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In vivo observations of cell trafficking in allotransplanted vascularized skin flaps and conventional skin grafts

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Summary

The problem of allogeneic skin rejection is a major limitation to more widespread application of clinical composite tissue allotransplantation (CTA). Previous research examining skin rejection has mainly studied rejection of conventional skin grafts (CSG) using standard histological techniques. The aim of this study was to objectively assess if there were differences in the immune response to CSG and primarily vascularized skin in composite tissue allotransplants (SCTT) using *in vivo* techniques in order to gain new insights in to the immune response to skin allotransplants.

CSG and SCTT were transplanted from standard Lewis (LEW) ad Wistar Furth (WF) to recipient transgenic green fluorescent Lewis rats (LEW–GFP). *In vivo* confocal microscopy was used to observe cell trafficking within skin of the transplants. In addition, immunohistochemical staining was performed on skin biopsies to reveal possible expression of class II major histocompatibility antigens.

A difference was observed in the immune response to SCTT compared to CSG. SCTT had a greater density cellular infiltrate than CSG ($p < 0.03$) that was focused more at the center of the transplant ($p < 0.05$) than at the edges, likely due to the immediate vascularization of the skin. Recipient dendritic cells were only observed in rejecting SCTT, not CSG. Furthermore, dermal endothelial class II MHC expression was only observed in allogeneic SCTT. The immune response in both SCTT and CSG was focused on targets in the dermis, with infiltrating cells clustering around hair follicles (CSG and SCTT; $p < 0.01$) and blood vessels (SCTT; $p < 0.01$) in allogeneic transplants.

This study suggests that there are significant differences between rejection of SCTT and CSG that may limit the relevance of much of the historical data on skin graft rejection when applied to composite tissue allotransplantation. Furthermore, the use of novel *in vivo* techniques identified

characteristics of the immune response to allograft skin not previously described, which may be useful in directing future approaches to overcoming allograft skin rejection.

Keywords

Skin; Composite tissue; Allotransplant; Rejection; Confocal; Dendritic

Modern transplantation immunology was born out of observations on the problem of conventional skin allograft rejection.¹ The particular susceptibility of allograft skin to immune rejection was a major barrier to the development of clinical composite allotransplantation (CTA). It has only been in the last decade, following the development of more potent immunosuppressants, that there have been the first successful clinical applications of composite tissue allotransplantation. However, skin rejection remains a significant obstacle with more than two-thirds of hand transplants undergoing an acute skin rejection episode in the first year.² This has made research investigating the mechanisms of skin rejection and finding means to overcome the immune attack on the skin on CTA of particular importance.

Much of our understanding of allograft skin rejection has been derived from histology studies examining biopsies taken during rejection of nonvascularized, conventional skin grafts (CSG). However, both the method of observation and the type of transplant used limit the application of these findings to skin within composite tissue transplants (SCTT) that is primarily vascularized with the composite graft. The use of histological specimens to examine the rejection mechanism is restricted by artifact from fixation techniques and the frequency that biopsies can be taken. Consequently, only subjective comparisons can be made as there are not enough observations to reach statistical significance. In addition, it is possible that there are differences between CSG and SCTT in their interaction with the immune system, making observations of CSG not directly applicable to SCTT.

The aim of this study was to objectively assess if there were differences in the immune response to CSG versus SCTT using *in vivo* imaging techniques. In addition, this study sought to identify unique characteristics of the immune response to SCTT that may be useful for directing the development of approaches to overcome the rejection of composite tissue transplants and, specifically the skin component of the allografts.

Materials and methods

Overview

Conventional skin grafts and skin as part of a composite tissue transplant were performed across a full major histocompatibility barrier. *In vivo* confocal microscopy was used to observe cell trafficking into allogeneic skin and observing the targets in the allografted skin. This study was approved by the Institutional Animal Care and Use Committee of Massachusetts General hospital and followed the policies outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Animals

Skin was transplanted from non-fluorescent donor rats to recipient rats that were transgenic for GFP. This allowed for selective imaging of fluorescent recipient cells infiltrating the non-fluorescent transplanted skin using confocal microscopy. A total of 18 rats were used in this study. Donor animals ($n = 9$) were 8–12 week-old Lewis (LEW; RT-1^l) and Wistar Furth (WF; RT-1^u) rats (180–220 g) (Harlan Sprague Dawley Inc., Indianapolis, IN). Recipient animals ($n = 9$) were 8 week to 6-month-old Lewis GFP transgenic rats (Rat Resource and Research Center (RRRC), Columbia, MO); these rats were derived from Lewis rats obtained from

Harlan. Syngenicity between the donor Lewis rats (from Harlan) and recipient Lewis GFP transgenic (LEW-GFP) rats (from RRRC) was confirmed by observing CSG survival between the two strains (LEW → LEW-GFP) for greater than 100 days in this study.

