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Research Article

Immuno-modulatory Role of Indoleamine 2, 3-Dioxygenase in Allogeneic Islet and Skin Transplantation

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Abstract

Indoleamine 2, 3-dioxygenase (IDO) is a naturally occurring immunomodulatory agent that has been found to play a key role in preventing rejection of the semi-allogeneic fetus during pregnancy. It is a cytosolic monomeric hemoprotein enzyme that degrades tryptophan, the least available essential amino acid in the body, through the kynurenine metabolic pathway. This causes a regulatory effect on T cells by, as it has been proposed, causing toxic tryptophan metabolites to accumulate and a tryptophan deficient microenvironment to form. By helping with the induction of T cell unresponsiveness, IDO has been shown to establish immune tolerance and control autoreactive immune responses. Thus, the application of IDO's ability to do this may have a huge potential in improving the rates of acceptance of insulin producing islet cell or skin graft transplants in patients suffering from conditions such as type I diabetes or burn injuries, respectively. The discovery of an agent able to do this is especially important since currently used systemic immunosuppressive drugs carry many side effects and cause complications that make the long term use of these agents problematic after transplantation. This review discusses the immunomodulatory role of IDO and the very promising results of studies done by our research group on the application of this enzyme in islet and skin transplantation.

Keywords: Indoleamine 2, 3-dioxygenase, skin transplantation, islet transplantation.

Introduction

There have been many problems and side effects associated with the use of systemic immunosuppressors, such as the increased incidence of malignancy and susceptibility to opportunistic infections (Li *et al* 2004), which make the long-term use of these drugs problematic, especially after transplantation.

Efforts have been undertaken to study potential new therapies involving locally expressed immunomodulatory or graft-protecting molecules that can aid in the acceptance of allografts, such as allogeneic islet and skin transplants, without using these long-term non-specific immunosuppressive drugs. Several local immunosuppressive factors, including

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interleukin-10 (IL-10) (Brauner *et al* 1997), transforming growth factor β (TGF- β) (Raju *et al* 1994), and Fas ligand (FasL) (Li *et al* 1998) have been studied for this aim. However, despite advances made in the past decade, these molecules have low therapeutic efficiency and thus a more specific and potent local immunosuppressive molecule to protect allogeneic engraftment is needed (Li *et al* 2004). Our research group's recent studies of using an enzyme, indoleamine 2, 3-dioxygenase (IDO), as a potential new anti-inflammatory therapy for engraftment of allogeneic islets and skin transplantation has, however, shown very promising results. IDO catalyzes the first step in the breakdown of the least available essential amino acid in the human body, tryptophan, through the kynurenine metabolic pathway (Sugimoto *et al* 2006). It is associated with a natural immunoregulatory mechanism in the body allowing for immunotolerance to the semi-allogeneic fetus during the course of pregnancy (Munn *et al* 1998). As such the potential use of this enzyme as an immunomodulatory factor is likely to minimize the need for currently used immunosuppressive treatments and improve the outcomes of transplantation. Additionally it may reduce the need to wean transplant recipients of their drugs, a complication that often results from long-term use of systemic immunosuppressors. (Lob and Konigsrainer 2009).

In this review, a brief background of IDO including its structure, expression, and regulation, role in tryptophan metabolism, history, and immunosuppressive potential will be given. Following this, possible applications of the immunosuppressive potential of IDO specifically in allogeneic islet and skin transplantation will be reviewed.

Background of Indoleamine 2, 3-Dioxygenase

Structure

IDO is widely expressed in mammalian cells when induced by pro-inflammatory cytokines. The mature IDO protein is a monomeric enzyme with a molecular weight of 42-45 kDa (Jalili *et al* 2007). It is a cytosolic enzyme and primarily inducible. IDO is ubiquitously expressed in extra hepatic cells and contains heme as its sole prosthetic group (Jalili *et al* 2007). Ferric iron ($Fe3+$) is bound to the enzyme when it is in its inactive form and tryptophan metabolism can only proceed if redox active compounds such as superoxide are present to generate the active $Fe2+$ form. Large amounts of these redox active compounds are produced by activated leukocytes, which suggests that this mechanism is limited to sites of infection or inflammation (Hirata and Haiyaishi 1975, Haiyaishi 1996, Thomas and Stocker 1999).

The tertiary structure of recombinant human IDO has been defined using X-ray crystallography and shows that the hemoprotein is folded into two distinct alpha helical domains: one small and one large (Sugimoto *et al* 2006). The heme prosthetic group is positioned in between the two (Sugimoto *et al* 2006).

