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ORIGINAL ARTICLE

Studying the immunosuppressive role of indoleamine 2,3-dioxygenase: tryptophan metabolites suppress rat allogeneic T-cell responses *in vitro* and *in vivo*

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Summary

Pregnancy is a natural model of successful tolerance induction against allogeneic tissues. Recent studies pointed to a role of indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme expressed in the placenta, in mediation of T-cell suppression. We want to apply to organ transplantation what nature has developed for suppression of fetal rejection during pregnancy. Here we analyze whether IDO-induced tryptophan metabolites are able to suppress the allogeneic T-cell response and allograft rejection in rats. Rat lymphocytes were stimulated with allogeneic dendritic cells *in vitro* in the presence of increasing amounts of tryptophan metabolites (kynurenine, 3-hydroxykynurenine, anthranilic acid, 3-hydroxyanthranilic acid and quinolinic acid) and T-cell proliferation was determined. The findings showed that kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid strongly suppress the T-cell response, whereas anthranilic and quinolinic acid are noneffective. Vital staining of cells with subsequent fluorescence-activated cell sorter analyses demonstrated that suppression is mediated by T-cell death. Thereafter, the action of metabolites was analyzed in a skin allograft model (BN $\rightarrow$ LEW). Lewis recipients received daily s.c. injections of tryptophan metabolite mixture (kynurenine + 3-hydroxyanthranilic acid), cyclosporin A (positive control), or no treatment (negative control). The metabolites induced a significant prolongation ($P = 0.0018$) of graft survival. We conclude that IDO-induced tryptophan metabolites suppress the T-cell response and prolong allograft survival in rats.

Introduction

A natural model of tolerance against allogeneic tissues is the acceptance of the fetus during pregnancy. If the exact mechanism of immunosuppression in pregnancy were known, it might indicate a way of tolerance induction in clinical transplantation. Recent studies showed that during pregnancy a placental enzyme called indoleamine 2,3-dioxygenase (IDO) plays a key role in suppressing the maternal T-cell response against the fetus [1]. By blocking the IDO function, fetal rejection was induced in a murine model [2]. IDO has been found in various cells and can be induced by cytokines. Dendritic cells (DC) are well known for their immunostimulatory capacity [3]. Many observations pointed to the existence of a suppressive DC subpopulation [4]. In recent experiments a subpopulation of IDO-producing DC with T-cell suppressive properties was identified [5,6] indicating that IDO might be involved in central and peripheral tolerance induction against autoantigens.

The IDO is a gene which codes for an enzyme that degrades tryptophan and other biological compounds. The enzyme acts on the indole ring catalyzing its oxidative cleavage. In case of tryptophan the primary metabolite is kynurenine followed by other metabolites finally leading to nicotinamide metabolism. As IDO destroys tryptophan and because the latter is an essential amino acid required for cell proliferation, it has been speculated

that IDO induces T-cell suppression by depriving these cells of tryptophan [7]. In a recent publication [8] we challenged this hypothesis by raising the question whether tryptophan metabolites might suppress the T-cell response. Our studies showed that certain metabolites are able to strongly inhibit the human T-cell response *in vitro*. These findings lead us to the idea to use these 'mediators' of the inhibitory IDO-producing DCs for suppression of rejection in organ transplantation.

In the current series of experiments we analyze whether tryptophan metabolites are able to suppress the allogeneic T-cell response in rats and whether they have an influence on rat skin allograft survival.

Material and methods

Isolation of splenocytes

Spleens were harvested from male Lewis (LEW; $RT1^1$) rats under sterile conditions and cut into small pieces. The tissue suspension was then gently pressed through a 70 μm nylon cell strainer (Becton Dickinson, Heidelberg, Germany), the cell suspension was washed and the lymphocytes were isolated by gradient centrifugation on lymphocyte cell separation medium (Lymphodex; Inno-Train Diagnostik GmbH, Kronberg, Germany).

