4 Characterisation of innate immune responses shortly after intranasal immunisation of model antigens.

4.1 General introduction

The nasal route for vaccination offers some important opportunities for the prophylaxis of many diseases. It is a particularly attractive approach due to the functionality of the CMIS, i.e. induction of immune responses at local sites can lead to effector responses at distant mucosal sites. Consequently, the rational design of nasal vaccines for clinical use depends on the availability of information about the mechanisms that lead to a mucosal immune response after intranasal vaccination (Ogra, et al. 2001).

The role of lymphoid tissues in respiratory tract defences includes antigen uptake and processing and presentation for the induction of mucosal immune responses. This has been found to occur in the secondary organised lymphoid aggregates, called the BALT, situated in the bronchial wall, and the NALT, which is situated at the nasal entrance to the pharyngeal duct. Other important parts of the respiratory tract that play roles in immune development include the draining LNs. In fact, it is the NALT that is the first point of contact for many inhaled antigens, and consequently plays a major role in both induction and effector immune responses, which are then further amplified in the draining CLN. Reviewed in (Bienenstock and McDermott 2005). In humans, the nasopharyngeal region also contains a high density of immune competent cells similar to the NALT, most notable in the Waldeyer's ring which consists of the tonsils and adenoids (Perry and Whyte 1998). As described in Chapter 1, NALT is a well organised structure, consisting of B and T-cell areas with surrounding APC, which are covered by an epithelial layer containing M-cells (Asanuma, et al. 1997; Karchev and Kabakchiev 1984; Kuper, et al. 1990). See Figure 4.1 for location and structure of the NALT.

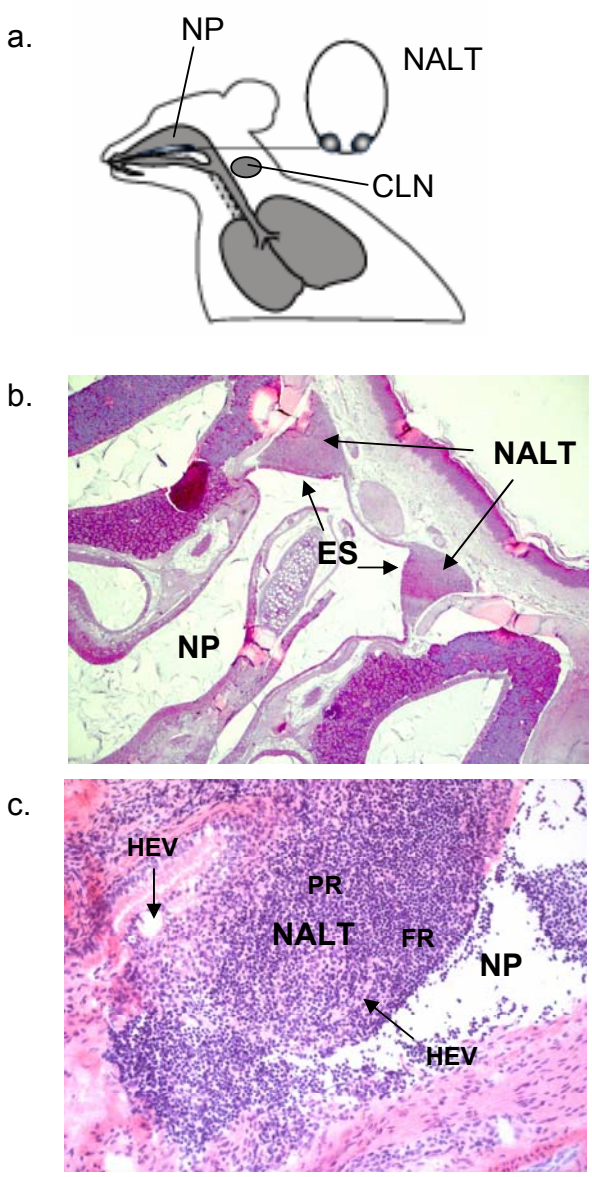


Figure 4.1: Location and structure of the NALT

a. Schematic diagram showing the anatomical location of murine NALT, NP (nasal passages) and CLN. b. Anatomic locations of NALT in naive Balb/c mice after hematoxylin-eosin (H+E) staining. Magnification = 5. c. Frozen NALT section from naive Balb/c mice after H+E staining. FR indicates follicular regions; PR, parafollicular regions; HEV, high endothelial venules and; ES, epithelial sides. Magnification = 20

Intranasal administration of antigens to the mucosal surface can lead to different outcomes depending on the nature of the antigen and interactions at the inductive site. Factors such as dose, the ability to bind, the use of adjuvant, and frequency of administration are all contributing factors. After intranasal immunisation, both humoral and cellular immune responses can occur. Small soluble antigens can penetrate the nasal lumen directly and interact with underlying APC, such as DC and macrophages, whereas the specialised M-cells can allow selective transport of both soluble and particulate antigen across the epithelium for further interaction with a wide variety of leukocytes. Once APCs in the M-cell pocket take up antigen, they process the antigen and migrate into the interfollicular areas, where they present antigenic epitopes in the context of MHC molecules (Fujimura 2000). However, the detailed pathway for the antigen uptake and their migration within the NALT is still unclear. This antigen processing and presentation by DC and macrophages leads to T-cell activation of both T-helper and CTL. A number of studies have revealed a dominant T_H0 cytokine profile, indicating that these T cells are capable of becoming T_H1 or T_H2 cells immediately after antigen exposure through the nasal tract. This in turn leads to the release of cytokines and chemokines as well as B-cell proliferation, differentiation, and maturation into IgA and IgG producing plasma cells (Hiroi, et al. 2001; Hiroi, et al. 1998; Yanagita, et al. 1999). In fact, after intranasal immunisation, the antibody response induced in the NALT and the associated draining LNs can be strong and may retain long term memory (Liang, et al. 2001; Tamura, et al. 1998; Zuercher, et al. 2002).

The first point of call for draining antigen activated immune cells is the superficial CLN, which then drains into the posterior CLN. These LNs are organised bean-shaped organs consisting of an outer B-cell cortex region, the paracortex, which is particularly rich in T cells, and the medulla which contains both B lymphocytes, plasma cells and macrophages (see Section 1.4.4.3 for more details). After initial induction in the NALT, immune responses are further amplified in these draining LNs following an onset delay. The CLN show a more pronounced expansion of lymphocytes when compared to the NALT, particularly after viral infection (11-fold vs. 2-fold). Even more strongly than in the NALT, B cells within the draining LNs show high production of specific IgG antibodies, which after entering the blood leads

to the generation of systemic IgG after intranasal vaccination. Reviewed in (Zuercher 2003). From the CLN, specific effector cells are distributed via lymph fluid and blood to both mucosal and systemic sites around the body. In fact, numerous studies have reported antigen specific immune responses from NALT primed lymphoid cells at local and distant mucosal effector sites (e.g. the D-NALT, the genitourinary tract and the gut) operated by the CMIS (Kunkel, et al. 2003; Lazarus, et al. 2003). However, the mechanisms of homing to these sites are largely unknown to date.

The role played by the GALT, such as PPs and ILFs, in antigen sampling, innate immune responses and in the subsequent generation of antigen-specific immune responses is well-characterised. For a comprehensive review see (Nochi and Kiyono 2006). Despite a central role in mucosal immunity, little is known about the nasal immune system. The phenotype and functions of the different cellular subpopulations are not fully characterised. The majority of studies looking at the NALT and CLN have focussed on the induction of adaptive immunity, and have therefore tended to examine later time-points. However, the characterisation of innate immune responses early after intranasal administration of antigens/pathogens in the upper respiratory tract is not fully understood. Gueirard et al examined immune responses after intranasal administration of the respiratory pathogen *Bordetella bronchiseptica*. $CD11c^+$ leukocytes were present in the NALT subepithelium of naive mice, but were not found to be homogeneously distributed. At 5 and 24 hours after *B. bronchiseptica* delivery, an increased number of cells expressing CD11c were detected and located exclusively along the epithelial side of each NALT aggregate without specific localisation to the T cell area. However, by 48 hours post challenge $CD11c^+$ cells had migrated to these areas (Gueirard, et al. 2003). Another study examined the NALT and CLN after influenza virus infection, and found that three days post administration slightly higher numbers of $CD4^+$ and $CD8^+$ T cells and lower $B220^+$ cell numbers were detectable in the NALT. At 4 days post infection, IgA producing plasma cells were found in the NALT, whereas another two days was needed before antibody responses were detected in the CLN (day 6) (Liang, et al. 2001). Recently, Park and colleagues constructed a group A streptococcus (GAS) strain that expressed the model OVA epitope on the bacterial cell surface, and used this strain in adoptive transfer experiments to study CD4 T cell response to bacterial infection in NALT. After

intranasal infection of mice with the recombinant strain, OVA-specific CD4 T cells were detected in the NALT at three days. The level of CD69, an early activation marker, on OVA-specific T cells in NALT was also assessed at an earlier time point. 36 hours after infection, a great number of T cells in NALT and CLN expressed CD69. Both levels of $CD69^+$ expression on T cells and their frequency were higher in NALT than in CLN (Park, et al. 2004).

Given the rapid nature of the innate immune system, I hypothesise that intranasal immunisation of antigen and adjuvant will lead to the induction of significant early immune responses in a number of different innate cell populations in both local URT lymphoid tissues, i.e. the NALT and CLN. In addition intranasal immunisation will result in significant up regulation of CAMs and the generation of GC.

The aim of this work is to contribute to the phenotypic and functional description of NALT and CLN early after intranasal administration of antigen. Balb/c mice were intranasally immunised with; PBS (naïve), the adjuvant LT or LT plus the tuberculosis fusion antigen Ag85B-ESAT6. I isolated cells from both compartments in mice 5, 24 and 72 hours after immunisation, and using flow cytometry and confocal microscopy, determined the frequencies, localisation and activation status of innate immune cell populations including; DC, macrophages, neutrophils, and NKC. I also examined the expression of cell adhesion molecules (MAdCAM-1, PNAd, ICAM-1 and VCAM-1) and germinal centre formation.

I found striking differences between the cell surface phenotype of leukocytes and their pattern of distribution from the NALT and CLN at all time points tested after immunisation. Altogether, these data provide evidence indicating the distinctive and complex phenotypic and functional features that exist in the immune cell population at early time points after intranasal administration of antigen.

4.1.1 Technical Note

I had to develop an approach for removal and analysis of the NALT (see Section 2.3.1.1 for removal details). Unfortunately, decalcification and formalin fixing of mouse heads destroys many antigen markers and therefore I could not use this process for examining sections of the NALT. At first I tried immunohistochemical staining methods on our frozen NALT sections; however I found that the background staining was very high and therefore not suitable for our study. I consequently decided to utilise mAb staining with flurochromes and found this to be a far more superior method for staining of the NALT.

4.2 Identification of cell populations early after intranasal immunisation

4.2.1 Introduction

The innate immune system has the important function of identifying and eradicating microbes (i.e. heterologous antigens) and alerting the adaptive immune system to their presence. The faster-acting innate immune responses provide a necessary first line of defence because of the relatively slow nature of adaptive immunity. For comprehensive reviews see (Medzhitov and Janeway 2000; Medzhitov and Janeway 1997). Innate immune responses, among their many effects, leads to a rapid burst of inflammatory mediators which results in the infiltration of various cell types to the site of stimulation. Innate immune processes are conducted by cells relatively unrestricted in antigen specificity, including NKC, DC, neutrophils and macrophages. See Figure 4.2 for origin and differentiation of immune cells.

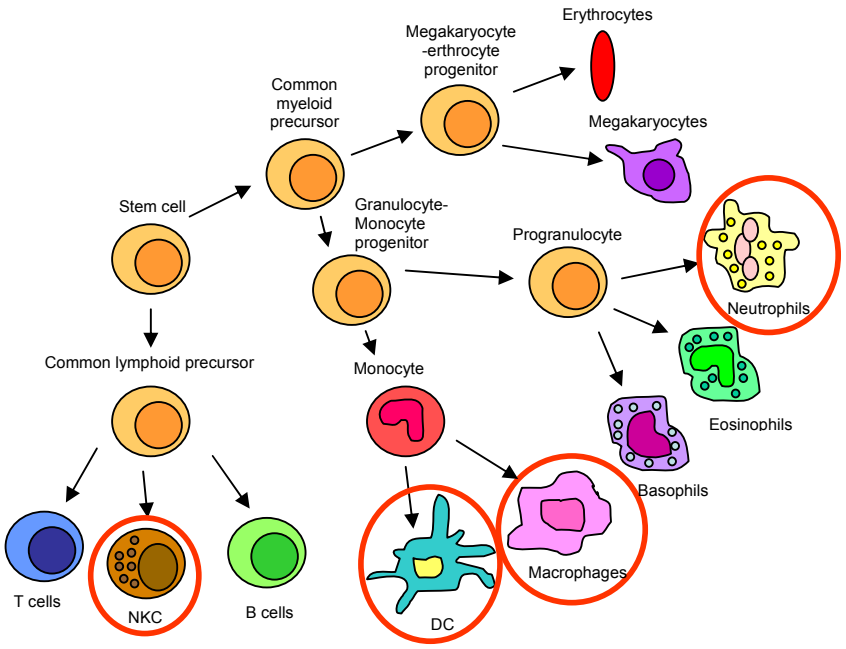


Figure 4.2: Origin and haematopoietic differentiation of immune cells

All hematopoietic cells are derived from pluripotent stem cells which give rise to two main lineages: one for lymphoid cells and one for myeloid cells. The common lymphoid progenitor has the capacity to differentiate into T cells, B cells or NKC depending on the microenvironment to which it homes. The myeloid cells differentiate into all other cells mentioned. Cells circled in red indicate the cell populations examined in this study.

Innate stimulation of DC triggers their differentiation into immunogenic APC, which consequently allows presentation of antigen material to T cells as well as providing the necessary inflammatory signals to modulate T cell differentiation (Reis e Sousa 2004; Steinman and Hemmi 2006). In bacterial infections, a newly discovered DC subpopulation termed iNOS/TNFα-producing "Tip"-DC has been reported to be critical, as demonstrated in a model of infection with *L. monocytogenes*, indicating that DC may also have a role to play in innate immune defences (Serbina, et al. 2003). Macrophages are essential effector cells of innate immunity that play a pivotal role in the recognition and elimination of invasive micro-organisms. Mediators released by activated macrophages orchestrate innate and adaptive immune host responses. As with DC, they also have a role to play in antigen presentation via MHC II, in addition to their other main phagocyctic role (Gordon and Read 2002). Neutrophils are an integral part of the innate immune response and are normally one of the first cell types

to arrive at an inflammatory site due to their high motility. As with macrophages, they are active phagocytes and ingest and destroy foreign antigens during a "respiratory burst" (Kobayashi, et al. 2005). NKC are a form of cytotoxic lymphocyte which constitute a major component of the innate immune system. NKC play a major role in the host-rejection of both tumors and pathogen infected cells and activated NKC also exhibit rapid secretion of several cytokines, notably IFN-γ (Young and Ortaldo 2006). Considering the above points I therefore speculated that intranasal immunisation may affect migration and activation of these cell populations within both the NALT and CLN at early time-points. As far as I know, this present study is the first to characterise the innate immune responses taking place in the upper respiratory tract lymphoid tissues. It will hopefully provide a better understanding of the inductive and effector factors involved in the early regulation of mucosal immunity which will help in the design of safer mucosal vaccines to elicit the appropriate protective immune response to a given pathogen.

