ORIGINAL ARTICLE

Activin inhibition limits early innate immune response in rat kidney allografts—a pilot study

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SUMMARY

Activins are members of the transforming growth factor-beta (TGF-β) superfamily of cytokines. They play critical roles in the onset of acute and chronic inflammatory responses. The aim of this study was to investigate how activin inhibition affects acute kidney injury and inflammation after transplantation. The study was carried out in kidney transplantation and renal ischemia-reperfusion models in the rat. Soluble activin type 2 receptor (sActRIIB-Fc) was used to inhibit activin signaling. Transplantation groups were as follows: (i) cyclosporine A (CsA) (ii) CsA + sActRIIB-Fc, (iii) CsA+ inactive protein control Fc-G1. IRI groups were as follows: (i) no treatment, (ii) sActRIIB-Fc. Serum activin B concentration was significantly elevated after transplantation and IRI, whereas activin A was produced locally in renal allografts. Activin inhibition efficiently limited neutrophil, macrophage, and dendritic cell infiltration to the allografts measured 72 h after transplantation. In addition, sActRIIB-Fc treatment modulated serum cytokine response after transplantation and reduced the early accumulation of fibroblasts in the graft interstitium. In conclusion activin inhibition reduces the innate immune response early after renal transplantation in the rat. It also limits the accumulation of fibroblasts in the graft suggesting that activins may be involved in the fibrogenic signaling already early after kidney transplantation.

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Key words
activin, innate immunity, ischemia-reperfusion injury, kidney transplantation, transforming growth factor-beta

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Introduction

In organ transplantation, the innate immune response is first activated in response to endothelial injury and activation after ischemia-reperfusion injury (IRI). Damage-associated molecular patterns (DAMPs) released and produced by injured cells bind toll-like receptors (TLRs) subsequently activating the innate immune response. TLR signaling in antigen presenting cells promotes inflammation and antigen presentation through upregulation of MHC class II antigens and costimulatory molecules and production of reactive oxygen species, chemokines, and cytokines [1]. Thus, the innate immune response aggravates graft injury and promotes the development of alloimmune response leading to acute and chronic rejection. IRI and the subsequent inflammatory microenvironment trigger repair processes predisposing the graft to fibrosis [2].

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Activins are members of the transforming growth factor-beta (TGF-β) superfamily of cytokines. Activins were initially named after their ability to activate the release of follicle-stimulating hormone from the anterior pituitary [3,4]. Later it has been discovered that activins are important regulators of inflammation and fibrosis [5,6]. The best studied members of the activin family are activins A and B. Activins bind to one of the two type 2 activin receptors (ActRIIA and ActRIIB) on the cell surface, which dimerise with a type 1 activin receptor (activin receptor-like kinase, ALK) [7,8]. Dimerization of the receptor complex activates SMAD 2/3 [9] and mitogen-activated protein kinase signaling pathways [10,11]. Follistatin is an endogenous activin-binding protein and an important inhibitor of its biological activity [12].

Activin A production is stimulated by TLR signaling and proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β [13–15]. Various inflammatory cells including monocytes, macrophages, dendritic cells and neutrophils are able to produce activin A [14,16,17]. Accordingly, serum activin A levels rapidly increase during septicemia in humans and also in experimental models in sheep and mice [15,18,19]. In mice, activin inhibition with follistatin has been shown to reduce inflammation during lipopolysaccharide (LPS) stimulated endotoxemia and enhance survival after a lethal dose of LPS [15]. Furthermore, activin A overexpression in the murine lung has been shown to cause an inflammatory lung injury that simulates the acute respiratory distress syndrome [20]. Thus, activin A seems to be an important regulator of the acute immune response.

Activins are particularly interesting in kidney transplantation, as exogenous follistatin has been shown to directly protect tubular cells from apoptosis after experimental renal IRI [21,22]. Follistatin treatment has also inhibited renal fibrosis in the unilateral ureteral obstruction model in the rat [23]. TGF-β1 stimulates activin A production in renal fibroblasts and activin inhibition reduces the fibrogenic effects of TGF-β1, suggesting that in fibrotic processes, activins are possible intermediates in TGF-β1 signaling [24,25].

As activins may increase acute tubular injury and inflammation and even promote the subsequent fibrotic processes, we hypothesized that inhibition of activin action could have beneficial effects already at the early phase after kidney transplantation. To investigate this effect, we used a soluble activin receptor 2 (s-ActRIIB-Fc) to block activins A and B signaling both in renal transplantation and in bilateral renal ischemia-reperfusion models in the rat.

Materials and methods

Animals

Permission for animal experiments was obtained from the State Provincial Office of Southern Finland (Helsinki, Finland). Inbred, male, fully major histocompatibility complex-mismatched Dark Agouti (DA) (AG-B4, $RT1^a$) and Wistar-Furth (WF) (AG-B2, $RT1^v$) rats (Harlan, Horst, the Netherlands) weighing 300–350 g were used. The animals received human care in compliance with the *Guide for the Care and Use of Laboratory Animal Resources* published by the National Institutes of Health and Office of Animal Care and Use (National Research Council, Washington DC, the National Academies Press, 2011), as well as specific national laws. The rats received food and water *ad libitum* and were not fasted before the surgery. All operations were performed under isoflurane (Isoflurane Baxter, Deerfield, IL, USA) anesthesia, and buprenorphine (Temgesic, Schering-Plough, Kenilworth, NJ, USA) was used for postoperative analgesia. After surgery, rats were kept warm under a heating lamp until recovery from anesthesia.

Activin inhibition

A soluble activin receptor sActRIIB-Fc was used to inhibit activins A and B signaling. The recombinant fusion protein containing the ectodomain of human ActRIIB fused to the Fc domain of human IgG1 (sActRIIB-Fc) was produced in-house as described previously [20,26]. The dose 2 mg/kg has been shown to be effective in limiting inflammation and fibrosis in earlier studies [20,26]. Control human IgG1-Fc fragment (Bio X cell, West Lebanon, NH, USA) was used as specificity control for sActRIIB-Fc activity.

