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Specialized Modalities

Modeling lung inflammation in 3D

A physiologically relevant model for studying interactions between immune cells and the lung epithelium using Lonza's Bronchial Air-Liquid Interface (B-ALI™) System

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In recent years, the compounding effects of widespread upper-respiratory viruses, such as COVID-19, influenza and RSV, have highlighted the dire need for physiologically relevant models that accurately represent interactions between multiple cellular systems, such as immune cells and the lung epithelium. Such models are an essential component of effective therapeutic testing and mechanistic analysis.

Here we discuss how we developed and optimized a co-culture model where immune cells (peripheral blood mononuclear cells; PBMCs) were co-cultured alongside respiratory pathway cells (normal human bronchial epithelial cells; NHBEs) using Lonza's B-ALI™ (Bronchial Air-Liquid Interface) system. We demonstrate that our optimized model supports physiologically functional epithelial membranes on the air interface, as NHBE cells polarized and differentiated to form cilia and tight junctions. In addition, the immune cell population in the liquid compartment was shown to behave as expected, with the T cell population exhibiting an activation response upon stimulation.

We then validated this co-culture as a physiologically relevant model for virally induced inflammation by stimulating the T cell population with viral antigens. The resulting co-culture demonstrated inflammatory responses by the T cell population through increased CD69 expression as well as an increase in the inflammatory factors granzyme B, TNF-α, and IL-6. Furthermore, we observed that NHBE membranes visibly thinned when co-cultured alongside PBMCs stimulated with viral antigens, which suggests that the inflammatory response by the PBMC fraction may have triggered necrosis in the epithelial lining above.

Taken as a whole, this model provides a straightforward, physiologically relevant system for studying interactions between the immune system and the lung epithelium.

Introduction

The recent national and global outbreaks of upper respiratory viruses, such as COVID-19, influenza, and RSV, along with the FDA's shifting emphasis towards xeno-free models for drug discovery research, have drastically increased the demand for culture systems that can better mimic the complex physiology of respiratory system damage than traditional 2D culture total submersion models.[1]

3D Air-Liquid Interface (ALI) culture using primary cells allows for the differentiation and unique polarization of human airway epithelial cells into the physiologically relevant cell types (e.g., mucin-producing goblet cells or ciliated cells) that allow for the proper functioning of the respiratory system *in vivo*.[1,2] This system provides the unique benefit of approximating the actual environmental conditions in which human airway cells exist *in vivo*, with the liquid compartment beneath the cells representing the bloodstream. This compartment contains a medium that induces differentiation and polarization of the seeded epithelial cells, causing them to mature into mucin- and cilia-producing cells and form tight junctions between cells like those found in the lining of the airway epithelium.[1,2]

Importantly, the use of primary human airway cells in an ALI culture system has a distinct advantage over immortalized cells, cancer cell lines, and even animal models in that they maintain the closest gene expression and disease susceptibility to *in vivo* conditions in the human body.[1-3] For example, both cancer cells and immortalized cells may lack the receptor expression found in their primary cell counterparts, which can change their susceptibility to diseases such as influenza.[3]

In addition, ALI culture allows for the co-culture of differentiated airway cells and immune cells, providing a system capable of modeling how respiratory capacity could be harmed by immune-mediated inflammatory effects during an infection or toxicological exposure.[1] For these reasons, ALI culture models using primary human airway cells serve as an excellent, physiologically relevant tool for exploring both disease pathology as well as drug discovery and toxicology.

One of the key challenges in developing a 3D ALI airway-immune cell co-culture model is balancing the media in such a way that airway cells can form a healthy, differentiated membrane with cilia and high integrity (i.e. with tight junctions) while also supporting the expansion, differentiation, and activation of the immune fraction residing in the basal chamber of the Transwell® plate.

Here, we present an easy-to-use airway-immune cell co-culture model using NHBE cells and PBMCs based in Lonza's B-ALI™ Media System. We show that the membranes developed using this protocol are healthy, robust, and differentiated, while the immune cell population displays appropriate proliferative and functional responses over the course of a 72-hour co-culture. Finally, we tested the inflammatory response of our co-culture by stimulating the immune (PBMC) fraction using viral antigens. PBMCs displayed a strong inflammatory reaction to the addition of viral antigens, while the NHBE membranes above thinned markedly compared to control conditions, suggesting that the underlying inflammatory immune response induced necrotic effects in the NHBE layer above. Overall, our NHBE-PBMC B-ALI™ Co-culture System is a highly-relevant model of lung physiology that can be utilized in a variety of applications from disease research to drug discovery.

