The Effect of Immunosuppression on Airway Integrity

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None of the authors have anything to disclose.

LIST OF ABBREVIATIONS

16HBE	Human Bronchial Epithelial cells
CLAD	Chronic Lung Allograft Dysfunction
EMT	Epithelial to mesenchymal transition
FCS	Fetal calf serum
HLMVEC	Human Lung Microvascular Endothelial cells
HPLC-MS/MS	High pressure liquid chromatography-mass spectrometry
IL-8	Interleukin-8
ISHLT	International Society for Heart and Lung Transplantation
LC-MS/MS	Liquid chromatography-mass spectrometry
LTx	Lung transplantation
MMF	Mycophenolate mofetil
TEER	Trans-epithelial electrical resistance

ABSTRACT

Background: Insults to the airway epithelium play a key role in constrictive bronchiolitis following lung transplantation (LTx), the typical hallmark of chronic rejection. Our hypothesis is that immunosuppressives might affect airway integrity.

Methods: A biculture of lung bronchial epithelial cells (16HBE) and lung microvascular endothelial cells (HLMVEC) was exposed to immunosuppressives (serum trough levels) for 24h or 4 days. Cytotoxicity, trans-epithelial electrical resistance (TEER) and permeability was measured after exposure to monotherapies and combination therapies. Apoptosis, oxidative stress, inflammation (IL-8), real time PCR for epithelial-to-mesenchymal-transition (EMT) and tight junction proteins were assessed in exposed cells.

Results: MMF and combination therapies including MMF, at serum trough levels and higher, are toxic for the 16HBE cells after 4 days exposure. Moreover, already after 24h, TEER of cells exposed to MMF decreases and permeability increases. MMF did not induce apoptosis, oxidative stress, loss of tight junctions or production of IL-8 after 24h, but possibly induces EMT in epithelial cells. MMF was detectable at both sides of the biculture and was also present in broncho-alveolar-lavage (BAL) of LTx patients. Other immunosuppressives were not toxic, neither changed TEER or permeability.

Conclusions: Our findings suggest that MMF is present in the airways of lung transplant patients and might affect the structural integrity of the airway, which needs further investigation and validation in the clinical setting.

INTRODUCTION

Lung transplantation (LTx) is the ultimate treatment option for patients with end-stage lung diseases. Survival after LTx remains poor with a median survival of 5.7 years, mainly due to chronic lung allograft dysfunction (CLAD)¹. The 2 phenotypes of CLAD recognized so far (restrictive allograft syndrome and bronchiolitis obliterans syndrome) show typical airway lesions on pathology, being constrictive bronchiolitis, which is small airway obliteration because of inflammation and subsequent fibrosis. Besides constrictive bronchiolitis lesions on histology, restrictive allograft syndrome is also characterized by persistent infiltrates on chest CT scan, which represent interstitial inflammation and/or fibrosis on histology.^{2,3} Chronic injury to the airway epithelium is believed to lead to an excessive immune response, overproduction of extracellular matrix and subsequent complete obliteration of the airway lumen⁴.

The causes of these injuries are only partly known. A possible mechanism is epithelial-to-mesenchymal-transition (EMT) in which bronchial epithelial cells transition to (myo)fibroblasts^{5,6}, thereby gaining properties of mesenchymal cells, including production of matrix metalloproteinases and deposition of extracellular matrix⁵.

To prevent CLAD, a triple drug regimen is used including a calcineurin inhibitor (cyclosporine (C) or tacrolimus (T)), antiproliferative agents (azathioprine (A) or mycophenolate mofetil (MMF or M) and corticosteroids (like dexamethasone (D))⁷. The most used triple drug regimen in our centre is a combination of dexamethasone-azathioprine-cyclosporine or dexamethasone-MMF-tacrolimus. Per the International Society of Heart and Lung Transplantation (ISHLT), the most used combination therapy at 1 year and 5 years after adult lung transplantation (LTx) is corticosteroids-MMF-tacrolimus, however long-term survival for this treatment and for the treatment regimen corticosteroids-MMF-cyclosporine is

worse compared to the treatment regimens without MMF and with azathioprine as antiproliferative agent ¹⁴.

Our hypothesis is that classical immunosuppressive drugs, used to control the immune system, may negatively affect the bronchial epithelium, contributing to constrictive bronchiolitis. Zacharias et al, for example, demonstrated a cytotoxic effect of high doses of mycophenolic acid in human retinal cells. Neuringer et al, demonstrated that both acute and chronic cyclosporine treatment of tracheobronchial epithelial cell cultures inhibited growth at clinically relevant concentrations ^{6,8,9}.

Our current in vitro research approach is to use cell lines of structural lung cells resembling the airway, allowing the study of the direct effects of the immunosuppressive drugs on airway integrity, using a biculture of human bronchial epithelial cells (16HBE) and lung microvascular endothelial cells (HLMVEC).

MATERIAL AND METHODS

Cell culture

Human Bronchial Epithelial cells, 16HBE14o⁺ (16HBE) (provided by Dr. Gruenert, University of California, San Francisco, USA) and Human Lung microvascular endothelial cells (HLMVEC) were purchased from the European Collection of Cell Cultures (Salisbury, UK) and cultured as previously described ¹⁰. 16HBE cells were used for experiments between passages 3-15, HLMVEC cells between passages 3-10. N-values of 6 were used (2 different passages of cells, 3 replicates per passage).

