Rac1 function in skin inflammation

RAC1 in keratinocytes regulates crosstalk to immune cells by Arp2/3 dependent control of STAT1

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Abstract

Crosstalk between keratinocytes and immune cells is crucial for the immunological barrier function of the skin and aberrant crosstalk contributes to inflammatory skin diseases. Using mice with a keratinocyte-restricted deletion of the RAC1 gene we found that RAC1 in keratinocytes plays an important role in modulating the interferon (IFN) response in skin. RAC1 mutant mice showed increased sensitivity in an irritant contact dermatitis model, abnormal keratinocyte differentiation, and increased expression of immune response genes including the IFN signal transducer STAT1. Loss of RAC1 in keratinocytes decreased actin polymerization in vivo and in vitro and caused Arp2/3 dependent expression of STAT1, increased interferon sensitivity and upregulation of aberrant keratinocyte differentiation markers. This is inhibitable by the AP-1 inhibitor tanshinone IIA. Loss of RAC1 makes keratinocytes hypersensitive towards inflammatory stimuli both in vitro and in vivo, suggesting a major role for RAC1 in regulating the crosstalk between the epidermis and the immune system.
Rac1 function in skin inflammation

Introduction

Collaboration and crosstalk between keratinocytes and resident immune cells is considered to be crucial for the skin function as an immunological barrier and impaired crosstalk is believed to contribute to many if not all inflammatory skin diseases. The complex relationship between keratinocytes and immune cells makes it difficult to understand the etiology of chronic skin inflammations (Lowes et al., 2007; Wagner et al., 2010): Do defects in keratinocyte barrier function or cytokine production by keratinocytes trigger activation of the immune system or is an abnormally activated immune system causing barrier defects and an altered cytokine profile of the keratinocytes?

RAC1 is a member of the Rho family of small GTPases, which controls various cellular processes such as ERK and AKT activity, actin polymerization, and ROS production (Bustelo et al., 2007). Previously, we and others showed that keratinocyte-restricted loss of RAC1 results in normal development and maintenance of the interfollicular epidermis, but loss of hair follicles and defective wound healing (Chrostek et al., 2006; Tscharntke et al., 2007; Castilho et al., 2007; Castilho et al., 2010). We report now that decreased Arp2/3 dependent actin polymerization in RAC1-null keratinocytes induces the IFN-γ activated transcription factor STAT1, sensitizes RAC1-null keratinocytes towards IFN-γ produced by immune cells, and induces aberrant keratinocyte differentiation.

These data reveal a novel role for RAC1 and actin polymerization in skin immunity by regulating the innate immune response in keratinocytes, thereby affecting the crosstalk between keratinocytes and immune cells.
Results

**RAC1 in keratinocytes controls sensitivity towards skin inflammation**

Mice with a keratinocyte-restricted deletion of the RAC1 gene are resistant towards 7.12-dimethylbenz(a)anthracene/ 12-0-tetradecanoyl-phorbol-13-acetate (DMBA/TPA) induced hyperproliferation and tumor formation in skin (Wang et al., 2010). Since DMBA/TPA induced skin tumors are dependent on a strong inflammatory response, we tested during our investigation, whether loss of RAC1 decreases TPA-induced inflammation. Surprisingly, microarray gene expression analysis of epidermal cells from TPA treated RAC1 ko and control mice revealed rather an increased inflammatory reaction in RAC1-null epidermis. Functional grouping of the genes up-regulated more than two-fold, using the DAVID program, revealed a significant enrichment of genes related to cytokines and inflammation, which included IL-6 (4 fold), CXCL10 (3.3 fold), CXCL1 (2.4 fold), TNFSF9 (2.3 fold), and CCL2 (2.1 fold) (Fig. 1A). This increased inflammatory response was not due to an increased activation of classical NF-κB signaling, as nuclear phosphorylation of NF-κB at S536 was unchanged between control and RAC1 ko epidermis treated or untreated with DMBA/TPA (Suppl. fig. 1A).

These data indicate that the TPA-induced skin inflammation does not require RAC1 function in keratinocytes, in contrast to TPA-induced hyperproliferation. They furthermore suggest that loss of RAC1 in keratinocytes might promote skin inflammation.

To directly address this question, we applied a contact dermatitis model, where the ears of the mice are painted with croton oil, a TPA containing irritant. Indeed, mice lacking RAC1 in keratinocytes showed a 3-fold increase in granulocyte infiltration after 8h, which resolved after 48h (Fig. 1B). Even in response to vehicle, RAC1 mutant mice showed an increased granulocyte infiltration, confirming an increased sensitivity of RAC1-null skin towards inflammation.

**Altered differentiation and increased expression of immune response related genes in RAC1-null epidermis**

To understand the molecular reason underlying the increased inflammation in mice lacking RAC1 in keratinocytes, we performed a gene expression analysis of epidermis of adult control and RAC1 mutant mice. We identified 231 genes up-regulated at least
Rac1 function in skin inflammation

2-fold in Rac1-null epidermis, excluding lowly expressed genes (basal expression level in Rac1-null below 100). Analyzing these genes by DAVID we found an enrichment of genes related to keratinocyte differentiation and inflammation, including many genes related to interferon response (CXCL10, OAS1D, OAS1A, OAS1E, OAS2, OASL2, ISG15, IFIT1, IFI27, STAT1; Fig. 1C; Suppl. table 1. To confirm the array results, we measured by qRT-PCR the mRNA amounts of 15 up-regulated genes related to inflammation (Sl100a9, Il1f6, IFI27, OAS1A, CCL1, CCL20, LCN2, CASP1), differentiation (FLG, SPRR2A), and other functions (CAR2, SERPINB3A, STF3A, ATP12A) in the epidermis of control and Rac1-mutant mice (Fig. 1D). All genes were stronger expressed in Rac1-null keratinocytes, although the variation was relatively high in the Rac1-null samples. These data confirm that loss of Rac1 in keratinocytes in vivo increases expression of immune response and keratinocyte differentiation genes.

Loss of Rac1 leads to changes in gene expression in both basal and supra-basal keratinocytes

Mouse epidermis contains basal keratinocytes, suprabasal keratinocytes, and immune cells. Since loss of Rac1 leads to a slight increase in suprabasal cells (Chrostek et al., 2006), the increased expression of differentiation markers could be an effect of an increased number of suprabasal cells rather than a change in gene expression of individual cells. Moreover, altered numbers or activation of immune cells might contribute to the observed changes in gene expression in epidermis of mice lacking Rac1 in keratinocytes.

We therefore sorted epidermal cells from adult control and Rac1 mutant mice into basal keratinocytes (α6 integrin high, CD45-), suprabasal keratinocytes (α6 integrin low, CD45-), and immune cells (CD45+) and analyzed in the keratinocyte fractions the expression of 12 genes, which are up-regulated in Rac1-null epidermis.