4.2.2 Results

4.2.2.1 Percentage, distribution and activation status of NALT and CLN innate cell populations after intranasal immunisation

In order to identify the cell populations involved in early immune responses after intranasal administration of antigen, cells were isolated 5, 24 and 72 hours from both the NALT and CLN of immunised mice. The number of cells recovered from isolated NALT of naïve Balb/c mice was 1.2 (± 0.8) x 10^5 cells for a single mouse. In contrast, the number of cells recovered from the CLN of naïve mice was over 20-fold higher at 2.8 (± 1.1) x 10^6 cells per mouse. The phenotype of NALT and CLN innate immune cells were analysed by flow cytometry as shown in Table 5.1. In the NALT of naïve mice, DC ($CD11c^+$), macrophages ($F4/80^+$), NKC ($DX5^+$) and neutrophils ($Ly6G^+$) were more abundant in terms to total percentage than in the CLN, however in terms of total cell numbers these cell types were more profuse in the CLN. The percentages/numbers of innate leukocytes observed in this present study, for both the NALT and CLN, are similar to those reported previously in Balb/c mice (Gueirard, et al. 2003; Liang, et al. 2001; Rodriguez-Monroy, et al. 2007; Tanigawa, et al. 2000; van Helvoort, et al. 2004; Woolard, et al. 2004; Wu, et al. 1997b; Yeo, et al. 2006).

Kinetic analysis of intranasal immunisation revealed that administration of LT plus Ag85B-ESAT6 differentially influenced several cell populations early after intranasal immunisation of antigen in both the NALT and CLN, and dramatic changes in cell number percentage were evident as early as 5 hours post-immunisation (Table 4.1 and Figure 8.1).

In order to confirm the flow cytometry data as well as examine the distribution of innate immune cell populations early after intranasal immunisation, serial frozen NALT and CLN sections of control (naïve) and immunised (LT + Ag85B-ESAT6) mice were stained for $CD11c^+$, $F4/80^+$ and $Ly6G^+$ cells. Unfortunately examination of $DX5^+$ (NKC) cells was not possible as no antibodies are currently commercially available for immunohistochemistry. Immunofluorescence analysis of $CD11c^+$, $F4/80^+$ and $Ly6G^+$ cells in the NALT and CLN indicated the same kinetics as the flow cytometric analysis (Figures 4.3A, 4.4A, 4.5A, 4.6A, 4.7).

To evaluate the effects of intranasal immunisation on the activation status of innate immune cells ($CD11c^+$, $F4/80^+$, $Ly6G^+$ and $DX5^+$) at early time-points, I examined both the number of cells expressing CD69, CD25, MHCII and VCAM-,1 and the Mean Fluorescence Intensity (MFI) of these markers by flow cytometry. The CD69 (Leu-23) antigen is a cell surface glycoprotein which is expressed on activated T and B lymphocytes, as well as NKC and neutrophils (Lanier, et al. 1988). CD69 expression is induced 4-6 hours after activation, reaches a peak at 18-24 hours and is still detectable on the cell surface 24 hours after stimulus is removed (Testi, et al. 1994; Testi, et al. 1989). Another marker that is expressed early after NKC activation is the IL-2 receptor (CD25). This antigen plays a role in IL-2 induced NKC proliferation and cytotoxic activity (Kehri, et al. 1988; Lanier, et al. 1985; Lanier, et al. 1988; Siegel, et al. 1987; Tsudo, et al. 1987). Class II MHC molecules are integral membrane proteins expressed at the cell surface, which bind peptide fragments of antigen and present them to T cells that express a complimentary T cell receptor for antigen. MHC class II molecules are only expressed on so-called “professional” APC, such as DC, macrophages and B lymphocytes. MHC II expression is upregulated after antigen stimulation and is therefore associated with APC activation (Santambrogio and Strominger 2006; Santana and Esquivel-Guadarrama 2006; Wetzel and Parker

2006). The MHC II expressed in Balb/c mice is known as I-A^d. VCAM-1 is also expressed at low levels on macrophages and DC, but can be induced after antigenic stimulation. This adhesion molecule can therefore be used an indicator for activation of these cell types (Balogh, et al. 2002; Rice, et al. 1991). From this analysis, it was determined that differences in expression of both MHC II and VCAM-1 expression on $CD11c^+$ and $F4/80^+$ cells were maximal in the NALT at early time-points with the CLN showing the greatest differences at later time-points (Figures 4.3B, 4.4B, 4.5B, 4.6B and Table 8.2).

The flow cytometry results are shown as percentages from 10,000 recorded events. I did not tie these percentages back to total cell numbers obtained after processing of both the NALT and CLN as it is very unlikely that the whole tissues could be dissected out. Therefore any data obtained from these calculations (i.e. percentages multiplied back to total cell numbers) would not be an accurate representation of the responses induced.

Table 4.1: Surface phenotype of isolated cells from NALT and CLN 5, 24 and 72 hours after intranasal immunisation.

Cells were isolated from NALT and CLN of Balb/c mice 5, 24 and 72hours after intranasal immunisation with PBS (naïve mice), 1µg LT or 1µg LT + 10µg Ag85B-ESAT6, and stained with flurochrome-labelled mAbs and analysed by flow cytometry in which 10,000 events were recorded. Data represents the mean percentage of expression ± standard deviation from groups of ten individual mice, from two independent experiments. The * indicates significant values of $p<0.05$ and **, $p<0.01$, as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Black represents those percentage values not significantly different to those seen in naïve mice; red indicates values significantly increased, and blue shows percentages significantly decreased in comparison to negative control mice.

Cell Marker	Immunisation	NALT %				CLN %			
		0hrs	5hrs	24hrs	72hrs	0hrs	5hrs	24hrs	72hrs
CD11c$^+$	LT	6.6 ± 1.5	5.2 ± 0.8*	5.3 ± 0.7*	5.9 ± 0.5	4.2 ± 0.3	4.4 ± 0.4	2.9 ± 0.8**	5.3 ± 0.7*
	LT + Ag	6.5 ± 1.1	5.3 ± 1.1*	7.9 ± 1.2*	6.8 ± 1.0	4.3 ± 0.6	4.3 ± 0.2	2.4 ± 0.3**	5.3 ± 0.9*
F4/80$^+$	LT	7.7 ± 1.2	6.2 ± 0.9*	6.0 ± 1.0*	7.8 ± 1.0	3.4 ± 0.8	3.3 ± 0.5	4.0 ± 0.3	5.7 ± 0.6**
	LT + Ag	7.6 ± 1.3	4.9 ± 1.1**	7.6 ± 1.2	7.9 ± 1.2	3.6 ± 0.7	3.2 ± 0.2	3.9 ± 0.6	6.7 ± 0.9**
DX5$^+$	LT	5.4 ± 1.6	5.6 ± 0.5	7.8 ± 2.1*	3.2 ± 0.8*	4.3 ± 0.9	5.8 ± 0.7*	8.2 ± 3.1**	4.7 ± 1.9
	LT + Ag	5.3 ± 1.2	5.0 ± 0.6	10.3 ± 3.1**	7.6 ± 2.1*	3.9 ± 1.4	10.4 ± 3.4**	8.9 ± 2.2**	3.6 ± 0.7
Ly6G$^+$	LT	7.6 ± 1.8	6.9 ± 1.7	9.0 ± 1.1*	7.0 ± 1.4	3.4 ± 0.7	3.0 ± 0.6	5.9 ± 1.2**	5.6 ± 1.2**
	LT + Ag	7.4 ± 1.6	4.8 ± 1.1**	5.8 ± 1.1*	7.8 ± 1.2	3.5 ± 0.7	5.5 ± 1.2**	3.2 ± 1.9	2.1 ± 0.3**

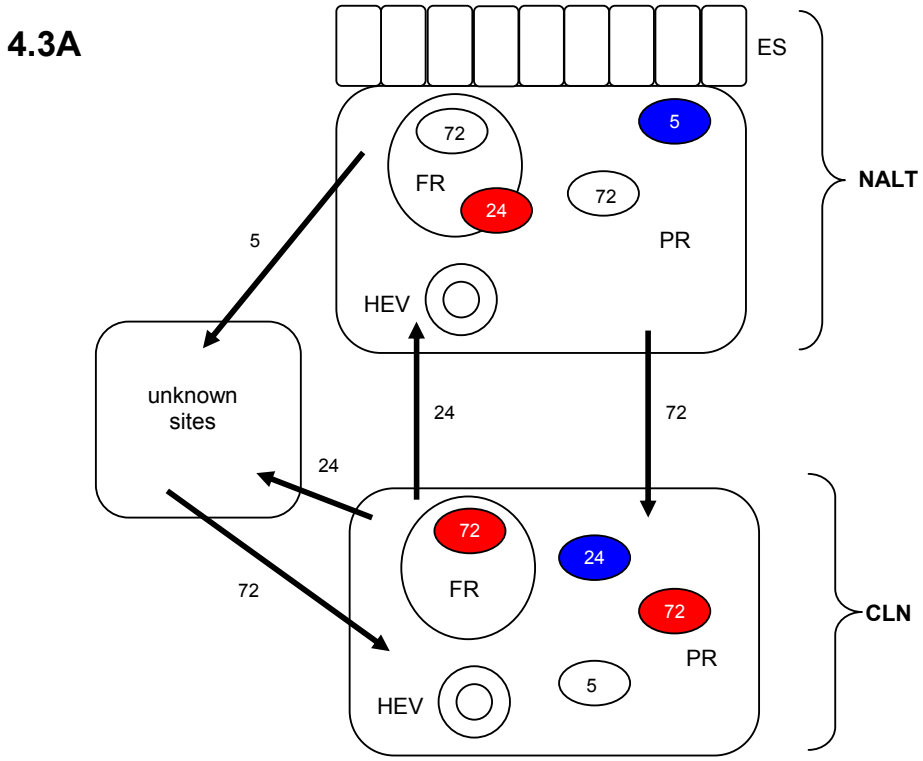
4.2.2.1.1 Dendritic Cells

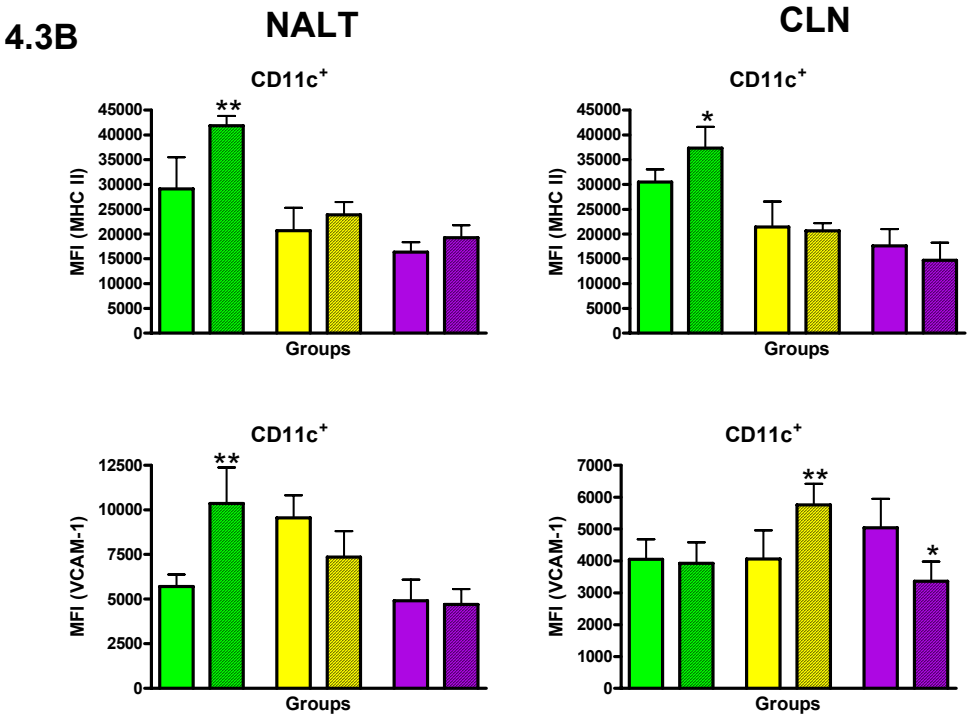
Figure 4.3: Schematic diagram showing an overview of DC movement and activation within the NALT and CLN 5, 24, and 72 hours after intranasal immunisation

A. Ovals represent cells and numbers within the ovals indicate individual time-points. White ovals represent that the percentage change is not statistically different (i.e. $p > 0.05$) from naïve animals, blue shows a significant decrease (i.e. $p < 0.05$) and red indicates a significant increase (i.e. $p > 0.05$) in the cell population when compared to control mice. The arrows indicate direction of movement at particular time-points. FR stands for follicular regions (i.e. B cell areas), PR is for parafollicular regions (i.e. T cell areas), ES is for epithelial sides, and HEV stands for high endothelial venules.

B. Cells were isolated from the NALT and CLN of control (PBS immunised) and immunised animals, 5, 24 and 72 hours after intranasal administration of antigen. Cells were analysed for $CD11c^+$ (Figure 4.3B) expression, as well as MHC II and VCAM-1 expression. For analysis, gates were set on the innate subset marker positive ($CD11c^+$) cells and the MFI of MHC II and VCAM-1 expression was determined. Data are presented as means ± SD with the * indicating significant values of $p < 0.05$ and **, $p < 0.01$, as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Green bars represent cells isolated at 5 hours, yellow at 24 hours and purple at 72 hours. Block colours represent naïve (PBS immunised) animals and, diagonal lines show animals intranasally immunised with adjuvant plus Ag85B-ESAT6. Scales are representational of individual activation marker MFI for each tissue – always refer to y-axis scale for each graph.

4.2.2.1.1 Dendritic Cells





4.2.2.1.1.1 5 hour time-point

Cells from the NALT of immunised mice at the 5 hour time-point were found to have a significantly lower ($p < 0.05$) percentage of $CD11c^+$ cells, as well as $CD11c^+$/VCAM-1^+ cells, when compared to PBS immunised animals (Tables 4.1 and 8.2A and Figure 8.1A). I also observed that both MHC II and VCAM-1 fluorescence increased ($p < 0.01$) significantly in all immunised mice on DC at this time-point (Figure 5.3B). However, the distribution of this cell type remained similar to that seen in naïve mice (Figure 4.7A). Within the CLN, as early as 5 hours post immunisation, DC were more widely distributed, this was in comparison to the DC pattern in naïve animals which appeared more clustered (Figure 4.7A). I also observed an increase in MHC II fluorescence ($p < 0.05$) on $CD11c^+$ cells from immunised animals compared to that of naïve mice (Figure 4.3B).

4.2.2.1.1.2 24 hour time-point

24 hours post intranasal immunisation, $CD11c^+$ and $CD11c^+$/$MHCII^+$ cells were significantly increased ($p < 0.05$) in the NALT (Tables 4.1 and 8.2A and Figure 8.1B). In addition I also observed a more wide-spread pattern of $CD11c^+$ cells in immunised mice compared to that seen in the naive mice (Figure 4.7A). Within the CLN, immunised mice were found to have a significantly lower percentage ($p < 0.01$) of $CD11c^+$ cells and the subpopulations of $CD11c^+$ cells positive for MHC II and VCAM-1 when compared to naïve animals (Tables 4.1 and 8.2A and Figure 8.1C). Conversely, I observed a significant increase in VCAM-1 expression on DC at 24 hours within the CLN of immunised mice (Figure 4.3B).

4.2.2.1.1.3 72 hour time-point

Even though the percentage of $CD11c^+$ cells were similar 72 hours after immunisation in all animals tested, I did also observe a more widespread distribution of DC within the NALT of immunised animals, particularly the appearance of DC within follicular regions (Table 4.1 and Figures 4.7A). I also noted that $CD11c^+$ cells expressing VCAM-1 significantly increased ($p < 0.01$) in those animals receiving adjuvant and antigen (Figure 4.3B). The CLN DC population as well as the $CD11c^+/MHCII^+$ and $CD11c^+/VCAM\text{-}1^+$ subpopulations were significantly increased ($p < 0.05$) in all immunised mice compared to PBS immunised animals at this final time-point (Tables 4.1 and 8.2A and Figure 8.1D). As observed in the NALT, I also saw DC within follicular and parafollicular regions (Figure 4.7A). In addition, VCAM-1 expression was observed to significantly decrease ($p < 0.05$) 72 hours after immunisation (Figure 4.3B). For overview see Figure 4.3.

