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Identification of Glucocorticoid-Induced TNF Receptor-Related Protein Ligand on Keratinocytes: Ligation by GITR Induces Keratinocyte Chemokine Production and Augments T-Cell Proliferation

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Abstract

Glucocorticoid-induced tumor necrosis factor (TNF) receptor-related protein ligand (GITRL) is a recently described co-stimulatory molecule expressed by antigen-presenting cells (APCs). Activated keratinocytes are known to engage intraepithelial T cells through co-stimulatory molecules. This study investigated the expression and function of GITRL in resting keratinocytes. We showed by immunofluorescence and flow cytometry that keratinocytes from Balb/C and C57Bl/6 mice, as well as PAM 212 murine cell line keratinocytes and human epidermal keratinocytes (HEK), express cell-surface GITRL. Stimulation of murine skin biopsies and HEK with GITR fusion protein (GITR: Fc FP) resulted in mRNA induction for chemoattractants: cutaneous T-cell-attracting chemokine (CTACK), thymus and activation-regulated chemokine (TARC), IL-8, monocyte chemoattractant protein-1 (MCP-1), and murine β-defensin 3 (MBD3). Immunofluorescent studies on mouse biopsies treated with GITR: Fc FP confirmed corresponding TARC and MCP-1 protein production by keratinocytes. Chemokine induction was shown to be NF-κB-mediated. T-cell proliferation was enhanced by the addition of keratinocytes. This was reversed by pretreatment with an anti-GITRL antibody. We conclude that keratinocytes express GITRL, and that through this important co-stimulatory molecule, they have the potential to influence T-cell numbers in the skin through chemokine production and through a direct cell–cell effect on T-cell proliferation.

INTRODUCTION

Keratinocytes are now recognized as not merely as forming an inert skin barrier but as creating a dynamic cellular interface between the host and its environment. They bind to microbes through Toll receptors (Begon *et al.*, 2007; Miller and Modlin, 2007). They

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CONFLICT OF INTEREST

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Discloure

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produce antimicrobial peptides (Braff and Gallo, 2006) and communicate with other cells of innate and adaptive immunity through direct contact and the release of signaling molecules (Wittmann and Werfel, 2006; Bogiatzi *et al.*, 2007; Ou and Huang, 2007).

Keratinocytes produce numerous chemokines, attracting multiple immune effector cells to the skin. In atopic dermatitis, cutaneous T-cell-attracting chemokine (CTACK) and thymus and activation-regulated chemokine (TARC) act as ligands for T cells expressing CCR10 and CCR4 and cooperate in the recruitment of memory T cells to sites of skin inflammation (Homey *et al.*, 2002; Saeki and Tamaki, 2006). Keratinocytes produce monocyte chemoattractant protein-1 (MCP-1) that attracts both monocytes and T cells to the skin (Gaga *et al.*, 2008). It can directly communicate with the T-cell populations that they attract into the skin. When activated by IFN-γ, keratinocytes upregulate the surface expression of major histocompatibility complex class I and II molecules (Griffiths *et al.*, 1989; de Bueger *et al.*, 1993) and co-stimulatory molecules, including ICAM-1 (Krueger *et al.*, 1990) and CD80 (B7–1) (Burns *et al.*, 2005). Hence, under conditions of skin inflammation, they may behave as nonprofessional APCs and present antigen (de Bueger *et al.*, 1993).

Glucocorticoid-induced tumor necrosis factor (TNF) receptor-related ligand (GITRL), a member of the TNF superfamily, is a recently described co-stimulatory molecule expressed by professional APCs, including macrophages, B cells, and dendritic cells (DCs) (Gurney *et al.*, 1999; Tone *et al.*, 2003). This molecule sends co-stimulatory signals to T cells through its receptor GITR, which is expressed at a low level on naive T cells (Gavin and Rudensky, 2003) and at higher levels on naturally occurring $CD4^+CD25^+$ regulatory T cells (nTreg cells) (McHugh *et al.*, 2002), and activated effector T cells (Shimizu *et al.*, 2002). Engagement of GITR by GITRL has been shown to enhance T-cell proliferation (Ronchetti *et al.*, 2007; Tuyaerts *et al.*, 2007; Igarashi *et al.*, 2008) and to alter T-cell cytokine production (Kanamaru *et al.*, 2004; Patel *et al.*, 2005; Mahesh *et al.*, 2006). In a series of different disease models, $GITR^{-/-}$ mice have been shown to have a reduced inflammatory response (Cuzzocrea *et al.*, 2004, 2005, 2006; Santucci *et al.*, 2007). There is a growing body of work showing that members of the TNF superfamily and their receptors have the ability to transduce signals through both the receptor and its respective ligand (Chen *et al.*, 2001; Chou *et al.*, 2001). This enables bidirectional communication between the cells. The term “reverse signaling” is used to refer specifically to signaling through the ligand (Sun and Fink, 2007). Consistent with this property of the TNF superfamily, there is evidence that after GITR/GITRL binding, reverse signaling occurs through GITRL, resulting in the generation of pro-inflammatory cytokines and chemokines by professional APCs (Agostini *et al.*, 2005; Bae *et al.*, 2008).

In this study, we hypothesized that keratinocytes, in their role as nonprofessional APCs, would express GITRL and communicate, through GITR/GITRL engagement, with T cells. Our work presented here shows, to the best of our knowledge, previously unreported GITRL expression by keratinocytes *in vivo* and *in vitro*. We explore the role of this molecule in skin inflammatory responses, showing that its ligation drives chemokine production by keratinocytes. We also show a role for keratinocyte-expressed GITRL in the promotion of T-cell proliferation.

RESULTS

GITRL is expressed on the surface of mouse keratinocytes

Previously published data have shown GITRL expression on the surface of APCs, including DCs and macrophages (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). As keratinocytes are known to behave as non-professional APCs and communicate with T cells (de Bueger *et al.*, 1993; Wittmann and Werfel, 2006), we hypothesized that mouse keratinocytes express GITRL. Keratinocytes in formalin-fixed 4-mm-thick skin biopsies taken from male Balb/C (Figure 1a) and male C57Bl/6 (Figure 1b) mice stained positive with fluorescent anti-GITRL antibody (Ab). No staining was observed with a corresponding isotype control Ab, suggesting that keratinocytes express GITRL.

