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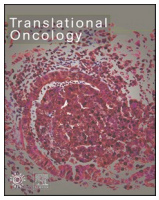

Activation of immune evasion machinery is a part of the process of malignant transformation of human cells

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ABSTRACT

Malignant transformation of human cells is associated with their re-programming which results in uncontrolled proliferation and in the same time biochemical activation of immunosuppressive pathways which form cancer immune evasion machinery. However, there is no conceptual understanding of whether immune evasion machinery pathways and expression of immune checkpoint proteins form a part of the process of malignant transformation or if they are triggered by T lymphocytes and natural killers (NK) attempting to attack cells which are undergoing or already underwent malignant transformation. To address this fundamental question, we performed experimental malignant transformation of BEAS-2B human bronchial epithelium cells and RC-124 non-malignant human kidney epithelial cells using bracken extracts containing carcinogenic alkaloid called ptaquiloside. This transformation led to a significant upregulation of cell proliferation velocity and in the same time led to a significant upregulation in expression of key immune checkpoint proteins – galectin-9, programmed death ligand 1 (PD-L1), indoleamine 2,3-dioxygenase (IDO1). Their increased expression levels were in line with upregulation of the levels and activities of HIF-1 transcription complex and transforming growth factor beta type 1 (TGF-β)-Smad3 signalling pathway. When co-cultured with T cells, transformed epithelial cells displayed much higher and more efficient immune evasion activity compared to original non-transformed cells. Therefore, this work resolved a very important scientific and clinical question and suggested that cancer immune evasion machinery is activated during malignant transformation of human cells regardless the presence of immune cells in microenvironment.

Introduction

Malignant transformation of human cells leads to their re-programming and is associated with inducing uncontrolled proliferation of these cells. There are several types of oncogene activation leading to malignant transformation [1]. The most common one is a mutation within a proto-oncogene which can cause a change in the protein structure, leading to an increase in protein (or an enzyme) activity or to its permanent activation [1]. One of such examples is permanent activation of H-Ras resulting from a mutation [2]. H-Ras is a small GTPase enzyme, named after "Harvey rat sarcoma virus", also known as transforming protein p21 [2,3]. H-Ras induces activation of both phosphatidylinositol-3 kinase (PI-3K) and MEK (mitogen-activated protein (MAP) and extracellular signal-regulating kinase (ERK) kinase) pathways and triggers cell proliferation [2,4]. H-Ras mutations are known to take place (and likely be a cause of malignant transformation) in a number of cancers, including the most dangerous types, such as pancreatic ductal adenocarcinoma and majority of digestive tract and lung cancers [5,6].

However, it is very important to understand whether this kind of re-

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programming is also responsible to induce cancer immune evasion machinery, or these networks are activated by the cells of immune system interacting with cancer cells, when they are trying to attack these cancer cells. Alternatively, the immune checkpoints and immune escape pathways may be activated during malignant transformation regardless the presence of the immune cells in microenvironment.

H-Ras mutations which cause its permanent activation are known to be induced by ptaquiloside, which is a plant alkaloid, highly present in fern called *Pteridium aquilinum* or bracken [7]. Bracken extracts and leaves containing ptaquiloside are known to cause H-Ras mutations leading to malignant transformation followed by development of cancers [7]. As such, ptaquiloside was reported to cause quite aggressive cancers of digestive tract (e. g. gastric and pancreatic cancers) [7].

Using ptaquiloside or bracken extracts is one of the ways to trigger natural malignant transformation of human cells rather than transfection with constitutively active H-Ras [3].

A number of *in vivo* studies were performed and it has become evident that ptaquiloside on its own and as a component of bracken extracts induces malignant transformation and causes various cancers [7–11]. It is however, unclear whether this transformation is associated with activation of cancer immune evasion machinery or the immune suppression pathways in cancer cells are triggered and induced by immune system.

Recent evidence clearly demonstrated that malignant cells operate immune evasion machinery, which contains immune checkpoint proteins and biochemical pathways controlling their expression [12]. For example, these could be proteins like galectin-9 and its receptor T cell immunoglobulin and mucin domain containing protein 3 (Tim-3), V-domain Ig suppressor of T cell activation (VISTA), which can act as both receptor and a ligand, programmed death ligand 1 (PD-L1) and others [13]. Also, these could be small molecular weight compounds, like amino acid L-kynurenine (produced from L-tryptophan with the help of enzyme called indoleamine-2,3-dioxygenase 1 (IDO1)) and some of its derivatives [14]. Expression of these immune checkpoint proteins can be induced through two major signalling pathways. These are transforming growth factor beta type 1 (TGF-β)-Smad3 as well as interferon gamma (IFN-γ) – Janus kinase (JAK)/signal transducer and activator of transcription 1 (STAT1) pathways [12,13,15].

Immune checkpoint proteins suppress anti-cancer activities of T lymphocytes and natural killer (NK) cells [12,16]. For example, galectin-9 can "opsonise" cytotoxic T cells binding to both Tim-3 and VISTA on their surface [17]. This affects their membrane potential and as a result leads to activation of granzyme B inside them which could trigger programmed cell death [18]. Actions of the immune checkpoint proteins like PD-L1 or VISTA prevent activation of anti-apoptotic machinery in immune cells and thus allow apoptosis to take place [13,19]. In T helpers, pre-opsonised with galectin-9, PD-L1, Tim-3 or VISTA prevent IL-2 production, required for activation of cytotoxic T cells [12, 13,20]. In addition, L-kynurenine acting as aryl hydrocarbon receptor (AhR) ligand [21,22] affects accumulation of hypoxia-inducible factor 1 (HIF-1) transcription complex, since both AhR and inducible alpha subunit of HIF-1 (HIF-1α) compete for aryl hydrocarbon receptor nuclear translocator (ARNT) also known as HIF-1β. This leads to exhaustion of T cells in hypoxic tumour microenvironment [14].

It is therefore unknown whether these immune evasion networks described above are induced by immune cells (since for example T cells clearly trigger immunosuppressive responses of cancer cells) or these pathways are enhanced and activated during the process of malignant transformation. And thus, we aimed to address this important question.

Here we report experimental malignant transformation of human epithelial cells using bracken extracts containing ptaquiloside. This transformation led to a significant upregulation of cell proliferation velocity and in the same time led to significant upregulation in expressions of key immune checkpoint proteins – galectin-9, PD-L1, IDO1. Their increased expression was in line with upregulation of the levels and activities of HIF-1 transcription complex and TGF-β-Smad3 signalling pathway. When co-cultured with T cells, transformed epithelial cells displayed much higher immune evasion activities compared to original non-transformed cells. These results resolve a very important scientific and clinical question and suggest that activation of cancer immune evasion machinery is a part of the process of malignant transformation of human cells regardless the presence of immune cells in microenvironment. These immune evasion networks display their activities as soon as the transformation occurs.

Materials and Methods

Materials

Media for cell culture, foetal bovine serum, supplements and basic laboratory chemicals were purchased from Sigma (Suffolk, UK). Microtiter plates for ELISA were obtained from Oxley Hughes Ltd (London, UK) and Nunc (Roskilde, Denmark). Rabbit antibodies against VISTA, galectin-9, granzyme B, phospho-S423/S425-Smad3, Smad4 and (tripartite motif containing 33) TRIM33 as well as goat anti-rabbit horseradish peroxidase (HRP) labelled secondary antibodies were purchased from Abcam (Cambridge, UK). Antibody against β-actin was purchased from Proteintech (Manchester, UK). Goat anti-mouse and anti-rabbit fluorescently-labelled dye secondary antibodies were obtained from Li-COR (Lincoln, Nebraska USA). Mouse anti-Smad3 antibody, ELISA-based assay kits for the detection of VISTA, galectin-9, PD-L1 and TGF-β as well as mouse anti-Smad3 antibody were purchased from Bio-Techne (R&D Systems, Abingdon, UK). Anti-Tim-3 mouse monoclonal antibody was described before [23]. All other chemicals used in this study were of the highest grade of purity and commercially available.

