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ORIGINAL ARTICLE

Clopidogrel reduces post-transplant obliterative bronchiolitis

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Summary

Survival after lung transplantation is mainly limited by the development of chronic lung allograft dysfunction (CLAD). The aim of this study was to investigate if platelet inhibition by clopidogrel has an influence on the formation of obliterative bronchiolitis, the histopathological correlate to bronchiolitis obliterans syndrome, present in the majority of patients suffering from CLAD. C57Bl/6 ($H2^b$) donor tracheas were orthotopically transplanted into CBA.J($H2^k$). Mice received different doses of clopidogrel alone or in combination with tacrolimus or everolimus. Grafts were analyzed by histology and immunofluorescence method on postoperative days 15, 30 or 60. Cytokines were analyzed by real-time polymerase chain reaction on postoperative day 21 and alloantibodies by FACS. Mice treated with 20 mg/kg/day clopidogrel for 30 days showed reduced obliteration [34.40 ± 3.76% (20 mg/kg/day clopidogrel) vs. 49.92 ± 2.11% (control), $n = 5$, $P < 0.05$]. Platelet inhibition resulted in significant lower infiltration of T cells and macrophages, and we also found significantly lower expression of IL-12, IL-4, IL-6, TNF-α, TGF-β, PDGFβ, MCP1, P-/E-selectin, ICAM1 and CD40L after treatment with clopidogrel. Combination of 1 mg/kg/day clopidogrel and 0.05 mg/kg/day everolimus or 12 mg/kg/day tacrolimus revealed a synergistic effect. Humoral immunity as manifested by donor-specific alloantibody secretion was also impaired after treatment with clopidogrel. Here, we can show that platelet inhibition by clopidogrel as a single treatment and in combination with tacrolimus or everolimus reduced the development of fibrosis and obliteration in tracheal allografts.

Introduction

Chronic lung allograft dysfunction (CLAD) remains the leading cause of mortality after lung transplantation and a majority of CLAD patients develop an obstructive pulmonary function, characterized by a bronchiolitis obliterans syndrome (BOS) phenotype [1,2]. BOS results in a persistent obstructive pulmonary function decline after lung transplantation and is the major obstacle to long-term survival occurring in approximately 50% of adult lung transplant recipients after 5 years [3]. On the cellular level, BOS is characterized by a diffuse infiltration of the bronchiolar epithelium and wall with mononuclear cells resulting in an inflammatory reaction [4] which in turn finally leads to a dense fibrosis of the lamina propria and lumen of the bronchioles as well as to a fibroproliferative remodelling of the blood vessels [5–7]. However, about one-third of the patients develop a restrictive pulmonary function, recently defined as a restrictive allograft syndrome phenotype, which is associated with worse survival after CLAD has been diagnosed [1,2]. In recent years, immunological functions of platelets became of increasing interest as their

interaction with endothelium induces significant changes in the adhesive and chemotactic properties of endothelial cells that trigger monocyte adhesion and transmigration. Furthermore, the role of platelets during the development of arteriosclerosis is meanwhile established [8,9] and in organ transplantation, platelets have been shown to increase the expression of adhesion molecules within the vessels of the transplant [10]. In the current study, platelet inhibition was achieved by the use of clopidogrel as already published [11]. Clopidogrel is a thienopyridine derivate that has become an important therapeutic agent for people suffering from coronary heart disease [12–15]. Monotherapy with clopidogrel effectively reduced the formation of transplant arteriosclerosis in a mouse model in previous investigations [11,16,17].

Hypothesis

The aim of this study was to elucidate if platelet inhibition alone or in combination with mTOR or calcineurin inhibition has an effect on obliterative bronchiolitis (OB), the histopathological correlate of BOS. The two clinically relevant drugs tacrolimus and everolimus were used for T-cell modulation. The orthotopic tracheal transplantation model in mice was employed as it is widely used for the investigation of immunological aspects during the development of OB, representing similar pathological features seen in human large airways affected by BOS [18–20].

Material and methods

Animals

C57Bl/6($H2^b$) and CBA.J($H2^k$) mice aged between 6 and 12 weeks at the time of experimental use were received from Charles River (Sulzfeld, Germany), bred and maintained in the central animal facility of the University of Erlangen-Nürnberg (Franz-Penzoldt-Zentrum) under specific pathogen-free conditions. This study was carried out in strict accordance with international guidelines for animal care and use and in accordance with the guidelines of the Animal Care and Use Committee of the Government of Bavaria (AZ 54-2532.1-14/10).

Orthotopic tracheal transplantation

Tracheal grafts were extracted from C57Bl/6($H2^b$) or CBA.J ($H2^k$) mice and transplanted orthotopically into CBA.J ($H2^k$). Briefly, the donor trachea was exposed via an anterior midline neck incision and preparation of the entire laryngotracheal complex. A five ring circumferential tracheal segment was excised and orthotopically transferred into the recipient [21]. Anastomoses were performed with 8-0 nylon sutures and skin was closed with a 4-0 absorbable thread (PGA Resorba, Nürnberg, Germany).

