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Composite Tissue Transplantation: A Rapidly Advancing Field

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Abstract

Composite tissue allotransplantation (CTA) is emerging as a potential treatment for complex tissue defects. It is currently being performed with increasing frequency in the clinic. The feasibility of the procedure has been confirmed through 30 hand transplantation, 3 facial reconstructions, and vascularized knee, esophageal, and tracheal allografts. A major drawback for CTA is the requirement for lifelong immunosuppression. The toxicity of these agents has limited the widespread application of CTA. Methods to reduce or eliminate the requirement for immunosuppression and promote CTA acceptance would represent a significant step forward in the field. Multiple studies suggest that mixed chimerism established by bone marrow transplantation promotes tolerance resulting in allograft acceptance. This overview focuses on the history and the exponentially expanding applications of the new frontier in CTA transplantation: immunology associated with CTA; preclinical animal models of CTA; clinical experience with CTA; and advances in mixed chimerism–induced tolerance in CTA. Additionally, some important hurdles that must be overcome in using bone marrow chimerism to induce tolerance to CTA are also discussed.

Transplantation of body structures composed of multiple tissues, derived from ectoderm and mesoderm, is known as composite tissue allotransplantation (CTA). The term was coined by Kleinert and Peacock.[1] CTA includes body structures such as the hand, larynx, joints, abdominal wall, tendons, and face. This is in contrast to solid organ transplantation, which involves transplantation of an organ (heart, lung, kidney, liver, and so on) with a relatively uniform structure. CTA aims to replace functional loss and improve the quality of life as compared with solid organ transplantation, which is life-saving in the majority of instances.

The 15th century legend of Saints Cosmos and Damien, who replaced the diseased leg of a man with one from a dead person, is often quoted as the first historical record of CTA (Fig 1). In fact, CTA was the initial goal of transplantation. World War II led to many British soldiers sustaining severe burn deformities. Reconstructing these defects with skin and other structures was the stimulus to the pioneering research led by Sir Peter Medawar.[2] Similarly, Joseph Murray, who performed the first successful kidney transplantations in 1954, was a plastic surgeon with a desire to perform skin and facial reconstruction.[3]

Unfortunately, the inability to address the intense antigenicity with the immunosuppressive agents at that time prevented further growth in the field of CTA. As new immunosuppressive drugs became available, the field of solid organ transplantation blossomed over the next 6 decades to achieve the high level of success it currently enjoys. This growth vastly improved

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understanding of the mechanisms underlying function of the immune system and led to the development of novel potent and less toxic immunosuppressive agents.

Following the classic experiments with skin grafting by Medawar, the next major milestone in CTA was the attempt at hand transplantation in Ecuador in 1964.[4] The transplant failed after 3 weeks due to rejection despite the use of the only 2 available immunosuppressive agents, azathioprine and steroids. This failure put a damper on further attempts at CTA due the perception that the antigenicity of skin and associated structures was insurmountable. It was the consensus that further attempts in CTA had to wait until better immunosuppressive agents and protocols became available. With the availability of cyclosporine and tacrolimus (FK506) by the 1990s, renewed efforts in CTA began. Following the report of success of limb allotransplantation in animals,[5] clinical efforts at transplanting hand (Lyon, France; Louisville, Kentucky, United States), larynx (Cleveland, Ohio, United States), and knee (Munich, Germany) were initiated.[6–9] It was the success at transplanting hands that brought the attention of the scientific community and the public to CTA.

A wide spectrum of transplants is currently labeled as CTA. More than 60 reports of such transplants are known. These include hand, knee and femoral diaphysis, abdominal wall, nerve, face and scalp, trachea, tendon, muscle, larynx, tongue, and penis.[10] This report reviews the immunology of CTA, preclinical models, challenges, and clinical progress.

THE IMMUNOLOGY OF CTA

CTA are composed of different tissues, including skin, subcutaneous tissue, muscle, bone, nerve, and blood vessels. Skin is thought to possess high immunogenicity.[11] Murray proposed a relative scale of antigenicity of tissues and organs and ranked skin the highest.[12] A CTA graft was thought to have the sum of immunogenicity of its different components and this high level of reactivity was perceived as a barrier to successful clinical application. However, studies have not fully borne out these assumptions. Lee et al[13] showed that the individual tissues in a vascularized limb transplant (ie, skin, subcutaneous tissue, muscle, bone, and blood vessel) were comparable in the elicited immune response. They also demonstrated that a whole limb allograft elicited a less intense immune response than did allografts of each of the individual components. The relative degree of antigenicity of the limb tissues varied according to the type of immune response measured (cellular or humoral) and the time that it was measured.[13]

Another concept relevant to CTA is split tolerance, a term coined by Billingham and Brent in 1959.[14] It refers to simultaneous tolerance to one tissue and rejection of another from the same donor. Skin grafts almost always fail in the transplantation setting, although as part of a limb graft they may not be rejected. This split tolerance was hypothesized to be due to a number of factors: (1) the secondary vascularization needed for the acceptance of conventional skin grafts upregulates cytokines, leading to rejection; (2) skin contains a larger number of antigen-presenting cells; and (3) skin-specific antigens are highly antigenic. However, some studies have found no difference in the acceptance of conventional skin allografts and primarily vascularized skin allografts.[13]

What is the explanation for the observation that the immunogenicity of the whole limb is less than that of skin alone? One possibility is that limb CTA (particularly limb transplants) function as a vascularized bone marrow transplant (VBMT). These transplants are an ideal form of bone marrow transfer as the graft itself provides the stromal microenvironment for proliferation of the donor cells.[15,16] This method has been shown to achieve stable chimerism with a low risk of graft-versus-host disease (GVHD).[17,18] The first evidence that the limb CTA functioned as VBMT was provided by Hewitt et al, who demonstrated the development of stable mixed chimerism across a major histocompatibility complex (MHC) haplotype mismatch in rats following limb transplantation.[19] This was subsequently demonstrated in a fully allogeneic

rat model that achieved equivalent results.[20] However, similar studies in large animals failed to demonstrate lasting chimerism.[21] In humans, short-lived peripheral blood microchimerism was noted in 2 hand recipients at Louisville, whereas the Lyon group documented early intragraft chimerism in skin biopsy specimens that later disappeared.[22,23] These data suggest that in humans, limb grafts do not function as VBMT. The reason may be the absence of a significant amount of hematopoietic marrow in the distal limb skeletal structures.

