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A novel rat full-thickness hemi-abdominal wall/hindlimb osteomyocutaneous combined flap: influence of allograft mass and vascularized bone marrow content on vascularized composite allograft survival

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Keywords
abdominal wall transplantation, allograft mass, allograft survival, combined flap, vascularized bone marrow, vascularized composite allotransplantation.

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Conflicts of interest
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Abstract

Vascularized bone marrow transplantation (VBMT) appears to promote tolerance for vascularized composite allotransplantation (VCA). However, it is unclear whether VBMT is critical for tolerance induction and, if so, whether there is a finite amount of VCA that VBMT can support. We investigated this with a novel VCA combined flap model incorporating full-thickness hemiabdominal wall and hindlimb osteomyocutaneous (HAW/HLOMC) flaps. Effects of allograft mass (AM) and VBMT on VCA outcome were studied by comparing HAW/HLOMC VCAs with fully MHC-mismatched BN donors and Lewis recipients. Control groups did not receive treatments following transplantation. Treatment groups received a short course of cyclosporine A (CsA), antilymphocyte serum, and three doses of adipocyte-derived stem cells (POD 1, 8, and 15). The results showed that all flaps in control allogeneic groups rejected soon after VCAs. Treatment significantly prolonged allograft survival. Three of eight recipients in HLOMC treatment group had allografts survive long-term and developed donor-specific tolerance. Significantly higher peripheral chimerism was observed in HLOMC than other groups. It is concluded that the relative amount of AM to VBMT is a critical factor influencing long-term allograft survival. Accordingly, VBMT content compared with VCA mass may be an important consideration for VCA in humans.

Introduction

Vascularized composite allotransplantation (VCA) describes the *en bloc* reconstruction of a recipient's anatomical unit, such as hand/forearm, abdominal wall or face, by replacing it with a corresponding part procured from a deceased donor [1–3]. Unlike solid organ transplants, VCAs characteristically contain multiple tissue types, such as skin, muscle, vessel, nerve, and often bone/marrow. Since 1998, over one hundred patients have

benefited from various kinds of VCA with impressive functional recovery for most cases. The technique has the potential to revolutionize reconstructive surgery but remains hindered by the requirement for lifelong nonspecific immunosuppressants and their attendant toxicities, some of which may even be fatal [4,5]. Conceivably, these problems could be solved by induction of donor-specific tolerance that allows complete withdrawal of immunosuppressants without harming VCA survival [6].

Bone marrow may have a pivotal role for inducing tolerance to allografts [7]. For VCA, the significance of bone marrow is suggested by clinical abdominal wall allotransplantation showing an increased rejection rate compared with hand allotransplantation [8,9].Vascularized bone marrow transplantation (VBMT) has been considered superior to conventional nonvascularized bone marrow transplantation, as marrow cells are maintained within their natural microenvironment and there exists a continuous supply of live bone marrow cells [10–13]. However, despite the availability of several animal VBMT models [12–14], it remains unclear whether the presence, or amount of, vascularized bone marrow is critical for tolerance induction.

In this study, we designed a rat two-component combined flap based on the common iliac vessels (CIVs). This comprised the previously reported VBMT of the hindlimb osteomyocutaneous flap (HLOMC) and a new full-thickness hemi-abdominal wall flap (HAW). The feasibility and reproducibility of the HAW/HLOMC model across a full major histocompatibility complex (MHC) mismatch were evaluated. Tolerance to the VCAs was induced with a protocol similar to our previously reported syngeneic adipocyte-derived stem cells (ADSCs)/ALS/short-term cyclosporine (CsA) regimen [15]. Allograft survival, peripheral chimerism, and peripheral blood panel were compared among HAW, HLOMC, and HAW/HLOMC recipients. It was found that the presence of VBMT was critical to the survival of VCAs. Furthermore, the ratio of allograft mass (AM) to VBMT also influenced VCA outcome. Hence, increasingly larger allografts, despite importing a constant amount of VBMT, correlated with lower peripheral chimerism and shorter survival.

Materials and methods

Animals

Male 8- to 12-week-old donor Brown-Norway (BN, $RT1A^n$) and recipient Lewis rats (LEW, $RT1A^l$), representing a full MHC mismatch, were purchased from the National Laboratory Animal Center, Taiwan. They were housed in pyrogen-free conditions under controlled temperature and lighting cycles with water and rat chow freely available at the Chang Gung Memorial Hospital Animal Center. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH, USA, and following the Institutional Animal Care and Use Committee (IACUC) protocol authorized by Chang Gung Memorial Hospital, Taiwan.

Experimental design

Transplantations were performed either as a single HAW flap, or a single hind limb osteomyocutaneous flap (HLOMC), which imports a constant amount of VBMT, or as a combined HAW/HLOMC flap. These are all based on the same source vascular pedicle (Fig. 1). Control VCA and syngeneic groups received no immunosuppressant treatment. VCA treatment groups received 0.5 ml antilymphocyte serum (ALS) 1 day before surgery and on postoperative day (POD) 10, CsA from day 0 to day 10 (16 mg/Kg/day), and intravenous infusions of ADSCs on POD 1, 8, and 15 (2×10^6/dose).