Treatment protocol

Clopidogrel (Plavix®; Sanofi-Aventis, Berlin, Germany) was obtained from the local hospital pharmacy and 75-mg tablets were dissolved in 0.9% saline under sterile conditions. This solution was then diluted appropriately in the following concentrations: 1 mg/kg clopidogrel equivalent of a human daily dose and 20 mg/kg clopidogrel, equivalent of a loading dose before a percutaneous intervention. Daily treatment with clopidogrel was started immediately after transplantation for 15, 21, 30 or 60 days. Because dissolved clopidogrel is unstable [22], the clopidogrel solution was freshly prepared every day and injected intraperitoneally immediately after preparation. The overall injection volume was 0.5 ml for each treatment group. In previous studies, we have shown that administration of 12 mg/kg/day tacrolimus (Prograf®; Astellas, München, Germany) and 0.05 mg/kg/day everolimus (Certican®; Novartis, München, Germany) resulted in appropriate plasma levels [17,23] comparable to human blood target levels of the respective medication [24,25]. Daily drug treatment started after transplantation and continued throughout the entire experimental protocol.

Platelet aggregation

For *ex vivo* platelet aggregation, blood was collected in 3.2% citrate.

Approximately 0.5 ml of blood could be obtained from each mouse, and samples were immediately processed after blood drawing. Platelet aggregation was evaluated by means of optical aggregometry in citrated blood samples at 37 °C using a 2-channel Chronolog aggregometer (Elvi Logos, Milan, Italy) as previously described by our group [11]. In brief, platelet-rich and platelet-poor plasma was prepared from citrated whole blood by means of centrifugation (100 *g* for 10 min). The final platelet count was adjusted to an average of 2.5×10^5 platelets/ml with autologous plasma. Twenty microliters of adenosine diphosphate (ADP; Sigma, St Louis, MO, USA) at a final concentration of 2×10^4 mol/l was added to induce platelet activation, and aggregation was recorded for at least 10 min. Maximal aggregation was mostly seen around 5 min and was used as a measurement of aggregation.

Analysis of the tracheal graft

Tracheal segments were recovered under anaesthesia on day 15, 21, 30 or 60 after transplantation. Grafts were perfused with normal saline and flash frozen in optimal cutting temperature medium (Tissue-Tek®, Sakura, Alphen aan den Rijn, The Netherlands) in liquid nitrogen to perform morphometric and immunohistochemical analysis.

Morphometry

At least five transverse sections (5 μm thickness) from different areas of each graft were stained with H&E and analyzed at an original magnification of ×100 using a conventional light microscope. A digital image of each section was captured and luminal obliteration was quantified as previously described by Reichenspurner *et al.* [26]. Briefly, luminal obliteration was defined as (1 – area luminal of the respiratory epithelium/area containing tissue luminal of the cartilage ring) × 100 (%). All image analyses were performed on a colour display monitor using ANAlysis® Image Analysis software (Olympus, Hamburg, Germany).

Immunhistochemistry

A minimum of five cryostat sections (5 μm thickness) from different areas of the graft were air-dried and fixed for 10 min in acetone. Slides were rehydrated and preincubated in staining buffer (0.1 Tris, pH 8.0 and 0.1% Tween 20) for 10 min and afterwards treated with 5% mouse serum (Invitrogen, Darmstadt, Germany) for 15 min in a chamber before incubation with primary antibodies for 1 h. After washing, slides were treated for 1 h with secondary antibody. Finally, anti-cytokeratin 18 antibody was applied and after three washes, slides were mounted using Vectashield Hardset Mounting Medium (VECTOR Laboratories, Burlingame, CA, USA). Sections were analyzed by epiflourescence microscopy (Olympus, Hamburg, Germany) under ×200 magnification. Numbers of positive cells in the subepithelial layer were manually counted under blinded experimental conditions and related to the analyzed area. Evaluation was performed regardless of the thickness of the subepithelial layer. The following antibodies and conjugates were used; anti-CD4, anti-CD8 (BD Bioscience, Heidelberg, Germany) and anti-macrophages F4/80 (AbD Serotec MorphoSys, Düsseldorf, Germany) as primary antibodies as well as mouse-anti-rat-IgG-Cy3 (Dianova, Hamburg, Germany) as secondary antibody. Tracheal epithelial cells were stained with a FITC-conjugated mouse anti-cytokeratin 18 antibody (SouthernBiotech, Birmingham, AL, USA).

Alloantibody detection in the serum

Blood from recipient mice was obtained during the harvesting procedure and centrifuged. Recovered serum was used for FACS analysis performed according to a previous published protocol by our group [27]. Briefly, 50 μl of recipient serum was added to 50 μl of the cell suspension and incubated for 30 min. After washing, 50 μl of BSA buffer was added as well as 1 μl of a FITC-labelled goat-anti-mouse-IgG-antibody (Sigma-Aldrich, Steinheim, Germany). Finally, FACS analysis (BD FACSCanto II; BD Biosciences, Heidelberg, Germany) was accomplished according to the manufacturer's protocol.

