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ORIGINAL ARTICLE

Lymphoid neogenesis in skin of human hand, nonhuman primate, and rat vascularized composite allografts

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Summary

The mechanisms of skin rejection in vascularized composite allotransplantation (VCA) remain incompletely understood. The formation of tertiary lymphoid organs (TLO) in hand transplantation has been recently described. We assess this phenomenon in experimental and clinical VCA rejection. Skin biopsies of human (n = 187), nonhuman primate (n = 11), and rat (n = 15) VCAs were analyzed for presence of TLO. A comprehensive immunohistochemical assessment (characterization of the cell infiltrate, expression of adhesion molecules) including staining for peripheral node addressin (PNAd) was performed and correlated with rejection and time post-transplantation. TLO were identified in human, nonhuman primate, and rat skin samples. Expression of PNAd was increased in the endothelium of vessels upon rejection in human skin (P = 0.003) and correlated with B- and T-lymphocyte numbers and LFA-1 expression. PNAd expression was observed at all time-points after transplantation and increased significantly after year 5. In nonhuman primate skin, PNAd expression was found during inflammatory conditions early and late after transplantation. In rat skin, PNAd expression was strongly associated with acute rejection and time post-transplantation. Lymphoid neogenesis and TLO formation can be uniformly found in experimental and human VCA. PNAd expression in vascular endothelium correlates with skin rejection and T- and B-cell infiltration.

Introduction

Ectopic lymphoid neogenesis and the formation of tertiary lymphoid organs (TLO) have been observed during chronic immune stimulation in autoimmunity [1–3] and microbial infection [4,5]. Recently, lymphoid neogenesis and TLO have also been described in solid organ allografts and were strongly associated with chronic rejection [6,7]. Although the mechanism by which TLO contribute to the chronic alloimmune response remains incompletely understood, experimental and clinical data suggest that TLO are local sites of alloantibody production and T-cell activation [8,9].

TLO are well characterized and structured de novo lymphoid tissues resembling the architecture of lymph nodes. They are composed of discrete T- and B-cell accumulations, high endothelial venules (HEV), antigen-presenting cells

and follicular dendritic cells, and occasional germinal centers [10,11]. HEV in peripheral lymph nodes and TLO express peripheral node addressin (PNAd), a glycoprotein which carries the MECA-79 epitope [12] and represents a ligand for L-selectin and α4β7 integrin mediating the extravasation of naïve lymphocytes [10,13]. In solid organ transplantation, the formation of TLO has been observed in kidney [7,8], heart [8], and lung allografts [14]. Presence of TLO closely correlates with chronic rejection and organ failure. This phenomenon has also been observed in animal models of cardiac [6] and vascular [8] transplantation.

Vascularized composite allotransplantation (VCA) is a young discipline with some 100 cases of hand and face transplantations performed to date [15,16]. Acute skin rejection may complicate the postoperative course in VCA and demands close monitoring of the allograft and high-dose immunosuppression (IS) to avoid graft loss. Efforts to understand the pathophysiology of rejection in a VCA identified transmigration and infiltration of alloantigen-specific T-lymphocytes into the skin as a key element of this process [17].

We have previously described a case of B-cell and antibody-mediated rejection (ABMR) after human forearm transplantation [18], where TLO were first discovered in human VCA and corresponded with detection of donor-specific alloantibodies (DSA). We herein investigate the phenomenon of lymphoid neogenesis in skin of human, nonhuman primate, and rat VCA to provide insights into the mechanisms of acute (and chronic) skin rejection. Our data indicate that lymphoid neogenesis and TLO may play a role in experimental and clinical skin rejection.

Materials and methods

Human hand allografts

Patients and sample collection

Six hand or forearm transplantations (five bilateral, one unilateral) were performed in five male and one female patient in Innsbruck, Austria ($n = 4$) or Valencia, Spain ($n = 2$), between 2000 and 2009. Detailed information on the patients, transplantation procedures, clinical courses, IS, complications, and side effects were published elsewhere [19–23]. All patients experienced episodes of acute rejection diagnosed by macroscopic skin alterations and/or histopathology of a skin biopsy. Rejection was successfully treated in all cases. Four-millimeter punch biopsies were collected as 'per protocol' at week 1, 4, 8, 12, month 6, 9, year 1, 1.5, 2, and annually afterward. 'For cause', biopsies were taken whenever rejection was clinically suspected. Skin specimens were collected under local anesthesia (2% lidocaine or 1% mepivacaine) from the skin lesion, if present. One hundred and eighty-seven skin biopsy specimens were analyzed of 11 hand/forearm allografts which were collected over a time-span of 12 years. The study was approved by the institutional review board of the Medical University of Innsbruck (AN 2013-0074 333/4.1).

Histology and immunohistochemistry

Tissue specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Hematoxylin-eosin staining was performed, and sections were graded for rejection as per Banff 2007 classification criteria [24]. In human skin biopsies, immunohistochemical labeling was used for routine markers on paraffin sections according to standard protocols. Adhesion molecules and PNAd were stained according to manufacturer instructions. Information on antibodies used is presented in Table 1. Immunohistochemical findings were read by a blinded pathologist. CD3, CD4, CD8, and CD20 staining was read as percentage of the cellular infiltrate. CD68, Foxp3, IDO, HLA class II-DR, and LFA-1 staining was graded as 0 (0–<10%), 1 (10–50%), and 2 (>50% stained infiltrating cells). Endothelial cell markers (C4d, ICAM-1, E-+P-selectin, VE-cadherin) were assessed as follows (0: no expression/few vessels showing noncircum-

Table 1. Antibodies used for immunohistochemistry.

