantages would be broader protection, as immunity would not be restricted to serotypes contained in the vaccine. Recombinant proteins can be expressed in bacteria making large-scale production at low cost feasible. Initial studies in mice and rats have already produced promising results (Austrian 2000). Several proteins, many of them surface exposed, provided protection against otitis media, invasive infection, sepsis and death in experimental models. Many reduced nasal carriage, an important characteristic for a successful pneumococcal vaccine candidate (Russell and Mulholland 2002, Adamou et al 2001). Unfortunately, some of the candidate proteins also display substantial sequence heterogeneity between different isolates compounding the development of a universally functional vaccine.

Similar to *S. pneumoniae*-derived proteins, also protein antigens isolated from NTHi and *M. catarrhalis* are currently being evaluated as vaccine candidates. Outer membrane proteins and secreted virulence factors such as toxins are generally the most likely candidates in the search for a good vaccine antigen as they are the bacterial components that are recognized by and interact first with the immune system. They should also be highly conserved and present in all strains.

A number of such possible vaccine antigens have been tested so far in various animal models (Poolman et al 2000, McMichael 2000). Results suggest that both humoral and cell-mediated immune mechanisms are necessary for protection. Several of the possible vaccine antigens were able to afford protection and allow rapid clearance of bacteria after challenge. In some cases this was due to elicitation of functional
antibodies, in others protection was achieved despite failure to elicit such antibodies (Poolman et al 2000, McMichael 2000).

Development of good humoral responses with high titres of high-affinity antibodies requires T cell help. Thus, a good vaccine candidate antigen has to elicit both humoral and cell-mediated immunity. Pre-clinical studies in the search for vaccine candidates for NTHi and *M. catarrhalis* have so far mainly relied on data from humoral immune responses, i.e. *in vitro* assays assessing the functional capacity of antibody from human serum or from experimental animal immunizations. Information from animal models is limited as both *M. catarrhalis* and NTHi are exclusive colonizers of humans and primates (Poolman et al 2000, McMichael 2000).

So far, no studies have been published investigated whether such protein vaccine candidates for otitis media can induce antigen-specific cell-mediated immunity. Dendritic cells initiate T-cell responses and are therefore of utmost importance for cellular immunity, their maturation being the key control point. Immature DCs may be able to reactivate memory T cells but only mature DCs expressing high levels of antigen-presenting and co-stimulatory molecules can stimulate primary responses by naïve T cells. DCs are the main APC present at the sites of infection in otitis media, i.e. the middle ear mucosa and the adenoids (Ichimiya et al 1997).

In the following chapter, we examine the interaction of human monocyte-derived DCs with various antigens derived from *S. pneumoniae*, non-typeable *Haemophilus influenzae* and *M. catarrhalis* in order to find antigens which have the capacity to elicit not only good humoral but also strong cellular immune responses in humans. The antigens used are all known to be targets of serum-derived antibody in humans and good antibody titres are present in pre-immune adult serum (Poolman et al 2000, McMichael 2000).

To allow systematic evaluation of the interaction between prospective vaccine antigens and human DCs we set up a DC-based culture system designed to test the effects on DCs themselves and on DC-mediated antigen presentation. This may be crucial to understanding whether such antigens would make appropriate vaccine
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antigens. In particular the ability to be presented by DCs to T cells derived from adults and elicit memory responses in adults indicated that the relevant antigen contains suitable T cell epitopes. Memory in adults may not in itself be proof of protection mediated via the specific antigen in question but is a marker – at least – of exposure, successful processing and immune recognition. This information may be particularly useful for the development of vaccines against pathogens whose major importance is during early life and childhood, as the presence of immune memory in adults may correlate with the reduced susceptibility to infection in adult life.

Our system for testing antigens at the pre-clinical stage may make the early screening of potential vaccine antigens a more useful one and may be used even before testing in animals. Access to a human model of immune response so early in vaccine development and antigen discovery could be a powerful tool for vaccine manufacturers and may become an integral part of vaccine candidate evaluation.
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6.2. *Streptococcus pneumoniae*-derived antigens

In the first part of this work, we examined the interaction between human monocyte-derived DCs and four *S. pneumoniae*-derived proteins and peptides (PhtD, its N-terminal and C-terminal fragments [N-ter PhD, C-ter PhtD] and the N-terminal fragment of CbpA [N-ter CpbA]). These antigens are outer membrane-derived proteins that had shown potential as vaccine candidates.

**Phenotypic maturation**

DCs were incubated with the various antigens for 24 hour and phenotypic maturation of DCs assessed by flow cytometry. As shown in Figure 6.1, antigens N-ter PhtD and C-ter PhtD potently induced strong upregulation of HLA-DR, CD86 and CD40 (data shown), CD83 and a further downregulation of the low levels of CD14 present on immature cells (data not shown). In contrast, full-length PhtD and N-ter CbpA had no detectable effect on these surface markers. PhtD- and N-ter CbpA-exposed DCs were immature cells with markers such as HLA-DR, CD86, CD40 (shown in figure 6.1), CD83 and CD14 remaining unchanged (data not shown).

**Clustering**

These results were reflected in the extent of clustering observed in the cultures. As depicted in Figure 6.2, N-ter PhtD and C-ter PhtD stimulated formation of many very large clusters, a phenomenon usually associated with DC maturation. Cluster formation was to the extent seen in LPS-exposed DCs. In contrast PhtD- or N-ter CbpA-pulsed DCs failed to cluster, confirming their immature status.

**Cytokine production**

A further characteristic feature of DC maturation is the induction of IL-12 production and a substantial increase in IL-10 production. Therefore we measured the levels of these two cytokines in the culture supernatants of DC incubated with the four antigens as above. As shown in figure 6.3, DCs secreted high amounts of both IL-12 and IL-10 upon exposure to N-ter PhtD and C-ter PhtD but not in response to the
other two antigens, confirming the highly stimulatory nature of the antigenic preparations of the N-terminal and C-terminal fraction of PhtD.

**Antigen-specific memory**

We next tested whether it was possible to detect a T cell memory response to any of these antigens in non-vaccinated individuals. This would indicate that these surface-exposed antigens could elicit an immune response during natural exposure through carriage. The Elispot technique was chosen to measure IFN-\(\gamma\) and IL-5 secretion by PBMCS after overnight incubation with the four antigens. These two cytokines were selected as representatives of a \(T_{H1}\)- or \(T_{H2}\)-biased T cell memory profile.