Cell lines

Cell lines used in this work were purchased from either European Collection of Cell Cultures, American Tissue Culture Collection or CLS Cell Lines Service GmbH. Cell lines were accompanied by authentication test certificates. BEAS-2B normal bronchial epithelium cells were cultured using DMEM medium supplemented with 10% foetal bovine serum, penicillin (50 IU/ml), and streptomycin sulfate (50 μg/ml). Jurkat T cells, RC-124 human kidney non-cancerous epithelial cells and HaCaT keratinocytes were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, penicillin (50 IU/ml), and streptomycin sulfate (50 μg/ml).

TALL-104 CD8-positive cytotoxic T lymphocytes, obtained from human acute lymphoblastic leukemia (TALL), were cultured according to the ATCC instructions. Briefly, ATCC-formulated Iscove's Modified Dulbecco's Medium was used. To make the complete growth medium we added 100 units/ml recombinant human IL-2; 2.5 μg/ml human albumin; 0.5 μg/ml D-mannitol and foetal bovine serum to a final concentration of 20% as well as penicillin (50 IU/ml), and streptomycin sulfate (50 μg/ml).

Bracken (Pteridium aquilinum) extracts

Bracken leaves from young and fully developed ferns collected in spring (May) time were frozen at -80°C and then homogenised in bidistilled water at room temperature in the ratio 1 g of leave tissue and 2 ml of water based on the principle described before [10]. Homogenates were centrifuged, immediately aliquoted and stored at - 20°C. Natural (not synthetic) ptaquiloside-containing bracken extracts are known to be highly active in inducing malignant transformation [7–11] and thus we used naturally occurring extract containing this compound and being highly active in permanently activating H-Ras. Extracts were added to the cells in the ratio 1:1000 and the medium was changed daily (refreshing the extract daily, since ptaquiloside is not stable for a long time). Ptaquiloside concentration was measured in the extracts as

described before using thin-layer chromatography approach [24,25] and was found to be 3.091 mg/ml (9.273 mg/g of the bracken tissue). Therefore, when extracts were added to the cells (1:1000 dilution), ptaquiloside concentration was 3.091 μg/ml, which is similar to the one reported for cell culture studies [11].

Western blot analysis

Levels of VISTA, granzyme B, galectin-9, Tim-3, phospho-S423/S425 Smad-3, Smad4 and TRIM33 were measured by Western blot and compared to the amounts of β-actin (protein loading control), as described previously [13,26].

Li-Cor goat secondary antibodies conjugated with infrared fluorescent dyes, were used as described in the manufacturer's protocol for visualisation of specific target proteins (Li-Cor Odyssey imaging system was employed). Western blot data were quantitatively analysed using Image Studio software and values were subsequently normalised against those of β-actin.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described recently [15]. Briefly, 5×10^6 cells subjected to the study were used for immunoprecipitation. Cross-linking was done using 1.42% formaldehyde followed by quenching with 125 mM glycine for 5 min. Cells were then washed twice with PBS and subjected to ChIP according to ChIP-IT high sensitivity kit (Active Motif) protocol. Immunoprecipitation was performed using mouse monoclonal anti-Smad3 antibody (R&D Systems, Abingdon, UK), and IgG isotype control antibody was used for a negative control IP. The Smad3 epitope recognised by this antibody does not overlap with DNA and co-activator binding sites of this protein. Immunoprecipitated DNA was then purified and subjected to quantitative real-time PCR (qRT-PCR) which was performed as outlined below. The following primers were designed using NCBI Primer-Blast primer designing tool: galectin-9 forward: 5'-TCTTGCAAAATATTCCCCTCTACAT-3'; reverse – 5'-AAATAATGG GGCTGGGCATTG-3'; IDO1 forward: 5'- GGAACGGGCAACTTGGTTT CT-3' and reverse: 5'- TCTAACTGTACCTGACTGCGG-3'; VISTA forward – 5'- GCCTACCACATACCAAGCCC-3' and reverse: 5'- ATCGGCAGTT-TAAAGCCCGT-3'; PD-L1 forward: 5'-CTAGAAGTTCAGCGCGGGAT-3' and reverse: 5'- GGCTGCGGAAGCCTATTCTA-3'. These primers allow amplification of the fragments of promoter regions of corresponding genes, which surround Smad3-binding sites.

qRT-PCR analysis

To detect HIF-1α, TGF-β, galectin-9 and IDO1 mRNA levels, we used qRT-PCR. Total RNA was isolated using a GenElute™ mammalian total RNA preparation kit (Sigma-Aldrich) according to the manufacturer's protocol, followed by reverse transcriptase–polymerase chain reaction (RT-PCR) of a target protein mRNA (also performed according to the manufacturer's protocol). This was followed by qRT-PCR. The following primers were used. HIF-1α: forward – 5′-CTCAAAGTCGGACAGCCTCA-3′, reverse – 5′-CCCTGCAGTAGGTTTCTGCT-3′; TGF-β: forward – 5'-TACCTGAACCCGTGTTGCTCTC-3', reverse – GTTGCTGAGGTATCGC-CAGGAA-3'; galectin-9: forward – 5′-CTTTCATCACCACCATTCTG-3′, reverse – 5′-ATGTGGAACCTCTGAGCACTG-3′; actin: forward – 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, reverse – 5′-CTAGAAGC ATTTGCGGTCGACGATGGAGGG-3′. Reactions were performed using a LightCycler® 480 qRT-PCR machine and SYBR Green I Master kit (Roche, Burgess Hill, UK). The assay was performed according to the manufacturer's protocol. Values representing VISTA mRNA levels were normalised against those of β-actin.

On-cell Western analysis

Cell surface levels of VISTA and PD-L1 proteins were analysed using on-cell Western analysis performed using a Li-COR Odyssey imager and the assay was performed in line with manufacturer's recommendations as previously described [13,26].

Enzyme-linked immunosorbent assays (ELISAs)

Secreted galectin-9, IL-2 and TGF-β were measured in cell culture media and PD-L1 was measured in cell lysates, by ELISA using R&D Systems kits (see Materials section) according to manufacturer's protocols.

To study recruitment of co-activators Smad4 and TRIM33 by Smad3 we used recently described ELISA-based assay [15] where we applied mouse anti-Smad3 antibody (R&D Systems) as capture one. Briefly, plates were coated with this antibody (1:500) overnight followed by blocking with 1% BSA (dissolved in phosphate buffered saline, PBS). Then cell lysates were applied and incubated for 2 h followed by 5 times washing with TBS (50 mM Tris–HCl, 140 mM NaCl, pH 7.3) containing 0.1% Tween 20 (TBST). Rabbit anti-TRIM33 or anti-Smad4 antibodies were used (1:1000, 2h incubation) to detect these proteins interacted with Smad3. Finally, the plates were washed 5 times with TBST and horseradish peroxidase (HRP) labelled goat anti-rabbit antibody was applied for 1 h at room temperature. The plates were washed 5 times with TBST followed by visualisation through the peroxidase reaction (ortho-phenylenediamine/H_2O_2). The reactions were quenched after 10 min with an equal volume of 1 M H_2SO_4 and the colour development was measured using a microplate reader as the absorbance at 492 nm.