Surgical procedures

Donor surgery

The HAW flap comprised the full thickness of half of the abdominal wall and measured 6×4 cm. This same dimension was maintained for all layers of the flap, including skin, fascia, rectus abdominis muscle, external oblique muscle, internal oblique muscle, transversus abdominis muscle, and peritoneum. The HAW flap was based on the CIVs, preserving the deep inferior epigastric vessels (DIEVs), the deep circumflex iliac vessels (DCIVs), and the superficial inferior epigastric vessels (SIEVs). An incision was performed in the groin 2 cm inferior and parallel to the inguinal ligament (IL), preserving the SIEVs. The dissection was continued laterally until the anterosuperior iliac spine. At this level, a laparotomy was performed (Fig. 1B) and a full-thickness dissection continued along the lateral border of the abdominal wall, the subcostal line, and the midline (Fig. 1C). The IL was detached from its lateral and medial insertions. For combined flap elevation, the femoral vessels (FVs), SIEVs, and distal branches to the hind limb were preserved (Fig. 1E). During the intra-abdominal dissection, all pelvic branches of the CIVs were ligated, except the external iliac vessels (Fig. 1D). Thus, the HAW flap (Fig. 1D) and the combined flap (Fig. 1E) were increased based on the ipsilateral CIVs. Flaps were flushed intra-arterially with heparinized saline until clear venous backflow was observed.

The single HLOMC flap was performed as previously described [16]. Osteotomies were performed to incorporate a constant distal 1/3 of femur and proximal half of the tibia within the transplant. The distal half of the thigh muscles and all the leg muscles were included in the flap. A lateral skin paddle of 3×3 cm was also preserved.

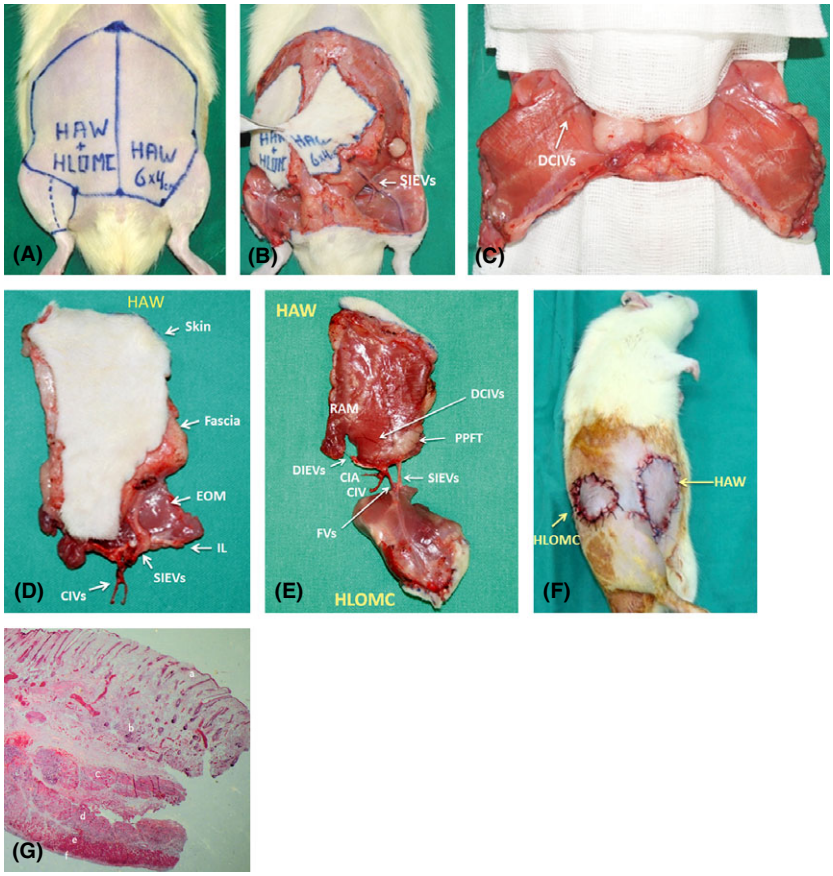

Figure 1 Graft harvesting for HAW and HAW/HLOMC. (A) Lewis rat skin markings for bilateral harvesting. Left side: HAW. Right side: HAW/HLOMC. (B) Preserved SIEVs and initial laparotomy. (C) Mid line split abdominal wall. (D) Components of the HAW. (E) Components of the full-thickness HAW/HLOMC flap. The thin bright peritoneum layer is observed on the deep surface of the HAW. (F) BN to LEW full-thickness HAW/HLOMC combined VCA, during immediate postoperative recovery. (G) H & E staining of a Group 3 HAW section collected on POD 7. All the abdominal wall layers can be identified: a. skin, b. fascia, c. external oblique muscle, d. internal oblique muscle, e. transversus abdominis muscle, f. peritoneum.

