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ORIGINAL ARTICLE

Targeting the Kv1.3 potassium channel for immunosuppression in vascularized composite allotransplantation – a pilot study

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Summary

Kv1.3-channels are critically involved in activation and function of effector memory T cells. Blocking Kv1.3-channels was investigated for its effect on skin rejection in a rat limb-transplantation-model. Animals received the Kv1.3-blocker correolide C systemically or locally as intra-graft-treatment in combination with tacrolimus. Systemic (intraperitoneal) administration of correolide C resulted in slight, but significant prolongation of allograft survival compared with untreated and placebo treated controls. In 4/6 correolide C treated animals, histology showed an intact epidermis and a mild infiltrate by day 10. High correolide C plasma trough levels correlated with prolonged allograft survival. A decrease in CD4+ and CD8+ effector memory T cells was observed in allograft skin, peripheral blood and the spleen on day 5. When applied subcutaneously in combination with systemic tacrolimus (30 days+/−anti-lymphocyte serum) detectable, but insignificant prolongation of graft survival was achieved. 2/5 animals showed an intact epidermis and a mild infiltrate until day 45. Tapering systemic tacrolimus and weaning on day 50 resulted in rejection by day 55, regardless of local correolide C treatment. Subcutaneous injection did not lead to systemic plasma levels. The Kv1.3-channel is a potential drug target worth exploring in more detail for immunosuppression in vascularized composite allotransplantation.

Introduction

Vascularized composite allotransplantation (VCA) serves as a suitable option for treatment of major tissue defects. Functional and esthetic outcomes in over 75 hand/forearm and 20 facial transplants to date are good to excellent [1–9]. However, life-long immunosuppression required to ensure graft survival is associated with a wide range of side effects [7,10–12] and introduction of immunosuppressive-sparing protocols is needed. Animal studies and clinical observations indicate that the skin is the principal target of rejection and therefore needs to be especially addressed when developing immunosuppression-minimization strategies [13–18].

The voltage-gated Kv1.3 potassium channel is expressed on peripheral blood T lymphocytes and is responsible for

regulation of the membrane potential and Ca2+ signaling [19,20]. Upon activation, expression of Kv1.3 is increased four- to fivefold, especially in effector memory T cells (Tem) [21]. In rats, T cells at the site of skin inflammation have been described as CD4+CCR7-CD45RC- Tem for delayed-type hypersensitivity (DTH) [22] and CD8+CCR7-CD45RC- Tem for acute contact dermatitis [23], both highly expressing the Kv1.3 channel. Kv1.3 inhibitors have been introduced as potential new therapeutics to treat T cell-mediated diseases [24–27]. The Kv1.3 inhibitor margatoxin for example was shown to inhibit a DTH reaction and a response to an allogenic challenge in miniswine [28].

Correolide was purified from the Costa Rican tree *Spachea correae* and is a natural occurring blocker of Kv1.3 channels on T lymphocytes [29]. Correolide C is a chemically modified analog of correolide with improved activity [30]. *In-vitro* studies showed inhibition of IL-2 production and proliferation of human and miniswine T cells by correolide C in a dose-dependent manner and diminished DTH responses in the miniswine [30]. A 4-week tolerability study in minipigs (5 mg/kg/day) resulted in plasma trough correolide C levels of 36.7 ± 11 ng/ml from days 4 to 28 and no side effects in blood chemistry, hematology and histology [H Glossmann, R Margreiter, unpublished data].

Skin rejection after hand/facial transplantation is mainly a T lymphocyte driven immune response towards the epidermis [31,32]. We investigated the effect of the Kv1.3 blocker correolide C on skin rejection and the kinetics of Tem in a rat hind-limb VCA model.

Methods

Animals and rat hind-limb transplant model

In a full major-histocompatibility-complex mismatch model, Lewis (LEW) male rats served as recipients and Brown-Norway male (BN) rats served as donors (200–250 g, Charles River, Sulzfeld, Germany). Experiments were performed under protocols approved by the Austrian Ministry of Science and Research. The rat hind-limb transplantation model was carried out as described before [33,34]. To minimize pain, buprenorphin was administered until postoperative day (POD) 3.

Experimental protocol and drug administration

The immunosuppressive effect of correolide C was evaluated for prevention of allograft rejection when administered (i) systemically (intraperitoneally, i.p. or intramuscularly, i.m., 5 mg/kg/day) or (ii) locally (subcutaneously, s.c., 3 mg/kg twice/week in combination with low-dose tacrolimus ± a lymphocyte-depleting antibody). Detailed information on experimental groups is presented in Table 1. Untreated animals, animals receiving a vehicle and animals treated with tacrolimus ± anti-lymphocyte serum (ALS) alone served as controls.