Biculture setup

To mimic the barrier between blood stream and epithelium of the lung (ie, to mimic drug delivery of immunosuppressive drugs to the lungs via the blood), a biculture of 16HBE cells and HLMVEC cells was set up as described in Luyts et al.¹⁰ (Figure S1, SDC, <http://links.lww.com/TP/B446>). When the medium of both compartments was renewed at day 1, 4 and 7, the supernatant of both compartments was collected (200 µL). The biculture was allowed to grow for 4 days, to reach submaximal Trans Epithelial Electrical Resistance (TEER), before experiments were started¹⁰.

Immunosuppressive drugs

Immunosuppressive agents were dissolved in dimethyl sulfoxide (DMSO) for azathioprine, ethanol for dexamethasone, tacrolimus and cyclosporine or acetone for MMF. Agents or their appropriate vehicle control were added. In all experiments, DMSO, ethanol or acetone never exceeded 0.1% as a final concentration in culture media. Immunosuppressive drugs cyclosporine, MMF, azathioprine and dexamethasone were purchased from Sigma-Aldrich (Bornem, Belgium). Tacrolimus (FK506) was kindly provided by Astellas Pharma Belux (Anderlecht, Belgium). For C, T and MMF, serum trough levels and levels 10 times lower and 10 times higher were tested. Trough levels were defined based on clinically targeted patients' trough levels (Table S1, SDC, <http://links.lww.com/TP/B446>). As serum trough levels are not determined for A, a concentration of 0.5 µg/mL was used in our experiments, based on the product information of Imuran. For dexamethasone, levels used were in accordance with daily doses used in human LTx recipients. Monotherapies as well as triple drug regimens were tested. The cells in the biculture set up were exposed via the basolateral compartment (endothelial side) (Figure S1, SDC, <http://links.lww.com/TP/B446>).

Cytotoxicity assays

To mimic acute and chronic exposure, exposure for 24h or 4 days respectively to the immunosuppressive drugs was performed.

Upon reaching submaximal TEER, cells were exposed via the basolateral compartment to medium containing 0.2 % FCS supplemented with different concentrations of immunosuppressive drugs. After an incubation period of 24 hours or 4 days, the cytotoxic effect of immunosuppressive drugs on 16HBE cells was assessed, using a WST-1 assay as described in Luyts et al.¹⁰

To confirm cytotoxicity, a LDH assay (ThermoFisher Scientific, Merelbeke, Belgium) was performed. Therefore, 15 000 16HBE cells were seeded into a 96 well-plate and incubated overnight in 37°C in a 100% humidified atmosphere containing 5% CO₂. Cells were then exposed to MMF (0-0.25-2.5-25 µg/mL) for 24h or 4 days. As LDH released into the supernatant is measured, medium was not changed for the 4 days exposure, but fresh medium was added after 2 days. LDH assay was performed per manufacturer's instructions.

Trans-epithelial electrical resistance (TEER)

TEER was measured using the EVOM² voltohmmeter with the STX2 electrode as described in the instruction manual of the EVOM2 meter (World Precision Instruments, Inc., Berlin, Germany). Corrections for the surface area were made.

Permeability assay

A permeability assay was performed on bicultures exposed to MMF for 24h. After 24h exposure with MMF, FITC-dextran (4 kDa) (ThermoFischer Scientific) was added to the epithelial side at a final concentration of 1 mg/mL. Cells were incubated 4 hours in the dark.

Afterwards, medium from the basolateral compartment was transferred to a black 96 well-plate and the amount of FITC-dextran that passed through the biculture was measured with a fluorescence multi-well plate reader (FluoStar Omega, BMG Labtech, Ortenberg, Germany) (excitation and emission wavelengths: 485 nm and 530 nm, respectively).

Epithelial-to-mesenchymal transition (EMT)

A monoculture of 16HBE cells was exposed to MMF (2.5 µg/mL) when confluency was reached. After 24h or 4 days exposure, RNA extraction was performed. Cells were lysed with Trizol Reagent (Thermo Fischer Inc.) and RNA was extracted via Phenol-chloroform Extraction per manufacturer's instructions (Qiagen, Venlo, The Netherlands). Amount of RNA was measured via NanoDrop (Isogen Life Science, Utrecht, The Netherlands). A human EMT PCR Array (Qiagen), looking at the expression of 84 key genes that either change their expression during EMT or regulate those gene expression changes, was performed per the manufacturer's instructions (n=3). Analysis of the assay was performed using the $\Delta\Delta C_t$ -method. Corrections for multiple testing were performed using the 'false discovery rate' test in the statistical program R.

To confirm epithelial-to-mesenchymal transition, also measurements of E-cadherin (Assay biotech, Sunnyvale, USA) and vimentin (Abnova, Germany) levels in exposed and unexposed cells were done via sandwich ELISA on cell lysate after 24h or 4 days exposure to 2.5 µg/mL MMF according to manufacturer's instructions. Samples were diluted 1/50 for the E-cadherin ELISA. For the vimentin ELISA, samples were not diluted.