Phospho-S2448 and phospho-S423/S425-Smad3 in cell lysates were also detected by ELISA as previously described [27]. Briefly, plates were coated with mouse anti-mTOR antibody or mouse anti-Smad3 antibody. The plates were then blocked with 1 % BSA. Cell lysates were added to the wells and kept at room temperature for at least 2 h. After extensive washing with TBST buffer, anti-phospho-S2448 mTOR (or anti-phospho-S423/S425-Smad3) antibody was added and incubated for 2 h at room temperature. The plates were then washed five times with TBST buffer and incubated with 1:1,000 HRP-labeled goat anti-rabbit IgG in TBST buffer and, after extensive washing with TBST, bound secondary antibodies were detected by the peroxidase reaction (ortho-phenylenediamine/H_2O_2). Reactions were quenched after 10 min with an equal volume of 1 M H_2SO_4 and colour development was measured in a microplate reader (absorbance at 492 nm).

Analysis of HIF-1 DNA-binding activity

HIF-1 DNA-binding activity was measured by the recently described method [26]. Briefly, 96-well Maxisorp™ microtitre plates were coated with streptavidin and blocked with BSA. A volume of 2 pmol/well biotinylated 2HRE (HRE – hypoxia response element) containing oligonucleotide was immobilised by 1 h incubation at room temperature. The plate was then washed five times with TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20), followed by 1 h incubation with cell lysate at room temperature. The plate was again washed with TBST buffer and mouse anti-HIF-1α antibody (1:1 000 in TBS with 2% BSA) was added. After 1 h of incubation at room temperature the plate was washed with TBST buffer and then incubated with Li-Cor goat anti-mouse secondary antibody labelled with infrared fluorescent dye. After extensive washing with TBST, the plate was scanned using a Li-Cor fluoroimager.

Granzyme B activity assays

Granzyme B activity in cell lysates was measured using a fluorometric assay [14] based on the ability of the enzyme to cleave the fluorogenic substrate Ac-IEPD-AFC (Sigma-Aldrich). The in-cell activity of granzyme B (granzyme B catalytic activity in living cells) was measured as described previously (Yasinska et al., 2020) by incubating living cells with 150 μM Ac-IEPD-AFC (granzyme B substrate) for 1 h at 37°C in

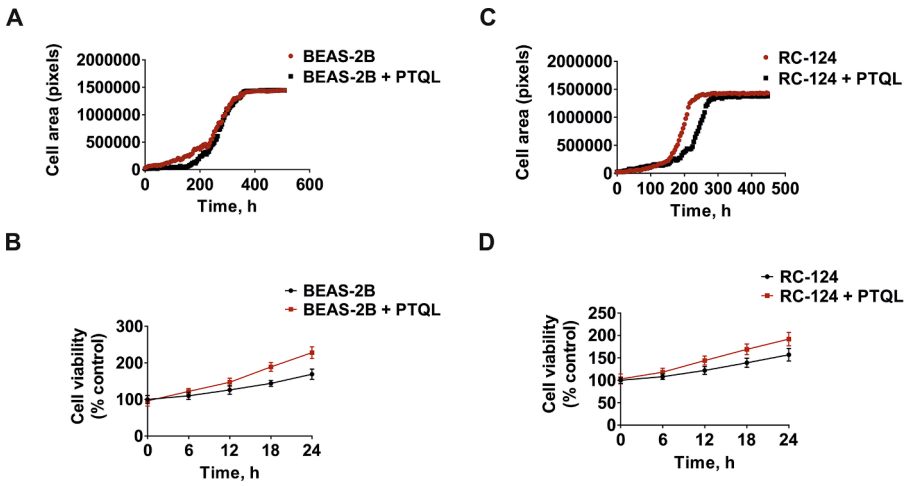

Figure 1. Malignant transformation of BEAS-2B with bracken extracts. (A) BEAS-2B cells were cultured for 10 days in the absence or presence of ptaquiloside containing bracken extracts (abbreviated as PTQL) followed by 5 days of culturing in PTQL-free medium. Cell proliferation was observed in real time using live cell imaging (see Materials and Methods). (B) Upon completion of the process (10 days with PTQL followed by 14 days in PTQL free medium) cell proliferation was analysed during 24 h using MTS test in comparison with BEAS-2B cells which were cultured in PTQL free medium for the whole period of time (24 days – control BEAS-2B cells). (C) RC-124 cells were cultured exactly as described above in the legend for panel A. (D) As for BEAS-2B cells, upon completion of the process (10 days with PTQL followed by 14 days in PTQL free medium) cell proliferation was analysed during 24 h using MTS test in comparison with RC-124 cells which were cultured in PTQL free medium for the whole period of time (24 days – control RC-124 cells). Panels A and C – in each case the graph shows one representative growth curve out of three which gave similar results. Panels B and D: data are mean values ± SEM of 4 independent experiments.

sterile PBS. Total cell fluorescence was measured in living cells using the excitation and emission wavelengths recommended by the Ac-IEPD-AFC manufacturer (Sigma) protocol. An equal number of cells that were not exposed to the granzyme B substrate were used as controls.

IDO1 activity measurement (detection of enzymatic generation of N-formylkynurenine)

IDO1 activity (or enzymatic conversion of L-Trp into N-formylkynurenine), which was then further converted into LKU, was measured using a previously described method [14,28] with minor modifications. Briefly, the cell or tissue lysates were added to the reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5), 20 mM ascorbate, 100μg/mL catalase and 2 mM L-Trp. The reaction was carried out at 37°C for 60 min and terminated by adding 10 μL of 30% (v/v) trichloroacetic acid to 160 μL of sample. Further operations were performed as previously described.

Detection of LKU

LKU was measured based on its ability to react with 4-dimethylaminobenzaldehyde [14]. Briefly, we took 160 μL of cell culture medium or blood plasma was added 10 μL 30% (v/v) trichloroacetic acid to each sample, and incubated the samples for 30 min at 50°C in order to hydrolyze N-formylkynurenine to LKU. The samples were then centrifuged at 3000 g for 10 min. 100 μl of supernatants were transferred to wells of a 96-well flat-bottom plate and mixed with 100 μL of freshly prepared Ehrlich's reagent (1.2% w/v 4-dimethylaminobenzaldehyde in glacial acetic acid) followed for 10 min incubation at room temperature. Absorbance was measured using a microplate reader at 492 nm.

Detection of PI-3K activity

PI-3K activity was detected using spectrophotometric method based on detection of substrate (PI-4,5-diphosphate) phosphorylation, as previously described [29].

Fluorescent microscopy and flow cytometry

Cells were cultured overnight on 12 mm cover slips in 24-well plates and then fixed/permeabilised for 20 min with ice-cold MeOH/acetone (1:1) [18]. Cover slips were blocked for 1h at RT with 10% goat serum in PBS. Cells were stained with anti-CK8 antibody overnight at 4°C. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The preparations were analysed using Olympus microscope as described previously [18]. Images were collected and analysed using proprietary image acquisition software.

Flow cytometry experiments were performed as previously described [18]. Briefly, cells were collected and fixed with 2% paraformaldehyde and permeabilised with 0.1% TX-100. Cells were stained with mouse anti-CK antibody Mean fluorescence intensity of stained cells was measured and analyzed using a FACSCalibur analyser and the CEllQuest Pro Software (Becton Dickinson, USA).

Live cell imaging

The effects of bracken extracts on cell proliferation and morphology of BEAS-2B and RC-124 cells was monitored and analysed using a Cell-IQ® v2 MLF integrated platform (CM Technologies Oy, Tampere Finland) for continuous real-time live cell microscopy. The instrument was equipped with a phase-contrast microscope (Nikon CFI Achromat phase-contrast objective with 10x magnification) and a camera. BEAS-2B and RC-124 were allowed to adhere to the bottom of 6-well plates (day 0) in a corresponding medium. Next day cells were placed into medium containing bracken extracts for 10 days as for all other experiments. Then the cells were washed and incubated for further 5 days without bracken extracts. In control wells, cells were incubated without bracken extracts during the whole experiment and culture medium changes were performed as described above. Phase contrast images were automatically captured every 4 hours during 15 days. Captured images were analysed by AnalyserTM (CellActivision) software.

