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Title: Non-vascularized human skin chronic allograft rejection

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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 leucocyte antigen

MFI: mean fluorescence intensity

PBMC: peripheral blood mononuclear cells

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

SPA: solid-phase assay

STSG: split-thickness skin allograft

VCA : vascularized composite allotransplantation

Abstract :

A 65 year-old male suffered from extensive lower legs burns in 1991, at the age of 40. He was treated by non-vascularized 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 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.

1-Introduction:

In the history of modern transplantation and transplantation immunology, skin grafting has 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.

Nowadays, total skin allografts are generally considered as 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 nowadays relatively well controlled, but the development of circulating anti-HLA 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 suffered from 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].

Skin autografts are standards of care for the management of burned patients [11]. In addition, non vascularized 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 non vascularized allogeneic STSG at the age of 40, 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 which has not been described previously.

2-Material and methods:

2.1 Clinical data:

In 1991, a 40 year-old male suffered from severe burns, involving 73% of his total body surface area (Fig. 1A). Allogeneic STSG transplantations (allograft STSG) were used to cover his lower extremities. The non-vascularized allogeneic STSG originated from a brain dead HIV-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 two 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 (Fig. 1B). Eventually, 25 years after transplantation, his skin texture had progressively become thicker, significant larger ulcers developed and a surgical debridement on lower extremities (Fig.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 grangrenosum.

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

2.2 Histological analysis and tissue processing

Histological analyses were performed from large samplings of the removed allograft skin. Ulcerated and non-ulcerated 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 (Ventana) 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, Novocastra, UK), CD68 (1:200, clone PG-M1, monoclonal, Dako, Ely, UK), 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 three 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 leucocytes antigens (HLA) class I type (Lymphoscreen HLA-ABC 60, Biotest, Germany) and a panel of B-lymphocytes from 26 donors of known HLA class II type (Lymphoscreen HLA DR30, Biotest, Germany) 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 (SPA) (Luminex, Austin, Texas) [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 polymerase chain reaction (PCR) Sequence specific oligonucleotides (SSO) method on peripheral blood mononuclear cells (PBMC) extracted 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 (Fig.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 (Fig.2B-C). Immunohistochemistry examination showed lymphocytic infiltrates which were composed

by T (CD3+) lymphocytes concentrated around dermal and hypodermal capillaries (Fig.2C). At the hypodermal interface, T (CD3+) and B (CD20+) lymphocytes were 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 (Fig.3A,B). Striking linear and circumferential extensive and diffuse C4d deposits were detected in most capillaries and arterioles endothelia (Fig.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 approximately 50% (Fig.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 FISH 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 more than 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 greater than 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 more than 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).

As 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. The full HLA donor pattern could however 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), i.e. an anti-HLA class II circulating donor-specific antibody (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 two months after the skin

transplantation procedure. No clinical acute rejection was reported by the clinical team (although initial punch biopsies during the first four 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, i.e. associated with a circulating DSA in 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" (Fig2 A). 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 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 which 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 antibody-mediated rejection had developed in the patient over the years. Among the lymphocyte

populations within the dermis, we observed the presence of Treg 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), i.e. 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.

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, i.e. true (robust) tolerance was probably never achieved. Interestingly, it should be mentioned that other different clinico-pathologic 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 (e.g. 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 which 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*.

Figure legends

Fig. 1A:
The lower extremities of the patient were severely burned.

Fig. 1B:
Deceased skin allograft. Small ulcers (arrow) developed 10 years after transplantation and were treated conservatively without immunosuppression.

Fig. 1C:
25 years after transplantation, larger ulcers led to extensive surgical debridement and excision of all the residual allograft deceased skin, which was macroscopically very thick.

Fig. 2A:
Allograft skin 25 years after transplantation: The skin was very thick with epidermal acanthosis and a dense fibrotic tissue. H&E 20x.

Fig. 2B:
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 200x.