FACS analysis confirmed an increase in suprabasal cell numbers in Rac1-null epidermis from 27.5% ±4.1% in control to 40.8% ±2.2% in Rac1-null (n= 4/4). Of 12 genes tested, 5 showed an increase in both basal and suprabasal fractions (SPRR2A, STF3A, Sl100a9, IFI27, OAS1A; Fig. 2A), while 7 genes were mainly increased in the suprabasal fraction (FLG, LCN2, ATP12A, CAR2, SERPINB3A, CCL1, IL1F6; Fig. 2A). These data indicate that both basal and suprabasal keratinocytes show signs of
aberrant differentiation and increased expression of immune response related genes in the absence of \textit{RAC1} and that the phenotype observed in \textit{RAC1}-null epidermis cannot be explained by an increased percentage of normally differentiated suprabasal cells.

\textbf{Increased activation, but no increased numbers of immune cells in \textit{RAC1}-null epidermis}

Infiltration of immune cells is a hallmark of clinical inflammation. We therefore assessed, whether loss of \textit{RAC1} in keratinocytes leads to an increased number of immune cells in the epidermis of adult mice older than 2 months. It should be noted that the immune cells in our mice all express RAC1, since the deletion of the \textit{RAC1} gene is restricted to the keratinocytes.

FACS analysis of CD45+ cells in the epidermis of adult mice did not indicate a significant increase in leukocytes in the absence of \textit{RAC1} in keratinocytes (control: 4.1 \% \pm 1.9\%; \textit{RAC1} ko: 5.2\% \pm 1.1\%; n= 14/14). Furthermore, checking the gene expression of markers for specific leukocyte subsets by microarray suggested unchanged numbers of T-cells (\textit{CD4, CD8, CD3}), and macrophages (\textit{MAC1}) in the epidermis (Fig. 2B). The marker for Langerhans cells (\textit{CD207}) was not changed, but the dendritic cell marker \textit{CD11C} appeared to be slightly increased (Fig. 2B).

Expression of T-cell activation markers \textit{ICOS, CTLA4, IL17A, CD69, CCR6, CD28 and CD5} and of the T-cell expressed integrins \textit{CD18 CD11A} was increased in \textit{RAC1}-null epidermis (Fig. 2B). We noticed also a ~ 4-fold increase in \textit{IFN-\gamma} expression, yet at a very low absolute level.

To validate this increase in \textit{IFN-\gamma} message, we performed qRT-PCR analysis of different cell fractions of control and \textit{RAC1} mutant epidermis. \textit{IFN-\gamma} was about 3-fold elevated in immune cells isolated from \textit{RAC1}-ko epidermis, confirming the result from the microarray analysis (Fig. 2C). No \textit{IFN-\gamma} mRNA was detected in basal and suprabasal keratinocyte fractions of control and \textit{RAC1} mutant mice. \textit{IL-1\beta} and \textit{IL6} mRNA were undetectable or low in keratinocytes and unchanged in keratinocytes and immune cells (Suppl. fig. 2A, B).

We then assessed by qRT-PCR the contribution of immune cells to the increased expression of \textit{LCN2, CCL1}, and \textit{S100A9} in \textit{RAC1}-null epidermis. These genes have previously been described to be expressed by immune cells (Borregaard et al., 2007; Wiener et al., 2008; Yang et al., 2010). \textit{LCN2} and \textit{S100A9} displayed an increased
expression in immune-cells isolated from \textit{RAC1} ko epidermis, but the levels of expression were much lower than in keratinocytes (Fig. 2C). \textit{CCL1} mRNA was strongly expressed, but not increased in immune cells in mutant mice compared to control mice (Suppl. fig. 2C).

These data show that loss of \textit{RAC1} in keratinocytes leads to activation of resident immune cells and a Th1 cytokine profile.

\textbf{No observable changes in barrier function of \textit{RAC1} ko mice}

To reveal the mechanism of how loss of \textit{RAC1} in keratinocytes leads to activation of immune cells we tested different hypotheses. First, we assessed whether it was possible to observe histological signs such as aberrant tight junctions that would support a defective barrier function of the epidermis, which could promote pathogen infiltration and immune cell activation. To optimally visualize the tight junctions at the transmission electron microscope, other than the routinely used OsO4, we utilized additional ruthenium and lanthanum based heavy metals in the post fixation process. We noticed in semithin sections that in the absence of the epidermal barrier, RuO4 diffused into the tissue resulting in black colorations (Fig. 3 a, a'). This was primarily observed at the cut edges of the samples and to a very small extent in hair follicles. In the rest of the epidermis, black colorations did not diffuse deeper than the surface of the stratum corneum, suggesting a normal barrier function in \textit{RAC1}-null skin (Fig. 3a, a'). Only in one isolated occasion, in one \textit{RAC1} ko sample, deeper black colorations were observed. However, serial sections of the same sample as well as of two other \textit{RAC1} ko and of one control sample revealed no diffusion from the surface and demonstrated the local restriction of this spot, which was probably due to sectioning of a peripheral region of the specimen.

For the ultrastructural analysis, as it is effectively the first tight junction barrier that exogenous factors would encounter in penetrating the epidermis, we concentrated our focus on the last layer of the stratum granulosum immediately deep to the stratum corneum. Here, for consistency, apical tight junctions located above desmosomes were studied and compared in both groups. Tight junctions with normal morphology and arrangement, with sharply defined kissing points of the membranes were constantly found in \textit{RAC1}-null epidermis (Fig. 3b, c, b', c'). Only sporadically (<5%), the kissing points of a tight junction were not visualized above the corresponding desmosome in \textit{RAC1} ko tissue. We also examined the distribution of lamellar bodies
and found that they can be consistently seen in both control and mutant epidermis to a comparable extent, although their amount may vary among the individual cells of the same epidermis (Suppl. fig.3a, a’). Also, their striation was similarly evident in both control and Rac1-null sections (Suppl. fig.3b, b’). Accordingly, vesicles of secretion of lipids in to the intercellular spaces (i.e. vesicles at the cell borders) and presence of secreted lipids between the upper epidermal cells were seen to a similar extent in control and ko (Suppl. fig.3c, c’). These data, which correlate with the normal lifespan and lack of spontaneous wounding and blistering of RAC1 ko mice, do not support a primary physical barrier defect as the driving factor behind the increased inflammatory response in mice with a keratinocyte specific ko of RAC1.

Loss of RAC1 induces IFN response genes in keratinocytes in the absence of immune cells

We then tested the hypothesis, whether the aberrant differentiation and the increased expression of immune response genes observed in RAC1-null skin is secondary to the transient influx of inflammatory cells into the dermis of 14d old RAC1 ko mice, which leads to the removal of the lower part of the hair follicles (Chrostek et al., 2006). We therefore studied gene-expression in full skin of 3d old control and RAC1 mutant mice. At this time point the deletion of the RAC1 gene is complete, but no obvious hair follicle defect is visible in hematoxylin-eosin stained back skin sections and no sign of immune cell infiltration is observed (Chrostek et al., 2006). By microarray, we identified 697 genes up-regulated more than 2-fold in skin from RAC1 mutant mice. Remarkably, only two of these genes, the differentiation marker SPRR1B and the stress response gene KRT16, showed increased expression in the epidermis of adult RAC1-mutant mice. Grouping of the upregulated genes according to their function using the DAVID program did not identify any increase in groups related to keratinocyte differentiation or immune response. Instead, several groups of nuclear proteins were found to be elevated in skin of RAC1-mutant mice (Suppl. fig. 4A) The lack of any major signs of inflammation in 3d old RAC1 ko mice gave us the opportunity to culture keratinocytes independent from immune cells and any external inflammatory stimuli and study alterations in gene expression.