Role in Tryptophan Metabolism

IDO catabolizes compounds containing indole rings (Johnson *et al* 2009). This includes tryptophan, the least available essential amino acid in the body. IDO catalyzes the first and rate-limiting step of oxidative tryptophan metabolism (Sugimoto *et al* 1006) as seen in Figure 1. Tryptophan is required by all forms of life for protein synthesis and other important functions (Jalili *et al* 2007) and serves as a precursor for the production of several very important molecules such as serotonin and melatonin (Lob and Konigsrainer 2009). The tryptophan metabolism pathway is not active constitutively in all cells under homeostatic conditions (Johnson *et al* 2009).

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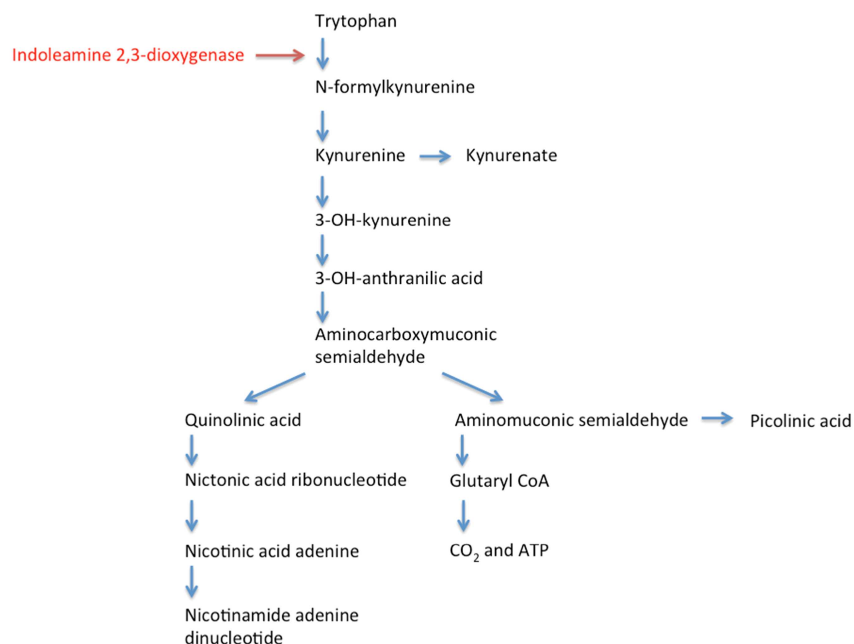

Figure 1: Tryptophan Catabolism through the Kynurenine Metabolic Pathway. Kynurenine, Quinolinic Acid, and Picolinic Acid are Directly Toxic to T Cells.

IDO hastens the oxidative cleavage of the pyrrole ring of L-tryptophan to which it has a high affinity (Km of approximately 0.02 mM) (Jalili *et al* 2007) to produce *N*-formylkyureneine. In this way IDO can quickly deplete its local tissue microenvironment of tryptophan. The *N*-formylkynurenine then rapidly degrades to formic acid and kynurenine, a stable end product. The activity of IDO can be measured by assessing the levels of kynurenine in serum or tissues using high performance liquid chromatography (HPLC) analysis (Johnson *et al* 2009). The kynurenine metabolic pathway eventually leads to the synthesis of nicotinamide adenine dinucleotide (NAD). Tryptophan 2, 3-dioxygenase, a constitutively expressed hepatic enzyme, also catabolizes the same step as IDO (Muller and Prendergast 2007). IDO, however, is the main enzyme involved in the kynurenine pathway, metabolizing 99% of the dietary tryptophan not used in protein synthesis within inflammatory conditions (Grohmann *et al* 2003).

Elevated levels of IDO and downstream metabolites such as *N*-formylkynurenine are usually what occur when the tryptophan metabolism pathway is deregulated. *N*-formylkynurenine is readily hydrolyzed to kynurenine and enzymatically converted to many different metabolites. One of these metabolites includes quinolinic acid, an excitotoxin whose production has been linked to the pathogenesis of neuroinflammatory, neurodegenerative disorders such as Alzheimer's and depression, age-related cataract, and HIV encephalitis (Heyes *et al* 1992, Guillemin *et al* 2005, Wichers and Maes 2004, Vazquez *et al* 2004, and Sardar and Reynolds 1995).

