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Anti-HLA sensitization in extensively burned patients: extent, associated factors, and reduction in potential access to vascularized composite allotransplantation

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Conflicts of interest
As principal investigators, Drs. Hivelin and Duhamel had full access to all study data and take responsibility for data integrity and accuracy of analysis. The authors of this manuscript have no conflict of interest to disclose. We hereby certify that no financial support or benefits have been received by any co-author, by any member of our immediate family, or by any individual or entity with whom or with which we have a relationship from any commercial source which is related directly or indirectly to the scientific work which is reported in the article. We understand that an example of such a financial interest would be a consulting relationship or stock interest in any business entity which is included in the subject matter

Summary

Extensively burned patients receive iterative blood transfusions and skin allografts that often lead to HLA sensitization, and potentially impede access to vascularized composite allotransplantation (VCA). In this retrospective, single-center study, anti-HLA sensitization was measured by single-antigen-flow bead analysis in patients with deep, second- and third-degree burns over ≥40% total body surface area (TBSA). Association of HLA sensitization with blood transfusions, skin allografts, and pregnancies was analyzed by bivariate analysis. The eligibility for transplantation was assessed using calculated panel reactive antibodies (cPRA). Twenty-nine patients aged 32 ± 14 years, including 11 women, presented with a mean burned TBSA of 54 ± 11%. Fifteen patients received skin allografts, comprising those who received cryopreserved (n = 3) or glycerol-preserved (n = 7) allografts, or both (n = 5). An average 36 ± 13 packed red blood cell (PRBC) units were transfused per patient. In sera samples collected 38 ± 13 months after the burns, all patients except one presented with anti-HLA antibodies, of which 13 patients (45%) had complement-fixing antibodies. Eighteen patients (62%) were considered highly sensitized (cPRA≥85%). Cryopreserved, but not glycerol-preserved skin allografts, history of pregnancy, and number of PRBC units were associated with HLA sensitization. Extensively burned patients may become highly HLA sensitized during acute care and hence not qualify for VCA. Alternatives to skin allografts might help preserve their later access to VCA.

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Introduction

Vascularized composite allotransplantation (VCA) is a therapeutic option that restores function and appearance of damaged tissues beyond the limits of conventional procedures, thereby improving the quality of life of patients with trauma, burns, or infections [1–4]. VCA involves grafting of appropriate tissue from donors matched for blood group, sex, and morphology. Previous studies carried out worldwide, on 60 upper extremities and 28 face transplantations, including one-third of burns, indicated encouraging outcomes [5–7].

Our experience in VCA included seven face transplant patients of eight waitlisted and over 30 assessed. Two burned patients had been either not included or withdrawn from the waiting list after 18 months, due to high PRA levels [3]. It appeared that immunologic issues related to anti-HLA sensitization were a major limitation in providing VCA to patients and enhanced the shortage of VCA procurement [3,5].

Presence of anti-HLA donor-specific antibodies (DSA) precludes VCA as this can lead to life-threatening complications [3,8,9]. As reported recently, a sensitized burned recipient of a face transplantation with a positive preoperative cross-match showed signs of antibody-mediated rejection (AMR) within 5 days, despite plasmapheresis and a high standard of care, and resolved only after multiple and complex treatments [9]. Another case of AMR after VCA had been reported at 9 years after forearm transplantation [10], with high level *de novo* DSA and B-cell deposits that required the administration of rituximab to completely disappear. A review on sensitization in VCA insisted on the multiple blood transfusion or skin grafts received by potential candidates, resulting in the formation of alloantibodies and the associated risks of AMR in VCA [11]. Those limited clinical data are supported by scarce experimental studies of allosensitization in VCA. The study of the humoral immune response in a rat limb transplantation model undergoing multiple episodes of acute rejection, treated with pulsed immunosuppression, did not resulted in serum antidonor antibodies or complement deposition in VCA [12]. In another study of VCA or kidney transplantations in CMH-I-mismatched rats sensitized by skin grafts, VCA underwent accelerated CMR, while renal allografts underwent hyperacute AMR [13].

Studies on VCA performed for burns sequels [3,14] have shed new light on two important concerns in the management of extensively burned patients: the involvement of hands and face, previously considered when defining the limits between intensive care and "futile" therapy [15,16], and HLA sensitization [3,9]. Burned patients are likely to become sensitized due to the risk factors acquired during intensive care; however, as VCA was not an option for these patients previously, no study reported the prevalence, extent, and intensity of this sensitization and the foreseeable difficulties to access to VCA.

We retrospectively studied anti-HLA sensitization and its impact on the access to subsequent VCA in extensively burned patients admitted at our specialized burn treatment center. Factors associated with HLA sensitization were investigated, and strategies for acute care and analysis of donor/recipient matching that could help increase the access to transplants are discussed.

