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Review

Tolerance induction in IDO expressing skin substitute allotransplant

T. A. Curran, S. Salimi, and A. Ghahary*

Department of Surgery, University of British Columbia, Vancouver, BC, Canada

ABSTRACT

Burn injuries are the 4th most common type of trauma experienced worldwide with severe burns accounting for more than 300,000 deaths every year. Debridement and wound coverage with an autologous graft is the gold standard of treatment but this is complicated in extensive surface area burns by a paucity of donor sites. Ready-made allogeneic skin substitutes are a promising treatment option in these instances although immune mediated rejection prevents them from acting as permanent wound coverage. Immune cell activation by antigens is crucial for immune-protection against pathological invasion however regulation of this interaction and induction of tolerance is essential in terms of autoimmunity, allergy, pregnancy and transplantation. Over the past decades we have seen advances in our knowledge of the molecular and cellular pathogenesis of tolerance. Indoleamine 2,3 dioxygenase (IDO) is one area where extensive success had been achieved in terms of understanding the mechanisms behind immune tolerance. IDO regulates the immune response through its negative effect on effector cell survival and upregulation of the regulatory T cell (Treg) population as a result of its catabolism of the essential amino acid tryptophan and accumulation of the tryptophan metabolites. It is expressed in a number of cell populations but facultative expression can be induced in skin cells, with the inflammatory mediator IFN-γ and other cytokines. Furthermore we have constructed skin substitutes populated with these IDO expressing fibroblasts. In this review we present a background on the mechanism of IDO governed immune tolerance, our methodology of IDO transduction and importantly our experience of tolerance with our IDO/ fibroblast skin substitute.

KEYWORDS: IDO, skin substitute, immune tolerance

ABBREVIATIONS

IDO, indoleamine 2,3 dioxygenase; IFN-γ, interferon-γ; Treg, regulatory T cells; TBSA, total burn surface area; GCN2, general control non-depressible 2; CHOP, C/EBP homologous protein; PBMC, peripheral blood mononuclear cells; MHC, major histocompatability complex; PCR, polymerase chain reaction; FACS, fluorescence-activated cell sorting; GAG, glycosaminoglycans; 1-MT, 1-methyl tryptophan; MMP, matrix metalloproteinase

INTRODUCTION

Millions of people are disabled and disfigured by burns every year with severe burns accounting for more than 300,000 deaths worldwide (www.who.int). Survivors often experience physical and psychological

*Corresponding author and reprint requests:
Dr. Aziz Ghahary, Director,
Burn and Wound Healing Research Lab,
4th Floor, The Blusson Spinal Cord Centre,
818 West 10th Ave, Vancouver, BC,
V5Z 1MQ, Canada.
aghahary@interchange.ubc.ca

consequences as a result of disability and disfigurement and primary health care for one inpatient can range from US$3000 to US$5000 per day (www.who.int). A burn destroys the protective skin armour and without this barrier the individual has no defence against the external environment. The gold standard of treatment is operative escharotomy and closure with an autologous skin graft harvested from an appropriate donor site. In instances of extensive surface area burn (>50% TBSA), suitable donor sites may be limited for autologous harvest and early wound coverage may thus be sub-optimal [1]. Early wound coverage is imperative to avoid local and systemic complications in addition to preventing the development of hyperproliferative healing if coverage is delayed more than 21 days [2]. An alternative and promising therapy is the use of readily available, skin substitutes consisting of dermal and epidermal cells [3]. The major disadvantage of these engineered tissue-allografts is that they can only provide temporary wound coverage as they are ultimately rejected by the immune response and thus their use in wound healing is limited [4, 5]. Therefore our research group are focusing on a local targeted immuno-suppressive agent which would prevent immune rejection and guarantee tolerance.

Indoleamine 2,3-dioxygenase (IDO) is a haem-containing enzyme that is expressed intracellularly and catabolises tryptophan to kynurenine [6]. Degradation of essential amino acids in the environment is an ancient survival property that has been conserved in evolution for defence and similarly the deficiency of tryptophan inhibits the survival of pathogens. Additionally this enzyme generated micro-environment inhibits the survival of effector immune cells and thus plays a role in immune tolerance for example by regulating maternal T-cell immunity during pregnancy [7] and in the immune resistance to tumours [8]. Based on the immune-protective property of IDO our group hypothesized that skin cells could be transduced with an IDO gene and these cells then used to populate a pre-formed skin substitute scaffold. In this review will discuss the role of IDO in allogeneic skin substitutes, our methodology of transduction and our *in vivo* experience.