Cell viability assay

Cell viability was measured using an MTS assay kit (Promega) according to the manufacturer's protocol.

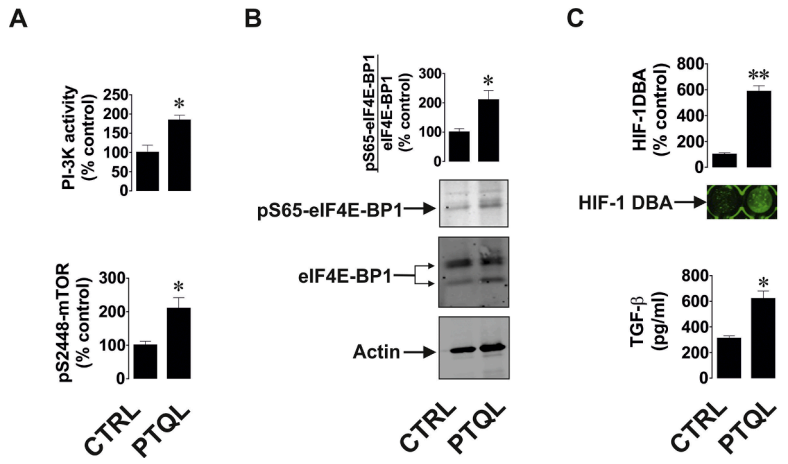

Figure 2. Malignant transformation is associated with permanent activation of PI-3K/mTOR pathway and HIF-1/TGF-β axis. BEAS-2B cells were cultured for 10 days in the absence or presence of ptaquiloside containing bracken extracts (PTQL) followed by 14 days of culturing in PTQL-free medium. Upon completion of the whole process, we detected: (A) PI-3K activity and activating phosphorylation of mTOR, (B) mTOR-dependent phosphorylation of eIF4E-BP1, and (C) HIF-1 DNA-binding activity as well as the levels of secreted TGF-β. Images are from one experiment representative of 4 which gave similar results. Quantitative data are mean values ± SEM of 4 independent experiments.

Statistical analysis

Each experiment was performed at least three times and statistical analysis, was conducted using a two-tailed Student's *t*-test. Statistical probabilities (p) were expressed as * when $p<0.05$; **, $p<0.01$ and *** when $p<0.001$

Results

Malignant transformation is associated with activation of immune evasion pathways regardless the presence of immune cells in microenvironment

Bracken extracts containing ptaquiloside were used to trigger natural malignant transformation of human epithelial cells. We used BEAS-2B

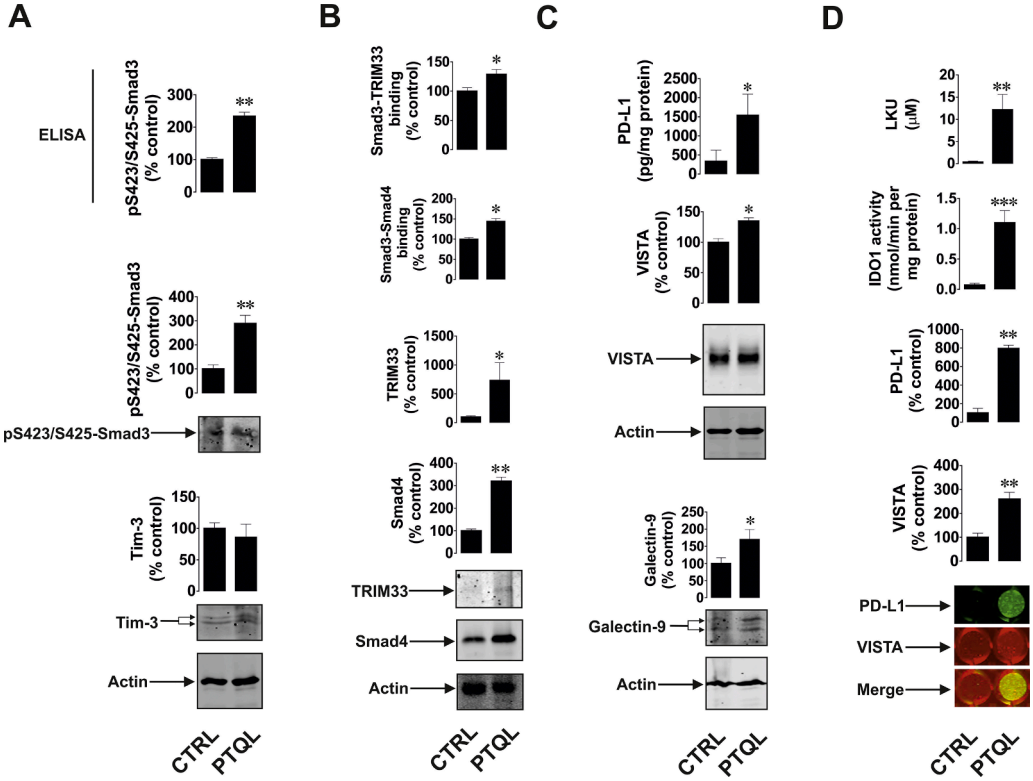

Figure 3. Malignant transformation leads to activation of immune evasion machinery. BEAS-2B cells were transformed as described above. Upon completion of the whole process, we analysed: (A) phospho-S423/S425-Smad3 levels were assessed by ELISA and Western blot, Tim-3 levels were detected by Western analysis; (B) levels of Smad4 and TRIM33 were analysed by Western blot and their recruitment to Smad-3 was measured by ELISA as outlined in Materials and Methods. (C) Levels of galectin-9 and VISTA were measured by Western blot analysis and PD-L1 was quantitated using ELISA. (D) Surface presence of VISTA and PD-L1 was analysed by on-cell Western; IDO1 activity and LKU release were analysed using biochemical assays described in Materials and Methods. Images are from one experiment representative of 4 which gave similar results. Quantitative data are mean values ± SEM of 4 independent experiments.

