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β2AR Antagonists and β2AR Gene Deletion Both Promote Skin Wound Repair Processes

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Skin wound healing is a complex process requiring the coordinated, temporal orchestration of numerous cell types and biological processes to regenerate damaged tissue. Previous work has demonstrated that a functional β-adrenergic receptor autocrine/paracrine network exists in skin, but the role of β2-adrenergic receptor (β2AR) in wound healing is unknown. A range of *in vitro* (single-cell migration, immunoblotting, ELISA, enzyme immunoassay), *ex vivo* (rat aortic ring assay), and *in vivo* (chick chorioallantoic membrane assay, zebrafish, murine wild-type, and β2AR knockout excisional skin wound models) models were used to demonstrate that blockade or loss of β2AR gene deletion promoted wound repair, a finding that is, to our knowledge, previously unreported. Compared with vehicle-only controls, β2AR antagonism increased angiogenesis, dermal fibroblast function, and re-epithelialization, but had no effect on wound inflammation *in vivo*. Skin wounds in β2AR knockout mice contracted and re-epithelialized faster in the first few days of wound repair *in vivo*. β2AR antagonism enhanced cell motility through distinct intracellular signalling mechanisms and increased vascular endothelial growth factor secretion from keratinocytes. β2AR antagonism promoted wound repair processes in the early stages of wound repair, revealing a possible new avenue for therapeutic intervention.

INTRODUCTION

Wound healing is a complex process requiring the coordinated, temporal orchestration of numerous cell types and processes to initiate repair (Shaw and Martin, 2009). The epidermis can synthesize and secrete a number of proteins (Boyce, 1994) including epinephrine (Schallreuter *et al.*, 1992; Pullar *et al.*, 2006b), a ligand for the β-adrenergic receptors (βARs): β1-adrenergic receptor (β1AR), β2AR, and β3AR (Wallukat, 2002). They are G protein–coupled receptors highly expressed on all cell lineages in the skin (McSwigan *et al.*, 1981; Steinkraus *et al.*, 1992; Izeboud *et al.*, 1999; Iaccarino *et al.*, 2002; de Coupade *et al.*, 2004);

therefore, an autocrine and paracrine βAR network exists in the epidermis and dermis, respectively.

Previously, nonselective activation or blockade of the βARs has revealed a regulatory role for βARs in keratinocyte migration and re-epithelialization. βAR activation decreased the rate of human keratinocyte (HK) migration *in vitro* (Pullar *et al.*, 2003, 2006a; Pullar and Isseroff, 2005), whereas a βAR antagonist promoted HK migration, by blocking the autocrine epinephrine-mediated decrease in migration (Pullar *et al.*, 2006b). In excised human skin, βAR activation delayed wound re-epithelialization (Pullar *et al.*, 2006a), whereas βAR antagonism promoted skin re-epithelialization (Pullar *et al.*, 2006b) in an *ex vivo* model of chronic wound re-epithelialization (Kratz, 1998).

In murine skin wound models, stress-induced increases in epinephrine delayed wound repair (Sivamani *et al.*, 2009; Romana-Souza *et al.*, 2010a), whereas, conversely, βAR antagonism enhanced re-epithelialization in a murine skin burn model *in vivo* (Sivamani *et al.*, 2009) and accelerated skin barrier recovery (Denda *et al.*, 2003). In addition, a nonselective βAR antagonist improved wound healing in diabetic (Romana-Souza *et al.*, 2009a) and burn-injured (Romana-Souza *et al.*, 2008) rats and chronically stressed mice (Romana-Souza *et al.*, 2010b), while delaying repair in rat acute skin wounds (Souza *et al.*, 2006; Romana-Souza *et al.*, 2009c).

Here the effect of β2AR antagonism and loss of the β2AR on the main processes in wound repair were investigated. The *in vitro*, *ex vivo*, and *in vivo* models allowed a thorough

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mechanistic investigation of skin wound repair at both the cellular and the tissue level, using a combination of pharmacological and genetic approaches.

RESULTS

β2AR antagonism promotes angiogenesis

Angiogenesis is an essential process in acute wound healing (Tonnesen *et al.*, 2000), and endothelial cell (EC) migration is a critical process in angiogenesis (Eming *et al.*, 2007). To determine whether a β2AR antagonist altered EC migration, single-cell migration (SCM) assays were performed with primary human dermal microvascular ECs (HDMECs) in the presence and absence of β2AR antagonist; however, there was no effect on migration rate (Figure 1a).

To determine whether β2AR antagonism could alter cell outgrowth from the cut edge of rat aorta (Smith and Staton, 2006), aortic pieces were embedded into the basement membrane extract and incubated in the presence or absence of a β2AR antagonist for 5 days. β2AR antagonism increased aortic outgrowth by 66% after 5 days (Figure 1b).

To investigate angiogenesis in a more complex, multi-cellular environment *in vivo*, the chick chorioallantoic membrane (CAM) assay, a model of embryonic angiogenesis (Smith and Staton, 2006), was selected. β2AR antagonist treatment increased angiogenesis by 48% in the CAM assay, between days 5 and 9 post fertilization (Figure 1c).

Finally, to determine whether β2AR antagonism could modulate wound angiogenesis *in vivo*, two full-thickness 6-mm wounds were created in the dorsal skin of wild-type (β2AR$^{+/+}$) and β2AR knockout (β2AR$^{-/-}$) mice (Chruscinski *et al.*, 1999). The wounds were treated immediately after wounding and then daily, topically, with hydrogel alone or hydrogel containing 0.1% β2AR antagonist, and photographed daily. To evaluate the amount of angiogenesis in the dermis, 7-μm-thick sections from the wound center, excised after 5 days, were immunostained with an antibody to an EC marker (CD31), revealing the degree of angiogenesis in the mouse wounds (Yoon *et al.*, 2009). β2AR antagonist treatment increased angiogenesis by 26% (Figure 1d). The amount of angiogenesis in β2AR$^{-/-}$ wounds was equal to that observed in control β2AR$^{+/+}$ wounds (Figure 1d), whereas β2AR antagonist treatment of β2AR$^{-/-}$ wounds had no effect (results not shown).

