

## OPEN

# Small Molecule Tyrosine Kinase Inhibitor Nintedanib Reduces Development of Cardiac Allograft Vasculopathy in Murine Aortic Allografts

Annika Gocht,<sup>1</sup> Bernd Spriewald, MD, DPhil,<sup>2</sup> Jörg H.W. Distler, MD,<sup>2</sup> Martina Ramsperger-Gleixner, PhD,<sup>1</sup> Stephan M. Ensminger, MD, DPhil,<sup>3</sup> Michael Weyand, MD,<sup>1</sup> and Christian Heim, MD<sup>1</sup>

**Background.** Nintedanib is a small molecule tyrosine kinase inhibitor that blocks the action of the platelet-derived growth factor receptor (PDGFR), the vascular endothelial growth factor receptor (VEGFR) and the fibroblast growth factor receptor. All of these receptors have been shown to be involved in the development of cardiac allograft vasculopathy (CAV) after heart transplantation. We therefore hypothesized that blocking these tyrosine kinase receptors with nintedanib could prevent CAV. **Methods.** CBA/JRj (H2<sup>k</sup>) mice underwent an abdominal aortic transplantation with a graft derived from fully allogeneic C57BL/6JRj (H2<sup>b</sup>) mice. Nintedanib was given daily from the first day after transplantation until harvest on day 14 for polymerase chain reaction analysis of intragraft cytokine expression or harvest on day 30 for histological analysis of the graft. **Results.** Nintedanib treatment resulted in significantly reduced neointima formation in the aortic graft compared with untreated control allografts. Interestingly, the immigration of smooth muscle cells into the neointima was markedly reduced while graft infiltrating macrophages and T cells were not altered in nintedanib-treated animals. The expression of the growth factor PDGF was significantly reduced in the nintedanib group going along with a distinctly reduced expression of the corresponding receptors PDGFR  $\alpha$  and  $-\beta$ . **Conclusions.** Treatment with nintedanib caused a significant reduction of CAV development after aortic transplantation in mice. We hypothesize the attenuated neointima formation in nintedanib-treated animals to be mediated by a direct inhibition of intimal smooth muscle cell proliferation via reduced expression of PDGF and the appropriate receptors PDGFR  $\alpha$  +  $\beta$ .

(*Transplantation Direct* 2018;4: e367; doi: 10.1097/TXD.0000000000000804. Published online 18 June, 2018.)

Nintedanib (formerly known as BIBF 1120) is a small molecule tyrosine kinase inhibitor that blocks signal transduction of platelet-derived growth factor receptors  $\alpha$  and  $\beta$  (PDGFR $\alpha$  +  $\beta$ ), vascular endothelial growth factor receptors

1 to 3 (VEGFR1-3) and fibroblast growth factor receptors 1 to 4 (FGFR1-4) which contain tyrosine kinase domains as part of their molecular structure and important for the signal transduction pathways.<sup>1-3</sup> Initially, nintedanib was investigated as an anticancer drug because of its antiangiogenic properties and it is now approved for the treatment of non-small-cell lung cancer under the name VARGATEF. In addition, nintedanib showed good antifibrotic properties and is therefore also approved for the treatment of idiopathic pulmonary fibrosis as OFEV (Böhringer-Ingelheim).

Heart transplantation is the last treatment option for patients with end stage heart disease. Survival rates after transplantation are strongly limited by the development of cardiac allograft vasculopathy (CAV). Ten years after heart transplantation, this pathology can be found in about 50% of the patients and it is one of the most common causes of death.<sup>4</sup> Cardiac allograft vasculopathy is a chronic disease that affects the vessels of the transplanted heart and leads to diffuse concentric narrowing of the vessel lumen due to neointima formation which predominantly consists of smooth muscle cells (SMCs) and leukocytes.<sup>5-8</sup>

All 3 classes of tyrosine kinase containing growth factor receptors affected by nintedanib (PDGFRs, VEGFRs, and FGFRs) are involved in the pathogenesis of CAV.<sup>9-11</sup>

Platelet-derived growth factor receptor is long known as one of the most potent stimulators of SMC migration and

Received 9 May 2018. Revision requested 14 May 2018.

Accepted 15 May 2018.

<sup>1</sup> Department of Cardiac Surgery, University Hospital Erlangen, Erlangen, Germany.

<sup>2</sup> Department of Internal Medicine, University Hospital Erlangen, Erlangen, Germany.

<sup>3</sup> Department of Cardiac Surgery, HDZ NRW, Bad Oeynhausen, Germany.

The authors declare no conflicts of interest.

This work was supported by Adumed foundation.

A.G., J.H.W.D., B.S., M.W., and C.H. drafted the study design. A.G. conducted the animal experiments and data collection. A.G., M.R.-G., and C.H. participated in data analysis and interpretation. A.G., M.R.-G., and C.H. participated in writing of the article including literature research. J.H.W.D., B.S., and S.M.E. enabled construction of the present manuscript by critical revising of the intellectual content from.

Correspondence: Christian Heim, MD, University Hospital Erlangen, Department of Cardiac Surgery, Krankenhausstraße 12, 91054 Erlangen, Germany. (christian.heim@fau.de).

Copyright © 2018 The Author(s). *Transplantation Direct*. Published by Wolters Kluwer Health, Inc. This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

ISSN: 2373-8731

DOI: 10.1097/TXD.0000000000000804

proliferation,<sup>12</sup> which in turn are central pathological features for neointima development in CAV. Expression of PDGF ligands and receptors is strongly upregulated in pathological states as CAV.<sup>10</sup> Although a deleterious effect of PDGF was detected, different ligand and receptor subtypes were assumed to be responsible.<sup>13–15</sup> Similarly, VEGF could be linked to CAV by several studies including some with human patients<sup>9,16</sup> as well as experimental animal studies.<sup>17,18</sup> Different ligand and receptor subtypes were found to contribute to the pathogenesis of CAV, especially VEGF-A<sup>9,16,17</sup> and VEGF-C.<sup>9,19</sup> Finally, for the 2 most abundant and relevant FGFR ligands FGF1 (acidic FGF/aFGF) and FGF2 (basic FGF/ bFGF)<sup>20</sup> reports also exist concerning their involvement in CAV.<sup>11,21,22</sup>

These observations led us to the hypothesis that treatment with nintedanib might prevent the development of CAV in an experimental mouse aortic allograft model.