Immunohistochemical antibody	Company	Dilution
Routine markers		
CD3	Daco, Vienna, Austria	1:100
CD4	Menarini Diagnostics, Vienna, Austria	1:10
CD8	Daco, Vienna, Austria	1:50
CD20	Daco, Vienna, Austria	1:700
CD68	Daco, Vienna, Austria	1:100
HLA class II-DR	Ab-Direct, Oxford, UK	1:300
C4d	Biomedica, Vienna, Austria	1:40
Foxp3	Biocare Medical, Concord, CA, USA	1:50
IDO (indoleamine 2,3-dioxygenase)	Chemicon, Temecula, CA, USA	1:25
Adhesion molecules		
LFA-1 (lymphocyte function-associated antigen-1)	Ab-Direct, Oxford, UK	1:100
ICAM-1 (intercellular adhesion molecule-1)	Novocastra, Newcastle upon Tyne, UK	1:20
E-selectin	Novocastra, Newcastle upon Tyne, UK	1:20
P-selectin	Novocastra, Newcastle upon Tyne, UK	1:25
VE-cadherin	AbD serotech, Oxford, UK	1:100
Psoriasin	Novocastra, Newcastle upon Tyne, UK	1:50
Lymphoid neogenesis		
PNAd (MECA-79 antibody)	Novus biologicals, LTD, Cambridge, UK	1:100

ferential staining; 1: mild staining in the majority of vessels; 2: intense, circumferential staining in most vessels). PNAd staining was graded for percentage of vessels staining positive (0: no expression; 1: 1–10% positive vessels; 2: 11–50% positive vessels; 3: >50% positive vessels) and staining intensity (0: no staining; 1: mild staining, 2: intense, noncircumferential staining; 3: intense, circumferential staining). PNAd expression was correlated with rejection severity, composition of the cell infiltrate, expression of adhesion molecules, and time after transplantation (very early: first 3 months; early: 3 months to 1 year after surgery; late: 1 year to 5 years after surgery; very late: after year 5).

Small and large animal vascularized composite tissue allografts

Nonhuman primate model of heterotopic facial segment allotransplantation

Cynomolgus monkeys (*Macaca fascicularis*, male and female) weighing between 2.8 and 8 kg were used for facial VCA. Matching of donor/recipient pairs was performed by ABO blood group compatibility and HLA class I mismatch. The transplant procedure was developed by Barth *et al.* [25] and described elsewhere. IS consisted of daily tacrolimus (trough blood level 30 ng/ml for 28 days and 15–25 ng/ml afterward) and mycophenolate mofetil (MMF, 50 mg/kg). Allograft skin biopsies were taken under propofol sedation with a 4-mm punch. A total of 11 skin biopsies collected from 11 transplanted animals between postoperative day (POD) 15 and 364 were analyzed.

Orthotopic rat hind-limb allotransplantation model

For rat hind-limb allotransplantation, a full MHC-mismatch model (Brown Norway to Lewis male rats, 200 g) was utilized. The procedure was carried out as described previously [26]. A total of 15 hind-limb transplantations were performed. IS included cyclosporine A (CsA, 10 mg/kg for 21 days) ± induction with antilymphocyte serum (ALS) on days -4 and +1 (0.5 ml) and IL-2 (1 μg/day). Fifteen skin and muscle specimens taken between POD 21 and 150 were included.

All research protocols were approved by the local (University of Maryland School of Medicine and University of Pittsburgh) Institutional Animal Care and Use Committee. Experiments were performed in accordance with the National Research Council' Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act.

Histology and immunohistochemistry

Nonhuman primate and rat samples were fixed in 4% paraformaldehyde. Hematoxylin-eosin staining was performed on paraffin embedded samples and read by a blinded pathologist. Rejection severity was assessed according to the Banff 2007 guidelines [24]. Immunohistochemical staining was performed for endothelial expression of PNAd (dilution 1:100; Novus biologicals, Cambridge, UK) according to manufacturer instructions. PNAd staining was graded as follows: 0: no expression; 1: mild staining in few vessels; 2: mild staining in >10% of vessels; 3: intense staining in >10% of vessels. PNAd expression was correlated with rejection, IS, and time post-transplantation.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). Correlation analyses were performed according to Spearman. Analysis of variance (ANOVA) was utilized to compare the differences between groups, and Student's *t*-test was used if appropriate. Correction of multiple comparisons was performed according to Bonferroni. A *P*-value of <0.05 was considered statistically significant.

Results

Human study

PNAd expression suggests lymphoid neogenesis in skin of human hand allografts

Of 187 allograft skin biopsies, 83 samples (44.4%) were collected per protocol and 104 (55.6%) for cause. Immunohistochemistry using the MECA-79 antibody revealed 52 samples (27.8%) with PNAd+ vessels, whereas 135 samples (72.2%) did not show endothelial PNAd expression. Of those samples staining positive for PNAd, 43 (82.6%) showed expression grade 1 (=1–10% PNAd+ vessels), and 9 (17.4%) grade 2 (=11–50% PNAd+ vessels). As a control, vessels were negative for PNAd staining in all recipient skin samples investigated (Fig. 1a). PNAd staining was observed in allograft vessels of the superficial and deep dermis, and occasional in venules and capillaries. While the PNAd+ vessels were accompanied by a diffuse cell infiltrate or single infiltrating cells located around the vessels in most cases (Fig. 1b and c), three samples showed typical nodular cell infiltrates, which were composed of T- and B-lymphocytes. These nodular infiltrates were located in the deep dermal compartment. A germinal center and lymphatic vessels associated with these structures were not observed. PNAd staining intensity was variable (Fig. 1d and e). Twenty-four samples (46.2%) were scored with 1 (=faint/mild, circumferential or noncirumferential), 24 samples (46.2%) with 2 (=intensive, noncircumferential), and four samples (7.6%) with 3 (=intensive, circumferential).

