Cystic Fibrosis Gene Therapy: Methods for the optimisation of CFTR gene delivery.

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4 The effect of Leptomycin B on gene transfer *in vivo*

4.1 Can the inhibition of the innate anti-retroviral immune response alter transduction efficiency: The effect of Leptomycin B on lentiviral mediated gene transfer

Responses elicited by target cells when infected with a HIV-1 derived lentivirus vector may decrease the level of gene transfer achieved, therefore, this chapter describes the testing of leptomycin B (LMB), and whether this inhibitor of the innate anti-retroviral immune response increases the amount of gene transfer achieved in nasal airway epithelia.

This study was divided into four parts;

1) The effect of LMB on lentivirus
2) Establish a dose-response for LMB in enhancement of gene transduction
3) Determine the optimal delivery method for LMB using the concentration established in 2)
4) Transfer information gained from 2 and 3 into a gene transfer protocol in CFTR knockout mice using a virus containing the therapeutic CFTR gene

4.2 Effect of Leptomycin B on lentivirus

A co-incubation study showing the effect of Leptomycin B on lentivirus vector viability was completed [189]. The results showed that LMB did not have a significant effect on the titre of lentivirus following co-incubation (Figure 4-1). The 500 ng/mL group showed a trend towards decreasing viral titre. All concentrations tested (final concentration of 5-500 ng/mL) were used for further investigation and *in vivo* studies.

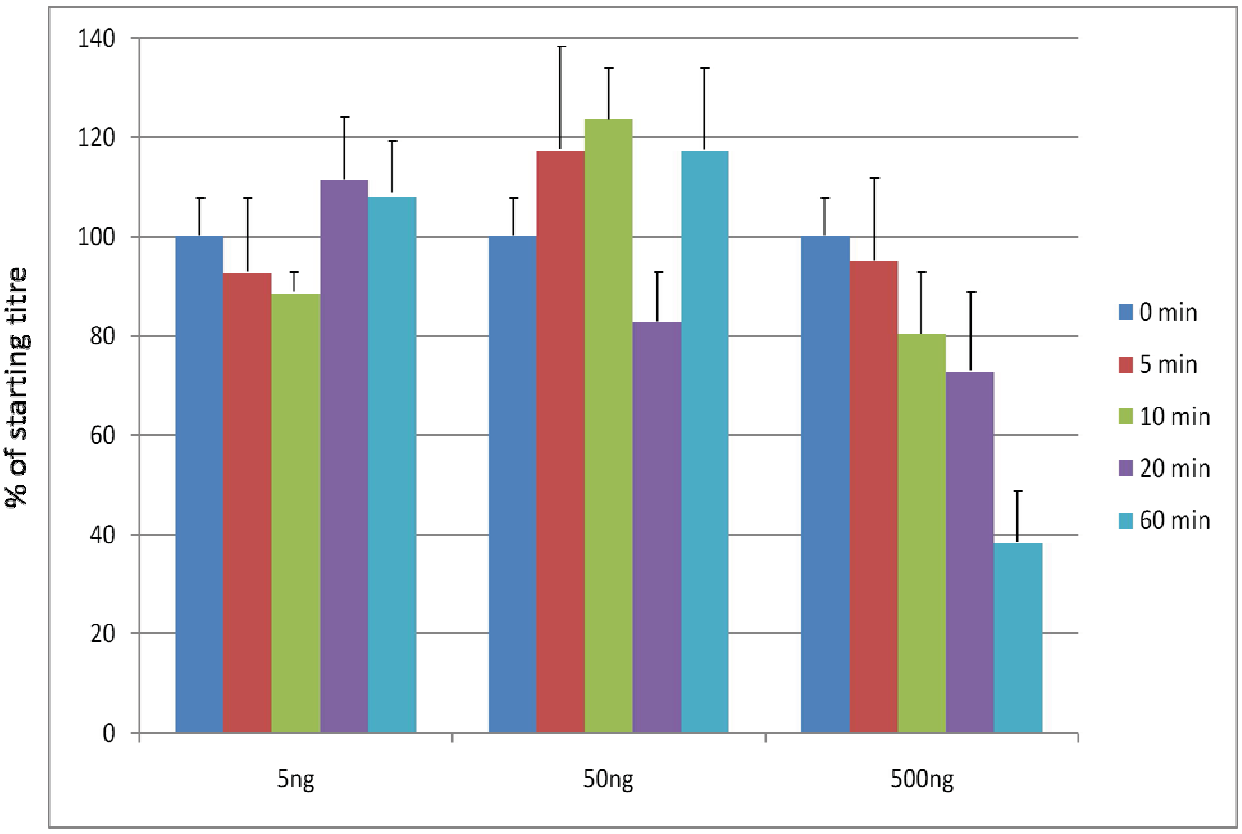

Figure 4-1: Effect of Leptomycin B on lentivirus titre *in vitro*

The effect of LMB on virus titre *in vitro* for each test concentration n=3 samples per timepoint + SEM

4.3 Effect of concentration (Dose/response) of LMB on gene transfer in vivo

The concentrations tested in the co-incubation study described previously (5 ng/ml, 50 ng/mL and 500 ng/mL) were tested *in vivo*. This experiment involved using the gene transfer protocol as described in 2.3.8. LMB was included in both the LPC and virus dose at the stated concentration, with LV-LacZ titre 8.64 x 10^8 TU/mL. Results showed that the highest level of gene transfer was observed in mice treated with the highest concentration of LMB tested- 500 ng/mL. However, this was not significant compared to the other concentrations tested or the standard dosing protocol. The trend seen though was considered enough to support further testing of 500 ng/mL LMB in the gene transfer protocol as the quantum increase in gene transfer seen was large enough, approximately 3-fold compared to our standard gene transfer protocol [189], to be of phenotypic benefit in CF gene therapy (Figure 4-2). Regression analysis showed the R^2 value to be 0.9615, which supported a decision to move forward incorporating LMB into the gene therapy protocol. In addition no difference in the distribution of gene transfer when LMB was included in the treatment protocol was observed compared to the standard gene transfer protocol.

4.4 Optimal delivery method of LMB in vivo

The next step was to investigate the optimal delivery method *in vivo*. This involved the delivery of LMB in the LPC pretreatment only, in the LV only and in both the LPC and LV. Controls included the same volume of carrier (70% methanol) or PBS added to both the LPC and LV. These two separate control groups were used to investigate the effect 70% methanol (the LMB carrier) may have on gene transfer, and the highest amount of gene transfer that could be obtained using a virus diluted (PBS control) compared with our standard gene transfer protocol.

