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Immunological Considerations for Inducing Skin Graft Tolerance

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1. Introduction

While the first uses of skin transplantation may be found in anecdotal images involving the use of foreign tissue to cover burns and damaged skin, the modern era of skin grafting attempts to understand the phenomenon scientifically. One well documented case describes Joseph E. Murray's surgical procedure to use skin from a recently deceased person to cover the skin of a pilot who suffered disfiguring burns in 1944 (Murray, 1992) The transplanted skin survived for more than thirty days, despite any predictions of rejection that might have destroyed the tissue in two weeks or less. Murray hypothesized that the procedure was successful because of the weakened state of the recipient's immune system. Murray continued his interest in transplantation immunology, and ten years later his team was the first to engraft an organ from a living donor, (Murray, 1992).

In 1944 Medawar described rejection of allografts, that is, organs or tissues exchanged between individuals of the same species (Medawar, 1944). His pioneering work described the consequences of allograft and autograft transplantation in exquisite detail. He used the rabbit skin model to show differences in acceptance of autografts, using the same individual or same strain as the tissue source, vs. allografts that exchanged skin between different animals of the same species. He further used meticulously measured tissue size as a surrogate marker for skin antigen dose and documented the process of healing for autographs opposed to the "actively acquired immune reactions" that resulted in the necrosis of allografts. R. E. Billingham (1959) reviewed the fate of engrafted tissue that is rejected by the host in light of the then recently discovered major histocompatibility complex in the mouse (MHC). The MHC genes encode cell surface proteins that allow the immune system to discriminate between its own cells and foreign cells. Billingham referenced the use of isogenic mouse strains wherein each animal of the strain has identical MHC genes, and these animal models facilitated the understanding of graft versus host disease, rejection mediated by immunocompetent cells within a graft (Billingham, 1959).

Billingham, Brent, and Medawar (1953) described immunological tolerance that is actively acquired following prenatal exposure to foreign tissue in a mouse model. This phenomenon described the survival of engrafted tissue, whereas the same tissue generated immunity resulting in graft destruction when transplanted in the adult. After eight weeks, the tolerant mice were challenged with adult skin of the same donor origin as the primary transplant, in order to evaluate the persistence of tolerance. These experiments documented the utility of

mouse models as a practical means to help us understand immunological processes. Five years later, Billingham and Silvers (1958) induced specific tolerance to the male chromosome associated Y antigen in neonatal female mice, again using the skin graft model. Researchers continue to use these basic methods to uncover the underlying mechanism of tolerance and immunity.

Skin grafts, with their enhanced antigenicity and abundant supplies of antigen presenting cells, remain a vigorous test of any tolerance induction system (Murray, 1971, Nasir et al., 2009, Tobin et al., 2009, Akdis, 2010, van den Berg et al., 2011, Kaplan, 2010, del Rio et al., 2010). Some of the skin associated antigens include autoantigens found on keratinocytes and appear to induce T lymphocyte reactivity that results in skin eruptions (Jackman et al., 2002). Antigenicity of skin has remained a concern for those promoting translational medicine, including those who promote the use of composite tissue allografts (Swearingen et al., 2008). The grafting of composite tissue is often used in limb replacement surgery. These grafts consist of complex combinations of multiple tissue types, including skin, adipose tissue, bone and others (Siemionow et al., 2009). The complexity of these composite tissue allografts is such that a new classification system was devised in order to accurately describe their attributes. Tissue antigenicity is one of the criteria on which the classification system is based (Gordon et al., 2009). These historical experiments helped to usher in the era of modern skin transplantation.

2. Generalized Immunosuppression to facilitate skin graft acceptance

Medical science has pursued a multiplicity of scientific directions in order to utilize solid organ transplantation as a potential cure for life-threatening diseases and as a treatment for sever disfigurement. Organ transplantation has been referred to as "One of the most remarkable achievements of medicine during the 20th century" (Goldstein, 2011). Scientific advances gleaned from the use of skin grafts and other tissue and organ grafts have restored tissue function, extended the life, and promoted the general well-being of the graft host. The spoken or unspoken hypothesis behind the use of generalized immunosuppression may be stated as follows: If the immune system in an otherwise healthy individual is capable of recognizing foreign tissue and mounting an attack against it, then significantly reducing all such recognition may allow for engrafted tissue to remain intact and functional. Many of these procedures required protracted or life-long treatments with unanticipated or negative sequelae (Ingvar et al., 2010, Berardinelli et al., 2009). The goal of these broad-based protocols was to protect the viability of the graft in order to protect the life of the host. Some notable examples of the use of generalized immunosuppressive agents are described below.

2.1 Three categories of drugs targeting lymphocytes have been described

Information gleaned from reviews in major journals describes three general categories of immunosuppressive agents (Halloran, 2004, Lindenfeld et al., 2004, Gonzalez Posada, 2006, 1970). These include antibodies directed against lymphocytes, steroids, and agents that interfere with metabolism or that have toxic effects on cells. The first category is often referred to as antilymphocyte or antithymocyte globulin (ALG or ATG). ALG in combination with an intrathymic dose of donor spleen cells was shown to induce graft survival in a rat model (Shen et al., 1996). Lewis-Brown Norway cardiac allografts showed extensive survival in Lewis rats; whereas skin grafts from the same donor strain were

rejected at a similar rate as third party grafts, that is, grafts bearing transplantation antigens different from those of the donor or host. These experiments demonstrated further the increased antigenicity of skin in relation to other tissues. The author hypothesizes that additional non-MHC skin antigens may be responsible for the rejection. Additional aspects of skin antigenicity are described above (Murray, 1971, Nasir et al., 2009, Tobin et al., 2009, Akdis, 2010, van den Berg et al., 2011, Kaplan, 2010, del Rio et al., 2010).

