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Research Paper

Inflammatory Response and Barrier Properties of a New Alveolar Type 1-Like Cell Line (TT1)

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Purpose. To evaluate the inflammatory response and barrier formation of a new alveolar type 1-like (transformed type I; TT1) cell line to establish its suitability for toxicity and drug transport studies.
Methods. TT1 and A549 cells were challenged with lipopolysaccharide (LPS). Secretion of inflammatory mediators was quantified by ELISA. The barrier properties of TT1 cells were evaluated by transepithelial electrical resistance (TEER), fluorescein sodium (flu-Na) apparent permeability (P_{app}) and staining of zona occludens-1 (ZO-1).
Results. LPS stimulated similar levels of secretion of IL-6 and IL-8 in TT1 and A549 cells. TNF-α was not produced by either cell line. In contrast to A549 cells, TT1 cells did not secrete SLPI or elafin. TT1 cells produced maximal TEER of ~55 Ω cm^2 and flu-Na P_{app} of ~6.0×10^{-6} cm/s. ZO-1 staining was weak and discontinuous. Attempts to optimise culture conditions did not increase the barrier properties of the TT1 cell layers.
Conclusions. The TT1 cell line models the alveolar inflammatory response to LPS challenge and provides a valuable complement to cell lines currently used in toxicity assays. However, under the experimental conditions used the TT1 cell line did not form the highly restrictive tight junctions which exist *in vivo*.

KEY WORDS: alveolar type 1-like cell line; barrier properties; inflammatory response; LPS; tight junctions.

INTRODUCTION

The alveoli of the lung are lined by a continuous epithelium formed predominantly by type 1 (AT1) alveolar epithelial cells held together by tight junctions. AT1 cells are very thin with protruding nuclei and cover approximately 95% of the alveolar surface (1). Besides providing a large area for gas exchange, AT1 cells are involved in ion and protein transport (2–5) which is important for the regulation of lung liquid homeostasis. Type 2 (AT2) alveolar epithelial cells cover only 5% of the alveolar surface (1) and are responsible for surfactant production and cellular proliferation by differentiating into AT1 cells as a repair or replacement mechanism (6). The alveolar epithelium is an important part of the innate immune system (7) and cell lines are widely used to study the inflammatory response of the alveolar epithelium to inhaled agents. To date, the A549 adenocarcinoma cell line is most frequently used to model the alveolar epithelium for toxicological testing (8). However, A549 is an AT2-like cell line, whereas an AT1 epithelial cell line representing the cell type that contributes the majority of the alveolar epithelial surface may provide a more relevant *in vitro* model of the alveolar epithelium.

Until the AT1-like (transformed type-1; TT1) cell line was reported recently by Kemp and co-workers (9), no human AT1 cell line had been available. The TT1 cell line was derived from AT2 cells by immortalization using transduction with the catalytic subunit of telomerase (hTERT) and a temperature sensitive mutant of simian virus 40 large-tumour antigen and displays an AT1-like phenotype. The immortal TT1 cells are negative for the AT2 cell markers alkaline phosphatase and thyroid transcription factor-1, do not contain lamellar bodies, display a flattened morphology and contain caveolae. These results indicate that the immortalization has driven the AT2 cells towards an AT1-like phenotype. This cell line, therefore, promises to obviate some of the shortcomings of the *in vitro* systems currently used for evaluating the toxicological response of the alveolar region to inhaled agents, including nanoparticles and inhaled drug products. However, the responsiveness of the TT1 cell line to inflammatory stimuli requires characterisation to allow the potential of this cell line to be evaluated.

Besides their role in the alveolar immune response, AT1 cells are thought to play a key role in the absorption and

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ABBREVIATIONS: AT1, alveolar type 1; AT2, alveolar type 2; flu-Na, fluorescein sodium; P_{app}, apparent permeability coefficient; SLPI, secretory leukoprotease inhibitor; TEER, transepithelial resistance; TT1, transformed type 1; ZO-1, zona occludens protein-1.

transport of inhaled drugs across the alveolar epithelial barrier. *In vitro* models of alveolar epithelial cells are widely used for the study of drug absorption and transport in the lung. Due to the inadequacy of current alveolar epithelial cell lines to generate tight junctions (11–15), most research is performed using primary AT2 cells that transform over time in culture into an AT1-like cell phenotype and form a tight polarized cell monolayer (10). Several animal species have been used as a source of cells for primary culture *in vitro* models of the alveolar epithelium (16–18). However, the use of human cells is preferred, as questions over extrapolation of results obtained from non-human drug absorption models are avoided. Human primary AT2 cells have been isolated from normal lung tissue after lobectomy and have been cultured and used in drug transport studies (18,19). However, ethical and logistical considerations related to human tissue limit the widespread application of primary human cell cultures. This is further restricted by the limited ability of AT2 cells isolated from adult lungs to proliferate *in vitro* which means that they cannot be subcultured. If they are able to form functional tight junctions and a tight monolayer, the TT1 cells could provide a regular source of human alveolar epithelial cells for investigations of drug transport in the peripheral lung. It is important, however, to evaluate thoroughly the barrier function of putative cell culture drug absorption models to prevent inappropriate use for drug transport studies (10).