In contrast to RAC1-null keratinocytes from adult mice, RAC1-deficient keratinocytes from 3d old mice spread and grew initially. After 4d in culture, however, they showed signs of differentiation and stopped proliferation, corresponding to previous reports.
Rac1 function in skin inflammation (Benitha et al., 2005). In order to investigate the changes in gene expression, we performed a microarray analysis of control and RAC1 ko keratinocytes after 2d culture in vitro. 557 genes were found to be up-regulated more than 2-fold in RAC1-null keratinocytes and DAVID analysis indicated an increased expression of genes related to epidermal differentiation (Fig. 4A). 65 of the 231 genes up-regulated in adult epidermis were also up-regulated in 2d cultured RAC1 ko keratinocytes (Suppl. table 2), including S100A9 (3.5-fold), which is strongly up-regulated in several human inflammatory diseases, SPRR2A (3.1-fold), which is a early skin differentiation marker, and the cysteine protease inhibitor STFA3 (3.5-fold). Surprisingly, also interferon response genes such as IFIT1 (4.3 fold), ISG15 (4.6 fold), CXCL10 (1.6 fold), and STAT1 (3.2 fold) were increased in cultured RAC1-null keratinocytes. Importantly, STAT1 is not only an IFN response gene, but also a transcription factor activated by IFN signaling (Najjar et al., 2010). CXCL10 is a known STAT1 target gene and an important activator of T-cells (Groom et al., 2011). IFN-γ expression, however, was undistinguishable from background levels and similar in control and RAC1-null keratinocytes. qRT-PCR confirmed a significantly increased expression of S100A9, SPRR2A, STFA3, CXCL10, and STAT1 (Fig. 4B). Western blot analysis demonstrated that STAT1 is also increased at protein level (Fig. 4C). These data suggest that the increased expression of IFN-γ response genes in cultured RAC1-null keratinocytes is independent of IFN-γ, but maybe related to the increased expression of the IFN-γ signal transducer STAT1. Therefore, we checked more carefully the expression of STAT1 and its target gene CXCL10 in RAC1-null epidermis in vivo. qRT-PCR revealed increased expression of both STAT1 and CXCL10 in RAC1-null skin already in 5d and 9d old mice, thus preceding the transient influx of macrophages at 2w of age (Fig. 4D). Expression levels in mutant samples were always higher than in control, but had a high variation. In adult epidermis, STAT1 and CXCL10 mRNA were particularly elevated in suprabasal keratinocytes lacking RAC1 (Fig. 4E). Western blot analysis indicated significantly increased protein levels of STAT1 and CXCL10 in adult RAC1-null epidermis (Fig. 4F). Finally, immunofluorescent staining of backskin sections confirmed increased amounts of nuclear STAT1 in the absence of RAC1 in vivo (Fig. 4G).
These data show that increased expression of STAT1 is an early event in vivo that might contribute to the increased production of IFN-γ target genes such as CXCL10.

**The increase of STAT1 is a cell autonomous effect independent of classical interferon signaling**

To exclude that the increase in IFN response genes observed in cultured RAC1-null keratinocytes is a delayed onset phenotype dependent on interaction with immune cells in vivo, we isolated not recombined, RAC1 conditional keratinocytes from adult RAC1 fl/fl mice and induced the deletion of the RAC1 gene by transfection with a cre and GFP expressing plasmid. Two days after transfection, GFP+ cells were sorted and replated. After two days in culture, we could detect a strong reduction of RAC1 protein (Fig. 5A) and a significant increase of STAT1 mRNA and protein as assessed by qRT-PCR and Western Blotting (Fig. 5B). This experiment confirms that the increase of STAT1 in RAC1-null keratinocytes is cell autonomous. This increased expression of STAT1 in RAC1-null keratinocytes was unexpected, as RAC1 has not previously been described to regulate STAT1 levels. A known pathway to induce STAT1 expression is IFN-γ signaling. Binding of IFN-γ to the IFN-γ receptor leads to phosphorylation of the intracellular kinases JAK1 and JAK2, which activates STAT1. Activated STAT1-homodimers then translocate to the nucleus and induce expression of IFN-γ target genes including STAT1 (Najjar et al., 2010).

To investigate whether JAK1/2 activation is involved in the increased STAT1 expression observed in RAC1-null keratinocytes we performed Western blotting for the phosphorylated forms of JAK1, JAK2 in lysates from 2d cultured keratinocytes, isolated from 3d old control and RAC1 mutant mice. The levels of pJAK1 and pJAK2 were similar in control and RAC1 ko keratinocytes (Fig. 5C). These experiments suggest that the increase of STAT1 in RAC1 ko keratinocytes is not caused by an increase of classical interferon signaling.

**RAC1 ko keratinocytes show an increased interferon response after IFN-γ stimulation**

The increase of STAT1 in keratinocytes might not only promote the expression of IFN response genes in keratinocytes, but could also increase the sensitivity of
Rac1 function in skin inflammation

keratinocytes towards IFN-γ. To test this possibility, we stimulated control and RAC1 ko keratinocytes for 6h with two different concentrations of IFN-γ and thereafter tested by qRT-PCR the expression of the interferon response genes CXCL10 and IFIT1. At all concentrations tested, RAC1 ko keratinocytes showed a stronger response than control cells (Fig. 5D). This was observed both with keratinocytes isolated from 3d old RAC1-null mice and with in vitro generated RAC1-null keratinocytes. IFN-γ treatment, however, did not affect expression of SPRR2A, STFA3 or S100A9 (Suppl. fig. 4B), suggesting that expression of these aberrant keratinocyte differentiation markers is independent of STAT1. The increased expression of IFN-γ response genes correlated with an increased phosphorylation of STAT1 at tyrosine 701 (Fig 5E.), although STAT1 can activate gene expression also independent of this phosphorylation site (Cheon et al., 2009), e.g. by acetylation (Krämer et al., 2009), sumoylation (Begitt et al., 2011) other phosphorylations (Nguyen et al., 2001). These data indicate that the increase of STAT1 in RAC1-null keratinocytes is functionally relevant for the keratinocyte response to IFN-γ.

