Bβ_{15-42} Attenuates the Effect of Ischemia-Reperfusion Injury in Renal Transplantation

Inga Sörensen,* Song Rong,* Nathan Susnik,* Faikah Gueler,† Nelli Shushakova,‡ Melanie Albrecht,‡ Anna-Maria Dittrich,‡ Sibylle von Vietinghoff,‡ Jan Ulrich Becker,‡ Anette Melk,§ Andrea Bohlmann,* Sonja Reingruber,¶ Peter Petzelbauer,¶ Hermann Haller,* and Roland Schmitt*

Departments of *Nephrology and Hypertension, †Pediatric Pneumology, Allergology, and Neonatology, ‡Institute of Pathology, and §Pediatric Nephrology and Gastroenterology, Medical School Hannover, Germany; ¶Fibrex Medical, Inc., Vienna, Austria; and ¶SERD at Department of Dermatology I, Medical University of Vienna, Vienna, Austria

ABSTRACT
Renal ischemia-reperfusion contributes to reduced renal allograft survival. The peptide Bβ_{15-42}, a breakdown product of fibrin, attenuates inflammation induced by ischemia-reperfusion in the heart by competitively blocking the binding of leukocytes to endothelial VE-cadherin, but whether it could improve outcomes in renal transplantation is unknown. Here, we tested the ability of Bβ_{15-42} to ameliorate the effects of renal ischemic injury during allogenic kidney transplantation in mice. In our renal transplantation model (C57BL/6 into BALB/c mice), treatment with Bβ_{15-42} at the time of allograft reperfusion resulted in significantly improved survival of recipients during the 28-day follow-up (60% versus 10%). Bβ_{15-42} treatment decreased leukocyte infiltration, expression of endothelial adhesion molecules, and proinflammatory cytokines. Treatment significantly attenuated allogenic T cell activation and reduced cellular rejection. Moreover, Bβ_{15-42} significantly reduced tubular epithelial damage and apoptosis, which we reproduced in vitro. These data suggest that Bβ_{15-42} may have therapeutic potential in transplant surgery by protecting grafts from ischemia-reperfusion injury.


Ischemia/reperfusion (IR) is an unavoidable consequence of the renal transplant procedure. Histologic signs of IR injury in early renal allograft biopsies are associated with a proinflammatory state and have been linked to decreased early and late allograft function.1–3 In general, renal IR injury is characterized by initial endothelial and epithelial cell damage, leading to the activation of inflammatory pathways and the recruitment of leukocytes into the postischemic kidney. Several studies have demonstrated a renoprotective effect of targeting post-IR leukocyte recruitment.4–7 Infiltration of leukocytes into the postischemic kidney involves a complex sequence of events, including leukocyte rolling, adhesion to the endothelium, and transmigration.8,9 Leukocyte adhesion is mediated through interaction with surface molecules that are upregulated on activated endothelial cells such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1). Another endothelial surface protein, VE-cadherin (or vascular endothelial cadherin), which is constitutively expressed, mediates endothelial cell-cell contacts at intercellular junctions.10 VE-cadherin can function as a receptor for fibrin through the NH2-terminal portion of the fibrin β-chain.11,12 If bound to VE-cadherin, fibrin fragments have open binding sites...
that can interact with leukocytes, which leads to endothelial
attachment and transmigration.\textsuperscript{13} \( \beta_{15–42} \) is a breakdown product of fibrin that corresponds to the VE-cadherin binding sequence of the fibrin \( \beta \)-chain but which lacks the leukocyte
binding site.\textsuperscript{13} As a consequence, \( \beta_{15–42} \) binding to VE-cadherin can competitively inhibit endothelial leukocyte
adhesion.\textsuperscript{14–16} In 2005, it was shown in rats that \( \beta_{15–42} \) (also called FX06) can protect from myocardial reperfusion damage, which was attributed to its capacity to prevent IR-induced leu-
kocyte infiltration.\textsuperscript{14–16} This result could be confirmed in a
subsequent clinical phase II trial in patients suffering from
acute myocardial infarction.\textsuperscript{16–19} Additionally, \( \beta_{15–42} \) bind-
ing to VE-cadherin was shown to prevent stress-induced RhoA
activation in a Fyn-dependent manner, leading to endothelial
stabilization and preservation of the endothelial barrier.\textsuperscript{14} 
These anti-inflammatory and vasculoprotective effects of \( \beta_{15–42} \) prompted us to test its therapeutic potential in renal
transplantation and allograft rejection.

