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Title:

Manipulating Stress Receptor Signaling to Enhance Immunosuppression and Prolong Survival of Vascularized Composite Tissue

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One Sentence Summary: Enhancing immunosuppression by adrenergic receptor manipulation using a β_2 -agonist enhances VCA graft survival without increasing toxicity.

Abstract: Vascularized composite tissue allotransplantation (VCA) can replace severely damaged body parts but the unavoidable toxicity of high doses of immunosuppressive drugs, such as tacrolimus, required results in significant morbidity. Here we tested whether we could suppress immune activity in a mouse model of VCA by mimicking the natural immune suppression generated by nervous system-induced signaling of adrenergic receptors (AR) by using a safe and well-studied β -AR agonist (terbutaline). Using wild-type and β_2 -AR-knockout (KO) mice, we found that increased β_2 -AR signaling results in delayed rejection in VCA recipients, even with subtherapeutic doses of tacrolimus, and this was associated with changes in immune contexture, expression of pro-inflammatory cytokines and chemokines, and function of endothelial adhesion molecules. We propose that β -AR agonists can be used safely to mimic the natural suppression of immune responses generated by adrenergic stress signaling and thereby reduce the dose needed of other more toxic immunosuppressive drugs.

Introduction

Remarkable improvements in on-site emergency care have led to increased survival of patients with previously fatal traumatic injuries resulting from accidents, gun-shots or explosions in both civilian and military settings¹⁻³. However, these surviving patients must endure life-long difficulties associated with the loss of legs, arms, hands or other functional units (including even faces) with a significant decline in quality of life. This difficult situation has generated interest in testing new transplant protocols using body parts from cadaveric donors in a procedure known as vascularized composite tissue allotransplantation (VCA). With more than 90 patients receiving VCA over the past decade, this novel transplantation is raising hope for patients with devastating deformities and complex tissue defects⁴⁻⁷. In large part, these successes have been achieved both by improved microsurgical techniques (*e.g.*, anastomoses of vessels and nerves) and the use of large doses of potent pharmacological agents to induce immunosuppression, including cyclosporine A, tacrolimus and mycophenolic acid⁸⁻¹¹. However, despite the excitement surrounding VCA, significant challenges prevent its widespread acceptance and use. First and foremost is that tissues and organs recovered from cadaveric donors are a scarce resource with few instances of HLA-match with the recipient¹². As a result, very heavy doses of immunosuppressive medications are required, exposing the patient to opportunistic infections, hyperglycemia, hepatotoxicity, nephrotoxicity, cancer and reproductive toxicity¹³. Additionally, *in vivo* and *in vitro* studies suggest that the immunosuppressants cyclosporine and tacrolimus can promote carcinogenesis and cancer progression through production of transforming growth factor- β , increasing tumor angiogenesis and metastasis^{14,15}. Adverse reactions and toxicity often necessitate reduction, and even complete withdrawal, of immunosuppressive drugs leading to a tragic graft rejection and loss. As an example of this scenario, a patient who received the first face transplantation in 2005 suffered from two different types of cancer as a consequence of potent

immunosuppression and subsequently lost her lips due to graft rejection¹⁶. Although, VCA can provide significant improvement in quality of life, far too many of these patients experience either graft rejection or increased risk of additional health problems, including cancer, from the chronic use of high dose immunosuppressive drugs¹³. This has led to significant ethical concerns about using this type of transplant in patients when it is not medically “life-saving” unlike the situation for patients requiring solid organ transplants such as liver or heart, which is medically required to save their lives. Moreover, in the latter case, there is often a better possibility to achieve a closer donor HLA match. So, it is clear that there is a serious and unmet medical need for new strategies to improve graft survival after VCA and lessen the risk of life-threatening morbidities from toxic immunosuppressive drugs.

The nervous and immune systems have been found to interact closely in host defense and stress responses¹⁷. Although the relationship of the hypothalamus-pituitary-adrenal (HPA) axis and cortisol has been well studied^{18,19}, the natural role of the sympathetic nervous system in regulating immune responses is not fully understood^{20,21}. Sympathetic and parasympathetic nerves are found innervating immune organs and near immune cells throughout the body. Extensive research now shows that neurotransmitter interactions between norepinephrine (NE) and β -adrenergic receptors (AR) regulate the immune system and that dysregulation of neuro-immune signaling pathways contributes to the pathogenesis of immune disorders²²⁻²⁴.

Recently, we have shown that pharmacologic blockade of the β -AR with the common pan- β -blocker propranolol reduces tumor growth rates by interfering with the suppressive activity of adrenergic stress on adaptive immune responses. Specifically, we found that blocking β -AR with propranolol increased glucose uptake and glycolysis needed for metabolic reprogramming during T cell activation and anti-tumor immunity²⁵⁻²⁸. We also found that β_2 -AR has an important role for immune modulation of T cells and myeloid-derived suppressor cells (MDSC)²⁹. The strong, naturally occurring immunosuppressive potential of β -AR

signaling is consistent with our observations that adrenergic stress or addition of β -AR agonists can suppress graft versus host disease (GVHD) following bone marrow transplantation (BMT)²⁹⁻³¹.

These data led us to investigate whether targeting the natural ability of β -AR to regulate immunity in response to adrenergic stress signaling could be used to more safely promote an immunosuppressive environment in transplanted grafts following VCA, thus allowing a reduction of the dose of the more toxic immunosuppressant drug tacrolimus. Here, we investigated the impact of targeting β_2 -AR using the β_2 -blocker terbutaline, on graft rejection rate and immune contexture using wild-type and β_2 -AR-knockout (KO) mice. We found that increased β_2 -AR activation results in delayed rejection responses in VCA recipients without detectable toxicity and this occurred through mechanisms involving pro-inflammatory cytokines and chemokine suppression as well as inhibition of endothelial adhesion molecules. More importantly, we were able to extend graft survival using a subtherapeutic dose of tacrolimus combined with β_2 -AR agonist. Together, these data reveal an immediately feasible strategy which can be tested in patients receiving VCA or other types of allotransplants.

Results

Safety and cross-reactivity with tacrolimus of selective β_2 -AR agonist

Our previous work has established a reliable and consistent platform using a pre-clinical murine model of hind limb VCA³² to investigate novel therapies to prolong transplant survival. The transplanted graft consisted of skin, fat, muscle, bone and blood vessels (Supplementary Fig. 1a), a complex combination of tissues similar to those which are often used in VCA.

While we know from the literature and its clinical safety profile that terbutaline is considered to be a safe drug, we wanted to be sure that there were no cardiac physiology problems generated by this β_2 -AR agonist alone, or in combination with the drug tacrolimus,

a drug that causes significant immunosuppression and commonly used in the transplant setting^{13,33}. We assessed blood pressure (BP) and heart rate (HR) with a non-invasive tail cuff system³⁴. Normal mice were acclimated to the device for 10 days prior to collecting readings. No statistical difference was detected in systolic/diastolic BP or HR between mice treated daily with β_2 -AR agonist or vehicle over a course of 11 days (Supplementary Fig. 1b). We also measured plasma levels of tacrolimus in the mice treated with two different doses; full dose (fTac, *i.e.*, a dose known to generally maintain allografts long term³²), and half dose (hTac, *i.e.*, a dose that only delays graft rejection) were measured. As expected, significantly higher drug concentrations were measured in the fTac injected group compared to the hTac group, but there was no significant difference in tacrolimus concentrations between mice treated with tacrolimus alone and mice treated with tacrolimus and β_2 -AR agonist combination (Supplementary Fig. 1c). In addition, no changes of body weight and physical appearance were detected suggesting that it is safe to use terbutaline alone and in combination with tacrolimus without drug interaction.

Decreased rejection responses and T cell infiltration into grafts with β_2 -AR agonist alone and in combination with tacrolimus

Next we assessed the impact of β_2 -AR agonist on VCA survival by evaluating the impact of terbutaline in recipients employing histological hematoxylin and eosin (H&E) and immunohistochemistry (IHC) stains performed on transplanted grafts 5, 7 and 10 days after VCA to measure rejection grades and CD4⁺ T, CD8⁺ T and Foxp3⁺ cell infiltration into grafts.

