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Transgene-specific host responses in cutaneous gene therapy: the role of cells expressing the transgene

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SUMMARY

A major issue in long-term gene therapy is host immune responses to therapeutic cells when transgene encodes a potential antigen. The nature of these responses depends on several factors including the type of cell and tissue expressing the transgene. Keratinocytes and fibroblasts, which are known to display distinct immunogenic profiles, are both potential targets for transgene expression in cutaneous gene therapy. However, whether there is an immunological advantage in targeting one cell type over the other is not known. To study the effect of cell type on transgene-specific host responses independent of antigen levels or methods of gene transfer and transplantation, we used a skin transplantation model in which transgene expression can be targeted transgene to either keratinocytes or fibroblasts. Although targeting an antigen to either cell type resulted in the induction of immune responses, these responses differed significantly. Transgenic keratinocytes were rejected acutely by a dominant Th2 response, while in the majority of grafted animals transgenic fibroblasts failed to induce acute rejection despite the induction of Th1 type inflammation in the graft. In a small number of mice, transgenic fibroblasts persisted for at least 20 weeks despite elicitation of antigen-specific responses. Therefore, fibroblasts may be an immunologically preferred target over keratinocytes for cutaneous gene therapy.

Keywords

Skin; Gene therapy; Fibroblast; keratinocytes; Immune responses

INTRODUCTION

A number of debilitating skin diseases, especially those caused by inherited single gene mutations, are candidates for gene therapy. As these diseases are life-long disorders, any gene-based therapy will require long-term expression of the corrective gene.1,2 Despite advances in gene transfer methodologies, a major issue for long-term expression is the potential for host responses against the cells expressing the therapeutic gene product, especially if the disease causing mutation results in no protein or a truncated protein.2-4The

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first report of a successful trial in humans was for junctional epidermolysis bullosa (EB) and used autologous, genetically modified keratinocytes.5 In this instance, the disease causing mutation was both a null and a point mutation in the LAMB3 gene resulting in a deficiency of laminin-5. Fortunately, no immune responses were reported to the gene-altered cells one year after gene transfer mainly because of the patient's compound mutation allowed residual synthesis making the patient tolerant to transgene product.5 Nevertheless, host responses to neoantigen production are likely to become central issues in future trials for this and other genodermatoses. In this study we report on differential host responses to neoantigen production depending on whether the gene-altered cells are keratinocytes or fibroblasts.

The nature of host responses in gene therapy depends on various factors including the immunogenicity of the transgene product, the method of gene transfer and the type of cell or tissue producing the gene product.6,7 To study host responses specifically in the case of cutaneous gene therapy, we have developed models for both *ex vivo* and *in vivo* transduction to skin components in immunocompetent mice.8,9 Direct retroviral mediated gene transfer to epidermis (*in vivo* method) or orthotopic transplantation of *ex vivo*-transduced keratinocytes to transgene-tolerant mice results in long-term transgene expression in epidermis. In non-tolerant mice however, transgene-specific responses lead to rejection of the genetically modified keratinocytes regardless of the method of gene delivery.8,9 Although transduced cells are rejected acutely whether the method of gene delivery is *ex vivo* or *in vivo*, the mechanism of immune rejection appears to differ depending on whether an *ex vivo* or an *in vivo* method of gene transfer was used. The loss of transgene expression following direct gene transfer to mouse skin is associated with Th1-type responses, while implantation of *ex vivo*-transduced keratinocytes expressing an intracellular antigen induces a Th2-biased response. This Th2 response is highlighted by an increased secretion of IL-4 and IL-5 by T lymphocytes and infiltration of mixed infiltrates including numerous eosinophils into the transduced skin.10 Keratinocytes are known to express a wide variety of soluble mediators of inflammation that regulate immune responses by influencing multiple different resident cell types including macrophages, dendritic cells (DC), neutrophils, lymphocytes, eosinophils and mast cells.11 Whether Th2/allergic inflammatory responses to antigenic transgene expression in keratinocytes are specific to this cell population or to the skin microenvironment, or are a characteristic of the *ex vivo* gene transfer is not clear.

