



Human keratinocyte carcinomas have distinct differences in their tumor-associated macrophages



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ABSTRACT

Cutaneous squamous cell carcinomas (SCCs) and basal cell carcinomas (BCCs) have different clinical behaviors, despite both being keratinocyte carcinomas mainly caused by ultraviolet radiation. Whether these distinct features are associated with tumor-associated macrophages (TAMs) is largely unknown. The main goal of this study was to conduct a comprehensive analysis of density and polarization states of TAMs in SCCs versus BCCs. The role of lactic acid in TAM polarization in SCC versus BCC was examined. We found that SCCs have a higher density of CD68 + TAMs compared to BCCs. TAMs in SCCs express higher levels of TAM-associated markers (arginase-1, MMP9, CD40 and CD127) than those in BCCs. Interestingly, differential expression of TAM-associated markers between SCCs and BCCs was reproduced in human monocytic THP-1 cells stimulated with SCC- or BCC-conditioned media. Analysis of soluble factor(s) in these tumors further revealed that SCCs have a significantly higher concentration of lactic acid than BCCs, and lactic acid was sufficient to upregulate TAM markers. Our results demonstrate that TAMs in SCCs versus BCCs differ in density and polarization states, which can be determined by soluble factors including tumor-derived lactic acid. These differences in TAMs may contribute to the distinct clinical behaviors of SCCs versus BCCs. This work was supported by grants from the National Institutes of Health and the Doris Duke Charitable Foundation.

Research in context: Few studies have studied tumor-associated macrophages in the context of SCC versus BCC. It has been demonstrated that macrophages mobilize to the epidermis after being exposed to ultraviolet-B radiation and produce interleukin-10 (IL-10). It has also been shown that the production of IL-10 results in the evasion of T cell-mediated immunity in BCCs and SCCs. However, the relationship between TAMs and the clinical behaviors of SCCs and BCCs remains largely unclear. Our study shows that despite their similar origins, human cutaneous SCCs and BCCs are considerably different in their TAMs. To our knowledge, these results provide the first evidence of differential TAM density and polarization in SCCs versus BCCs, which may contribute to their characteristic clinical behaviors. Future studies are necessary to elucidate the mechanisms by which TAMs influence these cancers with the goal of developing therapies tailored to each type of malignancy.

1. Introduction

Cutaneous squamous cell carcinomas (SCCs) and basal cell carcinomas (BCCs) are the most common skin cancers in the United States. Approximately, 5.4 million SCCs and BCCs are diagnosed annually and their incidence is rising [1]. Both SCCs and BCCs are keratinocyte

carcinomas (KCs) and exposure to ultraviolet (UV) radiation is the most important environmental factor involved in their initiation [2]. Despite the similarities in their origin and cause, SCCs and BCCs have distinct clinical features: SCCs are more aggressive and more likely to metastasize at late stages, while BCCs are more indolent and rarely metastasize [3]. Considerable effort has been devoted to elucidating the genetic events

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that drive the pathogenesis of cutaneous SCCs and BCCs. For example, UV-induced *TP53* gene mutations are an early event in SCC development, while aberrant activation of the Hedgehog signaling pathway through acquired mutations in the *PTCH1* and *SMO* genes is a pathognomonic feature of BCC development [4, 5]. It was proposed that the difference in cellular capacity for DNA repair, apoptosis, and proliferation plays an important role in the development of UV-induced SCCs and BCCs [6, 7]. Further, the importance of the immune system in skin cancer has been long recognized. UV radiation can induce more regulatory T cells than effector T cells in the skin, leading to an immunosuppressive microenvironment that is closely correlated with the development of cutaneous malignancies including KCs [8]. However, little is known about how tumor-infiltrating immune cells shape the clinical differences between SCCs and BCCs.

