Pravastatin prolongs graft survival in an allogeneic rat model of orthotopic single lung transplantation

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Abstract

Objectives: MHC class II molecules play central roles in immune recognition and rejection. As statins have been shown to inhibit the production of these molecules, we analyzed the possible immunosuppressive effect of pravastatin, using our rat model of orthotopic lung transplantation.

Methods: Single orthotopic lung transplantation was performed in a Fischer 344-to-Wistar Kyoto strain combination. One group received pravastatin i.p. after transplantation, controls NaCl. Statin serum levels were analyzed by high performance liquid chromatography (HPLC). Animals were sacrificed on postoperative day (POD) 14 and 21. At sacrifice, samples were obtained for histology, immunohistochemistry, flow cytometry and real-time RT-PCR analysis of CD25, TNF-α, and MHC class II expression. Rejection was graded via histology, using a system based on the working formulation of The International Society of Heart and Lung Transplantation. Immunohistochemistry was performed for expression of MHC class II, T-cell receptor, CD25, CD4/8 cell, NK cells, granulocytes and monocytes in naïve lungs and grafts from donor and recipient animals. Flow cytometric analysis of recipient peripheral blood mononuclear cells (PBMC) was used to analyze expression of CD3, CD4, CD8, and RT1B in both groups. In vitro analyses of MHC class II expression were performed in parallel. Results: HPLC confirmed effective delivery of pravastatin. Recipients treated with pravastatin showed significantly less rejection on POD 14 and on POD 21, when compared to controls. Immunohistochemistry showed specific differences, suggesting a delay in rejection in the pravastatin group. Flow cytometric analyses showed a higher expression of CD4 in the control group on POD 21. Results of real-time RT-PCR analyses for MHC class II expression showed a significant decrease in expression in the statin-treated group. Flow cytometric analysis of γ-IFN stimulated rat PBMC showed an inhibition of upregulation of MHC class II expression by pravastatin in vitro. Conclusions: Pravastatin prolongs graft survival in our allogeneic rat model of orthotopic lung transplantation. We assume that the underlying mechanism for this effect is the inhibition of upregulation of MHC class II molecule synthesis, thus blocking downstream effector mechanisms of the immune system.

1. Introduction

Lung transplantation (LTx) has evolved from an experimental endeavor into a standard treatment for patients suffering from end-stage lung diseases [1]. Recipients enjoy improved quality of life and prolonged survival. Accordingly, waiting lists have increased dramatically and the number of patients waiting has outgrown the number of potential donors by far. Over the years, survival has continually increased, reflecting achievements in lung preservation, surgical technique, and immunosuppression. However, when compared to other solid organ transplants, long-term results after LTx remain unsatisfactory with a 5-year survival rate of 45% compared to 72% in liver recipients, and a 10-year survival rate of only 23% [2]. Thus, two important barriers remain today. One is the poor long-term survival rate as a result of chronic allograft rejection and rejection, the second is the shortage of suitable donor supply [3].

Statins, 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors, are used in clinical practice as lipid-lowering agents. As such, they have been used in patients after heart or other solid organ transplantation to lower increased serum lipid levels. Since increased serum lipid levels are known to play a role in the induction of coronary artery disease, it came as no surprise that heart transplant recipients showed increased survival after statin administration. Surprisingly,
other patients, e.g., after liver or kidney transplantation [4], also showed increased organ survival. This cannot necessarily be explained by an inhibition of vascular disease, as this is not the main cause of chronic rejection in these patients. Since a variety of effects of statins other than simple decrease in serum lipid levels have been described [5,6], the increase in graft survival may be caused by such a lipid level lowering independent property of statins. In this context, it has been demonstrated that statins inhibit the production of MHC class II molecules on a transcriptional level [7—9], leading to a significant decrease or inhibition of de novo synthesis of MHC class II molecules. This leads to a decrease in presentation of internalized peptides by antigen presenting cells and thus indirectly suppresses T-cell activation. The mechanism of this inhibition is a result of reduced activation of the inducible promoter IV on the transactivator CIITA [10,11] and has been observed in a variety of cells [10]. Additionally, statins cause a reduction in Fc-γ-R-mediated immune-complex trafficking, degradation, and internalization. The above effects may lead not only to a reduction of the cell surface density of MHC class II molecules but also to a decrease in the amount of donor-derived peptides being presented [12]. Since MHC class II molecules are central to allo-recognition and rejection as they are required for antigen presentation and T-cell activation through the T-cell receptor, application of statins may cause a decreased T-cell activation in vivo.