PRECLINICAL ANIMAL MODELS OF CTA

Extensive preclinical laboratory work has been performed in rodent (rat, mouse, and hare), swine, and nonhuman primate models of CTA. The CTA models have been largely limb allotransplants and have included both functional and nonfunctional transplants as well as orthotopic and heterotopic models. In addition, experimentation has included hemifacial allotransplant models.[24] The research has evolved in a stepwise fashion. The questions that needed to be answered were as follows: (1) Is CTA feasible considering the perceived high antigenicity of the skin component? (2) Can CTA be performed without the use of highly toxic doses of immunosuppression? and (3) Can tolerance be achieved in CTA so as to enable reduction or elimination of immunosuppression?

A rat model was used in 1984[25] to demonstrate that limb transfer between different strains of rats was possible. The strains of rats used were BUF and LEW. Cyclosporine A alone was sufficient to prevent the rejection of transplanted limbs. However, concerns remained regarding the toxic side effects of high doses of the drug and the need for long-term use of the agent. Moreover, this is a relatively weak donor/recipient strain composition.

The clinical success of combination therapy (eg, cyclosporine and mycophenolate mofetil [MMF]) in reducing the immunosuppression-related toxicity of high doses of single agents was then tested in CTA. A rat hind limb allotransplantation model was used to test the efficacy of a combination of low doses of cyclosporine A and MMF. The study involved the orthotopic transfer of mid-femur limb transplants from Brown-Norway rats to MHC-disparate Fischer 344 recipients. The combination therapy succeeded in preventing acute rejection with a much lower toxicity profile.[26]

Similar success was shown using a different approach by Kanaya et al.[27] Costimulatory blockade at the time of antigen exposure was implemented to arrest the rejection cascade. Hind limbs from male ACI rats (RT1) were transplanted heterotopically into female Lewis rats (RT1). Costimulatory blockade was achieved with the intravenous administration of recombinant adenovirus carrying CTLA4Ig (AxCTLA4Ig) or CD40Ig (AxCD40Ig) following the limb transplantation. These agents used individually achieved mean survival time of 39.4 ± 6 and 13 ± 2.9, respectively, whereas the simultaneous use of both agents resulted in significant prolongation of survival to 49.2 ± 6.6 days. This benefit was attained without an increase in complications.

To address the larger issues of toxicity of immunosuppression, which precludes wide clinical application of CTA, numerous attempts have been made to promote graft/host tolerance in experimental models. Mixed allogeneic chimerism was proposed as an approach to achieve tolerance. Initial experiments in CTA used WF (RT1Au) and ACI (RT1Aa) rats, with MHC incompatibility.[28] WF rats were conditioned with a total body radiation dose of 500 to 700 cGy and treated with a single dose of ALS (10 mg) 5 days before and tacrolimus (1 mg/kd/d) started 1 day prior to the bone marrow transplant comprised of a mixture of T-cell–depleted syngeneic (WF) and allogeneic (ACI) marrow. One year following the establishment of mixed allogeneic chimerism, hind limb allotransplantation was performed. It was found that the degree of chimerism influenced the acceptance of the graft. Donor chimerism levels >60% led

to acceptance of the graft without rejection for the entire study period of 100–200 days, whereas the animals with chimerism levels of <20% resulted in moderate rejection.

A major problem to be addressed in the clinical use of mixed allogeneic chimerism to induce tolerance to CTA is GVHD. This is commonly seen after the transplantation of unmanipulated donor specific limbs. Gorantla et al[29] performed an elegant experiment in which (ACI→WF) chimeras received a limb from WF (syngeneic), Fisher (third-party), irradiated (1050 cGy) ACI, or nonirradiated ACI rats. The chimeric rats with >85% chimerism exhibited rejection-free survival of the donor specific hind limbs. However, 100% of these animals developed lethal GVHD approximately 22 days after transplantation. The group that received irradiated ACI or syngeneic WF limbs showed no signs of rejection or GVHD at 5 months. These data demonstrated that established chimeras could be susceptible to GVHD caused by immunocompetent donor cells transferred with the graft and that pretransplantation irradiation of the CTA allograft inactivates these cells, permitting both a rejection and GVHD-free survival of the transplant. Subsequent studies found that the GVHD was more likely due to lymph node burden accompanying the graft rather than the bone marrow.[30]

Another major deterrent to using conventional conditioning regimens to achieve chimerism is the toxicity of the radiation. Alternate strategies to lower the radiation dose have included the use of lymphocyte-depleting agents and CD-28 blockade. Foster et al[31] used CD28 blockade along with a combination of tacrolimus, antilymphocyte serum, and total body irradiation (300 cGy) used as conditioning prior to T-cell–depleted bone marrow transplantation in a rat vascularized hind limb allotransplantation model. There was acceptance of the grafts without acute or chronic rejection and long-term survival of allogeneic skin transplants.