In vitro and in vivo measurements of immunosuppressives in BAL

The prodrug MMF was added to the cells, and the active drug mycophenolic acid (MPA) was measured. To test whether MPA could translocate to the epithelial cells, MPA in

the supernatant at both sides of the biculture was measured using high throughput HPLC-MS/MS. Similarly, MPA was measured using HPLC-MS/MS in BAL of stable lung transplant patients (10 patients taking MMF vs. 10 patients not taking MMF) and tacrolimus was measured with an immuno-assay method and LC-MS/MS method on the Waters TQD LC-MS/MS, performed in the Clinical Laboratory of the University Hospital Leuven (5 patients taking tacrolimus vs. 5 not taking tacrolimus). Briefly, BAL is performed with 2 aliquots of sterile saline (50 mL) of which the returned fractions were pooled and centrifuged

¹¹.

Statistical Analysis

Graph prism 4.0 software (San Diego, CA, USA) was used for statistical analysis. Results are presented as mean $\pm$ SEM and analyzed using the MANN-Whitney U-test. A $p < 0.05$ was considered statistically significant.

RESULTS

MMF impairs epithelial integrity

None of the tested immunosuppressive drugs (tacrolimus, cyclosporine, azathioprine, MMF or dexamethasone) were toxic for the epithelial cells in biculture at trough levels after an acute exposure of 24h (Figure 1A). After a chronic exposure of 4 days, only MMF was toxic for the cells (viability $< 80\%$) at trough levels and concentrations 10 times higher (Figure 1A).

To validate cytotoxicity, an LDH assay on 16HBE cells was performed after 24h or 4 days exposure to MMF. This test showed similar results as the WST-1 assay: no toxicity after 24h exposure to MMF, but after 4 days of exposure to MMF, LDH in the supernatant increased significantly starting from a concentration of 2.5 $\mu\text{g/mL}$, which is indicative of cytotoxicity (Figure 1B).

Although there was no toxic effect of MMF after 24h exposure, TEER was significantly lower after 24h compared to control cells (Figure 1A) and permeability was also higher (Figure 1C). TEER for the bicultures exposed to the other immunosuppressive drugs did not decrease after 24h (Figure 1A).

Also, combination therapies (dexamethasone-cyclosporine-azathioprine, dexamethasone-tacrolimus-MMF, dexamethasone-cyclosporine-MMF and dexamethasone-tacrolimus-azathioprine) were tested. Combination therapies including MMF showed the same results as MMF alone (ie, no toxicity after 24h exposure, but toxicity after chronic exposure of 4 days). Combination of dexamethasone-cyclosporine-MMF was borderline toxic at trough levels and higher, while the combination dexamethasone-tacrolimus-MMF was already toxic for epithelial cells at trough levels (Figure S2A and S2B, SDC, <http://links.lww.com/TP/B446>). Although there was no toxic effect after 24h exposure, TEER was significantly lower for cells exposed to combination therapies including MMF, compared to unexposed cells (Figure S2C and S2D, SDC, <http://links.lww.com/TP/B446>). Combination therapies without MMF were not toxic for cells after acute or chronic exposure and TEER did not decrease after 24h exposure (data not shown).

To investigate the effect of MMF on epithelial cells, we performed multiple experiments (see online supplement, SDC, <http://links.lww.com/TP/B446>). We demonstrated that MMF had no effect on apoptosis (Figure S3, SDC, <http://links.lww.com/TP/B446>), IL-8 or 8-isoprostane concentration (Figure S4 and S5, SDC, <http://links.lww.com/TP/B446>), nor on loss of tight junction proteins (ZO-1 and occludin) (Figure S6, SDC, <http://links.lww.com/TP/B446>) after 24h exposure, compared to control cells.

EMT

Without correction for multiple testing, human EMT PCR Array after 24h exposure showed significant (2 fold) upregulation of 9 genes, and a nonsignificant trend for 3 other genes, involved in EMT. Also, 1 gene normally down regulated during EMT, was significantly down regulated compared to unexposed cells (Table S2, SDC, <http://links.lww.com/TP/B446>). After correction for multiple testing, however, none of the genes remained statistically significant.

In contrast, the same assay performed after 4 days exposure showed significant upregulation of 11 genes (VIM, MAP1B, MMP3, SNAI2, CALD1, CAMK2N1, FOXC2, BMP7, BMP2, ZEB1, PLEK2) involved in EMT after correction for multiple testing. Also, 2 genes (CDH2 and COL5A2) showed a nonsignificant trend. One gene (FGFBP1) normally down regulated during EMT, was significantly down regulated compared to unexposed cells and there was a nonsignificant trend for 2 other genes (KRT14 and DSC2). Furthermore, there were also genes (DESI1, ITGB1) that were significantly upregulated or down-regulated of which we know they are involved in EMT, but it is at present not clear what their function in EMT is and if they should be down- or upregulated for EMT to be present. There were also 2 genes (TMEFF1 and RGS2) that acted in the opposite way as expected for EMT. RGS2 was upregulated in the EMT PCR assay, but should be down regulated in EMT and TMEFF1 was down regulated in the assay and should be up regulated during EMT (Figure 2 A and B, Table S2, SDC, <http://links.lww.com/TP/B446>).

Genes were mainly involved in differentiation and development, migration and motility, formation of the cytoskeleton and extracellular matrix and cell adhesion.

To validate our findings, we also performed an ELISA to measure the protein levels of E-cadherin and vimentin in cell lysate of the epithelial cells exposed to serum trough levels of MMF for 24h or 4 days. All samples were below detection limits for the vimentin ELISA (data not shown). For E-cadherin, after 24h exposure, there is no difference in E-cadherin levels of MMF exposed compared to unexposed cells, but after 4 days exposure, E-cadherin levels are significantly lower in MMF exposed compared to unexposed cells (Figure 2C).

