Immunomodulatory properties of meningococcal outer membrane vesicles

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Abstract

Neisseria meningitidis (Nm) causes potentially fatal bacterial sepsis and meningitis in susceptible individuals, but induces protective immunity in asymptomatic carriers. Nm naturally sheds outer membrane vesicles (NOMVs) that contain a high proportion of lipopolysaccharide (LPS) and outer membrane proteins. NOMVs are known to induce serum bactericidal antibodies in mice and humans, but their inherent adjuvanticity of humoral responses toward protein and carbohydrate antigens has not been studied in detail. This thesis investigation has determined that NOMVs are potent intraperitoneal and intranasal adjuvants for model T-dependent antigens such as Ovalbumin and T-independent antigens such as dinitrophenol-ficoll. Surprisingly NOMVs had little or no effect on the primary antibody responses towards several clinically relevant capsular polysaccharides from *Streptococcus pneumoniae* regardless of the route of immunisation. NOMVs induced the proliferation of splenic B cells, and the production of cytokines from splenocytes and bone marrow-derived dendritic cells from not only wild-type but also LPS-hyporesponsive mice which indicates that both LPS and non-LPS components have immunomodulatory properties. Despite the potential of NOMVs inducing proinflammatory responses, preliminary results showed that intranasally applied NOMVs suppressed allergen induced or respiratory syncytial virus-induced airway eosinophilia in mouse models. Together our results provide important insights into the role of NOMVs in inducing protective immunity against meningococcal diseases, and their immunomodulatory properties that could be useful separately or synergistically in adjuvant development or immunotherapy.

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Common Abbreviations

APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BCR	B cell receptor
BMDCs	Bone-marrow derived dendritic cells
BMDMs	Bone-marrow derived macrophages
BSA	Bovine serum albumin
CBA	Cytokine bead array
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CPS	Capsular polysaccharide
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.n.	Intranasal
i.p.	Intraperitoneal
LAL	Limulus amoebocyte lysate
LBP	Lipopolysaccharide-binding protein
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
mAb	monocloncal antibody
MAC	Membrane attack complex
MBL	Mannose binding lectin
mg	milligram
µg	microgram
MHC	Major histocompatibility complex
mins	minutes
ml	millilitre
µl	microlitre

NO	Nitric oxide
NOMVs	Natural outer membrane vesicles
OMPs	Outer membrane proteins
OMPC	Outer membrane protein complex
Opa	Opacity-associated proteins
OPD	O-phenylenediamine dihydrochloride
Ova	Ovalbumin
PBS	Phosphate buffered saline
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
RSV	Respiratory syncytial virus
SEM	Standard error of the mean
TCR	T cell receptor
TD	T-dependent
Th	T helper
TI	T-independent
TLR	Toll-like receptor
TNF	Tumour necrosis factor

CHAPTER 1

Introduction

1.1 General Introduction

Neisseria meningitidis (Nm) is a human pathogen that is a major cause of septicaemia and meningitis worldwide. It is an encapsulated, Gram negative diplococcus that colonises the nasopharynx. Between one and 12 cases of meningococcal disease per 100,000 population occur every year with most cases seen in children aged six months to two years (Kolb-Maurer *et al.*, 2001; Pollard, 2001; Unkmeir *et al.*, 2002). The rate of asymptomatic carriage of meningococci during endemic disease is thought to be 10-30% of the general population (Caugant *et al.*, 1994; Yazdankhah *et al.*, 2004). Rates of transmission and carriage tend to be higher among populations living in confined areas (military recruits and students) (Stephens, 1999). In most cases Nm colonises the human nasopharynx stimulating natural immunity by inducing bactericidal antibodies in serum (Goldschneider *et al.*, 1969a; Goldschneider *et al.*, 1969b). It is thought that this is essential for protection (Saunders *et al.*, 1999). However in a number of cases colonization of Nm can lead to dissemination from local tissues into the blood causing meningococcal disease (Quakyi *et al.*, 1999; Katial *et al.*, 2002).

Meningococci are traditionally classified based on the antigenicity of surface structures with the serogroup of the organism being determined by the polysaccharide capsule it expresses. Further classification depends on

antigenic differences in the major outer membrane proteins PorB and PorA, and on lipopolysaccharide (LPS) structures that define the 12 immunotypes of the organism (Morley and Pollard, 2002). More recently multi-locus enzyme electrophoresis (MLEE), which measures the electrophoretic mobility of cytoplasmic enzymes (Caugant *et al.*, 1986) and multi-locus sequence typing (MLST), which uses molecular sequence data from housekeeping genes (Maiden *et al.*, 1998), are used to determine evolutionary changes in the meningococcus due to phase variation and high variability of antigens.

In developed countries the majority of meningococcal disease is caused by serogroup B and C meningococci, with serogroup B accounting for approximately 50% of these cases. Meningococcal disease caused by serogroup A is most frequently seen in Sub-Saharan Africa where epidemics have a regular cyclic pattern occurring every five to eight years. However, the first serogroup W135 meningococcal outbreak was reported in 2002 (Nicolas *et al.*, 2005).

A high level of mortality (30-60%) is associated with meningococcal disease, however both early recognition of the disease and the introduction of antibiotic therapy have lowered these levels. The use of some antibiotics has also been shown to decrease the carriage rate of meningococci (Hart *et al.*, 1993; Yazdankhah *et al.*, 2004). Nevertheless, antibiotic resistant strains exist and these could pose a problem for future treatment of meningococcal disease (Van Esso *et al.*, 1987; Hart *et al.*, 1993). It is for this reason that although for the time being we are able to treat meningococcal disease, prevention of the disease by vaccination would be better.

Vaccines to serogroups A, C, Y and W135 have been developed based on the capsular polysaccharides (CPSs). The importance of anti-polysaccharide antibodies in humans against serogroup A and C meningococcal disease has been demonstrated (Goldschneider *et al.*, 1969a; Goldschneider *et al.*, 1969b). It was shown that highly purified, high molecular weight polysaccharides (Gotschlich *et al.*, 1969c) were immunogenic in adults (Gotschlich *et al.*, 1969a; Gotschlich *et al.*, 1969b) however they were poorly immunogenic in infants and young children (Gold *et al.*, 1975) and provided no protection one-year post vaccination (Reingold *et al.*, 1985).

The immunogenicity of these vaccines has improved through conjugation to protein carriers, although there is a need to further understand polysaccharide vaccines, as they are not effective in young children. At present there is no universally effective vaccine to serogroup B meningococci, which is the major cause of meningococcal disease in Europe and the United States of America.

1.1.1 Capsular polysaccharides

Capsular polysaccharides (CPSs) are essential virulence factors that are used to classify meningococcal serogroups. There are 13 reported serogroups: A, B, C, D, 29E, H, I, K, L W135, X, Y and Z. Serogroups A, B, C, W135 and Y cause more than 90% of meningococcal disease worldwide. The CPSs vary in chemical structure. The capsules of serogroups B, C, Y and W135 all contain *N*-acetylneuraminic acid (sialic acid), whereas the capsule of serogroup A is composed of *N*-acetylmannosamine-1-phosphate.

The CPS protect from the actions of complement in the absence of CPS-specific antibodies. CPS-specific serum IgG antibodies induce complement mediated lysis of serogroups A, C, W135 and Y and opsonophagocytic killing of serogroup B. It has been suggested that enhanced colonisation of the human nasopharynx occurs due to a loss of capsule expression (Hammerschmidt *et al.*, 1996).

1.1.2 Polysaccharide and Conjugate vaccines

CPS-based vaccines have been formulated and are effective against serogroups A, C, W135 and Y. Two vaccines are routinely used covering either serogroups A and C, or A, C, Y and W135. However their usefulness has been hampered due to being poorly immunogenic in infants and the duration of immunity is limited. In order to improve the immunogenicity of these vaccines, polysaccharide capsules were conjugated to protein carriers. The *Haemophilus influenza* type B vaccine (Hib) showed the potential of conjugate vaccines by eliciting a high protective efficacy (Heath, 1998). Meningococcal conjugate vaccines were first described in the early 1980s, however the method of improving immunogenicity of polysaccharide capsules by conjugation to protein carriers was first demonstrated in the 1930s (Avery and Goebel, 1931). Jennings and Lugowski (1981) described the coupling of serogroup A, B and C polysaccharides to tetanus toxoid. They found the conjugate vaccines for polysaccharides A and C produced high levels of polysaccharide-specific bactericidal antibodies in mice and rabbits, whereas the conjugate polysaccharide B vaccine did not. A conjugate vaccine composed of meningococcal A and C polysaccharides and

a non-toxic mutant of diphtheria toxin (MenAC) was first to be tested in human trials. Phase 1 trials showed the vaccine to be immunogenic and to significantly increase antibodies to A and C meningococcal CPSs (Costantino *et al.*, 1992). Due to the low prevalence of serogroup A meningococcal disease in the UK, attention was focused on developing a vaccine for serogroup C disease (MenC). Trials were carried out with the MenC vaccine and it was found to be immunogenic after a three-dose schedule in infants (Richmond *et al.*, 1999) and after a single-dose in toddlers (Richmond *et al.*, 2001). It was established that some disease isolates lacked O-acetyl groups (OAC-ve) found in the polysaccharide capsule, therefore an alternative vaccine was produced that contained OAC-ve polysaccharide capsule coupled with tetanus toxoid (MenC-TT). This vaccine also proved to be immunogenic in adults after a single dose (Richmond *et al.*, 1999) and to produce bactericidal antibodies to OAC+ve and OAC-ve isolates in infants (Richmond *et al.*, 2001).

In 1999, the UK became the first country to introduce routine immunisation with MenC. A three-dose course was introduced for infants, and a single dose for children aged 1-17 years. Within 18 months an 81% reduction of confirmed meningococcal C disease was observed (Miller *et al.*, 2001) and vaccine efficacy was calculated at 93% for teenagers (Bose *et al.*, 2003), 92% for toddlers (Ramsay *et al.*, 2001; Balmer *et al.*, 2002) and 91.5% for infants (Balmer *et al.*, 2002). The impact of the MenC vaccine was vast due to a dramatic decrease in the incidence of serogroup C disease and fatalities.

The development of the conjugate vaccine against meningococcal serogroups A, C, Y and W135 (MenACYW) has the potential to tackle serogroup A meningococcal infection in sub-Saharan Africa as well as disease associated with serogroup Y which is prevalent in the USA (Snape *et al.*, 2005). Initial results with a single immunisation with MenACYW conjugated to diphtheria toxoid (MCV-4) in adults (Campbell *et al.*, 2002) and toddlers (Rennels *et al.*, 2002) were immunogenic and well tolerated. However the vaccine was only moderately immunogenic in infants (Rennels *et al.*, 2004). In January 2005, the US Food and Drug Administration licensed MCV-4 for use in adults aged 11 to 55 years (Plotkin *et al.*, 2006).

1.1.3 Serogroup B CPS

The chemical composition of the CPS of serogroup B meningococci is structurally and antigenically similar to glycans on the human neural cell adhesion molecule (N-CAM) (Finne *et al.*, 1983), therefore purified group B CPS is weakly immunogenic and an autoantigen in humans (Wyle *et al.*, 1972). Antibodies to the CPS have the potential to bind with host cells, and it is not known whether this would be harmful therefore it has not been used in a CPS-based vaccine. It has also been shown that IgM antibodies to group B CPS are naturally present in humans, however these antibodies are of low avidity and have low bactericidal activity and therefore their importance in protective immunity against serogroup B meningococci is disputed (Leinonen *et al.*, 1982). It is due to these reasons that the focus for developing a vaccine for serogroup B meningococci has turned to the outer membrane proteins (OMPs) and LPS.

The success of the MenC vaccine could not be extrapolated to serogroup B disease. It appears that conjugation of the polysaccharide from serogroup B to a protein carrier does not elicit a T cell dependent response to the polysaccharide. Although bactericidal activity and IgG responses have been seen in animal studies (Bartoloni *et al.*, 1995; Devi *et al.*, 1997), the bactericidal activity was not due to the serogroup B polysaccharide but was attributed to non-capsular antigens such as OMPs and LPS which show a great deal of antigenic variability limiting their use as vaccine candidates (Jones *et al.*, 1979).

At present there is no universally effective vaccine to serogroup B disease however several vaccines have been produced by using naturally occurring vesicles containing OMPs and LPS.

1.2 Non-capsular meningococcal surface antigens

1.2.1 Class I, II and III OMPs: PorA, PorB2 and PorB3

Neisserial porins are in abundance on the outer membrane of Nm and are potential vaccine and adjuvant candidates. They are co-expressed trimeric proteins which function as pores and are essential to the survival of the bacteria because they mediate the exchange of ions between the surrounding environment and the bacteria (van der Ley *et al.*, 1991; Mackinnon *et al.*, 1999). Meningococcal porins can be separated into three classes: class I (PorA, approximately 45kDa), and class II or III (PorB2 and PorB3, approximately 40-42kDa and 37-39kDa respectively). Meningococcal porins share 60-70% amino acid homology to gonococcal porins. Each porin

is made up of three single polypeptides of approximately 35kDa and contains a high proportion of β-pleated sheets (Massari P, 2003). PorA and PorB show antigenic variability and are used to determine serosubtypes of Nm. Studies of these proteins have determined that there are eight surface-exposed hydrophilic loop structures, with the sites of most variability corresponding to loops I and IV (Wright *et al.*, 2002; Clarke *et al.*, 2003; Vermont *et al.*, 2003).

Neisserial and other Gram negative bacterial porins are able to induce inflammation in mice, as well as releasing cytokines from human cells and activating both human and murine B cells. Investigations using human monocyte-derived dendritic cells (mo-DC) indicated that T cell proliferation could be induced after stimulation with meningococcal outer membranes deficient in LPS, implying that non-LPS components could be involved in the activation of DCs (Al-Bader *et al.*, 2003). Further investigations by the same group demonstrated maturation of mo-DC as well as increased expression of chemokines such as IL-8 and RANTES by recombinant (r)PorA. Low levels or no secretion of pro-inflammatory cytokines such as TNF-α, and anti-inflammatory cytokines, such as IL-10 were secreted from mo-DC after stimulation with rPorA indicating that PorA is more likely involved with the recruitment and activation of immune cells (Al-Bader *et al.*, 2004).

Activation of B cells and DCs by porins has been shown to be dependent on TLR2 and MyD88 by using both TLR2 and MyD88 knockout mice (Mackinnon *et al.*, 1999; Massari *et al.*, 2002; Massari P, 2003; Singleton *et al.*, 2005).

PorB, particularly PorB3 is less accessible to antibodies and therefore less at risk to immunological attack, due to a reduced size of the surface-exposed loop structure. Binding of monoclonal antibodies (mAbs) to PorB3 on live and heat- or ethanol-killed meningococci revealed that PorB3 mAbs were able to bind to killed but not live meningococci, indicating that PorB3 is buried in the outer membrane not accessible to antibody binding (Michaelsen *et al.*, 2001). Estabrook and colleagues (2004) have demonstrated that PorB binds mannose-binding lectin (MBL) and purified PorB can inhibit the binding of MBL to meningococci. MBL is a serum collectin resembling IgM and C1q, which can bind repeating sugar moieties on microorganisms such as encapsulated serogroup B and C meningococci, activating the lectin complement pathway leading to increased bacterial killing (Jack *et al.*, 2001).

Neisserial porins have also been shown to upregulate CD86, a costimulatory molecule on the surface of B cells and DCs, increasing the involvement of T cells through CD28 (Wetzler *et al.*, 1996; Mackinnon *et al.*, 1999). Studies using PorB conjugated with group C meningococcal CPS have shown that blocking CD86 greatly reduces the IgG response to the CPS, indicating that porins are able to provide crucial second signals for T cell-dependent induction of IgG. Thus neisserial porins are potential carrier and adjuvant candidates (Mackinnon *et al.*, 1999).

1.2.2 Class IV OMP: Reduction modifiable protein

The class IV OMP is called reduction modifiable protein (Rmp) because of its change in mobility in SDS-PAGE in the presence of a reducing agent. Rmp

is closely related to protein III (PIII) of *N. gonorrhoeae* with 96% homology in their DNA sequences (Lytton *et al.*, 1986; Klugman *et al.*, 1989). Both Rmp and PIII are constitutively expressed, antigenically invariable and associate with PorA and PorB, which is thought to be essential for outer membrane localisation and stability (Jansen *et al.*, 2000). The function of Rmp in the pathogenesis of Nm is relatively unknown, however mAbs against Rmp have been reported to block the serum bactericidal activity (SBA) of other antibodies against meningococci (Munkley *et al.*, 1991). It has also been demonstrated that Rmp can form complexes with the lactoferrin receptor (LbpA), the transferrin receptor (TbpA) and the siderophore receptor (FrpB), resulting in the stabilisation of oligomeric forms of the iron-regulated proteins (Prinz *et al.*, 2000).

1.2.3 Class V OMP: Opa and Opc proteins

Nm expresses colony opacity-associated (Opa) proteins involved in adhesion and invasion of host epithelial cells. Meningococci are able to encode three or four Opa proteins and at any one time, may express zero, one or many, whereas gonococci can encode up to 11 Opa proteins (Dehio *et al.*, 1998; Billker *et al.*, 2000). Unlike pili, Opa proteins are not required for colonization of the host, but have been known to interact with human cells leading to host cell invasion. In circumstances where there is sialylated LPS or an encapsulated meningococcal strain, Opa has been shown to be a poor mediator of bacterial adhesion (Pollard, 2001; Hauck *et al.*, 2003). According to their binding specificity for human surface receptors and structural analysis, Opa proteins have been grouped into two major classes: the Opa_{HS}

(heparan sulphate recognising) and the Opa_{CEA}-type (carcinoembryonic antigen recognising) proteins (Virji *et al.*, 1996; van Putten *et al.*, 1998). CD66 proteins are a subset of the CEACAM receptor family. The function of CEACAM receptors, expressed on epithelial, endothelial and phagocytic cells, is largely unknown however some members are known to mediate cell-cell adhesion and have a possible role as regulatory and sensory molecules in the adhesion events (Billker *et al.*, 2000; de Jonge *et al.*, 2004). Over 95% of meningococcal isolates are able to bind CD66a as are strains expressing capsule and sialylated LPS (Virji *et al.*, 1996). Opa binding to CD66a correlates with bacterial induction of an oxidative burst (Naids *et al.*, 1991; Gray-Owen *et al.*, 1997), which is preventable by Opa-specific antibodies and purified Opa proteins. Recently it has been shown that MBL binds surface-exposed constant regions of Opa, however the effect of MBL binding remains to be investigated (Estabrook *et al.*, 2004).

The Opc OMP is expressed at high levels in certain meningococcal strains. It has been shown to be structurally and antigenically different from Opa proteins (Achtman *et al.*, 1988). Merker and colleagues (1997) devised a structural model for Opc containing 10 transmembrane strands and five surface-exposed loops. Adhesion and invasion of endothelial and epithelial cells can be mediated by Opc (Virji *et al.*, 1993) and it has been found to bind to heparin and heparan sulphate proteoglycan receptors found on epithelial cells (de Vries *et al.*, 1998).

1.2.4 Iron-limited proteins

Iron acquisition is vital for all bacteria for its pathogenicity and iron-regulated proteins are essential for iron uptake. Survival of bacteria inside the host can depend on the ability of the bacteria to scavenge for essential nutrients such as iron. It has been well established that the growth of Nm depends on the availability of iron. The majority of iron in the human host is stored intracellularly in the form of ferritin and haemoglobin. Extracellular iron sources include transferrin found in body fluids such as serum and lymph, and lactoferrin in milk and secretions. Nm has to be able to survive in order to cause disease in various environments in the body containing a variety of forms and concentrations of iron therefore it has evolved iron acquisition systems to utilise the iron sources.

1.2.4a Transferrin binding proteins

During iron-limited growth, the acquisition of iron from transferrin is by two transferrin binding proteins (Tbps), Tbp1 and Tbp2 with molecular masses of approximately 98 and 65-90 kDa respectively, depending on the strain (Schryvers *et al.*, 1988). These Tbps function as a transferrin receptor, localised on the surface of Nm (Ala'Aldeen *et al.*, 1993). It has been demonstrated that antibodies to Tbp are sparsely detected in sera from healthy volunteers, whereas a clear antibody response is seen in sera from carriers and individuals with meningococcal disease. Antibodies to both Tbp1 and Tbp2 were detected, and anti-Tbp antibodies were cross-reactive between strains (Gorringe *et al.*, 1995).

The addition of Tbps in future outer-membrane based meningococcal vaccines has been suggested due to their surface accessibility and potential of generating bactericidal antibodies.

1.2.4b Lactoferrin binding proteins

The lactoferrin binding proteins, LbpA and LbpB make up the lactoferrin receptor. Using affinity isolation LbpA was identified as a 98kDa protein and under more stringent conditions, LbpB an 84kDa protein (Schryvers *et al.*, 1998). Lactoferrin binding and expression of the protein can be prevented by insertional inactivation of *lbpA* in Nm (Quinn *et al.*, 1994; Schryvers *et al.*, 1998). The lactoferrin receptor has been found on all meningococcal strains tested and is thought to be a major virulence factor as lactoferrin is found predominantly in the nasopharynx, which is the entry site for Nm. It has been recently demonstrated that both LbpA and LbpB are accessible at the cell surface and are targets for bactericidal antibodies. These antibodies were shown to have limited cross-reactivity, and a vaccine based on these proteins would require a number of variable Lbps to be effective against circulating strains (Pettersson *et al.*, 2006).

1.2.5 Lipopolysaccharide

Lipopolysaccharide is a major glycolipid and is the most abundant component on the outer membrane of Nm. Meningococcal LPS is distinct from many other Gram negative bacteria as it lacks O-antigens and can therefore be more properly described as lipooligosaccharide (LOS). There are approximately 12 immunotypes of meningococcal LPS, controlled by

highly regulated genes which can undergo phase variation (Stern *et al.*, 1987). Figure 1.1 shows possible LPS structures from *N. meningitidis* strain 44/76.

Due to the abundance of LPS on the outer membrane it is accessible to antibodies and with serum levels of bactericidal antibodies to LPS shown to correlate with protection against meningococcal disease, LPS has been considered as a possible vaccine candidate. In addition, monoclonal antibodies (mAbs) to two LPS immunotypes have shown to be protective in an infant rat model, therefore antibodies to LPS may be important in protection from meningococcal disease (Andersen *et al.*, 2002). However, as previously mentioned LPS exhibits endotoxic properties due to its lipid A moiety, and on removal of this portion a decrease in immunogenicity is seen (Preston, 1996; Quakyi *et al.*, 1997; Saunders *et al.*, 1999; Andersen *et al.*, 2002).

Drabick *et al.* (2000) undertook a study using an intranasal (i.n.) vaccine composed of NOMVs. It was well tolerated, even though LPS was present, as no harmful side effects were observed. Bactericidal antibodies to LPS were also induced, suggesting that the nasal mucosal surface is insensitive to LPS and i.n. immunisation could therefore provide another means of investigating NOMV immunogenicity.

The functional activity of the murine mAb B5, which recognises a conserved LPS epitope was investigated by Plested *et al.* (2003). They found that mAb B5 was able to protect infant rats from bacteremia caused by certain strains of Nm serogroup B but not others. They concluded that an inner core LPS epitope could provide a target for

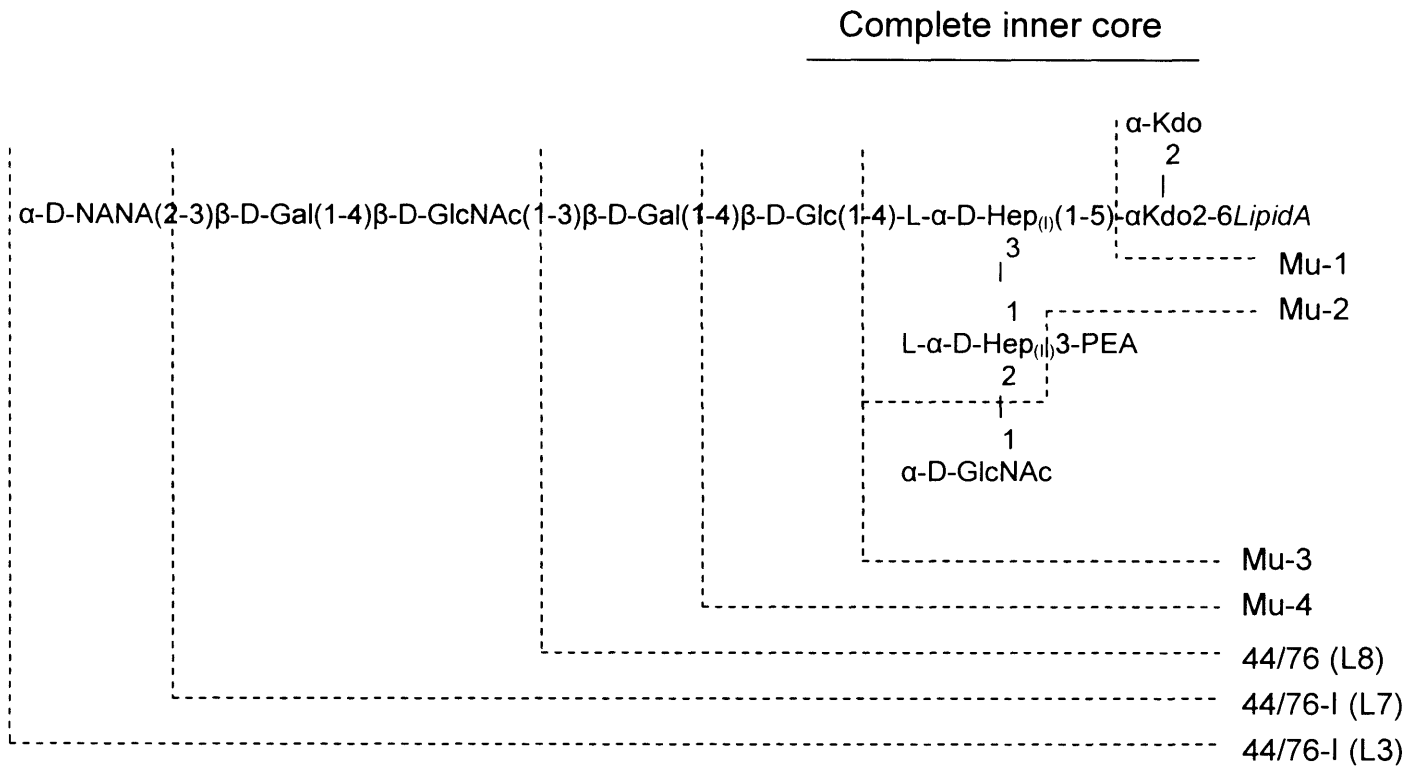

Figure 1.1: LPS structures from *N. meningitidis* strain 44/76

Modified from (Andersen *et al.*, 2002)

protective immunity, however a mAb with greater affinity would be more useful.

1.2.6 Other OMPs

Neisserial surface protein A (NspA) is a highly conserved membrane protein that is reported to be accessible at the surface of all intact meningococcal strains. Data indicates that it could be a promising vaccine candidate as the recombinant NspA protein induced an immune response in mice that protected against lethal meningococcal infection, by producing serum bactericidal and protective antibodies against meningococcal serogroups A, B and C (Martin *et al.*, 1997; Cadieux *et al.*, 1999). However a study by Moe and colleagues (1999) demonstrated differences in surface accessibility of NspA in different serogroup B strains, therefore a vaccine based on NspA may have to be supplemented with additional antigens.

Another iron-regulated protein is the 37kDa ferric binding protein, FbpA (Mietzner *et al.*, 1984). It is conserved in the genus *Neisseria* and has been found to be immunogenic and surface exposed (Gomez *et al.*, 1996) although no significant bactericidal activity directed to FbpA has been demonstrated (Gomez *et al.*, 1998).

Screening of the meningococcal serogroup B genome led to the discovery of NadA, a novel surface antigen. It was found to bind host cells and was postulated to belong to a novel class of adhesins (Comanducci *et al.*, 2002). NadA induces strong bactericidal antibodies and was found to be protective

in an infant rat model (Comanducci *et al.*, 2002). It was also found to react with 60% of serogroup B and C isolates from Brazilian patients (Fukasawa *et al.*, 2003).

1.3 Natural outer membrane vesicles

During the growth of Nm, natural outer membrane vesicles (NOMVs) are released from the cell surface. They are often referred to as 'blebs' and contain a high proportion of LPS and OMPs, which is representative of the meningococcal outer membrane (figure 1.2) (Devoe *et al.*, 1973; Frasch *et al.*, 1982). These blebs have been found to be highly immunogenic, although potentially toxic due to their high LPS content however removal of the LPS and phospholipids by using detergents reduces the toxicity (Saunders *et al.*, 1999; Andersen *et al.*, 2002). Haneberg *et al.* (1998b) showed that application of detergent solubilised OMVs (DOMVs) on to mucosal surfaces in human volunteers induced both mucosal and systemic immune responses, therefore proving that OMVs possess the necessary components to elicit a potentially protective response against meningococci. Due to the immunogenicity of NOMVs they have been used as vaccines in several countries.

1.3.1 NOMV vaccines

Due to the high incidence of meningococcal disease in Norway during the 1970-80s, a vaccine against serogroup B meningococcal disease was prepared from a B:15:P1.7,16 strain by detergent extraction and absorbed to aluminium hydroxide adjuvant. It was found to be highly immunogenic in

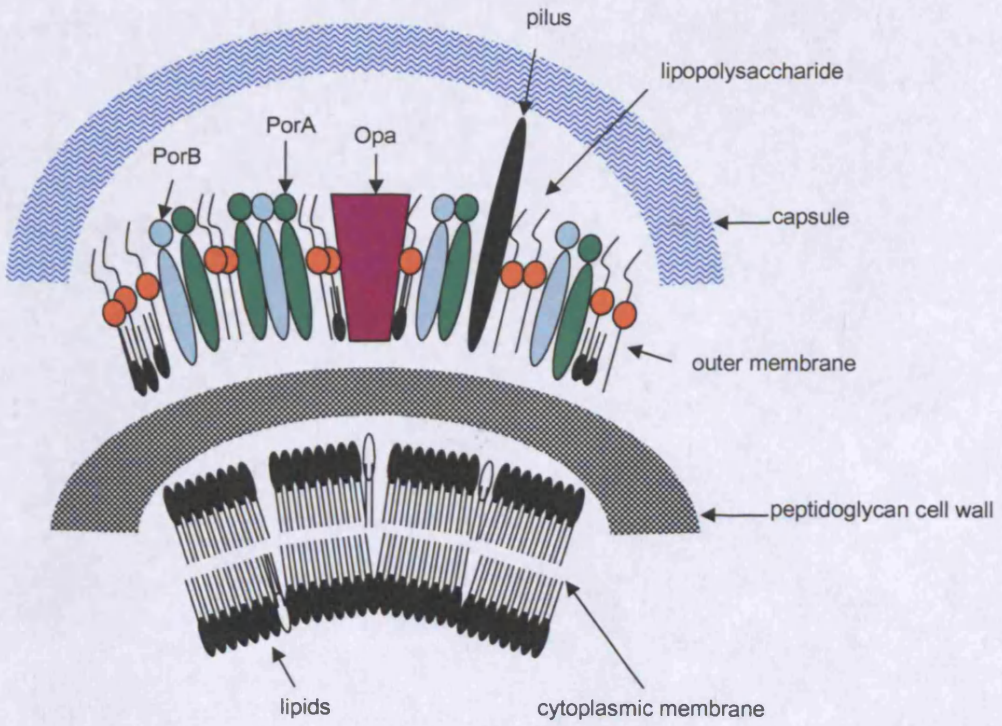

Figure 1.2

Surface structure of *Neisseria meningitidis (modified)*

(Pollard, 2001)

both mice and humans (Fredriksen *et al.*, 1991) and showed significant protection against systemic serogroup B meningococcal disease in teenagers (Bjune *et al.*, 1991). IgG levels were shown to be dramatically increased and opsonic activity (Aase *et al.*, 1995; Aase *et al.*, 2003) and a booster response were also observed (Aase *et al.*, 1995; Rosenqvist *et al.*, 1995; Haneberg *et al.*, 1998a). T cell responses to OMVs and PorA were also observed after intramuscular (i.m.) and i.n. immunisation (Naess *et al.*, 1998; Oftung *et al.*, 1999).

The Cuban OMV vaccine, VA-MENGOC-BC, contains purified CPS of serogroup C meningococci, and is absorbed with aluminium hydroxide gel. Two trials were performed during which the efficacy and safety of the vaccine was established. Due to the results of both of the trials, children aged between three months and six years in the most affected provinces of Cuba received the vaccine (Sierra *et al.*, 1991) and it has also been used during an epidemic in São Paulo, Brazil (de Moraes *et al.*, 1992; Milagres *et al.*, 1994). The vaccine has been shown to induce a characteristic Th1 immune response indicated by the presence of IFN-γ and IL-2 mRNA after *in vitro* OMV challenge of peripheral blood mononuclear cells from immunised subjects (Perez *et al.*, 2001).

The Dutch OMV vaccine is based on PorA. Two vaccine strains were produced each expressing three PorA proteins absorbed to aluminium hydroxide (Claassen *et al.*, 1996). Bactericidal antibodies were found to be PorA specific in immunised humans, and half of the volunteers showed a four-fold or higher increase in bactericidal antibody titres (Peeters *et al.*, 1996). A follow up study by van der Voort and colleagues (1997) examined

B and T cell responses in adult volunteers. They observed strong IgG levels after the first immunisation, however further immunisations did not increase the response. T cell responses determined by T cell proliferation were observed after the first and/or second immunisation but not after the third immunisation.

The vaccine used in Iquique, Chile consisted of purified meningococcal group B OMP (B:15:P1.3:L3,7) noncovalently complexed to group C CPS absorbed to alum. After further purification, the vaccine contained only approximately 0.1% LPS and was well tolerated with minor side effects in volunteers.

Although NOMV and DOMV vaccines have been widely used, they have limited immunogenicity against strains that the vaccine is not produced against, and are not well characterised in how they interact with the innate immune system.

1.4 Innate immunity to meningococcal disease

The innate immune system provides the first line of defence against infection and depends on germline–encoded receptors to recognise features common to many pathogens. The components of innate immunity are generally present before the onset of infection and do not require a prolonged period of induction. Physical barriers such as epithelial surfaces and mucous membranes are the first line of defense against pathogens providing a physical barrier. Physiological barriers such as pH, temperature and chemical mediators can also prevent pathogens from crossing epithelial surfaces. On penetration of these barriers phagocytic cells of the innate

immune system such as macrophages and neutrophils play an important part of innate immunity by eliminating foreign material.

Pathogens are recognised by the innate system by receptors known as pattern-recognition receptors (PRRs), which recognise features common to many pathogens also known as pathogen-associated molecular patterns (PAMPs) (Medzhitov *et al.*, 1997). Many different PRRs have been identified. Some are soluble circulating proteins such as mannose-binding lectin (MBL), C-reactive protein (CRP), complement and LPS-binding protein (LBP) whereas others such as Toll-like receptors (TLRs) and scavenger receptors (SRs) are found on the membrane of cells such as macrophages and DCs.

1.4.1 Macrophages and dendritic cells and their role in meningococcal infection

Macrophages and DCs are professional antigen presenting cells (APCs). They are specialised phagocytes that are involved in the clearance of microbial products and host cells. They are found throughout the body in areas where they are likely to encounter pathogens and microbial products. Not only are they efficient in the capture and processing of antigens, but they are also able to release large amounts of cytokines inducing responses upon binding to specific receptors.

DCs are present in most tissues and have a central role in the initiation of adaptive immune responses (Janeway, 2001) as well as the modulation of immune responses (Banchereau *et al.*, 1998). DCs are derived from myeloid progenitors within the bone marrow. From the bone marrow they migrate to

peripheral sites via the blood. At this stage they are considered to be immature and express low levels of MHC proteins and costimulatory molecules, therefore unable to stimulate naïve T cells. However, they are able to take up antigens by phagocytosis (Inaba *et al.*, 1993), macropinocytosis (Sallusto *et al.*, 1995) and endocytosis (Sallusto *et al.*, 1994). These immature DCs are then stimulated by infection and migrate to lymphoid tissue such as the spleen and lymph nodes, leading to an upregulation of costimulatory molecules and also activation of antigen-specific T cells (Banchereau *et al.*, 2000). The DCs at this stage are said to be 'mature' and have a reduced capacity to phagocytose antigens, however they are able to present peptides from proteins acquired from infection due to high-level expression of MHC class I and class II (Janeway, 2001). Both inflammatory products and infectious agents are able to bring about these changes in DCs (Banchereau *et al.*, 1998).

Many studies have shown that human and murine DCs can be stimulated by wild-type Nm to induce maturation characterised by an increase in surface expression of costimulatory molecules such as CD40, CD86 and MHC II, and the production of proinflammatory cytokines IL-6, TNF-α and IL-1α, that correlate with severe meningococcal disease (Dixon *et al.*, 2001; Kolb-Maurer *et al.*, 2001; Al-Bader *et al.*, 2003).

The ability of meningococci to adhere to and be phagocytosed by DCs has been investigated. Several studies have shown that the meningococcal capsule prevents the adherence of the bacteria to DCs therefore preventing phagocytosis and killing of the bacteria (Kolb-Maurer *et al.*, 2001; Unkmeir *et al.*, 2002). It has also been elucidated that although inactivated wild-type Nm

is readily phagocytosed by DCs, LPS-deficient mutants and live encapsulated wild-type bacteria are not and therefore internalisation of the bacteria is dependent on bacterial expression of LPS in intact Nm (Uronen-Hansson *et al.*, 2004a; Uronen-Hansson *et al.*, 2004b).

The production of IL-12 by DCs has been shown with wild-type meningococci (Dixon *et al.*, 2001; Al-Bader *et al.*, 2003) and is again dependent on internalisation of the whole bacteria as the addition of purified LPS to a LPS-deficient mutant, *lpxA* did not reconstitute the cytokine response by DCs (Uronen-Hansson *et al.*, 2004b).

The interactions of DCs with components of meningococci have also been investigated. Singleton and colleagues (2005) determined the effect of PorB on murine splenic and bone-marrow derived DCs (BMDCs). They found that PorB was able to upregulate the surface expression of costimulatory molecules on DCs and to produce IL-6 by MyD88- and TLR2-dependent mechanisms. In contrast, although recombinant PorA (rPorA) has been shown to induce maturation of human monocyte-derived DCs, low levels or no secretion of proinflammatory cytokines was observed and upregulation of TLR4 mRNA was induced whereas levels of TLR2 mRNA were unchanged (Al-Bader *et al.*, 2004).

Macrophages are the main phagocytic cells of the immune system. They are distributed throughout every organ, where they form a layer beneath the surface of epithelial cells in the gut, lung, kidney and skin. It is here where they recognise a range of antigens through a variety of cell-surface receptors for microbial constituents, such as the mannose receptor and CD14 – a

molecule that recognises and binds LPS (Aderem *et al.*, 1999). Resting macrophages have few MHC class II molecules on their surface and do not express B7 molecules. However the expression of these molecules is induced by ingestion of microorganisms. Once bound, microorganisms are engulfed and degraded in endosomes and lysosomes, generating peptides that can be presented by MHC class II on the cell surface. At the same time MHC class II and B7 molecules are induced on the surface of macrophages (Janeway, 2001).

There have been few reports studying the effects of meningococci on macrophages. Pridmore and colleagues (2001) showed that ten times more TNF-α was produced by stimulation of human monocyte-derived macrophages by wild-type Nm than by the *lpxA* mutant. Activation of macrophages by meningococcal LPS has been shown to be CD14/TLR4 dependent. This was shown by a decrease in TNF-α production when antibodies to TLR4 and CD14 were used to block LPS activation. A result of the activation of macrophages by meningococcal LPS is the release of cytokines, chemokines, nitric oxide and reactive oxygen species (Zughaier *et al.*, 2004).

1.4.2 Soluble factors involved in the recognition of pathogens

1.4.2a The complement system

The complement system helps to fight infection by opsonising pathogens and inducing inflammatory responses. It consists of more than 30 plasma and cell surface proteins and can be activated via three pathways dependent on

different molecules: (1) the classical pathway, (2) the mannose-binding (MB) lectin pathway and (3) the alternative pathway.

The classical pathway can be initiated by binding of C1q, which is part of the C1 complex, to the surface of certain pathogens as well as directly to antigen:antibody complexes. IgM, IgG3 and to a lesser extent IgG1 are able to activate complement. IgM is more efficient at activating complement than IgG due to its pentameric structure allowing the binding of two or more globular heads of the C1q molecule, which activates it. Binding induces a conformational change in the C1s-C1r-C1r-C1s complex, which leads to the activation of enzymatic activity in C1r cleaving the associated C1s (Arlaud *et al.*, 2001). Components C4 and then C2 are cleaved by C1s to produce C4b and C2b which form the C3 convertase (Kerr, 1980). This complex is linked to the surface of the pathogen, where it cleaves C3 molecules to produce C3b molecules that bind to the surface of the organism and act as opsonins (Rawal *et al.*, 2003). The other cleavage product, C3a promotes inflammatory responses.

The membrane attack complex (MAC) is initiated by binding of C3b to the C3 convertase to form a C5 convertase, which is cleaved to produce the anaphylatoxin C5a (DiScipio, 1992) and C5b which initiates the 'late' events of the complement cascade by the terminal complement components. The MAC is formed by the terminal proteins C5, C6, C7, C8 and C9. The C8 component mediates binding to C9, which forms pore-like structures in the membranes of some pathogens that may lead to lysis (Gotze *et al.*, 1970; Scibek *et al.*, 2002).

The MB lectin pathway uses mannose-binding lectin (MBL), a member of the collectin family (Turner, 1996), and ficolins, similar to C1q to trigger the complement cascade. They are able to recognise a range of carbohydrate residues on the surface of bacteria, for example mannose and LPS on Gram negative bacteria and eliminate pathogens by acting as opsonins (Turner, 1996). MBL forms a complex with serine proteases known as MBL-associated serine proteases (MASPs) (Matsushita *et al.*, 1992; Thiel *et al.*, 1997). Once MBL is bound to the surface of bacteria, the MASPs become activated and cleave C4 and C2 to form the C3 convertase and to activate the complement system in a similar way to the classical pathway. The binding capacity of MBL to meningococcal isolates has been investigated. It was initially observed that encapsulated meningococcal strains significantly inhibited binding of MBL (van Emmerik *et al.*, 1994). However, further studies have suggested that the level of MBL binding is determined more by LOS structure and sialylation. Jack and colleagues (1998) demonstrated that isogenic mutants of a serogroup B strain with truncated LOS bound MBL more strongly than organisms with intact LOS and sialylation of LOS prevented binding. This was further supported by an investigation by Devyatyarova-Johnson *et al.* (2000) in *N. gonorrhoeae* and studies using encapsulated strains of Nm serogroup C (Jack *et al.*, 2001).

The alternative pathway can proceed in the absence of specific antibody and does not depend on a pathogen-binding protein for its initiation. The pathway is initiated by binding of C3b to the C3 convertase on to the surface of an activator. These activators include a wide range of pathogens. Factor B then binds to the activator linked to C3b to form C3bB which becomes

susceptible to proteolysis by the serine protease factor D. Factor B is then cleaved into two fragments, Ba which is released, and Bb, which remains bound to C3b forming C3bBb, which is the C3 convertase of the alternative pathway (Xu *et al.*, 2001). The next stages are the same as for the classical pathway in that C3b binds to the surface of pathogens to signal the destruction of the pathogen by phagocytosis and C5 initiates the formation of the MAC complex.

The complement system has been shown to be important in meningococcal disease. Deficiencies, especially of the late complement components, increase the risk of meningococcal infection, indicating complement to be important in the defense against this pathogen (Ross *et al.*, 1984; Fijen *et al.*, 1999).

1.4.2b C-reactive protein

C-reactive protein (CRP) is an acute phase protein, which has been long recognised as an opsonin (Du Clos *et al.*, 1997). It is a pattern recognition molecule made up of five identical subunits each with a high binding affinity for phosphocholine (PC) when in the presence of Ca^{2+} (Szalai, 2002). PC is the dominant epitope of the cell wall polysaccharide, C-polysaccharide, on *Streptococcus pneumoniae*. Gram negative bacteria such as *Haemophilus influenzae* and Nm have also been found to bear the PC epitope (Kolberg *et al.*, 1997). Levels of CRP in blood have been shown to dramatically rise during inflammation and to complement activation and cytokines. CRP has binding sites for effector molecules that bind with C1q to activate complement and the high affinity receptors for IgG, FcγRI and FcγRII on neutrophils and

monocytic cells (Marnell *et al.*, 1995; Bharadwaj *et al.*, 1999). It has also been demonstrated that CRP can down-regulate the response to LPS by binding to FcγR, stimulating the production of IL-10 and blocking IL-12 production (Mold *et al.*, 2002), therefore possibly enhancing the clearance of meningococci.

1.4.3 Cell associated receptors involved in the recognition of pathogens

1.4.3a Toll-like receptors

Toll-like receptors (TLRs) have been shown to have a crucial role in the first line of defence against pathogens (Akira *et al.*, 2004). To date 11 have been identified (TLR1-TLR11). TLR family members are characterised by leucine-rich repeats and a Toll/IL-1 receptor (TIR) domain. The TIR domain has been shown to be similar to that of the IL-1 receptor family. All members of the TLR family appear to signal through the same molecules, including MyD88, IL-1 receptor-associated kinase (IRAK), TNF receptor associated factor (TRAF) 6, TIR-domain-containing adapter-inducing IFN-beta (TRIF), mitogen-activated protein (MAP) kinases and to activate nuclear factor (NF)-κB (Akira, 2003). Figure 1.3 shows the signalling pathways for TLRs 1, 2, 6, 5, 9 and 4. Individual TLRs recognise distinctive structural components of pathogens, and TLR expression is varied depending on location in the body and the cell type. For example, urogenital immunity is associated with TLR11, whereas TLRs 2, 3, 4, and 5 have roles in defence of the bronchial and gastrointestinal epithelia (Hopkins *et al.*, 2005).

