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ORIGINAL ARTICLE

SDF-1/CXCR4/CXCR7 is pivotal for vascular smooth muscle cell proliferation and chronic allograft vasculopathy

Michael N. Thomas,[1] Aivars Kalnins,[1] Martin Andrassy,[2] Anne Wagner,[1] Sven Klussmann,[3] Markus Rentsch,[1] Antje Habicht,[4] Sebastian Pratschke,[1] Manfred Stangl,[1] Alexandr V. Bazhin,[1] Bruno Meiser,[4] Michael Fischereder,[5] Jens Werner,[1] Markus Guba[1] and Joachim Andrassy[1]

1 Klinik für Allgemeine-,Viszeral-, Transplantations-, Gefäß- und Thoraxchirurgie, Klinikum der Universität München, Ludwig-Maximilians Universität, München, Germany
2 Innere Medizin III: Kardiologie, Angiologie und Pneumologie, Universitätsklinikum Heidelberg, Rupprecht-Karls Universität, Heidelberg, Germany
3 NOXXON Pharma AG, Berlin, Germany
4 Transplantationszentrum, Klinikum der Universität München, Ludwig-Maximilians Universität, München, Germany
5 Nephrologisches Zentrum, Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, Ludwig-Maximilians Universität, München, Germany

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Correspondence
PD Dr. med. Joachim Andrassy, Chirurgische Klinik und Poliklinik der Universität München, Campus Großhadern, Marchioninistrasse 15, 81375 München, Deutschland, Germany.
Tel.: +49 (0) 89/4400-7-2656;
fax: +49 (0) 89/4400-7-5655;
e-mail: joachim.andrassy@med.uni-muenchen.de

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Summary

Chronic rejection remains a major obstacle in transplant medicine. Recent studies suggest a crucial role of the chemokine SDF-1 on neointima formation after injury. Here, we investigate the potential therapeutic effect of inhibiting the SDF-1/CXCR4/CXCR7 axis with an anti-SDF-1 Spiegelmer (NOX-A12) on the development of chronic allograft vasculopathy. Heterotopic heart transplants from H-2bm12 to B6 mice and aortic transplants from Balb/c to B6 were performed. Mice were treated with NOX-A12. Control animals received a nonfunctional Spiegelmer (revNOX-A12). Samples were retrieved at different time points and analysed by histology, RT-PCR and proliferation assay. Blockade of SDF-1 caused a significant decrease in neointima formation as measured by intima/media ratio (1.0 ± 0.1 vs. 1.8 ± 0.1, $P < 0.001$ AoTx; 0.35 ± 0.05 vs. 1.13 ± 0.27, $P < 0.05$ HTx). *In vitro* treatment of primary vascular smooth muscle cells with NOX-A12 showed a significant reduction in proliferation (0.42 ± 0.04 vs. 0.24 ± 0.03, $P < 0.05$). TGF-β, TNF-α and IL-6 levels were significantly reduced under SDF-1 inhibition (3.42 ± 0.37 vs. 1.67 ± 0.33, $P < 0.05$; 2.18 ± 0.37 vs. 1.0 ± 0.39, $P < 0.05$; 2.18 ± 0.26 vs. 1.6 ± 0.1, $P < 0.05$). SDF-1/CXCR4/CXCR7 plays a critical role in the development of chronic allograft vasculopathy (CAV). Therefore, pharmacological inhibition of SDF-1 with NOX-A12 may represent a therapeutic option to ameliorate chronic rejection changes.

Introduction

With the introduction of powerful immunosuppressive protocols, early graft loss, due to therapy refractory rejection, has become a rare event in solid organ transplantation. However, current long-term graft survival rates have not significantly improved over the past years [1]. In the US, half-life of deceased renal transplants only modestly increased from 6.6 to 8.8 years within a time frame of 16 years. Most of the improvement was made in immunological high-risk patients, while half-lives in low-risk patients remained unchanged [2]. Similar results were reported for the half-lives of liver, lung, heart, intestine and pancreas transplantation [3]. Death with functioning graft mostly due to cardiovascular events, infections, malignancy, direct toxic effects of the immunosuppressants and chronic rejection processes remains the major problem after organ transplantation. Current ISHLT data indicate

that chronic allograft vasculopathy (CAV) is responsible for 14–20% of the deaths of heart transplant recipients who survived more than 1 year. [4].

Chronic allograft vasculopathy is caused by a continuous immune response against the endothelium and media [5,6]. A variety of different cells have been made responsible for these changes. Experimentally, CD8+ and CD4+ T helper cells, B cells and monocytes amongst others were shown to contribute to the neointima formation [7–9]. In clinical settings, HLA class I and II antibodies were repetitiously linked with chronic vascular changes [10,11]. Importantly, whatever the causative cell/antibody or agent may be, the proliferation of the smooth muscle cells (SMCs) and the remodelling of the extracellular matrix (ECM) are the key targets leading to the histological picture of CAV.