## MATERIALS AND METHODS

### Animals

C57BL/6JRj (H2<sup>b</sup>) mice and CBA/JRj (H2<sup>k</sup>) mice were originally purchased from Janvier (Saint Berthevin, France). C57BL/6JRj (H2<sup>b</sup>) mice were used as donors and CBA/JRj mice (H2<sup>k</sup>) as recipients of aortic allografts. Male and female mice were equally present in all experimental groups. All mice used in this study were aged between 8 and 12 weeks at the time of experimental use and were bred (C57BL/6 mice) and maintained (both lines) at the Preclinical Experimental Animal Center (PETZ) at the University of Erlangen-Nuremberg under specific pathogen-free conditions and treated in accordance with institutional and state guidelines. All animal experiments were approved by the responsible government (Regierung von Unterfranken in Würzburg) considering the demands of German law (“Tierschutzgesetz” and “Verordnung zum Schutz von zu Versuchszwecken verwendeten Tieren”) under the license number 55.2 to 2532.1-62/14.

### Abdominal Aortic Transplantation

The procedure was performed with the microscope (OPMI 1 FC; Zeiss) using a modified technique initially described by Koulack et al.<sup>23</sup> In brief, the donor thoracic aorta was isolated and resected. The recipient abdominal aorta was revealed and clamped and then transected with sharp microvascular scissors between the clamps. Two end-to-end anastomoses with interrupted single sutures were performed connecting the proximal and distal ends of the recipient aorta with the donor aorta graft.

### Treatment Protocol

Nintedanib (Hölzel Diagnostika, Köln Germany) was dissolved in distilled water and 60 mg/kg nintedanib in 0.1-mL solution were given via gavage once a day. Treatment started on day 1 after transplantation and was continued until sacrifice. As a control group we treated allografts with pure distilled water (0.1 mL). Groups are therefore designated “nintedanib” and “untreated” in the following. The treatment dose of 60 mg/kg was selected from literature research. Wollin et al<sup>3</sup> received good results with this dosage in a mouse model of fibrotic lung disease. Similarly, Huang et al<sup>24</sup> found a good efficiency of the same dosage in the context of systemic sclerosis in mice and Bonella et al<sup>25</sup> state 30 to 100 mg/kg orally once daily as the effective dose for mice with good tolerance after application for 10 to 30 days.

### Analysis of the Aortic Graft

Aortic grafts were removed under anesthesia on days 14 and 30 after transplantation for polymerase chain reaction (PCR)-analysis and histological analysis respectively. The experimental timeframe was chosen with regard to published data, which showed day 14 as the time point of highest cytokine expression and day 30 as an appropriate time point for fully developed vasculopathy in the murine aortic transplant model.<sup>26</sup> Grafts were perfused with saline and were flash frozen in OCT medium (Tissue-Tek, Sakura, Netherlands) in liquid nitrogen for histologic analysis of 7- $\mu$ m cryostat sections or were stored in RNAlater (Qiagen) at  $-20^{\circ}\text{C}$  for further gene analysis.

### Morphometry

The quantitative measurement of neointima proliferation was performed on sections stained with Elastika-van-Gieson. Five sections distributed over the length of each graft were analyzed by 2 independent examiners blinded to the experimental conditions at an original magnification of  $\times 40$  using a conventional light microscope (Olympus BX51). A digitized image of each section was captured and the areas within the lumen and the internal and external elastic lamina were circumscribed manually and measured as previously described.<sup>26</sup> Interobserver differences were minimal and mean values were used for interpretation. All image analyses were carried out using cellSens Dimension image analysis software (Olympus, Germany).

### Antibodies

For this study we used the following antibodies: anti-FGFR1, anti-FGFR2, anti-VEGFR2, anti-PDGFR $\alpha$ , and anti-SMA (Abcam, Cambridge, UK); antimacrophage F4/80 and anti-CD205 (AbD Serotec MorphoSys, Duesseldorf, Germany); anti-CD4 (BD Bioscience, Heidelberg, Germany); anti-PDGFR $\beta$  (eBioscience, San Diego, CA) and anti-VEGFR1 (Novusbio, Littleton, CO). Furthermore the secondary antibody mouse antirat IgG-Cy3 (Dianova, Hamburg, Germany) was used for detection of anti-FGFR1, anti-FGFR2, anti-VEGFR1, anti-VEGFR2, anti-PDGFR $\alpha$ , anti-PDGFR $\beta$ , anti-macrophage F4/80, anti-CD205, and anti-CD4. Anti-SMA was visualized with the secondary antibody goat anti-rabbit IgG-Alexa Fluor 555 (Invitrogen; Eugene, OR) and endothelial cells were detected with CD31-FITC (BD Biosciences, Heidelberg, Germany). All slides were covered with Vectashield Hard Set Mounting medium with DAPI (Vector Laboratories, Burlingame, CA) for nucleus staining.

### Immunofluorescence

Aortic grafts were removed and frozen as described in the morphometric analysis above. Seven-micron cryostat sections were placed on slides and fixed in acetone for 10 minutes. Before application of the detecting antibodies, slides were rehydrated in staining buffer (1 $\times$  Tris-buffered saline and 0.05% Tween 20%). After that, standard protocols involve preincubation with heat-inactivated serum to minimize un-specific antibody binding before applying the primary and secondary antibody. All staining variations involved the detection of endothelial cells with CD31-FITC. Some protocols contained an additional permeabilization procedure with 0, 1% Triton X-100 before overnight incubation with the primary antibody to receive the best results (PDGFR $\alpha$ , VEGFR2, and FGFR2). All steps were conducted in a

humidified chamber at room temperature. The slides were mounted with Vectashield Hard Set Mounting medium. Analysis was done with epifluorescence microscopy (Olympus, Germany). Quantification of the intragraft cellular infiltrate on day 30 after transplantation was performed with computerized image analysis using cellSens software and an original magnification of 100 $\times$ .

### Analysis of Intragraft mRNA Expression

RNA was isolated from whole aortic grafts removed on day 14, which was previously established as the time point of highest cytokine expression.<sup>26</sup> Grafts were flushed with sterile saline and stored in RNAlater at  $-20^{\circ}\text{C}$  until analysis. RNA isolation and complementary DNA (cDNA) synthesis was performed according to standard protocols. Real-time quantitative PCR amplification was performed in triplets using the StepOne Real-Time PCR System and the TaqMan Gene Expression Master Mix (Applied Biosystems Forster City, CA). To generate PCR standards, the respective PCR product was cloned into a TOPO cloning vector (Invitrogen, Karlsruhe, Germany). The exact identity of the cloned amplicons was backed up by sequence analysis. Standard curves with known concentrations of template copy numbers were used to determine the expression of the amplified target. In each experimental setup, we also analyzed the 18S rRNA expression as a housekeeping gene by quantitative PCR. Afterward, the samples were normalized against the expression of the housekeeping gene (the ratio between the copies of the target gene cDNA and copies of the 18S rRNA multiplied with 1000000). The results are given as relative copy numbers.

