

$\gamma\delta$ T cell–induced hyaluronan production by epithelial cells regulates inflammation

Julie M. Jameson, Gabrielle Cauvi, Leslie L. Sharp, Deborah A. Witherden, and Wendy L. Havran

The Scripps Research Institute, La Jolla, CA 92037

Nonhealing wounds are a major complication of diseases such as diabetes and rheumatoid arthritis. For efficient tissue repair, inflammatory cells must infiltrate into the damaged tissue to orchestrate wound closure. Hyaluronan is involved in the inflammation associated with wound repair and binds the surface of leukocytes infiltrating damaged sites. Skin $\gamma\delta$ T cells play specialized roles in keratinocyte proliferation during wound repair. Here, we show that $\gamma\delta$ T cells are required for hyaluronan deposition in the extracellular matrix (ECM) and subsequent macrophage infiltration into wound sites. We describe a novel mechanism of control in which $\gamma\delta$ T cell–derived keratinocyte growth factors induce epithelial cell production of hyaluronan. In turn, hyaluronan recruits macrophages to the site of damage. These results demonstrate a novel function for skin $\gamma\delta$ T cells in inflammation and provide a new perspective on T cell regulation of ECM molecules.

CORRESPONDENCE

Wendy L. Havran:
havran@scripps.edu

Abbreviations used: DETC, dendritic epidermal T cells; IEL, intraepithelial lymphocyte; ECM, extracellular matrix; GAG, glycosaminoglycan; HAS, hyaluronan synthases; FGF-7, keratinocyte growth factor-1; FGF-10, keratinocyte growth factor-2.

Wound repair is a multifaceted biological process that involves the participation of numerous cell types and factors to accomplish the three phases of wound healing: inflammation, tissue formation, and tissue remodeling. Skin $\gamma\delta$ T cells, also called dendritic epidermal T cells (DETC), are a prototypical $\gamma\delta$ intraepithelial lymphocyte (IEL) that recognize an, as yet unidentified, antigen expressed by damaged, stressed, or transformed keratinocytes in the epidermis (for reviews see references 1, 2). These intraepithelial $\gamma\delta$ T cells are thought to provide a first line of defense through the recognition of self-antigens expressed on damaged or stressed cells. Although the exact nature of the stress-induced keratinocyte antigen is unknown, it induces DETC to proliferate via the canonical V γ 3 V δ 1 TCR (2). DETC play roles in keratinocyte proliferation during wound repair (3), epithelial cell homeostasis (4), and have been implicated in contact hypersensitivity (5), however, their roles in inflammation and extracellular matrix (ECM) modulation are unknown.

Rapid and complete wound repair cannot occur without the infiltration of peripheral leukocytes into the wound site to help stave off infection, break-down tissue, and regenerate the epidermis (6–11). The first of these leukocytes are neutrophils, which are rapidly re-

cruited within 1 d of injury, and are one of the most abundant inflammatory cell types at this time-point (12). Macrophages, essential to the normal wound repair process, enter the site 3 d after injury (13–15). Macrophages phagocytose debris, produce extracellular matrix molecules, and secrete growth factors at the wound site (6, 16). Lymphocytes enter the dermis near the wound by day 7 and are the most common inflammatory cell at the wound site 14 d after injury (12). Defects in inflammation can destroy the delicate balance of these cell types and soluble factors necessary for complete wound repair.

Glycosaminoglycans (GAGs) are a major component of connective tissue and are regularly present in healing wounds. They commonly bind the cell surface of infiltrating leukocytes that migrate into the damaged site and play several functional roles in the inflammation associated with wound repair (17). It has been reported that GAG binding is not necessary for chemokine action, but it can augment the activity of low levels of chemokines (18). One of the best characterized GAGs is hyaluronan or hyaluronic acid, which is produced by many different cell types and is heavily distributed in the ECM during wound repair (19). Hyaluronan binds cell surface receptors including CD44 (20) and has been shown to play a key role in the inflammatory cell migration associated with wound repair (21). Addition of hyaluronan to

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acute membrane perforations accelerated the closure of the wounds in rats (22). Furthermore, hyaluronan oligomers have been shown to activate endothelial cells and dendritic cells via TLR4, which may be important for inflammation (23, 24). The role of T cells in hyaluronan deposition is unknown, but it may be used by resident T cells to regulate cellular infiltration (25).

Hyaluronan polysaccharides are extruded into the extracellular space simultaneous with their synthesis at the inner surface of the plasma membrane by three hyaluronan synthases (HAS), HAS 1–3 (26). The HAS genes are essential (27) and highly conserved (26). Several reports have shown that even though murine HAS 1–3 share 55–71% sequence identity, they present distinct patterns of expression and related but distinct enzymatic properties (26). Their functions are still being investigated, but it is known that they produce different sizes of hyaluronan. HAS 1 and 2 synthesize 200–2,000-kD HA polysaccharides and HAS 3 produces smaller size polymers of 40–100 kD (26). Their elongation rates are also different with HAS 1 being the fastest and HAS 3 being the slowest (28). Overexpression of HAS 2 in keratinocytes confers the ability to control their migration toward empty space or an *in vitro* wound site (29). Such functions for HAS 1 and HAS 3 have not been studied. Keratinocyte growth factor-1 (FGF-7), which is produced by DETC after activation and during wound repair (3, 30), binds the FGFR2-IIIb receptor expressed by keratinocytes and upon binding increases hyaluronan synthesis through HAS 2 and HAS 3 up-regulation (31).

To determine whether resident DETC play a role in hyaluronan deposition and inflammation we monitored the infiltration of leukocytes after full-thickness wounding of mice lacking $\gamma\delta$ DETC, TCR $\delta^{-/-}$ mice. We provide evidence that DETC play a critical regulatory role in early inflammation during wound repair by inducing keratinocytes to produce hyaluronan. Furthermore, DETC have the capacity to make hyaluronan themselves. We show that the hyaluronan, in turn, directs macrophages to the wound site. These results demonstrate a novel function for skin $\gamma\delta$ T cells in inflammation, involving a mechanism that provides a new perspective on the role of T cells in the regulation of ECM molecule production. This induction of epithelial cell gene expression by $\gamma\delta$ T cells, demonstrates the importance of these cells in tissue maintenance and homeostasis.

RESULTS

$\gamma\delta$ T cells are not required for rapid neutrophil migration to wounded skin

Inflammatory cells make their way to the epidermal compartment via the blood stream. The recruited cells traverse into the hypodermis of the skin via blood vessels. Next they migrate up out of the hypodermis into the reticular dermis, finally passing the dermal papillae into the epidermis. To determine if skin $\gamma\delta$ T cells play a role in this recruitment, TCR $\delta^{-/-}$ mice were wounded and observed for defects in

inflammatory cell infiltration. Neutrophils are one of the first cell populations to enter wild-type wound sites, usually infiltrating within 12 h and exiting via the scab by day 3 (12). Neutrophil migration into the wound site of TCR $\delta^{-/-}$ mice was examined in paraffin sections using antibodies specific for the Gr-1 marker. Neutrophils entered rapidly by day 1 after wounding in both wild-type and TCR $\delta^{-/-}$ mice and were observed in the hypodermis, dermis, and epidermis (Fig. 1, A and B). The dark Gr-1 staining around the scab and directly next to the wound site was of similar intensity in the wild-type and TCR $\delta^{-/-}$ mice (Fig. 1, A and B). Sections examined at 6 and 12 h likewise showed no difference in Gr-1 staining (unpublished data). Positive cells were no longer evident by day 5 in either mouse strain (unpublished data). No differences in the timing of neutrophil entry or exit from the wound site were detected between TCR $\delta^{-/-}$ and wild-type mice.

