5 Natural killer cell depletion influences antigen specific cytokine and antibody responses after intranasal immunisation

5.1 Introduction

NKC, a component of the innate immune system, mediate cellular cytotoxicity and produce chemokines and inflammatory cytokines, such as IFN-γ and TNF-α (Anegon, et al. 1988; Arase, et al. 1995; Arase, et al. 1996; Kim, et al. 2000; Kim and Yokoyama 1998; Ortaldo and Young 2003; Trinchieri 1989). They are an important constituent of innate resistance to viruses, bacteria and certain parasites as well as providing immune surveillance against the development of tumours (Biron, et al. 1989; French and Yokoyama 2003; Karre, et al. 1986; Lieke, et al. 2006; Lodoen and Lanier 2006; Roetynck, et al. 2006; Schafer and Eisenstein 1992; Storkus, et al. 1989; Yang, et al. 2006). NKC also interface with adaptive immunity by stimulating DC and by promoting T cell responses. NKC are bone marrow-derived granular lymphocytes, distinct from T and B cells that are widespread throughout the body being present in both lymphoid organs and non-lymphoid peripheral tissues. For a comprehensive review see (Hamerman, et al. 2005). NKC express a range of distinct receptors on their surface that are involved in recognising and responding to pathogens. There are two general types of NKC receptors, i.e., inhibitory and activation receptors (Lanier 1998). NKC express inhibitory receptors that monitor for normal expression of host proteins, and their function underpins the 'missing self' hypothesis. The best characterised are those receptors that bind classical and non-classical MHC class I, the expression of which is often targeted by pathogens or malignancies to subvert immune responses by T cells (Lanier 2005). The term 'NKC activating receptor' generally refers to those receptors that trigger release of cytolytic granules and typically induce cytokine production. Cellular ligands for NKC activating receptors are normally absent on healthy cells, but can be induced in response to cellular stress, such as infection. In addition, NKC express receptors that are a form of pathogen-associated pattern recognition system. The mouse Ly49H NKC receptor has been found to bind to a product *m157* of mouse cytomegalovirus (MCMV), and activation through these receptor ligand interactions enable NKC cells to limit early-stages of

MCMV infection and to undergo considerable proliferation (Arase, et al. 2002; Smith, et al. 2002). NKC have also been reported to express most of the known Toll-like receptors (TLR), including TLR3, TLR5 and TLR9. However, the role and the expression pattern of TLR on NKC has not been well characterised (Yokoyama and Scalzo 2002). Activated NKC exhibit rapid secretion of several cytokines, including IFN- γ, as well as other immunoregulatory mediators, including TGF-β, TNF-α, TNF-β, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1α, IL-1, IL-2, IL-3, IL-5, IL-8 and IL-10 (Biron 1997; Scharton-Kersten and Sher 1997; Warren, et al. 1995). Additionally, infected or activated macrophages and DC produce cytokines and chemokines such as IFN-α/β, IL-12, IL-15 and IL-18 that stimulate NKC to rapidly produce other cytokines (Andrews, et al. 2005; Biron, et al. 1999; Walzer, et al. 2005; Zitvogel, et al. 2006). Recent work suggests that crosstalk between DC and NKC may influence the adaptive immune response (Ferlazzo, et al. 2003; Kalinski, et al. 2005; Walzer, et al. 2005; Zitvogel, et al. 2006). Activation of NKC *in vivo* may be in part due to receptor-ligand interactions with DC, as well as DC-derived cytokines. Communication between these two cell types is not just unidirectional, as NKC can also stimulate maturation of DC by up-regulation of MHC complexes and co-stimulatory molecules, and therefore may represent a key mechanism to bridge NKC response to the stimulation of T cell responses (see Figure 5.1) (Raulet 2004).

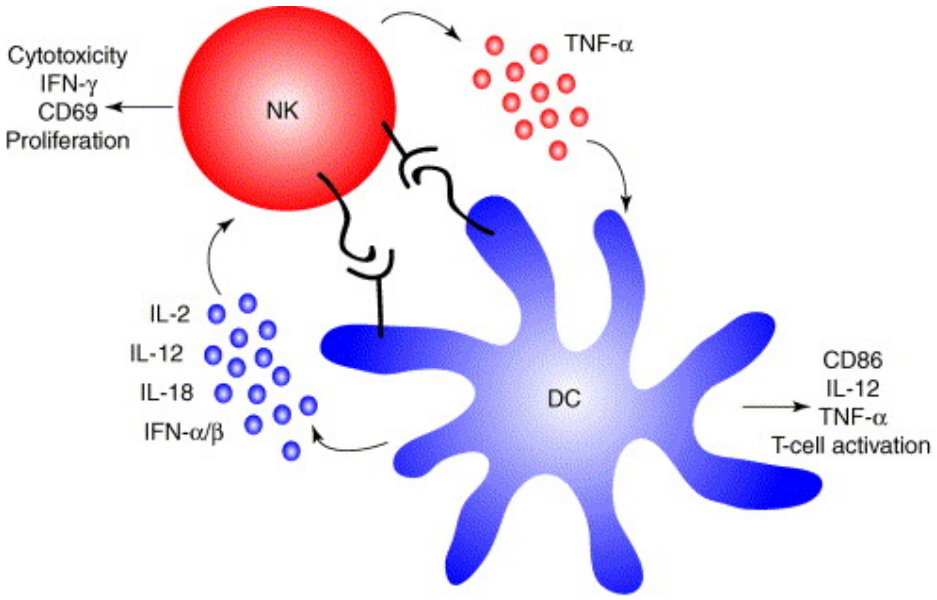


Figure 5.1: Crosstalk between NKC and DC.

NKC and DC have the ability to reciprocally activate one-another, both *in vitro* and *in vivo*. This crosstalk includes cell contact involving unknown receptor-ligand pairs and soluble mediators produced by the two cells. The cytokines, TNF-α, IL-2, IL-12, IL-18 and IFNs, have all been implicated in this process. The end result of these interactions is NKC activated for cytotoxicity, IFNγ production, and proliferation, and DC that have matured and are capable of cytokine production and T cell activation (Hamerman, et al. 2005).