Decreased F-actin polymerization in keratinocytes induces STAT1 expression, aberrant differentiation, and increased sensitivity towards IFN-γ

To understand the molecular pathways mediating the RAC1 dependent control of STAT1 expression and aberrant differentiation, we next investigated, which signaling pathways are altered in RAC1-null keratinocytes cultured in vitro. In vivo, we reported earlier that RAC1 is crucial for hyperproliferation and the hyperactivation of PAK1/2, AKT and ERK in response to TPA treatment of the skin, though dispensable for normal activation of these molecules in untreated skin (Wang et al., 2010). Since the in vitro culture conditions correspond to hyperproliferative conditions in vivo, we tested these pathways in RAC1-null keratinocytes in vitro. Indeed, we found decreased activation of PAK1/2, AKT and ERK, as revealed by Western blotting for phosphorylated forms of these molecules (Fig. 6A). Interestingly, total amounts of ERK and PAK1 were increased, whereas PAK2 and Akt were not changed (supp Fig. 5). Since RAC1 is known to control Arp2/3 dependent actin polymerization via the WAVE complex (Steffen et al., 2004), we then assessed the organization of the actin cytoskeleton in 2d cultured RAC1-null keratinocytes. RAC1 ko keratinocytes were smaller and more elongated, which correlated with an impaired ability of RAC1 ko
keratinocytes in forming lamellipodia (Fig. 6B). Correspondingly, the average amount of F-actin per cell as determined by FACS analysis of keratinocytes stained with fluorescently labeled phalloidin was decreased in the absence of RAC1 (Con: 874±166; RAC1 ko: 558±102; n=5/2; p=0.06) and the percentage of F-actin low cells was increased (Con:14,2±6; RAC1 ko:28,5±0.7; n=5/2; p=0.02). These data indicate that loss of RAC1 decreases actin polymerization in keratinocytes in vitro.

To investigate, how each of these pathways is influencing STAT1 expression and aberrant differentiation in keratinocytes, we used different inhibitors to block activation of PAK (PAK18, IPA3), ERK (MEK inhibitor PD98059), AKT (PI3K inhibitor LY294002), and actin polymerization (Lantrunculin A) in wildtype keratinocytes in vitro. After 24h incubation, we tested expression of SPRR2A, STFA3, S100A9, STAT1 and CXCL10 by qRT-PCR to detect aberrant differentiation and increased expression of immune response genes. Inhibitors of PAK1/2, MEK and PI3K had either no or even an inhibitory effect on the expression of SPRR2A, STFA3, S100A9 and STAT1. CXCL10 expression was not affected by PAK18 or MEK inhibition, but increased by IPA3 and the PI3K inhibitor. However, disrupting actin dynamics by lantrunculin increased the expression of all genes tested (Fig. 6C).

While lantrunculin inhibits all actin polymerization, RAC1 is regulating particularly Arp2/3-mediated actin polymerization via the WAVE complex. To test whether Arp2/3 dependent actin polymerization is involved in regulation of gene expression, we applied the Arp2/3 inhibitor CK548 to wildtype keratinocytes in vitro and measured the mRNA expression of STFA3 and S100A9 and both mRNA and protein of STAT1 after 24h. Arp2/3 inhibition significantly increased expression of all three genes (Fig. 6D 6E). Interestingly, CK548 was less effective in upregulation of STFA3 and S100A9 than lantrunculin or deletion of the RAC1 gene (cf. Fig. 6D with 6C and 4C). As Arp2/3 inhibition increased STAT1 levels we tested if Arp2/3 treatment also makes cells more sensitive towards IFN-γ stimulation. Indeed, IFN-γ induced expression of STAT1 and CXCL10 was increased in CK548 treated control cells. (Fig. 6F)

These data show that inhibition of Arp2/3 increases the expression of genes upregulated in RAC1-null cells and suggest that aberrant differentiation, increased expression of immune response related genes, and increased sensitivity towards interferons in RAC1 null keratinocytes are caused by changes in F-actin. In addition,
inhibition of AKT or PAK1/2 might contribute to the increased expression of CXCL10 in \textit{RAC1} ko keratinocytes.

\textbf{RAC1 controls F-actin polymerization in keratinocytes in vivo}

To assess, whether \textit{RAC1} is crucial for actin polymerization in keratinocytes also in vivo, we analyzed F-actin content in freshly isolated keratinocytes from 3d and adult control and \textit{RAC1} mutant mice. Average F-actin levels per cell were decreased both in 3d old (Con: 215±34; \textit{RAC1} ko: 157±32; n=7/3; p=0.03) and in adult mice (Con: 232±25; \textit{RAC1} ko: 157±12; n=3/3; p=0.01). Similarly, the percentage of F-actin low cells was increased in \textit{RAC1} null keratinocytes at both time points (Fig. 7A), yet more pronounced in adult mice, where these changes were significant. Immunofluorescent staining for F-actin confirmed a decreased level of F-actin in epidermis of adult \textit{RAC1} mutant mice compared to control (Fig.7B).

This decreased actin polymerization in vivo corresponded to the increased expression of keratinocyte differentiation markers and immune response genes in adult \textit{RAC1}-null epidermis.

\textbf{Reduced actin polymerization stimulates expression of STAT1 and keratinocyte differentiation in a tanshinone dependent manner}

To explore the mechanism how inhibition of actin polymerization affects gene expression in keratinocytes we tested different pathways. Retinoic acid (RA) signaling was reported to upregulate STAT1 expression in a IFN\(\gamma\) independent manner (Wong et al., 2002; Shang et al., 1999; Kolla et al., 1997; Kolla et al., 1996). We therefore assessed whether inhibition of actin polymerization increases RA responsive element (RARE) dependent luciferase reporter expression and whether exogenous all-trans RA (ATRA) stimulates STAT1 expression in primary keratinocytes. While latrunculin treatment increased RARE activation more than two-fold, the Arp2/3 inhibitor CK548 did not show an obvious effect (Suppl. fig. 6A). ATRA treatment increased STAT1 expression in the presence of latrunculin, but neither alone, nor in the presence of CK548 (Suppl. fig. 6B). These data do not support a major role of RA signaling in controlling STAT1 expression in response to RAC1-Arp2/3.
Rac1 function in skin inflammation

We then investigated the role of the AP-1 transcription factor, which promotes keratinocyte differentiation (Angel et al., 2001). AP-1 is a heterodimer of JUN and FRA family members and it was shown previously that inhibition of F-actin formation increases c-Jun expression by a posttranscriptional mechanism (Polak et al., 2006). In 3d old mice, no significant difference in c-Jun protein levels was observed (Fig. 8A). In adult RAC1-null epidermis, however, c-Jun protein amounts were three-fold increased (Fig. 8A). Microarray gene expression analysis indicated no significant change in c-JUN mRNA in adult RAC1-null keratinocytes.

To inhibit AP-1 function we treated cultured keratinocytes with the AP-1 dimerization inhibitor tanshinone IIA. Tanshinone blocked latrunculin induced upregulation of STAT1, and largely prevented the increase in S100A9 and STFA3 (Fig. 8B). In the presence of tanshinone, the latrunculin induced increase of S100A9 and STFA3 was dramatically reduced (Fig. 8B). CK548 dependent regulation of STAT1 on mRNA and protein level and STFA3 expression was completely inhabitable by tanshinone, while S100A9 expression was not affected (Fig. 8B, 8C). Treatment with tanshinone alone did not alter gene expression (Fig. 8B and D).

