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ORIGINAL ARTICLE

Histopathologic characterization of mild rejection (grade I) in skin biopsies of human hand allografts

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Summary

Mild skin rejection is a common observation in reconstructive transplantation. To enlighten the role of this inflammatory reaction we investigated markers for cellular and antibody mediated rejection, adhesion molecules and tolerance markers. Forty-seven skin biopsies (rejection grade I) of human hand allografts were investigated by immunohistochemistry (CD3, CD4, CD8, CD20, CD68, C4d, LFA-1, ICAM-1, E-selectin, P-selectin, VE-cadherin, HLA-DR, IDO, and Foxp3). Expression was read with respect to time after transplant. The infiltrate was mainly comprised of CD3+T-lymphocytes. Among these, CD8+cells were more prominent than CD4+cells. CD20+B-lymphocytes were sparse and CD68+macrophages were found in some, but not all samples (approximately 10% of the infiltrate). The CD4/CD8-ratio was increased after the first year. C4d staining was mainly positive in samples at time-points later than 1 year. Adhesion molecules LFA-1, ICAM-1, E-selectin, P-selectin, and VE-cadherin were found upregulated, and for P-selectin, expression increased with time after transplant. IDO expression was strongest at 3 months–1 year post-transplant and a tendency toward more Foxp3+ cells at later time points was observed. Mild skin rejection after hand transplantation presents with a T-cell dominated dermal cell infiltrate and upregulation of adhesion molecules. The role of C4d expression after year one remains to be elucidated.

Introduction

Skin rejection is frequently observed after human hand and face allotransplantation [1–10]. According to the latest update of The International Registry on Hand and Composite Tissue Transplantation, 85% of recipients experienced at least one acute rejection episode within the first year post-transplant [11]. All of them were reversible upon topical or systemic immunosuppressive treatment. Moderate and severe skin rejection can be diagnosed upon visual inspection while diagnosis of mild rejection requires skin biopsies. The clinical relevance of mild skin rejection and an effect on graft function and outcome has not yet been precisely defined. Histopathologic evaluation of more than 170 skin biopsies of five human hand- or forearm transplant recipients revealed that mild skin rejection (grade I) was most frequently seen [12]. In fact, in 52.5% of all samples showing rejection, a perivascular dermal infiltrate was found, which is defined as grade I skin rejection according to the Banff 2007 guidelines for skin containing composite tissue allografts [13]. So far, little is known about the mechanisms of the very early stages of skin rejection.

We herein investigate the phenotype of the dermal immune cell infiltrate as well as markers for cellular and antibody mediated rejection, adhesion molecules, and tolerance markers in grade I skin rejection to enlighten the role of this inflammatory reaction after composite tissue allotransplantation (CTA).

Materials and methods

Skin biopsies

Six patients (male = 5, female = 1; Innsbruck = 4, Valencia = 2) were given a bilateral hand- and/or forearm transplant between 2000 and 2009. At current, postoperative courses are between 11 and 1.5 years. The clinical courses of the first five patients including the immunosuppressive regimen, complications, and rejection episodes have been described elsewhere [3,7,9,10,14,15]. The fourth patient transplanted in Innsbruck experienced two mild rejection episodes at 14 days (grade II) and 9 months (grade I) after transplantation. Otherwise, the postoperative course has been unremarkable (manuscript in preparation).

Allografts were examined clinically at a regular basis and 4-mm punch biopsies of the skin were collected as per protocol (at short intervals during the first 6 months post-transplant and at more prolonged intervals thereafter, $n = 22$) or from the apparent lesion whenever rejection was suspected (for cause biopsy, $n = 25$).

