

Suppression of acute rejection by administration of prostaglandin E₂ receptor subtype 4 agonist in rat organ transplantation models

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Abstract

Background: Prostaglandin E₂ (PGE₂)–PGE₂ receptor subtype 4 (EP4) signaling is known to modulate the inflammation process. Several studies have demonstrated the potential utility of EP4-selective agonists for the management of autoimmune and inflammatory diseases. In the present study, we assessed the immunosuppressive efficacy of a selective EP4 agonist in experimental rat organ transplantation models.

Methods: A selective EP4 agonist (CAY10580) was continuously injected by subcutaneous insertion of infuser pumps into recipient rats that underwent heterotopic heart and small bowel transplantation.

Results: The administration of EP4 agonist significantly delayed cardiac allograft survival and delayed the onset of rejection in both the cardiac and intestinal allografts. Expression of pro-inflammatory cytokines of interferon- γ (IFN- γ) was suppressed by the treatment compared with the vehicle-treated group. Furthermore, the expression of suppressor of cytokine signaling-1 (SOCS-1), a known intracellular regulation factor of IFN- γ , was also down-regulated compared with the control group.

Conclusions: These results suggest that selective EP4 agonists represent a novel class of immune-modulator drugs that could be useful for the management of acute allogeneic rejection.

Keywords: Prostaglandin E2 receptor subtype 4 (EP4) agonist, Acute rejection, Inflammation, Interferon- γ (IFN- γ), Heart transplantation, Small bowel transplantation

Abbreviations

BN: Brown Norway

CNIs: Calcineurin inhibitors

EP: PGE₂ receptor subtype

EP4: CAY10580

IL: Interleukin

INF: Interferon

JAK: Janus kinase

LEW: Lewis

PGE₂: Prostaglandin E₂

SOCS: Suppressor of cytokine signaling

STAT: Signal transducer and activator of transcription

Introduction

Despite advances in surgical procedures and immunosuppressive agents that have allowed for the establishment of organ transplantation for treatment of life-threatening diseases, acute and chronic rejection remains a major problem in this field. Current immunosuppressive regimens comprising several agents, such as calcineurin inhibitors (CNIs), antimetabolites, steroids, and antibody drugs, accomplish better short-term outcomes. However, long-term administration of immunosuppressive agents, especially CNIs, leads to an increased risk of unfavorable side effects such as hypertension, nephrotoxicity, neurotoxicity, post-transplant malignancy, and metabolic deterioration (1). It is therefore highly desirable to identify new immunosuppressants with novel mechanisms and alternative strategies that have the potential to replace or minimize CNIs.

Prostaglandin E₂ (PGE₂) is produced during inflammatory responses and mediates a variety of both innate and adaptive immune responses through PGE₂ receptor subtypes (EP) 1–4, which have distinct and potentially antagonistic signaling cascades (2). Recent studies show that PGE₂–EP4 signaling is an important regulator of the immune response, and EP4 has emerged as an alternative therapeutic target for several autoimmune and inflammatory diseases (3-5). Our group also reported that immune-mediated mouse liver injuries are ameliorated by the administration of PGE₂ and selective EP4 agonists caused by suppression of interferon (INF)- γ production in natural killer T cells (6). Moreover, studies involving murine colitis models of inflammatory bowel diseases

revealed that EP4 works to suppress innate immunity and facilitate the proliferation of regulatory T cells (7). Selective EP4 agonists have also been reported to inhibit the production of chemokines and cytotoxic cytokines from immune cells, which suppress helper T₁ cell differentiation, and promote epithelial cell survival and growth by activating anti-apoptotic and proliferative cellular signaling pathways (3,8,9).

Consistent with those findings, PGE₂–EP4 signaling will be an alternative target to protect the alloimmune response after organ transplantation. However, the role of selective EP4 agonist administration to modulate immunological responses remains unresolved with the exception of a few studies of a murine cardiac transplantation model (10,11). The objective of this study was to investigate the possibility that a selective EP4 agonist protects against alloimmune rejection in rat organ transplantation models including heart and small bowel transplants based on the fact that PGE₂–EP4 signaling is a modulator of immunity.

Materials and Methods

Animals

All animal care and experimental protocols used in this study were approved by the local ethics committee of Kyoto University, Institute of Frontier Medical Sciences. The experiments were conducted using male Brown Norway (BN) rats (RT-1ⁿ) and Lewis (LEW) rats (RT-1^l) weighing 200 to 300 g and 7 to 10 weeks old, obtained from SLC (Kyoto, Japan).

