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Defects in Skin γδ T Cell Function Contribute to Delayed Wound Repair in Rapamycin-Treated Mice

Robyn E. Mills, Kristen R. Taylor, Katie Podshivalova, Dianne B. McKay, and Julie M. Jameson

Disruptions in the normal program of tissue repair can result in poor wound healing, which perturbs the integrity of barrier tissues such as the skin. Such defects in wound repair occur in transplant recipients treated with the immunosuppressant drug rapamycin (sirolimus). Intraepithelial lymphocytes, such as γδ T cells in the skin, mediate tissue repair through the production of cytokines and growth factors. The capacity of skin-resident T cells to function during rapamycin treatment was analyzed in a mouse model of wound repair. Rapamycin treatment renders skin γδ T cells unable to proliferate, migrate, and produce normal levels of growth factors. The observed impairment of skin γδ T cell function is directly related to the inhibitory action of rapamycin on mammalian target of rapamycin. Skin γδ T cells treated with rapamycin are refractory to IL-2 stimulation and attempt to survive in the absence of cytokine and growth factor signaling by undergoing autophagy. Normal wound closure can be restored in rapamycin-treated mice by addition of the skin γδ T cell-produced factor, insulin-like growth factor-1. These studies not only reveal that mammalian target of rapamycin is a master regulator of γδ T cell function but also provide a novel mechanism for the increased susceptibility to nonhealing wounds that occurs during rapamycin administration. The Journal of Immunology, 2008, 181: 3974–3983.

The ability of epithelial tissues to rapidly repair and regenerate in the face of damage is fundamental to barrier maintenance. During normal wound healing, a delicate balance of factors and cell types orchestrates the process in a precisely timed manner. Disruptions can result in the debilitating condition of a chronic, nonhealing wound. Much effort has been directed toward determining which factors affect wound repair processes in chronic wounds, but there is less known about what may regulate the cellular sources of the secreted factors important in proper wound healing (1–5).

Nonhealing wounds are exhibited by patients administered the immunosuppressant rapamycin (also called sirolimus) (6–10); however, the mechanisms contributing to this wound healing defect are unclear. Rapamycin is commonly administered for the prophylaxis of acute rejection of transplanted solid organs, used to coat arterial stents, and it is currently being examined in clinical prophylaxis of acute rejection of transplanted solid organs, used to coat arterial stents, and it is currently being examined in clinical trials for the treatment of hematological cancers (11–14). Rapamycin is known to be a potent inhibitor of certain effector αβ T lymphocyte populations (15–19), while other populations such as regulatory T cells exhibit a selective survival (20). In other studies, cytokine-driven responses by peripheral naive T cells are not affected by rapamycin, suggesting a reliance on other signaling molecules such as Pim-1 and Pim-2 (21). However, the effects of rapamycin on intraepithelial lymphocytes (IELs)3 such as skin γδ T cells have not been evaluated.

Closely associated with the epithelia is an interdigitating population of resident lymphocytes expressing the γδ TCR (22). Skin γδ T cells express a canonical Vγ8Vδ1 TCR that recognizes an unidentified self Ag expressed on damaged or stressed keratinocytes (23, 24). Intraepithelial γδ T cells are thought to act as primary responders to damage or disease due to their ability to sense and respond to epithelial damage or disruption (25–27). Specialized roles have been attributed to skin γδ T cells in the regulation of wound repair, epithelial homeostasis, cutaneous malignancy, and contact hypersensitivity (27–30).

Skin γδ T cells produce cytokines, chemokines, and growth factors with both autocrine and paracrine functions. Homeostatic production of growth factors such as insulin-like growth factor 1 (IGF-1) by skin γδ T cells maintains skin homeostasis (30). IGF-1 is a peptide hormone known to regulate both keratinocyte and skin γδ T cell migration and survival (30–33). In the context of wound healing, production of factors such as TNF-α, IGF-1, and keratinocyte growth factor-1 (KGF-1) by skin γδ T cells promotes wound closure, reepithelialization, and inflammatory cell recruitment to the wound site (27, 30, 34, 35). To perform these functions normally, dendritic skin γδ T cells retract their dendrites, adopt a rounded morphology, and migrate to the site of trauma where they proliferate locally (27). Unlike naive αβ T lymphocytes, skin γδ T cells reside in the epidermis in a preactivated state. They exhibit constitutive IL-2 promoter activation (36) and expression of activation markers CD25 (IL-2 receptor α) and CD69, suggesting that they are primed for a rapid response. However, little is known about the signaling pathways that regulate this response.

3 Abbreviations used in this: IEL, intraepithelial lymphocyte; IGF-1, insulin-like growth factor-1; KGF-1, keratinocyte growth factor-1; mTOR, mammalian target of rapamycin; mTORC, mTOR complex.