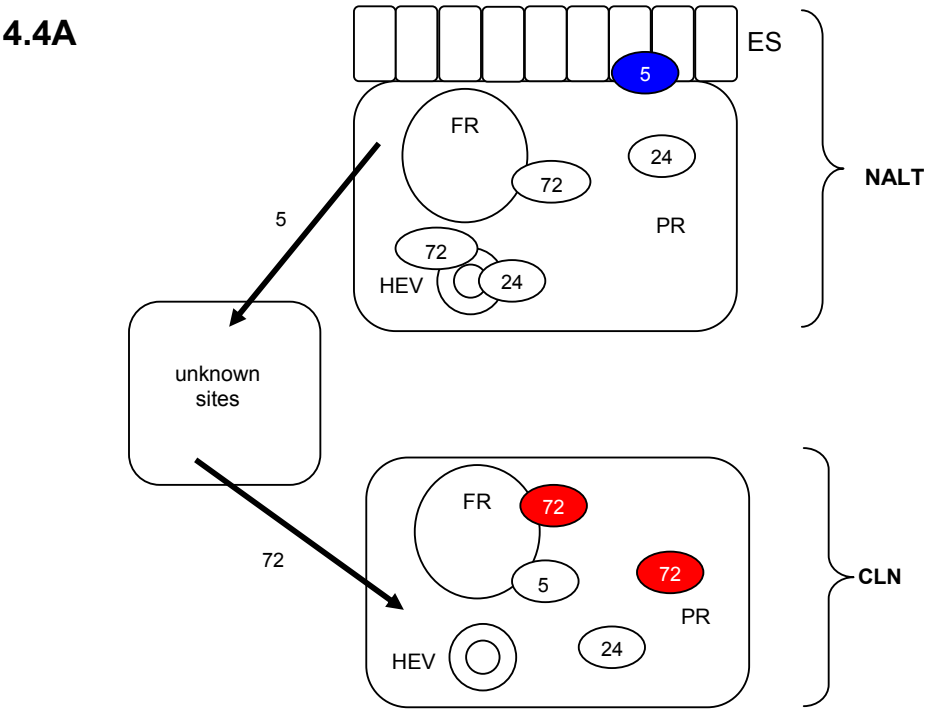
4.2.2.1.2 Macrophages

Figure 4.4: Schematic diagram showing an overview of macrophage movement and activation within the NALT and CLN 5, 24, and 72 hours after intranasal immunisation

A. Ovals represent cells and numbers within the ovals indicate individual time-points. White ovals represent that the percentage change is not statistically different (i.e. $p > 0.05$) from naïve animals, blue shows a significant decrease (i.e. $p < 0.05$) and red indicates a significant increase (i.e. $p > 0.05$) in the cell population when compared to control mice. The arrows indicate direction of movement at particular time-points. FR stands for follicular regions (i.e. B cell areas), PR is for parafollicular regions (i.e. T cell areas), ES is for epithelial sides, and HEV stands for high endothelial venules.

B. Cells were isolated from the NALT and CLN of control (PBS immunised) and immunised animals, 5, 24 and 72 hours after intranasal administration of antigen. Cells were analysed for $F4/80^+$ (Figure 4.4B) expression, as well as MHC II and VCAM-1 expression. For analysis, gates were set on the innate subset marker positive ($F4/80^+$) cells and the MFI of MHC II and VCAM-1 expression was determined. Data are presented as means ± SD with the * indicating significant values of $p < 0.05$ and **, $p < 0.01$, as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Green bars represent cells isolated at 5 hours, yellow at 24 hours and purple at 72 hours. Block colours represent naïve (PBS immunised) animals and, diagonal lines show animals intranasally immunised with adjuvant plus Ag85B-ESAT6. Scales are representational of individual activation marker MFI for each tissue – always refer to y-axis scale for each graph.

4.2.2.1.2. Macrophages



4.2.2.1.2.1 5 hour time-point

The percentage of F4/80$^+$ cells (macrophages) and those cells also positive for VCAM-1 were observed to significantly decrease ($p < 0.05$) within the NALT in intranasally immunised mice as early as 5 hours (Tables 4.1 and 8.2A and Figure 8.1A). The F4/80$^+$ cells from both naïve (PBS immunised) and immunised animals at this time-point were mostly located around the epithelial sides of the tissue; however a small number of macrophages in the NALT from immunised animals were also located in parafollicular T cell areas (Figure 4.7B). In addition, F4/80$^+$ cells also had a significant increase ($p < 0.01$) in MHC II expression as early as 5 hours after immunisation (Figure 4.4B). The F4/80$^+$ population in the CLN of immunised mice was similar to that seen in naive mice, however I did observe a significant increase in F4/80$^+$/VCAM-1$^+$ cells and MHC II expression at this early 5 hour time-point (Tables 4.1 and 8.2A and Figure 4.4B).

4.2.2.1.2.2 24 hour time-point

At 24 hours post administration of antigen, F4/80$^+$ cells had started to return to similar levels observed in the NALT of naïve mice (Table 4.1). In addition, the macrophages present were located even more centrally, as well as surrounding HEV in immunised mice (Figure 4.7B). Again as described for the F4/80$^+$ population at 5 hours within the CLN, the percentages of these cells were comparable to control mice (Table 4.1). However, I did observe that macrophages positive for MHC II and VCAM-1 were actually decreased ($p < 0.01$) in all immunised mice 24 hours post administration of antigen (Table 8.2A). I also noted that the fluorescence intensity of VCAM-1 significantly increased ($p < 0.01$) on CLN macrophages (Figure 4.4B). In terms of their distribution, the macrophages within CLN of immunised were mice were also observed in more central areas compared to the more peripheral pattern seen in naive animals 24 hours after immunisation (Figure 4.7B).

4.2.2.1.2.3 72 hour time-point

By 72 hours all immunised mice appeared to have similar numbers of NALT macrophages as naive animals, with most surrounding HEV (Table 4.1 and Figure 4.7B). Within the CLN I observed the same diffuse pattern as at 24 hours and in addition there was a significant increase ($p < 0.05$) in $F4/80^+$ cells as well as the subpopulations, $F4/80^+/MHCII^+$ and $F4/80^+/VCAM\text{-}1^+$ at this final time-point (Tables 4.1 and 8.2A and Figures 8.1D and 4.7B). I also observed a significant decrease ($p < 0.05$) in VCAM-1 expression 72 hours after immunisation on $F4/80^+$ cells within the CLN (Figure 4.4B). For overview see Figure 4.4.

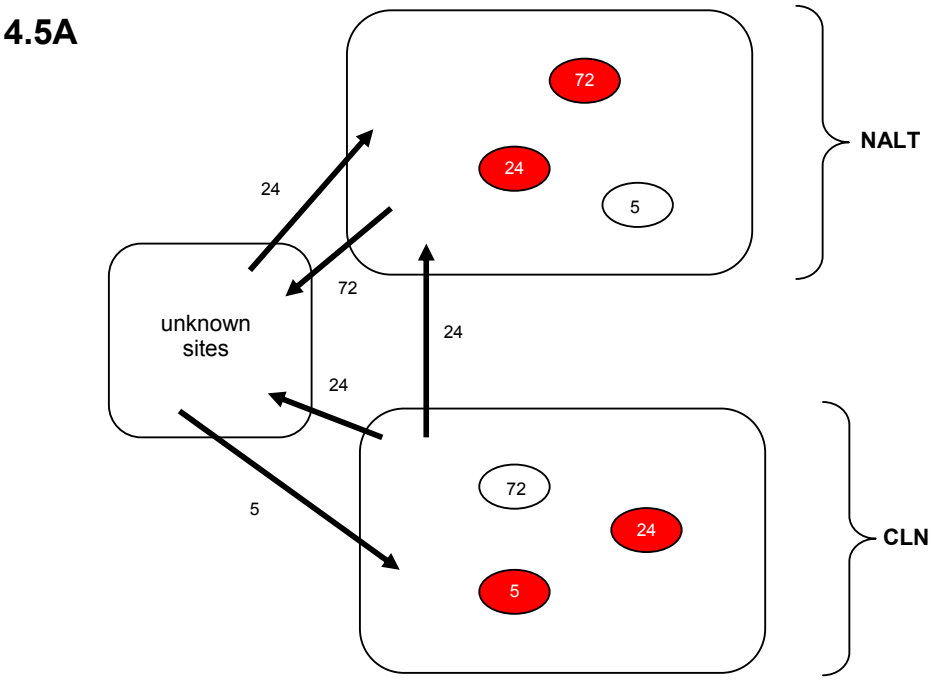
4.2.2.1.3 Natural Killer Cells

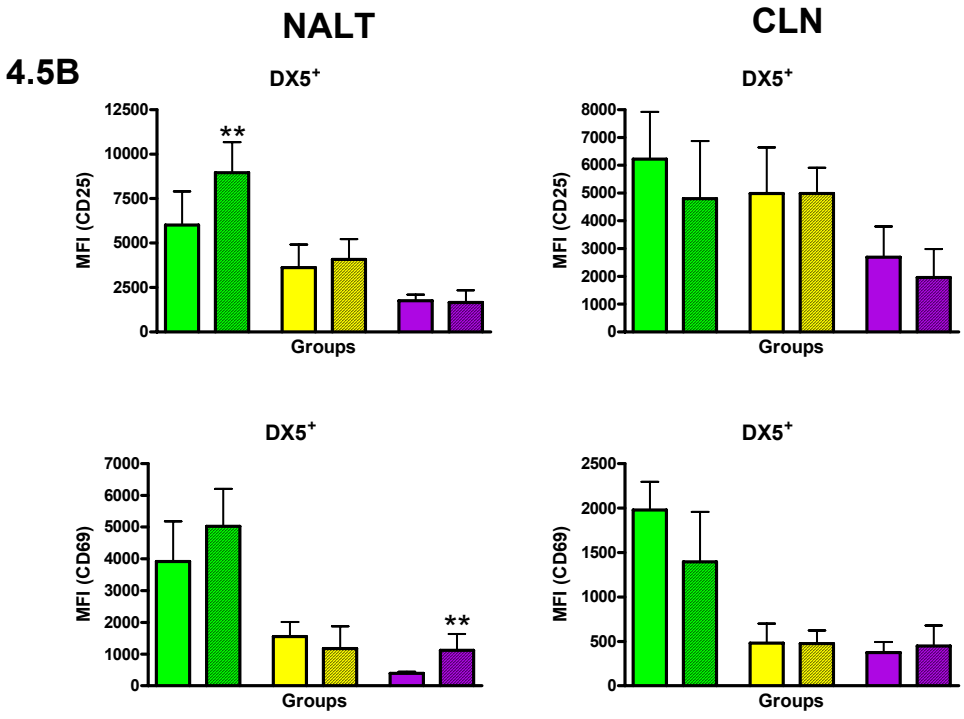
Figure 4.5: Schematic diagram showing an overview of NKC movement and activation within the NALT and CLN 5, 24, and 72 hours after intranasal immunisation

A. Ovals represent cells and numbers within the ovals indicate individual time-points. White ovals represent that the percentage change is not statistically different (i.e. $p > 0.05$) from naïve animals, blue shows a significant decrease (i.e. $p < 0.05$) and red indicates a significant increase (i.e. $p > 0.05$) in the cell population when compared to control mice. The arrows indicate direction of movement at particular time-points. FR stands for follicular regions (i.e. B cell areas), PR is for parafollicular regions (i.e. T cell areas), ES is for epithelial sides, and HEV stands for high endothelial venules.

B. Cells were isolated from the NALT and CLN of control (PBS immunised) and immunised animals, 5, 24 and 72 hours after intranasal administration of antigen. Cells were analysed for $DX5^+$ expression as well as CD25 and CD69 expression (Figure 4.5B). For analysis, gates were set on the innate subset marker positive ($DX5^+$) cells and the MFI of CD25 and CD69 expression was determined. Data are presented as means ± SD with the * indicating significant values of $p < 0.05$ and **, $p < 0.01$, as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Green bars represent cells isolated at 5 hours, yellow at 24 hours and purple at 72 hours. Block colours represent naïve (PBS immunised) animals and, diagonal lines show animals intranasally immunised with adjuvant plus Ag85B-ESAT6. Scales are representational of individual activation marker MFI for each tissue – always refer to y-axis scale for each graph.

4.2.2.1.3 Natural Killer Cells





4.2.2.1.3.1 5 hour time-point

The percentage of $DX5^+$ cells within the NALT was found not to be significantly different ($p > 0.05$) from those seen in naïve mice 5 hours after immunisation (Table 4.1). I also observed increased ($p < 0.05$) CD25 expression on $DX5^+$ cells, but significantly lower ($p < 0.01$) number of $DX5^+/CD69^+$ cells at 5 hours within the NALT (Table 8.2B and Figure 4.5B). The CLN percentage of NKC and $DX5^+/CD25^+$ cells in immunised mice were increased ($p < 0.01$) by more than 2x compared to naive mice (Tables 4.1 and 8.2B and Figure 8.1H). Conversely to what was observed in the NALT, all animals immunised with adjuvant and antigen showed an increase ($p < 0.01$) in $DX5^+/CD69^+$ cells 5 hours after immunisation within the CLN (Table 8.2B).

4.2.2.1.3.2 24 hour time-point

By 24 hours the percentage of $DX5^+$ and $DX5^+/CD25^+$ cells found within the NALT was significantly increased ($p < 0.01$) in immunised mice, with an approximately 2x higher percentage of NKC than naïve animals (Tables 4.1 and 8.2B and Figure 8.1F). Within the CLN, the percentages of $DX5^+$ and $DX5^+/CD25^+$ cells in immunised mice were still found to be significantly higher ($p < 0.01$) than naïve animals 24 hours after intranasal immunisation (Tables 4.1 and 8.2B and Figures 8.1I).

4.2.2.1.3.3 72 hour time-point

Within the NALT, 72 hours post immunisation, there continued to be a greater percentage ($p < 0.05$) of NKC, $DX5^+/CD25^+$ and $DX5^+/CD69^+$ cells compared to naïve mice but the percentage was lower than that seen at 24 hours (Tables 4.1 and 8.2B and Figure 8.1G). In addition, I observed a significant increase in surface CD69 expression on these cells (Figure 4.5B). For the CLN $DX5^+$ population I observed that NKC percentages in immunised mice returned to those seen for naïve animals ($p > 0.05$) (Table 4.1). However, there was still a significant increase ($p < 0.01$) in the $DX5^+/CD25^+$ subpopulation in immunised mice at this final time-point (Table 8.2B). For overview see Figure 4.5.