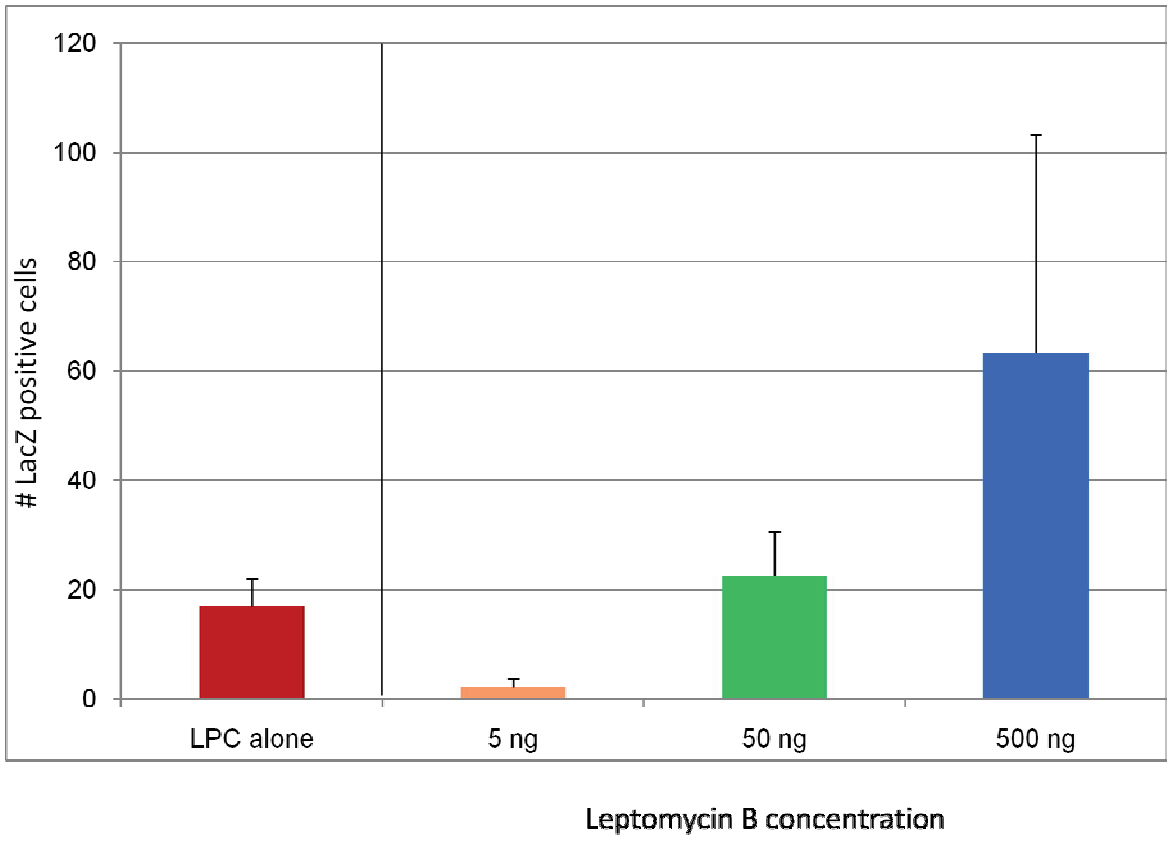

Figure 4-2: Effect of Leptomycin B concentration on gene transfer *in vivo*

Test concentrations of LMB (5ng, 50ng and 500ng) delivered in both LPC and LV, compared to LMB delivered in LPC only. n=3, + SEM. ANOVA p=0.309, regression analysis R^2 = 0.9615

The results showed that the optimal method of delivery was for the LMB to be included in both the LPC and the LV. Interestingly it also showed that the addition of 70% methanol had a negative effect on the level of gene transfer observed, however, this decrease was not significant (ANOVA, p= 0.755) (Figure 4-3).

Addition of LMB to both LPC and LV gave a non-significant increase in the amount of gene transfer compared to that seen in the control groups (70% Methanol and PBS) of 2.7 fold (t-test, p= 0.84) and 1.7 fold (t-test, p= 0.724) respectively. As the delivery of LMB appeared to have a positive effect on the amount of gene transfer achieved almost exactly reproducing the effect seen in the first experiment, addition of LMB to both the LPC pre-treatment and LV dose was used for further experiments. Because the difference between the addition of LMB to only LPC or only LV was small when compared to LMB addition to both LPC and LV, it was thought that any increase in the level of gene transfer seen warranted transferring this treatment into CFTR knockout mice to try and achieve the highest amount of gene transfer possible.

Again, there was no difference seen in the distribution of gene transfer when LMB was included in the treatment protocol. Results from this experiment could not be combined with the results from 4.3 as a new virus preparation was used for this experiment.

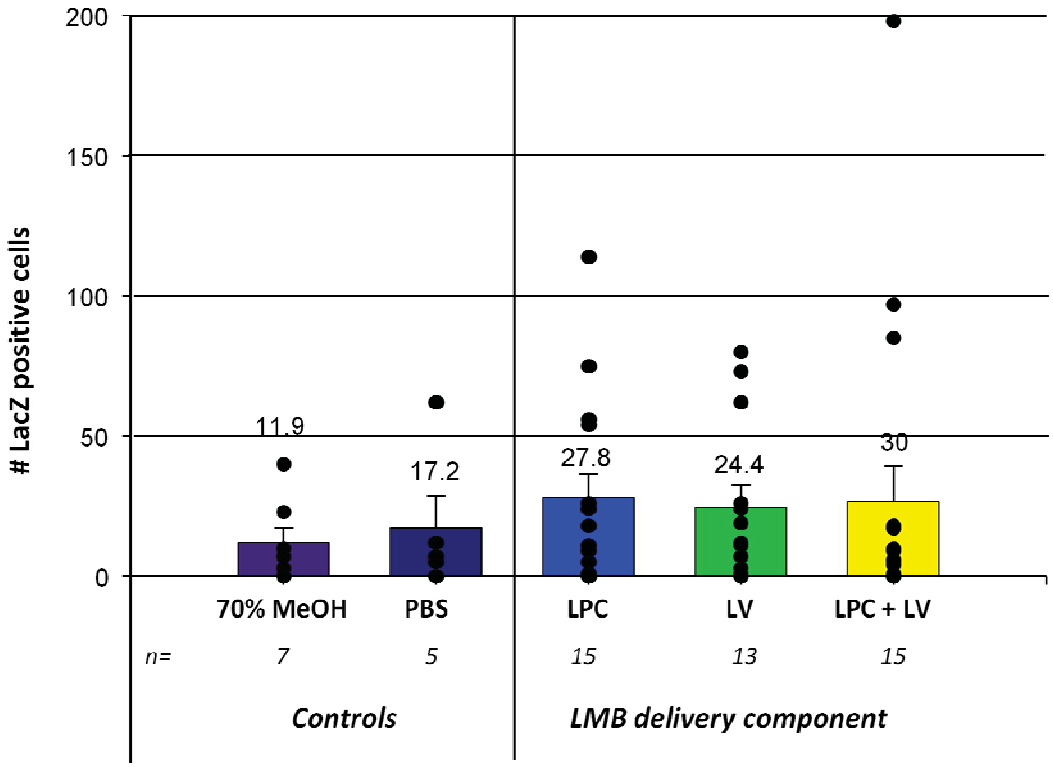

Figure 4-3: Effect of Leptomycin B delivery component on gene transfer *in vivo*

Effect of the delivery component on the level of gene transfer compared to equivalent volumes of PBS or 70% MeOH added to both LPC and LV. N values given on the graph, + SEM

p=0.755 ANOVA (comparison of all experimental groups)

4.5 Leptomycin B delivery to enhance gene transfer in CFTR knockout mice in vivo

Two groups of transgenic mice, *cftr*^{tm1Unc} (CFTR knockout, described previously-chapter 1), were treated, one with our LPC pre-treatment containing 500 ng/mL LMB followed 1 hour later by a LV containing a human CFTR (huCFTR) gene (LV-CFTR) and 500 ng/mL LMB and the other with the standard protocol without LMB. The only functional test for the presence or absence of the CFTR chloride channel, TPD measurement (as described in section 2.4.2.4) was performed on all mice at time points of 1 week, 1 month, 3 months and 6 months post treatment. The TPD measures the electrical potential of the epithelia when solutions containing high chloride concentration and low chloride concentration are infused into the treated nasal airway. Amiloride - a compound that blocks the epithelial sodium channel (ENaC) was added to the high and low chloride solutions that were infused into the nasal epithelia at a concentration of 0.1 mM. The use of amiloride in the solutions means that only chloride channel function is measured, which is important as CFTR transports chloride ions.