Both fibroblasts and keratinocytes are potential targets for therapeutic gene transfer depending on the nature of the disease. In instances such as correction of dystrophic EB when either fibroblasts or keratinocytes could be modified to supply therapeutic protein, fibroblasts have been proven to be the preferred target over keratinocytes, as the gene-corrected fibroblasts supplied higher amount of collagen VII to the basement membrane that the gene corrected keratinocytes.12 Although the nature of host responses to genetically modified fibroblasts has not been studied in detail, the known differences in susceptibility of dermal and epidermal allografts to rejection following transplantation suggest key differences between the immunogenic profiles of keratinocytes and fibroblasts.13-16 Unlike keratinocytes, fibroblasts have long been considered to be minimally immunogenic.17,18 *In vitro* data support immunosuppressive effects of dermal fibroblasts on allogenic T cell proliferation,19 although the immunoregulatory effects of fibroblasts *in vivo* are more

controversial. Some studies have reported that allogeneic fibroblasts are accepted across the histocompatibility barriers after transplantation,15,16,20 while others have shown that fibroblasts sensitize the host to alloantigens or secreted antigens.17,21-24

To compare host responses to transgene expression in fibroblasts and keratinocytes independent of antigen levels, gene transfer technique or transplantation method, we used an orthotopic murine skin transplantation method in which a mixture of keratinocytes and fibroblasts are implanted onto an excised wound bed on the back of a syngeneic mouse to regenerate skin.9 We used enhanced green fluorescent protein (GFP) as a model antigen and targeted expression to either keratinocytes or fibroblasts. In this way we were able to analyze transgene-specific immune responses as well as the fate of the transgenic cells. Our studies showed that, although transgene targeted to either cell population induced transgene-specific responses, the nature of these immune responses and the kinetics of immune rejection were significantly different for these two cell types.

RESULTS

Analysis of transgene expression in fibroblasts and keratinocytes before and after transplantation

To obtain comparable levels of transgene expression in keratinocytes and fibroblasts, GFP-expressing cells were isolated from FVB-Nagy-GFP mice in which expression of GFP is controlled by actin promoter and therefore is expressed in all nucleated cells.25 Analysis of GFP expression in cultured fibroblasts and keratinocytes by western blot (Fig. 1A) and flow cytometry (Fig. 1B) verified comparable levels of expression.

We have previously shown that epidermal cells maintained for more than 5 days in culture were completely depleted of antigen presenting cells (APCs), thereby reducing the possibility of direct antigen presentation by transplanted GFP-expressing APCs.9 There are resident populations of APCs in dermis including macrophages and dermal DCs. To examine the levels of contaminating APCs in cultures established from dermal cells, levels of CD11c transcript, a monocyte/dendritic cell-specific marker, were analyzed in primary and secondary cultures of dermal fibroblasts by RT-PCR. As shown in Fig. 1C, significant levels of CD11c transcripts were detected in primary cultures of fibroblasts (Fig. 1C, P0) confirming contamination of dermal preparations with APCs. However, CD11c transcript levels dropped precipitously to undetectable levels after passaging (Fig.1C, P1 and P2) indicating a progressive loss of APCs in culture. Therefore, to limit GFP expression to fibroblasts and avoid direct antigen presentation by APCs, cells recovered from mouse dermis were cultured and passaged twice before transplantation.

Previously we had shown that primary cultures of dermal fibroblasts support skin reconstitution in orthotopic transplants. To determine whether cultured fibroblasts maintain that capacity, GFP-expressing fibroblasts (FB-GFP) were mixed with non-transgenic isogenic keratinocytes (H-2^q) and used to reconstitute skin on GFP-tolerant mice (Gad-GFP, H-2^q) in which GFP is expressed in brain but not skin.9,26 In some animals, GFP-expressing keratinocytes (KC-GFP) were mixed with non-labeled isogenic fibroblasts and implanted onto GFP-tolerant mice as described previously.9 Skin grafts were examined

weekly for surface GFP expression using a fluorescent stereoscope. As shown in Fig. 1D, cultured transgenic fibroblasts contributed to skin formation by repopulating the dermal compartment of the regenerated skin. Surface GFP was detected whether GFP was targeted to keratinocytes or fibroblasts (Fig. 1D, top panels), although the signal from KC-GFP was brighter due the relative position and cell density in the epidermis. Analysis of tissue sections obtained at 6 weeks post-transplantation by fluorescent microscopy demonstrated that cultured fibroblasts survived *in vivo*, contributed to the formation of dermis, and did not migrate beyond the graft boundaries (Fig. 1D). Moreover, GFP expression persisted for the entire observation period of 20 week (Fig. 2) indicating that stable and long-term engraftment of genetically modified fibroblasts is achievable in the absence of transgene-specific immune response.

