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Review Article

Induction of Tolerance Through Mixed Chimerism for Composite Tissue Allotransplantation: Insights, Problems and Solutions

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Abstract

Composite tissue allotransplantation (CTA) research faces two critical challenges. Firstly, the most applicable experimental model(s) in which CTA tolerance induction regimens should be characterized and tested requires clarification. Secondly, it has not been determined what would constitute a suitable endpoint for clinical trials of such methodologies before progression toward wider clinical application could be considered appropriate. Currently, the most reliable method to induce CTA tolerance in animals is to establish mixed hematopoietic chimerism using bone marrow transplantation (BMT) from an allogeneic donor. This approach has three important constraints: (i) the requirement for toxic myeloablative conditioning; (ii) a prerequisite 28-day delay period between BMT and CTA; and (iii) the potential for inducing graft-versus-host disease (GVHD). We review the history of chimerism induction for CTA, the strategies that have been proposed to circumvent CTA-related problems, and the insights that have been gained from our own research into these issues. The benefits of vascularized BMT (VBMT) over conventional BMT for inducing CTA tolerance are highlighted. The establishment of mixed chimerism and the induction of tolerance require further research and refinement before they can be applied clinically. A safe and robust method of tolerance induction encourages wider application of reconstructive CTA with fewer ethical obstacles. (*Tzu Chi Med J* 2008;20(2):101–108)

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1. Introduction

Composite tissue allotransplantation (CTA) has the potential to revolutionize reconstructive surgery for complex tissue defects and lost limbs. The feasibility and benefits of CTA have been demonstrated in animals, in 26 human recipients of hand transplants and in two human recipients of partial face transplants (1).

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CTA recipients currently require nonspecific immunosuppression to prevent transplant rejection. The toxicities associated with these agents are substantial, including opportunistic infections, increased rates of malignancies and end-organ failure [2]. Furthermore, these agents do not control against chronic rejection, which remains the most common cause of late graft loss [3]. CTA is generally indicated for functional and/or esthetic enhancement, not for prolonging or saving lives. Ethical considerations regarding the cost as well as morbidity and mortality rates associated with nonspecific immunosuppression have limited the widespread clinical application of CTA.

2. Tolerance to CTA through mixed chimerism

Various methods to induce donor-specific transplantation tolerance have been reported for experimental solid organ, composite tissue allotransplants and other allotransplants. The successful induction of donor-specific tolerance in CTA recipients might theoretically overcome the limitations noted above. One robust approach to induce donor-specific tolerance is through bone marrow transplantation (BMT). Bone marrow chimerism results from the engraftment of hematopoietic stem cells (HSCs) [4]. Chimerism refers to the harmonious coexistence of tissues from different animals of the same or different species [5]. The first demonstration of this type of tolerance was reported in 1953 by Billingham, Brent and Medawar [6]. Immunosuppression is not required to prevent transplant rejection once engraftment has been established. Critical to this concept is the fact that bone marrow chimerism confers rejection-free transplant acceptance for highly antigenic tissues such as those from allogeneic skin, heart, and lung [7–9].

Donor-specific transplantation tolerance through chimerism can usually be achieved by one of two following approaches: full chimerism and mixed chimerism. Their differences are important to CTA. The level of chimerism in animals refers to the percentage of donor cells in peripheral blood lymphocytes as determined by flow cytometric analysis. In full chimerism, transplantation of major histocompatibility complex (MHC)-disparate bone marrow cells into ablated recipients results in donor-specific transplantation tolerance. However, recipients are relatively immunoincompetent and readily susceptible to graft-versus-host disease (GVHD) [10,11]. In addition, full chimerism is achieved at the cost of recipient ablation with significant morbidity and mortality [11]. In contrast, in mixed chimerism, the pluripotent HSCs of both the recipient and the donor coexist. Mixed chimerism, with levels of donor chimerism as low as 1%, also results in donor-specific transplantation tolerance and offers a number of advantages over full chimerism [5]. Mixed chimeras exhibit superior immunocompetence for primary immune responses and are more resistant to GVHD. Moreover, mixed chimerism can be established with less toxic myeloablative conditioning. Native bone marrow provides host antigen presenting cells (APC) that are essential for full immunocompetence, while allogeneic bone marrow provides a persistent source of antigens for induction and maintenance of tolerance [12].

