Local Expression of Indoleamine 2,3, Dioxygenase Prolongs Allogeneic Skin Graft Take in a Mouse Model

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Background and Objective: Despite the effectiveness of skin autotransplantation, the high degree of immunogenicity of the skin precludes the use of allografts and systemic immunosuppression is generally inappropriate for isolated skin grafts. Indoleamine 2,3 dioxygenase (IDO) is a potent immunoregulatory factor with allo- and autoimmune suppression and tolerance induction properties. This study examines the potential use of locally expressed IDO to prolong the allogeneic skin graft take in a mouse model.

Approach: Syngeneic-fibroblasts were transfected with noncompetent IDO viral vector and the level of Kynurenine (Kyn) in conditioned medium was measured as an index for IDO activity. Either 1 or 3×10^6 IDO-fibroblasts were introduced intra/hypo-dermally to the mouse skin. The expression, localization, and functionality of IDO were then evaluated. The cell-injected areas were harvested and grafted on the back of allogeneic mice. The graft survival, immune-cells infiltration, and interaction with dendritic cells were evaluated.

Results: The results showed a significant improvement in allogeneic graft take injected with 1×10^6 IDO-fibroblasts (18.4 ± 3.3 days) compared with control (12.2 ± 1.9 days). This duration increased to 35.4 ± 4.7 days in grafts injected with 3×10^6 IDO-expressing cells. This observation might be due to a significantly lower T cells infiltration within the IDO-grafts. Further, the result of a flow cytometric analysis showed that the expression of PD-L1/PD-L2 on CD11c+/eFluor+ cells in the regional lymph nodes of injected skin areas was significantly higher in IDO groups compared with control.

Conclusion: These data suggest that allogeneic skin graft survival outcome can be prolonged significantly by local overexpression of IDO without any systemic effect.

Keywords: skin transplantation, IDO, allogeneic grafts, allogeneic fibroblasts, cell therapy

INTRODUCTION

SKIN GRAFTING IS frequently used for the management of extensive skin defects and injuries, such as high total body surface area burns, severe exfoliating skin disorders (e.g., toxic epidermal necrosis), and large chronic wounds. Currently, the gold standard

of care for extensive wounds such as burn injuries is early excision and autologous skin grafting.¹ Each year, more than 40,000 hospitalized burn victims,² in addition to more than 5 million individuals with chronic wounds in North America, are potential candidates for skin grafting

procedures.^{3,4} However, in wounds such as large burns, harvesting autologous skin may not be an option. Allogeneic (cadaver) skin grafts are an alternative treatment in these cases.^{5,6} Unfortunately, as a result of extreme immunogenicity of the skin they only offer temporary wound coverage.

Skin is a protective barrier to the external environment, and it is not surprising that there is an abundance of antigen-presenting cells residing in the dermis and epidermis.^{7,8} This makes skin one of the most antigenic tissues in nature. The potent immunogenic nature of skin allografts and the demand for treatment of skin injuries highlight the need for discovering strategies that permit tolerance to skin allografts, with minimal systemic immune suppression, preferably with either minimal or no systemic immunosuppression.

Indoleamine 2,3 dioxygenase (IDO) is a potent immunomodulatory enzyme with allo- and auto-immune suppression and tolerance induction properties in a variety of physiologic and pathologic settings.^{9,10} For instance, IDO is capable of suppressing the maternal T lymphocyte immune response against semi-allogeneic fetal tissues, during the course of pregnancy through various mechanism such as producing immune-inhibitory metabolites (*e.g.*, Kynurenine [Kyn], Kynurenic Acid [KynA]) or generating a tryptophan-deficient environment.¹¹ In addition, it has been shown that IDO-expressing dendritic cells (DCs) do not sequester antigens or fail to present them. Instead, IDO activity in DCs alters the context in which antigens are presented, so that activated T cells undergo apoptosis, become anergic, or differentiate into T regulatory cells, instead of undergoing clonal expansion and differentiating into effector cells.^{12,13} Hence, it has been speculated that the immunoregulatory capacity of IDO can be therapeutically exploited to dampen allo-reactive immune responses. Our group previously showed that IDO overexpression prevents murine islet allograft rejection.¹⁴ This suggests that IDO expression can potentially protect allo-transplantation of solid organs without the use of systemic immunosuppressive medication.

