# Immunoproteasome inhibition prevents chronic antibody-mediated allograft rejection in renal transplantation

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**Chronic antibody-mediated rejection is the major cause of fading allograft function and loss after renal transplantation. Currently, pharmacological agents for the suppression of chronic antibody-mediated rejection are lacking. Non-selective proteasome inhibitors suppress antibody-mediated allograft rejection. However, extensive adverse side effects of these inhibitors severely limit their application. In contrast, immunoproteasome inhibition is effective in preclinical models of autoimmune diseases and was applied over weeks without obvious adverse side effects. ONX 0914, an immunoproteasome subunit LMP7 (β5i)-selective inhibitor, impeded the chronic rejection of kidneys transplanted from Fischer to allogeneic Lewis rats. ONX 0914 inhibited immunoproteasome induction both in immune organs and renal allografts. Selective immunoproteasome inhibition reduced the numbers of B and plasma cells, and suppressed donor-specific alloantibody production. The infiltration of T cells, B cells and macrophages as well as interferon-γ, interleukin-17, IgG and complement deposition were reduced in renal allografts of ONX 0914-treated recipients. Chronic nephropathy was ameliorated and renal allograft function preserved, enabling long-term survival of recipients. Thus, our studies define a critical role of the immunoproteasome in chronic kidney allograft rejection and suggest immunoproteasome inhibition as a promising therapeutic approach to suppress chronic antibody-mediated rejection.**

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Renal transplantation is the most effective therapeutic approach for the loss of kidney function. Immunosuppressive agents have effectively inhibited T cell–mediated acute rejection after kidney transplantation.[1,2] However, the long-term survival rate of renal allografts is still limited by chronic rejection. In recent years, alloantibody-mediated humoral immunity has been identified as the major cause of chronic allograft rejection.[3] Nevertheless, an effective approach to suppress humoral immunity and prevent chronic rejection is lacking.

Bortezomib, a broad spectrum proteasome inhibitor, inhibits alloantibody production through induction of apoptosis in plasma cells from patients with kidney transplantation.[4] Bortezomib inhibits the active sites of all types of 20S proteasomes, which contain 2 copies of 7 different α and 7 different β subunits each. In most human tissues the constitutive proteasome containing the 3 active site subunits β1, β2, and β5 is the predominantly expressed type of 20S proteasome.[5,6] The 20S proteasome fulfills numerous functions ranging from signal transduction to cell cycle control and from transcription regulation to apoptosis.[7–10] Consequently, nonselective proteasome inhibition results in dose-limiting adverse effects and allograft injury.

A much more attractive alternative would be to selectively inhibit proteasomes in cells of the immune system involved in graft rejection after organ transplantation. In T and B lymphocytes, antigen presenting cells, and inflamed tissues that are exposed to interferon (IFN)-γ or tumor necrosis factor (TNF), the catalytic subunits of the constitutive 20S proteasome are replaced by their cytokine-inducible counterparts β1i (LMP2), β2i (MECL-1), and β5i (LMP7) during the assembly of the 20S immunoproteasome.[5,11,12] Apart from its role in antigen processing, the immunoproteasome regulates the production of pro-inflammatory cytokines and the differentiation of Th17/Th1 cells involved in the pathogenesis of autoimmunity and transplant rejection.[13] The inhibition of immunoproteasomes with the LMP7-selective irreversible epoxyketone inhibitor ONX 0914 (formerly designated PR-957) prevents the development and exacerbation of numerous

autoimmune diseases.[14–18] Remarkably, bortezomib and ONX 0914 reduces the number of antibody-secreting plasma cells and anti-DNA antibodies in lupus-prone mice, and bortezomib does so in humans as well.[17,19,20] Nevertheless, the role of the immunoproteasome in alloantibody-mediated chronic rejection after transplantation has not been investigated.

In this study, we tested the effect of immunoproteasome inhibition on chronic rejection after kidney transplantation in the rat. Selective immunoproteasome inhibition suppressed the humoral immune response, which alleviated chronic allograft nephropathy and prolonged the long-term survival of recipients. We conclude that the immunoproteasome is centrally involved in chronic renal allograft rejection and propose the immunoproteasome as a promising new therapeutic target for the avoidance of chronic allograft rejection after kidney transplantation.