RESULTS

\( \beta_{15–42} \) Improves Outcome in a Life-Supporting
MHC-Mismatched Transplantation Model

To test the effect of \( \beta_{15–42} \) in transplantation-related IR, we
used a fully MHC-mismatched transplant model from
C57BL/6 to BALB/c mice without immunosuppressive ther-
apy.\textsuperscript{20} Ischemic allograft injury was induced by a prolonged
cold ischemia time of 60 minutes and a warm ischemia time of
30 minutes. In this model, the left kidney of the recipient is
removed and replaced during transplantation. The right kid-
ney is resected 4 days after transplantation and survival is lim-
ited by rejection of the life-supporting allograft.\textsuperscript{20} The first
group of mice was treated by allograft flushing with a \( \beta_{15–42} \)-
containing solution (100 \( \mu \)g \( \beta_{15–42} \)/ml) in combination with
a systemic intravenous (iv) injection (3 mg/kg) at the time of
allograft reperfusion and another bolus after 5 minutes
(\( \beta_{15–42} \)-renal + iv). A dose of 3 mg/kg and the double-injec-
tion mode was chosen according to previous myocardial re-
perfusion studies.\textsuperscript{15,16,21} The second group only received al-
lograft flushing (\( \beta_{15–42} \)-renal). The third group only received
systemic treatment (\( \beta_{15–42} \)-iv). The control group was treated
with allograft flushing using scrambled control peptide (100
\( \mu \)g/ml) plus a systemic control peptide injection (3 mg/kg) at the time of
allograft reperfusion and 5 minutes after reperfusion. Survival curves for 28-
day follow-up, \( n = 20 \). *\( P < 0.05 \), **\( P < 0.001 \).

\( \beta_{15–42} \) Treatment Protects from IR-Induced Acute
Kidney Injury

In the heart \( \beta_{15–42} \) has been demonstrated to protect from early
myocardial inflammation and structural damage after transient
coronary artery occlusion.\textsuperscript{15,16} On the basis of these data, we
hypothesized that the beneficial effects of \( \beta_{15–42} \) seen in the renal
transplantation model were mediated by protection from sur-
gery-induced IR damage. To test this hypothesis, male C57BL/6
mice were subjected to transient renal ischemia. After 27 minutes
of bilateral renal arterial occlusion, vascular clamps were released
and mice were injected intravenously with \( \beta_{15–42} \) or control
peptide followed by a second injection 5 minutes after reperfu-
sion. During the first 3 days of follow-up, renal function in both
groups of mice sharply declined, as shown by an increase in levels
of serum creatinine and urea (Figure 2, A and B). However, mice
that were treated with \( \beta_{15–42} \) were partially protected from renal
dysfunction, and mortality in \( \beta_{15–42} \)-treated mice was signifi-
cantly reduced (Figure 2C).

\( \beta_{15–42} \) Treatment Reduces Endothelial Activation and
Inflammatory Infiltration

To explore the underlying mechanisms of \( \beta_{15–42} \)-dependent
renal protection in IR, additional mice were subjected to tran-
sient renal ischemia, treated with \( \beta_{15–42} \) or control peptide,
and sacrificed 24 hours post-IR. Histologic examination of