Moreover, tanshinone also inhibited the increased interferon sensitivity observed after CK548 treatment (Fig. 8D). These data indicate that the increase in STAT1 expression is required for the CK548 induced increase of the IFN-γ response.

Despite the strong effect of the AP-1 inhibitor tanshinone, we could not observe a CK548 induced increase in c-Jun expression, suggesting a c-Jun independent mechanism at least in vitro (Suppl. fig. 7A).

Finally, we tested whether tanshinone is able to prevent the upregulation of STAT1, S100A9, and STFA3, and the increased sensitivity towards interferons in primary RAC1-null keratinocytes cultured in vitro. Since freshly isolated neonatal keratinocytes show already certain changes, such as the reduced level of F-actin (Fig. 7A), it was not clear to what extent it would be possible to rescue the RAC1-null phenotype.

Similar to the CK548 treated cells, tanshinone strongly reduced the increase in STFA3, but showed no significant effect on S100A9 (Fig. 8E). Average levels of STAT1 mRNA were reduced and Western blot analysis confirmed a reduction of STAT1 protein in tanshinone treated RAC1-null keratinocytes (Fig. 8F). Importantly, also the increased interferon response was inhibited by tanshinone treatment (Fig. 8G). These
data strongly suggest that RAC1-Arp2/3 dependent regulation of actin polymerization controls expression of the keratinocyte differentiation markers, STAT1, and interferon sensitivity by a mechanism sensitive to tanshinone.
Rac1 function in skin inflammation

Discussion

Keratinocytes are able to produce a large number of different cytokines and chemokines such as IL1-β, IL1-α, IL6, IL10, IL18, TNF-α, Il1f6, CCL20, CXCl9, CXCL10 and CXCL11. These mediators promote the activation and infiltration of immune cells, which in turn secrete cytokines that affect keratinocyte gene expression and function. This positive feedback allows quick and efficient activation of the immune system in case of wounding or infection of the epidermis (Nestle et al., 2009).

Clearly, immune cells can trigger this crosstalk, as shown by the induction of psoriasiform, inflammatory skin lesions in mice by transfer of CD4+CD45RBhi T cells into T cell deficient Rag2-/− mice (Leon et al., 2006). However, also keratinocytes are able to initiate immune system activation. For example, overexpression of a constitutively active form of STAT3 in keratinocytes is sufficient to induce a psoriasis-like skin phenotype including infiltration of lymphocytes and neutrophils (Sano et al., 2005). Immune cell produced cytokines such as IL-6 or IFNs, on the other hand, are strong activators of STAT3 (Sano et al., 2005). Furthermore, keratinocyte-restricted deletion of the transcription factor AP-1 genes JUNB and C-JUN is causing a psoriasis-like inflammatory skin disease in mice by decreased expression of TIMP-3, which results in increased shedding of TNFα (Guinea-Viniegra et al., 2009). Finally postnatal loss of the transcription factor SRF resulted in a hyperproliferative skin disease with psoriasis-like lesions (Koegel et al., 2009).

We now describe an additional pathway, how keratinocytes can activate the immune system and contribute to skin inflammation. Loss of RAC1 in keratinocytes induced cell autonomously the expression of the IFN-γ signal transducer STAT1, which conceivably increased the expression of immune cell activating proteins and chemokines such as CXCL10. In addition, the increased levels of STAT1 made RAC1-null keratinocytes hypersensitive to IFN-γ produced by immune cells, promoting the positive feedback loop by which keratinocytes increase immune cell activation. Many signaling pathways such as growth factor receptors, integrins, and cytokine receptors regulate RAC1 activation. RAC1-GTP might therefore be a signal integrator, collecting information from different sources, which then determines the sensitivity of the keratinocytes toward IFN-γ.
Unexpectedly, we found that RAC1 regulates STAT1 expression and interferon sensitivity by Arp2/3 mediated actin polymerization, which RAC1 promotes by interacting with the WAVE complex (Ladwein and Rottner, 2008). The RAC1 downstream effectors PAK, ERK, and AKT, which are all less activated in RAC1-null keratinocytes cultured in vitro, however, seem not to affect STAT1 expression. Since F-actin formation is controlled by many other molecules in addition to RAC1, it is conceivable that other pathways contribute to the regulation of skin sensitivity. However, the similar effect of latrunculin, which inhibits all actin polymerization, CK548, which blocks Arp2/3 dependent actin polymerization, and RAC1 knockout on the expression of STAT1 and the sensibility towards interferons suggests a major, non-redundant function for RAC1-Arp2/3.

Actin polymerization controls STAT1 expression and interferon sensitivity in a tanshinone regulated manner, as the inhibitor efficiently blocked the effects of latrunculin, CK548, and Rac1 deletion on STAT1 expression and the increased interferon sensitivity. In vivo, loss of RAC1 caused a posttranscriptional upregulation of c-Jun, similar to a latrunculin induced posttranscriptional upregulation of c-Jun in cell lines (Polak et al., 2006). In cell culture, cjun expression was not upregulated in RAC1 ko cells or in arp2/3 treated cells. This suggests that either it is a different member of the AP-1 transcription factor family which is regulating the response in vitro or it is a matter of AP-1 activation rather than absolute levels. Finally, off-target effects of tanshinone have to be considered.

Arp2/3 dependent actin polymerization is also regulating the expression of STFA3 and S100A9. S100A9 is a multifunctional molecule with chemokine-like, pro-inflammatory functions (Gebhardt et al., 2006), which probably further increases the hypersensitive status of the RAC1-null skin. However, the Arp2/3 inhibitor CK548 is much less stimulating compared to latrunculin and loss of RAC1, which is in contrast to the control of STAT1 expression where latrunculin CK548 and RAC1 ko induced STAT1 to a similar extent. This might indicate the involvement of Arp2/3 independent actin polymerization in the regulation of S100A9 and STFA3. Furthermore, Arp2/3 and RAC1 dependent regulation of S100A9 expression is not significantly blocked by tanshinone. These findings reveal that different molecular mechanisms are mediating the F-actin dependent regulation of gene expression in keratinocytes. They furthermore confirm that expression of aberrant keratinocyte differentiation markers in RAC1-null cells is not simply a consequence of increased STAT1 expression.
Rac1 function in skin inflammation

Which additional mechanisms might be involved? One additional mechanism could be RA signaling, which we found to be increased in keratinocytes treated with latrunculin. Classical NF-κB signaling, however, which is altered in different skin inflammation models (Wullaert et al., 2011), was not changed in \textit{RAC1}-null keratinocytes.

Already earlier RAC1 has been described to regulate gene expression in epithelial cells by controlling actin polymerization (Busche et al., 2008; Busche et al. 2010). In that case, RAC1 induced actin polymerization released the transcriptional co-factor MAL from G-actin, which translocates to the nucleus, binds to the transcription factor SRF, and induces MAL-SRF dependent gene expression. This MAL dependent gene expression is completely inhibitable by latrunculin, which leads to depolymerization of the actin cytoskeleton. In contrast, we found that latrunculin treatment or loss of \textit{RAC1} induced STAT1 expression in keratinocytes. It is therefore very unlikely that SRF is involved in the increased STAT1 expression.