Vascular endothelial growth factor (VEGF) is a potent pro-angiogenic factor in acute wounds (Barrientos *et al.*, 2008), and βAR activation can increase VEGF expression in human choroid ECs (Steinle *et al.*, 2008). To determine whether β2AR antagonism increased VEGF secretion from HDMECs, HKs, human dermal fibroblasts (HDFs), neutrophils, and macrophages, ELISAs were performed on control and β2AR antagonist–treated supernatants. VEGF secretion was not detected in HDMECs or HDF supernatants. Although VEGF secretion was detected in the supernatants of neutrophils and macrophages, β2AR antagonist treatment had no effect (results not shown). However, βAR antagonist treatment increased the amount of VEGF secreted from HKs after 24 hours by 23% (Figure 1e).
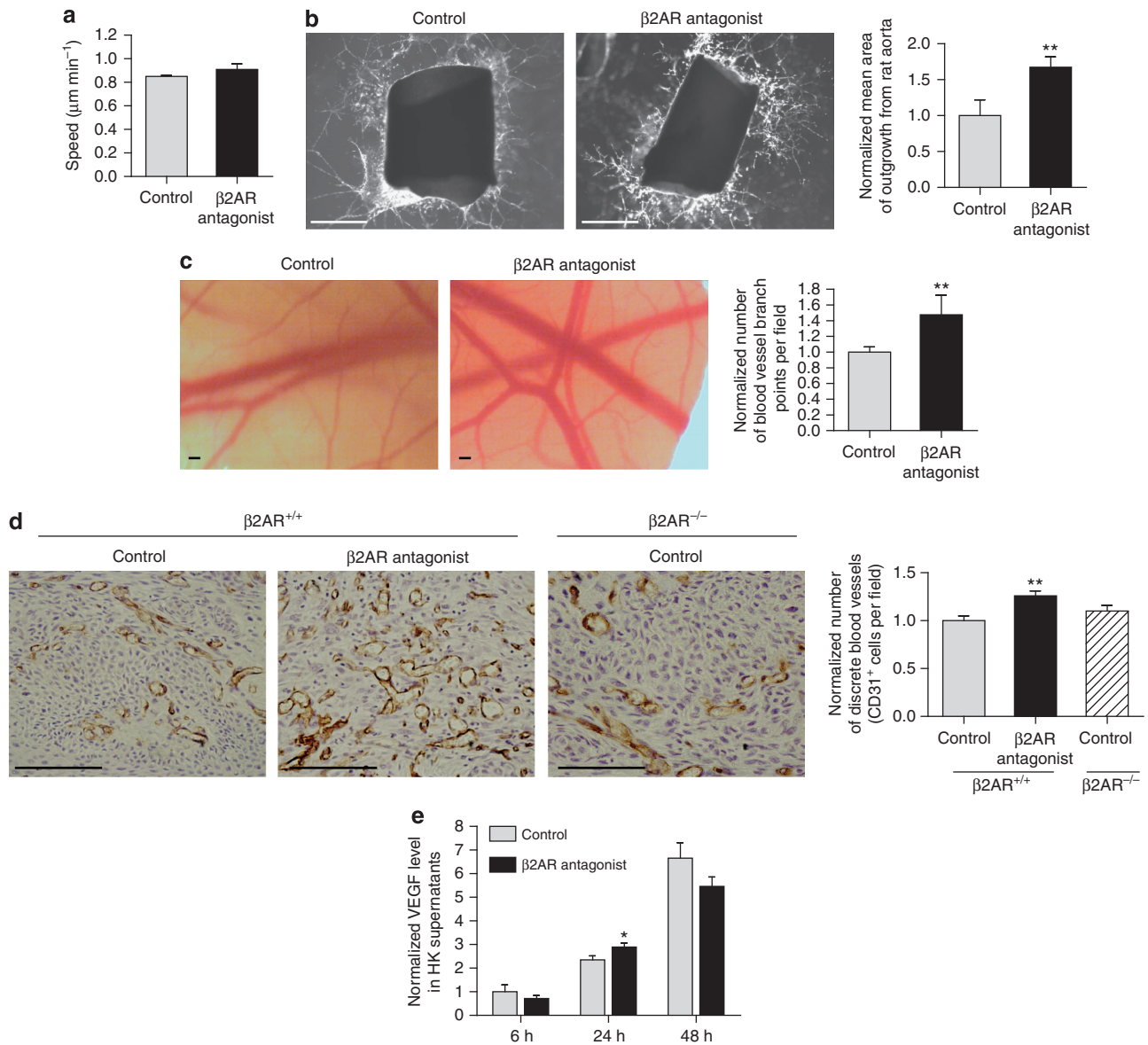
β2AR antagonism promotes human dermal fibroblast migration *in vitro* and function *in vivo*

Within a few days of wounding, fibroblasts proliferate and migrate into the wound bed (Shaw and Martin, 2009). A nonselective βAR agonist increased HDF migration via the src-dependent transactivation of EGFR (Pullar and Isseroff, 2006). To determine the effect of β2AR antagonism on HDF migration, SCM was performed in the presence and absence of a highly selective β2AR antagonist. β2AR antagonism increased the speed of HDF migration by 27% (Figure 2a). In addition, DFs were isolated from the skin of β2AR$^{+/+}$ and β2AR$^{-/-}$ mice. Although a β2AR antagonist increased the migration speed of β2AR$^{+/+}$ murine DFs (MDFs) by 49%, the speed of β2AR$^{-/-}$ MDFs was increased by 46% compared with β2AR$^{+/+}$ MDFs (Figure 2b). The β2AR antagonist had no additional effect on β2AR$^{-/-}$ MDF migration speed, as expected (Figure 2b). Extracellular signal–regulated kinase (ERK) has an important role in cell migration (Klemke *et al.*, 1997). β2AR antagonism increased β2AR$^{+/+}$ MDF ERK phosphorylation by 90% (Figure 2c).