Evidence from a number of studies have also suggested that rapid IFN-γ production by NKC may affect the characteristics of antigen-specific immune response, particularly by promoting T_H1 polarisation (Fearon and Locksley 1996; Korsgren, et al. 1999; Martin-Fontecha, et al. 2004; McKnight, et al. 1994; O'Leary, et al. 2006; Romagnani 1992; Scharton-Kersten and Scott 1995; Scharton and Scott 1993; Wang, et al. 1998). Collectively these data have led to a broadly based consensus that early NKC responses may have a profound effect on later adaptive immune responses. However, little is know about the role of NKC in induction of immune responses after mucosal administration of antigen. Previously I have shown that NKC are the most abundant innate cell type recruited to both the NALT and CLN early after intranasal immunisation. These $DX5^+$ cells were observed to express high levels of the markers CD69 and CD25, suggesting that these NKC were also activated. Given this influx of NKC post intranasal immunisation, I hypothesised that these innate immune cells may

be involved in processes that lead to antigen-specific immune responses, i.e. adaptive immunity. Specifically, this study examines whether NKC depleted mice have differences in both antigen-specific B and T cells responses through examination of antibody subtypes, their titres and cytokine production, when compared to control animals.

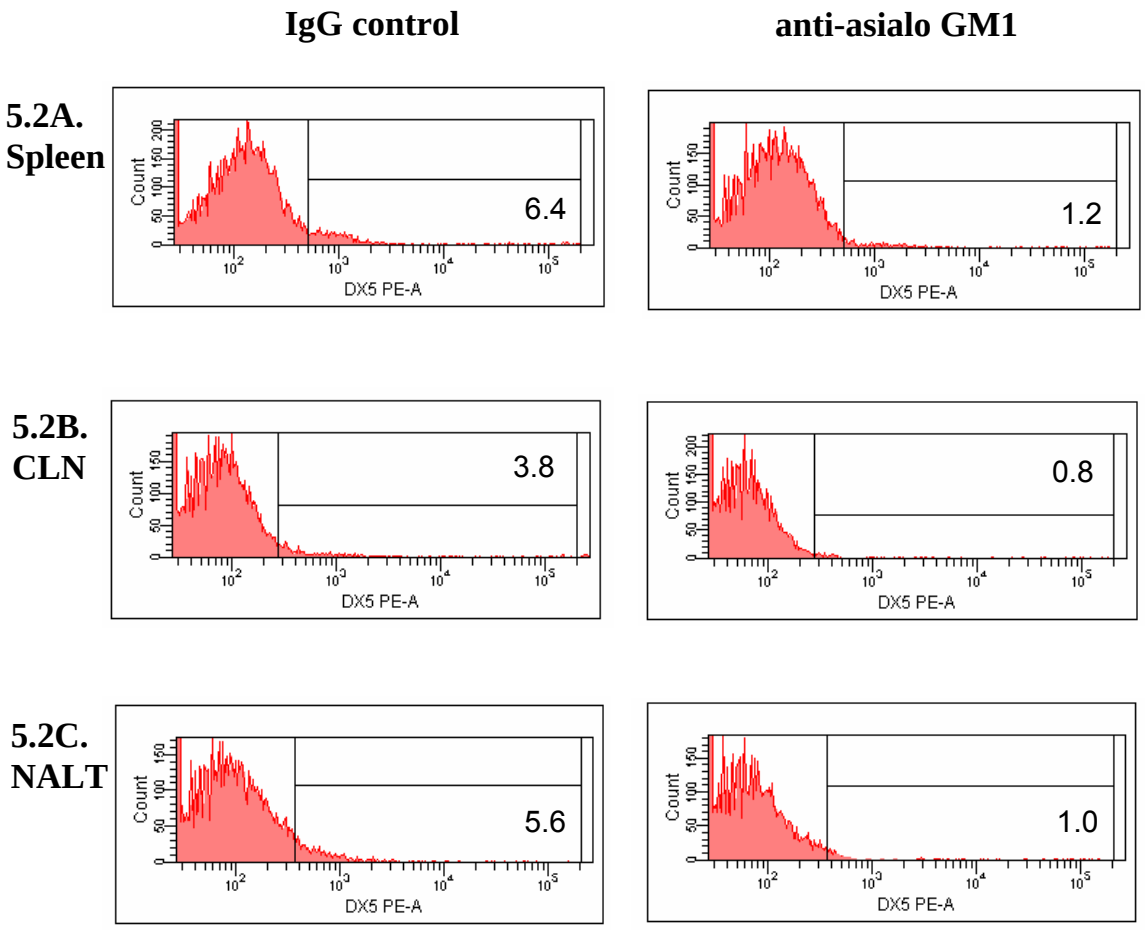
5.2 Results

5.2.1 Anti-Asialo GM1 treatment successfully depletes Balb/c mice of NKC

In order to study the effects of NKC on the induction of antigen-specific immune responses I employed chronic administration of polyclonal anti-asialo GM1 antibody to Balb/c mice. Virtually all murine NKC express this marker and, as widely demonstrated, this approach effectively depletes functional and phenotypic NKC activity *in vivo* (Yu, et al. 1992). In addition, this antibody does not activate NKC when binding. Controls were treated with an equivalent concentration of normal rabbit IgG. NKC depletion was effective as shown by histogram plots of $DX5^+$ cells within the spleens, CLN and NALT of antibody depleted and control mice (Figure 5.2A, B and C). This marker is also expressed at low levels on murine macrophages and NK T cells, so in order to make sure these cell types were not being reduced I examined the percentages of $F4/80^+$ cells and $\alpha\beta TCR^+/DX5^+$ cells within spleens of naive mice. I did not observe any significant reduction ($p > 0.05$) in these cells types in depleted mice when compared to control animals (Figure 5.3). To ensure NKC depletion, antibody treatment was given at 3-4 day intervals, beginning 7 days before intranasal immunisation and continuing until sacrifice. I also selected mice from within each cohort at the end of the study (day 21) to confirm that NKC remained at undetectable levels (Figures 5.2D, E and F).

Figure 5.2: Impact of *in vivo* administration of anti-asialo GM1 on NKC percentages in Balb/c mice.