Our data suggest that signaling pathways regulating RAC1 or actin polymerization in keratinocytes can modulate the sensitivity towards inflammatory skin diseases. Based on our study, it will be interesting to test RAC1 activation levels in skin of patients suffering from inflammatory skin diseases.

In conclusion, we demonstrate for the first time a link between RAC1 activation, Arp2/3 dependent actin polymerization, STAT1 expression and IFN-γ signaling in keratinocytes, which might play an important role in the crosstalk between keratinocytes and immune cells in inflammation related skin diseases. Actin polymerization is also involved in RAC1 dependent, aberrant keratinocyte differentiation, indicating that changes in the actin cytoskeleton can influence multiple signaling pathways.
Rac1 function in skin inflammation

Materials and Methods

Mice

Mice with keratinocyte restricted deletion of the RAC1 gene (RAC1 fl/fl K5 cre) on a 129Sv/C57BL6 outbred background were described previously (Chrostek et al., 2006). Adult mice were 2-6 mo old. Litter mates were used as controls.

Keratinocyte isolation and culture

Keratinocytes were isolated according to Lichti et al. (2008) and either directly processed (“in vivo”) or cultured (“in vitro”) following standard procedures. Size and circularity of keratinocytes were determined with Image J (http://rsbweb.nih.gov/ij/). For inhibitor experiments keratinocytes were treated for 24h or indicated times with 0.5μM latrunculin A, 10μM PAK18, 10μM IPA-3, 50μM PD98059, 50μM LY294002, 50μM CK584 (all Sigma), 3.5 μM tanshinone IIA (TOCRIS) or 0.001 % DMSO (Sigma). For interferon stimulation, keratinocytes were treated for 6h with 100U or 200U INF-γ (Peprotec). For ATRA stimulation, cells were treated for 24h with 1 μM all-trans retinal (Sigma).

Inflammatory skin models

TPA induced skin inflammation was carried out as described earlier (Wang et al., 2010). For croton oil induced irritant dermatitis adult mice were anesthetized by isoflurane and 10 μl 2% croton oil (Sigma) in a 4:1 acetone/olive oil mixture was applied on both sides of the right ear. As a control, the left ear was treated only with 4:1 acetone/olive oil mixture. Mice were sacrificed after 8h, and infiltration of granulocytes was assessed by immunofluorescent staining of cryosections as described below. All animal studies were carried out according to Danish rules of animal welfare.

Histological analysis

7μm sections of ear and back skin were performed on a cryostat and stained as described previously (Lefever et al., 2010). The following antibodies were used: rat anti Ly-6G (Gr-1), FITC-conjugated rat anti CD49f (clone GoH3; all BD Biosciences), rabbit anti STAT1 rabbit anti c-Jun (Cell Signalling). As secondary
agents Cy3-conjugated goat anti-rabbit and Cy5-conjugated streptavidin were used (all Jackson ImmunoResearch). F-actin was detected by Alexa-Flour-488-coupled phalloidin (Invitrogen). Nuclear counterstaining was performed with DAPI (Sigma). Images were analyzed with a DM RXA2 microscope, equipped with 20X HC PL Apo (NA 0.70), 40X HCX PL APO (NA 1.25-0.75) and 63X HCX PL APO (NA 1.40-0.60) objectives controlled by Leica Microsystems confocal software (version 2.61 Build 1537; all Leica Microsystems). Images of human samples were obtained by a Zeiss AxioImager M2 upright microscope using a Plan-Apochromat 20x/0.8 objective.

**Deletion of the RAC1 gene in vitro**

Primary keratinocytes from RAC1 fl/fl and, as control, RAC1 fl/+ mice were transfected at 30-40% confluency with pRRLSIN-Cre-IRES-EGFP (kindly received from Didier Trono, EPFL, Lausanne, Switzerland) using TransIT-Keratinocyte Transfection Reagent (Mirus) following the instructions of the manufacturer.

**Ultrastructural analysis**

After sacrifice, small pieces of back skin were taken from 1 control and 2 RAC1 ko, 30-week-old-mice and fixed in 4% paraformaldehyde, 2% glutaraldehyde, in 0.1M Na-cacodylate buffer pH 7.4 supplemented with 2mM CaCl2. For post-fixation, in addition to the traditional 1% OsO4 treatment, also protocols with different combinations of metals including, other than 1% OsO4, also 0.25% RuO4 with 0.25% K3Fe(CN)6, and, 2% La(NO3)3 were used. After dehydration in a graded ethanol series, samples were embedded in Agar low viscosity resin according to standard protocol (Jackson et al., 2011).

Semithin sections of 1µm were cut with a Reichert-Jung ultramicrotome and stained with toluidine blue. All semithin sections were examined using Leica-Leitz DMRXE Confocal Microscope and images were captured using a Leica DFC 300 FX camera with accompanying software. In total, 7 samples from the control mouse and 14 samples from the RAC1 ko mice were examined.

Ultrathin sections of 90-95nm were cut with a Reichert-Jung ultramicrotome and collected on 200 mesh formvar-coated copper grids for extensive analysis of the superficial tight junctions. Sections were stained with uranyl acetate and lead citrate in a Leica EM AC20 stainer. Sections were then examined with a Hitachi H-7000 Electron Microscope fitted with a 1K Hamamatsu Digital Camera. Images were
Rac1 function in skin inflammation

captured using AMTV542 Image Capture Engine software. In total, 6 samples from the control mouse and 10 samples from the RAC1 ko mice were examined.

**Microarray gene expression analysis and qRT-PCR**

RNA from epidermal lysates was isolated using the GeneElute Mammalian Total RNA miniprep kit (Sigma). Full skin samples were stored in RNA later (Sigma) and homogenized by a Dounce homogenizer before applying to the GeneElute kit. A proteinase K step was included according to protocol of the manufacturer. Microarray gene expression analysis was carried out at the Copenhagen University Hospital Microarray Center using the GeneChip Mouse Genome 430 2.0 Array (Affymetrix). Functional grouping of the upregulated genes was carried out using the DAVID program (Huang et al., 2009).

For qRT-PCR analysis, RNA was reverse transcribed using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas). qRT-PCR was performed on the Applied Biosystems 7300 Real Time PCR system using SYBR green incorporation following standard protocols. The Ct value was calculated based on duplicates and normalized to the housekeeping gene CYCA.

**Biochemical analysis**

Western blotting was performed according to standard protocols. The following antibodies were used: mouse anti RAC1 (clone 102; BD Biosciences), rabbit anti STAT1, rabbit anti pSTAT1 (Tyr 701), rabbit anti pPAK1/2 (Thr423/Thr402), rabbit anti pAKT (S473), rabbit anti pERK (Thr 202/Tyr 204), rabbit anti pNFkB (S536), rabbit anti pJAK2 (Y1007/1008), rabbit anti pJAK1 (Y1022/1023), rabbit anti c-JUN (all Cell Signalling), goat anti CXCL10 (R&D systems), and mouse anti tyrosinated tubulin (Y/L1/2; kindly provided by J. Wehland, Braunschweig, Germany). As secondary agents horseradish-peroxidase-coupled goat anti rabbit, goat anti mouse, and donkey anti goat antibodies were used (all Jackson ImmunoResearch). All results were quantified using TotalLab TL100 software (Nonlinear Dynamics). Tubulin was used to normalize for different protein amounts.