Recipient surgery

After dissecting the groin, end-to-end anastomoses were performed between common FVs of the recipient and CIVs of single HAW flap, CIVs of combined HAW/HLOMC flap, or FVs of single HLOMC flaps. For HAW or HAW/HLOMC flap inset, a matching abdominal wall skin-fascia defect was created starting from the groin. HAW flaps were inset heterotopically, immediately superficial to the abdominal wall muscles of recipients. For HLOMC flaps (or the HLOMC component of HAW/HLOMC combined flaps), a tunnel was created from the groin to the ipsilateral flank where a skin-fascia defect was created for the flap to be inset with 4–0 nylon.

Follow-up

VCAs were evaluated daily with an established semi-quantitative rejection grading system that ranges in severity from grade 0 to 4 as follows: grade 0, no rejection; grade 1, pink or slightly erythematous; grade 2, frank erythema; grade 3, erythema or purplish discoloration with blister formation or partial hair loss; and grade 4, dark purplish discoloration with blister formation and major hair loss. Rejection was defined when 80% of the VCA reached grade 4 [17]. For histopathological evaluation, full-thickness biopsies were taken upon appearance of clinical signs of rejection. Biopsies were fixed in 10% formalin and embedded in paraffin for hematoxylin and eosin (H&E) staining.

ADSC preparation

The procedure for ADSC preparation has previously been detailed [15]. Briefly, the inguinal fat pad from LEW rats was harvested sterile, washed, minced, and digested with type IV collagenase (Life Technologies, Grand Island, NY, USA) and hyaluronidase (Sigma-Aldrich, St Lewis, MO, USA). The pelleted stromal vascular fraction (SVF) containing ADSCs was resuspended and plated in stromal

media containing low-glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated at 37 °C in 5% CO_2 atmosphere. The media was replaced every 2–3 days and cells passed when 80–90% confluence was reached. ADSCs were ascertained to be $CD90.1^+CD29^+CD73^+CD45^-CD79a^-CD11b/c^-$ cells by flow cytometry before being infused to VCA recipients.

Flow cytometry

Venous blood was collected for analysis. White blood cells were collected following erythrocytes lysis and stained with the following antibodies: APC-conjugated anti-CD4, PerCP-conjugated anti-CD8, PE-conjugated anti-TCR, PE-conjugated anti-CD161, FITC-conjugated anti-CD45 (BD Biosciences, San Jose, CA, USA), and APC-conjugated anti-CD11b/c (BioLegend, San Diego, CA, USA). BN-derived cells were stained specifically with RT1Ac antibodies (AbD Serotec, Kidlington, UK). For regulatory T cells (Treg), cells were stained with anti-CD4 and PE-conjugated anti-CD25 (BD Biosciences), followed by permeabilization for 18 h at 4 °C with commercial kit (eBioscience, San Diego, CA, USA) and staining with PerCP-Cy5.5-conjugated anti-FoxP3 (eBioscience). Antibody-bound cells were analyzed by FACSCanto II flow cytometer (BD Biosciences).

Mixed lymphocyte reaction (MLR)

Rat spleens were harvested under sterile conditions. After erythrocyte lysis, $CD4^+$ and non-T cells in LEW and BN splenocytes, respectively, were collected via sorting by auto-MACS Pro (Miltenyi, Bergisch Gladbach, Germany) for one-way mixed lymphocyte reaction. The $CD4^+$ cells isolated from naïve or tolerant LEW rats were labeled with 1% fluorescent Violet Proliferation Dye 450 (VPD, BD Biosciences) at 37 °C for 10 min followed by washing with medium, before coculturing with allogeneic BN or third-party Sprague-Dawley (SD) stimulator cells that had been treated with mitomycin C for 30 min. Cells were cultured at 37 °C for 4 days before collection for analysis with flow cytometry.

Skin grafting

Dorsal cutaneous defects, superficial to panniculus carnosus, were created in recipients with long-term surviving VCA for insetting BN-origin full-thickness tail skin grafts (2 cm × 1 cm). These were fixed with tie-overs for 5 days, and successful grafts evaluated daily for rejection for another 60 days. Rejection was suggested if erythema, edema, scaling of the skin, hair loss, epidermolysis, and desquamation occurred; destruction of more than 80% of the graft defined rejection.

Histological exam

Long-term surviving allografts were embedded in specialized tissue-freezing medium for whole-body sectioning (Leica, Wetzlar, Germany) and cryosectioned with a Leica CM3600 macrotome. The sections on the whole-rat sized slide were stained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclei and set on the custom-made whole-rat sized stage of the TissueFAXS Plus™ (TissueGnostics, Vienna, Austria) and panoramic micro-imaged with 200× magnification. Histology was also evaluated by microscopy after H&E staining.