Patients and methods

Study population

This single-center, cross-sectional, noninterventional study was carried out at the Percy Military Hospital, Clamart, France, and approved by the Institutional Review Board (CPP Ile de France IX; 05/11/2011; No. 11-014). Patients with deep, second- and third-degree burns covering more than 40% TBSA similar to burns that led to VCA indications [3,5,8,9] treated between 2007 and 2010, and willing to participate in the study were included (Fig. 1). All included patients provided an informed consent. Data on demography, pregnancy, transfusion history, treatment, and surgical management including skin grafts, and length of stay in the intensive care unit (ICU) were extracted from institutional databases. The well-accepted units for estimating burned surface were used: The TBSA or percentage of body surface burned (BSB) does not include first-degree burns (that do not require surgical care). Unit burns standard (UBS) allows assessment of the increased morbidity

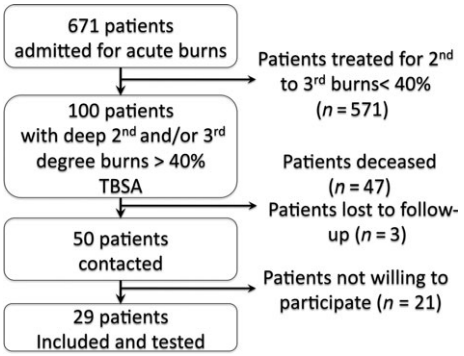

Figure 1 Study flow chart.

related to whole skin loss (WSL) and is defined as follows: UBS = % BSB + (3 × % WSL) [17].

Skin allografts

Glycerol-preserved and cryopreserved skin allografts were used. Glycerol-preserved human skin allografts (GPSA) were supplied by the Euro Skin Bank (Beverwijk, the Netherlands) and were maintained at room temperature (RT). Cryopreserved human skin allografts (CPSA) were processed by our tissue bank (CTSA: Centre de Transfusion Sanguine des Armées, Clamart, France) following a conventional cryopreservation protocol [18]. Briefly, skin allografts were recovered from deceased tissue donors and transferred to the CTSA in a chilled (4 °C) αMEM solution (Cryokit®) containing antibiotics for cryopreservation. Collected samples were incubated at RT for 2 h with continuous stirring in 14.9% glycerol containing 0.7% human albumin and antibiotics. Samples were placed in sterile pouches (Maco Biotech Freezing®, MacoPharma, Tourcoing, France) and subjected to stepwise cryopreservation (DigitCool®, IMV Technologies, France) with cooling at a rate of −1 to −2 °C/min between 10 °C and −40 °C, followed by −5 °C/min up to −130 °C, and then stored in liquid nitrogen (−150 °C to −170 °C) until use. For grafting procedures, the frozen pouches containing CPSA were thawed rapidly in a water bath at 37 °C for 2 min. Before use, both CPSA and GPSA were rinsed six times in Ringer's lactate solution at RT. The two types of allografts were considered equivalent, the choice being based on their availability.

HLA typing, detection of anti-HLA antibodies, and assessment of C1q fixation

Blood samples were collected from all included patients and used for HLA typing, HLA class I- and II-positive IgG antibodies (Abs) detection, and C1q complement fixation capacity of these Abs. ABO typing had been performed during the acute care at the transfusion center (Centre de Transfusion Sanguine des Armées, Clamart, France). The histocompatibility laboratory (Laboratoire d'histocompatibilité, Saint Louis Hospital, Paris, France) performed HLA typing using molecular biology methods (BAG Histo Spot® SSO System; BAG Health Care GmbH, Lich, Germany), anti-HLA Ab detection by SAFB testing (LABScreen single-antigen, One Lambda, Inc., CA, USA) using a Luminex® (LABScan 100®, One Lambda, Inc., CA, USA) platform, and data analysis (Fusion® software, One Lambda, Inc., CA, USA). Normalized mean fluorescence intensity (MFI) values were obtained with Fusion software after background subtraction using negative controls that were set to MFI <10 [19,20]. Due to the lack of a worldwide consensus on the MFI threshold value to consider an antibody response as positive, the MFI thresholds were set at 500, 1000, and 3000.

The serum levels of C1q-binding anti-HLA Ab were measured using a commercially available kit (C1qscreen Kit™, One Lambda, CA, USA) following the manufacturer's recommendations. Briefly, the complement component (C1q) bound by the antigen–antibody complex was detected with an R-phycoerythrin (PE)-labeled anti-C1q antibody. The fluorescence intensity was measured on the Luminex® platform and analyzed using Fusion® software. MFI values >300 were considered positive. The data on patients with unacceptable HLA specificities (HLA-A, HLA-B HLA-DR, and HLA-DQ) were transferred to the French national database, CRISTAL, to calculate cPRA.