Analysis of the intragraft mRNA expression

Grafts were harvested, flushed with sterile saline and stored in RNAlater (Qiagen, Hilden, Germany). RNA isolation and cDNA synthesis were performed according to standard protocols. Real-time polymerase chain reaction (RT-PCR) amplification was conducted in triplets by applying the StepOne RT-PCR System and the TaqMan Gene Expression Master Mix (Applied Biosystems, Forster City, CA, USA). Oligonucleotide sequences for TNF-α, IL-4, IL-6, IL-12, TGF-β, IFN-γ, ICAM-1, E-/P-selectin, PDGFβ, MCP1 and CD40-ligand (CD40L) were previously published [16,28]. To generate PCR-standards, the respective PCR product was cloned into a TOPO cloning vector (Invitrogen, Darmstadt, Germany) and identity of the cloned amplicons was confirmed by sequence analysis. Standard curves of known concentrations of template copy numbers were used to determine the expression of the amplified target and 18srRNA expression was used as a housekeeping gene. Samples were normalized against the housekeeping gene expression and results are expressed in relative copy numbers.

Statistical analysis

Results are given as the mean per group ± SEM which derived from the mean per graft. Data were analyzed using a two-tailed unpaired Student's *t*-test and a one-way ANOVA followed by a Bonferroni correction. $P < 0.05$ was considered as significant.

Results

Platelet aggregation was effectively inhibited after clopidogrel administration

Ex vivo blood samples were harvested on days 15 and 30 to ensure sufficient platelet aggregation inhibition within the CBA/J recipient mice. Aggregation was determined by means of light transmittance aggregometry in response to ADP (2×10^4 mol/l), as previously published by our group [11]. Blood drawn from recipient mice treated with 1 and 20 mg/kg clopidogrel showed significantly reduced platelet aggregation on day 15 [16 ± 5% (1 mg/kg/day clopidogrel) vs. 7 ± 4% (20 mg/kg/day clopidogrel) vs. 55 ± 7% (control), $n = 5$ per group, $P \leq 0.05$ both treatment groups versus control] and on day 30 [10 ± 5% (1 mg/kg/day clopidogrel) vs. 6 ± 3% (20 mg/kg/day clopidogrel) vs. 52 ± 8% (control), $n = 5$ per groups, $P \leq 0.05$ both treatment groups versus control]. Blood

from untreated control animals showed unimpaired platelet function throughout all time points. In addition, we have previously shown in a kinetic analysis that from day 14 onwards, platelet aggregation in a variety of clopidogrel dosages was significantly inhibited compared to untreated controls [11].

Single therapy with clopidogrel and in combination with tacrolimus or everolimus resulted in reduced levels of luminal obliteration

Orthotopic tracheal allografts were histologically analyzed on days 15, 30 and 60 after transplantation (Fig. 1a). It has previously been shown that the major lesions within the tracheal allografts develop during this timeframe [29]. After 30 days of treatment, mice receiving 20 mg/kg/day clopidogrel revealed significantly reduced luminal obliteration compared to untreated allografts [luminal obliteration: 42.25 ± 5.74% (1 mg/kg/day clopidogrel) vs. 34.40 ± 3.76% (20 mg/kg/day clopidogrel) vs. 49.92 ± 2.11% (control), $n = 5$ per group, $P < 0.05$ 20 mg/kg/day clopidogrel versus control]. After treatment with clopidogrel for 60 days, both experimental groups displayed a significant reduction in luminal obliteration compared to untreated allografts. Interestingly, at this time point recipients treated with 20 mg/kg/day clopidogrel showed no additional benefit regarding the amount of luminal obliteration compared to the 1 mg/kg/day clopidogrel group [luminal obliteration: 41.83 ± 5.25% (1 mg/kg/day clopidogrel) vs. 37.71 ± 6.28% (20 mg/kg/day clopidogrel) vs. 54.51 ± 5.57% (control), $n = 5$ per group, $P < 0.05$ both treatment groups versus control] (Fig. 1b).

Single treatment with tacrolimus and everolimus revealed reduced luminal obliteration compared to controls. However, combination of tacrolimus or everolimus with clopidogrel resulted in significantly less luminal obliteration compared to untreated controls on day 30. Everolimus combined with 20 mg/kg/day clopidogrel showed further inhibition of luminal obliteration compared to animals treated with 20 mg/kg/day clopidogrel alone but not compared to everolimus monotherapy (Fig. 2a–h).

Treatment with clopidogrel alone or in combination with tacrolimus or everolimus substantially reduced the amount of cellular infiltration

Immunofluorescence analysis of tracheal grafts on day 30 revealed substantially fewer CD4+ T cells in both clopidogrel treatment groups. Combination of clopidogrel and everolimus further decreased the overall amount of infiltrating cells and revealed significantly fewer CD4+ T cells in mice receiving 1 mg/kg/day clopidogrel and everolimus compared to clopidogrel alone (Fig. 3a–d). CD8+ T-cell infiltration showed a similar pattern and was reduced in both clopidogrel treatment groups. clopidogrel combined with tacrolimus significantly reduced CD8+ T-cell