Fate of antigen-expressing fibroblasts and keratinocytes in immunocompetent mice

To determine the fate of dermal fibroblasts expressing an antigen, FB-GFP were mixed with syngeneic non-labeled keratinocytes and used to reconstitute skin on the back of adult FVB mice (H-2^q) or Gad-GFP mice. For a comparison, KC-GFP were mixed with non-labeled fibroblasts and implanted onto FVB mice as described above. Skin grafts were examined weekly for surface GFP expression and clinical signs of graft rejection. Analysis of surface GFP in tolerant mice demonstrated continued GFP expression in all grafted mice for the entire period of observation with no reduction in GFP levels or graft size (Fig. 2 and Supplementary Fig.S1). As expected, FVB mice grafted with KC-GFP showed progressive loss of surface GFP starting at 3 weeks post-grafting with a complete loss of grafted skin in 4-5 weeks post-grafting (Fig. 2). The loss of surface GFP in this group was accompanied by clinical signs of acute graft rejection, including inflammation, erythema, contracture, necrosis and hair loss (Fig. 4 M). In mice grafted with FB-GFP however, there was significant variation in the kinetics of GFP loss (Fig. 2). In 25% of grafted mice (n=5), surface GFP was lost at week 3 post-grafting with no obvious change in appearance of grafted skin (Supplementary Fig. 1S). In the remaining animals (n=15) transgenic fibroblasts failed to induce acute rejection and surface GFP continued to be detected for at least 7 weeks. In these animals GFP was lost either between 7-10 weeks post-grafting (n=11), or persisted for the entire observation period of 20 weeks (n=4) (Fig. 2) suggesting long-term engraftment of transgenic fibroblasts in a small number of animals. The chronic loss of transgenic fibroblasts in the majority of skin grafts in normal mice but not in GFP-tolerant mice suggests immune-mediated clearance of transgenic fibroblasts, although the slow kinetics of rejection suggests a different type of immune response was responsible for rejection of keratinocytes.

Analysis of antigen-specific immune responses to transgenic fibroblasts and keratinocytes

To characterize host immune responses to antigen expressed in fibroblasts, GFP-specific immune responses were analyzed in mice grafted with FB-GFP and compared to those induced against KC-GFP. ELISA analysis of sera collected at 4, 6 or 10 weeks post-grafting for anti-GFP immunoglobulin G (IgG) showed substantial quantities of GFP-specific antibodies at 4 weeks post-transplantation with a steady increase in antibody titers in all FVB mice grafted with FB-GFP, regardless of the kinetics of GFP loss (Fig. 3A). Antibody

titers however, were significantly lower (about three fold) in mice grafted with transgenic fibroblasts as compared to those grafted with KC-GFP (Fig. 3A).

To analyze cell-mediated responses, splenocytes were isolated from mice grafted with FB-GFP or KC-GFP and T cell proliferative responses to GFP were assessed in a mixed lymphocyte reaction. As shown in Fig. 3B, there was a significant increase in proliferation of T cells from FVB mice grafted with either KC-GFP or FB-GFP but not those isolated from grafted GFP-tolerant mice. These data indicate that expression of an intracellular antigen in fibroblasts is sufficient to prime T cells and generate humoral and cell-mediated responses.

Histological analysis of skin grafts containing transgenic keratinocytes or fibroblasts

The local inflammatory responses to antigen expression by either fibroblasts or keratinocytes were examine in histological skin samples obtained at 4 weeks post-grafting. The results indicate substantial differences in tissue architecture as well as in intensity and type of inflammatory cells between the two cell types (Fig. 4, I and O). Rejection of KC-GFP grafts was accompanied by a mixed inflammatory infiltrates, epidermal hyperplasia and loss of hair follicle integrity and tissue architecture (Fig. 4O). In contrast, skin containing FB-GFP showed a mildly hyperplastic epidermis. Hair follicles were intact despite significant inflammatory infiltrates around the follicles and dermal papilla (Fig. 4I). The infiltrating cells included CD4+ cells, CD8+ T cells, and macrophages, and were present in all FVB mice grafted with FB-GFP including those which failed to exhibit acute rejection (Fig.4.G-L). This infiltrate however, was specific to antigen expression (i.e., GFP) as it was completely absent from grafts of Gad-GFP mice in which GFP is not an antigen (Fig. 4C-F). In these mice, the number and distribution of inflammatory cells were comparable to that of the normal skin (Fig. 4 C-F). Comparative analysis of inflammatory cells infiltrating skin grafts containing transgenic fibroblasts or keratinocytes showed a comparable number of CD4+ and CD8+ T cells but a significant reduction in the numbers of macrophages and B cells, and a complete lack of eosinophilic infiltrates when GFP was targeted to fibroblasts rather than keratinocytes (Table 1). These data indicate that expression of a single antigen in either fibroblasts or keratinocytes initiates an inflammatory reaction but recruits different types of immune cells to the site of transgene expression.

