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CASE REPORT

Nonvascularized human skin chronic allograft rejection

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A 65-year-old man had extensive burns of the lower legs in 1991, at the age of 40 years. He was treated by nonvascularized and de-epithelialized, allogeneic split-thickness skin allograft and cyclosporine monotherapy for 2 months. Ulcers developed between 10 and 25 years after transplantation and a surgical debridement on the lower extremities was required. Analyses of the removed tissue allografts showed chronic antibody-mediated and cellular rejection with extensive and dense fibrosis, and diffuse capillary C4d deposits. An anti-DRB1*08:01, donor-specific antibody was present. A unique clinical condition with late immunopathological features of human skin chronic allograft rejection is reported.

KEYWORDS

accommodation, alloantibody, clinical research/practice, ethics and public policy, organ transplantation in general, pathology/histopathology, rejection: antibody-mediated (ABMR), surgical technique, tolerance, vascularized composite and reconstructive transplantation

1 | INTRODUCTION

In the history of modern transplantation and transplantation immunology, skin grafting has had a special place since the initial scientific studies of T. Gibson and P. Medawar involving human skin allografts during World War II.[1] Indeed, the analysis of human skin allograft rejection in a patient with extensive burns indicated that human tissue rejection was an immunologic phenomenon. These observations were undoubtedly a key stimulus for P. Medawar and others to subsequently develop the entire field of transplantation immunology.

Currently, total skin allografts are generally considered vascularized composite allotransplantation (VCA).[2-5] The normal epidermis has numerous dendritic cells, which play a significant role in rejection.[3] This characterizes the skin as one of the most immunogenic tissues and VCA as one of the most challenging types of allografts,[2,3,5] and skin allografts are virtually always rejected unless adequate long-term immunosuppression is administered.[2,6] With current immunosuppressive regimens, solid organ transplantation (SOT) rejections are currently relatively well controlled, but the development of circulating anti-HLA donor-specific antibody (DSA) in the recipient plays a major role in the late loss of SOT.[7,8]

VCA has emerged as a possible option to treat patients who have lost their arms, face, or who have had large body skin defects.[2,9] The management of the recipients after VCA includes skin biopsies, and grades of rejection are diagnosed according to the Banff classification.[10]

Abbreviations: AMR, antibody-mediated rejection; CEA, cultured epithelial autograft; CyA, cyclosporine A; DNA, deoxyribonucleic acid; DSA, donor-specific antibody; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; MFI, mean fluorescence intensity; PCR, polymerase chain reaction; PCR-SSO, polymerase chain reaction and sequence specific oligonucleotide; PRA, panel reactive antibody; PRA-CDC, panel reactive antibody and complement-dependent microcytotoxicity assay; SOT, solid organ transplantation; STSG, split-thickness skin allograft; VCA, vascularized composite allotransplantation.

Samuel Rotman and Nathalie Koch contributed equally as first authors; Wassim Raffoul and Manuel Pascual contributed equally as last authors.

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Skin autografts are standards of care for the management of burned patients.[11] In addition, nonvascularized and de-epithelialized, split-thickness skin allograft (STSG) have been used as temporary substitutes to provide a complete functional restoration in severe cases of extensive full-thickness burn injury.[12-14] STSGs thus lack donor vasculature at the outset, which may influence the nature of the rejection process. In such cases, short-term cyclosporine (CyA) treatment has been administered to prevent rejection.

We present here the late clinical and immunopathological features of a severely burned patient who successfully received nonvascularized allogeneic STSG at the age of 40 years, but who eventually developed progressive and severe chronic allograft rejection of the skin. The characteristics of this case represent a distinct long-term clinical condition that has not been described previously.

2 | MATERIALS AND METHODS

2.1 | Clinical data

In 1991, a 40-year-old man had severe burns, involving 73% of his total body surface area (Figure 1A). Allogeneic STSG transplantations (allograft STSG) were used to cover his lower extremities. The nonvascularized allogeneic STSG originated from a brain-dead human immunodeficiency virus–negative elderly female organ donor, after informed consent was obtained from the family (personal communication, Dr L. Wiesner, Lausanne, Switzerland, September 12, 2017).[13] The skin allograft was composed of epidermis, dermis, and some very limited hypodermis tissue. No vascular structure from the allograft was isolated (no vascular anastomoses were performed). The skin graft was placed directly on the recipient legs after debridement. The skin allograft was then de-epithelialized using a shaver 18 days later. There was no spontaneous de-epithelialization. Cultured epithelial autografts (CEA) were prepared in the CHUV Dermatology tissue culture laboratory. The de-epithelialized allogenic STSG was covered by CEA 2 days and 14 days after de-epithelialization for the right and left legs, respectively. Written informed consents were obtained from the patient and his wife for publication of this case report and any accompanying images.