Ligation of GITRL on keratinocytes stimulates chemokine mRNA production

APCs have been shown to produce cytokines (TNF-α, IL-6) and chemokines (including IL-8 and MCP-1) as a result of reverse signaling through surface GITRL, ligated by both GITR fusion protein (GITR: Fc FP) and anti-GITRL Ab (Agostini *et al.*, 2005; Bae *et al.*, 2008). To explore the functionality of GITRL on the surface of keratinocytes, we looked for evidence of mRNA induction for TARC, CTACK, and MCP-1 in mouse skin biopsies stimulated with GITR: Fc FP. These chemokines are produced by keratinocytes and are involved in the development of numerous skin inflammatory conditions (Loetscher *et al.*, 1994; Uguccioni *et al.*, 1995; Kakinuma *et al.*, 2001, 2003a, b; Leung *et al.*, 2003). We also looked for GITR: Fc FP-mediated induction of mRNA for murine β-defensin (MBD)2 and MBD3, murine antimicrobial peptides with chemoattractant properties for subsets of murine T cells and immature DCs (Oppenheim *et al.*, 2003). Skin biopsies from male Balb/C mice were stimulated with either GITR: Fc FP (1 or 10 μg ml^{-1}) or with a corresponding amount of control fusion protein (Control: Fc FP). The biopsies were then harvested and analyzed for mRNA expression by real-time PCR. A significant increase in the TARC mRNA level was observed after 6 hours of treatment with 10 μg ml^{-1} of GITR: Fc FP compared with resting cells ((mean±SD): 1.0±0.8 vs 0.4±0.3 ng TARC per ng 18S RNA) and with cells treated with Control: Fc FP (1.0±0.8 vs 0.4±0.2 ng TARC per ng 18S RNA; $P<0.05$)) (Figure 2a). This increase was no longer evident at the 24-hour time point (Figure 2b). There was no significant increase in CTACK or MCP-1 mRNA levels after 6 hours incubation with 10 μg ml^{-1} of GITR: Fc FP, although there was a trend for CTACK upregulation at this time point (Figure 2c and e). After 24 hours treatment with GITR: Fc FP (10 μg ml^{-1}), mRNA levels for both CTACK and MCP-1 were significantly increased compared with resting cells ((mean±SD): 489±202 vs 205±116 ng CTACK per ng 18S RNA, $P<0.05$; and 12.6±6.0 vs 3.3±1.4 ng MCP-1 per ng 18S RNA, $P<0.01$) and compared with cells treated with Control: Fc FP (489±202 vs 221±101 ng CTACK per ng 18S RNA, $P<0.05$; and 12.6±6.0 vs 5.6±5.0 ng MCP-1 per ng 18S RNA, $P<0.05$, respectively; Figure 2d and f). The addition of GITR: Fc FP at a concentration of 1 μg ml^{-1} had no effect on mRNA levels of the aforementioned chemokines. In contrast, MBD3 mRNA levels increased significantly after 6 hours of treatment with either 1 μg ml^{-1} of GITR: Fc FP ((mean±SD): 0.15±0.06 vs 0.03±0.02 ng MBD3 per ng 18S RNA, $P<0.05$) or 10 μg ml^{-1} of GITR: Fc FP (0.11±0.01 vs 0.03±0.02 ng MBD3 per ng 18S RNA, $P<0.05$)) (Figure 2g). There was no significant difference between MBD3 mRNA levels produced in the presence of 1 μg ml^{-1} of GITR:

Fc FP compared with 10 μg ml^{-1} of GITR: Fc FP. This increase in the mRNA level had subsided by the 24-hour time point (Figure 2f). No significant induction in the MBD2 mRNA level was observed (data not shown). We also showed that 6 hours of incubation with 10 μg ml^{-1} of GITR: Fc FP caused a significant increase in the GITRL mRNA level compared with resting cells or cells treated with Control: Fc FP ($P<0.05$) (data not shown). Thus, it appears that GITRL may autoregulate its own expression upon cellular stimulation through GITRL.

Ligation of GITRL on keratinocytes activates chemokine production by these cells

In the previous experiment, it was shown that reverse signaling through keratinocyte expressed GITRL resulted in an increase in mRNA levels for TARC, MCP-1, CTACK, and MBD3. In the following experiment, we examined whether, under similar experimental conditions, chemokine protein production was upregulated. To this end, mouse skin biopsies were formalin-fixed after incubation with GITR: Fc FP or Control: Fc FP (10 μg ml^{-1}) for 24 or 36 hours and stained with fluorescent Abs against TARC and MCP-1. Upon treatment with GITR: Fc FP for 24 hours, a significant increase in the expression of TARC by the keratinocyte layer of the biopsies was observed (Figure 3a and b). In addition, a significant increase in the MCP-1 level was determined in the keratinocyte layer after 36 hours of GITR: Fc FP treatment (Figure 3a and b). This increased intensity in staining for MCP-1 was not apparent at the 24-hour time point (data not shown). No change in either TARC or MCP-1 expression was observed after treatment of skin biopsies with Control: Fc FP.

PAM 212 keratinocytes release TARC due to signaling through surface-expressed GITRL

To further define the role of GITRL in keratinocytes, we performed a series of experiments using a murine keratinocyte cell line, PAM 212. We first sought to define the expression of GITRL in this cell type. The expression of GITRL by PAM 212 murine keratinocytes was shown first by flow cytometry both before and after fixation of cells with paraformaldehyde to show that this procedure does not affect the binding of anti-GITRL Ab (Figure 4a). The average mean fluorescent intensity for cells stained with anti-GITRL Ab (eBioscience) before fixing in 1% paraformaldehyde was 43±9 vs 21±8 when compared with that for cells stained with the corresponding isotype control; and after fixing was 43±6 vs 24±8 for cells stained with the corresponding isotype control. GITRL expression by PAM 212 cells was then confirmed by immunofluorescence after staining with a different anti-GITRL Ab (Santa Cruz Biotechnology) (Figure 4b). The slides were analyzed using regular fluorescent microscope, we do not exclude the possibility of both surface and intracellular expression of GITRL by keratinocytes (Figure 4b).

To confirm that keratinocytes express functional GITRL, PAM 212 cells were stimulated with GITR: Fc FP (1 or 10 μg ml^{-1}) or with Control: Fc FP (10 μg ml^{-1}) for 24 hours. Quantitative analysis of the harvested supernatants by ELISA showed a significant induction in TARC production after 24 hours stimulation with 10 μg ml^{-1} of GITR: Fc FP compared with resting cells or treatment with Control: Fc FP or 1 μg ml^{-1} of GITR: Fc FP (Figure 4c). This finding is consistent with the increase in TARC expression induced by GITR: Fc FP in the keratinocyte layer of mouse biopsies (see Figure 3a).

Primary human epidermal keratinocytes produce CTACK and IL-8 mRNA in response to stimulation through surface-expressed GITRL

In the next set of experiments, we confirmed GITRL expression and functionality in undifferentiated human epidermal keratinocytes (HEK). It was first shown by flow cytometry that undifferentiated HEK express GITRL (Figure 5a). The average mean fluorescent intensity for cells stained with anti-GITRL Ab was 42±9 vs 4.8±1.1 compared with that for cells stained with the corresponding isotype control. GITRL expression by HEK was then confirmed by immunofluorescence (Figure 5b).

To explore whether the GITRL-mediated chemokine release observed in murine keratinocytes also occurred in a human model, HEKs were stimulated with GITR: Fc FP (1 or 10 μg ml^{-1}) or with Control: Fc FP (10 μg ml^{-1}) for 6 and 24 hours. The cells were then harvested and analyzed for mRNA expression by real-time PCR. A significant increase in CTACK and IL-8 mRNA levels was observed after 6 hours of treatment with 10 μg ml^{-1} of GITR: Fc (Figure 5c), as compared with Control: Fc FP.