The role of chemokines and their receptors for the rejection process have been studied extensively [12]. A particular significance for the development of CAV could be attributed to CXCR3 and MCP-1 [13,14]. SDF-1α/CXCR4 was shown to be involved in the process of SMC recruitment and proliferation after mechanical injury to large vessels [15–17]. Furthermore, CXCR4 is expressed on cells important for CAV development (T cells, monocytes, neutrophils, ECs and SMCs) [18]. Recently, another receptor for the SDF-1 has been identified [19,20]. Similar to CXCR4, the CXCR7 is expressed on vascular ECs and participates in the regulation of immunological mechanisms and stem cell trafficking [21].

With the SDF-1 inhibiting compound NOX-A12, a potent substance is available to block the SDF-1/CXCR4/CXCR7 pathway [22]. NOX-A12 is a so-called Spiegelmer®, a mirror-image RNA oligonucleotide that binds its target SDF-1 with subnanomolar affinity through its three-dimensional structure. The mode of binding is comparable to an antibody that recognizes an antigen. Due to its mirror-image nature, a Spiegelmer oligonucleotide cannot be recognized by naturally occurring nucleases resulting in an increased biostability [23]. Proven to be well tolerated in healthy individuals in early clinical trials [24], NOX-A12 was furthermore shown to be highly efficacious, blocking the migration of lymphoid cell lines [25].

The aim of this study was to investigate the effect of a Spiegelmer-based inhibition of SDF-1/CXCR4/CXCR7 on the development of CAV in two different murine models.

Methods

Animals

C57 Bl/6 (H^{2b}) and Balb/c (H^{2d}) mice were obtained from Charles River (Sulzfeld, Germany). H2-bm12 mice were purchased from Jackson Laboratory (Bar Harbour, ME, USA) and used at the age of 6–8 weeks. Animals were housed in filter-toped cages with unlimited access to food and water for the duration of the study. All procedures involving animals were performed according to the guidelines of the German animal welfare act (TierSchG).

Aortic transplantation (AoTx)

Murine aortic transplantation was performed using the modified nonsuture-cuff technique as previously described [26]. Briefly, a segment of the thoracic aorta of Balb/c ($H2^{d}$) mice was transplanted end to end to the right common carotid artery of C57Bl/6 ($H2^{b}$) mice. Aortic grafts were harvested 4 weeks after transplantation.

Heterotopic Heart transplantation (HTx)

Murine heart transplantation was performed as previously described using a slightly modified technique [27]. Briefly, hearts of H-2bm12 ($H2^{bm12}$) mice were transplanted heterotopically into the abdominal cavity of C57Bl/6 ($H2^{b}$) mice with vascular anastomoses on the abdominal aorta and inferior V. cava. Grafts were checked by daily palpation. After 40 days, grafts were harvested for further analyses.

Treatment

The SDF-1 antagonistic Spiegelmer NOX-A12 consisting of the primary sequence 5′-GCGUGGUGUGAUCUAGAUG UAUUGGCUGAUCCUAGUCAGGUACGC-3′ and modified with 40-KDa polyethyleneglycol (PEG) at its 5′-terminus as well as the nonfunctional Spiegelmer revNOX-A12 (5′-UAAGGAAACUCGGUCUGAUGCGGUAGCGCUGUG CAGAGCU-3′) equally modified were synthesized at NOXXON Pharma AG (Berlin, Germany). The treatment groups received either 15.5 mg/kg body weight NOX-A12 (based on molecular weight of the oligonucleotide part) or nonfunctional revNOX-A12 (serving as a control) intraperioneally (i.p.) every other day.

Histology

Four weeks after transplantation, aortic transplants were harvested and frozen for further histological analyses. Ten samples were cut from different sections of each graft and stained with haematoxylin/eosin (H&E) and Elastica van Gieson (EvG). The ratio of neointima/media was evaluated in double-blinded fashion as described elsewhere [28].

Immunohistochemistry

Paraffin-embedded sections of transplanted aortas were incubated with SM22 antibody (Abcam, Cambridge, UK)

and visualized with donkey polyclonal secondary antibody to rabbit IgG (Abcam, Cambridge, UK). Nuclei were counterstained with Hoechst 33258 (Sigma Aldrich, Taufkirchen, Germany) and analysed under fluorescence microscopy.

For staining of $CD3^+$ cells, frozen sections of aortic grafts were incubated over night with anti-mouse CD3 antibody (Pierce Biotechnology, Rockford, IL, USA) followed by secondary biotinylated antihamster IgG (Vector Labs, Burlingame, CA, USA). $CD3^+$ cells were visualized using the Vectastain ABC-AP kit (Vector Labs, Burlingame, CA, USA).