Transplant allocation, virtual cross-match, and cPRA calculation for VCA

The French "Agence de la Biomedicine" (ABM), similar to the Organ Procurement and Transplantation Network (OPTN) in the United States, regulates transplant allocation in France. VCA allocation requires recipient registration on the national waiting list and donor–recipient ABO blood group compatibility. VCA allocation priorities are registered in the national database (CRISTAL). ABM provides an estimation of anti-HLA sensitization with calculated panel reactive antibodies (cPRA) after virtual cross-matching [21]. In VCA, as in kidney transplantations, profiles of the so-called unacceptable HLA antigen haplotypes (HLA-A, HLA-B, HLA-DR and HLA-DQ) and virtual cross-matching are based on single-antigen flow bead (SAFB) analysis [22]. The cPRA represents the percentage of potential donors who would present unacceptable HLA antigens that hence will have to be preemptively declined. For VCA, as for all organ transplantation, the immunologic data included in the CRISTAL database are gathered by the histocompatibility laboratory

and transferred automatically to the CRISTAL immunologic file for each patient. This file included data on the blood group, and HLA typing, peak cytotoxic PRA values, and anti-HLA specificities (SAFB) for sensitized patients. Following standard procedures, blood samples are collected every 3 months from all the patients on the waiting list. HLA sensitization is defined as history of at least one anti-HLA class I or class II Ab. ABM maintains data for the previous 5 years on blood group type and unacceptable HLA specificities in donor populations from the geographical region of this study population (HLA-Cw and HLA-DP were not included). These attributes are analyzed using established algorithms [21,23] for determining the cPRA percentages based on this donor population [24]. Patients with cPRA≥85% are considered hyperimmunized and hence would not qualify for allotransplantation.

Calculated panel reactive antibodies values were currently determined for the study population and were categorized according to the MFI thresholds for SAFB (>500, >1000, >3000), and C1q fixation (>300 MFI).

For face or hand transplantations, in addition to ABO compatibility and immunologic cross-match, donor selection is based on matching of size/morphology of the graft and skin color. However, neither size nor color is included in CRISTAL database. Morphological matching and ethical concerns led us to opt for gender matching [3]. We finally assessed the consequences of including gender match among donor selection criteria on cPRA. Gender is not a consideration in calculating cPRA conventionally, and to assess the impact of the gender matching in VCA on the access to transplants, we also calculated cPRA on donors pool restricted to the same gender (Fig. 2).

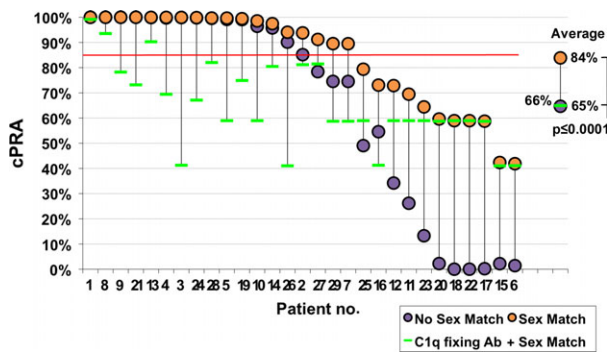

Figure 2 cPRA values in the study population. In patients with anti-HLA Ab reaction MFI>1000, cPRA values were determined after donor/recipient matching for ABO blood group and with gender matching (orange circles) or without gender matching (violet circles). cPRA scores determined for C1q-fixing anti-HLA Ab in gender-matched transplantations are also presented (green dashes). Red line marks the 85% cPRA threshold above which the recipients are considered highly sensitized. Mean cPRA values are plotted. The cPRA values with and without gender matching were compared by Wilcoxon test (*P*-values presented).

Comparison between the study population and patients on waiting list for kidney transplantation

To determine the potential access to compatible VCA transplants, we compared the cPRA percentages in the study population with those of a reference population (considered as not exposed to the risks related to extensive burns care) that consisted of all patients waitlisted for a kidney transplantation in the same geographical area (Ile de France) on July 27 2012 (data provided by the ABM). The sex ratio, age, and blood groups distribution were also compared.

Statistical analysis

JMP 7.2 software (SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. A *P*-value <0.05 was considered as statistically significant. Continuous variables were expressed as mean ± SD for normally distributed data and as median [Q1; Q3] otherwise. Categorical variables were expressed as frequencies and percentages and analyzed with Mood's median and chi-squared tests, respectively. Paired data were compared by Wilcoxon test. The number of anti-HLA specificities was determined for categorizing patient. CPRA values for patients categorized by HLA class were calculated for ABO-compatible and gender-matched donor–receiver pairs. Calculations of cPRA without gender matching or based on C1q-fixing anti-HLA Abs were also performed.

The relative risk (RR) with 95% confidence interval (CI) to be hypersensitized was determined by comparison of the study population with the population waitlisted for kidney transplantation (reference population). Bivariate analysis with linear regression was performed between the number of anti-HLA specificities (class I, II, or both (I + II)) or cPRA value as dependent variables, and acute care characteristics (number of blood transfusions, type of allograft (CPSA or GPSA)), and number of pregnancies as independent variables. Multiple regression was not considered due to the small population size.

Results

Patient demographics and clinical history

In this retrospective study, 29 extensively burned patients were analyzed, including 11 women, with a mean age of 32 ± 13 years (Table 1). The mean overall burned TBSA was 54 ± 11%, including 37 ± 13% TBSA with third-degree burns, and the mean UBS score was 165 ± 45.