GITRL induces chemokine production in keratinocytes through NF-κB activation

Having concluded that reverse signaling through GITRL had similar effects in both *ex vivo* and *in vitro* models, we sought to understand the signaling pathway engaged by GITRL, leading to chemokine production. In the first experiment, PAM 212 cells were stimulated with GITR: Fc FP (10 μg ml^{-1}) for 20 or 40 minutes and then fixed and stained for NF-κB p50. Figure 6a shows that at 20 minutes after stimulation, NF-κB remains inactivated in the cytoplasm. After 40 minutes of GITRL stimulation, translocation of NF-κB to the cell nuclei is apparent. To confirm NF-κB activation by GITRL ligation, PAM 212 cells were stimulated with GITR: Fc FP or Control: Fc FP (10 μg ml^{-1}) or with TNF-α (50 ng ml^{-1}) for 30 or 60 minutes. Nuclear extracts were isolated and assayed for p50 binding to its consensus-binding site by ELISA. There was a significant increase in optical density from nuclear extracts stimulated with GITR: Fc FP (10 μg ml^{-1}) for 30 or 60 minutes compared with nuclear extracts from cells stimulated with Control: Fc FP (Figure 6b). To explore the dependence of chemokine production upon GITRL ligation on NF-κB activation, the cells were cultured with the NF-κB activation inhibitor: cell-permeable quinazoline compound that acts as a highly potent inhibitor of NF-κB transcriptional activation (IC_{50} = 11 nM in Jurkat cells) and lipopolysaccharide-induced TNF-α production (IC_{50} = 7nM in murine splenocytes) (Tobe *et al.*, 2003). The cells were incubated with NF-κB activation inhibitor (10 or 100 nM) for 1 or 3 hours before being stimulated with GITR: Fc FP (10 μg ml^{-1}) for 24 hours. A higher concentration of NF-κB activation inhibitor (1 μM) was found in preliminary experiments to induce cell detachment and death (data not shown). Analysis of the supernatants for TARC production by ELISA showed a significant reduction in TARC production in all conditions containing NF-κB activation inhibitor compared with the treatment with GITR: Fc FP alone (P<0.01/P<0.001) (Figure 6c). Pre-incubation of murine skin biopsies with NF-κB activation inhibitor (100 nM) similarly inhibited the GITR: Fc FP-induced upregulation of TARC mRNA (P<0.05) (Figure 6d). Also, treatment of skin biopsies with NF-κB inhibitor abolished GITRL mRNA induction by GITR: Fc FP treatment (data not shown).

Murine T-cell proliferation is enhanced by the presence of keratinocytes

Multiple reports have shown that co-stimulation by professional APCs expressing GITRL enhances proliferation of GITR expressing T cells (Tone *et al.*, 2003; Ronchetti *et al.*, 2007; Tuyaerts *et al.*, 2007; Igarashi *et al.*, 2008). We sought to further show the functionality of GITRL on keratinocytes by exploring its effect on T cells contacting with keratinocytes. Purified murine $CD4^+$ T cells from Balb/C mice cultured with irradiated APCs were activated with anti-CD3 Ab (0.5, 0.1, 0.05, and 0.01 μg ml^{-1}) in the presence or absence of murine keratinocytes, fixed in 4% formaldehyde (1×10^4 keratinocytes per well) for 72 hours. Preliminary experiments determined that 1×10^4 per well of keratinocytes was the optimum concentration to use (data not shown). T-cell proliferation increased significantly in the presence of keratinocytes at anti-CD3 Ab concentrations of 0.5 μg ml^{-1} ($P<0.05$), 0.1 μg ml^{-1} ($P<0.01$), and 0.05 μg ml^{-1} ($P<0.01$) compared with treatment with anti-CD3 Ab alone. There was no difference in proliferation observed at the lowest concentration of anti-CD3 Ab used (0.01 μg ml^{-1}) (Figure 7a). As keratinocytes can express multiple other surface molecules that could be co-stimulatory for T cells, in order to show that the effect on proliferation was GITRL-specific, keratinocytes were pre-incubated with an anti-GITRL blocking Ab (1 μg ml^{-1}) or a matching isotype control before co-culture with T cells stimulated with anti-CD3 Ab (0.5 μg ml^{-1}). The addition of fixed keratinocytes to T cells increased proliferation counts from (mean±SD) 3,110±461 to 8,623±1,399 c.p.m. ($P<0.05$). Pre-incubation of keratinocytes with anti-GITRL Ab significantly reduced T-cell proliferation compared with T-cell proliferation in the presence of isotype control-treated keratinocytes (4,736±381 vs 8,186±1,742 c.p.m.) ($P<0.05$) (Figure 7b).

DISCUSSION

Keratinocytes represent the major cellular constituents of the skin and are being increasingly recognized as dynamic mediators of skin inflammation. Communication between keratinocytes and intraepithelial T cells is known to occur through the production of chemokines (Homey, 2005) and cytokines (Albanesi *et al.*, 2005). Some direct cell–cell contact is also described after IFN-γ-induced upregulation of surface molecules such as major histocompatibility complex class II (Griffiths *et al.*, 1989; de Bueger *et al.*, 1993), ICAM-1 (Krueger *et al.*, 1990), and CD80 (Burns *et al.*, 2005).

In this work, the expression of GITRL, a key co-stimulatory molecule for T cells, was shown in keratinocytes from murine skin biopsies, in the murine keratinocytic cell line, PAM 212, and in primary human keratinocytes by immunofluorescence and by flow cytometry. To our knowledge this is a previously unreported observation. We showed that GITRL engagement on the keratinocyte surface activates chemokine production by keratinocytes and also provides direct co-stimulatory signal for T-cell proliferation. The presence of this molecule on keratinocytes extends significantly the potential for signaling between the epithelium and GITR-expressing T cells. It is important to note that the immunostaining data in the present study do not exclude the possibility of intracellular expression of GITRL as well. However, our functional data shown in Figure 4 suggests surface expression of GITRL by keratinocytes.

GITRL, similar to most other members of the TNF family, is a type II transmembrane protein (Bodmer *et al.*, 2002). Previously, its ligation with anti-GITRL Ab or GITR: Fc FP, in the macrophage model, has been shown to induce "reverse signaling," resulting in the production of cytokines and also MCP-1 and IL-8 (Agostini *et al.*, 2005; Sun and Fink, 2007; Bae *et al.*, 2008). To test the functionality of GITRL on keratinocytes, we stimulated mouse skin biopsies with GITR: Fc FP and showed that reverse signaling through GITRL can be triggered in mouse skin biopsies, resulting in the induction of MCP-1 mRNA. Human keratinocytes stimulated with GITR: Fc FP showed induction of IL-8 mRNA. We also showed that through GITRL activation, mRNA for CTACK could be induced in both murine and human models, and mRNA for MBD3 and TARC in the murine model. A corresponding increase in protein production was shown in the murine skin biopsy model stained for TARC and MCP-1. The increased staining for was determined in the keratinocyte layer only, indicating that these cells were the only source of newly produced TARC and MCP-1 in response to GITR: Fc FP.

TARC, CTACK, IL-8, and MCP-1 all have a central role in skin inflammatory diseases, including atopic dermatitis (Kakinuma *et al.*, 2001; Stephens *et al.*, 2004), mycosis fungoides (Kakinuma *et al.*, 2003b), and psoriasis (Campanati *et al.*, 2007). MBD3 attracts T cells and immature DCs that express CCR6 into inflamed skin (Oppenheim *et al.*, 2003). This exhibition of GITRL-induced chemokine production in keratinocytes suggests a potential role for this molecule in skin pathology. Presently, it would be important to address its expression in skin inflammatory conditions.