Despite the significant strides made in the CTA rat model, the conventional chimerism protocols require a delay period between bone marrow transplantation and limb allotransplantation, making such protocols impractical for clinical application. By performing mixed allogeneic chimerism induction and rat hind limb allotransplantation “simultaneously,” Prabhune et al[32] found that infusion of donor bone marrow cells into conditioned hosts immediately after limb transplantation, plus immunotherapy (tacrolimus and MMF) for 28 days, resulted in stable mixed chimerism, robust tolerance, and reliable limb allograft survival.

Novel approaches have been attempted to simplify the procedure of rat hind limb transplantation with the development of nonfunctional heterotopic models. In one model, the skin of the hindlimb was removed to the ankle level before transplanting along with the vascularized epigastric skin heterotopically to the groin of the recipient.[33] Osteotomy and intramedullary fixation, which are part of the conventional CTA models, are eliminated, thereby lowering the risks of blood loss and infection. In 2007, Adamson et al described a rat heterotopic osteomyocutaneous flap containing all components of a limb transplant to evaluate tolerance induction.[34] The nonfunctional CTA model permits tolerance studies in the rat with a lower rate of complications.

Mouse models offer many potential advantages to study CTA in the laboratory. The availability of many transgenic strains, monoclonal antibodies to delineate transcription routes, cytokines products, and so on make the study of immunologic mechanism easier. In 2003, a new hindlimb transplantation mouse model was developed[35] involving syngeneic hindlimb transplantation in Swiss-Webster mice. However, the model demands a high level of technical expertise, including venous anastomosis to be done across a stent and a fine arterial anastomosis with 11-0 nylon sutures. This has deterred wider usage of this model.

CTA research using larger animals followed the earlier success in rat experiments. In 2000, Ren et al[36] used swine in CTA experiments. CTA flaps were transplanted from MHC-mismatched donors. Although acute rejection followed, the authors demonstrated the

feasibility of using the model to evaluate the efficacy of immunotherapy. The lack of significant morbidity to the recipient permits long-term evaluation to continue.

The use of nonhuman primates in CTA research has been more recent. In 2005 a sensate osteomyocutaneous radial forearm flap was established in monkeys.[37] Nonimmunosuppressed allografts were rapidly rejected, showing a perivenular T-cell infiltrate, which was associated with subsequent alloantibody formation. This led to graft thrombosis without prominent dermal infiltration. Subtherapeutically immunosuppressed animals also developed alloantibodies and rejected their CTA in a delayed fashion, exhibiting a marked dermal lymphocytic infiltrate similar in magnitude and distribution to previously reported human cases. The authors concluded that the CTA was well tolerated by nonhuman primates, although there was allosensitization that was responsive to immunosuppression. The model permitted the evaluation of CTA histology and holds promise for the evaluation of therapeutic strategies in CTA.

FACE ALLOTRANSPLANTATION

A rat hemifacial allotransplantation model was used to investigate functional tolerance across major histocompatibility complex barriers.[38] This CTA transplant, including ear and scalp, was performed between Lewis-Brown Norway ($RT1^{1+n}$) and Lewis ($RT1^{1}$) rats. Single agent immunosuppression with tapering doses of cyclosporine A was used. Excellent results were noted, with 5 of 6 face allografts surviving up to 240 days without rejection. The interesting finding was the demonstration of donor-specific chimerism at 21 days (1.11% CD4 and 1.43% CD8) in the peripheral blood of recipients.

A rabbit model to study facial allotransplantation has been described.[39] New Zealand rabbits served as donor and livid blue rabbits as recipients. Hemifacial CTA flap was harvested based on the external carotid artery along with the external mandibular and auricularis magna branches. Immunosuppression included cyclosporine A, azathioprine, and prednisone. The technical feasibility of the concept was aptly demonstrated with the success of the graft. The expertise that accrued with the above research laid the foundation for the clinical application of CTA to be discussed below.

CLINICAL EXPERIENCE WITH CTA

A wide spectrum of transplants falls under the umbrella term of CTA. These include the well-known hand and face transplants and others as well: abdominal wall, knee, nerve, flexor tendon apparatus, larynx, skeletal muscle, tongue, and penis. The cumulative worldwide experience is summarized in Table 1.

NERVE ALLOGRAFTS

Mackinnon et al published the initial report of successful peripheral nerve transplantation.[40] At present, 7 patients with long segment nerve loss in either the lower extremity (3 patients) or upper extremity (4 patients) have received nerve grafts.[40] Grafts were procured from ABO-matched deceased donors and preserved at 5°C for 7 days to lower the expression of MHC class II antigens. Sural nerve autografts were used in addition to the allografts in 5 of the 7 patients. Immunosuppression included cyclosporine (5 patients) or tacrolimus (2 patients), azathioprine, and tapering doses of steroids. Based on experimental evidence, which showed that as nerve regeneration occurs, donor antigenic determinants in the nerve allograft are replaced with host components,[41] immunosuppression was stopped completely 6 months after evidence of nerve regeneration beyond the graft. The mean duration of immunosuppression was 18 months. One patient rejected the allograft 5 weeks postoperatively due to inadequate immunosuppression. Sensory recovery was noted in the remaining 6 patients, but motor

recovery was seen only in 3. These mixed results are difficult to interpret due to the concomitant use of autografts.

FLEXOR TENDON TRANSPLANTATION

Two cases of a human vascularized allotransplantation of a complete digital flexion system were reported in 1992.[42] Restoration of function was seen as early as 4 months posttransplantation. Wrist swelling decreased progressively, and because the patient had no active motion preoperatively, the functional result—a range of motion in flexion of 80° in the proximal interphalangeal joint, no extension defect, and 55° of flexion in the distal interphalangeal joint with an extension defect of 35°—was considered excellent.