VSMC Culture

Primary mouse smooth muscle cells were isolated from the thoracic aorta of untreated Balb-c mice and cultured as described before [29]. Cells were cultured in DMEM F12 + 10% FCS and penicillin/streptomycin. For experiments, cells in passages 2–8 were used.

Proliferation Assay

Primary mouse vascular smooth muscle cells (VSMCs) were seeded on 96-well plates (1×10^4 cells/well) and cultured in DMEM/F12 containing 10% FCS. After 24 h, the media was replaced by serum-free media for 48 h to achieve synchronous growth arrest. Cells were then stimulated with 10% FCS containing medium with or without Spiegelmers or SDF1-α and with BrdU working solution.

NOX-A12 and revNOX-A12 were added at a concentration of 100 μg/ml and SDF1-α at a concentration of 10 ng/ml for 24 h.

BrdU uptake was determined using the cell proliferation ELISA BrdU assay (Roche, Penzberg, Germany). BrdU incorporation into DNA was measured after 24 h by photometric analysis using a microplate reader (Bio-Rad, Hercules, CA, USA). Experiments were repeated three times in triplicate.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from heart samples using Trizol (Invitrogen, Carlsbad, CA, USA). To determine the mRNA expression levels, 1 μg of total RNA was used to perform reverse transcription and quantitative real-time PCR using LightCycler (Roche) as described previously [30]. The primer sequences were as follows: IL-6 forward 5′-GATGC TACCAAACTGGATATAATC-3′ and IL-6 reverse: 5′-GG TCCTTAGCCACTCCTTCTGTG-3′; TNF-α forward 5′-CC ATTCCTGAGTTCTGCAAAG-3′ and TNF-α reverse 5′-GC AAATATAAATAGAGGGGGGC-3′; TGF-β forward 5′-GCA CTGCGCTGTCTCGCAAG-3′ and TGF-β reverse 5′- ACG CCGGGTAGCGATCGAGTG-3′; and β-actin forward 5′-C CCTAAGGCCAACCGTGAAA-3′ and β-actin reverse 5′-AC GACCAAGGCATACAGGGA-3′.

Expression of IL-6, TNF-α and TGF-β was normalized against β-actin as housekeeping gene.

Statistical analysis

All data are represented as mean ± SD. Statistical analysis was performed using Graph Pad Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA) for Mac using ANOVA. A P value < 0.05 was considered statistically significant.

Results

Inhibition of SDF-1 ameliorates vasculopathy after heart and aortic transplantation

C57Bl/6 aortas were transplanted into Balb/c mice. In untreated mice, extensive neointimal hyperplasia was observed 4 weeks after transplantation (Fig. 1a). These

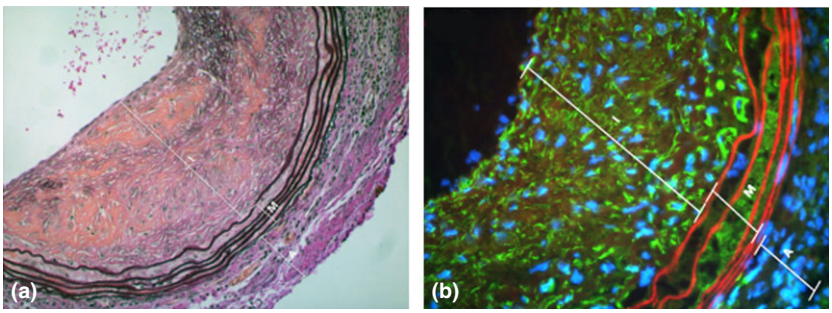

Figure 1 (a) Elastika van Gieson staining of aortic grafts 4 weeks after transplantation (BALB/c to C57BL/6), showing a significant thickening of the neointimal layer (20× magnification). (b) Immunohistochemistry for SM22 of aortic grafts 4 weeks after transplantation (BALB/c to C57BL/6), showing a significant thickening of the neointimal layer consisting primarily of vascular smooth muscle cells. Nuclei (blue) were stained with Hoechst 33258. (I: neointima; M: media; A: adventitia) (20× magnification).

neointimal lesions mainly consisted of vascular smooth muscle cells as demonstrated by SM22 staining [31] (Fig. 1b). Inhibition of SDF-1 with NOX-A12 resulted in a significant reduction in the intima/media ratios compared to the control mice (1.0 ± 0.1 (NOX-A12; $n = 6$) vs. 1.8 ± 0.1 (revNOX-A12; $n = 6$); $P < 0.001$ (Fig. 2)).