Methods

3D B-ALI™ NHBE-PBMC co-culture

A detailed protocol for establishing an optimized 3D B-ALI™ NHBE-PBMC co-culture can be found on the Lonza website.[4] In brief, Lonza NHBE cells (Lonza part no. CC-2540S;

n = 3 donors) were thawed in a 37°C water bath until only a sliver of ice remained, gently resuspended, and then pipetted into a T-75 flask with 25 mL of B-ALI™ Growth Medium that had been allowed to equilibrate to 37°C. This plate was incubated at 37°C for 2 – 4 days and cells harvested at 50 – 90% confluence, with a medium change on day 1.

On the day of NHBE harvest, Corning® Transwell® plate inserts were coated with 30 µg/mL collagen solution, washed with PBS, and then left to dry prior to cell plating.[4] NHBE cells were then trypsinized, harvested, counted, and then diluted using B-ALI™ Growth Medium in order to seed them at a density of 50,000 cells/well. Cells were then incubated for 3 – 4 days in 100 µL of B-ALI™ Growth Medium in the top chamber and 500 µL of B-ALI™ Growth Medium in the basal chamber. The medium was changed on day 1 and again on day 3 if proper morphology was not yet obtained. Once the cell layers displayed the proper morphology on day 3 or 4 (filling in the surface of the well, Figure 1), cells were exposed to air by removing media from the top chamber of the Transwell® above the membrane. The B-ALI™ Growth Medium in the basal chamber was replaced with 500 µL of B-ALI™ Differentiation Medium and then cells were cultured for another 14 days at 37°C, with medium changes every 2 – 3 days.

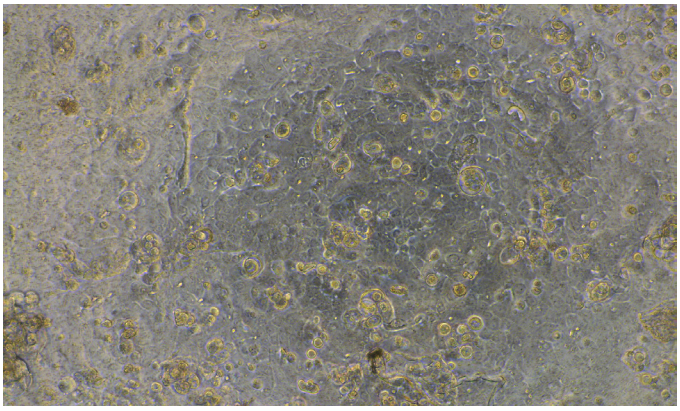

Figure 1.
NHBE morphology before airlift, day 3 – 4 of culture. Imaged at 10x magnification.

We established co-cultures with PBMC's (Lonza part no. CC-2703; n = 3 donors; allogenic with NHBEs; Donors 1 – 3 hereafter) 14 days after airlifting the NHBE cells. The PBMCs were thawed, counted, and resuspended into one of three media treatments (B-ALI™ Differentiation Medium, X-VIVO® 15, or a 50/50 mix). We seeded PBMCs into the basal chamber for all co-culture wells or an empty 24-well plate for a PBMC-only control at a density of 5×10^5 cells/well (1×10^6 cells/mL). NHBE-only controls were also maintained alongside all treatments with no added PBMCs. The B-ALI™ Inducer was added to all media conditions to support NHBE differentiation, and all B-ALI™ SingleQuots® Supplements were added at full strength to all media conditions. Viable T cell activation was established by adding IL-2 and anti-CD3/CD28 antibody to the PBMCs and medium after counting but before seeding for all treatments.

We incubated the co-cultures for 72 hours at 37°C, changing medium at 2 days (accomplished by removing the PBMCs and medium from each well, centrifuging and removing the medium, then resuspending the PBMCs in fresh medium and returning to the appropriate well). NHBE membrane measurements included trans-epithelial electrical resistance (TEER; a measure of tight junction formation or membrane integrity), mucin production (via Alcian blue staining), and membrane thickness (via sectioning and hematoxylin and eosin (H&E) staining). We assessed PBMC population viability and functionality via immunophenotyping using flow cytometry.

Co-culture inflammatory response to viral antigen stimulation

We used a different set of n = 4 unique NHBE and n = 4 unique PBMC donors (allogenic pairs; Donors 4 – 7 hereafter) to examine model responses to viral inflammation. PBMCs were pre-screened for responsiveness to the cytomegalovirus, Epstein-Barr, and influenza (CEF) peptide pool. We then we repeated the above protocol using the following modifications. First, we used only the 50/50 medium, as we found this to have the best balance between an optimal membrane and PBMC population.[4] Second, when the PBMC co-culture was established, we added CEF MHC Class 1 Peptide to the cell culture medium in each inflammation treatment well ("Virus"), bringing the final concentration of each peptide up to 0.5 µg/mL. Finally, in order to establish both positive and negative controls for T cell stimulation, we cultured PBMCs without NHBEs, and then exposed both PBMCs and NHBE-PBMCs to the following treatments: no stimulants (IL-2 alone; negative control), IL-2 + Virus, and IL-2 + Virus + anti-CD3/CD28 antibody (positive control; "CD3/28").