Expression and Regulation

IDO is primarily an inducible intracellular protein and is expressed in different tissues. IDO is most prominently expressed by some subsets of antigen presenting cells (APCs) of lymphoid organs and the placenta (Jalili *et al* 2007). However, it is also expressed in

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eosinophils (Odemuyiwa *et al* 2004), endothelial cells (Varga *et al* 1996 and Beutelspacher *et al* 2006), and lung epithelial cells (Hayashi *et al* 2004) suggesting that it may play an important role in allergic inflammation. IDO is only constitutively expressed in the male epididymis and lower gastrointestinal tract (Yoshida *et al* 1981). The actual presence or absence of functional IDO enzymatic activity is tightly regulated by specific maturation and activation signals. Conceptually this ability to up regulate or down regulate IDO in response to external stimuli seems logical, given the need for APCs to sometimes present antigens in an activating fashion and sometimes in a tolerating fashion, depending on the context (Munn and Mellor 2007).

A single gene located on the short arm of chromosome 8 (8p12-8p11) encodes the IDO protein (Burkin *et al* 1993). 10 exons are spread over 1.5 kbp of DNA (Burkin *et al* 1993). In cell types such as dendritic cells (DCs), macrophages, eosinophils, epithelial and endothelial cells, the IDO gene promoter is driven by a combination of locally produced potent pro-inflammatory stimuli. It is most strongly activated by the T helper 1 (Th-1) type cytokine interferon gamma (IFN-γ) (Jalili *et al* 2007). In addition to IFNs, CD40 ligand (CD40) up regulation on the T lymphocytes is also important for IDO expression (Munn *et al* 1999 and Hwu *et al* 2000).

Cells usually do not express IDO unless they are induced to do so by treating the cells with IFN- γ, which is produced in response to inflammation. All mammalian IDO genes studied so far have IFN-γ response elements including one interferon-gamma activated sequence (IGAS) and two interferon stimulated response elements (ISREs) (Jalili *et al* 2007 and Johnson *et al* 2009). IGAS is specific for IFN-γ, whereas the ISREs are nonspecific and can respond to IFN-α, IFN-β, and IFN-γ. IFN-γ is usually up to 100 times more potent at inducing IDO expression than either IFN-α or IFN-β but this depends on the cell type being cultured (Taylor and Feng 1991). Following ligation of IDO inducers, intracellular signaling occurs along the JAK-STAT pathway and nuclear factor kB (NF-kB) (Mellor and Munn 2004 and Thomas *et al* 1999) to result in the expression of the IDO protein (Tone *et al* 1990). After the IDO protein has been encoded, further signals are needed for the IDO protein to be activated.

IDO gene transcription can also occur in response to cytokines such as toll-like receptor (TLR) ligation (e.g. through lipopolysaccharide) (Mahanonda *et al* 2007 and Furset *et al* 2008), tumour necrosis factor α (TNF-α) (Werner-Felmayer *et al* 1989), glucocorticoid-induced TNF receptor (GItR) ligand (Grohmann *et al* 2007), and histone deacetylase inhibitors (HDACS) (Reddy *et al* 2008). T helper 2 (Th-2) type cytokines such as interleukin 4 (IL-4) and interleukin 13 (IL-13) inhibit the expression of IDO (Chaves *et al* 2001 and Musso *et al* 1994).

Recently another protein involved in tryptophan metabolism called IDO2 was also discovered (Ball *et al* 2007). The IDO2 gene is also located on chromosome 8 and its amino acid sequence is 43% similar to that of IDO (which is now also known as IDO1) (Ball *et al* 2007). However, it only shows 3-5% of the enzymatic activity of IDO when expressed as a transgene in bacteria or eukaryotic cells (Ball *et al* 2007).

IDO and Immunosuppression

The immune system continuously modulates the balance between responsiveness to pathogens and tolerance to non-harmful antigens, although the exact mechanisms are not understood. IDO has been suggested in taking part in the counter-regulatory mechanisms that prevent the immune system from causing inappropriate or excessive responses, which could potentially lead to damaging effects (Kyewski and Klein 2006). Munn *et al* (1998) were the first to report the potential role of IDO in immunosuppression.