Experimental design

The effect of activin inhibition on acute rejection and acute kidney injury was studied in a kidney transplantation model in the rat. Kidney transplantations were performed from DA to WF rats as described [27] using a modified microsurgical technique of Fisher and Lee. The donor right kidney was perfused with 5 ml cold PBS containing 50 IU/ml heparin and removed with a segment of aorta and vena cava. The kidney was preserved in ice-cold PBS before transplantation. The total ischemia time was 45 min. The recipient's right native kidney was removed at the time of transplantation, but the left native kidney was left untouched. In the

transplantation model, we had three study groups. All allografts were treated daily with cyclosporine A (CsA) 1.5 mg/kg s.c (Novartis, Basel, Switzerland). The groups were as follows: (i) CsA control group where transplant recipients received only CsA ($n = 5$), (ii) CsA + sActRIIB-Fc group where transplant recipients received ActRIIB-Fc 2 mg/kg intraperitoneally (i.p.) 2 h before transplantation ($n = 5$), (iii) CsA + Fc-G1 group where transplant recipients received inactive protein control (human Fc-G1 2 mg/kg i.p.) 2 h before transplantation ($n = 3$).

A bilateral renal clamping model was used to study the effect of activin inhibition on IRI without alloimmune response. Renal ischemia was induced in WF rats. Both renal pedicles were clamped for 45 min. During ischemia, the rats were kept under isoflurane anesthesia and the abdomen was closed temporarily. After the clamp was removed, the kidney was inspected for recovering blood flow and the abdomen was closed. In the IRI model, we had two study groups: (i) sActRIIB-Fc group where sActRIIB-Fc 2 mg/kg was given to the rat i.p. 2 h before the procedure ($n = 3$), (ii) the control group where the rats did not receive any medication ($n = 3$).

Both transplantation and IRI rats were followed for 72 h after the procedure. Serum samples for ELISA analyses (acute kidney injury markers and proinflammatory cytokines) were gathered 3, 8, 24, and 72 h after the blood circulation had been released to the transplant or to the clamped kidneys. In the IRI model, serum creatinine was measured 24 and 72 h after the procedure. The transplantation model was not graft dependent, and thus, serum creatinine was not measured. In the transplantation model, the kidney transplant and in the IRI model the right kidney were recovered for further analysis.

Half of each kidney sample was incubated in 2% paraformaldehyde for 24 h and then routinely fixed for paraffin blocks. The other half was embedded in O.C.T (Tissue-Tek; Miles Inc., Elkhart, IN, USA), snap-frozen in liquid nitrogen, and stored at −70 °C.

Histopathology

For analysis of acute kidney damage, paraffin-embedded renal specimens were cut into 2-μm-thick sections and stained with Mayer's hematoxylin–eosin, Masson's trichrome, and diastase-periodic acid-schiff stains. Tubular necrosis, dilatation, flattening, and casts were all graded on a semi-quantitative scale from 0 to 3 as follows: grade 0 = no damage, grade 1 = mild damage, grade 2 = moderate damage, grade 3 = severe damage. A pathologist performed the analysis in a blinded manner.

Immunohistochemistry

To study the renal expression of activins, inflammatory cell populations and fibroblasts immunohistochemistry were used.

Paraffin-embedded and frozen specimens were cut into 4-μm-thick sections. For epitope retrieval, the paraffin sections were heated in a microwave oven for 20 min in sodium citrate buffer (pH 6.0) and then allowed to cool down at room temperature for 20 min. Immunohistochemical stainings were performed using the peroxidase ABC method (Vectastain Elite ABC Kit; Vector Laboratories Inc., Burlingame, CA, USA), and the reaction was revealed by 3-amino-9-ethylcarbazole (AEC; Vector Laboratories). Counterstaining was performed using Mayer's hemalum. Endogenous peroxidase activity was blocked with a 20-min incubation using a 1% hydrogen peroxidase/phosphate-buffered saline solution. For specificity controls, the primary antibody was omitted. None of these specificity controls showed any immunoreactivity.

Mouse monoclonal antibodies against human activin A and activin B (AI006, 18/16 for activin A/INHBA and AI005, 12/9A for activin B/INHBB) were generated by AnshLabs LLC (Webster, TX, USA). The antibodies were selected for immunohistochemistry based on their specificity in Western blots and specific reactivity toward granulosa cells in human ovaries and macrophages and alveolar epithelium during lung injury in an earlier study carried out by our colleagues [26]. Immunohistochemical stainings were also used to detect FSP-1/S100A4$^+$ fibroblasts, MPO$^+$ neutrophils, ED1$^+$ monocytes/macrophages, ED3$^+$ activated macrophages, OX-62$^+$ dendritic cells, CD4$^+$ T-cells and CD8$^+$ T cells in the kidneys and allografts. The following antibodies and dilutions were used: CD169 (ED3) (5 μg/ml, MCA343GA; AbD Serotec, Kidlington, UK), CD4 (5 μg/ml, 554835; BD Pharmingen, San Diego, CA, USA), CD8 (5 μg/ml, 554854; BD Pharmingen), ED1 (5 mg/ml, 22451D; BD Pharmingen), S100A4 (FSP-1) (1:100, A5114; DakoCytomation, Glostrup, Denmark), myeloperoxidase (MPO) (20 mg/ml, ab9535; Abcam, Cambridge, UK), and OX-62 (10 μg/ml, MCA 1029G; Serotec).

For quantification of the results, positive cells for each immunohistochemical staining were counted from four random fields of each quadrant of the sections' parenchyma with 400× magnification. The results are

given as the mean number of positive cells per square millimeter. In addition to quantification of glomerular activin A staining, the positive cells were counted from 20 glomeruli. The results are given as the mean number of positive cells per glomerular cross section. The results were analyzed in a blinded manner.