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On the molecular level, rapamycin inhibits the serine/threonine kinase mammalian target of rapamycin (mTOR). mTOR is a central protein in a complex network that regulates amino acid and nutrient sensing in particular cell types through the formation of two separate protein complexes, mTORC1 (mTOR complex 1) and mTORC2 (37–39). Each multiprotein complex regulates distinct pathways of the signaling network. Rapamycin binds to the cellular protein FKBP12 and subsequently induces the dissociation of mTORC1, which consists of mTOR, raptor, and mLST8 (37, 40). This complex has downstream effects that include the regulation of cell cycle, translation machinery, and autophagy (41). The second complex, mTORC2, is involved in cytoskeletal rearrangement (42). mTORC2 is comprised of mLST8, SIN1, and rictor, and it is not immediately disrupted following rapamycin treatment in many types of cells (42). However, prolonged treatment of some cell lines with rapamycin can result in reduced levels of mTORC2 through reduction of rictor-bound mTOR, as well as decreased phosphorylation of Akt Ser473, the downstream target of mTORC2 (43). It has been controversial whether rapamycin results in anergy or apoptosis of transplant-specific effector T cells (19, 44, 45). Some groups suggest that mTOR regulates certain types of αβ T cells by impairing their ability to receive costimulation and cytokine signals (46, 47).

Herein we establish a murine model for the examination of wound healing defects mediated by rapamycin. Data presented here indicate that rapamycin negatively affects skin γδ T cell function during wound repair. Rapamycin arrests the IEL in G1 phase and blocks proliferative responses to cytokines such as IL-2, rendering them anergic. Our data show that skin γδ T cells do not undergo apoptosis when cytokine signals are suppressed by rapamycin. Instead, skin γδ T cells treated with rapamycin enter autophagy in an attempt to survive in the absence of cytokine signaling. As the skin IEL become anergic and autophagic, they exhibit impaired homoeostatic and activation-induced functions. This dysfunction is indicated by a diminished ability to produce soluble factors such as IGF-1 and TNF-α and delayed activation-induced cell migration. These effects are mediated through mTOR, as we observe reduced phosphorylation of both mTORC1 and mTORC2 downstream targets in skin γδ T cells treated with rapamycin. Finally, the addition of the skin γδ T cell-produced factor IGF-1 is able to successfully restore normal wound closure in rapamycin-treated mice. These studies demonstrate the selective inhibition of γδ T cell function in the skin of rapamycin-treated animals resulting in defective wound repair and they represent a novel pathway in the regulation of skin γδ T lymphocytes.

Materials and Methods

**Cell culture**

The skin γδ T cell line 7-17 was maintained in complete RPMI 1640 (Mediatech) supplemented with 10% heat-inactivated FBS (Omega Scientific) and 20 μM recombinant IL-2. The keratinocyte line PAM 2-12 was maintained in complete RPMI 1640 (Mediatech) supplemented with 10% heat-inactivated FBS (Omega Scientific) and 20 U/ml recombinant IL-2. The keratinocyte line PAM 2-12 was maintained in complete RPMI 1640 (Mediatech) supplemented with 10% heat-inactivated FBS (Omega Scientific) and 20 U/ml recombinant IL-2.

**Animals and wounding procedure**

TCRδ⁺/⁻ mice on the C57BL/6 background were purchased from Jackson ImmunoResearch Laboratories. C57BL/6 and C57BL/6 TCRδ⁺/⁻ mice were bred at The Scripps Research Institute and housed in specific pathogen-free conditions according to The Scripps Research Institute Institutional Animal Care and Use Guidelines. Mice were used between 10 and 16 wk of age. For rapamycin administration, mice were injected i.p. with 200 μl containing 1% rapamycin (Sigma-Aldrich) in 0.2% carboxymethylcellulose (Sigma-Aldrich) and 0.25% Tween 80 (Sigma-Aldrich) in distilled H₂O or with vehicle control daily. Since rapamycin is originally diluted in ethanol, the vehicle control contains equal amounts of diluent. For wound healing experiments, mice were administered rapamycin or vehicle control for 3 days before wounding, and daily administration was continued. Wounding was performed on mice anesthetized with isoflurane as previously described (27, 34). Briefly, the dorsal surface of the mouse was shaved, back skin and panniculus carnosus was pulled up, and one to two sets of sterile full-thickness wounds were generated using a sterile 2-mm punch tool. Wounds were left uncovered, and mice were housed individually with sterile paper bedding. In some experiments 100 ng of recombinant IFN-γ (Sigma-Aldrich) or buffer alone was applied to each wound site immediately postwounding and daily thereafter. Wounds on at least six mice were examined per condition in at least three independent experiments. For wound closure kinetics, images were acquired with a Nikon Coolpix S4 and wound size was monitored using ImageJ software (National Institutes of Health). To examine rounding of skin γδ T cells at the wound site, full-thickness wounds were generated in mouse ears using a 1-mm punch tool and wounded tissue was harvested 2 h later.