Heart and small bowel transplantation; treatment regimens

For evaluation of the immunosuppressive effects of EP4 agonist, two types of rat organ transplantation were performed: heterotopic heart-lung and heterotopic small bowel transplantation.

Intra-abdominal heterotopic heart-lung transplantation with one artery anastomosis method was performed as described by Muranaka et al. (12) with some modification. LEW rat heart-lung was heterotopically transplanted into BN rats or synergistically from LEW to LEW rats. After the donor rats were anesthetized and the chest wall was opened, the azygos vein with the left superior and right superior venae cavae was ligated and divided, followed by ligation and division of the inferior vena cava. The ascending aorta was dissected and transected under the portion of the left common carotid artery. An end-to-side anastomosis was performed with 9-0 nylon between the donor's ascending aorta and the recipient's aorta. The beating of the cardiac graft was monitored by daily palpitation through the recipient's abdominal wall. Rejection was defined as the time of cessation of graft

beating as confirmed by direct inspection (n=8, per each group), and histological examination of cardiac grafts rejection were evaluated 7 days after transplantation from another sets of experimental groups (n=6, per each group). Recipient animals were continuously administered an EP4 agonist of CAY10580 (100 µg/kg/day; Sigma-Aldrich, St. Louis, MO, USA) or control vehicle (10% DMSO-PBS) between days 0 and 14 after transplantation via a subcutaneously inserted infuser pump (ALZET osmotic pump, Model 2002; Cupertino, CA, USA) (n = 8 each).

Heterotopic small bowel transplantation was performed according to the modified methodology described by Monchik and Russel (13). A BN rat small intestine was heterotopically transplanted in a LEW rat. After the donor rats were anesthetized, an approximately 30-cm intestinal segment was removed from the donor using the superior mesenteric artery with the aortic cuff and portal vein as a vascular pedicle. An end-to-side anastomosis was performed with 9-0 nylon between the donor's aortic cuff and the recipient's aorta and between the donor's portal vein and the recipient's infrarenal vena cava. A Thiry-Vella loop was placed in the right abdominal flank. Acute rejection was evaluated by histological examination 6 days after transplantation. Recipient animals were continuously administered an EP4 agonist of CAY10580 (100 µg/kg/day; Sigma-Aldrich) or control vehicle (10% DMSO-PBS) between days 0 and 14 after transplantation via a subcutaneously inserted infuser pump (ALZET Model 2002) (n = 5 each). An additional control group comprised BN rats that were neither transplanted nor exposed to any procedure (n = 5).

Analysis of plasma creatine phosphokinase

7days after heart-lung transplantation, blood samples were obtained via inferior vena cava puncture, immediately centrifuged at 1500 *g* for 5 min, and stored at -80°C until analysis. Plasma creatine phosphokinase activities were measured using a standard clinical automatic analyzer.

Histologic analysis

Each excised graft was immediately fixed in 10% formaldehyde solution, embedded in paraffin, sectioned at 5- μ m thickness, mounted on glass slides, and deparaffinized. For histological analysis, slices were stained using hematoxylin-eosin standard techniques and examined in cross sections at 40 $\times$ and 100 $\times$ magnifications under a microscope. The area ratio of myocardial cell infiltration (affected/entire area) was calculated with NIH Image J 1.44 imaging software (NIH, Bethesda, MD, USA). The grading of acute rejection of intestinal grafts was performed as proposed and established by Wu et al. (19). To observe intestinal Paneth and goblet cells, Alcian blue and periodic acid-Schiff combined staining was used. In brief, following incubation in 1% Alcian blue for 15 minutes, sections were incubated in 0.5% periodic acid solution for 5 minutes and in Schiff reagent for 15 minutes. Alcian blue-positive Paneth cells and periodic acid-Schiff-positive goblet cells were counted over the size of a 0.5-mm grid at original magnification and at 100 $\times$ in 10 fields randomly selected from each slide.

Immunohistochemistry

Immunohistochemical studies of heart and intestinal tissues were performed on formalin-fixed, paraffin-embedded sections. These sections were deparaffinized before staining by xylene extraction, followed by extraction with ethanol and water. CD3 (1:1500; Dako, Glostrup, Denmark) and MAC387 (1:400; Novocastra, Newcastle, UK) antibodies were separately added onto the slides and incubated for 1 hour at room temperature. Tissue sections were then sequentially incubated with goat anti-rabbit biotinylated IgG and ExtrAvidin-conjugated horseradish peroxidase. Staining was developed with diaminobenzidine substrate, and nuclei were counterstained with hematoxylin.