In order to study the possible effects on graft survival after lung transplantation, and to analyze, whether the inhibitory effect on the upregulation of MHC class II molecules is responsible, we employed our model of orthotopic LTx in the rat. We hypothesized, that the effect of pravastatin would be observed best in a strain combination with little or no MHC class I and II differences but different backgrounds. Rejection in such a combination depends significantly on presentation of donor-derived peptides in the context of MHC class II molecules, meaning, rejection should be directly dependent on (a) the successful synthesis of MHC class II molecules and (b) the incorporation of donor peptides into the synthesized molecule. Accordingly, Fisher 344 (F344, RT1\(^{10}\)) and Wistar Kyoto (WKY, RT1\(^{11}\)) rats were chosen as donors and recipients, a combination which is MHC class Ia and II identical but differs in background.

2. Material and methods

All animals received human care in compliance with the Principles of Laboratory Animal Care formulated by the German Society for Medical Research after approval by the State Animal Studies Committee.

2.1. Groups and treatment

F344 (RT1\(^{10}\)) rats served as donors, WKY (RT1\(^{11}\)) rats as recipients. A total of 48 male rats underwent allogeneic (F344-to-WKY, \(n = 33\), one recipient died perioperatively) or syngeneic (WKY-to-WKY, \(n = 15\)) orthotopic left LTx. Animals were obtained from Charles River Germany (Sulzfeld, Germany) and guaranteed specific pathogen free. Animals were housed in groups of four and fed ad libitum. Preoperative preparation included assessment of health status, and 1—2 weeks of aclimatization to overcome the stress of transportation. Mean weight of the donor rats was 220 ± 10 g, recipients weighed 250 ± 10 g at time of Tx. After LTx, allogeneic recipients were randomly assigned to the control (\(n = 16\)) and treatment groups (\(n = 16\)).

No baseline immunosuppression was administered in either group. The treatment group received 15 mg/kg per day pravastatin i.p. (a generous gift from Bristol Meyer Squibs) from postoperative day (POD) until sacrifice. Controls received the same volume 0.9% NaCl i.p. Randomly selected animals were sacrificed on POD 14 and 21.

2.2. Lung transplantation

Organ harvests and transplantations were carried out under clean, but not sterile conditions. For anesthesia all animals were premedicated by ether inhalation, followed by intramuscular injection of atropine sulfate (0.2 mg/kg) and intraperitoneal injection of pentobarbital (60 mg/kg). After induction, animals underwent orotracheal intubation and were mechanically ventilated with 100% oxygen (respiratory rate 50 breaths/min, tidal volume 2.5 cm\(^3\)) using a Harvard rodent ventilator model 683 (Harvard Apparatus, Hugo Sachs). Intraoperative anesthesia was performed via isoflurane. Donor animals were placed in a supine position. After a median sternotomy and intravenous application of 1500 IU of heparine, the pulmonary artery was cannulated via the right ventricle. The atrial appendices were incised and the lungs were flushed by gravity (30 cm) with 20 cm\(^3\) of low molecular dextran solution (Perfadex®, Vitro Life, Göteborg, Sweden) at 8 °C, excised and immediately immersed in cold saline at 4 °C for storage. Implantations were performed using an operating microscope (magnification 16×). Recipient animals were placed in a right lateral position and a left sided thoracotomy was performed in the fourth intercostal space. After hilar dissection and left-sided pneumectomy, implantation began with the anastomoses of the pulmonary artery and veins (continuous suture, polypropylene 10-0). After removal of the vascular clamp, the donor and recipient bronchi were anastomosed (polypropylene 8-0 continuous suture). After careful inflation of the graft, the ventilation was adjusted and the airway anastomosis was examined for leakage. Following implantation, the thorax was closed. During closure, a chest tube was inserted, which was removed as soon as the spontaneous ventilation was to be sufficient. At this time, the recipient animal was extubated. Ischemia time was less than 1 h, no immunosuppression was applied perioperatively. Postoperatively, each animal was observed for the next several hours. Recipient animals were housed separately to exclude infection. Health status and weight was checked daily.

