Transplant International

Transplant International ISSN 0934-0874

REVIEW

Advances in the development of experimental composite tissue transplantation models

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Keywords
composite tissue allograft, experimental models, large animal models, small animal models.

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Received: 19 June 2009
Revision requested: 13 July 2009
Accepted: 30 July 2009

doi:10.1111/j.1432-2277.2009.00948.x

Summary

The preclinical experimental models of composite tissue allograft (CTA) have rapidly developed in the past years. When microsurgical techniques were established, researchers focused on immunomodulatory protocols that overcome the immunologic barrier between the allogenic donor and recipient. To test immunologic response, functional recovery, and technical feasibility, experimental CTA has been performed in different models, including rodents, large animals, and nonhuman primates. In the experimental studies, researchers are focused on tolerance-inducing strategies based on immunosuppressive protocols allowing for widespread application in the clinic. In this review, authors analyzed the current knowledge of immunologic aspects and tolerance-inducing strategies in CTA experimental models, including single components such as skin or vascularized bone allograft versus CTA containing multiple tissues such as experimental limb and face transplants, and emphasized their relevance and applicability to the clinical scenario.

Introduction

Composite tissue allograft (CTA) is currently accepted as an alternative therapy in selected cases of reconstructive surgery. Since 1998, when the first successful hand transplantation was performed in Lyon, France, hand transplantation programs have been launched in the United States, Austria, China, Italy, Belgium, and Poland. According to the International Registry on Hand and Composite Tissue Transplantation (IRHCTT; http://www.handregistry.com), a total of 46 hands/digits were transplanted in 34 patients. In addition, a considerable number of composite tissue transplantations have been performed around the world, including the femoral diaphysis ($n = 3$), the knee ($n = 5$), the larynx ($n = 31$), the uterus, the abdominal wall ($n = 10$), vascularized tendons ($n = 3$), peripheral nerves ($n = 7$), and most recently, partial face transplants ($n = 7$). Thus, the need for CTA transplants and potential applications have been amply documented worldwide, and confirmed the need for continuation of experimental research.

In most applications, CTA contains multiple components of tissues; however, each individual component of CTA, including skin, muscle, tendon, nerve, bone, and joint, has been already transplanted individually in the experimental animal models and clinically in patients [1–3]. Each individual component of CTA possesses unique immunologic characteristics that ultimately contribute to the successful outcome of the CTA. To prevent rejection and overcome the immunologic barrier, all CTA transplants require life-long immunosuppression. A number of studies on tolerance induction in CTA were reported mainly in animal models [4,5], and despite a recent report on tolerance induction in clinical renal transplantation [6], there are currently no protocols available that are applicable to clinical CTA in humans.

To test immunologic response, functional recovery, and technical feasibility, many different types of CTA models were developed. In these models, not only microsurgical techniques were improved, but extensive research was also carried out to overcome the immunologic barrier between the allogenic donor and recipient.

In the experimental studies, the most widely used models of single component of CTA are skin allograft and isolated vascularized bone marrow transplant (VBMT). Experimental skin allograft includes: vascularized skin allograft (VSA) and subcutaneous tissue and nonvascularized skin allograft (non-VSA). In the preclinical studies, VBMT is represented by isolated femoral bone transplant and by maxilla allograft. In the clinic, vascularized skin allograft (VSA) models are represented by abdominal wall transplant and scalp, whereas VBMT is represented by femoral diaphysis or knee joint transplants [7–11].

Experimental CTAs, which are composed of multi-tissues, differ in the content and number of transplanted tissues. These CTAs may include VSA and bone, and/or VSA, lymph nodes, and bone component (face allograft with calvaria or face allograft with mandible). The limb allograft model is most often used for immunologic assessment and tolerance induction in CTA and it contains vascularized skin, bone, lymph nodes, muscles, nerves, and tendons. In the clinic, these models are represented by unilateral and bilateral hand transplants, and by face transplants [12,13].

In this review, we have analyzed the current knowledge of immunologic aspects and tolerance-inducing strategies in CTA experimental models and emphasized their clinical relevance and applicability.

The models carrying a single tissue component of the CTA

Skin allograft

Skin is the largest organ in the human body, containing a specific immunologic microenvironment formed by cells and humoral compounds with precise organization, and represents a natural barrier with the ability to respond to foreign antigens with innate (inflammatory) and adoptive (specific) immune responses. Skin as an immunologic organ possesses an active defense function, which is attained by a specific immune system known as skin immune system (SIS) [14]. The complexity of immune response of the transplanted skin is regulated by cellular components of the SIS, which include: antigen presenting cells (APCs) such as Langerhans cells (LC) in the epidermis; dermal dendritic cells (DDC) in the dermis; skin-resident T lymphocytes, keratinocytes, and fibroblasts; dermal microvascular unit; and neural immunologic network. Moreover, transplanted skin may contain skin draining lymph nodes abundant with memory T cells.