H&E-histology and immunohistochemistry

A total of 185 tissue samples were fixed in 4% paraformaldehyde, paraffin-embedded and stained with hematoxylin-eosin (H&E) according to standard procedures. Rejection grade was assessed as per Banff 2007 guidelines for skin containing composite tissue allografts [13]. A total of 47 skin biopsies showed rejection grade I (= mild rejection characterized by a perivascular dermal infiltrate). Samples were investigated by immunohistochemistry using antibodies for CD3 (Dako, Vienna, Austria, dilution 1:50), CD4 (Menarini Diagnostics, Vienna, Austria, dilution 1:10), CD8, CD20, CD68 (all Dako, Vienna, Austria, dilution 1:50, 1:700 and 1:100), C4d (Biomedica, Vienna, Austria, dilution 1:40), LFA-1 (Ab-Direct, Oxford, UK, dilution 1:100), ICAM-1, E-selectin (both Novocastra, Newcastle upon Tyne, UK, dilution 1:20), P-Selectin (Novocastra, Newcastle upon Tyne, UK, dilution 1:25), VE-cadherin, HLA class II-DR (both Ab-Direct, Oxford, UK, dilution 1:100 and 1:300), Indoleamine 2,3-dioxygenase (IDO, Chemicon, Temecula CA, USA, dilution 1:25), and Foxp3 (Biocare Medical, Concord CA, USA, dilution 1:50). Procedures were performed as per the manufacturer's instructions.

Grading and interpretation of data

A pathologist blinded to the clinical observations and experienced in reading skin rejection assessed marker expression using light microscopy at ×10 to ×40 magnification. Expression of CD3, CD4, CD8, and CD20 was described as percentage of the infiltrating cells. CD68 staining was graded as 0 (0–10%), 1 (10–50%), and 2 (>50% of infiltrating cells). Markers expressed in the vascular endothelium were assessed as per the following scheme: 0 = no or few vessels showing noncircumferential staining; 1 = mild staining in the majority of vessels; 2 = intense, circumferential staining in most vessels. IDO, Foxp3, and HLA-DR staining was graded semiquantitatively as 0 (no staining), 1 (mild staining or few positive cells), 2 (intense staining or several positive cells). Expression levels were correlated with time after transplantation: very early (the first 3 months, $n = 11$), early (3 months–1 year after surgery, $n = 8$), and late (after year 1, $n = 28$). Expression of adhesion molecules, IDO, and Foxp3 was compared with skin biopsy specimens showing no signs of rejection.

Results

Phenotype of the perivascular immune cell infiltrate

In all biopsy specimens showing mild skin rejection (grade I, Fig. 1a) the majority of the cells were identified as CD3+ T lymphocytes (78.64 ± 5.46%, Fig. 1b). Among these, CD8+ T cells were more prominent than CD4+ T cells (52.57 ± 6.00% and 36.62 ± 5.82%, Fig. 1c,d). CD20+ B lymphocytes were sparse. CD20 was absent in 89.29% of biopsy samples, in the remaining 10.71% of biopsy specimens <5% of the cells stained positive for CD20. Macrophages were found in 46.51% comprising approximately 10% of the infiltrate (Fig. 1e). In 9.30% of samples, CD68+ cells comprised more than 50% of infiltrating cells. Intense HLA-class II DR expression within the infiltrate was observed in 90.99% of all samples (Fig. 1f).

With regard to time after transplantation there was a tendency toward a higher percentage of CD3+ T lymphocytes at later time points (very early: 71.25 ± 11.25%, early: 75.00 ± 12.25%, late: 83.82 ± 7.42%). Interestingly, about 35% of these cells were negative for CD4 and CD8 at very early time points, whereas at later time points nearly 100% were positive for either CD4 or CD8. The CD4/CD8 ratio increased in biopsy specimen taken later than 1 year post-transplant (very early: 0.64, early: 0.56,