To explore whether β2AR antagonism could modulate wound DF function, two full-thickness 6-mm wounds were created in the dorsal skin of β2AR$^{+/+}$ and β2AR$^{-/-}$ mice and treated daily. Wound area was calculated daily until excision to determine the rate of wound contraction (Galiano *et al.*, 2004a), as described in the Materials and Methods section. Wound contraction was accelerated 4-fold in β2AR antagonist–treated β2AR$^{+/+}$ wounds and 5-fold in control β2AR$^{-/-}$ wounds, 24 hours after wounding (Figure 3a). Wound contraction was still significantly enhanced by 2-fold in β2AR antagonist–treated β2AR$^{+/+}$ wounds and almost 3-fold in control β2AR$^{-/-}$ wounds, 48 hours post wounding. After 3 days, contraction was increased by 19% in β2AR antagonist–treated β2AR$^{+/+}$ wounds and 40% in control β2AR$^{-/-}$ wounds, but after 4 days, contraction of both β2AR antagonist–treated β2AR$^{+/+}$ wounds and control β2AR$^{-/-}$ wounds occurred at the same rate as control β2AR$^{+/+}$ wounds. β2AR antagonist–treated β2AR$^{-/-}$ wounds demonstrated the same rate of wound contraction as control β2AR$^{-/-}$ wounds (Figure 3a).

Expression of α-smooth muscle actin (SMA) is a reliable marker of myofibroblast differentiation, and the rate of wound contraction can directly correlate with SMA expression (Hinz *et al.*, 2001). Sections (7 μm thick) from the wound center, excised after 5 days, were immunostained with an antibody to SMA. Populations of SMA-stained fibroblasts were observed below the wound epithelial margins, 5 days post wounding. The area of SMA staining was increased by 60% in β2AR antagonist–treated wounds in β2AR$^{+/+}$ mice and was similar in control β2AR$^{-/-}$ and β2AR$^{+/+}$ wounds (Figure 3b).

Myofibroblasts synthesize collagen III early in the wound repair process, which is later replaced by collagen I in more mature scars (Hinz, 2007). Sections (7 μm thick) from the wound center, excised after 5 days, were immunostained with an antibody to collagen III. β2AR antagonist treatment increased the area of collagen III–stained neo-dermis by 20% in β2AR$^{+/+}$ wounds (Figure 3c), whereas control β2AR$^{-/-}$ and β2AR$^{+/+}$ wounds had similar areas of
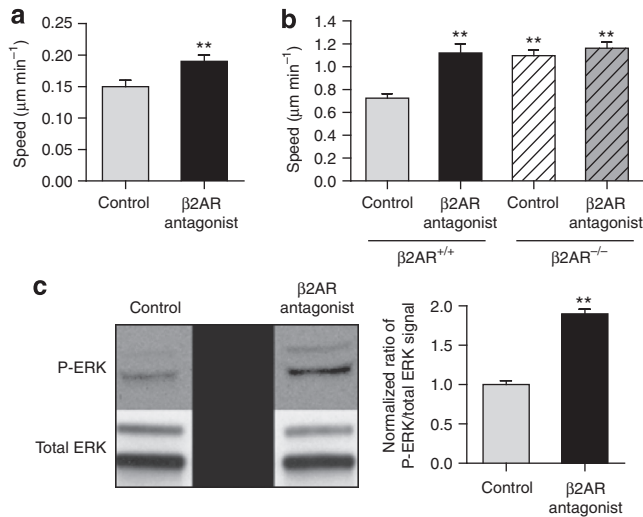
Figure 1. **β2-Adrenergic receptor (β2AR) antagonism promoted wound angiogenesis.** (**a**) Human dermal microvascular endothelial cells (HDMECs) were plated in the presence or absence of 10 nM βAR antagonist, and single-cell migration (SCM) was performed for 1 hour; see Materials and Methods (control, $n=200$; antagonist, $n=82$). (**b**) Rat aortic sections were prepared, incubated in the presence or absence of 10 μM βAR antagonist, and imaged every 24 hours; see Materials and Methods. Representative images of aortas 5 days post-treatment are shown, bar = 1 mm. The area of outgrowth was measured using the Volocity software (control, $n=4$; antagonist, $n=4$). (**c**) Fertilized chicken eggs were prepared and chick chorioallantoic membranes (CAMs) were treated with vehicle alone or with vehicle containing 100 μM β2AR antagonist, from days 5 to 9, and imaged every 24 hours; see Materials and Methods. Representative day 9 images are shown, bar = 1 mm (control, $n=16$; antagonist, $n=3$). (**d**) Paraffin-embedded wound sections (7 μm) from control and 0.1% βAR antagonist–treated wild-type (β2AR$^{+/+}$) mice and control β2-AR knockout (β2AR$^{-/-}$) mice day 5 post wounding were immunostained with an anti-CD31 antibody; see Materials Methods. Representative images are shown, bar = 100 μm ($n=10$–14). (**e**) Human keratinocytes (HKs) were plated for in the presence or absence of 10 nM β2AR antagonist and prepared for vascular endothelial growth factor-A (VEGF-A) ELISA; see Materials and Methods. Data are representative of five independent experiments from two separate cell strains. Values plotted are the means ± SEM. *$P<0.05$; **$P<0.01$.

collagen III staining (Figure 3c). To examine this further, sections were stained with picrosirius red to assess total collagen deposition in the neo-dermis (Junqueira *et al.*, 1979). β2AR antagonist treatment increased the area of collagen-stained neo-dermis, within 1 mm^2 of the wound edge, by 29% in β2AR$^{+/+}$ wounds (Figure 3d), whereas control β2AR$^{-/-}$ and β2AR$^{+/+}$ wounds had similar areas of collagen staining (Figure 3d).

β2AR antagonism has no effect on wound inflammation *in vivo*

Neutrophils and macrophages are recruited to the wound site to quell bacterial infections, where they secrete a cocktail of growth factors and cytokines to activate resident keratinocytes and fibroblasts (Martin, 1997).