Surgical technique

Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.). The abdomen and the lower limb of the donor and the recipient were shaved. The donor was also depilated with a commercially available depilatory cream, Nair® to reduce artifact caused by hair during imaging. All procedures were performed using sterile technique.

Musculocutaneous flap transplant—A musculocutaneous transplant was used as the SCTT. This novel model was used because, like many composite tissue allotransplants, it contains other tissues as well as skin. The presence of other tissues within the transplant may affect the immune response as these other tissues can often be more tolerogenic resulting in a state of ‘split tolerance’ in certain models (tolerance to the other allografted tissues but eventual rejection of skin).^{3,4} In a split tolerant state, it is possible that skin survival may be prolonged by virtue of being immediately adjacent to tolerant tissue (‘bystander suppression’).^{5,6} This will not occur with a CSG that does not contain other tissue types.

In donor animals, a quadrangular skin flap was raised with the underlying subcutaneous tissues⁷ and isolated on the epigastric vessels. The gastrocnemius muscle was isolated on the sural and femoral vessels. The composite flap was removed from the donor by dividing the femoral vessels at the inguinal ligament (Figure 1a). The donor animals were euthanized while fully anesthetized by injection of pentobarbital (200 mg/kg i.p.).

In recipient animals, an incision was made overlying the groin crease on the contra-lateral side to which the musculocutaneous flap had been harvested from the donor. The femoral vessels were isolated and divided distal to the inguinal ligament. Using microsurgical technique the donor and recipient femoral vessels were anastomosed end-to-end using 10-0 nylon suture. The composite musculocutaneous allograft was positioned and secured, with the muscle and skin components apposed, using 6.0 vicryl sutures (Figure 1b). All animals received buprenorphine 0.03 mg/kg subcutaneously every 12 h for 3 days post-operatively as analgesia.

Conventional skin graft transplant—On both donor and recipient animals a 2 × 2 cm rectangular piece of skin was raised over the posterior thorax, taking care to remove the panniculus carnosus. The skin raised from the donor was then secured to the margins of the wound bed of the recipient using 5-0 prolene interrupted sutures. A gauze tie-over dressing was applied to compress the skin graft onto the wound bed.

Injection of Evans blue dye—Evans blue dye was used to visualize the vasculature and assess the relationship of recipient cells around the graft vessels. A 2.5 ml aliquot of 1% (w/v) solution of Evans blue dye in phosphate buffered saline mixed in equal parts with a 5% (w/v) solution of bovine albumin. aliquot of 1% (v/v) was injected intravenously into the tail vein.

Experimental groups

To compare SCTT and CSG transplanted across isogeneic and allogeneic barriers, animals were divided into four experimental groups (see Table 1). Group 1 ($n = 3$) received a SCTT across an isogeneic barrier (Lew → Lew-GFP). Group 2 ($n = 2$) received a SCTT across a full MHC mismatched allogeneic barrier (WF → Lew-GFP). Group 3 ($n = 2$) received a CSG across an isogeneic barrier (Lew → Lew-GFP). Group 4 ($n = 2$) received a CSG across a full MHC mismatched barrier (WF → Lew-GFP). A key strength of this study was the use of noninvasive imaging that allowed for multiple images to be obtained from the same animal,

thus controlling for variability between animals. Each animal was imaged in multiple areas and depths in each imaging session, with multiple imaging sessions over consecutive days. To obtain an equivalent data-set with invasive imaging techniques (e.g. biopsies and histology), 50–100 times more animals would be required, introducing a significant source of variability in to the experiment.