The aim of this study was to evaluate the potential of the TT1 cell line for toxicity assays and drug transport studies. To investigate the inflammatory response of the TT1 cell line, cells were stimulated with increasing concentrations of lipopolysaccharide (LPS). Cytokines (interleukin-6; IL-6, and tumour necrosis factor-α; TNF-α), chemokine (interleukin-8; IL-8), and antiproteinases [secretory leukoprotease inhibitor (SLPI), and elafin] were quantified by enzyme-linked immunosorbent assay (ELISA). Results were compared to those in LPS-stimulated A549 cells. To evaluate whether the TT1 cell line forms a tight monolayer suitable for drug transport studies, the effect of culture conditions on epithelial barrier function was studied using (1) a range of seeding densities, (2) different culture media and supplements, (3) dexamethasone as a potential barrier enhancer, and (4) air interface culture *versus* liquid immersed culture. The barrier formation was monitored by transepithelial electrical resistance (TEER) measurements, transport of the paracellular marker fluorescein sodium (flu-Na) and immunocytochemical staining of tight junction protein, zona occludens-1 (ZO-1) (21).

MATERIALS AND METHODS

Cells and Culture Conditions

The TT1 cell line was generated from normal primary human AT2 cells which were isolated from normal regions of lung tissue following lobectomy for carcinoma (9). Transformation was accomplished by retroviral transduction with the catalytic subunit of human telomerase (hTERT) and a temperature sensitive mutant of simian virus 40 large antigen (U19tsA58). Passages 46–60 were used in this study.

To evaluate the inflammatory response to LPS, TT1 cells were cultured as described previously for these cells (9). The cells were grown in DCCM-1 medium (React Scientific, Ayr, UK) supplemented with 10% New Born Calf serum (NCS; Invitrogen, Paisley, UK), 1% penicillin/streptomycin/glutamine (PSG; Invitrogen) and 0.5 mg/ml gentacin (G148; Sigma Aldrich). A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS, 1% glutamine, 1% non essential amino acids and 0.1% gentamycin. Both cell lines were cultured in 24-well culture plates (Greiner Bio One, Stonehouse, UK) at a seeding density of 1.5×10^5 cells/well. Cells were incubated in humidified 5% CO_2/95% air at 37°C.

To investigate the barrier properties of the TT1 cell line, cells were seeded onto 1.13 cm^2 tissue culture-treated polycarbonate cell culture supports with a pore size of 0.4 μm (Transwell®, Costar, Birmingham, UK) at various seeding densities, and grown in DCCM-1 medium, Minimal Essential Medium (Sigma-Aldrich) or Small Airway Growth Medium (SAGM; Lonza Bioscience, Slough, UK), supplemented with 10% NCS or various concentrations of Fetal Bovine Serum (FBS; Sigma-Aldrich), 1% PSG and 0.5 mg/ml G148. Cells were grown as submerged cultures or as air-interface cultures (AIC). For AIC, cells were grown in the absence of apical medium from day 3 of the culture period. In addition, cells were incubated with dexamethasone (1 μM, Sigma-Aldrich) added to the culture medium from day 2 of the culture period.

Calu-3 cells were grown as submerged cultures in DMEM/Ham's Nutrient Mixture F 12 supplemented with 10% FBS, 1% glutamine, 1% non essential amino acids and 0.1% gentamycin as a positive control for tight junction formation (35). The cells were incubated in humidified 5% CO_2/95% air at 37°C and the medium was replaced every 2 days.

LPS Stimulation of TT1 Cells and A549 Cells

TT1 and A549 cells were seeded and cultured for 24 h before transfer to serum-free culture medium or serum-rich (10% NCS) culture medium for 24 h before addition of LPS (*Escherichia coli* 055:B5, Sigma-Aldrich). The medium was removed and the cells were incubated with fresh medium containing LPS at concentrations of 0, 1, 10 and 100 ng/ml. Each treatment was performed in duplicate. The resulting conditioned medium was aspirated and any secreted IL-6, TNF-α, IL-8, SLPI and elafin were measured by enzyme-linked immunosorbent assay (ELISA).

Measurement of Cytokines, Chemokine and Antiproteinases

IL-6, TNF-α and IL-8 were quantified in the cell supernatants obtained from each experimental condition using commercially available, quantitative sandwich ELISA kits (Quantikine®; R&D systems, Abingdon, UK) according to the manufacturer's recommendations. Measurements were performed in duplicate. The limits of detection were 0.7 pg/ml for IL-6, 3.5 pg/ml for IL-8 and 1.6 pg/ml for TNF-α.

SLPI was quantified by sandwich ELISA using monoclonal anti-human SLPI antibody-coated 96-well plates. Biotinylated anti-human SLPI antibody was used as detection antibody. Recombinant human SLPI was used as a standard (all obtained from R&D Systems, UK). The limit of detection for recombinant human SLPI was 5 ng/ml. A DuoSet kit

(R&D systems) was used to quantify elafin. Both assays were performed according to the manufacturer's recommendations with measurements performed in duplicate.