2.3. High performance liquid chromatography (HPLC)

Serum levels of pravastatin after i.p. administration were analyzed by isocratic high performance liquid chromatography with photometric detection (HPLC-UVIS) according to a protocol modified from Siekmeier et al. [13]. Briefly, WKY rats (\(n = 9\)) weighing 310—330 g were used as sample animals. Animals received 15 mg/kg pravastatin i.p., sequential blood samples were drawn at 5, 15, 30, 60, 90, and 120 min after
injection. Plasma samples were prepared by centrifugation, aspirated an immediately frozen at $-20^\circ$C. Individual sample size was 500 $\mu$L/bleed. Due to the limited amount of collectable rat blood, the original method was adapted to 0.5 ml serum instead of 5 ml. All chemicals were of best available analytical grade from Merck (Darmstadt, Germany), Sigma-Aldrich (Deisenhofen, Germany) or Fluka (Buchs, Switzerland). Briefly, 0.5 ml serum was added to 0.5 ml ultrapure water and 100 $\mu$l aqueous internal standard (triamcinolone acetonide, in-assay concentration: 1.25 mg/l). Samples were mixed and slowly passed through cyclohexyl solid phase extraction columns (J.T. Baker, Deventer, The Netherlands) fitted to an SPS 24 system (Varian, Darmstadt, Germany) and preconditioned with methanol, acetonitrile, ultrapure water, and 0.05 M potassium hydroxide buffer (pH 7) (5 ml of each solvent). The columns were washed with 5 ml ultrapure water, 5 ml 0.05 M potassium hydroxide buffer (pH 7) and 1 ml washing solution (acetic acid (0.1 M, pH 3.5)/acetonitrile/methanol (62:30:8, v/v/v)) and then dried by centrifugation for 10 min at 3400 $\times$ g. Analytical batches were eluted with 3 ml acetone and evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 200 $\mu$l Tris-buffered saline solution (0.1 M, pH 7.4) and right (naïve) lungs filled with O.C.T. (Tissue Tek, Sakura, Zoeterwoude, The Netherlands), snap frozen in liquid nitrogen immediately and stored at $-70^\circ$C until further use.

Histological analysis (hematoxylin–eosin) was performed using 4 $\mu$m cryostat sections. Graft rejection was graded according to a revised version of the working formulation of the International Society of Heart and Lung Transplantation [14]. Acute rejection was graded in A0–A5 according to the presence and the extent of perivascular and interstitial mononuclear cell infiltrates, with A5 representing a fully rejected graft, showing complete tissue fibrosis. In addition, the presence of accompanying lymphocytic bronchiolitis and bronchitis was scored from B0–B4 according to the extent and intensity of the airway inflammation. To avoid nonspecific changes mimicking rejection, the presence of infection was excluded via histological observation (localization of an infiltration process, exclusion of granulation tissue in the air spaces, interstitial pneumonia, normal right lung tissue) and daily clinical observation. Histological scoring was performed by a single investigator in a blinded fashion. Results were double checked by cross-referencing with two additional blinded investigators, who graded a random sample batch in parallel.

### 2.5. Immunohistochemistry

In order to differentiate cellular infiltration and observe intra-graft expression of cell surface molecules, standard one and two color immunohistochemistry was performed on cryostat sections. Briefly, 8 $\mu$m cryostat sections were fixed in acetone at $-20^\circ$C for 10 min. Following a wash cycle (3 x 2 min in Tris-buffered saline solution), they were incubated with the primary mAb for 30 min at room temperature. After a wash cycle in TBS-Tween, the sections were counterstained with hematoxylin. Control sections were included in which one or both primary antibodies were omitted.

The following mouse anti-rat monoclonal antibodies (mAb) were used: Primary: W3/25 (anti-CD4), 8/8 (anti-CD8), and D6.6 (anti-IgG2a, mouse; in TBS-Tween) was added the sections incubated for 30 min followed by addition of the substrate Fast Blue (Sigma, Deisenhofen, Germany). For the second color, the incubation with the primary mAb was performed for 45 min followed by an identical procedure with Fast Red (Sigma) as the substrate. Thus, derived sections were counterstained with hematoxylin. Control sections were included in which one or both primary antibodies were omitted.

Semiquantitative grading of immunohistochemistry analyses was performed on a scale of 0–5 (0 = no difference and 5 = massive cellular infiltration), compared to nontransplanted control animals as described previously [15]. Scoring was performed as discussed above.

**Grading:**

- 0 = no difference
- 1 = little more infiltrating cells
- 2 = difference clearly visible
- 3 = difference immediately visible in every microscopic field
- 4 = striking difference, dense infiltration
- 5 = massive cellular infiltration

### 2.6. Flow cytometric (FCM) analysis

Primary antibodies used were as follows: CD45RA (ox-33, mouse anti-rat, IgG1K, purified), CD3 (G.14, mouse anti-rat, IgG3K), CD8a (ox-8, mouse anti-rat, IgG1K), CD4 (ox-35, mouse anti-rat, IgG2aK), CD25 (ox-39, mouse anti-rat, IgG1K), and RT1B (ox-61, mouse anti-rat, IgG1K).