It was seen that in mammals during pregnancy, the expression of IDO at the interface between the fetus and the mother prevents abortion of the allogeneic fetus. IDO prevents maternal T cells from causing potentially harmful responses to paternally inherited fetal alloantigens by inhibiting the proliferation of these T cells in the placenta (Munn *et al* 1998). Munn *et* al (1998) also showed that the administration of 1-methyl tryptophan (1-MT), a commonly used competitive inhibitor of IDO, led to fetal rejection. 1-MT causes a reversal of the IDO induced suppression of T cells. IDO has also been shown to play an important role in setting up immunologically privileged parts of the body, such as the anterior chamber of the eye (Malina and Martin 1993) and the brain (Sardar and Reynolds 1995 and Hansen *et al* 2000).

Previous studies showed that IDO plays an important role in the induction of T cell unresponsiveness, thereby establishing immune tolerance and controlling autoreactive immune responses. The regulatory effect of IDO on T cells is due to the expression of this enzyme by DCs, monocytes, and macrophages. An inverse correlation has been found between IDO expression and the number of CD8 $^{+}$ (Brandacher *et al* 2006 and Inaba *et al* 2009) and CD3^{+} (Ino *et al* 2008) T lymphocytes. In particular, DCs, which are specialized to acquire, process, and present antigens to stimulate naïve T cells, can cause potent and dominant T cell and natural killer (NK) cell suppression (Johnson *et al* 2009). Exactly how IDO causes immunosuppression is unclear, but it has been proposed that IDO causes toxic tryptophan metabolites to accumulate and a tryptophan deficient microenvironment to form (Jalili *et al* 2007). The tryptophan metabolites, in particular kynurenine, quinolinic acid, and picolinic acid as shown in Figure 1, are directly toxic to T cells and NK cells (Soliman *et al* 2010). Depletion of the essential amino acid tryptophan causes immunosuppression by making the T lymphocytes more prone to cell cycle arrest at the G1 phase (Munn *et al* 1999, Frumento *et al* 2009 and Terness *et al* 2002), anergy (Munn *et al* 2004), and apoptosis (Fallarino *et al* 2002). Tryptophan starvation also causes naïve CD4+ T lymphocyte conversion to immunosuppressive regulatory T cells (Tregs) (Fallarino *et al* 2006) and activation of mature CD4+ CD25+ Foxp3+ Tregs (Sharma *et al* 2007). An increase in the number of Tregs causes an antigen-specific impairment of T-cell priming (Jalili *et al* 2010). The IDO overexpression also induces a Th2 immune response shift and generates an anti-inflammatory cytokine profile (Jalili *et al* 2010). IDO has also been known to be an innate defense mechanism. By depleting the local microenvironment of tryptophan, it has been able to help limit the growth of viruses, bacteria, and intracellular pathogens (de Jong *et al* 2012).

Our research group has been interested in the immune-protective role of IDO for allogeneic engraftment of a skin substitute. Li *et al* also showed that immune rejection of an allogeneic skin substitute could be prevented by using IDO expressing fibroblasts used as the cellular component of this skin substitute (Li *et al* 2004). It was then shown that the expression of this enzyme suppresses the major histocompatibility (MHC) class I antigens due to depletion of tryptophan (Li *et al* 2004). When tryptophan or an IDO inhibitor was added, MHC class I levels were restored (Li *et al* 2004). MHC class I antigens serve as targets for allogeneic immune rejection and as such, down regulation of these antigens seems to be a part of the mechanism underlying IDO mediated local immunosuppressive effects (Li *et al* 2004).

The immune privilege created by locally expressed IDO may not always be beneficial to the host and can lead to pathological effects, similar to those resulting from the use of other long-term immunosuppressant agents. Suppression of T cells can cause chronic infections to escape attack even if T cell antigens are present (Johnson *et al* 2009). At the sites of chronic inflammation, a potent and dominant T cell suppression has been studied (Johnson *et al* 2009). The same

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problem can result with developing malignancies. Cancer cells expressing IDO can deplete their local microenvironment of tryptophan, preventing T cell proliferation (Mellor and Munn 1999), and cause toxic metabolites to accumulate, further inhibiting T cell activation (Mellor *et al* 2002). As in DCs, monocytes, and macrophages, an inverse correlation has been found in many tumour types between IDO expression and the number of tumour infiltrating CD8+ T cells (Brandacher *et al* 2006, Inaba *et al* 2009, Ino *et al* 2008, and Uyttenhove *et al* 2003). Overexpression of IDO by malignant cells is an important cancer induced immune escape mechanism and has been associated with poor prognoses for survival (Brandacher *et al* 2006, Inaba *et al* 2009, Ino *et al* 2008, Risenberg *et al* 2007, Inaba *et al* 2010, and Pan *et al* 2008). This finding, however, has increased the potential of using IDO inhibitors, such as 1-MT, in cancer treatments. Studies in mice have shown that the combination of 1-MT with chemotherapeutic agents is more effective against tumours than the use of both therapies on their own (Hou *et al* 2007 and Qian *et al* 2009). It should be noted though that is has been shown in certain cancers, such as ovarian cancer, only the levo-1-MT (L-1MT) isomer is effective in blocking IDO expression (Qian *et al* 2009). The dextro-1-MT (D-1MT) isomer is inefficient (Qian *et al* 2009). IDO Increased expression of IDO has also been associated with inflammatory bowel disease (IBD) (Ciorba 2013).