Statistics

Results from flow cytometry were analyzed by one-way ANOVA and Tukey-HSD for *post hoc* pairwise comparison. Median VCA survival time was acquired by the product limit method of Kaplan–Meier. A probability value <0.05 was considered statistically significant.

Results

The HAW/HLOMC surgery is illustrated with a syngeneic control in Fig. 1. Ischemia time was 28 ± 3 min for all transplantations as only one set of anastomoses was required for all groups. As demonstrated in Table 1, allograft masses of HAW and HLOMC flaps were similar ($P > 0.05$) and approximately half that of combined flaps. All syngeneic flaps survived the follow-up period of 150 days without any signs of rejection.

The study groups and respective transplant survival times are shown in Table 2. The average rejection-free survival of flaps in all control allogeneic groups was 6 days. For combined flaps, as both the HAW and HLOMC parts contained skin (Fig. 1F), each component could be monitored independently; however, no notable differences were observed in the rejection courses between the two components.

The immunosuppressant protocol prolonged the survival of all VCA groups. However, the extent of prolongation for single HLOMC group was much longer than that of the single HAW and combined HAW/HLOMC groups. The median survival time of treated allogeneic flaps was 34 days

Table 1. Comparisons of the three VCA models.

VCA	Graft weight (g)	Vascularized bone	Flap harvest time (min)
HAW	10.7 ± 1.2	No	38 ± 4
HAW/HLOMC	22.4 ± 2.3	Yes	49 ± 3
HLOMC	11.6 ± 1.3	Yes	40 ± 5

Table 2. Study groups and corresponding immunosuppressant treatment and median allotransplant survival time.

Group	Donor-recipient combination	VCA	Immunosuppressive treatment	Recipient number	Median survival time ± standard error (days)
1	LEW→LEW	HAW	No	6	150 ± 0
2	LEW→LEW	HAW/HLOMC	No	4	150 ± 0
3	BN→LEW	HAW	No	6	14 ± 2.5
4	BN→LEW	HAW/HLOMC	No	4	14 ± 0.8
5	BN→LEW	HLOMC	No	4	11 ± 0
6	BN→LEW	HAW	ALS (0.5 ml, POD -1, 10)/CsA (16 mg/Kg, POD 0-10)/ ADSC POD1, 8, 15	8	34 ± 2.4
7	BN→LEW	HAW/HLOMC	ALS (0.5 ml, POD -1, 10)/CsA (16 mg/Kg, POD 0-10)/ ADSC POD1, 8, 15	7	48 ± 3
8	BN→LEW	HLOMC	ALS (0.5 ml, POD -1, 10)/CsA (16 mg/Kg, POD 0-10)/ ADSC POD1, 8, 15	8	57 ± 21