PNAd expression is increased during and correlates with acute rejection

In human skin biopsies, rejection ranged between grade 0 and IV (mean 0.76 ± 0.075). While 103 samples (55.1%)

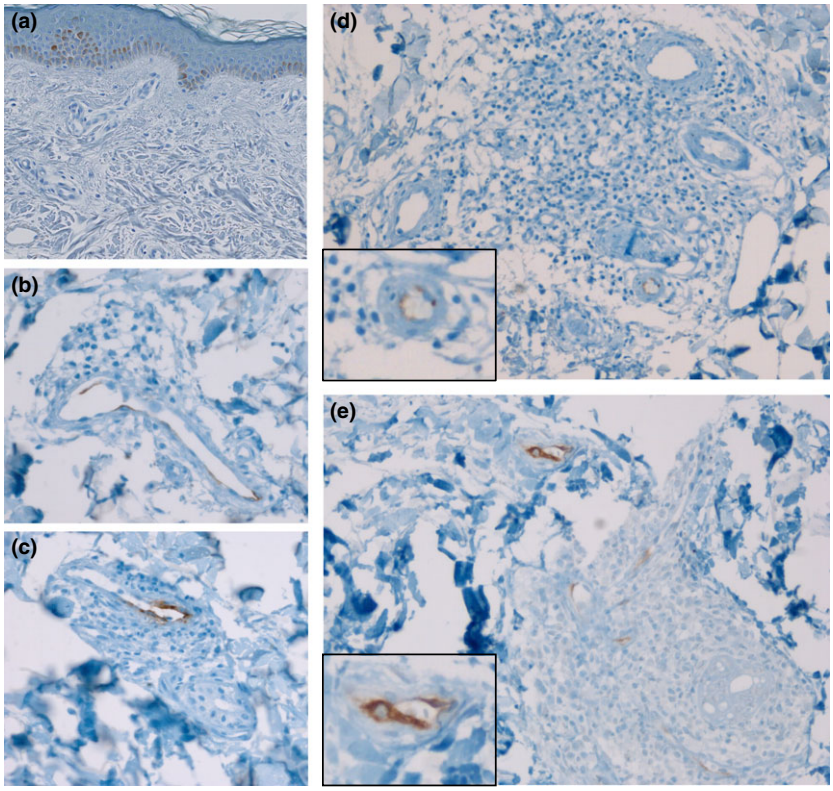

Figure 1 PNAd staining using the MECA-79 antibody in skin of human hand allografts. In recipient skin of hand transplanted patients, PNAd staining was negative in dermal vessels (a, at 9 years post-transplant, ×20). In some cases, only mild PNAd staining of single endothelial cells was observed (b, rejection grade I, ×40), while others showed intensive, but noncircumferential PNAd staining of dermal vessels (c, rejection grade I, ×40). Most often, PNAd positive vessels were accompanied by a diffuse cellular infiltrate, which mainly consisted of lymphocytes (faint staining: d, grade II, ×40; faint and intense staining: e, grade I, ×40). PNAd positive vessels were found in the superficial and deep dermis.

did not show histopathological signs of rejection, 45 samples (24.1%) exhibited grade I, 24 samples (12.8%) grade II, 11 samples (5.9%) grade III, and four samples (2.1%) grade IV. Table 2 shows the numbers of skin samples for PNAd scoring for all rejection grades. Overall, endothelial PNAd expression in dermal vessels was significantly increased during rejection (grades I–IV), compared to normal skin (nonrejection: 0.21 ± 0.05 vs. rejection: 0.49 ± 0.08; $P = 0.003$, Fig. 2a). In fact, the highest increase was observed in samples during rejection grades II and IV (grade I: 0.33 ± 0.08 vs. grade II: 0.75 ± 0.19 vs. grade III: 0.45 ± 0.16 vs. grade IV: 0.75 ± 0.48; Fig. 2b), which was statistically significant for grade II samples ($P = 0.001$). Also, PNAd staining intensity was stronger in rejecting skin samples (nonrejection: 0.33 ± 0.07 vs. rejection: 0.60 ± 0.09; $P = 0.026$, Fig. 2c). Again, PNAd staining intensity was increased during all rejection grades, with the highest increase in grade II samples (grade 0: 0.33 ± 0.07 vs. grade I: 0.44 ± 0.12 vs. grade II: 0.83 ± 0.21 vs. grade III: 0.73 ± 0.27 vs. grade IV: 0.50 ± 0.29, Fig. 2d). All of the three biopsies showing a typical nodular cell infiltrate revealed acute rejection grade II and most intensive endothelial PNAd staining of the vessels within the nodular cell structures. Furthermore, correlation analysis was performed between the percentage of PNAd+ vessels and rejection severity. A significant correlation at the 0.01 level [correlation coefficient (cc) = 0.249] was found between PNAd staining and rejection. In summary, endothelial PNAd expression and staining intensity were increased in human skin samples during acute allograft rejection and correlated with acute rejection.

PNAd expression correlates best with the presence of T- and B-lymphocytes and the adhesion molecule LFA-1

PNAd expression was correlated with markers characterizing the cellular infiltrate (CD3, CD4, CD8, CD20, CD68, and HLA class II-DR), markers indicative for ABMR (CD20 and C4d), tolerance markers (IDO and Foxp3), and adhesion molecules (LFA-1, ICAM-1, E-selectin, P-selectin, VE-cadherin, and Psoriasin). Strongest correlations were observed for CD3+ T-lymphocytes

Table 2. Relationship between PNAd expression/PNAd staining intensity and rejection severity.