The expression of different TLRs has been determined through a number of studies. It has been shown that different subsets of DCs express different

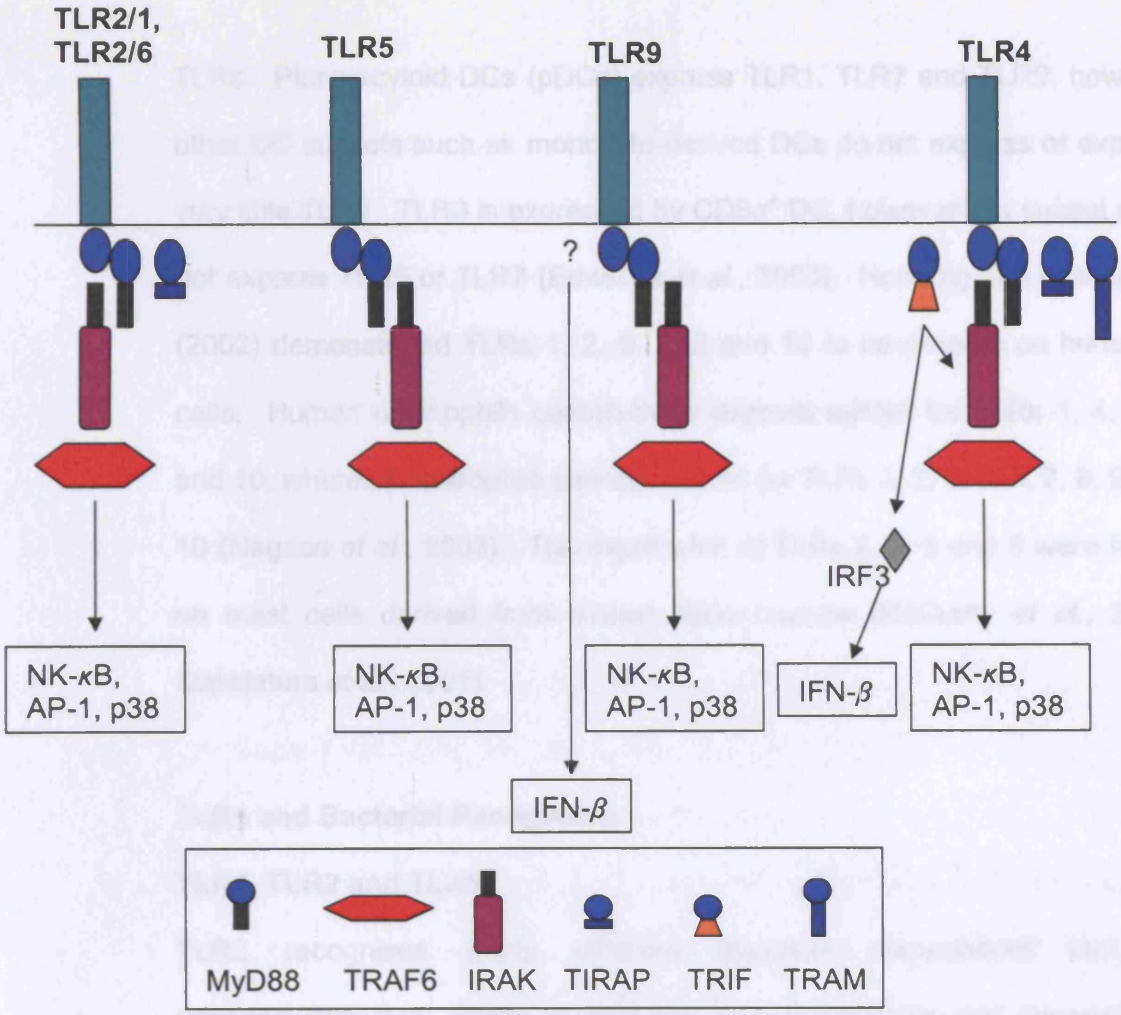

Figure 1.3: Schematic of TLR signalling pathways

TLRs. Plasmacytoid DCs (pDCs) express TLR1, TLR7 and TLR9, however other DC subsets such as monocyte-derived DCs do not express or express very little TLR9. TLR3 is expressed by $CD8\alpha^+$ DC, however this subset does not express TLR5 or TLR7 (Edwards *et al.*, 2003). Hornung and colleagues (2002) demonstrated TLRs 1, 2, 6, 7, 9 and 10 to be present on human B cells. Human eosinophils constitutively express mRNA for TLRs 1, 4, 7, 9 and 10, whereas neutrophils express mRNA for TLRs 1, 2, 4, 5, 6, 7, 8, 9 and 10 (Nagase *et al.*, 2003). The expression of TLRs 2, 4, 6 and 8 were found on mast cells derived from mouse bone marrow (McCurdy *et al.*, 2001; Supajatura *et al.*, 2001).

TLRs and Bacterial Recognition

TLR1, TLR2 and TLR6

TLR2 recognises many different microbial components including peptidoglycan from Gram positive bacteria, lipoproteins and lipopeptides, lipoarabinomannan from mycobacteria, porins from Nm and zymosan, a yeast cell-wall component (Takeda *et al.*, 2003). Ligand recognition by TLR2 is generally in conjunction with TLR1 and TLR6. TLR2 and TLR6 have been suggested to physically interact within the cell. Studies using TLR2-deficient mice highlighted the importance of TLR2 in the recognition of peptidoglycan and lipoproteins however a recent study has demonstrated that highly purified peptidoglycan are not recognised through TLR2, TLR2/1 or TLR2/6 and this loss of recognition is most likely due to the removal of lipoproteins and lipoteichoic acids during purification (Travassos *et al.*, 2004).

TLR4

Studies using C3H/HeJ (hyporesponsive to LPS) mice, which have a point mutation in the intracellular region of the *Tlr4* gene, and the generation of TLR4-deficient mice demonstrated that TLR4 is an essential receptor in the recognition of LPS (Akira, 2003; Takeda *et al.*, 2003). The recognition of LPS by TLR4 also relies on several other molecules. LPS initially binds to LPS-binding protein (LBP) found in serum. This complex is then recognised by CD14, which is present on the surface of mononuclear phagocytes. MD-2, which associates with the extracellular portion of TLR4, is also required. It has been shown that macrophages, DCs and B cells from MD-2-deficient mice have severely limited responses to LPS.

The cell-surface protein, radioprotective 105 (RP105) is a TLR-like molecule which has a functional association with TLR4 and is also required in the recognition of LPS. It contains a structurally related LRR domain found in the extracellular portion of TLRs, but lacks a Toll/IL-1 receptor domain and B cells from mice deficient in RP105 have impaired responses to LPS (Miyake *et al.*, 1995). Expression of RP105 has been shown to be dependent on coexpression of MD-1 (Miyake *et al.*, 1998; Nagai *et al.*, 2002). RP105 was once thought to be primarily expressed on the surface of B cells, however it has been recently shown that RP105 is also expressed on human monocytes, myeloid DCs, murine peritoneal macrophages, splenic DCs and BMDCs. In the same study it was also shown that RP105 can negatively regulate TLR4 signalling (Divanovic *et al.*, 2005).

TLR4 has also been reported to recognise several other ligands such as Taxol, endogenous ligands such as heat shock proteins and extracellular matrix components including fibronectin, hyaluronic acid and heparan sulphate.

TLR5

Flagellin, the main component of bacterial flagella, has been shown to signal through TLR5 (Hayashi *et al.*, 2001). Flagellin extends out from the outer membrane of Gram negative bacteria and is a known virulence factor. Flagella act as propellers to move bacteria through their aqueous environment as well as facilitating invasion by the attachment of bacteria to host cells.

TLR9

TLR9 is essential in the recognition of and responses to bacterial DNA and synthetic oligodeoxynuceotides containing unmethylated CpG dinucleotides (CpG DNA). Proliferation of splenocytes, cytokine production from macrophages and maturation of DCs were abolished in TLR9-deficient mice after stimulation with CpG DNA, a potent activator of immune cells, indicating the importance of TLR9 in these responses (Hemmi *et al.*, 2000). Unmethylated CpG motifs found within bacterial DNA have immunostimulatory activity. These motifs are rarely found in vertebrates, and if present they are generally methylated and lack immunostimulatory activity. CpG DNA is a possible adjuvant candidate as it has been shown to activate

DCs and produce cytokines characteristic of a Th1 immune response (Wagner, 2001) and to trigger B cell proliferation (Krieg *et al.*, 1995).

TLR11

TLR11 is expressed strongly in the liver and kidney, indicating a specific role in these organs and at low levels in the spleen. It was established that cells expressing TLR11 respond to uropathogenic bacteria and this was supported by studies that showed TLR11 deficient mice to be highly susceptible to infections with uropathogenic bacteria (Zhang *et al.*, 2004).

1.4.3b Scavenger receptors

Scavenger receptors (SRs) are glycoproteins that are able to bind polyanionic ligands including low-density lipoproteins (LDLs) such as oxidised LDL and acetylated LDL (Emi *et al.*, 1993; Kraal *et al.*, 2000). They were originally identified on macrophages, however endothelial cells and smooth muscle cells also express SRs. There are at least six molecular forms that have been classified into subgroups according to their structure. These subgroups are SR-A (SR-AI, SR-AII and MARCO), SR-B (CD36 and SR-BI), SR-C, SR-D (CD68), SR-E and SR-F. The class A SR (SR-A) was initially characterised by two isoforms (SR-AI and SR-AII) which are splice variants of the same gene (Emi *et al.*, 1993). A third variant, SR-AIII has been described along with another member of the subclass, the macrophage receptor with collagenous structure (MARCO). MARCO has a similar structure to SR-AI and is expressed constitutively on defined subsets of macrophages (van der Laan *et al.*, 1999). It is able to bind Gram positive

and Gram negative bacteria as well as bacterial CpG DNA (Zhu *et al.*, 2001), and is rapidly induced by BCG infection, bacterial sepsis or *in vitro* with bacteria or LPS (Kraal *et al.*, 2000; Kvell *et al.*, 2006). Meningococci have been shown to be able to bind bone-marrow derived macrophages (BMDMs) in mice through SR-A and that the ligand for SR-A mediated phagocytosis was not the lipid A component of LPS (Peiser *et al.*, 2002). Recent studies by Mukhopadhyay and colleagues (2004; 2006) demonstrated that mouse and human MARCO can bind Nm independently of LPS. They also indicated that the release of TNF-α and NO is not dependent on binding to MARCO or SR-A, therefore proposing that MARCO enhances the recognition and uptake of organisms such as Nm.

1.4.3c C-type lectins

C-type lectins and lectin-like receptors are expressed on the surface of APCs. Many of the lectins are members of the calcium-dependent 'C-type' lectin family and bind sugars via conserved carbohydrate recognition domains (CRDs). Examples of C-type lectins are the mannose receptor (MR) and DC-specific ICAM-3-grabbing non-integrin (DC-SIGN).

The CRD of the MR is structurally similar to that of MBL. The MR is an 180kDa type I transmembrane protein expressed on macrophages, DCs (Sallusto *et al.*, 1995) and hepatic endothelial cells containing eight CRDs that bind mannose, fucose, N-acetylglucosamine and glucose molecules found frequently on the surface of bacteria (Taylor *et al.*, 1990). The MR is able to bind a variety of pathogens such as *Mycobacterium tuberculosis* (Schlesinger, 1993), *Trypanosoma cruzi* (Kahn *et al.*, 1995) and

Pneumocystis carinii (O'Riordan *et al.*, 1995). Zamze and colleagues (2002) demonstrated that the MR was able to bind purified CPSs from *S. pneumoniae* and LPS but not the CPS from *Klebsiella pneumoniae.* The MR is an important receptor in innate immunity as it mediates phagocytosis (Stahl *et al.*, 1998) and endocytosis of soluble antigens to enhance the presentation of these antigens to T cells (Engering *et al.*, 1997). The MR has been described to have an additional role in the clearance of pituitary hormones such as lutropin and thyrotropin in order to prevent desensitisation of the target cell receptors (Simpson *et al.*, 1999).

DC-SIGN is a novel C-type lectin highly expressed on DCs. It is a type II transmembrane protein containing one CRD and ligand binding is calcium dependent. DC-SIGN binds ICAM-3 assisting in the adhesion of DCs with T cells (Geijtenbeek *et al.*, 2000). It has also been shown to recognise carbohydrate structures such as Lewis X and D-Mannose and to bind a broad range of pathogens such as hepatitis C virus (Pohlmann *et al.*, 2003), Dengue virus (Tassaneetrithep *et al.*, 2003), Ebola virus (Alvarez *et al.*, 2002), Mycobacteria (Geijtenbeek *et al.*, 2003), *Helicobacter pylori* and *Leishmania mexicana* (Appelmelk *et al.*, 2003). It has been demonstrated that pathogens may target DC-SIGN in order to alter the Th1 versus Th2 balance. The expression of DC-SIGN has been linked to Th2-type cytokines (Relloso *et al.*, 2002) and results have indicated that mycobacteria can suppress DC function and alter immune responses by shifting the Th1 versus Th2 balance by targeting DC-SIGN (Geijtenbeek *et al.*, 2003).

A murine homologue of human DC-SIGN has been isolated called SIGN-R1. It is one of five mouse genes to be identified and is found within a subset of

macrophages in the marginal zone of the spleen and lymph node medulla (Geijtenbeek *et al.*, 2002; Kang *et al.*, 2003). SIGN-R1 has been shown to be involved in the uptake of pneumococci and its CPS by marginal zone macrophages (MZM) (Kang *et al.*, 2004) and mice lacking SIGN-R1 are more susceptible to pneumococcal infection as the MZMs are unable to bind and phagocytose pneumococci efficiently (Lanoue *et al.*, 2004).

1.4.3d Complement receptors

Phagocytic cells express complement receptors (CRs) to facilitate the uptake and destruction of pathogens. These CRs bind pathogens coated in complement components such as C3b and C4b. Complement receptor 1 (CR1/CD35) is expressed on macrophages, follicular DCs, eosinophils and B cells and binds a host of microbial opsonins including C3b, C4b and MBL (Ghiran *et al.*, 2000). CR2 (CD21), although not described as a phagocytic receptor, is expressed on B cells and binds inactivated forms of C3b (iC3b). CR3 (CD11b/CD18, Mac-1) is expressed on similar cells to CR1 and recognise iC3b among with other non-complement ligands (Ehlers, 2000). CR4 (CD11c/CD18) has not been well characterised, but is expressed on DCs (Underhill *et al.*, 2002).

1.5 Adaptive Response

Defense against meningococcal disease involves both innate and adaptive mechanisms. Natural humoral immunity in the majority of individuals is successful in preventing meningococcal disease, however some individuals

are more susceptible and the protective role of antibodies after immunisation has been demonstrated.

Adaptive immunity is essential when innate mechanisms have been eluded or overwhelmed. The adaptive immune response takes several days to be initiated. As previously described DCs are important in the initiation of an adaptive response by the activation of antigen-specific naïve T lymphocytes. T cells are needed for cell-mediated immune responses, which depend on interactions between T cells and cells bearing the antigen that the T cells recognise. Cytotoxic T cells express CD8 on their cell surface. They function by targeting cells that have been infected by pathogens. CD4 T cells can be divided into two subsets: Th1 cells which are important in the control of intracellular bacterial infections and Th2 cells whose role is in the destruction of extracellular pathogens by activating B cells.

1.5.1 T-dependent and T-independent antigens

Antigens have been classified on their ability to stimulate antibody production as either T cell dependent (TD) or T cell independent (TI) (Mond *et al.*, 1995a; Mond *et al.*, 1995b). TD antigens, such as proteins and peptides, are dependent on T cells for antibody production. Antigen bound to the B cell receptor (BCR) is internalised and returned to the cell surface bound to MHC II molecules which is recognised by T cell receptors (TCRs) recognising the MHC:peptide complex. The interaction between CD40 on the B cell and CD40L on the T cell provide an essential second signal for B cell activation as well as for a sustained immune response including isotype switching.

TI antigens are able to stimulate naïve B cells in the absence of T cell help. TI antigens can be divided into two major categories: TI type 1 (TI-1) and TI type 2 (TI-2). TI-2 antigens are unable to stimulate B cell responses in neonatal mice or CBA/N mice (X-linked B cell defect) whereas these mice respond normally to TI-1 antigens and produce an antibody response (Amsbaugh *et al.*, 1972).

TI-1 antigens are polyclonal activators of B cells, as they are able to cause proliferation and differentiation of most B cells regardless of their specificities. They can stimulate responses in both mature and neonatal B cells. An example of a TI-1 antigen is LPS, which after binding with Lbp and CD14 associates with TLR4 on B cells (Hoshino *et al.*, 1999). TI-1 antigens are unable to induce affinity maturation and memory B cells, both requiring antigen-specific T cell help.

TI-2 antigens are predominantly polysaccharides that are large molecular weight antigens with highly repetitive structures and a long half live when injected *in vivo*. TI-2 antigens can only activate mature B cells, which would explain why infants are unable to make antibodies against polysaccharides, as most of their B cells are immature (Mond *et al.*, 1995b). B cell activation is achieved by cross-linking a specific number of BCRs specific for the antigen.

1.5.2 B cell mediated immunity

Another arm of the adaptive response is via the humoral immune response, in which the spread of infection is prevented by the production of antibodies by B cells. Activation of B cells into antibody-secreting plasma cells is triggered by antigen and generally involves T cell help. Antibodies can

protect the host in three ways. They can inhibit adherence of the pathogen by binding to the pathogen in a process called neutralisation. By binding to the pathogen, antibodies are able to encourage opsonisation as well as triggering complement activation.

1.5.2a Structure and assembly of antibody

Antibodies are the secreted form of the BCR constructed from paired heavy and light polypeptide chains. The variable region is made up of the first domains of the heavy and light chain and enables the binding of specific antigen. There are two types of light chain, lambda (λ) and kappa (κ) with no functional differences between them. Immunoglobulins (Igs) have either of these chains but never one of each and the ratio of the two types of chain varies between mammals.

The class and therefore the effector functions of antibodies are defined by the structure of the heavy chain. They are denoted by μ, δ, γ, α, and ε, representing the five main classes or isotypes: immunoglobulin M (IgM), IgD, IgG, IgA and IgE. Some of the isotypes have several subtypes, for example in the mouse IgG has subclasses IgG1, IgG2a, IgG2b and IgG3 and in humans IgG1, IgG2, IgG3 and IgG4.

1.5.2b Isotype switching

The constant region of the heavy chain defines the class of Ig, which is important for determining its effector function. Naïve mature B cells express both IgM and IgD on their surface. Upon activation by antigen, IgD expression is lost and the first antibody produced in an immune response is IgM. Activated B cells often switch to express a different heavy chain

constant region, resulting in a change of antibody class and effector function. Switching of antibody class is carried out by a deletional DNA recombination event called switch recombination. This occurs by looping-out and deletion of circular DNA containing the sequences between short tandem repeated sequences called switch regions (von Schwedler *et al.*, 1990; Stavnezer, 1996). Switch regions are located between the J gene segments on the heavy chain and the C_μ gene, and at similar sites for the other heavy-chain isotypes, except IgD.

The class of antibody expressed by mature B cells is influenced by antigen exposure. Signals from both T cells and cytokines can induce isotype switching to TD antigens, whereas TI antigens also respond to signals from cytokines and from cross-linking of surface Ig. It has been shown that IL-4, TGF-β and IFN-γ have roles in regulating isotype switching. In both humans and mice IL-4 can induce IgE, additionally in humans it can induce IgG4 and in mice IgG1. The addition of IFN-γ to activated B cells can induce switching in murine B cells to IgG2a. In contrast it inhibits the induction of IgE by activated B cells plus IL-4. Switching to IgA can be induced by the addition of TGF-β in activated mouse and human B cells (Stavnezer, 1996).

A study has shown a role for IL-27 in the induction of class switching. IL-27 is a member of the IL-12 family and is a heterodimeric cytokine. It is produced by activated APCs and is able to induce proliferation of naïve $CD4^+$ T cells (Pflanz *et al.*, 2002). Splenic B cells were found to express functional IL-27R and activated B cells stimulated with IL-27 were able to induce the expression of transcription factor T-bet and IgG2a, but not IgG1. Class switching to IgG2a was abolished in T-bet-deficient B cells activated with

LPS suggesting a critical role for IL-27 in the expression of T-bet and IgG2a (Yoshimoto *et al.*, 2004).

Isotype switching in human B cells can also be induced by B cell-activating factor (BAFF) and A proliferative-inducing ligand (APRIL), two members of the tumour necrosis factor family (Litinskiy *et al.*, 2002). They have more recently been shown to influence isotype switching in murine B cells inducing IgG1, IgA and IgE (Castigli *et al.*, 2005).

1.5.2c Functional differences

Antibodies are distributed throughout the body and have their particular effector function, which is determined by their isotype. IgM is the first antibody to be produced in a humoral immune response because it can be expressed without isotype switching. These antibodies tend to be of low affinity, however IgM molecules are secreted as pentamers and bind simultaneously to multivalent antigens. Due to the large size of the pentamers, IgM is mainly found in the blood. It is also efficient in activating the complement system, as it is able to bind multiple epitopes on the pathogen surface.

The other isotypes – IgG, IgA and IgE are smaller and therefore can more easily diffuse out of blood into tissues. IgG and IgE are always produced as monomers, whereas secreted IgA can occur as either monomers or dimers. Dimerisation of IgA monomers is required for transport through the epithelia.

The principle isotype found in the blood and extracellular fluid is IgG. There are four human IgG subclasses: IgG1, IgG2, IgG3 and IgG4. IgG1, IgG3 and IgG4 are able to cross the placenta and therefore have a role in protecting

the developing foetus. IgG3 is the most efficient at activating the complement system, whereas IgG1 and IgG3 are able to mediate opsonisation by binding Fc receptors on phagocytic cells.

IgA is the principle isotype found in secretions, but unlike IgG is a weak activator of complement and a less potent opsonin and therefore functions mainly as a neutralising antibody at mucosal surfaces. Neutralising antibodies bind toxin molecules secreted by bacteria. This in turn prevents the binding of the toxin to the cell and protects the cell. IgA is secreted in breast milk and is therefore passed from a mother to her baby helping to protect against newly encountered pathogens.

IgE is found at very low levels in the blood and extracellular fluid. It mediates hypersensitivity reactions that are responsible for the symptoms of hay fever and asthma for example. IgE binds to Fc receptors on the membrane of basophils and mast cells found beneath the skin and mucosa. The basophils and mast cells then release chemical mediators found in granules when antigen binds the cell-associated IgE giving rise to allergic manifestations.

1.5.2d Antibody-mediated effector functions

As described in section 1.4.2a, the classical complement pathway can be initiated by antibodies binding to the complement component C1q. In humans both IgM and IgG antibodies can bind C1q, when the antibodies are bound to the surface of a pathogen. In mice IgM, IgG2a, IgG2b and IgG3 have been found to be able to fix complement (Klaus *et al.*, 1979; Neuberger *et al.*, 1981). This then leads to the activation of enzymatic activity in C1r, which triggers the complement cascade.

Antibodies can promote phagocytosis of antigens by macrophages, DCs and neutrophils and trigger nonphagocytic cells such as NK cells and eosinophils to secrete stored mediators when Fc receptors are bound by antibody coated pathogens. Fc receptors are cell-surface molecules that recognise the Fc portion of Igs. Different cell types express Fc receptors for different isotype antibodies, therefore the antibody isotype determines which cell type will be engaged. Once the antibody-coated pathogen binds Fc receptors on a phagocyte, the surface of the phagocyte extends around the surface of the pathogen enclosing the pathogen in a phagosome. This then fuses with lysosomes to become a phagolysosome, allowing the release of lysosomal enyzymes and toxic nitrogen and oxygen-derived products to destroy the pathogen.

1.5.2e Natural IgM and B cells

Natural antibodies can be found in the sera of mice and humans prior to infection. They are generally of the IgM isotype, and can bind a specific antigen or pathogen even if the host has never experienced the pathogen. Natural antibodies are secreted by the B1 subset of B cells. They are long-lived, self-renewing cells, which differ from B2 cells by the way they differentiate during foetal and neonatal development. Natural antibodies are polyreactive to conserved structures such as nucleic acids, heat shock proteins, carbohydrates and phospholipids (Kantor *et al.*, 1993; Hardy *et al.*, 1994). Complement activation can be achieved through the binding of natural IgM to bacteria, demonstrating its importance during a primary immune response (Boes *et al.*, 1998). B1 cells can predominantly be found

in pleural and peritoneal cavities, although they make up a significant proportion of the splenic B cell population in foetal and neonatal mice. Natural IgM has been reported to be of low antigen-binding affinity due to the lack of somatic mutation in the V region segment.

B1 cells and marginal zone B cells share functional characteristics, and are thought to be involved in immune responses to TI antigens (Martin *et al.*, 2001). It has been recently shown that B1-a cells are the main source of natural IgM to pneumococcal CPS and that B1-b cells are the major population producing antibodies after CPS immunisation (Haas *et al.*, 2005).

1.5.2f Memory B cells

Immunological memory allows a more rapid and effective response to pathogens that have been previously encountered. It is thought that memory is sustained by long-lived antigen specific cells that were induced during the first contact with a pathogen and persist until the next exposure.

Memory B cells are differentiated germinal center B cells that were once stimulated by antigen and proliferated in the germinal center. They divide very slowly and express surface Ig but secrete none or very little antibody. At lower doses of antigen, memory B cells are able to produce antibody of higher affinity.

Although there is a lot of information on vaccine candidates and the role of antibodies in protection against meningococci, the mechanisms of natural immunity is not clear. It is thought to be induced through prolonged or intermittent colonisation at mucosal surfaces of the nasopharynx. Davenport

and colleagues (2003) reported that T cells from the tonsils are able to proliferate in response to NOMVs and proliferation was not dependent on LPS. They also found that high proliferative responses were associated with NOMV-specific IgG responses, but not SBA.

Non-capsular antigens are important targets for vaccines to serogroup B meningococci due to poor immunogenicity of the CPS, and inducing immunity against a mucosal pathogen, mucosal immunisation would be sensible.

1.6 Mucosal immunity

The primary site of invasion and colonization of Nm is the nasopharynx. Nm is able to adhere to the epithelial cells of the mucosa via pili and OMPs found on the surface of meningococci. Several factors can lead to the invasion of the mucosal surface, including a change in the hydrophobicity and antigenic variability of the cell-surface components involved with adhesion and invasion.

The upper respiratory tract is an important site for host defence against pathogens. In mice, the nasal-associated lymphoid tissue (NALT) is thought to resemble the Waldeyers's ring in humans, having a cellular composition similar to that of Peyer's patches. The NALT is composed of two components, the O-NALT and the D-NALT. The O-NALT is composed of a pair of organised lymphoid aggregates and has been suggested to function as a mucosal inductive site, and the D-NALT, which lines the nasal passages, is composed of less well organised lymphoid tissue and functions as an effector site. Previous studies have shown NALT to contain major

populations of both naïve B and T cells, with the majority of T cells expressing the αβ TCR (Liang *et al.*, 2001; Zuercher *et al.*, 2002).

The delivery of vaccines via a mucosal route would be advantageous, as it would provide a simple way of administering and inducing local immune responses at the natural site of colonization and infection. Protein vaccines administered i.n. do not induce strong immune responses and have therefore been enhanced by using mucosal adjuvants.

Recent studies of OMV vaccines administered i.n. have demonstrated that in both mice and humans, mucosal and systemic antibody responses with substantial bactericidal activity and antigen-specific T cell responses can be induced (Haneberg *et al.*, 1998b; Oftung *et al.*, 1999; Guthrie *et al.*, 2004). The OMV vaccines proved to be safe and well tolerated when i.n. administered.

Studies carried out by Etchart and colleagues (2006) have shown that NOMVs enhanced mucosal antibody responses toward influenza virus and respiratory syncytial virus (RSV) following i.n. immunisation. In particular their results showed that NOMVs were able to raise long-lived serum RSV-specific IgG. Although the mechanism for the NOMVs adjuvant effect has not been investigated, it would likely involve the interactions and responses of APCs upon exposure to meningococcal NOMVs.

Immunisation by the mucosal route is the preferred route for a number of reasons including the potential to induce both systemic and mucosal immunity and the elimination of needles. Subunit vaccines are preferable over more complex cell- or extract-based vaccines as they may be safer and

more effective, however induction of immune responses through mucosal immunisation is dependent on the use of an appropriate adjuvant.

1.7 Adjuvants

Adjuvants are in general compounds or substances that are able to enhance antibody responses against an antigen, as well as being able to bias the immune system towards either a Th1 or Th2 immune response (Marciani, 2003). Adjuvants used in human vaccines must meet rigorous requirements such as being non-toxic, able to stimulate strong humoral responses, not induce autoimmunity and be stable, therefore there are a limited number of them. Aluminium based adjuvants and MF59 are examples of adjuvants licensed for use in humans (Alving, 2002).

Adjuvants exert their effects by different mechanisms. For example, alum, which is an insoluble adjuvant, stays around the injection site and is released over a period of time to stimulate APCs (Marciani, 2003). Jordan *et al.*, (2004) recently described how immunisation of mice with alum primed splenic B cells and caused the accumulation of a previously unknown population of IL-4 producing Gr1+ cells which are required for optimal antibody production. Freund's complete adjuvant (FCA) is a potent adjuvant too toxic for use in humans but is commonly used in animal research. However its use in animals is also being discouraged. FCA's mineral oil components serve as an antigen depot and its mycobacterial cell wall component activates the immune system in a non antigen-specific manner (Billiau *et al.*, 2001).

In the search for new adjuvants, microbial products and modifications of such products have been studied. A big proportion of these have been TLR

ligands, for instance LPS, a TLR4 ligand and poly(I:C) a synthetic double stranded RNA ligand for TLR3 (Lien *et al.*, 2003).

Type 1 interferons (IFNs) are induced by a number of stimuli such as viral and bacterial infection. Enhanced antibody responses to poorly immunogenic proteins as well as immunological memory suggest type 1 IFNs to possess strong adjuvant properties (Le Bon *et al.*, 2001). Dendritic cells have also been proposed as adjuvants due to their ability to capture antigens and to control the magnitude of the immune response (Steinman *et al.*, 2002).

The type of immune response can be determined by adjuvants. A Th2 response can be generated by alum characterised by the production of cytokines such as IL-4 and IL-5, and IgG1 and IgE antibodies, but an absent CTL response. Adjuvants such as CpG DNA are able to induce strong CTL responses and a more Th1 response characterised by the secretion of IFN-γ, TNF-α and antibodies such as IgG2a (Weeratna *et al.*, 2000).

A large number of infections occur at mucosal surfaces therefore mucosal immunisation has been suggested as an alternative route of immunisation. However problems have arisen in being able to generate strong mucosal IgA immune responses, which could be important in blocking the attachment of bacteria and viruses. There are very few vaccines administered mucosally, one being the oral polio vaccine. Cholera toxin (CT) and *E. coli* heat labile enterotoxin (LT) are widely used experimental mucosal adjuvants in animals. They have been shown to be powerful mucosal adjuvants, however they are

considered too toxic for human use (Holmgren *et al.*, 2003). They work by using a combination of actions including enhancing antigen presentation by a variety of cell types and encouraging isotype switching in B cells.

As previously mentioned CpG DNA has been shown to be a strong adjuvant. Reports have been made on the use of CpG DNA as a mucosal adjuvant. These reports have indicated CpG DNA to be a potent inducer of immune responses to Hepatitis B surface antigen (McCluskie *et al.*, 1998; McCluskie *et al.*, 1999) and influenza virus (Moldoveanu *et al.*, 1998) when immunised i.n. in mice indicating CpG DNA as a promising new adjuvant. Its mechanism of action as an adjuvant has not been fully resolved but it has been hypothesised that it signals through TLR9 leading to cytokine secretion (Freytag *et al.*, 2005).

Another bacterially derived product which has been shown to induce mucosal and systemic antigen-specific antibodies following i.n. immunisation is monophosphoryl lipid A (MPL) (Baldridge *et al.*, 2000; Childers *et al.*, 2000). MPL is isolated from the LPS of *Salmonella Minnesota* R595 and its activity is associated with its ability to activate APCs and signal through TLRs to produce cytokines (Freytag *et al.*, 2005).

The use of meningococcal OMVs as a mucosal adjuvant has not been extensively studied. van de Verg and colleagues (1996) demonstrated that OMPs of serogroup B Nm to be an effective mucosal adjuvant for LPS of *Brucella meliensis* in mouse and guinea pig models, producing secretory IgA and IgG in the lungs and high levels of IgG in serum.

The potential of OMVs as a mucosal adjuvant to inactivated influenza virus was first shown by increases in systemic and salivary antibody responses

(Haneberg *et al.*, 1998a). It was then established that whole heat and formalin-inactivated meningococci were also able to enhance serum IgG and serum and salivary IgA responses to inactivated influenza virus (Berstad *et al.*, 2000). In a recent study, Bizanov and colleagues (2005) described protection against replication virus in the nasal cavity of mice after i.n. immunisation with formalin-inactivated influenza whole virus with OMVs from serogroup B meningococci indicating the effectiveness of bacteria-derived products as adjuvants.

1.8 Project Aims

NOMVs are known to induce serum bactericidal antibodies specific for meningococcal antigens in mice and humans however the adjuvanticity of NOMVs has not been extensively studied. By studying the adjuvant properties of NOMVs toward different antigens *in vitro* and *in vivo* we hope to establish whether NOMVs are a safe vaccine with inherent mucosal adjuvanticity and to gain insights into the role of NOMVs in natural immunity or disease following meningococcal colonisation.

The main aims of this thesis are to investigate:

- the adjuvanticity of NOMVs toward protein and carbohydrate antigens via different immunisation routes
- the immunomodulatory properties of NOMVs
- the contribution of meningococcal LPS and non-LPS components towards the immunomodulatory properties of NOMVs

- the immunomodulatory effect of NOMVs in a mouse model of allergen and RSV induced airway eosinophilia

The insights provided in this investigation will further our knowledge of NOMV-induced modulation of immune responses and contribute to the development of new mucosal adjuvants.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Antibodies

2.1.1a Antibodies used in ELISA assays

Product	Conjugate	Supplier	Dilution	Catalogue number
Goat anti-mouse (γ chain specific) IgG	Horseradish peroxidase	Jackson Immunoresearch	1/500	115-035-164
Goat anti-mouse (μ chain specific) IgM	Horseradish peroxidase	Jackson Immunoresearch	1/500	115-035-020
Goat anti-mouse IgA	Horseradish peroxidase	SouthernBiotech	1/1000	1040-05
Goat anti-mouse IgG1	Horseradish peroxidase	SouthernBiotech	1/1000	1070-05
Goat anti-mouse IgG1	Horseradish peroxidase	SouthernBiotech	1/1000	1070-05
Goat anti-mouse IgG2a	Horseradish peroxidase	SouthernBiotech	1/1000	1080-05
Goat anti-mouse IgG2b	Horseradish peroxidase	SouthernBiotech	1/1000	1090-05
Goat anti-mouse IgG3	Horseradish peroxidase	SouthernBiotech	1/1000	1100-05

2.1.1b Antibodies used in flow cytometric analysis

Target molecule	Isotype	Conjugate	Company	Catalogue number
Mouse CD11c	Hamster IgG1	PE	BD Pharmingen	553802
Purified CD16/CD32 (FcγIII/II receptor)	Rat IgG2b	Purified	BD Pharmingen	553142
Mouse CD45R/B220	Rat IgG2a	APC	BD Pharmingen	553092
Mouse CD3e	Hamster IgG1	PE	BD Pharmingen	553063
Purified rat IgG_1, κ isotype control	Rat IgG1, κ	Purified	BD Pharmingen	554682

2.1.2 Immunological kits

Target Molecule	Company	Catalogue Number
Griess Reagent System	Promega	G2930
Mouse Inflammation Cytometric Bead Array	BD Pharmingen	552364
Mouse Th1/Th2 Cytokine Cytometric Bead Array	BD Pharmingen	551287
Mouse eotaxin/CCL11 ELISA Development kit	R&D Systems	DY420
Mouse IgE ELISA set	BD Pharmingen	555248
Mouse Interferon Alpha ELISA Kit	Biosource	KMC4011
Mouse Interferon Beta ELISA Kit	Biosource	KMC4041
Dynal Mouse B Cell Negative Isolation Kit	Dynal Biotech	114.21

2.1.3 Biochemical reagents

Reagent	Company	Catalogue number
ABTS (2,2 Azino-bis(3-ethylbenthiazoline-6-sulfonic acid)	Sigma	A9941
Accustain Giemsa stain	Sigma	GS-500
Accustain Wright stain	Sigma	WS-16
Accutase	PAA Laboratories	L11-007
Acetic acid	BDH	153103D
Acrylamide/bisacrylamide 30% (v/v) solution	Bio-rad	161-0156
Ammonium hydroxide	Sigma	A6899
Ammonium persulphate	Bio-rad	161-0700
Betaplate scintillant	Wallac	SC/9200/21
Bio-safe Coomassie Stain	Bio-Rad	161-0786
Bovine serum albumin	Sigma	A4503
Bromophenol blue	Sigma	B8026
Carboxyfluorescein diacetate succinimidyl ester (CFSE)	Molecular Probes	V12883
Citric acid	Sigma	C0759
Dried skimmed milk	Marvel	Off shelf
Ethanol	BDH	15338
Ethylenediaminetetraacetic acid (EDTA)	Sigma	E5134
Formaldehyde (37% solution)	Sigma	F1635
Glycerol	Sigma	G6279
Hydrogen Peroxide	Sigma	H1009
Ionomycin	Sigma	I0634
Ketamine	Sigma	K2753
Laemmli sample buffer	Bio-Rad	161-0737
Lithium acetate dehydrate	Sigma	L4158
Methanol	BDH	10158BG
2-Mercaptoethanol 50mM	Gibco Invitrogen	31350-010

N, N, N, N, -tetra-methyl-ethylenediamine (TEMED)	Bio-Rad	161-0800
Naphthylethylenediamine	Sigma	N9125
o-Phenylenediamine Dihydrochloride (OPD) tablet sets	Sigma	P9187
Periodic acid	Sigma	P5463
Phenol	Sigma	P5566
Phorbol myristate acetate (PMA)	Sigma	P8139
Phosphate buffered saline (PBS) 10x, without magnesium or calcium	Life Technologies	14200-067
Phosphoric acid	Sigma	P5811
p-Nitro-phenyl-phosphate (pNpp)	Sigma	N1891
Potassium hydroxide	Sigma	P5958
Sodium chloride	Sigma	S7653
Sodium hydroxide	Sigma	S8045
Sulphuric acid	BDH	102761C
Tween 20	Sigma	P7949
Vybrant CFDA SE Cell Tracer Kit	Molecular Probes	V-12883
Xylazine	Sigma	X1251

2.1.4 Tissue culture reagents

Reagent	Company	Catalogue number
Foetal bovine serum Lot 40G2027K	Gibco Invitrogen	10270-169
Gentamicin	Life Technologies	15750-037
Penicillin/Streptomycin	Gibco Invitrogen	15140-122
RBC lysing buffer	Sigma	R7757
RPMI 1640 (1x) with Glutamax I, 25mM HEPES	Life Technologies	72400-054
Trypan blue	Sigma	T8154
Recombinant mouse GM-CSF	R&D Systems	415-ML
Recombinant murine M-CSF	R&D Systems	416-ML-010

2.1.5 Plastics

Item	Company	Catalogue number
Beckman centrifuge bottle with cap (50ml)	Beckman	357001
Cell strainer 0.4uM	Falcon	2340
Cell scrapers 25cm	Fahrenheit	3086
24-well plates	Falcon	3043
96-well immunoplates	NUNC	DIS-971-010P
96-well U bottomed plates	Nunclon	163320
15ml round conical tubes	Falcon	2096
50ml round conical tubes	Falcon	2070

2.1.6 Miscellaneous

Reagent	Company	Catalogue Number
DNP-ficoll	Biosearch Technologies	F-1200-10
Pneumococcal cell wall polysaccharide	Statens Serum Institute	3459
Albumin from chicken egg white, grade VI	Sigma	A2512
Type 3 purified pneumococcal polysaccharide	LGC	169-X
Type 4 purified pneumococcal polysaccharide	LGC	173-X
Type 14 purified pneumococcal polysaccharide	LGC	197-X
Type 19F purified pneumococcal polysaccharide	LGC	205-X

2.2 Methods

2.2.1 Preparation of NOMVs (supervised by Dr G Frith)

Mutant-4 *N. meningitidis* strain 44/76 was plated onto BHI agar plates containing 1% horse serum and incubated overnight at 37°C in a 5% CO_2 atmosphere. Broth cultures were prepared by scraping the bacteria off the plates and incubating flasks overnight at 37°C in an incubator shaking at 120-140rpm. The bacteria was then harvested and centrifuged at 12,000 x g for 30 mins at 4°C using a Beckman centrifuge with a JA-10.500 rotor. To kill any live bacteria in the supernatant, 1% phenol was added and incubated for 1-2h. An aliquot of supernatant was plated onto BHI agar and incubated overnight at 37°C, 5% CO_2 to check for any remaining live bacteria. Once established there was no live bacteria, the culture supernatants were harvested and put through a continuous flow ultrafiltration system to prepare NOMVs. Prior to use the filtration system was washed with 20% ethanol and water, and finally autoclaved. A peristaltic pump was used to feed the supernatant through a loop of plastic tubing which is connected to a size exclusion column (molecular weight cut off of 500kDa), until approximately 90% of the media was removed leaving a concentrated suspension of NOMVs. The concentrated suspension was centrifuged at 12,000 x g for 20 mins at 4°C (Beckman rotor JA-30.50) to remove cell debris. The supernatant was collected and centrifuged again at 80,000 x g for 2h at 4°C to further concentrate the NOMVs. The supernatant was then discarded and the pellet was re-suspended in sterile distilled water and subjected to ultra-centrifugation at 100,000 x g for 3h at 4°C. The remaining pellet was then re-suspended in Milli Q water and stored at 4°C.

2.2.2 BCA protein assay

The protein content of samples was measured using the Micro BCA™ Protein Assay Reagent Kit (Pierce Chemical Company). The assay was carried out according to manufacturer's instructions using the microwell plate protocol. Briefly, a standard curve was produced using bovine serum albumin (BSA) diluted in running buffer. The samples were diluted, in duplicate at appropriate concentrations, to which working reagent was added to the samples and the standards. The plate was then incubated for 2h at 37°C, after which the optical density was read at 562nm on a plate reader [Spectra MAX 340 (Molecular Devices)]. Using the standard curve, the protein concentration of the samples was determined.

2.2.3 Characterisation of humoral responses

2.2.3a Protocol for model antigen immunisations

Mice

Female or male C57Bl/6 and Balb/c mice were obtained from Charles River, UK (Margate, Kent, UK) or the SPF unit of the Institute for Animal Health, Compton. The animals were kept under specific pathogen free conditions and the mice were used between 6-10 weeks of age.

Intranasal protocol

Before i.n. immunisation, mice were anaesthetised by administering i.p. 150µl of ketamine/xylazine mixture (90mg/ml ketamine and 20mg/ml xylazine in PBS, diluted 1 in 10 in PBS before use). Once anaesthetised, mice were held with their heads tilted back and 50µl of sample was placed onto the

entrance of the nasal passage by using a Gilson P20 pipetter. After immunisation the mice were placed on their sides in their cages and hot lamps were placed over them until they regained consciousness.

Immunisations

Groups of mice were immunised either by the intraperitoneal (i.p.) or i.n. route with the model protein antigen Ovalbumin (Ova) or the T-independent type II antigen DNP-ficoll, in the presence or absence of NOMVs. Ova was used at a dose of 0.5mg and DNP-ficoll at 5µg in a 500µl or 50µl volume of sterile PBS when immunised i.p. or i.n., respectively. Control mice were given PBS only. The schedule for immunisations and blood sampling is shown in figure 2.1.

2.2.3b Protocol for *S. pneumoniae* CPS immunisations

Mice

Female or male C57Bl/6 and Balb/c mice were obtained from either Charles River, UK (Margate, Kent, UK) or the SPF unit of the Institute for Animal Health, Compton. The animals were kept under specific pathogen free conditions and the mice were used between 6-10 weeks of age.

Immunisations

Groups of mice were immunised either by the i.p. or i.n. route with pneumococcal CPSs (capsular serotypes 3, 4, 14 and 19F) obtained from the Statens Serum Institute, Copenhagen, Denmark in the presence or absence

of NOMVs. A range of doses of CPSs were used (5-20µg per mouse) in a 500µl or 50µl volume of sterile PBS when immunised i.p. or i.n., respectively. Control mice were given PBS only. The schedule for immunisations and blood sampling is shown in figures 2.1 and 2.2.

2.2.3c Preparation of mouse serum

Mice were bled from either the tail vein or the chest cavity. Blood was left for 1h at room temperature and left overnight at 4°C. To clarify serum from coagulated blood, samples were centrifuged at 14,000rpm for 20 mins in a Beckman microfuge. Serum was then removed and placed into new tubes and stored at -18^{o}C until required.

2.2.4d Characterisation of serum antibody responses to NOMVs by ELISA

Immunoplates were coated overnight with 100µl/well of 4µg/ml NOMVs in 50mM sodium carbonate-bicarbonate buffer, pH 9.6 and incubated at 4°C. Plates were washed with PBS + 0.05% Tween 20 (PBS-T) and were blocked for 1h with 150µl of PBS + 0.5% BSA. Plates were then washed again and sera were serially diluted starting at a 1 in 50 dilution, and 50µl was added to duplicate wells and incubated at room temperature for 2h in a moist box. The plates were washed again, and 50µl/well of either peroxidase-conjugated affinipure goat anti-mouse IgM or peroxidase-conjugated goat anti-mouse IgG (subclasses 1+2a+2b+3) (Jackson ImmunoResearch, Inc.) diluted 1 in 500 in PBS-T were added to the plates and incubated for a further 2h at room

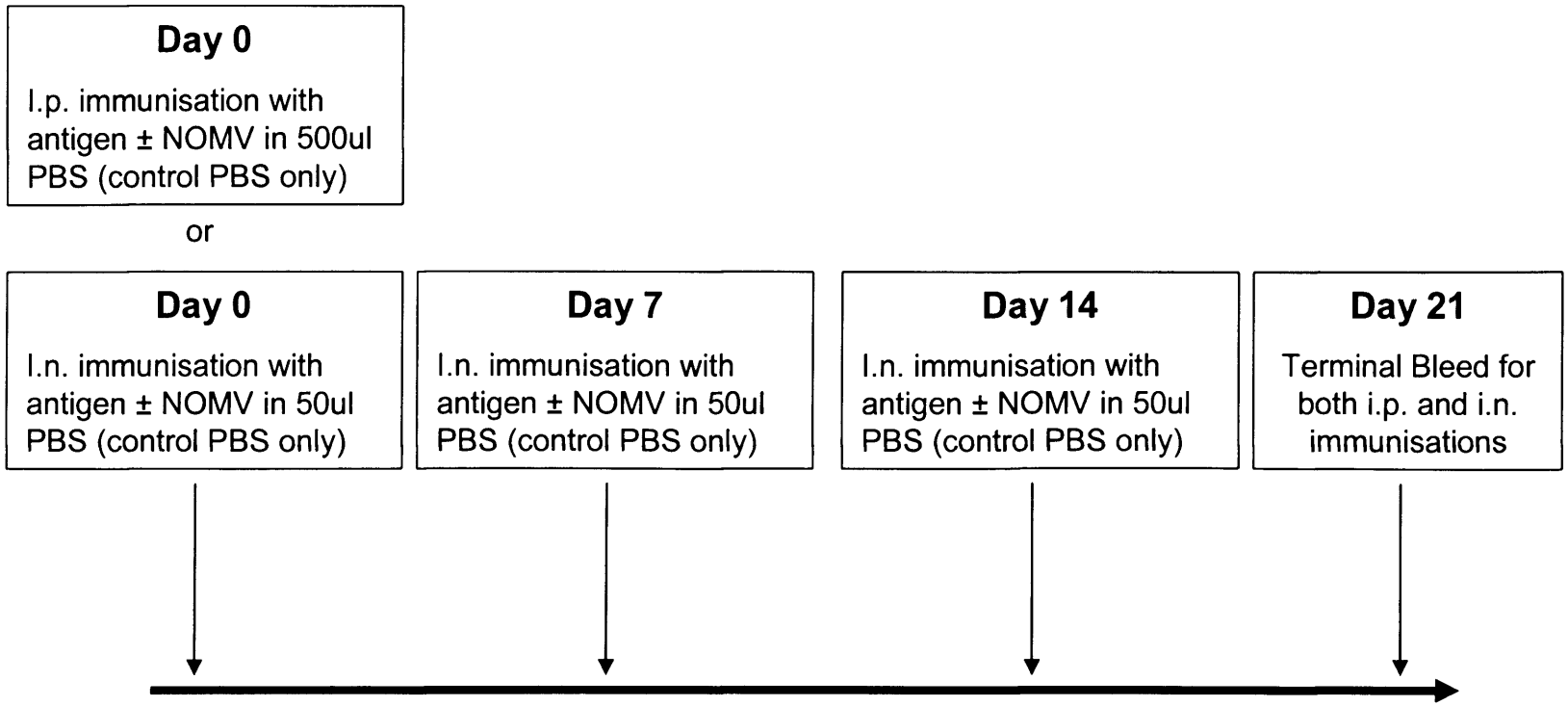

Figure 2.1 Schedule for i.p. and i.n. immunisation of mice with model antigens or *S. pneumoniae* CPSs with or without NOMV and serum collection

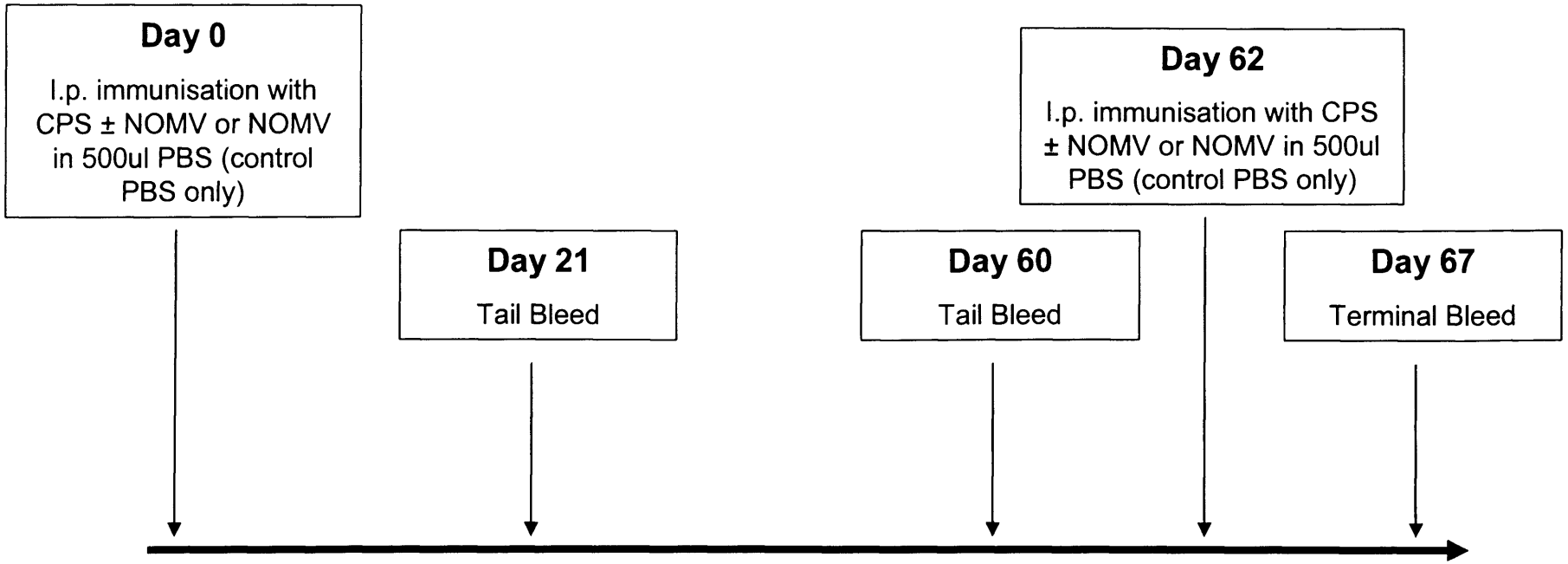

Figure 2.2 Schedule for immunisation and serum collection of mice with *S. pneumoniae* CPSs with or without NOMV for memory and boosting effect

temperature. The plates were washed again and 50µl/well of the substrate solution o-phenylenediamine dihydrochloride [OPD (a horseradish peroxidase substrate, Sigma)] was added. The reactions were stopped with 15µl/well of 3M H_2SO_4. Optical densities were then read at 490nm on a plate reader.