IDO mechanism of tolerance

IDO was first described in rabbit intestine in 1967 through the work of Higuchi and Hayaishi in the degradation of D-tryptophan [6]. We know now that it is the rate limiting enzyme in tryptophan metabolism along the kynurenine pathway [9]. It can also degrade serotonin, L-tryptophan, 5-hydroxytryptophan and melatonin [9]. It does this through oxidative cleavage of the indole ring in these compounds. IDO is expressed in a number of tissues in the body including trophoblast cells [7], monocytes, macrophages [10] and dendritic cells [11]. Additionally, expression can be induced, for example in fibroblasts, through stimulation with interferon-γ as part of the inflammatory response; the invasion of tissues with infectious agents incites the inflammatory cells that accumulate to release interferon-γ. The IFN-γ then triggers the synthesis of IDO intra-cellularly. The resultant catabolism of the essential amino acid tryptophan in the environment and the accumulation of tryptophan metabolites, collectively known as kynurenines, impairs proliferation of pathogens; viruses and bacteria.

It was as a result of the pivotal research from Munn and Mellor that we appreciate the role of IDO in immune tolerance [12]. Using 1-methytryptophan, a potent inhibitor of IDO in an allogeneic pregnant murine model they found all that all the conceptus were rejected [7]. They thus proposed that IDO expression at the maternal-foetal interface and subsequent tryptophan deficiency is necessary to prevent immunological rejection. In explanation of this tolerance our research group have shown that the IDO generated microenvironment has an immune-suppressive property through impairment of the survival of CD8+ lymphocytes [13], CD4+ lymphocytes, B-cells and THP-1 monocytes [14, 15]. One possible mechanism for this effect is the activation of stress pathways such as the mTOR kinase pathway, a nutrient sensitive target of Rapamycin, in bystander cells [16] interestingly however, inhibitors of TOR do not mirror the profound arrest seen with IDO mediated immune suppression [17]. Also important is the activity of Pim-1 and Pim-2 kinases which promote the rapamycin-resistant survival of lymphocytes [17] which may be the mechanism

that ensures survival of Treg. Another stress pathway that may play a role is the general control non-derepressible 2 (GCN2) stress response pathway. T-cells that lack GCN2 appear to proliferate in the presence of IDO and do not display anergy [18]. Our research group found that CD8+ T cells are more sensitive to low levels of tryptophan than CD4+ cells and that CHOP expression, a down-stream signal for the GCN2 kinase pathway, was significantly elevated in stimulated CD8+ T cells but not CD4+ T cells giving credence to this mechanism in immune suppression [13] (Figure 1). Adenoviral transduction of the IDO gene into dermal fibroblasts was also sufficient to generate an environment in which PBMCs, CD4+, CD8+, B-cells, Jurkat cells and THP-1 monocytes were unable to survive [15] (Figure 2). The advantage of expression of IDO in dermal fibroblasts is that, as non-professional antigen presenting cells fibroblasts also express major histocompatibility (MHC) class I receptors which can activate CD4+ lymphocytes, including Treg. Theoretically a specific population of Treg would proliferate in the favourable IDO environment. Incidentally, we also showed that IDO transduction is associated with down-regulation of MHC-I levels on the surface of IDO expressing keratinocytes which could diminish this activation potential [15].