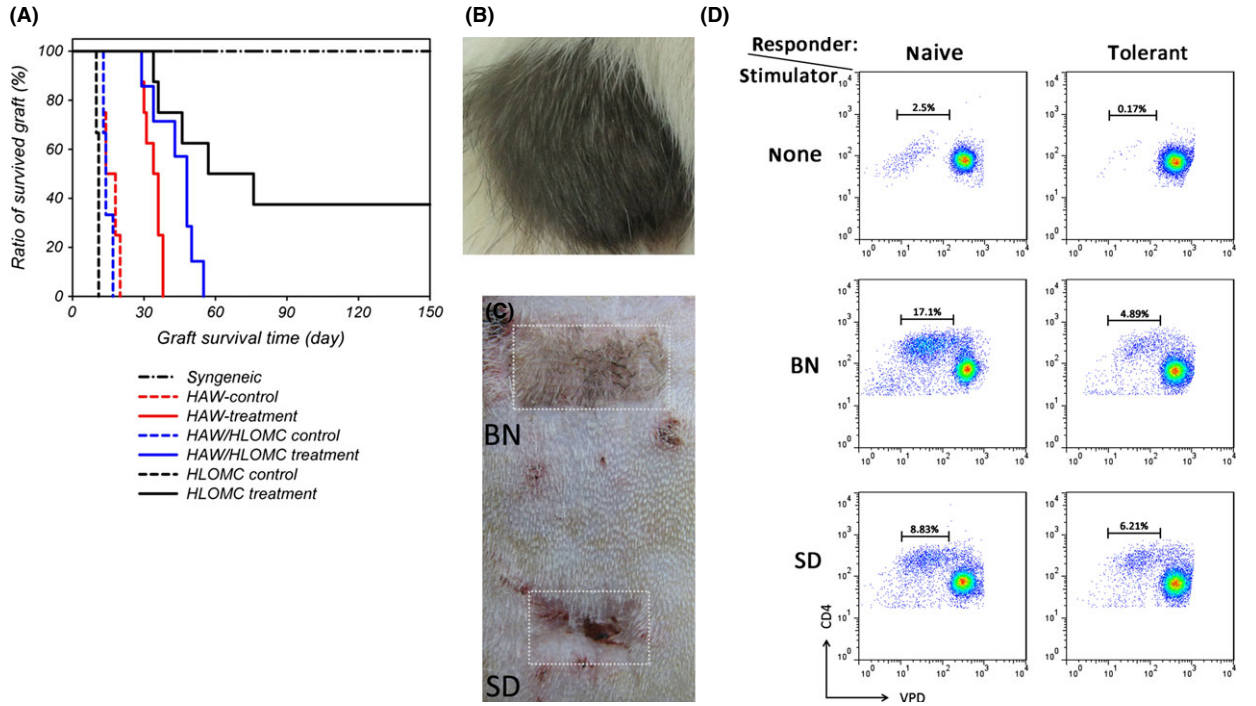

Figure 2 Tolerance was achieved for HLOMC VCA. (A) Kaplan–Meier survival curves of VCAs. Dotted lines represent control groups and solid lines represent treatment groups. (B) Representative BN HLOMC VCA of a tolerized LEW recipient at post-VCA day 150. (C) Representative accepted skin graft from BN (upper) and rejected skin graft from SD (lower) of a LEW recipient with a long-term accepted BN HLOMC VCA. Both grafts were on the same recipient and the photograph was taken at 31 days after skin grafting. The white dotted lines mark the boarders of the grafts. (D) In response to BN antigen, responder splenocytes from VCA-tolerant LEW rat proliferated significantly less than splenocytes derived from naïve LEW in one-way MLR. Conversely, splenocytes from naïve and tolerant LEW rats responded similarly to SD alloantigens. The fluorescent range marked with brackets was derived from proliferated responder cells.

for HAW, 48 days for HAW/HLOMC, and 57 days for HLOMC groups ($P < 0.001$). Survival curves for all groups are shown in Fig. 2. Treatment of ADSCs was beneficial to survival as the median survival time for the HLOMC recipients that were administered with CsA and ALS without ADSCs was about 32 days, as reported previously [15]. Some HLOMC recipients (three of eight) exhibited no signs of rejection for the entire observation period (150 days). Furthermore, only the recipients of long-term surviving VCA accepted a second alloantigen challenge in the form of a BN graft; these subsequently grew hair normally. Conversely, concurrent third-party skin grafts from SD rats were rejected; the residual wound healed secondarily with wound contraction (Fig. 2C). The *in vitro* mixed lymphocyte reaction also showed that cells from the tolerant recipient were hypoproliferative toward BN antigen