The highly immunogenic character of skin poses a significant challenge for skin allograft acceptance. For this reason, skin allograft is the most frequently used model for tolerance induction studies. From the historical perspective, acquired tolerance to allogenic skin was first reported by Billingham and his colleagues in the mouse model. There have been reports on the role of chimerism induction via hematopoietic cell transplantation into neonatal mice, and as a result, the adult mice accepted skin grafts from the donor strain and rejected third-party skin grafts [15]. Since that time, different tolerance-inducing strategies have been employed in experimental studies of skin allograft acceptance; however, the mechanism of skin allograft rejection still remains poorly defined.

Several studies demonstrated prolonged skin allograft survival; however, the efficacy and time of skin allograft survival were related to the pattern of skin vascularity, size of the skin allograft, and immunomodulatory protocols.

Nonvascularized skin allograft

In the studies by Cober *et al.* in a fully allogenic rat model, recipients of a skin allograft were preconditioned with antilymphocyte (ALS) serum and intrathymic infusion of donor bone marrow (BM) cells. A full-thickness skin graft from the BM donor was transplanted 3 weeks after recipient preconditioning. Authors reported prolonged skin allograft survival (median 24 days); however, tolerance was not achieved [16]. A different strategy for non-VSA survival was reported by Bartlett *et al.* Authors introduced immunosuppression using blockade of the CD40-CD154 co-stimulatory pathway and/or sirolimus. This resulted in a 64-day median survival time in full-thickness skin allograft model between Dark Agouti (DA) donors and ACI recipients [17]. These results documented that co-stimulatory blockade and effect of sirolimus reduced T-cell activation, leading to a prolongation of skin allograft survival.

Induction of tolerance to skin allografts without immunosuppression was studied by Petit *et al.*, where donor BM cells and subsequently non-VSA were applied in a fully MHC-mismatched rat model. Authors created hematopoietic chimeras after neonatal injection of donor BM cells and donor-origin epidermal cells. Two months after hematopoietic chimeric host creation, a skin allograft originating from the BM donor was transplanted and authors reported prolonged skin allograft survival (mean 15.5 days) without irradiation, myeloablation, or immunosuppression. However, despite the fact that the recipients were neonatally presensitized with donor BM cells and donor skin epidermal cells, macrochimerism was not detected [18].

Different strategies of immunomodulatory protocols used in the full-thickness skin allograft model were not effective in tolerance induction [16–18]. Moreover, donor preconditioning or application of myeloablative or non-myeloablative therapy, or chimera creation used in non-VSA models is not clinically relevant in a CTA, which represents vascularized allograft transplants.

Vascularized skin allograft

In clinical practice, reconstructive CTA transplantation, such as hand, face, and abdominal wall transplant, skin and musculocutaneous flaps are often needed for coverage of large defects. Therefore, in the CTA scenario, VSA rather than a small non-VSA transplant is required. To test the role of vascularity of the transplanted skin in allograft acceptance, we transplanted VSA from the groin region of the donor rat to the groin of the allogenic recipient across a full MHC barrier from ACI donor to Lewis recipient. In this model, we have tested the efficacy of short-term immunodepletive protocol using anti-αβ-T-cell receptor (anti-αβ-TCR) monoclonal antibody in combination with calcineurine inhibitors, either Cyclosporine A (CsA) or Tacrolimus, and we assessed skin allograft survival [19]. Our model differs from the models described above, as immunosuppressive therapy was given for 7 days only, and vascularized (not full-thickness) skin allograft was transplanted simultaneously, without recipient conditioning. Under this protocol of anti-αβ-TCR/CsA, significant extension of skin allograft survival was observed for up to 84 days post-transplantation. Tolerance to skin allograft was not confirmed; however, this immunosuppressive protocol given on the day of VSA transplantation has potential clinical applicability.

Recently, we have compared different sizes of VSA and non-VSA and efficacy of skin allograft survival based on low maintenance dose (2 mg/kg/day) of CsA monotherapy. In this study, we have documented in the rat model that vascularization and size of skin allograft may contribute to both skin allograft survival and donor chimersim induction [20]. We hypothesized that the vascularization pattern of CTA transplant may modify the immunologic response in a recipient. The immunologic responses to non-VSA and VSA may differ based on the length of the graft ischemia and relevant ischemic damage, and on the type of interaction between the skin allograft and the recipient immune system. In the scenario of VSA, reconstruction of blood supply within 1–2 h after donor–recipient vessels anastomosis minimizes ischemic damage. In contrast, after non-VSA transplant, the time of relative ischemia is extended to up to 2–3 days, which is required for revascularization of the graft within the recipient bed [21]. The differences in progress of vascularization are important in the skin allograft as skin is an abundant source of immunocompetent cells such as Langerhans cells and dermal dendritic cells, with antigen presenting cell function, and T lymphocytes. When non-VSA is placed in the recipient body, it is not vascularized during the first post-transplant days. During this early period, there is sprouting of new vessels from the recipient's background and neighboring recipient skin to reach the graft, and there is no direct connection between the donor-origin cells from the graft and the recipient's immune system via blood circulation. In contrast, after transplantation of VSA, graft-origin cells rapidly migrate into the recipient's blood circulation, and recipient immunocompetent cells invade the allograft immediately after pedicle anastomosis. These differences in dynamics of skin allograft vascularization process in non-VSA versus VSA, as well as graft size, were found to have an effect on the development of donor chimerism. We observed donor chimerism induction in both vascularized and nonvascularized skin grafts; however, larger graft size correlated positively with chimerism level only in the VSA recipients. In contrast, in non-VSA recipients, larger skin diameter correlated inversely with blood chimerism level [20]. In this model, under low nontoxic maintenance dose of CsA monotherapy, significant extension of VSA acceptance up to 290 days was achieved in large diameter VSA compared to 132 days in non-VSA model of the same graft size of 6 cm × 6 cm.