IDO transduction into fibroblasts

Although our initial results with fibroblasts transduced with an *adenoviral* IDO vector showed efficacy of IDO activity [14] the immunogenicity and transient gene expression of adenoviral vectors is not ideal for translation into clinical use and thus we graduated to using *lentiviral* vectors. Lentiviral vectors have been found to efficiently express target genes *in vivo* for more than 4 years [19]. A lentiviral construct for expressing the IDO gene was generated using a pLC-E vector and modified from the lentiviral backbone FUGW [20] (Figure 3). The human IDO gene template forward: 5'-GGGGACAAGTTTGTACAAAAA AGCAGGCTTCACCATGGCACACGCTATGG AAAACTCCTGG-3' and reverse primer: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT CCTAACCTTCCTTCAAAAGGGATTTCTC-3' (NM_002164; a gift from Dr. J. M. Carlin of Miami University). The transduced IDO cell population was enriched with fluorescent- activated cell sorting (FACS) to maximise efficacy of transduction and expression was confirmed by PCR of c-DNA and western blot and spectrometry for kynurenine [20]. We found that the presence of polybrene increased the transduction ratio approximately two fold when compared without ($P<0.01$, n=3) [20].

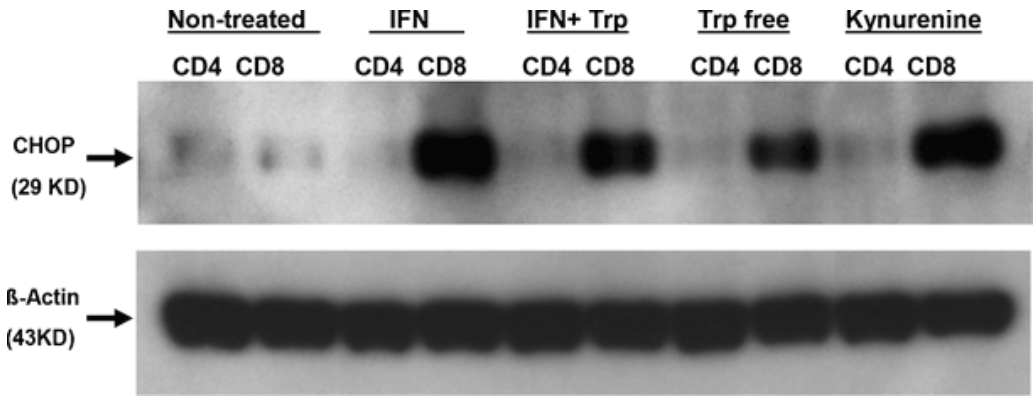

Figure 1. Western blot of cell lysate for the expression of CHOP. Comparison of CHOP expression in cell lysate after 4 day two chamber co-culture of CD4+ cells or stimulated CD8+ cells with fibroblasts, which had been either pre-treated with IFN-γ or not, in the absence or presence of media supplemented with extra tryptophan. Cell lysate from monoculture of CD4+ or CD8+ cells in the presence of tryptophan free media or in the presence of kynurenine was also analysed (n=3).

