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BRIEF COMMUNICATION

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Subcutaneous administration of a neutralizing IL-1β antibody prolongs limb allograft survival

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Cytokine-expression profiles revealed IL-1ß highly upregulated in rejecting skin of limb allografts. We investigate the effect of intragraft treatment with a neutralizing IL-1β antibody in limb transplantation. Following allogenic hind-limb transplantation, Lewis rats were either left untreated[1] or treated with anti-lymphocyte serum + tacrolimus (baseline)[2]; baseline immunosuppression + anti-IL-1β (1 mg/kg once/week, 6-8 subcutaneous injections) into the transplanted[3] or contralateral[4] limb. Endpoint was rejection grade III or day 100. Graft rejection was assessed by histology, immunohistochemistry, flow cytometry phenotyping of immune cells, and monitoring cytokine expression. Anti-IL-1β injections into the allograft or contralateral limb resulted in a significant delay of rejection onset (controls: 58.60 ± 0.60; group 3: 75.80 ± 10.87, $P = .044$; group 4: 73.00 ± 6.49, $P = .008$) and prolongation of graft survival (controls: 64.60 ± 0.87; group 3: 86.60 ± 5.33, $P = .002$; group 4: 93.20 ± 3.82, $P = .002$), compared to controls. Although the phenotype of the graft infiltrating immune cells did not differ between groups, significantly decreased skin protein levels of IL-1β, IL-4, IL-13, IP-10, MCP-1, and MCP-3 in long-term-survivors indicate an overall decrease of chemoattraction and infiltration of immune cells as the immunosuppressive mechanism of anti-IL-1β. Inhibition of IL-1β with short-term systemic immunosuppression prolongs limb allograft survival and represents a promising target for immunosuppression in extremity transplantation.

KEYWORDS
basic (laboratory) research/science, immunosuppressant, rejection: acute, rejection: T cell-mediated (TCMR), vascularized composite and reconstructive transplantation

Abbreviations: ALS, anti-lymphocyte serum; BN, Brown Norway rat; CT, comparative threshold cycle; CTLA-4, cytotoxic T lymphocyte-associated protein-4; H&E, hematoxylin & eosin; IFN-γ, interferon-gamma; ip, intraperitoneal; IHC, immunohistochemistry; IS, immunosuppression; LEW, Lewis rat; POD, postoperative day; RTqPCR, quantitative real-time polymerase chain reaction; sc, subcutaneous; SEM, standard error of the mean; TNF-α, tumor necrosis factor-alpha; VCA, vascularized composite tissue allograft/vascularized composite tissue allotransplantation.

Theresa Hautz and Johanna Grahammer contributed equally to this work.

1 | INTRODUCTION

With more than 150 performed cases of vascularized composite tissue allotransplantation (VCA) worldwide,[1] the skin has come into focus, both as a site of immune reaction,[2] and also as a target for therapeutic intervention. Exploring novel anti-skin rejection therapies would fulfill an important clinical need: reducing the recipient's exposure to chronic, systemic immunosuppression (IS).

A recent study investigating cytokine expression in the skin of VCAs has shown highly upregulated IL-1β levels during allograft rejection.[3,4] IL-1β constitutes a proinflammatory signal inducing T cell infiltration, memory CD4 + T cell activation, IL-6 expression, and Th-17 differentiation.[5,6] Blockers of IL-1 and IL-1β show significant effects in patients with autoinflammatory syndromes and have

TABLE 1 Experimental groups

Group	Systemic treatment	Local treatment	Number
1	No treatment	No	5
2	ALS 0.5 mL, days 0 + 3 and tacrolimus 0.3 mg/kg daily, ip, until day 50	No	5
3	ALS 0.5 mL, days 0 + 3 and tacrolimus 0.3 mg/kg daily, ip, until day 50	anti-IL-1β 1 mg/kg weekly, sc, into transplanted limb, days 35-100	5
4	ALS 0.5 mL, days 0 + 3 and tacrolimus 0.3 mg/kg daily, ip, until day 50	anti-IL-1β 1 mg/kg weekly, sc, into contralateral, nontransplanted limb, days 35-100	5

ip, intraperitoneal; sc, subcutaneous.

TABLE 2 Macroscopic rejection scoring

Grade	Macroscopic alterations
0	No signs of rejection
I	Erythema
II	Erythema and edema
III	Epidermolysis
IV	Mummification and necrosis

TABLE 3 Histopathologic rejection scoring for allograft skin (according to the Banff guidelines, 13) and muscle

Grade	Histopathologic alterations
Skin	
0	No signs of rejection
I	Perivascular dermal cell infiltrate
II	Diffuse dermal cell infiltrate, interface reaction, sporadic cell infiltration of epidermis
III	Moderate to severe cell infiltration of epidermis, epidermal cell necrosis
IV	Major epidermal necrosis, loss of epidermis
Muscle	
0	No signs of rejection
I	Mild, localized perivascular cell infiltrate
II	Diffuse cell infiltrate
III	Localized muscle cell necrosis and vasculopathy
IV	Major necrosis

TABLE 4 Rejection onset (=macroscopic grade I rejection) and graft survival (=progressive grade III rejection or POD 100) in experimental groups and individual animals

Group	Animal	Rejection onset	Graft survival	Category
1	1	POD 4	POD 8	
1	2	POD 4	POD 8	
1	3	POD 4	POD 7	
1	4	POD 4	POD 7	
1	5	POD 4	POD 8	
1	**Mean**	**POD 4.00 ± 0.00**	**POD 7.60 ± 0.26**	
2	1	POD 60	POD 64	
2	2	POD 58	POD 64	
2	3	POD 60	POD 68	
2	4	POD 58	POD 63	
2	5	POD 57	POD 64	
2	**Mean**	**POD 58.60 ± 0.60**	**POD 64.60 ± 0.87**	
3	1	POD 70	POD 82	Responder
3	2	POD 75	POD 82	Responder
3	3	no	POD 100	Long-term survivor
3	4	no	POD 100	Long-term survivor
3	5	POD 34	POD 69	
3	**mean**	**POD 75.80 ± 10.87**	**POD 86.60 ± 5.33**	
4	1	no	POD 100	Long-term survivor
4	2	POD 65	POD 86	Responder
4	3	POD 64	POD 100	Long-term survivor
4	4	POD 60	POD 100	Long-term survivor
4	5	POD 76	POD 80	Responder
4	**mean**	**POD 73.00 ± 6.49**	**POD 93.20 ± 3.82**	

Responder: delayed rejection onset and progression of rejection.
Long-term-survivor: animal reaches endpoint POD 100 macroscopically rejection-free or with rejection grade I or II.