Primers for tumor necrosis factor (TNF) $\alpha$ , IL-6, and PDGF-B are published in previous work of our group,<sup>27</sup> whereas primers for INF $\gamma$ , monocyte chemoattractant protein (MCP)-1, and CD40L are used as published by Overbergh et al.<sup>28</sup> The sequence from the primers and probe for platelet endothelial cell adhesion molecule (PECAM)-1 were designed with primer3 output and synthesized by Eurofins MWG Operon (Ebersberg, Germany) (PECAM-1: forward 5'-GAA CCC ATC AGG AGT GAA TAC GT-3'; reverse 5'-TGG ACC AAG TGT GTC ACT TGA AC-3' and probe: 5'-CGT GCA GGA GTC CT-3').

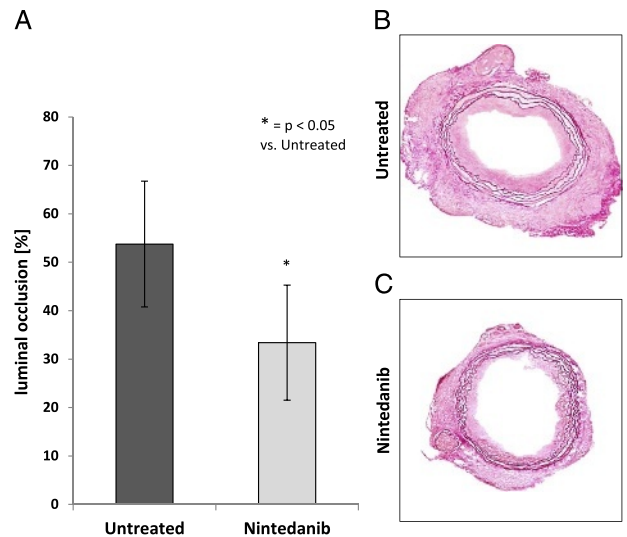
### Statistical Analysis

Results are given as the mean per group  $\pm$  SEM, which was derived from the mean per graft. Statistical analysis was done using a one-way analysis of variance followed by a Bonferroni correction using the SPSS software for windows (version 21). Differences between groups are considered as significant when *P* value is less than 0.05.

## RESULTS

### 30-day Treatment With Nintedanib Significantly Reduces Neointima Proliferation and SMC Migration

Main characteristic of CAV is the immigration of leukocytes and SMCs resulting in neointima formation.<sup>6,7</sup> We quantified the amount of vascular lumen that is occluded by this neointima and found a significantly reduced level of neointima in the group of nintedanib-treated animals in comparison with untreated controls 30 days after transplantation ( $33.4\% \pm 11.9\%$  vs  $53.8\% \pm 13.0\%$ ; *P* = 0.004; Figure 1). Congruously, immigration of smooth muscle cells into the neointima detected by immunofluorescence was significantly lower in nintedanib-treated grafts compared to untreated control



**FIGURE 1.** Treatment with nintedanib prevents intimal proliferation. Percentage of luminal occlusion was measured as described in section 2.5. on Elastica-van-Gieson stained graft sections from untreated control allografts and from nintedanib-treated allografts retrieved 30 days after transplantation (A). Isografts do not develop any neointima and therefore are not included into the diagram. B, C, Examples of analyzed microscopic images for untreated control allografts and nintedanib-treated allografts respectively. The pink layer between the open lumen and the darker curly structures of the media fibers represents the neointima. (n = 8 animals for each group, values given as mean  $\pm$  SD).

allografts ( $6528 \mu\text{m}^2 \pm 4863 \mu\text{m}^2$  vs  $34051 \mu\text{m}^2 \pm 8868 \mu\text{m}^2$ ; *P* < 0.001; Figure 2).

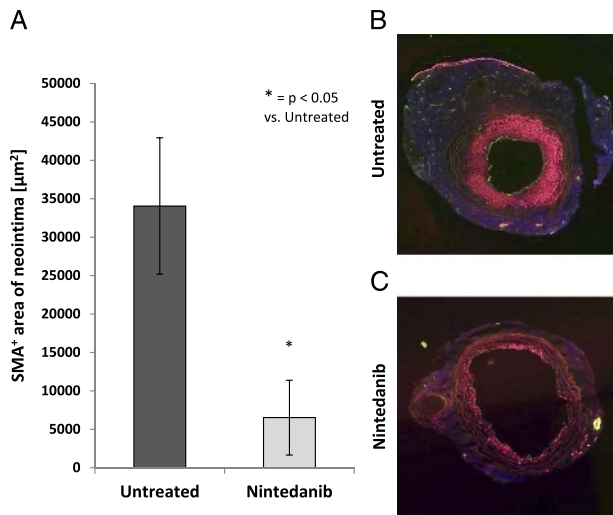
### Nintedanib Reduces the Infiltration of CD4<sup>+</sup> T Cells and Dendritic Cells, But Not the Infiltration of Macrophages Into the Graft Neointima

CD4<sup>+</sup> T cells, dendritic cells (DCs) and macrophages are all important contributors to the development of CAV.<sup>5,29-32</sup> We therefore measured their presence and distribution in the graft neointima by immunofluorescence staining. Comparing the area of neointima stained positive for CD4<sup>+</sup> T cells or DCs in nintedanib-treated and untreated allografts showed reduced values but without statistical significance for CD4<sup>+</sup> T cells in nintedanib-treated animals ( $1904 \mu\text{m}^2 \pm 1321 \mu\text{m}^2$  vs  $3646 \mu\text{m}^2 \pm 2496 \mu\text{m}^2$ ; (*P* = 0.12) (Figure 3A)) and lower values with a strong statistical significance for DCs ( $3539 \mu\text{m}^2 \pm 3175 \mu\text{m}^2$  vs  $10885 \mu\text{m}^2 \pm 1001 \mu\text{m}^2$ ; (*P* = 0.0023) (Figure 3B)). Amounts of macrophages did not differ in aortic grafts from nintedanib-treated animals and untreated controls ( $5185 \mu\text{m}^2 \pm 1132 \mu\text{m}^2$  for nintedanib group vs  $4993 \mu\text{m}^2 \pm 2073 \mu\text{m}^2$  for control (*P* = 0.85; Figure 3C)).