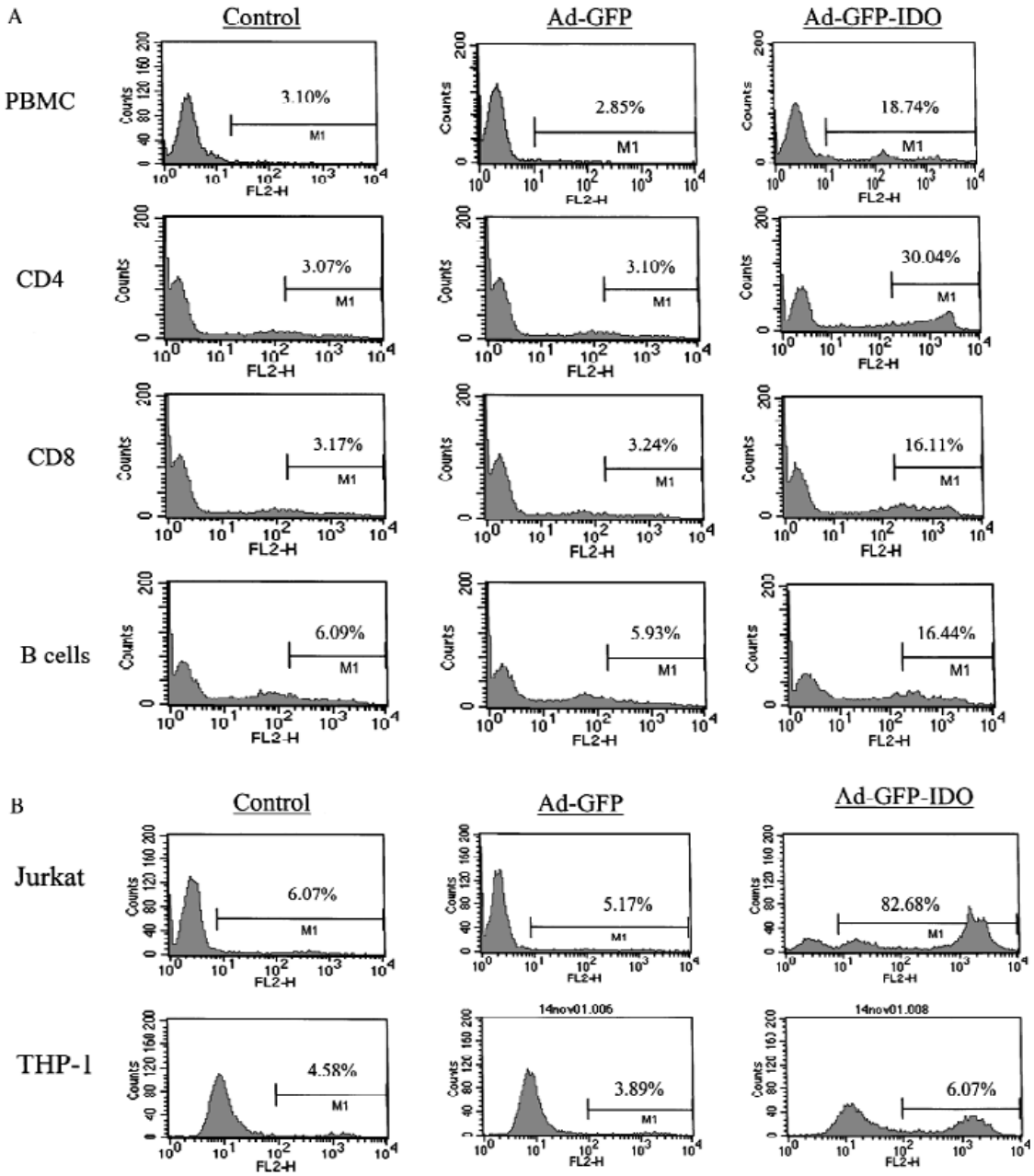

Figure 2. Flow cytometric analysis for PI positive bystander immune cells. A. PI staining of either human PBMC, CD4+, CD8+ or B-cell enriched immune cells after 5 day co-culture with non-viral infected, pre-infected fibroblasts with either an empty vector (Ad-GFP) or IDO adenoviral vector (Ad-GFP-IDO). B. PI staining of Jurkat and THP-1 after 5 days co-culture with control fibroblasts, empty vector (Ad-GFP) or IDO adenoviral vector (Ad-GFP-IDO).

Construction of fibroblast/ keratinocyte populated skin substitute

In deep dermal burns, the dermal tissue is destroyed and granulation tissue is formed by fibroblasts in the fascia and remaining dermis. The resultant platform for setting the graft in these deep burns is absent of the dermal scaffold and therefore has poor mechanical function. The primary advantage of a bi-layer skin substitute composed of both keratinocytes and fibroblasts is accelerated