Figure 1. \( \beta_{15–42} \) improves survival in a model of life-supporting
MHC-mismatched allogenic kidney transplantation. The first
group of mice (\( \beta_{15–42} \)-renal + iv) was treated with allograft flushing using a
(100 \( \mu \)g \( \beta_{15–42} \)/ml) in combination with a
systemic injection (3 mg/kg) at the time of allograft reperfusion and
5 minutes after reperfusion. The second group (\( \beta_{15–42} \)-renal) only
received allograft flushing. The third group (\( \beta_{15–42} \)-iv) only received
systemic treatment. The control group (control) was treated
with allograft flushing using the control peptide (100 \( \mu \)g/ml) plus a sys-
temic control peptide injection (3 mg/kg) at the time of allograft
reperfusion and 5 minutes after reperfusion. Survival curves for 28-
day follow-up, \( n = 20 \). *\( P < 0.05 \), **\( P < 0.001 \).
IR kidneys revealed a diminished influx of inflammatory leukocytes and reduced tubular injury in B\textsubscript{β15–42}-injected mice (Figure 3A). By immunostaining, we found significantly reduced numbers of infiltrating leukocytes (CD45-positive), macrophages (F4/80-positive), and neutrophils (Ly-6G-positive) (Figure 3, B and E). Most of these inflammatory cells were located within the tubulointerstitial space outside of blood vessels. Similarly, there were significantly fewer CD8-positive T cells in kidneys from B\textsubscript{β15–42} treated mice, as shown by reduced intrarenal CD8 protein abundance (Figure 3, D and G). In parallel, endothelial activation was significantly reduced, as reflected by decreased expression of ICAM-1, VCAM-1, and E-selectin (Figure 3, C, D, F, and G). Tubular epithelial cells were less damaged, as shown by diminished expression of tubular injury markers kidney injury molecule-1 (KIM-1) (Figure 3 D, F, and G) and neutrophil gelatinase-associated lipocalin (NGAL) (Figure 3F).22 Together these data suggest that exogenous administration of the fibrin breakdown product

![Figure 2](image)

**Figure 2.** B\textsubscript{β15–42} improves renal function and survival after IR caused by bilateral renal clamping for 27 minutes. (A) Serum creatinine, (B) serum urea, and (C) survival curves for mice that received B\textsubscript{β15–42} or control peptide shortly before vascular clamp release and 5 minutes after reperfusion (each injection 3 mg/kg into the tail vein). n = 12. Means ± SEM; *P < 0.05.

![Figure 3](image)

**Figure 3.** B\textsubscript{β15–42} reduces the influx of inflammatory cells, tubular injury and endothelial activation after IR caused by unilateral renal clamping for 27 minutes. Kidney examination 24 hours after renal clamping in animals treated with B\textsubscript{β15–42} or control peptide (n = 8). Representative (A) hematoxylin/eosin renal histology and immunostaining for (B) F4/80 and (C) ICAM-1. (D) Western blots for ICAM-1, CD8, and KIM-1 of representative animals and (G) corresponding densitometry relative to glyceraldehyde 3-phosphate dehydrogenase (GADPH). (E) Quantification of CD45-, F4/80-, and Ly-6G-positive cells in outer medullary region. (F) Quantitative PCR showing the relative expression levels of KIM-1, NGAL, VCAM-1, and E-selectin. Magnification in A through C, ×400. Data are means ± SEM; *P < 0.05, **P < 0.001.
Bβ15–42 reduces IR-induced endothelial activation, inflammatory cell influx, and subsequent tissue damage.

Bβ15–42 Has a Direct Antiapoptotic Effect on Renal Tubular Epithelial Cells

Tubular epithelial apoptosis was significantly reduced in Bβ15–42-treated kidneys as shown by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Figure 4, A and B). We therefore asked whether Bβ15–42 might have a direct protective, antiapoptotic effect on renal epithelial cells. To partially mimic the in vivo situation, mouse tubular cell lines (murine proximal tubule epithelial [mPT] and inner-medullary collecting duct [IMCD]) were treated with Bβ15–42 or control peptide and then subjected to ATP depletion or staurosporine exposure. ATP depletion and staurosporine caused a rapid increase in epithelial cell apoptosis and lactate dehydrogenase release as well as an upregulation of KIM-1 in both groups, but this increase was significantly milder in Bβ15–42-treated cells (Figure 4, C through H). According to current knowledge, VE-cadherin is the only transmembrane ligand of Bβ15–42. However, because VE-cadherin is absent from mPT and IMCD cells as confirmed by PCR (data not shown), these results suggest an additional mechanism of action through which Bβ15–42 exerts protective effects in a VE-cadherin-independent manner.