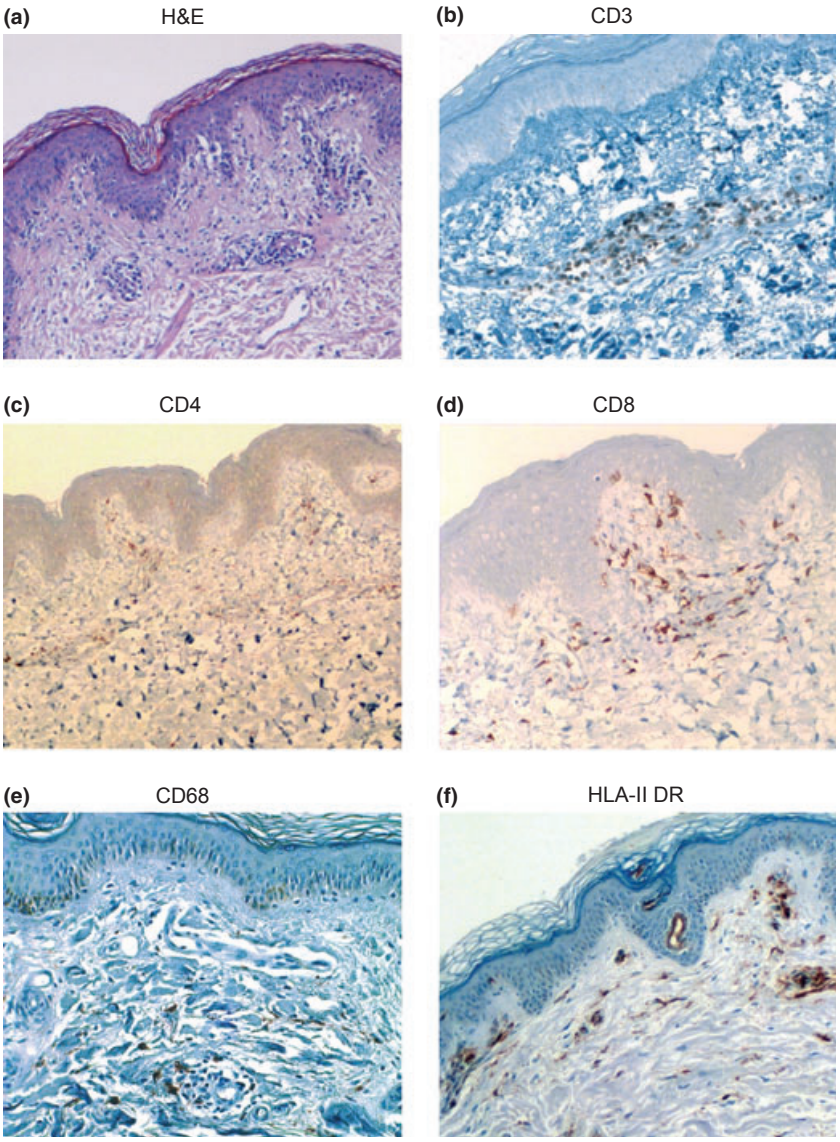

Figure 1 H&E characteristics of skin rejection grade I and immunohistochemical phenotype of the perivascular infiltrate. Grade I skin rejection is characterized by a perivascular dermal infiltrate (a), which was mainly comprised of CD3+ T-lymphocytes (b). Among these, CD8+cells were more prominent than CD4+cells (c,d). CD68+ macrophages were observed in the perivascular infiltrate (about 10%) and also scattered in the interstitial dermis (e). HLA-II DR staining was most often very intensive and found in the perivascular area as well as in a spotted pattern in the interstitial dermis (f).

late: 0.75). CD20+ B lymphocytes were sparse to absent at all time points. Macrophages were detected at all time-points.

Endothelial C4d deposits

In 42.42% of samples showing rejection grade I, mild endothelial C4d staining was observed, and in 18.18% of samples an intense, often circumferential staining pattern was found in the majority of vessels (Fig. 2a). The remaining 39.40%, grade I skin biopsies were negative for C4d. In skin samples free of a cellular infiltrate (rejection grade 0), 39.40% showed mild C4d staining, only 3.03% revealed intense stained C4d deposits, and 57.57% were negative for C4d. Overall, the number of C4d-positive samples significantly increased in grade I rejection skin biopsies compared with unaffected skin (60.6% vs. 42.4%, $P = 0.003$). C4d expression pattern changed with time after transplantation. In samples showing rejection grade I, C4d deposits increased with time after transplantation. Soon after transplantation (0–3 months) no C4d was detected. In contrast, 42.86% of tissue specimens taken between 3 months and 1 year post-transplant were positive for C4d and in biopsies taken after the first year 72.73% showed C4d-positive deposits (Fig. 2b). This phenomenon is also reflected by mean scores of C4d expression (very early: 0.00, early: 0.57 ± 0.30, late: 0.95 ± 0.15). In nonrejecting skin, C4d deposits were mainly found between 3 months and 1 year after transplantation (100% positive), whereas 33.3% and 52.4%