Balb/c mice were treated with 50µg anti-asialo GM1 antibody or 50µg rabbit IgG antibody and spleens, CLN and NALT were assessed for $DX5^+$ cells (NKC) after 2 doses of antibody (day 0) and after 8 doses of antibody (day 21) by flow cytometry. Figure 5.2A represent naïve spleens from IgG control and anti-asialo GM1 depleted mice at beginning of experiment (day 0). Figure 5.2B shows the CLN and C represents the NALT also at beginning of experiment (day 0). Figures 5.2D-F represents naive spleen, CLN and NALT, but at the end of the experiment (day 21). Histogram plots represent the $DX5^+$ population within each tissue and the number on the right hand side indicates the total percentages present. Plots shown are from five individual representative mice, and the mean values are indicated.



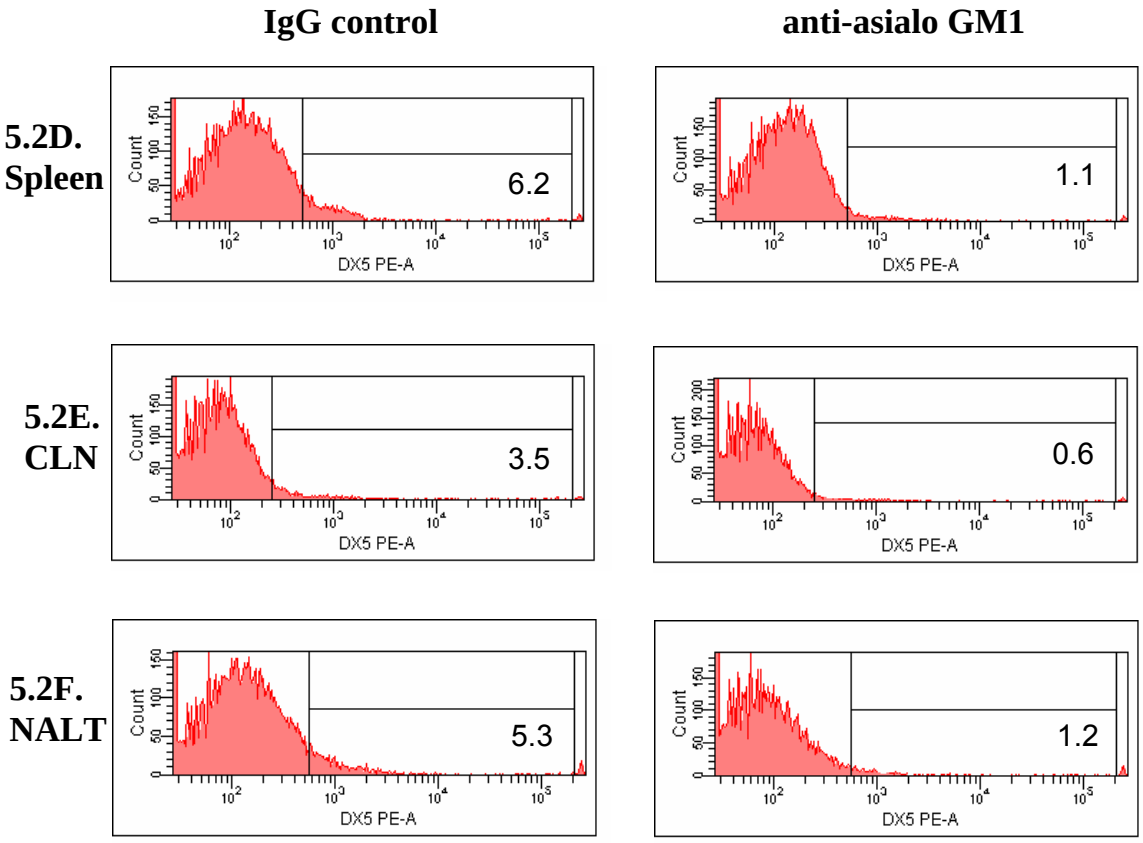
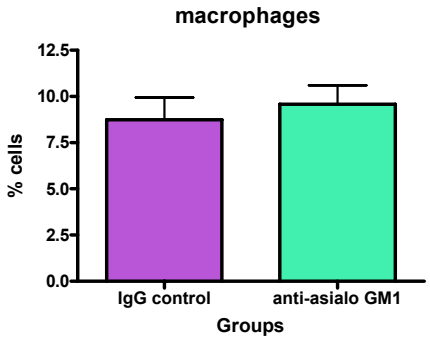

IgG control
anti-asialo GM1
5.2D. Spleen
5.2E. CLN
5.2F. NALT
Count
DX5 PE-A
6.2
1.1
3.5
0.6
5.3
1.2

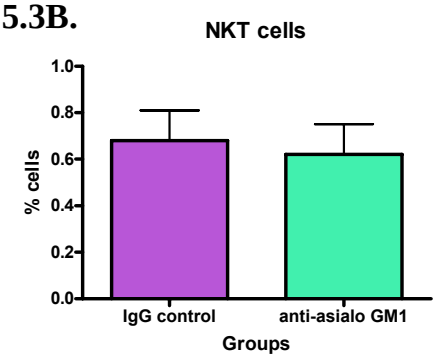
Figure 5.3: Percentages of macrophages and NKT cells in naïve normal IgG and anti-asialo GM1 treated mice.

Balb/c mice were treated with 50µg anti-asialo GM1 antibody or 50µg rabbit IgG antibody and spleens were assessed for $F4/80^+$ macrophages cells and $\alpha\beta TCR^+/DX5^+$ cells after 2 doses of antibody by flow cytometry. Figure 5.3A represent percentages of $F4/80^+$ cells from naïve spleens of IgG control and anti-asialo GM1 depleted mice. Figure 5.3B shows $\alpha\beta TCR^+/DX5^+$ cells from naïve spleens of IgG control and anti-asialo GM1 treated mice. Columns represent the mean (± SD) percentages of cells from five animals per group.

5.3A.



5.3B.