The same endpoints were measured in these trials as the first set of experiments; however, results from Donor 4 led us to also include additional endpoints to further clarify the inflammatory response of viral-activated PBMCs. For Donors 5 – 7, we also assessed inflammatory factors (granzyme B, TNF-α, and IL-6) released by the T cell population via ELISA to determine whether or not the T cell population exhibited an inflammatory response to the viral peptides.

Results

Optimizing the 3D B-ALI™ co-culture model

We undertook three rounds of optimization and found that using a 50/50 mix of B-ALI™ Differentiation Basal Medium with X-VIVO® 15 Serum-free Hematopoietic Cell Medium (with all B-ALI™ Supplements and Inducer added at full strength to the mix) and performing a medium change the day after co-culture initiation produced the best balance between a robust membrane and a healthy, functional

immune cell population over the course of a 72-hour co-culture.

The 50/50 medium produced the thickest membranes with the most visible cilia (Figure 2) for all three donors compared to either B-ALI™ Differentiation Medium or X-VIVO® 15 Medium alone (data not shown). While, in some cases, membranes formed with X-VIVO® 15 alone had higher overall transepithelial/transendothelial electrical resistance (TEER) values (data not shown), the TEER values for all three donors cultured with the 50/50 medium were well above the passing threshold value of 550 Ω*cm² for a physiologically healthy membrane (Figure 3). TEER values were also improved by performing a medium change the day after co-culture initiation (tested with Donor 2; utilized in Donor 3 as the only 50/50 media treatment). No difference in mucin production was seen among treatments (data not shown).

T cell populations exhibited a healthy activation response across the 72-hour co-culture (Figure 4), with the highest levels of T cell Activation (population of $CD8^+CD69^+$ PBMCs) in the X-VIVO® Medium treatment, followed by the 50/50 medium treatment for all three donors. There was no difference in either lymphocyte viability (ranging between 45 – 83% viability for 50/50 medium) or cytotoxic T cell population fractions (ranging between 16 – 46% $CD8^+$ expression) among treatments, indicating that all media were capable of supporting a healthy population of lymphocytes and that any differences between experiments were a result of donor variation (data not shown).

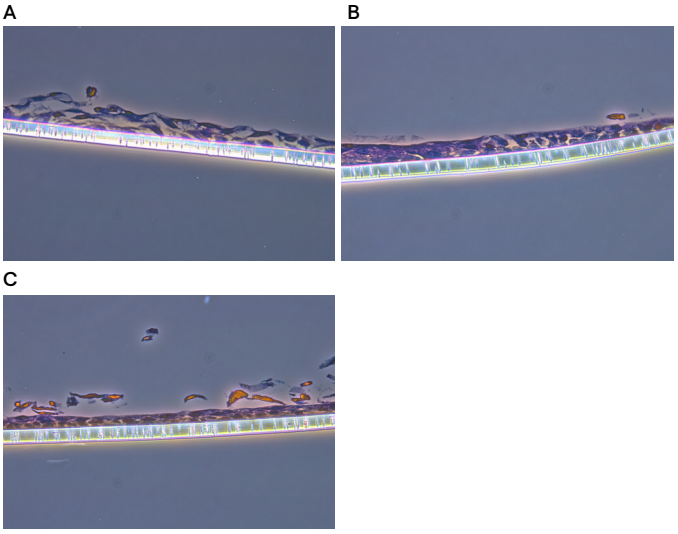

Figure 2.
Representative images of H&E stained NHBE membrane sections from Donor 1 (A), Donor 2 (B), and Donor 3 (C) for the 50/50 medium treatment. This treatment formed multiple layers of cells, with visible ciliation (see panel B in the top left of the membrane). Disconnected membrane fragments (e.g., panel C) are the results of fragmentation during sectioning in preparation for H&E staining. Membranes were harvested after 72 hours of co-culture. All images taken at 40x magnification.

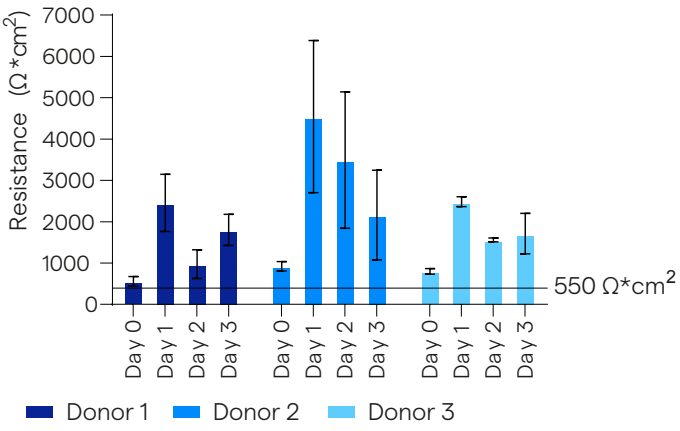

Figure 3.
TEER values of NHBE membranes for all three donors grown in co-culture with PBMCs using the 50/50 media treatment. Membranes were harvested after 72 hours of co-culture (day 0 – day 3). The black bar indicates the minimum passing threshold TEER level for healthy membrane integrity (550 Ω*cm²).