Colleagues who had experience in this technique scored traces blinded to group and animal. Traces were rejected if hypopolarisation was absent under amiloride perfusion or if traces were electrically unstable. ΔPD was used as the measure of CFTR function.

One week post treatment showed no correction of CFTR function (ΔPD) in either group. The group treated with LV-CFTR + LMB showed a level of CFTR function equivalent to that of untreated CFTR knockout mice. Mice treated with LV-CFTR only showed a trend towards correction, but due to variation within the treatment group, remained significantly different to the het (normal) control group. The difference- or lack thereof -of the treated groups to the untreated CFTR knockout mice is the comparison most relevant to correction. Both groups were seen to be significantly different to the

normal control group- het mice from the same colony (LMB p=0.044, standard p=0.004, t-test, Figure 4-4).

After measurement of TPD at 1 month, the group treated without LMB showed partial correction, as this group was not significantly different from the het control group (p=0.081 T-test), however, they were also not significantly different to the CF untreated group. The group that received LMB in their treatment were significantly different to the het group (p=0.022, t-test), indicating that there was no significant correction, of phenotype, however, the ΔPD value of this group decreased, indicating a trend toward normal CFTR function.

Testing 3 months post treatment showed that mice treated without LMB were not significantly different to the untreated CFTR knockout group, and were no longer significantly different to the het group (p=0.092, t-test). This change from the 1 month data can in part be attributed to the deaths of 2 mice in the group, one of which (indicated by the magenta point on the graph Figure 4-4) which showed correction of CFTR function at previous time points. Mice treated with LMB were not found to be significantly different to the untreated CFTR knockout group, however, showed partial correction as they were no longer seen to be significantly different to the het group (p=0.114, t-test).

The results at 6 months showed that mice treated without LMB were statistically different from the untreated CFTR knockout group (p=0.010, t-test), indicating correction of the CFTR chloride channel defect. They also were not significantly different to het mice (p=0.207, t-test) which also confirms they are showing correction of CFTR function. The statistics indicate that this result is correction of the CFTR defect, however, as shown in Figure 4-4, the ΔPD value is still higher than that seen in the het group, showing that although the statistics indicate correction of the CFTR defect has been achieved in this group at 6 months, the actual result indicates only partial correction of the defect. Mice that received LMB did not show any correction of chloride ion channel function (p=0.036, t-test). However, there is

only a small 'n' value in both 6 month groups and statistical power of 0.509 which is below the desired power of 0.8, the data should be interpreted carefully. However, 2 out of the 3 mice in the non-LMB group showed correction to the CFTR chloride ion channel, with one mouse not showing this correction. There was one mouse (indicated with the brown point in Figure 4-4) from the LV-CFTR + LMB group that from 1 week to 3 months was showing a trend towards correction, however at the 6 month time point, this mouse was no longer showing any sign of CFTR correction. It is hard to speculate why this is the case, and if it maybe due to the turnover of epithelial cells in the nasal airways within the 3 month time window between TPD measurements and the inherent variability of the TPD technique. Numbers of subjects in both groups that were measured out to 6 months were decreased in both LMB and non-LMB treated mice. The CF knockout mice are known to be less robust than normal mice due to the CFTR knockout causing gut problems, because of this they are known to have a decreased life span. Repeated anesthesia to perform the TPD measurements in a relatively short period of time undoubtedly had an impact on the mice and their ability to recover following each procedure. In total 4 mice in each group died throughout the experiment, decreasing the numbers in the 6 month groups to 2 for the LV-CFTR + LMB group and 3 for the LV-CFTR group.

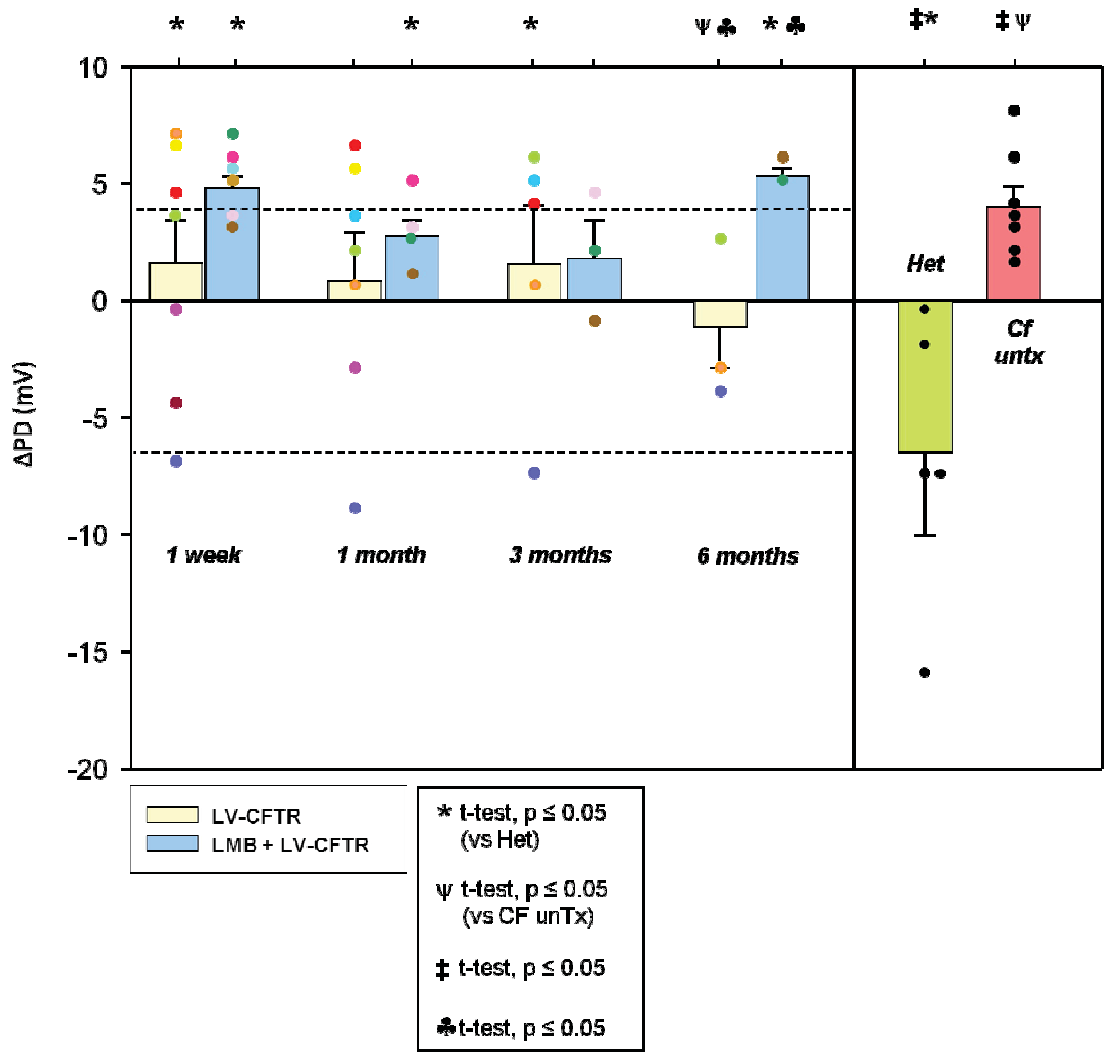

Figure 4-4: Transepithelial Potential Difference measurement of LV+/- Leptomycin B

Potential difference measured as Low Cl^- + amiloride – Basal + amiloride, n values are indicated by the coloured circles representing individual mouse TPD values for each treatment group over time. Various statistical tests are shown within the graph.