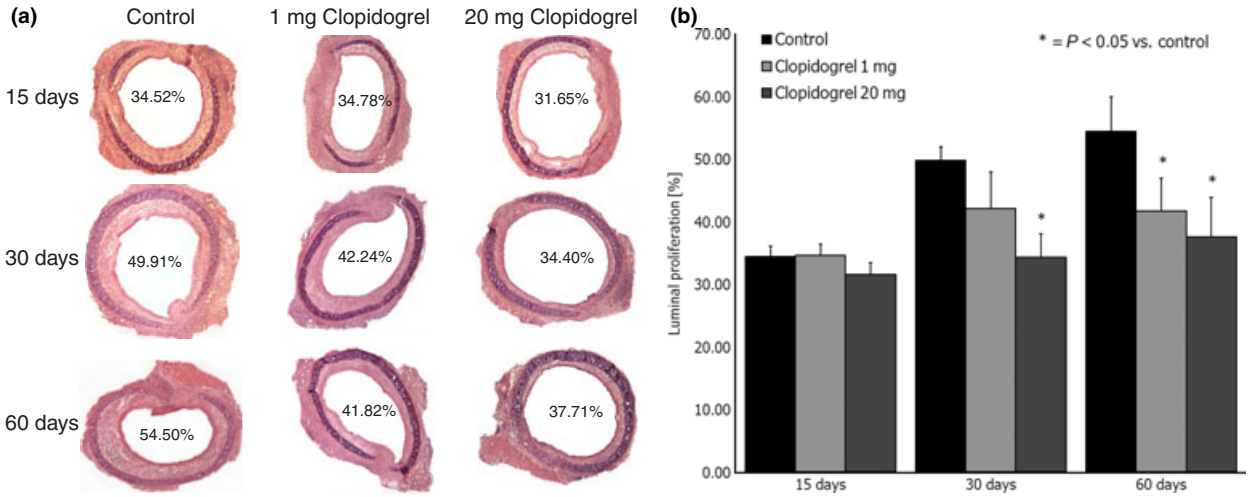

Figure 1 Histopathological evaluation of the morphology of untreated orthotopic tracheal allograft compared to single clopidogrel treatment groups with 1 or 20 mg/kg/day each harvested on day 15, 30 or 60, respectively (a). Tissue was snap-frozen and representative sections were stained with H&E stain. Pictures were taken at an original magnification of ×100. Quantification of luminal obliteration (%) defined as the area containing tissue luminal of the cartilage ring (b). Because of the fact that native tracheas have an epithelium layer and subepithelial connective tissue, the luminal obliteration measured according to methods described by Reichenspurner *et al.* [26] corresponds to about 15% within native grafts. Briefly, luminal obliteration was defined as (1 – area luminal of the respiratory epithelium/area containing tissue luminal of the cartilage ring) × 100 (%). Morphologically, clopidogrel decreased the amount of luminal obliteration significantly on day 60, but did not show any differences between high-dose (20 mg/kg/day) and low-dose (1 mg/kg/day) clopidogrel during all observed time points.

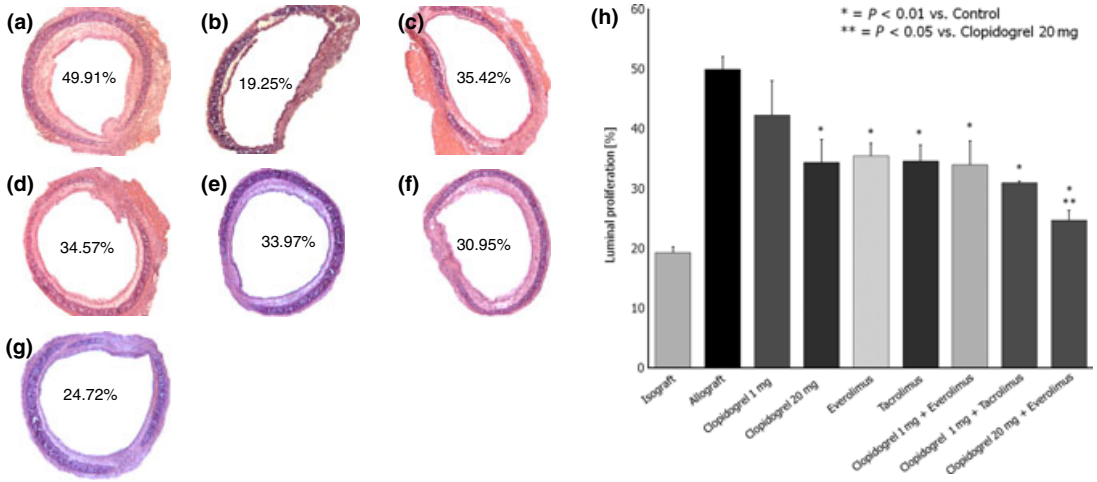

Figure 2 Histological overview (H&E staining) on groups treated either with 0.05 mg/kg/day everolimus (c) or 12 mg/kg/day tacrolimus (d), 1 mg/kg/day clopidogrel and 0.05 mg/kg/day everolimus (e), 20 mg/kg/day clopidogrel and 0.05 mg/kg/day everolimus (g) or 1 mg/kg/day clopidogrel and 12 mg/kg/day tacrolimus (f) for 30 days compared to untreated allograft (a) and untreated isograft (b). All recipients showed significantly less luminal obliteration than the untreated allograft (a) but the amount of fibroproliferation in the subepithelial layer was still higher compared to untreated isograft (b). Quantification of luminal obliteration (%), defined as the tissue containing area luminal of the tracheal cartilage ring, after 30 days of treatment with either single treatment of 1 or 20 mg/kg/day clopidogrel or combined with or single treatment with 0.05 mg/kg/day everolimus or 12 mg/kg/day tacrolimus, respectively. Combining clopidogrel with everolimus or tacrolimus resulted in an additional effect on the amount of luminal obliteration after 30 days. Luminal obliteration was obtained according to a method previously published by Reichenspurner *et al.* [26].

infiltration (Fig. 3e–h). Clopidogrel also resulted in significantly lower levels of macrophage (F4/80+) infiltration in a dose dependent manner. Combination of clopidogrel with everolimus or tacrolimus revealed an additional decrease in macrophages (F4/80+) on day 30 (Fig. 3i–l and m–o).