ELISA

Enzyme-linked immunosorbent assays (ELISA) were used to measure serum concentrations of acute kidney injury markers neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) and the key proinflammatory cytokines IL-1β, IL-6, and TNF-α. The analyses were performed according to the manufacturers' instructions. The following kits were used: rat NGAL (KIT046; BioPorto Diagnostics, Gentofte, Denmark), rat TIM-1/KIM-1/HAVCR (RKM100; R&D Systems, Minneapolis, MN, USA), rat TNF-α (RTA00; R&D Systems), rat IL-6 (R6000B; R&D Systems), and rat IL-1 beta/IL-1F2 (RLB00; R&D Systems).

Mouse activin B ELISA (AL-156) and activin A ELISA (AL-110) kits were used from AnshLabs LLC (Webster, TX, USA) to measure the serum concentrations in the rat models. Samples were diluted 1:20 and 1:5, respectively, prior to assay in mouse activin B and activin A ELISAs. The assays were performed according to manufacturer's instructions (http://www.anshlabs.com/product-category/immunoassays/). The assays measure total activin concentrations and exhibit minimal or negligible cross-reactions with different activin or inhibin ligands or other TGFbeta family members (see "instructions for use" of the assay kits, at the manufacturer's Web site indicated above).

Statistical analyses

Statistical analyses were performed with SPSS version 20.0 (IBM). In statistical analysis of the transplantation model, the CsA group was compared to the CsA + sActRIIB-Fc group. The additional control group receiving inactive protein control (CsA + Fc-G1) was compared to the CsA group in a separate analysis to confirm the specificity of sActRIIB-Fc. In the IRI model, the two study groups were compared with each other.

The semiquantitative results (tubular injury) were analyzed using Mann–Whitney *U*-test. To compare quantitative results between the groups, Student's *t*-test was used. Analysis of variance (ANOVA) for repeated-measures was used to analyze results that were obtained from multiple time points. Probability (*P*)-value of <0.05 was accepted as significant. The results are expressed as mean ± standard error of the mean (SEM).

Results

Serum activin B concentration is significantly elevated after transplantation and bilateral renal IRI, whereas activin A is produced locally in renal allografts

In normal rats, serum activin B concentration was 396 ± 39 pg/ml (Fig. 1). In rats receiving only CsA, serum activin B concentration was elevated from 3 h after transplantation and reached over 25-fold increase by 8 h (Fig. 1a). sActRIIB-Fc treatment efficiently inhibited this increase (Fig. 1c). Control Fc-G1 did not affect serum activin B concentration after transplantation (Fig. 1c). Also, bilateral renal IRI increased serum activin B concentration in the control rats with no treatment (Fig. 1b). The increase was not, however, as intense as measured after transplantation (Fig. 1a and b). In rats treated with sActRIIB-Fc, serum activin B levels remained low after bilateral IRI, but the difference to the control group was not statistically significant (Fig. 1c). Activin A concentration in normal rat serum was 258 ± 123 pg/ml (Fig. 1). Transplantation or bilateral renal IRI did not affect serum activin A concentration (Fig. 1a and b).

In immunohistochemical analysis of normal rat kidneys, only very mild glomerular and tubular activin A staining was present. In the bilateral IRI model, renal activin A expression was similar to normal kidneys 3 days after the procedure. sActRIIB-Fc did not modulate this mild basal activin A expression. In contrast, 3 days after transplantation, moderate activin A expression was detected in the glomerular cells of the allografts treated with CsA only (Fig. 2a and b). Also interstitial cells expressed activin A but at lesser levels. Tubular staining was nearly nonexistent. sActRIIB-Fc significantly reduced activin A expression in the glomeruli after transplantation (Fig. 2a and c). Control Fc-G1 did not modulate activin A expression when compared to the group receiving CsA only ($P = 0.920$) (Fig. 2a). No activin B was detected in normal kidneys or allografts after transplantation.

Activin inhibition with sActRIIB-Fc reduces macrophage, dendritic cell, and neutrophil infiltration in the renal allografts during acute rejection

Three days after transplantation, a substantial infiltration of all studied inflammatory cell subsets was detected in the CsA-treated control allografts (Figs 3