Generation of monocyte-derived dendritic cells

Dendritic cells were generated as previously described [9] with minor modifications. Briefly, blood of male Brown-Norwegian (BN) ($RT1^n$) rats was collected on heparin and diluted in equal volumes of RPMI-1640 medium (Promocell, Heidelberg, Germany). Mononuclear cells were isolated by gradient centrifugation, washed and the cell number was adjusted to 2.5×10^6 cells/ml. The cells were incubated in Petri dishes (Nunc GmbH & Co., Wiesbaden, Germany) at 37 °C and 5% CO_2 for 90 min. The adherent monocytes were resuspended in medium containing 300 U/ml rMu granulocyte macrophage colony-stimulating factor and 250 U/ml rRa interleukin 4 (Promocell). On day 6, 10 ng/ml rRa tumor necrosis factor α (Promocell) and 1 μg/ml prostaglandin E_2 (Sigma-Aldrich, Tanfkirchen, Germany) was added to the cultures. By day 9 large numbers of cells with DC type morphology were seen floating in the cultures. Suspension cells were harvested and stored for further use. Before use, the phenotype of DC was verified by fluorescence-activated cell sorter (FACS) analysis ($MHCII^{+++}$, $CD80^{++}$, $CD86^{++}$, ICAM-1^{++}, $OX62^{+}$, $ED2^{-}$).

Allogeneic T-cell stimulation

Dendritic cells ($n = 20\,000$) were co-incubated with allogeneic spleen lymphocytes ($n = 100\,000$) in 0.2 ml RPMI 1640 medium plus supplement (Promocell). Positive controls consisted of DCs plus allogeneic lymphocytes, whereas negative controls consisted of DCs or lymphocytes only. After 5 days ^{3}[H]thymidin was added for 12 h and the number of counts per minute (c.p.m.) was determined in a β-counter (Inotech Biosystems, Lansing, MI, USA).

Tryptophan metabolites

The heme enzyme IDO oxidizes the pyrrol moiety of tryptophan utilizing superoxide anion radical as both substrate and co-factor. The latter has the ability to reduce the inactive ferric-IDO to the active ferrous form. The main metabolites resulting from IDO-induced degradation of tryptophan are generally known as kynurenines and comprise *N*-formyl-kynurenine, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, anthranilic acid and quinolinic acid (Fig. 1). The first metabolite *N*-formyl-kynurenine is a short-lived, very instable compound. To determine the inhibitory concentration, tryptophan metabolites were tested in titration series. Kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, anthranilic acid, and quinolinic acid (Sigma-Aldrich) with a purity ≥98% were dissolved in RPMI 1640 medium and added to the cell cultures in different concentrations and combinations.

Detection of cell death

The isolated lymphocytes were cultured in the presence or absence (control) of kynurenine (750 μM), 3-hydroxykynurenine (350 μM), 3-hydroxyanthranilic acid (250 μM). After 3 days, the tested metabolites were washed out and the number of dead cells was determined using 7-AAD (BD Biosciences, Heidelberg, Germany) as a dead cell marker analyzed by a FACScan™ (BD Biosciences, Heidelberg, Germany).

Skin transplantation

Donor skin was collected from BN rats whereas LEW rats served as recipients. The abdominal skin was harvested and thinned for transplantation (graft size approximately 1 cm × 1 cm). Skin from the left side of the thorax of LEW rats was removed parallel with the dermal vascular plexus without injuring it. Donor skin was grafted, sutured and bandaged. The skin grafts were examined daily until rejection. Kynurenine and 3-hydroxyanthranilic acid were dissolved in saline. The mixture was administered intraperitoneally at a concentration of 15 mg/kg/day ($n = 6$). This dose was defined based on previous studies of suppression of allogeneic