Bioelectric Measurements

The formation of a tight cell monolayer was monitored by measuring TEER as a function of time in culture. TEER was measured daily using an EVOM epithelial voltohmmeter equipped with STX2 chopstick electrodes (World Precision Instruments, Stevenage, UK) and calculated by subtracting the resistance contributed by the Transwell cell culture support and correcting for the surface area of the Transwell. Before measuring TEER in cultures maintained at the AIC, fresh warmed medium was added to the apical chamber and culture plates were returned to the incubator at 37°C. TEER was measured after 1 h of equilibration, a period after which the permeability properties of the cell layer are stabilised following medium change (35). After the measurement, the medium was aspirated from the apical chamber.

Permeability of Cell Layers

The permeability of confluent immortal human alveolar epithelial cell layers on day 8 in culture was determined using fluorescein sodium (flu-Na; Sigma-Aldrich) according to the methods of Grainger and coworkers (35) with confluent Calu-3 cell layers (day 8 in culture) used as a positive control. Briefly, the cell layers were washed twice with warm Hank's balanced salt solution (HBSS; Sigma-Aldrich; 37°C). HBSS was introduced to the apical (0.5 ml) and basolateral chamber (1.5 ml) and cells were returned to the incubator at 37°C for 1 h to equilibrate. The paracellular marker, flu-Na, was dissolved in warm HBSS to produce a 0.1 mg/ml test solution. The TEER was measured immediately before experimentation. HBSS in the apical chamber was aspirated and the experiment was initiated by introducing 600 μl of test solution to the apical donor chamber. The initial concentration in the donor fluid was assayed by removing a 100 μl sample directly after adding the test solution. The cells were incubated at 37°C and stirred using an orbital shaker at 100 rpm during the experiment. Eight samples of 100 μl each were serially drawn from the receiver compartment over 2 h, with samples being replaced with 100 μl of fresh warmed HBSS. At the end of the experiment, a 100 μl sample was drawn from the donor compartment and TEER was measured after the final sample was taken. Each experiment was performed in triplicate.

For analysis of flu-Na transfer, all samples were transferred to a black 96-well plate (Nunc, through Fisher Scientific, Leicestershire, UK) and 100 μl of 40 mg/ml NaOH aqueous solution was added to each sample. Fluorescence was measured at excitation and emission wavelengths of 485 and 530 nm, respectively, using a fluorometer (Cytofluor, Series 4000, Foster City, CA, USA).

Flux (J) was determined by plotting the cumulative amount of flu-Na in the receiver chamber *versus* time. Apparent permeability coefficients (P_{app}) were calculated according to the equation $P_{app} = J/(A * C_0)$, where C_0 is the initial starting concentration of flu-Na in the donor fluid, and A is the surface area of the Transwell cell culture support (1.13 cm^2). To calculate the contribution of the cell layer to the P_{app} and therefore correcting for the P_{app} of the cell-free Transwell support the following equation was used: cell contribution $(\%) = 100 - [(1/P_T)/(1/P_{TC})*100]$, where P_T is the P_{app} of the cell-free Transwell support, and P_{TC} is the P_{app} calculated for the cell-covered Transwell support (22).

Immunocytochemical Staining

TT1 cell layers were stained for ZO-1 on day 8 in culture, to visualise expression of the tight junction protein. Calu-3 cell layers were used as a positive control (35). Cell layers were washed twice in PBS and fixed for 10 min using freshly prepared 3.7% paraformaldehyde at room temperature. The cells were washed with PBS and prepared for staining by permeabilization with 1% Triton X-100/0.5% FBS for 60 min followed by a 10 min blocking step with 50 mM ammonium acetate in PBS. After washing with PBS, cells were incubated at 37°C with rabbit anti-zona occludens-1 (ZO-1; 1 μg/ml; Zymed, Cambridge BioScience, Cambridge, UK) in PBS containing 1% (*w*/*v*) BSA for 60 min. Staining was visualized by incubation with AlexaFluor 488 chicken anti-rabbit IgG (10 μg/ml; Invitrogen) in PBS containing 1% BSA for 60 min at room temperature. The cell layer was washed again and counterstained with 1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) in water. After 10 min incubation, cells were washed again with PBS and the Transwell® cell culture support was cut from the plastic support, mounted on a microscope slide using 10% glycerol and sealed. Slides were stored at 4°C and viewed within a week using a Leica DMIR E2 confocal microscope (Leica Microsystems, Milton Keynes, UK). Fluorescent emissions from DAPI (λ_{ex}=205 nm; λ_{em}= 430–480 nm) and Alexa 488 (λ_{ex}=488 nm; λ_{em}=510–570 nm) were collected using separate channels at a magnification of ×40. Instrument gain and offset values remained constant for all experiments. Images obtained from each scan were pseudo-coloured blue (DAPI) and green (ZO-1), then overlapped afterwards to obtain a multicoloured composite image. Results shown depict a representative image from at least n=4 of each sample.