Figure 6.4 shows that N-ter PhtD and C-ter PhtD indeed induced a \(T_{H1}\)-type response with high-level, antigen-specific IFN-\(\gamma\) and no IL-5 production. This profile was seen in all six donors tested. The response to PhtD and N-ter CbpA was more difficult to interpret due to low numbers of responder cells and considerable variation between donors. The donor shown in Figure 6.4 appeared to produce slightly more IL-5 in response to PhtD and N-ter CbpA than to N-ter PhtD and C-ter PhtD. However, this trend was not observed in all donors tested. When data from all six donors were pooled, there were no significant differences in IL-5 production between any of the proteins.
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Figure 6.1. Effect of PhtD, N-ter PhtD, C-ter PhtD and N-ter CbpA on DC phenotype. Immature DCs were incubated for 24 hr with 5 μg/ml PhtD, N-ter PhtD, C-ter PhtD or N-ter CbpA or 0.5 μg/ml LPS and assessed by FACS for expression of HLA-DR, CD83 and CD86. Plots are representative of 3 independent experiments.
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Figure 6.2. Effect of PhtD, N-ter PhtD, C-ter PhtD and N-ter CbpA on DC clustering. Immature DCs were incubated for 24 hours with 5μg/ml PhtD, N-ter PhtD, C-ter PhtD or N-ter CbpA or 0.5μg/ml LPS and visualized using an inverted light microscope (x32, phase contrast). Images are representative of three independent experiments.

Figure 6.3. Effect of PhtD, N-ter PhtD, C-ter PhtD and N-ter CbpA on DC cytokine production. Immature DCs were incubated for 24 hr with 5μg/ml PhtD, N-ter PhtD, C-ter PhtD or N-ter CbpA or 0.5μg/ml LPS and cytokine levels in culture supernatants assessed using standard IL-10 and IL-12 ELISA. Data are representative of 3 independent experiments.
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Figure 6.4. Cytokine production by PBMC in response to stimulation with PhtD, N-ter PhtD, C-ter PhtD or N-ter CbpA. Freshly isolated adult PBMC were incubated with increasing concentrations of PhtD, N-ter PhtD, C-ter PhtD or N-ter CbpA for 24 hrs and then transferred to coated IFN-γ and IL-5 Elispot plates for a further 24 hrs for detection of cytokine-producing cells (spot-forming cells [SCFs]). The number of spots represents the number of cytokine-secreting cells. Hatched lines in the graphs indicate negative (lower lines, medium alone) and positive controls (upper lines, PMA/ionomycin-stimulated). Data from one donor presented.
6.3. Non-typeable Haemophilus influenzae-derived antigens (PD, LB1)

We next examined the interaction between DCs and two NTHi-derived antigens, the major outer membrane protein PD and a peptide derived from this protein, LB1. DCs were incubated overnight with the antigens and maturation assessed by upregulation of phenotypic markers and clustering.

As shown in Figure 6.5, incubation with PD or LB1 did not induce any phenotypic changes. Neither HLA-DR nor CD86 expression was altered and CD83 expression actually appeared to decrease slightly. This correlated with the clustering of the cells in culture. Figure 6.6. shows that after incubation with either antigen DCs remained single, immature cells with typical immature morphology and did not form clusters.

In order to detect T cell memory responses, PBMC Elispots were performed using PBMC from five healthy adult donors. Again, PBMCs were incubated overnight on a pre-coated IFN-γ Elispot plate with the antigens directly added to the wells. As shown in Figure 6.7, none of the volunteers produced IFN-γ production in response to PD or LB1, even at very high doses of antigen, whereas all responded to the control antigen tetanus toxoid (TT).

Thus, we explored whether monocyte-derived DCs as professional antigen-presenting cells would be able to induce responses to PD or LB1. DCs were loaded with antigen and co-cultured with autologous T cells 6 days, transferred to a pre-coated IFN-γ Elispot plate and incubated for a further 24 hours before detection of cytokine-secreting cells. Figure 5.8. shows that, again, no response to either LB1 or PD could be observed whereas good antigen-specific responses to TT were observed in the same individuals. When LPS was used to activate the DCs during antigen loading, all responses were enhanced, even those to PD and LB1. However, whereas the response to TT-loaded, LPS-matured DCs was significantly higher than that with LPS alone, the responses to PD- or LB1-pulsed, LPS-matured DCs were at the same level as responses to LPS alone. They are therefore unlikely to be specific for the test antigens (Figure 5.8).
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**Figure 6.5. Effect of PD and LB1 on DC phenotype.** Immature DCs were incubated for 24 hr with 5μg/ml PD, LB1 or 0.5μg/ml LPS and assessed by FACS for expression of HLA-DR, CD83 and CD86. Data are representative of 3 independent experiments.

**Figure 6.6. Effect of PD and LB1 on DC clustering.** Immature DCs were incubated for 24 hr with 5μg/ml PD or LB1 and visualized using a Zeiss Axiocvert inverted light microscope with x10 (upper panels) and x40 (lower panels) phase lenses.
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Figure 6.7. IFN-γ production by PBMC in response to stimulation with PD or LB1. Freshly isolated adult PBMC were incubated with increasing concentrations of PD or LB1 for 24 hr on pre-coated IFN-γ Elispot plates for 24 hrs for detection of cytokine-producing cells (spot-forming cells [SFCs]). Hatched lines in the graphs indicate negative (lower lines, medium alone) and positive controls (upper lines, PMA/ionomycin-stimulated). Means and standard errors are shown (n=7).

Figure 6.8. IFN-γ production by PBMC in response to DC loaded with PD or LB1. Immature DCs were pulsed overnight with medium alone (no antigen), 10 μg/ml PD, LB1 or TT in the absence (filled bars) or presence (empty bars) and incubated with autologous PBMC at a ratio of 1:10 for 6 days. Cells were transferred to a pre-coated IFN-γ Elispot plate and visualised after a further 24 hours incubation. Bars represent means and standard errors of 5 independent experiments. Control cultures of T cells, unpulsed DCs or pulsed DCs alone were included and always showed between 0 and 5 spots (data not shown).
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6.4. *Moraxella catarrhalis*-derived antigens (CopB, CD, UspA)

We went on to study interaction between DCs and three *M. catarrhalis* outer membrane proteins, CopB, CD and UspA, again focussing on the induction of DC maturation and presentation to autologous T cells.

Again, DCs were first incubated overnight with these antigens and their phenotypic maturation and cluster formation assessed by flow cytometry and microscopy respectively. As shown in Figure 6.9, both CopB and CD induced strong upregulation of HLA-DR, CD83 and CD86, indicating full DCs maturation. Upregulation was as strong as with LPS, a very potent maturation stimulus for DCs. Some upregulation in marker expression was seen when immature DCs were pulsed with UspA but this was not to the same extent as seen with CopB and CD (Figure 6.9).

In agreement with the phenotypic data, CopB- or CD-pulsed DCs formed large clusters and became strongly adherent to plastic, a characteristic of mature DCs (Figure 6.10). In contrast, only little cluster formation and attachment was observed in the cultures exposed to UspA.

Elispots for the detection of IFN-γ-producing memory T cells were performed using the PBMC from 5 healthy adult donors. As shown in Figure 6.11, IFN-γ was produced by all donors in response to both CopB and CD whereas none of them responded to UspA. While IFN-γ production after stimulation with CopB and CD was dose-dependent, UspA did not induce any response over the whole range of concentrations.