To substantiate these results, we used an established murine model of chronic rejection. In this model, hearts from H-2bm12 were transplanted heterotopically into the abdominal cavity of C57Bl/6 mice. The H-2bm12 and C57Bl/6 differ at the I-A locus of MHC class II antigen but are identical at MHC class I and minor MHC loci. After 40 days, the hearts were harvested. All hearts were still beating at this time. In line with our findings for the aortic transplants, the histological analysis revealed a significant decrease in the intima/media ratios of NOX-A12-treated animals compared to the control (0.35 ± 0.05 (NOX-A12; $n = 6$) vs. 1.13 ± 0.27 (revNOX-A12; $n = 6$); $P < 0.05$) (Fig. 3).

Inhibition of SDF-1 has a direct inhibitory effect on VSMC proliferation *in vitro*

We next examined the effects of NOX-A12 on VSMC proliferation *in vitro*. Primary VSMCs were isolated and cultured from thoracic aortas of Balb/c mice. After 48 h of starvation, VSMCs were stimulated for 24 h with serum containing 10% FCS, which led to a significant proliferation of VSMCs (0.083 ± 0.02 (Starve) vs. 0.365 ± 0.033 (revNOX-A12), $P < 0.0001$). When these cells were treated with NOX-A12 at a dose of 100 µg/ml, a significant decrease in proliferating cells could be observed (0.365 ± 0.033 (Ctr) vs. 0.19 ± 0.014 (NOX-A12), $P = 0.0002$).

Addition of 10 ng/ml SDF-1α (Roche, Penzberg, Germany) showed a trend towards more proliferating cells, without reaching significant levels (0.365 ± 0.033 (Ctr) vs. 0.395 ± 0.035 (SDF1), $P = 0.533$) (Fig. 4).

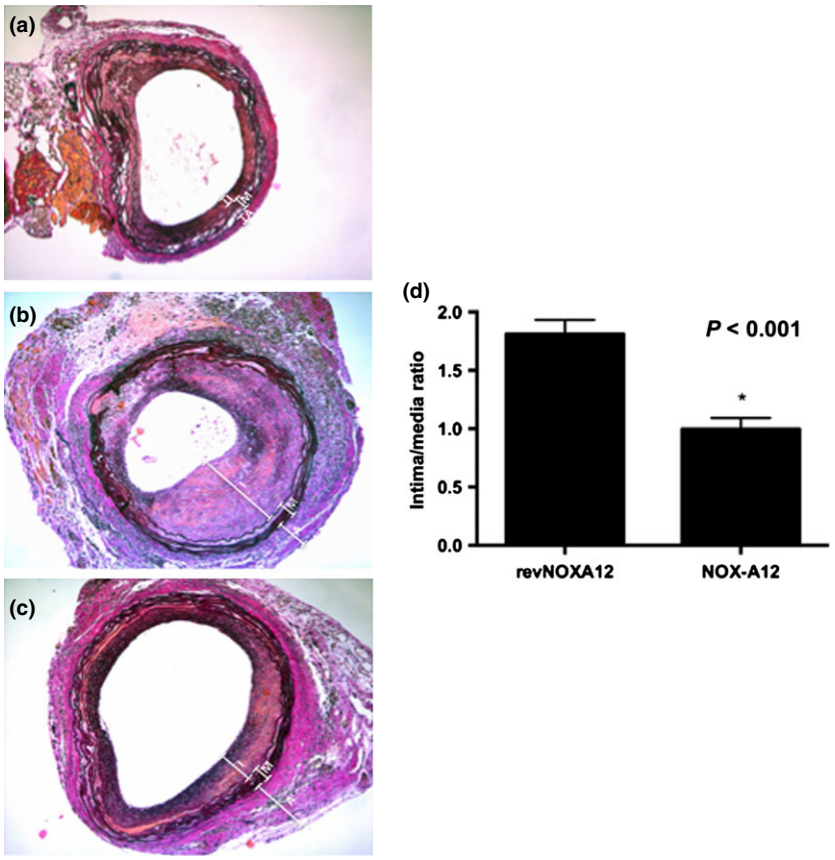

Figure 2 (a-c) Elastica van Gieson staining of aortic grafts 4 weeks after transplantation. Pictures are representative of 4 animals; I: neointima, M: media and A: adventitia. (a: syngeneic group (BALB/c to Balb/c), b: revNOX-A12 control group (BALB/c to C57BL/6), c: NOX A12-treated group (BALB/c to C57BL/6)). (d) Histomorphometric analysis of intima/media ratio of aortic allografts harvested at 4 weeks after transplantation ($n = 4$ for each group, *$P < 0.001$ revNox A12 vs. Nox-A12 treatment).