In the absence of $\gamma\delta$ T cells, mice have defects in macrophage homing to damaged skin

To examine the role of skin $\gamma\delta$ T cells in the migration of macrophages to the damaged site, skin sections from wounded and nonwounded wild-type and TCR $\delta^{-/-}$ mice were stained with antibodies specific for either MHC class II or the macrophage marker, F4/80 (Fig. 1, C–J). Sections directly adjacent to the wound were examined to compare the degree of inflammation in wild-type and TCR $\delta^{-/-}$ mice. MHC class II and F4/80-positive cells were delayed in entering the wound site of TCR $\delta^{-/-}$ mice as compared with wild-type mice. In wild-type mice, large numbers of MHC class II-positive cells were detected from the hypodermis up to the dermis directly beneath the hyperthickened, damaged epidermis by day 3 (Fig. 1 C). By day 5, the cells expressing MHC class II accumulated in great numbers just under the damaged epidermis and throughout the entire dermis (unpublished data). In contrast, wounds isolated from TCR $\delta^{-/-}$ mice had virtually no MHC class II-expressing cells in the dermis on day 3 after injury (Fig. 1 D) and those that entered on day 5 remained in the hypodermis (unpublished data). Large numbers of MHC class II-expressing cells finally entered the dermis around the wound site by day 7 in TCR $\delta^{-/-}$ mice (unpublished data).

To further characterize the MHC class II-positive infiltrating population, an anti-F4/80 antibody was used to detect macrophages. Macrophages were largely absent from nonwounded tissue in wild-type and TCR $\delta^{-/-}$ mice (Fig. 1, E and F). 3–5 d after injury, massive macrophage infiltration dominates the dermis near the wound site of wild-type mice (Fig. 1, G and I), whereas very few macrophages enter the dermis of TCR $\delta^{-/-}$ mice at this time (Fig. 1, H and J). These results indicate that macrophages are the major MHC class II-positive population infiltrating these wounds.

We quantified the number of F4/80-positive cells in the hypodermis or upper dermis of the wound site from wild-type and TCR $\delta^{-/-}$ skin sections (Fig. 1, K and L). These re-

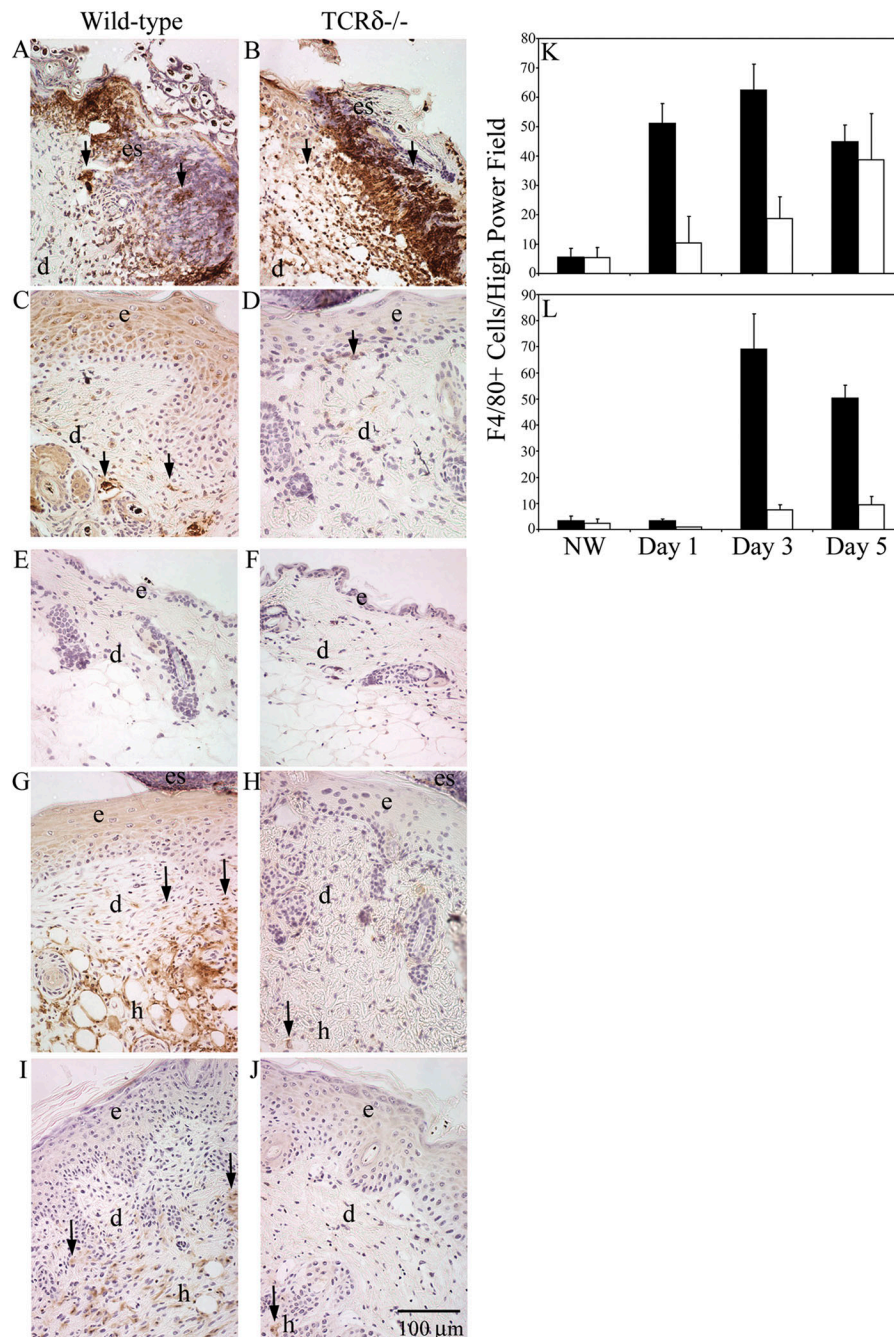


Figure 1. $\gamma\delta$ T cells are required for the rapid migration of macrophages, but not neutrophils, into the wound site. Nonwounded and wounded skin from wild-type (A, C, E, G, and I) and TCR $\delta^{-/-}$ (B, D, F, H, and J) mice were embedded in paraffin and 5 μ m sections cut for immunohistochemistry. Shown are sections stained with antibodies specific for Gr-1 on day 1 after wounding (A and B), MHC class II on day 3 after wounding (C and D), F4/80 on nonwounded sections (E and F), F4/80 on day 3 after wounding (G and H), or F4/80 on day 5 after wounding (I and J). The epider-

mis is oriented on the top and images represent the area directly adjacent to the wound site. Epidermis (e), dermis (d), hypodermis (h), and eschar (es) are labeled. Representative positive cells are marked by arrows. Digital images of representative wound sites were acquired ($\times 200$). Macrophage infiltration of the hypodermis (K) or upper dermis (L) of wild-type (solid bars) or TCR $\delta^{-/-}$ (open bars) mice was quantified by enumerating F4/80-positive cells in 1,000 \times fields. Error bars represent the SD.