Gross and histologic VCA rejections were graded using the Banff 2007 Working Classification of Skin-Containing Composite Tissue Allograft Pathology (Fig. 1a and Supplementary Table 1)³⁵. No gross rejection was observed in any graft prior to 5 days post-VCA, but over 85% of the vehicle injected recipients showed grade 3 or 4 rejection 7 days after VCA. In contrast, β_2 -AR agonist-treated recipient mice had less severe rejection (grade 2) at

day 7, which was further improved by the addition of subtherapeutic dose of tacrolimus (hTac), with some grafts surviving with grade 2 rejection at day 10 (Fig. 1b). Although no evidence of gross rejection was observed at day 5, various histologic rejection grades were detected with H&E. β_2 -agonist treatments delayed rejection responses with/without subtherapeutic dose of tacrolimus compared to the vehicle injected group (Fig. 1c).

To determine whether infiltrating cells originated from the donor or the recipient, we took advantage of differential CD45 isoform usage of C57BL/6 recipient (CD45.1) and BALB/c donor (CD45.2) mice. By day 7 post-VCA, over 90% of leukocytes within grafts (Supplementary Fig. 2a) and peripheral blood (Supplementary Fig. 2b) were from recipients. Reduced lymphocyte infiltration into the graft at an early phase of rejection would be expected to correlate with the observed enhanced graft survival. While moderate perivascular inflammation was detected at day 5 in the β_2 -agonist treated group (pathology grade 2), epithelial apoptosis and dyskeratosis were distinct in the vehicle treated group (pathology grade 3) (Fig. 2a, b and Supplementary Fig. 3a, b). Infiltrating lymphocytes (CD4⁺ T, CD8⁺ T, and Foxp3⁺ cells) were concentrated in the donor, but not recipient tissue, in both vehicle (Fig. 2a) and β_2 -agonist (Fig. 2b) treated mice, however, fewer lymphocytes infiltrated the grafts in the β_2 -agonist group (Fig. 2c). Although significantly less T cell infiltration was detected in β_2 -agonist treated grafts, compositions of CD4⁺/CD8⁺ central memory (CM) and effector memory (EM) T cell populations did not show a statistical difference by flow cytometry 5 days after VCA in the grafts (Fig. 2d). By day 7, the histological delineation between epidermis and dermis was lost and tissue rejection was nearly complete in the vehicle (Supplementary Fig. 3c) compared to the β_2 -agonist group (Supplementary Fig. 3d) and infiltrated CD4⁺ T, CD8⁺ T and Foxp3⁺ cells found in vehicle controls were declining resulting in no statistical difference with the β_2 -agonist treated group (Supplementary Fig. 3e). The majority of T cells in the grafts 7 days after VCA were EM cells in both groups, and these values were higher than healthy

donor (pre-transplanted grafts) control specimens (Supplementary Fig. 3f).

To test whether we could use a β -AR agonist to mimic natural NE and β -AR interactions and reduce the dose of tacrolimus needed for immunosuppression, we tested tacrolimus at half dose (hTac) in combination with either vehicle or β_2 -agonist. Grade 4 graft rejection was observed 10 days after VCA in the hTac+vehicle group, while the hTac+ β_2 group showed mainly only Grade 2 with partial Grade 3 rejection with intact skin histology (Fig. 2e). Abundant CD4⁺ T and CD8⁺ T cell infiltration was present 7 days after VCA in the hTac+vehicle group, and then the values dropped significantly 3 days later. β_2 -agonist treatments significantly decreased the number of infiltrating CD4⁺ T and CD8⁺ T cells at day 7 compared to the hTac+vehicle group, but more CD4⁺ T cell infiltration was found in the β_2 -agonist treated group than the vehicle injected group at day 10 representing remnant immune responses in the graft (Fig. 2f). There was no statistical difference in the proportion of CM and EM T cell populations in grafts 7 days after VCA between two groups using hTac (Fig. 2g).

Changes in memory T and Th1 cells with a β_2 -AR agonist treatment in recipients

In contrast to observations within grafts by flow cytometry (Fig. 2d, g), β_2 -AR agonist treatment significantly decreased the representation of CD4⁺ and CD8⁺ memory (CM and EM) T cell populations in the recipient's systemic blood compartment (spleen) at day 5 (*i.e.*, before the emergence of signs of rejection; Fig. 3a), however, by day 7 (*i.e.*, once gross signs of rejection are apparent) these differences were lost (Fig. 3b). This finding was more pronounced in the EM T cell population in mice treated with hTac. Additional decreases in the CD4⁺ T and CD8⁺ T EM populations were found with β_2 -agonist treatment at day 7 (Fig. 3c), and these differences are lost by day 10 (Fig. 3d). β_2 -agonist decreased the compositions of Th1 population significantly compared to the vehicle group at day 5 and 7 without tacrolimus (Fig. 3e) and at day 7 and 10 with hTac (Fig. 3f). Interestingly, higher Treg (CD4⁺CD25⁺Foxp3⁺) levels in the graft (Fig. 2c) and the body (spleen; Fig. 3e) did not predict a better prognosis for

transplanted grafts. There were more Foxp3⁺ cell infiltration in grafts and a greater Treg population systemically along with more infiltrated T cells in grafts. The Treg population was directly proportional to the severity of T cell infiltration in grafts after VCA. Accordingly, a smaller Treg population was found in grafts and the recipients treated with fTac (Supplementary Fig. 4). Thus, Treg populations, which are thought to have immunosuppressive effects, may have increased as a consequence of a fulminant immune response to the highly antigenic VCA.

Analyses of inflammatory cytokine levels after β_2 -AR modulation in recipients

Luminex analyses were used to determine if differences in the expression of inflammatory cytokines are associated with graft rejection after VCA followed by vehicle or β_2 -agonist injections. Systemic cytokines in recipient's plasma were measured. Significantly decreased IFN- γ , IL-6, and TNF- α levels with increased IL-13 levels were found in the β_2 -agonist group compared to the vehicle group 5 days after VCA. The elevated cytokines (IFN- γ , IL-6, IL-18, and TNF- α) decreased 2 days later was indicative of their likely role in graft rejection responses before phenotypic rejection was noted (Fig. 3g).

Augmentations of subtherapeutic immunosuppression using tacrolimus in combination with β_2 -AR agonist

β_2 -AR agonist was evaluated for its effects on the survival of grafts after cessation of immunosuppression. Recipients were given with fTac every day for 14 days, and then tacrolimus injection was stopped, and vehicle or β_2 -agonist injections were initiated and continued until grafts showed rejection (Fig. 4a). Distinct effects of β_2 -agonist treatment on suppression of memory T cell populations were not detected in the blood (Fig. 4b), and there was no survival benefit of grafts given β_2 -agonist following cessation of tacrolimus (Fig. 4c). However, with a scenario using hTac with either vehicle or β_2 -agonist (Fig. 4d), the dose of tacrolimus was reduced by half 14 days after a full dose of tacrolimus daily treatment (fTac to

hTac). Even though no statistical difference on CD4⁺ and CD8⁺ memory T cell populations was detected between two groups in the blood (Fig. 4e), there was a significant graft survival benefit in the β_2 -agonist group compared to the vehicle group (Fig. 4f).

Effects of β_2 -AR modulation in donor tissue

A well-studied adrenergic receptor knockout (AR KO) mouse strain as donors and recipients were used to investigate the specific role of β_2 -AR in the treatments of either donor or recipient²⁹. Grafts were harvested from BALB/c AR KO mice as donors, and transplanted into C57BL/6 wild-type (WT) recipients (Fig. 5a-d). Although the graft showed less severe gross rejection as grade 1 with β_2 -agonist compared to the vehicle injection (Fig. 5a), no statistical difference was found in the compositions of CD4⁺ and CD8⁺ memory T cell populations at day 5 in the spleen (Fig. 5b). However, the composition of CD4⁺ and CD8⁺ EM T cell populations were significantly lower in the β_2 -agonist group than the vehicle group at day 7 (Fig. 5c), and significantly less Th1 and Treg were detected with β_2 -agonist compared to vehicle at day 5 (Fig. 5d). In parallel, WT grafts were transplanted to C57BL/6 AR KO recipients with either vehicle or β_2 -agonist injections (Fig. 5e-h). Significant decreases in CD4⁺ and CD8⁺ memory T cell populations were detected at day 5 (Fig. 5f) and 7 (Fig. 5g) with gross grade 0 rejection in the β_2 -agonist treated group compared to the vehicle group (grade 3). In addition, β_2 -agonist decreased Treg population significantly compared to vehicle injection at day 5 (Fig. 5h). Furthermore, significant decreases in CD4⁺ T cell and Treg infiltration were detected with β_2 -agonist treatment in AR KO recipients bearing the WT grafts (WT $\rightarrow$ KO) compared to WT recipients bearing AR KO grafts (KO $\rightarrow$ WT) (Fig. 5i).