CLINICAL PROBLEM ADDRESSED

Burns and chronic wounds comprise nearly two-thirds of the advanced wound care sector, which account for nearly \$20 billion for healthcare system worldwide. Two of the currently used procedures in the management of extensive wound injuries are (1) The use of autologous grafting for the management of soft tissue injuries, such as extensive traumas or infections (*e.g.*, necrotizing fasciitis)

and (2) Allogeneic skin grafting mainly from the cadaver that acts as a temporary coverage.

Despite the effectiveness of skin autotransplantation, limitation in healthy autologous grafts in extensive skin injuries is always challenging. On the other hand, the high degree of immunogenicity of the skin precludes the use of allografts and systemic immunosuppression is generally inappropriate for isolated skin grafts. IDO is a potent immunoregulatory factor with allo- and autoimmune suppression and tolerance induction properties.

Thus, in this study, we examine the potential of introducing IDO-expressing fibroblasts to the dermis and hypodermis of allogenic skin grafts. The results revealed the feasibility and immune-protective function of IDO expressing allogenic skin graft transplants. To the best of our knowledge, this is the first time that IDO-expressing fibroblasts within full-thickness skin grafts have been used as a strategy for prolongation of allogeneic grafts survival.

MATERIALS AND METHODS

IDO-expressing cell preparation

As reported above, C57BL/6 dermal fibroblasts were transfected with a IDO-mCherry lentiviral vector carrying human IDO cDNA. IDO expression and activity in the transfected fibroblasts was determined by flow cytometry to detect mCherry red fluorescent protein and also by using Kyn assays, as explained previously.³⁰

Kyn assay

Measuring the amount of tryptophan metabolites in the fibroblast-conditioned medium was used as the indicator of biological activity of IDO cells. This was done before the cells being injected to the transplant grafts or back of the skin. The level of Kyn was measured according to a previously established protocol.^{31,32} Briefly, trichloroacetic acid was used for precipitating proteins in the conditioned medium. Subsequently, 0.5 mL of centrifuged supernatant was incubated with 0.5 mL of Ehrlich's reagent at room temperature for 10 min. A standard curve of defined Kyn concentration (0–20 mg/mL) was used to calculate the concentration of Kyn in the conditioned medium. This reaction mixture was measured with spectrophotometer at 490 nm.

Animals

C57BL/6 (H-2b) mice and female Balb/c (H-2d) were used as graft donors and recipients, respectively. Mice were purchased from Jackson laboratories (Indianapolis, IN). All experimental and surgical procedures were reviewed and approved by the University of British Columbia Animal Care Committee.

Immunofluorescent histological analysis

Tissues embedded in paraffin were sectioned and stained with hematoxylin and eosin (H&E). Grafts were harvested at indicated time points (day 7, 14, and graft rejection day), fixed in 10% formalin solution, then embedded in paraffin. The skin specimens were sectioned 5- μ m thick from the graft area and stained with H&E. Immunofluorescence staining was performed for cluster of differentiation (CD)3. Sections were rehydrated and blocked for nonspecific binding. Sections and primary antibodies were incubated overnight at 4°C. After being washed, the slides were incubated with the secondary antibodies for 1 h. Primary immune fluorescent antibodies used in this study were goat anti-CD3 antibody (1:200 dilutions; Bio-Rad). Secondary antibodies (1:1,000 dilution) also used were FITC goat anti-guinea pig IgG (Abcam), rhodamine goat anti-rabbit IgG (#7074 Cell signaling), and rhodamine goat anti-rat IgG (1:2,000; Dako Laboratories, Ontario, Canada).