Bβ15–42 Reduces Early Endothelial Activation and Inflammation in Allografts

To characterize the protective mechanisms of Bβ15–42 in renal allografts, additional mice were transplanted and treated with a combination of intrarenal and iv Bβ15–42 administration. Mice were sacrificed at 24 hours or at day 6 when mortality peaked in our model because of acute allograft rejection.20 For these experiments, the model was modified with one host kidney left in place to avoid death of recipients. Similar to the effects observed in the renal IR model, tubular cell morphology was better conserved in Bβ15–42-treated allografts at 24 hours after transplantation (Figure 5, A and B). Bβ15–42 treatment was associated with significantly fewer infiltrating leukocytes (Figure 5, C, D, G, and I) and a significant reduction in endothelial activation, as shown by diminished expression of adhesion molecules ICAM-1, VCAM-1, and E-selectin (Figure 5, H and I). Bβ15–42-treated allografts had better tubular cell integrity as seen by diminished KIM-1 (Figure 5, E, F, H, and I), NGAL expression (Figure 5H), and less epithelial apoptosis (Figure 5J). On day 6, we found massive allograft inflammation and tubulointerstitial damage representing acute rejection in both groups (Figure 6, A and B). However, these changes were clearly attenuated in Bβ15–42-treated allografts. Histopathological evaluation of acute allograft rejection revealed a trend for reduced peritubular infiltrates, less interstitial edema and necrosis, and diminished endothelialitis/arteritis in the Bβ15–42-treated group (Figure 6, A and B; Supplemental Figure C). Mild tubulitis was found in all allografts with no clear difference between treatment groups.
Data are means ± SEM; *P < 0.05, **P < 0.001.

Similar to the observations at 24 hours, Bβ15–42 treatment was associated with diminished numbers of CD45-, CD8-, F4/80-, Ly-6G-positive infiltrating cells (Figure 6, D, E, G, and H) and lower ICAM-1, VCAM-1, and E-selectin expression (Figure 6, H and I). Renal cytokine expression of IL-1β and IL-12 was decreased (Figure 6, J and K), whereas IL-10 and TGF-β were significantly upregulated in allografts of the Bβ15–42-treated group (Figure 6K). Tubular cell apoptosis and KIM-1 expression at day 6 was also significantly reduced with Bβ15–42 treatment (Figure 6, H and L). These data suggest that the protective effect of Bβ15–42 is importantly mediated through an attenuated inflammatory response resulting from decreased endothelial activation, reduced leukocyte infiltration, and better tubular cell protection.

The Protective Effects of Bβ15–42 Depend on the Time Point of Administration

To better characterize the protective effect of Bβ15–42 in the transplant setting, an additional group of mice was treated with delayed Bβ15–42 administration. In this group allografts were flushed with control peptide, and Bβ15–42 was given at 24 hours postreperfusion. Hematoxylin/eosin staining on day 6 suggested that allografts treated with delayed Bβ15–42 administration still had a tendency for reduced inflammatory peritubular infiltrates (Figure 6C) whereas little effect was observed on interstitial edema and epithelial cell integrity. By immunostaining, we could demonstrate that delayed Bβ15–42 administration significantly diminished the numbers of F4/80-positive infiltrating cells (Figure 6G) whereas quantification of CD45-, Ly-6G- and CD8-positive cells was not different from controls (Figure 6, F through H). In parallel, expression of renal E-selectin was significantly reduced, but ICAM-1 and VCAM-1 were unaffected (Figure 6, H and I). Changes in the expression of IL-1β, IL-12, IL-10, and TGF-β that were seen in the early Bβ15–42-treated group could not be observed anymore (Figure 6, J and K). Delayed Bβ15–42 treatment had no significant effect on tubular cell apoptosis or on KIM-1 expression (Figure 6, H and L). These results indicate that Bβ15–42 loses most of its protective properties on tubular cell integrity, inflammation, and acute rejection when administered at 24 hours postreperfusion.

Bβ15–42 Treatment Diminishes Alloreactive T Cell Priming

Alloreactive T cell priming is a key step in initiating the alloimmune response and promoting acute renal rejection. To exclude the possibility that Bβ15–42 has a direct effect on T cell activation, we added Bβ15–42 to standard in vitro proliferation assays using ovalbumin (OVA)-specific T cell receptor (TCR) transgenic splenocytes. In this antigen-dependent assay no significant effect of Bβ15–42 was found on CD4 or CD8 T cell activation after stimulation with C57BL/6 antigen-presenting cells (Supplemental Figures E and F). Although this argues against a direct effect of Bβ15–42, we found a significant inhibition of recipient T cell activation after Bβ15–42 treatment in vivo. Mixed lymphocyte reactions of recipient splenocytes at day 6 after transplantation showed that the proliferative response of T cells to allogeneic C57BL/6 stimulation was significantly attenuated after Bβ15–42 treatment (Supplemental Fig...
ure D). These data fit the hypothesis that Bβ15–42 indirectly modifies allospecific T cell activation by protecting from renal IR damage and by suppressing early leukocyte recruitment to the allograft, which reduces subsequent priming of antigen-specific T cells.