**Antibodies and flow cytometry**

FITC-, PE-, or allophycocyanin-conjugated mAbs specific for γδ TCR (GL3), CD25 (PC61), and Thy1.2 (53-2.1) were purchased from BD Biosciences. Other Abs used for flow cytometry include goat anti-anti-IFN-γ (G-17) (Santa Cruz Biotechnology), rat anti-CD69 (H1.2F3) (eBioscience), BrdU flow kit (BD Biosciences), and an annexin V apoptosis kit (BD Biosciences). Rabbit Abs specific for S6 kinase, p-S6 kinase (The389), Akt, and p-Akt (Ser473) were purchased from Cell Signaling Technology. Rat anti-Ki-67 Ag (DakoCytomation) was used for immunohistochemistry with biotin-conjugated mouse anti-rat secondary Ab (Jackson Immunoresearch Laboratories). Abs specific for CD3ε (500A2) (1 μg/ml) were used for stimulation of 7-17 cells and skin γδ T cells in epidermal sheets. Other secondary Abs used include HRP-conjugated goat anti-rabbit (Southern Biotech) and FITC-conjugated donkey anti-goat (Jackson ImmunoResearch Laboratories). For flow cytometry, a Cytofix/Cytoperm kit (BD Biosciences) was used for intracellular cytokine/growth factor staining. Cells were acquired with CellQuestPro on a BD FACS Calibur HTS (BD Biosciences) and analyzed with FlowJo software (Tree Star).

**Skin organ culture**

Skin organ cultures from C57BL/6 and TCRδ⁺/⁻ mice were established as previously described (27, 34). Briefly, gel foam (Pfizer) was soaked in media. Full-thickness biopsy wounds (2 mm) were generated and placed dermis side down on gel foam in 10% DMEM supplemented with rapamycin or ethanol control in 24-well plates. In some cases 7-17 skin γδ T cells were incubated in the presence of 20 ng/ml rapamycin or ethanol control for 15 h, stimulated for 2 h with anti-CD3ε, washed thoroughly, and plated at a density of 3 × 10⁵ cells per well. Recombinant IFN-1 was added at a concentration of 100 ng/ml to some wells. Images of wounds were acquired and kinetics of closure quantified using ImageJ software.

**Western blot analysis**

7-17 cells were incubated in starvation media for 2 h followed by culture in the presence of 20 ng/ml rapamycin or ethanol control for 2 h (p-S6 kinase) or 24 h (p-Akt). Next, cells were stimulated for various time points with 40 U/ml IL-2 and harvested in lysis buffer containing 62.5 mM Tris HCl (pH 6.8), 2% (v/v) SDS, 50 mM DTT, 10% glycerol, and 0.01% bromphenol blue. After lysis, insoluble material was removed by centrifugation at 12,000 × g for 10 min. Samples were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 1× TBS, 0.02% sodium azide, 3% BSA, and 10% goat serum. Primary Abs diluted in 1× TBS, 1% BSA, 0.2% Tween 20, 0.02% sodium azide, and 3% goat serum were incubated with the membrane overnight at 4°C. HRP-conjugated secondary Abs and ECL (Pierce Chemical) were used to detect primary Abs.

**Epidermal sheet immunofluorescence**

Wounded or nonwounded ears from rapamycin or vehicle-treated mice were excised, peeled into halves, and digested in 3% ammonium isothiocyanate (Sigma-Aldrich). Epidermal sheets were peeled and stained with PE-conjugated anti-γδ TCR, LysoTracker Green, and/or DAPI (Sigma-Aldrich). Digital images were acquired (Zeiss AxiosCam HRc). To examine migration of skin γδ T cells, ear skin was cultured in complete DMEM supplemented with 10% FBS and 20 ng/ml rapamycin in 24-well plates. For these experiments 1 μg/ml anti-CD3e (500A2) Ab or 100 ng/ml IFN-1
was added to culture. After culture, epidermal sheets were washed with PBS and processed as described above. Quantifications of dendrite number and autophagosomes were performed using Photoshop CS2 software (Adobe). More than 200 cells were counted per mouse and at least three mice were examined per condition.

**Proliferation assays**

Cells were cultured between 5 and 8 h in 10% complete DMEM supplemented with rapamycin or ethanol before addition to wells coated with stimulatory anti-CD3ε or γδ TCR Abs. In some cases, 100 ng/ml IGF-1, 40 U/ml IL-2, or 5 μg/ml Con A (Sigma-Aldrich) was added. Cells were cultured for 14–16 h before addition of 1 μCi/well [3H]thymidine (MP Biomedicals) for 8–10 h. Samples were harvested and [3H]thymidine incorporation was determined by a β-counter. To examine cell cycle, the keratinocyte line PAM 2-12 or the skin-derived system of skin organ culture (27, 49, 50), the early stages of repair has been described in humans (6–8) and rats (9, 48); however, repair has been ascribed to this defect. Wild-type C57BL/6J mice were administered rapamycin as compared with vehicle control (A). Rapamycin-treated mice exhibited a delay in wound closure as compared with those treated with vehicle. Data are means ± SEM and are representative of three experiments. At least six mice per condition have been examined in each experiment. B, Representative images from wounds acquired 4 days postwounding. C, Percentage of observed wounds remaining open 10 days postwounding. Data are means ± SD. D, Rapamycin impairs wound closure in skin from wild-type mice to a similar degree to skin from untreated TCRδ−/− mice using a skin organ culture system. E, Rapamycin inhibits the ability of 7-17 skin γδ T cells to restore normal wound closure to TCRδ−/− skin in organ culture. Data in skin organ cultures are means ± SEM and are representative of at least three experiments. *, p < 0.05 and **, p < 0.005 vs vehicle control (two-tailed, unpaired Student’s t test).