Fibroblast injection

C57BL/6 mice were injected intra/hypo-dermally with either 10^6 IDO-mCherry positive C57BL/6 fibroblasts or 10^6 regular C57BL/6 mouse dermal fibroblast resuspended in 100 μ L phosphate buffered saline (PBS), using a 27-gauge needle ($n=4$ mice/cell group). The injection was performed by first piercing the skin, then directing the needle as superficially as possible back upward toward the surface; this commonly led to formation of a well-demarcated papule in the center of the injected area. To identify the area of injection during the subsequent time points, the back skin was tattooed in six single spots around every area of injection on the back of the mouse. To measure the level of Kyn and KynA and investigate the probable systemic and local activity of IDO in injected grafts, the skin and serum samples were processed and subjected to mass spectrometry using the previously mentioned preanalytical sample processing protocols.³²

Viability and functionality of fibroblasts upon intra/hypo-dermal injection

To evaluate the viability and functionality of fibroblasts upon intradermal (ID) and hypodermal (HD) injection, 1×10^6 cells either IDO or regular (Control) B6 mouse cherry fibroblasts, were injected ID/HD on the back of Balb/C mice in four different points. The injected sites were harvested after 3 days, and the samples were subsequently subjected to fluorescent microscopy, flow cytometry, or mass spectrometry for tryptophan metabolite (Kyn, KynA) measurements. One area out of four was used to isolate the injected cells from the skin, using aforementioned method. Next, isolated cells were

stained with anti H2K antibody and calcein and subjected to flow cytometry.

To isolate skin cells, cell injected skin samples were incubated at 37°C in Dispase I (SIGMA D4818) for 1 h, with subsequent separation of the epidermis and dermis. Dermal sheets were incubated in 0.25% trypsin, 0.02% EDTA solution (SIGMA 59428C) for 30 min, and the remaining tissues were minced into small pieces and incubated in collagenase type 5 (3 mg/mL) and DNase for 30 min. Isolated cells were washed with 10% fetal bovine serum in PBS, and FACS was used for analysis.

In vivo assay for migrated skin DCs

At each indicated time point (weeks 1, 2, 4, and 8), the four injected areas of the skin of each individual mouse were shaved, followed by application of 100 μ L of 100 μ g/mL eFluor 670 (65-0840-90; eBioscience) dissolved in 1:1 acetone. After 24 h, inguinal and axillary lymph nodes (LNs) (back-skin draining LNs), in addition to spleen and mesenteric LNs (distant reticuloendothelial tissues), were isolated by incubating the tissue samples in 0.1% DNase I (MP Biomedicals) and 1 mg/mL collagenase D (Roche) for 30 min. Single cell suspensions were incubated with Fc block (FcRII/III mAb 2.4G2) for 15 min and stained with antibodies to CD11c and programmed death ligand 1 (PDL1), PDL2, and CD86.³³ Stained cells were washed and gated for CD11c⁺ eFluor 670⁺, and the percentage of PDL1-, PDL2-, and CD86-expressing IDO-fibroblasts was determined and compared with control fibroblasts. As a control, cells isolated from back-skin draining lymph nodes (axillary and inguinal), in addition to distant lymph nodes (mesenteric), and spleen were evaluated by flow cytometry, using the same procedure done for test samples.

Skin transplantation procedure

Preparation of graft bed. Recipient mice (Balb/c or B6) were anesthetized and secured on a surgical board and shaved using a clipper and depilatory cream. The area was subsequently prepared with a sterile technique (povidone-iodine and 70% alcohol). The graft beds of the ear, tail, and back were made using a 6 mm punch device to mark the area and curved scissors to remove a circular full thickness defect (6–7 mm) in three different sites on the back of the recipient mice. A preparation of the graft beds for each type of skin (ear, tail, and back) was performed immediately before the application of skin graft to avoid the potential of desiccation of site.