Correolide C (L-000778262) was a kind gift from Merck&Co., Inc, Rahway, NJ, USA. 1.2 mg correolide C was dissolved in 25 μl dimethyl sulfoxide (DMSO Hybri-Max; Sigma-Aldrich Company Ltd, Irvine, Ayrshire, UK), 125 μl Solutol-HS15 (BASF, Ludwigshafen, Germany), 125 μl ethanol (70%) and 2225 μl phosphate-buffered-saline (Dulbecco's PBS1x without Ca + Mg; PAA-Laboratories GmbH, Pasching, Austria) and stored at 4 °C. Correolide C was injected systemically (i.p. or i.m.) or locally (s.c., distributed equally in the subcutaneous compartment of the allograft). Low-dose systemic immunosuppression for animals treated with local correolide C consisted of tacrolimus (Prograf; Astellas Ireland Co. Ltd., Country Kerry, Ireland), given i.p. at 0.30 mg/kg/day for 30 days, ±a polyclonal T cell depleting antibody, ALS (rabbit anti-rat lymphocyte, Accurate Chemical & Scientific Corporation, Westbury, NY; 0.5 ml), administered i.p. on days 0 and 3. In one group, tacrolimus was tapered to 0.10 mg/kg until day 50. In addition to animals listed in Table 1, 14 animals were transplanted to assess the kinetics of memory T cells [untreated controls (n = 5), systemic correolide C 5 mg/kg/day i.p. (n = 5), local correolide C 3 mg/kg twice/week s.c + ALS + tacrolimus 0.3 mg/kg/day i.p. until day 30 (n = 4)].

Graft rejection

Skin rejection was graded per appearance as follows: no evidence of rejection: grade 0, erythema: grade I, erythema and edema: grade II, epidermolysis: grade III, necrosis/mummification: grade IV. The endpoint of the study was rejection grade III. Skin biopsies were taken at regular intervals and upon signs of skin rejection. Animals were sacrificed and the allograft amputated once the endpoint was reached. Skin specimens and limb transections were preserved in 4% paraformaldehyde and paraffin-embedded. Sections (4 μm) were stained with hematoxylin and eosin according to standard procedures. Immunohistochemistry was performed using a polyclonal rabbit anti-rat CD3 antibody and a monoclonal mouse anti-rat CD68 antibody (both abcam, Cambridge, UK, 1:100). Staining was performed as per manufacturer's instructions. A pathologist blinded to the treatment evaluated the samples for lymphocytic infiltration, interface reaction, dermal-epidermal separation and necrosis.

Tacrolimus whole blood levels

Whole blood was collected from the tail vein of the animals receiving tacrolimus every 5 days, starting on POD 30. Tacrolimus trough levels were measured by tandem mass-spectrometry preceded by a two-dimensional liquid

Table 1. Experimental groups.

Group	Systemic treatment	Local treatment	Number
Systemic administration of correolide C			
1	No treatment	No	6
2	Correolide C 5 mg/kg daily i.p.	No	6
3	Correolide C 5 mg/kg daily i.m.	No	5
4	Placebo daily i.p.	No	2
local administration of correolide C			
5	Tacrolimus 0.3 mg/kg daily i.p. until day 30	No	4
6	Tacrolimus 0.3 mg/kg daily i.p. until day 30	Correolide C 3 mg/kg 2×/week s.c.	5
7	Tacrolimus 0.3 mg/kg daily i.p. until day 30 and 0.1 mg/kg until day 50	Correolide C 3 mg/kg 2×/week s.c.	4
8	ALS 0.5 ml, day 0 + 3 and tacrolimus 0.3 mg/kg daily i.p until day 30	No	4
9	ALS 0.5 ml, day 0 + 3 and tacrolimus 0.3 mg/kg daily i.p until day 30	Correolide C 3 mg/kg 2×/week s.c.	5

i.p. intraperitoneal; i.m. intramuscular; s.c. subcutaneous; ALS anti-lymphocyte serum.

chromatography system (online-SPE-LC-MS/MS), as described by Seger *et al.* [35].

Measurement of correolide C blood levels

Whole blood was withdrawn in EDTA from the tail vein of transplanted animals every 5 days and at the endpoint (trough levels) and plasma stored at −80 °C. To evaluate the short time kinetics of single-dose systemic correolide C in rats, two additional animals received i.p. correolide C 5 mg/kg, EDTA blood was collected every hour from 1 to 8 h after administration and plasma prepared for further analysis as described below. Assuming first-order simple kinetics data were ln -transformed and fitted by linear regression.

Previously we had measured correolide C EDTA plasma levels in minis wine in a volume of 200 µl of EDTA plasma [31]. In order to adapt to the much smaller volumes needed for the rat experiments, we developed a new method requiring only 10 µl plasma. Levels were measured on an online-SPE-LC-MS/MS instrument as described by Seger *et al.* [35]. Sample preparation of plasma calibrators, quality control (QC) samples and rat specimens relied on a protein precipitation protocol [10 µl sample, 50 µl H_2O, 250 µl precipitation solution (0.1 M ZnSO4:MeOH = 1:2 (v/v))] spiked with the internal standard cyclosporine D. The 2D chromatography setup (injection volume 50 µl precipitation supernatant) combined the solid phase extraction step over a RP polymer material (Oasis HLB 20 × 2.1 mm, 25 µm particle size; Waters, Milford, MA, USA; solvent: H_2O:MeOH = 9:1 (v/v), 0.4% formic acid, 4 ml/min flow) with isocratic reversed phase chromatography using a RP C-18 HPLC column (Zorbax Eclipse XDB-C18 100 × 3 mm, 3.5 µm particle size, Agilent Technologies, solvent: H_2O:MeOH = 3:97 (v/v), 0.1% AcOH, 10 mM NH_4Ac, 0.8 ml/min flow). An API4000Qtrap instrument (Applied Biosystems/MDS Sciex, Toronto, Canada) operating in the ESI-SRM mode (correolide C quantifier SRM: 918.3 to 755.3 *m/z*; internal standard SRM: 1233.9 to 1198.7 *m/z*) served as detector. Quantification was based on the area ratio analyte to internal standard. A six-level calibrator ranging from 20 ng/ml (lower limit of quantification) to 1000 ng/ml (upper limit of quantification) was used, the linear calibration function was weighting with 1/x. Quantitative results were confirmed by a qualifier ESI-SRM experiment (918.3 to 449.3 *m/z*). The inter-batch precision (expressed as relative standard deviation) of the assay was 8% for the medium and high QC material (300 and 750 ng/ml respectively) and 18% for the low QC material (30 ng/ml) over the study period. The accuracy of the assay (expressed as relative bias) was $< \pm 2\%$.