Tissue responses to antigenic transgene expression targeted to keratinocytes or fibroblasts

Significant differences in the histopathology of skin grafts containing antigenic fibroblasts or keratinocytes suggests different types of immune responses mediating rejection of transgenic cells. To further evaluate tissue immune responses, transcript levels of several cytokines including IFN-γ and TNF-α (Th1 cytokines), IL-4 and thymic stromal lymphopoietin (TSLP) (Th2 cytokines), and TGF-β (Treg cytokine) were evaluated in the grafted tissue samples by quantitative RT-PCR. GFP-expressing grafts of GFP-tolerant mice served as controls. As shown in Fig. 5, significant levels of Th1 cytokines, IFN-γ and TNF-α were detected in the grafts whether antigen was targeted to keratinocytes or fibroblasts, although IFN-γ levels were significantly higher in the latter indicating a dominant Th1 response to antigen-expressing fibroblasts. Expression of Th2 cytokines, IL-4 and TSLP was

restricted to FVB mice grafted with KC-GFP, consistent with a dominant Th2 responses/ allergic inflammatory responses.10 TGF-β which is expressed by regulatory T cells and plays an immunosuppressive role,27 was significantly suppressed in grafted skin of FVB mice containing either KC-GFP or FB-GFP when compared to the grafted skin of GFP-tolerant mice. These data indicate that the majority of T cells infiltrating the grafted keratinocytes or fibroblasts are activated effector T cells. Despite a distinct Th1 polarized responses to transgenic fibroblasts, these cells survive acute rejection. Furthermore, Th2 type responses are induced only to antigen expression in keratinocytes.

In vitro studies have shown that dermal fibroblasts inhibit allogenic T cell activation at least partly through IFN-γ-mediated induction of indolamine 2,3-deoxygenase (IDO) in fibroblasts.19 Therefore, chronic rejection of fibroblasts may be related to IFN-γ-mediated induction of IDO in transgenic fibroblasts. However, analysis of the grafted tissue samples for IDO transcript levels demonstrated that despite the higher levels of IFN-γ in skin grafts containing FB-GFP, IDO expression was comparable to that of control grafts and was significantly lower than that of KC-GFP grafts (Fig. 5). IFN-γ-induced expression of IDO in human keratinocytes in the skin lesions of patients with atopic dermatitis or psoriasis have also been reported and may function as a feedback mechanism to control inflammation.28

DISCUSSION

Using a model of *ex vivo* cutaneous gene therapy, we compared immune responses to an antigenic transgene product expressed in two major skin cell types, fibroblasts and keratinocytes, and noted distinct immunogenic profiles. Expression of an intracellular antigen in either fibroblasts or keratinocytes was sufficient to prime T cells and to induce antigen-specific responses. However, there was a significant difference in the nature of these responses and the kinetics of transgene loss. Transgenic keratinocytes were rejected relatively rapidly by a dominant Th2 inflammatory response, while transgenic fibroblasts induced a dominant Th1 type inflammation in the graft. Therefore, the induction of Th2/ allergic inflammation to antigenic transgene expression is unique to keratinocytes not to the method of gene transfer or transplantation.

The dose of antigen is a determinant of the Th1/Th2 decision, with high doses inducing Th1 polarization and low doses inducing Th2 polarization.29 However, the observed differences in host responses to fibroblasts and keratinocytes could not be simply explained by antigen dose since the same number of fibroblasts and keratinocytes were implanted and GFP levels were comparable or even slightly higher in keratinocytes (Fig. 1). Moreover, our previous studies with keratinocytes in which transgene expression was controlled by a cellular promoter and there was a six fold difference in antigen expression levels, or when only a fraction of keratinocytes (10-20%) were transduced, did not show a significant impact on the intensity and type of transgene-specific host response.10

Our experimental model was designed to examine host responses to an intracellular transgene product. Whether similar Th1/Th2 polarization occurs when either a membrane-associated antigen or a secreted antigen is expressed by fibroblasts or keratinocytes remains to be determined. The sub-cellular localization of expressed protein has been shown to

influence the nature of the induced immune responses in DNA immunization.30,31 However, direct gene transfer results in transfection of APCs and differential processing of cell-associated and secreted proteins.32,30 In the absence of direct presentation of antigenic transgene by donor APCs in our transplantation model, T cell priming and activation is likely mediated by cross-presentation of antigen released from either fibroblasts or keratinocytes to the host APCs.33 It is clear that some DC subsets bias Th1/Th2 polarization. As skin contains numerous APCs including Langerhans cells, dermal DC, and macrophages, the uptake and processing of antigen from keratinocytes and fibroblasts by different subset of APCs may explain the polarization of immune responses elicited to antigen expression by these two cell types.34-36 Among cells that drive Th2 differentiation are TSLP-activated DCs.37 TSLP represents a key keratinocyte-derived cytokine that creates a Th2-permissive microenvironment and directly triggers DC-mediated allergic inflammation.37-39 The specific upregulation of TSLP transcript in skin grafts containing antigenic keratinocytes may explain induction of Th2 polarized responses to antigen expression in keratinocytes but not fibroblasts.