Suturing technique. In this study, we used a surgical microscope and surgical loupes (4 $\times$) to aid

in raft preparation and application. The 8 mm grafts were placed into the 6 mm recipient bed and sutured at 90-degree intervals using 7-0 Prolene sutures (12, 3, 6 and 9 o'clock). The intervening gaps were then addressed using simple interrupted sutures with 8-0 Nylon sutures (6–8 sutures).³⁴

Tissue optical clearing

Skin tissues were cleared by fructose solutions of varying concentrations (20%, 40%, 60%, 80%, 100%, and 115% wt/vol), which were generated by dissolving D-(-)-fructose (JT Baker, Center Valley, PA) in milliQ water with 0.5% α -thioglycerol (Sigma-Aldrich, St. Louis, MO) to prevent browning.³⁵ Tissues were equilibrated to increasing concentrations of fructose by incubating in each formulation for at least 24 h under gentle shaking at room temperature. Unclearing, or reversal to PBS, was performed by equilibrating samples in fructose solutions of decreasing concentrations under the same conditions.

Imaging

To quantify the migration of cherry red fibroblasts to the surrounding graft bed area, cleared skin tissue from the grafted area (2×2 cm) was harvested (day 0, week 1, 2) and cleared with the aforementioned method. Utilizing the tiling feature the whole tissue was scanned by confocal microscopy (Zeiss, Cell Observer SD) at the depth of 180–200 μ m from the epidermis. The grafts and graft bed areas were fully scanned in high magnification (100×) and mapped as a single image. Next, the borders of the grafts were marked on the image. Subsequently, to investigate the presence of Cherry red fibroblast outside the borders of the grafts, Z stacks (180–250 μ m depth with 1 μ m slices) were acquired in high magnification starting from the field adjacent to the graft in the graft bed area. Z-stacks in the recipients' graft bed areas were checked for fibroblasts from the center to the distant areas until no red cherry expressing fibroblasts were detected. This was done in eight directions from the center of the graft and the acquired Z-stacks of each field were 3D-reconstructed to fully visualize the cherry red cell presence in any layer of the image. The distance between the center of the graft and the center of furthest field positive for cherry red fibroblast was considered as migration distance.

Evaluating the graft survival

To study the effects of the injected IDO-expressing fibroblasts in prolonging allogeneic graft survival, a categorical grading system was used to quantify the degree of graft rejection: 0—intact graft; 1—onset of graft rejection, <25% rejection or shrinkage; 2—

>25% rejection or shrinkage; 3— >50% rejected or more than 50% shrinkage of its original size; 4— >75%; and 5—complete rejection (>95%).³⁶

RESULTS

Viability and function of the fibroblasts were preserved after intra/hypo-dermal injection

The results of the *in vivo* viability test showed that more than 90% of allogeneic injected IDO-expressing B6 cells (H2K+) and control fibroblasts were viable and alive upon injection, as evaluated by Calcein staining. Fluorescent microscopy results also confirmed the presence of efluor 670⁺ cells within the dermal area of skin grafts (Fig. 1A, B).

Measurement of Kyn and KynA as an index for IDO activity *in vivo*

Kynrenine and Kynurenic acid (Kyn and KynA) are two important products of enzymatic degradation of tryptophan by IDO, which suppress effector T cells and induce regulatory T cells.¹⁵ Results of mass spectrometry of injected skin sites showed that Kyn and KynA levels were significantly higher in the IDO-fibroblast group in comparison with control fibroblasts and uninjected skin samples. Although the Kyn/Tryptophan ratio was significantly higher in the IDO group, there was no significant difference in the absolute level of tryptophan among the study groups (Fig. 1C).

Intra/hypo-dermal injection of IDO-expressing fibroblasts prolonged allogeneic skin graft survival

To address the question of whether the presence of IDO-expressing fibroblasts within the dermis can prolong skin allograft survival, two experiments were conducted. In the first experiment, skin allografts were prepared by a one-time injection of 1×10^6 IDO-expressing fibroblasts and regular fibroblasts to the B6 mouse skin. The IDO expressing and control skin grafts were subsequently transplanted on the back of allogeneic BALB/C mice ($n=5$ /group). Using Kaplan–Maier analysis, the results showed a significant prolongation in survival of grafts containing IDO-expressing cells up to 18 ± 2.3 days in comparison with the non-IDO-expressing fibroblast group (11 ± 1.7 days, p -value <0.01) and noncell-injected group (10 ± 2.1 days, p -value = 0.021) (Fig. 2A, C and D).