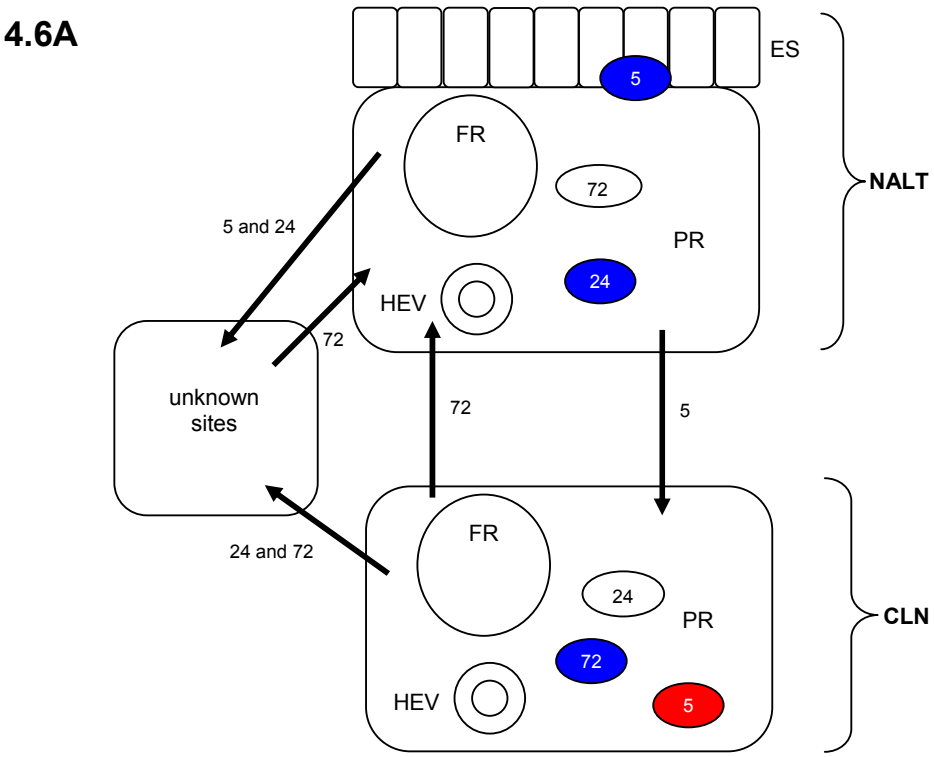
4.2.2.1.4 Neutrophils

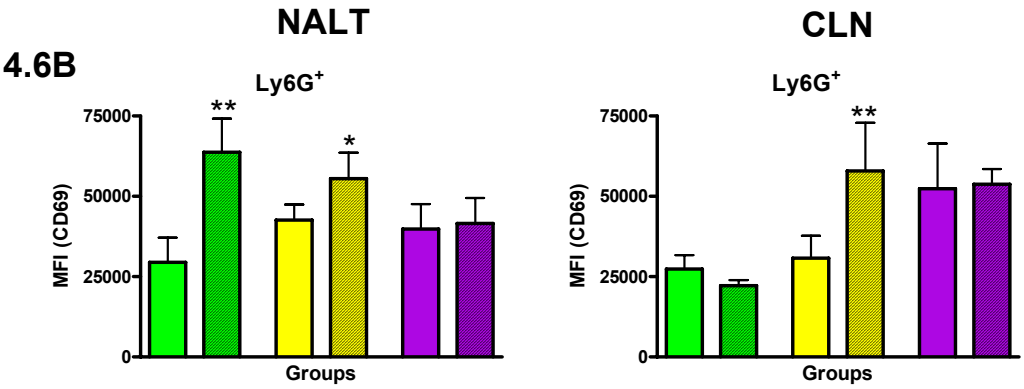
Figure 4.6: Schematic diagram showing an overview of neutrophil movement and activation within the NALT and CLN 5, 24, and 72 hours after intranasal immunisation

A. Ovals represent cells and numbers within the ovals indicate individual time-points. White ovals represent that the percentage change is not statistically different (i.e. $p > 0.05$) from naïve animals, blue shows a significant decrease (i.e. $p < 0.05$) and red indicates a significant increase (i.e. $p > 0.05$) in the cell population when compared to control mice. The arrows indicate direction of movement at particular time-points. FR stands for follicular regions (i.e. B cell areas), PR is for parafollicular regions (i.e. T cell areas), ES is for epithelial sides, and HEV stands for high endothelial venules.

B. Cells were isolated from the NALT and CLN of control (PBS immunised) and immunised animals, 5, 24 and 72 hours after intranasal administration of antigen. Cells were analysed for $Ly6G^+$ (Figure 4.6B) expression, as well as CD69 expression. For analysis, gates were set on the innate subset marker positive ($Ly6G^+$) cells and the MFI of CD69 expression was determined. Data are presented as means ± SD with the * indicating significant values of $p < 0.05$ and **, $p < 0.01$, as determined by one-way ANOVA followed by Dunnett's Multiple Comparison Test compared to negative control animals (i.e. PBS immunised). Green bars represent cells isolated at 5 hours, yellow at 24 hours and purple at 72 hours. Block colours represent naïve (PBS immunised) animals and, diagonal lines show animals intranasally immunised with adjuvant plus Ag85B-ESAT6. Scales are representational of individual activation marker MFI for each tissue – always refer to y-axis scale for each graph.

4.2.2.1.4 Neutrophils





4.2.2.1.4.1 5 hour time-point

Neutrophil numbers were observed to significantly decrease ($p < 0.05$), but the MFI of CD69 significantly increased ($p < 0.01$) on these cells in immunised animals within the NALT, compared to control mice at the 5 hour time-point (Table 4.1 and Figures 8.1E and 4.6B). The distribution of remaining cells was still similar to control animals (Figure 4.7C). I also observed that neutrophils ($Ly6G^+$) positive for CD69 decreased ($p < 0.01$) in all immunised mice after 5 hours within the NALT (Table 8.2B). Contrary to what I observed within the NALT, immunised animals had increased percentages ($p < 0.01$) of $Ly6G^+$ cells within their CLN at this early 5 hour time-point (Table 4.1 and Figure 8.1H).

4.2.2.1.4.2 24 hour time-point

By 24 hours, mice immunised with LT + Ag85B-ESAT6 had an increase in the percentage of $Ly6G^+$ cells compared to the percentage seen at 5 hours, however this was still significantly lower ($p < 0.05$) than that seen for naïve animals (Table 4.1 and Figure 8.1F). I also observed that CD69 expression was still higher ($p < 0.05$) in these animals when compared to controls (Figure 4.6B). I also observed movement of $Ly6G^+$ cells from the periphery into more central locations after immunisation (Figures 4.7C). Within the CLN mice receiving adjuvant and antigen showed an increase in CD69 expression on their $Ly6G^+$ cells when compared to naïve animals (Figure 4.6B). However, in terms of the percentage of $Ly6G^+$ cells I did not observe a significant difference ($p > 0.05$) between immunised and naïve animals (Table 4.1).

4.2.2.1.4.3 72 hour time-point

By 72 hours the $Ly6G^+$ NALT population was similar in all animals tested, but neutrophils of immunised mice still showed a more central wide-spread distribution than observed in control animals (Table 4.1 and Figure 4.7C). Within the CLN, I observed a depletion of the $Ly6G^+$ population in immunised mice (Table 4.1 and Figure 8.1J). In terms of distribution, I observed a similar pattern of $Ly6G^+$ cells in all immunised and naïve animals at all time-points tested within the CLN, i.e. within the parafollicular region (Figure 4.7C). For overview see Figure 4.6.

Figure 4.7: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT + Ag85B-ESAT6) Balb/c mice were compared. Figure 4.7A represents staining of frozen sections for CD11c in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling of cell nuclei by Hoechst (blue) and CD11c (red). There was no staining using isotype control mAb (not depicted). Magnification = 28. Arrows indicate particular areas such as; B-cell areas (follicular regions, FR), T-cell areas (parafollicular regions, PR), HEV and epithelial sides (ES). For H + E image reference see Figure 4.1.

Figure 4.7A:

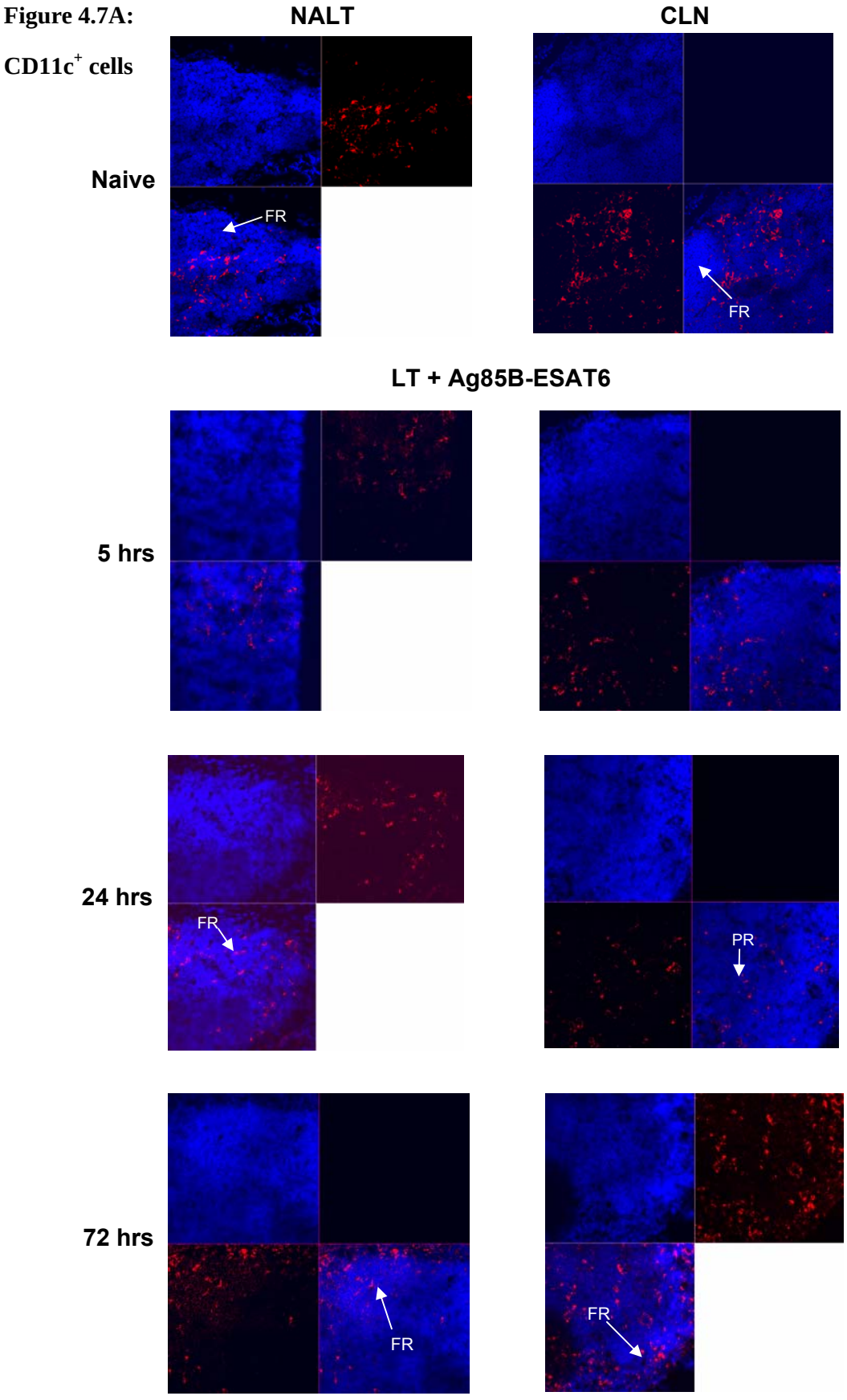

NALT
CLN
CD11c+ cells
Naive
FR
FR
LT + Ag85B-ESAT6
5 hrs
24 hrs
FR
PR
72 hrs
FR
FR

Figure 4.7: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT + Ag85B-ESAT6) Balb/c mice were compared. Figure 4.7B represents staining of frozen sections for F4/80 in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling of cell nuclei by Hoechst (blue) and F4/80 (green). There was no staining using isotype control mAb (not depicted). Magnification = 28. Arrows indicate particular areas such as; B-cell areas (follicular regions, FR), T-cell areas (parafollicular regions, PR), HEV and epithelial sides (ES). For H + E image reference see Figure 4.1.

Figure 4.7B:

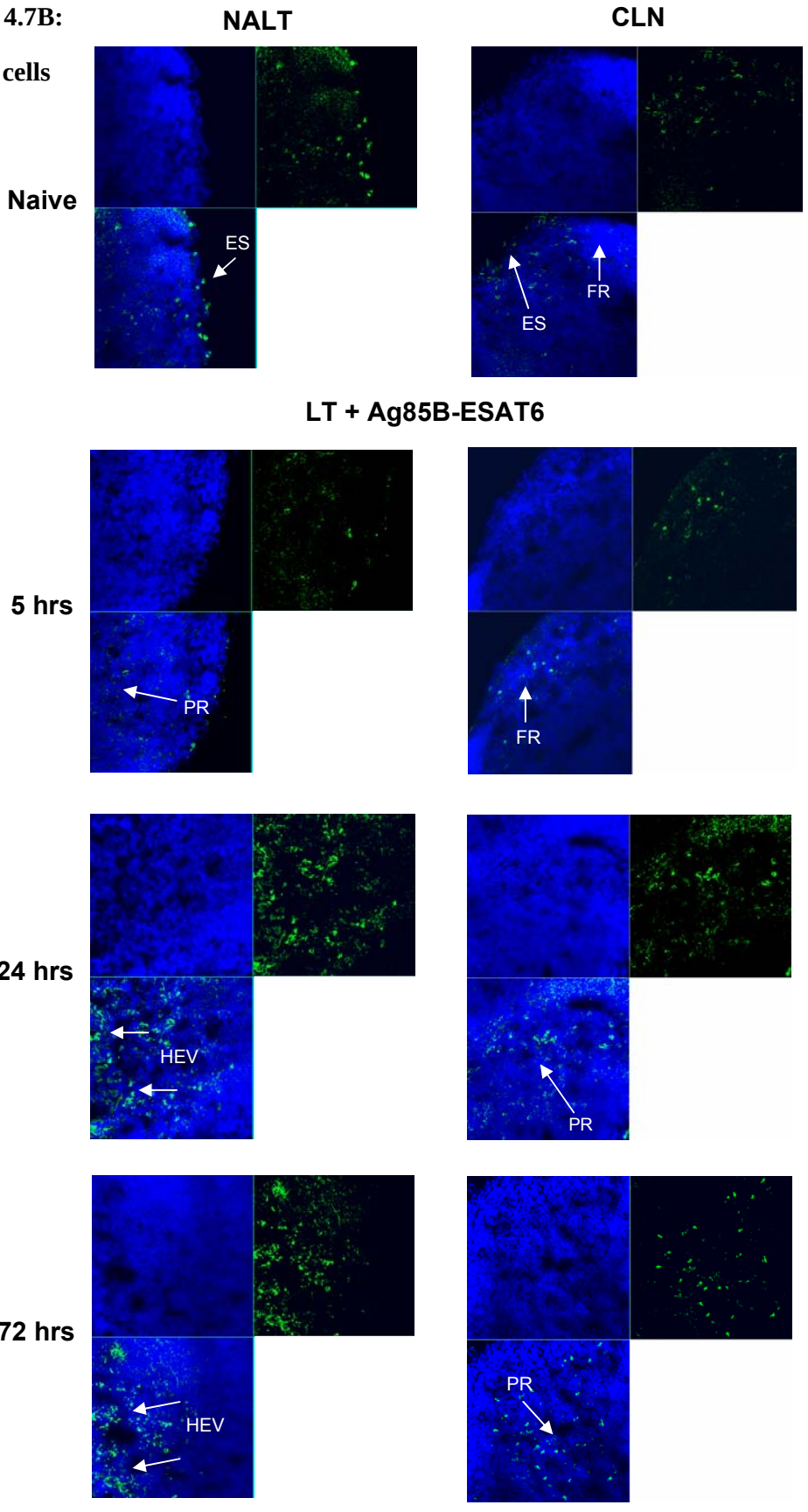
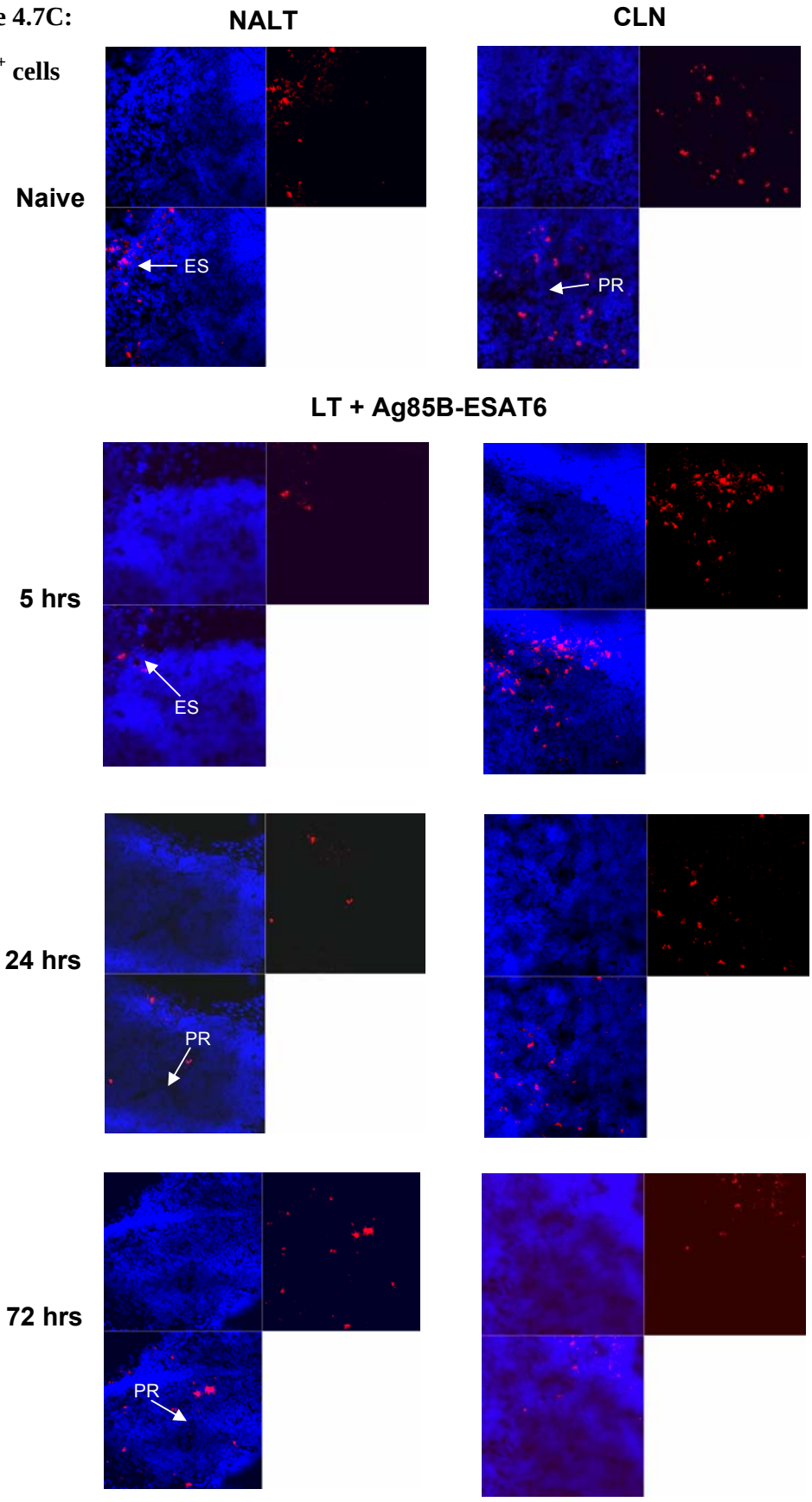