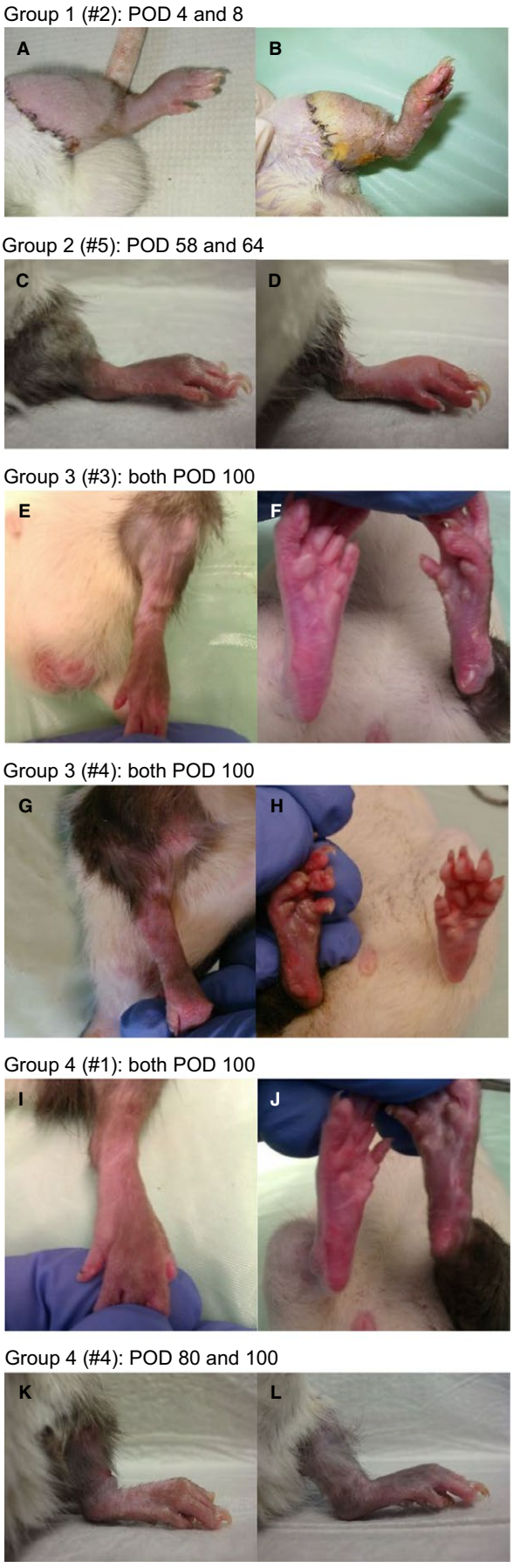


FIGURE 1 Macroscopic signs of allograft rejection and survival in transplanted rat hind-limbs. (A, B) Untreated controls (group 1, animal 2 exemplarily shown) already presented with edema and erythema (grade II) on POD 4 (A), which progressed to severe edema and epidermolysis (grade III) on POD 8 (B). (C, D) Animals treated with baseline IS (50 days tacrolimus+ALS, group 2, animal 5 exemplarily shown) developed erythema as a first sign of rejection (grade I) on POD 58 (C). On POD 64, severe edema and erythema together with hair loss and first signs of epidermolysis (grade III) were observed mainly at the forefoot of the limb allograft (D). (E-H) Two of 5 animals that were additionally treated with subcutaneous intragraft injections of anti-IL-1β (group 3) once a week were still rejection free on POD 100. Animals 3 (E, F) and 4 (G, H) showed neither erythema nor edema by then. Both BN forefoots were still hairy; hair loss was observed around the skin biopsy sampling sites on the BN thigh only (E, G). Even after 100 days, the skin of the foot soles was comparable to nontransplanted LEW foot soles and without desquamation and dryness (F, H). (I, J) One of 5 animals that were additionally treated with subcutaneous anti-IL-1β injections into the contralateral limb (group 4, animal 1) were free of macroscopic signs of allograft rejection on POD 100. (K, L) Another 2 of 5 animals in this group (animal 4 exemplarily shown) presented with mild rejection (erythema and mild edema on the BN forefoots) between POD 65 and 100 (K – POD 80, L – POD 100) [Color figure can be viewed at wileyonlinelibrary.com]

already been approved for treatment.[7-9] Disrupting IL-1β function is therefore expected to decrease skin rejection in VCA.

Here we tested the effect of an IL-1β blocking antibody on graft survival, rejection, cell infiltration, immune phenotype, and cytokine expression in an experimental rat hind-limb transplant model.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Brown Norway rats (BN) served as donors and Lewis rats (LEW) as recipients (Charles River, 200-250 g), representing a full MHC mismatch model in transplantation. Animals were housed under standard conditions with access to chow and water ad libitum. Experimental protocols were approved by the Austrian Federal Ministry of Science/Research.

2.2 | Experimental design

After orthotopic allogenic rat hind-limb transplantation, animals were treated as follows (Table 1): no immunosuppression (IS; group 1, n = 5); baseline IS with anti-lymphocyte-serum (ALS, Accurate Chemical&Scientific Corporation; 0.5 mL days 0 + 3 intraperitoneal [ip]) and tacrolimus (Prograf, Astellas; 0.30 mg/kg/day until day 50 ip; group 2, n = 5); baseline IS (see group 2) combined with a low-endotoxin, acid-free-purified anti-mouse/rat IL-1β monoclonal antibody (Clone B122, BioLegend; 1 mg/kg/week), administered subcutaneously (sc) into the transplanted limb (group 3, n = 5); or contralateral limb (group 4, n = 5). The immunosuppressive regimen was designed to overcome the immediate inflammation in response to ischemia/reperfusion and prevent an early and aggressive acute rejection. This IS regimen has been proven to be suitable in establishing

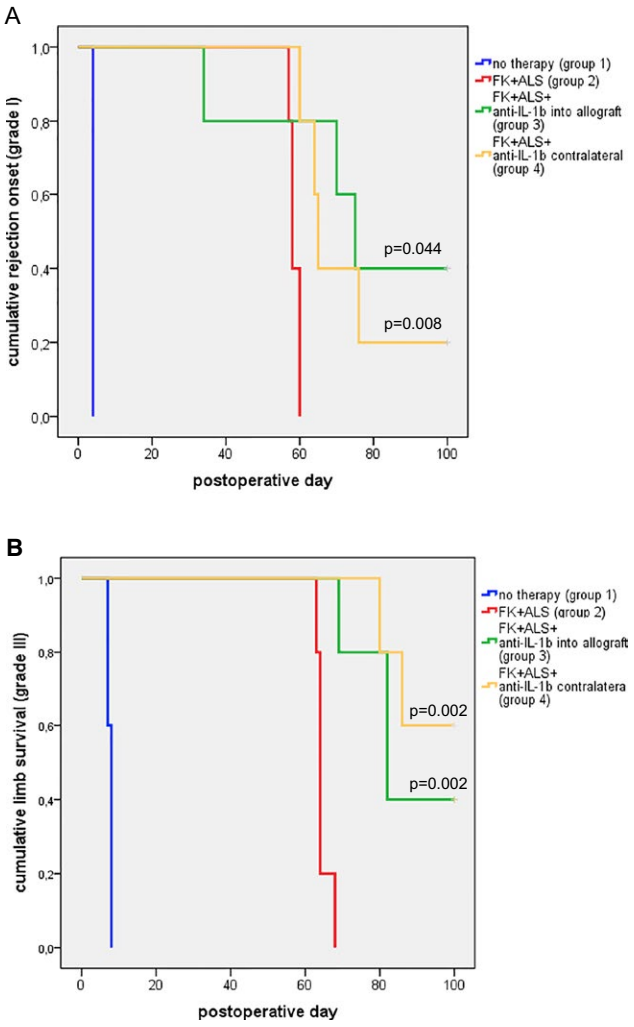


FIGURE 2 Cumulative rejection onset (A, grade I) and limb allograft survival (B, progressive grade III rejection or POD 100). Rejection onset was significantly delayed when anti-IL-1β was either given subcutaneous intragraft (group 3) or subcutaneously into the contralateral, nontransplanted limb (group 4), compared to the control group 2 (mean POD control group 2: 58.60 ± 0.60; group 3: 75.80 ± 10.87, *P* = .044; group 4: 73.00 ± 6.49, *P* = .008; A). Three of 10 animals of the anti-IL-1β treatment groups did not even develop macroscopic mild rejection until POD 100. In addition, limb allograft survival was significantly prolonged in both groups receiving weekly subcutaneous anti-IL-1β injections (groups 3 + 4), compared to control group 2 (mean POD control group 2: 64.60 ± 0.87; group 3: 86.60 ± 5.33, *P* = .002; group 4: 93.20 ± 3.82, *P* = .002; B). Five of 10 animals of groups 3 and 4 did not develop grade III rejection or were rejection-free on POD 100. Overall, 9/10 animals responded positively to subcutaneous anti-IL-1β treatment. Untreated animals started to macroscopically reject their limbs on POD 4.00 ± 0.00 (A) and displayed macroscopic grade III rejection on POD 7.60 ± 0.26 (B) [Color figure can be viewed at wileyonlinelibrary.com]

the environment for testing the effect of intragraft targeted therapy in limb transplantation.[10] Anti-IL-1β treatment was initiated on postoperative day (POD) 35, prior to weaning and cessation of tacrolimus therapy on POD 50 and continued once/week until POD 100. Tacrolimus blood trough levels have been shown to be below

TABLE 5 Histopathologic rejection grades of allograft skin and muscle of individual animals at the study endpoint (= macroscopic progressive grade III rejection or POD 100)

Group	Animal	Rejection grade skin	Rejection grade muscle	Category
1	1	IV	IV	
1	2	IV	IV	
1	3	IV	III	
1	4	IV	III	
1	5	IV	IV	
2	1	II	III	
2	2	III	III	
2	3	III	III	
2	4	III	0	
2	5	III	III	
3	1	II	III	Responder
3	2	II	II	Responder
3	3	I	I	Long-term survivor
3	4	I	I	Long-term survivor
3	5	III	III	
4	1	0	I	Long-term survivor
4	2	I	II	Responder
4	3	II	II	Long-term survivor
4	4	I	II	Long-term survivor
4	5	II	I	Responder

Responder: delayed rejection onset and progression of rejection.
Long-term survivor: animal reaches endpoint POD 100 macroscopically rejection-free or with rejection grade I or II.

detection limits at 5 days after cessation.[11] The antibody was delivered and equally dispensed in the subcutaneous compartment of the graft by 6-8 individual injections using a 27-gauge needle, distributed over the allograft/contralateral limb including the thigh, dorsum, and planta pedis.