sults indicate that macrophages enter the hypodermis of wild-type mice in great numbers by day 1 after wounding, whereas the numbers of macrophages entering TCR $\delta^{-/-}$

skin slowly increase until day 5 when they finally reach wild-type levels (Fig. 1 K). The macrophages that infiltrate the wounded TCR $\delta^{-/-}$

mis, unlike wild-type mouse skin in which macrophages infiltrate the entire hypodermis of the wound. In wild-type mice, macrophages leave the hypodermis for the upper dermal compartment by day 3, however, few F4/80-positive cells are found in the $\text{TCR}\delta^{-/-}$ mouse at this time-point (Fig. 1 L). Taken together our results indicate that there is at least a 4-d delay in macrophage infiltration of wounds in the absence of $\gamma\delta$ T cells.

Hyaluronan deposition is defective at the wound site of $\text{TCR}\delta^{-/-}$ mice

To investigate the mechanism involved in this delay in macrophage infiltration, we examined GAG deposition in the mouse wounds. GAGs play important roles in macrophage migration. Some GAGs are sulfated (dermatan sulfate, chondroitin sulfate, heparin sulfate), whereas others are not (hyaluronan). Wounded skin was excised from wild-type or $\text{TCR}\delta^{-/-}$ mice at various time-points. The wound sites were examined using alcian blue, pH 3.0, staining, which is

commonly used to detect both neutral and sulfated GAGs. As expected, in nonwounded skin there was light alcian blue staining indicating that there is a low level of constitutive GAG expression in the ECM (Fig. 2, A and B). In wild-type mice, 3-d-old wounds had intense alcian blue staining directly under the wound site emanating from the damaged area (Fig. 2 C). By day 5 after wounding the level of staining in wild-type skin decreased and was contained in the hypodermis (unpublished data). In contrast, the $\gamma\delta$ -deficient mouse skin displayed less intense alcian blue staining restricted to the hypodermis near the wound site 3 d after injury indicating reduced GAG expression (Fig. 2 D). By day 5 after wounding, the alcian blue staining in the $\text{TCR}\delta^{-/-}$ skin intensifies under the injured site, appearing at levels similar to the wild-type day 3-wounded skin but less localized (unpublished data). This delay in GAG expression is consistent with the delay in macrophage entry (Fig. 1) and the delay in wound repair we have previously reported for mice lacking $\gamma\delta$ DETC (3).

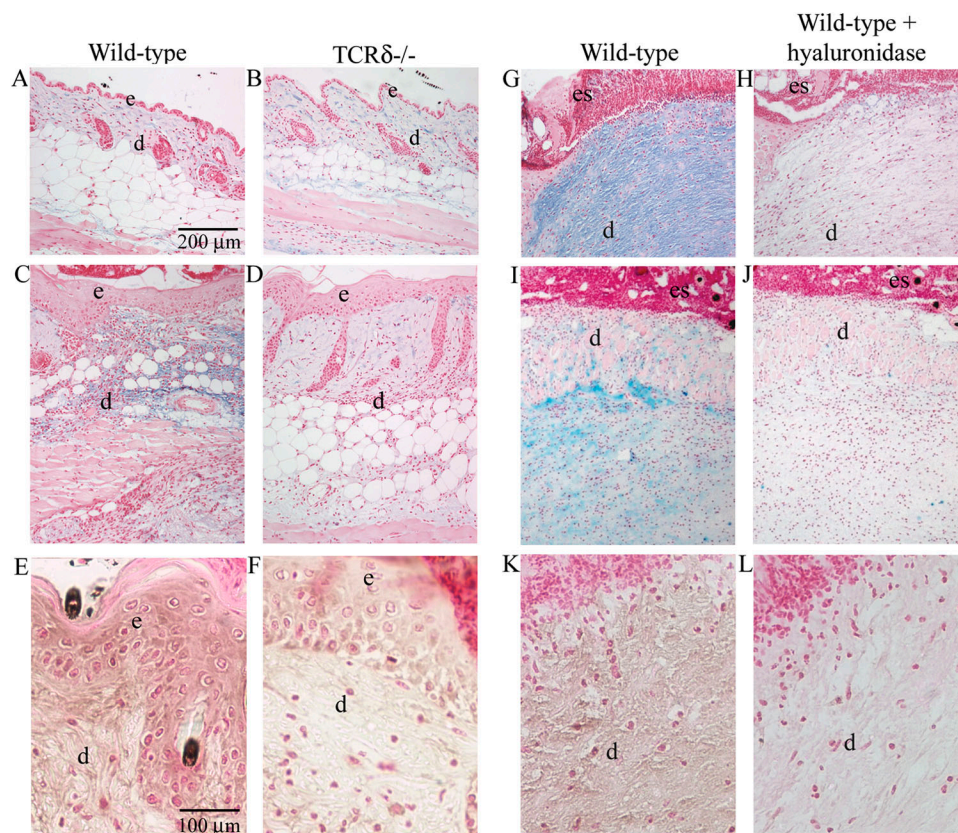


Figure 2. Decreased levels of hyaluronan are deposited in the ECM of $\text{TCR}\delta^{-/-}$ mice 3 d after wounding. Alcian blue staining was performed on 5 μm paraffin sections from nonwounded (A and B) or day 3 (C and D) wild-type (A and C) and $\text{TCR}\delta^{-/-}$ (B and D) mouse wounds. Blue stain represents GAG deposition. To identify the GAG as hyaluronan, biotinylated hyaluronan binding protein was used to stain wild-type (E) or $\text{TCR}\delta^{-/-}$ (F) day 3-wounded skin. Wild-type (G–L) or $\text{TCR}\delta^{-/-}$ (not depicted) wounded skin was preincubated with or without testicular (G and H) or *Streptomyces*

(I–L) hyaluronidase to further identify hyaluronan as the main GAG component stained by alcian blue (G–J) or biotinylated hyaluronan binding protein (K and L) during this time-point after injury. The epidermis is oriented to the top and images represent the area directly adjacent to the wound site. Epidermis (e), dermis (d), and eschar (es) are labeled. Digital images of representative wound sites were acquired at $\times 100$ (A–D) or $\times 200$ (E–L) magnification. Experiments were performed at least three times using three to five mice of each strain per time-point.

In wild-type mice, hyaluronan is heavily distributed at the wound site soon after tissue damage (21). To determine whether hyaluronan is the key GAG distributed in wild-type wounds 3 d after injury, a biotinylated hyaluronan binding protein was used to stain skin sections. Hyaluronan was detected in similar regions of the wounded skin as the alcian blue (Fig. 2 E). Staining with the hyaluronan binding protein confirmed a defect in hyaluronan deposition in $\text{TCR}\delta^{-/-}$ mice (Fig. 2 F). Furthermore, addition of either testicular (Fig. 2 H) or *Streptomyces* (Fig. 2, J and L) hyaluronidase to the skin sections before staining with alcian blue or hyaluronan binding protein diminished staining intensity to background levels. The low levels of GAGs observed in the hypodermis of $\text{TCR}\delta^{-/-}$ day 3-wounded skin can also be digested by either type of hyaluronidase (unpublished data). Taken together these results suggest that the GAGs distributed in wild-type skin 3 d after wounding are mostly composed of hyaluronan (Fig. 2, G-L), thus the $\text{TCR}\delta^{-/-}$ mice have at least a 2-d delay in hyaluronan deposition directly at the wound site. Hyaluronan is known to enhance cellular infiltration during wound repair (32), and as such may contribute to macrophage migration to the wound site.