Table 1. Clinical characteristics of the study population of extensively burned patients ($N = 29$).

Population characteristics	Value	Range
Age, years (mean ± SD)	32.3 ± 13.6	10–58
Male:Female, *n* (%)	18 (62%): 11 (38%)	–
Burns severity, %TBSA, (mean ± SD)		
Overall	54.3 ± 11.4	40–72
2nd degree: Superficial	2.1 ± 4.6	0–22
2nd degree: Deep	16 ± 12.1	0–61
3rd degree	37 ± 13	9–55
UBS	165.2 ± 45.3	68–231
Procedures*/patient, (mean ± SD)	6.1 ± 2.5	2–13
Patients receiving skin allografts, *n* (%)	15 (52%)	–
GPSA, n (%)	7 (24%)	–
CPSA, n (%)	3 (10%)	–
GPSA+CPSA, n (%)	5 (17%)	–
Allograft units/patient receiving allograft, (mean ± SD)	3.3 ± 2.3	1–7
Allograft surface area, cm^2, (mean ± SD)	6887 ± 3695	0–13200
Blood transfusions, (mean ± SD)		
Transfusion sessions/patient	15.3 ± 5.8	7–31
PRBC units/patient	35.9 ± 13.5	13–64
Pregnancies/woman, (mean ± SD)	1.9 ± 1.04	1–4
ICU stay, days, (mean ± SD)	92.8 ± 37.8	50–194
Time to SAFB testing, month, (mean ± SD)	38.14 ± 13.74	17–59

*all surgical procedures (wound cleansing, eschar debridement, skin grafting) until the last follow-up session.
CPSA, cryopreserved skin allografts; GPSA, glycerol-preserved skin allografts; PRBC, packed red blood cells; SD, standard deviation; TBSA, total body surface area; UBS, unit burns standard.

Data on pre-injury alloimmunization status of the patients were not available. At admission, none of the patients had a history of previous transplantations, although all the women had a history of pregnancy. The screening by the Etablissement Français du Sang (EFS) allowed confirming that none of the patient studied had any history of blood transfusion, (or transplantation) before the burns. The acute care included an average transfusion of 36 ± 13 units of packed red blood cells (PRBC) per patient, an average of six surgical procedures per patient, and an average ICU stay of 92 ± 37 days. Of the 15 patients who required skin allografts, three patients received cryopreserved allografts, seven patients received glycerol-preserved allografts, and the remaining five patients received both types of allografts. None of the patients received blood transfusion or skin allografts during the follow-up period after the acute care.

HLA sensitization in burned patients

Blood samples were collected from all 29 patients, 38 ± 13 months after the burn injury (Table 1). The study of the HLA antibodies was a transversal one performed after pooling the 29 survivors of the extensive burns injuries at the Percy Military Hospital, explaining why patients presented with different delay between the sensitizing events and serum analysis. Of these, 28 samples (97%) contained Abs against HLA class I or class II (Table 2). SAFB analysis indicated intense and broad sensitization, including C1q-fixing anti-HLA Abs (Fig. S1). Two patients were sensitized to HLA class I alone, one patient to HLA class II alone, while the majority were sensitized to both classes. The frequency of anti-HLA Ab with a MFI >3000 was 52% for class I (15/29), 45% for class II (13/29), and 57% for class I + II (17/29). The average number of HLA specificities per patient corresponding to an MFI ≥3000 was 12.2 for class I, 9.7 for class II, and 21.9 for class I + II. Twelve patients (41%) presented C1q-fixing Abs targeting HLA

Table 2. Reactivity and titers of anti-HLA antibodies from extensively burned patients ($n = 29$).

				HLA Ab/patient	
HLA Class	SA	MFI threshold*	Patients with HLA Ab, *n* (%)	Mean ± SD	Range
I	SA	>3000	15 (52%)	12.2 ± 17.3	0–50
		>1000	24 (83%)	18.2 ± 23.5	0–63
		>500	27 (93%)	22.7 ± 25.7	0–71
	SA C1q	>300	12 (41%)	3.1 ± 5.8	0–22
II	SA	>3000	13 (45%)	9.7 ± 16.3	0–57
		>1000	24 (83%)	16.4 ± 19.8	0–71
		>500	26 (90%)	22.4 ± 21.3	0–73
	SA C1q	>300	8 (28%)	2.1 ± 4.9	0–21
I + II	SA	>3000	17 (59%)	21.9 ± 31.5	0–95
		>1000	28 (97%)	34.7 ± 39.3	0–123
		>500	28 (97%)	45.1 ± 41.5	0–136
	SA C1q	>300	13 (45%)	5.3 ± 8.1	0–24

*Luminex® single-antigen flow bead (SAFB) analysis with MFI thresholds set at the indicated values.
Ab, antibody; SA, single-antigen analysis of anti-HLA Ab; SA C1q, single-antigen analysis of C1q-fixing anti-HLA Ab; SD, standard deviation.

class I, eight patients (28%) HLA class II, and thirteen patients (45%) for both classes.