Detection of memory to CopB and CD and their potent DC stimulatory effect prompted us to use DCs for professional presentation of the three antigens to T cells. Here, DCs were incubated with the antigens overnight in the absence or presence of LPS and then co-cultured with autologous T cells for 6 days. The Elispot technique was then used to evaluate IFN-γ production by the T cells. Only one experiment has been done so far. Figure 6.12. shows that this donor responded strongly to both CopB
and CD even in the absence of LPS. The response to UspA in the absence of LPS was barely detectable. All responses were enhanced when LPS was used to activate the DCs during pulsing with antigen and under these conditions UspA responses reached levels of the responses to CopB and CD, levels far above the response to LPS alone. This suggests that this increased response is at least in part due to enhanced antigen presentation of this antigen to specific T cells rather than a non-specific increase in DC-T interaction (see Figure 6.8. for comparison).
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Figure 6.9. Effect of CopB, CD and UspA on DC phenotype. Immature DCs were incubated for 20 hr with 1 μg/ml antigen (filled profiles) or left unchallenged (empty profiles) and assessed by flow cytometry for expression of HLA-DR, CD83 and CD86. One representative experiment of three is shown.

Figure 6.10. Effect of CopB, CD or UspA on DC clustering. Immature DCs were incubated for 24 hr with 1 μg/ml CopB, CD or UspA or medium alone and visualized using a Zeiss Axiovert inverted light microscope with x10 (upper panels) and x40 (lower panels) phase lenses.
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Figure 6.11. IFN-γ production by PBMC in response to stimulation with CopB, CD or UspA. Freshly isolated adult PBMC were incubated with increasing concentrations of CopB, CD or UspA for 24 hr on pre-coated IFN-γ Elispot plates for 24 hrs for detection of cytokine-producing cells (spot-forming cells [SFCs]). Hatched lines in the graphs indicate negative (lower lines, medium alone) and positive controls (upper lines, PMA/ionomycin-stimulated). Means and standard errors are shown (n=7).

Figure 6.12. IFN-γ production by PBMC in response to DC loaded with CopB, CD or UspA. Immature DCs were pulsed overnight with medium alone (no antigen), 1μg/ml CopB, CD, UspA or 10μg/ml TT in the absence (filled bars) or presence (empty bars) of LPS and incubated with autologous PBMC at a ratio of 1:10 for 6 days. Cells were transferred to a pre-coated IFN-γ Elispot plate and visualised after a further 24 hours incubation. One representative experiment of two is shown. Control cultures of T cells, unpulsed DCs or pulsed DCs alone were included and always showed between 0 and 5 spots (data not shown).
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6.5. DISCUSSION

The outcome of the encounter between DCs and T cells depends on the maturational state of the DC with delivery of both MHC-TCR stimulation (signal 1) as well as costimulation (signal 2). It also depends on the differentiation state of the T cell. Naïve T cells require high levels of co-stimulation and therefore only mature DCs can prime them. Presentation of antigen in the absence of co-stimulation results in deletion, functional anergy or tolerance of the responding T cell clone. In contrast, activated, memory (central and polarized) and effector cells rapidly respond to low doses of antigen even in the absence of co-stimulation and can therefore be activated by immature DCs and other antigen-presenting cells. Therefore, the ability of vaccine antigens to induce cell-mediated immunity depends on their ability to induce DC maturation when given as a vaccine as well as their immunodominance during infection or carriage so as to induce T cell memory.

In this chapter, I developed a system for evaluation of human cellular immune responses based on the use of monocyte-derived DCs in vitro. This model was used to examine a variety of vaccine candidate antigens with regard to their effects on DCs maturation and antigen presentation. Memory responses in the same donors were evaluated in order to obtain an indication as to the differentiation state of the T cells that responded to the antigen-loaded DCs in vitro. I used protein antigens derived from three bacteria that are carried as part of the normal flora by a large part of the population but may cause disease in risk groups such a very young infants. As these organisms are part of the normal flora for most individuals, presence of serum antibody and/or T cell memory gives a good indication as to the immunodominance of the various pathogen-derived proteins during natural exposure.

Serum antibody levels have been determined for some of the proteins used in this study. T cell memory responses have to our knowledge not been sought so far for any of the proteins.
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S. pneumoniae-derived proteins

In the first part of this work, I used the DC-based in vitro system to evaluate the potential of several S. pneumoniae derived proteins to interact with DCs. Four proteins were included in the study: PhtD, its N-terminal and C-terminal fragments (N-ter PhD, C-ter PhtD) and the N-terminal fragment of CbpA. PhtD (pneumococcal histidine triad) is an outer membrane protein that is able to protect mice against sepsis and death after challenge with at least certain pneumococcal serotypes and appears immunogenic during natural infection in humans as PhtD-specific antibody titres increase after infection. However, immunogenicity depends on the age of patients at infection, as very young children (2-3 months) do not appear to make antibodies (Adamou et al 2001). CbpA is choline-binding protein A, which also protects mice to some degree against challenge with a highly virulent strain and is immunogenic in colonized human subjects as determined by a significant rise in serum IgG titre (McCool et al 2002). It also reacts with human convalescent antisera (Rosenow et al 1997).

Neither the full-length PhtD nor the N-terminal fragment of CbpA were able to induce DC maturation as determined by failure to upregulate surface markers, to cluster and secrete cytokines. None of six individuals tested had T effector cell memory to these antigens as judged by the failure to produce IFN-γ or IL-5. The lack of response in the PBMC Elispot suggests there was no pre-existing memory to these two proteins.

These findings are in contrast to the observations that both proteins are recognized during infection or experimental carriage and both induce increased antibody titres. The reasons for this are unclear. They might have real biological basis but they may also simply be due to the limitations of the assays used. Failure to detect IL-5 in the PBMC cultures does not exclude presence of Th2 effectors. Other cytokines, in particular IL-4, may be involved and it is always technically challenging to detect Th2 cytokines.
In contrast to PhtD and N-ter CbpA, the two fragments of PhtD potently induced DC maturation. This raised the possibility that maturation may have been due to LPS contamination. The exact LPS levels in the two antigen preparations are not known but indicated as significant by the supplier. When PBMCs were stimulated with the two antigens for a short period for detection of effector memory, significant levels of IFN-γ and no IL-5 were observed. However, there was no dose-response effect, supporting the hypothesis that these preparations may contain high amounts of LPS. This highlights the need for very stringent purification procedures if vaccine candidates are to be examined in a system based on the use of DCs.

