8 Appendix

Figure 8.1: Scatter plots of cell populations early after intranasal immunisation.

Innate immune cell composition of NALT and CLN 5, 24 and 72 hours after intranasal immunisation. NALT and CLN cells were isolate from immunised Balb/c mice. Single cell suspensions (1 x 10^6/sample) were stained with flurochrome-labelled mAbs and analysed by flow cytometry. Figures 8.1A and B show $CD11c^+$ and $F4/80^+$ cells in the NALT 5 and 24 hours after immunisation, respectively with Figures 8.1C and D showing the same cells both in the CLN after both 24 and 72 hours. Figures 8.1E-G show $DX5^+$ and $Ly6G^+$ cells in the NALT, 5 (5.1E), 24 (5.1F) and 72 (5.1G) hours post immunisation and Figures 8.1H-J show these time-points in the CLN. Plots shown are from ten individual representative mice, and the mean values are indicated. Numbers in the upper plots refer to percentages of cells within leukocyte gate.

Figure 8.1A: $CD11c^+$ and $F4/80^+$ cells in NALT 5hrs after immunisation

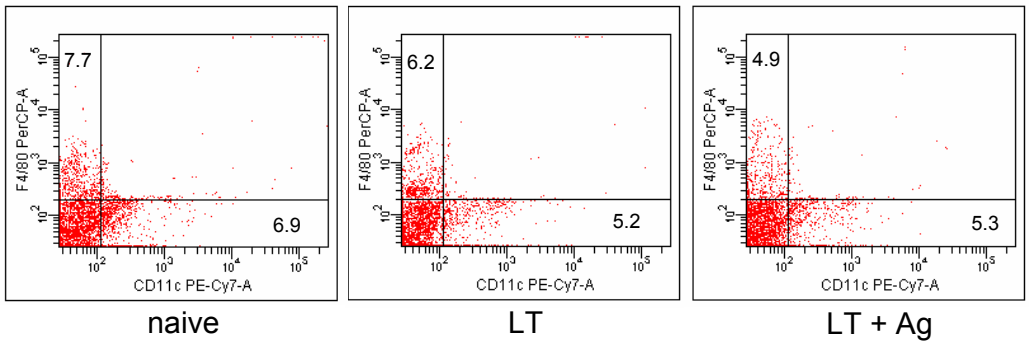


Figure 8.1B: $CD11c^+$ and $F4/80^+$ cells in NALT 24hrs after immunisation

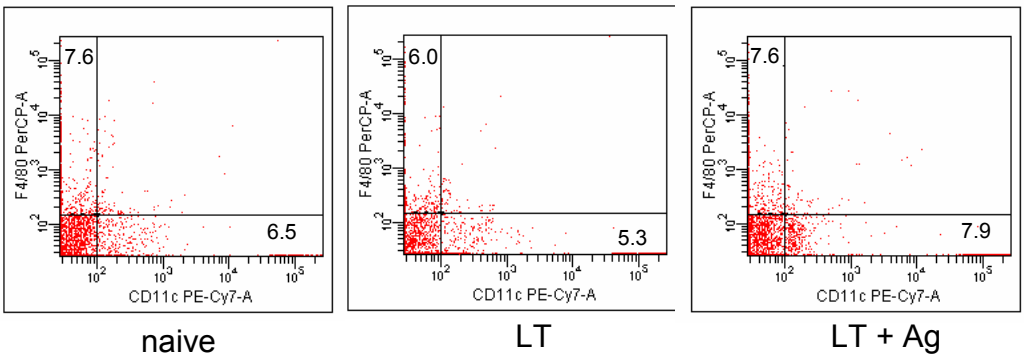