To determine whether a β2AR antagonist could alter the guidance of inflammatory cells to a wound, a zebrafish wound model was used. Indeed, zebrafish are known to have

Figure 2. **β2-Adrenergic receptor (β2AR) antagonism, or the absence of β2AR, increased dermal fibroblast (DF) migration.** (**a**) Human dermal fibroblasts (HDFs) were plated, single-cell migration (SCM) was performed for 1 hour, and the mean migration speed was calculated (HDF control, $n=107$; β2AR antagonist, $n=112$). (**b**) Murine dermal fibroblasts (MDFs) were isolated from β2AR knockout (β2AR$^{-/-}$) and wild-type (β2AR$^{+/+}$) neonates and SCM performed (MDF: β2AR$^{+/+}$ control, $n=77$, antagonist, $n=73$; MDF: β2AR$^{-/-}$ control, $n=60$). (**c**) Confluent 35-mm^2 dishes of MDFs were stimulated for 10 minutes at 37 °C with media alone (control) or with media containing 10 nM ICI 118,551, samples were prepared, and western blotting performed for phosphorylated (P-) and total extracellular signal–regulated kinase (ERK); see Materials and Methods. The data shown are representative of three independent experiments. Three blots from separate experiments were scanned and densitometry performed; see Materials and Methods. Values plotted are the means ± SEM. **$P<0.01$.

two β2AR genes (Steele *et al.*, 2011), but their role in tissue regeneration is unknown. Zebrafish larvae are transparent and zebrafish neutrophils are identifiable 48 hours post fertilization. Upon transection of the caudal fin, neutrophils are guided to the wound within minutes, but peak neutrophil recruitment occurs after approximately 6 hours (Renshaw *et al.*, 2006). When the wounded zebrafish larvae were immersed in pond water containing a β2AR antagonist, there was no effect on neutrophil guidance to the caudal fin wound (Figure 4a).

To determine whether β2AR antagonist treatment altered wound inflammation in the mouse excisional skin wound model, 7-μm-thick sections from the wound center, excised after 3 days, were immunostained with either an antibody to a polymorphonuclear cell marker (Ly6G-6C) (Fleming *et al.*, 1993) or a macrophage marker (F4-80) (Austyn and Gordon, 1981). Topical β2AR antagonist treatment or the absence of the receptor (β2AR$^{-/-}$) had no effect on the number of polymorphonuclear cells or macrophages recruited to the wound site either 3 or 5 days post wounding (Figure 4b and c; day 5 results not shown).

β2AR antagonism promotes keratinocyte migration *in vitro* and re-epithelialization *in vivo*

Keratinocyte migration from the wound edges is a critical process to restore the barrier function of the epidermis, and

they are a valuable source for growth factors in the wound (Shaw and Martin, 2009).

A β2AR antagonist increased keratinocyte migration significantly by 12% (Figure 5a). To confirm that murine keratinocytes (MKs) respond similarly to a β2AR antagonist, MKs were isolated from newborn β2AR$^{+/+}$ and β2AR$^{-/-}$ mice and the effect of a β2AR antagonist on motility was determined. A β2AR antagonist enhanced MK SCM by 28% (Figure 5b). β2AR$^{-/-}$ MKs migrated 16% faster than β2AR$^{+/+}$ MKs, whereas a β2AR antagonist had no additional effect on their migration, confirming the absence of the β2AR (Figure 5b). A β2AR antagonist–mediated 2.5-fold increase in ERK phosphorylation, known to have a pivotal role in pro-migratory signaling pathways (Klemke *et al.*, 1997), underpinned its effect on MK motility (Figure 5c).

Tyrosine hydroxylase, the enzyme controlling the rate-limiting step for catecholamine biosynthesis (Nagatsu *et al.*, 1964), was expressed in MKs (Figure 5d), and 100 ± 10 and 75 ± 8 pg mg^{-1} protein of epinephrine was measured by enzyme immunoassay in extracts from MKs. MKs similar to HKs (Pullar *et al.*, 2006b), therefore, can also synthesize epinephrine, generating an autocrine and paracrine adrenergic network in the murine epidermis and dermis, respectively.

Finally, to determine the effect of a β2AR antagonist on wound re-epithelialization, 7-μm-thick sections from the wound center, excised after 3 and 5 days, were stained with hematoxylin and eosin, and the linear distance covered by new epithelium was determined. β2AR antagonist treatment increased β2AR$^{+/+}$ wound re-epithelialization by 60 and 56% within 3 and 5 days post wounding, respectively (Figure 5e). In addition, re-epithelialization of β2AR$^{-/-}$ wounds was increased by 64% 5 days post wounding (Figure 5e).

DISCUSSION

β2AR antagonism enhanced angiogenesis *ex vivo* and *in vivo* and increased VEGF secretion from HKs. β2AR antagonism increased the migration rate of HDFs *in vitro*, while increasing SMA expression, collagen III expression, and contractile ability in murine wounds *in vivo*. In addition, β2AR$^{-/-}$ wounds contracted faster in the first few days post wounding. In contrast, β2AR antagonism had no effect on wound inflammation *in vivo*. Finally, β2AR antagonism or loss of β2AR increased keratinocyte migration *in vitro* and promoted re-epithelialization *in vivo* within 5 days of wounding. In summary, β2AR antagonism promoted wound repair, as summarized in the Supplementary Appendix S1 online.