Adding ALG to a protocol that includes azathioprine and prednisolone may reduce the toxic effects of ALG. Corticosteroids such as prednisolone reduce the inflammatory response, interfere with protein synthesis and are cytotoxic for lymphocytes. Despite the lymphocyte toxicity, these reagents were not found to prolong skin graft survival. The cytotoxic and antimetabolite drugs appeared to target DNA function, activity, or synthesis. Cyclophosphamide is a strong DNA cross-linker and alkylating agent. Methotrexate is a folic acid inhibitor that blocks DNA synthesis, and therefore interferes with cell division in lymphocytes. Azathioprine and 6-mercaptopurine are two related purine analogs that have been show to delay graft rejection. The effect of all of these drugs was variable depending on the species treated and the regimen used. Optimum regimen included using the drugs in combination with the specific antigen in order to have a more prolonged graft-protecting effect rather than a temporary dampening of the body's ability to mount an immune response.

2.2 Protection of rabbit skin by phenothiazine derivatives

Eyal and associates (1965) used phenothiazine related products to suppress immune responses to rabbit skin allografts. The team focused their attention on the action of three different compounds: chlorpromazine, perphenazine, or promethazine. They chose these reagents because of reports that these compounds could minimize cell death and tissue necrosis, albeit a protective effect was not witnessed with the engraftment of guinea pig skin. Eyal and colleagues hypothesized that an optimal dose and specific compound combination would be protective. These compounds produced different degrees of graft protection in controlled experiments where they were tested against normal saline in otherwise untreated animals. Both treatment and control groups of rabbits were given ear skin allografts of approximately equal sizes. They found that the most beneficial compound was promethazine, followed by perphenazine; and that the least protective was chlorpromazine. They attribute the extended skin graft survival of treated animals to a membrane protective effect of the drugs on graft recipient cells and to a reduced loss of donor antigen from the graft itself.

2.3 Prolongation of graft survival mediated by methylhydrazine derivatives

Floersheim (1967) used methylhydrazine derivatives to induce tolerance in MHC disparate adult mice and reported significant improvement in graft prolongation when the treatment was combined with an infusion of donor specific cells derived from spleen or from kidneys and liver. Graft survival was increased to at least twenty percent when this combined therapeutic approach was used. According to the US Environmental Protection Agency (EPA) these drugs may have a negative impact on liver and kidney primarily, and on blood and spleen secondarily (EPA, 2007). Acute but not chronic effects have been reported for humans (EPA, 2007). Thus despite some benefits in terms of graft survival, potentially harmful side effects limited more widespread use.

2.4 The use of urethane in combination with X-irradiation to protect skin grafts

Cole and Davis (1962) used a reagent known as a DNA antagonist and an inhibitor of mitosis in order to prolong survival of skin grafts in sub-lethally X-irradiated mice. They were aware of studies that demonstrating that high doses of radiation could protect a graft by suppressing an immune response. Cole and Davis hypothesized that a lower radiation dose in combination with urethane treatment would be immunoprotective and showed that sixty percent of the mice had significant skin graft survival. Mean graft survival in the treated animals was 40 days vs. 18 days for untreated mice of the same strain. Their study included an early attempt to develop irradiation bone marrow chimeric mice. A subsequent study induced skin graft tolerance that endured for more than 130 days and was specific for transplantation antigens of the bone marrow donor, as third party grafts were rejected (Davis and Cole, 1963). In such experiments, the native bone marrow is ablated, in whole or in part, by radiation, and specific donor derive bone marrow is used to replace the ablated cells of the host. Additional examples of irradiation bone marrow chimerism appear below.

More recently, urethane or polyurethane has been used as a dressing to promote wound healing of split thickness skin graft donors (Cigna et al., 2009), and as a component of negative pressure dressings for young burn patients who receive skin grafts (Psoinos et al., 2009).

2.5 Calcineurin inhibitors

Calcineurin is a protein phosphatase found in eukaryotic cells whose activity is significantly blocked by cyclosporine A and Fk506, now referred to as tacrolimus (Rusnak and Mertz, 2000). The once-popular calcineurin inhibitors (CNI) fell out of favor due to their observed nephrotoxicity (Groetzner et al., 2004). Additional complications of the use of CNI drugs include associated malignancies. Doesch et al. (2010) studied the development of neoplasias in cardiac patients treated with immunosuppressive drugs. They found a strong correlation between the use of CNIs or the drug azathioprine and the development of malignancies. These drug-associated malignancies include multiple forms of skin cancer, including squamous cell carcinoma (Wu et al., 2010), but exclusive of melanoma (Signorell et al., 2010). In contrast, the rate of malignancies was reduced in patients treated with non-CNI based agents such as mammalian target of rapamycin (m-TOR) inhibitors (Doesch et al., 2010).

3. Pre-engraftment reduction of tissue antigenicity

3.1 Cultured tissues and antibodies

Jacobs and Uphoff (1974) reviewed a variety of methodologies used to reduce the potential immune reaction between the donated tissue or organ and the graft recipient and reported that phenotypic changes including reduced antigenicity may be observed in cultured cells. These changes vary with species. The advantage of using these techniques may be a reduced dependence on the use of immunosuppressive agents. Antibodies may be used to treat the graft donor or the tissue itself. They summarized the experiments of Hellman and Duke (1967) who used a skin allograft model to demonstrate the reversal of tolerance to syngeneic skin when the tissue was incubated with skin of a MHC disparate donor. Unpredictably, pre-incubating donor and recipient skin together did not result in prolonged skin allograft survival.

3.2 Preconditioning with allogeneic RNA or DNA

In agreement with this data, the benefits of altering donor skin antigenicity was further demonstrated when Guttman et al. (1964) prevented acceptance of syngeneic skin grafts by pretreating mouse skin in vitro with allogeneic RNA. In contrast to these results, Lemperle et al. (1968) demonstrated that pre-treating donor tissue with RNA or DNA facilitated extended skin graft survival in MHC disparate donor and host combinations.