CTAs are comprised of a combination of skin, muscle, nerves, tendons, and/or bone and bone marrow and may pose a formidable antigenic barrier to tolerance induction [13]. The first mixed chimerism experiment involving CTA was performed in a rat model; chimerism was prepared using nonmyeloablative conditioning with 500–700 cGy total body irradiation (TBI), anti-lymphocyte globulin (ALG) and tacrolimus. Sequential limb allografts were placed 12 months after BMT. In animals with >60% early chimerism, no signs of rejection of the CTA were observed [13]. In sharp contrast, all animals with early chimerism, <20% of the animals, developed moderate rejection clinically and histopathologically [13]. The results of these studies clearly demonstrated that chimerism could induce tolerance to highly antigenic CTA.

3. Problems of CTA through mixed chimerism

Mixed chimerism is the most reliable method of inducing experimental transplantation tolerance. However, this approach has three important constraints: (i) the requirement for toxic myeloablative conditioning; (ii) a prerequisite 28-day delay period between BMT and CTA; and (iii) the potential for inducing GVHD. These limitations must be addressed if mixed chimerism is to be applied effectively for CTA tolerance induction in the clinical setting.

Recipient conditioning for HSC transplantation requires two essential elements: (i) cytoreduction of the recipient marrow to create a space or niche [14]; and (ii) immunosuppression to prevent residual host cells from rejecting the donor transplant [15]. Prior to infusing the host with donor bone marrow cells, the host must first be *conditioned* to create a *space* for engraftment and the chimerism induction. This *conditioning* is toxic to the host and often leads to immunoincompetence [16]. Myeloablative conditioning has thus been met with little enthusiasm amongst clinical transplantologists. However, great advances have been made by hematologists/oncologists in their quest to develop less myeloablative conditioning strategies [17]. These efforts have opened new doors to novel and less toxic antigen-specific approaches of conditioning recipients for engraftment of HSCs. Another method is to

manipulate the recipient to become hyporesponsive to the transplanted bone marrow until it can *take hold* and induce a self-perpetuating state of deletional tolerance [18]. Manipulation of the recipient cell components responsible for alloreactivity will create a more tolerogenic milieu for bone marrow engraftment. In a normal mouse model, chimerism has been achieved by nonmyeloablative conditioning using low-dose TBI, alkylating agents, and monoclonal antibody (mAb) treatments [18–20].

Costimulatory blockade during BMT may further reduce the need for myelotoxic therapy during conditioning. It is well established that T cells require at least two signals from APC to be activated. In this situation, signal 1 is in the form of antigen presentation, and signal 2 involves the interaction of costimulatory molecules and their receptors between T cells and APC [21]. Delivery of signal 1 without signal 2 induces anergy and immune deviation toward tolerance. By specifically targeting costimulatory molecules expressed on the T cells, peritransplant anergy can take place. CD28 is constitutively expressed on T cells and is upregulated during T cell activation. Blockade of CD28 on T cells or its ligand B7 (CD80 or CD86) on APCs (signal 2) induces recipient hyporesponsiveness by way of functional inactivation, regulation, or clonal deletion in solid organ allograft recipients. A number of agents, such as anti-CD154 (CD44L) and anti-CD28, which act by non-overlapping mechanisms, have been tested alone and in combination with the overall goal to eliminate the need for TBI [22,23].

Conventional protocols for preparing mixed chimerism in animal studies involve sequential steps, namely, host conditioning, donor BMT, characterization of chimerism by flow cytometry (at 28 days) and CTA [24]. The delay period has been considered a requirement for engraftment and repopulation of donor BM cells in the host. If allotransplantation is performed before successful engraftment of donor BM, it may interfere with the establishment of tolerance. This delay period might be possible for living solid-organ transplantation, as a delay between bone marrow infusion and organ transplantation is permitted. For hand transplantation, the hand is always harvested from a cadaveric donor; therefore, both the bone marrow and the hand would need to be transplanted simultaneously. This is why the 28-day delay presents an important limitation for clinical application in CTA.

One aspect of CTA that distinguishes it from most other forms of transplantation is the lymphoid burden in the allotransplant itself. The allogeneic lymphoid tissue will influence the conditioning approach for establishing chimerism. If tolerance induction by simultaneous placement of marrow plus CTA is considered, GVHD is a significant potential hazard [25]. GVHD is caused by the reactions of large numbers of immunocompetent donor cells in the lymphoid tissue such as those present in the transplanted donor limb. Further research is urgently needed to circumvent the problems associated with mixed chimerism.