In previous studies, a nonselective βAR antagonist, propranolol, improved healing in hospitalized burn patients, because of its ability to decrease energy expenditure and muscle protein catabolism (Mohammadi *et al.*, 2009) and improved wound repair in rabbit skin wounds (Zhang *et al.*, 2009), diabetic rats (Romana-Souza *et al.*, 2009b), and burn-injured rats (Romana-Souza *et al.*, 2008). The studies presented here use a highly selective β2AR antagonist, ICI 118,551 (Bilski *et al.*, 1983), demonstrating for the first time that selective blockade of the β2AR promotes wound
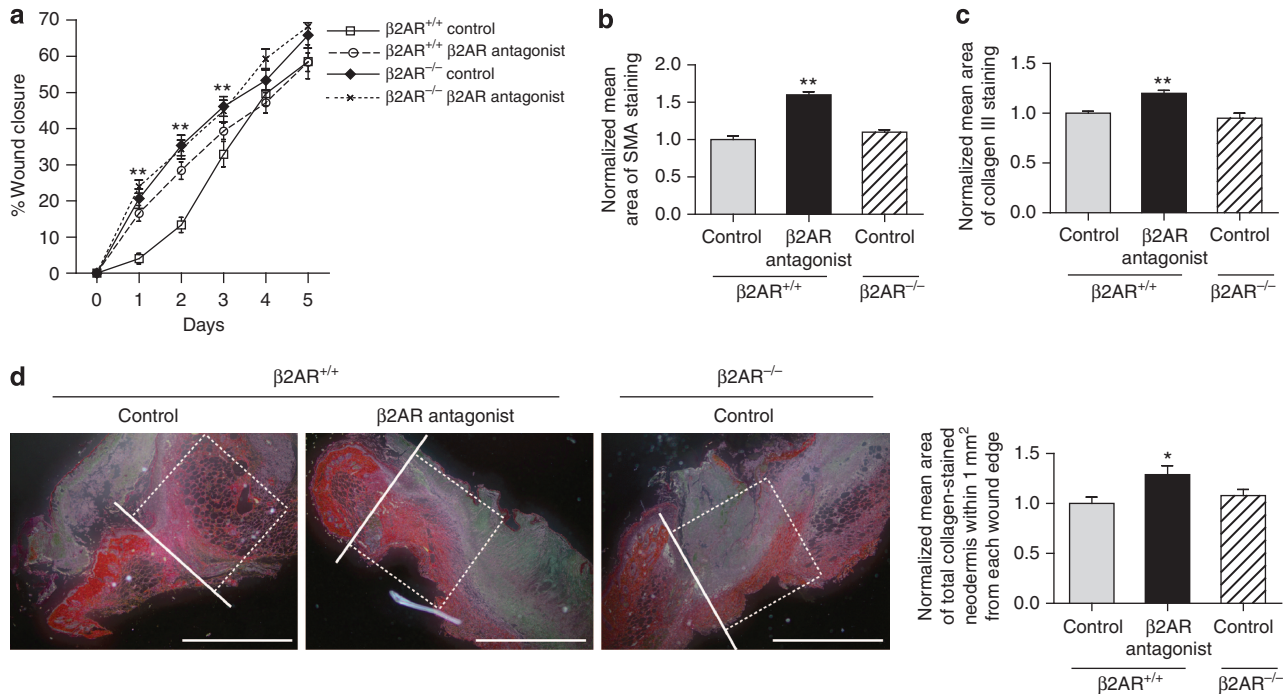
Figure 3. **β2-Adrenergic receptor (β2AR) antagonism, or the absence of β2AR, increased dermal fibroblast function *in vivo*.** (**a**) Two full-thickness 6-mm excisional wounds, created in the dorsal skin of β2-AR wild-type (β2AR$^{+/+}$) and β2-AR knockout (β2AR$^{-/-}$) mice, were treated and photographed daily, and the area of each wound was calculated; see Materials and Methods. Sections (7 μm), day 5 post wounding, were stained with (**b**) an anti-smooth muscle actin (SMA) antibody or (**c**) an anti-collagen III antibody. The mean area of positive staining was calculated; see Materials and Methods. (**d**) Representative images of picrosirius red staining are shown. The mean area of collagen-stained neo-dermis within (white square) 1 mm^2 of the (white line) wound edge was calculated; see Materials and Methods. Bar = 1 mm (n = 10–14). Values plotted are means ± SEM. *P < 0.05; **P < 0.01.

processes, including angiogenesis, DF function, and re-epithelialization, thus accelerating healing.

The mechanism underpinning the β2AR antagonist–mediated increase in angiogenesis could be partly attributed to the β2AR antagonist–mediated increase in VEGF secretion from HKs, known to be an important source of VEGF (Rossiter *et al.*, 2004) and other growth factors in wounds (Werner and Grose, 2003; Barrientos *et al.*, 2008). Indeed, the addition of autologous cultured keratinocytes can aid healing of chronic venous leg ulcers (Liu *et al.*, 2004) and the addition of VEGF promotes healing in chronic diabetic wounds (Galiano *et al.*, 2004b; Brem *et al.*, 2009); therefore, the β2AR antagonist–mediated increase in HK VEGF secretion could be highly beneficial for wound repair. In the complex wound environment, β2AR antagonist treatment could also increase the secretion of other pro-angiogenic factors such as fibroblast growth factor 2 or basic fibroblast growth factor or platelet-derived growth factor (Battegay *et al.*, 1994), from wound cells, which could contribute to the enhanced angiogenesis observed *in vivo*. In contrast, post-ischemic angiogenesis was impaired in β2AR$^{-/-}$ mice (Ciccarelli *et al.*, 2011).

The increase in wound contraction observed in β2AR$^{-/-}$ wounds (Figure 3a) occurred in the absence of an increase in SMA-expressing cells in the wound bed (Figure 3b), in contrast to the observed increase in both wound contraction and SMA expression in the β2AR antagonist–treated β2AR$^{+/+}$ wounds. Although the rate of wound contraction is thought to correlate directly with SMA expression (Hinz *et al.*,

2001), there are several reports describing that wound contraction can occur in the absence of SMA-expressing myofibroblasts. Wounds in vanadate-treated rats (Ehrlich *et al.*, 1999) and rat wounds treated with a Smad blocker, SB-505124 (Au and Ehrlich, 2010), contracted normally, but SMA-expressing cells were not detected in the granulation tissue. Finally, a personal communication from JJ Tomasek reports that SMA knockout mice heal normally (Hinz, 2007). It appears that wound contraction is not correlated with SMA expression in β2AR$^{-/-}$ mouse wounds and only β2AR antagonist treatment can increase SMA expression (Figure 3b).

In addition, blockade of autocrine, endogenously synthesized epinephrine (Figure 5d), inhibitory to keratinocyte migration (Pullar *et al.*, 2003, 2006a), likely contributed to the ability of the β2AR antagonist to promote MK migration *in vitro* and re-epithelialization *in vivo*.

Although β2AR antagonist treatment did not alter acute wound inflammation or the secretion of VEGF from inflammatory cells, β2AR blockade has been demonstrated to attenuate the hyper-inflammatory response to traumatic injury in mice (Rough *et al.*, 2009). It is possible, therefore, that β2AR antagonist treatment could reduce persistent or hyper-inflammation, which will be tested in a chronic wound model.