Numerous studies have demonstrated that macrophages are involved in all stages of carcinogenesis, including initiation, growth, invasion, and metastasis [9, 10]. While macrophages are generally characterized by two distinct polarization states, namely M1 and M2, most tumor-associated macrophages (TAMs) have an M2-like polarization state and their density positively correlates with a poor prognosis [9, 10, 11]. The published studies analyzing TAMs in cutaneous SCCs or BCCs are few: two studies showed that macrophages infiltrate into human epidermis upon exposure to UVB radiation and resultant TAMs, in both SCCs and BCCs, produce IL-10 leading to evasion of the local T cell-mediated immune response [12, 13]. Recently, TAMs in cutaneous SCCs were found to be a major source of vascular endothelial growth factor-C (VEGF-C), a critical lymphangiogenic factor [14]; while the number of TAMs in BCCs is directly correlated with depth of invasion, microvessel density, and induction of cyclooxygenase-2 [15]. Interestingly, significantly enhanced tumor growth has been reported in a BCC mouse model (induced in conditional *Ptch^{lox/lox}ERT2^{+/-}* mice) when cutaneous macrophages were depleted using clodronate liposomes [16]. While the published studies have used SCC or BCC cell lines or mouse models, the role of TAMs in the distinct clinical behaviors of human SCCs and BCCs has yet to be determined.

To pursue these questions, we conducted a comprehensive comparison of TAMs in human cutaneous SCC and BCC. Our data revealed that SCCs have a higher density and polarization state of TAMs than BCCs and express higher levels of TAM-associated markers. Moreover, tumor-derived lactic acid was demonstrated to be one critical soluble factor involved in the functional polarization of TAMs in SCCs.

2. Materials and methods

2.1. Keratinocyte carcinomas

De-identified, discarded tissue was obtained consistent with the general requirements of the protocol approved by the Yale Human Investigations Committee (HIC# 200002048). Histology was evaluated by board-certified dermatopathologists to ensure that tumors were cutaneous SCCs and BCCs prior to being included in experiments.

2.2. Immunohistochemistry

SCC and BCC formalin-fixed paraffin-embedded samples were sectioned (5µm thickness) and the slides were stained with haematoxylin and eosin and anti-CD68 antibody. CD68 + macrophages were visualized at 40x magnification with OTIPHON Nikon microscope. Numbers of TAMs per 40x high power fields were counted with ImageJ by two independent researchers.

2.3. Preparation of single cell suspensions

The freshly excised tumor samples were mechanically dissociated and digested with 10 µg/ml LiberaseTM and 100 µg/ml DNase I in 20 ml PBS with calcium and magnesium at a 37 °C shaking incubator (180 r.p.m.)

for 20–30 min. 200 µl 0.5 M EDTA was added to stop the enzymatic reaction. After filtrating through a 70 µm nylon cell strainer, cells were collected and washed with cold FACS buffer (0.5% FBS in PBS) and centrifuged (1,000 r.p.m.) at 4 °C. Red blood cells were lysed with ACK lysing buffer, then washed with FACS buffer. The samples were kept on ice throughout the rest of the staining process.

2.4. Flow cytometry

Human FcR Binding Inhibitor (eBioscience) was added to the samples (1:200) and incubated for 20 min on ice to block non-specific Fc-receptor binding. After, cells were washed with FACS buffer and stained with surface antibodies, which included anti-CD45 (HI30, eBioscience); anti-CD40 (5C3, BioLegend); and anti-CD127 (A019D5, BioLegend), at room temperature for 15 min in the dark. Samples were washed twice with FACS buffer. Intracellular staining with anti-CD68 (eBioY1/82A, eBioscience), anti-arginase-1 (R&D systems), and anti-MMP9 (R&D systems) was performed using the Fixation and Permeabilization Solution kit (BD Biosciences). Dead cells were excluded using 7-AAD staining. Samples were run on BD LSRII flow cytometer and data were analyzed using FACSDiva software and FlowJo.

2.5. Tumor-conditioned media and THP-1 stimulation

Fresh human cutaneous SCC and BCC specimens were dissociated with surgical scissors and razor blades. The minced samples were resuspended with complete RPMI 1640 media and centrifuged at 1,000 r.p.m. (4 °C) for 5 min. Cells were counted and equal number of live cells from each tumor was incubated with complete RPMI 1640 media in a humidified tissue culture incubator (37 °C) for 24 h. The tumor supernatant was carefully aspirated, centrifuged, and filtered with a sterile syringe filter (0.45 µm). The tumor-conditioned and control (RPMI 1640) media were stored at –80 °C for future experiments. For *in vitro* stimulation of THP-1 (human monocytic) cells, 1×10^6 THP-1 cells were incubated with 1 ml of tumor-conditioned or control media in sterile six-well Petri dishes for 48 h. Then, THP-1 cells were then harvested for flow cytometry as described above.