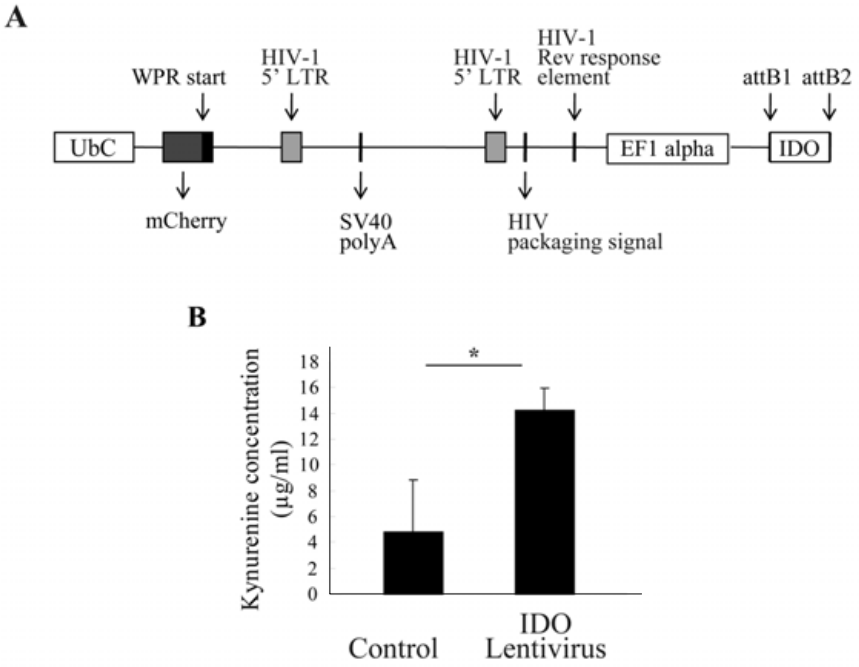

Figure 3. Schematic of lentiviral-based vector for transduction of IDO gene. A. IDO-m Cherry lentiviral construct. B. Kynurenine assay. Determination of function of IDO transfected cells by analysing the kynurenine content in the conditioned media compared with non transduced cells (n=3).

reepithelization and the combination is essential for restoration of good mechanical function of the tissue [3]. This is in part due to the growth of anchoring fibrils at the dermal-epidermal junction and are present at 3 months post-grafting but not seen on epidermal to muscle grafts [21]. Our skin substitute is composed of a type 1 bovine collagen and glycosaminoglycan (GAG) scaffold as described by Dr. S. T. Boyce [22] and populated with stratified layers of keratinocytes above and dermal fibroblasts inferiorly [23]. Manipulation of the collagen concentration is important as pore size must permit migration of the dermal cells in addition to the thickness of the substitute to allow regulation of the volume and concentration of starting materials. The 3-D collagen matrix permits the organisation of the collagen fibrils acting as a dermal substitute with the keratinocytes simulating the epidermal component of skin tissue matrix. Construction of our bi-layered skin substitute takes 14 days from inoculation [23].

IDO expressing fibroblast skin substitute *in vivo*

In our experience with either adenoviral or lentiviral tranduced fibroblasts, we have seen a potential functional and practical role of IDO expressing skin substitutes *in vivo*. In regard to its immune tolerant property we found that transduction of keratinocytes with the IDO vector results in downregulation of MHC-class I surface receptor [24]. Fibroblasts and keratinocytes are non-professional antigen presenting cells and this reduced expression can serve as an additive to immune tolerance of the allogeneic skin antigens. The key factor in this downregulation appears to be dependent on tryptophan depletion as opposed to an increase in kynurenine [24]. Importantly we showed that although IDO can induce apoptosis in local immune cells, it has no significant adverse effects on skin cells [25]. We also found as expected, that granulation tissue deposition was more abundant and the number of infiltrated inflammatory cells was less and showed linear-like distribution in the histology of our IDO composite grafts compared to the non-IDO skin substitutes which conversely showed massive random infiltration of lymphocytes at the reticular dermis after day 14 [14]. Incidentally, there was evidence of CD3+ cells around the IDO skin substitute but these were few and may contribute

to the faster wound healing seen in the IDO substitutes [14]. Additionally, proliferation of lymphocytes was reduced 5-fold in the IDO skin substitute environment and this effect was completely reversed upon supplementation with 1-methyl tryptophan (1-MT) [23] (Figure 4).

In relation to the wound healing properties of IDO skin substitutes we found a significant increase in the number of capillary-like vessels stained with CD31 after day 8 and this persisted up to day 28 at the end of the study [14]. Epidermal thickness and scar elevation were dramatically reduced in the IDO skin substitutes compared to controls giving credence to a role of IDO in reduction of dermal fibrosis. In conjunction with this anti-fibrotic effect we also found reduced MMP-1 levels. Overall wounds that were treated with IDO skin substitutes healed faster, within 8 days with reduced inflammatory response compared to untreated wounds or control skin substitutes [14, 23].