		PNAd expression		PNAd staining intensity	
Rejection grade	Score	Number	%	Number	%
Grade 0	0	83	80.6	83	80.6
	1	18	17.5	7	6.8
	2	2	1.9	12	11.7
	3	0	0.0	1	1.0
Grade I	0	32	71.1	32	71.1
	1	11	24.4	7	15.6
	2	2	4.4	5	11.1
	3	0	0.0	1	2.2
Grade II	0	12	50.0	12	50.0
	1	8	33.3	6	25.0
	2	4	16.7	4	16.7
	3	0	0.0	2	8.3
Grade III	0	6	54.5	6	54.5
	1	5	45.5	2	18.2
	2	0	0.0	3	27.3
	3	0	0.0	0	0.0
Grade IV	0	2	50.0	2	50.0
	1	1	25.0	2	50.0
	2	1	25.0	0	0.0
	3	0	0.0	0	0.0

(cc = 0.247) and CD20+ B-lymphocytes (cc = 0.268). Among the adhesion molecules, LFA-1, expressed on infiltrating lymphocytes, correlated best with PNAd expression (cc = 0.256). Correlation coefficients for all markers are shown in Table 3. When correlation analysis was performed only for PNAd+ samples, an even stronger correlation was found between PNAd expression and CD20+ B-lymphocytes (cc = 0.613). Also, C4d expression correlated well with PNAd expression (cc = 0.299), suggesting an association between PNAd expression and an antibody-mediated immune response. In PNAd+ samples, a correlation was also observed with number of macrophages, CD68+ (cc = 0.405), and expression of the adhesion molecule P-selectin (cc = 0.349, Table 3).

PNAd expression is significantly increased after 5 years post-transplant

Of 187 samples, 44 biopsies (23.5%) were collected during the very early postoperative period. Forty-three samples (23.0%) were taken early, 77 samples (41.2%) late, and 23 samples (12.3%) very late after transplantation. PNAd expression was observed at all time-points after human hand transplantation. A significant increase of PNAd+ vessels was observed very late after transplantation (very early: 0.32 ± 0.09 vs. early: 0.21 ± 0.07 vs. late: 0.27 ± 0.05 vs. very late: 0.83 ± 0.21, Fig. 3a). This phenomenon holds also true for PNAd staining intensity (very early: 0.45 ± 0.12 vs. early: 0.30 ± 0.10 vs. late: 0.36 ± 0.07 vs. very late: 1.00 ± 0.24, Fig. 3b). In fact, 12 of 23 samples (52.2%) taken very late showed endothelial PNAd expression at various degrees. Expression ranged from only mild,

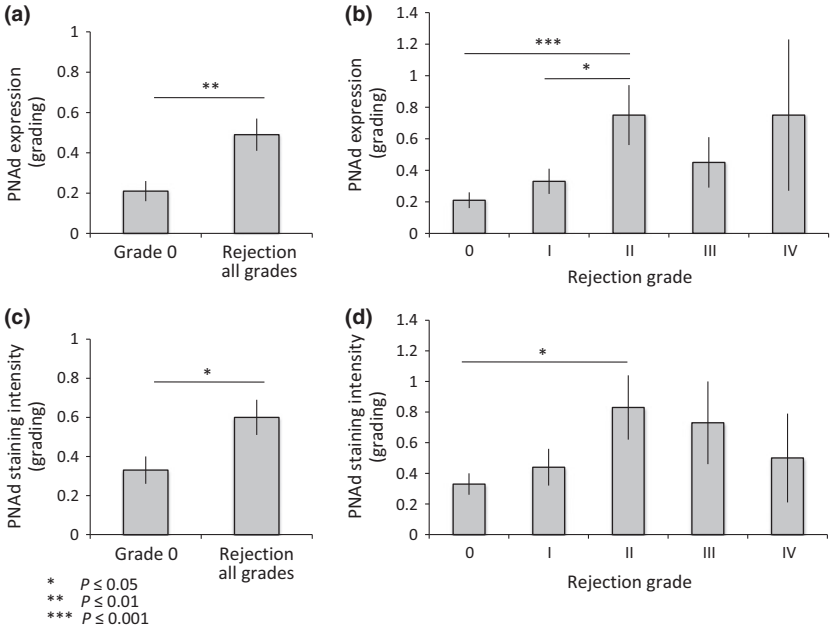

Figure 2 Lymphoid neogenesis after human hand transplantation is associated with acute rejection. PNAd expression was found significantly increased during skin rejection of human hand allografts, when compared to nonrejected skin (a). PNAd expression was increased during all stages of rejection (grade I–IV). The most prominent, statistically significant increase was found during grade II rejection (b). PNAd staining intensity was as well increased during rejection (c). Again, PNAd staining intensity was increased during all rejection grades, compared to grade 0, and strongest during grade II rejection (d).

Table 3. Correlation between PNAd expression and rejection markers, adhesion molecules and tolerance markers.

Marker	All samples Correlation coefficient	PNAd+ samples Correlation coefficient
CD3	0.247	−0.190
CD4	0.176	0.255
CD8	−0.118	−0.004
CD20	0.268	0.613
CD68	0.049	0.405
HLA class II-DR	0.084	0.190
C4d	0.125	0.299
LFA-1	0.256	0.192
ICAM-1	0.047	0.110
E-selectin	−0.013	0.159
P-selectin	0.068	0.349
VE-cadherin	0.025	0.274
Psoriasin	0.131	−0.036
IDO	0.131	0.271
Foxp3	0.113	0.244

noncircumferential to very strong and circumferential staining.