In conclusion, here we report that β2AR antagonism promotes wound angiogenesis, DF function, and re-epithelialization, enhancing wound repair, thus revealing a possible new avenue of therapeutic intervention. To our knowledge, this is previously unreported.
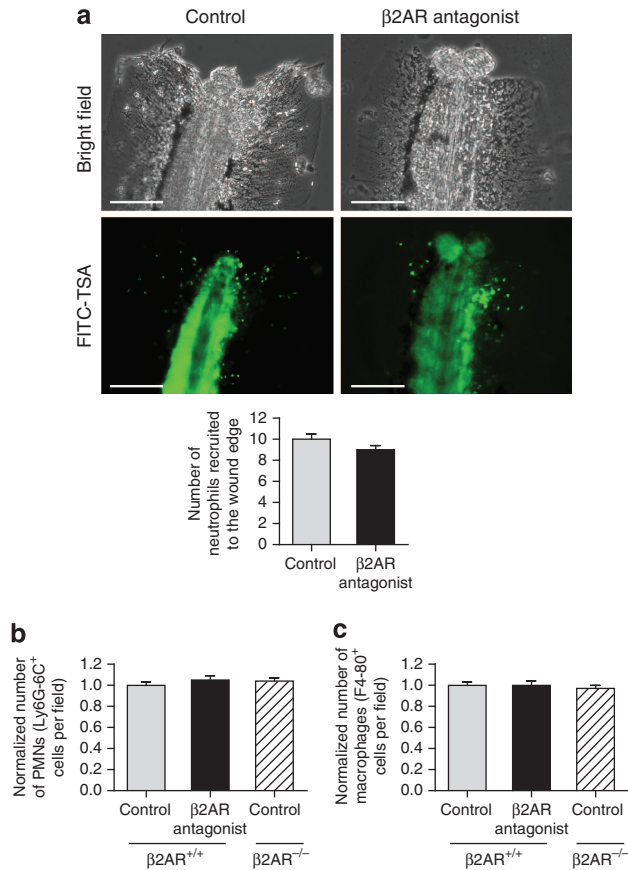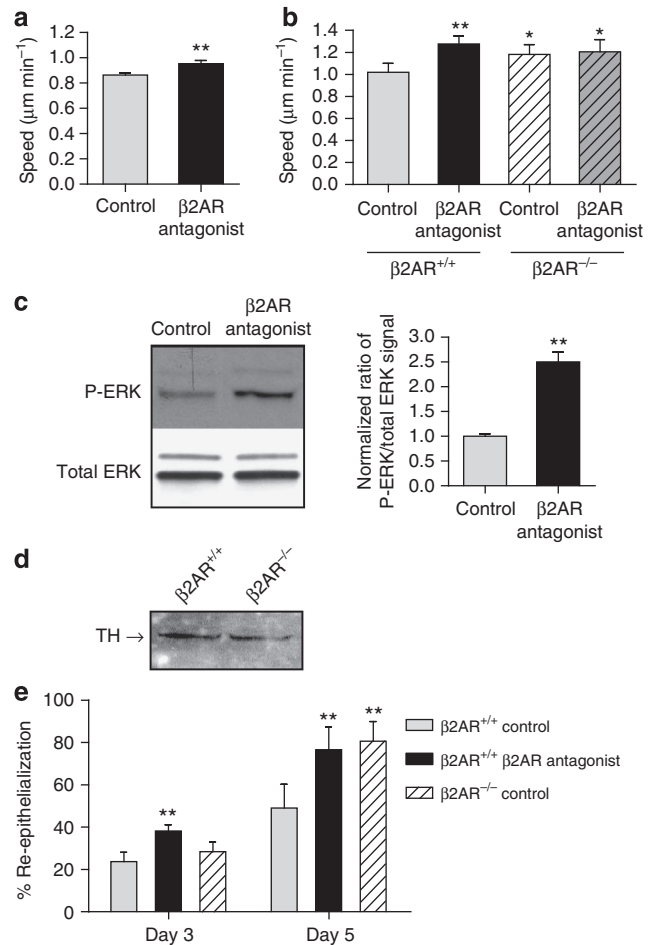
Figure 4. **β2-Adrenergic receptor (β2AR) antagonism has no effect on wound inflammation.** Zebrafish larvae were wounded, untreated, or treated with 500 μM β2AR antagonist and imaged using fluorescein-tyramine signal amplification; see Materials and Methods. (**a**) The number of neutrophils recruited to each wounded tail was recorded. Bar = 100 μm (n = 15–30). Paraffin-embedded murine wound sections (7 μm), day 3 post wounding, were immunostained with an antibody that recognizes (**b**) neutrophils (Ly6G-6C) or (**c**) macrophages (F4-80). The positive cells per field were counted and the data analyzed from 10 images of each wound section; see Materials and Methods (n = 10–14). Values plotted are means ± SEM. FITC, fluorescein isothiocyanate; PMNs, polymorphonuclear cells; TSA, tyramide signal amplification.

MATERIALS AND METHODS

Animals

The β2AR-deficient FVB/N (β2AR$^{-/-}$) mice were a kind gift from Dr Brian Kobilka, Stanford University (Stanford, CA) (Chruscinski *et al.*, 1999). The congenic controls, FVB/N (β2AR$^{+/+}$) mice, were purchased from The Jackson Laboratory (Bar Harbor, ME). The genotypes of the β2AR$^{+/+}$ and β2AR$^{-/-}$ mice were confirmed by PCR, using published primers (Chruscinski *et al.*, 1999). All animals used in the study were females between 8 and 12 weeks of age. The University of California, Davis Institutional Animal Care and Use Committee (IACUC) approved all animal protocols.

Adult zebrafish were maintained, as described (Westerfield, 1994), in compliance with the Animals (Scientific Procedures; www.legislation.gov.uk/ukpga/1986/14/contents) Act, 1986. Embryos were harvested and raised in egg water (Westerfield, 1994), at 28.5 °C, until the required developmental stage. Staging was performed in accordance with (Kimmel *et al.*, 1995).