Images were obtained using a DP-2 digital camera connected to an AX 80 light microscope (both from Olympus, Tokyo, Japan). Settings for image acquisition were identical for control and experimental tissues. Four ocular fields per section were captured, and a threshold optical density that best discriminated staining from the background was obtained using NIH Image J 1.44 imaging software (NIH, Bethesda, MD, USA). The total pixel intensity was determined and data were expressed as optical density using a counting grid at 40× magnification.

Extraction of RNA and real-time quantitative polymerase chain reaction analysis

Total RNA was purified from heart and intestinal tissues using the RNAeasy Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Complimentary DNA (cDNA) was generated from 1 ng of whole RNA using a SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). cDNA was analyzed for content using a SYBR-Green based quantitative

fluorescent PCR method (Applied Biosystems, Foster City, CA, USA). Fluorescence was detected with the Applied Biosystems 7500 Real-time PCR system (Applied Biosystems). Primers from Roche Universal ProbeLibrary (Roche Diagnostics, Basel, Switzerland) and Shen et al. (20), summarized in **Table 1**, were used. The following PCR conditions were used: 95°C for 15 min, followed by 40 cycles at 94°C for 15 sec, 60°C for 30 sec, and 72°C for 1 min, followed by 72°C for a final 10 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Fold induction was calculated using the C_t method, $\Delta\Delta C_t = (C_t^{\text{target gene}} - C_t^{\text{housekeeping gene}})^{\text{target sample}} - (C_t^{\text{target gene}} - C_t^{\text{housekeeping gene}})^{\text{control sample}}$, and the final data were derived from $2^{-\Delta\Delta C_t}$.

Plasma cytokine quantification by ELISA

Sandwich ELISA for rat plasma IFN- γ and IL-6 were performed using ELISA kits (Qiagen, Dusseldorf, Germany) according to the manufacturer's protocol.

Statistical analysis

Data are presented as means $\pm$ standard error of the mean. Comparisons between two groups were carried out using a two-tailed Student's t test and between multiple groups using one-way analysis of variance with Tukey's test of significance between individual groups. Survival data were plotted using Kaplan-Meier methods and analyzed using the log-rank test. Differences with p values of <0.05 were considered significant.

Results

Treatment with selective EP4 agonist prohibited acute rejection and prolonged graft survival after cardiac transplantation

The administration of a selective EP4 agonist of CAY10580 significantly prolonged cardiac allograft survival compared with vehicle-treated controls (n = 8, 11.0 ± 1.0 vs 8.0 ± 1.0 days, $p < 0.01$, by log-rank test) (Fig. 1A). The serum creatine phosphokinase concentration was significantly decreased in the CAY10580-treated group compared with the vehicle-treated controls (n = 8, $p < 0.01$) (Fig. 1B). In the vehicle-treated controls, cardiac allografts were macroscopically enlarged (Fig. 1C), and myocardial cell infiltration was observed on day 7 (n = 6, $20.1\% \pm 7.8\%$). However, the CAY10580-treated group showed attenuation of the development of myocardial cell infiltration on day 7 (n = 6, $7.3\% \pm 2.5\%$, $p < 0.01$) (Fig. 1D, E). These results suggest that EP4 agonist treatment ameliorates acute cardiac allograft rejection.

Treatment with selective EP4 agonist attenuated acute rejection in intestinal allografts

Graft intestinal tissues were obtained on day 6 in all groups. Histopathological evaluation revealed that the intestinal graft in the vehicle-treated allogeneic group showed severe acute rejection; conversely, the graft in the CAY10580-treated group showed moderate acute rejection (Fig. 2A, a-f). Alcian blue and periodic acid-Schiff staining revealed that epithelial mucosal destruction was significantly attenuated in the EP4-treated group (n = 5, $p < 0.05$) (Fig. 2A, g-i).

These findings in the EP4 agonist-treated group indicate moderate acute rejection of the intestinal graft.

Treatment with selective EP4 agonist attenuated infiltration of inflammatory cells and inhibited apoptotic reaction in allografts

Immunohistological analysis revealed that vehicle-treated cardiac (Fig. 3A) and intestinal grafts (Fig. 3B) showed CD3 (marker for pan-T cells)-positive cells and MAC387 (marker for a subset of reactive/infiltrating monocytes/macrophages)-positive cells in the infiltrated area. However, EP4 treatment significantly diminished infiltration of these positive cells ($p < 0.05$). These results suggest that EP4 treatment suppresses lymphoplasmacytic infiltration in both cardiac and intestinal allografts and apoptotic reaction of epithelial cells in intestinal allografts.