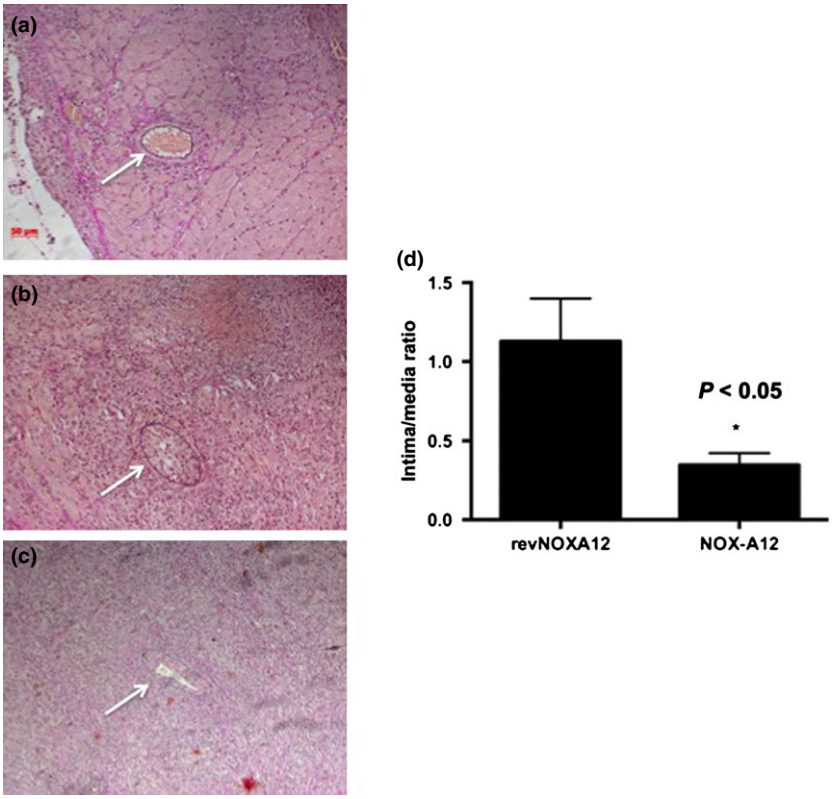

Figure 3 (a-c) Elastica van Gieson staining of heart grafts 40 days after transplantation (H-2bm12 to C57BL/6). Pictures are representative of 4 animals. (a: syngeneic group (C57BL/6 to C57BL76), b: revNOX-A12 control group (H2bm12 to C57BL/6), c: NOX-A12 treated group (H2bm12 to C57BL/6)/20× magnification)). (d) Histomorphometric analysis of intima/media ratio of heart allografts harvested at 40 days after transplantation ($n = 4$ for each group, $*P < 0.05$ vs. control).

Blockade of SDF-1 results in a decrease of 'pro-fibrotic' cytokine transcription levels in cardiac allografts

Many cells, cytokines, chemokines and antibodies have been shown to contribute to the development of CAV. After we had demonstrated a direct effect of SDF-1 on the proliferation of VSMCs, we next aimed to evaluate its immune modulatory effect, more specifically its effect on specific cytokines. B6 mice received a heart transplant from H-2bm12 and were treated with either NOX-A12 or control Spiegelmer (revNOX-A12). Forty days after transplantation, grafts were harvested and the expression levels of the cytokines TNF-α, TGF-β, and IL-6 were determined by quantitative RT-PCR.

Transcription levels of all three cytokines were significantly reduced under SDF-1 inhibition. Whereas TNF-α (Fig. 5a: revNOX-A12 3.42 ± 0.37 vs. NOX-A12 1.67 ± 0.33, $P < 0.05$, $n = 4$) and TGF-β (Fig 5b: revNOX-A12 2.18 ± 0.37 vs. NOX-A12 1.0 ± 0.38, $P < 0.05$, $n = 4$) transcription levels were reduced by >50%, IL-6 (Fig. 5c: revNOX-A12 2.18 ± 0.26 vs. NOX-A12 1.6 ± 0.1, $P < 0.05$, $n = 4$) was only reduced by about 25%.

Inhibition of SDF-1 results in a decrease in infiltrating $Cd3^+$ cells to the adventitia of aortic allografts

As T-cell activation and infiltration to the allografted vasculature are known to contribute to transplant vasculopathy and SDF-1 functions as a strong chemoattractant factor for T-cell recruitment, we further investigated the effect of Nox-A12 treatment on graft infiltrating cells.

Here, NOX-A12 treatment led to a significant decrease in infiltrating $CD3^+$ cells to the adventitia of transplanted aortic grafts compared to the animals treated by the control Spiegelmer revNOX-A12 (2.17 ± 0.27 (NOX-A12; $n = 4$) vs. 9.52 ± 2.39 (revNOX-A12; $n = 4$); $P = 0.038$ (Fig. 6)).