Cells were analyzed using one- and two-color FCM analyses on a FACScan (Becton Dickinson). Each analysis included isotype-matched negative and positive control mAb. Dead cells were excluded from analyses on the basis of propidium iodine retention and forward scatter.

Peripheral blood mononuclear cells (PBMC) were drawn by retroorbital bleeding and prepared using a water lysis protocol. Collected samples were frozen in 90% FCS,
supplemented with 10% DMSO at –70 °C until further use. For analyses, samples were thawed by gentle agitation in warm water, washed and counted. Viability of thawed samples was above 85 ± 5%. Thus, prepared cells were aliquoted at 1 x 10⁶ cells/tube and stained using a standard protocol. A minimum of 10,000 viable cells were analyzed per sample. Obtained data were analyzed using CellQuest (Becton Dickinson, USA) and Winlist software (Verity Software House Inc., ME, USA).

2.7. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed on an iCycler real-time PCR system (BIO-RAD, Munich, Germany). Total RNA was prepared using the Rneasy Midi Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. 100 ± 50 ng total RNA was applied for reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer’s instructions. PCR was performed using HotStarTaq (Qiagen) and 100 ± 50 ng cDNA. For quantification, 0.25 μl 1:200 SYBR Green I (10,000 x supplied concentration in DMSO, Molecular Probes/Invitrogen GmbH, Karlsruhe, Germany) was added. PCR reactions not resulting in a single product as indicated by the melting curve were not included in the study. The rat housekeeping genes β-actin and 18S were used to normalize different cDNA samples amounts. cDNA reactions without reverse transcriptase were performed in parallel to exclude contaminations with genomic DNA. Further negative controls were performed without template. Animals were not pooled, analyses of each sample was performed at least in duplicate.

The MHC class II primer specific PCR was cycled at 95 °C, 15 min, followed by 35 cycles 94 °C, 15 s, 59 °C, 25 s and 72 °C, 20 s, and 90 cycles for melting curve determination, beginning with 50 °C for 10 s and followed by increasing setpoint temperatures by 0.5 °C at each cycle, and a final holding temperature at 4 °C, until optional analysis by agarose gel electrophoresis. The TNF-α primer specific PCR was cycled at 95 °C, 15 min, followed by 35 cycles 94 °C, 15 s, 61 °C, 25 s and 72 °C, 20 s, and 90 cycles for melting curve determination, beginning with 50 °C for 10 s followed by increasing setpoint temperatures by 0.5 °C every cycle, and a final holding temperature of 4 °C, until optional analysis by agarose gel electrophoresis. IL-2 receptor specific PCR was performed using the protocol for the TNF-α specific primer with 45 cycles at 94 °C and a different annealing temperature of 60 °C.

Primers were designed using NCBI Genebank for mRNA sequence information, Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3_www.cgi#disclaimer) primer design and verified using the Basic Local Alignment Search Tool (NCBI). Specificity was verified via normal RT-PCR.

The following primers are used for real-time PCR:

Rat RT1.B(l) Prime: AGCGGGGCTCCCTACAGTGAC, Tm 61.4 °C and TCTGACAGAGAAATGCTG, Tm 57.3 °C, PCR product size 243 bp;
Rat CD25 Prime: CATTCCAGTTCTTCTCCTCG, Tm 57.3 °C and CCTGCAAGTTCTCCTG, Tm 59.4 °C, product size 385 bp;

The relative expression level of each gene in each sample (e.g., one specific gene) was calculated as follows: relative expression = SQspec. gene/SQhousekeeping gene. To allow for inter animal comparison, data are normalized to the housekeeping gene (delta-delta Ct). The relative gene expression level of the graft is then compared to the gene expression level of the remaining naïve lung of the recipient animal.

Data is presented as percentage of expression in the naïve lung in order to exclusive the influence/bias of potential infections or unspecific immune reactions. Determination of the gene expression levels of the remaining naïve right lung before and after allotransplantation of a left lung allowed for detection of potential systemic consequences of allogeneic immunoreactions.