Figure 8.1C: CD11c$^+$ and F4/80$^+$ cells in CLN 24hrs after immunisation

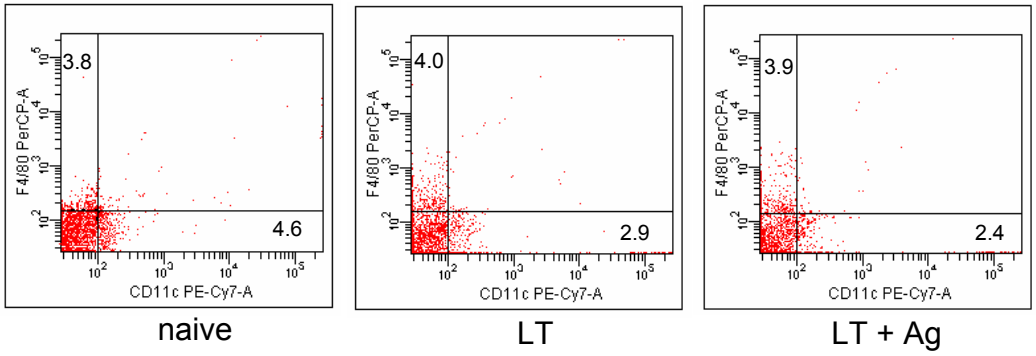


Figure 8.1D: CD11c$^+$ and F4/80$^+$ cells in CLN 72hrs after immunisation

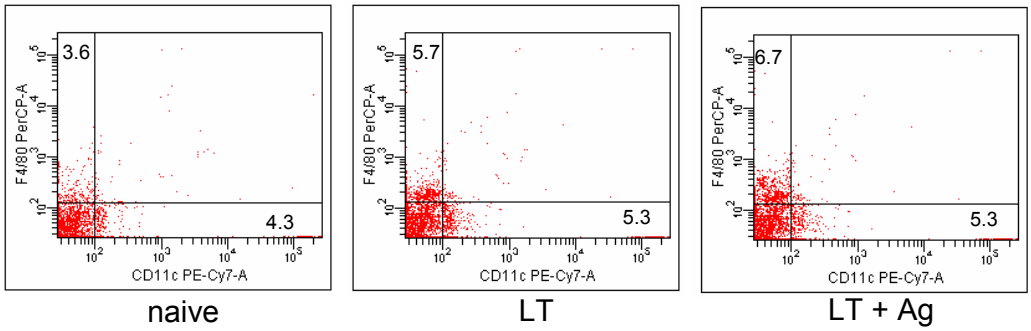


Figure 8.1E: DX5$^+$ and Ly6G$^+$ cells in NALT 5hrs after immunisation

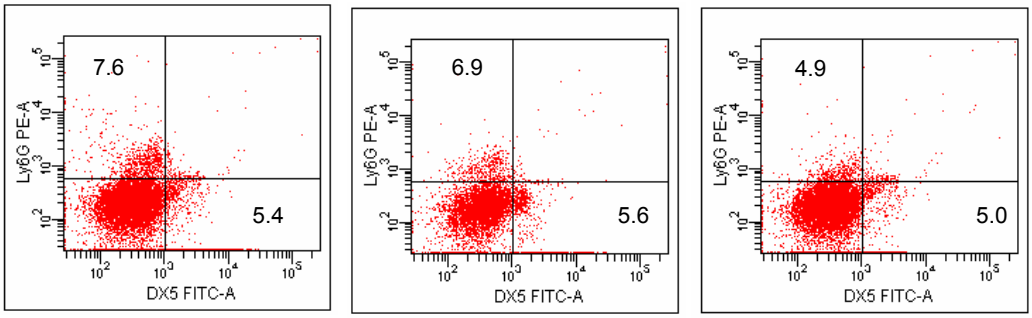


Figure 8.1F: DX5$^+$ and Ly6G$^+$ cells in NALT 24hrs after immunisation

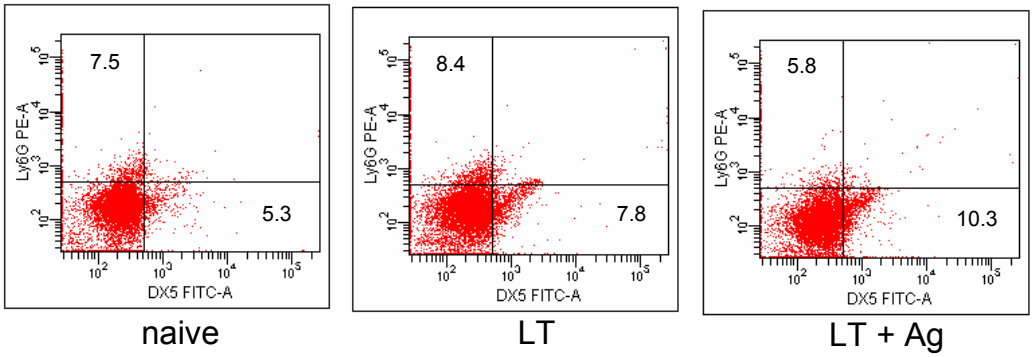


Figure 8.1G: $DX5^+$ and $Ly6G^+$ cells in NALT 72hrs after immunisation

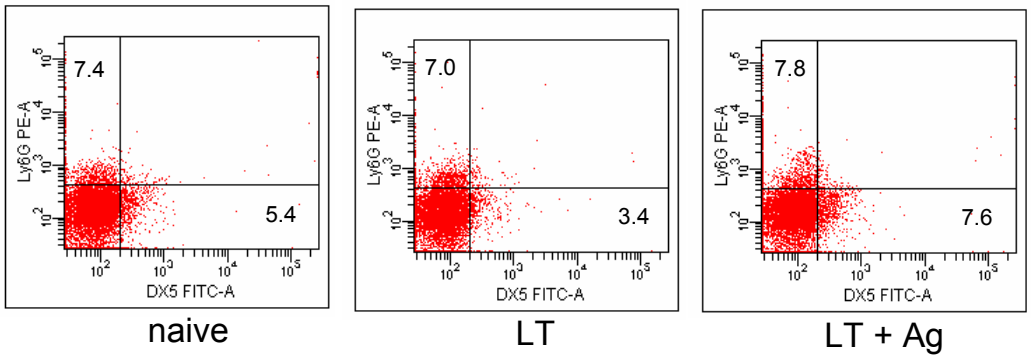


Figure 8.1H: $DX5^+$ and $Ly6G^+$ cells in CLN 5hrs after immunisation

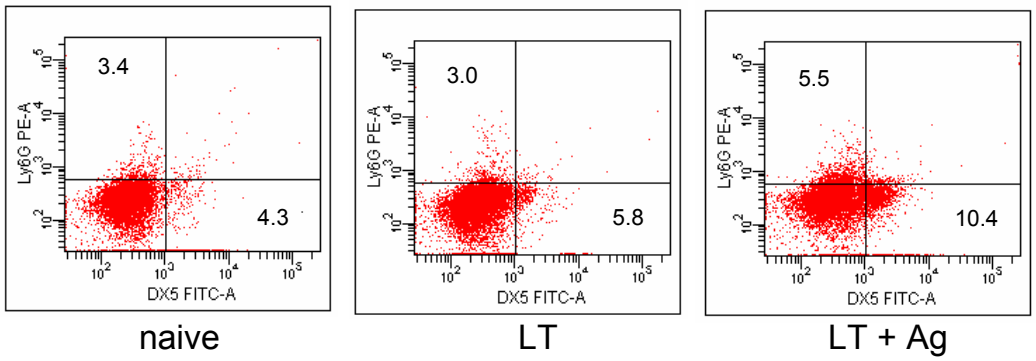


Figure 8.1I: $DX5^+$ and $Ly6G^+$ cells in CLN 24hrs after immunisation

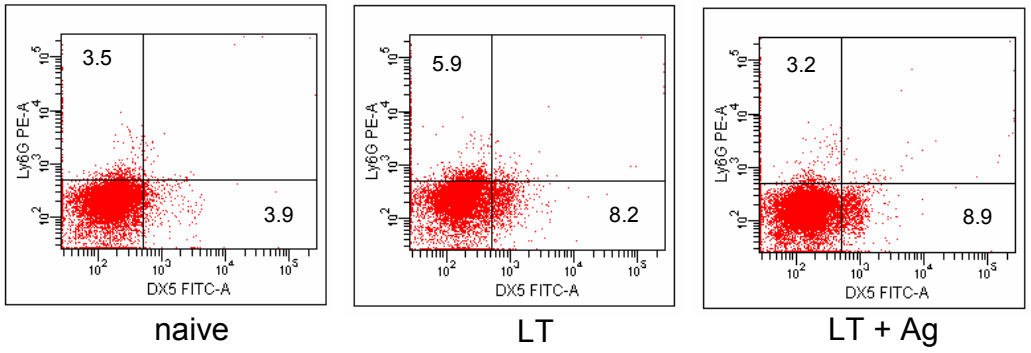


Figure 8.1J: $DX5^+$ and $Ly6G^+$ cells in CLN 72hrs after immunisation

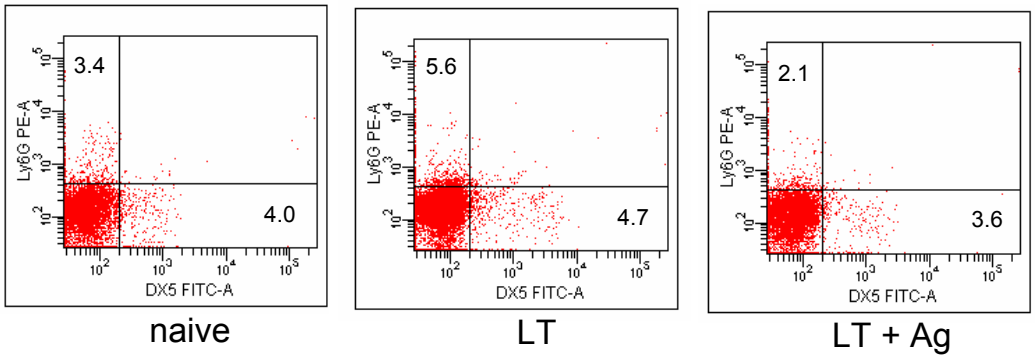


Figure 8.2: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT) Balb/c were compared. Figure 8.2A represents staining of frozen sections for CD11c in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling 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).