4. The use of chimerism to protect engrafted tissues

4.1 Bone marrow chimerism induced by whole body Irradiation

An early model of irradiation induced bone marrow chimerism was developed by two scientist working at the National Institute of Allergy and Infectious Disease (Liacopoulos and Goode, 1964). Liacopoulos and Goode were aware that overwhelming the immune system of potential tissue donors with a bolus of strong antigens from different sources may block reactivity to a specific unrelated antigen. This procedure is known as protein overloading and is directed towards the graft donor. The group tested the hypothesis that spleen cells, bone marrow or skin from animal models given a large dose of antigen would be tolerated in another animal. The graft recipients were irradiated and pretreated with donor strain derived cells prior to skin grafting. Rabbit gamma globulin or Limulus hemocyanin served as the strong antigens. The authors used both a mouse allograft model and a concordant rat-to-mouse xenograft model (Liacopoulos and Goode, 1964).

Owen et al. (1945) had observed earlier that naturally occurring tolerance could develop between dizygotic calves in utero. Since these calves each had a different immunological makeup, the prenatal exposure to each other's disparate antigenic makeup was undoubtedly the source of the immunological unresponsiveness. Stone et al. (1965) expanded the initial findings by engrafting skin between dizygotic twins known to be chimeric at the erythrocyte level. The discovery of chimerism in these calves provided the scientific basis for adapting this concept to the development of irradiation bone marrow chimerism as a model for the induction of transplantation tolerance.

Following these discoveries, a series of studies tested the hypothesis that the phenomenon of chimerism could be experimentally induced in order to prolong allograft survival (Mathe' et al., 1963, Seller, 1967, Lubaroff and Silvers, 1973, Buckley, 1975). Mathe' et al. (1965) applied this concept in order to protect a patient from leukemia. The patient was given whole body irradiation followed by a bone marrow transplant comprised of tissues from six different male and female donors, all of whom were related to the host. The bone marrow graft restored the myeloid and erythroid compartments. A skin graft from one of the male bone marrow donors remained intact and viable for more than seven months, while skin grafts from other donors were rejected. These results implied that the donor and the host were more histocompatible. More recent applications of irradiation bone marrow chimerism are discussed below.

Tolerance to allografts following whole body irradiation has been reviewed (Strober et al., 1979, Slavin et al., 1985, Sprent et al., 1993, Monaco, 2004). A potential mechanism to explain the success of these grafts was suggested by Okada and Strober (1982)who used a mixed lymphocyte reaction (MLR) to demonstrate that spleen cells from animals given whole body irradiation induced the proliferation of large numbers of cells directed against specific antigenic targets. This work extended earlier studies that identified two distinct populations of suppressor cells induced by these methods. One population responded specifically to

bovine serum albumin (BSA) as an antigen, while the other responded non-specifically (Slavin and Strober, 1979).

4.2 The induction of chimerism without myeloablation

Several investigators have reported the induction of allogeneic bone marrow chimerism without myeloablation (Sykes, 1996, Pan et al., 2003, Fuchimoto et al., 2000, Shapira et al., 2003, Matthews et al., 2004, Ciurea and Andersson, 2009, Przepiorka et al., 1999). Myeloablation refers to the massive radiation induced destruction of the host's bone marrow cells, especially the T cell lineages, and the use of allogeneic bone marrow to replace the destroyed cells. Because of the radiation, with or without other treatments, the host is unable to reject the donated bone marrow cells. The major side effects associated with lethal whole body irradiation, have caused some investigators to develop non-myeloablative procedures (Sykes, 1996).

4.2.1 Chimerism to induce tolerance to allografts and xenografts

Sykes used low dose (3 Gy) whole body irradiation in combination with a higher dose (7 Gy) irradiation of the thymus and antibodies that deplete $CD4^+$ and $CD8^+$ T cells to generate specific tolerance to the cells and tissues of the donor (Sykes, 1996). Sykes used this protocol to show tolerance to allografts in the mouse, and tolerance of rat skin in murine hosts. She used the swine–to–mouse model to show that the protocol could be expanded to a discordant model, that is, one involving hyperacute reactions between widely disparate host and donor species. She depleted T cells and natural killer cells (NK) in thymectomized mice, then engrafted the thymus of a fetal pig. These mice were shown to accept a skin graft from the same donor strain as the thymus graft. Despite being described as "non-myeloablative, the procedure combines low dose total body irradiation with focused high level irradiation targeted to the thymus.

4.2.2 Irradiation-free chimerism in a murine allograft model

Pan et al. (2003) used a combination of costimulation blockade and a metabolism antagonist to reduce the donor T cell population in donor bone marrow to induce chimerism in mice. Depletion of donor T cells reduced the risk of graft vs. host disease (GVDH) wherein donor T cells initiate an immune response against the new host. Pan and colleagues used a combination of fludarabine, described below, the immunosuppressive drug cyclophosphamide, and interruption of the CD40/CD154 T cell activation pathway to facilitate donor bone marrow engraftment. The protocol resulted in mixed chimerism in the host and allowed donor origin skin grafts to survive.

4.2.3 Irradiation-free chimerism in a swine animal model

Fuchimoto and colleagues (2000) developed a technique to create a hematopoietic system composed of both donor origin and host origin cells, referred to as "mixed chimerism" to distinguish the results from full chimerism that often occurs when the host is given lethal irradiation followed by an allogeneic bone marrow transplant. They used high doses of stem cells separated from peripheral blood to induce mixed chimerism. The chimeric host accepted skin grafts from a donor whose transplantation antigens (swine leukocyte antigens, SLA) matched those of the bone marrow, but did not accept skin grafts from a third party donor, one that matched neither the bone marrow donor nor the new host.