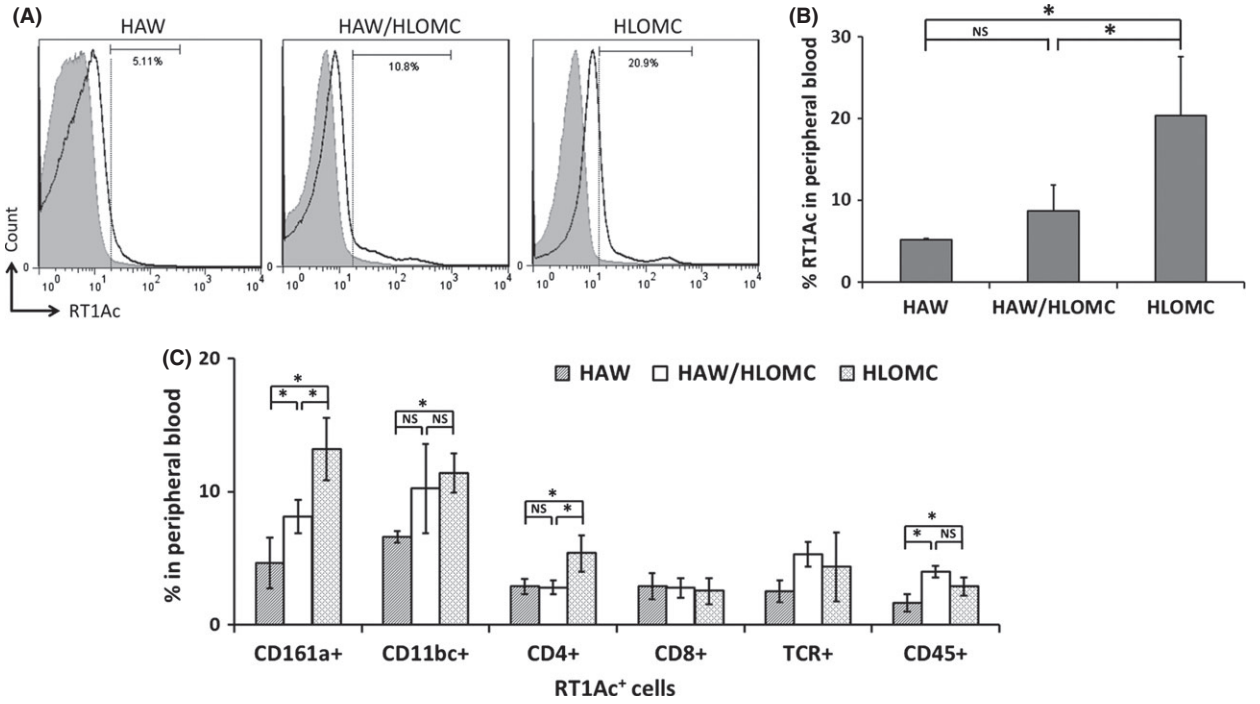

Figure 3 Peripheral chimerism of treatment groups at POD 28. (A) Representative flow cytometric histograms from each group. Shaded area represents signals derived from isotype control. (B) Group data of peripheral chimerism at POD28. (C) Peripheral chimerism of different cell types at POD28. Data for each cell type were analyzed with ANOVA followed by *post hoc* pairwise comparison when the significance of ANOVA was lower than 0.05. The data of $CD8^+$ and TCR^+ were not statistically significant. *: $P < 0.05$; NS, no significant difference.

compared with cells from naïve LEW (Fig. 2D). On the other hand, cells of naïve and tolerant LEW rats behaved similarly against third-party SD alloantigens. These data demonstrated that donor-specific tolerance had developed in recipients of long-term surviving HLOMC. Peripheral blood from recipients of all treatment groups were collected 4 weeks after transplantation and analyzed by flow cytometry. Statistically significant differences between groups emerged regarding peripheral chimerism. Single HLOMC recipients, on average, had 20% BN-derived cells within their circulation, in contrast to <10% peripheral chimerism in the other two groups (Fig. 3A). Further analyses of BN-derived leukocytes in the recipient circulation showed that every subpopulation, including T cells ($CD4^+$, $CD8^+$, TCR^+, Treg cells), B cells ($CD45^+$), dendritic cells ($CD11b/c^+$), and NK cells ($CD161^+$), had varying degrees of chimerism. As demonstrated in Fig. 3C, $CD161^+$, $CD11b/c^+$, $CD4^+$, and $CD45^+$ cells showed statistically significant differences when analyzed by ANOVA. When *post hoc* pairwise comparison was performed, $CD11b/c^+$ cells were higher in the HLOMC than the HAW group. $CD45^+$ cells in HLOMC and HAW/HLOMC groups were increased compared with those in the HAW group. Furthermore, $RT1Ac^+CD161^+$ and $RT1Ac^+CD4^+$ cells were significantly higher in the single HLOMC group than the other two groups (Fig. 3C).

When HLOMC VCAs survived over 150 days, peripheral lymphocyte panels of recipients were analyzed with flow cytometry. Compared with those acquired from syngeneic transplant recipients, naïve LEW rats and recipients that rejected their HLOMC allografts, it was found that firstly, no statistically significant differences were found in the levels of $CD45^+$, TCR^+, and $CD4^+$ cells among the four groups. Secondly, the levels of $CD161^+$ and $CD11b/c^+$ cells were significantly higher in VCA recipients, although no differences were found between rejection and tolerant recipients. Finally, tolerant recipients possessed significantly higher levels of $CD4^+CD25^+FoxP3^+$ cells than recipients of rejected VCA or syngeneic grafts. In contrast, the level of $CD8^+$ cells was significantly lower in the tolerant recipients than in the other three groups (Fig. 4).

Long-term surviving HLOMC VCAs were isolated with surrounding tissues and stained with DAPI. As shown in Fig. 5, the vascularized bone marrow within the allograft contained intact cell nuclei with DNA similar to that in the recipient.

Discussion

Firstly, we demonstrate that it is feasible to increase and transplant the combined HAW/HLOMC VCA based on the