In the second set of experiments, we evaluated the effect of an increased number of injected cells by threefold (3×10^6 , cells/graft). The results revealed a remarkable improvement in the survival of triple-injected grafts (35.4 ± 4.7 days) in com-

Figure 1. Insets in the upper row ($\times 25$) show selected areas magnified on lower row ($\times 200$). (A) Intradermal injection of IDO-Fibs. in syngeneic subjects before harvesting grafts 3 days postinjection: (a) Regular fibroblast (b) IDO fibroblast (c) Uninjected skin. (B) Live assay of cells isolated from grafts and stained with Calcein. (C) Kyn and tryptophan levels and Kyn/Tryp ratio in tissue extracts of injected grafts, and level of Kyn in the plasma of injected mice. IDO, indoleamine 2,3 dioxygenase. Kyn, kynurenine. (** $p < 0.01$; *** $p < 0.001$). Color images are available online.

parison with a single injection (18.4 ± 3.3 days, p -value < 0.01) and control groups (12.2 ± 1.9 days, p -value < 0.01) (Fig. 2B).

To minimize the variability between control and treated subjects, an individual mouse underwent double grafting ($n = 4$), with one IDO-graft and one control fibroblast injected graft. The results con-

firmed the effect of IDO expression in prolongation of graft survival (36.4 ± 4.1 days vs. 14 ± 2 days).

Infiltration of CD3⁺ cells were significantly less in the IDO-expressing transplanted grafts

Graft tissues were harvested at either the end point of the study (42 days) or at the time of graft

Figure 2. Skin graft transplantation results. **(A)** Results in different groups after one-time intradermal injection of IDO or regular fibroblasts ($n=5/\text{group}$). **(B)** Results after three intra/hypo-dermal injections in days -7 , -5 , and -3 to the same region of the grafts and transplantation in day 0. ($n=5/\text{group}$). **(C)** Grafts survival in different groups. **(D)** Results of graft survival were subjected to statistical analysis and compared to each other. (** $p < 0.01$; *** $p < 0.001$). Color images are available online.

rejection. Tissue samples were then subjected to histological evaluation. Consistent with the experiment shown for prolonged graft survival, IDO-expressing grafts revealed a more intact skin structure (Fig. 3A, B). Furthermore, significantly less infiltration of CD3⁺ cells were found within the IDO grafts (Fig. 3C).

ID/HD injected cells migrated outside of their injection site

To address whether injected cells migrate to the outside of injection sites, a confocal microscopic study on skin samples taken from the cell-injected sites was performed. The result revealed the presence of IDO-expressing cells within the graft bed,

Figure 3. *Inset in (A1)* shows selected area magnified on *(A2)*, and *inset in (A2)* shows selected area magnified on *(A3)*. H&E and immunofluorescent (IF) microscopy of grafts post-transplantation. **(A)** H&E and IF (CD3) staining of IDO transplanted grafts harvested at end point time (day 35). *Inset in (B1)* shows selected area magnified on *(B2)*, and *inset in (B2)* shows selected area magnified on *(B3)*. **(B)** H&E and IF (CD3) staining of allograft with regular fibroblast harvested at day of rejection (day 13) stained for CD3⁺ cells. **(C)** Average number of CD3⁺ cells infiltrated into the Control (regular) versus IDO fibroblast grafts (10 High Power Fields/graft). H&E, hematoxylin and eosin. Color images are available online.

even outside the borders of the grafts, indicating that cells are capable of local migration. To evaluate the time when the cells are migrating, a combination of tissue clearing techniques and confocal microscopy was utilized as explained in the methods (Fig. 4A, B). The results confirmed the migration of IDO-fibroblasts to the graft bed within the first week of transplantations and no additional migration was observed from week 2 to 3. (Fig. 4C). However, the migration score between IDO and

non-IDO-expressing cells was not significantly different (Fig. 4B).