4.2.4 Irradiation-free chimerism in humans

Shapira et al. (2003) reported an attempt to induce chimerism with no radiation in high risk patients who were not able to tolerate whole body radiation. The use of the drugs fludarabine and busulfan had a myeloablative effect on the host bone marrow cells. Fludarabine has been reported to minimize the development of neoplasias as well as to suppress metabolism (Matthews et al., 2004). Busulfan has been described as a myeloablation agonist that has been used to engraft bone marrow and stem cells (Ciurea and Andersson, 2009, Przepiorka et al., 1999). Eight percent of the patients developed full chimerism as evidence by donor origin hematopoietic cells. Regrettably, graft-versus-host disease (GVHD) was identified in most patients despite a brief low dose treatment of cyclosporine-A, and mortality was high.

5. Costimulation blockade to interrupt the process of T cell activation

If the events reported by Stone's group represent naturally occurring prenatal tolerance (Stone et al., 1965), how then may we induce tolerance in an adult? One answer may be found by dissecting the pathway for the development of immunity. These experiments tested the hypothesis that interference with one of the key steps leading to an immunological response would prevent the downstream events from occurring. This type of tolerance is referred to as peripheral tolerance as it leaves the host with the ability to respond to other immunological perturbans. Exposure of untreated animal models to a source of foreign antigen initiates the process of immunity and may provide the stimulus to recruit potentially alloreactive or xenoreactive cells from their histological sites. Subsequent interference with the reaction between CD40 and CD40L (CD154) or the reaction between B7 molecules and cytotoxic T lymphocyte-associated antigen (CTLA)-4 provides a scenario wherein cells that recognize the foreign antigen receive the initiating signal that recruits them to the site of antigen deposit, but not the required secondary signals. Costimulation blockade may be mediated by monoclonal antibodies directed against specific cell surface molecules, by the use of agonistic monoclonal antibodies to prevent reactivity induced by donor origin cells within the graft, referred to as graft-versus-host disease, (Albert et al., 2005, Yu et al., 2003, Yu et al., 2000) by the use of anti-sense RNA to facilitate apoptosis (Yu et al., 2004), or by deletion of cellular components expressing costimulation molecules. In systems where CD4 cells are important, graft survival may be accompanied by the expansion of $CD4^+$ CD25 Foxp3 regulatory cells.

When the elements of one's own immune system come into contact with particles, agents, cells or tissues not belonging to the host, those foreign agents are recognized as non-self. Immunity is the complex process of activating the body's adaptive immune defenses in order to protect the body from these specific invading elements. T cells are components of the adaptive immune system, and as such may be geared up to protect the body from foreign invaders, including pathogenic organism and transplanted cell and tissue grafts. They are increasingly protective upon re-exposure of the organism to the same antigen (Janeway C.A. et al., 2001).

An important feature of immunity is the activation of thymus derived lymphocytes or T cells. Grakoui et al. (1999) provided a detailed study of the environment and the molecular interactions required for T lymphocyte activation. In a host with an intact immune system, immunity would result in the destruction of engrafted tissue or organs from another individual who is not an identical twin. T cell activation is an essential feature of immunity,

and thus a significant player in the acceptance or rejection of a graft. A hypothesis utilizing this information might be stated as follows: If T cell activation is important for immunity and graft rejection, then interruption of the T cell activation cascade will prevent or delay graft rejection. Preventing an immune reaction between graft and host would provide an environment conducive to transplantation tolerance. In particular, preventing an immune response to the specific graft-associated antigens, often described as anergy, would be more beneficial than a generalized immunosuppressive response because the host immune system would still be capable of protecting the body from other invaders.

Modern transplantation immunology owes a great debt to the pioneering work of Jenkins and Schwartz (1987) who demonstrated that antigen presentation devoid of a MHC context failed to stimulate T cell clones in vitro or T cells in vivo. They used a chemical crosslinking agent to affix pigeon cytochrome peptides to the surface of spleen cells. If properly presented with the cell's MHC surface molecules, the process would have resulted in T cell proliferation, IL-2 production (Jenkins et al., 1987) and antigen recognition (Jenkins and Schwartz, 1987). The inappropriate antigen presentation resulted in T cells unresponsive to their cognate (recognizable) antigen; and thus the authors concluded that the mechanism involved was the deletion of antigen-specific T cells (Jenkins and Schwartz, 2009). Their in vitro blockade of T cell activation was reproduced in the mouse model.

In 1993, Boussiotis et al. (1993) published their research implicating the costimulatory molecule B7 as a contributor to allograft immunity. Using a transfection model, the authors showed that interrupting the B7:CD28/CTLA4 pathway was consistent with an anergy model of immune unresponsiveness to one specific human derived transplantation antigen, HLA-DR7. In contrast, blocking the reaction between the intercellular adhesion molecule 1 (ICAM1) and lymphocyte function antigen 1 (LFA1) appeared to be associated with immunosuppression. Important contributors to T cell activation have been reviewed by Wingren et al. (1995) and include the work of Ford and Larsen (2009). The work of Jenkins, Schwartz, and colleagues has shown antigen specific T cell unresponsiveness (Jenkins et al., 1987, Jenkins and Schwartz, 1987, Jenkins and Schwartz, 2009). Further research led to clarification of the mechanisms of T cell signaling and activation. One set of stimulatory reactions was a consequence of the binding of B7 (CD80 or CD86) with CD28; while a different cascade of events followed the binding of CD40 with CD154 (CD40L). Many features of these pathways were elaborated by Bluestone et al. (1995) and have been reviewed by Lenschow et al. (1996). The three main pathways of T cell activation have been described by ML Ford and CP Larsen (2009).