2.8. In vitro stimulation assay

For in vitro stimulation assays PBMC were isolated from freshly prepared WKY rat blood, following lysis and subsequent adherence to plastic culture flasks (90 min, 37 °C). Nonadherent cells were cultured in RPMI-1640 medium (PAA) supplemented with 10% FCS (FCS; Gibco, Basel, Switzerland) in 24-well plates at 1 x 10⁷ cells/well. Cultures were stimulated for 24, 36, 48, or 72 h with IFN-γ (100 ng/ml, rRat IFN-γ, Peprotechec Ltd., UK). Randomly chosen wells were blocked with pravastatin (20 μM). After stimulation, cells were aspirated gently, washed and analyzed for surface expression of MHC class II via single color FCM analysis.

2.9. Statistical analysis

Numeric data are expressed as mean values ± standard deviation. Student’s t-test was used to calculate the differences; p-values ≤0.05 were considered statistically significant.

3. Results

During the course of the experiment, all animals remained healthy.

3.1. Pravastatin serum levels

The HPLC protocol developed allowed for detection of a minimum concentration of 20 ng/ml pravastatin (Fig. 1). Serum pravastatin levels of the time dependent sequential analyses show a maximum concentration of 132 μg/l of pravastatin was achieved 15 min after injection (Fig. 1).
3.2. Suppression of upregulation of expression of MHC class II molecules

Using naïve PBMC from recipient strain (WKY) rats, we demonstrate inhibition of IFN-γ induced upregulation of cell surface expression of MHC class II molecules by pravastatin in vitro (Fig. 2).

Analyses included PBMC as a recipient specific analysis. Fig. 3 depicts an exemplatory FCM analysis on PBMC prepared from recipient animals on POD 21. Untreated animals show a higher level in MHC class II molecule high expressing cells. Immunohistochemistry of the grafts on POD 14 and 21 shows a significant upregulation of MHC class II surface expression in both groups (POD 14, n = 8, p < 0.001; POD 21, n = 8, p < 0.001), when compared to nontransplanted controls (not shown). However, comparison between the two transplanted groups shows a significant difference in MHC class II expression with the level of expression being higher on POD 14 and lower on POD 21 in pravastatin treated animals (POD 14: n = 8, p = 0.003; POD 21, n = 8, p < 0.001) (Fig. 4).

The intra-graft expression showed a distinct pattern when compared to expression in remaining naïve lung. Naïve lungs from transplanted animals (both groups) showed no significant difference in expression when compared to nontransplanted controls (not shown). To determine whether the observed inhibitory effect was due to a block of MHC class II

Fig. 1. HPLC analysis: (upper panel) sample chromatograms showing the elution profile of a typical rat plasma sample; (lower panel) plasma concentrations of pravastatin after i.p. administration 15 mg/kg.

Fig. 2. FCM analysis: depicted is an exemplary result of the in vitro analysis. The results show a suppression of upregulation of cell surface MHC class II molecule expression in IFN-γ activated PBMC.

Fig. 3. FCM analysis: depicted is an exemplatory FCM analysis of MHC class II cell surface expression on recipient PBMC. Statin-treated animals show a lower percentage of MHC class II expressing cells.

Fig. 4. Immunohistochemical analyses: analysis of expression of MHC class II molecules in allografts showing distinct differences at POD 14 and 21.

Fig. 5. Real-time RT-PCR: depicted are the results of the real-time RT-PCR analyses. At both time points, the level of gene expression is significantly lower in the pravastatin-treated animals, showing blocking of expression on a molecular level.
mRNA transcription, we performed quantitative real-time RT-PCR analyses on the grafts and naïve lungs in all animals demonstrating that the inhibition did indeed occur on the transcriptional level. While expression of MHC class II is elevated in both groups when compared to the right sided naïve lung, controls shows a 9.3-fold increase of relative expression on day 14 versus a 2.6-fold increase in the statin-treated group. On day 21, the ratio was 4.94 (control) versus 2.5 (statin) times above normal (Fig. 5). Expression in right-sided naïve lungs is not elevated above nontransplanted controls.

3.3. Pravastatin reduces the level of acute rejection and lymphocytic bronchitis after LTx

Fig. 6 depicts the results of the histochemistry demonstrating a significantly lower grade of acute rejection in grafts in pravastatin-treated animals on POD 14 with a mean of 3.38 in the statin group versus 4.69 in the control group (n = 8, p < 0.001). On POD 21, the statin group showed a mean of 3.31 versus 4.42 (control) (n = 8, p = 0.001). Grading for lymphocytic bronchitis showed significant differences, also, with a grade of 3.06 (statin group) versus 4.06 (control) on POD 14 (n = 8, p < 0.001) and 2.94 versus 3.93 (control) (n = 8, p < 0.001) on POD 21.