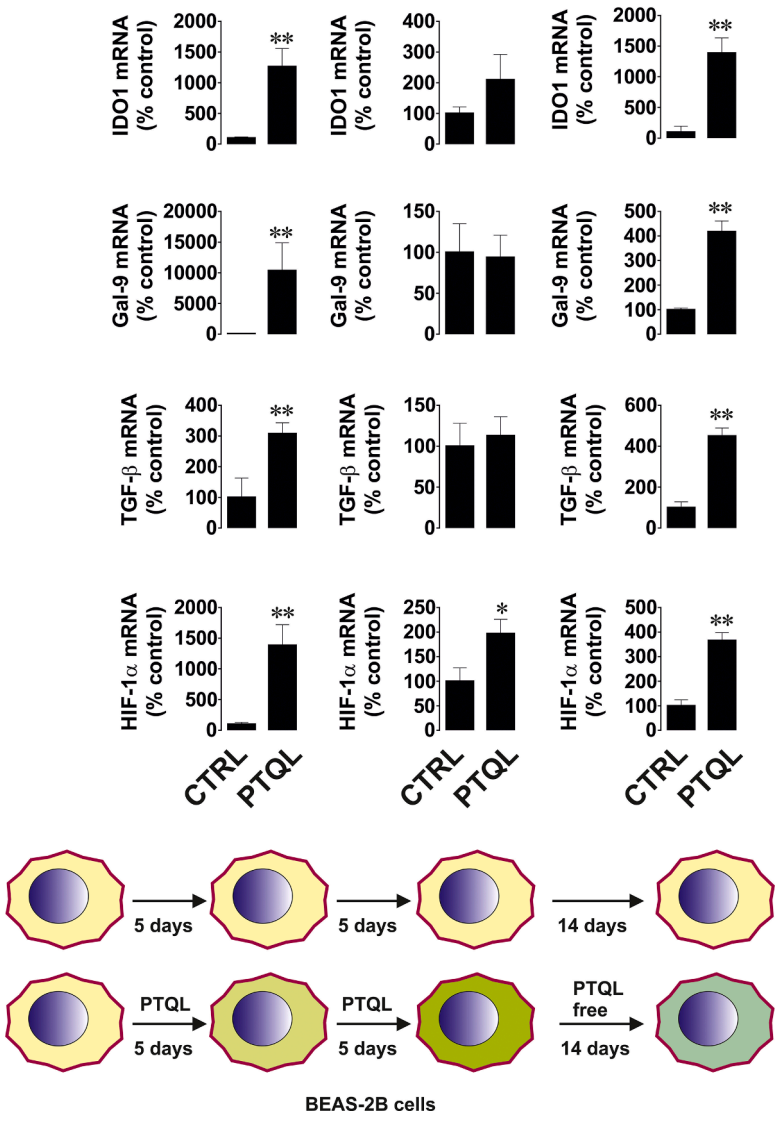

Figure 4. Malignant transformation leads to permanent upregulation of HIF-1α, TGF-β, galectin-9 and IDO1 mRNA levels in BEAS-2B cells. BEAS-2B cells were transformed as described above. After 5th and 10th days of culturing the cells in the absence or presence of PTQL and after day 14 of culturing them in PTQL-free medium we measured mRNA levels of HIF-1α, TGF-β, galectin-9 and IDO1. Data are mean values ± SEM of 4 independent experiments.

bronchial epithelium cells as they are known to be an excellent model for malignant transformation studies for several decades [3].

Bracken extracts permanently activate cell proliferation pathways

Cells were cultured for 10 days in the presence of bracken extract (dilution 1:1000). Bracken extract (ptaquiloside) containing medium was changed every day. Cells were split upon reaching 90 % confluence. Upon completion of 10 day exposure to bracken extracts, cells were cultured in normal medium (bracken extract (ptaquiloside) free) for 14 days and the investigations were conducted. In parallel, we cultured BEAS-2B cells in exactly the same way but in the absence of bracken extracts.

First of all, it was clear that when bracken extracts were present, this slowed cell proliferation which was monitored using live cell imaging. However, later on (Fig. 1A) the process has caught up and went much quicker (see Supplementary videos 1 and 2). Upon completion of the alteration process, transformed cells were proliferating much quicker compared to original non-transformed cells (Fig. 1B).

To verify the obtained results, we repeated the transformation experiment using RC-124 human kidney non-malignant cells performing exactly the same treatments for the same periods of time as described for BEAS-2B. Proliferation wise we observed the same pattern as in BEAS-2B: in the first instance – delay in proliferation, then upregulation of this process (Fig. 1 C and D, and Supplementary videos 3 and 4).

H-Ras activates PI-3K *via* interaction with this enzyme [2]. We found that PI-3K activity was significantly upregulated in transformed BEAS-2B cells and the same applied for the levels of phospho-S2448 mTOR activated by PI-3K pathway (Fig. 2A). Respectively, mTOR activity was also significantly higher in transformed cells as seen from phosphorylation of S65 residue of mTOR substrate eIF4E-BP (Fig. 2B). As a result, the DNA-binding activity of HIF-1 transcription complex was increased, which resulted in significant upregulation of TGF-β levels (Fig. 2C).

Malignant transformation is associated with activation of immune evasion machinery

TGF-β displays autocrine and paracrine activities, which resulted in constitutive upregulation of Smad3 phosphorylation levels in transformed BEAS-2B cells as measured by both Western blot and ELISA methods (Fig. 3A). Tim-3 levels remained unchanged in transformed

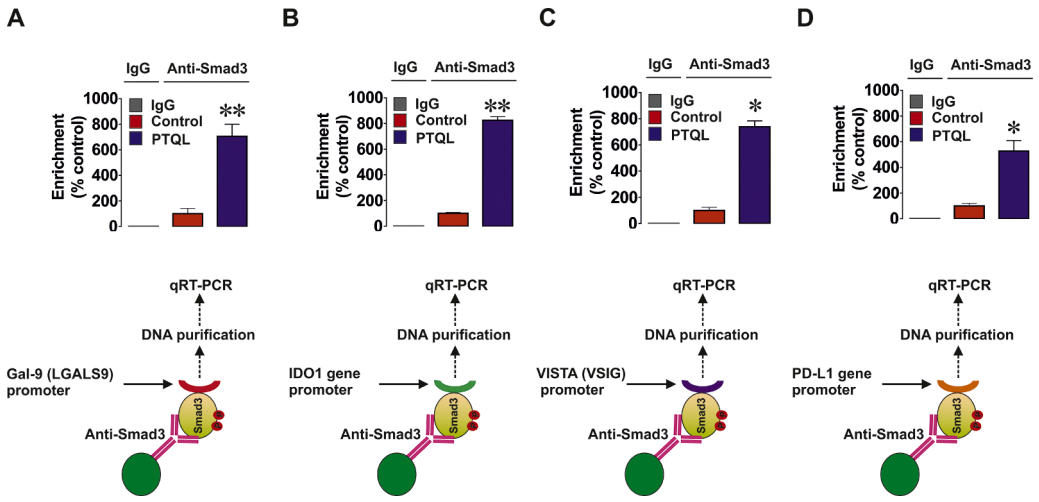

Figure 5. Smad3 induces expressions of galectin-9, IDO1, VISTA and PD-L1 on genomic level. Transformed and non-transformed BEAS-2B cells were subjected to ChIP followed by qRT-PCR. Smad3 was immunoprecipitated and presence of fragments of promoter regions of (A) galectin-9, (B) IDO1, (C) VISTA and (D) PD-L1 genes was analysed. Data are mean values ± SEM of 4 independent experiments.

cells compared to non-treated cells (Fig. 3A). Importantly, levels of Smad3 co-activator proteins, Smad4 and TRIM33, were significantly higher in transformed cells compared to controls. Respectively, ELISA-based recruitment assays showed clear upregulation of Smad3-Smad4 and Smad3-TRIM33 interactions in transformed cells compared to non-treated BEAS-2B cells.

As a result, expression levels of PD-L1, VISTA and galectin-9 were significantly upregulated by transformation (Fig. 3C). However, the levels of PD-L1 which was translocated onto the cell surface were much higher in transformed cells. In the same time, only marginal change in cell surface presence was observed for VISTA (Fig. 3D). It is important to mention that in control cells, VISTA levels on the cell surface were barely detectable despite very high expression levels of this protein. This suggests that VISTA has some intracellular function which remains to be studied. In the same time, IDO1 activity in transformed cells and respectively LKU release were highly upregulated in transformed cells, compared to those cultured in the absence of bracken extracts (Fig. 3D).

Transcriptions of key genes (mRNA levels of several proteins shown in the Fig. 2 and Fig. 3) were assessed using qRT-PCR during the process of transformation. Tests were done on the day 5 and day 10 of exposure to bracken extracts and day 14 post exposure. In parallel we tested control BEAS-2B cells which were cultured under the same conditions except presence of bracken extract in the medium. We found that HIF-1α, TGF-β, galectin-9 and IDO1 mRNA levels were highly upregulated on the day 5 of exposure and was in all cases (except HIF-1α – this was still upregulated) brought back to the control levels on the day 10. However, on the day 14 post exposure, all tested mRNAs were highly upregulated in cells treated with bracken extracts compared to non-treated cells (Fig. 4).