Larsen et al. (1996) showed the importance of interrupting CD40/CD28 binding in skin and cardiac allografts, although mechanistic details remained for future investigations. Some mechanisms may include the induction of anergy, as mentioned above, the reduction of alloreactive T cell populations (Iwakoshi et al., 2000), the induction of regulatory T cells and the interactions of other cells with regulatory functions (Gordon and Kelkar, 2009), as discussed below, or diverting the proliferation of a specific T cell type (Li et al., 2009). Pree et al. have been advocates of the translational aspect of costimulation blockade. In a clinical setting, these protocols may facilitate mixed chimerism and prevent a GVH reaction that may otherwise ensue (Pree et al., 2009, Pan et al., 2003).

5.1 The B7/CD28 pathway and skin grafts

Preventing binding of B7 molecules on B cells to their ligands, CD28 on T cells has been shown to be effective for interrupting T cell activation. Shiao et al. (2007) used a humanized

antibody directed against the T cell CD28 molecule to prevent the expansion of alloreactive T cells. The experiments were done in beige/SCID mice bearing human skin grafts and infused with peripheral blood cells expressing different human transplantation antigens (HLA) than those of the skin donor. Liu et al. (2007) used a gene therapy model to infuse antisense B7.1 in order to suppress alloreactivity to rat spleen cells. This model was not associated with GVHD as measured by examination of intestines, liver, skin, or other tissues(Liu et al., 2007). Rulifson et al. (2002) demonstrated that Langerhans cells resident in the skin of the donor were such effective antigen presenting cells (APCs) that they could prime T cells for activation independently of either the B7 or CD40 activation pathways. In their hands, the strength of this direct antigen presentation reaction limited the success of tolerance induction mediated through the interruption of either B7-CD28 or CD40-CD154 pathway. These findings are consistent with the work of Tao et al. (1997) who concluded that weak T cell receptor (TCR) signaling primed $CD4^+$cells in a CD28/B7 dependent manner. Early experiments using the skin allograft model were important for demonstrating that additional costimulatory molecules must be important for completing the T cell activation cascade (Kawai et al., 1996). Their report demonstrated suppressed proliferation and cytokine production by CD28 depleted T cells, nonetheless, these cells were capable of initiating skin allograft rejection.

5.2 Interrupting CD40 and B7 ligation

The combined effect of blocking the CD28 and CTLA4 ligation reactions was tested in a murine skin graft model (Li et al., 2006). The combined treatment was delivered via a replication incompetent adenovirus vector. Significant skin graft survival was demonstrated in the treated mice in contrast to rapid rejection of skin grafts in mice that were untreated or given non-expressing vectors. A detailed study outlining the efficacy of CTLA4Ig produced by different sources as an efficient tool for interrupting costimulatory signals produced by the ligation of B7/CD28 molecules was completed (Najafian and Sayegh, 2000). CTLA4Ig's higher affinity for B7 competitively inhibits B7/CD28 ligation, therefore interfering with the delivery of costimulatory signals. Other investigators found that CTLA4 signaling facilitated allograft survival of skin and islet grafts in mice treated with an infusion of donor derived antigen plus ant-CD154 monoclonal antibody (mAb) (Zheng et al., 1999). Tung et al. (2008) showed the combined effect of blockading both the B7 and CD40 pathways in a series of limb graft experiments. Balb/c male mice were donors of limbs engrafted onto C57Bl/6 female mice heterotopically. Costimulation blockade was mediated by anti-CD154 antibody with or without CTLA4-Ig. Anti-CD154 alone resulted in limb survival of 75 days, but graft survival was extended to 120 days by the combined therapy. Larsen et al. (1996) demonstrated the advantage of blocking both of these costimulatory pathways in order to facilitate the engraftment of skin and cardiac tissue. Both in vivo and in vitro confirmation of the effect was discussed.

Protracted survival of murine skin allografts in thymectomized mice treated with an infusion of donor derived spleen cells and anti-CD154 mAb was achieved only in mice expressing $CD4^+$ T cells, gamma interferon, and CTLA4 (Markees et al., 1998). In contrast, the studies by Gordon et al. (1998) using the concordant rat-to-mouse xenotransplantation model, showed no requirement of $CD4^+$ T cells or for gamma interferon for prolonging the survival of rat skin and islets in mice. The role of B7/CD28 ligation in providing costimulatory signals to effect T cell activation was confirmed by the studies of Onodera et

al. (1997). They pre-sensitized Lewis rats with Brown-Norway skin grafts and evaluated the survival of cardiac grafts when recipients were treated with CTLA4Ig with or without an infusion of donor antigen. They hypothesized that graft survival was mediated by either clonal anergy or the deletion of alloreactive $CD8^+$ cells.

5.3 Interfering with CD40/CD154 ligation

Although the first study of the CD40/CD154 pathway to promote graft survival utilized a concordant rat-to-mouse islet xenograft model (Markees et al., 1996), the first use of this system to facilitate skin graft acceptance was reported by Larsen at al. (1996), and described aspects of the costimulatory process related to both CD28-B7 ligation and CD40-gp39 ligations. The "gp39" designation was later changed to CD154 in keeping with the system for naming cell surface molecules. In their hands, each set of costimulatory molecules provides necessary but not identical signals resulting in fully activated T lymphocytes. Interrupting both of these pathways provided conditions for prolonged survival of heart tissue and skin allografts.

Interrupting the reaction between CD40 and CD154 has been confirmed as an effective method for limiting T cell costimulation. This pathway is required for the generation of T cells that are fully activated against alloantigen (Yamada and Sayegh, 2002), and interruption of this pathway promotes graft survival or tolerance. Moodycliffe et al. showed that the binding of these molecules encouraged the migration of dendritic cells from the skin to the lymph nodes for effective antigen presentation (Moodycliffe et al., 2000). Their model assessed dendritic cell (DC) function in wild type C57Bl/6 mice or those devoid of CD154 molecules. A combined therapy consisting of a bolus infusion of donor derived spleen cells and a brief course of treatment with anti-CD154 mAb resulted in prolonged survival of rat skin grafts in mice. Graft survival was enhanced significantly in mice devoid of $CD4^+$ T cells (Gordon et al., 2001).