Fig. 2C:
Hypodermis (arrow shows numerous adipocytes) and a mononuclear cell infiltrate within hypodermal fibrosis. H&E 200x.

Fig. 3A:
Immunohistochemistry using CD4:
Mild T cell capillaritis and venulitis were observed. 400x.

Fig. 3B:
Immunohistochemistry using CD3:
Lymphocytic endothelialitis (arrows) in the arterioles. 200x.

Fig. 3C:
Immunohistochemistry using C4d:
Endothelial cells within capillaries showed extensive and diffuse complement C4d deposits. 200x.

Fig. 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. 400x.

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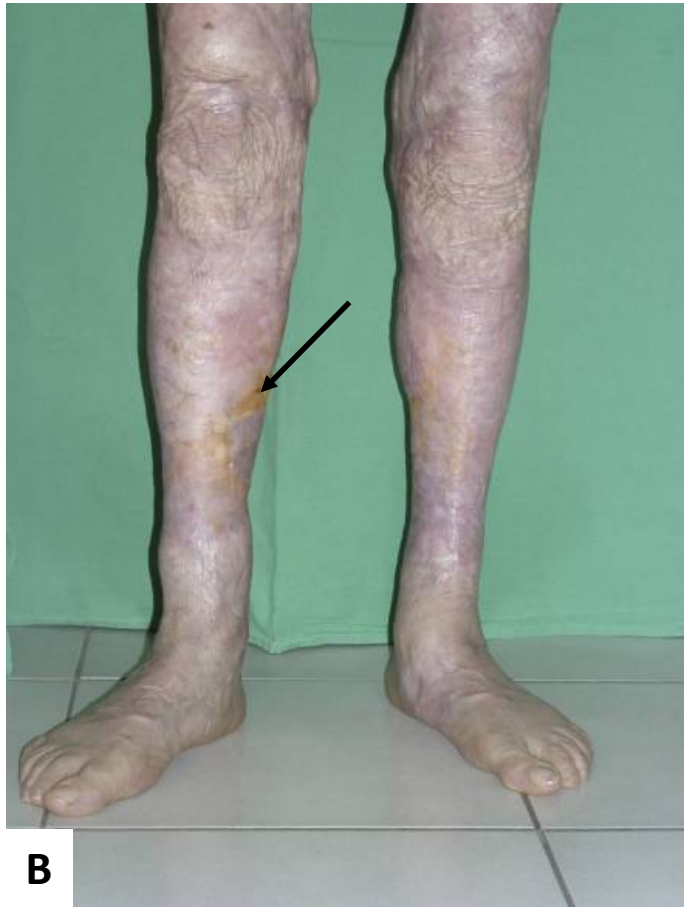
Table 1:

PRA I (cytotoxicity)	0%	
PRA II (cytotoxicity)	92%	
Single Ag class I (Luminex)	**Specificity**	**MFI**
	B8	9190
	B76	2353
	B42	2082
	B54	2080
	B39	2058
Single Ag class II (Luminex)	**Specificity**	**MFI**
	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	