Important actions of β_2 -AR agonist on donor stromal cells after VCA

β_2 -AR modulation in donors suppresses rejection responses after VCA, so to better determine the responsible populations of cells, bone marrow (BM) and stromal cells of the donor were examined. WT donors were exposed to 8 Gy (gray) irradiation the day before VCA

to eradicate BM cells, and then grafts including a femur was transplanted to β_2 -AR KO recipients followed by vehicle or β_2 -agonist injections. Both groups showed mild xerosis of grafts with intact skin anatomy (Fig. 6a), and distinct effects of β_2 -agonist treatment were lost in the $CD4^+$ and $CD8^+$ memory T cell (Fig. 6b), Th1 and Treg (Fig. 6c) populations 7 days after VCA. However, significantly lower numbers of $CD4^+$ and $CD8^+$ T cell infiltrations were found in the β_2 -agonist treated graft than the vehicle group (Fig. 6d). Further, β_2 -AR KO donor was irradiated, and then WT BMT was performed to generate a chimeric model including β_2 -AR KO stromal cells and WT BM, and vice versa (Fig. 7a). Grafts composed of β_2 -AR KO stromal with WT BM (KO+WT) showed more severe rejection than WT stromal with AR KO BM (WT+KO) grafts in AR KO recipients (Fig. 7a, b; KO+WT gross grade 2 and histologic grade 3, WT+KO gross grade 1 and histologic grade 2). Greater amounts of $CD4^+$ T and $CD8^+$ T cell infiltration were found in the KO+WT grafts than the WT+KO grafts (Fig. 7b). Additionally, significant decreases of $CD4^+$ and $CD8^+$ memory T cell populations were detected in the spleen of mice bearing WT+KO grafts compared to mice bearing KO+WT grafts (Fig. 7c) with no significant difference on Th1 and Treg populations between groups (Fig. 7d). It means that β_2 -AR signals in donor's stromal cells have critical roles on recipient's T cell trafficking.

Inhibition of endothelial adhesion molecules and T cell trafficking chemokines with β_2 -AR agonist

Leukocyte extravasation is one of the essential and first steps during the initiation of cell-mediated rejection and ICAM-1/2 and VCAM-1 are the endothelial adhesion molecules that mediate firm adhesion just prior to cell extravasation³⁶. To investigate changes of endothelial adhesion molecules in donor's vessels after β_2 -agonist treatment, WT donor's grafts in WT recipients were stained 5 days after VCA. β_2 -agonist suppressed ICAM-1 and VCAM-1 expression in graft vessels compared to vehicle, while the expression of ICAM-2 was increased in the β_2 -agonist treated group (Fig. 8a) by immunofluorescence. Also, recipient's

plasma levels of the relevant T cell trafficking chemokines CXCL-1, CXCL-10, CCL2, CCL3, CCL4, CCL5, CCL7 and CCL11 increased significantly at day 5, and resolving by day 7. Under β_2 -agonist treatment, levels of CXCL-1, CXCL-10, CCL3, CCL4, CCL5 and CCL7 which are pro-inflammatory chemokines that regulates leukocytes trafficking³⁷ were decreased significantly compared to the vehicle injection (Fig. 8b).

Effects of donor preconditioning prior to VCA with β_2 -AR agonist

β_2 -agonist treatment before VCA was examined as a pre-conditioning regimen as BALB/c WT mice were given β_2 -agonist for 2 weeks prior to VCA (Pre-VCA) and compared to the previous experiments injecting β_2 -agonist after VCA (Post-VCA) (Supplementary Fig. 5a). Although similar gross and histologic rejection grades were detected in the Pre-VCA group (grades 1 and 3 respectively) compared to the Post-VCA group (grades 0 and 3 respectively) 7 days after surgery, the immunosuppressive effects on memory T cell populations in recipients were lost in the setting of Pre-VCA (Supplementary Fig. 5b). But, suppression of the Th1 population and CD8⁺ T cell trafficking in Pre-VCA were comparable to Post-VCA (Supplementary Fig. 5c, d).

Discussion

Here, we tested a strategy that mimics the natural ability of nerves and adrenergic stress signaling to suppress T cell mediated immune responses to prolong survival of a complex tissue allograft. Importantly, we found that we could reduce the dose of a standard immunosuppressant drug tacrolimus to half by supplementing it with a β_2 -AR agonist, while obtaining significant immune modulatory effects favoring graft survival. Selective β_2 -AR targeting drugs have been safely and extensively used for decades for other indications, particularly for cardiovascular and pulmonary manipulation³⁸. Furthermore, at the dose of terbutaline that we used here to prolong graft survival, no changes in HR and BP were observed

with daily use, and no drug interaction with tacrolimus was found, thus supporting the safety of this approach. VCA composes of multiple tissues with different immunogenic and functional properties, including skin, muscles, bones and nerves from cadavers. Also, these cadaveric donors have little chance to match HLA types with the recipients. As a result, these donor tissues are highly antigenic requiring very large and toxic doses of drugs such as tacrolimus^{12, 39,40}. Mimicking the natural (and temporary) suppressive function of nerves on the immune response to promote survival of VCA, (or even that of more traditional solid organ transplants) has never been tested previously in either preclinical or clinical settings.

In non- β_2 -AR agonist treated animals, $CD4^+$ T and $CD8^+$ T cells were increased significantly in transplanted grafts at the time of rejection. Treatment with β_2 -AR agonist treatment was associated with significantly fewer numbers of infiltrated T cell, and decreased EM T and Th1 cell populations in recipients corresponding to the prolonged survival benefit. Although several clinical and preclinical studies have shown that Tregs modulate immune responses and that a high level of Treg population can predict better prognosis after solid organ and hematopoietic cell transplantation⁴¹, in our VCA model, increased numbers of Tregs were detected in the presence of strong immune responses after transplant. Thus, recipients with greater levels of Treg populations showed severe gross rejection at early time points and eventual resolution of the population in the recipient's blood and grafts after severe gross rejection was well underway. This suggests that Treg levels may influence or predict rejection of highly antigenic VCA grafts as opposed to those used in solid organ transplants, particularly at early time points. VCA was also associated with an increased immunosuppressive MDSC population compared to the non-transplanted animals, and a significant increase in MDSC was noted with β_2 -agonist treatment. This increase appeared consistent with β_2 -agonist effects in GVHD after BMT²⁹, and may be a more potent contributor to immunosuppression than Treg induction.

In terms of immune modulatory impact of addition of a β_2 -AR agonist, terbutaline delayed rejection responses in association with significant decreases in CD4⁺/CD8⁺ EM T and Th1 cell populations, cytokines and chemokines such as IFN- γ , IL-6, TNF- α , CXCL-1/10 and CCL3/4/5/7 in recipients. Additionally, β_2 -AR agonist treatment increased the anti-inflammatory cytokine, IL-13, which was associated with concomitant down-regulation of TNF- α production, a phenomenon observed in other studies^{42,43}. IL18, CCL2 and CCL11 were particularly noted as unresponsive to β_2 -agonist treatment indicating that these cytokine and chemokines could be additional targets to suppress rejection responses after VCA.