FUTURE DIRECTIONS

As discussed, we have generated an allogeneic skin substitute that is capable of inhibiting effector cell infiltration and subsequent immune rejection. The greatest obstacle in the practical application of this IDO expressing skin substitute is the use of adenoviral or lentiviral transduction methods. We have matured our method from using adenoviral vectors to a stable and more effective lentiviral transduction method however the use of viruses in clinical practice is not acceptable. Ideally, harnessing cells that have the ability to express IDO ubiquitously or are

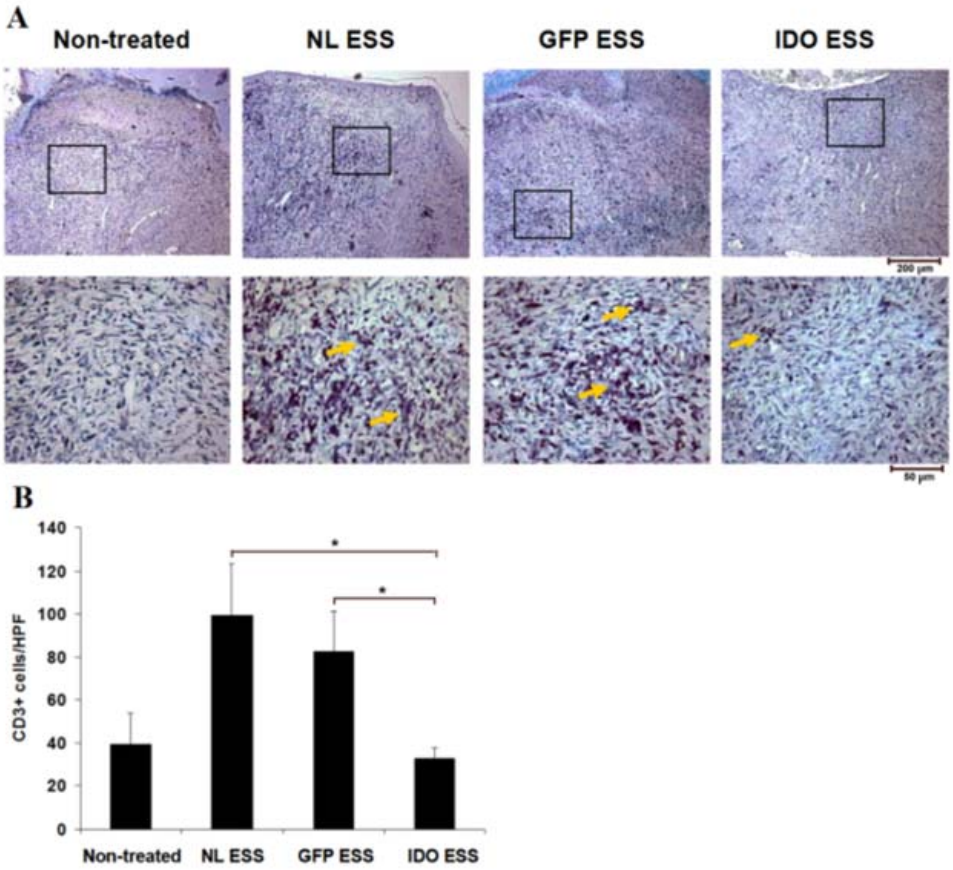

Figure 4. CD3+ lymphocyte staining in wound sections after engraftment. A. CD3+ T cell immune-histochemical staining of wounds grafted with either un-transduced cell skin substitute (SS), GFP transduced cell SS or IDO transduced cell SS 7 days engraftment. CD3+ T cells are shown by yellow arrows. (Scale bar equals 200um in upper row and 50ul in lower row). B. Numbers of infiltrating CD3+ T cells per high power field at magnification of 400x in each wound (P value < 0.001; n=5 HPF/slide; n=3 slides/condition).

facultative expressors into a skin substitute model could be an alternative method. Incidentally, only short-term expression of IDO is required for allogeneic skin substitute tolerance as it is assumed that the body will naturally replace the cells within the substitute as they die, eventually repopulating the dermis and epidermis with autologous cells. Of importance, and an area where understanding is incomplete, is the mechanism by which the IDO environment induces angiogenesis and the potential to harvest this property in therapeutics where vascularity is compromised. In conclusion, once a safe method of IDO expression in skin substitutes has been established, allogeneic IDO skin substitutes can be an alternate method for closure of full thickness burn wounds in practice.

CONFLICT OF INTERESTS

The authors state no conflict of interest.

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