Statistical Analysis

Data are presented as mean±standard deviation (SD) or mean±standard error (SE). A one-way ANOVA, with a post hoc Tukey–Kramer test for multiple comparisons, was used to analyse the concentration-dependent effects of LPS on cytokine, chemokine and antiproteinase release from TT1 and A549 cells. Significance was established at $p<0.05$.

RESULTS

Cytokine, Chemokine and Antiproteinase Secretion

The TT1 cells and A549 cells showed a concentration-dependant release of IL-6 ($p<0.05$) with TT1 cells producing significantly more IL-6 than A549 under unstimulated conditions ($p<0.0001$; Fig. 1a). The maximum IL-6 release in serum rich conditions, approximately 650 pg/ml, was equivalent for TT1 cells and A549 cells. The presence of serum in the culture medium sensitized both cell types, however, this effect

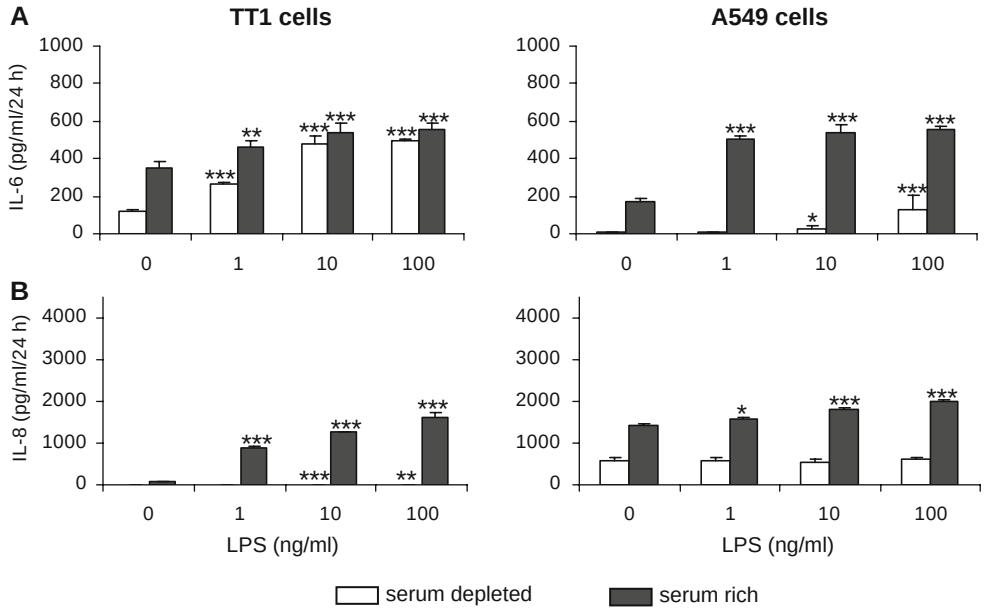

Fig. 1. LPS-induced dose-dependent release of IL-6 and IL-8 by TT1 cells and A549 cells. Cells were cultured with increasing doses of LPS for 24 h. Conditioned media were aspirated and IL-6 and IL-8 release was assayed by ELISA in duplicate. Data represent mean±SD (n=4). The *asterisks* depict significant differences between the control and LPS-stimulated cells: *p<0.05; **p<0.01; ***p<0.0001.

was more prominent for A549 cells. Neither cell line secreted TNF-α under serum rich nor serum depleted conditions (data not shown).

TT1 cells showed a dose dependant release of IL-8 (p< 0.0001) and were sensitized by serum in the culture medium. The TT1 cells secreted significantly less IL-8 compared to A549 under unstimulated conditions (p<0.0001; Fig. 1b). LPS did not induce IL-8 release by A549 cells cultured in serum depleted conditions. TT1 cells did not secrete SLPI or elafin under any of the experimental conditions, including LPS stimulation and serum sensitisation (Fig. 2a). A549 cells did secrete SLPI under basal conditions, although this release was not induced by LPS or affected by the presence or absence of serum. LPS-stimulated A549 cells showed a dose-dependent release of elafin when cultured in serum rich conditions (p<0.0001). In the absence of serum, A549 cells constitutively secreted elafin which was inducible by LPS. The maximal concentration of elafin secreted by A549 cells was approximately eightfold less than SLPI.

Transepithelial Electrical Resistance of Cell Layers

When cultured according to conditions described by Kemp *et al.* (9), the TEER of TT1 cells increased as a function of time to reach a plateau (approximately 41 Ω cm^2) at day 6 post-seeding (Fig. 3). Culture conditions were adjusted in an attempt to obtain tighter cell layers. Seeding density had no impact on TEER values (data not shown) and, of the culture media investigated [DCCM-1 (9) MEM and SAGM], cell layers cultured in SAGM produced a marginal increase in TEER to ~60 Ω cm^2 (data not shown). Therefore, SAGM was used as basal medium in further experiments to optimise culture conditions. None of the other strategies to increase the TEER such as increasing the concentration of serum to 20% *v*/*v*, the use of AIC, addition of dexamethasone (1 μM), or combinations of these conditions, produced higher TEER values (Table I). Calu-3 cells were used as a positive control and produced characteristic TEER of >1,000 Ω cm^2 under liquid covered culture (Table I).