Selective EP4 agonist administration altered pro-inflammatory cytokine mRNA expression

The mRNA expression levels of pro-inflammatory (interleukin [IL]-1 β , IL-2, IL-6, tumor necrosis factor- α , and IFN- γ) and anti-inflammatory (IL-10 and transforming growth factor- β 1) cytokines in the cardiac and intestinal grafts were estimated by quantitative RT-PCR. EP4 treatment inhibited mRNA expression of IL-6 and/or IFN- γ compared with the vehicle-treated cardiac (Fig. 4A) and intestinal (Fig. 4B) allografts, but significant differences in mRNA expression levels of anti-inflammatory cytokines between the two groups were not observed (* $p < 0.05$, ** $p < 0.01$).

IFN- γ expression was inhibited by selective EP4 agonist administration in the intestinal allografts

Because the mRNA expressions of pro-inflammatory cytokines were down-regulated by EP4 treatment, we estimated the plasma concentrations of IFN- γ and IL-6 in the intestinal transplantation groups. EP4 treatment significantly decreased plasma IFN- γ levels ($p < 0.05$) (Fig. 5A). Furthermore, because IFN- γ and IL-6 are responsible for induction of suppressor of cytokine signaling (SOCS)-1 and SOCS-3, respectively, we investigated these mRNA expressions in allografts and plasma levels of IFN- γ and IL-6. The EP4-treated group showed significant suppression of SOCS-1 expression ($p < 0.05$) (Fig. 5B). These results suggest that the allorejection suppression effects of EP4 treatment occur secondary to suppression of the IFN- γ signaling pathway.

Discussion

In this investigation, we demonstrated that continuous subcutaneous injection of EP4 agonist ameliorated acute rejection in both rat heart and small bowel transplantation models. Immunohistochemical analysis revealed that EP4 agonist significantly reduced lymphoplasmacytic infiltration in allografts and suppressed intestinal epithelial cell apoptosis. These activities were accompanied by a decrease in the expression of pro-inflammatory cytokines of IFN- γ , and quantitative RT-PCR analysis revealed that SOCS-1, which is a intracellular regulation factor responsible for IFN- γ , was also significantly down-regulated by the EP4 treatment. These findings imply that the reduced development of transplant allogeneic acute rejection by EP4 agonist administration is related to the attenuation of pro-inflammatory cytokine of IFN- γ .

Several studies and our previous investigations have demonstrated that EP4-selective agonists possess anti-inflammatory properties, and previous studies revealed that exogenous PGE₂ administration delayed the onset of rejection in experimental rat transplantation models (16-19). PGE₂ has EP1–4 receptors, and PGE₂–EP2/4 signaling has been reported those immunosuppressive reaction of PGE₂. According to these findings and the findings of a few recent reports suggesting that EP4 receptor activation prolonged cardiac allograft survival in murine transplantation models, we considered that EP4-selective agonists represent a novel class of immune-modulator drugs that could be useful for the management of not only autoimmune inflammatory diseases, but also acute

rejection after allogeneic transplantation. To the best of our knowledge, this study was the first to evaluate the effects of EP4 agonist as an immune suppressor in rat heart and small bowel transplantation models.

Although the anti-inflammatory mechanisms of PGE₂-EP4 signaling have not been clearly established, many papers have suggested that PGE₂-EP4 signaling modulates pro- and anti-inflammatory cytokine expressions and is correlated with T-lymphocyte differentiation. In our previous mice studies and in the present study, suppression of IFN- γ expression was consistently observed with the administration of EP4 agonist. Therefore, we focused on IFN- γ -Janus kinase (JAK) and the signal transducer and activator of transcription (STAT) signaling pathways, which both play important roles in the transcriptional control of pro-inflammatory cytokines. Chinen et al. recently reported that SOCS-STAT signaling was activated by PGE₂-EP4 signals and that this signaling suppressed promotion of the inflammatory process in mice colitis models (19). In the present study, we could not determine the presence of a relationship between the SOCS-STAT-1 activation (data not shown), but its possibility will be considered in further investigations.

As discussed above, several studies have recognized that PGE₂-EP4 signaling is an important regulator of the immune response. Therefore, a human clinical trial of an EP4 agonist for inflammatory bowel disease treatment was recently conducted and showed promising therapeutic effects (20). However, it was also reported that systemic exposure of EP4 agonists caused some side

effects, such as diarrhea and hypotension, because PGE₂-EP4 signals also play significant physiological roles in several organs other than immunological reactions. Further investigations, not only of the biological mechanisms but also of the pharmacological and pharmaceutical aspects of the ability of EP4 agonists to minimize unwanted physiological effects, are desirable for evaluation of clinical applications.