ALLOGENEIC VASCULARIZED KNEE TRANSPLANTATION

Knee transplantation is indicated for extensive loss of cartilage and bone with a deficient extensor mechanism and soft tissue and skin defect. The first report was published in 1997.[43] The group from Germany has thus far performed 6 allogeneic vascularized knee transplantation. ABO compatibility and a negative crossmatch were required in all cases. The first 5 patients were induced with antithymocyte globulin (ATG). Cyclosporine, azathioprine, and a tapered regimen of steroids were used for immunosuppression. In the 6th patient, tacrolimus and MMF were used. This patient also had a sentinel skin graft to monitor rejection.

Results in the first 5 patients were poor: 1 graft loss occurred at 5 weeks due to infection; 1 graft loss at 3 years from rejection due to noncompliance; and 3 recipients developed late rejection at 15, 16, and 24 months, respectively, and eventually lost the grafts following stress fractures and bone necrosis. It is a unclear whether the long cold ischemia times (range, 18–25 hours) had an impact on the long-term outcome. The last patient (on tacrolimus and MMF) had a functioning graft at 4 years. This patient had 1 episode of steroid responsive acute rejection at 28 months.

VASCULARIZED ALLOGENEIC SKELETAL MUSCLE TRANSPLANTATION

A 56-year-old renal transplant recipient on prednisone and cyclosporine[44] underwent resection of a recurrent squamous cell carcinoma of the scalp. This resulted in an 11×14 cm^2 defect involving exposed outer skull cortex. Four months later, allogeneic scalp reconstruction was performed using a haplomatched abdominal muscle flap composed of rectus abdominis and external oblique. ATG and methylprednisolone were used for induction immunosuppression. Acute rejection occurred 2 weeks postoperatively and was successfully treated with MMF, which was discontinued 4 months later. The patient had no further events during a follow-up of 1 year.

Simultaneous abdominal wall transplantation along with intestinal transplantation to provide cover for the allointestinal graft has been reported from the University of Miami.[45] The updated series includes 10 grafts performed in 9 patients.[46] Induction was with alemtuzumab followed by maintenance immunosuppression with tacrolimus and a steroid taper. Five grafts are reported lost due to sepsis (3 grafts), primary nonfunction (1 graft), and rejection (1 graft). Acute rejection episodes of the abdominal graft were noted in 3 patients and were successfully treated with a steroid bolus.

LARYNGEAL TRANSPLANTATION

A 40-year-old man received the first successful human laryngeal transplant at the Cleveland Clinic in 1998.[47] An HLA-matched laryngopharyngeal complex, including thyroid, parathyroids, and 5 rings of trachea, was transplanted along with anastomosis of both superior

and 1 of the recurrent laryngeal nerves. The patient was induced with anti-CD3 antibody and was maintained on cyclosporine, MMF, and steroids. Following a brief episode of rejection at 15 months, tacrolimus replaced cyclosporine. One episode of *Pneumocystis carinii* tracheobronchitis occurred at 15 months. At a follow-up of more than 7 years, the patient has had no further transplant-related problems. He has normal swallowing, good phonation, and high quality of life.[48] Tintinago from Columbia has performed over 14 laryngopharyngeal transplantations to date and with good success.[10]

HAND TRANSPLANTATION

The success of the first hand transplantation by the team at Lyon, France in 1998 focused intense attention on CTA.[6] Despite initial controversies and skepticism, hand transplantation was rapidly replicated successfully in the United States, Austria, China, Italy, and Belgium.[49] As per the second report of the International Registry on Hand and Composite Tissue Transplantation, 18 male patients have undergone 24 hand/forearm/digit transplantations (11 unilateral and 4 bilateral hand, 2 bilateral forearm, and 1 thumb) up to February 2006.[50] Cold ischemia time ranged from 30 minutes to 13 hours.

Postoperative immunosuppression included induction with antithymocyte globulin in 11 patients and basiliximab in 5 patients. Maintenance immunosuppression included the following: tacrolimus, MMF, and steroids in 15 patients; tacrolimus and steroids alone in 1 patient; rapamycin and MMF in 1 patient; and rapamycin with topical steroid and tacrolimus in 1 patient. Patient survival is 100%. The first hand transplant recipient lost his graft due to rejection from noncompliance.[51] Five other Chinese patients lost their grafts due to inability to continue immunosuppression. Acute rejection was seen in 12 patients, most of them between 7 to 14 weeks posttransplantation. All the episodes were reversed with the use of intravenous steroids/lymphocyte-depleting agents, alemtuzumab or ATG/basiliximab and/or topical tacrolimus/corticosteroid. As experience accumulates, a more conservative approach to diagnosing rejection has evolved (ie, grade 1 and 2 infiltrates are not aggressively treated, and grade 3 often with topical treatment only). Other important complications included cytomegalovirus (CMV) infection in 2 patients, herpetic blisters, cutaneous mycosis, ulnar osteitis due to *Staphylococcus aureus*, and metabolic complications (diabetes, renal impairment, Cushing syndrome, and avascular necrosis of the hip). Functional recovery has been excellent. Protective sensation was present in all patients by 6 to 12 months, 88% had onset of discriminative function, and 90% returned to work. The quality of life improved in 83% of recipients. Figure 2 shows the world's second hand transplant recipient at 8 years follow-up.