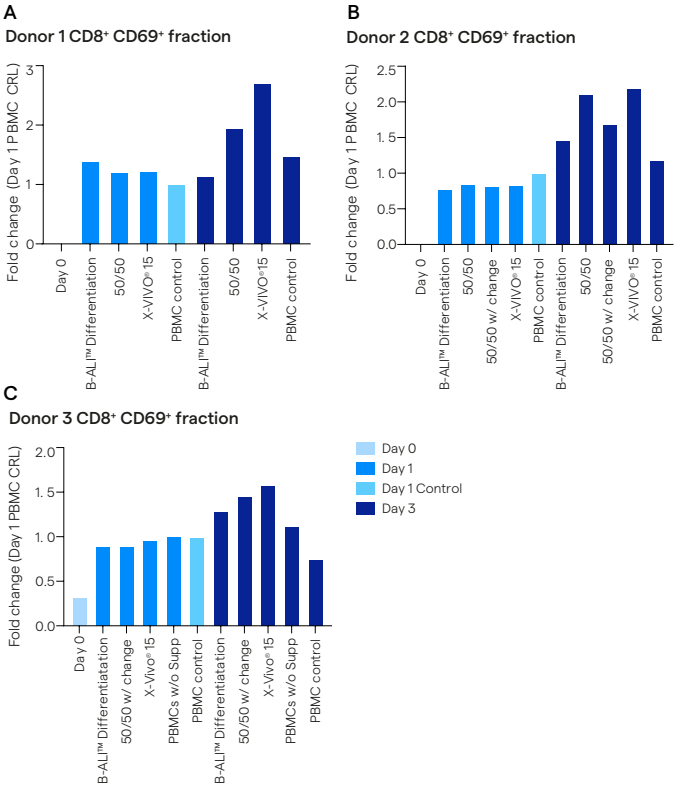

Figure 4.
T cell population activation for each donor (graphs A – C) from day 0 (initiation of NHBE-PBMC co-culture) to day 3 (72 hours of co-culture) for each medium. Data consists of fold change for each population relative to the PBMC monoculture control population size on culture day 1 (Day 1 PBMC Control). T cell population was determined by flow cytometric detection of the PBMC population subset expressing $CD8^+CD69^+$, indicating a cytotoxic T cell population that has been stimulated by the anti-CD3/CD28 antibody addition. Note that, for Donor 2, we added an additional media change for 50/50 medium after one day (panel B) for comparison. As it improved endpoints, we implemented this media change as standard procedure for Donor 3 (panel C).

The effect of viral-induced inflammation on 3D B-ALI™ NHBE-PBMC co-culture

We used four different allogenic donor pairs of NHBEs and PBMCs in the viral inflammation experiments. PMBC donors were selected by prescreening for response to the CEF peptide pool to ensure effects of inflammation were present to be observed. Initial T cell population size varied between these 4 donors (Table 1).

PBMC donor	Day 0 % CD3⁺ expression (T cells)
Donor 4	72.6
Donor 5	79.7
Donor 6	52.1
Donor 7	27.3

Table 1.
Initial percent of PBMC population expressing CD3⁺ (T cells) for each of the four donors co-cultured with NHBEs in this trial. Note the wide range of variation, with Donor 7 being especially depleted in comparison to the other three donors.

When stimulating the PBMC population using viral peptides (CEF peptides; primarily impact the cytotoxic MHC-I restricted T cell population), CD69⁺ expression, an early-stage indicator of CD8⁺ cytotoxic T cell activation, increased when PBMCs were co-cultured with NHBEs versus unstimulated controls (NHBE-PBMC co-culture + IL-2 only; Figure 5). This same pattern of increased activation in the co-culture was seen in comparison to PBMCs cultured in monoculture and exposed to viral particles for all four donors used in this study (Figure 5).

CD25⁺ expression, a later-stage indicator of activation in CD8⁺ cytotoxic T cells, followed a similar trend, though the effect was not consistent across all four donors (Figure 5). Positive controls (CD3/28 antibody) for T cell stimulation and activation were consistently much higher for both activation markers when PBMCs were co-cultured with NHBEs when compared to PBMCs in monoculture (Figure 5).

Similarly, the production of inflammatory factors by immune cells was higher in co-culture when compared to PBMCs in monoculture, and was typically higher when viral peptides were added versus the negative control (NHBE-PBMC co-culture + IL-2 only; Figure 6). The release of inflammatory cytokines and lytic enzymes generally reflected the size of the initial T cell population of the PBMCs, with the highest concentrations (and most significant differences between viral-activated co-culture and controls) released by Donor 5 (highest initial T cell population, Table 1) and decreasing with Donor 6 and 7 (Figure 6).