2.2.3e Characterisation of serum antibody responses to model antigens by ELISA.

Immunoplates were coated overnight with 50µl/well of 40µg/ml Ova in ELISA coating buffer pH 9.6 or 10µg/ml DNP-ficoll in 0.9% (w/v) NaCl and sealed in parafilm before incubation in a moist box at 37°C. Plates were then washed three times with PBS-T, and were blocked for 1h with 100µl of PBS-T + 4% dried skimmed milk. Plates were then washed and sera were serially diluted starting at a 1 in 50 dilution, and 50µl was added to duplicate wells and incubated at room temperature for 2h in a moist box. The plates were washed again, and 50µl/well of either peroxidase-conjugated affinipure goat anti-mouse IgM or peroxidase-conjugated goat anti-mouse IgG (subclasses 1+2a+2b+3) (Jackson ImmunoResearch, Inc.) diluted 1 in 500 in PBS-T were added to the plates and incubated for a further 2h at room temperature. The plates were washed again and 50µl/well of the substrate solution OPD was added. The reactions were stopped with 15µl/well of 3M H_2SO_4. Optical densities were then read at 490nm on a plate reader.

2.2.3f Characterisation of serum antibody responses to *S. pneumoniae* CPSs by ELISA.

Pneumococcal C polysaccharide or cell wall polysaccharide (CW-PS), which resides in the pneumococcal cell wall can be found in the purified pneumococcal CPSs. Sera were preabsorbed with CW-PS to remove antibodies to the cell wall polysaccharide when testing for antibodies to pneumococcal CPS antigens (Aaberge *et al.*, 1993). Mouse sera were absorbed with 100µg/ml CW-PS (Statens Serum Institute, Copenhagen, Denmark) for 1h at room temperature, at a 1 in 100 serum dilution in PBS-T, prior to further dilution in buffer without CW-PS.

Immunoplates were coated overnight with 50µl/well of CPS at 10µg/ml in 0.9% (w/v) NaCl and sealed in parafilm before incubation in a moist box overnight at 37°C. Plates were then washed three times, and in some cases plates were blocked for 1h with 100µl of PBS-T + 4% milk. The CW-PS absorbed sera were serially diluted starting at 1 in 50, and 50µl was added to duplicate wells and incubated at room temperature for 2h in a moist box. The plates were washed again, and 50µl/well of either peroxidase-conjugated affinipure goat anti-mouse IgM or peroxidase-conjugated goat anti-mouse IgG (subclasses 1+2a+2b+3) (Jackson ImmunoResearch, Inc.) diluted 1 in 500 in PBS-T were added to the plates and incubated for a further 2h at room temperature. The plates were washed again and 50µl/well of substrate solution OPD was added. The reactions were stopped with 15µl/well of 3M H_2SO_4. Optical densities were then read at 490nm on a plate reader.

2.2.4 In vitro cell assays

Mice

Female or male C3H/HeN (H-2k) and C3H/HeJ [LPS hyporesponsive (H-2k)] mice were obtained from Harlan UK Limited (Blackthorn, UK). The animals were kept under specific pathogen free conditions and the mice were used between 6-12 weeks of age.

2.2.4a Cell proliferation assay using ^{3}H-thymidine

Spleens from naïve mice were removed aseptically and placed into RPMI. Using the barrel of a 5ml syringe, a single cell suspension was produced by pushing the spleen through a cell strainer (40µm) into a 50ml Falcon tube. The cells were then centrifuged (Sorvall RT7 plus) for 5 mins at 1,000rpm at 4°C. After centrifugation, the supernatant was poured off and the red blood cells were lysed with red blood cell lysis buffer. Approximately 40ml complete RPMI [c. RPMI (RPMI 1640 with 1% penicillin/streptomycin and 10% FCS)] was added to the cells and the cells were washed by centrifugation as above. The cells were then re-suspended in 5ml c.RPMI, and a cell count was performed. Cells were then re-suspended to a density of 2 x 10^6 cells/ml and 100µl of the cell suspension was added to wells of a sterile 96 U-bottomed well plate. The cells were stimulated with 100µl of the test samples diluted in c.RPMI and added in triplicate. Phorbol myristate acetate (PMA) at 5µg/ml and ionomycin (200ng/ml) were used as a positive control, whereas medium alone was used as a negative control of cell proliferation. The cells were then incubated at 37°C, 5% CO_2 for 48h. Tritiated thymidine (1µCi in 20µl c.RPMI) (Amersham Pharmacia Biotech)

was then added to each well and incubated overnight. The plates were harvested using a TOMTEC 96-well harvester. The filters were dried and sealed in a plastic bag with Betaplate scintillant. Incorporated thymidine was measured using a MicroBeta TRILUX counter (Wallac, Turku, Finland).

2.2.4b CFSE labelling of mouse splenocytes

Spleens were aseptically removed from mice and placed in PBS as previously described. The cells were prepared as described in the proliferation assay and re-suspended at $1x10^7$ cells/ml in PBS + 0.01% BSA. A 5mM stock solution of carboxyfluorescein diacetate succinimidyl ester (CFSE) in DMSO was added to the cells at a final concentration of 5µM and incubated at 37°C for 5 mins. After incubation, the cells were washed three times with cold PBS + 5% FCS. The cells were then re-suspended in c.RPMI and counted.

2.2.4c Negative isolation of mouse B cells

B cell proliferation was measured using the Dynal® Mouse B cell Negative Isolation Kit (Dynal Biotech) and CFSE labelling as described above. The assay was carried out according to the manufacturer's instructions (Dynal Biotech). Briefly, spleens from naïve mice were aseptically removed and placed in c.RPMI. Using the barrel of a 5ml syringe, a single cell suspension was produced by pushing the spleen through a cell strainer (40µm) into a 50ml Falcon tube. The cells were then placed in cold c.RPMI and centrifuged for 10 mins at 1,000rpm at 4°C. After centrifugation, the

supernatant was poured off and the red blood cells were lysed with red blood cell lysis buffer. Approximately 40ml RPMI with 1% penicillin/streptomycin and 1% FCS was added to the cells and the cells were washed by centrifugation as above. The supernatant was again poured off and the cells were re-suspended to a density of 1 x 10^8 cells/ml in PBS/0.1% BSA. To the desired number of cells (1 x 10^7 per 100µl), 20µl of heat inactivated FCS and 20µl of antibody mix was added and incubated for 20 mins at 4^0C. The cells were then washed in PBS/0.1% BSA and centrifuged for 8 mins at 1,000rpm at 4^0C. After centrifugation the cells were re-suspended in PBS/0.1% BSA and pre-washed Mouse Depletion Dynabeads were added and incubated for 15 mins at room temperature with gentle tilting and rotation. The bead-captured cells were then gently re-suspended by pipetting and PBS/0.1% BSA was added to the cells. The tube was then placed in a magnet for 2 mins and the supernatant containing the negatively isolated B cells was transferred to a new tube.

2.2.4d Bone marrow derived macrophages

Mice were culled by increasing concentrations of carbon dioxide and hind limbs were removed into sterile PBS. Excess fat and tissue were removed after which the ends of the bones were cut off. Bone marrow was isolated by flushing the bone with c.RPMI using a 5ml syringe (Terumo) and 25G needle (Kendall). The bone marrow was then transferred to a 50ml Falcon tube and a single cell suspension was made by pipetting. The cell suspension was left to stand for 5 mins to allow debris to settle and the supernatant was harvested and centrifuged for 5 mins at 1,500rpm. The pellet was then re-

suspended in 10ml c.RPMI with 10ng/ml rmM-CSF (R&D Systems) and a cell count performed. Aliquots of 1-3 x 10^7 cells were placed into 75cm^3 tissue culture flasks and incubated at 37°C and 5% CO_2 for 16h. Following incubation, the non-adherent cells were transferred into two 75cm^3 flasks and 15ml c.RPMI with 10ng/ml rmM-CSF added. The flasks were incubated for two weeks, with a further 10ml c.RPMI with 10ng/ml rM-CSF added every three days until the cells were harvested.

2.2.4e Bone marrow derived dendritic cells

Hind limbs were prepared as mentioned in section 2.2.4c. The red blood cells were then lysed with red blood cell lysis buffer for approximately 4 mins, after which 40ml c.RPMI was added. The cells were again centrifuged, and the pellet was re-suspended in 10ml c.RPMI. A cell count was performed and 2 x 10^6 cells/10ml were put into Petri dishes (non culture treated, Falcon) in the presence of 20ng/ml rmGM-CSF (R&D Systems) and incubated at 37°C, 5% CO_2. On day three, 10ml of 20ng/ml rmGM-CSF in c.RPMI was added to each dish. On day six, 10ml of medium was taken from each dish, spun down and re-suspended in fresh medium containing 10ng/ml rmGM-CSF. The cells were then incubated for a further two days, and then the cells were harvested. The non adherent cells were collected by pipetting the medium from the plate, and the adherent cells were collected by adding 2ml Accutase per dish and incubating for 15 mins at 37°C, then being collected using a 1ml pipette.

2.2.4f Cytokine release assay

Mouse splenocytes were prepared and stimulated following the same protocol for thymidine proliferation assays. Samples were tested in duplicate and supernatant was harvested after 18h, 24h and 48h. Supernatants were stored at -18°C until used for cytokine analysis.

2.2.4g Griess Assay

Nitric oxide (NO) production was measured using the Griess Reagent System (Promega), which measures nitrite, a breakdown product of NO. The assay was carried out according to the manufacturer's instructions. Briefly, a nitrite standard reference curve was produced using the nitrite solution provided. The samples were added to the plate in duplicate, and sulfanilamide solution was added to both the standards and the samples and incubated in the dark, at room temperature for 5 mins. After this time N-1-napthylethylenediamine dihydrochloride (NED) was added to the plate and incubated for a further 5 mins. The optical density was then measured within 30 mins at 520nm on a plate reader.

2.2.4h Cytokine Cytometric Bead Array (CBA) assay

Cytokines present in the supernatants from stimulated mouse splenocytes, BMDCs, and lung fragment cultures were measured using the mouse inflammation and Th1/Th2 cytokine bead array (CBA) kits (BD Biosciences, Pharmingen). The mouse inflammation CBA kit was used to detect IL-12p70, TNF-α, IFN-γ, MCP-1, IL-10 and IL-6, and the mouse Th1/Th2 kit to measure

IL-2, IL-4, IL-5, IFN-γ and TNF-α. The assay was carried out according to manufacturer's instructions. Samples were examined by flow cytometry using a FACScalibur flow cytometry. Data was analysed using BD CBA software (BD Biosciences, Pharmingen).

2.2.5 Allergen-induced eosinophilia model

Ova mouse model

Female C57Bl/6 mice aged 6-8 weeks were given two-weekly i.p. injections of 0.1mg Ova (Sigma) in 500µl PBS. Seven days after the last i.p. injection, sensitised mice were anaesthetised by administering 150µl of xylazine/ketamine mixture i.p. (90mg/ml ketamine and 20mg/ml xylazine in PBS, diluted 1:10 in PBS before use) and immunised i.n. with 50µl of either PBS, 0.1mg OVA, 5µg NOMV or 0.1mg OVA + 5µg NOMV. After a further 7 days, mice were culled by administration of a fatal dose of avertin, and bronchoalveolar lavage (BAL) fluid, lungs and sera were harvested. A further i.n. challenge of 0.1mg OVA was given to some mice 7 days after the last i.n. immunisation. These mice were then culled 7 days after the challenge, as described above. The BAL was collected as briefly described. After the administration of avertin, the thoracic cavity was opened by careful dissection. The trachea was then exposed, and a small transverse incision was made. BAL was then carried out using three doses of 0.8ml PBS + 0.1% BSA, and the samples were stored individually on ice until processing. The cells were then centrifuged at 1000rpm for 5 mins, and the supernatant was removed. The cells were then counted and re-suspended to a density of 1×10^6 cells/ml. BAL eosinophils and other inflammatory cells were

enumerated by differential counts following cytospin preparations and Wright-Geimsa staining. The percentage cell type was calculated by determining the number of different cell types in fifteen high-powered fields and dividing this number by the total number of cells. To obtain the absolute number of each cell type in the BAL fluid, the percentages were multiplied by the total number of cells recovered from the BAL fluid. The lungs were stored in PBS and frozen immediately on dry ice until needed for measurement of cytokines. Serum was prepared as previously described. See figure 2.3 for immunisation schedule.

Respiratory syncytial virus mouse model

Female C57Bl/6 mice aged 6-8 weeks were given an intramuscular (i.m.) injection of 50µl β-propiolactone-inactivated RSV (iRSV). Control mice were given PBS only. Two weeks later, mice were anaesthetised as described above and immunised i.n. with 50µl of iRSV with or without 5µg NOMVs once a week for either one or three weeks. After a further 2 weeks, mice were intranasally challenged with live RSV. After a further 7 days, the mice were culled and samples were harvested as above. See figure 2.4 for immunisation schedule

2.2.6 Statistics

Data were analysed using software packages Microsoft Excel 2002 and Minitab 13 for windows. Statistical analysis was performed according to directions given by the University of Reading Statistical Services Centre. Statistical significance between two independent groups was performed

using a 2-test t-test whereas multiple groups were analysed using a General Linear Model ANOVA.

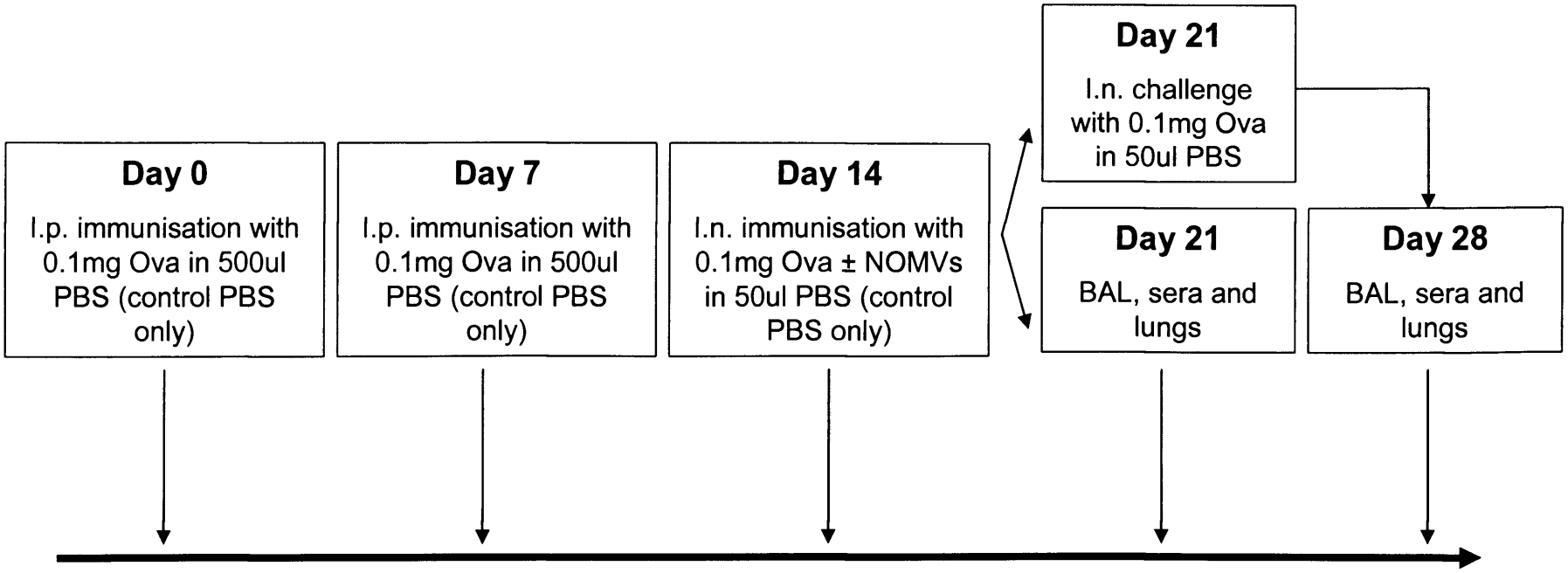

Figure 2.3 Schedule for immunisation of mice for Ova-induced eosinophilia model and BAL fluid, lung and serum collection of mice

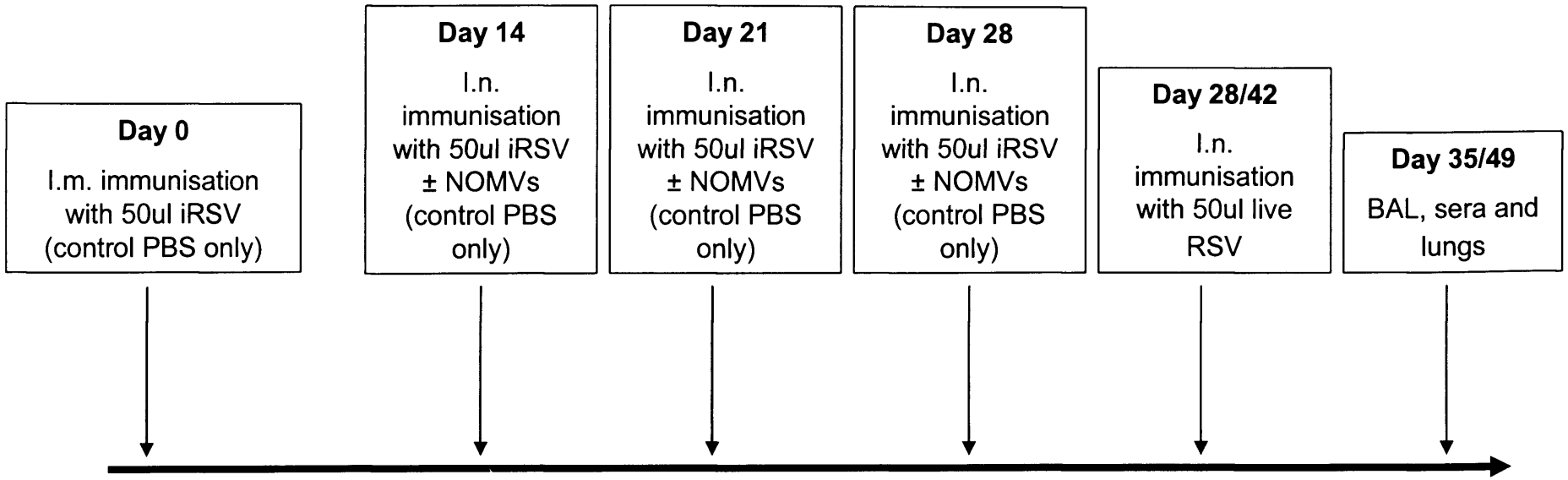

Figure 2.4 Schedule for immunisation of mice for iRSV-induced eosinophilia model and BAL fluid, lung and serum collection of mice

CHAPTER 3

The adjuvant properties of natural outer membrane vesicles from *N. meningitidis* serogroup B

3.1 Introduction

There is considerable interest in identifying adjuvant candidates suitable for human vaccines because of the generally poor immunogenicity of purified subunit vaccines and of the lack of adjuvants approved for human use. Alum has until recently been the only one adjuvant used with human vaccines due its safety record and its proven ability to enhance humoral responses to protein antigens and glycoconjugates. However there is a dire need for adjuvants that can enhance T cell responses and ones that can be applied via mucosal routes. We are interested in the adjuvant properties of NOMVs not only because it is a potential mucosal adjuvant candidate for human vaccines but also because its role in the establishment of natural immunity to meningococcal infection is not clear.

It has been established that NOMVs possess adjuvant properties, and modified forms of NOMVs have been applied as an adjuvant in approved and experimental vaccines. One of the first clinical uses of meningococcal outer membrane components was in the vaccine for *Haemophilus influenzae* type b (Hib). The outer membrane protein complex (OMPC) of Nm serogroup B was

used to enhance antibody responses to the CPS, polyribosylphosphate (PRP). OMPC was covalently attached to PRP providing a classical protein carrier effect in eliciting T cell help to the polysaccharide-specific B cell response. Although the reason for the OMPC adjuvant effect was not investigated in detail, it was shown that OMPC has mitogenic activity (Donnelly *et al.*, 1990; Liu *et al.*, 1992). It was later demonstrated that OMPC enhanced IgG antibody production to PRP in Balb/c and C3H/HeJ mice as well as the expression of costimulatory molecules, CD86, CD80 and CD40 on purified human B cells. The production of IFN-γ and TNF-α by human blood mononuclear cells after stimulation of OMPC was also established (Perez-Melgosa *et al.*, 2001). Therefore OMPC enhances anti-PRP antibody responses by stimulating innate cells as well as lymphocytes. It has recently been shown that Hib-OMPC conjugate vaccines binds human TLR2 expressed on human embryonic kidney (HEK cells) and mouse TLR2 on BMDCs. Binding was shown to be MyD88 dependent as the production of TNF-α was abolished in MyD88 knockout mice (Latz *et al.*, 2004).

A more recent application of meningococcal components is the use of very small size proteoliposomes (VSSPs) to enhance immune responses toward tumours. VSSPs are different from NOMVs in the preparation method that includes the incorporation of gangliosides, sialylated glycosphingolipids, from the plasma membrane of mammalian cells (Estevez *et al.*, 1999). Despite the high content of LPS, the safety and immunogenicity of VSSPs have been shown in phase I trials in melanoma and cancer patients (Carr *et al.*, 2003). VSSPs have been

shown to induce strong antibody responses to a model protein antigen (Ova), and to increase the expression of costimulatory molecules on murine BMDCs and human moDC grown in vitro. Upregulation of maturation markers were also found on DCs from C3H/HeJ mice indicating that VSSPs are composed of a variety of PAMPs, which can bind TLR4 as well as other TLRs or non-TLR receptors. Since the start of this thesis investigation, T cells have also been shown to proliferate and to secrete IFN-γ upon stimulation with VSSPs in the presence of Ova (Mesa *et al.*, 2004). From these studies, it is clear that modified NOMVs have strong B and T cell stimulating activities. However, it is uncertain whether VSSPs and NOMVs have identical adjuvant properties.

Since NOMVs are a natural product produced by shedding of membrane 'blebs' from meningococci during colonisation of the human nasopharynx, a few studies have investigated its use as a mucosal adjuvant. Meningococcal OMPs of serogroup B induced strong antibody responses toward LPS of *Brucella melitensis* in a mouse and guinea pig i.n. immunisation model. OMPs were effective in enhancing secretory IgA and IgG responses in the lungs, and high serum IgG levels of predominantly IgG1 subclass (Van De Verg *et al.*, 1996). More recent studies investigated the use of meningococci and NOMVs as a mucosal adjuvant to boost immune responses to the influenza virus. Results showed that after four weekly i.n. doses of virus plus whole killed meningococci, serum IgG and IgA and salivary IgA responses to the virus were significantly increased in comparison to immunisation with the virus alone (Berstad *et al.*,

2000). Furthermore, a recent study showed that replication of influenza virus in the nasal cavity could be prevented by i.n. immunisation of mice with NOMVs and inactivated influenza virus (Bizanov *et al.*, 2005). The specificity of the antibody response was not determined, but it is clear from these studies that OMPs or NOMVs have effective mucosal adjuvants for bacterial and viral antigens.

With respect to meningococcal disease, i.n. administered NOMVs have been shown to be immunogenic and induce strong systemic bactericidal antibody responses that are similar to those after i.p. immunisation as well as inducing IgA responses in the lung (Saunders *et al.*, 1999). A further study by Dalseg *et al.*, (1999) also demonstrated enhanced serum IgG levels and NOMV-specific IgA in saliva in a dose-dependent manner after four weekly immunisations of NOMVs via the mucosal route in the absence of the mucosal adjuvant cholera toxin. More importantly, despite the presence of LPS, NOMVs appear to be safe and immunogenic when immunised i.n. in humans (Drabick, 2000; Katial *et al.*, 2002). These studies demonstrated that after three i.n. doses of NOMVs low titres of long lasting bactericidal antibodies, cross-reactive against other group B strains were induced. Recently the same group demonstrated that rabbits immunised i.n. with NOMVs developed substantial levels of serum bactericidal antibodies to a wide range of antigens. They also observed mucosal responses characterised by IgA in saliva, nasal washes and lung lavages, indicating that

the rabbit model could be useful for testing NOMV vaccines (Shoemaker *et al.*, 2005).

It has been suggested that i.n. immunisation of NOMVs could provide an effective way of inducing both mucosal and systemic immunity and studies in mice and humans as described above support this theory. The importance of antibody responses to CPSs in protection against serious human infections with extracellular bacteria prompted us to investigate the effect of i.n. administered NOMVs on humoral responses toward TI antigens. CPSs from *Streptococcus pneumoniae* were selected for our studies for the following reasons: (1) *S. pneumoniae* is responsible for the morbidity and mortality of millions of people each year as it is a major cause of pneumonia, meningitis and bacteremia, affecting the young, the elderly and immunocompromised individuals; (2) like Nm it colonises the nasopharynx, and prevention of pneumococcal disease requires systemic and mucosal immune responses; and (3) the major virulence factor is the CPS, and anti-CPS antibodies correlate with protection.

CPSs from *S. pneumoniae* have been characterised extensively. They are made up of units of repeating oligosaccharides (AlonsoDeVelasco *et al.*, 1995). Differences in the polysaccharide structure of CPSs account for the 90 different pneumococcal serotypes, however most invasive disease is associated with 11 serotypes. Pneumococcal CPSs are classified as TI antigens due to their ability to elicit antibody responses in nude mice (Mosier *et al.*, 1982; van de Wijgert *et al.*, 1991). They are further classified as type 2 TI antigens (TI-2) due to their

inability to induce antibody responses in CBA/N mice (Amsbaugh *et al.*, 1972). TI-2 antigens are poorly metabolised, their responses are age dependent and characterised by limited isotype switching, low affinity antibodies and the inability to generate memory (Mosier *et al.*, 1982). A 23-valent pneumococcal CPS vaccine is currently licensed. It is effective in most adults, however it is poorly immunogenic in children under 18 months of age and therefore does not provide protection against infection. To combat the problem, TD protein carriers were conjugated to the CPSs, resulting in the recently licensed heptavalent pneumococcal conjugate vaccine (Butler *et al.*, 1995; Rennels *et al.*, 1998). These have proved successful as shown by a decline in the rate of invasive pneumococcal disease in children due to serotypes included in the conjugate vaccine. There are nevertheless concerns that other serotypes will become more prevalent and of the difficulties of manufacturing conjugate vaccines that provide coverage for all disease-causing serotypes. Conjugate vaccines are also unaffordable to populations in the developing world where the burden is highest. Adjuvants that are able to improve anti-CPS antibody responses would provide further options against *S. pneumoniae* and other encapsulated bacterial pathogens.

3.2 Objectives

The aim of the work described in this chapter was to investigate the adjuvanticity of NOMVs by using model TD and TI antigens including four variably

immunogenic CPSs from *S. pneumoniae,* which are clinically relevant TI-2 antigens.

3.3 Results

3.3.1 The humoral response to NOMVs in mice immunised i.p. and i.n.

To investigate whether NOMVs could act as an adjuvant to different antigens, initial experiments were carried out to assess the serum IgM, IgG and IgA antibody response to NOMVs in C57Bl/6 and Balb/c mice. Previous studies have shown that NOMVs immunised both i.p. and i.n. generate NOMV-specific antibodies but it is not known whether or not NOMVs act as an adjuvant for different types of antigens. For all experiments, unless stated otherwise, mice were immunised with 5μg NOMVs regardless of immunisation route. Mice immunised by the i.p. route were bled 21 days post immunisation and serum was collected. Mice immunised by the i.n. route were immunised once a week for three consecutive weeks and serum was collected seven days after the final immunisation. The antibody response to NOMVs was assessed by ELISA as described in section 2.2.4d.

3.3.1.1 Serum antibody responses to NOMVs in C57Bl/6 and Balb/c mice after i.p. immunisation

Figure 3.1 shows the serum IgM, total IgG and IgG subclass response to NOMV antigens in C57Bl/6 mice immunised i.p. with NOMVs or PBS. IgM levels were

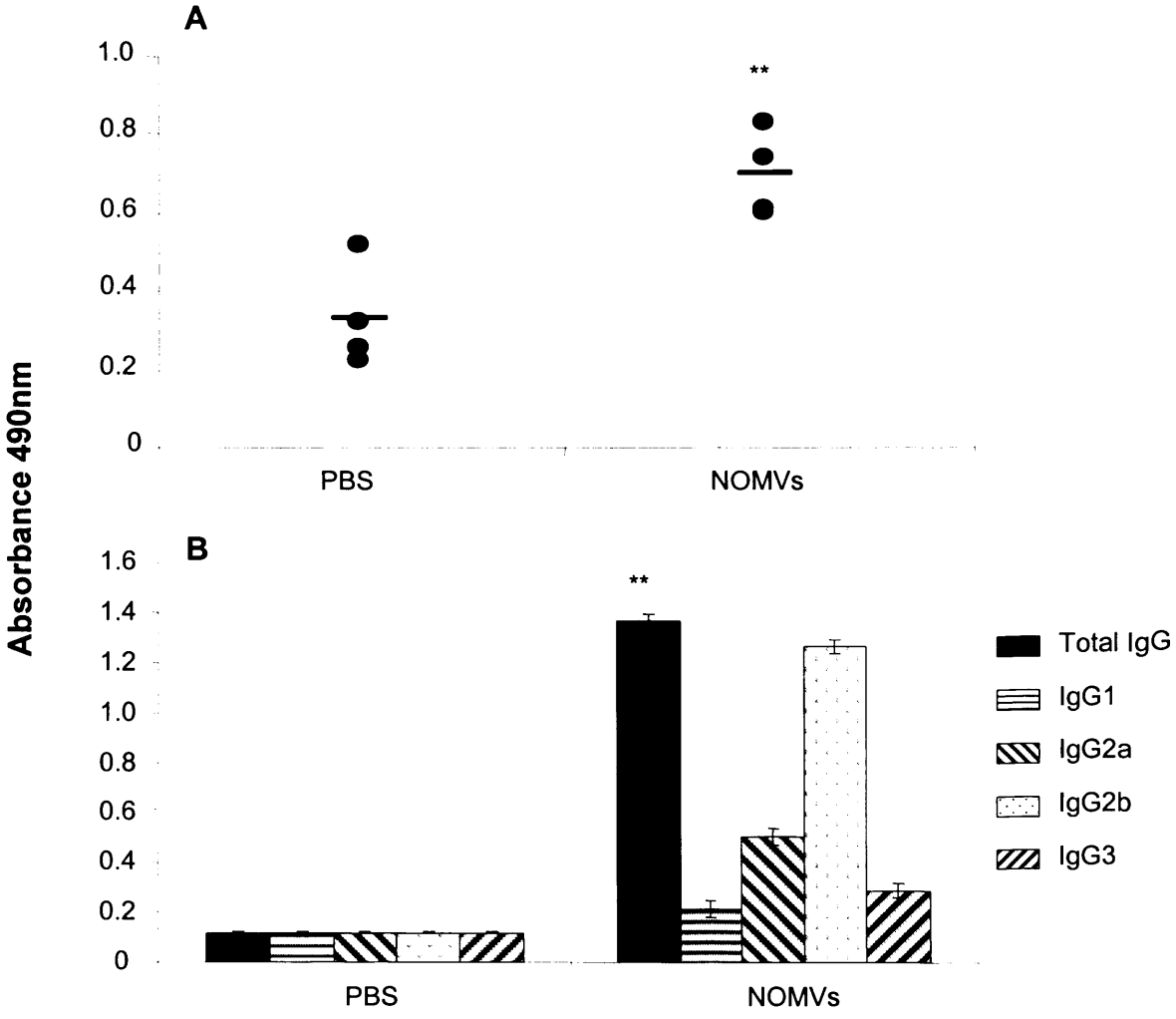

Figure 3.1 A comparison of serum antibody responses to NOMVs in C57Bl/6 mice after i.p. immunisation.

Groups of four C57Bl/6 mice were immunised i.p. with PBS or 5µg of NOMVs. Mice were bled 21 days after immunisation, and serum was analysed for (A) IgM and (B) total IgG and IgG subclasses by ELISA. IgM was detected in serum of individual mice (1 in 400 dilution) with goat anti-mouse IgM HRP conjugate and IgG was detected (1 in 1,600 dilution) with goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 HRP conjugates. OPD was used as a substrate. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one representative study out of two. Significant differences are indicated as ** $p < 0.015$.

significantly higher in serum from NOMV immunised mice than in PBS control mice. This was also true for the total IgG response. Mice immunised with NOMVs predominantly produced the IgG subclass IgG2b and lower levels of IgG2a.

Comparable experiments were also carried out in Balb/c mice (figure 3.2) and results were similar to those in C57Bl/6 mice. However IgG2b levels were approximately half the level of those in C57Bl/6 mice, but still significantly higher than in PBS immunised mice.

3.3.1.2 Serum antibody responses to NOMVs in C57Bl/6 and Balb/c mice after i.n. immunisation

The NOMV-specific antibody responses after i.n immunisation were then assessed. Figure 3.3A shows that C57Bl/6 mice immunised i.n. with NOMVs have significantly higher levels of IgM than those of mice immunised with PBS alone. Similar results were also found in Balb/c mice (figure 3.4A). The levels of total IgG (figure 3.3B and 3.4B) were consistently and significantly higher in both C57Bl/6 and Balb/c mice that received NOMVs compared to control mice. As with the mice immunised i.p., IgG2b was the subclass predominantly found after i.n. immunisation with NOMVs. Absorbance levels for IgG2b were lower in Balb/c mice than C57Bl/6, indicating lower amounts of NOMV-specific IgG2b.

As fairly high levels of NOMV-specific IgG were observed, serum IgA responses were also investigated. Figure 3.3C shows that sera from NOMV immunised C57Bl/6 mice exhibited fairly high levels of IgA and approximately two fold less

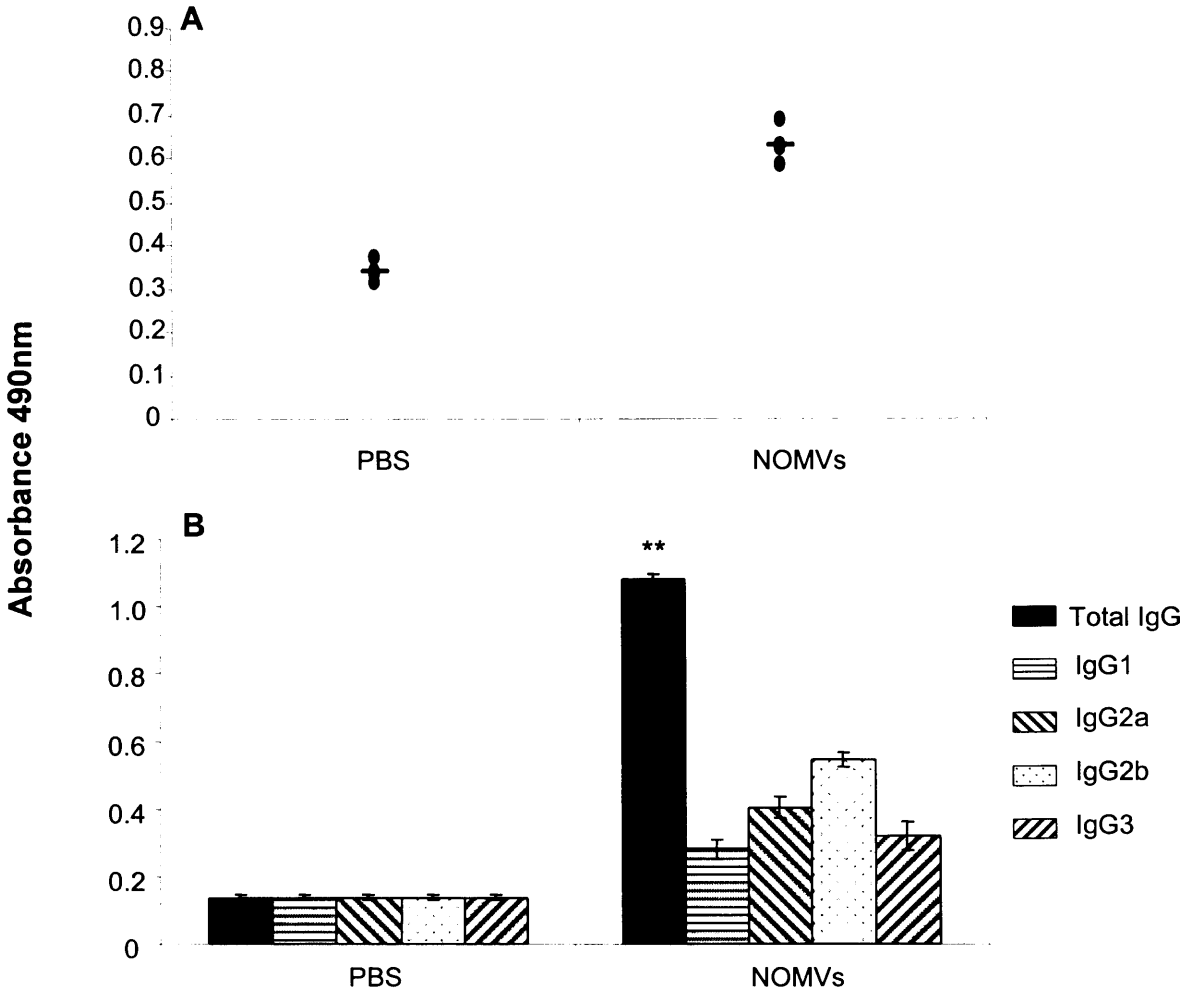

Figure 3.2 A comparison of serum antibody responses to NOMVs in Balb/c mice after i.p. immunisation.

Groups of four Balb/c mice were immunised i.p. with PBS or 5μg of NOMVs. Mice were bled 21 days after immunisation, and serum was analysed for (A) IgM and (B) total IgG and IgG subclasses by ELISA. IgM was detected in serum of individual mice (1 in 400 dilution) with goat anti-mouse IgM HRP conjugate and IgG was detected (1 in 1,600 dilution) with goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 HRP conjugates. OPD was used as a substrate. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one representative study out of two. Significant differences are indicated as ** $p < 0.015$.

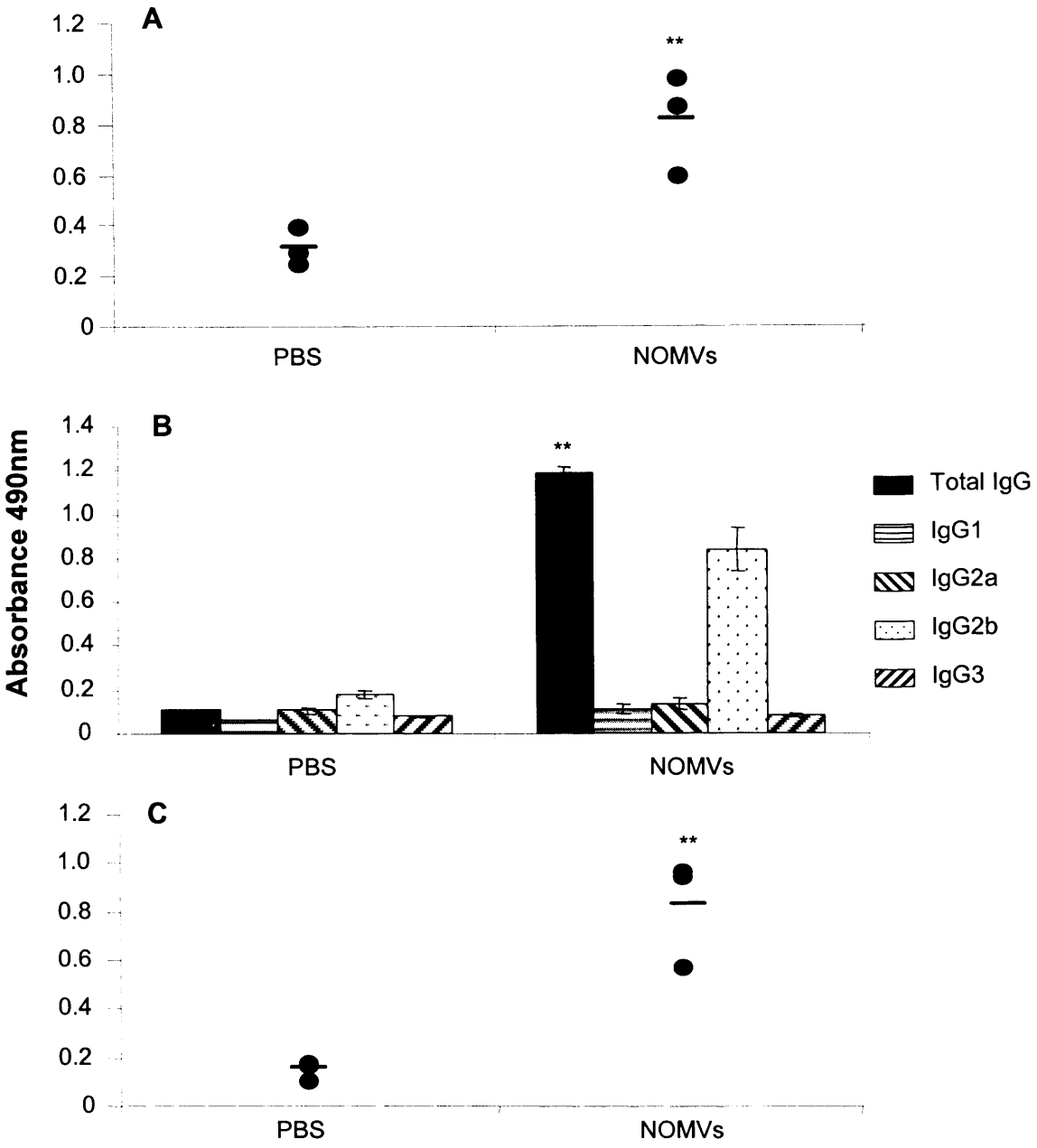

Figure 3.3 Serum antibody responses to NOMVs in C57Bl/6 mice after i.n. immunisation

Groups of three C57Bl/6 mice were immunised i.n. with 50µl of either PBS or 5µg NOMV once a week for three weeks. Mice were bled 7 days after the last immunisation, and serum was analysed for (A) IgM; (B) total IgG and IgG subclasses and (C) IgA by ELISA. IgM was detected in serum from individual mice (1 in 400 dilution) with goat anti-mouse IgM HRP conjugate. IgG was detected (1 in 3,200 dilution) with goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 HRP conjugates and IgA was detected (1 in 100 dilution) with goat anti-mouse IgA HRP conjugate. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one representative study out of two. Statistical differences are indicated as ** $p < 0.015$.

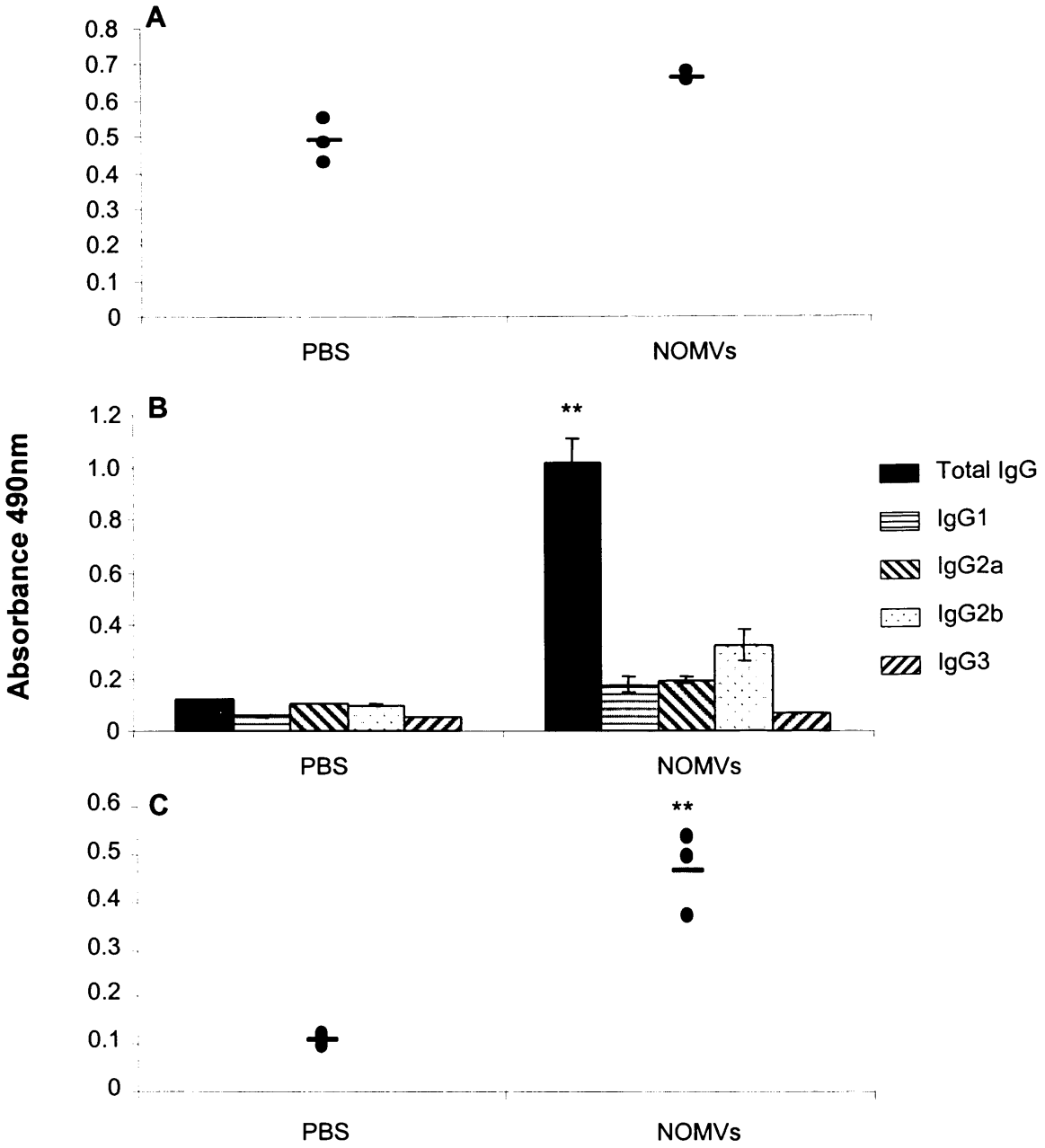

Figure 3.4 Serum antibody responses to NOMVs in Balb/c mice after i.n. immunisation

Groups of three Balb/c mice were immunised i.n. with 50µl of either PBS or 5µg NOMV once a week for three weeks. Mice were bled 7 days after the last immunisation, and serum was analysed for (A) IgM; (B) total IgG and IgG subclasses and (C) IgA by ELISA. IgM was detected in serum from individual mice (1 in 400 dilution) with goat anti-mouse IgM HRP conjugate. IgG was detected (1 in 3,200 dilution) with goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 HRP conjugates and IgA was detected (1 in 100 dilution) with goat anti-mouse IgA HRP conjugate. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one representative study out of two. Statistical differences are indicated as ** $p < 0.015$.

was found in Balb/c mice (figure 3.4C), compared to control mice, in which IgA responses were undetectable.