Translocation of MMF

To test the translocation of the active drug, MPA, from the endothelial to the epithelial side, we measured MPA at both sides of the biculture. MPA was detected in the supernatant at epithelial side at an average concentration of $50.9 \text{ ng/mL} \pm 5.9 \text{ ng/mL}$ (Figure 3A), while MPA in the supernatant at the endothelial side had an average concentration of $122.8 \text{ ng/mL} \pm 8.2 \text{ ng/mL}$. (Figure 3B).

In LTx patients receiving MMF, MPA was detectable in BAL samples with an average of $3.9 \text{ ng/mL} \pm 1.3 \text{ ng/mL}$ (Figure 3C), which was significantly higher compared to patients not taking MMF ($p < 0.0001$). There was, however, no correlation between serum trough levels and concentration MPA measured in BAL. Also, tacrolimus was detected in the BAL samples of patients taking tacrolimus, although at a very low concentration ($0.21 \text{ ng/mL} \pm 0.02 \text{ ng/mL}$). Tacrolimus concentrations in BAL samples of LTx patients not taking tacrolimus were below detection limit.

DISCUSSION

To our knowledge, this is the first in vitro study investigating the direct effect of immunosuppressive agents and combination therapies on a biculture of human bronchial epithelial cells and lung microvascular endothelial cells.

MMF at trough levels, although not toxic for epithelial cells after an exposure of 24h, significantly impairs epithelial integrity, while 4 days exposure to trough levels of MMF or higher concentrations, severely decreased viability. None of the other tested immunosuppressive drugs showed toxicity after 24h or 4 days exposure. Therefore, we further focused on possible mechanisms of MMF-induced impairment of the bronchial epithelium, but could not demonstrate a role for apoptosis, tight junction proteins ZO-1 and occludin, oxidative stress or inflammation (see online supplement, SDC, <http://links.lww.com/TP/B446>). However, we found evidence that EMT might be (at least partially) responsible for decreased epithelial integrity, occurring possibly already after 24h but clearly visible after 4 days exposure.

Indeed, our results suggest that MMF evokes EMT in 16HBE epithelial cells, which allows epithelial cells to migrate through the underlying basement membrane and to accumulate in the interstitium of the tissue, where they ultimately dispose all their epithelial markers and gain a fully fibroblastic phenotype^{3,12}. On the one hand, already after 24h, different genes involved in EMT were differently expressed in MMF exposed cells compared to unexposed cells. However, after correction for multiple testing, no significance was reached for these genes. After 4 days, on the other hand, even after correction for multiple testing, 16 genes were differentially regulated, suggesting that MMF can evoke EMT. However, we acknowledge that there are also 2 genes (TMEFF1 and RGS2) differentially expressed in the opposite way, which would favor against EMT. However, given the large majority of differentially expressed genes supports the EMT hypothesis, together with the E-cadherin results, we suspect that MMF could evoke EMT.

It is already known that bronchial epithelial cells undergo EMT when stimulated with TGF- β 1⁵. Although, TGF- β 1 is seen as the main orchestrator of the remodeling process of the

airways after LTx, it has been described that air pollutants and immunosuppressive drugs can also induce EMT³.

Moreover, Felton et al, showed that rat bronchial epithelial cell line (RL-65) can undergo partial EMT due to cyclosporine or MMF, with loss of cell-cell adhesions and upregulation of fibronectin and vimentin expression⁶. There may be several reasons why we did not see EMT following cyclosporine exposure: first, cells in the study of Felton et al, were exposed apically, whereas our cells were exposed at the basolateral side of the biculture set-up. We suspect that not all the cyclosporine is able to cross the endothelial barrier and the membrane before exerting its effects. Moreover, Felton et al, used a rat cell line in their study, which shows a difference in drug absorption, movement of drugs from the interstitium to blood, plasma/serum protein composition, plasma protein binding which limits extrapolation from rat to human¹⁴. We did not look at EMT for cyclosporine as we did not see an effect on cytotoxicity or TEER when cells were exposed to cyclosporine. A study of Tomei et al, performed in human type II pneumocyte derived A549 and 2 bronchial cell lines also looked at EMT of these cells after exposure to tacrolimus or everolimus. Doses of tacrolimus were comparable with our study and did not induce EMT. They, however, observed dose-dependent effects of everolimus on these cells, also pointing out the importance of administering the right dosage and avoiding, wherever possible, drug over-exposure¹⁵. Since we only rarely use everolimus in our hospital and worldwide use is also limited, we did not investigate the effects of everolimus in our model. There is also another study by Copeland et al, performed on proximal tubular epithelial cells that demonstrated an inhibitory effect of MMF on EMT in vitro¹⁶. They concluded that MMF may result in less fibrosis and a better long-term survival. However, concentrations they used to expose the cells with were between 1 and 50 ng/mL, which is much lower than we used in our experiments (2.5 µg/mL). We choose to use a concentration like the serum through levels to accurately reflect the in vivo situation.

In other organs, similar deleterious effects of MMF were shown as Qasim et al, used Caco-2 (epithelial colorectal) cells to investigate the effects of MMF ¹⁷. They demonstrated that MMF concentrations used (although higher than in our study) did not cause significant apoptosis or cell death. However, TEER of Caco-2 cells exposed to MMF decreased and tight junctions disappeared together with appearance of increased paracellular openings between adjacent cells after 72 hours ¹⁷. With a concentration of MMF 40 times higher than the amount we used, Zacharias et al, showed an increase in caspase-3/7 activity in ARPE-19 (retinal pigment epithelium) cells ⁸.