Small and large animal studies

PNAd expression in skin of nonhuman primate facial VCAs

Eleven skin biopsy samples from 11 transplanted animals collected between days 15 and 364 were analyzed for vascular PNAd expression. In three of 11 samples (27.3%), endothelial PNAd expression in HEV-like vessels was observed, whereas eight samples (72.7%) were negative for PNAd staining. PNAd staining was observed early and late after transplantation in samples with moderate (grade II rejection) to severe inflammation. Animal #1 had to be sacrificed on POD 15 due to technical graft failure. No acute rejection was recorded. Upon graft procurement, the allograft skin showed severe signs of hypoperfusion, massive infiltration of granulocytes, thrombotic vessels, and necrosis (Fig. 4a). Intensive, circumferential endothelial PNAd staining was observed in vasa vasorum around a larger thrombotic vessel in the deep tissue (Fig. 4b). Animal #4 lost its graft due to acute rejection on POD 36. Histology revealed severe rejection (grade IV). Intensive PNAd staining was found in HEV-like vessels of a lymphocytic cell aggregate (Fig. 4c and d). Animal #10 experienced two episodes of acute rejection until POD 323. After cessation of IS and subsequent acute rejection, intensive stained PNAd+ HEV-like vessels were observed in lymph-node-like structures in the subcutaneous tissue on POD 332 (Fig. 4e and f). Table 4 provides an overview on tissue sampling time-points, IS, acute rejection episodes, histology, and PNAd staining of all nonhuman primate samples analyzed.

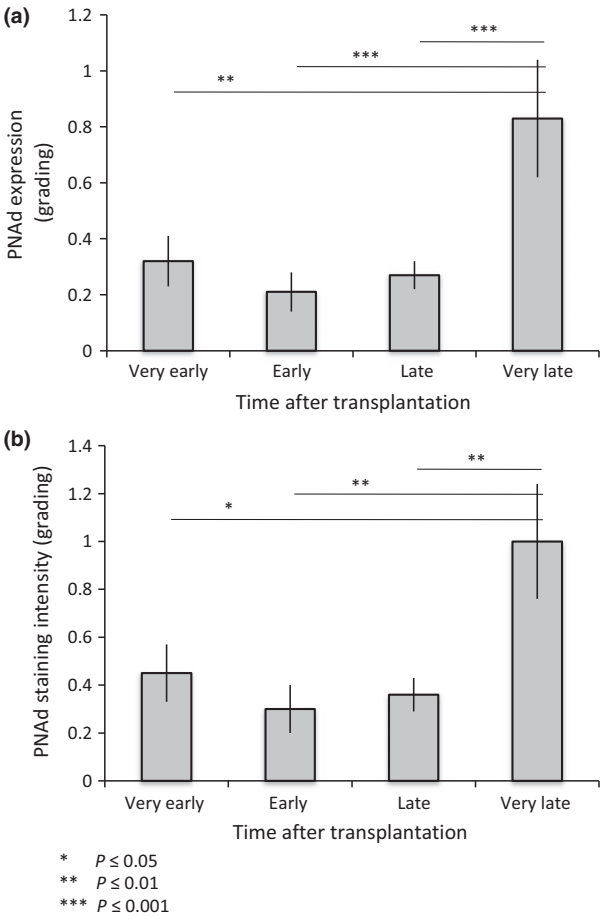

Figure 3 Lymphoid neogenesis in skin of human hand allografts at various time-points post-transplantation. Endothelial PNAd expression was significantly increased in biopsies taken at 5 years or later after hand transplantation (very late), when compared to biopsies taken within the first 3 months (very early), between month 3 and the first year (early) and between year 1 and 5 (late) after transplantation (a). A similar tendency was observed for PNAd staining intensity, which was found to be strongest in samples taken very late (b).

Acute skin rejection in rat VCAs is associated with PNAd expression

PNAd expression was evaluated in 15 skin samples collected from 15 rat hind-limb allografts at time-points ranging between POD 21 and 150. Additional information on skin biopsy samples with respect to IS, the number of acute rejection episodes, and the rejection grade at the sampling time-point is provided in Table 5. While PNAd expression was not found in isograft control skin ($n = 5$; Fig. 5a) or in normal, nonrejecting allograft skin (Fig. 5b), it was significantly increased in samples with rejection (nonrejection 0.00 ± 0.00 vs. rejection 1.64 ± 0.09; $P = 0.008$; Fig. 5c–e). To evaluate PNAd positivity with regard to time post-transplantation, skin biopsies were classified either as 'early' biopsies (taken before POD 50, $n = 7$) or as 'late' biopsies