3.3.1.3 Comparison of serum antibody responses to NOMVs in different strains of mice

As shown in figures 3.1 and 3.2, after i.p. immunisation there was no difference in the antibody isotype response to NOMVs in C57Bl/6 and Balb/c mice. The only difference was in the magnitude of the IgG and specifically the IgG2b response, which was greater in C57Bl/6 mice than Balb/c mice. This was also true for the IgG response after i.n. immunisation. IgA levels were also higher in C57Bl/6 mice than Balb/c mice, however the responses in both strains of mice were significantly increased compared to NOMV-specific IgA responses in PBS control mice.

3.3.2 The adjuvant effect of NOMVs on the humoral response to a TD antigen

To investigate the adjuvant effect of NOMVs, antibody responses to the model protein antigen Ova were examined after immunisation by the i.p. and i.n. route. For the following experiments, groups of C57Bl/6 mice were either immunised with PBS as a control or 0.5mg Ova in the presence or absence of 5μg NOMV. Mice immunised i.p. were bled 21 days post immunisation. Mice immunised i.n. received one 50μl dose once a week for three weeks, and were bled seven days

after the final immunisation. Antibody responses were measured by ELISA as described in section 2.2.3e.

Figure 3.5 shows the Ova-specific IgM, total IgG and IgG subclass response after i.p. immunisation. It was observed that there was no significant increase in the IgM response (figure 3.5A) in the sera of mice that received Ova plus NOMVs than those immunised with Ova alone. In comparison, a significant increase in Ova-specific IgG (figure 3.5B) was observed in mice immunised with Ova plus NOMVs compared to immunisation with the antigen alone. The Ova-specific IgG subclasses were also determined and IgG1 was predominantly found in sera from mice immunised with Ova alone and Ova plus NOMVs, however levels were higher in the mice immunised with adjuvanted Ova.

As shown in figure 3.6, IgM levels were not significantly different in sera from mice immunised i.n. with Ova alone or Ova plus NOMVs, however IgG levels were significantly increased in mice that received Ova plus NOMVs in comparison to mice immunised with Ova alone. Examination of Ova-specific IgG subclasses revealed that mice immunised with Ova alone produced predominantly low levels of IgG1. Whereas, mice that received Ova plus NOMVs produced a much higher level of IgG1 as seen after i.p. immunisation. Serum IgA antibodies to Ova were also investigated. Low but significant levels of Ova-specific IgA antibodies, indicated by low absorbance values, were found in mice immunised with Ova plus NOMVs compared to mice immunised with Ova alone.

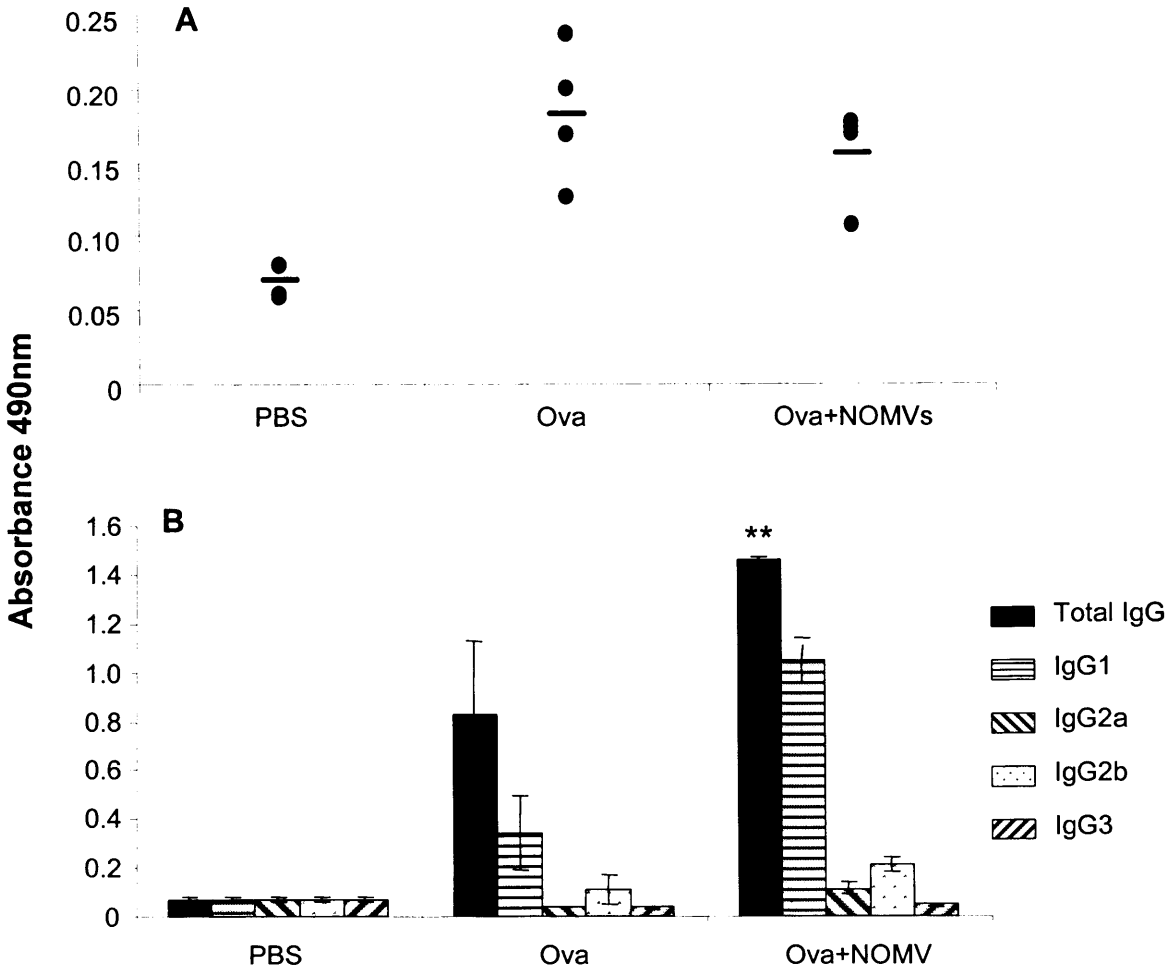

Figure 3.5 Serum antibody responses to Ova in C57Bl/6 mice after i.p. immunisation

Groups of four C57Bl/6 mice were immunised i.p. with 0.5mg of Ova with or without 5μg NOMV. Mice were bled 21 days post immunisation, and serum was analysed for (A) IgM and (B) total IgG and IgG subclasses by ELISA. IgM was detected in serum from individual mice (1 in 200 dilution) with goat anti-mouse IgM HRP conjugate and total IgG and IgG subclasses (1 in 100 dilution) with goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 HRP conjugates. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one representative study out of two. Significant differences are indicated as ** $p < 0.005$.

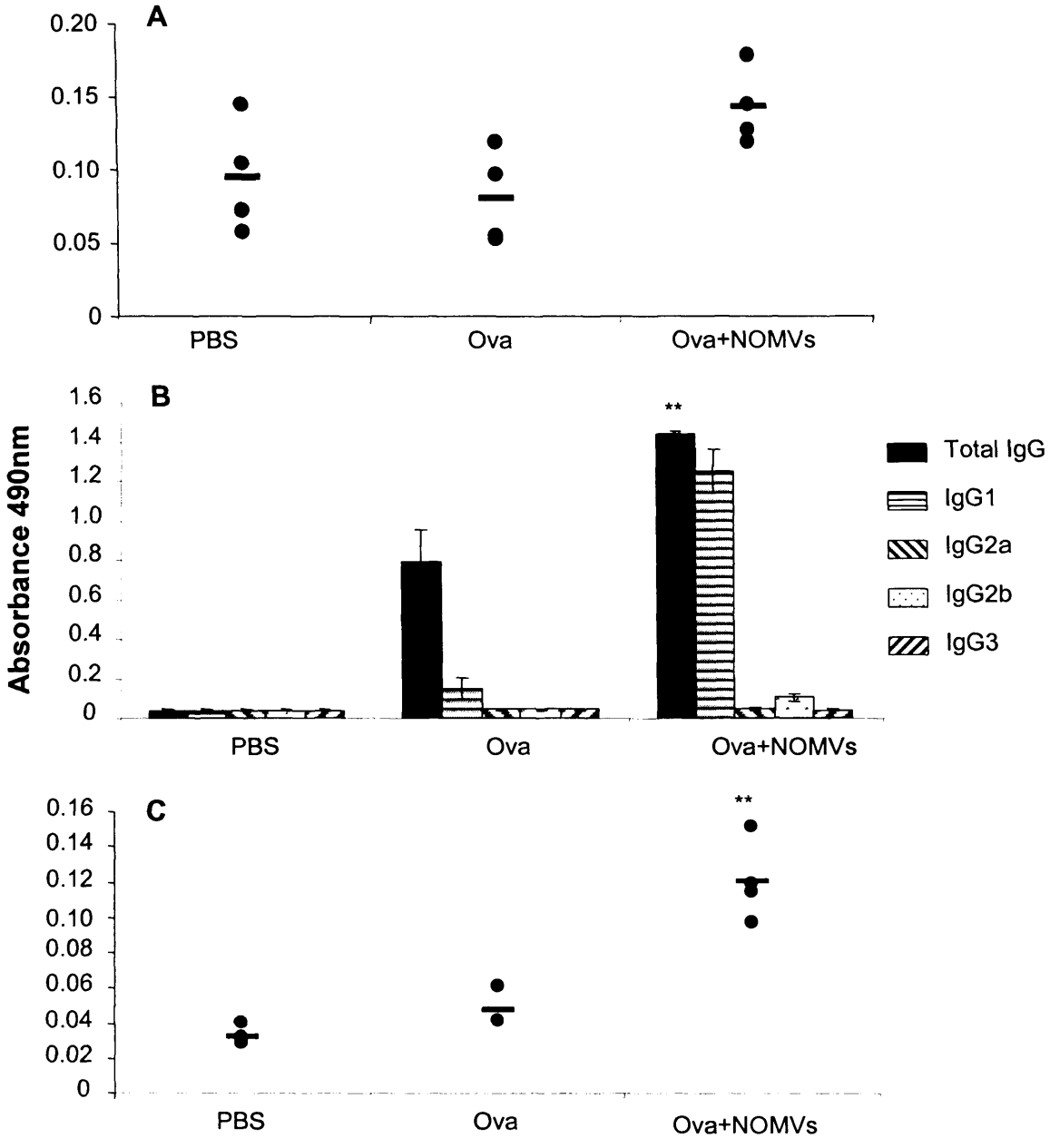

Figure 3.6 Serum antibody responses to Ova after i.n. immunisation in C57Bl/6 mice.

Groups of four C57Bl/6 mice were immunised i.n. with 0.5mg of Ova with or without 5µg NOMVs once a week for three weeks. Mice were bled 7 days after the last immunisation, and serum was analysed for (A) IgM; (B) total IgG and IgG subclasses and (C) IgA by ELISA. IgM was detected in serum from individual mice (1 in 200 dilution) with goat anti-mouse IgM HRP conjugate, IgG (1 in 12,800 dilution) with goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 HRP conjugates and IgA (1 in 100 dilution) with goat anti-mouse IgA HRP conjugate. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one representative study out of two. Significant differences are indicated as ** $p < 0.015$.

3.3.3 The adjuvant effect of NOMVs on the humoral response to a model TI-2 antigen

As improved IgG responses to Ova were seen after immunisation with Ova plus NOMVs, the adjuvant potential of NOMVs to a model TI-2 antigen, DNP-ficoll were examined after immunisation by the i.p. and i.n. route.

3.3.3.1 The adjuvant effect of NOMVs on DNP-ficoll after i.p. and i.n. immunisation

Serum DNP-ficoll-specific IgM, total IgG and IgG subclass responses in mice immunised i.p. with DNP-ficoll in the presence or absence of NOMVs are shown in figure 3.7A and 3.7B, respectively. Mice immunised with DNP-ficoll plus NOMVs did not show a significant increase in absorbance therefore no significant increase in IgM compared to mice immunised with DNP-ficoll alone. In contrast a significant increase in the DNP-ficoll specific IgG response was seen in mice immunised with DNP-ficoll plus NOMVs. Analysis of IgG subclasses specific for DNP-ficoll after immunisation with the antigen alone revealed the production of IgG2b and IgG3. A marked increase in both these subclasses was observed when mice were immunised with DNP-ficoll plus NOMVs.

DNP-ficoll specific antibody responses were also determined in sera from i.n. immunised mice. Figure 3.8 shows the (A) IgM; (B) total IgG and IgG subclasses and (C) IgA responses to DNP-ficoll after immunisation with DNP-

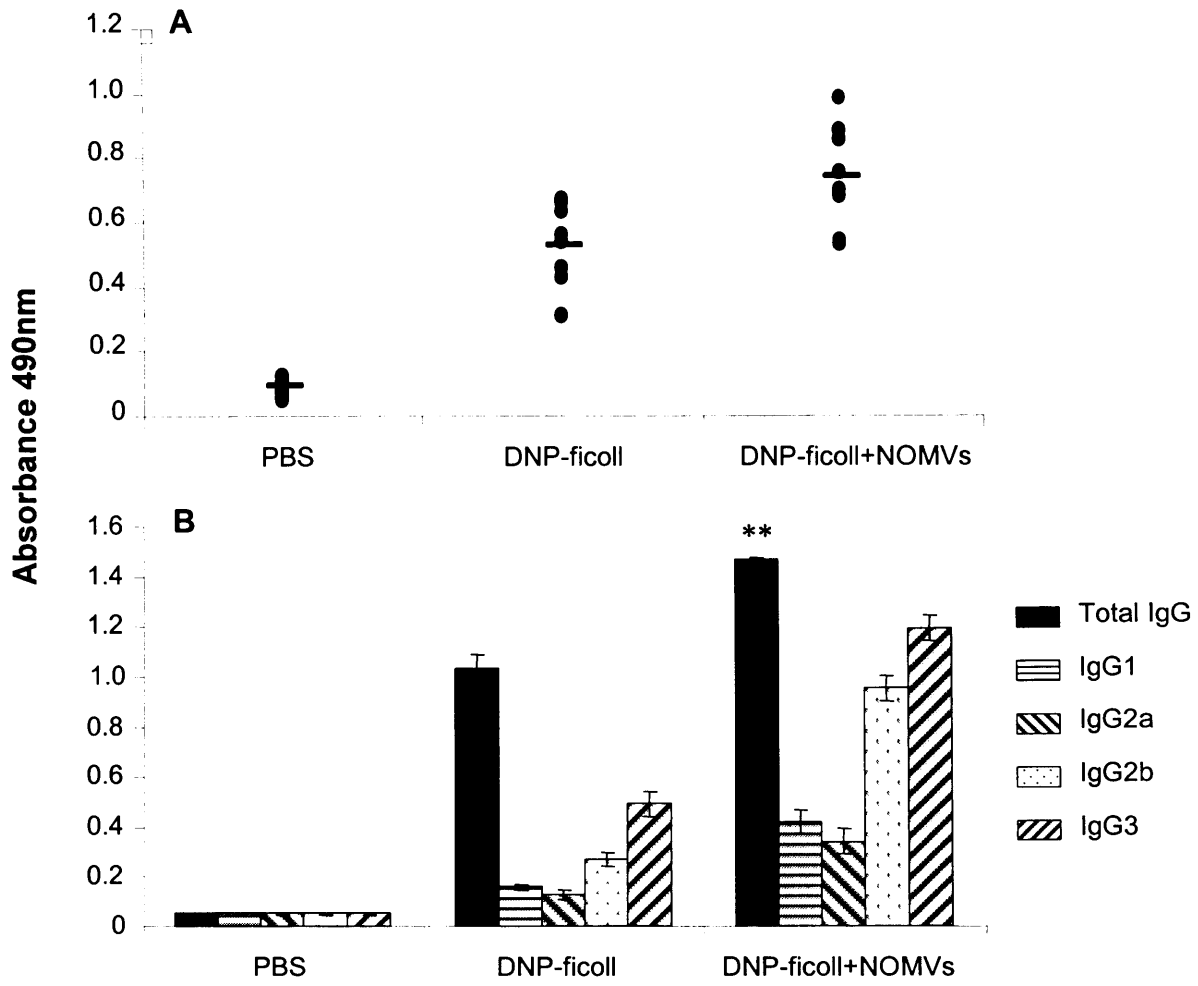

Figure 3.7 A comparison of serum antibody responses to DNP-ficoll after i.p. immunisation in C57Bl/6 mice using NOMVs as adjuvant.

Groups of eight C57Bl/6 mice were immunised i.p. with 5μg of DNP-ficoll with or without 5μg NOMVs. Mice were bled 21 days post immunisation, and serum was analysed for (A) IgM and (B) total IgG and IgG subclasses by ELISA. IgM was detected in serum of individual mice (1 in 200 dilution) with goat anti-mouse IgM HRP conjugate and IgG and subclasses (1 in 100 dilution) with goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 HRP conjugates. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one representative study out of two. Significant differences are indicated as ** $p < 0.05$.

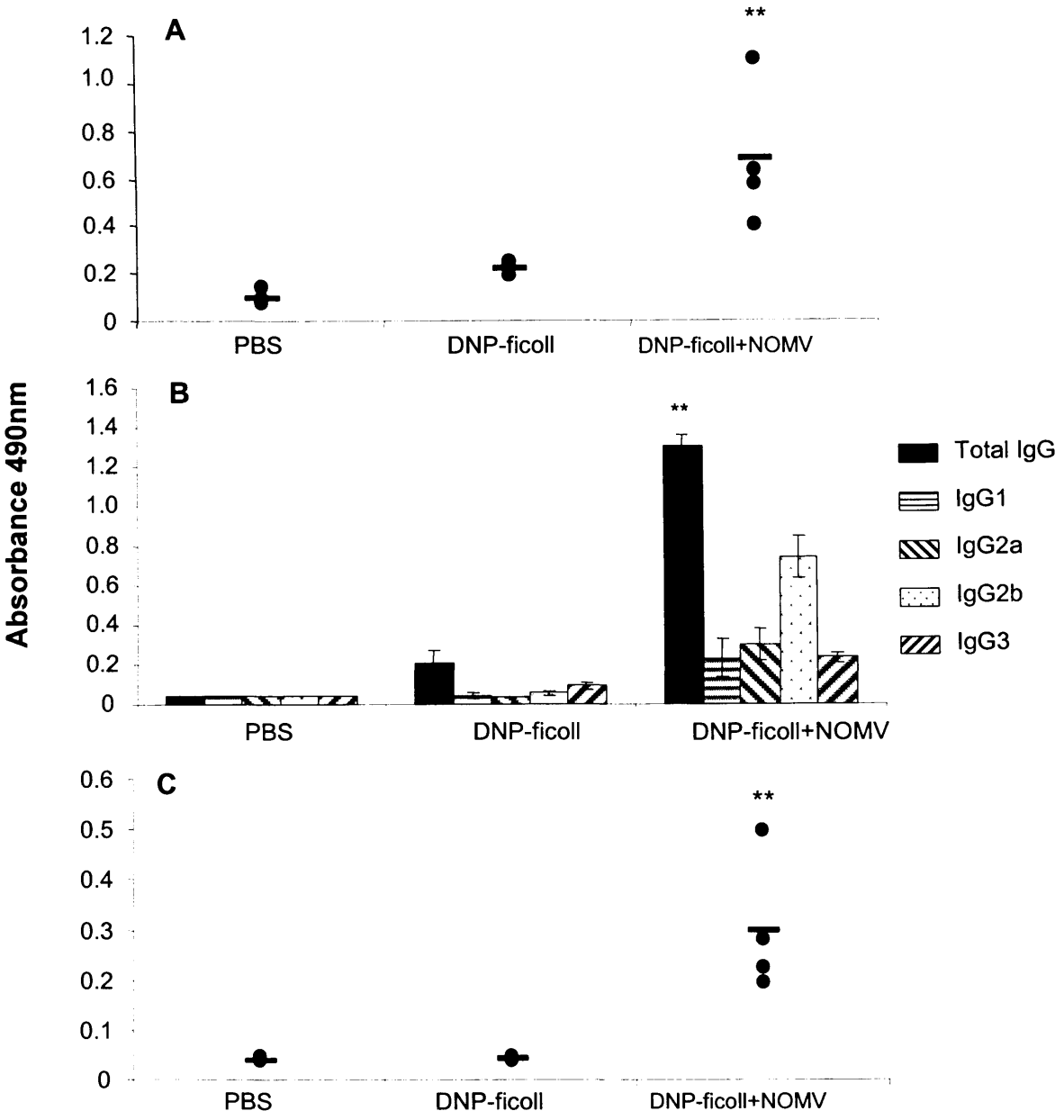

Figure 3.8 Serum antibody responses to DNP-ficoll after i.n. immunisation in C57Bl/6 mice using NOMVs as adjuvant.

Groups of four C57Bl/6 mice were immunised i.n. with 5μg of DNP-ficoll with or without 5μg NOMVs once a week for three weeks. Mice were bled 7 days after the last immunisation, and serum was analysed for (A) IgM; (B) total IgG and IgG subclasses and (C) IgA by ELISA. IgM was detected in individual mice (1 in 200 dilution) with goat anti-mouse IgM HRP conjugate, IgG (1 in 100 dilution) with goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 HRP conjugates and IgA (1 in 100 dilution) with goat anti-mouse IgA HRP conjugate. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one representative study out of two. Significant differences are indicated by ** $p < 0.015$

ficoll alone and DNP-ficoll plus NOMVs. Mice immunised with DNP-ficoll plus NOMV had significantly elevated IgM, total IgG and IgA antibody responses in comparison to mice immunised with DNP-ficoll alone. Examination of individual IgG subclasses revealed that sera from mice immunised with DNP-ficoll alone had primarily low levels of all IgG subclasses. In comparison, sera from mice immunised with DNP-ficoll plus NOMVs produced in the main IgG2b, with lower levels of IgG1, IgG2a and IgG3.

3.3.4 The adjuvant effect of NOMVs on the humoral responses toward clinically relevant TI antigens

The antibody responses toward CPSs from *S. pneumoniae* were investigated given that NOMVs were able to enhance antibody responses to DNP-ficoll, a model TI antigen, after i.p. and i.n. immunisation. For all experiments, unless stated otherwise, C57Bl/6 and Balb/c mice were immunised with either 5μg of type 3, 4, 14 or 19F CPS in the presence or absence of 5μg NOMVs. Previous work done in the laboratory has shown that 5μg of CPS to be adequate in eliciting an antibody response therefore this dose was administered (Hannah E Jones, PhD thesis). Mice immunised by the i.p. route were bled 21 days post immunisation and serum was collected. Mice immunised by the i.n. route received three 50μl doses at weekly intervals and were bled seven days after the final immunisation. The CPS-specific antibody responses were assessed by ELISA as described in section 2.2.3f.

3.3.4.1 The presence of natural IgM antibodies *S. pneumoniae* CPSs in serum of naïve mice

Serum from naïve mice has been shown to contain natural IgM antibodies to microbial carbohydrates. These antibodies are an important part of innate immunity and their presence may affect the antibody response towards CPSs therefore it is important to determine the levels of natural IgM to the CPSs used in this study (Goldblatt *et al.*, 1992). Analysis of sera from non-immunised C57Bl/6 and Balb/c mice showed that with the exception to type 4 CPS, the levels of natural IgM tended to be higher in Balb/c mice (figure 3.9). It is important to include data from PBS control mice due to the difference in levels of natural IgM specific for CPS serotypes in different strains of mice when investigating antibody responses to CPSs.

3.3.4.2 The adjuvant effect of NOMVs on the humoral response to *S. pneumoniae* CPSs in mice after i.p. and i.n. immunisation

To examine the adjuvant activity of NOMVs to clinically relevant TI antigens, the IgM and IgG antibody responses to *S. pneumoniae* CPSs type 3, 4, 14 and 19F were determined.

Figure 3.10 shows the CPS-specific IgM antibody response to type 3, 4, 14 and 19F CPSs after i.p. immunisation. In both C57Bl/6 and Balb/c mice only type 4 and type 19F CPSs elicited an IgM response. At day 21, for all CPSs tested and in both strains of mice, there was no significant increase in IgM levels after immunisation with the CPSs plus NOMVs compared to CPSs immunised alone.

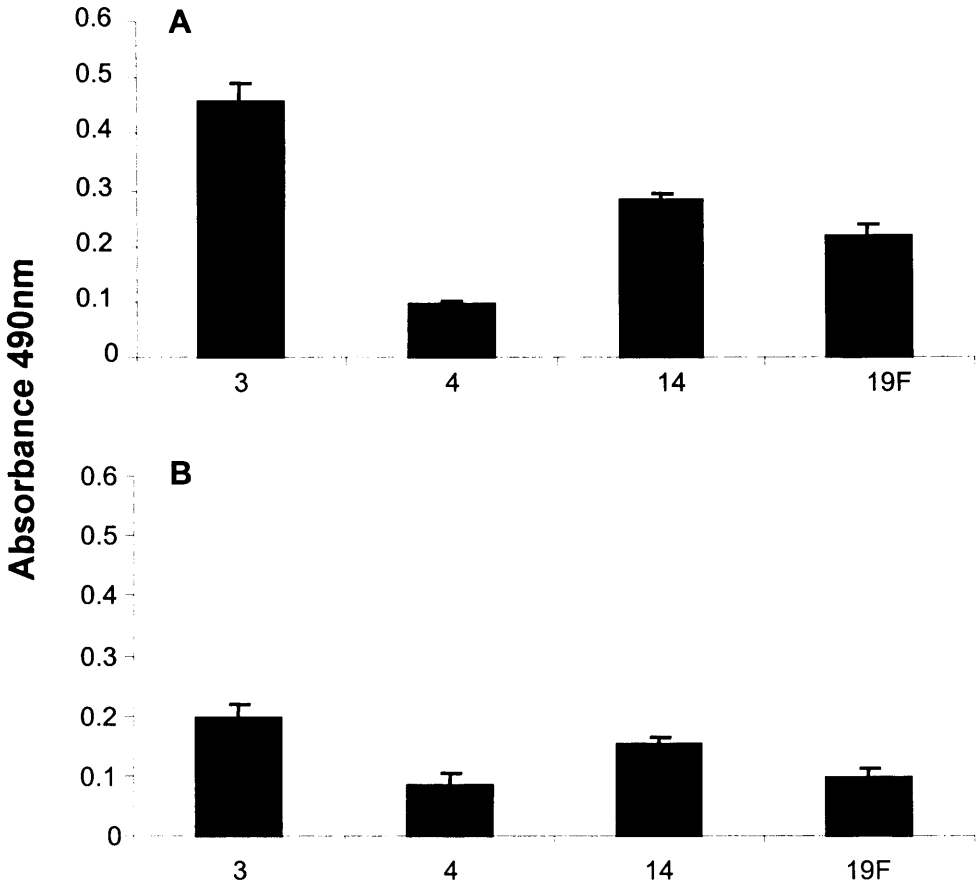

Figure 3.9 Serum IgM levels to *S. pneumoniae* in naïve C57Bl/6 and Balb/c mice.

Sera from naïve (A) Balb/c and (B) C57Bl/6 mice were analysed for IgM to four different CPSs by ELISA at a 1 in 400 dilution and preabsorbed with 100µg/ml of CW-PS. Goat anti-mouse IgM HRP conjugate was used to detect IgM and OPD was used as a substrate. Data represents the mean of eleven to fifteen mice ± s.e.m.

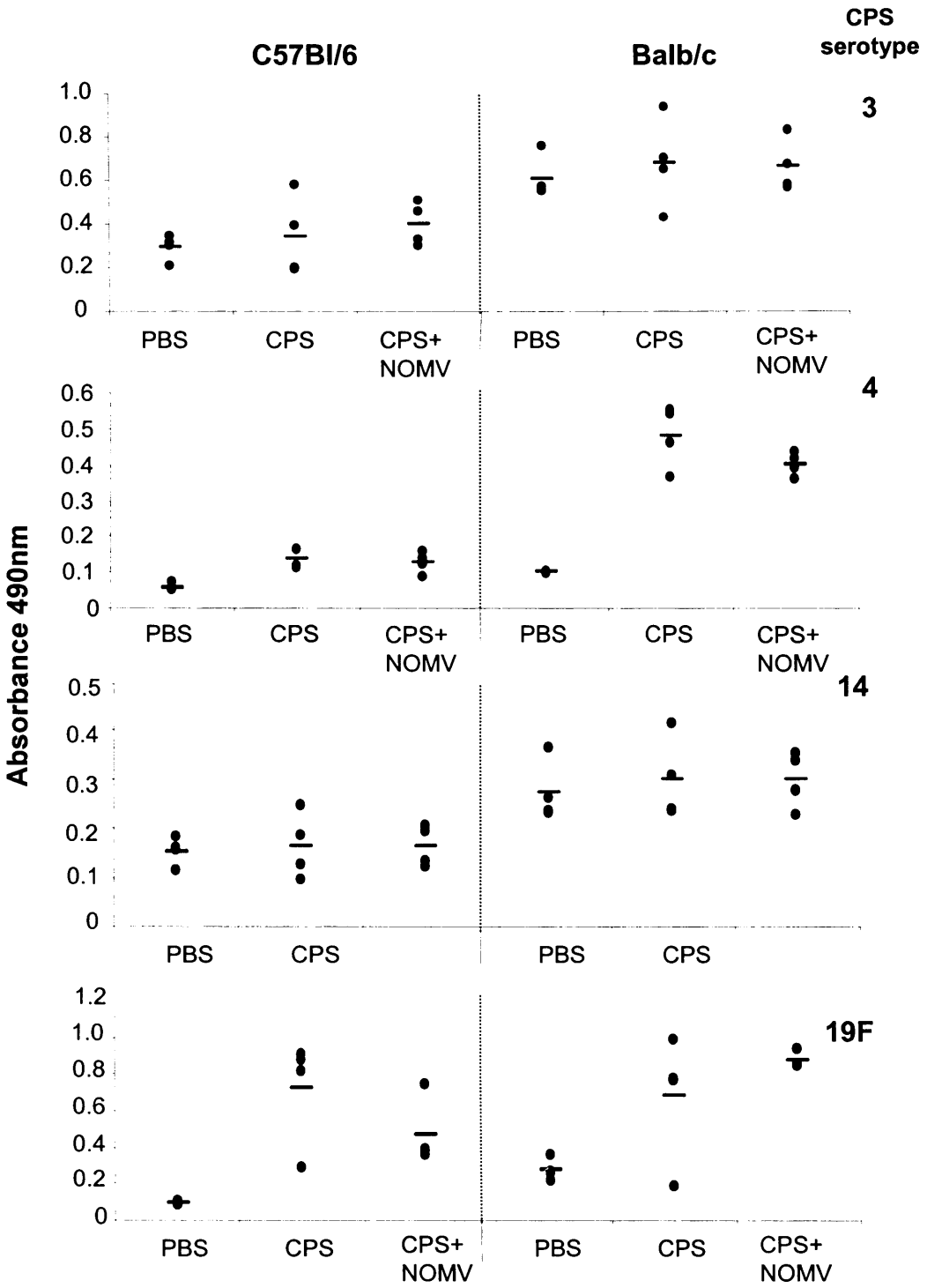

Figure 3.10 A comparison of serum IgM responses to *S. pneumoniae* CPSs after i.p. immunisation in C57Bl/6 and Balb/c mice using NOMVs as an adjuvant.

Groups of four C57Bl/6 (left panels) and Balb/c (right panels) mice were immunised i.p. with 5µg of CPS serotypes 3, 4, 14 19F with or without 5µg NOMVs (C+N or CPS respectively) or with PBS only. Mice were bled 21 days post immunisation and serum was analysed for IgM to the CPS by ELISA at a 1 in 400 dilution of serum. Goat anti-mouse IgM HRP conjugate was used to detect IgM and OPD was used as a substrate. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one representative study out of two.

It is evident from the data in this figure that absorbance values and therefore antibody responses to the CPSs are variable. CPS-specific IgG antibody responses were also investigated. Only IgG antibodies specific to type 3 and type 14 CPS were found in sera, and these were of isotypes IgG3 and IgG1 (data not shown).

Mice were also immunised i.n. with CPSs in the presence or absence of NOMVs. Figure 3.11 shows the IgM response to CPSs with NOMVs as adjuvant. Seven days after the final immunisation, the IgM response to the CPSs tested showed there was no improvement in the antibody response when mice were immunised with the CPSs plus NOMVs in comparison to CPSs alone. Not all the CPSs immunised i.n. elicited an IgM response that was greater than the natural IgM response to the CPS. In C57Bl/6 mice, only type 4 CPS gave a CPS-specific IgM response. In Balb/c mice, none of the CPSs produced a greater antibody response compared to the PBS control mice after i.n. immunisation.

3.3.4.3 Dose response to type 3 and type 4 CPSs immunised i.n.

Very few studies have investigated the humoral response to *S. pneumoniae* CPSs after i.n. immunisation in mice. As the CPS-specific IgM response in mice after i.n. immunisation was low or undetectable, mice were immunised with type 3 or type 4 CPS at a range of doses (5 - 20µg). As with the previous experiments groups of C57Bl/6 and Balb/c mice were immunised once a week

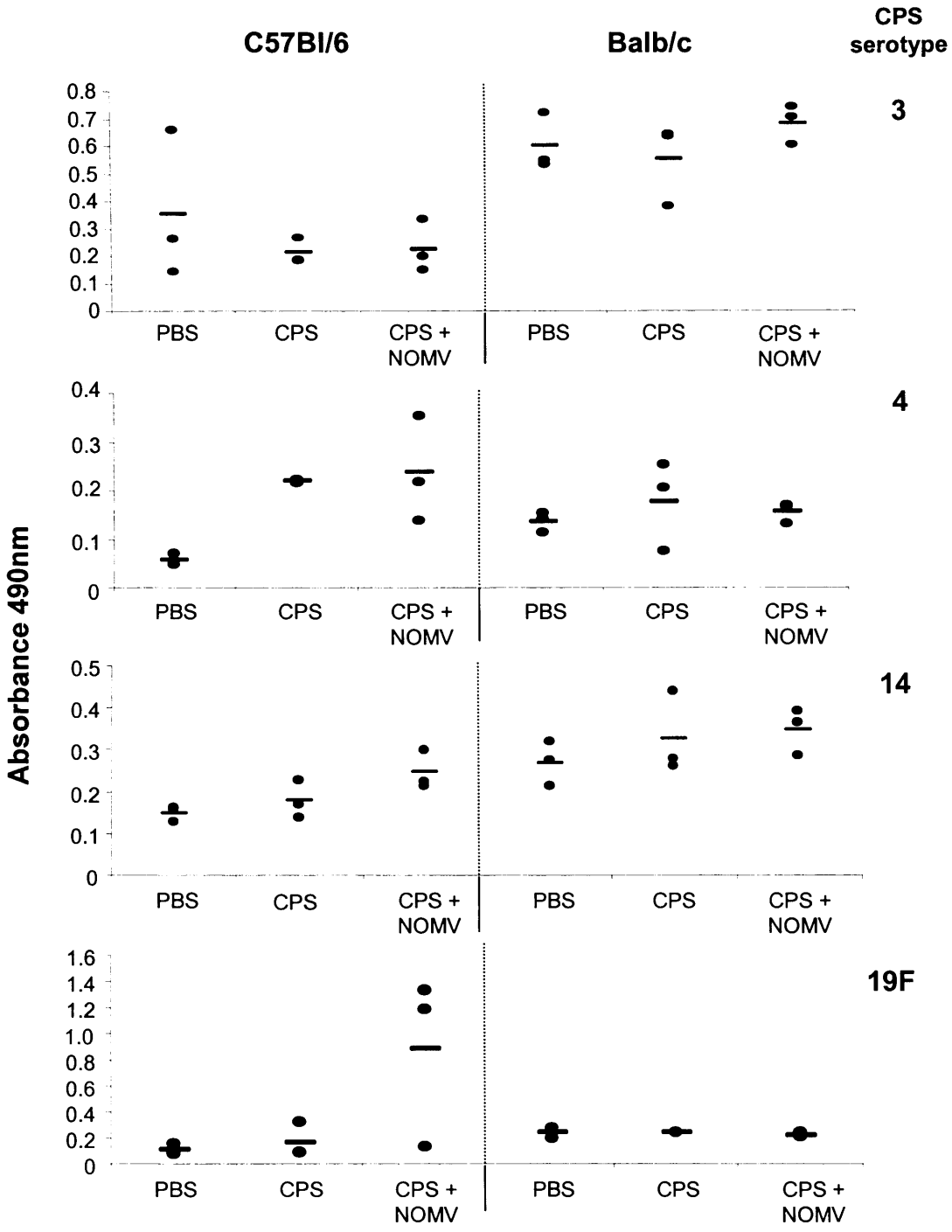

Figure 3.11 A comparison of serum IgM responses to *S. pneumoniae* CPSs after i.n. immunisation in C57Bl/6 and Balb/c mice using NOMVs as an adjuvant.

Groups of three C57Bl/6 (left panels) and Balb/c (right panels) mice were immunised i.n. with 50µl of 5µg type 3 (A); type 4 (B); type 14 (C) and type 19F (D) CPS with or without 5µg NOMVs or PBS alone, once a week for 3 weeks. Blood was taken 7 days after the last immunisation and serum IgM to the serotypes was analysed by ELISA. IgM was detected in individual mice at a 1 in 400 dilution of serum with goat anti-mouse IgM HRP conjugate. OPD was used as a substrate. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells.

for three weeks and blood was taken seven days after the last immunisation. Serum was then analysed for IgM and IgG antibodies by ELISA. Figure 3.12 shows the dose response to type 3 CPS. For each dose the absorbance was compared and any increase in absorbance reflected an increase in IgM levels in the serum. In both strains of mice, there was no increase in absorbance with an increase in dose of the CPS. This was also repeated for type 4 CPS (data not shown) and similar results were obtained. The CPS-specific IgG response was also examined, and the results are shown in figure 3.13. As shown in this figure, in both strains of mice, only a few mice in some of the groups produced IgG antibodies specific to the immunised CPS.

3.3.4.4 The effect of a booster immunisation and memory response to *S. pneumoniae* CPSs

The effect of a booster immunisation and the memory response to the CPSs was also investigated. In order to investigate the memory response to the immunised CPS, groups of mice were immunised i.p. as previously described (see section 3.5) and then again at day 62 but with the CPS alone. The mice were then bled five days later (see immunisation protocol 2.2). Figure 3.14 shows the serum IgM responses to the CPSs at day 21, day 60 and five days after the second immunisation. Sixty days after the primary immunisation with either the CPS alone or CPS plus NOMVs, IgM antibodies to all CPSs tested were at the same level, as indicated by similar absorbance levels as at day 21 showing that the antibodies are relatively long lived. After a second

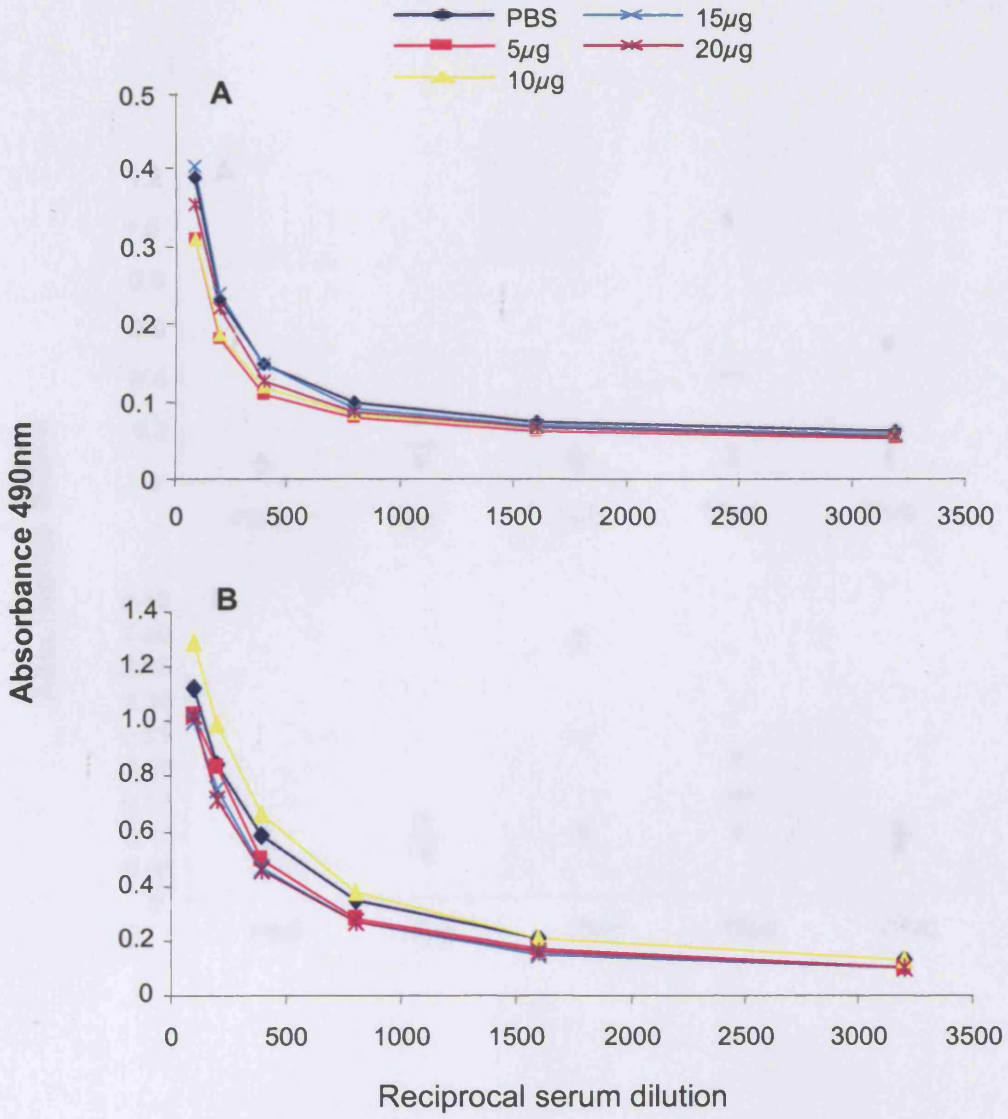

Figure 3.12 Comparison of serum IgM responses to *S. pneumoniae* type 3 CPS at different doses in C57Bl/6 and Balb/c mice.

Groups of four (A) C57Bl/6 and (B) Balb/c mice were immunised i.n. with 50μl of type 3 CPS at a range of doses (5 – 20μg in sterile PBS) or PBS alone, once a week for 3 weeks. Blood was taken 7 days after the last immunisation and analysed for type 3 specific IgM by goat anti-mouse IgM HRP conjugate and OPD as a substrate. These data are the means of duplicate wells.

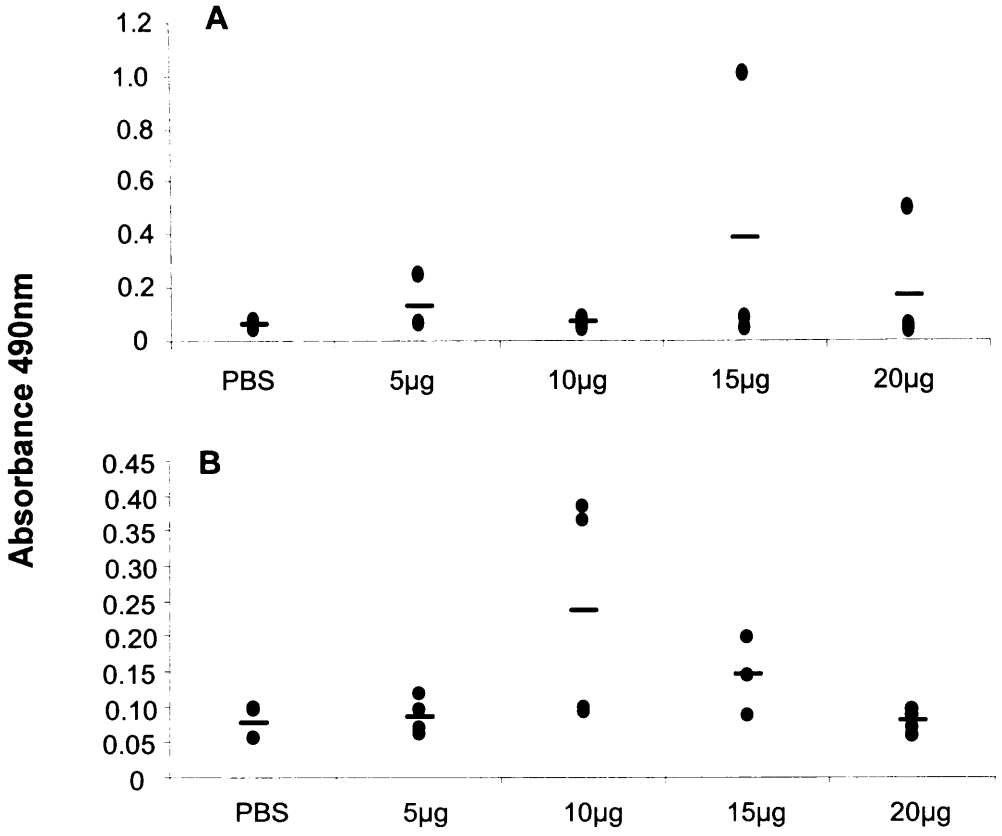

Figure 3.13 A comparison of serum IgG responses to *S. pneumoniae* type 3 CPS at different doses in C57Bl/6 and Balb/c mice after i.n. immunisation.

Groups of four (A) C57Bl/6 and (B) Balb/c mice were immunised i.n. with 50µl of type 3 CPS at a range of doses (5 - 20µg in sterile PBS) or PBS alone, once a week for three weeks. Blood was taken seven days after the last immunisation and serum IgG to serotype 3 was analysed by ELISA. IgG was detected using goat anti-mouse IgG HRP conjugate and OPD as a substrate. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells.