The differences in the immune response to non-VSA and VSA were also recently tested by Horner *et al.* in skin allograft transplantation performed from isogenic (Lewis) or allogenic (WF) rats to transgenic green fluorescent Lewis (LEW-GFP) recipients [22]. Four days of observation of cell trafficking between the donor and the recipient skin compartment indicated that VSA is more vulnerable to recipient dendritic cells infiltration, expression of MHC class II molecules in the dermal vessel endothelial cells, and more susceptible to skin allograft rejection compared with non-VSA.

In contrast to Siemionow's studies [20] where VSAs, under low nontoxic dose of CsA therapy, were more permissive for tolerance induction, Horner's study was performed without any immunosuppressive therapy, and as such explored possible mechanism of skin allograft rejection.

These data indicate that the mechanism of skin antigenicity and possibility of rejection may differ under different experimental conditions. The difference in the time of rejection, graft size, and donor-reactive T-cell ratio may be dependent on immunomodulation. A larger size of the skin allograft may down-regulate the immune response by stimulation of stronger regulatory T-cell response of the recipient. It means that a skin allograft recipient may generate adequate response to donor-reactive T cells to induce rejection of the small graft, but the threshold of initiating T-cell response required for rejection of a larger allograft is not achieved [21,23].

Studies on tolerance induction to an allogenic skin graft were performed in a preclinical miniature swine model across MHC barrier [24]. In this study, authors induced donor hematopoietic cells engraftment by partial T-cell depletion, low dose of irradiation, and donor

hematopoietic cell infusion, under a 45-day course of CsA. This was followed by the transplant of nonvascularized split-thickness skin allografts and VSA from the donor to the chimeric recipients. Tolerance was confirmed only in animals receiving VSA from the hematopoietic stem cell donor. This study confirmed our observations on the role of vascularity of the skin graft and immunomodulatory function of donor hematopoietic cells in tolerance induction. However, in contrast to our protocol, the protocol requiring recipient preconditioning with donor BM cells, followed by delayed skin allotransplantation, is clinically not applicable in CTA.

Vascularized bone marrow transplant

Bone is a unique component of CTA and may contain immunocompetent BM cells, which theoretically may give rise to graft-versus-host disease (GVHD). However, the presence of BM cells of donor-origin within transplanted CTA may also have advantages in developing donor-specific chimerism, which in many experimental models of CTA was permissive for tolerance induction. To study the immune response of single component of CTA, Suzuki *et al.* developed a model of isolated VBMT [25]. This model differs from the standard intravenous bone marrow transplantation (BMT) procedure in that the BM cells are delivered within the stromal microenvironment, which accelerates immune reconstitution and chimerism development of the recipient [26,27].

To study the immune contribution of a VBMT to the host and mechanism of tolerance induction, we have developed unilateral and bilateral VBMT models [28–30]. We found that a vascularized femur; rich with BM cells at different stages of development, without other tissue components, is a reliable model to study donor cell engraftment and chimerism development. We have recently introduced engraftment of immature donor-origin cells into BM compartment and multi-hematolymphoid organs of VBMT recipients across fully MHC barrier [30]. Engraftment of donor-origin cells was confirmed as early as 1 week post-transplant and donor-origin cells with immature phenotype were detectable in the BM compartment of the recipient at day 100 post-transplant. However, during follow-up period, a progressive decrease in the hematopoietic activity of allografted bone was observed and this corresponded with bone fibrosis. To further test the biological factors responsible for fibrosis within the allografted bone, we assessed the role of osteopontin. We found that fibrosis within the allografted bone was associated with overexpression of osteopontin at the fibrotic net and at the interface of bone trabeculae and BM [31]. Despite bone fibrosis, donor-specific chimerism was detectable in the peripheral blood of VBMT recipients, which was permitted by the repopulation of donor-origin cells within the BM environment of the recipient [30,31]. Proliferative potential of donor-origin cells was also confirmed by clonogenic activity *ex vivo* using colony-forming units assay [30].

These results suggested that VBMT is an efficient source of donor BM cells for donor cell engraftment, repopulation, and chimerism maintenance, and as such may have tolerogenic properties.

The models of CTA containing multiple tissues

The immunologic characteristics of CTA composed of multi-tissues raise new challenges for transplant immunologists. Most experimental models, such as limb or face allograft, except skin and bone with BM, contain additional immunocompetent tissues such as lymph nodes, muscles, and vessels. Due to the heterogeneity of transplanted tissues in limb or face transplant models, CTAs possess the potential to stimulate a potent alloreactive response directly, especially when CTA contains active hematopoietic cells.