β_2 -AR signal modulation either in recipients or donors was sufficient to delay gross rejection responses, but the modulation of donor cell β_2 -AR resulted in a more potent effect. Specifically, donor cell AR modulation in tissue stromal cells was necessary to suppress recipient T cell trafficking into grafts. Leukocyte extravasation is a prerequisite for acute rejection in grafts, and their migration into the tissue requires the expression of adhesion molecules on the surface of activated endothelium^{44,45}. The endothelial adhesion molecules ICAM-1 and VCAM-1 are known as the central mediators of leukocyte adhesion to and transmigration across the endothelium³⁶, and β_2 -AR agonist suppressed ICAM-1 and VCAM-1 expression with concomitant decreased T cell trafficking into grafts. Of note, ICAM-2 expression was increased, but did not compensate to allow T cell trafficking. As previously reported, during longer periods of inflammation, endothelial cells respond to inflammatory mediators by massive up-regulation of adhesion receptors such as ICAM-1 and VCAM-1, whereas ICAM-2 decreases⁴⁶⁻⁴⁸. A possible explanation for our observed increase in ICAM-2 is that it might represent a compensatory mechanism for decreased ICAM-1 or an additional effect of β_2 -AR modulation. The β_2 -AR agonist, terbutaline, stimulated angiogenesis on endothelial cells derived from the central nervous system⁴⁹, and ICAM-2 has been associated with this process⁵⁰. By regulating ICAM-2 and increasing angiogenesis, β_2 -agonist treatment

could reduce ischemic injury in harvested organs. Graft salvage effects of β_2 -agonist suggest that regulation of endothelial adhesion molecules partially contributes for the prevention of chronic rejection after VCA (Fig. 4). Another possible mechanism underlying benefits of a β_2 -agonist is that it can increase cAMP (cyclic adenosine monophosphate) levels; cAMP is known as a potent negative regulator in T cells, which dampens T-cell immune function through the cAMP/protein kinase A signaling pathway⁵¹.

β_2 -AR activation in donor BM cells was necessary for the observed immunosuppressive effects including suppression of IFN- γ levels in recipients. Although the donor's hematopoietic cell (HPC) was replaced by the recipient's HPC within 5 days after VCA in our model (Supplementary Fig. 2), it is likely that a transient mixed chimerism developed in the recipients which was sufficient for the induction of allograft tolerance^{52,53}. While the study of transplant tolerance is beyond the scope of this study, β_2 -agonist treatment could be a mechanism to facilitate the induction of tolerance and could replace at least some of the more toxic immunosuppressive drugs currently being used.

Preconditioning donors with β_2 -agonist suppressed T cell trafficking into grafts resulting in delayed acute rejection responses, even though systemic immunosuppressive effects such as decreases in CD4⁺/CD8⁺ memory T and Th1 cell populations were missing. Interestingly, in a previous study, cadaveric kidney donors who were treated with dopamine and NE to maintain their BP and HR while intensive cares resulted in reduced acute rejection and improved graft survival after transplantation^{54,55}, and our current findings may be the mechanism for this observation. β_2 -agonist addition to preservation solutions may be a relatively simple measure to improve organ transplantation, in addition to treatment of recipients.

One problem with our approach is that terbutaline is a relatively short acting β_2 -agonist. It is likely - that the effects of β_2 -AR modulation with longer acting agonists (*e.g.*, bambuterol,

salmeterol) could be superior to terbutaline. Another limitation is that our study is focused upon T lymphocytes and their role in graft rejection. Even though current immunosuppressive therapies for solid organ transplantation appear to be dominated by T cell mechanisms (making the information presented here quite pertinent), other immune cell subtypes including components of the innate immune system and B cells are also associated with graft rejection and may be contributory to some of the observed findings. These additional mechanisms should be addressed in future studies.

In conclusion, there is a significant need to reduce the amounts of standard immunosuppressant drugs currently being given to patients receiving VCA. We have demonstrated here that by targeting adrenergic stress pathways ordinarily used by nerves of the autonomic nervous system, we could not only delay rejection responses but more importantly, allow for reduction of the dose of a standard immunosuppressive drug in various VCA scenarios. We have also identified several relevant mechanisms by which this prolongation of the survival of VCA occurs. While future studies will certainly further optimize the dose and scheduling of applications of β -agonists to prolong graft survival, this approach could be tested immediately as a safe strategy for reducing the amount of highly toxic immunosuppressive drugs given to patients requiring VCA.

Methods

Mice

Female C57BL/6 (H-2^b), C57BL/6 (H-2^b, CD45.1), and BALB/c (H-2^d) mice aged 7-8 weeks were purchased from Charles River (Kingston, NY) and The Jackson Laboratory (Bar Harbor, ME) as recipients and donors respectively. β_2 -AR KO mice on BALB/c and C57BL/6 background are bred in-house from an established colony. Mice were fed a standard laboratory diet and housed under standard light and accommodation conditions. All animal experiments

were done with the approval of Roswell Park Comprehensive Cancer Center Animal Care and Use Committee IACUC.

VCA surgery

All procedures were carried out under sterile conditions by one investigator (MK) as described in our previous published work³². Briefly, a donor's abdominal aorta and femoral vein were used for revascularization with a recipient's common carotid artery and external jugular vein respectively using a non-suture cuff technique. We used BALB/c background strain as donors and C57BL/6 background strain as recipients because we have revealed that a BALB/c strain had a higher anatomical mutation rate on the Circle of Willis than C57BL/6 strain³².

Drug treatments

Immunosuppression was induced in mice using tacrolimus (Sigma-Aldrich, St. Louis, MO) in doses of 2 or 4 mg/kg (in DMSO; Sigma-Aldrich, St. Louis, MO) injected subcutaneously with a micro syringe (Hamilton, Reno, NV) daily. β_2 -AR activation was achieved using daily intraperitoneal injections of 2 mg terbutaline (200 μ L in DPBS; Corning Inc., Corning, NY). The same DPBS was used for vehicle.

HR and BP measurement

A noninvasive blood pressure monitoring system (CODA, Kent Scientific corporation, Torrington, CT) was used to measure HR and BP in mice⁵⁶. Mice were acclimated with the system for 10 days prior to initiating experimental measurements.

Blood collection

Blood was collected from the right superficial temporal vein (STV) using a sterile 5-mm animal lancet (Medipoint, INC., Mineola, NY) after anesthesia induction. The STV is a large vessel positioned posterior to the eye, which can be traced one eye length back and one eye width up from the sebaceous gland⁵⁷. Concentrations of tacrolimus were measured in

plasma prepared from the collected blood samples by VITROS 5.1 FS (Ortho Clinical Diagnostics, Inc., Rochester, NY).

H&E and IHC staining

Following standard euthanasia, grafted tissue was harvested and fixed in 10% formaldehyde (Thermo Scientific, Waltham, MA), and then tissue was embedded in paraffin. Formalin fixed paraffin sections were cut at 4 μ m, placed on charged slides, and dried at 60°C for one hour. Slides were cooled to room temperature and added to the Leica Bond Rx, where they were deparaffinized with Bond Dewax Solution (Leica, Allendale, NJ) and rinsed in water. Bond Epitope Retrieval 2 (Leica, Allendale, NJ) was used for target retrieval for 30 minutes. Slides were blocked using peroxide block from Bond Polymer Refine Detection kit (Leica, Allendale, NJ) for 5 minutes. Slides were incubated with CD4 Antibody (Abcam, Cambridge, United Kingdom) at 1/1000 or CD8 (Abcam, Cambridge, United Kingdom) at 1/1000 or FOXP3 (Boster Biological Technology, Pleasanton, CA) at 1/50 for 20 minutes followed by Rabbit Envision (Agilent Technologies, Santa Clara, CA) for 30 minutes. DAB (Diaminobenzidine) from the Bond Polymer Refine Detection kit (Leica, Allendale, NJ) was applied for 10 minutes for visualization. Slides were counterstained with Hematoxylin from the Bond Polymer Refine Detection kit (Leica, Allendale, NJ) for 8 minutes then placed into water. After removing slides from the Bond they were dehydrated, cleared, and cover-slipped.

Immunofluorescence histology

OCT (Sakura Finetek, Tokyo, Japan)-embedded tissue cryosections (9 μ m-thick) were fixed at -20°C in methanol/acetone (3:1), blocked using 10% serum, and stained with primary antibodies anti-mouse ICAM-1, ICAM-2, VCAM-1 antibodies (BD Biosciences, San Jose, CA) and anti-mouse CD31 antibody (Abcam, Cambridge, United Kingdom). Images of at least 5 consecutive fields (unit area of each field, 0.34 mm²) were captured by observers blinded to sample identity. Identical exposure times and image settings were used within each experiment.