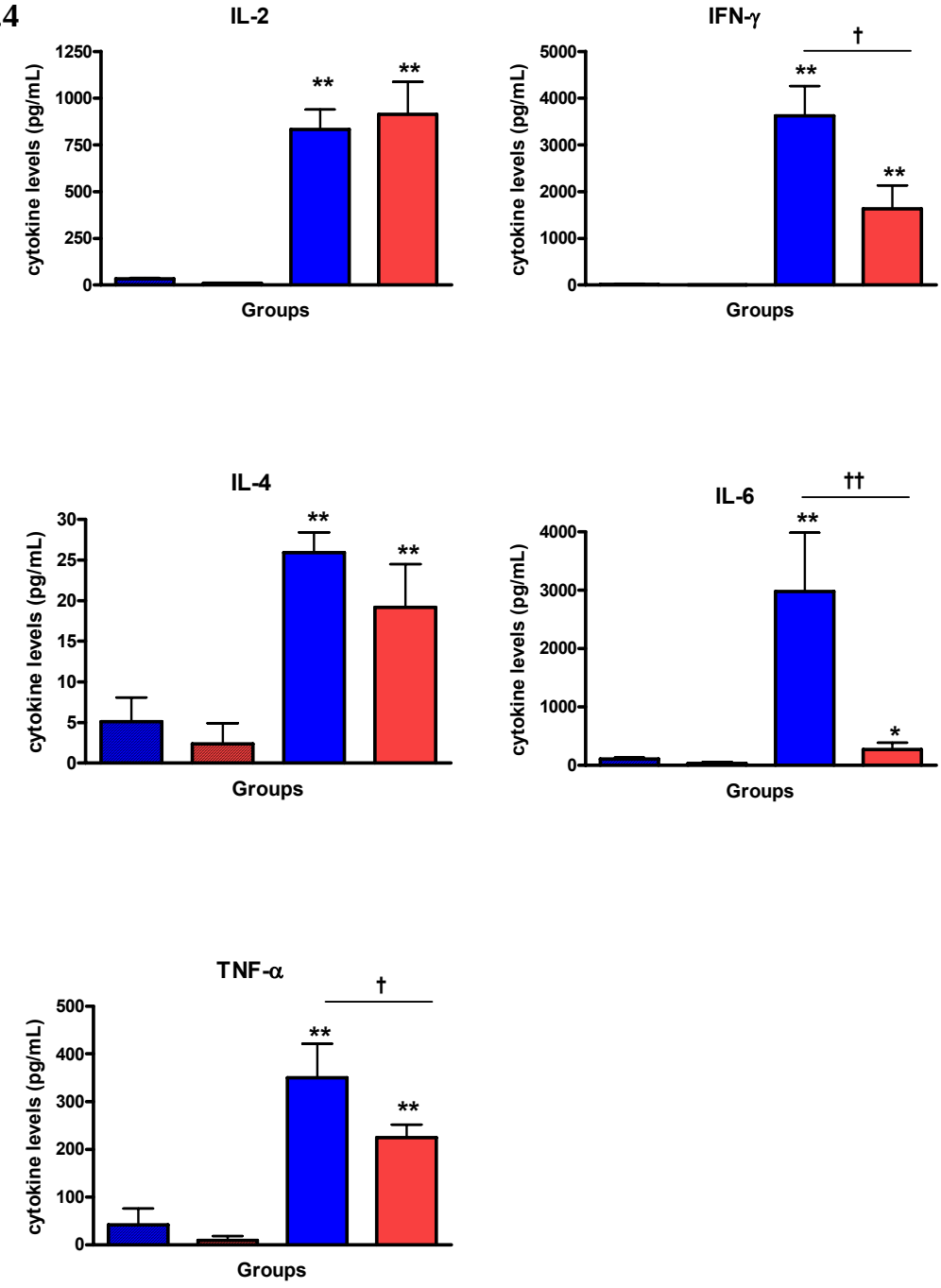
5.2.2 *In vivo* depletion of NKC affects induction of antigen-specific cytokine responses

Balb/c mice treated with anti-asialo GM1 and controls treated with an equivalent concentration of normal rabbit IgG were immunised with adjuvant and Ag85B-ESAT6 to assess the intensity of T_H1 and T_H2 associated cytokine synthesis (IL-2, IFN-γ, TNF-α, IL-4 and IL-6) As shown in Figure 5.4 all immunised mice had significantly higher levels ($p < 0.05$) of each cytokine when compared to their controls, i.e. naïve IgG control mice compared to immunised IgG control mice and naïve (PBS immunised) NKC depleted mice compared to adjuvant and antigen immunised NKC depleted mice. When IgG control immunised mice were compared to anti-asialo GM1 treated immunised mice, both IL-2 and IL-4 levels were found not to be significantly different ($p > 0.05$) between these cohorts. However, IFN-γ, TNF-α and IL-6 were all found to be significantly lower in immunised NKC depleted mice when compared to IgG control immunised animals ($p < 0.05$), with the reduction in IL-6 levels being the most significant ($p < 0.01$ vs. $p < 0.05$).

Figure 5.4: Anti-asialo GM1 antibody treatment alters the induction of antigen-specific cytokines.

Balb/c mice were treated with anti-asialo GM1 or normal rabbit IgG on days -7, -4, -2 then intranasally immunised with 1µg LT and 25µg Ag85B-ESAT6 or PBS on day 0. Mice were further treated ever 3-4 days, before sacrifice on day 21 when spleens were removed for T-cell assays. Cytokine responses were measured upon *in vitro* stimulation with Ag85B-ESAT6 (5µg/mL) for 36-42 hours. Cells were also stimulated with conA (positive control) and $RPMI^{+}$ media (negative control) (data not shown). Columns represent the mean (± SD) stimulation indices of splenocytes from five animals per group. The differences in cytokine levels between either control, or NKC depleted naïve and immunised animals is indicated by *, $p < 0.05$; and **, $p < 0.01$ with differences in immunised IgG control or anti-asialo GM1 treated animals shown by †, $p < 0.05$ and ††, $p < 0.01$ using a Kruskal-Wallis test followed by Dunn's Multiple Comparison test. The sensitivities of the CBA was > 1pg/mL for each cytokine. Blue indicates rabbit IgG control treated mice and red shows anti-asialo GM1 treated animals with diagonal lines and block colours representing naïve and immunised groups, respectively.