In vivo video rate confocal microscopy

Recipient cell trafficking in skin flaps and grafts were analyzed using fluorescence confocal microscopy (Figure 1c). Between 717 and 1012 images were analyzed in each group (summarized in Table 1). In anaesthetized animals,⁸ high-resolution images were obtained at 30 frames-per-second, with 30 frame averaging, through intact rat skin at depths of up to 275 μ m from the surface using a 30 $\times$ 0.90NA water-immersion objective lens (Lomo, St. Petersburg, Russia). GFP positive recipient cells were excited with a solid-state laser at 491 nm (Dual Calypso, Cobolt AB, Stockholm, Sweden) and Evans blue dye with a helium-neon laser at 638 nm (Radius, Coherent Inc., Santa Clara, CA); these were then detected with a photo-multiplier tube at 507 nm and 670 nm respectively through bandpass filters transmitting 500–550 nm (Chroma, Rockingham, VT) and 667.5–722.5 nm (Omega Optical, Brattleboro, VT).

Images were obtained within 4 h of transplantation, on days 1–4, and then on alternate days. Isogeneic transplants were imaged to at least 10 days after transplantation. Allogeneic transplants were not imaged after day 4 because of auto-fluorescence due to cell death from rejection.

Previous ischaemia-reperfusion studies have demonstrated that the distal edges of a primarily vascularized skin flap have the poorest blood supply initially.^{9,10} This property was utilized to assess the importance of the vasculature as a route for cellular influx. Images were obtained from areas at the center of the transplanted skin as well as areas along the edges furthest from the pedicle at each time-point. Comparison was then made between the center and edge of the transplanted skin for variations in cellular influx secondary to differences in blood flow.

Evaluation of infiltrating cell numbers was made by identifying the depth of greatest cell density and then counting the number of cells per field at that depth. Evaluation of clustering of infiltrating cells around vasculature and hair follicles was performed by merging the position of vessels and hair follicles in a stack of images taken at a single location on to a single image. The numbers of cells in areas adjacent to these adnexal structures were then compared with identical sized areas that were not adjacent to adnexae.

Histology and immunohistochemistry

Biopsies were taken from center and the edges of the donor tissues of each experimental animal at sacrifice. These were placed immediately into either cryomedia (Tissue-Tek. Sakura Finetek U.S.A, Inc. Torrance, CA) and stored at -8°C or fixed in 4% paraformaldehyde solution. Hematoxylin and eosin stains were obtained for all specimens. Specimens were immunostained with mouse monoclonal antibodies [3D6] to MHC Class II and [15-11C5] to CD8 (Abcam, Cambridge, MA) to examine MHC class II expression in the vasculature. All histology and immunohistochemistry results were examined by a qualified pathologist in a blinded fashion.

Statistical analysis

Statistical analysis was performed using paired and unpaired student *t*-tests.

Results

Influx

More recipient cells infiltrate SCTT than CSG due to immediate vascularization

—There were up to twice as many cells infiltrating the center of each isogeneic and allogeneic SCTT than the respective isogeneic (Figure 2a cf. 2c; $p < 0.03$) and allogeneic (2b cf. 2d; $p < 0.01$) CSG at each time-point. To investigate why there was more cellular influx in SCTT compared to CSG, the influx of cells at the center and the edge of each CSG and SCTT were compared. CSG, in which there is no blood supply initially, showed no significant difference in recipient cell numbers at the center compared to the edge at all time-points (Figure 2a and b; $p < 0.1$). In contrast, all SCTT had significantly more infiltrating recipient cells at the center (solid line) than the edge (dotted line) from day 1 onwards (Figure 2c and d; $p < 0.05$) indicating that the vasculature (which supplies the center more richly than the edge) was a major route for recipient cellular influx.

There is greater cellular trafficking into allografts than isografts—Greater numbers of recipient cells infiltrated the allografts compared to the isografts. In SCTT there were greater numbers of recipient cells in all allotransplants compared to isografts from two days following transplantation ($p < 0.01$) and thereafter. It wasn't until three days after transplantation of CSG that there were greater numbers of recipient cells in allotransplants compared to isografts ($p < 0.02$; Figure 2).

MHC II was only expressed on rejecting SCTT dermal vascular endothelium—

Class II MHC expression and the immune cell types infiltrating the graft were characterized by immunohistochemical staining of biopsy samples. Staining revealed Class II MHC was expressed on the endothelium of all allogeneic SCTT but not on isogeneic SCTT or CSG four days after transplantation (Figure 3). The cells infiltrating isogeneic and allogeneic CSG and SCTT included MHC class II positive and CD8 positive lymphocytes.