**Immunohistochemistry**

Wounded skin was excised from rapamycin or vehicle-treated mice, fixed in ethanol, and embedded in paraffin. Sections were prepared and stained with biotinylated Abs to Ki-67 followed by peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories). The presence of positive cells was revealed by incubation in metal-enhanced diaminobenzidine (Pierce Chemical) and counterstained with hematoxylin (Sigma-Aldrich). Cells with more than two positively staining vesicles were defined as autophagic.

**Results**

**Rapamycin impairs the ability of skin γδ T cells to mediate wound closure**

To examine how the mTOR inhibitor rapamycin affects wound repair, we established a murine model of wound healing in which mice were administered rapamycin daily. The defect in wound repair has been described in humans (6–8) and rats (9, 48); however, this has not been investigated in mice, and no mechanism has been ascribed to this defect. Wild-type C57BL/6J mice were administered daily with rapamycin or vehicle control for 3 days before wounding, and treatment was continued as wound closure was monitored. Wound size was measured during a period of 14 days (Fig. 1, A and B). Similar to findings in humans, rapamycin-treated mice exhibited delays in the rate of wound closure as compared with those treated with vehicle control. This defect was especially evident on days 4, 7, and 10 postwounding. Furthermore, significantly fewer wounds had completely closed on day 10 in rapamycin-treated animals as compared with wounds from vehicle control-treated animals (Fig. 1C). A delay in complete wound closure of 0–3 days was observed, similar to the delay observed in mice lacking γδ T cells (TCRδ−/− mice) (27). These data confirm that rapamycin has a negative impact on wound closure.

Given the potent immunosuppressive nature of rapamycin and the similarity of wound closure kinetics to TCRδ−/− mice, the contribution of skin γδ T cell dysfunction to impaired wound healing in rapamycin-treated mice was examined. Using a well-established system of skin organ culture (27, 49, 50), the early stages of wound healing were monitored in vitro. In cultures supplemented with rapamycin, wound closure in this skin organ culture model was inhibited, similar to our findings in vivo (Fig. 1D). The kinetics of closure observed in wounded skin treated with rapamycin were similar to those observed in skin from TCRδ−/− mice (Fig. 1D). Additionally, there was no further defect in wound repair in TCRδ−/− skin upon rapamycin treatment (Fig. 1D). To identify whether rapamycin impairs the ability of skin γδ T cells to mediate wound repair, we utilized a model we have previously established to assess skin γδ T cell wound healing functions. In this model, the addition of activated skin γδ T cells to ex vivo cultures of wounded skin from TCRδ−/− mice restores normal wound closure kinetics (27). Here, we examined whether rapamycin treatment impairs the ability of skin γδ T cells to affect early wound closure. When skin from TCRδ−/− mice was cultured with activated skin γδ T cells, early wound closure was restored as we previously reported (27) (Fig. 1E). However, rapamycin pretreatment of activated skin γδ T cells before their addition to TCRδ−/− skin organ culture impaired the ability of skin γδ T cells to restore early wound closure (Fig. 1E). These results indicate that rapamycin...
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**FIGURE 2.** Rapamycin treatment of skin γδ T cells directly inhibits IL-2 signaling through mTOR. A, IL-2 (40 U/ml) stimulates phosphorylation of p70 S6 kinase at Thr389 (a marker of mTOR activation) within 30 min, while rapamycin inhibits phosphorylation of this site. 7-17 skin γδ T cells were treated with rapamycin or ethanol control for 2 h following 2 h of starvation and were then stimulated with IL-2 for various time points. B, Akt is phosphorylated at Ser473 in skin γδ T cells stimulated with IL-2. Prolonged (24-h) rapamycin treatment inhibits this response, suggesting that activity of mTORC2 is inhibited. 7-17 skin γδ T cells were treated with rapamycin for 24 h. During the last 2 h, cells were also starved before stimulation with IL-2. Lysates were prepared for Western blot analysis.

impacts skin γδ T cell function, diminishing their capacity to mediate wound closure. This implicates the mTOR signaling cascade in wound healing functions mediated by skin γδ T cells.