The extensive tissue damage associated with rejection of transgenic keratinocytes was not observed during rejection of transgenic fibroblasts, even in the small number of mice in which transgenic fibroblasts were rejected acutely in week 3-4 post-grafting (Supplementary Fig. S1). The extensive tissue damage associated with the rejection of transgenic keratinocytes is a hallmark of Th2 responses. Th2 lymphocytes produce cytokines such as IL-4 and IL-5 that promote eosinophil infiltration and degranulation.29 Eosinophils exert cytotoxic activity through the release of several molecules such as leukotriene, superoxides, major basic protein, and eosinophil cationic protein that mediate tissue destruction.40 Noteworthy, we have previously shown that suppression of eosinophilic infiltrates in mice grafted with transgenic keratinocytes results in suppressed tissue destruction and delayed rejection of transgenic keratinocytes suggesting a key role for eosinophils in acute rejection of transgenic keratinocytes.10

The majority of mice transplanted with antigenic fibroblasts failed to induce acute rejection, although a significant number of $CD4^+$ cells, $CD8^+$ T cells and macrophages infiltrated the regenerated dermis. In these mice, rejection of fibroblasts followed a slow kinetics over a period of 7-10 weeks post-grafting and in about 20% of grafted animals, transgenic fibroblasts persisted for the entire observation period of 20 weeks. There was no correlation however, between the induction of immune responses (using antibody responses as an indicator) and long-term survival of transgenic fibroblasts. Although, histological analysis of grafted tissue surviving after 20 weeks showed no significant inflammatory infiltrates, these animals showed sustained and high levels of serum anti-GFP IgG comparable to cohort mice in which grafts were lost (data not shown). Considering that intense inflammatory infiltrates were present in all grafts analyzed at 4 weeks post-grafting (n=5), it is likely that initial tissue infiltrates were present in surviving grafts but were cleared at a later time. Although the mechanism of survival of fibroblasts in some animals remains to be investigated, the inability of mice with long-term surviving grafts to accept second grafts of GFP-expressing keratinocytes argues against induction of antigen-specific tolerance (data not shown). Furthermore, the lack of a significant difference in intragraft IDO, TGFβ, IL10 and FoxP3 transcript levels between keratinocytes and fibroblast grafts at 4 weeks post-grafting does

not support a role for Treg cells in suppressing immune responses to antigen expression in fibroblasts (Figure 5 and data not shown). Similar results were seen when fibroblasts expressing human FIX were implanted subcutaneously in rabbits. In this study 2 out of 15 animals showed long-term expression of transgene despite measurable titers of FIX-specific antibodies.22 Whether this is the result of active immune suppression, ignorance, or inefficient killing of fibroblasts needs further investigation.

In conclusion, our studies have demonstrated that despite the low immunogenic profile of fibroblasts, antigen targeted to these cells could prime T cells and induce humoral and cellular immune responses, although the nature of these responses was different than those induced to keratinocytes. Our data suggest that different strategies will be required to modulate or control destructive immune responses in gene therapy targeted to different cell types in a tissue.

MATERIALS AND METHODS

Animals, cells and skin transplantation

FVB/NJ, FVB-GadGFP and FVB-GFPNagy transgenic lines were purchased from the Jackson Laboratories (Bar Harbor, MA, USA). All strains of mice used for transplantation were male and between 6 to 8 week of age. Animal studies were performed in accordance with the institutional guidelines set forth by the State University of New York. Epidermal and dermal cells were prepared from 1-2 day old mouse skin using standard procedures.9 Epidermal cells were seeded on collagen coated plates in keratinocyte-serum free media (Invitrogen, Grand Island, NY, USA) containing 0.3mM calcium for 6 hours to allow attachment, then media was changed to 0.05 mM calcium to allow optimum condition for cell proliferation. Five to seven days later keratinocytes were harvested for transplantation. Dermal cells were cultured in DME containing 10% fetal bovine serum and passages twice before harvested for transplantation.

For transplantation, 4×10^6 cultured epidermal cells were mixed with 4×10^6 cultured dermal fibroblasts (isolated either from FVB or Nagy-GFP mice) and implanted as a slurry (150 μl volume) onto the fascia but under a silicon chamber implanted onto the back of an anesthetized mouse.9 After one week, chambers were removed and wounds were allowed to heal. A well-developed skin appeared 7-10 days thereafter.

Detection of GFP expression in skin

GFP expression in cultured cells was determined either by flow cytometry using a FACSCaliber (BD Immunocytometry system, San Diago, CA) or by western analysis. Equal amounts (40 μg) of total keratinocyte and fibroblast lysates were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes, probed with a polyclonal anti-GFP antibody (Clontech, Mountain View, CA) and developed with chemiluminescent detection system (ECL kit, Amersham Biosciences, Piscataway, NJ). As a control 25 ng of recombinant GFP was included in the western analysis.

