BRIEF COMMUNICATION

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

Theresa Hautz1 | Johanna Grahammer1 | Dominik Moser1 | Nadine Eberhart1
Bettina Zelger2 | Bernhard Zelger3 | Michael J. Blumer4 | Astrid Drasche1
Dolores Wolfram5 | Jakob Troppmair1 | Dietmar Öfner1 | Stefan Schneeberger1

1Daniel Swarovski Research Laboratory (DSL), Department of Visceral, Transplant and Thoracic Surgery, Center of Operative Medicine, Medical University of Innsbruck, Innsbruck, Austria
2Department of Pathology, Medical University of Innsbruck, Innsbruck, Austria
3Department of Dermatology, Medical University of Innsbruck, Innsbruck, Austria
4Department of Anatomy, Histology and Embryology, Division of Clinical and Functional Anatomy, Medical University of Innsbruck, Innsbruck, Austria
5Department of Plastic, Reconstructive and Aesthetic Surgery, Center of Operative Medicine, Medical University of Innsbruck, Innsbruck, Austria

Correspondence
Theresa Hautz
Email: theresa.hautz@i-med.ac.at

Cytokine-expression profiles revealed IL-1β 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 or treated with anti-lymphocyte serum + tacrolimus (baseline); baseline immunosuppression + anti-IL-1β (1 mg/kg once/week, 6-8 subcutaneous injections) into the transplanted or contralateral 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 chemotraction 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.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2018 The Authors. American Journal of Transplantation published by Wiley Periodicals, Inc. on behalf of The American Society of Transplantation and the American Society of Transplant Surgeons.
1 | INTRODUCTION

With more than 150 performed cases of vascularized composite tissue allotransplantation (VCA) worldwide, the skin has come into focus, both as a site of immune reaction, 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. IL-1β constitutes a proinflammatory signal inducing T cell infiltration, memory CD4+ T cell activation, IL-6 expression, and Th-17 differentiation. Blockers of IL-1 and IL-1β show significant effects in patients with autoinflammatory syndromes and have

| 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.

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

| Grade | Histopathologic alteration |
|-------|----------------------------|
| 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            |

| 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|        | 4.00 ± 0.00    | 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|        | 58.60 ± 0.60   | 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 Mean|        | POD 34         | POD 69        |          |
| 3 Mean|        | 75.80 ± 10.87  | 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 Mean|        | POD 76         | POD 80        | Responder |
| 4 Mean|        | 73.00 ± 6.49   | 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.
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...
the environment for testing the effect of intragraft targeted therapy in limb transplantation. 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 detection limits at 5 days after cessation. 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.

### 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                      |          |
| 3     | 1      | II                   | III                    | Responder|
| 3     | 2      | II                   | III                    | 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.

### 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. 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 x 5 mm) was collected from the allograft thigh for...
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), Lumines (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 Lumines (5 × 5 mm each). Blood was obtained by heart puncture for Lumines (0.5 mL, serum) and flow cytometry (0.5 mL, heparinized). Samples for RTqPCR and Lumines 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) by a blinded pathologist using light-microscopy. Immunohistochemical labeling for CD3, CD20, CD68, CD80, CD86, and cytotoxic T lymphocyte-associated
% of CD4+ cells in CD45+CD3+ T-cells
% of CD8+ cells in CD45+CD3+ T-cells
% of CD4+CD25+Foxp3+ cells in CD3+ T-cells

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4

Skin                      Muscle

1    2    3    4            1    2    3    4
2.5 Cell isolation and flow cytometry

Skin and muscle were isolated, also of heparinized peripheral blood, as described before. 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 FACSCalibur (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 x 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
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 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
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, MCP-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-18 highly up-regulated in rejecting skin of limb allografts, 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. It is secreted by macrophages and keratinocytes and involved in the pathogenesis of inflammatory dermatoses. Excessive IL-1 signaling directly activates keratinocytes to produce TNF and chemokines, resulting in attraction of T cells toward the epidermis—the key-mechanism in VCA rejection. Pharmacological antagonists of IL-1β are available for treatment of rheumatoid arthritis and cryopyrin-associated periodic syndromes.

Building on a previous trial, where a small-molecule blocker against E-selectin inhibited limb allograft rejection when administered subcutaneously, 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.

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. 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, 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; however, recently also B cell dominated rejections of VCA have been reported. In an experimental study by Liu et al., 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)
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. 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, and might be attributed to several factors including different in vivo half-lives of proteins and mRNAs and various mechanisms for posttranscriptional modification.

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.

ACKNOWLEDGMENTS

The study was supported by a MUI START grant from the Medical University of Innsbruck, dedicated to Theresa Hautz, MD, PhD. The authors would like to thank Evelyn Schlögl for excellent technical assistance.

DISCLOSURE

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

ORCID

Theresa Hautz http://orcid.org/0000-0003-0388-0202

REFERENCES

1. Shores JT, Brandacher G, Lee WP. Hand and upper extremity transplantation: an update of outcomes in the worldwide experience. Plast Reconstr Surg. 2015;135(2):351e-360e.

2. Kanitakis J, Jullien D, Petruzzo P, et al. Clinicopathologic features of graft rejection of the first human hand allograft. Transplantation. 2003;76(4):688-693.

3. Wolfram D, Morandi EM, Eberhart N, et al. Differentiation between acute skin rejection in allotransplantation and T-cell mediated skin inflammation based on gene expression analysis. Biomed Res Int. 2015;2015:259160.