Then it was aimed to confirm if the immune checkpoint proteins studied were upregulated through Smad3. We performed ChIP followed by qRT-PCR and found that Smad3 was binding to promoter regions of galectin-9, IDO1, VISTA and PD-L1 encoding genes. The levels of enrichment were significantly higher in the cells which underwent treatment with bracken extracts (containing ptaquiloside, Fig. 5). Analysis was conducted 14 days after completion of 10-day treatment with bracken extracts.

Taken together, these results show upregulation of the immune checkpoint proteins during malignant transformation regardless the presence of immune cells in microenvironment. Also, this malignant transformation effect achieved, seemed to be irreversible since it was preserved permanently upon completion of the transformation process. When transformed cells were cultured, they preserved their properties described above even after freezing and thawing.

Malignant transformation leads to development of immunosuppressive and growth potentials

Transformed cells obtained using 10-day exposure to bracken extracts followed by 14 days of culturing in extract free medium were co-cultured with TALL-104 cytotoxic T cells for 16 h followed by detection of granzyme B in BEAS-2B cells by Western blot analysis and granzyme B catalytic activity in these cells. In parallel, we co-cultured control (non-treated BEAS-2B) cells with TALL-104 cells and subjected them to the same tests. Cells were co-cultured in the ratio 1:1. We found that both types of BEAS-2B cells at rest did not express granzyme B protein and did not display any granzyme B activity (Fig. 6A). However, when co-cultured with TALL-104 cells, much higher levels of granzyme B were injected into control BEAS-2B cells compared to transformed cells as seen from both Western blot and granzyme B activity assays (Fig. 6A). Respectively, viability of TALL-104 cells exposed to control BEAS-2B cells was significantly higher than the one of those transformed using bracken extracts (as measured by MTS test; Fig. 6B, upper panel). In the same time, T lymphocytes significantly reduced viability of control BEAS-2B cells and did not affect the one of transformed cells suggesting that transformed cells display higher immunosuppressive activity compared to the control cells (Fig. 6B, lower panel).

When co-cultured in the ratio 1:1 for 16 h with Jurkat T (display T helper properties) or TALL-104 cells both, control and transformed BEAS-2B cells suppressed IL-2 production by Jurkat T cells (Fig. 6 C). In the same time, only transformed BEAS-2B cells led to significant upregulation of TGF-β production when co-cultured with both Jurkat T or TALL-104 cells (Fig. 6 C). This is in line with observations shown in the Fig. 6, where it was shown that transformed BEAS-2B cells reduced viability of T cells. And dying/stressed T cells are known to release TGF-β. On the other hand, transformed BEAS-2B cells also could have reacted to the presence of T cells by increased TGF-β production.

Finally, both types of T cells studied (Jurkat T and TALL-104) triggered galectin-9 secretion by BEAS-2B cells, phenomenon, which we have recently reported [13]. In both cases, transformed BEAS-2B released significantly higher levels of galectin-9 (Fig. 6 C).

We looked if any of galectin-9 receptors expressed by BEAS-2B (Tim-3 or VISTA) are responsible for galectin-9 carrier function. For this purpose we collected conditioned medium after culturing transformed BEAS-2B for 16 h or co-culturing them with primary human Jurkat T cells (when galectin-9 release takes place). We then immunoprecipitated

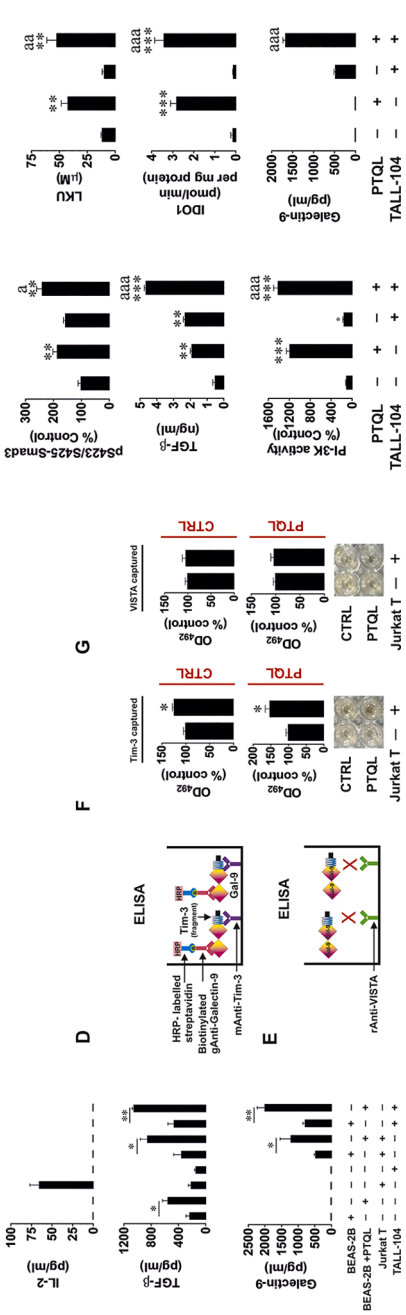

Figure 6. Malignant transformation highly increases immune evasion potential of BEAS-2B and RC-124 cells. Non-transformed and PTQL-transformed BEAS-2B cells were co-cultured with TALL-104 cytotoxic T cells in the ratio 1:1 for 16 h. (A) Granzyme B protein levels (Western blot) and catalytic activity (biochemical assay) were measured as outlined in Materials and Methods. (B) Viabilities of all the cells co-cultured were measured using MTS assay. (C) Non-treated and PTQL-transformed BEAS-2B cells were co-cultured for 16 h in the ratio 1:1 with Jurkat T cells (CD4-positive helpers) or TALL-104 CD8-positive cytotoxic T cells followed by detection of secreted levels of galectin-9, TGF-β and IL-2 in the conditioned media. To investigate if Tim-3 or VISTA is used as a carrier protein in galectin-9 secretion process in BEAS-2B cells, normal and PTQL-transformed BEAS-2B cells were co-cultured with Jurkat T cells for 16 h. Then Tim-3 (D) or VISTA (E) were immunoprecipitated using ELISA plate (see Materials and Methods) and galectin-9 (if bound to Tim-3 or VISTA) was measured using Biotechne ELISA kit. (F) Galectin-9 was detectable when Tim-3 was immunoprecipitated and (G) undetectable on the immunoprecipitated VISTA. Normal and PTQL-treated RC-124 cells were co-cultured for 16 h with TALL-104 cells in the ratio 1:1. (H) PI-3K activity was measured using spectrophotometric assay and TGF-β release and phospho-S423/S425 Smad3 were measured by ELISA as outlined in Materials and Methods. (I) Galectin-9 release was measured by ELISA, and IDO1 activity as well as LKU release, were detected using colorimetric assays described in Materials and Methods. Images are from one experiment representative of 4 which gave similar results. Quantitative data are mean values ± SEM of 4 independent experiments.

Tim-3 or VISTA from the conditioned medium on the ELISA plate (Fig. 6 D and E) and then measured galectin-9 bound to it using biotinylated galectin-9 detection antibody followed by visualisation using HRP-labelled streptavidin (see Materials and Methods for more details). Galectin-9 was detectable in the wells where Tim-3 was immunoprecipitated (Fig. 6 F) and was not detectable in the wells containing VISTA (Fig. 6 G). These results suggest that Tim-3 and not VISTA acts as a carrier/trafficker for galectin-9 when it is being secreted by non-transformed and transformed BEAS-2B cells.