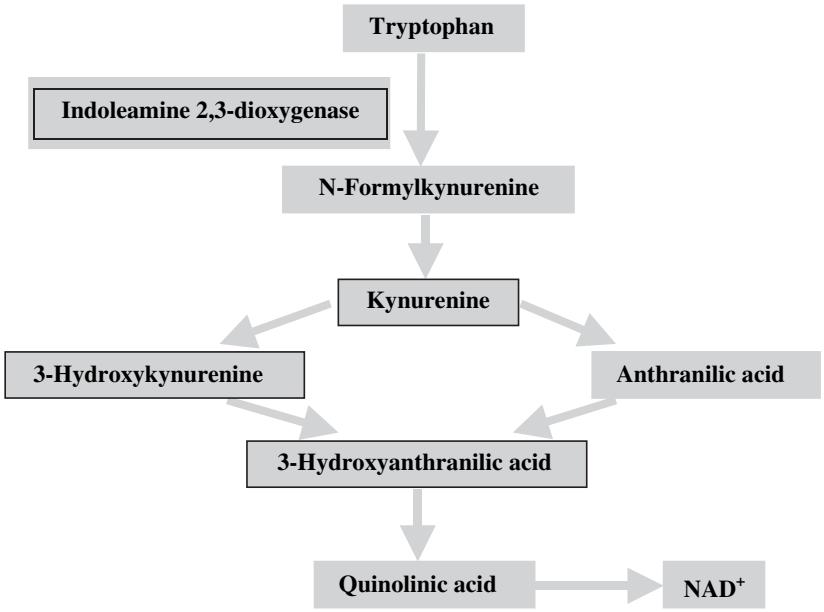

Figure 1 Tryptophan degradation along the kynurenine pathway with the initial and rate limiting catalysing activity of indoleamine 2,3-dioxygenase. The T-cell active compounds kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid were marked. Alternative pathways and secondary enzyme activity were not mentioned.

T-cell reaction in a lymph node assays. Cyclosporin A (Sandimmun; Sandoz [Novartis Pharma GmbH, Nurnberg, Germany]) ($n = 6$) was used in a concentration of 15 mg/kg. Negative controls received saline (placebo)($n = 5$) or no treatment ($n = 10$). In all treated groups, animals received daily injections (once a day) of 1 ml substance from the day of transplantation until graft rejection. Rats were randomized and evaluation of rejection was performed under blinded conditions. Rejection was monitored by examining the graft size and the extension of necrosis (color, dry necrosis, wet necrosis, adhesion), hair growth, secretion beneath the graft and revascularization. Necrosis of >80% of the graft was defined as complete rejection.

Animal protection and statistics

The 'Principles of Laboratory Animal Care' (NIH publication No. 86–23, revised 1985), as well as the German Law on the Protection of Animals were followed. All cell culture tests were performed in triplicates and results were given as mean ± SD. For calculating the half-inhibitory (I_{50}) concentration of substances, mean proliferation (c.p.m.) of stimulated (100%) and completely suppressed (0%) cells was determined. Thereafter, the concentration of substance giving 50% proliferation was calculated by regression. Graft survival was represented by Kaplan–Meier curves and statistical significance calculated by log-rank test with Bonferroni corrections.

Results

The tryptophan metabolites kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid inhibit rat T-cell proliferation *in vitro*

We analyzed the T-cell inhibitory effect of metabolites in an allogeneic cell culture model. Responder lymphocytes were isolated from spleen of LEW rats and stimulator cells (DCs) were generated from peripheral monocytes of BN rats. The two cell subpopulations were co-cultured in the presence of various concentrations of tryptophan metabolites and cell proliferation was measured at the end of the culture. Whereas kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid inhibited the T-cell response (Fig. 2a–c), anthranilic and quinolinic acid had no effect (Fig. 2e,f). The T-cell inhibitory concentrations (I_{50}) of active metabolites were as follows: kynurenine = 170 μM, 3-hydroxykynurenine = 17 μM, and 3-hydroxyanthranilic acid = 71 μM.

Under physiological conditions tryptophan metabolites do not act as single substances but as a mixture of all compounds. An interesting question is whether the combination of active metabolites is more effective than single substances. As *in vivo* kynurenine is quickly degraded to 3-hydroxykynurenine resulting in a mixture of both substances, we mixed kynurenine with 3-hydroxyanthranilic acid. It is expected that this mixture finally result in kynurenine + 3-hydroxykynurenine + 3-hydroxyanthranilic acid. As shown in Fig. 2d the above combination acts at lower concentrations ($I_{50} = 47$ μM) than single