5.4


IL-2
cytokine levels (pg/mL)
1250
1000
750
500
250
0
**
**
Groups
IFN-γ
cytokine levels (pg/mL)
5000
4000
3000
2000
1000
0
†
**
**
Groups
IL-4
cytokine levels (pg/mL)
30
25
20
15
10
5
0
**
**
Groups
IL-6
cytokine levels (pg/mL)
4000
3000
2000
1000
0
††
**
*
Groups
TNF-α
cytokine levels (pg/mL)
500
400
300
200
100
0
†
**
**
Groups

5.2.3 Mice depleted of NKC have reduced antigen-specific antibody responses

To assess antigen-specific antibody production in both control (i.e. rabbit IgG treated mice) and anti-asialo GM1 treated immunised mice, I measured both LT and Ag85B-ESAT6 specific total Ig, IgG, IgG1, IgG2a, IgG2b, IgG3, IgA, IgE and IgM levels in plasma using ELISA at day 14 and day 21 after intranasal immunisation. Figure 5.5A shows that immunised anti-asialo GM1 treated animals have a modest decrease in total both LT and Ag85B-ESAT6-specific Ig titres when compared to immunised rabbit IgG treated control animals on day 14. However, this decrease was not found to be significant ($p > 0.05$). By 21 days post immunisation, it is clear that all mice have made a vigorous total Ig anti-LT and anti-Ag85B-ESAT6 response, regardless of whether they were treated with anti-asialo GM1 or normal rabbit IgG, suggesting that depletion of NKC does not affect total Ig responses at this later time-point (Figure 5.5B).

I also did not observe a difference ($p > 0.05$) in Ag85B-ESAT6-specific IgG responses at either time-point between immunised rabbit IgG treated control mice or anti-asialo GM1 treated mice (Figure 5.5A and B). Examination of the IgG subtype titres, IgG1 and IgG2a, showed that NKC depleted mice were able to mount an apparently normal IgG1 response to Ag85B-ESAT6, whereas I could not detect serum IgG2a anti-Ag85B-ESAT6 ($p = 0.008$) above pre-immune levels throughout the experiment in any of the anti-asialo GM1 treated mice (Figures 5.5A and B). Interestingly, mice treated with anti-asialo GM1 were not able to mount a detectable LT-specific IgG response, including IgG1 and IgG2a ($p = 0.008$), 14 days after intranasal immunisation (Figure 5.5A). However by day 21, I did observe anti-LT IgG and IgG1 responses in anti-asialo GM1 treated animals, but as observed for anti-Ag85B-ESAT6 response I did not observe any detectable IgG2a titres ($p = 0.008$) (Figure 5.5B). On day 21 after immunisation I had more serum as it was the end of the experiment, compared to the tail bleeds performed on day 14, therefore I decided to examine both antigen-specific IgG2b, IgG3, IgE, IgA and IgM responses in control and NKC depleted mice to further characterise the *in vivo* antibody responses. As with IgG2a responses I did not detect any anti-LT or anti-Ag85B-ESAT6 IgG2b responses ($p = 0.008$) in all of the animals treated with anti-asialo GM1 (Figure 5.5B). I

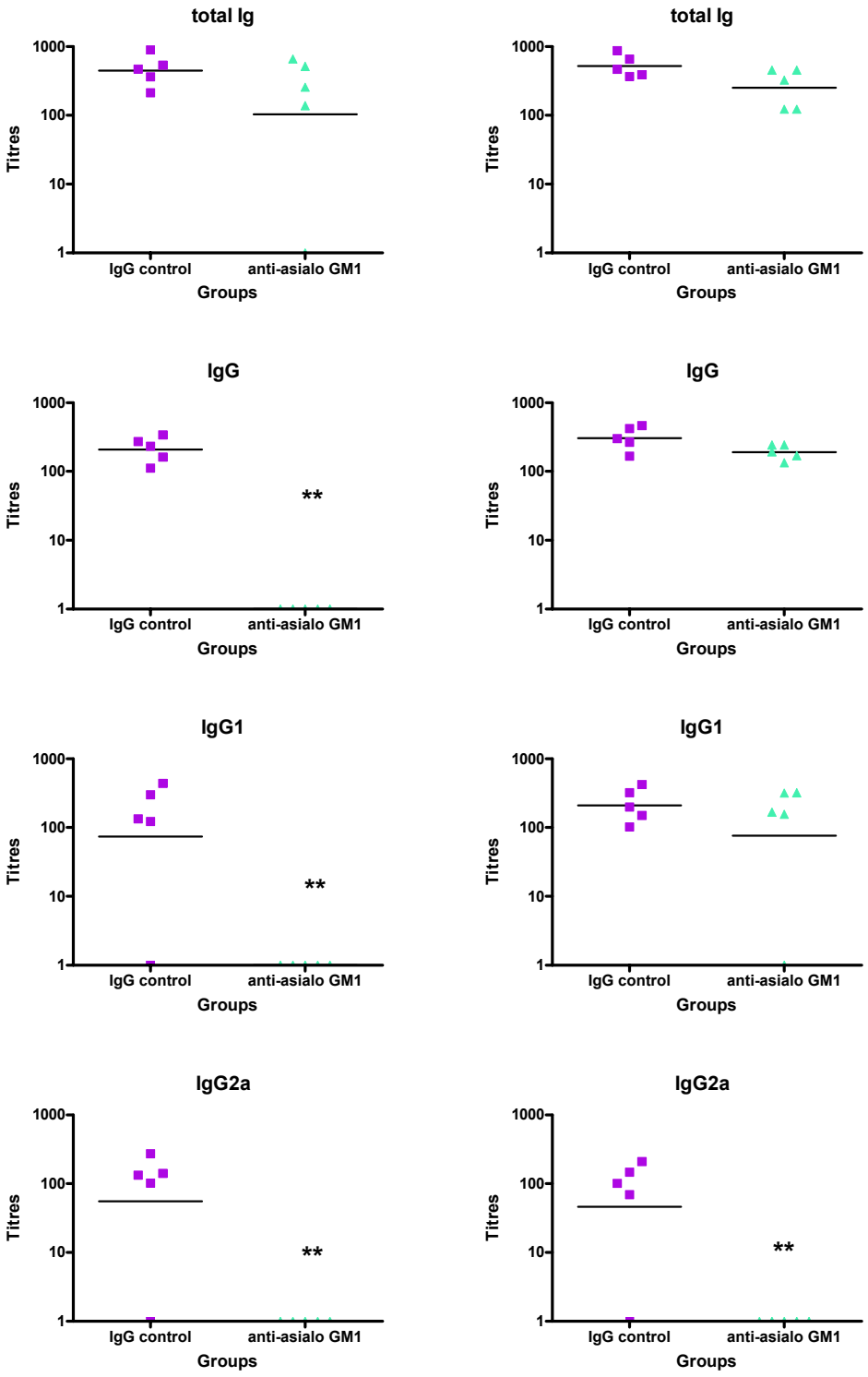
observed that control mice mounted both a modest IgE and IgM antigen-specific response after intranasal immunisation with LT and Ag85B-ESAT6 (Figure 5.5B). Conversely, I could not detect any anti-LT IgE and IgM responses in mice treated with anti-asialo GM1 ($p = 0.008$). Immunised control animals also had significantly higher ($p = 0.008$) IgE anti-Ag85B-ESAT6 titres compared to anti-asialo treated animals, though one of the five mice depleted of NKC did have detectable anti-Ag85B-ESAT6 IgE titres (Figure 5.5B). Notably, anti-Ag85B-ESAT6 IgM responses did not appear to be affected by anti-asialo GM1 treatment (Figure 5.5B). I could not detect any serum antigen-specific IgG3 or IgA in either rabbit IgG treated, or anti-asialo GM1 treated immunised animals (data not shown). Collectively, these data argue that anti-asialo GM1-mediated NKC depletion impacts the development of antigen specific immune response to antigens and adjuvant after intranasal immunisation.