**FACS analysis**
Epidermal cell preparations were isolated as described above and stained following standard procedures using PE-conjugated rat anti CD45.2, and FITC-conjugated rat anti CD49f. Fc receptors were blocked by rat anti CD16/32 (all BD Biosciences). F-actin was detected by Alexa-Flour-488-coupled phalloidin (Invitrogen). The stained cells were sorted on a pre-cooled FACSaria Cell sorter (BD Biosciences).

**Luciferase assay**

Primary keratinocytes were transfected at 30-40% confluency with pGL3-RARE-luciferase construct (Addgene 13548; Hoffman et al., 2006) and pRL-TK-renilla construct (Promega) using TransIT-Keratinocyte Transfection Reagent (Mirus) following the instructions of the manufacturer. Two days later cells were harvested and renilla and firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) following the instructions of the manufacturer using a Lumat CB9597 (Berthold Technologies).

**Statistics**

Data are presented as means ± standard deviation, with error bars representing standard deviation. Statistical significance was determined by the two-tailed Student’s t-test. Significant differences are indicated by asterisks.
Acknowledgments
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Author contribution
EP planned and performed experiments, analysed data and wrote the manuscript. ZW, AS, LR, TW, and KP performed experiments and analysed data. FQ and CB planned experiments, analysed data, and wrote the manuscript.

Conflict of interest
There is no conflict of interest for any of the authors.
Rac1 function in skin inflammation

References


Rac1 function in skin inflammation


Rac1 function in skin inflammation


Rac1 function in skin inflammation


Rac1 function in skin inflammation

Figure legends

Fig. 1: Increased inflammatory response in TPA and croton oil treated RAC1 ko skin
(A) Functional grouping of 83 genes increased more than two fold in RAC1 ko epidermis treated for 2w with TPA compared to TPA treated epidermis of control mice using the DAVID program. Gene groups related to inflammation are marked in bold and italics. “N” indicates the number of genes in the group. “Fold” indicates the fold enrichment of the group compared to a similar number of random genes (n=2/2). (B) Croton oil induced irritant dermatitis. Representative pictures of 8h croton oil stimulated ears from control and RAC1 ko mice stained for granulocytes (Gr-1) and nuclei (DAPI). Asterisks mark non-specifically stained sebaceous glands (scale bar 20µm). Quantification of Gr-1+ cells per field after 8h and 48h (three pictures per ear; n=12/12; *: p<0.05, **: p<0.001). (C) Functional grouping of 231 genes increased more than two-fold in the epidermis of at least 3 out of 4 adult RAC1 ko mice compared to control mice using the DAVID program. Bold style indicates gene groups related to keratinocyte differentiation. Gene groups related to inflammation are marked with bold and italics style. “N” indicates the number of genes in the group. “Fold” indicates the fold enrichment of the group compared to a similar number of random genes (n=4/4). (D) Gene expression analysis by qRT-PCR in epidermis of control and RAC1 ko mice of 14 genes identified by microarray as up-regulated in RAC1 ko mice (n=4/4; *: p<0.05, **: p<0.001, ***: p<0.0001).

Fig. 2: Loss of RAC1 in keratinocytes alters gene expression in basal and suprabasal keratinocytes and in epidermal immune cells
(A) Gene expression of 12 genes upregulated in RAC1-null epidermis by qRT-PCR in FACS-enriched basal (α6high CD45-) and suprabasal (α6low CD45-) keratinocytes of control and RAC1-null skin (n=4-5/4-5; *: p<0.05, **: p<0.001, ***: p<0.0001). (B) Microarray gene expression analysis of selected immune cell markers in epidermis of RAC1 ko mice compared to control mice identified by microarray (n=4/4). (C) Gene expression analysis by qRT-PCR of LCN2, S100A9 and INF-γ in
FACS enriched immune cells (CD45+) isolated from control and RAC1-null epidermis (n(LCN2, S100A9)=4/4; n(IFN-γ)=6/9; *: p<0.05).

**Fig. 3: No obvious ultrastructural signs of defective skin barrier of RAC1 deficient mice**

a, a’: Semithin sections of Control (a) and RAC1 ko (a’) mice after Os/Ru/La based post-fixation. Colouration was consistently seen in the surface of the stratum corneum (arrow) and also at the edges of the sample (white arrow head). No differences in the level of penetration of the heavy metals from the surface or the edges of the tissue sample can be seen in the control and ko samples. (scale bar = 80 microns).

b, c, b’, c’: Ultrathin sections after Os/Ru/La based post-fixation. Tight junctions (black arrowhead pointing at the brackets) are clearly recognizable in the stratum granulosum (SG) in both control (b, c) and RAC1 ko mice (b’, c’). Magnification in b is the same as shown in b’ (scale bar = 500nm). Magnification in c is the same as shown in c’ (scale bar = 100nm). SC = stratum corneum.

**Fig. 4: Increased interferon response in vivo and in vitro in RAC1 null cells**

(A) Functional grouping of 557 genes increased more than two-fold in RAC1-null keratinocytes isolated from 3d old mice cultured for 2d compared to control cells using the DAVID program. Bold style indicates gene groups related to keratinocyte differentiation. “N” indicates the number of genes in the group. “Fold” indicates the fold enrichment of the group compared to a similar number of random genes (n=3(pooled)/3(pooled)).

(B) Gene expression analysis by qRT-PCR of S100A9, STFA3, SPRR2A, STAT1, and CXCL10 in keratinocytes isolated from 3d old control and Rac1 mutant mice cultured for 2d in vitro (n(STAT1)=3/3; n(S100A9, STFA3, SPRR2A)=5/5; n(CXCL10)=6/6).