In this work, we have shown that NF-κB activation is involved in GITRL-driven production of chemokines by keratinocytes. Inhibition of GITRL-stimulated TARC mRNA induction was achieved in mouse skin biopsies with the addition of NF-κB activation inhibitor. Prevention of TARC protein production under similar conditions was also shown by ELISA in the PAM 212 model. Currently, there is minimal data on the signaling molecules involved in reverse signaling through TNF family members. Experiments to date suggest that different ligands provoke different signaling pathways. Activation of T cells through CD40L induces tyrosine phosphorylation of Lck and phospholipase C, which further activates protein kinase C (Sun and Fink, 2007). CD40L cross-linking has also been shown to induce NF-κB activation (Pontrelli *et al.*, 2006). It has been shown that p38 mitogen-activated protein kinase is involved in TNF-related apoptosis-inducing ligand-mediated co-stimulation (Sun and Fink, 2007). Consistent with our findings, previous work in a macrophage model found that GITRL stimulation induced extracellular signal-regulated kinase-1/2 phosphorylation and subsequent activation of NF-κB (Bae *et al.*, 2008).

It remains to be clarified, what overall effect is achieved through GITRL activation. Other groups have shown, consistent with our data, that reverse signaling through GITRL induces a repertoire of pro-inflammatory cellular products (Shin *et al.*, 2002a, b; Lee *et al.*, 2003; Bae *et al.*, 2008). In contrast, activation of GITRL has also been shown to initiate the immunoregulatory pathway of tryptophan catabolism in murine plasmacytoid DCs (Grohmann *et al.*, 2007). Furthermore, recent data indicate that the administration of GITR: Fc FP to an *in vivo* murine spinal injury model subdues the inflammatory process by preventing pro-inflammatory signals through GITR without evidence of GITRL

activation (Nocentini *et al.*, 2008). This apparent conflict may be related in part to GITR: Fc FP concentration. We have shown in our model system that concentrations <10 μg ml^{-1} did not signal the production of most chemoattractants examined. We have also made the observation that at concentrations >10 μg ml^{-1}, chemokine induction by GITR: Fc FP is diminished (data not shown). The differential findings may also be related to the unique structure of GITRL. It has recently been shown to be able to adopt multiple oligomerization states. These different states have been shown to progressively control GITR-mediated signaling with the GITRL dimer being inhibitory and the supercluster being hyperstimulatory (Zhou *et al.*, 2008). We hypothesize that, reverse signaling with GITR: FC FP may be critically dependent on the GITRL oligomerization state.

Numerous studies have shown that T-cell proliferation is enhanced through interaction with GITRL expressed on the surface of professional APCs (Ronchetti *et al.*, 2007; Tuyaerts *et al.*, 2007; Igarashi *et al.*, 2008). It has also been shown that the GITRL–GITR interaction inhibits the suppressor function of nTreg cells. However, it is now generally accepted that it is the ligation of GITR on activated T effector cells that directly affects their proliferation (Stephens *et al.*, 2004) and not its ligation on the surface of nTreg cells (Shimizu *et al.*, 2002; Kanamaru *et al.*, 2004). In the experiments presented here, enhanced proliferation of purified murine splenic $CD4^+$ T cells was shown in the presence of GITRL-expressing murine keratinocytes. The beneficial effect of keratinocytes was abolished in the presence of an anti-GITRL Ab but not reversed in the presence of an isotype control. This is to the best our knowledge previously unreported demonstration of enhanced T-cell proliferation by naturally expressed epithelial GITRL. Prior to this, Mahesh *et al.* (2006) showed that retinal pigment epithelial cells transfected with GITRL enhanced T-cell proliferation.

In summary, we have shown that GITRL is expressed by murine and undifferentiated human keratinocytes. Importantly, its ligation results in augmentation of anti-CD3-induced T-cell proliferation and in the stimulation of multiple chemoattractants production. Our current data suggest a potential role for keratinocyte-expressed GITRL in skin inflammation through the expansion of skin T-cell populations through chemokine release and enhanced proliferation.

MATERIALS AND METHODS

Mice

Male Balb/C and C57Bl/6 mice aged 6–12 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal protocols were approved by the Institutional Animal Care and Use Committee at National Jewish Health in Denver.

Reagents

Rabbit anti-mouse GITRL Ab, goat anti-mouse TARC Ab (clone N-20), and NF-κB p50 (clone D-17) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human GITRL monoclonal Ab (clone109114) and mouse IgG were purchased from R&D Systems (Minneapolis MN). Rabbit anti-mouse MCP-1 polyclonal Ab (ab7202) was purchased from Abcam (Cambridge, MA). Phycoerythrin (PE)-conjugated anti-mouse

GITRL Ab (clone eBioYGL386) was purchased from eBioscience (San Diego, CA). GITR (mouse): Fc (human) (recombinant) fusion protein [GITR: Fc FP] and control: Fc fusion protein (human) (recombinant) [Control: Fc FP] were purchased from Alexis Biochemicals (San Diego, CA). Cy3- and FITC-conjugated goat anti-rabbit, goat anti-mouse, and rabbit anti-goat secondary Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). NF-κB activation inhibitor was purchased from Calbiochem (San Diego, CA). Nuclear and cytoplasmic extracts from cells were prepared with an NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford, IL). TransAM NF-κB p50 kits were purchased from Active Motif (Carlsbad, CA).

Cell culture

PAM 212 mouse cells were kindly supplied by Dr Ansel's lab at University of Colorado Denver (Denver, CO). They were cultured in the RPMI 1640 medium (Cellgro, Manassas, CA) containing 10% heat-activated fetal calf serum (Gemini, West Sacramento, CA), 40 mmol l^{-1} L-glutamine, 100 U ml^{-1} penicillin, and 100 U ml^{-1} streptomycin. They were plated overnight in 24-well tissue culture plates (1×10^5/ml) before being stimulated with GITR: Fc FP or Control: Fc FP (1 or 10 μg ml^{-1}) for 6 or 24 hours. NF-κB activation inhibitor was pre-incubated for 1 or 3 hours at concentrations of 10 nM, 100 nM, and 1 μM (IC_{50} = 11 nM in Jurkat cells). For immunofluorescence studies, PAM 212 cells were plated in eight-well chamber slides (Lab-Tek II, Thermo Fischer Scientific, Rochester, NY), pre-coated with poly-L-lysine. They were incubated for 24 hours before staining.

HEKs were purchased from Cascade Biologics (Invitrogen, Carlsbad, CA) and cultured in serum-free media with Penicillin G (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and amphotericin B (0.25 μg ml^{-1}). They were plated overnight in 48-well plates (0.5×10^4 in 500 μl) before being stimulated with GITR: Fc FP or Control: Fc FP (1 or 10 μg ml^{-1}) for 6 and 24 hours. For immunofluorescence studies, HEK cells were plated in eight-well chamber slides (Lab-Tek II) pre-coated with poly-L-lysine. They were incubated for 24 hours before staining.

Ex vivo experiments

Male Balb/C and C57Bl/6 mice were shaven along their backs. Further hair removal was achieved with Nair (Church & Dwight, Princeton, NJ). At 3 days later, the mice underwent CO_2 euthanasia. The shaved skin was dissected from the bodies and tape-stripped twice. Punch biopsies of 4mm were then taken along the skin. There was no visible inflammation evident at the time the biopsies were being preformed. The biopsies were incubated in 200 μl of RPMI 10% fetal calf serum with GITR: Fc FP or Control: Fc FP (1 or 10 μg ml^{-1}) for 6 or 24 hours.