To assess GFP expression in grafted skin of live animals, animals were anesthetized and placed under a fluorescent stereoscope (Bio 2M, Zeiss Inc., Thornwood, NY) equipped with

a mercury lamp and wide band filter set for GFP. Images were captured using a Nikon Coolpix 995 (Nikon Instrument Inc. Melville, NY) and processed using Adobe Photoshop. For detection of GFP expression in tissue sections, samples were fixed in cold 4% paraformaldehyde for 30 min prior to embedding in OCT. Frozen sections were dried and rehydrated, mounted in Vectashield mounting media with DAPI (Vector Laboratories, Inc. Burlingame, CA, USA), examined and photographed with an epifluorescent Eclipse 800 microscope (Nikon Instrument Inc. Melville, NY) equipped with image analyzing software.

Immunofluorescent analysis

Cryosections were rinsed with PBS, dried and fixed with cold acetone for 2 min. Fixed tissue sections were blocked in 5% non-fat milk in PBS and stained with 1 μg/ml of rat anti-mouse CD4, CD8a, B220 and F4/80 monoclonal antibodies (BD-Pharmingen, San Diego, CA) for 60 min. Antibodies were detected using the Alexa 594-conjugated goat anti-rat antibody (Molecular probes, Eugene, OR). Cellular infiltrates were assessed in 5 randomly selected fields at 400X magnification.

Analysis of GFP-specific responses in grafted mice

Antibody responses to GFP were determined by ELISA as described previously 10 To determine GFP-specific lymphocyte proliferation, splenocytes were incubated in 96-well plates (triplicates, 2×10^5 cells/well) for 72 hrs with an equal number of mitomycin-treated GFP-labeled or non-labeled splenocytes. Cells were pulsed with 1 μCi ^{3}H-thymidine/well for the last 16 hrs and harvested using a semi-automatic harvester (Skatron Instrument., Lier, Norway). Thymidine uptake was measured with a scintillation counter.

RNA analysis

For detection of low levels of contaminating APCs in culture, total RNA was isolated from primary and secondary cultures of fibroblasts using Trizol reagent (Gibco/BRL, Grand Island, NY). RNA samples (0.2 ug) were analyzed by RT-PCR (OneStep RT-PCR kit, Qiagen, Hilden, Germany) using primers specific to cd11c gene, a marker for dendritic cells. 9 Pgk-1 primers were used as controls to ensure RNA integrity and equal loading. PCR products were analyzed by 1 % agarose electrophoresis with ethidium bromide staining. Relative quantification of cytokine mRNA expression in RNA isolated from skin biopsies was performed by quantitative real-time PCR using a 7300 Real Time System (Applied Biosystem, Foster City, CA). The PCR reaction was run with Syber green Taq polymerase for 45 cycles of: 95°C, 15 s and 60°C, 1 min. All samples were run in triplicate and non template controls were included in each run. The RNA levels of the target genes were normalized against Pgk-1 transcript levels and the comparative $C_T(^{2-\Delta\Delta CT})$ method41 was used for calculating relative cytokine mRNA expression. The PCR efficiencies, as determined by assaying serial dilutions of RNA, were approximately equal for the target genes and the housekeeping genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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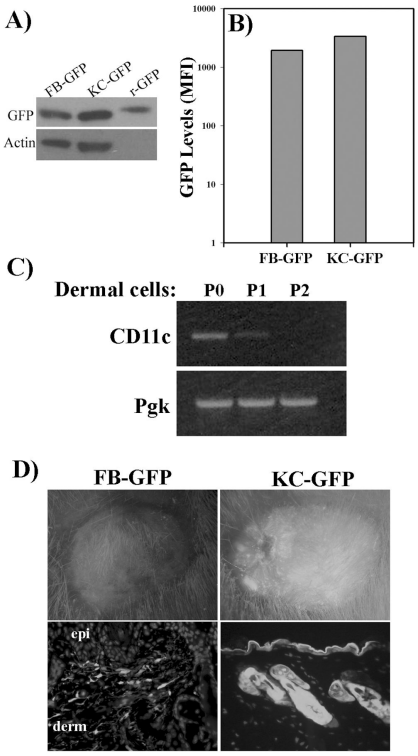

Figure 1. Analysis of GFP expression and stable engraftment of fibroblasts and keratinocytes

A) Keratinocytes (KC-GFP) and fibroblasts (FB-GFP) were isolated from Nagy-GFP mice and total cell lysates were analyzed for GFP and actin by western blot. Recombinant GFP (r-GFP) was included to indicate size and concentration of GFP. B) GFP levels in intact cells were quantified by flow cytometry using median fluorescent intensity (MFI). C) RT-PCR analysis of dermal cultures for contaminating APCs. Total RNA isolated from primary cultures of dermal cells (P0), after one passage (P1) or two passages (P2) was analyzed by RT-PCR using primers specific for CD11c (upper panel) or phosphoglycerate kinase-1 (lower panel). D) Skin reconstitution by cultured FB-GFP or KC-GFP mixed with non-labeled keratinocytes or fibroblasts respectively, on Gad-GFP mice (GFP-tolerant). Surface GFP (upper panels) and tissue epifluorescent (lower panels) at 6 weeks post-transplantation are shown. Sections were counterstained by DAPI (nuclear staining).