2.6. Determination of lactic acid concentration

Human cutaneous SCC or BCC samples were mechanically dissociated, enzymatically digested as above, and washed with cold PBS. Live cells were counted and lactic acid concentration was measured using the Lactate Colorimetric Assay Kit II (BioVision) following the manufacturer's instructions. 500,000 live cells in lactate assay buffer (50 µl/well) were added to a 96-well plate. 50 µl of reaction mix was added into each well and incubated for 30 min at room temperature. Absorbance was measured at 450 nm using a SpectraMax M5 microplate reader (Molecular Devices), then background absorbance was subtracted. A standard curve was made, and lactic acid concentration (mM) of each sample was calculated by applying the sample reading to the standard curve. The final lactic acid concentration was shown on a per-cell basis (nmol/cell).

2.7. THP-1 stimulation with lactic acid and quantitative PCR

1×10^6 THP-1 cells were incubated with complete RPMI media containing 0 mM (control) or 25 mM lactic acid in a 12-well plate for 24 h (37 °C). Total RNA was extracted from the stimulated cells and cDNA was synthesized with the SMARTer PCR cDNA Synthesis kit (Clontech). Quantitative PCR (qPCR) was performed using an Mx3000P Thermocycler (Stratagene) with 3 µl of cDNA plus 6 µl of Perfecta SYBR Green Supermix (Quanta BioSciences) and 3 µl (10 µM) of specific primers: *ARG1*: FP, 5'-GCAAGGTGGCAGAAGTCAAGA-3'; RP, 5'-TGGCATGGC-CAGAGATGCTT-3'. *MMP9*: FP, 5'-GCCTCTGGAGGTTCCACGTG-3'; RP, 5'-GGGAAGTACACGCGCCAGTAG-3'; *CD40*: FP, 5'-GCTGGCACTGTAC-GAGTGAGGC-3'; RP, 5'-CTTATTGGTTGGCTTCTTGGC-3'; *IL7R*: FP, 5'-

CGCCAGGAAAAGGATGAAAAC-3'; RP, 5'-GACTCCATTCACCTCA-GAAGCCT-3'; *ACTB*: FP, 5'-GTCCACACCCGCCGCGCCAG-3'; RP, 5'-CGGGGGGCATCGTCG-3'. All samples were run in triplicate and fold change in gene expression was calculated using the reference sample (housekeeping gene *ACTB*).

2.8. Statistics

Statistical difference in values between two experimental groups was determined using the unpaired Student's *t* test (2-tailed distribution) and nonparametric Mann-Whitney test. Statistical differences in values between three experimental groups were determined by using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Human cutaneous SCCs have a higher TAM density than BCCs

To characterize TAMs in SCCs and BCCs, we collected specimens of human cutaneous SCCs and BCCs and stained the slides of biopsy-proven SCCs ($n = 5$) and BCCs ($n = 6$) with CD68, a marker of human macrophages. Our results show that SCCs consistently had an approximately 6-fold higher density of CD68 + TAMs than BCCs (Fig. 1A). We also quantified the number of TAMs in SCCs and BCCs per high-powered field (Fig. 1B, $P < 0.0001$). To further confirm the difference in TAM-density, we analyzed CD45 + CD68 + TAMs in a second set of SCCs ($n = 6$) versus BCCs ($n = 6$) by flow cytometry. As shown in Fig. 1C, SCCs have a higher percentage of TAMs than BCCs. The difference was statistically significant (Supplementary Figure 1, $P = 0.0005$). Together, these data demonstrate that SCCs have a significantly greater density of TAMs than BCCs.

3.2. TAMs in human cutaneous SCCs express higher levels of TAM-associated polarization markers than TAMs in BCCs

TAM-like polarization of macrophages has been associated with an increase in various expression of markers including arginase-1, MMP-9, CD127 and CD40 [9, 10, 11]. Considering the difference in clinical outcomes observed in SCCs versus BCCs, we hypothesized that TAMs in these skin cancers had different phenotypic characteristics. To test this, we analyzed the expression of TAM-associated markers on BCC ($n = 9$) and SCC ($n = 13$) TAMs. Compared to BCC TAMs, SCC TAMs expressed higher levels of the TAM-associated markers including arginase-1, MMP-9, CD40 and CD127 (Fig. 2A). Traditionally, arginase-1 and MMP9 are M2-associated markers while CD40 and CD127 are M1-associated markers. Therefore, our results support the observation that macrophages in human cutaneous SCCs exhibit both M1- and M2-associated markers [17].