A



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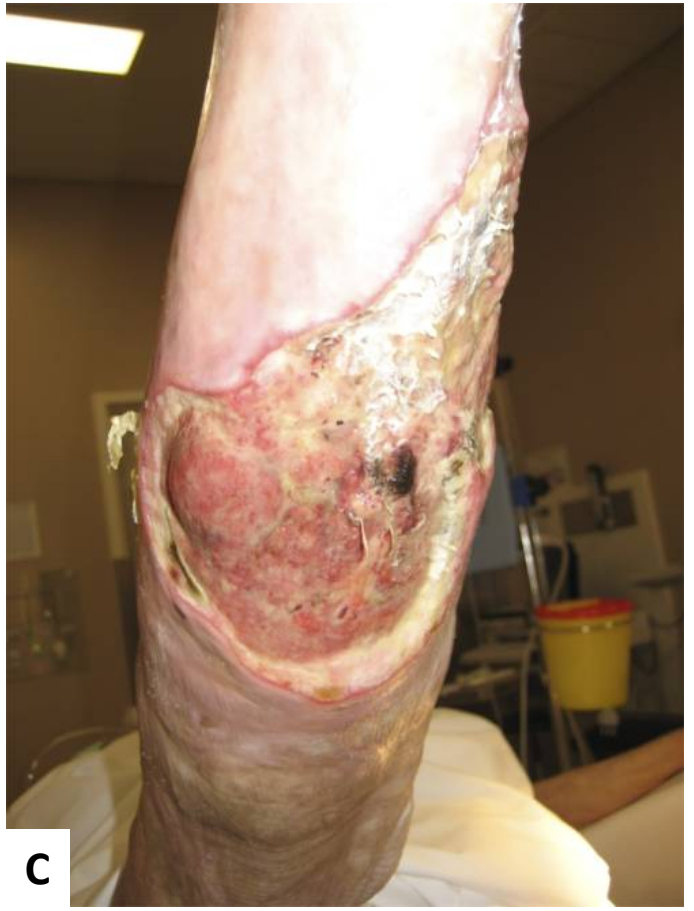


C

Fig. 1

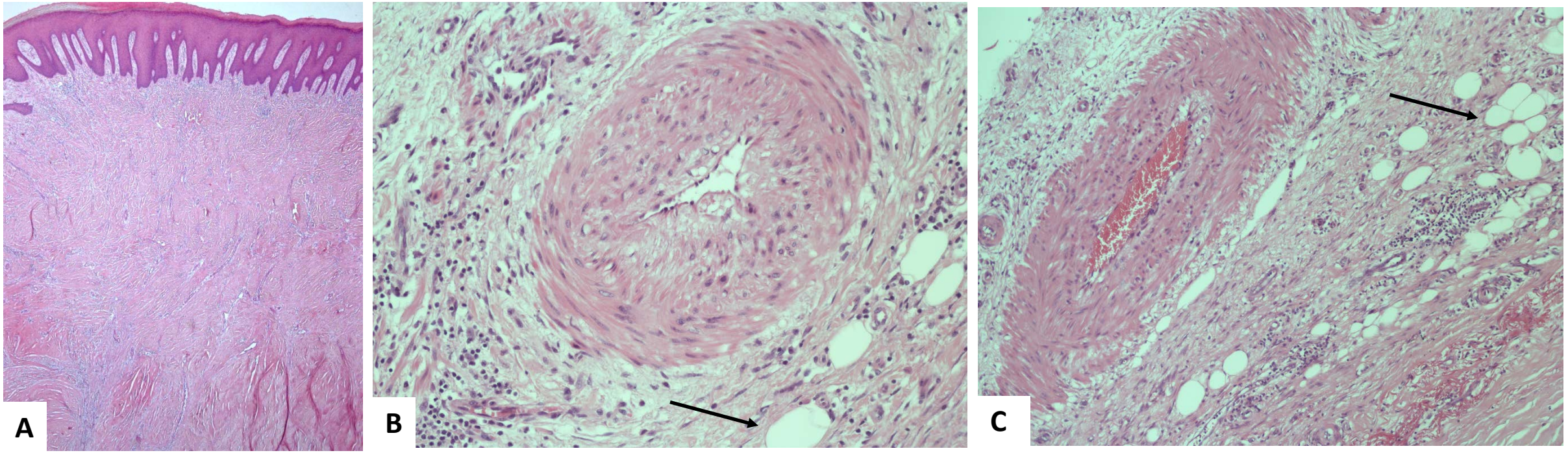
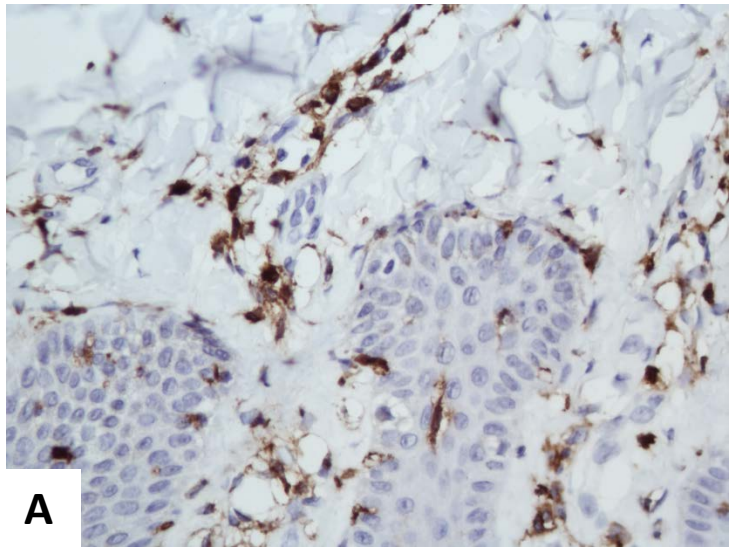