Monotherapy with clopidogrel decreased intragraft cytokine- and adhesion molecule-mRNA expression levels

Time point of intragraft analysis of cytokines and adhesion molecules was chosen in analogy with our experiences with the aortic allograft model [30] and results from cellular graft infiltration during our morphometric time-course analysis of tracheal allografts. Recipients treated either with 1 mg/kg/day clopidogrel or 20 mg/kg/day clopidogrel were compared to untreated allografts and syngeneic grafts. Both treatment groups expressed significantly reduced levels of TNF-α, TGF-β, PDGFβ, IL-12, IL-6, IL-4, MCP1 and INFγ compared to untreated allografts. In addition, we observed a decrease in adhesion molecule expression such as P-/E-selectin and ICAM-1 as well as CD40L (Fig. 4a–l).

Clopidogrel alone and in combination with everolimus or tacrolimus significantly decreased the amount of donor-specific alloantibody production

After treatment with 1 mg/kg/day clopidogrel and 20 mg/kg/day clopidogrel significantly lower levels of circulating alloantibodies were detected in the peripheral venous blood on day 30 (and day 15 and 60) compared to control (Fig. 5a and b). Single treatment with everolimus and tacrolimus also revealed reduced alloantibody production. Combination of clopidogrel and everolimus or tacrolimus resulted in an additional inhibition of alloantibody production, but we could not observe a significant reduction compared to the tacrolimus or everolimus alone treatment groups. Interestingly, when clopidogrel was combined with everolimus or tacrolimus maximum suppression of alloantibody production was already achieved at a dose of 1 mg/kg/day clopidogrel and there was no additional effect in the high-dose clopidogrel group.

Discussion

Previous studies have shown that platelets contribute to the development of arteriosclerosis [8,9] via interaction with the endothelium resulting in early inflammation during the process of arteriosclerosis within the vessel wall [31,32]. Data of the current study show that, treatment with clopidogrel after orthotopic tracheal transplantation results in reduced levels of T cell and macrophage infiltration associated with lower expression levels of inflammatory cytokines, adhesion molecules and alloantibody secretion finally accompanied by diminished luminal obliteration. The orthotopic tracheal transplant model is characterized by only one large airway; however, the basic intragraft