$\text{TCR}\delta^{-/-}$ mice have impaired hyaluronan synthase expression in the epidermis 1 d after wounding

HAS 1–3 synthesize hyaluronan at the plasma membrane and HAS expression correlates with increased levels of hyaluronan (26). To identify whether the defect in hyaluronan deposition observed in $\text{TCR}\delta^{-/-}$ mice was due to decreased production by epidermal cells, we examined HAS expression levels in the epidermis after wounding in wild-type and $\text{TCR}\delta^{-/-}$ skin (Fig. 3). Epidermis was isolated from the wounds of mice, RNA was extracted, and semi-quantitative RT-PCR for each HAS was performed. Mice lacking $\gamma\delta$ T cells have decreased levels of HAS 1 at 12 h and 1 d after wounding and decreased levels of HAS 2 and 3 at 1 d after wounding as compared with wild-type mice. Using densitometry, HAS gene expression was corrected for β -actin expression and wild-type HAS 1–3 expression was found to be 1.35, 1.5, and 1.46 times greater, respectively, than the $\text{TCR}\delta^{-/-}$ expression at day 1 after wounding. These results indicate that there is a defect in epidermal cell HAS expression during wound repair in $\text{TCR}\delta^{-/-}$ mice, which may result in decreased hyaluronan deposition at the wound site.

Hyaluronan is digested by hyaluronidases, therefore it is possible that the defect in hyaluronan deposition in $\text{TCR}\delta^{-/-}$ skin is due to increased levels of hyaluronidases. Therefore we examined hyaluronidase expression in wounded $\text{TCR}\delta^{-/-}$ mouse epidermis. There were decreased levels of hyaluronidase 1 expression, similar levels of hyaluronidase 3 expression, and increased levels of hyaluronidase 2 expression in the epidermis of $\text{TCR}\delta^{-/-}$ mice as compared with wild-type mice (Fig. S1). Thus the defect in hyaluronan deposition in the $\text{TCR}\delta^{-/-}$ mice does not appear to be caused by a

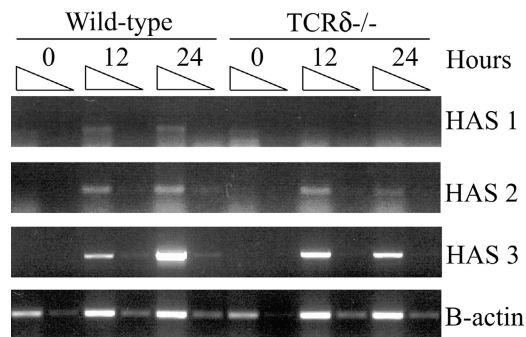


Figure 3. $\text{TCR}\delta^{-/-}$ mice have defects in HAS expression in the epidermis 1 d after wounding. Epidermis was isolated from nonwounded skin or at various times after wounding from wild-type or $\text{TCR}\delta^{-/-}$ mice. RNA was isolated and RT-PCR for HAS 1–3 performed. cDNA was diluted 1:10 for semiquantitation and compared with β -actin housekeeping gene levels. Using densitometry (Adobe Photoshop), HAS gene expression was corrected for β -actin expression and wild-type HAS 1–3 expression was 1.35, 1.5, and 1.46 times greater, respectively, than $\text{TCR}\delta^{-/-}$ 1 d after wounding. These results are representative of at least three separate experiments with reproducible results.

marked up-regulation in hyaluronidases. However, the increased hyaluronidase 2 expression may further exacerbate the defect in hyaluronan deposition.

To determine whether the defect in epidermal cell HAS expression during wound repair is mediated by the keratinocytes, skin $\gamma\delta$ T cells, or both, we examined HAS gene expression in vitro. We first studied whether DETC express HAS 1, 2, or 3 upon TCR stimulation. $\alpha\beta$ T cells have been reported to express HAS 1 and 3 constitutively (33). We examined RNA from the skin $\gamma\delta$ T cell line, 7–17, for HAS expression using RT-PCR. 7–17 DETC express HAS 1 and 3, and this expression is inducible upon stimulation with antibodies specific for the CD3 complex (Fig. 4 A). HAS 1 expression is up-regulated within 30 min after stimulation and is maximally expressed by 1 h. HAS 3 is up-regulated by 30 min after stimulation with sustained expression until 2.5 h (Fig. 4 A). DETC-expressed HAS 1 and 3 may partially account for the defect in HAS expression during wound repair in $\text{TCR}\delta^{-/-}$ mice. However, HAS 2 does not appear to be expressed by DETC (Fig. 4 A) and because there are decreased levels of HAS 2 expression in the $\text{TCR}\delta^{-/-}$ epidermis (Fig. 3), we examined whether DETC up-regulate HAS expression by keratinocytes.

DETC induce keratinocytes to synthesize hyaluronan through keratinocyte growth factors

We next used a transwell coculture assay to test the impact of DETC on hyaluronan synthase expression by keratinocytes. 7–17 DETC were seeded in the inserted transwell, whereas the keratinocyte cell line, PAM, was cultured in the bottom chamber. The PAM keratinocytes were incubated alone, with resting 7–17 DETC, or with anti-CD3-activated 7–17 DETC. After 8 h of coculture PAM kerati-

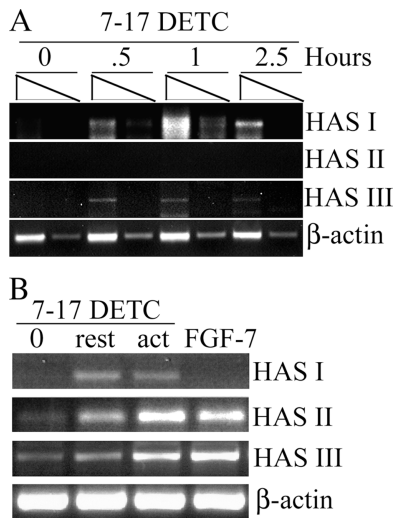


Figure 4. DETC can express HAS genes and up-regulate keratinocyte HAS gene expression. The DETC cell line 7-17 expresses HAS 1 and 3 upon anti-CD3 stimulation (A). RT-PCR was performed on 7-17 DETC RNA isolated 30 min, 1 h, and 2.5 h after stimulation. DETC induce HAS expression by PAM keratinocytes in transwell culture (B). Coculture of 7-17 DETC in the upper chamber and PAM cells in the lower chamber were performed. In some experiments FGF-7 was added. RT-PCR was performed on keratinocyte RNA isolated either before or following coculture with resting or activated 7-17 DETC or recombinant FGF-7. cDNA was diluted 1:10 for semiquantitation and compared with β -actin housekeeping gene levels. These results are representative of at least three separate experiments.