The active form of MMF, MPA could be detected at both sides of the biculture, proving that MPA is present at the epithelial side after exposure of the endothelial side of the biculture to the prodrug MMF. Whether it is the prodrug MMF or its active compound MPA that is able to cross the endothelial/epithelial barrier cannot be concluded based on this study. Moreover, even in vivo, we could detect MPA in BAL fluid of LTx patients. The presence of MPA in the BAL fluid proves that MPA, and its metabolites, are present in the airway lumen and can exert deleterious effects on the airway epithelium. To our knowledge, this is the first time that MPA could be detected in BAL samples of LTx recipients. We also tested if other immunosuppressive agents could translocate to the epithelium and indeed we detected the presence of tacrolimus in BAL-fluid of LTx patients, although at a very low concentration (average of 0.208 µg/L, below detection limit). We acknowledge that concentration measured below detection limit is not accurate, but there was no peak visible at the retention time of tacrolimus for patients not taking tacrolimus (control) and there was a clear peak for patients taking tacrolimus, however with a low concentration. This provides proof that tacrolimus is present in the BAL fluid of patients. We suspect that the high dilution factor of BAL is the cause of the very low concentrations found and the fact that there is a lower trough level of tacrolimus compared to MMF (10 ng/mL compared to 2.5 µg/mL) in patients.

The amount of MPA and other agents found in BAL is much lower compared to what is found in serum, which is probably a dilution effect as the instilled fluid is mixed with the epithelial lining fluid. Determination of the percentage of epithelial lining fluid and hence the concentration of constituents in this epithelial lining fluid is therefore difficult.^{18,19} The amount of MPA found in the BAL samples therefore does not reflect the amount of MPA that can be found in the epithelial lining fluid. However, the fact that MPA can be found in BAL of these patients does prove that at least the active form of MMF, MPA can transmigrate into the airway lumen and encounters airway epithelial cells. If it is the prodrug MMF that transmigrates into the airway lumen or its active component MPA cannot be concluded based on our experiments.

One of the limitations of our study is that we used cell lines instead of primary cells. However, we specifically chose to use a cell line because we wanted to establish large scale experiments which are producible and physiological relevant. It has been described that the risk of infections and contamination for cell lines is much lower than for primary cells; variability is lower, reproducibility is higher and more standardized and controlled results can be achieved. Another limitation of our study is that we used serum trough levels of immunosuppressive drugs to expose our cells with. This is probably not the exact number of immunosuppressive drugs that reaches the epithelial cells, given that serum peak levels may be much higher. To our knowledge, the concentration of immunosuppressive drugs present in the epithelial lining fluid has not been studied yet. To take this into account, we also tested concentrations 10 times higher than these serum trough levels.

Although MMF was toxic on cell culture at trough levels and is detectable in BAL fluid, patient outcome, nevertheless, demonstrates no difference in incidence of acute rejection, chronic rejection or overall survival between MMF and azathioprine treatment²⁰.

Moreover, MMF can prevent acute and chronic rejection by suppression of the recipient's immune system. Maybe it is only in the very long run (survival > 5 years) that chronic MMF exposure towards the structural cells starts to play a role, which needs further investigation. However, our findings might open a discussion about the dose of MMF given to LTx patients and raises questions of current overdosing MMF to transplant patients, which could be prevented with therapeutic drug monitoring of MMF.

In conclusion, we found that MMF could decrease the epithelial integrity, possibly by EMT. These findings need further attention and consideration given the wide-spread use of MMF to transplant patients.

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FIGURE LEGENDS

Figure 1: A. Cytotoxic effect of immunosuppressive agents (cyclosporine, tacrolimus, MMF, azathioprine and dexamethasone) on a biculture of 16HBE cells and HLMVEC endothelial cells after 24 hours or 4 days. Trough levels and concentrations 10 times higher and 10 times lower were used. Cytotoxicity is defined as a viability below 80% compared to control. TEER measurement of the epithelial cells in the biculture exposed to trough levels of the immunosuppressive agents for 24 hours. The solid line represents the TEER of control cells and the dashed line represents the TEER of the cells exposed to the immunosuppressive agents. TEER measured right before exposure was set as reference (100%). N=6 (2 different passages of cells, 3 replicates per experiment). B. LDH assay for 16HBE cells exposed to MMF for 24h (left) and 4 days (right). n= 12 (2 different passages, 6 replicates per passage). C. Permeability assay on biculture after addition of MMF at the HLMVEC side for 24h. Flux of 4KDa FITC dextrans is shown, expressed as a percentage compared to control cells. ***: $p < 0.01$, Mann-Whitney U-test. N=6 (2 different passages of cells, 3 replicates per experiment).

Figure 2: A. Genes upregulated or downregulated in the real-time PCR EMT assay and their function after 4 days exposure to MMF. The grey colored genes are borderline significant. A p value < 0.05 is significant; Statistical test used is 'False Discovery Rate' to correct for multiple testing. B. Heat map of the genes that were significantly differently expressed in MMF exposed cells compared to control cells. Blue: genes that are upregulated. Red: genes that are down regulated. Black: genes involved in EMT, but not mentioned to be up- or down regulated in the assay. There is 1 gene, RGS2, that is up regulated and should be down regulated for EMT. There is also 1 gene, TMEFF1 that is upregulated in the assay and should be down-regulated for EMT. N=3. C. Validation of EMT via E-cadherin ELISA for cells

exposed to MMF for 24h or for 4 days. *: $p < 0,05$; Mann-Whitney U-test, $n=9$ (3 different passages of cells, 3 replicates per passage).