4. Wolfram D, Starzl R, Hackl H, et al. Insights from computational modeling in inflammation and acute rejection in limb transplantation. PLoS ONE. 2014;9(6):e99926.

5. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol. 2009;27:519-550.

6. Muhr P, Renne J, Schaefer V, et al. Primary human keratinocytes efficiently induce IL-1-dependent IL-17 in CCR6 + T cells. Exp Dermatol. 2010;19(12):1105-1107.

7. Cohen S, Hurd E, Cush J, et al. Treatment of rheumatoid arthritis with anakinra, a recombinant human interleukin-1 receptor antagonist, in combination with methotrexate: results of a twenty-four-week, multicenter, randomized, double-blind, placebo-controlled trial. Arthritis Rheum. 2002;46(3):614-624.
8. Ilowite NT, Prather K, Lokhnygina Y, et al. Randomized, double-blind, placebo-controlled trial of the efficacy and safety of rilonacept in the treatment of systemic juvenile idiopathic arthritis. *Arthritis Rheum*. 2014;66(9):2570-2579.

9. Lachmann HJ, Kone-Paut I, Kuenzerme-Deschner JB, et al. Use of canakinumab in the cryopyrin-associated periodic syndrome. *N Engl J Med*. 2009;360(23):2416-2425.

10. Hautz T, Zelger B, Grahammer J, et al. Molecular markers and targeted therapy of skin rejection in composite tissue allotransplantation. *Am J Transplant*. 2010;10(5):1200-1209.

11. Hautz T, Krapf C, Grahammer J, et al. Targeting the Kv1.3 potassium channel for immunosuppression in vascularized composite allotransplantation - a pilot study. *Transpl Int*. 2013;26(5):552-561.

12. Sacks JM, Kuo YR, Horibe EK, et al. An optimized dual-surgeon simultaneous orthotopic hind-limb allotransplantation model in rats. *J Reconstr Microsurg*. 2012;28(1):69-75.

13. Cendales LC, Kanitakis J, Schneeberger S, et al. The Banff 2007 working classification of skin-containing composite tissue allotransplantation pathology. *Am J Transplant*. 2008;8(7):1396-1400.

14. Hautz T, Wolfram D, Eberhart N, et al. The impact of skin type and area on skin rejection in limb transplantation. *Vascu Compos Allotransplant*. 2014;1(1):42-49.

15. Drenth JP, van der Meer JW. The inflammasome—a frontline of innate defense. *N Engl J Med*. 2006;355(7):730-732.

16. Uchi H, Terao H, Koga T, et al. Cytokines and chemokines in the epidermis. *J Dermatol Sci*. 2000;24(suppl 1):S29-S38.

17. Fenini G, Contassot E, French LE. Potential of IL-1, IL-18 and inflammasome inhibition for the treatment of inflammatory skin diseases. *Front Pharmacol*. 2017;8:278.

18. Renne J, Schafer V, Werfel T, et al. Interleukin-1 from epithelial cells fosters T cell-dependent skin inflammation. *British J Dermatol*. 2010;162(6):1198-1205.

19. Nakajima A, Matsu T, Komine M, et al. TNF, but not IL-6 and IL-17, is crucial for the development of T cell-independent psoriasis-like dermatitis in Il1rn-/- mice. *J Immunol*. 2010;185(3):1887-1893.

20. Dhimolea E. Canakinumab. *MAbs*. 2010;2(1):3-13.

21. Gillespie J, Mathews R, McDermott MF. Rilonacept in the management of cryopyrin-associated periodic syndromes (CAPS). *J Inflamm Res*. 2010;3:1-8.

22. Schneeberger S, Gorantla VS, van Riet RP, et al. Atypical acute rejection after hand transplantation. *Am J Transplant*. 2008;8(3):688-696.

23. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol*. 2007;7(10):803-815.

24. Carman CV, Martinelli R. T lymphocyte-endothelial interactions: emerging understanding of trafficking and antigen-specific immunity. *Front Immunol*. 2015;6:603.

25. Chandraker A, Arscott R, Murphy G, et al. The management of antibody-mediated rejection in the first presensitized recipient of a full-face allotransplant. *Am J Transplant*. 2014;14(6):1446-1452.

26. Weissenbacher A, Hautz T, Zelger B, et al. Antibody-mediated rejection in hand transplantation. *Transpl Int*. 2014;27(2):e13-e17.

27. Liu K, Vergani A, Zhao P, et al. Inhibition of the purinergic pathway prolongs mouse lung allograft survival. *Am J Respir Cell Mol Biol*. 2014;51(2):300-310.

28. Sutton CE, Lalor S, Sweeney CM, et al. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity*. 2009;31(2):331-341.

29. Lichtinghagen R, Musholt PB, Lein M, et al. Different mRNA and protein expression of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases 1 in benign and malignant prostate tissue. *Eur Urol*. 2002;42(4):398-406.

30. Greenbaum D, Colangelo C, Williams K, et al. Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol*. 2003;4(9):117.

---

**How to cite this article:** Hautz T, Grahammer J, Moser D, et al. Subcutaneous administration of a neutralizing IL-1p antibody prolongs limb allograft survival. *Am J Transplant*. 2018;18:2029-2042. [https://doi.org/10.1111/ajt.14765](https://doi.org/10.1111/ajt.14765)