Nikolic et al. used a skin graft model to differentiate between models that blocked immunity to alloantigens vs. those that interrupted autoimmunity (Nikolic et al., 2010). They used normoglycemic non obese diabetic mice (NOD) that received whole body irradiation, T cell depletion, and anti-CD154 mAbs with or without bone marrow cells from C57Bl/6 mice. The treatment delayed or prevented hypoglycemia, but only prevented isletitis in mice that received bone marrow cells as well as the other conditioning treatments.

Xu et al. (2010) studied the role of minor histocompatibility antigens in bone marrow grafts. They found that when they used B10.BR skin to sensitize AKR mice to alloantigens, donor origin cells were cleared more rapidly than in unsensitized mice of the same strain. Recipient mice treated with antibodies to CD154 did not produce antibodies to minor histocompatibility antigens of the donor. These studies suggest that blockade of CD40-CD154 ligation may facilitate bone marrow transplantation and reduce the risk of reactivity to minor histocompatibility antigens.

Although blockade of CD40 and CD28 activation pathways did not produce the vasculopathy shown with other methods, translating this method into a clinical protocol was unexpectedly problematic because of the development of thromboembolisms (Yamada and Sayegh, 2002). Scientists have since analyzed the cause for the pathophysiological problems identified clinically. Their reports help to identify platelets as a source for soluble CD154 or CD40 ligand and demonstrate a correlation between increased circulating levels of this molecule and increased thrombus formation (Yacoub et

al., 2010, Yuan et al., 2010). These studies show further that CD154's concentration is increased in hypertensive patients included non-diabetic and pre-diabetic persons with metabolic syndrome (Unek et al., 2010).

In an effort to overcome these complications, Gilson et al. (2009) compared two different isotypes of anti-CD40 monoclonal antibodies. Isotypes are any one of the five main structurally and functionally distinct categories of antibodies (Janeway C.A. et al., 2001). Gilson et al. (2009) found that the IgG2b, but not the IgG1 antibody had a synergistic effect on graft survival when combined with CTLA-4-Ig, and hypothesized that the use of alternative antibody isotypes may encourage the development of new reagents that are as effective as anti-CD154 antibodies, but without harmful clinical effects.

6. The induction of regulatory T cells (Tregs)

In 1975, Gelfand and Paul (1975) used an anti-thy1.2 antibody to facilitate C57Bl/6 skin grafts on Balb/c mice. The antibody, which was directed against thymocytes, resulted in prolonged skin graft survival in comparison to untreated mice. The study demonstrated infectious tolerance as the effect could be transferred to new hosts given cells of the treated mice. The authors thus hypothesized that the treatment generated a population of suppressor cells that regulated the processes responsible for graft survival. In the same year, Gershon hypothesized the existence of suppressor T cells that down modulated immune responses and detailed predominant requirements for their functionality (Gershon, 1975). These concepts were controversial at the time that they were advanced.

During the 1980's, the concept began to take hold that there existed a specific population of regulatory T lymphocytes with the capacity to specifically suppress immune responses. Maki et al. showed that B6AF1 mice pretreated with ALS and given both BMC and skin grafts from CH3 donor mice had suppressed reactivity against donor antigen as demonstrated by in vitro assays (Maki et al., 1981a, Maki et al., 1981b). Streilein and Niederkorn (1985) described the induction of a suppressor T cell population that helps to explain the phenomenon of the anterior chamber of the eye functioning as an immunologically privileged site. The suppressor activity was associated with improved skin graft survival and other host responses. The suppressor cells were described as Thy1.2 and L3T4 expressing $CD4^+$ T lymphocytes.

Subsequent studies by Subba and Grogan (1986) using rat skin allografts implanted in the anterior eye chamber resulted in minimal alloreactivity as measured in vitro. Treatment with cyclophosphamide or removal of the allograft reversed the immunosuppression seen in mixed lymphocyte reactions (MLR), thus implicating the involvement of suppressor cells.

Based on a wealth of more recently generated data, the scientific community no longer needs to be convinced that a regulatory cell population exists or can be induced. Regulatory T cells have been identified or induced by some of the methods shown in Table 1 below. We now accept the description of the predominant category of regulatory cells responsible for allograft survival, the generation of chimerism and mixed chimerism, protection from autoimmunity and reduced risk of graft vs. host disease (GVHD) and host vs. graft disease (HVG) as being $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T lymphocytes. These Tregs may be naturally occurring in the thymus or adaptively induced in response to foreign antigen and costimulation blockade (Guo et al., 2008, Schwartz, 2005, Sakaguchi, 2005, Coenen et al.,

2005, von Boehmer, 2005). The Tregs express the forkhead transcription factor Foxp3 (Fontenot and Rudensky, 2005), and there is some evidence that the expression level of this transcription factor may be a key indicator of the level of immunosuppression potential (Chauhan et al., 2009) The immunosuppressive effect of these cells has been shown by in vivo and in vitro experiments.