3.4. Graft infiltrating cells

Immunohistochemical analysis shows no difference in cell surface expression of CD4 in grafts on POD 14 (n = 8, n.s.), while on POD 21, a significant decrease in expression of CD4 in grafts of pravastatin-treated animals can be observed (n = 8, 1.85 versus 2.68, p = 0.016). The level of expression of CD8 did not differ at either time points (n = 8/8, n.s./n.s.). On day 21, the CD4+ cells in the pravastatin-treated group show a pattern of perivascular and peribronchial infiltration, while grafts from control animals show a ubiquitous infiltration (Fig. 7a). While no difference of TCR expression on POD 14 could be observed (not shown), on POD 21, grafts from statin-treated animals show a lower level of TCR expression when compared to controls (p = 0.011) (Fig. 7b).

No difference was observed in the level of granulocytic infiltration (not shown).

Analyses of monocytic infiltration show no difference on POD 14 but a significantly lower infiltration in the pravastatin group on POD 21 (p < 0.05) (Fig. 7c). No difference in of NK-cell infiltration was observed (Fig. 7c).

3.5. Circulating cells

One- and two-color FACScan analyses of recipient PBMC demonstrated a significantly higher number of CD3+/4+ cells in control animals on POD 21 while no differences could be observed in CD CD3+/8+ and CD4+/8+ cells as well as CD45RA (not shown).

3.6. Expression of IL-2 receptor

Immunohistochemistry revealed an increase of IL-2 receptor expression in grafts on POD 14, which failed to reach significance (Fig. 7b). Thus, no significant differences in the level of expression of CD25 were observed in grafts (not

![Pravastatin reduces the level of acute rejection and lymphocytic bronchitis after LTx](image)

**Fig. 6.** Histochemistry (upper panel): depicted are typical slides of naïve (left) control (middle) and statin-treated (right) animals. While the control animal has almost completely rejected, the alveolar structures are clearly visible in the statin animal. There is marked lymphocytic infiltration in the statin animal too, albeit rejection is at a lower level. Semiquantitative analysis of histochemistry (lower panel): depicted are the results of the histochemical analyses, according to the score, based on the ISHLT criteria (A1–5, B1–4). At both time points, statin-treated animals show significantly less lymphocytic rejection as well as peribronchial infiltration.
However, CD25 mRNA expression was significantly elevated in both groups at both time points (Fig. 8). In contrast to the immunohistochemistry, CD25mRNA expression on POD 14 in the pravastatin treatment group shows an eightfold upregulation when compared to naïve/right lung controls, while control animals show a 4.8-fold increase ($n=7$, $p<0.05$) (Fig. 8). On POD 21, the overall level of expression is lower in both groups, with the control group showing a 4.4-fold increase of expression and the statin group showing a 2.7-fold increase ($n=7$, $p<0.05$) over naïve controls (Fig. 8). In contrast to intra-graft data (mRNA and immunohistochemistry), FCM analysis of PBMC on POD 21 showed a higher level of CD25 expression in statin-treated animals in the peripheral blood (not shown).

3.7. Transcription of TNF-α

Analysis by real-time RT-PCR demonstrates a significantly higher level of transcription in grafts from control groups on POD 14 ($n=7$, $p>0.05$), on POD 21 no differences were observed (Fig. 8).

4. Discussion

Lung transplantation has become a standard treatment for patients suffering from end-stage pulmonary failure.
Accordingly, waiting lists have increased dramatically and the number of patients waiting has outgrown the number of donors by far. In spite of significant progress in surgical technique (e.g., minimal invasive technique), therapy of rejection, and management of pulmonary infections, postoperative survival times after LTx are still significantly shorter when compared to those after transplantation of other parenchymal organs. This suggests that alternative treatment approaches to current immunosuppressive regimes may be necessary in order to further increase survival times.

Hypercholesterolaemia is an adverse effect of immunosuppressive therapy and hence, a common finding after transplantation [16]. Therefore, statins were commonly prescribed to recipients who developed hypercholesterolaemia. However, statin administration for patients after LTx may show pleiotropic beneficial effects. At present, the evidence of benefit from statin prescription is mainly confined to heart transplant recipients but it is likely that recipients of lung transplants would also benefit and there is data showing increased organ survival as a side effect of the statin therapy in patients after liver or kidney transplantation [4], also. One may explain the increase in organ survival after heart, but not after kidney transplantation, by an inhibition of vascular disease caused by elevated serum lipids, as this is not the cause of chronic rejection and graft failure in these patients. Thus, lipid lowering independent side effects of the statins have been proposed to cause graft protection and a number of effects of statins have been described [5,6]. In this context, it has been demonstrated that statins inhibit the upregulation of cell surface density of MHC class II molecules by blocking transcription of mRNA [8,9]. This transcriptional block prevents effective processing of internalized molecules in antigen presenting cells, thereby indirectly suppressing T-cell activation.