4. Solutions to the problems, including our own experiences

We used a rat CTA model in the transplantation of hind limb osteomyocutaneous (HOMC) flaps for the study of chimerism and tolerance with reference to the problems noted above [26]. Briefly, HOMC flap CTA harvest started with a longitudinal incision along the medial aspect of the lower leg from the ankle to the thigh (Fig. 1A). Circumferential skin incisions were made at the level of the mid-thigh and the ankle joints to preserve the cutaneous paddle (3×5 cm) in the lateral part of the lower leg. The femoral vessels were mobilized individually from the superficial epigastric vessels to the inguinal ligament. Both the femur and tibia were then amputated distally to completely mobilize the lower limb segment based solely on the femoral vessels. The cutaneous part of the HOMC flap was based on the vascular supply from the lateral leg to facilitate insetting with the skin externalized. The limb and bone marrow in the medullary cavity were then flushed with heparinized saline. The HOMC flaps, weighing an average of 8 ± 1.5 g, were wrapped with wet gauze and placed in iced water. The recipient operation was started with a transverse incision in the inguinal region to expose and mobilize the femoral artery and vein just distal to the superficial epigastric vessels and proximally up to the muscular branch to the gracilis. A 2×4 cm defect was created in the gluteal area to position the HOMC flap with sutures. The femoral vessels were anastomosed using microsurgical technique (10-0 nylon). The skin was closed using absorbable suture (5-0 Monocryl; Ethicon, Sommerville, NJ, USA). The CTA study protocols of conventional BMT and our recently developed vascularized BMT (VBMT) for chimerism and tolerance induction are depicted in Figs. 1B and 1C, respectively, for comparative purposes.

The rat offers significant advantages over the mouse for CTA study for the following reasons: (i) lower cost; (ii) hind limb transplantation is technically more feasible; and (iii) rats are more prone to develop GVHD than mice (making an evaluation of GVHD more similar to humans). In the rat, transplantation of unmodified marrow results in rapidly lethal GVHD [27]. Rat models have been previously utilized for evaluating tolerance to lung [9], heart [28], trachea [29], and limb transplants [13].

In a previous rat model, a minimum dose of 850 cGy TBI was required to achieve engraftment in 100% of rat recipients of marrow depleted of $\alpha\beta$-T cell receptor $(TCR)^+$ T cells [30]. The addition of ALG (10 mg on