In summary, the present results show for the first time that systemic administration of an EP4 agonist can effectively suppress acute rejection in rat heart and small bowel transplantation models. EP4 agonists may be a potential therapeutic candidate for combination with CNIs following organ transplantations that require high therapeutic doses of CNIs, such as heart and small bowel transplantations.

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Legends for Figures

Figure 1. Selective EP4 agonist of CAY10580 (EP4) treatment attenuated allogeneic rejection in the rat heart-lung transplantation model. **(A)** Survival of cardiac allografts (Lewis [LEW] to Brown Norway [BN]). Rats treated with EP4 (solid line) showed prolonged allograft survival compared with the vehicle-treated rats (dotted line) ($n = 8$, 11.0 ± 1.0 vs 8.0 ± 1.0 days, $p < 0.01$, by log-rank test). **(B)** The serum creatine phosphokinase concentration was significantly decreased in the EP4-treated group compared with the vehicle-treated controls ($n = 8$, 140 ± 53 vs 386 ± 107 IU/L, $p < 0.05$). **(C)** Representative gross findings and **(D,a-f)** histologic findings (hematoxylin-eosin staining) of cardiac allografts on day 7. In the vehicle-treated controls, cardiac allografts were macroscopically enlarged, and myocardial cell infiltration was observed (**D,b,e**, $n = 6$, $20.1\% \pm 7.8\%$). However, the EP4-treated group showed attenuation of the development of myocardial cell infiltration on day 7 (**D,c,f**, $n = 6$, $7.3\% \pm 2.5\%$, $p < 0.01$). Scale bars = $200 \mu\text{m}$. * $p < 0.05$, ** $p < 0.01$.

Figure 2. Selective EP4 agonist of CAY10580 (EP4) treatment also attenuated allogeneic rejection in the rat small bowel transplantation model. Graft intestinal tissues were obtained on day 6 in all groups, and representative histological sections were examined microscopically after hematoxylin-eosin and Alcian blue and periodic acid-Schiff staining. **(A, a-f)** The intestinal graft in the vehicle-treated allogeneic group showed severe acute rejection; in contrast, the graft in the EP4-treated group showed moderate acute rejection. **(A, g-i)** Alcian blue and periodic

acid-Schiff–positive epithelial mucosal cell counts were significantly different between the vehicle-treated and EP4-treated groups ($n=5$, 7 ± 3 cells vs 146 ± 22 cells per 0.5-mm square; $*p < 0.05$, $**p < 0.01$). Scale bars = 300 μm (a-c). Scale bars = 200 μm (d-i).

Figure 3. Representative CD3 and MAC387 immunohistochemistry of cardiac and intestinal allografts. **(A)** Vehicle-treated cardiac allografts showed many CD3- and MAC387-positive cells in infiltrated areas. However, EP4 treatment attenuated infiltration of these positive cells ($n=6$, $*p < 0.05$. Scale bars = 200 μm) . **(B)** In intestinal allografts, CD3-positive cells remarkably infiltrated the submucosal area in vehicle-treated allografts; in contrast, this infiltration of positive cells was attenuated by EP4 treatment. Significant differences between the number of MAC387-positive cells in the vehicle-treated and EP4-treated groups were not observed ($n=5$, $*p < 0.05$. Scale bars = 200 μm).

Figure 4. Expression of mRNA levels of pro-inflammatory and anti-inflammatory cytokines in the **(A)** cardiac ($n=6$) and **(B)** intestinal allografts ($n=5$) as estimated by quantitative RT-PCR. EP4 treatment significantly inhibited mRNA expressions of IL-6 and IFN- γ compared with the vehicle-treated cardiac allografts and significantly inhibited mRNA expression of IFN- γ compared with the vehicle-treated intestinal allografts ($*p < 0.05$).

Figure 5. EP4 treatment suppressed the IFN- γ signaling pathway and nuclear-factor (NF)- κB activation in the intestinal allografts. **(A)** Plasma concentrations of IFN- γ and IL-6 in the intestinal

transplantation groups were estimated by ELISA. EP4 treatment significantly decreased plasma IFN- γ levels (n=5, 610.2 ± 80.1 vs 462.1 ± 22.9 pg/ml, *p < 0.05). **(B)** Suppressor of cytokine signaling (SOCS)-1 and SOCS-3 mRNA expressions in intestinal allografts were measured by quantitative real-time PCR. The EP4-treated group showed significantly suppressed SOCS-1 mRNA expression (n=5, *p < 0.05).