nocytes were harvested, RNA was isolated, and RT-PCR was performed to detect the levels of HAS expression. Low to undetectable levels of HAS genes were detected in keratinocytes grown without DETC in the insert suggesting that the keratinocytes do not express much HAS 1-3 constitutively (Fig. 4 B). This correlates with the low to undetectable levels of HAS expression observed in nonwounded skin (Fig. 3). However, resting DETC stimulate PAM to express low levels of all three HAS genes, whereas activated DETC stimulated even greater expression of HAS 2 and 3 (Fig. 4 B). Transwell experiments were also performed using a fluorescent-labeled hyaluronan binding protein. Hyaluronan was detected at higher levels in keratinocytes cultured with DETC (Fig. 5, B and C) as compared with keratinocytes cultured without DETC (Fig. 5 A). This staining is diminished to background levels with the addition of *Streptomyces* hyaluronidase. Taken together these results demonstrate that DETC induce HAS expression and subsequent HA production by keratinocytes. This highlights a new role for $\gamma\delta$ T cells in the regulation of epithelial cells.

A likely candidate for the induction of HAS 2 and 3 is FGF-7. This growth factor has recently been shown to induce HAS 2 and 3 expression in rat keratinocytes (31). FGF-7 and keratinocyte growth factor-2 (FGF-10) bind the same receptor expressed on keratinocytes, FGFR2IIIb (34-36).

Both factors are produced by DETC upon TCR stimulation and act in a paracrine fashion on keratinocytes (3, 30). DETC-produced keratinocyte growth factors play a key role in keratinocyte proliferation during wound repair (3) and have been shown to be the most important DETC-produced factors for keratinocyte proliferation (30). To determine whether FGFR2IIIb binding results in up-regulated HAS 2 and 3 expression in mouse keratinocytes, recombinant FGF-7 was added to PAM cells. RT-PCR revealed that both HAS 2 and 3 are up-regulated by FGF-7 (Fig. 4 B). This correlates well with the previously published results in rat keratinocytes (31).

To examine whether this increase in hyaluronan synthase expression results in increased production of hyaluronan, we cultured the keratinocytes with recombinant FGF-7 and stained for hyaluronan using the fluorescent-labeled hyaluronan binding protein. Addition of FGF-7 resulted in increased hyaluronan production, even at concentrations as low as 20 ng/ml (Fig. 5 E). The other FGFR2IIIb ligand, FGF-10, is also produced by DETC upon TCR stimulation, albeit at much lower levels (3; unpublished data). FGF-10 also induced hyaluronan production by keratinocytes when added in vitro (Fig. 5 G). To determine whether keratinocyte growth factors are the major DETC-produced inducers of keratinocyte hyaluronan synthesis, a keratinocyte growth factor receptor blocking peptide was added to the transwell assays (30). This peptide is derived from a unique region of FGFR2IIIb, and has been previously shown to neutralize keratinocyte growth factors (30, 37). The peptide blocked hyaluronan production by stimulated 7-17 DETC (Fig. 5 D), whereas vehicle alone did not (Fig. 5 H). To verify the specificity of the peptide for the keratinocyte growth factor receptor, it was used to block FGF-7-induced hyaluronan production (Fig. 5 F). Taken together these results identify keratinocyte growth factors as the major hyaluronan inducers produced by DETC.

Activated DETC or recombinant FGF-7 induce hyaluronan deposition in TCR $\delta^{-/-}$ skin organ culture

To investigate whether DETC-produced factors increase hyaluronan production by keratinocytes in situ, we performed organ cultures using skin from TCR $\delta^{-/-}$ mice. Skin was excised from the backs of TCR $\delta^{-/-}$ mice and immediately placed dermis-side down on media for 2 d. The skin was then embedded in paraffin and sections were stained for hyaluronan using alcian blue. We detected low level hyaluronan deposition throughout the dermis and hypodermis in nonwounded skin cultured in vitro for 2 d possibly due to fibroblast activation by serum in the culture media (Fig. 6, A and D). To examine whether DETC produce factors that stimulate hyaluronan production in cultured skin tissue, resting 7-17 DETC or DETC stimulated for 15 h with either concanavalin A or antibodies specific for CD3 were added to the bottom of the culture dish beneath the TCR $\delta^{-/-}$ skin. Although the presence of resting