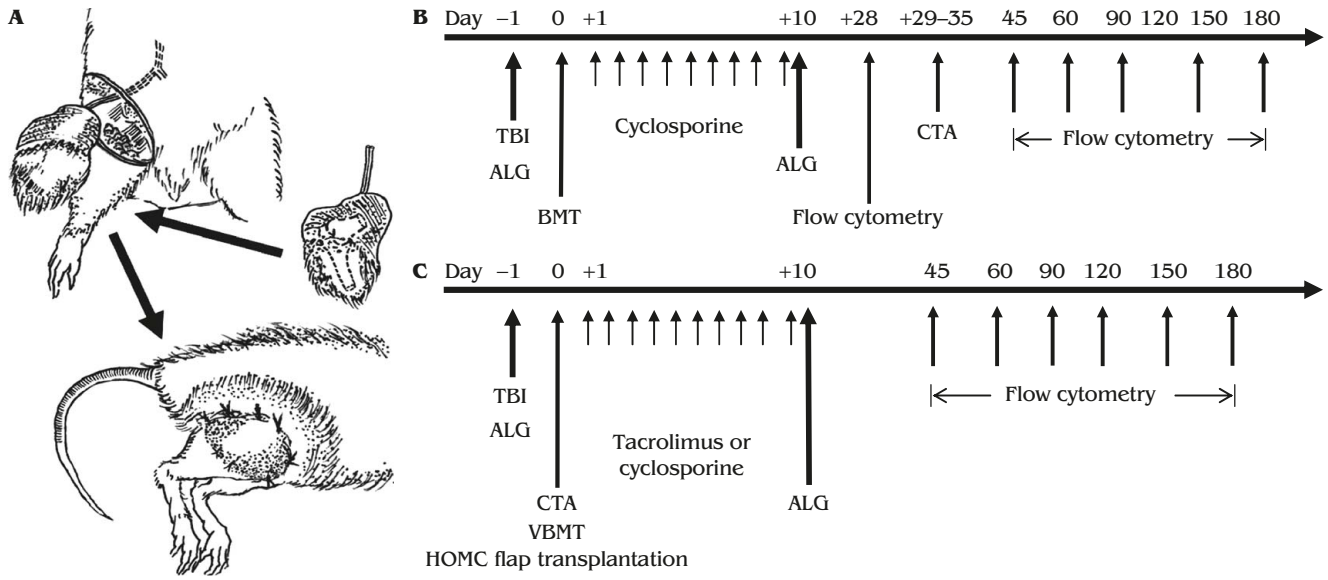

Fig. 1 — (A) Schematic presentation of the transplantation of hind limb osteomyocutaneous (HOMC) flaps from a donor Brown Norway ($RT1A^C$) rat to a recipient Lewis ($RT1A^l$) rat. The hind limb flap was harvested and tailored to the osteomyocutaneous flap with the preservation of a 3×2 cm skin paddle in the lower posterior limb. Femoral vessels as the recipient vessels were anastomosed. The flap of the donor rat was inset in the back of the recipient with easy access for daily monitoring of rejection. See the text for details. (B) In conventional CTA protocol of BMT to induce chimerism and tolerance, recipient rats were conditioned with total body irradiation (TBI), transplanted with 100×10^6 bone marrow cells with T-cell depletion from donors, and given cyclosporine (16 mg/kg/day 0 to +10) plus ALG (5 mg on days 1 and 10). HOMC flap transplantation was carried out between days 29 and 35. Characteristics of engraftment and chimerism level in recipient rats were monitored periodically until the end of the observation period. (C) In the protocol of vascularized BMT (VBMT) to induce chimerism and tolerance, recipient rats were conditioned with TBI, transplanted with VBMT from donor HOMC flap on day 0, and given tacrolimus (1 mg/kg/day) or cyclosporine (16 mg/kg/day 0 to +10) plus ALG (5 mg on days 1 and 10). Characteristics of engraftment and chimerism level in recipient rats were monitored periodically until the end of the observation period.

day 5) plus FK506 (1 mg/kg/day −1 through to day +10) reduced the minimum TBI dose to 500 cGy [31]. The minimum dose of TBI could also be significantly reduced if an anti-NK mAb was administered [32]. The recent concept of costimulatory blockade during BMT may be used to reduce the need for myelotoxic therapy further during conditioning [33]. In a rat CTA model with CD28 blockade by administrating CTLA4-Ig at 2 mg/kg per day (alternate days in combination with tacrolimus at 1 mg/kg daily from day 0 through to day +10, and a single dose of 10 mg ALG on day +10), the required TBI conditioning was as low as 300 cGy on day −1 [33]. Using the same rat surgical model, we took further specific measures in order to circumvent the problems associated with CTA, as follows.

4.1. Reducing alloreactivity can eliminate toxicity of myeloablative conditioning

Since the toxicity associated with these myeloablative agents remains a concern, the development of a less toxic nonmyeloablative conditioning is one of our main goals in CTA tolerance induction. Accordingly, the addition of cyclosporine (16 mg/kg/day 0 to +10) plus ALG (5 mg on day −1 and day +10) could help donor marrow cells to engraft and create mixed chimerism. Cyclosporine can aid in the induction of mixed chimerism when using TBI as low as 200 cGy [34] (Fig. 2). Notably, our results showed a better acceptance rate of CTA at the dose of 400 cGy than previous results [31].

In our other study, fludarabine phosphate (Flu) was used to reduce the dose of TBI. Flu is one of the purine nucleoside analogs that have immunosuppressive activity against lymphocytes by inhibiting DNA synthesis [35] and by inducing apoptosis [36]. CD4 and CD8 T cells are more sensitive to the effects of Flu than B cells [37,38]. By adding Flu, tolerance to CTA can be achieved by further reduction of TBI down to 200 cGy [34] (Fig. 3). The development of less toxic immunosuppressive and/or tolerizing strategies render the recipient hyporesponsive to the donor at the time of transplantation, thereby theoretically allowing the reduction of TBI or dose of the myelotoxic agent. In the future, the use of costimulatory blockade, infusion of *ex vivo* expanded immature dendritic cells cocultured with donor T cells, or facilitating cells (FLs) [10],