Figure 8.2A:

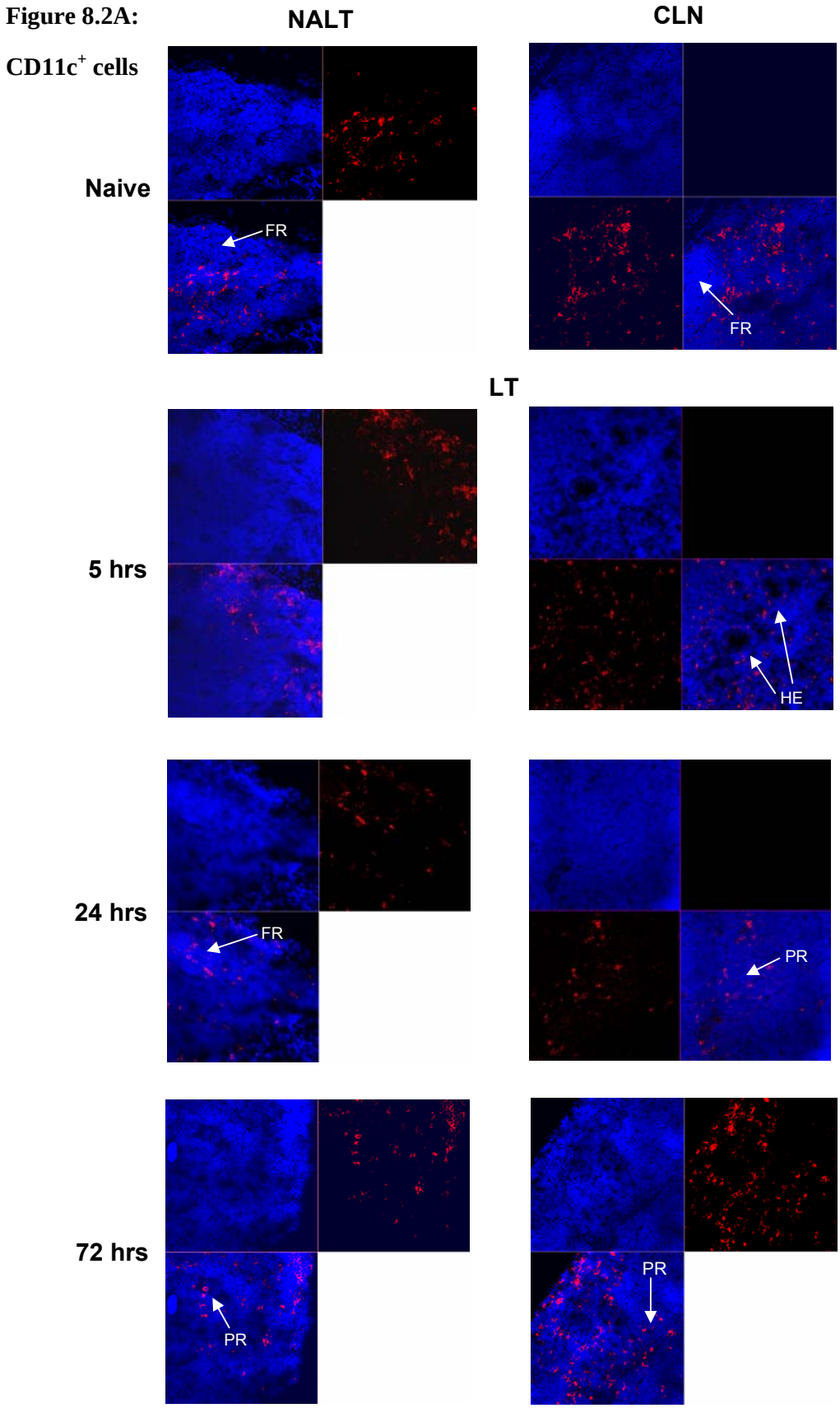

NALT
CLN
CD11c+ cells
Naive
FR
FR
LT
5 hrs
HE
24 hrs
FR
PR
72 hrs
PR
PR

Figure 8.2: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT) Balb/c were compared. Figure 8.2B 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).

Figure 8.2B:

F4/80^{+} cells

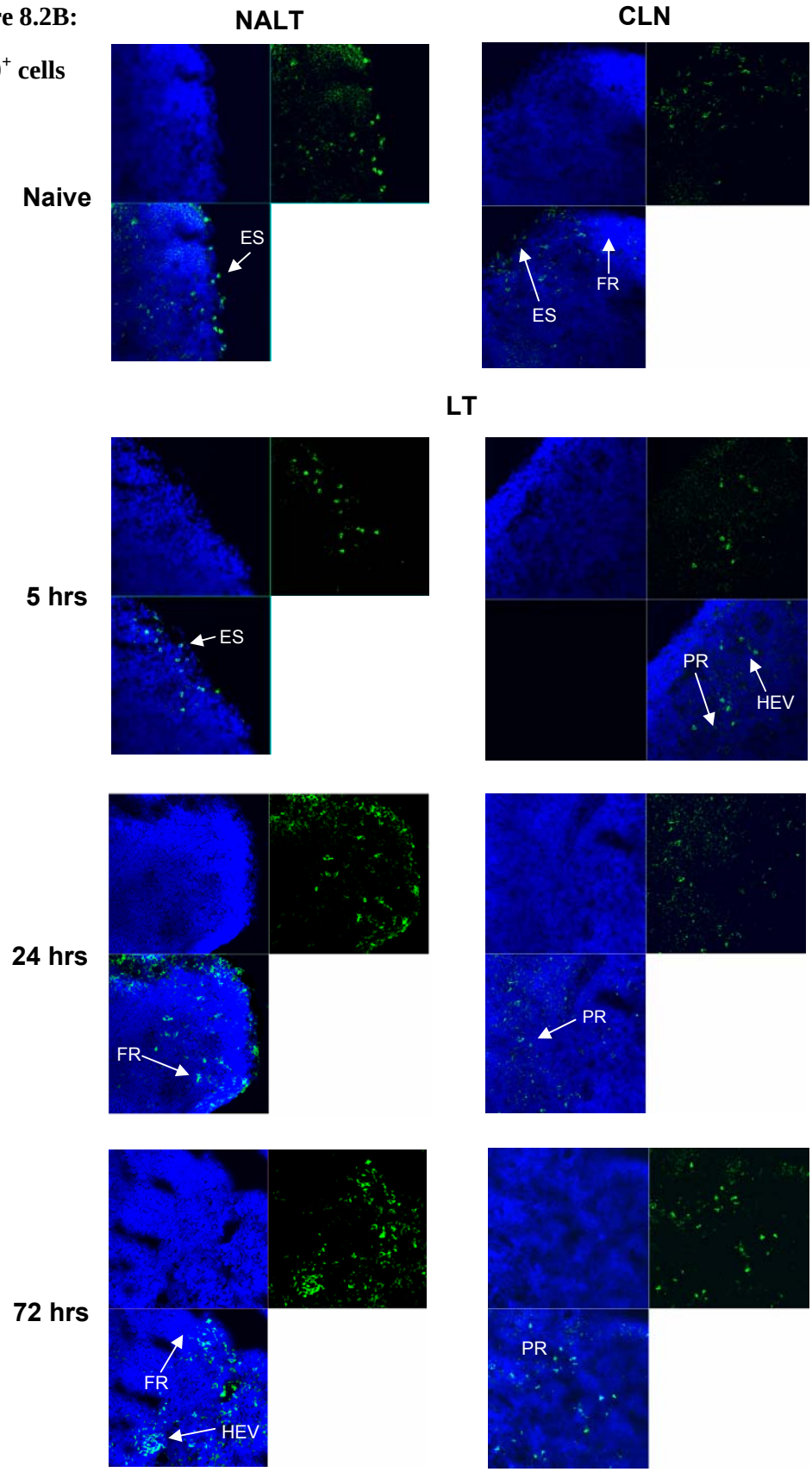


Figure 8.2: Immunofluoroscent analysis of NALT and CLN early after intranasal immunisation. Both naïve (PBS immunised) and immunised (LT) Balb/c were compared. Figure 8.2C represents staining of frozen sections for Ly6G in the NALT and CLN, 5, 24 and 72 hours post immunisation. Double immunolabeling 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).