Figure 5.5: Anti-asialo GM1 treatment influences the development of antigen-specific antibody responses.

Balb/c mice were treated as described in Material and Methods (purple squares represent normal rabbit IgG treatment and green triangles indicate anti-asialo GM1 treatment) and intranasally immunised with Ag85B-ESAT6 and LT, then bled on days 14 and 21 for evaluation of primary antigen-specific total Ig, IgG, IgG1, IgG2a, IgE and IgM synthesis by ELISA. Figures 5.5A and B show anti-LT and anti-Ag85B-ESAT6 titres 14 and 21 days after immunisation, respectively. Data reflect five individual mice per group (minus background naïve animal titres) expressed as total antibody titre using a cut off of OD 0.3., with the black bar showing the geometric mean. Significant differences as calculated with the Mann-Whitney U-Test are marked with **; $p = 0.008$.

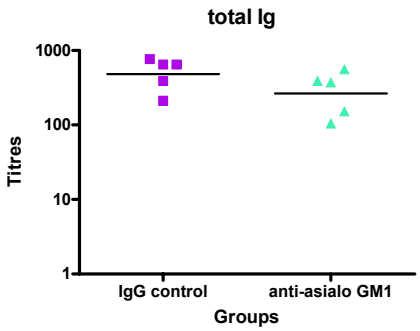
Day 14

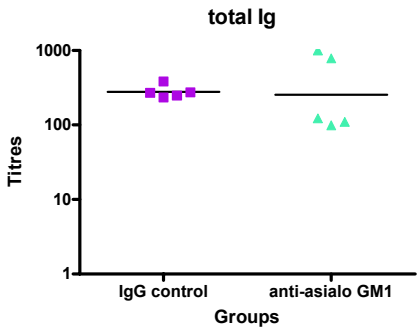
5.5A. **LT-specific titres** **Ag85B-ESAT6-specific titres**

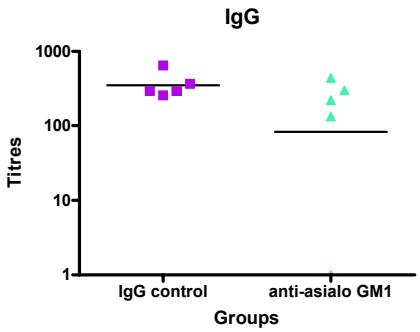


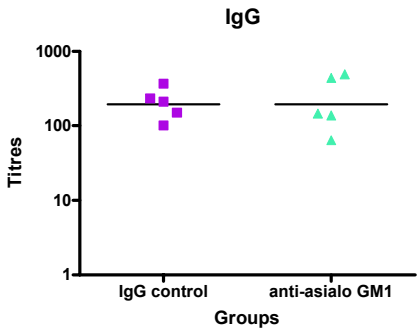
Day 21

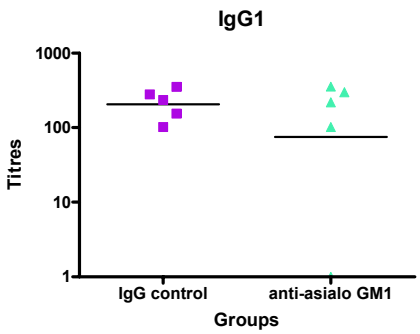
5.5B. LT-specific titres **Ag85B-ESAT6-specific titres**