Cell isolation and flow cytometry analysis

To assess the kinetics of memory T cells in allograft skin, spleen and peripheral blood, samples were collected from 14 additionally transplanted animals on day 5 [untreated controls ($n = 5$) and systemic (i.p.) correolide C ($n = 5$)] and day 35 [correolide C + 30 days tacrolimus + ALS ($n = 4$)], respectively. Skin samples were stored in dispase (1 mg/ml, Roche Diagnostics GmbH, Vienna, Austria) overnight. After washing with complete medium, (10% FCS and 1% pen/strep in RPMI 1640; LONZA, Basel, Switzerland) skin samples were digested in specific collagenase D (1 mg/ml; Roche) for 1 h at 37 °C. Next, the cell suspension was strained through a Nylon cell strainer and washed twice. For lymphocyte isolation, the cell suspension was slowly placed on histopaque (histopaque 1083; Sigma-Aldrich Company Ltd, Irvine, Ayrshire, UK) and centrifuged at 800 rcf for 30 min at 20 °C without breaks. The white blood cell ring fraction was transferred into PBS, washed three times and used for flow cytometry analysis. The spleen was stored in complete medium overnight. The tissue was homogenized by straining through a Nylon cell strainer and then continued with the protocol described above. Heparinized peripheral blood was slowly placed on histopaque and continued with the protocol described

above. For three/four-color flow cytometry cell suspensions were incubated at 4 °C for 30 min with appropriate dilutions of directly labeled monoclonal rat/mouse antibodies against CD3 (PE, G4.18), CD4 (FITC, OX35), CD8a (PE-Cy7, OX8), CCR7 (APC, 4B12; all eBioscience) and CD45RC (PE, OX22; BD). Prior to CCR7 staining the FcgIIIR/FcgIIR was blocked with purified CD16/32 (eBioscience). Fluorescence intensity was analyzed on a FACS Calibur (BD) and data analysis was performed using CellQuestPro software.

Statistical analysis

Graft survival was assessed by Kaplan–Meier log rank survival analysis. Values are expressed as mean ± standard deviation (SD) or standard error of mean (SEM, correolide C plasma levels, facs data). Analysis of variance (ANOVA) was utilized to compare the differences between groups. Correction of multiple comparisons was performed according to Bonferroni. A P-value of <0.05 was considered statistically significant.

Results

Systemic administration of correolide C

Survival

Untreated animals presented with first visible signs of allograft rejection (appearance grading rejection grade I) after 3.33 ± 1.03 days, which progressed to rejection grade II on day 5.00 ± 0.89 (Fig. 1a). After 8.83 ± 0.98 days all limbs displayed epidermolysis (rejection grade III), which progressed to severe necrosis by day 10 (Fig. 1b). No difference was detected between untreated and placebo treated animals ($P = 0.894$). Systemic (i.p.) administration of correolide C resulted in statistically significant prolongation of limb allograft survival: 4/6 limbs did not show any signs of rejection by day 5 (Fig. 1c). Animals developed rejection grade II by day 9.20 ± 1.10, which further progressed to grade III on day 10.50 ± 1.38 (Fig. 1d, $P = 0.037$; Fig. 1e). However, when given i.m. correolide C did not prolong graft survival (rejection grade III after 8.40 ± 0.55 days). Table 2 shows the mean values of trough levels for correolide C after i.p. and i.m. injection. High correolide C trough levels correlated with prolonged limb allograft survival – animal 1 and 2 (i.p. correolide C) which had levels above 500 ng/ml revealed longest limb allograft survival.

No severe complications or side effects were observed after administration of systemic correolide C. Animals gained weight during the observation period and no abnormal behavior was recorded. 3/6 animals receiving i.p. injections of correolide C presented with signs of anemia, in fact the color of their eyes and ears was pale compared with the other animals. This phenomenon was not observed in any placebo-treated animals.

Histology

Histology of animals in the control group displayed severe changes in limb skin, muscle, vessels, subcutaneous tissue