Figure 8.2C:

Ly6G$^+$ cells

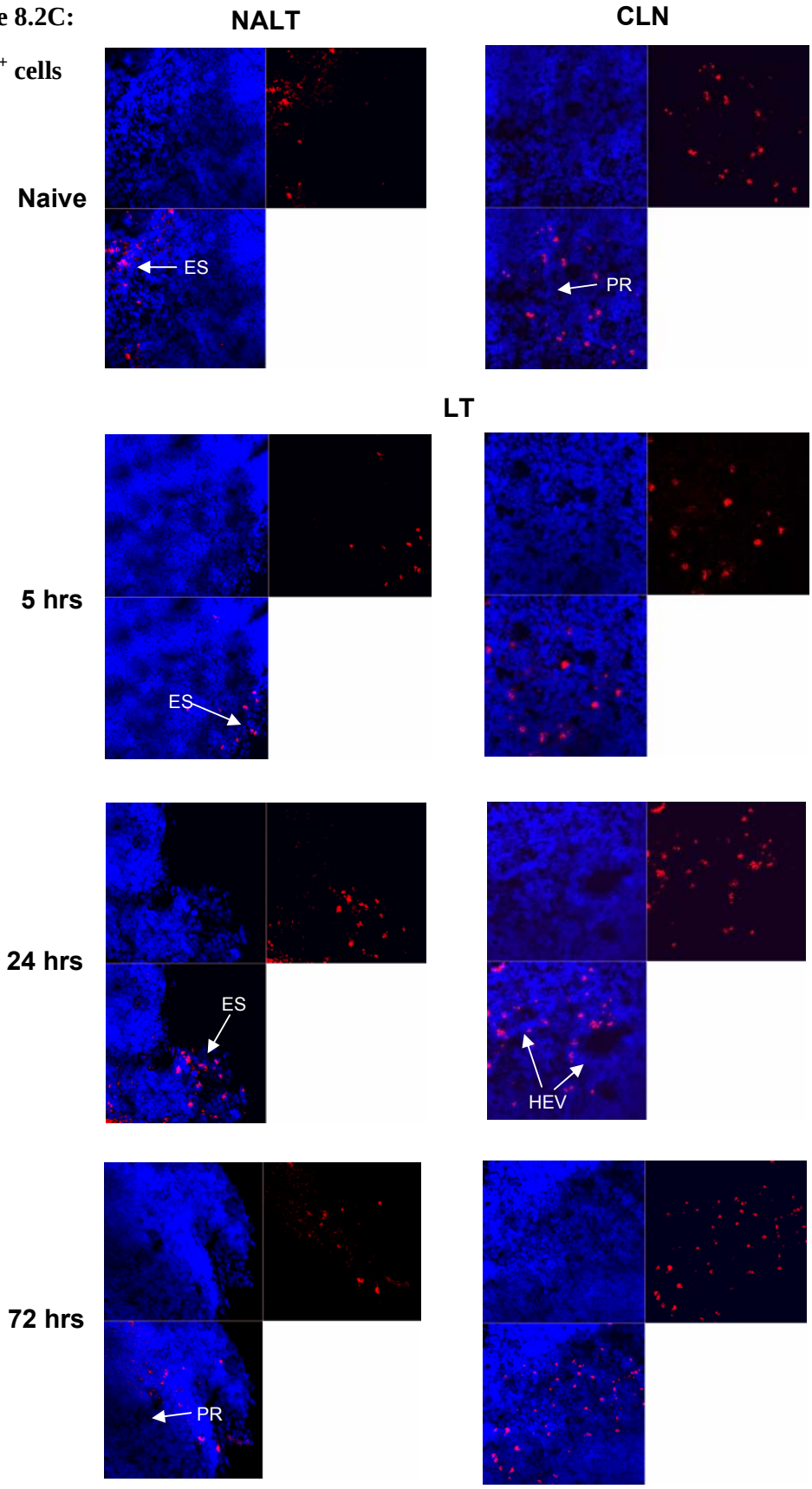


Table 8.1: Activation marker expression on NALT and CLN innate immune cells from immunised Balb/c mice.

Percentage expression of activation markers MHCII and VCAM-1 on $CD11c^+$ and $F4/80^+$ cells (Table 8.2A) and CD25 and CD69 on $DX5^+$ and CD69 on $Ly6G^+$ cells (Table 8.2B) of NALT and CLN from immunised Balb/c mice. Numbers represent the mean percentage of expression ± SD of groups of ten individual mice, from two independent experiments. The mean percentage values indicating the frequency of either, $CD25^+$, $CD69^+$, $MHCII^+$ or $VCAM\text{-}1^+$ cells, were calculated individually for each gated cell population expressing the corresponding surface marker ($DX5^+$, $Ly6G^+$, $CD11c^+$ or $F4/80^+$). 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.

Table 8.2A.