Recipient dendritic cells are present in allogeneic SCTT, but not allogeneic CSG

—Recipient cells infiltrating the transplanted skin could also be identified and characterized *in vivo* with confocal microscopy by virtue of their fluorescence. Recipient dendritic cells (rDCs) were observed in the dermis in allogeneic SCTT two days after transplantation (Figure 4a) but not in allogeneic CSG at any time-point (Figure 4b). In contrast, rDCs were observable in both isogeneic SCTT from seven days after transplantation, and isogeneic CSG from eight days after transplantation.^e

Target

Infiltrating cells cluster around vessels and hair follicles in the superficial dermis of allotransplants

—Rat epidermal thickness was determined to be ~40 μ m from measurements made on the biopsy samples. *In vivo* imaging revealed minimal cellular infiltrate within 50 μ m of the surface of the skin in all animals at all time-points compared to the dermis ($p < 0.01$).

Hair follicles and blood vessels were identified *in vivo* within the skin using confocal microscopy. Hair follicles could be localized by auto-fluorescence of the hair within the follicle, and blood vessels could be identified by injection of Evans blue dye. SCTT were examined four days after transplantation for evidence of clustering of infiltrating cells. There was clustering of infiltrating cells around both hair follicles (Figure 5a; $p < 0.01$) and blood

^eThere is no immunohistochemical stain that is specific for dendritic cells. A standard method to distinguish DCs by their unique morphology (from which they derive their name) was used to identify DC.

vessels (Figure 5b; $p < 0.01$) in allogeneic SCTT but no significant clustering around either structure in isogeneic SCTT ($p > 0.1$).

There was clustering of infiltrating cells around hair follicles in allogeneic CSG ($p < 0.01$; Figure 5c) but no significant clustering in isogeneic CSG ($p > 0.1$). Only scattered blood vessels were visible in skin grafts at four days after transplantation following injection of Evans blue dye. Confirmation that sufficient dye had been injected was provided by imaging blood vessels in the ear (Figure 5d). Due to the limited number of blood vessels visible in CSG, clustering of infiltrating cells was only examined around hair follicles.

Discussion

Since the first successful hand allotransplant in 1998, there have been over 50 composite tissue allotransplants including more than 34 hand transplants,¹¹ 8 abdominal walls,¹² a scalp,¹³ leg, and three partial faces.¹⁴ More widespread application of these techniques is limited by the high doses of immunosuppression required to suppress allorejection and the increased susceptibility of skin to reject.¹⁵ Much of our knowledge about the mechanism of skin rejection has been gathered from *in vitro* observations of histological specimens taken from rejecting CSG. However, those observations may not accurately represent the *in vivo* clinical situation for the primarily vascularized skin in SCTT. This study indicates there are significant differences between SCTT rejection and CSG rejection, limiting the relevance of much of the historical data on skin graft rejection when applied to composite tissue allotransplantation. The use of novel *in vivo* techniques in this study identified characteristics of the immune response to allografted skin that have not been previously described, which may be useful in directing future approaches to overcoming skin rejection.

Influx includes the vascular route, and is earlier and more intense in skin flaps compared to skin grafts

This study indicates that, in contrast to CSG, the vascular route of cellular influx is important for primarily vascularized SCTT, accounting for up to half of the recipient cells found within the skin over the first four days after transplantation. Consequently, there are significantly more infiltrating cells within allogeneic SCTT than CSG. Furthermore, there was an earlier observable rejection response with increased cellular infiltration of allogeneic SCTT from day two post-transplantation compared to day three for CSG.

The earlier, more intense rejection response seen in SCTT compared to CSG at first seems to contrast with previous reports that primarily vascularized allogeneic skin transplants may have a slight survival advantage over CSG.^{16,17} One explanation may be that many of the infiltrating cells in SCTT are not involved in the rejection response as indicated by the large numbers of cells infiltrating isogeneic SCTT. To further delineate the contribution of the rejection process to cellular infiltration it would be useful to examine CSG and SCTT control groups, receiving allogeneic grafts (CSG or SCTT) under immunosuppressive cover. Another reason why allogeneic primarily vascularized skin transplants may have a slight survival advantage over CSG is that SCTT may be more resistant to the effects of rejection than CSG due to SCTT having more extensive vasculature initially. This possibility is supported by the observations that the rejection response in CSG is primarily due to infarction of the microvasculature,¹⁸ whereas vessel infarction has only been seen in cases of severe rejection in human composite tissue allotransplants.¹⁹

MHC class II was only expressed on SCTT, not CSG dermal vascular endothelium

MHC class II was only expressed on the dermal vascular endothelium of SCTT, not CSG. This difference may be a function of the later vascularization of CSG compared to SCTT. The

induced expression of MHC class II observed on the endothelium during rejection may be one reason for more cellular infiltrate in allogeneic SCTT compared to isogeneic SCTT.