4.5.1 Analysis of CFTR in sacrificed mice

Two methods for the analysis of CFTR gene transfer were investigated in treated excised nasal epithelia. The nasal septum was removed from the mouse, with some septums being used for immunohistochemistry and some for real time PCR. The rationale behind the two methods of analysis were that each analysis provides different information as to the localization of the CFTR transgene, which cells have been transduced and the real time PCR giving an indication of the average copy number of the CFTR transgene per cell. While real time PCR does not allow us to determine which cells have been transduced in the nasal airway, it provides a qualitative measure of transduction with our lentivirus. Immunohistochemistry however, can give us an image of which cells have been transduced with the CFTR gene to determine if the correct respiratory cells are being targeted as is desired.

Following sacrifice of each mouse, the nasal septum was removed as described in section 2.4.2.6. The nasal septum underwent one of two procedures. It was either digested in Viagen TM ® tail lysis buffer, supplemented with 0.4 mg/mL Proteinase K to extract genomic DNA from the nasal airway epithelial cells (for real time PCR), or snap frozen in OCT (optimal cutting temperature) compound for use in immunohistochemistry. Both types of samples were stored at – 80 °C.

4.5.1.1 Immunohistochemistry to visualize CFTR *in vitro* and *in vivo*

When using a therapeutic gene such as CFTR for gene delivery, it can be difficult to assess whether the gene has been delivered and integrated into the host cell, if the gene product (CFTR) is functional, and which cells it has been expressed in. CFTR function can be measured as described previously *via* TPD measurement. Presence of the gene can be detected using real time PCR (as described in 2.3.5.2). The real time PCR is limited in this capacity as it gives an average copy number, which can be used if a whole organ is being analysed, however in this case where a piece of tissue is being excised, it is difficult to remove the exact same piece of tissue each time. The nasal septum is a small and delicate

piece of tissue which makes not only its removal difficult, but there is a high chance of causing damage to the tissue during the removal process. This makes analysis of real time PCR results difficult to compare between samples, as there is no guarantee that each piece of tissue removed is exactly the same as the next piece. A further limitation is neither of these methods show which cell types have been transduced. While it would not be expected that the pattern of transduction would differ greatly to that seen with our lentivirus carrying the LacZ transgene, this is still an assumption. Apart from immunohistochemistry, no other methods of analyzing CFTR allow assessment of the presence and expression of the gene on a cell by cell basis.

Good antibodies to the CFTR gene have been difficult to make or find commercially. There are many antibodies available, however, there has been little success using them on tissues that have been excised from treated subjects due to a lack of specificity or sensitivity. A monoclonal antibody made by John Riordan [174] was used in this study- MM19-596.1.1 (596) (Ascites). This antibody is specific to the human CFTR gene, the transgene used in these studies and recognizes the nuclear binding domain 2 (NBD2). The 596 antibody was chosen as it was reported to have an increased specificity towards CFTR of 10-1000 times compared to other antibodies, and has been successfully used in immunohistochemistry studies [174].

4.5.1.1.1 In vitro optimisation and testing of mouse 596 antibody

CHOK-1 cells were transduced with 5 µL of concentrated LV-CFTR and allowed to grow to confluency (courtesy of Nicole Wood,). CHOK-1 cells and CHOK-1 cells transduced with LV-CFTR in culture were plated in a multi-chamber slide and incubated for 4 hours to allow the cells to attach to the substratum. The cells were then fixed with 4% PFA prior to antibody binding. A dilution of the 596 mouse monoclonal antibody in 10% normal goat serum was added to the cells at room temperature and incubated for 2 hours in a humidified container (to ensure the slides do not dry out). A secondary

biotinylated goat anti-mouse antibody (Vector Laboratories, Cat #BA-9200), diluted 1:1000 in 1% normal goat serum was incubated with the cells at room temperature for 45 minutes, followed by a tertiary antibody, streptavidin Alexa 568 (Invitrogen, Cat # S-11226) diluted 1:500 from stock in 1% normal goat serum, incubated at room temperature for 45 minutes, to visualize CFTR. Cells were counterstained with DAPI to visualize cell nuclei.

When observed under fluorescence both transduced and untransduced CHOK-1 cells showed DAPI stained nuclei (visualized at excitation 360 nm, emission 460 nm) and low level signal from the streptavidin Alexa antibody (visualized at excitation 578 nm and emission 603 nm). It is not clear whether the background Alexa fluorescence observed in the untransduced control cells is due to cross-recognition of CHO CFTR or is non-specific. Therefore, the technique was also assessed using the LV-CFTR +/- LMB treated mouse nasal septums that had been snap frozen in OCT. The 596 antibody had previously been used to visualize CFTR in treated mouse tissue.

Immunohistochemistry utilizing the 596 antibody to the human CFTR gene was tested to visualize CFTR expression in the mouse airway epithelia.

Nasal septums embedded in OCT were cut on a cryostat and mounted on d-poly-lysine coated slides. Tissue sections on the slides were fixed with methanol for 5 minutes immediately after cutting. Background IgG staining was reduced by the addition of FAB fragment (Affinipure FAB fragment Goat anti mouse IgG) and incubated for one hour at room temperature. Tissue was then blocked by incubation with 10% v/v normal goat serum prior to probing with the 596 mouse monoclonal antibody. The 596 antibody was used at 1:1000 dilution in 5% normal goat serum was incubated on the tissue sections for 1 hour. The secondary antibody used was a biotinylated goat anti-mouse in a 1:1000 dilution, and the tertiary antibody used was streptavidin alexa 568 as a 1:500 dilution, with both the secondary and tertiary antibodies incubated with the tissue sections for 45 minutes in a humidified

chamber. Slides were coverslipped with Vectashield containing DAPI to counterstain cell nuclei. Septums were then observed under fluorescence- using the excitation wavelengths for both DAPI and streptaviden alexa 568 described previously.

Again, as was seen in the transduced and untransduced CHOK-1 cells, a high background was observed. Control samples from untreated CFTR knockout mice and heterozygous mice, both of which should not show fluorescence to the CFTR antibody as the antibody is reportedly specific for human CFTR, were also analysed. Both groups of control samples showed fluorescence at the same levels as that seen in the LV-CFTR +/- LMB treated mice. In the treated mice, if CFTR is present, it should be seen as a definite point of red fluorescence seen clearly in only some of the epithelial cells- as not all cells of the septum will be transduced by the LV-CFTR. The fluorescence that was observed was faint and in all cells visible in the section, which was seen in both the untreated and treated sections (see Figure 4-5).

Repeated attempts were made to optimize the immunohistochemistry protocol to reduce the background seen in untreated tissues. Differing fixation solutions, including 100% ethanol, 95% methanol + 5% acetic acid and no fixation, were tested with a range of dilutions of both primary and secondary antibodies (no antibody, 1/50, 1/100 and 1/200 dilutions of each antibody). Optimisation tests were carried out on CHOK-1 cells both untransduced and cells transduced with LV-CFTR plated in a 96 well plate. Observation of cells under fluorescence showed no difference in the amount of fluorescence seen in the untransduced cells compared to the LV-CFTR transduced cells (data not shown).