Figure 5. **β2-Adrenergic receptor (β2AR) antagonism, or the absence of β2AR, increased keratinocyte migration and re-epithelialization.** (**a**) Human keratinocytes (HKs) and (**b**) murine keratinocytes (MKs), isolated from β2-AR knockout (β2AR$^{-/-}$) and wild-type (β2AR$^{+/+}$) neonates, were plated and single-cell migration (SCM) performed; see Materials and Methods (HK: control, n = 224, antagonist, n = 179; MK: β2AR$^{+/+}$ control, n = 128, β2AR antagonist, n = 102; β2AR$^{-/-}$ control = 105). MKs were treated with media alone (control) or with media containing 10 nM β2AR antagonist, and western blotting for extracellular signal–regulated kinase (ERK) was performed; see Materials and Methods. (**c**) Blots were scanned and densitometry performed using NIH Image J. (**d**) MK lysates were prepared and western blotting for tyrosine hydroxylase (TH) was performed. The data shown are representative of three independent experiments. Control and β2AR antagonist–treated β2AR$^{+/+}$ wounds and control β2AR$^{-/-}$ wounds were excised, fixed, and stained. The percentage of wound re-epithelialization, at days 3 and 5 post wounding, was calculated using Image J; see Materials and Methods (n = 10) (**e**). Values plotted are means ± SEM. *P < 0.05; **P < 0.01. P-ERK, phosphorylated-ERK.

Culture of primary cells

Primary MKs were isolated from newborn mouse pups, as described previously (Isseroff *et al.*, 1983), and cultured in MK growth media (Epilife and keratinocyte growth supplement), 10 ng ml^{-1} murine EGF, 0.1 nM cholera toxin (Invitrogen, Paisley, UK), and 0.5% antibiotic solution (25 U ml^{-1} penicillin and 25 μg ml^{-1} streptomycin (Sigma Aldrich, Poole, UK), murine keratinocyte complete growth medium). To isolate MDFs, the remaining dermal pieces were minced with sterile scissors and transferred to 100-mm plastic cell culture dishes from Falcon Labware (BD Biosciences, Oxford, UK).

Dermal pieces were allowed to attach for 5 minutes, before the addition of 4 ml of fibroblast growth medium, which consists of DMEM (basal medium), 0.5% antibiotic solution (25 U ml^{-1} penicillin and 25 μg ml^{-1} streptomycin; Sigma Aldrich), and 10% fetal calf serum (Invitrogen). The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 hours before adding an additional 6 ml of fibroblast growth medium per dish.

Primary human neonatal DFs were purchased from Invitrogen and cultured as described previously (Pullar and Isseroff, 2006).

Primary HDMECs were purchased from Invitrogen. Cells were grown in EC growth media supplemented with microvascular growth supplement (PromoCell, Heidelburg, Germany) and antibiotics (25 U ml^{-1} penicillin, 25 μg ml^{-1} streptomycin; Invitrogen) at 37 °C with 5% CO_2/95% air in a humidified atmosphere. Cells were grown on attachment factor containing 0.1% gelatin (Invitrogen), and experiments were conducted using cell passages between 3 and 7.

Primary neonatal HKs were purchased from Invitrogen and cultured as described previously (Pullar and Isseroff, 2005). At least three keratinocytes strains, between passages 3 and 6, were used in all experiments.

SCM assay

Plastic cell culture dishes (35 mm) were coated with collagen I (60 μg ml^{-1}) (Invitrogen) in phosphate-buffered saline for 1 hour at 37 °C. MDFs, isolated from both β2AR$^{-/-}$ and β2AR$^{+/+}$ mice, and HDFs were plated at a density of 25 cells per mm^2 in fibroblast growth medium, whereas MKs, HKs, and HDMECs were plated at a density of 50 cells per mm^2, in their appropriate complete media (see culture methods), for 2 hours at 37 °C. Cells were incubated with complete media alone (control) or with media containing 10 nM β2AR antagonist (ICI 118,551; Tocris Biosciences, Bristol, UK) at time 0. The 35-mm dishes were placed in a heating chamber, designed to maintain the medium at 37 °C, and images were taken every 10 minutes for 1 hour with the Improvision software (Perkin Elmer, Cambridge, UK), as described previously (Pullar *et al.*, 2006b).

Cell treatments for immunoblotting

MKs or MDFs plated at a density of 1×10^6, isolated from both β2AR$^{-/-}$ and β2AR$^{+/+}$ mice, were incubated with either murine keratinocyte complete growth medium (MK) or fibroblast growth medium (MDF) alone (control and lysates for catecholamine synthesis enzyme detection), or with media containing 10 nM B2AR antagonist (ICI, 118551), for 10 minutes. Lysates were prepared and electrophoresed, as previously described for phosphorylated and total ERK (Pullar *et al.*, 2003) and tyrosine hydroxylase (Pullar *et al.*, 2006b).

Enzyme immunoassay for the quantitative determination of epinephrine in small sample volumes

MKs (1×10^7), isolated from both β2AR$^{-/-}$ and β2AR$^{+/+}$ mice, were extracted in 100 μl 0.1 N HCl and sonicated on ice for 10 minutes. Extracts were tested in triplicate in an epinephrine enzyme immunoassay (Invitrogen) according to the manufacturer's instructions.

ELISA

Cells were plated on collagen I (30 μg ml^{-1})–coated 6-well plates and incubated for 24 hours in the appropriate complete media to reach 70/80% confluence. Cells were washed with Hank's balanced salt solution and serum starved for 24 hours in the appropriate basal media. Cells were then incubated with either basal media alone or media containing 10 μM β2AR antagonist for 6, 24, or 48 hours. After each time point, media were collected and stored at −80 °C for analysis with a Duoset VEGF-A ELISA kit (R&D Systems, Abingdon, UK).

Rat aortic ring assay

Animals were killed using an approved schedule 1 method. The abdominal and aortic arcs were extracted from adult Wistar rats and placed into sterile phosphate-buffered saline. The fibro-adipose tissue was then removed, and the aortas were washed in warm, sterile phosphate-buffered saline three times. Aortas were cut into uniform, 1-mm sections, placed into 80 μl of basement membrane extract (R&D Systems), and incubated at 37 °C for 30 minutes. Aortas were incubated in EC basal media supplemented with 2% microvascular growth supplement for 3 days, and then with media alone or media containing 10 μM β2AR antagonist (ICI 118,551) for a further 5 days. Aortic endothelial outgrowth was imaged every 24 hours, using a Nikon eclipse microscope and the Improvision Openlab software. The area of outgrowth was analyzed using the Improvision Volocity software.