Images were analyzed with ImageJ software (NIH, Bethesda, MD) for determination of the relative fluorescence staining intensity; regions of interest were defined based on CD31 fluorescence, and each pixel in identified regions was assigned a fluorescence intensity value (based on a scale from 0 to 255).

Flow Cytometry

Spleens were mechanically disrupted and directly passed through a 70 μ m nylon cell strainer (Alkali Scientific, Pompano Beach, FL) followed by lysing red blood cells with hypotonic lysis buffer (Gibco, Gaithersburg, MD). Single-cell suspensions were created from whole tissue transplanted grafts using the Medimachine tissue disruption system (Beckton Dickinson, Franklin Lakes, NJ), followed by leukocyte isolation using Lymphoprep (Stemcell Technologies, Vancouver, Canada). Prepared cells were stained with different antibodies for extracellular and intracellular markers. Antibodies of CD45 (BUV395, clone; 30-F11), CD45.1 (BUV395, clone; 20), CD45.2 (BV605, clone; 104), CD3 (Alexa Fluor 700, 17A2), CD4 (PerCp, clone; RM4.5), CD8 (Alexa Fluor 488, clone; 53-6.7), CD25 (APC, clone; PC61), CD44 (V450, clone; IM7), CD62L (PE, clone; MEL-14), Foxp3 (PE, clone; MF23), IFN- γ (PE-CF594, clone; XMG1.2), IL4 (APC, clone; 11B11), and IL17 (BV421, clone; TC11-18H10) were used (BD Bioscience, San Jose, CA). All data were collected on an LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA) and analyzed with WinList 9.0 software (Verity Software House, Topsham, ME).

Luminex assay

Plasma was prepared from collected blood after a 20-minute centrifuge at 800g without using a brake. Mouse 11-plex cytokine and 9-plex chemokine were performed by Flow and Imaging Cytometry Shared Resource, Luminex Division at Roswell Park Comprehensive Cancer Center per the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Bone marrow chimeras

Chimeras were generated between BALB/c WT and β_2 -AR KO mice as donors. Recipient mice were lethally irradiated with 8.0 Gy of total body irradiation (Cesium¹³⁷ source). One day after irradiation, BM was reconstituted with the intravenous injection via a tail vein of 10×10^6 donor cells. Reconstituted mice were used 8 weeks after BMT.

Assessment of rejection grade

Gross rejection grades and pathology rejection grades were evaluated by M.K. and P.N.B. respectively based on the Banff 2007 working classification of skin-containing composite tissue allograft pathology³⁵.

Statistical analysis

Comparison between groups were performed using Student's t test, and statistical significance was accepted with $p < 0.05$. Log-rank (Mantel-Cox) test was used for survival comparisons between groups using GraphPad Prism, Version 8 software (GraphPad Software, Inc., La Jolla, CA).

List of Supplementary Materials:

Fig. 1. A schematic illustration of our VCA model and the safety of a selective β_2 -adrenergic receptor (AR) agonist drug, terbutaline.

Fig. 2. Pathologic findings 5 and 7 days after VCA with either vehicle or β_2 -agonist injections.

Fig. 3. The composition of donor's and recipient's leukocytes in donor grafts and recipient blood 5 and 7 days after VCA.

Fig. 4. Numbers of Foxp3⁺ cell in transplanted grafts and the composition of Treg (CD4⁺CD25⁺Foxp3⁺) population with fTac injections after VCA.

Fig. 5. Preconditioning in donors with β_2 -AR agonist delays rejection responses through suppression of T cell trafficking in the grafts.

Table S1. Visual and histologic grading system for assessment of rejection after VCA

References:

- 1 Fleming, M. E., Bharmal, H. & Valerio, I. Regenerative medicine applications in combat casualty care. *Regen Med* **9**, 179-190, doi:10.2217/rme.13.96 (2014).
- 2 Fleming, M., Waterman, S., Dunne, J., D'Alleyrand, J. C. & Andersen, R. C. Dismounted complex blast injuries: patterns of injuries and resource utilization associated with the multiple extremity amputee. *J Surg Orthop Adv* **21**, 32-37 (2012).
- 3 Gawande, A. Casualties of war--military care for the wounded from Iraq and Afghanistan. *N Engl J Med* **351**, 2471-2475, doi:10.1056/NEJMp048317 (2004).
- 4 Petit, F., Minns, A. B., Dubernard, J. M., Hettiaratchy, S. & Lee, W. P. Composite tissue allotransplantation and reconstructive surgery: first clinical applications. *Annals of surgery* **237**, 19-25, doi:10.1097/01.SLA.0000041228.23111.30 (2003).
- 5 Siemionow, M. & Kulahci, Y. Facial transplantation. *Seminars in plastic surgery* **21**, 259-268, doi:10.1055/s-2007-991196 (2007).
- 6 Devauchelle, B. *et al.* First human face allograft: early report. *Lancet* **368**, 203-209, doi:10.1016/S0140-6736(06)68935-6 (2006).
- 7 Dubernard, J. M., Owen, E. R., Lanzetta, M. & Hakim, N. What is happening with hand transplants. *Lancet* **357**, 1711-1712, doi:10.1016/S0140-6736(00)04846-7 (2001).
- 8 Gilbert, R. Transplant is successful with a cadaver forearm. *Med Trib Med News*, 20-23 (1964).
- 9 Gupta, A., Kumer, S. & Kaplan, B. Novel immunosuppressive strategies for composite tissue allografts. *Current opinion in organ transplantation* **19**, 552-557, doi:10.1097/MOT.0000000000000135 (2014).
- 10 Dubernard, J. M. *et al.* Human hand allograft: report on first 6 months. *Lancet* **353**, 1315-1320, doi:10.1016/S0140-6736(99)02062-0 (1999).

552 11 Jones, J. W., Gruber, S. A., Barker, J. H. & Breidenbach, W. C. Successful hand
553 transplantation. One-year follow-up. Louisville Hand Transplant Team. *N Engl J Med*
554 **343**, 468-473, doi:10.1056/NEJM200008173430704 (2000).

555 12 Kueckelhaus, M. *et al.* Vascularized composite allotransplantation: current standards
556 and novel approaches to prevent acute rejection and chronic allograft deterioration.
557 *Transplant international : official journal of the European Society for Organ*
558 *Transplantation* **29**, 655-662, doi:10.1111/tri.12652 (2016).

559 13 Petruzzo, P. *et al.* The International Registry on Hand and Composite Tissue
560 Transplantation. *Transplantation* **90**, 1590-1594, doi:10.1097/TP.0b013e3181ff1472
561 (2010).

562 14 Basu, A. *et al.* Overexpression of vascular endothelial growth factor and the
563 development of post-transplantation cancer. *Cancer research* **68**, 5689-5698,
564 doi:10.1158/0008-5472.CAN-07-6603 (2008).

565 15 Gallagher, M. P. *et al.* Long-term cancer risk of immunosuppressive regimens after
566 kidney transplantation. *Journal of the American Society of Nephrology : JASN* **21**, 852-
567 858, doi:10.1681/ASN.2009101043 (2010).

568 16 in *The Daily Telegraph* (2016).

569 17 Chavan, S. S., Ma, P. & Chiu, I. M. Neuro-immune interactions in inflammation and
570 host defense: Implications for transplantation. *American journal of transplantation :*
571 *official journal of the American Society of Transplantation and the American Society*
572 *of Transplant Surgeons* **18**, 556-563, doi:10.1111/ajt.14515 (2018).

573 18 Smith, S. M. & Vale, W. W. The role of the hypothalamic-pituitary-adrenal axis in
574 neuroendocrine responses to stress. *Dialogues Clin Neurosci* **8**, 383-395 (2006).

575 19 Herman, J. P. *et al.* Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress
576 Response. *Compr Physiol* **6**, 603-621, doi:10.1002/cphy.c150015 (2016).

577 20 Sundman, E. & Olofsson, P. S. Neural control of the immune system. *Advances in*
578 *physiology education* **38**, 135-139 (2014).