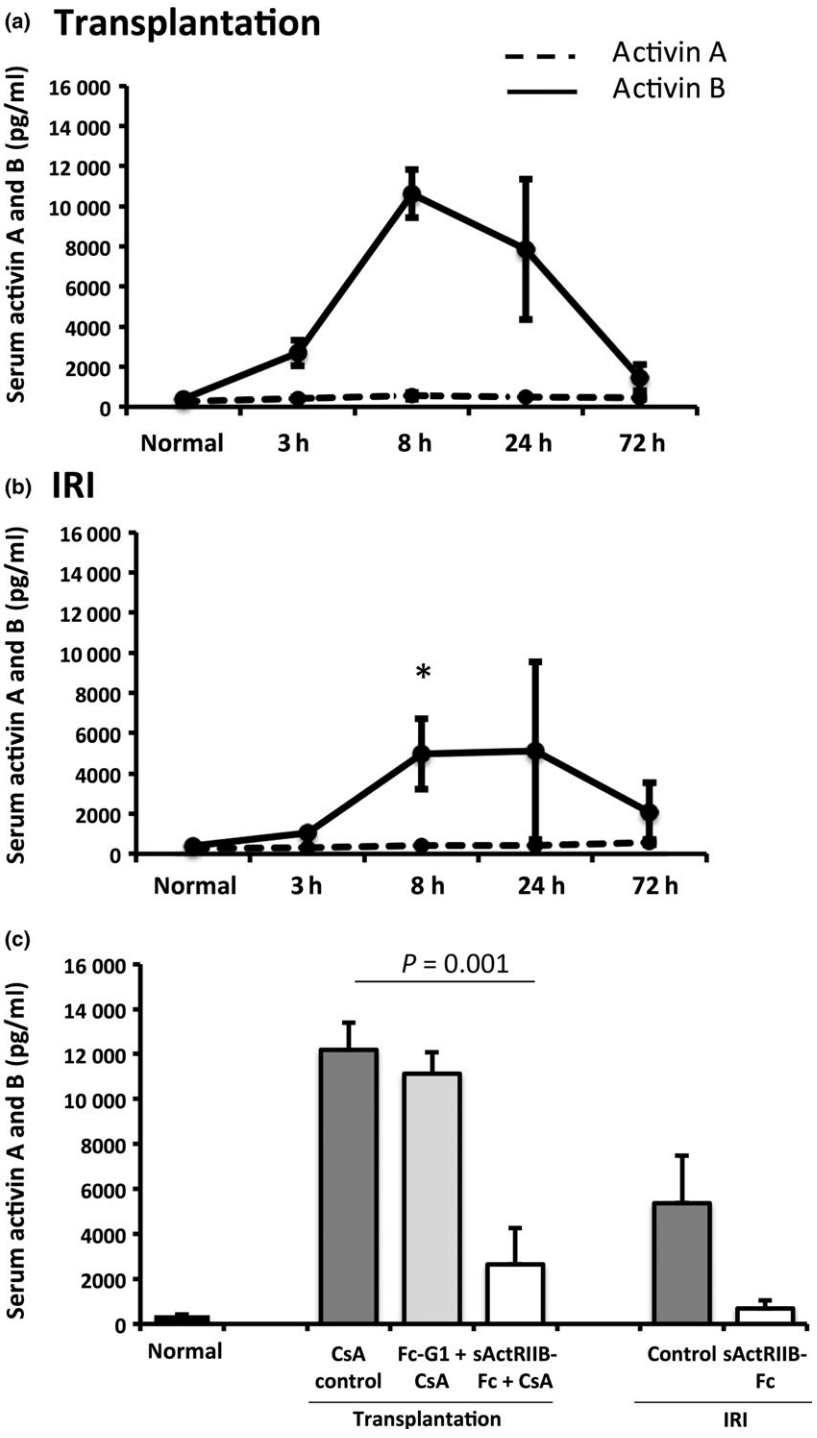

Figure 1 (a) Serum activins A and B concentration after transplantation in rats treated only with CsA (n = 5). Serum activin B concentration increased significantly after transplantation, whereas activin A concentration remained in the normal level. (b) Serum activins A and B concentration after bilateral ischemia-reperfusion injury (IRI) in rats with no treatment (n = 3). Activin B concentration increased significantly after IRI. However, at 8 h, the concentration was significantly lower in the IRI model than in the transplantation model at the same time-point (*P = 0.033). Bilateral renal IRI did not affect serum activin A concentration. (c) sActRIIB-Fc treatment significantly inhibited serum activin B concentration after transplantation (n = 4). Control Fc-G1 did not affect serum activin B concentration (n = 3). sActRIIB-Fc treatment did not have significant effect on serum activin B levels after bilateral IRI (n = 3). The results are given as mean ± SEM.

and 4). Activin inhibition with sActRIIB-Fc significantly reduced the number of MPO^+ neutrophils (P = 0.013), $ED3^+$ activated macrophages (P = 0.034), and $OX\text{-}62^+$ dendritic cells (P = 0.036) in the graft when compared to the CsA control group (Figs 3 and 4). In $ED1^+$ monocytes/macrophages, the inhibitory effect did not reach statistical significance (Fig. 3d). Activin inhibition did not modulate the CD4 or CD8 T-cell response (Fig. 3e and f). Inactive protein control human Fc-G1 did not affect monocyte (P = 0.398), macrophage (P = 0.319), neutrophil (P = 0.550), nor dendritic cell (P = 0.738) infiltration when compared to the group receiving only CsA (Fig. 3a–d).

In the bilateral renal IRI model, the inflammatory cell response detected by immunohistochemistry was mild both in the control and in the sActRIIB-Fc group

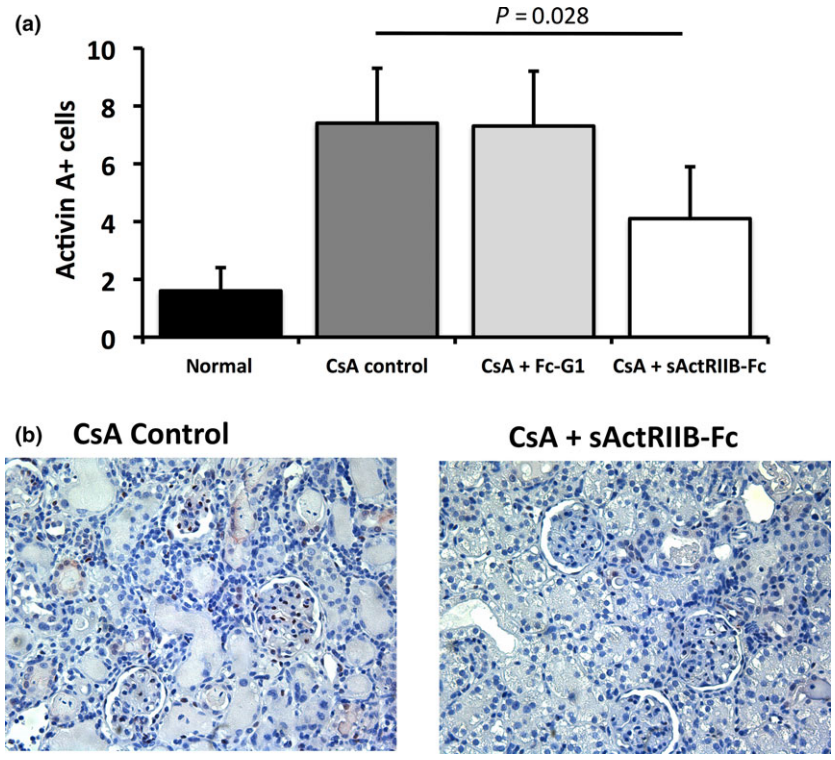

Figure 2 Renal activin A expression was analyzed from immunohistochemical stainings 3 days after transplantation. Activin A expression was induced in the glomeruli and to some extent in interstitial cells in allografts treated with CsA only (a and b). sActRIIB-Fc treatment significantly reduced the number of activin A-positive cells in the glomeruli (activin A+ cells/glomerular cross section) (a and c). Control Fc-G1 did not affect activin A expression (CsA control versus CsA + Fc-G1, $P = 0.920$) (a). The data are given as mean ± SEM. Normal ($n = 3$), CsA control ($n = 5$), CsA + Fc-G1 ($n = 3$), CsA + sActRIIB-Fc ($n = 5$). The original magnification for the photomicrographs was ×400.