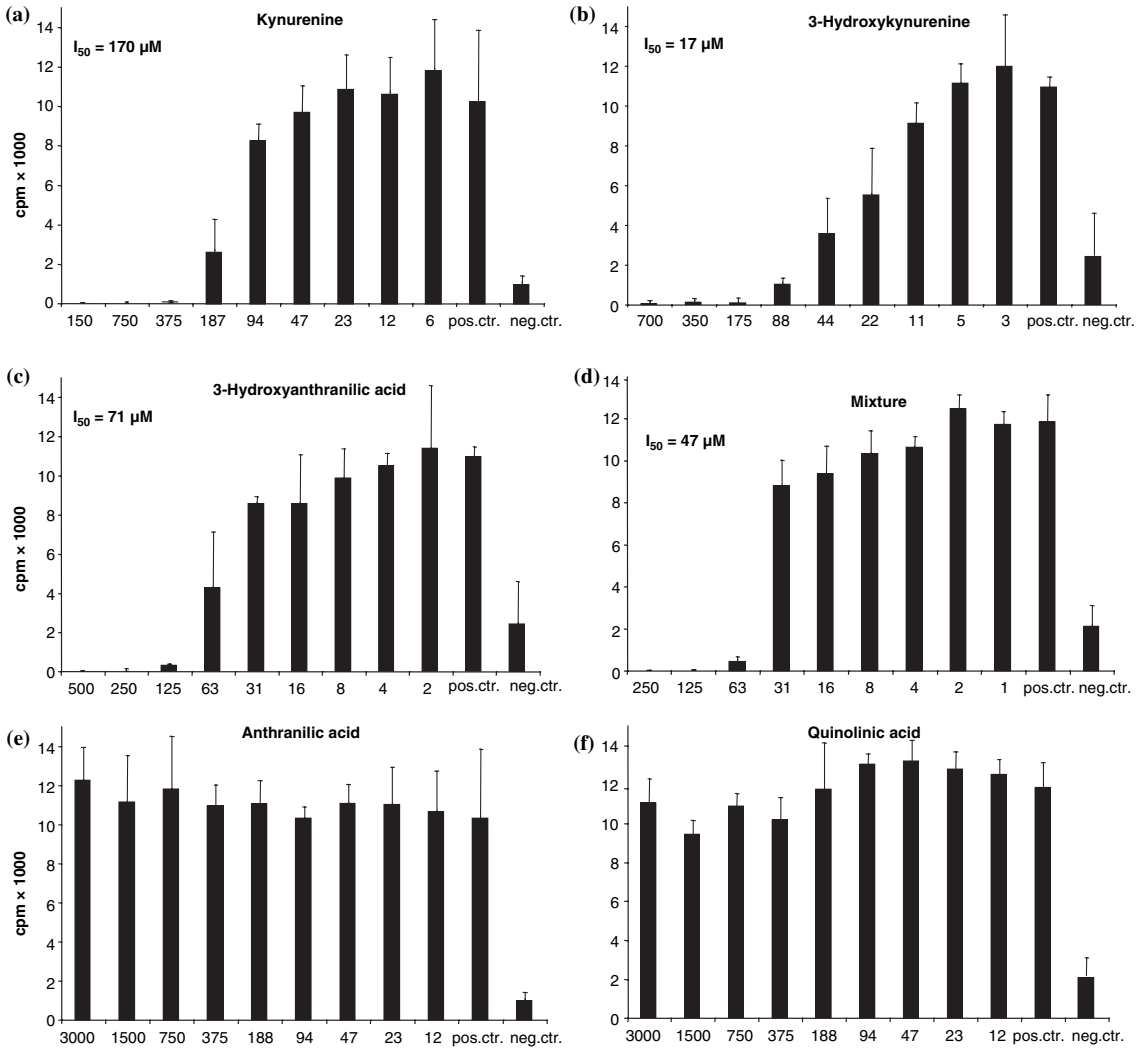

Figure 2 The effect of IDO-induced tryptophan metabolites on allogeneic T-cell response. Lymphocytes were stimulated with allogeneic DCs in the presence of various amounts (abscissa) of (a) kynurenine, (b) 3-hydroxykynurenine, (c) 3-hydroxyanthranilic acid, (d) kynurenine plus 3-hydroxyanthranilic acid (mixture), (e) anthranilic acid, or (f) quinolinic acid. The positive control consisted of lymphocytes plus allogeneic DC and the negative control consisted of unstimulated lymphocytes. T-cell proliferation was determined by ^{3}[H]thymidin incorporation (c.p.m.)(ordinate) after 5 days of culture.

doses of kynurenine (I_{50} = 170 µM) or 3-hydroxyanthranilic acid (I_{50} = 71 µM) (Fig. 2a,c).