### Nintedanib Treatment Results in the Reduced Expression of PDGF Receptors $\alpha$ and $\beta$

To investigate whether continuous treatment with nintedanib influences the expression of the targeted tyrosine kinase containing receptors we analyzed the receptor distribution within the neointima of the aortic graft using immunofluorescence. For the PDGF receptors, PDGFR $\alpha$  and PDGFR $\beta$  were significantly reduced in nintedanib-treated animals ( $3596 \mu\text{m}^2 \pm 2867 \mu\text{m}^2$  vs  $15970 \mu\text{m}^2 \pm 6522 \mu\text{m}^2$  (*P* = 0.00062) for PDGFR $\alpha$  (Figure 4A) and  $10662 \mu\text{m}^2 \pm 7272 \mu\text{m}^2$  vs  $19440 \mu\text{m}^2 \pm 3467 \mu\text{m}^2$  (*P* = 0.048) for PDGFR $\beta$  (Figure 4B)). Expression of FGF receptors 1 and 2





**FIGURE 2.** Treatment with nintedanib reduces neointimal SMA quantity. SMA-positive area of the neointima was measured in grafts immunohistological stained with anti-SMA as described in the methods section: Immunofluorescence. A, Grafts of untreated control allografts and of allografts treated with nintedanib for 29 days were analyzed. B, C, Examples of analyzed microscopic images for untreated control allografts and nintedanib-treated grafts, respectively, with red indicating SMA, green indicating endothelium and blue marking cell nuclei ( $n = 5$  animals for each group, values given as mean  $\pm$  SD).

and VEGF receptor 2 was not altered by nintedanib treatment ( $3993 \mu\text{m}^2 \pm 1542 \mu\text{m}^2$  (nintedanib) vs  $4709 \mu\text{m}^2 \pm 560 \mu\text{m}^2$  (allografts) for FGFR1 ( $P = 0.49$ ; Figure 5C);  $11120 \mu\text{m}^2 \pm 5435 \mu\text{m}^2$  (nintedanib) vs  $10399 \mu\text{m}^2 \pm 1468 \mu\text{m}^2$  (allografts) for FGFR2 ( $P = 0.84$ ; Figure 5D) and  $6053 \mu\text{m}^2 \pm 1590 \mu\text{m}^2$  (nintedanib) vs  $6621 \mu\text{m}^2 \pm 2581 \mu\text{m}^2$  (allografts) for VEGFR2 ( $P = 0.76$ ; Figure 5B)). Interestingly, VEGFR1 expression was increased in nintedanib-treated animals though no significance was reached ( $6777 \mu\text{m}^2 \pm 1368 \mu\text{m}^2$  vs  $3942 \mu\text{m}^2 \pm 2574 \mu\text{m}^2$  ( $P = 0.17$ ; Figure 5A)).

### Treatment With Nintedanib Increases the Expression of Some Cytokines But Significantly Decreases the Expression of PDGF Within the Graft

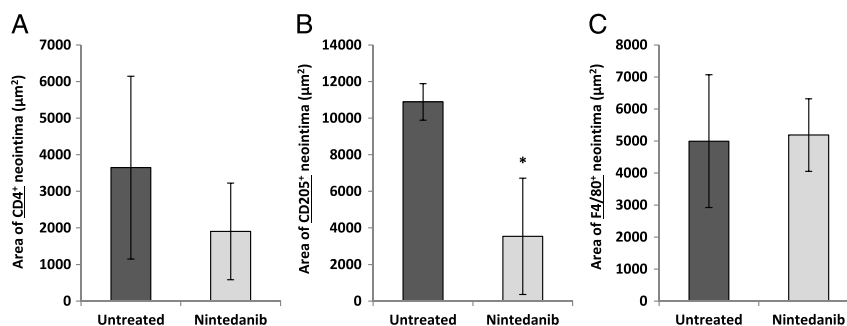
The development of CAV is driven by the release of proinflammatory cytokines from several cell types, predominantly macrophages and T cells, but also from nonimmune cells like SMCs or endothelial cells.<sup>5,6</sup>

We analyzed the expression of a variety of proinflammatory cytokines in the aortic graft and found statistically significant increases for nearly all of them in allografts compared to isografts, as was expected. After nintedanib treatment only PDGF and PECAM-1 showed a significantly decreased expression compared to untreated allografts and CD40L was slightly but statistically not significantly reduced. The expression levels of important cytokines with promoting effects on CAV development like  $\text{TNF}\alpha$ , IL-6,  $\text{INF}\gamma$ , and MCP-1 were not reduced as compared to untreated controls (Figure 6).