Immunofluorescence staining

Mouse tissue samples were fixed in 10% phosphate-buffered saline (PBS)-buffered formalin, embedded in paraffin, and processed for histology. The samples were blocked for 30 minutes using SuperBlock (Skytec, Logan, UT) and 10% goat serum (Jackson ImmunoResearch Laboratories) before being incubated overnight at 4 °C with primary Abs against GITRL, TARC, and MCP-1. A secondary Ab was added for 1 hour at room temperature along with

4′−6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Sigma, St Louis, MO) before the slides were mounted in 12-oxo-phytodienoic acid (Sigma). PAM 212 cells and HEK cells cultured on poly-L-lysine-coated slides were fixed in 4% paraformaldehyde in PBS before being labeled as described above. All slides were analyzed by fluorescent microscopy (Leica Microsystems, Wetzlar, Germany) with imaging software Slidebook (Intelligent Imaging Innovations, Denver, CO).

Flow cytometry

PAM 212 cells and HEKs were detached from 24-well tissue culture plates using HyQtase (Hyclone, Logan, UT). The supernatants were removed and 350 μl of HyQtase was added per well for 10 minutes at 37 °C. When cell detachment was confirmed by microscopy, the cell suspension was removed from the wells and spun down at 1,000 r.p.m. for 5 minutes at 4 °C. PAM 212 cells were labeled with PE-conjugated anti-GITRL Ab. In some experiments, cells were fixed in 1% paraformaldehyde before staining. HEKs were both stained with an FITC-conjugated anti-GITRL Ab and blocked in PBS+2% BSA+10% goat serum, stained overnight with anti-GITRL Ab, and then with an FITC-conjugated secondary Ab. Murine and human keratinocyte samples were then analyzed by flow cytometry (FACScan Becton-Dickinson Cytometer, Franklin Lakes, NJ) and CellQuest Pro software (Becton Dickinson).

Real-time PCR

Total cellular RNA was isolated by the acid guanidinium thiocyanate–phenol–chloroform method, according to the manufacturer's guidelines (Qiagen, Valencia, CA), transcribed into cDNA, and analyzed by real-time PCR using the dual-labeled fluorigenic probe method on an ABI Prism 7000 Sequence detector (Perkin-Elmer Applied Biosystems, Foster City, CA). Primers and probes for murine TARC, CTACK, MCP-1, MBD2, MBD3 and GITRL, and human CTACK, IL-8, and 18S mRNA were purchased from Applied Biosystems. Standard curves were generated using the fluorescent data from two-fold serial dilutions of total cDNA of the highest expression sample. Quantities of target gene expression in test samples were normalized to the corresponding 18S RNA transcript.

ELISA

Cell supernatants were harvested and frozen at −80 °C. They were later analyzed using the TARC Quantikine Elisa kit (R&D Systems) according to the manufacturer's instructions.

NF-κB immunofluorescence assay

PAM 212 cells were plated on chamber slides (5×10^4/ml) as before and stimulated with GITR: Fc FP for 20 and 40 minutes. The slides were washed once in PBS, fixed in methanol, and then allowed to air dry. They were blocked in PBS, 0.05% Tween, 1% BSA (Sigma), and 2% donkey serum (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature and incubated with an anti-mouse NF-κB p50 Ab overnight at 4 °C before being labeled with a secondary Ab (rabbit anti-goat cy3 Ab) for 1 hour at room temperature.

NF-κB activation assay

PAM 212 cells were plated in six-well dishes (0.5×10^6 per well) for 24 hours. At that time, they were stimulated with GITR: Fc FP or Control: Fc FP (10 μg ml^{-1}), or with TNF-α (50 ng ml^{-1}) for 30 or 60 minutes. The cell cultures were detached using HyQtase (Hyclone) and pelleted. Nuclear and cytoplasmic extracts were isolated according to the manufacturer's protocol and frozen at −80 °C. NF-κB activation was analyzed using a TransAM kit (Active Motif), which assays p50 binding to the consensus-binding site using a colorimetric readout.

Murine spleen $CD4^+$ T-cell isolation

Male Balb/C mice aged between 6 and 12 weeks were killed by CO_2 euthanasia and their spleens were removed and crushed. Negative selection of $CD4^+$ T cells was carried out by treating cells with immunomagnetic beads (by depletion of $CD8^+$, $CD11b^+$, $CD16^+$, $CD19^+$, $CD36^+$, and $CD56^+$ cells) with the Untouched $CD4^+$ T cell Isolation Magnetic Bead Kit (Miltenyi Biotec, Auburn, CA).

Proliferation assay/keratinocyte T-cell co-culture

Purified mouse spleen $CD4^+$ T cells were cultured at 5×10^4 cells per well (in a 1:1 ratio) with irradiated (3 000 rad) APC (depleted $CD4^-$ population). Cells were stimulated with anti-CD3 Ab (0.5, 0.1, 0.05, 0.01 μg ml^{-1}; Beckton Dickinson, Franklin Lakes, NJ) in the presence or absence of 4% paraformaldehyde-fixed PAM 212 murine keratinocytes (10 000 per well). An anti-GITRL blocking Ab (1 μg ml^{-1}) or a mouse IgG_1 isotype control (1 μg ml^{-1}) (R&D Systems, Minneapolis, MN) was added to keratinocytes for 1 hour prior to proliferation assays. Keratinocytes were then washed three times in PBS before the addition to T-cell cultures to remove any extra anti-GITRL Ab from the solution. The cells were cultured for 72 hours at 37 °C. At 18 hours before harvesting, cell cultures were pulsed with 1 μCi per well of tritiated thymidine (ICN Biomedicals, Costa Mesa, CA), and cell-incorporated tritium was counted in a liquid scintillation counter.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism (version 4.03, GraphPad Software, La Jolla, CA). Data were analyzed by Student's *t*-test or one-way analysis of variance and significant differences were determined by the Tukey–Kramer test. Differences were considered significant at $P \leq 0.05$. A minimum of three independent experiments were conducted to allow for statistical comparison.