The patient who did not present with any anti-HLA Ab (with MFI>500) was an 18-year–old male (blood group 0, Rh+), with 43% third-degree burns, 65% TBSA burned, and a high UBS score (194). He had been hospitalized for 78 days during which he received a 2600 cm^2 GPSA in a single session, 36 PRBC in 14 transfusion sessions, and five surgical procedures. Thus, his medical condition and clinical course were similar to those of other included patients in this study, except that he had a single skin allograft procedure as against the average of about three allograft procedures per patient.

Access to VCA transplants

The potential accessibility of the study population to matched VCA transplants from the local donor population was determined in terms of cPRA score (Fig. 2). Overall, cPRA calculations retrieved 18 highly sensitized patients (cPRA>85%) for gender-matched transplantations (Figs 2 and S3). Thus, the entire study population, except one patient, was HLA sensitized, and 62% of the population was hypersensitized. Paired analyses using Wilcoxon's test showed that gender-matching, C1q-fixing anti-HLA Abs, and MFI threshold had a statistically significant influence on accessibility to compatible transplants (Figs 2 and S3). When gender match was disregarded, the number of highly sensitized patients (cPRA>85%) significantly decreased from 18 to 15 (Fig. 2). If only the C1q-fixing Abs were considered among the unacceptable HLA Abs, then the average cPRA of the population studied would significantly decrease to 66% from 85% (Fig. S3c). ABO matching (identical vs. compatible) had no significant effect on the access to transplantation (data not presented).

Factors associated with HLA sensitization

Factors potentially associated with HLA sensitization were examined in the study population by bivariate analysis (Table 3). The severity of burns (third-degree burns or UBS score) was not associated with HLA sensitization significantly. Factors that were found to be associated significantly with HLA sensitization were number of pregnancies, number of skin allografts, number of PRBC units transfused, and the percentage of burned TBSA. Pregnancies were associated with anti-HLA class I, while skin allograft and PRBC units with class II. The use of CPSA, but not GPSA, was associated with anti-HLA sensitization. The number of procedures and the length of ICU stay (both related to the use of skin allografts) were also significantly associated with HLA sensitization, but not the length of time interval between the intensive care and the assessment for HLA sensitization.

Table 3. Analysis of factors associated with HLA sensitization in the study population of burned patients ($N = 29$): *P*-values for comparison of variable *vs.* anti-HLA Ab reactions of MFI >1000.

	Anti-HLA Class			
Variable	I	II	I + II	cPRA*
Age	0.53	0.94	0.69	0.99
Gender	0.20	0.60	0.20	0.09
Pregnancy				
Yes	0.20	0.60	0.08	0.09
No. of pregnancies	*0.0004*	0.08	*0.003*	0.13
Burns severity, %TBSA				
Overall	0.56	0.07	0.21	0.18
2nd degree: Superficial	0.35	0.84	0.51	0.70
2nd degree: Deep	0.73	0.75	0.71	0.98
3rd degree	0.58	0.24	0.36	0.12
Unit burns standard (UBS)	0.53	0.14	0.26	0.09
No. of procedures†	0.15	*0.024*	*0.046*	*0.029*
Skin allografts				
Yes	0.58	0.2	0.2	0.18
No. of procedures	0.21	*0.013*	*0.047*	0.07
Allograft surface area	0.18	*0.01*	*0.037*	0.06
GPSA	0.88	0.88	0.88	0.87
CPSA	0.35	*0.01*	*0.01*	*0.008*
Blood transfusions				
Transfusion sessions/patient	0.22	0.053	0.087	0.07
PRBC units/patient	0.21	*0.014*	*0.047*	*0.044*
ICU stay length	*0.005*	*0.016*	*0.003*	*0.033*
Time to SAFB	0.65	0.86	0.86	0.57

*cPRA value calculated for ABO-compatible and gender-matched transplantations, considering anti-HLA Ab with MFI>1000. Significant results are presented in italics and underlined.

†all surgical procedures (wound cleansing, eschar debridement, skin grafting) until the last follow-up session.

cPRA: calculated panel of reactive antibodies, CPSA: cryopreserved skin allografts, GPSA: glycerol-preserved skin allografts, PRBC: packed red blood cells, SD: standard deviation, TBSA: total body surface area.