Table 1: Primers used in quantitative real-time PCR analysis

m RNA	Forward	Reverse
IL-1 β	5'- TGTGATGAAAGACGGCACAC -3'	5'- CTTCTTCTTTGGGTATTGTTTGG -3'
IL-2	5'- AAAC TCCCATGATGCTCAC -3'	5'- GAAATTTCCAGCGTCTTCCA -3'
IL-6	5'- CCGGAGAGGAGACTTCACAG -3'	5'- ACAGTGCATCATCGCTGTTC -3'
IL-10	5'- AGTGGAGCAGGTGAAGAATGA-3'	5'- TCATGGCCTTGTAGACACCTT -3'
IFN- γ	5'- TGAAAGCCTAGAAAGTCTGAAGAAC -3'	5'- CGTGTACCGTCCTTTTGC -3'
TNF- α	5'- TGAAC TTCGGGGTGATCG -3'	5'- GGGCTTGTCACCTCGAGTTTT -3'
TGF- β_1	5'- GCAACACGTAGAACTCTACCAGAA -3'	5'- CAGCCACTCAGGCGTATCA -3'
SOCS-1	5'- TGGTAGCACGTAACCAGGTG-3'	5'- GAAGGTGCGGAAGTGAGTGT-3'
SOCS-3	5'- CCTTTGAGGTTTCAGGAGCAG-3'	5'- CGTTGACAGTCTCCGACAA-3'
GAPDH	5'- AGCTGGTCATCAATGGGAAA -3'	5'- ATTTGATGTTAGCGGGATCG -3'

IL, interleukin; *IFN- γ* , interferon- γ ; *TNF- α* , tumor necrosis factor- α ; *TGF- β_1* , transforming growth factor- β_1 ; *SOCS*, suppressor of cytokine signaling; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase

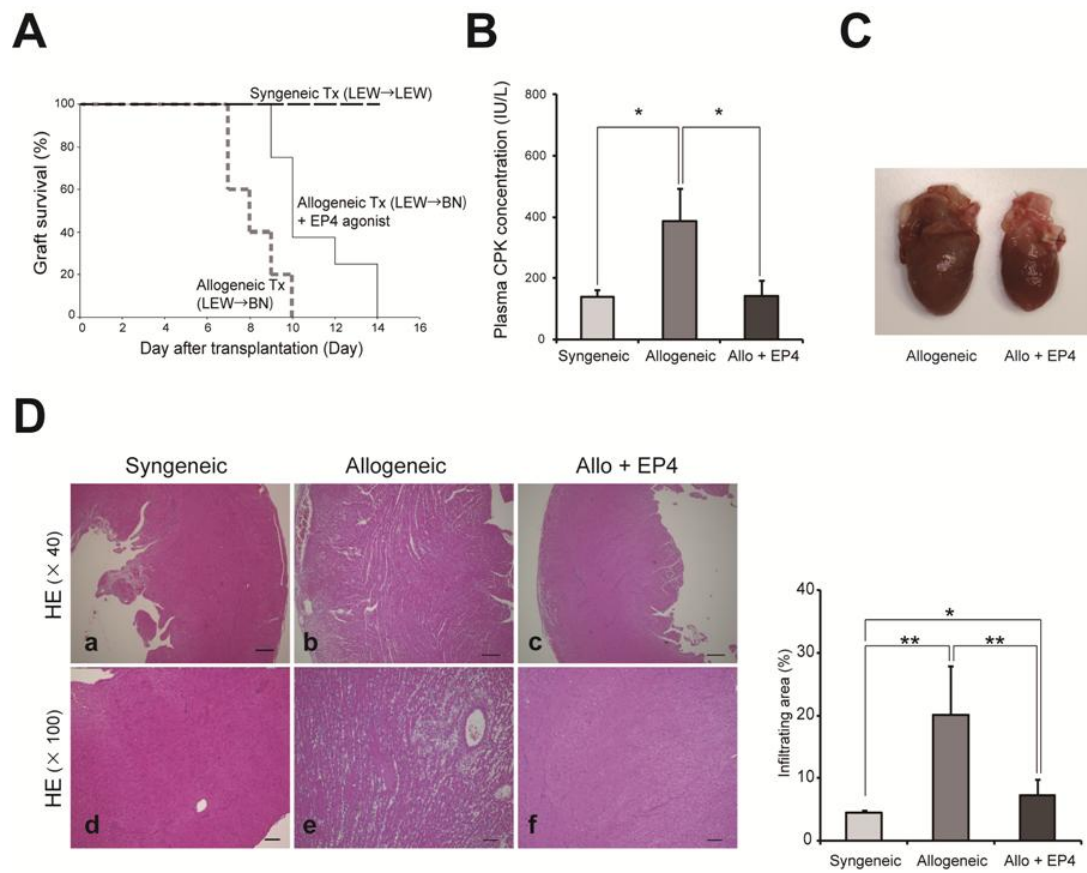


Figure 1

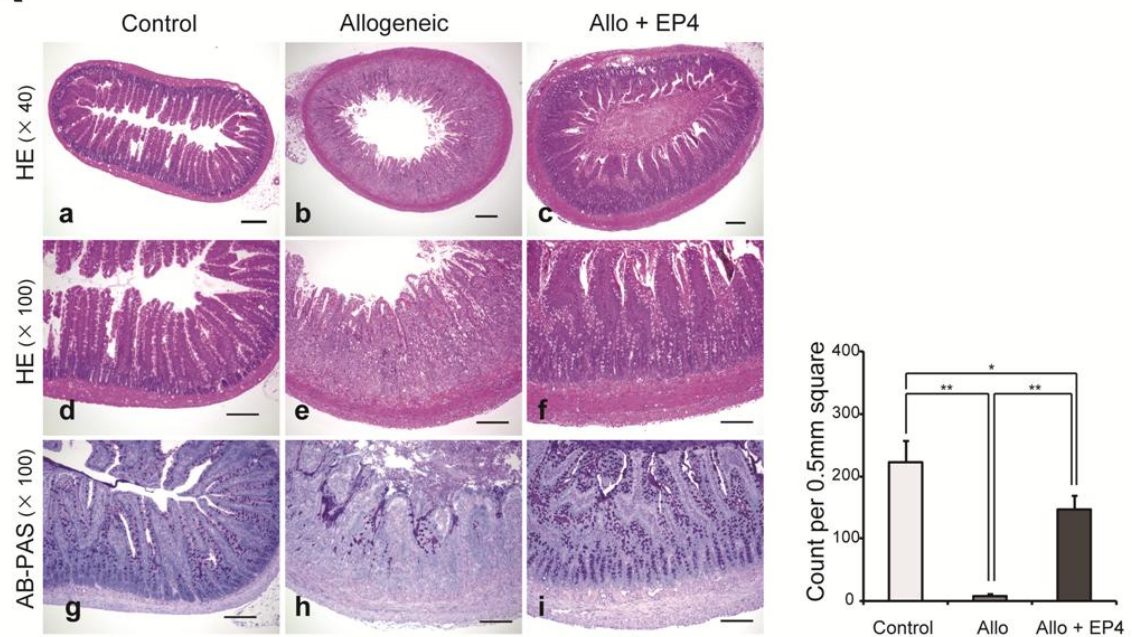
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Figure 2

