RAS-activated PI3K/AKT signaling sustains cellular senescence in experimental models of psoriasis via P53/P21 axis

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Abstract

Psoriasis is a chronic immune-mediated skin disease in which upper epidermal keratinocytes exhibit a senescent-like phenotype. In psoriatic skin, a variety of inflammatory cytokines can activate intracellular pathways including phosphatidylinositol 3-kinase (PI3K)/AKT signaling and RAS effectors. AKT and RAS participate to cellular senescence, but currently their role in senescence responses occurring in psoriasis have not yet been investigated. Here, we found AKT hyperactivation associated to the upregulation of senescence markers in psoriatic keratinocyte cultures subjected to multiple passages to promote senescence in vitro, as well as in skin lesions of patients affected by psoriasis. AKT-induced senescence was sustained by constitutive RAS activation, and PI3K/AKT pharmacological inhibition contrasted senescence processes induced by cytokines in psoriatic keratinocyte cultures. Additionally, RAS overexpression in the suprabasal epidermis in a murine model of psoriasis was accompanied by AKT upregulation, increase of senescence marker expression and by skin inflammation. In this model, both senescence and inflammation were significantly reduced by selective AKT inhibition.

Therefore, targeting RAS-AKT pathway could be a promising novel strategy to counteract multiple psoriasis symptoms.

Introduction

Psoriasis is a chronic immune-mediated skin disease characterized by multiple skin manifestations, as erythematous, scaling papules and plaques, provoked by alterations in skin homeostasis and immune responses. Th1, Th17, and Th22-lymphocyte-derived cytokines, including IFN-γ, IL-17A, IL-22, and TNF-α, have been identified as key drivers of psoriasis pathogenesis and can activate convergent intracellular pathways in psoriatic lesions resulting in epidermal hyperplasia, skin inflammation, and angiogenesis [1–3].

Phosphatidylinositol 3-kinase (PI3K)-dependent molecular pathway is hyperactivated in psoriatic skin and involved in several cellular processes contributing to psoriasis pathogenesis [4–9]. In human keratinocytes, cytokines induce the activation of multiple PI3K downstream effectors, such as AKT, phosphoinositide-dependent kinase-1, and mTOR [4, 5, 10], which in turn control the secretion of inflammatory mediators [11]. AKT also increases cell proliferation and impairs terminal differentiation of human keratinocytes [12], as well as it prevents cytokine-induced cellular apoptosis and promotes senescence-like growth arrest in psoriasis [4]. In psoriatic plaque, keratinocytes of the middle and suprabasal epidermal layers are characterized by a typical senescent state displaying resistance to apoptosis, release of inflammatory molecules, and expression of specific markers of senescence. On the contrary, keratinocytes in basal layers are characterized by a high proliferative potential [13–15].

It is known that senescence determines permanent cell growth arrest and upregulation of multiple proteins of cell cycle. In senescent cells, P53 is hyper-activated, resulting in the induction of the expression of several genes, such as the inhibitor cyclin-dependent kinase (CDK) complexes
p21CIP1/WAF1 (P21), which causes cell cycle arrest [16–18]. Additionally, cell growth block in senescence also depends on tumor-suppressor pathways controlled by the CDK inhibitor p16INK4a (P16) and Retinoblastoma protein (Rb) [19, 20].

Furthermore, senescent cells display a senescence-associated secretory phenotype (SASP), consisting in the hypersecretion of soluble and insoluble factors, such as cytokines, chemokines, growth factors and proteases, which reinforce the senescent cell cycle arrest and inflammation by acting in a paracrine and autocrine manner [21–23].

Previous reports showed that dysregulation of AKT drives senescence cell switch. For example, the expression of constitutively active AKT enhances cellular senescence in human endothelial cells via P53/P21-dependent pathway [24]. In mouse embryonic fibroblasts, AKT mediates RAS-induced premature senescence, and it is also involved in replicative senescence by increasing intracellular levels of reactive oxygen species [25]. In human fibroblasts, the chronic AKT up-regulation leads to P53 translation, and interferes with P53 ubiquitination and degradation, resulting in P53 accumulation associated with a senescent-like cell phenotype [26]. Consistently, we previously reported that the pharmacological PI3K/AKT inhibition by Ly294002 renders psoriatic keratinocytes more susceptible to pro-apoptotic signals induced by Th1/Th17-released cytokines [4].

PI3K/AKT pathway is a crucial effector of RAS protein [27–29], a small GTPase involved in multiple pathways and proliferation [30]. Activating mutations of the RAS GTPases are the most common genetic alterations identified in human tumors [31].

RAS and its effectors are up-regulated in human psoriatic skin [32–34], likely being involved in driving replicative senescence processes.

Thus, the existing data provides the basis to sustain the hypothesis that an AKT-dependent mechanism could be responsible for the senescent state of suprabasal keratinocytes observed in psoriatic plaques. However, to date, the role of AKT in this context is still unclear.

Herein, we explored AKT expression and function in sustaining senescence in psoriasis. We found that AKT hyperactivation is mediated by RAS activation and associates to high expression of typical senescence markers, both in vivo in skin psoriatic lesions and in vitro in psoriatic keratinocyte cultures undergoing senescence. Of note, senescence and skin inflammation recapitulated in experimental in vitro and in vivo models of psoriasis were significantly reverted by pharmacological PI3K/AKT inhibition.