When comparing membrane quality across all four treatments (Co-culture + IL-2, Co-culture + IL-2 + Virus, Co-culture + IL-2 + Virus + CD3/28, and NHBE monoculture) between the first and last day of co-culture, it was noticeable that membranes had thinned over the course of the four days for co-culture treatments that were exposed to the viral peptides (Figure 7, column 3 vs. 1) or viral peptides and the anti-CD3/CD28 positive control (Figure 7, column 4 vs. 1). This trend was less noticeable in Donor 7, which overall produced a thin, poor-quality membrane (Figure 7, row D). No significant differences were seen between treatments for TEER or mucin production (data not shown), though it should be noted that at no point did the TEER values for Donor 7 exceed the 550 Ω*cm² threshold indicating a healthy, intact membrane.

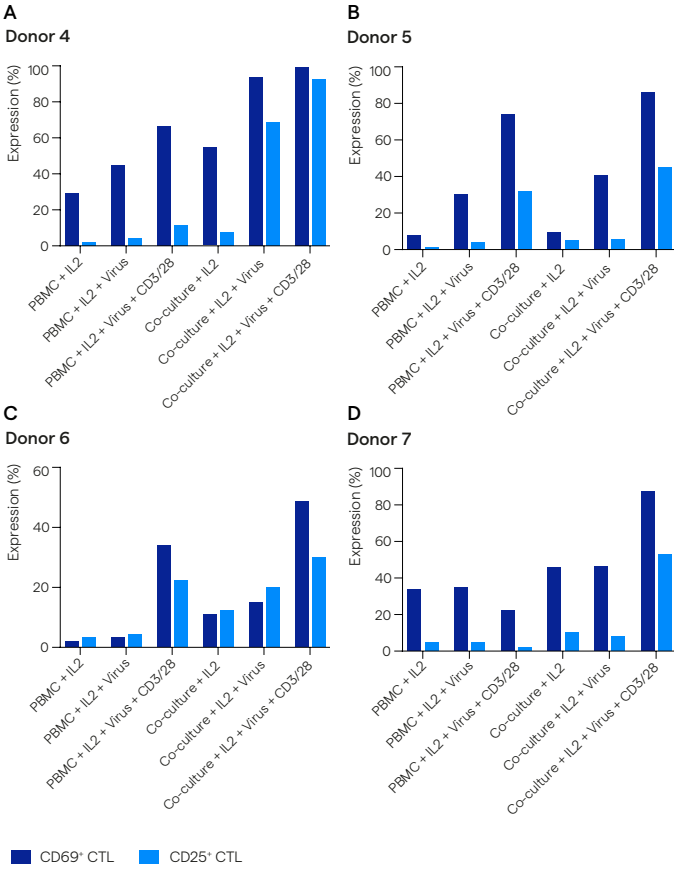

Figure 5.
Percent expression for CD69⁺ and CD25⁺ among the CD8⁺ population of PBMCs (cytotoxic T lymphocytes; CTL) after 72 hours of monoculture (PBMC) or co-culture (Co-culture) for all four donors (graphs A-D, respectively) and for all treatments (IL-2, IL-2 + viral antigens ("Virus"), and IL-2 + Virus + CD3/28 antibody positive control). CD69⁺ is a marker of early CD8⁺ T cell activation, while CD25⁺ is a late-stage indicator of activation. Higher expression of either marker indicates an inflammatory immune response.