In general, depending on the application of IDO, the expression and inhibition of IDO might be helpful in generating local immune privilege for allogeneic engraftment or disabling the cancer cells to protect themselves against the host immune system. Our research group has used the expression of IDO in skin cells to immuno protect either allogeneic skin cells or insulin producing islets. Using this approach, a tryptophan deficient environment has been generated within which the activated immune cells, mainly CD8+ T cells, cannot survive and attack the allogeneic cells.

Role of IDO Expression in Allogeneic Islet Transplantation

IDO expression has great potential in helping patients suffering from autoimmune disorders such as type I diabetes. Since the discovery of insulin, daily insulin injections have been the only way of treating patients with type I diabetes. Islet transplantation has been thought to be a feasible and attractive alternative therapeutic approach (Ricordi *et al* 2008 and Fiorina and Secchi 2007). However, patients need to receive a systemic immunosuppressive drug for their entire lifetime, which is problematic due to its side effects, and some immunosuppressive drugs are also prodiabetic (Froud *et al* 2006, Zahr *et al* 2007, Drachenberg *et al* 1999, and Vantyghem *et al* 2007). Our research group conducted a series of novel studies showing the potential use of three-dimensional islet grafts equipped with IDO-expressing dermal fibroblasts to prevent islet allograft rejection without systemic immunosuppressive agents. We showed that IDO genetically modified islets transplanted into mouse kidney capsules survive longer than those islets transplanted without IDO expressing fibroblasts (Jalili *et al* 2010). This prolongation was due to the expression of IDO preventing T cell proliferation and infiltration into transplanted allogeneic islets (Jalili *et al* 2010).

Later studies by our research group (Jalili *et al* 2010) investigated the potential use of composite 3-dimensional islet grafts. These grafts were engineered by embedding allogeneic mouse islets and adenoviral transduced IDO-expressing syngeneic bystander fibroblasts within collagen gel matrices (Jalili *et al* 2010). As shown in Figure 2 (Jalili *et al* 2010), T cells accumulate at the margins of IDO-expressing grafts but are not able to actually invade the islets.

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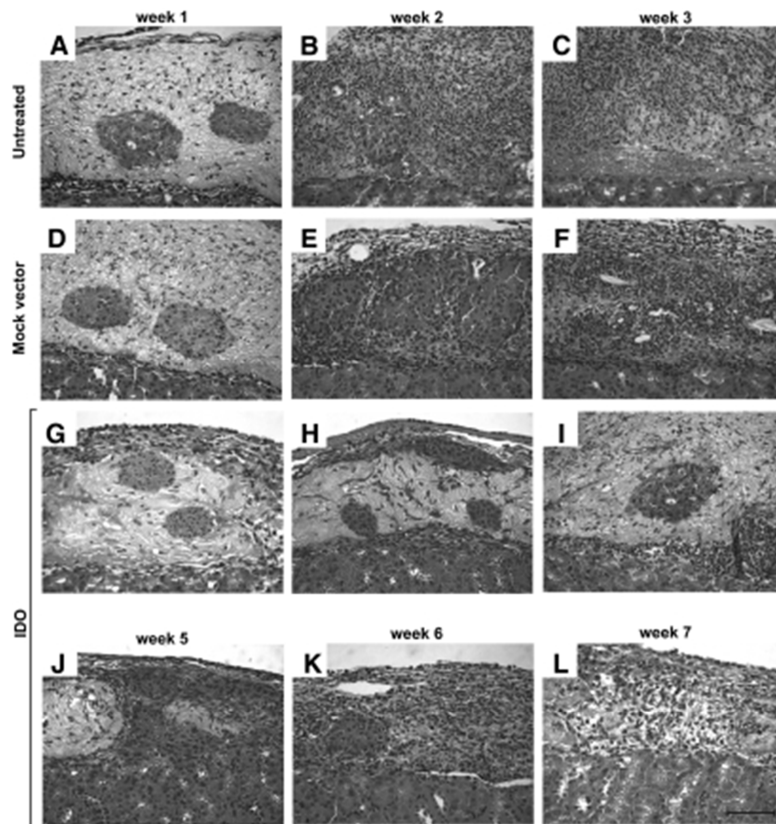