Materials and Methods

Human Subjects

Skin biopsies were obtained from patients (aged 40–70 years) affected by moderate-to-severe chronic plaque psoriasis (n = 10), afferent to the Integrated Center for Research in Psoriasis (CRI-PSO) and from
healthy donors undergoing plastic surgery ($n = 10$). Biopsies were taken from skin plaques at sites overlapping lesional (LS), and non lesional (NLS) sites [15]. Patients were enrolled in the study after giving their written and signed consent, with the approval of the IDI-HRCCS Local Ethics Committee (Prot. N. CE-475) and according to the Declaration of Helsinki Guidelines.

Keratinocyte Cultures and Treatments

Human primary keratinocytes were isolated as previously reported [35,36]. Serial propagation of cell cultures were carried out to obtain senescence as described [37]. Colony Forming Efficiency (CFE) and aborted colonies formation were obtained and examined as previously reported [38–40]. Pre-senescent cultured psoriatic keratinocytes were cultured and stimulated as previously described [9]. When necessary, keratinocyte cultures were pre-treated with 5µM MK2206 or Ly294002 (both from Selleckchem Huston, TX, USA) for 1 h before adding cytokines [9].

Senescence-associated β-galactosidase activity assay

Keratinocyte Cultures and Treatments

β-galactosidase (SA-β-gal) activity measurements were performed as previously reported [15,41]. The development of blue color was followed under a 200X magnification microscope.

Retroviral-mediated gene transfer

L(RAS-V12)SN was constructed by cloning H-RAS-V12 (a gift from Manuel Serrano, CNIO, Madrid, Spain) into the EcoRI site of LXSN retroviral vector. The amphotropic producer cell lines Am12/L (RAS-V12)SN and Am12/LXSN (empty vector) were generated as described previously [37,42]. Keratinocyte infections were carried out as described previously [38].

H-RAS-induced murine psoriasiform model

InvtTA mice [43] were crossed with a homozygous tetOHRAS-V12 line [44] to give tetORAS (ST) and double transgenic (DT) InvtTA/ tetOHRAS-V12 offspring, and genotyped. Transgene induction was suppressed by doxycycline as described previously [45]. Mice were on C57BL/6 and FVB/n background. DT mice received daily topical administration of MK2206 ($n = 6$) or only vehicle solution ($n = 6$) for 5 consecutive days, as described [9]. ST mice ($n = 3$) received only control vehicle. On day 6, full thickness back skin biopsies of the treated area were collected and analyzed as reported [45]. All mouse procedures were carried out in accordance with institutional standard guidelines and in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals following protocols approved by The Pennsylvania State University IACUC.

Immunohistochemistry

Paraffin-embedded sections were obtained from biopsies of healthy or psoriatic skin, including LS and NLS zones. IHC analyses were performed using the following primary antibodies (Abs): anti-phosphorylated-AKT (Ser473 #3787) (Cell Signaling), anti-P53 (#sc-126), anti-P21 (#sc-6246) (both obtained from Santa Cruz, Dallas, TX, USA). Paraffin-embedded sections were analyzed by IHC as
described (36). Abs used were: anti-phosphorylated-AKT (Ser473, #3787S) (Cell Signaling), anti-P53 and Ly6G (#550291, BD Biosciences, Franklin Lakes, NJ, USA), CD3 (#A0452, Dako, Glostrup, Denmark), anti-CD8 (#217344, Abcam, Cambridge, United Kingdom). Immunoreactivities and measurements were performed as described [9].

**RNA Isolation and Real-Time Polymerase Chain Reaction (PCR)**

Total RNA was extracted from mouse paraffin-embedded skin biopsies, reverse-transcribed and analyzed as reported [9]. The primers used are listed in Table 1. *Il-1β, Cxcl1, Il-23, Cxcl16* mouse genes were analyzed by the TaqMan gene expression assay (Thermo Fisher Scientific, Rome, Italy) (assay ID: Mm00434228-m1; Mm04207460_m1; Mm00518984_m1; Mm00469712_m1, respectively). mRNA levels were normalized to *HPRT1* and *Beta-2 microglobulin (B2M)* mRNA expression. The values obtained from triplicate experiments were averaged, and data presented as mean $2^{-\Delta\Delta CT} \pm SD$. 
Table 1
List of primer sequences used for real-time PCR analysis on human and mouse samples.