**Nontypeable H. influenzae-derived proteins**

In the second part of this work, I used two antigens derived from NTHi, protein D (PD, also called P5 protein) and a derivative of this protein, LB1. PD is a heat-modifiable major outer membrane protein. It may play a role in NTHi pathogenesis as an adhesin by binding to respiratory mucin. Antibodies against this protein were bactericidal when elicited in mice but not in rabbits. A further limitation is the variability in the surface exposed regions of this protein observed during persistent infection or at different anatomical sites. LB1 is a synthetic chimeric peptide derived from the P5 protein by fusing potentially immunoreactive regions of P5 to a ubiquitous T-cell epitope from the measles virus. This peptide was able to inhibit adherence of NTHi to the tracheal mucosal epithelium in the chinchilla model (Poolman et al 2000).

Neither of these two proteins was able to induce DC maturation or elicit IFN-γ production after presentation on antigen-loaded DCs. These antigen-loaded but immature cells would only have been able to activate memory cells (effector and possibly central memory cells, as well) and again, lack of such responses in both the co-culture as well as the PBMC-based assays suggests lack of T_11-type memory in the individuals tested in this study. It is not known whether normal, unvaccinated adults have anti-P5 antibody in the serum.
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Failure to detect type 1 memory T cell responses does not exclude the possibility that there is memory which may be limited to type 2 T effectors and B cell memory. It may also be a technical issue and the antigen formulations used in this study may have lost their T cell epitopes during the production process. However, this is unlikely as LB1 was constructed by fusing a P5 peptide to a ubiquitous T cell epitope derived from the measles virus.

Interpretation of the data in this chapter is limited by the fact that only IFN-γ was measured. This serve well as a marker for T cell activation but only evaluation of a variety of other cytokines such as IL-2, IL-4, IL-5, IL-13 and/or IL-10 will allow detection of T cell memory of more subtypes.

*M. catarrhalis*-derived proteins

In the last part of this work, I examined the interaction between DCs and three proteins derived from the outer membrane of *M. catarrhalis*, CopB, CD and UspA. All three proteins are integral outer membrane proteins. CopB has a role in iron acquisition; CD is a porin but also appears to mediate adhesion, as does UspA (McMichael 2000).

We found that these proteins have different capacities for inducing DC maturation, with CopB and CD both being potent stimuli for upregulation of surface markers and cluster formation whereas UspA only weakly induced those hallmarks of DC maturation.

As DCs are highly sensitive to LPS, there is always the possibility that maturation is due to LPS contamination in the antigen preparation rather than the antigen itself. However, LPS contamination is unlikely to be the reason for the potent activity of CopB and CD. Levels of LPS as measured by the limulus amebocyte lysate endotoxin assay were <4.4 pg/ml for CopB and 72 pg/ml for CD. While 72 pg/ml are considerable levels of LPS, they are still far below the levels of LPS needed to induce DC maturation to the extent observed after exposure to CopB and CD. As shown in dose-response curves (Figure 3.2), 100 pg/ml had no or little effect on
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expression of HLA-DR, CD83 and CD86. Full upregulation was achieved with 500 pg/ml LPS. Therefore we believe that the effect of CopB and CD is due to the properties of the antigens themselves and not LPS contamination.

Maturation of DCs is pivotal to efficient antigen presentation. Considering their potent effects on DC maturation, we therefore expected DCs loaded with CopB and CD to induce strong effector responses. This was found to be true for all individuals tested. The T cells responding to antigen-loaded DCs during such co-cultures are likely to be central or polarized memory cells. The number of cells secreting IFN-γ in response to these two proteins increased with increasing antigen dose, suggesting that these responses are indeed antigen-specific and not due to LPS (cf. the PBMC responses to the N-terminal and C-terminal fragments of pneumococcal PhD).

Loading of DCs with CopB or CD in the presence of LPS enhanced T cell responses. The antigen-loaded, mature DCs may now activate more effector cells and production of IL-12 may also drive more central memory cells towards Th1 polarisation and IFN-γ production.

Examination of acute-phase and convalescent humoral immune responses in patients with *M. catarrhalis* infection have shown that CopB is a major target of the humoral immune response during natural exposure (Helminen et al 1995). Combining these data with our finding that this protein elicits potent DC maturation and T cell responses in vitro may suggest that CopB is a very good vaccine candidate, which even in the absence of adjuvant would be able to elicit both humoral and cellular immunity. The only drawback is the serological variability between strains resulting in two or possibly three different serotypes (McMichael 2000).

In contrast to CopB, CD is highly conserved. This protein elicits high antibody titres in mice. These, however, are not functional, i.e. lack bactericidal and opsonophagocytic activity. Despite the failure to elicit functional antibodies, immunization of mice with a recombinant form of this protein does appear to enhance clearance of bacteria after pulmonary challenge (McMichael 2000). This suggests that clearance in this model is due to a mechanism other than antibody mediated killing. Considering the excellent type 1 T helper cell responses observed
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in our *in vitro* model, clearance in this case might rely on cell-mediated immunity involving type 1 helper cells and macrophages.

In contrast to CopB and CD, UspA-loaded DCs were unable to elicit IFN-γ production by autologous T cells, presumably due to lack of memory cells in the PBMC preparations that would respond to antigen-loaded, immature DCs. When DCs had been loaded with UspA in the presence of LPS, T cell responses could be elicited after 6 days co-culture with such antigen-loaded, mature DCs. Maturation may now have allowed the DCs to present UspA to central memory or even naive T cells and drive their development into Th1 effector cells, suggesting that there was no type 1 helper T cell memory in these individuals. This was corroborated by the lack of responsiveness in the PBMC Elispot.

Specific antibody against UspA has been found in the human pre-immune serum suggesting that this antigen can induce memory responses during natural exposure. However, this antigen may be particularly suited to induce humoral immunity with Th2 responses and B cell memory. Future experiments including measurement of other cytokines as mentioned above will allow more thorough interpretation of the data. Assays for evaluation of T cell responses have already been extended to include IFN-γ intracellular cytokine staining and CFSE staining to measure proliferation. We have also advanced in the process of establishing intracellular cytokine staining and Elispots for IL-5 and IL-10. Intracellular cytokine staining is now also combined with CD4 surface staining to allow further characterization of the responding T cell populations. These assays will now be added to our DC-based system of pre-clinical evaluation of human T cell responses to potential vaccine candidate antigens.

In conclusion, evaluation of the interaction between DCs and potential vaccine antigens may be crucial to understanding why certain antigens would make appropriate vaccine antigens. Our system also allows simultaneous assessment of the effect of adjuvants on antigen presentation. This system for testing antigens at the pre-clinical stage may make the early screening of potential candidates more useful and could be used even before testing in animals. This may be particularly important for pathogens, such as *M. catarrhalis*, NTHi or *S. pneumoniae*, which are exclusive
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colonizers of man limiting the information that can be derived from animal models. Access to a human model of immune response so early in vaccine development and antigen discovery could be a powerful tool for vaccine manufacturers.
Chapter VII

DISCUSSION
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VII. DISCUSSION

The DC is one of the most important immune cells, bridging innate and adaptive immunity, with a crucial role in not only the initiation but also the maintenance and polarisation of adaptive immune responses. Knowledge about the life cycle of the DC and its function has exploded in recent years revealing the tremendous potential for utilising this cell to enhance immune responses in vaccination and immunotherapy. However, while our understanding of the complex biology of DCs has advanced dramatically, their contribution in a number of key areas such as early life immunity and responses to polysaccharide antigens is still unclear.