2.3 | Surgical procedure

Anesthesia was performed with isoflurane inhalation anesthesia (3-4% for induction, 0.5-1.5% for maintenance). In addition, midazolam (Dormicum, 2.0 mg/kg), medetomidine (Domitor, 0.15 mg/kg), and fentanyl (Fentanyl-Janssen, 0.005 mg/kg) were given. Surgical details have been described previously.[12] Postoperative analgesia included buprenorphine (0.1 mg/kg) and carprofen (4.0 mg/kg) twice/day until POD 5 and 7, respectively. Grafts were monitored daily for macroscopic signs of rejection (Table 2). On POD 58, animals were anesthetized with isoflurane inhalation and a skin biopsy (5 × 5 mm) was collected from the allograft thigh for

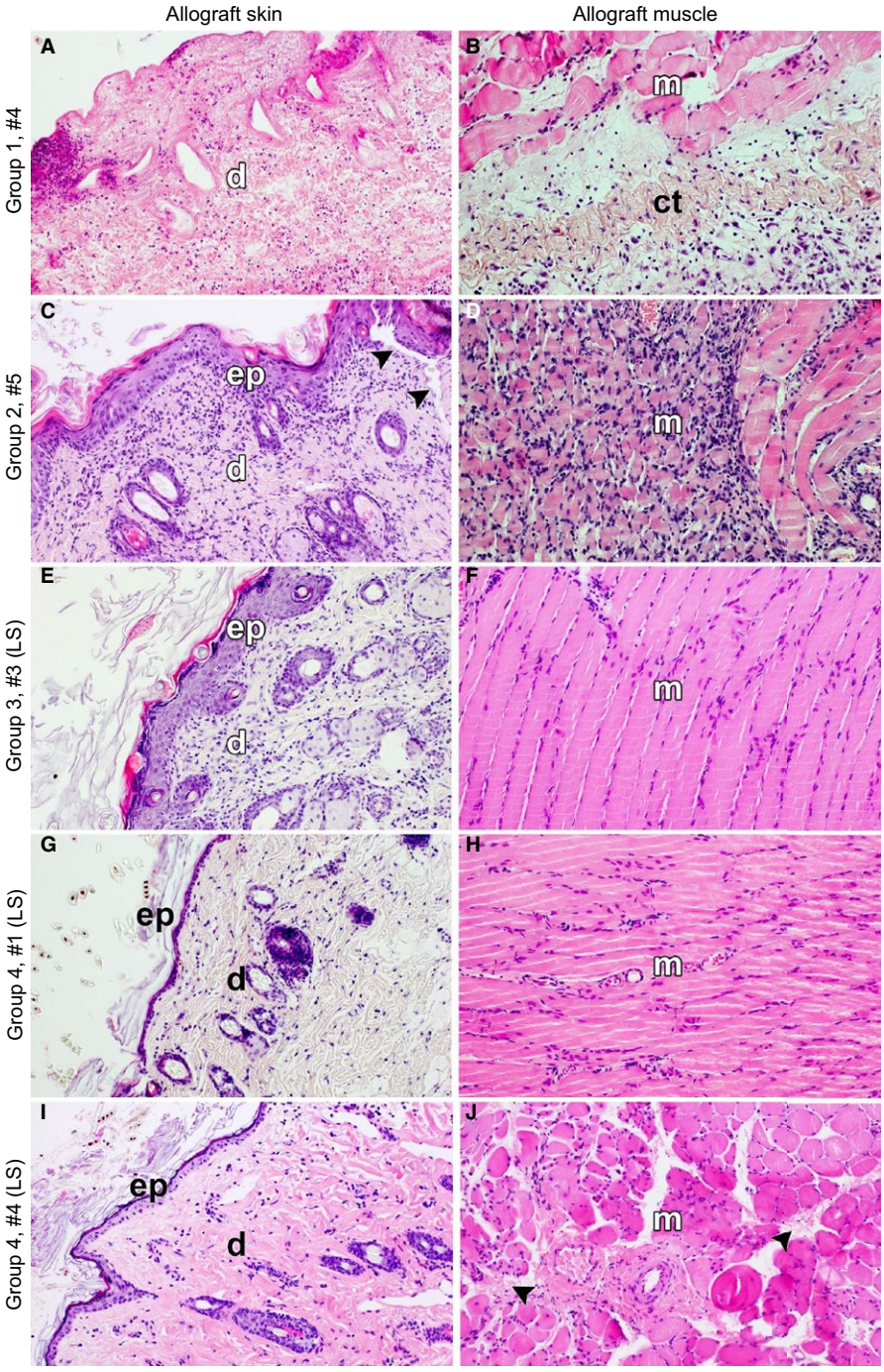
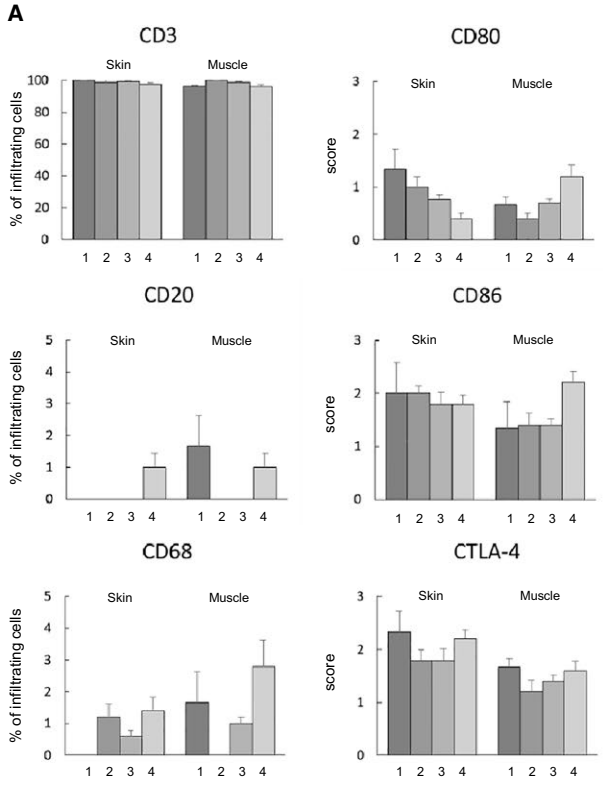


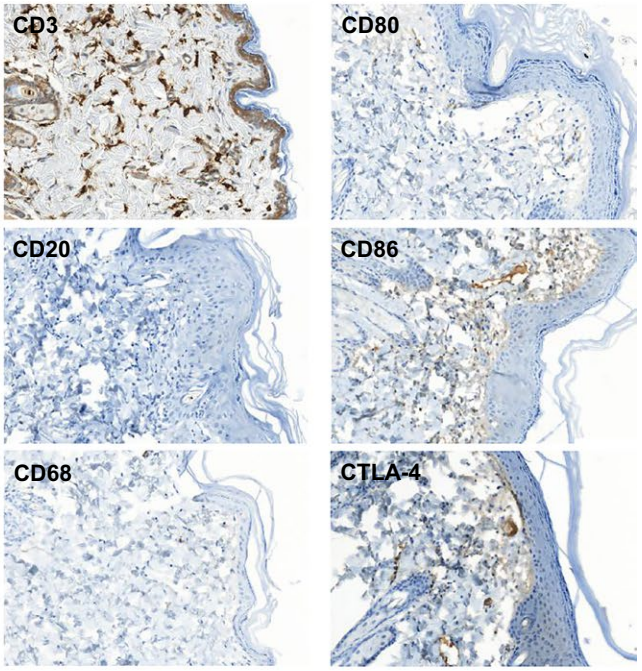
FIGURE 3 Histopathologic observations in allograft skin (A, C, E, G, I) and the underlying muscle (B, D, F, H, J) collected at the study endpoint. In untreated animals (group 1), the epidermis (ep) and adjacent dermis were completely necrotic (d) as a sign of massive rejection on POD 8 (A). In underlying muscle (m), necrotic tissue has partially been replaced by connective tissue (ct) (B, animal #4 exemplarily shown in A and B). Group 2 animals revealed severe cell infiltration in the dermis (d), at the dermal-epidermal interface, and in the epidermis (ep) as well as the beginning of epidermolysis (arrowheads) at the study endpoint (=macroscopic grade III rejection) (C). In the muscle (m) severe cell infiltration together with a loss in tissue architecture were observed (D, animal 5 exemplarily shown in C and D). One long-term survivor of group 3 (animal 3) showed a mild dermal cell infiltrate in allograft skin on POD 100 with an otherwise intact epidermal layer (E). Also, in allograft muscle (m), a mild cell infiltrate was observed, mainly located in the perivascular areas(F). One of 3 long-term survivors of group 4 (animal 1) did not show histopathologic signs of rejection in allograft skin (G) and only a mild cell infiltrate in allograft muscle (m) on POD 100 (H). Another long-term survivor of this group (animal 4) displayed mild perivascular dermal cell infiltration in the allograft skin on POD 100 (I), whereas the underlying muscle (m) was more severely affected including cell infiltration, muscle fiber shrinkage, and evidence of early fibrosis (arrowheads) (J). All sections have the same magnification (×200). LS, long-term survivor [Color figure can be viewed at wileyonlinelibrary.com]

histopathologic examination. At the end-point (either grade III rejection or POD 100), animals were sacrificed and donor hind-limb tissues were collected. Skin obtained from the thigh was divided into 4 pieces for histology/immunohistochemistry (IHC), quantitative real-time polymerase chain reaction (RTqPCR), Luminex (5 × 5 mm each), and flow cytometry (2 × 1 cm). An additional skin biopsy for histopathology was taken from the dorsum (5 × 5 mm). The anterior tibialis muscle was divided into 3 pieces for histology/IHC, RTqPCR, and Luminex (5 × 5 mm each). Blood was obtained by heart puncture for Luminex (0.5 mL, serum) and flow cytometry (0.5 mL, heparinized). Samples for RTqPCR and Luminex were stored in RNA-later (Sigma-Aldrich) at −80°C. For all experiments n = 5 samples/group were collected.