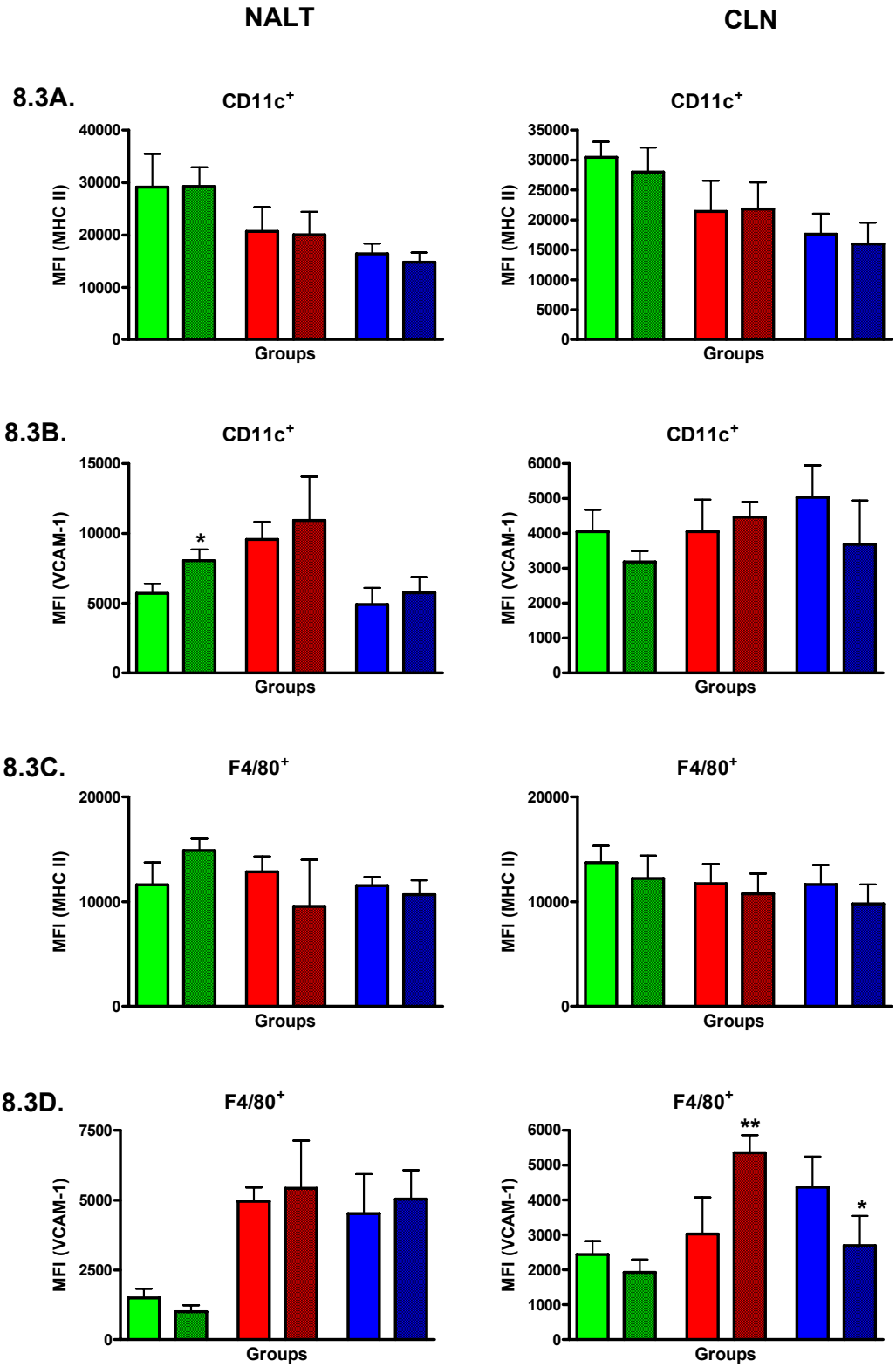
			5 hrs	24 hrs	72 hrs	5 hrs	24 hrs	72 hrs
Leukocyte	**Tissue**	**Immunisation**	**MHC II**			**VCAM-1**		
$CD11c^+$	NALT	Naive	4.6 ± 1.0	4.5 ± 0.4	4.4 ± 0.2	3.1 ± 0.8	3.2 ± 0.5	3.4 ± 0.4
		LT	4.2 ± 1.1	4.0 ± 0.8	4.1 ± 0.4	2.6 ± 0.7**	4.4 ± 0.5**	3.1 ± 0.4**
		LT + Ag	4.0 ± 0.8	5.5 ± 0.8**	4.0 ± 0.3	2.6 ± 0.6**	4.0 ± 1.1	4.0 ± 0.8**
$CD11c^+$	CLN	Naive	2.3 ± 0.2	2.1 ± 0.3	2.1 ± 0.2	1.7 ± 0.1	1.6 ± 0.3	1.8 ± 0.3
		LT	2.9 ± 0.3**	1.7 ± 0.3**	2.6 ± 0.2**	1.9 ± 0.3	1.4 ± 0.2	2.5 ± 0.5**
		LT + Ag	2.6 ± 0.4	1.5 ± 0.3**	2.9 ± 0.4**	1.7 ± 0.1	1.2 ± 0.2**	2.4 ± 0.4*
$F4/80^+$	NALT	Naive	5.5 ± 1.1	5.4 ± 0.8	5.3 ± 0.5	3.8 ± 0.5	3.5 ± 0.6	3.8 ± 0.6
		LT	5.9 ± 1.2	5.9 ± 1.3	5.7 ± 0.6	3.1 ± 0.3**	3.3 ± 0.6	3.8 ± 0.9
		LT + Ag	4.7 ± 0.8	4.4 ± 0.3	4.8 ± 0.3	2.9 ± 0.2**	3.9 ± 0.7	4.4 ± 0.8
$F4/80^+$	CLN	Naive	2.5 ± 0.3	2.6 ± 0.1	2.3 ± 0.3	1.6 ± 0.1	1.7 ± 0.2	1.6 ± 0.1
		LT	2.7 ± 0.3	2.2 ± 0.1**	2.8 ± 0.2**	2.1 ± 0.2**	1.4 ± 0.1**	2.5 ± 0.5**
		LT + Ag	2.1 ± 0.4	2.0 ± 0.2**	2.7 ± 0.3*	2.3 ± 0.4**	1.2 ± 0.1**	2.2 ± 0.3**

Table 8.2B.

			5 hrs	24 hrs	72 hrs	5 hrs	24 hrs	72 hrs
Leukocyte	**Tissue**	**Immunisation**	**CD25**			**CD69**		
$DX5^+$	NALT	Naive	2.3 ± 0.3	2.1 ± 0.4	2.0 ± 0.2	1.6 ± 0.1	1.4 ± 0.3	1.4 ± 0.4
		LT	1.7 ± 0.1**	2.3 ± 0.2	1.5 ± 0.1	1.2 ± 0.1**	1.4 ± 0.4	1.1 ± 0.2
		LT + Ag	2.0 ± 0.2	4.3 ± 1.1**	3.1 ± 1.1**	1.1 ± 0.1**	1.3 ± 0.5	2.3 ± 0.4**
$DX5^+$	CLN	Naive	1.3 ± 0.4	1.1 ± 0.2	1.0 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
		LT	1.5 ± 0.3	1.9 ± 0.6**	1.5 ± 0.4**	2.5 ± 0.8**	0.9 ± 0.3	0.9 ± 0.2
		LT + Ag	3.8 ± 1.2**	1.6 ± 0.5*	1.1 ± 0.1	3.0 ± 1.1**	0.6 ± 0.1	0.7 ± 0.1
$Ly6G^+$	NALT	Naive				2.2 ± 0.2	2.3 ± 0.5	2.2 ± 0.5
		LT				1.2 ± 0.1**	3.1 ± 1.1**	2.6 ± 0.5
		LT + Ag				0.8 ± 0.1**	1.9 ± 0.2	2.7 ± 0.6
$Ly6G^+$	CLN	Naive				0.7 ± 0.1	0.8 ± 0.2	0.8 ± 0.1
		LT				0.5 ± 0.1	1.9 ± 0.3**	1.2 ± 0.4*
		LT + Ag				0.9 ± 0.3	1.0 ± 0.1	0.6 ± 0.1

Figure 8.3: Mean Fluorescence Intensity (MFI) of activation markers on innate immune cell populations early after intranasal immunisation.

Cells were isolated from the NALT and CLN of control (PBS immunised) and LT immunised Balb/c mice, 5, 24 and 72 hours after intranasal administration of antigen. Cells were analysed for $CD11c^+$ and $F4/80^+$ expression as well as MHC II and VCAM-1 expression (Figure 8.3A $CD11c^+/MHCII^+$; 8.3B $CD11c^+/VCAM\text{-}1^+$; 8.3C $F4/80^+/MHCII^+$ and 8.3D $F4/80^+/VCAM\text{-}1^+$). $DX5^+$ populations were examined for CD25 and CD69 expression with $Ly6G^+$ cells examined for CD69 expression (Figure 8.3E $DX5^+/CD25^+$; 8.3F $DX5^+/CD69^+$ and 8.3G $Ly6G^+/CD69^+$). For analysis, gates were set on the innate subset marker positive ($CD11c^+$, $F4/80^+$, $DX5^+$ and $Ly6G^+$) cells and the MFI of MHC II, VCAM-1, 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, red at 24 hours and blue at 72 hours. Block colours represent naïve (PBS immunised) animals and, hatched lines show animal's intranasally immunised adjuvant alone.