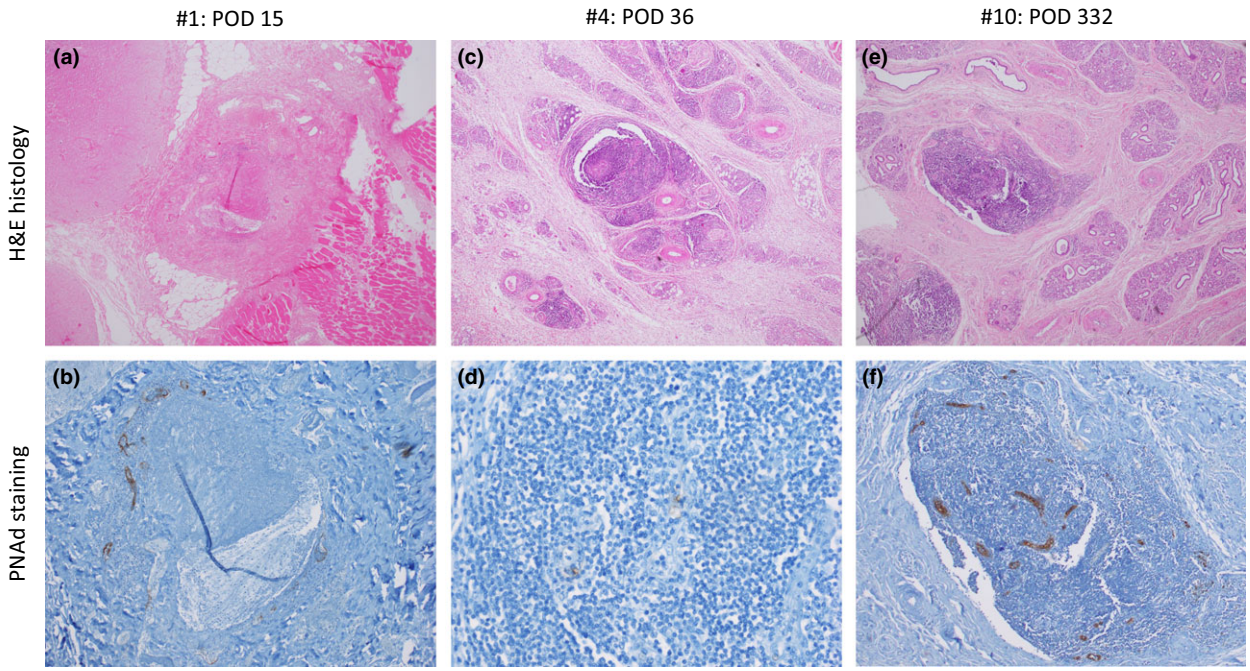

Figure 4 PNAd expression in skin of nonhuman primate facial vascularized composite allografts. In three of 11 animals, endothelial PNAd expression was observed, which was related to moderate to severe rejection and inflammation. Animal #1 lost its allograft due to technical failure on POD 15. Histology showed severe necrosis, infiltration of granulocytes, and signs of hypoperfusion (a, ×4). The vasa vasorum stained positive for PNAd around a larger thrombotic vessel (b, ×10). Animal #4 revealed grade IV rejection on POD 36, and histology showed a lymphocytic accumulation in the deep subcutaneous tissue (c, ×4). PNAd staining was found in small HEV-like vessels within the lymphoid-like structure (d, ×40). In the biopsy of animal #10, two lymphocytic cell aggregates were found during grade II rejection on POD 332 (e, ×4) with intensively stained PNAd+ HEV-like vessels (f, ×10).

Table 4. Characteristics of nonhuman primate VCA samples.

Animal	Tissue sampling POD	IS	AR episodes	Rejection grade	PNAd score	Comments
1	15	tac/MMF	0	IV	2	Technical graft failure
2	23	tac/MMF	1	IV	0	Graft without vascularized bone marrow – lost to AR
3	24	tac/MMF	0	I	0	Technical graft failure
4	36	tac/anti-CD28	1	IV	1	Graft lost to AR
5	37	tac/MMF	0	I	0	Technical graft failure
6	42	tac/MMF	1	IV	0	Graft without vascularized bone marrow – lost to AR
7	58	tac/MMF	0	I	0	Animal sacrificed due to weight loss
8	93	tac/MMF	0	II	0	Animal sacrificed due to ureteral obstruction
9	104	tac/MMF	0	I	0	Animal sacrificed due to weight loss
10	332	tac/MMF	2	II	3	IS withdrawn on POD 323, AR
11	364	tac/MMF	2	IV	0	IS withdrawn on POD 322, AR

POD, postoperative day; IS, immunosuppression; AR, acute rejection; Tac, tacrolimus; MMF, mycophenolate mofetil.

(taken on or after POD 50, $n = 8$). A significant increase of PNAd expression was found in late biopsies, compared to early biopsies (early: 0.29 ± 0.11 vs. late: 2.00 ± 0.09; $P < 0.001$; Fig. 5f). While only one early-taken biopsy was positive for PNAd expression, all of the eight late-taken biopsies showed PNAd+ HEV-like vessels to various extent in the dermis (Table 5). No clear correlation was observed for PNAd expression and IS in a limited number of animals. Endothelial PNAd staining was also observed in the underlying muscle of those animals with vascular PNAd

Table 5. Characteristics of rat VCA samples.

Animal	Tissue sampling POD	IS	AR episodes	Rejection grade	PNAd score
1	21	CsA (21ds)	0	I	0
2	21	CsA (21ds)	0	I	0
3	21	ALS D-4, 1, CsA (21Ds)	0	0	0
4	21	ALS D-4, 1, CsA (21Ds)	0	0	0
5	21	ALS D-4, 1, CsA/IL-2 (21Ds)	0	0	0
6	21	ALS D-4, 1, CsA/IL-2 (21Ds)	0	0	0
7	37	ALS D-4, 1, CsA/IL-2 (21Ds)	0	I	2
8	55	ALS D-4, 1, CsA/IL-2 (21Ds)	1	III	1
9	60	ALS D-4, 1, CsA/IL-2 (21Ds)	1	III	2
10	60	ALS D-4, 1, CsA/IL-2 (21Ds)	1	III	2
11	80	ALS D-4, 1, CsA/IL-2 (21ds) + top tac Ds 21-60	0	I	3
12	115	ALS D-4, 1, CsA/IL-2 (21Ds)	1	II	2
13	125	ALS D-4, 1, CsA/IL-2 (21Ds)	1	III	2
14	150	ALS D-4, 1, CsA/CD200 (21Ds)	0	I	3
15	150	ALS D-4, 1, CsA/CD200 (21Ds)	0	I	1

POD, postoperative day; IS, immunosuppression; AR, acute rejection; CsA, cyclosporine A; ALS, antilymphocyte serum; D, day; top tac, topical tacrolimus.