The tryptophan metabolites kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid induce T-cell death

The registered lack of proliferation may have resulted from T-cell areactivity or cell death. In order to clarify the underlying mechanism rat lymphocytes were co-incubated in the presence of inhibitory concentrations of kynurenine, 3- hydroxykynurenine, or 3-hydroxyanthranilic acid with allogeneic DC as described in the previous experiment. Cytotoxicity was evaluated by FACS analyses following vital staining with 7-AAD. As shown in Fig. 3 the active metabolites induce T-cell death.

Tryptophan metabolites prolong allogeneic skin graft survival

The BN rats served as donors and LEW rats as recipients. LEW rats received daily s.c. injections of a metabolite mixture (kynurenine + 3-hydroxyanthranilic acid) or cyclosporin A (positive control). The negative control group received no active substance. After skin transplan-

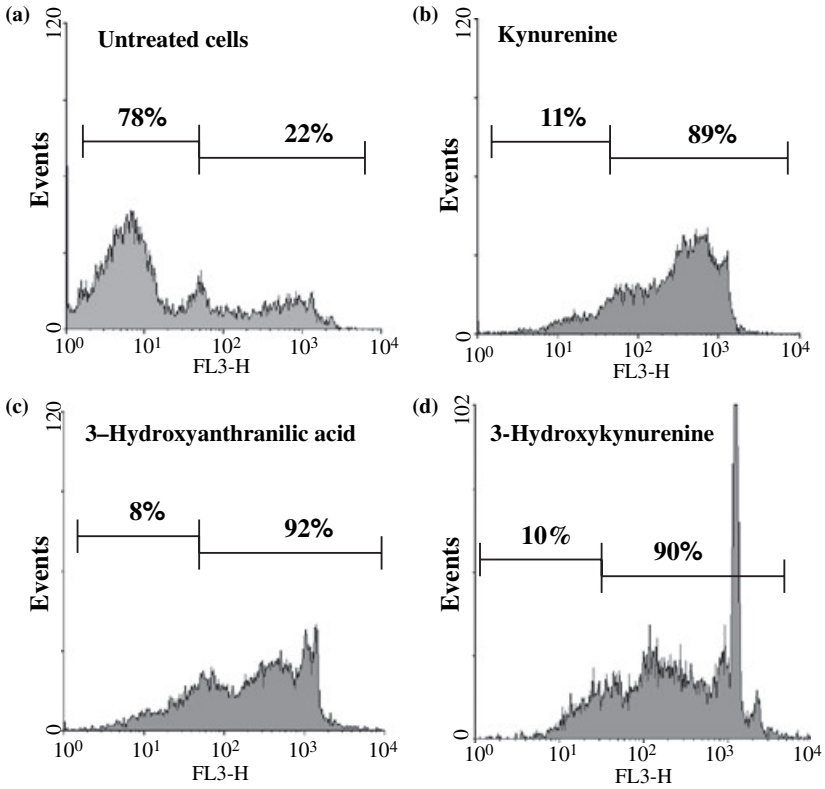

Figure 3 Cell death induction by the active tryptophan metabolites. (a) Untreated cells. Lymphocytes were co-incubated with allogeneic DC in the presence of suppressive doses of (b) kynurenine (750 μm), (c) 3-hydroxyanthranilic acid (250 μm) or (d) 3-hydroxykynurenine (350 μm). The negative control consisted of cell culture without metabolites. After 3 days the cells were washed and the percentage of 7-AAD-positive cells was determined in a FACScan™.