The patient received a total of 35 blood transfusions. The mid-term results of this case have been reported in 1994.[13] The immunosuppressive regimen only consisted of CyA monotherapy during the first 2 months after transplantation.

During the first 4 months, a total of 5 punch biopsies were performed. Each of them showed acute cellular rejection on both legs. Early signs of fibrosis within dermis and hypodermis of the skin allograft were observed after 6 weeks. No C4d deposits were demonstrated on these first biopsies. The first sign of epithelialization of the epidermis was observed 20 days after the placement of CEA on the skin allograft. The surface of the skin allograft was completely covered by an autograft epidermis 2 months after the placement of CEA. After 12 months, superficial ulcers were noted and, thereafter, the skin showed decreased elasticity by palpation. Ten years after transplantation, new small ulcers developed on his lower extremities, which were treated conservatively (Figure 1B). Eventually, 25 years after transplantation, his skin texture had progressively become thicker, significant larger ulcers developed, and a surgical debridement on lower extremities (Figure 1C) was required. Allograft rejection was the likely cause of his leg ulcers. Cultures and all histological analyses for fungi were negative. Microbiology cultures were also negative. There were no clinical arguments for pyoderma gangrenosum.

Unfortunately, in the ensuing months the patient developed refractory ischemic cardiac failure, and he died at home at the age of 66 years.

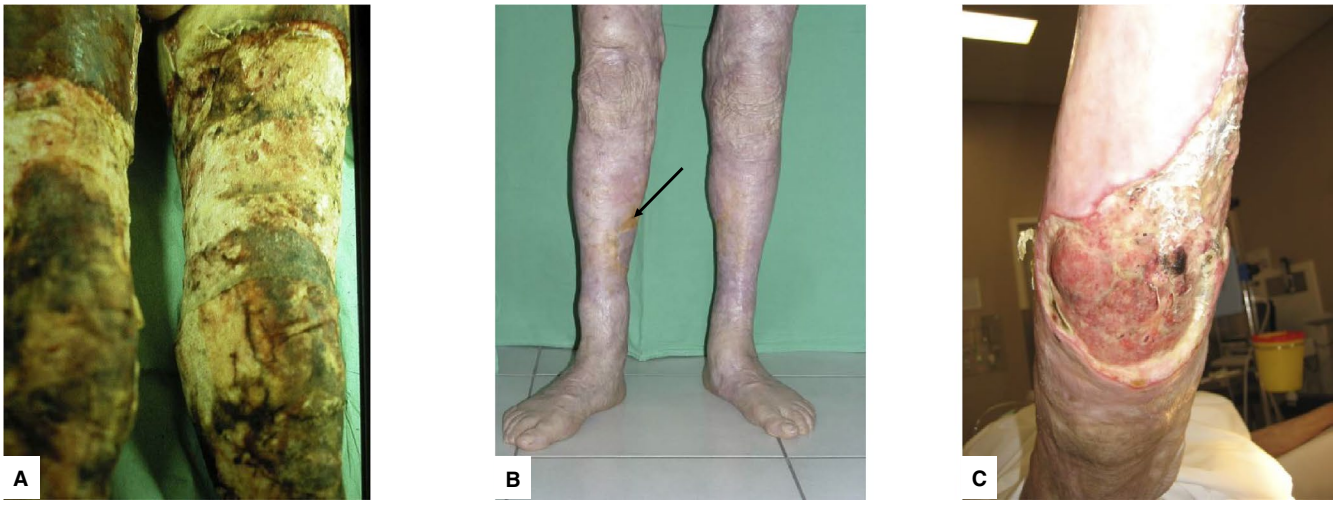

FIGURE 1 A, The lower extremities of the patient were severely burned. B, Deceased skin allograft. Small ulcers (arrow) developed 10 years after transplantation and were treated conservatively without immunosuppression. C, Twenty-five years after transplantation, larger ulcers led to extensive surgical debridement and excision of all the residual allograft deceased skin, which was macroscopically very thick