The questions we asked originally:

- Does the pronounced functional impairment of neonatal DCs persist into infancy?
- Are DCs involved in responses to polysaccharides?
- Does their functional impairment in early life play a role in the defective polysaccharide response during this period in ontogeny?
- Can assessing interactions with DCs in vitro contribute to the pre-clinical evaluation of candidate vaccine antigens?

7.1. Impaired maturation of infant DC

Detailed examination of DC function at birth, performed in both our laboratory and those of others, provided the direct basis for the first part of the thesis, the investigation of DC function in the postnatal period. Despite the limited availability of material, I have been able to show in this thesis that the impaired LPS responsiveness observed in cord-blood derived DCs does persist into infancy. Infant DCs appear to have increased responsiveness as compared to cord blood but still do not display fully functional maturation as seen in adult DCs. They undergo the morphological and phenotypic changes typically associated with DC maturation.
However, they display a markedly reduced tendency to cluster and their IL-12-producing capacity is severely diminished.

Concurrent with the generation of monocyte-derived DCs *in vitro*, some of the infant blood samples used in this thesis were sufficiently large to allow assessment of blood DCs directly by flow cytometry, work done by a colleague, Dr. Stephen Hughes. Blood DCs were defined as HLA-DR$^\text{Lin}$ cells, the lineage cocktail containing antibodies against B and T cells, NK cells, monocytes/macrophages and neutrophils. The frequency of blood DCs appeared to be similar in adult and infant samples, with DCs representing approximately 1% of the PBMCs, and their expression of CD86 and HLA-DR was comparable, too (Dr. Stephen Hughes, personal communication). These data indicate that *in vivo* infant DCs are phenotypically similar to *in vitro*-derived DCs used for my studies, suggesting that the functionality of DCs after *in vitro* differentiation is likely to be representative of the physiological situation.

The experiments presented in this thesis show that 'mature' DCs derived from infant peripheral blood are equipped with the surface molecules necessary for productive dialogue with T cells and should be able to activate even naïve T cells. However, due to the absence of the crucial $T_h1$-driving cytokine IL-12, infant DCs may not be able to support differentiation into $T_h1$-type effector cells allowing the preferential development of $T_h2$-type effector cells or even regulatory cells.

The potent synergistic effect of IFN-$\gamma$ on IL-12 production appears to allow infant DCs to overcome the impaired LPS responsiveness suggesting that, given the appropriate signals, early life DCs may be able to induce $T_h1$ development. This of course has important implications for vaccine development, as $T_h1$ responses are harder to elicit in infants than $T_h2$ responses. Hence, successful induction of $T_h1$ responses relies on the use of agents that deliver very strong activation signals to DCs and are able to elicit IL-12 production by infant DCs (Kovarik et al 1999). Elucidation of the exact molecular events during IL-12 induction in adults may also allow more direct manipulation of this process in infants to improve vaccine success.
A number of different adjuvants are being explored with regard to their capacity to induce potent T_{H1} responses in early life. In mice, complete Freud's adjuvant (CFA) has been shown to have very potent T_{H1}-driving activity (Forsthuber et al 1996). Unfortunately CFA contains mycobacterial components and is highly inflammatory and therefore restricted to use in animals. The only currently approved adjuvants for human vaccination are aluminium compounds but they are poor stimulators of T_{H1} responses (Gupta and Siber 1995) and therefore not ideal, in particular for early life vaccination. Live replicating agents can also potently activate T_{H1} effectors but they are of limited value to vaccinology due to obvious safety concerns.

A variety of novel adjuvants are being explored, one of the most promising for early life vaccination being CpG-containing DNA (Siegrist 2001). Such oligonucleotides can potently induce DC maturation and IL-12 production. When used as adjuvants in mice, they can circumvent the T_{H2} polarisation in early life and induce adult-like T_{H1} responses to vaccine antigens such as tetanus toxoid or measles (Kovarik et al 1999). These compounds were even able to enhance responses to pneumococcal conjugates vaccines (Chu et al 2000, Kovarik et al 2001).

The postnatal period is characterized by a general immaturity of both the innate and adaptive immune system. Whether this is a 'remnant' of a foetal immune system required during pregnancy, developmentally beneficial to enable establishment of tolerance in the newborn or simply a detrimental consequence of neonatal inexperience remains a question of controversy.

Answering this question is not only of great interest to immunologists in general but of more direct need for vaccinologists. If the maturation of infant immune system is delayed in order to allow establishment of tolerance, then disturbing this balance by means of a strongly T_{H1}-skewing vaccination may lead to autoimmune manifestations later in life. However, it is often suggested that the infant immune system is skewed towards T_{H2} at birth and then normally re-directs this bias through natural exposure to T_{H1}-driving stimuli during natural infection and environmental exposure. It has been proposed that intense vaccine schedules during early life with vaccines that nearly exclusive induce T_{H2}-biased responses may prevent this re-
tuning of the immune system although no evidence for this has been presented. This highlights the need to fully elucidate the nature of the immune response to early life infections and then design novel vaccine formulations that induce immune response closely resembling natural responses. Such formulations may be optimal for preventing infection while at the same time allowing appropriate development of the infant immune system.

These findings highlight the importance of seeking a deeper understanding of DC function in early life and the need to study vaccine formulations and existing and novel adjuvants with regard to their effects on DCs and the induction and polarization of adaptive immunity.

7.2. Direct interaction of DCs with pathogen-derived capsular polysaccharides

Pneumococcal capsular polysaccharides are typical examples of T-independent type 2 antigens, which classically do not recruit on conventional T cell help and therefore do not induce affinity maturation, isotype switching or memory. While it is true that such responses can be initiated in the absence of conventional T cells, it is also clear that they can be greatly enhanced if B cells receive such help. This was thought to originate from non-conventional T cells probably via the production of cytokines such as IFN-γ. However, the role of the DC in this setting was never evaluated and discounted very early because one study published in 1991 reported that polysaccharides do not bind to MHC class II. However, since then a much deeper understanding of the complexities of antigen presentation has developed and shown that there are many possibilities for antigens other than proteins to be presented in the context of classical as well as newly discovered antigen-presenting molecules.

This therefore re-opened the question of the role of the DC in responses to polysaccharides, which I have addressed in this thesis. I reasoned that DCs might be involved indirectly in polysaccharide responses through passive transport of the molecules to the appropriate site and B cell population (e.g. the germinal centers of
lymph nodes, acting as a “trojan horse”) or more directly through active uptake and processing. The experiments presented in this thesis provide some evidence for the latter possibility.