3.3. SCC- and BCC-conditioned media differentially drive polarization of human THP-1 cells

We and others have demonstrated that TAM polarization is profoundly affected by the tumor microenvironment [18, 19] Given that TAMs in human cutaneous SCCs and BCCs have different polarization states, we hypothesized that soluble factors in the tumor microenvironment lead to this difference. To test this, we generated SCC- and BCC-conditioned media to act as *ex vivo* models of the tumor microenvironments in SCCs and BCCs, respectively. THP-1 human monocytic cells were stimulated with SCC- versus BCC-conditioned media, and compared to control RPMI 1640 media. Their activation states were analyzed after 48 h. Compared to those cells stimulated with BCC-conditioned or control media, THP-1 cells stimulated with SCC-conditioned media showed an increase in TAM-like polarization,

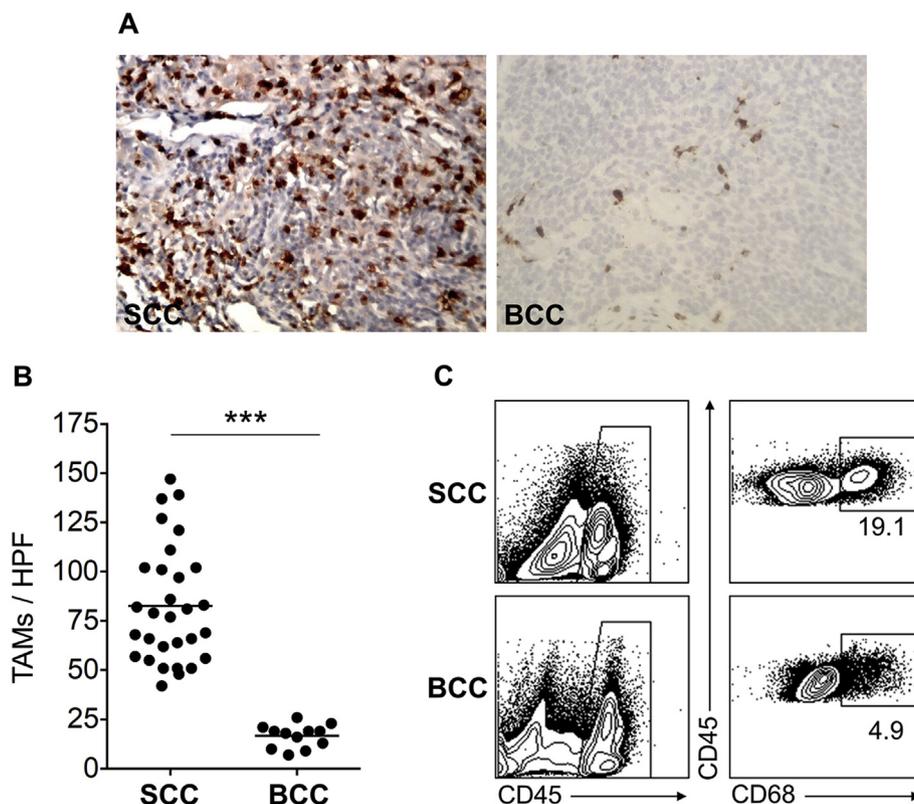


Fig. 1. Human cutaneous SCCs have a greater TAM density than BCCs. A, Representative histologic images of SCC versus BCC comparison staining with anti-human CD68 antibody (40X). B, Number of TAMs per 40x high power fields were counted. ***: $P < 0.001$. SCC: $n = 5$; BCC: $n = 6$. C, CD45⁺CD68⁺ TAMs in human SCCs ($n = 6$) and BCCs ($n = 6$) analyzed by flow cytometry. Data are representative of at least four independent experiments.