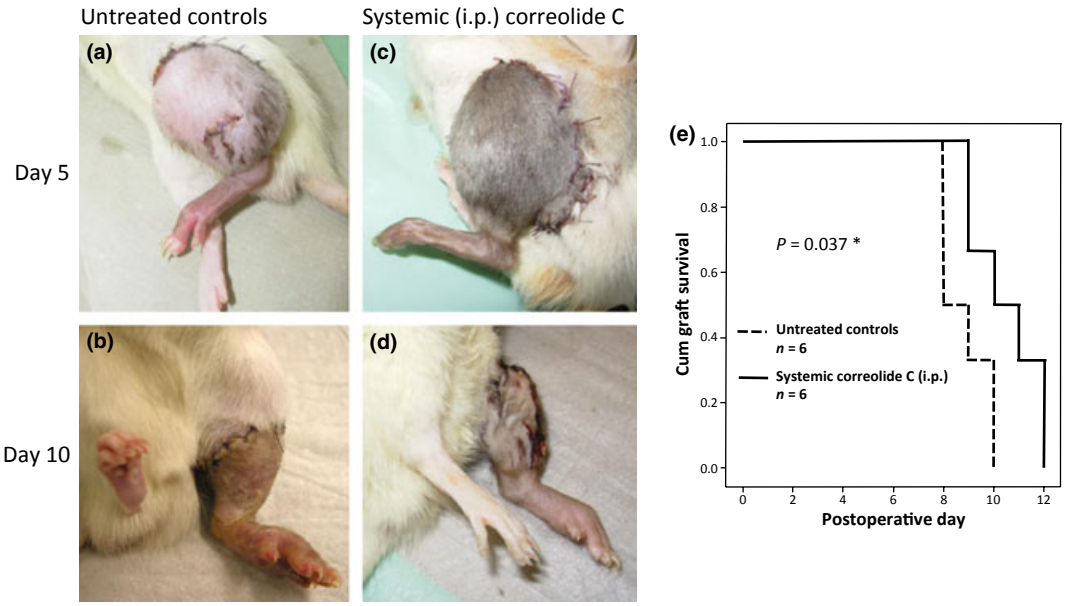

Figure 1 Systemic (i.p.) correolide C treatment – appearance of rejection on days 5 and 10 and graft survival. Untreated controls already showed mild signs of rejection on day 5 (erythema and mild edema, a) and severe epidermolysis and skin necrosis on day 10 (b). In contrast, 4/6 i.p. correolide C treated animals revealed unaffected limbs on day 5 (c; picture animal 2) and only mild to moderate signs of rejection on day 10 (d; picture animal 2, mild edema). Overall, systemic (i.p.) correolide C treatment resulted in significant prolongation of graft survival, compared with untreated controls (e).

Table 2. Trough plasma levels of correolide C after intraperitoneal (i.p.) and intramuscular (i.m.) injections.

		Correolide C (ng/ml)	
Animal	Days	Mean (ng/ml)	SEM (ng/ml)
Intraperitoneal (i.p.)			
1	5,10,15	559.4	295.6
2	5,10,14	539.8	319.7
3	5,10,12	104.5	40.4
4	5,9,14	33.5	8.0
5	3,8,13	41.7	9.8
6	6,11	55.8	2.2
Intramuscular (i.m.)			
1,2,3	5,10	59.9	31.2

and nerve on day 8.83 (mean, endpoint of the study – appearance rejection grade III). A dense cellular infiltrate was observed in both dermis and epidermis (Fig. 2a), together with epidermal vacuoles as signs of keratinocyte apoptosis and necrosis (Fig. 2b). The muscle revealed an intense infiltrate and myocyte necrosis. Infiltrating cells were also observed in the vessel walls and the perineural sheaths. Histopathologic changes of placebo treated animals were similar to untreated controls. 4/6 animals treated with i.p. injections of correolide C did not show any histopathologic signs of rejection in the skin on day 5. Skin biopsies of 2/6 animals revealed a mild perivascular cellular infiltrate at this point. On day 10, a mild diffuse dermal lymphocytic infiltrate was found in 2/6 animals (Fig. 2c). In addition, single vacuolized keratinocytes were observed in skin biopsies of another two animals with an otherwise intact epidermal layer (Fig. 2d). One animal demonstrated a dense lymphocytic infiltrate in the epidermis and dermis and another one presented with necrosis of the epidermis. After limb retrieval and sample collection at the endpoint, histological alterations of tissues were comparable to those found in limbs of controls. Immunohistochemistry of high-grade rejection samples from all groups identified only 5–20% of infiltrating cells as CD3+ T cells (Fig. 2e). The majority of infiltrating cells were identified as CD68+ macrophages (Fig. 2f). Histology of samples from animals treated with correolide C i.m. injections was indifferent to controls. However, in 2/5 animals abscesses were found in the medial muscle groups of the contralateral limb at the site of i.m. injection.

Local administration of correolide C

Survival

Animals treated with i.p. injections of tacrolimus for 30 days developed first evidence of rejection (appearance grading rejection grade I) on day 34 (mean 34.25 ± 0.96) and progressed to rejection grade III within 10 days after cessation of tacrolimus (mean day 40.50 ± 1.00, Fig. 3a).