Skin/bone marrow transplantation

To test the role of donor hematopoietic cells in skin allograft acceptance, we developed new techniques of simultaneous donor BM and skin allotransplantation. Bone marrow was transplanted in a different form including unprocessed, whole BM cells, 'crude' BM, and as VBM transplantation [32,33]. Simultaneous skin allotransplantation with direct donor BMT delivered into the recipient bone cavity in the 'crude' form under 35 days of selective T-cell depletion with anti-αβ-TCR/CsA protocol significantly prolonged skin allograft survival (mean 68 ± 4.9 days) compared with that of anti-αβ-TCR/CsA protocol alone (20.4 ± 1.1 days) without BM cells. The presence of donor BM cells in the recipient BM compartment resulted in chimerism development, and corresponded with skin allograft acceptance. These data indicated that transplantation of BM cells, including stromal cells, in their natural microenvironment is an effective method for donor cell engraftment and repopulation, which allowed for chimerism maintenance.

The CTA transplantation of vascularized BM is believed to be a superior technique facilitating donor cells engraftment and reconstitution, when compared with standard intravenous delivery of BM cells [34]. To test this hypothesis, we developed a microsurgical technique of simultaneous skin and VBMT allotransplantation based on the same femoral artery and femoral vein [35]. This model was used to test the therapeutic effect of donor BM on rat skin allograft survival under our well-established 1-week protocol of anti-αβ-TCR/CsA. In this study, a

skin allograft transplanted with bone component was accepted up to 125 days after immunosuppressive protocol withdrawal. In contrast, in a group where VSA alone, without bone component, was transplanted, allografts were accepted up to 61 days after immunosuppressive protocol withdrawal [33]. Simultaneous transplantation of the vascularized skin and vascularized bone with BM cells, modified by a short-term immunodepletive protocol, confirmed their beneficial effect on skin allograft acceptance, and this was associated with the development of donor-specific chimerism.

As simultaneous transplantation of vascularized bone, as a supportive therapy for CTA, would not be clinically practical, we have previously reported that BM cells transplanted directly into the bone cavity resulted in a better donor cell engraftment and chimerism maintenance compared with that of standard intravenous BM infusion in a rat model across MHC barrier [36]. These observations encouraged us to use the method of direct intrabone BM cells delivery for skin allograft survival across strong MHC barrier between ACI donors and Lewis recipients. We found that 1-week immunomodulatory protocol of anti-αβ-TCR/CsA, simultaneous transplantation of donor BM cells, and boost therapy of anti-αβ-TCR/CsA given 4 weeks after vascularized skin transplantation significantly prolonged skin allograft acceptance up to 90 days, when donor BM cells were transplanted directly into the bone cavity, whereas in a group with standard intravenous BMT, skin allografts were accepted up to 78 days [our unpublished data].

In these models of skin allograft transplants supported with different forms of BMT and short-term immunodepletive protocol, significant extension of skin allograft acceptance was observed, but tolerance was not achieved.

A series of experiments of BMT as a strategy for mixed chimerism and tolerance induction in the skin allografts were introduced by Wekerle research group in the mouse model [37–40]. Authors modulated alloresponsiveness to donor antigens by using co-stimulatory blockade protocol, with an anti-CD154(CD40L)mAb, with or without fusion protein CTLAIg, and subsequently donor BMT. Co-stimulation blockade used in these studies inhibits CD40-CD40L and CD28-CD80/86 interactions between T cells and APC, and in combination with donor BMT leads to extrathymic deletion of donor-reactive T cells and this mechanism may participate in tolerance induction. Transient immunosuppression without cytoreductive conditioning resulted in mixed chimerism induction (20–90%), and tolerance to full-thickness skin allograft and immunocompetence to the third-party skin allograft were confirmed.

The model of vascularized skin/bone transplant has a direct relevance to the clinical scenario of CTA containing bone component, such as hand transplant [41] or face transplant model (http://www.usatoday.com/news/health/2008-12-16-face-transplant). Donor BMT as a supportive therapy was also successfully used in the experimental clinical trial of kidney transplants [6] and was performed in the first case of partial face transplant [42,43]. As confirmed by clinical hand transplants, human hand is not abundant with active BM cells, and in some hand transplant recipients, only transient chimerism was detected [44,45], and only transient microchimerism was confirmed in face allograft recipients [13]. This may be explained by the fact that a hand or face allograft with bone component contains only small amounts of functionally active donor BM cells and this amount is not sufficient to affect human allograft recipients.

The limb allograft model

The most commonly used CTA model for tolerance-inducing protocols is the limb allograft. Limb allografts were introduced as both functional and nonfunctional transplants, such as orthotopic and heterotopic models [4,5,46,47].

Experimental limb allograft models have been performed in rodents (rat, mouse), in large animals (swine), and in nonhuman primates. These models differ in their responses to immunomodulatory protocols and tolerance induction.

Rodent model of limb allograft

During the past 25 years of experimental experience with limb transplants, researchers are working not only on improvement of microsurgical techniques but also on different strategies for tolerance induction. This section briefly summarizes the experimental experience of tolerance induction in limb allograft model based on different immunosuppressive and immunomodulatory protocols.