NALT
CLN
F4/80+ cells
Naive
ES
ES
FR
LT + Ag85B-ESAT6
5 hrs
PR
FR
24 hrs
HEV
PR
72 hrs
HEV
PR

Figure 4.7: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT + Ag85B-ESAT6) Balb/c mice were compared. Figure 4.7C represents staining of frozen sections for Ly6G in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling of cell nuclei by Hoechst (blue) and Ly6G (red). There was no staining using isotype control mAb (not depicted). Magnification = 28. Arrows indicate particular areas such as; B-cell areas (follicular regions, FR), T-cell areas (parafollicular regions, PR), HEV and epithelial sides (ES). For H + E image reference see Figure 4.1.

Figure 4.7C:


NALT
CLN
Ly6G+ cells
Naive
ES
PR
LT + Ag85B-ESAT6
5 hrs
ES
24 hrs
PR
72 hrs
PR

4.2.3 Discussion

The innate immune response reacts rapidly to foreign antigens with activation and migration of its cellular components (Janeway and Medzhitov 2002; Medzhitov and Janeway 2000). The relevance of these individual components to host survival against pathogen infections is beyond doubt, with the protective contribution of DC, macrophages, NKC and neutrophils, as well as various cytokines being demonstrated in a number of infections (Bodnar, et al. 2001; Harshan and Gangadharam 1991; Murphy, et al. 2004; Pedrosa, et al. 2000; Rogers and Unanue 1993; Samsom, et al. 1997; Schafer and Eisenstein 1992; Weissman and Fauci 1997). This has lead to an appreciation in recent years that innate immunity is central to protection against infectious diseases and the induction of adaptive immune responses. However, an integrated picture of innate responses after mucosal vaccination has not been established. The current study addresses this by characterising changes in defined cell populations, their activation status, cell adhesion molecule expression and GC formation in the first few hours and days following intranasal immunisation with a mucosal adjuvant and model antigen. Hopefully this study will accentuate the importance of understanding the nature of innate immune responses at the mucosae for the design of improved vaccines.

4.2.3.1 5 hour time-point

The earliest changes in NALT cell populations detected were a rapid and significant reduction in the total percentages of DC, macrophages and neutrophils and a concomitant increase in CLN neutrophils along with an increase in NKC. The 2-fold increase in NKC percentages that occurred 5 hours after intranasal immunisation transformed this minor CLN population to the most abundant innate cell type in the CLN. NKC contribute to innate immunity by the production of cytokines, particularly IFN-γ, along with other immunomodulatory signals early after their activation. In addition to the increase of $DX5^+$ cells seen within the CLN, I also observed a significant increase of $DX5^+CD69^+$ and $DX5^+/CD25^+$ cells. As already discussed, the CD69 and CD25 antigens are some of the earliest markers expressed on activated NKC following stimulation by a variety of mitogenic agents. Our *in vivo* observations in immunised mice show that even though the percentage of these cells increases,

their MFI does not, possibly indicating that this influx of NKC cells in the CLN precedes their activation. Contrary to the activation status observed in the CLN NKC, I did observe a significant increase in CD25 expression on NALT NKC, suggesting that the cells already present within this lymphoid tissue may be activated. The earlier activation status of these cells within the NALT highlights the fact that the NALT is the first area that uptake of soluble antigen occurs before it can gain access to nose-draining LNs such as the superficial CLN. Although I did observe migration of NKC cells into the CLN, I did not see a concurrent reduction in percentages within the NALT, suggesting that influx of NKC within the CLN is due to migration of this innate cell population from another site, as yet unidentified. In this present study I removed the O-NALT; however there is also another, less organised, lymphoid site situated within the nasal passages called the D-NALT (Kuper, et al. 1992). Studies to date suggest that the O-NALT is rich in unswitched, naive B cells and naive T cells, suggesting that it is a mucosal inductive site, whereas the D-NALT may function as an effector site (Hiroi, et al. 1998; Wu, et al. 1996). With these points in mind it may be that the other cell populations undergoing changes during this early 5 hour time-point are in fact trafficking to this area to 'pick-up' and process antigen before moving back to the inductive O-NALT or other lymphoid tissues. Whereas this may be true for the macrophage and DC populations, with regards to the $Ly6G^{+}$ population, it appears that the disappearance of neutrophils from the NALT probably results in the migration and influx of this cell population into the CLN 5 hours after immunisation. As described for the up regulation of CD25 on NKC, I also observed a significant increase of CD69 on neutrophils suggesting that these cells were activated within the NALT, but not the CLN. Neutrophils are usually thought of as the leukocyte population involved in acute inflammatory responses, acting as a first line of defense against invading microorganisms. They are also able to synthesise cytokines in response to a variety of inflammatory stimuli. For a comprehensive review see (Nathan 2006). Therefore, activation of this innate cell population may induce further cellular and vascular innate immune mechanisms at very early time-points after immunisation. In addition to the decrease in percentages of APC within the NALT, I also observed an increase in activation marker expression (i.e. MHC II and VCAM-1), suggesting that the NALT cells present may be activated and ready to process antigen. In lymphoid tissues, mature FDC play key roles in organising the

formation of GC. FDC interact avidly with B cells by virtue of the adhesion molecules ICAM-1 and VCAM-1 on the FDC, which interact with $\alpha_L\beta_2$ and $\alpha_4\beta_1$ integrins on the B cell. These physical interactions are crucial for the survival of B cells proliferating in the GC (Koopman, et al. 1994; Tew, et al. 2001). Therefore, the presence of DC expressing VCAM-1 to high levels may indicate why I observe GC formation as early as 5 hours post immunisation within these lymphoid tissues (see Section 5.4). I also noted that distribution of APC in immunised NALT was very similar to that seen in naïve animals, i.e. surrounding the edge of the tissue. This localisation of these DC is comparable to another study which examined $CD11c^+$ cell distribution early after *B. bronchiseptica* challenge (Gueirard, et al. 2003). Even though cell percentages of the APC, DC and macrophages did not change in the CLN of immunised mice 5 hours post immunisation, I did observe an increase in the MFI of MHCII on both $CD11c^+$ and $F4/80^+$ cells present, indicating that these cell types may in fact be activated. These activated cells may have already processed antigen or become activated via immunomodulatory signals secreted from the NKC and neutrophils in the O-NALT or D-NALT, and have consequently migrated down to the CLN. In addition, I also observed the movement of these cells to more central areas within the CLN, possibly indicating the involvement of these APC in presenting antigen via MHC II to lymphocytes. Several studies show that LT toxin exerted detectable regulatory effects on DC and macrophages, altering both their surface phenotype and their ability to process and present protein antigens (Martin, et al. 2002; Petrovska, et al. 2003).These observations may explain why I see increased activation of these cell types as early as 5 hours post immunisation. The FACS plots (Figure 8.1) had some diagonal smearing which may be due to the presence of dead cells. In order to confirm and exclude this data a live/dead marker could be added next time to allow this differentiation.

4.2.3.2 24 hour time-point

By 24 hours post immunisation I observed a dramatic increase in the percentages of NKC within the NALT when compared to naïve animals. I also again observed an increase in this cell population with the CLN; however the increase was not as great as that seen 5 hours after immunisation. Therefore, it is tempting to speculate that the

influx of NKC observed within the NALT may be due to migration of these $DX5^+$ cells from the CLN. Neutrophils within the NALT immunised animals were again decreased in comparison to the percentage seen in control (PBS immunised) animals however, percentages seen in the CLN were back to those observed in naïve animals. The reduction in the $Ly6G^+$ population within the NALT was not as significant as that seen at the 5 hour time-point, and this may account for the percentage observed within the CLN at 24 hours. It was also noted that the neutrophils present in these immunised animals appeared to be activated due to up regulation of the CD69 antigen. Within the NALT, $F4/80^+$ cells from immunised mice had returned to those seen in naïve mice and DC percentages were significantly increased. The increase in these cell populations may be due to macrophage and DC returning from the D-NALT ready to present antigen, in fact the APC present in the NALT of these mice were located within both B and T cell areas supporting this hypothesise. As already discussed, NKC and major producers of IFN-γ and the influx of NKC within the CLN at both 5 and 24 hours post immunisation may indicate why I do not see any movement of macrophages out of this tissue. IFN-γ can induce production of macrophage migration inhibitory factor which in turn can prevent macrophages from leaving the tissue. Even though I did not see any differences in percentages of $F4/80^+$ cells within the CLN at the 24 hour time-point, I again observed a more diffuse staining pattern, again highlighting that those macrophages present may in fact be processing and presenting antigens to lymphocytes. I also observed that the macrophages and DC within the CLN had up regulated expression of VCAM-1. APC expressing this cell adhesion molecule are known to increase presentation of antigen to GC B cells and this subset of cells may be involved in the induction of antigen-specific humoral immune responses shortly after intranasal immunisation. DC populations within the CLN decreased within the CLN of immunised animals, including the $CD11c^+/MHCII^+$ and $CD11c^+/VCAM\text{-}1^+$ subset. This migration of DC may be to the spleen for further induction of immune responses, however as I did not examine splenocytes populations I can not speculate further.

4.2.3.3 72 hour time-point

At the final time-point examined (i.e. 72 hours), there was a decrease in the NKC population within the NALT in those animals immunised, when compared to that seen at 24 hours after immunisation. The reduction in percentages of NKC may signal their movement from the CLN to other secondary lymphoid organs within the mucosal immune systems, as well as movement systemically. The $DX5^+$ cells present also had significantly more expression of the CD69 antigen when compared to control animals. As already discussed, CD69 is an early marker of NKC activation, however high expression is still seen up to 48-72 hours after stimulus is removed. Therefore, this high CD69 expression on $DX5^+$ cells may show the population of NALT NKC cells that were activated very shortly after intranasal immunisation, or the cells may in fact be a newly activated population that has migrated in from another site. In addition, previous work on IL-12 stimulation of NKC cells suggests that CD69 expression also identifies cells in a state of anergy post function, not cells that are preactivated and ready to function (Craston, et al. 1997). Consequently this may be another explanation as to why there are $DX5^+/CD69^+$ cells 72 hours after immunisation. The CLN $DX5^+$ cell population were back to those percentages seen in control mice by 72 hours, with those cells present having no significant increase in their CD69 or CD25 expression. This may indicate that this cell population has returned to basal levels by 72 hours, as other populations are now beginning to modulate immune responses, e.g. lymphocytes. I also observed a return to those percentages consistent with naïve animals in the $Ly6G^+$ NALT population. As mentioned above, neutrophils are one of the first cell types to migrate to sites of inflammation and consequently are also one of the first to disappear after stimulus has been removed. This cell type only has a very short life-span, 6-48 hours, after their activation and this may in part explain why I no longer observe changes in the percentage of neutrophils within the NALT at this later time-point. Conversely, I did observe changes in the percentages of $Ly6G^+$ cells with the CLN 72 hours after immunisation. This may simply be due to the onset delay, through trafficking of local NALT immune responses, to the CLN after initial induction within the NALT. As described for neutrophils responses within the NALT at 72 hours, I also no longer observed any changes in both the DC and macrophage populations when compared to naïve animals. Examining tissue sections of the NALT I did see that both populations were still in more central areas rather than surrounding

the periphery, which is the case in PBS immunised animals, suggesting that these APC are still processing and presenting antigen to lymphocytes. I also observed macrophages surrounding HEV, indicating the migration of this cell type from the NALT through the lymphatics to other lymphoid tissues for induction of further immune responses. The increase in number of $CD11c^+$ DC, and $F4/80^+$ macrophage cells in the CLN by 72 hours is impressive, and their position in the T and B cell areas might reflect their involvement in immunologic reactions taking place in this lymphoid tissue.

4.2.3.4 Summary

In summary, I demonstrate for the first time that intranasal immunisation with adjuvant and antigen results in the dramatic changes in innate cell populations within both the NALT and CLN. APC and neutrophil percentages decrease within the NALT as early as 5 hours post immunisation, and it isn't until day 3 that levels return to those seen in control animals. A reduction in DC CLN percentages is not observed until 24 hours after immunisation, but a significant APC influx is observed by 72 hours. Neutrophils accumulate within 5 hours in the CLN and remain present until day 3. Both macrophages and DC start to migrate into more central areas of these lymphoid tissues by 24 hours indicating their involvement in the linking of adaptive and innate immune responses. The innate cell population showing the most drastic changes in percentage are NKC. These cells migrate into the CLN first, before moving up to the NALT. Immunisation also results in a rapid and transient activation of all cells first in the NALT, and then in the CLN, highlighting the fact that the NALT is the first point of call for inhaled antigens. Overall the innate immune response after intranasal immunisation is a complicated one, with cells rapidly moving to and fro between both the NALT and the CLN, as well as yet undefined tissues. Further studies are needed to understand the functional implications of all this leukocyte migration early after intranasal immunisation.