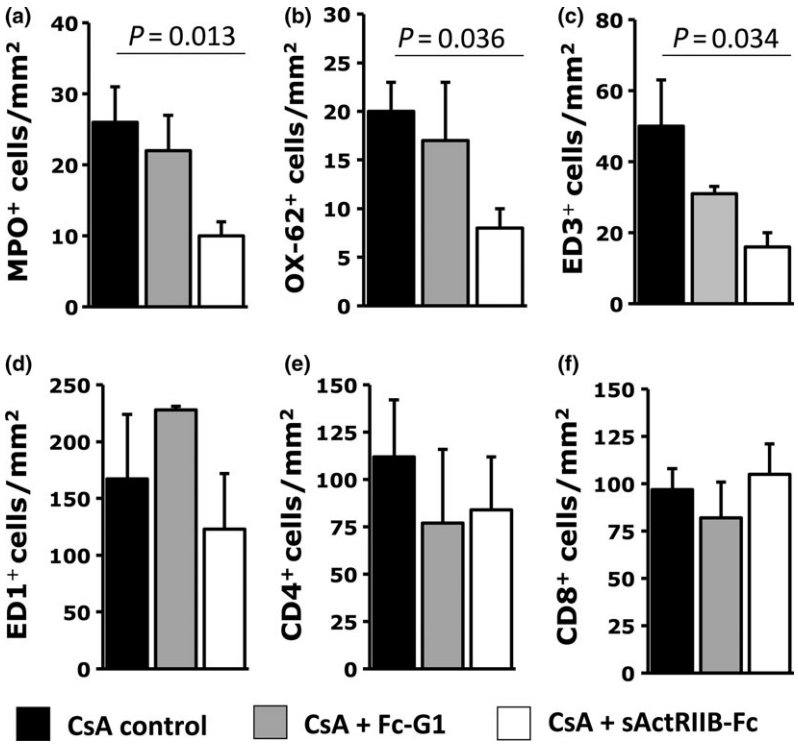

Figure 3 Activin inhibition with sActRIIB-Fc reduced the innate immune response after rat renal transplantation. sActRIIB-Fc treatment significantly reduced the number of MPO^+ neutrophils (a), $OX\text{-}62^+$ dendritic cells (b), and $ED3^+$ macrophages (c) in the graft 3 days after transplantation. The inhibitory effect of sActRIIB-Fc on cells positive with the pan monocyte/macrophage marker ED1 did not reach statistical significance (d). sActRIIB-Fc did not modulate the T-cell response (e and f). CsA control ($n = 5$), CsA + Fc-G1 ($n = 3$), CsA + sActRIIB-Fc ($n = 5$). The results are given as mean ± SEM.

3 days after IRI. This was expected, because in this experimental model, inflammation starts to decline 24 h after IRI as there is no alloimmune response to maintain the inflammatory environment. There were no statistical differences observed between the IRI study groups (Fig. S1).