This is the first time that MHC class II has been observed on endothelium in rat dermis. However, it is consistent with previous studies indicating that rat endothelium does not express MHC class II constitutively,²⁰ but expression can be induced in retina, brain, liver, kidney^{21, 22} and on heart allotransplants²³ by immune upregulation.

Presence of rDCs in SCTT, but not CSG

A major difference between allogeneic SCTT and CSG was that rDCs were observed by day 2 in SCTT but not at any time-point in allogeneic CSG. This observation has not been previously reported. It is possible that the early presence of rDC in rejecting SCTT could be exploited to accomplish skin tolerance in SCTT. For example, recipient dendritic cells have been used to achieve skin tolerance in murine bone marrow transplant tolerance induction models.²⁴ However, only prolonged skin survival has been achieved with donor dendritic cells²⁵ indicating that recipient dendritic cells can also have a crucial role in preventing the rejection process.

The target of the rejection response is the vasculature and adnexae

There was clustering of infiltrating cells in allogeneic transplants around vasculature and hair follicles, but not the epidermis. Previous explanations for the susceptibility of skin to reject have often focused on skin-specific antigens. Skin-specific antigens have been described in mouse allotransplantation²⁶ and rat to mouse xenotransplantation²⁷ models. However, skin-specific antigens have only been identified on epidermal cells in the skin, not around adnexal structures or blood vessels in the dermis. The observation of no epidermal clustering suggests that skin-specific antigens may not provide a full explanation for skin's susceptibility to rejection. The observation of statistically significant clustering around hair follicles and blood vessels also objectively confirms previous subjective observations that skin rejection may be focused in the superficial dermis, around adnexal glands and the vasculature.^{19,28,29}

The final element in the initiation of the immune response is cellular efflux and antigen presentation in lymphoid tissues. Donor cell efflux was examined in this model via microsatellite repeat PCR analysis (data not shown). As in previous studies, the efflux of donor cells were below the level of detection (<1%). To obtain a complete picture of the mechanism of rejection the efflux of donor and recipient cells from the allograft, the precise trafficking patterns to lymphoid tissues, and antigen presentation to T-cells needs to be examined. However, this would require modifications to this model. Efflux of donor cells could be imaged by allotransplanting vascularized skin from a GFP positive rat to a GFP negative rat with *in vivo* imaging of target lymphoid tissues. Likewise, efflux of recipient cells targeting could be followed by an injection of recipient type immature GFP labeled dendritic cells at the time of transplantation with *in vivo* imaging of the targeted lymphoid tissues.

In conclusion, this study identifies differences in cell trafficking into and within conventional skin grafts in comparison to skin as part of a composite tissue graft. In contrast to CSG, the vascular route in SCTT is important for initial cellular influx where there is trafficking of rDCs and there is an earlier and more intense cellular infiltrate seen in the SCTT. In addition, there is an early presence of rDC in rejecting SCTT but not CSG. Finally, the rejection response has dermal targets rather than the epidermis as has been often assumed previously. These observations challenge previous dictums that have directed skin transplant rejection research, and also give direction to future research in to ways to avoid skin rejection in CTA.