Permeability

The permeability of the TT1 cell layers cultured according to Kemp *et al.* (9) was measured initially as P_{app} flu-Na $3.4\pm0.6\times10^{-6}$ cm/s. P_{app} for the cell layers cultured using SAGM was $6.3\pm1.0\times10^{-6}$ cm/s. Cell layers cultured in higher serum concentration, AIC or with the addition of dexamethasone, showed P_{app} values of approximately 6.0×10^{-6} cm/s. Furthermore, the combinations of the culture conditions described above had no significant impact on the permeability of the cell layers (Table I). The relative contribution of the cells to the P_{app} was calculated to correct for contribution of the Transwell support. The Calu-3 cells provided almost the entire permeability barrier (200-fold greater contribution to barrier compared to support) whereas the contribution of TT1 cells to the permeability barrier was much lower; only three times that of the culture support.

Immunofluorescence Staining of Tight Junctions

ZO-1 was detected in TT1 cells and Calu-3 cells by an anti-ZO-1 antibody (Fig. 4). In contrast to the high level and continuous staining pattern of ZO-1 expression in Calu-3 cells, the tight junctions in TT1 cells were less heavily stained and the staining was discontinuous. The addition of dexamethasone to the culture medium produced more distinct and regular staining patterns in the TT1 cells, but there was no ZO-1 staining, even with the addition of dexamethasone, when cells were cultured at the air-interface.

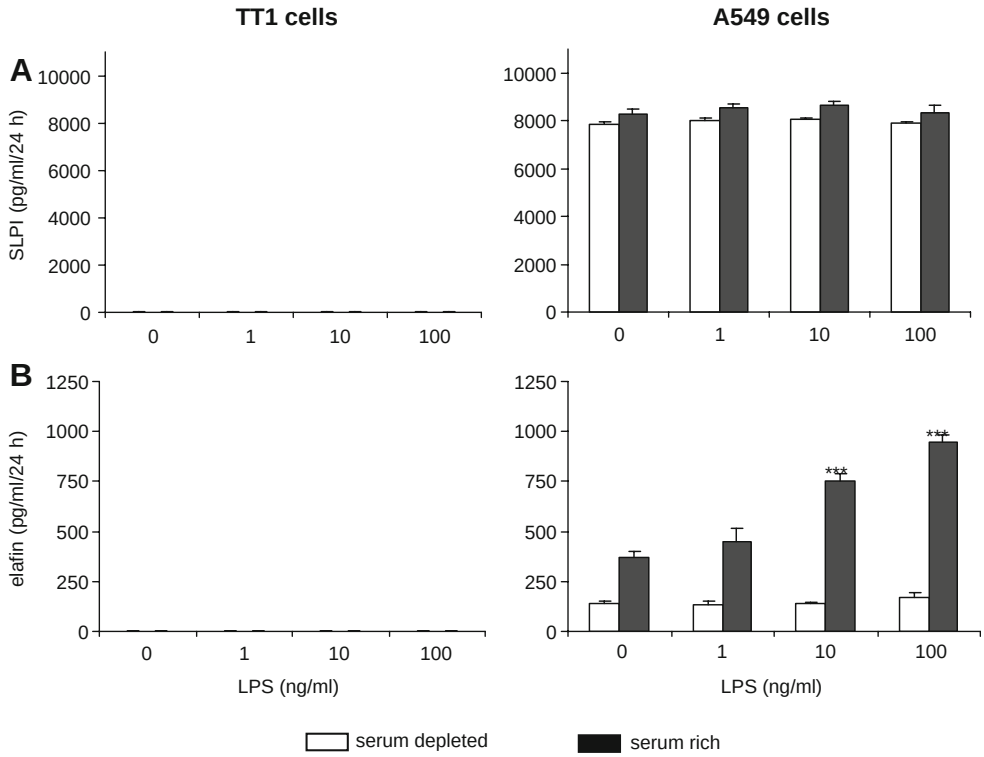

Fig. 2. LPS-induced release of SLPI and elafin by TT1 cells and A549 cells. Cells were cultured with increasing doses of LPS for 24 h. Conditioned media were aspirated and antiproteinase release was assayed by ELISA in duplicate. Data represent mean±SD (n=4). The *asterisks* depict significant differences between the control and LPS-stimulated cells: *p<0.05; **p<0.01; ***p<0.0001.

DISCUSSION

This is the first study to evaluate the inflammatory response and barrier function of a new transformed human AT1-like cell line (TT1) and thereby examine its applicability in toxicity assays and drug transport studies. LPS-stimulated TT1 and A549 cells secreted IL-6 and IL-8 similarly, but proteinase inhibitors, SLPI and elafin, were produced only by A549 cells. The TT1 cell line produced cell layers with low TEER and high permeability due to poor tight junction formation under a variety of cell culture conditions. These results demonstrate that the TT1 cell line may be used to study the toxicological response to inhaled agents, but indicate that the cell line requires further development (e.g. lower passage number cells; identification of favourable culture conditions) before it can be used to measure trans-epithelial solute flux.