Rapamycin treatment inhibits phosphorylation of the mTOR targets p70 S6 kinase and Akt in skin γδ T cells

Little is known about the signaling cascades activated in intraepidermal γδ T cells. In an effort to determine whether T cell trophic factors such as the cytokine IL-2 signal via mTOR in skin γδ T cells, we examined the molecular targets of the two mTOR complexes. Signaling through mTORC1 induces the phosphorylation of p70 S6 kinase at many sites, including Thr389 (37). To evaluate mTOR activity, the skin γδ T cell line 7-17 was stimulated with IL-2 in the presence and absence of rapamycin. Phosphorylation at Thr389 was evident in IL-2-stimulated skin γδ T cells within 15 min and was observed at higher levels by 30 min. This activation-induced phosphorylation is repressed in the presence of rapamycin (Fig. 2A), demonstrating that IL-2-mediated phosphorylation of S6 kinase is dependent on mTOR.

mTOR is a component of another complex, mTORC2, that mediates cytoskeletal rearrangement (42). Signaling through mTORC2 induces phosphorylation of Akt at Ser473 (43, 51). Although the mTORC2 complex was originally reported to be rapamycin resistant (38, 42), the phosphorylation of Akt Ser473 by mTORC2 is diminished in particular cell types after 24 h of rapamycin treatment (43). In an effort to determine whether mTORC2 activity is present in skin γδ T cells and whether this activation is rapamycin-sensitive, we treated the skin γδ T cell line with and without rapamycin, stimulated the cells with IL-2 or anti-CD3ε Abs, and evaluated Akt phosphorylation. Upon IL-2 treatment or TCR stimulation of skin γδ T cells, Akt is phosphorylated at Ser473 within 15 min. After 24 h of rapamycin treatment this phosphorylation is severely impaired (Fig. 2B). Similar to previous reports, rapamycin was unable to inhibit Akt Ser473 phosphorylation at time points earlier than 24 h of treatment (data not shown).

Skin γδ T cells are unresponsive to IL-2 or mitogen stimulation in the presence of rapamycin

IL-2-stimulated proliferation of certain αβ T lymphocyte subsets induces the phosphorylation of p70 S6 kinase in a rapamycin-sensitive manner (52). However, peripheral regulatory T cells are resistant to rapamycin treatment, suggesting that they rely on alternate pathways for proliferation (20). To examine whether skin γδ T cell proliferation is dependent on mTOR, skin γδ T cells were treated with various concentrations of rapamycin before stimulation with Abs specific for CD3ε (Fig. 3A), Con A (Fig. 3C), or γδ TCR (data not shown), or addition of IL-2 (Fig. 3B, left panel). Rapamycin treatment inhibited proliferation of the skin γδ T cell line 7-17 to either anti-CD3ε or cytokine stimulation at rapamycin concentrations as low as 10 ng/ml. Similar results were obtained with γδ T cells isolated from the skin of C57BL/6 mice (Fig. 3B, right panel). This proliferative defect could not be restored in the presence of rapamycin by supplementing the stimulus with IL-2 or IGF-1 (Fig. 3C). Removal of the inhibitor from the culture for several days restores normal skin γδ T cell proliferation (data not shown). To identify at which stage of the cell cycle rapamycin-treated skin γδ T cells arrest, BrdU incorporation and DNA content analyses were performed. Rapamycin treatment blocks skin γδ T cells from exiting the G1 phase of the cell cycle, preventing entry into synthesis (S) phase (Fig. 3D). These results suggest that skin γδ T cells depend on mTOR signaling as a major pathway for exit from G1 phase.

The bulk of the epidermis consists of keratinocytes. To examine whether rapamycin affects the ability of keratinocytes to proliferate, the PAM 2-12 cell line was incubated with BrdU for 48 h in the presence of rapamycin (Fig. 3E). In contrast to skin γδ T cells, keratinocytes retain the ability to proliferate during treatment with rapamycin. Similar results were obtained with keratinocytes pulsed with BrdU for 16 or 24 h (data not shown). Since previous studies have identified defects in keratinocyte stem cell proliferation in the presence of rapamycin (53), we monitored keratinocyte proliferation in vivo. We performed biopsy punch wounds on the skin of rapamycin- or vehicle-treated mice and stained skin sections for Ki-67 expression to identify proliferating cells (Fig. 3F). Keratinocyte proliferation at the wound site was comparable between rapamycin- and vehicle-treated mice. Using either BrdU or monitor keratinocyte turnover or Ki-67 staining to identify proliferating cells, no defect in keratinocyte proliferation was found in the presence of rapamycin. Thus, at clinically relevant doses, rapamycin specifically inhibits skin γδ T cell proliferation in the epidermal compartment. These results demonstrate cell-specific regulation of proliferation via mTOR signaling within the epidermis.

Rapamycin induces autophagy, not apoptosis, of skin γδ T cells

In several T cell populations, such as double-positive thymocytes or effector T cells, rapamycin has been shown to inhibit proliferation and increase the susceptibility to apoptosis (18, 54). In other cases, lymphocyte populations undergo macroautophagy, a process in which the cell catabolizes proteins and organelles as a survival mechanism (55). Having observed the diminished proliferative capacity of rapamycin-treated skin γδ T cells, we examined whether these cells were undergoing increased levels of apoptosis. For these experiments, skin γδ T cells were treated with rapamycin and apoptosis was examined by annexin V binding and propidium iodide staining. No increase in apoptosis was evident in skin γδ T cells treated with rapamycin (Fig. 4A). Furthermore, apoptosis is not exacerbated when rapamycin-treated skin γδ T cells are stimulated to undergo activation-induced cell death or are cytokine-starved (Fig. 4A). Additionally, rapamycin does not induce skin γδ T cells to undergo apoptosis when administered for several days in skin organ culture (data not shown). In fact, rapamycin treatment does not seem to affect the viability of skin γδ T cells either in vitro or in vivo. γδ TCR-bearing cells were quantified in epidermal sheets via immunofluorescent staining, and no measurable decrease in the number of cells per square millimeter was detected.
after up to 14 days of rapamycin treatment (Fig. 4B). Taken together, these results suggest that rapamycin treatment does not induce or exacerbate apoptosis in skin γδ T cells.