Zebrafish caudal fin wound model

Zebrafish larvae, 72 hours post-fertilization, were immersed in pond water containing 2% tricaine, and complete transection of the tail was performed with a sterile scalpel blade. After wounding, the larvae were incubated in the presence or absence of 500 μM β2AR antagonist (ICI 118,551) for 6 hours, and washed and stained using a fluorescein-tyramide signal amplification method (Perkin Elmer) before fixation in 4% paraformaldehyde. Tails were mounted and photographed on a Nikon TE-2000E inverted microscope at ×20 magnification using a fluor objective. The number of neutrophils recruited to each wounded tail was recorded.

CAM assay

Fertilized eggs were obtained, 1 day post fertilization, from local hatcheries and incubated at 37 °C in a humidified environment for 48 hours. On day 3, approximately 5 ml of albumin was removed from the obtuse poles of the eggs using a 21 G cannula. A square window (2×2 cm^2) was opened into the shell and shell membrane using a Dremel tool (Dremel, Uxbridge, UK). The window was then sealed with Parafilm and incubated horizontally for 48 hours at 37 °C. On day 5, water or 100 μM β2AR antagonist (ICI 118,551) in water was evaporated onto the center of a sterile 13-mm coverslip and then placed face down onto the CAM. CAMs were photographed through the coverslip center, every 24 hours, until day 9 using a stereomicroscope (Prior Scientific, Cambridge, UK). Angiogenesis was analyzed by counting the total number of branch points per field of view through the center of the coverslip.

Murine wound model

β2AR$^{-/-}$ and β2AR$^{+/+}$ mice were anesthetized by intraperitoneal injection of ketamine (100 mg kg^{-1})/xylazine (10 mg kg^{-1}) (Pfizer, Sandwich, UK). Back skin was shaved and two circular, full-thickness 6-mm excisional wounds were created 2 cm apart, in the center of the back, using a sterile 6-mm biopsy punch (SMS, Camberley, UK) to mark the skin for surgical excision. Wounds were treated topically with 100 μl of hydrogel alone (Duoderm, ConvaTec, Uxbridge, UK) or containing 0.1% (1 mM) selective β2AR antagonist (ICI 118,551)

(a concentration previously demonstrated to accelerate mouse epidermal barrier recovery; Denda *et al.*, 2003) immediately after wounding and daily thereafter until harvesting ($n = 5$–7 mice per group, two wounds per mouse). Each mouse was housed separately after wounding until wound harvest. Wounds were left uncovered and digitally photographed, daily, to determine wound contraction over time. Wounds were harvested 3 and 5 days post wounding by carefully applying an 8-mm punch (SMS) around the original wound and lightly pressing to form an outline on the skin. Scissors were used to excise the wound without damaging the delicate wound bed.

For histological analysis, the wounds were fixed in an IHC zinc fixative (BD Biosciences). The zinc-fixed biopsies were bisected, to ensure that sections were taken from the center of the wound, dehydrated through an ethanol–xylene series, and embedded in paraffin. Cross-sections, 7 μm thick, were stained with the hematoxylin–eosin technique or with picrosirius red, to visualize collagen in the neo-dermis (Junqueira *et al.*, 1979), as described by Dapson *et al.* (2011). Sections were immunostained with an antibody against SMA (Dako, Ely, UK), collagen III (Rockland, Lorne Laboratories, Reading, UK), Ly6G-6C (a neutrophil marker; BD Pharmingen, Oxford, UK), F4-80 (a macrophage marker; Serotec, Kidlington, UK), or CD31 (an EC marker; BD Pharmingen), followed by DAB detection (BD Pharmingen) according to the manufacturer's protocols.

Specimens that were damaged in the histological process or otherwise non-interpretable were excluded from the study. Re-epithelialization was determined by examining all hematoxylin- and eosin-stained sections by light microscopy, using a Q-imaging Retiga-EX camera attached to a Nikon T-100 inverted microscope, controlled by the Improvision software. Image J was used to measure the linear distance covered by new epithelium and the linear distance between the original wound edges, to determine the percentage of re-epithelialization. Wound closure was quantified by measuring the wound area of each wound from the digital pictures taken every day with Image J. Each picture was calibrated individually. Images of picrosirius red–stained sections were captured on an Olympus BX51 upright microscope equipped for dark field. The area of the collagen-stained neo-dermis was analyzed by measuring the red-stained area within 1 mm^2 of each wound edge on the dark-field pictures with the Image J software. Images of the SMA-stained wounds were captured on a Nikon SMZ-U upright microscope with the Nikon ACT-1 software. Images of the collagen III–stained wounds were captured on a Nikon Eclipse inverted microscope with the NIS-Elements software. NIH Image J was used to determine the area of SMA and collagen III staining in each wound to calculate the mean area of SMA and collagen III staining in each group. Images of the CD31- (× 40 magnification), Ly-6G-6C-, and F4-80-stained (× 60 magnification) wounds were also captured on the Nikon Eclipse microscope. The number of stained cells/vessels in each image was counted in a double-blind manner, and the average cell/vessel number was calculated for each group. For all image analysis, 10 fields were selected from the dermis below the wound margins and across the wound bed using a template to ensure that images were captured from similar areas in each wound.

Statistical analysis

Unless mentioned in a specific method, categorical variables were compared with a two-tailed Fisher's exact test or a one-way analysis of variance, followed by the Dunnett's test, whereas each continuous variable test group was compared with the control using the two-tailed Student's *t*-test for unpaired data with unequal variance. $P < 0.05$ (*) was significant and $P < 0.01$ (**) highly significant.

Author contributions

CEP and RRI initiated the concept for the murine wound study. RRI contributed to the initial analysis of the murine wound study and the proof-reading of the final article. CEP initiated the concept for all mechanistic studies, designed, acquired, analyzed, and interpreted the majority of the data, and drafted and revised the article. GSLP, APO, SE, and BB substantially contributed to the design of some experiments, the acquisition, analysis, and interpretation of the majority of the associated data, and either the drafting or the proofreading of the article.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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