<table>
<thead>
<tr>
<th>Human</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL8</td>
<td>Fw 5’-CCC CTA AGA GCA GTA ACA GTT CCT- 3’</td>
</tr>
<tr>
<td></td>
<td>Rv 5’-GGT GAA GAT AAG CCA GCC AATC- 3’</td>
</tr>
<tr>
<td>CCL2</td>
<td>Fw 5’-CAC CAG CAG CAA GTG TCCC- 3’</td>
</tr>
<tr>
<td></td>
<td>Rv 5’-CCA TGG AAT CCT GAA CCC AC- 3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Fw 5’-TCG CCA GTG AAA TGA TGG CT- 3’</td>
</tr>
<tr>
<td></td>
<td>Rv 5’-TGG AAG GAG CAC TTC ATC TGT T- 3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>Fw 5’-GGC ACT GGC AGA AAA CAA CC-3’</td>
</tr>
<tr>
<td></td>
<td>Rv 5’-CAC CAG GCA AGT CTC CTC AT-3’</td>
</tr>
<tr>
<td>CCL20</td>
<td>Fw 5’-GTG CTG CTA CTC CAC CTC TG- 3’</td>
</tr>
<tr>
<td></td>
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<tr>
<td>CXCL1</td>
<td>Fw 5’-CCTCAATCCTGCATCCC- 3’</td>
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<td></td>
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<tr>
<td>HPRT1</td>
<td>Fw 5’-TGACACTGGCAAAAACAATGCA-3’</td>
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<td></td>
<td>Rv 5’-GGTCTTTTTACCAGCAAGCT- 3’</td>
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<table>
<thead>
<tr>
<th>Mouse</th>
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<tbody>
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<td></td>
<td>Rv 5’-CTT CAT TGC GGT GGA GAG TC- 3’</td>
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<tr>
<td>Tnf-α</td>
<td>Fw 5’-GTC CCC AAA GGG ATG AGT T- 3’</td>
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<tr>
<td></td>
<td>Rv 5’-GGG TCT GGG CCA TAG AAG T- 3’</td>
</tr>
<tr>
<td>Ifn-γ</td>
<td>Fw 5’-AGC AGG GCG AAA AGG GAT GC- 3’</td>
</tr>
<tr>
<td></td>
<td>Rv 5’-GGG TTG TTG ACC TCA AAC TTG- 3’</td>
</tr>
</tbody>
</table>
### Immunoblotting and Densitometry

Total protein extracts by cell cultures, immunoblotting, and densitometric analyses were performed as described [9,35,36,46]. The Abs used were: anti-p-AKT (S473, #9271), anti-AKT (#9272), anti-p-Rb (Ser807/811, 9308), all provided from Cell Signaling, anti-P53 (#sc-126), anti-P21 (#sc-6246), anti-P16 (#sc-467), anti-RB (#sc-50), anti-cyclin D (#sc-8396) and anti-β-actin (#sc-1615) all purchased from Santa Cruz.

### Ras Activity Assay

Ras activity assay was carried out as previously reported [4].

### Statistical Analysis

Statistical analysis was performed by Student’s t test, Mann–Whitney U as specified in the figure legends. All analyses were conducted using Prism v.5.0 (GraphPad Software, La Jolla, CA, USA). Values were expressed as mean ± S.D., and statistical significance was assumed at a p value of 0.05 or less.

### Results

**RAS and AKT are hyper-activated in psoriatic keratinocytes and associate with a senescent-like phenotype**

AKT hyperactivation in psoriatic skin [4,12,47] could be involved in driving senescence processes in the epidermis of psoriatic skin [15, 16]. To verify this hypothesis, we evaluated the expression of
phosphorylated-AKT (p-AKT) in association to the levels of P16 and P21 senescence markers [15,48,49], during the serial culture passages of both healthy and psoriatic keratinocytes, leading to a senescence phenotype. As shown in Fig. 1A, we observed an increase of p-AKT expression throughout the culture passages, together with an increase of P21 and P16 expression, and a reduction of phosphorylated-Rb protein (p-Rb). Of note, psoriatic keratinocytes, expressing higher levels of p-AKT compared to healthy strains, displayed an increased SA-β-GAL activity and percentage of aborting colonies, both key markers of cellular senescence, starting from the first culture passage (Supplementary Fig. 1A and B).

PI3K/AKT pathway is a crucial effector of RAS [27–29], and alterations in RAS and AKT activities have been observed in the epidermis of psoriatic skin [34]. Similarly to AKT, RAS activity was high in psoriatic keratinocytes at the first passage of culture, and persisted throughout all the culture passages. In contrast, RAS activity was undetectable in healthy keratinocytes from the first-to-fourth culture passages, and increased only at sixth-passage culture in association to senescence markers and AKT activation (Fig. 1A).

To evaluate whether RAS hyperactivation in keratinocytes could sustain AKT phosphorylation and P53, and in turn the downstream P21 and P16 markers, we performed retroviral-mediated gene transfer of a mutant active form of H-RAS (H-RAS-V12, mutated at codon 12) into healthy keratinocyte cultures. As shown in Fig. 1B, H-RAS-V12-transduced keratinocytes (H-RAS V12) at second-passage culture displayed senescent features, and an increase of p-AKT and P53/P21 pathways compared to cells transduced with empty vector (EV) at the same culture passage. In keeping with our previous findings [37], P16 expression was not involved in RAS-V12-mediated senescence in primary keratinocytes. On the contrary, P16 expression was evident in replicative senescent keratinocytes (Fig. 1A, Healthy KC and Fig. 1B, EV at culture passage 10). Notably, keratinocyte stem cells, which escaped RAS-V12-mediated senescence (H-RAS-V12 at culture passage 24) [37], displayed low levels of p-AKT, P53 and P21.

We next evaluated the SASP in keratinocytes isolated from psoriatic and healthy skin at different passages of culture, in terms of mRNA expression of a set of cytokines and chemokines released by senescent cells. In line with the previous results, mRNA expression of the SASP molecules, such as CXCL8, CCL2, IL-6, IL-1β and CXCL1, increased during senescence progression, with a significant higher expression in psoriatic keratinocytes compared to healthy controls (Fig. 2).