(C) Expression of STAT1 protein in keratinocytes isolated from 3d old control and RAC1 mutant mice cultured for 2d in vitro. A representative Western blot is shown (n=6/6; *: p<0.05, **: p<0.001, ***: p<0.0001). (D) Gene expression analysis of STAT1 and CXCL10 in full skin samples from 5 day and 9 day old RAC1 ko and control mice (n=6/6;*: p<0.05). (E) Gene expression analysis for STAT1 and CXCL10 in FACS-enriched basal (α6high CD45-) and suprabasal (α6low CD45-) keratinocytes of control and RAC1-null skin (n=4-5/4-5; *: p<0.05, **: p<0.001). (F) Western blot
Rac1 function in skin inflammation

analysis for STAT1 and CXCL10 in epidermal lysates from adult RAC1 mutant mice and control mice. Representative Western blots are shown (n=9/12; *: p<0.05 , **: p<0.001) Immunostaining for STAT1 on cryo-sections of back skin from adult control and RAC1 mutant mice. The dashed line marks the dermal-epidermal junction. Representative pictures are shown (n=7/7). (F)

Fig. 5: Loss of RAC1 in keratinocytes cell autonomously increases STAT1 expression and enhances sensitivity to IFN-γ

(A) Efficient loss of RAC1 protein in GFP+, cre transfected RAC1 fl/fl and RAC1 fl/+ keratinocytes 4d after transfection. Shown is a representative Western blot and quantification of 3 independent experiments. (B) Gene expression analysis of GFP+, cre transfected RAC1 fl/fl and RAC1 fl/+ keratinocytes 4d after transfection for mRNA by qRT-PCR (upper panel) and protein by Western blot (lower panel) for STAT1 and CXCL10. Representative Western blots for 3d are shown (n=3-5/3-5). (C) Western blot analysis of pJAK2 (Tyr1007/1008) and pJAK1 (Tyr1022/1023) expression in keratinocytes isolated from 3d old control and RAC1 mutant mice cultured for 2d in vitro. Shown is a representative Western blot and quantification of 3 independent experiments. (D) Gene expression analysis by qRT-PCR of CXCL10 and IFIT1 mRNA in keratinocytes isolated from 3d old control and RAC1 mutant mice cultured for 2d in vitro and stimulated for 6h with indicated amounts of INF-γ (n = 5/6; *: p<0.05, **: p<0.001). (E) Western blot analysis of pSTAT1 (Tyr 701) on subconfluent keratinocytes isolated from 3d old control and RAC1 ko mice cultured for 3d in vitro and stimulated with 100U/ml IFN-γ for 6h (n=6/8 *; p<0.05)

Fig. 6: Alterations in actin cytoskeleton correlate with increased STAT1 expression in keratinocytes in vitro

(A) Western blot analysis for pPAK1/2 (Thr423/Thr402), pERK1/2 (Thr202/Tyr204), and pAKT (Ser473) of lysates from keratinocytes isolated from 3d old control and RAC1 ko mice cultured for 2d in vitro (n=3-6/3-6). (B) Phalloidin staining for F-actin by fluorescently labeled phalloidin in keratinocytes isolated from 3d old control and RAC1 ko mice cultured for 2d in vitro. Nuclear counterstaining by DAPI. Shown are representative pictures (n=1/6; scale bar 20µm). Cell area and cell circularity (circle=1) were measured from 260 control and mutant keratinocytes. (C) Gene
expression analysis of STAT1, CXCL10, STFA3, S100A9, and SRRR2A by qRT-PCR in subconfluent control keratinocytes from adult mice treated for 24h with either 0.5μM latrunculin A, 10μM Pak18, 10μM IPA-3, 50μM PD98059 or 50μM LY294002 (n=7–10/7–10; *: p<0.05, **: p<0.001, ***: p<0.0001). (D) Gene expression analysis of STAT1, STFA3, and S100A9 by qRT-PCR in subconfluent control adult keratinocytes treated for 24hr with 50μM CK584 (n=11/11*: p<0.05, **: p<0.001, ***: p<0.0001) (E) Western blot analysis for STAT1 in adult control keratinocytes after 24hrtreatment with 50μM CK548 (n=6/6; ***: p<0.0001) (F) Gene expression analysis of STAT1 and CXCL10 on 24 h 50μM CK548 treated adult control keratinocytes after 6h stimulation with 100U/ml INF-γ (n=8/9; *: p<0.05, **: p<0.001)

Fig. 7: Decreased actin polymerization in RAC1-null epidermis of adult mice.

(E) Total F-actin levels in keratinocytes from 3d old and adult control and RAC1 ko mice determined by FACS analysis of primary keratinocytes stained with fluorescently labeled phallolidin and FITC-conjugated antibodies against α6 integrin (n(3d)=6/3; n(adult)=3/3); **: p<0.001). (F) F-actin levels in back skin of adult control and RAC1-mutant mice determined by staining with fluorescently labelled phallolidin. Representative pictures are shown (n=3/3; scale bar 20μm).

Fig. 8: RAC1 regulates actin polymerization-dependent gene expression in a tanshinone inhibitable manner

(A) Western blot analysis for c-JUN in epidermal lysates from 3d old (n=4/4) and adult (n=5/5) RAC1 ko mice. (B) Gene expression analysis of STAT1, S100A9, and STFA3 by qRT-PCR in subconfluent adult control cells treated with 3.5 μM tanshinone, 0.5μM latrunculin, or 50μM CK584, as indicated (n=7/7; *: p<0.05,**: p<0.001, ***: p<0.0001). (C) Western blot analysis of STAT1 expression in 24h 50μM CK548 and 3.5μM tanshinone treated subconfluent adult control cells (n =6/6; **: p<0.001). (D) Gene expression analysis of STAT1 and CXCL10 in 50μM CK548 and 3.5μM tanshinone treated subconfluent adult control cells stimulated for 6h with 100U/ml IFN-γ (n=9/8 ; *: p<0.05,**: p<0.001) (E) Gene expression analysis of STAT1, S100A9, and STFA3 in subconfluent keratinocytes from
3d old control and RAC1 ko mice cultured for 2d in vitro and treated for 24h with 3.5 μM tanshinone (n=3/3). (F) Western blot analysis for STAT1 expression in subconfluent keratinocytes isolated from 3d old control and RAC1 ko mice treated, cultured for 2d in vitro and treated for indicated times with 3.5 μM tanshinone (n=3/3; *: p<0.05; **: p<0.001.) (G) Gene expression analysis of STAT1 and CXCL10 in subconfluent keratinocytes from 3d old control and RAC1 mutant mice cultured for 3d in vitro and treated for 48h with 3.5 μM tanshinone and stimulated for 6h with 100U/ml IFN-γ (n=5/8; *: p<0.05, **: p<0.001)
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### B

Control  
Gr-1 DAPI  
RAC1 ko

### C

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### D

[Bar chart showing fold increase for various genes]
Fig. 3

Control

RAC1 ko

SC

SG

SC

SG

SC

SG
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### Figure D

**STAT1**

- **CXCL10**

**Fold increase**

- Con  vs  Rac1 ko

**Figure E**

**STAT1**

- **CXCL10**

**Fold increase**

- Con  vs  Rac1 ko

**Figure F**

- **STAT1**

**Fold increase**

- Con  vs  Rac1 ko

**Figure G**

- **Control**

- **STAT1**

- **RAC1 ko**
Figure 5

A. Western blots showing the expression of RAC1 and Tubulin. The fold increase is shown for fl/+ cre and fl/fl cre conditions.

B. mRNA expression of STAT1 in fl/+ cre and fl/fl cre conditions over 2d and 3d cult periods.

C. Western blots showing the expression of pJAK2 and Tubulin under Con and Rac1 ko conditions.

D. Fold increase of IFIT1 and CXCL10 after treatment with 100U and 200U IFN-γ in Con and Rac1 ko conditions.

E. Western blots showing the expression of pSTAT1 (701) in Con and Rac1 ko conditions after treatment with 100U IFN-γ.