Interestingly, similar results were obtained with RC-124 cells. We found that transformed RC-124 cells also showed higher PI-3K activity and upregulated Smad3 phosphorylation, as measured by ELISA (Fig. 6 H). They were releasing higher levels of TGF-β compared to non-treated RC-124 cells. In line with this and upregulation of Smad3 phosphorylation, IDO1 activity and respectively LKU release were significantly higher in transformed RC-124 cells. When co-cultured with TALL-104 cytotoxic T cells, transformed RC-124 cells released much higher levels of galectin-9 compared to non-treated RC-124 cells (Fig. 6 H and I).

Malignant transformation upregulates cell proliferative and competition potentials

It was then sought to explore proliferative and competition potential of both control and transformed BEAS-2B cells and co-cultured them with non-malignant human keratinocytes (HaCaT) in the ratio 1:1. Competitive cell proliferation was controlled visually and also by on-cell Western analysis detecting the levels of cytokeratin 8 (CK8) on their surface. Overall expression of CK8 in BEAS-2B and HaCaT cells was measured using fluorescence microscopy and FACS analysis (Fig. 7 A and B). We found that HaCaT cells expressed much higher levels of CK8 compared to BEAS-2B. Surface presence of CK8 in non-transformed and transformed BEAS-2B cells was measured using on-cell Western analysis. It was confirmed that surface levels of CK8 were significantly higher in HaCaT cells compared to both types of BEAS-2B (Fig. 7C). We found that when HaCaT cells were co-cultured with control BEAS-2B, bronchial epithelium cells were competed out by keratinocytes. But with transformed BEAS-2B, we observed the opposite effect (Fig. 7C). Importantly, secretion of galectin-9 was not detectable in any of the samples confirming that this galectin-9 secretion phenomenon, which we reported previously, is induced just by T cells and not the other epithelial cells.

Taken together, our results have shown that malignant transformation is associated with activation of immune evasion machinery regardless the presence of immune cells. Appearance of T cells in the microenvironment leads to a rapid and efficient immunosuppressive response.

Discussion

Malignant transformation of human cells is associated with their reprogramming which results in uncontrolled cell proliferation and in the same time activation of immunosuppressive pathways which form cancer immune evasion machinery. However, there is no conceptual understanding on whether immune evasion machinery pathways and expression of immune checkpoint proteins form a part of the process of malignant transformation or they are triggered by lymphocytes and natural killers attempting to attack cells undergoing malignant transformation. To address this fundamental question, we performed experimental malignant transformation of BEAS-2B human bronchial epithelium cells and RC-124 non-malignant human kidney epithelial cells. One of the ways of malignant transformation is permanent activation of H-Ras GTPase, which induces transformation of human cells and often lies at the core of malignancies affecting human digestive system – e. g. gastric and pancreatic cancers [1–6]. Experimental malignant transformation of BEAS-2B cells was first reported in 1985 and was achieved by transfecting them with H-Ras oncogene [3]. We

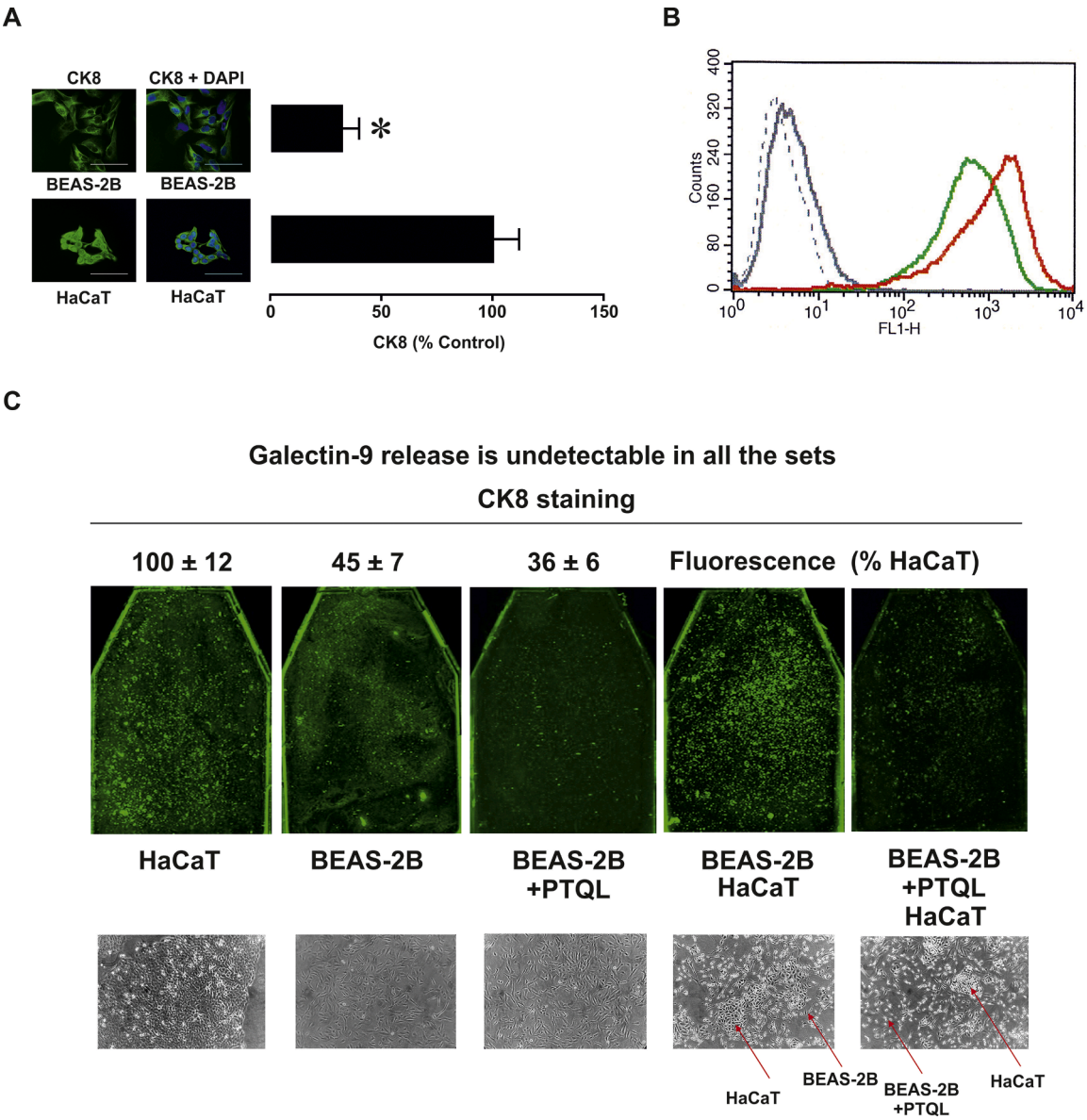

Figure 7. Malignant transformation of BEAS-2B cells increases their proliferative and competition potentials. Levels of CK8 were assessed using fluorescence microscopy (A) in permeabilised BEAS-2B and HaCaT cells (scale bars equal 100 μm each). Also, CK8 was measured by flow cytometry (B) in both cell types after permeabilisation- (grey dotted plot – unstained HaCaT cells; grey plot – unstained BEAS-2B cells; green plot – CK8 in BEAS-2B cells; red plot – CK8 in HaCaT cells). (C) Normal and PTQL-transformed BEAS-2B cells were co-cultured in the ratio 1:1 with HaCaT keratinocytes. Cells were put together at the same time into culture and assessment was performed in 40 h. Levels of CK8 on the cell surface were assessed by on-cell Western (HaCaT cells, as one can see, display much higher levels of this protein on their surface compared to BEAS-2B) and cells were observed using light microscopy. Images and flow cytometry plots are from one experiment representative of 4 which gave similar results. Quantitative data are mean values ±SEM of four independent experiments.

performed natural transformation (without stressing the cells by the process of transfection) using bracken extracts containing ptaquiloside, which is a natural agent causing malignant transformation of human epithelial cells by forming adducts with DNA and inducing mutation/constitutive activity of H-Ras.