**Phosphorylated-AKT is enhanced in psoriatic skin and correlates with senescence marker expression**

AKT activation was evaluated in skin biopsies obtained from LS and NLS skin of psoriatic patients and healthy donors. Immunohistochemical staining showed an enhanced p-AKT expression in epidermal keratinocytes of psoriatic LS skin (Fig. 3, c) compared to healthy and NLS skin biopsies (Fig. 3, a and b). p-AKT was intensely expressed throughout the epidermis of LS skin, with a predominant positivity in the nuclei and perinuclear areas of keratinocytes of the spinous layer (Fig. 3, c). In contrast, p-AKT staining
was weak in the epidermis of NLS skin (Fig. 3, b) and totally absent in healthy skin (Fig. 3, a). p-AKT was also observed in infiltrating immune cells accumulating in LS skin, as well as in other resident skin cells, such as dermal endothelial cells, also present in healthy and NLS skin (Fig. 3, a-c). Of note, a higher number of P53- and P21-positive keratinocytes was observed in spinous and granular epidermal layers of LS compared to NLS skin (Fig. 3, e, f, i), whereas P53 staining was faintly detected in healthy skin (Fig. 3, d). Consistently with p-AKT and P53 expression, P21 staining was weak in NLS skin and absent in healthy controls (Fig. 3, g and h).

### Senescence is sustained by inflammatory cytokines and downregulated by PI3K/AKT inhibition in psoriatic keratinocytes

We evaluated whether Th1/Th17-released cytokines could induce AKT up-regulation mediated by RAS in psoriatic keratinocytes. To this aim, RAS and AKT activation, in association to senescence markers, were examined in senescent psoriatic keratinocytes obtained by serial passage cultures and 4-h stimulated with IL-22, IL-17A or TNF-α alone or in combination. IL-22 did not influence RAS activation, nor p-AKT and P53 expression, whereas it upregulated P21 and P16 proteins in senescent psoriatic keratinocytes (Fig. 4A). In contrast, both IL-17A and TNF-α induced a significant activation of RAS and p-AKT, with a concomitant increase of P53, P21 and P16 expression (Fig. 4A). IL-17A and TNF-α also showed synergistic effects on senescent psoriatic keratinocytes, especially in terms of RAS and p-AKT activation (Fig. 4A). We next determined whether the upregulation of senescence-related proteins induced by IL-17A/TNF-α was mediated by AKT activation. To this end, senescent psoriatic keratinocytes were pre-treated for 1-h with a selective AKT1/2/3 inhibitor, MK2206, or with Ly294002, a pan-PI3Ks inhibitor, and then stimulated with IL-17A and TNF-α. Both inhibitors decreased p-AKT levels in psoriatic keratinocytes at basal condition and in response to cytokines. P53 and P16 content was strongly reduced by Ly294002 upon stimulation with IL-17/TNF-α, and weakly by MK2206 (Fig. 4B). P21 levels were reduced by both inhibitors in all cytokine treatment settings (Fig. 4B). Then, we evaluated the effect of AKT inhibition on SASP in psoriatic senescent keratinocytes. MK2206 interfered with CXCL1 and IL-6 expression, suggesting a direct role of AKT in the transcriptional regulation of these genes (Fig. 5A), both in absence and presence of cytokines. Moreover, MK2206 also reduced the expression of CCL20 induced by IL-17A/TNF-α. PI3K inhibition by Ly294002 significantly reduced CXCL8 and IL-6, both at basal level and after cytokine treatment (Fig. 5A), whereas IL-17A/TNF-α-induced CCL2, IL-1β and CXCL1 mRNA expression was reduced only by Ly294002. On the other hand, CCL20 was not influenced by Ly294002 in activated keratinocytes.

Finally, in line with these data, we found that both MK2206 and Ly294002 significantly reduced the percentage of SA-β-GAL-positive cells in both untreated and IL-17A/TNF-α-treated senescent psoriatic cultures (Fig. 5B).
RAS over-expression in the suprabasal keratinocytes of murine epidermis leads to spontaneous psoriasis-like disease mediated by AKT activation

Overexpression of H-RAS-V12 in suprabasal keratinocytes of adult mice causes the development of a psoriasiform dermatitis, characterized by keratinocyte hyperproliferation, acanthosis, hyperkeratosis, intra-epidermal neutrophil microabscesses and increased skin infiltration of Th1/Th17 and Tc1/Tc17 cells [45].