NALT
CLN
8.3A.
CD11c+
MFI (MHC II)
Groups
8.3B.
CD11c+
MFI (VCAM-1)
Groups
8.3C.
F4/80+
MFI (MHC II)
Groups
8.3D.
F4/80+
MFI (VCAM-1)
Groups

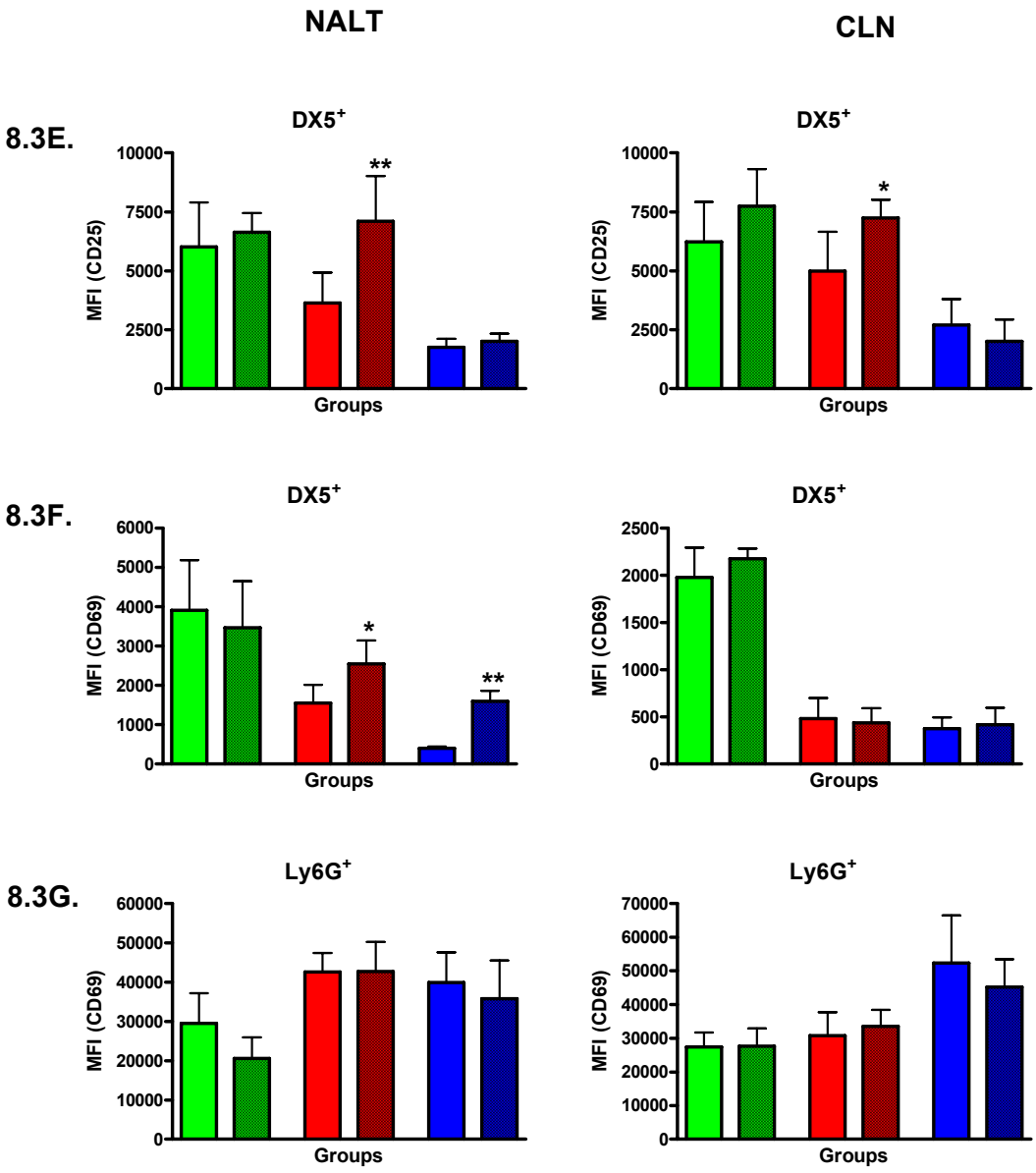

NALT
CLN
8.3E.
DX5+
MFI (CD25)
Groups
8.3F.
MFI (CD69)
8.3G.
Ly6G+

Figure 8.4: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 8.4A represents staining of frozen sections for MAdCAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT 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 8.4A:

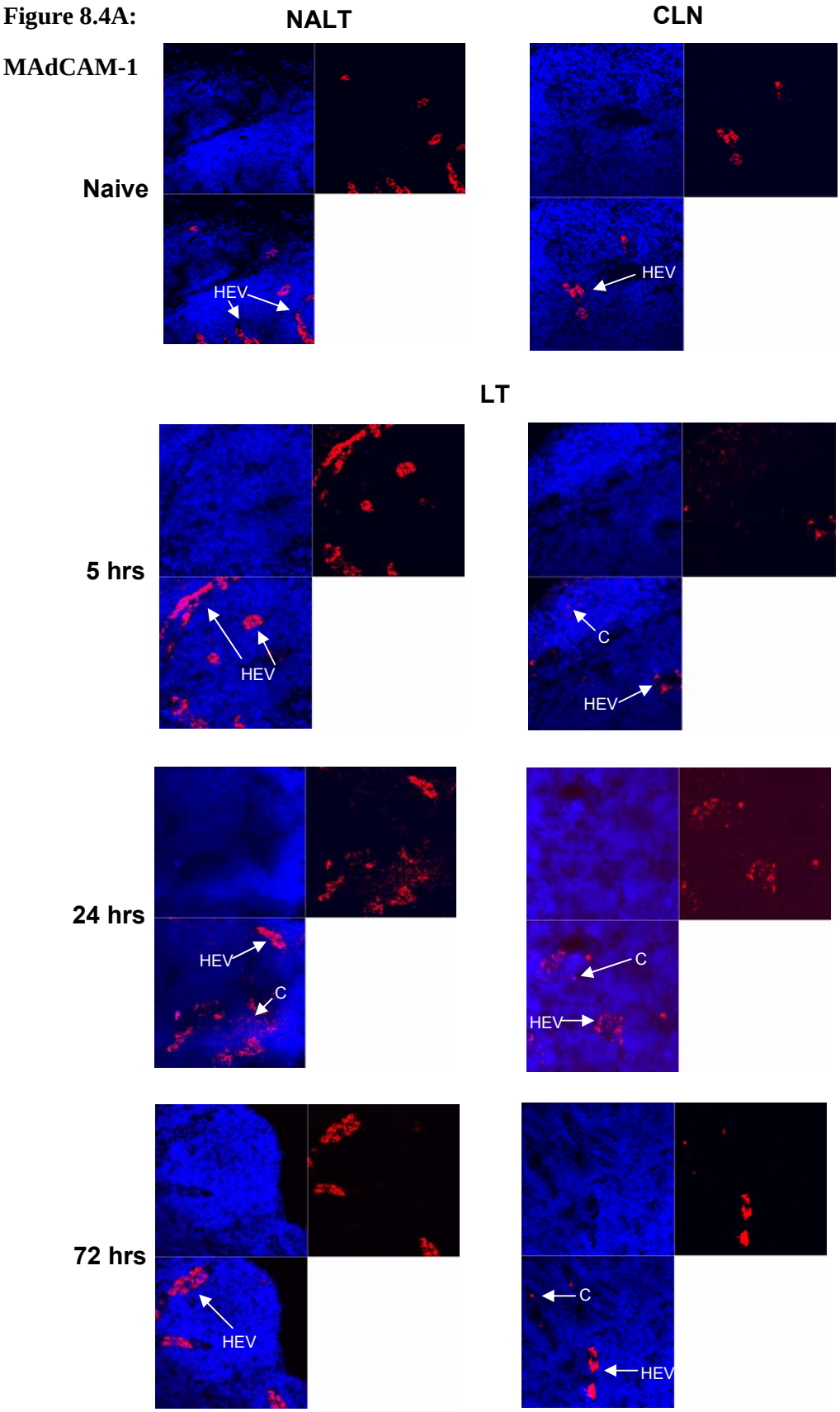

NALT
CLN
MAdCAM-1
Naive
HEV
HEV
LT
5 hrs
HEV
C
HEV
24 hrs
HEV
C
C
HEV
72 hrs
HEV
C
HEV

Figure 8.4: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 8.4B represents staining of frozen sections for PNAd in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT 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 8.4B :

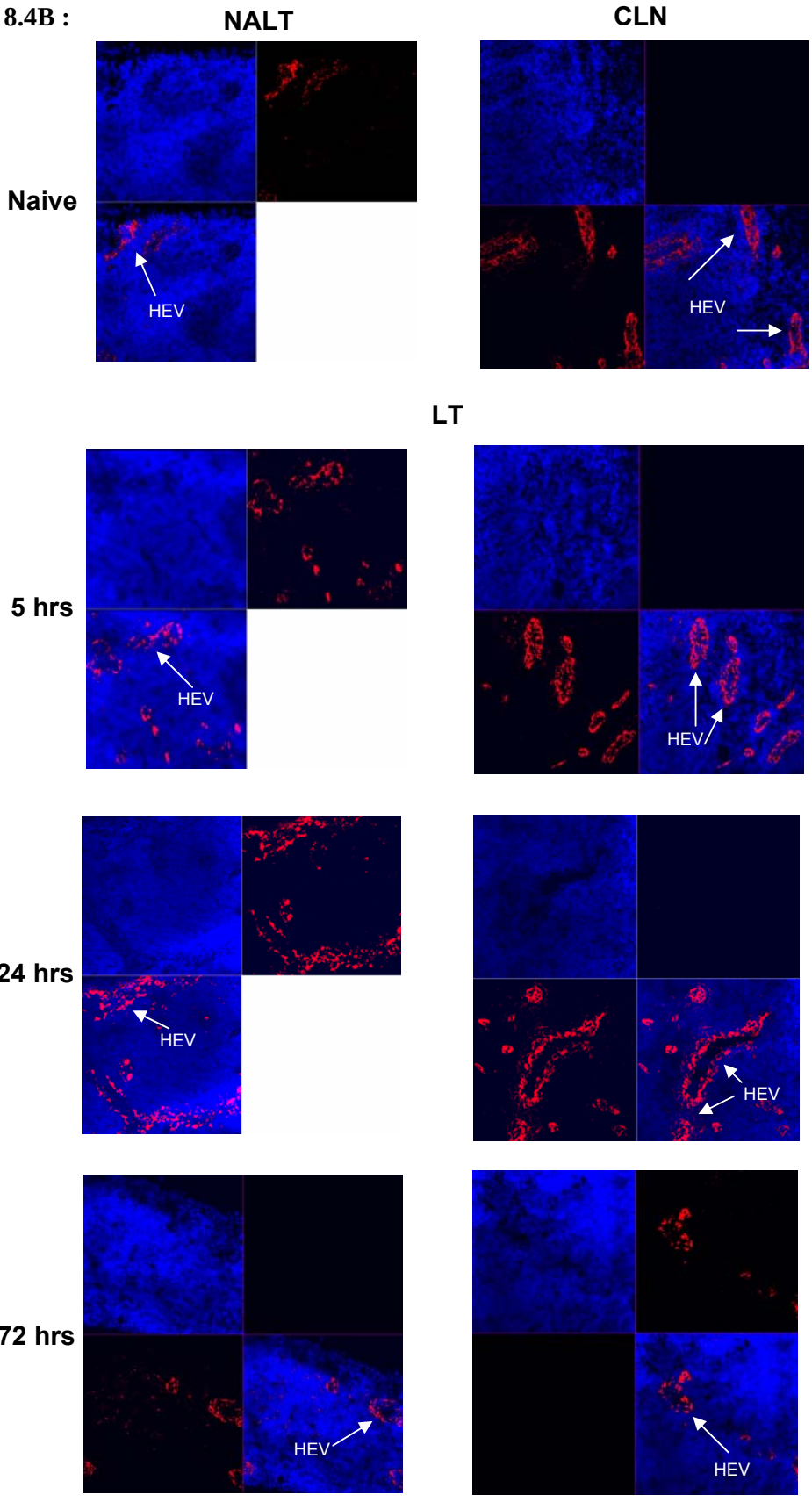

NALT
CLN
PNAd
Naive
HEV
HEV
LT
5 hrs
HEV
HEV
24 hrs
HEV
HEV
72 hrs
HEV
HEV

Figure 8.4: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 8.4C represents staining of frozen sections for ICAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT 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 8.4C :