Accordingly, we hypothesized that statins should increase organ survival after LTx by inhibiting the upregulation of MHC class II molecules. In order to analyze this question, we employed our model of left-sided, orthotopic LTx in the rat. Theoretically, the effect of pravastatin should be observed best in a strain combination with little or no MHC class I and II differences but different breeding backgrounds, since rejection in this combination would depend on the presentation of donor-derived peptides in the context of donor or recipient MHC class II by antigen presenting cells. The strain combination Fisher 344 (F344) and Wistar Kyoto (WKY) provides such a setting, being MHC class Ia and II syngeneic but of different non-MHC background.

Of the many HMG-CoA reductase inhibitors, we decided to use pravastatin because others (e.g., lovastatin, simvastatin, atorvastatin) are substrates of the cytochrome P450-3A4 enzyme and their metabolism would be significantly inhibited by a variety of drugs, routinely used after solid organ transplantation (e.g., azole antifungal agents, macrolide antibiotics and cyclosporine A). Pravastatin is not significantly metabolized by cytochrome P450 enzymes and therefore is largely unaffected [17,18]. In addition, it has been demonstrated that the risk of rhabdomyolitic myopathy is significantly elevated, if lipophilic simvastatin is administered, compared to hydrophilic pravastatin [19].

We first adapted an analytical HPLC protocol of measurement of pravastatin concentrations, which allowed for sensitive detection of statin concentrations and demonstrated effective delivery via i.p. injection. Next, we conducted in vitro stimulation experiments, to demonstrate that pravastatin inhibits the upregulation of MHC class II molecules in recipient strain PBMC. Analyses of PBMC obtained from statin-treated recipients animals corroborated these results.

Based on the above, our model should allow us to analyze, whether the proposed immunosuppressive effect of pravastatin has a graft-protective effect after LTx. As expected, control animals rejected their grafts. Statin-treated animals developed rejection also, but there was a significant difference in the appearance of grafts at time of necroscopy already. Grafts from pravastatin-treated animals obtained at POD 14 or 21, generally, had a better overall appearance. Histochemical analyses confirmed a significant reduction in cellular rejection and accompanying bronchiolitis in grafts from treated animals, when compared to controls both on POD 14 and 21. In order to analyze, whether pravastatin reduced the expression of MHC class II molecules, we performed immunohistochemical stainings showing that control animals expressed significantly higher levels of cell surface MHC class II molecules than statin treated recipients on POD 21. On POD 14, control animals showed less expression of MHC class II, which may be explained by different dynamics in cellular surface expression and production of new class II molecules. Quantitative real-time RT-PCR analyses, performed in parallel, demonstrated that there was indeed an inhibition of MHC class II expression on a transcriptional level. In this context, it is of interest to note that the upregulation of MHC class II was confined to infiltrating as well as autochthonous cells of the grafts. In naïve lungs, no significant difference to nontransplanted controls could be observed.

Analyses of infiltrating cells showed no differences in CD3+ cell infiltration on POD 14. On POD 21, the infiltration by CD3+ cells and monocytes was significantly reduced. Granulocytic infiltration was unaffected. Differentiation of lymphocytic cells revealed no difference in infiltration of cytotoxic CD8+ T cells at either time point. However, the infiltration of CD4+ T-helper cells was significantly reduced in the statin treated group on POD 21, showing a different pattern of infiltration, also. Since CD4+ T cells play important roles in MHC class II driven indirect recognition processes, this reduction corroborates the hypothesis, that the suppression of cell surface upregulation of MHC class II is the cause of the observed effect.

As it has been shown that statins cause a reduction in FcyR-mediated immune complex processing, these data are important as internalized immune-complexes are degraded and presented in the context of MHC class II. The lower level of CD4+ T cell and monocyte infiltration and the decreased MHC class II molecule synthesis and expression within statin-treated grafts suggest that specific cellular immune responses, as well as nonspecific inflammatory responses may be inhibited by statins. The finding that control animals show a significant elevation in circulating CD4+ T cells corroborates these data.