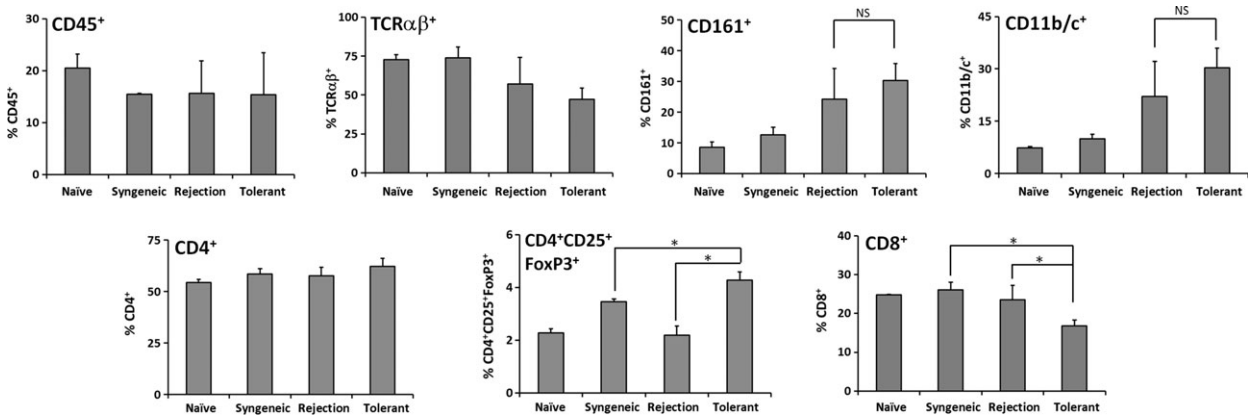

Figure 4 Comparison of peripheral levels of lymphocytes. Percentage of different cell types in peripheral lymphocytes among LEW recipients that developed tolerance with long-term surviving HLOMC (survival), rejected the HLOMC (rejection), the LEW recipients with syngeneic HLOMC (syngeneic) and untreated LEW (naïve) were shown. Overall significance was acquired with one-way ANOVA. Pairwise comparisons between tolerant and rejection groups, or between tolerant and syngeneic groups, were performed only when the significance of ANOVA was lower than 0.05. *: $P < 0.05$; NS, no significant difference.

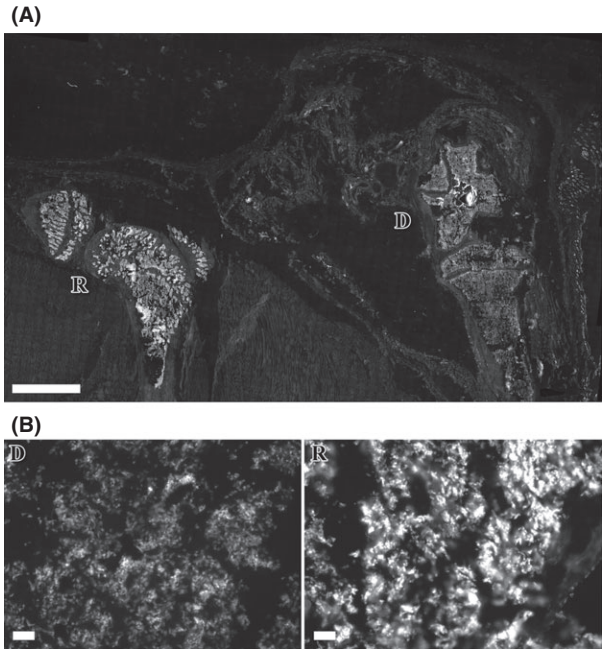

Figure 5 DAPI-stained long-term surviving HLOMC allograft with surrounding tissues. Both donor (labeled 'D') and recipient (labeled 'R') bone marrow showed intact cell nuclei. (A) scale bar: 5 mm; (B) scale bar: 50 μm.

iliac vessels. The HAW flap in this model contains skin, fascia, muscles, and peritoneum derived from full-thickness abdominal wall (Fig. 1D). In view of the clinical significance of abdominal wall VCA in humans [8,18,19], this flap provides a novel and valid animal model for clinically relevant VCA research. One advantage of the single HAW flap design is the dual blood supply from the deep (DIEVs-DCIVs) and superficial vascular (SIEVs) systems, which allows flexibility in adjusting the amount of skin or muscle within the flap independently.

Bone marrow transplantation has been applied to achieve immunological tolerance to organ allografts [20,21] as well as VCAs [22,23]. VBMT provides additional advantages such as instant engraftment and a natural stromal supporting microenvironment [11]. Barth *et al.* [24] demonstrated that under therapeutic immunosuppression, inclusion of vascularized bone marrow within VCA prevented rejection, graft loss, and prolonged VCA survival in nonhuman primates. The donor bone marrow viability persisted and became chimeric for both donor and recipient cells. Vascularized bone marrow may convey its effects via suppression of alloantibody production. This work showed that donor vascularized bone marrow continued to function immunologically in recipients undergoing maintenance immunosuppression. Lin *et al.* also demonstrated that VCA was more permissive to tolerance induction than full-thickness skin grafts due to the presence of vascularized bone marrow. Recipients that were tolerant to VCA showed highest peripheral mixed chimerism at POD30. Degree of chimerism then gradually declined to the background level at POD90 [25]. The level of $CD4^+FoxP3^+$ Tregs was significantly higher in recipients tolerant to VCA. These studies demonstrated well the advantage of including vascularized bone marrow in VCA for maintaining allograft survival and developing donor-specific tolerance. However, the interactions of vascularized bone marrow and other aspects of the allograft, such as the influence of its mass, were not clearly identified.