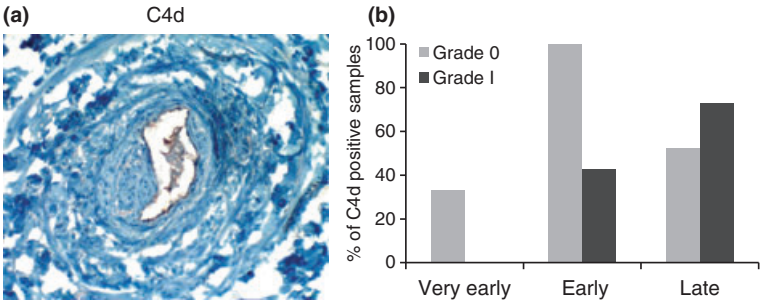

Figure 2 C4d deposits in mild skin rejection. In about 60% of all grade I skin biopsies C4d staining was detected in the endothelium of allograft vessels (a). In nonrejecting skin, C4d deposits were mainly found between 3 months and 1 year post-transplant, whereas in grade I samples C4d deposits were mainly observed at time-points later than 1 year (b).

were positive within the first 3 months and after year 1 postoperative, respectively (Fig. 2b). However, differences between groups (grade 0 vs. grade I) with regard to time after transplantation were not statistically significant.

Expression of adhesion molecules

In mild rejection, expression of LFA-1, which was detected within the perivascular infiltrate, was significantly upregulated (mean score grade 0: 0.28 ± 0.06, grade I: 0.58 ± 0.11). Overall, 45.0% of samples showing grade I rejection were positive for LFA-1 staining, whereas only 23.6% samples showing normal skin (grade 0) were LFA-1 positive ($P = 0.014$, Fig. 3). Expression of ICAM-1 and E-selectin in the vascular endothelium was also highly upregulated when compared with samples without signs of rejection (mean score ICAM-1: grade 0: 0.14 ± 0.04, grade I: 0.46 ± 0.09 and E-selectin: grade 0: 0.09 ± 0.03, grade I: 0.38 ± 0.11, Fig. 3). The percentage of samples staining positive for both ICAM-1 and E-selectin was significantly higher during grade I rejection when compared with not affected skin (ICAM-1: 43.6% vs. 12.8%, $P < 0.001$ and E-selectin: 29.7% vs. 9.3%, $P = 0.005$, Fig. 3). P-selectin staining in vessels had slightly increased in grade I skin biopsies (mean score grade 0: 0.43 ± 0.07, grade I: 0.59 ± 0.12,). In total, 46.2% of grade I skin biopsies were positive for P-selectin staining, however, expression was also found in 35.1% of skin samples without rejection ($P = 0.251$, Fig. 3). The same tendency was observed for VE-cadherin expression (mean score grade 0: 0.69 ± 0.07, grade I: 0.88 ± 0.12). Positive stained endothelial cells were detected in 65.0% of samples during mild rejection, but also in a high number of samples without rejection (55.5%, $P = 0.317$).

With respect to time after transplantation, expression pattern of adhesion molecules was relatively heterogenous (with the exception of P-selectin). Mean scores of lymphocyte trafficking markers are displayed in Table 1. P-selectin was significantly increased in samples collected after year one, whereas levels of E-selectin expression were similar at all time-points.

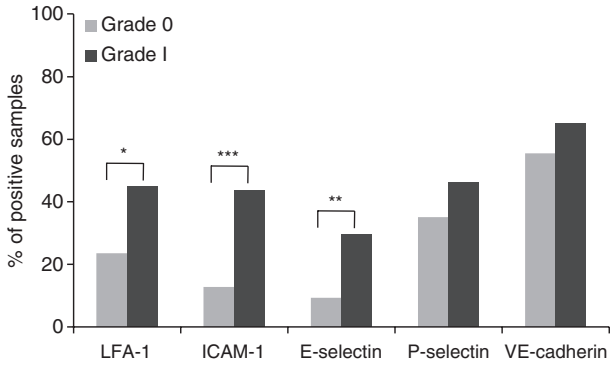

Figure 3 Expression of adhesion molecules in non-inflamed skin and grade I rejection. During mild skin rejection, all adhesion molecules investigated were upregulated. Strongest increase was found for LFA-1, ICAM-1, and E-selectin expression. P-selectin and VE-cadherin expression was also observed in normal skin and slightly increased in samples showing grade I rejection.