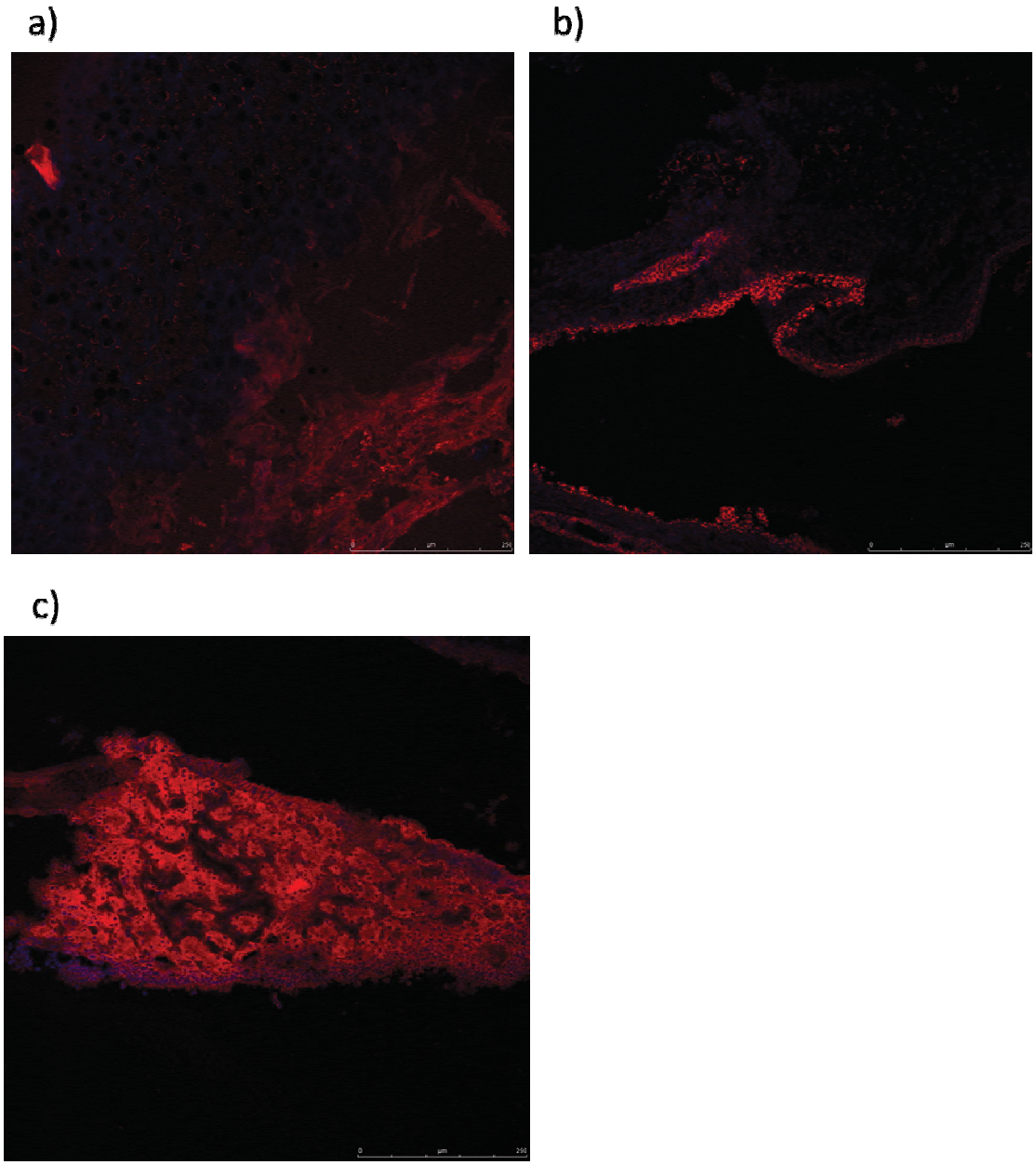

Figure 4-5: Immunohistochemistry images of mouse nasal septums.

a) LV-CFTR treated

b) CFTR Knockout untreated

c) Heterozygous

As repeated attempts to optimise the immunohistochemistry method failed to reduce the background, septums that were embedded in OCT were removed from OCT at room temperature and washed in 0.9% NaCl to remove excess mounting media before digestion for gDNA in Viagen tail lysis buffer.

These septums then underwent RT-PCR analysis for vector copy number with the other septum DNA samples.

4.5.1.2 Real-Time Polymerase Chain Reaction (RT-PCR) analysis of vector copy number in excised mouse nasal septa

Mouse nasal septa gDNA was analysed *via* RT-PCR for vector copy number as described in section 2.3.5.2.

The results of RT-PCR showed not all gDNA from LMB treated or standard protocol treated mice gave a result, as there was no amplification of either PCR reaction (mouse transferrin or Gag). This is presumably due to the concentration of gDNA or DNA quality being too low for successful amplification or due to the presence of inhibitors in the sample. To assess this, the same volume of sample used for RT-PCR (5 µL) was run on a 1.5% agarose gel. Six of 13 samples were not visible on the gel, with all of these six samples not giving a result in the PCR analysis. Unfortunately, as the nasal septum of the mouse is small, and the whole amount of tissue was digested in the original reaction, it was not possible to prepare any more gDNA that could be used for RT-PCR analysis.

RT-PCR results showed the copy number in LMB treated mice to be on average 0.1077 vector copies per cell ± 0.122. The mice that did not receive LMB in their gene delivery protocol had an average of 0.0445 vector copies per cell ± 0.0234. However, the difference between the two treatment groups was not seen to be significant (t-test, $p=0.417$).

A correlation between the PD result achieved for each mouse and the corresponding vector copy number show no relationship between the two parameters, as mice that responded well using the TPD analysis, did not give a high vector copy number as may have been expected.

4.5.2 *In vivo* Delivery of LMB II

In an attempt to resolve the effect of LMB on gene transfer *in vivo*, it was decided to repeat the *in vivo* studies with larger sample sizes. In addition, in the initial *in vitro* studies with LMB, it was found that the carrier solution for the LMB, 70% MeOH, had a small but discernible negative effect on the amount of gene transfer observed. As the aim of this project was to enhance the level of gene transfer, another carrier solution for LMB was sought. Eventually a supplier offering LMB dissolved in ethanol (EtOH) (LC Laboratories) was found. As the LMB in EtOH was available at a higher starting concentration- 540 µg/mL compared to our original stock at 5 µg/mL, (Sigma # L2913) this had the additional advantage that the final concentration of the solvent delivered with the LV to the mouse was 100 fold lower than that delivered with the previous study using LMB in 70% MeOH. In addition, ethanol also has lower toxicity than methanol.

Normal C57Bl/6 mice were instilled using the standard protocol described in section 2.4.1. Mice were divided into 3 groups, n=6, receiving either standard LPC + LV-LacZ, 500 ng/mL LMB in both LPC and LV-LacZ or the equivalent dilution of carrier solute, ethanol, in both LPC + LV-LacZ. Mice were sacrificed after one week using the protocol described in section 2.4.2 and analysed for LacZ positive cells.