IDO-expressing fibroblasts survived within allograft and remained functional

To further clarify the mechanism(s) underpinning immunomodulatory effect of ID/HD injected IDO cells, we studied the fate and function of these cells within allografts for up to 8 weeks. To this end, we dermally injected 1×10^6 cherry red IDO/

Figure 4. Migration of allogeneic IDO-expressing fibroblasts to graft bed area. **(A)** The grafts and graft beds areas were tiled with a confocal microscope and the graft areas were highlighted (*green circle*). **(B)** Z-stack images of the fields outside of the graft zone (*red squares*) and the presence of cherry red fibroblasts outside the graft area. The distance between the center of the graft and the center of the furthest field with cherry red cells is considered the migration distance. **(C)** Migration distances at different time points were subjected to statistical analysis. Color images are available online.

control B6 fibroblasts into four spots on the back of Balb/c mice ($n=3/\text{group}$). The injected skin with surrounding tissues along with regional and distant lymph nodes and spleens were harvested at different time points (weeks 1, 2, 4, and 8).

The immunofluorescent microscopy confirmed the presence of allogeneic fibroblasts in the samples at all time points (Fig. 5A). As an indicator of IDO activity, the levels Kyn, KynA, and tryptophan were measured by mass spectrometry and the re-

Figure 5. Studying the fate and whereabouts of IDO-expressing fibroblasts injected in allogeneic dermis and hypodermis. *Insets in the upper row (x25) show selected areas magnified on lower row (x100).* **(A)** Immunofluorescent microscopy showing cherry red cells in allogeneic skin at different time points. **(B)** Kyn/Tryptophan ratio of tissue extracts and at different time points in IDO and Ctrl groups **(C)** Tracking cherry red fibroblasts in skin-draining lymph nodes at different time points. Color images are available online.

Figure 6. Statistical analysis of flow cytometry results for different surface markers on efluor 670+/CD11⁺ cells in skin-draining lymph nodes. Black bar: IDO-fibroblasts; Gray bar: Regular fibroblasts (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

sults showed a significantly high level of Kyn expression and KYN/tryptophan ratio within the injected skins (Fig. 5B).

Migration of IDO-fibroblasts to regional lymph nodes

Different lymphatic tissues (dorsal skin draining, mesenteric, and spleen) were isolated and evaluated for the presence of cherry red⁺ cells by flow cytometry. The number of red cherry present in the regional skin draining lymph nodes were markedly higher in IDO-expressing fibroblast-treated animal group on week 1 post-transplantation. However, no cherry red cell was detected at week 2 postinjection (Fig. 5C). The result was confirmed by fluorescent microscopy.

ID/HD injection of IDO-expressing fibroblasts modified the phenotype of recipient DCs in and around the injection region

We investigated the *in vivo* interaction between allogeneic fibroblasts with host DCs (Fig. 6). Cherry red⁺ IDO or control C57BL/6 fibroblasts were injected Intra/Hypo-dermally into four spots (1×10^6 cells/spot). An acetone-dissolved fluorescent dye (efluor 670) was applied on three of the injection sites 24 h before harvesting skin-draining regional and distant lymphatic tissues. Based on a previously established method, uptake of this fluorescent dye by skin resident antigen-presenting cells tags these cells.¹⁶ Flow cytometric analysis showed that the expression of programmed death ligand 1 and 2 (PD-L1 and PD-L2) on CD11c⁺/eFluor⁺ cells in the regional lymph nodes of injected skin areas was significantly higher in IDO groups compared with those of control fibroblasts injected and non-injected groups at all time points (1, 2, 4, and 8 weeks) postinjection. No difference in DC phenotype was found in distant lymph nodes (mesenteric) or spleen.

Additionally, no statistically significant difference was observed in the expression of CD86 between the experimental groups.