Table 1. Expression of adhesion molecules at various time-points after transplantation.

	0–3 months (very early)		3 months–1 year (early)		>year one (late)	
	Mean score	SEM	Mean score	SEM	Mean score	SEM
LFA-1	0.75	0.31	0.62	0.26	0.50	0.14
ICAM-1	0.25	0.16	0.88	0.23	0.39	0.10
E-selectin	0.38	0.26	0.38	0.18	0.38	0.15
P-selectin	0.25	0.25	0.25	0.16	0.83	0.15
VE-cadherin	1.12	0.13	0.12	0.12	1.04	0.17

SEM, standard error of the mean.

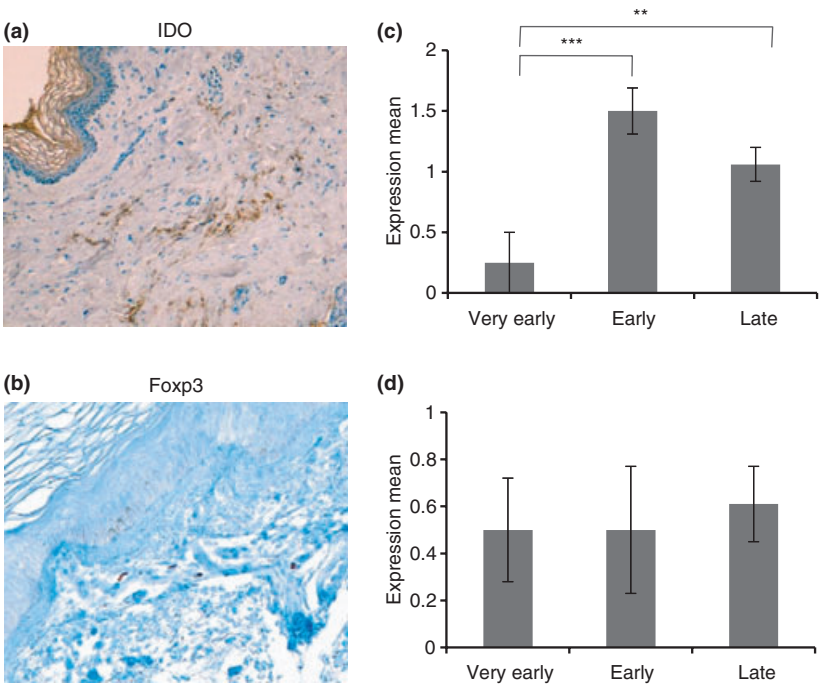

Figure 4 Indoleamine 2,3-dioxygenase (IDO) and Foxp3 staining and expression levels at various time-points after transplantation. In grade I skin biopsies IDO+ cells were mostly found scattered in the dermis (a) and expression was significantly increased after 3 months post-transplant (b). Approximately 5% of infiltrating cells were positive for Foxp3 (c). A discrete increase of Foxp3+ cells was observed in samples taken after the first year postoperative (d).

Indoleamine 2,3-dioxygenase and Foxp3 staining

The IDO staining was found in 72.73% of biopsies showing low-grade rejection. When compared with normal skin, IDO expression was found increased upon rejection grade I (mean score grade 0: 0.42 ± 0.07, grade I: 0.97 ± 0.13). IDO+ cells were not restricted to the perivascular area, but also found in the interstitial dermis (Fig. 4a). IDO expression was low in samples taken during the first 3 months after transplantation and highly increased in skin biopsies obtained thereafter (mean score very early: 0.25 ± 0.25, early: 1.50 ± 0.19, late: 1.06 ± 1.14, Fig. 4b).

The Foxp3 expression was found in 41.46% of tissue specimens and Foxp3+ cells comprised about 5% of the infiltrating cells (Fig. 4c). In tissue samples showing normal skin Foxp3 expression levels were very low and increased in biopsies showing mild rejection (mean score grade 0: 0.10 ± 0.03, grade I: 0.56 ± 0.12). A discrete increase of Foxp3 infiltrating cells was observed with time after transplantation (mean score very early: 0.50 ± 0.22, early: 0.50 ± 0.27, late: 0.61 ± 0.16, Fig. 4d).