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Abbreviations:

Ab	antibody
APC	antigen-presenting cell

Control: Fc FP	control fusion protein
CTACK	cutaneous T-cell-attracting chemokine
DAPI	4′−6′-diamidino-2-phenylindole, dihydrochloride
DC	dendritic cell
***GITR*: Fc FP**	GITR fusion protein
GITRL	glucocorticoid-induced TNF receptor-related protein ligand
MBD2	*murine β-defensin 2*
MBD3	*murine β-defensin 3*
MCP-1	monocyte chemoattractant protein-1
nTreg cell	*naturally occurring CD4+CD25+ regulatory T cell*
PBS	phosphate-buffered saline
TARC	thymus and activation-regulated chemokine
TNF	tumor necrosis factor

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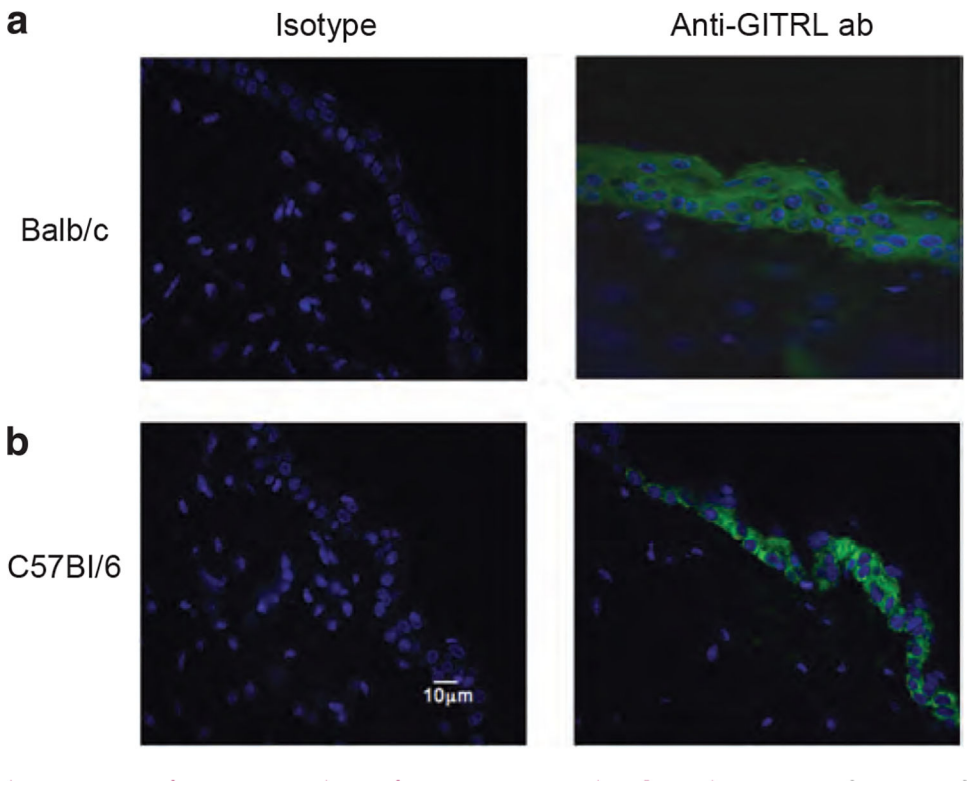

Figure 1. Surface expression of GITRL on murine keratinocytes.

Skin punch biopsies obtained from (**a**) male Balb/C mice and (**b**) male C57Bl/6 mice were immunostained with anti-GITRL Ab (right panel, green) or with control IgG (left panel). The sections were counterstained with DAPI (blue). Representative data out of three independent experiments is shown (bar = 10 μm).

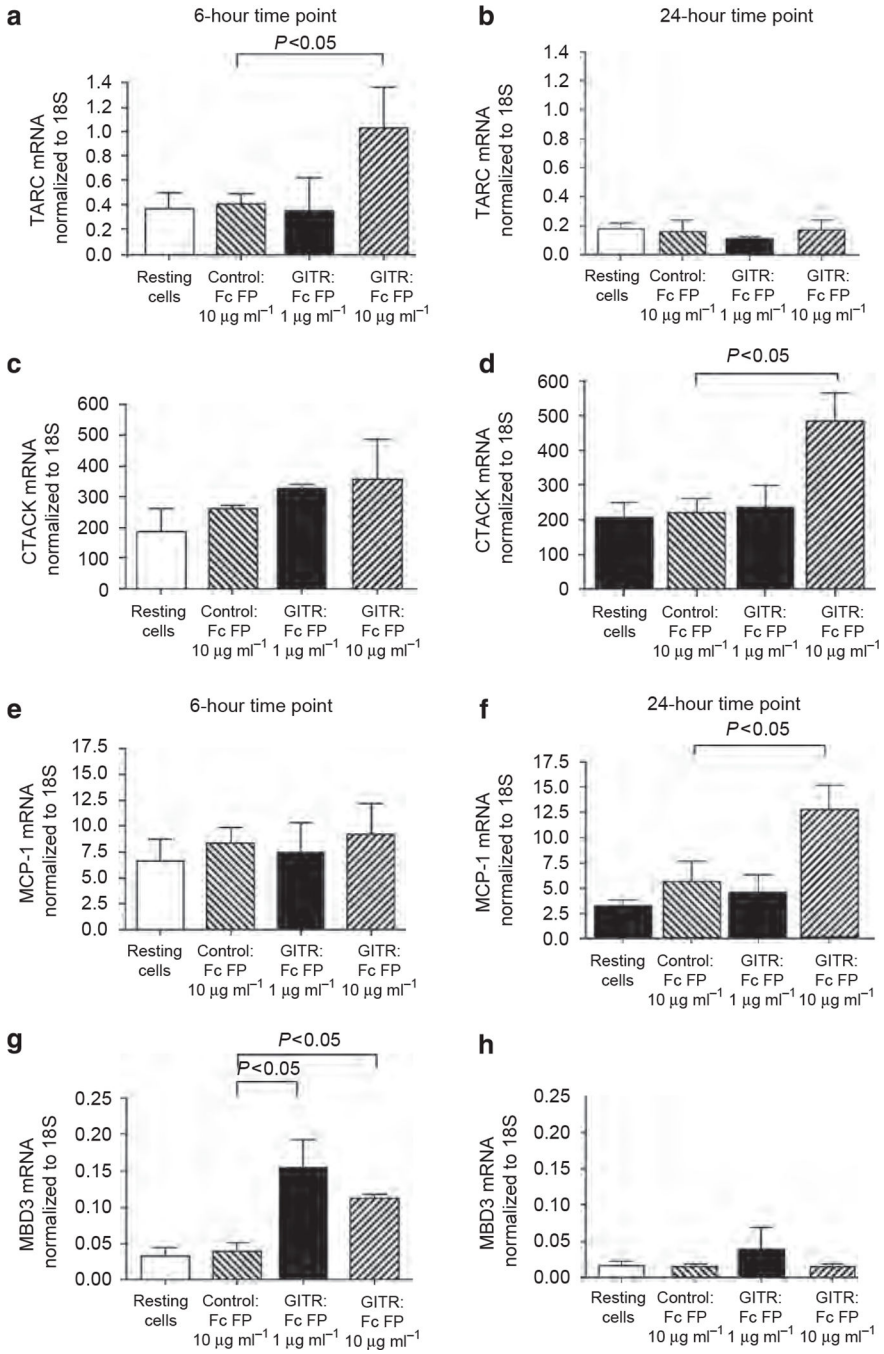

Figure 2. Chemokine-induced transcription via GITRL stimulation.

Keratinocytes regulate the expression of TARC (**a** and **b**), CTACK (**c** and **d**), MCP-1 (**e** and **f**), and MBD3 (**g** and **h**) mRNA levels after reverse stimulation through GITRL. Skin biopsies from male Balb/C mice were incubated for 6 hours (**a**, **c**, **e**, and **g**) or 24 hours (**b**, **d**, **f**, and **h**) in the presence or absence of GITR: Fc FP (1 or 10 μg ml^{-1}) or Control: Fc FP, then harvested and analyzed for mRNA expression by RT-PCR using primers specific for TARC, CTACK, MCP-1, MBD3, and 18S RNA. Values indicate the mean±SEM of six independent experiments.