PENILE TRANSPLANTATION

A 44-year-old man with traumatic penile defect received a penile transplant from a 22-year-old deceased donor. The recipient could urinate in a standing position at 10 days. However, the graft was amputated at the end of 2 weeks secondary to psychological issues.[52]

FACE TRANSPLANTATION

The maxillofacial surgery team at Amiens, France performed the world's first human face allotransplantation.[53] A 38-year-old woman received a central and lower facial transplant. A sentinel skin graft was placed in the left infra mammary area to monitor rejection episodes. Immunosuppression included induction with thymoglobulin and maintenance with tacrolimus, MMF, and prednisone. An acute rejection episode occurred at the end of 3 weeks and required intravenous steroids for control. Early functional results are promising.

TONGUE TRANSPLANTATION

In 2003 Rolf Ewer (Vienna) performed the world's first tongue transplantation on a 42-year-old man with tongue cancer.[54] The graft showed no signs of rejection and had some useful sensation enabling the patient to swallow fluids during a reported follow-up of 8 months.

DILEMMA REGARDING EXPANSION OF CTA

As evidenced by the clinical outcomes in CTA (Table 1), there has been considerable progress in the field in the past decade. Despite the success so far, CTA (particularly hand transplantation) remains controversial. The main deterrents are the long-term problems associated with immunosuppression including infections (CMV, Epstein-Barr virus), neoplasms, and metabolic complications (diabetes, osteoporosis, renal toxicity, hyperlipidemia, and so on). The price of immunosuppression is perceived by many to be justified in the case of life-saving transplants, such as the heart and liver, but not so in CTA where the main benefit is restoration of function and/or an improvement of quality of life. Approaches to eliminate the requirement for these agents would be a significant advance. The way forward seems to be an attempt to achieve donor-specific tolerance.

CTA AND TOLERANCE

Tolerance has been defined as the long-term acceptance of a transplanted graft displaying normal histological characteristics and function in a nonimmunosuppressed recipient who retains immunocompetence to reject a third-party graft but to accept a second donor-specific allograft.[55] Achieving tolerance will have a profound impact on the clinical application of CTA, likely greater than even solid organ transplantation. Both central and peripheral mechanisms are important in inducing and maintaining tolerance. Central deletion of clones of reactive lymphocytes will achieve tolerance. In addition, regulatory lymphocytes in the periphery may help to suppress the activity of the effector lymphocytes that escaped the deletion process.

One proven method of establishing tolerance is by achieving hematopoietic stem cell chimerism.[56] Chimerism refers to the stable coexistence of tissues from 2 genetically disparate beings in a single organism. Chimerism may be achieved by actively infusing hematopoietic elements or seen as a collateral effect of a solid organ transplant. The first is called macrochimerism and is seen when bone marrow is transplanted into a recipient whose marrow has been incompletely or fully ablated, establishing the take of the infused hematopoietic cells. The conditioning mainly plays a role in preventing rejection of the transplanted marrow.[57] The donor stem cells engraft in the recipient's marrow and produce all the blood-derived cell lineages. Newly produced T lymphocytes that react against donor or host antigens are removed by clonal deletion in the thymus, leading to the development of a new hybrid immune system with the establishment of reciprocal bidirectional donor: host tolerance. Ildstad et al[58] has shown that even a very low level of chimerism (1%) is sufficient to induce donor-specific tolerance.

The other type of chimerism is microchimerism, referring to the presence of donor leukocytes in the peripheral blood or tissues of the recipient. Passenger leukocytes present in the donor organ migrate into the recipient and account for the detection of microchimerism.[59] The absence of engraftment of pluripotent donor hematopoietic stem cells and the lack of a conditioning regime distinguish microchimerism from macrochimerism. The low levels of donor cells found in the recipient's blood causes one to wonder whether there is any relationship at all between microchimerism and tolerance. It has been suggested that clonal exhaustion with donor-specific tolerance may result from the interaction of the passenger and recipient leukocytes.[60] In a study of long-term survivors of human liver or kidney allotransplantation,

microchimerism was identified in one or more peripheral locations using molecular testing and immunohistochemistry.[61] However, no relationship has been conclusively shown between the occurrence of tolerance/rejection and the presence/absence of microchimerism, because microchimerism has also been detected in individuals undergoing graft rejection.[62,63]

Macrochimerism can exist in 2 forms: (1) fully allogeneic chimerism, and (2) mixed allogeneic chimerism. Full chimerism is the result of complete myeloablation of the recipient's marrow, resulting in near complete replacement with donor marrow cells. This is the standard therapy for leukemias and other immunohematological disorders. Two facts that have emerged from the bone marrow transplantation experience for hematological malignancies have led to a revised view of the role of conditioning. First, remission seen with allogeneic bone marrow transplantation for malignancies may be linked to the immunotherapeutic potential of the donor lymphocytes rather than the ablative chemo-radiation conditioning regimen.[64,65] Second, conditioning may function more to immunosuppress the host and prevent graft rejection rather than to prepare vacant niches in the bone marrow.[66] This has resulted in the pursuit of nonmyeloablative conditioning to lower the morbidity and mortality associated with conventional conditioning.[67] Nonmyeloablative conditioning has the potential to radically alter the outcomes of both CTA and solid organ transplantation.