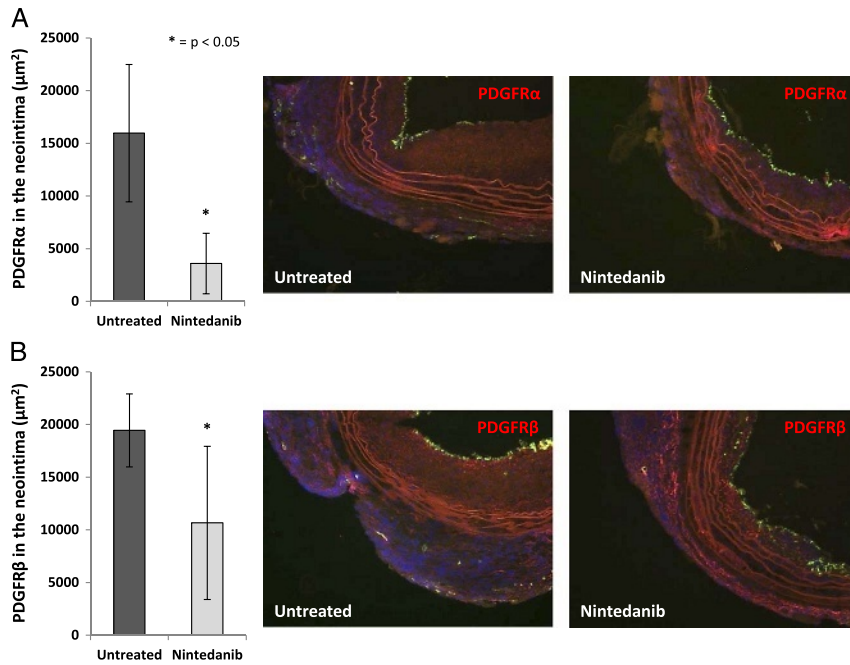
## DISCUSSION

Cardiac allograft vasculopathy still is the most important long term complication after heart transplantation and many efforts are taken to find a way to prevent its development.<sup>33</sup> In the current study, we tested nintedanib, a tyrosine kinase inhibitor with antagonistic effects on different growth factor receptors, for its impact on CAV. Results of our study revealed that treatment with nintedanib (1) significantly reduced CAV development after allogeneic aortic transplantation in mice, (2) which was characterized by significantly reduced amounts of neointimal smooth muscle cells accompanied by a decreased immigration of DCs and T cells into the neointima, and (3) by reduced expression of PDGF receptors  $\alpha$  and  $\beta$  as well as their ligand PDGF-B. This proposes a direct inhibition of SMC accumulation in the neointima via blocking their PDGF receptors combined with reduced DC immigration and subsequently decreased T-cell amounts in the neointima as the mechanisms of action.

The accumulation of smooth muscle cells in the intima with resulting intimal thickening is the most important feature of CAV.<sup>5,7,34</sup> Nintedanib significantly inhibited this process in our mouse model which can be explained by the effects of the targeted tyrosine kinase receptors (TKRs).  $\text{PDGFR}\alpha + \beta$  strongly mediate SMC proliferation and migration after ligand binding,<sup>11,30,31</sup> while VEGFR1 + 2 only mediate cell migration,<sup>32,33</sup> but additionally seem to be able to potentiate the mitogenic effect of FGF.<sup>32</sup> FGF again is a very potent factor for SMC proliferation<sup>34</sup> via its receptors FGFR1 and FGFR2.<sup>35</sup> Therefore blocking the activity of these receptors holds a great potential of inhibiting SMC migration and proliferation. Nintedanib is effective in our model of CAV development with regard to significantly reduced neointimal thickness and decreased SMC amounts.



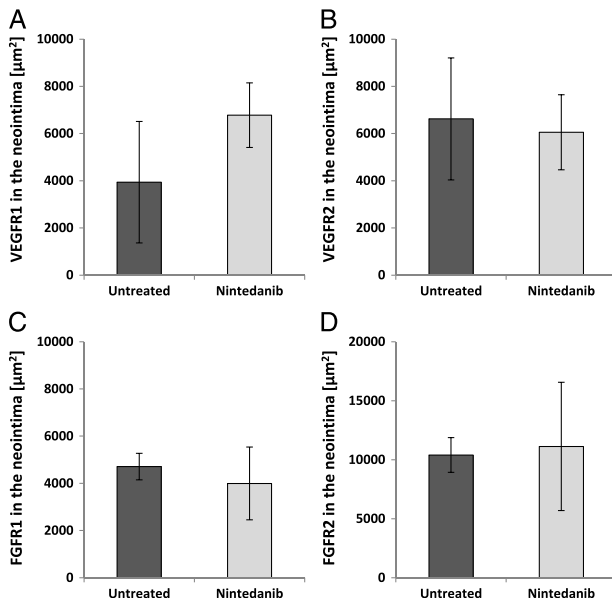
**FIGURE 3.** Effect of nintedanib treatment on graft infiltrating leukocytes. Grafts of untreated control allografts and nintedanib-treated allografts retrieved 30 days after transplantation were immunohistological stained with specific markers for  $\text{CD4}^+$  T cells (CD4; A); DCs (CD205; B) and macrophages (F4/80; C). Area of the graft stained positive for the respective receptor was measured as described in the methods section: Immunofluorescence ( $n = 6$  animals for each group (treated allografts and control allografts) and each leukocyte population, values given as mean  $\pm$  SD;  $*P < 0.05$  vs untreated control allograft).



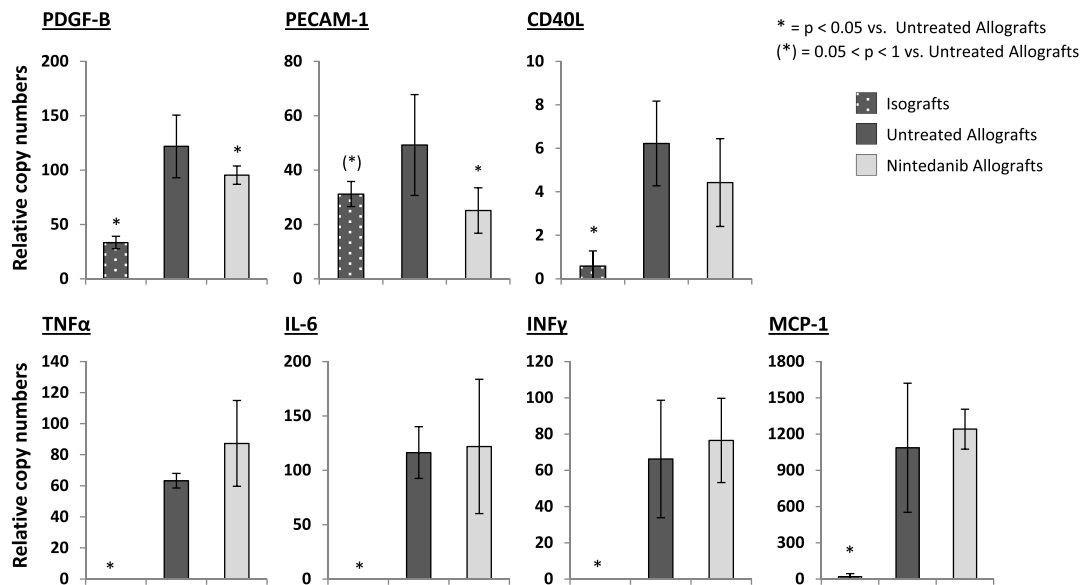
**FIGURE 4.** Expression of PDGF receptors in the neointima. Depicted in the diagrams is the area of neointima (μm<sup>2</sup>) immunohistologically stained positive for the respective receptors (PDGFRα antibody or PDGFRβ antibody) in both untreated control allografts and nintedanib-treated allografts and measured as described in the methods section: Immunofluorescence. Red granular staining in the neointima marks the respective PDGF receptor, green color indicates endothelium and blue color marks cell nuclei (n = 5 animals for each group (treated allografts and control allografts) and each receptor, values given as mean ± SD).