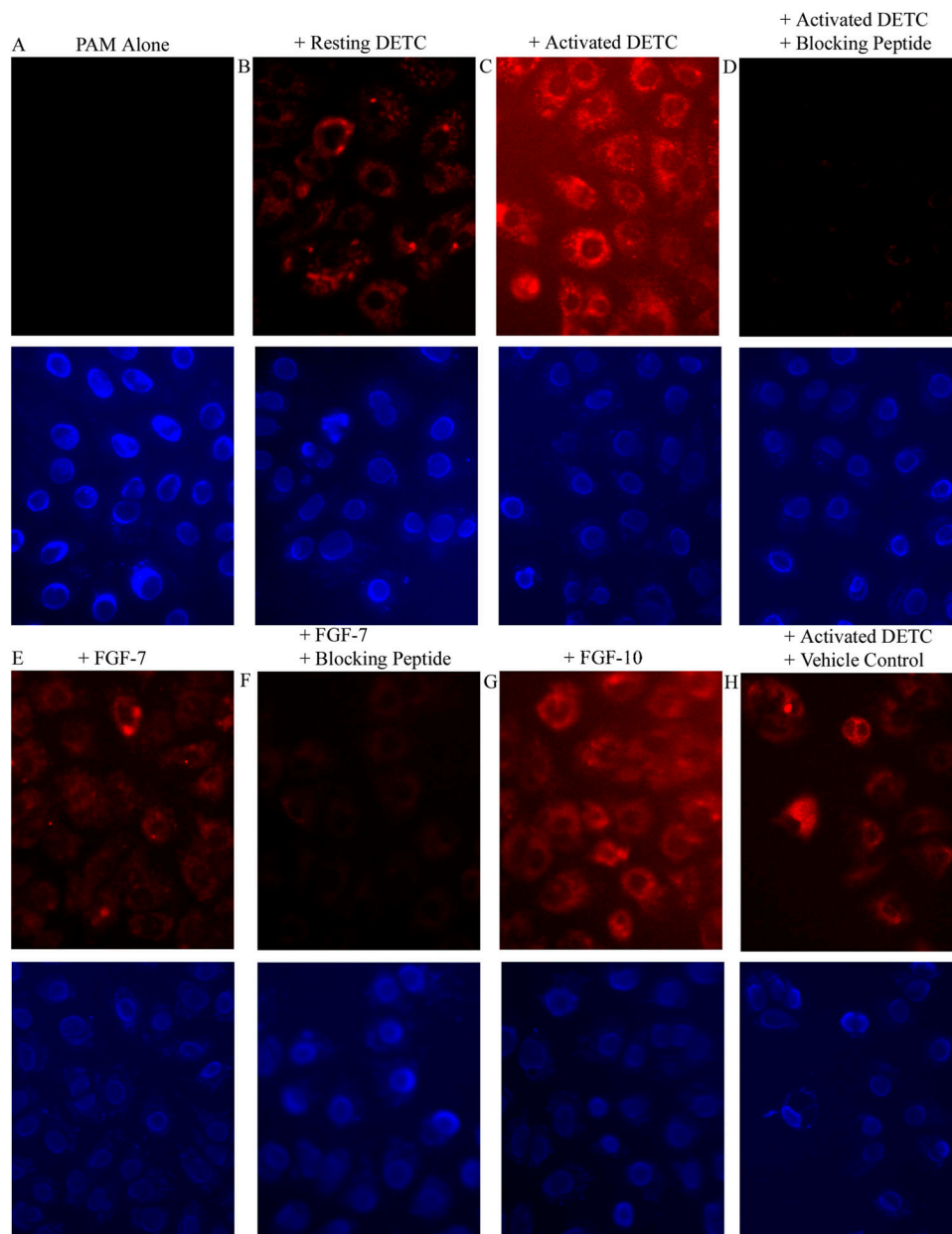


Figure 5. DETC-produced keratinocyte growth factors induce hyaluronan production by keratinocytes. PAM keratinocytes were cultured on coverslips in the lower chamber of a transwell alone (A), with resting 7–17 DETC (B), with activated 7–17 DETC (C), with 10 ng/ml FGF-7 (E), or with 200 ng/ml FGF-10 (G). In some cases the keratinocyte growth factor recep-

tor blocking peptide at 10 μ g/ml (D and F) or vehicle control (H) was added. Cells were stained with phycoerythrin-labeled hyaluronan binding protein (top) and DAPI (bottom). Digital images of representative cells from at least three experiments were acquired at $\times 200$ magnification.

DETC did not alter hyaluronan deposition compared with $\text{TCR}\delta^{-/-}$ skin alone (unpublished data), the addition of activated DETC resulted in increased hyaluronan production, shown as intense alcian blue staining in the dermis and hypodermis of $\text{TCR}\delta^{-/-}$ skin (Fig. 6, B and E). Digestion with hyaluronidase identified the GAG as hyaluronan (unpublished data). The results from this qualitative assay strengthen the argument that DETC produce factors in-

involved in the induction of hyaluronan production by skin cells. To identify whether FGF-7 induces hyaluronan deposition in $\text{TCR}\delta^{-/-}$ skin, recombinant FGF-7 was added to the skin organ culture. Addition of FGF-7 increased hyaluronan production in skin from $\text{TCR}\delta^{-/-}$ mice (Fig. 6, C and F). These experiments suggest that DETC are involved in hyaluronan deposition likely via the production of keratinocyte growth factors.

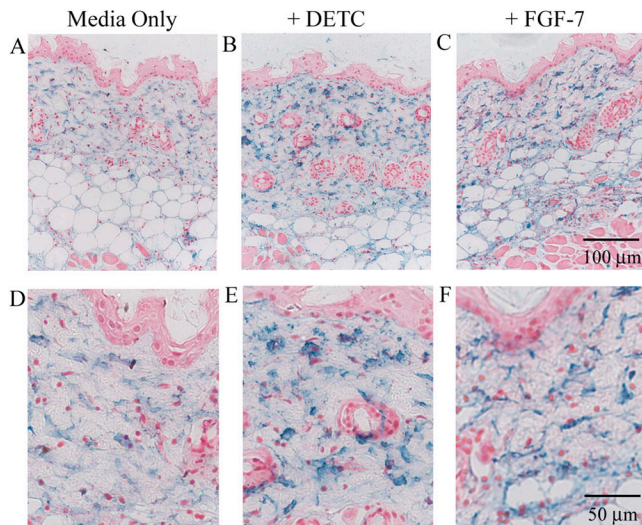


Figure 6. DETC or FGF-7 up-regulate hyaluronan in $TCR\delta^{-/-}$ skin organ culture. $TCR\delta^{-/-}$ back skin was cultured in media for 2 d alone (A and D), with activated 7-17 DETC (B and E), or FGF-7 (C and F). Shown are sections stained with alcian blue. The epidermis is oriented on the top and images represent the area directly adjacent to the wound site. Epidermis (e) and dermis (d) are labeled. Representative positive cells are marked by arrows. Digital images of representative wound sites were acquired at $\times 200$ (A-C) or $\times 400$ (D-F) magnification.

Hyaluronan or FGF-7 restores macrophage recruitment to the wound site in $\gamma\delta$ T cell-deficient mice

Macrophages migrate in response to hyaluronan injected into the peritoneal cavity (38). We applied hyaluronan to the wounds of $TCR\delta^{-/-}$ mice in an attempt to restore macrophage recruitment to the wound site. Application of hyaluronan directly to the excision wounds increased the levels of hyaluronan as evidenced by increased alcian blue staining in the wound (Fig. 7 B) and restored macrophage recruitment to the wound site (Fig. 7, E and H). This increase in the number of inflammatory cells entering the upper dermis from the hypodermis indicates that hyaluronan can induce large inflammatory infiltrates. Wild-type mice showed no difference in macrophage migration whether or not they received hyaluronan (Fig. S2). Thus, hyaluronan deposition in the wound site is sufficient to allow macrophage recruitment to the damaged region of the skin in mice lacking $\gamma\delta$ T cells.

DETC isolated from the wound site produce FGF-7 within 2 d after injury (3). FGF-7 plays a key role in the induction of keratinocyte-produced hyaluronan in vitro (31). To examine the potency of FGFR2IIIb ligands in vivo we applied FGF-7 to wounds on $TCR\delta^{-/-}$ mice. Consistent with our findings in vitro, application of FGF-7 to wounds on $TCR\delta^{-/-}$ mice resulted in increased levels of hyaluronan (Fig. 7 C) and increased numbers of macrophages homing to the wound edge (Fig. 7, F and I). Quantitation of macrophages per high power field demonstrated that there were only 10 ± 5 macrophages per field in mice receiving buffer alone, whereas there were 43 ± 7 macrophages per field upon addi-