The first successful limb transplantation between MHC-mismatched BUF and Lewis rat strains under CsA protocol was introduced by Kim *et al.* [46]. In this study, authors reported successful limb allograft survival under a continuous dose of CsA (10 mg/kg/day) administration. Within 1 week after CsA cessation, animals rejected the transplanted limb. Shortly thereafter, Black *et al.* reported that application of a moderate dose of CsA (8 mg/kg/day) for 20 days, followed by a maintenance dose given twice weekly, resulted in indefinite limb allograft survival in the transplant performed between semi-allogenic LBN donors and Lewis recipients [48].

Our first studies on limb allografts were performed between Lewis–Brown–Norway (LBN) ($RT1^{l+n}$) donors and Lewis ($RT1^{l}$) recipients under a combined protocol of systemic CsA (4 mg/kg/day) monotherapy with topical

application of fluocinolone acetonide (6 mg/cm^2/day). In this study, synergistic therapeutic effect of topical steroids and low dose of CsA resulted in the extension of limb allograft survival for up to 51 days post-transplant [49].

These data indicate that CsA monotherapy is very effective to suppress rejection of rat limb allograft as long as low dose CsA treatment is continued without side effect. However, in all reports, after CsA treatment cessation, limb allografts were rejected.

In the clinical scenario, CTA is difficult to control without maintenance of immunosuppression. To overcome the issues of toxicity of immunosuppression, numerous studies have been performed to promote tolerance to the allograft, and one of the proposed approaches for tolerance induction is the development of mixed donor-specific chimerism.

In limb allograft models, favorable effect for allograft survival was accomplished by using vascularized bone with BM cells as an integral component of the CTA model. The presence of hematopoietic cells within transplanted limb allograft under favorable immunosuppressive conditions is imperative for chimerism induction and may lead to development of tolerance. Chimerism induction is one of the most often tested strategies to achieve donor-specific tolerance. Numerous studies were performed in different centers to induce tolerance to limb allografts in rodent models. Mixed chimerism and transplantation tolerance to CTA may be induced by donor BMT without lethal irradiation, cytoreductive conditioning under short-term immunsuppression, co-stimulation blockade, or transient nonselective or selective immunodepletion.

The role of chimerism in limb allograft survival was investigated by Hewitt *et al.* in a parental-to-hybrid one-way strain combination from Lewis to LBN, in immunologically unmodified limb allograft recipients [50]. Authors documented that the presence of 60.2 ± 14.5% of donor chimerism was associated with development of GVHD, whereas mixed T-cell chimerism level below 18.3 ± 3.9% was associated with tolerance induction in most of limb allograft recipients.

However, the reports on the role of chimerism in CTA acceptance and rejection are conflicting in terms of chimerism effect on immune modulation of recipients. Foster *et al.* created mixed chimeras by transplantation of syngenic (WF) and allogenic (ACI) T-cell depleted BM cells into WF recipients. Selective depletion of αβ-TCR was performed *in vitro*, before BMT, and WF recipients were conditioned with a single dose of ALS (10 mg) given 5 days before BMT and 500–700 cGy of total body irradiation. Post-transplant therapy was maintained for 10 days by tacrolimus (FK506). Limb allografts were transplanted at 12 months after mixed allogenic chimeras were established. In contrast to Hewitt studies, chimerism level exceeding 60% was associated with tolerance induction to limb allografts, whereas low level of chimerism, below 20%, was associated with moderate signs of rejection [51].

Prabhune *et al.* found that a high and stable level of donor chimerism (>80%) was associated with long-term limb allograft survival in the conditioned host [52]. Fully MHC-mismatched rat chimeras (ACI to WF) were created by transplantation of donor BM depleted *in vitro* from αβ-TCR cell. Limb allografts from lethally irradiated (1050 cGy) ACI donors were transplanted at 50–70 days after chimera creation. This study demonstrated that irradiation of CTA before transplantation into chimeric host inactivates donor immunocompetent cells present within the graft, permitting long-term limb allograft acceptance without GVHD. In contrast, chimeric hosts of nonirradiated limb allografts developed GVHD. A second protocol was designed with simultaneous transplantation of *in vitro* T-cells depleted donor hematopoietic cells and limb transplants from ACI donors to WF recipients preconditioned with 950 cGy total body irradiation [52]. Infusion of donor BM into conditioned recipients simultaneously with CTA resulted in stable chimerism, robust tolerance, and limb allograft survival.

Introduction of BM carrying CTA into the clinical scenario could also bear the risk of GVHD because of the lymphocyte-rich component of the grafts. To reduce the number of immunocompetent cells within the CTA, lymph nodes from composite limb allograft were surgically removed and lymphadenectomized limbs were transplanted across MHC barrier from ACI donors to WF chimeric hosts created by 950 cGy of total body irradiation, and subsequent *ex vivo* T-cell depleted BMT [53]. No clinical or histologic signs of GVHD or rejection were observed within 5 months after transplantation.

Prevention of GVHD in chimeric hosts by lymphadenectomy of the allograft may be an alternative procedure to pretransplant irradiation, as mature T cells responsible for immune response reside in the lymph nodes, not in the BM component.