Graft or Target	Authors	Method of Induction
Skin grafts Autoimmunity	(Oderup et al., 2006)	Il2Rbeta-/- recipients
Heart	(Oderup et al., 2006)	Rag 1 -/- given anti-154 and anti-B7
Chimerism	(Iudaev et al., 1975)	Sublethal WBI and BMC
Hy antigen and HVG	(Weng et al., 2007)	Sublethal WBI
Chimerism Skin allografts	(Yamazaki et al., 2007)	BMC, DST and anti-CD154
Skin graft	(Chai et al., 2005)	Foxp3 transduced expression in naïve $CD4^+$ $CD25^-$
Skin graft	(Banuelos et al., 2004)	DST and anti-CD154 in CD4, CD8, or CD25 depleted skin and islet graft recipients
Skin graft	(Sanchez-Fueyo et al., 2007)	Wt or Class II – mice
Skin graft Mixed chimerism	(Pilat et al., 2010)	Rapamyacin and costimulation blockade;
Skin graft	(Kim et al., 2011)	Anti-CD154, Non cytolytic anti-CD4
Composite tissue allograft	(Bozulic et al., 2011)	Anti-TCR, WBI

Legend: Table 1 describes some milestones in the history of regulatory T cell recognition. Abbreviations: WBI, whole body irradiation, BMC, bone marrow cells; HVG, host vs. graft reaction; Hy, male specific antigen; DST, donor specific antigen; wt, wild type; TCR, T cell Receptor

Table 1. The Induction of $CD4^+$ $CD25^+$ Regulatory T Cells

7. Other cells with regulatory functions

7.1 Double negative T cells

Double negative T cells, described as $CD3^+$ T lymphocytes bearing typical αβT cell receptors, but expressing no CD4, CD8, or NK1.1 surface molecules. These cells use the process of trogocytosis to gain access to the alloantigen, xenoantigen, or self peptides that are then expressed on their cells surfaces (Ford McIntyre et al., 2008). Trogocytosis is the process of utilizing broken pieces of plasma membrane as an antigen capture mechanism (Hudrisier et al., 2007). B and T cell receptors as well as some costimulatory co-receptors appear to be involved in this process.

7.2 $CD8^+$ T cells

$CD8^+$ T cells have been shown to promote skin allograft survival in fully mismatch donor-host combinations. Graft survival was initiated by rapamycin treatment of graft hosts (El Essawy et al., 2011). El Essawy and colleagues found that C57Bl/6 mice devoid of CD4 molecules handily rejected DBA mouse skin allografts, but showed prolonged (median survival, >100 days) survival of these grafts following rapamycin treatment of the grafts hosts. These regulatory/suppressor T cells were $CD28^+$ but showed low levels of IFN-γ, IL-2, and IL-10.

7.3 Natural Killer T (NKT) cells

Natural Killer T (NKT) cells are lymphocytes bearing TCR T cell receptors. A regulatory role for these cells was described in mice devoid of the Jα18 segment of the TCR and in mice lacking the CD1 restricting element associated with NKT cells following costimulation blockade mediated by a transfusion of donor antigen and a short course of anti-CD154 mAb (Gordon and Kelkar, 2009). Graft survival was abbreviated in the Jα18 and in the CD1 deficient mice as compared to wild type mice of the same strain (Gordon and Kelkar, 2009).

7.4 Induction of tolerogenic dendritic cells

Adorini and Penna (2009) report on the use of vitamin D receptor (VDR) agonist as a means of inducing tolerogenic dendritic cells (DCs). These DC are associated with an immunosuppressive effect. The use of secosteroid hormones that function as VDR agonist help to induce DCs that then facilitate the induction of CD4(+) CD25(+) Foxp3(+) regulatory T cells.

8. The humanized mouse as a model for the study of the human immune system

The humanized mouse model takes advantage of the existence of basic cellular deficiencies in non-obese diabetic (NOD) and CB-17 *scid/scid* strains of mice. Some strains are crosses between the two and bear the deficiencies of each parental type. The severe combined immunodeficient substrain lacking a functional IL-2 receptor gamma gene has become a more advanced mouse model. These mice may be infused with human hematopoietic stem cells that reconstitute a functional human immune system in the murine environment. These genetically altered mice provide important tools for studying

autoimmunity, cellular and molecular interactions of the human immune system, solid tumors and other forms of cancer, and may provide models for studying human retroviruses or other infections where no appropriate animal model exists. Many of these strains have been developed by Lenny Schultz and others at Jackson Laboratories (Bar Harbor, ME). Some phenotypic characteristics of the most recent strain include the lack of T cells, B cells, and NK cells. There is no IL-2R γ chain, both the innate and adaptive compartments of the immune system are defective, and most antibody responses are very low. Salient features of these mice including timelines for their development have been reviewed recently (Van Duyne et al., 2009, Ishikawa et al., 2008, Hill et al., 1991). In the section below, we review the use of this important model for investigation in several areas of biomedical research.

8.1 Humanized mouse system for the study of skin grafts and wound healing

Racki et al. (2010) compared usage of NOD-scid IL2rgamma(null) mouse (NSG) vs. the CB17-scid bg (SCID.bg) mouse to study skin allograft survival vs. rejection. They found that components of the human immune successfully engrafted into the NSG mouse, but that skin graft survival was poor. In contrast, the SCID.bg mouse was less successful in engrafting human cells, but showed improved skin graft survival over the NSG model. Their results showed that infiltration of transplanted human skin by glucocorticoid receptor isoform 1 (GR-1$^+$) cells impaired graft survival. GR-1$^+$ cells are immature myeloid cells that may differentiate into dendritic cells, have been identified in human bone marrow recipient, and are characterized as having an immunosuppressive effect (Li et al., 2004). When Racki's team treated NSG mice with antibodies to GR1, the cellular infiltrate decreased and skin graft survival was improved. Additional studies that impact skin graft survival and wound healing used an alternative model, the nude mouse (Escamez et al., 2004). This mouse lacks a thymus, and therefore has no mature T lymphocytes. They found that treating laboratory engineered "skin equivalent" with keratinocyte growth factor improved wound healing.