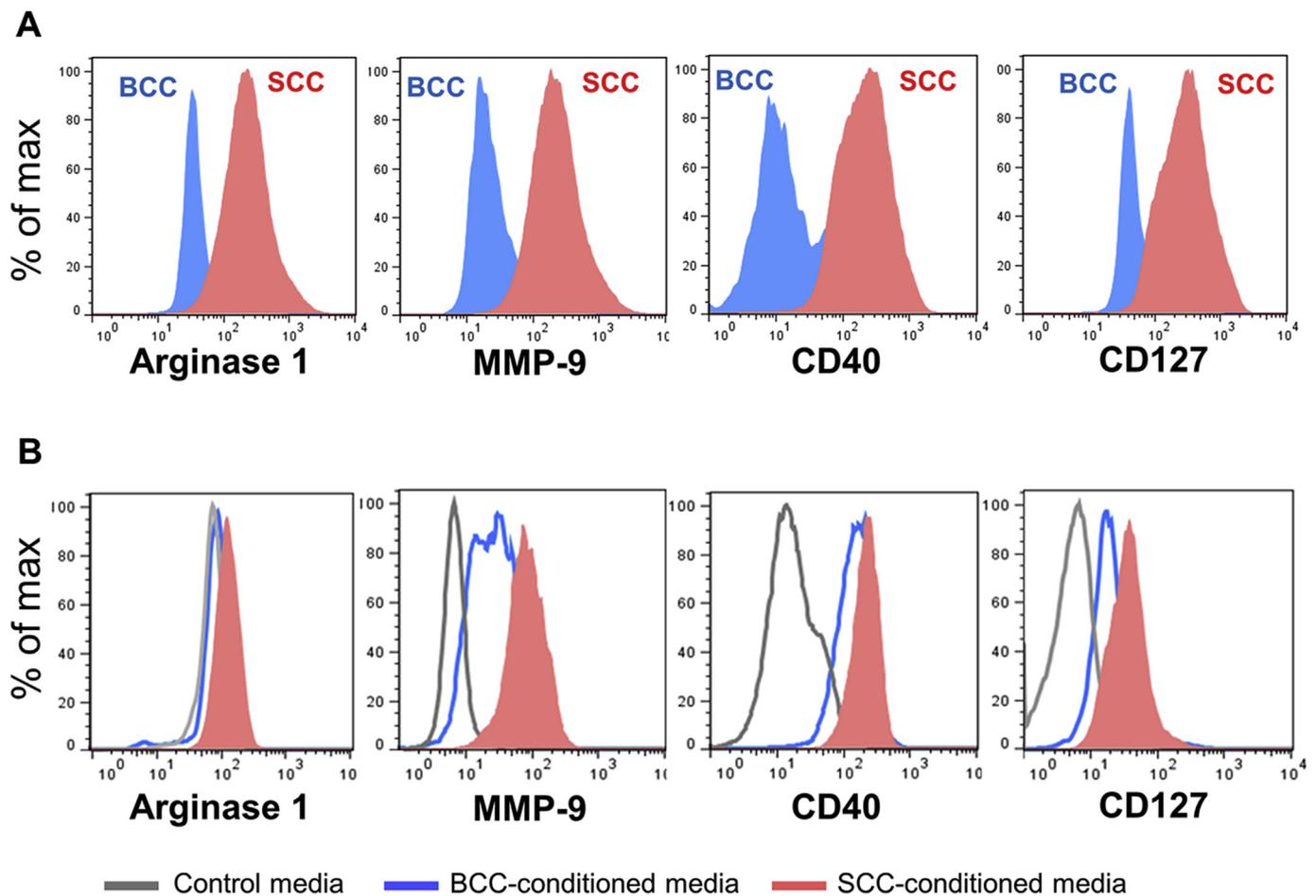


Fig. 2. TAMs in SCCs versus BCCs or THP-1 cells incubated with SCC- or BCC-conditioned media are different in their polarization states. A, Biopsy-proven human cutaneous SCCs ($n = 13$) and BCCs ($n = 9$) were dissociated by enzyme and the expression of arginase-1, MMP9, CD40, and CD127 on TAMs ($CD45 + CD68+$) were analyzed by flow cytometry. B: THP-1 cells were incubated with SCC- or BCC-conditioned media, or control RPMI media for 48 h. Cells were then collected for flow cytometry analysis of arginase-1, MMP9, CD40, and CD127. Data are representative of at least three independent experiments.

which was demonstrated by higher levels of arginase-1, MMP-9, CD40 and CD127 (Fig. 2B). The differences in MMP-9 and CD127 were statistically significant (Supplementary Figure 3, $P = 0.03$, $P = 0.02$, respectively). In THP-1 cells stimulated with SCC-conditioned media, arginase-1 trended upward compared to those stimulated with BCC-conditioned media ($P = 0.15$). These results suggest that soluble factor(s) present in the tumor microenvironment are able to partially drive TAM polarization through contact-independent, paracrine communication; however, cell-to-cell contact may play a more important role in TAM polarization.