Figure 2: Histology of Composite Islet Grafts. Graft-Recipient Mice were Killed at Indicated Time Points Post Transplantation. Composite Islet Grafts were then Retrieved and Stained with Hematoxylin and Eosin. Untreated (A-C) and Mock Vector Infected (D-F) Fibroblast Grafts after 1, 2, and 3 Weeks Post Transplantation, Respectively. G-L: IDO-Expressing Fibroblast Grafts after 1-3 and 5-7 Weeks Post Transplantation. Note that Inflammation and Cellular Infiltration into the Graft Started in Control Groups in the Second Week but, in the IDO Group, Delayed until the Sixth Week Post Transplantation. Scale Bar: 100 µm.

The IDO expression also protects the islets through reducing the level of donor specific alloantibodies (Jalili *et al* 2010). It does not negatively affect islet function as shown in results from Jalili *et al* (2010) in Figure 3.

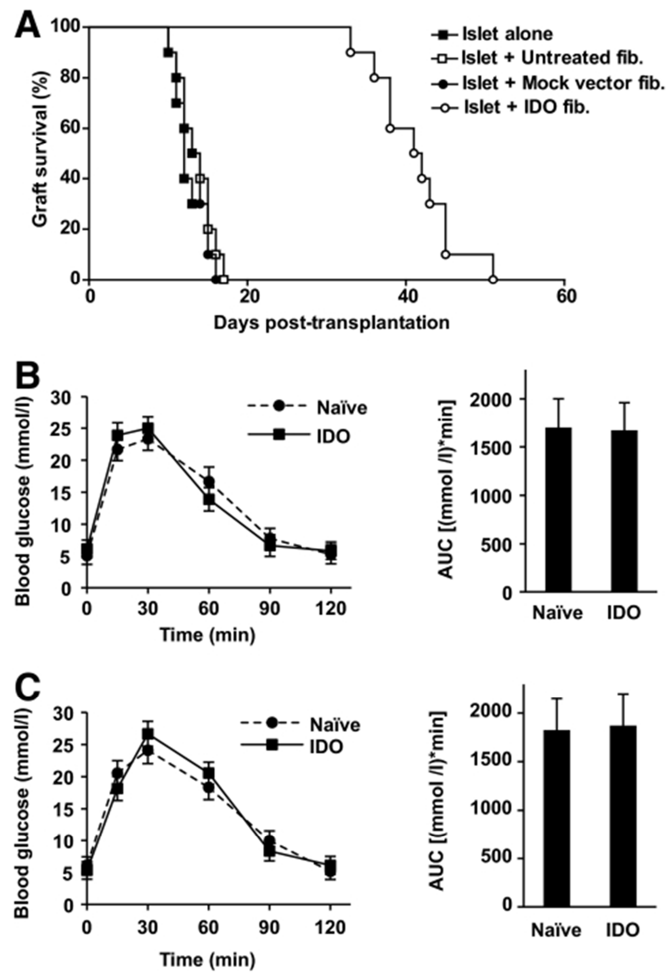

Figure 3: Islet Graft Survival and Function after Transplantation. A: Kaplan-Meier Survival Curve Shows Prolongation of IDO-Expressing Grafts Survival (Solid Line) Compared with Islet Alone (Dash-Dot Line), Untreated (Dashed Line), and Mock Virus-Infected (Dotted Line) Grafts (n=10). Intraperitoneal Glucose Tolerance Test (IPGTT) after 2 Weeks (B) and 4 Weeks (C) Post Transplantation Confirmed Normal Glucose Responsiveness in Graft-Bearing Mice (Solid Line) vs. Naïve Mice (Dashed Line) (n=3). Bar Charts on the Right Panels Show Area under the IPGTT Curves. Error Bars Indicate SEM.