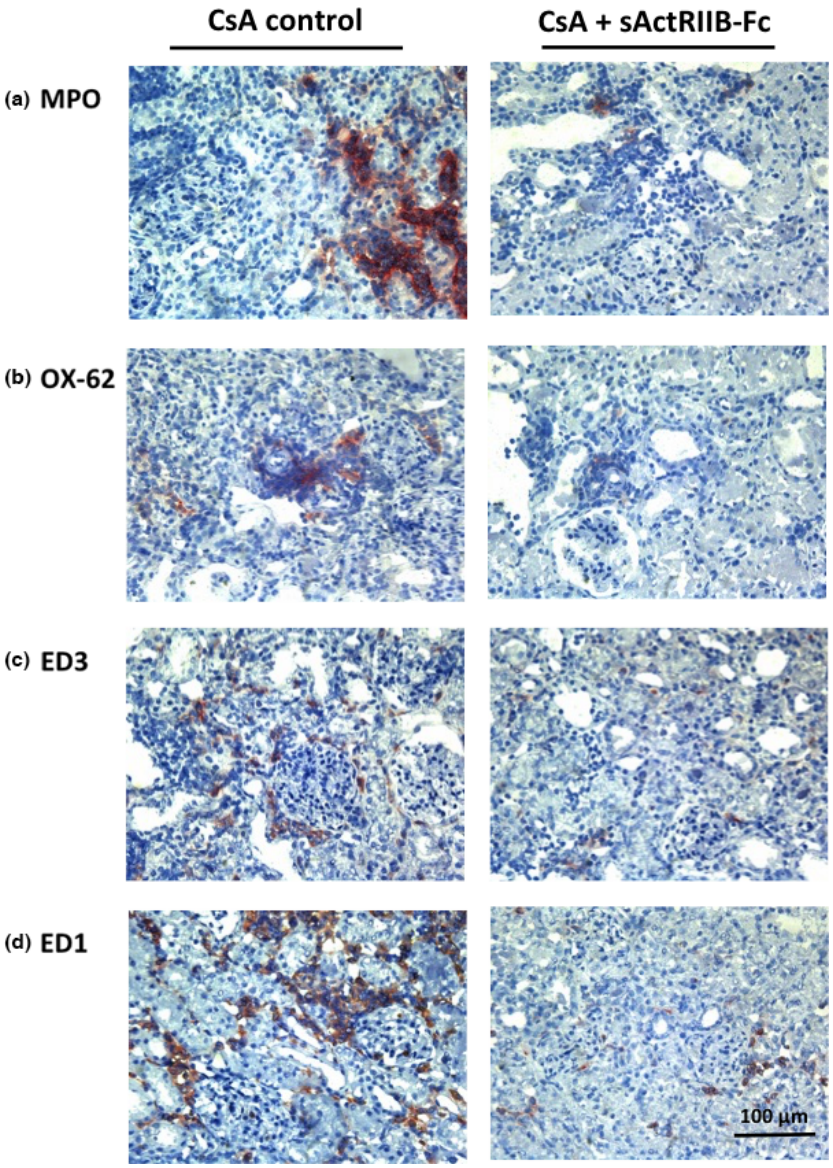

Figure 4 Immunohistochemical stainings of myeloperoxidase$^+$ (MPO$^+$) neutrophils (a), OX-62$^+$ dendritic cells (b), ED3$^+$ macrophages (c), and ED1$^+$ monocytes/macrophages (d) in rat renal allografts 3 days after transplantation. Representative photomicrographs of allografts treated with CsA only are shown in the left column and allografts with sActRIIB-Fc treatment in the right. The original magnifications were ×400.

Activin inhibition modulates cytokine response after transplantation

To further investigate the role of activin in acute inflammation early after renal transplantation, we examined the effect of sActRIIB-Fc treatment on serum concentrations of the key proinflammatory cytokines IL-1β, IL-6, and TNF-α. The serum cytokine concentrations were measured with ELISA 3, 8, 24, and 72 h after transplantation.

In the CsA control group, the IL-1β plasma concentrations peaked at 8 h and diminished thereafter (Fig. 5a). IL-6 and TNF-α concentrations were at the highest 3 and 8 h after transplantation and then gradually decreased (Fig. 5b and c). Activin inhibition with sActRIIB-Fc altered the cytokine profile. Peak serum concentration of IL-1β was significantly reduced by sActRIIB-Fc treatment (CsA versus CsA + sActRIIB-Fc; 52.9 ± 9.4 pg/ml vs. 19.4 ± 5.1 pg/ml, $P = 0.012$) (Fig. 5a). In contrast, sActRIIB-Fc treatment enhanced IL-6 peak serum concentration (474.4 ± 34.3 pg/ml vs. 700.0 ± 95.7 pg/ml, $P = 0.033$) (Fig. 5b). sActRIIB-Fc did not significantly affect TNF-α peak concentration (9.6 ± 1.3 pg/ml vs. 6.6 ± 0.4 pg/ml, $P = 0.110$) (Fig. 5c). Inactive protein control human Fc-G1 did not affect serum interleukin peak concentrations when compared to the CsA group (Table S1).

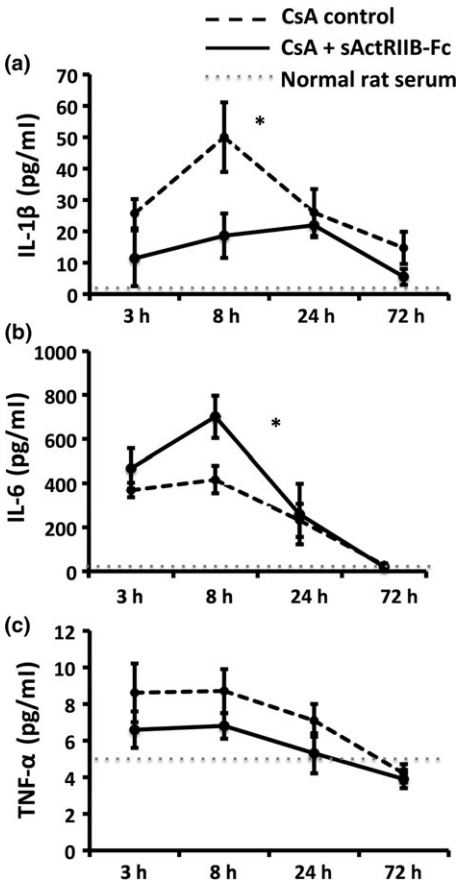

Figure 5 Activin inhibition modulates cytokine response after transplantation. (a) sActRIIB-Fc treatment reduced IL-1β serum peak concentration after transplantation (*CsA control versus CsA + sActRIIB-FC, $P = 0.012$). (b) In contrast, sActRIIb-Fc treatment increased IL-6 serum peak concentration (*CsA control versus CsA + sActRIIB-FC, $P = 0.033$). (c) Activin inhibition did not affect TNF-alpha production after transplantation. CsA control ($n = 5$), CsA + sActRIIB-Fc ($n = 4$), normal rats ($n = 3$). The results are given as mean ± SEM.

sActRIIB-Fc does not prevent acute tubular necrosis after transplantation or bilateral renal IRI, but it limits the early accumulation of fibroblast to the graft interstitium

The effect of activin inhibition on acute kidney injury was quantified by following serum levels of acute kidney injury markers NGAL and KIM-1. In addition, histopathological analysis of the renal allografts and IRI kidneys was performed at the end of the experiment.

In CsA-treated control allografts, serum NGAL and KIM-1 levels rose quickly after transplantation, peaked at 24 h, and decreased thereafter. Activin inhibition with sActRIIB-Fc did not significantly alter the NGAL or KIM-1 levels (Fig. 6a and b). In histopathological analysis, moderate tubular injury was seen in all allografts. sActRIIB-Fc treatment did not reduce tubular injury after transplantation (Table S2). However, activin inhibition with sActRIIB-Fc halved the amount of FSP-1/S100A4$^+$ fibroblasts in the graft 72 h after transplantation when compared to the CsA control group (Fig. 7).