DISCUSSION

Allografts are being used as temporary coverage for different types of skin defects. The drawback of using allografts lies in the susceptibility of the graft to immune rejection.^{6,17} The grafting of allogeneic skin initiates a chain of reactions by the recipient's immune system, leading to destruction of the graft. Here, we investigated whether a highly antigenic tissue such as allogeneic skin can be immunoprotected by introducing IDO-expressing fibroblasts. This might be a potential method for prolonging allogeneic tissues survival including skin grafts from recipient immune cells. Although cell therapy with collagen-expressing fibroblasts for treating epidermolysis bullosa is in a phase three clinical trial,¹⁸ to the best of our knowledge, this is the first time that engineered fibroblasts within full-thickness skin grafts have been used as a strategy for the prolongation of graft survival.

The findings revealed that local IDO expression suppresses cellular alloimmune responses against skin allograft and significantly prolongs their survival without systemic antirejection treatments in immune-competent mice. This prolongation is likely to be due to the expression of IDO. As it has been previously shown, IDO is an immunomodulatory enzyme that either directly effects the level of essential amino acid, tryptophan, or induces immunosuppressive properties by production of tryptophan metabolites such as Kyn and KynA or a combination of both within the allografts that received IDO-expressing fibroblasts.

The result of immunofluorescence microscopy confirmed the presence of IDO-expressing fibro-

blasts in dermal and hypodermal layers of Balb/c mice at different time points. The mass spectrophotometry results further showed a higher ratio of both Kyn and KynA/tryptophan ratio in the IDO group in comparison with the control groups. This signifies that injected IDO-expressing cells are healthy and able to express IDO, which leads to the production of its metabolites. However, we found no difference in the level of tryptophan among experimental groups. This might be explained by the fact that blood flow and extracellular fluid are a rich source of tryptophan and its deficiency induced by IDO expression can be compensated by replenishing the amino acid supply. Indeed, it has been shown that Kyn/tryptophan ratio plays a superior significance in suppressing the immune response in comparison to either tryptophan or Kyn concentration individually.^{19,20}

To investigate the effect of the number of IDO-expressing fibroblasts on allograft survival, B6 allograft received three separate injections (each 10^6 cells) (days 7, 5 and 3 before transplantation) of IDO-expressing fibroblasts and subsequently transplanted them on the back of Balb/C mice. The result showed a “dose-dependent” effect between the number of injected IDO-expressing cells (*i.e.*, level of IDO within the graft) and the extent of graft survival. Specifically, triple injections (3 repeats of 10^6 cells/injection/graft) of IDO cells at different time points before transplantation prolong the grafts survival more robustly in comparison to a single injection (10^6 cells/graft). This finding indicates that the expression of IDO and the level of tryptophan metabolites are critically important for prolonging allograft take. In fact, our findings demonstrated a higher Kyn and KynA/tryptophan ratio in the IDO group compared with those of control fibroblast group and normal skin. However, the level of these metabolites in serum samples taken from IDO-treated and controls was not significantly different, indicating that locally expressed IDO plays a critical role in prolongation of allogeneic engraftment. This finding was also confirmed by showing a significantly lower number of infiltrated CD8⁺ cells in the IDO-fibroblasts grafts in comparison with that of controls.

Interestingly, injected IDO-expressing fibroblasts showed a capacity to migrate to the regional, but not distant, lymph nodes within the first week of injection. This finding is important as it is well documented that fibroblasts can act as nonprofessional antigen-presenting cells via the expression of major histocompatibility complex (MHC) class II molecules. As these cells can interact with naive

KEY FINDINGS

- Robust expression of IDO within allograft can be achieved by introducing engineered IDO-fibroblasts.
- IDO expression within the allograft can efficiently prolong the graft survival and significantly delay the rejection process.
- IDO cells significantly decrease the number of CD3⁺ T cells infiltration into the graft and also may affect the function of infiltrated DCs by expressing tolerogenic markers.