Gilet et al. (2009) used SCID mice reconstituted with human PBLs and engrafted with human skin to study T cell subset recruitment mediated by the cytokine CCL17. Erdage and Morgan (2004) used the same animal model (huPBMC-SCID) to study the differences in allo- and xenoreacivity to bioengineered skin modified with or without keratinocyte growth factor. The team used the same model to study the survival of human fetal vs. neonatal skin grafts. They found that the lower MHC Class I and Class II expression in fetal skin was associated with longer graft survival than that of neonatal skin (Erdag and Morgan, 2002). Issa et al. (2010) separated regulatory T cells from human PBLs and used them to control immune responses to skin allografts in a humanized mouse model.

8.2 The humanized mouse system and autoimmunity

The humanized mouse model has become an important research tool for the study of type 1 diabetes and psoriasis. Pearson et al. (Pearson et al., 2008) described the NOD-Rag1null Prf1null Ins2Akita spontaneously hyperglycemic mouse model. This model has no autoimmune defect, but may be used to study type 1 diabetes in an environment that avoids any toxicity associated with chemically induced diabetes models. King et al. (2008) describe the engraftment of peripheral blood mononuclear cells (PBMC) vs. human stem cells. They

induced diabetes and showed that an infusion of allo-PBMCs leads to islet graft rejection, thus they proposed the use of this model to study mechanisms of human islet allograft rejection. Other aspects involving the use of these animal models to study diabetes have been reviewed (King et al., 2008, Shultz et al., 2007).

Humanized mouse models of psoriasis have been developed that use bioengineered skin derived from biopsies and from patients with psoriasis (Guerrero-Aspizua et al., 2010). A psoriasis pathology was duplicated by injecting cultivated T cell populations along with IL-17and IL-22, and depriving the skin of its stratum corneum. This model will be used to assess the pathophysiology of the disease and to suggest alternative therapeutic targets, and to test new hypothesis related to patient outcomes in pre-clinical investigations.

Bhagavathula et al. (2005) used the severe combined immunodeficient (SCID) mouse to demonstrate the effectiveness of humanized neutralizing mAbs directed against transduced amphiregulin as a therapeutic agent reversing some phenotypic characteristics of psoriasis in transplanted skin. The mAb decreased epidermal thickness in vivo and reduced keratinocyte growth in vitro.

Because TNFα antagonists have been used to counter the effects of psoriasis, Gordon et al. (2005) used a humanized mAb directed against TNFα to dissect the mechanism of action of these reagents. They injected the reagent into otherwise unaffected skin engrafted onto the SCID-HU mouse model and they injected highly activated T cells in order to simulate an acute psoriasis phenotype. The concentration of epidermal Langerhans cells (LCs) in the plaques began to increase within one week of the mAb treatment, and thus the loss of LCs was associated with plaque development. These studies help to clarify the method of action of the TNFα antagonist and suggest a method for reversing the course of this disease in patients.

8.3 The humanized mouse system and immunity: Graft vs. Host Disease (GVHD)

This model has been used to study GVHD. Soluble FasL was used to treat graft recipients in order to prevent alloreactivity of immunocompetent donor T cells that recognize the host antigens (Bohana-Kashtan et al., 2009). Pinot et al. (2010) describe the development of the NSG mouse model and its use in studying xeno-GVHD. Vlad et al. (2009) used this mode to demonstrate the prevention of GVHD through the use of an immunoglobulin component, the immunoglobulin-like transcript 3-Fc protein, that induces $CD8^+$ suppressor cells.

8.4 The humanized mouse system as a source for induced pluripotent stem cells

Hanna et al. (2007) used a humanized mouse model of sickle cell anemia to describe the benefits of using induced pluripotent stem cells (IPS) derive from adult fibroblast cells. This "knock-in" model replaced mouse globin genes with mutated human Aγ and β^S globin genes. The homozygous mutant developed many phenotypic characteristics of the sickle cell disease. Tail snips from these mice were used as a source of fibroblast cultures. Retroviral vectors were used to transduce expression of the four transcription factors needed to reprogram the adult mouse cells or to express selectable markers. The investigators used homologous recombination to repair the genetic defect responsible for sickle cell disease. The investigators anticipate future studies that eliminate the use of retroviral vectors and oncogenes in applications of IPS for human use.

9. Conclusions

The modern world of skin grafting, arising from its historical roots to new depths of scientific understanding, will help to advance translational science and medicine. Skin grafts allow us to understand specific immunological functions, help dissect the mechanisms responsible for tissue and organ specific tolerance, identify additional regulatory cells or functions required for transplantation tolerance, test the development of mouse models of the human immune system and help answer fundamental questions related to the use of stem cells as curative agents for human disease.

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Skin Grafts - Indications, Applications and Current Research
Edited by Dr. Marcia Spear

ISBN 978-953-307-509-9
Hard cover, 368 pages
Publisher InTech
Published online 29, August, 2011
Published in print edition August, 2011

The procedure of skin grafting has been performed since 3000BC and with the aid of modern technology has evolved through the years. While the development of new techniques and devices has significantly improved the functional as well as the aesthetic results from skin grafting, the fundamentals of skin grafting have remained the same, a healthy vascular granulating wound bed free of infection. Adherence to the recipient bed is the most important factor in skin graft survival and research continues introducing new techniques that promote this process. Biological and synthetic skin substitutes have also provided better treatment options as well as HLA tissue typing and the use of growth factors. Even today, skin grafts remain the most common and least invasive procedure for the closure of soft tissue defects but the quest for perfection continues.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Ethel J. Gordon (2011). Immunological Considerations for Inducing Skin Graft Tolerance, Skin Grafts - Indications, Applications and Current Research, Dr. Marcia Spear (Ed.), ISBN: 978-953-307-509-9, InTech, Available from: http://www.intechopen.com/books/skin-grafts-indications-applications-and-current-research/immunological-considerations-for-inducing-skin-graft-tolerance

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