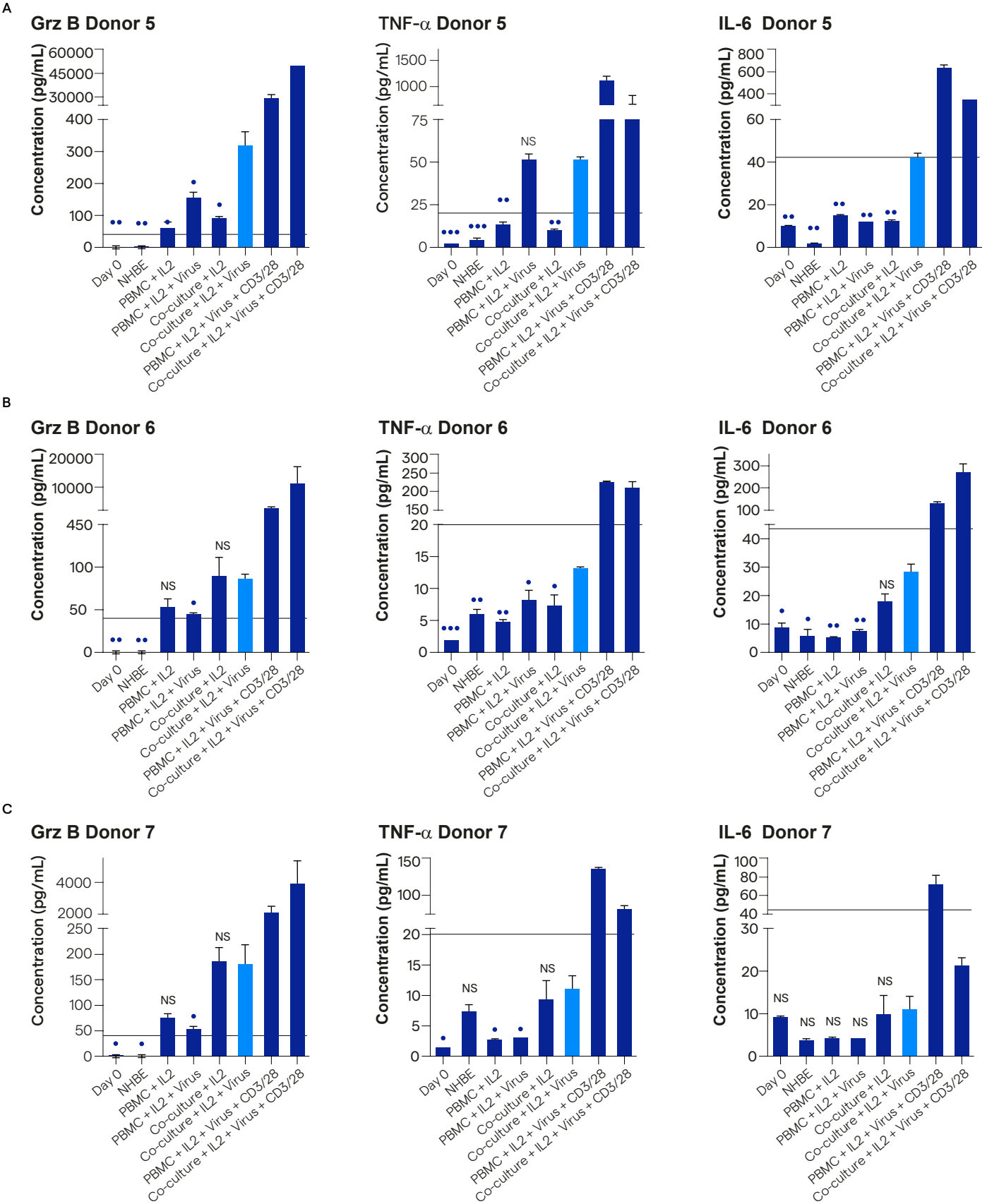

Figure 6.
Intracellular inflammatory cytokine and lytic enzyme concentrations for Donors 5 – 7 (rows A – C, respectively) for each treatment. Day 0 values were measured upon initiation of co-cultures. All other values were measured after 72 hours. Statistical differences indicated were determined by T tests comparing the viral stimulated co-culture (Co-culture + IL2 + Virus; light-colored) against all other treatments except the two positive controls (treatments with CD3/28 antibody addition). The overall response profile is directly reflective of the size of the initial $CD3^+$ T cell population. Donor 5 had the highest T cell population of the donors tested and displays the highest level of inflammatory factors while the reverse is true with Donor 7. Note that cytokine release was not measured for Donor 4. The maximum intracellular concentration found in healthy individuals is shown by the horizontal black line in each panel.[5–7] NS = not significantly different from viral-stimulated co-culture, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