2.4 | Histopathology and immunohistochemistry

Skin and muscle were fixed in 4% paraformaldehyde and embedded in paraffin. Hematoxylin and eosin (H&E)–stained sections (4 μm) were graded (Table 3, for skin samples in accordance with the Banff guidelines for skin-containing VCAs)[13] by a blinded pathologist using light-microscopy. Immunohistochemical labeling for CD3, CD20, CD68, CD80, CD86, and cytotoxic T lymphocyte-associated


A
CD3
Skin
Muscle
% of infiltrating cells
CD80
score
CD20
CD86
CD68
CTLA-4


B
CD3
CD80
CD20
CD86
CD68
CTLA-4

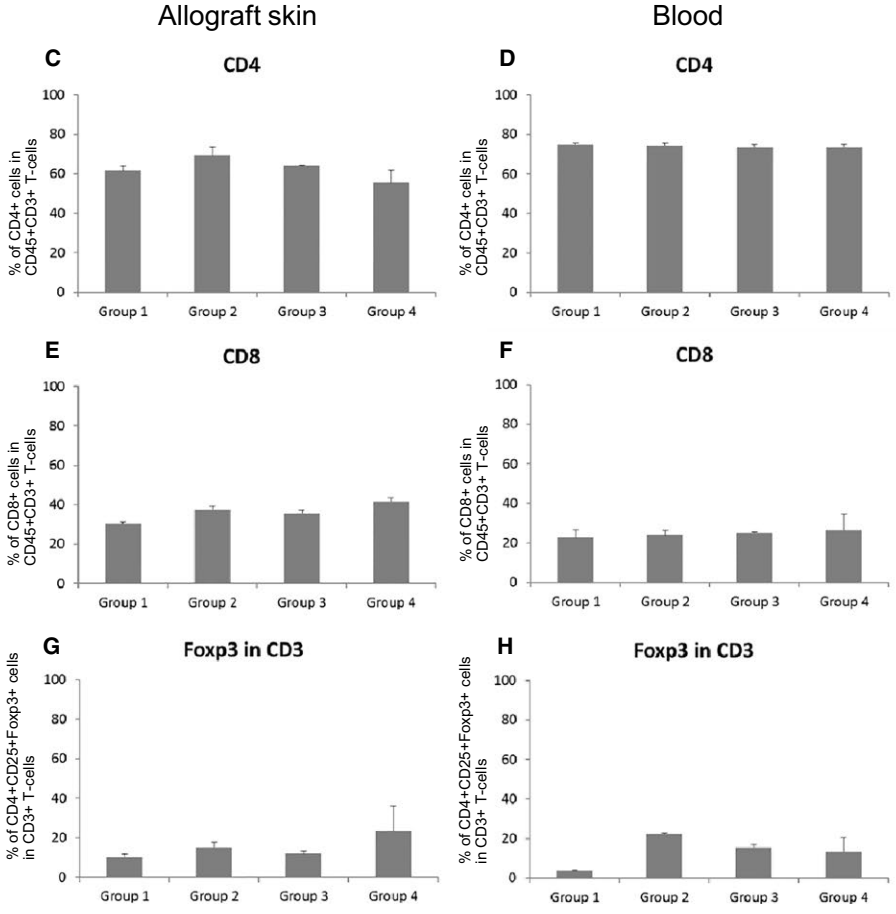

Allograft skin
Blood
C
CD4
% of CD4+ cells in CD45+CD3+ T-cells
D
E
CD8
% of CD8+ cells in CD45+CD3+ T-cells
F
G
Foxp3 in CD3
% of CD4+CD25+Foxp3+ cells in CD3+ T-cells
H
Group 1
Group 2
Group 3
Group 4

FIGURE 4 Characterization of the cell infiltrate using immunohistochemical stains (allograft skin and muscle) and flow cytometry analysis (allograft skin, blood). Monoclonal antibodies against CD3 (1F4, dilution 1:100), CD68 (KP1, dilution 1:200), CD80 (3H5, dilution 1:100; all ThermoFisher Scientific), CD86 (D6, dilution 1: 100), CD20 (0.N.85, dilution 1:100), and CTLA-4 (F8, dilution 1:100; all Santa Cruz Biotechnology, Inc.) were used for characterization of the cellular infiltrate in allograft skin. CD3, CD20, and CD68 staining was read as percentage of the cellular infiltrate. CD80, CD88, and CTLA-4 staining was graded as follows: 0 (0%), 1 (1-10%), 2 (10%-50%), and 3 (>50% positive stained infiltrating cells). A, The graphs show results of immunohistochemical staining in allograft skin and muscle for groups 1 – 4 (abscissa). Overall, no significant differences in the phenotype of infiltrating cells was observed between anti-IL-1β-treated groups 3 and 4 and group 2 controls. B, Immunohistochemical staining for CD3, CD20, CD68, CD80, CD86, and CTLA-4 in the allograft skin exemplarily shown for animal 4 of anti-IL-1β-treatment group 4 on POD 100 (long-term survivor). All sections have the same magnification (×200). Skin and blood T cells were isolated ex vivo. Skin samples stored in Dispase overnight at 4°C were washed with complete medium (10% FCS, 1% pen/strep in RPMI 1640, Lonza) and digested in collagenase D (1 mg/mL, Roche) for one hour at 37°C. The cell suspension was strained through a Nylon cell strainer, washed twice, and then slowly placed on Histopaque (1083, Sigma-Aldrich) and centrifuged at 2500 rpm for 30 minutes at 20°C without breaks. The white blood cell ring fraction was transferred into phosphate-buffered saline, washed 3 times, and used for flow cytometry. Heparinized peripheral blood was slowly placed on Histopaque and continued as described earlier. For detection of intracellular Foxp3, a Foxp3 Fixation/Permeabilization Kit (00-5521, eBioscience) was used according to the manufacturer's instructions. Cell suspensions were incubated at 4°C for 30 minutes with appropriate dilutions of directly labeled monoclonal rat antibodies either against CD45 (APC, OX1), CD3 (PE, G4.18), CD4 (FITC, OX35), and CD8a (PE-Cy7, OX8) or against CD3 (PE, G4.18), CD4 (FITC, OX35), CD25 (APC,OX39), and Foxp3 (PE-Cy7, FJK-16s; all eBioscience) and analyzed on a FACS Calibur. C – H No significant difference of CD45+ CD3+ CD4+ T cells, CD45+ CD3+ CD8+ T cells, and CD3+ CD4+ CD25+ Foxp3 T regulatory cells within the CD3+ T cell pool was found in allograft skin (C, E, G) and blood (D, F, H) between anti-IL-1β-treated groups 3 and 4 and group 2 controls [Color figure can be viewed at wileyonlinelibrary.com]

protein-4 (CTLA-4) was performed on paraffin sections according to standard protocols. Photos were taken with a PL-A642 camera (Pixelink) mounted on a BX50F4 microscope (Olympus).

2.5 | Cell isolation and flow cytometry

Skin was placed in Dispase (1 mg/mL, Roche) overnight at 4°C and cells were isolated, also of heparinized peripheral blood, as described before.[14] Cell suspensions were stained with antibodies against CD45, CD3, CD4, CD8a, CD25, and Foxp3 (all eBioscience) for 4-color flow cytometry. Fluorescence intensity was analyzed on a FACS Calibur (BD). Data analysis was performed using CellQuestPro software.