Continuous targeting of a receptor might change its expression levels, and we therefore analyzed the expression of TKRs after 30 days of treatment with nintedanib. The efficiency of nintedanib to dose-dependently inhibit TKRs was demonstrated in vitro for PDGFRs, VEGFRs, and FGFRs<sup>2,3</sup> and also in vivo in mice treated with 60 mg/kg p.o. for PDGFRs α and β.<sup>3</sup> Tyrosine kinase receptors are known to be internalized

and consequently downregulated after activation through ligand binding.<sup>35,36</sup> The activity of the receptor tyrosine kinase seems to be important for mediating this effect although the extent of its contribution is discussed controversially and seems to vary between different TKRs.<sup>37-39</sup> Few reports exist that describe the effect of tyrosine kinase inhibition on TKR expression, and although 1 study found reduced internalization and degradation of epidermal growth factor receptor after treatment with the tyrosine kinase antagonist genistein,<sup>40</sup> this result is contradicted by another study that found unchanged epidermal growth factor receptor internalization after inhibition of the receptor tyrosine kinase.<sup>41</sup> Observations of our own study were also diverse and partially even differed markedly between subtypes of the same TKR. Although the expression of both PDGFRα and PDGFRβ was clearly downregulated in nintedanib-treated grafts, the expression of FGFR1, FGFR2, and VEGFR2 was not influenced by nintedanib and interestingly the expression of the VEGFR1 was even elevated. This supports the notion that the expression of TKRs is not regulated uniformly, but differs among the individual receptors. Regarding the elevated expression of VEGFR1 it should be kept in mind that the VEGFR1 has a 10-fold lower tyrosine kinase activity than VEGFR2<sup>42,43</sup> and effects of the classical ligand VEGF-A are predominantly mediated via VEGFR2.<sup>43</sup> Therefore, high levels of VEGFR1 do not necessarily cause an amplified VEGF action. On the other hand, downregulation of both PDGFR subtypes in our experimental model might be the mechanistic link between nintedanib treatment and inhibition of SMC proliferation. Suesskind-Schwendi et al<sup>44</sup> used nintedanib in a rat lung transplantation model and also found receptor down-regulation (PDGFRα and VEGFR2), but interestingly did not reveal decreased fibrosis development after lung transplantation. This controversy to our results can be explained by a different



**FIGURE 5.** Expression of VEGF and FGF receptors in the neointima. Depicted is the area of the neointima (μm<sup>2</sup>) immunohistologically stained positive for the respective receptors in grafts of untreated control allografts and of nintedanib-treated allografts retrieved 30 days after transplantation (n = 5 animals for each group (treated allograft and control allografts) and each receptor, values given as mean ± SD).



**FIGURE 6.** Expression of inflammatory cytokines in aortic grafts. Intra-graft gene expression analysis was done by quantitative real time PCR as described in Materials and methods (Analysis of intra-graft mRNA expression), and each diagram shows relative copy numbers of the respective measured gene expression in untreated control isografts, untreated control allografts and nintedanib-treated allografts retrieved 14 days after transplantation ( $n = 6$  animals for each group (isografts, untreated allografts and nintedanib-treated grafts) and for each gene, values given as mean  $\pm$  SD).

pathomechanism in the aortic transplant model: neointima formation in CAV initially is driven by SMC proliferation and accumulation as well as leukocyte infiltration whereas fibrosis represents only a minor aspect in CAV. Further studies would be necessary to specify the regulation of receptor expression.

Beside SMCs, leukocytes are of great importance in the pathogenesis of CAV. Dendritic cells as “professional” antigen presenting cells<sup>29</sup> promote the development of CAV by initiating the activation of T cells.<sup>45</sup> We found significantly reduced graft infiltrating DCs after nintedanib treatment compared with untreated control allografts which might be another mechanism of action of nintedanib in addition to inhibiting SMC proliferation. Reports concerning the existence of TKRs on DCs though are rare and can only be found for VEGFRs which are supposed to play a role in DC differentiation<sup>46</sup> and the inhibition of T cell activation.<sup>47</sup> One might therefore hypothesize that upregulated VEGFR1 after nintedanib treatment reduces DC ability to activate T cells. Additionally, decreased amounts of DCs found in the neointima of allografts after nintedanib treatment might also suggest a role for tyrosine kinases in the migration of DCs.

Of the several distinct subtypes of T cells especially CD4<sup>+</sup> T cells are associated with the promotion of CAV and subsequent neointima formation.<sup>30,31,48,49</sup> Our results show reduced CD4<sup>+</sup> presence in grafts of nintedanib-treated animals, which is possibly associated with reduced stimulation by DCs as a cause of their decreased numbers as mentioned above. Another link to reduced T-cell numbers can be seen in the lowered intra-graft expression of CD40L in the nintedanib group, because binding of lymphocyte CD40L is an important costimulatory signal for T-cell activation.<sup>50</sup> Additionally, reduced T-cell amounts could also derive from a direct effect of nintedanib on T cells. Though the connection between T cells and growth factor receptors is relatively poorly explored, several studies demonstrate the existence of PDGFRs,<sup>10,51</sup>

VEGFR1<sup>52,53</sup> and FGFRs<sup>54</sup> on T cells and ascribe them functions in inhibition of T cell activity,<sup>10,51</sup> promotion of T cell recruitment<sup>52</sup> and chemotaxis,<sup>53</sup> promotion of IL-10 production and possibly inhibition of INF $\gamma$  release<sup>53</sup> as well as costimulation in T-cell activation.<sup>54</sup> Additionally, some studies describe concrete observations made for the influence of nintedanib on T cells. Wollin et al<sup>3</sup> found that nintedanib reduces lymphocyte counts in the bronchoalveolar lavage of mice with bleomycin or silica induced pulmonary fibrosis, but no further division between T and B lymphocytes and their respective subtypes was made.

Macrophages play, like DCs and CD4<sup>+</sup> T cells, a central role in the pathogenesis of CAV.<sup>30,31,48,49,55</sup> Therefore, we examined their occurrence in the neointima of the different experimental groups. Interestingly, we found no alteration regarding the number of graft infiltrating macrophages after nintedanib treatment. This is in contrast to reported results from different mouse fibrosis models, where nintedanib prevented the pathogenesis associated increase of macrophages.<sup>24,56</sup> Huang et al<sup>24</sup> showed in vitro as well as in vivo that the inhibitory effect of nintedanib is due to blocking macrophage polarization into the M2 subtype. M1 macrophage counts were not affected by nintedanib in this experiment. These findings implicate a dominant role for M1 macrophages in the inflammatory processes of CAV, while M2 macrophages are important in the development of fibrosis.<sup>57</sup> The above-mentioned findings may also explain some of our cytokine expression results described in the following section.