Discussion

The skin is the major target of rejection after CTA [16,17]. Diagnosis, relevance, and therapy of mild rejection are still challenging and mainly based on clinicopathological correlation. Findings of a recent study in rats assessing the immune responses to skin in CTA and in conventional skin grafts indicate that skin rejection in CTA is focused on dermal targets in the superficial dermis, around adnexal glands, and the vasculature [18]. This initial inflammatory reaction in the dermis is considered to originate from microvascular damage because of a yet unknown mechanism and followed by transition of dermal fluid to the epidermis resulting in spongiosis. These subtle changes can be found in early stages of all immune-mediated dermatoses, where inflammatory cells initially enter the skin via blood vessels and thus present with the histopathologic pattern of 'superficial perivascular dermatitis' (e.g. spongiotic dermatitis, psoriasis, drug/viral eruption, erythema multiforme, etc.) or 'superficial and deep perivascular dermatitis' (arthropod bite reaction, deep variant of figurated erythema, polymorphic light eruption, etc.). Kanitakis *et al.* [19] described the overall changes during skin rejection in CTAs as not specific, and discussed possible dermal differential diagnoses not only for grade I rejection but also for more advanced stages of rejection. The differential diagnosis for 'superficial perivascular dermatitis without epidermal involvement and lymphocyte predominance' seen in grade I skin rejection includes a wide range of inflammatory skin diseases: tinea versicolor, dermatophytosis, erythrasma, pitted keratolysis, vitiligo, Schamberg's disease, viral exanthems, drug eruptions, urticaria (late stage), figurated erythema (superficial variant) [20]. Hence, there is a need for precise characterization of the inflammatory reaction in grade I rejection after human CTA to better delineate mild rejection from inflammatory skin diseases.

In our study, in depth immunohistochemical investigation of 47 skin biopsies showing grade I skin rejection taken from 11 human hand allografts revealed that the perivascular dermal infiltrate in mild rejection is composed of mainly CD3+ T lymphocytes. This is in line with observations published by other groups [14,19]. In our study, CD8+ T cells showed predominance over CD4+ T cells in grade I skin rejection, which contrasts previous findings [6,21–24]. With progression of rejection, the proportion of CD4+ and CD8+ T cells changed, and CD4+ T cells were predominantly found in severe rejection [12]. About 10% of the infiltrate in grade I rejection was CD68+ macrophages and no or only few B-lymphocytes were detected. In this context, it might be relevant that Clark *et al.* [25] reported a quite large number of T-cells present in normal human skin. It is assumed that these cells may comprise of a skin-specific immune system [26,27].

Capillary C4d deposition is regarded as a valuable marker of antibody-mediated rejection. Our study reveals that 60.6% of all grade I rejection biopsies were positive for C4d staining in the vascular endothelium. This differs from a previous trial, where only 49.1% of samples showed vascular C4d deposits in samples of all rejection grades [12].The C4d deposits were also found in samples without evidence of rejection, however, expression was significantly less. Furthermore, positive C4d staining in grade I rejection was mainly observed in samples taken after at least 1 year post-transplant. This is in contrast to what was observed in nonrejecting skin samples where C4d deposits were mainly detected within the first year after transplantation. In this context, it is important to note that no donor specific alloantibodies were detected at any time-point. In a previous trial, Kanitakis *et al.* [28] reported complete absence of C4d deposition in a series of 60 mucocutaneous biopsy specimens from four patients with CTA obtained 7 days–7 years post-transplant. Recently, however, four C4d positive rejection episodes (grade I and II) were reported in two CTA patients by Landin *et al.* [14] and also detected in the absence of (i) HLA antibody production, (ii) histologic changes of allograft injury, and (iii) clinical rejection, underlining it's unclear clinical utility as surrogate marker of rejection. In solid organ transplantation it is known that different transplanted organs have different susceptibilities to antibody mediated rejection [29]. Troxell *et al.* [30] showed that C4d staining also occurred in biopsies of small intestine allografts in the absence of rejection. Twenty-seven percent of cases were positive for C4d in the capillaries without evidence of rejection, and 36% of cases were found with signs of acute allograft rejection. Recently, Magro *et al.* [31] reported that C4d staining was also observed in inflammatory skin diseases. Positive C4d staining in blood vessels was detected upon vasculopathic conditions (e.g. porphyria and vasculitis), dermatomyositis and systemic lupus erythematosus, Therefore, it may also be hypothesized that C4d deposition in the skin may be an unspecific marker of skin inflammation.