Three VCAs, namely single HLOMC (incorporating VBMT), single HAW, and HAW/HLOMC combined flaps, were performed to evaluate whether the presence of VBMT is sufficient to promote long-term allograft survival. The first two are similar in AM (indicated by weight) but different in that HAW does not contain VBMT. HAW/HLOMC combined flaps contain approximately double the AM and a similar amount of VBMT as the HLOMC flap. Without treatment, all three VCAs showed similar survival times ($P > 0.05$). The tolerogenic immunosuppressant regimen prolonged allograft survival for all VCAs; however, a significantly longer survival time was observed in HLOMC group compared with the other two ($P < 0.05$). Such differences are in part explained by the highest chimerism level of the single HLOMC group determined at 4 weeks post-transplantation before rejection was observed clinically for all treated subjects. These results are in accordance with previous literature, which stated that chimerism is associated with induction of tolerance [26–28]. However, when AM was doubled using the HAW/HLOMC flap, allograft survival time was closer to that of the single HAW despite the presence of VBMT. This suggests that the beneficial effects of VBMT on VCA survival may be limited by a certain mass of allograft that it can support. This may have significance for future clinical VCA practice, in that the inclusion of vascularized bone within the VCA, or by heterotopic transplantation, may be beneficial to VCA outcome.

Our observations are supported by the results of the study by Ulusal *et al.* [29]. In that study, VCA survival after 1 week CsA monotherapy was not statistically different between unilateral and bilateral groin allotransplantation (100% increase in AM, survival time 19 vs. 19.5 days, T-cell chimerism <1%). However, a combined groin flap including the upper half of the hindlimb (containing VBMT) resulted in increased AM and prolongation of VCA survival to 27 days (approximately 42% improvement when compared with the unilateral groin flap). The level of T-cell chimerism was also increased to 6–19%. Both our study and that of Ulusal *et al.* support a relationship between AM and VBMT content on VCA survival.

Engraftment and reconstitution of multiple hematopoietic lineages following allotransplantation may be critical for induction of tolerance to VCAs [26]. In the current study, the higher degree of chimerism in HLOMC recipients was reflected by the increased level of donor-derived $CD161^+$ NK cells as well as $CD4^+$ cells relative to the other cell types. Accumulating evidence indicates that NK cells are heterogeneous with specific combinations of cell surface molecules and play essential roles in transplantation immunology, including the processes of rejection as well as tolerance induction and maintenance [30]. The tolerogenic effects of NK cells were mediated by secretion of IL-10, activation of Tregs as well as elimination of APCs [31]. Our data suggested NK cells of donor origin may play a role in the early phase of tolerance induction, which is worthy of further investigation.

When peripheral lymphocytes were inspected in naïve and syngeneic recipients and recipients that rejected or tolerated HLOMC allografts, only $CD8^+$ and $CD4^+CD25^+FoxP3^+$ Tregs showed significant differences between rejected and tolerant recipients. The tolerant recipients had a significantly higher level of Tregs and lower $CD8^+$ cells. An inverse relationship between $CD8^+$ cells and Tregs has been reported previously. For example, Guo *et al.* reported that the prolongation effects of IL35 and decitabine on mice cardiac allograft survivals were associated with the increase of Tregs and decrease in $CD8^+$ T cells [32]. When inducing tolerance using CD3 antibody, a different pattern of T-cell reconstitution was observed in the allograft as well as the draining lymph nodes. The tolerogenic treatment prevented accumulation of $CD8^+$ cells while inducing an increase in Treg cells [33]. Furthermore, increases in $CD8^+$ cell expansion and infiltration in allografts are closely associated with acute rejection [34]. As the total level of TCR did not show significant changes among all four groups, the rise and decline of Tregs and $CD8^+$ cells, respectively, suggested a decreased Teff/Treg ratio, which is consistent with earlier reports that a decreased Teff/Treg ratio is correlated with induction of tolerance to allografts [35]. Furthermore, as the donor bone marrow stays viable within long-term surviving VCAs (as indicated by the intact cell nuclei), the continuous interplays between donor bone marrow-derived cells and recipient $CD8^+$ or Tregs will be investigated in future studies.

A classification system was previously proposed to categorize various VCAs based on their relative complexity [36,37]. The concomitant VCAs, which refer to transplanting multiple VCAs from one donor to one recipient, exhibit maximum complexity and remain to be performed successfully in the clinical setting [37,38]. With similar ischemia times and anastomosis requirements, the HAW/HLOMC combined flap can be viewed as an ideal animal model for concomitant transplantation of two flaps in one operation. Features of the single flaps may interact with each other and new cellular interactions may emerge that have significant influences on VCA outcome. For example, although the outcome of the combined flap was similar to that of the single HAW, the differences in cellular panels (e.g. $CD161^+$ and $CD45^+$ cells in Fig. 3C) suggested that diverse cellular processes could be involved in rejection of the HAW/HLOMC versus HAW flaps. Relinquishment of the benefits provided by VBMT in the HAW/HLOMC VCA indicated future studies are warranted to discover the optimal immunosuppression or cell therapy regime to maintain long-term allograft survival for VCAs with different AM and VBMT compositions.

Authorship

AER, WWL, CJW, YLW, LYS, CFL and SHC: performed research and collected data. AER, HYC and FCW: designed study and analyzed data. AER, HYC, CGW and FCW: wrote the paper.

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