Discussion

In this study, we could show in two experimental models that the inhibition of the SDF-1/CXCR4/CXCR7 pathway significantly reduces chronic vasculopathy. The observed effects may have been caused by a direct inhibitory effect on proliferating VSMCs and also by direct immune modulation resulting in decreased levels of 'pro-fibrotic' cytokines and

infiltration of CD3-positive cells. The pharmacodynamic effect of NOX-A12 remained stable over time and seemed devoid of unwanted side effects, as reported in other studies [26].

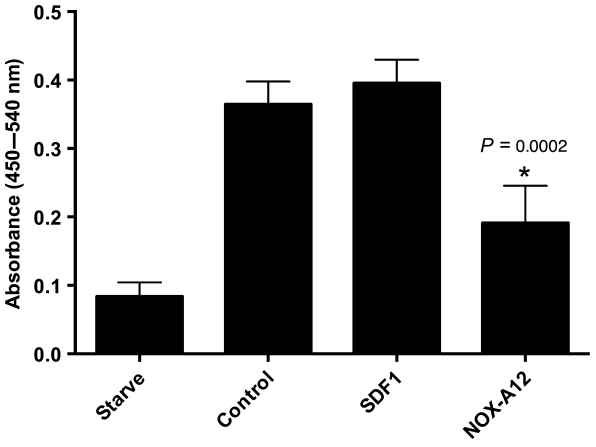

Figure 4 Magnitude of absorbance after BrdU incorporation of primary vascular smooth muscle cells (VSMC). NOX-A12 (100 μg/ml) led to a significant decrease in proliferating mVSMC compared to the control group. *P = 0.0002 control vs. Nox-A12 treatment.

Chronic rejection is a problem of varying intensity with respect to the transplanted organ. Renal transplant trials, for example, showed that severe chronic allograft nephropathy characterized by progressive renal dysfunction with chronic interstitial fibrosis, tubular atrophy, vascular occlusive changes and glomerulosclerosis was present in 58.4% of patients, with sclerosis in 37.3% of glomeruli 10 years after transplantation [32]. Minor chronic rejection changes (Banff Grade 1) were seen in 94% of the grafts and in 100% of the grafts 1 year and 5 years after transplantation, respectively. Bronchiolitis obliterans syndrome (BOS) in lung transplantation develops in 37% and in 58% after 5 and 10 years of the surviving lung transplant recipients, respectively [4]. Current ISHLT data indicate that CAV is one of the major reasons for late death of the HTx patients [4], and 90% of the heart transplant recipients show robust CAV 10 years after transplantation [18].

Chronic allograft vasculopathy is characterized by the formation of an occlusive neo-intimal layer in the vessels, consisting primarily of vascular smooth muscle cells and an accumulation of extracellular matrix ultimately resulting in graft fibrosis and cardiac hypertrophy [30,33]. The pathogenesis of CAV is still not fully understood. One of the problems may be that many studies use animal models in

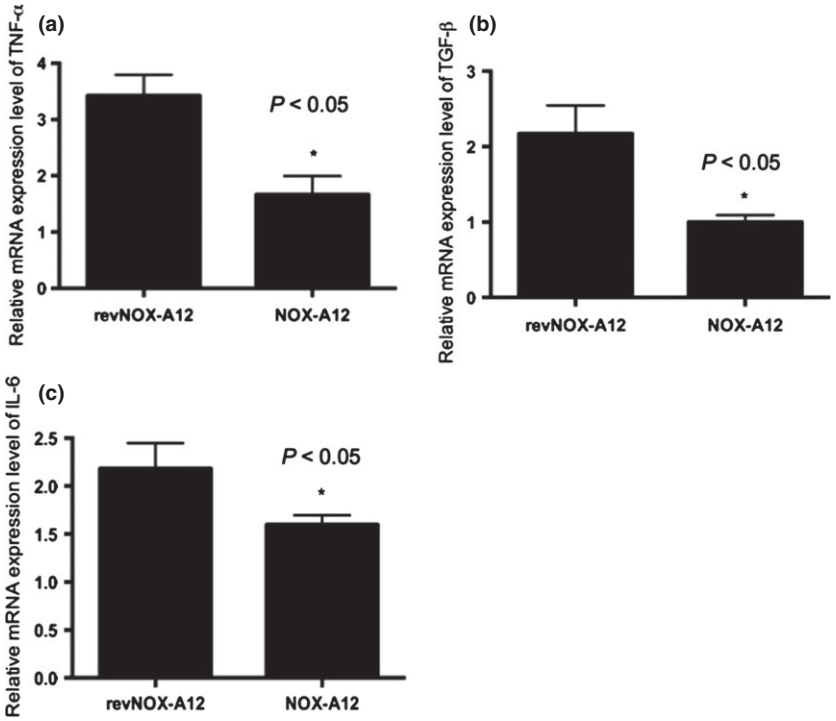

Figure 5 TNF-α (a), TGF-β (b) and IL-6 (c) mRNA levels of heart grafts 40 days after transplantation, showing a significant reduction under NOX-A12 treatment compared to the revNOX-A12 control group. As negative controls, heart samples after syngeneic heart transplants from B6 were used. The cytokine transcript levels were normalized against ß-actin and show the fold increase over the negative control (n = 4 for each group, *P < 0.05 vs. control).