Figure 3: Concentration of the active drug mycophenolic acid (MPA) (ng/mL) present in supernatant of 16HBE side of the biculture (A) ($n=6$), the supernatant of HLMVEC side of the biculture after exposure to $2.5 \mu\text{g/mL}$ of the prodrug MMF (B) ($n=6$) and in BAL of patients (C) ($n=10$), measured via high throughput HPLC-MS/MS. *: $p < 0.05$, ***: $p < 0.0001$; Mann-Whitney U-test.

A

Upregulated during EMT	Function	Fold upregulation vs. control	p-value
VIM	Cytoskeleton	12.70	0.03
MAP1B	Cytoskeleton	3.35	0.008
MMP3	Extracellular matrix & cell adhesion	4.34	0.02
SNAI2	Transcription factor	3.82	0.003
CALD1	Migration & motility	2.52	0.05
CAMK2N1	Protein kinase inhibitor	2.48	0.03
FOXC2	Extracellular matrix & cell adhesion	3.53	0.05
BMP7	Cell growth & proliferation	5.03	0.04
BMP2	Cell growth & proliferation	23.41	0.008
ZEB1	Transcription factor	6.00	0.05
PLEK2	cytoskeleton	1.46	0.04
CDH2	Extracellular matrix & cell adhesion	3.77	0.09
COL5A2	Differentiation & Development	2.98	0.08

Downregulated during EMT	Function	Fold downregulation vs. control	p-value
FGFBP1	Cell growth & proliferation	7.06	0.03
KRT14	Differentiation & development	3.34	0.053
DSC2	Extracellular matrix & cell adhesion	1.75	0.09

B

C

Online supplement

MATERIALS AND METHODS

Apoptosis assays

Using the biculture, after 24h (acute) and 4 days (chronic) exposure to serum through levels of MMF, supernatant of epithelial cells was collected and centrifuged for 10 min to pellet detached cells. The cells still attached to the biculture, were rinsed twice with ice cold DPBS. Afterwards, 200 μ L cell extraction buffer (ThermoFisher Scientific) supplemented with 1 mM Phenylmethylsulfonyl fluoride and 1 tablet PhosSTOP (Roche) was added to the epithelial side of the biculture. After scraping cells, cell extraction buffer containing scraped cells was added to the pelleted detached cells and vortexed. Samples were stored in -80°C until the start of the ELISA for caspase 3 (ThermoFisher Scientific). The APO-1/FAS/CD95 ELISA (ThermoFisher Scientific) was performed on cell culture supernatant that was collected after 24h exposure. ELISA's were performed according to the manufacturer's instructions Using a 1/5 sample dilution for caspase 3 and 1/2 for APO-1/FAS/CD95.

IL-8 release and oxidative stress (8-isoprostane)

After exposure for 1, 4 and 7 days, IL-8 (CXCL8) and 8-isoprostane release in culture media of the apical (16HBE cells) and the basolateral (HLMVEC cells) compartment of the biculture were determined via sandwich ELISA (Invitrogen) and competitive ELISA (Cayman Chemical, Michigan, USA) respectively. ELISA was performed per manufacturer's instructions. Cell culture supernatant was diluted 1/5 and 100 μ L was used.

Tight junction proteins (ZO-1 and occludine)

Bicultures of 16HBE epithelial cells and HLMVEC endothelial cells were exposed to MMF for 24 hours or 72 hours (in this case, medium was changed once and new MMF was added) at serum concentration (2.5 $\mu\text{g}/\text{mL}$) at the endothelial side of the membrane. After incubation, immunofluorescent staining, as described by Luyts et al, on the biculture membrane was performed with the Alexa Fluor 488-labeled mouse antihuman ZO-1 antibody and the Alexa Fluor 594-labeled mouse antihuman Occludin antibody (ThermoFisher Scientific)⁹. Nuclei were counterstained with 4',6-diamino-2-phenylindole (DAPI) (ThermoFisher Scientific). Only epithelial cells were stained. A confocal microscope was used to visualize the stainings (Leica SP8x).

RESULTS

MMF does not induce apoptosis, oxidative stress or inflammation

Cells exposed to MMF for 24h or 4 days were tested for apoptosis via APO-1/FAS in supernatant of epithelial cells in biculture and caspase-3 on cell lysate of epithelial cells from the biculture. APO-1/FAS was below detection limit for all tested samples (data not shown). For the active caspase-3 assay, there was no difference between controls and MMF treated cells (Figure S3).

The results of the 8-isoprostane ELISA are shown in Figure S4. After 4 days of exposure to MMF, 8-isoprostane levels seem to be decreased compared to cells without exposure to MMF. However, we could clearly see under the microscope that cells exposed to MMF after 24h began to release from the culture flask. Medium of the cells exposed to MMF for 4 days was renewed after 2 days. Therefore, the released cells were washed out and less cells were still present on the insert. This explains the drop in 8-isoprostane compared to cells without MMF exposure. We could therefore not conclude that there is a difference in 8-isoprostane levels for MMF exposed cells compared to unexposed cells.