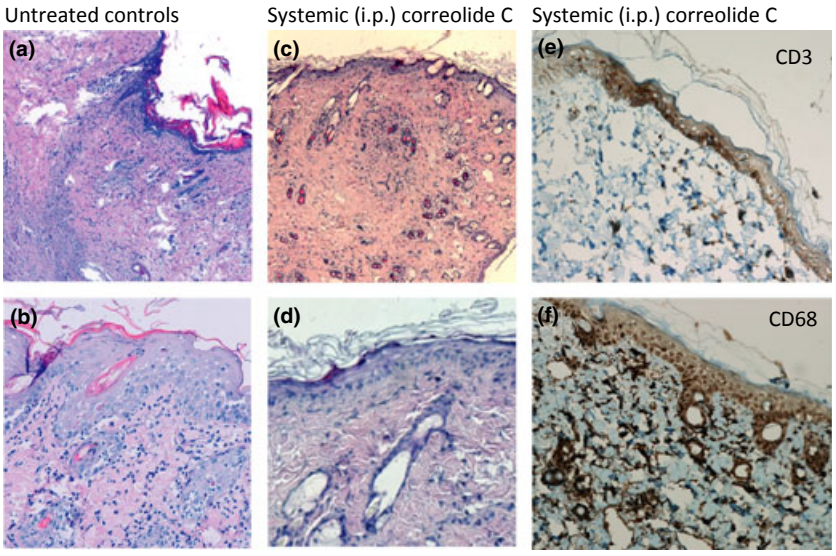

Figure 2 Histopathologic and immunohistochemical findings in skin biopsies of untreated controls and correolide C (i.p.) treated animals. On day 8 an intense infiltrate was found in the superficial and deep dermis of untreated control animals (a). Infiltrating cells were also found in the epidermis as well as single vacuolized necrotic keratinozytes (b). On day 10 only a mild dermal infiltrate was observed in 2/6 i.p. correolide treated animals without affecting the epidermis (c; picture animal 2). Another 2/6 animals revealed single vacuolized keratinocytes with an otherwise intact epidermal layer (d; picture animal 6). Immunohistochemical staining for CD3 and CD68 demonstrated that only 5–20% of infiltrating cells were positive for CD3 (T lymphocytes, e). The cellular infiltrate was dominated by CD68+ macrophages in all groups (f; pictures i.p. correolide C treated animal, day 10). CD3+ and CD68+ cells were not only found scattered in the dermis, but single cells also migrated to the epidermis.

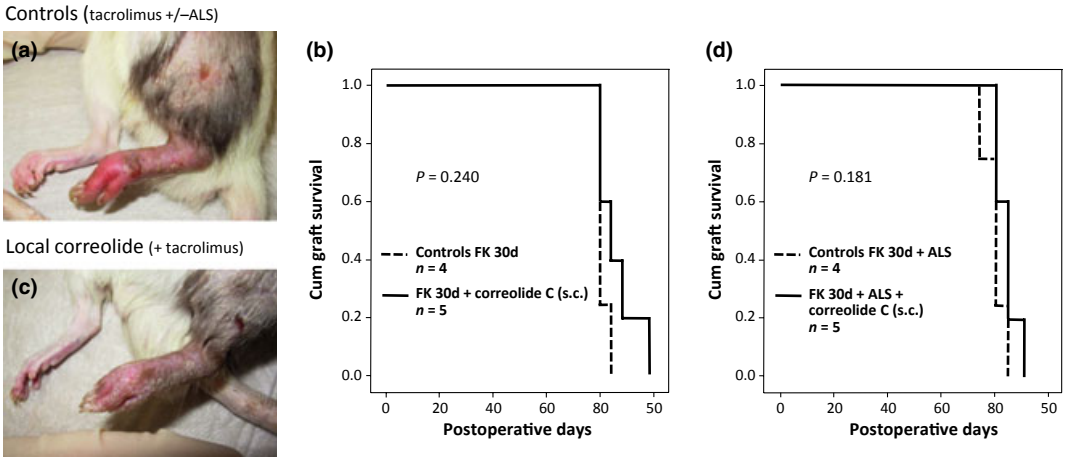

Figure 3 Local (s.c.) correolide C treatment – appearance of rejection on day 40 and graft survival. Controls (tacrolimus 30 days ± induction with ALS) showed epidermolysis and severe erythema by day 40 (a). Additional treatment with intragraft (s.c.) injections of correolide C resulted in a slight delay of the rejection process (b). One animal only revealed mild erythema on day 40 (c) and the allograft survived until day 49. Local intragraft administration of correolide C together with a short course of tacrolimus and induction with ALS also resulted in slight, but statistically insignificant prolongation of limb allograft survival (d).

Additional treatment with local correolide C resulted in slight, but statistically insignificant prolongation of graft survival (appearance grading rejection grade III: mean day 43.00 ± 3.74; $P = 0.240$, Fig. 3b). Rejection was first observed on day 37.2 ± 2.17, signs of rejection grade II appeared by day 40.00 ± 2.74 and progressed to grade III within the following 3 days. One animal only showed mild erythema on day 40 (Fig. 3c) and did not show signs of epidermolysis until day 49. Local correolide C injections in combination with stepwise reduction in systemic tacrolimus (0.30 mg/kg until day 30, 0.10 mg/kg until day 50) resulted in rapid allograft rejection after day 50. All animals developed rejection grade III by day 55.

Anti-lymphocyte serum given on days 0 and 3 in addition to daily tacrolimus did not affect the outcome and graft survival was not different to animals receiving tacrolimus only (mean day 39.7 ± 2.06). In combination with local correolide C treatment, a minor prolongation of graft survival could be observed (mean day 41.80 ± 2.05, $P = 0.181$, Fig. 3d). Correolide C plasma levels were below quantification level (<10.0 ng/ml) between days 30 and 60 after s.c. application. No side effects as a result of local correolide C treatment were observed.