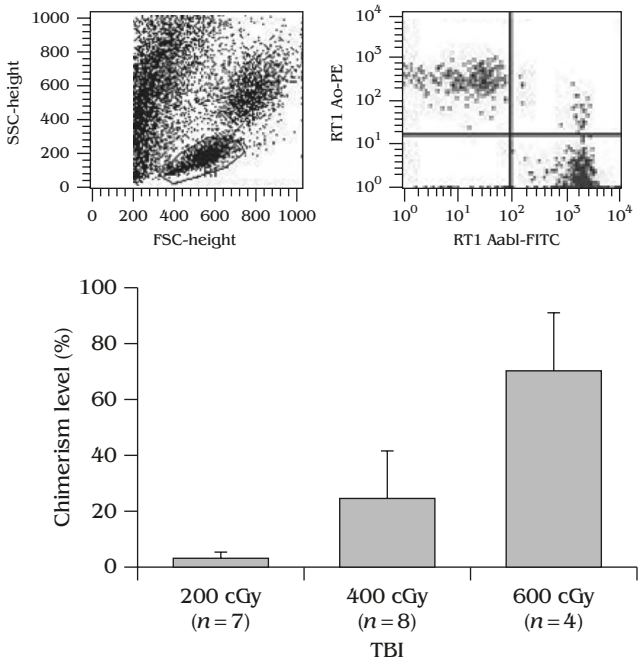

Fig. 2 — Cyclosporine replaces tacrolimus-based nonmyeloablative conditioning. We used donor Brown Norway ($RT1A^C$) and recipient Lewis ($RT1A^l$) strains. The level of chimerism refers to the percentage of donor cells in peripheral blood lymphocytes based on dual-color flow cytometric analysis, as exemplified in the upper right corner (*upper frame*). Characteristics of engraftment and chimerism level in rats 1 month after being conditioned with TBI at 200, 400 and 600 cGy, transplanted (i.p.) with 100×10^6 bone marrow cells T cell depletion (TCD) from donor rats, and given 16 mg/kg cyclosporine (s.c.) between day 0 and day 10, and 5 mg ALG (i.p.) at day −1 and at day +10 (*lower frame*). Note that the levels of donor chimerism correlate with the amount of conditioning.

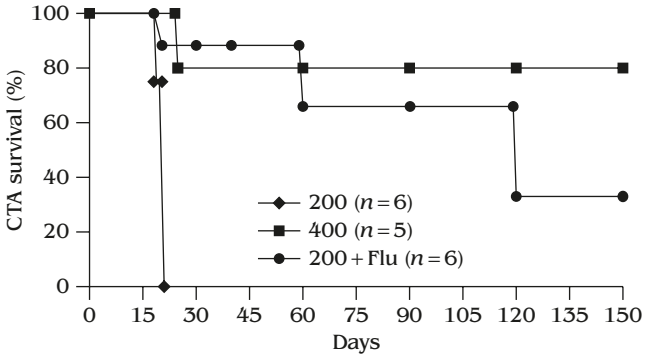

Fig. 3 — Reducing alloreactivity can eliminate toxicity of myeloablative conditioning. Flu acts against CD4 and CD8 T cells. Injection of Flu at day −1 can further reduce TBI dose down to 200 cGy, and induce tolerance to CTA as effectively as the use of TBI alone at a dose of 400 cGy.

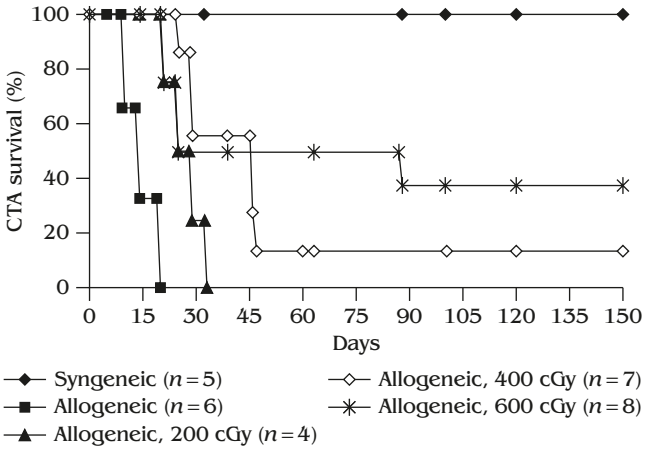

Fig. 4 — VBMT can simultaneously induce mixed chimerism and tolerance to CTA. VBMT can induce tolerance to allotransplants after nonmyeloablative immunomodulation. The survival of each allotransplant was analyzed and recorded. The syngeneic transplant was not rejected clinically, whereas the allogeneic transplant was rejected within 14 days. When tolerance was created by immunomodulation, the survival of grafts could be prolonged until the end of the observation period indicated at the doses of 400 and 600 cGy TBI.

or infusion of donor-specific regulatory T cells (Treg) sorted from stable chimera could be used to render host-versus-graft (HVG) reactivity in the recipient more hyporesponsive. In this way, the myelotoxic TBI dose can be largely reduced.