I have shown here that pneumococcal capsular polysaccharides interact directly with human DCs, that they are internalized and trafficked to late endosomal compartments. This appears to have no effect on DC maturation by any of the parameters I assessed. These polysaccharides appear to be completely inert with regard to their effect on DCs. The reasons for this are still unclear. Most antigens, including proteins, are similar in that they do not induce DC maturation (cf. TT, LB1, PD, UspA used in this thesis). Turley et al have shown that such antigens are stored in their native form inside DCs until these receive a second signal that induces their maturation. Only then will antigen processing and MHC loading commence (Turley et al 2000).

This agrees with ideas proposed in the so-called danger model by Polly Matzinger, i.e. the immune system generally appears not to distinguish antigens as self and non-self but recognises antigens as non-self only when seen in combination with a second stimulus that is clearly not self, such as the highly conserved ‘pathogen-associated molecular patterns’, molecules that have no homology with any host component (Matzinger 2002).

Most self-antigens in the host are glycosylated and there is a danger that strong effector responses to polysaccharides may result in autoimmune reactions due to cross-reactivity between pathogen- and host-derived carbohydrates. Therefore, one might ask whether polysaccharide antigens are too similar to such ubiquitous glycosylated self-antigens so that any response, in particular DC maturation as the origin of potent effector responses, must be avoided in order to prevent autoimmunity. Based on this idea, one might also speculate whether responses to polysaccharides in infancy are particularly hard to elicit because they are particularly dangerous. The infant immune system may not yet have encountered such antigens in the context of infection and has therefore not yet ‘learned’ to distinguish between pathogen- and host-derived carbohydrates.
Using specific inhibitors, I have shown that internalisation of pneumococcal polysaccharides is likely to involve both macropinocytosis and receptor-mediated endocytosis. That raises the question whether uptake may be mediated by a specific receptor. Zamze and colleagues (Zamze et al 2002) have recently shown that pneumococcal polysaccharides bind to the purified macrophage mannose receptor (MR) in vitro. However, DCs express a very wide range of receptors with affinity for carbohydrate-containing ligands, such as DC-SIGN or other C-type lectins. It is possible that some of these also recognize capsular polysaccharides. Binding of the polysaccharides to such receptors may have serious consequences for the ensuing immune response as suggested by the recent discovery that a number of such antigen receptors are capable of negative regulation of TLR signalling and modulate DC maturation in response to TLR-mediated signals.

7.3. Modulation of DC maturation as an immune escape mechanism

The experiments presented in this thesis have shown that uptake of pneumococcal polysaccharides in itself does not induce DC maturation or cytokine production but dramatically alters the response of DCs to challenge with a potent maturation stimulus such as LPS. Activation of monocyte-derived DCs with LPS normally induces strong upregulation of MHC Class II, co-stimulatory and adhesion molecules as well as the release of a variety of pro- and anti-inflammatory cytokines, among them IL-12 and IL-10.

Pre-incubation with pneumococcal polysaccharides still allows phenotypic maturation of DCs but predisposes them to release very high levels of IL-10 upon stimulation with LPS. This potent immunosuppressive cytokine is very important in the induction of adaptive regulatory T cells, which can suppress effector cells in an antigen-specific manner. Secretion of high levels of IL-10 by DCs may have profound effects on DC-induced T cell differentiation. Induction of anergy or immunoregulation by polysaccharides may help to explain the hyporesponsiveness observed in some cases after vaccination with pure polysaccharides.
The type of downregulation suggested above has indeed been shown for cellular responses in the later stages of tuberculosis. In this phase, macrophages infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis, release high amounts of the mycobacterial cell wall component lipoarabinomannan in its mannosylated form (ManLAM). ManLAM, which is structurally very similar to naturally occurring bacterial polysaccharides, binds its main *in vivo* receptor DC-SIGN with high affinity (Geijtenbeek et al 2003) but is also recognized by other receptors such as the MR, CD11b and CD11c (Kaufmann and Schaible 2003). Binding of ManLAM to DC-SIGN and MR delivers a negative signal to DCs resulting in the release of high levels of IL-10 and reduced levels of IL-12 respectively in response to LPS (Geijtenbeek et al 2003, Nigou et al 2001). Such 'tolerogenic DCs' may then drive the generation of regulatory cells which may be responsible for the suppression of antigen-specific effector responses observed in the later stages of tuberculosis (Engering et al 2002b).

A number of other studies have suggested that binding of certain ligands to antigen receptors activates an immunosuppressive program in DCs. CR3 for example is targeted by *Bordetella pertussis*-derived filamentous haemagglutinin (FHA) to induce high levels of IL-10 production and inhibit IL-12 production in DCs in response to LPS (McGuirk et al 2002). The CR3 receptor (also called Mac-1) consists of CD18 and CD11b, the latter being recognised also by ManLAM.

Intriguingly, it has recently been shown that cross-linking of the mannose receptor on DCs by a specific anti-MR monoclonal antibody resulted in the development of phenotypically mature DCs that produce high levels of IL-10 and reduced levels of IL-12. T cells co-cultured with such DCs initially proliferated (Chieppa et al 2003). This pattern is highly similar to the one we saw upon incubation with pneumococcal polysaccharides and LPS. Chieppa et al reported that the T cells that had initially proliferated later became anergic and behaved as suppressor/regulatory T cells. A number of natural MR ligands (MUC III, biglycan and ManLAM) had the same effect on DC function and T cell activation while others (mannan, dextran) had no significant effect.
These observations show that the appropriate engagement of certain antigen receptors by defined ligands induces an immunosuppressive program in human monocyte-derived DCs that allows them to preferentially produced IL10 which may then dampen inflammation and inhibit the generation of potentially harmful Th1 responses. This has been suggested to be a powerful new immune escape mechanism (Engering et al 2002b).

This raises the question of what the biological need for such a system may be. One might speculate that – as for the TLR family – antigen receptor-mediated negative signalling may have evolved first to serve the host. TLRs recognize evolutionary conserved molecular patterns in microbial components and activate signalling pathways that induce antimicrobial effector responses and inflammation. However, many of them do not only bind microbial components but also self antigens, released by cells and tissues during distress such as injury or inflammation. This has led to the idea that perhaps TLRs originally evolved as receptors for injury-related signals and that microbes subsequently evolved mechanisms to use these receptors for their own benefit (Matzinger 2002).