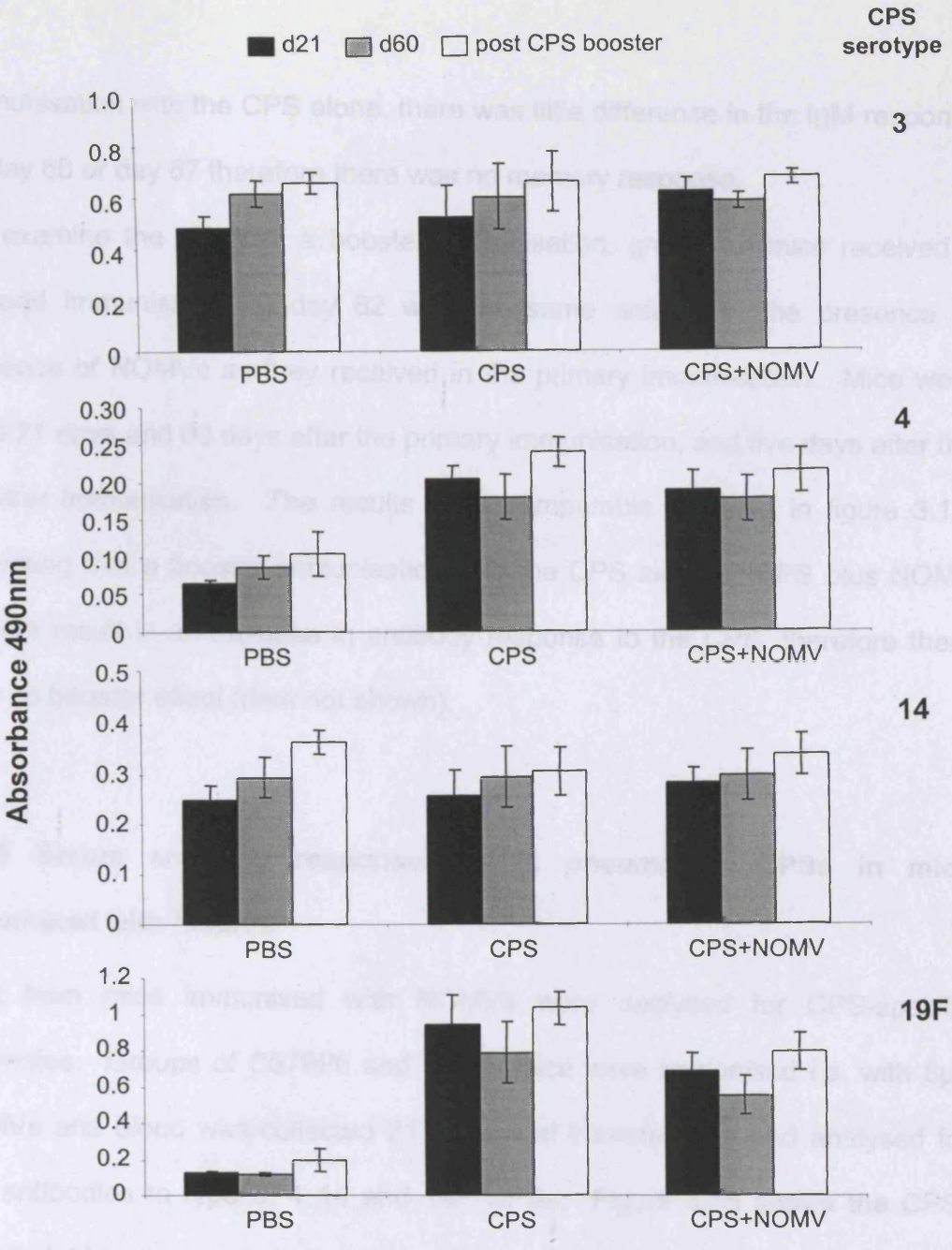

Figure 3.14 Serum IgM responses to *S. pneumoniae* CPSs in C57Bl/6 using NOMVs as an adjuvant.

Groups of four C57Bl/6 mice were immunised i.p. at day 0 with 5µg of CPS serotype 3, 4, 14 or 19F with or without 5µg NOMVs or with PBS and at day 61 with CPS or PBS only. Mice were bled at day 21 and 60 after the first immunisation and seven days after the second injection. Serum was analysed for IgM to the CPS by ELISA at a 1 in 200 dilution of serum. Goat anti-mouse IgM HRP conjugate was used to detect IgM and OPD was used as a substrate. Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one study.

immunisation with the CPS alone, there was little difference in the IgM response at day 60 or day 67 therefore there was no memory response.

To examine the effect of a booster immunisation, groups of mice received a second immunisation at day 62 with the same antigen in the presence or absence of NOMVs as they received in the primary immunisation. Mice were bled 21 days and 60 days after the primary immunisation, and five days after the booster immunisation. The results were comparable to those in figure 3.12, indicating that a booster immunisation with the CPS alone or CPS plus NOMV did not result in an increase in antibody response to the CPS, therefore there was no booster effect (data not shown).

3.3.5 Serum antibody responses to *S. pneumoniae* CPSs in mice immunised with NOMVs

Sera from mice immunised with NOMVs were analysed for CPS-specific antibodies. Groups of C57Bl/6 and Balb/c mice were immunised i.p. with 5µg NOMVs and blood was collected 21 days post immunisation and analysed for IgM antibodies to type 3, 4 14 and 19F CPSs. Figure 3.15 shows the CPS-specific IgM responses in both PBS control and NOMV immunised mice. In both strains of mice there was no significant difference in absorbance levels and therefore no difference in IgM levels between naïve and NOMV immunised mice to the CPSs.

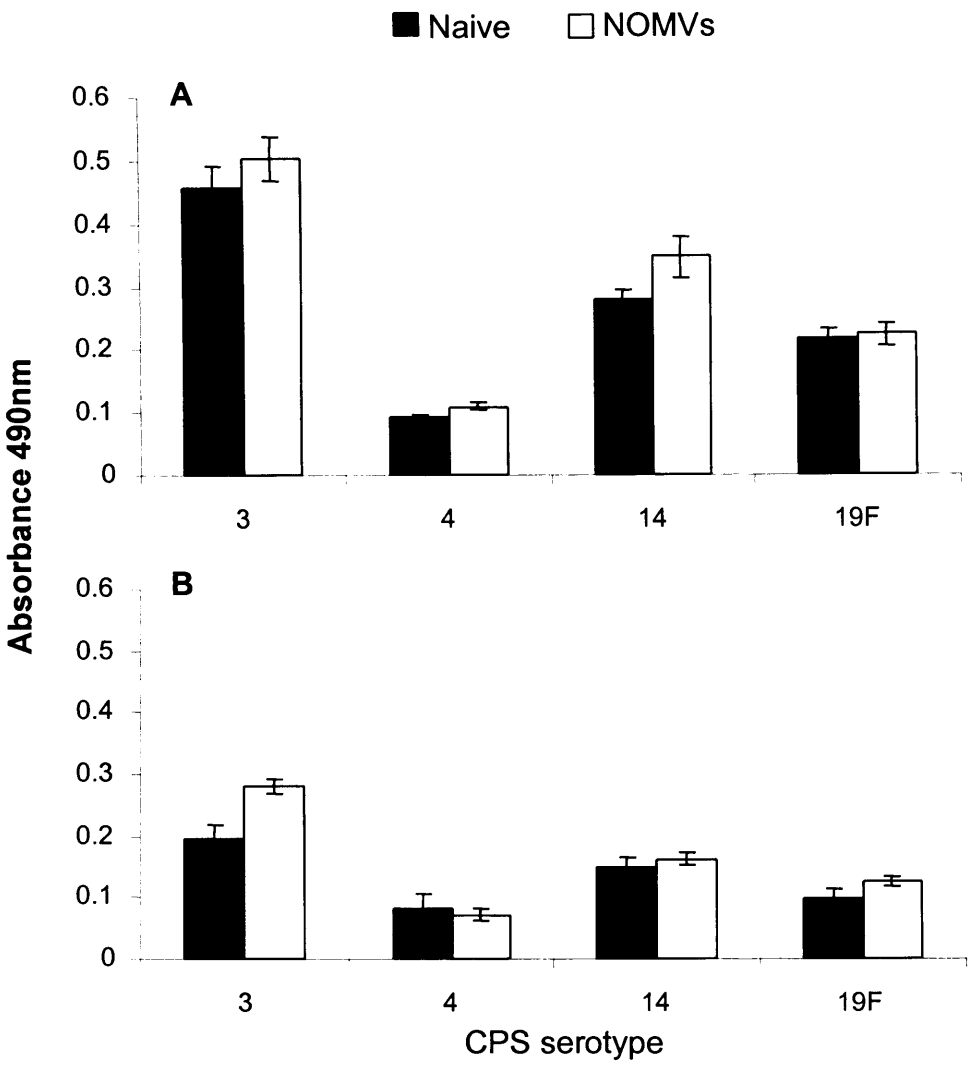

Figure 3.15 Serum IgM levels in NOMV immunised C57Bl/6 and Balb/c mice to *S. pneumoniae* CPSs.

Sera from (A) Balb/c and (B) C57Bl/6 mice immunised with 5µg NOMVs were analysed for IgM to four different CPSs by ELISA at a 1 in 400 dilution and preabsorbed with 100µg/ml of CW-PS. Goat anti-mouse IgM HRP conjugate was used to detect IgM and OPD was used as a substrate. Data represents the mean of four to eleven mice ± s.e.m.

3.3.5.1 Booster effect of NOMV immunisation

CPS-specific antibodies in the sera of NOMV immunised mice after a booster immunisation with NOMVs was also investigated. Briefly, mice were immunised on day 0 and then at day 62 with either PBS or 5μg NOMV. The mice were bled at day 21 and day 60, and five days after the booster immunisation. Figure 3.16 shows the CPS-specific IgM antibodies in sera from C57Bl/6 and Balb/c mice after 21 days and after the booster immunisation. In both strains of mice, and for all CPS serotypes examined, an increase in IgM levels was observed after a booster immunisation with NOMVs.

3.4 Discussion

Previous reports have demonstrated that NOMVs from serogroup B meningococci to be highly immunogenic and display adjuvant properties, therefore NOMVs in one form or other have been used in vaccines. The immunogenicity of NOMVs has been attributed to components such as LPS and porins. Neisserial porins can induce inflammation and activate B cells and DCs, which has been shown by upregulation of the costimulatory molecule B7-2 (CD86), increasing the involvement of T cells via CD28. The activation of B cells and DCs was shown to be dependent on TLR2 and MyD88 by using knockout mice (Mackinnon *et al.*, 1999; Massari *et al.*, 2002; Massari P, 2003; Singleton *et al.*, 2005). LPS has been shown to have a major effect on the immune system via TLR4 and therefore possesses adjuvant properties

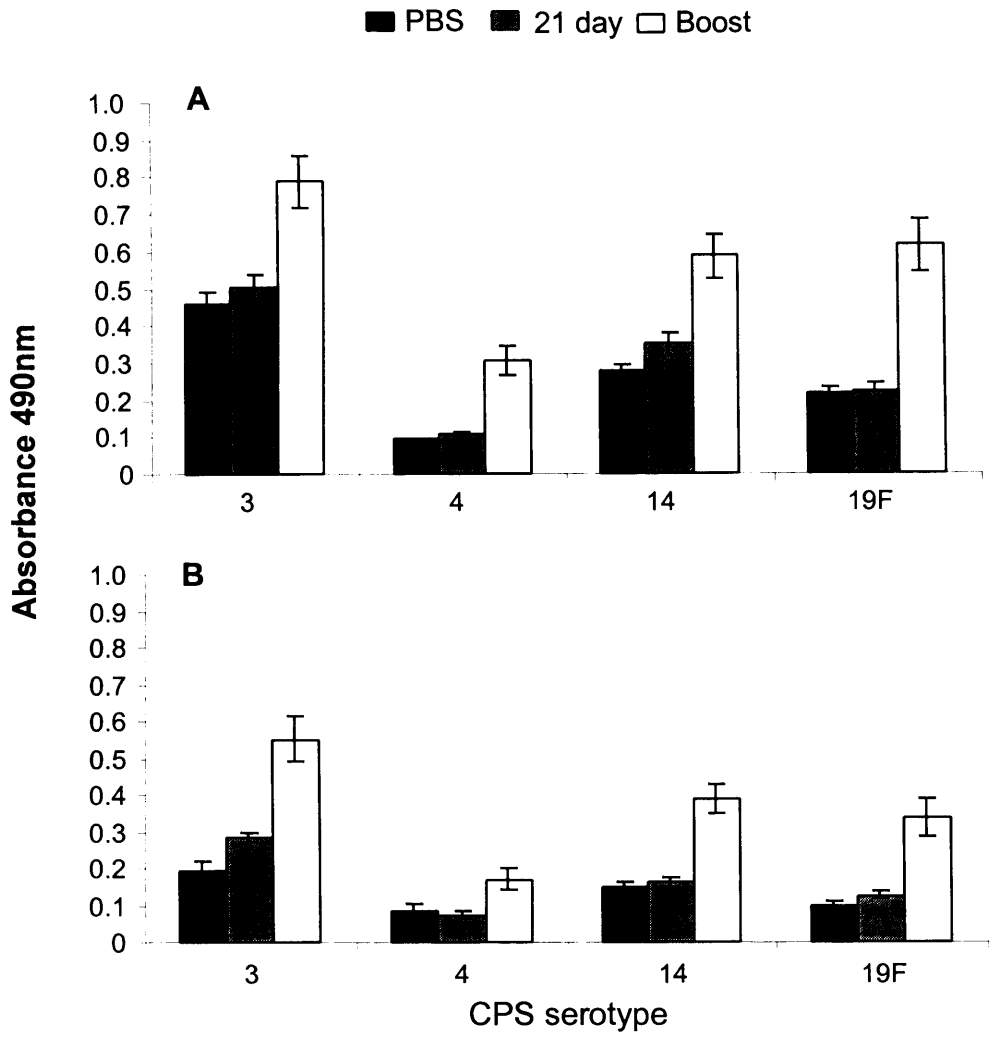

Figure 3.16 Serum IgM levels in NOMV immunised C57Bl/6 and Balb/c mice to *S. pneumoniae* CPSs before and after booster immunisation.

Groups of three or four (A) Balb/c and (B) C57Bl/6 mice were immunised with 5μg NOMV or PBS only. A booster was given at day 61 (same as the first immunisation). Mice were bled at day 21 and day 67 and serum was analysed by ELISA to four different CPSs at a 1 in 400 dilution. Goat anti-mouse IgM HRP conjugate was used to detect IgM and OPD was used as a substrate. Data represents the mean of eight to twelve mice ± s.e.m.

(Armerding *et al.*, 1974; Skidmore *et al.*, 1975). In this chapter, the adjuvanticity of NOMVs was further investigated using model and clinically relevant antigens.

Before assessing the adjuvanticity of NOMVs with model TD and TI antigens and CPSs, the primary antibody response to NOMVs was established in C57Bl/6 and Balb/c mice. By both immunisation routes NOMV-specific IgM and IgG levels were significantly higher in sera from NOMV immunised mice than in PBS control mice. Sera from NOMV-immunised mice contained high levels of IgG2b and low levels of IgG2a (figures 3.1 – 3.4), which are the primary antibodies associated with a Th1 response and that mediate complement fixation in mice. After i.n. immunisation, NOMVs also induced fairly high levels of serum IgA. These results are consistent with other studies in the literature including one from this laboratory investigating antibody responses to NOMVs after i.n. immunisation (Guthrie *et al.*, 2004).

With the primary antibody response to NOMVs determined, the adjuvanticity of NOMVs to the model protein antigen Ova was established in C57Bl/6 mice. Immunisation of mice with Ova in the presence or absence of NOMVs by i.p. and i.n. routes induced similar levels of Ova-specific IgM, however levels of Ova-specific IgG were much higher in serum from mice that received both the antigen and adjuvant (figures 3.5 and 3.6). Mice immunised with Ova alone exhibited low levels of Ova-specific IgG1, the isotype primarily induced by protein antigens in mice and humans, and IgG2b. Mice that were also

immunised with NOMVs displayed enhanced IgG1 responses. Low levels of Ova-specific IgA antibodies were also found in the sera from mice immunised i.n. with Ova plus NOMVs. These results confirm that NOMVs have the ability to enhance antibody responses to a model TD antigen such as Ova.

To further establish the ability of NOMVs as an adjuvant, the effect of NOMVs on the antibody responses toward DNP-ficoll were investigated in C57Bl/6 mice. After i.p. immunisation, DNP-ficoll specific IgM responses were similar in mice that received either DNP-ficoll alone or with NOMVs and the responses were greater than that in the sera of PBS control mice. In contrast the IgM response to DNP-ficoll after i.n. immunisation was significantly higher when DNP-ficoll was immunised with NOMVs than DNP-ficoll alone. The IgG response to DNP-ficoll was significantly higher in mice that received DNP-ficoll plus NOMVs by both immunisation routes, with improved IgG2b and IgG3 responses. Serum IgA to DNP-ficoll was absent in mice immunised with DNP-ficoll alone, however high levels were detected in sera from mice immunised with DNP-ficoll plus NOMVs. These data show that NOMVs can act as a mucosal adjuvant for DNP-ficoll, a TI-2 antigen that is often used as a model for anti-CPS antibody responses.

Polysaccharide vaccines are in general weakly immunogenic in children less than two years old and in the elderly due to their TI nature. The immune response to CPSs is also age dependent and studies have shown that despite conversion to TD forms through conjugation to proteins, polysaccharide induced

responses are still deficient in many aspects and therefore additional adjuvant is added to the CPS and carrier proteins. Previous studies have shown that type 14 and type 19F CPSs are only weakly immunogenic in Balb/c mice when not conjugated to a carrier protein (Mawas *et al.*, 2000) as is type 3 CPS which induces weak IgM and IgG3 responses (Dullforce *et al.*, 1998). The immune response to type 3 CPS has also been shown to be dependent on complement characterised by low IgM levels and a lack of isotype switching in C3- and complement receptor 2-deficient mice (Pozdnyakova *et al.*, 2003). Antibody responses to type 19F and 6B CPSs after conjugation to a non-toxic mutant of diphtheria toxin CRM_{197} were enhanced, characterised by increasing titres of serum IgG2a and IgG3, by the addition of CpG DNA which has been shown to be an effective adjuvant (Chu *et al.*, 2000). Moreno-Fierros and colleagues (2003) described the antibody response to type 6B CPS in the presence and absence of recombinant Cry1Ac protoxin from *Bacillus thuringiensis* as a carrier protein and adjuvant after i.n. immunisation. Their results described higher systemic and mucosal responses with the CPS plus Cry1Ac. However there was an absence of CPS-specific IgG antibodies.

With encouraging results with NOMVs as an adjuvant to DNP-ficoll, we were interested in investigating whether NOMVs could act as an adjuvant to clinically relevant polysaccharides. The polysaccharide capsule of *S. pneumoniae* protects the bacteria from innate immune defences and therefore the antibody response to the capsule is crucial. Before investigating the differences in

antibody responses to type 3, 4, 14 and 19F CPSs with or without NOMVs, the levels of natural serum IgM antibodies to the CPSs were determined in C57Bl/6 and Balb/c mice. Natural antibodies are generally of the IgM isotype and are produced by B-1a cells, which are mostly generated in the spleen. Wardemann *et al.*, (2002) have shown that in mice the spleen is essential for the generation and survival of B-1a cells and that mice lacking B-1a cells have a low level of natural IgM. Natural IgM antibodies were found to all serotypes tested, but levels differed not only between serotypes but also in the different strains of mice (figure 3.9). The levels to type 4 CPS were very low in both C57Bl/6 and Balb/c mice. In comparison in Balb/c mice the level of natural IgM to type 3 CPS was fairly high.

After determining the levels of natural IgM, the primary antibody responses to the CPSs tested in the presence or absence of NOMV were determined in C57Bl/6 and Balb/c mice by i.p. and i.n. immunisation routes. The CPSs used in the study were selected because they vary in immunogenicity as indicated by a previous study done in the laboratory (Jones et al., submitted manuscript). The IgM response was found to vary depending on the mouse strain, the route of immunisation and the response in individual mice. Via the i.p. route, and in both strains of mice, type 4 and 19F CPS produced an IgM response to the CPS that was greater than the natural IgM response to the CPS. However the response was not enhanced by immunisation with the CPS plus NOMVs. After i.n. immunisation apart from type 4 CPS in C57Bl/6 mice, none of the CPSs

immunised alone produced an IgM response greater than the natural levels, and again this response was not improved by immunisation with the CPS plus NOMVs. Mice also didn't respond in a dose-dependent manner to type 3 or 4 CPS after i.n. immunisation. The spleen, and importantly marginal zone B cells have been shown to have a greater capacity to respond to TI-2 antigens, which could explain the failure to see enhanced antibody responses after i.n. immunisation. However antibody responses did not improve after i.p. immunisation with the CPSs plus NOMVs. IgG responses were rarely observed, which is consistent with previous results on a large panel of CPS that were carried out in our laboratory (Jones *et al.*, submitted manuscript). However where an IgG response was detected, IgG3 and IgG1 subclasses dominated which is consistent with previous reports (Mosier *et al.*, 1982; Dullforce *et al.*, 1998; Mawas *et al.*, 2000). This is in contrast to immunisation with DNP-ficoll, which always produced high IgG responses.

It was unexpected that immunisation of NOMVs with the CPSs would fail to enhance IgM and IgG responses due to enhanced antibody responses observed after immunisation with DNP-ficoll plus NOMVs. Although DNP-ficoll is considered as a model T-I 2 antigen, DNP is a hapten and ficoll is a synthetic polysaccharide. Epitope size and conformation of DNP-ficoll are different to that of CPSs and its interaction with the innate immune system could also be different from that of CPSs. Antibody responses to DNP-ficoll have also been shown to be influenced by APCs and T cells (Boswell *et al.*, 1980; Mond *et al.*,

1980). Meltzer and Goldblatt (2006) have recently shown that pneumococcal CPSs can interact directly with human DCs by binding receptors such as the macrophage mannose receptor described by Zamze *et al.* (2002) or other receptors such as DC-SIGN. They also demonstrated that uptake did not induce DC maturation unless in the presence of a second signal such as LPS. A study has also determined the requirement of CD1 molecules, found on the surface of APCs and $CD8^+$ T cells to provide help for antibody production to TI-2 antigens (Kobrynski *et al.*, 2005). Therefore antibody responses to TI antigens could be influenced by the nature of the antigen. This has been demonstrated in a study using CpG motifs as an adjuvant to TI-2 antigens. Enhanced antibody responses to TNP-ficoll were seen when CpG was coadministered i.p., whereas CpG failed to enhance responses to 18 CPSs unless the pneumococcal serotypes were conjugated to tetanus toxoid. They suggested that this might be due to the degree of involvement of immune cells such as APCs and T cells after stimulation with the adjuvant (Kovarik *et al.*, 2001). This could be also true for NOMVs in the studies carried out.

As the CPSs immunised i.n. failed to produce an antibody response greater than the natural IgM response, the dose of the CPSs was increased. It was established that mice did not respond in a dose dependent manner to type 3 or type 4 CPS after i.n. immunisation and once again the IgG response was variable in individual mice. For some of the CPSs, IgM responses were greater after i.p. immunisation than i.n.. This is most likely due to the presence of B1-a

cells in the spleen producing natural IgM and Igs to the CPSs after i.p. immunisation and not after i.n. immunisation. MZ B cells of the spleen have also been implicated in response to TI-2 antigens, and it has been shown that mice possessing B1-a cells but not MZ B cells have impaired responses to TNP-ficoll after i.p. immunisation (Guinamard *et al.*, 2000). This suggests that the spleen and route of immunisation is also important in the production and enhancement of humoral responses to TI-2 antigens.

For all CPSs tested the level of IgM to the CPSs was sustained for at least 60 days indicated by similar absorbance levels, supporting data previously generated in our laboratory (Jones *et al.*, submitted paper). This is probably due to the fact that pneumococcal CPSs have a high molecular weight with a relatively long half-life therefore they degrade slowly *in vivo* sustaining antibody levels (Sela *et al.*, 1972).

A booster immunisation of CPS given 62 days after the initial immunisation with either the CPS alone or CPS plus NOMV had no effect on the antibody levels to the CPSs used. This is not unusual and is consistent with other studies that a booster effect or memory response was not observed with polysaccharide antigens (Baker *et al.*, 1971; Mond *et al.*, 1995a; Mond *et al.*, 1995b).

It was seen that serum from mice immunised i.p. with NOMVs displayed a similar level of CPS-specific IgM to that of naïve mice. A second immunisation with NOMVs 62 days after the initial immunisation markedly increased the CPS-specific IgM response to all four CPSs tested that included cross-reactive

antibodies. This indicates that immunisation with NOMVs may confer cross-protection to the CPSs tested in this study, however this needs to be further investigated in more detail. It is clear from the results with NOMV that anti-CPS antibody responses are poorly induced and are difficult to enhance via the intranasal route. This raises the question to the identity of the protective antigen induced by natural colonisation of Nm, even though CPS-protein conjugate vaccines induce protective immunity in vaccinated individuals.

There were certain limitations with the assays carried out in this chapter. One limitation was that controls were not included when determining whether NOMVs could act as an adjuvant to different antigens. Controls would have enabled us to assess whether there was any variability during immunisation with the different antigens. This could have been determined when measuring the antibody responses by ELISA, as any difference in the response to the control could indicate variability in the other samples. In order to determine whether NOMVs could act as an adjuvant, antibody responses were assessed by measuring changes in the OD of the serum samples from the different immunised mice. Different experiments were not analysed by ELISA in the same assay therefore it was not possible to directly compare the effect of NOMVs on different antigens. It could have been more accurate to use antibody standards so that OD's could have been converted to concentrations, which would have taken into account the variability of measuring OD's.

In contrast to CPS antigens, previous studies performed in this laboratory have shown that mice immunised i.n. with NOMVs can induce anti-LPS antibodies, which are cross-reactive between LPS variants (Andersen *et al.*, 2002). Thus, poor anti-CPS antibody responses in the presence of NOMV are unlikely due to the insufficient stimulation of APCs, but may result in the lack of number or activation of a different B cell subset. B cell subsets for DNP and similarly small epitopes are potentially different from those present on CPS. The mechanism of B cell activation by purified CPS requires further investigation. Understanding of the antibody response towards carbohydrate epitopes is of interest in the development of vaccines against group B meningococcal disease because LPS is one of the subcapsular antigens being assessed as a potential vaccine candidate (Plested, 2003; Gidney *et al.*, 2004). Plested and colleagues (2003) have provided proof-of-principle of a strategy of using only inner core epitopes of LPS conjugated to a protein carrier to induce protective antibodies that are cross-reactive to full-length LPS on live meningococci B strains, but the anti-LPS antibody generated were not optimal in protection studies. We therefore have tried to isolate high affinity antibodies against inner core LPS after immunisation with NOMV preparations derived from Nm strain 44/76 (Mu-4). We have analysed several high affinity anti-mut 4 LPS antibodies but found that these antibodies were non-cross reactive with wild-type meningococci (MacKenzie *et al.*, unpublished data). Although our results indicate that NOMVs are a good adjuvant for inducing high affinity anti-LPS antibodies, the strategy of using inner core LPS epitopes in the context of NOMVs to generate a universal serogroup B

vaccine requires optimisation and identification of inner core epitopes that are exposed on live Nm.

In conclusion, although NOMVs increase primary antibody responses to model antigens such as Ova, DNP-ficoll and LPS it does not appear to act as an adjuvant towards CPSs from *S. pneumoniae*. However as shown with Ova and DNP-ficoll, NOMVs could prove to be suitable as a mucosal adjuvant boosting antibody responses for TD and TI-1 antigens via the i.n. route. Naturally occurring microbial products such as NOMVs would also provide an avenue to understand mucosal immune responses generated i.n. under more physiologically relevant conditions. Finally, natural immunity induced by NOMV-shedding Nm in serogroup B or other groups may not involve the induction of anti-CPS antibodies in the nasopharynx.

CHAPTER 4

The possible mechanisms of adjuvant action and immunomodulatory properties of NOMVs

4.1 Introduction

Adjuvants can be described as any component or product that when co-administered with a vaccine enhances the immunogenicity of the vaccine antigen via humoral or cell-mediated immunity. The mechanism of action of some adjuvants is poorly defined however the effect they can have on the immune system is better documented. Aluminium adjuvants (e.g. Alum) are an example of adjuvants that are approved for human use. Their mechanism of action is thought to be through a depot effect where the antigen and adjuvant stay around the injection site to produce a prolonged effect targeting APCs. Alum is typically effective in enhancing antibody responses and producing a predominantly Th2 response to protein antigens.

Particulate adjuvants, such as immune stimulating complexes (ISCOMs) are used in veterinary vaccines and induce strong Th1 and Th2 responses. They form aggregates by binding antigens, which are then engulfed by APCs. However more effective targeting of APCs is achieved by using adjuvants whose ligands are recognised by receptors on APCs.

Microbial products have also been shown to be strong adjuvants. For example complete Freund's adjuvant (CFA) is a potent adjuvant composed of paraffin oil and heat-killed mycobacteria. It is frequently used in experimental animal models but is deemed too toxic for human use (Billiau *et al.*, 2001).

Immune responses to pathogens can be enhanced by the activation of the immune system through TLRs. Binding of PAMPs such as LPS, flagellin and bacterial CpG DNA to TLRs leads to the activation of signalling pathways and results in the production of pro-inflammatory cytokines and enhanced expression of costimulatory molecules on immune cells. This ultimately affects both the innate and acquired immune systems. Hence TLRs are potent mediators of adjuvant activities. For example LPS, one of the best-characterised PAMPs can influence and stimulate the production of IL-12 and NO by binding and signalling through TLR4. Bacterial CpG DNA has also been shown to have adjuvant properties when administered with an antigen. Its activity comes from binding to TLR9 which in mice results in a predominantly Th1 response (Krieg, 2002). It has also been shown to be effective as a mucosal adjuvant promoting mucosal IgA production (McCluskie *et al.*, 2000).

Mucosal immunisation is thought to have advantages over traditional immunisation including the ability to induce local/mucosal as well as systemic immunity, and the production of secretory IgA to specific pathogens at mucosal surfaces to prevent the binding of pathogens and therefore prevent infection (Freytag *et al.*, 2005). The elimination of the need of using needles for vaccination would also be of a practical benefit. The most investigated mucosal adjuvants are cholera toxin (CT) and *E. coli* heat labile enterotoxin (LT). They are strong mucosal adjuvants although they are too toxic for human use. The mechanism of action has been attributed to enhanced presentation to a variety of cell types especially APCs, the induction of

isotype switching in B cells to increase the production of IgA, influencing cytokine production and enhancing the uptake of the co-administered antigen (Holmgren *et al.*, 2003).

As mentioned in the previous chapter, NOMVs and its components have been shown to have immunomodulatory properties, including enhancing antibody responses, inducing cytokine production and upregulation of costimulatory molecules on immune cells (Saunders *et al.*, 1999; Sprong *et al.*, 2001; Al-Bader *et al.*, 2003; Massari P, 2003). The mechanism by which NOMVs exert their action is not fully understood and we sought to determine further the immunomodulatory potential of NOMVs by investigating the role of LPS and non-LPS components.

4.2 Objectives

The aim of the work in this chapter was to investigate the possible mechanisms of adjuvant action and the immunomodulatory properties of NOMVs.

4.3 Results

4.3.1 Proliferation of splenocytes from C3H/HeN and C3H/HeJ mice in response to NOMVs.

Studies were carried out to investigate the proliferation of splenocytes by NOMVs using splenocytes from C3H/HeN and C3H/HeJ mice. By using C3H/HeJ mice, which are hyporesponsive to LPS, the effects that are not due to LPS were also examined. Figure 4.1A shows the proliferation of

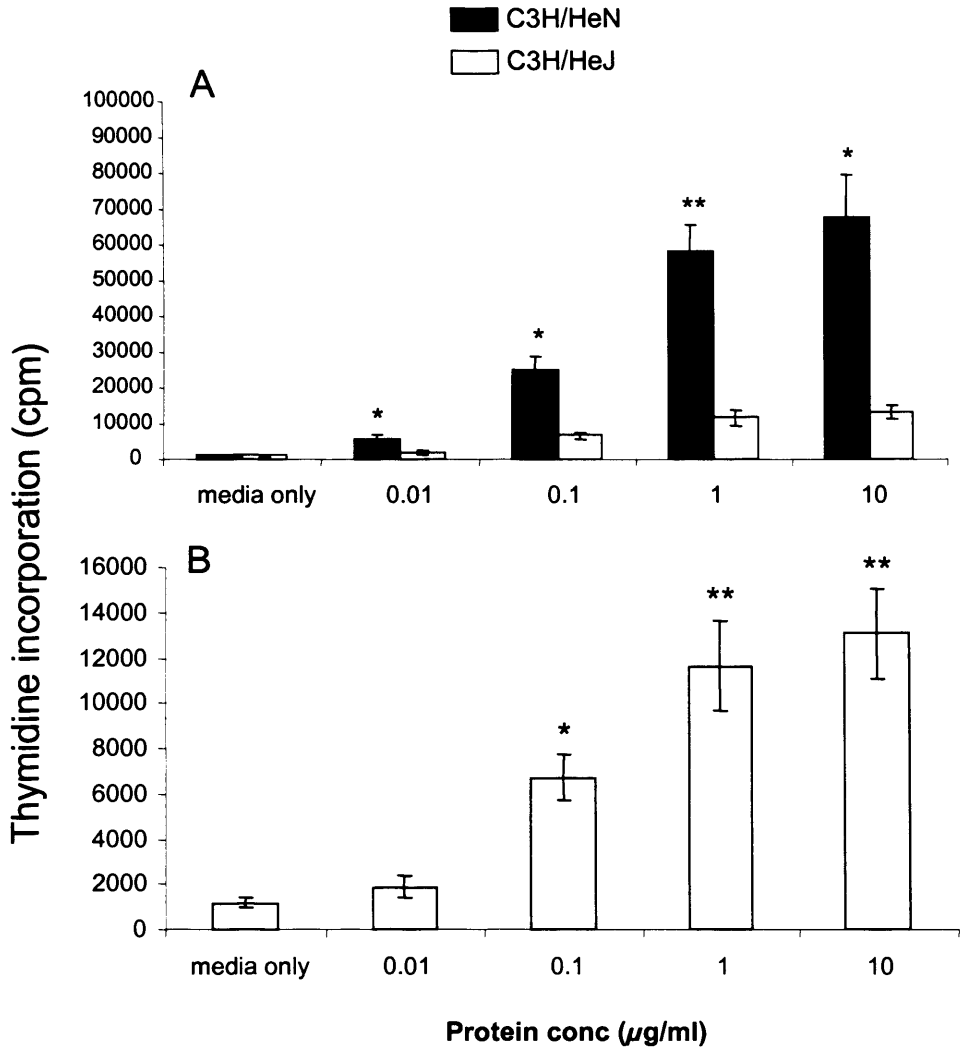

Figure 4.1 Proliferation of splenocytes from C3H/HeN and C3H/HeJ mice stimulated with NOMVs.

Spleens were taken from naïve C3H/HeN and C3H/HeJ mice and cell suspensions were prepared. Cells (2 x 10^5 /well) were cultured with medium alone or NOMVs (0.01-10μg/ml). Cells were pulsed with tritiated thymidine after 48h of culture and were frozen 18h later. Upon thawing proliferation was determined using a scintillation counter. Panel (A) compares splenocyte proliferation between C3H/HeN and C3H/HeJ mice, and panel (B) proliferation of splenocytes from C3H/HeJ mice. Data represents the mean of three experiments ± SEM. For (A) C3H/HeN ≠ C3H/HeJ and (B) media only ≠sample; * $p<0.05$, ** $p<0.005$.

C3H/HeN and C3H/HeJ splenocytes after stimulation for 48h with different concentrations of NOMVs. Proliferation of splenocytes was measured by the incorporation of tritiated thymidine into DNA of dividing cells. Both C3H/HeN and C3H/HeJ splenocytes proliferated in a dose dependent manner to NOMVs and proliferation between the two strains of mice was significantly different ($p < 0.05$) when stimulated with different concentrations of NOMVs. Although proliferation in C3H/HeJ mice (figure 4.1B) was also dose dependent, the level of proliferation was approximately six fold less, indicating that proliferation is not wholly dependent on LPS.

4.3.2 Proliferation of C3H/HeN and C3H/HeJ T and B cells in response to NOMVs.

It has been shown that splenocytes from C3H/HeN and C3H/HeJ are able to proliferate after culture with NOMVs. In order to determine which cell type was proliferating, splenocytes were labelled with CFSE. After labelling, the cells were cultured for 48h with NOMVs, and then labelled with anti-CD3 and anti-B220 antibodies, markers for T and B cells, respectively. As cells proliferate, the intensity of CFSE labelling reduces, which is shown by individual peaks. PMA and ionomycin were used as a positive control to show that splenocytes from C3H/HeN and C3H/HeJ mice are able to proliferate. PMA and ionomycin are commonly used to induce the proliferation of T cells. Figure 4.2 shows the lack of proliferation of T cells after stimulation with NOMVs. T cells from both C3H/HeN and C3H/HeJ mice did not show any proliferation in response to NOMVs, and their profiles are similar to those of splenocytes after stimulation with media only.

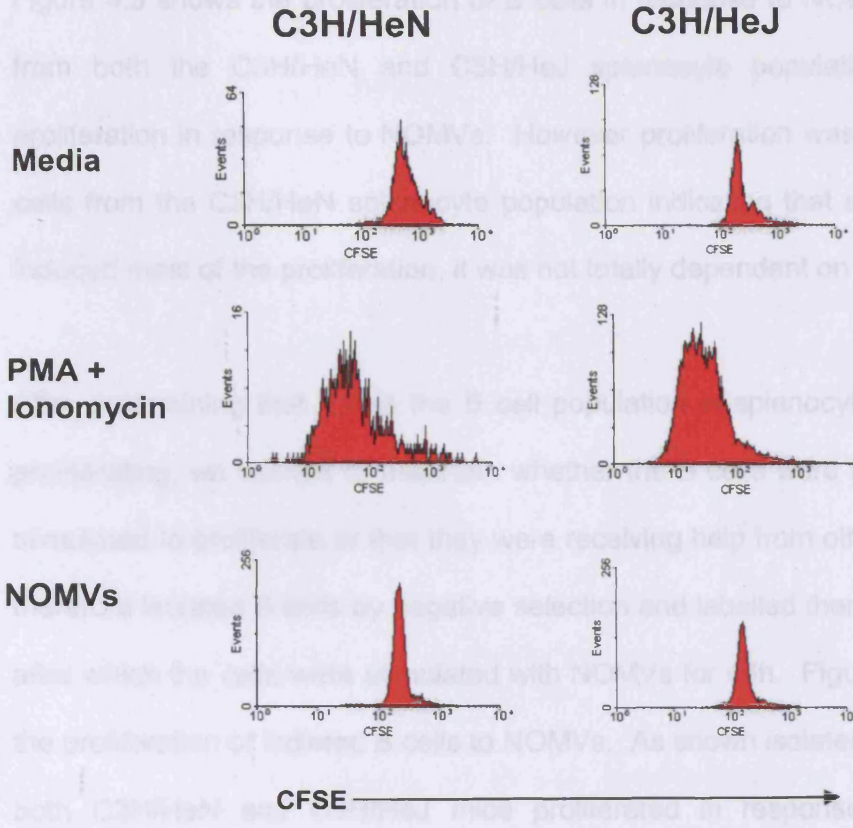

Figure 4.2 CFSE analysis of T cell proliferation in response to NOMVs

Spleens from naïve C3H/HeN and C3H/HeJ mice were taken and cell suspensions were prepared. Cells (1 x 10^7 cells/ml) were labelled with 5μM CFSE at 37^{0}C for 10mins. Cells were then cultured with either NOMVs, PMA (5ng/ml) + ionomycin (200ng/ml), or media alone. After 48hr, cells were labelled with anti-CD3 antibody and examined by flow cytometry. The CD3 positive population was gated on to determine proliferation.

Figure 4.3 shows the proliferation of B cells in response to NOMVs. B cells from both the C3H/HeN and C3H/HeJ splenocyte populations showed proliferation in response to NOMVs. However proliferation was greater in B cells from the C3H/HeN splenocyte population indicating that although LPS induced most of the proliferation, it was not totally dependent on LPS.

After determining that it was the B cell population in splenocytes that were proliferating, we wanted to ascertain whether the B cells were being directly stimulated to proliferate or that they were receiving help from other cells. We therefore isolated B cells by negative selection and labelled them with CFSE, after which the cells were stimulated with NOMVs for 48h. Figure 4.4 shows the proliferation of isolated B cells to NOMVs. As shown isolated B cells from both C3H/HeN and C3H/HeJ mice proliferated in response to NOMVs indicating that both LPS and non-LPS components can induce proliferation and that there is direct stimulation of the cell population, indicating that other cell types are not necessary.

4.3.3 NOMVs stimulate the production of bactericidal NO by BMDMs.

Binding of bacterial products to macrophages stimulates phagocytosis, producing toxic products such as NO, which are directly toxic to bacteria. In order to investigate whether NOMVs can stimulate the production of NO, BMDMs from C3H/HeN and C3H/HeJ mice were used. BMDMs were cultured for 48h with different concentrations of NOMVs after which the supernatant was removed and NO production was measured using the

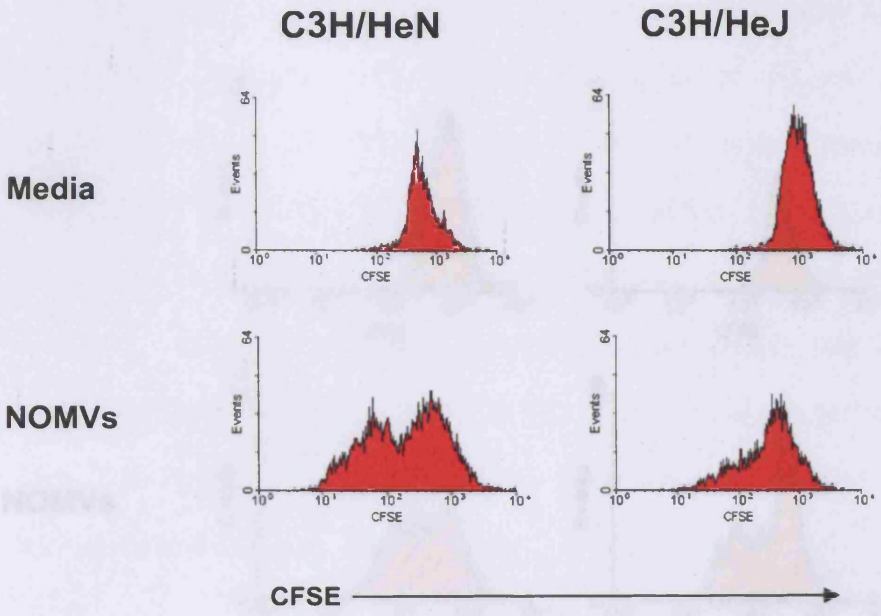

Figure 4.3 CFSE analysis of B cell proliferation in response to NOMVs

Spleens from naïve C3H/HeN and C3H/HeJ mice were taken and cell suspensions were prepared. Cells (1 x 10^7 cells/ml) were labelled with 5μM CFSE at 37^{0}C for 10mins. Cells were then cultured with either NOMVs or media alone. After 48hr, cells were labelled with anti-B220 antibody and examined by flow cytometry. The B220 positive population was gated on to determine proliferation.

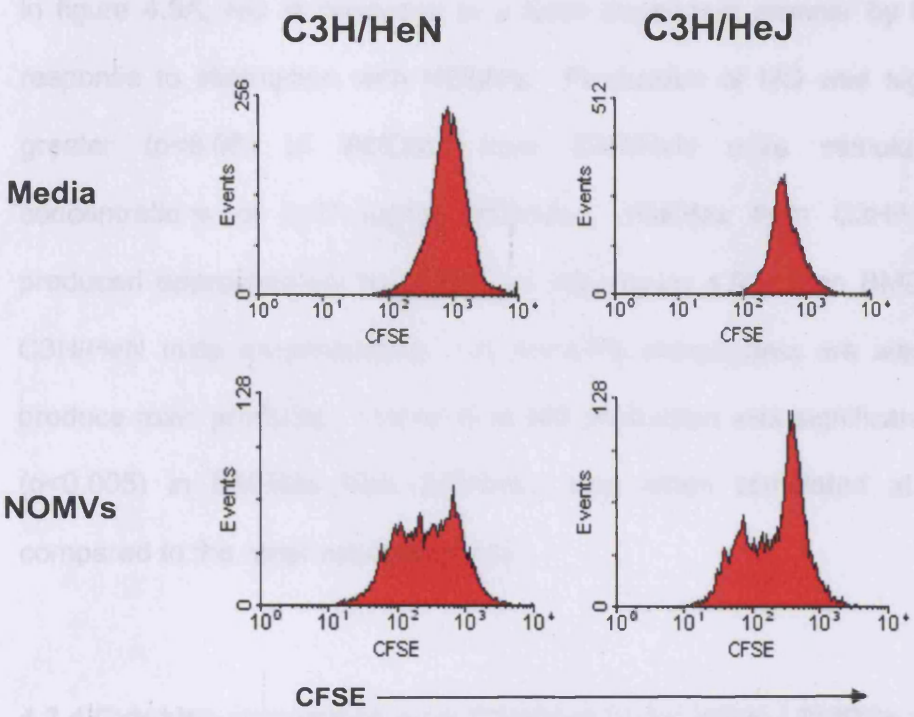

Figure 4.4 CFSE analysis of isolated B cell proliferation in response to NOMVs

Spleens from naïve C3H/HeN and C3H/HeJ mice were taken and B cells were isolated using a Dynal B cell negative isolation kit. The B cells (1 x 10^7 cells/ml) were then labelled with 5μM CFSE at 37^0C for 10mins. The cells were then cultured with either 5μg/ml NOMVs or media alone. After 48hr, cells were labelled with anti-B220 antibody and examined by flow cytometry. The B220 positive population was gated on to determine proliferation.

Griess assay, which measures nitrite, a breakdown product of NO. As shown in figure 4.5A, NO is produced in a dose dependent manner by BMDM in response to stimulation with NOMVs. Production of NO was significantly greater ($p<0.05$) in BMDMs from C3H/HeN mice stimulated with concentrations of 0.01-1μg/ml NOMVs. BMDMs from C3H/HeJ mice produced approximately two fold less NO (figure 4.5B) than BMDMs from C3H/HeN mice demonstrating that non-LPS components are also able to produce toxic products. The level of NO production was significantly higher ($p<0.005$) in BMDMs from C3H/HeJ mice when stimulated at 10μg/ml compared to the other concentrations.