Role of IDO Expression in Engraftment of an Allogeneic Skin Substitute

In cases of burn injuries where skin transplantations is required, engineered epidermal substitutes using patients keratinocytes have been developed and used as an adjunctive therapy for permanent skin replacement (Gallico *et al* 1984 and Green *et al* 1993). Though desirable, it is unlikely to have an autologous engraftment for patients who suffer from extensive skin loss from a variety of conditions including large and severe thermal injury. The autologous culture method also needs several weeks for cell growth and graftable epidermal substitute production. To overcome these problems, cultured keratinocyte allografts

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from unrelated donors have been suggested as functional skin replacements for large burn injuries as well as diabetic, elderly, and immunocompromised patients who suffer from non-healing complications. Despite the absence of leukocytes and Langerhans cells, the findings showed that cultured epidermal allografts are still rejectable. These epidermal substitute allografts are more immunogenic than previously thought (Rouabhia *et al* 1993, Aubock *et al* 1988, and Phillips 1991) and therefore exploring an allogeneic, nonrejectable, and readily available skin substitute may provide a better means of improving wound coverage. Our research group hypothesized that IDO expression should overcome these problems and has worked to develop and apply a novel non-rejectable skin substitute to function not only as wound coverage but also as a rich source of wound-healing promoting factors.

In a series of studies, we have demonstrated that overexpression of IDO by fibroblasts suppresses immune responses against allogeneic engrafted skin substitutes prepared from both the collagen populated fibroblasts and epidermal layer of keratinocytes (Chavez-Munoz *et al* 2012). Further we have shown that IDO expression down regulates MHC class I antigens in transfected keratinocytes which can promote tolerance of the skin substitute (Li *et al* 2004). Apoptotic rates of different immune cells including CD4+ and CD8+ are also shown to increase significantly (Forouzandeh *et al* 2008a) while primary dermal fibroblasts and keratinocytes remain resistant to these apoptotic effects of IDO (Forouzandeh *et al* 2008a, Forouzandeh *et al* 2008b, and Ghahary *et al* 2004). When IFN-γ treated fibroblasts were used to induce IDO expression, similar results were found. Immune cells undergo higher rates of apoptosis while primary skin cells do not undergo these changes (Forouzandeh *et al* 2008a).These results are summarized in Figure 4 (Bahar *et al* 2012).

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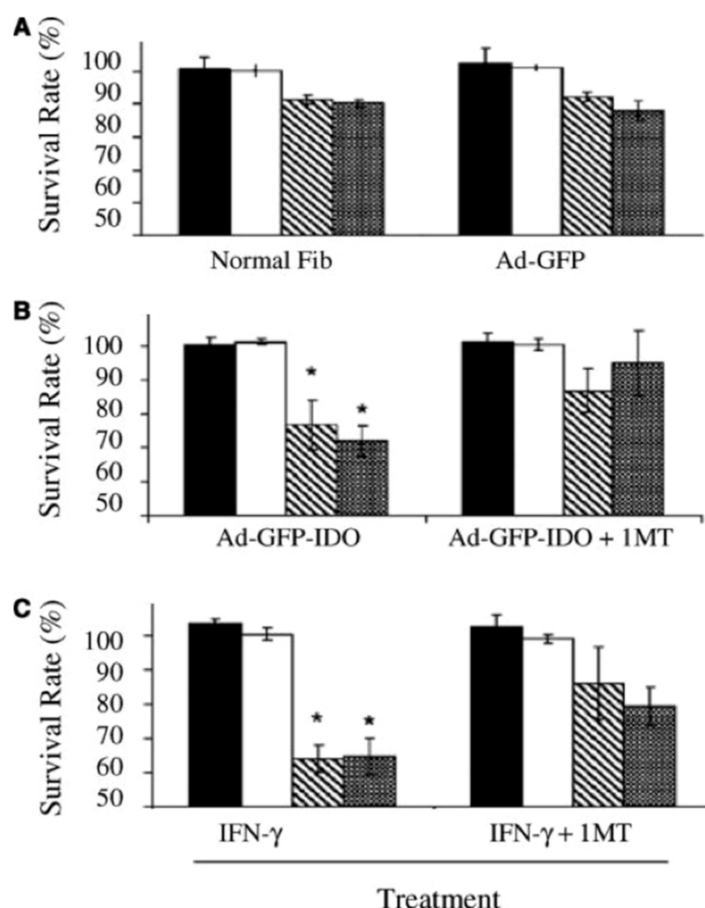