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References

- Gibson T, Medawar PB. The fate of skin homografts in man. *J Anat* 1943;77:299–310. [PubMed: 17104936]
- Lanzetta M, Petruzzo P, Dubernard JM, et al. Second report (1998–2006) of the international registry of hand and composite tissue transplantation. *Transpl Immunol* 2007;18:1–6. [PubMed: 17584595]
- Mathes DW, Randolph MA, Solari MG, et al. Split tolerance to a composite tissue allograft in a swine model. *Transplantation* 2003;75:25–31. [PubMed: 12544866]
- Hettiaratchy S, Melendy E, Randolph MA, et al. Tolerance to composite tissue allografts across a major histocompatibility barrier in miniature swine. *Transplantation* 2004;77:514–21. [PubMed: 15084927]
- Chen ZK, Cobbold SP, Waldmann H, et al. Amplification of natural regulatory immune mechanisms for transplantation tolerance. *Transplantation* 1996;62:1200–6. [PubMed: 8932256]
- Davies JD, Leong LY, Mellor A, et al. T cell suppression in transplantation tolerance through linked recognition. *J Immunol* 1996;156:3602–7. [PubMed: 8621893]
- Nishikawa H, Manek S, Green CJ. The oblique rat groin flap. *Br J Plast Surg* 1991;44:295–8. [PubMed: 1711909]
- Sipkins DA, Wei X, Wu JW, et al. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature* 2005;435:969–73. [PubMed: 15959517]
- Carroll WR, Esclamado RM. Ischemia/reperfusion injury in microvascular surgery. *Head Neck* 2000;22:700–13. [PubMed: 11002326]
- Kuntscher MV, Schirmbeck EU, Menke H, et al. Ischemic preconditioning by brief extremity ischemia before flap ischemia in a rat model. *Plast Reconstr Surg* 2002;109:2398–404. [PubMed: 12045567]
- Lanzetta M, Petruzzo P, Margreiter R, et al. The international registry on hand and composite tissue transplantation. *Transplantation* 2005;79:1210–4. [PubMed: 15880072]
- Levi DM, Tzakis AG, Kato T, et al. Transplantation of the abdominal wall. *Lancet* 2003;361:2173–6. [PubMed: 12842369]
- Jiang HQ, Wang Y, Hu XB, et al. Composite tissue allograft transplantation of cephalocervical skin flap and two ears. *Plast Reconstr Surg* 2005;115:31e–5e. [PubMed: 15622228]
- Kanitakis J, Badet L, Petruzzo P, et al. Clinicopathologic monitoring of the skin and oral mucosa of the first human face allograft: report on the first eight months. *Transplantation* 2006;82:1610–5. [PubMed: 17198245]
- Lee WP, Yaremchuk MJ, Pan YC, et al. Relative antigenicity of components of a vascularized limb allograft. *Plast Reconstr Surg* 1991;87:401–11. [PubMed: 1998012]
- Steinmuller D. The enigma of skin allograft rejection. *Transplant Rev* 1998;12:42–57.
- Bushell, A.; Liddington, ML.; Morris, PJ. Directly vascularized skin grafts are more immunogenic than whole organ allografts: a novel study in an CD4 tolerance model; Abstracts of the 4th Basic Sciences Symposium of the Transplantation Society; Noordwijkerhout. 1995.
- Dvorak HF, Mihm MC Jr, Dvorak AM, et al. Rejection of first-set skin allografts in man. the microvasculature is the critical target of the immune response. *J Exp Med* 1979;150:322–37. [PubMed: 379265]
- Cendales LC, Kirk AD, Moresi JM, et al. Composite tissue allotransplantation: classification of clinical acute skin rejection. *Transplantation* 2006;81:418–22. [PubMed: 16477229]

20. Choo JK, Seebach JD, Nickleit V, et al. Species differences in the expression of major histocompatibility complex class II antigens on coronary artery endothelium – implications for cell-mediated xenoreactivity. *Transplantation* 1997;64:1315–22. [PubMed: 9371674]
21. Wang Y, Calder VL, Lightman SL, et al. Antigen presentation by rat brain and retinal endothelial cells. *J Neuroimmunol* 1995;61:231–9. [PubMed: 7593559]
22. Ustinov J, Loginov R, Bruggeman C, et al. CMV-induced class II antigen expression in various rat organs. *Transpl Int* 1994;7:302–8. [PubMed: 7916933]
23. Forbes RD, Gomersall M, Darden AG, et al. Multiple patterns of MHC class II antigen expression on cellular constituents of rat heart grafts. Lack of correlation with graft survival, but strong correlation with vasculitis. *Transplantation* 1991;51:942–8. [PubMed: 2031276]
24. Beriou G, Pêche H, Guillonnet C, et al. Donor-specific allograft tolerance by administration of recipient-derived immature dendritic cells and suboptimal immunosuppression. *Transplantation* 2005;79:969–72. [PubMed: 15849552]
25. Markees TG, Phillips NE, Gordon EJ, et al. Prolonged skin allograft survival in mice treated with Flt3-ligand-induced dendritic cells and anti-CD154 monoclonal antibody. *Transplant Proc* 1999;31:884–5. [PubMed: 10083387]
26. Boyse EA, Old LJ. Loss of skin allograft tolerance by chimeras. *Transplantation* 1968;6:619. [PubMed: 4876502]
27. Silverman MS, Chin PH. Differences in the rejection of skin transplants and blood cells of donor marrow origin by radiation-induced chimeras. *Ann N Y Acad Sci* 1962;99:542–9. [PubMed: 13992944]
28. Dvorak HF, Mihm MC Jr, Dvorak AM, et al. The microvasculature is the critical target of the immune response in vascularized skin allograft rejection. *J Invest Dermatol* 1980;74:280–4. [PubMed: 6993571]
29. Bhan AK, Mihm MC Jr, Dvorak HF. T cell subsets in allograft rejection. In situ characterization of T cell subsets in human skin allografts by the use of monoclonal antibodies. *J Immunol* 1982;129:1578–83. [PubMed: 6213716]