The AT2-like A549 adenocarcinoma cell line is the most widely used to model the alveolar epithelium in toxicity assays (8). However, a human AT1 cell line would be more relevant than, or at least complimentary to, the use of A549 cells to evaluate the toxicity of inhaled agents as the alveolar epithelial surface is formed predominantly by AT1 cells (1). If evenly distributed in the lung, deliberately inhaled medicinal aerosols or incidentally inhaled environmental aerosolised particles will deposit predominately on to the surface of ATI cells. Thus, to replicate the situation that occurs *in vivo* and predict inflammatory events or absorptive clearance, an AT1 culture system is essential. The current drive to develop nanoparticles as delivery systems for inhaled drugs and concomitant concerns over their safety reinforces the necessity for suitable *in vitro* models to evaluate the safety of such delivery systems (36,37). Inimical properties of nanoparticles (25,26) have been detected *in vitro* by measuring IL-6 and IL-8 release from A549 epithelial cells and this study indicates that the TT1 cell line has the capacity for this functional response. LPS is a standard challenge agent which has been used to produce a proinflammatory response in primary or continuous AT2 or AT2-like cells *in vitro* (23,24,28). Indeed, human AT2 cell precursors to the TT1 cell line described herein secrete significant quantities of IL-6 and IL-8 following

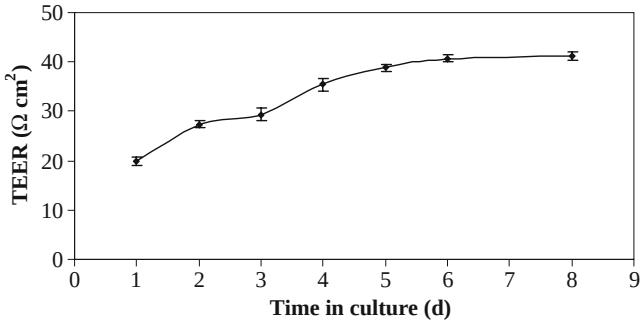

Fig. 3. Transepithelial resistance of TT1 cells cultured according to conditions used by Kemp *et al.* and plotted as a function of time from three separate experiments. Cells were grown on 1.13 cm^2 cell culture supports. Data represents mean±SE (n=9).

Table I. Transepithelial Electrical Resistance (TEER), Apparent Permeability (P_{app}) of Fluorescein Sodium (including mass balance for recovery) across TT1 cells on Transwell filters, and the contribution of cell layer to the P_{app} value

Culture condition	$TEER_{max}$ (Ω cm^2)	P_{app} ($\times 10^{-6}$ cm/s)	Recovery (%)	Cell contribution (%)
Kemp *et al.* 2008	40.7±0.7	3.4±0.6	99.6±0.2	78.0
10% FBS	54.6±0.5	6.3±1.0	103.4±1.5	72.2
10% FBS + 1 μM Dex	56.3±0.2	5.9±0.9	101.7±0.5	74.1
10% FBS, AIC	55.2±0.4	6.5±1.3	101.9±1.0	71.6
20% FBS	58.1±0.5	5.8±0.9	101.1±0.7	74.6
20% FBS + 1 μM Dex	54.7±0.0	6.0±1.0	101.7±1.2	73.6
20% FBS, AIC	57.7±0.3	5.9±1.0	100.3±0.8	74.1
20% FBS + 1 μM Dex, AIC	55.4±0.3	6.3±1.2	102.3±0.9	72.4
Calu-3	1113.5±23.8	0.12±0.0	98.8±0.3	99.5

Cells were cultured in small airway growth medium except for the culture condition according to Kemp *et al.* (9) (DCCM-1 medium) and for the positive control, Calu-3 cells (DMEM). Data from three experiments; mean±SE (n=9)
FBS fetal bovine serum, *Dex* dexamethasone, *AIC* air interface culture

LPS challenge (28). LPS-stimulated A549 cells secrete IL-6 (24), and IL-8 (23,24) *in vitro*, but LPS has minimal effects on the TNF-α gene expression (27). The TT1 cells secreted IL-6 and IL-8 in response to LPS release similarly to A549 cells, a response that was strongly augmented in both cell lines by the presence of serum. TNF-α was not detected in the medium from TT1 or A549 cells. Primary human AT2 cells are reported to produce low levels of TNF-α in response to LPS (28) and the absence of this response in A549 cells raises questions over the suitability of this cell line as an AT2 cell model for immunotoxicity studies. However, it is unknown whether AT1 cells secrete TNF-α in response to LPS exposure *in vitro* or *in vivo*, thus absence of TNF-α release is not necessarily an indictment of the TT1 cell line, and it may be entirely appropriate that this cell line does not secrete TNF-α in response to LPS *in vitro*.