4.3.4 Cytokine production from C3H/HeN and C3H/HeJ BMDCs after stimulation with NOMVs.

BMDCs from C3H/HeN and C3H/HeJ mice were cultured with NOMVs and the cytokine profile was determined. Supernatants were removed after 24 and 48h and analysed using a cytokine Cytometric Bead Array (CBA) assay. Figure 4.6 shows the cytokine release from BMDCs. The production of IL12p70, IFN-γ and IL-10 is shown in figure 4.6A. Stimulation of BMDCs with NOMVs for 24h increases the production of IL12p70 and IL-10 in both C3H/HeN and C3H/HeJ mice. After 48h the levels of both of these cytokines is still slightly increased in NOMV stimulated cells than in cells stimulated with media alone. Figure 4.6B shows the production of TNF-α, MCP-1 and IL-6. A vast increase is seen in the production of TNF-α and IL-6 after stimulation with NOMVs in both strains of mice. There is considerable

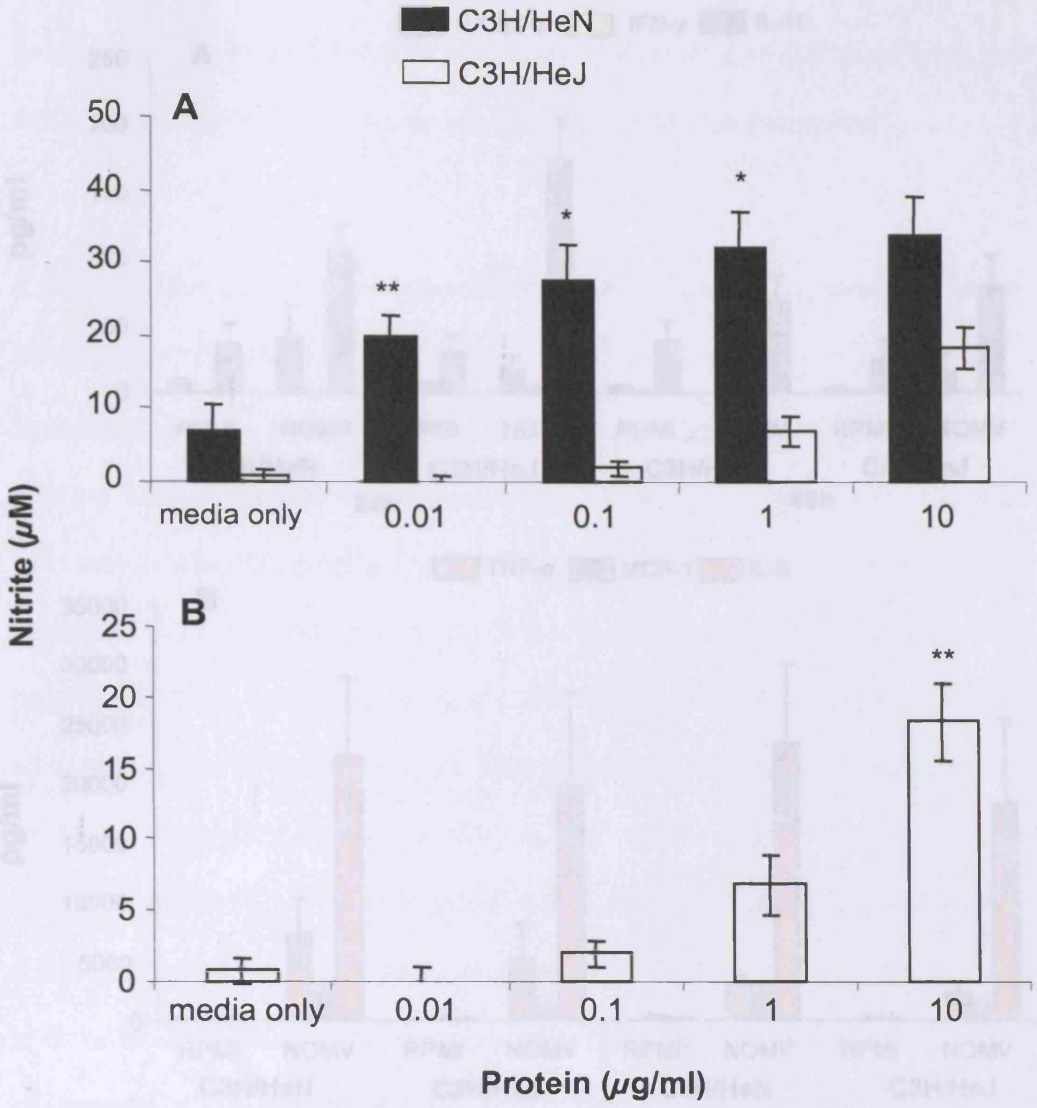

Figure 4.5 Nitrite production from BMDMs from C3H/HeN and C3H/HeJ mice stimulated with NOMVs.

BMDMs from C3H/HeN and C3H/HeJ mice were stimulated with medium alone and NOMVs. After 48hrs, cell culture supernatant was removed and the Griess assay was used to measure nitrite as an indicator of NO production. Panel (A) compares nitrite production from BMDMs of C3H/HeN and C3H/HeJ mice, and (B) nitrite production from C3H/HeJ mice. Data represents the mean of three experiments ± SEM. For (A) C3H/HeN ≠C3H/HeJ and (B) media only ≠sample; * $p<0.05$, ** $p<0.005$.

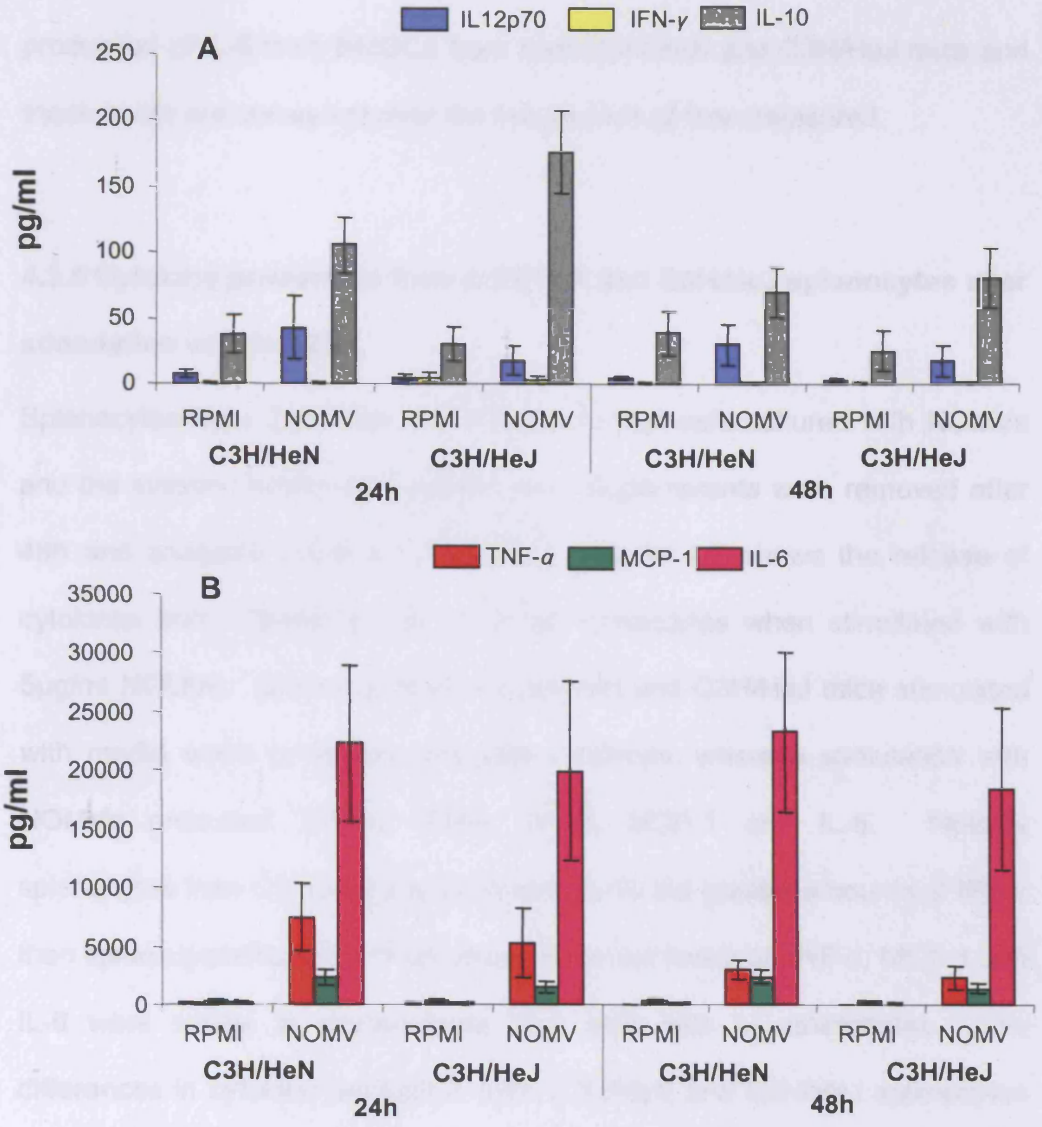

Figure 4.6 Cytokine release from C3H/HeN and C3H/HeJ mouse BMDCs after culture with NOMVs.

BMDCs from naïve C3H/HeN and C3H/HeJ mice were prepared and (2 x 10^6/tube) cultured with 5μg/ml of NOMVs. Supernatant was removed from each tube after 24 and 48h. Cytokines IL12p70, IFN-γ, IL-10 (A) TNF-α, MCP-1 and IL-6 (B) were analysed by a cytokine Cytometric Bead Array (CBA) assay (BD Biosciences, Pharmingen). Data represents the mean of five experiments ± s.e.m.

production of IL-6 from BMDCs from both C3H/HeN and C3H/HeJ mice and these levels are consistent over the two periods of time measured.

4.3.5 Cytokine production from C3H/HeN and C3H/HeJ splenocytes after stimulation with NOMVs.

Splenocytes from C3H/HeN and C3H/HeJ mice were cultured with NOMVs and the cytokine profile was determined. Supernatants were removed after 48h and analysed using a CBA assay. Figure 4.7 shows the release of cytokines from C3H/HeN and C3H/HeJ splenocytes when stimulated with 5μg/ml NOMVs. Splenocytes from C3H/HeN and C3H/HeJ mice stimulated with media alone produced very little cytokines, whereas stimulation with NOMVs produced TNF-α, IFN-γ, IL-10, MCP-1 and IL-6. Notably splenocytes from C3H/HeJ produced less IL-10 but greater amounts of IFN-γ than splenocytes from C3H/HeN mice. However levels of TNF-α, MCP-1 and IL-6 were similar in supernatants from both sets of splenocytes. The differences in cytokine production from C3H/HeN and C3H/HeJ splenocytes could be as a result of the effect of LPS as C3H/HeJ mice are hypo-responsive to LPS. The production of IL12p70 was not detectable in splenocytes from either strain of mice after 48h, therefore IL12p70 levels were studied at various time points (18h and 24h), however production of the cytokine was not detected by the CBA assay.

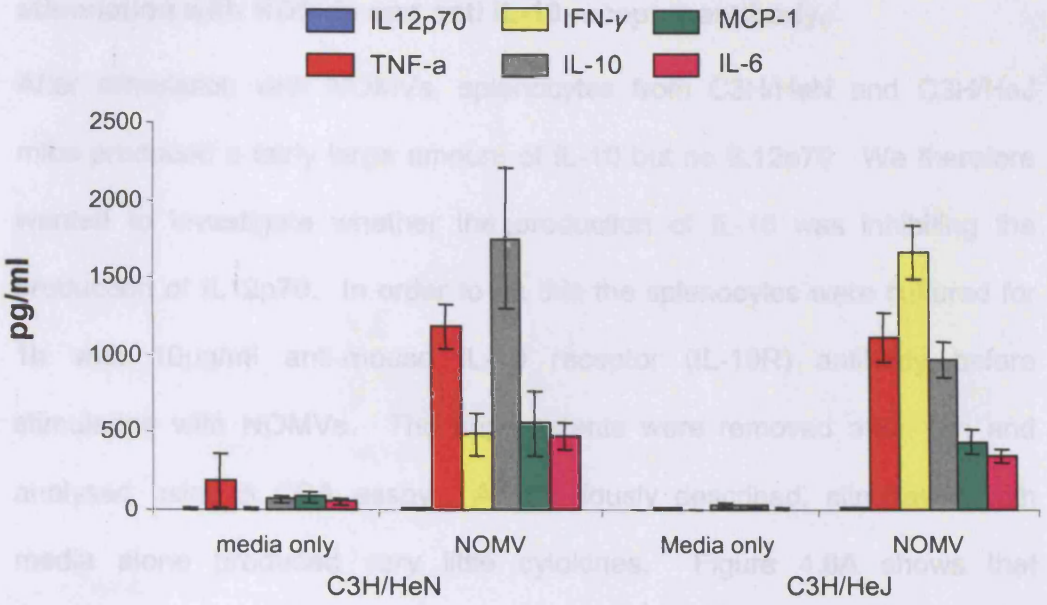

Figure 4.7 Cytokine release from C3H/HeN and C3H/HeJ mouse splenocytes after culture with NOMVs.

Spleens from naïve C3H/HeN and C3H/HeJ mice were removed and single cell suspensions were prepared. Cells (2 x 10^5/well) were cultured with 5μg/ml of NOMVs. Supernatant was removed from each well after 48h. Cytokines were analysed by a cytokine Cytometric Bead Array (CBA) assay (BD Biosciences, Pharmingen). Data represents the mean of three experiments ± s.e.m.

4.3.6 Cytokine production from C3H/HeN and C3H/HeJ splenocytes after stimulation with NOMVs and anti IL-10 receptor antibody.

After stimulation with NOMVs, splenocytes from C3H/HeN and C3H/HeJ mice produced a fairly large amount of IL-10 but no IL12p70. We therefore wanted to investigate whether the production of IL-10 was inhibiting the production of IL12p70. In order to do this the splenocytes were cultured for 1h with 10μg/ml anti-mouse IL-10 receptor (IL-10R) antibody before stimulation with NOMVs. The supernatants were removed after 48h and analysed using a CBA assay. As previously described, stimulation with media alone produced very little cytokines. Figure 4.8A shows that splenocytes from both strains of mice cultured with IL-10R antibody before stimulation with NOMVs produced more TNF-α, INF-γ and IL-6. The splenocytes from C3H/HeN mice cultured with the IL-10R antibody produced a three-fold increase of IL12p70, and the splenocytes from C3H/HeJ mice a two-fold increase (figure 4.8B). Splenocytes from both C3H/HeN and C3H/HeJ mice produced cytokines in a dose dependent manner when stimulated with IL-10R at concentrations from 0.001 – 10μg/ml and NOMVs (data not shown).

4.3.7 The production of type 1 interferons from splenocytes stimulated with NOMVs and anti IL-10R antibody.

The supernatant from splenocytes in the above study were also assayed for IFN-α and IFN-β using ELISA kits. Figure 4.9 shows the production of IFN-β from splenocytes of C3H/HeN and C3H/HeJ mice. After 24h stimulation with

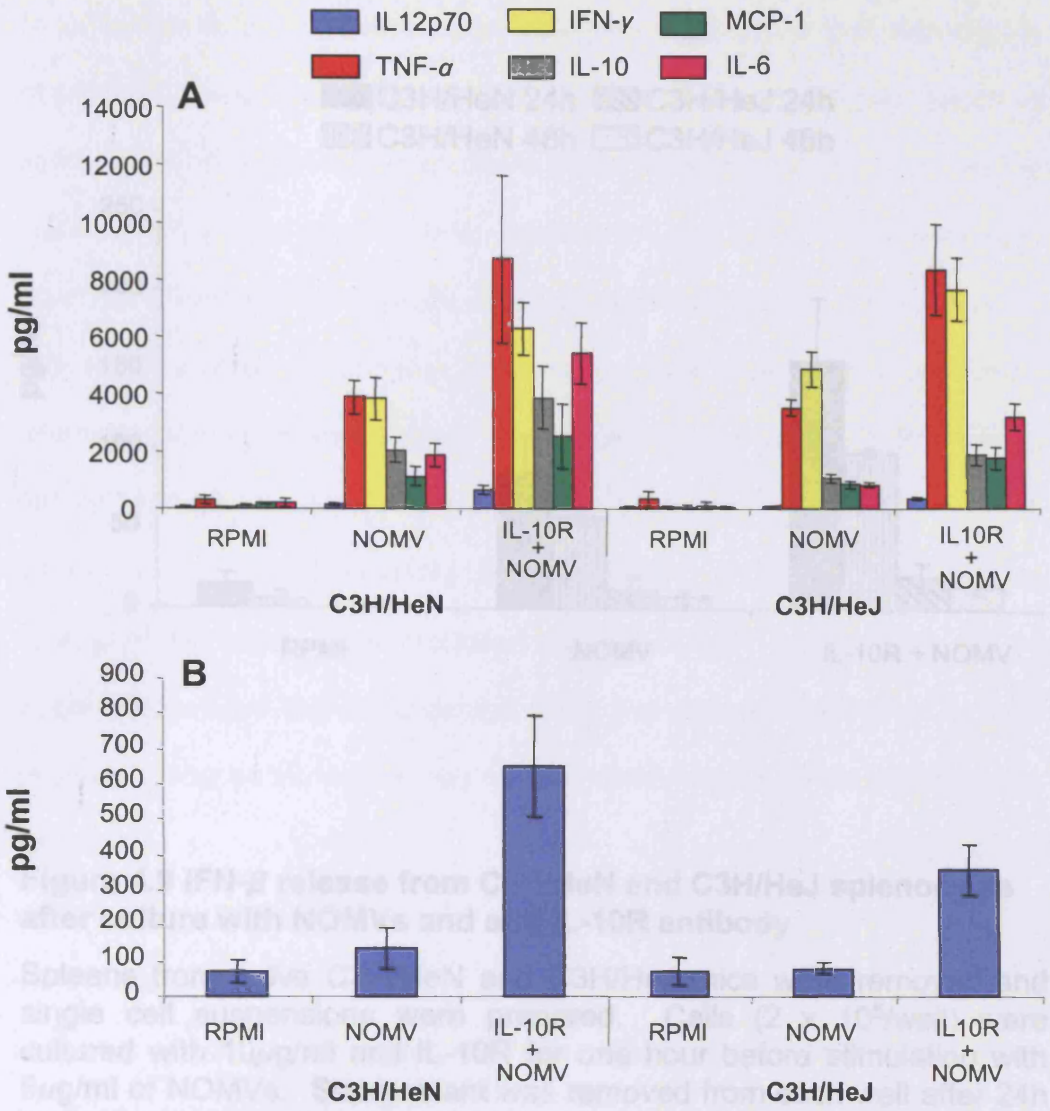

Figure 4.8 Cytokine release from C3H/HeN and C3H/HeJ mouse splenocytes after culture with NOMVs and anti IL-10R antibody

Spleens from naïve C3H/HeN and C3H/HeJ mice were removed and single cell suspensions were prepared. Cells (2 x 10^5/well) were cultured with anti IL-10R (10μg/ml) for one hour before stimulation with 5μg/ml of NOMVs. Supernatant was removed from each well after 48h and cytokines IL12p70, IFN-γ, MCP-1, TNF-α, IL10 and IL-6 (A), or IL-12p70 (B) were analysed by a cytokine CBA assay (BD Biosciences, Pharmingen). Data represents the mean of three experiments ± s.e.m.

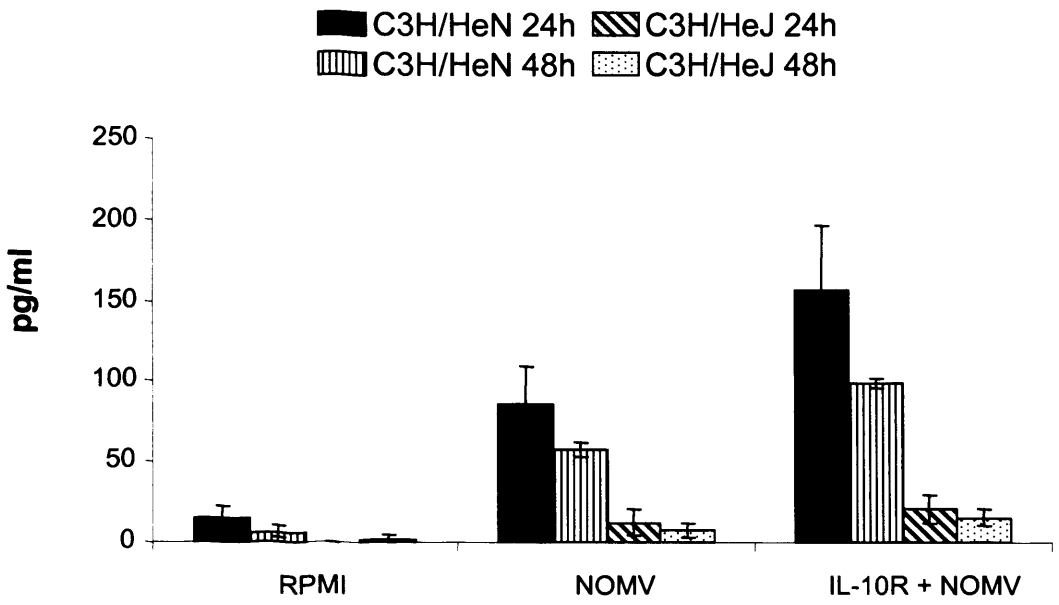

Figure 4.9 IFN-β release from C3H/HeN and C3H/HeJ splenocytes after culture with NOMVs and anti IL-10R antibody

Spleens from naïve C3H/HeN and C3H/HeJ mice were removed and single cell suspensions were prepared. Cells (2 x 10^5/well) were cultured with 10μg/ml anti IL-10R for one hour before stimulation with 5μg/ml of NOMVs. Supernatant was removed from each well after 24h and 48h. IFN-β was analysed by a mouse interferon beta ELISA kit (Biosource). Data represents the mean of four experiments ± s.e.m.

NOMVs, the splenocytes from C3H/HeN mice produced IFN-β, with slightly decreased levels after 48h stimulation. After 24h and 48h stimulation of splenocytes from C3H/HeJ mice, there was no detection of IFN-β. A two-fold increase in IFN-β production from splenocytes of C3H/HeN mice was seen after 24h stimulation with IL-10R antibody and NOMVs than after stimulation with NOMVs alone. There was no significant difference in IFN-β production when splenocytes from C3H/HeJ mice were cultured with IL-10R antibody before stimulation with NOMVs than after stimulation with NOMVs alone, indicating that LPS influences the production of IFN-β. Splenocytes stimulated with media alone produced very little IFN-β.

Supernatants from the same assays were also assayed for IFN-α. In both strains of mice, and after 24h and 48h stimulation with NOMVs no IFN-α was detected.

4.4 Discussion

Previous results in this thesis have indicated that NOMVs are able to augment antibody responses to certain antigens therefore possessing adjuvant and immunomodulatory activity. This chapter examined the effect of NOMVs on different cell populations from C3H/HeN and C3H/HeJ mice. NOMVs induced the proliferation of splenocytes from both strains of mice, shown by thymidine incorporation, indicating that proliferation was not dependent on LPS. Proliferation was approximately five times higher in splenocytes from C3H/HeN mice than C3H/HeJ mice and the response was shown to be dose dependent. Further experiments were carried out to determine which cell population was proliferating. By CFSE-labelling of

splenocytes it was shown that B cells, but not T cells were proliferating in response to NOMVs, in both strains of mice. PMA and ionomycin was used as a control to measure T cell proliferation. Ionomycin is a calcium ionophore that is used in conjunction with PMA. PMA activates protein kinase C, which is important in cell division and can only induce proliferation in the presence of calcium ions.

Proliferation of B cells was shown to be by the direct action of NOMVs and that other cell types were not involved, by negatively isolating B cells from splenocytes of both strains of mice. Greater proliferation was most likely seen in total splenocytes and B cells from C3H/HeN mice, as LPS is a known polyclonal activator of B cells. However these studies have also shown that proliferation can also occur independently of LPS. Previous studies have shown that meningococcal outer membrane preparations consisting of mainly porins are able to act as B cell mitogens (Melancon *et al.*, 1983). A later study by Wetzler and colleagues (1996) confirmed that purified neisserial porins can induce B cell proliferation, and that proliferation was not as a result of LPS contamination by using isolated B cell populations from C3H/HeJ mice. They also described upregulation of the costimulatory molecule, B7-2 on the surface of B cells suggesting a possible mechanism of adjuvant activity. The ability of porins to stimulate B cells and to upregulate the expression of B7-2 was shown to be dependent on TLR2 and MyD88 by using TLR2- and MyD88-knockout mice (Massari *et al.*, 2002).

The work in this chapter and published findings by other groups suggests that NOMVs are able to activate immune cells through TLR2 and TLR4.

Work carried out in collaboration with Valerie Quesniaux and colleagues further established that NOMVs could signal through either of these receptors. This was shown by the production of TNF-α and NO by BMDCs and BMDMs from $TLR2^{-/-}$ and $TLR4^{-/-}$ knockout mice after stimulation with NOMVs. The response in double knockout mice was little to absent implying that signalling occurs mostly through TLR2 and TLR4.

The ability of NOMVs to stimulate the production of NO was further established using BMDMs from C3H/HeN and C3H/HeJ mice. Phagocytosis of pathogens by macrophages and neutrophils produces a variety of toxic products including NO and hydrogen peroxide to help kill the pathogen. NO is produced by inducible nitric oxide synthase, iNOS2. NO production was seen in the supernatant of BMDMs from both strains of mice and in a dose dependent manner. Levels of NO production were approximately two fold less in BMDMs from C3H/HeJ mice, indicating that LPS to an extent can influence the production of NO. Previous reports have shown NO production by peritoneal macrophages stimulated with Nm. This response was also shown to be mostly dependent on LPS by using C3H/HeJ mice (Mukhopadhyay *et al.*, 2004). An earlier study indicated both protective and pathological roles of NO. This study showed that although NO could increase levels of TNF-α induced by meningococcal LPS, which is known to initiate endotoxic shock, NO may also have a beneficial role for macrophages anti-Nm bactericidal activity (Padron *et al.*, 1999). The production of NO has also been shown to be regulated by IFN-γ, which can induce iNOS catalysing the oxidation of arginine to produce NO (Nathan, 1997).

The effect of NOMVs on the release of cytokines from BMDCs of C3H/HeN and C3H/HeJ mice were investigated after culture for 24 and 48h. Moderate levels of IL-10, low levels of IL12p70 and no IFN-γ were found in the supernatants of BMDCs from both strains of mice after stimulation with NOMVs for 24h in comparison to cells cultured with media alone. After 48h levels of IL-10 decreased by approximately two fold. At both time points very high levels of IL-6 were exhibited in both strains of mice. TNF-α production was also fairly high after 24h in both strains of mice, with slightly lesser amounts produced after 48h. These results show that the profile of cytokine production by BMDCs is not dependent on LPS. The activation of human monocyte-derived DCs by components of the meningococcal outer membrane has been described and demonstrated by an increase in the production of proinflammatory cytokines and the expression of costimulatory molecules such as CD80 and CD86 (Dixon *et al.*, 2001; Kolb-Maurer *et al.*, 2001; Al-Bader *et al.*, 2003). A recent paper has also described the ability of PorB to induce the maturation of murine splenic DCs and BMDCs as shown by increased expression of CD86, MHC II and the production of IL-6. The activation of DCs was found to be dependent on TLR2 and MyD88 (Singleton *et al.*, 2005). This work further demonstrates the immunomodulatory properties of non-LPS components of meningococci.

Cytokine release from splenocytes of C3H/HeN and C3H/HeJ mice was also investigated after culture with NOMVs. Splenocytes from C3H/HeN mice produced high levels of TNF-α and IL-10, and lower levels of IFN-γ, MCP-1

and IL-6 in response to stimulation with NOMVs. In comparison splenocytes from C3H/HeJ mice produced similar amounts of TNF-α, MCP-1 and IL-6, but higher levels of IFN-γ and lower amounts of IL-10. Culture of splenocytes from both strains of mice with media only induced very little amounts of any of the cytokines. Our findings are in agreement with others, which demonstrate that LPS is not the only component of meningococci that can induce cytokine production (Uronen *et al.*, 2000; Sprong *et al.*, 2001).

The production of IFN-γ by cells stimulated with NOMVs could partly explain why there was production of IgG3 and IgG2a when mice were immunised with NOMVs in the previous chapter, as IFN-γ is known to induce isotype switching to these subclasses. IL-6 has also been reported to be involved in the differentiation of B cells to antibody producing plasma cells, which also induces isotype switching (Hirano, 1998). The production of IL-6 was seen from BMDCs and splenocytes from C3H/HeN and C3H/HeJ mice after stimulation with NOMVs. IL-6 has been shown to be produced by several cell types including APCs, T cells and B cells and can induce the proliferation of B cells, which could explain the splenocyte proliferation seen in this study.

There was no production of IL12p70 by splenocytes from either strain of mice. IL-12 is a heterodimeric cytokine composed of p40 and p35 subunits. The p40 subunit is produced in greater quantities than the p35 unit, which requires coexpression of the p40 subunit for secretion of the biologically active cytokine. Free p40 can be present as monomers or homodimers in mice, and both have been proposed as inhibitors of biologically active IL-12 (Gillessen *et al.*, 1995). In our investigation we only measured IL12p70, therefore large amounts of IL12p40 could have been produced inhibiting the

production of IL-12. IL-12 induces T cells and NK cells to produce several cytokines including IFN-γ. Surprisingly, even though there was no IL-12 production, there was still production of IFN-γ.

IL-18 has been described as an IFN-γ-inducing factor, that is similar to IL-12 and that is required to produce IFN-γ (Okamura *et al.*, 1995). It is generally thought that IL-18 alone is unable to stimulate IFN-γ production and the presence of both IL-12 and IL-18 is required for the efficient production of IFN-γ. However reports have indicated IL-12-independent IFN-γ production, which is dependent on IL-18 (Xing *et al.*, 2000; Muller *et al.*, 2001). Another member of the IL-12 family, IL-27, has also been discovered that synergises with IL-12 to produce IFN-γ in T cells. IL-27 is a heterodimeric protein composed of Epstein-Barr virus (EBV)-induced gene 3 (EBI3), a p40-related protein and p28 (Pflanz *et al.*, 2002). Therefore in future studies it may be necessary to measure IL-18 and IL-27 in order to determine whether these cytokines are also influencing IFN-γ production.

The production of IL-12 is regulated by several mechanisms. Negative regulation of IL-12 production can be due to the production of IL-10, which is a potent inhibitor of IL-12 (D'Andrea *et al.*, 1993; Aste-Amezaga *et al.*, 1998). As seen in figure 4.6, splenocytes from both strains of mice cultured with NOMVs produced IL-10, although levels were greater in C3H/HeN mice than in C3H/HeJ mice. In order to determine whether the production of IL-10 was having a negative effect on the production of IL-12, before stimulation of the splenocytes with NOMVs, the cells were cultured with IL-10R antibody therefore preventing the binding of IL-10 to its receptor and not the production of IL-10. The cytokine profile from the splenocytes from both

strains of mice indicated that pre-incubation with the IL-10R antibody before stimulation with NOMVs increased the production of IL12p70 in comparison to cells cultured with NOMVs or media alone. An increase in IFN-γ production was also observed in the supernatants from splenocytes treated with IL-10R antibody and NOMVs. The production of IL12p70 by splenocytes was shown to be dose dependent on the amount of IL-10R cultured with the cells indicating that even the smallest amount of IL-10 can inhibit the production of IL-12. This dose dependency was also seen to a certain extent for the production of IFN-γ, indicating that IL-12 production could have a positive influence on IFN-γ production.

Type 1 interferons (IFN-α/β) are most commonly known to be produced in response to viral infection however they can also be expressed as a result of bacterial infection or by components of bacteria such as LPS and bacterial DNA (Bogdan, 2000). A recent study has shown that type I IFNs signal as a link between innate and adaptive immunity by demonstrating that not only are type I IFNs produced by DCs, but they can cause the maturation of DCs (Le Bon *et al.*, 2002). In humans the type 1 IFNs consist of 14 IFN-α subtypes and IFN-β, -δ, –τ, –ω, –κ and limitin. Our results showed NOMVs induced the production of IFN-β from splenocytes of C3H/HeN mice, but not IFN-α. The production of IFN-β was shown to be dependent on LPS, as there was very little to none produced from splenocytes of C3H/HeJ mice. It has been previously shown that LPS induces predominantly IFN-β (Toshchakov *et al.*, 2002) and that it can regulate the differentiation or activation of most effector cells including DCs and B cells (Bellardelli *et al.*, 1996). Analysis of

supernatants from splenocytes cultured with IL-10R antibody and NOMVs showed an increase in IFN-β production implying that levels of IFN-β can be influenced by the production of IL-12, whose production is increased after blocking the action of IL-10. A recent study has demonstrated that type 1 IFN is required for the production of IL-12 in an autocrine manner (Gautier *et al.*, 2005). Further studies in our laboratory (Durand *et al.*, manuscript in preparation) demonstrated that i.m. injection of NOMVs in C3H/HeN mice produced a rapid and short-lasting production of IFN-β in sera 2h post immunisation, and this production was not seen in C3H/HeJ mice, supporting the work carried out *in vitro*. Lower levels of IFN-α were demonstrated in sera from both C3H/HeN and C3H/HeJ mice however the levels declined rapidly after 2h. NOMVs were also able to induce cross-priming against Ova indicated by a significant increase in $CD8^+$ T cells after i.m. immunisation. Cross-priming was found to be dependent on the IFN-α/β pathway indicated by a decrease in $CD8^+$ T cells in IFN-α/βR-deficient mice.

Our results indicate that NOMVs have the potential to be an adjuvant as they are able to target cells of the immune system inducing mechanisms that link innate and adaptive responses. NOMVs are able to stimulate the production of cytokines that can target APCs. For example TNF-α can induce DC maturation, influencing the migration of DCs to T cell areas in tissues and to present antigen (McWilliam *et al.*, 1996). The production of cytokines by DCs and other cell types is important in T cell differentiation, for instance IL-12 is crucial in the development of Th1 responses (Trinchieri, 1993; Cella *et al.*, 1996).

In conclusion, our results suggest that NOMVs have adjuvant properties that stimulated APC functions and proliferation, and enhanced production of cytokines that are important for B and T cell responses. More importantly NOMVs' effects are not wholly dependent on LPS. Having shown this we wanted to investigate the effect of NOMVs on mucosal immunity that operates in mainly a Th2-biased microenvironment.

CHAPTER 5

The effect of NOMVs on lung inflammation and immunity

5.1 Introduction

Asthma is a common chronic disease in children and a major risk factor for asthma is allergy. Allergic asthma is a Th2-associated disease characterised by chronic airway inflammation, the secretion of IL-4, IL-5 and IL-13 by Th2 lymphocytes, the infiltration of eosinophils in response to certain antigens and elevated levels of serum IgE (Savelkoul *et al.*, 2000; Barends *et al.*, 2004).

Lung eosinophilia is a characteristic of allergic asthma and it is thought that the infiltration of eosinophils is key to the pathogenesis of the disease. Eosinophilia is regulated in three ways: (1) adhesion receptors that allow firm adhesion of eosinophils to the vascular endothelium; (2) factors such as cytokines and chemokines which influence the recruitment and migration of eosinophils to the inflammatory site; and (3) leukocytes which regulate the expression and release of cytokines and chemokines. Eosinophils are able to cause tissue damage by releasing inflammatory mediators such as eosinophil peroxidase and eosinophil-derived neurotoxin, as well as being a source of the majority of Th2 cytokines (Boehme *et al.*, 2004; Zhu *et al.*, 2004).

A strong correlation has been shown between the level of cytokines produced by activated T cells and the degree of damage in the lung by

eosinophils. IL-4 is a principle Th2 cytokine, which drives Ig isotype switching by B cells towards IgE synthesis and whose main role is during the initial priming of Th2 effector cells (Herrick *et al.*, 2003). IL-5, primarily produced by activated T cells, promotes the differentiation, maturation and recruitment of eosinophils (Sanderson, 1992; Delayre-Orthez *et al.*, 2004; Kay *et al.*, 2004).

Chemokines such as eotaxin (CCL11), RANTES (CCL5) and monocyte chemoattractant protein (MCP-1/CCL2) have also been shown to be involved in the development of asthma (Wohlleben *et al.*, 2003). Eotaxin was first identified in BAL fluid of Ova-sensitised guinea pigs (Jose *et al.*, 1994), however human eotaxin (Ponath *et al.*, 1996a; Ponath *et al.*, 1996b) and murine eotaxin (Gonzalo *et al.*, 1996) have also been described. Eotaxin has been shown to bind CCR3, which is highly expressed on eosinophils and basophils, but is also present on T cells and epithelial cells. It is able to induce the migration of eosinophils as well as attract basophils and Th2 lymphocytes (Daugherty *et al.*, 1996; Teran, 2000; Lukacs, 2001).

The incidence of asthma is steadily increasing and the reason for this is unknown. Changes in the environment, such as exposure to microbial compounds and improved health care and hygiene practice have led to the hygiene hypothesis. This hypothesis states that decreased exposure to infections early on in life is increasing the prevalence of allergic disease as lack of exposure to infectious diseases hinders the establishment of Th1 biased immunity (Herz *et al.*, 2000). The prevalence of allergic disease tends to be higher in the developed world rather than the developing world

(Hawrylowicz *et al.*, 2005). Infection with heat-killed *Listeria monocytogenes*, a Gram positive bacteria, has been shown to provoke a Th1-dominated response that is able to reduce airway inflammation and eosinophilia in a murine model of asthma (Yeung *et al.*, 1998; Hansen *et al.*, 2000). Similarly Erb and colleagues (1998) demonstrated using a murine model of Ova-induced airway eosinophilia that i.n. infection with *Mycobacterium bovis*-Bacillus Calmette-Guérin (BCG) four or 12 weeks before allergen airway challenge resulted in a significant reduction in eosinophilia in the lungs. They reported that Th2 responses were suppressed by IFN-γ produced in response to BCG infection and they have demonstrated similar results using heat killed BCG (Major *et al.*, 2002). More recently heat-killed *Bordetella pertussis* has been shown to inhibit Ova-induced allergic responses in a murine model, again characterised by a decrease in eosinophil accumulation in the airway and down-regulation of Th2 cytokines. This was found to be due to unmethylated CpG motifs in the DNA of the bacteria activating the TLR9 pathway (Kim *et al.*, 2004). Previous to these studies it has been shown that administration of unmethylated CpG oligodeoxynucleotides (ODNs) before allergen challenge could decrease allergen-induced eosinophilia and the number of allergen-specific IgE-producing cells, and that IFN-γ has a crucial role in aiding these effects (Broide *et al.*, 1998; Kline *et al.*, 1998; Sur *et al.*, 1999). The mechanism of how this is achieved has been partly described in a recent study by Hessel *et al.*, (2005). They described two distinct pathways. The first is by preventing the induction of cytokines by Th2 cells, which is achieved by APCs being unable to present antigen to Th2

cells, but not Th1 cells. The second pathway is by inhibiting cytokines such as IL-4 that influence the production of IgE.

Experiments carried out by Etchart and colleagues (2006) in mice sensitised with iRSV demonstrated that i.n. immunisation of NOMVs were able to prevent the accumulation of eosinophilia in the lungs of mice after live RSV challenge. Viral respiratory infection by RSV in infants has been described as a risk factor in acquiring allergic asthma, as well as frequently causing bronchiolitis and pneumonia in infants and young children (Connors *et al.*, 1992; Schwarze *et al.*, 1998). It has been shown that mice immunised with iRSV produce a predominantly Th2-like response, characterised by a high ratio of IL-4 to IFN-γ mRNA in the lungs (Waris *et al.*, 1996). It has also been shown that IL-4 and IL-5 both have a major role in lung eosinophilia during RSV infection, as mice treated with anti-IL-5 prevented eosinophilia and IL-4 deficient mice were devoid of eosinophils (Schwarze *et al.*, 2000).

It is not just the effects of bacteria or bacterial products, which have been shown to prevent allergic inflammation. IL-15 has been previously shown to accelerate allergic sensitisation in mice. By blocking the effects of IL-15 using a soluble IL-15 receptor antibody, the production of allergen-specific IgE and eosinophil recruitment were reduced (Ruckert *et al.*, 2005).

Recent studies have also indicated that Th2 allergic responses could be regulated by T regulatory (Tr) cells. Naturally occurring Tr cells originate in the thymus and represent 5-10% of $CD4^+$ T cells in humans and mice. It has been proposed that they do this by secreting large amounts of IL-10, which

has been shown to protect mice from allergen-induced Th2 responses. IL-10 is able to suppress both total and allergen-specific IgE but can also induce IgG4 production. It can also down-regulate eosinophil function and activity as well as inhibiting the production of IL-5. Several reports have described this possible association in humans and as Tr cells are major producers of IL-10 this has been proposed as a possible mechanism, however there have also been some contradictory results suggesting that IL-10 has no effect on Th2 responses (Erb *et al.*, 2002; Robinson *et al.*, 2004; Hawrylowicz *et al.*, 2005).

Taking into account previous reports and our preliminary data we wanted to determine whether i.n. immunisation of NOMVs could have the same effect as shown with CpG ODNs and BCG and be able to reduce lung inflammation pathology for example by inhibiting the characteristics of allergic asthma, such as the development of eosinophilia by using murine models of Ova-induced and iRSV-induced eosinophilia.

5.2 Objective

The aim of the work in this chapter was to investigate the effect of i.n. immunisation of NOMVs on lung inflammation using murine models of allergen-induced allergy.

5.3 Results

5.3.1 The effect of NOMVs on the development of lung inflammation

To address whether i.n. immunisation with NOMVs would have an effect on the development of lung inflammation, C57Bl/6 mice were immunised in a model of Ova-induced allergy (see figure 2.3 for immunisation schedule). In order to determine whether NOMVs have an effect the number of eosinophils, lymphocytes and macrophages was assessed. Figure 5.1 shows the morphology of the cell types, which were enumerated after BAL and Wright-Geimsa staining. The percentage cell type and absolute cell numbers in the BAL fluid were calculated as described in the methods (section 2.2.5).

Figure 5.2A shows that Ova-sensitised C57Bl/6 mice immunised i.n. with NOMVs either in the presence or absence of Ova showed a significant decrease ($p<0.017$) in the percentage of eosinophils in comparison to mice immunised with Ova alone. There was no statistically significant difference in the percentage of macrophages and lymphocytes among the different immunised groups of mice (figure 5.2B and 5.2C respectively) and the percentage of macrophages dominated over the number of lymphocytes found in the BAL fluid.

Figure 5.3 shows the total number of the different cell types in the BAL fluid. As shown in figure 5.3A, a significant decrease ($p<0.005$) in the number of eosinophils was observed after i.n. immunisation with NOMVs in the presence or absence of Ova. The number of macrophages and lymphocytes were slightly increased after immunisation with Ova plus NOMV however this increase was not statistically significant.

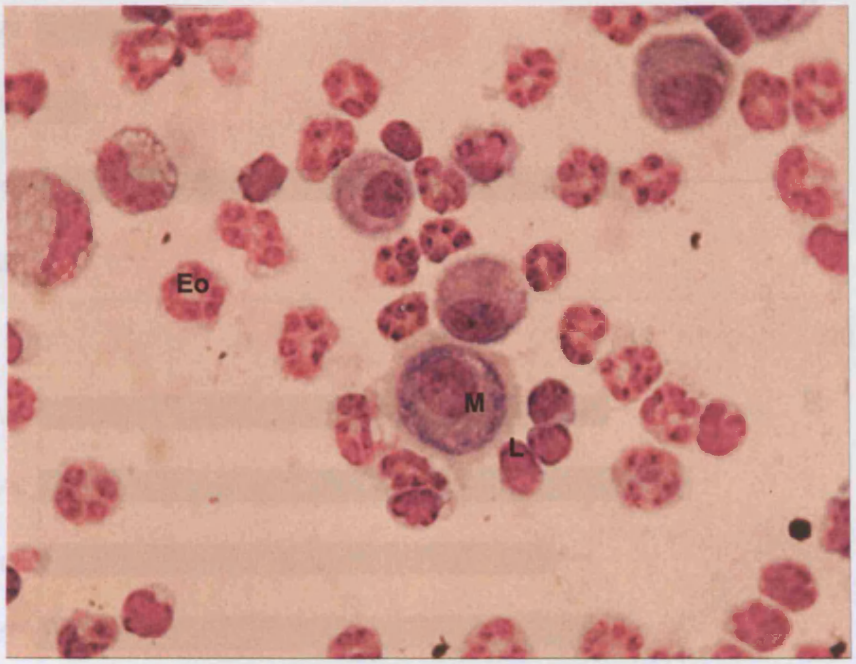

Figure 5.1 Morphology of cell types after BAL and Wright-Giemsa staining

C57Bl/6 mice were immunised i.p. with either PBS or Ova once a week for two weeks. Two weeks later the mice were then split into five groups and immunised i.n. with either PBS, Ova, NOMVs or Ova+NOMVs. After a further seven days the mice were culled and BAL was carried out. The number of eosinophils (Eo), macrophages (M) and lymphocytes (L) was enumerated by Wright-Giemsa staining.

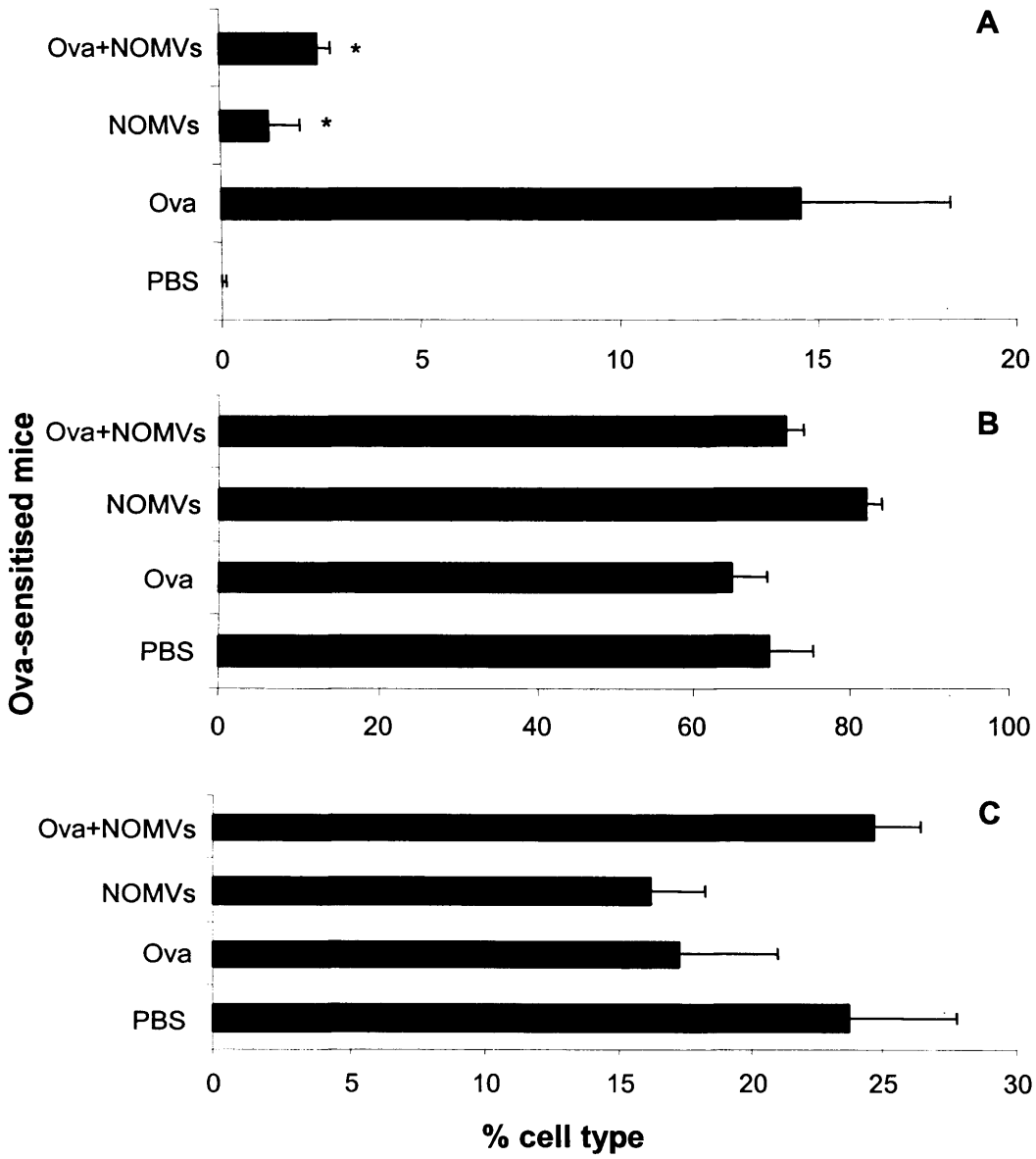

Figure 5.2 The effect of NOMVs on the percentage of Ova-induced cell infiltrates in the lungs.