Histology

Biopsies of animals receiving tacrolimus showed normal skin without evidence of rejection until day 30. Typical histopathologic signs of high-grade rejection were observed by day 40 (Fig. 4a). When correolide C was administered locally in addition to tacrolimus, in 3/5 animals a dense inflammatory infiltrate was observed in the epidermis, dermis and in underlying tissues by day 40 (Fig. 4b) as well as

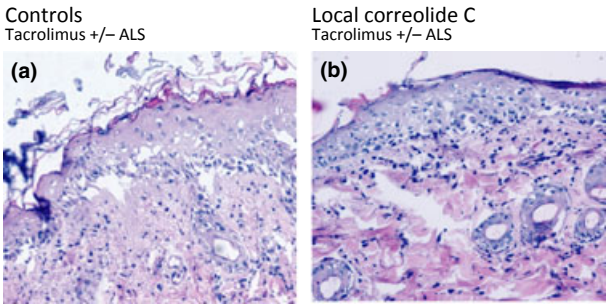

Figure 4 Histopathologic findings in skin biopsies of controls and local (s.c) correolide C treated animals on day 40. After 30 days of tacrolimus treatment ± induction with ALS a cellular infiltrate was observed in the dermis, epidermis and along the dermal-epidermal interface together with signs of dermal-epidermal separation on day 40 (a). Animals additionally treated with intragraft (s.c.) injections of correolide C revealed a dermal cell infiltrate also affecting the dermal-epidermal interface (b). However, no dermal-epidermal separation was observed by then.

vacuolization and necrosis of keratinocytes. 5–30% of the infiltrating cells stained positive for CD3. Again, the infiltrate was dominated by CD68+ macrophages. No difference regarding the composition of the infiltrate was observed between the local correolide C treatment and control group. Two animals were free of rejection as per histology of the skin until day 40 and only showed a mild cellular infiltrate on day 45. However, on day 50 both limbs showed massive cell infiltration in all layers of various tissues, vasculopathy and necrosis. Between days 30 and 50 a mild diffuse dermal infiltrate was detected in skin biopsies of the tacrolimus-weaning group. By day 55 also the epidermis was affected by infiltrating lymphocytes.

Histology of limbs from animals treated with tacrolimus + induction with ALS and tacrolimus + ALS + local correolide C did not differ from animals treated with tacrolimus only.

Tacrolimus blood levels

When given at a dose of 0.30 mg/kg/30 days, tacrolimus whole blood trough level on POD 30 was 3.2 ± 0.8 ng/ml. In animals treated with low-dose tacrolimus until day 50 (0.10 mg/kg/20 days) whole blood trough levels were 1.4 ± 0.6 ng/ml on day 50. Five days after cessation, tacrolimus levels were below quantification level (<0.6 ng/ml) in all animals.

Single dose pharmacokinetics of i.p. correolide C

After i.p. injection of 5 mg/kg correolide C peak levels after 1 hour in two rats were 344.8 ng/ml and 315.3 ng/ml, respectively. To determine plasma elimination of correolide C i.p. in these two animals, the linear regression analysis of the ln –transformed data yielded elimination constants of 0.181 ($R^2 = 0.9065$) and 0.152 ($R^2 = 0.9356$) per hour, respectively. Thus, the estimated elimination half-lives are 3.9–4.6 h.

Phenotype of T cells and presence of memory T cells

To identify the phenotype of skin allograft infiltrating T cells, a flow cytometry analysis of mononuclear cell suspensions from the skin, blood and spleen of correolide C treated animals (i.p. and local injections) and untreated controls was performed. After i.p. correolide C treatment 44.70 ± 1.94% of CD3+ T cells showed a CD4 and 47.40 ± 7.67% a CD8 phenotype on day 5. In untreated controls 55.52 ± 3.82% were identified as CD4+ and 44.06 ± 3.86% as CD8+ T cells, CD3+. Assessment of the Tem phenotype (negative for the leukocyte common antigen CD45RC and the lymph node homing receptor CCR7 [21]) within the subsets of CD4+ and CD8+ T cells in allograft skin demonstrated a decrease in Tem after i.p. correolide C treatment on day 5, compared with untreated controls (CD4 Tem 27.35 ± 5.49% and CD8 Tem 19.84 ± 3.83% vs CD4 Tem 38.38 ± 2.76% and CD8 Tem 25.31 ± 1.91%, Fig. 5a). Also, in the peripheral blood and the spleen the percentage of Tem was decreased for the CD4+ and CD8+ T cell populations after correolide C treatment compared with untreated controls (peripheral blood: correolide C 8.65 ± 1.75% and 5.54 ± 2.13% vs. controls 14.94 ± 4.36% and 6.27 ± 2.014%, Fig. 5b; spleen: correolide C: 28.43 ± 7.58% and 14.73 ± 4.18% vs. controls: 37.81 ± 1.27% and 18.35 ± 4.87%, Fig. 5c). When compared with controls, local (s.c.) correolide C treatment resulted in reduction in Tem in allograft skin (CD4 Tem 26.61 ± 2.67% and CD8 Tem 13.99 ± 2.81%), peripheral blood (CD4 Tem 10.96 ± 3.78% and CD8 Tem 5.38 ± 2.58%) and spleen (CD4 Tem 10.59 ± 1.13% and CD8 Tem 9.78 ± 3.43%) on day 35 (Fig. 5a–c).

The central memory T cell (Tcm) population (CD45RC-CCR7+ [21,36]) was increased in graft infiltrating CD4+ and CD8+ T cells after i.p. and local correolide C treatment as compared with controls (Fig. 5d). In the peripheral blood, a decreased CD4+ Tcm population after i.p. correolide C treatment and CD8+ Tcm population after local correolide C treatment was observed (Fig. 5e). In the speen, the percentage of CD4+ and CD8+ Tcm was increased after i.p. correolide C injections, but not after local treatment (Fig. 5f).