2.2 | Histological analysis and tissue processing

Histological analyses were performed from large samplings of the removed allograft skin. Ulcerated and nonulcerated skin lesions were sampled as well as tissue from deep dermis and hypodermis. Standard serial sections were processed using paraffin-fixed tissue and stained with hematoxylin-eosin and Masson trichrome. With frozen tissue, serial sections were processed and stained with hematoxylin-eosin. Results were graded using the VCA-Banff 07 classification.[10]

2.3 | Immunohistochemistry and immunofluorescence

For each sampling, immunochemistry on paraffin-fixed tissue was performed using C4d, CD3, CD4, CD8, CD20, CD68-PGM1, Foxp3, S100, CD79a, CD103, TIA-1, and immunofluorescence on frozen tissue was performed using IgM, IgG, C4d, C3, and C5-9.[15,16]

Immunochemistry was done by using automat (Roche, Basel, Switzerland) following our protocol for C4d (1:75, polyclonal antibody, Ventana), CD3 (Ready to use; Novocastra, UK), CD4 (Ready to use, clone SP35, monoclonal antibody, Ventana), CD8 (1:30, clone C8/144B, monoclonal antibody; Dako, Ely, UK), CD20 (1:400, clone L26, monoclonal antibody), CD68 (1:200, clone PG-M1, monoclonal, Dako), FoxP3 (1:50, clone 236A/E7, monoclonal antibody; Abcam, Cambridge, UK). C4d deposits were scored by analogy following the Banff 2013 classification for renal transplantation.[16,17]

All biopsies were evaluated separately by 3 independent pathologists (SR, IR, and RC).

2.4 | Immunological and serological analyses

The presence of lymphocytotoxic antibodies were detected by panel reactive antibody (PRA) and complement-dependent microcytotoxicity assay (PRA-CDC). Briefly, a panel of T-lymphocytes from 56 donors of known human leukocytes antigens (HLA) class I type (Lymphoscreen HLA-ABC 60; Biotest, Dreieich, Germany) and a panel of B-lymphocytes from 26 donors of known HLA class II type (Lymphoscreen HLA DR30, Biotest) was screened with recipient sera. The percent of panel lymphocytes lysed by the recipient serum in the presence of complement was reported.

Presence of anti-HLA class I and II antibodies was also tested using the multiplex technology solid-phase assay (Luminex, Austin, TX).[18] The cut-off level was defined as a baseline normalized 2000 mean fluorescence intensity units (MFI).

2.5 | Molecular HLA typing

Recipient HLA typing was performed using the polymerase chain reaction (PCR) sequence specific oligonucleotides (SSO) method on peripheral blood mononuclear cells extracted deoxyribonucleic acid (DNA). Briefly, amplified DNA was hybridized using LABType kit on the Luminex System (Ingen–One Lambda Inc, Canoga Park, CA).

Donor HLA typing was performed postoperatively from stored frozen skin biopsy samples and paraffin-fixed skin biopsy samples. Purification of genomic DNA from tissue was obtained by proteinase K treatment following the EZ1 DNA tissue kit protocol (Qiagen, Switzerland). HLA typing was performed by PCR-SSO technique.

3 | RESULTS

3.1 | Histological and immunopathological results

Microscopic examination of ulcerated skin tissue samples showed strikingly dense fibrosis of the dermis with total loss of adnexa. The fibrosis extended within the subcutaneous tissue to reach 1 cm of thickness (Figure 2A). There was also grade IV acute cellular rejection according to Banff VCA criteria,[10] with deep skin ulcerations, lymphocytic and neutrophilic perivascular infiltrates, and graft vasculopathy within the dermis and the hypodermis (Figure 2B,C). Immunohistochemistry examination showed lymphocytic infiltrates that were composed of T ($CD3^+$) lymphocytes concentrated around dermal and hypodermal capillaries (Figure 2C). At the hypodermal interface, T ($CD3^+$) and B ($CD20^+$) lymphocytes were