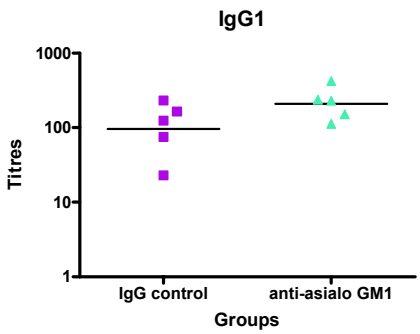


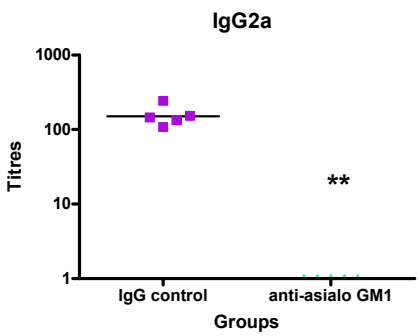


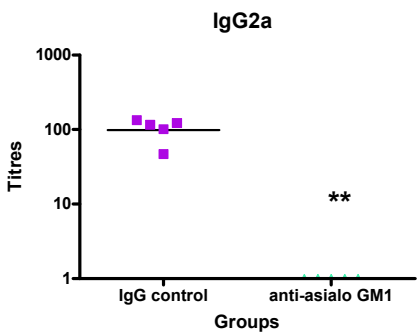






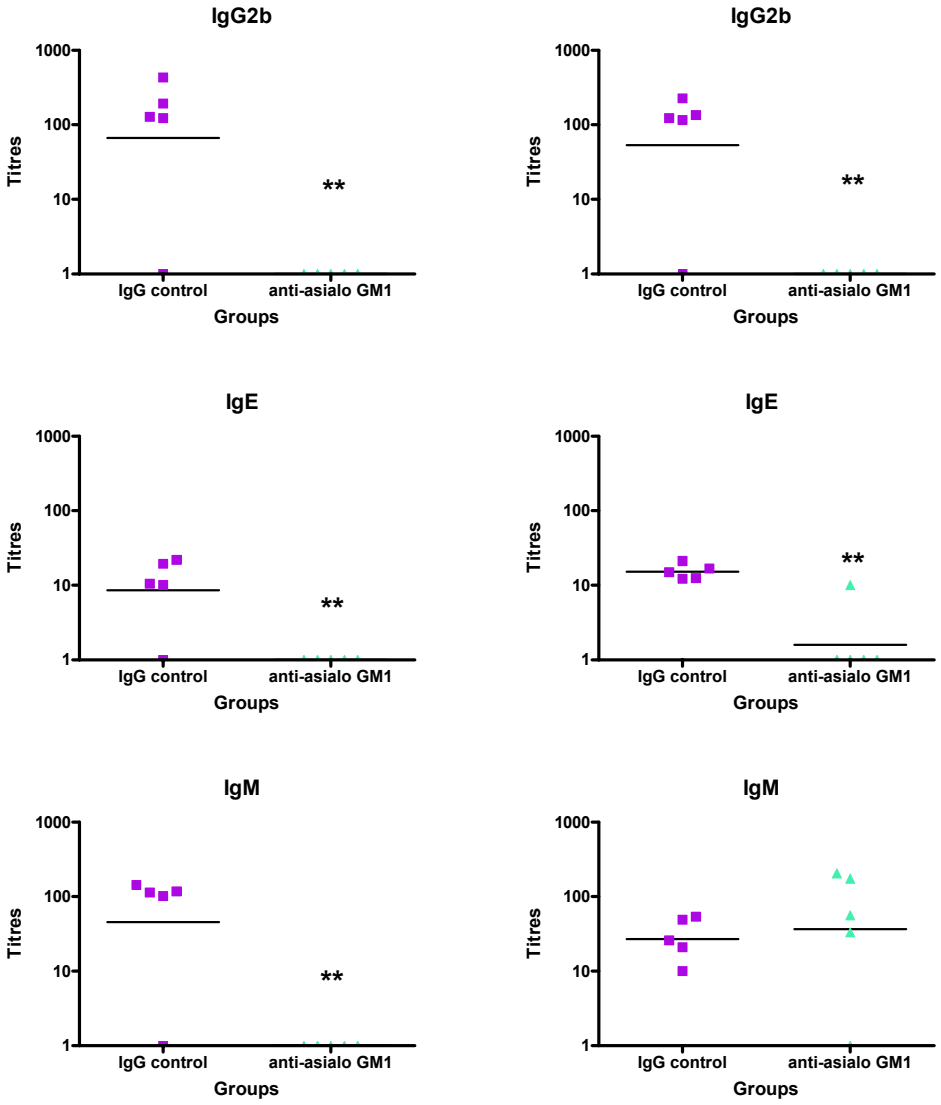






Day 21

5.5B. LT-specific titres Ag85B-ESAT6-specific titres



5.3 Discussion

In this study I provide evidence that NKC, a major arm of innate immunity, participate in the development of antigen-specific immune responses after mucosal immunisation. The requirement of NKC in the induction of adaptive immunity was reflected by the significantly decreased production of IFN-γ, TNF-α and IL-6 in anti-asialo GM1 treated mice when compared to IgG control animals. NKC were also involved in antigen–specific antibody responses, as mice depleted of NKC failed to mount both IgG2a and IgG2b responses. Interestingly, examination of specific adjuvant and antigen antibody responses gave diverse results, i.e. early (day 14) anti-LT antibody responses lacked any specific IgG titres. However, this was not the case for anti-Ag85B-ESAT6 responses as I observed both IgG and IgG1 at this time-point. Additionally, adjuvant antibody responses at day 21 failed to elicit any detectable IgM, whereas this was not found for antigen antibody responses.

Antibody depletion was utilised in this study for the removal of NKC as there are, as yet, no animal models in which NKC activities are genetically and selectively deficient. Most mutations inserted into mice to study the role of NKC affect the granules of many other cell types (e.g. CTL) and do not ablate other NKC activities such as cytokine production (Kim, et al. 2000). Most recently, some transgenic mice have been constructed that express the diphtheria toxin receptor under the control of the NKp46 promoter. Addition of diphtheria toxin to these mice results in death of only the cells expressing the toxin receptor. However, this data is still unpublished and the mice are not currently available. For *in vivo* depletion of NKC in Balb/c mice I utilised the anti-asialo GM1 antibody. This antibody has been employed in many previous NKC depletion experiments with effective results. However, asialo GM1 is also expressed at low levels on the surface of other cell types. NKC and NKT cells show different susceptibilities to *in vivo* treatment with anti-asialo GM1, possibly due to the difference in their expression of asialo GM1 (Sonoda, et al. 1999). By taking advantage of this difference, I could selectively deplete NKC without affecting NKT cells. Asialo GM1 is also expressed at low levels on the surface of macrophages, however this present study and previous ones have shown that treatment with anti-asialo GM1 does not affect macrophage numbers or their activity (Keller, et al. 1983). These data therefore suggest that only NKC are being depleted in mice and that the

observations noted throughout the study are a direct cause by the absence of NKC and not any other cell type.

The mechanisms by which NKC modulate adaptive immune responses are not fully understood. Factors that affect APC and determine the differentiation of naïve T cells into either T_H1 (IL-2, IFN-γ, TNF-α), or T_H2 (IL-4, IL-10) phenotypes and B cells into antibody producing cells would be expected to have an impact on the development of antigen specific immune responses after intranasal immunisation. NKC contribute to the innate immune defence through the early release of IFN-γ and TNF-α. Our results show that levels of IFN-γ and TNF-α from stimulated splenocytes were significantly lower in NKC depleted mice than in IgG control animals. These data indicate that NKC may be a major source of these cytokines after mucosal immunisation, and consequently may drive T_H1 adaptive immune responses. These data are also consistent with other findings that show that NKC depletion reduces IFN-γ production after systemic administration of antigen/pathogen (Korsgren, et al. 1999; Martin-Fontecha, et al. 2004; Sayeh, et al. 2004; Scharton and Scott 1993). As already discussed, NKC are also involved in crosstalk and activation of DC. TNF-α is also produced by activated DC, and this may also explain why I observed a significant decrease in production of this cytokine in anti-asialo GM1 treated animals. Studies have failed to detect IL-4 production from NKC, however it has been hypothesised that NKT cells may be an important source of IL-4 and, therefore in the development of T_H2 type immune response. In this study I showed that NKT cells were not depleted in anti-asialo GM1 treated animals, possibly explaining why I did not observe any significant decrease in antigen-specific IL-4 production from NKC depleted mice. No significant reduction in IL-4 levels was also observed by Satoskar and colleagues in NK^-T^+ mice. However, they also did not detect a significant reduction in IFN-γ levels (Satoskar, et al. 1999). IL-6 is an important mediator of the immune response during acute phases of stimulation. Levels of IL-6 were significantly higher in non-depleted controls than in anti-asialo GM1 treated animals after stimulation of splenocytes by antigen. As IL-6 is a cytokine produced by activated macrophages and DC, these data corroborate the involvement of NKC in promoting APC activation (Akira and Kishimoto 1996; Banchereau, et al. 2000; Grohmann, et al. 2001). IL-2 is produced by T cells and enhances NKC cytotoxicity and cytokine production as well as inducing proliferation of activated T and B cells.