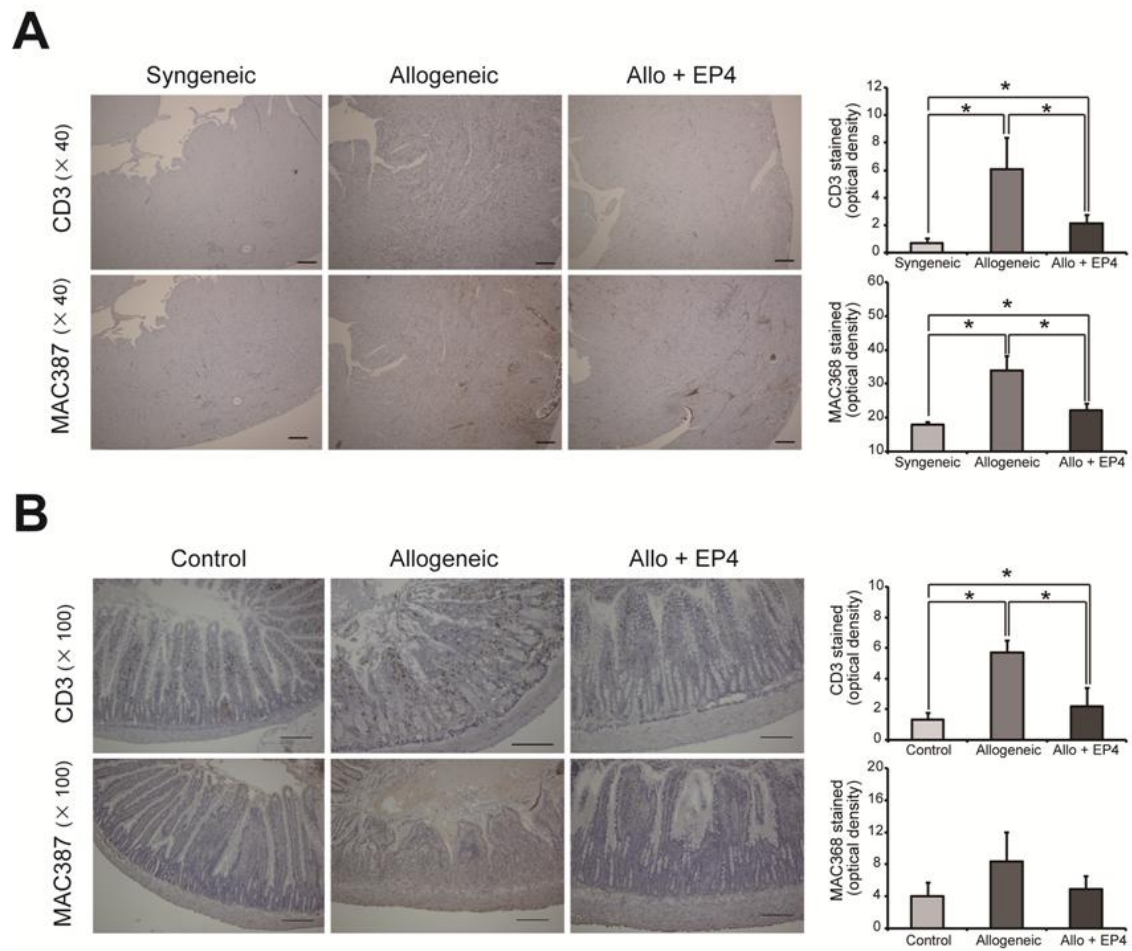


Figure 3

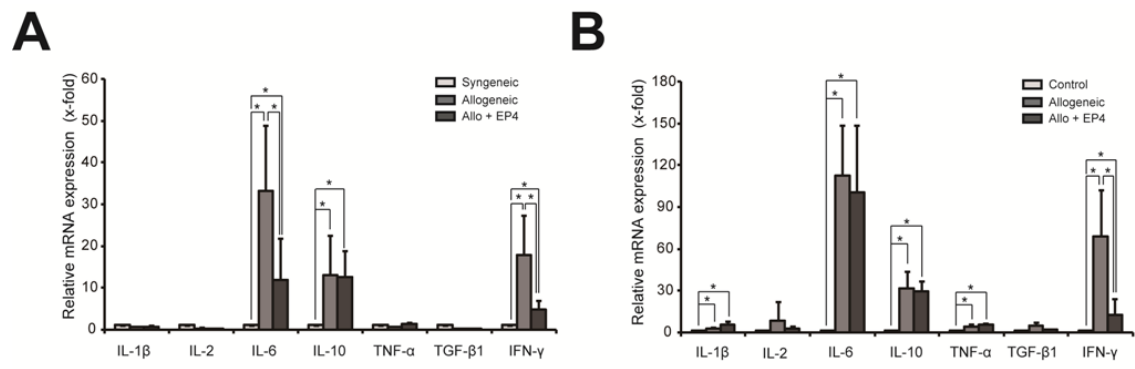


Figure 4

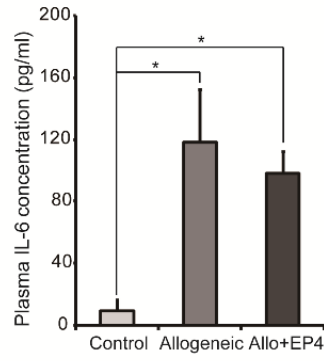
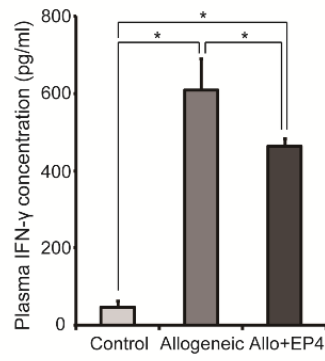
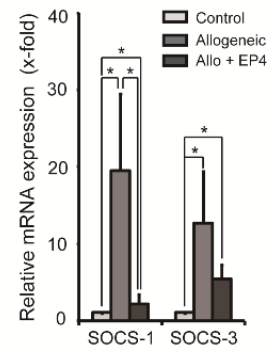
A**B**

Figure 5