Intra-graft expression analysis of multiple proinflammatory cytokines revealed increased levels in the untreated control allografts compared to isografts, as was expected. Similar high values could be found after nintedanib treatment (eg, TNF $\alpha$ , IL-6, INF $\gamma$  and MCP-1), which confirms that rather M1 macrophages with their typical inflammatory mediators are the important subpopulation in our transplant model. Moreover, several cytokines exert a positive feedback and

promote macrophage immigration, for example MCP-1,<sup>45</sup> IL-6<sup>46</sup> or TNF $\alpha$ .<sup>47</sup> Interestingly, there was a significantly reduced expression of PDGF, ligand for the PDGFR, and one of the most potent stimulators of SMC proliferation<sup>58</sup> after treatment with nintedanib. This supports the hypothesis that inhibition of PDGF signaling finally leads to the drastically reduced intimal SMC accumulation. This mechanism even seems strong enough to not be overruled by high levels of inflammatory cytokines.

## CONCLUSIONS

Treatment with nintedanib significantly reduced the development of CAV in a murine model of aortic transplantation. As the mechanism of action we assume a direct inhibition of migratory and proliferative signals on SMCs caused by blocking their TKRs combined with the reduced expression of PDGF. Additional positive influence could be mediated by the inhibited immigration of DCs and T cells. The result of this study includes translational implications, because nintedanib is already approved for human treatment.

## ACKNOWLEDGMENTS

The authors acknowledge support by Deutsche Forschungsgemeinschaft and Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU) within the funding program Open Access Publishing. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

## REFERENCES

- Wollin L, Wex E, Pautsch A, et al. Mode of action of nintedanib in the treatment of idiopathic pulmonary fibrosis. *Eur Respir J*. 2015;45:1434–1445.
- Hilberg F, Roth GJ, Krssak M, et al. BIBF 1120: triple angiokinase inhibitor with sustained receptor blockade and good antitumor efficacy. *Cancer Res*. 2008;68:4774–4782.
- Wollin L, Mailet I, Quesniaux V, et al. Antifibrotic and anti-inflammatory activity of the tyrosine kinase inhibitor nintedanib in experimental models of lung fibrosis. *J Pharmacol Exp Ther*. 2014;349:209–220.
- Lund LH, Edwards LB, Dipchand AI, et al. The Registry of the International Society for Heart and Lung Transplantation: thirty-third adult heart transplantation Report—2016; focus theme: primary diagnostic indications for transplant. *J Heart Lung Transplant*. 2016;35:1158–1169.
- Rahmani M, Cruz RP, Granville DJ, et al. Allograft vasculopathy versus atherosclerosis. *Circ Res*. 2006;99:801–815.
- Ramzy D, Rao V, Brahm J, et al. Cardiac allograft vasculopathy: a review. *Can J Surg*. 2005;48:319–327.
- Libby P, Pober JS. Chronic rejection. *Immunity*. 2001;14:387–397.
- Heim C, Eckl S, Preidl R, et al. Delayed therapy with clopidogrel and everolimus prevents progression of transplant arteriosclerosis and impairs humoral alloimmunity in murine aortic allografts. *Eur J Cardiothorac Surg*. 2015;47:180–187.
- Daly KP, Seifert ME, Chandraker A, et al. VEGF-C, VEGF-A and related angiogenesis factors as biomarkers of allograft vasculopathy in cardiac transplant recipients. *J Heart Lung Transplant*. 2013;32:120–128.
- Raines EW. PDGF and cardiovascular disease. *Cytokine Growth Factor Rev*. 2004;15:237–254.
- Miller GG, Davis SF, Atkinson JB, et al. Longitudinal analysis of fibroblast growth factor expression after transplantation and association with severity of cardiac allograft vasculopathy. *Circulation*. 1999;100:2396–2399.
- Nelson PR, Yamamura S, Mureebe L, et al. Smooth muscle cell migration and proliferation are mediated by distinct phases of activation of the intracellular messenger mitogen-activated protein kinase. *J Vasc Surg*. 1998;27:117–125.
- Mancini MC, Evans JT. Role of platelet-derived growth factor in allograft vasculopathy. *Ann Surg*. 2000;231:682–688.
- Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*. 2004;84:767–801.
- Meliss RR, Pethig K, Schmidt A, et al. Cardiac allograft vasculopathy: ad-ventitial immunoreactivity for PDGF-B and PDGFR-beta in extra- versus intramural coronary arteries. *Transplant Proc*. 2001;33:1579–1580.
- Reinders ME, Fang JC, Wong W, et al. Expression patterns of vascular endothelial growth factor in human cardiac allografts: association with rejection. *Transplantation*. 2003;76:224–230.
- Lemstrom KB, Krebs R, Nykanen AI, et al. Vascular endothelial growth factor enhances cardiac allograft arteriosclerosis. *Circulation*. 2002;105:2524–2530.
- Roux N, Leveque S, Freguin-Bouilland C, et al. A kinetic study of SDF-1, VEGF and MCP-1 blood and tissue levels after aortic transplantation in mice. *Acta Histochem*. 2012;114:636–638.
- Nykanen AI, Sandelin H, Krebs R, et al. Targeting lymphatic vessel activation and CCL21 production by vascular endothelial growth factor receptor-3 inhibition has novel immunomodulatory and antiarteriosclerotic effects in cardiac allografts. *Circulation*. 2010;121:1413–1422.
- Itoh N, Omitz DM. Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. *J Biochem*. 2011;149:121–130.
- Zhao XM, Citrin BS, Miller GG, et al. Association of acidic fibroblast growth factor and untreated low grade rejection with cardiac allograft vasculopathy. *Transplantation*. 1995;59:1005–1010.
- Luo W, Liu A, Chen Y, et al. Inhibition of accelerated graft arteriosclerosis by gene transfer of soluble fibroblast growth factor receptor-1 in rat aortic transplants. *Arterioscler Thromb Vasc Biol*. 2004;24:1081–1086.
- Koulack J, McAlister VC, Giacomantonio CA, et al. Development of a mouse aortic transplant model of chronic rejection. *Microsurgery*. 1995;16:110–113.
- Huang J, Maier C, Zhang Y, et al. Nintedanib inhibits macrophage activation and ameliorates vascular and fibrotic manifestations in the Fra2 mouse model of systemic sclerosis. *Ann Rheum Dis*. 2017;76:1941–1948.
- Bonella F, Stowasser S, Wollin L. Idiopathic pulmonary fibrosis: current treatment options and critical appraisal of nintedanib. *Drug Des Devel Ther*. 2015;9:6407–6419.
- Ensminger SM, Spriewald BM, Witzke O, et al. Kinetics of transplant arteriosclerosis in MHC-Class I mismatched and fully allogeneic mouse aortic allografts. *Transplantation*. 2002;73:1068–1074.
- Abele S, Spriewald BM, Ramsperger-Gleixner M, et al. Attenuation of transplant arteriosclerosis with clopidogrel is associated with a reduction of infiltrating dendritic cells and macrophages in murine aortic allografts. *Transplantation*. 2009;87:207–216.
- Overbergh L, Giulietti A, Valckx D, et al. The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *J Biomol Tech*. 2003;14:33–43.
- Costello JP, Mohanakumar T, Nath DS. Mechanisms of chronic cardiac allograft rejection. *Tex Heart Inst J*. 2013;40:395–399.
- Kitchens WH, Chase CM, Uehara S, et al. Macrophage depletion suppresses cardiac allograft vasculopathy in mice. *Am J Transplant*. 2007;7:2675–2682.
- Liu Z, Fan H, Jiang S. CD4(+) T-cell subsets in transplantation. *Immunol Rev*. 2013;252:183–191.
- Raimondi G, Thomson AW. Dendritic cells, tolerance and therapy of organ allograft rejection. *Contrib Nephrol*. 2005;146:105–120.
- Heim C, Gocht A, Weyand M, et al. New targets for the prevention of chronic rejection after thoracic organ transplantation. *Thorac Cardiovasc Surg*. 2018;66:20–30.
- Nagano H, Mitchell RN, Taylor MK, et al. Interferon-gamma deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. *J Clin Invest*. 1997;100:550–557.
- Goh LK, Sorkin A. Endocytosis of receptor tyrosine kinases. *Cold Spring Harb Perspect Biol*. 2013;5:a017459.
- Miaczynska M. Effects of membrane trafficking on signaling by receptor tyrosine kinases. *Cold Spring Harb Perspect Biol*. 2013;5:a009035.
- Sorkina T, Huang F, Beguinot L, et al. Effect of tyrosine kinase inhibitors on clathrin-coated pit recruitment and internalization of epidermal growth factor receptor. *J Biol Chem*. 2002;277:27433–27441.
- Sorkin A, Westermarck B, Heldin CH, et al. Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF beta-receptor. *J Cell Biol*. 1991;112:469–478.
- Sorokin A, Mohammadi M, Huang J, et al. Internalization of fibroblast growth factor receptor is inhibited by a point mutation at tyrosine 766. *J Biol Chem*. 1994;269:17056–17061.
- Yang EB, Wang DF, Mack P, et al. Genistein, a tyrosine kinase inhibitor, reduces EGF-induced EGF receptor internalization and degradation in human hepatoma HepG2 cells. *Biochem Biophys Res Commun*. 1996;224:309–317.