2.6 | RNA isolation and RTqPCR

Skin and muscle were homogenized with a TissueRuptor (Qiagen) and total RNA was isolated using the RNAeasy-mini-kit (Qiagen) including DNAse treatment according to the supplier's instructions. One micrograms of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). RTqPCR for IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-17A, interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α) was performed on a 7500 real-time PCR system (Applied Biosystems) with Sybr green (Qiagen). Quantification of gene expression was carried out using the ΔΔCT method. Comparative threshold cycle (CT) values were normalized to beta actin as reference gene.

2.7 | Protein isolation and Luminex

Proteins from skin and muscle were isolated using a disperser (T10, basic ULTRA-TURRAX, IKA) with 1 mL 1xCell Lysis Buffer (Cell Signaling) on ice. Proteins were quantified after homogenization using the BCA Protein Assay Kit (Pierce Biotechnology). Cytokine/chemokine protein levels were measured in skin, muscle, and serum using the Cytokine&Chemokine 22-Plex Rat ProcartaPlex Panel (EPX220-30122-901, Invitrogen) in a Luminex MAGPIX (Luminex Corporation) and analyzed by xPonent 4.2 Rev.2 (Luminex Corporation).

2.8 | Skin transplantations

Rejection-free animals were transplanted with an additional full-thickness skin graft (1.5 × 1.5 cm) from BN and Buffalo rats (Charles River) onto the back on POD 100. Grafts were inspected daily for rejection over 20 days.

2.9 | Statistical analysis

Graft survival was assessed by Kaplan-Meier log-rank survival analysis. Data are expressed as mean ± standard error of the mean (SEM). Analysis of variance was used to compare differences between groups. Group 2 served as an adequate control group for anti-IL-1β-treatment groups 3 and 4. The post hoc test according to Bonferroni was utilized for correction of multiple comparisons. A *P*-value of <.05 was considered statistically significant.

3 | RESULTS

3.1 | Rejection onset and allograft survival

Detailed information on rejection (macroscopic grade I) and graft survival (macroscopic grade III rejection) for individual animals and treatment groups are summarized in Table 4. In brief, untreated animals (group 1) presented with grade III rejection on POD 7 and 8, respectively (Figure 1A,B). Although group 2 animals developed grade III rejection between 13 and 18 days after tacrolimus weaning (Figure 1C,D), intragraft anti-IL-1β injections (group 3) resulted in a significant delay of rejection onset ($P = .044$, Figure 2A) and

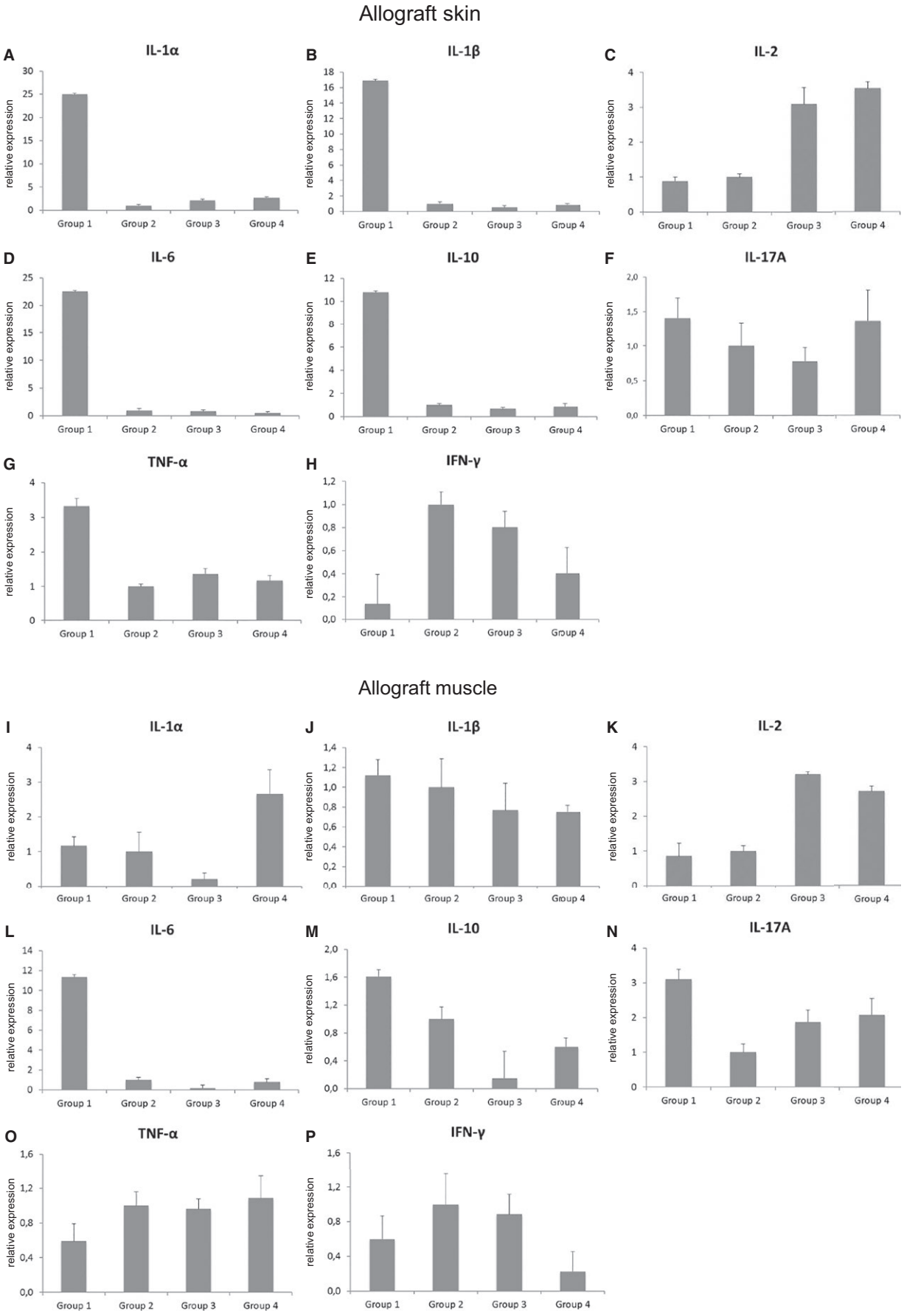

Allograft skin
A
IL-1α
B
IL-1β
C
IL-2
D
IL-6
E
IL-10
F
IL-17A
G
TNF-α
H
IFN-γ
Allograft muscle
I
IL-1α
J
IL-1β
K
IL-2
L
IL-6
M
IL-10
N
IL-17A
O
TNF-α
P
IFN-γ
relative expression
Group 1
Group 2
Group 3
Group 4

FIGURE 5 RTqPCR analysis showing relative gene expression levels of IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-17A, TNF-α, and IFN-γ in allograft skin (A – H) and muscle (I – P). For RTqPCR, a total volume of 15 µl per reaction was used containing 50 ng cDNA template, 1.5 µl QuantiTect Primer Assay (Qiagen), 7.5 µl QuantiTect SYBR green PCR kit (Qiagen), and 5 µl ddH2O. Primers for IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α were obtained from Qiagen. Cycling conditions included a hot start activation (5 minutes, 95°C), 40 cycles of 10 seconds denaturation (95°C), and annealing and extension (30 seconds, 60°C). Amplicons were quantified with the comparative threshold cycle (CT) method; data acquisition was performed using the 7500 System SDS Software Version 2.0.5 (Applied Biosystems). Amplification specificity was checked using melting curve according to the manufacturer's instructions. All samples were measured in duplicate and respective results were averaged. A–P, In allograft skin (A–H) and muscle (I–P) no significant differences in relative gene expression of cytokines was observed between anti-IL-1β-treatment groups 3 and 4 and group 2 controls. Expression levels of all markers were normalized to those of group 2

significantly prolonged limb survival ($P = .002$, Figure 2B), compared to controls (group 2). In group 3, 2/5 animals were identified as responders, defined by delayed rejection, but eventually developing moderate/severe rejection (1 + 2). Another 2/5 animals reached the study endpoint POD 100 and were therefore classified as long-term survivors (Figure 1E-H, 3 + 4). Both animals were macroscopically rejection free at this time-point. Subcutaneous anti-IL-1β injections into the contralateral limb (group 4) also resulted in a significant delay of rejection onset ($P = .008$, Figure 2A) and significantly prolonged graft survival ($P = .002$, Figure 2B), compared to control group 2. Two of 5 animals were identified as responders and 3/5 animals reached POD 100 and were considered long-term survivors. Animal 1 was rejection-free during the entire observation period and showed a well-perfused allograft with unaffected hair growth (Figure 1I,J). Two long-term survivors presented mild rejection between POD 65 and 100 (3 + 4). Epidermolysis was never observed. (Figure 1K,L). No obvious side effects (anemia, fatigue, or abnormal behavior) due to anti-IL-1β administration nor skin irritation/inflammation at the injection-sites was observed.