4.3 Cell adhesion molecule expression early after intranasal immunisation

4.3.1 Introduction

4.3.1.1 Leukocyte trafficking and cell adhesion molecules

The trafficking of immune cells in blood and lymph into secondary lymphoid tissues is an important part of the immune response that is required for an efficient and targeted response to antigenic challenge (Butcher and Picker 1996). During innate immune responses, inflammatory mediator such as cytokines; including IL-1β, TNF-α and IFN-α/β, are released in response to foreign antigens. Along with these cytokines, various chemokines (such as IL-8, monocytes chemoattractant proteins and macrophage inflammatory proteins) are released and their receptors are expressed on the surface of activated cells. As a result, vascular endothelial cells may alter their surface expression of selectins and intracellular adhesion molecules, leading to the extravasation and selective retention of some leukocytes at the inflamed site (Fabbri, et al. 1999). Leukocytes exit the blood at specialised HEV, and are regulated in part by multistep cascades, involving sequential leukocyte/endothelial adhesion and activation events (Gowans and Knight 1964). At sites of chronic inflammation, HEV-like vessels are often induced and allow large numbers of leukocytes to emigrate from blood and accumulate in extra lymphoid tissues (Freemont 1987). The first of these interactions causes the leukocyte to roll along the vessel wall (Bargatze, et al. 1994). Within seconds, the rolling leukocyte becomes firmly adherent, and the leukocyte transmigrates from the blood vessel to tissue (Warnock, et al. 1998; Warnock, et al. 2000). These cascades can be tissue-specific, with much of the specificity determined by selective expression of adhesion molecules by endothelia in various tissues, and of their ligands by circulating leukocytes (Butcher, et al. 1999). There are four major families of cell adhesion molecules; the Ig superfamily cell adhesion molecules (CAMs), integrins, cadherins, and selectins. A selection of cell adhesion molecules and the receptors involved in each step of leukocyte extravasation to lymphoid tissues are listed in Table 4.2.

Table 4.2: Cell Adhesion Molecules and Their Receptors

Molecule	Expression/Distribution	Ligand
MAdCAM-1	HEV and DC	$\alpha_4\beta_7$ integrin
PNAd	HEV	L-Selectin
ICAM-1 (CD54)	Monocytes; Endothelial cells; DC; Fibroblasts; Epithelium; Synovial cells and T lymphocytes	$\alpha_L\beta_2$ integrin (LFA-1, CD11/CD18)
VCAM-1 (CD106)	Monocytes; DC; Synovial cells and Activated Endothelial cells	$\alpha_4\beta_1$ integrin (VLA-4)

4.3.1.2 Cell adhesion molecule expression in the NALT and CLN

As already discussed, the murine NALT and CLN are involved in the generation of local immune responses within the upper respiratory tract. Previous intranasal immunisation studies have shown that the CLN drain the NALT, and therefore for this present study cell adhesion molecule expression will be examined in the both these upper respiratory tract lymphoid tissues to determine expression profiles. All NALT HEV express PNAd, either associated with MAdCAM-1 or alone, while NALT FDC express both MAdCAM-1 and VCAM-1. Interestingly, these expression profiles are distinct from those of the gut mucosal inductive site PP, where predominately MAdCAM-1-$\alpha_4\beta_7$ interactions mediate leukocyte homing (Csencsits, et al. 1999; Csencsits, et al. 2002). Intracellular adhesion molecule-1 (ICAM-1) expression within the NALT, and the epithelium overlying the NALT, has been shown to be constitutively low in naïve mice (Hussain, et al. 2001). Within the CLN, HEV also utilise PNAd-L-selectin interactions and MAdCAM-1-$\alpha_4\beta_7$ interactions for leukocyte binding, although not all HEV express MAdCAM-1 (Csencsits, et al. 2002; Wolvers, et al. 1999). ICAM-1 is also constitutively expressed at low to moderate levels on HEV of LNs, and all other vascular endothelium. In the CLN, staining for diffuse MAdCAM-1 is found to correlate with the expression of MAdCAM-1 on its HEV, and is located within the follicular stromal elements. The CLN also displays diffuse staining of VCAM-1 and ICAM-1 within its follicles. Evidence from cell ligand blocking experiments and the Stamper-Woodruff *ex vivo* binding assays, indicates that PNAd-L-selectin interactions mediate the majority of naive-leukocyte binding in both the NALT and CLN, with lesser roles for MAdCAM-1-$\alpha_4\beta_7$ and VCAM-1-$\alpha_4\beta_1$ interactions (Csencsits, et al. 2002).

4.3.2 Results

4.3.2.1 Intranasal immunisation increases both expression and distribution of cell adhesion molecules at early time-points in the NALT and CLN.

In order to identify the cell adhesion molecule expression profile early after intranasal immunisation, serial frozen NALT and CLN sections of control (naïve) and immunised (LT + Ag85B-ESAT6) mice were investigated for the distribution and expression of MAdCAM-1, PNAd, ICAM-1 and VCAM-1 at 5, 24 and 72 hours. As described in previous studies for the NALT and CLN, the HEV of naïve (PBS immunised) mice were found to express both MAdCAM and PNAd (Figures 4.8A and 4.8B). However, no diffuse MAdCAM-1 expression was observed in either the NALT or CLN (Figure 4.8A). ICAM-1 was observed mainly in the vascular endothelium, and weak expression of ICAM-1 was also shown around micro-vessels as well as on the surface of some cells in the NALT (Figure 4.8C), whereas ICAM-1 expression was found to be restricted to HEV and microvessels in the CLN (Figure 4.8C). Weak diffuse VCAM-1 expression was observed in the NALT and was localised on the surface of cells as well as around blood vessels, but not on HEV within the NALT of naïve animals (Figure 4.8D). Within CLN, VCAM-1 was observed on venular endothelium, vessels, and in addition on cells within this tissue (Figures 4.8D). After intranasal immunisation, I visually observed a greater intensity of MAdCAM-1 expression, which was mainly located on the vascular endothelium of HEV, and was higher than that seen in naïve animals in both the NALT and CLN at all time-points (Figure 4.8A). In addition to venular endothelium, MAdCAM-1 was also shown on the surface of infiltrating cells in the inflamed tissues after only 5 hours in the NALT (Figure 4.8A). All immunised mice were found to have some degree of diffuse MAdCAM-1 staining at all time-points tested within the CLN (Figure 4.8A). An increase in both PNAd expression and the number of HEV expressing this cell adhesion molecule was seen in the NALT of mice as early as 5 hours after intranasal immunisation (Figure 4.8B). This expression profile was still present at 24 hours, although by day 3 (72 hours) expression levels of PNAd were back down to that seen in naïve animals (Figure 4.8B). Expression of this addressin in the CLN was also found to have a similar profile to that seen in the NALT (Figure 4.8B). In intranasally immunised mice, higher expression of ICAM-1 was observed on HEV, micro-vessels,

and on the surface of cells within the NALT (Figure 4.8C). The pattern of diffuse ICAM-1 staining was also more widespread and of greater intensity than that seen in naïve animals at both 24 and 72 hours in this tissue (Figure 4.8C). Expression of ICAM-1 was also up-regulated in the CLN following immunisation, on both vascular endothelium, and with extension to many cell surfaces within CLN at all time-points examined (Figure 4.8C). As with ICAM-1 expression, VCAM-1 was also observed to be more intense and widespread on HEV, blood vessels and cells in the NALT of immunised mice (Figure 4.8D). Intranasal immunisation also increased VCAM-1 expression and distribution in CLN, especially at 24 hours (Figure 4.8D). Expression of this cell adhesion molecule was also more widely distributed at both 5 hours and 72 hours within the CLN, however levels of expression were only moderately increased at the 72 hour time-point when compared to naïve animals (Figure 4.8D).

Figure 4.8: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 4.8A represents staining of frozen sections for MAdCAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT + Ag85B-ESAT6 in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and MAdCAM-1 (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 4.8A:

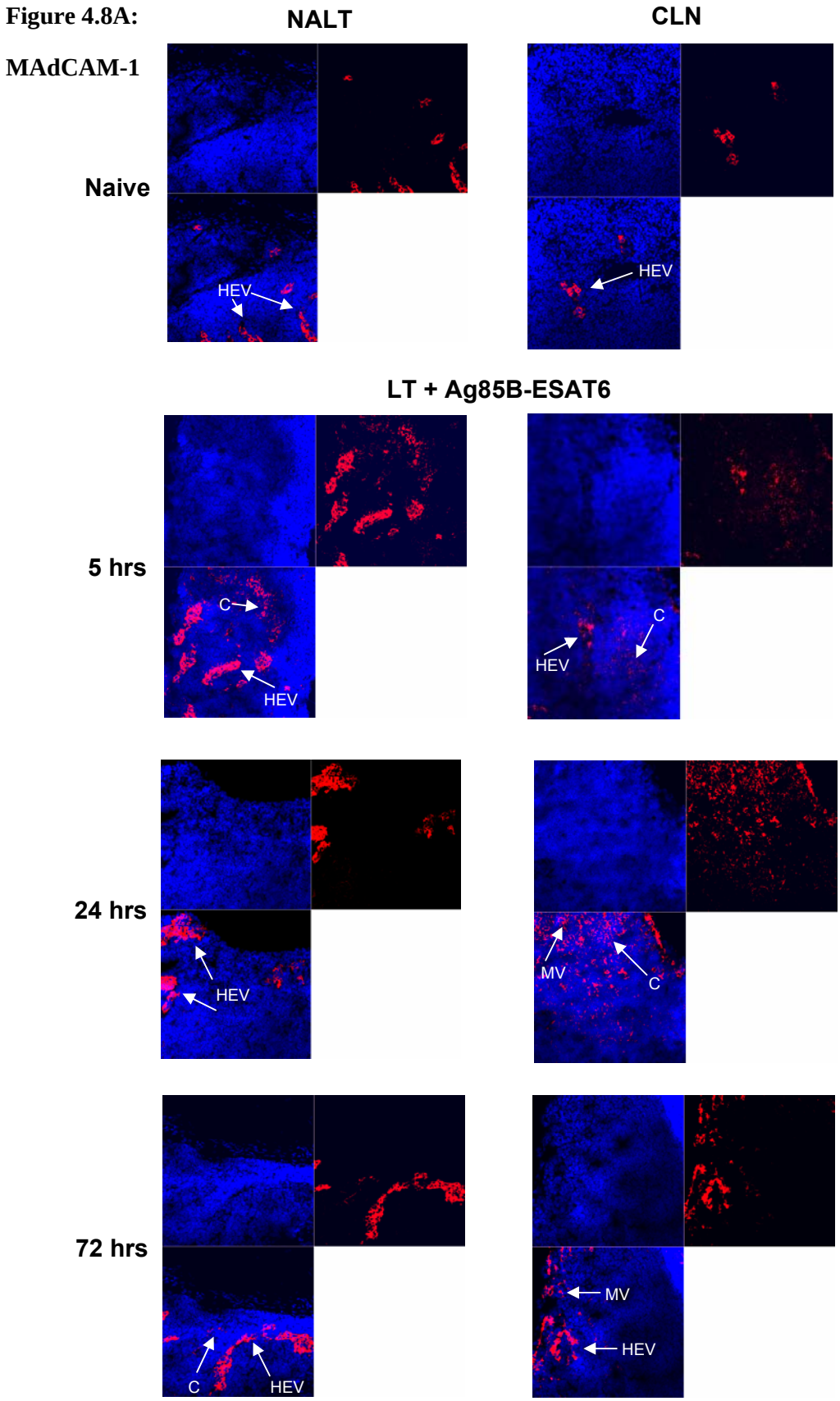

NALT
CLN
MAdCAM-1
Naive
HEV
HEV
LT + Ag85B-ESAT6
5 hrs
C
HEV
HEV
C
24 hrs
HEV
MV
C
72 hrs
C
HEV
MV
HEV

Figure 4.8: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 4.8B represents staining of frozen sections for PNAd in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT + Ag85B-ESAT6 in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and PNAd (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 4.8B:

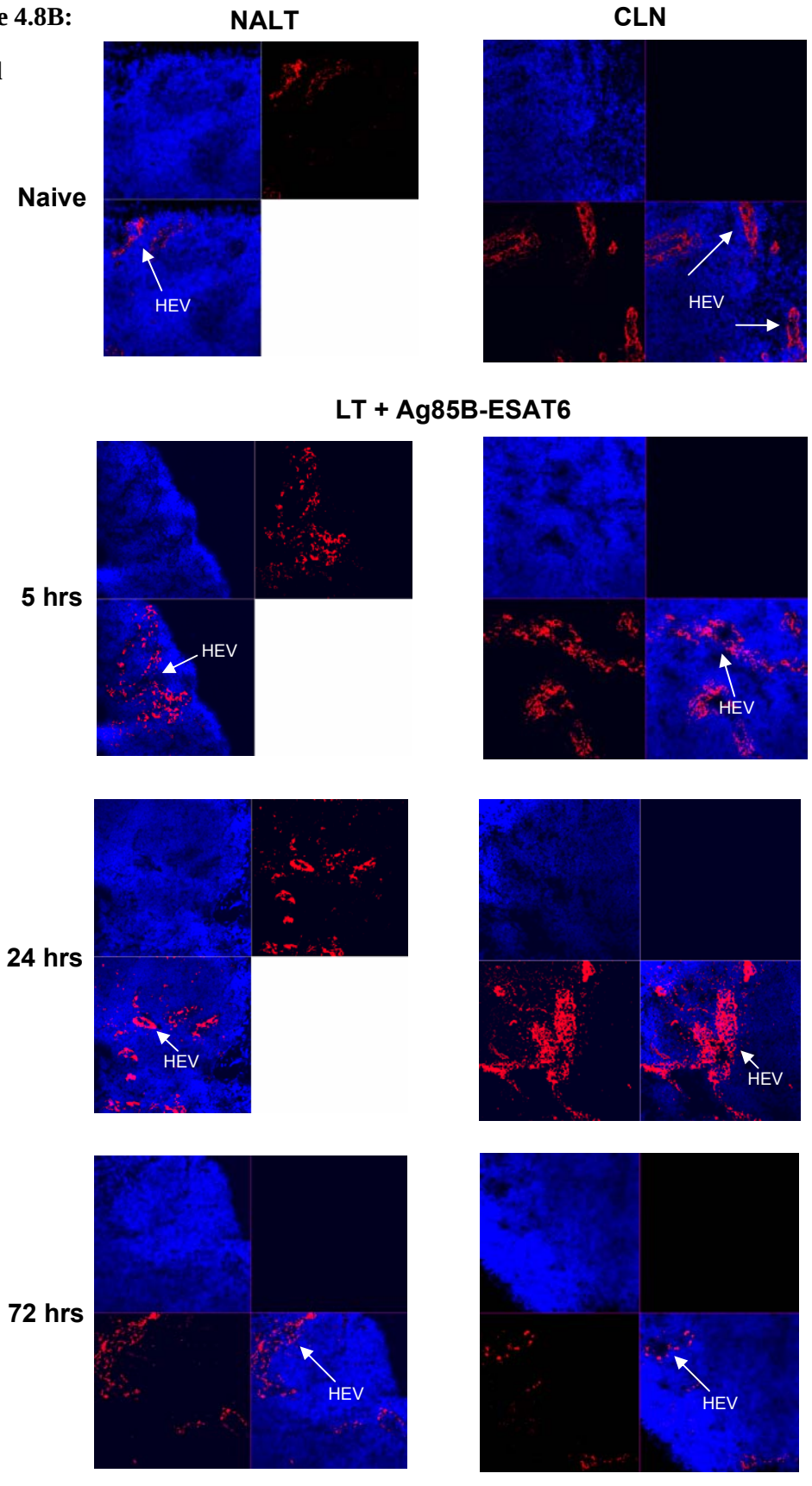

NALT
CLN
PNAd
Naive
HEV
HEV
LT + Ag85B-ESAT6
5 hrs
HEV
HEV
24 hrs
HEV
HEV
72 hrs
HEV
HEV