C57Bl/6 mice were immunised i.p. with Ova once a week for two weeks. Two weeks later the mice were then split into four groups and immunised i.n. with either PBS, Ova, NOMVs or Ova+NOMVs. After a further seven days the mice were culled and BAL was carried out. The number of (A) eosinophils; (B) macrophages and (C) lymphocytes were enumerated by counting 15 random fields after Wright-Giemsa staining. The results are expressed as the mean and SEM of four mice from one representative experiment out of three. Asterisks indicate a value significantly different from that observed after i.n. infection with Ova, * $p < 0.017$

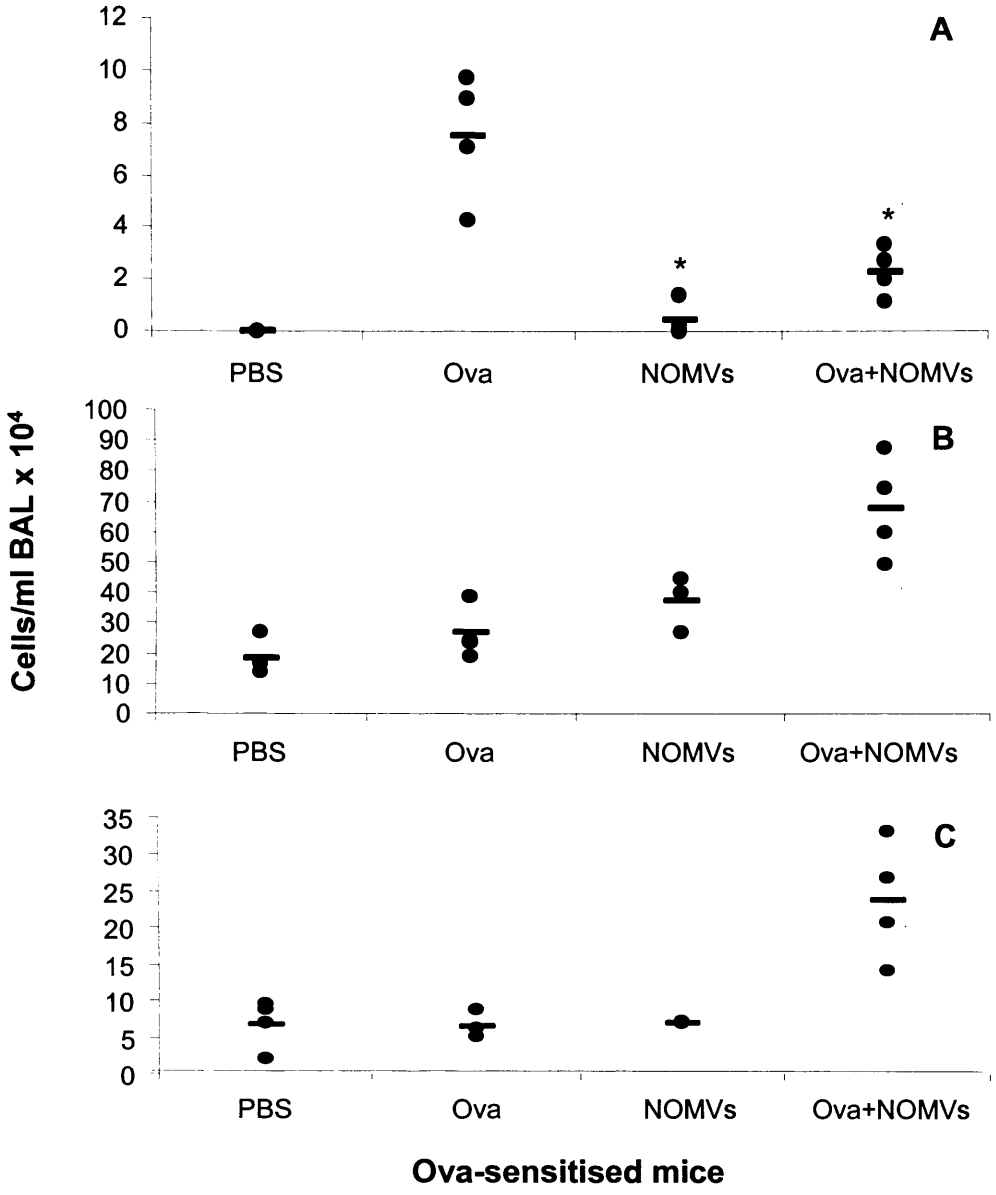

Figure 5.3 The effect of NOMVs on the total number of Ova-induced cell infiltrates in BAL fluid.

C57Bl/6 mice were immunised i.p. with Ova once a week for two weeks. Two weeks later the mice were then split into four groups and immunised i.n. with either PBS, Ova, NOMVs or Ova+NOMVs. After a further seven days the mice were culled and BAL was carried out. The number of (A) eosinophils; (B) macrophages and (C) lymphocytes were enumerated by Wright-Giemsa staining. Individual mice are indicated by (•) and the mean as (—). These data represent one experiment out of three. Asterisks indicate a value significantly different from that observed after i.n. infection with Ova, * $p < 0.005$.

5.3.2 The effect of NOMVs on preventing lung inflammation after Ova challenge

Preliminary experiments indicated a decrease in eosinophil numbers after i.n. immunisation with NOMVs either in the presence or absence of Ova, therefore we wanted to determine whether i.n. immunisation with NOMVs before Ova airway challenge would reduce or prevent further eosinophilia. Figure 5.4 shows the percentage of eosinophils, lymphocytes and macrophages in BAL fluid. An increase in the percentage of eosinophils was seen in mice that were immunised with Ova before challenge with Ova, compared to control mice. I.n. immunisation with NOMVs in the presence or absence of Ova before Ova challenge brought about a significant decrease in eosinophil numbers ($p=0$) in the BAL fluid in comparison to Ova-only immunised mice. As previously observed, the percentage of macrophages dominated over the numbers of eosinophils and lymphocytes (figure 5.4B). The percentage of macrophages stayed constant among the different groups of immunised mice, and although the percentage of lymphocytes differed slightly among the groups the difference was not statistically significant. The total number of cells in the BAL fluid was calculated as previously described. Again a significant decrease ($p<0.002$) in the number of eosinophils was seen in mice immunised with NOMVs or Ova plus NOMVs before i.n. Ova challenge. As shown in figure 5.5B and 5.5C there was no significant difference in the number of macrophages and lymphocytes after i.n. challenge with Ova, however a slightly greater number of these cell types was found after immunisation with NOMVs.

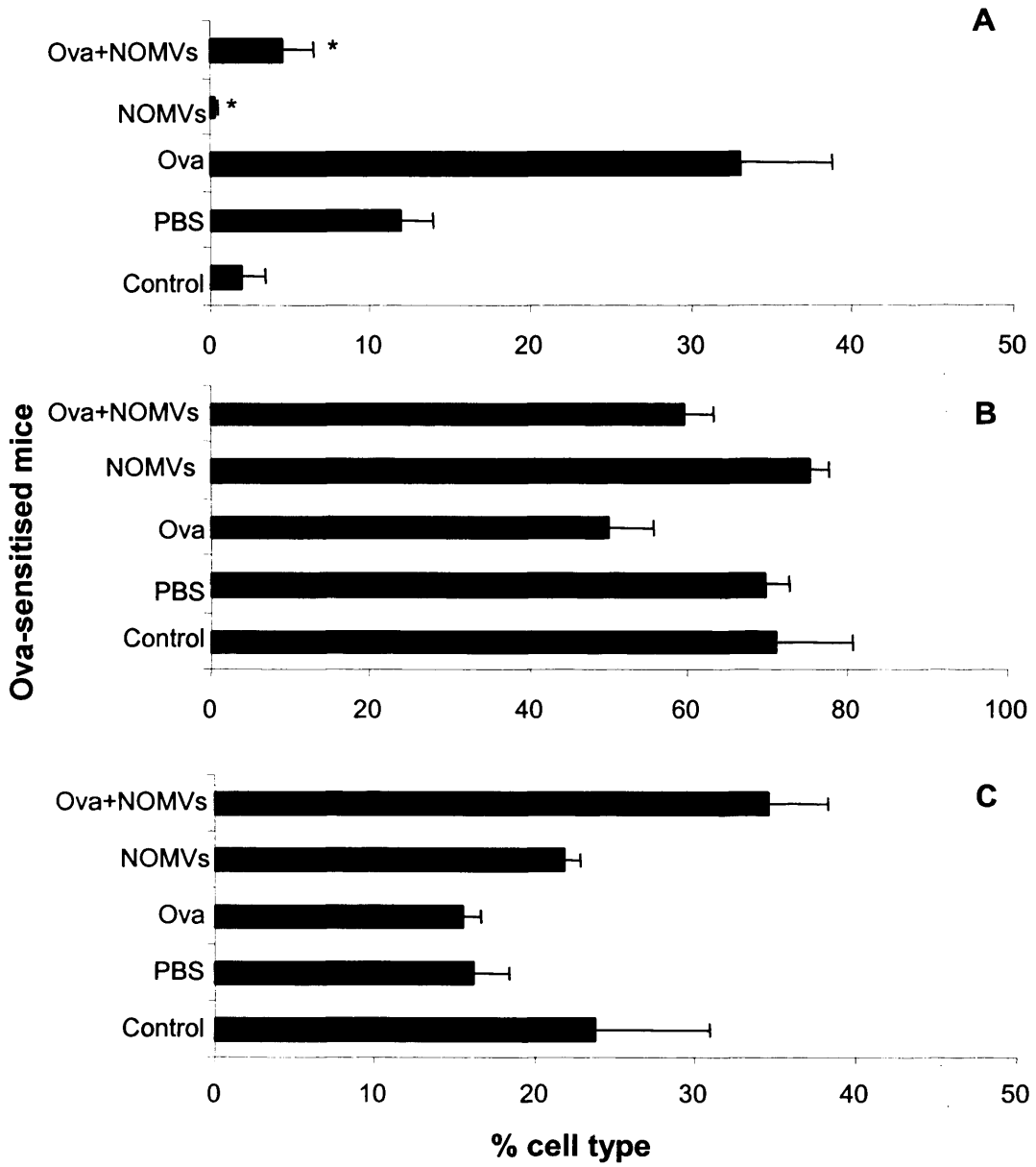

Figure 5.4 The effect of NOMVs on the percentage cell type in the lungs of mice after sensitisation and challenge with Ova.

C57Bl/6 mice were immunised i.p. with Ova or PBS (control) once a week for two weeks. Two weeks later the mice were then split into five groups and immunised i.n. with either PBS, Ova, NOMVs or Ova+NOMVs. After a further two weeks the mice were all immunised i.n. with Ova and seven days later the mice were culled and BAL was carried out. The number of (A) eosinophils; (B) macrophages and (C) lymphocytes were enumerated by counting the cell types in 15 random fields after Wright-Giemsa staining. The results are expressed as the mean and SEM from four mice and are representative of one experiment out of three. Asterisks indicate a value significantly different from that observed after i.n. infection with Ova, * p = 0.

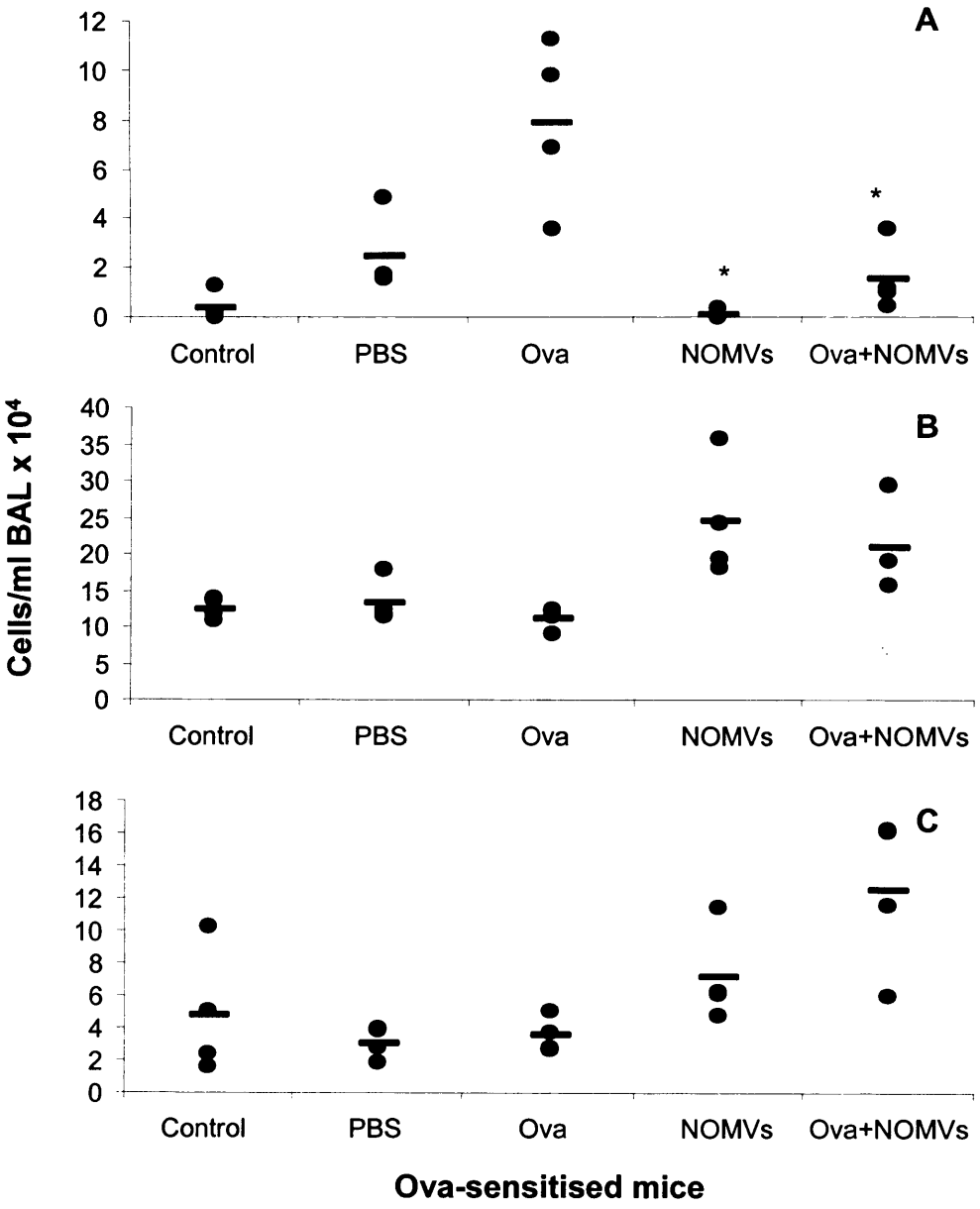

Figure 5.5 The effect of NOMVs on the total cell number in the BAL fluid of mice sensitised and challenged with Ova.

C57Bl/6 mice were immunised i.p. with Ova or PBS (control) once a week for two weeks. Two weeks later the mice were then split into five groups and immunised i.n. with either PBS, Ova, NOMVs or Ova+NOMVs. After a further two weeks the mice were all immunised i.n. with Ova and seven days later the mice were culled and BAL was carried out. The number of (A) eosinophils; (B) macrophages and (C) lymphocytes were enumerated by Wright-Giemsa staining. Individual mice are indicated by (•) and the mean as (—). These data represent one experiment out of three. Asterisks indicate a value significantly different from that observed after i.n. infection with Ova, * $p < 0.002$.

5.3.3 The effect of NOMVs on the production of eotaxin and IgE antibodies.

The results in the above section suggest that immunisation with NOMVs can suppress the development of eosinophilia. We were then interested to examine the levels of eotaxin in the supernatant of lung homogenates and the levels of total IgE in the sera of infected mice, which is shown in figure 5.6. As demonstrated in figure 5.6A the amount of eotaxin found in each of the groups of mice was fairly constant, indicating that NOMVs did not have an effect on eotaxin levels. The average amount of total serum IgE was also found to be consistent and fairly low in all groups of mice (figure 5.6B), however there was a considerable degree of variation in the amount of IgE produced ranging from 0 to 22ng/ml between individual mice of the different groups.

5.3.4 The effect of NOMVs on lung inflammation using a RSV infection model.

As a decrease in lung eosinophilia was observed after immunisation of NOMVs in a murine model of Ova-induced lung eosinophilia, we then wanted to determine the effect of NOMVs after i.n. immunisation with RSV which induces a strongly Th2-biased environment (see figure 2.4 for immunisation schedule).

Figure 5.7 shows the percentage of eosinophils, macrophages and lymphocytes in BAL fluid of iRSV-sensitised mice, after i.n. immunisation with NOMVs in the presence or absence of iRSV prior to challenge with live RSV. A significant decrease ($p<0.04$) in the percentage of eosinophils was

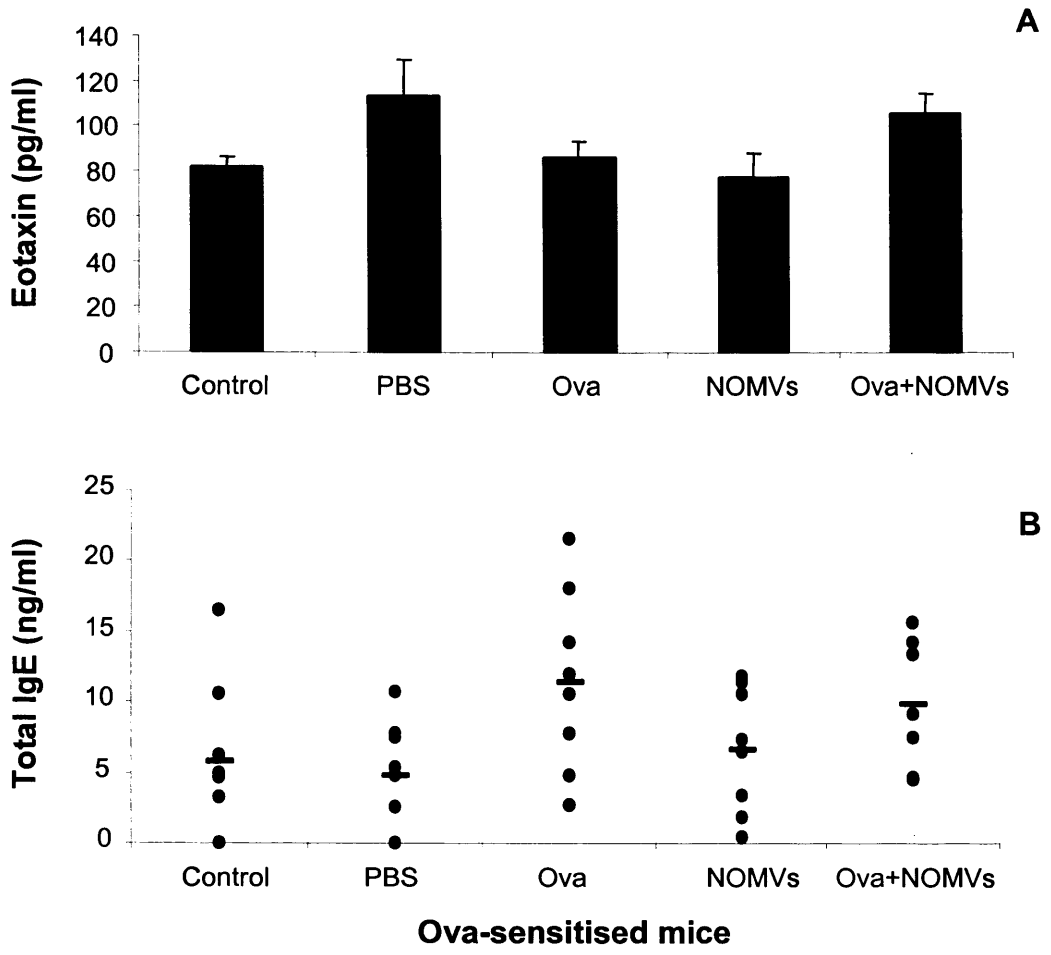

Figure 5.6 The effect of NOMVs on the production of eotaxin and IgE antibodies after sensitisation and challenge with Ova.

C57Bl/6 mice were immunised i.p. with Ova or PBS (control) once a week for two weeks. Two weeks later the mice were split into five groups and immunised i.n. with either PBS, Ova, NOMVs or Ova+NOMVs. After a further seven days the mice were all immunised i.n. with Ova and seven days later the mice were terminally bled and the lungs were removed. Supernatants from lung homogenates were assayed for (A) eotaxin (1 in 10 dilution) and sera was assayed for (B) total IgE (1 in 50 dilution). Individual mice are indicated by (•) and the mean as (—). These data are the means of duplicate wells from one representative study out of two.

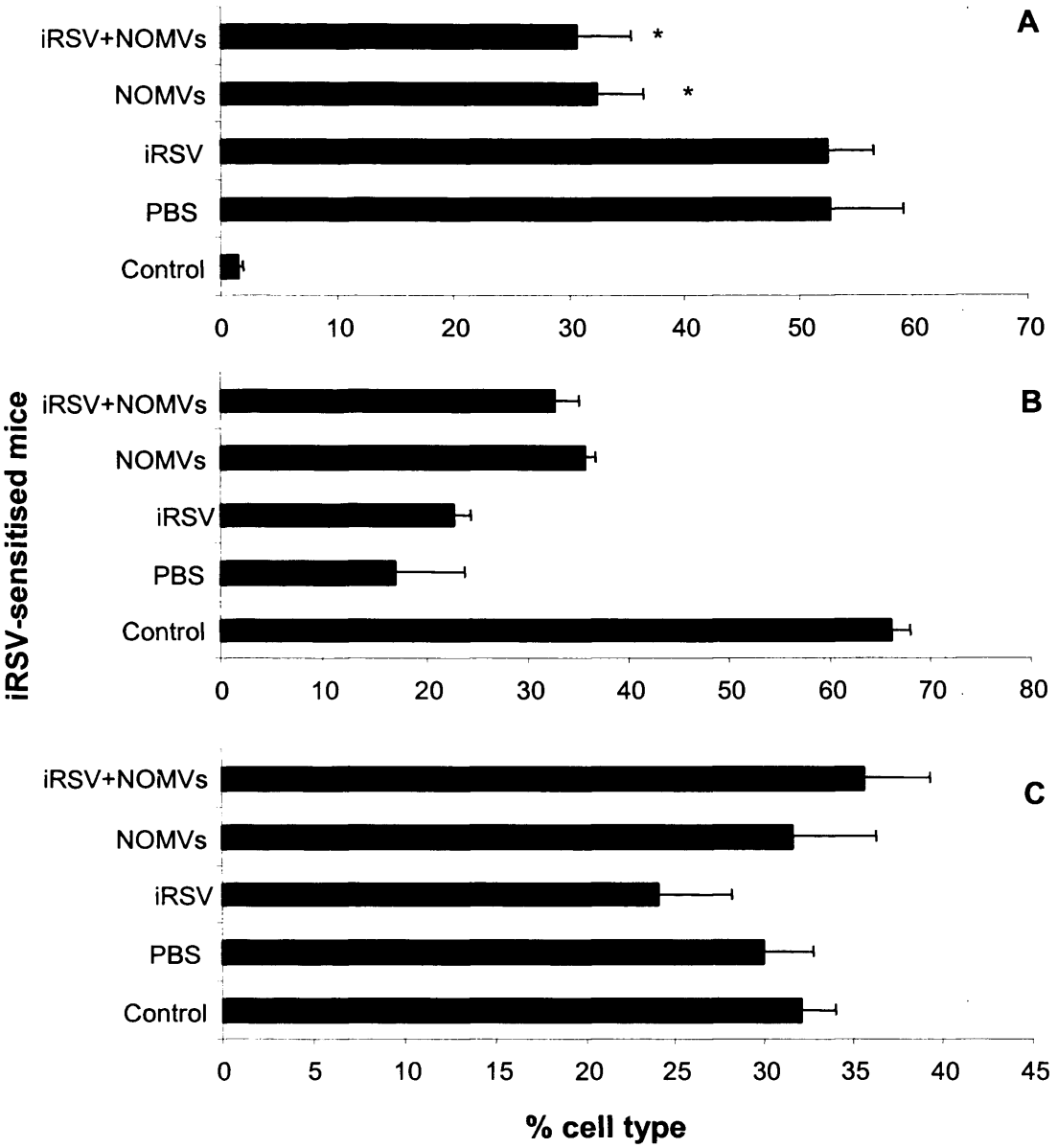

Figure 5.7 The effect of NOMVs on the percentage of iRSV-induced cell infiltrates after iRSV sensitisation and challenge with live RSV.

C57Bl/6 mice were immunised i.m. with iRSV or PBS (control) once a week for two weeks. Two weeks later the mice were then split into five groups and immunised i.n. with either PBS, iRSV, NOMVs or iRSV+NOMVs. After a further two weeks the mice were all immunised i.n. with live RSV and seven days later the mice were culled and BAL was carried out. The number of (A) eosinophils; (B) macrophages and (C) lymphocytes were enumerated by counting 15 random fields after Wright-Giemsa staining. The results are expressed as the mean and SEM from four mice. Asterisks indicate a value significantly different from that observed after i.n. infection with iRSV before live RSV challenge, *$p < 0.04$.

observed in mice that received NOMVs alone or with iRSV before live RSV challenge compared to mice i.n. immunised with iRSV alone. The percentage of macrophages was almost twice the amount in control mice than in mice that were sensitised i.m. with iRSV. However there was no statistically significant difference between the percentage of macrophages or lymphocytes among the different immunised groups of mice.

The absolute number of each cell type in the BAL fluid was calculated and as figure 5.8 shows, when cell numbers were calculated via this method there was no statistically significant decrease in the number of eosinophils after immunisation with NOMVs either in the presence or absence of iRSV. The average number of macrophages and lymphocytes (figure 5.8B and 5.8C) was again constant among the different infected groups of mice, however there was significant mouse-to-mouse variation in some of the groups.

In order to determine further whether NOMVs could significantly decrease lung inflammation using this model, the mice were immunised i.n. for three consecutive weeks instead of one week with PBS, iRSV, NOMVs or iRSV plus NOMVs before live RSV challenge. Once again a significant decrease ($p<0.025$) in the percentage of eosinophils was observed after immunisation with NOMVs alone or NOMVs plus iRSV compared to immunisation with iRSV alone (figure 5.9A). The percentage of macrophages and lymphocytes observed after three i.n. immunisations were similar to that after one immunisation (figure 5.9B and 5.9C). The total number of cells per ml of BAL fluid also indicated a significant decrease ($p<0.04$) in the number of eosinophils after immunisation with NOMVs with or without iRSV (figure 5.10A). The average number of macrophages was consistent among the

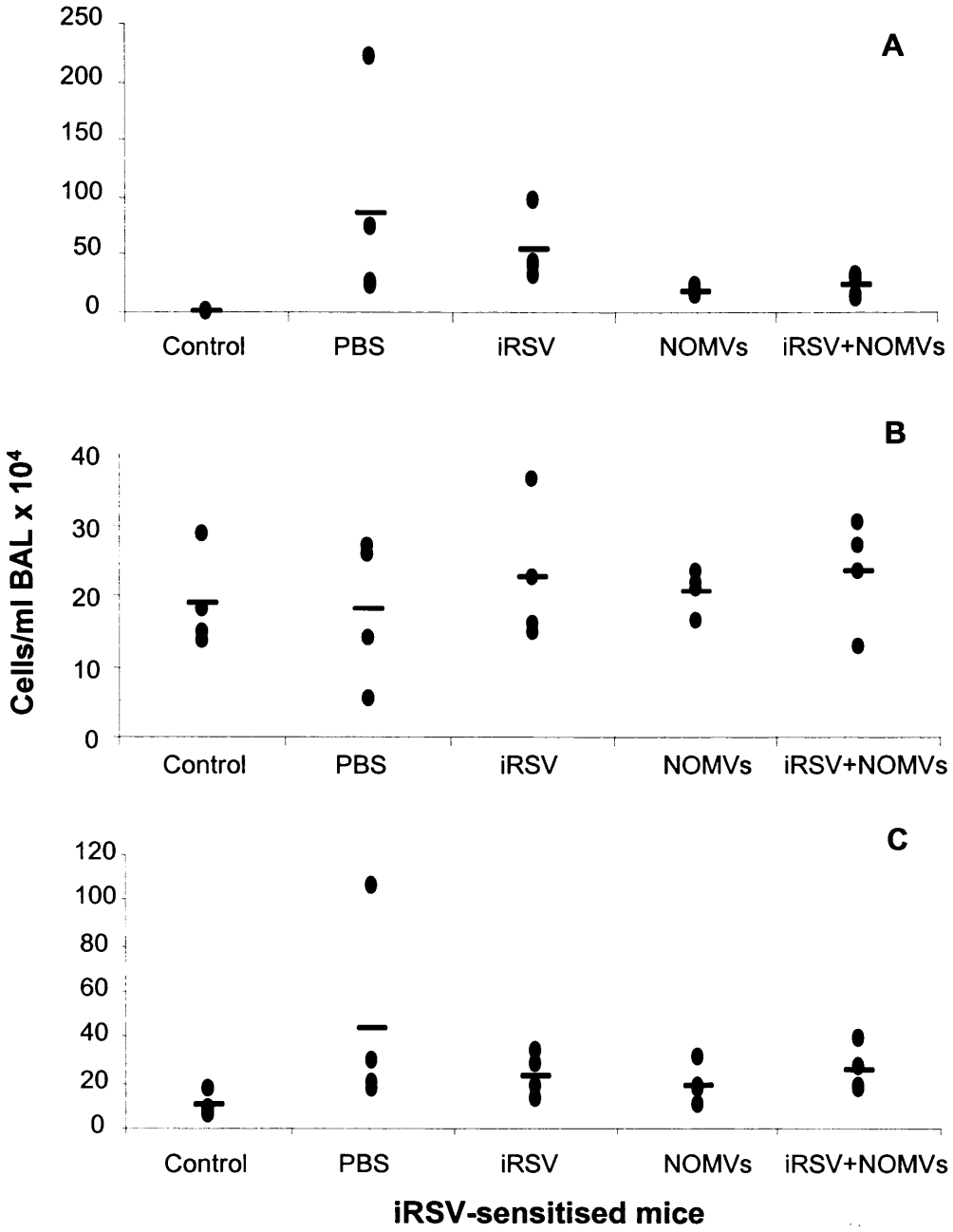

Figure 5.8 The effect of NOMVs on the total cell numbers in BAL fluid of mice sensitised and challenged with RSV.

C57Bl/6 mice were immunised i.m. with iRSV or PBS (control) once a week for two weeks. Two weeks later the mice were then split into five groups and immunised i.n. with either PBS, iRSV, NOMVs or iRSV+NOMVs. After a further two weeks the mice were all immunised i.n. with live RSV and seven days later the mice were culled and BAL was carried out. The number of (A) eosinophils; (B) macrophages and (C) lymphocytes were enumerated by Wright-Giemsa staining. Individual mice are indicated by (•) and the mean as (—).

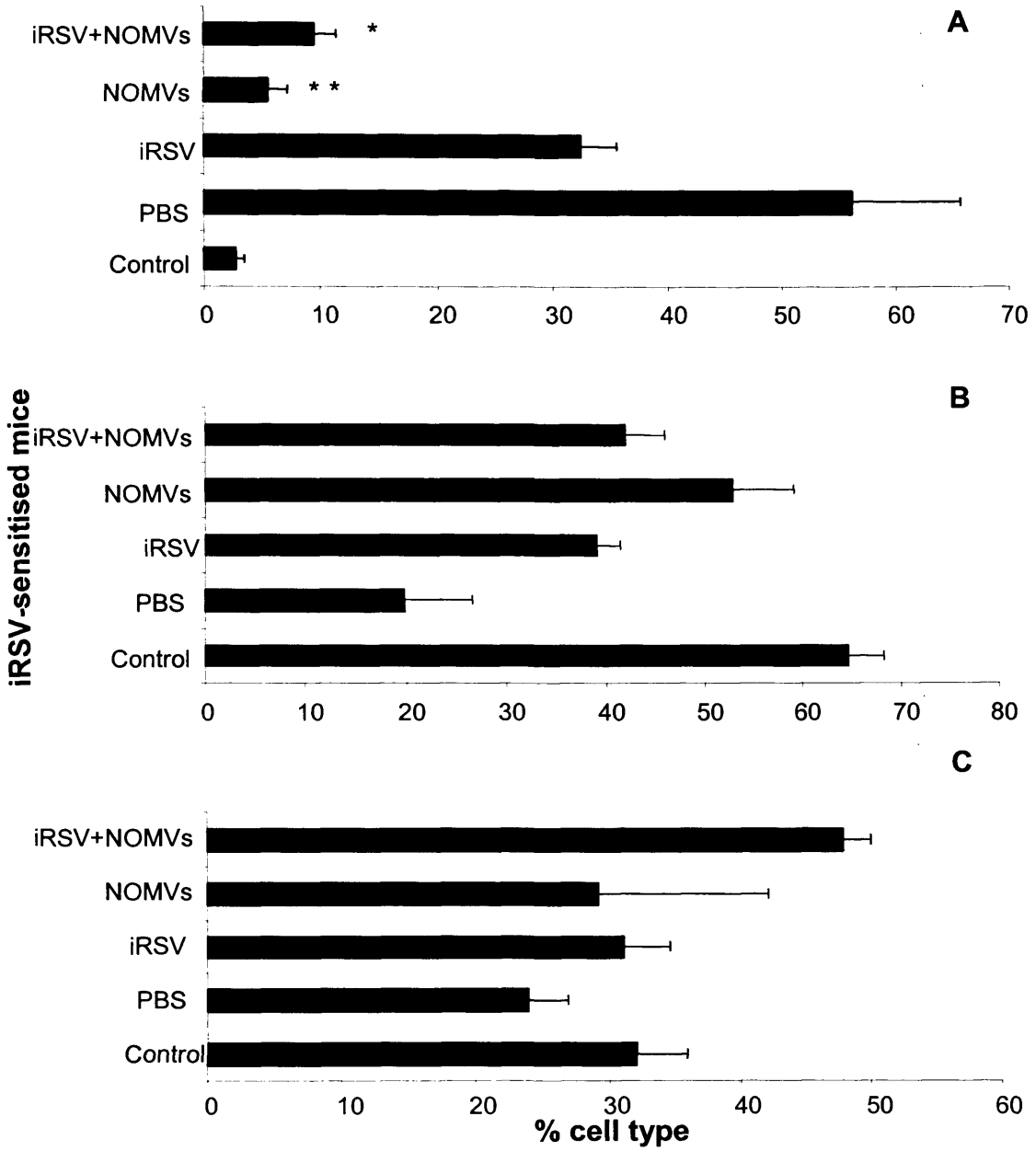

Figure 5.9 The effect of repeated exposure of NOMVs on the percentage of iRSV-induced cell infiltrates after sensitisation and challenge with RSV.

C57Bl/6 mice were immunised i.m. with iRSV or PBS (control) once a week for two weeks. Two weeks later the mice were then split into five groups and immunised i.n. once a week for three weeks with either PBS, iRSV, NOMVs or iRSV+NOMVs. After a further two weeks the mice were immunised i.n. with live RSV and seven days later the mice were culled and BAL was carried out. The number of (A) eosinophils; (B) macrophages and (C) lymphocytes were enumerated by counting 15 random fields after Wright-Giemsa staining. The results are expressed as the mean and SEM from four mice. Asterisks indicate a value significantly different from that observed after i.n. infection with iRSV before challenge with live RSV immunisation, * $p < 0.025$ and ** $p_{174} = 0.007$.

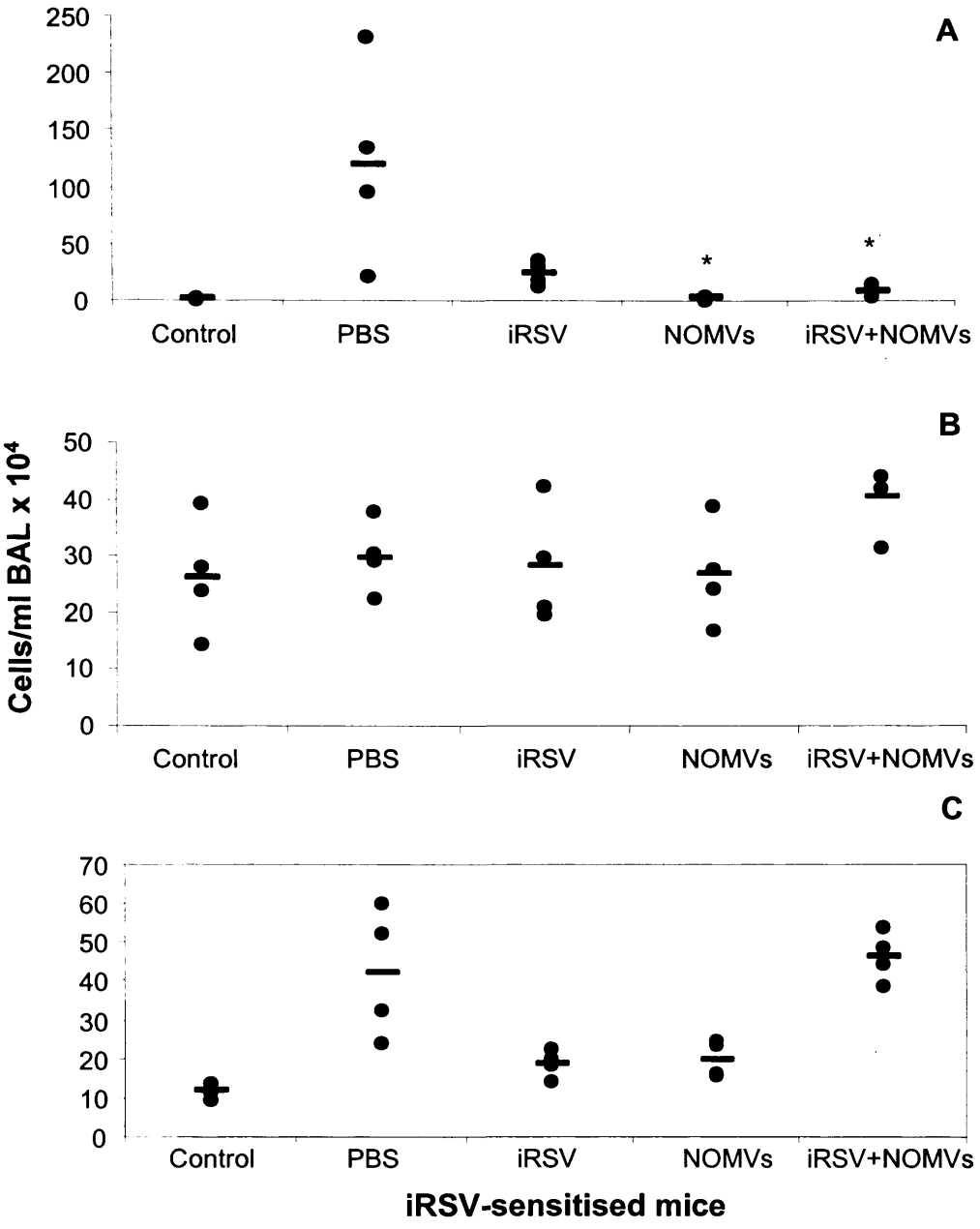

Figure 5.10 The effect of repeated exposure of NOMVs on the total cell numbers in BAL fluid of mice sensitised and challenged with RSV.

C57Bl/6 mice were immunised i.m. with either iRSV or PBS (control) once a week for two weeks. Two weeks later the mice were then split into five groups and immunised i.n. once a week for three weeks with either PBS, iRSV, NOMVs or iRSV+NOMVs. After a further two weeks the mice were challenged i.n. with live RSV and seven days later the mice were culled and BAL was carried out. The number of (A) eosinophils; (B) macrophages and (C) lymphocytes were enumerated by Wright-Giemsa staining. Individual mice are indicated as (•) and the mean as (—). Asterisks indicate a value significantly different from that observed after infection with iRSV before challenge with live RSV, * $p < 0.04$.

different immunised groups of mice (figure 5.10B), however the average number of lymphocytes in the groups immunised with PBS or NOMVs plus iRSV before live RSV challenge were slightly higher than the other groups, but this difference was not statistically significant. There was again significant mouse-to-mouse variation in the groups as observed previously.

5.3.5 The effect of NOMVs on the production of eotaxin.

The level of eotaxin in the supernatant of lung homogenates was determined after one and three i.n. immunisations with NOMVs in the presence or absence of iRSV before i.n. challenge with live RSV. After one i.n. immunisation (figure 5.11A) there was slightly less eotaxin found in the supernatant of mice that received NOMVs alone or NOMVs plus iRSV compared to mice receiving PBS or iRSV, however this decrease was not statistically significant. The level of eotaxin after three i.n. immunisations was fairly consistent among the different immunised groups, with levels between 40 – 60pg/ml as shown in figure 5.11B.

5.3.6 The effect of NOMVs on anti-NOMV antibody responses.

The amount of total IgE in serum was determined in individual mice of the different groups after one or three i.n. immunisations before challenge. Figure 5.12 shows that similar amounts of total IgE were found in sera of mice that received either one or three i.n. immunisations before challenge with live RSV. IgE antibodies were barely detected in mice that were immunised i.n. with PBS before live RSV challenge. The levels of IgE varied

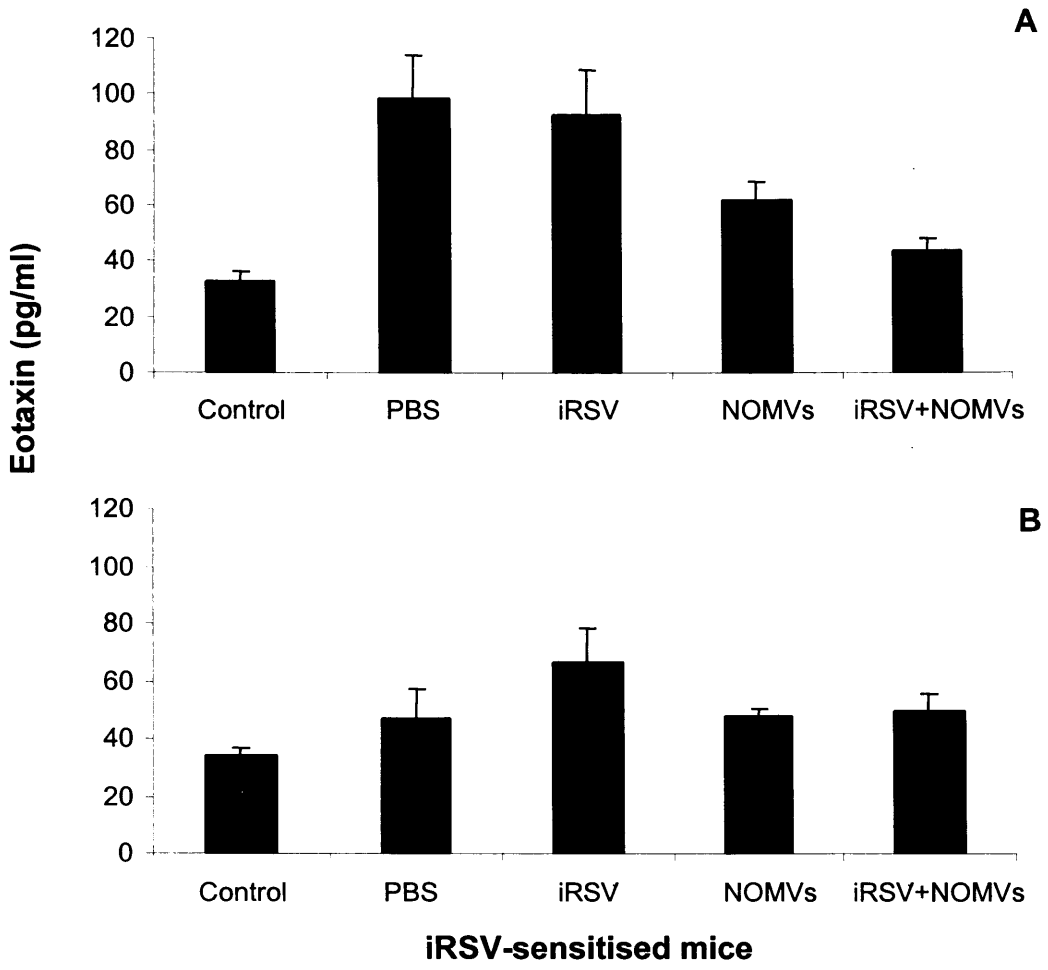

Figure 5.11 The effect of NOMVs on the production of eotaxin after sensitisation and challenge with RSV.

C57Bl/6 mice were immunised i.m. with iRSV or PBS (control) once a week for two weeks. Two weeks later the mice were split into five groups and immunised i.n. with PBS, iRSV, NOMVs or iRSV+NOMVs (A) once a week, or (B) once a week for three weeks. After a further two weeks the mice were all immunised i.n. with live RSV and seven days later the mice were culled and the lungs were removed. Supernatants from lung homogenates were assayed for eotaxin (1 in 10 dilution) using a mouse Eotaxin ELISA kit (R&D systems). Error bars represent the S.E.M.

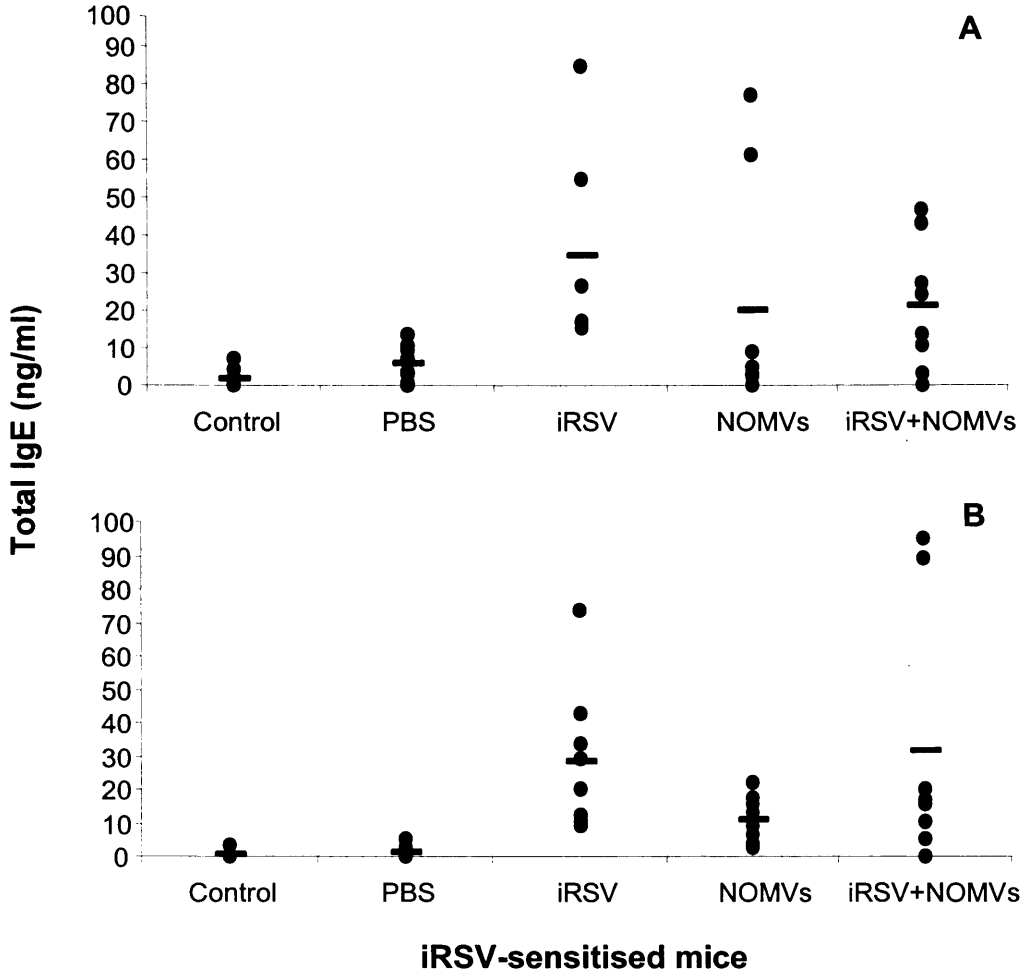

Figure 5.12 The effect of NOMVs on the levels of IgE antibodies in the serum of mice sensitised and challenged with RSV.

C57Bl/6 mice were immunised i.m. with either iRSV or PBS (control) once a week for two weeks. Two weeks later the mice were split into five groups and immunised i.n. with either PBS, iRSV, NOMVs or iRSV+NOMVs for (A) one week or (B) once a week for three weeks. After a further two weeks the mice were all immunised i.n. with live RSV and seven days later the mice were terminally bled. Serum from individual mice were assayed for total IgE (1 in 50 dilution) using a mouse IgE ELISA set (BD Pharmingen). Individual mice are indicated by (•) and the mean as (—).

quite considerably in individual mice of groups that were immunised with NOMVs in the presence or absence of iRSV.