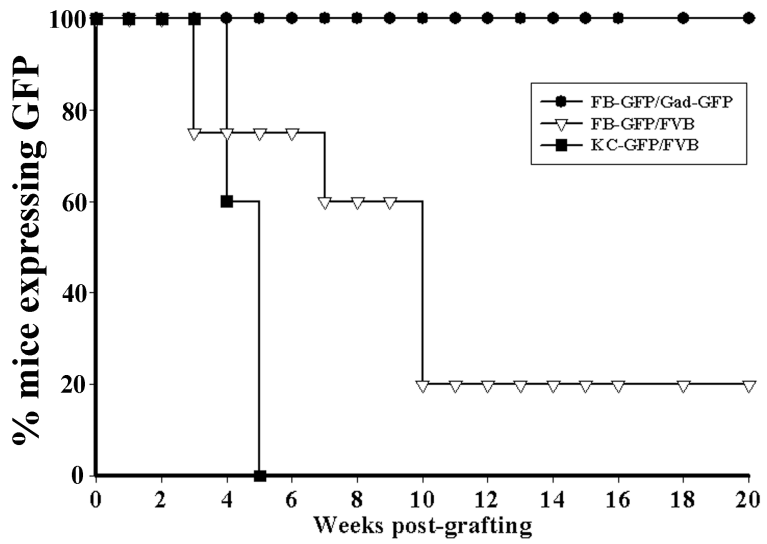

Figure 2. Kinetics of graft rejection in mice grafted with transgenic fibroblasts or keratinocytes

Survival curve of grafted GFP^+ cells during a 20 week period after transplantation of transgenic fibroblasts (FB-GFP/FVB, n=20) or keratinocytes (KC-GFP/FVB, n=10) onto FVB mice. Surface GFP was monitored using a fluorescent stereoscope. As a control, FB-GFP grafts were transplanted onto Gad-GFP mice that are tolerant to GFP (n=5).

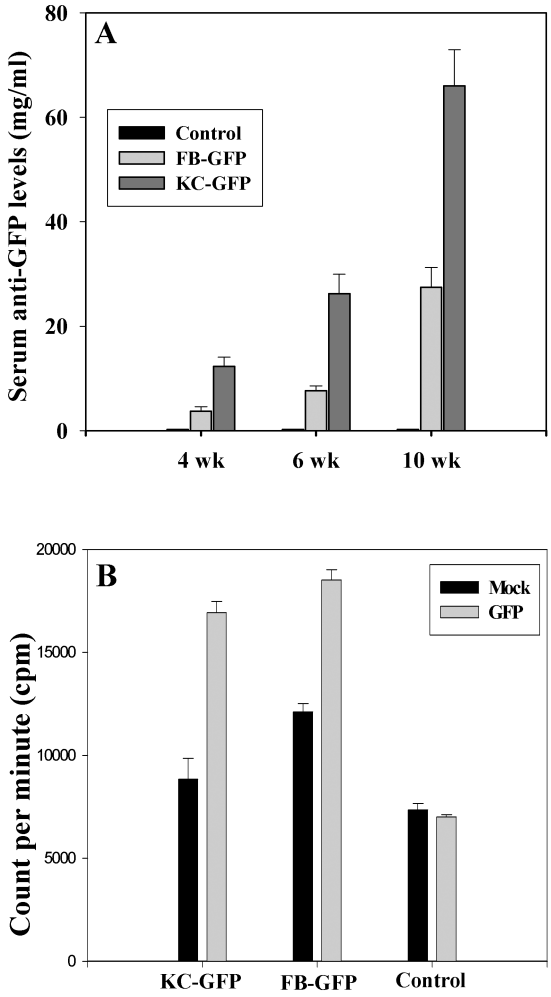

Figure 3. Immune activation in FVB mice grafted with transgenic fibroblasts expressing an antigen

A) Sera were collected at 4, 6 or 10 weeks post-grafting and assessed for the presence of anti-GFP IgG by ELISA using a monoclonal anti-GFP antibody as a standard. Error bar represent SE. *P<0.005 for KC-GFP and FB-GFP grafted FVB mice (n=10). B) GFP-specific lymphocyte proliferation at 4 weeks post-grafting. Splenocytes were isolated from grafted mice and cultured for 72 hr with mitomycin-treated GFP-expressing splenocytes or mock splenocytes. Lymphocyte proliferation was determined by measurement of ^{3}H-thymidine incorporation during the last 16 hrs of culture (n=3/group). Error bar represent SE. *P<0.05 for GFP-stimulated and mock-stimulated.