If this idea is extended to carbohydrate-binding antigen receptors, these may have evolved originally to recognize host-associated glycosylated molecules and function in adhesion, migration and even antigen uptake. Pathogens may then subsequently have evolved mechanisms to recognize such receptors and again use them for their own benefit. DC-SIGN for example assists in adhesion of DCs and T cells and internalises antigen for presentation (Geijtenbeek et al 2000b, Engering et al 2002a). However, it is also targeted by a large number of pathogens, such as HIV, Mycobacterium tuberculosis, Ebola virus, dengue virus and Schistosoma mansoni and has been shown to interfere with DC function in a variety of different ways (Geijtenbeek et al 2000a, Geijtenbeek et al 2003, Simmons et al 2003, Tassaneentrithep et al 2003, Van, I et al 2003). The same is true for MR, which binds lymphatic epithelium and mediates uptake of antigen for presentation, and the integrin CR3, which binds ICAM-1 as well as the complement component C3b and extracellular matrix proteins (Irjala et al 2001, Prigozy et al 1997, Sallusto et al 1995). Again, both molecules are targeted by certain microbial components which
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has an effect on DC function (Chieppa et al 2003, Nigou et al 2001, McGuirk et al 2002).

If this were true, why would such receptors be capable of intracellular signalling and negative regulation of TLR signalling? One might speculate that this function evolved to prevent pathology due to excessive activation of cell-mediated immune responses, in particular Th1-biased effector cells.

TLR receptor triggering potently induces DC maturation and release of pro-inflammatory cytokines. IL-12 secretion, triggered via toll-like receptors 2 and 4 (TLR2 and TLR4), is of crucial importance for the generation of Th1 versus Th2 effector responses. In both injury- and infection-induced TLR-signalling, counterregulatory mechanisms must exist to avoid chronic inflammation and limit pathology. Such counterregulation might be initiated by molecules such as DC-SIGN and MR, which are capable of intracellular signalling as suggested by the presence of immunoreceptor tyrosine-based activation motifs (ITAMs) in their intracellular domains. As mentioned before, these might function normally in adhesion to other host cells and in migration. Their signalling function may have evolved to allow counterregulatory IL-10 production upon interaction with normal tissues and induction of regulatory cells that can dampen the immune response. However, pathogens such as M. tuberculosis, HIV and possibly encapsulated bacteria may hijack this system to subvert immune responses directed against them by releasing components that can bind to such molecules resulting in IL-10 production and the induction of antigen-specific anergy.

Recently published reports investigating the function of a number of molecules involved in intracellular signalling have provided fascinating new data that may explain how antigen receptor can negatively regulate TLR signalling. Phosphoinositide 3-kinase (PI3-K) has been found to be an endogenous suppressor of IL-12 production that acts by suppressing some of the signalling pathways downstream of TLRs (Fukao and Koyasu 2003). PI3-K is also involved in the uptake of many pathogens and other foreign material, including pneumococcal polysaccharides (see Chapter IV) and is activated rapidly upon first encounter with
these antigens. Therefore, binding of pathogens to certain antigen receptors may activate PI3-K, which may then mediate the negative regulation of TLR signalling.

It is likely that a balance between the strength of signalling from adhesion molecules/antigen receptors versus TLRs signalling would determine final outcome of the interaction. It was suggested recently that this may be the basis for immune escape by certain infectious agents (Kaufmann and Schaible 2003). However, this idea could be extended to include a counterregulatory function to prevent excessive pathology after inflammation or infection. Thus, in the initial stages of inflammation, TLR signalling might be stronger due to the high levels of injury-related molecules but as inflammation is being cleared, the influx of such molecules decreases and the interaction with normal tissues via C-type lectins prevails, inducing high level IL-10 production and allowing DCs to specifically suppress autoreactive effector cells. In infection, DCs will initially interact with pathogens in the context of infected and inflamed tissues and host- and pathogen-derived factors are likely to induce strong TLR signalling. Some pathogen-derived molecules may then bind to C-type lectins and stronger C-type lectin signalling may initiate counterregulatory IL-10 production and induction of adaptive, suppressive regulatory cells.

Such a system would rely on the fine-tuning of a very fine balance between stimulatory and inhibitory signals. Disruption of this delicate balance might help to explain the failure of the pneumococcal polysaccharide vaccine in certain subgroups. Vaccination of HIV-infected individuals in Uganda for example did not only show complete lack of efficacy of the vaccine but also revealed potential vaccine-associated harm, as evidenced by a significant rise in pneumonia in the recipients of the pneumococcal vaccine but not the controls (French et al 2000).

In such a situation, the combined suppressive effect of HIV and capsular polysaccharides may prove stronger than the TLR signalling and tip the fragile balance towards immunosuppression. Lack of efficacy of the unconjugated vaccine has also been observed in a number of vaccine trials in the elderly for both the pneumococcal and the meningococcal C polysaccharide. Hyporesponsiveness after vaccination with either of these polysaccharide formulation has also been observed in

The failure of the polysaccharide vaccines in these settings has been unexplained although some have suggested, that there is some form of B cell depletion as a result the cross-linking of B cell receptors by the polysaccharides and subsequent B cell apoptosis but this mechanism has not been substantiated. However, in the light of the data obtained in this thesis, one may speculate that specific immune suppression may also play a role in these vaccine failures.

In summary, DCs have a crucial role in the balance between induction of immunity or tolerance. Modulation of the maturation state of DCs is thus an indispensable tool for relaying decisions to the effector arm of the immune system. A wide variety of pathogens (and also cancers) take advantage of this system and modulate DC maturation to direct immune responses favourable to them. While it is difficult to extrapolate directly from in vitro data to a real in vivo effect, our data warrant further investigations into the effect of using large doses of pure polysaccharide vaccines on subsequent immune responses, particularly in high-risk groups.

7.4. Interaction between DCs, polysaccharides and B cells

While the interaction between DCs, polysaccharides and B cells was not investigated in this thesis, recent advances in the understanding of DC and B cell biology would provide the basis for very interesting new aspects to the work presented in this thesis. DCs were thought to be unable to interact directly with B cells and provide help only through activation of an intermediary helper T cell. However, a number of reports published in recent years have shown that DCs can initiate and maintain certain B cell responses directly through release of soluble factors, without activation of the normally required intermediary T helper cell (Saeki et al 2000, Balazs et al 2002, Litinskiy et al 2002).
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Balasz et al recently published very interesting data on the murine immune response to encapsulated *S. pneumoniae* (Balazs et al 2002). The only two blood cell populations to take up bacteria after systemic infection were neutrophils and CD11c<sup>lo</sup> blood DCs. The entire compartment of the blood DCs then migrated to the marginal zone of the spleen where they co-localized with B1 cell and marginal zone B cells and supported antigen-specific B cells by providing critical survival signals and promoting their differentiation into Ig-M secreting plasmablasts. In the absence of such signals, antigen-activated B cells undergo apoptosis resulting in severely impaired antibody production.