The results of the IL-8 measurements are shown in Figure S5. It can clearly be seen that there is no difference in IL 8 between exposed and nonexposed cells.

ZO-1 and occludine staining on biculture

The decrease in TEER of bicultures (and hence barrier function) exposed to MMF for 24 hours, could not be explained by a decrease in tight junction expression. However, after 24h, the tight junction proteins ZO-1 and occludine (Figure S6) were unchanged. There was no clear difference between control cells and cells exposed to MMF throughout the whole surface of the stained insert. For the staining of tight junction proteins ZO-1 and occludine 72 hours after exposure with MMF, clear differences between MMF-exposed cells and control cells were seen, as cells exposed to MMF had detached, consequently tight junctions had disappeared, and regions without cells were present (data not shown).

FIGURE LEGEND

Figure S1: *In vitro* set up of a biculture of human bronchial epithelial cells (16HBE; apical side) and human lung microvascular endothelial cells (HLMVEC; basolateral side). Immunosuppressive agents are added at the basolateral side.

Figure S2: A. Viability assay of combination therapy dexamethasone-tacrolimus-MMF on the biculture after 24h or 4 days exposure. B. Viability assay of the combination therapy dexamethasone-cyclosporine-MMF on biculture after 24h or 4 days exposure. C. TEER measurement of cells exposed to dexamethasone-tacrolimus-MMF at trough levels for 24h. D. TEER measurement of cells exposed to dexamethasone-cyclosporine-MMF at trough levels for 24h. Cytotoxicity is defined as a viability below 80% compared to control. TEER measured right before exposure was set as reference (100%). N=6 (2 different passages of cells, 3 replicates per experiment). *: $p < 0.05$; Mann-Whitney U-test. 0: control, 1: 1/10 serum trough levels, 2: serum trough levels, 3: 10x serum trough levels.

Figure S3: Active Caspase 3 apoptosis assay on 16HBE cells in a biculture after exposure of 24h to the serum trough level of MMF (2.5 $\mu\text{g/mL}$). N=6 (2 different passages of cells, 3 replicates per experiment).

Figure S4: 8-isoprostane ELISA on the supernatant of 16HBE cells in biculture with HLMVEC cells after 24h and 4 days. Cells exposed to MMF were exposed to serum trough levels of MMF (2.5 $\mu\text{g/mL}$). Exposure was on the HLMVEC side of the biculture.

Figure S5: IL-8 ELISA on the supernatant of 16HBE cells in biculture with HLMVEC cells after 24h and 4 days. Cells exposed to MMF were exposed to serum trough levels of MMF (2.5 $\mu\text{g/mL}$). Exposure was on the HLMVEC side of the biculture.

Figure S6: Staining of the 16HBE cells on the insert in biculture with HLMVEC cells for nonexposed cells (left) or after exposure to 2.5 $\mu\text{g/mL}$ MMF for 24h (right). The blue color is DAPI and the green color is ZO-1 protein.

FIGURES

Figure S1:

FIGURE S2:

FIGURE S3:

FIGURE S4:

FIGURE S5:

FIGURE S6:

TABLE LEGEND

Table S1: Different immunosuppressive agents and serum trough levels that were used in the experiments.

Table S2: : Results of the EMT real-time PCR array for all the tested genes after 24h or 4 days exposure to serum concentrations of MMF. Uncorrected p values are calculated using a Mann-Whitney U-test comparing cells without MMF exposure and cells with MMF exposure (2.5 µg/mL). Corrected p values were reached after correcting for multiple testing with a 'False discovery rate' test. A $p < 0.05$ was considered significant. Fold upregulation is >1 when the gene of the MMF exposed cells is upregulated compared to the same gene in unexposed cells. Fold upregulation <1 when the gene of the MMF exposed cells is down regulated compared to the same gene in unexposed cells. Green indicates genes that are upregulated during EMT, red indicates genes that are down regulated during EMT.

Table S1:

Immunosuppressive agent	Levels used in the experiments
Cyclosporine (C)	0.3 µg/mL
Tacrolimus (T)	0.01 µg/mL
Mycophenolate mofetil (MMF, M)	2,5 µg/mL
Azathioprine (A)	0.5 µg/mL
Dexamethasone (D)	2 µg/mL

Table S2:

Gene	24h exposure			4 days exposure		
	Uncorrected p	Corrected p after FDR	Fold upregulation	Uncorrected p	Corrected p after FDR	Fold upregulation
AHNAK	0.11	0.42	0,67	0.05*	0.14	0,44
AKT1	0.45	0.78	1,19	0.08 [#]	0.21	0,64
BMP1	0.63	0.98	1,15	0.11	0.26	0,56
BMP2	NA	NA	NA	0.0004*	0.008*	23,41
BMP7	NA	NA	NA	0.006*	0.04*	5,03
CALD1	0.09 [#]	0.42	2,20	0.009*	0.05*	2,52
CAMK2N1	0.06 [#]	0.33	1,70	0.002*	0.03*	2,48
CAV2	0.88	1	0,96	0.70	0.75	0,95
CDH1	0.21	0.53	1,45	0.60	0.68	1,15
CDH2	0.56	0.89	1,25	0.03*	0.09 [#]	3,77
COL1A2	NA	NA	NA	0.15	0.31	0,55
COL3A1	0.95	1	1,03	0.34	0.54	3,86
COL5A2	0.47	0.81	1,26	0.02*	0.08 [#]	2,98
CTNNB1	0.30	0.58	1,80	0.70	0.75	0,93
DSC2	0.28	0.58	1,32	0.03*	0.09 [#]	0,57
DSP	0.20	0.53	1,27	0.84	0.87	0,95
EGFR	0.88	1	0,97	0.73	0.77	0,92
ERBB3	0.97	1	1,01	0.06 [#]	0.17	0,51
ESR1	NA	NA	NA	0.42	0.57	2,14
F11R	0.73	1	1,07	0.03*	0.11	0,55
FGFBP1	0.01*	0.18	0,45	0.003*	0.03*	0,14
FN1	0.13	0.42	1,28	0.51	0.64	0,84
FOXC2	0.92	1	0,96	0.009*	0.05*	3,53
FZD7	0.03*	0.24	1,76	0.72	0.77	0,93
GNG11	0.26	0.56	0,80	0.96	0.97	0,99
GSC	NA	NA	NA	0.49	0.62	1,86
GSK3B	0.67	1	0,91	0.44	0.59	0,89
IGFBP4	0.31	0.58	1,25	0.41	0.57	1,29
IL1RN	0.77	1	1,10	0.59	0.68	1,08
ILK	0.91	1	0,98	0.05*	0.14	0,60
ITGA5	0.86	1	0,97	0.03*	0.09 [#]	0,52
ITGAV	0.004*	0.18	1,98	0.04*	0.14	0,63
ITGB1	0.85	1	0,93	<0.0001*	0.003*	0,66
JAG1	0.03*	0.24	1,46	0.56	0.67	1,15
KRT14	0.21	0.53	1,56	0.01*	0.054 [#]	0,30
KRT19	0.42	0.76	0,79	0.53	0.65	1,15
KRT7	0.71	1	1,10	0.65	0.73	1,11
MAP1B	0.04*	0.28	1,88	0.0004*	0.008*	3,35
MMP2	0.12	0.42	1,50	0.08 [#]	0.21	0,61
MMP3	0.83	1	0,94	0.001*	0.02*	4,34
MMP9	0.02*	0.20	1,83	0.46	0.60	1,12
MSN	0.36	0.67	0,75	0.37	0.56	0,08
MST1R	0.37	0.67	1,23	0.18	0.35	0,75

NODAL	0.12	0.42	1,61	0.40	0.57	0,63
NOTCH1	0.78	1	1,09	0.34	0.54	1,46
NUDT13	0.22	0.53	1,56	0.13	0.28	0,51
OCLN	0.92	1	1,03	0.29	0.48	1,17
PDGFRB	0.25	0.56	1,43	0.16	0.31	2,48
PLEK2	0.12	0.42	1,41	0.004*	0.04*	1,46
DES1	0.16	0.47	1,43	0.008*	0.05*	1,73
PTK2	0.64	0.98	0,91	0.86	0.88	0,97
PTP4A1	0.16	0.47	1,28	0.24	0.41	1,15
RAC1	0.30	0.58	1,24	0.18	0.35	1,17
RGS2	0.04*	0.26	4,82	0.003*	0.03*	6,44
SERPINE1	0.26	0.56	1,52	0.13	0.28	1,76
GEMIN2	0.02*	0.20	1,72	0.11	0.26	1,42
SMAD2	0.52	0.87	1,15	0.38	0.57	0,89
SNAI1	0.22	0.53	2,04	0.46	0.60	0,79
SNAI2	0.02*	0.23	2,24	<0.0001*	0.003*	3,82
SNAI3	0.90	1	1,03	0.69	0.75	1,09
SOX10	0.13	0.42	2,21	0.35	0.54	0,54
SPARC	0.25	0.56	1,30	0.02*	0.09	0,43
SPP1	0.83	1	1,08	0.97	0.97	1,02
STAT3	0.56	0.89	1,12	0.57	0.67	0,89
STEAP1	0.14	0.42	2,70	0.11	0.26	1,76
TCF3	0.12	0.42	1,20	0.19	0.35	1,33
TCF4	0.86	1	1,04	0.41	0.57	0,86
TFPI2	0.009*	0.18	3,80	0.01*	0.054 [#]	2,65
TGFB1	0.29	0.58	1,24	0.35	0.54	0,81
TGFB2	0.75	1	0,93	0.13	0.29	1,13
TGFB3	0.20	0.53	1,58	0.25	0.41	0,59
TIMP1	0.02*	0.20	1,44	0.64	0.73	1,04
TMEFF1	0.006*	0.18	0,44	0.004*	0.03*	0,63
TMEM132A	0.09 [#]	0.42	1,69	0.05*	0.14	0,66
TSPAN13	0.07 [#]	0.38	1,56	0.02*	0.08 [#]	1,25
TWIST1	0.54	0.89	1,17	0.52	0.65	0,89
VCAN	NA	NA	NA	0.18	0.34	2,23
VIM	0.009*	0.18	2,56	0.003*	0.03*	12,70
VPS13A	0.13	0.42	1,52	0.40	0.57	0,83
WNT11	NA	NA	NA	0.11	0.26	5,00
WNT5A	NA	NA	NA	0.20	0.35	2,62
WNT5B	0.09 [#]	0.42	0,78	0.19	0.35	0,71
ZEB1	NA	NA	NA	0.009*	0.05*	6,01
ZEB2	0.99	1	1,00	0.14	0.30	0,61