579 21 Forsythe, P. The nervous system as a critical regulator of immune responses underlying
580 allergy. *Current pharmaceutical design* **18**, 2290-2304 (2012).

581 22 Madva, E. N. & Granstein, R. D. Nerve-derived transmitters including peptides
582 influence cutaneous immunology. *Brain, behavior, and immunity* **34**, 1-10,
583 doi:10.1016/j.bbi.2013.03.006 (2013).

584 23 Padgett, D. A. & Glaser, R. How stress influences the immune response. *Trends in*
585 *immunology* **24**, 444-448 (2003).

586 24 Glaser, R. & Kiecolt-Glaser, J. K. Stress-induced immune dysfunction: implications for
587 health. *Nature reviews. Immunology* **5**, 243-251, doi:10.1038/nri1571 (2005).

588 25 Eng, J. W. *et al.* Housing temperature-induced stress drives therapeutic resistance in
589 murine tumour models through beta2-adrenergic receptor activation. *Nat Commun* **6**,
590 6426, doi:10.1038/ncomms7426 (2015).

591 26 Bucsek, M. J. *et al.* beta-Adrenergic Signaling in Mice Housed at Standard
592 Temperatures Suppresses an Effector Phenotype in CD8(+) T Cells and Undermines
593 Checkpoint Inhibitor Therapy. *Cancer research* **77**, 5639-5651, doi:10.1158/0008-
594 5472.CAN-17-0546 (2017).

595 27 Qiao, G. *et al.* beta-Adrenergic signaling blocks murine CD8(+) T-cell metabolic
596 reprogramming during activation: a mechanism for immunosuppression by adrenergic
597 stress. *Cancer Immunol Immunother* **68**, 11-22, doi:10.1007/s00262-018-2243-8
598 (2019).

599 28 Qiao, G., Chen, M., Bucsek, M. J., Repasky, E. A. & Hylander, B. L. Adrenergic
600 Signaling: A Targetable Checkpoint Limiting Development of the Antitumor Immune
601 Response. *Front Immunol* **9**, 164, doi:10.3389/fimmu.2018.00164 (2018).

- 29 Mohammadpour, H. *et al.* beta2 adrenergic receptor-mediated signaling regulates the immunosuppressive potential of myeloid-derived suppressor cells. *J Clin Invest* **129**, 5537-5552, doi:10.1172/JCI129502 (2019).
- 30 Leigh, N. D. *et al.* Housing Temperature-Induced Stress Is Suppressing Murine Graft-versus-Host Disease through beta2-Adrenergic Receptor Signaling. *J Immunol* **195**, 5045-5054, doi:10.4049/jimmunol.1500700 (2015).
- 31 Mohammadpour, H. *et al.* beta2-Adrenergic receptor activation on donor cells ameliorates acute GvHD. *JCI Insight* **5**, doi:10.1172/jci.insight.137788 (2020).
- 32 Kim, M., Fisher, D. T., Powers, C. A., Repasky, E. A. & Skitzki, J. J. Improved Cuff Technique and Intraoperative Detection of Vascular Complications for Hind Limb Transplantation in Mice. *Transplant Direct* **4**, e345, doi:10.1097/TXD.0000000000000756 (2018).
- 33 Vasilic, D. *et al.* Risk assessment of immunosuppressive therapy in facial transplantation. *Plast Reconstr Surg* **120**, 657-668, doi:10.1097/01.prs.0000270316.33293.ec (2007).
- 34 Zhao, X. *et al.* Arterial Pressure Monitoring in Mice. *Curr Protoc Mouse Biol* **1**, 105-122, doi:10.1002/9780470942390.mo100149 (2011).
- 35 Cendales, L. C. *et al.* The Banff 2007 working classification of skin-containing composite tissue allograft pathology. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **8**, 1396-1400, doi:10.1111/j.1600-6143.2008.02243.x (2008).
- 36 Panes, J., Perry, M. & Granger, D. N. Leukocyte-endothelial cell adhesion: avenues for therapeutic intervention. *Br J Pharmacol* **126**, 537-550, doi:10.1038/sj.bjp.0702328 (1999).

626 37 Olson, T. S. & Ley, K. Chemokines and chemokine receptors in leukocyte trafficking.
627 *Am J Physiol Regul Integr Comp Physiol* **283**, R7-28, doi:10.1152/ajpregu.00738.2001
628 (2002).

629 38 Barisione, G., Baroffio, M., Crimi, E. & Brusasco, V. Beta-Adrenergic Agonists.
630 *Pharmaceuticals (Basel)* **3**, 1016-1044, doi:10.3390/ph3041016 (2010).

631 39 Schneeberger, S. *et al.* Achievements and challenges in composite tissue
632 allotransplantation. *Transplant international : official journal of the European Society*
633 *for Organ Transplantation* **24**, 760-769, doi:10.1111/j.1432-2277.2011.01261.x
634 (2011).

635 40 Howsare, M., Jones, C. M. & Ramirez, A. M. Immunosuppression maintenance in
636 vascularized composite allotransplantation: what is just right? *Current opinion in organ*
637 *transplantation* **22**, 463-469, doi:10.1097/MOT.0000000000000456 (2017).

638 41 Wood, K. J. & Sakaguchi, S. Regulatory T cells in transplantation tolerance. *Nature*
639 *reviews. Immunology* **3**, 199-210, doi:10.1038/nri1027 (2003).

640 42 Cosentino, G. *et al.* IL-13 down-regulates CD14 expression and TNF-alpha secretion
641 in normal human monocytes. *J Immunol* **155**, 3145-3151 (1995).

642 43 Albanesi, C. *et al.* IL-4 and IL-13 negatively regulate TNF-alpha- and IFN-gamma-
643 induced beta-defensin expression through STAT-6, suppressor of cytokine signaling
644 (SOCS)-1, and SOCS-3. *J Immunol* **179**, 984-992, doi:10.4049/jimmunol.179.2.984
645 (2007).

646 44 Issekutz, A. C., Meager, A., Otterness, I. & Issekutz, T. B. The role of tumour necrosis
647 factor-alpha and IL-1 in polymorphonuclear leucocyte and T lymphocyte recruitment
648 to joint inflammation in adjuvant arthritis. *Clin Exp Immunol* **97**, 26-32,
649 doi:10.1111/j.1365-2249.1994.tb06574.x (1994).

650 45 Turner, M. D., Nedjai, B., Hurst, T. & Pennington, D. J. Cytokines and chemokines: At
651 the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta* **1843**,
652 2563-2582, doi:10.1016/j.bbamcr.2014.05.014 (2014).

653 46 McLaughlin, F. *et al.* Tumor necrosis factor (TNF)-alpha and interleukin (IL)-1beta
654 down-regulate intercellular adhesion molecule (ICAM)-2 expression on the
655 endothelium. *Cell Adhes Commun* **6**, 381-400, doi:10.3109/15419069809109147
656 (1998).

657 47 Lehmann, J. C. *et al.* Overlapping and selective roles of endothelial intercellular
658 adhesion molecule-1 (ICAM-1) and ICAM-2 in lymphocyte trafficking. *J Immunol*
659 **171**, 2588-2593, doi:10.4049/jimmunol.171.5.2588 (2003).

660 48 Lyck, R. & Enzmann, G. The physiological roles of ICAM-1 and ICAM-2 in neutrophil
661 migration into tissues. *Curr Opin Hematol* **22**, 53-59,
662 doi:10.1097/MOH.0000000000000103 (2015).

663 49 Lemmens, S., Kusters, L., Bronckaers, A., Geurts, N. & Hendrix, S. The beta2-
664 Adrenoceptor Agonist Terbutaline Stimulates Angiogenesis via Akt and ERK
665 Signaling. *J Cell Physiol* **232**, 298-308, doi:10.1002/jcp.25483 (2017).

666 50 Huang, M. T. *et al.* Endothelial intercellular adhesion molecule (ICAM)-2 regulates
667 angiogenesis. *Blood* **106**, 1636-1643, doi:10.1182/blood-2004-12-4716 (2005).

668 51 Vang, T. *et al.* Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent
669 protein kinase inhibits signaling through the T cell receptor. *J Exp Med* **193**, 497-507,
670 doi:10.1084/jem.193.4.497 (2001).