While simultaneous transplantation of donor hematopoietic cells and CTA may be clinically relevant, creation of the chimeric host a few weeks or months before CTA would be not clinically applicable.

Elimination of memory T lymphocytes or inhibition of T-cell activation constitutes a critical mechanism of transplantation tolerance. The nonselective depletion of T cells is the most widely used protocol in many experimental models and in the clinic, and is accomplished by targeting of all T cells, not only alloreactive T cells, by either polyclonal (antilymphocyte sera) or monoclonal antibodies (mAb) (anti-CD3, anti-CD52).

Working on tolerance-inducing strategies in the rat limb allograft model, we have tested the efficacy of combined polyclonal antilymphocyte (ALS) serum and CsA. A course of 21 days of ALS/CsA successfully induced tolerance to the limb allograft in a semiallogenic rat model, wherein it was transplanted from LBN donors to Lewis recipients. Tolerance was confirmed *in vivo* by acceptance of the donor skin graft, and immunocompetence by rejection of the third-part skin allograft; and *ex vivo* by hyporesponsivenes to donor antigens by MLR assay [5]. When this protocol of 21 days of ALS/CsA was applied in a fully MHC-mismatched rat model, prolonged limb allograft survival for up to 56 days was achieved, but tolerance to limb allograft was not induced [54].

To further expand our investigation of tolerance-inducing protocol, we have developed a new strategy with selective targeting of potentially alloreactive T cells in a limb transplant model. Initial studies using mAb against αβ-TCR were introduced by Heidecke *et al.* in a rat heart allograft model. Authors reported long-term cardiac allograft survival after pretransplant treatment with monoclonal antibody R73 (mouse anti-rat αβ-TCR) [55].

Selective depletion of alloreactive T cells reduces initial alloreactive response by inhibition of specific antigenic peptides, such as αβ-TCR, which delivers the first signal of activation. Moreover, anti-αβ-TCR mAb selectively targets only alloreactive T cells, and spares other T cells, such as γδ T cells, natural killer (NK) cells, and other leukocytes including monocytes, and in this way prevents innate immunity [56].

To test the efficacy of short-term combined immunodepletive protocol with anti-αβ-TCR/CsA, we have investigated the effect of different treatment intervals (35, 21, 7, and 5 days) of immunosuppression administration for chimerism development and tolerance induction in a semiallogenic and fully MHC-mismatched rat model [4,57–59].

Under this combined anti-αβ-TCR/CsA protocol, the 5-day treatment was as successful as the 35-day protocol for long-term limb allograft survival. Indefinite limb allograft survival (over 720 days) and functional recovery were associated with the presence of stable level of donor-specific chimerism in CD4 and CD8 T-cell subpopulations. Tolerance to donor antigens and immunocompetence to foreign antigens were confirmed *in vivo* by skin grafting and *ex vivo* by MLR assay. In this model, combined anti-αβ-TCR/CsA protocol resulted in over 95% of depletion of αβ-TCR-positive cells as early as 1-week post-transplantation, and T cell repopulated to the preoperative value approximately 2 months after treatment cessation [57,58]. Prolonged deletional effect of anti-αβ-TCR/CsA protocol can be explained by the fact that donor hematopoietic cells engraft in the recipient and provide donor antigen to the thymus, leading to lifelong negative selection of newly developed donor-reactive thymocytes via central deletional mechanism [60]. This study confirmed that the short-term protocol of anti-αβ-TCR/CsA therapy is sufficient for the maintenance of immunologic unresponsiveness of the newly developed repertoire of T lymphocytes [59].

To assess the mechanism of tolerance induction, we performed limb transplants between LBN donors and euthymic and thymectomized Lewis rat recipients using 7-day of anti-αβ-TCR/CsA protocol without maintenance therapy [59]. Combined protocol of anti-αβ-TCR/CsA applied to thymectomized Lewis recipients extended MST of limb allografts up to 51 days, whereas application of αβ-TCR/CsA to euthymic recipients resulted in indefinite limb allograft survival (MST = 370 days). Indefinite limb allograft survival in the euthymic rats was associated with the presence of stable chimerism in the peripheral blood, thymus, lymph nodes, and spleen of long-term survival limb allograft recipients. Only transient chimerism was observed in the thymectomized rats. This study confirmed that thymus is an essential organ for tolerance induction in limb allograft models and transient immunodepletive protocol of anti-αβ-TCR/CsA may facilitate engraftment of donor cells into the thymus, leading to negative selection of newly developing alloreactive host T cells.

Chimerism induction in limb allograft model may be accomplished by BM cells from intact femur and tibia, but may be enhanced by donor hematopoietic cells delivery. In our studies on limb allografts, we have tested the role of selected fraction of immature donor BM cells CD90 for chimerism induction without immunosuppressive manipulation of the recipient. We found that direct intrabone donor CD90 cells delivery prolonged limb allograft survival up to 15 days without any immunosuppression. This was associated with the presence of donor-origin cells (16%) in the peripheral blood of limb allograft recipients [61].