Figure 4.8: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 4.8C represents staining of frozen sections for ICAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT + Ag85B-ESAT6 in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and ICAM-1 (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 4.8C:

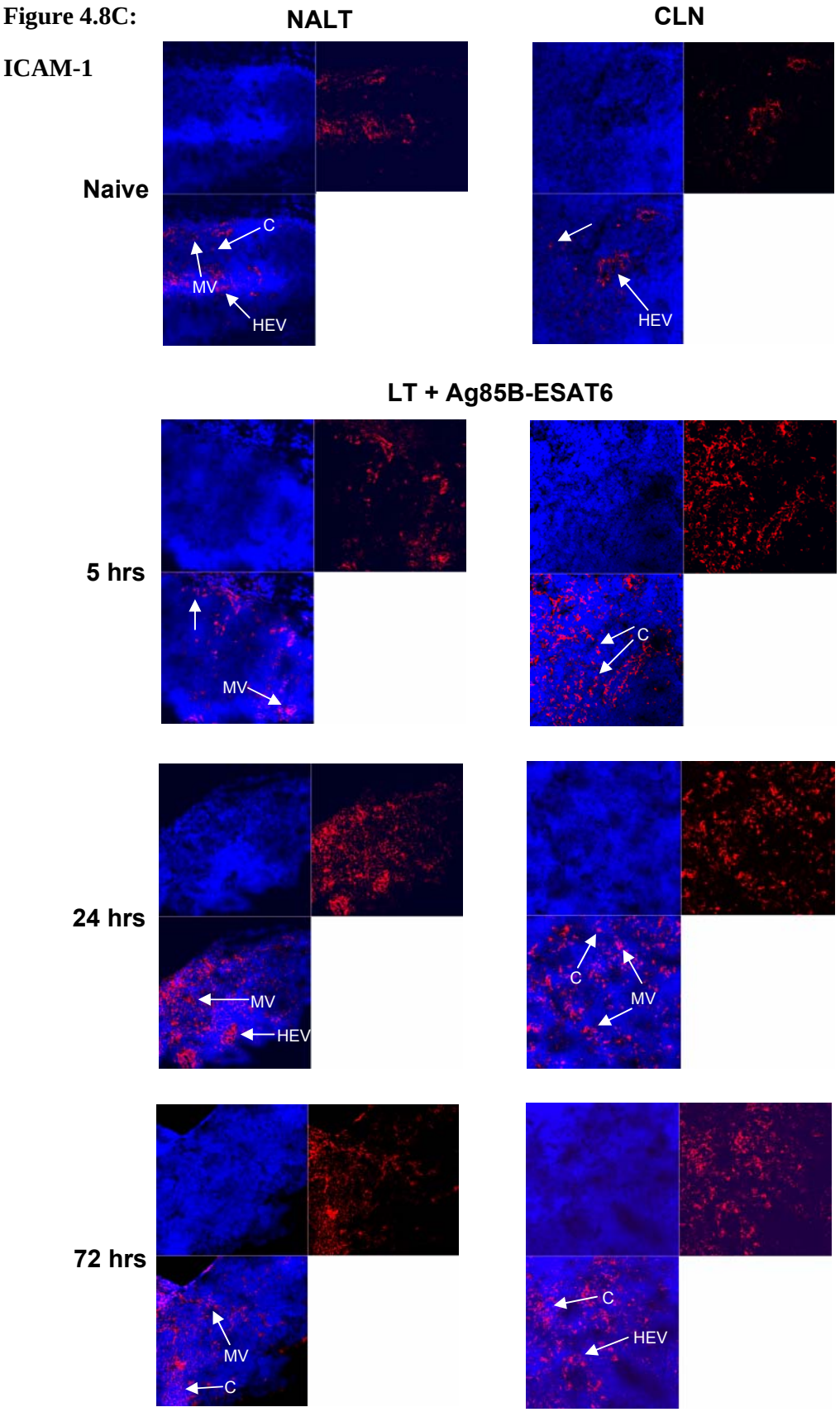
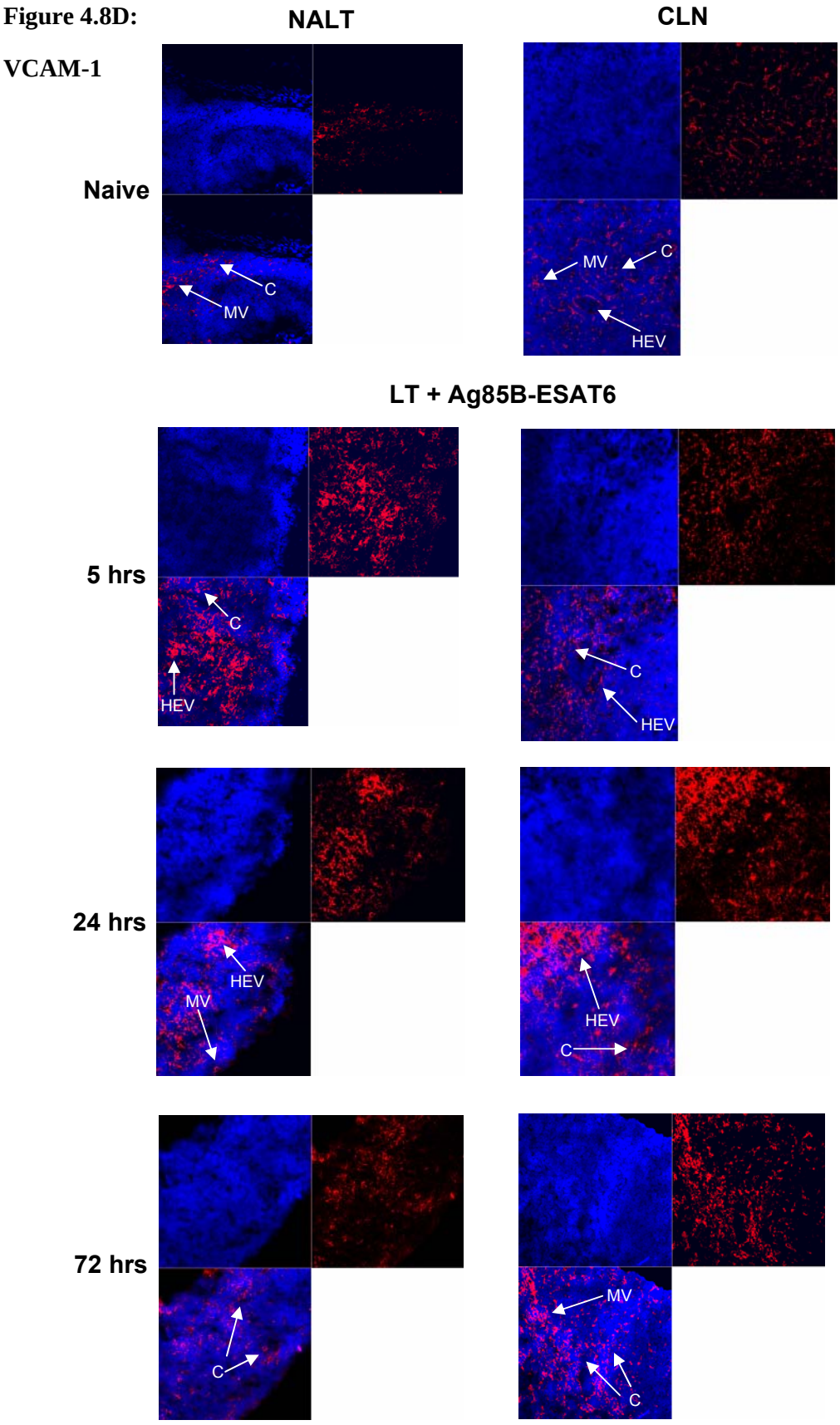

NALT
CLN
ICAM-1
Naive
C
MV
HEV
HEV
LT + Ag85B-ESAT6
5 hrs
MV
C
24 hrs
MV
HEV
C
MV
72 hrs
MV
C
C
HEV

Figure 4.8: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 4.8D represents staining of frozen sections for VCAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT + Ag85B-ESAT6 in Balb/c mice. Double immunolabeling of cell nuclei by Hoechst (blue) and VCAM-1 (red). White arrows and letters indicate particular structures i.e. HEV, MV (micro-vessel/blood vessel) and C (cells). There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 4.8D:


NALT
CLN
VCAM-1
Naive
C
MV
MV
C
HEV
LT + Ag85B-ESAT6
5 hrs
C
HEV
C
HEV
24 hrs
HEV
MV
HEV
C
72 hrs
C
MV
C

4.3.3 Discussion

The impact of immunisation on the expression of mucosal homing receptors on circulating immune cells, as well as MAdCAM-1 expression on endothelium, has been rather well studied, particularly with regards to the gut (Kantele, et al. 2005; Kantele, et al. 1999; Lindholm, et al. 2004; Quiding-Jarbrink, et al. 1997; Rydstrom and Wick 2007). The migration of antigen-sensitised cells is preferentially determined by the concurrent expression of homing-specific adhesion molecules in the tissue endothelium, and the specific receptors (integrins) expressed on the activated lymphoid cells. Oral (intestinal) mucosal exposure to antigen seems to stimulate expression of $\alpha_4\beta_7$ integrins, and intranasal immunisation has been shown to induce expression of L-selectin as well as $\alpha_4\beta_7$ integrins (Quiding-Jarbrink, et al. 1997). However, it is still unknown whether this homing of specific cells is mediated by altered cell adhesion molecule expression after intranasal vaccination in the upper respiratory tract lymphoid tissues (i.e. NALT and CLN). In this present study I show that cell adhesion molecule expression of MAdCAM-1, PNAd, ICAM-1 and VCAM-1 is both increased, and more widely distributed, early after intranasal immunisation in both the NALT and CLN.

It has been demonstrated that both NALT and CLN express MAdCAM-1 and PNAd on their HEV (Csencsits, et al. 1999; Csencsits, et al. 2002). I observed expression of both these addressins on HEV in naïve mice confirming these previous studies. In addition, I found that as early as 5 hours post intranasal immunisation, expression of MAdCAM-1 and PNAd was further upregulated on HEV in both tissues. The changes in CAM expression between naïve and immunised mice was by visual assessment only. This increased MAdCAM-1 expression is similar to that observed in other studies after either oral immunisation or challenge in the gut mucosa (Lindholm, et al. 2004; Quiding-Jarbrink, et al. 1997; Rydstrom and Wick 2007). Significant induction of PNAd also takes place during gut inflammation, allowing the entrance of leukocytes to inflamed mucosal sites (Salmi, et al. 1994; Salmi, et al. 1993). It is likely that the increased MAdCAM-1 and PNAd expression in the respiratory mucosa after immunisation is one of the mechanisms controlling the recruitment of leukocytes to the site of inflammation. This is supported by studies that show expression of the homing receptors $\alpha_4\beta_7$ and L-selectin on leukocytes that are found in both the NALT

and CLN (Csencsits, et al. 2002). The presence of MAdCAM-1 staining within the NALT and CLN may suggest a mechanism for the entrance of intestinal leukocytes, which have been shown to preferentially express $\alpha_4\beta_7$ and enter lymphoid tissues primarily through MADCAM-1-$\alpha_4\beta_7$ interactions (Bargatze, et al. 1995; Streeter, et al. 1988). Though the HEV of the NALT and CLN do express MAdCAM-1, most cell binding, as well as short- and long-term leukocyte homing, appears to be mediated through PNAd-L-selectin interactions (Csencsits, et al. 1999). In this present study I also observed that expression of PNAd is more abundant than MAdCAM-1 on HEV in both the NALT and CLN (particularly in CLN) of both naïve and immunised mice, again highlighting the importance of peripheral addressins in leukocyte trafficking to and from the nasal passages and LNs. As well as MAdCAM-1 expression on HEV, diffuse staining was also observed in both the NALT and CLN after intranasal immunisation. This expression appears to be on the surface cells and may be on infiltrating immune cells, or on existing cell populations within these lymphoid tissues. This diffuse MAdCAM-1 staining has been observed previously in both the NALT and CLN within B cell areas, as well as on FDC in the NALT. The fact that this diffuse MAdCAM-1 staining was only observed after intranasal immunisation supports other studies that have shown that addressin expression by interfollicular DC and macrophages is important in leukocyte recruitment and retention in LNs (Szabo, et al. 1997).

In addition to MAdCAM-1 and PNAd, I observed that both ICAM-1 and VCAM-1 were constitutively expressed in the normal NALT and CLN, supporting the findings of previous studies (Csencsits, et al. 1999; Csencsits, et al. 2002; Hussain, et al. 2001). I also found that expression was dramatically induced, following intranasal immunisation, on both vascular endothelium and cell surfaces. Other studies have also shown that upon inflammatory stimuli, both ICAM-1 and VCAM-1 become strongly up-regulated on endothelial cell lines (up to 40-fold), and are found in increased levels in a variety of inflamed tissues (Dustin, et al. 1986; Henninger, et al. 1997; Meager 1999). Leukocytes migrating into sites of both mucosal and systemic inflammation are also found to express the $\alpha_L\beta_2$ and $\alpha_4\beta_1$ integrins that direct adherence to endothelial cells expressing ICAM-1 and VCAM-1, respectively (Engelhardt, et al. 1994; Steffen, et al. 1996). It therefore appears that leukocyte trafficking, after

intranasal immunisation, to both the NALT and CLN may also depend on the interactions of $\alpha_L\beta_2$ and $\alpha_4\beta_1$ cells with vascular ICAM-1 and/or VCAM-1 in addition to MAdCAM-1 and PNAd. It is interesting to note that the same molecules, i.e. ICAM-1 and VCAM-1, are involved in trafficking in the genitor-urinary tract, which may explain why high levels of antigen-specific immune responses are induced in the genital tract after nasal immunisation (Perry, et al. 1998). In addition to ICAM-1/VCAM-1 staining on vascular endothelium, I also observed diffuse staining on the surface of cells, particularly after intranasal immunisation in both the NALT and CLN. The adhesion molecules ICAM-1 and VCAM-1 are expressed basally at low levels on the surface of macrophages and DC, and can be induced to high levels after antigenic stimulation (Colic and Drabek 1991; Perretti, et al. 1996; Rice, et al. 1991; Yasukawa, et al. 1997). It has been suggested that leukocytes migrate, within tonsils in humans and NALT in rodents, via B cells binding directly to FDC through $\alpha_4\beta_1$/VCAM-1 and $\alpha_L\beta_2$/ICAM-1 interactions. This prevents apoptosis of B cells and is thus crucial for their fate (Csencsits, et al. 1999; Koopman, et al. 1994; Koopman, et al. 1991). A similar adhesion mechanism using $\alpha_4\beta_1$/VCAM-1 interactions has been demonstrated to be involved in the migration of T cells into GC, and this may play a role in B cell retention within GC (Schriever, et al. 1997). In fact, increased expression of VCAM-1 on FDC has been found to coincide with GC formation in both the spleen and peripheral LNs (Balogh, et al. 2002). However, VCAM-1, as a counter-receptor for both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ heterodimers, suggests that the $\alpha_4\beta_7$ leukocytes might also bind in GC through this route as well (Ruegg, et al. 1992). Interaction of $\alpha_L\beta_2$/ICAM-1 also facilitates membrane antigen recognition by B cells (Carrasco, et al. 2004). In addition, ICAM-1 binding to $\alpha_L\beta_2$ on T cells also forms part of the stable T cell-APC immunological synapse, which is essential for antigen presentation (Dustin and Shaw 1999; Monks, et al. 1998). This increased expression of VCAM-1 and ICAM-1 on the surface of leukocytes indicates a possible role in early recruitment and retention of immune cells, enabling increased antigen presentation, and the consequent induction of antigen specific immune responses in the NALT and CLN after intranasal immunisation.