The amount of NOMV-specific total IgG and IgG subclasses was also examined. Figure 5.13A shows a three-fold increase in the amount of total IgG in mice that were immunised with NOMVs in the presence or absence of iRSV. After a single i.n. immunisation, the IgG2b subclass predominated. Similar results were observed after three i.n. immunisations (figure 5.13B), however the levels of total IgG were slightly increased and the levels of IgG2b increased by approximately three-fold. The levels of IgG1, IgG2a and IgG3 were also slightly increased in mice that received NOMVs with or without iRSV after three i.n. immunisations.

5.3.7 The effect of NOMVs on the cytokines produced in response to lung inflammation.

We then determined whether NOMVs influenced the cytokine profile by measuring cytokine production in the supernatant of lung homogenates after one or three i.n. immunisations using a Th1/Th2 CBA assay. An increase in TNF-α production was seen after an increase of i.n. immunisations (figure 5.14B) however the pattern of cytokine release in the groups was not consistent between the two experiments. The level of IFN-γ also varied among the different immunised groups, however the levels were consistent after either one or three immunisations.

Figure 5.15 shows the amount of IL-2, IL-4 and IL-5 in the supernatant of the lung homogenates of individual mice. The level of IL-2 was low in all groups after one immunisation and did not increase after three immunisations. This

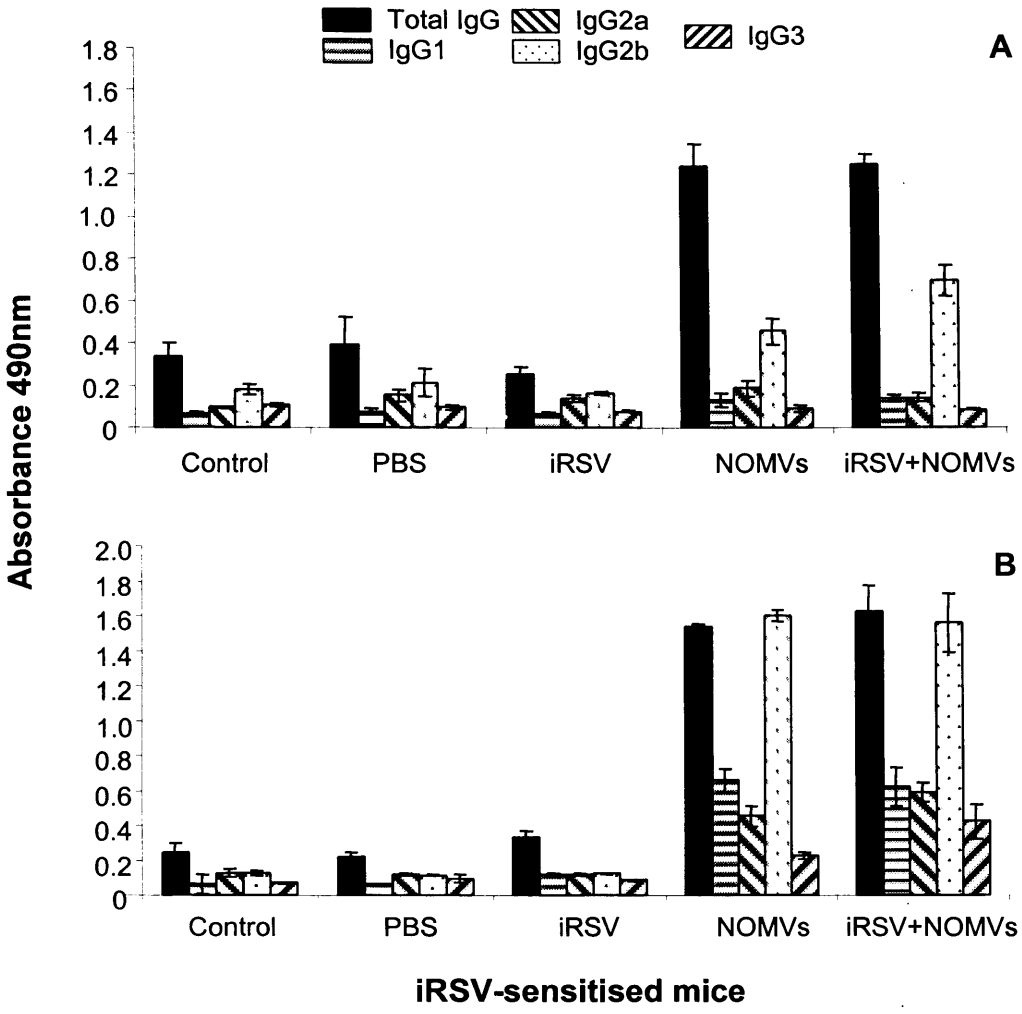

Figure 5.13 The effect of NOMVs on the production of NOMV-specific total IgG and IgG subclasses after sensitisation and challenge with RSV.

C57Bl/6 mice were immunised i.m. with iRSV or PBS (control) once a week for two weeks. Two weeks later the mice were split into five groups and immunised i.n. with PBS, iRSV, NOMVs or iRSV+NOMVs for either (A) one week, or (B) once a week for three weeks. After a further two weeks the mice were all immunised i.n. with live RSV and seven days later the mice were terminally bled. Serum from individual mice were assayed for total IgG and IgG subclasses (1 in 400 dilution).

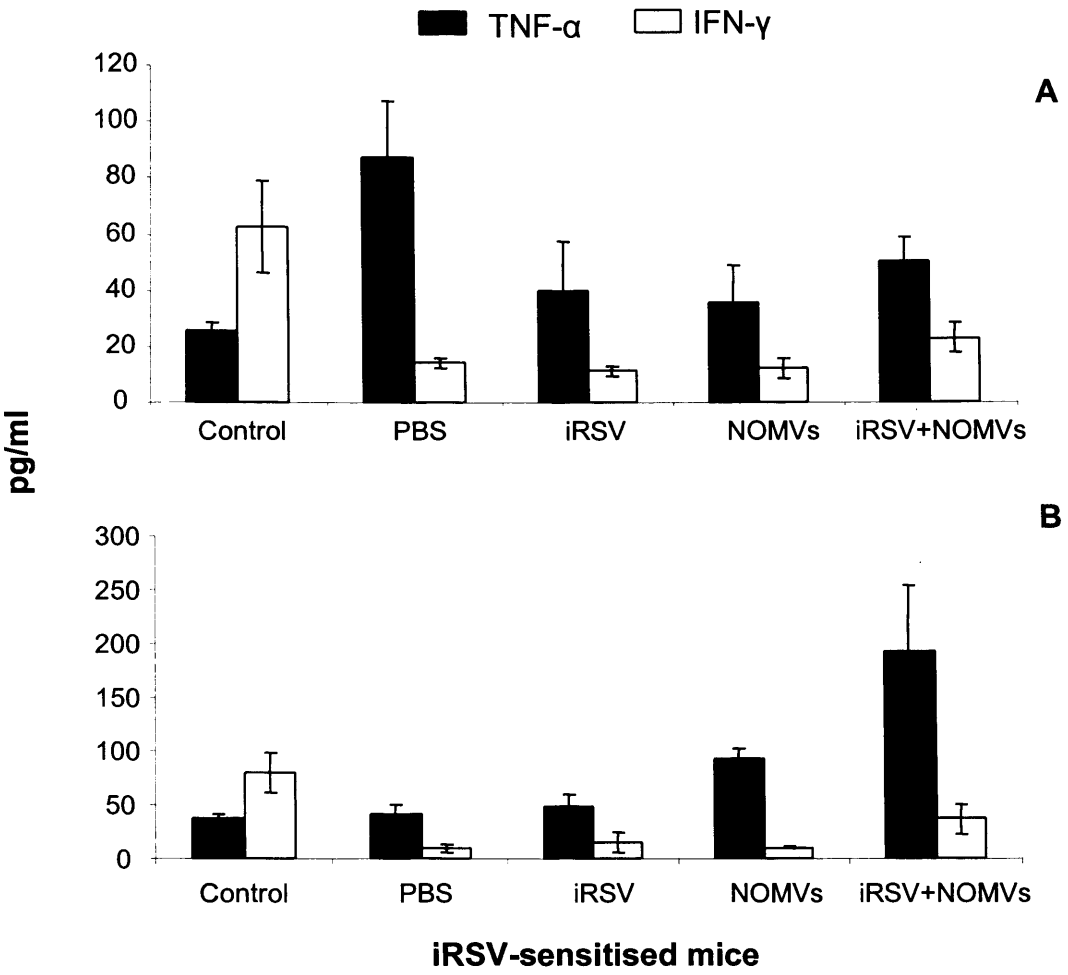

Figure 5.14 The effect of NOMVs on the production of Th1 cytokines during sensitisation and challenge with RSV.

C57Bl/6 mice were immunised i.m. with iRSV or PBS (control) once a week for two weeks. Two weeks later the mice were split into five groups and immunised i.n. with PBS, iRSV, NOMVs or iRSV+NOMVs for either (A) one week, or (B) once a week for three weeks. After a further two weeks the mice were all immunised i.n. with live RSV and seven days later the lungs were removed. The lungs were homogenised and the supernatant was used to measure cytokines using an inflammation cytokine bead array (CBA) kit. Error bars represent the S.E.M.

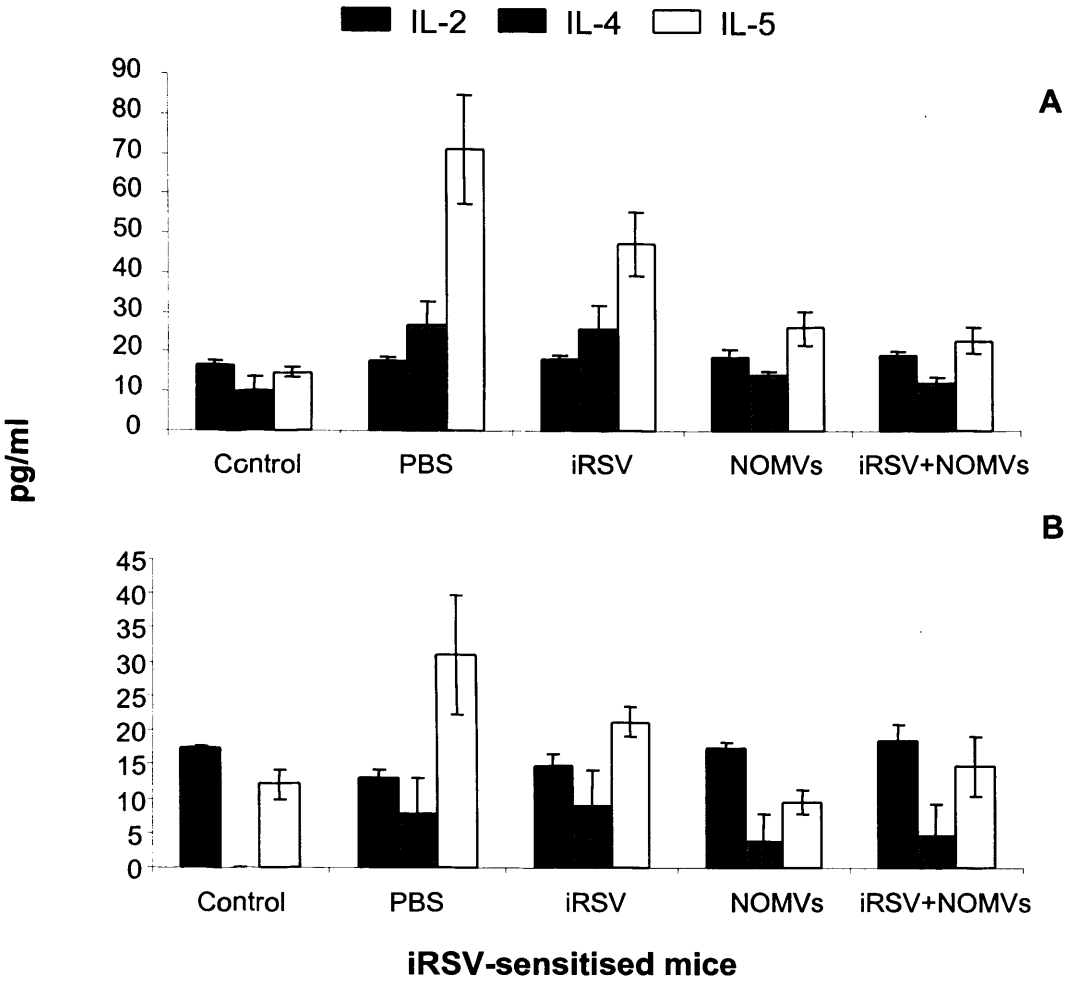

Figure 5.15 The effect of NOMVs on the production of Th2 cytokines during sensitisation and challenge with RSV.

C57Bl/6 mice were immunised i.m. with iRSV or PBS (control) once a week for two weeks. Two weeks later the mice were split into five groups and immunised i.n. with PBS, iRSV, NOMVs or iRSV+NOMVs for either (A) one week, or (B) once a week for three weeks. After a further two weeks the mice were all immunised i.n. with live RSV and seven days later the lungs were removed. The lungs were homogenated and the supernatant was used to measure cytokines using an Th1/Th2 cytokine bead array (CBA) kit. Error bars represent the S.E.M.

was also true for IL-4. The levels of IL-5 were higher in groups that received one i.n. immunisation with either PBS or iRSV before live RSV challenge. However these levels were not significantly decreased after immunisation with NOMVs in the presence or absence of iRSV. The amount of IL-5 was found to be approximately two fold less after three i.n. immunisations and responses tended to be higher in the groups that did not receive NOMVs, however again this difference was not statistically significant.

5.4 Discussion

It has been previously shown in animal models of allergen-induced asthma that exposure of the lung to bacteria or bacterial products can inhibit the development of lung inflammation and increase Th1 immune responses. We have previously shown and it has been reported in the literature that NOMVs are able to produce a Th1-biased immune response characterised by the production of IL-12 and IFN-γ. Experiments carried out by Etchart and colleagues (2006) demonstrated that i.n. immunisation of NOMVs reduced and/or prevented lung pathology caused by immunisation with RSV. Therefore experiments were carried out using two murine models of allergen-induced eosinophilia to further examine the effect of i.n. immunisation of NOMVs on lung inflammation and pathology.

Initial experiments were carried out using a model of Ova-induced eosinophilia. Mice i.n. immunised with NOMVs in the presence or absence of Ova showed a significant decrease in the percentage ($p < 0.017$) and the total number of eosinophils ($p < 0.005$) present in the BAL fluid compared to

mice immunised with Ova alone. This supports previous observations that bacteria and bacterial products are able to inhibit the accumulation of eosinophils in the lungs of mice infected with allergens (Erb *et al.*, 1998; Sur *et al.*, 1999). The percentage of each cell type in fifteen high-powered fields and the absolute number of each cell type in the BAL fluid were both calculated to take into account the variation in the total number of cells recovered from the mice during BAL.

The total number of macrophages and lymphocytes were slightly increased in the BAL fluid from mice immunised i.n. with Ova plus NOMVs compared to the other groups (figure 5.3B and 5.3C). It has been previously shown that macrophages infiltrate the lung during the sensitisation phase and after Ova challenge (Drazen *et al.*, 1996; Gonzalo *et al.*, 1996) and that the number of macrophages can increase after non-specific stimulation (Fokkens, 1999) therefore accounting for why we observed an increase of this cell type.

I.n. immunisation of NOMVs before allergen challenge prevented a further increase of eosinophilia in the lungs. This was demonstrated by a significant decrease in the percentage ($p = 0$) and the absolute number ($p < 0.002$) of eosinophils in the BAL fluid from mice that were immunised with NOMVs in the presence or absence of Ova compared to mice immunised with Ova alone. As expected the group of mice that were i.n. immunised with Ova before challenge with the allergen had the highest percentage and number of eosinophils (figure 5.4A and 5.5A) in the BAL fluid. As observed previously, higher numbers of macrophages and lymphocytes were observed in mice immunised with NOMVs, either in the presence or absence of Ova before

challenge with Ova, however this increase was not statistically significant. The total number of the different cell types varied among mice in the same immunised group, which could be due to factors beyond our control such as variation in the amount of sample reaching the lungs of mice due to sneezing or swallowing of the sample after i.n. administration.

An internal control group of mice were also included in these experiments to determine whether a single i.n. immunisation with Ova following sensitisation with PBS would encourage lung inflammation and therefore eosinophilia. A small percentage and therefore small number of eosinophils were found in the BAL fluid of these mice, indicating that sensitisation with Ova was needed to develop eosinophilia. These mice exhibited similar levels of macrophages and lymphocytes as the group of mice that were sensitised with Ova.

The results of these experiments demonstrated that i.n. immunisation of NOMVs are able to influence lung inflammation in mice characterised by the suppression of eosinophilia, which has not been previously demonstrated in literature and supports previous findings with other bacterial products.

Eotaxin has been reported to have a key role in allergic inflammation in particular its effect on eosinophils. Gonzalo and colleagues (1996) showed that neutralisation of eotaxin results in a decrease in the recruitment of eosinophils and therefore lung inflammation. As shown in figure 5.6, eotaxin levels were similar among all groups of mice, indicating that i.n. immunisation of NOMVs in the presence or absence of Ova did not have an effect in this model. Previous reports have determined the amount of eotaxin or the effect of eotaxin in several different ways. For example, some groups measured

the expression of eotaxin in the lung by Northern blot analysis using total RNA extracted from the lung (Gonzalo *et al.*, 1996), whereas others have used an anti-eotaxin antibody or eotaxin-knockout mice to assess the effects of eotaxin on the recruitment of eosinophils under different conditions (Boehme *et al.*, 2004). Therefore it may be better in future experiments to examine the effect of NOMVs on eotaxin levels by using one of these approaches as it could prove to be more sensitive.

As well as eosinophilia and eotaxin production being characteristic of allergen-induced asthma, another characteristic of a Th2-biased response is high levels of IgE. IgE plays an important role in allergic disease as antigen-crosslinking IgE, which is bound by high affinity receptors on mast cells and basophils causes the release of inflammatory mediators. The production of IgE is up-regulated by IL-4 and IL-13, which enhance the proliferation and class switching of B cells (Sutton *et al.*, 1993; Gould *et al.*, 2003). The levels of total IgE were found to be similar in all groups of mice, however there was considerable variation in IgE levels among mice within each group (figure 5.6B). The difference in levels of total IgE were not statistically significant between mice that were or were not immunised with NOMVs, although we did not investigate the possibility that IgG antibodies which are found in higher concentrations could be competing for binding with IgE antibodies as suggested by Lewkowich *et al.* (2004a; 2004b). The same group also describe how other researchers have developed ELISA systems that require extensive optimisation for each antigen examined which should be noted if in future studies the amount of Ova-specific and NOMV-specific IgE is to be measured.

As shown using the Ova model, i.n. immunisation of NOMVs can diminish lung inflammation and pathology demonstrated by a significant decrease in lung eosinophilia. We then examined whether NOMVs were able to produce a similar response in a murine model using iRSV. The immune response induced by viruses is usually characterised by the secretion of IFN-γ by NK cells and T cells which has the potential to inhibit Th2 responses, however infection with RSV has been found to increase allergen-induced airway eosinophilia identifying a possible link between RSV infection and allergic asthma (Schwarze *et al.*, 1997; Schwarze *et al.*, 1999).

I.n. immunisation of mice with NOMVs following sensitisation with iRSV and prior to challenge with live RSV significantly reduced the percentage of eosinophils in BAL fluid than in mice immunised with iRSV alone (figure 5.7). However a statistically significant decrease however was not observed when the percentage of eosinophils was calculated as the absolute number of eosinophils in the BAL fluid. From figure 5.8A it is apparent that there was mouse-to-mouse variation among the group that were i.n. immunised with iRSV before challenge with live RSV, and this variation could have occurred due to reasons previously described in this chapter. The total number of macrophages and lymphocytes among the groups stayed fairly constant, however mouse-to-mouse variation was again apparent in some groups. Unlike in the Ova-model, NOMVs did not bring about a statistically significant decrease in the total number of eosinophils. We have shown in Chapter one and previous studies have indicated that immune responses to NOMVs after i.n. immunisation can benefit from repeated exposure. Experiments carried out by Etchart and colleagues (2006) indicated that mice received three i.n.

immunisations with NOMVs in the presence or absence of iRSV before challenge with live RSV, therefore we carried out further experiments to determine whether repeated immunisation of NOMVs would decrease eosinophilia. Using this method a significant decrease in both the percentage of eosinophils and the absolute number of eosinophils was seen in mice that received NOMVs i.n. before live RSV challenge, indicating that repeated immunisation with NOMVs can significantly decrease lung pathology, in particular the accumulation of eosinophils. As previously observed the total number of macrophages stayed constant among the different immunised groups, and although the numbers of lymphocytes varied this was not statistically significant.

The level of eotaxin in the supernatant of homogenised lungs from individual mice among the different immunised groups after one or three i.n. immunisations was not significantly different however levels were slightly lower in mice that were immunised with NOMVs. It has been shown that eotaxin levels are upregulated after i.n. immunisation with RSV (Haeberle *et al.*, 2001) and a recent study by Matthews *et al.*, (2005) demonstrated that neutralisation of eotaxin reduced the accumulation of eosinophils in the lung as well as decreasing the production of IL-5. Therefore as well as measuring the levels of eotaxin, it could be advantageous in future experiments to determine the effect of NOMVs on eotaxin levels by neutralising eotaxin and then measuring the level of eosinophilia.

Unlike the Ova model where we did not observe a difference in total IgE serum responses among the different groups of mice, the levels in serum from mice in the RSV model varied among the groups. Very little IgE was detected in the sera of mice from the control group or the group that were sensitised with iRSV and then i.n. immunised with PBS before being challenged with live RSV. In the other groups, the amount of IgE varied from mouse-to-mouse (0 – 100ng/ml) therefore there was no statistically significant difference among the groups. It has been proposed that virus specific T cells producing IL-4 and IL-5 may induce the production of virus-specific IgE, therefore this could explain why we have detected slightly more IgE production in the mice immunised with iRSV. However it has also been reported that infection with RSV does not affect titres of total IgE in the serum of mice (Barends *et al.*, 2002; Barends *et al.*, 2004). In order to examine further the effect of NOMVs on the production of IgE it would also be beneficial to measure RSV-specific IgE responses.

The levels of NOMV-specific total IgG and IgG subclasses were also determined. Mice that received NOMVs either in the presence or absence of iRSV, demonstrated a significant increase in total IgG levels. These levels were slightly increased in mice that received three i.n. immunisations compared to one immunisation before challenge with live RSV. The subclass that dominated after immunisation with NOMVs was IgG2b, which was previously observed and shown in Chapter three, and is the subclass predominantly associated with a Th1 response. The levels of IgG2b were much higher after three immunisations and increased levels of IgG1, IgG2a

and IgG3 were also seen. Increased levels of IgG2a and IgG3 could be due to isotype switching induced by IFN-γ released by Th1 cells in response to stimulation with NOMVs, whereas an increase in IgG1 could have been induced by the production of IL-4 by Th2 cells, as a result of immunisation with RSV. In future experiments it would be advantageous to measure RSV-specific antibody production, allowing us to determine if NOMVs are able to influence the IgG subclasses produced after RSV immunisation and whether NOMVs can reduce the level of Th2 cytokines or increase the level of Th1 cytokines inhibiting the production of IgG1 and IgE antibodies.

In order to fully understand the antibody responses, the production of cytokines must be examined in order to determine their influence on the subclasses produced. Levels of Th1 and Th2 cytokines were measured in the supernatant of lung homogenates to determine whether immunisation with NOMVs had shifted a predominantly Th2 response associated with iRSV immunisation to a more Th1 response. There was no trend in the production of TNF-α or IFN-γ among the groups of mice after either one or three i.n. immunisations before challenge with live RSV. However there was a slight increase in TNF-α production in mice that received three immunisations with NOMVs either in the presence or absence of iRSV. After one i.n. immunisation, mice in all of the groups displayed similar amounts of IL-2 and IL-4, however increased levels of IL-5 were seen in mice that were sensitised and immunised with PBS or iRSV, but that did not receive NOMVs before challenge with live RSV. This trend was also observed after three i.n. immunisations however the levels were decreased by approximately two-fold. This indicates that immunisation of mice with NOMVs either in the presence

or absence of iRSV can slightly inhibit the production of IL-5, which could explain why there was a decrease in the accumulation of eosinophils in the lungs, as IL-5 mediates the recruitment and activation of eosinophils. It has also been shown in animal models that inhibiting IL-5 using neutralising antibodies prevents the terminal differentiation of eosinophils (Coffman *et al.*, 1989; Mauser *et al.*, 1995), over expression of IL-5 promotes long lasting eosinophilia and IL-5-deficient mice are unable to increase the number of eosinophils in response to antigens (Sanderson, 1992; Foster *et al.*, 1996; Kopf *et al.*, 1996).

Previous reports have determined cytokine levels in BAL fluid (Matthews *et al.*, 2005) or by measuring mRNA expression of different cytokines in BAL fluid (Waris *et al.*, 1996; Barends *et al.*, 2004), which could prove to be more sensitive and therefore more accurate. It has also been described in a recent report that treatment of mice with an anti-eotaxin antibody affected IL-5 levels, but not levels of IFN-γ (Matthews *et al.*, 2005). Therefore this could imply that although a change in Th1 cytokines may not be observed in mice immunised with NOMVs a change in Th2 cytokines would.

As previously mentioned IL-10 has been shown to have an important role in allergic responses possibly through inducing IL-10-secretory Tr cells. The results in Chapter Four indicate that NOMVs are able to stimulate IL-10 production *in vitro* and *in vivo* after i.m. injection, therefore this could indicate another possible mechanism in the suppression of allergic Th2 responses. The production of IL-10 in mice after i.n. immunisation with NOMVs could also be investigated by measuring the levels in sera after each immunisation

to determine when maximal production occurs, and see if that correlates with a decrease in allergic Th2 responses.

As demonstrated in this Chapter, NOMVs have been shown to have an effect on lung inflammation after i.n. immunisation in the presence or absence of allergens. In general, the most obvious effect was the influence of NOMVs on the level of eosinophilia in the lungs of mice. Cytokine, chemokine and antibody responses were also determined, however the results were not conclusive and further investigation is needed. There are many novel therapeutic agents and adjuvants being described as being able to inhibit airway inflammation and although the mechanism of how this is achieved is not fully understood, a couple of recent studies have indicated possible mechanisms. For example a recent study by Patel and colleagues (2005) described how the synthetic bacterial lipopeptide Pam3CSK4, a TLR2 agonist, could reduce eosinophilia and Th2 cytokine production by an IL-12 dependent mechanism. This has been followed by another recent study describing how lipoprotein I of *Pseudomonas aeruginosa* could decrease eosinophilia and modulate Th2 effector cells by stimulating TLR2 and TLR4 signalling pathways (Revets *et al.*, 2005). Delayre-Orthez and colleagues (2004) examined the effect of LPS on allergen sensitisation and challenge in a murine model. They found that LPS administered during the sensitisation phase prevented airway eosinophilia as seen in previous studies (Gerhold *et al.*, 2002a; Gerhold *et al.*, 2002b), whereas LPS given during challenge increased airway eosinophilia (Tulic *et al.*, 2000). This suggests that LPS may be an important factor in the severity of the disease, which should be

taken into consideration when assessing whether NOMVs could be used as a vaccine or adjuvant.

Many groups have examined the expression of TLRs on epithelial cells of the airway and it has been shown that epithelial cells express mRNA for TLRs 1 – 10 (Becker *et al.*, 2000; Muir *et al.*, 2004; Greene *et al.*, 2005). TLR2 is expressed predominantly on the cell surface along with TLR1 and TLR9 whereas TLR3, 4 and 5 reside intracellularly or at low-level surface expression. Both TLR4 and TLR5 have been shown to be able to move to the membrane following stimulation (Monick *et al.*, 2003; Adamo *et al.*, 2004). Airway inflammation is usually associated with bacterial or viral agents, and therefore it is very possible that TLRs are activated in order to produce a rapid and effective innate response and that changes in TLR function could influence inflammatory lung diseases.

It is plausible that NOMVs are also acting through TLR2 and/or TLR4 pathways in order to exert its effects. We have shown in the previous chapter that LPS and non-LPS components of NOMVs are able to cause cell proliferation and release cytokines therefore this is something that should be further studied and by using TLR knockout mice we could ascertain which component/s contribute to the findings in this chapter.

CHAPTER 6

Final Discussion

6.1 The immunomodulatory properties and possible mechanisms of action of NOMVs

In this thesis investigation the immunomodulatory properties of NOMVs from serogroup B meningococci were determined in order to assess the potential use of NOMVs and their components as a mucosal adjuvant, their ability to modify immune responses and to gain further insights into their role in establishing immunity against Nm.

Alum, the only adjuvant approved for human vaccines for many years, has been shown to enhance mainly humoral responses to protein antigens and glycoconjugates. However for a number of viruses and tumours, cell-mediated immunity correlates with protection. Thus there is an urgent need of identifying adjuvants that can enhance T cell responses for subunit vaccine candidates for viral infections and cancer. It is also a key aim in vaccine development that adjuvants and antigens can be administered mucosally because there are many infections occurring at mucosal surfaces where immune protection mechanisms need to be activated. It is important that new adjuvants must meet rigorous safety requirements including having low toxicity, being stable and well defined. Most importantly adjuvants need to be able to stimulate strong and appropriate

immune responses. It has been established that initiation of an immune response requires the activation of APCs by danger signals derived from foreign or host cells. Not surprisingly microbial products, mainly TLR ligands, have been extensively investigated as adjuvant candidates. For example, CpG DNA on binding TLR9 enhances immune responses resulting in a predominantly Th1 response and LPS has been shown to be highly immunogenic and display adjuvant properties on binding TLR4.

NOMVs from serogroup B meningococci are an interesting adjuvant candidate. They are produced naturally by Nm and are exposed to mucosa and nasal associated lymphoid tissues (NALT) during Nm colonisation. They contain a high proportion of LPS and OMPs, both TLR agonists, and therefore they display adjuvant properties (Wetzler *et al.*, 1996; Quakyi *et al.*, 1997; Al-Bader *et al.*, 2003; Al-Bader *et al.*, 2004). In the absence of added adjuvant, NOMVs have been shown to be effective in inducing bactericidal antibodies when administered i.n. (Drabick, 2000; Guthrie *et al.*, 2004). The mechanism of action of NOMVs has not been defined clearly, but the engagement of LPS and porins with TLRs are thought to contribute significantly to their effects. LPS is generally considered to be toxic due to the lipid A moiety, however the removal of this portion brings about a decrease in immunogenicity including anti-LPS antibody responses. Modified forms of NOMVs such as DOMVs and VSSPs with reduced LPS content and its associated toxicity have been used in vaccines against serogroup B Nm in favour of NOMVs (Bjune *et al.*, 1991; Fredriksen *et*

al., 1991; Sierra *et al.*, 1991; Boslego *et al.*, 1995). However studies in humans and animals using NOMVs i.n. have shown them to be well tolerated with no harmful side-effects (Drabick, 2000).

The use of NOMVs as a mucosal adjuvant has not been extensively studied, but it has been demonstrated that i.n. immunisation can induce both systemic and mucosal immunity (Dalseg *et al.*, 1999; Saunders *et al.*, 1999; Guthrie *et al.*, 2004). In this thesis investigation, it was shown that NOMVs were able to enhance serum antibody responses to model TD and TI antigens. I.n. immunisation of C57Bl/6 mice with Ova plus NOMVs significantly increased Ova-specific IgG and IgA levels compared to immunisation with the antigen alone. NOMVs were also able to significantly enhance IgG and IgA responses to DNP-ficoll, a model TI-2 type antigen after i.n. immunisation. In a parallel study in the Carbohydrate Immunology group, it was demonstrated that the requirement for complement in IgM antibody responses to 11 of 12 CPSs tested was not identical to DNP-ficoll (Jones *et al.*, submitted manuscript). We thus wished to determine whether or not NOMVs could enhance antibody responses toward clinically relevant CPS antigens as they did to DNP-ficoll, a prototypical TI-2 antigen.

We focused our studies on antibody responses to CPSs because they have been shown to be important in protection against infection with extracellular bacteria. We chose to study CPSs from *S. pneumoniae* because this organism

is important to world health and new pneumococcal vaccines and improvement to existing vaccines are needed. Developing vaccines against *S. pneumoniae* is problematic due to the presence of many serotypes that cause disease, and antibody responses are variable with their responses being serotype and age dependent. However, antibody responses to pneumococcal CPSs are characteristic of TI-2 antigens. Anti-CPS antibodies are generally of low affinity with limited isotype switching, and CPS immunisation does not generate memory (Mosier *et al.*, 1982). Although conjugate pneumococcal vaccines have proved successful, even in children, to the serotypes included in the vaccine, there are nevertheless concerns that other serotypes not covered by the current conjugate vaccines will become prevalent. Conjugate vaccines are also unaffordable to populations where the burden is highest. Adjuvants are being investigated to improve anti-CPS responses in young children that will provide alternative means of protecting against *S. pneumoniae* and other encapsulated bacterial pathogens. NOMVs having demonstrated adjuvant effects on protein and LPS antigens from NOMVs as well as model TD and TI antigens were expected to have similar effects on anti-CPS antibody responses.

Serum antibodies to the CPSs were mostly of the IgM subclass after both i.p. and i.n. immunisation and the response varied due to the difference in immunogenicity of the CPSs. Natural IgM antibodies to the CPSs were seen in naïve mice and the levels varied depending on the strain of mice. B-1a cells from the spleen have been shown to be a source of natural IgM and to respond

rapidly to TI antigens such as CPSs (Hayakawa *et al.*, 2000), which could account for why responses to some CPSs were higher after i.p. than i.n. immunisation. Immunisation of the CPSs in the presence of NOMVs by both immunisation routes did not enhance the IgM response to the individual CPSs.

However it was found that immunisation with the CPSs in the presence or absence of NOMVs induced relatively long-lived antibody responses, which was demonstrated by the presence of IgM antibodies specific to the CPSs in serum two months post immunisation. Pneumococcal CPSs have a relatively long half-life, therefore they degrade slowly which would account for the sustained antibody levels seen (Sela *et al.*, 1972).

Antibody responses to type 3 and type 4 CPSs after i.n. immunisation were not dependent on dose and IgG production was rare and varied between individual mice. Where CPS-specific IgG antibodies were detected after i.p. or i.n. immunisation, the IgG3 subclass predominated, which is the subclass that has been shown to be protective against pneumococcal infection in mice (Perlmutter *et al.*, 1978; Peeters *et al.*, 1991). A booster immunisation with CPS did not have any effect on the antibody response to the CPSs, which is consistent with literature that TI antigens are unable to induce memory responses. Mice immunised i.n. with NOMVs alone displayed levels of CPS-specific IgM that were similar to that of naïve mice however further immunisation with NOMVs produced a marked increase in CPS-specific IgM indicating an antigen-independent mechanism of activation of CPS-specific B cells and/or of B cells

with cross-specificity for CPS structures. Cross-reactivity is often observed among polysaccharide antigens containing similar oligosaccharide residues, and the ability to produce a broad rather than CPS serotype-specific IgM response for anti-bacterial defences is beneficial. It is possible that immunisation with NOMVs stimulates a B cell population that has already been pre-selected or -activated by CPSs or cross-reacting antigens.

Evidence suggests that natural immunity to meningococcal disease may also occur due to colonisation with the non-pathogenic *Neisseria lactamica*, which shares cross-reactive antigens with Nm. It is worth noting that OMVs from *N. lactamica* only differ to meningococcal NOMVs in that they do not contain PorA (Sanchez *et al.*, 2001; Oliver *et al.*, 2002; Braun *et al.*, 2004). A recent study has shown that i.n. immunisation of mice with OMVs from *N. lactamica* to induce cross-reactive protective antibodies to Nm and to enhance antibody responses to the model antigen HBsAg (Sardinas *et al.*, 2006). Data on natural immunity to Nm by OMPs is limited, and it is not clear whether antibody responses need to be directed toward one particular antigen or several antigens. Antibody responses to serogroup B CPSs may not be important in immunity against Nm in our studies as we have shown that NOMVs are able to stimulate antibody responses to protein and LPS as well as model TD and TI antigens, but not pneumococcal CPS antigens. This indicates the importance of non-capsular antigens and responses to multiple antigens would be beneficial against an organism such as Nm that demonstrates antigenic variation. Natural immunity is

thought to occur at mucosal surfaces and therefore both mucosal and systemic immune responses are thought to be involved. Davenport and colleagues (2003) demonstrated mucosal T cell responses to meningococci following *in vitro* studies with cells from palatine tonsils. In addition i.n. immunisation of NOMVs has been shown to induce mucosal immunity in the NALT with the D-NALT being a major site for anti-meningococcal antibody forming cells, as well as other mucosal surfaces (Guthrie *et al.*, 2004; Sardinas *et al.*, 2006). It would therefore be appropriate to investigate further mucosal immune responses in future studies to complement findings described in this thesis.

Our results indicate that enhancement of antibody responses by NOMVs is dependent on the nature of the TI-antigen, as humoral responses to DNP-ficoll were enhanced by NOMVs whereas responses to pneumococcal CPSs were not. Our results support earlier findings by Kovarik *et al.*, (2001) who reported similar results but using CpG-ODN, TNP-ficoll and CPSs following s.c. immunisation. The group only observed enhancement of antibody responses to CPSs after conjugation of the CpG motifs to the CPSs, which was in agreement with a study where enhanced antibody responses were only seen after CpG-ODN was conjugated to Hib polysaccharide (von Hunolstein *et al.*, 2000). Polysaccharide-protein conjugates are generally more immunogenic than polysaccharides on their own as they can recruit T cell help. This enables class switching of antibodies from IgM to IgG and the induction of a stronger and broader antibody response. In future studies it would therefore be worth

investigating whether conjugation of NOMVs or addition of meningococcal OMPC to pneumococcal CPSs would help to enhance antibody responses. Unmodified OMPC was an effective adjuvant and protein carrier for the polysaccharide, PRP, in the Hib vaccine (Heath, 1998).

The possible mechanisms of how NOMVs are acting as an adjuvant were explored. NOMVs had pleiotropic effects on several immune cell types as suggested by the production of key cytokines and factors involved in innate and adaptive immunity. These include NO, interferons, TNF-α, IL-10 and IL-12. NOMVs were shown to cause the release of NO, a versatile molecule in host defence, from C3H/HeN and C3H/HeJ BMDMs. The activation of human DCs with meningococci has been well documented as shown by upregulation of TNF-α, IL-6 and IL-8 and costimulatory molecules such as CD86 and MHC II molecules (Dixon *et al.*, 2001; Kolb-Maurer *et al.*, 2001; Unkmeir *et al.*, 2002; Al-Bader *et al.*, 2003). In this study we showed the production of IL12p70, IL-10, TNF-α, MCP-1 and IL-6 in the supernatant of BMDCs from C3H/HeN and C3H/HeJ mice stimulated with NOMVs. In contrast, stimulation of splenocytes induced IFN-γ as well as the other cytokines mentioned, but not IL12p70. IFN-γ is produced by a variety of cells including NK cells and Th1 cells. It plays an important role in both innate and adaptive immunity as IFN-γ can modulate cellular immunity mediated by Th1 cells and humoral immunity by inducing the production of IgG2a and IgG3, which is characteristic of a Th1 response. This makes IFN-γ produced after stimulation with NOMVs an important factor in

NOMVs being used as an adjuvant due to the effects it can have on the immune system. Immunisation of mice with CPSs plus NOMVs led to the production of IgG3, which could have been as a result of the induction of IFN-γ. Splenocytes cultured with IL-10R prior to stimulation with NOMVs, induced the production of IL12p70 in both C3H/HeN and C3H/HeJ mice indicating that IL-10 was negatively regulating the production of IL12p70. Thus identification of the source of IL-10 and the mechanism of its induction by NOMVs would be important for the application of NOMV or its components as adjuvant for cell-mediated Th-1 responses.

In addition to IFN-γ, NOMVs also stimulated the production of IFN-β but predominantly from splenocytes of C3H/HeN mice, indicating that production is dependent on LPS. Further work carried out in the group showed the production of IFN-β as well as IFN-α in sera from C3H/HeN mice after i.m. immunisation with NOMVs (Durand *et al.*, manuscript in preparation). Type I interferons are not only important in innate immunity but have also been found to enhance T and B cell responses (Le Bon *et al.*, 2002). In this thesis, it was shown that NOMVs were able to stimulate proliferation of B cells, but not T cells, in both wild-type and LPS-hyporesponsive mice, indicating that LPS and non-LPS components are capable of inducing B cell proliferation. The induction of IL-6, IL-10 and type I interferons by NOMVs is likely to contribute significantly to the quality and magnitude of the antibody response, although we have also shown that NOMVs are able to induce B cell proliferation directly.

Our studies confirm and build on those already published that LPS and non-LPS components of NOMVs contribute to its immunomodulatory and adjuvant properties. Preliminary investigations into which components were responsible for these properties were carried out. Preparative SDS-PAGE (Bio-Rad Model 491 Prep Cell) was used to separate the components of NOMVs. Analysis of the gels by silver staining revealed that many of the fractions showed multiple bands indicating that some of the components were not separated sufficiently. Therefore we were unable to determine which individual components had immunomodulatory properties because several attempts of purifying individual components by preparative SDS-PAGE were unsuccessful. To truly establish separation of individual components, optimisation of this method or the use of an alternative approach such as HPLC would be required.

Having demonstrated that NOMVs have the ability to activate APC, induce characteristics of Th1 immune responses and stimulate B cell responses, we went on to investigate its effect on Th2 immune bias responses. Since allergen-induced airway inflammation is associated with Th2 cells that promote airway eosinophilia, we studied the effects of NOMVs using murine models of allergen-induced eosinophilia.

6.2 The effect of NOMVs on lung inflammation and immunity

Allergy is a main risk factor for asthma and is a chronic disease in children. Allergic asthma is a Th2-associated disease characterised by airway inflammation, the infiltration of eosinophils, elevated levels of serum IgE and the production of IL-4 and IL-5. The incidence of asthma is steadily increasing and it has been hypothesised that this is partially due to a decrease in exposure, early on in life, to infections that generally elicit Th1-biased immunity. There has been a lot of attention on novel treatments for decreasing allergic responses using bacteria and bacterial products that induce Th-1 responses. We have previously shown that NOMVs are able to produce a characteristic Th1 response as indicated by the production of IL-12p70 from BMDCs and splenocytes and IFN-γ from splenocytes. Therefore the main aim of this part of the thesis was to determine whether i.n. immunisation of NOMVs can affect lung inflammation in murine models of allergen-induced asthma by biasing the immune response.

Our studies demonstrated that NOMVs were able to significantly decrease eosinophilia in the lungs of mice from both infection models, and challenge with the allergens did not result in an increase in eosinophilia. The levels of eotaxin and total IgE were found to be similar among all the groups of mice in the Ova-induced allergy model. In contrast in the RSV model, the levels of eotaxin were slightly reduced in mice that received NOMVs and serum IgE levels were higher in mice that received iRSV in the presence or absence of NOMV compared to

control mice, although the responses differed among mice of the same immunisation group. High levels of NOMV-specific IgG2b were seen after i.n. immunisation with NOMVs or iRSV plus NOMVs, which supports the findings in chapter three. In future experiments it would be important to examine RSV-specific antibody responses to determine whether NOMVs are able to influence the subclass of antibody produced by iRSV therefore influencing either a Th1 or a Th2 response. The cytokines produced in response to immunisation with NOMVs and the allergens can also influence the subclass of antibody produced. The production of IL-4 would drive isotype switching of B cells toward the production of IgE, whereas IFN-γ would encourage the production of IgG2a and IgG3, which are characteristic of a Th1 response. However, serum levels of IFN-γ were found to be similar among all the groups of mice, as were levels of IL-4 in the supernatant of lung homogenates, indicating that immunisation with NOMVs either in the presence or absence of iRSV did not influence cytokine production. It may have been better to measure cytokine responses in BAL fluid as other groups have demonstrated this method to be more sensitive and reliable and therefore this approach should be used in future investigations.

We have previously shown that components of NOMVs are able to produce Th1 associated cytokines and that they are able to activate immune cells through TLR2 and TLR4. TLR2 is expressed on the surface of epithelial cells of the airway and TLR4 is expressed intracellularly although it has been shown to move to the membrane following stimulation, therefore indicating that the

interaction of NOMVs with TLR2 and TLR4 could favour the generation of a Th1 rather than a Th2 immune response. In order to examine this further and to dissect the mechanism of action of NOMVs in preventing lung inflammation it would be interesting to carry out similar experiments as described in chapter five but in TLR2- and TLR4-deficient mice that were not available during the course of this thesis investigation.

As well as generating adaptive immune responses to pathogens, recognition of PAMPs by TLRs can initiate the production of T regulatory (Tr) cells, which have been shown to suppress airway inflammatory responses. This was shown in a study where mice pre-treated with killed mycobacteria resulted in the development of a $CD4^+$ Tr population (Zuany-Amorim *et al.*, 2002). A further study also showed this response following infection with *Bordetella pertussis* and that filamentous haemagglutinin induced the production of IL-10 by DCs, which can induce the development of Tr cells from naïve T cells (McGuirk *et al.*, 2002). Studies in this thesis indicated the production of IL-10 *in vitro* by BMDCs and splenocytes and *in vivo* after injection with NOMVs. This could provide another mechanism of how responses to NOMVs are suppressing the effects seen in the allergy-induced models. Therefore it would be valuable in future studies to determine whether Tr cell populations are being induced.

The production of IL-10 has been reported as a consequence to stimulation with LPS (Tulic *et al.*, 2000) and as previously described in Chapter five, LPS has

been reported to have differing effects on allergic responses, which needs to be taken into account if NOMVs are going to be used as an adjuvant or novel therapy. It is a possibility that immunisation with NOMVs could also cause inflammatory responses and tissue destruction but this does not appear to be the case via i.n. immunisation in humans. IL-10 and TGF-b contribute to the anti-inflammatory, immunosuppressive environment of the upper and lower respiratory tract, and there was no indication that NOMV applied i.n. can overcome a Th-2 bias. It is also not certain what would happen after a longer time interval before rechallenge with the allergen. These are aspects that should be examined in future experiments involving NOMVs and compared with other approaches in the literature such as CpG ODNs or BCG.

6.3 Final Summary

The studies in this thesis have built on the previous literature describing immunomodulatory properties of NOMVs. We have shown that NOMVs are immunogenic when administered by the i.n. route and display adjuvant properties by increasing antibody responses against a model TD and a model TI antigen. In contrast, an adjuvant response by NOMVs was not observed after immunisation with CPSs from *S. pneumoniae,* which are clinically relevant TI-2 antigens.

Both LPS and non-LPS components appear to be responsible for the adjuvant and immunomodulatory properties of NOMVs by their ability to stimulate BMDCs

and splenocytes through TLR2 and TLR4 pathways. The immune response was seen to be Th1 biased, in agreement with previous literature.

Pathogens and microbial components associated with producing a Th1-biased immune response have been used to study lung inflammation, allergic responses and their effect on Th2 associated responses. The work shown in this thesis indicates the potential of NOMVs as an adjuvant to prevent eosinophilia in murine allergy-induced models. The mechanism of how this is achieved needs further investigation but one possibility is that immunisation with NOMVs enhances the maturation of APCs via TLR2 and TLR4 pathways to prime and differentiate some naïve T cells to T regulatory cells. Further studies need to be carried out to determine the full effect of NOMVs in these models with respect to mucosal antibody and cytokine responses, the components of NOMVs that are responsible for the effects and the potential dangers of inducing Th1 responses via the i.n. route in order to enhance cell-mediated immunity or to attenuate lung inflammation.

Together these results indicate that NOMVs are able to produce immunity against meningococcal disease, and their immunomodulatory properties can be utilised in future adjuvant development and immunotherapy.

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