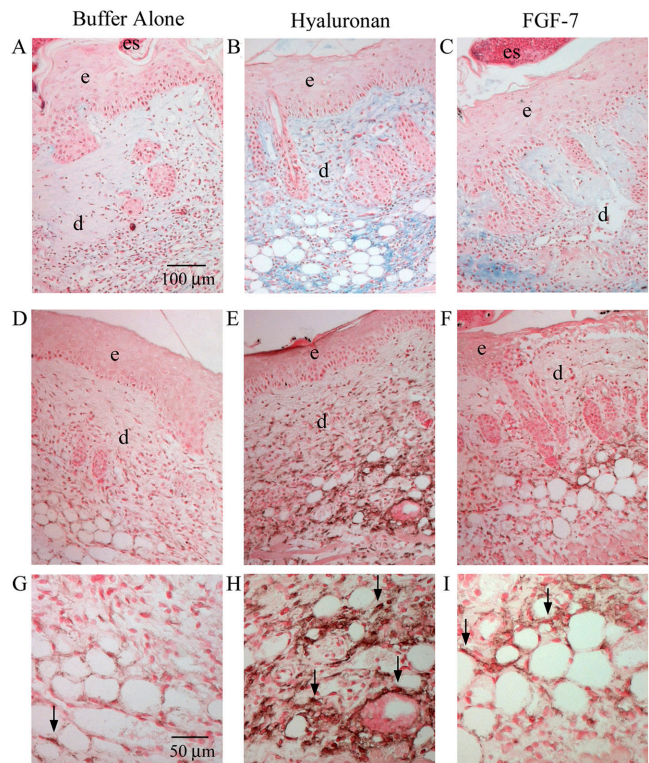


Figure 7. Application of hyaluronan or FGF-7 restores macrophage infiltration and hyaluronan deposition in $TCR\delta^{-/-}$ mice. $TCR\delta^{-/-}$ mice were wounded and buffer (A, D, and G), hyaluronan (B, E, and H), or FGF-7 (C, F, and I) was applied topically to the wounds on days 0 and 1. Wounds were harvested on day 3, embedded, and stained with alcian blue (A-C) or with antibodies specific for F4/80 (D-I). The number of F4/80-positive cells in the upper dermis of the skin was quantified demonstrating 10 ± 5 positive cells per field for buffer alone and 43 ± 7 positive cells per field for either KGF-1 or HA addition. The epidermis is oriented on the top and images represent the area directly adjacent to the wound site. Epidermis (e), dermis (d), and eschar (es) are labeled. Representative positive cells are marked by arrows. Digital images of representative wound sites were acquired at $\times 200$ (A-F) or $\times 400$ (G-I) magnification.

tion of either KGF-1 or HA. Wild-type mice receiving KGF-1 or HA showed no differences in macrophage migration at this time-point (Fig. S2). Taken together this data supports a role for $\gamma\delta$ T cell-produced keratinocyte growth factors in mediating hyaluronan deposition and subsequent macrophage migration to the wound site. Mice deficient in $\gamma\delta$ T cells not only lack $\gamma\delta$ T cell-produced hyaluronan, but also the rapid mobilization of keratinocyte growth factors to the epithelial cells that induces keratinocyte production of hyaluronan.

DISCUSSION

$\gamma\delta$ T cells are considered by many to bridge the gap between innate and adaptive immunity. Adaptive responses by $\alpha\beta$ T cells have been well characterized including their ability to rapidly eradicate pathogens. Less understood is the capacity of T cells to maintain homeostasis of other cells or to repair tissue. Recent studies have characterized the ability of $\gamma\delta$ T

cells to regulate epithelial cell proliferation or apoptosis, and $\alpha\beta$ T cell migration within a tissue as examples of T cell maintenance of tissue homeostasis (3–5). Here we describe a novel mechanism of tissue maintenance in which skin $\gamma\delta$ T cells regulate inflammation by modulating epithelial cell hyaluronan production. In this way $\gamma\delta$ T cells, activated by damaged keratinocytes, can control the timing and location of macrophage entry into a site of epithelial injury. As such $\gamma\delta$ T cells not only regulate epithelial cell proliferation but also have the ability to regulate epithelial cell-induced recruitment of inflammatory cells to injured tissue. Our results identify a novel mechanism used by T cells to localize inflammatory cells to a specific site in the tissue.

Our findings reveal a critical connection between lymphocytes and the ECM for the regulation of inflammation. Inflammation is a highly dynamic process that involves many different cell types, factors, and ECM molecules. Rapid leukocyte infiltration is necessary for efficient and complete wound healing (6–11). Once neutrophils, macrophages, and infiltrating T cells arrive at the wound site, they can protect against pathogens, produce proteases for tissue break-down, secrete factors for tissue regeneration, and even produce chemokines to augment the inflammation. It is critical to determine which tissue-resident cell populations are involved in this phase of wound healing. Our examination of mice lacking $\gamma\delta$ T cells has uncovered a key role for DETC in the recruitment of macrophages to wounds.

During wound repair there is a coordinated and ordered deposition of ECM molecules. GAGs have been implicated in the inflammatory phase of wound repair in several capacities (for reviews see references 17, 21). One of the key roles for GAGs involves cell adhesion. Inflammatory cells may be tethered to the ECM adjacent to the wound site and migrate toward the wound. This would explain why we found intense staining for GAGs just beneath the wound site in wild-type mice, where the inflammatory cells are needed. Hyaluronan is a secreted GAG that is associated with early wound repair (21). The intact hyaluronan molecule signals migrating cells via the hyaluronan receptors CD44, ICAM-1, and RHAMM to produce cytokines or enhance infiltration (32, 39), whereas the rapidly degraded hyaluronan binds TLR4 to signal endothelial cells and dendritic cells to produce chemokines (23, 24). Therefore, it is likely that molecules in addition to hyaluronan are also directly involved in macrophage recruitment. Hyaluronan may also stabilize the chemokine gradient, as has been shown for other GAGs (18), allowing the infiltrating leukocytes to migrate in the correct direction. The delay in macrophage entry in the $\gamma\delta$ -deficient mice may be due to decreased adhesion of macrophages to hyaluronan in the ECM or defects in chemokine gradient maintenance. Being an extremely hygroscopic molecule hyaluronan also has hydrating properties that allow for cell migration (40). Neutrophil infiltration occurs before hyaluronan deposition in wounds from wild-type mice. Therefore the delay in hyaluronan production we describe in $\text{TCR}\delta^{-/-}$ mice does not

result in delayed neutrophil entry. In contrast, macrophage entry into wild-type wounds occurs by day 3, concurrent with hyaluronan deposition. In this study we observe defects in macrophage entry to the wound site in $\text{TCR}\delta^{-/-}$ mice that can be restored with the addition of high molecular weight hyaluronan into the wound site. This formally demonstrates that hyaluronan is a key mediator of inflammation and macrophage migration during wound repair.

Hyaluronan is synthesized at the plasma membrane by HAS 1–3, which have been shown to be regulated by several different factors (31, 42). Although we found very little, if any, hyaluronan synthase expression in the epidermis before wound administration, by day one after wounding sustained increases in HAS 1–3 were evident in the wild-type mouse epidermis. There are several possible candidates for the induction of hyaluronan synthesis including EGF, IGF-1, and FGF-7. EGF is known to induce keratinocyte proliferation (41) and up-regulate HAS 2 (42), however, it is not produced by activated DETC (unpublished results). The role of IGF-1 in hyaluronan induction is somewhat controversial (43, 44). However, it did not induce hyaluronan synthase expression by PAM keratinocytes in our study. We have previously demonstrated that DETC isolated directly from the wound site at this time-point express FGF-7 and FGF-10 (3). Both factors bind FGFR2IIIb (34–36) and blocking FGFR2IIIb blocks the keratinocyte proliferation induced by DETC coculture with keratinocytes (30). This suggests that the major DETC-produced epithelial growth factors are keratinocyte growth factors (30). Our observations in this study suggest that FGFR2IIIb binding factors are also necessary for DETC-induced keratinocyte hyaluronan production. Because DETC express FGF-7 at much higher levels than FGF-10, FGF-10 may play a redundant role in terms of the induction of hyaluronan synthesis. Keratinocyte growth factors act in a paracrine manner, therefore keratinocytes rely on production by cells such as DETC and fibroblasts. In this way, DETC may act as early inducers of hyaluronan production, thereby regulating macrophage wound infiltration. Understanding this mechanism helps clarify the complicated relationship between T cells and the extracellular matrix.