Fig. 2



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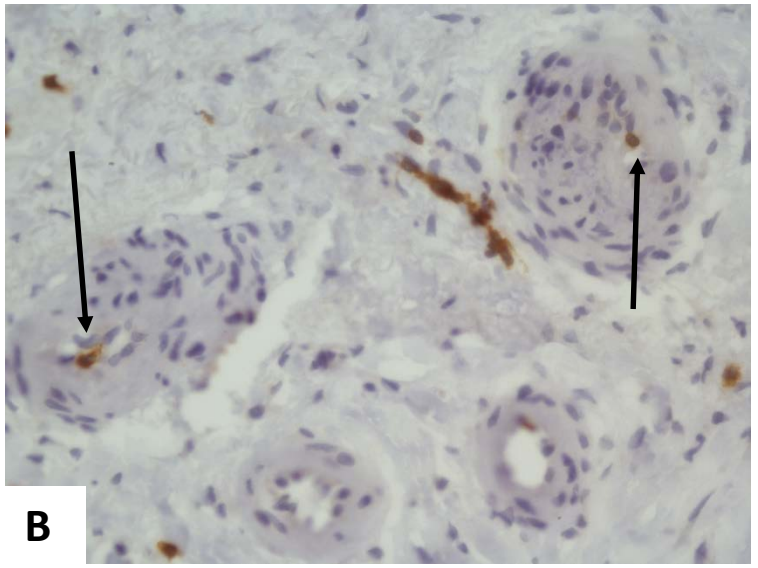

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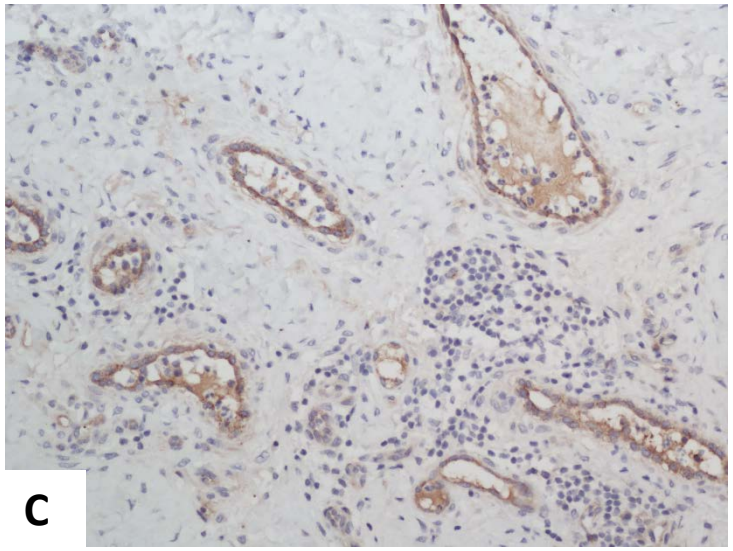