Although inhibiting mTOR does not affect skin γδ T cell survival, it is possible that this inhibition causes the cells to undergo other cellular changes that affect activation-induced functions, such as the lysosomal degradation process called autophagy. mTOR can act as a sensor to regulate the autophagy pathway (56, 57). CD4+ T cells treated with rapamycin undergo autophagy in vitro, implicating the process in T cell homeostasis (55). Autophagy is indicated by the presence of large autophagosomes in the cytoplasm. To determine whether rapamycin-mediated mTOR inhibition induces autophagy in skin γδ T cells, we used several common methods to detect autophagosomes in skin γδ T cells. First, cells were treated with rapamycin or vehicle control and stained with acridine orange to monitor the number of autophagic vesicles. Rapamycin treatment significantly increased the incidence of skin γδ T cells undergoing autophagy as compared with the basal level observed in vehicle control-treated cells (Fig. 4C).

To identify whether skin γδ T cells also undergo autophagy during rapamycin treatment in vivo, we used LysoTracker Green staining to identify the number of cells with acidic vesicles in the epidermis. In mice administered rapamycin, there were increased numbers of skin γδ T cells exhibiting large numbers of acidic vesicles (Fig. 4D). These data indicate that mTOR inhibition of skin γδ T cells not only inhibits proliferation, but also induces autophagy.

Activation-induced morphology changes in skin γδ T cells are inhibited by rapamycin

The process of autophagy preserves the cytoskeleton, preventing its degradation while recycling cellular macromolecules in an effort to stave off cell death (57). To identify whether blocking mTORC2 function with rapamycin affects the capacity of skin γδ T cells to alter their cellular morphology upon activation, skin from rapamycin-treated mice was subsequently cultured with stimulating Abs specific for CD3ε. Skin γδ T cells normally exhibit a highly dendritic morphology. Upon TCR ligation, the skin γδ T cells normally become rounded within several hours and initiate migration (Fig. 5A). However, skin γδ T cells in the epidermis of mice treated with rapamycin did not exhibit this characteristic morphology change (Fig. 5A). These results were replicated using rapamycin treatment in vitro, and once
again neither IL-2 nor IGF-1 was able to restore normal cell rounding and migration (data not shown).

One of the primary responses of skin γδ T cells to wounding is activation-induced rounding of these characteristically dendritic cells before migration to the wound edge and proliferation at the wound site (27).

**FIGURE 4.** Rapamycin promotes autophagy rather than apoptosis in skin γδ T cells. A, Inhibition of mTOR with rapamycin does not induce or augment apoptosis. 7-17 skin γδ T cells were left untreated, stimulated with Abs specific for CD3ε, or starved for 6 h in the presence of rapamycin or ethanol control. Cells were then stained with annexin V and propidium iodide (PI) to assess apoptosis and analyzed by flow cytometry. B, Similar numbers of skin γδ T cells remain in the epidermis of mice treated with rapamycin for 14 days. Epidermal sheets were prepared from mice administered rapamycin or vehicle control and stained with Abs specific for the γδ TCR. The number of γδ T cells was quantified per mm². C, Rapamycin induces skin γδ T cells to undergo autophagy. An increased number of cells treated with rapamycin exhibit more than two autophagosomes as compared with ethanol control-treated cells. D, An increased number of skin γδ T cells undergo autophagy in vivo after rapamycin administration. Epidermal sheets were prepared from mice administered rapamycin as compared with vehicle control. Sheets were stained with fluorescent Abs to the γδ TCR to identify skin γδ T cells and LysoTracker Green to identify autophagosomes. The number of autophagosomes was quantified per γδ T cell, and cells with more than two autophagosomes were considered autophagic. Arrows indicate autophagosomes within skin γδ T cells. Data represent means ± SD. *, p < 0.05 vs vehicle control (two-tailed, unpaired Student’s t test).

**FIGURE 5.** Rapamycin treatment inhibits activation-induced morphology changes. A, Skin γδ T cells from mice administered rapamycin exhibit a decreased ability to “round-up” in response to stimulation. Epidermal sheets were isolated from mice treated with rapamycin or vehicle control and stimulated with Abs to CD3ε before immunofluorescent staining. The numbers of dendrites per skin γδ T cell were quantified in at least 300 cells per mouse and are representative of at least six mice per condition. Data represent the means ± SD. *, p < 0.05 vs vehicle control (two-tailed, unpaired Student’s t test). B, Skin γδ T cells exhibit a delay in morphology changes at the wound site in rapamycin-treated mice. Epidermal sheets were prepared 2 h postwounding in mice treated with rapamycin and stained with fluorescent Abs specific for the γδ TCR. The white line indicates the wound edge. At least 150–200 cells were quantified from wounds in mice from three separate experiments.
this response in vivo, skin γδ T cells were examined from wounds isolated from rapamycin- and vehicle-treated mice. In rapamycin-treated mice, skin γδ T cells exhibited a rounded morphology, while cells from control-treated mice exhibited a dendritic morphology (Fig. 5B). Furthermore, skin γδ T cells isolated from the wound site have a reduced capacity to produce factors such as TNF-α (data not shown). These results provide further evidence that activation-induced functions of skin γδ T cells are negatively regulated by rapamycin. It is possible that normal homeostatic functions are similarly altered.