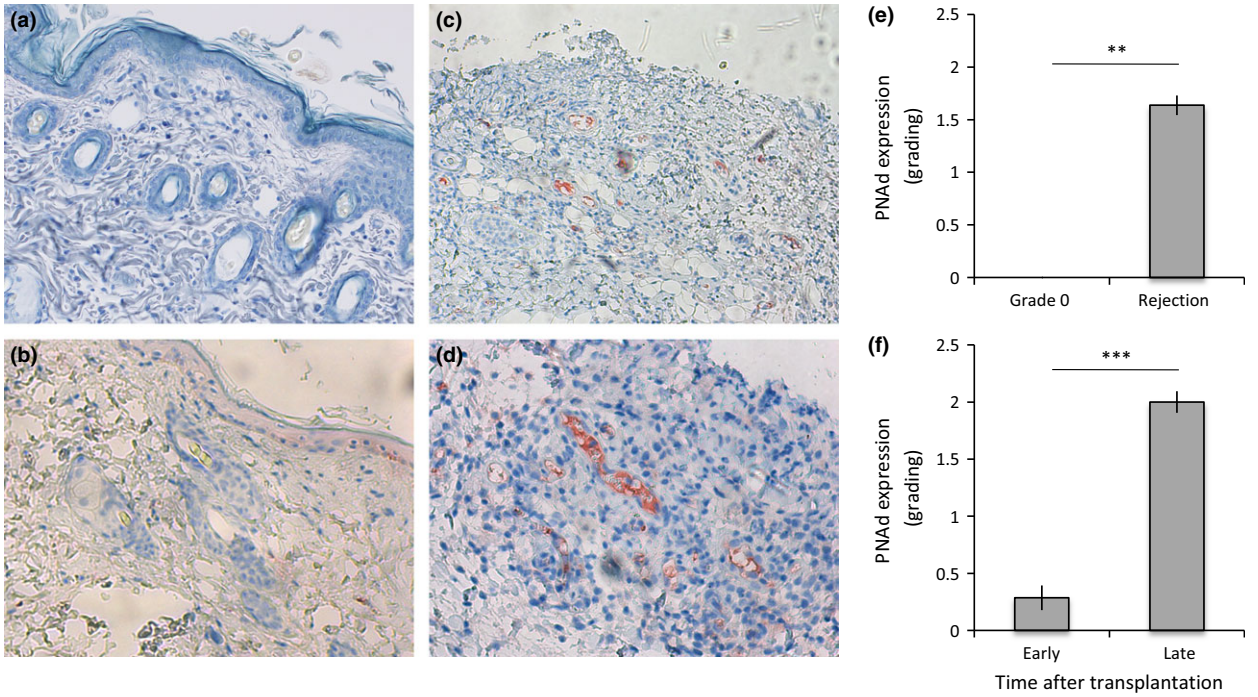

Figure 5 PNAd expression in skin of rat hind-limb vascularized composite allografts. PNAd expression was not found in skin of rat hind-limb isografts (a, ×20) or allografts without any signs of rejection (b, ×20, sample taken on POD 21). Upon acute rejection, intensive PNAd staining was observed in the endothelium of vessels and capillaries (c, ×10; d ×40). Analysis of 15 rat allograft skin biopsies showed that PNAd expression was restricted to rejecting samples and significantly upregulated during acute allograft rejection (e). Similar to human skin biopsies, PNAd expression was significantly increased in biopsies taken late (on or after POD 50) post-transplantation (f).

expression in skin samples (data not shown). In summary, the data suggest an association of PNAd expression with acute rejection and time post-transplantation in rat VCA, indicative for lymphoid neogenesis.

Discussion

We herein report PNAd expression, lymphoid neogenesis, and formation of TLO in skin of human hand allografts.

PNAd expression in vascular endothelial cells correlated with acute rejection and was increased with time post-transplantation. PNAd expression was also observed during inflammatory conditions, mainly acute rejection, in nonhuman primate and rat VCA.

De novo induction of endothelial L-selectin ligands (e.g., PNAd), which recruit lymphocytes to inflamed tissues by mediating cell extravasation, has first been described in the course of acute rejection of solid organ allografts by Renkonen and colleagues. Using the MECA-79 antibody, they found a strong correlation between PNAd expression and acute rejection of human heart [27] and kidney [28] allografts. The authors report PNAd staining in capillaries and venules, but they did not observe classical TLO, which is a highly organized form of lymphoid neogenesis (lymphoid-like structure with defined B- and T-cell zones and PNAd+ HEV). This highly organized form of lymphoid neogenesis was only observed in a small set of biopsies ($n = 3$) taken from our human hand allografts. In a report on lymphoid neogenesis in murine cardiac allografts undergoing chronic allograft rejection, Baddura *et al.* [6] showed that endothelial PNAd expression was either observed in HEV of organized TLO or lacking organized lymphoid accumulations, as observed in the majority of our PNAd+ human skin samples. The formation of *de novo* lymphoid tissue with PNAd+ HEV, but without organized TLO, was also shown in chronically rejected lung allografts [14]. Interestingly, the degree of tertiary lymphoid formation has been shown to be related to the immune activation status of the host [29]. Therefore, it can be speculated that those recipients of organ or vascularized composite tissue allografts with a highly activated immune status more likely develop a well-organized form of lymphoid neogenesis. This hypothesis is supported by the fact that most well-organized TLO described in the literature were found during chronic allograft rejection, which reflects an ongoing, chronic state of immune activation, whereas a more disorganized form of lymphoid neogenesis was also associated with acute rejection.