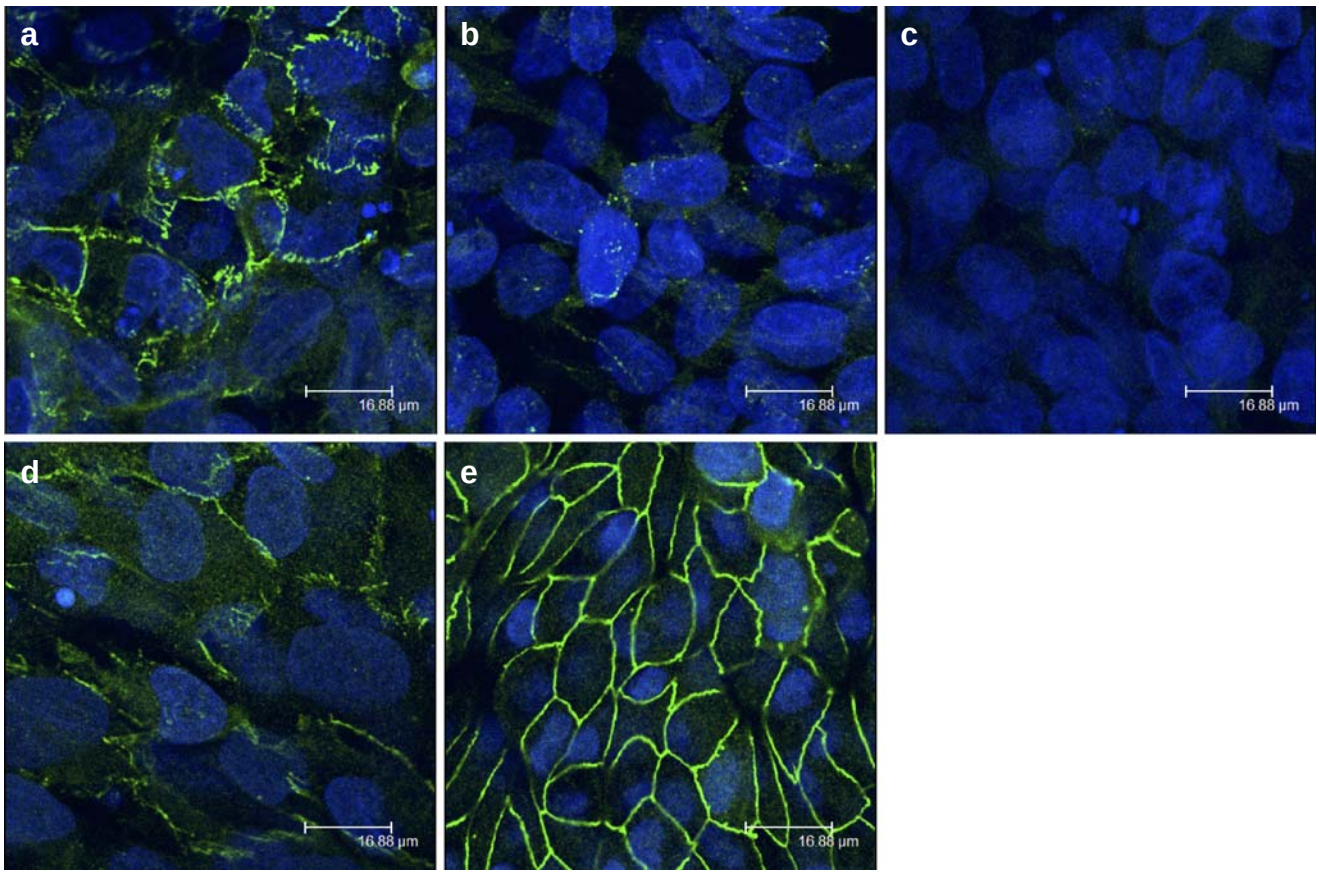

Fig. 4. Distribution of ZO-1 of TT1 cells. TT1 cells were grown on 1.13 cm^2 Transwell® cell culture supports and stained for ZO-1 at day 8 in culture. The following culture conditions were applied: **a** LLC containing 1 μM dexamethasone in SAGM, **b** AIC containing 1 μM dexamethasone in SAGM, **c** AIC in SAGM alone and **d** LLC cells grown in DCCM-1 medium. **e** Calu-3 cell layers were used as a positive control.

In addition to the cytokine and chemokine profile, the release of antiproteinases by LPS-stimulated TT1 cells and A549 cells was measured. Antiproteinases such as SLPI and elafin are secreted locally at sites of injury to prevent the harmful effects of proteolytic enzyme release from inflammatory cells (29). In addition to elastase-inhibitory effects, SLPI and elafin have been shown to have antimicrobial properties *in vitro* (30,31). Antiproteinase secretion by primary AT2 cells and A549 cells has been described (32,33), however, data regarding secretion from AT1 cells is limited. In contrast to A549 cells, the TT1 cells did not produce SLPI or elafin in response to LPS, and serum components were required for the activation of A549 cells to produce elafin. It may be that AT2 cells rather than AT1 cells are responsible for alveolar SLPI and elafin release *in vivo*, and these *in vitro* observations reflect this. The transformed nature of the TT1 cell line must be remembered, however, when interpreting these results. Little is known, about antiproteinase release by AT1 cells; this warrants further investigation.