3.4. Lactic acid is sufficient to polarize human macrophages to a TAM-like state

Using murine tumor models, we demonstrated that tumor-derived lactic acid induces TAM-like polarization in macrophages in a hypoxia-inducible factor 1 α (HIF1 α)-dependent manner [18, 20]. In this study, we asked whether human cutaneous SCCs and BCCs have different concentrations of lactic acid as a functional difference in their ability to polarize macrophages. Lactic acid concentrations in primary human SCCs and BCCs were measured and revealed that SCCs have an approximately four-fold higher concentration of lactic acid than BCCs (Fig. 3A). We next asked whether lactic acid could be a determinant of TAM polarization in SCCs and BCCs. To address this, we stimulated THP-1 cells with lactic acid. Compared to the control media (0 mM lactic acid), 25 mM lactic

acid, a concentration observed in numerous cancers [18], significantly induced the expression of the TAM-associated markers arginase-1, MMP-9, CD40, and CD127 (Fig. 3B, C, D, E); this phenotypic change was consistent with what we observed when THP-1 cells were stimulated with SCC-conditioned media (Fig. 2B). Taken together, these data demonstrate that human cutaneous SCCs have higher level of lactic acid than BCCs and that lactic acid is sufficient to induce a heterogeneous polarization state of TAMs.

4. Discussion

A majority of malignancies are primarily initiated by genetic alterations or mutations, but their subsequent development and behaviors requires support from the surrounding cells, such as TAMs [9, 10, 21]. In this study, we found that cutaneous SCCs have an approximately 6-fold higher TAM density than BCCs. Given that SCCs are clinically more aggressive than BCCs, this finding highlights the observation that a greater density of TAMs is associated with tumors with a worse prognosis. We do note that there exists a subset of immature myeloid dendritic cells (mDCs) that are CD68-positive and may impact the interpretation of the TAM densities [22, 23]; however, mDCs are found in both cutaneous SCCs and BCCs and we do not expect that the relative difference between the two tumor sets would be changed [24, 25]. Interestingly, TAMs in cutaneous SCCs not only express arginase-1 and MMP-9, two functional M2-associated markers, but also highly