Comparison between study population and patients on waiting list for kidney transplantation

The waiting list for kidney transplantation from the same geographical area as the study population included 1055 patients (Fig. S4). The sex ratio was identical to the one of the burns population (62% male patients), with an older age (50 vs. 32 yo). Their transfusion or pregnancy histories are not included in CRISTAL database. However, 20% of them had received a previous solid organ transplant (SOT). Their blood groups distribution was similar to the one of the burns patients. Of these, 15% were hyperimmunized. The median [Q1; Q3] cPRA score was 0% [0%; 57%]. In contrast, in the study population of extensively burned patients, the cPRA values were 85% [19.7%; 99.7%] and 51% were hyperimmunized (Figure 3). The median cPRA value was comparatively significantly higher for the study population ($P < 0.0001$). The relative risk (RR) of being

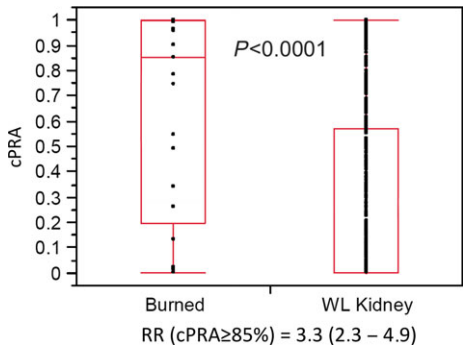

Figure 3 HLA sensitization in the study population of extensively burned patients (n = 29) compared with that in patients on waiting list for kidney transplant (n = 1055) from the same geographical area. Box-and-whisker plot of cPRA percentages. ABO-compatible and gender-matched populations were analyzed. Bottom/top: first/third quartiles, inside band: Median, whiskers: minimum/maximum. RR = relative risk of being hypersensitized (cPRA≥85%) for an extensively burned patient compared to a patient waitlisted for kidney transplant.

hyperimmunized (cPRA≥85%) in the study population was 3.3 times higher (range, 2.3–4.9) than those on waiting list for kidney transplantation.

Discussion

This study demonstrates that in extensively burned patients, anti-HLA alloimmunization is common and at high levels, which reduces their eligibility for VCA. We report that a majority of the patients presenting third-degree or deep second-degree burns, with >40% TBSA affected, and who are therefore likely to be considered for reconstructive transplantation using VCAs, are highly HLA sensitized.

While over 6000 people sustain major burn injuries in the United States every year [25], potential candidates for face and/or hand transplantation are rare, which is supported by the analysis of 29 burned patients presented in this study. Despite the improved survival of burned patients, numerous patients go through extensive functional and esthetic sequels. Among the 28 patients who received face transplantation worldwide since the first procedure in 2005, about one-third (8 patients) had burn injuries [7]. The involvement of hands and face [26,27] affects the quality of life of the burned patients and leads to treatment limitation [28,29], which makes VCA an attractive option in these patients [3,14]. However, repeated blood transfusions and skin allografts [30] are two of the major contributors to anti-HLA sensitization that precludes VCA. Typically, early excision and skin graft closure require transfusion of about 14 units of packed red blood cells (PRBC) [31]. Despite autologous micrografting techniques, patients with major burns over 40% of total body surface area (TBSA) present fewer skin graft donor sites and hence need skin allografts [32].

Sensitization of burned patients and access to VCA transplants

In the present analysis, 28 of the 29 extensively burned patients were anti-HLA immunized, and the majority was hyperimmunized. In this study population, over 60% (18/29) were considered as highly sensitized (cPRA ≥85%). The cPRA scores were close to 60% in five patients and 40% in two patients. Due to the limited reports on VCA procedures so far[5], the effects of DSA and AMR on VCA outcomes are usually extrapolated from kidney transplantation experience where the risk for AMR and graft loss directly correlates with high titer of anti-HLA DSA[20]. Kidney transplant recipients with MFI >3000 for anti-HLA Abs had a 60-fold higher risk of AMR than patients with MFI <500 [20]. There is no rationale to ascertain if highly immunized VCA candidates would benefit from the desensitization strategies reported for solid organs [9]. The application of desensitization protocols is controversial [33], and hence the issue of HLA sensitization needs to be addressed before considering VCA. The histologic restoration was obtained in the reported case of VCA AMR [9] only after a combination of treatment with a complement fraction C5 blocker (eculizumab), a proteasome inhibitor (bortezomib), and a lymphocyte CD52 glycoprotein-binding monoclonal antibody (alemtuzumab), all potentially associated with serious adverse effects [34–36].

The experience in kidney transplantation suggests that those highly sensitized patients would have reduced access to VCA. Approximately 30% of patients on the current US kidney transplant waiting list show evidence of sensitization, and only 6.5% of highly sensitized patients receive a transplant per year [37]. The shortfall in VCA donation [3] is also likely to limit the number of potential donors. For severely burned patients with potential VCA indication, an inclusion in a national prioritization program for highly sensitized patients might also help preserve a satisfactory donor flow [38] and decrease average transplant waiting time [39]. Sensitized VCA recipients would be submitted to intensive clinical, histologic, and immune monitoring to detect and treat acute AMR.

Class I and Class II anti-HLA specificities

The differential association of class I and II with anti-HLA sensitization observed in the study population could not be analyzed further because of the small size of the population. The kidney transplantation experience has indicated an association between anti-HLA class I DSA and early AMR, and between anti-HLA DSA class II and microvascular

injury and slower progression to graft loss [40]. However, in practice, patients are cosensitized to both classes, and those with DSA against only one class of HLA are rare. Assessing the tendency of HLA Ab titers to decrease over time would require a long-term study involving repeated testing and a long follow-up. However, a decrease in anti-HLA Ab titers has little impact on the access to the transplants as the identification of unacceptable HLA specificities, and cPRA calculations are based on anti-HLA Ab peaks from the patient's history [39].