The barrier presented by absorptive epithelia can be modelled using polarized epithelial cell layers that form tight junctions *in vitro*. However, to date there has not been an animal or human alveolar cell line that can perform this function, and the only models of the alveolar epithelium are based on freshly isolated primary type II cells that have been cultured for several days to form tight polarised AT1-like cell monolayers (10). The TT1 cell line is reported to be AT1-like and express tight junction proteins in culture (9); the essential credentials for an alveolar epithelial barrier-forming cell-line. However, when cultured using the conditions reported for the establishment of the cell line (9), TT1 cells did not produce physiologically-relevant restrictive tight junctions.

Tight junction formation by respiratory epithelial cells is highly dependent on culture conditions such as seeding density, culture medium and supplements, culture method and cell passage. The TT1 cell line was developed using DCCM-1 medium (9), whereas typically MEM has been used for rat alveolar cells (2,3) and SAGM for human alveolar cells (18,19). When DCCM-1, MEM and SAGM were used as a basal medium to culture the TT1 cells in preliminary experiments, TEER were low (ranging between 40–60 Ω cm^2) and P_{app} were relatively high (>3.4×10^{-6} cm/s). Similarly, neither changing seeding density nor a low concentration of dexamethasone, 0.1 μM, had any beneficial effect on the barrier function of TT1 cultures. In further experiments, the effect of serum, dexamethasone 1 μM and AIC on barrier formation by TT1 cells were investigated (Table I). Media supplements are important for cell growth and differentiation; serum has been reported to influence tight junction formation (12,34) and dexamethasone has been described as a possible barrier enhancer (12,20). AIC has been reported as a factor that can improve the poor tight junction formation by A549 cells (11), with air exposed-A549 cell layers having more regular ZO-1 staining and low solute P_{app}, although TEER values remained low.

In this study, no beneficial effect on the barrier formation of TT1 cell layers compared to control was achieved by changing the culture conditions (Table I). Although the addition of dexamethasone appeared qualitatively to increase ZO-1 expression (Fig. 4), cell layer TEER and P_{app} were unchanged, suggesting that any enhancement of enhanced ZO-1 expression was insufficient to impact functionally on the cell barrier. The maximum TEER achieved during culture ($TEER_{max}$) of the TT1 cells was approximately 55 Ω cm^2 which is extremely low compared to TEER reported for alveolar cell layers generated from primary human AT2 cells that differentiated over time in culture to an AT1 phenotype (>1,400 Ω cm^2) (19). In addition, P_{app} of flu-Na in TT1 cell layers (6.0×10^{-6}) compared to human primary alveolar cell layers (1.2×10^{-7}) (38) are relatively high, indicating that the TT1 cell line produces leaky cell layers. The leakiness of the inter-cellular junctions would be more apparent from the P_{app} and proportion of cell contribution of the TT1 cells to the P_{app} value (Table I) were it not for the exceptionally large size of TT1 cells, approximately 40 μm^2 (9), which results in a reduced tight junction:cell surface ratio.

The lack of functional tight junction formation by the TT1 cells was not resolved by employing culture conditions that typically promote tight junction formation. Other strategies that have been employed previously, such as the coating of the culture support and medium supplementation with calcium, were considered less propitious and were not attempted in this study. However, it is more likely that at higher passage number the TT1 cells suffer the phenotypic drift which has been reported for other cell lines to result in impaired differentiation and deficient tight junction formation (39,40). This possibility was not explored due to unavailability of low passage cells. Finally, the immortalization technique may have engendered critical alterations in cell differentiation or protein expression. Hopfer *et al.* (41) reviewed the effects of immortalizing epithelial cells and concluded that early passage transformants usually retain tight junctions, but cell lines often enter crisis after 15–20 passages resulting in poorly differentiated cells (39), although the use of hTERT and SV40 LT antigen to immortalize primary human cells offers the prospect of being able to explore the normal characteristics of primary human cells without the limitation of a limited lifespan and confounding effects of cellular senescence (42).

In summary, we have demonstrated that TT1 cells release cytokines and chemokines after LPS stimulation, indicating that the TT1 cell line is suitable to study inflammatory responses of the alveolar epithelium. Given that hitherto no satisfactory human AT1 cell line has been available for biomedical research, these *ab initio* studies herald the potential of TT1 cells to constitute a much-needed AT1 cell line to complement the AT2 cell lines that are used currently in toxicological assays. In addition, this study is the first to our knowledge to suggest that AT2 cells and not AT1 cells may be responsible for LPS-induced SLPI and elafin secretion; the protective mechanism of the alveolar epithelium against proteolytic attack. Disappointingly, the high passage TT1 cells used in this study did not generate the tight cell monolayers required for modelling drug transport and epithelial barrier function *in vitro*. Further development or modification of the TT1 cell line will be required for the cells to be used for this application.

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