3.2 | Histopathology

An overview on histopathologic scores of allograft skin and muscle samples of individual animals is presented in Table 5. In short, group 1 animals displayed severe inflammation and necrosis in the histopathological assessment of skin and muscle at the study endpoint (Figure 3A,B). In group 2, 4/5 limbs showed histopathologic skin rejection grade III (Figure 3C) and one animal rejection grade II upon tissue procurement. The muscle was affected to a lesser extent in group 2 animals (Figure 3D). Animals responding to anti-IL-1β treatment of groups 3 and 4 showed a milder histopathological rejection in skin and muscle compared to group 2 controls. Although long-term-survivors of groups 3 and 4 mostly revealed a mild perivascular cell infiltrate (grade I) in skin and muscle on POD 100, one long-term survivor of group 4 (macroscopically rejection-free) was histopathologically free of dermal cell infiltration (grade 0) on POD 100 (Figure 3E-J).

Histopathologic scores of skin samples collected from the dorsum of the forefoot were consistent with those taken from the thigh of the allograft. Control skin biopsies of group 3 and 4 animals collected on POD 58 were rejection-free or displayed mild perivascular dermal cell infiltration (grade I), while biopsies of control group 2 revealed rejection grade II at this time-point (data not shown).

3.3 | Immunohistochemistry

Immunohistochemical characterization of the cell infiltrate in allograft skin and muscle, whether it was mild or severe, consisted of nearly 100% CD3+ T cells and sparse CD20+ B cells and CD68+ macrophages/monocytes in all groups. Membrane proteins involved in costimulatory signaling of T cells (CD80, CD86, CTLA-4) were positive in 10-50% of infiltrating cells with no difference between groups 3 and 4 and group 2 controls ($P > .05$, Figure 4A,B). The mild infiltrate found in long-term survivors did not differ significantly from group 2 animals (data not shown).

3.4 | Local (allograft skin) and systemic (blood) T cell phenotypes

No significant difference was found for the proportions of skin and blood CD4+ and CD8+ T cells within the CD3+ CD45+ T cell pool between groups 3 and 4 and group 2 controls (Figure 4C-F). This was also true for CD3+ CD4 + CD25+ Foxp3+ T regulatory cells (Figure 4G-H).

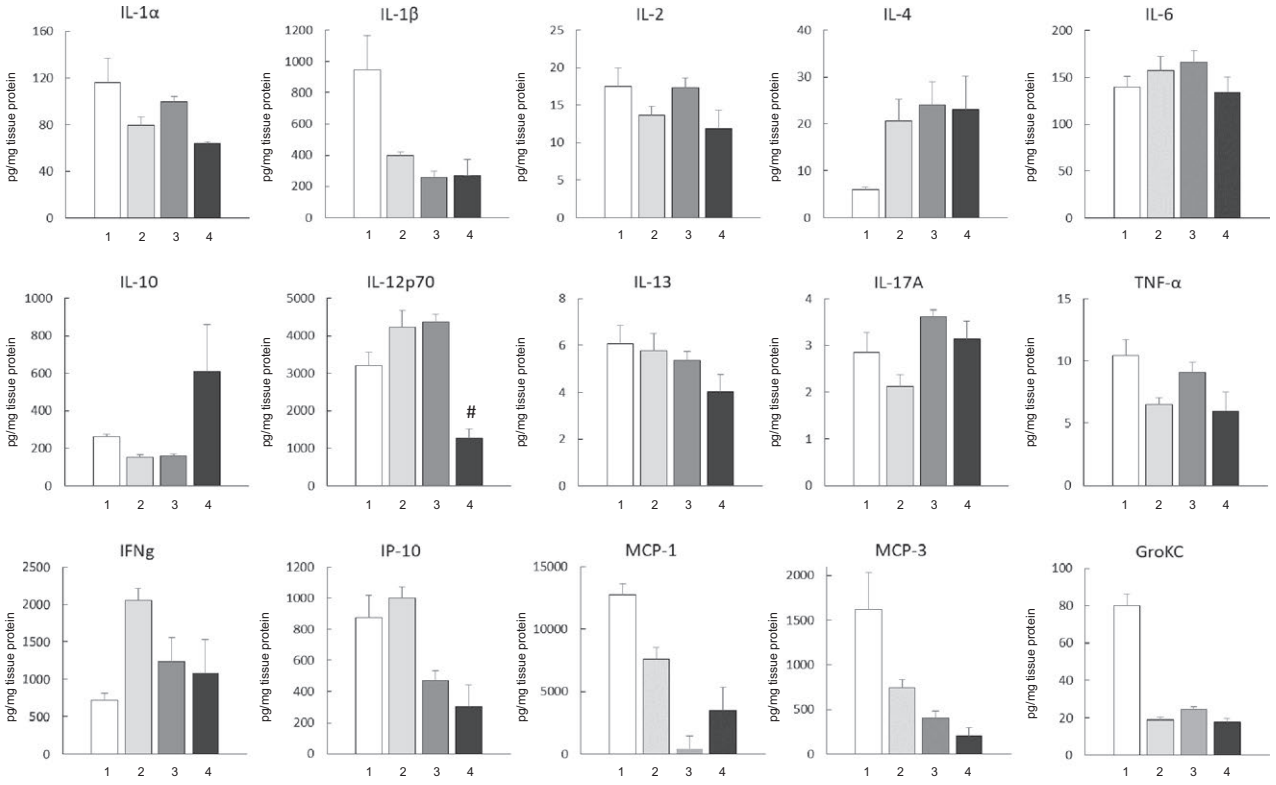
3.5 | Cytokine mRNA levels in allograft skin and muscle

Relative gene expression levels were normalized to those of group 2. On the mRNA level, no significant differences in relative expression of cytokines analyzed in skin and muscle were observed between groups 3 and 4 and group 2 controls (Figure 5). Of interest, the expression pattern in the skin of untreated animals[1] differed significantly from all other groups. When comparing long-term survivors with group 2 controls, IL-1α in skin ($P = .017$) and IL-2 ($P = .010$) and IL-17A ($P = .049$) in muscle showed a significant increase in relative gene expression (data not shown).

3.6 | Cytokine/chemokine protein levels in allograft skin, muscle, and serum

Protein levels of 15 cytokines in skin and muscle are depicted in Figure 6. Of note, IL-1β protein levels were slightly decreased in both skin and muscle after subcutaneous IL-1β blockade in groups 3 and 4, compared to group 2 controls. Systemically, a slight increase in IL-1β levels was observed in these groups (Figure 7A). When analyzing