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Fig. 3

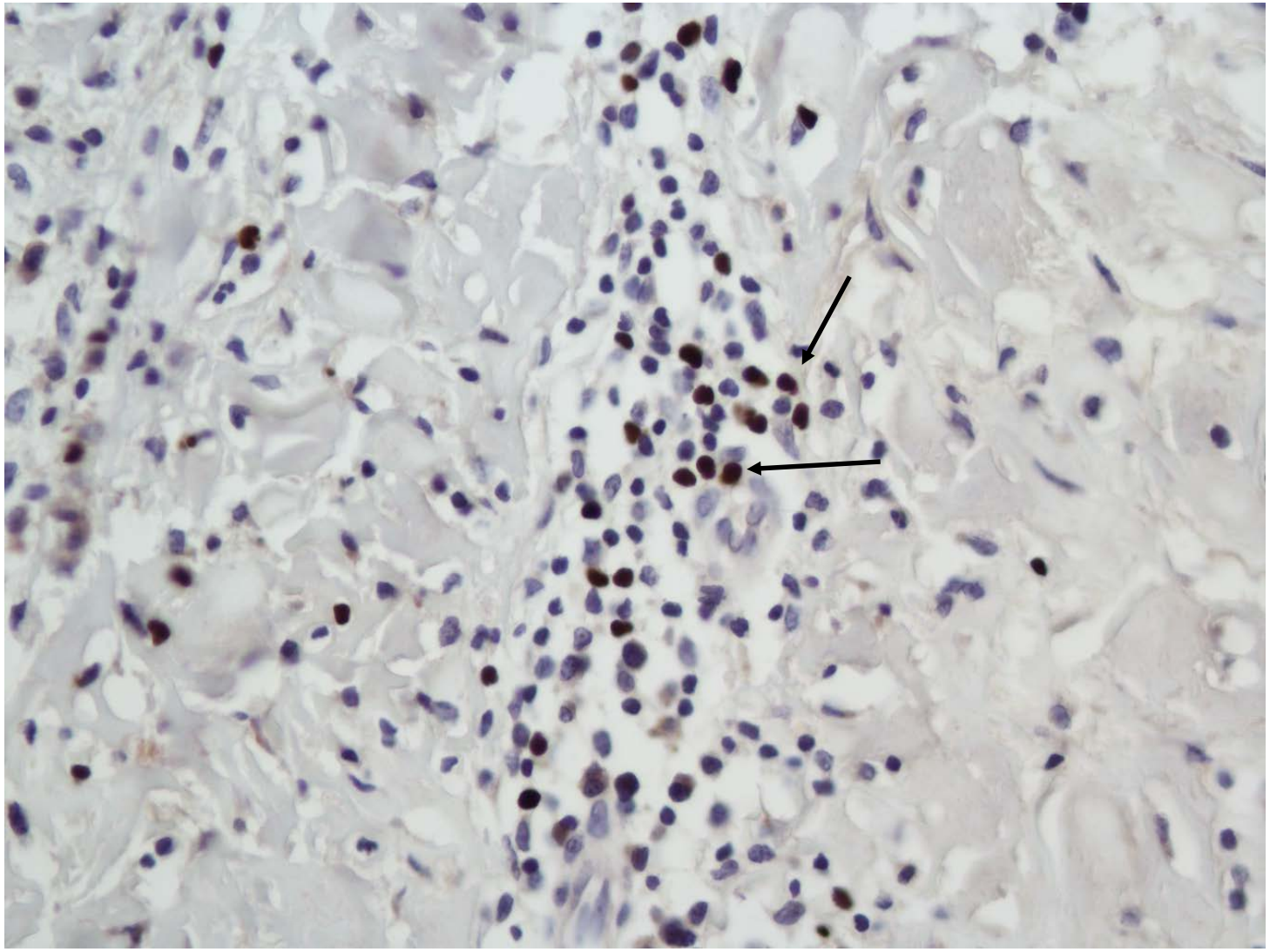

Fig. 4