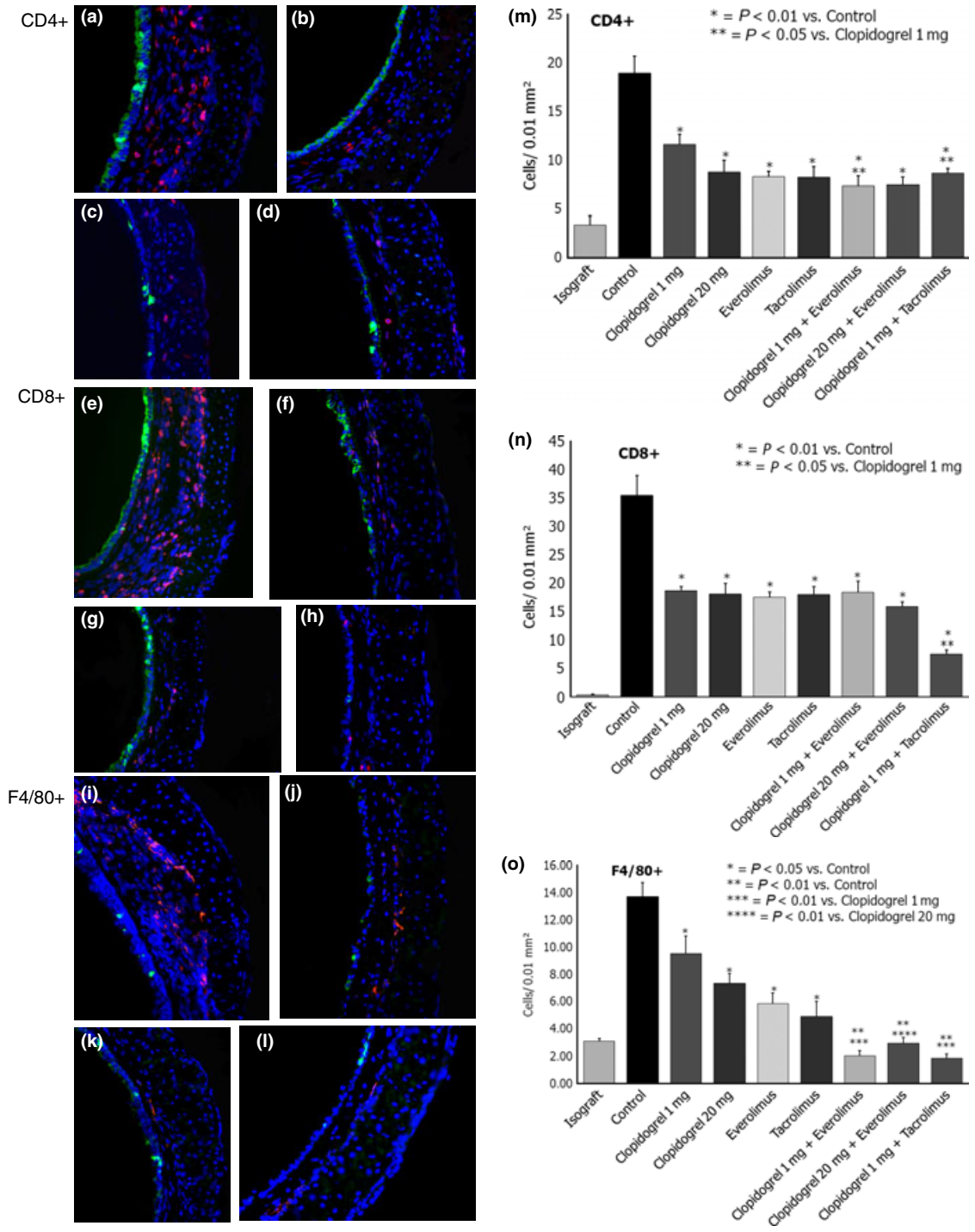

Figure 3 Extraction of immunofluorescence photomicrographs of cryostat sections from orthotopic tracheal allografts day 30 after transplantation (a–l). Mice treated with 20 mg/kg/day clopidogrel (b, f, j) showed a significant decrease in CD4+ T cells (a–d, m), CD8+ T cells (e–h, n) and macrophages (F4/80+) (i–l, o) compared to the respective controls (a, e, i). Animals treated with 1 mg/kg/day clopidogrel and 0.05 mg/kg/day everolimus (c, g, k) showed additional effects with regard to T cell and macrophage infiltration. Recipients treated with 1 mg/kg/day clopidogrel and 12 mg/kg/day tacrolimus (d, h, l) showed significant lower levels of CD8+ T cell (h), CD4+ T cell (d) and macrophage (F4/80+) infiltration (l) within the graft. Quantification of intragraft-cellular infiltration in cryostat immunofluorescence sections was performed for CD4+ cells (m), CD8+ cells (n) and macrophages (F4/80+) (o). Tracheal allografts were analyzed at a magnification of ×200. Positive stained cells per area in the graft were counted on a magnification of ×200 (n = 5 animals per group). (Green: Cytokeratin 18, red: CD4+ T cells, CD8+ T cells, F4/80+ cells, respectively, blue: nucleus).