DETC have been shown to inhibit $\alpha\beta$ T cell migration (5) and induce epithelial cell proliferation (3) as direct mechanisms of cell regulation. However, in our studies we describe a regulatory role in which DETC regulate the tissue in which they reside to support macrophage migration. $\gamma\delta$ IELs in other tissues such as the lung have also been implicated in the initiation of inflammatory cell infiltration (45) and may also induce epithelial hyaluronan production. Furthermore, therapies that stimulate epithelial $\gamma\delta$ T cells to rapidly produce keratinocyte growth factors and mobilize inflammatory cells to the damaged site may be useful in patients with wound healing complications, such as diabetics. Our findings demonstrate a novel interaction between the immune system and wound repair, which may aid in the development of future therapies for chronic nonhealing wounds.

MATERIALS AND METHODS

Animals and wounding procedure. TCR $\delta^{-/-}$ mice on the C57Bl/6 background were purchased from the Jackson ImmunoResearch Laboratories. C57Bl/6 (wild-type) and TCR $\delta^{-/-}$ 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 at 10–14 wk of age. Wounding was performed on mice anesthetized with isoflurane. In brief, the mouse backs were shaved, back skin and *panniculus carnosus* was pulled up, and a sterile 3-mm punch tool was used to create two or three sets of full-thickness wounds as described previously (3). Wounds were left uncovered. In some experiments 50 μ g high molecular weight hyaluronic acid from bovine vitreous humor (Sigma-Aldrich), 10 μ g recombinant FGF-7 (Amgen), or buffer were added in 10 μ l to the wounds on days 0 and 1, and wounds were harvested on day 3. These reagents all had minimal endotoxin levels as determined by LAL test (Cambrex).

Cell lines. The DETC cell line 7–17 was maintained in complete RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Omega Scientific, Inc.) and 20 U/ml IL-2. The PAM cell line was maintained in complete DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum. All cells were maintained in 37°C under 5% CO₂.

Isolation of epidermis. Epidermis was prepared from the skin of C57Bl/6 and TCR $\delta^{-/-}$ mice as described previously (3). In brief, skin from wounded mice was excised including a 3-mm border around the wound. Skin was incubated in a 0.3% bovine pancreatic trypsin, type XI (Sigma-Aldrich) solution for 1–2 h at 37°C and the epidermis was separated from the dermis and placed directly in Trizol reagent (Invitrogen).

RNA preparation and RT-PCR. Total RNA extracts were performed in TRIzol reagent (Invitrogen) as per manufacturer's instructions. 1 μ g RNA was reverse transcribed in 30 μ l using reverse transcriptase (Invitrogen) as per manufacturers directions and 1 μ l cDNA was amplified using PCR conditions previously described for HAS 1, HAS 2 (42), HAS 3, hyaluronidase 1–3 (33), and β -actin (3). PCR products after 35 cycles, except β -actin at 20 cycles and HAS 2 at 31 cycles, were separated by electrophoresis through 1.3% agarose and visualized with ethidium bromide.

Transwell coculture of DETC and keratinocytes. 8×10^5 PAM keratinocytes were cultured in the bottom chamber of a 0.4- μ m pore size 12-well transwell assay plate (Corning), whereas 10^5 DETC (cell line 7–17) were cultured in the upper chamber insert in complete DMEM (Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum for 7 h. 7–17 DETC were either rested in media alone or stimulated for 18 h with antibodies specific for CD3 ϵ at 1 μ g/ml (500A2 clone) and washed in PBS before addition to upper transwell. In some cases recombinant FGF-7 (Amgen) or FGF-10 (Cell Sciences) was added at concentrations ranging from 20 ng/ml to 50 μ g/ml.

Immunofluorescent staining. To examine hyaluronan production using immunofluorescent staining, PAM keratinocytes were cultured on coverslips overnight to adhere in complete DMEM supplemented with 5% heat-inactivated fetal bovine serum. *Streptomyces* hyaluronidase (Sigma) was added (10 U/ml) for 30 min at 37°C before stimulation with DETC or growth factors. Cells on coverslips were stimulated by incubation with either 7–17 DETC in a transwell insert or recombinant growth factors for 4 h at 37°C. Next coverslips were fixed in 4% paraformaldehyde for 20 min, permeabilized in 0.3% Triton X-100 in PBS with 3% BSA for 20 min, incubated with 3 μ g/ml biotinylated hyaluronan binding protein for 90 min, washed with PBS, and subsequently incubated with 1 μ g/ml streptavidin-phycoerythrin (Biomed) for 45 min. Slides were fixed in mounting media with DAPI (Oncogene) and digital images were acquired. In some experiments keratinocyte growth factor receptor peptide inhibitor (10 μ g/ml) or control vehicle (1% DMSO) were added before stimulation (30, 37). Results are representative of at least three separate experiments.

Immunohistochemistry. Mice were killed and the complete wounds including 2 mm of the epithelial margins were excised, fixed in ethanol, and embedded in paraffin. Sections were prepared and stained with biotinylated antibodies to Gr-1 (BD Biosciences), CD3 (BD Biosciences), F4/80 (Caltag), MHC class II (M5/114; BD Biosciences), or biotinylated hyaluronan binding protein (Caltag) followed by peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories). The presence of positive cells was revealed by incubation in metal-enhanced DAB (Pierce chemical Co.) and sections were counterstained with hematoxylin (Sigma-Aldrich) or nuclear fast red (Biomed). Slides were mounted with DPX mounting media (Sigma-Aldrich). Control staining was done without the primary antibody or with 10 U/ml *Streptomyces* hyaluronidase (Sigma-Aldrich) added before staining. Enumeration of F4/80-positive cells was performed by counting the number of positive cells per 1,000 \times high power field. At least two wounds from each of three to five wild-type and TCR $\delta^{-/-}$ mice at each time-point were paraffin-embedded for histological analysis, immunostaining, and counting. Experiments were performed at least three times.

Alcian blue staining. Paraffin sections were stained with alcian blue (Sigma) pH 2.5 (3% acetic acid) or pH 3.1 (0.5% acetic acid) for 5 min. The slides were counterstained with nuclear fast red (Biomed) and mounted with DPX mounting media. Staining was performed at least three times for each time-point and strain of mouse. For GAG digestion, 0.5 mg/ml testicular hyaluronidase (Sigma-Aldrich) or 10 U/ml *Streptomyces* hyaluronidase was added for 30 min at 37°C before alcian blue staining. At least two wounds from each of three to five wild-type and TCR $\delta^{-/-}$ mice at each time-point were paraffin-embedded for alcian blue staining.

Skin organ culture. Gel-foam (Pfizer) was soaked in complete media for 15 min on each side before addition of skin. Back skin from TCR $\delta^{-/-}$ mice was excised for culture on gel-foam in a 24-well plate in DMEM with 10% FCS. In some cases 7–17 DETC either stimulated for 24 h by concanavalin A (Sigma-Aldrich) or by antibodies specific for CD3 ϵ were plated at the bottom of the well at a density of 2×10^5 cells per well. Recombinant FGF-7 was added at a concentration of 50 μ g/ml to some wells. Skin was harvested 2 d later, embedded for histological examination, and stained with alcian blue.

Online supplemental material. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20042057/DC1>.

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