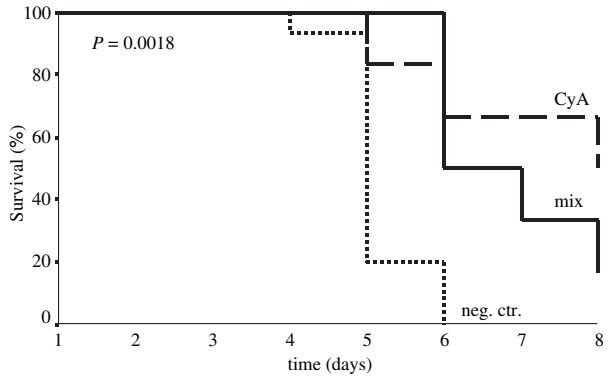

Figure 4 The effect of tryptophan metabolites on rat skin allograft survival. Skin was transplanted from BN to LEW rats. Recipients received daily i.p. injections with 15 mg/kg metabolite mixture (kynurenine and 3-hydroxyanthranilic acid; $n = 6$) or cyclosporin A (positive control; $n = 6$). Negative controls received saline solution or no treatment ($n = 15$). Kaplan–Meier survival curves are shown. The metabolite mixture significantly prolonged graft survival ($P = 0.0018$).

tation, the grafts were daily inspected for typical rejection signs. Kaplan–Meier survival curves are shown in Fig. 4. The treatment with metabolite mixture significantly prolongs graft survival when compared with the negative control.

Discussion

In this study we analyzed the impact of tryptophan metabolites on rat T cells and tested their action on skin allograft survival. Previous findings of Munn *et al.* [1] and Mellor and Munn [2] demonstrated a central role of IDO in suppression of fetal rejection. As pregnancy is a successful model of tolerance induction, IDO may be useful for the development of novel therapeutic strategies in organ transplantation. The hypothesis of Munn *et al.* [7] concerning the lack of tryptophan as mediator of IDO-induced suppression was challenged by us. Therefore, we analyzed the immunosuppressive properties of metabolites resulting from tryptophan degradation. Our recent findings [8] showed that the resulting kynurenines strongly inhibit T-cell proliferation. One possibility of using the IDO mechanism for suppression of rejection would be to treat graft recipients with the immunosuppressive tryptophan metabolites. We chose an allogeneic skin transplant model in rats which is known to be a highly immunogenic system. In order to use this rat model, we first tested the action of active kynurenines on rat T cells *in vitro*. The results were similar to those of our previous human cell cultures, showing that rat T cells are suppressed by the metabolites. We also found that the combination of metabolites is more effective than single substances. This

is relevant for the *in vivo* situation where they do not act as single compounds but as a combination of all substances. The decision of using a metabolite mix for controlling allograft rejection was also based on the wish to provoke minimal side effects. The used combination of kynurenine and 3-hydroxyanthranilic acid has the advantage of avoiding the administration of neurotoxic tryptophan metabolites. Quinolinic acid is an agonist of *N*-methyl-D-aspartate receptors which causes neuronal damages, whereas 3-hydroxykynurenine generates free radicals also leading to neuronal defects [10,11].

As skin allograft rejection is difficult to control, we first took this model for testing the effectiveness of the metabolites. If they were effective, so went the reasoning, we would expect far better results in other transplant models. Our data showed a significant prolongation of graft survival although the classical immunosuppressive agent cyclosporin A was more effective. This finding was in line with our observation of suppressed popliteal lymph node reaction upon injection of BN cells into the food pads of LEW rats treated with the same metabolites (data not shown). In contrast to what we expected from our *in vitro* findings and from the murine IDO-mediated abortion models [2], the impact of tryptophan metabolites on graft survival was rather moderate. Of course, a detailed dose-effect study is required to further optimize the suppressive effect of these compounds. On the contrary, the metabolites apparently act locally, partially losing their activity by systemic administration. This speculation is based on the observation that in pregnancy their effect is limited to the placenta without affecting the immune system of the fetus. This is in line with previous observations suggesting that IDO mediates immunosuppression by generating redox active specimens [12,13]. It is known that such molecules are quickly inactivated and consequently only locally active. Regarding IDO-mediated tolerance in pregnancy it is possible that the strong immunosuppression results from the combined action of metabolites and locally reduced tryptophan concentrations. The metabolites may also induce cytokine production in the placenta which plays a role in immunosuppression.

In conclusion, our data show that IDO-induced tryptophan metabolites induce rat T-cell suppression and prolong skin allograft survival.

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