We evaluated the impact of AKT inhibition in the psoriasiform murine model induced by RAS overexpression. As previously described, InvTA x tetORAS double transgenic (DT) mice deprived from dox for 7 days displayed phenotypic psoriasis-like changes (45). DT/-dox mice were 5-day treated topically with MK2206 or with control vehicle. In parallel, in a group of DT mice, dox administration was maintained, and mice were treated only with vehicle as healthy control mice (n = 3). As shown in Fig. 6A, MK2206 administration in DT/-dox mice resulted in the amelioration of several histological parameters associated to psoriasis, with a decrease of ~ 50% of acanthosis (Fig. 6A and B), and a reduction in the number of dermal infiltrating immune cells (about 50% decrease) (Fig. 6A and B). Stratum corneum thickness was also reduced (about 55% reduction). Furthermore, in skin of DT/-dox mice, a strong expression of p-AKT in the suprabasal layers of epidermis, where H-RAS-V12 is overexpressed, was observed, whereas p-AKT staining was almost undetectable in DT + dox/control mice (Fig. 6C, i and ii). Moreover, p-AKT expression (Fig. 6C, iii) was significantly reduced in skin of DT/-dox mice treated with MK2206 in comparison to untreated DT/-dox mice. Of note, DT/-dox mouse skin showed a high content Ly6G+ neutrophils present in intra-epidermal microabscesses (Fig. 6C, v), which was inhibited by MK2206 treatment (Fig. 6C, vi). Consistently, DT + dox/control mice did not present Ly6G+ cells in epidermis (Fig. 6C, iv). Suprabasal RAS overexpression also caused an increase in the amount of skin infiltrating CD3+ and CD8+ T cells, with all CD8+ T cells infiltrating the epidermis (Fig. 6C, viii, xi). Both CD3+ and CD8+ cells were undetectable in control mice and in DT/-dox mice treated with MK2206 (Fig. 6C, vii, x, ix, xii).

We next analyzed mRNA expression of a panel of inflammatory cytokines and chemokines in whole back skin samples. As shown in Fig. 7, the expression of all these genes strongly increased in DT/-dox mice in comparison to DT/+dox control mice. MK2206 strongly reduced the expression of Cxcl1, G-csf, Gm-csf, Cxcl16 genes, all involved in the neutrophil activation and recruitment (Fig. 7), whereas it determined a minor effect on IFN-γ expression and not significant changes in IFN-γ-dependent genes, such as Ccl2 and Cxcl10 (data not shown). Furthermore, mRNA expression of Cdkn1a and Trp53, but not Cdkn2a, was up-regulated in DT/-dox mouse skin and it was reduced by MK2206 (Fig. 7).

Discussion

Senescence is a cellular program involved in physiological and homeostatic processes, as well as in pathological conditions, and characterized by a permanent cell growth arrest in G1- or in G2-cell cycle phase, pointed to prevent the proliferation of damaged cells [50,51]. Senescence is evoked by cellular
stress and by tumor suppressive mechanisms that must be overcome to promote malignant transformation.

Psoriatic keratinocytes are characterized by a senescent-like phenotype, manifesting a peculiar resistance to apoptosis and a high expression of P21 and P16 senescence markers, which is supported by several intracellular pathways [4, 15, 16].

In this study, we explored the role of PI3K/AKT pathway in epidermal senescence of psoriatic skin. We found that keratinocyte cultures from psoriatic patients and undergoing cellular senescence exhibit higher levels of p-AKT during in vitro culture passages, compared to keratinocytes from healthy skin. Hyperactivated AKT correlates with high levels of P53, P21 and P16 senescence markers and SA-β-gal activity and with a reduced expression of p-Rb protein.

Together with RAF/ERK axis, PI3K and AKT are effectors of RAS molecule. RAS-RAF-MAPK pathway is upregulated in psoriatic skin [32,33], and induces thickening and inflammation in the epidermis of murine models of psoriasis [52,53]. However, to date, the role of RAS/PI3K/AKT signaling in the mechanisms underlying senescence of psoriatic keratinocytes are not being proven. To this matter, we showed that psoriatic keratinocytes show a lower replicative rate compared to healthy cells and are characterized by a constitutive RAS hyperactivation that correlates with AKT/P53/P21 upregulation. This observation suggests a link between RAS and PI3K/AKT axis in senescence programs occurring in psoriatic keratinocytes and could depend on the continue exposition to cytokine milieu of psoriatic skin during disease evolution. The genetic and epigenetic status also predisposes psoriatic keratinocytes to a pre-senescent status and to respond aberrantly to cytokines.

PI3K/AKT are not the only effectors of cellular senescence. Additional intracellular mediators of senescence have been identified, including the negative regulator of RAS/ERK signaling, neurofibromin 1 (NF1), in human fibroblasts undergoing AKT-induced senescence [54]. Interestingly, NF1 is downregulated in psoriatic skin, thus resulting in the hyper-activation of RAS [55].

In addition, psoriatic keratinocytes exhibit a strong upregulation of P16, which drives senescence programs in parallel to P21, most likely in response to Th1/Th17 cytokines present in the microenvironment of psoriatic plaques. In particular, pro-inflammatory cytokines can activate various intracellular pathways, including PI3K/AKT axis, that result in the upregulation of the downstream P53 and P16 effectors [56]. Activated P53 induces in turn P21, which impedes cell-cycle progression by inhibiting cyclin E/CDK2 complex. P16 also induces cell-cycle arrest by targeting cyclin D/CDK4 and cyclin D CDK6 complexes. Both P21 and P16 inhibit Rb phosphorylation, thus resulting in S-phase arrest [57,58]. Consistently, Th1/Th17-released cytokines enhance RAS activation, and induce AKT phosphorylation, as well as P53, P21, and P16 expression levels. A strong effect was exerted by TNF-α and IL-17A alone and by their combination. In contrast, IL-22 induced a significant induction of P16, not dependent on RAS and AKT up-regulation. This finding suggests a novel pro-senescent function for IL-22, a pathogenic cytokine known to impair the terminal differentiation of human keratinocytes. We hypothesize that IL-22 could sustain the oncogene-induced senescence in psoriatic keratinocytes,
differently to what observed in hepatic cells, where IL-22 induces a senescence mediated by P53/P21 axis [59].