4.2. VBMT can simultaneously induce mixed chimerism and tolerance to CTA

To overcome the 28-day delay for tolerance induction to CTA in rats, we used VBMT to simultaneously induce mixed chimerism and tolerance to CTA. We treated the recipient rats using TBI at different doses (600, 400, 200 cGy) alongside tacrolimus and ALG. Complete acceptance of syngeneic transplants and short-term survival of allogeneic transplants without any immunosuppression were observed, and tolerance was induced in 37.5% of the animals using 600 cGy TBI and in 16.7% of the animals using 400 cGy TBI. These animals with tolerant CTAs survived until the end of the 150-day observation period [38] (Fig. 4). Tolerance was induced in 33% of the rats using 200 cGy in VBMT and in 0% of the rats using 200 cGy TBI in BMT [39] (Fig. 5). Our results indicated that donor bone marrow cells, which are released from vascularized bone transplants, may have also contributed to the induction of mixed chimerism. In addition, HSCs in the bone marrow cavity of VBMTs downmodulated host immune responses and induced tolerance. Collectively, VBMT in CTA directly offers bone marrow niches for HSCs and auto-induces

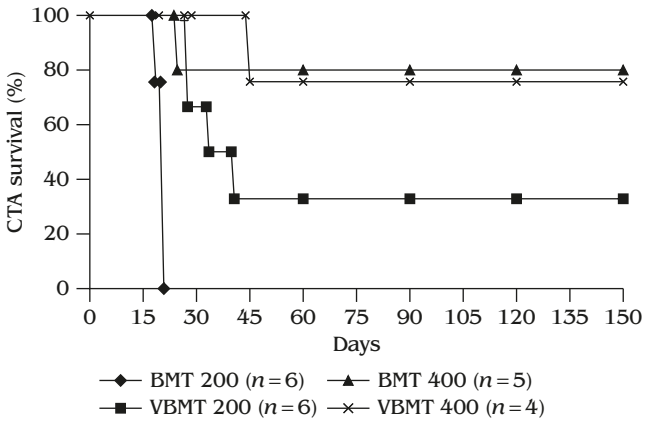

Fig. 5 — VBMT offers stromal space and allows the reduction of myelotoxic agents for tolerance strategies. Recipient rats were preconditioned with 200 cGy and 400 cGy total body irradiation (TBI) on day −1. Experimental groups were: Group I: 200 cGy plus BMT, Group II: 400 cGy plus BMT, Group III: 200 cGy plus VBMT, Group VI: 400 cGy plus VBMT. All recipients were treated with cyclosporine 16 mg/kg/day between day 0 and day 10, and ALS 5 mg on day −1 and day+10. Tolerance was induced in 33% of the rats with 200 cGy in VBMT, but 0% of the rats with 200 cGy TBI.