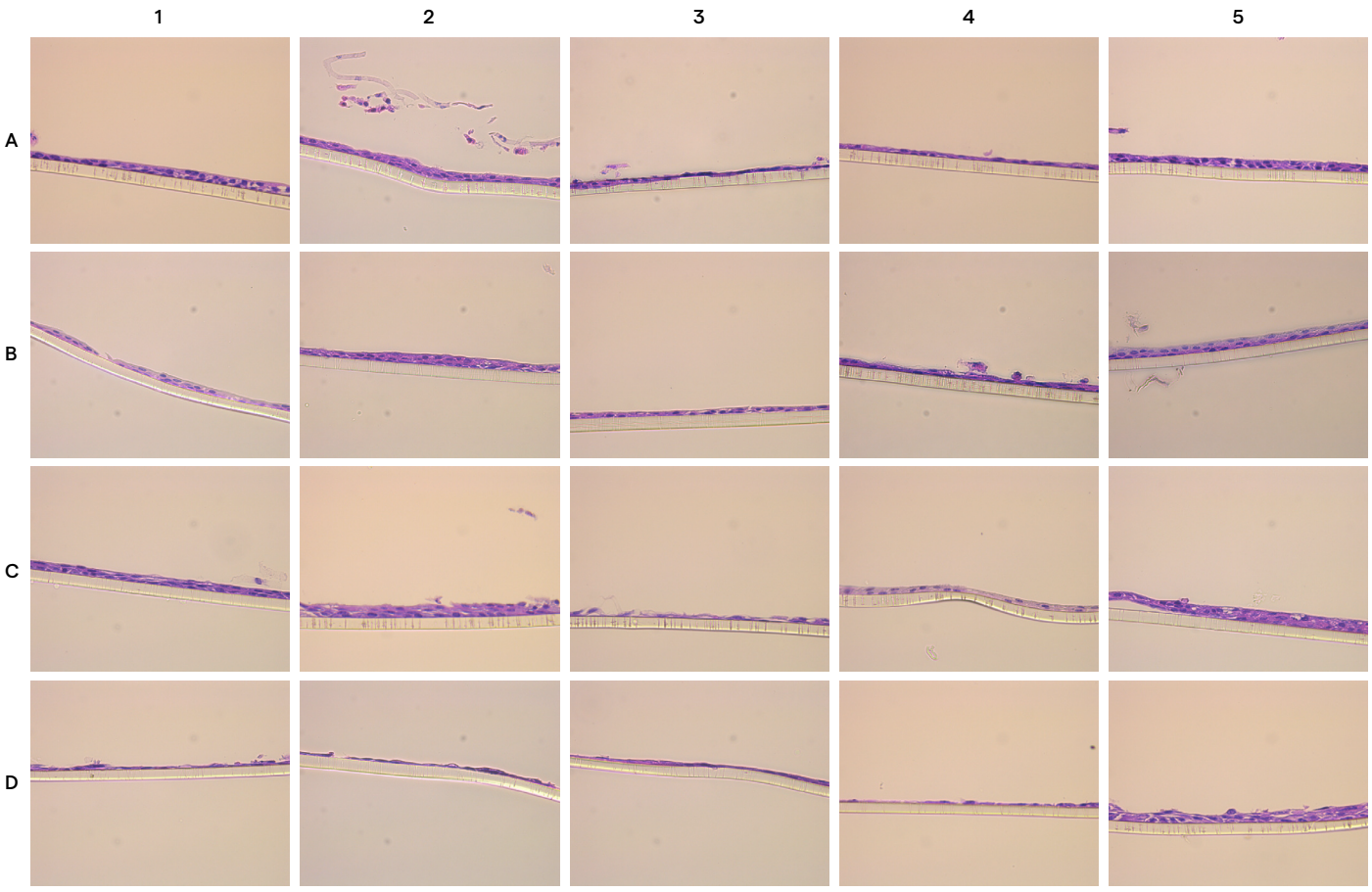

Figure 7.
H&E stained membrane sections for Donors 4 – 7 (rows A – D, respectively) collected on day 0 (initiation) of co-culture (column 1), and day 3 (fourth day of co-culture) for the following treatments: column 2: Co-culture + IL-2; column 3: Co-culture + IL-2 + viral antigens; column 4: Co-culture + IL-2 + anti-CD3/CD28 antibody (positive control); and column 5: NHBE monoculture. Disconnected membrane fragments (e.g., panel A5) are the results of fragmentation during sectioning in preparation for H&E staining. All images taken at 40x magnification.

Discussion

Our results demonstrate the successful creation of a co-culture model using Lonza's B-ALI™ System with primary NHBEs and PBMCs. The model mimics the inflammatory responses seen in the respiratory system when triggered by viral antigens and driven by cytotoxic T lymphocytes.

In our first set of experiments, we found that, overall, a 50/50 mix of B-ALI™ Medium and X-VIVO® 15 medium (specifically formulated to support immune cells) best supported the co-culture model. To formulate the 50/50 media mix, we chose to incorporate the B-ALI™ SingleQuots® Supplements at full strength (adding to the combined 50/50 medium rather than adding supplements to the B-ALI™ Differentiation Medium base and then splitting) in order to best support the quality of the NHBE fraction.

These supplements contain components, such as epinephrine and hydrocortisone, which could suppress inflammatory responses. Indeed, we found some evidence that cytotoxic T cell activation could have been partially influenced by the B-ALI™ Supplements, as the PBMC monoculture control had lower activation when compared to the PBMC monoculture without the B-ALI™ Supplements (Figure 4C; only tested in Donor 3).

However, in all cases, the interactive effects of co-culturing PBMCs with NHBE cells was demonstrated by the high degree of cytotoxic T cell activation when the PBMC fraction was stimulated with anti-CD3/CD28 antibody in the presence of the NHBE membrane (Figure 4), near 100% in some cases. This indicated that chemical crosstalk between the NHBE and PBMC fractions primed immune cells for activation and negated any dampening effect by the B-ALI™ Supplements.