The role of supportive therapy with donor BM cells in chimerism induction and limb allograft survival was also studied by simultaneous transplantation of donor VBM or BM. In this study, limb allografts between BN donors and Lewis recipients were performed under triple immunosuppressive protocol given for 12 weeks. Authors reported that induction of donor chimerism in the peripheral blood and lymphoid organs of the recipients was accomplished via central and peripheral mechanisms and this promoted the acceptance of the hindlimb allografts [62].

Large animal model of limb allograft

Tolerance induction to limb allograft was successfully achieved in rodents. However, limb allografts in large animal models require stronger immunosuppression for allograft acceptance and therapeutic levels allowing for

allograft survival may cause serious toxicity. Several studies were performed in large animals (swine) and in nonhuman primates. These models differ in response to immunomodulatory protocols and tolerance induction.

The effect of a 12-day course of CsA monotherapy (13 mg/kg/day) was tested by Lee *et al.* in a MHC-matched, minor antigen-mismatched, miniature swine model of musculoskeletal allograft [63]. Authors reported tolerance induction to the donor skin graft and rejection of third-party control skin graft in long-term surviving musculoskeletal allograft recipients. In contrast, MHC-mismatched recipients rejected musculoskeletal allograft despite 12-day course of CsA. In this study, authors emphasized that both the immunosuppressive regimen and MHC matching play an important role in CTA survival. Follow-up studies performed in MHC-matched, minor antigen-mismatched, miniature swine limb allografts, under 12-day course of CsA immunosuppression, demonstrated indefinite survival of the musculoskeletal portion of the limb allograft, whereas only prolonged skin survival was reported [64].

The protocol with transient T-cell depletion was applied in a fully MHC-disparate, miniature swine model. Tolerance to musculoskeletal elements of limb allograft was induced, whereas skin component was rejected [65].

In these models, independent of the immunomodulatory protocols, contrasting immune responses to different tissue types from the same donor were reported as a split tolerance.

Recently, a sensate osteomyocutaneous radial forearm flap was transplanted in a preclinical nonhuman primate model. However, subtherapeutically immunosuppressed animals, under combined immunotherapy of FK506, MMF, and prednisone, developed alloantibodies and rejected transplanted forearm grafts [66].

The studies of limb allografts performed in large animal models indicate that extensive immunomodulation of the recipient immune system is required to achieve long-term survival, which would be a prerequisite to utilize these protocols in clinical application.

Experimental face transplant models

Face allograft is one of the most developed experimental transplantation procedures in the last years. Skin constitutes a major component of face transplant, and has been recognized both experimentally and clinically as one of the most immunogenic tissues of CTA. In the transplant scenario, this poses a perplexing challenge as it requires life-long immunosuppression to overcome the problem created by a significant MHC barrier. The complexities involved in the effective transplantation of skin as an organ can be attributed to the fact that skin represents its own immune system with many specialized immune cells, capable of responding to foreign antigens. Therefore, after transplantation without appropriate immunosuppression, skin is likely to be the principal target of acute and chronic rejection.

Rat model is the most commonly used experimental face allograft model is [67–72]. However, recently, the face allograft was also performed in large animal models (rabbit, swine) [73,74] and in nonhuman primates as a heterotopic transplant [75,76]. Experimental models of face allograft differ in the content of transplanted tissues and may include skin and soft tissue components only, or additionally, skin elements may also contain bone and cartilage.

Rodent model of face allograft

Composite face/scalp and hemiface allograft model

Since 2000, Microsurgery Laboratory of The Cleveland Clinic had introduced and developed different rat experimental models of face allograft transplants. First reports documenting successful composite face/scalp flap transplantation in the rat model was introduced in 2003 and tested the functional outcome and survival time of facial allografts [67,68]. Long-term face allograft acceptance was accomplished under a low maintenance dose of CsA monotherapy (2 mg/kg/day) without side effects. To further assess the immunologic aspects of face allograft survival, Siemionow group developed a hemiface transplant model between semiallogenic (LBN) and fully MHC-mismatched (ACI) donors to Lewis recipients [69,70]. Immunosuppressive protocol of CsA monotherapy (2 mg/kg/day) permitted long-term face allograft survival in both models (over 330 days) and was associated with the presence of donor-specific chimerism maintained by CD4 and CD8 T-cell subpopulations. The presence of chimerism in face transplant model may be explained by the fact that skin is a rich organ containing dermal T lymphocytes. Moreover, face allograft contains not only skin, subcutaneous fat tissue, muscles, nerves, cartilage, and vessels, but also numerous lymph nodes, a source of donor immunocompetent T cells and B cells, which may contribute to chimerism development.

Composite hemiface/calvaria model

The clinical need for coverage of extensive craniomaxillofacial deformities, including bone and soft tissues, encouraged us to develop a rat model of composite hemiface/calvaria allograft [71]. Long-term follow-up, up to 220 days, under a low maintenance dose of CsA, and subsequent histologic and immunologic assessment, proved viability of bone component of composite hemiface/calvaria allograft with viable BM cells, and the presence of peripheral blood chimerism. In contrast to face transplant

model without bone component, in hemiface/calvaria model, chimerism was predominated by B lymphocytes.