Results shown in Figure 4-6 indicate that the standard gene transfer method resulted in an average of 258 LacZ positive cells. The group receiving LMB in the treatment showed an average on 169.3 LacZ positive cells, significantly lower than the standard LV gene transfer (t-test p=0.029). Surprisingly however, the group that received EtOH in the LPC + LV-LacZ showed increased (an average of 293 LacZ

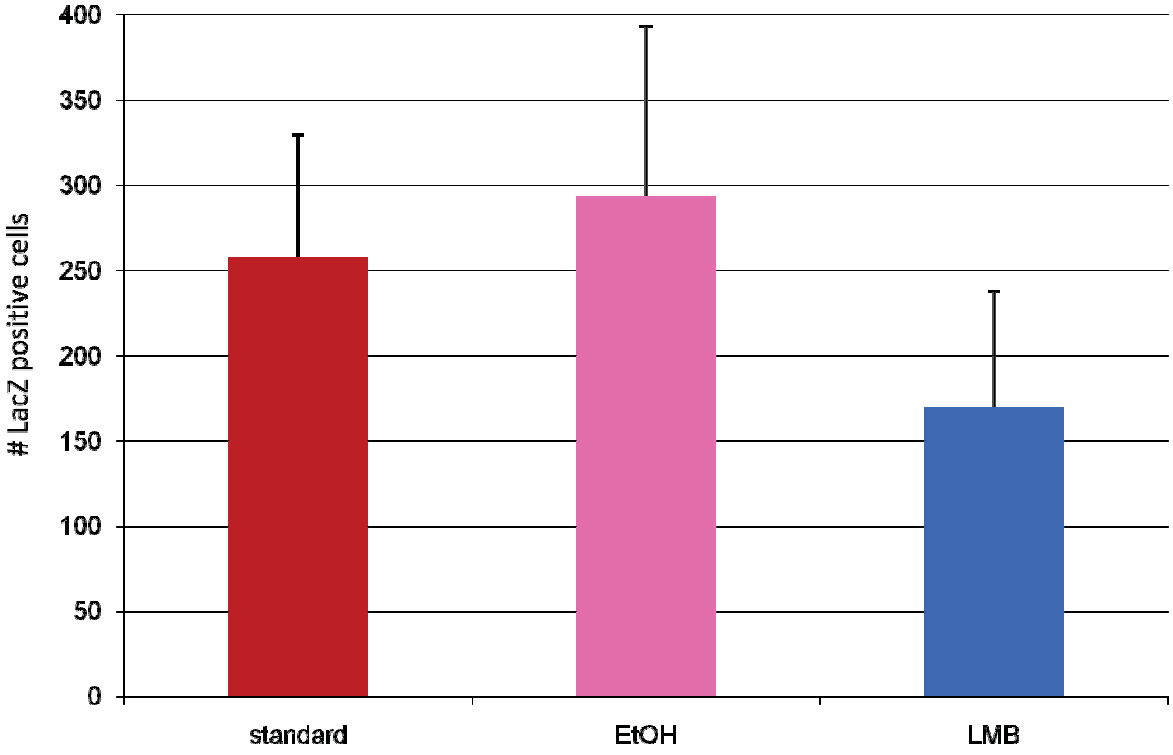

Figure 4-6: Effect of Leptomycin B and Ethanol on gene transfer

Mice (n=6 /group) were instilled with either our standard gene transfer protocol, or with the addition of EtOH or LMB to both components of therapy. A significant difference in #LacZ positive cells per mouse were seen (p=0.03, ANOVA) and further tests showed a significant difference between EtOH and LMB treated groups (p=0.017, t-test). Data shown +SEM

positive cells, approximately 11.5% of the airway epithelia) gene transfer from our standard protocol. However, this result was not significantly different from the standard delivery method (t-test, p=0.589) but was significantly different from the LMB treated group (t-test, p=0.017). An ANOVA test showed there to be a significant difference between the groups, (p=0.03) with the significant difference being between the EtOH treated group and the LMB treated group as also shown by the T-test.

4.5.3 Testing the activity of Leptomycin B in cell culture

As the *in vivo* experiments using the LMB in ethanol did not increase the amount of gene transfer seen in the mouse nasal airways, an experiment was designed to test the activity of the LMB *in vitro*.

LMB inhibits Rev dependent export of mRNA [145]. Rev is a regulatory protein that is essential in HIV-1 replication. Via interaction with the Rev Response Element, Rev controls the export of unspliced and singly spliced mRNA's from the nucleus to the cytoplasm of the cell. These mRNA's encode Gag, Pol and Env.

Therefore, an experiment was conducted to test the effect of LMB at nanomolar concentrations has on p24 production from a Gag expression construct. This approach was derived from an experiment conducted by Wolff *et al* (1997), where LMB was tested as a low molecular weight inhibitor of Rev nuclear export, with the result that LMB was found to inhibit Rev function and HIV-1 replication [145].

The effect of both stocks of Leptomycin B on p24 production was assessed.

Transfection of 293T cells was carried out using the plasmid pcDNA3-gagstop/RRE/Intron (provided by D. Anson, unpublished) that requires Rev for Gag production. Five hundred nanograms of plasmid were transfected into 293T cells using the calcium phosphate precipitation method described in 2.3.2. Eight

hours post transfection, media was changed with media containing 0.1 nM, 1 nM and 10 nM LMB from both old and new (70% MeOH vs EtOH) stocks of LMB.

Forty-eight hours post transfection, cell media containing p24 was collected, 0.2 µm filtered and a p24 assay to measure Gag protein was performed (as described in 2.3.7). Results showed that the old stocks of LMB dissolved in 70% methanol did not inhibit Gag production, whereas new stocks of LMB dissolved in ethanol appeared to decrease Gag production in a dose-response manner as would be expected (see Figure 4-7).

4.6 Discussion

4.6.1 Leptomycin B to improve LV delivery *in vivo*

The results of initial studies into the use of LMB to enhance gene delivery appeared promising, with LMB enhancing gene transfer compared to PBS and 70% MeOH controls. The increase in gene transfer compared to the controls was not significant; however, there was a trend towards increasing gene transfer when LMB was incorporated into the gene therapy protocol.

This study also looked at the method of delivery of LMB and in which component of our gene therapy protocol it should be delivered in, to have the greatest effect on the level of gene transfer. The results showed incorporating LMB into both the LPC and lentivirus as being the best method to increase gene transfer. Although the increase was not significantly higher when using the LMB in both components of our gene transfer protocol, it showed a trend towards increased gene transfer when compared with LMB delivered in the LPC or the LV-LacZ alone. Despite the large 'n' values of 15 mice per treatment group, significance was not achieved due to the variation between mice in each treatment group. There was a fold increase of 1.7 and 2.7 seen in the LMB delivered in LPC and LV treatment group