Figure 1.

Isolation, placement and imaging of skin within a composite tissue transplant. The skin supplied by the epigastric vessels and gastrocnemius muscle were isolated on the common femoral vessels (1a). The flap was depilated with Nair® hair removing ointment prior to isolation, whereas the recipient groin site was only shaved, facilitating differentiation of donor and recipient tissues on imaging (1b). The skin flap underwent *in vivo* confocal imaging (1c) initially daily with the animal under sedation.

KEY

Error Bars indicate standard error of the mean

Figure 2.

Representative examples of recipient cell influx into skin flaps and skin grafts transplanted across isogenic and allogeneic barriers. There was no significant difference in the numbers of cells observed at the depth of greatest cell density at the center and edge of CSG at any time-point (isogenic CSG (2c) $p > 0.1$; allogeneic CSG (2d) $p > 0.1$). In contrast, there were significantly more cells in the highly vascularized center of SCTT than either the skin flap edge (isogenic SCTT (2a) $p < 0.05$; allogeneic SCTT (2b) $p < 0.05$) or the center of comparable isogenic (Figure 2a cf. 2c; $p < 0.03$) or allogeneic (Figure 2b cf. 2d; $p < 0.01$) CSG. There was greater infiltrate in to both the center and edge of rejecting allogeneic than isogenic SCTT (2b cf. 2a; $p < 0.01$) and CSG (2d cf. 2c; $p < 0.02$).

Figure 3. Endothelial class II expression on vasculature of rejecting skin. MHC Class II expression was observed on immunohistochemical staining of biopsies taken from allogeneic SCTT taken 4 days after transplantation (3a), but not on isogeneic SCTT (3b) ($\times 40$ magnification).

Figure 4.

Recipient cell types within transplanted skin Infiltrating recipient cells included dendritic cells, which were observable on confocal microscopy in allogeneic flaps from two days after transplantation (4a), but not observable at any time-point in CSG (4b). Both CD8+ (4c) and Class II positive (4d) lymphocytes were identified in allogeneic and isogeneic CSG and SCTT on immunohistochemical staining ($\times 40$ magnification).

Figure 5.

Clustering of infiltrating cells observed around blood vessels and hair follicles in allogeneic skin transplants. Clustering of infiltrating cells was observed around blood vessels (5a; vessels colored red, arrows indicating perivascular cell clustering; $p < 0.01$) and hair follicles (5b; arrows indicating cells palisading around hair base; $p < 0.01$) in allogeneic SCTT, and around hair follicles in allogeneic CSG (5c; arrows indicating cells palisading around hair base; $p < 0.01$), but not in isogeneic SCTT ($p > 0.1$) and CSG ($p > 0.1$), 4 days after transplantation. Adequate Evans blue dye injection to image CSG vessels was confirmed by good vessel detection in the ear (5d). However, few vessels were visible in CSG 4 days after transplantation, preventing assessment of cell clustering around CSG vessels.

Table 1

Experimental groups.

Group	Rats (<i>n</i>)	Images (<i>n</i>)	SCTT/CSG	Transplant barrier
1	3	1012	SCTT	LEW→LEW-GFP
2	2	962	SCTT	WF→LEW-GFP
3	2	717	CSG	LEW→LEW-GFP
4	2	732	CSG	WF→LEW-GFP