T cells through MHC class II expression,^{21–23} concurrent IDO expression can induce Treg characters or anergy in the T cells.^{24,25} In this case, IDO-fibroblasts may also suppress the immune cells more effectively. Moreover, IDO expression is a crucial requisite of tolerogenic antigen-presenting cells, specifically some subtypes of DCs.^{26–28} Thus, IDO and MHC II expression may enable IDO-fibroblasts to generate a specific immune suppression against recipient alloreactive T cells.^{24,25} After the first week, tracking of the non IDO fibroblast in the lymph nodes (regional or distant) did not show any migration of those cells from the skin to the lymph nodes.

Finally, as shown previously, the interaction of fibroblasts and DCs results in the expression of co-inhibitory molecules by DCs.²⁹ The immunomodulatory effects of fibroblasts on expression of co-inhibitory molecules (PDL-1, PDL-2, and B7H4) and costimulatory molecules (CD86 and CD80) on DCs were also studied. High expression of co-inhibitory molecules on the migratory DCs from the area of IDO-fibroblast injection site might be in favor of indicating their role in inducing tolerogenic features in neighboring DCs. To further confirm this potential mechanism, a new series of experiments need to be conducted.

In conclusion, this study confirms the feasibility of a functional IDO-expressing allogeneic skin graft and supports the idea that IDO significantly improves skin allograft survival. This finding demonstrates that the immunosuppressive effect of IDO can be utilized to prolong allograft survival and may decrease numerous difficulties related to shortage of skin autografts without the need for immunosuppressive treatment regimens that compromises the functionality of the immune system.

INNOVATION

To the best of our knowledge, this is the first time that IDO-expressing fibroblasts within full-thickness skin grafts have been used as a strategy for prolongation of allogeneic grafts survival. The

data from this study provide evidence that local immunosuppression can be provided by the delivery of IDO-expressing fibroblasts in allogeneic skin transplantation, which can result in significant improvement in allograft survival. This “cell based” approach to localized immunosuppression can also provide potential opportunities to autoimmune skin disorders such as Alopecia Areata.

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AUTHOR DISCLOSURE AND GHOSTWRITING

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ABOUT THE AUTHORS

Mohammadreza Pakyari, MD, is currently a PhD candidate at the University of British Columbia (UBC). His thesis involves improving the outcome of allogeneic and autologous skin grafting. His fields of interest are transplantation immu-

nology and wound healing, and he has published several peer-reviewed articles in these fields. **Ali Farokhi, PhD**, received his degree from UBC where he studied the field of wound healing for the development of non-rejectable wound coverage. He developed a new detergent-free method for decellularizing the skin while keeping the structural components in the skin scaffold relatively intact. This work has significant application in the treatment of burn injuries and chronic wounds. **Reza B. Jalili, MD, PhD**, is a principal investigator at ICORD and a scientist in the Department of Surgery at UBC. Dr. Jalili works on finding innovative ways to treat chronic wounds. In particular, his research is aimed at developing regenerative strategies by identifying the optimal combination of progenitor cells and extracellular matrix that can lead to effective healing of chronic wounds. **Ru-hangiz T. Kilani, PhD**, has been closely involved in the wound healing area for the last 30 years. She has 65 peer-reviewed articles published in international journals. She is actively involved in a few groundbreaking discoveries which led to three patents. **Erin Brown, MD, PhD, FRCS**, is a clinical professor in the Department of Surgery at UBC. He is the Research and Fellowship Director for the Division of Plastic Surgery. Aziz has 7 patents, one of which is related to identifying a high-level of a biomarker for early detection of rheumatoid arthritis (RA). This technology has so far generated 3 worldwide patents including its therapeutic use in treating RA patients.

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Abbreviations and Acronyms

CD	= cluster of differentiation
DC	= dendritic cells
HD	= hypodermal
H&E	= hematoxylin and eosin
ID	= intradermal
IDO	= indole amine 2–3 dioxygenase
Kyn	= kynurenine
KynA	= kynurenine acid
LN	= lymph nodes
MHC	= major histocompatibility complex
PBS	= phosphate buffered saline
PDL	= programmed death ligand