**Rapamycin negatively affects skin γδ T cell homeostatic functions**

Since mTOR has the capacity to regulate transcription and translation through p70 S6 kinase and eIF-4E binding proteins (58) and to regulate skin γδ T cell autophagy, there may be consequences of rapamycin treatment on normal cellular homeostasis. Although long-term treatment with rapamycin does not impair surface expression of activation markers such as CD25 or CD69 (Fig. 6A), skin γδ T cells isolated from mice treated with rapamycin express a reproducible reduction in levels of TCR as compared with mice administered vehicle alone (Fig. 6A). Down-regulation of TCR could have negative implications for T cell activation. Additionally, skin γδ T cells isolated from rapamycin-treated mice have reduced levels of homeostatic IGF-1 production (Fig. 6B). Constitutive IGF-1 production by skin γδ T cells has been shown to play an important role in skin homeostasis (30) by promoting keratinocyte migration and survival of both γδ T cells and keratinocytes.

**Addition of skin γδ T cell-produced growth factors such as IGF-1 restores wound closure in rapamycin-treated mice**

Since our data demonstrated that rapamycin has a negative impact on skin γδ T cell function in vivo, we examined the possibility that the addition of growth factors produced exclusively by skin γδ T cells in the epidermal compartment would restore rapid wound closure in rapamycin-treated mice. To examine this, recombinant IGF-1 was added to wounded skin in organ culture and wound closure was assessed. Addition of IGF-1 to skin treated with rapamycin did indeed restore wound closure (Fig. 7A). To assess whether addition of IGF-1 would have the same effect in vivo, mice were administered rapamycin for 3 days before wounding and daily thereafter. Upon wounding, either recombinant IGF-1 or buffer alone was applied directly to the wound. Treatment of wounds in rapamycin-treated mice with IGF-1 restored normal rates of wound healing (Fig. 7B). Notably at days 4, 7, and 10, when the greatest difference between rapamycin-treated and vehicle-treated mice was evident (Figs. 1A and 7B), addition of IGF-1 to rapamycin-treated wounds clearly restored wound healing kinetics to those of untreated animals (Fig. 7B). These studies indicate that dysfunctional wound repair caused by rapamycin can be rescued by the addition of factors normally produced by skin γδ T cells.

**Discussion**

Rapamycin is a powerful clinical therapeutic administered for the prophylaxis of allograft rejection, coatings on arterial stents, and it
is currently used in clinical trials for the treatment of cancer. Several studies have described the potent inhibitory action of rapamycin on various αβ T lymphocyte populations, as the mechanism employed to prevent rejection of transplanted solid organs (59). Herein we show that similar to allograft αβ T lymphocytes, intraepithelial γδ T lymphocytes are suppressed by rapamycin treatment. Due to the inhibitory effects of rapamycin on cell cycle and function, there has been speculation that rapamycin renders effector T cells anergic (19, 45). Anergy has been described as a mechanism by which lymphocytes are functionally inactive following stimulation, but survive in a nonresponsive state (60). Skin γδ T cells are present but with diminished functional capacity in rapamycin-treated mice. Our results show that γδ TCR expression is reduced, cytokine and growth factor production are decreased, and proliferation and cell cycle initiation are inhibited. This may be caused by a block in mTOR-mediated costimulatory responses (signal 2) or a defect in IL-2 signals (signal 3). IL-2 receptor signaling is key for the proliferation and cell cycle progression of skin γδ T lymphocytes (61). We show that IL-2 responsiveness is diminished in rapamycin-treated skin γδ T cells, which may be exacerbated by the reduced levels of TCR expression. Upon removal of rapamycin, normal skin γδ T cell function is restored, suggesting that rapamycin inhibition is reversible.

In the presence of rapamycin, skin γδ T cells survive in the hyporesponsive state for a prolonged time. Instead of undergoing apoptosis, skin γδ T cells appear to undergo autophagy as they attempt to survive with diminished mTOR signaling. This mechanism of survival may be vital for cell types such as skin γδ T cells, which are only seeded in the murine skin from the fetal thymus and cannot be repopulated by the adult thymus (62). Since treatment with rapamycin impairs the ability of skin γδ T cells to proliferate or migrate in response to T cell growth factors such as IL-2 or IGF-1, the skin γδ T cell may become autophagic in an attempt to survive in the absence of cytokine/growth factor signaling. Although most cells undergo a basal level of autophagy, this process is induced during nutrient deprivation in an attempt to break down macromolecules and recycle the components (57, 63). With skin γδ T cells undergoing autophagy and unable to proliferate, the number of skin γδ T cells in the epidermal compartment may eventually diminish. In our studies a decrease was not observed after 2 weeks of rapamycin treatment, but long-term, sustained rapamycin administration, as would occur in transplant recipients, may impact skin T cell numbers. The increased level of autophagy may also impair the cellular machinery involved in activation, leading to inhibition of downstream effector functions. Rapamycin treatment directly affects pathways involved in nutrient sensing and cellular growth through inhibition of mTORC1 and mTORC2, but increased levels of autophagy may also induce degradation of signaling molecules involved in other independent pathways. It is still unclear whether particular proteins are specifically targeted for degradation during autophagy.