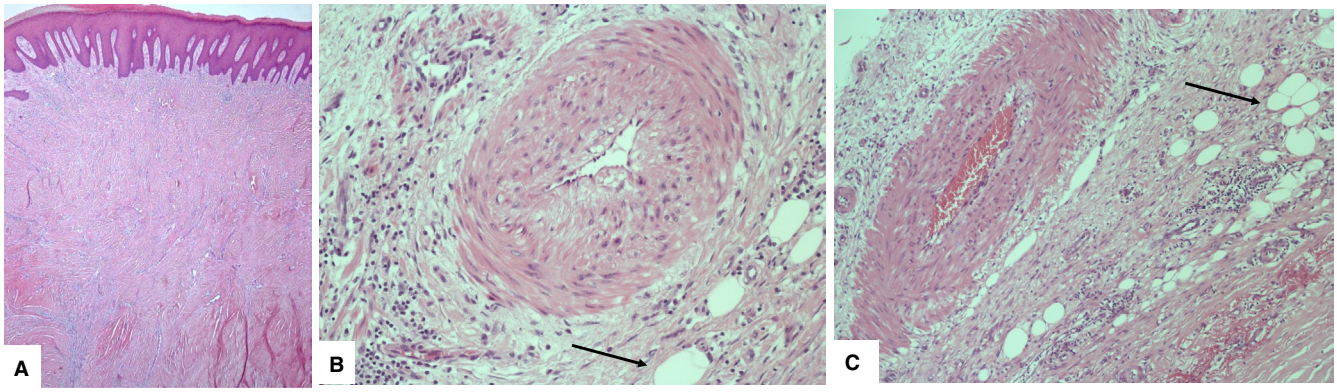

FIGURE 2 A, Allograft skin 25 years after transplantation: The skin was very thick with epidermal acanthosis and a dense fibrotic tissue. H&E ×20. B, Graft vasculopathy. Perivascular mononuclear infiltrates were observed within the dermis and the hypodermis (arrow shows adipocytes). The arteriole is occluded by intimal myofibroblastic proliferation. H&E ×200. C, Hypodermis (arrow shows numerous adipocytes) and a mononuclear cell infiltrate within hypodermal fibrosis. H&E ×200

organized as lymphoid infiltrates surrounding capillaries. Among the T CD3$^+$ population, T CD4$^+$ tended to be slightly predominant as compared to T CD8$^+$. T CD4$^+$ lymphocytes infiltrated some capillaries to form capillaritis and venulitis (Figure 3A,B). Striking linear and circumferential extensive and diffuse C4d deposits were detected in most capillaries and arterioles endothelia (Figure 3C). C5-9 deposits were also observed with the same pattern as the C4d deposits, and immunofluorescence showed endothelial IgM deposits within some arterioles, but no IgG deposits were observed (not shown). Some rare plasma cells (CD79a+) and macrophages (CD68$^+$) were observed within these infiltrates. T reg (CD3$^+$/FoxP3$^+$) lymphocytes were numerous as compared to T CD4$^+$ and CD8$^+$ populations, concentrating around capillaries. The FoxP3/CD8 ratio estimated comparing both infiltrates (T CD4$^+$ and T CD8$^+$) was of ≈50% (Figure 4). Few dendritic cells (S100$^+$) were observed within rete ridge of the epidermis. Arteries and arterioles showed myointimal proliferation, and were infiltrated by lymphocytes and neutrophils. Of note, on a graft biopsy, 24 years after the transplant procedure, we could identify donor cells (XX) by the fluorescence in situ hybridization technique, but without CD34 and CD45 expression (not shown). It was not possible to characterize these cell types further due to the lack of available tissue.

3.2 | Serological and molecular results

High levels of circulating anti-HLA class II antibodies, with >10 HLA-DR specificities between 5000 and 10 000 MFI by Luminex, were present in the recipient serum. This high pattern of sensitization was confirmed by a PRA-CDC class II result >90%. Low-level anti-HLA class I sensitization was also present with 5 specificities mainly directed against HLA-B antigens, with the anti-HLA-B8 antibody present at a significant high level of >5000 MFI (Table 1).

Recipient HLA class I DNA analysis showed the HLA-A*01, A*30; HLA-B*13, B*57 alleles and the HLA class II DRB1*07:01; DRB1*07:01 and DQB1*02:02, DQB1*03:03 alleles, respectively (Table 1).

Because the deceased donor in this specific procedure was not HLA typed at the time of transplantation in 1991, we isolated DNA from skin biopsies, and analyzed by PCR-SSO the donor's HLA type. Biopsy-isolated DNA analysis confirmed the presence of the recipient HLA DRB1*07:01 allele-specific DNA, but it also demonstrated the presence of an HLA-DRB1*08:01 allele. As the recipient was homozygote on the HLA-DRB1*07:01 allele, the HLA-DRB1*08:01 specificity was therefore of donor origin. However, the full HLA donor pattern could not be determined due to insufficient DNA (mainly dense fibrosis) within the available allograft tissue samples, and in view of the recipient high immunization with anti-HLA class II specificities (HLA-DR, see Table 1), only HLA-DR typing was performed.