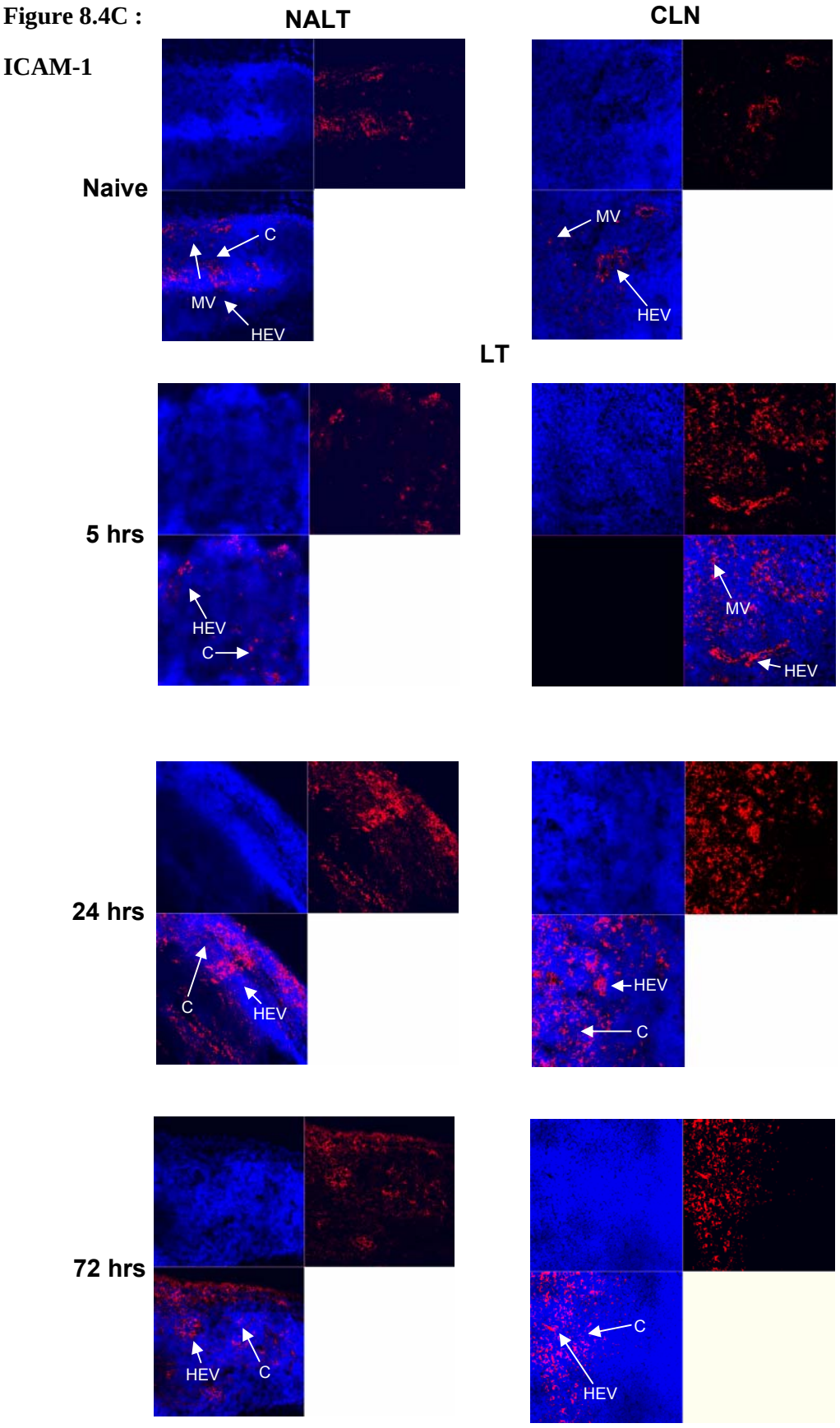

NALT
CLN
ICAM-1
Naive
C
MV
HEV
MV
HEV
LT
5 hrs
HEV
C
MV
HEV
24 hrs
C
HEV
HEV
C
72 hrs
HEV
C
C
HEV

Figure 8.4: Differential expression of cell adhesion molecules in the NALT and CLN after intranasal immunisation.

Figure 8.4D represents staining of frozen sections for VCAM-1 in the NALT and CLN, 5, 24 and 72 hours post immunisation with LT 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 8.4D :

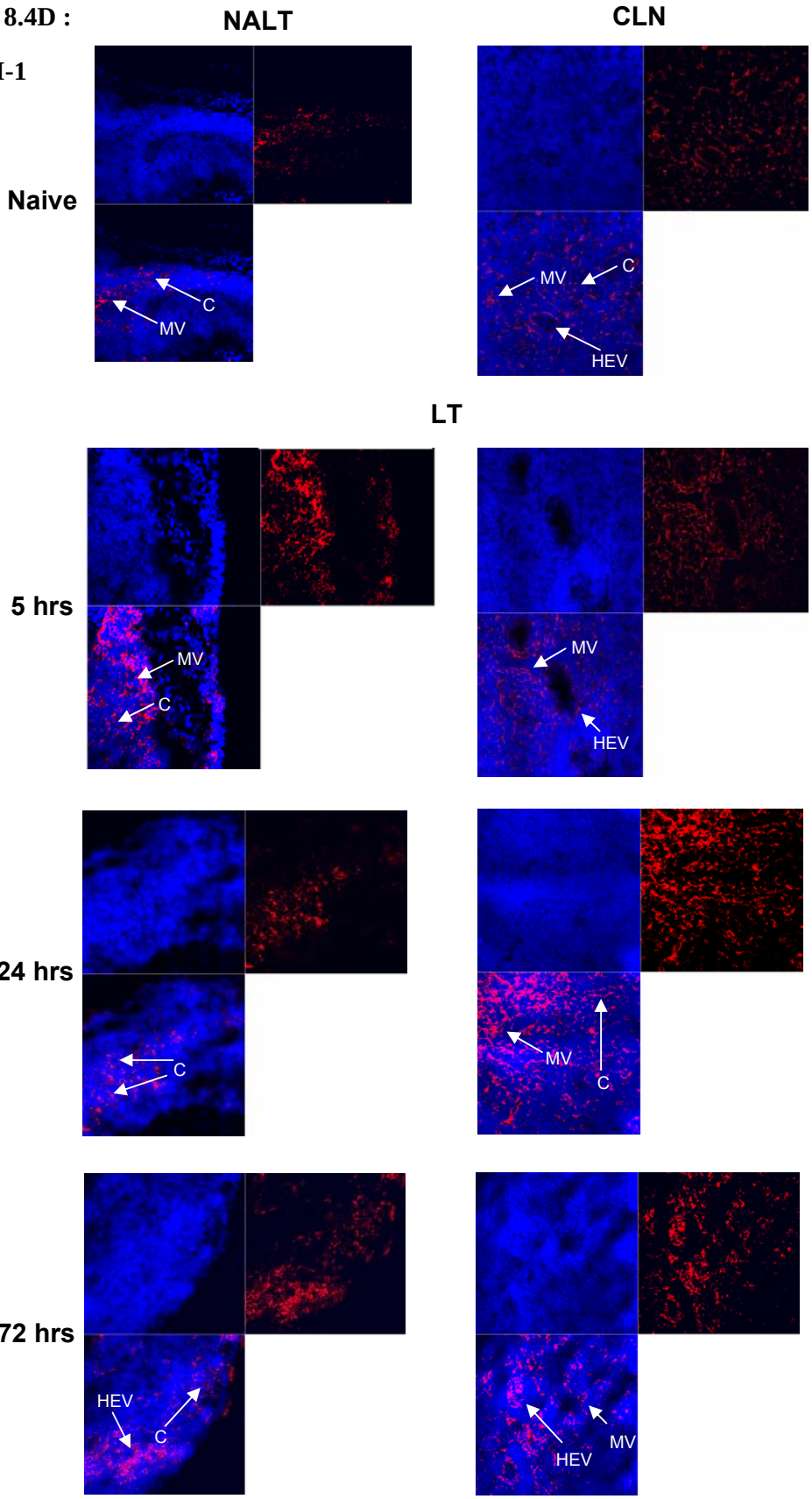

NALT
CLN
VCAM-1
Naive
C
MV
MV
C
HEV
LT
5 hrs
MV
C
MV
HEV
24 hrs
C
MV
C
72 hrs
HEV
C
HEV
MV

Figure 8.5: Immunohistochemical 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. Figure 8.5 reveals the presence of GC in the NALT and 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 8.5: GC