Constitutively active H-Ras directly binds PI-3K [2] leading to its constant activation and also triggers activation of MAP kinase pathway, which induces proliferative activity. As one can see (Fig. 1, supplementary videos 1 - 4), exposure to ptaquiloside-containing bracken extracts finally upregulated proliferative activity of BEAS-2B and RC-124 cells. Transformation also led to increased cell competition potential (Fig. 7). PI-3K and mTOR activities were highly upregulated at any moment of time in transformed cells and respectively, activation of these pathways and increased proliferative activity was in line with upregulated HIF-1 activity and increased levels of secreted TGF-β (Fig. 2). Higher levels of TGF-β led to significant upregulation of levels of phospho-S423/S425-Smad3 and its co-activators – Smad4 and TRIM33 (Fig. 3 A and B). In the same time, levels of galectin-9, VISTA, PD-L1 and IDO1 (and respectively LKU release) immune checkpoint proteins were significantly upregulated (Fig. 3C and D). Importantly, among the checkpoint proteins belonging to B7 family (VISTA and PD-L1) only PD-L1 was translocated onto the cell surface (Fig. 3D). VISTA was mainly present inside the cells which means, that it has some intracellular function in these cells which remains to be identified. Further experiments confirmed that expression of galectin-9, IDO1, VISTA and PD-L1 was upregulated on genomic level and the process was Smad3-dependent (as verified by ChIP followed by qRT-PCT, Fig. 5). Interestingly, the immune checkpoint protein mRNA levels (TGF-β, galectin-9 and IDO1) were highly upregulated after 5 days of exposure to bracken extract, went back to the control level after 10 days, however,

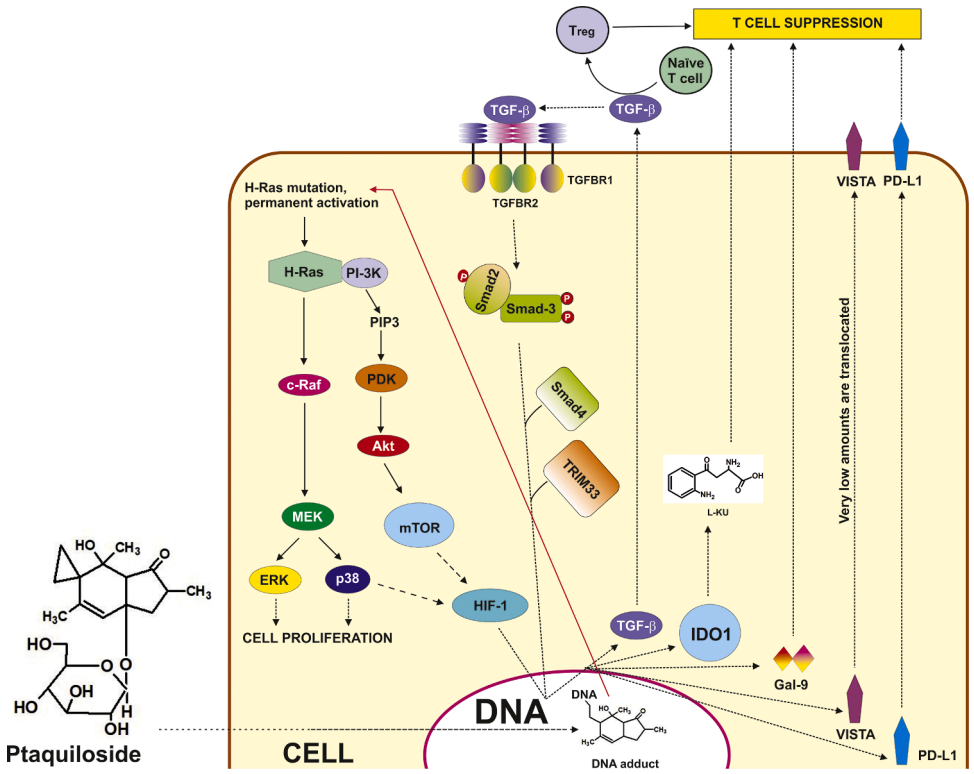

Figure 8. Mechanism of ptaquiloside-induced malignant transformation associated with activation of immune evasion machinery. The scheme illustrates the mechanism of malignant transformation induced by ptaquiloside and achieved through permanent activation of H-Ras GTPase. The scheme demonstrates known mechanisms leading to cell proliferation and in the same time cross-links with pathways inducing activation of immune evasion machinery regardless the presence of immune cells in microenvironment.

when the agent was removed, we again observed highly upregulated levels of these checkpoints. Only HIF-1α mRNA level was constantly upregulated meaning that this stress protein is crucial for adaptation to stress associated with the process of transformation (see Fig. 4 for results).

As a result, it is clear that immune checkpoint proteins are upregulated by TGF-β-Smad3 pathway, where Smad3 clearly involves its co-activators. As we know from our previous studies [19], HIF-1 can upregulate TGF-β expression in the first instance and then TGF-β-Smad3 pathway, being an autocrine signalling network, triggers self (TGF-β) expression [26]. However, this pathway leads to upregulation of expression of immune checkpoint proteins, which are crucial for cancer immune evasion machinery. This takes place in absolute absence of any immune cells.

When the process was completed and transformed cells faced T cells in co-culture (Fig. 6), it was obvious that the immunosuppressive machinery was much more active in the cells which underwent exposure to ptaquiloside-containing bracken extracts. And this appeared to be the case for both BEAS-2B and RC-124 cells (Fig. 6).

Taken together these results demonstrate for the very first time that activation of immune evasion machinery takes place in human cells during malignant transformation regardless the presence of immune cells in microenvironment. As an example we demonstrated the process of experimental transformation using agent known to cause H-Ras mutations. As we can see, this resulted in upregulation of PI-3K/mTOR pathway and hypoxic signalling, which is known to trigger TGF-β expression [26]. TGF-β being autocrine growth factor keeps its own expression (TGF-β-Smad3 pathway is known to directly induce TGF-β expression) on the high level [26]. This results in upregulated levels of immune checkpoint proteins, expressions of which are controlled by Smad3 – galectin-9, PD-L1, IDO1 and respectively LKU levels and when facing T cells, these crosslinking pathways suppress their activities. Importantly, transformation led to upregulation of the protein amounts and levels of recruitment of coactivators of Smad3 (Smad4 and TRIM33) by this protein. This also means that transcriptional processes regulated by Smad3 during malignant transformation were involving de-repression or similar kinds of events leading to activation of gene expression from scratch. This conclusion can be drawn since Smad4 is used by Smad3 to enhance the expression of active genes, while TRIM33 is known to be used for the purpose of activation of expression of “inactive” genes [29]. In addition, TGF-β itself is known to induce differentiation of naïve T cells into immunosuppressive regulatory T cells (Tregs) [30,31]. These mechanisms are schematically summarised in the Fig. 8.

Our results have now addressed the fundamental question discovering that activation of immune evasion machinery and immunosuppressive checkpoint proteins and pathways forms a part of the process of malignant transformation. These processes take place regardless the presence of immune cells in microenvironment. This concept needs to be further investigated to clarify the best biochemical targets for prevention of malignant transformation through the influence on development of cancer immune evasion machinery.

Author Contribution Statement

MA, IMY, SS and VS performed majority of the experiments and analysed the results; SR, SMB, DC, MM, AB and EFK completed the work associated with live cell imaging, flow cytometry and fluorescent microscopy as well as helped with co-cultures of epithelial cells with cytotoxic T cells. EFK and VVS wrote the manuscript. VVS designed the study and supervised the whole project.

Declaration of Competing Interest

The Authors have no conflicts of interest to declare

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2023.101805.

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