Finally, our results indicate that there are differences in the kinetics of cell adhesion molecule expression early after intranasal immunisation. This data presents interesting implications for the complex nature of immune cell trafficking in both the NALT and CLN and the induction of immune responses. Based on what is known about the role of these ligands and leukocyte receptors in leukocyte extravasation, I expect that induction of the addressins precedes cell entry into both the NALT and CLN. However, as early as 5 hours post intranasal immunisation I could not separate the onset of cell influx with the induction of cell adhesion molecules, suggesting that these two events are very closely linked. In fact, it has been shown that soluble factors released by activated macrophages or lymphocytes may regulate PNAd and MAdCAM-1 expression on HEV in LNs (Hendriks, et al. 1987; Hendriks and Eestermans 1983; Mebius, et al. 1993; Panes and Granger 1998). Similarly, VCAM-1 expression on endothelial cells is induced by several inflammatory factors (Aplin, et al. 1998; Aplin, et al. 1999; Hanada and Yoshimura 2002). With these points in mind, it is notable that I observed increased expression of MAdCAM-1 and VCAM-1 as early as 5 hours in the NALT, but this was not seen in the CLN until 24 hours post immunisation. This may be due, in part, to the earlier activation of leukocytes in the NALT, the site of antigen administration, before these cells drain into the CLN, therefore inducing immune responses slightly later. In addition, the low frequency of L-selectin ligand (PNAd)-expressing vessels 72 hours after intranasal immunisation suggests the possibility that other adhesion molecules, including MAdCAM-1, ICAM-1 and VCAM-1, could mediate later stages of the inflammation. These results are similar to studies performed using the radiolabeled mAb technique, which have revealed that inflammatory stimuli (e.g., cytokines, endotoxin) elicit a time-dependent increase in the expression of all endothelial CAMs in different tissues of the digestive system (Eppihimer, et al. 1996; Henninger, et al. 1997; Panes and Granger 1998).

The work presented here suggests that the recruitment and retention of immune cells early after intranasal immunisation relies in part on modulation of expression of the cell adhesion molecules; PNAd, MAdCAM-1, ICAM-1 and VCAM-1. The expression profile observed indicates the complex nature of leukocyte trafficking after intranasal administration of antigen, and may provide insights into strategies to regulate effector cell populations after mucosal vaccination.

4.4 Germinal centre formation early after intranasal immunisation

4.4.1 Introduction

After antigen challenge, GC are formed within the secondary lymphoid organs (LNs, PP, spleen or tonsils/NALT). GC are the site where B cells proliferate and differentiate into Ig-producing plasma cells, generate high-affinity antigen-specific B-cell receptors by affinity maturation, and differentiate into memory cells (Kelsoe 1996; Kosco-Vilbois, et al. 1997a; Kosco-Vilbois, et al. 1997b; Thorbecke, et al. 1994). The major cell populations encountered in GC are centroblasts and centrocytes, B cells that can be labelled with peanut agglutinin (PNA) (Rose, et al. 1980). Within GC, PNA^+ centrocytes are associated with FDC and T cells (Kosco-Vilbois, et al. 1997b). These FDC retain native antigen and present it to centrocytes, resulting in selection and activation of antigen-specific B cells with high-affinity Ig receptors. Outside the GC, B cells with receptors of high affinity differentiate to plasma cells or memory cells (Kelsoe 1996).

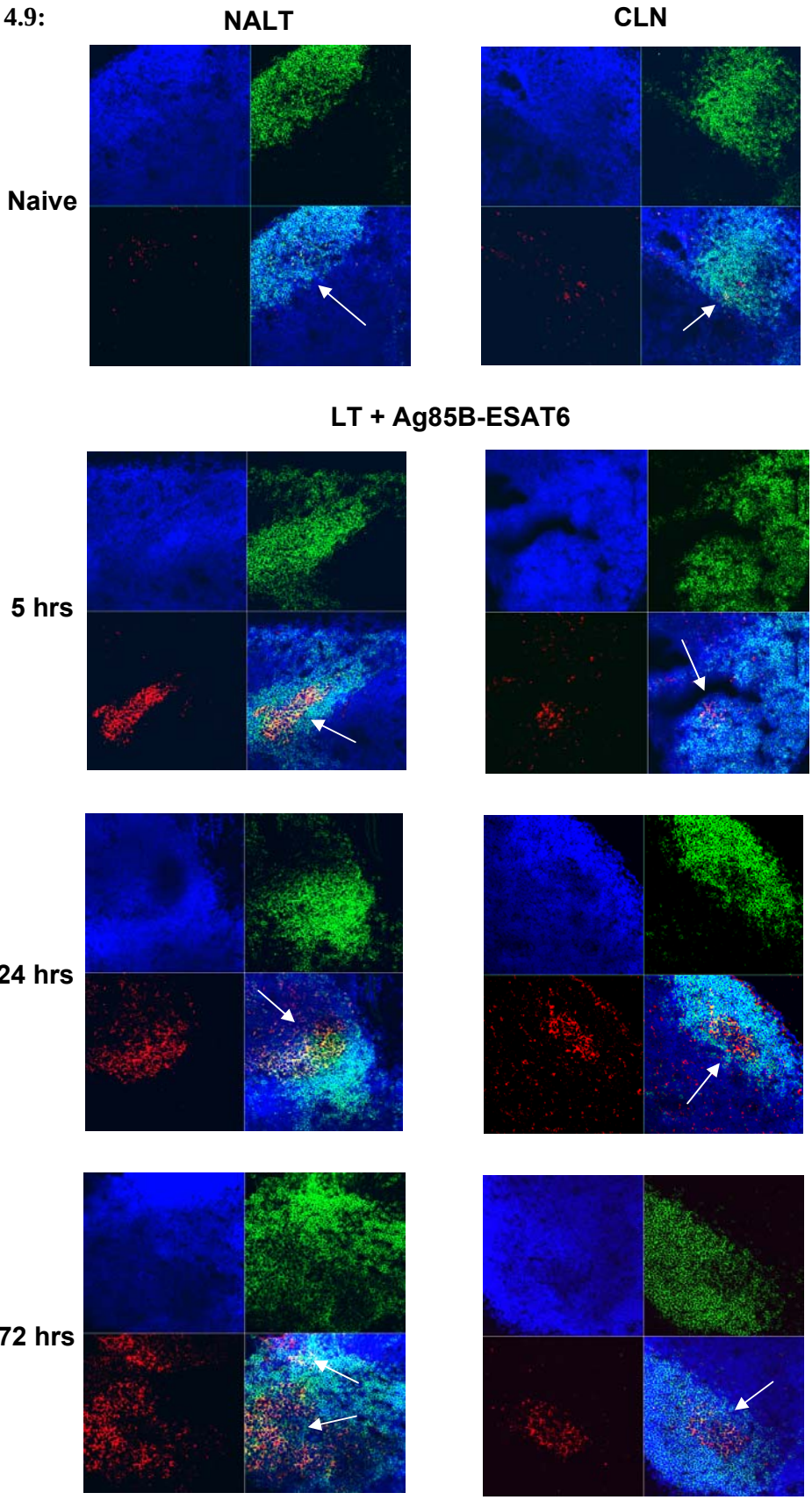
4.4.2 Results

To assess the formation of GC occurring in situ early after intranasal immunisation, cryosections were prepared from both the NALT and CLN, 5, 24 and 72 hours post immunisation. As early as 5 hours post intranasal immunisation, GC were seen in both the NALT and CLN of immunised mice and were still present at completion of the experiment on day 3, as indicated by PNA-binding cells (in red), which are representative of GC B cells (Figure 4.9). At the 5 hour time-point, at least 1 GC was seen per CLN/NALT, whereas those from unvaccinated animals displayed minimally reactive or no GC (Figures 4.9). By 24 hours, vaccinated animals contained a larger number of expanded GC (approximately 2 per tissue) in both lymphoid tissues (Figures 4.9). Finally, 3 days after intranasal immunisation GC were still found to be present, both in larger size and number (3 per NALT and 3-4 per CLN) (Figures 4.9).

Figure 4.9: Immunofluorescence evaluation of GC in both CLN and NALT from immunised Balb/c mice.

Balb/c mice were immunised with either PBS (naïve) or 1µg LT + 25µg Ag85B-ESAT6. Figure 4.9 reveals the presence of GC in the NALT and the CLN of immunised mice at 5, 24 and 72 hours post intranasal immunisation. Triple immunolabeling of B220-positive cells (green), PNA binding cells (red) and cell nuclei by Hoechst (blue) is shown for all images. White arrows indicate GC, or lack of in naïve animals. There was no staining using isotype control mAb (not depicted). Magnification = 28.

Figure 4.9: GC



4.4.3 Discussion

Antigenic stimulation induces the formation of GC in lymphoid tissue. GC represent critical sites within organised lymphoid tissues, in which B cell responses to antigen are amplified and refined in specificity (Guzman-Rojas, et al. 2002). Mucosal but not peripheral, B cells are driven into GC through interaction of innate immune receptors with microbial antigens independent of B cell receptor specificity. This process requires T cells, and recruitment depends on innate immune mechanisms (Casola, et al. 2004). In this present study, I observed the formation of $PNA^{+}B220^{+}$ cells within B cell areas as early as 5 hours post intranasal immunisation. These GC continued to grow and expand in size and number up until the end of the experiment (72 hours) in both the NALT and CLN. This provides direct evidence for the induction of a regional response as GC reactions were induced in both these lymphoid tissues following immunisation. Previous studies have also shown GC formation in the NALT and CLN, but not until 3 days or later after intranasal immunisation or challenge (Asakura, et al. 1998; Eriksson, et al. 2004; Shimoda, et al. 2001; Zuercher, et al. 2002). However, Savransky et al have shown GC formation as early as 6 hours post intranasal immunisation with the Staphylococcal Enterotoxin B in the both the lungs and the spleen (Savransky, et al. 2003). In addition, 72 hours post immunisation, the PNA^{+} cells appeared to be moving away from the B cell areas, particularly within the NALT. Previous studies have shown that more B cells expressing switched transcripts are actually found outside GC, before seeding to other mucosal areas (Kelsoe 1996). This suggests that maturing and differentiating plasma cells are already moving away from B cell follicles at this time-point. The B7 molecules present on APC and B cells play a critical role in both isotype switching and the formation of GC (Han, et al. 1995). Previous studies have shown that *in vivo* adjuvant properties of LT appeared to be mediated by both B7-1 and B7-2, possibly explaining why I see development of GC at these early time-points (Martin, et al. 2002; Petrovska, et al. 2003). Formation of these GC in both the NALT and the CLN highlights the possible mechanisms responsible for induction of humoral immunity following intranasal immunisation.

4.5 Summary discussion

The work presented in this chapter provides insights into the complex nature of innate immune responses induced following intranasal immunisation within the upper respiratory tract. I show that a significant influx and efflux of cells occurs in both the NALT and CLN as early as 5 hour post immunisation, which correlates with the increased expression of cell adhesion molecules also at this time. Examination of a number of activation markers indicates that the innate cell types examined (i.e. DC, macrophages, NKC and neutrophils) are stimulated. These activated cells may be releasing pro-inflammatory cytokines that are enabling vascular endothelial cells to increase their expression of adhesion molecules such as PNAd, MAdCAM-1, ICAM-1 and VCAM-1. In addition, antigen-derived stimuli combined with host cell factors, such as cytokines and chemokines, may recruit and activate additional cells. Macrophages, DC, neutrophils and NKC are probably part of the first wave of direct responders, since I observed increases and decreases of these cell populations as early as 5 hours within the NALT and CLN. Leukocyte homing to both normal tissues and sites of inflammation is, in part, regulated by differential expression of cell surface homing receptors and their selective interactions with tissue selective vascular adhesion molecules at sites of leukocyte recruitment. The differential expression of these cell adhesion molecules shortly after intranasal immunisation may be consistent with the homing of leukocytes into and from both the NALT and CLN from various sites in a time-dependent manner. In order to characterise this migration more fully I can examine the specific integrins on the cells present in these tissues to determine their origin. Leukocytes present in both the NALT and CLN were found to move from peripheral areas into B and T cell areas after intranasal immunisation, suggesting their involvement in the initiation of adaptive immune cell responses; this was especially true for both DC and macrophages. Increased expression of MHC II and movement of these cell types into parafollicular regions within the NALT and CLN suggests their interaction with $CD4^+$ T_H cells. In addition, I observed increased expression of VCAM-1 and ICAM-1 on APC cells, which may correlate with the generation of GC within both lymphoid tissues and subsequent antigen-specific antibody responses. Antigen administered intranasally would not be expected to persist for very long as uptake by APC would result in its rapid breakdown ready for presentation to T cells. In fact, when I probed both the NALT and CLN with mAb against ESAT-6 I did not

observe any protein within the tissues as early as 5 hours, therefore supporting this hypothesis.

As already postulated in the Chapters 3, the differences observed in immune responses between animals immunised with adjuvant alone or adjuvant plus antigen may be down to the differences in the amount of antigen the mouse is receiving rather than a modulating effect by the model antigen (i.e. 1µg LT compared to 1µg LT plus 25µg Ag85B-ESAT6). These data suggest that the variation in innate responses we observe in mice immunised with adjuvant alone compared to those animals receiving adjuvant plus antigen may, to some extent, be influenced by the differential amplification of early innate factors through antigen dose. As already discussed the adjuvant LT is known to modulate innate responses, particular through its interaction and activation of professional APC. However, we cannot exclude the possibility that the fusion antigen is also modulating these early immune responses. Recently, Latchumanan and colleagues observed that when bone marrow cells are incubated with either Ag85B or ESAT-6 they differentiate into DC-like APC, with high expression of surface MHC and co-stimulatory molecules (Latchumanan, et al. 2005; Latchumanan, et al. 2002). In addition, Olsen et al have shown that ESAT-6 can induce the production of IFN-γ from bovine NKC with the response mediated through stimulation of APC (Olsen, et al. 2005). This indicates that both proteins may actually have adjuvant properties similar to those seen in LT. Ouhara *et al*, have reported that the outer membrane protein 100 of *Actinobacillus actinomycetemcomitans* binds to fibronectin inducing antimicrobial peptides production via the MAP kinase pathway (Ouhara, et al. 2006). As already discussed, Ag85B has previously been shown to bind selectively to fibronectin. It is tempting to speculate that Ag85B may also induce antimicrobial peptide production through fibronectin binding interactions. Antimicrobial peptides are important components of innate immune defence. They exhibit large cationic patches on their molecular surface which enables them to depolarise and/or pierce bacterial cell membranes. In addition to their innate antibacterial role, they have been demonstrated to have a number of immunomodulatory functions, including the ability to alter host gene expression, act as chemokines and/or induce chemokine production, promote wound healing, and modulate the responses of DC and lymphocytes (reviewed in (Hancock and Sahl 2006). This work suggests that if Ag85B could promote antimicrobial peptide

production through fibronectin binding and both Ag85B and ESAT6 can activate DC, then it is possible that the fusion protein is also modulating innate immune responses along with LT. However, as already discussed ESAT-6 may also attenuate the innate immune response by dampening production of IL-12p40, TNF-α and NO (Stanley, et al. 2003). In addition some very recent work has shown that ESAT-6 inhibits activation of transcription factor NF-κB and interferon regulatory factors (IRFs) after TLR signaling (Pathak, et al. 2007). Hence, it may be the differences we are seeing in early immune responses in those animals receiving adjuvant and the fusion protein is actually a direct result of the dampening down of TLR induced innate immune responses. These data therefore provide some other possible elucidations as to why we see variations in early immune responses between adjuvant and adjuvant plus antigen immunised mice.

It is hoped that this current research will provide new insights into the function of upper respiratory tract mucosal tissues and the interplay of innate and adaptive immune responses that results in immune protection at mucosal surfaces. Characterisation of these innate immune response mechanisms involved early after intranasal immunisation may help clarify the concepts and provide the tools that are needed to exploit the full potential of mucosal vaccines