A Allograft skin



B Allograft muscle

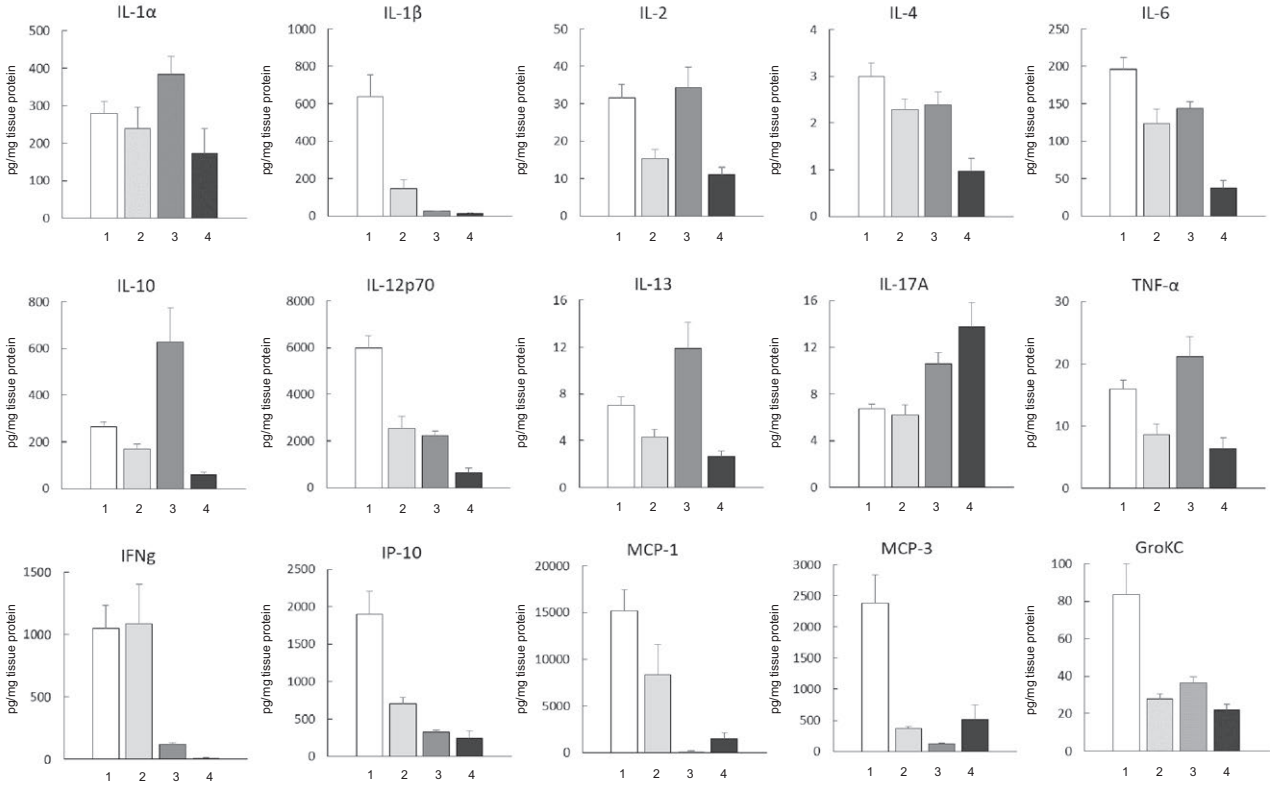


FIGURE 6 Cytokine protein levels analyzed by Luminex Technology in allograft skin and muscle. Protein levels in skin, muscle, and serum were analyzed with Luminex as described in Materials and Methods. Results were read in pg/mL and normalized to total mass of sample protein for skin and muscle samples (pg inflammatory mediator/mg protein). All samples were measured in duplicate and respective results were averaged. If there was a deviation of more than 20% between the duplicates, the higher value was included in the analysis. Any analyte with a concentration outside the linear range of the Luminex assay was excluded from analysis (NA). The rat panel commercially available for Luminex analysis included a total of 22 cytokines. We have decided to show data on 15 cytokines relevant in skin inflammation and skin rejection, as shown by Wolfram et al[4] previously (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17A, TNF-α, IFN-γ, IP-10, MCP-1, MCP-3, GRO/KC). A, B, Protein levels in pg/mg tissue protein are displayed for 15 cytokines found in allograft skin (A) and muscle (B). Anti-IL-1β-treatment groups 3 and 4 are compared to control group 2. *$P < .05$ group 3 vs control group 2; # $P < .05$ group 3 vs group 4; abscissa: group

protein levels in allograft skin of long-term survivors, a significant decrease in IL-1β levels was observed in long-term survivors, compared to controls ($P = .038$) and responders ($P = .005$). This correlated with a decreased expression of IL-4, IL-13, IP-10, monocyte chemoattractant protein (MCP)-1, and MCP-3 (Figure 7B). Moreover, serum protein levels of IP-10, MCP-1, and MCP-3 were significantly lower in these animals (data not shown).

3.7 | Skin grafts

On POD 100, full-thickness skin grafts were transplanted from BN and Buffalo rats to 3 macroscopically rejection-free long-term survivors of groups 3 and 4. Both skin grafts were rejected within 20 days in all animals (grade IV; Figure 8A,B). This confirms that despite long-term allograft survival, a uniform tolerance toward the donor antigens has not been established.

4 | DISCUSSION

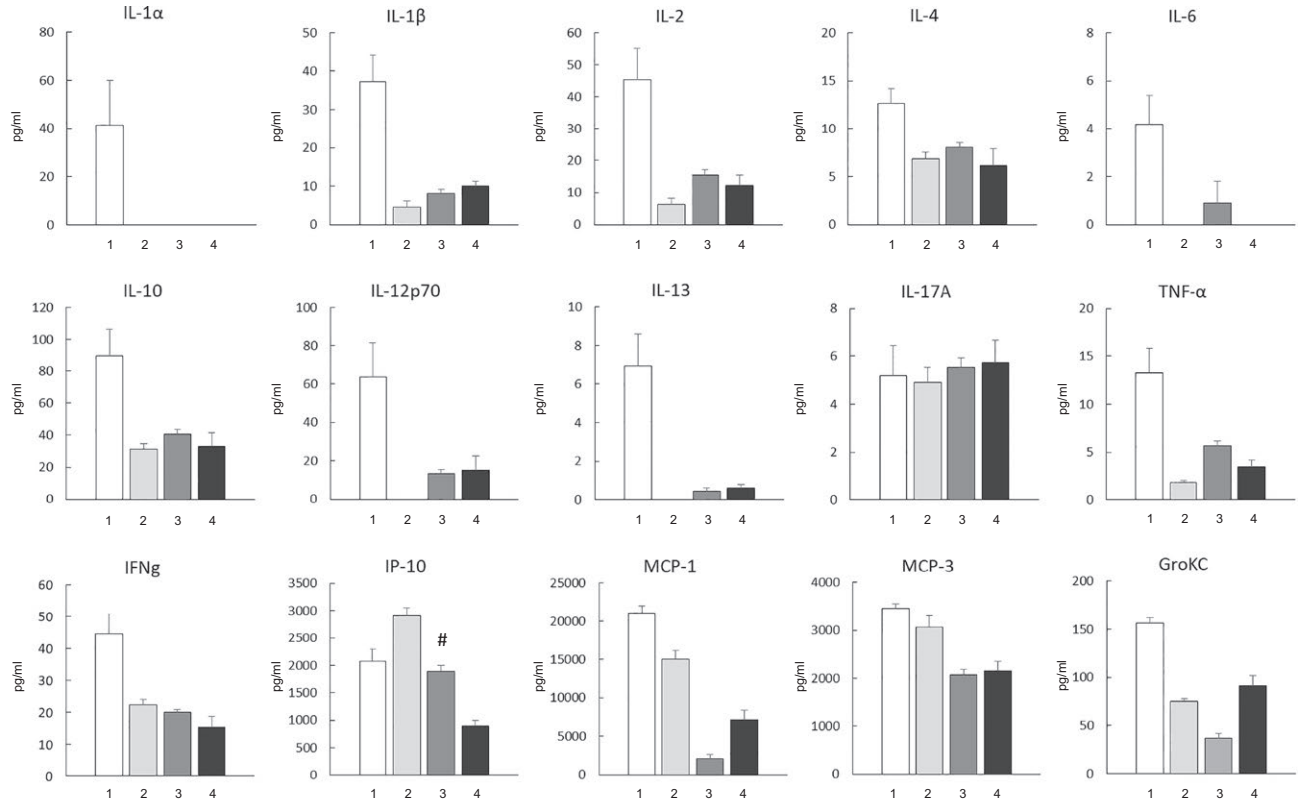
Because cytokine-expression profiles revealed IL-1ß highly upregulated in rejecting skin of limb allografts,[3,4] the effect of an intragraft administered neutralizing IL-1β-antibody was herein investigated in a rat limb-transplant model. IL-1β is a product of the inflammasome-protein complex.[15] It is secreted by macrophages and keratinocytes[16] and involved in the pathogenesis of inflammatory dermatoses.[17,18] Excessive IL-1 signaling directly activates keratinocytes to produce TNF and chemokines, resulting in attraction of T cells toward the epidermis[19]—the key-mechanism in VCA rejection.[2] Pharmacological antagonists of IL-1β are available for treatment of rheumatoid arthritis[7] and cryopyrin-associated periodic syndromes.[20,21.]

Building on a previous trial, where a small-molecule blocker against E-selectin inhibited limb allograft rejection when administered subcutaneously,[10] the monoclonal IL-1β-antibody used here resulted in a significant delay of rejection onset and prolongation of graft survival when injected directly into the allograft. Moreover, we observed that subcutaneous anti-IL-1β injections in a more distant site of the allograft (contralateral limb) showed an effect similar to intragraft injections. This finding indicates that the antibody causes a systemic response to prevent rejection of the limb allograft in a distant area. This is underscored by the observation that systemic protein levels of IP-10, MCP-1, and MCP-3, which are cytokines involved in recruitment and attraction of T cells, NK cells, macrophages, and monocytes to sites of inflammation/infection, were significantly decreased in long-term survivors of anti-IL-1β-treated groups 3 and 4. For clinical practice, this suggests that subcutaneous or dermally delivered antirejection therapy in VCA might also be placed in an area close to the allograft (for example, in hand transplant recipients on/into the recipient′s own skin at the upper arm), thereby protecting the donor skin against multiple injections and skin irritations, which poses the risk of subsequent infection and might per se act as a trigger of inflammation and rejection, as observed in some human hand transplants exposed to mechanical stress.[22]

The overall cell infiltrate in anti-IL-1β-treated groups 3 and 4 was diminished at the study endpoint as shown in H&E stains, especially in long-term survivors. This might result from less activation of endothelial cells, which is normally initiated by cytokines such as TNF-α or IL-1.[23] Because IL-1β protein levels were significantly diminished in allograft skin of long-term survivors of groups 3 and 4, this might have resulted in less endothelial cell activation and hence decreased expression of adhesion molecules and chemokines, thereby preventing recruitment and attraction of circulating leukocytes to the allograft dermis/epidermis as the most vulnerable site of rejection in VCA. Endothelial cells can activate T cells during inflammation,[24] which might be prohibited when blocking key cytokines such as IL-1β and thus activation of endothelial cells.