671 52 Sachs, D. H., Kawai, T. & Sykes, M. Induction of tolerance through mixed chimerism.
672 *Cold Spring Harb Perspect Med* **4**, a015529, doi:10.1101/cshperspect.a015529 (2014).

673 53 Oura, T., Hotta, K., Cosimi, A. B. & Kawai, T. Transient mixed chimerism for allograft
674 tolerance. *Chimerism* **6**, 21-26, doi:10.1080/19381956.2015.1111975 (2015).

- 54 Schnuelle, P., Lorenz, D., Mueller, A., Trede, M. & Van Der Woude, F. J. Donor catecholamine use reduces acute allograft rejection and improves graft survival after cadaveric renal transplantation. *Kidney Int* **56**, 738-746, doi:10.1046/j.1523-1755.1999.00567.x (1999).
- 55 Schnuelle, P. *et al.* Impact of donor dopamine on immediate graft function after kidney transplantation. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **4**, 419-426, doi:10.1111/j.1600-6143.2004.00331.x (2004).
- 56 Wang, Y., Thatcher, S. E. & Cassis, L. A. Measuring Blood Pressure Using a Noninvasive Tail Cuff Method in Mice. *Methods Mol Biol* **1614**, 69-73, doi:10.1007/978-1-4939-7030-8_6 (2017).
- 57 Regan, R. D., Fenyk-Melody, J. E., Tran, S. M., Chen, G. & Stocking, K. L. Comparison of Submental Blood Collection with the Retroorbital and Submandibular Methods in Mice (*Mus musculus*). *J Am Assoc Lab Anim Sci* **55**, 570-576 (2016).

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Participated in critical revision: M.K., J.J.S., and E.A.R.

All authors discussed results and commented on the manuscript.

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Fig. 1

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Fig. 1: $\beta 2$ -AR agonist delays rejection responses after VCA. a Examples of each clinical and histologic rejection grade in a murine heterotopic hind limb transplant performed in a total

728 MHC-mismatch. Scale bar; 50 μ m. **b** Gross (clinical) rejection grades depending on different
 729 treatments after VCA. Vehicle; $n=8$, β_2 ; $n=4$, hTac; $n=4$, hTac+ β_2 ; $n=4$, fTac; $n=6$. **c**
 730 Histologic rejection grades depending on different treatments after VCA, $n=4$ /group.

Fig. 2

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Fig. 2: β_2 -AR agonist decreases T cell infiltration in transplanted grafts along with lower numbers of Foxp3 positive cells compared to the vehicle injected group. a, b Representative figures for H&E and IHC with CD8, CD4, and Foxp3 antibodies with either vehicle or β_2 -agonist treatment for 5 days. The borderline (black dotted line) differentiates recipient and donor, scale bar; 400 μ m. **c** Numbers of CD4, CD8, and Foxp3 positive cells in grafts 5 days after VCA. Over 8 fields from 3 mice/group *, $p < 0.05$ by Student's t test, error bar; standard error of the mean. **d** Compositions of CD4⁺/CD8⁺ central memory (CM) and effector memory (EM) T cell populations in transplanted grafts 5 days after VCA. Control; non-vascularized donor grafts, $n = 5$, ns; not significant, *, $p < 0.05$ by Student's t test, error bar; standard error of the mean. **e** Representative figures for H&E stain at different time points after VCA. Scale bar; 200 μ m. **f** Numbers of CD4⁺ and CD8⁺ T cells in grafts 5, 7 and 10 days after VCA in the hTac+vehicle and the hTac+ β_2 -agonist treated groups. Over 5 fields from $n = 3$ /group, *, $p < 0.05$ by Student's t test, error bar; standard error of the mean. **g** Compositions of CD4⁺/8⁺ CM and EM T cell populations in transplanted grafts 7 days after treatment either with hTac or hTac+ β_2 -agonist. $n = 3$, ns; not significant, error bar; standard error of the mean.

Fig. 3

Fig. 3: β₂-AR agonist decreases CD4⁺/CD8⁺ EM T and Th1 cell populations, and significantly decreases cytokine levels such as IFN-γ, IL-6 and TNF-α compared to the vehicle group in recipients' blood. a, b Compositions of CD4⁺/CD8⁺ CM/EM T cell

751 populations in the blood 5 and 7 days either with vehicle or β_2 -agonist after VCA without
752 tacrolimus. Day 5; $n \geq 9$, Day 7; $n = 5$, control; non-transplanted animals, ns; not significant, *;
753 $p < 0.05$ by Student's t test, error bar; standard error of the mean. **c, d** Compositions of
754 $CD4^+/CD8^+$ CM/EM T cell populations in the blood 7 and 10 days with either vehicle or β_2 -
755 agonist after VCA with a half dose of tacrolimus (hTac). Day 7; $n = 4$, Day 10; $n \geq 12$, ns; not
756 significant, *; $p < 0.05$ by Student's t test, error bar; standard error of the mean. **e, f** Th1 and
757 Treg cell populations in $CD4^+$ T cells without and with tacrolimus after VCA with either vehicle
758 or β_2 -agonist treatments. Day 7; $n \geq 6$, Day 10; $n \geq 4$, ns; not significant, *; $p < 0.05$ by Student's
759 t test, error bar; standard error of the mean. **g** In the setting of wild type (WT) donors and WT
760 recipients VCA without tacrolimus, various cytokine levels were analyzed 5 and 7 days after
761 transplant by Luminex assay. $n = 6$; pooled data from duplicate samples, *; $p < 0.05$ by Student's
762 t test, error bar; standard error of the mean.

Fig. 4

Fig. 4: β 2-AR agonist injected group achieves significantly longer graft survival than the vehicle injected group with a suboptimal dose of tacrolimus. **a** WT recipients having WT donor grafts were treated with a full dose (optimal) of tacrolimus (fTac) for 14 days, and then treated with either vehicle or β 2-agonist after cessation of fTac. **b** CD4⁺/CD8⁺ CM and EM T cell compositions were analyzed in the blood 21 days after VCA, 7 days after treatment with

769 either vehicle or β_2 -agonist. $n=6$, ns; not significant, *, $p<0.05$ by Student's t test, error bar;
770 standard error of the mean. **c** Graft survival curves. $n=5$, ns; not significant by Log-rank test
771 using GraphPad Prism. **d** WT recipients were treated with a half dose (subtherapeutic) of
772 tacrolimus along with vehicle or β_2 -agonist after 14-day fTac injections. **e** $CD4^+/CD8^+$ CM and
773 EM T cell compositions were analyzed in the blood 28 days after VCA, 14 days after treatment
774 with either vehicle or β_2 -agonist. $n=5$, ns; not significant by Student's t test, error bar; standard
775 error of the mean. **f** Graft survival curves. $n=5$, *, $p<0.05$ by Log-rank test with GraphPad
776 Prism.

Fig. 5

Fig. 5: Modulation of β_2 -AR signaling in donors is more effective to delay rejection responses than the modulation in recipients. a BALB/c β_2 -AR KO donors were transplanted to C57BL/6 recipients with either vehicle or β_2 -agonist injections. **b, c** Compositions of

781 CD4⁺/CD8⁺ CM/EM T cell populations in the spleen 5 and 7 days with either vehicle or β_2 -
 782 agonist after VCA without tacrolimus in WT recipients receiving BALB/c β_2 -AR KO grafts.
 783 Day 5; $n=6$, Day 7; $n\geq 6$, ns; not significant, *; $p<0.05$ by Student's t test, error bar; standard
 784 error of the mean. **d** Th1 and Treg cell populations in CD4⁺ T cells after vehicle or β_2 -agonist
 785 treatments in WT recipients receiving BALB/c β_2 -AR KO grafts. $n\geq 4$, ns; not significant, *;
 786 $p<0.05$ by Student's t test, error bar; standard error of the mean. **e** BALB/c WT donors were
 787 transplanted to C57BL/6 β_2 -AR KO recipients with either vehicle or β_2 -agonist injections. **f, g**
 788 Compositions of CD4⁺/CD8⁺ CM/EM T cell populations in the spleen 5 and 7 days with either
 789 vehicle or β_2 -agonist after VCA without tacrolimus in β_2 -AR KO recipients receiving BALB/c
 790 WT grafts. Day 5; $n\geq 3$, Day 7; $n\geq 6$, ns; not significant, *; $p<0.05$ by Student's t test, error
 791 bar; standard error of the mean. **h** Th1 and Treg cell populations in CD4⁺ T cells with either
 792 vehicle or β_2 -agonist treatments in β_2 -AR KO recipients receiving BALB/c WT grafts. $n\geq 3$,
 793 ns; not significant, *; $p<0.05$ by Student's t test, error bar; standard error of the mean. **i**
 794 Representative figures for IHC with CD4, CD8 and Foxp3 antibodies with β_2 -agonist treatment
 795 for 5 days in WT recipients bearing AR KO donor's grafts (KO→WT) and AR KO recipients
 796 bearing WT donor's grafts (WT→KO). Over 9 fields from $n=3$ /group, ns; not significant, *;
 797 $p<0.05$ by Student's t test, error bar; standard error of the mean, scale bar; 400 μ m.