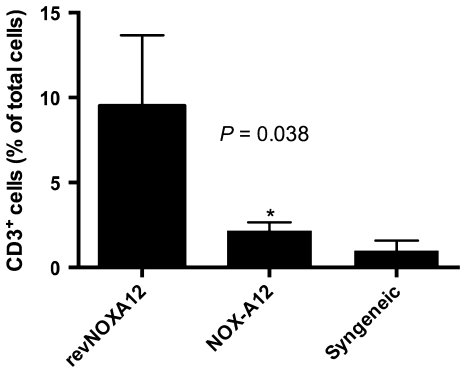

Figure 6 Immunohistochemical staining of aortas 28 days after transplantation, showing a significant reduction in $CD3^+$ cells under NOX-A12 treatment compared to the revNOX-A12 control group. As negative controls, aortic samples after syngeneic aortic transplantation were used. (n = 3 for each group). *P = 0.038 revNoxA12 vs. Nox-A12 treatment.

which the chronic rejection process does not necessarily reflect the changes seen in humans. The pathology usually develops over months and years in human transplantation, whereas CAV in the rodent models is already present at 4-8 weeks. Furthermore, the rodent models often lack continuous immunosuppression, which may result in a more vigorous immune response towards endothelial and SMCs. This may be responsible for the differences observed regarding the origin of the ECs and SMCs: Over 90% of these cells found in CAV lesions are from the host in rodent models [33] compared to 3–15% in human transplants [34,35].

Early experiments using carotid artery transplants in genetically modified mice indicate that CD4 + T helper cells, macrophages and B cells primarily contribute to transplant vasculopathy development, whereas cytotoxic CD8 + and natural killer cells seem rather not involved [36].

Alloreactive T cells are activated by endothelial and vascular smooth muscle cells of the transplanted graft by the expression of MHC I and II molecules which are up-regulated in response to inflammatory cytokines, in particular TNF- α and IFN-γ. This in turn functions as a positive feedback loop boosting alloantigen presentation and attack of effector cells [37]. Activated T cells further migrate into allograft arteries and contribute to vascular damage by direct cytotoxic effects or through the production of cytokines, mainly INF-γ and TGF-β, which induce cellular changes leading to tissue remodelling (reviewed in [38]). Therefore, although the exact pathogenesis of chronic rejection remains unclear, increasing evidence highlights the need to control activation and migration of T cells into the allograft in order to maintain long-term allograft survival [39]. Although we do not show specific data to alloreactivity and function of T cells, they were nonetheless significantly reduced in number when animals were treated with Nox-A12. This effect was specific for T cells. Control F4/80 stains to measure macrophage infiltration did not yield an effect of this treatment (data not shown).

Various experimental models identified cell signalling via cytokines and chemokines as important for CAV development and chronic rejection. In particular, the classical Th1-cytokine IFN-γ seems pivotal as blockade of this cytokine abrogated chronic-type lesions completely (reviewed in [18]). However, it is not only the IFN-γ but also downstream events as the TNF-α signalling that are relevant for this process. Thus, mice deficient for both forms of TNF receptors were protected from CAV. Interestingly, protection from vasculopathy was evident only when donor but not host cells was deficient for TNF [35]. Also, the TGF-β along with downstream molecules as connective tissue growth factor (CTGF) and IL-6 promote facets of chronic rejection [40]. Particularly, TGF-β is a cytokine of extraordinary importance for a variety of other physiological processes. Knocking out TGF-β causes severe developmental complications, which often result in preterm death. It is necessary for immune regulation and is also involved in chronic rejection. Interestingly, it seems that TGF-β is interlinked with the interleukin IL-6. IL-6 was reported to enhance TGF-β signalling by altering the receptor localization in the cell membrane [41] and to influence its effect. Furthermore, IL-6 itself was found to participate in the chronic rejection processes [42]. In accordance with the expectations, in our experiments, cytokine mRNA levels measured by RT-PCR for TGF-β, TNF-α and also IL-6 were significantly reduced in our heart transplant samples under the conditions of SDF-1 inhibition with NOX-A12.