In the control group of bilateral renal IRI model, the serum NGAL and KIM-1 levels rose slowly and reached the highest level at 72 h after IRI (Fig. 6c and d). Serum creatinine was clearly elevated 24 h after IRI, but at 72 h, the renal function was close to normal (Fig. 6e). Activin inhibition did not have significant effects on serum NGAL, KIM-1 nor creatinine levels (Fig. 6). In the histopathological analysis 3 days after IRI, the tubular injury was moderate and similar to that seen in the transplantation model. sActRIIB-Fc treatment did not affect tubular injury after experimental IRI (Table S2).

Discussion

Here, we demonstrate that kidney transplantation and renal IRI induce a strong systemic activin B response, whereas activin A is produced locally in renal allografts. Inhibition of activin activity with a soluble receptor sActRIIB-Fc efficiently reduced the infiltration of innate immune cells into the renal allografts early after transplantation. It also modulated the proinflammatory cytokine response. In addition, sActRIIB-Fc treatment limited the early accumulation of fibroblasts in the graft interstitium. Thus, our results indicate that activins regulate acute inflammation and are involved in fibrogenic signaling already in the early phase after renal transplantation.

Our results suggest that activins regulate the innate immune response soon after renal transplantation. Activin inhibition efficiently reduced the dendritic cell, macrophage, and neutrophil infiltration into the graft. Also earlier studies suggest that activins may have a key role in initiating the innate immune response. Overexpression of activin A in mouse airways causes marked pulmonary macrophage and neutrophil infiltration, which is later followed by lymphocytes [20]. Accordingly follistatin treatment almost completely inhibits neutrophil and macrophage infiltration to airways during experimental cystic fibrosis in mice [28]. Moreover, activins have been shown to induce high-mobility group box-1 production, which is a central DAMP initiating the TLR signaling and innate immune response [20]. In experimental kidney transplantation, impaired TLR signaling increases allograft survival [29,30]. Thus, inhibition of the innate immune response might be beneficial in the transplant setting. Our current results are in

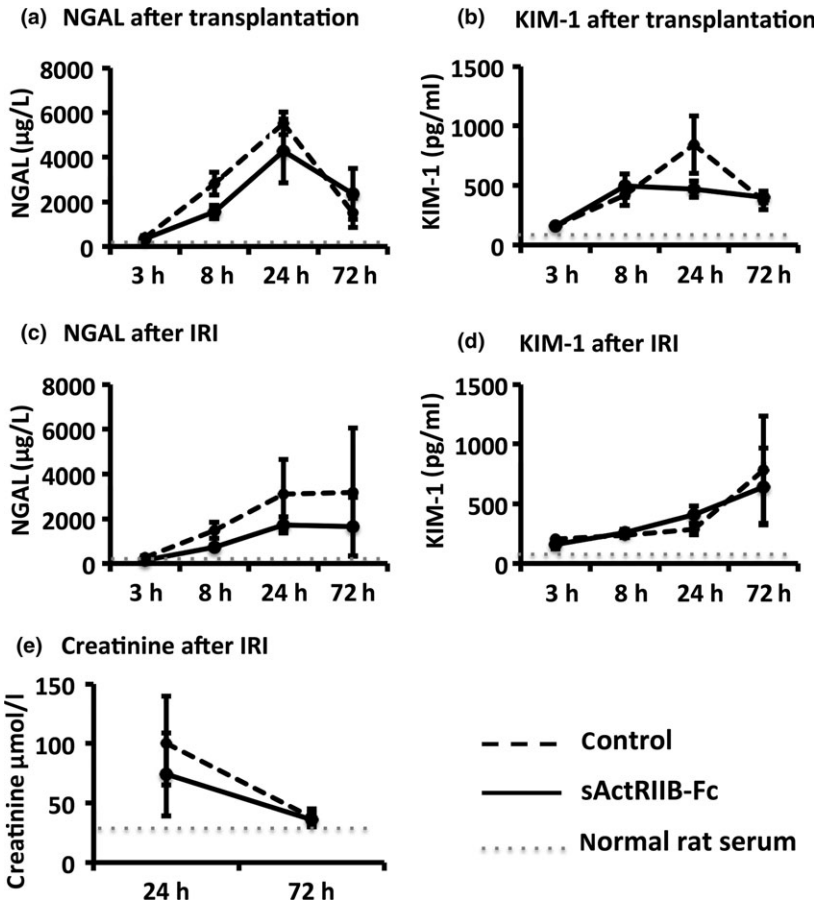

Figure 6 Activin inhibition with sActRIIB-Fc did not alter the serum concentration of acute kidney injury markers neutrophil gelatinase-associated protein (NGAL) nor kidney injury molecule-1 (KIM-1) after transplantation (a and b) or after bilateral renal ischemia-reperfusion injury (c and d). Activin inhibition did not affect serum creatinine after ischemia-reperfusion injury (IRI) (e). $n = 5$ in the transplantation groups and $n = 3$ in the IRI model groups. Transplantation: CsA control ($n = 5$), CsA + sActRIIB-Fc ($n = 5$); IRI: control ($n = 3$), sActRIIB-Fc ($n = 3$), normal rats ($n = 3$). The results are given as mean ± SEM.