Skin-resident γδ T cells are known to play roles in the early stages of wound repair (27, 34). Similar to TCRδ T− mice, the delay in wound healing observed in rapamycin-treated mice occurs within the first 3 days of wound healing. The precise timing of wound repair is critical as key growth factors, cytokines, and chemokines participate in the entry, exit, and function of resident and inflammatory cells at the wound site. Although rapamycin has been shown to potentiate inhibit αβ T lymphocytes, peripheral αβ T lymphocytes do not infiltrate the wound site until 7 days postwounding, and they play roles in the later stages of wound repair (64). Thus, the early timing of the wound healing defect observed during rapamycin treatment makes it unlikely that αβ T cell inhibition by rapamycin is responsible for the delay in wound closure.

The cell-specific nature of rapamycin inhibition is a testament to the complex and sometimes redundant nature of the mTOR pathway. In contrast to allograft-specific αβ T cells, which undergo anergy or apoptosis (19, 45, 65), rapamycin treatment leads to an accumulation of CD25+ regulatory T lymphocytes in mice (66) and humans (17). Additionally, some tumor cell lines are susceptible to apoptosis upon rapamycin treatment (67, 68). Since keratinocytes compose the bulk of the epidermis, their proliferative capacity is essential to wound repair. However, during wound repair, we show that keratinocytes retain their ability to proliferate in the presence of rapamycin. Although signaling through PI3K is important for keratinocyte proliferation (69), it must not depend on mTOR. Additionally, IGF-1 application rescues normal wound closure rates, suggesting that keratinocytes can respond to growth factors normally in the presence of the inhibitor. In the epidermis, IGF-1 is exclusively produced by skin γδ T cells (30), but its receptor is widely expressed among cells in the epidermal compartment including keratinocytes. IGF-1 plays key roles in both keratinocyte and skin γδ T cell migration and survival (30–33). Addition of this growth factor restores wound closure upon rapamycin treatment, presumably by overcoming the impaired function of the skin γδ T cells and acting upon neighboring keratinocytes to promote migration. Thus, supplementing the wound environment with exogenous IGF-1 restores the paracrine effects of skin γδ T cell-derived IGF-1 on other epidermal cells, thereby reestablishing wound closure.

Rapamycin acts by binding FKBP12 and inhibiting the serine/threonine kinase mTOR. Although there appears to be an ever-expanding list of downstream targets for mTOR, the best studied readout of mTOR function in mTORC1 is phosphorylation of p70 S6 kinase (70). S6 kinase is involved in proliferative responses of αβ T lymphocytes, and our results indicate that IL-2 signaling induces mTOR phosphorylation of S6 kinase in skin γδ T cells as well and that this phosphorylation is inhibited by rapamycin. The second complex formed with mTOR has been shown to regulate spatial aspects of cell growth. mTORC2 affects cytoskeletal reorganization in yeast and is implicated as an upstream regulator of Rho GTPases in mammalian cells (42). Although originally reported as a rapamycin-resistant complex (42), recent evidence shows that in particular cell types mTORC2 is sensitive to prolonged rapamycin treatment (43). Skin γδ T cells exhibit rapamycin-sensitive phosphorylation of Akt Ser 473, suggesting that mTORC2 is not functional during rapamycin treatment in these cells. The defect we observe in the capacity of skin γδ T cells to undergo rapid activation-induced morphology changes, both in vitro and in vivo, in the presence of rapamycin highlights the capacity for the drug to inhibit cytoskeletal functions. To our knowledge, this is the first time that a T cell has been shown to exhibit mTORC2 function and cytoskeletal changes that are rapamycin-sensitive.

Both αβ and γδ TCR-expressing cells are found in human skin. Similar to the mouse, human skin T cells exhibit a restricted TCR repertoire specific to the cutaneous environment (71, 72) and are able to produce TNF-α and IFN-γ upon stimulation (73). Skin γδ T cells in the human epidermis are also able to produce IGF-1 (our unpublished data). Additionally, human skin γδ T cells have tumoricidal capabilities much like the mouse γδ T cell (73). Our studies suggest that prolonged rapamycin treatment would result in skin γδ T cell dysfunction and an inability to respond to injury. Herein we have identified a key signaling pathway vital for skin γδ T cell function and a novel mechanism that may contribute to
wound healing defects observed during treatment with rapamycin (6–10).

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Disclosures

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