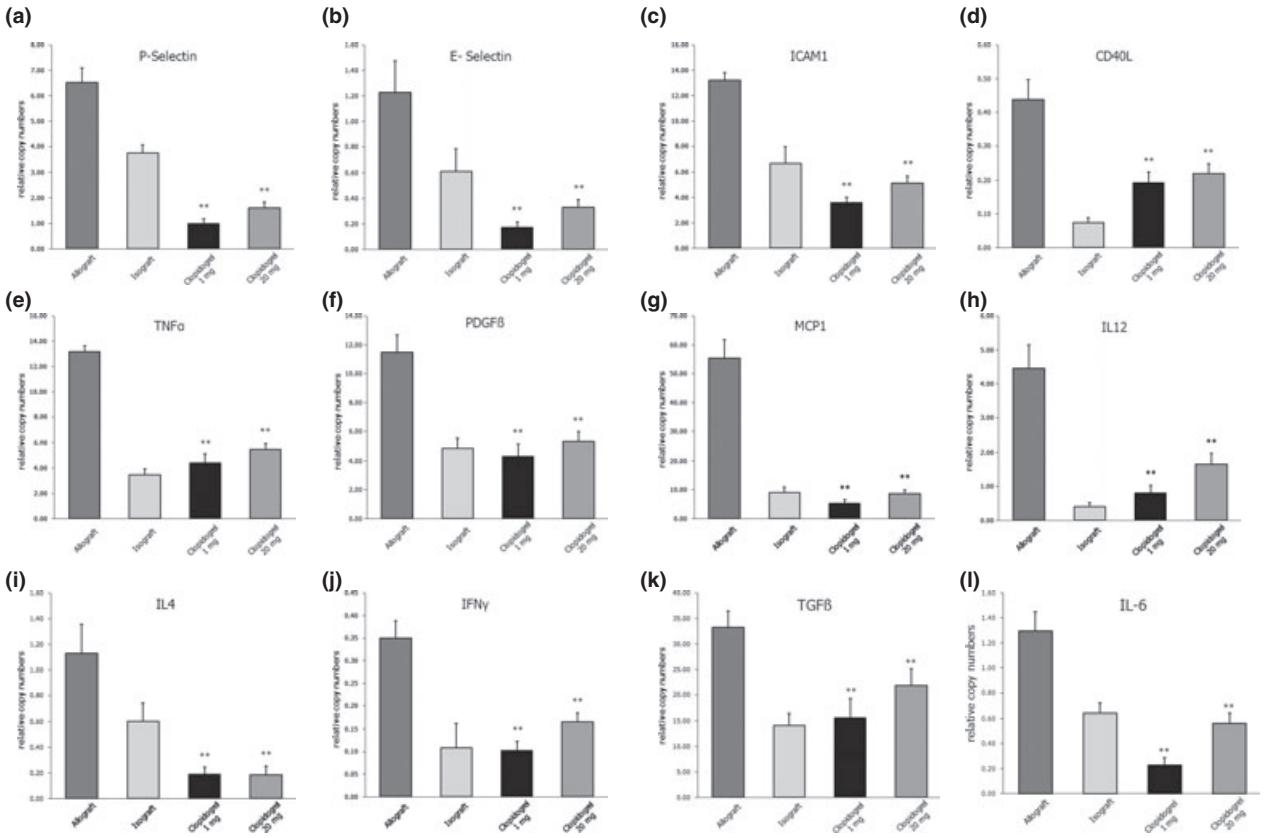

Figure 4 Quantitative RT-PCR analysis was performed for cellular adhesion molecules such as P-selectin (a), E-selectin (b), ICAM1 (c), CD40L (d) and intragraft cytokine production such as TNF-α (e), platelet derived growth factor (PDGF)β (f), monocyte chemoattractant protein (MCP)1 (g), IL-12 (h), IL-4 (i), INFγ (j), TGF-β (k), IL-6 (l) production. Orthotopically transplanted tracheal grafts were analyzed after 21 days of treatment with 1 or 20 mg/kg/day clopidogrel and compared to untreated controls and isografts harvested after 21 days. Data are shown as the mean of five animals from each group ($n = 5$, $^{**}P < 0.05$ vs. control).

immune mechanisms leading to luminal obliteration are comparable because of the ventilation which is, for example, absent in the heterotopic transplant model. Although, the histopathological changes found in orthotopically transplanted tracheas are similar to those found in different stages of BOS in humans [19], there are differences in function and status of epithelial cells in small airways in humans representing a limitation of this model [18]. However, it is generally accepted as a suitable experimental model to investigate the basic intragraft immune mechanisms leading to luminal obliteration [18,33]. Moreover, the orthotopic transplant model is especially useful not only to study pharmacological effects on the development of luminal obliteration but also to determine the role of microcirculatory perfusion reduction going along with increased ischemia as shown in patients after lung transplantation [34,35]. Although the orthotopic tracheal transplant model does not reflect the exact degree of airway obliteration as found in clinical patients after lung transplantation and measurements of lung function as in clinical lung transplantation are not performed, the reproducible development of subepithelial fibrosis of the large airways within a relatively short time frame underlines the suitability of this experimental model and supports its further use as an exemplary model for the development of OB, the histopathological correlate of BOS.

Studies by Henn *et al.* [31] have shown that activated platelets express CD40L on their surface, which is subsequently cleaved generating soluble (s)CD40L and capable of inducing the expression of chemokines (MCP1), cytokines (IFN-γ, IL-6) and adhesion molecules (ICAM-1, E-selectin) [31] by ligating CD40 on endothelial cells and monocytes [36]. It has recently been demonstrated that anti-CD40L significantly attenuates the development of BOS [37,38]. Data of the current study reveal reduced CD40L and E/P-selectin mRNA expression within orthotopic tracheal allografts of transplant recipients after treatment with clopidogrel confirming an anti-inflammatory effect even in a rejecting fully

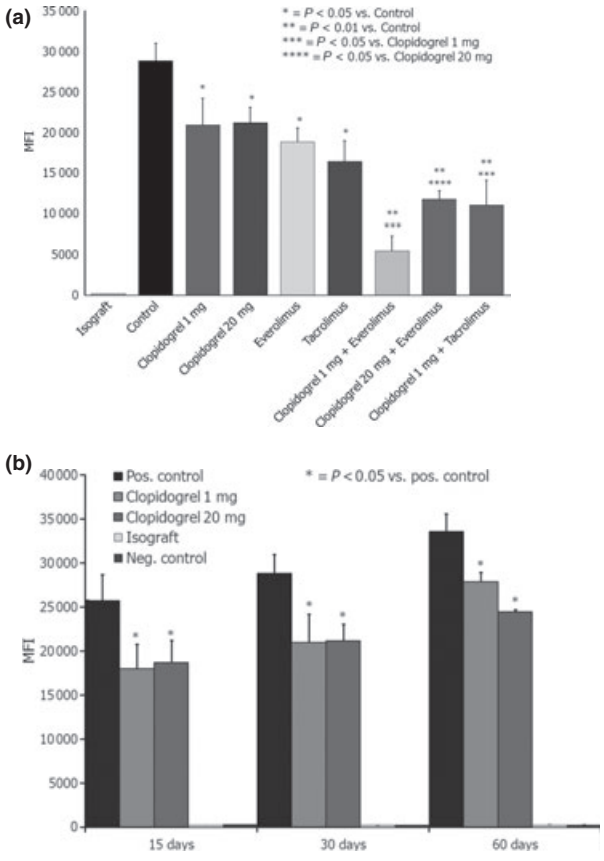

Figure 5 Quantification of alloantibody secretion measured by FACS 30 days (a) and additionally 15 as well as 60 days (b) after transplantation of tracheal grafts from C57BL/6($H2^b$) donors into CBA. J($H2^k$) recipients and before transplantation (negative control). Levels of alloantibodies within the venous blood were significantly lower in the clopidogrel treatment groups compared to controls and further reduced by combination with either tacrolimus or everolimus (a). Alloantibodies are expressed as levels of MFI (mean fluorescence intensity).

mismatched tracheal allograft model in the absence of any additional immunosuppression.