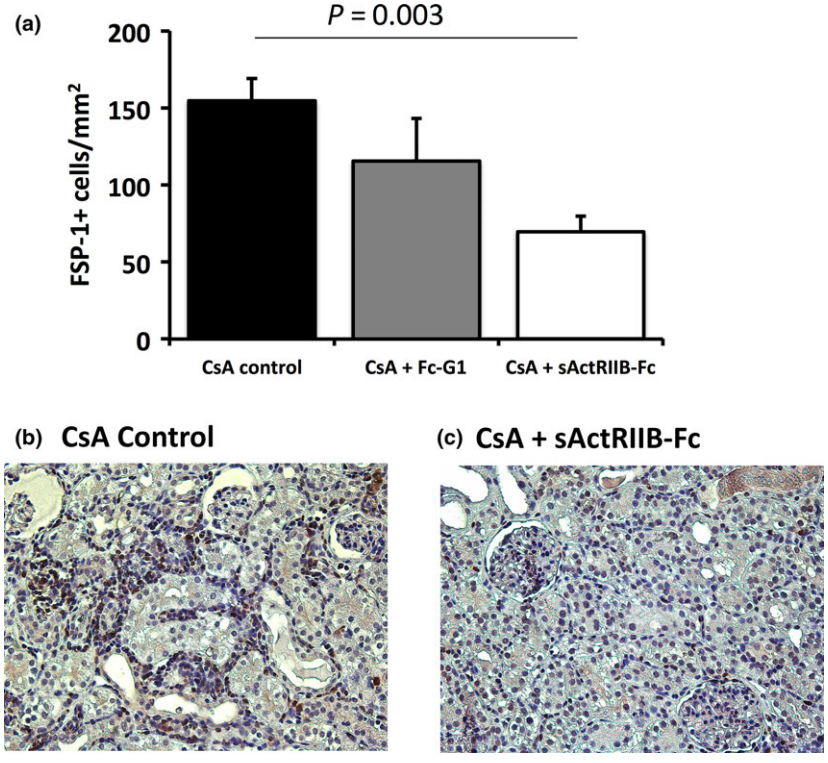

Figure 7 Activin inhibition with sActRIIB-Fc limited the early accumulation of FSP-1+ fibroblasts in the graft interstitium (a) suggesting that activin inhibition reduces early fibrotic processes in the renal transplant. Representative FSP-1 stained kidney section of (b) an allograft treated with CsA only (c) and of an allograft with sActRIIB-Fc treatment (c). n(CsA control) = 5, n(CsA + Fc-G1) = 3, n(CsA + sActRIIB-Fc) = 5. The data are given as mean ± SEM. The original magnification for the photomicrographs was ×400.

accordance with the previous knowledge and further suggest that activins could be interesting therapeutic targets in limiting the innate immune response in kidney transplantation.

In our study, serum activin A levels were not affected by IRI or transplantation, which contrasts the earlier study in mice reporting a fivefold to 10-fold increase in serum activin A after administration of LPS [15]. Instead, we found a significant increase in the serum activin B levels after transplantation and IRI. Furthermore, in our study, sActRIIB-Fc treatment efficiently inhibited the increase in activin B levels after transplantation, which was accompanied by reduced serum level of IL-1β and increase in serum IL-6. A recent study has reported a similar strong and immediate increase in serum activin B levels after renal IRI in mice. The rise in activin B levels was later followed by a modest increase in serum activin A concentration [31]. Although activin A is a key regulator of inflammatory responses, our study and new emerging knowledge suggests that the less well-known activin B may have a central role in serum cytokine response after renal transplantation and IRI.

An earlier study by Maeshima *et al.* [21] demonstrated that activin A expression is strongly upregulated in tubular cells after experimental renal ischemia-reperfusion injury in the rat. In addition, follistatin treatment has been shown to inhibit tubular cell apoptosis and improve renal function [21]. There is also *in vitro* evidence that activin A serves as an autocrine regulator of tubular cell apoptosis and proliferation [22]. In our study, tubular activin A production was not induced 3 days after transplantation or bilateral renal IRI. Furthermore, activin inhibition with sActRIIB-Fc did not limit acute tubular necrosis in either transplantation or IRI model. Thus, our results challenge the existing knowledge and further investigations are needed to specify the role of each follistatin target molecule in the pathogenesis of tubular injury.

According to our results, activin inhibition also reduced the early accumulation of fibroblasts in the graft. Thus, activins may be involved in kidney allograft fibrosis already in the early phases after transplantation. Activins may also regulate the further development of renal fibrosis, as activin inhibition with follistatin has been shown to reduce fibrosis in the unilateral ureteral obstruction model in the rat [23]. *In vitro* studies indicate that activin A promotes fibrosis by enhancing renal fibroblast proliferation and transdifferentiation into myofibroblasts. In addition, activin A induces the formation of extracellular matrix both in renal fibroblasts and in glomerular mesangial cells. [24,32] Activin inhibition reduces TGF-β1-induced type I collagen and α-smooth muscle cell expression in fibroblasts suggesting that activin A in its own part mediates some of the fibrogenic effects of TGF-β1 [24,25]. In our study, activin inhibition reduced early macrophage infiltration to the graft, which may also limit the further development of renal fibrosis. High activin transcript levels have been shown to be associated with a profibrotic alternatively activated macrophage phenotype in mouse renal allografts [33,34]. Thus, according to previous reports and our results, activins function at multiple levels to regulate fibrotic processes in the kidney. However, further studies are needed to explore the ultimate role of activin inhibition in the development of renal transplant fibrosis.

Taken together, our study is the first report on the effects of activin inhibition in a transplantation setting. Activin inhibition efficiently reduces the innate immune response after transplantation in the rat. Interestingly although no effect on IRI was seen, activin inhibition reduced early fibroblast expression suggesting that activins may also be involved in the regulation of renal transplant fibrosis. Thus, this study suggests that activins may have potential as future therapeutic targets in the transplantation process.

Authorship

NP and JS: participated in research design, performance of the research, data analysis, and writing of the manuscript. AP: contributed important reagents and participated in research design and writing of the manuscript. JR: participated in research design, performance of the research, and writing of the manuscript. BK, SM, AK and M-PR: participated in the monoclonal antibody generation, and assay development of the activin A and activin B ELISA assays as well as processing of the samples in the activin ELISAs. HH: participated in research design, data analysis, and writing of the manuscript. OR: contributed important reagents and participated in overall research design and writing of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. In the bilateral renal IRI model the inflammatory cell response was mild in both control and sActRIIB-Fc groups three days after IRI.

Table S1. Fc-G1 did not affect serum cytokine concentrations after transplantation.

Table S2. Histopathological evaluation of the kidneys three days after transplantation or bilateral renal clamping induced ischemia-reperfusion injury.

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