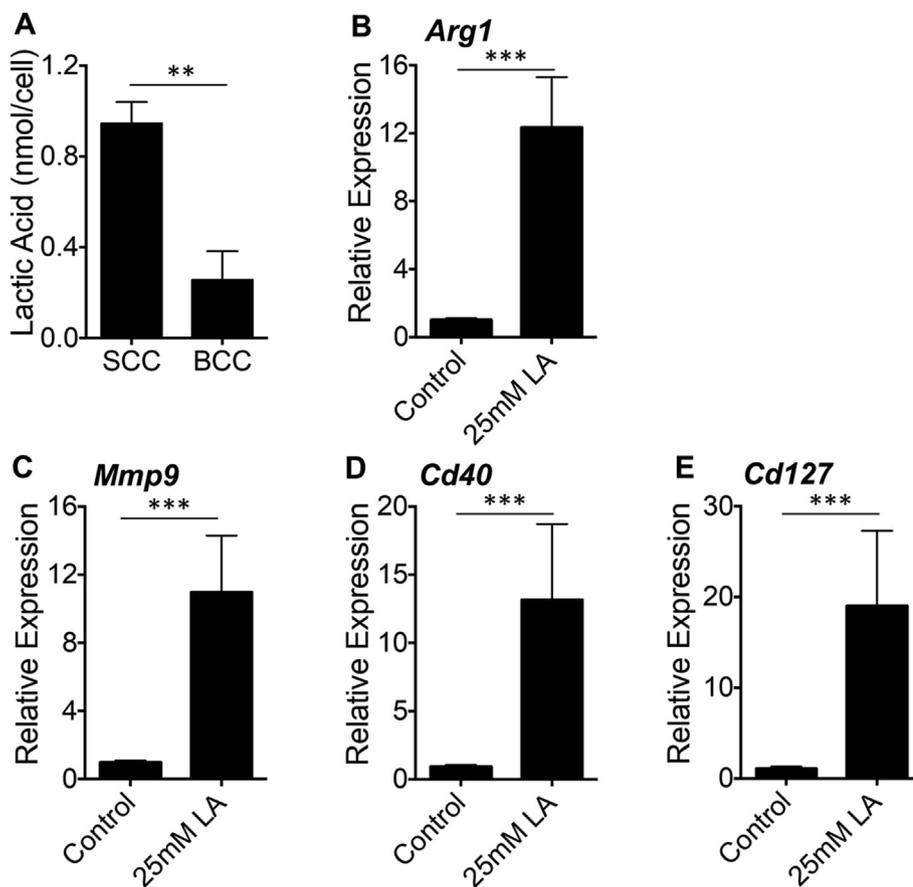


Fig. 3. Human cutaneous SCCs have a greater concentration of lactic acid than BCCs and lactic acid is sufficient to induce arginase-1, MMP9, CD40, and CD127 in THP-1 cells. A, Biopsy-proven SCCs and BCCs were digested as described. 500,000 live cells were used to measure intracellular concentrations of lactic acid using Lactate Colorimetric Assay Kit II. Data were showed in nmol/cell (means \pm S.E.M.). **: $P < 0.01$. SCCs: $n = 5$; BCCs: $n = 5$. B–F, Gene expression analysis (B, *Arg1*; C, *Mmp9*; D, *Cd40*; E, *Cd127*) on THP-1 cells stimulated with 0 mM (control) or 25 mM lactic acid was performed by qPCR and relative expressions were showed with means \pm S.E.M. ***: $P < 0.001$. $n = 9$. Data are representative of at least three independent experiments.

up-regulate the M1-associated markers CD40 and CD127. Indeed, macrophages with combinations of M1- and M2-associated markers within human skin cancers have been described by others [17, 26], indicating that macrophages have dynamic polarization states in response to different microenvironments within tumors. Acidic and/or hypoxic microenvironment induced by vigorous glycolysis of tumor cells has been demonstrated to promote M2-like macrophage polarization [19, 27]. Due to a higher growth rate, SCCs may have more areas with an acidic and/or hypoxic microenvironment than BCCs, leading to more TAM-like macrophages polarization. TAM-derived arginase-1 is a key enzyme that is involved in the production of polyamines, which can be in turn utilized by tumor cells for cellular proliferation and growth [28]. MMP-9 contributes to many aspects of tumor development including angiogenesis, local tissue invasion, and metastasis [29]. Therefore, the difference in levels of arginase-1 and MMP-9 produced by TAMs in SCCs and BCCs may be one important reason for the distinct clinical behavior of SCCs and BCCs.

Remarkably, the *in vivo* immunophenotype of TAMs in human cutaneous SCCs and BCCs was partially reproduced by *in vitro* stimulation of a human monocytic cell line (THP-1) with SCC- and BCC-conditioned media. This suggests that monocytes recruited to tumors are exposed to many soluble factor(s) in the tumor microenvironment, leading to TAM polarization through contact-independent, paracrine communication. Given the heterogeneity and complexity of the tumors, the source of such signaling factors could be derived from a variety of cells in the microenvironment, including tumor cells, Th1/Th2 cells, other immune cells and fibroblasts. Here we demonstrate that cutaneous SCCs have a higher concentration of lactic acid than BCCs and, in line with our findings shown in mouse tumor models [18], lactic acid stimulates THP-1 cells to upregulate TAM-associated molecules including arginase-1 and MMP-9 in a dose-dependent manner. Interestingly, we also observed a

significant upregulation of more classical M1-like markers including CD40 and CD127. These data suggest that tumor-derived lactic acid is important in inducing a heterogeneous polarization state of TAMs within the tumor microenvironment. However, our study does not exclude other soluble factors that might also act through a paracrine manner to induce an intricate phenotype in TAMs that involves both M1- and M2-like activation.

Our findings demonstrate that TAMs in human cutaneous SCCs and BCCs have different densities and functional immunophenotypes, potentially explaining their distinct clinical behaviors. Soluble factor(s) including tumor-derived lactic acid might be the key in determining TAM activation state within the tumor microenvironment. Our data provides the first evidence, to our knowledge, of different TAM density and polarization states in human cutaneous SCCs *versus* BCCs. We believe a better understanding of TAM polarization in SCCs *versus* BCCs will provide novel insights for therapies targeting keratinocyte carcinomas.

Declarations

Author contribution statement

Xiaodong Jiang, Nika Cyrus: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Mike Wang, Diana Yanez, Richard Lacher, Anne Marie Rhebergen, Carolyn Brokowski, Anjela Galan: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Samuel Book: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Oscar Colegio: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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