Mixed allogeneic chimerism involves the coexistence of donor and recipient hemopoietic systems in the recipient and was first shown in conditioned adults transplanted with a T-cell–depleted (TCD) mixture of syngeneic and allogeneic marrow. This results in donor-specific tolerance.[58,68] This approach for inducing donor-specific tolerance has resulted in graft acceptance in a wide spectrum of allografts, including lung,[69] heart,[70] pancreatic islets,[71] and CTA allografts, such as skin,[58] trachea,[72] and esophagus.[73] Clinical success with bone marrow transplant–induced mixed chimerism has been reported with skin[74] and kidney allografts.[75–77]

The advantages of inducing mixed chimerism over full chimerism are the diminished incidence and severity of GVHD and the preservation of immunocompetence for primary immune responses.[78,79] Additionally, mixed chimerism can be established with nonmyeloablative conditioning, allowing a significant reduction in toxicity.[80] Hence, mixed allogeneic chimerism is a more suitable approach to reach the goal of durable transplantation tolerance, as well as for treatment of a number of nonmalignant disorders of the hematopoietic system.

FACILITATING CELLS

The major obstacle to the widespread application of donor marrow infusion to achieve mixed chimerism is GVHD. This risk is particularly high when unmodified bone marrow is used from mismatched donors. The severity of GVHD is directly related to the degree of HLA mismatch.[81] GVHD is caused by donor cytotoxic $CD8^+$ T cells and NK cells.[82] The use of TCD donor bone marrow resulted in a reduced incidence of GVHD in animal models and humans.[83] However, there was an associated decrease in engraftment of the donor bone marrow (BM).[83] This might be caused by the elimination of cells promoting engraftment by the T-cell depletion process. The mechanism of T-cell depletion induced graft failure is now known. $CD8^+/TCR^-$ graft facilitating cells (FC) are a tolerogenic cell population that promotes engraftment in mismatched recipients.[84] There are 5 major subpopulations of FC: precursor plasmacytoid dendritic cells (p-preDC FC), NK FC, CD 19^+ FC, and $CD14^+$ FC.[85] Further studies have identified the function of FC to reside in class II^+, $Thy1^+$, $CD5^+$, and $CD2^+$ cells in mice marrow.[86] The FC population makes up only 0.4% of the total BM and comprises <1.6% of the total lymphoid gate. An ablated recipient survives when 1000 syngeneic purified stem cells are transplanted. However, the same recipient does not survive after infusion of 10,000 allogeneic purified stem cells due to failure of engraftment. The addition of 30,000

CD8/TCR facilitating cells from the same donor enables allogeneic purified stem cells to engraft (Fig 3).[85] The p-pre-DC FC population is critical to FC function because removal of this subpopulation completely abrogates the FC effect (Fig 3). However, p-pre-DC FC do not replace FC in the full biologic effect.

ROLE OF REGULATORY T CELLS AND DC IN CTA

It is now established that regulatory T cells (T_{reg}), a certain subset of T lymphocytes, may play a crucial role in immunoregulation of innate and adaptive immune responses. The best characterized subpopulation of T_{reg} express CD4 and CD25.[87] However, $CD8^+/CD25^-$, TNK, and $CD4^-/CD8^-$ regulatory T cells have also been described.[87] Foxp3 expression, a transcription factor that induces a regulatory feedback loop via hypomethylation, is considered vital for the development and function of T_{reg}.[88] T_{reg} cells have been identified both in transplanted organs as well as in the peripheral blood. T_{reg} cells have been described in kidney transplant recipients[89] and increased Foxp3 RNA levels in urine have been linked to improved outcomes in renal transplant recipients with acute rejection.[90] T_{reg} cells are interesting from the point of view that they may play a role in modulating the recipient's immune response to donor-specific antigens by establishing a regulatory feedback loop. If this process can be amplified, T_{reg} cells may hold the key to achieving donor-specific tolerance and prolonging the life of allografts.

Inducible T_{reg} secrete interleukin-10 (IL-10) and are known to suppress allograft rejection by regulating alloresponsive T cells. Hara et al showed that IL-10 blocking antibodies facilitated the rejection of skin allografts via a T_{reg}-dependent mechanism.[91] Regulatory T cells are important in the induction and maintenance of allograft tolerance. Anergic T cells have been adoptively transferred via renal allograft to rhesus monkey recipients resulting in tolerance induction in 3 of 6 monkey recipients.[92]

Regulatory T cells have been characterized in recipient lymphoid tissues as well as in transplanted allografts. T_{reg} in the recipient lymphoid tissue may protect the allograft from initial attack, whereas T_{reg} at the graft site help down-regulate the effector cells that have infiltrated the graft. Long-term surviving allografts have been associated with infiltration T_{reg}. Treatment of rhesus monkeys with humanized anti-CD154 monoclonal antibody (mAb) was associated with a mild but persistent lymphocyte infiltration in skin biopsy specimens of long-term full-thickness skin and renal allografts.[93–95] The lymphocyte infiltration in long-term renal allograft recipients is associated with increased levels of the TGF-β1 in nonhuman primates, whereas the disappearance of these cells is associated with graft rejection.[96] A recent study suggests that naturally occurring $CD4^+/CD25^+$ T_{reg} mediate their suppressive effects via a cell-contact–dependent mechanism involving TGF-β.[97]

Studies in human hand transplantation have demonstrated the presence of graft-infiltrating cells with Fox P3 expression in the allograft skin up to 6 years after transplantation.[98] These Foxp3-expressing cells had increased IL-10 and TGF-β messenger RNA (mRNA) levels, and when introduced into an MLR, were able to completely inhibit the donor-directed T-cell responses, suggesting that these cells were protecting the graft from rejection.[98] Our own studies in hand transplant recipients have shown that high numbers of $CD4^+/CD25^+/Foxp3^+$ T cells infiltrate the donor skin from 4 months to as far out as 8 years.[99]

Although labeled as early rejection by the conventional biopsy scoring nomenclature (grade 1 or 2), this infiltrate may in fact be protective. Further research is needed to develop a mechanistically rational approach to characterizing and treating rejection. In kidney transplantation, when Foxp3 expression has been noted in acute rejection, there is simultaneous elevation of other cytokines such as perforin, TNF-α, and IFN-γ. In contrast, when Foxp3 mRNA expression occurs in the absence of elevation of perforin, TNF-α, or IFN-γ, but with

an increase of TGF-β or IL-10, it may indicate ongoing regulatory immunomodulation.[90] Interestingly, culture of naïve human peripheral blood (PB) lymphocytes in the presence of TGF-β promotes the generation of $CD4^+/CD25^+$ regulatory cells in vitro.[100] These data suggest that a long-lasting immune response involving regulatory cell infiltrates may be required for tolerance induction and long-term allograft survival.