In line with these observations, PI3K/AKT inhibition significantly reverted senescent phenotype of psoriatic keratinocytes. In particular, Ly294002, a pan-PI3K inhibitor, significantly inhibited SASP expression, with the exception of CCL20 chemokine, whereas MK2206, a selective AKT1/2/3 inhibitor, weakly reduced SASP profile. This result could depend on the involvement of PI3K in several intracellular pathways upstream of AKT/mTOR axis. Indeed, Hart demonstrated that pharmacological PI3K inhibition prevented STAT3 phosphorylation induced by a Tec kinase in human transformed cells, suggesting an interplay between PI3K and STAT3 (60). Similarly, PI3Kδ isoform sustains IL-22-induced STAT3 activation in psoriatic keratinocytes [9]. However, PI3K/AKT also activates NF-κB pathway, and its inhibition reduces the NF-κB-dependent inflammatory responses in human keratinocytes [61].

In this study, we showed for the first time that AKT hyper-activation mediated by RAS overexpression in the suprabasal epidermal layers is crucial for the establishment of psoriasis phenotype. Mice overexpressing the H-RAS-V12 transgene in the upper epidermis layers show increased levels of p-AKT, together with P21 and P53 senescence markers. In line with in vitro and in vivo findings, the employment of the AKT1/2/3 inhibitor MK2206 in murine psoriasiform model significantly attenuates the disease symptoms by reducing the epidermal thickness and dermal accumulation of immune cell infiltrate. Of note, RAS overexpression in mice did not influence P16 expression, in line with what observed in vitro in healthy keratinocyte cultures transduced with H-RAS and characterized by a senescence status [37] associated to the upregulation of the only AKT/P53/P21 axis. These data contrast with the high P16 expression observed in human psoriasis lesions and in psoriatic keratinocyte cultures undergone senescence, where genetic background could play a critical role. In addition, the low expression levels of P16 could depend also on the absence of IL-22 in RAS-induced disease model. In line with the human disease condition, P21 and P53 levels were upregulated in RAS-induced disease model, and were strongly reduced by MK2206.

Of note, MK2206 drastically inhibited the recruitment and infiltration into the skin of different leukocyte subpopulations responsible for induction of psoriasiform phenotype in RAS-induced model. Among them, skin-infiltrating neutrophils and CD3+CD8+ T cells were reduced by AKT inhibition, together with molecules involved in neutrophilic chemoattraction and activation (i.e. CXCL1, GM-CSF, G-CSF, CXCL16), and with cytokines released by Th17, γδT and Tc1/Tc17 cells (i.e. IL-17, IL-23 and IFN-γ) and by dendritic cells (i.e. IL-1β, IL-23 and TNF-α).

In conclusion, we propose a novel model of RAS/AKT-induced senescence (Fig. 8), where cytokine-induced RAS upregulation in keratinocytes determines the basal AKT/P53/P21 and P16 activation. In turn, these pathways determine a strong decrease of cell growth rate, and induction of hypersecretory phenotype in keratinocytes, all processes responsible for aberrant epidermal thickening and skin chronic inflammation observed in psoriatic plaque. SASP exhibited by epidermal keratinocytes is implicated in the amplification of inflammatory circuits through paracrine effects on skin infiltrating immune cells and
dermal vasculature, and by autocrine action on keratinocytes themselves (Fig. 8). Senescence could also contribute to the onset of various comorbidities, that frequently affect psoriatic patients [62]. Indeed, the secretion of SASP components can cause chronic inflammation not only locally in the skin, but also systemically [14,50,63,64]. However, in psoriatic skin, senescence represents a response to chronic stress stimuli and aberrant cell division, thus it is thought to protect psoriatic lesions from tumorigenesis [65].

Targeting AKT pathway could be a promising novel strategy to counteract multiple psoriatic symptoms, taking account of senescence-associated pathological signs.

Declarations

Acknowledgements

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Contributions

LM designed experiments, performed all in vitro investigations, analyzed in vitro and in vivo data, and wrote the original draft; JB conducted in vivo experiments; ABG critically read the manuscript; ED provided RAS-overexpressing cellular model and contributed to reviewing the draft; CS contributed to in vitro experiments; SP provided human skin biopsies; CA critically reviewed and corrected the draft; SM conceived the research, designed the study and critically corrected the manuscript. The paper has been read and approved by all named authors.

Conflicts of Interest

The authors state no conflict of interest.

Ethics approval

All animal experiments were performed in accordance with institutional standard guidelines and in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals following protocols approved by The Pennsylvania State University IACUC. Patients were enrolled with the approval of the IDI-IRCCS Ethics Committee (Prot. N. CE 475) and according to the Declaration of Helsinki Guidelines.