Fig. 6

Fig. 6: β ₂-AR agonist has effects with donor's stromal cells to inhibit T cell trafficking into transplanted grafts. **a** BALB/c WT mice as a donor was exposed to 8 Gy irradiation the day before surgery, the composite graft was transplanted to C57BL/6 β ₂-AR KO recipients with either vehicle or β ₂-agonist treatments without tacrolimus. **b**, **c** Compositions of CD4⁺/CD8⁺

CM and EM T cell populations and Th1 and Treg populations. $n \geq 3$, ns; not significant, * ; $p < 0.05$ by Student's t test, error bar; standard error of the mean. **d** IHC with CD4/CD8 antibodies, and cells were counted by ImageJ program. Over 5 fields from $n = 3$ /group, ns; not significant, * ; $p < 0.05$ by Student's t test, error bar; standard error of the mean, scale bar; 400 μm .

Fig. 7

808 **Fig. 7: β_2 -AR agonist has effects with donor's stromal cells to inhibit T cell trafficking**
 809 **into transplanted grafts. a** BALB/c AR KO and WT donors were exposed to 8 Gy irradiation,
 810 and then BALB/c WT and AR KO BM were transplanted respectively. After 8-week BM
 811 transplantation (BMT), VCA was performed on C57BL/6 AR KO recipients followed by β_2 -
 812 agonist treatment for 7 days. **b** H&E and IHC with CD4 and CD8 antibodies, 6 fields from $n=$
 813 3/group, *, $p < 0.05$ by Student's t test, error bar; standard error of the mean, scale bar; 200 μm .
 814 **c, d** Systemic compositions of CD4⁺/CD8⁺ CM/EM T cell populations and Th1 and Treg cell
 815 populations in CD4⁺ T cells 7 days after VCA with β_2 -agonist treatment in C57BL/6 AR KO
 816 recipients having a graft from either BALB/c β_2 -AR KO with WT BMT (KO+WT) or BALB/c
 817 WT with AR KO BMT (WT+KO). $n \geq 3$ ns; not significant, *, $p < 0.05$ by Student's t test, error
 818 bar; standard error of the mean.

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donors and recipients followed by either vehicle or β_2 -agonist injections. ICAM-1, VCAM-1, ICAM-2 and CD31 antibodies were used. Histograms depict quantification of IF intensity of each antibody in all CD31⁺ vessels; numbers denote Micro-Flow Imaging (MFI). All data are representative of duplicate experiments, $n= 3$, scale bar; 100 μm . **b** Using Luminex assay, in the setting of WT donors and WT recipients VCA without tacrolimus, various chemokine levels were analyzed 5 and 7 days after transplant. $n= 6$; pooled data from duplicate samples, ns; not significant, *; $p < 0.05$ by Student's t test, error bar; standard error of the mean.

Representative data between 2 different experiments, $n= 5$, ns; not significant, $*$; $p< 0.05$ by Student's t test, error bar; standard error of the mean.

Supplementary Fig. 2

Supplementary Fig. 2: The composition of donor's and recipient's leukocytes in donor grafts and recipient blood 5 and 7 days after VCA. a, b Source of infiltrated T cells in transplanted grafts and recipient's blood between recipient (CD45-1) and donor (CD45-2). Control; non-vascularized grafts, $n\geq 3$, ns; not significant, $*$; $p< 0.05$ by Student's t test, error bar; standard error of the mean.

Supplementary Fig. 3

Supplementary Fig. 3: Pathologic findings 5 and 7 days after VCA with either vehicle or $\beta 2$ -agonist injections. **a, b** Representative H&E images revealed epithelial dyskeratosis (arrowhead) and apoptosis (arrow) in the vehicle injected group (**a**; rejection grade 3) not in the $\beta 2$ -agonist injected group (**b**; rejection grade 2) 5 days after VCA, scale bar: 50 μ m. **c, d**

Representative figures for H&E and IHC with CD8, CD4, and Foxp3 antibodies either with vehicle or β_2 -agonist treatment for 7 days. **e** Numbers of CD4, CD8, and Foxp3 positive cells in grafts 7 days after VCA. 9 fields from 3 grafts per group, ns; not significant, error bar; standard error of the mean. **f** Compositions of CD4⁺/CD8⁺ CM and EM T cell populations in transplanted grafts 7 days after VCA. Control; non-vascularized grafts, $n=5$, ns; not significant, *; $p<0.05$ by Student's *t* test, error bar; standard error of the mean, scale bar; 400 μ m.

Supplementary Fig. 4

Supplementary Fig. 4: Numbers of Foxp3⁺ cell in transplanted grafts and the composition of Treg (CD4⁺CD25⁺Foxp3⁺) population with fTac injections after VCA. **a Representative figure for H&E and IHC with numbers of CD4, CD8 and Foxp3 cells 10 days after VCA. 5 fields from 3 grafts, error bar; standard error of the mean, scale bar; 400 μ m. **b** The Treg**

887 population was analyzed with recipient's spleen 30 days after VCA. Control; a mouse without
 888 VCA, $n=4$, ns; not significant, error bar; standard error of the mean.

Supplementary Fig. 5

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Fig. 5: Preconditioning in donors with β_2 -AR agonist delays rejection responses through suppression of T cell trafficking in the grafts. **a** BALB/c donor mice were injected with β_2 -agonist for 2 weeks before VCA (Pre-VCA), and then β_2 -agonist treatment was stopped after the surgery in C57BL/6 AR KO recipients. Representative figures, scale bar; 400 μ m. **b** Systemic compositions of CD4⁺/CD8⁺ CM and EM T cell populations 7 days after VCA. **c** Th1 and Treg cell populations in CD4⁺ T cells. $n \geq 4$ mice *; $p < 0.05$ by Student's t test, ns; not significant, error bar; standard error of the mean. **d** Numbers of CD4 and CD8 positive cells in grafts 7 days after VCA. Over 10 fields from 3 mice/group *; $p < 0.05$ by Student's t test, ns; not significant, error bar; standard error of the mean, scale bar; 400 μ m. Data with empty circles (Post-VCA); historical data.

Supplementary Table 1: Visual and histologic grading system for assessment of rejection after VCA

	Grade	Visual assessment	Histologic assessment
	0: No rejection	Normal epidermal and dermal appearance without evidence of rejection.	No or rare inflammatory infiltrates.
	1: Indeterminate/supposed rejection	Very early visual signs of rejection. Focal (<25%) inconspicuous changes in the skin color.	Mild. Mild perivascular infiltration. No involvement of the overlying epidermis.
	2: Mild rejection	Clear visual signs of rejection. Diffuse (25%-50%) clear changes in color and texture of skin.	Moderate. Moderate-to-severe perivascular inflammation with or without mild epidermal and/or adnexal involvement (limited to spongiosis and exocytosis). No epidermal dyskeratosis or apoptosis.
	3: Moderate rejection	Extensive (>50%) and advances alteration of skin quality such as desquamation, scabs, skin crusts.	Severe. Dense inflammation and epidermal involvement with epithelial apoptosis, dyskeratosis and/or keratinolysis.
	4: Severe rejection	Destruction of epidermis with intact dermis or complete destruction of the skin.	Necrotizing acute rejection. Frank necrosis of epidermis or other skin structures.