Our data suggest that anti-IL-1β treatment does not immediately influence the composition of graft-infiltrating cell populations. Even if the overall cell infiltrate in anti-IL-1β-treated groups 3 and 4 was diminished at the study endpoint, the proportions of CD4+ and CD8+ T cells, T regulatory cells, B cells, and macrophages did not differ significantly between these groups and group 2 controls. Altogether, the composition of infiltrating cells is similar to what has been observed in skin of most human hand allografts during acute rejection[10]; however, recently also B cell dominated rejections of VCAs have been reported.[25,26] In an experimental study by Liu et al,[27] selective inhibition of the purinergic pathway reduced acute rejection and the total amount of inflammatory cells in murine lung allografts, and they found few alterations in the composition of the cell infiltrate (CD4+ T cells, Th17 cells, T regulatory cells), similar to what we observed in our animals. However, they noticed that T cell activation (number of effector memory T cells) was inhibited in allografts by blocking purinergic receptors. Further studies

A Serum



B Allograft skin – Group 2 (controls) vs Long-term-survivors (LS) vs Responders (RE)

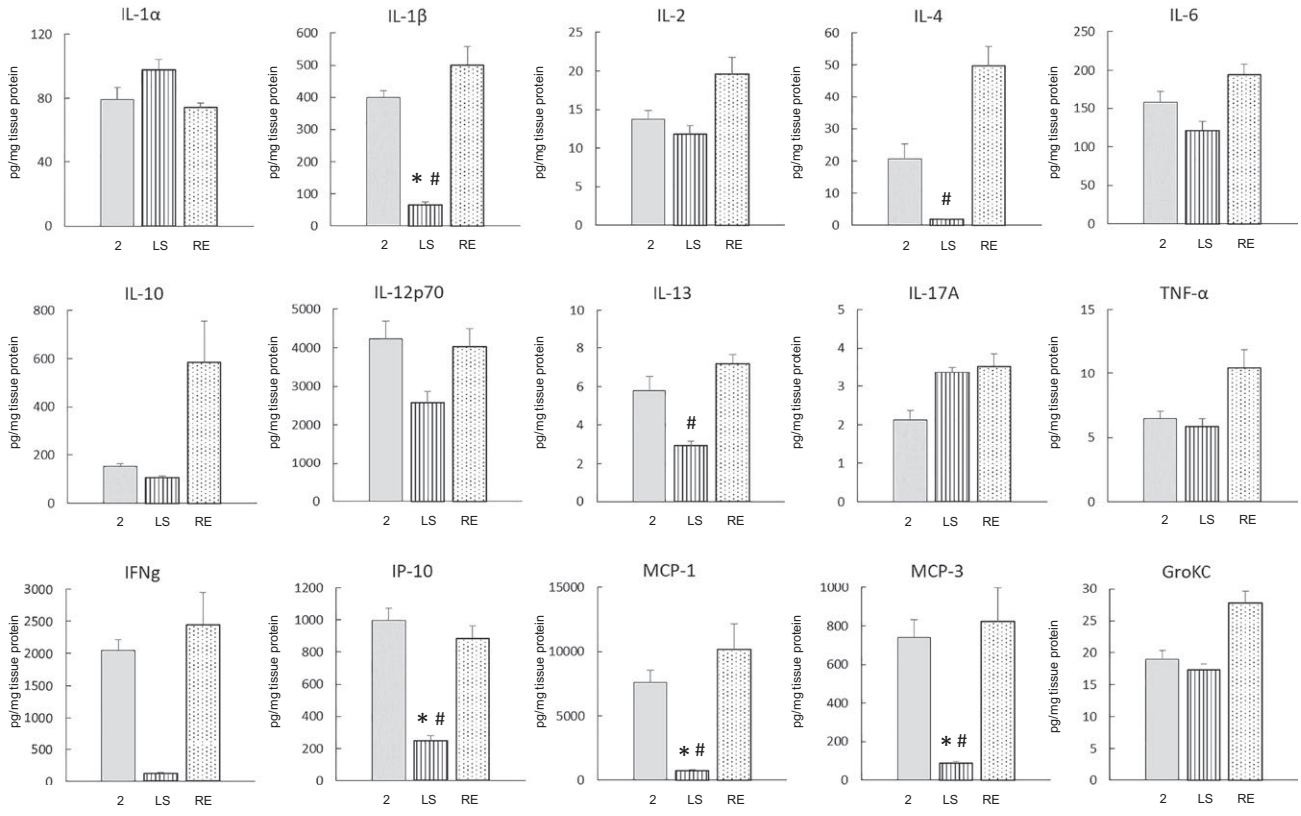


FIGURE 7 Cytokine protein levels analyzed by Luminex Technology in serum and allograft skin. A, Protein levels in pg/mL are displayed for 15 cytokines in serum. Anti-IL-1β-treatment groups 3 and 4 are compared to control group 2. *P < .05 group 3 vs control group 2; #P < .05 group 3 vs group 4; abscissa: group. B, Protein levels in pg/mg tissue protein are displayed for 15 cytokines isolated from allograft skin. Long-term-survivors (LS) of groups 3 and 4 are compared to responders (RE) and control group 2. *P < .05 LS vs control group 2; #P < .05 LS vs RE; abscissa: group

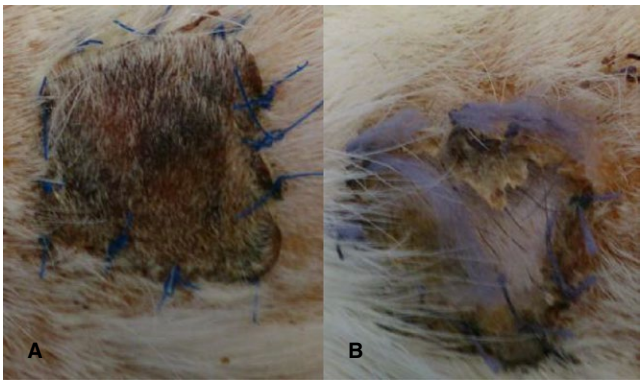


FIGURE 8 Macroscopic signs of severe rejection of full-thickness skin grafts 20 days after transplantation. BN (A) and third-party skin grafts (B) showed severe necrosis or desquamation (B) 20 days after transplantation (skin grafts of animal 4, group 3 exemplarily shown) [Color figure can be viewed at wileyonlinelibrary.com]

could evaluate whether decreased numbers of effector memory T cells, myeloid suppressor cells, and activated/mature dendritic cells contributed to the positive effect of anti-IL-1β treatment in our study.

When evaluating intragraft cytokine expression on the mRNA and protein level, no significant differences were observed between anti-IL-1β-treated groups 3 and 4 and the control group 2. This might be attributed to the fact that both anti-IL-1β treatment groups included animals that eventually displayed grade III rejection and moderate to severe cell infiltration and rejection, while the onset of rejection was delayed and graft survival significantly prolonged. This implicates a strong immune response with high cytokine levels. We therefore analyzed mRNA and protein cytokine levels in long-term survivors and responders of groups 3 and 4 separately and compared them to group 2. Hence, the therapeutic effect of the IL-1β-antibody in long-term-survivors was correlated with a decreased protein expression of IL-1β, IL-4, IL-13, IP-10, MCP-1, and MCP-3 in allograft skin. IL-1β together with IL-23 has been shown to play an important role in IL-17A production by γδ T cells, and the IL-1β/IL-23-IL-17A axis is critical for the onset and amplification of inflammatory responses.[28] Interrupting this axis by IL-1β blockade might also have contributed to the positive effect observed in our animals.

A discrepancy between mRNA and protein levels was observed for some cytokines; however, this problem has also been described by others, especially in complex in vivo situations,[29] and might be attributed to several factors including different in vivo half-lives of proteins and mRNAs and various mechanisms for posttranscriptional modification.[30]

Our results indicate that targeted treatment strategies applied subcutaneous intragraft or into the recipient skin, such as inhibition of IL-1β with short-term systemic IS, are feasible in VCA and hold great potential in the development of low-dose immunosuppressive regimen. As the inflammatory process in the skin is a complex event with numerous cytokines and chemokines involved, a combination of 2 or 3 agents targeting key molecules promoting skin rejection might further enhance the efficacy of such an approach.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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