Discussion

The Kv1.3 voltage-gated potassium channel is critically involved in the counterbalance of calcium influx at T cell receptor activation and effector function [37]. Thus, Kv1.3 regulates the membrane potential and is expressed four- to fivefold in activated CD4+ and CD8+ Tem, compared with inactivated T cells [20,21,24]. Previous studies demonstrated an increased number of Kv1.3+ infiltrating disease-associated autoreactive T cells in type 1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis and psoriasis [22,38]. Based on these findings, the Kv1.3 channel was introduced as a novel target for treatment of autoimmune diseases with the aim to selectively suppress Tem without affecting function and proliferation of naïve T cells and Tcm.

In an attempt to identify novel agents suitable to prevent T cell activation and limit the need for calcineurin inhibitors for immunosuppression in transplantation, we investigated the effect of a Kv1.3 channel blocker, correolide C, on skin rejection in a rat VCA model. In this study, monotherapy with a Kv1.3 channel blocker administered i.p. resulted in moderate prolongation of limb allograft survival. Progression to rejection grade II was delayed from day 5 (controls) to day 9.2 (correolide C i.p. treated animals). Most interestingly, the two animals, which revealed very high plasma levels (up to 1100 ng/ml) after i.p. injection showed the most prolonged graft survival (grade III on day 12), whereas four other i.p. treated animals with levels between 23.5 and 175 ng/ml demonstrated less effect. Our findings therefore suggest a dose-effect correlation and an effect on allograft survival. Our data reveal that there is a plasma concentration dependency of correolide C to delay rejection, but they do not allow determination of the threshold level required. Based on extrapolations from the existing data, it is assumed that the target range may be above 200–300 ng/ml (about 22–33 nM). Extensive PK-analysis (including AUC) remain to be performed and the esti-

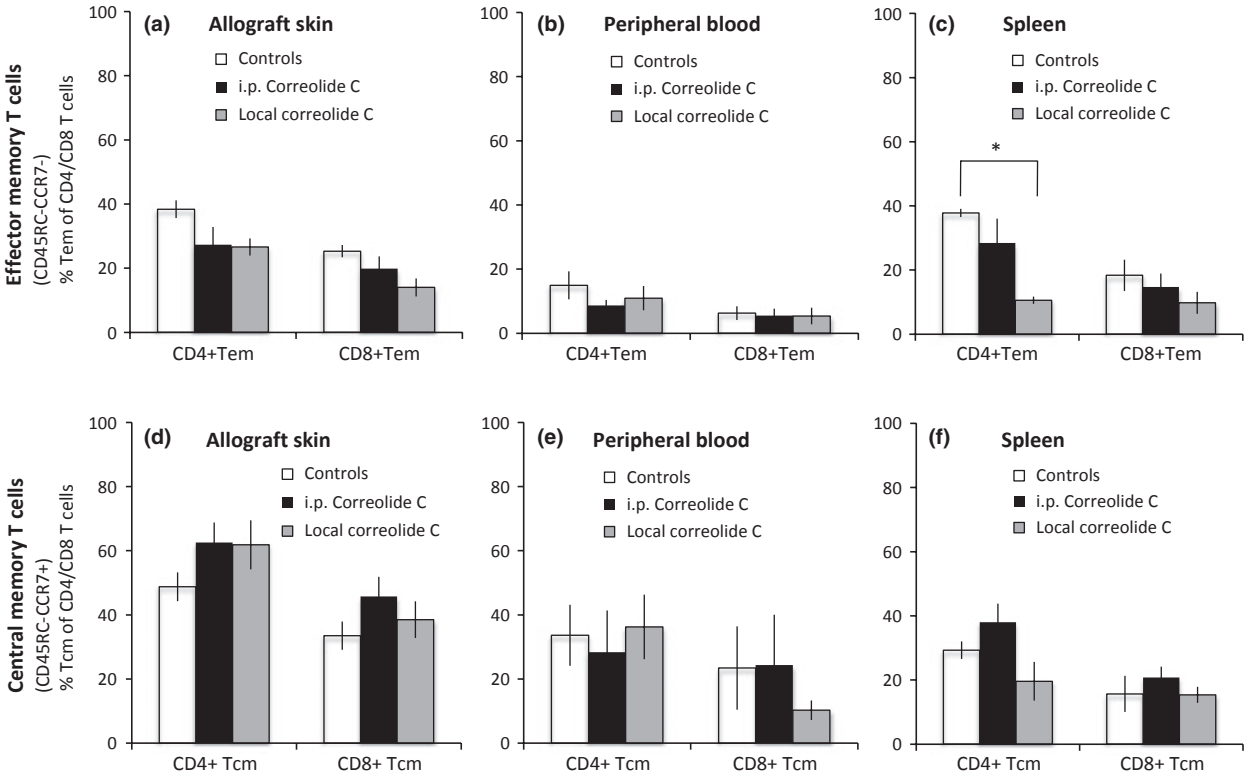

Figure 5 Presence of effector memory (Tem, CD45RC-CCR7-) and central memory (Tcm, CD45RC-CCR7+) T cells within the CD4+ and CD8+ T cell subsets. Flow cytometry analysis of mononuclear cell suspensions prepared from allograft skin of correolide C treated animals (i.p. injections: $n = 5$; local injections: $n = 4$) and untreated controls ($n = 5$) demonstrate that the proportion of Tem was diminished after i.p. and local correolide C treatment for both, the CD4+ and CD8+ T cell phenotype (a). In peripheral blood (b) and the recipients' spleen (c) the percentage of Tem was also found to be decreased for the CD4+ and CD8+ T cell populations after i.p. and local correolide C treatment. The proportion of Tcm was increased for the CD4+ and CD8+ T cell phenotype in allograft skin after i.p. and local correolide C treatment (d). A decreased CD4+ Tcm population after i.p. correolide C treatment and CD8+ Tcm population after local correolide C treatment was observed in the peripheral blood (e). In the spleen, CD4+ and CD8+ Tcm were increased after i.p. correolide C injections, but not after local treatment (f).