Activated platelets release P-selectin [39] and platelet derived growth factor (PDGF) [40] which can contribute to a systemic inflammatory response in various pathological conditions including chronic rejection [41,42]. Clopidogrel reduced P-selectin and PDGF expression levels within the tracheal transplant. Previous data have already highlighted the importance of P-selectin [43] and clinical studies revealed an association between the presence of P-selectin-positive platelet aggregates and primary graft dysfunction after lung transplantation [44,45].

Platelets can also induce enhanced surface expression of ICAM-1 [46] and E-selectin [47] on endothelial cells that mediate adhesion and transmigration of leukocytes through the vascular wall. Recent studies suggest that a functionally unimpaired microvasculature is critical in the maintenance of normal airway architecture [34,48]. Untreated orthotopic tracheal allografts undergo a decrease in vascular perfusion associated with a loss of endothelial cells in the early days after transplantation whereas isografts and immunosuppressed allografts were preserved [34]. These results implicate that obliteration of airway microvasculature, affecting tracheal allografts after transplantation, results in diminished blood perfusion of the graft and finally leads to tissue hypoxia enhancing inflammation and rejection of the transplant [34,48]. Considering previous studies of our group proving the inhibitory effect of clopidogrel in mice on platelet activation [11] we speculate at this stage, that beside immunosuppressive effects, targeting platelets by clopidogrel may also mitigate the process of OB by improving allograft perfusion and therefore reduce intragraft tissue hypoxia as platelet inhibition is well known to have a beneficial effect on hemodynamics in particular within the microvasculature. This might explain the additional reduction in luminal obliteration in the high dose clopidogrel treatment group in the early phase after transplantation (day 30) compared to lower dose treatment group. Reduction in OB after treatment with clopidogrel was characterized by a significant drop in graft-infiltrating macrophages associated with decreased intragraft MCP-1 expression, a major chemoattractant for macrophages during inflammation [49]. Interestingly, tracheal infiltration with CD4+ and CD8+ T cells was also diminished after treatment with clopidogrel and accompanied by lower intragraft expression of IL-4, IL-12 and CD40L. However, in a previous study of our group to characterize the mode of action of clopidogrel on the development of transplant arteriosclerosis, in particular immunomodulating effects, we used monoclonal antibodies against GP-Ib and GP-VI [50,51]. As treatment with anti-GP-Ib and anti-GP-VI mAbs showed no significant difference in the amounts of transplant arteriosclerosis compared with untreated controls, these results suggested that reduction in the development of transplant arteriosclerosis by clopidogrel was not entirely caused by its ability to decrease ADP-induced platelet aggregation [16]. Taken together, these results and data of the current study suggest that this might also hold true for the reduction in OB by clopidogrel in this experimental tracheal transplant model.

Accumulating evidence suggests that allogeneic antibody responses play an important role in acute lung rejection and in the development of BOS. Recipients with pre-existing anti-MHC antibodies show a significantly higher risk for early graft dysfunction and poor prognosis [52]. Our data show a strong correlation between the level of donor-specific alloantibodies and the formation of luminal obliteration during treatment with clopidogrel. Numerous reports have looked at the role of donor-specific

alloantibodies and an association was demonstrated in several murine models [53,54], whereas other groups using different models of experimental transplantation failed to demonstrate an association [55,56].

Combined treatment with everolimus or tacrolimus and clopidogrel dramatically reduced the development of OB, implying a synergistic effect. Tacrolimus blocks T- and B-cell proliferation and represents a backbone of current immunosuppressive therapy regimen following lung transplantation [57]. Recently, mTOR inhibitors such as everolimus have been introduced in clinical lung transplantation and showed some promising results in particular with regard to low nephrotoxicity [58,59]. The survival rate for our experiments was over 90%, and no infectious complications or bleedings were observed in single or combination treatment groups with the exception of the high dose clopidogrel and tacrolimus group which was therefore prematurely stopped. However, apart from this experimental group, the toxicity of these drugs alone and in combination seems to be tolerable which is in line with previously published clinical data [60]. Therefore, in clinical lung transplantation, combination of clopidogrel with the established drugs like tacrolimus or everolimus may be a very promising approach. Further preclinical as well as clinical studies with longer follow-up are necessary to identify the optimal treatment strategy especially with regard to the rather varying data of everolimus and so far no convincing evidence of reduction in BOS for either drug alone has been demonstrated [58]. In addition, the pathological role of platelets and platelet inhibition in the field of CLAD with its hallmark feature BOS needs to be further elucidated. Therefore, there is also a need for further studies in different animal models, investigating if the effect of clopidogrel seen on large airways also persists in smaller airways displaying a different function of the epithelium.

Conclusion

In conclusion, we have shown that clopidogrel alone or with everolimus or tacrolimus can dramatically reduce the development of OB in an experimental orthotopic tracheal allograft model. As all these drugs have an established clinical safety profile, patients suffering from BOS after lung transplantation may, after further clinical evaluation, benefit from this new treatment strategy.

Authorship

RHMP, MW and SME: designed the study. RHMP, SE, NK, BMS and MR-G: performed the experiments and collected data. RHMP and SME: analyzed the data. RHMP and SME: wrote the manuscript.

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