Litinskiy et al have demonstrated that these critical survival signals are BLyS (B lymphocyte stimulator) and APRIL (A PRoliferation Inducing Ligand), soluble ligands for the B cell receptor TACI (Transmembrane Activator and Calcium modulator cyclophilin ligand Interactor). BLyS and APRIL are released by DCs and monocytes in response to IFN-α, IFN-γ, CD40L and LPS. In the presence of appropriate cytokines, they can induce CD40-independent class switching. IL-10 or TGF-β co-stimulate switching from IgM to IgG and or IgA, while IL-4 supports switching from IgM to IgE (Litinskiy et al 2002).

In the light of these findings, it would greatly enhance our understanding of the interactions between human DCs and polysaccharide to study release of BLyS and APRIL after pulsing with polysaccharides and the above-mentioned stimuli and assess whether such DCs may be able to support B cell responses to polysaccharides in vitro.

7.5. *DC-S.pneumoniae* interactions during natural exposure

A number of studies have suggested that natural exposure to *S. pneumoniae* bacteria (and also *H. influenzae* type b) induces the generation of polysaccharide-specific memory, which is why the risk of disease with these pathogens decreases with age. This was inferred from the observation that vaccination of previously unvaccinated adults with a conjugate vaccine appears to re-activate memory cells instead of
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inducing a primary responses as expected (Baxendale et al 2000, Hougs et al 1999). This suggests that these volunteers developed polysaccharide-specific memory after natural exposure through carriage. In the pneumococcal study of Baxendale et al, polysaccharide-specific memory B cells secreted IgG and IgA while in a different study Carsetti et al (ref) suggested a particular subset of IgM-memory B cells are important in controlling *S. pneumoniae* infection.

A small number of *in vivo* and *in vitro* studies of the immune response to *S. pneumoniae* bacteria have been published (Colino et al 2002, Snapper et al 2001, Wu et al 1999). However, most of these used unencapsulated bacteria and the polysaccharide-specific responses assessed in those reports were in fact directed against the cell-wall polysaccharide phosphocholine (PC), which may be very different from the capsular polysaccharides. The unencapsulated bacteria potently induced phenotypic DC maturation and high levels of cytokine production. All *in vivo* responses, i.e. to protein as well as PC, were dependent on viable DCs and it was also shown that – while largely T-independent – the anti-PC response strongly required presence of CD40L. This may suggest that – as mentioned above – DCs were involved and had to be stimulated via CD40L to produce BLyS and APRIL, which would allow generation of B cell memory to the bacteria in the absence of T cell help.

In contrast to the data in the reports mentioned above, encapsulated bacteria were used in this thesis and great care was taken to ensure that bacteria were encapsulated in order to assess the effect of capsular polysaccharides when encountered in this context. Such bacteria had only a weak stimulatory effect on DCs in our system, with only partial upregulation of surface markers and no/little cytokine production (with the exception of serotype 19F which induced both IL-12 and IL-10). Thus, the capsule appears to not only shield bacteria from the innate immune system but in addition appears to hinder the development of adaptive immunity by preventing full DC maturation. Taking into account our knowledge about DC biology and interactions with T cells in general, one might speculate that the partial upregulation of surface markers may allow DCs to activate naïve T cells and support their differentiation to a certain extent, to central memory cells. These are not yet
polarized but can rapidly differentiate into effector cells upon re-activation. Polarization is then still possible in the presence of the appropriate cytokines. Whether DCs that have internalised encapsulated pneumococci would be able to support B cell differentiation, antibody production and B cell memory formation directly remains an open question.

7.6. DCs in vaccination

Vaccination is one of the most successful health interventions that – together with the provision of clean water supplies – has had the most impact on reducing morbidity and mortality due to infectious disease.

Vaccination is very complex. Formulations must be immunogenic enough to provide long-lasting immunity but should have minimal side effects and of course not induce disease-like symptoms. They must be immunogenic and at the same time safe also in risk groups. Or course they should be also be cheap and stable. It is becoming more and more apparent that vaccines not only have to induce an immune response that protects from subsequent infection but they should also induce the “right” type of immunity, i.e. $T_H1$ versus $T_H2$ or systemic versus mucosal, an aim that is often not fulfilled by the currently existing vaccines.

As mentioned before, DCs are instrumental in the initiation, maintenance and polarisation of immune responses. Suboptimal DC function is therefore likely to have a severe impact on vaccination and may be part of the reasons for vaccine failure in certain settings and groups. This is highlighted by a number of observations described in this thesis:

The impairment of DC maturation in early life is likely to play an important role in the observed bias away from $T_H1$-like responses and appropriate manipulation, for example during vaccination, may mimic natural development and restore the correct balance.
Modulation of DC maturation by pneumococcal capsular polysaccharides may partly explain the immunological hyporesponsiveness in certain groups after vaccination and again manipulation at this key control point may re-direct the balance and restore responsiveness. Our data were obtained in an *in vitro* system and it is always difficult to extrapolate directly from the test tube to the situation in humans. However, the data presented in this thesis do suggest a more thorough investigations of the immune response induced by certain vaccines would be interesting.

Generally, evaluation of the immunogenicity of vaccine formulations in humans relies on measurements of the humoral response. However, it is important to extend the investigations to assessment of cell-mediated immune responses. The experiments presented in this thesis show that – thanks to the recent advances in DC cell culture – it is feasible to establish an *in vitro* system for the evaluation of cellular immune responses to vaccine antigens. In this thesis, such a system was used to assess cellular memory responses in unvaccinated individuals, i.e. memory established through natural exposure. Using a variety of antigens derived from several different human pathogens, we have shown that the capacity of the antigens to induce DC maturation and IL-12 secretion *in vitro* correlates very well with their capacity to induce *Th*1-type memory *in vivo* (cf. CopB and CD induced DC maturation *in vitro* and memory was easily detectable, whereas UspA, PD and LB were unable to mature DCs *in vitro* and memory responses were not detectable with our assays). Hence, such a human model of immune responses so early in vaccine development and antigen discovery could be a very valuable in the pre-clinical evaluation of vaccine candidates.
7.7. Concluding remarks

While the work presented in this thesis has generated answers to some of the original questions, it has raised many more; the answers to these will deepen our understanding of DC biology and may provide useful targets for manipulation of DC function in vivo:

Questions that remain unanswered

- How much further does the observed functional deficit of infant DCs persist into childhood?
- What are the factors that trigger the step-wise development of DCs during ontogeny?
- Can stimuli that allow infant DC maturation in vitro enhance Th1 responsiveness in vivo? Would this lead to detrimental effects on establishment of peripheral tolerance in early life?
- What are the exact molecular events involved in the modulation of DC maturation by pneumococcal capsular polysaccharides?
- Is this modulation responsible for the hyporesponsiveness observed in certain groups after pure polysaccharide vaccine?
- Can polysaccharides induce the development of immunoregulatory T cells via modulating DC maturation?
- Can DCs directly enhance antibody secretion by polysaccharide-specific B cells?
- Can early life responses to polysaccharide and conjugate vaccines be enhanced by manipulating DCs in vivo?


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