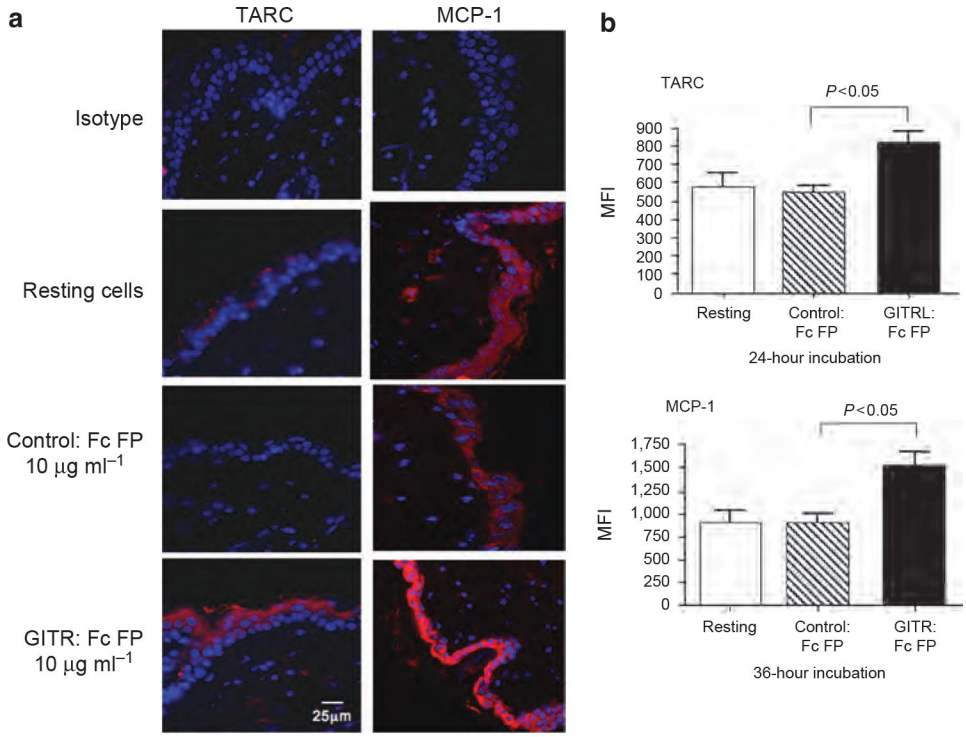

Figure 3. Increased expression of chemokines by murine keratinocytes after engagement of GITRL with GITR: Fc FP.

Skin punch biopsies obtained from male Balb/C mice were incubated with or without GITR: Fc FP or Control: Fc FP for 24 or 36 hours. (**a**) After formalin fixed biopsies were immunostained with anti-TARC Ab or anti-MCP-1 Ab, or with isotype control (red). The sections were counterstained with DAPI (blue). Representative data from three independent experiments is shown (bar = 25 μm). (**b**) Mean fluorescent intensity of TARC (24-hour time point) and MCP-1 (36-hour time point) staining in murine skin biopsies.

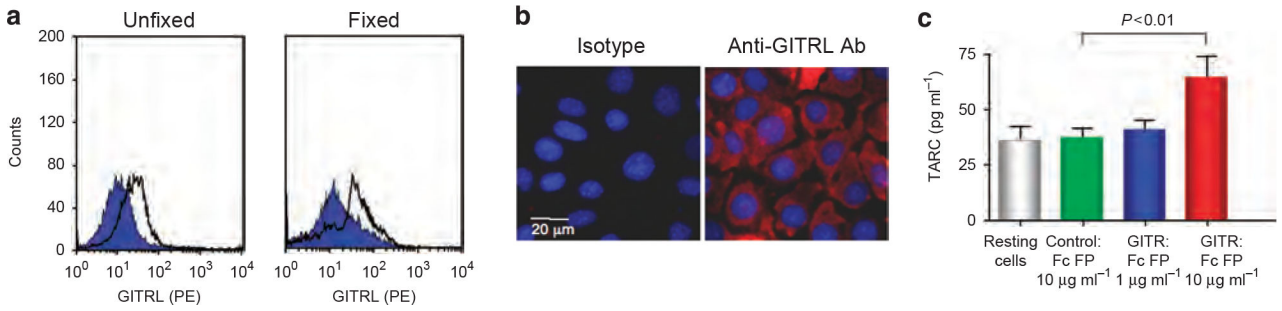

Figure 4. PAM 212 keratinocyte cell line expresses GITRL.

(**a**) Cells were stained using PE-conjugated anti-GITRL Ab before and after fixing with 1% paraformaldehyde after overnight culture and analyzed by flow cytometry. The histogram represents one of five independent experiments. The black histogram denotes anti-GITRL Ab and the blue histogram denotes the isotype. (**b**) Cells were stained with PE-conjugated anti-GITRL Ab (red) and DAPI (blue) and analyzed by immunofluorescence (bar = 20 μm). (**c**) PAM 212 cells were incubated with GITR: Fc FP (1 or 10 μg ml^{-1}) or with Control: Fc FP (10 μg ml^{-1}) for 24 hours. Supernatants were harvested and TARC production was quantified by ELISA. Values indicate the mean±SEM of six independent experiments.

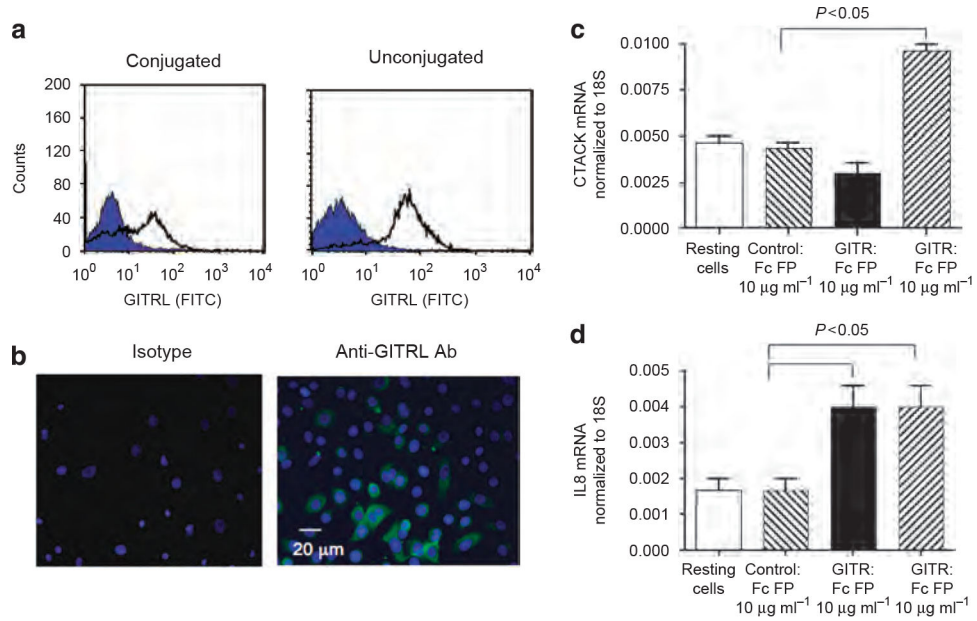

Figure 5. HEK expresses GITRL.