DISCUSSION

Endothelial activation and leukocyte-dependent inflammation play instrumental roles in the pathogenesis of renal IR injury and early allograft damage.9,24 Our results suggest that Bβ15–42 conveys structural and functional protection in renal IR by reducing postischemic inflammation through inhibition of endothelial activation, reduced leukocyte recruitment, and tubular epithelial protection. In MHC-mismatched transplantation this is associated with a reduction in allogenic T cell priming and a sustained attenuation of allograft rejection.

Previous studies have indicated that the protective effect of Bβ15–42 is mediated through an inhibition of leukocyte-endothelial interactions.13,16 By binding to VE-cadherin, Bβ15–42 blocks potential binding sites for fibrin E1, which is a plasmin digest of crosslinked fibrin.13 E1 fragments consist of the three N-terminal segments of the fibrin α-, β-, and γ chains. The β-chain sequence has a binding site for VE-cadherin, whereas the α-chain sequence can bind CD11c. By binding to VE-cadherin and in parallel to CD11c, fibrin E1 can function as a bridge molecule between endothelial cells and leukocytes.
Bβ_{15-42} competes for the VE-cadherin binding site and thus prevents endothelial leukocyte attachment and transmigration. Consistent with this blocking mechanism, we found that early inflammatory cell influx was significantly reduced in the kidneys of Bβ_{15-42} treated mice. According to another mechanism of action that has recently been described, Bβ_{15-42} binding to VE-cadherin can induce endothelial signaling via the src kinase Fyn.\(^{14}\) Fyn interaction with p190RhoGAP leads to the inactivation of RhoA, causing better preservation of endothelial barrier function.\(^{14}\) In our experiments, Bβ_{15-42} inactivation of RhoA, causing better preservation of endothelial barrier function.\(^{14}\) In our experiments, Bβ_{15-42} treatment reduced the upregulation of the endothelial adhesion receptors ICAM-1, VCAM-1, and E-selectin, pointing to a possible link to RhoA, which is a known inducer of these adhesion molecules.\(^{25,26}\) In addition to these anti-inflammatory and vasculo-protective effects, we found that Bβ_{15-42} treatment was associated with a significantly better preservation of tubular epithelial integrity and reduced epithelial apoptosis. Although these effects could be secondary to reduced tissue inflammation, our in vitro data suggest an additional direct effect of Bβ_{15-42} as a protective and antiapoptotic molecule for renal epithelial cells. Because renal epithelial cells do not express VE-cadherin, which is the only receptor for Bβ_{15-42} known so far, further research is warranted to identify the underlying signaling pathway. Interestingly, we found that the combination of allograft flushing and recipient injection with Bβ_{15-42} acted in a partially additive fashion. Although this might reflect a dose effect, it could alternatively fit the concept of a VE-cadherin-independent mechanism of action. For example, our data would be consistent with possible extrarenal effects of Bβ_{15-42}, in which epithelial protection of other tissues might help to avoid “remote” organ damage, which has an important effect on the outcome of acute kidney injury.\(^{27}\)

In renal transplantation, surgical trauma and IR lead to an initial proinflammatory state.\(^{1-3}\) Endothelial cells are activated and T cells, along with other inflammatory cells, enter the allograft. This may serve as a stimulus for rejection if MHC differences are present.\(^{29}\) It has consistently been shown across many studies that more severe IR damage is associated with a stronger inflammatory response and an increased risk of subsequent rejection.\(^{1,4,29,30}\) Despite the suggestive clinical data, it has been difficult to directly test the contribution of initial IR injury to allograft rejection in experimental studies.\(^{23}\) Our results, in conjunction with several recent intervention studies,\(^{20,31}\) strongly support the hypothesis that therapeutic targeting of nonalloimmune IR injury can efficiently contribute to minimizing subsequent alloimmune processes and renal rejection.