41. Wang Q, Villeneuve G, Wang Z. Control of epidermal growth factor receptor endocytosis by receptor dimerization, rather than receptor kinase activation. *EMBO Rep.* 2005;6:942–948.
42. Shibuya M. Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) signaling in angiogenesis: a crucial target for anti- and pro-angiogenic therapies. *Genes Cancer.* 2011;2:1097–1105.
43. Yao J, Wu X, Zhuang G, et al. Expression of a functional VEGFR-1 in tumor cells is a major determinant of anti-PlGF antibodies efficacy. *Proc Natl Acad Sci U S A.* 2011;108:11590–11595.
44. von Suesskind-Schwendi M, Boxhammer E, Hirt SW, et al. The activity of nintedanib in an animal model of allogenic left lung transplantation resembling aspects of allograft rejection. *Exp Lung Res.* 2017;43:259–270.
45. Deshmane SL, Kremlev S, Amini S, et al. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res.* 2009;29:313–326.
46. Scheller J, Chalaris A, Schmidt-Arras D, et al. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta.* 1813;2011:878–888.
47. Parameswaran N, Patial S. Tumor necrosis factor- $\alpha$  signaling in macrophages. *Crit Rev Eukaryot Gene Expr.* 2010;20:87–103.
48. Szeto WY, Krasinskas AM, Kreisel D, et al. Depletion of recipient CD4+ but not CD8+ T lymphocytes prevents the development of cardiac allograft vasculopathy. *Transplantation.* 2002;73:1116–1122.
49. Shi C, Lee WS, He Q, et al. Immunologic basis of transplant-associated arteriosclerosis. *Proc Natl Acad Sci U S A.* 1996;93:4051–4056.
50. Elgueta R, Benson MJ, de Vries VC, et al. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev.* 2009;229:152–172.
51. Daynes RA, Dowell T, Araneo BA. Platelet-derived growth factor is a potent biologic response modifier of T cells. *J Exp Med.* 1991;174:1323–1333.
52. Zhang J, Silva T, Yarovinsky T, et al. VEGF blockade inhibits lymphocyte recruitment and ameliorates immune-mediated vascular remodeling. *Circ Res.* 2010;107:408–417.
53. Shin JY, Yoon IH, Kim JS, et al. Vascular endothelial growth factor-induced chemotaxis and IL-10 from T cells. *Cell Immunol.* 2009;256:72–78.
54. Byrd VM, Kilkenny DM, Dikov MM, et al. Fibroblast growth factor receptor-1 interacts with the T-cell receptor signalling pathway. *Immunol Cell Biol.* 2003;81:440–450.
55. Belperio JA, Ardehali A. Chemokines and transplant vasculopathy. *Circ Res.* 2008;103:454–466.
56. Ackermann M, Kim YO, Wagner WL, et al. Effects of nintedanib on the microvascular architecture in a lung fibrosis model. *Angiogenesis.* 2017;20:359–372.
57. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity.* 2016;44:450–462.
58. Li L, Blumenthal DK, Terry CM, et al. PDGF-induced proliferation in human arterial and venous smooth muscle cells: molecular basis for differential effects of PDGF isoforms. *J Cell Biochem.* 2011;112:289–298.