Therefore, among the numerous anti-HLA class II antibody specificities detected in the recipient's serum, an antibody against DRB1*08:01 could be demonstrated (with an MFI value by Luminex of 11373), ie, an anti-HLA class II circulating DSA was present in the recipient serum.

4 | DISCUSSION

We report the clinical and immunopathological features of late and chronic human skin allograft rejection 25 years after transplantation, in a patient who received a non-vascularized de-epithelialized allogeneic STSG after suffering from severe burns in 1991.[13] Immunosuppression with CyA was administered only during 2 months after the skin transplantation procedure. No clinical acute rejection was reported by the clinical team (although initial punch biopsies during the first 4 months showed acute cellular rejection), and the only possible subclinical sign of rejection was decreased skin elasticity. Subsequently, between 10 and 25 years after transplantation, the skin gradually became very thick, and large skin ulcers developed. Interestingly, the analysis of the removed tissue allografts showed a pattern of chronic mixed skin allograft rejection, ie associated with a circulating DSA in the recipient's serum, but with a component of acute cellular rejection. The ulcerations were also associated with signs of cellular rejection. Extensive fibrosis within the dermis was present, displaying "scleroderma-like features" (Figure 2A). Arteries showed myofibroblastic hyperplasia within intima and media, very similar to the graft vasculopathy that is observed in chronic allograft rejection after SOT and in VCA.[17,19,20] Capillaries and arterioles showed T-cell (CD3$^+$,CD4$^+$) lymphocytic

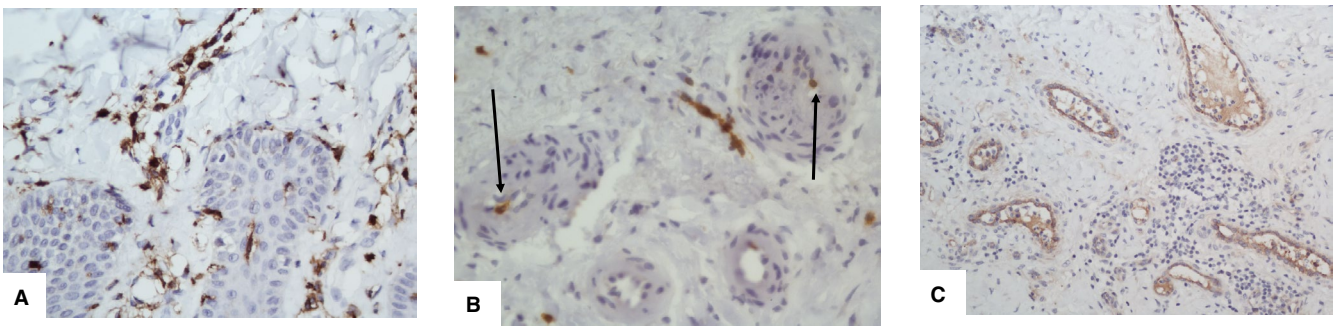

FIGURE 3 A, Immunohistochemistry using CD4: Mild T cell capillaritis and venulitis were observed. ×400. B, Immunohistochemistry using CD3: Lymphocytic endothelialitis (arrows) in the arterioles. ×200. C, Immunohistochemistry using C4d: Endothelial cells within capillaries showed extensive and diffuse complement C4d deposits. ×200

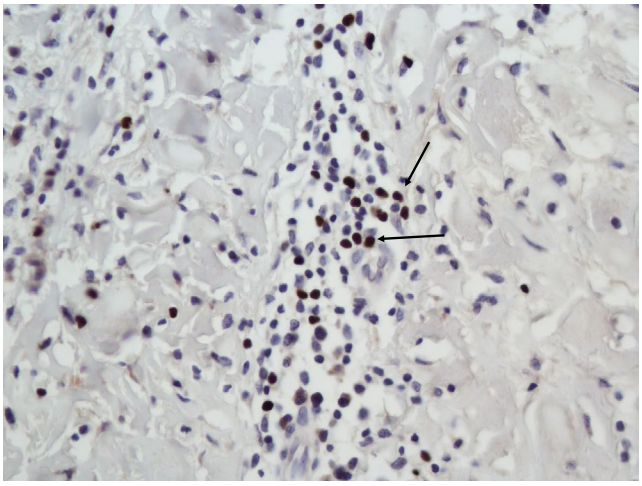

FIGURE 4 Immunohistochemistry using Foxp3: Within lymphocytic infiltrates and among T cells, T reg (Foxp3^{+}) cells were relatively numerous. T reg are shown by arrows with their dark black nucleus. ×400