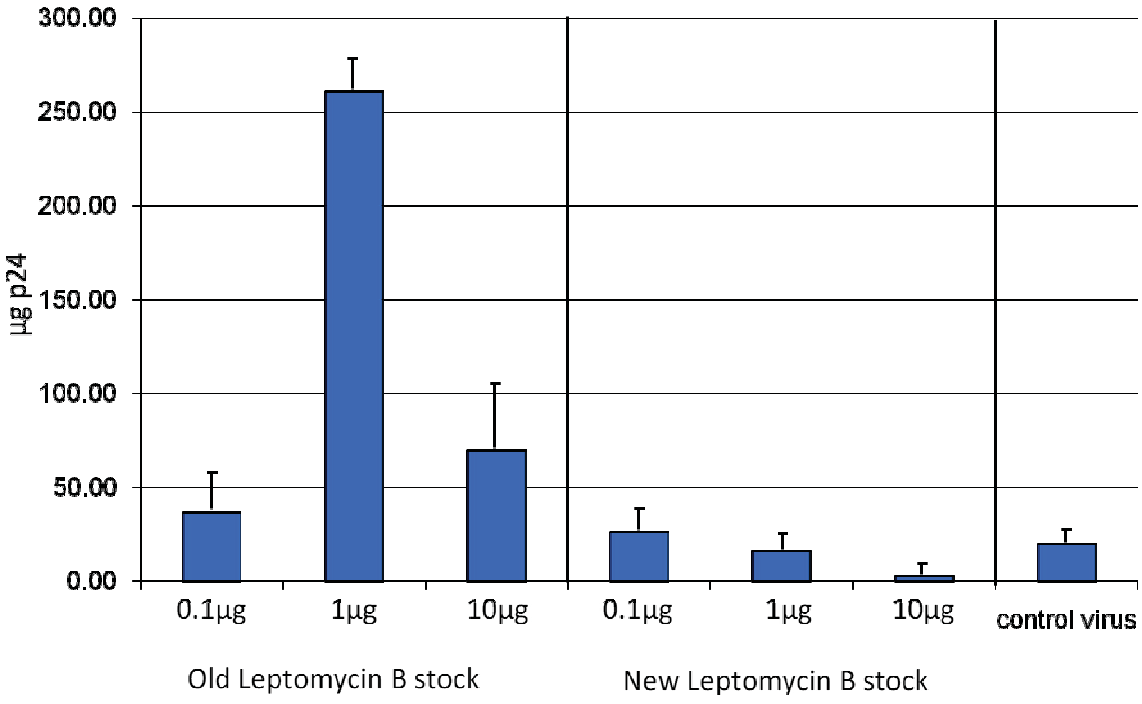

Figure 4-7: Effect of old and new Leptomycin B stocks on gag (p24) production

Old and new stocks of LMB were tested for their effect on p24 production. Results show the old LMB stock does not inhibit p24 production where the new LMB stock does show inhibition of p24 production.

compared with the PBS and 70% MeOH control groups respectively. Although neither increase was significant (p=0.84, p=0.724 for MeOH, PBS comparisons respectively) the increase seen in gene transfer was thought to be of a quantum that would be beneficial in increasing the response to gene transfer when using the therapeutic CFTR gene in CFTR knockout mice and therefore worthy of further investigation.

Accordingly, this method of LMB delivery was then tested in CFTR knockout mice, using a lentivirus containing the huCFTR gene. However, the standard gene transfer protocol showed greater correction of TPD at all time points compared to the mice that received LMB. The mice treated with LMB showed no correction of Cl- channel function at 1 week and 6 months post instillation, however there was a decrease in ΔPD when mice were tested at time points of 1 and 3 months. The magnitude of the decreases seen, were not as great as the change in ΔPD seen in mice that received no LMB in the treatment protocol. This does not correlate with the LacZ data where mice treated with LMB showed a trend towards increased gene transfer. The lack of correction seen in LMB treated mice compared with the non-LMB mice may be due to the effect the carrier solute- 70% methanol- has on gene transfer. In the LacZ studies, it was seen that the 70% methanol appeared to have a non-significant negative effect on the level of gene transfer seen. This negative effect of the carrier solute may have more of an effect on gene transfer than was initially thought.

Mice that did not receive any LMB showed correction at 6 months of the CFTR ion channel by TPD measurement. This correction is statistically significant, compared to the untreated CFTR knockout group (p=0.010) and not significantly different to the het control group (p=0.207). Although the statistics indicate that there is correction of the CFTR channel, when looking at the actual ΔPD values achieved by this group, they still do not reach the levels of the het mice, indicating that only partial correction has been achieved. In this treatment group there was at least one mouse (indicated in

Figure 4-4, dark blue point) that showed consistent correction over the time of the experiment, however at the 6 month time point, the level of correction had decreased.

The Transepithelial Potential Difference measurement is the only method of testing the function of the CFTR gene, and this technique does have large variability in the results achieved due to the relatively challenging nature of placing the canular into the nasal airway. It is however the only tool to measure the function of the CFTR chloride ion channel in the nasal airways of treated mice. The technique is quite difficult due to the positioning of the cannular into the nasal airway without being able to visualize where it is placed meaning that the exact positioning will change from mouse to mouse or in subsequent assessments of the same mouse. This also means it is not possible to determine which cells- respiratory, olfactory, transitional epithelial- are being targeted. However, once experienced in the technique, it is probably the most valuable tool for assessing outcomes in *in vivo* CFTR gene transfer experiments in CF knockout mice.

Due to the limitations of TPD analysis a second, independent and quantitative measure of CFTR gene transfer, with less variability, was sought, and it was thought that a possible immunohistochemistry would provide a useful method to assess CFTR gene transfer and expression in a manner that would give a result similar to that obtained with the LacZ marker gene. It was hoped that the information gained from the use of immunohistochemistry would help to identify which cells we are targeting with our vector, and if this differs from our marker gene studies. It was also thought it would give a better indication of the correct cannular placement to best be able to target transduced cells for electrophysiological analyses.

The process for the immunohistochemistry was developed from protocols received from John Riordan's laboratory (University of North Carolina, Chapel Hill). Initial testing on transduced and CHOK-1 cells untransduced with a lentiviral vector expressing CFTR showed that both cell types showed equal

fluorescence Although steps were taken to try and reduce the background staining by using different fixative solutions and a range of concentrations of both primary and secondary antibodies none of these where effective in decreasing the level of fluorescence seen in the untransduced CHOK-1 cells compared to the transduced cells.

The immunohistochemistry was however transferred to our *in vivo* system as it was not known if there was endogenous CFTR in CHOK-1 cells that could be cross-reacting with the 596 antibody. Western blotting would determine if the antibody was reacting to another specific protein. Unfortunately this was not able to be completed for this thesis but should however be carried out before using the antibody further. The 596 antibody has been used in western blotting at recommended dilutions of 1:1000 to 1:5000 (UNC CFTR antibody distribution program).

As treated mouse nasal septums had been removed from sacrificed mice and frozen in OCT, thin sections were cut and incubated with the 596 antibody. As seen with the initial tests on the CHOK-1 cell lines, signal from the CFTR antibody reaction was seen in both treated and untreated mouse tissues to be non-specific, with no distinct bright cells indicating CFTR transduced cells in treated tissues. All samples, both treated and untreated looked the same as all samples showed low level fluorescence. Tissues were again stained using a range of dilutions of reagents and using various fixation methods. Again these made no difference to the background signal seen in untreated tissues.

It was not only the background signal from the untreated mouse nasal septa that was a problem with the immunohistochemistry approach. From the embedding process to the staining process there were a number of other issues to be considered.

Firstly, when embedding the thin piece of nasal septum in OCT, the orientation of the tissue is important. Various orientations of the tissue were tried, with the full face of the treated side being cut

or with the septum being embedded "on end" to allow a section containing both the treated and un-treated sides to be exposed to the antibody (as we see with our marker gene studies). Neither of these orientations for embedding gave a section of tissue that could be recognized and orientated to identify what area was being observed due to the small size of the tissue which led to difficulty in embedding, and the thin and delicate nature of the tissue making it difficult to obtain an intact piece of tissue following cryostat sectioning.

Finally, in mouse nasal septum, from antibody stained thin sections it was not easy to determine where in the tissue the section represented. The sections did not allow easy identification of the cell types that were visible and which cells had dual CFTR/DAPI signal as the processing of the samples appeared to have damaged the tissue. A more specific "counterstain" than DAPI would be useful in identifying the specific cell types in the airway epithelia.

Other members of the laboratory also tried using this antibody on transduced and untransduced cell lines (CHOK-1, A549, NIH3T3, 293T) and reported similar findings as those presented here. A large background staining was seen in all cell lines, whether transduced with our LV-CFTR or untransduced. This suggests that there may be a problem with the antibody itself. Unfortunately, further samples of the same antibody, or of other antibodies to huCFTR, were not available to further investigate and develop the immunohistochemical analyses.