This is the first study showing that Bβ_{15-42} protects from acute kidney injury. In renal allotransplantation, the tissue-protective effect was associated with a reduced alloimmune response and prolonged graft survival. In conjunction with recent clinical trials showing an excellent tolerability and safety of Bβ_{15-42} in patients,\(^{17,18}\) our data suggest that intrasurgical administration of this peptide could be used to prevent IR-triggered alloimmune processes and reduce the risk of rejection in renal transplantation. Compared with other experimental strategies that try to prevent IR injury and rejection by blocking individual adhesion molecules or chemokines, the use of Bβ_{15-42} might be more promising because of its broader mechanism of action. Recipients of high-risk allografts (e.g., extended-criteria donor kidneys), which are more likely to develop relevant IR damage and delayed graft function, could especially benefit from additional protection including Bβ_{15-42}.

**CONCISE METHODS**

**Animals**

Male C57BL/6 (H\(^{2b}\)) and female BALB/c (H\(^{2d}\)) mice were supplied by Charles River (Sulzfeld, Germany) and were housed under standard conditions. Mice that were 10 to 12 weeks old weighing between 20 and 30 g were used for all experiments. For T cell proliferation experiments, DO11.10 (BALB/c-Tg[DO11.10]10Loh/J) mice bearing an OVA-reactive transgenic TCRs were used. All experimental procedures were in agreement with institutional and legislative regulations and were approved by the local authorities.

**IR Protocol**

Renal IR injury was induced through bilateral clamping of renal pedicles as described previously.\(^{32}\) Male C57BL/6 were anesthetized with isoflurane. After median laparotomy, renal pedicles were dissected and a nontraumatic vascular clamp was applied for 27 minutes. Bβ_{15-42} or random control peptide was administered shortly before vascular clamp release and 5 minutes after reperfusion by two injections of 3 mg/kg into the tail vein. The dose of 3 mg/kg was chosen based on previous experiments in models of myocardial IR.\(^{15,16}\) Blood samples were taken on days 0, 1, 3, 5, and 7, and renal function was estimated by serum creatinine and urea measurements using an automated method (Beckman Analyzer, Beckman Instruments GmbH, Munich, Germany). In a second group of mice, renal pedicle clamping was only performed on the left side. These animals were sacrificed after 24 hours and kidneys were harvested for further examination.

**Allogeneic Kidney Transplantation**

Vascularized kidney transplantation from male C57BL/6 to female BALB/c mice was performed as described previously.\(^{20}\) Briefly, mice were anesthetized with isoflurane and the donor kidney, ureter, and bladder were harvested en block, including the renal artery with a small aortic cuff and the renal vein with a small caval cuff. Cold ischemia time was 60 minutes, and warm ischemia time was 30 minutes. After explantation, kidneys were stored in vehicle solution at 4°C for 45 minutes. These ischemia times induce a moderate degree of IR injury in this model. Fifteen minutes before implantation, donor kidneys were flushed with vehicle solution containing 100 μg Bβ_{15-42}/ml or vehicle solution containing random control peptide through the renal artery. After left nephrectomy of the recipient, vascular cuffs were anastomosed to the recipient abdominal aorta and vena cava, respectively, below the level of the native renal vessels. The ureter was directly anastomosed into the bladder. Before reperfusion, Bβ_{15-42} or random control peptide was administered intravenously, followed by a second injection (3 mg/kg each) after 5 minutes. The right native
kidney was removed on posttransplantation day 4 so that survival became graft dependent. Blood was taken on days 0, 5, 14, 21, and 28, and renal function was estimated by serum creatinine and urea measurements. For pathology studies, allografts were flushed with vehicle solution containing 100 μg Bβ15–42/ml or vehicle solution containing random control peptide and then transplanted with one host kidney left in place to stabilize the recipient and avoid premature death. Recipients were treated with two iv injections of Bβ15–42 or random control peptide (3 mg/kg each) before reperfusion and after 5 minutes. Mice were sacrificed at 24 hours to analyze allografts and at day 6 to investigate allografts and to determine T cell priming using spleen cells. In an additional group, allografts were flushed with vehicle solution containing 100 μg/ml random control peptide and recipients were treated with two iv injections of Bβ15–42 (3 mg/kg each, 5 minutes apart) at 24 hours posttransplant surgery. Allografts and spleens were harvested on day 6.