We have previously shown that dermal perivascular inflammation can be observed in approximately 25% of numerous skin biopsies investigated [12]. However, as mentioned, histopathologic findings indicative of grade I rejection does not always correlate with the definitive clinical diagnosis of mild rejection. The present study revealed that 22 of 47 grade I skin biopsies were taken as per protocol while clinical signs for rejection were absent. This is in agreement with a report by Kanitakis *et al.* [32], who had described mild histopathologic signs of rejection with absence of clinical signs of rejection. This makes it difficult for the treating physician to judge if rejection grade I indicates (i) the onset of 'true' rejection with subsequent progression to more severe stages, (ii) a residue of a previous rejection episode, (iii) the presence of an equilibrium between the alloimmune response and a 'tolerogenic' counterresponse, (iv) skin-resident T cells that can be evident to a large number in non-inflamed skin and be recruited upon immunregulation or –modulation [27] or (v) the onset/presence of a nonrejection related disorder. As neither the phenotype of the infiltrate nor the expression of adhesion molecules help to predict the progression of a possible rejection-related process, the dynamics of the clinical observation together with repeat biopsy are the best and most reliable tools helping in decision making. Interestingly, biopsy samples taken after healing in clinically visible rejection have been reported to show persistent perivascular immune cell infiltration consistent with grade I rejection, as in many cases [14]. At present it seems unclear if and how aggressive this persistent subclinical infiltration should be treated. This is similar to patients with atopic dermatitis (AD), where clinically unaffected skin has underlying subclinical inflammation which is thought to account for the recurrent flare-ups. Notably, for patients with AD this has lead to a shift in treatment concepts such as long-term, low-dose, intermittent anti-inflammatory topical therapy with topical corticosteroids or topical calcineurin inhibitors [33,34]. Therefore, if an ongoing subclinical inflammation is routinely observed in hand transplant patients this would favor a continuous topical therapy.

Our observations indicate that adhesion molecules are upregulated during mild skin rejection. Especially expression of LFA-1, ICAM-1, and E-selectin was upregulated in these biopsy specimens when compared with nonrejecting skin samples. Among all adhesion molecules investigated ICAM-1, and E-selectin expression correlated best with severity of rejection. Skin homing of T lymphocytes

mediated by adhesion molecules has been shown to play a central role in the pathomechanism of a variety of inflammatory skin diseases. In psoriatic lesions, for example, upregulation of E-selectin, ICAM-1, VCAM-1, and HLA class II have been reported [35]. Monoclonal antibodies and chemical small molecule inhibitors against these molecules have been developed and introduced as novel options for treatment [36,37]. First efforts by our group to locally block E- and P-selectins in an experimental rat hind-limb transplantation model revealed promising results to overcome skin rejection [12].

A tolerogenic phenotype of a proportion of the infiltrating cells (IDO or Foxp3 expression) was observed during grade I rejection and the percentage of these cells increased at later time-points. This observation suggests that regulatory mechanisms are present within the skin of the allograft and may contribute to self-limitation of the alloimmune response.

In summary, our findings may help to better characterize mild skin rejection after human hand allotransplanation. Findings indicate a T-cell dominated immune response with upregulation of a pattern of adhesion molecules. To fully understand the dynamics of the onset of skin rejection in human hand and face transplantation, further investigations are required.

Authorship

TH: designed research, performed research, analyzed data, and wrote the article. BZ: performed immunohistochemistry (IHC), analyzed data. GB: contributed to research design and data interpretation. HM: reviewed and interpreted IHC data. JG: collected data and performed analysis. BZ: reviewed histology and IHC data. WPAL: contributed to research design, data interpretation, and discussion. PC: contributed to sample collection, review of data, and interpretation. RM: developed research design, helped with data interpretation, supervision. JP: contributed to interpretation and discussion of results. SS: designed research, oversaw the experiments, and wrote the article.

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