C1q fixation

To our knowledge, this is the first report on C1q-fixing anti-HLA Abs in extensively burned patients. Selecting potential donors on preformed HLA antibodies specificity and strength as in kidney transplantation led to a dramatic reduction of the access to transplants. A critical issue for those widely sensitized candidates for VCA transplantation is to discern which of the detected antibodies might be pathogenic. The ability of Abs to bind C1q [41,42] or C4d [43] fraction of the complement can be determined for the different HLA specificities. Although the significance of C4d deposition in skin is controversial [44], it has been included in the Banff CTA-07 criteria for the categorization of VCA graft rejection [45]. As early AMR is considered to be complement mediated, we hypothesized the study of C1q-fixing ability of HLA Abs in SAFB may represent an interest for donors selection. In the present study, we found that 45% of the burned patients presented C1q-fixing anti-HLA Abs and hence would be at a high risk of aggressive transplant destruction and rejection. Among the HLA specificities considered as unacceptable, if only those capable of C1q fixing are considered for cPRA calculation, then this would result in a significant increase in the number of patients with potential access to transplants.

Such approach might suffer limitations, as conflicting results exist about the C1q-fixing DSA in clinical transplantation. First evidences to support the clinical relevance of C1q-binding DSA, to assess the risks of humoral rejection and transplant loss, came from the group who developed the C1q test [42,46]. Two studies reported preformed C1q-fixing DSA to be more specific than IgG DSA to predict early AMR (eAMR) in cardiac transplantation [47] and delayed kidney transplant function [48]. Correlations had been reported between *de novo* C1q DSA and reduced graft survival of heart transplants [47] or AMR of kidney transplants [41,49]. In adversarial larger kidney transplant studies, preformed C1q-fixing DSA did not correlate with rejection, transplant function, or transplant loss [50–52], while the strength of pretransplantation DSA did so as *de novo* C1q DSA [51].

The number and intensity of C1q-fixing DSA were closely correlated with IgG DSA MFI (C1q requires a minimal density of IgG to bind [53]), and IgG assay might seem sufficient for donors' exclusion [50,52]. Nevertheless, this trend to predict the complement-fixing ability of a given antibody from its high MFI results in IgG assay is not a rule [42,54–56] nor recommended [57].

Moreover, antibodies are also related to CMR through complement independent pathways such as ADCC [58–60]. As an instance, terminal complement blockade only partially reduced the incidence of eAMR after positive cross-match kidney transplantation [61]. While the C1q SAFB assay detected complement-fixing IgM [55,57], it does not detect multiple low-titer DSA (that individually do not bind complement) or non-HLA antibodies.

Whether C1q-fixing DSAs are associated with a higher risk of antibody-mediated damage remains to be determined in VCA. Excluding only the donors presenting HLA specificities targeted by C1q-fixing Abs would result in more donors for VCA recipients; however, whether the potential donors spared by C1q-fixing preformed DSA would allow for acceptable outcomes remains to be studied. As for kidney transplantation, the donors targeted by high MFI IgG should still be avoided to prevent eAMR.

Factors associated with HLA sensitization

Among the factors found to be associated with anti-HLA sensitization in the present study, blood transfusions, pregnancies, and skin allograft are known to cause HLA alloimmunization [30,62]. In contrast, third-degree burns surface or UBS score was not associated with anti-HLA class I or II Abs (MFI>1000) or with cPRA score, despite their association with higher requirement of transfusions and skin allografts, which may be explained by the small population size. HLA sensitization resulted from the combined effect of multiple blood transfusions and skin allograft. As we do not have a third group with blood transfusion only, it would be difficult to objectively quantify the sole effect of allograft skin application on HLA sensitization.

Impact of skin allograft preservation conditioning on HLA sensitization

Skin allografts are known to promote an alloimmune response, and skin is considered as the most antigenic tissue [63]. Clinically, rejection rate is expected to be similar with both CPSA and GPSA, although *in vitro* and animal studies have indicated a weaker immune response with GPSA [64,65]. In the present study, GPSA were seen to be less likely to promote anti-HLA Ab development than CPSA, but this correlation is not conclusive because of the small size of the population. Information on the HLA type

of the donor skin allografts was not available, and hence association between the donor HLA and anti-HLA Abs specificities could not be studied. It would seem premature to promote the use of GPSA over CPSA from this study, although GPSA has been reported to perform as well as CPSA [64,66] or better [67].

Impact of omitting gender match from VCA allocation criteria

As an elective procedure, VCA allows for donor selection based on ABO compatibility, negative cross-match, and size/morphological match including skin color matching. However, information on color and size is not included in the French national database, CRISTAL. Although gender mismatch has been avoided in VCA for morphological, ethical, and societal considerations [68], in view of the shortage of VCA transplant donors, we assessed the consequences of gender mismatch on the access to transplantation.