As the immunohistochemistry analyses could not be made to work, samples embedded in OCT were removed and digested to extract gDNA. Real-time PCR was carried out on these samples to allow detection of the lentivirus vector. The RT-PCR shows incorporation of the vector into the treated tissues, but does not give an indication as to whether the incorporated gene is expressed or functional. However, it does give a quantitative measure of the amount of gene transferred into treated tissues.

The PCR results were somewhat surprising as they showed that mice treated with LMB had a 2.3 fold higher vector copy number per cell when compared to tissues from mice who received our standard LPC/LV treatment, however the difference was not significant This result does not correlate with the TPD measurements which showed at the final time point correction in only mice treated with the standard LPC/LV not containing LMB. This unusual result could be attributed to the LMB or methanol having an effect on the distribution of gene transfer by diluting the LPC and LV rendering each solution less viscous. This may alter where the gene was delivered and therefore, the TPD measurement being taken in areas where the gene was not present, but able to be detected *via* RT-PCR.

Given the lack of reproducibility on the effect of LMB on gene transfer *in vivo* it was important to check the biological activity of the stocks used. It is interesting to consider these tests of LMB activity on Gag production, as the LMB used in the *in vivo* LV-CFTR experiment showed no inhibition of Gag production, which indicates it is likely to be inactive. The apparent inactivity of the LMB may have had an effect on the gene transfer observed, however, it is difficult to determine whether this was a factor, as the experiments were not carried out at the same time. Other important factors to consider are the original LMB stock was over 2 years old, and LMB in solvent is only stable for 2 years. The LMB had also been dried down under nitrogen after the original *in vivo* experiments had been performed to allow it to be resuspended in the more favourable solvent ethanol. Subsequently literature was found that reported that this was likely to render the LMB inactive. It should also be noted that this LMB had been used in many experiments and exposed to light. However, the importance of the experiment was that it showed that the new stock, which did not show the same enhancement of LacZ gene transfer *in vivo* as the original stock, was biologically active when the experiments were done.

It is also important to look at the results from the RT-PCR carefully, as the vector copy number is an underestimate of the true amount of vector present. This is due to the whole septum being removed from the mice, including both the treated and non-treated sides. To gain a more representative value

for copy number it would be ideal to remove only the treated side of septum, however, this is technically very difficult as the mouse nasal septum is approximately 1 mm thick, and to divide this into treated and non-treated sides without damaging the epithelia is near impossible.

When looking at the second *in vivo* instillation of LMB dissolved in EtOH carrier solution, the results are surprising with the group receiving EtOH in the delivery protocol giving the highest amount of gene transfer, and also being significantly different from the LMB treatment group p=0.017. This result may be due to the properties of EtOH and the effects on the mobilization of the LPC or the LV-LacZ in the nasal airways, however, as the LMB treated group was found to give less gene transfer than both the standard group and the EtOH treated group, these studies were no longer continued as it became evident that LMB was not giving reproducible functional increases in the level of gene transfer achieved in normal mice treated with LacZ virus, or in CFTR knockout mice that received LV-CFTR.

4.7 Conclusions

4.7.1 Using LMB to enhance gene transfer

The inhibitor of the innate antiretroviral immune response- LMB- was tested to see the effect it would have on our lentiviral gene transfer protocol, with the anticipation of increasing the level of gene transfer achieved *in vivo*.

The initial results indicated a trend towards LMB increasing gene transfer when delivered at 500 ng/mL in both the LPC pre-treatment and the LV dose. However, when this was transferred into CFTR knockout mice, using LV-CFTR, the functional correction of CFTR was not evident in the LMB treated group. In retrospect, it was important to look at the decrease in gene transfer seen when the equivalent volume and concentration of methanol was used as a control in the early experiments. The

negative effect of the methanol may have had a more profound effect on the level of gene transfer than was first considered.

When a new supplier of LMB in EtOH was found, the levels of gene transfer increased, however, the EtOH appeared to have a greater effect on the enhancement of gene transfer compared to the LMB treated group. This result has led to the conclusion of this experiment as LMB does not appear to have the increased effect on the levels of gene transfer that had been expected.

The results obtained in these experiments did not give either definitive or statistically significant data to suggest that LMB dissolved in either 70% methanol or ethanol was going to enhance gene transfer *in vivo*. With the results obtained, further experiments using LV-CFTR in CFTR knockout mice, to gain significance would require >50 mice per treatment group, which technically is impractical and near impossible to carry out due to the difficult nature of the TPD testing technique. It would not seem practical to conduct this experiment when the increase in gene transfer compared to receiving no LMB is negligible.

The paper these experiments were derived from showed *in vitro*, gene transfer increased 3-fold when cells had been treated with LMB prior to LV transduction [140]. These results however, cannot be compared with our *in vivo* data as gene transfer in cell culture is different to gene transfer in an intact biological system such as the mouse nasal airways, as the virus is not sitting on the cells to be transduced for a known period of time, and there are other processes for "living" occurring that have unknown effects on gene transfer.

The *in vitro* testing of LMB activity showed the original LMB to be inactive, it is not known whether this was caused by the age of the stock, or whether it was due to the repeated use and therefore exposure to light. In hindsight, this may have influenced the results of the latter experiments using LV-CFTR in

CFTR knockout mice. If the LMB stock used was inactive, this may account for the lack of enhancement of gene transfer seen when LMB was incorporated into the gene therapy protocol. This is also likely to explain in part, the positive effect seen when using the LMB with LV-LacZ in normal mice, as the LMB stock was new, whereas when it was used for the CFTR experiments, the stock had been used a number of times, exposing it to light, which may render it inactive.

4.7.2 Methods for the analysis of CFTR gene transfer

Immunohistochemistry using the 596 mouse monoclonal antibody to the huCFTR gene was investigated as a potential method for visualizing expression of the CFTR transgene delivered to the nasal airways. Although attempted optimization was undertaken in both cell culture and on excised mouse nasal septum, there was no difference seen between control cells or tissues that did not contain the huCFTR transgene and those that had been treated with the huCFTR transgene. A western blot to determine if the antibody does bind to huCFTR should be carried out if the antibody is to be used in the future.

To gain some information from the nasal septum sections taken from all the treated mice, all septums were digested and the resulting gDNA was used in a RT-PCR reaction to determine the copy number of the transgene per cell. From this analysis, data was able to be generated, however, it conflicted with the data gained from the TPD analysis, as the TPD analysis showed the non-LMB treated group to have more CFTR function, whereas the RT-PCR analysis showed the LMB treated group to have a higher vector copy number per cell. It must be remembered that this RT-PCR analysis did not include all the samples from every treated mouse (from both treatment groups) as the septum is quite small, and from some samples no gDNA was recovered. The RT-PCR can also be seen as giving a diluted result as the copy number is measured per cell in the sample. The sample contains both the treated and

untreated sides of the nasal septum, which will cause the sample to be diluted, and does not give any indication of the types of cells transduced.

The analysis of the function of CFTR *in vivo via* TPD and the post-mortem analysis of CFTR integration *via* immunohistochemistry and RT-PCR have proven to be difficult to optimize and gain consistent results from during the course of this experiment, and further work to optimize the immunohistochemistry aspect of analysis would be of most benefit to visualize where and to which cells the CFTR transgene has been delivered.

Ideally, it would be beneficial to use the TPD analysis for the functional analysis of Cl- channel function, and immunohistochemistry on sections of treated nasal airways to visualize incorporated CFTR, to allow analysis of numbers of cells transduced and types of cells transduced by our vector, allowing direct correlation of the two measures of CFTR gene expression.