When further investigating characteristics associated with PNAd staining in human hand transplantation, we observed highest correlations for the presence of T- and B-lymphocytes and PNAd expression. As T-lymphocytes have been identified as key players during acute skin rejection after VCA [17,30], our finding that lymphoid neogenesis is associated with cellular rejection fits well with this idea. Best correlation was observed for B-lymphocytes, which is interesting, because B-lymphocytes are usually only present at a very small number during rejection of a VCA. Moreover, presence of CD20+ B-lymphocytes correlated at a very high level with PNAd staining. Correlation was also observed for C4d staining, an established marker for ABMR in kidney transplantation. Little is known about ABMR and the relevance of DSA, B-lymphocytes, and C4d staining in VCA. This specific type of rejection has been recently described in a patient after forearm transplantation [18]. Nine years after bilateral forearm transplantation, the patient developed allograft rejection and tested positive for presence of DSA for the first time. Large lymphoid aggregates predominantly composed of B-lymphocytes with PNAd+ HEV-like vessels typical for classical TLO formation were found in the deep dermis. Dermal vessels also stained positive for C4d. The patient was treated with the monoclonal CD20 antibody rituximab and has been free of rejection since then. There is growing evidence that the formation of ectopic lymphoid tissue supports a local humoral immune response. In a rat aortic interposition model, it was shown that lymphoid nodular infiltrates of chronically rejected aortic allografts produced antibodies directed against donor MHC-I molecules [8]. Local alloantibody production in TLO was also observed in human renal allografts, which had to be retrieved after chronic ABMR [31]. Moreover, it has been shown that blocking lymphotoxin signaling, a pathway essential for the development and maintenance of lymphoid tissue and TLO, inhibits TLO formation in murine cardiac allografts and abrogates effector humoral responses [32]. Based on our observations, we speculate that PNAd expression and lymphoid neogenesis might be indicative for ABMR in VCA and can be targeted pharmacologically.

While the diagnosis of chronic rejection is well defined in solid organ transplantation, little is known about chronic rejection in VCA. Lacking a clear definition of chronic allograft rejection in VCA, it is far-fetched to establish any correlation between PNAd expression and chronic rejection in our samples. Allograft vasculopathy, as the main feature of chronic rejection in solid organ allografts, has only been reported by one center performing clinical VCA [33]. In a rat model of hind-limb transplantation, Unadkat *et al.* [34] demonstrated that multiple acute rejection episodes lead to vasculopathy and muscle fibrosis. It is hypothesized that multiple (untreated) acute rejection episodes imitate a state of chronic inflammation, which may trigger myointimal proliferation and occlusion of allograft vessels. Recently, Mundinger and colleagues have shown in face [35] and fibula [36] transplant models of nonhuman primates that long-term survivors had typical features of chronic rejection, such as neointimal proliferation, vasculopathy, fibrosis of vessel walls, luminal occlusion, and presence of TLO. In contrast to our findings, TLO were found in the absence of acute rejection. Even if PNAd expression after human hand transplantation was found at all time-points after transplantation, it is interesting that PNAd expression was significantly increased in biopsies 5 years following

transplantation. This observation supports the findings of Mundinger *et al.* [35] that a chronic inflammation status might be present in these cases.

While the results of this study clearly demonstrate PNAd expression and evidence for lymphoid neogenesis/TLO as well as a correlation with acute rejection after VCA, our findings do not allow conclusions regarding the functionality of the cells and structures involved. Especially B-lymphocyte clusters have been described to act like functional ectopic germinal centers under specific conditions, triggering an aggressive local humoral immune response [37]. Furthermore, it has been shown that effector and memory immune responses are generated within tertiary lymphoid tissues, suggesting that intragraft TLO are indeed immunologically active and contribute to the rejection process [9]. Our study is limited by the fact that the tissue samples were analyzed retrospectively and that in some cases, only small pieces of tissue were available. As discussed by Thaunat [38], methods for analysis are limited in such small biopsy samples and the methods used in our study do not allow to draw conclusions regarding the functionality of TLO. Functional assays, however, often require larger pieces of tissue. In addition, TLO formation is suggested to be a patchy process and it is most likely that TLO formation may be missed when collecting a biopsy at a defined location. Hence, our data do not clarify the quantitative distribution and extent of lymphoid neogenesis in an entire hand allograft or allow to judge whether the process is restricted to certain areas or spotted over the whole allograft.

In essence, the correlation between PNAd expression, acute rejection, chronic rejection (or chronic inflammation), and time after transplantation is nonexclusive in our view. PNAd expression and/or TLO formation may be a consequence of a repeat and/or chronic cellular and/or antibody-mediated immune response. We therefore remain cautious with our conclusions indicating that we believe that the presence of PNAd and TLO is meaningful. The pathogenesis may not be exclusive for one cause or the other. In depth, studies are needed to further assess the role and function of lymphoid neogenesis during acute and chronic rejection/inflammation in VCA.

Authorship

TH: designed research, performed research, analyzed data, wrote the article. BGZ and BZ: analyzed histology and immunohistochemistry. IWN: performed rat experiments. GSM, RNB and EDR: performed nonhuman primate experiments. GB: contributed to review of data and data interpretation. AW and PC: contributed to human data collection. RM: contributed to research design and data interpretation. WPAL and JP: contributed to data interpretation and discussion. FGL: contributed to research design, data interpretation, and discussion. SS: designed research, supervision, wrote the article.

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