Next to modulation of the extracellular matrix, proliferation of vascular smooth muscle cells of the intima or media of allograft arteries plays a key role in the development of transplant vasculopathy [43]. Here, SDF-1 is known to be involved in the recruitment of peripheral blood progenitor cells and is highly expressed in SMCs of the neointimal layer [17]. Blockade of SDF-1 attenuated transplant vasculopathy in a murine model of chronic allograft vasculopathy by significantly decreasing the number of circulating haematopoetic stem cells [44].

To further investigate the effect of SDF1-inhibition by NOX-A12, we tested the potential of this drug to inhibit the proliferation of SMCs and found indeed a significant decrease.

The role of chemokines and their receptors for CAV development have also been intensively investigated [34]. Chemokines are small molecules (8-10 kDa) and share 20–50% homology. Their main function is to guide the migra-

tion of cells. Chemokines are divided into four different types with respect to the spacing of their first two cysteine residues: 1.) CC-, 2.) CXC- 3.) C- and 4.) CX_3C chemokines.

Stromal cell-derived factor-1 (SDF-1) is a CXC chemokine, which was originally identified as a bone marrow stromal cell-secreting factor, supporting the development and proliferation of B cells [45]. Mice genetically deficient for SDF-1 or its receptor die *in utero* with defects of B lymphopoiesis, myelopoiesis, embryonic organ vascularization or cardiogenesis [46]. It regulates the migration and mobilization of bone marrow-derived stem and progenitor cells (HSPCs) [47].

CXCR4, originally thought to be the only receptor of the SDF-1, is expressed on different tissues during the development including the immune and central nervous system. It has an important function for the migration of resting leucocytes and hematopoietic progenitor cells [48]. Disruption of the SDF-1/CXCR4 axis results in a rapid egress of HSPCs from the bone marrow. This effect is used for the mobilization of these cells prior to hematopoietic stem and progenitor cell transplantation [49]. In the immune system, it is expressed in monocytes, B cells and naïve T cells. CXCR4 is upregulated in response to hypoxia through HIF-1 and VEGF. This may be responsible for the elevated levels of CXCR4 in a variety of different tumours (reviewed in [50]). Finally, CXCR4 was found to be expressed on VSMCs [33,51].

The other receptor for SDF-1, the CXCR7, has been discovered more recently [20]. It binds SDF-1 as well as I-TAC (CXCL11). CXCR7 regulates several important biological processes including cell survival, cell adhesion and tumour development. $CXCR7^{-/-}$ mice have cardiac defects, indicating a role for cardiogenesis [52].

Its role for CAV has yet to be determined, although it is clear that it is expressed on activated endothelial cells that are involved in building neovasculature.

A series of papers have investigated the role of SDF-1 for neointima formation following arterial mechanical injury [15,53,54]. Although these papers specifically looked at SDF-1α/CXCR4, the repetitive message was nonetheless that neointima formation can be ameliorated by the inhibition of this axis. In a human trial investigating histological samples of renal transplants, SDF-1 levels were elevated in biopsies, showing signs of chronic allograft nephropathy (CAN) [55]. Similar to these data, SDF-1α was upregulated in the adventitia and media of transplanted vasculopathic aortic grafts in a murine model of chronic rejection [44]. In this trial, the frequency of CXCR4-positive HSCs in the peripheral blood was increased and contributed to the neointima formation. We could confirm these data using a different approach. Along with a similar aortic transplant model, we used an MHC class II discordant fully vascularized heart transplant model as an established murine model of chronic rejection. Furthermore, we used a novel pharmacological approach with designed structured mirror-image oligonucleotide that blocks not only the interaction of SDF-1α and other isoforms with CXCR4 but also the interaction of SDF-1α and other isoforms with the other SDF-1-receptor CXCR7. Due to the use of non-natural mirror-image nucleotides [23], Spiegelmers are biostable and also do not induce type I interferons, as recently described for certain natural and synthetic RNAs [56], and are considered to be immunologically passive.

In conclusion, we could confirm a pivotal role of the SDF-1/CXCR4/CXCR7 axis for CAV development. Our data indicate that SDF-1 inhibition exerts its beneficial effect by reducing the expression of pro-fibrotic inflammatory cytokines as well as the blockade of activated T-cell migration to the allograft arteries. In addition, a direct antiproliferative effect on vascular smooth muscle cells could be demonstrated.

Inhibition of the chemokine SDF-1 by NOX-A12 may be a promising pharmacological approach to selectively target a key-signalling pathway involved in the deleterious effects of chronic rejection.

Authorship

MT, AK, MA, AW and JA: performed the experiments. MT, MF, AVB, MA and JA: contributed to the research design. MT and JA: wrote the manuscript. All authors contributed to careful review of the paper and approved its submission.

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