In this present study I did not observe any differences in IL-2 production in anti-asialo GM1 treated mice compared to IgG control immunised mice. Consequently it appears that NKC do not affect T cell proliferation, whereas they do appear to modulate antigen-specific cytokine production.

NKC have also been shown to regulate B cell differentiation and antibody production. However, their role in regulating antibody secretion, both *in vitro* and *in vivo*, is uncertain as many studies contradict one another, some suggesting that NKC down-regulate antibody production, others proposing that they actually enhance it (Arai, et al. 1983; Jensen, et al. 2004; Khater, et al. 1986; Korsgren, et al. 1999; Robles and Pollack 1989; Satoskar, et al. 1999; Snapper, et al. 1994; Wang, et al. 1998; Wilder, et al. 1996; Yuan, et al. 2004). Many different methods were utilised in these studies to deplete NKC and this may, in part, explain these inconsistent findings. These problems may be overcome if genetically modified mice with a specific deletion of NKC can be constructed, however this has yet to be achieved. The fact that the IgG2a subclass of antibody was the one most affected by anti-asialo GM1 treatment is not surprising, given the fact that its production is sensitive to levels of IFN-γ in the milieu (Finkelman, et al. 1988; Snapper and Paul 1987; Stevens, et al. 1988). IFN-γ is one of the cytokines secreted by NKC, and IL-2 activated NKC have been shown *in vitro* to enhance IgG2a secretion by LPS-stimulated B cells, in a manner dependent upon IFN-γ secretion (Amigorena, et al. 1990; Michael, et al. 1991). In accordance with these observations I also noted a significant decrease in IFN-γ production from antigen stimulated splenocytes, and this may account for the lack of any detectable IgG2a titres. These results are consistent with other studies that have also shown that changes in IgG2a levels are NKC-dependent (Jensen, et al. 2004; Korsgren, et al. 1999; Satoskar, et al. 1999; Wilder, et al. 1996). In relation to the effects observed on IgG2b concentrations in NKC-depleted mice, it has been proposed that IFN-γ also regulates production of IgG2b to T cell dependent antigens (Snapper and Mond 1993). Again the significant reduction of IFN-γ observed in this present study may account for this disparity in IgG2b titres. I did not observe any significant differences in anti-Ag85B-ESAT6 IgG1 levels in anti-asialo GM1 treated animals when compared to control mice. This may correlate with our cytokine data, as IFN-γ has been shown to inhibit IgG1, whereas production of these antibody subtypes is supported by IL-4 (Finkelman, et al. 1988; Yoshimoto and Paul 1994). A number of

studies have also shown that NKC depletion does not affect antigen-specific IgG1 responses after challenge/immunisation (Satoskar, et al. 1999; Wilder, et al. 1996). Animals treated with anti-asialo GM1 were not able to mount either an anti-LT or anti-Ag85B-ESAT6 IgE response. As already described, I also observed a significant reduction in IL-6 production from antigen stimulated splenocytes. In addition to IL-4, IL-6 is also known to modulate IgE class-switching, and a reduction in IL-6 levels may explain why I do not see any significant IgE titres in NKC depleted mice (Maggi, et al. 1989). Korsgren et al show a reduction in antigen specific IgE responses in NK1.1 treated mice when using a murine model of allergic asthma (Korsgren, et al. 1999). In the immune response to a foreign protein, the first antibodies to appear are of the IgM class. Shaping of the secondary antibody repertoire is generated by means of class-switch recombination, which replaces IgM with other isotypes such as IgA, IgE and IgG. One reason I may not be detecting any LT-specific IgG responses at day 14 may be that it is too early for LT specific antibody class-switching, and therefore the main subtype present would be IgM. Unfortunately, I did not have enough serum at day 14 to perform these IgM ELISAs and therefore I cannot confirm this hypothesis. I did perform IgM ELISAs at day 21 and found that mice treated with anti-asialo GM1 were found to have similar levels of anti-Ag85B-ESAT6 IgM compared to control animals, but undetectable anti-LT IgM levels on day 21. The failure of mice depleted of NKC to mount an efficient anti-LT IgM response at this time-point may explain why I see no anti-LT IgG responses at day 14, i.e. most IgM has now been class-switched to other isotypes.

The differences in anti-adjuvant and anti-antigen antibody responses suggest that there is a mechanism of NKC involvement associated with activation events initiated by adjuvant. The actions of LT are multifactorial, involving both direct activation of various APC, as well as secondary activation via cytokine secretion (Martin, et al. 2002; Petrovska, et al. 2003). Our findings here indicate that NKC may be implicated in these processes. Part of the action of the adjuvant may be to activate the NKC directly, or may be the stimulation of NKC through other cell types e.g. DC. In fact, one study has shown that LT can bind directly to asialo GM1 therefore substantiating the theory that these adjuvants may be inducing direct effects on NKC (Fukuta, et al. 1988). However, further studies will have to be performed in order to substantiate these theories.

In conclusion, the present data indicate that NKC have a critical role in mucosal immunisation and subsequent development of adaptive immune responses. These new data suggest the possibility of a central role for the increased numbers of NKC observed in the NALT and CLN shortly after intranasal immunisation, and suggest that NKC activity may be one of the factors governing the development of both antigen-specific cytokine and antibody responses, particularly the T_H1 response, after mucosal vaccination.