**Synthesis of Peptides**
Bβ15–42 (amino acid sequence, GHRPLDKKREEAPSLRAPPPISSGGGGYR) and the control scrambled peptide (amino acid sequence, DRGAPAHRPGRPSGRSTPEKEKLLPG) were produced by solid-phase peptide synthesis and purified with reverse-phase HPLC as described previously.16

**Histology, Immunostaining, and TUNEL**
After harvesting, a representative part of each kidney was fixed immediately in PBS-buffered 4% paraformaldehyde and embedded in paraffin. Four-micrometer sections were used for immunostaining and for hematoxylin/eosin staining to evaluate histologic damage. Allografts were semiquantitatively scored for acute rejection by a blinded qualified pathologist classifying signs of acute tubulitis, acute vascu- litis, interstitial infiltrate and necrosis, edema, and hemorrhage. Immunostaining was performed using the following primary antibodies: monoclonal rat anti-mouse F4/80 (Serotec, Oxford, United Kingdom), monoclonal rat anti-mouse CD45 (BD Pharmingen, BD Biosciences, Santa Cruz, CA), affinity-purified rat anti-mouse Ly-6G/Gr-1 (eBioscience, San Diego, CA), goat anti-rat ICAM-1 (R&D Systems, Minneapolis, MN), and rabbit anti-human KIM-1 (Novus Biologicals, Littleton, CO). Deparaffinized kidney sections were boiled in citrate buffer for antigen retrieval, blocked with 5% milk, and incubated overnight at 4°C with primary antibodies. This was followed by antibody visualization using Alexa 488/Alexa 547 secondary antibodies (Molecular Probes/Invitrogen, Carlsbad, CA) or the ABC Vectastain kit (Vector Laboratories, Burlingame, CA). Quantification of CD45-, F4/80-, and Ly-6G-expressing cells was done by counting of positive cells in ten randomly chosen, nonoverlapping fields (magnification, ×400) in outer medulla. A fluorescence in situ cell death detection kit was used according to the manufacturer’s instructions for TUNEL assay (Roche Applied Science, Mannheim, Germany). TUNEL-positive tubular cells and total DAPI (4’,6-diamidino-2-phenylindole)-positive tubular cells were counted in ten nonoverlapping fields of outer medulla in each sample (magnification, ×400). Data are presented as a percent ratio of TUNEL-positive epithelial cells versus total DAPI-positive epithelial cells.

**Cell Culture**
Immortalized mPT or inner medullary collecting duct (IMCD) cells (both kindly provided by Dr. L. Cantley, Yale School of Medicine, New Haven, CT) were grown to 60% to 70% confluence, treated with Bβ15–42 or control peptide (each at 30 μg/ml), and then subjected to ATP depletion using glucose-free medium containing 5 mM sodium cyanide and 5 mM 2-deoxy-d-glucose (Sigma-Aldrich, St. Louis, MO). In parallel experiments, mPT and IMCD cells were treated with 0.2 μM staurosporine (Sigma-Aldrich, St. Louis, MO) or vehicle to induce apoptosis. Apoptosis was determined after 16 hours by TUNEL assay (Roche Applied Science, Mannheim, Germany) and caspase-3 assay (GenScript, Piscataway, NJ); additionally a lactate dehydrogenase cytotoxicity assay (BioVision, Mountain View, CA) was performed with the same conditions. All assays were done according to the manufacturers instructions.

**Western Analysis**
Western analysis was performed as described previously.33 In brief, a representative part of each kidney was frozen in liquid nitrogen immediately after harvesting. Tissue was homogenized and protein electrophoresis was performed as described previously.33 Proteins were transferred to polyvinyldene difluoride membranes, blocked with 5% milk in TBS-Tween, and probed overnight at 4°C with the following primary antibodies: monoclonal rat anti-mouse CD8 (provided by A. Dittrich), goat anti-rat ICAM-1 (R&D Systems, Minneapolis, MN), and rabbit anti-human KIM-1 (Novus Biologicals, Littleton, CO). Antibody binding was visualized by chemiluminescence (SuperSignal West Pico Chemiluminescent, Thermo Scientific, Rockford, IL). Rabbit anti-mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Sigma Aldrich, St. Louis, MO) was used as an internal loading control and for normalization of protein quantification. Immunoblots were scanned and quantified using ImageJ densitometry software.