capillaritis or venulitis. Donor cells were identified on graft biopsy tissue 24 years after transplantation. Diffuse and extensive complement C4d and C5-9 deposits were present on the vascular endothelium of capillaries, and these findings were consistent with a process of antibody-mediated rejection (AMR) associated with chronic allograft injury. Luminex technology demonstrated anti-HLA antibodies in serum with high MFI values, particularly for class II antibodies, with more than 10 DR specificities. Biopsy-isolated DNA analysis revealed the presence of HLA-DRB1*08 of donor origin and the recipient had a circulating anti-HLA-DRB1:08 antibody that was donor specific. In recent years, anti-HLA class II DSA have been associated with chronic rejection of VCA, and HLA class II mismatching with worse allograft outcomes of SOT.[21] By analogy to VCA, the presence of diffuse capillary C4d deposits and endothelialitis, associated with circulating anti-HLA class II DSA in recipient serum, suggests that chronic AMR had developed in the patient over the years. Among the lymphocyte populations within the dermis, we observed the presence of T reg cells in high numbers which, interestingly, may have played a role in the unusually long-term survival of the skin allografted tissue. Overall, the findings derived from the case reported here are in accordance with the admitted role of anti-HLA class II antibodies in chronic allograft rejection and injury, with chronic vascular changes. Anti-HLA antibodies probably cause allograft injury by various mechanisms, some being associated with their capacity to activate the complement system, hence, the presence of C4d and C5-9 deposits, and others being complement-independent.

It should be noted that E. Morelon et al have recently reported the findings of subacute rejection of VCA (face transplantation), ie, they demonstrated that the vascular compartment of a vascularized facial allograft is also susceptible to chronic antibody-mediated rejection.[2,19-22] Their previous observations in vascularized skin allotransplants together with our findings suggest that, similarly to SOT, both antibody-mediated and T cell–mediated graft injury can occur or coexist late after skin transplantation in humans.

TABLE 1 Serological data

Tests	Specificity	MFI
PRA I (cytotoxicity)	0%	
PRA II (cytotoxicity)	92%	
Single Ag class I (Luminex)		
	B8	9190
	B76	2353
	B42	2082
	B54	2080
	B39	2058
Single Ag class II (Luminex)		
	DRB1*03	12718
	DRB1*13	12307
	DRB1*15	12034
	DRB1*16	11970
	DRB1*14	11718
	DRB1*08	**11373**
	DRB1*11	11101
	DRB1*01	10807
	DRB1*01:03	10582
	DRB1*12	10279
	DRB1*04	10184
	DRB1*18	9625
	DRB3*02	8971
	DRB1*10	8876
	DRB5*02	8524
	DRB1*12	8330
Recipient's HLA		
HLA class I	A*01	
	A*30	
	B*13	
	B*57	
HLA class II	DRB1*07:01	
	DRB1*07:01	

Abbreviations: HLA, human leukocyte antigen; MFI, mean fluorescence intensity; PRA, panel reactive antibody.
Anti-HLA DRB1*08 is a DSA with a MFI value of 11373 in bold.

In our patient, the absence of overt signs of acute clinical rejection during the first 10 years, without any long-term immunosuppression, is also quite remarkable and it may suggest some degree of recipient allograft tolerance (or "hyporesponsiveness") to the skin transplant. However, the early time course biopsies indicated that cellular rejection took place in the first months after transplantation, ie, true (robust) tolerance was probably never achieved. Interestingly, it should be mentioned that other different clinicopathologic presentations or clinical conditions such as chronic cutaneous graft-versus-host disease (GVHD) can also have significant fibrosis of the dermis, including of the deep dermis, and in rare cases scleroderma-like changes have been reported such as those

observed in our case. Therefore, it may be that some pathophysiological mechanisms of injury and disease (eg, the presence of growth factors or cytokines) may be common in such conditions.[23,24]

In conclusion, the current report details the features of the very late development of chronic mixed cellular and humoral allograft rejection of the skin, in a unique case after STSG. It also provides new clinical and immunopathological evidence that may be relevant to the current debates on the indications and risks of skin allograft transplants in humans, which can be successful in the early years after the procedure, but which may have dramatic consequences in the long term.

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DISCLOSURE

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

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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