Whether donor mismatch between NHBE cells and PBMCs played a part in activating cytotoxic T cells is not clear, but due to the nature of the Transwell® Membrane plate, the immune cells are prevented from coming into contact with the NHBE cells, thus making an allogenic response less likely.[8,9]

Overall, when all endpoints were weighed against each other, the thickness and degree of differentiation of the membrane produced with the 50/50 medium (Figure 2) indicated that the 50/50 medium was the best medium to use for this co-culture, as it also promoted high membrane integrity (TEER) (Figure 3) as well as a healthy, functionally-responsive T cell population (Figure 4).

Our second set of experiments validated the performance of the co-culture as a model for virus-induced inflammatory effects on the lung epithelium. Here we used a different set of donors that had been pre-screened for responsiveness to the CEF peptide pool. Once again, we observed a general "priming" effect of NHBEs on PBMCs in co-culture, as the expression of both early (CD69) and late (CD25) $CD8^+$ cytotoxic T cell markers (Figure 5) as well as inflammatory factor production (Figure 6) tended to be higher in co-culture than when PBMCs were in monoculture.

Regardless, stimulation of co-cultures with viral antigens generally increased T cell activation markers (Figure 5) and the release of inflammatory factors (Figure 6) when compared to co-cultures with IL-2 alone, though there was variation across donors for these effects that correlated with the initial T cell population sizes (Table 1). For example, Donor 5 had the highest T cell population of the donors tested and displayed the highest level of inflammatory factors while the reverse is true with Donor 7 (Table 1, Figure 6). This evidence suggests that an inflammatory response is occurring within the PBMC population as a result of viral stimulation, which is further supported by the fact that NHBE membranes co-cultured with PBMCs induced by CEF peptides thinned noticeably over the entire course of the co-culture (Figure 7 column 3) when compared to NHBE membranes cultured without PBMCs (Figure 7 column 5).

Membranes from viral-stimulated co-cultures most closely resembled those from co-cultures activated by the anti-CD3/CD28 antibody control (Figure 7 column 4), which stimulated drastic inflammatory responses across all immune cell endpoints (Figures 5 and 6). Of note, Donor 7 could be considered a "low responder" as the PBMC population that had the lowest starting T cell population size (Table 1). To further examine models of minimal inflammation, PBMC donor lots can be pre-screened for a lack of response to the CEF peptide pool. Donors with minimal response will not produce the same heightened levels of inflammatory factors or display the same activation markers in a monoculture pre-screen environment. Utilizing these lots will create an environment with minimal necrotic effects.

Similar to our results, other studies have shown that inflammatory, pro-apoptotic cytokines released by activated immune cells lead to necrotic tissue death of the surrounding tissue.[10] It should be noted that, in any ALI model using a Transwell® Plate with sub-cell sized pores, any inflammatory-driven tissue necrosis would only be accomplished through chemical messengers such as cytokines, as the membrane itself bars immune cells from infiltrating and directly contacting the epithelial tissue layer above.

Conclusions

We present a 3D NHBE-PBMC B-ALI™ Co-culture system that is both easy to implement and successfully models the physiologically relevant pathological effects of inflammation on the airway epithelium when stimulated with common viral peptides.

Lung epithelial membranes developed using this protocol are healthy, robust and differentiated. We demonstrated that this unified system undergoes noticeable shifts in phenotype for both lung and immune compartments after antigen administration.

We tested the inflammatory response of our co-culture by stimulating the immune (PBMC) fraction using viral antigens. In response, the PBMCs displayed a strong inflammatory reaction and the NHBE membranes thinned markedly compared to control conditions, suggesting that the underlying inflammatory immune response induced necrotic effects in the NHBE layer above.

The use of differentiated, primary NHBE cells in the B-ALI™ System better represents *in vivo* conditions and patient physiology than 2D submerged systems, reducing the need for animal models by offering increased translational value of experiments across a number of applications including immunotoxicity, vaccine development, small molecule therapies, and biologic therapies.[1-3]

Overall, our NHBE-PBMC B-ALI™ Co-culture system has proven to be a highly relevant model of lung physiology that has numerous important applications from disease research to drug discovery.

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Ordering information

Catalog no.	Description	Size
CC-2540S	Cryopreserved Human Normal Bronchial Epithelial (NHBE) Cells for B-ALI™ Culture	≥ 500,000 cells
CC-2703	Cryopreserved Human Peripheral Blood Mononuclear Cells (PBMCs)	≥ 100 million cells
00193516	B-ALI™ Growth Basal Medium	250 mL
00193517	B-ALI™ Differentiation Basal Medium	500 mL
00193515	B-ALI™ SingleQuots® Supplement Kit	SingleQuots®
00193514	B-ALI™ Bronchial Air-Liquid Interface Medium BulletKit®	BulletKit®
02-060Q	X-VIVO® 15 Serum-free Hematopoietic Cell Medium, w/ L-Glutamine, gentamicin, and phenol red	1 Liter
CC-5024	HEPES Buffered Saline Solution	500 mL
CC-5012	Trypsin/EDTA	100 mL
CC-5002	Trypsin Neutralizing Solution	100 mL

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