Data availability
The authors declare that all other data supporting the findings of the current study are available within the article or in its Supplementary Information files or are available from the corresponding author on reasonable request. No datasets were generated or analyzed during this study, thus data sharing is not applicable to this article. Uncropped WB are reported in Supplementary Figure 2.

References

13. Wrone-Smith T, Mitra RS, Thompson CB, Jasty R, Castle VP, Nickoloff BJ. Keratinocytes derived from psoriatic plaques are resistant to apoptosis compared with normal skin. Am J Pathol.


Figures
RAS and AKT activity is enhanced in psoriatic keratinocytes at baseline and during senescence compared to healthy cells and associates with senescence markers expression.

A Representative western blot (WB) showing GTP-RAS, phosphorylated AKT (p-AKT)/AKT, P53, P21, P16, p-Rb and b-actin (for normalization) expression levels in healthy and psoriatic keratinocytes (Healthy KC and Pso KC) undergoing cellular senescence by serial culture propagation. Figures show results at
different culture passages (C.P. 1, 4, 6). The graphs show the densitometry intensity (DI) of P53, P21, P16 and RAS-GTP expression levels and the ratio of p-AKT level to total AKT level, and p-Rb to total Rb protein content after normalization to the level of b-actin protein (n = 3 independent experiments); data are expressed as means ± SD; ***p < 0.001, **p < 0.01 and *p < 0.05, as determined by Student's t test. Representative WB show H-RAS-V12, p-AKT, P53, P21, P16 and b-actin expression in Healthy H-RAS-V12 transduced at culture passage 2 and 24 (C.P. 2, 24). Empty-vector-transduced keratinocytes (EV) at culture passage 2 and 10 were used as negative control. The bar graphs show the D.I. values of H-RAS-V12, P53, P21 and P16 and the ratio of p-AKT level to total AKT level (n = 3 independent experiments; the means ± SD) **p < 0.01 and *p < 0.05, as determined by Student's t test.

**Figure 2**

**SASP gene expression is higher in Pso KC compared to Healthy KC and increases during senescence.**

mRNA expression of SASP molecules as *CXCL8, CCL2, IL-6, IL-1β* and *CXCL1*, were detected by Real time (RT)-PCR in Healthy and Pso KC undergoing senescence through serial propagation in culture, analyses were conducted at different culture passages (C.P. 1, 4, 6). The graphs show data normalized to *HPRT1*
mRNA expression levels and expressed as mean values ± SD (n = 3 independent experiments); **p < 0.01 and *p < 0.05 as calculated by Student’s t test.

**FIGURE 3**

**Figure 3**

**Phosphorylated-AKT expression is enhanced in psoriatic skin lesions and correlates with senescence markers expression.**

Representative staining of immunohistochemical (IHC) analyses of p-AKT, P21 and P53 (red-brown stained), performed on paraffin-embedded sections of biopsies obtained from psoriatic skin (n = 10),
including non-lesional (NLS), lesional (LS) zones of evolving plaques, as well as skin from healthy donors ($n = 10$). Sections were counterstained with Mayer’s H&E. Bars, 100 μm. For each specimen three sections were analyzed, and positive cells were evaluated in five adjacent fields, at a 200X magnification. A semiquantitative, four-stage scoring system was applied, ranging from negative immunoreactivity (0) to strong immunoreactivity (4+) for p-AKT. Analyses were conducted by a researcher blind to the experimental groups of patients. Graphs show the mean of four-stage score values for p-AKT epidermal expression, or the mean values of the number of P21-positive cells. Results are shown as mean and ± SD. **$p < 0.01$ and *$p < 0.05$, as determined by Mann–Whitney U test.

**FIGURE 4**
Figure 4

Cellular senescence in psoriatic keratinocytes is sustained by psoriasis-related inflammatory cytokines and is affected by specific PI3K/AKT inhibitors.

A Representative WB showing GTP-RAS, p-AKT/AKT, P53, P21, P16, and b-actin (for normalization) expression levels in Pso KC at C.P. 4, left untreated or stimulated for 4 hours with inflammatory cytokines, such as IL-22 (50 ng/ml) alone, IL-17A (50 ng/ml) and TNF-a (50 ng/ml) alone or in combination. Graphs show the D.I. of GTP-RAS, P53, P21 and P16 expression levels and the ratio of p-AKT expression level to total AKT protein content after normalization to b-actin protein level (n = 3 independent experiments); data are expressed as means ± SD; ***p < 0.001, **p < 0.01 and *p < 0.05, as determined by Student’s t test. B Representative WB of p-AKT/AKT, P53, P21, P16, and b-actin (for normalization) expression in Pso KC at C.P. 4, left untreated or stimulated for 6 hours with a combination of IL-17A and TNF-a after a pretreatment of 1 hour with MK2206 or Ly40002 inhibitors. The graphs show D.I. of P53, P21 and P16 expression levels and the ratio of p-AKT expression level to total AKT protein level after normalization to b-actin protein content (n = 3 independent experiments). Data are expressed as mean values ± SD; ***p < 0.001, **p < 0.01 and *p < 0.05, as calculated by Student’s t test.
**Figure 5**