Figure 4: Effect of IDO Expression on Survival Rate of Human Skin and T Cells. Fibroblasts were Either Left Untreated or Treated with Various Adenovirus (MOI: 100) or Interferon- γ (100 U/ml) for 48 Hours. Cells were then Washed and Cocultured with the Indicated Cell Type in Two-Chamber Coculture System for 4 Days, in the Absence or in the Presence of the IDO Inhibitor 1-Methyl-Tryptophan (800μM). Cell Viability was then Analyzed by Fluorescent-Activated Cell Sorting (FACS) Using 7-AAD (Panels A-C). Bar Charts Corresponding to the Survival Rate of Each Bystander Cell Type in Each Treatment Group was then Compared with that of Non-Cocultured Cells that Constitutes 100% Survival rate for Each Cell Type. The Results of this Comparison are Shown for Fibroblasts (Solid Bars), Keratinocytes (Open Bars), Isolated Purified CD4+ T Cells (Hatched Bars), and CD8+ T Cells (Checked Board Bars). The Significant (P<0.001) Differences have been Indicated by Asterisks for Three Independent Experiments.

We further showed that IDO-expressing fibroblasts embedded within a collagen gel accelerates wound healing by promoting neovascularization in an animal model (Li *et al* 2006). As shown in Figure 5, wounds that received IDO-expression skin substitutes healed significantly faster than those of controls (Bahar *et al* 2012).

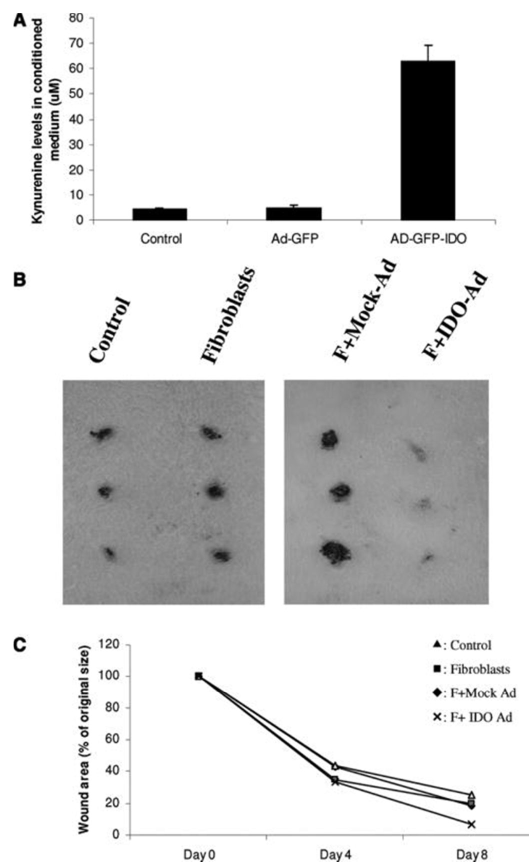

Figure 5: Grafting IDO Genetically Modified Human Fibroblasts Populated Collagen Gels Improves Wound Healing in Rats. A, Kynurenine Levels in Conditioned Media from Non-Infected Fibroblasts (Control) and Ad-GFP or Ad-GFP-IDO-Infected Cells were Measured and Compared. To Achieve this, the Free Viral Particles were Removed by Washing the Cells with PBS after 30 Hours, and Fresh Medium was Added. Conditioned Medium was then Collected from the Same Number of Infected and Noninfected Cells at 72 Hours Post Transfection. B, This Panel Shows the Clinical Appearance of Triplicate Wounds Receiving Either Nothing (Control), Skin Substitute with Non-IDO Expressing Fibroblasts (Fibroblasts), Empty Vector Transected Fibroblasts (F+Mock-Ad), or IDO Expressing Fibroblasts (F+IDO-Ad). C, The Surface Areas of Wounds Receiving Different Treatments Shown in (B) were Quantified on Days 0, 4, and 8 after Wounding and Compared (n=6).

Conclusion

IDO expression by a cellular component of the allogeneic graft or bystander cells is a promising new therapeutic approach in prolonging the survival and acceptance of allogeneic islet transplants and skin grafts in patients who suffer from autoimmune disorders such as type I diabetes or have had burn or other non-healing injuries. This new concept that cells expressing IDO can suppress T cell response and promote immune tolerance may help with the acceptance of transplants by hosts without the side effects seen with currently used systemic immunosuppressive drugs. As such, this promising finding reveals that the use of IDO therapy has a significant potential in improving the quality of life of patients who need these kinds of transplantations.

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