**Mixed Lymphocyte Reactivity**
Priming of alloantigen-specific T cells from kidney graft recipients was investigated by performing mixed lymphocyte reactivity assays based on the measurement of BrdU incorporation during DNA synthesis. The responder spleen cells were obtained from naive BALB/c mice or from allograft recipients at day 6 after transplantation. Stimulator cells were prepared from the spleens of syngenic (i.e., BALB/c) and allograft donors (i.e., C57BL/6). Stimulator cells were treated with 50 μg/ml mitomycin C for 30 minutes, washed, and added to responder cells and cultured for 48 hours. Afterward, BrdU was added, and 18 hours later responder cell proliferation was quantified using a colorimetric cell proliferation ELISA kit (Roche Diagnostics GmbH) in accordance with the manufacturer’s instructions. Syngenic stimulator cells were used as background controls and were subtracted from allogeneic responses. To test for a direct effect of Bβ15–42 on antigen-specific T cell activation, co-cultures were conducted using OVA-specific TCR transgenic splenocytes. Transgenic CD4- or CD8-positive T cells (OT-II, OT-I cells) were stimulated with C57BL/6 antigen-presenting cells and OVA peptide in the presence of different concentrations of Bβ15–42 or random control peptide. After 2, 3, and 4 days, T cell proliferation was determined by carboxyfluorescein di-
acetate succinimidyl ester dilution measurement using flow cytometry as described above.

Quantitative Real-Time PCR
RNA was isolated from frozen kidney tissue using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Reverse transcription was performed with M-MLV reverse transcriptase (Promega, Madison, WI) and random primers. Amplified cDNA was used as a template for quantitative PCR. The levels of mRNA expression were determined by quantitative real-time PCR using a Roche Lightcycler 480 system with SYBR green master mix and the following specific primers: NGAL forward TGAGGAAAGTTTCACCCCGCTTTG, NGAL reverse ACAGGAAAGATGGAGTGGCAGACA; KIM-1 forward AAACCAAGTATCCCAACAGC, KIM-1 reverse GTGGTGCTCTCTAGTAGCC; TGF-β1 forward CTGGATCGGACCCTGCAGGGAAC, TGF-β1 reverse ATGCTCAATCAGGCTCTTC; IL-12-35 forward GACCTCGAGATAGCC; IL-12-40 reverse CTAGGATCGGACCCTGCAGGGAAC, IL-12-35 forward GACCTCGAGATAGCC; VCAM-1 forward TGACAAGTCCCCATCGTGTAC; VCAM-1 reverse ACCTCGCGACGGCATAATT; E-selectin forward TGAGGATCGGACCCTGCAGGGAAC, E-selectin reverse GGGCTGCTTCCTGGAAGAT; TGF-β forward CAACAAATTCCTGGCGTTACCTTGG, TGF-β reverse GAAAGCCCTGTATTCCGTCTCCTT; IL-1 reverse AGTGGTGCTCTCTAGTAGCC; actin forward AGCCATGCAAACCTTTGAC; IL-10 forward TGAATTCCCTGGGTGAGAAG, IL-10 reverse GCTCCACTGCCTTGCTCTTA; actin forward AGCCATGCAAACCTTTGAC; actin reverse CTCTCAGCTGTTGGGTGTTAA.

Melting curves were examined to verify that a single product was amplified. For quantitative analysis, relative mRNA levels were calculated according to the 2−AΔCt method; all samples were normalized to actin gene expression.

Statistical Analysis
Results are expressed as means ± SEM. Statistical significance was assessed by unpaired t test or by log-rank tests performed on Kaplan–Meier survival curves (GraphPad Software, San Diego, CA). P < 0.05 was considered to be statistically significant.

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DISCLOSURES
S.R. and P.P. are founders and shareholders of Fibrex Medical, Inc.

REFERENCES


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