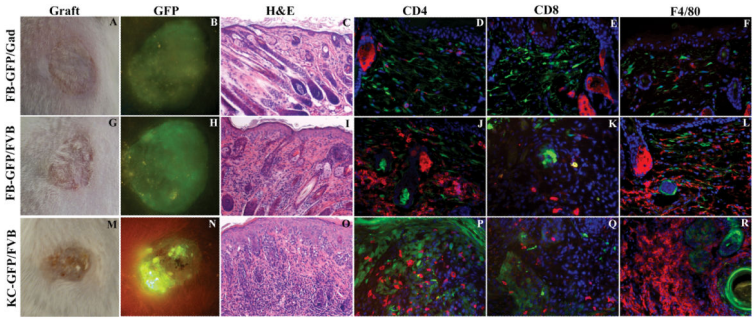

Figure 4. Histological and immunochemical analysis of skin grafts containing transgenic fibroblasts or keratinocytes

Gross appearance and surface GFP expression in grafts containing FB-GFP are shown in Gad-GFP mice (A-B) and FVB mice (G-H) or in grafts containing KC-GFP in FVB mice (M-N) at 4 weeks post-transplantation. Hematoxylin/eosin histological sections (C, I, O) and immunostaining with anti-CD4 (D, J, P), anti-CD8 (E, K, Q) or anti-F4/80 antibodies (F, L, R) of FB-GFP (C-F, I-L) or KC-GFP (O-R) grafted skin taken at 4 weeks post-grafting from Gad-GFP (D, E, F) or FVB (D, F, H). In sections stained by immunofluorescence, GFP-expressing cells are indicated by green and antibody staining in red. Red staining in sebaceous glands is non-specific. Sections were counterstained with DAPI to visualize tissue structure and nuclei. Magnification is 100X for (C, I, O) and 400X for (D-F, J-L, P-R).

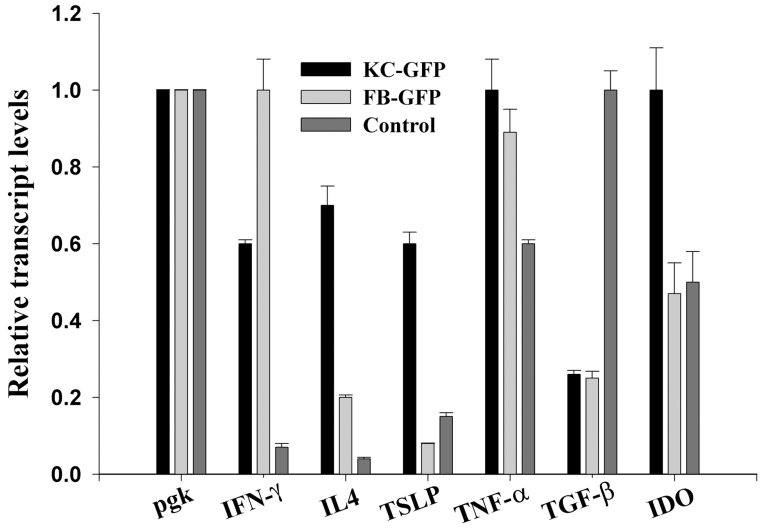

Figure 5. Profile of cytokine gene expression in transplanted tissue

Quantitative RT-PCR analysis on the transcript levels of indicated cytokines in the grafted skin tissues harvested at 4 weeks post-grafting. Transcript levels were normalized to phosphoglycerate kinase (PGK). Error bar represent SE.

Table 1

Summary of distribution of inflammatory cells in grafted skin

Cell Type	Gad-GFP	FB-GFP	KC-GFP
CD4+ Cells	13±4.8	105±38	123±30
CD8+ Cells	2±0.5	8.5±3	12±5
F4/80 + Cells	52±11	266±63	400±94
B220+ Cells	3±1.2	10±3	42±8
Eosinophils	1±1	1±1	29±7

Summary of distribution of inflammatory cells in skin reconstituted from transgenic fibroblasts (FB-GFP) or keratinocytes (KC-GFP) on FVB mice or from transgenic fibroblasts on GFP-tolerant mice (Gad-GFP). Cellular infiltrates were assessed in 5 randomly selected fields at 400X magnification and are expressed as Mean±SEM.