After the initiation of allogeneic immune responses, T-cell mediated rejection is significantly driven by cytokines, such as IL-2. T cells become sensitive to IL-2 by a transient elevation in IL-2 receptor expression. This elevation is antigen specific. Accordingly, we investigated differences in IL-2 receptor synthesis and expression between the two
groups. While we could not demonstrate significant differences in immunohistochemistry, there was an increase on the transcriptional level in both groups. On POD 21, control animals showed a significantly higher level of transcription than statin-treated animals, an observation that matches with our above findings. Surprisingly, on POD 14, statin-treated animals show a significant increase in transcription of CD25, when compared to controls and on POD 21 cell surface expression of CD25 was increased in PMBC of the statin-treated group. While we do not have a conclusive explanation for these data, we believe that these findings may represent different dynamics in the rejection process in the two groups and fall in line with the findings for MHC class II.

This is the first report demonstrating that pravastatin decreases rejection after orthotopic allogenic LTx in an animal model and that this effect is caused by a lipid lowering independent, immunosuppressive property of HMG-CoA reductase inhibitors. While we believe the suppression of molecular production on a transcriptional level to be the underlying cause for the immunosuppressive effect of pravastatin, the second hypothetical mechanism should be mentioned. Differing from data showing direct inhibition of MHC class II [20–22], some groups propose that the disruption of lipid rafts which are critical components in a variety of immune processes essential for antigen presentation and subsequent T-cell activation, may explain the downregulatory effects of statins on cell-surface expression of MHC class II and other immunoregulatory molecules [12]. Such disruption could lead to insufficient loading MHC class II molecules, thereby further increasing the immunosuppressive effect by downregulating indirect antigen presentation.

We conclude that the administration of pravastatin after lung transplantation may significantly downregulate immunological responses, driven by indirect recognition in clinical recipients by inhibition of MHC class II molecule expression and intracellular loading and that this may be the underlying cause for graft protective effects observed in recipients after solid organ transplantation. Thus, pravastatin could be used as an additional, selective immunosuppressive drug, in order to increase survival times after lung transplantation.

While there is currently no experimental or clinical data demonstrating that other statins have similar graft protective immunosuppressive effects, all statins do inhibit MHC class II expression, albeit to a different degree. Therefore, it is probable that other statins may have similar effects. The analysis of these and the different statins are part of our ongoing efforts to further elucidate the effects of this interesting group of drugs.

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References


Appendix A. Conference discussion

Dr R. Bonser (Birmingham, United Kingdom): The dose of pravastatin that you were using is about 20- to 30-fold higher than you would use in a human being; and therefore, you’re likely to see more immunological effect. So why did you choose that dose and why didn’t you try a clinical comparable dose?

Dr Simon: The dose was chosen actually based on experimental data we had at that time from companies using statins in animal experimental models.

We thought about using a dose that was comparable to humans; however, the results in our retrospective study mirror the effects probably on chronic rejection. And just to see the effect, we thought that we would probably be better off using a high dose in a model that we could analyze easily and not wait for 100 days. Accordingly, we tried to develop a model that would give us the effect of the statin maybe after 14 or 21 days.

However, you’re absolutely right. And we’re actually moving into this model, decreasing the dose and increasing the observation period. To show the effect just as a proof of principle, we tried high dose, short time.

Dr W. Klepetko (Vienna, Austria): I was impressed by the difference of survival that you have seen in your clinical correlation, which would make the statins the most potent immunosuppressant agent that is currently available on the market. Could you comment on this enormous difference between the two groups.

Dr Simon: To tell you the truth, when we first saw these curves, we thought that there was something wrong with our analysis, because we couldn’t believe what we were seeing. So we redid the analyses with the statistician of our hospital. For instance, in one analysis, we omitted all patients transplanted in the pre-pravastatin era. Additionally, analyses were performed based on periods of transplantation.

But no matter what you do, you always get the same result. You have an enormous survival benefit in the group receiving pravastatin in our retrospective analysis. These are about 73 patients out of 500. But no matter what you do, you always get the same result. The curves don’t change.

Dr A. Kappetein (Rotterdam, The Netherlands): The curves don’t change, which means there are no other factors that influence the outcome.

Dr Simon: As far as we have been able to find out, no. Of course, we are looking into other variables like immunosuppression via CyA of Tacrolimus or azathioprine versus MMF. But basically, the groups are comparable.

Dr Kappetein: Nowadays every patient receives a statin. So wasn’t it the case that all the patients that were operated 10 years ago were the ones without statins? And all the patients that were operated more recently received statins.

Dr Simon: Partially, of course, this is true. But in our cohort, not everybody receives statins. Patients receive statins once they got elevated serum lipid levels and 500 the patients in the retrospective analysis, only 70 received statins.