mated half-life of about 4–5 h suggests that a dosing interval of 12 h for i.p. injections with correolide C in this rat limb transplant model may exhibit a more profound effect. I.m. application exhibited mean trough levels of about 60 ng/ml not exceeding 160 ng/ml and did not show any biological effect.

Subcutaneous drug application resulted in slight, but insignificant prolongation of limb allograft survival in the rat model. Plasma levels never exceeded the detection level of the LC-MS/MS assay (10 ng/ml). The doses and administration intervals for s.c. were based on tolerable volumes of adhesion molecule blockers performed by our working group in a similar setting ([31] and unpublished data). The data obtained from the groups receiving local correolide C treatment emphasize that the administration intervals of local correolide C (3 mg/kg twice/week) may not be sufficient as only slight prolongation of allograft survival was achieved. Moreover, a higher dosage was selected for the systemic injections (5 mg/kg) compared with the local injections (3 mg/kg).

Encouraging results have been shown using Kv1.3 blockers in models of skin inflammation. Systemically administered margatoxin revealed a 65% inhibition of the induration of the immune response in a minis wine model of DTH [28]. Matheu *et al.* [39] also demonstrated inhibition of DTH responses and suppression of antigen-specific proliferation of Tem by systemic Kv1.3 blockade with Shk-183 (Stichodactyla helianthus neurotoxin). Local targeting of Tem with intradermal injections of ShK has been shown to be effective in a psoriasiform SCID mouse model by Gilhar *et al.* [38]. Efficacy of topical administration of a small-molecule blocker of Kv1.3, PAP-1, was also demonstrated by Azam *et al.* [23] in a rat model of allergic contact dermatitis (ACD). PAP-1 treatment decreased infiltration of CD8+ T cells and production of the inflammatory cytokines IFN-g, IL-2 and IL-17, when compared with vehicle treatment. However, PAP-1 selectively affected the antigen-specific Tem responses but did not suppress mast cell-mediated and unspecific inflammation. Interestingly, Kv1.3 blockade with PAP-1 had no effect on TNF-a production

[23]. ShK, however, inhibited expression of IFN-g and TNF-a in the graft [38], but failed to induce complete recovery of disease or complete suppression of the inflammatory response. The authors explain this observation by the fact that TNF-a and a battery of other proinflammatory cytokines can also be produced by other cell populations present in the skin, such as keratinocytes, Langerhans cells and mast cells [40,41]. So far there is no evidence that these cell populations are affected by Kv1.3 inhibitors.

Further to the suboptimal dosing interval discussed elsewhere in the article this may help to understand why correolide C did ameliorate, but not abrogate the alloimmune response in our model. Based on our flow cytometry findings showing a decrease in the proportion of Tem within the CD4+ and CD8+ T cell populations after correolide C i.p. treatment in allograft skin, peripheral blood and spleen on POD 5 (mild stage of rejection), we suggest that correolide C has an effect on Tem in the setting analyzed here. This effect was also observed after local treatment of mild rejection in allograft skin (day 35). However, immunohistochemistry revealed a high number of macrophages in advanced stages of skin rejection. As macrophages are not affected by Kv1.3 blockers it might be speculated that this cell type together with other immunocompetent cells (cytotoxic T cells, NK cells, mast cells, Langerhans cells) might limit the effect of correolide C alone.

In summary, the results of this study clearly demonstrate prolongation of allograft survival after rat hind-limb transplantation, when a Kv1.3 inhibitor was given systemically as monotherapy. However, its efficacy seems to be critically depending on high systemic drug concentrations and adequate dosing intervals to keep plasma levels above a threshold. Moreover, the effect of a Kv1.3 blocker alone might not be sufficient to completely abrogate rejection when given as monotherapy systemically or as topical agent after only a short initial course of systemic immunosuppression. Our findings strongly suggest that the Kv1.3 channel is a novel target worth exploring in more detail to eliminate conventional immunosuppression in VCA.

Authorship

TH: designed research, performed research, analyzed data, wrote the article. CK: performed research, analyzed data. JG: performed research, analyzed data. BZ: analyzed histology and IHC. TH: contributed to sample collection. CS: LC-MS/MS method development and measurement of correolide C EDTA-plasma levels and tacrolimus whole blood levels. NE: performed cell isolation and facs analysis. CW: performed research. FM: contributed to sample collection. KK: contributed to cell isolation method design and oversaw facs analysis. AG: contributed to LC-MS/MS method design, data interpretation and discussion. GB: contributed to review of data and data interpretation. WPAL: helped with data interpretation and discussion. RM: contributed to research design and data interpretation. JP: contributed to interpretation and discussion of results. HG: contributed to research design, provided correolide C, expertise in pharmacokinetics and supervision. SS: designed research, oversaw the experiments, wrote the article.

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