Maxilla model

Another experimental model tested the application of heterotopic rat maxilla allotransplantation for coverage of midfacial or maxillary deformities [72]. This model differed from other face allograft models in the content of transplanted tissues. Rat maxilla model contains mainly bone and mucosal tissues without skin component. Histology confirmed viability of all allografted tissues, including bone, nasal septum, and teeth, at day 105 post-transplant.

Composite hemiface/mandible/tongue model

To further test feasibilities of reconstruction of low- or midface defects, we recently introduced the challenging model of heterotopic transplantation of composite hemiface/mandible/tongue allograft in the rat [77]. In this model, mandible, which represents vascularized bone with active BM cells, was transplanted as one component with hemifacial skin flap. The presence of BM within the transplanted composite hemiface/mandible/tongue graft facilitates chimerism induction, which is permissive for long-term allograft acceptance. Moreover, engraftment of donor-origin cells into BM compartment and lymphoid organs of composite hemiface/mandible/tongue recipients was confirmed [unpublished data, manuscript in preparation].

Long-term face allograft acceptance in our models was accomplished by low maintenance, nontoxic dose of CsA monotherapy. This protocol permitted 'prope' tolerance in the experimental rat model [78]. In the clinic, immunosuppression minimization was successfully used in solid organ transplant recipients [79,80]; however, CTA still requires stronger maintenance immunosuppression because of the complexity of transplanted tissues.

Hemiface model for functional recovery

Functional recovery after hemiface CTA was assessed in syngenic (Lewis to Lewis) and allogenic (BN to Lewis) rat models. Authors reported that hemiface transplants with motor and sensory nerve appositions showed significant evidence of motor function return, and positive cortical response to stimulation of the whiskers, in contrast to face transplants without nerve appositions [81]. This allograft was maintained at the moderate dose of CsA (13 mg/kg/day) until postoperative day 80 and at the maintenance dose of 10 mg/kg/day during the follow-up period.

Large animal model of face transplantation

To restore the extensive craniomaxillofacial defects in humans by allograft, experimental studies in larger animal models are required; however, they are still very limited.

The rabbit model of composite orthotopic hemiface/calvaria allograft was reported [74]. In this model adequate immunosuppression of combined treatment with maintenance dose of CsA (4 mg/kg/day) and prednisone (2 mg/kg) for 2 weeks resulted in prolonged, over 120 days, osteocutaneous allograft survival. Authors introduced surgical feasibility of composite hemiface/calvaria allograft in the larger rabbit model; however, functional recovery and nerve regeneration were not assessed as authors did not perform nerve coaptation.

Large animal models, such as swine or nonhuman primate, offer better opportunity for preclinical studies of CTA. These models have an advantage because of the similarity of MHC antigens to humans; however, direct applicability of these models to clinical trials in humans is not possible.

The effect of hemiface transplantation was tested in a miniature swine model between Hwa-Ban strain and Lan-Yu strain [73]. Facial allograft included skin, muscle, lymphoid gland tissue, ear cartilage, and sensory nerve. Short-term CsA treatment of 10 mg/kg/day for 2 weeks tapered to 5 mg/kg/day for the next 2 weeks, and resulted in delayed rejection between 38 and 49 days post-transplantation. The limited time of facial allograft survival in this study indicated that a stronger immunomodulatory protocol is necessary to extend survival and for clinical relevance.

A modern immunosuppressive regimen of induction therapy with antithymocyte globulin (ATG), methylprednisolone, and maintenance therapy with FK506 and rapamycin was applied for heterotopic transplantation of facial allograft in cynomolgus monkeys [75]. Flaps composed of skin, muscle, and mandible bone were transplanted to the groin region of the recipients. Allograft survival range was from 6 to 129 days post-transplantation. Limited number (three of seven cynomolgus monkeys) of primates used in the group under a stronger immunosuppressive protocol demonstrated technical success of heterotopic facial allograft transplantation.

Different immunosuppressive protocols, based on tacrolimus monotherapy, were applied in another heterotopic face allograft study in nonhuman primates. A segment of mandible, masseter, and overlying skin was transplanted to the lower abdominal wall of an allogenic recipient. Authors reported rejection-free allograft survival ranging from 60 to 177 days. A major limitation of this immunosuppressive procedure was that five of six animals developed post-transplant lymphoproliferative disease without clinical evidence of graft rejection [76].

Both primate studies of composite face allograft confirmed that in the clinical scenario, safer immunosuppressive protocols should be considered with the goal to achieve long-term survival without side effects of immunosuppressive therapy.

Conclusion

Transplant tolerance in CTA can be achieved in experimental rodent models; however, attempts of tolerance induction in the preclinical larger animal models are still challenging. The most promising strategy for tolerance induction is via development of donor-specific chimerism. However, this approach in clinical scenario is limited to living related donors, and as such will not apply to human CTA.

However, some strategies of tolerance induction, such as irradiation or donor bone marrow transplantation, were recently reported in clinical cases of face transplantation, indicating that further modification of the existing protocols and development of new immunomodulatory strategies are needed to justify broader application of CTA procedures in the clinic.

Authorship

MS and AK: participated in review design, in the data analysis, and in writing the paper.

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