## RESULTS

### ONX 0914 inhibits immunoproteasome induction after kidney transplantation

In this study, we applied the LMP7 inhibitor ONX 0914 to explore the role of the immunoproteasome in chronic renal allograft rejection in rats. First, we subcutaneously injected different doses of ONX 0914 in Lewis rats to determine the appropriate dose for *in vivo* treatment. Guided by the dose of ONX 0914 required for selective LMP7 inhibition in mice,[14] 0 mg/kg (vehicle), 3 mg/kg, and 5 mg/kg of ONX 0914 were subcutaneously injected. One day after treatment, rats were killed to purify 20S proteasomes from spleens and bone marrow. The activities of proteasome subunit β5c and immunoproteasome subunit β5i (LMP7) were tested using their specific fluorogenic substrates Ac-WLA-AMC and Ac-ANW-AMC, respectively. In comparison with vehicle, treatment with 5 mg/kg inhibited LMP7 activity >80% in spleen and >90% in bone marrow but only inhibited <10% of β5c activity (Figure 1). Bortezomib inhibited antibody-mediated chronic renal allograft rejection[21] and therefore served as positive control. Our data suggest that ONX 0914 selectively and sufficiently inhibited LMP7 *in vivo* at a dose of 5 mg/kg.

At 3 weeks after allogeneic kidney transplantation from F344 rat donors to Lewis rat recipients, when alloantibodies are emerging in this model, vehicle, ONX 0914 (5 mg/kg), and bortezomib (0.2 mg/kg) were injected twice weekly into recipients for 7 weeks. At 10 weeks after transplantation, recipients were killed and the expression of the immunoproteasome was analyzed in spleens, bone marrow, and renal grafts. In contrast to samples from syngeneic recipients, the expression of LMP2 and LMP7 was significantly increased in spleens, bone marrow, and renal grafts from allogeneic recipients. Treatment with ONX 0914 or bortezomib significantly reduced the increase in the amounts of LMP2 and LMP7 after transplantation (Figure 2a) most likely because immunoproteasome expression in the kidney is cytokine (mainly IFN-γ)-dependent, and bortezomib and ONX 0914 were shown to reduce IFN-γ production by Th1 cells[14] (see also Figure 5b below). Immunohistofluorescence staining further demonstrated that the immunoproteasome was induced in renal allograft. The evaluation of the microscopic images confirmed that treatment with ONX 0914 or bortezomib effectively suppressed expression of LMP2 and LMP7 in renal allografts (Figure 2b). In parallel, the activities of LMP2 and LMP7 as determined via the hydrolysis of LMP2- and LMP7-selective fluorogenic substrates by 20S proteasomes purified from transplanted kidneys were also upregulated in spleens, bone marrow, and renal grafts from vehicle—but not from bortezomib—or ONX 0914–treated allogeneic recipients. Apparently ONX 0914 and bortezomib inhibited the IFN-γ and TNF-responsive upregulation of LMP2 and LMP7 expression and activity after transplantation (Figure 2c).

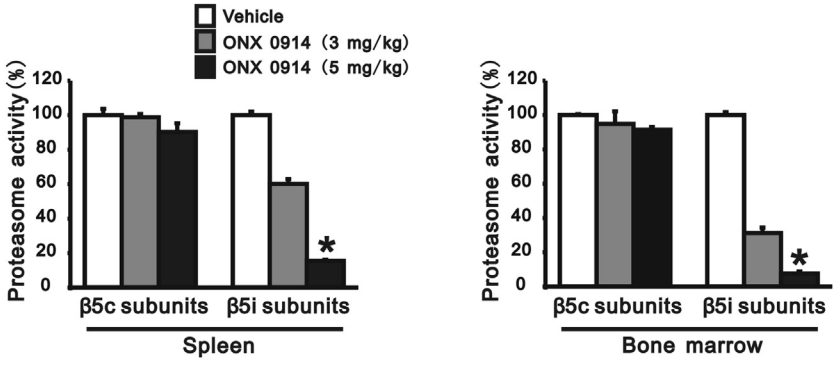


**Figure 1 | Titration of immunoproteasome inhibition by ONX 0914 in rats.** Vehicle, 3 mg/kg, and 5 mg/kg of ONX 0914 were subcutaneously injected into naïve Lewis rats 1 day before killing. Spleens (left panel) and bone marrow (right panel) were then collected to purify the 20S proteasome. Activities of β5c and β5i (LMP7) subunits from each group of spleens and bone marrow were measured by specific substrates. The activity percentages of β5c and β5i from each group were normalized to vehicle group. Treatment with 5mg/kg of ONX 0914 *in vivo* significantly inhibited more than 80% of the activity of LMP7 both in spleens (left panel) and bone marrow (right panel) but inhibited less than 10% of the activity of β5c. Data are expressed as mean ± SEM of each group ($n = 5$) from 3 separate experiments. *$P < 0.05$ versus vehicle group.