Similarly, in our preclinical rat model, we have found that when WF rats (RT1Au) were conditioned with 600 cGy total body irradiation, infused with TCD BM cells from MHC-mismatched ACI donors and transplanted with an ACI osteomyocutaneous hind-limb, skin from tolerant animals showed increased numbers of $CD4^+/Foxp3^+$ T regulatory cells (Fig 4A). Cells in rejecting and naïve tissues were $CD4^+$, with no detectable levels of Foxp3 observed (Fig 4B and 4C).

DC play a central role in innate and acquired immunity.[101,102] The functional maturation state of the DC may determine whether tolerance induction occurs. Survival following heart allotransplantation was significantly prolonged with the administration of immature DC.[103] In addition, it has been shown that preDC expressing $CD8^+/TCR^-$ facilitating cells induced donor-specific tolerance to skin allografts.[85] Although the mechanism is not completely understood, immature DC have the capacity to promote transplant tolerance via generation of regulatory T cells.[104] When naïve $CD4^+$ T cells were repeatedly stimulated with allogeneic immature DC, Jonuliet et al demonstrated generation of T_{reg}-like cells that had low proliferative capacity, secreted IL-10, and were able to inhibit allo-antigenic-specific immune responses.[105] On the other hand, maturation of plasmacytoid DC promotes their ability to prime $CD4^+/CD25^+$ T_{reg} in a human in vitro model.[106] Current efforts have focused on generating T_{reg} by inhibiting (NF)-κβ and oxidative pathways in immature DC with anti-oxidative vitamins.[107] Similarly, inhibition of DC maturation with vitamin D3 and MMF enhances the frequency of $CD4^+/CD25^+$ T_{reg} that can adoptively transfer transplant tolerance.[102,108,109]

Rapamycin inhibits DC maturation and the capacity to stimulate T cells both in vivo and in vitro.[110] Horibe et al showed that when rapamycin conditioned, alloantigen-pulsed DC were administered 7 to 14 days posttransplantation, indefinite survival of vascularized skin allografts was observed. When recipients were challenged with donor or third-party skin grafts, only the donor grafts survived. Interestingly, recipient spleen, graft-associated lymph nodes, and the skin graft itself contained increased levels of $CD4^+/CD25^+/Foxp3^+$ T_{reg}.[111] Similarly, Turnquist et al demonstrated that when maturation-resistant, alloantigen-pulsed, rapamycin-conditioned DC were infused into recipient mice with a short maintenance course of low-dose rapamycin, indefinite heart allograft survival was observed. Resistance to rejection was conferred when $CD4^+$ T cells from long-term acceptors were adoptively transferred. Interestingly, rapamycin-conditioned DC facilitated the proliferation of alloantigen-specific, $CD4^+/CD25^+/Foxp3^+$ T_{reg} compared with non-T_{reg}. Although rapamycin targets effector T cells, T_{reg} suppressive function is conserved. These data suggest that immature DC support T_{reg} function, and this sustainability decreases with DC maturation.[109] Taken together, a strong association between immature DC and T_{reg} suggests that targeting both these immune system regulators might ultimately be the most successful method in attaining indefinite allograft survival.

CONCLUSIONS

The success of clinical hand transplantation has brought CTA to center stage. The fears related to the intense antigenicity of CTA (particularly of skin) have largely been overcome with the use of immunosuppression currently used in solid organ transplantation. However, wider application of CTA will require further reduction of risks associated with long-term immunosuppression. The induction of donor-specific tolerance will alter the risk benefit ratio

in favor of CTA. One of the promising methods of inducing tolerance is by achieving mixed allogeneic chimerism through BM transplantation. In addition, CTA provides an ideal platform to study the role of T_{reg} and DC in transplantation tolerance. Easy visibility and the ability to permit safe biopsies make CTA (particularly hand) suitable in this regard. The day may not be far when CTA becomes the standard of care for tissue replacement by plastic and reconstructive surgeons.

Acknowledgments

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Fig 1.
The legend of Saints Cosmos and Damien, who replaced the diseased leg of a man with one from a dead person.

Fig 2.
The world's second and longest surviving hand transplant at 8 years follow-up.