(**a**) Cells were stained using both directly and indirectly FITC-conjugated anti-GITRL Ab and analyzed by flow cytometry. The histogram represents one of five independent experiments. The black histogram denotes anti-GITRL Ab and the blue histogram denotes the isotype. (**b**) Cells were stained with FITC-conjugated anti-GITRL Ab (green) and DAPI (blue) and analyzed by immunofluorescence (bar = 20 μm). HEKs regulate the expression of (**c**) CTACK (**d**) and IL-8 mRNA level after reverse stimulation through GITRL. Human keratinocytes were incubated for 6 hours in the presence or absence of GITR: Fc FP (1 or 10 μg ml^{-1}) or Control: Fc FP, then harvested and analyzed for mRNA expression by RT-PCR using primers specific for CTACK, IL-8, and 18S RNA. Values indicate the mean±the SEM of three independent experiments.

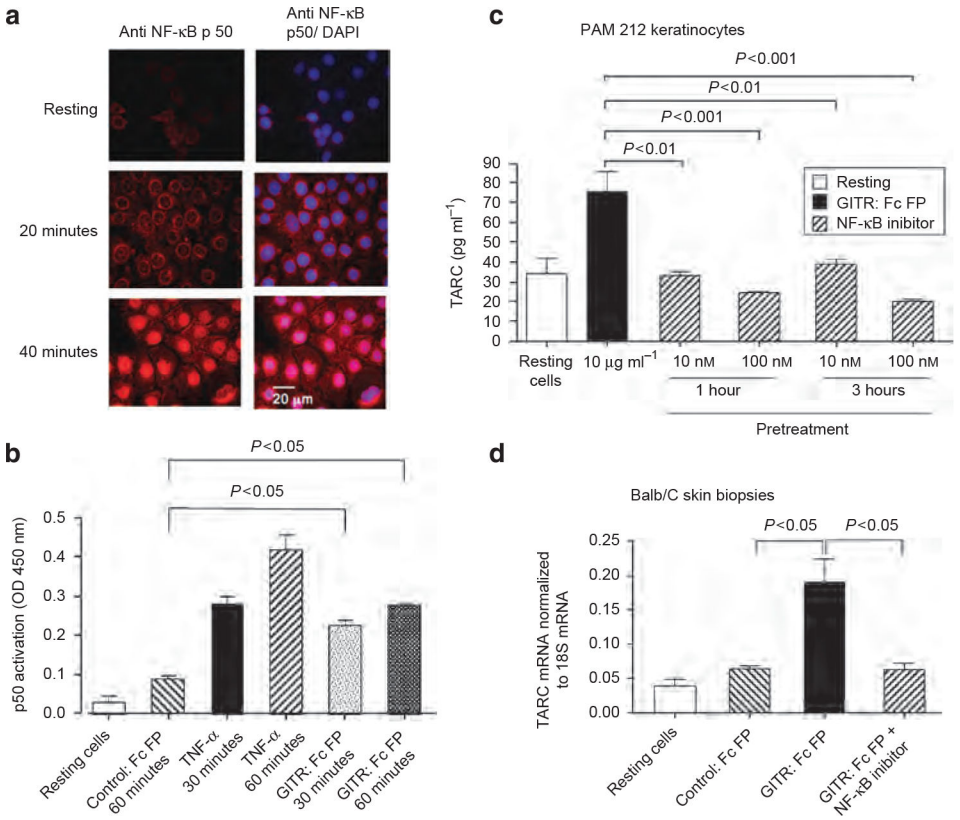

Figure 6. GITRL induces chemokine production in keratinocytes through NF-κB activation.

(**a**) PAM 212 cells were stimulated with GITR: Fc FP for the stated time periods and then fixed and stained with anti-NF-κB Ab (red). In the right column, DAPI nuclear staining (blue) is superimposed (bar = 20 μm). (**b**) PAM 212 cells were stimulated with GITR: Fc FP or Control: Fc FP (10 μg ml^{-1}) or with TNF-α (50 ng ml^{-1}) for 30 or 60 minutes. Nuclear extracts were isolated, and NF-κB activation was analyzed by assessing p50 binding to its consensus-binding site by ELISA. (**c**) PAM 212 cells were pre-incubated for 1 or 3 hours with NF-κB activation inhibitor (10/100 nM) before stimulation with GITR: Fc FP. Supernatants were analyzed for TARC by ELISA. (**d**) Murine skin biopsies pre-incubated with NF-κB activation inhibitor (100 nM for 1 hour), then stimulated with GITR: Fc FP. TARC mRNA was quantified. Values indicate the mean±SEM of three separate experiments.

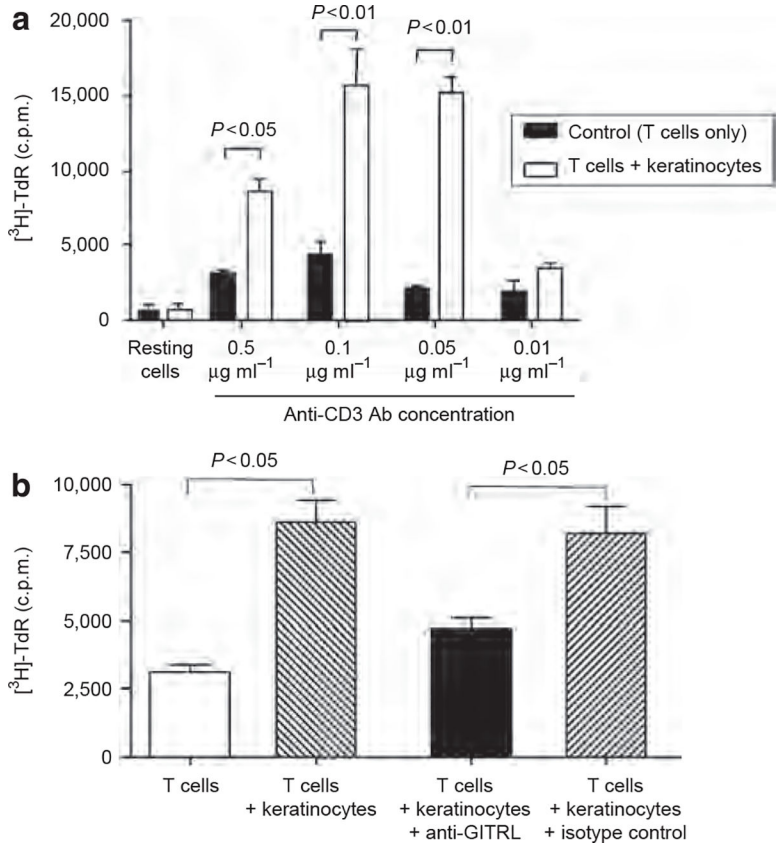

Figure 7. Cell proliferation is enhanced by GITRL-expressing keratinocytes.

Murine $CD4^+$ T cells were isolated from mouse spleens and stimulated with anti-CD3 Ab in the presence or absence of keratinocytes. (**a**) Keratinocytes (1×10^4 well) enhance T-cell proliferation at increasing concentrations of anti-CD3 Ab (0.01–0.5 μg ml^{-1}). (**b**) Keratinocytes were cultured with anti-GITRL blocking Ab (1 μg ml^{-1}) or with mouse IgG^1 isotype control for 1 hour before their use in proliferation assays.