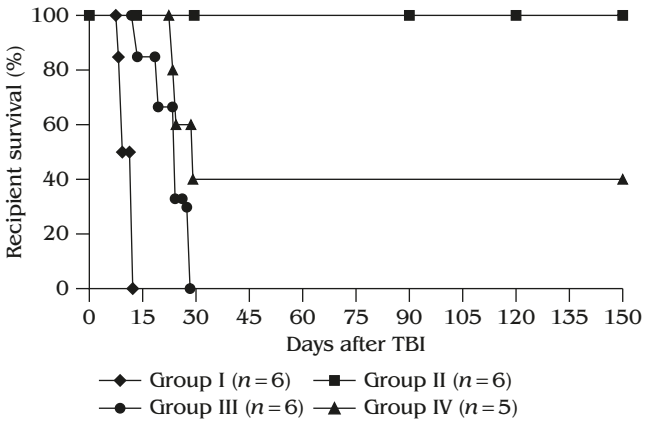

Fig. 6 — Immunomodulation of donor allotransplant plays an important role in avoiding GVHD. Recipients were preconditioned with 950 cGy TBI on day −1. Experimental groups were: Group I: irradiation without allotransplantation, Group II: syngeneic hind limb osteomyocutaneous (HOMC) flap transplantation, Group III: allogeneic HOMC flap transplantation, Group VI: allogeneic hind limb HOMC flap perfused with anti-TCR mAb into the graft before transplantation. All of the rats died within 12 days after 950 cGy. Syngeneic and allogeneic hind limb HOMC flap could rescue the rats after lethal irradiation. Allogeneic hind limb HOMC flap transplantation in Group III induced GVHD, which led to death at around 3 weeks after transplantation. In contrast, HOMC flap perfused with anti-αβ-TCR mAb avoided GVHD and created tolerance in the recipient rats. Thus, rats in Group IV experienced long-term survival of allotransplants.

donor-specific tolerance to its allotransplants without the 28-day delay period.

4.3. Immunomodulation of donor allotransplants plays an important role in reducing GVHD

GVHD is mediated by immunocompetent donor cells that initiate a rejection response against the recipient. T cells have been identified as the most important effector cellular subset in this reaction [40], although other cell populations may also participate [41]. Removal of mature T cells from the transplanted bone marrow graft has prevented GVHD effectively in mice, rats and humans [27].

GVHD can be initiated by mature T cells from the donor bone marrow or those present in the transplanted donor limb. We found that depletion of both αβ T cells and γδ T cells from the donor marrow inoculum prevented GVHD, implicating a role for both types of T cells as effectors in GVHD. Importantly, this approach to T cell depletion does not remove facilitating cells (FC) [10], nor does it compromise engraftment. Interestingly, the phenotype of FCs is not dissimilar to that of plasmacytoid DCs [42]. Plasmacytoid DCs are known to mediate antigen-specific tolerance and induce $CD4^+$ as well as $CD8^+$ regulating T cells *in vitro* [43,44]. For VBMT to induce tolerance, graft perfusion with anti-T cell receptor mAb can immunomodulate the bone graft to protect the lethally irradiated rats with CTA from GVHD and concomitantly induce long-term donor-specific tolerance to CTA [45] (Fig. 6).

There is much room for further research in the relief of GVHD. A great deal of attention has recently been paid to the preparation of specific cell populations that promote tolerance, notably plasmacytoid dendritic cells, HSC and mesenchymal stem cells (MSC). Infusion of large numbers of allogeneic FCs, HSCs or MSCs can make chimerism and tolerance to CTA less toxic [42,46]. Infusion of MSCs from a third party as the source has also been observed to lessen GVHD in allogeneic bone marrow transplantation in leukemia patients [47,48; Lung-Ji Chang, personal communication]. In fact, MSCs have been used to facilitate the induction of mixed hematopoietic chimerism and islet allograft tolerance without GVHD in rats [49]. Encouraging results obtained along these lines of investigation have affected a number of biomedical disciplines, including regeneration medicine, tissue engineering, hematology/oncology, cell therapy and transplantation. Lastly, it is also important to develop patient monitoring measures, which can be used to reflect a success or failure of tolerance induction to CTA during and after treatment for individual patients. One should be able to develop such tests based on

animal models of CTA via mixed chimerism. These tests should eventually be considered an integral part of the protocols to be used clinically for CTA.

5. Conclusions

CTA continues to face histocompatibility and immunogenicity problems. Modern immunosuppressive regimens are used to maintain the function and viability of the CTA. The side effects of chronic immunosuppression and the uncertainty of long-term outcomes due to chronic rejection have limited the clinical application of CTA. Tolerance induced by mixed chimerism, instead of relying on chronic immunosuppression, will be a major departure from the current clinical practice in CTA recipients. Effective means of depleting T cells, development of humanized mAbs to human T cells and to costimulatory molecules, and expansion of FCs and HSCs raise hopes that advancements made in rodent models will soon be applicable to large animal models and humans. Efforts should also be directed towards the generation of new biologicals through the collaboration of academic centers and biopharmaceutical companies. Further research is necessary to minimize CTA-associated immune response problems before CTA protocols can attain widespread clinical application. Our recent studies involving VBMT have offered an attractive surgical model to the study of CTA tolerance, which can be further manipulated in order to yield more satisfactory results. As early clinical trials lead to refinements, significant immunological and clinical benefits may soon be brought about for CTA patients.

Acknowledgments

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