Allowing a gender mismatch resulted in a significantly reduced cPRA score than if gender matching was imposed ($P \leq 0.0001$, Figs 2 and S3c) and thereby increased the VCA-eligible population by 20%. In the subset of hypersensitized patients, donor–receiver gender did not seem to influence the cPRA score, as the score was close to 100% regardless of gender match. Conversely, patients who had a cPRA score close to 0% if gender match was not considered showed a cPRA score of about 50% if gender matched with donors, as the remaining 50% would presumably be eliminated because of a gender mismatch. The gender match seems to have a higher impact on cPRA for less sensitized patients in terms of cPRA score; however, its influence on the access to transplant is the same, and this access could be doubled. Thus, allowing a gender mismatch may therefore be considered on a case-by-case basis to improve the access to transplants in highly sensitized patients.

Proposed modifications in acute care of burn patients in view of VCA

Whether phenotyped PRBC and platelets should be preferred for burned patients with extensive facial and hand injuries could not be assessed in this study, but this has been common practice in our intensive care unit for several years and may aid in reducing allosensitization.

In cardiovascular surgery (CVS), the transplantation of cryopreserved human heart valve led to broad and strong HLA class I and II DSA sensitization [69], while the majority of patients receiving decellularized allografts had significantly lower anti-HLA Abs than those receiving cryopreserved ones [70,71]. Indeed, artificial or biosynthetic dermal grafts do not induce anti-HLA DSA and appear to be attractive alternatives [72,73] that should be preferred in VCA when possible. Swine skin xenografts offer similar outcomes as GPSA or CPSA to overlay microskin autografts [74]. One of the concerns was that Abs produced against swine leukocyte antigens (SLA) may cross-react with human ones (HLA) as broad HLA-specific Abs cross-react with porcine lymphocytes [75]. However, studies on kidney transplantations suggest that sensitization after swine xenograft does not preclude a subsequent human transplant [76].

To summarize, of the 29 burn patients analyzed, all except one were sensitized to HLA antigens, about two-thirds of the population was hyperimmunized, and about half presented C1q-fixing anti-HLA Abs. The possibility of restoring appearance via VCA transplantation though attractive for extensively burned patients is jeopardized because of HLA sensitization caused by blood transfusions and skin allografts. An early anticipation and assessment of the possibility of VCA should be included in the management of extensively burned patients during the acute care phase. This could include opting for alternative allografts such as artificial, biosynthetic, or decellularized allografts. Further, if the calculation of cPRA percentages is performed with disregard to gender mismatch and inclusion of only C1q-fixing anti-HLA Abs instead of all anti-HLA Abs, then the cPRA percentage would be lower, thereby improving the eligibility for VCA. In conclusion, in order to keep the option of VCA open for extensively burned patients, efforts should be taken to minimize HLA sensitization, and the proposed changes in acute care may be considered in future treatment strategies.

Authorship

PD, EB, LL, and MH: participated in research design. PD, CS, BA, CJ, TL, LB, EB, and MH: participated in the performance of the research. CS, BA, DC, and CJ: contributed new reagents or analytic tools. PD, CS, PG, BA, CJ, and MH: participated in data analysis. PD, TL, CS, PG, LL, and MH: participated in the writing of the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Reactivity of anti-HLA antibodies in single-antigen flow bead analysis (Luminex®). Representative images from six patients are shown. Patients were ranked in decreasing order of their cPRA percentages. The mean fluorescence intensity (MFI) for reactions with all antibodies (blue), and with C1q-fixing antibodies (red) are presented as indicated.

Figure S2. Number and reactivity of HLA serotypes in the study population (n = 29). Patients were ranked in a decreasing order of their cPRA percentages. The mean fluorescence intensity (MFI) for reactions with different HLA classes (CL) and with C1q-fixing antibodies ($C1q^+$), categorized according to MFI threshold values of 500, 1000 and 3000, are presented as indicated.

Figure S3. cPRA values in the study population, according to the MFI threshold, gender match, and presence of C1q-fixing antibodies. All patients were matched for ABO blood groups and analyzed according to MFI threshold categories or considering only C1q+ Abs, (a) after gender matching, or (b) without gender matching. Patients were ordered by decreasing cPRA scores, and were considered as highly sensitized if cPRA score ≥85% (patients on the left of the vertical dashed line). The continuous line connects the cPRA values for the MFI≥1000 threshold (Luminex®) presented in Fig. 2. (c) Mean cPRA values (calculated according to MFI threshold categories or considering C1q+ Ab only, with or without gender matching), are plotted, and were compared using Wilcoxon's test (P-values presented).

Figure S4. Comparison of demographic characteristics and sensitizing events, between the studied burns patients population and patients on waiting list for kidney transplant from the same geographical area. The two populations presented the same sex ratio and blood group distribution.

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