**PI3K/AKT inhibitors counteract senescence-associated responses in psoriatic keratinocytes.**

A mRNA expression level of SASP molecules as *CXCL8, CCL2, IL-1β, IL-6, CCL20* and *CXCL1*, were detected by RT-PCR in Pso KC (C.P. 4) stimulated for 5 hours with IL-17A/TNF-a combined cytokines and pre-treated for 1 hour with MK2206 or Ly294002 inhibitors. The graphs show data normalized to *HPRT1* mRNA expression level and expressed as means ± SD (*n* = 3 independent experiments); **p < 0.001 and *p < 0.01 as calculated by Student’s *t* test. B Representative images of Pso KC (CP 4) cultures analyzed
for SA-β-gal activity detected by colorimetric staining (blue) in cells at passage P4, stimulated for 24 hours with IL-17A/TNF-a cytokine combination and pre-treated for 1 hour with MK2206 or Ly294002 inhibitors. Keratinocytes were fixed and incubated with SA-b-GAL staining solution overnight. The development of blue colour was detected under a 200X magnification microscope. Bars, 50 μm. Graphs show the means (n = 3 independent experiments) of the percentage of SA-β-gal positive cells ± SD, counted in two adjacent fields. ***p < 0.001, **p < 0.01 and *p < 0.05, as determined by Student’s t test.

**FIGURE 6**
**Figure 6**

**RAS over-expression in suprabasal keratinocytes of murine epidermis induces psoriasis-like phenotype mediated by AKT activation.**

**A** Representative H&E staining of skin from DT +dox/control mice, DT/-dox mice in which H-RAS-V12 was overexpressed and from DT/-dox H-RAS-V12 overexpressing mice treated with MK2206. Bars, 500 µm. **B** Graphs show the measurements of epidermal and stratum corneum thickness (mm), as well as skin infiltrating immune cell number, analyzed as parameters of skin acanthosis and inflammation. Data are reported as means ± SD per section ($n = 3$ sections) for each group ($n = 3$ mice for control group; $n = 6$ DT/-dox group and $n = 6$ DT/-dox + MK2206 group, **$p < 0.01$ and *$p < 0.05$, as assessed by Student’s $t$ test. **C** IHC staining (red-brown staining) of p-AKT, Ly6G, CD3 and CD8 performed on skin of DT +dox/control mice, DT/-dox and DT/-dox H mice treated with MK2206. Sections were counterstained with Mayer’s H&E and were visually evaluated by a pathologist experienced in dermatology. Bars, 500 µm. For each specimen three sections were analyzed, and positive cells were evaluated in five adjacent fields, at a 200X magnification. A semiquantitative, four-stage scoring system was applied, ranging from negative immunoreactivity (0) to strong immunoreactivity (4+) for p-AKT. Analyses were conducted by a researcher blind to the experimental groups of mice, who took five measurements per three sections for each mouse. Graphs show the mean of four-stage score values for p-AKT expression ± SD, and means of Ly6G-, CD3-, CD8-positive cell number ± SD ($n = 3$ sections) per all mice of each experimental group. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$, as assessed by the Mann–Whitney U test.
Figure 7

AKT inhibition counteracts RAS-induced psoriasis-associated inflammatory gene expression in murine epidermis.

*IL-1β, Cxcl1, Il-17a, Il-23, Tnf-a, Ifn-g, G-csf, Gm-csf, Cxcl16, Trp53 (P53), Cdkn1a (P21), Cdkn2a (P16)*

mRNA expression was detected by RT-PCR performed from dorsal skin biopsies of mice treated as indicated: DT +dox/control mice (n = 3), DT/-dox mice in which H-RAS-V12 was overexpressed (n = 6) and DT/-dox H-RAS-V12 overexpressing mice treated with MK2206 (n = 6). The graphs show mean values of
relative mRNA levels normalized to g B2m gene expression, ± SD. ***p < 0.001, **p < 0.01 and *p < 0.05, as assessed by the Mann–Whitney U test.

Figure 8

Graphical model of RAS-AKT-mediated senescence in psoriatic plaque.
A graphical scheme of the molecular mechanisms underlying senescence-responses occurring in keratinocytes of psoriatic lesional skin. We assume that in psoriatic skin RAS activation by inflammatory cytokines as IL-17A and TNF-α induces PI3K activity which in turn results in the activation of AKT pathway. Activated AKT sustains both P53/P21 axis and P16 activation leading to cell growth arrest through the reduction of Rb protein phosphorylation. In addition, IL-22 cytokine enhances P16 protein expression. PI3K/AKT molecular axis induces the expression of multiple genes belonging to the inflammatory SASP. We hypothesize that this molecular event could be mediated by PI3K-activated STAT3 induction (not dependent on AKT), and by NF-κB pathway triggered by AKT. The senescence-associated growth arrest and the hypersecretory phenotype of epidermal keratinocytes contribute to different aspects of psoriatic disease, as well as thickened epidermis, and tissue and systemic chronic inflammation. In particular, the over-secretion of SASP molecules is involved in the recruitment and activation of epidermal and dermal infiltrating immune cells, in aberrant angiogenesis, and in autocrine effects on keratinocytes by amplifying the autoinflammatory circuits occurring in psoriatic plaque. The image was made by using Biorender (biorender.com).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFIG1.tif
- SUPPLFIG2uncroppedWB.pdf
- ReproducibilitychecklistMercurioL.etal.pdf