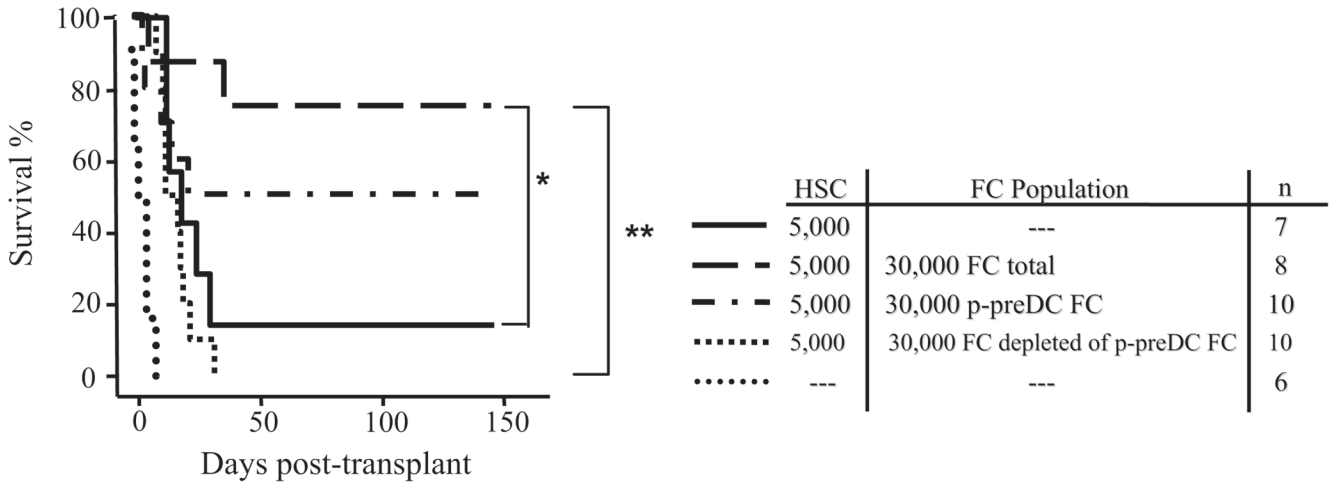

Fig 3.

In vivo assay for allogeneic FC. B10 mice were conditioned with 950 cGy TBI and transplanted with 5000 HSC alone (—) or in combination with 30,000 purified FC total (– –), p-preDC FC (– · –), or FC without p-preDC from B10.BR mice (▪▪▪▪▪). Some recipient mice were used as irradiation controls (…….). The cumulative survival of recipients was analyzed using the Kaplan-Meier method. Animals were followed up for 4 mo. *, $P = .0165$ between the HSC +FC group and the HSC group; **, $P = .0006$ between the HSC+FC group and the HSC+FC without p-preDC group.

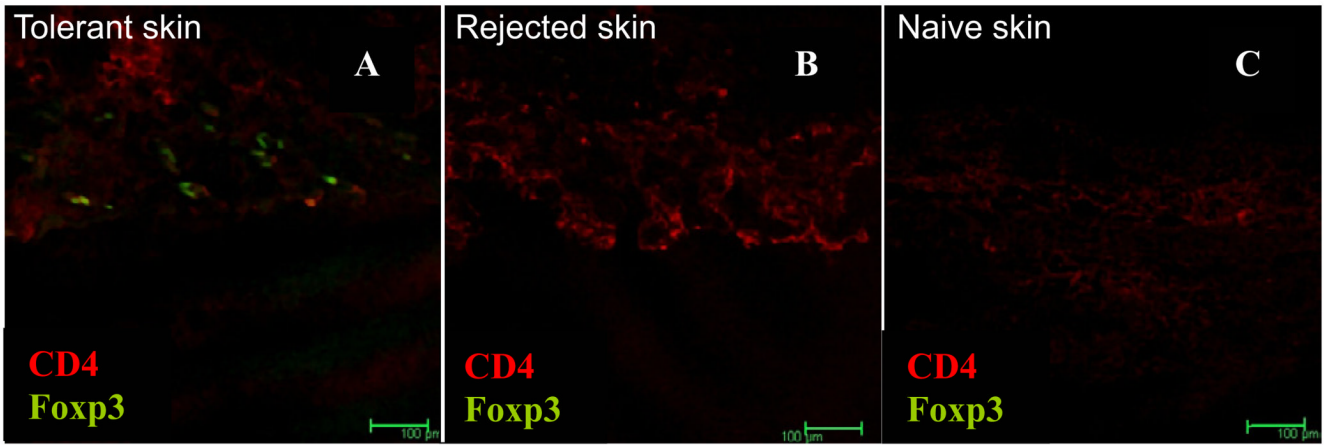

Fig 4.
Expression of Foxp3 in tolerant, rejected, and naïve skin tissues after CTA.

Table 1

Clinical Results of CTA

CTA	No.	Immunosuppression	Rejection	Other Complications	Results
Nerve[40]	7	CyA/FK+Aza+steroid taper	1	—	Sensory recovery – 6 mo Motor recovery – 3 mo
Flexor tendon transfer[42]	2	CyA for 6 mo	—	—	Excellent active and passive motion
Knee[112]	6	ATG+CyA (5)/FK (1)+Aza+steroid taper	5	Infection of graft	Graft loss, 5 Good function at 4 y, 1
Skeletal muscle[44]	1	ATG+CyA+prednisone	1	—	Viable at 1 y
Larynx[48]	1	Anti CD3+CyA+MMF+steroids	1	*Pneumocystis carinii* bronchitis	Good function at 7 y
Hand[50]	18	ATG (11)/Basiliximab (5) +FK+MMF +steroids	12	CMV infection, 2; herpes, 1; cutaneous mycosis, 1; and metabolic complications	90% return to work 62.5% improved quality of life
Tongue[54]	1	—	—	—	Function at 8 mo
Penis[52]	1	Daclizumab, CyA+MMF+steroids	—	Psychological issues	Amputated at 14 d
Face[53]	1	Thymoglobulin, FK+MMF+steroids	2	—	Sensory recovery – 6 mo Motor recovery – 10 mo
Abdominal wall[46]	9	Alemtuzumab+FK+MMF+steroid taper	3	Primary nonfunction, sepsis	5 deaths, 4 alive

Abbreviations: CyA, cyclosporine; FK, FK 506; Aza, azathioprine; ATG, antithymocyte globulin.